



# Fine-tuning root exudation sampling procedures– evaluating the effect of sampling solution volume and the suitability of Micropur as microbial activity inhibitor

Uxue Otxandorena-Ieregi · Michael Santangeli · David Aleksza · Stephan Hann · Eva Oburger

Received: 19 November 2023 / Accepted: 14 March 2024  
© The Author(s) 2024

## Abstract

**Background and aim** Root exudates are the main drivers of plant-microbes-soil interactions. There are several approaches to collect root exudates, but critical methodological evaluation remains limited. The goal of this study was to assess the effect of the applied sampling solution volume and to test the suitability of Micropur as microbial activity inhibitor during the exudate sampling process.

**Methods** Using *Zea mays* L. as model plant and the soil-hydroponic hybrid exudate sampling approach,

we tested the effect of different sampling solution volumes and different Micropur concentrations on maize root exudation. Additionally, a sterile hydroponic-only experiment was conducted to monitor potential effects of Micropur on maize physiology. Photometric and LC-TOF-MS analyses were used to reveal the effect of different conditions on root exudation and plant stress response.

**Results** Our results demonstrated that both, the sampling volume as well as the presence or absence of microbial activity inhibitor can significantly affect obtained exudation results. Carbon exudation rates were underestimated when using a small sampling volume relative to the root biomass. Even though the overall C exudation was minimally affected by microbial degradation, the addition of Micropur ( $\geq 5 \text{ mg L}^{-1}$ ) significantly reduced the decomposition of  $^{14}\text{C}$  citric acid. Maize plants did not show any stress symptoms upon exposure to different concentrations of MP but root membrane permeability and exudation were negatively affected when using MP concentrations  $>5 \text{ mg L}^{-1}$ .

**Conclusion** Our findings highlight the importance of thoroughly evaluating experimental procedures and delivers relevant insights supporting future experimental designs targeting root exudates.

Responsible Editor: Eusun Han.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11104-024-06630-w>.

U. Otxandorena-Ieregi · M. Santangeli · D. Aleksza · E. Oburger (✉)  
Institute of Soil Research, University of Natural Resources and Life Sciences, Tulln an der Donau 3430, Austria  
e-mail: eva.oburger@boku.ac.at

U. Otxandorena-Ieregi  
Department of Microbiology and Ecosystem Science,  
University of Vienna, Vienna 1030, Austria

M. Santangeli · S. Hann  
Institute of Analytical Chemistry, University of Natural Resources and Life Sciences, Vienna 1190, Austria

D. Aleksza  
Institute of Molecular Plant Biology, University of Natural Resources and Life Sciences, Vienna 1190, Austria

**Keywords** Rhizosphere · Dissolved organic carbon · Rhizodeposition · Methodology · Non-targeted metabolomics

## Introduction

Root exudates are soluble organic compounds that are released by living roots. They embrace a broad spectrum of molecules, from low-molecular-weight (LMW) compounds like organic acids, amino acids, carbohydrates or phenols to high-molecular-weight polysaccharides, proteins, and mucilage (Grayston et al. 1997; Farrar et al. 2003; Jones et al. 2004; Oburger and Jones 2018). To date, both, the general importance of root exudates and the methodological challenges involved in their investigation have long been recognised (Phillips et al. 2008; Neumann et al. 2009; Vranova et al. 2013; Oburger and Jones 2018; Williams et al. 2021), however, little attention has been paid to relevant procedural details in the exudate sampling process that can potentially cause a bias in obtained exudation results.

The collection of undisturbed root exudates (or gross exudation) from plants is challenging. This difficulty arises due to several soil processes triggered upon the release of these metabolites. Sorption by the soil matrix, microbial decomposition, as well as mass flow and diffusion, are known to quickly alter the quality and quantity of exudate in the soil solution. Additionally, freshly released compounds are further mixed with dissolved organic carbon derived from soil organic matter as well as metabolites of microbial origin (Todorovic et al. 2001; Oburger and Jones 2018; Pausch and Kuzyakov 2018; Canarini et al. 2019). The physical inaccessibility to roots without disturbance or damage to the plant presents another challenge in the sampling process (Phillips et al. 2008). Various methods have been developed to sample root exudates either from plants grown in nutrient solution (hydroponics) or soil-grown plants, as summarized by (Oburger and Jones 2018). In recent decades, hydroponic systems have been preferentially selected to investigate root exudates, as they facilitate access to the roots and provide an easily controllable environment (e.g., sterility and nutrient availability can be achieved more easily than in soil) (Krafczyk et al. 1984; Jones and Darrah 1996; Kuzyakov and Jones 2006; Lesuffleur and Cliquet 2010). However, the environment in which roots are growing strongly affects the quantity and quality of rhizodeposition (Carvalhais et al. 2011; Canarini et al. 2016). Although results of root exudates obtained from hydroponically grown plants may be

entirely appropriate depending on the research question, the ecological relevance will remain questionable until we verify the findings in soil. Among several approaches (for a detailed overview of exudation sampling techniques see the review of Oburger and Jones, 2018), the soil-hydroponic-hybrid setup is a feasible methodological compromise applicable in the laboratory/greenhouse and even in the field (Santangeli et al. 2024). In this simple and universally applicable sampling approach, plants are grown in soil (e.g., pot, rhizoboxes, field) and exudates are then collected hydroponically for a short period of time (2–4 h) after careful root washing. Despite potential root damage and the sudden change in environmental conditions (soil to solution), root metabolome, and therefore, root exudates can be expected to still reflect exudation occurring in natural soil if the sampling procedure is kept short (Oburger and Jones 2018).

Irrespective of the medium in which the plants are grown, exudates are mostly collected hydroponically in a water matrix basis (e.g. Groleau-Renaud et al. 1998; Oburger et al. 2014; Rosenkranz et al. 2021) as this avoids sorption to soil particles and potentially allows to control microbial activity during exudate collection. The sampling solution can be static or non-static. In the first approach, the same solution is in contact with the roots throughout the entire sampling period, in the latter, fresh solution is constantly passed through (Jones and Darrah 1993; Oburger et al. 2013). While exudate collection in static solution is operationally simpler, the build-up of metabolites (which are unlikely to occur in natural soil) will slow down passive diffusion and can thus bias the obtained exudate quantity. Whereas, the re-uptake of LMW compound by roots can also happen and will depend on the metabolic load on the surrounding solution (Jones and Darrah 1992, 1993, 1996; Farrar et al. 2003; Lesuffleur et al. 2007; Personeni et al. 2007; Jämtgård et al. 2008; Jones et al. 2009; Svennerstam et al. 2011; Warren 2015, 2016; Uscola et al. 2017; Tiziani et al. 2020). Considering the contribution of passive diffusion and the potential reuptake of metabolites to the overall exuded C, the length of the sampling period (Aulakh et al. 2001; McLaughlin et al. 2023) and the sampling solution volume, more precisely the root biomass to sampling solution volume ratio (RSVR, either g root dry weight or cm<sup>2</sup> root surface divided by L of sampling solution) are likely to be decisive factors, especially

in experiments involving plants with dissimilar root system sizes and morphologies (e.g., different plant species, soil texture and developmental stages). However, to date, little attention has been paid to potential biases resulting from procedural settings like the sampling solution volume in relation to the investigated root system size.

Next to the choice of sampling solution volume, microbial decomposition during the exudate collection period can significantly alter obtained metabolite concentrations resulting in an underestimation of exudation rates (Rangel-Castro et al. 2005; Jones et al. 2009). In contrast to sterile or semi-sterile hydroponically grown plants, soil-grown roots are expected to be heavily colonized by a wide range of microorganisms (Schmidt and Eickhorst 2013; Dietrich et al. 2022). To avoid the microbial decomposition of metabolites during exudate sampling, a variety of microbial activity inhibitors have been used: rifampicin/ tetracycline (Graham et al. 1981; Schwab et al. 1983; Imas et al. 1997), streptomycin/ chloramphenicol, cycloheximide/ streptomycin (Kuzyakov et al. 2003; Gent et al. 2005), Bronopol (Schindlegger et al. 2014), as well as the water purification product Puritabs (Gherardi and Rengel 2004) and the frequently used, Micropur (Hansen and Jolley 1995; Tomasi et al. 2012; Oburger et al. 2014; Valentinuzzi et al. 2015; Zwetsloot et al. 2018; Rosenkranz et al. 2021; Heuermann et al. 2023). Compared to other products, silver ion ( $\text{Ag}^+$ ) containing Micropur has both antibacterial and antifungal properties (Gibała et al. 2021) and as it is also provided in powder form, an accurate dosage can easily be applied in a wide range of experiments. While some comparative tests have been published (Schindlegger et al. 2014; Valentinuzzi et al. 2015), a detailed methodological assessment of the suitability of Micropur (or any other microbial activity inhibitor) in exudation sampling procedures is still missing. In addition to assessing the effectiveness of inhibiting microbial activity, it is essential to evaluate any potential adverse impacts on the plant and the exudation process itself to avoid experimental setup-driven biases.

Aiming at fine-tuning the (hydroponic) exudate collection procedure, we tested how different sampling solution volumes – or more precisely the root biomass to sampling solution volume ratio (RSVR) - affect obtained root exudation rates. Additionally, we evaluated the suitability of Micropur as a microbial

activity inhibitor during exudation sampling considering both its bacteriostatic efficiency and potential plant stress response upon root exposure to Micropur. While our primary focus was on the soil-hydroponic-hybrid exudate collection technique, our findings have broader applicability to any experimental setup aimed at collecting root exudates in a hydroponic system.

## Material and methods

In total, we conducted four different experiments addressing different aspects relevant to the (hydroponic) exudate collection procedure. Prior to the detailed experimental descriptions, we briefly outline the specific challenges addressed in each experiment.

### Experiment 1: Effect of exudate sampling solution volume on exudate quantity

When investigating root exudation of different species, environmental conditions, or plant developmental stages, root biomass can be expected to differ significantly between species and/or treatments, but typically, only one sampling solution volume (SV) is used throughout an experiment. When the SV is kept constant, the root to sampling solution volume ratio (RSVR) can be markedly different for different treatments/species depending on plant growth performance. This can potentially affect the amount of exudates released via passive diffusion, especially when low SVs are used as considerable metabolite concentrations can quickly accumulate in the sampling solution. Hence, in Experiment 1, we investigated whether different RSVRs can cause a bias in the quantity of exudates collected.

### Plant growth

In total, 24 *Zea mays* L. cv NK Falkone plants were grown in pots for 4 weeks, with one plant per pot. Seeds were germinated in moist tissue paper in the dark for three days before transferring them to soil-filled pots. The growth substrate was a mixture of quartz sand (83.3%) and loam soil (Haplic Phaeozem, 16.6%) sieved to <2 mm. The soil was fertilized with ( $\text{mg Kg}^{-1}$ ):  $\text{NH}_4\text{NO}_3$  (100),  $\text{K}_2\text{SO}_4$  (100),  $\text{MgCl}_2 \times 6\text{H}_2\text{O}$  (50),  $\text{CaSO}_4 \times 2\text{H}_2\text{O}$  (100),  $\text{CaHPO}_4$  (50),  $\text{MnSO}_4 \times \text{H}_2\text{O}$  (3.25),  $\text{Zn}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$

(0.79),  $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$  (0.5),  $\text{H}_3\text{BO}_3$  (0.17) and Fe-EDTA (3.25) (see also: Vetterlein et al. 2021). The pots contained 400 g of air-dried mixed substrate and they were watered each second day to 18% of volumetric water content (VWC). Plants were grown in the greenhouse with a 12 h photoperiod at  $350 \mu\text{M m}^{-2} \text{s}^{-1}$  photosynthetically active radiation (PAR), at 22/18 °C day/night, respectively, and relative humidity of 65%.

#### *Exudation sampling & plant harvest*

Root exudates were sampled according to the soil-hydroponic-hybrid method (Aulakh et al. 2001; Oburger et al. 2014; Williams et al. 2021; Santangeli et al. 2024) following the recommendations of Oburger and Jones (2018). Around 3 hours after the onset of light, the rooted soil block was removed from the pots and the roots were carefully rinsed with deionized water to remove soil particles. Next, roots were emerged for 5 minutes in fresh sampling solution (Milli-Q water (MQ) and  $10 \text{ mg L}^{-1}$  Micropur classic (Katadyn®, Switzerland)) to osmotically adjust the root cells and remove damaged cells as well as metabolite accumulation in the apoplast. Thereafter, exudates were sampled hydroponically in MQ water containing  $10 \text{ mg L}^{-1}$  MP for approximately 3 hours. The sampling period was kept relatively short to avoid changes in exuded metabolite patterns due to altered plant metabolisms when exposed to a new environment but long enough to have a measurable amount of exudates. While changes in plant gene expression might occur rather quickly, changes in the plant's metabolome can be expected to take multiple hours (Mangelsen et al. 2011). By keeping the sampling period short, we ensure that obtained exudation patterns still reflect what is occurring in the soil. Exudates were collected in six different sampling solution volumes (130, 200, 300, 400, 500 and 1000 mL), resulting in 4 biological replicates per RSVR (one blank per treatment was included with the corresponding sampling volume). After the exudate collection period, plants were removed from the sampling solution and the exudate solution was passed through  $0.2 \mu\text{m}$  membrane filters (Cellulose acetate, OE 66, Whatman, UK) and immediately frozen at  $-20 \text{ }^\circ\text{C}$ . Shoot and root dry weight (dw) was determined by drying the samples at  $60 \text{ }^\circ\text{C}$  in the oven for 72 hours.

#### Experiment 2: Effect of Micropur on exudate quality and quantity under sterile conditions

To reveal any impacts of the microbial activity inhibitor Micropur (MP) on plant physiology as well as on root exudation, we conducted a hydroponic experiment (experiment 2) where plants were grown, and exudates were sampled with increasing MP concentration (0, 1, 5, 10 and  $20 \text{ mg L}^{-1}$ ) under sterile conditions. Even though our focus lies on soil-based exudation sampling techniques, this proof-of-principle experiment requires sterile conditions – which are more easily maintained in hydroponics – as only then any changes in exudation or plant stress response can be attributed to the presence or absence of MP.

#### *Plant growth*

*Zea mays* L. cv NK Falkone seeds were sterilized (5% NaOCl for 15 min and rinsed 5 times with distilled sterile water) and germinated in Lysogeny Broth (LB) plates (0.75% agar) for 72 h. Sterile seeds were transferred to a two-chamber hydroponic system (Kawasaki et al. 2018). The system was fully sealed, the upper chamber contained the aerial part of the plant and the lower chamber had the nutrient solution and the plant root system. The plant was held between the two compartments with a perforated seed holder. The lower chamber contained an air inlet fitted with a sterile Syringe Filter ( $0.45 \mu\text{m}$  pore size, Cellulose acetate, Puradisc FP 30, Whatman, UK) to aerate the solution. The upper chamber air ventilation was regulated by two outlets fitted with sterile PTFE filters outside to ventilate the air in the upper chamber. We used half-strength Hoagland solution (Hoagland and Arnon 1950) of the following composition: 3 mM  $\text{KNO}_3$ , 0.5 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ , 2 mM  $\text{Ca}(\text{NO}_3)_2$ , 2.02 mM  $\text{MgSO}_4$ ,  $0.15 \mu\text{M}$   $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$ ,  $7.5 \mu\text{M}$   $\text{MnCl}_2 \times 4 \text{H}_2\text{O}$ ,  $0.37 \mu\text{M}$   $\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$ ,  $0.25 \mu\text{M}$   $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ,  $25 \mu\text{M}$   $\text{H}_3\text{BO}_3$  and  $50 \mu\text{M}$  Fe-EDTA. The nutrient solution within the two chamber systems was autoclaved at  $121 \text{ }^\circ\text{C}$  for 15 minutes. To maintain the sterility, all the manipulations were performed under the sterile conditions of a laminar flow hood. Plants were grown for 14 days at room temperature ( $21 \text{ }^\circ\text{C}$ ) in the lab under LED Growth Lights (ATUM HORTI 600 V2). The photoperiod was set at 12 h/12 h and the light source intensity was

450  $\mu\text{M m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation (PAR) at the top of the upper chamber.

#### *Exudation sampling & plant harvest*

Root exudates were collected in different sampling solutions having 0, 1, 5, 10 and 20  $\text{mg L}^{-1}$  MP in MQ water, while the sampling volume was 100 mL. In total, we had 23 sterile maize plants and 5 treatments, with 4–5 biological replicates each. Any work requiring the opening of the chambers was conducted under the laminar flow hood. The exudation sampling was carried out as described above (see section Exudation sampling & plant harvest of Experiment 1) without the root washing step. The root exudation sampling was performed in three batches due to the limited space in the laminar flow hood (treatments were distributed evenly across the batches). This resulted in a variation of the sampling period between 2.78 to 2.98 hours. Both rinsing and sampling solutions were autoclaved. Plants were placed below the lamps during the sampling period. Total exuded DOC was directly measured photometrically (see Total C of Exudate analyses section). Sterility was checked before and after exudation sampling to confirm that sterility was maintained throughout the entire experiment. For this, 100  $\mu\text{L}$  of nutrient solution, sampling solution and filtered (0.2  $\mu\text{m}$  pore size, CHROMAFIL® CA Syringe Filters, XH42.1, Germany) exudate solution was pipetted onto individual LB agar plates which were kept in the incubator at 25 °C for 7 days. An aliquot of the filtered exudate solution was frozen at –80 °C for further metabolomic analyses. Fresh biomass aliquots of 200 mg and 500 mg from root and shoot, respectively, were harvested from each plant and immediately frozen by dipping in liquid nitrogen and stored at –80 °C.

Experiment 3: Efficiency of Micropur to inhibit microbial activity and its effect on root exudation of soil-grown plants

In Experiment 3, we tested the efficiency of MP in inhibiting microbial activity when using the soil-hydroponic-hybrid exudation sampling setup. Increasing concentrations of MP (0, 1, 5, 10 and 20  $\text{mg L}^{-1}$ ) containing sampling solutions were spiked with  $^{14}\text{C}$ -labelled citric acid to trace any potential decomposition loss of a representative

LMW exudate compound from the sampling solutions during the exudate collection period. In addition to being reported as exuded by maize (Krafczyk et al. 1984; Ma et al. 2022), citric acid was chosen as a tracer molecule as it was shown that re-uptake of organic acids by plant roots is negligible (Warren 2016). Drops of the sampling solution were plated onto agar plates and monitored the growth of colony-forming units (CFU).

#### *Plant growth*

A total of 15 *Zea mays* L. cv NK Falkone were grown in a pot experiment as described above (Plant growth of Experiment 1) for 3 weeks.

#### *Exudation sampling & plant harvest*

Exudation sampling was conducted as described for Experiment 1 using 100 mL as sampling solution volume. Additionally, 0.5 mL of 1 mM  $^{14}\text{C}$ -citric acid (Perkin Elmer®, NEC160), which corresponded to an activity of  $2.775 \times 10^6$  cpm per sample, was added to each sampling solution that contained different concentrations of MP (0, 1, 5, 10 and 20  $\text{mg L}^{-1}$ ). Roots of soil-washed plants were submerged in radiolabelled sampling solution for approximately 3 hours and were kept under LED Growth Light (ATUM HORTI 600 V2) during the sampling period. After the sampling period, roots were removed from the sampling solution and the loss of  $^{14}\text{C}$  activity was immediately determined by liquid scintillation counting using UltimaGold™ XR scintillation fluid (PerkinElmer Corp., Shelton, USA) and a TriCarb 2910 TR liquid scintillation counter (PerkinElmer, Inc. Waltham, MA, USA) in relation to the initial  $^{14}\text{C}$  activity added. Total dissolved organic C concentrations were determined spectrophotometrically as described below.

#### *Incubation of sampling solution in agar Petri dishes*

To evaluate the efficacy of Micropur, 100  $\mu\text{L}$  of non-filtered exudate sample solutions (0, 1, 5, 10 and 20  $\text{mg L}^{-1}$  MP) of individual plants were collected immediately after the sampling period and incubated on LB agar. The exudate sampling solution was uniformly spread in the media by adding two sterile glass balls into each plate and horizontally shaking

them. After 24 hours at 30 °C, the growth of colony-forming units (CFU) of microorganisms was visually evaluated.

#### Experiment 4: Effect of Micropur on plant root cell permeability

This experiment was conducted to test a potential negative effect of MP on root membrane permeability.

##### *Plant growth*

In all, 9 *Zea mays* L. cv NK Falkone were grown in a pot experiment as described above (Plant growth of Experiment 1) for a duration of 4 weeks.

##### *Exudation sampling & plant harvest*

Exudation sampling with the sampling solutions containing either 0, 5 or 10 mg L<sup>-1</sup> MP. After the sampling period, a homogeneous subsample of fresh roots was taken to test the root membrane stability as described further below (see Membrane stability index section).

##### Exudate analyses

##### *Total dissolved organic C exudation*

The presence of ultraviolet (UV) absorbing bonds was used to estimate dissolved organic carbon (DOC) concentration in exudate samples (Baes and Bloom 1990; Brandstetter et al. 1996; Oburger et al. 2022). DOC was determined photometrically at 260 nm with a microplate reader (TECAN-Infinite™ 200 PRO) according to (Oburger et al. 2022) by pipetting 250 µL of prefiltered exudate solution, blanks, standards and quality controls into 96-microwell plates (Greiner UV-STAR® flatbottom). Calibration of standards was prepared with potassium phthalate (KHP, Elementar) with carbon concentrations ranging from 0.5 to 50 mg L<sup>-1</sup>. The C exudation rate results were expressed in µmol of C per gram of dry root biomass per hour of sampling period.

##### *Non-targeted metabolomic analysis*

Aliquots of exudate samples (40 mL) stored at -80 °C were freeze-dried (at -45 °C and 0.070 mbar,

Alpha 1–4 LSCplus, Christ, Germany). Subsequently, the dried samples were reconstituted in either 0.35 or 0.7 mL of LC-MS grade water in order to achieve a concentration of around 100 mg C L<sup>-1</sup>, thereafter, they were filtered through 0.2 µm filters (4 mm; cellulose acetate, Nalgene™, USA). Non-targeted metabolomic analysis was conducted according to Lohse et al., 2023. Briefly, Reversed-Phased Liquid Chromatography (RPLC) was performed using an Infinity II UHPLC system (Agilent Technologies, Santa Clara, USA), with an Atlantis T3 C18 column, 2.1×150 mm, 3 µm particle size (Waters Corporation, Milford, USA). The separation was performed through a gradient elution at a flow rate of 200 µL min<sup>-1</sup>. Mobile phase A was MQ water with 0.1% v/v formic acid, while mobile phase B was 100% methanol. Initially, the gradient conditions (100% A) were held for 2 minutes, followed by a decrease to 60% A over the next 8 minutes and held steady for 2 minutes. A cleaning step (100% B in 0.1 minutes, then held for 2 minutes), was followed by a quick decrease to 0% B and an appropriate re-equilibration period of 5.9 minutes. The entire analysis took 20 minutes per injection. The injection volume was 5 µL, and the column temperature was maintained at 40 °C.

MS acquisition was performed with a 6230b Time-of-Flight mass spectrometer equipped with a dual Jetstream ESI interface (Agilent Technologies, Santa Clara, USA). Mass spectra ranging between 90 and 1700 m/z were recorded in negative (-) polarity, using the 2 GHz extended dynamic range mode with a spectral acquisition rate of 2 Hz. TOF settings can be found in the SI of Lohse et al. 2023.

Data were acquired with the MassHunter acquisition software (version 10.1, Agilent Technologies, Santa Clara, CA), while MassHunter Profinder B.10.00 software (Agilent Technologies, Sta Clara, CA) was used for peak picking and chromatographic deconvolution with a Batch Recursive Feature Extraction (BRE, small molecules/peptides) workflow. Detailed information about the adopted BRE workflow can be found in the SI information of Lohse et al., 2023. Briefly, Retention time extraction was set to 2.00–14.00 minutes. The m/z range was restricted to 100–1000, and [M-H]<sup>-</sup> ion, while the maximum permitted charge state was set to two. The matching tolerance for feature alignment was set to ±10 ppm and ± 0.30 min for mass accuracy and retention time,

respectively. An absolute peak height threshold of 1000 counts was applied.

Samples were analysed in 4 randomised batches. Pool quality controls were prepared by combining equal volumes of each sample (50  $\mu\text{L}$ ) and injected every 5 samples to monitor the stability of the TOFMS system.

Data was further processed in Rstudio (version 2023.06.0+421). First, features that were absent in more than 25% of the samples in any given treatment were excluded. Then the measured intensities were normalized with the corresponding exudation rate ( $\mu\text{mol C g}^{-1}$  root dw  $\text{h}^{-1}$ ) and dilution factor, followed by log transformation and unit variance scaling. Finally, data was adjusted using the ComBat method, correcting the biases introduced by the measurement of samples in separate batches. The treatment dependent differences in obtained metabolite features were then evaluated by PCA and volcano plots. A molecular feature was considered unique if it was only present in one treatment.

Analyses of plant stress response and membrane stability index (MSI)

In order to determine whether exposure to MP has a negative effect on the plant's physiological status, the general stress response in frozen ( $-80\text{ }^{\circ}\text{C}$ ) roots and shoots of hydroponically grown maize plants (Experiment 2) was examined as follows: Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) concentration was measured, reactive oxygen species (ROS) that accumulate in tissues when organisms are exposed to stress (Buckner et al. 2000; Jones 2000). Additionally, antioxidant enzymes such as catalase (CAT) or superoxide dismutase (SOD) were quantified. These enzymes are components of the cellular defensive system, responsible for scavenging ROS (Apel and Hirt 2004). When the capacity of the defensive system is exceeded, ROS can cause cell damage, here we determined the content of malondialdehyde (MDA), a product of plant cell lipid damage (Dix and Aikens 1993). Finally, an assay determining the Membrane Stability Index (MSI) was conducted as described by Sairam et al. (1997) to assess the potential membrane leakage effect that MP has on the roots.

### *Hydrogen peroxide ( $\text{H}_2\text{O}_2$ )*

Hydrogen peroxide content was determined following the protocol of Sergiev et al. (1997). Frozen shoot (500 mg) and root (200 mg) tissues were milled to powder (25 Hz, RETSCH MM400 Mixer Mill) in Eppendorf tubes. As extractant, 1 and 1.2 mL of 0.1% (w/v) trichloroethanoic acid (TCA) was used, for shoots and roots, respectively. The samples were vortexed and centrifuged at 10,000 g for 25 minutes at  $4\text{ }^{\circ}\text{C}$  (Fresco™ 21 Microcentrifuge, Heraeus™, Thermo Scientific™). Thereafter, 300  $\mu\text{L}$  of the supernatant was added to 300  $\mu\text{L}$  10 mM potassium phosphate buffer (pH 7.0) and 600  $\mu\text{L}$  of 1 M potassium iodide. After the incubation at room temperature in the dark for 20 minutes, absorbance was read at 390 nm. The amount of  $\text{H}_2\text{O}_2$  was calculated using a calibration curve prepared with known concentrations of  $\text{H}_2\text{O}_2$  (10–200  $\mu\text{M}$ ) in 0.1% TCA and results were expressed as  $\text{H}_2\text{O}_2\ \mu\text{mol g}^{-1}$  plant fresh weight (fw).

### *Lipid peroxidation*

Malondialdehyde (MDA) content in fresh plant biomass was measured as the indicator of lipid peroxidation according to the method defined by Heath and Packer (1968). First, 200  $\mu\text{L}$  of plant extract (same TCA supernatant as in  $\text{H}_2\text{O}_2$ ) was transferred into a fresh Eppendorf tube, and 800  $\mu\text{L}$  of 0.5% (w/v) thiobarbituric acid (TBA) containing 20% (w/v) TCA was added. The acidic environment created by TCA is not enough to provoke the reaction of one molecule of MDA with two molecules of TBA. Thus, tubes have to be incubated at  $95\text{ }^{\circ}\text{C}$  for 15 minutes (lids were perforated with a needle to avoid explosions during the heat treatment). Samples were cooled down on ice for 5 minutes. The mixture was centrifuged at 10,000 g for 5 minutes at  $4\text{ }^{\circ}\text{C}$ , and the supernatant was used for photometric analyses. Standards made by MDA (1–50  $\mu\text{M}$ ) in 0.1% TCA were treated exactly as samples. MDA level was calculated by measuring the absorbance at 532 nm. A correction was made for nonspecific turbidity by subtracting the absorbance measured at 600 nm from 532 nm absorbance. Results were expressed as MDA  $\text{nmol g}^{-1}$  fresh plant tissue.

### Superoxide dismutase (SOD)

The activity of SOD (EC 1.15.1.1) was determined by quantifying the photoinhibition of nitroblue tetrazolium (NBT) by superoxide anions generated from the photoactivation of riboflavin (Beyer and Fridovich 1987). Frozen 500 mg of shoot and 200 mg of root tissues were milled to powder in Eppendorf tubes and 100 mM ice-cold phosphate buffer (pH=7.5) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) was used for the proteins' extraction (1 and 1.2 mL were used for shoots and roots, respectively). The samples were vortexed and centrifuged (10,000 g, 4 °C, 25 minutes). The same supernatant was also used for the catalase and protein determination assays.

Next, the following reaction mix was prepared: 1.5 mL of 100 mM phosphate buffer (pH 7.6), 850 µL of MQ water, 0.1 mL of 3 mM EDTA, 0.1 mL of 1.5 M NaCO<sub>3</sub>, 0.2 mL of 0.2 M L-methionine, 100 µL of 1.5 mM NBT, 0.1 mL of 60 µM riboflavin and 50 µL of tissue extract. The photoinhibition was carried out by shaking the reaction mix exposed to white light for 15 minutes at room temperature (controls without enzyme extract were run in parallel), and thereafter, absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% of inhibition of NTB. The results were then expressed in units SOD activity mg<sup>-1</sup> protein.

### Catalase (CAT)

The catalase (EC 1.11.1.6) activity was determined according to the procedure of Aebi (1984). The reaction mix contained: 1.5 mL of 100 mM potassium phosphate buffer (pH=7), 0.5 mL of 7.5 mM H<sub>2</sub>O<sub>2</sub> and 50 µL of plant extract. The mixture was brought to 3 mL by the addition of MQ water. The concentration of H<sub>2</sub>O<sub>2</sub> was determined using Beer's law, having 36.6 mM<sup>-1</sup> cm<sup>-1</sup> (Aebi, 1984) as the molar extinction coefficient. To achieve this, the absorbance values obtained from the microplate were normalized to cuvette absorbances using the path length of the former. Considering that one catalase unit is the amount of the enzyme that catalyses the conversion of one

µmol of H<sub>2</sub>O<sub>2</sub> per minute, results were given in units of catalase per mg<sup>-1</sup> of protein.

### Membrane stability index

The effect of MP in soil-grown plants was tested by calculating the Membrane Stability Index based on Sairam et al. (1997). Once terminated the sampling period of exudates, root subsamples were taken and immersed in MQ water (10 mg fw root mL<sup>-1</sup> MQ water). The electric conductivity (EC) of each sample was measured twice, first after 30 minutes at 40 °C and then after 10 minutes at 100 °C treatment using a conductivity meter (Cond 740, inoLab®, Germany). The membrane stability index (MSI) was calculated accordingly:

$$MSI = \left( 1 - \frac{EC_{40^{\circ}C, 30 \text{ min}}}{EC_{100^{\circ}C, 10 \text{ min}}} \right) \times 100$$

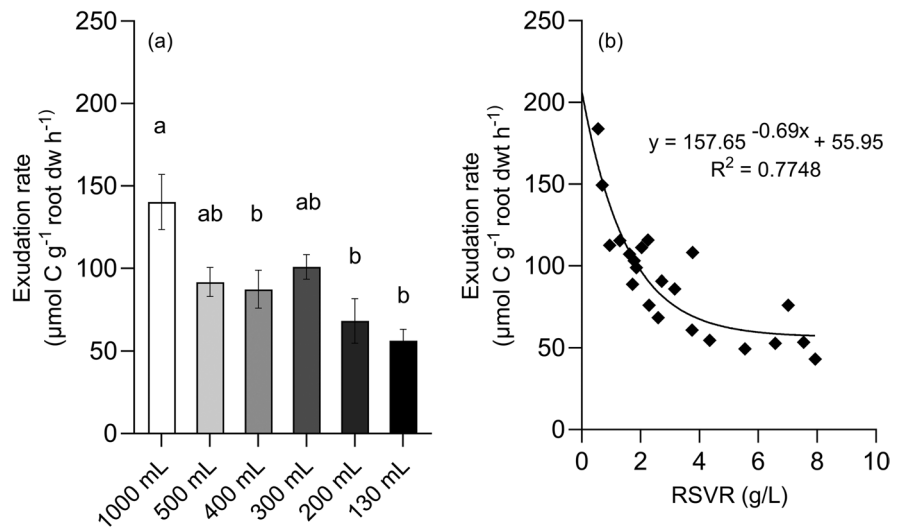
## Results

### Experiment 1: Effect of exudate sampling solution volume on exudate quantity

Root exudates were collected in different sampling volumes: 0.13, 0.2, 0.3, 0.4, 0.5 and 1 L were used which corresponded to RSVRs of 7.27±0.60, 4.35±0.85, 2.54±0.50, 2.48±0.73, 1.93±0.45 and 0.88±0.32 g L<sup>-1</sup>, respectively. There was no statistically significant difference in plant biomass between treatments (see SI Fig. S1). However, total C exudation rates differed significantly across the entire range of sampling volumes (p=0.00346, Fig. 1a). While exudation rates remained comparable when using a SV from 200 to 500 mL (corresponding to an RSVR range of 1.93–4.35 g root dw L<sup>-1</sup>), collecting root exudates in 1000 mL (RSVR 0.88 g L<sup>-1</sup>) resulted in C exudation rates that were 2.20 times higher than exudation rates obtained from using a SV of 130 mL (RSVR 7.72 g L<sup>-1</sup>). Plotting obtained C exudation rates against the RSVR of the individual plants revealed that the exudation rate change due to different RSVR could be fitted to a first-order decay equation (R<sup>2</sup> = 0.78) indicating a dependency of obtained exudation rates on the applied RSVR (Fig. 1b).



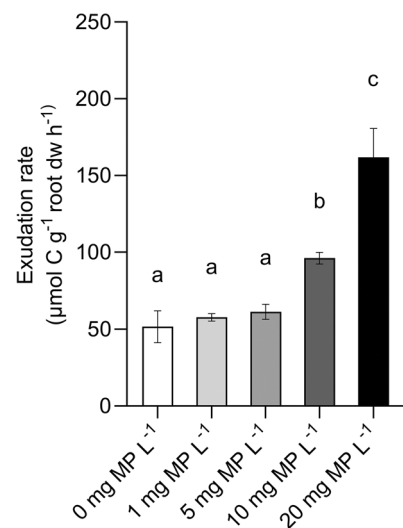
**Fig. 1** Total dissolved organic carbon (C) exudation rates ( $\mu\text{mol C g}^{-1}$  root  $\text{dw h}^{-1}$ ) of *Zea mays* L. cv NK Falkone depending on (a) sampling solution volume (mL) or (b) on Root biomass to Sampling Solution volume Ratio (RSVR,  $\text{g L}^{-1}$ ). Values in (a) represent mean  $\pm$  SEM of 4 biological replicates. Letters in (a) represent significant differences between the investigated treatments (ANOVA, Tukey  $p < 0.05$ ). The fitted model in (b) is a first-order exponential decay equation



#### Experiment 2: Effect of Micropur on exudate quality and quantity under sterile conditions

Testing the effect of different concentrations of MP (0–20  $\text{mg L}^{-1}$ ) in the sampling solution under sterile conditions revealed a rise in C exudation of maize with increasing concentrations of MP ( $p = 0.003$ , Fig. 2). No significant differences in exudation rates were found between 0 and 5  $\text{mg L}^{-1}$  of MP, while exposure to 10 and 20  $\text{mg L}^{-1}$  of MP increased C exudation rates by a factor of 1.86 and 3.13, respectively, when compared to the control treatment (0  $\text{mg L}^{-1}$  MP). Plating aliquots of nutrient and sampling solutions confirmed that sterility was maintained throughout plant growth and the exudate collection period. As there were no statistical differences in plant biomass between treatments (see SI, Fig. S2) and sterility was maintained throughout the experiment, observed changes in exudation can be solely attributed to the increased MP concentration in the sampling solution.

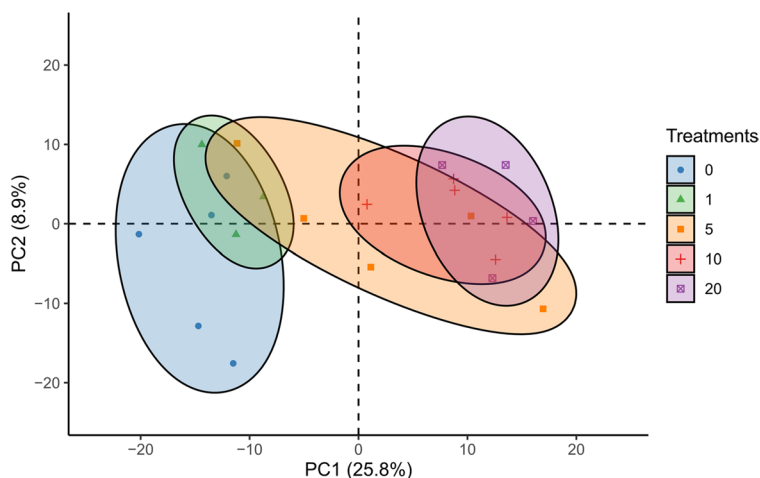
In addition to the total C exudate quantity, we observed a gradual change in root exudate composition with increasing concentrations of MP as determined by non-targeted metabolomic profiling, especially at 10 and 20  $\text{mg L}^{-1}$  MP (Fig. 3). Results of pairwise permutational multivariate analysis of variance (PERMANOVA) analyses based on Euclidean distance further confirmed differences in the root exudation metabolome as a result of MP treatments ( $F_{(4,17)} = 2.1397$ ,  $R^2 = 0.335$ ,  $p = 0.001$ ). Comparing the number of unique and shared features of



**Fig. 2** Exudation rates of total dissolved organic carbon of *Zea mays* L. cv NK Falkone ( $\mu\text{mol C g}^{-1}$  root  $\text{dw h}^{-1}$ ) grown hydroponically under sterile conditions and exposed to different Micropur concentrations (0, 1, 5, 10 and 20  $\text{mg L}^{-1}$ ) during the exudation sampling period (3 h). Values represent mean  $\pm$  SEM of 5 different biological replicates. Letters represent significant differences between the investigated treatments (ANOVA, Tukey  $p < 0.05$ )

control (zero MP) with 5  $\text{mg L}^{-1}$  MP and 20  $\text{mg L}^{-1}$  MP showed that the control treatment shared 88% of molecular features with 5  $\text{mg L}^{-1}$  MP (10% were only present in 5  $\text{mg L}^{-1}$  and 2% only in the control) (Fig. S5a). The use of 20  $\text{mg L}^{-1}$  further reduced the percentage of shared features to 77% of

**Fig. 3** Principal component analyses (PCA) of metabolite profiles exuded by *Zea mays* L. cv NK Falkone ( $\mu\text{mol C g}^{-1}$  root dw  $\text{h}^{-1}$ ) grown hydroponically under sterile conditions and exposed to different Micropur concentrations (0, 1, 5, 10 and 20  $\text{mg L}^{-1}$ ) during the exudation sampling period (3 hours). Each data point represents a single sample



total molecular features detected (18% unique for 20  $\text{mg L}^{-1}$  and 5% for control) (Fig. S5b). Volcano plots further revealed that in addition to unique features, 8% (5  $\text{mg L}^{-1}$ , Fig. S6) and 5% (20  $\text{mg L}^{-1}$ , Fig. S7) showed a significant fold change (greater than two) in intensities compared to the control treatment, rendering to 80% and 73% for 5  $\text{mg L}^{-1}$  and 20  $\text{mg L}^{-1}$ , respectively, the percentage of features with comparable intensities.

Interestingly, the plant stress assays did not show any significant response to MP exposure (Fig. 4). The concentration of root  $\text{H}_2\text{O}_2$  was analysed but results are not shown as values were below the limit of quantification (1.92  $\mu\text{M H}_2\text{O}_2$ ).

#### Experiment 3: Efficiency of Micropur to inhibit microbial activity and its effect on root exudation of soil-grown plants

Investigating the efficiency of different concentrations of MP to inhibit the decomposition of LMW exudate compounds by adding a small amount of  $^{14}\text{C}$  labelled citric acid to the exudate sampling solution, revealed that the absence of MP resulted in a statistically significant loss of about 53% of the originally added citric acid (Fig. 5) during 3 hours of exudation sampling. The addition of 1  $\text{mg L}^{-1}$  MP already resulted in a recovery of 80% of the added  $^{14}\text{C}$  citric acid, and with the use of  $>5$   $\text{mg L}^{-1}$  MP, more than 85% of the citric acid remained in the solution. Interestingly, the increase up to 20  $\text{mg L}^{-1}$  MP did not further increase citric acid recovery compared to 5  $\text{mg L}^{-1}$  MP.

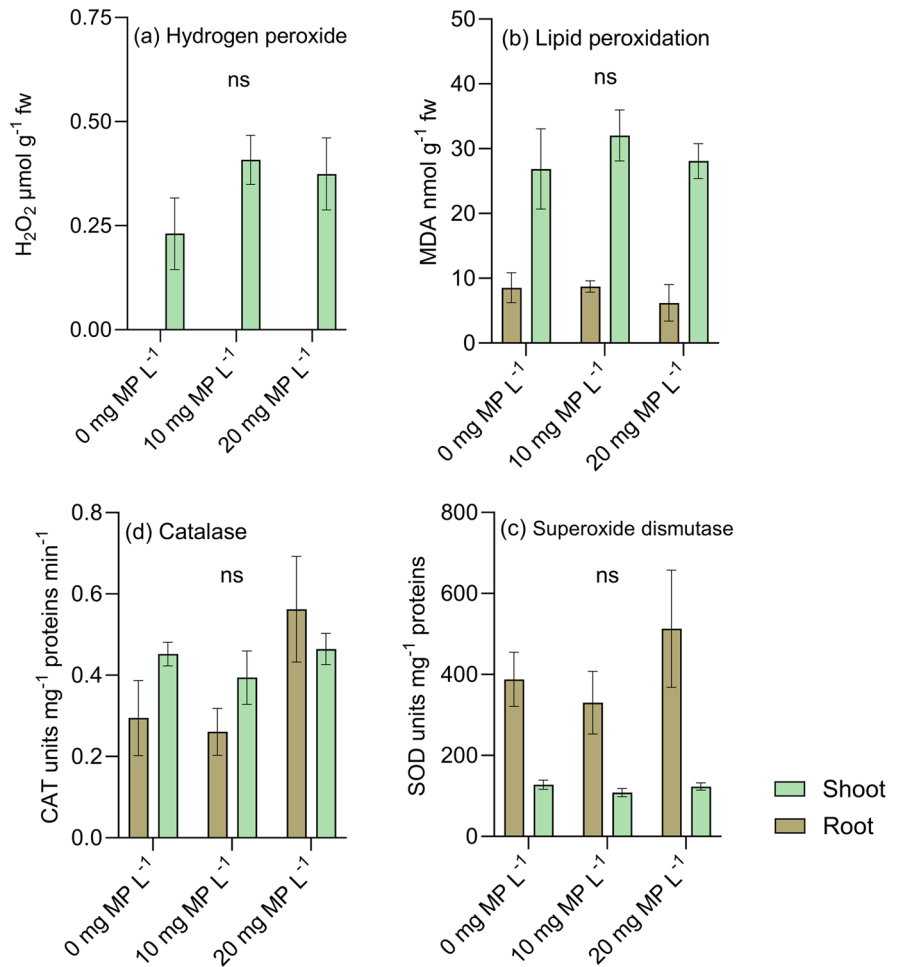
Plating and incubating small aliquots of the exudate solutions right after the sampling period onto agar allowed us to monitor the growth-inhibiting effect of MP (Fig. 6). The emergence of colony-forming units (CFU) after 24 hours of incubation decreased drastically when the sampling solution contained more than 5  $\text{mg L}^{-1}$  MP. However, even at the highest concentration tested, 20  $\text{mg L}^{-1}$  MP, a complete inhibition of microbial growth was not achieved.

Consistent with the findings from sterile grown plants (Experiment 2, Fig. 2), root exudates of soil-grown plants also showed altered C exudation rates ( $p=0.0299$ , Fig. 7) in the presence of different MP concentrations. Like in experiment 2, exudation rates were comparable when the sampling solution contained either 0, 1 or 5  $\text{mg L}^{-1}$  MP, while 10 and 20  $\text{mg L}^{-1}$  MP caused a significant increase in total C exudation rate by on average a factor of 2.0 compared to the control treatment.

#### Experiment 4: Effect of Micropur on plant root cell permeability

Testing root membrane stability on a small set of soil-grown plants after hydroponic exudation sampling with different concentrations of MP (0, 5, 10  $\text{mg L}^{-1}$ ) revealed that the membrane stability decreased significantly when exposing roots to 10  $\text{mg L}^{-1}$  MP while exposure to 5  $\text{mg L}^{-1}$  MP showed comparable results to the control treatment (Fig. 8b). As observed in the previous experiments,

**Fig. 4** Plant stress response of *Zea mays* L. roots and shoots upon exposure to different concentrations of Micropur during the hydroponic exudation sampling period (3 h). (a) Hydrogen peroxide ( $\mu\text{mol g}^{-1}$  fw). (b) lipid peroxidation stress in malondialdehyde (MDA) ( $\text{nmol g}^{-1}$  fw). (c) superoxide dismutase activity expressed in SOD units  $\text{mg}^{-1}$  of protein. (d) Catalase activity expressed in units  $\text{mg}^{-1}$  of protein  $\text{min}^{-1}$ . Values represent mean  $\pm$  SEM of 5 different biological replicates. “ns” stands for Not Statistically Significant (ANOVA,  $p < 0.05$ )



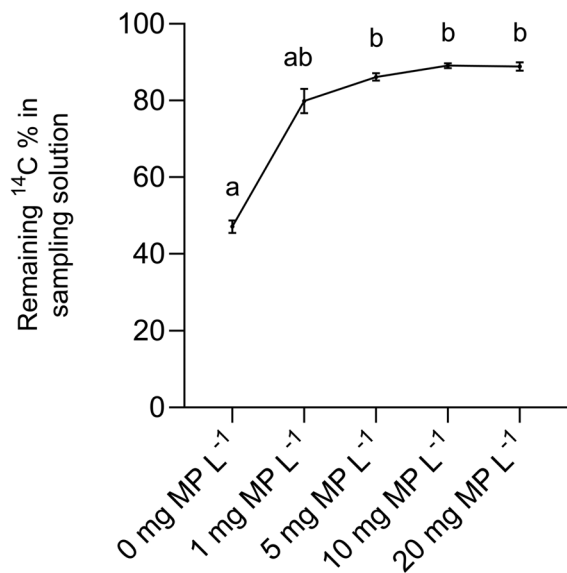
C exudation rates increased significantly in the presence of 10 mg L<sup>-1</sup> MP while remaining comparable to the control treatment at 5 mg L<sup>-1</sup> MP.

## Discussion

### Sampling solution volume affecting root exudation

Next to active secretion via membrane transporters, root exudation is also believed to be driven by diffusion due to the concentration gradient between the root cells and the surrounding soil environment (Nguyen 2003; Bertin et al. 2003; Jones et al. 2009; Canarini et al. 2019). To date direct proof of the latter is still missing, however, our results strongly suggest that passive diffusion from root cells into the surrounding environment contributes to overall

C secretion. Sampling exudates from plants with comparable root biomass (SI Fig. S1), we observed higher exudation rates when a larger sampling solution volume was used, with total C exudation rates being 2.2 times higher than when using 1000 mL rather than 130 mL (Fig. 1a). As diffusion-driven exudation depends on the loading of organic molecules in the surrounding environment, using a relatively small sampling volume can quickly lead to an accumulation of metabolites reducing diffusion with time, while the initial rate of diffusion will still be maintained in a larger sampling solution volume. Our results nicely demonstrate a decrease in dissolved organic C exudation rates with an increase in RSVR (Fig. 1b) indicating a saturation effect during the sampling period when the SV is low. This is further supported by previous findings of Jones and Darrah (1993), who reported that after a 10-day root

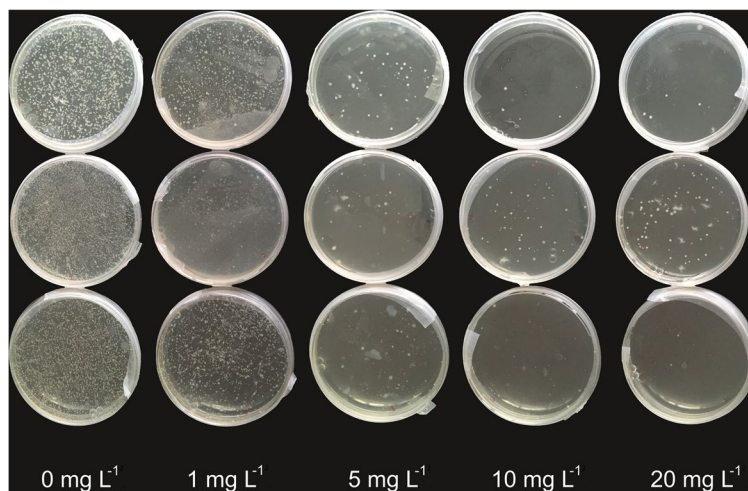


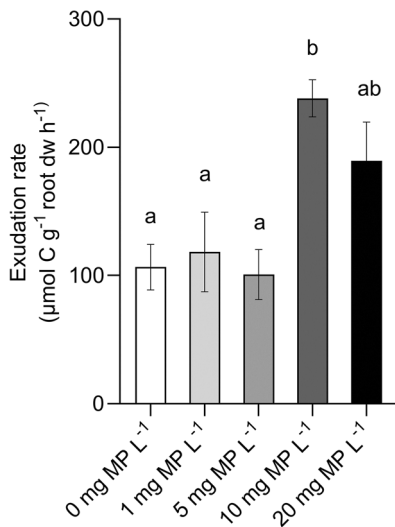
**Fig. 5** Recovery (%) of <sup>14</sup>C citric acid added to the sampling solution at the beginning of the exudate collection period after 3 hours of exudation sampling. Values represent the mean  $\pm$  SEM of 3 biological replicates. Different letters indicate significant differences according to one-way ANOVA (Tukey Post hoc test) ( $P < 0.05$ )

exudation sampling period, 8.7 times more C was retrieved from roots sampled in a solution that was daily replaced compared to when continuously keeping roots in the same solution. It is noteworthy, that re-uptake of metabolites during the exudate collection period can also occur further reducing obtained exudation rates (Jones and Darrah 1992, 1993, 1996;

Farrar et al. 2003; Lesuffleur et al. 2007; Personeni et al. 2007; Jämtgård et al. 2008; Jones et al. 2009; Svennerstam et al. 2011; Warren 2015, 2016; Uscola et al. 2017; Tiziani et al. 2020). It has been reported that a wide range of metabolites can be taken up again by the root (Warren 2015, 2016; Sasse et al. 2019), and despite its strong dependency on external concentration, the uptake of certain metabolites was found to even happen at very low external concentrations (around 0.5  $\mu$ M) (Warren 2015, 2016). Again, the overall C re-uptake is more likely to be reduced when using a larger SV as the concentration of metabolites will be lower compared to a smaller SV. Re-absorption cannot be entirely eliminated unless a non-static solution or constant rinsing of roots with fresh solution is applied. Nevertheless, employing constantly flowing solutions is methodologically more challenging compared to the use of static solutions, particularly when dealing with a large number of plants or working in the field. Furthermore, metabolite re-uptake can also be expected to occur in soil, therefore, it could be considered as part of the naturally occurring root exudation process when determining gross exudation rates. Nonetheless, it is possible to minimize the re-uptake of metabolites by keeping the sampling period short (Jones and Darrah 1993; Oburger and Jones 2018). Exposing soil-grown plants to hydroponic conditions for an extended time period (24–48 h) is also likely to alter plant metabolism, resulting in different exudation patterns than those occurring in soil (Oburger and Jones 2018).

**Fig. 6** Growth of colony forming units (CFU) on agar plate incubated for 24 hours at 30 °C after the addition of exudate sampling solutions containing different concentrations of Micropur (0, 1, 5, 10 and 20 mg L<sup>-1</sup>). Each plate represents one individual replicate from five treatments





**Fig. 7** Dissolved organic carbon (C) exudation rates ( $\mu\text{mol C g}^{-1}$  root dry weight  $\text{h}^{-1}$ ) of *Zea mays* L. cv NK Falkone of 21 days soil-grown plants. Values represent the mean  $\pm$  SEM of 3 different biological replicates. Letters represent significant differences between the investigated treatments (ANOVA, Tukey  $p < 0.05$ )

Different species, plant developmental stages and environmental growth conditions can result in a wide range of different root biomasses. For example, the same genotype can produce around 10 g root dry weight under optimal (control) conditions but only 2 g dry root biomass e.g. when the same growth substrate is contaminated with heavy metals (Zhang et al. 2019). Using a constant SV of e.g., 0.5 L would result in a RSVR of up to 20 ( $\text{g L}^{-1}$ ) for the control treatment but a RSVR of only 4 ( $\text{g L}^{-1}$ ) for the heavy metal treatment. Based on our observations (Fig. 1), the use of the same SV for both treatments would lead to an underestimation of root exudation rates under control conditions. To avoid such a bias, we therefore recommend aiming for a similar RSVR range for all investigated treatments. It is difficult to predict a plant's root weight on the day of sampling, and adapting the sampling volume on the same day is impractical (though possible) often causing a significant delay in the entire procedure. Therefore, we recommend setting up additional plants from the start (ideally one or two for each treatment), whose biomass can be assessed a day prior to the experiment. A previously estimated conversion factor (fresh to dry biomass) can then be used to estimate the dry biomass from root fresh weight. Apart from obtaining information

on root biomass, also other parameters like shoot fresh weight or dimensions of the root system can aid in fine-tuning the SV on the sampling day, especially if the root weight within one of the experiments is highly varying. Even if large SV might seem to be a simple solution to this, using large SVs typically results in very low metabolite concentrations posing a great analytical challenge, even if samples are pre-concentrated (e.g., freeze-drying). Keeping all these aspects in mind, we suggest choosing an SV that will result in an RSVR ranging between 2 and 4  $\text{g (root dw) L}^{-1}$  and keeping the exudate collection period short (2–3 hours). Our results from Experiment 1 demonstrate that within this RSVR range exudation rates are comparable (Fig. 1b), allowing for some natural variation in root biomass without introducing an experimental bias. Nevertheless, it is advisable to check obtained results for potential RSVR outliers potentially affecting obtained exudation rates.

#### Micropur as microbial activity inhibitor during root exudation sampling

It has been demonstrated that the half-life of low molecular weight root exudates in the rhizosphere is typically in the range of 2–3 hours (Gunina et al., 2017; Jones et al., 2009; Kuijken et al., 2015; Nguyen and Guckert, 2001; Oburger et al., 2009). These findings lead to the use of microbial activity inhibitors during root exudation sampling (Graham et al. 1981; Schwab et al. 1983; Hansen and Jolley 1995; Imas et al. 1997; Kuzzyakov et al. 2003; Gherardi and Rengel 2004; Gent et al. 2005; Oburger et al. 2014; Schindlegger et al. 2014; Rosenkranz et al. 2021; Heuermann et al. 2023). However, very few efforts have been made to thoroughly test their inhibitory efficacy or any negative effect they might have on the plant, and thus, on root exudation. To check the effectiveness of Micropur, radioactive citric acid was added to the sampling solution and its loss during the sampling period was quantified. After a 3-hour sampling period, about half (52.89%) of the  $^{14}\text{C}$  label initially added was lost when no Micropur was present in the exudate sampling solution (Fig. 5). Kuijken et al. (2015) incubated citrate in the solution of hydroponically grown tomato plants and obtained a comparable degradation rate (half-life time of 2.18 hours). Interestingly, Personeni et al. (2007) reported a significantly lower loss of  $1.5 \pm 1.6\%$  of  $^{14}\text{C}$ -glucose after

the incubation in root exudate solution for 3 hours. However, in this study,  $^{14}\text{C}$  glucose incubation was conducted after terminating root exudation sampling, hence in the absence of roots. Considering that the majority of microbes can be expected to be attached to the root surface, the differences in obtained results in our study, where roots were present, compared to the study of Personeni et al. (2007) are not surprising. The addition of  $5\text{ mg L}^{-1}$  MP resulted in the recovery of 86.15% of the initially added citric acid in the solution after the exudation. Even with higher concentrations, up to  $20\text{ mg MP L}^{-1}$ , we did not recover 100% of the added  $^{14}\text{C}$  from the solution. As organic acids were found not to be taken up by roots (Warren 2016), the unaccounted 13.84% of the  $^{14}\text{C}$  citric acid activity could be attributed to adsorption onto the rhizoplane or background microbial activity. Even though the formation of CFUs was significantly reduced when using  $>5\text{ mg MP L}^{-1}$ , complete inhibition of microbial growth was not achieved even at  $20\text{ mg MP L}^{-1}$  (Fig. 6). Bernards et al. (2014) also observed that microbial decomposition of phytosiderophores may happen even at  $100\text{ mg L}^{-1}$  of MP and that when using only water without MP, only a very low concentration of phytosiderophores was collected. Nevertheless, our study demonstrates that MP at the concentration of  $5\text{ mg L}^{-1}$ , is sufficient to significantly suppress microbial decomposition of low molecular weight compounds and to decrease the number of proliferating microorganisms, while an

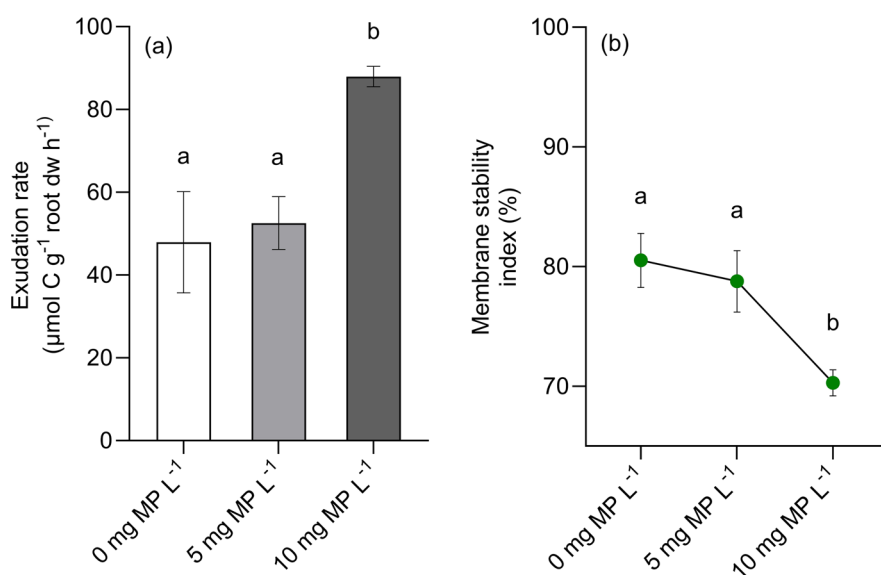
increase in MP concentration does not further inhibit microbial activity and growth.

Interestingly we found that total C exudation rates did not differ when MP concentrations between 0 and  $5\text{ mg L}^{-1}$  were applied irrespective of whether the plants were grown under sterile conditions (experiment 2, Fig. 2) or non-sterile conditions (experiment 3, Fig. 7 and experiment 4, Fig. 8a). This suggests, that despite microbial decomposition significantly affecting the recovery of individual, LMW metabolites, the overall amount of C exuded was minimally affected by microbial activity during the 3 hours exudate collection period. Consequently, when interested only in total C fluxes, a microbial activity inhibitor can potentially be omitted during the sampling process. However, our results also confirm that when interested in metabolite composition, the use of a microbial activity inhibitor is essential to avoid underestimation of metabolite-specific exudation rates.

#### Micropur affecting plant physiology

The active components of MP are silver ions ( $\text{Ag}^+$ ). Previous studies reported elevated ROS detoxification reactions (e.g. antioxidative enzymes and catalase content) in *Oryza sativa* and *Ricinus communis* when exposed to silver nanoparticles ( $30$  and  $60\text{ mg mL}^{-1}$ ) (Mirzajani et al. 2013; Yasur and Rani 2013) indicating potential negative effects of the Ag-containing MP on plant physiology. However, even

**Fig. 8** **a** Total dissolved organic carbon (C) exudation rates ( $\mu\text{mol C g}^{-1}\text{ root dw h}^{-1}$ ) and **(b)** Membrane Stability Index of roots of 4 weeks soil-grown *Zea mays* L. cv NK Falkone exposed to different concentrations of Micropure ( $0, 5$  and  $10\text{ mg L}^{-1}$  MP) during the exudate collection period (3 h). Values represent mean  $\pm$  SEM of 3 biological replicates. Letters represent significant differences between the investigated treatments (ANOVA, Tukey  $p < 0.05$ )



when exposed to the highest applied MP concentration ( $20 \text{ mg L}^{-1}$ ), our plants did not show any general stress responses (Fig. 4). This suggests that the tested MP concentration range ( $1\text{--}20 \text{ mg L}^{-1}$ ) did not negatively affect general plant metabolisms when keeping the sampling period relatively short (2–3 hours). Nevertheless, C exudation rates were still increased when exposed to MP concentrations greater than  $5 \text{ mg L}^{-1}$  in the sterile hydroponic experiment (experiment 2, Fig. 2), with C exudation being 1.86 and 3.13 times greater at 10 and  $20 \text{ mg L}^{-1}$  MP, respectively when compared to the zero MP control treatment. The same trend was also observed for soil-grown plants (Figs. 7 and 8a). As the hydroponic experiment was conducted under sterile conditions, the lower C exudation rates in the 0, 1, and  $5 \text{ mg L}^{-1}$  MP treatments cannot be attributed to microbial decomposition, hence MP ( $> 5 \text{ mg L}^{-1}$ ) must trigger increased C loss from exposed roots. Testing the effect of MP on root membrane permeability by determining the membrane stability index (MSI) revealed, that even though MP did not trigger general plant stress response reactions, MP concentration  $> 5 \text{ mg L}^{-1}$  caused a significant decline in MSI when compared to the control treatment (Fig. 8b). These results indicate that high concentrations of MP ( $> 5 \text{ mg L}^{-1}$ ), destabilize root membrane integrity, leading to an increasing C leakage to the sampling solution, and thus, to an overestimation of root exudation. Our findings are supported by reports from (Blamey et al. 2010) who observed that after 24 hours, root tips growing in  $\text{Ag}^+$  containing solutions ( $15$  to  $60 \text{ mg L}^{-1}$ ) showed ruptures in the rhizodermal and outer cortical layers of cells in the root elongation zone of cowpea seedlings. Exposure to high ( $> 5 \text{ mg L}^{-1}$ ) concentrations of MP did not only alter exudate quantity (Fig. 2) but also gradually altered the quality of exudates (Fig. 3). Valentinuzzi et al. (2015) observed that exposure of hydroponically grown white lupin to Micropur ( $1 \text{ mg L}^{-1}$ ) for 24 hours resulted in a shift of organic acid and flavonoid patterns obtained in the exudate sampling solution. However, in this study, sterility was not maintained throughout the experiment, hence both control and MP treatment could have been affected by microbial decomposition. Our experiments revealed that MP has an effect on exudation mainly in terms of quantity (Fig. 2), and it only had a minor (but still significant) impact on the quality of exuded metabolites with the number of shared molecular features

decreasing with increasing MP concentrations when compared to zero MP (Fig. 3 and Fig. S5–S8). Despite the observed changes in metabolite features with increasing MP concentration in the sampling solution, the application of  $5 \text{ mg L}^{-1}$  MP resulted in a share of 88% of detected metabolite features. Taken together, the application of  $5 \text{ mg L}^{-1}$  MP during exudation sampling allows an appropriate balance between targeting exudation profiles minimally affected by microbial decomposition and by the presence of MP itself.

## Conclusion

Although the biogeochemical importance of root exudates has been widely accepted, not enough attention has been paid to the biases potentially introduced by the selected sampling procedure. Here we showed that both, the sampling solution volume as well as the presence or absence of a microbial activity inhibitor can significantly affect obtained exudation results. We demonstrated that the root biomass to sampling solution volume ratio (RSVR) should be kept in a range between 2 and 4 g root dry weight per litre sampling solution to avoid exudation rates altered by experimental settings. Our results further revealed that Micropur is a suitable microbial activity inhibitor with  $5 \text{ mg L}^{-1}$  being the recommended application concentration. We saw that MP concentrations below  $5 \text{ mg L}^{-1}$  did not sufficiently inhibit microbial activity and growth, while concentrations above  $5 \text{ mg L}^{-1}$  MP negatively affected root membrane integrity triggering an increased loss of C from the roots. Interestingly, we found that the effect of microbial activity on total C recovered during the exudate sampling period was minimal in the absence of MP, despite detecting significant losses (52.89%) of individual LMW exudates, like citric acid. Hence, we conclude that the decision to include MP in an exudation sampling scheme will depend on the research question. When only interested in total C fluxes, MP (or any other microbial activity inhibitor) can be omitted from the sampling solution if the sampling period is kept short. Nevertheless, when exudate metabolite composition is of interest, we recommend the addition of  $5 \text{ mg L}^{-1}$  MP to the sampling solution to capture exudation profiles that are then only minimally biased by microbial decomposition

and the presence of MP itself. Taken together, this study highlights the necessity to critically assess experimental approaches and delivers relevant insights that will support informed choices when designing experiments targeting root exudates in the future.

**Acknowledgements** This work was funded by ERC Starting Grant PhytoTrace (project number 801954) and DFG, German Research Foundation (project number 403803214). We acknowledge Wieland Schweizer for helping with Experiment 1 and Mika Tarkka for the input of plant stress response assays. Further, we thank Dagmar Wobken and Stephanie A Eichorst from the University of Vienna for their discussion on this work. This work was supported by the team from The BOKU Core Facility Mass Spectrometry (CFMS).

**Authors' contribution** UOI: Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing - Original Draft. MS: Formal analysis, Data curation (non-targeted metabolomics), Writing - Review & Editing. DA: Methodology (plant stress assays), Writing - Review & Editing. SH: Resources, Writing - Review & Editing. EO: Conceptualization, Methodology, Investigation, Supervision, Funding acquisition, Writing - Review & Editing. All authors contributed to the article and approved the submitted version.

**Funding** Open access funding provided by University of Natural Resources and Life Sciences Vienna (BOKU).

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

## References

- Aebi H (1984) Catalase in vitro. *Meth Enzymol* 105:121–126. [https://doi.org/10.1016/S0076-6879\(84\)05016-3](https://doi.org/10.1016/S0076-6879(84)05016-3)
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399. <https://doi.org/10.1146/annurev.arplant.55.031903.141701>
- Aulakh MS, Wassmann R, Bueno C et al (2001) Characterization of root exudates at different growth stages of ten Rice (*Oryza sativa* L.) cultivars. *Plant Biol* 3:139–148. <https://doi.org/10.1055/s-2001-12905>
- Baes AU, Bloom PR (1990) Fulvic acid ultraviolet-visible spectra: influence of solvent and pH. *Soil Sci Soc* 54. <https://doi.org/10.2136/sssaj1990.03615995005400050008x>
- Bernards ML, Buxton EA, Jolley VD et al (2014) Maize hybrids differ in their 24-H patterns of Phytosiderophore release. *J Plant Nutr* 37:575–594. <https://doi.org/10.1080/01904167.2013.867984>
- Bertin C, Yang X, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256:67–83. <https://doi.org/10.1023/A:1026290508166>
- Beyer WF, Fridovich I (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal Biochem* 161:559–566. [https://doi.org/10.1016/0003-2697\(87\)90489-1](https://doi.org/10.1016/0003-2697(87)90489-1)
- Blamey FPC, Kopittke PM, Wehr JB et al (2010) Rhizotoxic effects of silver in cowpea seedlings. *Environ Toxicol Chem* 29:2072–2078. <https://doi.org/10.1002/etc.236>
- Brandstetter A, Sletten RS, Mentler A, Wenzel WW (1996) Estimating dissolved organic carbon in natural waters by UV absorbance (254 nm). *J Plant Nutr Soil Sci* 159:605–607. 0044–3263/19610612-0605 \$10.00 + ,2510
- Buckner B, Johal GS, Janick-Buckner D (2000) Cell death in maize. *Physiol Plant* 108:231–239. <https://doi.org/10.1034/j.1399-3054.2000.108003231.x>
- Canarini A, Merchant A, Dijkstra FA (2016) Drought effects on *Helianthus annuus* and *Glycine max* metabolites: from phloem to root exudates. *Rhizosphere* 2:85–97. <https://doi.org/10.1016/j.rhisph.2016.06.003>
- Canarini A, Kaiser C, Merchant A et al (2019) Root exudation of primary metabolites: mechanisms and their roles in plant responses to environmental stimuli. *Front Plant Sci* 10. <https://doi.org/10.3389/fpls.2019.00157>
- Carvalho LC, Dennis PG, Fedoseyenko D et al (2011) Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *J Plant Nutr Soil Sci* 174:3–11. <https://doi.org/10.1002/jpln.201000085>
- Dietrich M, Montesinos-Navarro A, Gabriel R et al (2022) Both abundant and rare fungi colonizing *Fagus sylvatica* ectomycorrhizal root-tips shape associated bacterial communities. *Commun Biol* 5:1261. <https://doi.org/10.1038/s42003-022-04178-y>
- Dix TA, Aikens J (1993) Mechanisms and biological relevance of lipid peroxidation initiation. *Chem Res Toxicol* 6:2–18. <https://doi.org/10.1021/tx00031a001>
- Farrar J, Hawes M, Jones D, Lindow S (2003) How roots control the flux of carbon to the rhizosphere. *Ecology* 84:827–837. [https://doi.org/10.1890/0012-9658\(2003\)084\[0827:HRCTFO\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2003)084[0827:HRCTFO]2.0.CO;2)
- Gent MPN, Parrish ZD, White JC (2005) Nutrient uptake among subspecies of *Cucurbita pepo* L. is related to exudation of citric acid. *J Am Soc Hortic Sci* 130:782–788. <https://doi.org/10.21273/JASHS.130.5.782>
- Gherardi MJ, Rengel Z (2004) The effect of manganese supply on exudation of carboxylates by roots of lucerne



- (*Medicago sativa*). *Plant Soil* 260:271–282. <https://doi.org/10.1023/B:PLSO.0000030182.11473.3b>
- Gibała A, Zeliszevska P, Gosiewski T et al (2021) Antibacterial and antifungal properties of silver nanoparticles—effect of a surface-stabilizing agent. *Biomolecules* 11:1481. <https://doi.org/10.3390/biom11101481>
- Graham JH, Leonard RT, Menge JA (1981) Membrane-mediated decrease in root exudation responsible for Phosphorus inhibition of vesicular-arbuscular mycorrhiza formation. *Plant Physiol* 68:548–552
- Grayston SJ, Vaughan D, Jones D (1997) Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. *Appl Soil Ecol* 5:29–56. [https://doi.org/10.1016/S0929-1393\(96\)00126-6](https://doi.org/10.1016/S0929-1393(96)00126-6)
- Groleau-Renaud V, Plantureux S, Guckert A (1998) Influence of plant morphology on root exudation of maize subjected to mechanical impedance in hydroponic conditions. *Plant Soil* 201:231–239. <https://doi.org/10.1023/A:1004316416034>
- Gunina A, Smith AR, Kuzyakov Y, Jones DL (2017) Microbial uptake and utilization of low molecular weight organic substrates in soil depend on carbon oxidation state. *Biogeochemistry* 133:89–100. <https://doi.org/10.1007/s10533-017-0313-1>
- Hansen NC, Jolley VD (1995) Phytosiderophore release as a criterion for genotypic evaluation of iron efficiency in oat. *J Plant Nutr* 18:455–465. <https://doi.org/10.1080/01904169509364915>
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. *Arch Biochem Biophys* 125:189–198. [https://doi.org/10.1016/0003-9861\(68\)90654-1](https://doi.org/10.1016/0003-9861(68)90654-1)
- Heuermann D, Döll S, Schweneker D et al (2023) Distinct metabolite classes in root exudates are indicative for field- or hydroponically-grown cover crops. *Front Plant Sci* 14. <https://doi.org/10.3389/fpls.2023.1122285>
- Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. *Circ Calif Agric Exp Station* 347. <https://ia801405.us.archive.org/24/items/waterculturemeth347hoag/waterculturemeth347hoag.pdf>
- Imas P, Bar-Yosef B, Kafkafi U, Ganmore-Neumann R (1997) Release of carboxylic anions and protons by tomato roots in response to ammonium nitrate ratio and pH in nutrient solution. *Plant Soil* 191:27–34. <https://doi.org/10.1023/A:1004214814504>
- Jämtgård S, Näsholm T, Huss-Danell K (2008) Characteristics of amino acid uptake in barley. *Plant Soil* 302:221–231. <https://doi.org/10.1007/s11104-007-9473-4>
- Jones A (2000) Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? *Trends Plant Sci* 5:225–230. [https://doi.org/10.1016/S1360-1385\(00\)01605-8](https://doi.org/10.1016/S1360-1385(00)01605-8)
- Jones DL, Darrah PR (1992) Re-sorption of organic components by roots of *Zea mays* L. And its consequences in the rhizosphere. *Plant Soil* 143:259–266. <https://doi.org/10.1007/BF00007881>
- Jones DL, Darrah PR (1993) Re-sorption of organic compounds by roots of *Zea mays* L. and its consequences in the rhizosphere - II. Experimental and model evidence for simultaneous exudation and re-sorption of soluble C compounds. *Plant Soil* 153:47–59. <https://doi.org/10.1007/BF00010543>
- Jones DL, Darrah PR (1996) Re-sorption of organic compounds by roots of *Zea mays* L. and its consequences in the rhizosphere. III. Characteristics of sugar influx and efflux. *Plant Soil* 178:153–160. <https://doi.org/10.1007/BF00011173>
- Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. *New Phytol* 163:459–480. <https://doi.org/10.1111/j.1469-8137.2004.01130.x>
- Jones DL, Nguyen C, Finlay RD (2009) Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant Soil* 321:5–33. <https://doi.org/10.1007/s11104-009-9925-0>
- Kawasaki A, Okada S, Zhang C et al (2018) A sterile hydroponic system for characterising root exudates from specific root types and whole-root systems of large crop plants. *Plant Methods* 14:1–13. <https://doi.org/10.1186/s13007-018-0380-x>
- Krafczyk I, Trolldenier G, Beringer H (1984) Soluble root exudates of maize: influence of potassium supply and rhizosphere microorganisms. *Soil Biol Biochem* 16:315–322. [https://doi.org/10.1016/0038-0717\(84\)90025-7](https://doi.org/10.1016/0038-0717(84)90025-7)
- Kuijken RCP, Snel JFH, Heddes MM et al (2015) The importance of a sterile rhizosphere when phenotyping for root exudation. *Plant Soil* 387:131–142. <https://doi.org/10.1007/s11104-014-2283-6>
- Kuzyakov Y, Jones DL (2006) Glucose uptake by maize roots and its transformation in the rhizosphere. *Soil Biol Biochem* 38:851–860. <https://doi.org/10.1016/j.soilbio.2005.07.012>
- Kuzyakov Y, Raskatov A, Kaupenjohann M (2003) Turnover and distribution of root exudates of *Zea mays*. *Plant Soil* 254:317–327. <https://doi.org/10.1023/A:1025515708093>
- Lesuffleur F, Cliquet JB (2010) Characterisation of root amino acid exudation in white clover (*Trifolium repens* L.). *Plant Soil* 333:191–201. <https://doi.org/10.1007/s11104-010-0334-1>
- Lesuffleur F, Paynel F, Bataillé MP et al (2007) Root amino acid exudation: measurement of high efflux rates of glycine and serine from six different plant species. *Plant Soil* 294:235–246. <https://doi.org/10.1007/s11104-007-9249-x>
- Lohse M, Santangeli M, Steininger-Mairinger T et al (2023) The effect of root hairs on exudate composition: a comparative non-targeted metabolomics approach. *Anal Bioanal Chem* 415:823–840. <https://doi.org/10.1007/s00216-022-04475-9>
- Ma J, Wang W, Yang J et al (2022) Mycorrhizal symbiosis promotes the nutrient content accumulation and affects the root exudates in maize. *BMC Plant Biol* 22:64. <https://doi.org/10.1186/s12870-021-03370-2>
- Mangelsen E, Kilian J, Harter K et al (2011) Transcriptome analysis of high-temperature stress in developing barley caryopses: early stress responses and effects on storage compound biosynthesis. *Mol Plant* 4:97–115. <https://doi.org/10.1093/mp/ssq058>
- McLaughlin S, Zhalnina K, Kosina S et al (2023) The core metabolome and root exudation dynamics of three phylogenetically distinct plant species. *Nat Commun* 14:1649. <https://doi.org/10.1038/s41467-023-37164-x>
- Mirzajani F, Askari H, Hamzelou S et al (2013) Effect of silver nanoparticles on *Oryza sativa* L. and its rhizosphere bacteria. *Ecotoxicol Environ Saf* 88:48–54. <https://doi.org/10.1016/j.ecoenv.2012.10.018>
- Neumann G, George TS, Plassard C (2009) Strategies and methods for studying the rhizosphere—the plant science toolbox. *Plant Soil* 321:431–456. <https://doi.org/10.1007/s11104-009-9953-9>

- Nguyen C (2003) Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie* 23:375–396. <https://doi.org/10.1051/agro:2003011>
- Nguyen C, Guckert A (2001) Short-term utilisation of  $^{14}\text{C}$ -[U] glucose by soil microorganisms in relation to carbon availability. *Soil Biol Biochem* 33:53–60. [https://doi.org/10.1016/S0038-0717\(00\)00114-0](https://doi.org/10.1016/S0038-0717(00)00114-0)
- Oburger E, Jones DL (2018) Sampling root exudates – Mission impossible? *Rhizosphere* 6:116–133. <https://doi.org/10.1016/j.rhisph.2018.06.004>
- Oburger E, Kirk GJD, Wenzel WW et al (2009) Interactive effects of organic acids in the rhizosphere. *Soil Biol Biochem* 41:449–457. <https://doi.org/10.1016/j.soilbio.2008.10.034>
- Oburger E, Dell'mour M, Hann S et al (2013) Evaluation of a novel tool for sampling root exudates from soil-grown plants compared to conventional techniques. *Environ Exp Bot* 87:235–247. <https://doi.org/10.1016/j.envexpbot.2012.11.007>
- Oburger E, Gruber B, Schindlegger Y et al (2014) Root exudation of phytosiderophores from soil-grown wheat. *New Phytol* 203:1161–1174. <https://doi.org/10.1111/nph.12868>
- Oburger E, Staudinger C, Spiridon A et al (2022) A quick and simple spectrophotometric method to determine total carbon concentrations in root exudate samples of grass species. *Plant Soil* 478. <https://doi.org/10.1007/s11104-022-05519-w>
- Pausch J, Kuzyakov Y (2018) Carbon input by roots into the soil: quantification of rhizodeposition from root to ecosystem scale. *Glob Chang Biol* 24:1–12. <https://doi.org/10.1111/gcb.13850>
- Personeni E, Nguyen C, Marchal P, Pagès L (2007) Experimental evaluation of an efflux-influx model of C exudation by individual apical root segments. *J Exp Bot* 58:2091–2099. <https://doi.org/10.1093/jxb/erm065>
- Phillips RP, Erlitz Y, Bier R, Bernhardt ES (2008) New approach for capturing soluble root exudates in forest soils. *Funct Ecol* 22:990–999. <https://doi.org/10.1111/j.1365-2435.2008.01495.x>
- Rangel-Castro JJ, Killham K, Ostle N et al (2005) Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. *Environ Microbiol*. <https://doi.org/10.1111/j.1462-2920.2005.00756.x>
- Rosenkranz T, Oburger E, Baune M et al (2021) Root exudation of coumarins from soil-grown *Arabidopsis thaliana* in response to iron deficiency. *Rhizosphere* 17:100296. <https://doi.org/10.1016/j.rhisph.2020.100296>
- Sairam RK, Deshmukh PS, Shukla DS (1997) Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. *J Agron Crop Sci* 178:171–178. <https://doi.org/10.1111/j.1439-037X.1997.tb00486.x>
- Santangeli M, Steininger-Mairinger T, Vetterlein D et al (2024) Maize (*Zea mays* L.) root exudation profiles change in quality and quantity during plant development – a field study. *Plant Sci* 338:111896. <https://doi.org/10.1016/j.plantsci.2023.111896>
- Sasse J, Kant J, Cole BJ et al (2019) Multilab EcoFAB study shows highly reproducible physiology and depletion of soil metabolites by a model grass. *New Phytol* 222:1149–1160. <https://doi.org/10.1111/nph.15662>
- Schindlegger Y, Oburger E, Gruber B et al (2014) Accurate LC-ESI-MS/MS quantification of 2'-deoxymugineic acid in soil and root related samples employing porous graphitic carbon as stationary phase and a  $^{13}\text{C}$ -labeled internal standard. *Electrophoresis* 35:1375–1385. <https://doi.org/10.1002/elps.201300551>
- Schmidt H, Eickhorst T (2013) Detection and quantification of native microbial populations on soil-grown rice roots by catalyzed reporter deposition-fluorescence in situ hybridization. *FEMS Microbiol Ecol*. <https://doi.org/10.1111/1574-6941.12232>
- Schwab SM, Menge JA, Leonard RT (1983) Quantitative and qualitative effects of phosphorus on extracts and exudates of sudangrass roots in relation to vesicular-arbuscular mycorrhiza formation. *Plant Physiol* 73:761–765. <https://doi.org/10.1104/pp.73.3.761>
- Sergiev L, Alexieva E, Karanov E (1997) Effect of spermine, atrazine and combination between them on some endogenous protective systems and markers in plants. *Compt Rend Acad BulgSci* 51:121–124
- Svennerstam H, Jämtgård S, Ahmad I et al (2011) Transporters in *Arabidopsis* roots mediating uptake of amino acids at naturally occurring concentrations. *New Phytol* 191:459–467. <https://doi.org/10.1111/j.1469-8137.2011.03699.x>
- Tiziani R, Pii Y, Celletti S et al (2020) Phosphorus deficiency changes carbon isotope fractionation and triggers exudate reacquisition in tomato plants. *Sci Rep* 10:1–12. <https://doi.org/10.1038/s41598-020-72904-9>
- Todorovic C, Nguyen C, Robin C, Guckert A (2001) Root and microbial involvement in the kinetics of  $^{14}\text{C}$ -partitioning to rhizosphere respiration after a pulse labelling of maize assimilates. *Plant Soil* 228:179–189. <https://doi.org/10.1023/A:1004830011382>
- Tomasi N, De Nobili M, Gottardi S et al (2012) Physiological and molecular characterization of Fe acquisition by tomato plants from natural Fe complexes. *Biol Fertil Soils* 49:187–200. <https://doi.org/10.1007/s00374-012-0706-1>
- Uscola M, Villar-Salvador P, Oliet J, Warren C (2017) Root uptake of inorganic and organic N chemical forms in two coexisting Mediterranean forest trees. *Plant Soil* 415. <https://doi.org/10.1007/s11104-017-3172-6>
- Valentinuzzi F, Cesco S, Tomasi N, Mimmo T (2015) Influence of different trap solutions on the determination of root exudates in *Lupinus albus* L. *Biol Fertil Soils* 51:757–765. <https://doi.org/10.1007/s00374-015-1015-2>
- Vetterlein D, Lippold E, Schreiter S et al (2021) Experimental platforms for the investigation of spatiotemporal patterns in the rhizosphere—laboratory and field scale. *J Plant Nutr Soil Sci* 184:35–50. <https://doi.org/10.1002/jpln.202000079>
- Vranova V, Rejsek K, Skene KR et al (2013) Methods of collection of plant root exudates in relation to plant metabolism and purpose: a review. *J Plant Nutr Soil Sci* 176:175–199. <https://doi.org/10.1002/jpln.201000360>
- Warren CR (2015) Wheat roots efflux a diverse array of organic N compounds and are highly proficient at their

- recapture. *Plant Soil* 397:147–162. <https://doi.org/10.1007/s11104-015-2612-4>
- Warren CR (2016) Simultaneous efflux and uptake of metabolites by roots of wheat. *Plant Soil* 406:359–374. <https://doi.org/10.1007/s11104-016-2892-3>
- Williams A, Langridge H, Straathof AL et al (2021) Comparing root exudate collection techniques: an improved hybrid method. *Soil Biol Biochem* 161:108391. <https://doi.org/10.1016/j.soilbio.2021.108391>
- Yasur J, Rani PU (2013) Environmental effects of nanosilver: impact on castor seed germination, seedling growth, and plant physiology. *Environ Sci Pollut Res* 20:8636–8648. <https://doi.org/10.1007/s11356-013-1798-3>
- Zhang F, Liu M, Li Y et al (2019) Effects of arbuscular mycorrhizal fungi, biochar and cadmium on the yield and element uptake of *Medicago sativa*. *Sci Total Environ* 655:1150–1158. <https://doi.org/10.1016/j.scitotenv.2018.11.317>
- Zwetsloot MJ, Kessler A, Bauerle TL (2018) Phenolic root exudate and tissue compounds vary widely among temperate forest tree species and have contrasting effects on soil microbial respiration. *New Phytol* 218:530–541. <https://doi.org/10.1111/nph.15041>

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.