Research paper

Enhanced root exudation of mature broadleaf and conifer trees in a Mediterranean forest during the dry season

Gilad Jakoby[1], Ido Rog [ID][1], Shacham Megidish[1] and Tamir Klein [ID][1,2]

[1]Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot 76100, Israel; [2]Corresponding author (tamir.klein@weizmann.ac.il)

Root exudates are part of the rhizodeposition process, which is the major source of soil organic carbon (C) released by plant roots. This flux of C is believed to have profound effects on C and nutrient cycling in ecosystems. The quantity of root exudates depends on the plant species, the period throughout the year, and external biotic and abiotic factors. Since root exudates of mature trees are difficult to collect in field conditions, very little is known about their flux, especially in water-limited ecosystems, such as the seasonally hot and dry Mediterranean maquis. Here, we collected exudates from DNA-identified roots in the forest from the gymnosperm *Cupressus sempervirens L.* and the evergreen angiosperm *Pistacia lentiscus L.* by 48-h incubations on a monthly temporal resolution throughout the year. We examined relationships of the root exudate C flux to abiotic parameters of the soil (water content, water potential, temperature) and atmosphere (vapor pressure deficit, temperature). We also studied relationships to C fluxes through the leaves as indicators of tree C balance. Root exudation rates varied significantly along the year, increasing from 6 µg C cm^{-2} root day^{-1} in both species in the wet season to 4- and 11-fold rates in *Pistacia* and *Cupressus*, respectively, in the dry season. A stepwise linear mixed-effects model showed that the three soil parameters were the most influential on exudation rates. Among biotic factors, there was a significant negative correlation of exudation rate with leaf assimilation in *Cupressus* and a significant negative correlation with leaf respiration in *Pistacia*. Our observation of enhanced exudation flux during the dry season indicates that exudation dynamics in the field are less sensitive to the low tree C availability in the dry season. The two key Mediterranean forest species seem to respond to seasonal changes in the rhizosphere such as drying and warming, and therefore invest C in the rhizosphere under seasonal drought.

Keywords: carbon balance, rhizosphere, root exudates, root secretion, total organic carbon.

Introduction

The plant root system has major essential functions, such as water and nutrient uptake, and anchorage in soils. In order to succeed in these tasks, the root interacts with its immediate soil environment, the rhizosphere (Neumann and Römheld 2007). One of the major processes facilitating interaction in the rhizosphere is rhizodeposition—the release of a variety of compounds into the rhizosphere by plant roots. These compounds originate from lysates of sloughed-off root cap and border cells, as well as compounds secreted as root exudates in passive and active ways from intact root tissues (van Dam and Bouwmeester 2016).

Through root exudation, a part of the carbon (C) that a plant assimilates by photosynthesis is transferred to the soil. This flux of C is believed to have profound effects on C and nutrient cycling in ecosystems. The estimated amount of C that is being released by root exudates is 5–21% of all C allocated into fine roots (Haichar et al. 2014), and can comprise up to 10% of net primary productivity in forests (Kannenberg and Phillips 2017). This flux is influenced by many factors, including plant species and age, and environmental conditions such as light

intensity, temperature, water availability and soil characteristics (Neumann and Römheld 2007, Meier et al. 2013, Vranova et al. 2013).

The development of a relatively easy and cheap method to collect and assess directly the flux of root exudates in the natural environment, particularly from mature trees, opened a new opportunity to better understand and quantify the process of rhizodeposition (Phillips et al. 2008). One of the advantages of this flux measurement is the potential to close the fundamental knowledge gap of tree and forest C balance research. The lack of a precise measurement of root exudates has caused researchers to use only an estimation of this flux. For example, Klein and Hoch (2015) constructed a full description of tree C allocation dynamics throughout a year in *Pinus halepensis*, using an indirect measurement of average annual belowground increase in soil C. The latter involves a number of other factors that do not represent the absolute amount of C released by the root such as organic matter from dead roots, leaf litter and microorganisms, calling for direct measurement of root exudation rate.

In recent years, the number of studies using this direct measurement has increased (Phillips et al. 2008, 2011, Brzostek et al. 2013, Yin et al. 2014, Tückmantel et al. 2017, Sun, Ataka, *et al.* 2017, Sun, Kominomi, et al. 2017, Nakayama and Tateno 2018). However, most studies focused on specific times throughout the year. For example, sampling during the growing season or during several months (Brzostek et al. 2013, Yin et al. 2014, Tückmantel et al. 2017, Sun, *et al.* 2017a, 2017b, Nakayama and Tateno 2018). An exception was Phillips et al. (2008), and (Phillips et al. 2011), who performed annual resolution (i.e., every 4–8 weeks throughout the year) and reported some seasonality in the quantity of root exudates produced by loblolly pine.

Climate change, such as warming and reduced precipitation, as predicted for the Mediterranean basin during the current century, could affect the C dynamics in dry regions and probably root exudation flux (Grünzweig et al. 2009, Ahlström et al. 2015). Preece and Peñuelas (2016) summarized drought studies that measured root exudation, mostly in controlled environments, and suggested that a low to moderate drought increases exudation, but this effect is more variable under extreme drought. The studies that do collect and assess root exudates in the natural environment are conducted in temperate ecosystems, with no representation to Mediterranean and semi-arid ecosystems. Rotenberg and Yakir (2010) reported that the time of gross primary productivity (GPP) peak is shifted from summer (July and August) in European temperate forests to early spring (March) in Mediterranean and semi-arid forests. However, it is unknown whether belowground activity of plants follows this aboveground shift.

Finally, it is known that trees release part of the C assimilated by photosynthesis through root exudation in order to control and shape the rhizosphere (Meier et al. 2013). The factors affecting the quantity of root exudates are determined by a combination of interacting endogenous and exogenous factors. Due to the complexity of this phenomenon and the numerous factors affecting it, it has been so far difficult to study. The objective of this research was to study the seasonal dynamics of organic C secreted by tree roots along a year and test how biotic and abiotic factors affect this flux. In the East Mediterranean forest, which is routinely exposed to seasonal drought (April to September), tree physiological activities are mostly synchronized with the wet season (October to March) in conifers and broadleaf species alike (Klein et al. 2013, Klein, et al. 2016a, 2016b, 2016C). Therefore, we hypothesized that root exudation should increase seasonally with soil moisture, also matching the higher tree C availability associated with it.

Materials and methods

Field study site and growth conditions

Field measurements of mature trees were conducted in a forest research plot in Harel forest, at the Judean foothills, Israel (Klein et al. 2013, Lapidot et al. 2019). Harel forest is located 4 km south-west of Beit Shemesh, Israel (31° 43′N, 34° 57′E, 320 m elevation) (Figure S1 available as Supplementary Data at *Tree Physiology* Online). The vegetation is dominated by the planted gymnosperm tree species *P. halepensis* and *Cupressus sempervirens*, and with the local Mediterranean angiosperm woody species *Quercus calliprinos*, *Ceratonia siliqua* and *Pistacia lentiscus*, accompanied by a variety of annual plants that thrive from winter to spring. In Harel forest, conifers were planted ∼45 years ago, with broadleaf species populating the afforestation soon after the conifer planting. The forest canopy is dominated by the relatively tall conifers, and especially the pines, with lower stature oak and carob, and *Pistacia* forming a forest understory (Table S1 available as Supplementary Data at *Tree Physiology* Online). The meteorological information was provided by the Israel Meteorological Service (IMS). Air temperature and relative humidity were measured at standard height of 2 m above ground at Beit Jimal weather station, located 1.4 km east of our measured forest plot and at the same elevation. The weather station provides 10-min averaged observations. Fifteen-year average minimum, mean and maximum temperature of the hottest month (August) were 19.5, 25.3 and 31.0 °C, respectively; average minimum, mean and maximum temperature of the coolest month (February) were 11.5, 16.0 and 20.5 °C, respectively. Thirty-year average annual precipitation is 509 mm, restricted to the period from September to May. Temperature and air humidity data were further used to calculate the vapor pressure deficit (VPD; as outlined in Lapidot et al. 2019). Soil water content (%, v/v) and soil water potential (MPa) were measured using a dielectric constant EC-5 soil moisture sensor and an MPS-6 soil water potential sensor, respectively, connected to an Em50 data logger (Decagon Devices Inc., Pullman,

WA, USA) which was programed to record observations at 30-min intervals. The sensors were located in two places in the center of the measured forest plot at two depths: 5 and 25 cm below ground surface. Over 95% of root measurements were performed in between these depths. The predominant soil type in the study area was terra rosa with neutral acidity (pH 6.9–7.2), consisting of an A horizon of 20.6 ± 0.7 cm soil depth and then C horizon which consists of soil that penetrates the cracks between the weathered limestone bedrock.

Study trees

Within the 1500 m^2 forest research plot, we identified four to five representative trees of each of the five studied species (Table S1 *available as Supplementary Data at* Tree Physiology *Online*). All measurements were performed repeatedly along the year around the trunk of these individuals, and tree species was verified by DNA barcoding for each sample, with misidentified roots being discarded from the analysis. We note that, while our original goal was to study the roots of all five tree species, root identification results (below) showed that the majority of accessible roots were belonging to the coniferous *C. sempervirens* and the broadleaf *P. lentiscus*, and hence the analysis was restricted to these two species.

Root exudate collection

Root exudates were collected from intact lateral fine roots (one to two roots per individual tree) using a non-soil syringe system modified from Phillips et al. (2008). Measurements were performed along 18 campaigns on different roots at each sampling, around the same four individual trees per species. Root tips with 0.5–4 mm diameter from the 5–30 cm of the A horizon were sampled. They remained attached to the target trees during the entire procedure until harvest. The fine roots were carefully placed into a paper bag filled with soil and were reburied. The reburial was intended to allow additional time for roots to recover from any potential injury or stress sustained during the excavation and rinsing process. After 24 h, we gently washed the intact fine roots with a spray bottle, using autoclaved C-free nutrient solution (0.5 mM NH_4NO_3, 0.1 mM KH_2PO_4, 0.2 mM K_2SO_4, 0.2 mM $MgSO_4$, 0.3 mM $CaCl_2$) and fine forceps to remove soil particles and other possible contaminants, and then the fine roots were placed into a 20 ml sterile plastic syringe and filled with 0.5–1.3 mm acid-washed glass beads and 10 ml autoclaved C-free nutrient solution. Then, the syringes were covered with aluminum foil and leaf litter to block sunlight and heat. After 48 h, the nutrient solution was collected from each syringe system. An additional 10 ml of the double distilled water was flushed through the syringe system to obtain a representative C recovery. One control per sample was included as the same process without a root. All solutions were filtered immediately through a 0.22 μm sterile

syringe filter (Millex PVDF, Millipore Co., Billerica, MA, USA) in the field and stored in the lab at -20 °C until analysis. The solutions were analyzed for dissolved organic C on a total organic carbon analyzer (Shimadzu VCPH—Carbon and Nitrogen analyzer, Kyoto, Japan). Root exudation rates were calculated as the total amount of C flushed from each root system over the incubation period, divided by root surface area of the investigated root strand, and hereafter referred to as specific exudation rate (μg C m^{-2} day^{-1}). After root exudate collection, roots were cut off the tree and stored at 4 °C for several days up to 1 week until processing. Root surface area was measured by flatbed scanner (HP color jet pro MFP m477fdn) and with ImageJ analysis software. Subsequently, root biomass was determined by oven drying (48 h, 60 °C) and weighing.

Testing the parameters of the root exudation collection method

We examined the degree to which root morphological variables, namely, root surface area and root dry weight, predicted exudation rate. There was a positive correlation between the two morphological variables ($R^2 = 0.88$, $P < 0.001$ and $R^2 = 0.69$, $P < 0.001$, for *C. sempervirens* and *P. lentiscus*, respectively) (Figure S2 available as Supplementary Data at *Tree Physiology* Online). Then, we found that exudation rate was well predicted by root surface area ($R^2 = 0.48$, $P < 0.001$; $R^2 = 0.3$, $P < 0.001$, for *C. sempervirens* and *P. lentiscus*, respectively) and, to lesser extent, by root dry weight ($R^2 = 0.37$, $P < 0.001$; $R^2 = 0.3$, $P < 0.001$, for *C. sempervirens* and *P. lentiscus*, respectively). These relationships were significant also when considering the wet and dry season values separately (Figure S3 available as Supplementary Data at *Tree Physiology* Online).

DNA extraction, amplification, sequencing and sequence analysis

In order to ensure the identification of the species, root samples were taken for DNA identification based on the rRNA second internal transcribed spacer 2 (ITS2) sequence. Identification via tracking the root from tip to its stem of origin was avoided in order to minimize damage to the stand by recurrent campaigns. DNA extraction was based on the method described in Wang et al. (1993) and was modified to woody plant species. Briefly, 400 μl 0.5 N NaOH +1.5% polyvinylpyrrolidone (PVP) was added to 3–4 mg of dried ground root sample. The samples were vortexed and incubated for 10 min at 60 °C, inverted gently every 150 s. Next, samples were centrifuged for 5 min at 20,000 relative centrifugal force. From each sample, 2 μl supernatant was transferred to 150 μl 100 mM Tris HCl solution (pH 8.0) and mixed well. Then 2 μl of each sample was

used for PCR amplification. PCRs were performed using 2X PCRBIO HS Taq Mix Red (PB10.23–02) in a total volume of 30 µl with the following program: 95 °C for 30 s, 56 °C for 40 s, 72 °C for 60 s; 35 cycles. The primer sequences ITS-p5 (5′-CCTTATCATTTAGAGGAAGGAG-3′) and ITS-u4 (5′-GGTTTCTTTTCCTCCGCTTA-3′) were used, based on Cheng et al. (2016). Purified amplicons were sequenced in both directions with the above primers using Sanger sequencing method on a 3730 DNA analyzer sequencer (Applied Biosystems, Foster City, CA, USA). Contig assembly and the generation of consensus sequences were performed using Sequencing Analysis Software v5.3 (Applied Biosystems). Full ITS sequences were subjected to Hidden Markov model (HMM; Keller et al. 2009) to remove the conserved 5.8S and 28S sequences and retain the correct position of the ITS2 within the full ITS sequence (Koetschan et al. 2012). Species identification was evaluated with BLAST1 method (Ross et al. 2008) on three different parts of the ITS sequence: full ITS sequence, primary structure of the ITS2 and secondary structure prediction of the ITS2 using tools from the ITS2 database (Schultz et al. 2006, Koetschan et al. 2012).

Leaf gas exchange

Leaf CO_2 respiration and assimilation rates were measured on leaves of the same trees measured for root exudation rates; within 1–10 days from the day of exudation collection, each measurement day is an average of three samplings during the day: morning, noon and afternoon. These measurements were performed using a leaf gas exchange system equipped with an infrared gas analyzer (IRGA; Walz, Effeltrich, Germany). Leaves were dark adapted for 30 min, and a single mature intact leaf was inserted into a larger measurement chamber. A standard leaf chamber (Walz 3010-S) with top LED light source (Walz 3040-L) were set to zero light. The CO_2 level was set to provide a stable concentration of 400 p.p.m.; flow rate was set to 750 µmol s^{-1} and the impeller to speed 7; the temperature was set at a level matching the outside conditions ±1 °C. In each measurement, a single intact mature leaf from the *Pistacia*, or a needle cohort of 10 adult needles from the *Cupressus*, was randomly sampled. Leaves were from sunlit branches in the middle of the canopy, which, in the case of *Cupressus*, was accessed using an extension stick reaching up to 6 m above ground. The leaf area relative to the chamber size was calculated and multiplied for each tree species. All measurements were taken after the IRGA values were stable. For C assimilation rate, net photosynthesis was measured on the same leaves. Conditions inside the chamber were set similarly to the dark respiration measurement, albeit with additional photosynthetically active radiation, at a level matching the outside conditions, between 250 and 1500 µmol m^{-2} s^{-1}, depending on the measurement day and reflecting the seasonal cycle.

Statistical analysis

Statistical analysis was done using R statistical software (R Development Core Team). We used one-way ANOVA, followed by a *post hoc* Tukey honest significant test ($P < 0.05$), to analyze differences in root exudation rates between the different sampling date for each species separately. We used *t*-test ($P < 0.05$), to analyze differences in root exudation rates between species for each sampling date separately. The lme4 package (Bates et al. 2014) was used to perform a linear mixed-effects analysis of the relationship between root exudation rate and different environmental conditions. As fixed effects, we entered soil water content, soil water potential and soil temperature at 5 and 25 cm depths, air temperature and daily maximum VPD (without interaction term) into the model. Although closely related, we used both soil water content and soil water potential, as they have discrete dynamics, also considering their dependence on layer-specific soil structure. As random effects, we had different sampling dates. Pearson's correlation analysis was used for all the other relationships analysis. All data met the normality and homoscedasticity assumption or were transformed properly before being applied to statistical analyses.

Results

Environmental conditions at the site during the study period

The investigation period had typical conditions for the site, with temperatures ranging between 4 and 40 °C, and mostly around 10–18 °C on winter days and 21–34 °C on summer days (Figure 1A). VPD was mildest in midwinter, ∼1.3 kPa, and highest during spring and autumn heatwaves, up to 6.5 kPa. Rainfall was mostly confined to October to April, with diurnal amounts of up to 33 mm. Water content at 5 and 25 cm below ground was 3–25 and 7–31%, respectively (Figure 1B). Water potential at 5 and 25 cm below ground were −7.7 to 0 and −2.4 to 0 MPa, respectively. The difference in water potential between the soil layers increased in autumn, with stable values at 25 cm and decreasing values in the topsoil. Soil temperatures at 5 and 25 cm below ground were 9–34 and 11–28 °C, respectively.

Root distribution and identification in Harel forest

We verified the tree species identity of the excavated roots, by DNA extraction and sequencing the ITS2. A total of 242 fine roots were excavated along the year in plot locations around four individual trees from each of the five major tree species in the plot. In spite of our balanced design, excavated roots were dominated by *C. sempervirens* and *P. lentiscus*, respectively (Figure 2). Moreover, around trunks of a specific species (5–30 cm soil depth and 50 cm radius from the trunk), the proportion of the root's species distribution was similar to the total plot
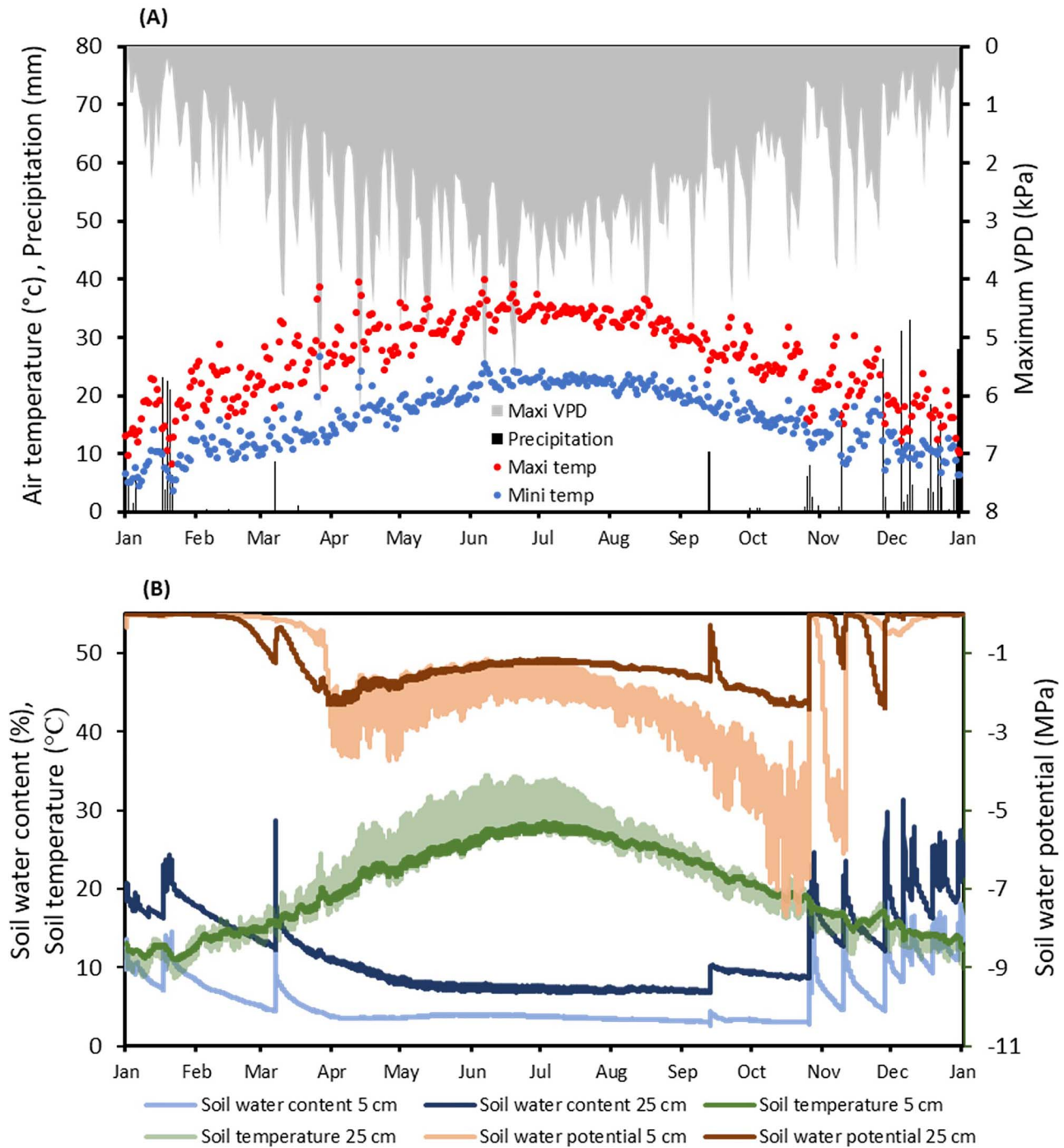
Figure 1. Meteorological and soil conditions in Harel forest 2017–2018. (A) Meteorological conditions at Beit Jimal, 1.4 km E of the study site. (B) Soil conditions in 5 and 25 cm depth.

distribution, except for the two conifer species: *P. halepensis* and *C. sempervirens* (Figure 2). Fine roots of *P. halepensis* were found only around these two conifers. Unexpectedly, across the research plot and particularly around the trunks of *Q. calliprinos* and *C. siliqua*, only one root of *Quercus* was found. Deeper excavation was not possible due to the shallow A horizon of 20.6 ± 0.7 cm soil depth.

Monthly dynamics of root exudates in Cupressus and Pistacia
Monthly mean root exudation rate of *C. sempervirens* and *P. lentiscus* changed significantly along the year, in spite of the large variations among samples of the same date (Figure 3). In addition, there was a significant difference in root exudation rate between the two species in October, November and January (Figure 3). In both species monthly mean root exudation
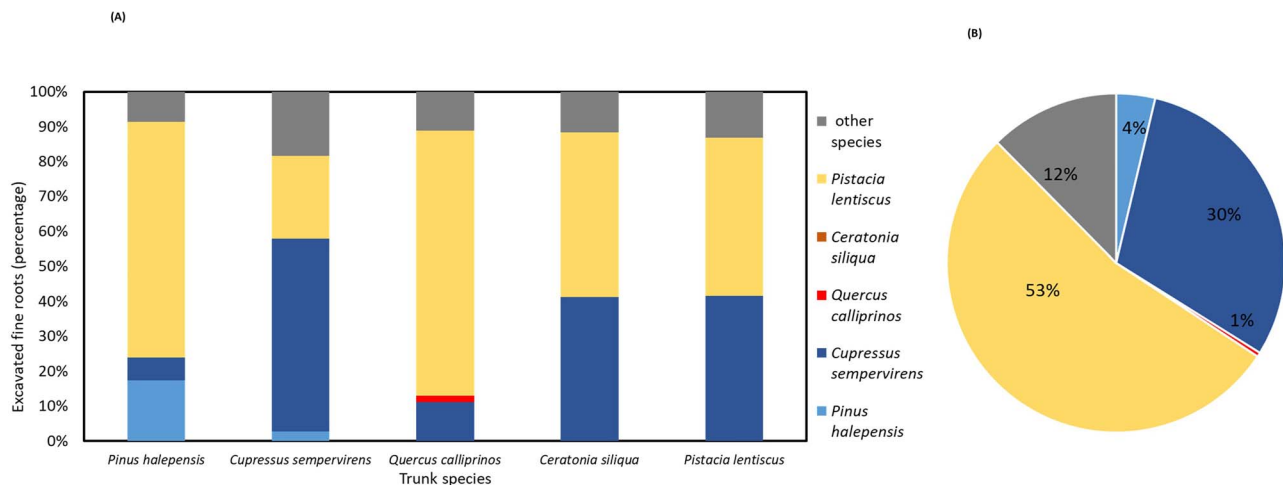
Figure 2. Percentages of excavated fine roots, sorted by species, in Harel forest during 2017–2018 based on DNA identification with ITS2 sequences. (A) Percentages of excavated fine roots of species around trunks of a specific species ($N = 4$ and for *P. lentiscus* $N = 5$). (B) Percentages of total identification of fine roots ($N = 242$).

rate tended to increase from the beginning of the year, peak during the dry season and then subsequently decrease. In *C. sempervirens*, monthly mean root exudation peaked at the end of September while in *P. lentiscus* at the end of June. *Cupressus. sempervirens* monthly mean root exudation ranged from 66.7 µg C cm^{-2} root day^{-1} in September 2017 to 6.2 in March 2017. *Pistacia lentiscus* monthly mean root exudation ranged from 31.5 µg C cm^{-2} root day^{-1} in July 2017 to 6.7 in February 2017.

Relationships between root exudation rate and seasonal changes in soil and atmosphere

Soil measurements included soil water content (m^3 m^{-3}), soil water potential (MPa) and soil temperature (°C) (Figure 1B), and atmospheric measurements included air temperature (°C) and daily maximum vapor pressure deficit VPD (kPa) (Figure 1A). Correlations with soil parameters (Figure 4) were generally better than those with meteorological parameters (Figure S4 available as Supplementary Data at *Tree Physiology* Online). Specifically, root exudation rate increased linearly with soil temperature, about 1.5 µg C cm^{-2} root day^{-1} increase per 10 °C (Figure 4). Similarly, exudation decreased by ~1.0 µg C cm^{-2} root day^{-1} per soil moisture increase of ~10% (v/v). Since multiple parameters correlated with the seasonal exudation trends, we used a stepwise linear mixed-effects model to test the contribution of each parameter, based on Akaike's information criterion (AIC) rank (Table 1). We applied the model with sampling date as a random effect and the environmental conditions as fixed effects. The model found that for *C. sempervirens*, the dominant parameters were soil water potential and soil temperature at the two depths (exudation rate = $0.26 \times \text{SWP}_{5cm} - 0.73 \times \text{SWP}_{25cm} - 0.36 \times \text{ST}_{5cm} + 0.56 \times \text{ST}_{25cm}$, $F(4,57) = 9.5$,

$P < 0.001$, $R^2 = 0.36$). In *P. lentiscus* the dominant effects were soil water potential and soil water content at the two depths (exudation rate = $6.74 - 23.48 \times \text{SWP}_{5cm} - 10.83 \times \text{SWP}_{25cm} + 0.14 \times \text{SWC}_{5cm} + 0.33 \times \text{SWC}_{25cm}$, $F(4,176) = 40.83$, $P < 0.001$, $R^2 = 0.47$).

Relationships between root exudation rate and leaf carbon assimilation and respiration

We examined the relationships between root exudation rate and leaf assimilation rate and leaf respiration rate, measured on the same trees at the site (Figure 5). Leaf assimilation represented C source strength, whereas leaf respiration was used as an index of C sink strength, associated with high metabolic demands for C. In a previous study on *P. halepensis* growing in a nearby site, leaf and root respiration rates had a similar seasonal dynamics (Klein and Hoch 2015). There was a negative correlation with leaf assimilation rate in *P. lentiscus* ($R^2 = 0.53$, $P = 0.001$), but not significantly for *C. sempervirens* ($R^2 = 0.29$, $P = 0.09$). Leaf respiration rate showed a strongly negative correlation with *C. sempervirens* ($R^2 = 0.76$, $P = 0.002$) but not with *P. lentiscus* ($R^2 = 0.04$, $P = 0.44$). Overall, root exudation rate tended to increase when both leaf assimilation and respiration were decreasing.

Discussion

Root exudation dynamics in a Mediterranean forest

Root exudation rate is influenced by multiple factors, including plant species and age, environmental conditions, the physiological status of the plants and biotic factors (Neumann and Römheld 2007, Meier et al. 2013, Vranova et al. 2013). Here, *C. sempervirens* and *P. lentiscus* exuded at similar rates in winter months; however, in autumn months *C. sempervirens*
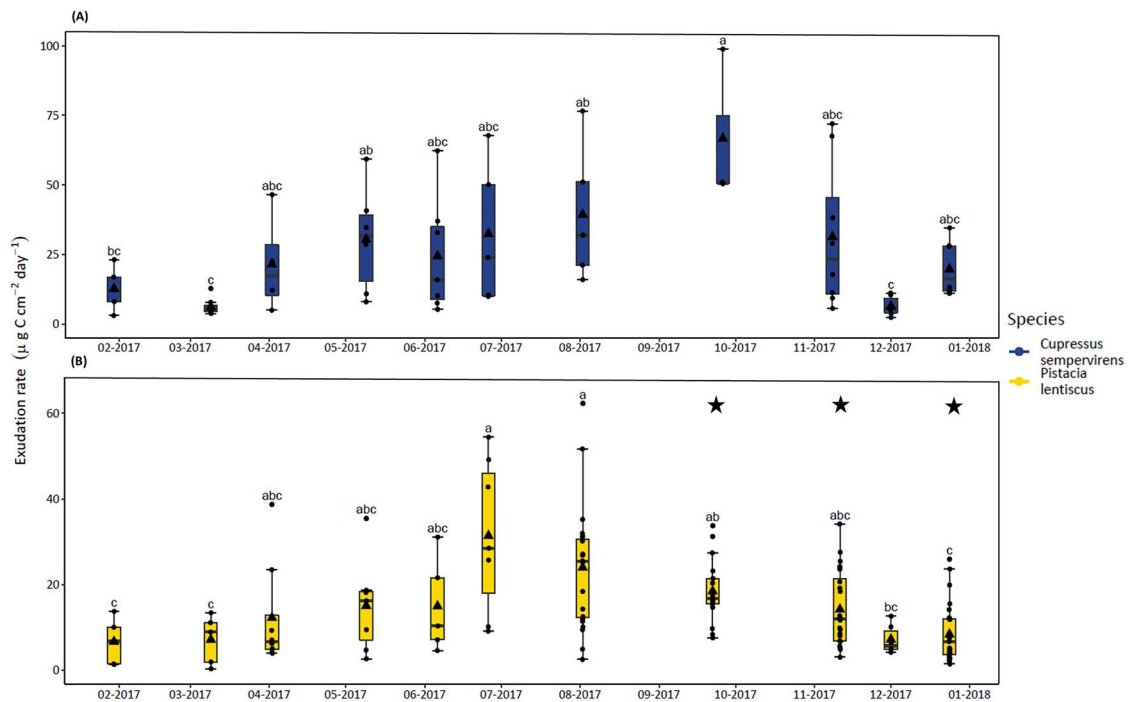
Figure 3. Monthly dynamics of root exudation rate ($\mu g\ C\ cm^{-2}$ root day^{-1}) at Harel forest from February 2017 to January 2018, in *P. lentiscus* (A) and *C. sempervirens* (B). Different lowercase letters represent significant difference between sampling date of each species separately based on Tukey honest significant difference test ($P < 0.05$). Asterisks represent significant difference between species for each sampling date separately based on *t*-test ($P < 0.05$). Lines in boxes represent median, top and bottom of boxes represent first and third quartiles, whiskers represent 1.5 interquartile range, triangles represent means and data points represent single observations. Blue and yellow denote *C. sempervirens* and *P. lentiscus*, respectively.
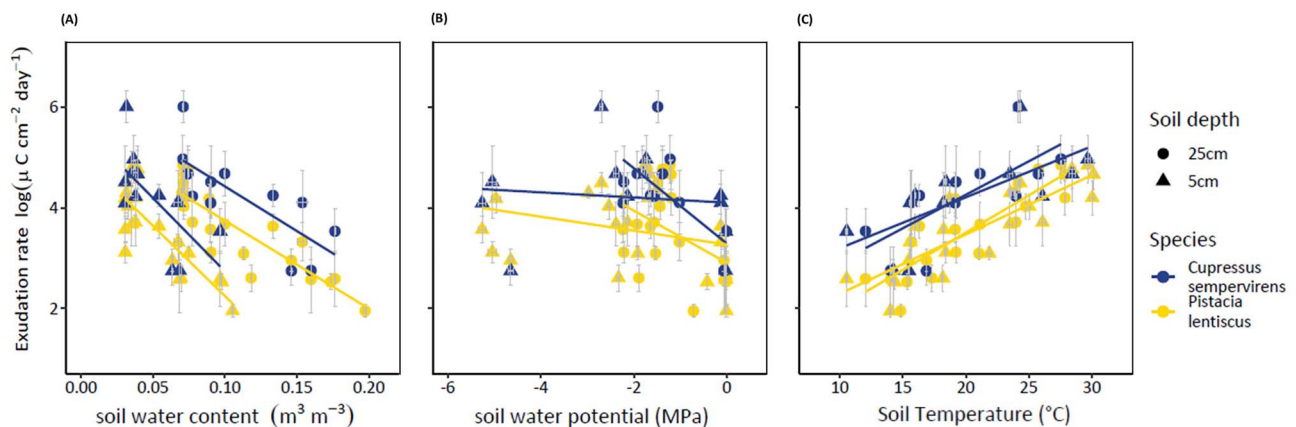

Figure 4. Relationship between monthly mean root exudation rate ($\mu g\ C\ cm^{-2}$ root day^{-1}) with (A) soil water content ($m^3\ m^{-3}$), at 5 cm soil depth $P = 0.018$, $R^2 = 0.45$ and $P < 0.001$, $R^2 = 0.68$ and at 25 cm soil depth $P = 0.004$, $R^2 = 0.59$ and $P < 0.001$, $R^2 = 0.76$ for *C. sempervirens* and *P. lentiscus*, respectively; (B) soil water potential (MPa), at 5 cm soil depth $P = 0.73$, $R^2 = 0.01$ and $P = 0.21$, $R^2 = 0.08$ and at 25 cm soil depth $P = 0.02$, $R^2 = 0.44$ and $P = 0.04$, $R^2 = 0.2$ for *C. sempervirens* and *P. lentiscus*, respectively; and (C) soil temperature (°C), at 5 cm soil depth $P = 0.01$, $R^2 = 0.47$ and $P < 0.001$, $R^2 = 0.71$ and at 25 cm soil depth $P = 0.008$, $R^2 = 0.52$ and $P < 0.001$, $R^2 = 0.75$ for *C. sempervirens* and *P. lentiscus*, respectively. Values are means (±SE). Triangles and circle represent soil depth of 5 and 25 cm below ground, respectively. Blue and yellow denote *C. sempervirens* and *P. lentiscus*, respectively.

exuded twofold more C than the coexisting, neighboring *P. lentiscus* (Figure 3). In addition, the annual peak exudation rate for *Pistacia* was in July, and for *Cupressus* it was in October. Other comparative studies at forest ecosystems also showed that species diverge in their root exudation rate (Yin et al. 2014, Sun, *et al.* 2017a), but not all (Brzostek et al. 2013).

Yet in the latter study, the authors noted that more extensive field sampling would have been able to resolve species level differences (the sampling duration was only 2 months). One explanation for the species differences in our study may be related to differences in species strategies for responding to drought stress. *Cupressus sempervirens* is characterized by
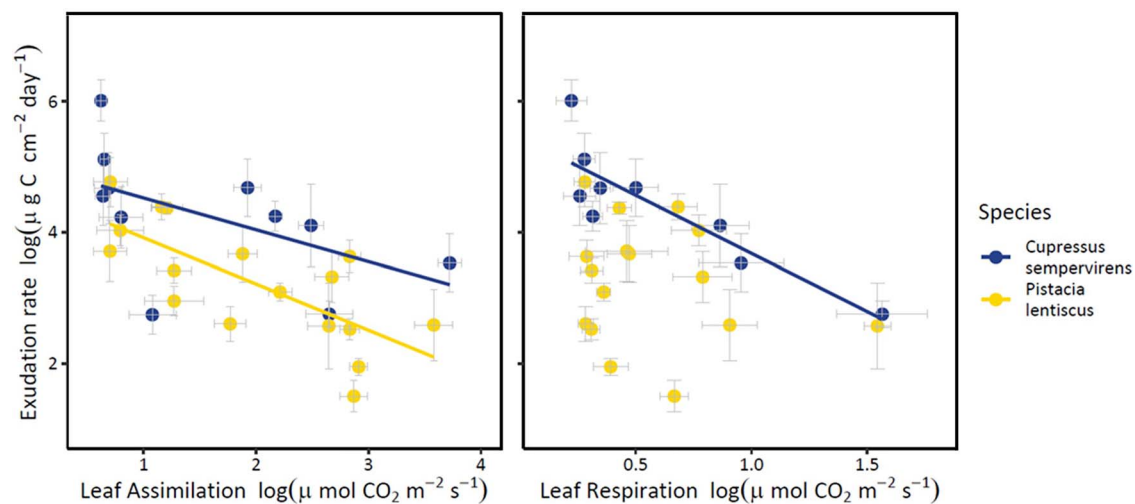
Figure 5. Relationship between monthly mean root exudation rate (µg C cm^{-2} root day^{-1}) with (A) monthly mean leaf assimilation (µmol CO$_2$ m^{-2} s^{-1}), exudation rate = 5.01–0.49 × leaf assimilation, $P = 0.09$, $R^2 = 0.29$ and exudation rate = 4.62–0.7 × leaf assimilation, $P < 0.001$, $R^2 = 0.53$, for *C. sempervirens* and *P. lentiscus*, respectively, and (B) respiration rate (µmol CO$_2$ m^{-2} s^{-1}), exudation rate = 5.45–1.77 × leaf respiration, $P = 0.002$, $R^2 = 0.76$ and exudation rate = 3.58–0.57 × leaf respiration, $P = 0.44$, $R^2 = 0.04$, for *C. sempervirens* and *P. lentiscus*, respectively. Values are means (±SE). Blue and yellow denote *C. sempervirens* and *P. lentiscus*, respectively.

anisohydric-like stomatal response (Froux et al. 2005), whereas *P. lentiscus* is characterized by isohydric-like stomatal response (Trifilò et al. 2015). Consequently, the conifer is relatively more active than the neighboring broadleaf in summer, which is in line with *Cupressus*' prolonged exudation response to the decreasing water availability.

Root exudation rates varied significantly along the year, with relatively low exudation rate in both species in the wet season and a tendency to increase in the dry season (Figure 2). This result negates our hypothesis that tree physiological activities are mostly synchronized, so root exudation should increase seasonally with soil moisture (the main limiting factor in this ecosystem), matching the higher tree C availability associated with the wet season in our region (Klein et al. 2013, Klein, et al. 2016*a*, 2016*b*). Yet, our observations generally corroborate the few previous studies that have measured root exudation under drought in a controlled environment, which have reported generally higher C release under water stress (Preece and Peñuelas 2016, Karst et al. 2017, Preece et al. 2018). Our stepwise linear mixed-effects model result also supports this finding, showing that soil parameters including soil water potential and soil temperature in *C. sempervirens* ($R^2 = 0.36$), and soil water potential and soil water content in *P. lentiscus* ($R^2 = 0.47$), explained the exudation dynamics best (Table 1). In *C. sempervirens*, as soil temperature increased and water potential became more negative, root exudation rate increased. Interestingly, while these relationships represented the root responses to conditions at 25 cm, relationships with the topsoil conditions were weaker and opposite in direction (Table 1). This is because topsoil water potential decreased

throughout the autumn (Figure 1B), when *Cupressus* exudation rates were already decreasing (Figure 3A). In *P. lentiscus* it seems that the exudation rate followed the trends of soil water potential and soil water content, but only partially. As the soil started to dry, the exudation rate tended to increase, but then decreased in August, while the soil continued to dry.

A major role of root exudation is the maintenance of functioning rhizosphere (Neumann and Römheld 2007, Meier et al. 2013, van Dam and Bouwmeester 2016), which is impacted dramatically by drought. The first possible explanation for increasing exudation rate during drought period may be the increase in lubrication in order to help the roots move through the dry soil and maintain root–soil contact (Nguyen 2003, Henry et al. 2007, Vranova et al. 2013). A second explanation could be the result of higher root mortality and lower cell membrane integrity, which causes an increased leakage of metabolites (Henry et al. 2007).

A third explanation could be related to microbial activity, which typically tends to increase with moisture and temperature (at least at the ranges relevant to a Mediterranean forest).

Considering this assumption, there is a possibility that the increase in exudation in the dry season is a tree response to lower microbial activity. The theory of microbial priming suggests that trees can control their rhizosphere microbiome composition and activity through root exudates (Meier et al. 2013, van Dam and Bouwmeester 2016). In turn, rhizosphere bacteria improve plant nutrient supply, biomass and survivorship in drying soil (Belimov et al. 2009, Kang et al. 2014, Timmusk et al. 2014, Naylor and Coleman-Derr 2018, Vargas et al. 2019).

Table 1. Mixed effect model results for root exudation rates in relation to environmental parameters, in *Cupressus* (top) and *Pistacia* (bottom)

Species	Model summary				Variable coefficients				
	F value	Pr > |F|	R^2	R^2 corrected	Effect	Estimate	Standard error	t Value	P > |t|
C. sempervirens	(4,57) 9.5	<0.001	0.4	0.36	Intercept	0	1.04	0	1
					Soil water potential 5 cm	0.26	0.11	2.49	0.02
					Soil water potential 25 cm	−0.73	0.21	−3.44	0.001
					Soil temperature 5 cm	−0.36	0.19	−1.9	0.06
					Soil temperature 25 cm	0.56	0.25	2.25	0.03
P. lentiscus	(4,176) 40.83	<0.001	0.48	0.47	Intercept	6.74	0.38	17.93	<0.001
					Soil water content 5 cm	−23.48	6.18	−3.8	<0.001
					Soil water content 25 cm	−10.83	3.68	−2.95	0.004
					Soil water potential 5 cm	0.14	0.05	2.83	0.005
					Soil water potential 25 cm	0.33	0.12	2.68	0.008

Root exudates are assumed to be one of the major sources of soil organic C, and up to 21% of all C allocated into fine roots is secreted into the rhizosphere through root exudates (Haichar et al. 2014). The mechanism that determines the rate of root exudation is probably affected by the amount of C the plant allocates to root components, together with root status properties such as cell membrane potential, membrane permeability and concentration gradients between roots and the soil solution (Farrar et al. 2003). Many studies investigated the mechanisms underlying root C allocation to exudation (Farrar and Jones 2000, Karst et al. 2017). There are two major hypotheses; under the 'push' hypothesis, the more C 'pushed' into roots, the more C is exuded from roots. In other words, if the tree allocates more C to the roots, the gradient difference will increase between the root and the soil solution, which results in increasing root exudation rate. For example, *Picea abies* trees in a free-air CO_2 enrichment in a temperate mixed forest have shown indirect evidence for increased root exudation in consequence of elevated CO_2 absorption (Klein, Bader et al. 2016). Similarly, Sun, Kominami et al. (2017) found that root–exudate flux of trees may be related closely to their photosynthetic capacity, by showing a positive correlation between leaf nitrogen content (as indicator of photosynthetic capacity) and root exudation flux in four different tree species. The 'pull' hypothesis suggests that root exudation of C is controlled by exogenous biotic and abiotic factors drawing C into the rhizosphere. For instance, root colonization by fungi can increase root exudation (Meier et al. 2013). While other studies found a linkage that supports the first hypothesis, our results might be regarded as supporting the 'pull' hypothesis, by showing a negative correlation between C gain of the overall system and secretion of C to the rhizosphere. One limitation of our study is that, without root C measurements, we assume that C gain at the whole tree scale corresponds with C availability in the roots, which might not always be the case. The incompatibility of our findings with previous studies can be explained by significant differences in the environmental settings and, consequently, the physiological activity of these plant species. For instance, Sun, *et al.* (2017a) conducted their research in a temperate forest in Japan (annual precipitation of 1449 mm), which causes different timing for physiological activity that manifests in photosynthesis activity that synchronizes with the warm period (May to July; Miyama et al. 2003). However, our experiment was conducted in a semi-arid forest in Israel (annual precipitation is 509 mm), which causes physiological activities to synchronize with the cool and wet season (October to March). In our study, it seems that root exudation was unrelated to the tree C status, either its source strength or sink strength. Rather, exudation peaked during the dry season, where both water and nutrients are in shortage.

The challenge of collecting root exudation in the forest

Discussion of the sampling methodology in our study can potentially help advance the research field. Studying the role of root exudates from mature trees in the forest requires a method that is able to collect and then investigate this process from an ecological perspective. While a single, ideal method for sampling root exudates does not yet exist, soil-based approaches provide more realistic insights into exudation dynamics in natural soil environments (Oburger and Jones 2018). Thus, the method of in situ trap solution with intact root segments (Phillips et al. 2008) was used here, with additional improvements in accordance with our research questions and sampling site. Still, the trap solution offers wetter conditions than the native rhizosphere, and particularly in summer, which could potentially change exudation rates. However, the low exudation rates of *Pistacia* in winter with wetter soils compared with the high rates in summer in drier soils are not supportive of such an artifact. We had to ensure that our root segments belong to the target tree species aboveground. Harel forest is a dense semi-natural mixed forest, with more than five woody species. Therefore, a fine root segment near to a trunk does not necessarily mean it belongs to that tree species (Figure 4A). To overcome this problem, we identified each root segment that was sampled, by developing a rapid DNA identification method. From this procedure we conclude that among the five selected species, *C. siliqua* and *Q. calliprinos* have deeper root systems beyond our excavation, while the other species that were selected had shallower root systems. Moreover, the roots of those species can reach a long distance horizontally, a few meters away from the trunk.

In summary, our study presents a new glimpse into the dynamics of root exudation in a natural environment characterized by a long seasonal drought. By using a field-adapted method, we were able to measure the rate of root exudation of two Mediterranean tree species along the year. The enhanced exudation flux during the dry season, at a time of otherwise low aboveground activity, indicates that exudation dynamics in the field are less sensitive to tree C availability. Instead, they might respond to seasonal changes in the rhizosphere. The combination of further physiological measurements (e.g., non-structural carbohydrates, root respiration, etc.) and characterization of microorganisms in the rhizosphere should shed more light on the role(s) and complexity of root exudation.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

Authors' contributions

Research was performed in situ by G.J. and led by T.K. I.R. and S.M. performed the soil and leaf measurements in situ. G.J. wrote the manuscript, guided by T.K.

Data accessibility

All data used in the paper are available in its tables and figures.

References

Ahlström A, Raupach MR, Schurgers G, Smith B, Arneth A, Jung M, Kato E (2015) The dominant role of semi-arid ecosystems in the trend and variability of the land CO2 sink. Science 348:895–899.

Bates D, Mächler M, Bolker B, Walker S (2014) Fitting linear mixed-effects models using lme4. arXiv preprint arXiv 1406:5823. doi: 10.18637/jss.v067.i01.

Belimov AA, Dodd IC, Hontzeas N, Theobald JC, Safronova VI, Davies WJ (2009) Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling. New Phytol 181:413–423.

Brzostek ER, Greco A, Drake JE, Finzi AC (2013) Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. Biogeochemistry 115:65–76.

Cheng T, Xu C, Lei L, Li C, Zhang Y, Zhou S (2016) Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. Mol Ecol Resour 16:138–149.

Farrar J, Hawes M, Jones D, Lindow S (2003) How roots control the flux of carbon to the rhizosphere. Ecology 84:827–837.

Farrar JF, Jones DL (2000) The control of carbon acquisition by roots. New Phytol 147:43–53.

Froux F, Ducrey M, Dreyer E, Huc R (2005) Vulnerability to embolism differs in roots and shoots and among three Mediterranean conifers: consequences for stomatal regulation of water loss? Trees 19:137–144.

Grünzweig JM, Hemming D, Maseyk K, Lin T, Rotenberg E, Raz-Yaseef N, Pete D, Yakir D (2009) Water limitation to soil CO2 efflux in a pine forest at the semiarid timberline. J Geophys Res Biogeogr 114:1–14.

Haichar FZ, Santaella C, Heulin T, Achouak W (2014) Root exudates mediated interactions belowground. Soil Biol Biochem 77:69–80.

Henry A, Doucette W, Norton J, Bugbee B (2007) Changes in crested wheatgrass root exudation caused by flood, drought, and nutrient stress. J Environ Qual 36:904–912.

Kang SM, Radhakrishnan R, Khan AL, Kim MJ, Park JM, Kim BR, Shin DH, Lee IJ (2014) Gibberellin secreting rhizobacterium, pseudomonas putida H-2-3 modulates the hormonal and stress physiology of soybean to improve the plant growth under saline and drought conditions. Plant Physiol Biochem 84:115–124.

Kannenberg SA, Phillips RP (2017) Plant responses to stress impacts: the C we do not see. Tree Physiol 37:151–153.

Karst J, Gaster J, Wiley E, Landhäusser SM (2017) Stress differentially causes roots of tree seedlings to exude carbon. Tree Physiol 37:154–164.

Keller A, Schleicher T, Schultz J, Müller T, Dandekar T, Wolf M (2009) 5.8S–28S rRNA interaction and HMM-based ITS2 annotation. Gene 430:50–57.

Klein T, Hoch G (2015) Tree carbon allocation dynamics determined using a carbon mass balance approach. New Phytol 205:147–159.

Klein T, Shpringer I, Fikler B, Elbaz G, Cohen S, Yakir D (2013) Relationships between stomatal regulation, water-use, and water-use efficiency of two coexisting key Mediterranean tree species. For Ecol Manage 302:34–42.

Klein T, Bader MKF, Leuzinger S, Mildner M, Schleppi P, Siegwolf RT, Körner C (2016a) Growth and carbon relations of mature *Picea abies* trees under 5 years of free-air CO2 enrichment. J Ecol 104:1720–1733.

Klein T, Cohen S, Paudel I, Preisler Y, Rotenberg E, Yakir D (2016b) Diurnal dynamics of water transport, storage and hydraulic conductivity in pine trees under seasonal drought. iForest 9:710.

Klein T, Rotenberg E, Tatarinov F, Yakir D (2016c) Association between sap flow-derived and eddy covariance-derived measurements of forest canopy CO 2 uptake. New Phytol 209:436–446.

Koetschan C, Hackl T, Müller T, Wolf M, Förster F, Schultz J (2012) ITS2 database IV: interactive taxon sampling for internal transcribed spacer 2 based phylogenies. Mol Phylogenet Evol 63:585–588.

Lapidot O, Ignat T, Rud R, Rog I, Alchanatis V, Klein T (2019) Use of thermal imaging to detect evaporative cooling in coniferous and broadleaved tree species of the Mediterranean maquis. Agric For Meteorol 271:285–294.

Meier IC, Avis PG, Phillips RP (2013) Fungal communities influence root exudation rates in pine saplings. FEMS Microbiol Ecol 83:585–595.

Miyama T, Kominami Y, Tamai K, Nobuhiro T, Goto Y (2003) Automated foliage chamber method for long-term measurement of CO2 flux in the uppermost canopy. Tellus B Chem Phys Meteorol 55:322–330.

Nakayama M, Tateno R (2018) Solar radiation strongly influences the quantity of forest tree root exudates. Trees 32:871–879.

Naylor D, Coleman-Derr D (2018) Drought stress and root-associated bacterial communities. Front Plant Sci 8:2223.

Neumann G, Römheld V (2007) The release of root exudates as affected by the plant physiological status. In: Pinton, R, Zeno V, and Paolo N (eds). The rhizosphere: biochemistry and organic substances at the soil-plant interface, CRC Press, Boca Raton, FL, USA, Vol. 2, pp. 23–72.

Nguyen C (2003) Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie, EDP Sciences, 23 (5–6), pp. 375–396.

Oburger E, Jones DL (2018) Sampling root exudates–mission impossible? Rhizosphere 6:116–133.

Phillips RP, Erlitz Y, Bier R, Bernhardt ES (2008) New approach for capturing soluble root exudates in forest soils. Func Ecol 22:990–999.

Phillips RP, Finzi AC, Bernhardt ES (2011) Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO2 fumigation. Ecol Lett 14:187–194.

Preece C, Peñuelas J (2016) Rhizodeposition under drought and consequences for soil communities and ecosystem resilience. Plant Soil 409:1–17.

Preece C, Farré-Armengol G, Llusià J, Peñuelas J (2018) Thirsty tree roots exude more carbon. Tree Physiol 38:690–695.

Ross HA, Murugan S, Sibon Li WL (2008) Testing the reliability of genetic methods of species identification via simulation. Syst Biol 57:216–230.

Rotenberg E, Yakir D (2010) Contribution of semi-arid forests to the climate system. Science 327:451–454.

Schultz J, Müller T, Achtziger M, Seibel PN, Dandekar T, Wolf M (2006) The internal transcribed spacer 2 database—a web server for (not only) low level phylogenetic analyses. Nucleic Acids Res 34: W704–W707.

Sun L, Ataka M, Kominami Y, Yoshimura K (2017a) Relationship between fine-root exudation and respiration of two Quercus species in a Japanese temperate forest. Tree Physiol. 37: 1011–1020.

Sun L, Kominami Y, Yoshimura K, Kitayama K (2017) Root-exudate flux variations among four co-existing canopy species in a temperate forest, Japan. Ecolo Res 32:331–339.

Timmusk S, El-Daim IAA, Copolovici L, Tanilas T, Kännaste A, Behers L, Nevo E, Seisenbaeva G, Stenström E, Niinemets Ü (2014) Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. PLoS One 9:e96086.

Trifilò P, Nardini A, Gullo MAL, et al. (2015) Diurnal changes in embolism rate in nine dry forest trees: relationships with species-specific xylem vulnerability, hydraulic strategy and wood traits. Tree Physiol 35:694–705.

Tückmantel T, Leuschner C, Preusser S, Kandeler E, Angst G, Mueller CW, Meier IC (2017) Root exudation patterns in a beech forest: dependence on soil depth, root morphology, and environment. Soil Biol Biochem 107:188–197.

van Dam NM, Bouwmeester HJ (2016) Metabolomics in the rhizosphere: tapping into belowground chemical communication. Trends Plant Sci 21:256–265.

Vargas R, Kenney AM, Bilinski T (2019) Variable influences of water availability and Rhizobacteria on the growth of *Schizachyrium scoparium* (little bluestem) at different ages. Front Microbiol 10: 1–16.

Vranova V, Rejsek K, Skene KR, Janous D, Formanek P (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.

Wang H, Qi M, Cutler AJ (1993) A simple method of preparing plant samples for PCR. Nucleic Acids Res 21:4153.

Yin H, Wheeler E, Phillips RP (2014) Root-induced changes in nutrient cycling in forests depend on exudation rates. Soil Biol Biochem 78:213–221.