RESEARCH ARTICLE

Combined effects of drought and simulated pathogen attack on root exudation rates of tomatoes

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Abstract

Background and aims Food production is threatened by direct climate change effects including drought. Indirect effects, including changes in plant-pathogen dynamics and increased susceptibility to pathogens, further exacerbate the risks. Root exudation, which plays a crucial role in plant defence against drought and pathogens, is influenced by both water stress and pathogens. However, the interactive effects of these abiotic and biotic factors are rarely studied.

Methods We conducted a controlled environment experiment to investigate the effects of moderate drought and simulated pathogen attack (using pipecolic acid, an inducer of systemic acquired resistance) on the rates of root exudation of total organic carbon (TOC) and total nitrogen (TN) of four tomato cultivars grown in potting soil.

Results Drought increased the exudation of TOC and TN per unit of root area, while pipecolic acid did not have any significant effect. Furthermore, there was no interaction observed between the abiotic and biotic factors. However, due to the reduction in plant and root biomass caused by drought, the total exudation per plant remained similar between control and water-limited plants. Additionally, pipecolic acid reduced the carbon-to-nitrogen ratio of exudates and increased the total exudation of TN.

Conclusion The increased exudation observed in drought-stressed plants may serve as a strategy to maintain root and rhizosphere activity despite reduced root growth. Notably, the impact of drought differed among the tested cultivars, highlighting their diverse levels of drought tolerance. This emphasises the importance of preserving a wide range of crop cultivars to ensure food security under increasing drought.

Keywords Rhizodeposition · Pipecolic acid · Total organic carbon · Total nitrogen · *Solanum lycopersicum*

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Introduction

Maintaining secure food supplies involves understanding how the plants we eat are affected by abiotic and biotic stress and, ultimately, selecting varieties that are more tolerant to such stress. Drought is an increasing problem for agriculture, as lower rainfall across large regions of the world means that there is less water available for irrigation. Around 75% of the global harvested area has experienced yield losses related to drought (Kim et al. 2019), leading to food shortages, which increase prices and threaten the nutrition of millions of people. At the same time, pathogens that attack crops reduce yields by up to 30%, depending on the crop and the location (Savary et al. 2019). While plants have in-built defence systems to fight pathogen attacks, the allocation of resources to this process can mean a decrease in growth due to growth–defence trade-offs (He et al. 2022). Moreover, plant pathogen infection risk is predicted to increase in some regions due to climate warming altering the geographical and seasonal niches of pathogens (Chaloner et al. 2021), leading to a higher requirement for pesticide use, with negative impacts on the environment and human health (Tang et al. 2021; Wuepper et al. 2023). New strategies to adapt our agricultural systems to be able to resist water stress and pathogen attack are needed.

Root exudation is a main link between plants and soils, with exuded compounds playing key roles in interactions with other plants, with microbes and fauna, and with soil properties (Bais et al. 2006; Sasse et al. 2018). Root exudation is the release of organic compounds into the soil (including sugars, organic acids, amino acids and structural carbohydrates). This represents a significant proportion of the carbon budget of a plant, making up approximately 11% of net fixed C (Jones et al. 2009), although this varies a lot between plant functional types and tending to be higher in younger plants (Gransee and Wittenmayer 2000; Pausch and Kuzyakov 2018). Root exudation patterns have been shown to be very variable, between species and individuals, and also to change (in quantity and composition) in response to biotic and abiotic factors (Walker et al. 2003; Bais et al. 2006; Gargallo-Garriga et al. 2018; Tiziani et al. 2022).

In relation to water availability, overall root exudation, usually measured as total organic carbon (TOC),

has been shown to increase in response to mild and moderate drought (Preece and Peñuelas 2016), and may represent a plant strategy to change the soil environment to reduce drought stress (Williams and De Vries 2020). The mechanisms for this may include exudation of the structural carbohydrate mucilage which keeps the soil near the roots moist and maintains hydraulic conductivity (Ahmed et al. 2014), the formation of a rhizosheath (soil aggregation on the surface of the root) (Ndour et al. 2020; Chai and Schachtman 2022), and the promotion of interactions with rhizosphere microbes that can improve water relations (Naylor and Coleman-Derr 2018). Other studies have indicated specific compounds in exudates that change under drought. For example, experimental drought stress increased exudation of proline and abscisic acid in barley (Calvo et al. 2017).

The role of root exudates in pathogen defence is also key (Haichar et al. 2014; Vives-Peris et al. 2019). Defence compounds in exudates may be released both constitutively (phytoanticipins) and in response to specific threats (phytoalexins) (Baetz and Martinoia 2014). There is a large diversity of antimicrobial compounds released by roots, with phenolics and terpenoids being particularly notable (Badri et al. 2013; Baetz and Martinoia 2014), including in tomato (Steinkellner et al. 2005). For example, various cinnamic acid derivatives (e.g., t-cinnamic acid) have anti-fungal activity, and have been shown to increase both inside barley root tissues and in root exudates, during infection by the soil fungal pathogen *Fusarium* (Lanoue et al. 2010). In a separate study with barley, roots infected with *Fusarium graminearum* recruited bacteria with antifungal properties, which was proposed to be linked to the root exudates (Dudenhöffer et al. 2016). Pathogen attack, even above ground, can also stimulate the release of root exudates that attract beneficial root microbes. For example, in Arabidopsis, infection by the foliar pathogen *Pseudomonas syringae* increases exudation of l-malic acid, which causes increased root colonisation by *Bacillus subtilis* which is a plant-beneficial rhizobacterium (Rudrappa et al. 2008). Similarly, tomato plants that were exposed to foliar pathogen attack released root exudates that attracted a beneficial fungus (*Trichoderma harzianum*) (Lombardi et al. 2018).

While the impact of individual abiotic and biotic stressors on exudation is well-documented, the interactive effects of multiple stressors remain largely

unexplored, creating a significant knowledge gap. The main objective of our study was to investigate the combined influence of moderate drought and simulated pathogen attack on root exudation rates in four tomato cultivars of varying drought tolerance. Tomato (*Lycopersicon esculentum*) was selected as the focal crop due to its global significance, ranking as the second most important vegetable crop worldwide, with a current production of approximately 189 million tons of fresh fruit from 5 million hectares (FAOstat 2021). Future climate projections indicate that increasing air temperatures and decreased water availability in major tomato-growing regions will limit production, potentially resulting in a 6% decrease by 2040 compared to the 1980–2009 period (Cammarano et al. 2022). Additionally, tomato is highly susceptible to various plant diseases caused by bacterial, fungal, and viral pathogens (Panno et al. 2021), and that can be increased due to water stress.

Our hypotheses were as follows: (1) Moderate drought would increase specific exudation rates (exudation per root area), consistent with previous studies in other plant species that have demonstrated increased exudation under drought conditions. (2) Simulated pathogen attack would also stimulate specific root exudation as a response to produce higher levels of antimicrobial compounds, thus (3) combined drought and simulated pathogen attack would therefore produce the greatest changes in exudation. Furthermore, we hypothesised (4) that there would be cultivar-specific variations in exudation rates due to different resource allocation patterns and varying levels of resistance to water stress and pathogen attack.

Methods

Plant material and initial growth conditions

Four cultivars of tomato (*Solanum lycopersicum* (L.) H.Karst.) were selected to represent commercially available varieties with different stated levels of drought tolerance, namely cv. Bella Rosa, Estrella del Balcón, Flor de Baladre and Valenciano. The cultivars that were stated (by the seed providers) as being more drought resistant were Estrella del Balcón and Valenciano, and the two cultivars that were more drought sensitive were Bella Rosa and Flor de Baladre. Seed was acquired from different comercial

providers (Bella Rosa: Agroleg Varo SRL, Bogata de Mures, Nr. 486D, Rumania; Estrella del Balcón: http://www.seedsgallery.shop; Flor de Baladre: Semilleros Corbalán CB, La Carrasquilla, Murcia, Spain, via suhuertoencasa.com; Valenciano: Fitoagrícola, Castellón de la Plana, Castellón, Spain). Tomato plants were grown from seed in the greenhouse of the Research Unit Environmental Simulation at Helmholtz Munich (Germany) in January 2020. Sowing of seed was staggered by cultivar, with a week in between, due to constraints on the space available in the 'VOC-SCREEN' platform (Jud et al. 2018) where the later stages of the experiment took place. Thus, for all parts of the experiment, cultivars were separated in time. Plants were grown in potting soil (Einheitserde CL-Topf, a peat-based mixture), which consisted of 70% organic material, and contained 340 mg L^{-1} nitrogen, 260 mg L^{-1} phosphorus, 330 mg L^{-1} potassium oxide, and 100 mg L^{-1} magnesium. Plants grew in 1 L pots, with optimum watering, until they were 14 days old. At that stage, 30 plants per cultivar were transferred to the 'ExpoSCREEN' (Vanzo et al. 2015) controlled climate chamber (27 ℃/20 ℃ day/night temperature with 13-hour photoperiod and 600μmol PPFD m^{-2} s^{-1}). Plants were kept in that chamber for 14 additional days, to acclimatise to being out of the greenhouse environment.

Experimental design

Two treatments of moderate drought and simulated pathogen attack were applied in a factorial design. Drought was applied by limiting watering and pathogen attack was simulated by the addition of pipecolic acid to the irrigation water. Pipecolic acid is a crucial regulator of plant systemic acquired resistance, that confers long-lasting protection against a broad spectrum of microorganisms (Ding et al. 2016). Therefore, pipecolic acid was used as a proxy for pathogen attack, being much more controllable than the addition of a soil pathogen. There were four treatment combinations: control, drought, pipecolic acid, and drought + pipecolic acid. For each cultivar, on day 28 at 16:00, 16 individuals were selected, being those that were healthy and most similar in size and appearance. The 16 selected plants were randomly assigned to one of the four treatments, with four replicates per treatment (see the schematic diagram in Supplementary Information Fig. S1, for a summary of the

experimental steps). Plants receiving pipecolic acid were separated from the other plants by placing them in a separate sub-chamber, to prevent the compound from affecting the other plants through the emission of stress-related volatile organic compounds (VOCs) induced by pipecolic acid. For each of those plants, 40 mL of 2.06 mmol L^{-1} pipecolic acid was applied to different points on the soil surrounding the stem. This made 82.4 μmol of pipecolic acid per pot, with this amount reflecting other experiments that have used pipecolic acid to study systemic acquired resistance (Lenk et al. 2019). The remaining plants were given 40 mL of deionised water instead.

On day 29, those 16 plants were photographed for determination of leaf area, by placing them on a rotating plate inside a cubicle with a blue background. Two fixed cameras were used to capture images: one was positioned above the plant and the other had a horizontal view. This setup allowed for standardized lighting conditions during image acquisition. Nine images were taken of each plant to capture the plant from all angles (360° rotation), and this was used later to calculate the total leaf area using the Leaf Area Calculator as described by Jud et al. (2018). Plants were then transferred to the VOC-SCREEN platform (Jud et al. 2018), which is a customised cuvettes system set-up in a climate chamber of the ExpoSCREEN facility. This platform consists of 24 cuvettes, equipped with two CO_2/H_2O gas analysers (LI-640, LICOR, Lincoln, Nebraska, USA) that measure the absolute CO_2 and H_2O concentration of the air at the inlet and outlet of the cuvettes, allowing calculation of carbon balance (assimilation and evapotranspiration rates).

In this platform, each plant was placed inside a glass cuvette (Supplementary Information Fig. S2), each with a volume of ~40 L (height of 60 cm and an inner diameter of 29 cm). The cuvettes were continuously purged with particle- and carbon-filtered air from the chamber, which had the ambient CO_2 concentration. The inlet flow was set to 10 L min^{-1} for Estrella del Balcón and Valenciano measurements but had to be adjusted to 12 L min^{-1} for Bella Rosa and Flor de Baladre to avoid condensation of water vapor in the cuvettes. Because the soil compartment in the cuvette is not separated from the above-ground space, four additional cuvettes contained pots that had only soil but no plant to correct for high CO_2 and H_2O gas exchange rates that might occur from the soil.

Plants were kept in the VOC-SCREEN cuvettes for seven days, and during that time, there were constant measurements of CO_2 exchange, air temperature and humidity (using the sensor DKRF400, Driesen + Kern GmbH, Bad Bramstedt, Germany), and soil temperature and humidity (using 5TM soil moisture and temperature sensors, Decagon Devices Inc., Pullman, WA, USA). Temperature did not vary between treatments with 27 °C/19 °C (day/night) temperatures and a 13-hour photoperiod with 600 μmol PPFD m^{-2} s^{-1}. Plants in the control watering treatment received 30 mL twice per day (morning and evening) totalling 420 mL of water during the seven days. The drought treatment was implemented by reducing the daily watering, but exact amounts of water addition varied slightly between cultivars, to ensure an effective drought treatment for all. This was evaluated by checking the visual status of the plants as well as gas exchange measurements. Therefore, Estrella del Balcón received 85 mL under drought (no watering during the first three days, then 25 mL on the fourth day and 20 mL on the remaining three days) whereas the other three cultivars received 120 mL under drought (no watering during the first three days, then 30 ml on the remaining four days). All plants from the drought treatment, across all four varieties, showed visual signs of drought stress (i.e., wilting) by the end of the experiment. On day 35, the plants were removed from the chamber and photographed for a second determination of leaf area (example images for each cultivar can be seen in the Supplementary Information Fig. S3).

Air humidity within the cuvettes was on average 51% (SE = ± 4%) in well-watered cuvettes and 42% in drought cuvettes (SE = ± 5%), when grouping together all cultivars. Mean soil volumetric water content over the seven days, across all cultivars, was 27.0% (SE = ± 6.2%) in well-watered cuvettes and 16.5% (SE = ± 6.6%) under drought. Therefore, there was a significant effect of drought on soil volumetric water content (linear model, $F = 72.8$, $p < 0.001$). Cultivar also had a significant effect on soil water content (linear model, $F = 6.9$, $p < 0.001$), but there was no interaction between cultivar and the drought treatment, with the drought consistently reducing water content. There was no significant effect of the pipecolic acid addition on air humidity or soil volumetric water content. Mean soil temperature was 22.5 °C

(SE$=\pm 2.5$ ºC) in well-watered cuvettes and 23.3 ºC (SE$=\pm 2.8$ ºC) in drought cuvettes.

Gas exchange measurements

During the last seven days of the experiment for each tomato cultivar, gas exchange measurements were performed in the VOC-SCREEN platform. Measurements were taken for one cuvette at a time, for 5 min and 20 s each time, before sampling the next cuvette. Net CO_2 assimilation and evapotranspiration rates were calculated according to von Caemmerer and Farquhar (1981) and related to leaf area, that was interpolated from the images taken at the beginning and the end of each experiment. The soil CO_2 gas exchange in the background (soil-only) cuvettes was very low and hence considered negligible. Daily sums for net photosynthetic CO_2 assimilation (mmol CO_2 m^{-2} d^{-1}, and later referred to as net-C assimilation) and evapotranspiration (mol H_2O m^{-2} d^{-1}) were estimated per cuvette during the daylight period by integrating each measurement for the time between two measurements. These daily sums were calculated for the five days where the full day was measured, which excluded the first and last day in the VOC-SCREEN, therefore included days 30–34.

Plant traits and root exudation measurements

To measure root exudation, we followed a modified version of the soil-hydroponic-hybrid method (Oburger and Jones 2018) detailed by Williams et al. (2021). For each intact plant, the roots were cleaned by submerging them in distilled water, but not removed from the rest of the plant. A subset of the roots was more carefully cleaned by further submerging them in distilled water and carefully removing any remaining pieces of substrate with tweezers. The subset of the roots for exudate analysis made up 30% of the total fresh root mass on average. Exudates were sampled from this subset, rather than the full root system to ensure a careful yet effective cleaning within a few hours at the end of each round of the experiment. It should be noted that a recovery period of several days has been shown to be beneficial in avoiding the measurement of carbon released from damaged root tissue (Williams et al. 2021). The roots for exudate analysis were briefly rinsed with water to remove metabolites released by the washing process.

They were then placed in a 50 mL falcon tube containing 45 mL of milli-Q water and secured in position so that roots were fully submerged. No microbial activity inhibitor was used, as this has been shown to modify exudation patterns, although this means that exudation rates may be slightly higher than measured due to potential microbial degradation of exudates.

Aluminium foil was used to cover the top of the tube to prevent anything else from entering the exudate solution. Plants were transferred to a growth chamber with constant light (600 µmol PPFD m^{-2} s^{-1}) and temperature (27 ºC) and remained there for two hours. After this time, roots were carefully removed from the solution, and the sample was filtered (0.22 µm), then stored in a freezer (-80 ºC) until further analysis. Total (non-purgeable) organic carbon (TOC) and total nitrogen (TN) were measured (Analytik Jena-Analyser multi N/C 3100, Analytik Jena, Jena, Germany). Root area was measured for the subset that was used for the exudate measure using a flatbed scanner with images later analysed using ImageJ software. All plant samples were dried at 60 ºC for two days and dry mass was measured. This was separated into root, stem and leaf fractions, with the root fraction further separated into the part that was used for the exudate measurement and the rest.

The specific rate of C and N released from the root to be calculated per root area, as µg C or N cm^{-2} root h^{-1}, or alternatively per dry root mass (mg C or N g^{-1} root h^{-1}). In addition to specific exudation rate, total exudation rate per pot (mg of TOC or TN per hour) was calculated by multiplying the exudation rate per root mass by the total mass of roots per plant. By measuring the root exudation rates immediately after the experiment we aimed to capture the effects of the drought and simulated pathogen treatments before the plant adapted to the post-experiment conditions. A drawback of this short acclimation time is that exudation may have been overestimated if roots had been subjected to minor wounding during the root washing process. However, any root damage was minimal, following visual assessment, and we therefore deemed this a worthwhile trade-off.

Statistical analyses

All statistical analyses were carried out using R v3.6.1 (R Core Team 2019). Plant biomass and root exudation data were log-transformed to correct for

positive skewness and to achieve normality of the residuals in subsequent models. The effect of drought and pipecolic acid on plant biomass was analysed using linear mixed-effects models with tomato cultivar as a random effect. The effect of the cultivar and the drought and pipecolic acid treatments on net-C assimilation and evapotranspiration was analysed in two different ways. Firstly, linear models were used with data from the final full sampling day (day 34), when differences between treatments were expected to be greatest, with cultivar and treatment as fixed effects. Secondly, mixed effects models were used to analyse the data from days 30–34, with cultivar and treatment as fixed effects and sampling day as a random effect. This was to account for the time series and the fact that different cultivars may have started the experiment with different sizes or rates of net-C assimilation and evapotranspiration.

Specific root exudation was calculated per root area and root mass, with results showing the same patterns. Data are presented for exudation per root area to allow us to explore the relationship between specific exudation and root biomass without the two variables being confounding variables. One data point was removed for exudation of TN as it was unusually low and deemed to be an outlier. The effect of cultivar on exudation rate of C and N per root area was tested with linear models. The overall effect of drought and pipecolic acid treatments on C and N exudation rates was analysed using linear mixed-effects models with tomato cultivar as a random effect. Subsequently, cultivars were also analysed separately, using linear models, to see the effect of drought and pipecolic acid on exudation, and a Tukey test was used to see if there were significant differences between treatments. To assess the relationship between two continuous variables (e.g., TOC and TN exudation, and TOC exudation and biomass) the function *r.squaredGLMM* (from the package MuMIn) was used and the conditional R^2 was reported.

The overall effects of drought and pipecolic acid on total exudation rate per pot were then tested using a linear mixed model as before, with cultivar as a random effect, and linear models and Tukey tests were used for testing effects within cultivars. Additionally, the C:N ratio of root exudates was calculated (using values for specific exudation). The effect of cultivar on C:N was tested using linear models, and the effects of drought and pipecolic acid were analysed with linear mixed effects models (cultivar as a random effect).

Results

Drought and simulated pathogen attack on plant biomass and gas exchange

Drought had a negative effect on total dry mass (linear mixed effects model, $t = -18.2$, $p < 0.001$). Plant biomass in the two treatments with adequate water was 1.8 times higher than in treatments including drought, with mean biomass ($\pm$ SE) being 2.98 ($\pm$ 0.11) g under drought compared with 5.2 ($\pm$ 0.18) g with adequate water. This pattern of a negative effect of drought on total biomass was the same for the root dry mass ($t = -11.7$, $p < 0.001$), stem dry mass ($t = -11.0$, $p < 0.001$), and leaf dry mass ($t = -20.2$, $p < 0.001$) (Supplementary Information Fig. S4). We observed no effect of pipecolic acid (and no interaction between pipecolic acid and drought) for plant biomass. Plant dry biomass varied strongly between the tomato cultivars (linear model, $F = 6.6$, $p < 0.001$), with mean ($\pm$ SE) total dry biomass being 3.37 ($\pm$ 0.27) g for Estrella del Balcón, 3.47 ($\pm$ 0.28) g for Valenciano, 4.59 ($\pm$ 0.36) g for Bella Rosa and 5.03 ($\pm$ 0.36) g for Flor de Baladre. However, there was no interaction with the drought treatment, such that plants under drought were always smaller than well-watered plants, within each cultivar.

Net-C assimilation on the final full day of measurements (after six days of the drought and pipecolic acid treatments) was strongly affected by both cultivar (linear model, $F = 17.7$, $p < 0.001$) and treatment (linear model, $F = 51.4$, $p < 0.001$) (Table 1). There was no interaction between cultivar and treatment. Net-C assimilation was lower in drought-treated cuvettes, providing evidence for the effectiveness of the drought treatment. Mean values ($\pm$ SE) of net-C assimilation on that final day, for all cultivars together, were 271 ($\pm$ 15) mmol CO_2 m^{-2} d^{-1} in well-watered cuvettes and 88 ($\pm$ 12) mmol CO_2 m^{-2} d^{-1} in drought cuvettes. The pipecolic acid treatment did not appear to affect net-C assimilation. The cultivar that had the highest C assimilation rates on day 34 was Estrella del Balcón, followed by Valenciano, and then with Flor de Baladre and Bella Rosa having the lowest rates (Table 1). Analysis of the five full days

Table 1 Net-C assimilation and evapotranspiration values on day 34 of the experiment for each of the four tomato cultivars under the different treatments

	Mean net-C assimilation mmol CO_2 m^{-2} d^{-1} ($\pm$ SE)	Mean evapotranspiration mol H_2O m^{-2} d^{-1} ($\pm$ SE)
Bella Rosa		
Control	247 ($\pm$ 29)	49 ($\pm$ 5)
Drought	39 ($\pm$ 20)	17 ($\pm$ 3)
PA	237 ($\pm$ 22)	53 ($\pm$ 5)
Drought + PA	11 ($\pm$ 11)	10 ($\pm$ 3)
Estrella del Balcón		
Control	334 ($\pm$ 78)	81 ($\pm$ 14)
Drought	208 ($\pm$ 38)	62 ($\pm$ 8)
PA	384 ($\pm$ 23)	77 ($\pm$ 2)
Drought + PA	142 ($\pm$ 18)	43 ($\pm$ 3)
Flor de Baladre		
Control	222 ($\pm$ 12)	42 ($\pm$ 2)
Drought	55 ($\pm$ 15)	20 ($\pm$ 1)
PA	193 ($\pm$ 18)	37 ($\pm$ 3)
Drought + PA	79 ($\pm$ 19)	24 ($\pm$ 3)
Valenciano		
Control	262 ($\pm$ 31)	54 ($\pm$ 4)
Drought	81 ($\pm$ 21)	28 ($\pm$ 3)
PA	292 ($\pm$ 31)	69 ($\pm$ 9)
Drought + PA	93 ($\pm$ 20)	34 ($\pm$ 3)

PA pipecolic acid

of sampling identified an interaction between cultivar and treatment (mixed effects model, *ChiSq* = 28.0, $p < 0.001$), with the cultivar Estrella del Balcón maintaining slightly higher C assimilation under the drought only treatment (Supplementary Information Fig. S5a).

Similar to net-C assimilation, evapotranspiration on day 34 was strongly affected by both cultivar (linear model, $F = 30.1$, $p < 0.001$) and treatment (linear model, $F = 30.0$, $p < 0.001$), with no interaction. Evapotranspiration was lower in cuvettes treated with drought, with means ($\pm$ SE) for all cultivars together being 58 ($\pm$ 3) mol H_2O m^{-2} d^{-1} in well-watered cuvettes and 30 ($\pm$ 3) mol H_2O m^{-2} d^{-1} in drought-treated cuvettes. There was no noticeable effect of pipecolic acid. Cultivar differences in evapotranspiration rates were very similar to those for C assimilation, with Estrella del Balcón having the highest values, followed by Valenciano, then Bella Rosa and Flor de Baladre having lower rates (Table 1). Analysis of evapotranspiration across the five sampling days found an interaction between cultivar and treatment (mixed effects model, *ChiSq* = 50.1, $p < 0.001$), again with the cultivar Estrella del Balcón maintaining

higher evapotranspiration under the drought only treatment (Supplementary Information Fig. S5b).

The effect of tomato cultivar on root exudation

There was a significant effect of cultivar on root exudation of TOC per root area (linear model, $F = 4.4$, $p < 0.01$), with mean ($\pm$ SE) exudation rates highest for Estrella del Balcón (15.9 $\pm$ 2.4 cm^{-2} root h^{-1}) and Valenciano (12.9 $\pm$ 1.1 cm^{-2} root h^{-1}) followed by Flor de Baladre (11.5 $\pm$ 1.7 cm^{-2} root h^{-1}), and with lowest TOC exudation for Bella Rosa (10.4 $\pm$ 3.2 cm^{-2} root h^{-1}). A significant difference in the specific TOC exudation rate was found between Bella Rosa and both Estrella del Balcón and Valenciano (Fig. 1).

Mean ($\pm$ SE) exudation rate of TN was highest for Estrella del Balcón (1.4 $\pm$ 0.9 cm^{-2} root h^{-1}), followed by for Flor de Baladre (1.1 $\pm$ 0.2 cm^{-2} root h^{-1}), then Valenciano (1.0 $\pm$ cm^{-2} root h^{-1}), and lastly Bella Rosa (0.8 $\pm$ 0.2 cm^{-2} root h^{-1}) (Fig. 1). Although there was no significant effect of cultivar in TN exudation per root area, there was a significant difference in exudation rates between Estrella del
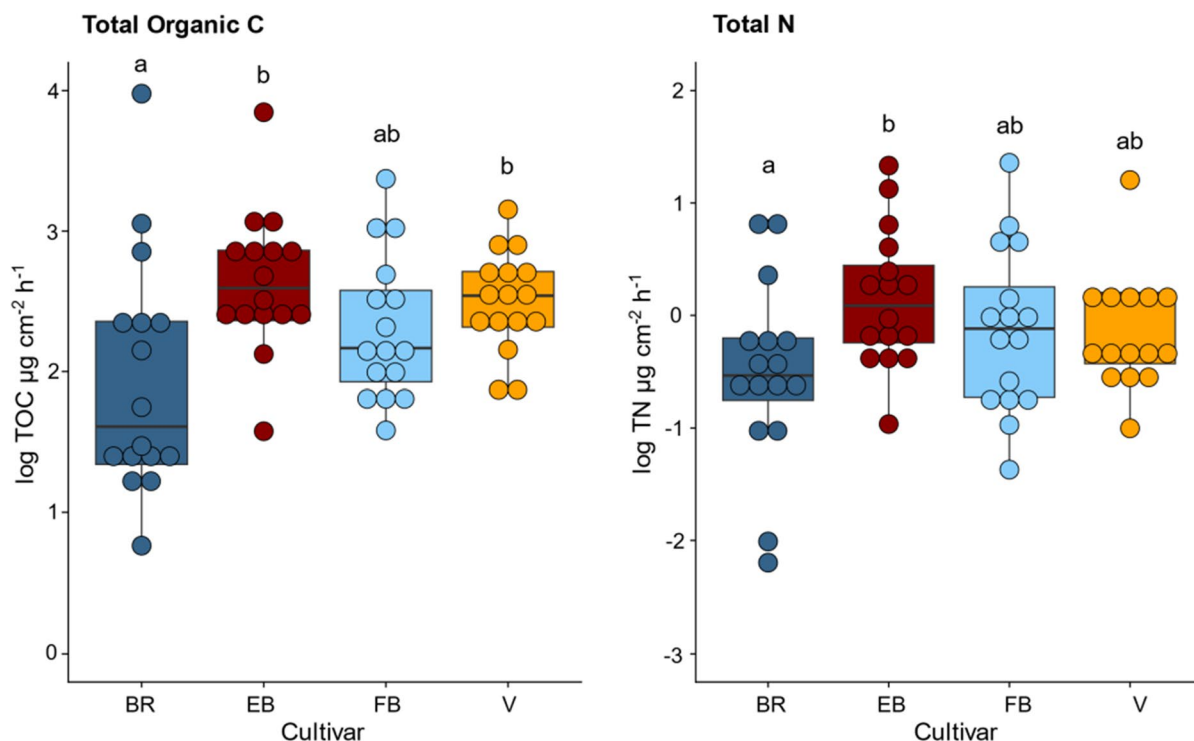
Fig. 1 Root exudation of TOC and TN per root area per hour (on a natural log scale) for the four tomato cultivars. Each point represents a plant. Letters a and b show significant differences ($p < 0.05$) from a Tukey Test. Cultivar names are abbreviated to: BR (Bella Rosa), EB (Estrella del Balcón), FB (Flor de Baladre) and V (Valenciano)

Balcón and Bella Rosa (Tukey Test, $p < 0.05$). There was a positive correlation between TOC and TN exudation rates per root area (linear mixed effect model, $t = 8.0$, $p < 0.001$, conditional $R^2 = 0.51$) (Supplementary Information Fig. S6).

Drought and simulated pathogen attack on exudation per root area

Drought had a positive effect on the specific exudation rate of TOC per root area (mixed effects model, $t = 3.8$, $p < 0.001$, Fig. 2a). Mean TOC exudation rate per root area was 1.7 times higher in the treatments with drought compared to those without (15.6 ± 1.9 cm^{-2} root h^{-1} compared with 9.4 ± 0.9 cm^{-2} root h^{-1}, respectively). However, there was no effect of the pipecolic acid treatment and no interaction between the drought treatment and the pipecolic acid application.

When considering the four cultivars separately, Bella Rosa showed a drought * pipecolic acid interaction (linear model, $t = -2.3$, $p < 0.05$), and Flor

de Baladre showed a positive drought effect (linear model, $t = 2.5$, $p < 0.05$), whereas the other two cultivars did not show significant effects (Fig. 3).

Results were very similar for the exudation rate of N per root area, with an overall drought effect (linear mixed effects model, $t = 5.0$, $p < 0.001$) but no interaction with pipecolic acid, or single pipecolic acid effect (Fig. 2b). Mean TN exudation rate per root area was 1.9 times higher in the treatments with drought compared to those without (1.42 ± 0.2 cm^{-2} root h^{-1} compared with 0.73 ± 0.1 cm^{-2} root h^{-1}). The results for the separate cultivars showed that drought increased specific exudation for Bella Rosa (linear model, $t = 3.9$, $p < 0.01$), and also for Flor de Baladre (linear model, $t = 2.8$, $p < 0.05$) (Fig. 4).

There was a strong negative correlation between exudation rate per root area and dry root mass, for both TOC (linear mixed effects model, $t = -5.6$, $p < 0.001$, conditional $R^2 = 0.41$) and TN (linear mixed effects model, $t = -5.7$, $p < 0.001$, conditional $R^2 = 0.39$) (Supplementary Information Fig. S7).
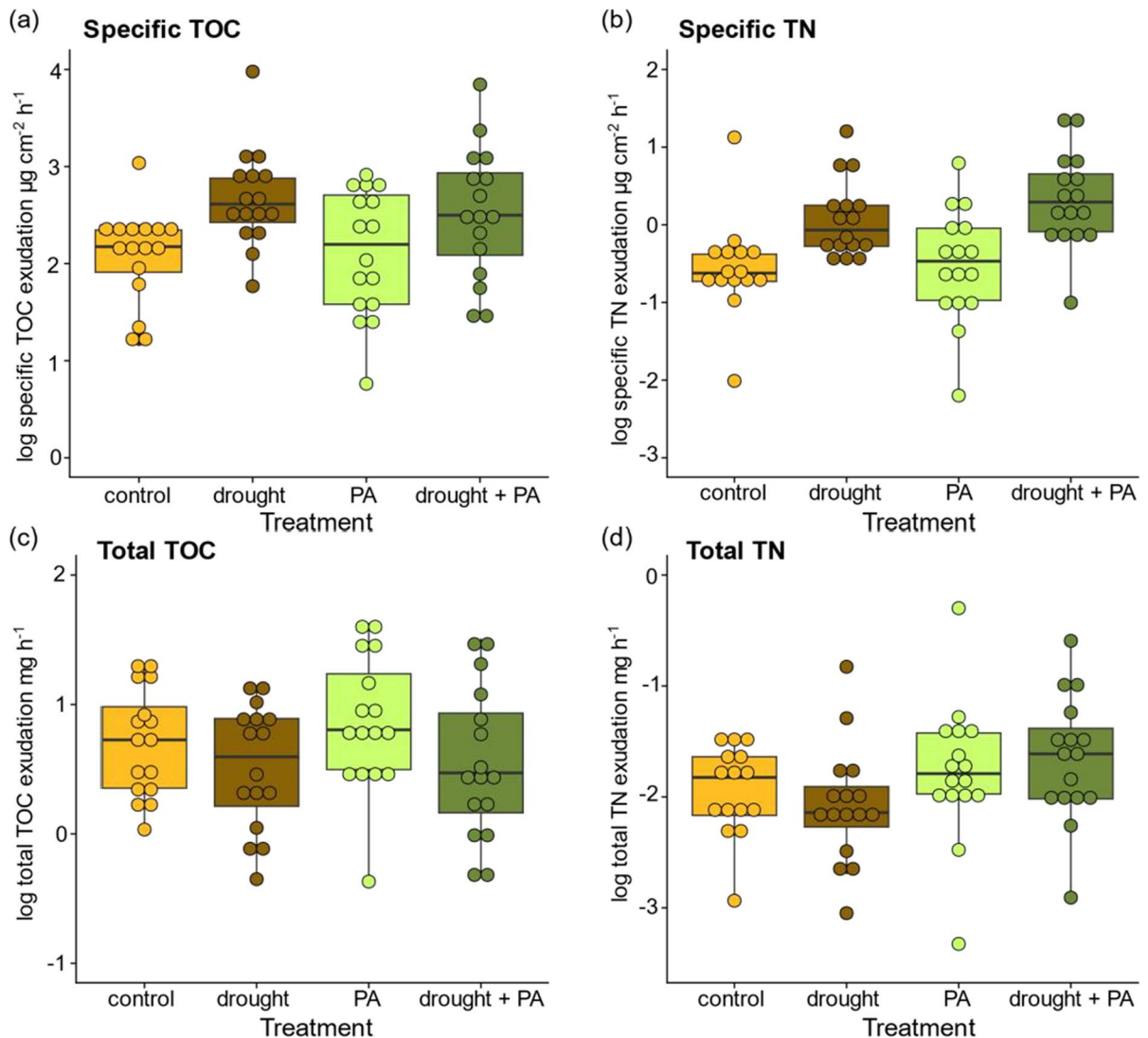
Fig. 2 Exudation rates by treatment, shown as specific exudation rate per root area of TOC (**a**) and TN (**b**), and total exudation rate per plant of TOC (**c**) and TN (**d**). Drought had a positive effect on specific exudation rates of TOC ($p < 0.001$) and TN ($p < 0.05$). Drought had a negative effect on total TOC exudation ($p < 0.05$) and pipecolic acid had a positive effect on total N exudation ($p < 0.05$). For all panels, each point represents a plant, and exudation is shown on a natural log scale, with all tomato cultivars grouped together

This negative relationship was not affected by either the drought or the pipecolic acid treatment.

Total exudation per plant

The total exudation rate of TOC per plant was also calculated, representing the product of the exudation rate per root mass and the total root mass of that plant. As already shown above, drought increased exudation rate per root area but decreased root mass, and total exudation per plant also showed a small negative effect of drought (linear mixed effects model, $t = -2.2$, $p < 0.05$) (Fig. 2c). However, there was a smaller effect size, with plants under drought releasing 1.3 times less TOC than plants receiving adequate water (total biomass was 1.8 times less). This indicated that the decrease in root (and overall plant) biomass was partially
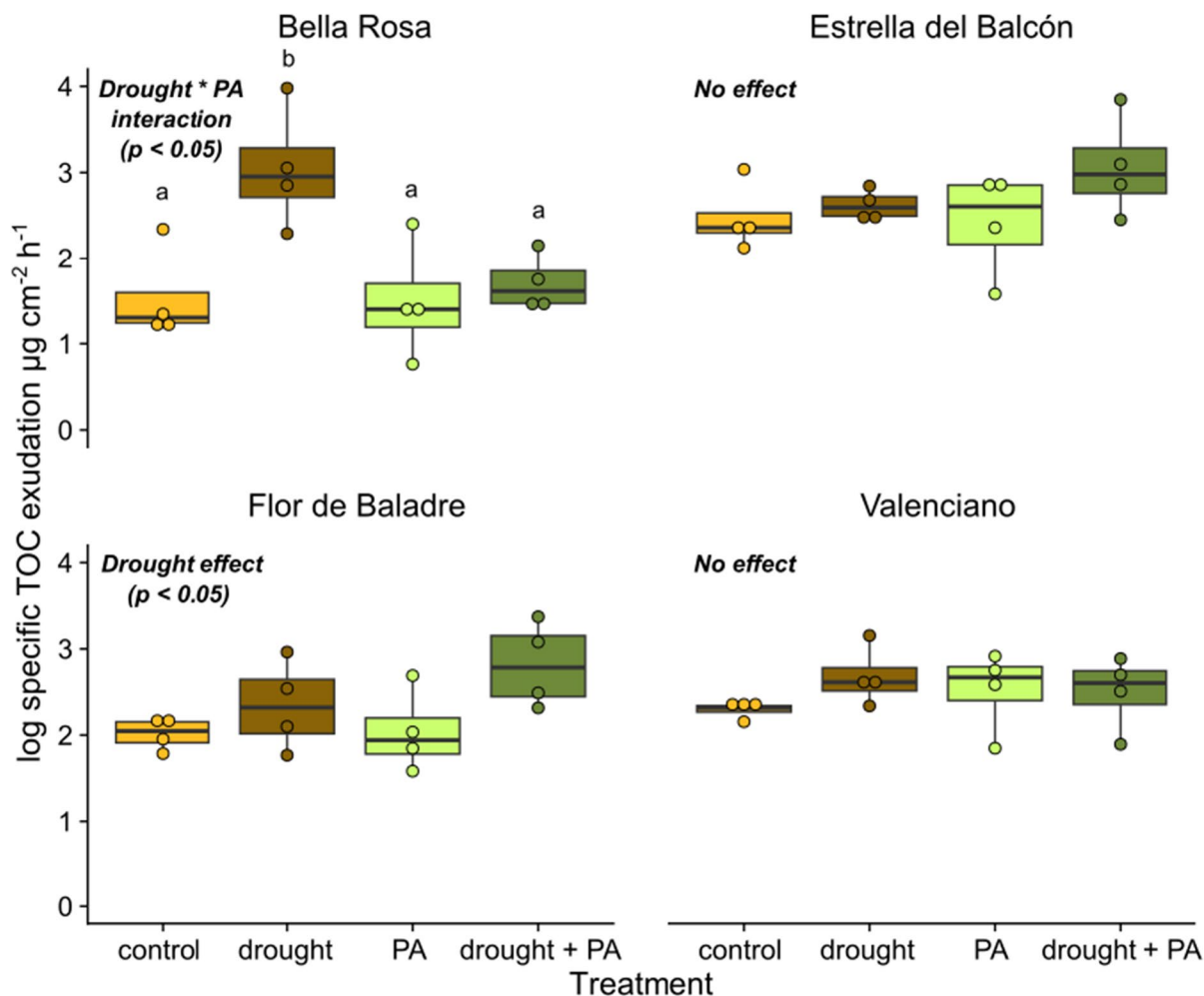
Fig. 3 Exudation rate of TOC per root area on a natural log scale for the four different treatments, with cultivars shown separately. Letters a and b show significant differences ($p < 0.05$) from a Tukey Test. Each point represents a plant

compensated for by the increase in the rate of specific exudation (summarised in Fig. 5).

There was also an effect of cultivar (linear model, $F = 3.9$, $p < 0.05$), with total exudation rate of TOC per plant being 1.7 times higher in Valenciano, the cultivar with highest exudation (2.8 mg h^{-1}) compared with Bella Rosa, the cultivar with lowest exudation (1.6 mg h^{-1}). Moreover, treatment effects varied by cultivar. Estrella del Balcón demonstrated a positive effect of pipecolic acid on total exudation of TOC (linear model, $F = 6.6$, $p < 0.05$), and this same pattern was shown for Flor de Baladre, but the effect was not significant (linear model, $F = 3.9$, $p = 0.07$) (Fig. 6). However, a significant negative effect of drought was found for Valenciano (linear model,

$F = 8.6$, $p < 0.05$). For Bella Rosa there was an interaction between drought and pipecolic acid, such that drought alone increased exudation, however, drought and pipecolic acid decreased exudation (linear model, $F = 4.9$, $p < 0.05$) (Fig. 6).

A different result was shown for total exudation of nitrogen per plant, with a positive effect of pipecolic acid (linear mixed effects model, $t = 2.2$, $p < 0.05$), when all cultivars were combined, and no effect of drought, nor any interaction (Fig. 2d). There was no significant effect of cultivar on total exudation of N, but cultivars nonetheless did behave differently from each other. In particular, Estrella del Balcón showed an interaction between drought and pipecolic acid (linear model, $F = 4.8$, $p < 0.05$), such that while
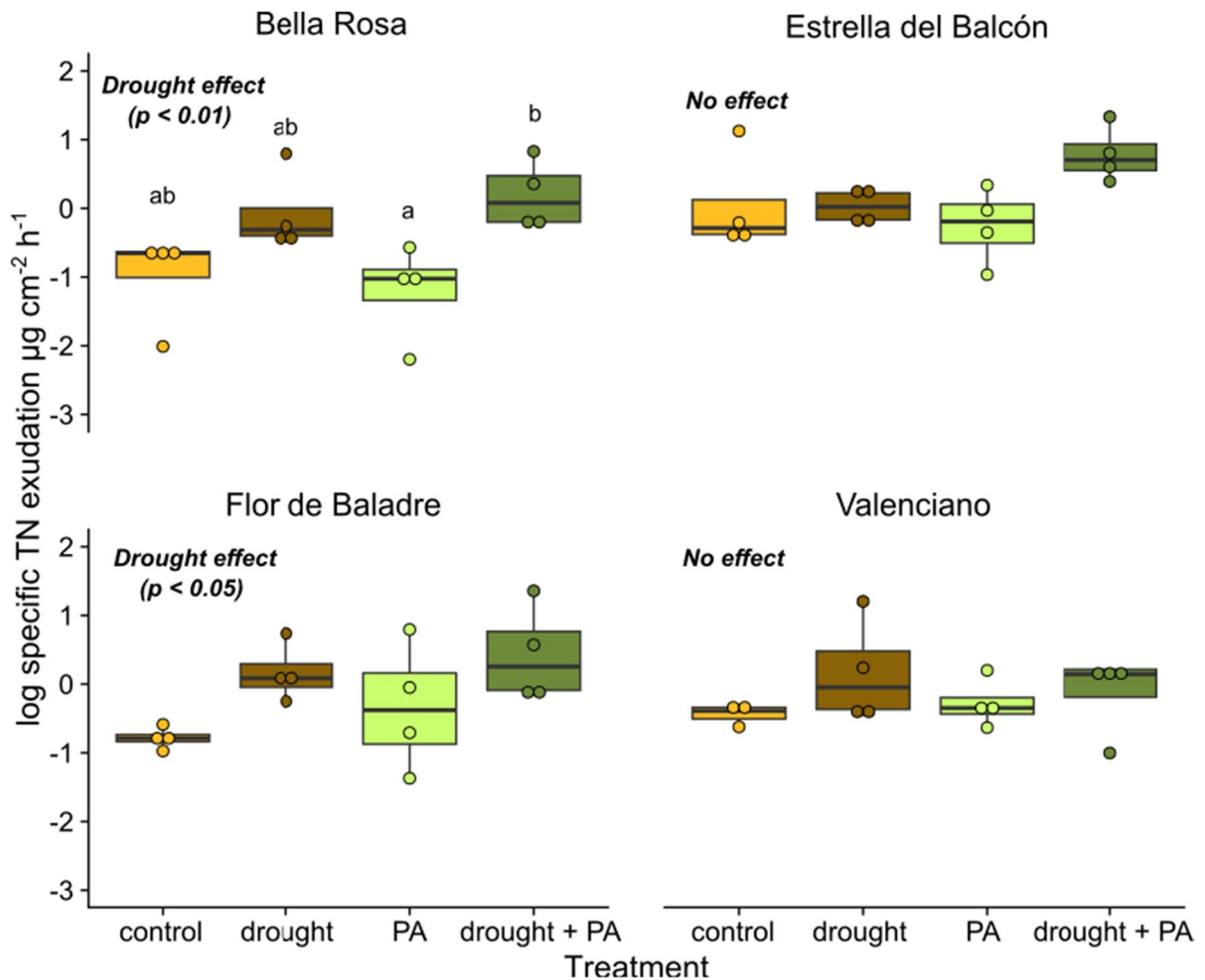
Fig. 4 Exudation rate of TN per root area on a natural log scale for the four different treatments, with cultivars shown separately. Letters a and b show significant differences ($p < 0.05$) from a Tukey Test. Each point represents a plant

drought alone decreased exudation, when drought and pipecolic acid were both added, exudation increased (Fig. 7). This was the same pattern shown for total TOC exudation for the same cultivar and in Flor de Baladre (Fig. 6).

C:N ratio of root exudates

The C:N ratio of root exudates was very variable between samples, ranging from 2.5 to 30.5. There was no cultivar effect, but there was a marginally significant interaction effect of the drought and pipecolic acid ($t = -1.9$, $p = 0.06$). This resulted from the combined drought and pipecolic acid treatment having a lower C:N composition than the other treatments,

signifying a relative decrease in TOC and a relative increase in TN in exudates (Supplementary Information Fig. S8).

Discussion

Biomass and net-C assimilation was decreased by drought

Changes in biomass are often found in experiments with abiotic and biotic stresses, and in this experiment, the drought treatment dramatically reduced the overall plant biomass across all tissue types (root, stem and leaf), as well as net-C assimilation. It is
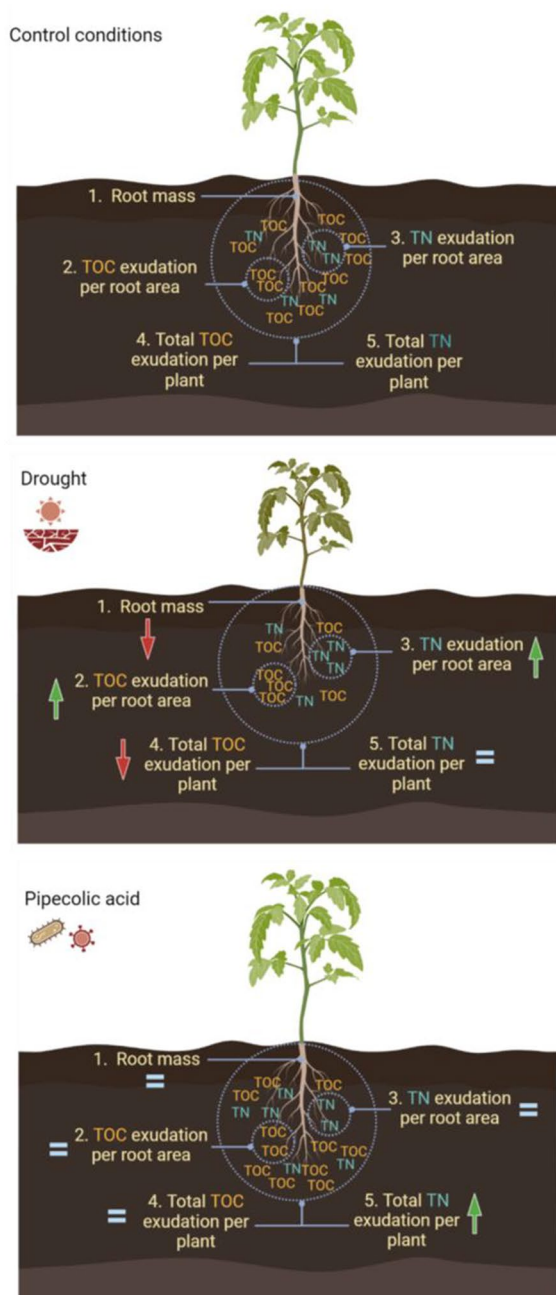
Fig. 5 Summary of the effects of drought and pipecolic acid on exudation of TOC and TN from tomato roots. Root biomass under drought was lower than under adequate watering (control conditions), but specific exudation of TOC and TN per root area was increased. Total exudation of TOC per plant was lower under drought but exudation of TN per plant was maintained at control levels. Addition of pipecolic acid did not affect root biomass or specific exudation but did increase total exudation of nitrogen per plant due to a decrease in the C:N ratio of exudates, particularly when drought and pipecolic acid were combined. There was no significant interaction between drought and pipecolic acid when all cultivars were grouped together

also expected that the induction of systemic acquired resistance (SAR) should result in lower allocation to biomass (Heil et al. 2000; Walters and Heil 2007), although SAR can be induced without having an impact on biomass. In this study, the pipecolic acid addition did not affect plant biomass or net-C assimilation which may indicate that the treatment was not fully effective in simulating a pathogen infection, or that the duration of the treatment was not long enough to see such an effect. However, this would have to be confirmed with a pathogen assay and molecular analysis of pathogenesis-related (*PR*) genes, which was not carried out in this experiment. Also, there were other effects of the pipecolic acid addition, particularly on the exudation of N, suggesting that it was effective.

Drought increased exudation of C and N per root area

The drought treatment was the dominant factor affecting the specific exudation rates of both TOC and TN, when considering all tomato cultivars together. Drought increased exudation rates, in line with our first hypothesis. Increases in exudation rates under water stress have been shown before in many studies with a variety of plant species and measurement techniques (Henry et al. 2007; Preece and Peñuelas 2016; Karlowsky et al. 2018; Preece et al. 2018). It is important to note that while there was this overall drought effect, when tomato cultivars were considered individually, results were more nuanced, and cultivar-specific effects were evident, in agreement with our fourth hypothesis. For specific exudation of both TOC and TN, two cultivars (Estrella del Balcón and Valenciano) did not show significant effects of the drought treatment (or pipecolic acid), indicating that drought responses vary between tomato cultivars and that these two cultivars are potentially more drought resistant. This agrees with the net-C assimilation and evapotranspiration measurements which also indicated that these two cultivars had higher gas exchange rates over all treatments. Further evidence that Estrella del Balcón was potentially the most resistant to drought, was that net-C assimilation and evapotranspiration did not decrease as much as in other cultivars. The other two cultivars, Bella Rosa and Flor de Baladre, did show increased exudation rates due to drought, although for TOC exudation in Bella Rosa this was only evident in the drought-only
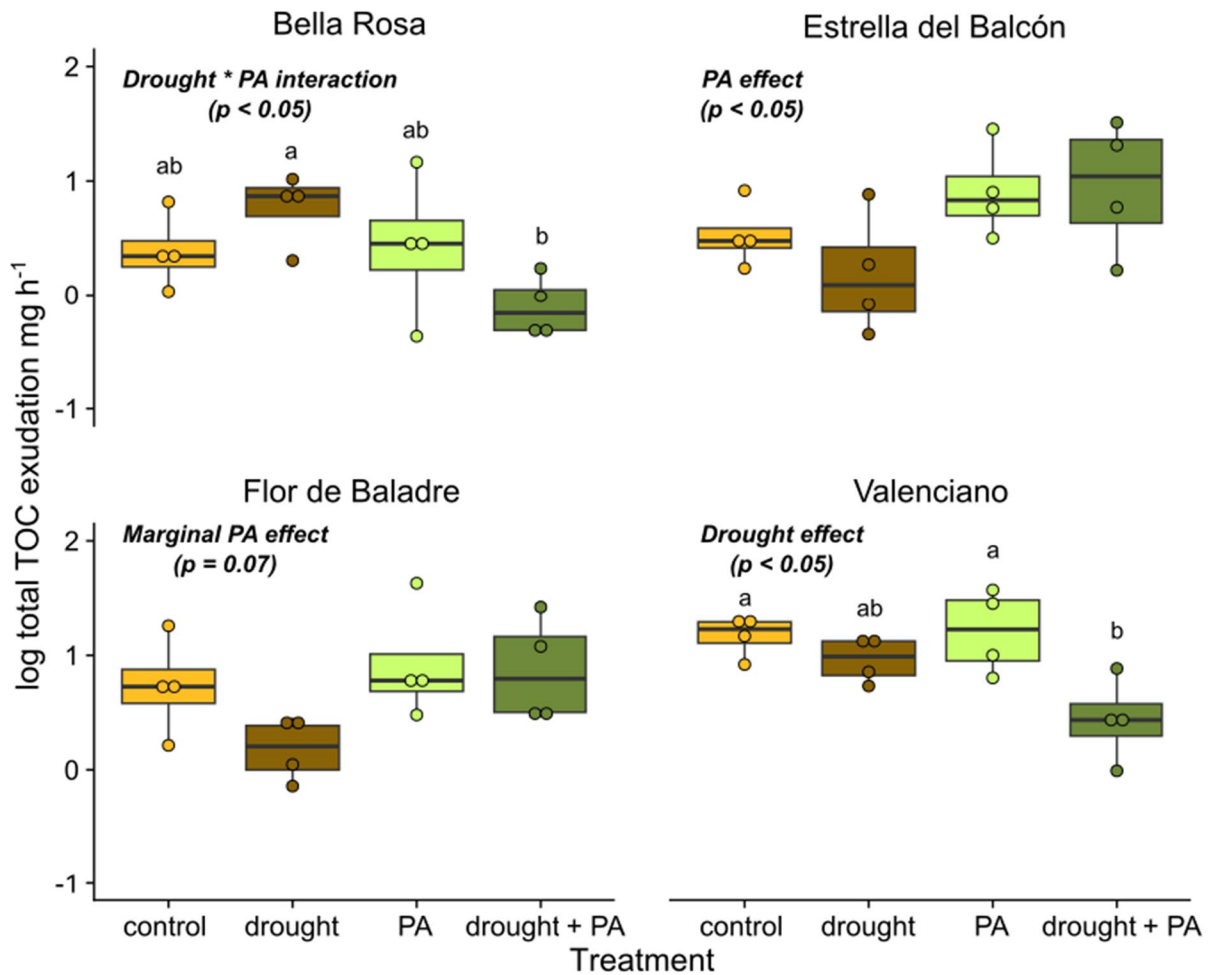
Fig. 6 Total exudation rate of TOC per plant on a logged scale for the four different treatments, with cultivars shown separately. Letters a and b show significant differences ($p < 0.05$) from a Tukey Test. Each point represents a plant

treatment, and when drought was combined with the pipecolic acid addition there was no change from the control.

Previous studies have searched for general patterns between root exudation and root morphology, but results are variable with both positive and negative relationships found between exudation rates and root length or root area (Wen et al. 2022). A recent study with 17 common perennial European grassland species showed positive relationships between exudation and root diameter which is linked to microbial collaboration and outsourcing of nutrient acquisition (Williams et al. 2022). In our experiment, the strong negative correlation between root biomass and root exudation per root area clearly indicates a trade-off

in allocation either to making roots or to increasing exudation. This implies that under drought plants are unable to allocate the same resources (in terms of carbon) to constructing roots, but by up-regulating root exudation in the roots that are present, the physiological activity of the roots can be maintained to some extent.

Our hypothesis that the addition of pipecolic acid would stimulate specific exudation rates was not confirmed, as there was no overall effect of the simulation of pathogen attack. This could indicate that the drought treatment had a stronger effect on plant physiology, at least at this scale. Clear evidence supporting the hypothesis that the combined treatment (drought and simulated pathogen attack) had the
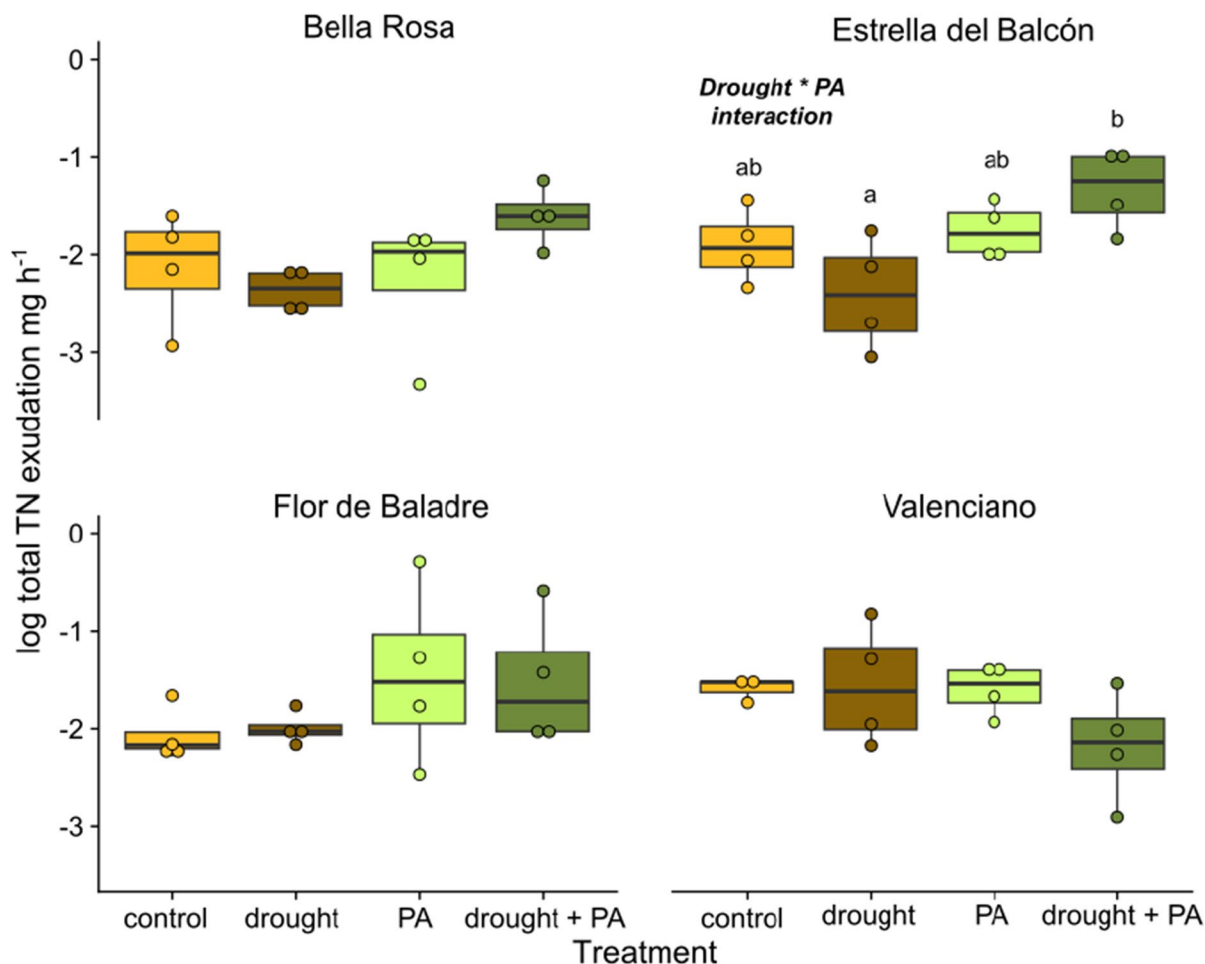
Fig. 7 Total exudation rate of TN per plant on a logged scale for the four different treatments, with cultivars shown separately. Letters a and b show significant differences ($p < 0.05$) from a Tukey Test. Each point represents a plant

strongest effect on root exudation (hypothesis 3) was not found. When interacting factors are present, such as in this study, the overall effects on plant physiological processes, such as exudation, are likely to be complex and potentially antagonistic at the level of overall exudation rates. Future studies that also consider changes in root exudate composition might provide a clearer understanding of these complexities. As we discuss below, results at the whole-plant level did reveal a response to pipecolic acid, in terms of higher total TN exudation.

It should be noted that all methods of measurement of root exudates are subject to drawbacks. In this case, the collection of exudates so soon after the experiment concluded (without allowing for a lengthy hydroponic recovery period as recommended

in Williams et al. 2021), might have led to an overestimation of exudation. This overestimation could be attributed to the additional measurement of carbon and nitrogen released from damaged roots during the root washing process. However, great care was taken to limit the manipulation of the roots and any minor damage would have been equal between the treatments and should not have affected the drought effect that we observed. More studies are warranted to further support these results.

Drought effects on total exudation of carbon and nitrogen are minimal

By calculating the total exudation per plant, we showed that, when all cultivars were pooled, increased

specific exudation rate of TOC under drought offsets the decrease in root biomass, resulting in total exudation rates per plant that were only slightly lower than under well-watered conditions. For nitrogen, this compensation effect was even greater, with no significant difference in total exudation found due to the drought treatment. This reflects results in trees, where the maintenance of total exudation has been shown following experimental drought in a *Fagus sylvatica* and *Picea abies* forest (Brunn et al. 2022). A review of drought effects on rhizodeposition also found that a drought-related reduction in root biomass was often the main driver of plant-level reductions in rhizodeposition (Preece and Peñuelas 2016).

As with other measured parameters, there were differences between cultivars, and while drought decreased total exudation at the species level, at the cultivar level, drought was only the main driver of total exudation of TOC for Valenciano. In contrast, for Estrella del Balcón and Flor de Baladre, pipecolic acid increased exudation (although only significant for Estrella del Balcón), and the two treatments interacted with each other in Bella Rosa. This underlines the important phenotype effect. The importance of differences between accessions has previously been shown for root exudate composition in genetically diverse accessions of Arabidopsis, further emphasising the importance of considering multiple populations (Mönchgesang et al. 2016). Given that only four tomato cultivars were tested here, all of which are usually grown under conditions with irrigation, it is likely that we would have found an even greater variability in responses if more cultivars had been included, such as those normally grown outside in rainfed systems. This also highlights the need to preserve diversity in crop cultivars, in order to increase the chances of identifying cultivars with advantageous root traits, such as for higher drought or pathogen tolerance.

Total exudation of nitrogen is increased under simulated pathogen attack

Pipecolic acid generally had fewer effects on plant growth and root exudation compared with drought, but, when considering all cultivars together, simulated pathogen attack did increase the total amount of nitrogen exuded per plant. This was apparently achieved, not by an increase in the specific exudation rate, but by a change in the composition of exudates, to have a higher proportion of N-containing compounds. This lowering of the C:N ratio in exudates was particularly driven by the combined treatment of both drought and pipecolic acid addition. A higher proportion of N in root exudates may indicate a shift towards exudation of amino acids, though this was not specifically measured in this study. Other studies have found exudation of amino acids to increase during drought (Bobille et al. 2019), during recovery following drought (Gargallo-Garriga et al. 2018), and following infection with the bacterial pathogen *Pseudomonas syringae* pv *tomato* (Yuan et al. 2018), and to decrease when plants are under N deficiency (Carvalhais et al. 2011).

Decreases in exudate C:N ratio increase the decomposability of root exudates, which constitute a labile fraction of soil organic matter. Lower C:N ratios in the labile soil fraction, including exudates, were found to be positively correlated with higher N mineralization in a N-limited grassland (Fornara et al. 2011). Labile exudates induce soil priming, leading to additional effects on soil microbial turnover and community structure (Liu et al. 2017; Zhou et al. 2021). Moreover, this change in the C:N ratio suggests that alterations in exudate composition are likely to be occurring even when there are no changes in exudation rates, as found in a recent study that observed microbial responses to exudates produced by drought-stressed plants, despite overall C content being accounted for (De Vries et al. 2019).

Significant effects of the treatments on total N exudation were more difficult to see at the cultivar level, likely due to the large variability in measurements and the relatively low replication rate. Only Estrella del Balcón showed a significant effect, with the combined drought and pipecolic acid treatment inducing more exudation of N per plant than the drought only treatment.

Conclusion

Under moderate drought conditions, the specific rates of TOC and TN exudation per root area increased when considering all four tomato cultivars. However, the simulated pathogen attack did not have a discernible effect on specific exudation. The total exudation of TOC per plant slightly decreased due to reduced root biomass caused by drought, but because of the higher specific exudation this was to a much lesser extent than the biomass differences. In contrast, the total exudation of nitrogen increased

following the addition of pipecolic acid, resulting in a shift towards a higher N content and lower C content in the exudates. Notably, there were noticeable variations among the different tomato cultivars in response to the treatments, both in terms of specific and total exudation rates. These differences indicate varying levels of resistance to water stress and pathogen attack among the cultivars. Overall, drought had a more pronounced impact on total C exudation, while the simulated pathogen attack exerted a greater influence on total N exudation.

Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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