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Maize roots modulate microbial functional traits in the rhizosphere to mitigate drought stress

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ABSTRACT

Drought affects soil C sequestration by altering the availability of nutrients to plants and microorganisms. However, the mechanisms of plant-microbe interactions and the potential role of root hairs, which enlarge the root-soil interface, in maintaining rhizosphere processes under drought remain uncertain. We investigated the effect of a 7-day drought on root gene expression in two maize plants, a root hair-deficient mutant and its corresponding wild-type, and its correlation with rhizosphere functions: microbial growth and enzyme kinetics related to organic matter decomposition. Under drought, roots reduced the expression of several *chitinase*, *acid phosphatase* and pathogenesis-related genes. In parallel, drought reduced the maximum enzymatic rate of β-glucosidase and acid phosphatase by 3.5- and 1.9-fold, respectively, while the affinity of these enzymes in the rhizosphere increased by 35 and 71 %, respectively, compared to the well-watered treatment. The effect of drought was more pronounced in the rhizosphere of wild-type maize than in that of the mutant. Notably, leucine aminopeptidase and N-acetylglucosaminidase did not respond to drought. Inhibition by high substrate concentrations was observed for β-glucosidase and acid phosphatase only under drought, highlighting the potential use of the substrate inhibition model as a complementary indicator of altered enzyme systems in response to environmental regulators. Finally, drought prolonged the microbial lag phase by up to 24 h and reduced the microbial specific growth rate by up to 36 % compared to the well-watered treatment. The maximum specific growth rate recovered after rewetting of the soil, demonstrating the sustainability of microbial function after a short-term drought.

1. Introduction

The impact of stressful conditions, such as drought periods, on soil organic matter dynamics and, consequently, on soil health, is becoming increasingly evident (Sang et al., 2022; Yakushev et al., 2023). Drought impacts the availability and turnover of nutrients in the rhizosphere of plants, where microbial communities are able to use root-derived organic compounds for growth (Zhang et al., 2023). Plant roots modulate microbial growth through rhizodeposition, which encompasses the release of cells, proteins, extracellular enzymes, and other metabolites into the rhizosphere (Jacoby et al., 2017). The components of rhizodeposits are involved in a variety of functions, including the modulation of phosphate (organic acids) or iron availability (siderophores), and the attraction or inhibition of rhizosphere bacteria by certain flavonoids, terpenes, or pathogenesis-related proteins (Dennis et al., 2010; De-la-Peña et al., 2010). Changes in rhizodeposition may, therefore, serve to mitigate the effects of drought on microbial functioning within the rhizosphere, as evidenced by corresponding alterations in gene expression levels within root cells (Maron et al., 2010; Ganther et al., 2022; Bilyera et al., 2022). Furthermore, roots may respond to drought by increasing root hair development, resulting in greater rhizosphere extension and water retention, thereby increasing microbial activity (Holz et al., 2018). However, the contribution of root hairs to nutrient cycling in the rhizosphere under drought is still unclear and could be investigated using root hair-deficient mutants. For example, by comparing a wild-type maize with its root hair deficient mutant *rth3*,

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root hairs significantly increased rhizosheath formation in maize by a factor of 1.8 (Burak et al., 2021). The rhizosheath is a specific environment at the closest root-soil interface, stimulated by the presence of root hairs and mucilage (Liu et al., 2019; Etesami, 2021; Zhang et al., 2020), which exhibits greater stability than adjacent soil to drought (Brown et al., 2017; Pang et al., 2017; Steiner et al., 2024). Therefore, processes within the rhizosheath may remain unaltered by drought, although drought may have an impact in the immediate vicinity, where the influence of the root is less pronounced than that of the abiotic stressor. In this way, the importance of root hairs in maintaining rhizosphere functions during drought could be elucidated by differentiating the soil directly attached to the root surface and the following soil fraction.

Transformation of organic C in soil is supported by extracellular hydrolytic enzymes that mediate the degradation of large organic polymers into labile monomeric sources of C (e.g. β-glucosidase), N (e.g. N-acetylglucosaminidase and leucine aminopeptidase) and P (e.g. acid phosphatase). These monomeric sources are subsequently available for microbial uptake (Zhang et al., 2019; Mavrodi et al., 2021). Changes in functional traits of hydrolytic enzymes in response to drought can be elucidated by assessing enzyme kinetics. The Michaelis-Menten equation (Aon and Colaneri, 2001) allows the affinity constant (K_m) and the maximum enzymatic rate (V_{max}) of enzymes to be estimated (Tian et al., 2020; Alves et al., 2021). However, this approach cannot discriminate between microbial and plant-derived enzyme sources (Castro et al., 2010; Pérez-Izquierdo et al., 2019; Staszel et al., 2022). Another significant aspect of the Michaelis-Menten approach, which is based on the range of increasing substrate concentrations, is that certain enzymes exhibit inhibition by elevated substrate levels. This serves as a regulatory feedback mechanism for the decomposition process (Haldane, 1965). Consequently, the sensitivity of enzymes to saturating substrate concentrations may elucidate the strength of the reaction regulation in response to environmental stressors. Despite this phenomenon is known in enzymology, the substrate inhibition model has yet to be described in studies on rhizosphere functions under drought.

Drought determines the concentration and localisation of substrates in soil pores, limiting enzyme accessibility to them and thus affecting microbial growth and respiration (Allison, 2005; Schimel et al., 2007). Periods of drought followed by rewetting of soils generate CO_2 pulses to the atmosphere, a phenomenon known as the Birch effect (Birch, 1958). These CO_2–C losses from the soil, resulting from stimulated microbial activity and growth, contribute to soil decarbonisation and greenhouse gas emissions (Fierer and Schimel, 2002; Xiang et al., 2008; Liu et al., 2022) and are accompanied by heat dissipation, which is considered a reliable indicator of microbial metabolic response and activity (Herrmann et al., 2014). Consequently, the functional properties of the dominant microbial groups following drying-rewetting events, including the maximum specific growth rate (μ_{max}) and the lag time before exponential growth, can be determined by heat release under unlimited growth conditions by adding an excess of available substrates and nutrients (Blagodatsky et al., 2000; Braissant et al., 2010; Wutzler et al., 2012). Isothermal microcalorimetry, which is based on the measurement of heat dissipation over time from microbial activity and growth, is a highly sensitive technique that requires only a small amount of soil, making it particularly useful for studies that are restricted by the availability of soil sample. The presence of root hairs can mitigate the impact of drought by extending the rhizosphere (Ma et al., 2018), as mentioned before. However, the precise interplay between drought and the presence of root hairs in influencing the response of microorganisms to soil rewetting awaits further investigation.

The objective of this study was to evaluate how soil organic matter transformation, which is regulated by plant-microbe interactions through extracellular enzyme activity, is affected by drought (abiotic factor). We also aimed to determine whether root hairs (biotic factor) are essential to mitigate the effects of drought on microbial growth and enzyme activity in different compartments of maize rhizosphere. To this end, we combined analysis of root gene expression levels associated with enzyme production by root cells with analysis of enzyme kinetics in the maize rhizosphere. This was prompted by the positive correlations observed in maize roots between transcriptomics and proteomics (Ebinezer et al., 2020) and between enzyme activity and acid phosphatase gene expression (Ganther et al., 2022). We also investigated the expression of maize pathogenesis-related proteins (De-la-Peña et al., 2010) as an additional interactive factor affecting microbial growth. For this purpose, a root hair deficient mutant of maize (*rth3*) and its corresponding wild-type (WT) were grown in soil columns and exposed to a seven-day drought period. We hypothesized that (1) drought will slow down microbial growth and decrease the V_{max} of enzymes because of the reduced accessibility to nutrients and substrates through reduced water content or due to changes in the production of enzymes; (2) the effect of root hairs on rhizosphere processes will be more pronounced under drought than under optimal soil moisture because root hairs exhibit benefits for the plants especially under drought stress (Holz et al., 2018). It was expected that these effects would be more pronounced in WT than in *rth3* maize, as the former showed higher shoot biomass production than the *rth3* mutant in the field (Vetterlein et al., 2022) and in an independent column experiment (Lippold et al., 2021). Therefore, WT plants might be exposed to a slightly higher stress intensity due to leaf water loss compared to *rth3*.

2. Materials and methods

2.1. Plant growth conditions and harvest: root and soil sampling

The experimental design was a two factorial (plant genotype and water level) randomized column design with six biological replicates per treatment. Two maize genotypes differing in root morphology, the root hair deficient mutant *rth*3 and its corresponding wild-type *Zea mays* L. B73 (WT) were grown in soil columns (25 cm height and 7 cm inner diameter) in a climate chamber under the following conditions: 12 h of daylight at 29 °C, 12 h of night at 20 °C, 45 and 58 % of relative humidity during day and night, respectively, and 350 μmol m^{-2} s^{-1} of photo-synthetically active radiation. Details of the experimental conditions can be found in Hartwig et al. (2025). Briefly, the columns were filled with a haplic Phaeozem soil with a maximum water holding capacity (MWHC) of 51 % (w/w), which was fertilized with N, P, K and Mg to obtain an adequate available nutrients content. All pots were adjusted to a water content in soil of 17.5 % (w/w), corresponding to 35 % of the MWHC, optimal for plant growth under these experimental conditions. Details on soil characteristics and fertilization are described in Vetterlein et al. (2021). Two water levels were applied: the well-watered and the drought treatment. In the well-watered treatment, plants were kept at 17.5 % of water content in soil until day 22. In the drought treatment, plants were grown under optimal watering until day 15; thereafter, plants were not watered between day 15 and 22. The duration of the drought stress was determined in a preliminary experiment in order to obtain, on the day of harvest, a level of stress severe enough to induce metabolic changes, but avoiding the limit of stress tolerance of the plants. The aim was to obtain maximum inhibition of transpiration rates for a few days, which was achieved.

On day 22, plants were harvested and soil from 8 to 12 cm depth was taken from each soil column. Root segments were gently removed to collect the bulk soil. Thereafter, fresh roots were shaken to collect the "loosely root-attached" (LRA) soil. Subsequently, roots were washed using a sterile solution of 0.3 % NaCl in a 1:10 ratio (g/mL), vortexed for 10 s, repeated two times, yielding the "rhizosphere". In this study, the rhizosphere fraction includes the rhizosheath, which represents soil directly attached to the root surface, therefore highly influenced by root hairs. In contrast, LRA soil was believed to have a low contribution of root hairs. The soil samples were stored at 4 °C and analysed within the following 4 days after the sampling. The water content % (w/w) of the bulk and LRA soil was measured with a HB43–H Halogen moisture

analyser Mettler Toledo (Greifensee, Switzerland). Dry weight of soil in the rhizosphere suspension was calculated by drying a 400 µl aliquot of each sample, in three technical replicates, at 55 °C overnight.

2.2. Root gene expression

Fresh washed maize roots from the 8–12 cm depth of four biological replicates per treatment were dried with sterile paper towel and frozen rapidly in liquid nitrogen, subsequently stored at −80 °C until extraction. Roots were powdered under liquid nitrogen and total RNA was extracted by NucleoSpin RNA Plant kit (Macherey-Nagel, Germany) using 50 mg of root powder per extraction. RNA quality and quantity were verified using a NanoDrop 1000 spectrophotometer and an Agilent 2100 Bioanalyser. All samples were of RNA Integrity Value over 8. NovaSeq 6000 (Illumina, US) was used to implement a 150 bp paired-end Illumina library and perform sequencing at the average depth of 20 million reads per sample, at Genewiz (Azenta, Leipzig, Germany). The raw sequences will be available as fastq files in the NCBI Short Read Archive and will be linked to the BioProject accession number PRJNA1190671. Low quality sequences and sequencing artefacts were removed with Trimmomatic v.0.36 (Bolger et al., 2014), and the processed Illumina reads were aligned against the Zea mays B73 RefGen_v5 reference genome using HISAT2 v.2.1.0 (Kim et al., 2015). Read counts were obtained using featureCounts of Subread v.1.6.3 (Liao et al., 2019). Count data was normalized and changes in gene expression were calculated by pairwise comparisons using DESeq2 v. 1.38.2 (Love et al., 2014) and Benjamini-Hochberg method to adjust the p-values. Genes were considered as differentially expressed with a p - adjust <0.05 and $1 <$ absolute log2 - foldchange (LFC) < -1. Permutational analysis of variance (PERMANOVA) was performed using the 'vegan' package (v.2.6–4) applying the 'Adonis'-test with Euclidian distances (Oksanen et al., 2022).

2.3. Extracellular enzyme kinetics

Fresh LRA soil and rhizosphere samples, six biological replicates per treatment, were analysed for kinetics of enzymes related to organic C, N, P, and microbial necromass turnover. Fluorogenic substrates for β-glucosidase (4-methylumbelliferone-β-D-glucoside), acid phosphatase (4-methylumbelliferone-phosphate), leucine aminopeptidase (L-leucine 7-amino-4 -methylcoumarin-hydrochloride), and N-acetylglucosaminidase (4-methylumbelliferone-N-Acetyl-β-D-glucosaminide) were purchased from Sigma Aldrich (Germany). A suspension of 0.2 g of LRA soil was prepared in 20 ml of MilliQ water using low-energy sonication (40 J s^{-1} output energy) for 1 min (German et al., 2012). The rhizosphere suspension washed from the roots was not further diluted and was directly used for the enzymatic assay. Enzyme activities were measured in 96-well microplates using 50 µl of the corresponding soil suspension, 50 µl of buffer (MES or Trizma, for 4-methylumbelliferone, MUF-, or 7-amino-4 -methylcoumarin-hydrochloride, AMC-based substrates, respectively), and 100 µl of substrate in a range of final concentrations: 0, 5, 20, 50, 75, 100, 200, and 400 µM. Fluorescence was measured at 360/465 nm excitation/emission wavelengths and at a bandwidth of 35 nm with a plate reader (TECAN Infinite F200 Pro) after 30, 90 and 150 min of incubation in darkness, at room temperature, and under continuous orbital shaking. Standard curves of MUF and AMC were obtained for the soil suspensions, to counteract for interactions between the product and soil particles, and enzyme activity was expressed as MUF or AMC release over time per gram of soil dry mass (nmol product g^{-1} dry soil h^{-1}).

The Michaelis–Menten function (Eq. (1)) was used to determine enzyme kinetic parameters: the potential maximum enzymatic rate (V_{max}), and affinity constant (K_m) of the analysed enzymes:

$$\upsilon = \frac{V_{max}\, S}{K_m + S} \tag{1}$$

where υ is the rate of the enzyme-mediated reaction, S is the substrate concentration (µM), and K_m is the affinity constant (µM) corresponding to the substrate concentration at half of the maximum enzymatic rate, V_{max} (nmol product g^{-1} dry soil h^{-1}). Additionally, inhibition of enzymes by saturating concentrations of substrate (Haldane, 1965; Kaiser, 1980), which may occur in soil samples, was estimated by the substrate inhibition model equation (Eq. (2)),

$$\upsilon = \frac{V_{max}\, S}{K_m + S\left(1 + \frac{S}{K_i}\right)} \tag{2}$$

where K_i represents the inhibition constant (µM), which corresponds to the concentration of substrate at which the activity of the enzyme decreases, not following the saturation pattern described by the Michaelis-Menten equation. Therefore, high K_i values indicate low sensitivity of enzymes to substrate inhibition, i.e. large amounts of substrate are required to achieve inhibition of the enzymatic reaction. The fitting of enzyme reaction curves to the models (Eqs. (1) and (2)) was performed with OriginPro 2023 (64-bit) 10.0.0.154 (Government) Copyright © 1991–2022 OriginLab Corporation.

Finally, the affinity constant (K_m) of enzymes was expressed as a function of the water content % (w/w) of the LRA soil to assess the sensitivity of the enzymatic systems in their affinity for the substrate to drought.

2.4. Microbial growth

Microbial growth was induced in 3 g of fresh homogenized bulk soil, six biological replicates per treatment, by the addition of glucose and nutrients, and measured by heat dissipation over time using an isothermal calorimeter TAM Air (TA Instruments, Germany) set at 20 °C. Three moisture levels were studied while keeping the same unlimiting substrate concentrations: (1) original soil moisture after 22 days of maize growth under drought and well-watered conditions; (2) rewetted soil to a water content of 15–17 % (w/w); and (3) highly rewetted soil to a water content exceeding 19 % (w/w), to simulate rainfall conditions. Substrate concentrations were as follows (in mg g^{-1} fresh soil): glucose 5.00, NH_4SO_4 0.95, K_2HPO_4 1.12, and $MgSO_4 \bullet 7H_2O$ 1.90, added homogeneously, by stirring, into the soil using a solid carrier (talcum 20 mg g^{-1} soil) in case (1), or sterile water in cases (2) and (3). Glucose was selected as the C source as it is a monomeric organic molecule, essential component of root exudates and microbial metabolites, thus naturally abundant in soil organic matter and readily available for microbial use (Papp et al., 2020; Blagodatskaya et al., 2021). In addition, glucose has been widely used in physiological approaches to measure microbial growth traits (Panikov, 1995; Anderson and Domsch, 1978).

Heat dissipation was measured over time and expressed per grams of dry soil (mW g^{-1} dry soil h^{-1}). During incubation time, soil moisture was unchanged. The microbial intrinsic trait - maximum specific growth rate (μ_{max}) - was calculated by the following exponential model equation (Eq. (3))

$$y(t) = \beta_0 + \beta_1 \exp^{\mu_{max} t} \tag{3}$$

where *y(t)* is the heat (mW) released over time, measured at time *t*; β_0 and β_1 are fitted coefficients corresponding to non-growth and growth-associated heat release; and μ_{max} is the maximum specific growth rate, i.e. potential maximum of active cells, growing under no limitations (Wutzler et al., 2012). As we use non-optimal moisture conditions for microbial growth, we will refer to it as "microbial specific growth rate" (µ). We calculated the lag time as the time point when heat release exceeded the mean upper confidence level, $\alpha = 0.05$, of the first 5 h after substrates addition, by 2-times its standard deviation.

2.5. Statistical analyses

Statistics were performed for a total of 4 treatments resulting from the combination of 2 factors: water level (well-watered, i.e. control, and drought) and maize genotype (WT and *rth3* mutant), each with a sample size of n = 6 biological replicates, and n = 4 biological replicates in the case of gene expression. For microbial growth and enzyme kinetics, the Shapiro-Wilk test was performed to ensure that the data were normally distributed, and Levene's test was performed to assess the homogeneity of variances within treatments. Two-way analysis of variance (ANOVA) followed by post-hoc Fisher LSD test was used to detect significant differences ($p < 0.05$) between treatments. All analyses were performed in RStudio/2023.09.1 + 494.

3. Results

3.1. Plant growth in the climatic chamber

Shoot growth of WT plants was greater than that of the *rth3* mutant, with WT plants exhibiting higher shoot dry weight and leaf area than *rth3* mutant plants on the day before the start of the drought treatment (Fig. S1). After seven days of drought, transpiration rates were 8 % (WT plants) and 10 % (*rth3* mutant) of those of the well-watered plants (Table S1). This correlated with lower soil moisture under WT than *rth3* plants in both treatments, well-watered and drought, as well as after rewetting of the soils (Table 1a).

3.2. Root gene expression

RNA sequencing of maize roots revealed an overall strong effect of drought on gene expression patterns, explaining 61.5 % of the variation in gene expression levels according to PERMANOVA analysis (Fig. S2). WT plants were more affected by drought in their gene expression levels than *rth3* mutants, with 5765 and 3427 differentially expressed genes, respectively, in drought compared to the well-watered treatment (Hartwig et al., 2025). In this study, we focused on the expression of genes related to enzyme production to assess the contribution of root-derived enzymes to rhizosphere hydrolytic activity. Three of the five *acid phosphatase* genes and two of the four *chitinase* genes analysed were down-regulated, and one of each gene group (*PAP2* and *CHN5*, respectively) was up-regulated under drought (Fig. 1a–i). In addition, one of the genes encoding the plasma membrane phosphate transporter PHT1, *PHT1;5*, was also up-regulated under drought compared to the well-watered treatment (Fig. 1j). We then assessed the expression of maize root genes related to plant defence and rhizodeposition as modulators of rhizosphere microbial community composition, which potentially affects their functions. Indeed, drought treatment reduced the expression of genes encoding pathogenesis-related proteins, genes involved in siderophore, flavonoid and benzoxazinoid biosynthesis, and ABC transporters (Table S2). This effect was modulated by the maize genotype, as the number of genes down-regulated by drought was lower in the *rth3* mutant compared to WT plants. A direct comparison of WT and *rth3* maize under drought revealed a higher relative expression of pathogenesis-related proteins and benzoxazinoid biosynthesis-related genes in *rth3* compared to WT roots. A detailed analysis is published elsewhere (Hartwig et al., 2025) and here we focused on gene expression levels related to rhizosphere functions.

Table 1
Soil water content % (w/w) (a), and microbial specific growth rates (b) in bulk soil collected under a root hair deficient mutant (*rth3*) and its corresponding wild-type (WT) maize grown for 22 days in well-watered and drought conditions, and after rewetting the soils. Values represent the mean of 4–6 biological replicates ± standard deviation. Different letters denote significant differences between treatments according to Fisher's LSD test after ANOVA ($p < 0.05$).

(a) Soil moisture % (w/w)

	Drought	Well-watered	Rewetted soil	Highly- rewetted soil
WT	6.10 ± 0.23^{a}	9.64 ± 0.30^{b}	15.34 ± 0.80^{c}	19.55 ± 0.74^{d}
rth3	7.11 ± 0.15^{e}	11.28 ± 0.61^{f}	16.45 ± 0.61^{g}	21.31 ± 0.48^{h}

(b) Microbial specific growth rates, μ (h^{-1})

	Drought	Well-watered	Rewetted soil	Highly- rewetted soil
WT	0.14 ± 0.02^{a}	0.19 ± 0.03^{b}	0.19 ± 0.01^{b}	0.13 ± 0.04^{a}
rth3	0.15 ± 0.03^{a}	0.18 ± 0.01^{b}	0.20 ± 0.01^{b}	0.15 ± 0.01^{a}

3.3. Extracellular enzyme kinetics

Two of the four enzymes tested were responsive to drought: β-glucosidase (BG) and acid phosphatase (AP). In the rhizosphere, drought reduced the V_{max} of BG by 3.5- and 1.3-fold under WT and *rth3* plants, respectively, compared to the well-watered treatment (Fig. 2a top). In addition, the plant genotypes differed in the drought treatment as shown by a 2.3-fold higher V_{max} of BG under *rth3* than WT maize. This greater reduction in BG V_{max} under WT plants was accompanied by a 35 % higher affinity (lower K_m) of BG enzymes in drought than in the well-watered treatment (Fig. 2a bottom). Drought reduced AP V_{max} by 86 % and increased AP affinity by 71 % under WT maize compared to the well-watered treatment, whereas no differences in AP kinetic parameters were observed for *rth3* maize (Fig. 2a). In LRA soil, drought decreased BG V_{max} by 2.8 and 2.4-fold and increased BG affinity by 2.4 and 1.9-fold under WT and *rth3* maize, respectively, compared to the well-watered treatment (Fig. 2b). AP V_{max} was not affected under drought, while affinity of enzymes increased by 58 % under WT plants, in LRA soil (Fig. 2b). Finally, no effect of drought was observed on AP kinetics under *rth3* maize in LRA soil (Fig. 2b). N-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP) showed no sensitivity to drought in their V_{max} in any of the studied soil compartments (Fig. S3). However, in the rhizosphere, affinity of LAP increased (lower K_m) by 52 % under *rth3* maize in the drought treatment compared to the well-watered (Fig. S3a bottom). In the rhizosphere and drought conditions, the affinity of NAG enzymes was a 3.4-fold higher under WT maize compared to the *rth3* mutant (Fig. S3a bottom). Finally, there was a 65 % decrease in the affinity of NAG enzymes in LRA soil, which was marginally significant at a p-value of 0.1 under *rth3* mutant (Fig. S3b bottom).

To compare more directly the effect of drought on the two drought-sensitive enzymes in the rhizosphere compartments, we calculated the percentage decrease in V_{max} of BG and AP under drought compared to the well-watered treatment. Drought reduced BG V_{max} to a greater extent than AP, and this effect was more pronounced in the rhizosphere than in the LRA soil (Fig. 2c). For instance, in the rhizosphere of WT and *rth3* maize, we found up to 11 and 3.5 % decrease in BG V_{max}, respectively, and up to 3 and 0.7 % decrease in AP V_{max}, respectively. Remarkably, a higher decrease of both V_{max} occurred under WT than *rth3* maize in the rhizosphere, while no genotypic differences were found in the LRA soil (Fig. 2c).

The substrate inhibition of BG, AP, LAP and NAG enzymes in the LRA soil was specific to the enzyme and treatment (Fig. 3) and did not occur in the rhizosphere (Fig. S4). For instance, BG showed substrate inhibition under WT plants in the drought treatment (Fig. 3a). This resulted in a reduction in the inhibition constant, K_i, indicating inhibition of activity at a substrate concentration of 407 ± 65 μM (Table 2). In contrast, inhibition of BG under WT plants did not occur in the well-watered treatment, with K_i values that were 10 times higher than those observed under drought conditions (Fig. 3a–Table 2). Inhibition of AP activity was observed under WT and *rth3* plants in the drought treatment, but not in the well-watered treatment (Fig. 3b), with K_i values which tended to be lower under drought than under well-watered conditions (Table 2). Finally, LAP and NAG enzymes showed substrate inhibition that was not dependent on drought (Fig. 3c and d). For more details about the K_i of individual replicates, see Table S3.

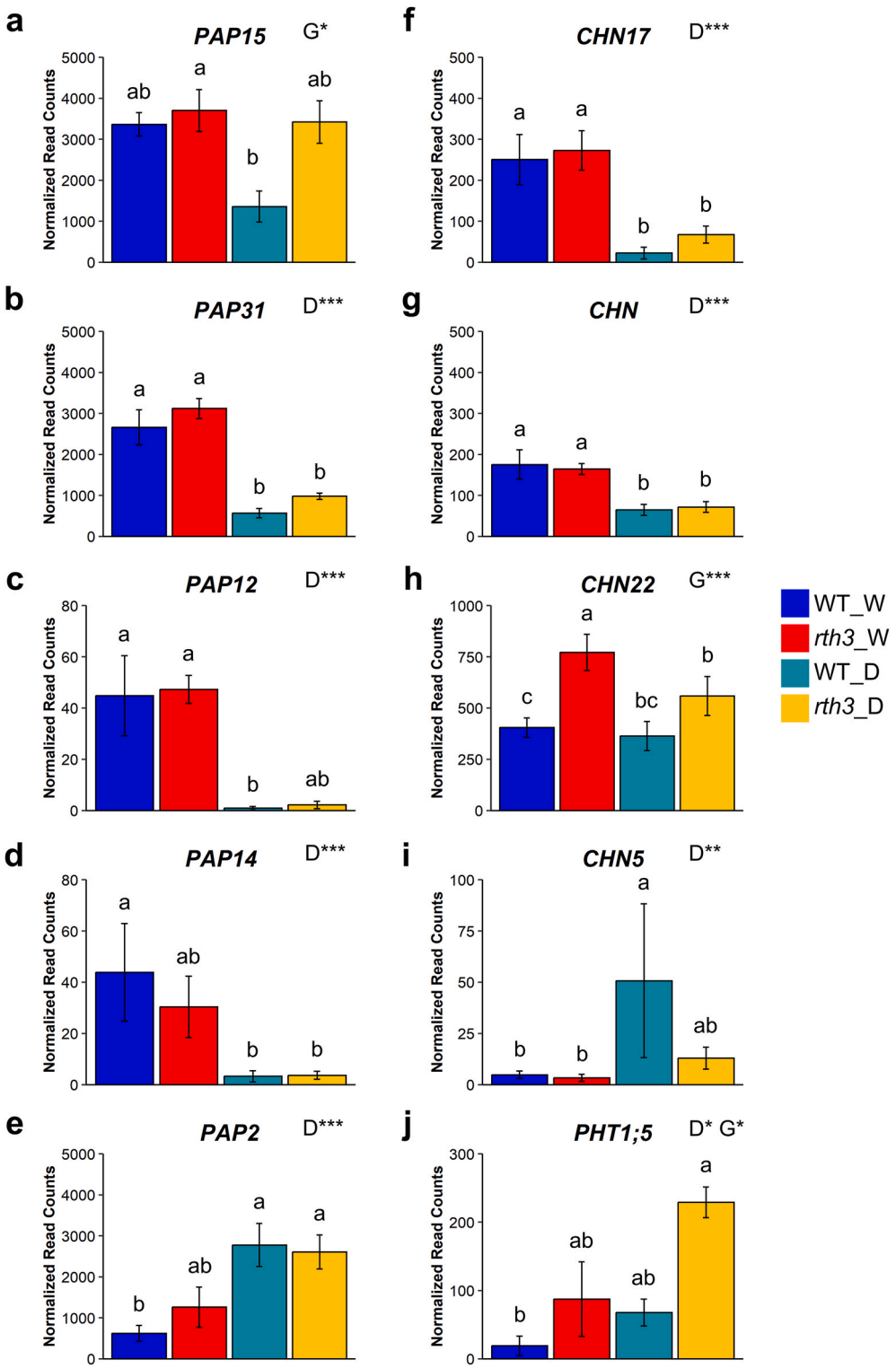

Fig. 1. Differentially expressed *acid phosphatase* (a–e), *chitinase* (f–i), and *plasma membrane phosphate transporter* (j) genes by drought or by maize genotype. Roots were subjected to RNA sequencing and the gene expression results were normalized using DESeq2. The genes are ordered according to their predicted function and expression pattern, and they are annotated according to the Maize GDB syntax. Different letters indicate significant differences according to Kruskal-Wallis and Dunn test ($p < 0.05$), $n = 4$ biological replicates. The letter D denotes the effect of drought (drought confronted to well-watered), and letter G the effect of maize genotype (WT confronted to *rth3*) on gene expression for $p < 0.05$ (*), 0.01 (**) or 0.001 (***). Abbreviations are: WT: wild-type maize, *rth3*: root hair deficient mutant, W: well-watered, D: drought.

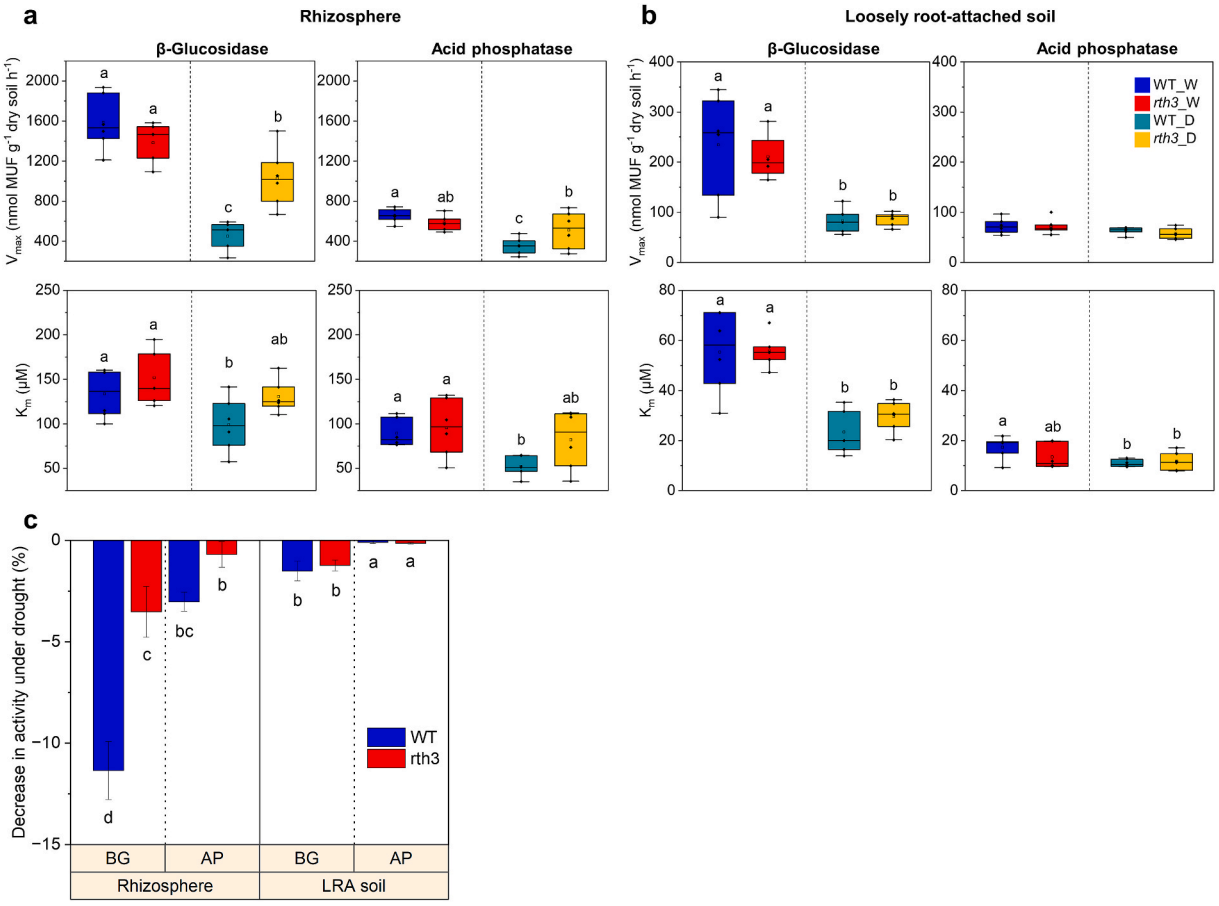

Fig. 2. Maximum enzymatic rates (V_{max}) and affinity constant (K_m) of enzymes in the rhizosphere (a) and loosely root-attached (LRA) soil (b) collected under a root hair deficient mutant (*rth3*) and its corresponding wild-type (WT) maize grown for 22 days in well-watered (W) and drought (D) conditions. Percentage of decrease in β-glucosidase (BG) and acid phosphatase (AP) V_{max} under drought compared to the well-watered treatment in the two soil compartments (**c**). Different letters denote significant differences according to Fisher's LSD test after ANOVA ($p < 0.05$), n = 5–6 biological replicates.

A linear correlation analysis revealed a lower K_m, indicating a higher affinity of BG enzymes, under drought conditions compared to the well-watered treatment in LRA soil (Fig. S5). However, the K_m of AP, LAP and NAG did not correlate to the soil moisture. Furthermore, no clear differences in how maize genotypes responded to drought were observed.

3.4. Microbial growth

Compared to the well-watered treatment, drought increased the microbial lag time by 15 and 24 h after glucose and nutrient addition in soil samples collected under *rth3* and WT plants, respectively (Fig. 4a). In addition, two of six biological replicates of soil samples from WT maize under drought showed no growth 70 h after nutrient addition (Fig. S6), both correlated to a slightly lower water content in soil compared to the other replicates. The rewetting of soil to a moisture between 15 and 17 % (w/w) mitigated the effects of drought and plant genotype, resulting in similar growth curves between the treatments with a lag time of up to 15 h (Fig. 4b). In addition, we observed a higher heat release during the first 10 h after nutrient and water addition and before the start of exponential growth under WT plants (blue line) compared to the *rth3* mutant (Fig. 4b). During the exponential growth phase, drought reduced microbial specific growth rates (μ) by 36 and 20 % under WT and *rth3* maize, respectively, compared to the well-watered treatment (Table 1b). This indicated a slower glucose metabolism under drought conditions. Despite the shorter lag time after rewetting of the soils, μ remained comparable to the well-watered treatment (Table 1b). When the soil was re-watered to a soil moisture exceeding 19 % (w/w), μ decreased to values comparable to those observed during drought (Table 1b). Correspondingly, a positive linear correlation between soil water content % (w/w) and μ was observed up to a moisture content of 16–18 % (w/w). The data indicated that moisture levels above 19 % (w/w) were associated with a reduction in microbial growth rate (Fig. 5).

4. Discussion

4.1. Root gene expression analysis detects potential modifiers of microbial growth and activity

The expression of several genes related to plant defence was down-regulated by drought, to a greater extent in WT than in *rth3* maize roots (Table S2). Maize roots can indirectly modify rhizosphere microbial growth and physiology through the secretion of antimicrobial proteins and exudation of secondary metabolites (Kudjordjie et al., 2019). Those were reduced under drought, probably as a plant strategy to prioritize other gene pathways related to primary functions. Despite this reduced expression of defence genes under drought, microbially mediated processes in the rhizosphere were slowed down, probably due to the low accessibility to substrates. This highlights the impact of water limitation on rhizosphere processes despite the presence of root hairs (Zhang et al., 2023; Canarini et al., 2021). Furthermore, the greater extent of drought-response in WT than in *rth3* maize could be explained by the larger shoot biomass of WT than *rth3* plants, thus higher water loss by evapotranspiration and a more severe drought intensity on the former. Several maize root genes encoding plant enzymes potentially involved in nutrient turnover in the rhizosphere were down-regulated under drought compared to the well-watered treatment (Fig. 1a–i).

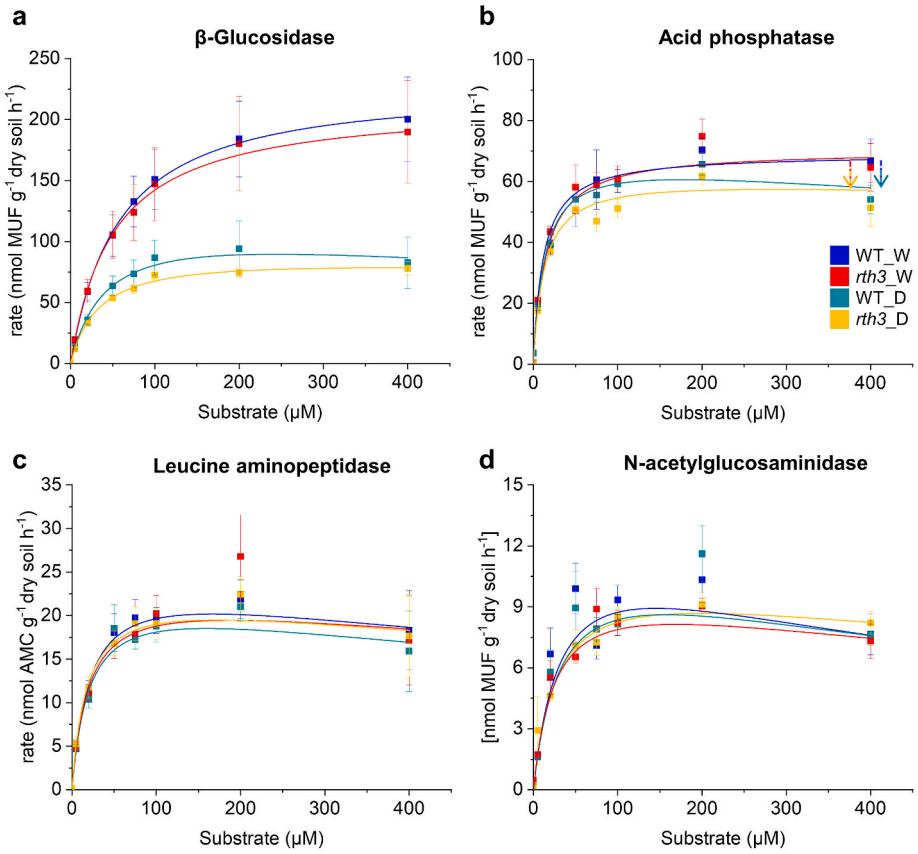

Fig. 3. Substrate inhibition curves of enzymes (a–d) in loosely root-attached soil collected under a root hair deficient mutant (*rth3*) and its corresponding wild-type (WT) maize grown under well-watered (W) and drought (D) conditions. Curves represent the mean of 4–6 biological replicates and error bars the standard errors. Arrows point at the inhibition of acid phosphatase activity under drought.

Table 2
Inhibition constant (K_i) obtained by applying the substrate inhibition model for β-glucosidase (BG), acid phosphatase (AP), leucine aminopeptidase (LAP) and N-acetylglucosaminidase (NAG) in loosely root-attached soil sampled under a root hair deficient mutant (*rth3*) and its corresponding wild-type (WT) maize grown for 22 days in well-watered (W) and drought (D) conditions. Values represent the mean of 3–6 biological replicates ± standard errors; different letters denote significant differences according to Fisher's LSD test after ANOVA ($p < 0.1$), n = 3–6 biological replicates.

	Inhibition constant (K_i)			
Treatment	BG	AP	LAP	NAG
WT_W	4054 ± 1688[a]	6518 ± 3124	677 ± 387	497 ± 225
WT_D	407 ± 65[b]	2547 ± 895	467 ± 405	223 ± 70
*rth3*_W	1893 ± 470[a]	3166 ± 1278	163 ± 140	1454 ± 674
*rth3*_D	3746 ± 1990[a]	2842 ± 1513	162 ± 107	439 ± 40

Interestingly, the down-regulation of *acid phosphatase* genes, particularly in WT plants, correlated with the greater reduction in AP activity in the rhizosphere of WT maize compared to that of the *rth3* mutant. This suggests a reduced contribution of plant-derived AP enzymes and a predominance of microbial-derived enzymes with a greater affinity for the substrate. We also found at least two *chitinase* genes which were down-regulated by drought in both maize genotypes (Fig. 1f and g). However, the step of chitin degradation performed by NAG, was maintained under drought in the rhizosphere and LRA soil, suggesting a prevalence of microbial-derived enzymes to maintain the activity. Experimental proof of these hypotheses would require an *in vitro* system free of microorganisms (Yun and Kaeppler, 2001).

The lack of drought effect on AP kinetics under *rth3* maize (Fig. 2) could be correlated to the maintenance of *PAP15* gene expression levels by the mutants under drought (Fig. 1a). Young maize seedlings improve P uptake by increasing the expression levels of RTH5 protein, which is associated with root hair development (Li et al., 2015). In contrast, root hair deficient mutants often show retarded growth (Brown et al., 2012) and slower P uptake than wild type (Gahoonia et al., 2001). Larger P uptake per unit of root surface area in WT than in *rth3* maize has also been confirmed at the field scale (Vetterlein et al., 2022). Alternatively, symbiosis with arbuscular mycorrhizae could have compensated for the lack of root hairs in the *rth3* mutant (Ma et al., 2021). This idea was not supported by experimental work, as the colonization rate of maize roots at the nine-leaf growth stage was very low and was not affected by the *rth3* mutation in the field (Vetterlein et al., 2022). In addition, the mycorrhizae can be disrupted under drought conditions, so plants need to maintain their own AP production, which was done by one of the five AP-related genes, *PAP15* in our study (Fig. 1a). The contribution of plant-derived AP enzymes, together with the microbial-derived enzymes, may have resulted in an unaffected AP kinetics in the rhizosphere compartments of *rth3* plants. Furthermore, the selective up-regulation of one of the 13 maize plasma membrane P transporters, PHT1; 5, observed under drought in the *rth3* mutant (Fig. 1j) did not result in increased P uptake, as reflected by the lower P content in *rth3* leaves compared to WT. Taken together, these results demonstrate the effect of the presence of root hairs on rhizosphere functions and the contribution of plants to the kinetics of the enzymes.

4.2. *Enzyme-specific response to drought*

The abiotic regulator drought was the main factor slowing down rhizosphere enzyme kinetics, confirming our first hypothesis. Moreover, plant-genotypic differences in soil enzyme kinetics appeared under

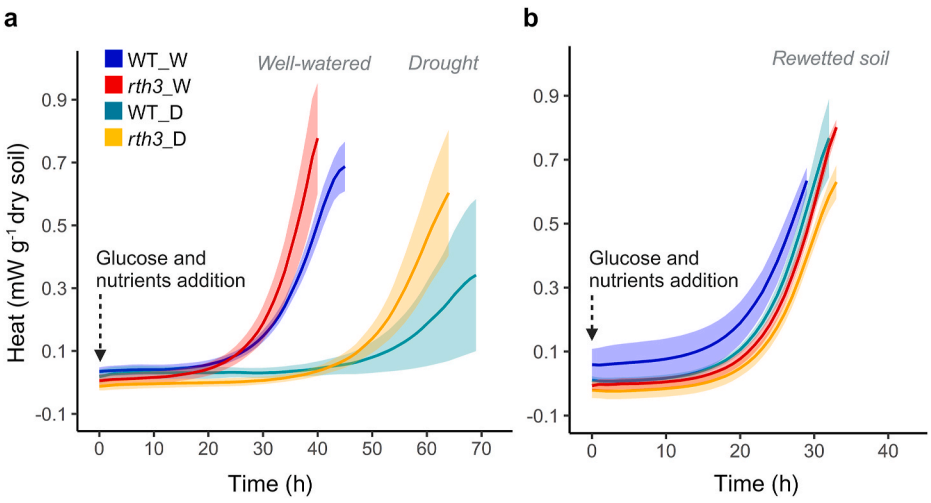

Fig. 4. Microbial growth induced by substrate addition in bulk soil collected under a root hair deficient mutant (*rth3*) and its corresponding wild-type (WT) maize grown for 22 days in well-watered (W) and drought (D) conditions (a), and after rewetting the soil to a water content between 15 and 17 % (w/w) (b). Curves were obtained by measuring heat dissipation over time. Curves represent the mean of 4–6 biological replicates, and the shaded area represents the confidence level for α = 0.05.

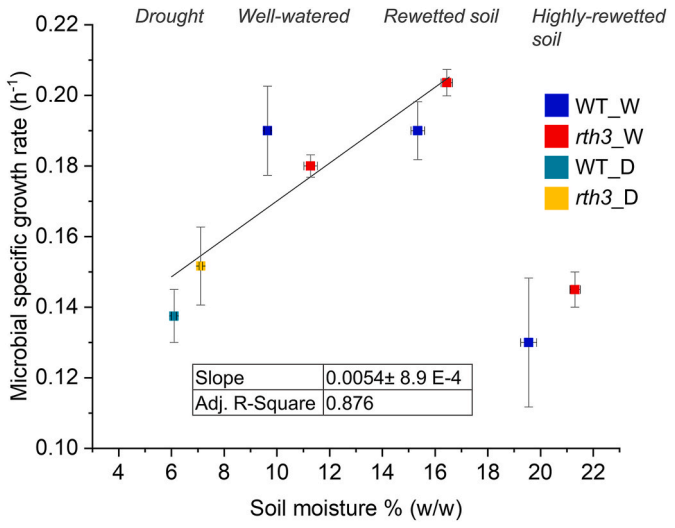

Fig. 5. Linear correlation between soil moisture % (w/w) and the microbial specific growth rate, μ (h^{-1}), in bulk soil collected under a root hair deficient mutant (*rth3*) and its corresponding wild-type (WT) maize grown for 22 days in well-watered (W) and drought (D) conditions, and following rewetting of the soils. Values represent the mean of 4–6 biological replicates and error bars the standard errors. Regression slope for the correlation line and R^2 are shown in the table.

drought conditions but not in the well-watered treatment, confirming our second hypothesis. Within the tested enzymes, we found a higher response of BG to drought than AP activity, and this effect was more pronounced under WT than *rth3* maize (Fig. 2). Enzyme kinetics is an effective indicator of drought tolerance in the rhizosphere compartments and changes in the active microbial community in response to it (Stone et al., 2012; Kujur and Kumar Patel, 2014; Wang et al., 2023). For instance, some drought-resistant wheat genotypes exhibited a 2-fold reduction in the V_{max} of BG simultaneously demonstrating enhanced BG and AP affinities, indicative of more efficient enzyme systems for nutrient mining in soil (Hosseini et al., 2024). Short-seasonal droughts revealed a reduction in the V_{max} of BG and AP, accompanied by an increase in the affinity of these enzymes (Yakushev et al., 2023). The results of our study revealed a greater response to drought, as evidenced by a reduction of BG and AP V_{max} in the rhizosphere, of WT plants compared to the *rth3* mutant. This was accompanied by a higher affinity of the enzymes in the former, whereas no change in affinity was observed under the *rth3* mutant (Fig. 2a). Such an ability of *rth3* mutant to maintain the enzyme systems (as evidenced by similar K_m values) within the rhizosphere suggests its tolerance to drought conditions. In contrast, the enzyme properties were notably influenced by drought conditions under the WT plants, indicating functional alterations in the microbial community (distinctive K_m values under drought conditions in comparison to the well-watered treatment). This functional alteration could result in the production of isoenzymes with a higher affinity for the substrates under drought conditions. One reason for this could be the greater drought intensity experienced by the WT plants compared to the mutant due to the higher shoot biomass, i.e. greater water loss by evapotranspiration, resulting in a lower soil moisture. For this reason, the inaccessibility of substrates due to low diffusion in the rhizosphere was more pronounced in the WT plants than in the *rth3* mutant. This could also be attributed to the altered contribution of plant-derived enzymes in the rhizosphere of WT plants under drought conditions (section 4.1). Furthermore, a higher P content was observed in WT leaves compared to *rth3* leaves under drought (Hartwig et al., 2025). This was also confirmed in a previous study using the same maize genotypes grown in similar columns and the same soil substrate (Lippold et al., 2021). This finding coincided with an effect of drought on AP kinetics in the rhizosphere of WT plants but not of the *rth3* mutant, demonstrating the influence of root hairs on soil processes through differential nutrient uptake. Finally, we did not observe differences in V_{max} of AP between the well-watered and drought treatments in LRA soil (Fig. 2b), supporting our assumption on strongly reduced effect of root hairs in this soil fraction.

As mentioned above, the different response of enzyme kinetics in soil may be related to different plant strategies to overcome drought. An increase in the rate of C and N exudation per root surface area under drought was observed in WT plants compared to the well-watered treatment (Hartwig et al., 2025). In a field experiment using the same maize genotypes and soil substrate, WT plants showed an increase in C exudation rate at the end of the growing season, which was suggested to be a mechanism to overcome increasing drought stress during plant growth (Santangeli et al., 2024). In contrast, the *rth3* mutant maintained a similar C and N exudation under drought (Hartwig et al., 2025), which also correlated with a non-significant increase in C exudation at the final growth stage of plants in the field (Santangeli et al., 2024). Overall, we suggest that WT plants would increase exudation rates to overcome the

intensity of drought stress. This correlated with the greater belowground response, as reflected by enzyme kinetics related to organic C and P cycling, under WT plants compared to *rth3* (Fig. 2). Exudation has also been demonstrated to increase soil microbial respiration rates, as labile C input can trigger fast-growing microorganisms (Hou et al., 2025). In our study, the bulk soil under WT plants showed higher heat dissipation after soil rewetting compared to soil under *rth3* (Fig. 4b), which may be related to the available plant-exuded C immobilized by the low soil water content during the drought.

The response to drought was enzyme specific, with no sensitivity of LAP and NAG, enzymes related to N acquisition, to drought (Fig. S3). Soil microorganisms can acquire N from organic sources such as amino acids or microbial necromass through, e.g., LAP and NAG activities, respectively (Li et al., 2019). These enzyme-mediated processes are tightly regulated in the rhizosphere compartments to maintain resource stoichiometry (Banerjee et al., 2016). This regulation can result in drought resistance of NAG activity (Baldrian et al., 2010) or even a 35–70 % increase in LAP and NAG activity under drought (Zhang et al., 2021). In our study, maize genotypes maintained (*rth3* plants) or increased (WT plants) C and N exudation under drought (Hartwig et al., 2025), which we consider to be a possible factor that sustained organic N degradation activity. Alternatively, the possible increase in microbial necromass under drought might have maintained NAG activity. In contrast, the positive correlation between the affinity of BG and soil moisture (Fig. S5) suggests a higher sensitivity of BG to drought compared to N-acquiring enzymes in the rhizosphere. This could also be related to the diversity and extent of BG and AP activity, which are widely expressed by soil microorganisms to degrade β-glucosides in the final step of cellulose degradation (Cañizares et al., 2011) or acquire P from organo-phosphates (Spiers and McGill, 1979). P turnover is closely related to C metabolism, as C is required as an energy source for microbial growth, and is part of P-containing compounds such as nucleic acids, ATP, phospholipids and other cell components (Chen et al., 2023). These molecules are related to cell growth and division, demonstrating the ecological implications of drought on rhizosphere functions. A relatively high activity of C- and P-acquiring enzymes was revealed in our study as more than 10-fold higher maximum rates of BG and AP compared to N-cycling enzymes in the rhizosphere (Fig. 2, Fig. S3, see y-axis scale). We hypothesize here that this could be due to a broader range of substrates for BG and AP compared to those for LAP and NAG.

4.3. Substrate inhibition of β-glucosidase and acid phosphatase under drought

The substrate inhibition of BG and AP activity was detected solely under drought in LRA soil (Fig. 3a and b), whereas that of LAP and NAG was drought-independent (Fig. 3c and d). These findings were in line with the enzyme kinetics, as BG and AP were sensitive to drought (Fig. 2), whereas LAP and NAG were not (Fig. S3). Substrate inhibition can occur when two substrate molecules bind to the enzyme simultaneously, resulting in a reduced or inhibited catalytic activity; or, less likely, when the substrate itself contains a chemically reactive functional group that acts as an inhibitor of the enzyme (Kaiser, 1980). The absence of substrate inhibition of enzymes in the rhizosphere could be explained by the greater amounts of enzyme molecules produced by roots and microorganisms in response to higher moisture, C and nutrient availability than in the LRA soil. Therefore, the possibility of two substrate molecules binding to the same enzyme under saturating substrate conditions is low. However, in LRA soil, the higher affinity of the enzymes for the substrates (Fig. 2 bottom), together with the likely lower number of enzymes, indirectly indicated by the V_{max}, may increase the chances of binding two substrate molecules simultaneously. This assumption was supported by the 6.8- and 8.5-fold higher V_{max} of BG and AP, respectively, in the rhizosphere compared to the LRA soil; and the 2.5- and 4.5-fold higher affinity of BG and AP enzymes, respectively, in LRA soil compared to the rhizosphere (Fig. 2). Remarkably, V_{max} of enzymes in the rhizosphere were higher than in LRA soil, even when comparing drought conditions in the rhizosphere with the well-watered conditions in LRA soil. This emphasises the importance of the rhizosheath (which belonged to the rhizosphere fraction in our study) in maintaining soil functions in the vicinity of the root under conditions of water limitation. Soil properties, such as soil texture and water holding capacity, can influence the response of enzymes to the substrate inhibition model. For instance, sandy soil showed up to 7-fold lower V_{max} of enzymes compared to loamy soil in a column experiment (Yim et al., 2022). This could be due to lower enzyme production in sandy compared to loamy soil, making inhibition by high substrate concentrations more likely and independent of drought treatment. Finally, BG activity curves showed a typical pattern of non-competitive inhibition, with V_{max} reduced by up to 4-fold (Fig. 3a). This higher impact of drought on BG activity was also visible as a higher decrease in BG activity in both soil compartments (Fig. 2c), which we attribute to a greater extent of C-related activity than organic N or P decomposition due to microbial stoichiometric requirements (Allison, 2005; Zhang et al., 2019).

4.4. Microbial substrate-induced growth was retarded by drought

The seven-day drought period resulted in an essential delay of microbial exponential growth following substrate addition, under WT and *rth3* maize in comparison to the well-watered treatment (Fig. 4a). This prolonged time for microbial activation may be attributed to the reduced substrate accessibility under water limitation (Manzoni et al., 2012), as evidenced by the accelerated growth observed following soil rewetting to a moisture level of 15–17 % (w/w) (Fig. 4b). In general, the response to drought stress can be manifested as altered metabolic activities or a changed community composition of active microorganisms (Castro et al., 2010; Engelhardt et al., 2019). For example, while some microorganisms modify their metabolic profiles in response to drought, others enter a dormant state (Schimel et al., 2007). Fungi are typically more resilient to low soil moisture than bacteria, exhibiting slower growth rates (Yuste et al., 2011; Wan et al., 2023; Zhuang et al., 2024). It can therefore be hypothesized that the long lag phase observed in this study might be related to microbiota reshaping, including the bacterial, archaeal and fungal fractions, targeting slow-growing microorganisms. This longer lag time corresponded to a lower μ under drought compared to the well-watered conditions (Table 1b), which is also associated with more efficient and slow-growing microorganisms (Andrews and Harris, 1986; Liu et al., 2022). Fungi have been demonstrated to display enhanced resilience to changes in precipitation over years in a grassland steppe (Yang et al., 2021). This could be attributed to the capacity of fungi to mobilize nutrients via the hyphal network (Cairney, 1992; Treseder et al., 2018), or to alter the community composition to be more resilient to drought (Preece et al., 2019). In our study, drought affected the composition of fungal communities more than bacterial and archaeal ones, by explaining the 10 % of the variance, compared to the 6 % for archaea/bacteria in the bulk soil (Hartwig et al., 2025). *Ascomycota* was the most responsive phylum, including the families *Ceratobasidiaceae*, *Trichocomaceae*, and *Aspergillaceae* under WT plants, whereas *Pseudeurotiaceae* was the most responsive family under *rth3* plants (Hartwig et al., 2025 Supplementary material). Given the relatively short duration of the drought, this may indicate that fungi are capable of rapid structural changes in dominant taxa to redirect metabolic patterns to maintain stability during prolonged droughts. Overall, we can confirm our first hypothesis stating that the microbial growth was slowed down under drought conditions.

The rewetting of the soils exhibited a lag time before microbial exponential growth that was up to 5 and 27 h shorter than that observed in the well-watered and drought treatments, respectively (Fig. 4b). Rewetting soils after a drought period may result in a short-term increase in microbial activity and CO_2 efflux due to the sudden accessibility of substrates that were previously immobilized within soil pores by slow diffusion (Schimel et al., 2007). Furthermore, the turnover of

microbial necromass formed due to osmotic stress during drying-rewetting events may also fuel the growth and metabolic processes of the active community, which is responsible for a CO_2 and heat pulse upon rewetting (Birch, 1958; Barnard et al., 2013; Blazewicz et al., 2014). This phenomenon is known as the Birch effect, and has been frequently observed as an increase in CO_2 efflux in grassland and agricultural soils after exposure to a rainfall event (Steenwerth et al., 2005). Our results are consistent with this observation, which predicts C losses from the soil and a contribution to greenhouse gas emissions (Sang et al., 2022). Soil microorganisms are often exposed to seasonal changes in precipitation rates, which may allow them to adapt to periods of drought and rewetting (Siebert et al., 2019; Wang et al., 2024). In addition, plant growth, involving water uptake from the soil, can lead to even drier soil conditions, increasing the intensity of the drought. In our study, the seven-day drought period had notable effects on plant physiology and growth (Fig. S1), microbial growth rates (Table 1b), and rhizosphere enzyme kinetics (Fig. 2). In addition, the intensity of the drought was greater in the WT plants, as confirmed by a lower soil water content, compared to the *rth3* mutant. However, the effect of drought or maize genotype on microbial growth disappeared after rewetting of the soil, and microbial growth was fully recovered (Fig. 4b). Thus, the drought period did not alter the maximum growth rate of the microbial community, demonstrating its ability to recover after a rewetting event (Barnard et al., 2013). This observed functional sustainability under a short-term drought demonstrates the presence of microbial self-regulatory mechanisms that should be considered in the development of soil management strategies aimed at reducing C losses from the soil, and maintaining soil health.

5. Conclusions

In conclusion, our results indicated a reduced contribution of plant-derived enzymes to rhizosphere degradation processes in maize under drought. The lower expression of *acid phosphatase* genes in WT maize may have influenced the overall reduction of AP activity in the rhizosphere. Additionally, plant fitness, C and N exudation, and nutrient uptake from the soil affected rhizosphere processes under drought. We suggest here that further research should focus on integrating proteomics, plant exudation profiles, soil nutrient analysis and enzyme kinetics to determine the plant-microbial contribution to soil biogeochemical cycles. In parallel, our study represents, to our knowledge, the first application of the substrate inhibition model of enzymes *in situ* in soil systems. We propose that this model can be used as a tool to elucidate the strength of feedback regulation of enzyme reactions or changes in this regulation due to environmental stressors such as drought. This model should be evaluated in different soil types considering contrasting soil texture, water holding capacity, as well as various root systems, which are critical factors in the application of models.

CRediT authorship contribution statement

María Martín Roldán: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Henrike Würsig:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Mika T. Tarkka:** Writing – review & editing, Supervision. **Roman P. Hartwig:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Monika A. Wimmer:** Writing – review & editing, Validation, Investigation, Conceptualization. **Evgenia Blagodatskaya:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.109837.

Glossary

AP	Acid phosphatase
BG	β-Glucosidase
C	Carbon
K_m	Michaelis-Menten affinity constant
LAP	Leucine aminopeptidase
μ	Microbial specific growth rate
N	Nitrogen
NAG	N-Acetylglucosaminidase
rth3	Root hair deficient mutant
V_{max}	Maximum enzymatic rate
WT	Wild-type

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