

Functional traits of hydrolytic enzymes are related to the extent of root-root interactions in the rhizosphere of maize

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ABSTRACT

The rhizosphere, a region of intensive interactions between roots and microorganisms, is shaped by biological competition and physical limitations of soil environment, which also affects decomposition of organic compounds via extracellular enzymes. This study examined the influence of contrasting intensities of root-to-root interactions and root-to-physical constraints on the kinetics of four hydrolytic enzymes in the rhizosphere of two *Zea mays* L. genotypes: wild type and root hair-defective *rth3* mutant grown in loam and sand substrates. The field setups included the following: i) unrestricted plot cultivation of maize with unhampered root-root interactions; ii) root window with root-root interactions and physical constraints; and iii) spatially restricted soil columns. *Root windows* demonstrated similar potential enzymatic activity to *plot cultivation*, but either 59.4% smoothed (N-acetylglucosaminidase) or 621% amplified (acid phosphatase) the effect of plant genotype. *Soil column* exhibited substantially higher activity of acid phosphatase than the other setups due to P-limited conditions induced by the restricted soil volume and elevated root length density (RLD). When normalized by RLD, *plot cultivation* showed higher specific V_{max} for C- and N-acquiring enzymes than soil column. Overall, the three field setups, which differed in neighboring plants, spatial restrictions, and sampling strategy, were associated with distinct rhizosphere enzymatic responses. Our findings further highlight the importance of considering RLD when interpreting rhizosphere enzyme activities, especially for P-mobilizing enzymes under P limitation and for C- and N-acquiring enzymes when scaling across field and controlled setups.

1. Introduction

Plant roots release various organic compounds into the surrounding soil, in an area known as the rhizosphere (Bais et al., 2006). Root exudates can stimulate the microorganisms to produce extracellular enzymes facilitating decomposition of specific organic compounds (Allison and Vitousek, 2005). Hydrolytic enzymes, such as β -glucosidase (BG), N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), and acid phosphatase (AP), are key enzymes involving in the decomposition of soil organic matter (Burns et al., 2013). Their activity can vary widely depending on root-root interactions between neighboring plants

(Schofield et al., 2019) and environmental conditions including nutrient availability, moisture, temperature, soil volume, and texture (Dong et al., 2022; Lee et al., 2025; Xiao et al., 2023).

Soil texture impacts rhizosphere processes by regulating aeration, water and nutrient retention, diffusion, and sorption dynamics of organic compounds. These factors then modulate root development, microbial activity, and the enzyme-mediated decomposition of organic matter (Berg and Smalla, 2009; Totsche et al., 2018). In addition, root hairs play a pivotal role in soil exploitation, enhancing nutrient and water uptake, particularly in coarser soils such as sand, where water retention is lower than in finer loams (Cai et al., 2021).

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Root-root interactions are belowground processes in which the roots influence each other directly through physical contact, the exudation of allelochemicals, competition for soil resources (e.g., water and nutrients), or the modification of the rhizosphere environment (Mommer et al., 2016). These interactions occur at two levels: intra-plant competition, which occurs within the root system of a single plant; and inter- (including intra-) plant competition, which occurs between the roots of neighboring plants and within the root system of an individual plant (Hodge, 2012; Maina et al., 2002). It is assumed that neighboring roots compete when their process-specific rhizosphere extents overlap. This can be approximated by comparing the extent of the rhizosphere with half the mean inter-root distance, which is derived from root length density (de Parseval et al., 2017; Schlüter et al., 2018). This increases the likelihood of direct physical contact between root segments as well as indirect interactions mediated by diffusible compounds or microbial communities within shared soil volumes. Thus, the degree of rhizosphere overlap acts as an emergent property that intensifies the strength of root-root interactions and modifies local resource availability (Raynaud, 2010; Schnepf et al., 2022). This study specifically investigates both i) maize plants experiencing intraspecific root-root interactions with neighboring conspecifics and ii) maize plants subjected to root-root interactions within their own root system.

While previous studies have primarily investigated the influence of plant-plant interactions on belowground enzyme activities (Gomola et al., 2018; Sun et al., 2021), few have directly compared the enzymatic traits in the rhizosphere of an isolated single plant with those of a plant surrounded by neighbors (Schofield et al., 2019; Xiao et al., 2023).

Plants grown in dense populations often behave differently than those grown alone (Freschet et al., 2021). For example, enzyme activity in the rhizosphere is often higher during plant-plant interactions than in an isolated single plant (Xiao et al., 2023). This is probably due to increased root mass and broader rhizosphere extension, which increases microbial activity through greater cumulative exudation (Chaboud and Rougier, 1991) and potential competition for nutrients among plants (Badri et al., 2012; Cavalieri et al., 2020).

Although plant-plant competition is influenced by biological interactions, it is also strongly affected by physical constraints, such as soil volume and spatial heterogeneity (Chen et al., 2020; Poorter et al., 2012). Soil volume limits the available space for root growth and accessible resources (Hodge, 2004; Schenk, 2006). Restricted soil volume can intensify the effects of competition by forcing roots to grow closer together. This can increase root overlap and alter the composition and diffusion of root exudates (Semchenko et al., 2007). Increased plant density usually increases microbial activity due to greater cumulative root exudation. However, severe volume restriction can limit root growth and exudate production per plant, reducing the expected increase in microbial activity per unit soil volume (Schenk, 2006). Therefore, it is essential to understand how physical soil constraints interact with root-root competition in order to interpret rhizosphere processes, especially in environments where soil volume is artificially restricted.

Experimental exploration of the aforementioned physical constraints (i.e., restricted soil volume) can also help to modulate the intensity of belowground interactions, for example by confining root growth and eliminating inter-plant competition. Pot and column experiments are a common approach in which single plants are used to eliminate the effects of plant-plant interactions, providing a controlled environment that facilitates repetition of experiments throughout the year (Poorter et al., 2016). However, extrapolation of results obtained from plants grown in a controlled environment (i.e., in a pot or column) to those grown in the field needs to consider the differences in environmental conditions (e.g., soil volume) and setup-related methodology of sampling. To bridge this gap, the present study evaluated the rhizosphere enzyme activities associated with individual maize plants using spatially restricted soil columns under natural field conditions. In columns, root growth was confined to a defined soil volume with no inter-plant

competition, enabling precise measurement of rhizosphere enzyme activity while maintaining field relevance (Santangeli et al., 2024). In parallel, the enzyme activity in the rhizosphere of soil columns was compared with that of two other field setups that represent contrasting intensities of belowground interactions: i) the root window enables direct imaging of individual roots but partly restricts belowground interactions in the frontal area due to a hard wall of root window that may induce local root accumulation and cause artificial mechanical impacts; and ii) plot cultivation represents natural field conditions, involving multiple intraspecific belowground interactions, with no individual volume limitations or mechanical barriers. These three experimental setups provided an opportunity to compare the influence of the sampling approach and belowground interaction intensity on rhizosphere enzyme activity in a field study. As each setup had a specific experimental purpose, the rhizosphere sampling strategy had to be adapted to the particular constraints and objectives of each system. Plot cultivation was employed to obtain general, field-based estimates (Vetterlein et al., 2021); root windows were designed to visualize belowground processes with minimal disturbance (Bilyera et al., 2022); and soil columns were utilized to study root exudation under controlled confinement prior to root washing (Santangeli et al., 2024). To ensure comparability across these contrasting setups, sampling was standardized by using similar sample masses (Ghaderi et al., 2025), despite the different tools and collection procedures used. The experimental design in this study contrasted two soil substrates (loam and sand) and two maize genotypes (the wild type (B73) and the root hair-defective *rth3* mutant), both across the three setups and within each individual setup, to investigate the effect of root hairs and soil texture on belowground processes and rhizosphere enzyme activity.

The main objectives of this study were: 1) to compare enzyme kinetics in the rhizosphere of maize monoculture across three experimental setups that differ in the intensity of root-root interactions and physical constraints; and 2) to test whether the effects of soil substrate and plant genotype vary across the three experimental setups. We acknowledge that the setups confound soil volume, planting density, root confinement, and sampling method. Therefore, causal attribution to any single factor is limited.

We hypothesized: **first**, we predicted that rhizosphere enzyme activity would increase with root density, whether arising from the dense root system of a single plant or from the overlapping roots of neighboring plants intersecting in their rhizosphere zones (e.g., in root window). This increase is expected to be particularly pronounced in soil columns due to the restricted belowground volume, which elevates intra-plant interactions and consequently raises the rhizosphere enzyme activity. **Second**, we expected the differences in nitrogen- and phosphorus-acquiring enzyme activities to be most pronounced between maize genotypes in soil columns under spatially restricted conditions. This is because root hairs create localized P-depletion zones, thereby increasing the demand for P-acquiring enzymes (Bilyera et al., 2022). Genotype-specific differences in root exudate composition can alter rhizosphere stoichiometry and stimulate microbial activity. In wild type, this stimulation elevates the production of nutrient-acquiring enzymes, indirectly increasing P and N availability by promoting P- and N-solubilizing microorganisms that release phosphatases (Manzoor et al., 2017; Ridgeway et al., 2024). In addition, it is expected that the demand for P-driven by root hairs will be more evident in sand than in loam, as the coarser texture promotes faster depletion zones (Bilyera et al., 2022; Lippold et al., 2025). This, in turn, is likely to result in a stronger rhizosphere phosphatase response, reflecting an adaptive investment in phosphorus mobilization under conditions of limited availability. **Third**, the impact of the experimental field setup on the specific enzyme activities (standardized per microbial biomass) will be more pronounced in sand, the soil substrate with lower nutrient availability, than in loam. The greater nutrient and organic matter content of loam, its higher microbial biomass, and its better moisture retention will mitigate the effects of root-root competition and spatial boundary

constraints.

2. Material and methods

Experimental setup. This project was conducted within the frame of the Priority Program 2089 “Rhizosphere Spatiotemporal Organization – a key to Rhizosphere Functions” at the research station in Bad Lauchstädt, Germany (51°22'0" N, 11°49'60" E). The details are described in Vetterlein et al. (2021). Generally, the seeds from two genotypes of maize (*Zea mays* L.): a wild type (WT; B73) (Hochholdinger

et al., 2008) and a root hair-defective *rth3* mutant (*rth3* mutant) (Wen and Schnable, 1994) were sown in the last week of April 2019 on two soil substrates: loam (L) and sand (S). Loam substrate was based on the Haplic Phaeozem and consisted of 32.5% sand, 47.9% silt, and 19.5% clay. Sand substrate was prepared by sieving and mixing 16.7% loam substrate with 83.3% quartz sand (WF 33, Quartzwerke Weferlingen, Germany). The resulting sand substrate consisted of 91.8% sand, 5.6% silt, and 2.6% clay. Both soil substrates were sieved through ≤ 4 mm and filled in the field plots, achieving bulk densities of 1.39 g cm^{-3} and 1.50 g cm^{-3} for loam and sand substrates, respectively. To compensate

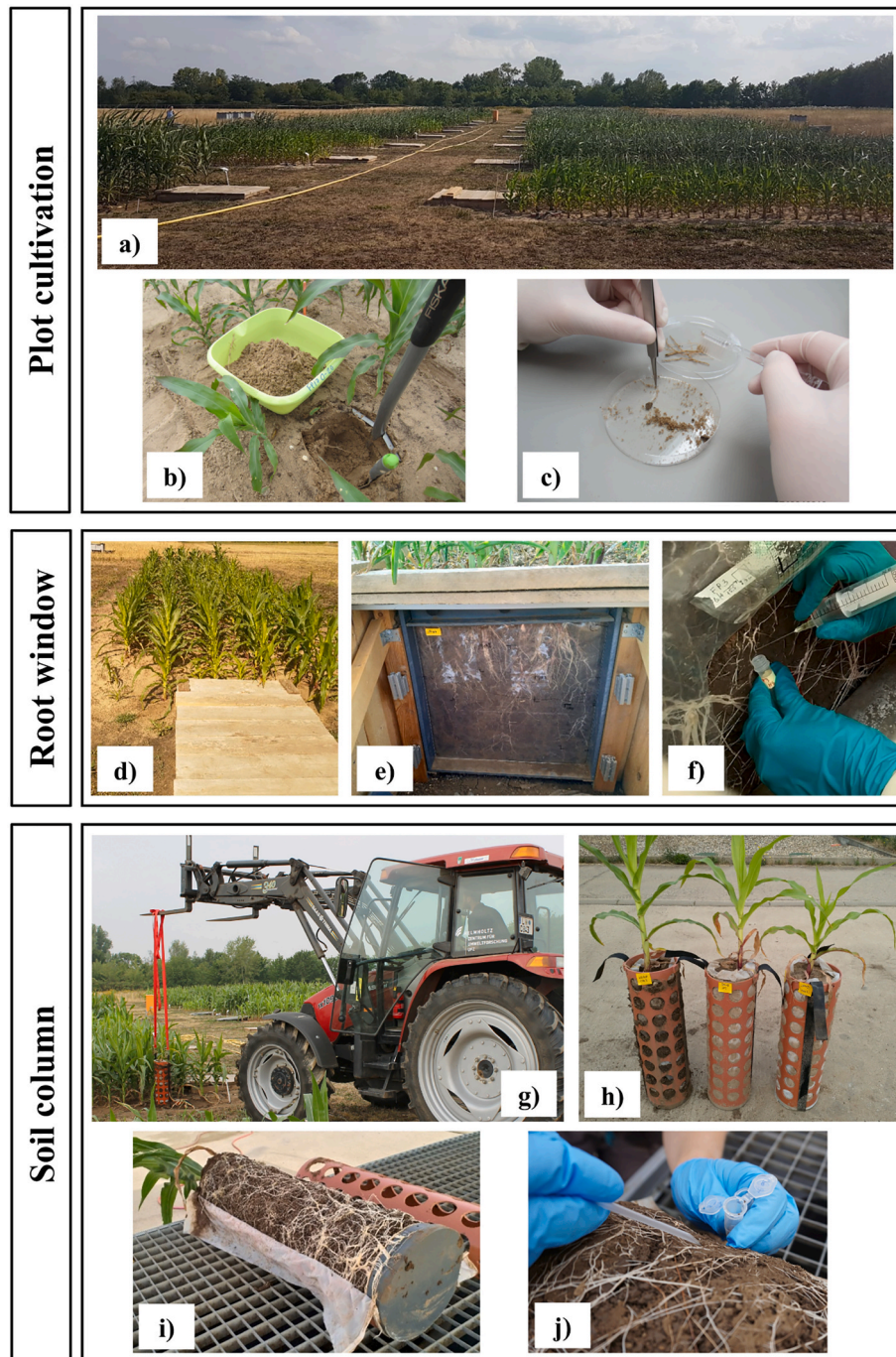


Fig. 1. Three field setups used to collect rhizosphere samples from *Zea mays* L. at the growth stage BBCH 59 in the field. **a)** the experimental design in plot cultivation with 24 field plots; **b)** sampling soil/root from plot, **c)** sampling rhizosphere with a sterile brush; **d)** the location of root window per plot; **e)** the observation area of root window at depth 10–50 cm covered with transparent acrylic glass plate; **f)** sampling rhizosphere from root window plants with a sterile wet needle; **g)** soil column retrieval; **h)** maize plant growing in the soil column; **i)** a soil column with an open side of the PE tube; **j)** sampling rhizosphere from soil column-grown plant with a sterile small tube. The photos are taken by the members of “SPP Rhizosphere 2089”.

for the higher initial nutrient content of the loam, sand was fertilized with double amounts of N, P, K, Mg, and Ca compared to loam to achieve similar shoot growth rates for the WT on both soil substrates (Vetterlein et al., 2021). Micronutrients (B, Mn, Zn, and Cu) were just applied to sand. Fertilizers were surface-applied with the amount of 50% before seeding and 50% at BBCH 14, corresponding to the four-leaves-unfolded vegetation stage (Vetterlein et al., 2022). The soil substrate properties and the amount of applied fertilizers are reported by Vetterlein et al. (2021). The maize plants were grown in the field in three distinct setups – plot cultivation, root window, and soil column – which were all incorporated into the field plots. All three field setups were established at the same time in the same field environment under identical climatic and developmental conditions, differing only in the degree of root confinement and mechanical restriction (i.e., intensity of root-root interactions and physical constraints) (Bilyera et al., 2022; Santangeli et al., 2024; Vetterlein et al., 2021). Sampling was carried out at the end of tassel emergence vegetative growth stage of maize (BBCH 59; 12-week-old) (Lancashire et al., 1991) in the first week of August 2019. The mean daily precipitation and air temperature during the growth period (April–August) were 1.11 mm and 17.53 °C, respectively. Overall, 2019 was reported as a dry year (Jorda et al., 2022). The weather data are available from the German Meteorological Service (DWD) located at the research station Bad Lauchstädt (ID 02878; https://opendata.dwd.de/climate_environment/CDC/observations_germany/climate/).

Plot cultivation. Maize plants growing in a randomized block design with six replicates on loam and sand soil substrates were taken from the field plots (1 × 3.1 × 11 m, height × width × length; 24 plots in total). Planting density per plot was 9.5 plants m⁻² (Fig. 1a). After cutting the shoots of the maize plants in the field, a 20 × 20 × 20 cm cube of soil was excavated next to the maize plants using a spade (Fig. 1b). First, the roots were shaken to remove bulk soil, then rhizosphere soil was collected randomly from the roots at two depths of 0–20 and 20–40 cm (Vetterlein et al., 2021). No significant depth effect was observed for the tested enzymatic activity. Therefore, the mean of the two depths was taken for each replicate. Thus, we ended up with six replicates for each soil substrate and maize genotype combination.

Root window (1 × 1 × 1.6 m, height × width × length) was established at the short end of each field plot with three replicates per soil substrate and maize genotype combination (totally 12 root windows). The root window was covered with a transparent removable acrylic glass plate (5 × 600 × 600 mm, height × width × length). Planting density was 2–5 plants per root window (Figs. 1d and e) (Bilyera et al., 2022).

Soil columns (20 × 60 cm, diameter × height) were localized in each field plot with four replicates per soil substrate and maize genotype combination (totally 16 columns; Fig. 1g). The inner part of the columns was covered by a 30 µm nylon mesh (SEFAR Nitex, Heiden, Switzerland), which allowed water, solutes, and microorganisms to pass through, while the root growth was restricted beyond the mesh barrier. The available soil volume per plant was 18850 cm³. The bulk density of each column was 1.43 g cm⁻³ and 1.65 g cm⁻³ for loam and sand substrates, respectively. Planting density was one plant per soil column (Fig. 1h) (Santangeli et al., 2024). Each column was taken out of the field plot, and the PE tube was removed from one side (Fig. 1i) to collect the rhizosphere samples.

Enzyme assay. Approximately 200 mg of rhizosphere samples were collected from each field setup. In plot cultivation, sampling was carried out by gentle brushing off the soil adhering to the roots using a sterile toothbrush (Fig. 1c). In root window and soil column, to avoid any disturbance for further analyses, sampling was randomly done from soil adhering to the root surface without destroying the aggregates, using a sterile needle (Fig. 1f) and a sterile small tube (Fig. 1j), respectively. Each sampling strategy was selected as the most appropriate and commonly applied approach for the specific constraints and design purpose of the respective experimental setup. Although different

sampling tools were used according to the constraints of each setup, the sampling mass was standardized to ~200 mg (Ghaderi et al., 2025). We acknowledge that these procedures may have captured different proportions of tightly versus loosely adhering rhizosphere soil, as well as soil from different distances from the root surface. Thus, absolute comparability of the sampled rhizosphere compartments cannot be guaranteed solely by mass standardization. Enzyme kinetics was measured upon the rhizosphere samples taken out of i) plot cultivation, ii) root window, and iii) soil column in six, three, and four replicates, respectively, per soil-genotype combination. For that we used the fluorogenically labeled substrates e.g., 4-methylumbelliferyl-β-D-glucoside (MUF-G) (Sigma-Aldrich, CAS: 18997-57-4), 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (MUF-NAG) (Sigma-Aldrich, CAS: 37067-30-4), L-leucine-7-amido-4-methylcoumarin hydrochloride (AMC-L) (Sigma-Aldrich, CAS: 62480-44-8), and 4-methylumbelliferyl-phosphate (MUF-P) (Sigma-Aldrich, CAS: 22919-26-2) to estimate the activities of β-glucosidase (BG; EC 3.2.1.21), N-acetylglucosaminidase (NAG; EC 3.2.1.14), leucine aminopeptidase (LAP; EC 3.4.11.1), and acid phosphatase (AP; EC 3.1.3.2), respectively. Soil suspension (i.e., mixture of fresh soil and deionized water at a ratio of 1:100 by weight) was sonicated for 1 min using the Ultrasonic Homogenizer UP200S (Hielscher Ultrasonics GmbH, Germany). Enzyme measurement was performed by pipetting 50 µL of soil suspension, 50 µL of MES (Sigma-Aldrich, ≥ 98%, CAS: 117961-21-4) buffer for MUF- or TRIZMA (Trizma hydrochloride, Sigma-Aldrich, ≥ 99%, CAS: 1185-53-1 and Trizma base, Sigma-Aldrich, ≥ 99%, CAS: 77-86-1) buffer for AMC-based substrates, and 100 µL of the range of substrate concentrations (5, 10, 20, 50, 80, 100, 200, and 400 µM) into a 96-well black microplate (PureGrade™, Germany) (German et al., 2012). Standard calibration for each soil substrate was performed by applying 50 µL of soil suspension, 50 µL of MES or TRIZMA (for MUF- or AMC-based substrates, respectively), and 100 µL of MUF and AMC solutions (for MUF- and AMC-based substrates, respectively) at concentrations of 0, 0.1, 0.2, 0.4, 0.5, 0.7, 0.8, and 1 µM. Fluorescence was detected using Infinite® F200 PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 30 min, 90 min, and 150 min using excitation and emission wavelengths of 360 nm and 465 nm, respectively. Enzyme activity was calculated by converting the measured fluorescence in the microplates to MUF or AMC concentrations using the standard calibration curves. The Michaelis-Menten equation (Michaelis and Menten, 1913) was used to determine enzyme kinetic parameters of K_m and V_{max} for each enzyme using Eq (1). The model provided good fits to the substrate saturation curves across all enzyme-substrate combinations.

$$v = \frac{V_{\max} \times [S]}{K_m + [S]}, \quad (1)$$

where v is the rate of the enzyme-mediated reaction, [nmol g⁻¹ h⁻¹]; $V_{\max}$ is the maximum rate of the reaction, [nmol g⁻¹ h⁻¹]; S is the substrate concentration, [µmol L⁻¹]; and K_m is an affinity constant that equals the substrate concentration at half of the maximum reaction rate, [µmol L⁻¹].

Root length density-normalized enzyme activity. To relate enzyme activity to root length density (RLD), the estimated $V_{\max}$ values in plot cultivation and soil column were assigned to the corresponding RLD. The RLD_{vol} was determined using Eq. (2) (Newman, 1969):

$$RLD_{vol} = \frac{RL}{V}, \quad (2)$$

where RLD_{vol} is root length density on a volumetric basis, [cm cm⁻³]; RL is total root length, [cm]; and V is soil volume, [cm³].

However, to standardize units for calculating an RLD-specific activity (usually estimated per g soil), the RLD_{vol} values were converted to a mass basis using the soil bulk density. The conversion was performed according to Eq. (3):

$$RLD_{mass} = \frac{RLD_{vol}}{\rho_b}, \quad (3)$$

where RLD_{mass} is root length density on a mass basis, [cm g^{-1}]; RLD_{vol} is root length density on a volumetric basis, [cm cm^{-3}]; and ρ_b is soil bulk density, [g cm^{-3}], obtained from the published data in Vetterlein et al. (2021) and Santangeli et al. (2024) for plot cultivation and soil column, respectively (Table S1).

The RLD_{mass} -specific activity was then calculated by dividing V_{max} values by RLD_{mass} according to Eq. (4) for plot cultivation and soil column:

$$RLD_{mass} - \text{specific activity} = \frac{V_{max}}{RLD_{mass}}, \quad (4)$$

where RLD_{mass} -specific activity is the enzyme activity per unit root length density on a mass basis, [$\text{nmol cm}^{-1} \text{h}^{-1}$]; V_{max} is the maximum rate of the reaction, [$\text{nmol g}^{-1} \text{h}^{-1}$]; and RLD_{mass} is root length density on a mass basis, [cm g^{-1}].

This mass-based normalization provides an operational RLD -specific V_{max} that enables relative comparisons across field setups with slightly different soil densities. Importantly, V_{max} was measured on localized rhizosphere samples, whereas RLD represents the average root abundance over a larger soil volume. RLD -normalized V_{max} is therefore used here as a comparative index across field setups, not as a mechanistic per-root rate.

The half-mean inter-root distance was calculated separately for plot cultivation and soil column using Eq. (5) (Gardner, 1960):

$$r_2 = \frac{1}{\sqrt{(\pi \times L_v)}}, \quad (5)$$

where r_2 is the half-mean inter-root distance, [cm]; and L_v is root length density on a volumetric basis, [cm cm^{-3}].

The mean RLD_{vol} and corresponding half-mean inter-root distance for plot cultivation and soil column were obtained from the published data in Vetterlein et al. (2022) and Santangeli et al. (2024), respectively (Table S1). The r_2 represents half of the average distance between neighboring root segments (Morris, 1996). In a large RLD_{vol} , the r_2 is small, which leads to significant overlap of neighboring root segments and their rhizosphere extent.

Statistical analysis was performed using R version 4.3.3 (R Core Team, 2024). The Kolmogorov-Smirnov test and Levene's test were applied to evaluate the normality of the data and homogeneity of variances, respectively. Data meeting the assumptions of normality and homogeneity were processed using a three-way ANOVA followed by a Pairwise t -test ($P < 0.05$) to evaluate the effects of field setup, plant genotype (root hairs effect), and soil substrate. For data that did not meet these assumptions, an aligned rank transformation (ART) was applied to enable ANOVA-style tests with nonparametric robustness, followed by Pairwise comparisons ($P < 0.05$).

3. Results

3.1. Comparison of three field setups

Despite the controlled design across substrates, genotypes, and fertilizer regimens, significant differences in root system development emerged. Plot cultivation had the largest soil volume. Consequently, it exhibited up to 78% lower volumetric- and mass-based RLD (RLD_{vol} and RLD_{mass} , respectively) and a larger half-mean inter-root distance (r_2) compared to soil column (Table S1) (Santangeli et al., 2024; Vetterlein et al., 2022).

3.1.1. Maximal potential enzymes activity (V_{max})

Overall, soil substrate was the dominant factor influencing V_{max} , accounting for up to 75.4% of the variation, followed by field setup and

plant genotype (Table S2). The effect of field setup on V_{max} was soil substrate-dependent and varied by enzyme. The activities of β -glucosidase (BG) and acid phosphatase (AP) were influenced by the field setup in sand, whereas the activities of N-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP) were affected in loam (see P values in Table S3). Conversely, the effect of field setup on V_{max} was generally greater in the $rth3$ mutant than in the WT (see P values in Table S3). Of the four enzymes tested, AP activity was the most affected by field setup, accounting for up to 41% of the observed variation (Table S2).

A comparative analysis revealed no consistent increase in the activity of all enzymes under plot cultivation versus the other setups (Fig. 2). Solely, up to 8.2 times higher V_{max} values were exhibited in plot cultivation for NAG in sand, regardless of genotype, compared to the other setups (Fig. 2b). Greater activity was mostly observed for AP in soil column, not only compared to the other setups (up to 35 times, except for S_{rth3} ; Fig. 2d) but also compared to the other enzymes (several orders of magnitude; Table S5).

Remarkably, when normalized for root length density on a mass basis (RLD_{mass}), the RLD_{mass} -specific potential enzyme activities (calculated as V_{max} per unit root length on a mass basis, adjusted for rhizosphere volume) of C- and N-acquiring enzymes (BG, NAG, and LAP) were consistently higher in plot cultivation than in soil column across all soil-genotype combinations (Figs. S1a, b, and c). This pattern arose from the substantially lower RLD_{mass} in plot cultivation, resulting in a greater half-mean inter-root distance (r_2) (Table S1). In contrast, the pattern for AP differed, with RLD_{mass} -specific activity remaining higher in soil columns than in plot cultivation only for the L_{rth3} and S_{WT} (Fig. S1), which was consistent with their absolute V_{max} trends (Fig. 2d). Meanwhile, the loam-grown WT showed higher RLD_{mass} -specific AP activity in plot cultivation than in soil column (Fig. S1d).

Soil substrate effect. On average, enzyme activity was up to 95 times higher in loam than in sand (pronounced for LAP in the WT of soil column; Fig. 2), except for AP, which exhibited an opposite pattern in the $rth3$ mutant across all setups (Fig. 2d). However, when considering the RLD_{mass} -specific AP activity, the pattern changed in soil column, with the sand-grown WT demonstrating higher activity than the loam (Fig. S1d).

Plant genotype (i.e., root hairs) effect was enzyme-specific and most pronounced in both soil substrates for AP in plot cultivation and root window (Fig. 2d). Remarkably, the $rth3$ mutant compensated for the lack of root hairs by up to 7.2-fold greater V_{max} of NAG in loam-grown maize in all setups (Fig. 2b).

3.1.2. Enzymes affinity to substrate (K_m)

In general, field setup was the main factor influencing K_m , accounting for up to 88.3% of the variability, followed by soil substrate (Table S6). Specifically, the effect of field setup on K_m was found to be more pronounced in loam than in sand (see P values in Table S7). Of the four enzymes tested, the substrate affinity of NAG exhibited the greatest sensitivity to field setup (Table S6). The increase in K_m values was enzyme-specific (Fig. 4; Table S5). Up to 17.6-fold higher K_m values for C- and N-cycling enzymes (BG and NAG) were observed in plot cultivation compared to the other setups. However, in soil column, this increase was confined to loam (Figs. 4a and b). On the other hand, soil columns demonstrated up to 30-fold higher K_m values for AP than the other setups (Fig. 4d). The pronounced difference in K_m between **soil substrates** was observed uniquely in soil column (Fig. 4). Furthermore, the variation in K_m between **plant genotypes** was most pronounced for NAG and AP in soil column and plot cultivation, respectively, in both soil substrates (Figs. 4b and d).

Detailed analyses of the effects of plant genotype and soil substrate within each individual field setup are presented and discussed in sub-headings Text S1 and Text S2 of the supplementary information, respectively.

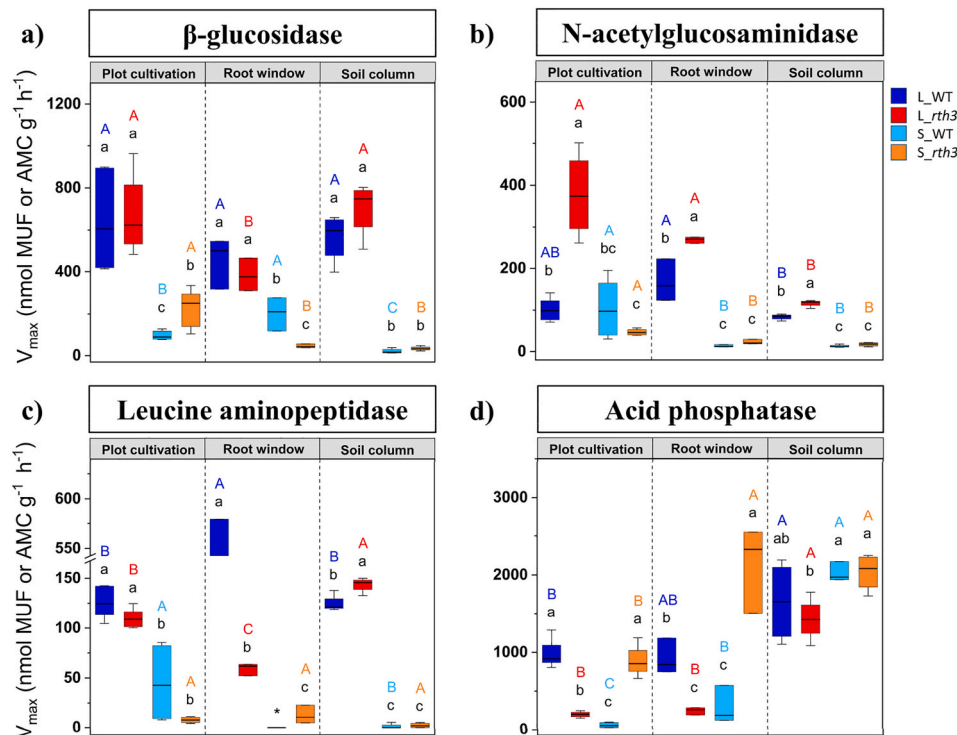


Fig. 2. Comparison of the activity (V_{max} , $\text{nmol MUF or AMC g}^{-1} \text{h}^{-1}$) of a) β -glucosidase, b) N-acetylglucosaminidase, c) leucine aminopeptidase, and d) acid phosphatase among the three field setups shown in boxplots. Upper case letters indicate significant differences at a level of $P < 0.05$ among setups for the same soil-genotype combination (shown by the same color). Lower case letters indicate significant differences at a level of $P < 0.05$ among soil-genotype combinations within each setup. Asterisk demonstrates the value below the detection limit. L, S, WT, and *rth3* represent loam, sand, wild type, and the root hair-defective *rth3* mutant, respectively. Data are means of six, three, and four replicates for plot cultivation, root window, and soil column, respectively. Whiskers extend to the most extreme data points within 1.5 times the interquartile range (IQR) from the box.

4. Discussion

4.1. Maximal potential enzymes activity (V_{max})

4.1.1. Comparison of three field setups

Pronounced soil substrate-specific variations in V_{max} across enzymes, specific to the soil substrate, revealed that the influence of the field setup was not uniform (Tables S2 and S3). The specific effects of field setup (the degree of root-root interactions and soil volume) on the activity of BG and AP in sand and of NAG and LAP in loam (Table S3) suggest that field setup impacts rhizosphere conditions and microbial responses in the context of soil texture (Daunoras et al., 2024; Yim et al., 2022). In sand, larger pores and weaker aggregation may amplify the effects of field setup-induced changes in the accessibility of organic substrates, root exudation, and microbial stoichiometry (Kravchenko et al., 2019; Witzgall et al., 2021). This results in facilitated activity of C- and P-cycling enzymes, which is potentially linked to the reduced physical protection of organic matter in coarse-textured soils, which makes substrates more accessible (Abdalla et al., 2016; Bragazza et al., 2021; Six et al., 2002), despite generally lower microbial biomass (Martín Roldán et al., 2025a). Conversely, the higher organic matter retention and structural stability of loam can buffer C- and P-enzyme activities while increasing the sensitivity of N-cycling enzymes to field setup-driven shifts in N availability or community composition (Merino et al., 2016). However, this pattern likely reflects context-dependent microbial resource allocation rather than strict functional partitioning among enzyme groups (Allison and Vitousek, 2005; Moorhead et al., 2016). These texture-dependent responses should therefore be interpreted as tendencies rather than fixed distinctions between enzyme classes. This nuance refines, rather than contradicts, the third hypothesis, emphasizing that sand and loam differ in how they mediate the effects of field setup on the rhizosphere.

Despite employing different sampling strategies, the overall findings based on the same soil substrates and maize genotypes (Table S4) were consistent with those obtained from experimental setups tested in our study (Fig. 2). As the sampling year was generally dry (Jorda et al., 2022), the similarity of enzymatic activity across setups could suggest some resilience to drought, which is consistent with the findings of Hartwig et al. (2025) on the same plant genotypes. The similarity in general enzyme activity patterns within each setup individually (larger activity in loam than in sand, with minor differences between plant genotypes, except for AP in soil column; Fig. 2) further suggested that the effect of the sampling strategy was rather minor. Nonetheless, as the sampling procedure was confounded with the experimental setup, the setup-associated differences reflect the combined influence of interaction intensity, soil volume, and the specific rhizosphere compartment rather than the isolated effect of any single factor.

The greater effect of field setup on V_{max} in the *rth3* mutant compared to the WT (see P values in Table S3) suggests that the absence of the root hair-mediated stabilization of rhizosphere processes may increase sensitivity to experimental setups. In the WT, elongated root hairs increase the exudation zone and microbial recruitment (Holz et al., 2020), thereby buffering the potential of enzymes in the rhizosphere against changes in root density (Bilyera et al., 2022) and soil volume (Yim et al., 2022) across different setups. Such a buffering effect could reduce fluctuations in organic substrate diffusion and microbial functioning, resulting in smaller, more consistent V_{max} values across different setups. In contrast, the *rth3* mutant, with its limited rhizosphere extent, may react more sensitively to variations in the interaction between plants and soil confinement (Würsig et al., 2025; Yim et al., 2022), which could lead to greater variability in V_{max} among setups.

The larger V_{max} values for NAG in sand under plot cultivation compared to the other setups (Fig. 2b) are likely driven by intense inter-plant interactions. In sand, having low nutrient availability, such

interactions may enhance root exudation, as plants under competitive growth might alter the release of organic compounds, such as sugars, amino acids, organic acids, and secondary metabolites, to mediate signaling and resource competition (Eroglu et al., 2026; Li et al., 2016). The increased exudation, thereby, could stimulate the activity of rhizosphere microorganisms, including bacteria and fungi capable of producing NAG (Blagodatskaya et al., 2021; Zhalnina et al., 2018). This cascade, from root signaling and exudation to microbial activation and enzyme synthesis, highlights the dynamic interactions between plant and functional traits of N- and C-acquiring enzymes (NAG) in the rhizosphere. Notably, NAG contributes to both nitrogen and carbon acquisition via fungal chitin and bacterial peptidoglycan degradation, reflecting its dual hydrolytic role in microbial nutrient mobilization and biomass turnover (Karamanos, 1997). However, the fact that the maximal $V_{\max}$ was only observed for NAG and not for the other enzymes suggests that NAG is particularly sensitive to competitive inter-plant interactions, whereas the other enzymes may respond to distinct organic substrates or be regulated by different environmental factors (Dijkstra et al., 2013; Merino et al., 2016).

Conversely, the strong effect of field setup on AP (Table S2), which exhibited larger $V_{\max}$ values in soil column versus the other setups (Fig. 2d), as well as its elevated activity compared to the other enzymes (Table S5), suggests a strong response to rhizosphere P-mobilization, which partially supported the first hypothesis. This response may have been more pronounced in soil column due to the restricted soil volume (Oliverio et al., 2020), which led to a higher root length density (RLD) than in plot cultivation (Table S1). This likely resulted in faster local phosphorus depletion in the rhizosphere and greater P-deficiency stress at the root level. Consequently, both plants and rhizosphere microorganisms in the columns likely contributed to the production of more AP and/or exhibited other P-mobilization strategies, such as the increased release of organic acids (Richardson et al., 2011), to overcome this limitation. As a result, phosphorus concentrations in young leaves remained comparable in both field setups ($1.8\text{--}2.5\text{ mg g}^{-1}$) (Santangeli et al., 2024; Vetterlein et al., 2022). Phosphate limitation occurred across all setups, as the plant's phosphate status was already below adequate levels compared to nitrogen and carbon from the earliest growth stage (BBCH 14) tested in both soil substrates (Santangeli et al., 2024; Vetterlein et al., 2022). The field studies demonstrated that the lack of plant-available phosphate increased further during the plant's development (BBCH 19 and BBCH 59), manifesting as purple discoloration of the maize leaves (Jorda et al., 2022; Vetterlein et al., 2022). In addition, compared to BBCH 19 under plot cultivation (Würsig et al., 2025), AP activity was up to 4.8-fold greater at BBCH 59 with comparable weather conditions in this study, supporting our interpretation. The measured AP activity mirrors both root and microbial phosphatases, which our design is unable to distinguish.

Accounting for RLD_{mass} leads to an RLD-normalized $V_{\max}$ that simplifies comparison across field setups with different root densities (Table S1). The lower RLD_{mass} in plot cultivation, where roots are more sparsely distributed (higher r_2 ; Table S1), may promote greater rhizosphere extent per unit root length, as the influence of individual roots could extend further without overlap (Delory et al., 2017; Wang et al., 2018). This might enable more soil volume per root to be primed by exudates, which could support elevated microbial activity. The higher RLD-normalized $V_{\max}$ for C- and N-acquiring enzymes in plot cultivation (Figs. S1a, b, and c) is consistent with reduced rhizosphere overlap and increased microbial stimulation under competitive inter-plant interactions, which are typical of open-field growth (Kuzakov and Blagodatskaya, 2015; Li et al., 2016). However, alternative explanations, such as differences in microbial biomass or sampling effects, remain equally plausible. Since $V_{\max}$ was measured in localized rhizosphere samples, while RLD represents the average root abundance over a larger soil volume, the ratio is merely a comparative index and cannot be interpreted as a direct measure of enzyme activity or investment per unit root length. The present data do not enable these mechanisms to be

distinguished. Previous studies using the same maize genotypes (the WT and *rth3* mutant) and soil substrates have demonstrated that soil texture, root architectural traits (including root hairs and root caps), and the spatial position along the root strongly influence the assembly of the rhizosphere microbiome and the activation of microbial biomass (Martín Roldán et al., 2025a; Rüger et al., 2023; Yim et al., 2022). As this study did not quantify microbial abundance and community composition directly, we present this interpretation as a hypothesis, suggesting that similar microbial dynamics may have contributed to the observed enzyme activity patterns. In contrast, the high RLD_{mass} of an isolated single plant grown in a column leads to substantial rhizosphere overlap throughout its root system. This could dilute the RLD_{mass} -specific activity as exudate-driven hotspots converge and intra-root competition for organic compounds intensifies within the confined soil volume (Semchenko et al., 2007). Conversely, the higher RLD_{mass} -specific AP activity for *L.rth3* and *S.WT* in soil columns than in plot cultivation (Fig. S1d, consistent with Fig. 2d), indicates that severe P limitation, intensified by restricted soil volume and rapid phosphate depletion, probably promoted greater per-root investment in phosphatase production (both by plant and microorganisms), despite high RLD_{mass} and rhizosphere overlap (Oliverio et al., 2020). However, for the loam-grown WT, the higher RLD_{mass} -specific AP activity in plot cultivation than in soil column (Fig. S1d) potentially reflects root hair-specific responses to soil substrate properties, or to milder P limitation stress in loam. This might allow competitive inter-plant interactions in field plots to enhance root-length-specific exudation and enzyme expression similarly to that observed for C- and N-acquiring enzymes (Li et al., 2016; Zhalnina et al., 2018). A conceptual overview of rhizosphere processes and the associated investment strategies in C-, N-, and P-acquiring enzymes is presented in Fig. 3. Furthermore, the relative RLD_{mass} for AP activity of each replicate demonstrated contrasting patterns in plot cultivation and in the soil column (Fig. S2). While there was no clear relationship between AP activity and RLD_{mass} in soil column (Fig. S2a), in plot cultivation, the relationship appeared significant and nearly linear after excluding very low activities in *S.WT* (Fig. S2b). AP is an enzyme for which a significant contribution from both plants and microorganisms can be expected, particularly under phosphorus-limiting conditions (Tadano and Sakai, 1991; Zhang et al., 2010). It is also plausible that a threshold was reached in soil column at an RLD_{mass} of approximately 1.5 cm g^{-1} , corresponding to a half-mean inter-root distance of about 0.4 cm (Fig. S2; Table S1). This is suggested to be the distance at which roots may begin to sense neighboring root segments within a root system of an individual plant (Lippold et al., 2021; Schlüter et al., 2018). The observed linear relationship between AP activity and RLD_{mass} in plot cultivation, consistent with the positive relationship between C- and N-acquiring enzymes and root length (Kanté et al., 2021), should be further investigated by future studies. This could be done by employing *in situ* soil zymography to visualize the spatial distribution of AP hotspots in the rhizosphere and to quantify how root proximity and inter-root distances influence enzyme secretion patterns under varying phosphorus availability.

Our results are consistent with the view that the level of root-root competition is closely modulated by soil substrate and volume. This interplay may shape the patterns of rhizosphere enzyme activity, resulting in elevated AP activity under spatial constraints, which could reflect the combined plant-microbial response to phosphorus limitation (Bilyera et al., 2022; Lee et al., 2025). Unlike plot cultivation, where spatial restrictions were minimal, soil column was the most restricted by soil volume and associated resources.

Overall, despite the same abiotic factors for all setups, such as weather, soil texture, and fertilization, the differences among the three field setups were enzyme-specific, with no consistent increase in $V_{\max}$ for all enzymes under any of the setups. Instead, higher $V_{\max}$ was primarily observed for NAG in plot cultivation, while AP showed higher activity in soil column than in the other setups (Fig. 2). This highlights the complexity of enzyme regulation in agricultural systems. The

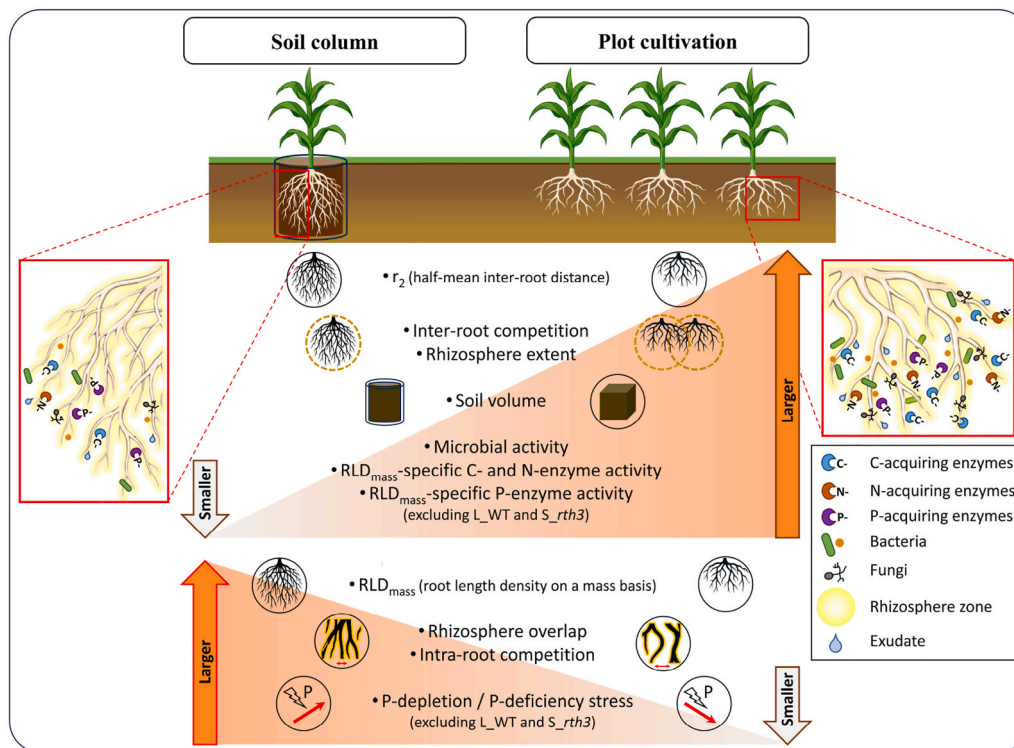


Fig. 3. Conceptual overview of rhizosphere processes and enzyme investment strategies in relation to root density, spatial competition, and microbial responses in plot cultivation compared to soil column. In plot cultivation, the lower RLD_{mass} and greater inter-root distances allow for a larger rhizosphere extent per root, with reduced overlap, promoting higher RLD_{mass} -specific C- and N-acquiring enzyme activities. In contrast, the high RLD_{mass} and confined soil volume in soil column result in substantial rhizosphere overlap and intensified intra-root competition, generally diluting C- and N-enzyme activities but enhancing RLD_{mass} -specific acid phosphatase (AP) activity (excluding L_WT and S_rth3) under severe P limitation.

increased NAG activity in plot cultivation is probably due to the greater abundance of soil fauna and chitin-rich residues resulting from the turnover of fungi and arthropods (Das et al., 2012), which are largely absent in confined column systems. Therefore, biotic interactions, such as plant-plant competition and rhizosphere microbial activity, are suggested to play a more dominant role in shaping enzyme behavior than uniform environmental conditions alone (Burns et al., 2013). These findings align with the laboratory observations, in which enzyme responses differed despite controlled conditions, emphasizing the importance of biological regulation over physicochemical constraints (Martín Roldán et al., 2025b; Spohn and Kuzyakov, 2013; Zhang et al., 2015).

4.1.2. Soil substrate effect

The higher enzyme activity observed in loam compared to sand in almost all setups (Fig. 2) could be related to the greater i) water holding capacity (Adamu and Aliyu, 2012; Vetterlein et al., 2021), ii) carbon exudation rate (Santangeli et al., 2024), iii) organic matter content (Moorhead et al., 2013; Vetterlein et al., 2021), iv) nutrient availability (Vetterlein et al., 2021), v) bacterial diversity (Yim et al., 2022), and vi) microbial biomass (Martín Roldán et al., 2025a) in loam compared to sand. Conversely, increased AP activity in the rhizosphere of the sand-grown *rth3* mutant compared to the loam-grown *rth3* mutant across all setups (Fig. 2d), which contradicted the second hypothesis, was attributed to the lower content of plant-available phosphate in sand compared to loam. Therefore, the need for phosphorus increased root length density in sand (Vetterlein et al., 2022), which likely facilitated increased AP activity released by the plant directly or by the rhizosphere microorganisms to mobilize phosphorus from organic sources in sand (Bilyera et al., 2022; Hummel et al., 2021). Remarkably, in our study, this mechanism was particularly pronounced in the *rth3* mutant, suggesting an intensified enzymatic system and a higher exudation rate compared to the WT (Santangeli et al., 2024), in order to obtain more

phosphorus and compensate for the lack of root hairs, which significantly contribute to plant P uptake at a field scale (Bilyera et al., 2022). Nevertheless, the shift in the pattern of AP activity after normalization by RLD_{mass} observed exclusively in soil column, exhibited higher activity in the sand-grown WT compared to the loam (Fig. S1d), supporting the second hypothesis. In sand, the lower buffering capacity and slower nutrient diffusion can increase P-depletion around root hairs (Bilyera et al., 2022; Lippold et al., 2025). These pronounced gradients might amplify the biochemical signal for AP production, leading to a greater investment in P-acquiring enzymes per unit root length on a mass basis in the WT. In contrast, the *rth3* mutant, lacking root hairs, does not benefit from the same intensification of the localized depletion zone in sand. Instead, it compensates through higher total root production and broadly elevated AP activity.

4.1.3. Plant genotype (i.e., root hairs) effect

The pronounced effect of the plant genotype on AP activity in both soil substrates under conditions of both multiple (plot cultivation) and partly restricted (root window) inter-plant interactions (Fig. 2d) refuted the second hypothesis. Specifically, plant genotypes characterized by the presence of root hairs in loam and the lack of root hairs in sand exhibited greater enzymatic activity when competing with neighboring plants (Fig. 2d), enhancing P mineralization from organic compounds (Bilyera et al., 2022). The increased effect of root hairs on AP activity in loam in the environments with inter-plant interactions (Fig. 2d) extended the findings of Vetterlein et al. (2022), presenting a higher P content in the shoots of loam-grown WT compared to the *rth3* mutant. Nevertheless, this pattern was not significant in soil column for either soil substrate in the study of Santangeli et al. (2024), which supports our findings (Fig. 2d). The higher NAG activity in the *rth3* mutant compared to the WT in loam under all setups (Fig. 2b) suggests that the *rth3* mutant may compensate for the lack of root hairs by increasing its NAG activity.

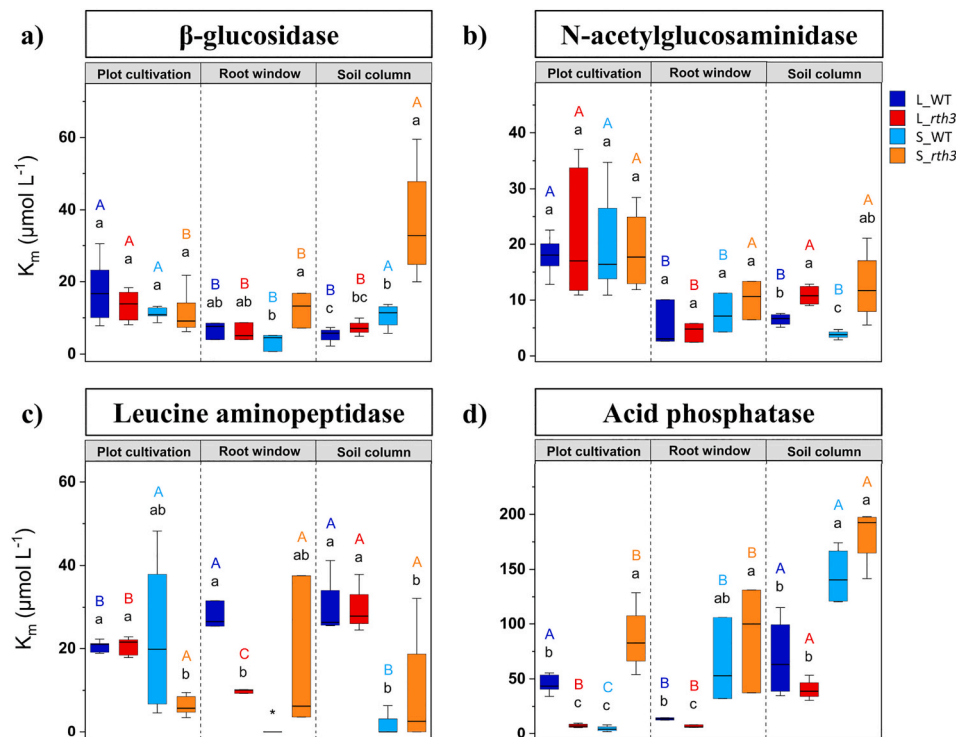


Fig. 4. Comparison of the affinity (K_m , $\mu\text{mol L}^{-1}$) of a) β -glucosidase, b) N-acetylglucosaminidase, c) leucine aminopeptidase, and d) acid phosphatase to substrate among the three field setups shown in boxplots. Uppercase letters indicate significant differences at a level of $P < 0.05$ among setups for the same soil-genotype combination (shown by the same color). Lowercase letters indicate significant differences at a level of $P < 0.05$ among soil-genotype combinations within each setup. Asterisk demonstrates the value below the detection limit. L, S, WT, and *rth3* represent loam, sand, wild type, and the root hair-defective *rth3* mutant, respectively. Data are means of six, three, and four replicates for plot cultivation, root window, and soil column, respectively. Whiskers extend to the most extreme data points within 1.5 times the interquartile range (IQR) from the box.

To achieve this, the *rth3* mutant releases more root exudates than the WT (Santangeli et al., 2024). This could result in faster microbial turnover and increased necromass formation (i.e., the production of organic substrates for NAG) (Meier et al., 2017; Phillips et al., 2011).

4.2. Enzymes affinity to substrate (K_m)

4.2.1. Comparison of three field setups

The significant effect of field setup on substrate affinity (K_m) of most enzymes (Fig. 4; Table S6) can be explained by the difference in root exudate composition and availability of organic substrate in various degrees of plant-plant interactions (Jandová et al., 2015; Li et al., 2016). Conversely, environments characterized by no plant-plant interactions, such as soil column, may have more isolated root exudation patterns and less enhanced organic substrate inputs (Ulbrich et al., 2022).

The greater influence of field setup on K_m in loam than in sand (see P values in Table S7) highlights that belowground interaction regimes can fundamentally alter enzyme-substrate affinity (Allison and Jastrow, 2006, 2006, 2006; Burns et al., 2013). Soil substrate thus modulated the setup-driven effects, likely due to its physicochemical properties amplifying the enzymatic response to belowground interaction intensity in soils with a finer texture (Bai et al., 2025; Sinsabaugh et al., 2008). The specific sensitivity of the substrate affinity of NAG to field setup (Table S6) might reflect increased microbial demand for chitin-derived nitrogen under competitive rooting conditions (Hamer et al., 2021; Schimel and Bennett, 2004).

The higher K_m values for BG and NAG in plot cultivation, compared to root window (mostly in both soil substrates) and soil column (mostly in loam) (Figs. 4a and b), reflect a possible shift in isoenzyme production (Razavi et al., 2016) and availability of organic compounds (Allison and Vitousek, 2005). In environments with multiple plant interactions, organic substrate levels are potentially higher and more diverse than in

environments with partly restricted or no inter-plant interactions due to greater root distribution and higher cumulative exudation (Cavaleri et al., 2020). This could favor fast-growing microbes (r-strategists) that produce enzymes with lower substrate affinity, which can be compensated for some enzymes by a higher maximum activity (V_{max}) (Schimel and Schaeffer, 2012), as was observed for NAG in our study (Figs. 2b and 4b). On the other hand, the elevated K_m values for AP in soil column compared to the other setups (Fig. 4d) may indicate a broader range of such isoenzymes with lower cumulative affinity in response to low phosphate availability under spatially restricted conditions (Yun and Kaeppler, 2001). The experimental setup with no inter-plant competition may enable individual plants or their associated rhizosphere microbiota to enhance phosphatase production, resulting in higher K_m (Nannipieri et al., 2011; Richardson et al., 2009).

The variation in the patterns of K_m values among enzymes for each soil-genotype combination (Table S5) suggests that different extracellular enzyme systems were active and stabilized in the rhizosphere in each setup. This likely reflects distinct plant-soil-microbe interactions, including shifts in microbial community composition, root exudate profiles, and nutrient acquisition strategies that develop under various environmental conditions (e.g., field setups). Individual plants shape their own specific microbiomes and associated enzyme systems (Allison and Vitousek, 2005; Nannipieri et al., 2011; Richardson et al., 2009). However, in conditions involving plant-plant interactions, microorganisms and enzymes may be exchanged among the plants. This could further influence the observed enzyme regulation and activity patterns in different setups (Kuzakov and Blagodatskaya, 2015; Somero, 1978).

4.2.2. Effect of soil substrate and plant genotype

The more pronounced difference in K_m between **soil substrates** for nutrient-cycling enzymes in soil column compared to the other setups (Fig. 4) could be due to decreased biotic interactions in the restricted soil

volume (Semchenko et al., 2007; Sillo et al., 2025) influencing substrate affinity of enzymes. In this confined setting, microbes may respond more directly to soil substrate properties, resulting in distinct patterns of enzyme affinity. The marked difference in K_m between **plant genotypes** for NAG and AP in soil column and plot cultivation, respectively (Figs. 4b and d), highlights the strong interaction between plant genotype and environment on the efficiency of the aforementioned enzymes (Burns et al., 2013) and warrants further experiments. Our results are consistent with the view that the presence or absence of plant-plant interactions and root hair development fundamentally alter the functionality of microbial and root-derived enzymes (Li et al., 2016).

5. Conclusion

Our study emphasized that enzyme dynamics in the rhizosphere is influenced by the combined effects of the degree of belowground interactions, physical constraints, and the sampling procedure. The integrated experimental design nevertheless provided a comparative framework for interpreting rhizosphere processes in various methodological and ecological contexts. Experimental setups act as critical modulators, either enhancing or weakening the plant-soil-microbe interactions. Importantly, methodological differences across setups did not lead to strong disagreement in the response patterns of most enzymes and parameters tested. Under **plot cultivation**, sparser root distributions were associated with greater RLD-normalized investment in carbon- and nitrogen-acquiring enzymes, which was likely mediated by an expanded rhizosphere influence and increased microbial activity. **Root window** adequately mirrored the patterns of enzyme activity observed in plot cultivation. However, it tended to alter the magnitude of the effect of the plant genotype, making NAG activity weaker and AP activity stronger in the rhizosphere of root hair-deficient versus wild type of maize. Therefore, root window is more relevant for the small-scale, 2D spatial resolution studies of rhizosphere processes than for estimating fluxes at the field scale. **Column-based studies** may overestimate AP activity, which can exaggerate the severity of phosphorus limitation. Conversely, in soil columns the effect of root hairs on AP activity may be underestimated because spatial confinement results in reduced phosphorus availability. This, in turn, may constrain the influence of root hairs on soil biochemical processes, thereby highlighting the critical role of spatial dynamics in genotype-environment interactions. Consequently, NAG activity, which was strongly influenced by inter-plant interactions, tended to be reduced in confined systems. When RLD_{mass} is accounted for, phosphorus limitation, intensified by high root length density in confined systems, elevated the phosphatase activity in a genotype- and soil substrate-dependent manner. Therefore, while robust findings appear across setups, this study helps bridge lab-based mechanistic insights to field-scale realities, demonstrating that individual plant data may be relevant for upscaling to plot conditions. Although RLD-normalized V_{max} is a useful comparative index, it remains an operational metric rather than a direct measure of per-root activity. Mechanistic interpretations of such RLD-normalized index require further tests quantifying enzyme activity and root traits at matched spatial scales. Although **column systems** are valuable for providing mechanistic insights under spatially restricted conditions, whether in laboratory or field experiments, their quantitative estimations of enzymatic activity should be interpreted with caution. The way in which nutrient stress responses are expressed in column-based studies may differ from the way they are expressed in unconfined field plots, where inter-plant interactions serve as a characteristic feature. Incorporating microbial community analysis across all setups in future studies could further clarify the respective contributions of root hairs and soil texture to nutrient cycling. Together, these findings emphasize the need to explicitly consider root length density and cultivation scale when scaling rhizosphere processes from individual plants to field ecosystems.

CRedit authorship contribution statement

Negar Ghaderi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Nataliya Bilyera:** Writing – review & editing. **Xuechen Zhang:** Writing – review & editing. **Michael Santangeli:** Writing – review & editing, Methodology. **Vusal Guliyev:** Writing – review & editing. **Zeeshan Ibrahim:** Writing – review & editing. **Mika Tarkka:** Writing – review & editing, Resources. **Doris Vetterlein:** Writing – review & editing, Methodology, Resources. **Eva Oburger:** Writing – review & editing, Methodology. **Bahar S. Razavi:** Writing – review & editing. **Evgenia Blagodatskaya:** Writing – review & editing, Supervision, Resources, Conceptualization, Funding acquisition.

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.rhisph.2026.101468>.

Data availability

Data will be made available on request.

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