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Effects of drought on enzyme activities and hotspot distribution along plant roots

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ABSTRACT

The frequency and severity of drought are projected to increase due to climate change, and Southeast Asia is no exception. Water scarcity hampers all biochemical processes in soil and induces stunted plant growth. While the rhizosphere harbors the most dynamic biochemical processes in the biosphere, the interaction mechanisms between residing microbes and plant roots under drought are poorly understood. In this research, soybean was planted in soil collected from the Red River Delta of Vietnam to test two hypotheses: (i) drought reduces rhizosphere enzyme activities and hampers the extent of the high enzyme activity along single root (from the root tips), and (ii) the turnover time of substrate by enzymes increases with decreasing soil moisture. The research aimed to characterize distributions of β-glucosidase and acid phosphatase enzymes in a distance from root tips. In addition, enzyme activities and plant root and shoot characteristics (length and weight) were investigated. The results demonstrated that shoot length was more impacted by drought than root length with the reduction of 25% for the former and 5% for the later. Meanwhile, the reduction in shoot weight was 61%, and root weight was 90% as the plant experienced drought conditions. The extent of a hotspot for enzymes along a single plant root, measured from the root tips, also decreased in response to drought. Furthermore, drought reduced both rhizosphere enzyme activities, resulting in a slower turnover time of β-D-glucopyranoside (MUF-G) and 4-methylumbelliferyl-phosphate(MUF-P) substrates. The research has shed light on the adverse impacts of drought on root-microbe interactions, which ultimately lead to poor crop growth.

Keywords: Zymography, enzyme visualization, water scarcity, Red River Delta, climate change.

1. Introduction

Water scarcity has been recently recognized as one of the most expensive natural disasters exacerbated by climate change (Andreadi et al., 2005). By 2050, it is projected to impact the lives of 57% of the world's population by 2050 (UN, 2018). The shortage of water exacerbates various

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preexisting socio-economic challenges, such as the outbreak of infectious diseases (Salvador et al., 2023), ecosystem disruption (de Fouw et al., 2016), and food insecurity (Epule et al., 2014). In Southeast Asia, drought has devastated rainfed crops over four decades, from 1980 to 2019 (Venkatappa and Sasaki, 2021). Vietnam, like other Southeast Asian nations, experiences seasonal drought due to its typical monsoon weather (Vu-Thanh et al., 2014; Tuan et al., 2022). Unfortunately, drought frequency is predicted to expand in Southeast Asia due to daily decreasing precipitation and rising temperatures (Naumann et al., 2018), particularly in Vietnam, the number of wet days during dry season demonstrated a reduction pattern (Ngo-Duc, 2023). Drought adversely affects various plant species' morphological, physiological, and biochemical characteristics. These effects include reductions in leaf size, stem extension, and root elongation, ultimately constraining plant growth and productivity (Anjum et al., 2011).

Consequently, there is a shift in the allocation of assimilated carbon (C) to favor the roots (Franco et al., 2011; Fenta et al., 2014; Hasibeder et al., 2014). An increase in root-to-shoot ratio serves as an indicator of worsening drought patterns. In addition to these effects, water deficit negatively impacts the roots' ability to uptake essential nutrients such as potassium (K) and phosphorus (P) (Sardans and Peñuelas, 2004; Ge et al., 2012), as well as their capacity to transport these nutrients from roots to shoots (da Silva et al., 2011). Roots play a pivotal role in mediating plant resistance and resilience to drought by interacting with soil microbes through their released root exudates and rhizodeposits. These exuded compounds foster the recruitment of beneficial microorganisms to the rhizosphere for soil organic matter decomposition. However, drought conditions can alter the root exudates' quality and quantity (Williams and de Vries, 2019), subsequently affecting microbial activities (de Vries et al., 2019). In general, microorganisms accelerate the decomposition of soil organic matter in the form of plant litters or root exudates by synthesizing various enzymes to catalyze the reaction. Among the common enzymes responsible for the C and P cycles, β-glucosidase and acid phosphatase are involved in degrading cellobiose's glycosidic bonds (Acosta-Martínez et al., 2019) and organic phosphate compounds (Tarafdar and Claassen, 1988), respectively.

Nevertheless, the activities of these enzymes are controlled by various biotic and abiotic factors (Holík et al., 2019), with water scarcity being one of the most critical factors, as soil moisture regulates soil physical condition (Puissant et al., 2018). Literature has consistently demonstrated the adverse effects of water stress on rhizosphere enzyme activities (Hosseini et al., 2022; Hoang et al., 2022), leading to reduced nutrient supply for plants. While the effects of drought on microbial activities are well-documented, the extent of enzyme distribution from the root tips and the turnover time of substrate due to microbial enzyme activities remains underexplored.

Soybean is one of four main staple crops (Kim et al., 2019) that provide human and livestock vegetable-derived calories. However, the growing water scarcity due to climate change is supposed to be the most critical threat to soybean yield globally (Kunert et al., 2016). Although water stress

has been shown to reshape root architecture, reduce root length, and accumulate dry biomass (Thu et al., 2014), there is limited understanding of how rhizosphere microbial processes coincide with a plant's response to drought. Therefore, this research aims to investigate the impact of drought on enzyme activities, substrate turnover time in the root zone, and enzyme distribution along soybean roots. Two hypotheses are proposed: (i) drought reduces enzyme activities and restricts their extent from root tips, and (ii) substrate turnover time is prolonged due to unfavorable conditions for soil organic matter decomposition under drought. The experiment involved two treatments: optimum moisture and drought. Soybean seeds were grown in rhizoboxes at optimum conditions with 60% water holding capacity (WHC) for 45 days before exposure to water stress at 30% WHC for 14 days. The zymography method was applied to visualize enzyme distribution before collecting soil samples in the root zone, enabling the measurement of enzyme kinetics using fluorescent substrates.

3. Materials and methodologies

3.1. Soil sample collection

Crop plant roots grow mainly up to 20 cm (Müller et al., 2021) and the soil microbial communities are associated with typical plant root signals (Hirsch et al., 2003). To ensure the resemblance of microbial characteristics related to soybean rhizosphere, soil samples were collected from the topsoil layer (0-20 cm) of a soybean field in Vu Thu district, Thai Binh province, Vietnam (20°25'04.8" N, 106°16'33.1"E, as depicted in Fig. 1). This field had a crop rotation system, with soybean being grown in the winter following two seasonal rice cultivation cycles.

The properties of the collected soil were detailed in Table 1 (as described by Shang et al. (2023)).

Table 1. Preliminary soil properties

Soil parameters	Value
pH_{H2O}	7.07
Total organic C	0.37%
Total N	0.12%
Total P (P_2O_5)	0.18%
Sand	79.5%
Silt	9.7%
Clay	10.8%

The soil was air-dried and 2 mm sieved to remove plant litter and gravel.

Figure 1. Soil sampling field

3.2. Soil water holding capacity

Soil water holding capacity (WHC) was determined following the method described by Naeth et al. (1991). Thirty grams of soil were placed in a 100 cm^3 cylinder and placed on a 20 cm sand layer within a larger container. The container was saturated with water for a minimum of 24 hours. Subsequently, the container was allowed to drain for 24 hours before the soil was placed in an oven at 105°C for 12 hours. WHC was calculated as follows:

$$\text{WHC (\%)} = \frac{(\text{Water saturated soil weight} - \text{dry weight}) * 100}{\text{Dry weight}}$$

3.3. Experiment setup

Sieved soil was packed in white mica rhizoboxes (20 × 20 × 3 cm) to attain a bulk density of 1.1 g/cm^3. The soil was then moistened to 60% WHC. All rhizoboxes were kept in a dark place with a constant 60% WHC to stabilize the microbial activities. Soybean seeds (*Glycine max* L. DT96) were selected for their drought-tolerant characteristics, provided by the Agricultural Genetics Institute of Vietnam. Seed sterilization was performed according to Hoang et al. (2022) using 70% ethanol, 10% hydrogen peroxide, and distilled water, respectively. The germinated seeds on a Petri dish were transplanted into the rhizoboxes on the third day of growth. To ensure optimum moisture levels (60% WHC), all soybean plants were monitored using a gravitricity weighing scale for 45 days. On the 46^{th} day, half of the rhizoboxes underwent reduced irrigation until the soil moisture reached 30% WHC (drought treatment), which was maintained for 14 consecutive days. The other half of the rhizoboxes remained at 60% WHC until the end of the experiment.

3.4. Enzyme hotspot distribution from the root tip

On day 60^{th} of plant growth, hotspots of enzymatic activity along single plant roots were depicted by applying a modified zymography method based on Razavi et al. (2016). Fluorescent substrates, including β-D-glucopyranoside (MUF-G) - a joint unit of cellobiose (Çankaya, 2015), and 4-methylumbelliferyl-phosphate (MUF-P), were employed to detect the distributions of β-glucosidase and acid phosphatase, respectively. Polyamide membrane filters (Tao Yuan, China) with a pore size of 0.45 μm were cut to fit the rhizobox door and immersed in the respective substrate solutions. The membranes were directly incubated on the root-exposed side and covered with aluminum foil to prevent quick drying. After one hour of incubation, the membranes were gently taken off, cleaned with a soft brush, and exposed to UV light with an excitation wavelength of 355 nm and an emission wavelength of 460 nm in a closed room. Hotspot intensity on the zymogram was calibrated by soaking individual 3 cm^2 membranes in MUF solutions at respective concentrations of 0.01, 0.2, 0.5, 1, 2, 4, 6, and 10 mM. These membranes were exposed to UV light in the same way as the samples. Calibrated values were used to quantify color intensity on the zymograms and relate enzyme activity to the gray values. Fluorescent signals of MUF on an area basis were calculated based on the volume of substrate solution taken up by a fixed membrane size.

To ensure image quality and processing uniformity, all images were captured with a digital camera (Canon EOS 6D, Canon Inc.) at a consistent distance. ImageJ was processed and analyzed by converting the original

images to 16-bit grayscale values, adjusting background and contrast levels, and converting gray values to enzyme activities (Razavi et al., 2016). In each plant rhizosphere, at least 3 single roots were selected to calculate hotspot extent from the root tips, with the highest enzyme activity (Ma X. et al., 2021). The hotspot values along the plant roots were determined as the top 25% of higher enzyme activities after subtracting background values at zero concentration from all zymograms (Ma et al., 2017). The area was measured from the root tip along the root center.

3.5. Enzyme kinetics

Following zymography, rhizosphere soil samples were collected by gently shaking the roots to remove the bulk soil. These collected samples were used to measure the kinetics of β-glucosidase and acid phosphatase using the fluorescent substrates mentioned earlier. These substrates contain fluorescent groups, and the energy emitted during enzymatic catalysis was monitored at an excitation wavelength of 355 nm and an emission wavelength of 460 nm by CLARIOstar plus (BMG LABTECH, Germany). Accordingly, 0.5 g of soil (dry weight equivalent) was weighed into an air-tight glass bottle and filled with 50 ml of sterilized water followed with a 20-minute shaking. A 96-well black plate (Puregrade, Germany) filled 50 µL of the soil slurry, 50 µL of MES buffer (pH 6.5), and 100 µL of the substrate solution. The plate was measured at four time points: 0, 30, 60, and 120 minutes after adding the substrate. To define the slope, a calibration line was prepared with MUF substrate at concentrations of 0, 10, 20, 30, 40, 50, 100, and 150 µM. Enzyme kinetic parameters were calculated based on the Michaelis-Menten equation (1):

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

In which, v is the reaction rate at the 120-minute-time point of the measurement; V_{max} is the maximum reaction rate at saturated substrate concentration (nmol MUF g^{-1} soil h^{-1}); [S] stands for the substrate concentration (µmol g^{-1} soil); K_m is the substrate concentration at which the reaction rate attains a half of the maximum. It's important to note that the K_m value is inversely correlated with substrate affinity. In other words, a higher K_m value indicates lower substrate affinity.

3.6. Substrate turnover time by enzyme activities

The substrate turnover time by enzyme activities (T_t, hours) was calculated using equation (2) as described by Panikov et al., 1992, where S is the substrate concentration relative to the V_{max}. In this study, S was set at 150 µmol L^{-1}.

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

3.7. Root and shoot characteristics

By the end of the experiment, plants were harvested and gently washed with deionized water. The root and shoot lengths (cm) were measured using ImageJ and calculated on average. Subsequently, the plant roots and shoots were separated and dried at 60°C for two days before being weighed to determine root and shoot biomass.

3.8. Statistical analysis

All treatments were replicated three times, and the data was subjected to statistical analysis using Sigma-Plot 14.0. One-way

ANOVA was applied to confirm hotspot dimension along plant roots after checking the normality and homogeneity of values.

Different enzyme activities and root, shoot characteristics between the optimum and drought treatments were tested using a T-test. A probability level of $p < 0.05$ indicated statistical significance, denoted in the figures using error bars.

4. Results

4.1. Characteristics of drought-suffered plants and hotspot distribution along single root (from root tip)

Drought negatively affected root architecture and shoot growth, which is visually depicted in Fig. 2. The lack of water resulted in stunted root and shoot growth, characterized by smaller sizes.

Figure 2. Plant characteristics are different between drought (6) and optimum treatment (8N)

Specifically, drought had a more pronounced effect on shoot length, with a reduction of 25% in shoot length compared to a 9% reduction in root length (Fig. 3). Furthermore, the weight of shoots decreased by 61%, while root weight decreased by 90%.

Consequently, hotspot distribution along single plant roots from the root tips also decreased in response to the drought effect. The reduction was 2.4 times in β-glucosidase and 2.9 times in acid phosphatase in the drought treatment (Fig. 4).

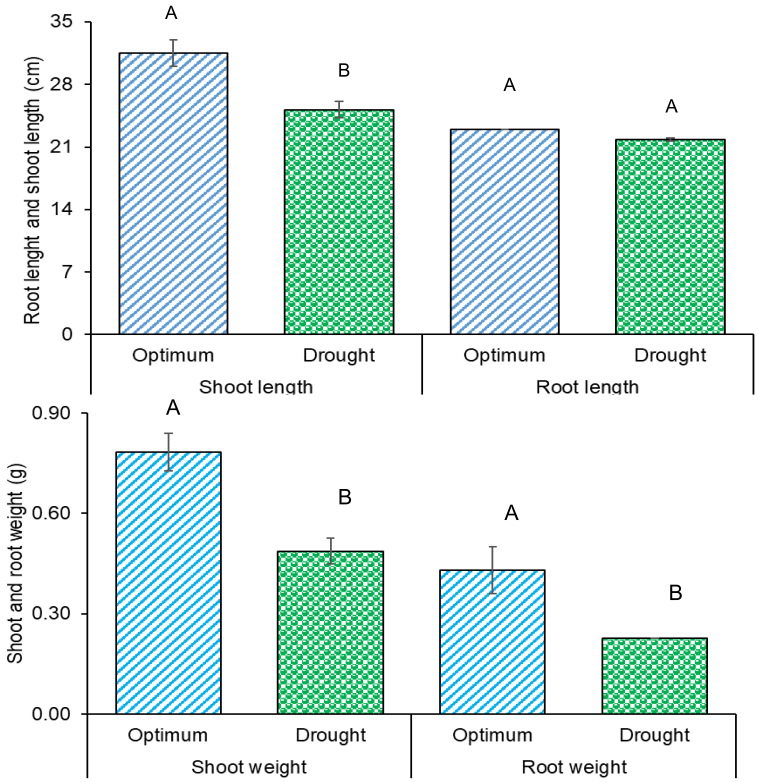

Figure 3. Effects of drought on plant were characterized in root and shoot lengths (cm), root and shoot weight (g). Letters demonstrate the significant difference between optimum and drought of the respective parameter ($p<0.05$). Bars show standard error.

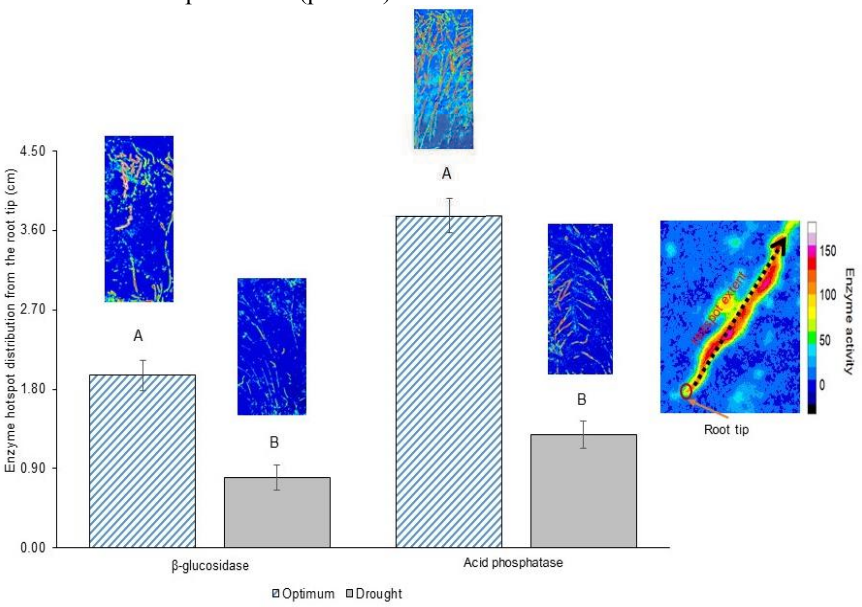

Figure 4. Enzyme hotspot distribution from the root tip. Letters show the significant difference between optimum and drought ($p<0.05$). Bars show standard error.

4.2. Enzyme kinetics and substrate turnover time

The activities of both β-glucosidase and acid phosphatase in the optimum treatments significantly decreased as the plants experienced drought effect ($p<0.05$). Specifically, acid phosphatase activity decreased by 22%, and β-glucosidase activity decreased by 67% (Fig. 5).

In contrast, the substrate turnover time increased by 54% for β-glucosidase and 21% for acid phosphatase in drought-treated plants compared to the optimum condition (Fig. 6).

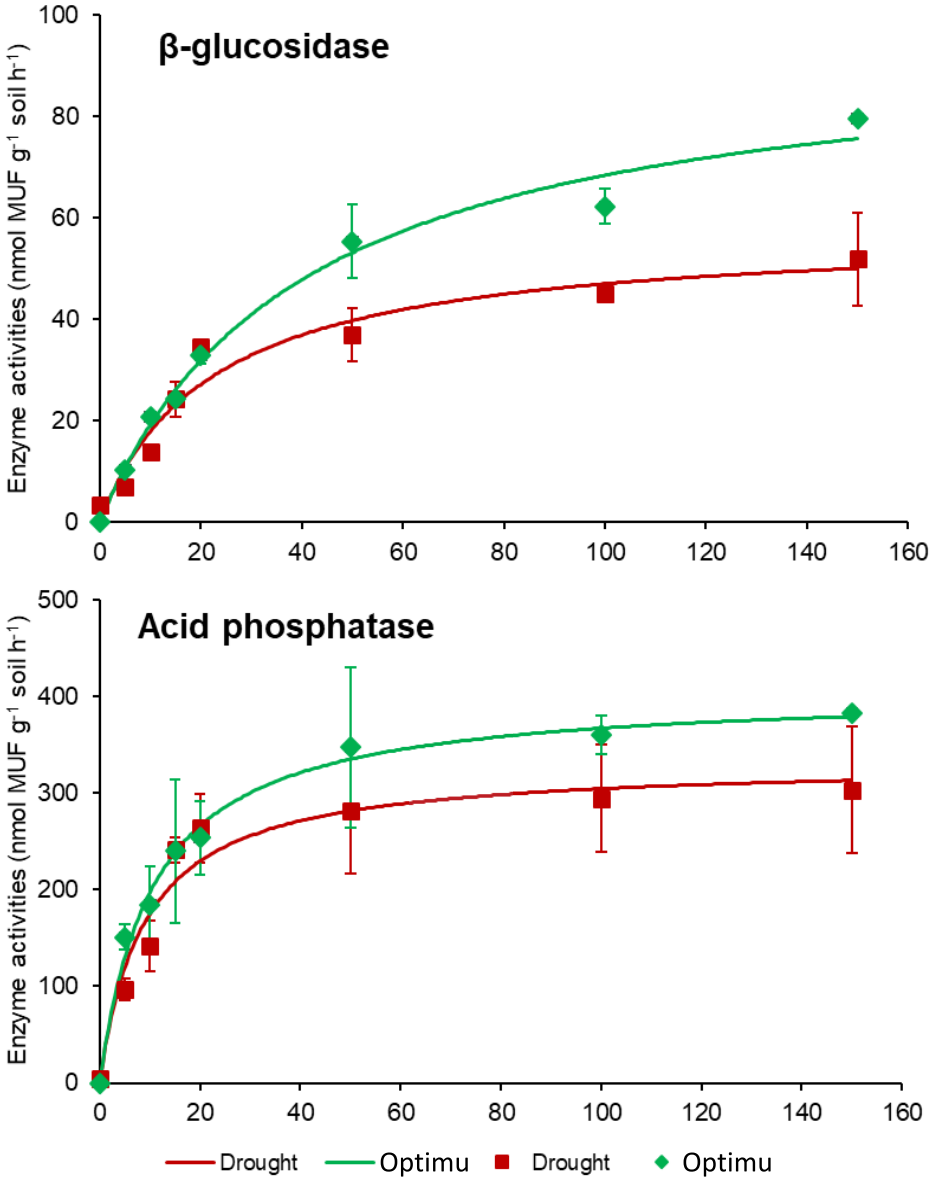

Figure 5. Kinetics of β-glucosidase and acid phosphatase at drought condition and moisture soil

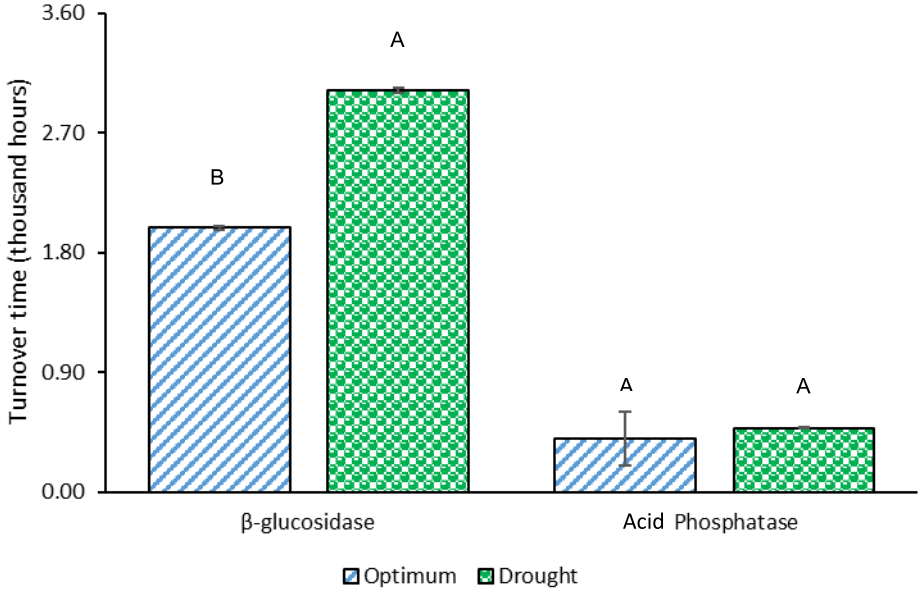

Figure 6. Turnover time of enzyme activities. Letters show the significant difference between optimum and drought

5. Discussions

5.1. Water stress effects on plant growth

Water deficiency substantially reduces overall plant growth (Ahluwalia et al., 2021), which is also associated with our finding in this experiment. The adverse effects of drought on root and shoot length, root and shoot weight have been previously demonstrated in rice (Usman et al., 2013) and many other plant species. These reductions are attributed to a series of hazardous drought impacts on plant physiology, such as restricted photosynthesis (Shi et al., 2019), disruption in mineral nutrition and ion homeostasis within plant cells (Kheradmand et al., 2014), and reduced mobility of P (Mariotte et al., 2020). In our experiment, the drought was artificially introduced at the beginning of the soybean flowering stage, which enhanced the plant's sensitivity to drought. Therefore, soybean plants subjected to drought exhibited wilting and harsh characteristics with shorter shoots and thinner roots. Notably, the severity of drought effects was more pronounced in plant shoots than in plant roots in our experiment. This suggests a possible water stress survival strategy employed by the plant: conserving water through reduced shoot growth while maintaining root length to access deeper soil layers (Poorter et al., 2012). Although root length remained relatively constant in water stress, root weight significantly decreased, indicating thinner root morphology compared to well-irrigated roots (Hazman and Brown, 2018).

Additionally, the relationship between the extent of enzymatic hotspots along single roots (β-glucosidase and acid phosphatase) and root length was found to be independent (Fig. 2). Root tips were identified as hotspots of glucose exudation (Hoang et al., 2022), which is synthesized directly by rhizosphere microbes and immediately activates microbial activities. In zymograms, root tips were visually demonstrated in dark red color. At an increasing distance from the root tips, the

color intensity decreased for β-glucosidase, while it remained constant for acid phosphatase under optimum conditions. However, during drought, the extent of enzyme activity from the root tips decreased compared to the optimum condition, regardless of the enzyme types. Drought likely led to a shorter localization of exuded labile sugars such as glucose or sucrose along a single root at a further distance from the root tips, which leads to a sharp decrease in enzyme activity in the longitudinal exuding root zone (Holz et al., 2019).

5.2. Enzyme kinetics and substrate turnover time

The microbial community residing in the rhizosphere directly benefits from the C exudation from plant roots, which stimulates the generation of enzymes. However, the low soil moisture hampers the activities of rhizosphere enzymes due to (i) restricted substrate diffusion to and from enzymes (Mganga et al., 2019) and (ii) increased osmotic stress (Chen et al., 2022). This explains the reduced enzyme activities of β-glucosidase and acid phosphatase under drought (Fig. 4). While Mariotte et al. (2020) found that short-term drought of one week did not affect phosphorus (P) availability, the extended two-week drought condition in our experiment may have resulted in decreased acid phosphatase activity due to restricted microbial activity in the rhizosphere (Zhu et al., 2022) and reduced plant P demand (Sardans et al., 2006). Furthermore, it's worth noting that photosynthesized C translocation from shoot to root was proposed to increase with decreasing soil moisture (Zang U et al., 2014) as an adaptation strategy of plants to drought. Nevertheless, these translocated C compounds were exuded in larger volume from drought-suffered plants than non-drought plants (Preece et al., 2018). This means that in the presence of more labile C sources in the rhizosphere, microbes produce less β-glucosidase activity to save energy and nutrient consumption.

Consequently, the reduction in β-glucosidase activity was three times more pronounced than the reduction in acid phosphatase as the plant was exposed to the same level of drought intensity. As a result, the substrate turnover time by enzymes was prolonged in drought conditions compared to the moist soil (Fig. 5), signifying that it takes more time to decompose soil organic matter under drought than under optimum conditions. Furthermore, the shorter turnover time of the P cycle compared to the C cycle in our findings demonstrates that drought causes a more substantial impact on the latter than the former.

6. Conclusions

Our results support the two hypotheses regarding enzyme activities and substrate turnover time. Drought triggers adverse effects on plant morphology, including a 25% reduction in shoot length, a 9% reduction in root length, a 61% reduction in shoot weight, and a 90% reduction in root weight. In addition, it shortens the root longitudinal allocation of enzymes up to 2.9 times. The restricted substrate diffusion to enzymes and reduced enzyme activity in the rhizosphere explain the significant decline in β-glucosidase and acid phosphatase activities under drought. Consequently, the turnover time of substrate in the rhizosphere prolongs with restricted soil moisture, which should be considered in the alteration of biogeochemical processes under climate change impacts.

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