



Drought tolerance of wheat genotypes is associated with rhizosphere size and enzyme system

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Abstract

Background and aims Utilizing drought-tolerant genotypes with appropriate adaptive characteristics is a crucial mitigation strategy to improve wheat productivity in dry conditions. Understanding rhizosphere processes (e.g., enzyme traits) involved in nutrient acquisition and adaptation to drought stress across different genotypes is critical for the development of drought-resistant genotypes.

Methods We grew three wheat genotypes with varying drought tolerance -Baran (rainfed drought-tolerant), Sirvan (drought-tolerant), and Marvdasht (non-drought-tolerant)- in rhizoboxes under drought stress. Through in-situ zymography and ex-situ enzyme

kinetic analysis, we examined the localization and dynamic behaviors of key enzymes, acid phosphatase (ACP) and β -glucosidase (GLU), in the rhizosphere and their relationship with root traits.

Results Baran displayed a more extensive root system with abundant lateral roots compared to other genotypes. Its rhizosphere exhibited a higher hot-spot of GLU and ACP than Sirvan (1.5- and 1.2-fold higher, respectively) and Marvdasht (2- and 2.7-fold higher, respectively). Baran also demonstrated a broader enzyme activity expansion in the rhizosphere, showcasing its superior nutrient exploration capability. Drought-tolerant genotypes displayed elevated GLU and ACP activity in the rhizoplane, indicating enhanced root exudation. Notably, V_{\max} values of GLU were approximately 2-fold lower in drought-tolerant genotypes than in Marvdasht, revealing an energy conservation strategy in dry conditions. Additionally, drought-tolerant genotypes exhibited a higher affinity of GLU and ACP to substrates, enabling efficient nutrient extraction from soil organic matter despite lower enzyme activity.

Conclusion Our findings demonstrate that drought-tolerant genotypes can better withstand water stress by having a broader rhizosphere extent and an effective enzyme system, both of which are primarily facilitated by lateral root growth.

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Introduction

Water scarcity is a significant abiotic stressor that considerably restricts wheat growth and productivity worldwide (Comas et al. 2013). With an expected increase in the world's demand for wheat by 26% by 2050 (Cui 2020), it is imperative to intensify efforts to mitigate the detrimental effects of drought on wheat yield. Developing and planting drought-tolerant genotypes is a cost-effective and efficient strategy to improve wheat productivity under dry conditions. Traditional plant breeding and crop genetic engineering have advanced various morphological, physiological, and biochemical traits to make plants more resilient to drought stress (Fleury et al. 2010). However, plant breeders and geneticists have primarily emphasized aboveground properties as target traits, while underground properties such as root morphology and rhizosphere microbiome (composition and processes) are also crucial for plant growth and productivity, efficient water uptake, nutrient acquisition, and plant drought tolerance (Comas et al. 2013; Corneo et al. 2016; Azarbad et al. 2018; Ober et al. 2021; Nerva et al. 2022). Therefore, it is necessary to place greater emphasis on underground properties to improve plant resistance to drought stress.

The rhizosphere plays a crucial role in root-microbial interactions, providing a high-intensity environment for various microbial processes to occur at a quicker rate than the total soil average (Kuzyakov and Blagodatskaya 2015). The secretion of enzymes by both plant roots and microorganisms is a crucial process that occurs within the rhizosphere and plays a vital role in enhancing plant drought tolerance (Song et al. 2012; Gianfreda 2015). Extracellular enzymes control a variety of reactions and metabolic procedures during the mineralization of soil organic matter (Nannipieri et al. 2012; Burns et al. 2013). Enzyme activity improves carbon and nutrient availability in the rhizosphere, allowing plants to combat abiotic environmental stress (Hinsinger et al. 2003; Kuzyakov and Razavi 2019; Zhang et al. 2022). For instance, β -glucosidase and acid phosphatase, derived from both plant roots and the soil microbiome, play crucial roles as key enzymes in the carbon and phosphorus cycles (Nannipieri et al. 2011; German et al. 2011). β -glucosidase breaks down cellobiose into glucose molecules, providing an additional energy source for microbes and plants during drought stress (Song

et al. 2012; Sami et al. 2016; Bilyera et al. 2021). Simultaneously, acid phosphatase hydrolyzes organic phosphorus compounds into phosphates (Nannipieri et al. 2011). These phosphates are crucial for various plant physiological processes, including energy transfer, nucleic acid synthesis, and cell division (Khan et al. 2023). The increased availability of energy and nutrients, such as phosphorus, can support metabolic processes even under drought stress, thereby improving the plant's drought tolerance. The spatial pattern of enzyme activity in the rhizosphere not only reflects the nutrient demands of plants and microorganisms, but it can also contribute to the plant's ability to cope with drought stress through the improved exploitation of soil resources (Hummel et al. 2021; Hoang et al. 2022; Song et al. 2022). Additionally, enzyme systems can be regulated by altering substrate affinity or isoenzyme expression, maintaining enzyme function efficiency at a high level during environmental stresses (Razavi et al. 2016a). Consequently, it is crucial to investigate these traits across different genotypes and integrate them into plant breeding programs for the development of drought-resistant genotypes.

Research studies have shown that there are differences in both the localization of enzyme activities and the efficiency of enzyme systems across various plant species or genotypes (Ma et al. 2018b; Holz et al. 2020; Rakotoson et al. 2020; Zhang et al. 2022; Schwerdtner et al. 2022). For instance, Ma et al. (2021) found that lupine had a significantly higher increase in the hotspot area of phosphatase activity in response to phosphorus deficiency than maize, indicating a more effective phosphorus acquisition strategy in lupine. In another study, Song et al. (2022) observed that the spatial distribution of enzyme activity in the rhizosphere soil and rhizoplane of spruce seedlings was more heterogeneous than that of beech seedlings, suggesting a better adaptation of spruce to extract nutrients from a more varied soil environment. However, our knowledge regarding spatial distribution and catalytic properties of enzymes in the rhizosphere of different wheat genotypes with varying levels of drought tolerance remains limited.

Differences in enzyme characteristics between different plant species or genotypes are related to alteration in quantity and quality of root exudates as well as modification in root morphology (Zhang et al. 2019; Bilyera et al. 2021; Song et al. 2022). Root exudates serve as a source of nutrients and energy for

microbes to thrive and synthesize their own enzymes (Neumann and Römheld 2002; Badri and Vivanco 2009). However, the regulatory effects of root exudates on enzyme properties in the rhizosphere are heavily dependent on root traits. This is because the production and composition of exudates are strongly influenced by root morphology (Holz et al. 2018; Kuzyakov and Razavi 2019). The roots are composed of discrete segments such as root cap, root tip, elongation zone, root-hair zone, mature zone, and lateral root that exhibit varying degrees of maturity and specialization, each possessing unique physiological and biochemical properties. As a result, exudates are distributed heterogeneously along the root due to these distinct characteristics (Pausch and Kuzyakov 2011; Sasse et al. 2020). Lateral roots are roots that emerge from the seminal root and grow horizontally, enabling the plant to explore a larger volume of soil and acquire water and nutrients more efficiently than the seminal root alone (Lloret and Casero 2002; Ahmed et al. 2016). Additionally, exudation is primarily concentrated in the root tips and emergence sites of lateral root (Van Egeraat 1975; McCormack et al. 2015; Holz et al. 2018; Bilyera et al. 2021). This concentration results in a higher density of microbes, greater microbial activity, and elevated enzyme production within these regions compared to other sections of root (DeAngelis et al. 2009). Our previous study also has demonstrated that the lateral root regions exhibit higher drought resistance of enzyme activity compared to the seminal root regions (Hosseini et al. 2022). This calls for investigating the effect of lateral roots on localization of enzyme activity and ultimately on the drought tolerance of wheat.

This study aims to investigate the spatial distribution (hotspot area and rhizosphere extent) and kinetic properties of enzymes in the rhizosphere of various wheat genotypes with varying levels of drought tolerance and their relationship to root properties (i.e., lateral and seminal roots). The findings of this research will contribute to our understanding of the interplay between enzyme traits in rhizosphere and drought tolerance of wheat, enabling the development of genotypes better adapted to drought conditions. We hypothesized that: i) wheat breeding manipulates the spatial distribution and catalytic properties of enzymes in the rhizosphere; ii) increasing wheat resistance to drought is associated with broader enzymatic hotspot areas and higher enzyme efficiency; iii)

wheat genotypes with more lateral roots have broader enzymatic hotspot areas and higher enzyme efficiency. To evaluate these hypotheses, a three wheat genotypes -Baran (rainfed drought-tolerant), Sirvan (drought-tolerant), and Marvdasht (non-drought-tolerant)- were grown in rhizoboxes under optimum condition, followed by drought stress equivalent to 30% of water holding capacity (WHC) for a duration of three weeks. To investigate the spatial distribution of acid phosphatase (ACP) and β -glucosidase (GLU) in the rhizosphere of different wheat genotypes and their relationship with root morphology, in-situ soil zymography was employed. Additionally, ex-situ kinetic assays of ACP and GLU were performed to examine the dynamic behavior of these enzymes and their potential linkage with the drought tolerance of wheat.

Materials and methods

Soil collection and experimental setup

The soil sample (a typical Haplocambid) was collected from the Ap-horizon of a wheat field (36° 15' N, 59° 40' E) placed in Mashhad, Iran. The physical and chemical characteristics of soil are given in our previous study (Hosseini et al. 2022). After removing the roots and stones, the soil was air-dried and sieved by a 2 mm mesh. Then soil was fertilized according to fertilizer recommendation with urea (320 mg kg⁻¹), triple superphosphate (350 mg kg⁻¹), and potassium sulfate (250 mg kg⁻¹) and prepared for pouring in rhizoboxes with an inner size of 21 × 18 × 2 cm (height × width × diameter). To prevent making soil layers and achieve a uniform soil surface, rhizoboxes were horizontally filled with soil.

Three wheat (*Triticum aestivum*) genotypes with different tolerance to drought -Baran (rainfed drought-tolerant), Sirvan (drought-tolerant), and Marvdasht (non-drought-tolerant)- were selected according to the recommendation of the Iranian Seed and Plant Improvement Institute. Three-day-old seedlings of each genotype were sown in separate rhizoboxes in four replicates. The rhizoboxes were retained in a greenhouse environment during the wheat's growing season. The day and night temperatures were 25 °C and 18 °C, respectively, and the light intensity was 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 12 hours per day. Rhizoboxes

were positioned slantwise (at a 45° angle) to direct root growth toward the rhizobox's lower wall. The soil water content was maintained at 70% of their WHC for the first 20 days of wheat growth. Then, drought stress was applied by drying the rhizoboxes to 30% of WHC. Wheat genotypes were grown for 20 other days at this moisture level. To ensure an even distribution of moisture throughout the rhizoboxes, distilled water was added daily from the top and bottom.

In-situ soil zymography

After plant growth for 40 days, the lower wall of rhizoboxes was removed and in-situ zymography was applied for mapping ACP and GLU in the rhizosphere according to the method described by Razavi et al. (2019). 4-methylumbelliferyl (MUF)- β -D-glucopyranoside and MUF-phosphate (all from Sigma Aldrich, Germany) were used as substrates for visualizing the activities of GLU and ACP. The substrate of each enzyme was dissolved to a concentration of 12 mM in the MES ($C_6H_{13}NO_4SNa_{0.5}$; Sigma Aldrich, Germany) buffer with pH 6.5. The polyamide membrane (0.45 μ m pore size and 20 cm diameter, Tao Yuan, China) was soaked in substrate solution and immediately attached to the soil surface for 2 hours. Afterward, the membrane was removed from the soil surface and carefully cleaned using a soft brush. A UV light source with an excitation wavelength of 355 nm and an emission wavelength of 460 nm was used to photograph the membrane using a digital camera, the Nikon D5300.

The standard calibration curve was made by photographing the 2×2 saturated polyamide membranes with an increasing concentration of MUF (0, 0.2, 0.5, 1, 2, 4, 6, 8, and 10 mM) under the UV light. The enzyme activity corresponding to gray values was calculated based on the size of the polyamide membrane, the volume of MUF absorbed by the membrane, and the concentration of MUF, and then expressed as pmol MUF mm⁻² h⁻¹.

Image processing

The Image-J program was used for all procedures involving the extraction of gray values from images and their conversion into enzyme activities. The original images were first changed into 8-bit grayscale

images. Then, the background value (zero concentration of standard calibration curve) was subtracted from all images. Modified gray values were converted to enzyme activities according to the standard calibration line. The mean+2SD (standard deviation) of enzyme activity in the background portion of the zymogram was considered as the threshold to distinguish hotspots of enzyme activity or areas with high enzyme activity from bulk soil or areas with low enzyme activity (Bilyera et al. 2020). We showed the hotspots of enzyme activity with red color in zymograms. The rhizosphere extent of enzyme activity was considered from the root center to the areas with at least 20% higher enzyme activity than in bulk soil. Different root regions were considered for determining rhizosphere extent. The rhizosphere extent was separately calculated for seminal and lateral roots and normalized by dividing it by root radius. Also, we measured rhizosphere extent for root tips (0 to 2 cm) and mature roots (> 3 cm from root tip) (Yu et al. 2016). Average enzyme activity in the rhizoplane and rhizosphere was determined by calculating the weighted average of the enzyme activity based on pixels located within these respective regions. The rhizoplane is defined as the root surface zone, while the rhizosphere encompasses the area from the root surface extending to the outer boundary of the bulk soil.

Ex-situ enzyme kinetics

After zymography, soil sampling from the rhizosphere was done to determine the enzyme kinetic properties using the fluorometric microplate assay (German et al. 2011). A range of concentration (0, 20, 40, 60, 80, 100, 200, and 400 μ mol) of the fluorogenic substrates such as MUF- β -D-glucopyranoside and MUF-phosphate was used to measure kinetics parameters of GLU and ACP, respectively. One gram of fresh soil was dispersed in 50 mL sterilized water and shaken for 30 min at 250 rpm. Prepared soil suspension (50 μ L), MES buffer (50 μ L), and substrate solution (100 μ L) were transferred by multichannel pipet into microplates. The fluorescence was assayed at four-time points (0, 30 min, 1 h, and 2 h after substrate addition) using a CLARIOstar Plus multi-mode microplate reader (BMG LABTECH, Germany). The fluorescence intensity was converted to enzyme activity (nmol MUF g⁻¹ soil h⁻¹) using a standard

calibration line. To determine V_{max} (maximum enzyme activity, nmol MUF g^{-1} dry soil h^{-1}) and K_m (Michaelis-Menten constant, nmol substrate g^{-1} dry soil), the enzyme activities were plotted by fitting the non-linear regression of the Michaelis-Menten Eq. (1) in SigmaPlot 12.3 software:

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

where V and S are enzyme activity and substrate concentration, respectively.

Catalytic efficiency (K_a , nmol MUF nmol substrate $^{-1}$ h^{-1}) and turnover time of added substrates (T_t , hour) were calculated using the Eq. (2) and (3), respectively:

$$K_a = \frac{V_{max}}{K_m} \quad (2)$$

$$T_t = \frac{K_m + [S]}{V_{max}} \quad (3)$$

As the drought conditions reduce the input of root exudate into the rhizosphere (Holz et al. 2018), hence we calculated T_t at substrate concentrations reflecting scenarios of substrate deficiency or low concentration, represented by $S = K_m$ (Hoang et al. 2022).

Shoot and root properties

After conducting zymography and collecting soil samples for enzyme kinetics analysis, the plant's shoots and roots were meticulously harvested. Subsequently, they were thoroughly rinsed with distilled water and subjected to a drying period of 72 hours at 70 °C, followed by precise weight measurement. The root morphology traits, including the length, surface area, volume, and diameter of seminal and lateral roots on the soil surface, were quantified by analyzing rhizobox images using the SmartRoot plugin within the Image-J software (Lobet et al. 2011).

Statistical analysis

The ANOVA analysis, Tukey's HSD test ($P < 0.05$), and Pearson correlation analysis were done in JMP8 software. One-way ANOVA was applied to test the differences in means among wheat genotypes or root

types. Also, principal component analysis (PCA) was done by GraphPad Prism 9 software to study the relationship between root and enzyme properties.

Results

Shoot and root properties

The length, surface, and volume of seminal roots for the Baran and Sirvan genotypes were significantly higher than for the Marvdasht genotype. The parameters of lateral roots including length, surface, and volume for the Baran genotype were higher than Sirvan and Marvdasht genotypes (Fig. 1a-c). The diameter of seminal and lateral roots did not differ among wheat genotypes (Fig. 1d). The root system of the Baran genotype created 1.6- and 2.6-fold more lateral root tips in comparison with the Sirvan and Marvdasht genotypes ($p < 0.05$, Fig. 1e). The shoot and root dry biomass and their ratio for the Baran genotype were considerably greater than for Marvdasht and Sirvan genotypes ($p < 0.05$, Fig. 1f, Fig. S1).

Spatial distribution of enzyme activities

Baran had the highest hotspot percentage of GLU and ACP among all genotypes, which were 1.5- and 1.2-fold greater than Sirvan and 2- and 2.7-fold greater than Marvdasht, respectively ($p < 0.05$, Fig. 2). Furthermore, the normalized hotspot percentage of GLU in Baran was greater than in Sirvan ($p < 0.05$; Fig. S2). Additionally, the normalized hotspot percentage of ACP in Baran was higher than that in other genotypes, but it was not statistically significant (Fig. S2).

In the Baran genotype, the rhizosphere extent of GLU activities in seminal roots was significantly broader than in lateral roots ($p < 0.05$, Fig. 3a). Conversely, in Marvdasht, an inverse pattern emerged, revealing that the rhizosphere extent of GLU activities in lateral roots was significantly higher than in seminal roots ($p < 0.05$, Fig. 3a). The normalized rhizosphere extent of GLU in the lateral roots of Marvdasht, as well as ACP in the lateral roots of Baran and Sirvan, exhibited a significantly greater presence compared to their corresponding seminal roots ($p < 0.05$, Fig. 3b-d). In drought-tolerant genotypes, the rhizosphere extent of GLU and ACP activities

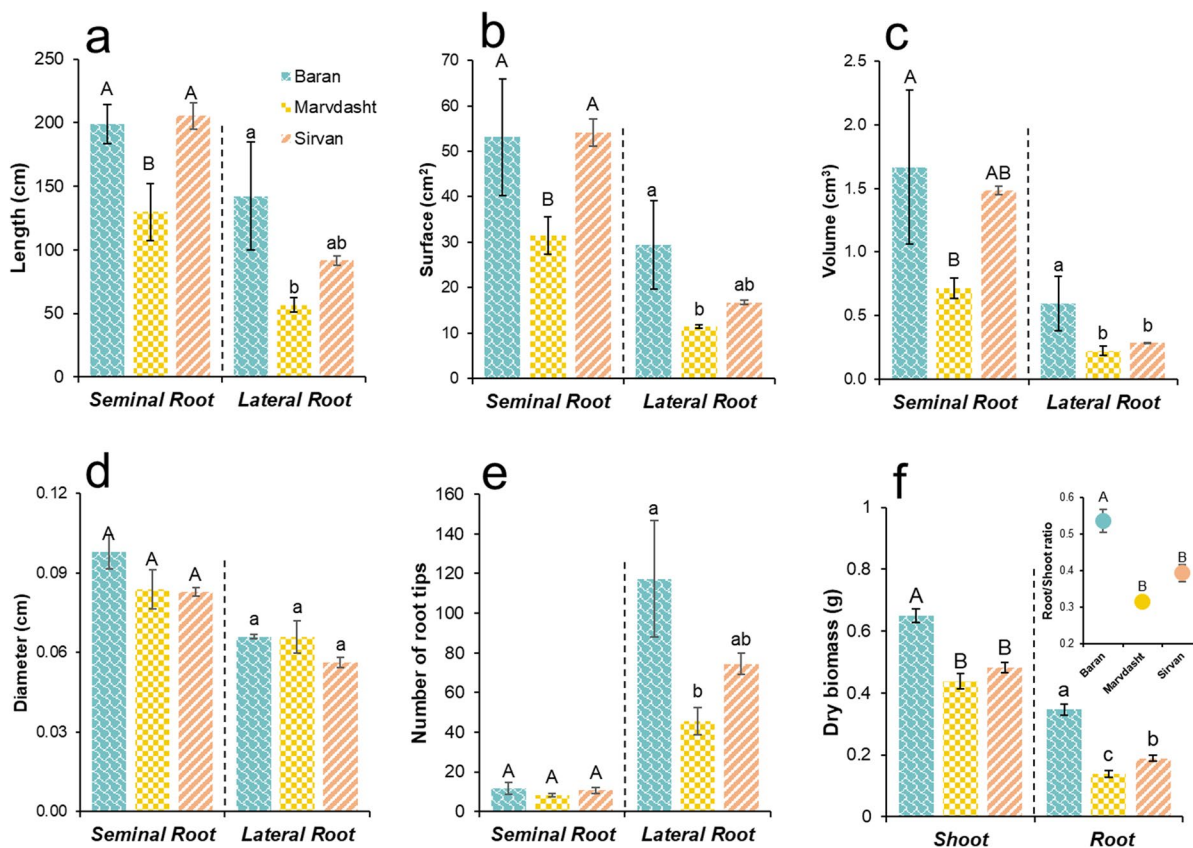


Fig. 1 Length (a), surface (b), volume (c), diameter (d), number of root tips (e) of seminal and lateral roots on the soil surface, and dry biomass of root and shoot and their ratio (f)

in various wheat genotypes. Different letters represent significant differences among wheat genotypes in each root type ($p < 0.05$). Error bars are standard deviation ($n = 4$)

for root tips was significantly higher than for mature roots ($p < 0.05$, FigS3a-b). Expansion of ACP activities in seminal root regions of Baran was 15 to 35% higher than in two other genotypes ($p < 0.05$, Fig. 3c). The rhizosphere extent of GLU in lateral root regions of Baran and Marvdasht was approximately 40% broader than in Sirvan ($p < 0.05$, Fig. 3a). The rhizosphere extent of ACP for the mature roots and root tips of Baran were 0.85 and 1.18 mm which were the highest among all wheat genotypes ($p < 0.05$, Fig. S3b).

Average enzyme activity in rhizoplane and rhizosphere of lateral roots was greater than seminal roots ($p < 0.05$; except for ACP in rhizoplane of Baran and GLU in rhizosphere of Marvdasht). Baran had the highest GLU and ACP activity in the rhizoplane, followed by Sirvan, while Marvdasht had the lowest one (Fig. S4a). We observed the opposite trend in the

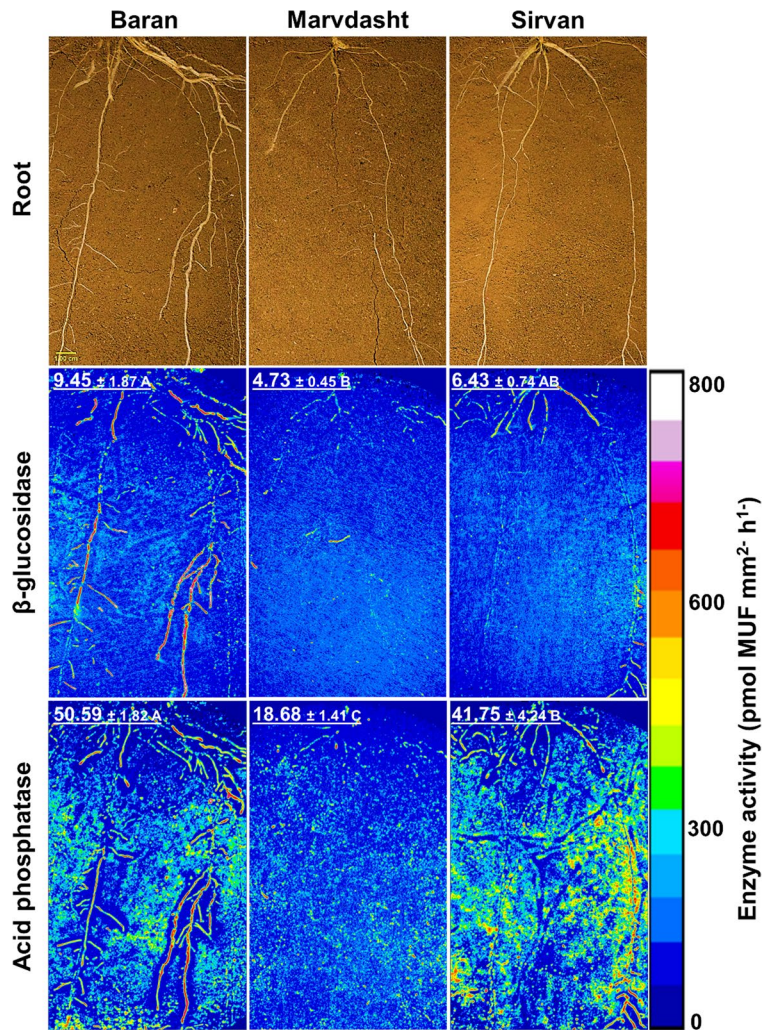
rhizosphere, Marvdasht had the highest ACP activity in seminal and lateral roots, followed by Sirvan and Baran (Fig. S5b). In the case of GLU, Sirvan exhibited the highest activity in lateral roots, whereas the GLU activity in seminal roots remained unaffected by genotypes (Fig. S5b).

Generally, the hotspot percentage, rhizosphere extent, and average enzyme activities in rhizoplane were based on the following order: Baran > Sirvan > Marvdasht.

Catalytic properties

The V_{\max} of GLU for Marvdasht was approximately 2-fold greater than Baran and Sirvan ($p < 0.05$, Fig. 4a). Also, the V_{\max} of ACP for Marvdasht and Sirvan was 1.8-fold higher than Baran ($p < 0.05$, Fig. 4b). The affinity of GLU and ACP to substrates

Fig. 2 Spatial distribution of GLU and ACP activities in the rhizosphere of various wheat genotypes. Hotspots or areas with high enzyme activities are represented by red color and areas with low enzyme activities are indicated by blue color. The color map is proportional to enzyme activity ($\text{pmol MUF mm}^{-2} \text{h}^{-1}$). Values on each zymogram represent: the hotspot percentage of enzyme activity \pm standard deviation ($n=4$). Different letters represent significant differences among wheat genotypes ($p < 0.05$)



in the rhizosphere of drought-tolerant genotypes were higher (the lower K_m) than non-drought-tolerant one ($p < 0.05$, Fig. 4c-d). The turnover time of substrates decomposed by GLU and ACP was faster in the rhizosphere of Baran and Sirvan than in the rhizosphere of Marvdasht (Fig. 5a-b). In the case of GLU, for example, the substrate turnover time for Baran and Sirvan was at least 30% shorter than for Marvdasht ($p < 0.05$, Fig. 5a). The catalytic efficiency of GLU and ACP for drought-tolerant genotypes was significantly higher than for non-drought-resistant ones ($p < 0.05$, Fig. 5c-d). In the case of ACP, the Baran genotype had 1.6-fold higher catalytic efficiency than Sirvan ($p < 0.05$, Fig. 5d).

The V_{max} of GLU and ACP had a negative correlation with hotspot percentage ($r = -0.54$, -0.70 ,

$p < 0.05$), rhizosphere extent of lateral roots (only for ACP $r = -0.86$, $p < 0.05$), rhizosphere extent of seminal roots ($r = -0.72$, -0.81 , $p < 0.05$) and a positive correlation with enzyme activity in the rhizosphere of lateral roots (only for ACP $r = 0.89$, $p < 0.05$) and seminal roots ($r = 0.52$, 0.54 ; Fig. 6).

Relationship between enzyme properties and root morphology

We found a positive correlation between hotspots and the catalytic efficiency of enzymes with different root traits. Among root properties, lateral root traits included the length, surface, volume, and number of root tips showed the strongest positive correlation with the hotspot percentage and catalytic efficiency of GLU and

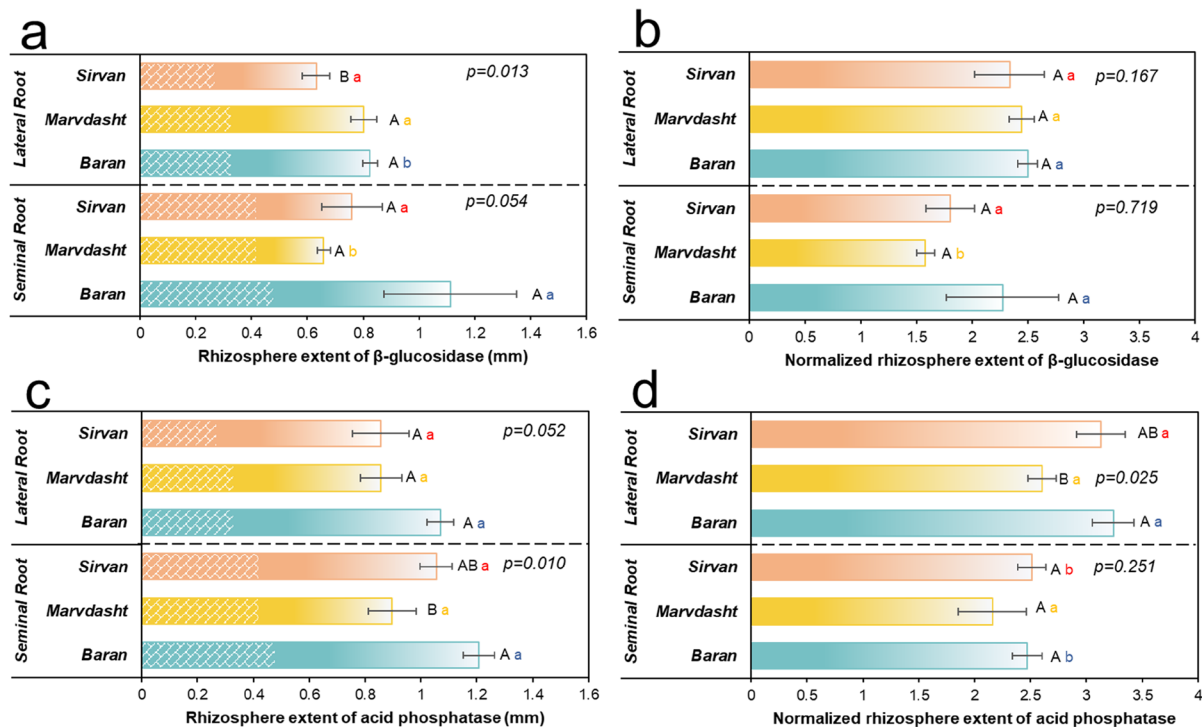


Fig. 3 Rhizosphere extent (**a** and **c**) and rhizosphere extent per root radius (normalized rhizosphere extent; **b** and **d**) of enzyme activity in seminal and lateral root regions of various wheat genotypes. Different capital letters represent significant dif-

ferences among wheat genotypes in each root type ($p < 0.05$). Different small letters indicate significant differences between seminal and lateral root in each genotype ($p < 0.05$). Error bars are standard deviation ($n = 4$)

ACP ($p < 0.05$; Fig. S5). The PCA analysis showed that the first and second PCs explain 71.87% and 14.32% of the total variance, respectively (Table S1). Root dry biomass, lateral root traits (length, surface, volume, and number of root tips), hotspot percentage, and catalytic efficiency of GLU and ACP had a strong positive correlation with PC1. Seminal root traits (length, surface, and diameter) and diameter of lateral roots were the main drivers of PC2 (Table S1, Fig. 7). The small angle of the loading vectors between lateral root traits (except for diameter) and hotspots of GLU and ACP suggests a positive correlation between them. Baran genotype was clearly separated from the two other genotypes and characterized by higher lateral root traits and a greater hotspot percentage of GLU and ACP (Fig. 7).

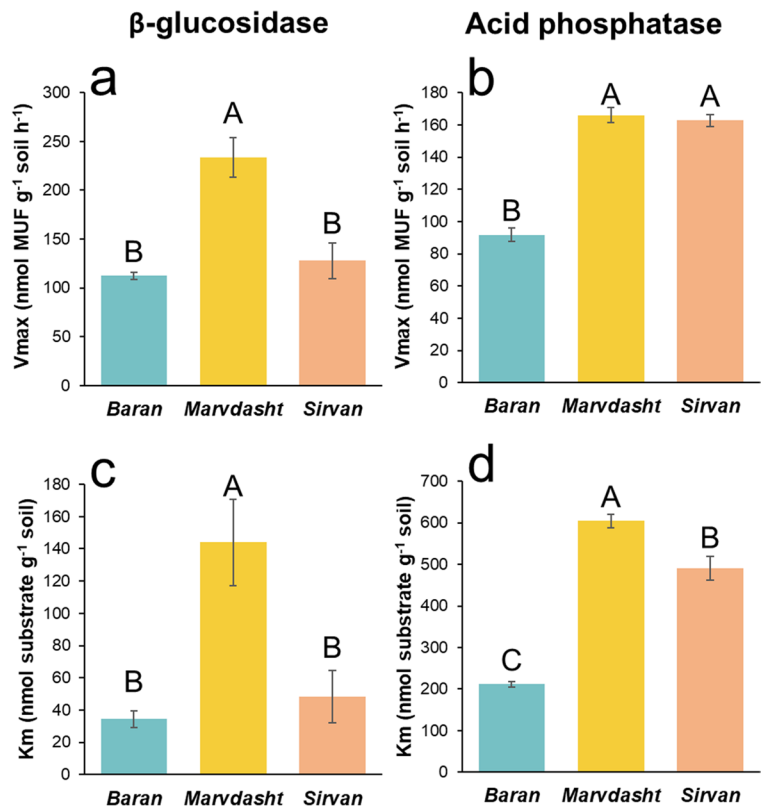
Discussion

In this study, drought-tolerant genotypes had a higher shoot dry biomass and an extensive root

system compared to the non-drought-tolerant genotype (Fig. 1, Fig. S1). The extensive root system and high root-to-shoot ratio are common traits of drought-tolerant plants (Ehdaie et al. 2012; Demelash et al. 2021). Furthermore, among drought-tolerant genotypes, Baran created more branched lateral roots compared to Sirvan which can increase the capacity of the root to explore the soil to uptake water and nutrients under drought stress (Ahmed et al. 2016).

Our result revealed that the spatial pattern of enzyme activity differed among wheat genotypes and correlated with root properties and plant genetics, confirming our first hypothesis. Among the wheat genotypes, Baran and Sirvan had the largest enzyme hotspot regions (GLU and ACP) (Fig. 2). Drought-tolerant species genetically create greater above-ground and below-ground biomass and perform photosynthesis normally under drought stress, resulting in more total root exudation per plant and a large surface area for microbes attachment than non-drought-tolerant ones (Song et al. 2012; Azarbad et al. 2018;

Fig. 4 Maximum catalytic activity (V_{max} ; **a** and **b**) and Michaelis-Menten constant (K_m ; **c** and **d**) of GLU and ACP in the rhizosphere of various wheat genotypes. Different letters represent significant differences among wheat genotypes ($p < 0.05$). Error bars are standard deviation ($n = 4$)



Bilyera et al. 2021). The development of enzymatic hotspots in the rhizosphere has been reported to be influenced by plant growth (Giles et al. 2018; Ma et al. 2018a; Hosseini et al. 2022). However, regardless of the root surface, the Baran represented higher normalized hotspots than other genotypes, indicating the great potential of this genotype to produce root exudates (Fig. S2). This is most likely due to the formation of more lateral roots and root tips in the Baran genotype because the root exudations occur mainly there than on other root parts (Ashford and McCully 1973; Lloret and Casero 2002; Holz et al. 2018; Ma et al. 2021). Formation of lateral root primordia leads to significant damage in the primary root cell cortex (Neumann and Römheld 2002), leaking out easily degradable organic carbon and sugars (Neumann and Römheld 2002; Rüger et al. 2021). Furthermore, root cell walls are not formed at the tips of young roots which may increment the release of various organic carbons (McCully and Canny 1985; Pausch and Kuzyakov 2011; Kuzyakov and Razavi 2019; Ma et al. 2021) and enlarge hotspots of enzyme activity. These findings are supported by higher enzymatic

activities at the rhizoplane of lateral roots and root tips than seminal and mature roots (Fig. S4a). Previous studies have also shown that plants with more lateral roots have a higher abundance, diversity, and microbial activity (Yu et al. 2021; Galindo-Castañeda et al. 2022). More lateral roots increase microbial attachment surfaces, endophyte entry points, and carbon rhizodeposition, which could provide a high capacity for the maintenance of microbial communities (Liu et al. 2006; Galindo-Castañeda et al. 2022). Yu et al. (2021) found that lateral roots are a crucial trigger for maintaining the spatial distribution of bacterial diversity in the maize rhizosphere. PCA and correlation analyses also demonstrated that hotspots of enzyme activity strongly correlated with lateral root traits (Fig. 7).

The rhizosphere extent of enzyme activities illustrates the response of plants and microbes to nutrient limitation or, in the case of the latter, the availability of rhizodeposits, which serve as their main source of energy (Burns et al. 2013; Song et al. 2022). The axial distribution of enzyme activity in different regions of the rhizosphere was genotype-specific owing to

Fig. 5 Turnover time (T_i ; **a** and **b**) and catalytic efficiency (K_a ; **c** and **d**) of GLU and ACP in the rhizosphere of various wheat genotypes. Different letters represent significant differences among wheat genotypes ($p < 0.05$). Error bars are standard deviation ($n = 4$)

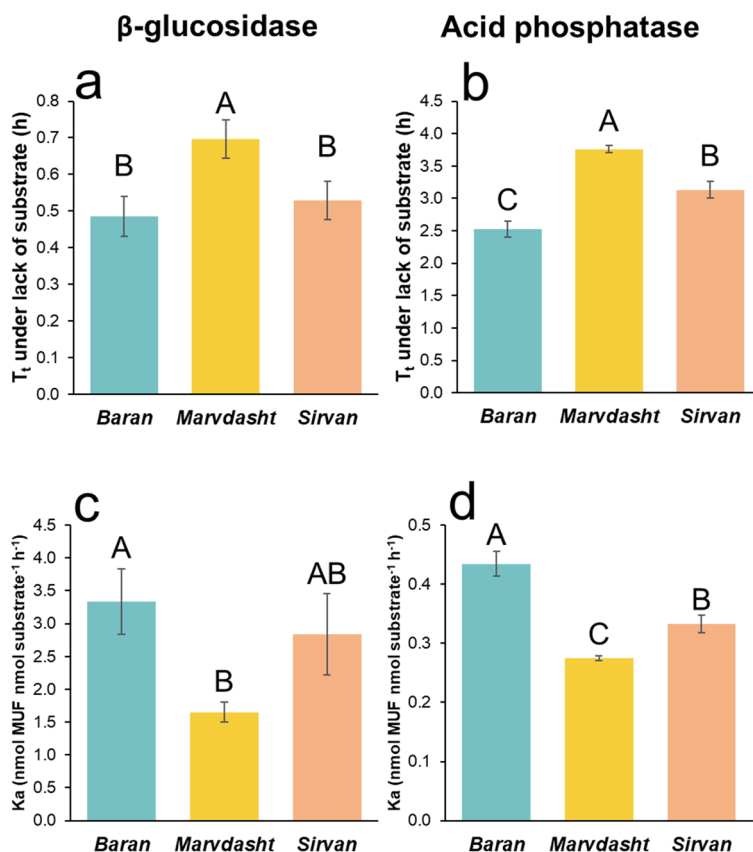
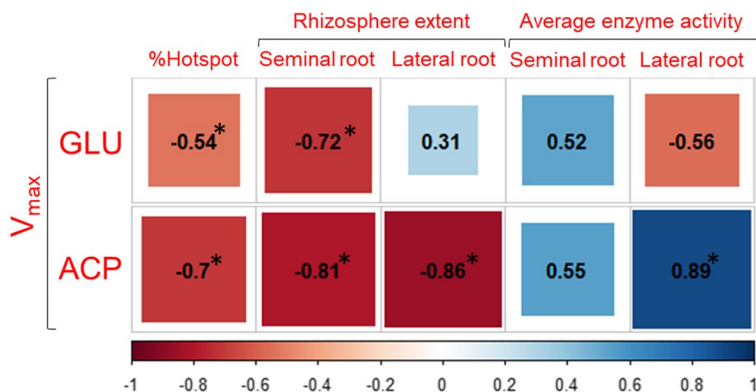


Fig. 6 Pearson's correlation coefficients between V_{max} , hotspot percentage, rhizosphere extent and average enzyme activity. Asterisks indicate that Pearson's correlation coefficients are significant at $p < 0.05$



different root morphologies (root radius) (Jungk 2001; Ma et al. 2018a, b; Holz et al. 2020). Additionally, the normalized rhizosphere extent varied among genotypes due to the various nutrient mobilization abilities and root exudation patterns (Razavi et al. 2016b; Bilyera et al. 2021; Ma et al. 2021; Song et al. 2022). The expansion of enzyme activity in the rhizosphere of Baran and Sirvan was broader than in Marvdasht,

supporting the higher enzymatic hotspots found in these genotypes (Fig. 3, Fig. S3). As the enzyme diffusion in the soil is very limited (Guber et al. 2018), root-derived enzymes are restricted to the rhizoplane (Bilyera et al. 2021). Therefore, a greater extension of the rhizosphere in drought-tolerant genotypes results from the high availability of rhizodeposits, which stimulate microorganisms to produce enzymes. This

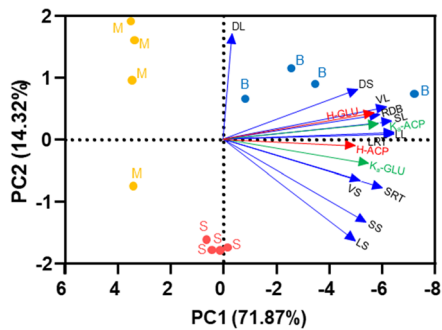


Fig. 7 Biplot of PCA for the relationship between root traits of three wheat genotypes and some enzyme properties. GLU, β -glucosidase; ACP, acid phosphatase; H, hotspot percentage; K_a , catalytic efficiency; RDB, root dry biomass; LS, length of seminal root; LL, length of lateral root; SS, surface of seminal root; SL, surface of lateral root; VS, volume of seminal root; VL, volume of lateral root; DS, diameter of seminal root; DL, diameter of lateral root; SRT, number of seminal root tip; LRT, number of lateral root tip; B, Baran; M, Marvdasht; and S, Sirvan

in turn increases the volume of soil in which these genotypes can explore for nutrients (Zhang et al. 2022; Zhou et al. 2022). Additionally, the lateral roots acquire nutrients more effectively than the seminal roots, as evidenced by a higher normalized rhizosphere extent in lateral roots (Fig. 3b-d).

The average enzyme activity in the rhizosphere of lateral and seminal roots (especially for ACP) was higher in Marvdasht than in two other genotypes (Fig. S4b), which was partially consistent with the results for maximum reaction rate (V_{max}) (Figs. 4a-b and 7). The increase in V_{max} of GLU and ACP in the Marvdasht is probably a strategy to compensate for the narrow size of the rhizosphere, as demonstrated by a negative correlation between V_{max} and rhizosphere extent or hotspot percentage (Fig. 7). In fact, the root and rhizosphere microbiome of Marvdasht in response to drought stress and nutrient limitation release more enzymes to break down soil organic matter and consequently meet their requirements (Burns et al. 2013; Liu et al. 2022). For example, the high ACP activity in root-adjacent soil intensifies the mineralization of organic phosphorus in the narrow rhizosphere and consequently improves the uptake of phosphorus by the plant (Holz et al. 2020). However, the high K_m or low affinity of GLU and ACP to substrate counteracts the high V_{max} , which inhibits enzymatic reactions and likely slows down the microbial

mineralization of soil organic matter (Zhang et al. 2022). These findings are supported by the longer turnover time of GLU and ACP in the Marvdasht rhizosphere (Fig. 5a-b). The higher K_m in Marvdasht reveals a shift in the functional structure of the microbiome toward microbial communities with low enzyme efficiency (Blagodatskaya et al. 2009; Zhang et al. 2020a; Hoang et al. 2022), as evidenced by lower K_a in our study (Fig. 5c-d).

In contrast, the low V_{max} of GLU in the Baran and Sirvan is likely a strategy of the soil microbial community to conserve energy in stress status (Fig. 4a). It is assumed that the more lateral roots in drought-tolerant genotypes provide a high content of easily degradable organic carbon such as glucose which is utilized directly by soil microorganisms for growth (Godin et al. 2015) and thus reduce the production of carbon-acquiring enzymes (Elser et al. 2003; Tischer et al. 2015). Furthermore, Baran lateral roots may increase the dissolution of inorganic phosphorus in the soil through the release of H^+ and organic acids (Rudolph-Mohr et al. 2017; Zhang et al. 2021), or stimulate phosphate-solubilizing microorganisms to mobilize phosphorus (Wei et al. 2019; Liu et al. 2022) and thus improve soil available phosphorus and decrease the ACP production in the rhizosphere compared to Sirvan and Marvdasht (Fig. 4b). The lower K_m of GLU and ACP in the rhizosphere of drought-resistant genotypes reflects the expression of various enzyme types (isoenzymes) by similar or different microbial communities (Fig. 4c-d). Previous studies have shown that the secretion of multiple enzyme isoforms would maintain the high catalytic efficiency and crucial functions under water stress (Razavi et al. 2016a; Wang et al. 2020). Thus, a higher K_a or enzyme catalytic efficiency in the rhizosphere of drought-tolerant genotypes indicates a redirection in carbon allocation towards the biosynthesis of isoenzymes, rather than microbial growth (Zhang et al. 2020b). This adaptation reflects an efficient microbial strategy in coping with drought stress (Dorodnikov et al. 2009). These findings are confirmed by the faster turnover time of GLU and ACP which accelerates the decomposition of soil organic matter and is a critical strategy for improving nutrient and carbon transformation under drought stress (Hoang et al. 2022). Taken together, the preferred microbial strategy in the rhizosphere of Baran and Sirvan (drought-tolerant genotypes) was to produce an enzyme pool

with higher stability and efficiency rather than a large amount of enzymes.

Conclusion

We found that wheat breeding manipulated root morphology, the spatial distribution of enzyme activities, and the catalytic properties of enzymes in the rhizosphere, which may lead to different capacities for acquiring nutrients and improving drought resistance. Non-drought-tolerant wheat (Marvdasht) invested more in enzyme production (high V_{\max}) rather than in root development, resulting in small areas of microbial hotspots with low enzyme efficiency (high K_m), which was not a viable strategy under drought conditions. In contrast, drought-tolerant genotypes, especially Baran, invested more in lateral root formation rather than in enzyme production. Lateral roots are characterized by higher exudation (more enzymatic activity in the rhizoplane of the lateral roots) and wider extension of the rhizosphere, which in turn led to the expansion of hotspots of enzymatic activities towards the bulk soil and thus increased the ability of drought-tolerant genotypes to acquire nutrients from the soil and cope with drought stress. Additionally, the preferred microbial strategy in hotspots of drought-tolerant genotypes was to produce an enzyme pool with a high affinity for substrates that maintain the high catalytic efficiency and crucial functions under drought stress.

Our results suggest that drought-tolerant genotypes used a combination of strategies to cope with water stress, which were mainly driven by lateral roots development. We, therefore, conclude that forming lateral roots, expanding rhizosphere extent or microbial hotspots, and producing enzymes with high efficiency can be incorporated as fundamental traits in breeding programs for the development of drought-resistant wheat genotypes.

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on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets are available from the corresponding author on request.

Declarations

Competing interests The authors declare no conflict of interest.

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