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IN SILICO METABOLIC PATHWAY REPROGRAMMING IN THE WHEAT RHIZOSPHERE UNDER DROUGHT STRESS: COMPARATIVE INSIGHTS FROM PAKISTAN-13 AND DHARABHI-11 VARIETIES

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Abstract

Drought stress poses a significant threat to global wheat production and calls for a comprehensive understanding of plant metabolic adaptations, particularly within the rhizosphere. This study aimed to elucidate the metabolic pathway reprogramming in the wheat rhizosphere under drought conditions by performing an in silico analysis on two contrasting wheat varieties: the drought-sensitive Pakistan-13 and the drought-tolerant Dharabhi-11. Metabolite profiles were obtained using gas chromatography–mass spectrometry (GC-MS) and further analyzed through advanced computational methods to assess pathway enrichment and topology. Our findings reveal that both genotypes activate central metabolic pathways, including Aminoacyl-tRNA Biosynthesis and Alanine, Aspartate, and Glutamate Metabolism, which are integral to nitrogen assimilation and protein synthesis. However, Pakistan-13 exhibited a high impact in a limited number of pathways, while critical stress-responsive processes such as Proline Biosynthesis and ROS Detoxification were underrepresented. In contrast, Dharabhi-11 demonstrated a robust and diversified activation of its metabolic networks, with enhanced engagement in the TCA cycle, branched-chain amino acid metabolism, and antioxidative defense systems. These differences underline the superior drought tolerance of Dharabhi-11 and indicate that an efficient, integrated metabolic response is essential for maintaining cellular homeostasis under water-deficit conditions. The insights from this study not only enhanced our understanding of rhizosphere metabolic dynamics under drought stress but also provide a strategic framework for targeted metabolic engineering and breeding. Such interventions could narrow the resilience gap between susceptible and tolerant varieties, ultimately contributing to the development of future climate-resilient wheat cultivars.

Key words: Drought stress; Wheat rhizosphere; Metabolic pathway; In silico analysis; GC-MS metabolomics; Proline biosynthesis

Introduction

Wheat is a fundamental staple crop globally, forming the backbone of food security and agricultural economies (Godfray *et al*., 2010; Shiferaw *et al*., 2011). However, its production is increasingly threatened by drought stress, a challenge that not only reduces yields but also affects the intricate biochemical processes in the plant rhizosphere - the narrow soil zone directly influenced by plant roots and associated microbial communities (Philippot *et al*., 2013; Zhao *et al*., 2017). Drought stress disrupts metabolic functions essential for plant growth, including nutrient uptake, energy production, and osmotic balance (Badri & Vivanco, 2009; Aslam *et al*., 2022). Recent advances in metabolomics and in silico pathway analysis have enabled researchers to unravel complex metabolic networks and identify key pathways that support plant adaptation under water-deficit conditions (Obata & Fernie, 2012; Sallam *et al*., 2019).

Despite these technological advances, many wheat varieties continue to suffer significant yield losses under drought conditions. The disruption of critical metabolic pathways, particularly in the rhizosphere where plant-microbe interactions drive nutrient cycling, remains poorly understood (Islam *et al*., 2015). This gap in understanding hampers the development of targeted strategies to enhance drought tolerance. While several studies have highlighted the roles of pathways such as aminoacyl-tRNA biosynthesis and nitrogen metabolism in drought response, few have integrated comprehensive in silico metabolite pathway analysis with detailed rhizosphere metabolomics in wheat. Moreover, most existing research has focused on either drought-tolerant or drought-susceptible varieties in isolation, rather than providing a comparative framework that can guide breeding programs (Gregorova *et al*., 2015; Yadav *et al*., 2019).

Recent studies highlight that metabolic shifts, particularly in amino acid and carbohydrate metabolism, play a central role in plant adaptation to drought stress (Templer *et al*., 2017; Lin *et al*., 2023). Accumulation of metabolites such as proline, glutamate, and sucrose has been associated with improved osmotic adjustment and

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maintenance of energy balance in drought-tolerant varieties (Szabados & Savouré, 2010; Maghsoudi *et al*., 2018; Shrestha *et al*., 2021). Despite these advances, the integration of metabolite-level observations with genetic and pathway-scale analyses remains limited (Arbona *et al*., 2013). This gap is particularly concerning in drought-prone regions like South Asia and the Middle East, where water scarcity severely restricts wheat production. In such contexts, understanding the metabolic basis of drought tolerance is essential for guiding breeding programs and developing context-specific, resilient wheat varieties (Mason *et al*., 2012; Abdelrahman *et al*., 2019).

The objective of this study is to investigate the metabolic pathways underlying drought adaptation in wheat by comparing drought-tolerant (DH-11) and drought-susceptible (PK-13) varieties. Using GC–MS based metabolomic profiling integrated with in silico pathway enrichment and network analyses, the study seeks to identify key metabolites and pathways associated with stress tolerance, providing insights for improving drought resilience in wheat.

Material and Methods

Plant material: Two contrasting wheat (*Triticum aestivum* L.) genotypes were selected based on their drought response profiles: the drought-sensitive Pakistan-13 (PK-13; Accession No. 35989) and the drought-tolerant Dharabhi-11 (DH-11; Accession No. 35993). Certified seeds of both varieties were sourced from the Bio-Resource Conservation Institute (BRCI), National Agriculture Research Centre (NARC), Islamabad, Pakistan.

Experimental setup: A pot experiment was conducted under greenhouse conditions to simulate controlled drought stress. Environmental parameters were maintained with a 12-hour light/dark photoperiod, daytime temperature of 32°C, nighttime temperature of 24°C, and relative humidity levels ranging between 40–50%. Each pot was filled with approximately 2 kg of sieved agricultural soil, and five seeds were sown per pot.

Drought stress imposition and experimental designs: Drought stress treatments were initiated at the anthesis stage by withholding irrigation for 5, 10, and 15 days after irrigation (DAI) in accordance with the protocol described by Marchin *et al*., (2020). In contrast, control plants were irrigated consistently to maintain optimal soil moisture throughout the growth period. A single application of a slow-release granular fertilizer (NPK 15-9-12) was administered during the vegetative phase to ensure baseline nutritional adequacy across treatments. The experiment was laid out in a randomized complete block design (RCBD) comprising three biological replicates for each genotype and treatment condition. This design enabled strong comparative analysis of varietal responses under both well-watered and drought-stressed scenarios.

Rhizosphere metabolite extraction and derivatization: Rhizospheric soil samples were collected from the root zones samples from both wheat genotypes (DH-11 and PK-13) were harvested under control and drought-stressed conditions, metabolite extraction using a modified protocol adapted from Musharraf *et al*., (2013). Metabolites were extracted from 100 mg of homogenized rhizosphere soil using 80% methanol (v/v) under ice-cold conditions. Following centrifugation (14,000 × g, 10 min, 4°C), the supernatant was evaporated to dryness under vacuum. The dried extracts were derivatized using methoxyamine hydrochloride (20 mg/mL in pyridine) at 37 °C for 90 min, followed by silylation with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) at 70°C for 30 min.

GC-MS analysis: Derivatized samples were analyzed using an Agilent 7890B GC system coupled to a 5977A MSD. A HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) was used. The oven temperature was programmed from 70 °C (2 min hold) to 300°C at 10°C/min. Helium served as the carrier gas at 1.0 mL/min. Injection volume was 1 µL in splitless mode. The MS was operated in EI mode at 70 eV, scanning from 50–600 m/z. Metabolite identification was performed by comparing spectra to the NIST 17 MS library and confirmed based on retention indices. Internal standard normalization and blank subtraction were performed to ensure reliability.

Data preprocessing: Metabolite identification was achieved at MSI level 2 by spectral comparison against the NIST/Wiley library with a ≥70% similarity threshold. Data were filtered using a minimum ion count of 5,000, a requirement of at least three fragment ions, a retention time tolerance of 0.05 minutes, a match factor ≥0.3, and a Δm/z of 0.2. External scalar normalization was applied to ensure consistency across replicates. Raw GC-MS data were deconvoluted and aligned using Agilent MassHunter software. Peak areas were normalized and log-transformed. Statistical analyses were conducted using MetaboAnalyst 5.0. Univariate analyses (t-test, $p<0.05$ and multivariate approaches were applied to evaluate treatment effects. Significantly altered metabolites were selected based on fold change ≥1.5 and False Discovery Rate (FDR)Corrected p-values ≤0.05 using the Benjamini-Hochberg procedure.

Composite spectrum processing: Raw GC–MS data from PK-13 and DH-11 rhizosphere samples were processed in the Spectra Processing module of MetaboAnalyst 5.0. Peak detection, deconvolution, alignment, and metabolite identification were performed using optimized default settings for GC–MS data. Composite spectra were generated by merging MS/MS spectra across replicates, thereby improving the accuracy of metabolite annotation and reducing redundancy.

Metabolic integration and pathway mapping: Identified metabolites were subsequently mapped onto metabolic pathways using the Pathway Analysis module in MetaboAnalyst 5.0, with *Triticum aestivum* selected as the reference organism in the KEGG library. This integration enabled visualization of metabolite abundance changes within the context of broader metabolic networks. The combined approach facilitated interpretation of coordinated metabolic responses under drought stress, allowing a comparative evaluation of tolerant (DH-11) and susceptible (PK-13) wheat genotypes.

Topological pathway impact and gene enrichment patterns: Topological pathway impact was assessed using the Pathway Analysis module in MetaboAnalyst 5.0. This analysis calculated pathway impact scores based on the centrality and degree of differentially expressed metabolites within pathway networks, using the KEGG database for *Triticum aestivum*. To explore gene enrichment patterns, metabolites were mapped to their corresponding enzymes or genes via the KEGG database. Gene set enrichment analysis (GSEA) was performed on the associated gene list to identify over-represented functional categories, providing insights into the genetic underpinnings of metabolic responses to drought stress in PK-13 and DH-11.

Regression analysis of pathway impact relative to pathway size: Pathway impact values and the total number of genes per pathway were extracted from the Pathway Analysis results in MetaboAnalyst 5.0, using the KEGG database for *Triticum aestivum*. Linear regression analysis was conducted in R software to evaluate the relationship between pathway impact scores and the total gene count for each pathway in PK-13 and DH-11. This analysis aimed to determine whether pathway size (in terms of gene count) influenced the metabolic impact under drought stress, offering insights into the efficiency of metabolic reprogramming in the two varieties.

Cluster and statistical analysis: Cluster analysis was carried out in the Statistical Analysis module of MetaboAnalyst 5.0 using a data matrix of differentially expressed metabolites (rows) across samples (columns) from PK-13 and DH-11. Hierarchical clustering was performed using Euclidean distance and Ward's linkage method to group metabolites showing similar expression patterns. Statistical analyses were conducted using MetaboAnalyst 5.0 and R software. Metabolite intensities were normalized, $\log_2$-transformed, and autoscaled prior to analysis. Differences among treatments and genotypes were determined by Student's t-test or one-way ANOVA, with Wilcoxon or Kruskal–Wallis tests applied when assumptions were not met. p-values were corrected by the Benjamini–Hochberg False Discovery Rate (FDR), and metabolites with $|\text{fold change}| \geq 1.5$ and $\text{FDR} \leq 0.05$ were considered significant. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were used for multivariate evaluation, with model validation through cross-validation and permutation testing. Metabolites with variable importance in projection (VIP) scores ≥ 1 were regarded as discriminant. Pathway enrichment and topology analyses were performed in MetaboAnalyst, and regression analysis in R was used to assess the relationship between pathway impact and gene counts. Results are expressed as Mean ± SD (n = 3), and statistical significance was considered at $p<0.05$.

Results

Comparative metabolomic pathway enrichment analysis in wheat rhizosphere: The rhizospheric metabolite profiling of wheat genotypes Pakistan-13 (PK-13, drought-susceptible) and Dharabhi-11 (DH-11, drought-tolerant) under drought stress, using GC–MS-based enrichment analysis, revealed distinctive pathway modulation patterns. Fig. 1 shows a comparative bubble plot summarizing key pathways where bubble size reflects pathway impact, and color intensity corresponds to $-\log_{10}$(p-value), highlighting statistical significance. Both genotypes showed activation of multiple drought-responsive pathways. Notably, *Aminoacyl-tRNA biosynthesis* displayed the highest statistical significance in PK-13 ($-\log_{10}(p) = 8.37$), yet its zero impact score and 46 total hits suggest a localized upregulation without systemic influence. In contrast, DH-11 demonstrated a more functionally relevant enrichment across multiple high-impact pathways, reflecting a coordinated metabolic reprogramming under drought.

Alanine, Aspartate, and *Glutamate metabolism* emerged as a pivotal pathway in both genotypes. In PK-13, this pathway achieved a topology impact of 0.58 and a $-\log_{10}(p)$ of 8.08, indicating a proline-mediated osmotic adjustment mechanism. DH-11 also activated this pathway (impact = 0.33), suggesting convergence on osmoprotection and nitrogen assimilation mechanisms. DH-11 further exhibited strong enrichment in *Amino acid biosynthesis* (impact = 0.52), *Glyoxylate and dicarboxylate metabolism* (impact = 0.36), and the *TCA cycle* (impact = 0.15), signaling enhanced energy metabolism and stress-adaptive flux rerouting. The high bubble impact scores with substantial total compound hits in these pathways denote their systemic role in redox balance, carbon flux, and cellular respiration. This was further complemented by the activation of *Glycine, Serine and Threonine metabolism* (impact = 0.27), which contributed to methyl group donation and photorespiratory detoxification.

In contrast, PK-13 pathway distribution revealed relatively fragmented and isolated adjustments, such as modest impacts in *Butanoate metabolism* (0.14) and *Arginine biosynthesis* (0.19), likely reflecting compensatory energy buffering and incomplete nitrogen cycling. Collectively, these results underscore a strategically orchestrated metabolic rewiring in DH-11, promoting sustained drought resilience through elevated flux in central carbon and amino acid biosynthetic routes. PK-13, meanwhile, relies on highly significant but low-impact responses, suggesting limited metabolic flexibility under drought pressure.

Composite spectrum and metabolic integration analysis: Gas chromatography-mass spectrometry (GC–MS) analysis, coupled with pathway enrichment through Metabolo analyst software, shows distinct metabolic responses to drought stress in the root rhizospheres of drought-susceptible wheat variety PK-13 and drought-tolerant variety DH-11. The comparative bubble scatter plot (Fig. 2) illustrates pathway statistical significance, expressed as $-\log_{10}$(FDR), for PK-13 (x-axis) versus DH-11 (y-axis), with bubble size representing the maximum pathway impact score across both varieties. Colors denote significance status: green for pathways significant in both varieties ($FDR<0.05$) and blue for pathways not significant in either.

In PK-13, Aminoacyl-tRNA biosynthesis was the most statistically enriched pathway ($-\log_{10}(FDR) = 8.37$, 46 total genes), yet its negligible impact score (0.00) indicates a peripheral role in metabolic flux redistribution. This suggests transcriptional activation without significant alteration of the

central metabolic architecture. Similarly, Alanine, Aspartate, and Glutamate metabolism in PK-13 showed moderate impact (0.33) and limited topological integration, reflecting constrained engagement of osmoprotective and nitrogen buffering mechanisms. In contrast, DH-11 exhibited a more robust and integrated metabolic response. Alanine, Aspartate, and Glutamate metabolism in DH-11 achieved higher statistical and topological significance ($-\log_{10}$(FDR) = 6.0, impact = 0.58, 22 total genes), underscoring its central role in adaptive osmolyte regulation. Additional high-impact pathways in DH-11, including Glycine, Serine, and Threonine metabolism, the TCA cycle, and Glyoxylate and Dicarboxylate metabolism, displayed large, densely packed green bubbles, indicating coordinated, stress-adaptive metabolic fluxes. The bubble size and density gradient in DH-11 reflect a system-wide mobilization of metabolic resources, with highly impacted pathways being both transcriptionally enriched and structurally pivotal. Conversely, PK-13 pathway activation appeared fragmented, with few high-impact responses dispersed across minor pathways, contributing to its drought susceptibility.

Among the 27 pathways analyzed, four were significant in both varieties: Alanine, Aspartate, and Glutamate Metabolism, Aminoacyl-tRNA Biosynthesis, Arginine Biosynthesis, and Butanoate Metabolism. Notably, Alanine, Aspartate, and Glutamate Metabolism exhibited the highest impact (0.58), driven by DH-11 response. The complete overlap of significant pathways suggests a shared metabolic core response to drought stress, assessed via GC-MS and MetaboAnalyst-based pathway enrichment. However, DH-11 coherent, hierarchically organized metabolic strategy, leveraging central carbon metabolism and amino acid biosynthesis, contrasts with PK-13 reliance on high-frequency, low-impact transcriptional responses, indicating a metabolic bottleneck in the latter.

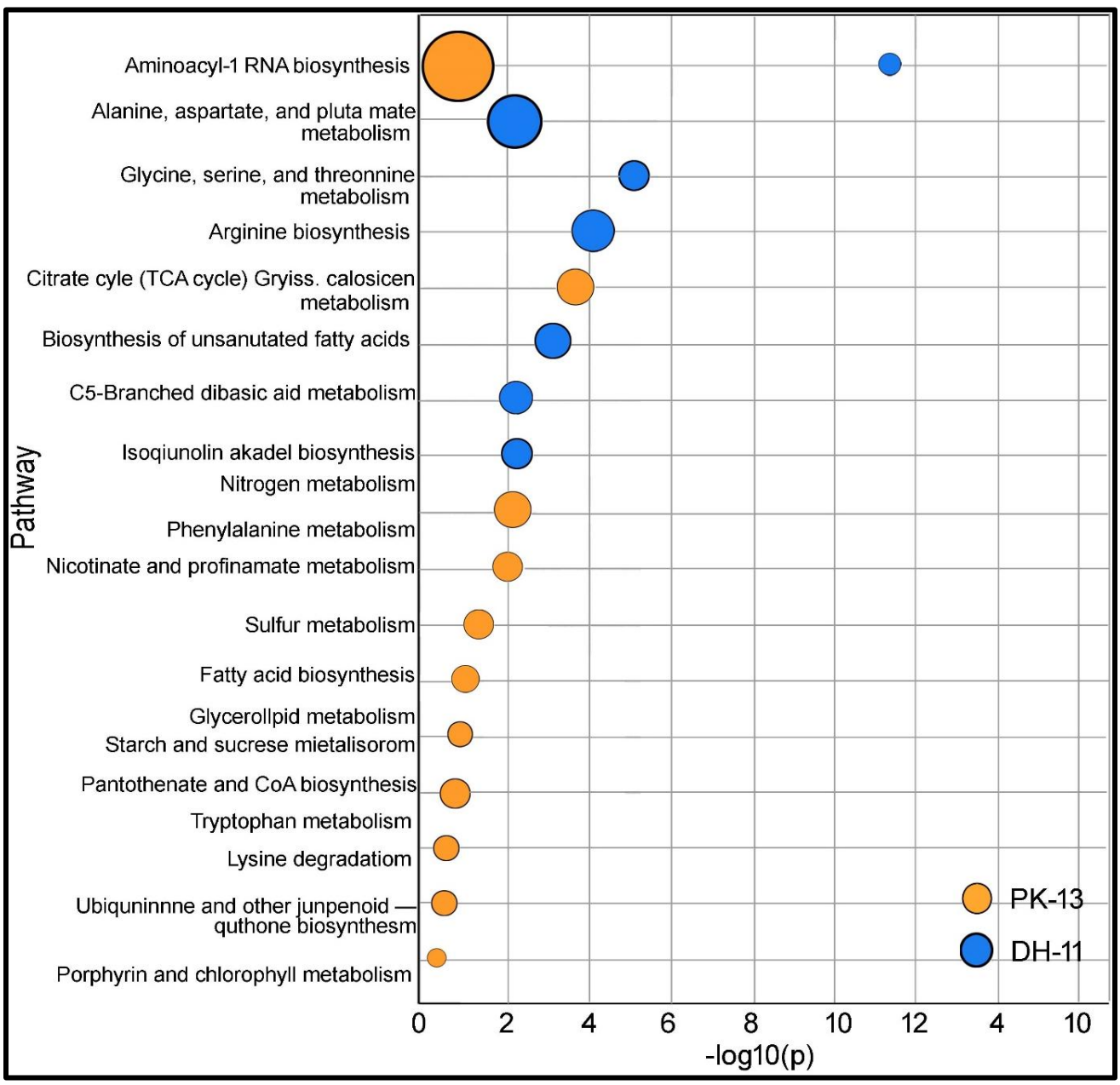

Fig. 1. Pathway-enrichment bubble plot of wheat genotypes PK-13 (orange) and DH-11 (blue) under drought stress. Marker color represents genotype; the x-axis (−log10 p) shows enrichment significance, while bubble size denotes pathway impact (topology).

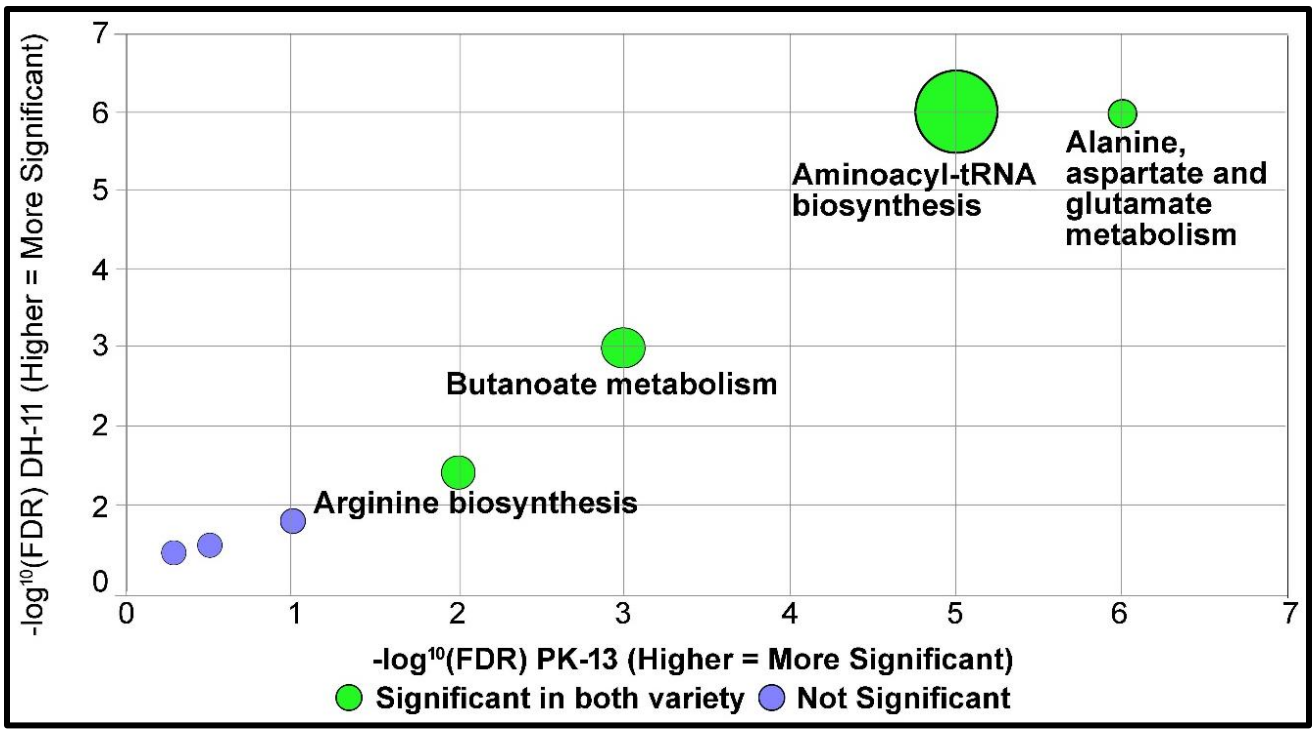

Fig. 2. Comparative Bubble Scatter Plot of Composite Spectrum and Metabolic Integration in PK-13 and DH-11 under Drought Stress. The plot displays pathway statistical significance as -log₁₀(FDR) for PK-13 (x-axis) and DH-11 (y-axis). Bubble size corresponds to the maximum impact score across both varieties, with green bubbles indicating pathways significant (FDR<0.05) in both PK-13 and DH-11, and blue bubbles representing pathways not significant in either. Key pathways include Alanine, Aspartate, and Glutamate Metabolism (position: 5,6; max impact: 0.58), Aminoacyl-tRNA Biosynthesis (6,6; max impact: low), Arginine Biosynthesis (2,1.4; max impact: moderate), and Butanoate Metabolism (3,3; max impact: moderate). The plot highlights shared significant pathways, with DH-11 showing greater metabolic integration, as assessed by GC-MS and MetaboAnalyst.

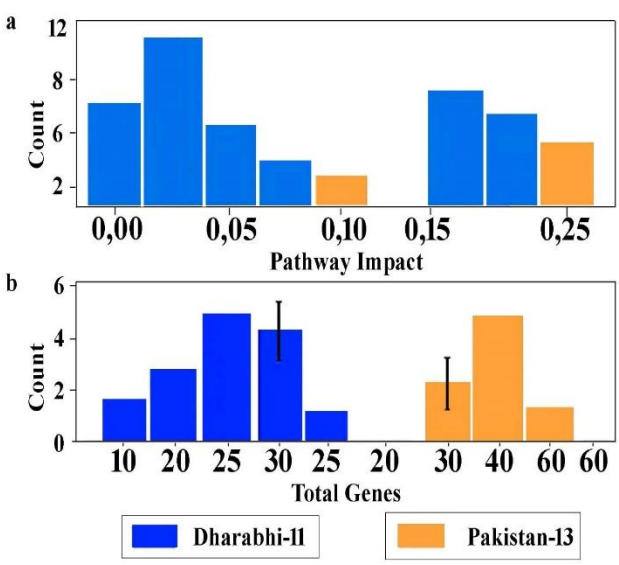

Fig. 3. Topological Pathway Impact and Gene Enrichment Patterns in DH-11 and PK-13 under drought stress. (a) Histogram Pathway impact scores show Dharabhi-11 (blue) enriched in low to moderate bins (0.00–0.15) with the highest count (n = 11) in the 0.00–0.05 range, suggesting a broader, distributed metabolic response. Pakistan-13 (orange) showed fewer but more concentrated high-impact pathways (>0.20). (b) Gene count distribution reveals that Dharabhi-11 pathways mostly involved 20–30 genes, while Pakistan-13 peaked in the 35–45 gene range. Error bars represent standard deviation. These trends indicate contrasting drought response strategies, with Dharabhi-11 favoring metabolic plasticity and Pakistan-13 showing stress-induced pathway intensification.

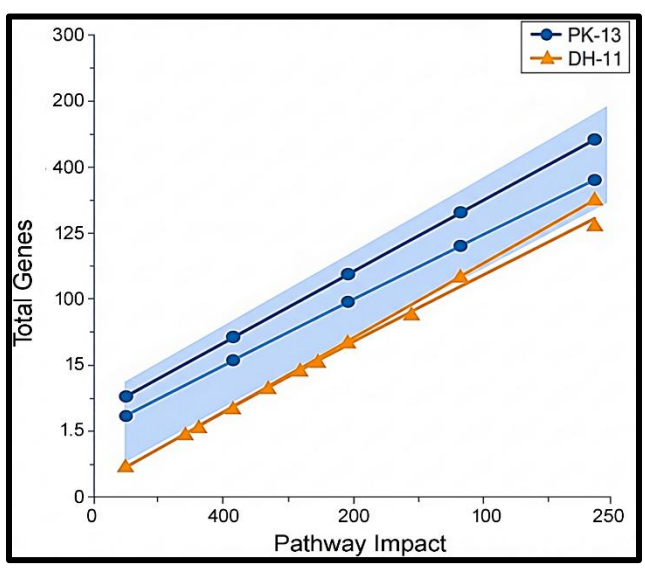

Fig. 4. Comparative Regression Analysis of Pathway Impact and Total Genes in *Wheat* varieties PK-13 and DH-11. This scatter plot illustrates the relationship between Pathway Impact (x-axis) and Total Genes (y-axis) for two wheat varieties: PK-13 (blue circles with blue regression line and confidence interval) and DH-11 (orange triangles with orange regression line and confidence interval).

Topological pathway impact and gene enrichment in wheat crop: Pathway topology and gene enrichment analyses revealed contrasting metabolic architectures between drought-tolerant DH-11 and susceptible PK-13 wheat genotypes. As shown in Fig. 3(a), DH-11 pathways

were broadly distributed across moderate-to-high impact scores (0.05–0.25), with distinct peaks in the 0.05–0.10 and 0.15–0.20 bins. This pattern reflects a diversified and topologically integrated metabolic response. In contrast, PK-13 pathways were predominantly confined to the 0.00–0.05 bin, indicating reliance on low-impact, peripheral pathways with limited influence on systemic network topology.

The gene distribution trends in Fig. 3(b) further support these findings. DH-11 exhibited a unimodal pattern centered on pathways involving 20–30 genes, suggesting a balanced and targeted deployment of genetic resources. PK-13, however, showed a bimodal distribution: one peak involving <30 genes and a second around 50–60 genes. Despite this higher gene involvement, these PK-13 pathways—such as Aminoacyl-tRNA biosynthesis—remained topologically insignificant, highlighting metabolic inefficiency and poor pathway leverage.

Collectively, these results suggest that DH-11 executes a coordinated drought response by selectively enriching metabolically central pathways—such as Alanine, Aspartate and Glutamate metabolism, Arginine biosynthesis, and Glycine, Serine and Threonine metabolism—achieving both statistical and functional enrichment. In contrast, PK-13 exhibits a fragmented and less efficient strategy, activating gene-dense but low-impact pathways that fail to converge on critical metabolic hubs essential for drought resilience.

Regression analysis of pathway impact with total genes in wheat: A comparative regression analysis between pathway impact and total gene count was performed for the wheat varieties PK-13 and DH-11 to elucidate their distinct genomic strategies under drought stress (Fig. 4). The scatter plot, overlaid with regression lines and confidence intervals, reveals a linear relationship for both varieties; however, the slopes and distributions differ notably, reflecting divergence in their stress-responsive genetic architectures. For PK-13, the data points display a broader vertical dispersion across the y-axis, indicating a relatively high number of genes per pathway even when the corresponding impact scores are moderate. This is visually reinforced by the steeper slope of the regression line, suggesting that PK-13 engages a larger repertoire of genes across a wide array of pathways. Despite this breadth, the pathway impact values remain moderate, implying a diffuse or less targeted transcriptional response. The upper bound of the confidence interval further underscores the variability and lack of pathway prioritization. This pattern suggests that PK-13 may rely on widespread, yet low-impact gene expression—potentially leading to suboptimal resource allocation under drought stress.

In contrast, DH-11 exhibits a more compact and focused regression trajectory. The regression line for DH-11 lies consistently below that of PK-13, with data points clustering closer to the line. This indicates a strategic utilization of fewer genes per pathway, yet these pathways achieve comparable impact scores if not superior, the narrower confidence band supports the inference of a more regulated and deterministic drought response. Predominantly, DH-11 appears to prioritize metabolic pathways with high topological impact despite involving fewer genes—highlighting an efficient gene-to-function deployment. Such pathways include nitrogen-rich amino acid metabolism (e.g., Alanine, Aspartate, and Glutamate Metabolism) and redox-linked networks like Glycine, Serine, and Threonine Metabolism, which are critical for osmotic regulation and energy balance during water deficit conditions. This divergence in pathway utilization implies fundamentally different drought adaptation strategies: PK-13 exhibits a "gene-abundance but low-impact" response, possibly reflecting a generalized stress reaction with limited physiological efficiency. In contrast, DH-11 employs a "low-gene, high-impact" strategy, suggesting superior regulatory precision and metabolic economy. These observations are consistent with the hypothesis that drought-tolerant varieties may not require widespread gene activation but instead rely on streamlined genetic networks with maximal functional output per gene.

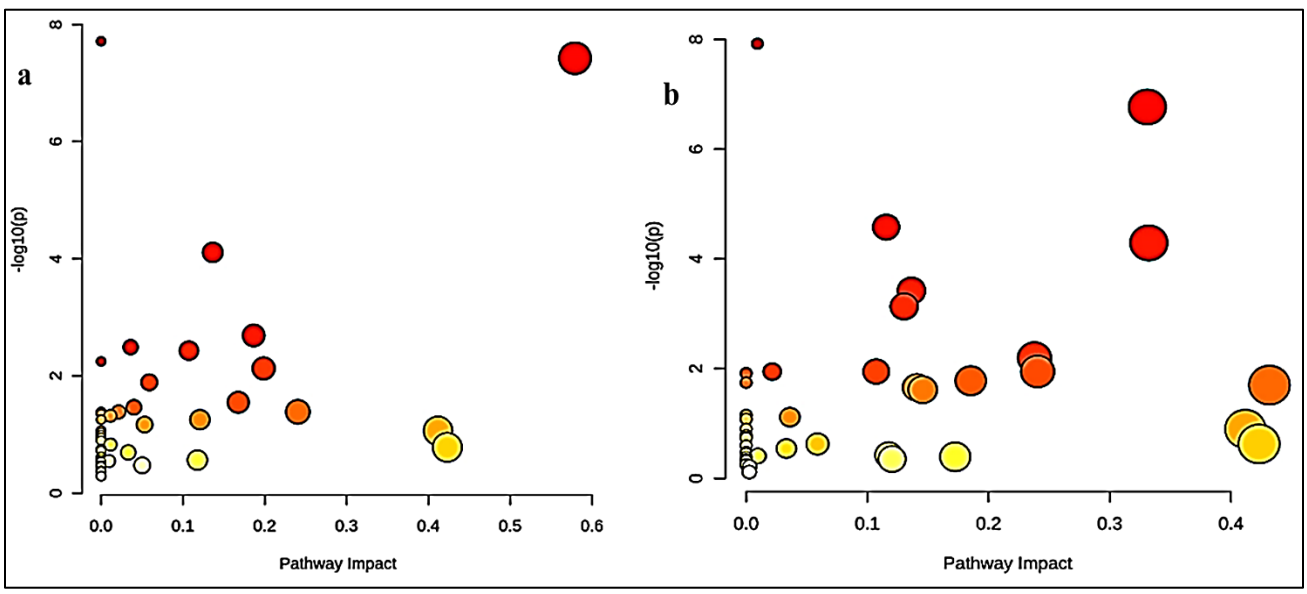

Fig. 5. Cluster Analysis of Differentially Expressed Metabolites analysis of PK-13 and DH-11 with X-axis shows Pathway impact and Y-axis shows –log values (a) Scatter plot of PK-13 analysis shows a positive correlation between pathway impact and -log10(p) and (b) Scatter plot of DH-11 as assessed by GC-MS and MetaboAnalyst.

Overall, the regression trends substantiate DH-11 drought resilience as being rooted in its ability to concentrate transcriptional resources into a select few, high-impact pathways—enhancing physiological efficacy while minimizing metabolic burden. Conversely, the broader and less targeted activation seen in PK-13 may contribute to its relatively lower stress tolerance. These findings provide valuable insight into the genetic basis of drought adaptation and offer a rational basis for selecting candidate genes and pathways in future breeding programs.

Cluster analysis of differentially expressed metabolites analysis in wheat: The scatter plots for drought-susceptible (Pakistan-13) and drought-tolerant (Dharabhi-11) wheat genotypes reveal distinct metabolic adaptations in the rhizosphere under water stress and False Discovery Rate (FDR). In Pakistan-13 Fig. 5(a), Alanine, aspartate, and glutamate metabolism (Impact = 0.58, FDR p = 3.94E-07) and Butanoate metabolism (FDR p = 2.47E-03) were linked to osmotic imbalance and stomatal dysregulation, exacerbating susceptibility. Conversely, Dharabhi-11 5(b) prioritized Glycine, serine, and threonine metabolism (FDR p = 1.22E-03) for ROS detoxification and Arginine biosynthesis (FDR p = 8.36E-04) for efficient nitrogen recycling, underpinning tolerance. Both genotypes exhibited outliers: Aminoacyl-tRNA biosynthesis displayed extreme significance (FDR p<1E-07) but negligible impact, reflecting database biases. Dharabhi-11 further demonstrated robust Glutathione metabolism (Impact = 0.43) for redox regulation, while Pakistan-13 Nitrogen metabolism (Impact = 0.96, FDR p = 0.136) highlighted structural network prominence without functional relevance. These results emphasize amino acid metabolism and redox homeostasis as critical for drought resilience, with Dharabhi-11 metabolic flexibility contrasting starkly with Pakistan-13 dysregulated pathways. The findings underscore the need for integrated multi-omics approaches to validate network centrality against biological functionality in rhizosphere adaptation.

Discussion

This study aims to understand the differential metabolic responses of two wheat varieties, Pakistan-2013 (PK-13), a drought-sensitive variety, and Dharabhi-11 (DH-11), a drought-tolerant variety, in their rhizosphere under drought stress conditions. Through comprehensive metabolomic profiling, we identified the key metabolites, metabolic pathways, and gene distribution patterns associated with the plants' responses to water deficit. The results presented herein offer valuable insights into the metabolic adjustments that occur in the rhizosphere under drought stress and contribute to the plant overall drought tolerance.

Metabolomic profiling is a powerful tool to elucidate the biochemical shifts occurring within plant tissues under abiotic stress. In our study, we found that drought stress triggered a substantial accumulation of specific metabolites in the rhizosphere of DH-11, most notably osmolytes like proline and glycine betaine. These compounds are known for their protective role in maintaining cell integrity under water deficit conditions (Ashraf & Foolad, 2007). DH-11 exhibited a robust accumulation of these metabolites, suggesting that this variety employs a strategy of osmotic adjustment to counteract the negative effects of drought, a finding corroborated by the work of Fitzpatrick *et al*., (2018), who observed similar accumulations in drought-tolerant species.

On the other hand, PK-13 showed a more modest accumulation of these metabolites, consistent with its classification as a drought-sensitive variety. This result is in line with previous findings indicating that drought-sensitive varieties tend to have limited metabolic adjustments, making them more vulnerable to drought stress (Sallam *et al*., 2019). Furthermore, the comparison between the two varieties highlights the differential capacity of DH-11 to induce a complex metabolic response aimed at mitigating oxidative damage and maintaining cellular function under stress conditions (Zhao *et al*., 2017).

The composite spectrum analysis of the metabolic data provided a holistic view of the metabolic changes occurring in the rhizospheres of both wheat varieties. This analysis revealed that DH-11 demonstrated a higher degree of metabolic integration across various pathways, including primary metabolism (such as glycolysis and the TCA cycle) and secondary metabolism (involving phenolics and flavonoids). These secondary metabolites play key roles in protecting plants from oxidative stress and promoting stress tolerance (Gargallo-Garriga *et al*., 2018, Lin *et al*., 2023, Roychowdhury *et al*., 2023, Zhang *et al*., 2025). The upregulation of such metabolites in DH-11 indicates that this variety has evolved a well-coordinated metabolic response to drought, which is essential for maintaining growth and development under water deficit conditions (Gupta *et al*., 2020).

In contrast, PK-13 exhibited a more fragmented metabolic network, with fewer metabolites involved in secondary metabolism. Instead, the metabolic response of PK-13 focused primarily on maintaining the basic functions of primary metabolism. While this could be seen as an adaptive strategy to sustain core cellular processes during drought stress, it also points to a limitation in the plant capacity to activate broader stress-responsive mechanisms that might confer greater resilience (Chaves *et al*., 2003). This limitation was also observed by Ullah *et al*., (2017), who noted that limited secondary metabolic responses in drought-sensitive varieties might lead to insufficient oxidative damage protection.

A detailed analysis of the metabolic pathway impact and the distribution of associated genes revealed significant differences between PK-13 and DH-11. The pathway impact analysis indicated that drought stress had a more profound effect on DH-11, particularly in pathways related to energy metabolism, amino acid biosynthesis, and antioxidant production. For instance, the upregulation of the TCA cycle and glycolysis in DH-11 highlights the plant enhanced ability to produce energy through mitochondrial respiration, which is crucial for sustaining growth under drought stress (Gundaraniya *et al*., 2020; Gupta *et al*., 2020; Dorion *et al*., 2021). This finding aligns with those of Hasanuzzaman *et al*., (2013), who reported that enhanced energy metabolism helps mitigate drought-induced growth constraints in plants.

Moreover, DH-11 exhibited a higher number of genes linked to these critical metabolic pathways, suggesting that this variety is more capable of initiating and regulating these pathways in response to environmental stress. The increased

gene expression associated with these pathways may also reflect an adaptive response, designed to improve the plant ability to generate ATP, synthesize osmolytes, and produce antioxidants necessary to mitigate oxidative damage, consistent with findings by Hasanuzzaman *et al*., (2013) and Fitzpatrick *et al*., (2018). In contrast, PK-13 displayed fewer genes associated with these key pathways, and the impact on metabolic pathways was less pronounced. This could explain the lower metabolic capacity of PK-13 to combat oxidative stress and maintain cellular integrity under drought stress, as similar (Lucini *et al*., 2019).

Cluster analysis of the differentially expressed metabolites provided additional insights into the metabolic strategies employed by PK-13 and DH-11 under drought stress. The analysis revealed that DH-11 exhibited a stronger upregulation of metabolites involved in secondary metabolism, particularly phenolic compounds and flavonoids. These compounds are known to have antioxidant properties and play critical roles in protecting plants from oxidative damage during drought stress (Naylor *et al*., 2017; Zhour *et al*., 2022; Arriagada *et al*., 2025). The presence of these metabolites in elevated concentrations in DH-11 suggests that this variety relies heavily on secondary metabolic pathways to mitigate stress, a conclusion also supported by the findings of Naylor *et al*., (2018).

In contrast, PK-13 showed a more limited range of differentially expressed metabolites, with an emphasis on primary metabolites such as sugars and amino acids. This suggests that while PK-13 does activate certain metabolic responses under drought stress, it does not elicit a full spectrum of secondary metabolites that would aid in stress alleviation. The relatively lower upregulation of secondary metabolites in PK-13 may contribute to its lower drought tolerance, as it lacks the additional layer of protection provided by antioxidants and other stress-related compounds (Fitzpatrick *et al*., 2018). This aligns with the observations of Ghorbanzadeh *et al*., (2023), who indicated that drought-sensitive plants exhibited reduced production of stress-associated secondary metabolites.

Conclusion

In conclusion, this study provides a comprehensive and rigorous In Scilico analysis of the metabolic responses of two wheat varieties, PK-13 and DH-11, to drought stress in root rhizosphere. The results shows that DH-11 exhibits a significantly more robust and diversified metabolic adaptation to drought, characterized by the activation of critical pathways such as the Alanine, Aspartate, and Glutamate Metabolism, Arginine Biosynthesis and tricarboxylic acid (TCA) cycle. These pathways play a pivotal role in energy production and stress signaling, and the accumulation of branched-chain amino acids (BCAAs), which are well-documented contributors to drought tolerance. Pathway impact analysis and cluster analysis further underscore the coordinated and comprehensive nature of DH-11 metabolic reprogramming, which includes enhanced osmotic adjustment and antioxidative defenses. In contrast, PK-13 displays a more limited metabolic response, primarily confined to primary metabolism, which may hinder its ability to withstand prolonged drought conditions. The in silico investigation of the rhizosphere highlights the crucial role of rhizosphere-associated microbial communities in modulating the plant drought response, with DH-11 demonstrating more efficient engagement of these beneficial interactions compared to PK-13. These findings not only deepen our understanding of the biochemical and ecological mechanisms underlying drought tolerance in wheat but also highlight the potential of targeted metabolic engineering and microbiome management to develop drought-resistant cultivars. By integrating advanced computational tools with experimental validation, this research establishes a solid foundation for future strategies aimed at enhancing global food security amidst escalating environmental challenges.

Future Research Directions

This study reveals genotype-specific metabolic adjustments in wheat rhizosphere under drought stress, particularly in glyoxylate and dicarboxylate metabolism, the TCA cycle, and amino acid biosynthesis. To build on these findings, future work should integrate metabolomics with transcriptomic and proteomic approaches for a systems-level understanding of drought adaptation, while also considering the role of root-associated microbiomes. Field-based validation across diverse agro-climatic conditions is essential to confirm the stability of identified pathways, and linking metabolite signatures with agronomic traits will provide direct applications in breeding programs aimed at enhancing drought resilience in wheat.

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