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Elucidating drought responsive networks in tef (*Eragrostis tef*) using phenomic and metabolomic approaches

Aiswarya Girija[1] | Jiwan Han[2] | Fiona Corke[1,3] | Jason Brook[1,3] | John Doonan[1,3] | Rattan Yadav[1,3] | Habte Jifar[4] | Luis A. J. Mur[1,2]

[1]Institute of Biological, Environmental and Rural Science, Aberystwyth University, Aberystwyth, Wales, UK

[2]Software College, Shanxi Agricultural University, Taigu, Shanxi, China

[3]The National Plant Phenomics Centre, Aberystwyth University, Aberystwyth, Wales, UK

[4]National Tef Improvement Program, Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia

Correspondence

Luis A. J. Mur, Institute of Biological, Environmental and Rural Science, Aberystwyth University, Aberystwyth, Wales, UK.
Email: lum@aber.ac.uk

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Abstract

Drought is a major abiotic stress that limits crop productivity and is driving the need to introduce new tolerant crops with better economic yield. Tef (*Eragrostis tef*) is a neglected (orphan) Ethiopian warm-season annual gluten-free cereal with high nutritional and health benefits. Further, tef is resilient to environmental challenges such as drought, but the adaptive mechanisms remain poorly understood. In this study, metabolic changes associated with drought response in 11 tef accessions were identified using phenomic and metabolomic approaches under controlled conditions. Computerized image analysis of droughted plants indicated reductions in leaf area and green pigments compared with controls. Metabolite profiling based on flow-infusion electrospray–high-resolution mass spectroscopy (FIE-HRMS) showed drought associated changes in flavonoid, phenylpropanoid biosynthesis, sugar metabolism, valine, leucine and isoleucine biosynthesis, and pentose phosphate pathways. Flavonoid associated metabolites and TCA intermediates were lower in the drought group, whereas most of the stress-responsive amino acids and sugars were elevated. Interestingly, after drought treatment, one accession Enatite (Ent) exhibited a significantly higher plant area than the others, and greater accumulation of flavonoids, amino acids (serine and glycine), sugars (ribose, myo-inositol), and fatty acids. The increased accumulation of these metabolites could explain the increased tolerance to drought in Ent compared with other accessions. This is the first time a non-targeted metabolomics approach has been applied in tef, and our results provide a framework for a better understanding of the tef metabolome during drought stress that will help to identify traits to improve this understudied potential crop.

1 | INTRODUCTION

An increasing global population and climate change poses an immense challenge to the agricultural sector and to future food security. With regard to climate change, drought is one of the major environmental stresses that negatively impact crop growth and productivity (Khan et al. 2021). The quality of the food produced can be further improved by developing nutritionally rich orphan crops that can survive under extreme environmental conditions. These crops can also possess desirable traits like tolerance to various abiotic and biotic stresses (VanBuren et al. 2020). One such crop that has been gaining popularity recently due to its resilience and nutritional significance is the African crop, tef. Tef (*Eragrostis tef*) is the most important staple gluten-free cereal crop of Ethiopia, which is grown in over three million hectares of land across the country by more than seven million households (Assefa et al. 2015; CSA 2020). It is the only species in the *Eragrostis* genus which is cultivated for human consumption. Tef seeds are ground to flour and used to prepare Ethiopian traditional flatbread "injera" and other foods and beverages (VanBuren et al. 2020). Tef straw can also be used as livestock forage due to its high digestibility

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and protein content (Mekuriaw et al. 2020) as well as for plastering of mud in local house construction. Despite its nutritional and adaptive traits, tef is still an understudied orphan crop due to low investment in its research and breeding (Chanyalew et al. 2019).

Tef is a C_4 allotetraploid ($2n = 4x = 40$) annual grass and is highly resilient to extreme environmental conditions (Muanenda et al. 2019). Compared with other cereals, tef is tolerant to abiotic stresses like drought, moisture-limited conditions, waterlogging, and can even survive in poorly drained soils (vertisols) (Martinelli et al. 2018). This general adaptability makes tef a promising crop for combating future food security and environmental constraints. Like any other crop improvement program, tef research is dependent on a good supply of varied genetic resources, but there is limited variability in some important traits such as lodging (Jifar et al. 2020) or drought tolerance, which makes the selection of appropriate genotypes even more challenging.

Tef naturally adapts to short episodes of drought, but prolonged and/or terminal drought affects its productivity (Kamies et al. 2017). Most of the drought studies in tef have highlighted the need to identify more tolerant genotypes and improve them further with marker-assisted and omics approaches (Abraha et al. 2017; 2018; Araya et al. 2011; Assefa et al. 2015; Degu et al. 2008). Over recent decades, more than 10 varieties have been released in Ethiopia with some degree of drought tolerance, but Tsedey (released in 1984) remains the most popular variety among farmers (Chanyalew et al. 2021). Water deficiency studies in Tsedey have shown that severe drought at the grain filling stage reduces yield (Mengistu 2009). Tsedey is the object of molecular studies as the regulatory role of microRNA's in the post-transcriptional regulation of drought responses has been defined in this cultivar compared with Alba (drought susceptible) (Martinelli et al. 2018). The recent availability of a tef genome (VanBuren et al. 2020) has opened more possibilities to characterize gene–metabolite–phenotype relationships. Such studies can be used to reveal information on agronomic and nutritional traits or to identify the key biochemical pathways involved in the synthesis of specific metabolites (Cao et al. 2017). These metabolomic approaches are particularly relevant to drought research as changes in osmolytes, antioxidant chemicals, and phytohormones are prominent (Fàbregas & Fernie 2019). To our knowledge, there are no metabolomic studies focusing on tef's response to drought, although this approach has been widely applied to crops such as rice, wheat, and maize (Bowne et al. 2012; Guo et al. 2020; Naing & Kim 2021; Yadav et al. 2021). In this study, we integrate imaging and untargeted metabolomics to elucidate the metabolic networks associated with drought response in 11 diverse tef seedlings. The present study will facilitate the development of drought-tolerant tef genotypes and further to improvement of this neglected crop.

2 | MATERIALS AND METHODS

2.1 | Plant material

Eleven diverse tef accessions (landraces/cultivars) obtained from NPGS-GRIN database, https://npgsweb.ars-grin.gov/ (Table 1) were screened for the drought study. Plants were germinated in compost (Levington F2 peat-based compost) and grown under controlled environmental conditions at the National Plant Phenomics Centre (NPPC), Aberystwyth, Wales, UK at 24/21°C ± 2°C, 14/10 h day/night photoperiod. Three-week-old tef seedlings were transferred to the Photon

TABLE 1 List of tef accessions used for drought study

	Accession number	Plant name	Location	Seed color
1	243908[a]	Magna	Ethiopia	White
2	524435	Alba	Ethiopia, 1700 m	White
3	524433	Ada	Ethiopia	White
4	524439[b]	Enatite	Ethiopia	White
5	557456[b]	DZ-01-354	Ethiopia	White
6	524437	Beten	Ethiopia	White
7	524443[a]	Manyi	Ethiopia	White
8	524438	Dabbi	Ethiopia	Red
9	524442	Karadebi	Ethiopia	Red
10	524440	Gea lamie	Ethiopia	Red
11	557457	Red dabbi	Ethiopia	Red

Note: The accessions were obtained from the US-GRIN germplasm collection.

[a] Magna is a common name for variety DZ-01-196 which also released in 1970 following selection from a landrace collection. Magna is also known as Manyi in some areas of Ethiopia. In US-GRIN germplasm database, Magna and Manyi were used to represent two different accessions 243908 and 524443 which they collected in 1957 and 1988 from different sources. We consider them to be segregants of the same land race.

[b] In Ethiopia, Enatite is the common name of DZ-01-354 which was released in 1970 through selection from landrace collections. In US-GRIN database Enatite and DZ-01-354 were given for two accession numbers 524439 and 557456 as they received in 1988 and 1990 from different sources. As our US-GRIN Enatite and DZ-01-354 behaved differently in our studies, we consider them to be segregants of the original 1970 released land race.

Systems Instruments (PSI) PlantScreen automated platform to impose highly regulated drought conditions. The experimental conditions imposed were well-watered control (65% soil water content [SWC] and severe drought, 15% SWC). Each pot contained two plants, at sampling, the aerial parts of these were pooled together as one biological replicate. Five biological replicates were used per accession for both control and drought treatments.

2.2 | Drought experiment

To characterize the impact of drought on tef, 11 diverse tef accessions were used for the water stress experiment under controlled conditions at the NPPC. All plants were uniformly watered prior to the start of the stress experiment. The drought experiment used the automated target weight watering of the PSI system, and the plants were differentially watered to achieve an SWC of 15% (severe drought) and 65% (control), respectively. When the pots had achieved these water targets, the levels were maintained for 5 days. This involved pot weight being monitored daily to target weights of 85 g (control) and 57 g (drought). The details of mean water daily use and pot weight obtained from the PSI system is given in the Appendix S1; Figure S1. After 5 days of severe drought treatment, the plants were imaged, and the aerial parts were sampled and flash frozen for further metabolite analysis.

2.3 | Imaging and feature extraction

Prior to sampling, images of both control and experimental plants were captured using Nikon D60 18–55 mm and exported as RGB color images. The images were processed to extract features like plant height, area, and color information (green, red, orange, and yellow). The data was extracted as pixel values; Figure 1 shows the image acquired and post-processing. The processing and extraction were done using C++ and OpenCV 2.4.9, an open-source computer vision library (Fisher et al. 2016).

2.4 | Metabolite profiling and statistical analysis

Five days after the drought treatment, aerial parts were harvested from well-watered—65% (control) and drought—15% (experimental) water content groups for the metabolite extraction. Two plants per accession were pooled together to make one biological replicate from each group. Forty milligrams of the fresh samples were extracted in 1 mL of chloroform:methanol:water (1:2.5:1, v/v/v). About 100 μL of the supernatant was transferred to an HPLC glass vial with a 0.2 mL microinsert. The detailed procedure for metabolite extraction is described in Allwood et al. (2006) and Kattupalli et al. (2021). Untargeted metabolite profiling was undertaken, and mass-ions (*m/z*) were acquired in both positive and negative ionization modes. FIE-MS data were normalized to the total ion count. The significant hits were extracted based on ANOVA, and the *m/z* was then identified based on accurate masses (5 ppm resolution), ionization patterns linked to that particular metabolite and associated isotopic forms. The annotation for the selected *m/z* features was obtained by comparing the obtained compound ID's with the *Oryza sativa japonica* (Japanese rice) database in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and also the Human metabolite (HMDB), PubChem, and ChEBI databases.

Statistical analyses were performed on $\log_{10}$-transformed values in Metaboanalyst 5.0 (https://www.metaboanalyst.ca/). Multiple comparisons and post hoc analyses used Tukey's Honestly Significant Difference (Tukey's HSD) Fisher LSD test ($P < 0.05$) to determine significant compounds. Principal component analysis (PCA), one-way analysis of variance (ANOVA), and hierarchical cluster analyses (HCA) for the identified metabolite features used the online R-based platform, Metaboanalyst 5.0 (Pang et al. 2021). All significant metabolites were mapped into metabolomic pathways based on the KEGG database (Kattupalli et al. 2021).

3 | RESULTS

3.1 | Image-assisted evaluation of morphological traits in tef accessions under drought and well-watered regime

The plants' initial responses to any stress are reflected in their morphology, which affects their growth and development. Here, we used an image-assisted feature extraction approach to identify the significant morphological variations between the well-irrigated and droughted plants. The drought response for 11 tef accessions was monitored based on the water use efficiency and phenotypic responses. Figure S1 shows the gradual decline in the pot weight between the control and drought groups during the drought treatment. The experimental time course started after the droughted plant achieved their target pot weight. Plants were imaged after 5 days of drought treatment, and features (plant area, height, green, red, purple, orange, yellow, blue, and cyan pixels) were extracted (Figure 2A). The PCA showed clear discrimination between the control and drought samples. Compared with well-watered conditions, area (mm) and height (pixel) were significantly decreased in the droughted plants (Figure 2B). The colored pixel values correspond to the accumulation of pigments like chlorophyll, and in our study, apart from the blue pixel, green pixel counts were significantly reduced, but the other colored pigments (red and purple) increased in the droughted plants (Figure 2B).

3.2 | Drought-induced metabolite changes in tef

We employed a high-throughput untargeted metabolite profiling to identify the metabolic responses in 11 diverse tef accessions. The whole metabolome was extracted from five biological replicates of

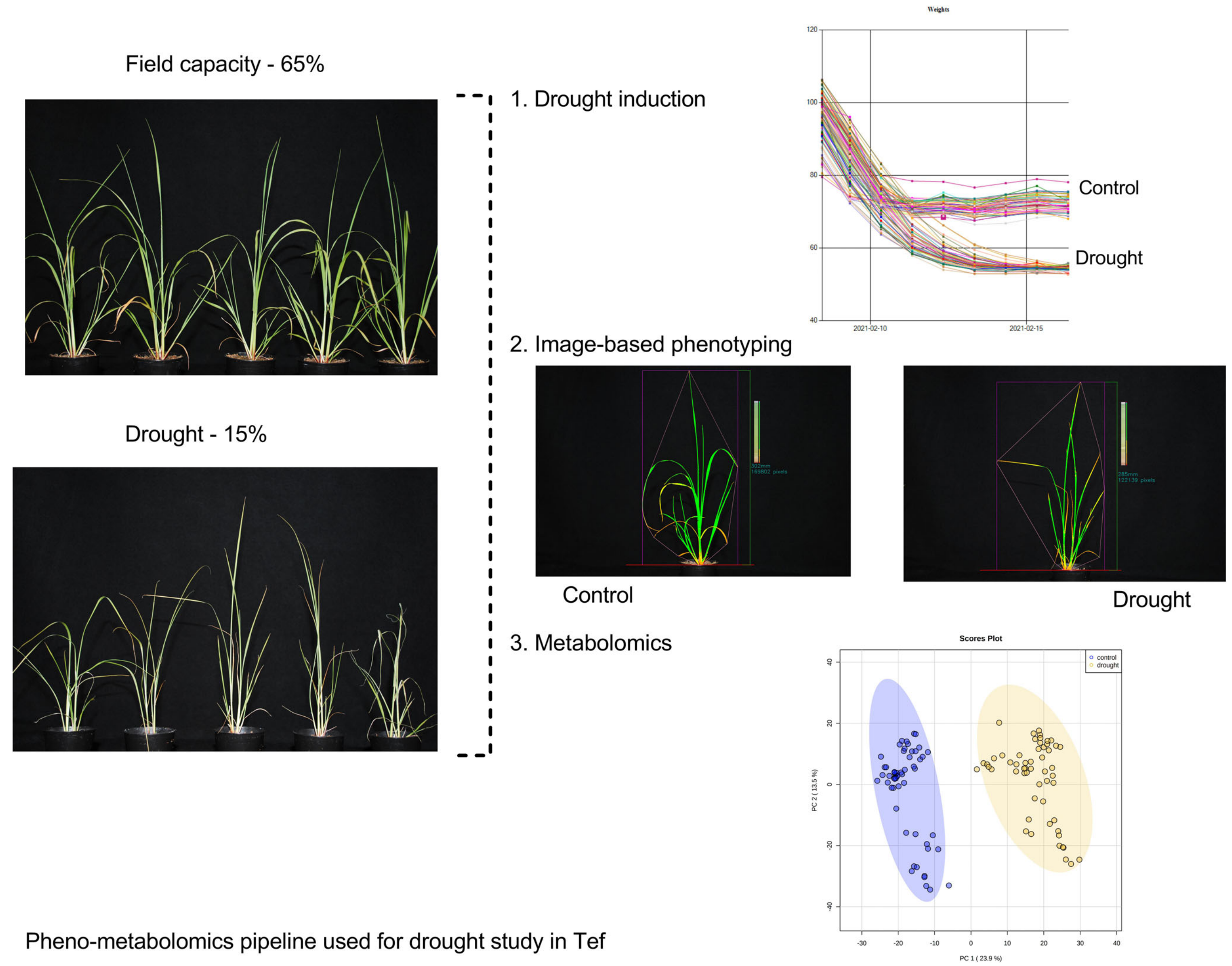


FIGURE 1 Overall representation of image-metabolomics pipeline used to study drought response in tef

shoots for each control and drought group and analyzed using FIE-MS. A total of 5200 *m/z* features were identified by ANOVA from both modes, of which 4881 were found to be significant between the control and experimental group. An unsupervised multivariate data analysis was performed on the *m/z* obtained from both ionization modes, positive and negative (Figure 3A). Overall, the PCA showed a clear separation between the control and droughted group along PC1, which described the major sources of variation. Between the tef accessions, there were metabolomic differences with distinct sub-clusters being observed (Figure 3B). The accessions, Manyi, Magna, and Ada were clustered together, Gea-lamie, Dabbi, and Alba formed another cluster, Karadebi, Red dabbi, Beten, Enatite, and DZ-01-354 showed similar metabolomes in control and droughted groups. We also assessed *m/z* features in each ionization mode separately. The PCA analysis of negative mode metabolites in the control and drought samples separated across the second PC (23.9% of total variation) (Figure 4A). The overall variation within the tef accessions is also shown by hierarchical clustering analysis in Figure S3.

The significant sources of variation between the groups were identified by ANOVA (corrected for false-recovery rates). The reference metabolome library of *Oryza sativa* (rice) (available in the KEGG library) was used to identify the significant *m/z* features and define the main pathway changes. The pathway enrichment analysis of significant features showed changes in pathways related to flavonoid biosynthesis, galactose metabolism, phenylpropanoid biosynthesis, and valine, leucine, and isoleucine biosynthesis Figure 4B. From these 166 *m/z* annotated features (M–H [−]) were identified for further assessment.

3.3 | Major differential metabolic shifts in tef are associated with flavonoids, amino acids, and sugar pathways

The selected M–H [−] metabolite features were annotated and classified according to three metabolomic super-pathways; "flavonoids," "amino acids," and "sugars/TCA" intermediates and compared between

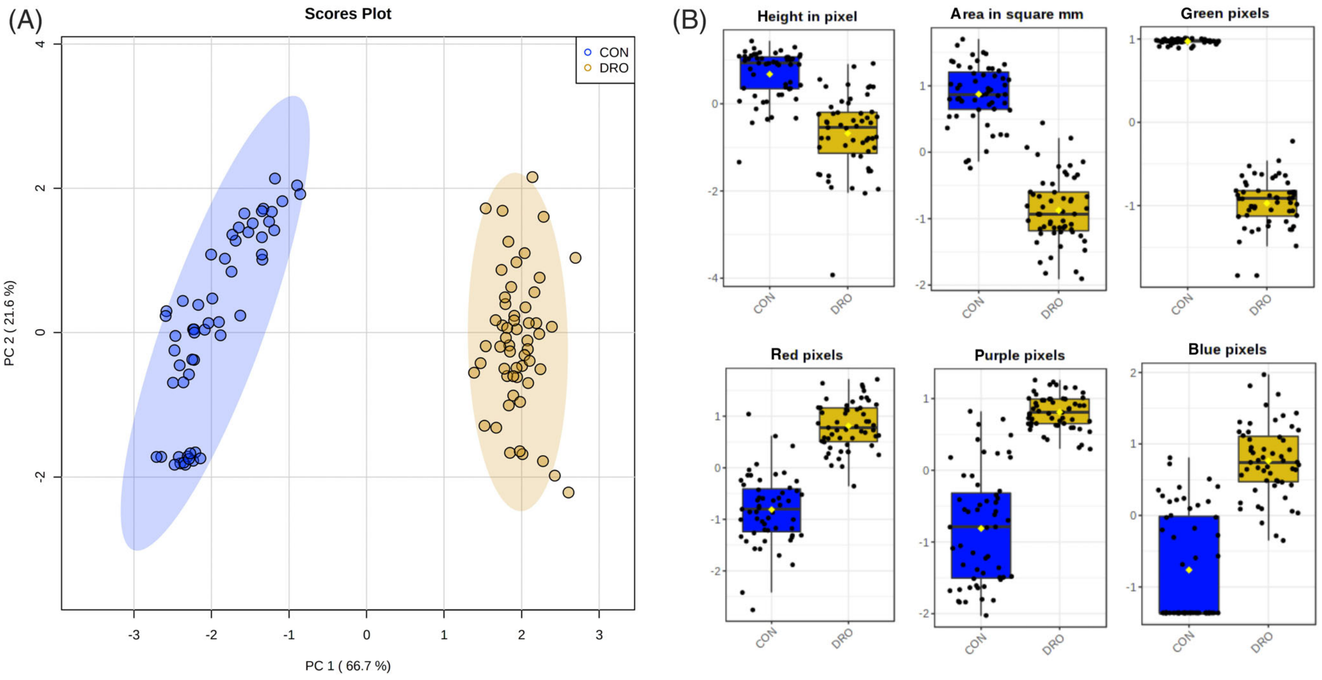


FIGURE 2 Image-based phenomics assessment of tef accessions. (A) PCA showing association of features extracted from image between control and drought group. Features extracted include plant height in pixel, area in square mm, plant colors in pixel (green, red, purple, orange, yellow, and blue). (B) Comparison of height, area, green, red, purple, and blue pixels in control and droughted group

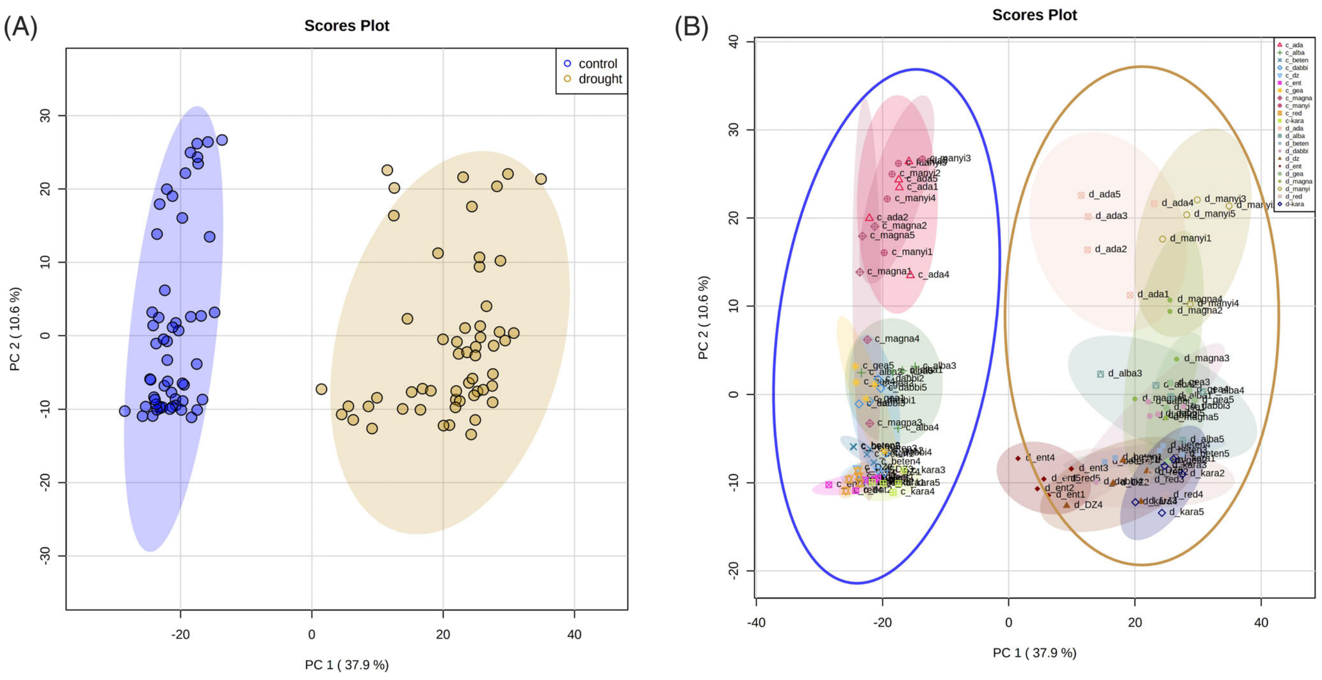


FIGURE 3 Drought induced global metabolic response in Tef. The *m/z* features from the positive and negative mode were extracted and analysed. (A) PCA representing overall metabolite variation in control and drought. (B) PCA showing metabolite response within genotypes

the genotypes using heatmaps (Figure 5). Some of the putatively identified phenylpropanoid/flavonoid related metabolites are shikimic acid, p-coumaroylquinic acid, pinobanksin, 4-coumaroylshikimate, dattelic acid, caffeyl alcohol, chlorogenic acid, kaempferol/luteolin, kaempferol-3-*O*-rutinoside, naringenin, afzelechin, quercetin kaempferol-3-O-glucoside, and rutin/quercetin 3-*O*-rhamnoside 7-*O*-glucoside (Figure 5A). We found that the accumulation of most of the flavonoid pathway-related compounds in the droughted group was

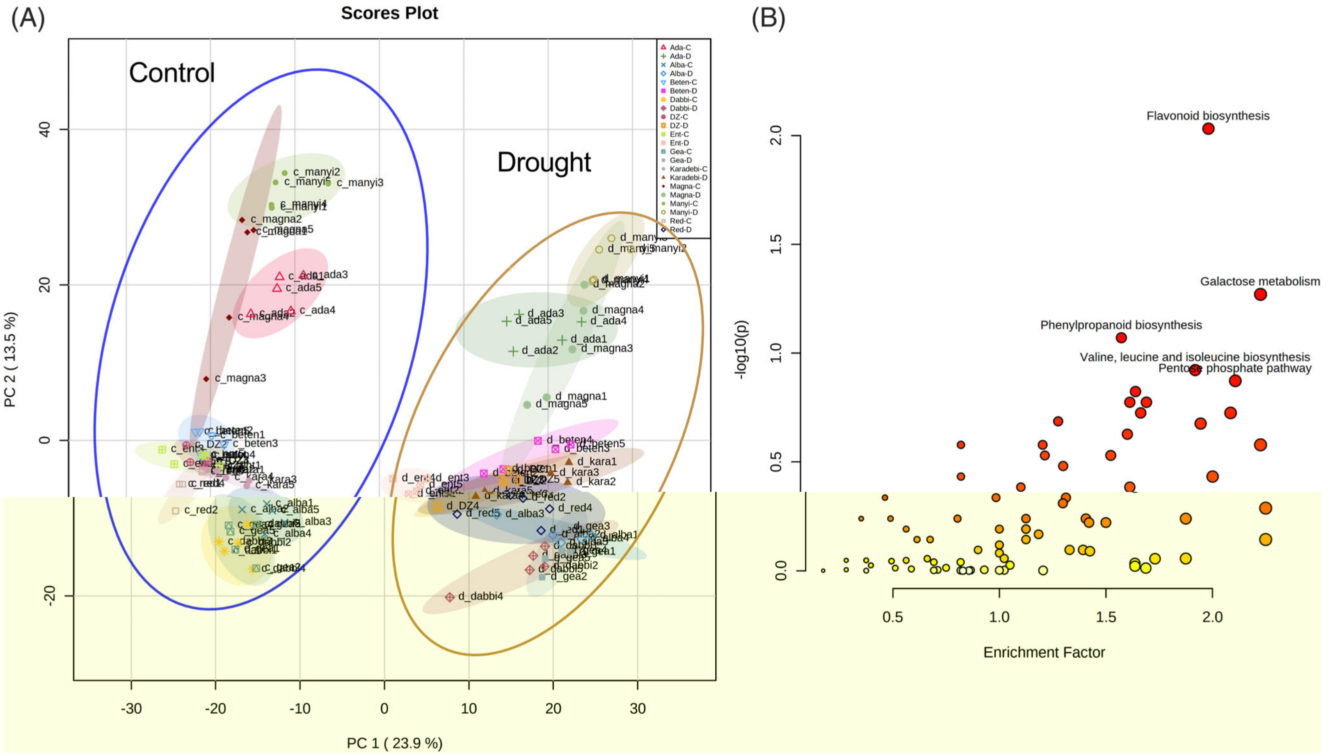


FIGURE 4 Metabolite distribution within tef cultivars under drought response. The *m/z* features from the negative mode were analysed and identified for biological functions against the reference *Oryza sativa* (rice) metabolome. (A) PCA representing metabolite variation in negative mode between genotypes within control and drought group. (B) The pathway enrichment showing significant metabolic pathways associated with drought response in tef

decreasing compared with the control group except for the accession, Enatite (Ent). Enatite-drought (Ent-D) was clustered together with the control group, and the level of some of the metabolites (e.g., kaempferol, quercetin, and rutin) are maintained between Enatite control (Ent-C) and drought (Ent-D).

We found that most amino acids accumulated to higher levels in droughted tef plants, but there were some key differences between the genotypes. Aspartic acid, glutamic acid, alanine, glycine, glutamine, and serine were more elevated in Dabbi, Gea-lamie, and Alba under control conditions, whereas only glutamine, glycine, and serine were highly accumulated in Ent-D and Ada-D (Figure 5B). As with the flavonoids, Ent-D was clustered together with the control group and showed decreased accumulation in amino acids except for serine, glutamine, glycine, gamma aminobutyric acid (GABA), aspartic acid, and glutamic acid.

The sugars, raffinose, sucrose, and ribose showed increased accumulation in droughted plants, whereas glycolysis/TCA intermediates like pyruvic acid, citric acid, succinic acid, and oxoglutaric acid were more abundant in the control group (Figure 5C). We observed similar metabolomic responses in the accessions except for Ent-D and Ada-D, which showed similar accumulation patterns. One of the interesting observations was that a key indicator of drought, myo-inositol, was higher in Ent-D and Ada-D. In general, the overall drought response in tef is mostly characterized by flavonoids, sugars, and amino-acid mechanisms, but genotype could influence this association (Figure 5).

3.4 | Elucidating the drought tolerance in tef seedlings using morphological–metabolite approaches

To identify tolerant varieties, we undertook a comparative analysis of phenotypic data between the 11 tef accessions. We found that only Ent-D had a greater leaf area than other accessions (Figure 6A), whereas height did not show any significant variation (Figure 6B). Therefore, we designated Ent-D as tolerant and the others as the susceptible group.

To further investigate the possible mechanism of drought tolerance in Ent, we compared the Ent metabolome to those of the “other” tef accessions (susceptible). From the PCA analysis of the negative ionization data, Ent-C was clustered together with “others” in the control group. In contrast, the metabolite pattern of Ent-D was distinct from other accessions in the droughted group (Figure 7A). The metabolite differences between Ent and other accessions were identified by ANOVA, and functional analysis of significant metabolite features was associated with branched-chain amino acid (BCAAs) biosynthesis, galactose metabolism, starch, and sucrose metabolism, phenylpropanoid biosynthesis, and ascorbate and aldarate metabolism (Figure 7B). The *m/z* M–H [−] features were identified and putatively annotated. The overall accumulation of annotated metabolites in Ent compared with other accessions is shown as a heat map (Figure 7C). This further indicated that the response of Ent to drought was distinctive.

Class-wise analyses based on amino acids, sugars/TCA/organic acid intermediates and fatty acids were carried out to highlight differences

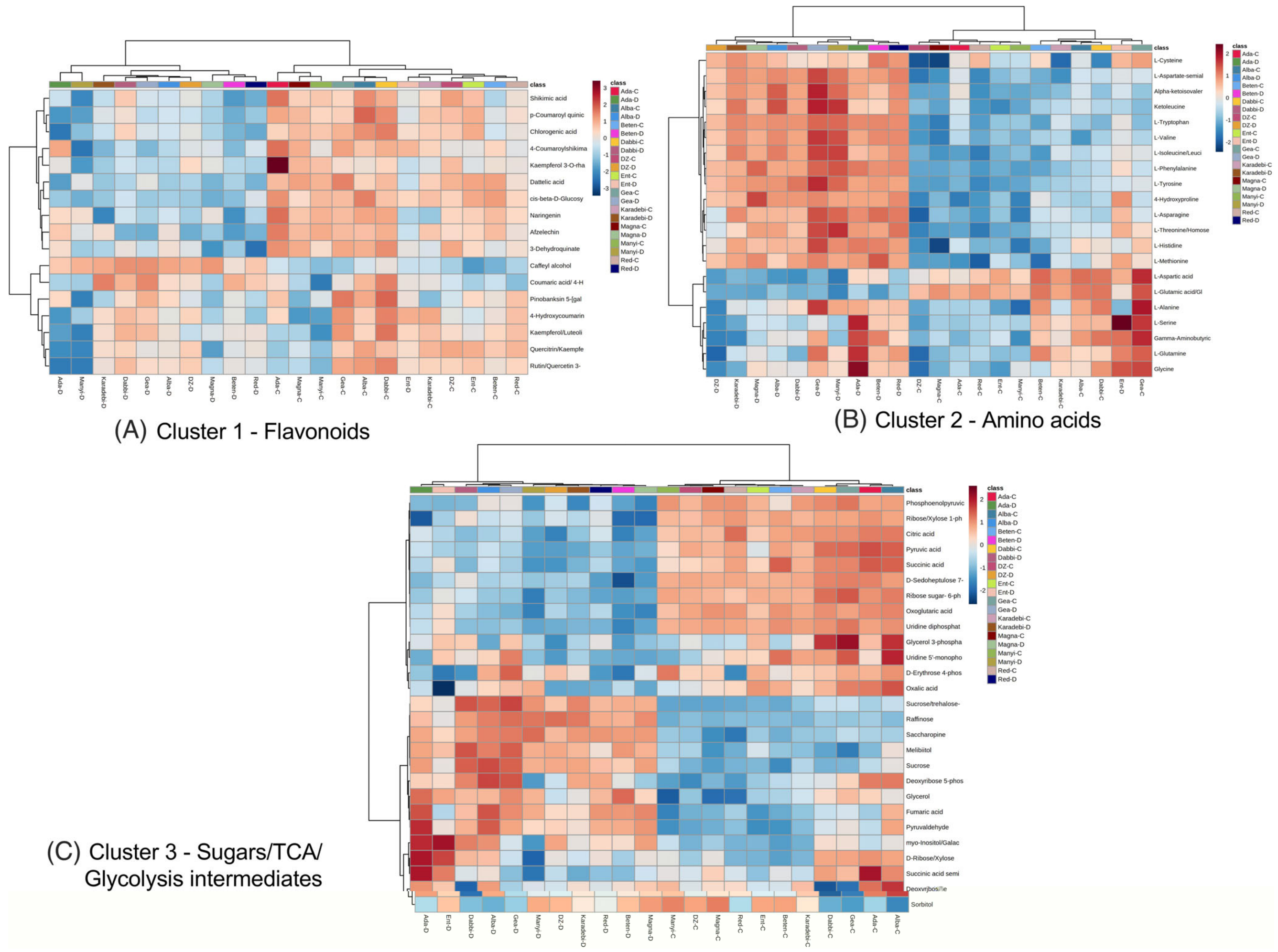


FIGURE 5 Major clusters showing variation during drought. The clusters are represented as individual heatmaps: (A) flavonoids, (B) amino acids, and (C) sugars/TCA intermediates

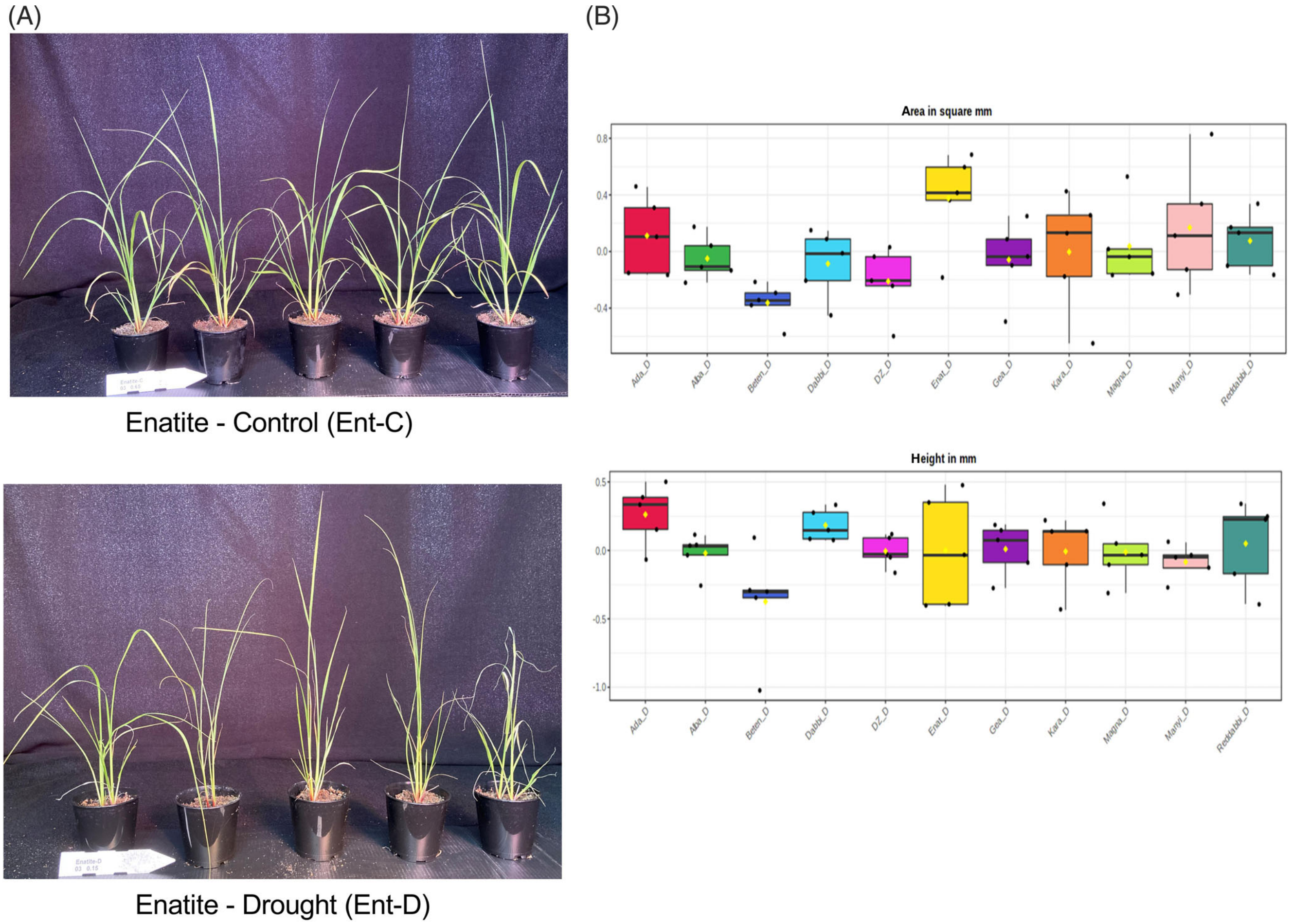
in key metabolites (Figure 8). In the amino acid class, except for glycine and serine, phenylalanine, tryptophan, tyrosine, valine, isoleucine/leucine, ketoleucine, and histidine was less abundant in Ent-D (Figure 8A). The levels of aspartic acid were maintained in both the control Ent (Ent-C) and drought (Ent-D). Among the sugars and TCA intermediates, ribose/xylose, myo-inositol, nicotinic acid, succinic acid semialdehyde, glyceric acid, and glycerol-3-phosphate showed increased accumulation in Ent-D, but sucrose, sucrose/trehalose phosphate, raffinose, fumarate, malate, isopropyl malate, and pyruvic acid were decreased (Figure 8B). Linolenic, linoleic, palmitic, and palmitoleic acids were significantly increased in Ent-D compared with the other genotypes (Figure 8C). The phenylpropanoids derivatives, caffeyl alcohol, quercetin rhamnoside, were decreased, but 4-coumaroyl shikimate, and fatty acids (palmitic acid, linolenic acid, and linoleic acid) increased in Ent-D (Figure 8C).

4 | DISCUSSION

The metabolome connects the phenome and genome (Großkinsky et al. 2015), given this, studies that incorporate phenomic and metabolomic assessments could develop our understanding of regulatory networks, which will further aid in the improvement of crops. Drought is one of the serious abiotic stresses faced by plants, and tolerance requires coordinated regulation of complex networks (Todaka et al. 2015). Tef can tolerate conditions like drought and waterlogging, but it is still affected by severe drought, so information on any adaptive mechanisms could aid the development of tolerant varieties. In this study, we used high-throughput imaging and metabolomics assisted approaches to describe the global response in tef subjected to drought stress under controlled conditions.

4.1 | Effect of water stress accurately defined by image-assisted pre-field phenotypic screening

Controlled experiments are important for selecting the candidates for stress tolerances as it reduces the risk of other environmental fluctuations (Dalal et al. 2019). Tef is sensitive to drought during the early seedling stage until it has developed a deep root system for later improved tolerance (Degu 2010). Thus, we concentrated on assessing



Enatite - Control (Ent-C)

Enatite - Drought (Ent-D)

FIGURE 6 Phenotypic response of Enatite genotype. (A) Morphological difference between Enatite control (Ent-C) and Enatite drought (Ent-D). (B) Difference in area and height in Ent-D with other droughted tef accessions

drought responses during the seedling stage under highly regulated environmental conditions. For this, 11 diverse tef accessions were selected, which were mostly landraces/cultivars that possess diverse phenotypic and agronomic features. We applied an image-assisted phenomics approach to understand the natural variation within tef accessions under drought stress. Such image-based studies have been undertaken in other monocot species such as maize, barley, wheat (Kim et al. 2020), and *Brachypodium* (Fisher et al. 2016) to elucidate the morphological responses to drought. Our phenotypic data analyses of tef plants under drought stress showed that the most effective indicators of drought response are plant area and plant color. After exposure to drought, droughted plants showed a significant decrease in plant height, area, green pixels but an increase in red and purple pixels (Figure 2). The changes in the pixel color could reflect the relationship with the accumulation of pigments, degradation of chlorophyll or water content. Also, a decrease in plant area and height is likely to reflect drought disrupting growth and development. Crucially, phenomic approaches targeted one accession, Enatite, which exhibited drought tolerance at the seedling stage (see below). Such observations underline the applicability of phenomics in plant breeding programs to target different phenotypes in a diverse population.

4.2 | Drought responsive metabolic networks in tef are associated with changes in flavonoids, amino acids, and sugars

Having defined the drought phenotypes, we next examined the metabolomic consequences of drought. This aimed to suggest why tef is reasonably well adapted to drought than other cereals. The assessment could also suggest if there are any genotypic differences during drought between various accessions.

Taken together, tef accessions showed differences in 166 differential metabolites under severe drought stress. The most pronounced changes were observed in flavonoid biosynthesis, galactose metabolism, phenylpropanoid biosynthesis, BCAA biosynthesis, and pentose phosphate pathway. Flavonoids are the major class of phenylpropanoids and are an important class of secondary metabolites that play a protective role in plant defense mechanisms (Tohge et al. 2018). Several transcriptomic and metabolomic studies have shown high levels of phenolics and flavonoids in *Brassica napus*, *Triticum aestivum*, rice, and potato are associated with drought stress (Yadav et al. 2021). They can function as antioxidants, maintain water homeostasis (Naing & Kim 2021), and improve the adaptability of

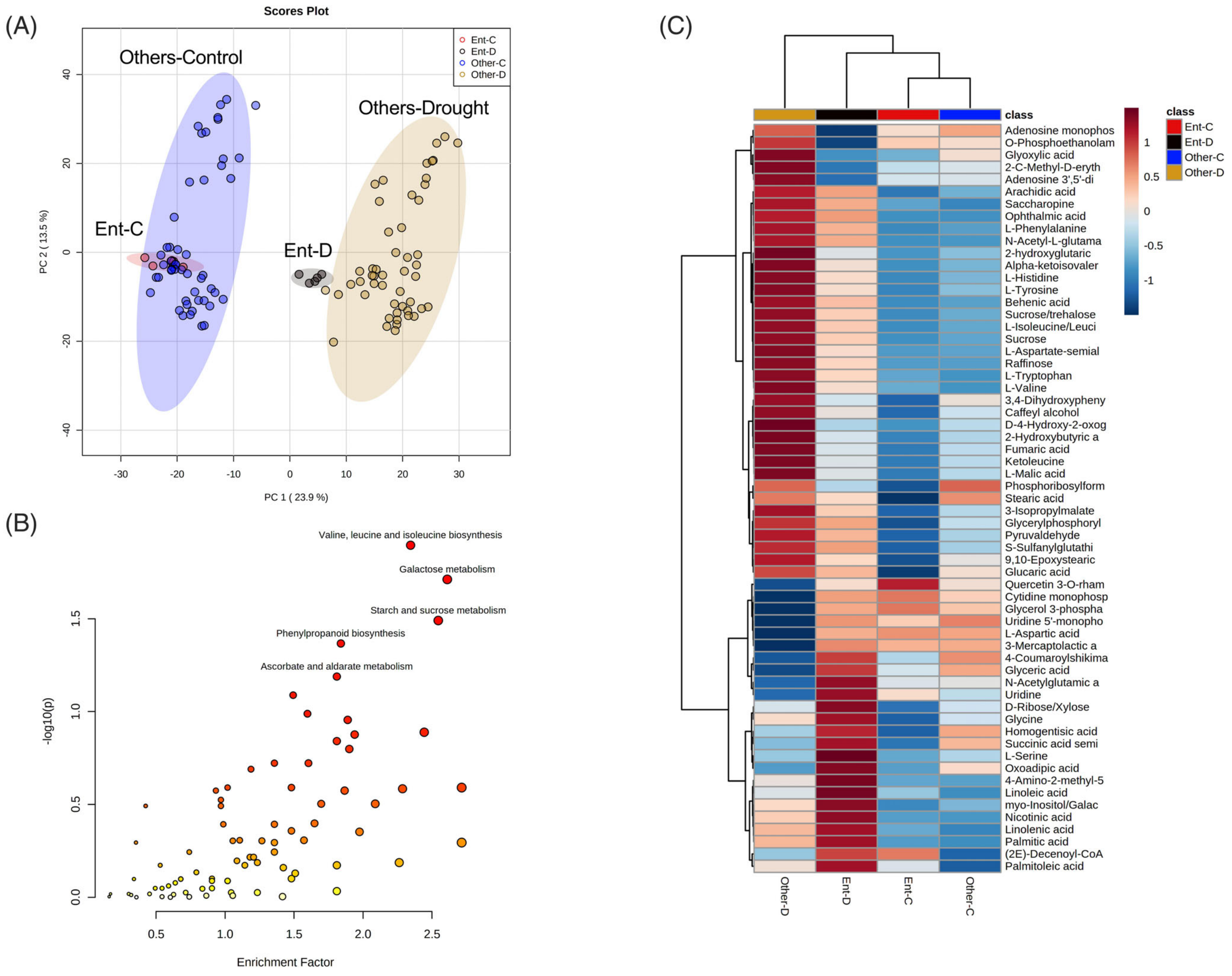


FIGURE 7 Metabolite response of Enatite with other accessions. (A) PCA distribution of metabolite variation between Enatite control (Ent-C), Enatite drought (Ent-D), others control (Other-C), and others drought (Others-D). (B) Key metabolite pathways showing variation in Ent-D with others. (C) Heat map showing distribution of differentially accumulated metabolites in Ent and other genotypes

plants to stress, but the exact mechanisms require characterization. One recent study in maize seedlings showed that flavonoids could facilitate drought tolerance by reducing oxidative damage and controlling stomatal movement (Li et al. 2021). Tef is found to be rich in flavonoids (Ravisankar et al. 2018) and this may be why this species can adapt to extreme environmental conditions. Interestingly, we found that most of the flavonoid related metabolites like shikimic acid, naringenin, 4-coumaroylshikimate, and p-coumaroyl quinic acid showed less accumulation in all the droughted accessions, whereas rutin, quercetin, and kaempferol/luteolin showed differential regulation within the accessions. Caffeyl alcohol, an intermediate of the lignin pathway, was consistently accumulated in all tef accession within the water-deficit regime (Figure 5A). Caffeyl alcohol is associated with the biosynthesis of monolignols, and the biosynthesis of lignin is further modulated by the phenylpropanoid pathway in grasses (Carrizo et al. 2021). Cell wall changes have been linked to drought tolerance (Lenk et al. 2019) and modulation of lignin could be one component of this, along with flavonoids and phenylpropanoid derivatives (Yan et al. 2018).

The accumulation of amino acids and sugars under drought stress has been described in many plant species. Amino acids accumulate later than sugars during drought stress (Fàbregas & Fernie 2019). The level of several amino acids was significantly increased in all tef accessions during drought stress. This included the BCAA's (valine, isoleucine, and leucine), hydroxyproline, aromatic amino acids (tryptophan, phenylalanine, and tyrosine), cysteine, methionine, and threonine. Drought-induced accumulation of proline BCAA's has been well studied in many plants (Živanović et al. 2020) and play osmoregulatory roles (Hayat et al. 2012). Furthermore, the aromatic amino acids, which are the precursors for secondary metabolite synthesis, have also been found to accumulate in maize leaves, rice, and wheat under drought (Michaletti et al. 2018).

Osmoregulatory changes based on sugars are also important responses to drought. During stress, the cellular homeostasis is maintained by adequate sugar transporters and sugar levels. Comparing the levels of sugars in drought imposed tef genotypes showed a high accumulation of sucrose, raffinose, and ribose along with myo-inositol (Figure 5C). The accumulation of soluble sugars like sucrose, glucose, fructose, and raffinose family have been reported during drought stress (Kaur et al. 2021). Sucrose is the major soluble sugar transported between plant sources and sinks, whereas raffinose and myo-inositol function as osmolytic responses to drought stress (Salvi et al. 2020). The TCA (citric acid, succinic acid) and glycolysis intermediates (glycerol-3-phosphate, pyruvic acid phosphoenolpyruvic acid) were reduced in the droughted group. Interestingly, they were evident variations in the level of some sugars, TCA and glycolysis intermediates within genotypes, for example, sorbitol, ribose, succinic acid semialdehyde, and glycerol-3-phosphate. In the present study, there was an accumulation of stress-induced amino acids (proline, BCAA's, and aromatic amino acids), sucrose, raffinose, sucrose/trehalose-6-phosphate, and myo-inositol, which could act as key components in the early drought response in tef, but this needs to be validated with more gene-expression and time-course studies.

4.3 | Tef accession, Enatite showed less sensitivity toward severe drought

As indicated previously, Enatite was phenotypically different and less sensitive to drought (Figure 6). Interestingly, drought tolerance indices suggest that Enatite had intermediate grain yield under moisture-stressed and non-stressed environments (Abraha et al. 2017). Based on the stress susceptibility index (SSI) and yield stability index (YSI), Enatite was found to be the most sensitive genotype at the anthesis stage (Ferede et al. 2020). Drought tolerance of Enatite at the seedling stage suggests that either this could be a feature associated with short-duration exposure to drought and /or was observable only under carefully regulated conditions. Examining possible metabolomic causes for this phenotype suggested that BCAA's, galactose metabolism, starch and sucrose metabolism, phenylpropanoid metabolism, and ascorbate and aldarate metabolism could be key differences Figure 7. Among the annotated metabolites, relative increases in flavonoid derivative, 4-coumaroyl shikimate, amino acids (glycine, serine), sugars (ribose, myo-insitol), and fatty acids (linoleic acid, nicotinic acid, linolenic acid, palmitic acid, and palmitoleic acid) were observed. Linolenic acid is the precursor to the biosynthesis of jasmonates, which are important signals with roles in drought responses (Riemann et al. 2015). However, it should be noted that we did not detect jasmonate accumulation in our metabolite profiles. Stress-induced BCAA's, proline, sucrose, and raffinose were lower in Ent-D compared with other genotypes. This could suggest that the osmoregulatory

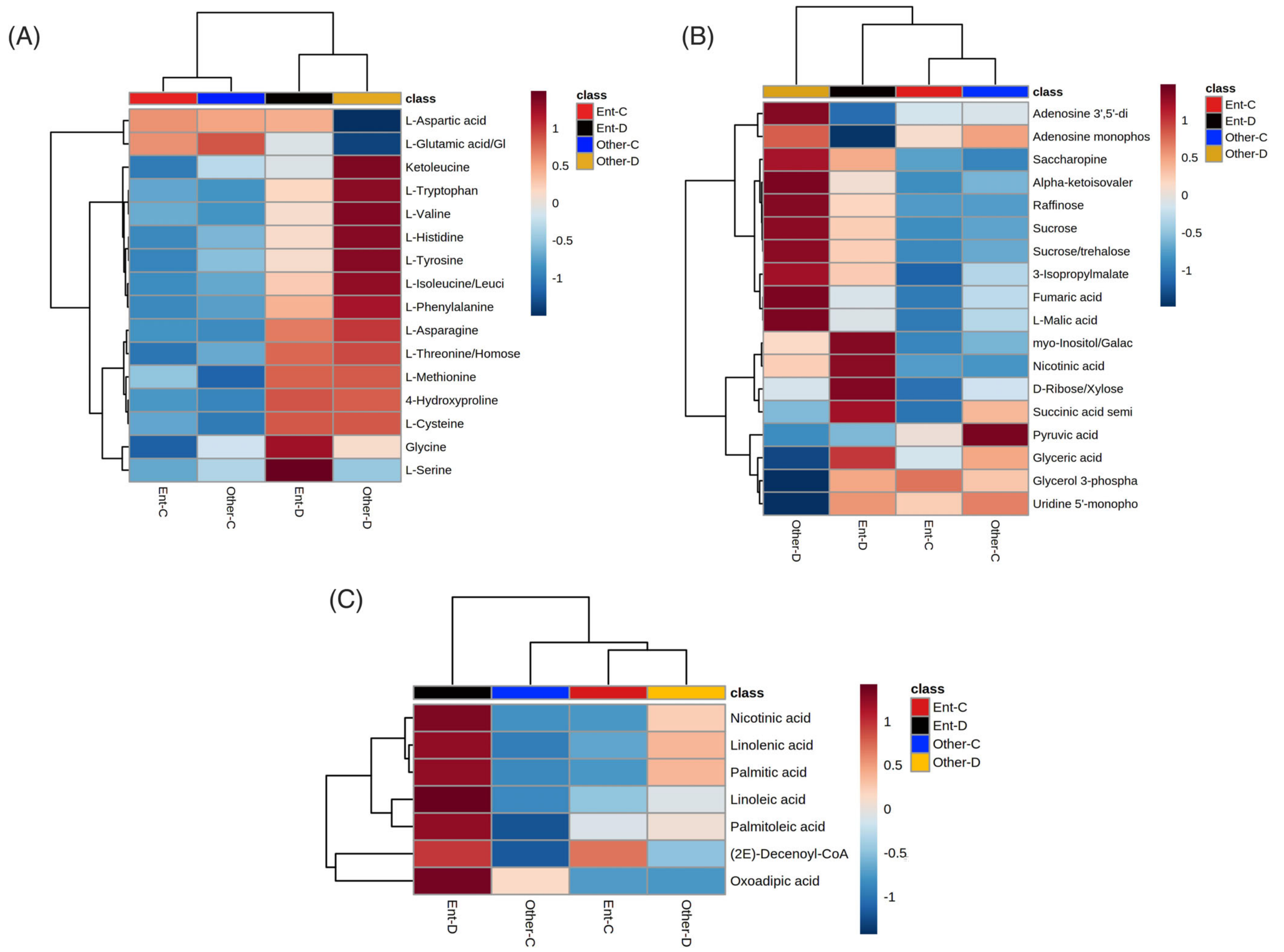


FIGURE 8 Distribution of amino acids, sugars, and fatty acids in Enatite compared with other accessions. (A) amino acids, (B) sugars, and TCAS intermediates (C) fatty acids and phenylpropanoid derivatives

mechanisms previously referred to are more prominent in Enatite, at least at the seedling stage. Previous studies in a drought-tolerant variety, Tsedey and susceptible variety (Alba), showed a significant role of micro-RNA's (miRNA) in regulating drought response (Martinelli et al. 2018). Our results suggest that there will be an influence of other factors, such as a possible role of other post-transcriptional regulation linked to the less sensitive nature of genotypes and is also apparently dependent on the genotype and developmental stage.

5 | CONCLUSIONS

Our initial imaging and metabolomic data showed that severe drought generally reduces plant growth and induces the accumulation of amino acids (proline, BCAA's, and aromatic amino acids) and sugars (sucrose and raffinose). In general, there were enhanced levels of primary metabolites, with decreased accumulation of secondary metabolites (flavonoids) which could propose the involvement of some reactive oxygen species (ROS) or sugar sensing signaling pathways under drought stress in tef. Enatite was found to be less sensitive, the effect of drought stress on Enatite was significantly different than others with increased accumulation of fatty acids, glycine, serine, and myo-inositol. Accumulation of fatty acids could be associated with jasmonate signaling and myo-inositol with ROS. These hypothesizes need to be further investigated in a large set of genotypes. This stated, our study has generated a new metabolomic resource for this understudied potential crop, which will further help in advancing more "omics" studies in tef.

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AUTHOR CONTRIBUTIONS

Aiswarya Girija, Luis A J Mur, and Fiona Corke designed the study. Aiswarya Girija performed the research. Jiwan Han performed the image feature extraction and analysis. Fiona Corke and Aiswarya Girija involved in phenomics data collection and interpretation. Aiswarya Girija performed the metabolite experiment. Aiswarya Girija and Luis A J Mur analysed the results. Aiswarya Girija prepared the manuscript. Fiona Corke, Jiwan Han, Habte Jifar, John Doonan, Rattan Yadav, and Luis A J Mur contributed toward discussion and completion of manuscript.

DATA AVAILABILITY STATEMENT

Phenomic and metabolite data used for the current study are provided as supplementary files.

ORCID

Luis A. J. Mur https://orcid.org/0000-0002-0961-9817

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