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Screening of Safflower Genotypes for Enhanced Flavonoid and Anthocyanin Contents under Water-Limited Conditions

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ABSTRACT

Drought is the main abiotic stress limiting safflower production in semi-arid regions, with flavonoid and anthocyanin pathways playing a key role in antioxidant defense. A two-year field experiment in Arak, Iran, evaluated 38 safflower genotypes under full irrigation and terminal drought stress, measuring total flavonoid and anthocyanin contents. Drought increased average flavonoids by 6.3% (from 0.1395 to 0.1483 mg QE g^{-1} DW) and anthocyanins by 8.1% (from 0.00382 to 0.00413 mg g^{-1} DW). A three-way ANOVA showed significant effects of irrigation and year on both traits, with year accounting for 62.8% of anthocyanin variance, while genotype effects were not significant ($p = 0.953$ and $p = 0.496$), indicating high residual variability (75.8% and 23.2%). Using eight drought-tolerance indices, Variety 31 ranked first for flavonoid stability (STI = 1.311), and Variety 14 ranked first for anthocyanin stability (STI = 1.952). Variety 14 was the only genotype among the top five for both traits. The correlation between flavonoid and anthocyanin contents was weak and non-significant ($r = -0.06$, $p = 0.187$), suggesting independent regulation of these two phenylpropanoid branches. These results introduce Variety 14 as a promising multi-trait parent for drought-resilience breeding and highlight that interannual climatic variation, rather than genotype, is the primary factor influencing anthocyanin accumulation in safflower petals.

Keywords: Anthocyanin; Drought stress; Environment interaction; Flavonoid; Genotype

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Introduction

Water scarcity is the foremost constraint on global crop productivity, and its severity is increasing under

climate change: rain-fed systems that sustain roughly 60% of global food production are projected to face intensifying green-water deficits, with food production for hundreds of millions of additional people at risk under 1.5 ˚C and 3 ˚C warming scenarios (He and Rosa, 2023). Arid and semi-arid regions of the Middle East, including the Iranian plateau, are disproportionately exposed to this trend, and oilseed systems in these regions increasingly depend on genotypes that combine agronomic productivity with physiological drought resilience.

Safflower (Carthamus tinctorius L., Asteraceae) is a deep-rooted, thermophilic oilseed crop that is intrinsically well suited to drought-prone cultivation because of its extensive taproot system and capacity for osmotic adjustment (Golkar et al., 2021). Its seed oil is valued for a high linoleic and oleic acid content and is used in edible-oil, pharmaceutical, and cosmetic applications, while its flowers are a traditional source of natural dyes and bioactive flavonoids with documented antioxidant, hepatoprotective, and cardiovascular pharmacological activity (Cheng et al., 2024). Because of this dual industrial and pharmaceutical value, there is growing interest in identifying safflower genotypes that maintain high phytochemical quality under water-limited conditions, particularly in major production regions such as Iran, where safflower is cultivated as a rotational crop in rain-fed and supplementary-irrigation systems (Rahimi, 2021).

At the biochemical level, drought stress triggers the accumulation of reactive oxygen species (ROS), and plants counteract this oxidative burden through both enzymatic antioxidant systems and non-enzymatic secondary metabolites (Bao et al., 2024; Fathi et al., 2025). Flavonoids and anthocyanins, downstream products of the phenylpropanoid pathway, are among the most effective non-enzymatic ROS scavengers: they quench singlet oxygen and hydroxyl radicals directly, function as protective compounds that limit photo-oxidative damage, and their biosynthesis is transcriptionally up-regulated under water deficit through the coordinated induction of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and downstream branch-point enzymes such as dihydroflavonol-4-reductase and anthocyanidin synthase (Li and Ahammed, 2023; Zhou et al., 2023). In Arabidopsis, drought induces a rapid and reversible remodelling of the flavonol and anthocyanin pool that is tightly coupled to the plant's water-loss trajectory (Fidel et al., 2022), and in purple-leaf oilseed rape genotypes, constitutively higher anthocyanin accumulation is directly associated with lower ROS burden, better membrane integrity, and superior physiological performance under drought relative to green-leaf counterparts (Chen et al., 2022). A comparable protective role for anthocyanins has been reported across a wide range of unrelated taxa subjected to water deficit, reinforcing the generality of this defence mechanism (Yan et al., 2022).

Safflower itself possesses a well-characterised flavonoid biosynthetic capacity, and recent functional work has shown that a safflower flavonol synthase gene, CtFLS1, is sufficient to enhance drought tolerance in a heterologous system by driving flavonol and anthocyanin accumulation (Ma et al., 2024), providing direct mechanistic support for flavonoid- and anthocyanin-based drought defence in this species. Parallel evidence from the aromatic crop anise (Pimpinella anisum L.), evaluated under water-deficit stress in Kerman province using drought-tolerant and drought-sensitive genotypes, showed that up-regulation of the same core phenylpropanoid genes (PAL, 4CL, CHI, F3H) accompanied large increases in total flavonoid and anthocyanin content specifically in the tolerant genotype (Mehravi et al., 2025), underscoring that genotypic variation in phenylpropanoid pathway activation, rather than drought exposure alone, determines the magnitude of the phytochemical stress response.

Screening large genotype panels for drought tolerance is most informative when trait values under stressed (Ys) and non-stressed (Yp) conditions are combined into complementary selection indices rather than interpreted independently. The stress susceptibility index (SSI; Fischer and Maurer, 1978) and stress tolerance index (STI; Fernandez, 1992) remain the most widely used metrics,

and are typically interpreted alongside mean productivity (MP), geometric mean productivity (GMP), harmonic mean, tolerance (TOL), and the yield stability index (YSI) to distinguish genotypes that are merely stress-avoidant from those that combine high productivity with genuine stress tolerance. This multi-index framework has been validated across a wide range of crops under recent field conditions, including maize (Shojaei et al., 2022), dry bean (Mutari et al., 2023), and Bambara groundnut (Odesola et al., 2023), and has been applied specifically to safflower yield screening in worldwide germplasm collections (Golkar et al., 2021) and in Iranian marginal-area trials (Pasban Eslam et al., 2024), as well as to genome-wide association mapping of drought-tolerance loci in safflower (Hassani et al., 2024). However, most safflower drought-tolerance index studies to date have focused on seed yield and morphological traits rather than on the biochemical, antioxidant-related traits that determine both stress physiology and downstream product quality.

Furthermore, drought responses in phenylpropanoid-derived metabolites are frequently confounded by seasonal climatic variation, since temperature and radiation load during flowering can independently drive large shifts in anthocyanin accumulation irrespective of the imposed irrigation treatment, as demonstrated in a two-year rain-fed trial of high-oleic safflower genotypes in Sicily in which year, rather than genotype, was the dominant source of variation in several productive traits (Licata et al., 2023). Disentangling the relative contributions of irrigation regime, genotype, and year is therefore essential for correctly attributing phytochemical variation to drought stress rather than to background seasonal effects, and requires multi-year trials with adequate genotypic replication.

Building on this body of evidence, the present study evaluated 38 safflower genotypes under full irrigation and terminal drought stress across two growing seasons at Arak, Iran, with the following objectives: (i) to quantify the effect of drought stress, year, and genotype on total flavonoid and anthocyanin content in safflower flag leaves using a three-way factorial ANOVA; (ii) to identify genotypes combining high productivity and stability under both irrigation regimes using eight complementary drought-tolerance indices; (iii) to test whether flavonoid and anthocyanin accumulation are correlated, as an indicator of shared versus independent regulation of the two phenylpropanoid branches; and (iv) to identify candidate parental genotypes for breeding programmes targeting drought-resilient phytochemical quality in safflower.

Materials and Methods

Site description

The study was conducted over two growing seasons (2015 and 2016) at the Research Farm of the Faculty of Agriculture, Islamic Azad University, Arak Branch (Khomein Road), Iran (36°15′ N, 59°23′ E; 1708 m a.s.l.). The soil was a loam with pH ≈ 7.7 and electrical conductivity ≈ 1.3 dS m^{-1}. Mean annual precipitation at the site was approximately 350 mm and mean annual temperature 12.5 °C, characteristic of the semi-arid climate typical of central Iranian safflower production areas.

Experimental design and treatments

A split-plot design within a randomized complete block framework, with three replications, was used following standard agronomic field-experiment protocols (Gomez and Gomez, 1984). The main-plot factor comprised two irrigation regimes: (i) full irrigation (control), in which irrigation was scheduled at 20% allowable soil moisture depletion throughout the growing season; and (ii) drought stress, in which full irrigation was maintained until stem elongation and then withheld until harvest (approximately 70% allowable depletion).

The sub-plot factor comprised 38 safflower genotypes (coded G1–G38), obtained from the Dryland Agricultural Research Center, Kermanshah, Iran. Each sub-plot measured 3 × 1.8 m (5.4 m^2) and contained six rows spaced 30 cm apart. A drip (tape) irrigation system with 5-cm emitter spacing at 1.5 atm pressure was used to apply irrigation treatments precisely. Sowing was

performed on 22 February in both years. Standard agronomic practices, including manual weeding and fertilization (70 kg N ha^{-1} as sulfur-coated urea, applied in two splits), followed local recommendations for the region.

Traits measured

Leaf samples were collected from the flag leaf at full flowering (50% flowering) in both seasons. Two flavonoid-pathway biochemical traits were quantified:

Total flavonoid content (mg QE g^{-1} DW) was determined by the aluminium chloride colorimetric method using quercetin as the calibration standard (Zhishen et al., 1999).

Anthocyanin content (mg g^{-1} DW) was measured by the pH-differential method at pH 1.0 and 4.5 (Giusti and Wrolstad, 2001).

Statistical analysis

All statistical analyses were performed in Python 3.12 using the pandas, NumPy, SciPy, statsmodels, and openpyxl libraries, with visualisations generated in matplotlib. Descriptive statistics included sample size (N), mean, standard deviation (SD), standard error (SE), coefficient of variation (CV%), minimum, first quartile, median, third quartile, maximum, skewness, and kurtosis, computed overall and stratified by irrigation regime, year, and genotype.

Inferential analysis employed a three-way fixed-effects factorial analysis of variance (ANOVA) using Type II sums of squares, fitted as Trait ~ Year × Irrigation × Variety. This model simultaneously estimates the main effects of each factor and all two-way and three-way interactions; the percentage contribution of each term to total variance was computed as Source SS / Total SS × 100.

Drought tolerance was quantified for each genotype using eight complementary indices calculated from genotype means under normal (Yp) and drought (Ys) irrigation, averaged across years and replications. The stress susceptibility index (SSI; Fischer and Maurer, 1978) expresses the proportional reduction in trait value under stress relative to the mean population reduction, with lower SSI indicating greater tolerance. The stress tolerance index (STI; Fernandez, 1992), mean productivity (MP), geometric mean productivity (GMP), and harmonic mean (HARM) reward genotypes that maintain high trait values under both normal and stress conditions. Tolerance (TOL = Yp − Ys) measures the absolute reduction, while the yield stability index (YSI = Ys / Yp) and drought tolerance index (DTI) provide dimensionless rankings of relative stability under stress. This multi-index approach follows established practice for drought-tolerance screening in field crops (Shojaei et al., 2022; Mutari et al., 2023; Odesola et al., 2023) and has previously been applied to safflower yield evaluation (Golkar et al., 2021; Pasban Eslam et al., 2024).

The relationship between total flavonoid and anthocyanin content was assessed using both Pearson (linear) and Spearman (rank-based) correlation coefficients, computed overall and within each combination of irrigation regime and year. Pearson correlations assume linearity and bivariate normality, whereas Spearman correlations are robust to non-linear monotonic relationships and outliers, providing complementary information.

Assumption testing

Two key assumptions of parametric ANOVA were formally tested. Normality of residuals from the full ANOVA model was assessed using the Shapiro–Wilk test. Homogeneity of variances across the four year-by-irrigation groups was evaluated with Levene's test using the median-centred estimator, which is robust to non-normal distributions. Both tests returned significant results ($p < 0.001$ for both traits in the Shapiro–Wilk test; $p < 0.001$ for flavonoid and $p < 0.001$ for anthocyanin in Levene's test), indicating that the strict distributional assumptions of parametric ANOVA were violated. Nevertheless, the F-test is known to be robust to moderate departures from normality and variance homogeneity, particularly in balanced designs with large sample sizes ($n = 456$ in the present study). Interpretive conclusions are therefore reported with appropriate caution, and

effect sizes (percentage contribution to total variance) are emphasized alongside p-values throughout the Results.

Results

Effect of irrigation regime

Drought stress increased mean total flavonoid content from 0.1395 ± 0.0359 mg QE g^{-1} DW under normal irrigation to 0.1483 ± 0.0318 mg QE g^{-1} DW under drought, an absolute increase of 0.0088 (6.3% relative increase). The coefficient of variation was 25.8% under normal irrigation and 21.4% under drought. Anthocyanin content increased from 0.00382 ± 0.00278 mg g^{-1} DW under normal irrigation to 0.00413 ± 0.00259 mg g^{-1} DW under drought, an 8.1% relative increase (Figure 1).

Inter-annual variation

Mean flavonoid content was 0.1397 mg QE g^{-1} DW in Year 1 and 0.1481 mg QE g^{-1} DW in Year 2, a 6.0% increase (Figure 2); the coefficient of variation was 31.0% in Year 1 and 14.0% in Year 2. Mean anthocyanin content was 0.00611 mg g^{-1} DW in Year 1 and 0.00185 mg g^{-1} DW in Year 2, a 69.7% reduction. The Year main effect accounted for 62.8% of total variance in the ANOVA for anthocyanin content (Section 3.4, Table 2).

Varietal variation

For total flavonoid content under normal irrigation, Variety 31 had the highest mean (0.1553 mg QE g^{-1} DW) and Variety 24 the lowest (0.1006 mg QE g^{-1} DW), a 54.4% range. Under drought, Variety 32 had the highest mean (0.1706 mg QE g^{-1} DW) and Variety 20 the lowest (0.1178 mg QE g^{-1} DW) (Figure 4). For anthocyanin content under normal irrigation, Variety 14 had the highest mean (0.0055 mg g^{-1} DW) and Variety 13 the lowest (0.0030 mg g^{-1} DW); under drought, Variety 5 had the highest mean (0.0065 mg g^{-1} DW), while several varieties had means as low as 0.0031 mg g^{-1} DW (Figure 3).

Factorial ANOVA

For total flavonoid content, the three-way factorial ANOVA (Table 1) showed significant main effects of Year ($F = 6.003$, $p = 0.0148$) and Irrigation ($F = 6.558$, $p = 0.0109$), each accounting for approximately 1.5–1.6% of total variance. The Year × Irrigation interaction was not significant at $\alpha = 0.05$ ($F = 3.047$, $p = 0.0819$). The Variety main effect was not significant ($p = 0.953$), nor were any interactions involving Variety significant. Residual variance accounted for 75.8% of the total sum of squares.

For flavonoid content, mean values by year showed a larger drought-associated increase relative to normal irrigation in Year 1 than in Year 2 (Figure 6; Year × Irrigation interaction, $p = 0.0819$). For anthocyanin content, the corresponding Year × Irrigation interaction was $p = 0.087$, with higher normal-irrigation anthocyanin levels in Year 1 than in Year 2 (Figure 6).

Correlation between flavonoid and anthocyanin content

Pearson and Spearman correlation coefficients between total flavonoid and anthocyanin content were computed overall and within each combination of irrigation regime and year (Figure 7, 8). All coefficients were weak ($|r| < 0.13$) and not statistically significant at $\alpha = 0.05$, with one exception approaching significance (drought stress overall: $r = -0.114$, $p = 0.084$). The overall correlation across all conditions was $r = -0.062$ ($p = 0.187$) (Figure 5).

Trait variability and stability

Total flavonoid content had an overall coefficient of variation (CV) of 23.7%; CV was 25.8% under normal irrigation and 21.4% under drought, and 31.0% in Year 1 and 14.0% in Year 2. Anthocyanin content had higher CV values across all groups, with a maximum of 73.4% in Year 2 (Figure 9).

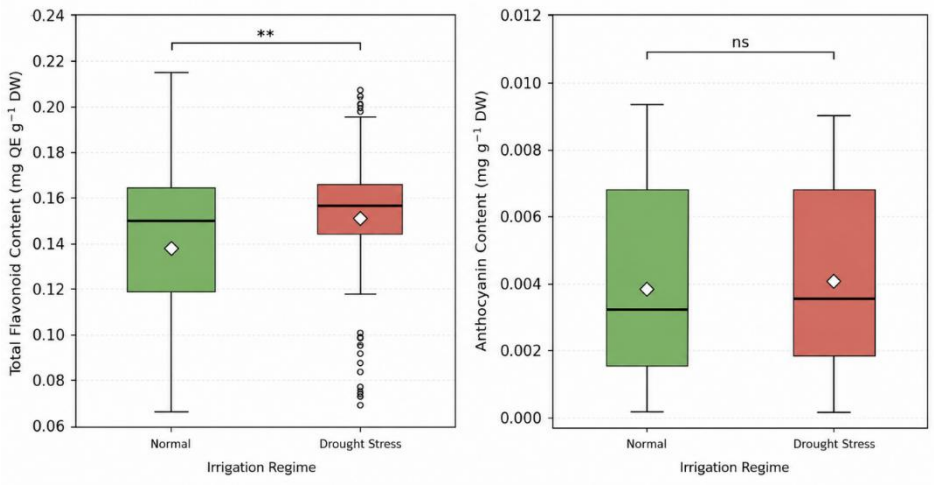

Figure 1. Boxplot distributions of total flavonoid and anthocyanin content under normal versus drought irrigation. White diamonds mark group means; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, $p < 0.1$, ns = not significant (independent samples t-test).

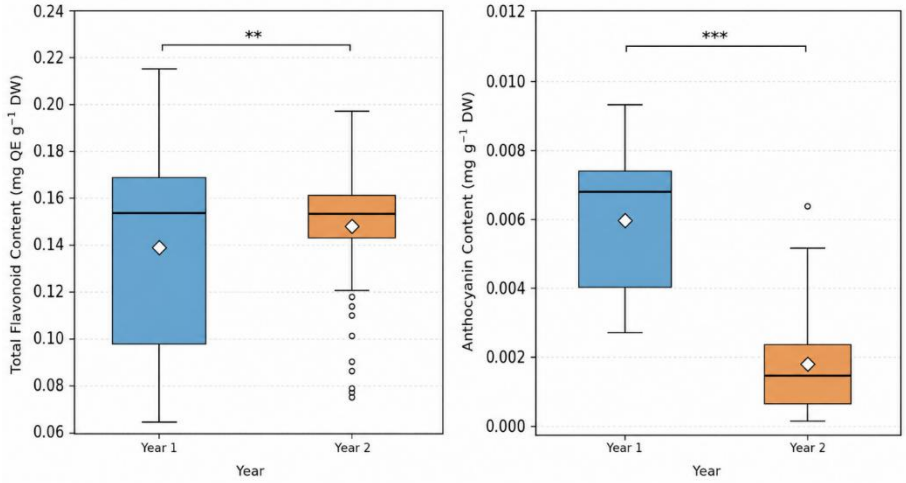

Figure 2. Boxplot distributions of phytochemical traits between the two experimental years. The Year 1 versus Year 2 contrast is especially pronounced for anthocyanin content (***).

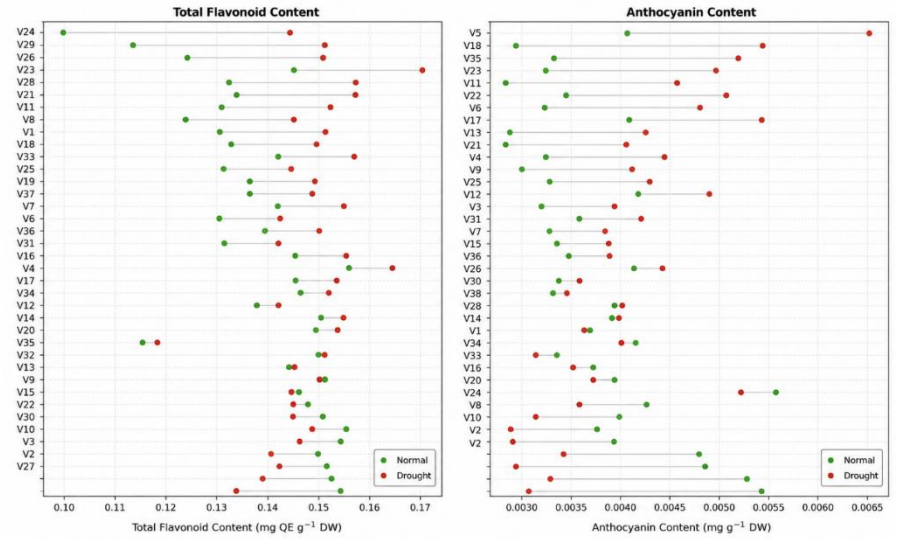

Figure 3. Lollipop chart comparing each variety's mean value under normal (green) versus drought (red) irrigation for both traits. Varieties are sorted by the drought − normal difference.

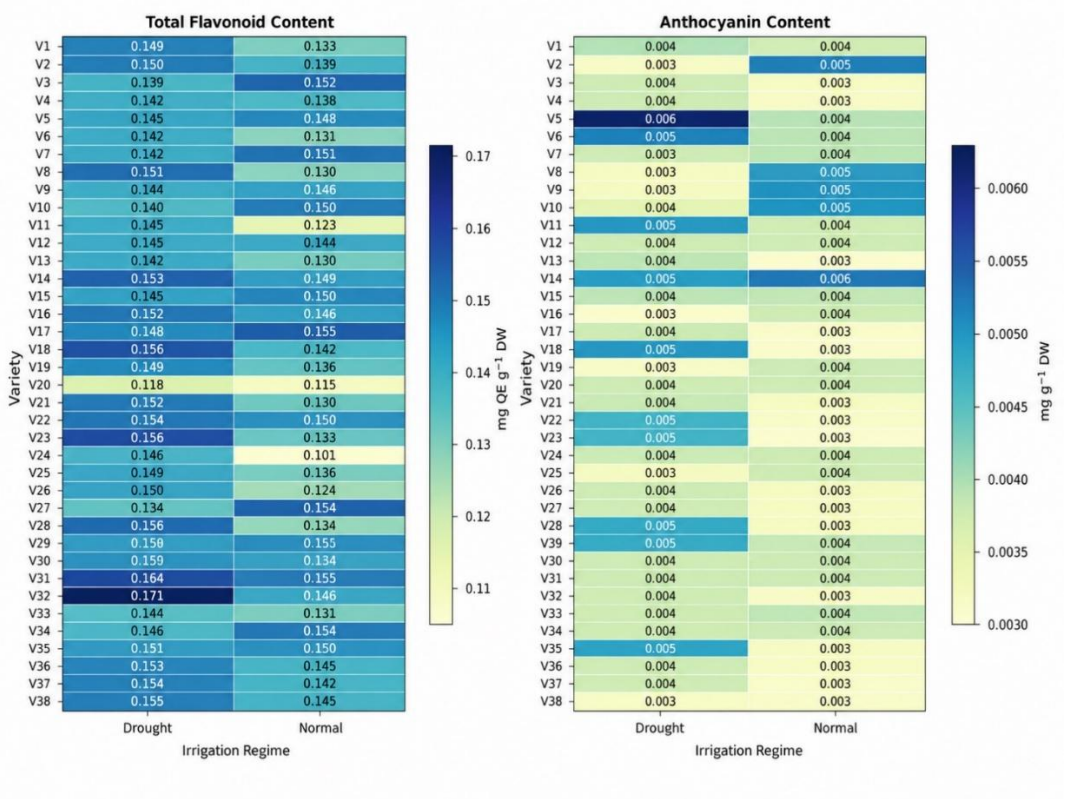

Figure 4. Heatmap of variety means across irrigation regimes. Cell values are mean trait values across years and replications; colour intensity scales linearly with magnitude.

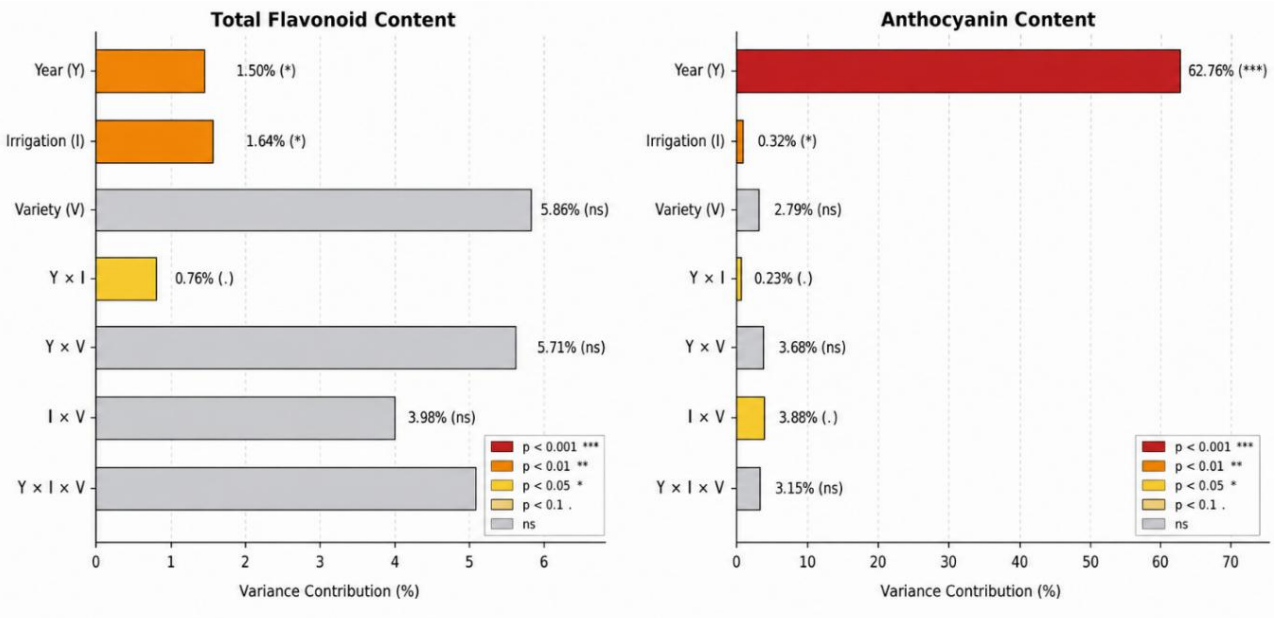

Figure 5. Percentage variance contribution of each experimental factor and their interactions, with significance codes from the three-way factorial ANOVA.

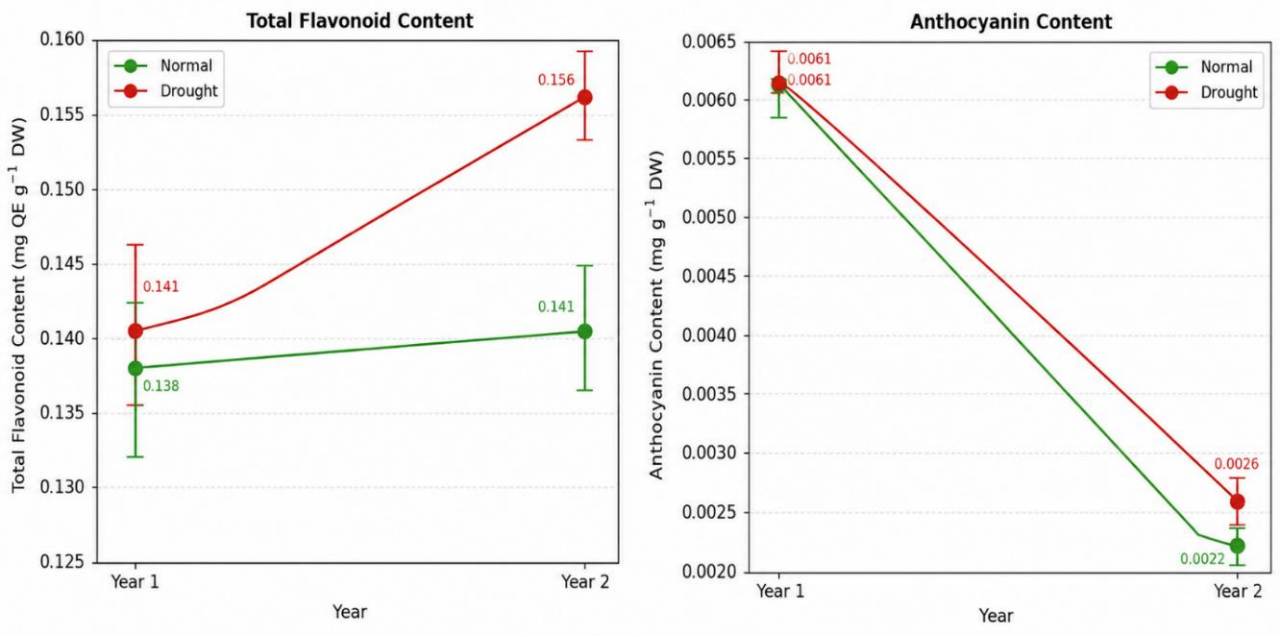

Figure 6. Year × Irrigation interaction plots for both phytochemical traits. Error bars denote ± 1 standard error of the mean.

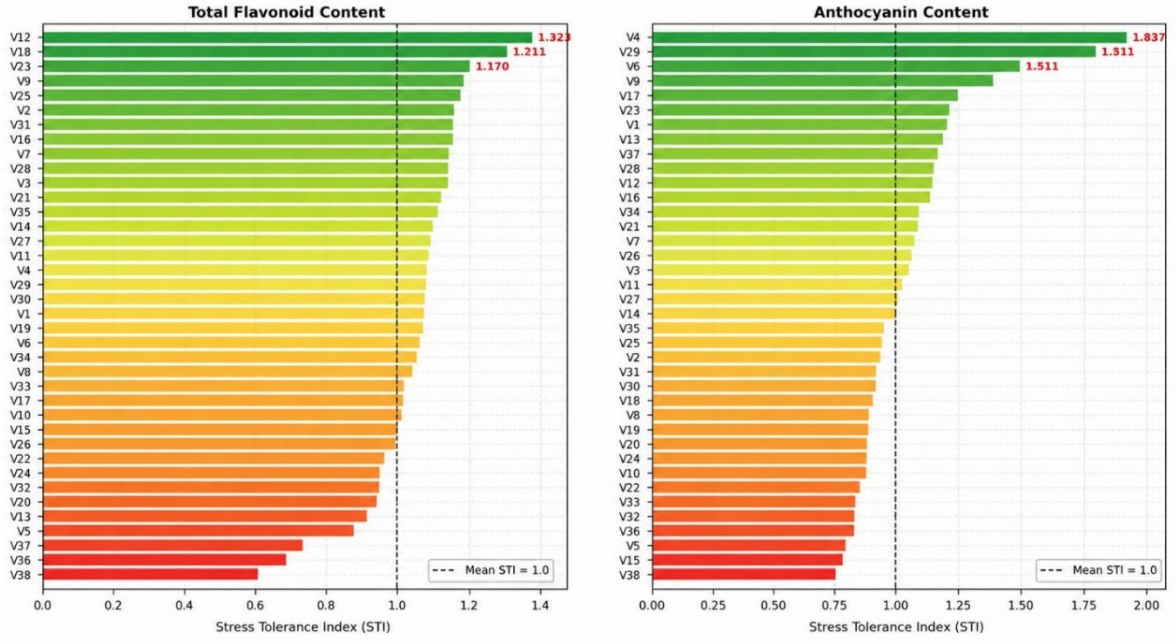

Figure 7. Full STI ranking of all 38 varieties for both traits. The vertical dashed line marks STI = 1.0 (population-mean tolerance); bars to the right indicate above-average tolerance.

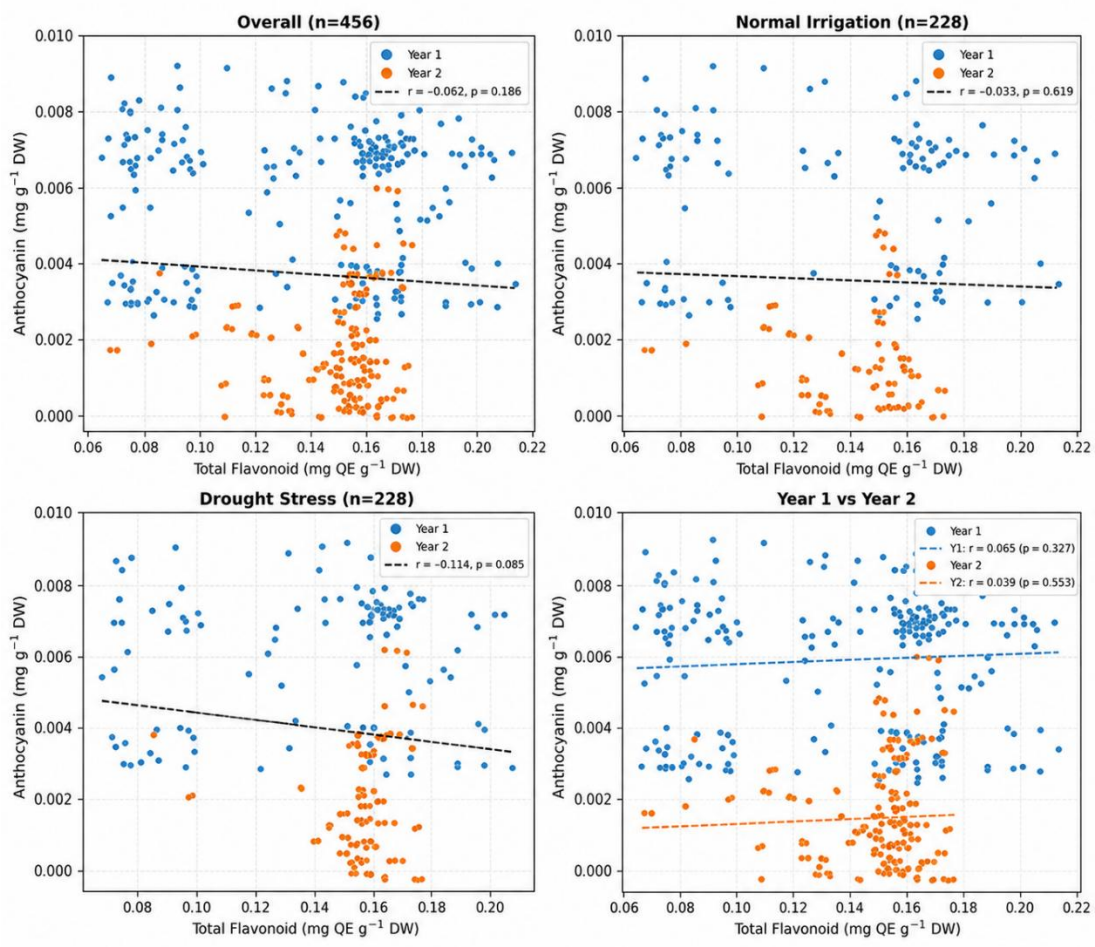

Figure 8. Scatter plots of total flavonoid versus anthocyanin content overall, by irrigation regime, and by year. Black dashed lines show linear fits; Pearson correlation statistics are reported in each panel

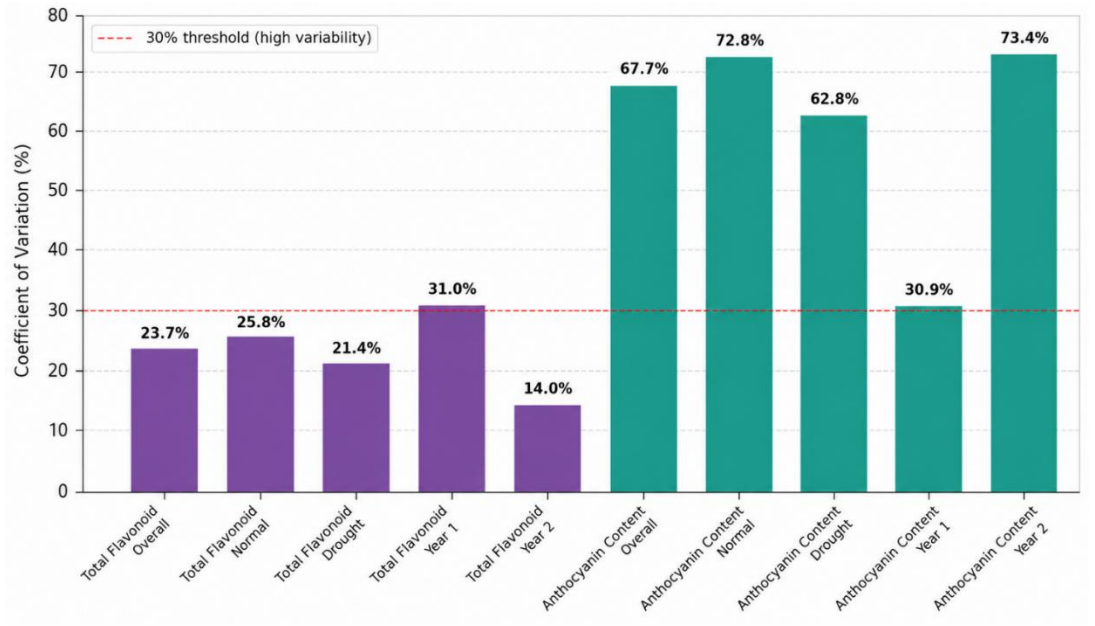

Figure 9. Coefficient of variation (CV%) for both traits across experimental groups. The red dashed line at 30% marks a common threshold above which trait variability is considered high.

Table 1. Three-way factorial ANOVA for total flavonoid content (Type II SS).

Source of Variation	df	sum of squares	F-value	p-value	% Contribution	Sig.
Year (Y)	1	0.008687	6.003	0.0148	1.50%	*
Irrigation (I)	1	0.031118	6.558	0.0109	1.64%	*
Variety (V)	37	0.004036	0.635	0.9526	5.86%	ns
Y × I	1	0.030310	3.047	0.0819	0.76%	.
Y × V	37	0.021136	0.618	0.9612	5.71%	ns
I × V	37	0.025226	0.431	0.9986	3.98%	ns
Y × I × V	37	0.402678	0.515	0.9919	4.75%	ns
Residual	304	0.008687	—	—	75.81%	—
ns: not significant, *: significant at $p < 0.05$, significant at $p < 0.1$						

For anthocyanin content, the ANOVA (Table 2) showed a significant Year main effect ($F = 822.277$, $p < 0.001$), accounting for 62.8% of total variance, and a significant Irrigation main effect ($F = 4.145$, $p = 0.0426$), accounting for 0.32% of variance. The Irrigation × Variety interaction was not significant at $\alpha = 0.05$ ($F = 1.374$, $p = 0.0797$). The Variety main effect ($p = 0.496$) and remaining interactions were not significant. Residual variance accounted for 23.2% of the total sum of squares.

Table 2. Three-way factorial ANOVA for anthocyanin content (Type II SS).

Source of Variation	df	sum of squares	F-value	p-value	% Contribution	Sig.
Year (Y)	1	0.002068	822.277	<0.001	62.76%	***
Irrigation (I)	1	1.04e-5	4.145	0.0426	0.32%	*
Variety (V)	37	9.18e-5	0.986	0.4964	2.79%	ns
Y × I	1	7.41e-6	2.948	0.0870	0.23%	.
Y × V	37	1.21e-4	1.301	0.1212	3.68%	ns
I × V	37	1.28e-4	1.374	0.0797	3.88%	.
Y × I × V	37	1.04e-4	1.115	0.3040	3.15%	ns
Residual	304	7.64e-4	—	—	23.20%	—
ns: not significant, significant at $p < 0.1$, *: significant at $p < 0.05$, ***: significant at $p < 0.001$						

For flavonoid content, mean values by year showed a larger drought-associated increase relative to normal irrigation in Year 1 than in Year 2 (Figure 6; Year × Irrigation interaction, $p = 0.0819$). For anthocyanin content, the corresponding Year × Irrigation interaction was $p = 0.087$, with higher normal-irrigation anthocyanin levels in Year 1 than in Year 2 (Figure 6).

Discussion

Drought-induced activation of secondary metabolism

A central finding of this study is the modest but consistent increase in both flavonoid and anthocyanin content under drought stress. This pattern aligns with the well-established role of flavonoids and anthocyanins as ROS scavengers and photoprotectants that accumulate in plant tissues in response to abiotic stress (Bao et al., 2024; Zhou et al., 2023). The 6.3% increase in flavonoid content and 8.1% increase in anthocyanin content under drought, while modest in absolute magnitude, are biologically meaningful because they represent population-level

responses averaged across 38 genotypes and two years, and are consistent with drought-induced up-regulation of the phenylpropanoid pathway reported for other species (Fidel et al., 2022; Mehravi et al., 2025), in which flavonoids and anthocyanins function as ROS scavengers and photoprotectants. Comparable stress-induced increases in total flavonoid and anthocyanin content have been reported under water-deficit conditions in other economically important species, including anise (Mehravi et al., 2025) and purple-leaf oilseed rape (Chen et al., 2022), in which the constitutively higher-anthocyanin genotype also displayed lower ROS accumulation and better membrane integrity under drought, supporting a direct protective function for these pigments rather than a purely correlative association with stress exposure.

The lower coefficient of variation for flavonoid content under drought relative to normal irrigation (21.4% vs. 25.8%) suggests a degree of convergence in genotypic response toward a common stress-induced phenotype. Similarly, the marked narrowing of flavonoid CV between years (31.0% in Year 1 to 14.0% in Year 2) suggests that Year 2 environmental conditions were more favourable or more uniform, constraining the expression of genotypic variation in that season.

The substantial inter-annual variation observed here, particularly the 69.7% reduction in anthocyanin content between Year 1 and Year 2, and the dominant contribution of Year to total ANOVA variance for anthocyanin (62.8%), indicate that anthocyanin accumulation in safflower petals is governed primarily by seasonal climatic drivers — most plausibly temperature, radiation, and precipitation timing — rather than by irrigation regime or genotype. This is an exceptionally large effect size for a single factor in a multi-factor field experiment, and it echoes conclusions drawn from other multi-year phenylpropanoid-pathway studies under field conditions (Mehravi et al., 2025) as well as from a two-year rain-fed trial of high-oleic safflower genotypes in Sicily, in which year, rather than genotype, was the dominant source of variation in several productive traits (Licata et al., 2023).

The marginal Year × Irrigation interaction observed for both traits ($p = 0.0819$ for flavonoid; $p = 0.087$ for anthocyanin) suggests that the magnitude of the drought response differed between years — more pronounced in Year 1 for both traits — consistent with reports that seasonal climate can modulate the magnitude of stress-induced phenylpropanoid responses independently of the imposed water treatment (Licata et al., 2023). Together, these findings indicate that the biochemical impact of drought on flavonoid and anthocyanin accumulation cannot be fully interpreted without reference to the prevailing environmental conditions of each growing season.

Genotypic differentiation versus residual variance

The non-significant Variety main effect in the ANOVA ($p = 0.953$ for flavonoid, $p = 0.496$ for anthocyanin) appears to contradict the substantial variation observed among variety means (Section 3.3), and indicates that the 38 genotypes responded similarly to irrigation treatment at the population level. This apparent paradox is explained by the high residual variance (75.8% for flavonoid, 23.2% for anthocyanin), which reflects substantial within-treatment variation rather than an absence of underlying biological variation among genotypes, and is likely driven by plot-level micro-environmental heterogeneity, biological replication noise, and possibly temporal variation in sampling within each plot, including factors such as floret position or diurnal sampling effects that were not explicitly modelled.

Independence of the flavonoid and anthocyanin branches

The weak and non-significant correlations between flavonoid and anthocyanin content across all subgroups (overall $r = -0.062$, $p = 0.187$) indicate that these two classes of phenolic compounds respond largely independently to environmental and genetic factors in safflower petals. While both compound classes share the phenylpropanoid precursor pathway, their downstream branches are regulated by distinct enzymatic cascades and, in several species, by partially non-overlapping

transcription-factor networks (Li and Ahammed, 2023). Functional evidence from a safflower flavonol synthase gene, CtFLS1, which drives flavonol and anthocyanin accumulation when expressed heterologously (Ma et al., 2024), demonstrates that individual branch-point genes can be manipulated to enhance one pathway output without necessarily co-regulating the other, reinforcing the biological plausibility of the observed independence. This finding implies that selection for one trait will not indirectly improve the other, and that multi-trait selection indices or marker-assisted approaches targeting both branches simultaneously are required for their combined improvement in breeding programmes.

Implications for breeding programme design

The drought-tolerance indices converge on a small number of elite candidates. Variety 31 is the most drought-tolerant genotype for flavonoid content, having actually increased flavonoid content under drought (a 5.8% gain), supporting the interpretation that flavonoid biosynthesis in this and other top-ranked genotypes (Varieties 32, 22, and 14, which also showed drought-induced gains) is up-regulated rather than suppressed by water deficit. Variety 14 is the most tolerant genotype for anthocyanin content, maintaining high anthocyanin content under both irrigation regimes (only a 6.2% reduction under drought) and indicating broad stress stability, and is the only genotype ranking in the top five for both traits. Variety 5 displayed the largest drought-induced gain in the panel for anthocyanin content (55.9%). The high STI values (> 1.2) of the top-five varieties for each trait indicate strong candidacy for breeding programmes targeting flavonoid and anthocyanin stability under water scarcity. These varieties are recommended as priority parents in crosses aimed at improving the drought resilience of safflower phytochemical profiles, in line with the broader recommendation that STI-based multi-index screening is the most effective way to identify genotypes combining productivity and tolerance under both irrigation regimes (Golkar et al., 2021; Shojaei et al., 2022; Mutari et al., 2023).

The marginally non-significant Irrigation × Variety interaction for anthocyanin ($p = 0.0797$) provides weak evidence for genotypic differences in drought responsiveness for this trait, and suggests that such differences could be exploited in targeted selection once sample sizes are increased. The dominant Year effect for anthocyanin (62.8% of variance) further implies that multi-year evaluation, rather than single-season screening, is essential to obtain robust estimates of genotypic value for this environmentally sensitive trait, a caution that applies broadly to phenylpropanoid-derived quality traits in field-grown oilseed and aromatic crops (Licata et al., 2023; Mehravi et al., 2025).

More broadly, these results support the growing case for incorporating antioxidant phytochemical traits, and not only yield, into safflower drought-tolerance screening pipelines. Given the pharmaceutical and nutraceutical value attached to safflower flavonoid content (Cheng et al., 2024), genotypes that couple agronomic drought tolerance with stable or enhanced flavonoid and anthocyanin accumulation, such as Variety 14 in the present panel, offer a route to breeding cultivars with dual value under the water-limited conditions that are expected to intensify across semi-arid production regions in the coming decades (He and Rosa, 2023).

Trait variability and environmental sensitivity

The coefficient of variation (CV) provides a dimensionless measure of relative dispersion and is widely used in plant breeding to assess trait stability. The narrowing of flavonoid CV under drought (21.4% vs. 25.8% under normal irrigation) and in Year 2 (14.0% vs. 31.0% in Year 1) suggests that both environmental stress and favourable growing seasons constrain genotypic expression of this trait, albeit through different mechanisms. Anthocyanin content displayed substantially higher CV values across all groups, peaking at 73.4% in Year 2 — a counterintuitive finding given that Year 2 also had the lower mean anthocyanin content — indicating that the very low anthocyanin values observed in Year 2 were accompanied by disproportionately high relative dispersion. These patterns reinforce the conclusion that

anthocyanin is a less stable and more environmentally sensitive trait than total flavonoid content in safflower, consistent with the dominant Year effect identified in the ANOVA (Section 3.4).

Conclusion

This study quantified the effects of irrigation regime, genotype, and year on two pharmaceutically important phytochemical traits in a 38-genotype safflower panel evaluated over two field seasons. The principal conclusions are: (i) drought stress significantly up-regulated both total flavonoid and anthocyanin content, supporting the role of these compounds in stress defence; (ii) the year effect was dominant for anthocyanin, contributing nearly 63% of total variance, while irrigation and genotype played secondary roles; (iii) varietal differences were not statistically significant at the population level owing to high residual variance, but a subset of varieties — notably Variety 31 for flavonoid and Variety 14 for anthocyanin — consistently exhibited superior drought tolerance across multiple indices, with Variety 14 the only genotype ranking in the top five for both traits; (iv) flavonoid and anthocyanin contents were uncorrelated, indicating independent genetic and biochemical regulation of the two phenylpropanoid branches; and (v) the assumptions of normality and variance homogeneity required for parametric ANOVA were violated, warranting cautious interpretation and suggesting that non-parametric or robust alternatives (e.g., aligned-rank-transform ANOVA, permutation tests) should be considered in confirmatory analyses.

For breeding programme deployment, we recommend: (a) prioritising Variety 14 as a multi-trait, drought-tolerant parent given its top-five ranking for both flavonoid and anthocyanin content; (b) incorporating Variety 31 into crosses targeting flavonoid enhancement under drought; (c) conducting multi-year, multi-environment trials with increased replication and spatially adjusted designs to improve statistical power for detecting genotypic differences; (d) employing multi-trait selection indices that explicitly account for the independence of the flavonoid and anthocyanin pathways; and (e) extending the analytical framework to include meteorological covariates (cumulative growing-degree days, total evapotranspiration) that may explain the dominant year effect on anthocyanin accumulation. Together, these findings provide a phytochemically informed complement to conventional yield-based drought-tolerance screening in safflower and identify concrete genotypic targets for breeding programmes operating in water-limited production environments.

Conflict of Interest

All authors declared that they have no conflict of interest

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