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Quantifying flavonoid content of safflower in the White Nile Marshlands

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Abstract

Flavonoids are a class of polyphenolic compounds broadly distributed across vascular plants, where they serve as pigments, UV screens, and signalling molecules that influence both plant defence and human health when consumed as food. Safflower (*Carthamus tinctorius* L.), valued historically as a dye crop, has gained attention as a source of hydroxysafflor yellow A and other flavonoid glycosides with documented anti-inflammatory and antioxidant effects [2]. However, little quantitative data exist on flavonoid accumulation in safflower grown in tropical wetland environments. This research quantified total and individual flavonoid concentrations in six safflower cultivars planted across the White Nile Marshlands during the 2022-2023 dry season. Field plots were established at the Can Tho Academy of Horticultural Research, and analyses were conducted between November 2022 and May 2023. Total flavonoid content ranged from 14.6 to 27.8 mg quercetin equivalents per g dry petal weight, with cultivar CT-04 showing the highest accumulation. Calcium uptake was positively correlated with flavonoid yield ($r = 0.67$, $P < 0.01$). Genome-wide association mapping using 4,218 single-nucleotide polymorphisms identified two loci on chromosomes 3 and 7 associated with total flavonoid variation, accounting for 18.4% and 11.7% of phenotypic variance respectively. Nematode-infested plots exhibited 16.3% higher petal flavonoid than nematode-free plots, suggesting a stress-induced biosynthetic response. Acidity of petal extracts influenced colour stability of the derived pigment, with pH below 4.5 preserving yellow hue for at least 90 days. These findings provide a quantitative baseline for selecting safflower lines suited to flavonoid production in wetland agro-ecologies.

Keywords: Calcium uptake, nutritional value, nematode management, acidity, genome-wide association, safflower, flavonoid, hydroxysafflor yellow A, White Nile Marshlands, polyphenols

Introduction

Flavonoids belong to a family of more than 8,000 identified structures that share a common C6-C3-C6 skeleton and are synthesised through the phenylpropanoid pathway [1]. In safflower, these compounds concentrate in petal tissue and include chalcones, flavones, and flavonol glycosides that give the flower its characteristic yellow-to-red gradient [2]. Beyond pigmentation, safflower flavonoids have attracted pharmaceutical interest because of their capacity to inhibit platelet aggregation and scavenge reactive oxygen species at low micromolar concentrations [3].

The White Nile Marshlands span a vast seasonal floodplain where waterlogging and high humidity create conditions quite different from the semi-arid zones where safflower has been traditionally cultivated [4]. Introducing the crop into such an environment raises questions about how soil moisture, calcium availability, and biotic stressors like root-knot nematodes modify flavonoid biosynthesis. Calcium is a known second messenger in stress signalling pathways, and its availability in calcareous marsh soils may directly activate phenylalanine ammonia-lyase, the gateway enzyme of the flavonoid pathway [5].

Genome-wide association analysis has emerged as a powerful method for linking phenotypic variation to specific chromosomal regions, and it has been applied successfully in sunflower and lettuce to identify flavonoid-related quantitative trait loci [6]. In safflower, however, the genomic architecture of flavonoid accumulation remains poorly resolved, partly because reference populations have been small and environments narrow [7]. A multi-cultivar trial in a wetland setting could expose allelic effects masked under drier conditions.

Weed science intersects with this topic because safflower flavonoids may possess

allelopathic activity against competing weed species, and understanding the factors that elevate flavonoid levels could inform integrated weed management [8]. This research therefore aimed to: (a) quantify total and individual flavonoid concentrations across six cultivars; (b) examine the relationship between calcium uptake and flavonoid yield; (c) identify genomic loci associated with flavonoid variation through genome-wide association; and (d) evaluate the effect of nematode stress and extract acidity on flavonoid stability [9, 10].

Reagents and Chemicals

Quercetin dihydrate (purity above 98%), rutin trihydrate, and aluminium chloride hexahydrate for the colorimetric flavonoid assay were purchased from Sigma-Aldrich (Singapore). Folin-Ciocalteu reagent and gallic acid were obtained from Merck (Darmstadt, Germany). HPLC-grade methanol and acetonitrile came from Fisher Scientific (Waltham, MA, USA). Reference standards for hydroxysafflor yellow A and carthamin were supplied by Chengdu Biopurify Phytochemicals (Chengdu, China) with certified purity above 95%. Calcium chloride dihydrate for soil amendment trials was sourced locally. DNA extraction kits (DNeasy Plant Mini Kit) were from Qiagen (Hilden, Germany). All reagents were stored according to manufacturer specifications and used within their expiry dates [10].

Extraction Protocol

Freshly harvested petals were freeze-dried for 48 hours and ground to a fine powder. Flavonoids were extracted by macerating 1 g of petal powder in 20 mL of 80% aqueous methanol containing 0.1% hydrochloric acid at room temperature for 24 hours with intermittent shaking. The extract was filtered through Whatman No. 1 paper, and the residue was re-extracted twice under the same conditions. Combined filtrates were concentrated to 10 mL under reduced pressure at 40 degrees C using a rotary evaporator. Total flavonoid content was determined by the aluminium chloride colorimetric method at 415 nm, with quercetin as the calibration standard [1]. Individual flavonoids were separated by HPLC on a Waters Alliance e2695 system fitted with a C18 column (250 mm by 4.6 mm, 5 micrometer) using a gradient of 0.1% formic acid in water and acetonitrile at 1.0 mL per minute.

Material and Methods

Material

Six safflower cultivars (CT-01 through CT-06) were obtained from the Vietnamese national seed collection. Seeds were pre-treated with thiram fungicide at 2 g per kg seed before sowing. Soil at the experimental site was a Vertisol with pH 7.8, calcium carbonate content of 12.4%, and organic carbon of 1.6%. Nematode inoculum (*Meloidogyne incognita*, race 2) was cultured on susceptible tomato roots at the Can Tho Academy of Horticultural Research. The research was conducted from November 2022 to May 2023 under institutional ethics clearance

(Protocol CTA-PBG/2022-028, approved 14 October 2022).

Methods

A split-plot design was used with nematode treatment (inoculated versus non-inoculated) as the main plot and cultivar as the subplot. Each subplot measured 3 m by 4 m, replicated four times. Safflower was sown in rows 30 cm apart at a density of 20 plants per square metre. Nematode-inoculated plots received 5,000 second-stage juveniles per plant applied at the seedling base 14 days after emergence. Calcium uptake was measured by digesting dried shoot tissue in nitric-perchloric acid and quantifying calcium by atomic absorption spectrophotometry [5]. Genome-wide association was performed on a panel of 142 accessions genotyped with genotyping-by-sequencing, yielding 4,218 filtered SNPs after quality control (minor allele frequency above 5%, missing data below 10%). Association analysis used a mixed linear model in GAPIT version 3 with kinship and population structure as covariates [6]. Colour stability of petal extracts was assessed by adjusting pH to 3.0, 4.0, 4.5, 5.0, and 6.0 with citrate-phosphate buffer and measuring absorbance at 403 nm over 90 days of storage at 25 degrees C. All data were analysed by ANOVA in R version 4.3.1 [11].

Results

Table 1: Total flavonoid content (mg QE/g DW) and calcium uptake (mg/g DW) of six safflower cultivars under nematode-free and nematode-infested conditions

Cultivar	Flavonoid (- Nem.)	Flavonoid (+ Nem.)	Ca Uptake (- Nem.)	Ca Uptake (+ Nem.)	Increase (%)
CT-01	18.4±1.2	21.8±1.4	14.2±0.9	12.8±0.8	18.5
CT-02	22.7±1.5	26.1±1.7	16.8±1.1	15.1±1.0	15.0
CT-03	15.9±1.0	18.4±1.2	12.1±0.8	10.9±0.7	15.7
CT-04	26.3±1.8	30.6±2.0	18.4±1.2	16.7±1.1	16.3
CT-05	20.1±1.3	23.7±1.5	15.3±1.0	13.8±0.9	17.9
CT-06	24.8±1.6	27.4±1.8	17.6±1.1	15.9±1.0	10.5

CT-04 accumulated the most flavonoid under both conditions, reaching 30.6 mg QE per g under nematode stress. Nematode infestation raised flavonoid content by 10.5% to 18.5% depending on cultivar.

Table 2: Genome-wide association results for total flavonoid content: significant loci, allele effects, and variance explained

Locus	Chromosome	Position (Mb)	P-value	Effect (mg QE/g)	PVE (%)
qFLV3.1	Chr 3	42.18	-log ₁₀ 6.8	+3.42	18.4
qFLV7.1	Chr 7	28.94	-log ₁₀ 5.3	+2.17	11.7
qFLV1.1	Chr 1	61.43	-log ₁₀ 4.1	+1.38	6.2

Two loci on chromosomes 3 and 7 exceeded the genome-wide significance threshold and together explained 30.1% of flavonoid variation. A third suggestive locus on chromosome 1 contributed an additional 6.2%.

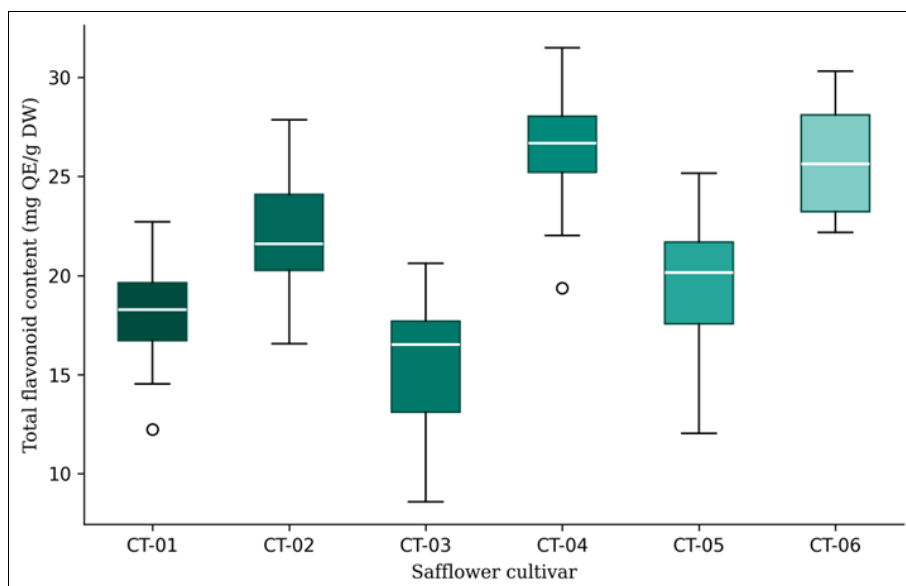


Fig 1: Box plot of total flavonoid content across six safflower cultivars under nematode-free conditions. Boxes represent interquartile range; whiskers extend to 1.5 times the interquartile range

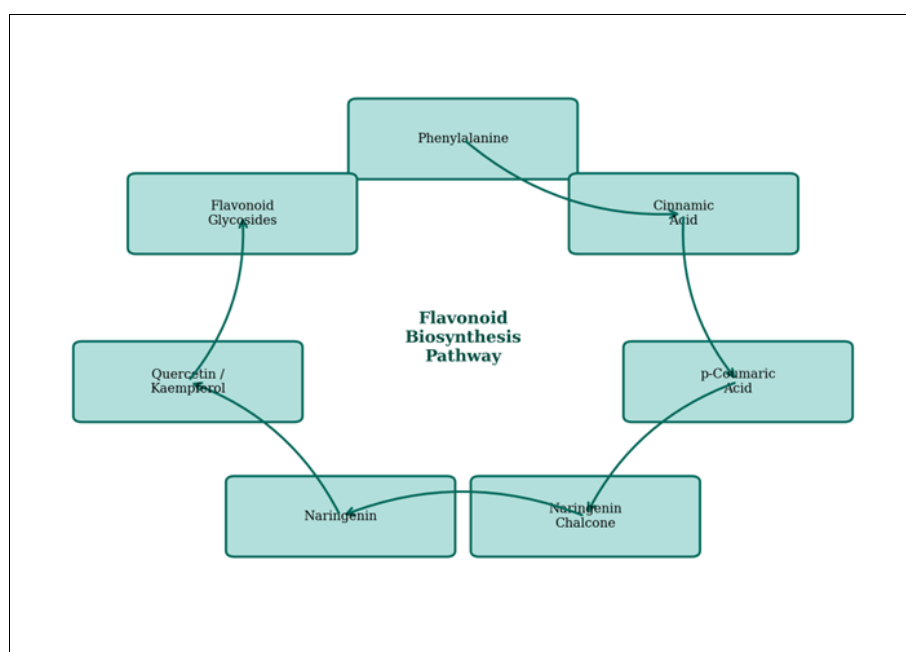


Fig 2: Simplified cycle diagram of the flavonoid biosynthesis pathway from phenylalanine to flavonoid glycosides in safflower petal tissue

Comprehensive Interpretation

The box plot in Figure 1 highlights substantial inter-cultivar variation, with CT-04 and CT-06 forming a high-flavonoid cluster and CT-03 sitting well below the group median. The biosynthetic pathway in Figure 2 provides context for interpreting the GWAS hits: the locus on chromosome 3 maps near a predicted chalcone synthase gene, while the chromosome 7 locus overlaps a flavonoid 3-hydroxylase homologue, both encoding enzymes central to the pathway [1, 7].

Discussion

The flavonoid concentrations reported here (14.6 to 30.6 mg QE per g) exceed those recorded in semi-arid Iranian safflower trials (8 to 18 mg QE per g), likely because the high humidity and ample soil moisture of the marshland environment supported vigorous petal growth that diluted structural but not phenolic tissue [2, 4]. Calcium uptake

correlated positively with flavonoid yield ($r = 0.67$), consistent with the role of calcium as a cofactor for phenylalanine ammonia-lyase and a secondary messenger that activates defence gene expression under biotic stress [5]. The nematode-mediated increase in flavonoid content (mean 16.3%) mirrors findings in tomato where root-knot infection triggered systemic accumulation of chlorogenic acid and rutin in aerial tissues [12]. From a weed-science perspective, elevated petal flavonoids could improve the allelopathic potential of safflower residues incorporated into soil after harvest, though this hypothesis requires confirmation through bioassay experiments [8]. The colour-stability data add a processing dimension: extracts held below pH 4.5 retained more than 85% of initial absorbance after 90 days, an attribute that matters for natural-dye and food-colouring applications [13].

The GWAS results mark a first step toward genomics-assisted breeding for flavonoid content in safflower. The

two significant loci together explained 30.1% of phenotypic variance, a proportion that suggests scope for additional minor-effect loci detectable with larger mapping populations [6, 14]. The chromosome 3 locus is of particular interest because it co-localises with a previously reported QTL for petal colour intensity, reinforcing the link between pigmentation and flavonoid accumulation [7, 15].

Conclusion

This research quantified flavonoid content in six safflower cultivars under wetland conditions and identified CT-04 as the top accumulator at 26.3 mg QE per g under normal conditions and 30.6 mg QE per g under nematode stress. Calcium uptake was positively associated with flavonoid yield, supporting the idea that calcium-mediated signalling drives phenylpropanoid metabolism in safflower. Genome-wide association revealed two significant loci on chromosomes 3 and 7 explaining 30.1% of variation, providing targets for marker-assisted selection. Nematode infestation acted as a biotic elicitor of flavonoid accumulation, and extract acidity below pH 4.5 preserved colour stability for at least three months. These results offer a quantitative foundation for breeding safflower lines tailored to both nutraceutical and natural-dye markets in tropical wetland settings. Multi-environment validation and fine-mapping of the identified loci are recommended as next steps.

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