RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
57 (1) 56-66, 2025
http://doi.org/10.54910/sabrao2025.57.1.6
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

GENES EXPRESSION AND BIOCHEMICAL COMPOUNDS RESPONSE TO FERTILIZERS IN ROSELLE (*HIBISCUS SABDARIFFA* L.)

M.M. AL-BAIK* and A.A. ALAMERY

Department of Field Crops, College of Agriculture, University of Kerbala, Kerbala, Iraq
*Corresponding author's emails: sabraoassociateeditors@gmail.com, memealnageb17@gmail.com
Email address of co-author: abas.hussian@uokerbala.edu.iq

SUMMARY

A field experiment undertaking in spring 2022 assessed the impact of seven NPK fertilizer combinations on the gene expression and biochemical compounds of two Roselle (*Hibiscus sabdariffa* L.) cultivars, held at the Kerbala Governorate, Iraq. The randomized complete block design (RCBD) layout had two factors and three replications. The first factor included NPK fertilizer combinations, while the second factor was the two Roselle cultivars (Iraqi-1 and Iraqi-2). The results showed cultivar Iraqi-2 contains missense mutations in codons 23 and 61 (CCA → CTA and ATT → AGT, respectively), which encode for amino acids (Pro → Leu and Ile → Ser, respectively) in the *CHS* gene. Missense mutations were also evident in codons 80 and 100 (TTG → TTC and CCA → TCA, respectively), encoding amino acids (Leu → Phe and Pro → Ser, respectively) in the *F3H* gene of the same cultivar. Cultivar Iraqi-1 proved superior in vitamin C, quercetin, hibiscetin, and gossypetin, with average values of 37.87, 0.387, 0.386, and 0.186 mg g^{-1}, respectively. In fertilizer combinations, combination 6 excelled in all the above-mentioned traits, with averages of 44.42, 0.410, 0.451, and 0.250 mg g^{-1}, respectively. The interaction between the factors was significant for all the traits under study.

Keywords: Roselle (*H. sabdariffa* L.), NPK fertilizer combinations, cultivars, gene expression, biochemical compounds

Key findings: The NPK combination (175:150:150 kg ha^{-1}) excelled in almost all the characteristics of Roselle (*H. sabdariffa* L.). The cultivar Iraqi-1 of Roselle was significantly superior for the studied characteristics. Therefore, before application, it is crucial to determine the balance among the macronutrients.

Communicating Editor: Dr. A.N. Farhood

Manuscript received: December 29, 2023; Accepted: May 05, 2024.

Citation: Al-Baik MM, Alamery AA (2025). Genes expression and biochemical compounds response to fertilizers in Roselle (*Hibiscus sabdariffa* L.). *SABRAO J. Breed. Genet.* 57(1): 56-66. http://doi.org/10.54910/sabrao2025.57.1.6.

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a medicinal plant, belonging to the family Malvaceae. It is an annual and perennial herbaceous plant reaching a height of 2.5–3 m. It is considerably one of the most important basic sources of active substances used in manufacturing medicines. Therefore, it occupies a great position in the agricultural and industrial fields (Rabo *et al*., 2015). It is a belief that the original home of the Roselle may be the tropical and subtropical regions of the African continent. However, it can also be prevalent in regions with a warm climate, i.e., India, the Kingdom of Saudi Arabia, Malaysia, Thailand, Vietnam, the Philippines, Mali, Mexico, and Egypt, with these countries also considered its original home (Tounkara *et al.,* 2011).

In Iraq, Roselle cultivation began in the 20th century, with an expanding cultivation in the Sunniyah District, Diwaniyah Governorate, Southern Iraq, due to the great demand for its calyx leaves, used as a refreshing juice during hot summers. It also has the best medicinal values in its ability to lower the blood pressure for people suffering from hypertension. Therefore, Agricultural Research Stations have been trying to expand its cultivation in Central and Southern Iraq (Al-Sarraf, 1991).

The leaves of the calyx are a source of glycosides, which are plant organic compounds decomposed by acids and by enzymes into one or more active non-sugary substances, referring to the physiological and medicinal effects of the plant (Villalobos-Vega *et al.,* 2023). Moreover, the calyx leaves, considered as the most important part of the plant, contain active substances, where these active substances contain large quantities of carbohydrates, proteins, ash, fats, fibers, water, carotene, vitamin C, thiamine, riboflavin, ascorbic acid, and niacin. Roselle also contains mineral elements, such as, phosphorus, manganese, iron, calcium, and sodium (Balarabe*,* 2019).

Fertilization with nutrients is considerably one of the crucial and essential nutrient provider in plants, giving necessary elements to encourage the physiological and biochemical processes and growth and development of crop plants **(**Alamery *et al.,* 2019; Lateef *et al.,* 2019). Feeding the Roselle plant with various nutrients, especially nitrogen, phosphorus, and potassium, has a major role in increasing plant growth and the active substance, such as, hepsin glyceride and anthocyanin (Fahmy and Hassan, 2019).

Iraq has calcareous soils and suffers from a decrease in the macronutrients (Alafeea *et al.,* 2019). The process of balancing nutrients is critical in obtaining a higher efficiency of the absorption process, which also play a primary role in plant growth and development (Alaarage and Alamery, 2023). Therefore, the presented study sought to determine the best combination of NPK fertilizers and its effect on the active substance of Roselle, as well as, knowing the performance of two cultivars of Roselle and their bioactive substances.

MATERIALS AND METHODS

A field experiment, undertaken in spring 2022, helped assess the impact of seven NPK fertilizer combinations on the gene expression and biochemical compounds of two Roselle (*Hibiscus sabdariffa* L.) cultivars, held at the Kerbala Governorate, Iraq. The randomized complete block design (RCBD) used had two factors and three replications. The first factor included seven NPK fertilizer combinations — C0 = 0 NPK kg ha^{-1}, C1 = 50:25:25 NPK kg ha^{-1}, C2 = 75:50:50 kg NPK ha^{-1}, C3 = 100:75:75 kg NPK ha^{-1}, C4 = 125:100:100 kg NPK ha^{-1}, C5 = 150:125:125 kg NPK ha^{-1}, and C6 = 175:150:150 kg NPK ha^{-1}. The second factor included two local varieties of hibiscus, i.e., cultivar Iraqi-1 (Red) and cultivar Iraqi-2 (White). Adopting reference varieties registered in the NCBI received designations as V1 and V2 for the purpose of comparison with the local varieties. The phosphorus and potassium addition ensued in two batches, one before planting, and the second batch combined with the second batch of nitrogen fertilizer, according to the transactions. Nitrogen fertilizers' application continued in two batches—first, after germination, and the second dose, before the plants reach the flowering stage.

RNA extraction

Five selected Roselle plants came from each experimental unit in each NPK combination and each replication. The aim of the selection was to verify the molecular identification of the *F3H* and *CHS* genes responsible for manufacturing the active substances in the Roselle cultivar plants. This proceeded in the laboratory at the Department of Field Crop Sciences, College of Agriculture, University of Kerbala, Kerbala, Iraq. A sample taken from each plant bore labels for RNA extraction at the beginning of the control results, with the control results monitored.

In Roselle plants, using mRNA sequencing primers for the *CHS* and *F3H* genes had the kit prepared by the American company, Zymo (Table 1). Performing the reverse transcription polymerase chain reaction (RT-PCR) for RNA samples continued on extracted leaves of both Roselle cultivars, using the Add Script RT-PCR SYBR Master Kit 2X, final volume. The sample gained supplementation with distilled water to 25 µl. The reaction mixture preparation ensued in a sterile tube (one tube for each gene, with a tube free of negative control DNA). Its components reached mixing using a micropipette, then placed in a centrifuge to maintain the final volume of the reaction mixture, and set in the instantaneous thermal polymerase device. Implementation of the program used the *CHS* and *F3H* genes (Table 2).

Reverse transcription polymerase chain reaction (RT-PCR)

The identification of *F3H* and *CHS* genes for the production of enzymes responsible for the synthesis of active substances in hibiscus reached detection using specialized primers (designed by the researchers using NCBI) (Table 2). The reaction mixture preparation continued in a sterile tube (one for each genotype, with a DNA-free tube, negative control), with its components mixed using a micropipette. The reaction mixture contains 10 µL of Taq PCR Premix, 1 µL of primer, and 1 µL of primer. For the reverse transcribe, the target gene had 5 µl of DNA and 8 µl of distilled water added, then placed in a centrifuge to preserve the final volume (25 µl) of the reaction mixture. Then, placing in a PCR machine, performing the reaction for amplification. In determining the diameters of the PCR products and the DNA ladder marker, employed electrophoresis after combining 1 g of agarose and dissolving in 100 g ml of TBE (1X), with the mixture heated until it reached boiling point.

Table 1. Specific primers for identifying *CH3* and *F3H* genes used in reverse transcription polymerase chain reaction (RT-PCR).

Gene name	Initiator code	Relay	Tm ℃	GC%	bp
CHS	CHS	AGAAATCCGCAAGGCACAAC '3 F5'	59.9	55	811
			59.5	50	
F3H	F3H	CTTCGGGAATCCGGTACTCT '3 F5'	59.5	50	888
			59.4	55	

Table 2. PCR conditions' program for amplification of the *CHS* and *F3H* genes.

Stage	Temperature (°C)	Time	Number of courses
Initial Denaturation	95	3 min	1
Denaturation-2	95	45 s	
Annealing	59	45 s	
Extension-1	72	2 min	35
Extension-2	72	7 min	1

By lowering the temperature to between 40 °C and 50 °C, adding 2 microliters of safe red dye followed. Meanwhile, 3 µl of PCR products reached combining with 5 µl of loading buffer. After preparing the gel pouring tray and positioning the comb to create wells within the agarose gel layer, pour the melted agarose into the prepared tray and allow it to solidify at room temperature. After the agarose had completely solidified, the comb needs careful removing without causing any distortion or breaking in any of the wells. Then, returning the tray to its correct place in the electrophoresis apparatus, pouring the TBE into the electrophoresis chamber continued until immersing the agarose layer to a height of approximately 1 mm. Finally, the PCR products' injection into each well of the agarose gel ensued, with a 5-µl (1 kb) ladder marked on the wells on the left side of the additional samples to determine the size of the PCR products more precisely. Afterward, turning on the power supply at 120 mA for an hour and a half. After electrophoresis, lifting the agarose layer underwent placement on a UV transilluminator.

Estimating the active ingredient content

Preparing a solution of the calyx leaves of the Roselle plant proceeded by taking 1 g of dry leaf powder, ground using an electric grinder, and adding (100) ml of Acetonitrile to it. Afterward, placing the solution in the ultrasound machine for 30 min at a temperature of 35 °C. Then, the solution's filtration used a filter paper, with the filtrate placed in the rotary evaporator at a temperature of 35 °C until dry. Then, adding 1 ml of Acetonitrile, the solution incurred filtering using a micro-filter with a size of 0.45 millimeter microns. Afterward, putting the solution in tightly sealed glass containers for making estimates of the materials later, as measured in the HPLC device (Kelly *et al.*, 1995).

Determination of active compounds

After completing the process of preparing the solution and preparing the samples with contents for estimation, a High-Performance Liquid Chromatography (HPLC) device served to estimate and diagnose the active compounds in the calyx of the Roselle plant. Injecting the 25 ml of the sample continued into the device manufactured by the Japanese company, Shimadzu (LC-10A). Separating the compounds and determining their type compared with the standard materials occurred on the separation column under the same conditions (Table 2) and with the same concentration of the separated materials in the examined sample using a special equation. The active materials' estimation transpired at the Department of Environmental and Water Research, Ministry of Science and Technology, Iraq. The calculation of concentration of the compounds was according to the following equation.

$$\text{Sample concentration} = \text{Standard Con.x}\frac{\text{Area of the sample package examined}}{\text{Standard form package space}}\text{x Number of dilution times}$$

Concentration of vitamin C

The concentration of vitamin C's measurement in the calyx leaves of the two Roselle cultivars followed the method of Kapur *et al.* (2012).

Preparation of solutions

Ammonium molybdate preparation comprised dissolving five grams of it in 100 ml of distilled water. Taking the oxalate acid (0.05M): 6.3 g of (Oxalic acid) with 5.845 grams of EDTA (0.02M) had the volume added to 100 ml of distilled water. Sulfuric acid (5%): 5 ml of sulfuric acid proceeded with the volume completed to 100 ml of distilled water. A mixture of glacial acetic acid and metaphosphoric acid, prepared by dissolving 80 ml of acetic acid with 30 ml of metaphosphoric acid, then, bringing the volume to 500 ml of distilled water.

Digestion of plant samples

Taking one gram of the calyx leaves of the two Roselle cultivars reached placing in a 25 ml

beaker, with 10 ml of oxalate acid ($C_2H_2O_4$) added to it. The samples remained in the shade for 24 hours, and afterward, retaining the filtrate after filtering it through filter paper.

Estimation of vitamin C

The 2.5 ml of the digested plant sample sustained mixing with 2.5 ml of oxalic acid solution, 0.5 ml of a mixture of metaphosphoric acid and glacial acetic acid, and 1 ml of ammonium molybdate solution. Increasing the volume to 25 ml, used a distilled water, with the samples read using a spectrophotometer at a wavelength of 760 nm.

Standard curve

Attaining the relationship between the concentration of vitamin C and the absorbance values for each concentration continued, with the standard curve drawn by taking concentrations of 0.5, 1, 1.5, 2, 2.5, and 3 ml L^{-1} of ascorbic acid.

RESULTS AND DISCUSSION

Diagnosis of genetic mutations in *CHS* gene

The results showed the presence of mutations in the studied Roselle (*Hibiscus sabdariffa* L.) cultivars (Table 3). It was evident that cultivar V2 contains a silent mutation in codons 23, 61, and 80 (CCA → CCC, ATT → ATC, and TGT → TGC, respectively) that encodes for amino acids (Pro, Ile, and Cys, respectively). Detecting silent mutations also resulted in the cultivar Iraqi-1 in codons 80, 85, and 99 (TGC → TGT, CCC → CCT, and TCA → TCC, respectively), encoding the amino acids (Cys, Pro, and Ser, respectively). As for the Roselle cultivar Iraqi-2, the diagnosis of missense mutations appeared in codons 23 and 61 (CCA → CTA and ATT → AGT, respectively), which encode for amino acids (Pro → Leu and Ile → Ser, respectively) (Figure 1).

***F3H* gene mutation diagnosis**

The outcomes revealed the presence of genetic mutations in the *F3H* gene in the studied Roselle cultivars (Table 4). It was apparent that cultivar V2 contains a silent mutation in codon 62 (GCG → GCA) that encodes the amino acid (Ala). Silent mutations also occurred in the cultivar Iraqi-1 in codons 25, 70, and 119 (GTG → GTA, GAG → GAA, and CTA → TTA, respectively), which encode amino acids (Val, Glu, and Leu). As for the Roselle cultivar Iraqi-2, silent mutations manifested in codons 62, 82, and 85 (GCG → GCA, AAG → AAA, and GTG → GTT, respectively), encoding amino acids (Ala, Lys, and Val, respectively). Other missense mutations diagnosed included codons 80 and 100 (TTG → TTC and CCA → TCA, respectively) that encoded amino acids (Leu → Phe and Pro → Ser, respectively) (Figure 2).

Table 3. Diagnosis of genetic mutations in the *CHS* gene for the two cultivars of Roselle under study.

Position	23		61		80		85		99	
	Codon	Amino Acid	Codon	Amino Acid	Amino Acid	Codon	Amino Acid	Codon	Amino Acid	Codon
KJ559430.1: (V1)	CCA	Pro	ATT	Ile		TGT	Pro	CCT	Ser	TCC
KJ605650.1: (V2)	CCC	Pro Silent mutation	ATC	Ile Silent mutation	Cys Silent mutation	TGC	Pro	CCT	Ser	TCC
Hibiscus: Cultivar Iraqi-1	CCA	Pro	ATT	Ile	Cys Silent mutation	TGC	Pro Silent mutation	CCC	Ser Silent mutation	TCA
Hibiscus: Cultivar Iraqi-2	CTA	Leu Missense mutation	AGT	Ser Missense mutation	Cys	TGC	Pro	CCT	Ser	TCC

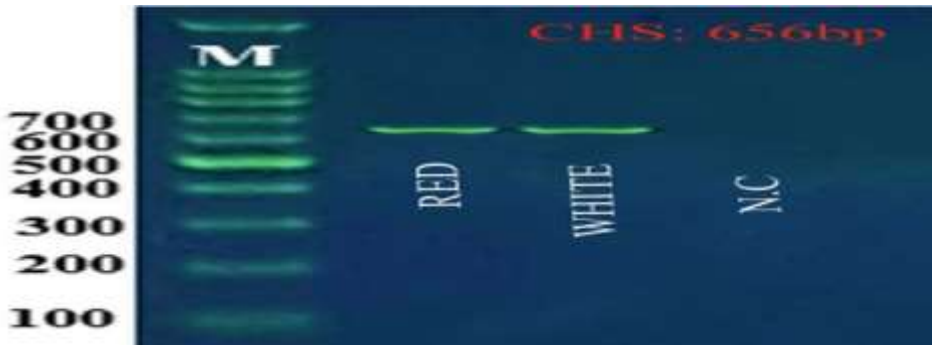

Figure 1. Migration of PCR products for *CHS* gene amplification products for the two cultivars of Roselle plant with the comparison treatment (N.C) in addition to the DNA ladder attached to the left side of the figure.

Table 4. Diagnosis of genetic mutations in the *F3H* gene for the two Roselle cultivars under study.

Position	25		62		70		80	
	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid
KJ559430.1: (V1)	GTG	Val	GCG	Ala	GAG	Glu	TTG	Leu
KJ605650.1: (V2)	GTG	Val	GCA	Ala Silent mutation	GAG	Glu	TTG	Leu
Hibiscus: Cultivar Iraqi-1	GTA	Val Silent mutation	GCG	Ala	GAA	Glu Silent mutation	TTG	Leu
Hibiscus: Cultivar Iraqi-2	GTG	Val	GCA	Ala Silent mutation	GAG	Glu	TTC	Phe Missense mutation
Position	82		85		100		119	
	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid
KJ559430.1: (V1)	AAG	Lys	GTG	Val	CCA	Pro	CTA	Leu
KJ605650.1: (V2)	AAG	Lys	CTG	Leu	CCA	Pro	CTA	Leu
Hibiscus: cultivar Iraqi-1	AAG	Lys	GTG	Val	CCA	Pro	TTA	Leu Silent mutation
Hibiscus: cultivar Iraqi-2	AAA	Lys Silent mutation	GTT	Val Silent mutation	TCA	Ser Missense mutation	CTA	Leu

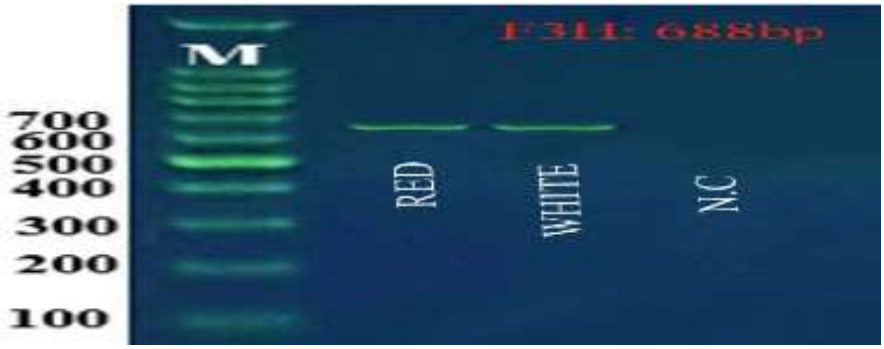

Figure 2. Migration of PCR products for *F3H* gene amplification products for the two cultivars of Roselle plant with the comparison treatment (N.C) in addition to the DNA ladder attached to the left side of the figure.

Table 5. Effect of NPK combinations on the vitamin C content in calyx leaves of two Roselle cultivars.

NPK Combinations	Cultivars		Means (mg g^{-1})
	Iraqi-1	Iraqi-2	
C0	26.67	25.04	25.85
C1	29.28	26.04	27.66
C2	34.30	29.47	31.88
C3	37.30	35.47	36.38
C4	42.99	39.67	41.33
C5	47.60	34.70	41.15
C6	46.99	41.86	44.42
Means (mg g^{-1})	37.87	33.17	
$LSD_{0.05}$ Cultivars: 2.546, NPK Combinations: 4.763, C x NPK Combinations: 6.736			

Vitamin C concentration

The results enunciated a significant difference between the two cultivars of Roselle (Table 5). The cultivar Iraqi-1 was superior in the vitamin C content of the calyx leaves, with an average of 37.87 mg g^{-1}, while the cultivar Iraqi-2 showed an average of 33.17 mg g^{-1}, with a percentage increase of 2.35%. The said trait proved genetically managed, and therefore, it varies according to the nature of the genetic makeup of the *H. sabdriffa* cultivars (Bamishaiye *et al.,* 2011). The observed genetic variations emerged in the sequences of the genes under study. This could explain the superiority of the cultivar Iraqi-1 for active substances, including vitamin C, due to the absence of genetic mutations in the sequence of the cultivar itself (Tables 3 and 4).

The findings further revealed the NPK fertilizer combinations caused a significant effect on the vitamin C content in the calyx leaves of the Roselle cultivars. The NPK combination C6 gave the highest average content of the sepal leaves of vitamin C, amounting to 44.42 mg g^{-1}. However, it did not differ significantly from the average of the combinations C5 and C4, which amounted to 41.15 and 41. 33 mg g^{-1}, respectively, with an increase of 7.47%, compared to the control treatment, showing an average of 25.85 mg g^{-1}. The NPK combinations C1, C2, and C3 provided the averages of 27.66, 31.88, and 36.38 mg g^{-1}, respectively.

The superiority of NPK combination C6 may be due to the elements it contains, which were essential for the vegetative growth of Roselle plants. Thus, the efficiency of the carbon assimilation process works to form various compounds, such as, amino acids, sugars, and secondary metabolites, in addition to vitamins (Fernández *et al.,* 2013). The results also disclosed a substantial interaction between the studied cultivars and the NPK fertilizer combinations. The maximum interaction effects were evident in the cultivar Iraqi-1 with C5 NPK combination (47.60 mg g^{-1}). However, it did not differ significantly from C4 and C6 for the same cultivar.

Quercetin content

The results exhibited a notable difference between the cultivars Iraqi-1 and Iraqi-2 of the Roselle (Table 6). The cultivar Iraqi-1 was superior in the content of calyx leaves, with an average of 0.387 mg g^{-1}, while the cultivar Iraqi-2 gave an average of 0.356 mg g^{-1}, with an increase rate of 8.70%. In cultivar Iraqi-2, the decrease in active substances (quercetin) may be due to the inhibition of the action of enzymes responsible for the synthesis of active substances, as a result of genetic variations in the *F3H* and *CHS* genes (Tables 3, 4). The findings further signified the NPK fertilizer combinations caused a remarkable effect on the calyx leaves' content of quercetin. The NPK combination C6 gave the topmost average content of quercetin (0.410 mg g^{-1}). But, it did not differ significantly from the average of NPK combination C5 (0.398 mg g^{-1}), by an increase of 3.01%, compared with the control treatment (0.331 mg g^{-1}).

The NPK combinations C1, C2, C3, and C4 gave quercetin averages of 0.334, 0.360, 0.379, and 0.388 mg g^{-1}, respectively. The reason for the increase could refer to the effectiveness of fertilizer combinations, which

Table 6. Effect of NPK combinations on quercetin content in calyx leaves of two Roselle cultivars.

NPK Combinations	Cultivars		Means (mg g^{-1})
	Iraqi-1	Iraqi-2	
C0	0.339	0.324	0.331
C1	0.368	0.301	0.334
C2	0.369	0.352	0.360
C3	0.400	0.358	0.379
C4	0.406	0.371	0.388
C5	0.414	0.382	0.398
C6	0.415	0.405	0.410
Means (mg g^{-1})	0.387	0.356	
$LSD_{0.05}$ Cultivars: 0.006, NPK Combinations: 0.012, C x NPK Combinations: 0.017			

Table 7. Effect of NPK combinations on hibiscetin content in calyx leaves of two Roselle cultivars.

NPK Combinations	Cultivars		Means (mg g^{-1})
	Iraqi-1	Iraqi-2	
C0	0.287	0.259	0.273
C1	0.300	0.273	0.286
C2	0.336	0.305	0.320
C3	0.392	0.322	0.357
C4	0.429	0.338	0.383
C5	0.473	0.379	0.426
C6	0.490	0.412	0.451
Means (mg g^{-1})	0.386	0.326	
$LSD_{0.05}$ Cultivars: 0.010, NPK Combinations: 0.019, C x NPK Combinations: 0.026			

lead to an increase in the leaf area because they contain more nitrogen (Hapsari and Setyaningsih, 2021). Thus, the leaves' increased interception of sunlight which helps the efficiency of the carbon assimilation process caused many compounds' production in the calyx leaves, containing active substances, including quercetin in the Roselle plants (Karim and Ihsan, 2015).

The results also showed a noteworthy interaction between the Roselle cultivars and NPK fertilizer combinations. The highest quercetin content resulted in the interaction of cultivar Iraqi-1 with C6 combination, with an average of 0.415 mg g^{-1}. However, it did not differ significantly from the average of the NPK combinations C3, C4, C5, and C6 with the Roselle cultivar Iraqi-2. The interaction with the least value of quercetin was the Roselle cultivar Iraqi-2 with NPK combination C2, and the average amounted to 0.301 mg g^{-1}.

Hibiscetin content

For hibiscetin content, a significant difference existed between the cultivars Iraqi-1 and Iraqi-2 of the Roselle (Table 7). The cultivar Iraqi-1 was superior in the hibiscetin content of the calyx leaves, with an average of 0.386 mg g^{-1}, while the cultivar Iraqi-2 gave an average of 0.326 mg g^{-1}, with an increase rate of 18.40%. The occurrence of genetic mutations in the sequence of the cultivar Iraqi-2 compared with the cultivar Iraqi-1, identified in codon 23 (CCA → CTA), encoded the amino acid (Leu → Pro). Likewise, in codon 61 (ATT → AGT), which encodes the amino acid (Ser → Ile), has worked, in one way or another, to reduce the gene expression of the *F3H* and *CHS* genes responsible for the synthesis of active substances, including hibiscetin. On the contrary, no influential mutations were evident in the sequence of the cultivar Iraqi-1, and this explains its superiority in its content of active substances (Tables 3 and 4).

The findings in the same table showed the NPK fertilizer combinations caused a considerable effect on the calyx leaves' hibiscetin content. The NPK combination C6 displayed the highest average of the hibiscetin content (0.451 mg g^{-1}) compared with the control treatment (0.273 mg g^{-1}). The NPK combinations C1, C2, C3, C4, and C5 gave hibiscetin content averages of 0.286, 0.320,

0.357, 0.383, and 0.426 mg g^{-1}, respectively. The reason for the increase in the hibiscetin content may be due to the fertilizer combinations containing crucial elements for plant growth, which worked to increase plant cell growth. This, in turn, influenced some physiological processes, cell division, and the effectiveness of the carbon assimilation process, thus, increasing metabolic products, including hibiscetin. Al-Mohammad *et al.* (2021) mentioned nitrogen fertilizer has a major role in enhancing plant growth and the concentrations of active substances, such as hepsin glycoside, flavonoids, and other active compounds.

The outcomes further disclosed for hibiscetin content, a significant interaction between the Roselle cultivars and the NPK fertilizer combinations. The highest hibiscetin content resulted in the interaction of NPK combination C6 and cultivar Iraqi-1, with an average of 0.490 mg g^{-1}. However, no notable difference occurred from the average for the C5 combination with the same cultivar. The lowest hibiscetin content appeared in the interference of Roselle cultivar Iraqi-2 with the control treatment (0.259 mg g^{-1}).

Gossypetin content

For gossypetin content, a pronounced difference appeared between the two cultivars of the Roselle plant (Table 8). The cultivar Iraqi-1 was superior in calyx leaves' content of gossypetin content, with an average of 0.186 mg g^{-1}. Meanwhile, the cultivar Iraqi-2 gave an average of 0.175 mg g^{-1}, with an increase rate of 0.186 mg g^{-1} (6.28%). Genetic mutations may have a negative or positive influence, and the genes responsible for the synthesis of enzymes were also necessary for the synthesis of active substances, including gossypetin content. It was evident that the Roselle cultivar Iraqi-2 contains several missense mutations in codons 23 and 61 (CCA → CCC and ATT→ ATC in sequence). These encode for the amino acids Pro→ Leu and ATT → AGT, respectively, which could have caused the decrease in the content of active substances, while detecting silent mutations appeared in the sequence of cultivar Iraqi-1 in codons 25, 62, 70, 80, 85, and 99 (Tables 3 and 4). The type of genetic mutations does not affect the nature of the synthesis of enzymes responsible (F3H and CHS) for the synthesis of active substances, which explains the superiority of the Roselle cultivar Iraqi-1 in containing active substances.

The NPK fertilizer combinations caused a significant effect on the gossypetin content, and the NPK combination C6 showed the highest average of gossypetin (0.250 mg g^{-1}) compared with the control treatment (0.088 mg g^{-1}). The NPK combinations C1, C2, C3, 4C, and C5 averages for gossypetin content reached 0.115, 0.154, 0.187, 0.234, and 0.236 mg g^{-1}, respectively. The reason for the increase was due to fertilizer combinations, referring to the fact that NPK fertilizers contain vital elements for plant growth. It enhanced the efficiency of physiological processes and cell division, and the effectiveness of the carbon metabolism process, thus, increasing metabolic products (Al-Mohammad *et al.*, 2021; Khedr *et al*., 2024; Panjaitan *et al*., 2024).

Table 8. Effect of NPK combinations on gossypetin content in calyx leaves of two Roselle cultivars.

NPK Combinations	Cultivars		Means (mg g^{-1})
	Iraqi-1	Iraqi-2	
C0	0.082	0.094	0.088
C1	0.097	0.133	0.115
C2	0.166	0.142	0.154
C3	0.210	0.165	0.187
C4	0.232	0.236	0.234
C5	0.254	0.219	0.236
C6	0.263	0.238	0.250
Means (mg g^{-1})	0.186	0.175	
$LSD_{0.05}$ Cultivars: 0.003, NPK Combinations: 0.006, C x NPK Combinations: 0.008			

The results of the same table indicated a significant interaction effects between the studied cultivars and NPK fertilizer combinations for gossypetin content. The interaction of the Roselle cultivar Iraqi-1 with NPK combination C6 revealed the highest average of gossypetin content (0.263 mg g^{-1}). Meanwhile, the lowest interference emerged in the cultivar Iraqi-2 with the control treatment, with an average of 0.082 mg g^{-1} for the cultivar Iraqi-1.

CONCLUSIONS

A difference was evident in the sequences of the Roselle cultivars Iraqi-1 and Iraqi-2 from the sequences of cultivar V2 under study. It was also notable that the missense mutations in the cultivar Iraqi-2 had a negative impact on the gene expression rate of *CHS* and *F3H*, responsible for synthesizing active substances, thus, reducing the content of active substances in this cultivar. Remarkably also, the NPK combinations C6 and C5 were superior in qualitative characteristics, such as, the content of the sepal leaves for active substances, i.e., vitamin C, quercetin, hibiscetin, and gossypetin.

REFERENCES

Alaarage SS, Alamery AA (2023). Role of bio-azotobacter and nitrogen fertilizers on growth and yield traits of sorghum (*Sorghum bicolor* L.). *SABRAO J. Breed. Genet.* 55(4): 1311-1320.

Alafeea RAA, Alamery AA, Kalaf IT (2019). Effect of bio fertilizers on increasing the efficiency of using chemical fertilizers on the yield component of maize (*Zea mays* L.). *Plant Arch*. 19(2): 303-306.

Alamery AA, Lateef SM, Almosawy AN, Alhassaany MH, Almosawy MM (2019). Effect of Phosphate Bio fertilizers on increasing the efficiency of the use of phosphate mineral fertilizers and its effect on some growth properties of broccoli (*Brassica oleracea* var. *italica*). *Biochem. Cell Arch*. 19: 27-51.

Al-Mohammad MH, Sachet TF, Al-dulaimi ZS (2021). Effect of phenylalanine, jasmonic acid and biofertilizer on growth, yield and anthocyanin pigments of roselle calyces. *IOP Conf. Ser. Earth Environ. Sci*. 910(1): 77-120.

Al-Sarraf AHM (1991). Guidance Bulletin on Gujarat Agriculture. General Authority for Agricultural Services. Agricultural Extension Department, Baghdad, Iraq, pp. 326.

Balarabe MA (2019). Nutritional analysis of *Hibiscus sabdariffa* L. (Roselle) leaves and calyces. *Plant J.* 7(4): 62-65.

Bamishaiye EI, Olayemi FF, Bamishaiye OM (2011). Effects of boiling time on mineral and vitamin C content of three varieties of *Hibiscus sabdariffa* drink in Nigeria. *World J. Agric. Sci.* 7(1): 62-67.

Fahmy AA, Hassan HMS (2019). Influence of different NPK fertilization levels and humic acid rates on growth, yield and chemical constituents of roselle (*Hibiscus sabdariffa* L.). *Middle East J. Agric. Res*. 8(4): 1182-1189.

Fernández V, Sotiropoulos T, Brown PH (2013). Foliar Fertilization: Scientific Principles and Field Practices. International Fertilizer Industry Association (IFA). Paris, France. pp. 141.

Hapsari BW, Setyaningsih W (2021). Methodologies in the analysis of phenolic compounds in roselle (*Hibiscus sabdariffa* L.): Composition, biological activity, and beneficial effects on human health. *Horticulturae* 7(2):31-35.

Kapur A, Hasković A, Čopra-Janićijević A, Klepo L, Topčagić A, Tahirović I, Sofić E (2012). Spectrophotometric analysis of total ascorbic acid content in various fruits and vegetables. *Bull. Chem. Technol. Bosnia and Herzegovina* 38(4): 39-42.

Karim HG, Ihsan SA (2015). Effect of varieties and spraying with compound fertilizer (NPK) on the growth yield and content of some active substances in kujarat (*Hibiscus sabdariffa* L.). *Al-Furat J. Agric. Sci*. 7(4): 30-37.

Kelly KL, Kimball BA, Johnston JJ (1995). Quantitation of digitoxin, digoxin and their metabolites by high- performance liquid chromatography using pulsed amperometric detection. *J. Chromatogr*. 711: 289-295.

Khedr NM, Ibrahim AA, El-Metwally M, Eldakroory S, Soliman MI (2024). Phytochemical analysis, antioxidant activity, antimicrobial

evaluation, and cytotoxicity effects of wild medicinal plants. *SABRAO J. Breed. Genet.* 56(4): 1552-1562. http://doi.org/10.54910/sabrao2024.56.4.21.

Lateef SM, Alamery AA, Alhassaany MH, Almosawy AN, Almosawy MM (2019). Role of Bio Fertilizers and Phosphate levels on some growth and yield properties of broccoli (*Brassica oleracea* var. *italica*). *Plant Arch.* 19(2): 1564-1568.

Panjaitan RGP, Gunadi AT, Titin, Raharjeng ARP (2024). Inventory of traditional medicinal plants in Kubu Raya Regency, Indonesia. *SABRAO J. Breed. Genet.* 56(5): 1970-1981. http://doi.org/10.54910/sabrao2024.56.5.20.

Rabo SA, Mustapha BS, Simon AB, Hamma IL (2015). Influence of NP fertilizer on the performance of Roselle (*Hibiscus sabdariffa* L.) insamaru Zaria. *Niger. J. Agric. Food Environ.* 11(3): 61-64.

Tounkara F, Amadou I, Wei Le G, Hui hi Y (2011). Effect of boiling on the physicochemical properties of Roselle seeds (*Hibiscus sabdariffa* L.) cultivated in Mali. *Afr. J. Biot.* 10(79): 18160-18166.

Villalobos-Vega MJ, Rodríguez-Rodríguez G, Armijo-Montes O, Jiménez-Bonilla P, Álvarez-Valverde V (2023). Optimization of the extraction of antioxidant compounds from Roselle *Hibiscus* Calyxes (*Hibiscus sabdariffa* L.), as a source of nutraceutical beverages. *Molecules* 28(6): 2-13.