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Original Article

Evaluation of Phytochemical and Biochemical Attributes of Saffron (*Crocus sativus* L.): Effects of Cultivation Environment

Saeed Hasanian-Badi[1], Javad Tabatabaeian Nimavard[2*] and Benyamin Torabi[3]

[1] *Department of Agronomy, Agricultural Sciences, Islamic Azad University (IAU), Ardestan Branch, Ardestan, Iran*
[2] *Department of Agricultural Sciences, Islamic Azad University (IAU), Ardestan Branch, Ardestan, Iran*
[3] *Department of Agronomy, Faculty of Agriculture, Gorgan University of Agricultural Sciences and Natural Resources, Golestan, Iran*

Corresponding author: Email: taba805@gmail.com

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ABSTRACT

Evaluating quantitative and qualitative variations in saffron across different regions, focusing on physiological, biochemical, and stigma-related traits, is vital for identifying the optimal cultivation areas. This work aimed to investigate the quantitative, qualitative, and biochemical characteristics of saffron grown at five research locations across Iran (Torogh Rood: RL-1, Natanz: RL-2, Kashan: RL-3, Qaenat: RL-4, and Badrood: RL-5) over two growing seasons. The results revealed that RL-2 and RL-4 significantly outperformed the yield and quality of other regions. Maximum flower count and dried stigma yields were recorded in RL-4, with 79.32 and 99.76 flowers/m² and 0.934 and 1.179 g/m² in the first and second years, respectively. Furthermore, RL-4 exhibited the highest concentrations of safranal (18.0 and 16.8 $E^{1\%}_{1\ cm}$), picrocrocin (110.9 and 133.2 $E^{1\%}_{1\ cm}$), and crocin (264.0 and 306.5 $E^{1\%}_{1\ cm}$) during the first and second years, respectively. Significant variations were observed across the regions in chlorophyll pigments, proline levels, total phenolic content, and antioxidant enzyme activity. A positive and significant relationship between yield, quality, and secondary metabolite content. Conversely, an important negative correlation was observed between the output and antioxidant enzyme activity levels. Higher levels of clay, silt, iron, and magnesium had a positive influence on the yield, quality, and secondary metabolite content. In contrast, elevated temperatures, sand content, and zinc levels were associated with decreased yield and quality. This study identifies RL-2 and RL-4 as optimal cultivation regions for saffron, attributed to their enhanced yield, superior quality, and elevated concentrations of key bioactive compounds, including safranal, picrocrocin, and crocin.

Keywords: Chlorophyll pigment, secondary metabolite, soil element content, stigma yields

ITRODUCTION

Crocus sativus L., commonly known as saffron and a perennial of the Iridaceae family, has a prominent position as one of the world's most expensive spices and a valuable medicinal plant [1]. Iran and several other countries, including India, Spain, Afghanistan, Morocco, and Greece, are major saffron-producing nations [2]. Iran is a global leader in saffron cultivation, contributing over 90% of the world's total production [3]. Stigma, a part of the saffron flower, is an economically valuable component and is renowned as one of the world's most expensive spices [4]. Several factors, including the stigma count per flower, the floral count per plant, harvesting efficiency, flower size, stigma length, and the compositional amounts of picrocrocin, safranal, and crocin significantly influence saffron yield and quality [5, 6]. Quality in saffron is largely dependent on the presence and concentration of secondary metabolites and their derivatives [7]. The reputation of saffron as a premium spice is attributed to three major organic compounds [2]. Crocin imparts a characteristic yellow color, picrocrocin contributes to bitter taste, and safranal is responsible for its distinct aroma [8]. Beyond their sensory attributes, these compounds exhibit significant antioxidant and anti-inflammatory properties, which play a vital role in the therapeutic benefits associated with saffron [9].

The differences among saffron ecotypes adapted to various regions and environmental conditions are influenced by multiple factors such as climate, soil, altitude, and cultivation history [10]. These differences are observed in traits such as flowering time, growth duration, stigma color, aroma and flavor, yield, and resistance to pests and diseases [2, 11]. The careful identification and selection of appropriate ecotypes for each region can greatly enhance saffron yield and product quality while also contributing to the preservation of genetic diversity [11]. For instance, an ecotype adapted to an area with cold winters may exhibit better cold resistance than one cultivated in a region with mild winters [12, 13]. One study revealed significant differences in the fresh and dry weights of stigmas, peroxidase (POX) activity, and anthocyanin content in petals among different saffron ecotypes, specifically Natanz and Zanjan [14]. Since saffron is grown in multiple locations and its adaptation to specific climatic conditions, diverse ecotypes have emerged across various areas. Parameters such as stigma length and dry weight, stigma yield, flower count, and the mass of the developing corms are influenced by the geographical origin of the corms [15]. The performance of ecotypes varies by region, indicating an interaction between ecotypes and their environment [14]. Farrokhi *et al.* [2] informed that the Torbat-e-Heydarieh location, at an altitude of approximately 1323.3 MASL, achieved the highest saffron stigma and flower yield, and safranal content (15.8%), picrocrocin (30.6%), and crocin (69.3%) content. They attributed this superiority to the low maximum summer temperature in this area. This study aimed to examine the quantitative, qualitative, and biochemical changes in saffron samples cultivated across various research locations in Iran over two years while examining their correlation with climatic and soil variations.

MATERIAL AND METHODS

Research Locations and Plant Materials

Saffron cultivation (three-year-old corms) was conducted from 2021 to 2023 in Torogh-Rood, Natanz, Kashan, Badrood (Isfahan Province), and Qaenat (South Khorasan Province), Iran. Soil samples (0-30 cm depth) were collected from selected farms with similar agronomic practices and analyzed for texture and physicochemical properties (Tables 1 and 2). Corms (2.5–3.5 cm diameter, 10–15 g weight) were planted in August on raised beds (80 cm wide, 30 cm high), with three rows per bed, 10 cm planting depth, 20 cm between rows, and 5 or 6.7 cm spacing within rows. Fields were left fallow the previous year, with manual weed control applied during the experiment.

Table 1 Geographical and climatic information for the selected research locations for the experiment, Iran

Locations cods	City	Province	Latitude (N)	Longitude (E)	Altitude (m)	Average rainfall (mm/year)	Average temperature (°C)	Climate
RL-1	Toroqh Rood	Esfahan	33°26′	51°47′	~1,372	250	17.5	Moderate to cold, mountainous
RL-2	Natanz	Esfahan	33°31′	51°54′	~1,656	300	15.1	Cold to temperate, mountainous
RL-3	Kashan	Esfahan	33°58′	51°26′	~942	200	18.2	Hot desert climate
RL-4	Qaenat	South Khorasan	33°43′	59°11′	~1,413	300	14.2	Semi-arid, continental
RL-5	Badrood	Esfahan	33°42′	51°48′	~1,372	250	17.1	Semi-arid

Table 2 The basic physical and chemical features of soil in the selected research areas for the experiment (two-year average)

Characteristics	RL-1	RL-2	RL-3	RL-4	RL-5
Texture	Sandy Loam	Loam	Sandy Loam	Loam	Sandy Loam
Sand	69.2	33.1	72	50	40
Silt	18.3	40.3	14	30	40
Clay	12.5	25.4	14	20	20
pH	7.33	7.59	7.66	7.77	7.95
Electrical conductivity ($dS.m^{-1}$)	1.46	1.58	2.08	2.1	1.95
OM (%)	0.65	0.88	0.99	0.95	1.11
N (%)	1.97	1.74	1.86	1.96	1.99
P (ppm of soil)	8.94	9.02	9.88	10.1	10
K (ppm of soil)	310	307	328	308	322
Iron (ppm of soil)	1.66	1.84	1.99	2.95	2.35
Manganese (ppm of soil)	2.75	3.07	3.11	3.77	2.1
Copper (ppm of soil)	0.45	0.49	1.19	0.71	1.01
Zinc (ppm of soil)	0.66	0.57	1.04	0.57	0.75

RL1: Toroqh Rood; RL-2: Natanz; RL-3: Kashan; RL-4: Qaenat; RL-5: Badrood

Measurement of Traits Related to Stigma and Flower

Flowering commenced in early November and continued for approximately 20 days. After harvesting the flowers and stigmas, we measured several related traits, incorporating the count of flowers, the yield of fresh blooms, and the yield of dried stigmas. The measurements were performed using a high-precision balance [16].

Determination of Saffron Quality

The quality of saffron was assessed according to the ISO 3632-1 [17] standard, utilizing both the stigma and style. The samples were measured when they had previously been heated in an oven (Model M-55E, Fanazma-Gostar Company, Iran) at 55 °C for 45 min [2]. Subsequently, aqueous extracts of saffron were analyzed using a spectrophotometer (PerkinElmer, USA) to quantify picrocrocin (the flavoring agent), safranal (the aromatic component), and crocin (the primary coloring agent). This analysis aimed to evaluate the bitterness, flavor strength, and coloring power of saffron. Data are expressed as a proportion of dry mass and reported as the absorbance of a 1% aqueous solution of dehydrated saffron across wavelengths ranging from 200 to 700 nm. Absorbance values at specific wavelengths—257 nm for picrocrocin, 330 nm for safranal, and 440 nm for crocin—were recorded as the absorbance of the 1% aqueous solution at these wavelengths and calculated using the following formula [17, 33]:

$$E^{1\%}_{1\,cm}(\lambda\ \max) = \frac{D \times 10000}{M \times (100 - wMV)}$$

Biochemical and Physiological Analyses

To assess biochemical and physiological parameters, including photosynthetic pigments, proline, anthocyanins, total phenols, malondialdehyde (MDA), total flavonoids, and the activity of antioxidant enzymes, flower samples were collected in triplicate using the following methods:

Measurement of Pigments

The content of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid was quantified using the methodology described by Lichtenthaler and Wellburn [19]. For this purpose, 0.25 g of plant leaves were ground with 5 mL of 80% acetone. The resulting solution was analyzed using spectrophotometry at wavelengths of 663, 646, and 470 nm. Pigment concentrations were then calculated based on specific equations.

Amount of Free Proline

The level of free proline was determined using the method that was described by Bates *et al.* [20]. Leaf samples, specifically 0.5 g, were homogenized in 10 mL of a 3% sulfosalicylic acid solution. Following filtration of this homogenate, 2 mL of the resulting solution was combined with ninhydrin reagent and acetic acid. The mixture was subsequently incubated in a water bath maintained at 100 °C for one hour. Following this incubation period, the mixture was cooled using an ice bath. The upper phase of the solution was utilized in order to measure the proline concentration at a specific wavelength of 520 nm by adding 4 mL of toluene and stirring for a period of 15–20 seconds.

Quantity of Total Phenol

To determine the quantity of total phenol utilizing the Folin-Ciocalteu method, either frozen or fresh samples, specifically 0.5 g, were homogenized with 80% ethanol. The resulting mixture was then centrifuged at 10,000 rpm for 20 min, and the supernatant was subsequently transferred to a new Falcon tube. The extract (0.5 mL) was subsequently mixed with 7 mL of distilled water, followed by the addition of 5 mL of Folin-Ciocalteu reagent to the resulting solution. A volume of 4 mL of a 1 M sodium carbonate solution was added after 3 min, and the mixture was allowed to stand in the dark for 15 min. In the final step, the optical absorbance of the samples was measured at 765 nm through a spectrophotometer [21]. For the preparation of a standard curve, gallic acid was employed at concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 $mg.mL^{-1}$ (Fig. 1).

Measurement of Malondialdehyde (MDA)

This passage describes a laboratory procedure to measure malondialdehyde (MDA) levels in leaf tissue. It involves homogenizing the tissue, centrifuging the extract, mixing the supernatant with specific chemical solutions, heating and cooling the mixture, centrifuging again, and finally measuring the absorbance at two different wavelengths using a spectrophotometer. A correction is then applied by subtracting the non-specific absorbance. The method is attributed to Heath and Packer [22].

Measurement of Flavonoids

To assess the flavonoid levels in the plant sample, an extraction was conducted using 0.5 g of the sample and 10 milliliters of an 80% ethanol solution. The extract that resulted from this process was then centrifuged, and the supernatant was subsequently collected. Following this, a 0.5 mL portion of the extract was combined with 1.5 mL of methanol, 0.1 mL of a 10% aluminum chloride solution, 1 M potassium acetate, and 2.8 mL of distilled water. This combination was then incubated at room temperature for a duration of 30 min, after which the absorbance was measured at a wavelength of 415 nm using a spectrophotometer [23]. Quercetin was employed for the purpose of preparing the standard curve at concentrations of 0, 0.5, 1, 1.5, 2, 2.5, and 3 $\mu g\ mL^{-1}$ (Fig. 2).

Measurement of Catalase, Peroxidase, and Superoxide Dismutase Activities

Catalase (CAT) activity was measured according to Dhindsa *et al*. [24]. Fifty microliters of enzyme extract were mixed with 1 mL of 50 mM potassium phosphate buffer (pH 7) containing 15 mM hydrogen peroxide. Absorbance at 240 nm was recorded over 1 min. CAT activity was calculated by multiplying the absorbance difference by 1000, dividing by 39.2, and then multiplying by 2.

Peroxidase (POX) activity was measured following Chance and Maehly [25]. A reaction mixture containing 50 µL enzyme extract, 3 mL of 0.1 M potassium phosphate buffer, 50 µL of guaiacol, and 50 µL of 3% hydrogen peroxide was monitored for absorbance changes at 436 nm every 15 seconds for 3 minutes. POX activity was calculated by multiplying absorbance by 1000, dividing by 26.6, and then multiplying by 2.

Superoxide dismutase (SOD) activity was determined using the method of Beauchamp and Fridovich [26], based on SOD's inhibition of nitroblue tetrazolium (NBT) photoreduction. A reaction mixture containing 50 µL enzyme extract, 1 mL of 50 mM potassium phosphate buffer (pH 7.8), 75 µM NBT, 13 mM L-methionine, 100 mM EDTA, and 2 µM riboflavin (added just before use) was exposed to light for 15 minutes. Absorbance was measured at 560 nm. SOD activity was calculated by multiplying absorbance by 1000 and then by 2.

Data Analysis

Data normality was confirmed using Kolmogorov-Smirnov and Shapiro-Wilk tests. Statistical analysis was performed with SAS (v9.2) and Minitab (v19) software using a completely randomized block design with three replicates. Means were compared by LSD test at $p \leq 0.05$. Additionally, correlation coefficients, principal component analysis (PCA), and cluster analysis were conducted using the same software.

RESULTS

Quantitative and Qualitative Characteristics of Saffron

The analysis of the data revealed that the samples from the Natanz region (RL-2) and Qaenat (RL-4) exhibited superior quantitative and qualitative traits, including flower number, fresh flower yield, dry stigma yield, and safranal, picrocrocin, and crocin content, compared to other research locations. Over the two years of experimentation, the highest flower numbers recorded were 79.32 and 99.76 flowers m^{-2} RL-4. Additionally, the fresh flower yields were 72.1 and 102.5 g m^{-2}, while the dry stigma yields were 0.934 and 1.179 g m^{-2}. The safranal contents measured were 18.0 and 16.8 $E^{1\%}_{1\ cm}$, with picrocrocin levels at 110.9 and 133.2 $E^{1\%}_{1\ cm}$ and crocin levels at 264.0 and 306.5 $E^{1\%}_{1\ cm}$, respectively, for RL-4. In contrast, the lowest flower number in the first year was observed in RL-1 (62.3 flowers m^{-2}), while during the subsequent year, RL-3 recorded the lowest count (80.0 flowers m^{-2}). The lowest fresh flower yields in both years were associated with RL-1, with 55.7 g m^{-2} in the first year and 70.05 g m^{-2} during the subsequent year. Furthermore, RL-5 exhibited the lowest dry stigma yield in the first year and RL-1 during the subsequent year, with values of 0.692 and 0.865 g m^{-2}, respectively. Moreover, RL-5 consistently showed the lowest safranal and picrocrocin content across both years, while the lowest average crocin content was recorded for RL-1 (Table 3).

Level of Photosynthetic Pigments

The results demonstrated remarkable differences in traits associated with the levels of photosynthetic pigments, specifically chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, among various research locations. Mean comparisons revealed that the highest levels of chlorophyll a (18.89 and 22.01 µg g^{-1} FW), chlorophyll b (11.28 and 10.96 µg g^{-1} FW), and total chlorophyll (30.17 and 32.97 µg g^{-1} FW) were observed in the RL-4 samples during both years of the study. Furthermore, RL-2 exhibited the highest average values for all traits, except chlorophyll b in the second year, across both experimental years. With respect to the quantities of chlorophyll a, b, and total chlorophyll, RL-5 samples recorded the lowest averages in both years, with values of 14.36, 17.38, 8.32, 8.12, 22.68, and 22.51 µg g^{-1} FW, respectively (Table 4).

Regarding the carotenoid level, RL-2 consistently had the highest average in both years (6.22 and 6.1 µg g^{-1} FW). Differently, RL-5 exhibited the lowest average in the first year, while RL-1 had the lowest average in the second year, with values of 4.67 and 2.83 µg g^{-1} FW, respectively (Table 4).

Proline Level

The study's outcomes pointed to marked differences in the average proline amounts detected in samples from various geographical locations. RL-5 samples exhibited the highest average proline levels in both years of the experiment, measuring 1.59 and 1.72 µmol g^{-1} FW. RL-4 samples showed the highest average for this trait, with a value of 1.71 µmol g^{-1} FW in the first year. Conversely, the least proline level was observed in RL-3 during both years, with average values of 0.56 and 1.29 µmol g^{-1} FW, respectively (Table 4).

Total Phenol Level

The results revealed marked differences in total phenolic levels. Specifically, RL-3 and RL-4 showed the highest average phenol content in both years (2.61, 2.69, 2.49, and 2.7 mg GA g^{-1} FW, respectively). Conversely, RL-5 in the first year and RL-1 and RL-2 during the subsequent year had the least average phenol content (1.24, 2.14, and 2.11 mg GA g^{-1} FW, respectively) (Table 4).

Malondialdehyde Content

Data analysis revealed that the MDA content did not differ significantly among samples from different research locations in the first year, but significant differences were observed during the subsequent year. In the second year, RL-1 had the maximum MDA content (3.15 nmol mL^{-1}), while RL-5 samples had the lowest average MDA content (1.7 nmol mL^{-1}) (Table 5).

Flavonoid Content

Flavonoid content varied significantly among samples in the first year, whereas no significant differences were observed between samples from different research locations during the subsequent year. In the first year, RL2 and RL4 had the highest average flavonoid content (27.46 and 31.49 mg QE g^{-1} FW, respectively). In contrast, the other three research locations had the lowest average flavonoid levels (18.85, 19.21, and 21.64 mg QE g^{-1} FW, respectively) (Table 5).

Antioxidant Enzyme Activity

The findings from the data analysis highlighted marked distinctions in the activities of CAT, POX, and SOD among samples from different research locations in both years of the study. In both years, RL-5 exhibited the highest activities of CAT (7.63 and 1.26 U mg $protein^{-1}$ min), POX (4.41 and 5.24 U mg $protein^{-1}$ min), and SOD (11.51 and 9.36 U mg $protein^{-1}$), respectively. Unlike, RL-1 showed the minimum activities of CAT (2.35 and 0.39 U mg $protein^{-1}$ min), POX (3.58 and 2.39 U mg $protein^{-1}$ min), and SOD (4.66 and 6.67 U mg $protein^{-1}$) in the initial and subsequent years (Table 5).

Table 3 Comparative analysis of average traits associated with quantitative and qualitative performance of saffron over two years across various research locations

Research locations	Flower number (m^2)		Fresh flower yield (g m^{-2})		Dry stigma yield (g m^{-2})		Safranal ($E^{1\%}_{1\,cm}$)		Picrocrocin ($E^{1\%}_{1\,cm}$)		Crocin ($E^{1\%}_{1\,cm}$)	
	2022	2023	2022	2023	2022	2023	2022	2023	2022	2023	2022	2023
RL-1	68.72 ± 2.07 b	81.2 ± 3.18 bc	55.7 ± 2.77 b	80.6 ± 3.97 b	0.725 ± 0.05 b	0.865 ± 0.071 b	13.5 ± 1.38 bc	14.0 ± 1.43 b	96.2 ± 2.55 b	112.1 ± 4.41 c	208.6 ± 4.36 c	231.4 ± 14.09 c
RL-2	77.8 ± 1.88 a	94.5 ± 2.68 ab	69.1 ± 4.47 a	102.6 ± 7.44 a	0.899 ± 0.045 a	1.178 ± 0.037 a	16.7 ± 0.9 ab	17.3 ± 0.88 a	108.3 ± 1.82a	135.6 ± 4.89 a	261.8 ± 8.39 a	289.7 ± 21.44 b
RL-3	66.15 ± 3.19 b	80 ± 7.37 c	63.8 ± 4.66 ab	93.1 ± 2.39 ab	0.753 ± 0.026 b	1.081 ± 0.041 a	11.9 ± 1.13 c	15.3 ± 0.68 ab	95.7 ± 3.22 b	117 ± 5.38 bc	234.7 ± 7.12 b	243.1 ± 7.17 c
RL-4	79.32 ± 1.77 a	99.76 ± 2.83 a	72.1 ± 1.59 a	102.5 ± 5.42 a	0.934 ± 0.027 a	1.179 ± 0.058 a	18.0 ± 0.49 a	16.8 ± 1.37 ab	110.9 ± 2.23a	133.2 ± 8.03 ab	264.0 ± 4.32 a	306.5 ± 1.81 a
RL-5	62.3 ± 3.28 b	81.1 ± 3.52 bc	58.2 ± 1.86 b	87.7 ± 4.71 ab	0.692 ± 0.034 b	0.998 ± 0.082 ab	11.8 ± 0.86 c	13.9 ± 0.95 b	96.9 ± 1.55 b	109.4 ± 3.04 c	238.5 ± 1.62 b	252.1 ± 7.49 bc
LSD =0.05	6.59	13.86	9.09	16.85	0.133	0.19	3.62	3.15	7.92	16.75	19.96	39.97

Means (± SE) sharing similar letters within each column showed a statistically significant difference at the 5% level, as determined by the Least Significant Difference (LSD) test.
RL1: Toroqh Rood; RL-2: Natanz; RL-3: Kashan; RL-4: Qaenat; RL-5: Badrood

Table 4 Variations in photosynthetic pigment content, proline levels, and total phenols in saffron plants cultivated at different research locations

Research locations	Chlorophyll a (µg g^{-1} FW)		Chlorophyll b (µg g^{-1} FW)		Total chlorophyll (µg g^{-1} FW)		Carotenoid (µg g^{-1} FW)		Proline (µmol g^{-1} FW)		Phenol (mgGA g^{-1} FW)	
	2022	2023	2022	2023	2022	2023	2022	2023	2022	2023	2022	2023
RL-1	15.65 ± 0.39 b	19.23 ± 1.84 bc	10.36 ± 1.27 ab	9.44 ± 1.91 a	26.01 ± 0.95 b	28.67 ± 0.53 ab	5.29 ± 0.3 bc	2.83 ± 0.74 b	0.84 ± 0.08 c	1.59 ± 0.16 ab	1.89 ±0.37 ab	2.04 ± 0.02 b
RL-2	18.86 ± 0.27 a	23.85 ± 2.42 a	11.22 ± 0.68 a	3.94 ± 1.12 b	30.08 ± 0.95 a	27.79 ± 1.32 ab	6.22 ± 0.11 a	6.1 ± 0.53 a	1.21 ± 0.14 b	1.08 ± 0.12 c	1.82 ±0.44 ab	2.11 ± 0.1 b
RL-3	15.16 ± 0.13 bc	17.02 ± 0.63 c	9.31 ± 0.53 ab	8.73 ± 0.2 a	24.47 ± 0.6 bc	25.76 ± 0.68 b	4.77 ± 0.22 c	5.58 ± 0.16 a	0.56 ± 0.07 c	1.29 ± 0.17 bc	2.61 ± 0.02 a	2.69 ± 0.06 a
RL-4	18.89 ± 0.48 a	22.01 ± 1.06 ab	11.28 ± 0.55 a	10.96 ± 2.82 a	30.17 ± 0.94 a	32.97 ± 3.19 a	6.13 ± 0.12 ab	3.34 ± 1.03 b	1.71 ± 0.07 a	1.18 ± 0.14 c	2.49 ± 0.39 a	2.7 ± 0.04 a
RL-5	14.36 ± 0.34 c	17.38 ± 0.86 c	8.32 ± 0.46 b	8.12 ± 0.49 ab	22.68 ± 0.3 c	25.51 ± 0.64 b	4.67 ± 0.45 c	3.47 ± 0.4 b	1.59 ± 0.1 a	1.72 ± 0.07 a	1.24 ± 0.13 b	2.73 ± 0.12 a
LSD =0.05	1.2	4.37	2.71	4.74	2.79	5.49	0.88	1.88	0.33	0.34	1.1	0.25

Means (± SE) sharing similar letters within each column showed a statistically significant difference at the 5% level, as determined by the Least Significant Difference (LSD) test.
RL1: Toroqh Rood; RL-2: Natanz; RL-3: Kashan; RL-4: Qaenat; RL-5: Badrood

Table 5 Mean levels of malondialdehyde, flavonoids, and antioxidant enzyme activity in saffron plants across different research locations

Research locations	MDA (nmol mL^{-1})		Flavonoids (mgQE g^{-1} FW)		CAT (U mg $protein^{-1}$.min)		POX (U mg $protein^{-1}$.min)		SOD (U mg $protein^{-1}$)	
	2022	2023	2022	2023	2022	2023	2022	2023	2022	2023
RL-1	2.86 ± 0.5 a	3.15 ± 0.42 a	18.85 ± 1.22 b	28.21 ± 6.11 a	2.35 ± 0.266 b	0.39 ± 0.06 b	3.58 ± 0.235 b	2.39 ± 0.16 c	4.66 ± 0.43 c	6.67 ± 1.32 b
RL-2	3.48 ± 0.73 a	1.89 ± 0.16 bc	27.46 ± 1.25 a	27.88 ± 4.37 a	2.63 ± 0.307 b	0.45 ± 0.01 b	4.45 ± 0.288 ab	2.59 ± 0.41 bc	5.59 ± 0.26 bc	8.53 ± 0.27 ab
RL-3	2.56 ± 0.3 a	1.8 ± 0.2 bc	19.21 ± 1.96 b	31.49 ± .19 a	2.68 ± 0.339 b	0.7 ± 0.28 b	5.97 ± 0.756 a	2.68 ± 0.22 bc	5.39 ± 0.73 bc	7.36 ± 0.22 ab
RL-4	2.31 ± 0.48 a	2.72 ± 0.31 ab	31.49 ± 1.19 a	24.15 ± 0.7 a	2.47 ± 0.39 b	0.42 ± 0.02 b	5.74 ± 0.14 a	3.46 ± 0.4 b	7.07 ± 0.67 b	6.25 ± 0.47 b
RL-5	2.26 ± 0.24 a	1.7 ± 0.25 c	21.64 ± 1.04 b	18.48 ± 7.39 a	7.63 ± 0.385 a	1.26 ± 0.16 a	4.41 ± 0.444 a	5.24 ± 0.75 a	11.51 ± 0.47 a	9.36 ± 0.85 a
LSD =0.05	1.71	1.01	4.63	16.98	1.69	0.48	1.45	1.01	1.93	2.51

Means (± SE) sharing similar letters within each column showed a statistically significant difference at the 5% level, as determined by the Least Significant Difference (LSD) test.
RL1: Toroqh Rood; RL-2: Natanz; RL-3: Kashan; RL-4: Qaenat; RL-5: Badrood

Table 6 Correlation among quantitative, qualitative, and biochemical attributes of saffron across various research locations over two years of experimentation

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1.00	**	**	**	**	**	**	ns	**	ns	ns	*	ns	ns	*	**	ns
2	0.82	1.00	**	*	**	**	**	*	*	ns	ns	*	*	ns	**	**	*
3	0.76	0.81	1.00	**	**	**	**	ns	**	ns	ns	*	*	*	*	**	ns
4	0.54	0.40	0.56	1.00	**	**	**	ns	**	ns	ns	*	ns	*	ns	ns	ns
5	0.76	0.83	0.74	0.52	1.00	**	**	*	*	ns	ns	*	ns	*	*	**	ns
6	0.67	0.66	0.69	0.56	0.74	1.00	**	ns	**	ns	ns	*	ns	ns	ns	*	ns
7	0.77	0.68	0.64	0.50	0.76	0.54	1.00	*	**	ns	ns	ns	ns	*	*	*	ns
8	-0.14	-0.26	-0.13	0.17	-0.42	0.04	-0.34	1.00	*	*	ns	ns	*	ns	*	*	ns
9	0.61	0.44	0.50	0.61	0.39	0.54	0.67	0.47	1.00	ns	ns	ns	*	*	ns	ns	ns
10	-0.11	-0.18	-0.06	0.19	0.09	-0.15	0.14	-0.36	-0.15	1.00	ns	ns	ns	ns	*	*	ns
11	0.15	0.17	0.12	0.16	0.07	0.04	0.15	-0.02	0.12	-0.17	1.00	ns	ns	ns	ns	ns	**
12	0.36	0.35	0.47	0.35	0.27	0.27	0.09	0.11	0.17	-0.19	-0.04	1.00	*	ns	ns	*	ns
13	-0.04	-0.24	-0.28	-0.11	-0.22	-0.11	0.02	0.40	0.34	-0.12	-0.22	-0.30	1.00	ns	ns	ns	*
14	0.24	0.21	0.29	0.32	0.32	0.13	0.27	-0.02	0.24	0.22	0.15	0.11	0.07	1.00	ns	ns	*
15	-0.35	-0.52	-0.37	0.17	-0.36	-0.15	-0.24	0.33	0.04	0.43	0.14	-0.08	0.04	0.13	1.00	**	*
16	-0.61	-0.75	-0.65	-0.18	-0.64	-0.32	-0.48	0.34	-0.19	0.38	-0.17	-0.28	0.23	-0.14	0.79	1.00	ns
17	0.13	0.24	0.12	0.21	0.18	0.07	0.18	0.02	0.19	-0.08	0.74	0.13	-0.24	0.29	0.37	-0.06	1.00

1. Number of flowers, 2. Flower fresh yield, 3. Stigma dry yield, 4. Safranal, 5. Picrocrocin, 6. Crocin, 7. Chlorophyll a, 8. Chlorophyll b, 9. Total chlorophyll, 10. Carotenoids, 11. Proline, 12. Total phenols, 13. MDA, 14. Flavonoids, 15. CAT, 16. POX, 17. SOD.

Table 7 Correlation among climatic and soil parameters of research locations with quantitative, qualitative, and biochemical traits of saffron

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Sand	-0.46	-0.47	-0.48	-0.45	-0.52	-0.59	-0.52	0.49	-0.21	-0.37	-0.54	0.45	0.18	-0.07	-0.15	-0.28	-0.13
Silt	0.36	0.36	0.37	0.34	0.41	0.51	0.43	-0.47	0.14	0.23	0.64	-0.51	-0.21	-0.07	0.15	0.37	0.20
Clay	0.62	0.69	0.70	0.63	0.71	0.72	0.68	-0.45	0.36	0.60	0.31	-0.26	-0.13	0.33	0.18	0.11	0.04
pH	-0.02	0.30	0.25	-0.05	0.05	0.42	-0.17	-0.15	-0.21	-0.09	0.61	0.23	-0.88	-0.28	0.38	0.87	0.48
Electrical conductivity	0.02	0.38	0.32	0.00	0.06	0.39	-0.24	0.31	-0.06	-0.08	0.18	0.80	-0.77	0.04	0.54	0.57	0.49
Organic matter	-0.12	0.31	0.24	-0.13	-0.01	0.32	-0.26	-0.28	-0.35	0.05	0.39	0.31	-0.96	-0.25	0.20	0.85	0.26
Nitrogen	0.36	0.54	0.55	0.45	0.52	0.25	0.52	0.59	-0.16	-0.97	0.64	0.05	-0.15	-0.61	0.42	0.44	0.73
Phosphorus	-0.04	0.26	0.20	-0.08	-0.03	0.36	-0.30	0.32	-0.11	-0.26	0.41	0.66	-0.80	-0.15	0.59	0.70	0.63
Potassium	0.78	0.35	0.42	0.75	0.68	0.49	0.85	0.25	0.83	-0.21	-0.16	0.34	-0.78	-0.52	-0.36	0.63	-0.21
Iron	0.46	0.52	0.50	0.40	0.40	0.73	0.21	0.54	0.42	-0.18	0.59	0.53	-0.41	0.17	0.92	0.40	0.88
Manganese	0.78	0.75	0.76	0.79	0.75	0.68	0.66	0.60	0.83	0.44	-0.36	0.69	0.33	0.92	0.62	-0.59	0.24
Copper	-0.13	-0.06	-0.14	-0.52	-0.43	-0.16	-0.68	-0.11	-0.62	-0.14	-0.03	0.55	-0.90	-0.36	-0.05	0.71	0.04
Zinc	-0.31	-0.31	-0.38	-0.47	-0.51	-0.33	-0.77	-0.16	-0.72	-0.08	-0.47	0.46	-0.57	-0.31	-0.43	0.33	-0.37
Latitude	-0.24	0.23	0.16	-0.22	-0.14	0.08	-0.43	0.08	-0.32	0.07	-0.21	0.80	-0.77	0.01	0.14	0.46	0.07
Longitude	0.73	0.60	0.61	0.67	0.62	0.78	0.51	0.77	0.79	-0.07	0.35	0.54	0.07	0.51	0.99	-0.10	0.82
Altitude	0.60	0.29	0.35	0.58	0.57	0.45	0.73	-0.21	0.52	0.25	0.41	-0.66	0.45	0.21	0.14	-0.24	0.11
Average rainfall	0.86	0.59	0.64	0.83	0.81	0.76	0.89	0.11	0.80	0.30	0.41	-0.27	0.41	0.49	0.52	-0.29	0.37
Average temperature	-0.65	-0.50	-0.43	-0.53	-0.42	-0.63	-0.40	-0.25	-0.57	-0.39	-0.33	-0.07	-0.24	-0.64	-0.70	0.24	-0.47

Coefficients less than 0.24: non-significant; between 0.25 and 0.45: significant at the 5% probability level (*); and greater than 0.46: significant at the 1% probability level (**).

1. Number of flowers, 2. Flower fresh yield, 3. Stigma dry yield, 4. Safranal, 5. Picrocrocin, 6. Crocin, 7. Chlorophyll a, 8. Chlorophyll b, 9. Total chlorophyll, 10. Carotenoids, 11. Proline, 12. Total phenols, 13. MDA, 14. Flavonoids, 15. CAT, 16. POX, 17. SOD

Correlation Analysis

The simple correlation analysis findings suggested that the quantitative, qualitative, and biochemical traits of saffron at different research locations, as presented in Table 6, showed significant positive interrelations between the traits. Dry stigma yield exhibited a strong, noteworthy positive correlation with the count of flowers, fresh flower yield, and qualitative traits, such as safranal, picrocrocin, crocin, total chlorophyll, phenolic compounds, and flavonoids. Conversely, a significant negative correlation was observed between dry stigma yield and MDA content and the activity of antioxidant enzymes, including CAT and POX. Additionally, the findings showed that the activity of antioxidant enzymes, particularly CAT and POX, was negatively and significantly correlated with yield-related traits, such as the count of flowers, fresh flower yield, and dry stigma yield, as well as saffron quality traits, such as crocin and picrocrocin.

The simple correlation between climate-related and soil parameters at the research locations and the phytochemical attributes of the plant is presented in Table 7, based on the mean data. The results indicated that among the soil parameters, silt and clay content, iron, and magnesium exhibited significant positive correlations with various phytochemical attributes of the plant, including flower count, fresh flower yield, dry stigma yield, and secondary metabolite content, such as crocin, picrocrocin, and safranal. Conversely, average temperature as a climatic parameter, along with sand content and Zn as soil parameters, demonstrated significant negative correlations with the aforementioned plant traits.

Regression and Ccluster Analysis

The results of the stepwise regression analysis are obtainable in Table 8, where dry stigma yield was treated as the dependent variable, and other quantitative, qualitative, and biochemical traits were considered as independent variables. Overall, 71.71% of the variation in dry stigma yield could be explained by flower yield, total chlorophyll, and phenolic content.

According to the cluster analysis results, five research locations were evaluated based on 17 quantitative, qualitative, and biochemical traits and categorized into three groups. Regions RL-1, RL-3, and RL-5 were included in the first cluster, showing no superiority for any specific trait. The second and third clusters, consisting of RL-2 and RL-4, respectively, exhibited similar means across most traits with slight differences. However, cluster three, containing RL-4, demonstrated superiority in most traits, except for chlorophyll a, carotenoids, and malondialdehyde, whereas cluster two exhibited superiorities (Table 9 and Fig. 3).

Table 8 Stepwise regression analysis for dry saffron stigma yield as the dependent variable across research locations

Term	Coef	SE Coef	T-Value	P-Value
Constant	0.047	0.128	0.36	0.018
Fresh flower yield (X1)	0.00574	0.00162	3.53	0.002
Total chlorophyll (X2)	0.01382	0.00829	1.67	0.007
Phenol (X3)	0.0809	0.0368	2.20	0.037
R-sq(adj) = 71.71%				
Y = 0.047 + 0.00574 (X1) + 0.01382 (X2) + 0.0809 (X3)				

Table 9 Cluster analysis of traits related to quantitative, qualitative, and biochemical yield of saffron grown in research locations

Variable	Cluster1	Cluster2	Cluster3
Number flower	73.255	86.180	89.540
Flower yield	73.246	85.875	87.345
Stigma yield	0.852	1.039	1.056
Safranal	13.445	17.028	17.443
Picrocrocin	104.586	122.000	122.092
Crocin	234.776	275.775	300.325
Chl-a	16.468	21.354	20.449
Chl-b	9.048	7.580	11.120
Total-Chl	25.516	28.934	31.570
Carotenoid	4.435	6.164	4.736
Proline	1.265	1.142	1.445
Phenol	2.200	1.964	2.594
MDA	2.388	2.683	2.516
Flav	22.980	27.669	27.819
CAT	11.977	11.539	35.524
POX	23.058	22.518	26.566
SOD	7.061	6.134	8.878
Number	3	1	1

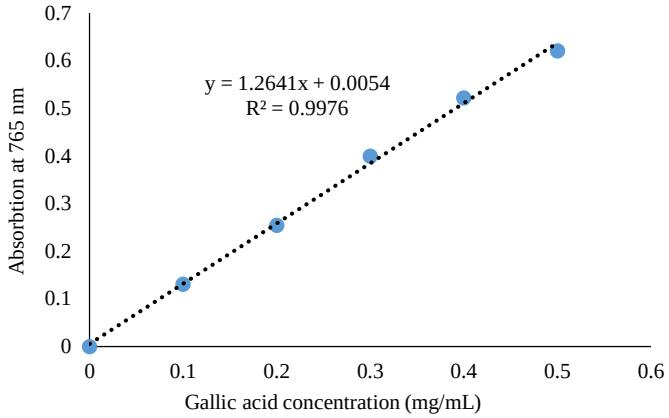

Fig. 1 Calibration curve for quantification of gallic acid based on absorbance measurements at 765 nm

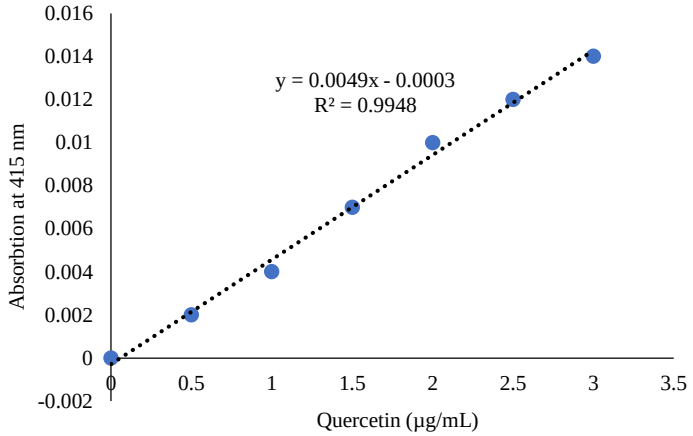

Fig. 2 Calibration curve for quantification of quercetin based on absorbance measurements at 415 nm

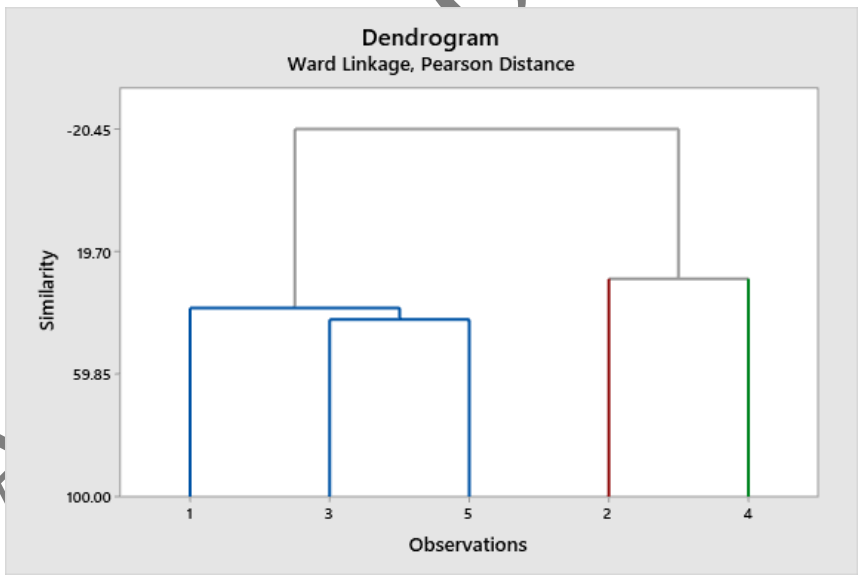

Fig. 3 Cluster analysis of five research locations based on phytochemical and biochemical traits of saffron

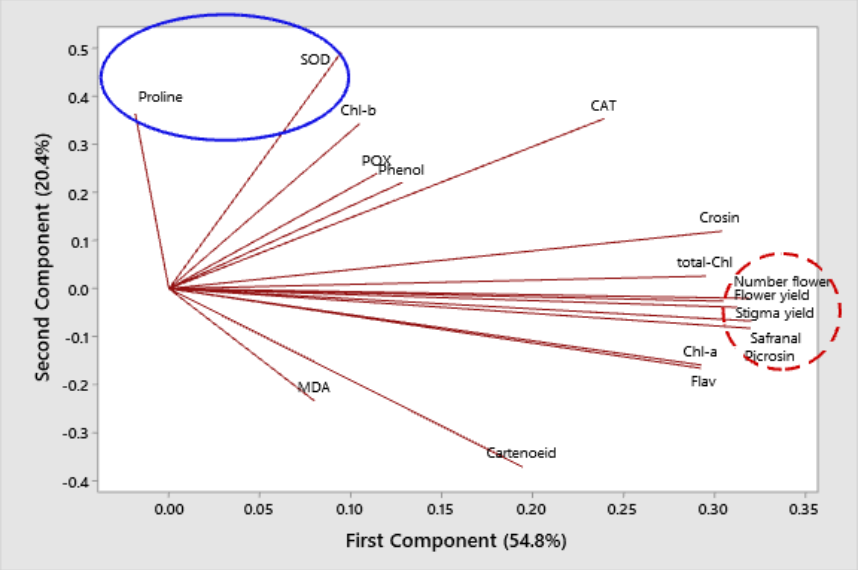

Fig. 4 Principal component analysis of phytochemical and biochemical traits of saffron

Analysis of Principal Components

The analysis of the two principal components related to the quantitative, qualitative, and biochemical traits of saffron across different research locations (Fig. 4) indicated that these components accounted for 75.2% of the variation. The first component, which explains 54.8% of the variation, is characterized by traits associated with flower count, flower yield, stigma yield, and the content of key saffron compounds, such as safranal and picrocrocin. Hence, this component can be labeled as both quantitative and qualitative. The second component, accounting for 20.4% of the variation, demonstrated superiority in traits related to proline content and superoxide dismutase enzyme activity. Therefore, it can be designated as a physiological and biochemical component.

DISCUSSION

This study aimed to evaluate the quantitative, qualitative, and biochemical variations in saffron across different research locations over two years. The results revealed that regard with both quantitative and qualitative attributes, such as flower count, stigma and flower yield, and quality-related parameters, including safranal, picrocrocin, and crocin content, samples from RL-2 and RL-4 exhibited significantly superior performance. According to Farrokhi *et al.* [27], saffron cultivation in North Khorasan and Razavi Khorasan provinces demonstrated a significant advantage in the average yield characteristics, such as flower yield, stigma yield, and the levels of safranal, picrocrocin, and crocin, with those observed in South Khorasan Province. This aligns with the findings of Rahimi *et al.* [28], who also reported significant variations in quantitative and qualitative traits across different saffron cultivation areas. The levels of key primary metabolites within saffron, along with the prevailing environmental conditions, determine its yield and overall quality [29]. By analyzing meteorological and soil data from the research locations and examining the correlations between these parameters and the biochemical attributes of saffron, it was found that an increase in soil properties, such as silt and clay content, nitrogen, potassium, iron, and magnesium levels, positively influenced the average quantitative and qualitative traits of saffron. In contrast, a higher sand content in the soil negatively affected these plant traits. Additionally, climatic parameters, such as average rainfall, altitude, and longitude, positively affected the quantitative and qualitative traits. In contrast, the average temperatures of the regions showed a negative impact. Supporting these findings, other researchers have reported that climatic parameters, particularly average rainfall and altitude, positively affect saffron performance traits. Increased averages of these parameters lead to enhanced flower and stigma yields as well as higher levels of phytochemical compounds in saffron [2, 27].

In the present study, samples from RL-4 (Qaenat, South Khorasan Province) also exhibited satisfactory average performances. Overall, North, Razavi, and South Khorasan provinces are considered major saffron producers in Iran and globally, with a long history of saffron cultivation. As a result, saffron from these regions is characterized by superior quantitative and qualitative traits. Given the genetic characteristics of these ecotypes, high performance in quantitative and qualitative traits from the samples collected in these areas was anticipated. Bayat *et al.* [30] suggested that improved climatic conditions and better field management led to higher saffron bulb quality and an increase in the number of flower buds. Similarly, Siracusa *et al.* [31] stated that ecological conditions and bulb sources meaningly influence flower number and stigma yield. Alavi Siney *et al.* [32] observed genetic differences among saffron ecotypes regarding flower yield and stigma yield.

Saffron quality is primarily determined by the levels of safranal, picrocrocin, and crocin, which are critical contributors to its distinctive flavor, aroma, and color. In the present study, samples from RL-1 and RL-5 exhibited the lowest average concentrations of these compounds, underscoring the significant influence of ecotypic variation on saffron quality. Conversely, samples RL-2 and RL-4 demonstrated the highest average levels of these traits, likely attributable to their superior adaptation to specific environmental factors such as soil composition, temperature, and humidity. These findings suggest that the superior quality of RL-2 and RL-4 results from their genetic potential and their unique responses to environmental conditions. However, the lower levels of qualitative compounds in RL-1 and RL-5 may be linked to adverse environmental conditions or nutrient deficiencies, which could disrupt physiological and biochemical processes, ultimately reducing saffron quality [2, 33].

Bayat *et al.* [30] stated that safranal is derived from picrocrocin during drying and storage and that its content is further influenced by climatic conditions that affect saffron stigmas. Secondary metabolites in saffron are highly dependent on climatic conditions and agricultural practices. Owing to its adaptability to diverse climatic environments, saffron has developed distinct ecotypes across different regions [34]. Prior research has shown notable differences in the levels of secondary metabolites among different regions. For example, Caballero-Ortega *et al.* [35] attributed regional variations in saffron composition to environmental factors, cultivar type, and cultivation practices. Moreover, researchers have identified higher concentrations of key bioactive compounds in Qaenat saffron, which are linked to the region's high altitude and unique climatic conditions [34, 36]. A notable feature of the Natanz region (RL-2) is its elevated altitude relative to other locations, which is associated with enhanced quantitative and qualitative traits in this ecotype. The positive influence of altitude on saffron's qualitative traits, including crocin, picrocrocin, and safranal, has been corroborated by several studies [2, 37]. In this study, picrocrocin content ranged from 95.7 to 135.6 $E_{1\ cm}^{1\%}$, while crocin levels ranged from 208.6 to 306.5 $E_{1\ cm}^{1\%}$ across the analyzed samples. According to the ISO/TS 3632 standards, saffron from RL-2 and RL-4 was classified as category I, indicating superior quality. Notably, significant differences in crocin, picrocrocin, and safranal concentrations have been observed between saffron grown in different countries and within regions under similar soil and climatic conditions [38].

The findings of this investigation demonstrated important differences among the research locations regarding physiological and biochemical attributes, including the content of photosynthetic pigments, proline, total phenols, flavonoids, MDA, and the activity of antioxidant enzymes. Alavi Siney *et al.* [32] highlighted that the physiological characteristics of saffron ecotypes vary across regions, which can be attributed to environmental conditions. These findings highlight the interplay between ecotypes and environmental factors. Variations in photosynthetic pigments, especially chlorophyll, among the saffron ecotypes, are associated with genetic diversity and adaptation to specific environmental conditions. Each ecotype may respond uniquely to climatic factors, soil type, and agricultural practices, leading to significant changes in chlorophyll content and, consequently, photosynthetic capacity [39].

This correlation analysis highlights the critical influence of both soil composition and climatic conditions on the phytochemical quality and yield of saffron. The positive relationships between silt, clay, iron, and magnesium with key plant traits suggest that richer, finer-textured soils with essential micronutrients support better flower production and higher concentrations of valuable secondary metabolites, like crocin, picrocrocin, and safranal [2]. On the other hand, the negative impact of higher average temperatures, sandy soils, and elevated zinc levels indicates that these factors may create less favorable growing conditions, possibly by affecting nutrient availability or plant stress responses [27].

The results suggested a negative covariation between the total chlorophyll content and proline levels. Specifically, RL-5, which displayed the lowest chlorophyll content, had the maximum proline level. The observed effect could stem from the competitive interaction between glutamine kinase, which facilitates proline synthesis, and glutamate ligase, the initial enzyme in the chlorophyll production pathway, within RL-5. This competition may divert glutamate (a precursor in chlorophyll and proline biosynthesis pathways) toward proline synthesis, thereby limiting chlorophyll biosynthesis [40]. Proline, a non-essential amino acid, plays a crucial character in saffron responses to stress. Differences in proline content among saffron ecotypes can result from environmental conditions, genetic diversity, or varying stress responses. Ecotypes growing in drier regions often produce higher proline levels to prevent cellular damage [41]. Consistent with these findings, variations in proline content have been reported among different plant ecotypes, such as *Silybum marianum* [42] and *Foeniculum vulgare* [43]. In another study, significant differences were observed among ten saffron ecotypes with respect to moisture content, total phenols, total flavonoids, picrocrocin, crocin, and safranal, supporting the findings of the ongoing research [44]. Recent research has indicated that climatic conditions and extraction methods significantly influence the total phenol content. Phenolic compounds in saffron are critically important compared with other food additives and spices [45].

MDA, generated during the process of lipid peroxidation in plant cells, is a critical marker for assessing oxidative stress and damage caused by biotic and abiotic stressors. Different saffron ecotypes may produce varying levels of MDA in response to environmental conditions. Factors influencing these differences include genetic characteristics, environmental conditions, and cultivation history [32]. In an experiment by Kabiri *et al.* [44], the highest total flavonoid level was reported in ecotype ES7 and then ES8, whereas the lowest was recorded in ES1. These findings align with the present study's observation of significant differences in flavonoid content among the saffron ecotypes. Regarding antioxidant enzyme activities (CAT, POX, and SOD), significant differences were observed among saffron samples from various regions, with the highest activity recorded in RL-5. These results highlight the pivotal role of antioxidant enzymes in improving environmental stress tolerance and maintaining physiological stability in saffron ecotypes, which may assist in selecting and enhancing stress-resistant ecotypes.

Stigma yield exhibited a noteworthy negative correlation with proline, total phenol, MDA, and flavonoid content. Overall, the findings revealed that saffron quality traits, including picrocrocin and crocin content, were negatively affected by traits such as SOD activity, total phenols, MDA, and flavonoids. In other words, increased levels of these parameters reduced the average quality traits of the saffron. Consistent with the present findings, Alavi Siney *et al.* [11] reported significant correlations among most functional traits of saffron, with stigma number and dry weight showing positive correlations with stigma yield. This indicates that the potential of ecotypes varies in flower production, and genotypes that produce more flowers also exhibit higher average stigma dry weights, ultimately leading to higher yields. Similarly, Amirnia *et al.* [46] reported that the strongest correlation between the studied traits and dry stigma yield in saffron was related to flower number and stigma dry weight.

CONCLUSION

The Natanz (RL-2) and Qaenat (RL-4) regions showed superior saffron performance in terms of flower number, yield, and key compounds (safranal, picrocrocin, and crocin). Significant regional differences were found in photosynthetic pigments, proline, phenols, antioxidant enzymes, and other biochemical traits. Positive correlations existed between yield, quality, and secondary metabolites, while yield negatively correlated with antioxidant enzyme activity. Soil silt, clay, iron, and magnesium positively influenced yield and biochemical

content, whereas higher temperature, sand, and zinc reduced saffron quality and yield. RL-2 and RL-4 were identified as optimal cultivation areas due to their favorable profiles.

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