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Original Article

The Changes in Yield, Biochemical Properties and Essential Oil Compounds of Saffron (*Crocus sativus* L.) Plants Treated with Organic and Inorganic Fertilizers under Dryland Farming System

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Abstract

The use of environmental friendly fertilizers is the main agricultural practice to improve the plant quality and quantity. The present study aims to assess the effect of organic and inorganic fertilizers on flower and stigma yield, biochemical properties and essential oil (EO) composition of saffron (*Crocus Sativus* L.) in research station of Hamand Absard, Iran. The plants were treated with cattle manure (20 t ha^{-1}) and foliar application of delfard (7 kg ha^{-1}) and floral P (2.5 kg ha-1) in a completely randomized block design (CRBD) with three replications under dryland farming system. The results showed significant increases of flower number and yield under all fertilizers compared to control. The yield of fresh and dry stigma under manure application was higher than floral and delfard. The response of chlorophyll (Chl) concentration was different under fertilizers. Floral and manure application led to higher Chl a compared to control. Chl b under manure and delfard was significantly higher than control and floral. TPC and TFC were significantly improved by organic and inorganic fertilizers. The main improvement of TPC and TFC was obtained under manure application followed by floral and delfard. GC/MS analysis showed the main EO composition were Methyl pentanoate (25.8- 62.15%) followed by Dihydro- β- ionol (17.7-34.79%), and hexadecyl acetate (4.9-9.4%), representing different responses to fertilizers. To sum up, the best fertilizer to improve the yield of saffron was manure followed by floral and delfard.

Keywords: Essential oil composition, Dryland farming system, Stigma yield, Delfard, Manure.

Introduction

Saffron (*Crocus sativus* L.) is a perennial herb and belongs to the Iridaceae family, which has been cultivated in Iran, Morocco, India, Greece, Italy, and Spain [1,2]. Iran with the best climate for saffron is considered to main saffron production [2]. The annual production of saffron is about 200 t per year all over the world, which Iran covers about 80% of this production. Khorasan province is the best site in the world for high quality saffron [3]. Stigma is a well-known part of saffron, which is very expensive spice and used as a food flavoring, a coloring agent, and as a traditional herbal medicine [4]. 144.responsible components of saffron color and taste are crocins, the glycoside derivatives of crocetin, picrocrocin (mainly responsible for the bitter taste), and safranal (monoterpene aldehyde). Safranal is formed by hydrolysis from picrocrocin during drying and storage [3, 5]. The pharmacologic activity of saffron are antioxidant, anti-tumor, analgesic, anti-inflammatory, anti-cough, insulin resistance etc [3].

Today, the remarkable increase in the world food production needs to use the high amount of chemical fertilizers. However, some unfavorable effects of excessive use of chemical fertilizers in conventional agricultural practices have been well reported [6,7].

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Chemical fertilizers via contaminating soil and water are the main risk for human being's health [8]. In agro-ecosystems, application of synthetic toxic chemical pesticides restricts the soil fertility and growth of cultivated crops [7]. In Iran, in order to compensate the deficiency of nutrients in soil, chemical fertilizers are being used in higher quantities. To minimize the accumulation of pollutants in agro-ecosystems, toxic chemicals especially chemical pesticides and fertilizers in agricultural process should be reduced. In this regard, organic products are considered as an alternative to sustainable agriculture development. Recently in Iran, the use of eco-friendly compounds has been raised. Organic inputs can grantee both agricultural production and nature conservation. The current approach is to find an appropriate substitute of chemical fertilizers by organic compounds that are cost-effective and eco-friendly [7,8]. Bio and organic fertilizers include living cells of microorganisms, which colonize the plant root and promote plant growth by converting nutritionally important elements such as N and P from unavailable to available form through biological process like nitrogen fixation and solubilization of rock phosphate [7,9].

Chlorophyll (Chl) as primary metabolite is directly involved in the metabolic pathways of an organism necessary for its growth, development, and reproduction [10]. Phenol, flavonoid and essential oil (EO) are secondary metabolites with a restricted occurrence in taxonomic groups, that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell with its environment, ensuring the survival of the organism in its ecosystem [10]. These kinds of secondary metabolites show valuable pharmacologic activity [11-13].

Inorganic and organic fertilizers can improve the quality and quantity of medicinal plants. The functional impact of organic fertilizer on *Satureja mutica* L. [9], *Satureja sahendica* Bornm. [14], *Drimiopsis maculata* Lindl. & Paxton [15]; and the helpful reaction of inorganic fertilizer on *Cucurbita pepo* L. [16]. Seyyedi *et al*. [17] showed the increased yield of saffron with mineral fertilizer. According to the literature review, there is little information to compare the effect of organic and inorganic fertilizers on saffron. Therefore, the purpose of study was to assess the effects of manure, delfard and floral P fertilizer on flower and stigma yield, chlorophyll (Chl), phenol and flavonoid concentrations, and essential oil (EO) composition of saffron under dry farming systems.

Material and Methods

Experimental site and design

A two-year field experiment was conducted at Hamand Absard research station (52º 21′ E and 35º 43′ N), belonging the research institute of Forests and Rangelands, Tehran province. It was carried out in completely randomized block design (CRBD) with three replications in 2015 and 2016. The minimum and maximum temperatures were respectively -15 °C in January–February and 35 °C in July–August with mean annual amount to be 12 °C. Mean annual rainfall was 340 mm. Fig. 1 represents the ambrothermic diagram during 2015 and 2016. Table 1 describes soil features of the case study.

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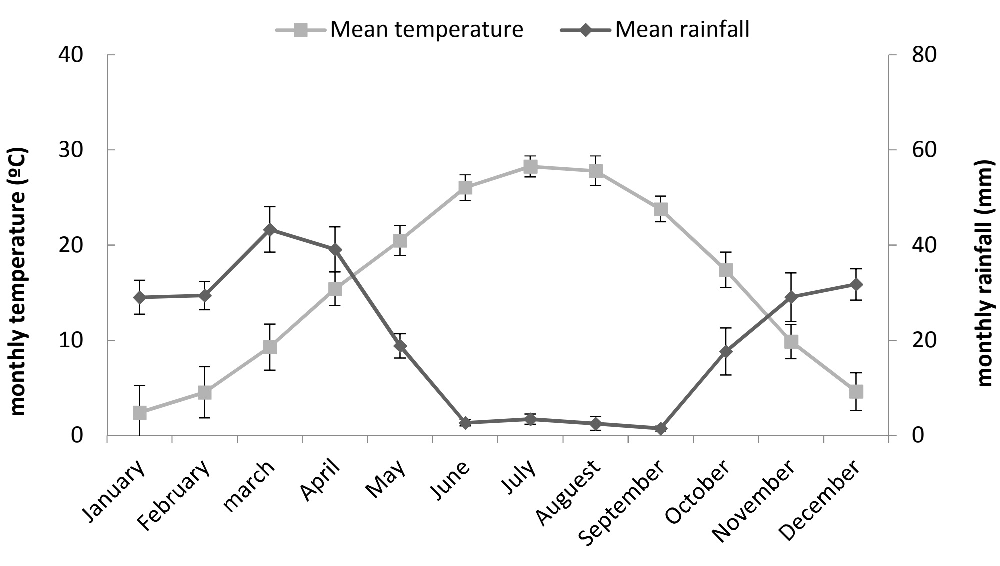

Fig. 1 The ombrothermic diagram in the case study

Table 1 The soil characteristics of the experimental field

Year	pH	EC	OC (%)	N (%)	P (mg/kg)	K (mg/kg)	Sand (%)	Silt (%)	Clay (%)
2015	8.3	0.84	1.2	0.96	18	272	21	45	32
2016	8.1	0.85	1.3	0.99	21	279	20	47	33

Table 2 The fertilizer characteristics used in the study

Fertilizer	N (%)	P (%)	K (%)	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
Manure	3.2	1.6	3	55.4	3456	378.5	203.6
delfard	12	8	4	500	2000	1000	1000
Floral P	11	20	11	500	1500	1000	1000

Agronomic Practices

The experimental field was prepared applying plow, disk, and leveler according to the local practice for saffron production. Each plot was 5 × 10 m. Planting depth was 12 cm, corm spacing on the row was 8 cm and row spacing was 35 cm. The average density was 35 corms per square meter. Weed management was carried out by hand-removal over growing seasons.

Fertilizer Application

Table 2 shows the fertilizer compounds. Before corm planting, cattle manure (20 t ha^{-1}) was scattered onto the soil surface and then mixed into the soil. Delfard (7 kg ha^{-1}) as a special fertilizer for saffron and floral P (2.5 kg ha^{-1}) as a main fertilizer in Iranian soils was used with foliar application at January 15 and March 15. At the end of phonological stage, the plants were harvested at the November 1 to November 20.

Flower and Stigma Weight

The stigmas were carefully separated from the flowers and dried at room temperature for 72 h, which is the Iranian traditional method widely used by saffron producers, while the other parts were dried at oven (75 °C). The flower and stigma weight were measure based on g per square meter.

Chlorophyll (Chl) Assay

The contents of chl a and b were extracted according to Arnon [18]. 200 mg of fresh samples were homogenized in 8 ml 80% acetone. After that, the mixture was centrifuged at 4 °C for 15 min (3000 rpm). Supernatants were used for analyzing chlorophyll content. Absorbance was determined at 645 and 663 nm by the spectrophotometer.

Determination of Total Phenolic Content (TPC)

Folin–Ciocalteu reagent was selected to measure TPC spectrophotometrically [19]. 100 µl of the MeOH solution of the precisely measured weight of investigated plant 1–10 (2.54, 2.58, 2.25, 4.03, 4.80, 2.13, 4.62, 1.47, 1.58, 15.05 mg mL^{-1} respectively) were mixed with 0.75 mL of Folin–Ciocalteu reagent and allowed to stay at 22º C for 5 min. The mixture was supplied with 0.75 ml of $NaHCO_3$. Absorbance was measured at 725 nm by UV–VIS spectrophotometer (Varian Cary 50) after 90 min at 22 ºC. Standard curve was calibrated by Gallic acid (0–100 mg ml^{-1}; r > 0.99). The results were represented as mg Gallic acid (GA) g^{-1} Dry weight.

Determination of Total Flavonoid Content (TFC)

The flavonoid levels were measured by aluminum chloride colorimetric method [20]. Briefly 0.5 ml of extract solution with 1.5 ml of 95% ethanol, 0.1 ml of aluminum chloride 10%, 0.1 ml of 1 M potassium acetate were mixed with 2.8 ml of distilled water. The mixture vortexed for 10 s and left to stand at 25 ºC for 30 min. The absorbance of the mixture was read at 415 nm. Quercetin concentrations (0 to 1200 µg ml^{-1}) were prepared and linear fit was used for calibration of the standard curve.

Essential Oil Extraction

In order to identify the EO content, during peak flowering season, 100 g of dried aerial parts from each treatment were hydrodistilled in the Clevenger type apparatus for 3 hr. Anhydrous sodium sulfate was used to dry EO samples, and finally the samples were stored at 4 °C to further analysis of GC and GC-MS [7].

Gas Chromatography (GC) Analysis

Thermo-UFM ultrafast gas chromatograph equipment with a ph-5 fused silica column (10m length × 0.1 mm id., film thickness 0.4 µm) was used to anlyze EOs. Oven temperature was maintained at 60 ºC for 5 min and then programmed to 285 ºC at a rate of 5 ºC min^{-1}; flame ionization detector (FID) and injector temperature were 290 ºC and 280 ºC, respectively; helium was applied as carrier gas with an inlet *pressure* of 0.5 kg cm^{-2}.

Gas Chromatography-mass Spectrometry (GC-MS)

GC-MS analyses were accomplished by Varian 3400 GC-MS system equipment with AOC-5000 auto injector and DB-5 fused silica capillary column (30 m × 0.25 mm i.d.;

film thicknesses 0.25 µm). Temperature was programmed from 60 °C to 250 °C with 3 °C min^{-1}; Injector and interface temperature were 260 °C and 270 °C, respectively; acquisition mass range of 40–340 amu; ionization voltage of 70 eV; the carrier gas was helium at a velocity of 45 *cm* sec^{-1}.

Component Identification

Homologous series of n-alkanes (C_7–C_{25}) determined the retention index for all volatile constituents. According to Adams, the components of oil were identified by matching their retention indices (RI) and mass spectra. EO components were identified by GC/MS spectroscopy [9].

Statistical Analysis

The data (n = 3) were subjected to one-way analysis of variance (ANOVA) and using the SAS software package for Windows (SAS, version 9.3, SAS Institute, Cary, NC). Duncan's multiple range tests showed the comparison of mean values. The data were statistically investigated at 5% probability level.

Results

Flower Number and Yield

The response of saffron plants was different under organic and inorganic fertilizers. We found significant increases of flower number and yield under all fertilizers compared to control. Flower number varied from 26.433 flowers in control to 46.33 flowers in manure application (Fig. 2 a). Flower yield significantly improved by organic and inorganic fertilizers. Compared to control, there were observed 45, 31, and 32% increases of flower yield in plant treated with manure, delfard, and floral, respectively (Fig. 2 b).

Stigma Yield

The response of saffron stigma to fertilizers was the enhancement of stigma weight. The yield of fresh stigma varied from 0.5 to 88 g per square meter. This trait in plants under manure application and floral was higher than delfard and control (Fig. 2 c). The yield of dry stigma was a little different, where there was no significant difference between delfard and floral. However, organic fertilizer resulted in greater dry weight yield compared to control and delfard (Fig 2 d).

Chl Content

The response of Chl concentration was different under fertilizers. Floral and manure application led to higher Chl a compared to control.

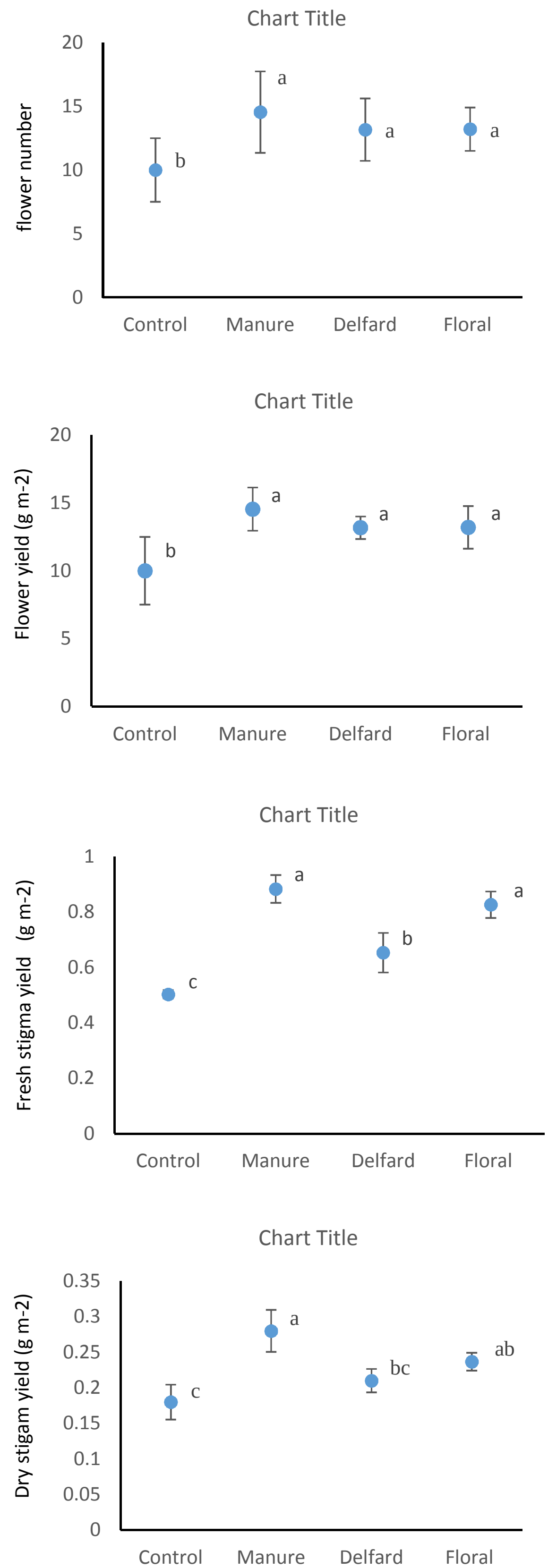

Fig. 2 Flower number and yield of flower and stigma under organic and inorganic fertilizers. The values are presented with mean ± SE (n=3)

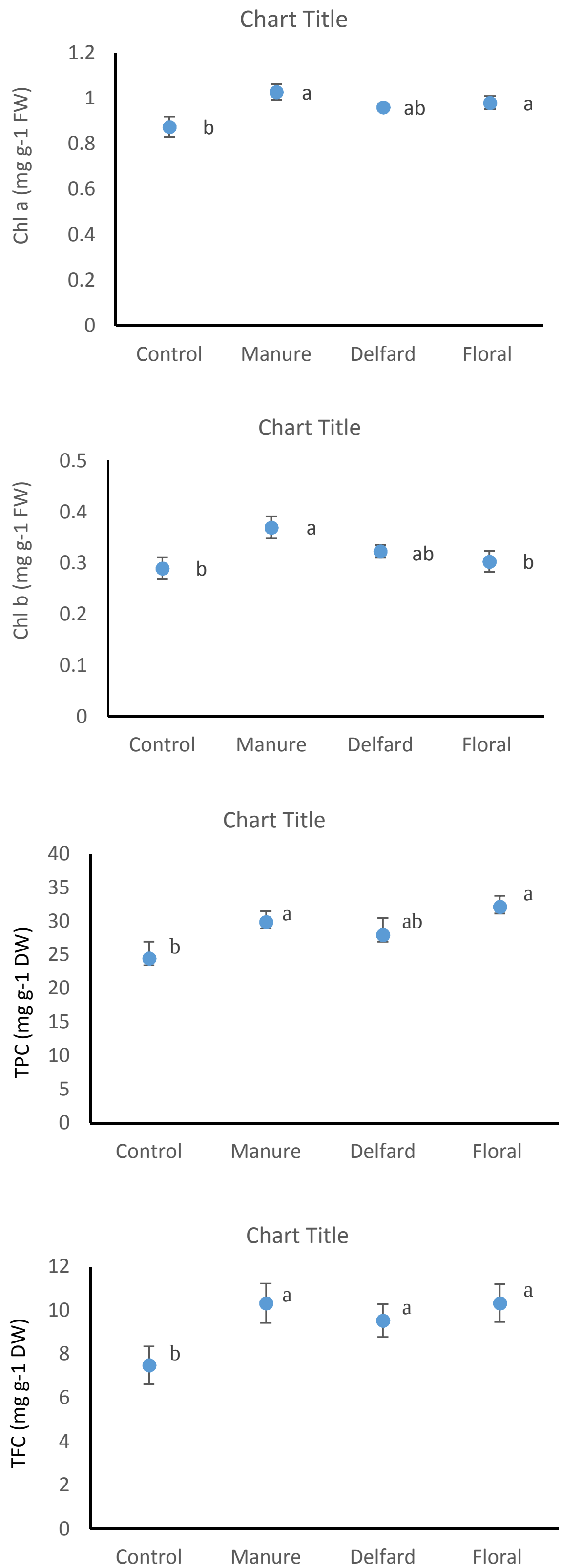

Fig. 3 Chlorophyll (Chl), total phenolic content (TPC) and total flavonoid content (TFC) under organic and inorganic fertilizers. The values are presented with mean ± SE (n=3)

However, there was no significant change among the fertilizers for Chl a (Fig. 3a). Chl b was also showed differences under experimental treatments. Manure, delfard, and floral improved Chl b by 27, 11, and 4% compared to control (Fig. 3b).

Phenol and Flavonoid Content

TPC and TFC were significantly improved by organic and inorganic fertilizers. TPC varied from 24.49 to 32.13 mg g^{-1} DW for control and floral, respectively (Fig. 3 c). TFC in all fertilizers treatments was higher than control plants. Manure, delfard, and floral increased TFC by 37, 27, 37% in comparison with control (Fig. 3d).

EO Composition

EO compounds are presented in Table 3. GC/MS analysis showed the main EO composition were Methyl pentanoate followed by Dihydro-β-ionol and hexadecyl acetate. Methyl pentanoate varied from 25.8% (control) to 62.15% (delfard). However, the other main component was Dihydro-β-ionol ranging from 17.7% (delfard) to 34.79% (control). The highest and lowest hexadecyl acetate was found in EO of plants under manure and floral, respectively.

Discussion

The organic and inorganic fertilizers improved the flower number and yield of saffron. Increasing the flower number due to the availability of essential nutrients in the existing fertilizers and increasing the available water improve the reproductive properties in the plant [9]. Increased number of flowers in saffron was reported by Khoramdel *et al.* [21] when plants were treated by Delfard fertilizer.

Similarly, Ghanbari *et al.* [22] obtained the increased flower number in semiarid areas under the influence of bio-organic fertilizers. The studied fertilizers increased nitrogen accumulation and increase stigma yield by improving the storage capacity of nutrients and increasing growth-regulating hormones and microorganisms [7,9]. In the present study, there was found a significant increase in flower and stigma yield of saffron under organic and inorganic fertilizers. Befrozfar *et al.* [23] stated that stimulating the production of auxin-like substances during the application of foreign fertilizers is the main reason of increased plant yield. Also, since the amino acid tryptophan is a precursor to the synthesis of indole acetic acid (IAA), the presence of zinc in the structure of this amino acid is essential, where the experimental fertilizers, especially Delfazed and manure, are rich in nutrients.

Table 3 Essential oil composition under organic and inorganic fertilizers

Compound	RI	Control	Delfard	Floral	Manure
Hexanal	802	-	2.3	1.5.33	-
Methyl pentanoate	821	25.8	62.19	57.64	37.8
3,3,5-trimethyl-cyclohexene	824	0.1	-	-	-
3,5,5-trimethyl cyclo hex-3-en-1-one	832	0.5	0.43	0.48	0.45
Heptanal	902	-	-	0.25	0.32
1-heptanol	971	-	-	0.48	0.21
β- pinene	980	-	-	0.62	-
2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde	1049	-	0.38	-	0.36
(Phenyl ethyl alcohol	1052	-	0.27	-	-
Isophorone	1061	-	0.34	0.81	0.32
6-camphenone	1091	-	0.2	-	-
2,2,6-trimethyl-1,4-cyclo hexanedione	1102	-	0.58	-	-
Dihydro-4-hydroxy-2 (3H)-furanone	1110	1.51	-	-	
2,6,6-trimethyl-1,4- cyclohexadiene-1- carboxaldehyde (isomer of safrana	1117	0.74	-	-	0.15
4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (isomer 1)	1171	-	0.42	-	-
2,3-dihydroxy naphthalene-1,4-dione	1241	0.43	-	-	-
4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde	1281	0.49	-	-	0.53
E-4-(2,6,6-trimethyl-cyclohexyl) but-3-en-2-one	1315	0.75	0.36	-	
10- undecenol	1361	-	1.03	-	0.32
Ethyl- (4E)- decenoate	1380	-	0.3	-	
Dodecanal	1409	1.01	-	-	1.8
Geranyl propanoate	1476	0.92	-	-	-
(E)-β- ionone	1488	-	0.73	-	0.84
β-himachalene	1497	1	-	-	-
Tridecanal 1509	1508	0.4	-	-	1.3
Germacrene B	1556	1.9	0.48	-	
(2E)- tridecen-1-al	1567	1.41	-	-	0.23
Hexyl octanoate	1581	0.36	-	-	0.45
Hexyl phenyl acetate	1623		0.32	-	-
α- cadinol 1652	1650	0.37	1.84	-	1.02
Safranal (= 2,6,6-trimethyl-1,3-cyclohexadien-1-carboxaldehyde)	1664	-	0.45	-	1.04
(2Z, 6Z)- farnesal	1683	-	0.62	-	-
6-oxoisophorone (= 2,6,6-trimethyl-2-cyclohexen-1,4-dione)	1703	1.39	-	-	0.78
Methyl eudesmate	1719	0.46	0.19	-	
Isobicyclogermacrenal	1733	0.34	-	-	0.67
epi- Cyclocolorenone	1774	1.39	-	-	-
Dihydro-4-oxoisophorone (= 2,6,6-trimethtl-1,4-cyclohexandione)	1781	1.04	0.66	-	1.6
(5Z, 9E)- farnesyl acetone	1892	0.61	-	-	-
Nonadecane	1900	-	1.15	-	-
Cyclohexadecanolide	1932	0.41	-	-	0.14
Dihydro- β- ionol	1967	37.49	17.7	33.82	35.8
2 α-acetoxy-amorpha-4,7(11)-diene-8-one	1981		0.39		-
n-eicosane	2004	-	-	-	-
Hexadecyl acetate		9.8	5.2	4.9	9.4
n-octadecanol	2080	0.37	0.45	-	-
2,6,6-trimethyl-4-oxo-2-cyclohexen-1-carboxaldehyde	2096	0.37	0.32	-	-
n-heneicosane	2111	0.41	0.12	-	-
Methyl octadecanoate	2123	0.35	0.15	-	-
Abieta-8 (14), 13(15)-diene	2154	-	-	-	-
Citronellyl anthranilate	2176	2.42	-	-	1.9
1-docosene	2184	3.83	-	-	-
(E)- methyl communate	2259	0.39	-	-	1.2
Tricosane	2296	0.75	-	-	-
Total	-	99.51	99.57	99	98.63

Therefore, it can increase the growth and consequently the yield of flowers and stigma by affecting the synthesis of hormones, especially auxin. Khorramdel *et al*. [21] reported an increase in saffron stigma yield under manure and foliar application with Delfard fertilizer so that the effect of manure was more than other fertilizers, which was in line with the results of the present study. In addition, Khoramdel *et al*. [21] showed that Delfard fertilizer increased flower fresh weight by 20%.

We obtained the increased Chl content under the experimental fertilizers. The application of organic fertilizers in the soil improves soil structure, moisture storage capacity, the possibility of preparing a more suitable substrate for root growth and increasing plant Chl, and thus improves the quality characteristics of the plant [24,25]. In addition to its role in the formation of proteins, nitrogen is an essential component of the Chl molecule and its supply is associated with high vegetative growth and dark green color. The adequate amounts of animal manure increase nutrients, especially nitrogen, for root rhizosphere during the synthesis of photosynthetic pigments in leaves. Besharati *et al*. [26] reported that fertilizer treatments significantly increased Chl a content in saffron compared to the control treatment.

Phenolic compounds have antioxidant properties that prevent oxidative stress or mitigate its effects on plant cells [9]. The application of various fertilizers, especially organic fertilizers and plant growth promoters, increases the production of phenolic compounds due to the increased access of plants to nutrients, especially carbon and nitrogen. Providing the resources needed for primary plant metabolism is closely related to the biosynthesis of secondary metabolites in biochemical pathways and increasing plant growth along with improving photosynthetic efficiency increases the supply of necessary substrates for metabolic pathways, which leads to optimal production. The compounds are mentioned. Increase of TPC of saffron stigma under biological and organic fertilizers was reported by Ghanbari *et al*. [22], being consistent with the results of the present study. Also in a similar study, Bakhtiari *et al*. [7] expressed an increase in TPC of *Satureja macrantha* C.A.Mey. under organic and inorganic fertilizers. Flavonoids have significant effects on plant cells by destroying and detoxifying free radicals [9]. The use of organic and inorganic fertilizers increases the biosynthesis of flavonoid compounds in plants. Oloyede *et al*. [27] reported increased TPC in squash under chemical fertilizers. There is a significant positive relationship between antioxidant power and phenolic and flavonoid compounds in plants [28]. The schemic acid pathway is one of the most important pathways for the production of secondary metabolites, especially phenols and flavonoids in plants, which are enhanced by organic and biological fertilizers [9] Similar to the present study, improved TPC and TFC were reported in saffron [22] and *Satureja mutica* Fisch. & C.A.Mey. [9] under organic fertilizers.

EO profile of saffron was significantly changed under fertilizer treatments. Kosar *et al*. [29] showed that 2,6,6-Trimethyl-1,3-cyclohexadien-1-carboxaldehyde is the main compound of volatile composition of saffron. The change of EO composition amount can be due to the biochemical pathways of producing the corresponded compounds that are strongly corresponded to external factors like soil and foliar application of organic and inorganic fertilizers [26]. Saki *et al*. [9] showed the significant changes under manure application in *S. mutica*. Farag *et al*. [30] identified 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde as the main component of saffron EO, while Condurso *et al*. [31] represented β-cyclocitral as the major component of EO composition in saffron.

Conclusion

Fertilizers have an important role in improving soil fertility, increasing yields and improving harvest quality. However, a significant portion of fertilizers are lost, increasing agricultural cost, wasting energy and polluting the environment, which are challenges for the sustainability of modern agriculture. To meet the demands of improving yields without compromising the environment, environmentally friendly fertilizers have been developed. Environmentally friendly fertilizers are fertilizers that can reduce environmental pollution from nutrient loss by retarding, or even controlling, the release of nutrients into soil. Most of environmentally friendly fertilizers are employed in the form of coated fertilizers. Here, organic fertilizer (animal manure) was determined as the most effective type of fertilizer to improve the yield and biochemical properties of saffron. After organic fertilizer, floral and delfard were respectively improve the plant quality. To reach the special essential oil composition, it is needed the comprehensive planning of fertilizer application to achieve the given component.

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