Pak. J. Bot., 55(4): 1287-1293, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-4(19)

EFFECT OF BIOSTIMULANTS ON LEAF AND STIGMA PROPERTIES OF SAFFRON (*CROCUS SATIVUS* L.)

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Abstract

In this study, the effects of biostimulants on the volatile components in the stigma, macro-micro elements and heavy metals in *Crocus sativus* L. (saffron) leaf were investigated. The amounts of safranal in the stigma were analysed by LC-MS/MS. Following applications of biostimulants to the corms, the highest amount of safranal was obtained in 4684.06 mg kg^{-1} for the application of BIO1 biostimulants in the first year of application and 9228.77 mg kg^{-1} for the application of BIO3 biostimulants in the second year of the study. Volatile fatty acids in the saffron stigmas were also determined by GC/MS-MS instrument. The highest fatty acid content (75.50%) in saffron corms was obtained with BIO7 biostimulants applied corms whereas the highest amount of fatty acid (60.07%) in the leaves was observed with the application of BIO5. In addition to these results, application of biostimulants to the both corms and leaves of saffron resulted in an increase in iron (Fe) amount above the heavy metal limits. Obtained results are believed to provide deep knowledge about applications and effects of biostimulants on the saffron quality for the food and pharmaceutical industry.

Key words: Biostimulants, *Crocus sativus* L., Fatty acid, Heavy metal, Minerals, Nutrient element.

Abbreviations: CHNS-O: Elemental analyzer, **B:** Boron, **C:** Carbon, **Ca:** Calcium, **Cu:** Copper, **Fe:** Iron, **H:** Hydrogen, **K:** Potassium, **Mg:** Magnesium, **Mn:** Manganese, **N:** Nitrogen, **P:** Phosphorus, **Na:** Sodium, **Zn:** Zinc, **LC-MS/MS:** Liquid chromatography–mass spectrometry, **GC/MS-MS:** Gas chromatography/mass spectrometry, **ICP-OES:** Inductively Coupled Plasma Mass Spectrometer, **LOD:** limit of detection, **LOQ:** limit of quantification

Introduction

Saffron (*Crocus sativus* L.) is grown for its dried stigma. There are more than 150 volatile components in the stigma of saffron including crocins, crocetin, picrocrocin, and safranal. Safranal is responsible for the distinctive scent and aroma of saffron. Saffron also contains proteins, sugars, carotenes-vitamins, flavonoids, amino acids, minerals, fatty acids, and other chemical compounds (Ebrahimzadeh *et al*., 2000; Ozdemir *et al*., 2004; Minaei *et al*., 2017). Safranal represents about 70% of the saffron flavour and is the most important pharmacological agent of saffron. Safranal is antioxidant, cytotoxic, antitussive, anticonvulsant, antinociceptive, neuroprotective, antidepressant, and has many pharmacological effects (Hazman & Bozkurt, 2015; Asil & Ayanoglu, 2018; Rameshrad *et al*., 2018; Asil, 2021a).

In saffron studies, there are not many studies on the use of biostimulants such as natural substance, microorganism, hormones, and plant growth regulators to increase plant performance and product quality characteristics. Research should be conducted on this subject to obtain sustainable and quality products of the production system. There is a growing interest in plant biostimulants (hormone and foliar fertilizers) and microbial fertilizers to protect the nutrition and health of plants (Rubio-Moraga *et al*., 2014). The effects of hormones and plant growth regulators on the production of bulbous plants (such as saffron) are not clearly known yet. Hormones and plant growth regulators have been reported to be effective for corm growth and its proliferation (Asil & Ayanoglu, 2018). In addition, the development of biological products including useful micro-organisms (*Rhizobium* spp., *Frankia* spp., *Streptomyces* spp., *Bacillus* spp., *Pseudomonas* spp., and mycorrhizal fungi) and plant growth-promoting rhizobacteria (PGPR) was beneficial to conserve the health and yield of the crops. The effects of this higher concentration of bioactive molecules, AMF (Arbuscular Mycorrhizal Fungi) and PGPR-hosted plants for the pharmaceutical and food industries should be investigated (Sharaf-Eldin *et al*., 2008; Caser *et al*., 2019). Biostimulants are products applied to plants or soil to make plants more efficient, regulate and improve the physiological processes of plants (Sut *et al*., 2020; Asil, 2021b). It is beneficial to know the possible toxic influences of hormones, plant growth regulators, bacterial isolates and chemical fertilizers that can be applied to saffron and medicinal plants. Physical and chemical properties of essential oils depend on the type and the number of fatty acids. Fatty acids are components of foods acting as an energy source for humans and animals (Duru & Konuskan, 2014; Chichiriccò *et al*., 2016; Asil & Konuskan, 2021).

The main purpose of this study was to investigate effects of certain biostimulants applied to the corm and leaf of saffron, on the quality, chemical content, volatile components of its stigma, and macro-micro elements and heavy metals in saffron leaf.

Materials and Methods

Obtaining the samples: Saffron-applied biostimulants were applied separately to the corm and leaf. Since some of the biostimulants have both leaf and corm applications, biostimulants with this feature were applied both to the leaf and to the corm. The treatments for applying biostimulants to corm and leaf are given in (Table 1).

Table 1. Treatment of applying biostimulants to corm and leaves of saffron.

Biostimulants application code	Content of the biostimulants product	Treatments (Corm or Leaf)
BIO1	Nitrogen (N) 9.0%, Phosphorus (P2O5) 6.0%, Potassium (K2O) 4.0%, Magnesium (Water Soluble Magnesium) 0.4%, Sulphur (Combined Sulfur) 1.0%, Contains Non Plant Food Ingredients: Microbial biopolymer Poly-β-hydroxybutyrate (PHB) 0.62%, Organic matter: Hydrolyzed biomass of beneficial soil bacteria (*Bacillus megaterium* and *Pseudomonas aureofaciens*) 20.0%	4 mL
BIO2	Pseudo-monasfluorescens bacteria isolate	2 mL
BIO3	*Bacillius subtilis* (10^9 KbE/ml), *Bacillus megaterium* (10^9 KbE/ml) and *Lactococcus* spp. (10^9 KbE/ml), zeolite, ZnO	5 mL
BIO4	*Endo mycorrhiza*, *Trichoderma harzianum*, *Bacillus subtilis*, *Bacillus megaterium*	2 mL
BIO5	As a plant nutrition product containing 6% Metallic iron (EDDHA) and Na	4 g
BIO6	Hormone	1000 mg kg^{-1}
BIO7	Liquid organomineral fertilizer with NK	2 mL
BIO8	Liquid organic fertilizer containing amino acids	2 mL
BIO7	Liquid organomineral fertilizer with NK	2 mL
BIO9	Liquid micro plant nutrient	4 mL
BIO10	NPK liquid foliar fertilizer	4 mL

Plant material and its growing: This work was performed in Kirikhan (36°31'45"N 36°22'45"E) district of Hatay province, Turkiye in September 2018-May 2020. The experiment was established under field conditions and saffron corms with an average weight of 8 g were used as production material. The experiment was conducted according to the design of the randomized plot with 3 replications. In the application of corm, 500 mL water was applied at the doses indicated for ornamental plants on the labels of Control, BIO1, BIO2, BIO3, BIO4, BIO5, BIO6 and BIO7 products, by keeping them for half an hour. In leaves application, Control, BIO1, BIO5, BIO6, BIO7, BIO8, BIO9, and BIO10 products were added to 500 mL of water at the doses indicated for ornamental plants on the labels, and after the plants reached a leaf length of 10 cm, the leaves were sprayed. 10 corms were planted in each parcel to a depth of 10 cm and the first watering was accomplished directly after planting. No other watering was done in field conditions. Weed control was done manually. No drugs or fertilizers were used (Asil, 2021b).

Materials: Stigmas were harvested and dried in both years of the experiment for further study. Saffron stigmas (saffron obtained from Hatay/Turkey) were used in the experiments. Safranal standard was purchased from Sigma Aldrich (W338907-SAMPLE-K). Absolute ethanol with 99.8% purity was used for the extractions. Biostimulants were purchased from the authorized dealers and the properties of the products are given in Table 1.

Preparation of Saffron Extract: 0.020 g of saffron stigma (0.020 g) was extracted in 5 mL of absolute ethanol at ambient temperature in the dark for 24 hours. Subsequently, ultrasonication was performed for one hour. Obtained extracts were stored in the refrigerator at 4° C (Verma & Middha 2010).

LC-MS/MS Analysis: LC-MS/MS – Thermo Scientific Quantum Access model device was used. Pinnacle II Cyano (5 µm. 150 × 2.1 mm) column was used with a flow rate of 200 µL min^{-1} in acetonitrile: water (70:30 v/v) mobile phase and 0.1% formic acid. Enhanced resolution (-ER), single ion (-Q1), multiple ions (-Q1MI), advanced product ions scans (-EPI) and -MRM were used during the study. Scitex Analyst-software (version-1.4.1) was used for data collection and processing. Turbo ion injection probe (0.012 mm stainless steel) was operated in negative mode. Safranal standards were pre-screened for respective -Q1 masses. The most intense peaks of the proton-free precursor and product ions were selected, and mass parameters were optimized for infusion and flow experiments (Verma & Middha 2010).

Gas chromatography-mass spectroscopy (GC-MS/MS) analysis: GC-MS/MS analysis was accomplished using the Hewlett-Packard 6890 series GC-MS/MS analyzer. HP-5MS fused silica column (5% phenyl methyl polysiloxane 30 m 0.25 mm i.d. Film thickness 0.25 µm) was used with Hewlett-Packard mass selective detector 6890. GC-MS/MS analysis was carried out according to the standard procedure (Göktürk & Asil, 2018). The oven of the istrument was first heated to 60°C and held on 1 minute. Then, the temperature was increased by 5 degrees per minute to 200°C and kept for 1 minute. Finally, the temperature was increased by 20 degrees per minute to 280°C and waited for 21 minutes. Helium (99.9999%) as a mobile gas with 1 mL min^{-1} flow rate was used. Injector temperature was kept at 200°C.

Leaf analysis: Biostimulants were applied to the saffron leaves when the plant leaf was grown around 10 cm. Leaf samples were collected after 2 months of biostimulant applications. Nutrients and heavy metal contents in the leaves of the saffron were investigated. Phosphorus was analysed with UV spectrophotometer about Barton method. C, H and N analyzes were performed with Elemental Analyzer (CHNS-O) according to DUMAS method. Na, K and Ca analyzes were carried out using Flame Photometer device. Samples were burnt with microwave oven for analysis. Micro element analyzes were carried out with ICP-OES instrument. Correlation coefficient (R2), limit of detection (LOD) and limit of quantification (LOQ) values for each micro element are given in (Table 2).

Table 2. LOD and SD values of Micro elements.

	B	Cu	Fe	Mg	Mn	Zn
AVG.	4.004733	0.938616	1.432946	1.643032	1.146783	2.257555
SD	200.0842	200.4058	197.0988	198.6491	200.0203	203.7827
LOD	228.1173	206.9762	207.1295	210.1503	208.0478	219.5856

Table 3. The amount of safranal found in saffron stigmas by LC-MS/MS and GC-MS/MS analysis after applications of biostimulants to the corms and leaf of saffron and Duncan groups.

Biostimulants	LC-MS/MS analyzed (mg kg^{-1})		GC-MS/MS analyzed (%)	
	Safranal	Safranal	Safranal	Safranal
	1st Year	2nd Year	1st Year	2nd Year
	Applications of corm			
Control	3494.37 b*	5275.18 d*	24.47 a*	39.29 a*
BIO1	4684.06 a*	1815.34 f*	11.70 b*	3.94 g*
BIO2	2465.92 e*	1699.85 h*	5.10 f*	6.83 f*
BIO3	3434.85 c*	9228.77 a*	8.17 c*	23.05 c*
BIO4	2371.20 f*	9197.66 b*	4.48 g*	26.03 b*
BIO5	3025.62 d*	5398.06 c*	7.51 d*	15.86 d*
BIO6	1866.22 g*	1792.15 g*	6.67 e*	2.25 h*
BIO7	1572.13 h*	4740.71 e*	8.17 c*	8.34 e*
	Applications of leaf			
Control		5275.18 e*		39.29 a*
BIO1		9876.73 a*		27.74 b*
BIO5		3921.77 f*		1.48 h*
BIO6		3623.84 h*		8.47 f*
BIO7		6646.77 c*		25.46 d*
BIO8		6193.73 d*		16.41 e*
BIO9		3809.51 g*		3.52 g*
BIO10		6660.93 b*		26.45 c*

*, **:ns, It is significant at 0.01 and 0.05 levels, not found

Data analysis

Statistical analysis was done using MSTATC software and mean comparisons were also performed using Duncan's multiple range tests at $p \leq 0.05$.

Results and Discussion

Evaluation of the influences of biostimulants on safranal amount (LC-MS/MS) and ratio (GC-MS/MS) in stigma after corm and leaf applications: It is difficult to reveal complete composition of the saffron with only one extraction due to the complexity of its chemical components (Verma & Middha, 2010). Therefore, the chemical composition analysis of saffron was tried to determine using different instrumentations such as GC/MS-MS and LC/MS-MS during the study (Table 3). The effects of some biostimulants applied to the corms and leaves of saffron plant on the amount of safranal in the stigma were determined using LC/MS-MS analyzes. Volatile components of saffron stigma were also established using GC/MS-MS analyzes.

Safranal amounts after applications of the biostimulants to the corms of saffron were determined by LC-MS/MS analysis and are summarized in Table 3. The safranal ratio and amount were significantly (p<1%) affected by applications of the biostimulants. The highest amount of safranal (4684.06 mg kg^{-1}), was found with application of BIO1 biostimulants to the corms of saffron in the first year of the experiment. In the second year of the experiment, the highest amount of safranal (9228.77 mg kg^{-1}), was observed with the application of BIO3 biostimulants. Saffron leaves appear with or after flowering hence the effects of biostimulants applied to the leaves of saffron can only be detected in the second year of experiment. The stigmas were analyzed in the second year. Table 3 summarizes the amount of obtained safranal determined by LC-MS/MS analysis after applications of some biostimulants to the leaves of saffron. The highest amount of safranal with 9876.73 mg kg^{-1} was obtained with the application of BIO1 biostimulants in the second year of the experiment (Table 3). To the best of our knowledge, no study was conducted for the determination of the safranal amount by LC-MS/MS analysis. In a study Ameri *et al*., (2019) determined the amount of safranal by using UV-Vis spectrophotometer. According to their results, BIO6 biostimulants caused highest amount of safranal 150 mg kg^{-1} (48.3 E% 330 nm).

Following applications of the biostimulants to the corm and leaf of saffron the volatile components in the stigma were also determined by GC-MS/MS analysis. The amounts of safranal (%) in saffron stigmas after addition of a variety of biostimulants are summarized in Table 4. When the effects of corm biostimulant applications on Safranal amount were compared, the highest safranal content was found in the control application with 24.47% for the first year and 39.29% for the second year of the application (Table 3).

Table 4. Fractions of volatile components (fatty acids) after biostimulants application to corm of Saffron and Duncan groups.

	Fatty acid	Biostimulants (%)							
		Control	BIO1	BIO2	BIO3	BIO4	BIO5	BIO6	BIO7
1st Year	Arabonic	1.42 ns	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.00 ns
	Decanoic	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	2.38 a*
	Tridecanoic	0.00 d*	0.00 d*	2.71 b*	0.00 d*	0.00 d*	2.43 bc*	4.90 a*	2.05 c*
	Palmitic	5.47 c	11.33 a	0.00 e	0.00 e	0.00 e	8.50 b	8.50 b	1.08 d
	Myristic	0.00 d*	0.00 d*	2.73 b*	0.00 d*	0.00 d*	2.43 bc*	4.90 a*	2.05 c*
	Eicosatrienoic	20.72 d*	11.59 e*	3.84 f*	35.45 a*	0.00 g*	0.00 g*	28.00 c*	30.36 b*
	Linoleic	9.54 a*	6.76 b*	0.00 e*	5.13 d*	0.00 e*	5.37 d*	6.17 c*	6.29 bc*
	Oleic amide	2.79 g	20.68 a*	14.43 f*	19.46 d*	20.51 ab*	19.88 c*	17.25 e*	20.35 b*
	Stearic	5.24 c*	2.51 f*	5.80 b*	3.03 e*	9.05 a*	3.62 d*	3.50 d*	9.12 a*
	Oleic	0.00 b*	4.43 a*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*
	Eicosane	0.00 d*	0.00 d*	2.94 b*	6.60 a*	0.00 d*	0.00 d*	2.10 c*	1.82 c*
	Pentacosane	0.00 d*	2.82 a*	1.36 c*	2.83 a*	2.14 b*	2.41 ab*	0.00 d*	0.00 d*
	Octacosane	2.37 a*	0.00 c*	0.00 c*	0.00 c*	1.00 b*	0.00 c*	0.00 c*	0.00 c*
	Total	**47.55 d***	**60.18 c***	**36.11 f***	**72.51 b***	**32.70 g***	**44.64 e***	**75.32 a***	**75.50 a***
2nd Year	Pentanol	0.00 b*	0.00 b*	0.00 b*	0.00 b*	1.93 a*	0.00 b*	0.00 b*	0.00 b*
	Tridecanoic	4.29 b*	0.00 e*	1.32 d*	2.08 c*	0.00 e*	6.04 a*	0.00 e*	0.00 e*
	Palmitic	4.73 b*	4.36 b*	1.53 c*	0.00 d*	6.90 a*	1.48 c*	1.23 c*	0.00 d*
	Myristic	0.00 c*	0.00 c*	1.32 b*	0.00 c*	0.00 c*	3.50 a*	0.00 c*	0.00 c
	Heptanoic	0.00 b**	0.00 b**	1.05 a**	0.00 b**	1.28 a**	0.00 b**	0.00 b**	0.00 b**
	Eicosatrienoic	12.62 e*	39.77 a*	34.16 c*	0.00 f*	23.38 d*	35.80 b*	0.00 f*	0.00 f*
	Linoleic	3.20 c*	5.51 a*	0.00 e*	4.68 b*	5.73 a*	4.98 b*	2.34 d*	0.00 e*
	Oleic amide	2.70 g*	20.59 b*	20.16 c*	10.32 e*	9.25 f*	9.30 f*	24.53 a*	14.03 d*
	Stearic	8.03 c*	8.95 b*	6.44 d*	11.37 a*	2.39 f*	7.96 c*	0.00 g*	2.87 e*
	Oleic	0.00 b*	1.00 a*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*
	Eicosane	0.00 b*	2.28 a*	0.00 b*	0.00 b*	1.94 a*	0.00 b*	0.00 b*	0.00 b*
	Docosane	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	3.40 a*
	Octacosane	0.00 b*	0.00 b*	2.19 a*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*
	Total	**35.57 d***	**82.48 a***	**68.18 b***	**28.45 e***	**52.80 c***	**68.79 b***	**28.10 e***	**19.94 f***

*, **:ns, It is significant at 0.01 and 0.05 levels, , not found

As a result of biostimulant applications, it was observed that the amounts of safranal were significantly decreased. Apart from the control, BIO1 application among all of the biostimulants applied to the corms in the first year, the highest amount of safranal with 11.70% was obtained. It was found to be 26.03% with the BIO4 application to the corms of saffron in the second year (Table 3). The highest amount of safranal with 27.74% was observed with the applications of BIO1 biostimulants to the leaves of saffron (Table 3). Hormone and foliar fertilization applied to the plant increases the growing crops, the number of flowers and the amount of crocin (Tajik and Nikman, 2015), but decreases the content of picrocrocin and safranal in saffron (Cardone *et al*., 2020). In our previous report, the amounts of safranal measured by GC-MS/MS analysis were found to be 70.4% and 67.4%, respectively, in Safranbolu and Kirikhan saffrons. The amount of Safranal varies with climate of the region and location of the plant (Asil, 2018). In a study comparing Iranian and Spanish saffron analyzed with SPME-GC-MS instrument, the amounts of safranal in Spanish saffron were found to be between 29.8% and 64.5% and safranal was found to be around 36.2% to 64.3% for Iranian saffron (Farag *et al*., 2020). The amount of safranal in the same or different locations varies depending on both agricultural applications and drying conditions (Urbani *et al*., 2015). Similar results were also observed in this study.

The amounts of volatile fatty acids after biostimulants applications according to GS-MS/MS Analysis Results (%): Fatty acids are important parts of health and nutrition. Fatty acids are effectively used in the treatment of many diseases. In this study, the effects of biostimulants applied to saffron plant on the number of fatty acids were also investigated. Fatty acids were significantly affected ($p \leq 5$) on applications of biostimulant. Following applications of some biostimulants to the corms and leaves of saffron, 16 volatile fatty acids were distinguished for corm application (Table 4) and 12 volatile fatty acids were observed for the leaves application (Table 5). According to the findings, 4 essential fatty acids possessed 18 carbons and 2 essential fatty acids contained 20 and 13 carbons in their structures. Saturated fatty acids were found to be as myristic acid, palmitic acid and stearic acid. Unsaturated fatty acids were oleic acid (omega w-9) and linoleic acid (omega w-6). Unsaturated fatty acids including linolenic (35.26%) and linoleic acid (13.45%) precursors of ω-3 and ω-6, which are considered as essential for the human body, are known as lipid-lowering oils for the human body (Chichiricco *et al*., 2016). The highest amount of linoleic acid (omega w-6) was found in the application of control among the biostimulants applied to the corm of saffron. Biostimulants applications to the corms reduced the amounts of linoleic acid (omega w-6). The highest amount of Linoleic acid (omega w-6) with 9.44% was obtained with the application

of BIO1 biostimulants to the leaves of saffron in the second year of the study.

Considering the effects of the biostimulants applied to the corms on the amounts of volatile fatty acids, the highest amount of fatty acid with 75.50% was obtained with the application of the BIO7 in the first year. The lowest amount of essential fatty acids with 19.94% was also observed with the application of BIO7 biostimulants in the second year. BIO1, BIO2, BIO4 and BIO5 biostimulants resulted in the increase of their activities after the first year of the experiment. However, control, BIO3, BIO6 and BIO7 biostimulants were found to reduce their activity in the second year of the study on the application of biostimulants to the leaves of saffron, the highest amount of fatty acid with 56.66% was obtained in the BIO5 application (Table 5). It was reported that applications of hormone (Tajik & Nikman, 2015) and foliar fertilization to the saffron plant decreased the amounts of picrocrocin and safranal (Cardone *et al*., 2020) and increased the amounts of other volatile components.

Assessment of Nutrients and Heavy Metal Contents after Applications of Biostimulants to the Saffron Leaves: Apart from saffron stigmas, leaves also have important pharmacological properties. Studies carried out to reveal ingredients of saffron leaves showed that leaves extract has antidepressant, antinociceptive, anti-inflammatory, free radical scavenging, and antityrosinase effects. Active components of the saffron leaves have not been extensively discovered compared to its stigma or corms (Li *et al*., 2004; Sánchez-Vioque *et al*., 2012). This antioxidant activity could potentially be used in food products and functional ingredients for dietary supplements due to biologically valuable components including flavonoids in the flowers and leaves of crocus species (Smolskaite *et al*., 2011). Saffron leaves are also potential sources for animal feed due to their protein, oil, ash, cellulose, and many Macro- and micro element contents (Fahim *et al*., 2012). Many metals are quite necessary for human metabolism. Some of these elements are found in enzymes to activate them, and therefore, they significantly influence biochemical processes in cells. These elements are essential for human health in very small amounts, which are Na, K, Mg, Mn, Cr, Fe, Co, Ni, Cu, Zn, and Cd (Esmaeili *et al*., 2013). The human health beneficial related elements are also found in saffron leaves. Application of biostimulants to the corms of saffron showed that the highest amounts of nutrients in the plant leaves were found as 2.15% nitrogen fraction with BIO1 application, 44.68% carbon fraction with BIO4 application, 5.97% hydrogen fraction with BIO4 application, 510.7 mg kg^{-1} phosphorus with BIO1 application, 504 mg kg^{-1} sodium with the control application, 8520 mg kg^{-1} potassium with BIO6 application and 6210 mg kg^{-1} Calcium with BIO7 application. In addition to that; 19.20 mg kg^{-1} boron with BIO7 application, 4.20 mg kg^{-1} copper with BIO1 application, 2690.4 mg kg^{-1} iron with BIO3 application, 5854.2 mg kg^{-1} magnesium with BIO3 application, 51.84 mg kg^{-1} manganese with BIO3 application and 9.42 mg kg^{-1} zinc with BIO7 application were obtained (Table 6).

Applications of biostimulants to the leaves of saffron, nutrient fractions in the leaves were found as 2.08% nitrogen (N) fraction obtained from the application of BIO7, 43.52% carbon (C) fraction was obtained from the application of BIO10. Whereas 5.88% hydrogen (H) fraction was obtained from the application of BIO10, 480.6 mg kg^{-1} phosphorus (P) from the application of BIO7, 570 mg kg^{-1} sodium (Na) was obtained from BIO9 application, 7674 mg kg^{-1} potassium (K) obtained from the application of BIO8, and 6618 mg kg^{-1} calcium (Ca) was obtained from BIO7 application. In addition to that, 21.24 mg kg^{-1} of boron (B) was obtained from the application of BIO8, 4.08 mg kg-1 of copper (Cu) was obtained from the application of BIO6, 2328.0 mg kg^{-1} of iron (Fe) was obtained from the application of BİO7. In addition to 5671.8 mg kg^{-1} of magnesium (Mg) was obtained from the application of BIO7, whereas 48.72 mg kg^{-1} of mangan (Mn) was obtained from the application of BIO7 and 11.94 mg kg^{-1} of zinc (Zn) was obtained from the application of BIO6 (Table 6) were obtained.

Table 5. Fractions of volatile components (fatty acids) after biostimulants application to leaf of Saffron and Duncan groups.

Fatty acid	Biostimulants (%)							
	Control	BIO1	BIO5	BIO6	BIO7	BIO8	BIO9	BIO10
Undecanoic	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	3.02 a*
Tridecanoic	4.32 a*	0.00 d*	1.03 c*	3.32 b*	1.22 c*	0.00 d*	0.00 d*	0.00 d*
Palmitic	4.76 f*	8.68 c*	7.32 d*	6.51 e*	0.00 g*	8.88 b*	0.00 g*	10.00 a*
Myristic	0.00 c*	0.00 c*	1.03 b*	3.32 a*	1.22 b*	0.00 c*	0.00 c*	0.00 c*
Eicosatrienoic	12.64 e*	21.25 b*	25.97 a*	0.00 f*	0.00 f*	0.00 f*	20.45 c*	16.22 d*
Linoleic	3.22 d*	9.44 a*	0.00 e*	3.19 d*	4.49 c*	5.97 b*	4.86 c*	0.00 e*
Oleic amide	2.73 h*	10.17 g*	15.66 c*	18.08 b*	11.50 f*	18.38 a*	14.76 d*	13.68 e*
Stearic	8.03 a*	2.25 h*	4.30 e*	7.54 c*	3.26 g*	5.02 d*	7.92 b*	4.00 f*
Eicosane	0.00 b*	0.00 b*	0.00 b*	0.00 b*	3.90 a*	0.00 b*	0.00 b*	0.00 b*
Docosane	0.00 c*	0.00 c*	1.35 b*	0.00 c*	0.00 c*	0.00 c*	2.24 a*	0.00 c*
Octacosane	0.00 b*	2.04 a*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*	0.00 b*
Total	**35.72 g***	**53.83 b***	**56.66 a***	**41.96 e***	**25.59 h***	**38.25 f***	**50.23 c***	**43.90 d***

*, **:^{ns}, It is significant at 0.01 and 0.05 levels, not found

Table 6. Evaluation of macro and micro element content after applications of corm and leaf biostimulants in saffron leaf.

Biostim-ulants	N (%)	C (%)	H (%)	P (mg kg^{-1})	Na (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	B (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
Applications of corm													
Control	1.54	41.62	5.73	367.3	504	7098	6096	16.26	2.88	1317.0	3960.0	29.22	6.72
BIO1	2.15	41.24	5.76	510.7	444	7908	5460	12.90	4.20	1468.8	3715.8	38.10	8.04
BIO2	1.56	37.55	5.39	315.7	378	5760	5574	14.64	1.98	1422.0	3609.0	25.32	6.00
BIO3	1.60	43.69	5.80	405.4	420	5010	6156	9.42	3.24	2690.4	5854.2	51.84	7.98
BIO4	1.81	44.68	5.97	482.8	378	4740	5538	15.48	3.18	1580.4	4130.4	33.96	7.50
BIO5	1.87	37.59	5.28	371.1	336	7038	5322	11.70	3.00	2593.8	5852.4	48.48	9.12
BIO6	2.02	41.45	5.53	463.0	426	8520	4776	15.12	3.78	1182.6	3271.8	22.62	7.98
BIO7	1.99	37.21	5.11	378.4	408	5454	6210	19.20	3.42	2089.8	5434.2	41.76	9.42
Applications of leaf													
Control	1.54	41.62	5.73	367.3	504	7098	6096	16.26	2.88	1317.0	3960.0	29.22	6.72
BIO1	1.55	39.73	5.31	327.0	378	5202	6270	12.78	1.74	1355.4	4080.6	25.98	4.86
BIO5	1.68	41.08	5.59	385.8	522	6660	5862	12.84	3.00	1725.0	4612.8	32.70	8.16
BIO6	1.88	40.33	5.63	426.9	366	6600	5322	16.14	4.08	1715.4	4650.0	37.56	11.94
BIO7	2.08	41.11	5.62	480.6	390	6288	6618	9.66	2.94	2328.0	5671.8	48.72	7.86
BIO8	1.64	41.72	5.76	394.1	438	7674	5574	21.24	3.24	844.2	2817.0	19.68	5.88
BIO9	1.61	41.89	5.68	383.1	570	6270	6234	21.00	1.68	784.2	2984.4	15.66	5.58
BIO10	1.63	43.52	5.88	417.1	450	5418	6192	18.60	1.92	845.4	2886.0	16.20	5.58

If saffron leaves are considered as an animal feed, the number of mineral substances in the feed must be sufficient and within the appropriate limits for animal health. The amounts of Magnesium, Calcium, and Phosphorus elements and their ratios to each other are very important for animal health. In other words, Ca/P and K/ (Ca+Mg) ratios in the feed or leaves should be determined (Polat and Bayraklı, 2019). After applications of biostimulants to the corms of saffron, the Ca/P ratios were found to be as control (16.6), BIO1 (10.7), BIO2 (17.7), BIO3 (15.2), BIO4 (11.5), BIO5 (14.3), BIO6 (10.3) and BIO7 (16.4). Furthermore, the applications of biostimulants to the leaves of saffron, the Ca/P ratios were obtained as control (16.6), BIO1 (19.2), BIO5 (15.2), BIO6 (12.5), BIO7 (13.8), BIO8 (14.1), BIO9 (16.3), and BIO10 (14.8). If the Ca/P ratio is higher than 2.0, animals can be poisoned after feeding. According to the findings, Ca/P ratios were found to be high in all biostimulants applications. If K/ (Ca+Mg) ratio becomes higher than 2.2, the risk of tetany disease could be observed in animals (Ayan *et al*., 2010). K/ (Ca+Mg) ratios for all applied biostimulants were found to be less than 2.2.

According to FAO/WHO reports, acceptable heavy metal limit values for plants must be as follows; Pb (2 mg/kg), Cd (0.5 mg/kg), Ni (5 mg/kg), Cr (0.5 mg/kg), Cu (5 mg/kg), Fe (30 mg/kg) and Zn (30 mg/kg) (FAO/WHO). Following the application of biostimulants to the leaves and corms of the saffron, the amounts of copper (Cu) and zinc (Zn) were found to be below the limits. The limit of toxicity of iron (Fe) in plants is higher than 1000 mg kg-^{1}. The amounts of iron (Fe) with applications of biostimulants to the corms of saffron were found to be higher than the limits.

Applications of BIO8, BIO9 and BIO10 biostimulants to the leaves of saffron resulted in lower amounts of Fe than the accepted limits (Table 6).

Conclusion

In conclusion, the effects of some biostimulants applied to the corms and leaves of the saffron plant were determined on the amount of safranal, volatile fatty acids, toxic volatile components and macro-micro elements and heavy metals in saffron leaf. Some of the biostimulants showed their effects in the first year of the study, and others were effective in the second year of application. This study provides an appropriate biostimulant selection for perennial and corm plants, especially Saffron, to gain the desired yield. The amount of safranal was increased by application of BIO1 biostimulants for the first year, and enhanced by application of BIO3, BIO4, BIO5 and BIO7 biostimulants for the second year. This study provides important information on the effects of some biostimulants for perennial and corm plants such as saffron. In addition to these results, application of biostimulants to both corms and leaves of saffron resulted in an increase in iron (Fe) amount above the heavy metal limits. The amounts of iron for the applications of biostimulants to the corms of saffron were found to be higher than the toxic limit values, but applications of BIO8, BIO9 and BIO10 biostimulants to the leaves of saffron showed the lower amounts of iron (Fe) below the toxic limits. Fortunately, the amounts of both copper (Cu) and Zinc (Zn) were found to be in acceptable heavy metal limits. The toxic effect and the amount of safranal and fatty acids of the stigmas were investigated depending on the types of biostimulants. The present results shall provide deep knowledge about applications and effects of biostimulants are the saffron for the food and pharmaceutical industry.

Acknowledgements

This work was supported by Hatay Mustafa Kemal University Coordinatorship of Scientific Research Projects (project # 18.M.092).

References

Ameri, R., M. Azizi and A. Mollafilabi. 2019. Effects of gibberellic acid and naphthalene acetic acid on saffron plant (*Crocus sativus* L.) under field conditions. *J. Appl. Hort.*, 21(2): 123-130. DOI: https://doi.org/10.37855/jah.2019.v21i02.21

Asil, H., and F. Ayanoglu. 2018 The effects of different gibberellic acid doses and corm cutting methods on saffron (*Crocus sativus* L.) yield components in Turkey. *Fresenius Environ. Bull.*, 27(12 A). 9222-9229.

Asil, H. 2018. GC-MS analysis of volatile components of Safranbolu and Kirikhan saffron (*Crocus sativus* L.) prepared by ultrasonic extraction. *Fresenius Environ. Bull.*, 27(12 B): 9557-9563.

Asil, H. 2021a. Evaluation of the effects of different storage times on pharmacological agents of Saffron (*Crocus sativus* L.) (safranal. crocin and crocetin) and their quality characteristics. *Celal Bayar Üniv. Fen Bilim. Derg,* 8(2): 263-269.

Asil, H. 2021b. The effect of different biostimulants applications on corm characters of saffron (*Crocus sativus* L.). Academic Reseach in Life Sciences for Sustainibility. Publisher: Published by Artikel Akademi

Asil, H. and D.B. Konuskan. 2021. Investigation of fatty acid compositions of obtained from different oilseeds by cold pressed method. *MKU.Tar.Bil. Derg.,* 26(3): 70-678, https://doi.org/10.37908/mkutbd.959699.

Ayan, I., H. Mut., O. Onal-Asci., U. Basaran and Z. Acar. 2010. Effect of manure application on the chemical composition and nutritive value of rangeland hay. *J. Anim. Vet. Adv.*, 9(13): 1852-185.

Cardone, L., D. Castronuovo., M. Perniola., N. Cicco and V. Candido. 2020. Saffron (*Crocus sativus* L.), the king of spices: An overview. *Sci. Hort.*, 272: 109560. https://doi.org/10.1016/j.scienta.2020.109560.

Caser M., S. Demasi., Í.M.M. Victorino, D. Donno, A. Faccio, E. Lumini and V. Scariot. 2019. Arbuscular mycorrhizal fungi modulate the crop performance and metabolic profile of saffron in soilless cultivation. *Agronomy,* 9(5): 232.

Chichiriccò, G., B. Lanza, P. Piccone and A. Poma. 2016. Nutrients and heavy metals in flowers and corms of the Saffron Crocus (*Crocus sativus* L.). *Med. Aromat. Plants*, 5(254): 2167-0412.

Duru, S. and D.B. Konuskan. 2014. Increasing level of oleic acid in vegetable oil and its effects on oil quality. *J. Food*, 39(6): 379-385.

Esmaeili, N., H. Ebrahimzadeh., K. Abdi., M. Mirmasoumi., N. Lamei and M.A. Shamami. 2013. Determination of metal content in *Crocus sativus* L. corms in dormancy and waking stages. *Iran. J. Pharm. Res.*, 12(1): 31.

Ebrahimzadeh, H., T. Radjabian and R. Karamian. 2000. In vitro production of floral buds and stigma-like structures on floral organs of *Crocus sativus* L. *Pak. J. Bot.*, 32(1): 141-150.

Fahim, N.K., S.F. Janati and J. Feizy. 2012. Chemical composition of agriproduct saffron (*Crocus sativus* L.) petals and its considerations as animal feed. *J. Food*, 37: 197-201.

Fallahi, H.R., M. Aghhavani-Shajari., H. Sahabi and H. Feizi. 2017. Mother corm weight and soil amendment improves the vegetative and reproductive growth of saffron (*Crocus sativus* L.). *J. Med. Spice Plants*, 22(3): 110-114.

Farag, M.A., N. Hegazi., E. Dokhalahy and A.R. Khattab. 2020. Chemometrics based GC-MS aroma profiling for revealing freshness, origin and roasting indices in saffron spice and its adulteration. *Food Chem.*, 331: 127358.

Göktürk, E. and H. Asil. 2018. GC-MS Analysis of Saffron (*Crocus sativus* L.) Stigma Extract Grown in Hatay / Kirikhan. *Türk Tarım Doğa Bilim. Derg.*, 5(3): 317-321.

Hazman, Ö. and M.F. Bozkurt. 2015. Anti-inflammatory and antioxidative activities of safranal in the reduction of renal dysfunction and damage that occur in diabetic nephropathy. *Inflammation*, 38(4): 1537-1545.

Khan, M.A., S. Naseer., S. Nagoo and F.A. Nehvi. 2011. Behaviour of saffron (*Crocus sativus* L.) corms for daughter corm production. *J. Phytol.,* 3(7): 47-49.

Li, C.Y., E.J. Lee and T.S. Wu. 2004. Antityrosinase Principles and constituents of the petals of *Crocus sativus*. *J. Nat. Prod.*, 67(3): 437-440. https://doi.org/10.1021/np0302854.

Minaei, S., S. Kiani., M. Ayyari and Ghasemi-Varnamkhasti. 2017. A portable computer-vision-based expert system for saffron color quality characterization. *J. Appl. Res. Med. Aromat. Plants*, 7: 124-130. https://doi.org/10.1016/j.jarmap.2017.07.004.

Ozdemir, C., Y. Akyol and E. Alcitepe. 2004. Morphological and anatomical studies on two endemic Crocus species of Turkey area. *Pak. J. Bot*., 36(1): 103-114.

Polat, H. and F. Bayrakli. 2019. Nutrient content and crude protein of some plants in natural rangeland in Konya Region. *J. Bahri Dağdaş Crop Res*., 8(1): 132-147.

Rameshrad, M., B.M. Razavi and H. Hosseinzadeh. 2018. Saffron and its derivatives, crocin, crocetin and safranal: a patent review. *Exp. Opin. Ther. Pat.*, 28(2): 147-165.

Rubio-Moraga, A., O. Ahrazem, R.M. Pérez-Clemente., A. Gómez-Cadenas., K. Yoneyama, J.A. López-Ráez and L. Gómez-Gómez. 2014. Apical dominance in saffron and the involvement of the branching enzymes CCD7 and CCD8 in the control of bud sprouting. *BMC Plant Biol.*, 14(1): 171.

Sánchez-Vioque, R., M.F. Rodríguez-Conde., J.V. Reina-Ureña., M.A. Escolano-Tercero., D. Herraiz-Peñalver and O. Santana-Méridas. 2012. *In vitro* antioxidant and metal chelating properties of corm, tepal and leaf from saffron (*Crocus sativus* L.). *Ind. Crops. Prod.*, 39: 149-153.

Sharaf-Eldin, M., S. Elkholy, J.A. Fernández, H. Junge, R. Cheetham, J. Guardiola and P. Weathers. 2008. Bacillus subtilis FZB24® affects flower quantity and quality of saffron (*Crocus sativus*). *Planta Med.*, 74(10): 1316.

Smolskaite, L., T. Talou., N. Fabre and P.R. Venskutonis. 2011. Valorization of saffron industry by-products: bioactive compounds from leaves. In: *Baltic Conference on Food Science and Technology Food Baltt*, Vol. 6:pp. 67-72.

Sut, S., I. Ferrarese, S.S. Shrestha, G. Kumar, A. Slaviero, S. Sello and S. Dall'Acqua. 2020. Comparison of biostimulant treatments in *Acmella oleracea* cultivation for alkylamides production. *Plants*, 9(7): 818.

Tajik, S. and V. Niknam. 2015. Effects of salicylic acid on carotenoids and antioxidant activity of saffron (*Crocus sativus* L.). *Appl. Food Biotechnol.,* 2(4): 33-37. https://doi.org/10.22037/afb.v2i4.9739.

Urbani, E., F. Blasi, C. Chiesi, A. Maurizi and L. Cossignani. 2015. Characterization of volatile fraction of saffron from central Italy (Cascia, Umbria). *Int. J. Food Prop.*, 18(10): 2223-2230. https://doi.org/10.1080/10942912.2014.968787.

Verma, R.S. and D. Middha. 2010. Analysis of saffron (*Crocus sativus* L. Stigma) components by LC–MS–MS. *Chromatographia*., 71(1-2): 117-123.

(Received for publication 18 March 2022)