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Effect of Tryptophan and Glutamic Acid on Phytochemical Traits of Iranian and Afghan Saffron

Naseer Mukhlis[1] and Azizullah Kheiry[2]

[1]Assistant, Professor, Department of Horticultural Sciences, Faculty of Agriculture, DaikuniUniversity, AFGHANISTAN.

[2]Asosciated Professor, Department of Horticultural Sciences, Faculty of Agriculture, Zanjan University, Zanjan, IRAN.

[1]Corresponding Author: naseer.bamyani@gmail.com

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ABSTRACT

In order to investigate the effect of Tryptophan and Glutamic acid amino acids on physiological traits of saffron, a factorial experiment was conducted in a randomized complete block design with three replications in 2019 at the research farm of Zanjan University. Experimental treatments include three genotypes (Iranian, Afghani 1 and Afghani 2) as the main treatment and the amino acid Tryptophan at two levels (1 and 2 mM) and Glutamic acid at two levels (1 and 2 mM) as sub-treatments, were considered with witnesses. The results showed that Tryptophan and Glutamic acid treatments had a significant effect about one percent (0.01 %) on the main traits including flower number, flower dry weight, vegetative body, phenol yield, antioxidant activity and crocin. Also, yield traits of phenol, flavonoids and antioxidants treated with two amino acids showed a significant difference about five percent (0.05 %). The maximum yields of phenol and flavonoids at 2 mM Tryptophan concentration were 0.35 and 0.026 mg / g, respectively, and the lowest yields at 1 mM Glutamic treatment were 0.34 and 0.02 mg / g, per hectare. In general, different levels of Tryptophan and Glutamic acid can play an effective role in improving the physiological traits and production of this product. Application of 1 mM Glutamic acid to produce the maximum vegetative body of the plant, treatment of 1 and 2 mM both amino acids to improve physiological parameters and 1 mM Glutamic acid for the performance of secondary metabolites is desirable and recommended.

Keywords- Amino acids, Antioxidants, Crocin, Flavonoids, Saffron.

I. INTRODUCTION

Saffron (*Crocus sativus*) is a perennial plant belonging to the Iridaceae family. Its leaves are 6 to 10 standing, saffron flowers are 1 to 2 purples in color and relatively large (Wendelbo and Mathew, 1975). Saffron is a triploid vegetable that is sterile with 24 chromosomes. The saffron plant has an underground stem and a solid bulb called corm, and its propagation is done only by cultivating the corm and creating a new daughter corm from the mother corm, due to its sterility. Gresta et al., 2009). Saffron is the most valuable agricultural and medicinal product in the world (Koocheki et al., 2011). Its stigma is used in the food industry due to its colored and aromatic substances. Saffron is one of the agricultural products of Iran and Afghanistan, which is important in improving the economic andsocial status of saffron growers even in dry and low-rain regions (Maqsoudi, 2009).

Saffron is widely used in traditional medicine and pharmaceutical industries. The dried red stigma of saffron is not only a spice but also a very popular medicinal plant in traditional medicine, which is used against muscle cramps, asthma, menstrual disorders, liver disease, cancer treatment and strengthening the digestive system (Ferrara et al., 2014). Currently, saffron is the most expensive spice in the world and Iran is the most important producer of this product. The importance of saffron in Iran can be examined from various aspects of the lack of water (compared to other agricultural products), social and political, job creation in terms of export development. In saffron genotypes, the observed differences are in different appearance traits. Paying

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attention to the amount of diversity is important in plant breeding, because the appropriate figures are provided. This diversity comes from the influence of environmental and genetic factors (Farshad Far, 2016).

The genotypes of cultivated saffron from the point of view of genetic diversity have been done by different researchers with different morphological and molecular methods, in which less protein has been used for diversity. Most of the SDS PAGE studies on saffron have been conducted with the aim of investigating a specific protein in this plant, and less have been used to investigate genetic diversity. Considering the economic importance of saffron as well as the wide genetic diversity of the genus Crocus in Iran, it is necessary to investigate this diversity and compare the similarity and difference between different species of this genus with C. sativus and also different genotypes. Saffron is a mutational phenomenon, and the morphological diversity of genotypes does not follow it. The environmental conditions of the flowering time and the length of the flowering period may have an effect. By using different molecular markers, no genetic variation has been observed between the genotypes of *Crocus sativus* (Fluch et al., 2009). Which is cultivated in some countries including Iran, Greece, Morocco, Spain, France, India, China, Pakistan and Afghanistan (Izadpanah F. A Kalantari et al., 2015). Iran is known as the largest and most important saffron producing country in the world (Rezvani Moghaddam, 2015). Saffron contains more than 150 volatile and fragrant substances. It also has a number of active and inactive carotenoid compounds including zeaxanthin, lycopene and various types of alpha and beta carotenes, and it has been proven that this plant is a rich source of phenolic contents with antioxidant activity (Goli et al., 2002). The saffron stigma contains large amounts (over 8% of dry weight) of the apocarenoid crostin and crocins (glycosylated forms of crostin. Crocin is responsible for the red color of the stigma, picrocrocin is responsible for the bitter taste, and safranal is responsible for the spicy aroma of saffron (Cabllero et al., 2007). Amino acids directly and indirectly affect the physiological activities, growth and development of plants (Faten et al., 2010). Amino acids are the building blocks of proteins that perform structural, metabolic and transport functions in plants (Liu et al., 2008). Amino acids are the precursors of plant hormones and other growth substances. Amino acids increase the productivity of plant metabolism and increase product quality and yield. Increasing the plant's tolerance and improving it in biological stress, facilitating the absorption of nutrients, transferring the use and increasing the quality characteristics of the product (Calvo et al., 2014).

Increasing the process of plant respiration, photosynthesis, protein synthesis, enhances plant growth and performance (Davies, 2010). Foliar spraying of radish plants with compounds containing amino acids increases the concentration of nitrogen in the branches. The use of amino acid increased potatovegetative growth, increased total nitrogen, phosphorus and potassium in strawberry shoots, as well as yield, weight, TSS, vitamin C and total sugar concentration of fruits (Ahmadi, 2018). In adverse environmental conditions, amino acid production is reduced or stopped. The aromatic amino acid phenylalanine and tryptophan in plants are not only essential components for protein synthesis, but also as biosynthetic precursors they promote the production of a wide range of plant secondary metabolites (Tzin and Galili, 2010).

Also, studies showed that tryptophan plays a role as an important precursor in the biosynthesis of auxin (indole acetic acid) (Ramaiah et al., 2003). Foliar spraying with tryptophan at a concentration of 100 mg/liter resulted in the highest growth parameters including plant height, flower dry weight, and the most components of plant yield and plant seeds, as well as chlorophyll (a+b) content of *Hibiscus subaerial* L. had (Gendy and Nosir, 2016). Glutamic acid is one of the amino acids that play a role in seed germination and as a precursor in the synthesis of chlorophyll, as well as in the synthesis of other amino acids, and prevent the blocking of air holes due to adverse environmental conditions. Tryptophan is a precursor for the biosynthesis of auxin hormone, which is one of the most basic factors of plant metabolism (Asadi, 2020).

II. MATERIALS AND METHODS

In order to investigate the effect of tryptophan and glutamic acid on the morphological and phytochemical traits of Iranian and Afghan saffron in the crop year of 2018 and 2019 in the form of a factorial experiment in the form of a randomized complete block design in three replications in the research farm of Zanjan University with a latitude of 35 degrees and 25 minutes and Longitude 47 and 10 minutes with an approximate height of 1663 meters above sea level. The test treatments included the amino acid glutamic acid and tryptophan at the level of 1 and 2 mM. At first, saffron seeds were planted, sorted, measured, and treated with amino acid glutamic acid and tryptophan each at two levels (1 and 2 mM), immersed in different concentrations. In order to sample the soil of the test site from a depth of 030 cm, it was transferred to the soil science laboratory of Zanjan University in order to determine the physical, chemical and texture characteristics of the soil. The physical and chemical properties of farm soil are listed in Table 1. Preparing the farm land, plowing and disking operations, leveling the land, removing stones and clods, weeds and plotting it 5 meters long and 2 meters wide, the distance between the rows 50 cm, the distance between the stumps 20 cm and planting depth the tubers were planted between 15 and 20 cm of soil.

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Table 1: Physical and chemical criteria of soil in the cultivation area

K (mg. kg^{-1})	P (mg.kg^{1-})	Total N	Texture of soil			PH	EC (dS.m^{-1})	Organic material (%)
			Sand (%)	Silty(%)	Clay (%)			
174	22	0.06	40	27	33	7.6	0.96	1.18

In each plot, 10 rows with a length of 2 meters with a density of 100 tubers were cultivated with three replications with 45 experimental units. The first irrigation was done after cultivation. Then the cell breaking operation took place. Irrigation was done in two stages during the growing season. Of course, due to the climatic conditions of the region, the second irrigation was done after collecting the flowers. Weed control and weeding was carried out manually. No chemical pesticides and herbicides were used during the experiment. The appearance of saffron flowers occurred on the firstof November and flowering continued for 30-50 days after the appearance of the first flowers. The flowers were collected by hand once a day and in order to investigate the effect of the treatments on different traits, samples were taken at the flowering stage. The flowers that appeared daily (between 6 and 8 in the morning) were collected, counted and transferred to the laboratory to measure the wet and dry weight of the stigma, and the flower head was separated from the stigmas, and then the stigmas were placed in the oven (at a temperature of 40 degrees Celsius for 24 hours) dry and their dry weight was weighed with a sensitive scale with an accuracy of 0.0001 g. In order to calculate the length of flower and stigma, the flowers harvested from each replicate were randomly selected daily and measured in centimeters using a ruler. And finally, the average length of flowers and stigmas during the flowering period was considered as the average length of flowers and stigmas for each plot.

III. MEASUREMENT OF PHYSIOLOGICAL TRAITS

To measure total phenol content, it was tested using Folin ciocaltue colorimetric method (Meda et al., 2005). First, 900 microliters of ionized water were prepared with 100 microliters of extract, and 250 microliters of Folin Ciocalto reagent was mixed and kept at room temperature for 5 minutes. Then 250 microliters of sodium carbonate were added and finally, after 30 minutes, it was kept at room temperature in the dark. Its absorbance was read using a spectrophotometer at a wavelength of765 nm. Gallic acid was used as a standard in this method. The results were reported based on milligrams of Gallic acid/gram of dry weight. Total flavonoid content was determined by aluminum chloride colorimetric method provided by (Kaiju et al., 2006). First, 1400 microliters of ionized water, 10 microliters of extract prepared with methanol (80%), 50 microliters of 10% aluminum chloride solution, and 50 microliters of 1 M potassium acetate solution were added. And after staying in the dark for 30 minutes, it was kept at room temperature and then it was read at a wavelength of 415 nm. In this method, quercetin was used as a standard. The results were expressed based on milligrams of quercetin/gram of dry matter. To determine the antioxidant activity of the stigma and petals of saffron, the method of measuring the reduction of radical capacity with the help of 2, 2 diphenyl 1 picrylhydrazyl (DPPH) was used (Turkmen et al., 2005). This compound changes color from purple to yellow by taking an electron from the antioxidant compound. The more the amount of antioxidant substance increases, the more DPPH is consumed and the purple color tends to yellow more (Haghirossadat et al., 2010). In test tubes with lids and covered with aluminum foil (to prevent the effects of light), 60 microliters of saffron stigma extract were mixed with 1440 microliters of DPPH 0.004% methanol and kept for 30 minutes at 25°C. It was kept in an incubator. And then its absorption was read with a spectrophotometer at a wavelength of 517 nm. The percentage of inhibition of free radicals was calculated with the following formula:

$$I= (Ac-As)/Ac*100$$

I: Percentage of free radical inhibition.
Ac: Absorption rate for the control sample.
As: Absorption rate of plant sample.

The main compounds of saffron, crocin, picrocrocin and safranal, were read by a spectrophotometer (UV 6505) using the standard method (ISO/TS 3632 2, 2003) ISO 3632. 5 mg of dry stigma was weighed with a scale with an accuracy of 0.0001 g in a watch glass and transferred to a 100 ml flask. Then 90 ml of ionized water was added to it. The prepared extract was placed in a balloon for 20 minutes on a magnetic stirrer at a speed of several hundreds of revolutions per minute. Then the said extract was brought to a volume of 100 ml and the lid was closed and mixed well until the same solution was obtained. This solution was quickly removed from light and filtered through a strainer. Distilled water was used as a control and regulator of the spectrophotometer. Then, the number of secondary metabolites of saffron, crocin, picrocrocin and safranal were measured with a spectrometer at wavelengths of 440, 257 and 330 nm, respectively (Vakili Qartaul, 2016). The amounts of each compound were calculated using the following formula:

$$A(\lambda max) = D*10000/m*(100-wMV)$$

λmax: The values of each combination are colored.
D: The absorption rate of each of the mentioned items.
M: Sample weight in grams.
WMV: The humidity level is typical.

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In order to analyze the statistical data obtained from the experiment, SAS 9.1.3 Portable software was used. Means were compared with each other using Duncan's test at a probability level of 5%. Excel software was used to draw graphs and figures.

IV. RESULTS AND CONTROVERSY

The results of analysis of variance (Tables 2 and 3) showed that glutamic acid and tryptophan treatment had a significant effect on the studied traits. So that the attributes of number of flowers, dry weight of flowers, total phenol, antioxidant activity and crocin were significant in the simple andreciprocal effects of glutamic acid and tryptophan treatment at the possible level of 1%. In the characteristics of corm fresh weight, flower fresh weight, and stigma dry weight, with the simple and reciprocal effects of glutamic acid and tryptophan treatment, they were significant at the possible level of 5%. Therefore, the traits of stigma length, flower length in simple effects were not affected by glutamic acid and tryptophan treatment. The results of analyzing the variance of the data of the morphological traits and also the performance of these traits per hectare (Table 2) showed that glutamic acid and tryptophan treatment had a significant effect on the studied traits. So that the traits of number of flowers, dry weight of flowers, total phenol, antioxidant activity and crocin in the simple and reciprocal effects of glutamic acid and tryptophan treatment at the probability level of 1%, as well as the traits of corm fresh weight, flower fresh weight, stigma dry weight, with simple effects and the interaction of glutamic acid and tryptophan treatment was significant at the possible level of 5%.

Table 2: Analysis of variance of Philological traits

S.O. V	(df)	Phenol (mg/g)	Flavonoids (mg/g)	Anti- Oxidant (%)	Crocin $A^{1\%}_{1\,cm}$ (λ_{400})
Replication	2	0/05 ^{ns}	0/06 ^{ns}	0/09 ^{ns}	15/63^{ns}
Genotype	2	0/108**	0/05 ^{ns}	0/01184 **	7/74 ^{ns}
Main plot error	4	0/01^{ns}	0/06^{ns}	0/07^{ns}	29/79^{ns}
Treatment	4	0/012^{ns}	0/029^{ns}	0/07^{ns}	70/30 ^{ns}
Genotype and Treatment	8	0/029*	0/016*	0/013*	193/80 **
Sub-plot error	24	0/08	0/015	0/04	51/75
(C.V)		0/86	16/41	12/40	27/25

ns and **: represent non-significant and significant at 5 % and 1 % level, respectively.*

Physiological traitsTotal phenol:

The results of analysis of variance (Table 2) showed that the simple effects of tryptophan and glutamic acid treatments on the significant increase in the number of phenolic compounds were significant at the probable level of 0.01. Also, the mutual effects of the treatments have a significantdifference at the probability level of 0.05. The highest amount of phenol with (0.351372) mg/g was observed in the treatment of 2 mM glutamic acid level. Other levels of mutual effects of the treatments also increased the amount of phenol compared to the control (Figure 1).

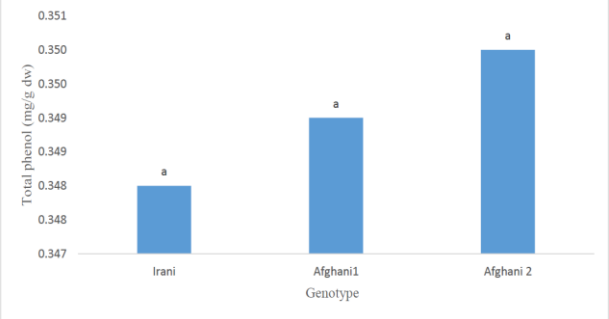

Figure 1: Simple effect of glutamic acid and tryptophan genotype on Total Phenol

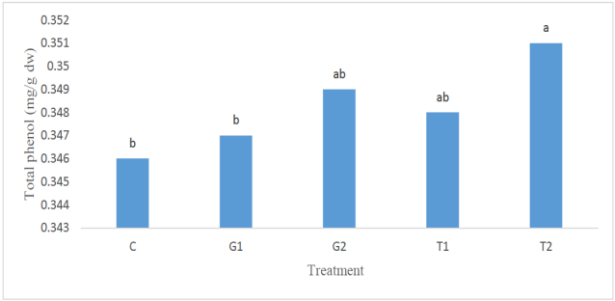

Figure 2: Simple effect of glutamic acid and tryptophan treatment on Total Phenol

Flavonoid:

The results of analysis of variance (Table 2) showed that the simple effects of genotype in glutamic acid and tryptophan treatments on the amount of plant flavonoid had a significant difference at the level of 0.05. The results obtained from the average comparison table show the positive effect of different levels of glutamic acid and tryptophan treatments and their mutual effects in total flavonoids. The highest level of flavonoid (0.02683) mg/g was obtained in the control (Figure 3). Flavonoid and phenolic compounds are considered as secondary metabolites of the plant in addition to being strong antioxidants. In general, one of

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the most important factors that affect the production of secondary metabolites in plants is environmental stress (Bernstein et al., 2010).

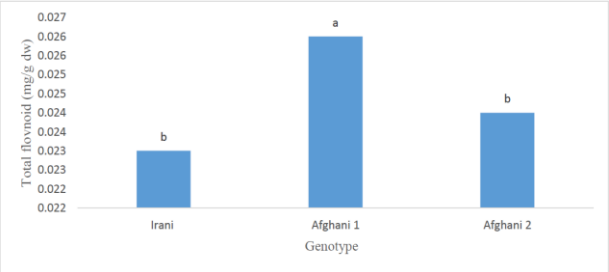

Figure 3: Simple effects of glutamic acid and tryptophan treatments on Flavonoids

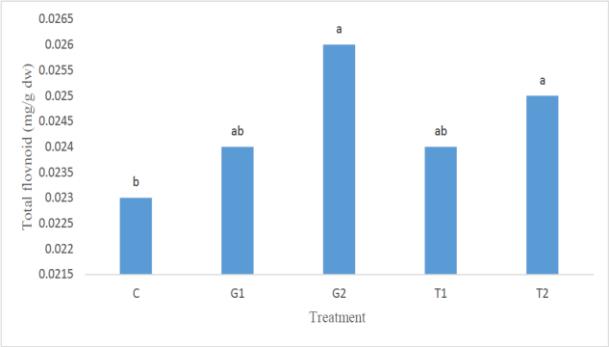

Figure 4: Simple effects of glutamic acid and tryptophan treatments on total flavonoids

Antioxidant activity:

Based on the results obtained from analysis of variance (Table 2), the simple effects of genotypes have a significant difference at the probability level of 0.01 and the treatment has a significant difference at the level of 0.05. Based on the results of comparing the simple effects of the treatments, the control treatment increased the antioxidant activity compared to the treatments. Although increasing the concentration of glutamic acid and tryptophan treatments decreased the antioxidant activity at some levels. The mutual effects of the treatments reduce the antioxidant activity comparedto the simple effects, so that the mutual effects of the second levels are slightly different, so the mutual effects of the first and third levels of both treatments were at the same level as the other treatments (Figure 5).

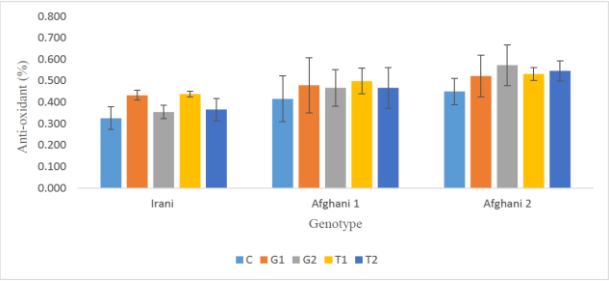

Figure 5: Interactions of glutamic acid and tryptophan on Anti-Oxidant activities

Crocin:

The results of analysis of variance (Table 2) of the interaction effects of genotypes in the treatment have a significant difference at the probability level of 0.01. The amount of color of genotypes is different. The results of comparing the mean of Figure (6) of the mutual effects of Afghani genotype1 treatments have the highest amount of color and the lowest amount belongs to Afghani genotype 1in tryptophan treatment. Crocin and picrocrocin are spectrally degraded in the stigma during the storage drying and extraction process. This action is dependent on temperature, humidity and light radiation.

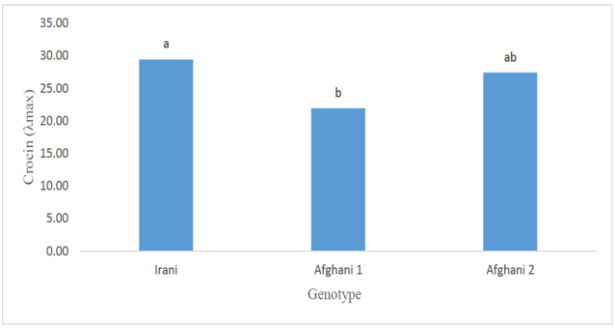

Figure 6: Simple effect of glutamic acid and tryptophan genotype on secondary metabolites Crocin

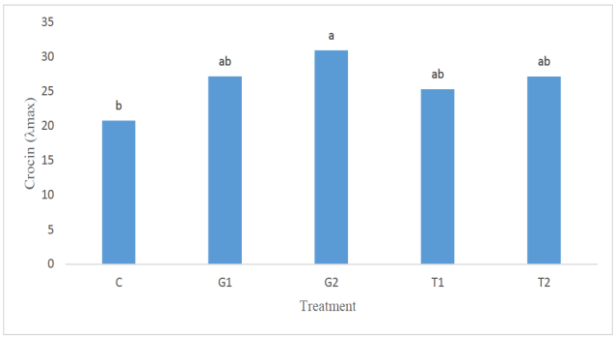

Figure 7: Simple effect of glutamic acid and tryptophan treatment on secondary metabolites Crocin

Physiological traits:

Phenolic compounds include a large group of secondary metabolites, which include many cyclic compounds such as phenol compounds, flavones, flavonoids, tannins, lignin, and even cyclic amino acids such as tryptophan and tyrosine. These compounds have many ecological and physiological roles such as defense and antioxidant roles (Andre et al., 2009). Based on the average comparison results (Figure 1), the interaction effects of genotypes of 2 mM glutamic acid treatment increased yield and total phenol in saffron plants.

Although the amino acid tryptophan had no effect on the total phenol of saffron plant. Reports showed that the total phenolic and flavonoid contents of saffron stigma are higher than those of plants (Kirimi et al., 2010). Purple mint (Perilla frutescens) extract had the

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highest amount of total phenolic compounds in the full flowering stage, while the highest DPPH free radical inhibitory power of the extract was observed in the early vegetative stages (Gai et al., 2017).

During the growth period of marjoram (*Origanum majorant* L), total phenolic concentration was affected by both phenological stages and weather factors and significantly changed with growth stage (Sellami et al., 2009). The results of other studies showed that the application of amino acid increasedthe amount of phenol content in thyme (Reda et al., 2005). This report is consistent with the results obtained in this research. A study on eucalyptus showed that under water stress conditions, phenolic compounds increased in the plant (Schwambach et al., 2008). reported that the phenolic compounds of the ethanol extracts of clove (Eugenia caryophyllata Thunb) and lavender (*Lavandula stoechas* L) buds were lower than those of Crocus (Gul chain et al., 2004). Flavonoids are also a large group of phenolic compounds with more than 3000 structures and one of the most important secondary compounds in plants, which are mainly found in most medicinal plants in different amounts. Also, the results comparing the average (Figures 2 and 3) of the simple effects of amino acid treatments, glutamic acid and tryptophan, did not show favorable effects compared to the control in saffron plants. Although the amino acid treatment of 2 mM glutamic acid increased total phenol in saffron plant. According to the results of many researchers, flavonoids are among the secondary metabolites whose biosynthetic pathways are altered under environmental conditions (Kutchan, 2001).

There are about 4000 types of compounds belonging to the group of flavonoids in plants, which are synthesized during the pathway of phenol propanoic synthesis in plants, and they mainly include flavone, flavanol, and anthocyanins (Siriamornpus et al., 2010). The results of various researches have shown that the effective substances in plant organs are never constant, they can be changed according to the stages of plant growth and different environmental conditions. It is completely dependent on environmental factors, flowering growth conditions, harvest time, genetic diversity and plant phenology (Dambolena et al., 2010). Flavonoids are bioactive compounds that include about 60% of polyphenolic compounds found in plants and are abundantly found in fruits, vegetables, seeds and nuts (Shahbazi et al., 2013).

Although the most antioxidant activity was shown in stigma tissues, followed by stem and leaves (Karimi et al., 2010). Antioxidants can include phenolic compounds (tocopherol, flavonoid and phenolic acid), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acid and amines), and carotenoid ascorbic acid (Hall and Cuppet, 1997). Based on the average comparison results (Figure 7), the interaction effects of genotypes in glutamic acid and tryptophan treatments caused a decrease in antioxidant activity on saffron plants. Although the treatment of control genotypes caused an increase in antioxidant activities on saffron plants. Therefore, amino acid treatments showed a relative reaction on coriander. They reported that amino acid foliar spray has a positive effect on the antioxidant activity of basil plant. The use of amino acid has increased total antioxidants. In another research, the antioxidant capacity of stigma extract is even higher than that of tomato and carrot plants (Nik naam et al., 2017).

Crocin turns into crocin and gentiobiose as a result of decomposition (Pham et al., 2000). The obtained results comparing the mean (Figure 18) of the mutual effects of the genotypes of the amino acid treatment of 1 mM glutamic acid caused an increase in the color of the saffron plant. Although amino acid tryptophan increased the taste of saffron candy. Crocin and picrocrocin are naturally degraded in the stigma during the storage drying and extraction process. This action is dependent on temperature, humidity and light. There may be changes in the ingredients. In another study, it has been reported that soil characteristics, moisture retention and nutrients are effective for the production and construction of secondary metabolites (Mandal et al., 2007). Amino acid foliar application increased essential oil in basil plants (Saburi et al., 2011).

V. CONCLUSION

The results obtained from this experiment showed that the use of amino acid glutamic acid and tryptophan had a positive and significant effect on the growth, physiological and biochemical characteristics of saffron plant. The effects of two amino acid levels (glutamic acid and tryptophan) were investigated. They left different effects on the pattern of growth and development of physiological traits. Amino acid concentration of 1 mM glutamic acid and 2 mM tryptophan, in addition to improving growth and development indicators, also increased flower and stigma performance. Amino acid foliar spraying (glutamic acid 1 mM and tryptophan 2 mM) led to increasedgrowth and production of vegetative bodies compared to the concentration of 1 mM tryptophan and 2 mM glutamic acid. Also, according to the results of amino acid, the concentration of 1 mM glutamic acid increased the metabolites although the result of this test, amino acid with a concentration of 2 mM tryptophan and 1 mM glutamic acid had a greater effect on the improvement of physiological indicators. Therefore, amino acid with a concentration of 1 mM glutamic acid and 2 mM tryptophanshowed the best effect on the yield of flowers, and a concentration of 1 mM glutamic acid achieved the highest vegetative body yield. In general, it can be said that amino acid is directly and indirectly effective on the physiological activities on the growth and development of plants. Considering the cultivation, both the production and the result of effective substances from

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the morphological and physiological traits, the application of amino acid in the form of foliar spraying can be effective andrecommendable.

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