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Research Paper

Changes in Total Phenol and Some Enzymatic and Non-Enzymatic Antioxidant Activities of Rose-scented Geranium (*Pelargonium graveolens*) in Response to Exogenous Ascorbic Acid and Iron Nutrition

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Abstract

The strong antioxidant activity of *Pelargonium graveolens* is well established. The question addressed in this study was whether different concentrations of exogenous ascorbic acid (AsA) and iron (Fe) could influence the antioxidant activity and total phenol content (TPC) of geranium. Thus, three levels of Fe (0, 20 and 40 µM) and three levels of AsA (0, 1 and 2 mM) in the nutrient solution were combined factorially based on a completely randomized design with six replications, and chlorophyll content, TPC, and antioxidant activities of the leaves were measured. The results showed that oil content, ascorbate peroxidase (APX), and catalase (CAT) activities were increased in leaf samples under Fe starvation, regardless of the AsA concentration. The highest peroxidase (POD) activity was observed in samples treated with 20 µM Fe and 1 mM AsA. The highest total chlorophyll content was produced in plants treated with 40 µM Fe along with 1 mM AsA. TPC was increased with an increase in Fe concentration. Despite the positive effect of AsA on the pigment contents, plants treated with AsA showed lower TPC under all Fe concentrations. In total, lower Fe nutrition increased oil content and reactive oxygen species (ROS) scavenging activity of geranium. AsA application increased oil content while decreased total phenol and antioxidant activity in this plant.

Keywords: Ascorbate peroxidase, Catalase, Essential oil, Peroxidase.

INTRODUCTION

Pelargonium graveolens is a well-known member of the Geraniaceae. It is native to South Africa and popularly known as rose scented-geranium. Around 500–750 tons of the essential oil of *P. graveolens* is annually produced by Egypt, Algeria, Morocco, Reunion Island, and India (Pandey and Patra, 2015). It has great importance in the perfume, food and pharmaceutical industries (Pandey and Patra, 2015). The essential oil and extract of the aerial parts of rose scented-geranium possess a number of biological properties including antimicrobial, anti-inflammatory, and antioxidant activities (Boukhris *et al*., 2013; Boukhris *et al*., 2015).

Biotic and abiotic stresses induce reactive oxygen species (ROS) in plant cell. ROS including hydrogen peroxide, superoxide anion, hydroxyl, singlet oxygen, perhydroxyl radical, and peroxyl causes oxidative damage to lipids, proteins, nucleic acids, photosynthetic pigments, and enzymes and impairs the normal functions of cells (Blokhina *et al*., 2003; Gill and Tuteja, 2010). To counteract the toxicity of ROS, plants have developed both enzymatic (superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase) and non-enzymatic (ascorbic acid, glutathione, α-tocopherol, β-carotene, and phenolic compounds) antioxidant defense mechanisms (Blokhina *et al*., 2003; Gill and Tuteja, 2010).

The study of antioxidant activities of essential oils and aqueous extracts shows that the essential oil and hydrosol of *P. graveolens* are good sources of antioxidants (Boukhris *et al*., 2012; Ćavar and Maksimović, 2012). It has been reported that hydrosol antioxidant activity is higher than those obtained from essential oil, and even ten times higher than thymol that is usually used as a positive probe (Ćavar and Maksimović, 2012). The relatively strong antioxidant capacity could be attributed in part to the presence of *b*-citronellol and geraniol in the essential oil as well as the phenolic compounds in the extracts (Boukhris *et al*., 2012).

Iron (Fe) plays a fundamental role in plant metabolisms, such as hormone formation, DNA synthesis (Zancan *et al*., 2008), respiration, and photosynthesis (Tewari *et al*., 2013). Fe availability is mostly a limiting factor for plant growth; however, it is abundant in the earth's crust (4.2%) and many soils (2-60%). Despite the important role of Fe in many aspects of cell metabolisms, the control of Fe availability is highly recommended by plant nutrition experts (Curie and Briat, 2003). A literature review revealed that Fe may be an important factor in the ROS level in organisms. In the presence of ROS, Fe can take part in the Fenton reaction, thereby inducing the generation of highly toxic ROS (Zancan *et al*., 2008). On the other hand, a study to determine Fe starvation under oxidative stress in *Anabaena* sp. showed that iron limitation led to the accumulation of ROS in *Anabaena* sp. strain PCC7120 and caused oxidative damage (Latifi *et al*., 2005).

Ascorbic acid (AsA) is found in extremely slight quantities in leaves, but it plays a major role in the prevention of oxidative damage by nullifying ROS, and as a consequence, producing its own free radical, monodehydroascorbate (Smirnoff, 1996; Guo *et al*., 2005). Antioxidants are important not only during the life of the plants but also for human health. Antioxidant defense systems may help the body to protect itself against the destructive effects of ROS, which are linked to a variety of diseases including cardiovascular diseases, cancers, atherosclerosis, diabetes, and aging (Ray *et al*., 2012). The medicinal plant antioxidants can, therefore, serve as a type of preventive medicine. Despite many papers on antioxidant activity of *Pelargonium graveolens* (Boukhris *et al*., 2015), little is known about the influence of micronutrient Fe on the antioxidant activity changes. Therefore, this comparative study was conducted to evaluate the antioxidant activity of *P. graveolens* in response to different levels of Fe and exogenous AsA.

MATERIALS AND METHODS

Greenhouse condition and experiment details

The experiment was conducted in an experimental greenhouse at the Faculty of Agricultural

Sciences, Lorestan University in 2013. During the study, the mean temperature, relative humidity, and light intensity were maintained at 22-32°C, 60-70% and 600 ± 100 µmol m^{-2} s^{-1}, respectively. Semi-hardwood cuttings with at least five nodes were obtained from mother plants in the same greenhouse in August 2013. The cuttings were rooted following insertion in a sand medium. Uniform rooted cuttings were then transplanted in plastic pots (25 cm diameter and height) filled with sand and grown hydroponically. The treatments were applied after the plants established with a height of about 12 cm and 4-6 leaves in October 2013. A factorial experiment was used based on the completely randomized design consisting of three levels of Fe (0, 20 and 40 µM) and three levels of AsA (0, 1 and 2 mM) in the nutrient solution with six replications. During the experiment, which lasted for about 90 days, the plant growth conditions were controlled regularly. The youngest fully expanded leaves of different individuals were sampled for enzyme activities and pigment assays. Afterward, the aerial parts of the rose-scented geranium were harvested, air-dried in the shade at room temperature, and then evaluated for oil content, antioxidant activity, and total phenolic content (TPC).

Essential oil isolation

Air-dried leaves were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, and essential oil content was recorded. The essential oil content was calculated as ml 100 g^{-1} leaf dry weight. The essential oil extraction was only performed on leaves because the preliminary experiments showed that the essential oil content of the stems was very low compared with that of the leaves.

Measurements of non-enzymatic antioxidant activity

1. Extraction process

For extraction, 1 g of air-dried and finely ground leaves (in each treatment) was incubated in methanol (80%). After two days at room temperature, it was filtered using a filter paper. The final extract was used for determination by 1-(2, 6-dimethylphenoxy)-2-(3, 4 dimethoxyphenylethylamino) propane hydrochloride (DPPH) radical scavenging assay and TPC.

2. DPPH radical scavenging assay

The total antioxidant capacity of the studied extracts was assessed by DPPH quenching ability (Burits *et al*., 2001). Briefly, 20 µl of the sample extract with methanol was adjusted to 100 µl. Then, 350 µl of freshly prepared DPPH was mixed with it. The mixture was then incubated for 20 min in darkness at room temperature. Afterward, the absorbance measurements were read at 517 nm using a UV 1800 spectrophotometer. A positive control (ascorbic acid) was prepared in the same way as the samples were made. The DPPH radical scavenging of each plant extract was calculated using the following equation (1).

$$\text{Scavenging activity (\%)} = [A_{\text{control}} - A_{\text{sample}}/A_{\text{control}}] \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance in the presence of extracts (Burits *et al*., 2001). The decrease in absorption at 517 nm was used to calculate the IC50 (mg/ml).

3. Total phenolic content

TPCs were analyzed using the Folin–Ciocalteu method as described by Singleton *et al*. (1999) with some modifications. A volume of 10 µl of the plant extract was mixed with 490 µl of the Folin-Ciocalteu reagent and then 500 µl of 1% Na_2CO_3 was added to a 5-mL test tube and

they were mixed. After two hours in darkness at room temperature, the absorbance of the reaction mixture was measured at 765 nm. The amounts of TPC in the extracts were expressed in gallic acid equivalents (mg GAE/g plant dry weight) according to the equation of the line for the gallic acid standard curve.

Chlorophyll and carotenoids determination

Chlorophyll and carotenoid contents were determined according to Lichtenthaler (1987). In brief, 0.1 g of leaf tissue was collected and ground with liquid N. The samples were mixed with 10 ml acetone and centrifuged for 15 min at 4000 rpm, and then the supernatants were collected. The absorbance of the supernatant liquid (3 ml) was measured at 470, 662 and 645 nm using a spectrophotometer for total carotenoids, chlorophyll *a* and chlorophyll *b*, respectively.

Measurement of enzymatic antioxidants

The peroxidase (POD) activity was assayed based on the method of MacAdam *et al*. (1992). So, 0.3 g of leaf tissue was ground with liquid N and mixed with 1.5 ml potassium phosphate buffer (pH 7.0). The homogenized tissue was centrifuged at 14,000 rpm and 4°C for 20 min. The supernatant was used for the POD assay. The POD activity was calculated by monitoring the decline in absorbance at 475 nm and expressed in µmol H_2O_2 reduction min g^{-1} FW.

The Catalase (CAT) activity was determined following the procedure of Chance and Maehly (1955) with some modifications. So, 0.3 g of leaf tissue was ground in liquid N and mixed with 1.5 ml of potassium phosphate buffer (containing EDTA and PVP). The samples were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was used for the CAT assay. The CAT activity was calculated based on the decrease in the absorption at 240 nm due to the decomposition of Mmol H_2O_2 reduction min^{-1} g FW^{-1}.

The ascorbate peroxidase (APX) activity was measured using the procedure of Nakano and Asada (1981). Basically, the decrease in ascorbate concentration was followed by a decline in optical density at 290 nm as a result of ascorbate oxidation. So, 0.1 g of leaf tissue was collected and ground in liquid N and mixed with 1.5 ml of potassium phosphate buffer (50 Mm and pH 7.0). The reaction mixture was centrifuged at 4000 rpm and 4ºC for 20 min, and then the supernatant was collected. The APX activity was determined in µmol ml^{-1} ascorbate oxidation min g^{-1} FW at 290 nm.

Statistical analysis

Data were subjected to the analysis of variance using the MSTAT-C software (Michigan State University, East Lansing, MI, USA). Means comparison was done using the Least Significant Difference (LSD) at $P<0.05$. Correlation coefficients were calculated using the MSTAT-C software.

RESULTS AND DISCUSSION

Essential oil content

The effect of different levels of Fe and ascorbic acid on the oil content of *P. graveolens* is shown in Fig. 1. The results revealed that the changes in oil content in response to Fe concentration significantly depended on the AsA application. In AsA-untreated plants, the oil content of the samples tended to decrease with an increase in Fe concentration. Many studies have revealed that the application of extra Fe diminished essential oil content of aromatic plants. An investigation on the effect of different Fe levels on *Mentha arvensis* showed that the increase in Fe supply enhanced leaf area, chlorophyll content, and photosynthetic rate. Further, plant height, the number of shoots, menthol content, and total oil yield were highest at 5.60 ppm Fe (Misra and Srivastava, 1990).

Similarly, the application of high Fe concentration reduced biomass and essential oil of Oregano plants grown in solution culture (Yeritsyan and Economakis, 2002). The production of essential oil in aromatic plants is mainly dictated by the combined influences of plant genetics, plant development stage, and environmental stresses. It seems that the reduction of Fe concentration in a nutrient solution acts as a kind of environmental stress and increases oil content. AsA as a growth regulator plays a major role in many biological processes in plants. It was shown that foliar application of AsA, thiamine, α-tocopherol or their combinations significantly increased the oil yield of *Jasminum grandiflorum* compared to the control plants (Eid *et al*., 2010). The results of our study strongly support this view as the application of 1 mM AsA increased oil content in the samples. However, oil content decreased under Fe starvation, regardless of AsA concentration (Fig. 1).

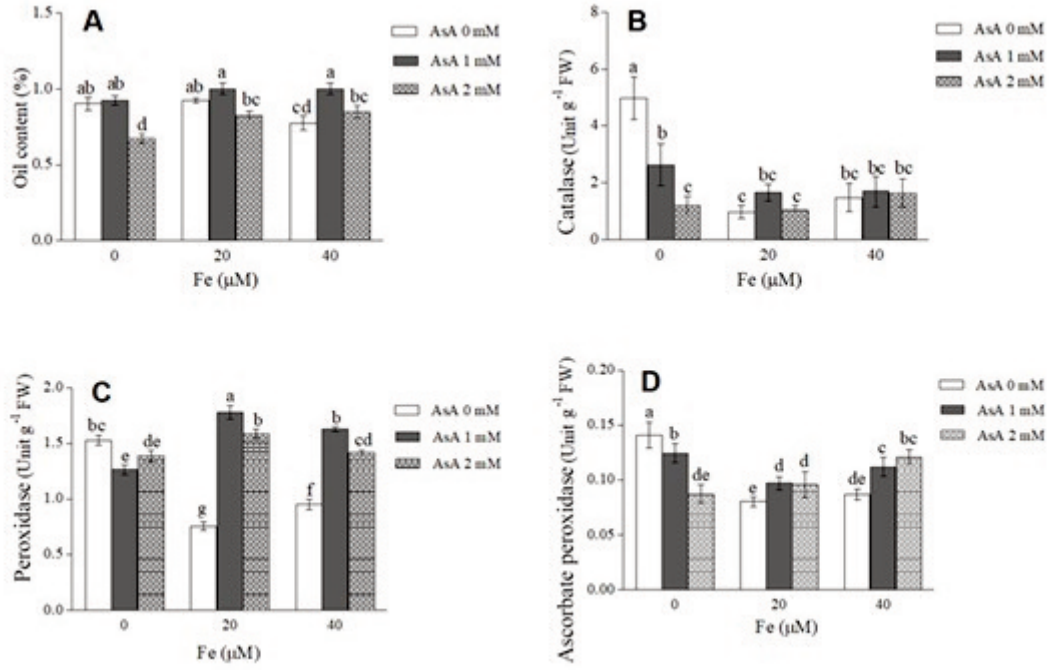

Fig. 1. The effects of different levels of Fe and ascorbic acid on oil content (A), catalase (B), peroxidase (C) and ascorbate peroxidase (D) activity in *Pelargonium graveolens*. Values are the mean of six replications ± standard error of the mean. Values with the same letter(s) are not significantly different at P<0.05 according to LSD test.

Antioxidant enzymes activity

The results as to the effect of exogenous AsA and Fe application on the POD, CAT and APX activities are shown in Fig. 1. The POD, CAT and APX activities were significantly decreased with the increase in AsA concentration in the Fe-starved plants. These results are in line with a study on canola in which ascorbic acid application improved photosynthesis and seed yield but reduced antioxidant enzyme activities of the plants (Bybordi, 2012). Qian *et al*. (2014) observed an inhibitory effect of the exogenous AsA on the activity of antioxidant enzymes, including superoxide dismutase, POD, CAT, and APX, by inhibiting the transcription of antioxidant enzymes biosynthesis genes. Although our results indicated that the activities of POD and APX in the Fe-treated (20 and 40 µM) plants were significantly enhanced by the application of AsA, the AsA treatment had no significant effect on the CAT activity in the Fe-treated plants. These findings are in agreement with those reported by Vansuyt *et al*. (1997), who showed that the expression of

ascorbate peroxidase gene can be controlled by Fe at the level of mRNA accumulation. Whereas Fe can catalyze the production of the ROS through the Fenton reaction, when *Brassica napus* seedlings were treated with Fe (III)-citrate (500 µM), a large increase was observed in APX mRNA level. But, the APX transcripts were hardly detectable in the control seedlings under Fe starvation. CAT and APX were highly correlated with each other (Table 1).

Chlorophyll and carotenoids contents

Leaf chlorophyll and carotenoid values were found to be significantly increased with the increase in Fe concentration (Fig. 2). Similarly, increases were reported in carotenoid synthesis in *Dunaliella salina* under Fe^{2+} and acetate treatment (Mojaat *et al*., 2008). Fe is one of the most important micro-elements affecting the productivity of photosynthetic plants due to its effect on the biosynthesis of chlorophylls and carotenoids and PSII efficiency (Hindt and Guerinot, 2012).

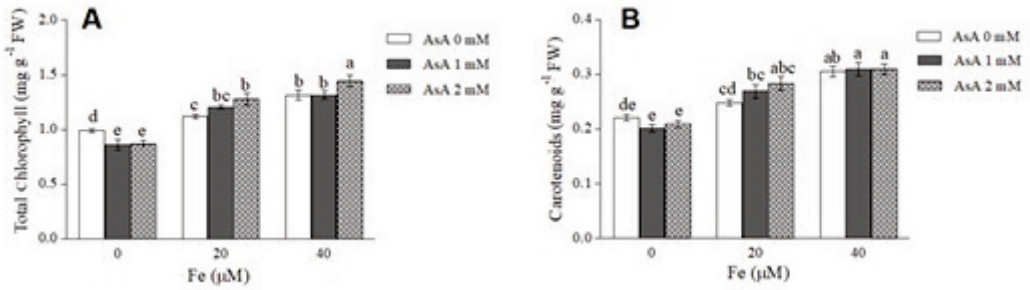

Fig. 2. The effects of different levels of Fe and ascorbic acid on total chlorophyll (A) and carotenoid (B) contents in *Pelargonium graveolens*. Values are the mean of six replications ± standard error of the mean. Values with the same letter(s) are not significantly different at P<0.05 according to LSD test.

Marsh *et al*. (1963) reported that Fe deficiency reduced chlorophyll synthesis in *Vigna sinensis* through decreasing δ-aminolevulinic acid synthesis. Machold and Stephan (1969) investigated the function of Fe in chlorophyll biosynthesis. They found that the activation of Fe led to the transformation of co-proporphyrin into protoporphyrin and increased chlorophyll synthesis. Our results revealed that chlorophyll and carotenoid contents in the Fe-treated and Fe-untreated samples may be significantly affected by AsA. The total chlorophyll and carotenoids were increased by using exogenous AsA in the plants treated with 20 and 40 µM Fe (Fig. 2). Bybordi (2012) showed that exogenous AsA improved photosynthesis and seed yield in canola under salt stress through its positive effects on chlorophyll *a* and *b* contents. In contrast, our results showed that the application of AsA reduced the total chlorophyll content of the plants under Fe deficiency (Fig. 2). These results are in agreement with the results of Qian *et al*. (2014) who showed that exogenous AsA imposed a type of stress that inhibited plant growth and decreased the chlorophyll content, thus reducing the photosynthetic efficiency in *Arabidopsis thaliana.* Therefore, AsA application in the present research might have caused further Fe starvation stress, decreasing the synthesis of chlorophyll and carotenoids. Chlorophyll was positively correlated with carotenoid and TPC (Table 1). Carotenoid was positively correlated with CAT and TPC (Table 1).

Total phenolic content and non-enzymatic antioxidant capacity

The results showed that the highest TPC was observed in the plants grown in 20 and 40 µM Fe, suggesting that Fe starvation decreased TPC (Fig. 3). Kabir *et al*. (2015) investigated the

mechanisms associated with differential tolerance to Fe deficiency in okra (*Abelmoschus esculentus* Moench.) and observed increases in TPC in both BARI-1 and *Orca onamica* roots in response to Fe deficiency. In the current research, TPC of geranium root was not measured. But, probably lower TPC in Fe-untreated aerial parts can be related to the leakage of phenols from the roots in response to Fe deficiency because phenolic compounds are the most frequently reported root exudates in response to Fe deficiency (Kabir *et al*., 2015). AsA had a strong negative effect on the plant TPC at different Fe concentrations. The highest TPC (37.32 mg GAE g^{-1} DW) was observed in 20 µM Fe with 0 Mm AsA (Fig. 3). TPC was positively correlated with total chlorophyll, carotenoids, and oil content (Table 1).

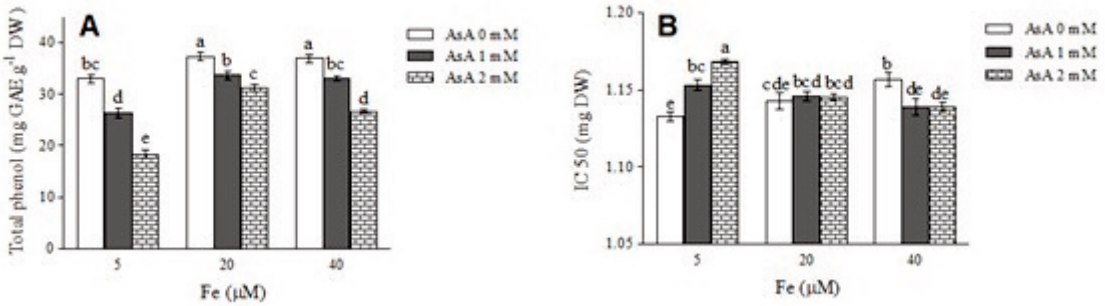

Fig. 3. The effects of different levels of Fe and ascorbic acid on total phenol (A) and antioxidant activity (as DPPH) (B) in *Pelargonium graveolens*. Values are the mean of six replications ± standard error of the mean. Values with the same letter(s) are not significantly different at P<0.05 according to LSD.

Table 1. The correlation coefficient of some biochemical characteristics of *Pelargonium graveolens*.

	Oil content	CAT	POD	APX	Total chl.	Carotenoids	TPC	DPPH
Oil content	1							
CAT activity	-0.053	1						
POD activity	0.209	0.136	1					
APX activity	0.081	0.591**	0.230	1				
Total chlorophyll	0.084	-0.197	0.081	-0.029	1			
Carotenoids	0.114	-0.283*	0.084	-0.149	0.913**	1		
TPC	0.403**	0.021	-0.262	-0.084	0.409**	0.387**	1	
DPPH	-0.431**	-0.320*	-0.136	-0.328*	-0.354**	-0.187	-0.384**	1

*, ** and ns: Significant at P < 0.05, P < 0.01 and insignificant, respectively. CAT: Catalase; POD: Peroxidase; APX: Ascorbate peroxidase; TPC: Total phenol content; DPPH: 1-(2, 6-dimethylphenoxy)-2-(3, 4-dimethoxyphenylethylamino) propane hydrochloride.

The ability of rose-scented extracts to scavenge DPPH was assessed on the basis of their IC50 values, defined as the concentration of the test sample necessary to decrease the initial DPPH radical concentration by 50%.

The results proved that the extract of Fe-starved samples had the lowest inhibitory activity on DPPH. Generally, higher Fe concentrations had positive effects on the antioxidant activities of the samples. The application of AsA promoted the antioxidant properties of the 40 µM Fe-treated samples. On the contrary, the AsA treatment had a strong negative effect on the antioxidant activ-

ities of the Fe-untreated samples while the AsA application had no significant effect on the IC50 value of 20 µM Fe-treated samples. There were negative correlations between CAT and APX activities and essential oil content with IC50 (Table 1). A stronger radical scavenging activity in AsA and Fe-untreated plants assay can be related to their superior CAT and APX activities and higher oil content.

The statistical analysis displayed a negative correlation between TPC and IC50; indicating that scavenging activity on DDPH radical was enhanced by the increase in TPC (Table 1). Many studies have demonstrated a positive correlation between TPC and antioxidant activity (Pawar *et al*., 2011; Ghahremani-majd *et al.,* 2012). According to Ghahremani-majd *et al*. (2012), the positive correlation between TPC and antioxidant activity underlies the fact that phenolic compounds of geranium samples contribute to their antioxidant capacities. Polyphenols antioxidant potential depends on their organization, the number of hydroxyl groups, and the extent of structural conjunction, as well as the presence of electron-donating and electron-withdrawing substituents in the ring structure (Miller and Rice-Evans, 1997).

CONCLUSION

The current study demonstrated that Fe starvation had a noticeable effect on DDPH radical scavenging activity. Furthermore, the highest oil content, APX and CAT activities were obtained under Fe starvation. The highest POD activity was observed in the samples treated with 20 µM Fe and 1mM AsA. It is noteworthy that the Fe and AsA treatments increased the synthesis of chlorophyll and carotenoids. TPC was enhanced by the higher Fe concentrations, and the AsA application reduced TPC in plants under both treatments (Fe utilization and starvation). In total, the results of the present research revealed that lower Fe application increased oil content and ROS scavenging activity of geranium. AsA application increased oil content while decreased TPC and antioxidant activity. Further research is recommended on the molecular and biochemical investigation of the effect of AsA on the total phenol synthesis in geranium.

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