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Research Article

Changes in antioxidant content of lemon fruits in response to zinc foliar application

Nasim Rastgoo and Mahdiyeh Gholami*

Department of Horticulture, College of Agriculture, Isfahan University of Technology, Isfahan, Iran
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Abstract

Agronomic biofortification is the process of enriching fruits during crop growth with required nutrients, such as Zinc (Zn). An experiment was conducted to evaluate the foliar fertilization effects of Zn on the nutritional quality of lemon (*Citrus limon* L. Burm.) juice. The treatment consisted of three levels of zinc concentration (0, 0.5 and 1 g L^{-1}). Trees were sprayed three times at a later stage of fruit expansion with foliar applications of zinc sulfate at the above-mentioned rates. The Zn spray significantly increased the concentrations of Zn in the leaves and fruits. Application of Zn resulted in significantly higher fruit ascorbic acid, total phenolic compounds, flavonoids, anthocyanin, carotenoids, and reduced glutathione contents. Activities of peroxidase and polyphenol oxidase enzymes in fruit juice were significantly decreased with 0.5 or 1 g Zn L^{-1} application. Although the mechanisms of changes in some juice phytochemicals are not well known, the derived data from this study could impact citrus growers and conclusively aid in the development of fruit with superior antioxidant quality.

Keywords: Biofortification, *Citrus limon*, Functional foods, Micronutrient, Nutraceutical

Introduction

Consumers see the connection between fruits and health and associate their diets with the prevention of these diseases. Numerous epidemiological and prospective studies have been undertaken that suggest a strong link between dietary intake of phytochemicals and human health, particularly in protecting against chronic degenerative diseases, such as cardiovascular disease, diabetes mellitus and cancers (Hajiaghaalipour *et al*., 2015). As the public becomes more aware of the health benefits of fruits, the demand for fruits specifically developed for their health benefits is increasing (Byrne, 2012). Such health-enhanced products that could be sold fresh or processed into extracts are natural sources of antioxidants, antimicrobials, or food colorants for the health and food foot industries (Cevallos-Casals *et al*., 2006). Using practical and new approaches to provide additional health benefits to consumers or animals by promoting the state of well-being and possibly reducing the risk of disease is called functional foods. It was mainly the advances in understanding the relationship between nutrition and health that resulted in the development of the concept of functional foods (Siro *et al*., 2008). When functional food aids in the prevention and/or treatment of disease(s) and/or disorder(s) other than anemia, it is called nutraceutical (Kalra, 2003). The use of dietary supplements, functional foods, and nutraceuticals is increasing as the industry is responding to consumers' demands. The markets for health food will certainly increase in the future.

Although it is known that genetic and environmental factors can affect the composition of fruits, the influence of nutrient availability on the phytochemical concentrations in plants namely citrus fruits is unassailable (Emami Bistgani *et al*., 2018). The fruit nutritional quality of cultivated strawberries was found to be affected by the fertilization regime (Di Vittori *et al.,* 2018). The findings of Estrada-Ortiz *et al*. (2013) suggested that supplying 30% phosphite in the nutrient solution activated defense mechanisms in the strawberry plants, which enhanced the anthocyanins concentration and improved fruit quality. Recently, Souguir *et al*., (2018) indicated that sesame plants supplemented with 100 mg L^{-1} calcium nitrate had the highest antioxidant activity compared to 0 mg L^{-1}. Tavallali *et al*. (2017) demonstrated that foliar zinc (Zn) application at a rate of 0.2% (w/v), considerably increased polyphenolic contents as well as the antioxidant activity of anise (*Pimpinella anisum* L.) fruits. According to Tshivhandekano *et al*. (2017) there was an enhancement in bush tea leaf polyphenols, total flavonoids, and total antioxidants in response to nitrogen (75 and 150 kg ha^{-1}) treatment.

Agronomic biofortification is the process of enriching fruits during crop growth with required nutrients such as Zn. Zn deficiency is commonly

*Corresponding Author, Email: mah.gholami@iut.ac.ir

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observed in citrus orchards in Iran. The major reason for the widespread occurrence of Zn deficiency in soils is the low availability of Zn to plant roots, rather than the low Zn content in soil (Tavallali, 2016). Two main theories could be proposed for the high Zn deficiency in *citrus* orchards. In one theory, the carbonate found in calcareous soils may have led to the adsorption of this element by calcium carbonate ($CaCO_3$) and the formation of an insoluble complex with Zn. The second theory is based on the reduction of Zn solubility up to 100-fold per unit increase in pH (Hasani *et al*., 2012). Foliar fertilization effectively supplies the nutrient demand of fruit trees during periods when 1- due to special soil conditions such as low or excess soil moisture, pH and salinity, soil-applied fertilizer is ineffective, 2- when nutrient fixation occurs in the soil, and 3- when tree demand for a specific nutrient is high (Lovatt, 2013).

Assuming that nutrient availability determines the nutritional quality of fruits, we hypothesized that the antioxidant parameters of lemon juice would be higher in trees treated with Zn. Therefore, in the present study, the effects of Zn foliar fertilization on the content of some health-promoting compounds (ascorbic acid (AsA), total phenolic compounds (TP), flavonoids, anthocyanin, carotenoids and reduced glutathione (GSH) contents) and the activity of peroxidase (POD) and polyphenol oxidase (PPO) enzymes in lemon fruits were investigated. Our purpose was to examine the nutraceutical quality of lemon fruit CV. Lisbon is affected by Zn fertilizer under the alkaline soil conditions of Iran. We assumed that derived data could be used to establish recommendations for lemon orchards' micronutrient fertilization in similar conditions.

Materials and methods

Experimental site and plant material: The experiment was carried out during 2015–2016 at a commercial 'Lisbon' lemon (*Citrus limon* L. Burm.) orchard in Larestan, Fars province, Iran (latitude 28°2′ N, longitude 53°9′ E). The area is in a semiarid zone with an average annual rainfall of about 601 mm, in which the average temperature of the coldest month is lower than 5 °C and that of the warmest month is higher than 42 °C. The trees were 7 years old, spaced at 8 × 8 m, in a sandy loam soil. They received standard horticultural practices, and disease and pest control.

Treatments and experimental design: Three levels of zinc (0, 0.5 and 1.0g L^{-1} Zn) were applied as a foliar application. Zinc sulphate ($ZnSO_4.7H_2O$), manufactured by Merck, was used as a source of Zn. The experiment was laid out in a randomized, complete block design with four replications. The zinc sulfate solution spray started at a later stage of fruit expansion (October 23, before the maturity stage) at intervals of 15 days (Dong *et al*., 2009). Foliar treatments were applied until the point of runoff between 7:00 am and 8:00 am. Control trees were sprayed with an equal volume of distilled water. For each treatment, 24 uniform trees with homogeneous fruit loads were selected, and the trees were grouped into 4 blocks with 6 trees per block. Fruit quality was determined on four replicates of 48 fruits (8 outside fruits per tree, 2 from each quadrant) collected from each treatment. Fruits from each of the 6 trees in a block were composited to make a sample of 48 fruits. Generally, 8–10 segments were available from each fruit. After washing, fruits were peeled and split in half by knife from stem to stylar end. One-half of the segments were juiced using a commercial kitchen juicer and used for quality analysis. Samples of the juice were placed into 15 mm × 110 mm vials sealed with aluminum foil, lyophilized and kept at -20°C until analysis. The rest of the fruit was dried to a constant weight in a forced-air oven at 70 °C.

Measurement and analysis: A slightly modified version of the method used by Kampfenkel *et al*. (1995) was applied to measure the total AsA content. Frozen lemon juice (0.1 g) was homogenized in 1 mL of 6% trichloroacetic acid (TCA). The extracts were centrifuged at 15,000 × *g* for 10 min at 4°C, and then the supernatant was used to assay the ASA. The reaction mixture (3 mL) contained 100 mM potassium phosphate buffer (pH 7.4), deionized water, 10% TCA (w/v), 44% H_3PO_4 (v/v), 3% $FeCl_3$ (w/v), 4% 2, 2-dipyridyl (w/v), and 0.2 mL of supernatant. The sample was incubated in a water bath at 42°C for 40 min and then the absorbance was read at 525 nm using a Shimadzu UV 160 A spectrophotometer (Shimadzu Corp., Kyoto, Japan). Using an AsA standard curve, the AsA content in juice samples was obtained.

TP was estimated by the Folin-Ciocalteu method with slight modifications (Savikin *et al*., 2014). Briefly, fruit juice was extracted with 80% methanol (1:10 v/v) for 2 hours in darkness. Then, a 0.2 mL sample was taken and mixed with 1 mL of a 10-fold diluted Folin-Ciocalteu reagent. This mixture was homogenized and incubated for 5 minutes, followed by the addition of 7.5% sodium carbonate. After 1 h at room temperature in the dark, the absorbance was measured at 750 using a UV-vis spectrophotometer. TP was quantified using a calibration curve obtained from measuring the absorbance of gallic acid standards and expressed in mg gallic acid 100 mL^{-1} of fruit juice.

Total flavonoid content was determined using the aluminum trichloride method as described by Wang *et al*. (2014). For this purpose, a lemon juice sample (1 mL) was mixed with 5% sodium nitrite solution (0.5 mL) and subsequently with 10% aluminum chloride (0.5 mL) and sodium hydroxide (1 M, 2 mL). The absorbance of the mixture was measured spectrophotometrically at 510 nm. The flavonoid concentration was calculated from a calibration curve using rutin as a standard and expressed as mg 100 mL^{-1} of fruit juice.

Anthocyanins were extracted from lyophilized samples (0.1 mL) with 10 mL acidified methanol (1% HCl) with continuous shaking for 30 min. The mixture

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was centrifuged at 4,500 × *g* for 5 min at 4 °C, and the extracted anthocyanins were collected. The extraction operation was repeated twice. The methanolic extracts were pooled and taken to a final volume with methanol. Anthocyanins were quantified by UV-visible spectrophotometry, measuring the absorbance at 530 nm, and expressed as mg cyanidin 3-O-glucoside equivalents per 100 mL of fruit juice (mg CGE 100 mL^{-1}) (Tierno *et al*., 2015).

To determine the total carotenoid content, 0.2 mL of sample was extracted using 20 mL of acetone (80% v/v), stirred for 15 minutes, and filtered (the extraction was repeated two times). The filtrate was then centrifuged at 10,000 × *g* for 15 minutes, and spectrophotometry was applied to define the carotenoid (CAR) content (Medina *et al*., 2011). Making use of the formula, the researcher calculated the concentration of the sample from the absorbance of the supernatant at 663, 648 and 470 nm.

GSH was measured by Cardoso *et al*. (2015). Aliquots (0.5 mL) of the juice sample were mixed with 0.5 mL of 10 mM DTNB in tubes containing 200 mM phosphate buffer (pH 8.0). The absorbance at 412 nm (Shimadzu spectrophotometer) was measured 5 minutes later. GSH was used as a standard, and results were expressed in µmoles L^{-1}.

A slightly modified version of the method suggested by Liu *et al*. (2007) was used to extract POD and PPO. Consequently, 2 mL juice was homogenized with 10 mL of ice-cold 50 mM sodium phosphate buffer (pH 7) having 0.2 g of polyvinyl polypyrrolidone (PVPP) and powdered at 4 °C. Centrifuging the homogenate was then done at 15,000 × *g* for 30 min at 4 °C; for the enzyme assay, the supernatant was used.

The changes in the absorbance at 470 nm were the basis to assay POD activity. 2.8 mL of substrate solution and 50 µL of the enzyme extract were inserted in the reaction cuvette. The substrate solution included 20 mM guaiacol and 25 mM hydrogen peroxide that were dissolved in 50 mM sodium phosphate buffer (pH 7.0). To determine PPO activity, every 3 mL reaction mix included 100 µL enzyme extract, 0.1 M catechol, and 0.1 M sodium phosphate buffer (pH 6.8). At 420 nm, an increase was observed in the absorbance.

The anti-radical ability of the sample juices was evaluated using the DPPH radical scavenging method based on Vinha *et al*. (2014). A 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol was prepared. 0.2 mL of fruit juice (diluted 10-fold) was added to 2.8 mL of DPPH solution. The solution was then stirred and kept in the dark for 30 min. The percentage scavenging effect was calculated as scavenging rate = (A0 – A1 /A0) × 100, where A0 was the absorbance of the control (without juice) and A1 was the absorbance in the presence of the juice at 517 nm. To do all spectrophotometric measurements, the researcher used Schimadzu UV-VIS AA 6300 spectrophotometer.

Zn contents in fruit and leaves: Tissue samples consisted of about 60 leaf blades and one-half of 48 harvested fruits. The leaf samples were taken at harvest from fully developed leaves, near the fruits. The leaves and fruit pulp were oven-dried to constant weight at 70 °C. About 0.5 g of dry, ground leaf tissue was ashed in a muffle furnace at 550 °C for 5 h. The ashed prepared was dissolved in diluted hydrochloric acid and then diluted with ion-exchanged water. The concentration of Zn was measured by atomic absorption spectrophotometry (Shimadzu, AA 6300, Japan) and expressed as mg kg^{-1} dry weight.

Statistical analysis: All data presented were means of four replicates. Analysis of variance (ANOVA) for various parameters was done following ANOVA test. When the F was significant at the $P \leq 0.05$ level, treatment means were separated using Duncun's multiple range test. The correlation was established by the CORR of SAS v 9.2 (SAS Inc., Cary, NC, USA).

Results

In the present study, the effects of Zn treatments on GSH, flavonoids, carotenoids, total phenolic compounds concentrations, DPPH scavenging ability and leaf and fruit Zn content were significant. Also, analyses of variance showed that there were no significant differences among Zn treatments for anthocyanin concentration (Table 1).

Both Zn spray treatments resulted in a significant increment of AsA content in the juice of 'Lisbon' lemon. The AsA was increased with an increase in Zn concentration. The application of the 0.5 g L^{-1} Zn was 52.5 mg 100 mL^{-1}, or a 55% increase; while the addition of 1 g L^{-1} Zn resulted in 59.2 mg 100 mL^{-1}, or 75% AsA increase (Figure 1-a). Similarly, TP was significantly increased in the juice of the lemon fruits treated with Zn compared with the fruit of those who received only distilled water (Figure 1-b). In 0.5 and 1 g L^{-1} Zn, TP content was 54 and 74% higher than the control, respectively. Zn application increased flavonoid concentration with equal potency in either 0.5 or 1 g L^{-1} (Figure 1-c). At harvest, the value of flavonoid in control fruits was about 17 mg 100 mL^{-1} juice for lemon, while in Zn-treated fruits it was about 28 mg 100 mL^{-1} juice. Anthocyanin accumulation of lemon juice was not affected significantly by the foliar application of Zn (Figure 1-d) and declined slightly compared with that measured in the control (0.49 mg CGE 100 mL^{-1}). Juice anthocyanin content did not significantly correlate with leaf or fruit Zn content (Table 1). As shown in Figure 1-e, pre-harvest foliar application of 0.5 or 1 gL^{-1} Zn significantly increased the content of carotenoids, corresponding to 2.2-fold and 2.6-fold, respectively. The results of this study show that an increase in the Zn content of fruit juice increases fruit GSH content at harvest. GSH concentration in control fruits was 54.5 µmol L^{-1}, while in 0.5 or 1 g L^{-1} Zn-treated fruits it was 62.4 and 86.5 µmol L^{-1}, respectively (Figure 1-f). The enzyme activities of POD and PPO were affected significantly by the foliar application of Zn (Figure 2);

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Table 1. Analyses of variance (ANOVA) of Zn application on some quality parameters

Source of variarion	df	GSH	Leaf Zn content	Fruit Zn content	Anthocyanins	DPPH	Flavonoids	Carotenoids	Total phenolic compounds	AsA	PPO activity	POD activity
Block	3	19585.8**	664.9**	26.2**	0.01**	1.49**	0.8**	3.56**	2260.9**	241.3**	13.21**	19.6**
Zn treatment	2	111657.5**	11965**	718.6**	0.001^{ns}	2.63**	1.62**	10.6**	5580.2**	695 **	15.6**	27.2**
Error	6	456.9	18.8	1.38	0.0006	0.02	0.01	0.09	45.6	4.7	0.69	1.15
CV (%)		13.1	3.3	3.4	5.2	5.2	5.1	5.6	6.7	4.4	6.7	6.9

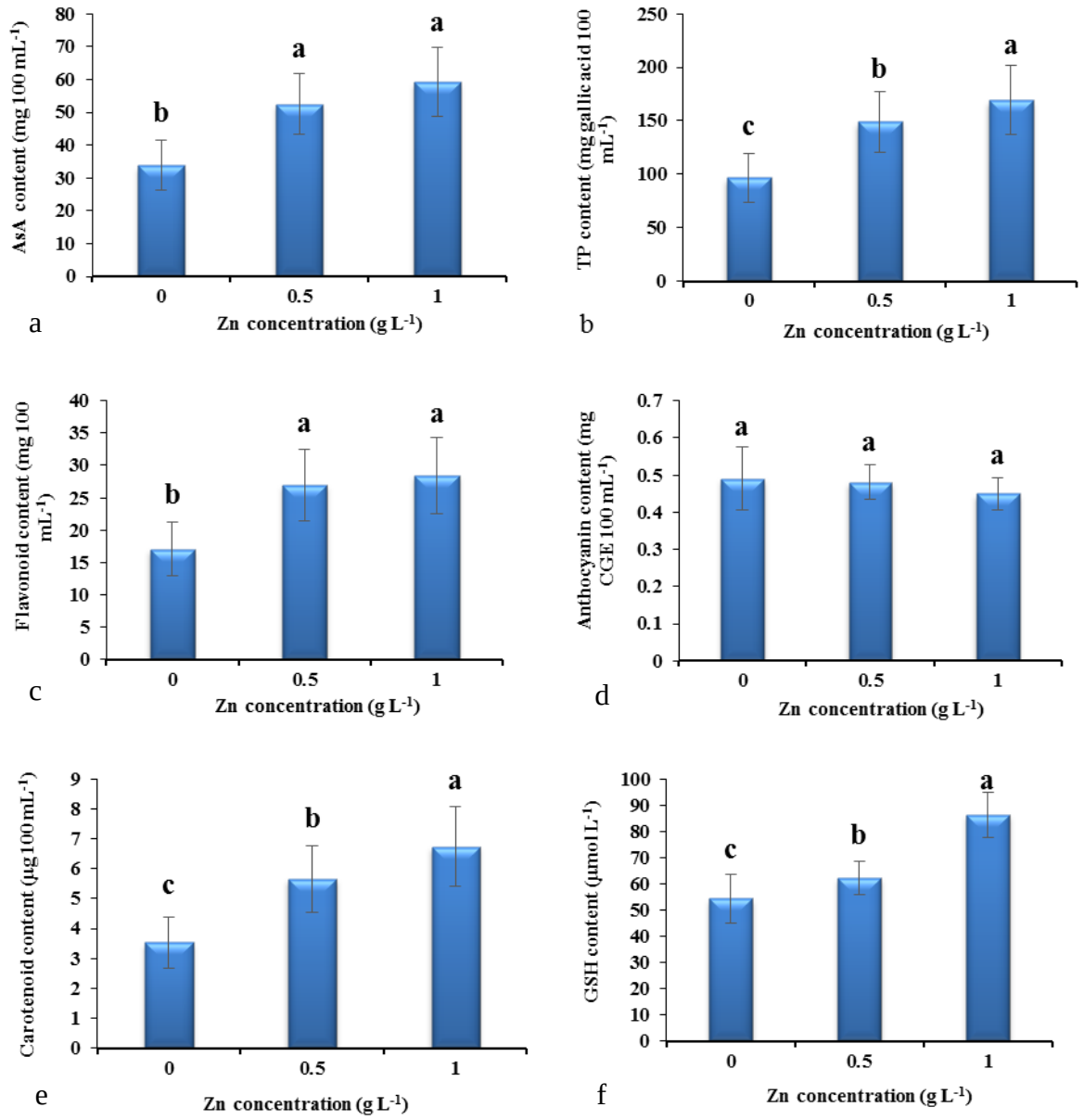

Figure 1. The content of a) ascorbic acid (AsA); b) total phenolic compounds (TP); c) flavonoids; d) anthocyanins; e) carotenoids and f) reduced glutathione (GSH) in the juice of the control and $ZnSO_4$-treated lemon fruits at harvest. The data are mean values ± SE of four technical replicates. Values followed by the same lowercase letters are not significantly different.

the highest was recorded in the control treatments. However, there was no difference in POD or PPO activity between 0.5 and 1 g L^{-1} Zn treatments. No significant regression was evident between the fruit enzyme activity and leaf or fruit Zn concentrations (Table 1). This suggests that fruit enzyme activity was

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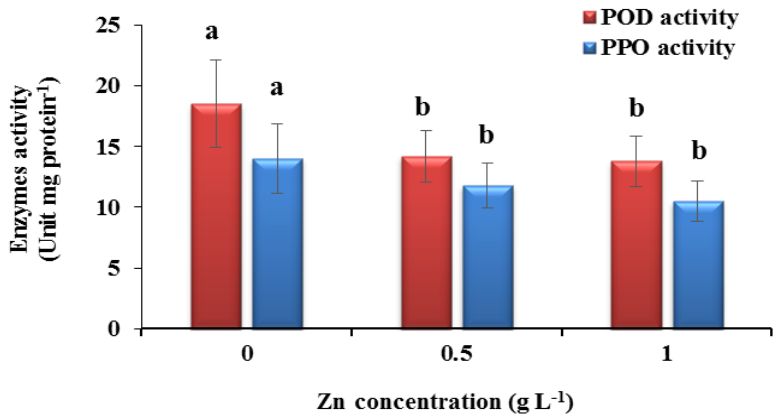

Figure 2. The activity of peroxidase (POD) and polyphenol oxidase (PPO) in the juice of the control and $ZnSO_4$-treated lemon fruits at harvest. The data are the mean values ± SE of four technical replicates. Values followed by the same lowercase letters are not significantly different.

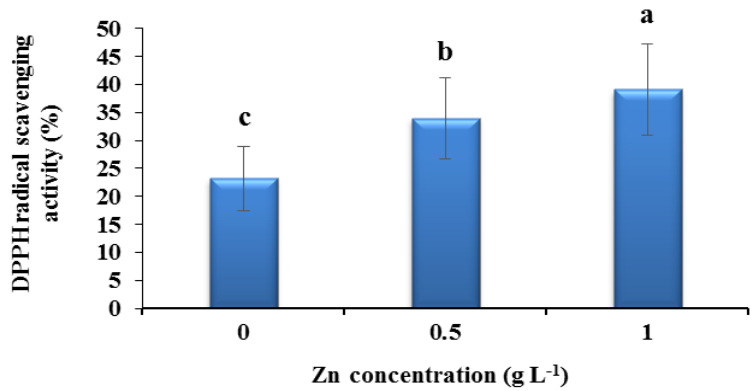

Figure 3. The inhibition percentage of DPPH in the juice of the control and $ZnSO_4$-treated lemon fruits at harvest. The data are the mean values ± SE of four technical replicates. Values followed by the same lowercase letters are not significantly different.

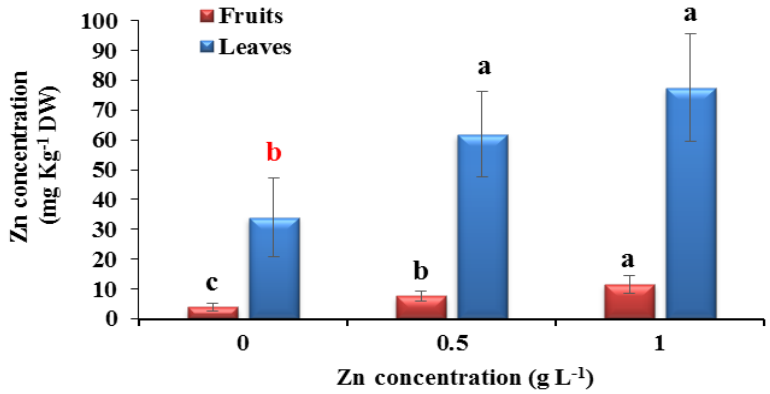

Figure 4. The content of zinc in the control and $ZnSO_4$-treated lemon leaves and fruits at harvest. The data are the mean values ± SE of four technical replicates. Values followed by the same lowercase letters are not significantly different.

independent of leaf Zn concentration in the range of 34-142 mg kg^{-1}. Plants treated with 1 g L^{-1} Zn recorded the highest juice anti-radical ability. In comparison to the non-Zn-treated fruits, DPPH radical-scavenging activity of 0.5 or 1 g L^{-1} Zn was almost 46.6 and 68.5% higher, respectively (Figure 3).

There was a significant difference between concentrations of Zn in leaves due to treatments (Figure 4). The increase in Zn concentrations in leaf tissues coincided with an increase in Zn concentrations in fruits (Figure 4). In Zn-treated plants at the higher Zn level (1 g L^{-1} Zn), there was a substantial increase in Zn concentration, corresponding to 2.3-fold in leaf and 2.9-fold in fruit. Leaf or fruit Zn contents increased with increasing Zn concentrations, and a higher Zn concentration was observed in the samples receiving 1 g

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Table 2. Pearson correlation analysis of the relationship between leaf and fruit Zn content and phytochemical contents of 'Lisbon' lemon juice after foliar application of zinc sulfate ($ZnSO_4$).

	ASA	Total phenolic compounds	Flavonoids	Anthocyanins	Carotenoids	GSH	DPPH	PPO activity	POD activity	Leaf Zn content	Fruit Zn content
Leaf Zn content	0.93**	0.91**	0.88**	-0.20^{ns}	0.93**	0.89**	0.88**	- 0.42^{ns}	- 0.43^{ns}	1.00	0.95**
Fruit Zn content	0.88**	0.87**	0.82**	- 0.10^{ns}	0.90**	0.93**	0.84**	- 0.45^{ns}	- 0.43^{ns}	0.95**	1.00

ns: not significant, ** significant correlations at P ≤ 0.01

L^{-1} Zn compared to the control and 0.5 g L^{-1} Zn treated ones. The Zn content of leaves was correlated with fruit Zn content, and these parameters correlated positively with juice antioxidant contents (Table 2).

Discussion

Many factors influence the nature and concentration of antioxidant compounds in fruits, including the chemical structure of the antioxidants, pre and post-harvest factors, and processing conditions. The pre-harvest factors that affect the antioxidant activity of *Citrus* fruits include environmental and agronomic conditions such as fertilizer (Zou *et al*., 2016). Although, based on studies on citrus, Zn application increases fruit quality and its antioxidant contents, to the best of our knowledge, no study has explored the potential role and the basis of Zn application to increase nutraceutical quality in lemon fruit.

The Zn application significantly improved fruit biochemicals (AsA, TP, flavonoids, anthocyanin, carotenoids and GSH contents). Zn plays an important role in photosynthesis, carbohydrate biosynthesis, protein synthesis, and nucleic acid metabolism, and it acts as a co-factor of many enzymes, controls the expression of genes required to protect cells from the detrimental effects of stress and promotes the activation of various enzymes required in these biochemical reactions (Nasir *et al*., 2016). ASA content in the lemon juice increased significantly with an increase in Zn concentration. Zn plays an active role in the production of auxin in plants and increased synthesis of auxins has been reported to increase the accumulation of ASA content in 'Kinnow' mandarin (Razzaq *et al*., 2013; Al-Obeed *et al*., 2017). Similarly, an increase in the level of ASA has been found in strawberry (Chaturvedi *et al*., 2005), mandarin (Khan *et al*., 2012) and apple (Rasouli and Koushesh Saba, 2018) fruits with foliar application of Zn. Also, minerals such as Zn are involved in sugar metabolism which directly are related to the synthesis of vitamin C (Nasir *et al*., 2016). Trees treated with 0.5 and 1 g L^{-1} Zn showed an increase in the level of TP about 1.5-fold and 1.7-fold higher than the control, respectively (Figure 2). The results are in line with the findings of Razzaq *et al*. (2013) and Khan *et al*. (2015) in 'Kinnow' mandarine, Song *et al*. (2015) in grape, and Davarpanah *et al*. (2016) in pomegranate but different from Aglar *et al*. (2016) in 'Jersey Mac' apples. According to Song *et al*. (2015), the increase in TP content was due to an increased expression of genes responsible for phenolic compound biosynthesis. In our study, foliar applications of Zn increased the total flavonoids in lemon juice. Both Zn and phenolic content changes have been the subject of many studies. However, to our knowledge, very little work has explored the potential role of exogenous Zn application to improve the biosynthesis of phenolics particularly flavonoids, in fruits. Due to the fact that sucrose is a positive regulator of the biosynthesis of flavonoids (Song *et al*., 2015), increased levels of flavonoids due to Zn sprays may be attributed to their effects on photosynthesis and sugar accumulation, which are involved in the biosynthesis of flavonoids. The critical attributes of Zn in plant cell function imply that leaves with Zn deficiency will display modified cellular metabolism, and consequently, a modified balance of flavonoid biosynthesis and accumulation. Results disagree with the findings of Manthey *et al*. (2000), who concluded that increased flavonoid concentrations were correlated with the increased levels of leaf TP occurring in blight-induced Zn-deficient citrus. Zn spray had no significant effect on anthocyanin content. When the effects of foliar sprays of Zn and boron were studied in pomegranate, Davarpanah *et al*. (2016) found that there was no significant effect on total anthocyanins with any of the Zn treatments. Similarly, anthocyanin content has not been increased in pomegranate received Zn spray (Hasani *et al*., 2012) and the mechanism of how Zn did not affect anthocyanin content is not wholly described. In contrast, the anthocyanin content of Zn-treated grapes was higher than that of the control group (Song *et al*., 2015). The highest increase in the level of carotenoid contents was recorded in the fruit harvested from the trees, which were sprayed with 1 g L^{-1} Zn before the maturity stage. This is possible because Zn deficiency significantly results in the deformation of chloroplast structure and the limitation of photosynthetic enzymes, consequently reducing the carotenoid contents (Fu *et al*., 2016). Increased levels of carotenoids due to Zn sprays may be attributed to their effect on increasing chlorophyll biosynthesis and carotenoids (Aglar *et al*., 2016). The variation of GSH in lemon juice showed that its content can be affected by external Zn treatments. Soleimani Aghdam and Bodbodak (2014) reported that enhanced glucose content in fruits may activate the oxidative pentose phosphate pathway and lead to higher NADPH. Glutathione reductase (GR) then utilizes NADPH to convert oxidized glutathione to GSH. Also, Liu *et al*. (2013) reported that glucose-6-phosphate

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dehydrogenase (G6PDH), as a rate-limiting enzyme in the pentose phosphate pathway, enhances GR and dehydroascorbate reductase enzyme activities, which led to enhanced AsA and GSH concentrations. In this connection, Razzaq *et al*. (2013) reported that the effect of Zn spray on increased fruit sugar contents might be due to its role in the activity of the aldolase enzyme, which is directly linked with the formation of sugars in fruits. Also, Azevedo *et al*. (2007) reported that long-term exposure to Zn enhanced the activity of G6PDH in the cells. Our results showed that DPPH radical-scavenging activity changes followed the same trend as AsA, TP, carotenoids and GSH, which was comparable to some early reports (Sida-Arreola *et al*., 2017; Rasouli and Koushesh Saba, 2018) but different from Davarpanah *et al*. (2016). Zn plays a major role in controlling the production and detoxification of reactive oxygen species (ROS), which can damage membrane lipids and sulfhydryl groups. Also, Zn-finger proteins are involved in small RNAs production, which is essential in the plant oxidative stress response. To protect the cell from oxidative stress, Zn may act synergistically with other antioxidants.

According to Zago *et al*. (2000), Zn could interact with antioxidants that act through the chelation of redox-active metals. In the present study, external Zn treatments decreased the POD and PPO activities of lemon juice. Once the cellular compartmentation is destroyed, the released POD and PPO in the cytoplasm oxidize phenolics and flavonoids into quinones and leads to a decrease in the content of TP and flavonoids after harvest. Many compounds can inhibit PPO activity directly or indirectly based on their mode of action, namely, complexing agents, reducing agents, enzyme inhibitors, acidulants, enzyme treatments, and chelating agents (Du *et al*., 2012). Higher content of flavonoid and TP as well as decreased activity of POD and PPO enzymes indicated that preharvest $ZnSO_4$ spray might help lemon fruits by protecting the antioxidants (Luo *et al*., 2018).

Zn contents in the leaves were higher than in the fruit juice. Zn concentrations in foliar-applied 0.5 or 1 g L^{-1} Zn leaves were 1.82 and 2.28-fold higher than control, respectively. On the other hand, in the fruits, the Zn concentration of the respective treatments was 1.91, or 2.91-fold higher than the control. It seems that sufficient foliar application of Zn caused the accumulation of Zn in the fruits. This could be attributed to the well Zn-supplied by foliar application. Gurel and Basar (2018) reported that Zn movement from leaves to fruit is limited, however, unless the concentration in the leaves is abundant. The results of Saa *et al*. (2018) demonstrate that absorption from foliar Zn fertilizers has a very low efficiency. Therefore, to improve the effectiveness of zinc foliar sprays, the application of three spraying per year was examined in this experiment. In the present study, the application of 1 g L^{-1} Zn as three sprays per year was found to raise Zn to the sufficiency level and thereby satisfy the Zn requirements of lemon trees grown on soils where Zn limits plant performance. It has been reported that the critical concentration of Zn in citrus leaves was 25–100 mg kg^{-1} (Khan *et al*., 2015), whereas the normal range of citrus leaf Zn concentration was also reported to be 20–110 mg kg^{-1} (Swietlik, 2002); another research reported that the normal range of citrus leaf Zn concentration was 25–50 mg kg^{-1} (Hippler *et al*., 2015). Therefore, the optimum leaf Zn concentration for lemon trees is uncertain. It is estimated that 1/3 of the world population is affected by Zn deficiency, which is associated with low dietary intake and illness and death in the developing world (Gurel and Basar, 2018); however, agronomic biofortification can cure human Zn deficiency. This study may provide a good strategy for increasing the juice antioxidants and Zn content of lemon trees, which show no Zn deficiency symptoms and a leaf Zn concentration is less than 35 mg kg^{-1} dry weight.

Conclusions

Despite the large number of papers about the antioxidant activity of citrus juices, less is known about the potential role of Zn application in increasing the nutritional quality of lemon. In conclusion, the two novel observations in this work are that three Zn sprays per year at a later stage of fruit expansion was found to raise Zn to the sufficiency level and that applying Zn significantly improved lemon juice's biochemical contents. Fruit harvested from trees treated with 1 g L^{-1} Zn exhibited higher AsA, TP, flavonoids, anthocyanin, carotenoids and GSH contents, and lower POD and PPO activity as compared to control. Therefore, when citruses are to be grown under certain conditions that interfere with Zn absorption in the soil solution, foliar fertilization may be critical to avoiding Zn deficiency damage. Foliar application of Zn to lemon trees is considered to be important in the enrichment efforts of food crops to promote human daily Zn intake. Further research is needed to investigate the effects of other factors, such as environment, soil characteristics and rootstock, on the nutraceutical properties of lemon juice to obtain new insights into future functional food programs.

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