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ORIGINAL ARTICLE

Soluble sugars, phenolic acids and antioxidant capacity of grape berries as affected by iron and nitrogen

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Abstract

Foliar nutrition is one of the effective cultural practices in vineyards. In this research, the effect of iron chelate (Fe-EDDHA) and urea, each in three levels of 0, 0.5 and 1%, was evaluated with an ANOVA completely randomized block in commercial vineyard (cv "Sultana") located in Bahareh village of Malayer city (Iran). Vines were sprayed in three stages: a week before bloom (8 June), 2 weeks after bloom (29 June) and 5 weeks after bloom (20 July) during the growth seasons in 2015 and 2016. The grapes harvesting was done in mid-September according to the maturity level of untreated vines. In comparison with the other treatments, moderate levels (0.5%) of fertilizers allow to reach the highest glucose and sucrose concentration at harvest. Foliar spray of high iron chelate doses in combined with 0.5% urea caused a considerable increase in berries putrescine and spermine concentration. However, combination effects of urea and Fe-EDDHA with moderate level (0.5%) were the most efficient for spermidine accumulation of 'Sultana' grapevine. For the moderate levels (Fe-EDDHA 0.5%) of fertilizers treatment, most phenolic acids and anthocyanidins reached a peak, and the highest free radical scavenging capacities (DPPH) of grape samples were achieved. The activity superoxide dismutase, guaiacol peroxidase, catalase and ascorbate peroxidase increased with moderate levels of Fe-EDDHA in combination with high levels of urea treatments. However, the maximum glutathione reductase was obtained with 1% urea in combination with Fe-EDDHA at 1% concentrations. Altogether, data showed that iron and nitrogen are highly efficient to manage quality and nutritional potential of grape berries.

Keywords Anthocyanidins · Glucose · Glutathione reductase · Grapes · Nitrogen · Nutrition

Introduction

At world scale, grapes (*Vitis vinifera* L.) are one of the most important fruit crops in terms of surfaces, yield and economic value. Among the 75.8 million metric tons produced in 2016, almost 50% worldwide were consumed as fresh grapes, raisins or fruit juice (OIV 2017). Grape quality is related to the balance between primary and secondary metabolites. Among primary metabolites, glucose and fructose are the main sugars in berries, while some sucrose can be found in cultivars other than *V. vinifera*. Amount of these primary metabolites can be affected by variety, harvesting time and berries sanitary status (Dai et al. 2011). Moreover, high sugar accumulation in berries can improve the volatility of aromatic compounds (Ali et al. 2010). Secondary metabolites such as flavonoids, anthocyanins, tannins and phenolic acids accumulate in berries at different developmental stages. As recently reviewed by Daglia et al. (2014) for fresh grapes, these phenolic compounds are considered to

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have various beneficial effects on human health, in relation with their antioxidant properties.

Mineral nutrition has an important effect on grape yield and quality (Keller et al. 2001). Among all nutrients, nitrogen (N) has a huge effect on grapevine vegetative growth, yield, and tissue composition (Jackson and Lombard 1993; Bell and Henschke 2005). Nitrogen is a basic element of some main biomolecules including chlorophyll, amino acids, nucleic acids and hormones which are critical for optimum plant metabolism and energy production (Keller 2015). For these reasons, low availability of nitrogenous reserves owing to insufficient fertilization in the previous growing period can retard or inhibit shoots growth and reproductive buds development (Celette et al. 2009) and, therefore, can lead to weak fruit set (Keller et al. 2001). The nitrogen reserves are depleted around bud break, reaching a low level during flowering or, sometimes, as late as veraison (Schreiner et al. 2006). Therefore, adequate leaf nitrogen range is needed to increase photosynthesis and subsequent crop yield and quality. Nitrogen effects on berry composition are complex and interact with other parameters as rootstocks and initial plant N status (Bell and Henschke 2005; Stockert et al. 2013; Habran et al. 2016). Organic acids, phenolic acids and anthocyanins are among the most affected compounds (Habran et al. 2016; Gutiérrez-Gamboa et al. 2017; Canoura et al. 2018).

Iron (Fe) is a key micronutrient in grapevine plants. It acts as a cofactor or component of some proteins and many enzymes involved in electron transfer system and reduction/oxidation reactions (Curie et al. 2008; Curie and Briat 2003). Moreover, iron mediates some of the main physiological processes such as photosynthesis and respiration and also enzyme activation. Biosynthesis of chlorophyll, assimilation of carbon, nitrogen, and sulfur, synthesis of phospholipid and abscisic acid and scavenging of oxygen free radicals are other roles of iron in plants (Curie et al. 2008). Iron deficiency is one of the main problems of growing grapevines in calcareous soils or high pH irrigation water. Lime-induced chlorosis decreases fruit production, reducing berry and cluster weight and yield and affecting berry quality indices such as total anthocyanin, polyphenol and resveratrol content (Bavaresco et al. 2001).

Fertilization, both at soil and foliar levels, is one of the main viticultural practices which can affect fruits yield and quality (Karimi 2017). Foliar application is one of the fastest and effective methods for providing plants with some specific requirements, especially at some critical stages of growth (Marschner 2011). The effect of foliar application on grape composition is highly dependent on the form of N used, the time of application and the variety. Whereas primary metabolites, except amino acids, are rarely directly affected, yeast assimilated nitrogen (YAN) and secondary metabolites as phenolics and aromas are usually improved (Delgado et al. 2004; Lacroux et al. 2008; Lasa et al. 2012; Garde-Cerdán et al. 2015; Gutiérrez-Gamboa et al. 2017).

Iron fertilization can affect fruit quality factors and all the components of yield in many fruit trees (Àlvarez-Fernàndez et al. 2006). Among small fruits, grapes have relatively low tolerance to iron deficiency (Àlvarez-Fernàndez et al. 2006) and foliar application of iron, particularly at specific phenological stages, is required to supply Fe quickly through plant leaf cuticles pores. The beneficial effects of Fe foliar spray on vineyard yield and berry sugar content were shown by many authors (Bacha et al. 1995; Shi et al. 2017). Several studies demonstrated that some N forms interact with Fe uptake and can enhance Fe deficiency chlorosis in plants (Smolders et al. 1997; Jiménez et al. 2007; Marschner 2011). However, there is little evidence on the interactive effects of iron and nitrogen fertilization on berry composition in terms of soluble sugars, phenolic acids and enzymatic and non-enzymatic antioxidant capacity of grapevine.

The aim of the present study was to compare the effects of foliar applications of nitrogen and iron delivered separately or in combination, on grape biochemical parameters. We investigated whether foliar applications of urea and Fe-EDDHA could affect soluble sugars, polyamines, phenolic acids and enzymatic and non-enzymatic antioxidant capacity of 'Sultana' grapevine fruits located in Bahareh village of Malayer city (Iran).

Materials and methods

Plant materials and treatments

The present experiment was conducted in 2015 and 2016, on 16-year-old grapevines (*Vitis vinifera* cv. 'Sultana') grown in a commercial vineyard located in Bahareh village of Malayer city (lat. 34°30′N, long. 48°85′E, alt. 1750 m), Iran. The plants were grown on their own root in a clay–loamy soil, with a pH of 8.3 and 8.7 at the depth of 0–30 cm and 30–60 cm, respectively, Soil analysis is provided in Supplemental Table 1. Vines with non-trellised canopies and planting density of 1.5 × 3 m were pruned on the 5th March with 8 canes of 12 buds besides 8 renewal spurs and watered every 12 days with furrow irrigation system. Base mineral fertilizers were applied at the rates of 150 N kg ha^{-1} (as ammonium sulfate; 20.5% N), 80 kg P_2O_5 ha^{-1} (as triple superphosphate; 48% P_2O_5), 100 kg K_2O ha^{-1} (as potassium sulfate; 50% K_2O),and 30 kg $MgSO_4 \cdot 7H_2O$ ha^{-1} (as magnesium sulfate; 18.3% Mg), and 4 kg $ZnSO_4 \cdot H_2O$ ha^{-1} (as zinc sulfate monohydrate; 35% Zn), respectively, taking into consideration soil nutrient content (Sing 2006).

The study was conducted using completely randomized block design, with three replications, and included two grapevine plants per experimental unit. The plants were

sprayed to run off with three levels of urea (46% nitrogen; 0, 0.5 and 1%) combined to three levels of iron chelate 6% [(ferric ethylenediamine di (*o*-hydroxy phenylacetic) acid (Fe-EDDHA)]; 0, 0.5 and 1%) on all leaves at three developmental stages including a week before bloom (8 June), 2 weeks after bloom (29 June) and 5 weeks after bloom (20 July) during the growth seasons in 2015 and 2016. The plants treated in 2015 were the same treated also in 2016. The different foliar treatments were named as N_1F_1 (control, urea and Fe-EDDHA at 0%); N_1F_2 (0% urea and 0.5% Fe-EDDHA); N_1F_3 (0% urea and 1% Fe-EDDHA); N_2F_1 (0.5% urea and 0% Fe-EDDHA); N_2F_2 (0.5% urea and 0.5% Fe-EDDHA); N_2F_3 (0.5% urea and 1% Fe-EDDHA); N_3F_1(1% urea and 0% Fe-EDDHA); N_3F_2 (1% urea and 0.5% Fe-EDDHA); N_3F_3 (1% urea and 1% Fe-EDDHA). These mineral elements have been given to the vines in addition to a regular fertilization made to soil. It should be noted that at each stage, 350 mL of each dose of fertilizer was sprayed on vines canopy. Therefore, in addition to soil application, a total amount of 5 g (for 0.5% dose of both urea and Fe-EDDHA) and 10 g (for 1% dose of both urea and Fe-EDDHA) of these fertilizers were added to each plant in each year. Fruits were harvested at mid-September based on maturity index of control untreated vines (°Brix of 22.8).

Leaf mineral contents

Samples of petioles from the leaves adjacent to fruit clusters located at the middle of growing shoots were collected 15 days after bloom (1 July) each year and their N, P, K, Mg, Ca, Zn, Mn and Fe concentrations were measured. Samples were dried in oven (72 °C for 72 h), then powdered and used for measurement of nutrient elements. Total N was assayed using the Kjeldahl method. Phosphor was determined using a spectrophotometer. K was flame photometrically analyzed. The sample extracts were measured for Mg, Ca, Fe, Zn and Mn using an atomic absorption spectrophotometer (Varian, 220).

Extraction and analysis of soluble sugars

After harvesting, the samples of berries were powdered (0.5 g) and homogenized with 10 mL 80% ethanol. After centrifugation (8000*g* for 15 min), the solution was filtered through a 0.2 µM filter (Shin et al. 2002). The filtered supernatants were applied for measurement of fructose, glucose and sucrose using a Crystal 200 series HPLC pump (ATI Unicam, Cambridge, UK) equipped with a SPD UV–Vis detector (Philips, Cambridge, UK) and a Spherisorb ODS-2 Column (0.3 µm, 150 mm × 4.6 mm i.d.) from Hichrom (Berkshire, UK). Sodium citrate (pH 5.5) and ultrapure acetonitrile (1:99, v/v) at a flow rate of 0.1 mL min^{-1} were used as the mobile phase. The injection volume was 10 µL (Comis et al. 2001). External standard solution calibrations of sucrose, glucose and fructose (Sigma, Australia) were used to integrate peaks. Sugar concentrations were expressed in µmol/g fresh weight (FW).

Extraction and analysis of polyamines

The berries' free polyamines extraction, separation, identification and measurement by direct dansylation and HPLC have been described according to Walter and Geuns (1987). Briefly, 250 mg of frozen berries was homogenized in 2 mL of 4% $HClO_4$ containing 1, 7 diaminoheptane-2HCl as internal standard. After 1 h at 4 °C, the homogenate was passed through a 0.45 µ filter. Then, 1 mL of carbonate buffer (pH 9) and 1 mL of dansyl chloride solution (10 mg mL^{-1} acetone) were added to 0.2 mL of homogenate. After warming for 1 h at 60 °C, the dansylated polyamines were extracted with 3 mL of toluene.

The extract was loaded on a 0.5 g silica gel column and washed with 5 mL of toluol and 5 mL of toluol–triethylamine (10/0.3). The dansylated polyamines were then eluted with 3 mL of ethyl acetate and the volume was reduced under N_2. Isocratic HPLC analysis with acetonitrile/H_2O (72/28, v/v) on a 10-cm long 3 mm octadecyl silica column took 8 min. Solvent flow was 2 mL min^{-1}. Dansylated putrescine, spermidine and spermine were injected as references.

Extraction and determination of phenolic acids and anthocyanidins

For analysis of phenolic acids and anthocyanidins, the ground powders of entire berries (1 g) of each treatment were boiled in 0.1 N HCL for 25–30 min. The filtrate was then separated with ethyl acetate and dissolved in water and the portion insoluble in water dissolved in 80% methanol and filtered through a Millex HA 0.45 µm filter (Milipore Crop.) before injection (Koponen et al. 2007). Chromatographic separation was done on a Hypersil ODS 5 µm column (4.6 × 250 mm) at 25 °C. Chromatography was performed with a Crystal 200 series HPLC pump (Unicam, Cambrige, UK) equipped with a UV–Vis detector, regulated at 254 nm. The mobile phase consisted of potassium dihydrogen phosphate and acetonitrile (80:20, v:v). The flow rate was 1 mL min^{-1}. Standard acids (gallic acid, catechin, caffeic acid, catechin hydrate, *p*-coumaric acid, epicatechin, ferulic acid, myricetin, resveratrol, quercetin and kaempferol) and anthocyanidins (delphinidin, cyanidin, pelargonidin, malvidin) were purchased from E. Merck. Stock solutions of the standard acids were prepared in a concentration of 1 g 100 mL^{-1} in pure methanol (Vekiari et al. 2008).

Total soluble protein

Total soluble protein content of berries was determined with the colorimetric method of Bradford (1976) by recording the samples absorbance at 595 nm, using bovine serum albumin as a standard and finally was expressed as mg g^{-1} FW of berry samples.

Antioxidant enzyme activities

One-hundred milligrams of frozen berries powder was homogenized in 1.0 mL sodium phosphate buffer (0.05 M, pH 7.8) containing 1.0 mM EDTA and 2% (w/v) polyvinylpolypyrrolidone. The obtained solution extract was centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatant was used for all enzyme activity measurements. All activities were performed at 4 °C.

Superoxide dismutase (SOD) activity was determined based on the method of Beauchamp and Fridovich (1971), which measures inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. Each reaction mixture (3 mL) contained 50 mM sodium phosphate buffer pH 7.8, 33 µM NBT, 10 mM L-methionine, 0.66 mM EDTA, and 0.0033 mM riboflavin. Each reaction was carried out at 25 °C at a light intensity of approximately 250 µmolm^{-1} s^{-1} for 5 min. One unit of SOD activity was defined as the quantity of SOD required to inhibit the reduction of NBT by 50%.

Catalase (CAT) activity was determined by measuring the decrease in the absorbance of H_2O_2 at 240 nm (Bergmeyer 1970). Each 3 mL reaction mixture contained 0.05 M sodium phosphate buffer, pH 7.0 with 1.0 mM EDTA and 3% (v/v) H_2O_2. The decrease in absorption at 240 nm was monitored for 3 min. One unit of CAT activity was defined as the amount of enzyme that resulted in 1.0 µmol of H_2O_2 degraded mL^{-1} min^{-1}.

Guaiacol peroxidase (GPX) activity was measured by following the oxidation of guaiacol by H_2O_2 at 470 nm (Herzog and Fahimi 1973). One mL of each crude leaf enzyme extract was added to a 3 mL reaction mixture containing 0.855 µL of 25 mM guaiacol and 1.355 µL of 30% (v/v) H_2O_2 in 3 mL of sodium phosphate buffer, pH 7.0. The reaction was initiated by adding the H_2O_2. One unit of GPX activity was defined as the amount of enzyme that degraded 1.0 µmol guaiacol mL^{-1} min^{-1}.

Ascorbate peroxidase (APX) activity was determined by measuring the decrease in absorbance at 290 nm for 1.0 min as ascorbate was oxidized (Nakano and Asada 1981). Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM ascorbate, 0.1 mM Na_2 EDTA, and 1.2 mM H_2O_2. One unit of APX activity was defined as the amount of enzyme that oxidized 1.0 µmol ascorbate mL^{-1} min^{-1}.

Glutathione reductase (GTR) activity was measured following the decrease in absorption at 340 nm due to NADPH oxidation (Foyer and Halliwell 1976). The reaction mixture contained 50 mM phosphate buffer (pH 7) with 2.5 mM MgCl2, 0.5 mM GSSG, 0.2 mM NADPH, and 0.3 mL enzyme extraction in final assay volume of 2.8 mL.

The specific activity of each antioxidant enzyme was then expressed in units mg^{-1} TSP.

Determination of antioxidant capacity

To determine the antioxidant activity of berries, free radical scavenging properties of the extracts were evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH (radical scavenging activity (Bozin et al. 2008). In brief, various concentrations of each extract were added to 1 mL of 90 µM DPPH solution and made up with methanol (95% v/v) to a final volume of 3 mL. The mixture was shaken immediately after adding DPPH solution and was allowed to stand for 1 h at room temperature in the dark and then the absorbance was read at 517 nm against the blank (the same solution with no added extract). Three replicates were recorded for each sample. The DPPH radical scavenging capacity (RSC) was calculated using the following equation:

$$\text{DPPH RSC}\,(\%) = \left[\left(A_{\text{blank}} - A_{\text{sample}}\right)/A_{\text{blank}}\right] \times 100.$$

Statistical analyses

Data were subjected to analysis of variance (ANOVA) using the GLM procedures of the SAS software package (SAS Institute Inc., Cary, NC, USA), and mean separation was done using Duncan's multiple range test at $P \leq 0.05$. Because of the non-significant effect of the year, the average data for both years were analyzed.

Results

Leaf mineral content

Urea and Fe-EDDHA application, and their interaction, significantly ($P \leq 0.001$) increased leaf petioles nutrient concentration in 'Sultana' grapevine plants. Nitrogen concentration increased with the graded levels of urea in comparison with control untreated vines (Table 1). Interestingly, N concentration of vines treated with urea and Fe-EDDHA was found to be higher in comparison to those treated with urea only; the highest concentration of N was detected in treated vines with Fe-EDDHA at 1% in combination with 1% urea (N_3F_3; Table 1). The highest K concentration was obtained with 1% urea in combination with Fe-EDDHA at

Table 1 Effect of urea and Fe-EDDHA foliar application on leaf petioles mineral composition of 'Sultana' grapevine cultivar

Fertilizers concentration	N (%)	K (%)	P (%)	Mg (%)	Fe (ppm)	Zn (ppm)	Mn (ppm)
N1F1	1.31 ± 0.06 f	1.60 ± 0.06 e	0.27 ± 0.03 c	1.27 ± 0.03 e	120 ± 4 e	32.5 ± 1 f	127 ± 2 e
N1F2	1.48 ± 0.07 e	1.72 ± 0.04 c	0.28 ± 0.04 c	1.32 ± 0.03 cd	144 ± 5 b	47.7 ± 4 c	135 ± 2 c
N1F3	1.50 ± 0.09 e	1.83 ± 0.04 b	0.32 ± 0.03 b	1.40 ± 0.04 b	176 ± 7 a	54.4 ± 2 b	142 ± 2 b
N2F1	1.68 ± 0.05 d	1.65 ± 0.05 d	0.36 ± 0.03 a	1.30 ± 0.04 d	118 ± 4 e	36.5 ± 3 e	125 ± 3 e
N2F2	1.78 ± 0.06 c	1.84 ± 0.06 b	0.37 ± 0.05 a	1.35 ± 0.05 c	134 ± 3 c	46.7 ± 3 c	136 ± 1 c
N2F3	1.81 ± 0.05 c	1.82 ± 0.08 b	0.32 ± 0.03 b	1.37 ± 0.03b c	141 ± 4 b	59.4 ± 2 a	143 ± 3 b
N3F1	1.96 ± 0.07 b	1.71 ± 0.06 c	0.27 ± 0.04 c	1.34 ± 0.04 c	126 ± 5 d	40.7 ± 1 d	142 ± 3 b
N3F2	2.15 ± 0.12 a	1.97 ± 0.04 a	0.33 ± 0.03 b	1.35 ± 0.04 c	135 ± 4 c	55.5 ± 2 b	132 ± 2 d
N3F3	2.17 ± 0.23 a	1.85 ± 0.05 b	0.36 ± 0.04 a	1.45 ± 0.05 a	136 ± 3 c	57.6 ± 2 ab	154 ± 4 a

N1 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

In each column, means with the same letters are not statistically different ($P \leq 0.05$) by Duncan's multiple range test. Values are means of three replicates ± SD

0.5 concentrations (N_3F_2). The lowest K concentration was achieved with control untreated plants (N_1F_1; Table 1).

Petioles phosphorus and Mg contents were significantly affected by iron and urea fertilization. The highest amounts of these nutrients were observed in N_2F_2 and N_3F_3, respectively (Table 1). The leaf petioles' Fe, Zn and Mn concentration was significantly enhanced by all treatments as compared with control. The maximum value of Fe was related to 1% Fe-EDDHA-treated vines. Leaf Zn concentration of both N_2F_3 and N_3F_3 treatments was found to be higher in comparison with control vines (Table 1).

Soluble sugars

The effect of urea and Fe-EDDHA and their interaction on berry soluble sugar concentration were significant ($P < 0.001$). The highest fructose concentration was obtained in N_2F_3 treatment although it did not significantly differ from N_2F_2. Moreover, the highest glucose concentration was found in N_2F_2 but did not significantly differ from N_2F_1 (Table 2). Sucrose concentration was higher in fruits on vines sprayed with N_2F_2 treatment (Table 2).

Free polyamines

Urea and Fe-EDDHA application, and their interaction, significantly ($P \leq 0.001$) increased fruit free polyamines concentration in 'Sultana' grapevine plants. Putrescine and spermine concentrations were found to be the highest in plants treated with N_3F_3 and N_2F_3 fertilizers, respectively (Table 2). However, the combination of urea and Fe-EDDHA in moderate level (0.5%) was the most efficient for spermidine accumulation of 'Sultana' grapevine. The lowest concentrations of putrescine were found in N_2F_2-treated vines and the lowest spermine and spermidine were found in control untreated vines (Table 2).

Table 2 Effect of urea and Fe-EDDHA foliar application on berries soluble sugars and polyamine values in 'Sultana' grapevines cultivar

Fertilizers concentration	Fructose (μmol g^{-1} berry)	Glucose	Sucrose	Spermine (nmol g^{-1} berry)	Spermidine	Putrescine
N1F1	24.2 ± 0.3 e	35.1 ± 0.4 f	0.8 ± 0.1 f	10.2 ± 0.1 g	9.0 ± 0.2 f	10.7 ± 0.4 b
N1F2	32.2 ± 0.5 b	45.5 ± 0.5 b	1.4 ± 0.1 b	14.4 ± 0.1 d	12.9 ± 0.2 d	7.5 ± 0.3 d
N1F3	29.6 ± 0.5 c	39.7 ± 0.5 d	1.3 ± 0.2 c	13.7 ± 0.2 d	12.9 ± 0.25 d	9.1 ± 0.3 c
N2F1	32.6 ± 0.6 b	46.1 ± 0.3 b	1.2 ± 0.6 e	16.3 ± 0.6 c	16.5 ± 0.4 b	7.2 ± 0.3 d
N2F2	36.1 ± 0.5 a	48.4 ± 0.35 a	1.9 ± 0.1 a	18.2 ± 0.1 b	22.1 ± 0.5 a	6.3 ± 0.3 e
N2F3	38.7 ± 0.3 a	32.1 ± 0.4 g	0.7 ± 0.1 f	21.9 ± 0.1 a	20.5 ± 0.3 a	12.0 ± 0.3 a
N3F1	22.8 ± 0.6 f	42.9 ± 0.7 c	1.2 ± 0.1 d	11.3 ± 0.1 f	8.5 ± 0.3 f	9.0 ± 0.4 c
N3F2	27.5 ± 0.6 d	37.8 ± 0.4 e	1.3 ± 0.2 c	12.4 ± 0.2 e	11.5 ± 0.2 e	10.5 ± 0.3 b
N3F3	30.1 ± 0.7 bc	33.5 ± 0.6 g	1.2 ± 0.1 d	14.3 ± 0.1 d	12.9 ± 0.2 c	12.5 ± 0.3 a

N1 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

In each column, means with the same letters are not statistically different ($P \leq 0.05$) by Duncan's multiple range test. Values are means of three replicates ± SD

Phenolic acids and anthocyanidins

The fruits phenolic acid concentration was affected significantly ($P \leq 0.01$) by urea and Fe-EDDHA application. Cyanidin and pelargonidin as anthocyanidins (Table 4) and most phenolic acids such as gallic acid, catechin hydrate, *p*-coumaric acid, resveratrol and myricetin were found to be higher in vines treated with 0.5% urea in combination with 0.5% Fe-EDDHA fertilizers as compared to other nutrition treatments (Table 3). The combination effect of urea at 0.5% and Fe-EDDH at 1% concentration was the most efficient on the concentration in epicatechin and other anthocyanidins including delphinidin and malvidin (Table 4). The highest concentrations in quercetin and ferulic acid were observed for the N_3F_1- and N3F3-treated plants, respectively. Catechin concentrations were the highest for N_2F_1-treated plants (Table 3). The maximum concentrations of kaempferol and caffeic acid were observed for 1% Fe-EDDH sprayed vines (N_1F_3). The lowest concentrations in delphinidin, cyanidin, pelargonidin (Table 4), gallic acid, epicatechin, ferulic acid, and quercetin were recorded for control vines. The lowest malvidin concentration was reported for Fe-EDDTA at 1% treatment (N_1F_3). The catechin concentration in fruits of N_3F_2-treated vines was the lowest in comparison to all the other treatments (Table 3). Caffeic acid, catechin hydrate, p-coumaric acid and resveratrol concentrations in fruits of N_3F_3-treated vines were the lowest in comparison to other treatments (Table 3).

Total soluble proteins

Urea and Fe-EDDHA application, and their interaction, significantly ($P \leq 0.001$) influenced the total soluble protein content of 'Sultana' grapevine fruits. As shown in Fig. 1a, total soluble proteins increased dramatically for both Fe- and N-treated vines and the highest values were found for 1% urea-treated vines in combination with all three levels of Fe-EDDHA. The lowest total soluble protein content was observed for control plants (Fig. 1a).

Antioxidant enzyme activities

SOD activity in fruits was significantly affected by N and Fe treatments. As shown in Fig. 1b, SOD activity in fruits increased dramatically with the concentration of Fe-EDDHA treatments when iron treatment was applied alone or combined with 0.5% urea. The highest SOD activity recorded in fruits developed on vines treated with Fe-EDDHA at 1% was more than twice that of the control plants.

As shown in Fig. 2a, the combination effect of Fe-EDDHA and urea on GPX activity was found to be significant ($P \leq 0.001$). The highest GPX activity was achieved in 0.5% urea in combination with 0.5 and 1% Fe-EDDHA

Table 3 Interaction effect of urea and Fe-EDDHA foliar application on phenolic acids (μg g^{-1} berry) values in 'Sultana' grapevine cultivar

Nutrient treatments	Gallic acid	Catechin	Caffeic acid	Catechin hydrate	P-coumaric acid	Epicatechin	Ferulic acid	Myricetin	Resveratrol	Quercetin	Kaempferol	Total phenolic acids
N1F1	2.0 ± 0.25 f	4.0 ± 0.21a	1.3 ± 0.23 c	6.4 ± 0.30 e	4.0 ± 0.18 f	0.7 ± 0.26 c	3.1 ± 0.23 f	0.6 ± 0.15 d	15.1 ± 0.24 de	1.7 ± 0.26 e	1.6 ± 0.36 c	40.5 ± 0.66 g
N1F2	5.6 ± 0.30 c	3.8 ± 0.24 ab	1.6 ± 0.28 b	10.3 ± 0.23b	7.0 ± 0.20 b	0.8 ± 0.33 bc	3.8 ± 0.22 e	0.9 ± 0.23 bc	27.1 ± 0.14 ab	4.2 ± 0.30 c	1.9 ± 0.32 ab	67 ± 0.43 b
N1F3	4.4 ± 0.20d	4.0 ± 0.21a	2.1 ± 0.10 a	8.1 ± 0.23 c	5.8 ± 0.27 c	1.3 ± 0.09 a	5.7 ± 0.22 cd	1.0 ± 0.11 b	19.6 ± 0.41 cd	5.1 ± 0.20 b	2.1 ± 0.28 a	59.2 ± 0.46 d
N2F1	5.2 ± 0.27 c	4.4 ± 0.20 a	1.5 ± 0.14 b	10.1 ± 0.36 b	7.3 ± 0.20 b	1.1 ± 0.28 ab	4.0 ± 0.30 e	0.8 ± 0.22 b	25.8 ± 0.28 b	3.5 ± 0.23 cd	1.5 ± 0.16 d	65.2 ± 0.34 c
N2F2	7.2 ± 0.30 a	3.5 ± 0.23 b	1.0 ± 0.46 d	11.6 ± 0.32 a	8.8 ± 0.21 a	0.9 ± 0.10 b	7.2 ± 0.17 b	1.3 ± 0.18 a	29.7 ± 0.37 a	2.3 ± 0.29 de	2.0 ± 0.32 a	75.5 ± 0.76 a
N2F3	6.3 ± 0.29 b	3.3 ± 0.29 b	0.9 ± 0.28 d	5.8 ± 0.25 ef	5.7 ± 0.26 c	1.4 ± 0.30 a	7.6 ± 0.17 b	0.9 ± 0.42 bc	12.2 ± 0.34 e	3.0 ± 0.32 d	1.7 ± 0.49 bc	48.8 ± 0.44 f
N3F1	5.7 ± 0.32 c	4.1 ± 0.20 a	1.4 ± 0.34 bc	8.9 ± 0.27 c	5.8 ± 0.21 c	1.0 ± 0.26 b	4.9 ± 0.32 d	1.1 ± 0.05 ab	23.4 ± 0.35 c	6.3 ± 0.20 a	1.8 ± 0.34 b	64.4 ± 0.36 c
N3F2	3.0 ± 0.26 e	3.2 ± 0.20 b	1.2 ± 0.33 c	7.2 ± 0.27 d	4.8 ± 0.25 e	1.0 ± 0.18 b	6.3 ± 0.29 c	0.8 ± 0.01 c	17.3 ± 0.30 d	5.6 ± 0.13 ab	2.2 ± 0.36 a	52.6 ± 0.47 e
N3F3	2.3 ± 0.24 f	3.9 ± 0.25a	0.9 ± 0.24d	5.4 ± 0.20 f	3.9 ± 0.15 f	0.9 ± 0.42 b	8.5 ± 0.19 a	0.7 ± 0.12 cd	10.6 ± 0.27 e	4.1 ± 0.17 c	1.3 ± 0.32 e	42.5 ± 0.18 g

N1 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

In each column, means with the same letters are not statistically different ($P \leq 0.05$) by Duncan's multiple range test. Values are means of three replicates ± SD

Table 4 Interaction effect of urea and Fe-EDDHA foliar application on individual anthocyanidins (mg g^{-1} berry) in 'Sultana' grape cultivar

Nutrient treatments	Delphinidin	Cyanidin	Pelargonidin	Malvidin
N1F1	0.9 ± 0.32 b	0.7 ± 0.04 c	0.7 ± 0.07c	0.9 ± 0.42 b
N1F2	1.3 ± 0.26 ab	1.0 ± 0.03 b	1.3 ± 0.50 ab	0.7 ± 0. 14 bc
N1F3	1.5 ± 0.29 a	1.0 ± 0.02 b	1.1 ± 0.31 b	0.6 ± 0.08 c
N2F1	1.1 ± 0.30 b	0.8 ± 0.04 c	1.3 ± 0.31 a	1.1 ± 0.25 ab
N2F2	0.9 ± 0.21 b	1.2 ± 0.33 a	1.4 ± 0.39 a	0.9 ± 0.31 b
N2F3	1.6 ± 0.32 a	1.1 ± 0.07 ab	1.0 ± 0.26 b	1.3 ± 0.40 a
N3F1	0.8 ± 0.16 c	1.2 ± 0.50 a	0.8 ± 0.11 c	0.8 ± 0.06 b
N3F2	0.9 ± 0.30 b	1.1 ± 0.07 ab	0.1 ± 0.10 b	0.9 ± 0.38 b
N3F3	0.9 ± 0.20 b	0.7 ± 0.04 c	0.1 ± 0.07 b	1.2 ± 0.24 a

N1 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

In each column, means with the same letters are not statistically different ($P \leq 0.05$) by Duncan's multiple range test. Values are means of three replicates ± SD

and the lowest activity was detected in control plants. The effect of increasing concentrations of iron on GPX activity was dramatically stronger than that of urea foliar application (Fig. 2a).

As shown in Fig. 2b, foliar spray of Fe-EDDTA and urea on CAT activity was statistically significant (0.1% level). Catalase activity in fruits of 'Sultana' grapevine dramatically increased with the graded levels of Fe-EDDHA in comparison with control vines. The most marked increase in CAT activity of the fruits was found to be related to a foliar application of 0.5 and 1% Fe-EDDHA in combination with urea at 0.5% concentration with activity of 5.9 and 5.8 unit mg^{-1} proteins, respectively (Fig. 2b).

Urea and Fe-EDDHA application, and their interaction, significantly ($P \leq 0.001$) affected APX activity of 'Sultana' grapevine berries. As shown in Fig. 2c, the activity of APX increased with graded levels of Fe-EDDHA and urea, except for N_3F_3 treatment characterized by a lower activity in comparison to N_3F_2. The highest APX activity (15.9 unit mg^{-1} protein) in fruits of treated vines was found for 1% urea combined with 0.5% Fe-EDDHA (N_3F_2), although it did not significantly differ with that recorded for 0.5% urea and 1% Fe-EDDHA-treated (N_2F_3) plants. The lowest APX activity (5.16 unit mg^{-1} protein) was related to control vines (Fig. 2c).

Urea and Fe-EDDHA application, and their interaction, significantly ($P \leq 0.001$) influenced GTR activity in 'Sultana' grapevine fruits. The maximum GTR was obtained with 0.5% urea in combination with 1% Fe-EDDHA treatment (N_2F_3). The lowest glutathione reductase activity was achieved with control plants (N_1F_1; Fig. 2d).

Antioxidant capacity

Urea and Fe-EDDHA application, and their interaction, significantly ($P \leq 0.001$) influenced DPPH radical scavenging capacity of 'Sultana' grapevine fruits. The application of Fe-EDDHA increased antioxidant activity as measured by DPPH, through its interaction with urea. Based on our results, the highest (39.6%) free radical scavenging capacity of grape samples was achieved for 0.5% Fe-EDDHA combined with 0.5% urea. The lowest antioxidant capacity (23.5%) was found for control vines (Fig. 3).

Discussion

Cultural practices such as fertilization have major effects on fruit productivity and its final quality. In this work, the interactive effect of urea and chelated iron (Fe-EDDHA) on soluble sugars, phenolic acids and enzymatic and non-enzymatic antioxidant capacity of grapevine berries were

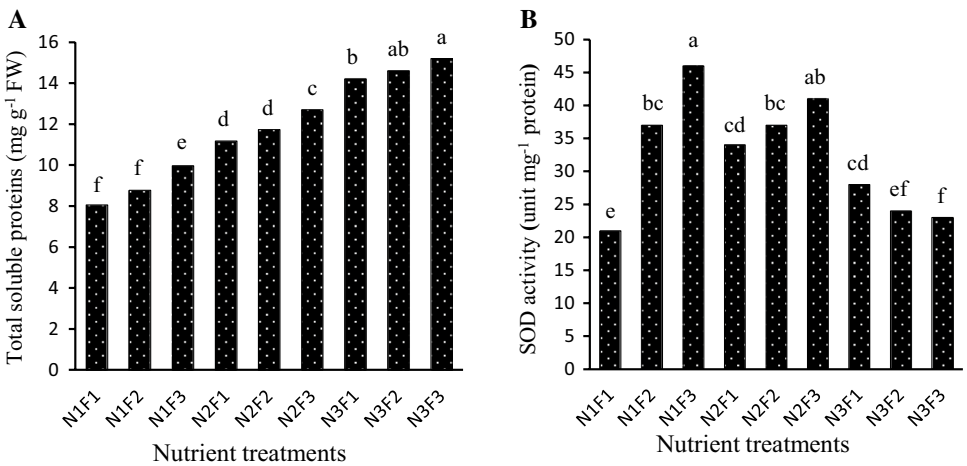

Fig. 1 Effect of urea and Fe-EDDHA foliar application on berries' total soluble protein content (**a**) and superoxide dismutase (SOD; **b**), activity values in 'Sultana' grapevine cultivar. Mean values marked with the different letters are significantly different ($P \leq 0.05$) by Duncan's multiple range test. *N1* 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

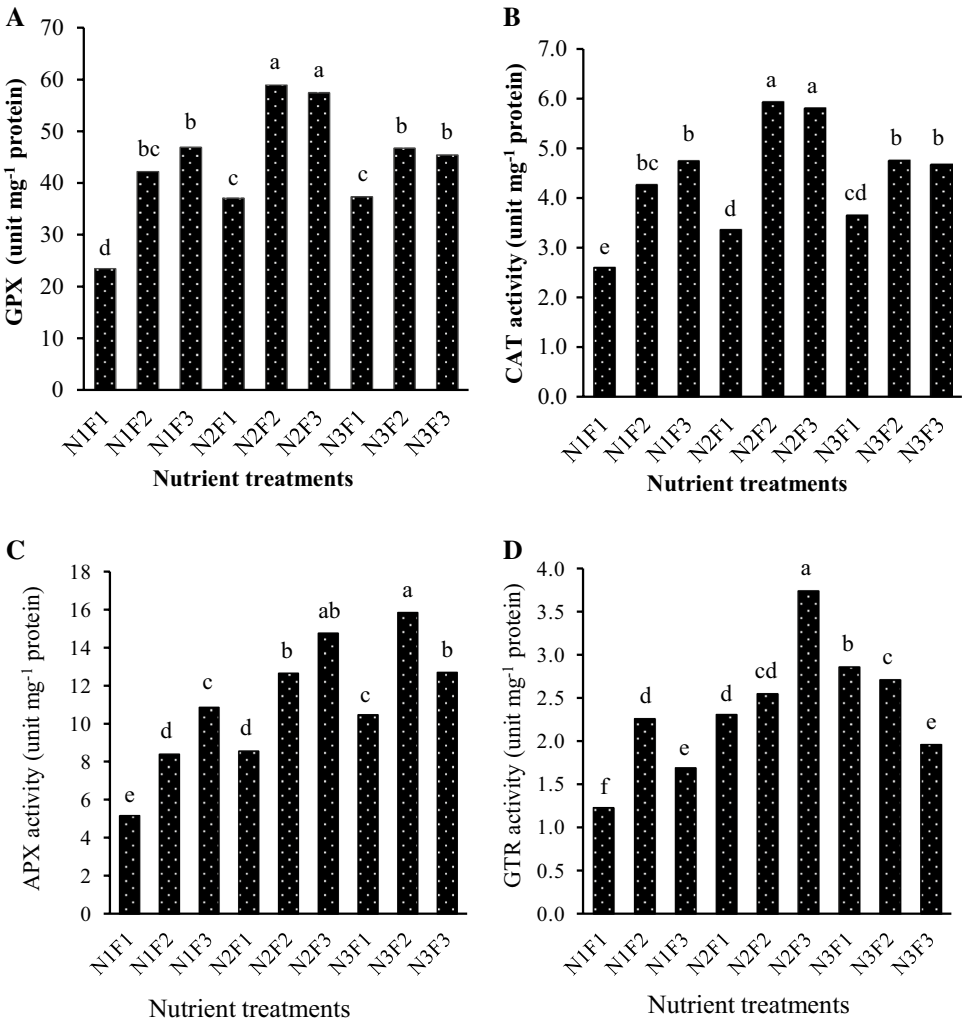

Fig. 2 Effect of urea and Fe-EDDHA foliar application on berries guaiacol peroxidase (GPD; **a**), catalase (CAT; **b**), ascorbate peroxidase (APX; **c**), and glutathione reductase (GTR; **d**) activities in 'Sultana' grapevine cultivar. Mean values for each enzyme activity marked with same lower case letter in each panel are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. *N1* 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

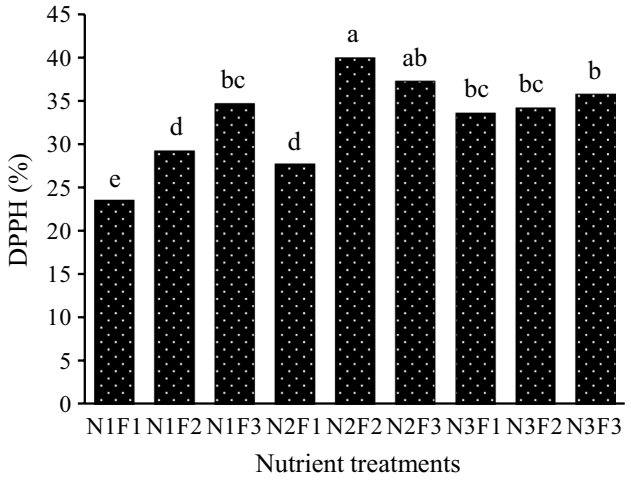

Fig. 3 Effect of urea and Fe-EDDHA foliar application on berries antioxidant capacity (measured by DPPH) in 'Sultana' grapevine cultivar. Mean values marked with the different letters are significantly different ($P \leq 0.05$) by Duncan's multiple range test. *N1* 0% urea, *N2* 0.5% urea, *N3* 1% urea, *F1* 0% Fe-EDDHA, *F2* 0.5% Fe-EDDHA, *F3* 1% Fe-EDDHA

evaluated under field conditions during two consecutive years.

Foliar application of urea and chelated iron, dramatically affected leaves' nutrient concentrations of 'Sultana' grapevine plants. The highest N and K concentration in leaf petioles, respectively, was related to N_3F_3- and N_3F_2-treated vines. Combined spray of both fertilizers, particularly at 1%, gave satisfactory improvement in nutrient concentration of Mg, P, Zn, Mn and Fe. This finding confirmed that foliar application of key nutrients at appropriate time during the growth season can affect the internal solubility of nutrients directly or indirectly. This is in agreement with the result of previous works (Delgado et al. 2004; Roosta and Mohsenian 2012; Askary et al. 2017). Observed changes in nutrient concentration may be interpreted by EDHHA-induced acidification of cell sap which promotes nutrient remobilization through providing an optimum physiological pH range. Due to the low Fe mobility in the phloem, repeated foliar sprays in chlorotic vines should be made to meet the Fe requirement during rapid shoot development (Rombolà et al. 2000).

Improved foliar nutrition has consequences on fruit composition. In grape, berries glucose and fructose are among the main sugars determining berries quality (Hufnagel and Hofmann 2008). For this reason, any factor changing sugar content can alter grape juice quality and taste. Nutrients such as nitrogen and iron have a major effect on sugar accumulation in grapes. In our study, the highest glucose and sucrose concentrations were found in grape berries treated with moderate levels (0.5%) of urea and Fe-EDDHA together compared to other treatments. The berry concentration of these two soluble sugars decreased for vines treated only with urea at the highest dose. The fructose concentration in 1% urea-treated vines was 58% lower than its concentration in fruits of N_2F_3-treated vines. The reduction of soluble sugar concentration with increasing nitrogen availability has been reported by other authors (Delgado et al. 2004) mainly through a positive effect on vegetative growth (Keller 2015). Abd El-Razek et al. (2011) hypothesized that increasing nitrogen supply reduced the availability of sugars such as sucrose for transport into the berries of 'Crimson Seedless' grape. These findings are in parallel with findings stating that excessive availability of N reserve can enhance shoot growth and canopy development and lead to poor fruit set and quality (Keller et al. 2001). Our results are also in agreement with those of Abdel-Salam (2016) who reported that treatment with Fe significantly increased total soluble solid and sugar and decreased acidity. Fe plays a key role in carbohydrate metabolism and fruit quality. Interestingly, foliar application of urea combined with chelated iron enhanced berries sugar content. Fe-EDDTA spray improved berries glucose, fructose and sucrose content and to a great extent alleviated the negative effect of excessive nitrogen supply on sugars accumulation in berries. Shi et al. (2017) postulated that increasing Fe-EDHHA fertilization improved reducing sugars of grape berries. Previous works have described the effects of suitable iron fertilization on photosynthesis efficiency, sugar transportation and accumulation (Ahmed et al. 1997; Álvarez-Fernández et al. 2003; Bertamini and Nedunchezhian 2005). We propose that berry soluble sugars were associated strongly with balanced ratio of nitrogen and iron supply in grapevine, through their effect on source–sink relationships.

Polyamines could serve as a nitrogenous source or as signal molecules regulating the reproductive development in the grapevine (Panagiotis et al. 2012). It is expected to increase in concert with rising urea doses in vines. Interestingly in our study their content was found to be higher in moderate combined doses of urea and Fe-EDDH-treated vines. This finding highlighted the interactions of iron and nitrogen metabolisms which have to be taken into account for vineyard fertilization programs in order to produce safe fruits with a lower free nitrate content. Therefore, putrescine may be a rather highly transportable nitrogenous molecule. Recently, Zhu et al. (2016) have indicated that putrescine plays an important role in the regulation of Fe deficiency responses in *Arabidopsis* plants. There are some evidences that nitrogen enhances polyamines content which in turn induces a rapid increase of nitric oxide production Zhu et al. (2016). Finally, nitric oxide has been demonstrated to play an important role in controlling Fe uptake and remobilization in plants.

In our work, soluble proteins were significantly increased by a combined spray of both urea and Fe-EDDHA, particularly at 1% dose. The significant effect of iron of soluble protein content was observed by Ranieri et al. (2001) in iron-deficient sunflower plants and in seeds of *Vigna unguiculata* (Salih 2013). A positive effect of foliar spray of urea and other micronutrients as boron on protein content was also reported in almond (Nezami 2012).

Grape phenolics contribute to color, flavor, texture and astringency of wine and to its antioxidant properties. The biosynthesis of soluble phenolics begins with the aromatic amino acid phenylalanine, a product of the shikimate pathway (Castellarin et al. 2013). Among viticultural practices, fertilization is well known to affect the proportion and the amount of phenolic compounds in berries, including anthocyanins and phenolic acids (Bavaresco et al. 2001; Soubeyrand et al. 2014). In the present study, the foliar application of urea alone increased the berry phenolic acid content. However, the combination of this fertilizer with Fe-EDDHA had the additive effect and increased the phenolic acid content indicating the involvement of iron in phenolic acid biosynthesis pathway. Our present results confirm that foliar application of urea at period of time before and after bloom, when shoot growth is slowed or stopped, enhances resveratrol concentration in berries in comparison with control as it was previously shown in Tempranillo vines (Garde-Cerdán et al. 2015). Consequently, it is likely that foliar treatments with urea favored the synthesis of amino acids including phenylalanine, a precursor of phenolic compounds including stilbenes (Garde-Cerdán et al. 2015).

Iron and nitrogen play important physiological roles as cofactor or component of some proteins and detoxification of reactive oxygen species (Keller et al. 2001; Curie et al. 2008). In the current work, the highest free radical scavenging capacity of grape berries, as measured by DPPH, was achieved in Fe-EDDHA at 0.5% in combination with urea at 0.5%. Iron is either a constituent or a cofactor of many antioxidant enzymes (Zhu et al. 2016), and enhances the stability and the activity of enzymes. This role was confirmed in the current study, especially when iron was supplied in combination with a moderate dose of urea as nitrogenous source. The highest SOD activity was observed in vines treated with Fe-EDDHA at 1% with no urea foliar application. As the intrinsic constituent or metal cofactor, iron is actively involved in cellular detoxification reactions catalyzed by CAT, GPX,

APX and Fe-SOD scavenging H_2O_2 and O_2^- (Ranieri et al. 2001). Heme proteins (i.e., CAT and GPX enzymes) and iron–sulfur proteins (i.e., isoenzymes of SOD) are two main groups of Fe-containing proteins (Marschner 2011) which can explain the higher antioxidant enzyme activities in the current study. All these observations confirmed that sufficient supply of iron and nitrogen may improve antioxidant capacity of fruits as documented by higher phenolics accumulation of berries in current study.

In conclusion, nitrogen and iron have several physiological functions in grapevine metabolism and any change in their concentration can affect yield and nutritional quality of berries. In the current study, the effect of combined foliar spray of urea and Fe-EDDHA was evaluated in bearing grapevine plants. Urea and Fe-EDDHA, and their interaction, significantly ($P \leq 0.001$) increased leaf nutrient concentration in 'Sultana' grapevine plants. Most berries phenolic acids, anthocyanidins and soluble sugars were found to be higher in vines treated with moderate levels of urea and Fe-EDDHA fertilizers as compared to other treatments. Moreover, putrescine and spermine concentrations were found to be the highest in fruits grown on plants treated with 0.5% urea combined with 1% Fe-EDDHA fertilizers. Interestingly, the highest free radical scavenging capacities (DPPH) of grape samples were achieved in Fe-EDDHA at 0.5% in combination with urea at 0.5%. The activity of approximately all antioxidant enzymes increased with moderate levels of Fe-EDDHA in combination with high levels of urea treatments and vice versa. The results showed that foliar application of iron chelate and urea especially at 0.5% during key stages of berry development is a key practice to improve the nutritional quality and antioxidant capacity of grapes, especially in the regions with calcareous nature and high pH soils.

Author contribution statement RK was the project supervisor and was the responsible for correspondence with the editors and reviewers. MK was MSc. student and responsible for field and laboratory experiments. NO participated in editing the manuscript.

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