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24-Epibrassinolide enhanced the quality parameters and phytochemical contents of table grape

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Summary

Enhancing the nutritional quality of fruits using safe and environmental friendly methods has become one of the most important targets in modern fruit production systems. Brassinosteroids, a new group of phytohormones with positive roles on human health, have been shown to modulate a wide range of plant activities and enhance fruit quality in some crops. This study was conducted to examine the effect of 24-Epibrassinolide (EBL), a synthetic brassinosteroid, on quality attributes and some active bio-compounds of 'Thompson seedless' table grapes. Grape vines and bunches were sprayed with EBL (at 0, 3 and/or 6 μmol L^{-1}) at three different stages (4 weeks after full bloom, at veraison stage and one day before harvest). As a novel finding in seedless grapes, exogenous EBL substantially enhanced soluble solids content, total organic acids, antioxidants, phenolics and ascorbic acid levels in treated berries. Also the activity of catalase and polyphenol oxidase enzymes was increased. There was no significant difference between the two tested brassinosteroid concentration levels in most cases. EBL showed a good potential for enhancing table grape phytonutrients, nutritional quality and phytochemical contents and can be introduced as a safe compound to be used in table grape production programs.

Keywords: brassinosteroid, polyphenol oxidase, phenolic compounds, phytochemicals, total antioxidant activity, 'Thompson seedless'

Introduction

Few fruits have garnered as much attention in the health research projects as grapes. In addition to being rich in some important bio-compounds necessary for human health including antioxidants, phenolics and anthocyanins, part of the reason for the importance of grapes may be their widespread presence in diets worldwide and profound economic importance. Grapes are cultivated in almost all countries and are consumed in different kinds of foods including fresh fruit, raisins, vinegar, juices, wines, seed oil and also medicinal and cosmetic products (EYDURAN et al., 2015). The health benefits of fresh grapes are mainly related to their phenolic compounds such as gallic acid, catechin, anthocyanins and resveratrol and a wide variety of procyanidins. These phytochemicals have been reported to have a wide range of pharmacological effects, including anti-carcinogenic, anti-atherogenic, anti-inflammatory, antimicrobial and antioxidant activities (LUAN et al., 2013; HARINDRA-CHAMPA, 2015). Improving the quality including phytonutrients, phytochemicals, antioxidants and vitamins of fruit not only results in enhanced marketability, nutritional and health improving properties of the berries, but also increases the quality of byproducts. Different quality parameters including nutritional properties are determined by genetic, environmental factors and cultural activities (HARINDRA-CHAMPA, 2015). For many years, the use of chemicals, including biocides against biotic stresses and inorganic nutrients for supplying essential elements for the plants, has been the main strategy to enhance crop quality during production. Different chemicals have been used to control diseases and disorders of crops and maintain the quality during handling and storage operations. However, for food safety issues and environmental concerns, recently the use of chemicals in food production systems has highly been restricted and organically produced foods, or foods with the least chemical residues, are preferred by the consumers (ASGHARI and SOLEIMANI-AGDAM, 2010; ROMANAZZI et al., 2016). It is well known that different plant growth regulators (PGRs) and phytohormones are the main players in different plant growth processes and new findings show that the use of different PGRs may be considered as an effective strategy to manage the plant growth and enhance the crop quality during growth stages (VARDHINI and ANJUM, 2015). While there is a little evidence about the effects of new plant growth regulators and phytohormones, the role of some classic PGRs such as ethylene, abscisic acid and gibberellins on yield, quality and responses of different table grape cultivars against different stresses has been widely studied. According to recent studies, among different phytohormones brassinosteroids (BRs) may have more crucial roles in development and ripening of table grapes (SYMONS et al., 2006; HARINDRA-CHAMPA, 2015; IŞÇI and GÖKBAYRAK, 2015). Brassinosteroids are plant-specific polyhydroxylated derivatives of 5α-cholestane, structurally similar to cholesterol-derived animal steroid hormones. Recent studies indicate the positive roles of BRs in human health including inhibition of herpes simplex virus type 1 (HSV-1) and arenavirus, measles, junin and vesicular stomatitis virus replication in cell culture (ESPOSITO et al., 2011). Brassinosteroids regulate the expression of specific plant genes and complex physiological responses related to different growth and defense mechanisms, cell division and enlargement, nutrient uptake, antioxidant systems, CO_2 enrichment, fruit quality and stress related responses (DIVI and KRISHNA, 2009; CHOUDHARY et al., 2012; ASGHARI and ZAHEDIPOUR, 2016).

Studies show that with the onset of ripening the concentration of natural BRs is increased in table grapes, indicating a positive role for these phytohormones in berry development and ripening (REES et al., 2012; HARINDRA-CHAMPA, 2015). According to the findings of SYMONS et al. (2006), exogenous BRs may enhance skin coloration and sugar accumulation and promote ripening process in table grapes, while brassinazole (an inhibitor of BR biosynthesis) significantly delays fruit ripening. Enhancing the natural defense systems of plants against pathogens and abiotic stresses is one of the most important and unique roles of BRs in plants. Increase in yield and quality of some horticultural crops has been reported after treatment with BRs and according to the reports the effects of BRs depend on plant growth stage, environmental conditions and BR concentrations (DIVI and KRISHNA, 2009; VARDHINI and ANJUM, 2015).

However, the positive effects of BRs on table grape production is not limited to fruit ripening. A wide range of different genes and enzymes may be regulated or affected by BRs. Some evidence indicate that BRs may enhance the synthesis of phytonutrients and activate resistance of grapes against different stresses. According to the findings of ZHU-MEI et al. (2013), external EBL has been shown to enhance the resistance related phytochemicals and subsequent resistance of 'Ziciuwuhe' table grape seedlings against chilling stress. The authors reported that EBL treatment has increased the gene

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expression and enzyme activity of some antioxidant and anti-stress enzymes. The positive effects of EBL on maintaining fruit quality, enhancing postharvest life and decreasing grey mold extension in table grapes has been reported by LIU et al., (2016). Treatment of strawberry seedlings with EBL simultaneously enhanced some growth parameters and disease resistance systems of strawberry plants, acting as a growth-promoting and relatively stress-mediating agent at low concentrations while strongly enhancing stress resistance mechanisms at higher doses (ASGHARI and ZAHEDIPOUR, 2016). The role of BRs in increasing leaf photosynthesis rate and improving the capacity of grape vines under stress conditions has also been reported by WANG et al. (2015). The important roles of BRs in promoting plant growth and enhancing natural stress related phytochemicals make it an appropriate candidate for organic crop production systems.

'Thompson seedless' is one of the most important table grape cultivars cultivated and exported worldwide. It is considered for relatively large berry size, high soluble sugars and capacity for both fresh consumption and processed to raisins, vinegar and wines. However, reports about BRs influencing the phytochemicals and different antioxidant fractions in grapes are few and there is no information about the effects of exogenous BRs on fruit quality parameters. On the other hand, in most of the studies on table grapes and other fruit crops, the effect of single spray with exogenous BRs have been studied. It has been well demonstrated that the effect of exogenous phytohormones on plant physiological traits and fruit quality is mostly dependent on crop species, phytohormone concentration, and the time and number of applications (IŞÇI and GÖKBAYRAK, 2015). Since a few days after application, the exogenous phytohormones are inactivated by plant cells, then it seems that in order to achieve the best results the hormone application should be repeated several times during plant and fruit growth stages. In this study, we examined the effect of spraying exogenous EBL at 3 successive stages on 'Thompson seedless' table grape quality parameters and some natural phytonutrient contents.

Materials and methods

Treatment of vines and bunches with EBL: The experiment was performed on 12-year old own rooted grapevines (*Vitis vinifera* L. cv. Thompson Seedless) planted at 2 m × 3 m spacing, trained on Cordon system in a commercial vineyard located in Urmia, Iran. The orchard was trained according to standard cultural practices including Guyot-training and drip irrigation. Nine vines (three vines per treatment) were chosen and uniform cultural practices were adopted according to recommendations (MAHINDRA, 2010). In order to determine the effects of exogenous EBL on fruit quality indices, vine canopy and bunches were sprayed with EBL solutions at 3 different growth stages: 1) four weeks after full bloom, 2) veraison stage, and 3) one day before harvest. EBL was obtained from Sigma (St. Louis, MO, USA) and different concentrations of solutions (0, 3 and 6 μmol L^{-1}) were applied. Appropriate amount of EBL to reach the desired concentration was dissolved in ethanol and then made to volume with distilled water. Final concentration of ethanol in each solution was about 0.1% (1 mL ethanol in 1000 mL of EBL solution) and the same concentration of ethanol was added to distilled water used for treating control vines. Table grapes were harvested at commercial maturity (when the control berries reached 18.5 °Brix) and immediately transferred to postharvest laboratory. Grape clusters were selected for uniformity of size, shape, color and freedom from blemishes and subjected to quality analysis.

Determination of total soluble solids (TSS), pH, total acidity (TA) and ascorbic acid (AA) content: All chemicals were purchased from Sigma (Sigma-Aldrich co. Germany). Berry TSS, pH and TA were determined according to the method described by AYALA-ZAVALA et al. (2007). 40 berries from each replicate, two berries from the shoulder, 2 from the middle and 1 from the bottom of each bunch, were wrapped in cheesecloth and squeezed with a hand press.

TSS was determined at 20 °C with an Atago DBX-55 refractometer (Atago Co. Ltd., Tokyo, Japan). pH was evaluated by a pH-meter (AZ-8601, China). TA was determined by diluting each 5 mL aliquot of grape berry juice in 95 mL of distilled water and then titrated to pH=8.2 using NaOH (4 g L^{-1}).

AA content was determined according to the method described by BALLENTINE (1941). The berry juice was extracted by pressing the berries and filtered using a muslin cloth, 5 mL of juice was added to 1 mL of 10% potassium iodide (KI) and 2 mL of 2 N sulfuric acid and the resulting solution was titrated with 0.01 N iodate until the starch was formed. 1 mL of 0.01 N Iodate corresponds to 0.88 mg of AA.

Evaluation of Catalase (CAT) and polyphenoloxidase (PPO) enzymes activity, total phenolics content (TPC) and total antioxidant activity (TAA): All enzyme extract procedures for whole berry flesh were conducted at 25 °C. CAT activity was measured according to BEERS and SIZER (1952) with slight modifications. The reaction mixture consisted of 2.5 ml sodium phosphate buffer (50 mmol L^{-1}, pH 7.0), 0.2 ml H_2O_2 (1%) and 0.3 ml enzyme. The decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm. The specific activity was expressed as U mg^{-1} protein, where one unit of catalase converts 1 mol of H_2O_2 per min.

The activity of PPO enzyme was determined using the method described by PIZZOCARO et al. (1993). Enzyme activity was assayed by determining the rate of increase in absorbance at 420 nm and 25 °C. The reaction mixture contained 0.5 mL of enzyme extract and 2.5 mL of buffered substrate (100 mmol L^{-1} sodium phosphate, pH=6.4, and 50 mmol L^{-1} Catechol). The linear section of the activity curve as a function of time was used to determine the PPO activity (U mg^{-1} protein min^{-1}). The unit for the PPO activity was defined as a change of 0.001 in absorbance at the conditions of the assay.

TPC of the whole berry extracts was determined by Folin-Ciocalteu method and was expressed as mg gallic acid kg^{-1} on a fresh weight basis. For extraction, 1 gr of berry sample was homogenized with a solution composed of methanol/HCl (V/V 2:28). Then, the mixture was centrifuged at 10000 g and 4 °C for 10 min. The supernatant was used for the assay of total phenolics content. For the assay 0.1 mL of this extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7 mL distilled water. After incubating for 2 min at room temperature in the dark, 1 mL of sodium carbonate saturated solution was added and the samples were incubated again for 2 h at room temperature. The absorbance of mixture was read at 760 nm using a spectrophotometer (model, Analytik Jena Specord 200, Germany) and the sample phenolics content was expressed as mg gallic acid 100 g^{-1} dry weight (DW) (PLESSI et al., 2007).

TAA of berry juice was determined by ferric ions reducing antioxidant power assay (FRAP) according to BENZIE and STRAIN (1996) with slight modifications. The stock solutions included 5 mL of a 10 mmol L^{-1} TPTZ (2, 4, 6-tripyridyl-s-triazine) with 40 mmol L^{-1} HCL plus 5.41 mL of $FeCl_3$ (20 mmol L^{-1}) and 50 mL of phosphate buffer, (0.3 mol L^{-1}, pH=3.6) and was prepared freshly and warmed at 37 ºC. Berry extracts (150 mL) were allowed to react with 2.85 mL FRAP solution and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37 ºC for 10 min. For construction of calibration curve five concentrations of $FeSO_4 7H_2O$ (1000, 750, 500, 250, 125 μmol L^{-1}) were used to obtain the calibration curves. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol L^{-1} $FeSO_4$. ($y = 0.0009 \times - 0.0275$, $R^2 = 0.995$).

Statistical analysis
The experiment was conducted as a completely randomized design with 3 EBL levels and 3 replicates (3 vines). 5 bunches were harvested from each vine (replicate) for quality analysis and bulked prior to analysis. The data were analyzed by repetitive measures analysis of variance using SAS (V 9.3, SAS Institute Inc., USA) package and means were compared by Duncan's multiple range test. Differences at P≤ 0.05 were considered significant.

Results

TSS, PH, TA and AA content: As shown in Fig. 1A, berries from vines sprayed with EBL had a significantly higher TSS than the control (p≤0.05) and a substantial increase in TSS content of treated berries was recorded. There was no significance difference between the two levels of EBL.

pH value of the berry juice was decreased as the result of treatment with EBL and the effect was concentration dependent (p≤0.05) (Fig. 1B.).

As shown in Fig. 1C, total organic acid content of the berries from treated vines was higher than the control and there was no significant difference between the two EBL levels (p≤0.05).

According to the data shown in Fig. 1D, EBL treatment had a significant effect on AA content of 'Thompson seedless' grape berries (p≤0.05) at the lower EBL concentration, but not for the higher one. Also the difference between EBL concentrations was not statically significant.

CAT and PPO enzymes activity, total phenolics and total antioxidants contents: CAT and PPO, as important antioxidant and anti-stress enzymes in plants, were significantly affected by pre-harvest EBL treatment (p≤0.01). As shown in Fig. 2A, B, the activity of these enzymes was significantly enhanced in response to EBL treatment. With increase in EBL concentration, the effect of phytohormones on CAT and PPO was not significantly increased.

As shown in Fig. 2C, D, EBL spray effectively enhanced fruit TPC and TAA (P≤0.01). While EBL in a concentration dependent manner enhanced the total phenolics content it was more effective on enhancing TPC and TAA at 3 µmol L^{-1} (significant difference between treatments for TPC, while not for TAA).

Discussion

Some bioactive compounds of the berries like phenolics, stilbenes and antioxidants are main factors determining the quality of both fresh and processed products of grapes (Xia et al., 2010). Increase in berry phenolics and other antioxidants are not only important for fresh table grapes but also directly affects the quality of byproducts. Also high quality berries have high storage capacity and are suitable for export (Jaakola, 2013). Increase in TSS and TA of the berries results in enhanced organoleptic property and nutritional quality. There are contradictory reports about the effect of BRs on AA content in plants and harvested crops. For example, exogenous BR has been reported to increase the AA levels in cultured cells of *Chorispora bungeana* under stress conditions (Liu et al., 2009). In contrast, BR treatment has been shown to decrease AA content in tomatoes and strawberry fruits (Hayat et al., 2012; Asghari and Zahedipour, 2016). According to our data, exogenous EBL enhanced the AA content of grape berries. BRs may enhance the synthesis of ascorbic acid in plant cells by enhancing the activity of L-galacton-1, 4-lacton dehydrogenase (L-GaLDH). This enzyme catalyzes the last step of ascorbic acid biosynthesis. According to the findings of Debolt et al. (2006), ascorbic acid is also used as a precursor for the synthesis of tartaric acid in grapes and the rate of ascorbic acid conversion to tartaric acid is increased during berry ripening. Since BRs play roles in signaling pathways of other plant hormones involved in ripening process, it has been demonstrated that BRs are the latest phytohormones implicated in the control of table grape berry ripening and anthocyanin accumulation (Luan et al., 2013).

Effects of EBL on enhancing fruit quality parameters and phytochemical contents including antioxidants, phenolics, ascorbic acid

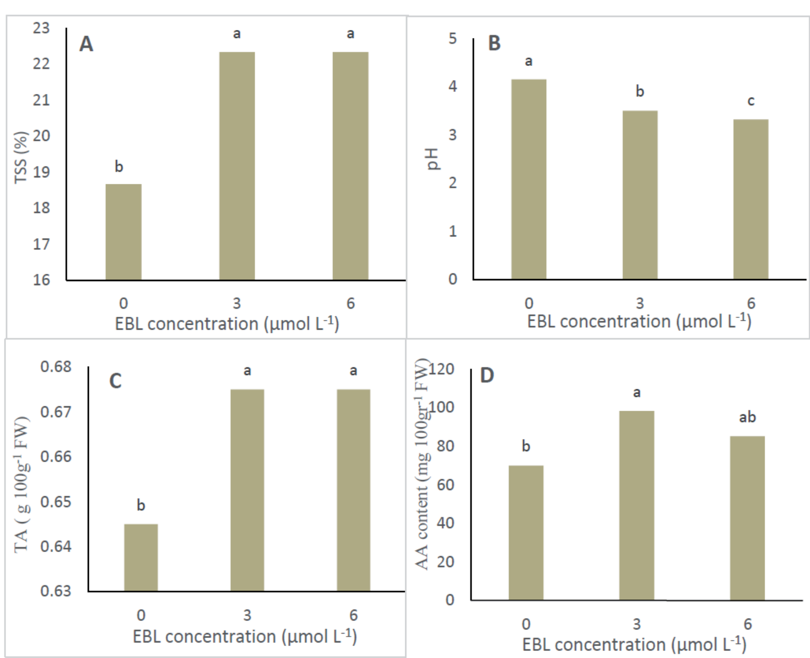

Fig. 1: Effect of EBL treatment on total soluble solid (TSS) content (A), pH (B), total acidity (TA) (C) and ascorbic acid (AA) content (D) in 'Thompson seedless' table grape. Different lowercase letters indicate the significance difference between the means (r=5) according to Duncan's Multiple Range Test (p≤0.05).

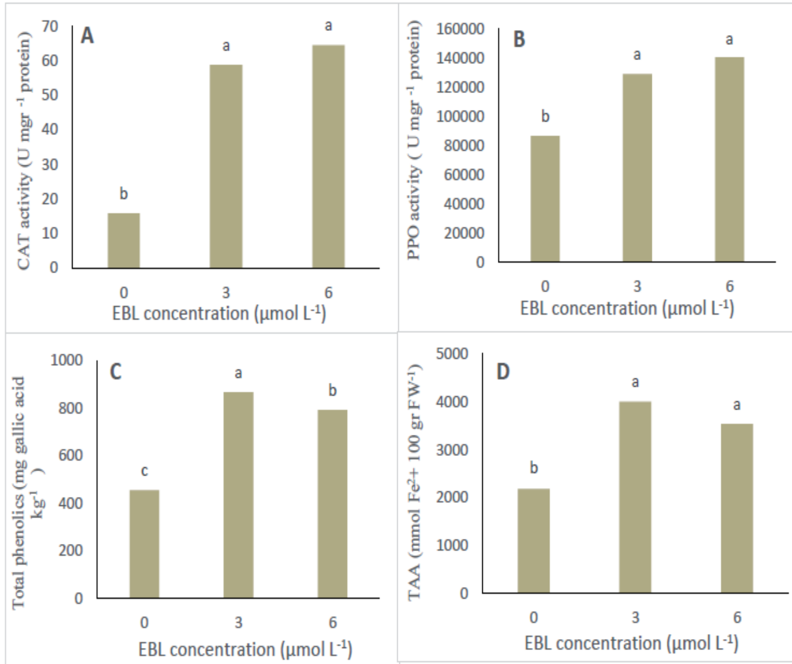

Fig. 2: Effect of EBL treatment on catalase (CAT) enzyme activity (A), polyphenoloxidase (PPO) enzyme activity (B), total phenolics content (TPC) (C) and total antioxidant activity (TAA) (D) in ‘Thompson seedless’ table grape. Different lowercase letters indicate the significance difference between the means (r=5) according to Duncan’s Multiple Range Test ($p \leq 0.01$).

(vitamin C) and soluble sugars is mainly due to effects on photosynthesis reaction. Exogenous BRs may substantially enhance the photosynthesis activity in different plants. BRs may enhance the net photosynthesis rate by enhancing chlorophyll and carotenoid development and playing crucial roles in gene expression and enzyme activity of some important photosynthetic enzymes such as Rubisco (Vardhini and Rao, 1998; Yu et al., 2004; Vardhini and Anjum, 2015; Asghari and Zahedipour, 2016). In addition, the role of BRs in increasing the absorption and transport of CO_2 in leaves and enhancing stomatal conductance has been reported in our previous studies on strawberry plants (Asghari and Zahedipour, 2016). Increase in photosynthesis rate results in enhanced carbohydrate production. Carbohydrates produced during photosynthesis reaction are not only used as precursors for different structures and bio-compounds during normal growth and development of the cells, but also modulate the growth and metabolic processes in plants via mediating gene expression and enzymes activity (Koch, 1996).

Reactive oxygen species (ROS) and free radicals are produced during normal cell metabolism and should immediately be removed after production. Chloroplasts, mitochondria and peroxisomes are the main sites of free radical and ROS generation during photosynthesis, respiration and photorespiration. Also free radicals and ROS are produced during normal metabolisms in human cells. ROS and free radicals, in the absence of antioxidant systems, are able to destroy the living cells by creating the oxidative burst. Different stresses, metabolic activities and physical and chemical conditions of the cells, such as antioxidant activity and pH, may substantially affect the oxidative burst (Metwally et al., 2003). Plant and human cells protect themselves against free radicals and ROS using different antioxidant systems, including enzymatic antioxidants such as CAT, superoxide dismutase (SOD), peroxidase (POD), and non-enzymatic ones such as ascorbic acid and phenolics. In fact, the production and activity of different antioxidants are necessary for suppressing oxidative damage in cells. CAT is one of the most important antioxidants scavenging H_2O_2. Elevated levels of H_2O_2 are toxic to the cells and CAT immediately converts this molecule to H_2O and O_2, leading to protection of cells from H_2O_2 damage (Gayatridevi et al., 2013). Effect of BRs on enhancing the antioxidant systems in plant cells and activating plant resistance against oxidative damage caused by pathogens and environmental conditions such as drought, salinity, heavy metal, high temperature and chilling stresses in some plants has been well demonstrated (Vardhini and Anjum, 2015). BRs have been shown to enhance total antioxidant capacity and some antioxidant fractions in some plants including ‘Zicuiwuhe’ table grape seedlings and some harvested crops (Zhu et al., 2010; Zhu-Mei et al., 2013).

Phenolics are important bio-chemicals in foods. Grapes are rich in different important phenolic compounds such as, catechins, epicatechins, procyanidins, proanthocyanidins, viniferones, quercetin, kaempferol, myricetin, isorhamnetin, caffeic acid, coumaric acid, ferulic acid and gallic acid, all providing the human body with antioxidant, anticancer, anti-inflammatory and anti-aging benefits (Paredes-Lopez et al., 2010). In addition to acting as powerful antioxidants, these compounds have been shown to play roles in a series of plant and fruit physiological processes including growth, color development and anti-stress responses (Asghari and Zahedipour, 2016). Exogenous application of some phytohormones and PGRs have been reported to affect the metabolism and biosynthesis of phenolics in plants (Luan et al., 2013). BRs enhance production of phenolic compounds and consequent resistance against biotic and abiotic stresses in different plants and harvested crops by increasing gene expression and enzyme activity of phenylalanine ammonia lyase (PAL), the main enzyme responsible for production of phenolics, and polyphenol oxidase (Asghari and Zahedipour, 2016; Gao et al., 2016). PPO (EC 1.10.3.1.) is a copper-containing enzyme catalyzing the oxidation of o-diphenols to o-diquinones. The main function of quinones in plants seems to be the mediation of resistance induction against pathogens and unfavorable conditions giving plants the ability of surviving and maintaining productivity under stress conditions. These compounds have been shown to have free-radical scavenging capacity, anti-coronary, anti-cancer, antivirus, antioxidant and anti-inflammation activities, prevent metabolic diseases and protect umbilical vascular endothelial cells in human body cells (Paredes-Lopez et al., 2010; Xia et al., 2010). As an important resistance related enzyme, PPO improves the resistance of fruits and plants against pathogens and pests by oxidizing phenolic compounds to quinines. Increase in PPO activity during growth stages not only results in high quality grapes but also is crucial for establishment of an efficient resistance network in plants and fruits, making the fruit more resistant against postharvest losses (Yoruk and Marshal, 2003; Zhu-Mei et al., 2013). Because phenolic compounds have antifungal and antibacterial effects, therefore, enhancing PPO activity and increasing total phenolics content of fruit with BRs may help producing fruit with no further need for the use of chemical biocides.

Conclusions

Exogenous brassinosteroid substantially enhanced 'Thompson seedless' berry quality attributes, natural phytochemicals, antioxidants and biochemical compounds. Increase in fruit biochemical content is of most important priority for food scientists. According to the data from this study we may conclude that EBL at 3 and 6 μmol L^{-1}, enhances berry quality indices and promotes health saving benefits of table grapes by enhancing phenolic componds biosynthesis and accumulation, PPO and CAT enzymes activity and total phenolics, different antioxidant fractions, total antioxidant capacity and ascorbic acid content. Increased TSS and total acidity and decreased pH as well as increased antioxidants, phenolics and ascorbic acid content of berries in an organic production system, without further need for the use of chemicals, results in enhanced nutritional property and medicinal quality of fresh berries and its byproducts. Interestingly, EBL acted as a growth enhancing, photosynthesis promoting and resistance mediating agent. Therefore, the increase in fruit quality parameters and phytonutrient contents is not at the expense of reduced crop yield and because of decreasing the need for disease control, the cost of EBL spray is economically acceptable. Increase in different phytochemicals and bio-compounds such as phenolics, total antioxidant capacity, antioxidant enzymes and ascorbic acid not only enhances the nutritional quality, which is very important for the consumers, but also promotes the fruit storage life. Since EBL treatment had no adverse effect on crop yield (unpublished data), we could recommend exogenous brassinosteroid treatment as a safe cultural activity for enhancing table grape quality, safety and nutritional property. Since no significant difference was seen between the two EBL levels in most cases, then 3 µmol L^{-1} is recommended for use in commercial scales.

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