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Effect of nitric oxide on biochemical and antioxidant properties of pomegranate fruit cv. Shishe-kab during cold storage

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Abstract
Pomegranate is a subtropical fruit that is widely consumed as fresh fruit and juice, however, its postharvest life is limited mainly due to storage disorders. The aim of this study was to determine the effect of nitric oxide (NO) on antioxidant activity and quality attributes of pomegranate fruit. The fruits were dipped for two minutes into different NO concentrations (0, 30, 100, 300 or 1000 µM). Then, the fruits were stored in cold room at 5 °C and RH 85%. After 90 days storage, changes in chilling injury, electrolyte leakage, titratable acidity (TA), total soluble solids (TSS), pH, antioxidant activity and total anthocyanin were evaluated. The results showed that 1000 µM NO application resulted in significant decrease in electrolyte leakage and TSS and maintained antioxidant activity and total anthocyanin in pomegranate fruit. However, no significant effect of NO treatment was observed on TA and chilling injury index. In conclusion, postharvest application of NO may be a promising method for maintaining quality of pomegranate fruit during cold storage.

Keywords: chilling injury, color, pomegranate, postharvest physiology, sodium nitroprusside.

Introduction

Pomegranate fruit (*Punica granatum* L.) is an ancient edible fruit, cultivated extensively around the world including Iran, Spain and Turkey. This fruit is generally consumed as fresh (arils) or juice, due to its potential health benefits (Holland and Bar-Ya'akov, 2008) such as, high antioxidant, anti-mutagenic and anti-hypertension activities of the fruit (Lansky *et al.*, 2000). A remarkable increase in the commercial farming of pomegranates has been observed during the last decade. The edible portion of pomegranate is an excellent dietary source, that contains a significant proportion of organic acids, soluble solids, polysaccharides, vitamins, fatty acids and mineral elements of nutritional significance (Fadavi *et al.*, 2006). Due to high level of dietary sources of antioxidant phenolics and anthocyanins, there has been an increasing interest in determining antioxidant properties of red fruits in recent years (Ozgen *et al.*, 2007).

It is well known that the quality of fruits and vegetables declines and many nutrients are lost rapidly following harvest. Decline in quality and occurrence of senescence are usually shown by morphological, biochemical and biophysical signs including loss of turgor and loss of important nutrients, such as sugar, vitamins and antioxidant components (Jones *et al.*, 2006). In spite of the low respiration rate in pomegranate

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fruits, it is a highly perishable product (Barman *et al.*, 2011). This Fruit is sensitive to low temperatures, and storage at cold temperatures such as 5 ℃ or lower would result in occurrence of chilling injury symptoms (Elyatem and Kader, 1984). Exposure of ripe pomegranate fruits to such circumstances, results in quality reduction by showing aril browning (Khodaei *et al.*, 2015). Shivashankara *et al.* (2004) showed that aril browning in pomegranate is attributed to the oxidative damage of membranes, leading to higher activities of certain enzymes such as polyphenol oxidase and peroxidase.

'Shishe-Kab' is a commercial pomegranate cultivar widely cultivated in Iran, especially in the South Khorassan province. However, as reported for most pomegranate cultivars due to chilling injuries, weight loss, decay, and husk scald of fruit (Caleb *et al*., 2012), postharvest life and consumption of this cultivar is still limited due to the quantitative and qualitative losses during postharvest handling and storage (Moradinezhad *et al*., 2013).

Nitric oxide is a bioactive molecule that with diverse signaling functions in plants. Previous studies have indicated due to effects of nitric oxide on respiration, ethylene biosynthesis, disease incidence, peel color and enzyme activity, it is effective in retarding the senescence and ripening in several fruits (Manjunatha *et al.*, 2010).

Several reports have shown that nitric oxide when applied at low concentrations can effectively extend postharvest life of various fruits, such as strawberry (Soegiarto and Wills, 2006), peach (Zhu *et al.*, 2006), longan (Duan *et al.*, 2007), plum (Singh *et al.*, 2009) and winter jujube (Zhu *et al.*, 2009). Recently, it is also reported that pre-harvest foliar application of sodium nitroprusside (SNP) as a nitric oxide donor compound can significantly reduce aril browning in pomegranate fruits (Khodaei *et al.*, 2015). However, no information has been reported on the effect of pre-storage nitric oxide dipping on improvement of pomegranate fruit quality particularly on Shishe-kab cultivar. Therefore, the main objectives of this study were to assess the effects of different concentrations of nitric oxide on biochemical and qualitative attributes of pomegranate fruit during prolonged cold storage, and to find whether nitric oxide can improve chilling resistance of pomegranate fruits under cold storage.

Materials and Methods

Preparing plant material and storage conditions

Pomegranate fruits (cv. Shishe-kab) were harvested in November 2015 from trees grown at the uniform conditions on a commercial orchard in Ferdows, South khorasan province, Iran. Fruits were harvested at full maturity stage (with firm texture and fully developed skin color), and immediately transported to laboratory. Diseased, sunburn, bruised and injured fruits were discarded and fruits with uniform size (300-350 g), shape and color were used for experiment.

Fruits were washed with distilled water and Tiabendazol 1% for one min and thereafter were subjected to different concentrations (0, 30, 100, 300 or 1000 µM) of sodium nitroprusside (SNP, nitric oxide donor). SNP was purchased from Merck Company. Different concentrations of SNP were applied by dipping the fruits into solutions consisting of different concentrations of SNP at ambient temperature for two min (Saadatian *et al.*, 2012). Fruits were stored for 90 days in a cold room (5±1 °C) with 85±5% relative humidity. Four replicates (each replication contained 6 individual fruits) were used for each treatment.

Chilling injury (CI) index

Occurrence of chilling injury and its intensity symptoms were visually recorded on a 4-point hedonic scale based on the percentage of husk surface affected by CI symptoms (dehydration, browning, and pitting): 1= (no CI symptoms), 2= (1–25% of surface

damaged), 3= (26–50% of surface damaged), 4= (>51% of the surface damaged) (Moradinezhad and Khayyat, 2014).

Electrolyte leakage (EL)

Leakage of ions from fruit husk (rind) disks was measured according to the method described by Li *et al*. (2014) with some modifications. Disks of fruit skin with 2.5 cm diameter were taken from fruit per each replication and were placed in 20 ml of deionized water at ambient temperature for 24 h. Electrical conductivity was measured with a digital electrical conductivity meter (C1). The same disks were kept in a boiling water bath (100 °C) for 1 h to release all electrolytes, then cooled at the ambient temperature and conductivity was recorded (C2). The EL was expressed in percentage using the following formula (Beckerson and Hofstra, 1980):

$$\mathrm{El} = \frac{C_1}{C_2} \times 100$$

Titratable acidity, pH and total soluble solids

Titratable acidity (TA) was determined by titration of two ml of juice with 0.1 M NaOH to an end point of pH 8.2 and results were showed as a percentage of citric acid. The pH was measured at room temperature using a pH meter. Total soluble solids (TSS) was determined with a hand-held refractometer (RF 10, Brix 0–32, Extech Co., USA) at 25 °C, and expressed as Brix.

Antioxidant activity

DPPH method was used to measure the antioxidant activities of pomegranate juices based on the evaluation of the free radical scavenging capacity of the juices (Blois, 1958). Briefly, one ml of juice was mixed with two ml of 0.1 mM DPPH in methanol. The absorbance was measured at 517 nm using spectrophotometer (Unico 2100, China). Antioxidant activity was expressed as the percentage decline in absorbance relative to the control, corresponding to the percentage of DPPH scavenged (%DPPH), which was calculated according to the following formula:

$$\mathrm{DPPH}(\%) = 1 - \frac{\mathrm{A(sample)}}{\mathrm{A(control)}} \times 100$$

Total anthocyanin

Total anthocyanins content (TAC) was determined spectophotometrically by the pH differential method (Lako *et al.*, 2007). Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5 using spectophotometer, and then calculated according to Equation (1).

$$A = [(A510 - A700)pH_{1.0} - (A510 - A700)pH_{4.5}] \quad (1)$$

Results were expressed as mg of cyanidin-3-glucoside per 100 ml of juice, using a molar absorptive coefficient (ε) of 26900 and a molecular weight of 449.2 and then total anthocyanin content was calculated according to Equation (2).

$$\text{Total anthocyanin}\,(\mathrm{mg/l}) = \frac{A \times MW \times DF \times 1000}{\varepsilon} \quad (2)$$

where A= absorbance, MW= molecular weight of cyanidin-3-glucoside, DF=the degree of dilution, and ε= molar absorptive coefficient.

Aril color measurement

Aril color of pomegranate fruits was determined using a colormeter (TES-135A, Taiwan) and recorded as L* (lightness), a* (-greenness to +redness) and b* (-blueness to + yellowness). The different color indices (hue and chroma) were calculated according to Equations (3) and (4).

$$\mathrm{hue}^{\circ} = \text{arc tang}\frac{b}{a} \quad (3)$$

$$\mathrm{Chroma} = \sqrt{a^2 + b^2} \quad (4)$$

Statistical analysis

The data were analyzed based on a completely randomized design with four replications. Data were analyzed using GLM (generalized linear model) procedure of SAS

program version 6.1. Mean value differences were analyzed using LSD's test to examine if differences were significant at $P< 0.05$.

Discussion and Results

Chilling injury index

Analysis of results showed that occurrence of chilling injury was higher in control compared to NO-treated fruits. Occurrence of chilling injury symptoms in fruits that treated with 1000 µM of NO was significantly lower than other NO concentrations (Table 1). In accordance with our results Wu *et al*. (2014) showed that chilling index of banana was lower in NO-treated fruits than control. It has been reported that low temperatures cause generation of reactive oxygen species (ROS), which induce oxidative stress and CI in fruits (Shi *et al*., 2007). Due to antioxidant properties of NO, it can down-regulate the generation of ROS, and reduces oxidative stress in pomegranate fruit during cold storage, which consequently alleviate the CI occurrences (Zaharah and Singh, 2011). Improving effects of NO on CI have been reported in several fruit species during cold storage (Xu *et al*., 2012).

Electrolyte leakage

The electrolyte leakage of pomegranate fruits was significantly affected by nitric oxide treatment (Table 1). The lowest value of electrolyte leakage was found in fruits treated with 1000 µM NO (47.525%) and the highest EL was obtained in control fruits (81.333%). Our results are in agreement with those that previously reported in banana (Wu *et al*., 2014) and peach (Zhu and Zhou, 2006). EL is usually used to evaluate chilling resistance of plant tissues (Zhao *et al*., 2009). Dysfunction of cell membranes at low temperature is the primary reason for membrane permeability and lipid peroxidation (Nishida and Murata, 1996). Flores *et al*. (2008) reported positive effects of NO on cell membrane integrity, which can be seen as a decrease in EL. Exogenous NO application can alleviate chilling injuries in banana fruits in cold-storages. This can be attributed to the enhancement of antioxidant defense system and accumulation of secondary metabolites (Wang *et al*., 2013).

Titratable acidity, pH and total soluble solids

The TA content and pH of pomegranate juice were not affected by the NO treatment but the effect of nitric oxide on TSS was significant (Table 2). TSS was decreased by application of NO. The highest TSS (18.37) was found in control fruit. In agreement, Asghari and Khamiri Sani (2010) reported that exogenous application of NO on grape caused a significant decrease in the amount of TSS during storage. Moreover, no significant effects of different concentrations of NO were reported on pear fruits (Liu *et al*., 2011). Decrease in respiration, decrease in synthesis and increase in use of metabolites decrease the conversion of carbohydrates to sugars, as a consequence TSS decreases (Rohani *et al*., 1997). Therefore, it seems that by slowing the respiration rate (Manjunatha *et al*., 2010) NO can influence TSS of pomegranate juice.

Table 1. Effects of different concentrations of nitric oxide on chilling injury (CI) and electrolyte leakage (EL) on pomegranate fruit cv. Shishe-kab after 90 days of storage at 5 °C

Nitric oxide (µM)	Chilling injury (CI)	electrolyte leakage (EL) (%)
0 (Control)	2.25 a†	81.333 a
30	2.25 a	71.221 ab
100	2 a	59.842 bc
300	2 a	51.510 c
1000	1.25 b	47.525 c

† Mean values followed by different letters, within the same column, are statistically different at $P< 0.05$.

Table 2. Effects of different concentrations of nitric oxide on titratable acidity (TA), pH and total soluble solids (TSS) of pomegranate fruit cv. Shishe-kab after 90 days of storage at 5 °C

Nitric oxide (µM)	TA (%)	pH	TSS (%)
0 (Control)	1.82 a†	6.25 a	18.37 a
30	1.78 a	6.17 a	17.37 b
100	2.03 a	5.98 a	17.25 b
300	2.40 a	5.97 a	17.75 ab
1000	2.24 a	6.05 a	17.50 b

† Mean values followed by different letters, within the same column, are statistically different at $P< 0.05$.

Antioxidant activity

The NO treatment at all concentrations significantly increased the antioxidant activity of pomegranate fruits (Table 3). The highest antioxidant activity (48.557%) was found in 1000 µM of NO, while, there was no significant difference among 1000, 300 or 100 µM of NO application. Application of different concentrations of NO caused a significant increase in antioxidant activity than the control. Accordingly, Saadatian *et al.* (2012) reported induction of antioxidant activity in kiwifruits by application of high concentration (1 and 1.5%) of NO. In our study, the lowest value of antioxidant activity (21.903%) was obtained in control fruits. In agreement with our results, persimmon (Shahkoomahally *et al.*, 2015) and litchi (Barman *et al.*, 2014) fruits which treated with SNP had higher antioxidant activity than the non-treated fruit during storage. The accumulation of ROS (reactive oxygen species) resulting from an altered balance between ROS production and scavenging capacities will reduce the storage quality and marketability of fruits and vegetables (Hodges, 2003). High activity of antioxidant compounds can reduce the accumulation of ROS, as a result decreases oxidative damage, and delays senescence (Wu *et al.*, 2012). The NO may act as an antioxidant (Dhindsa *et al.*, 1981) and the protective role of NO may be attributed to its contribution in the induction of expression of genes encoding ROS-scavenging antioxidants under stress conditions (Shi *et al.*, 2007). Since there is a correlation between antioxidant and senescence in plants (Hodges, 2003), increasing the activity of antioxidant compounds by NO treatment may be beneficial for extending postharvest life of fruits (Lai *et al.*, 2011).

The coordinated action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) is very important for scavenging ROS to protect cell membranes. This is thought to be a major mechanism of resistance to chilling stress (Sevillano *et al.*, 2009). Fruits of chilling-tolerant mandarin have a higher antioxidant enzyme system than the fruits of chilling sensitive cultivars (Sala, 1998). It has been showed that postharvest treatments that induced chilling tolerance and alleviated chilling injury, also enhanced antioxidant enzyme activity (Aghdam and Bodbodak, 2013).

In our experiment, NO increased the antioxidant activity of pomegranate fruit and decreased the amount of electrolyte leakage. NO can boost the activity of antioxidant enzymes such as ascorbate peroxidase and suoeroxide dismutase (Khodaei *et al.*, 2015).

Total anthocyanin

Total anthocyanin significantly affected by NO treatment (Table 3). The highest amount of anthocyanin was found fruits dipped in a 1000 µM NO solution (26.573%) and the lowest amount was found in control fruits (14.472). Our

finding is in agreement with Barman *et al.* (2014) who showed that litchi fruits that treated with SNP had higher retention of anthocyanin pigments in comparison with control fruits. Furthermore, Duan *et al.* (2007) reported that NO through reducing loss of moisture and lowering enzyme activities has a role in maintaining anthocyanin content in longan fruits.

It seems that application of NO through enhancing antioxidant system can protect plant against polyphenol oxidase (PPO), POD and H_2O_2. These components have a major role in degradation of anthocyanins (Khodaei *et al.*, 2015). The administration of NO donors to tobacco plants induced the expression of gene encoding phenylalanine ammonialyase (PAL) (Durner *et al.*, 1998), which is a key enzyme in the synthesis of anthocyanins and different compounds related to plant defense (Boss *et al.*, 1996). Therefore, it is likely that increase in anthocyanin following NO treatment could be due to the induction of PAL gene expression in the fruit (Villarreal *et al.*, 2009).

Aril color properties

Application of nitric oxide has no significant effect on lightness, b* value and hue angle of aril color (Table 4). However, the a* value (redness) and chroma components had the most obvious changes (Table 4). Application of NO significantly increased the amount of a* and chroma of aril color in comparison with the control. The lowest amount of a* value (17.065) and the lowest amount of chroma were found in control (18.384). Color is a human perception by definition, which has long been used in the assessment of fruit quality (Mutari and Debbie, 2011). Previous observations showed that the tomato fruits stored at high temperatures retained the color characteristics of fruits than the fruits stored at chilling temperatures (Tadesse *et al.*, 2015). The better color of arils may be attributed to the effectiveness of NO against chilling injuries and/or improving anthocyanin maintenance.

Table 3. Effects of different concentrations of nitric oxide on antioxidant activity and anthocyanin content of pomegranate fruit cv. Shishe-kab after 90 days of storage at 5 °C

Nitric oxide (µM)	Antioxidant (%)	Anthocyanin (mg/l)
0 (Control)	21.903 c†	14.472 c
30	36.199 b	17.899 bc
100	45.229 a	18.562 bc
300	45.572 a	21.603 ab
1000	48.557 a	26.573 a

† Mean values followed by different letters, within the same column, are statistically different at $P< 0.05$.

Table 4. Effects of different concentrations of nitric oxide on aril color properties of pomegranate fruit cv. Shishe-kab after 90 days of storage at 5 °C

Nitric oxide (µM)	L*	a*	b*	hue°	Chroma
0 (control)	25.685 a†	17.065 c	6.600 a	2.605 a	18.384 c
30	22.973 a	19.093 c	6.231 a	6.021 a	20.427 c
100	20.518 a	24.415 b	7.346 a	3.405 a	25.540 b
300	23.438 a	32.758 a	6.590 a	6.108 a	33.511 a
1000	26.175 a	34.818 a	8.967 a	4.714 a	36.112 a

† Mean values followed by different letters, within the same column, are statistically different at $P < 0.05$

Conclusion

In conclusion, antioxidant activity and anthocyanin contents were increased by application of high concentrations of nitric oxide. NO can improve a* and chroma indices of aril color of pomegranate fruits, resulting in aril with more attractive red color. Therefore, pre-storage application of nitric oxide represents an attractive approach for pomegranate industry. In addition, electrolyte leakage of pomegranate during cold storage period was decreased by pre-storage application of nitric oxide. Therefore, pre-storage application of proper concentration of nitric oxide could be used to maintain the pomegranate fruit quality during prolong cold storage. Further studies are necessary to determine optimum concentration and duration of NO application in order to achieve maximum benefit of this chemical in postharvest cold storage of pomegranate fruit.

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