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Effect of Salicylic Acid and Glycine Betaine on Postharvest Gray Mold Disease and Chilling Injury of "Wonderful" Pomegranate Fruit

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THE effect of salicylic acid (SA) and glycine betaine (GB) under different concentrations on gray mold disease caused by (*Botrytis cinerea*, Pers.: Fr.) fungus and chilling injury as well as weight loss, color, malondialdehyde (MDA) content, anthocyanin, vitamin C, electrolyte leakage (EL) and enzyme activities of pomegranate cv. Wonderful fruits were investigated during 2018 and 2019 seasons. The obtained results indicate that gray mold disease and chilling injury were significantly ($p<0.05$) reduced by glycine betaine at 40 mM (T7) followed by salicylic acid at 4 mM (T4) compared to control treatment (T1). The most effective treatment in decreasing MDA was the treatment T7 followed by T4. Therefore, SA and GB treatments could maintain normal cell membrane structure and function through down-regulating MDA content and up-regulating peroxidase and polyphenol oxidase enzyme activities to alleviate gray mold disease and chilling injury in pomegranate cv. Wonderful fruits.

Keywords: Pomegranate cv. Wonderful, glycine betaine, salicylic acid, *Botrytis cinerea,* malondialdehyde**.**

Introduction

Pomegranate (*Punica granatum* L., family *Punicaceae*) is one of the most essential subtropical fruits owing to its nutritional, pharmacological, and medicinal properties (Kahramanoğlu, 2019). There are many challenges in seeking to reach new markets for exporting with safety and phytosanitary controls (Ornelas-Paz *et al.,* 2017). Owing mainly to recorded health effects and enriched bioactive phytochemicals in pomegranate fruit global production and consumption significantly increased over the last decade (Koushesh Saba and Zarei 2019). According to the American Animal and Plant Health Inspection Service (APHIS) (Powell, 2003), pomegranate fruits should be free from the Mediterranean fruit fly *Ceratitis capitata* that is the most important defect especially for global exporting. Therefore, pomegranate fruits should be stored for at least 14 days at a temperature below 1.1°C. Nevertheless, these conditions often cause symptoms of chilling injury (CI), in which the optimal storage temperature for pomegranate fruits ranged from 5 to 7.5°C (Kashash *et al.*, 2019). Primarily, increasingly cold storage is used to convey fruit for long distances (Mustafa et al., 2018), to prolong the marketing season and maintain the quality of the fruit (Razavi et al.,2018), despite inducing reactive oxygen species (ROS) causes cell damage and death by cold storage (Foyer et al., 2017), by measuring the malondialdehyde (MDA), which reflects the free radical content in the cell and accompanied by a reduction of antioxidant enzyme activity, that means, increasing MDA content through

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the storage period is very harmful to the fruits, and thereby maintaining cell membrane structure and function is a crucial element for maintaining post-harvest fruits (Sun et al., 2020). Therefore, using alternative methods to scavenging ROS as well as improve fruit quality and upsurge the storability of fruit is inevitable (Batista Silva et al., 2018).

In addition to chilling injury, pomegranate fruits are vulnerable to infection by many fungal pathogens in fields, packinghouses and storage places which may cause a great reduction in fruit quality and storability. Pomegranate fruits are subjected to be infected by several pathogens such as *B. cinerea*, *Penicillium expansum*, *P. glabrum*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Pestalotia brevista* and *Pilidiella granati* (Thomidis, 2014). Gray mold disease caused by *B. cinerea* is considered the most the economic post-harvest disease of pomegranate fruits that cause yield losses which could vary from 30-50% based on growing season and storage conditions (Teksur *et al.,* 2014). Decay caused by *B. cinerea* is mainly originated from the latent infection of blossom, but also could occur when micro-wounds, injuries or any part of the skin is contaminated with the fungus. Unfortunately, the application of fungicides is still the main strategy used to control pomegranate fruit rots. However, searching for safe, effective and low-cost alternatives to fungicides are urgently needed. So, strategies must be found that remarkably attenuate chilling injury and decay and permit exposure to cold quarantine treatments against the Mediterranean fruit fly. Using resistance inductors (elicitors) to biotic and/or abiotic stresses is a way of preserving the consistency of postharvest fruits (Zhou et al., 2018), contributing factor for monitoring huge numbers of physiological processes and system resistance is salicylic acid (SA), which is considered an endogenous phenolic nature compound that acts as a plant growth regulator and the key signaling component of the systemic acquired response (SAR)(Chen et al., 2006). The role of SA in chilling injury by involving in the induction of numerous enzymes related-resistance such as phenylalanine ammonia lyase (PAL) and peroxidase (POD) besides inducing accumulation hydrogen peroxide which is involving in activation of systemic resistance, and scavenging of reactive oxygen species (Babalar et al. 2007). Salicylic acid may also be responsible for the development of defence compounds such as pathogenesis-related proteins (Shi et al., 2018). Some studies were reported in recent years which indicated that SA increases resistance to chilling injury and fruit decay, such as on pomegranate (Koyuncu et al., 2019 and Mansour et al., 2020) & guava fruit (Lo'ay & Taher, 2018) & orange fruit (Rasouli et al., 2019) & lemon fruit (Siboza et al., 2014) & apricot fruit (Ezzat et al., 2017) & mandarin fruit (Ennab et al., 2020) & sweet cherry fruit (Giménez et al., 2017) and plum fruit (Serrano et al., 2018). In general, plants use strategies of avoidance and tolerance chilling and decay caused by pathogens, including the synthesis of low molecular-weight compounds called compatible solutes, for instance, glycine betaine known as amino acid derivate (Sakamoto and Murata, 2002). However, endogenous GB aggregation is normally insufficient for mitigating postharvest senescence and chilling stress with oxidative aspect (Shan et al., 2016), and currently, there is no study about GB-treated pomegranate fruit under storage conditions.

Glycine betaine is a quaternary ammonium compound for osmotic adaptation that plays an important role in maintaining cell osmotic pressure, protecting protein or the function of enzymes and regulating stress reactions in higher plants by enhancing antioxidant enzymes activity (Figueroa-Soto and Valenzuela-Soto, 2018). As well as induction the phenylpropanoids activity pathway is crucial to cell wall strengthening, and play an essential part in fruits against various stresses such as biotic and abiotic which in turn lead to higher membrane unsaturated fatty acids (Sun *et al*., 2020), More attention is given recently to the post-harvest GB application in fruit. Chilling injury improvement and cold tolerance have been documented through GB treatments in some fruits, such as peach fruit (Wang et al., 2019), hawthorn fruit (Razavi et al., 2018), pear fruit (Sun et al., 2020), mango fruit (Awad et al., 2017), and loquat fruit (Zhang et al., 2016). Searching for cost-effective, convenient, and highly efficient alternatives to reduce the chilling injury and gray mold (*Botrytis cinerea*, Pers.: Fr.) disease of fruits is urgently needed to fulfil the world consumption through exportation and internal use of pomegranate fruits.

Although a well-studied application has been established by salicylic acid and glycine betaine, the comparison between them was not reported. In the present study, the main aim was to investigate the application of salicylic acid and

glycine betaine for minimizing postharvest losses on pomegranate fruit "wonderful" to alleviate CI along with reducing the gray mold disease caused by *B. cinerea*, with maintaining the quality of the fruits.

Materials and Methods

Pomegranate fruit sampling

Pomegranate fruit (*Punica granatum* L.), cv. 'Wonderful', were obtained from a private orchard in Kafr El-Sheikh governorate, Egypt. Fruits were harvested in mid-October 2018 and 2019 seasons at commercial maturity (Fawole and Opara 2013), with anthocyanin pigment (17%), vitamin C (12.80 and 13.95), MDA peel (3.00 and 2.40), and MDA arils (2.00 and 1.10), in both seasons respectively, and transported immediately in an air-conditioned vehicle to the postharvest handling laboratory at Horticulture Research Institute, Giza governorate, Egypt.

Isolation and identification of rot fungal pathogens

Pomegranate cv. Wonderful fruits with typical rot symptoms were collected at harvesting time (35 fruits in 2018 and 50 fruits in 2019 growing seasons) from a private orchard located in Kafr El-Sheikh governorate, Egypt. Methods of Leyronas *et al.*, (2012) were used for the isolation of causal pathogens responsible for rot symptoms of fruits. To isolate the causal pathogens, under aseptic conditions the rotted lesions of peel tissues (approx. 5 mm) were cut by flamed knife and placed immediately on acidified potato dextrose agar (2.5 ml 85 % lactic acid/litre nutrient medium) in Petri dishes and incubated at 23 °C for one week. Purification of fungal pathogens was done using hyphal tip technique (Leyronas *et al.* 2012). Identification of the causal pathogens was done according to the description of Ozcelik and Ozcelik, (1997) based on the morphological characters and microscopic examination at the Department of Agricultural Botany, Faculty of Agriculture, Kafrelsheikh University, Egypt. The most frequent pathogen during the isolation/ identification section was used for the next experiments.

Pathogenicity test and Koch's postulates

Pomegranate fruits healthy and free of visual symptoms of the diseases, insect damage and mechanical injury with regular shape, size and color were selected for the purpose of pathogenicity capability of the isolated fungus. Fruits were washed thoroughly in running tap water for 15 min and left to dry at room temperature (approx. 20±2 °C), then were immersed in 0.3% sodium hypochlorite for 5 min and washed thoroughly in sterilized distilled water several times. Purified fungus inoculum (growing margins) of 10 days old potato dextrose agar (PDA) cultures were inserted individually into holes in the fruits made by flamed cork borer (5 mm diameter). Removed peel pieces by cork borer were used to close the holes of the same fruits after the inoculation process according to Šernaitė *et al*., (2020). For the disease development, the inoculated fruits were kept in a plastic food tray enclosed with a polyethylene bag (disinfected with 70% ethanol and exposed to UV light for 20 min), closed and stored for 1 week at 23 °C with approximately 70% relative humidity. Pathogenic fungus was re-isolated onto PDA before use for further work experiments.

Application of SA and GB against B. cinerea and CI

Healthy pomegranate fruits and free of visual symptoms of diseases, and mechanical injury with regular shape, size and color were selected. Fruits were washed thoroughly in running tap water for 15 min, then were immersed in 0.3% sodium hypochlorite for 5 min and washed several times thoroughly in sterilized distilled water. Fruits were divided into 2 groups (1 for *B. cinerea* and 1 for CI assessments). The first group of fruits were inoculated according to methods described by Šernaitė *et al*., (2020) with the spores of purified *B. cinerea* 15 days old PDA cultures. To collect the spores, 5 ml of sterilized water were added to the grown *B. cinerea* on agar surface in Petri dishes and rubbed with a sterile glass rod. Suspension of collected spores was passed through the double layer of sterile cheese cloth, counted using a haemocytometr and adjusted to a concentration of 1×10^8 spores/litre. The spore suspension was sprayed inside the crown of pomegranate fruits after removing the sepals which close the crown. Artificially inoculated fruits were kept at room temperature and allowed to dry at air conditions for 24 hr before SA and GB treatments application. Fruits were treated by the following treatments :

- Inoculated fruits were treated by immersion in distilled water suspended with Tween-20 (v/v) 0.2% for 10 min at room temperature and served as control treatment (T1).
- Inoculated fruits were treated by immersion in SA (Oxford Laboratory Reagents, Mumbai,

India) under different concentrations 2, 3 and 4 mM (T2, T3 and T4), respectively suspended with Tween-20 (v/v) 0.2% for 10 min at room temperature.

- Inoculated fruits were treated by immersion in GB (Oxford Laboratory Reagents, Mumbai, India) under different concentrations 20, 30 and 40 mM (T5, T6 and T7), respectively suspended with Tween-20 (v/v) 0.2% for 10 min at room temperature.

Three replicates were used for each concentration, each replicate consisted of 10 fruits. Tween□20 (v/v) 0.2% was added to all the solutions as a surfactant. A randomized complete design was used in this experiment. For monitoring the disease development, inoculated and inoculated-treated fruits were kept in plastic food trays enclosed with a polyethylene bags disinfected with 70% ethanol and exposed to UV light for 20 min, closed and stored at 23°C with approximately 70% relative humidity. Decay severity percentage was scored at 9 and 15 days after inoculation (DAI) and electrolyte leakage (EL) was measured 1, 2 and 3 days after treatment (DAT).

In the second group, which designed for the chilling injury assessment, healthy fruits were treated as mentioned above with the first group. Then, all fruits were air-dried at room temperature for 30 min, and then placed in boxes and stored at 0 °C with 90% relative humidity for 21 days. Three replicates for each concentration, each replicate contains 20 fruits. The fruits of each treatment taken from cold storage every seven days' intervals and placed into (approx. 20±2°C) for another three days before assessment, to evaluate the chilling injury, and the quality attributes. Each treatment was replicated three times and the experiment was repeated twice in 2018 and 2019.

Gray mold development assessment

To monitor the development of *B. cinerea*, artificially inoculated fruits in the crown and treated were assessed after 9 and 15 days. Each fruit was scored for crown decay (percentage of skin surface coverage) based on the following 0-4 scale of Palou *et al*. (2007) as follows: 0 = no mycelium present, 1 = mycelium in the crown, 2 = mycelium with lesion ≤ 25% of fruit surface, 3 = lesion on 26-50%, 4 = lesion > 50%. Results were expressed as a percentage of decay severity and calculated using the following equation described by Descalzo *et al*. (1990) as follow: **DS** % = [∑ (a × b)/N × K] × 100 where DS = decay severity, a = number of infected fruits rated, b = numerical value of each grade scale, N = total number of examined fruits, K = the highest degree in the scale.

Electrolyte leakage assessment

Fungal inoculated and inoculated-treated pomegranate fruits were used to measure the EL (membrane permeability, integrity and cellular compartment indicator) 1, 2 and 3 days after treatment (DAT) application according to methods of Zhu *et al.* (2009). Twenty disc pieces (1 cm diameter, 1 mm thick) of peel were washed 3 times with deionized water (2-3 min.), then replaced with flasks containing 50 ml deionized water (Milli-Q 50, Millipore, Bedford Mass., USA). After 24 hr of shaking at room temperature, the EL was measured by an electrical conductimeter (Acromet AR20, Fisher Scientific, Chicago, IL) which used to record the initial conductivity for each sample vial. For inducing the cell tissue break, flasks contain samples were incubated at 80 °C for 1 hr in the water bath (Fisher Isotemp, Indiana, PA), then the flasks were replaced for shaking (Innova 2100 platform shaker) for 24 h at room temperature again. After the incubation period, the final conductivity was recorded for each sample vial. Electrolyte leakage percentage was calculated according to the formula of Szalai *et al*. (1996):

EL = EC1/EC2 × 100, where: EC1 = initial conductivity; EC2 = final conductivity

Chilling injury assessment

Chilling injury was estimated depending on 4 points hedonic scale of Sayyari *et al*. (2011) for husk browning of the fruit surface, using the following formula according to (Jannatizadeh, 2019):

CI (%) = ∑ [(value of hedonic scale) × (number of fruit at the CI scale)] / (4 × total number of fruit) × 100.

Fruit weight loss (%)

Changes in weight in the fruits were recorded at 7, 14 and 21 days after application of all treatments using a digital balance (EK600H-Japan) and fruit weight was calculated as the differences between initial weight and final fruit weight and expressed as weight loss percentage.

Color assessment

Changes in color of pomegranate fruit peel

and arils as indicated by chroma was measured with a calibrated Minolta Chroma Meter (Model CR-400/410, Minolta Corp, Osaka, Japan), as defined by (Mc Gire, 1992).

Fruit peel and arils MDA content assessment

Methods of thiobarbituric acid (TBA) reactions with slight modifications done by Zhao *et al*. (2007) were used to determine the malondialdehyde (MDA) content. To estimate the amount of accumulated MDA in tissue samples, the following equation was used: MDA (μmol g^{-1} FW) = [6.452 (OD532−OD600) – 0.559 OD450] * 10 ml / FW, where FW = fresh weight of sample fruit (g) and OD = optical density. In this reaction TBA forms complexes of reddish color with low molecular weight aldehydes, such as malondialdehyde. MDA content was expressed on a fresh weight basis, (μmol g^{-1} FW).

Total anthocyanin content

Total anthocyanin content (TAC) was determined according to Wrolstad (1993) using the pH differential method. TAC was expressed as cyanidin-3-glucoside and was calculated using the following equation:

$$\text{cyanidin -3- galactoside equivalents } (mg.l^{-1}) = A * MW * DF * 103 / \varepsilon * 1$$

where A = difference in absorbance at pH 1 (A520 – A700) – pH 4.5 (A520 – A700); MW (molecular weight) for cyanidin-3-glucoside = 449.2 g mol^{-1}; DF = dilution factor = 5; 1 = path-length in cm; ε = 29.600 molar extinction coefficient. Results were expressed as mg/100 ml of crude juice.

Vitamin C

Results were expressed as mg/100 g of fresh weight using a standard curve that was made by different concentrations of ascorbic acid (A.O.A.C. 2000).

Determination of enzyme activities

Three millilitres of 50 mM TRIS buffer (pH 7.8) with 1 mM EDTA-Na_2 and polyvinylpyrrolidone (7.5 %) were used for homogenization of 0.5 g pomegranate fruits. Centrifugation was carried out at 12000 rpm for 20 min. at 4 °C for all homogenates. The supernatant of each homogenate was replaced for measuring the total soluble enzyme activity using a spectrophotometer (UV-160A, Shimadzu, Japan). Enzyme activity was determined at 7, 14 and 21 days after application of all treatments. According to the protocol of Hammerschmidt *et al*. (1982), the peroxidase activity (POD) activity with the presence of guaiacol and hydrogen peroxide was measured. Peroxidase enzyme activity was calculated by changing of absorbance per min. per gram fresh weight. Peroxidase activity was expressed as changes in absorbance (optical density per min/g sample, OD/ min/g). According to the methods of Coseteng and Lee (1978), the polyphenol oxidase activity (PPO) activity was measured. Polyphenol oxidase enzyme activity was calculated by changing absorbance per min. per gram fresh weight. Polyphenol oxidase activity was expressed as changes in absorbance (optical density per min/g sample, OD/min/g).

Statistical analyses

Experiments were arranged as a randomized complete design with three replicates. Data were subjected to statistical analysis by (ANOVA) using SAS software. Mean were compared by Duncan's multiple range tests ($P < 0.05$).

Results

Isolation and identification of rot fungi

Certain fungal pathogens were frequently isolated during 2018 and 2019 seasons from pomegranate cv. Wonderful fruits visualized typical rot symptoms. Results of fungal pathogen identification depending on morphological characters and microscopic examination summarized that *B. cinerea* was the most frequently isolated pathogen (32 and 44 %) among all fungal pathogens isolated from the rotted fruits during 2018 and 2019 growing seasons, respectively, followed by *A. niger*, *P. expansum* and *A. alternata* (Table 1). The fungus *B. cinerea* was used for the pathogenicity test. Pathogenicity test through artificial inoculation of *B. cinerea* isolate to healthy Wonderful pomegranate fruits resulted in the appearance of symptoms similar to those of natural infection after 8 days from inoculation. Crown decay indicated that *B. cinerea* exhibited a quick mycelium surface coverage.

Effect of SA and GB treatments against B. cinerea (Decay severity %)

Post-harvest treatments of SA and GB under certain concentrations significantly ($P < 0.05$) suppressed the development of *B. cinerea* in the crown area of artificially inoculated Wonderful pomegranate fruits after 9 and 15 days compared to inoculated-untreated control treatment (Table 2). One-way ANOVAs indicated that the most effective treatment against the decay caused by *B. cinerea* was the treatment T7 (GB at 40 mM) followed by T4 (SA at 4 mM) in both 9 and 15 days during 2018 and 2019 seasons (Table 2). Treatment T7 (GB at 40 mM) was superior to T5 (GB at 20 mM) and T6 (GB at 30 mM), showing the benefit of concentration increasing as well as with SA. Treatment T7 exhibited the least decay percentage at 9 and 15 days (2 and 3.5%), respectively compared to the control treatment (9 and 16.17%) during 2018. Similar results were obtained with the same treatment T7 at 9 and 15 days (1.67 and 3.17%), respectively compared to the control treatment (8 and 16 %) during 2019 (Table 2). Following treatment against the decay caused by *B. cinerea* was T4 (SA at 4 mM) at both 9 and 15 days during 2018 and 2019 seasons. Among SA concentrations, T4 exhibited the least decay percentage at 9 and 15 DAI (2.83 and 4.67%), respectively compared to control treatment (9 and 16.17%) during 2018. Similar results were obtained with the same treatment T4 at 9 and 15 days (2.33 and 4.33%), respectively compared to the control treatment (8 and 16 %) during 2019.

TABLE 1. Frequency percentage of isolated fungal pathogens from pomegranate fruits showing rot symptoms.

Isolated fungal pathogen	Frequency (%)	
	Year 2018	Year 2019
Botrytis cinerea	32	44
Aspergillus niger	25	27
Penicillium expansum	24	29
Alternaria alternata	19	-

TABLE 2. Effect of postharvest treatments on decay severity % of "Wonderful" pomegranate fruit during 2018 and 2019 seasons.

Treatment	Year 2018 Decay severity (%)		Year 2019 Decay severity (%)	
	9 DAI	15 DAI	9 DAI	15 DAI
T1	9.00±1.73a	16.17±1.26a	8.00±1.80a	16.00±0.87a
T2	3.83±0.58b	7.00± 0.50b	3.50±0.50b	6.67±0.29b
T3	3.67±0.29c	6.17±0.29 cd	3.33±0.29bc	5.67±0.29c
T4	2.83±0.29cd	4.67±0.58 de	2.33±0.29cd	4.33±0.29d
T5	5.17±1.04b	7.83±1.26b	4.83±1.15b	6.83±0.29b
T6	3.17±1.04cd	5.67±1.15cd	3.17±0.29cd	4.83±1.04cd
T7	2.00±0.00d	3.50±0.50e	1.67±0.58d	3.17±0.29e

Treatments: (T1) control, (T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5) Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$), DAI = days after inoculation.

Effect of SA and GB treatments on electrolyte leakage

Effect of SA and GB treatments under certain concentrations on electrolyte leakage (membrane permeability, integrity and cellular compartment indicator) of artificially inoculated Wonderful pomegranate fruits were evaluated 1, 2 and 3 days (Fig. 1). One-way ANOVAs indicated that the most effective treatment in decreasing EL was the treatment T7 (GB at 40 mM) followed by T4 (SA at 4 mM) after 1, 2 and 3 days during 2018 and 2019 seasons (Fig. 1). Treatment T7 exhibited the least EL values at 1, 2 and 3 days (11.60, 15.22 and 19.57), respectively compared to control treatment (23.98, 25.91 and 37.26) during 2018. Similar results were obtained with the same treatment T7 followed by T4 during 2019 season (Fig. 1).

Effect of SA and GB treatments on chilling injury and weight loss (%)

As predicted, the percentage of chilled fruits was increased with the progress of storage periods, and it is worth noting that there is no chilled fruit after 7 days of storage as shown in Fig. (2) In addition, the findings revealed that all treatments significantly decreased the percentage of chilled fruit compared to the control treatment. However, At the end of storage, the most significant treatments in reducing chilled fruits were T4 (SA at 4mM), T6 (GB at 20mM) and T7 (GB at 40 mM), which exhibited the least percentage (9.44, 9.48 and 9.36%), compared to control treatment T1 (33.60%) during 2018 season and the same trend in the second one, which T6 and T7recorded the least values (7.07 and 6.76%) respectively.

Concerning weight loss percentage was also estimated 7, 14 and 21 days after application of SA and GB treatments and the obtained results summarized in Fig. (3) interpreted that, treatment T7 after 21 days from the application was the most effective one (6.28%) followed by T4 (6.90%) and T6 (6.97%) compared to control treatment T1 (7.99%). Obtained results presented in Fig. (3), at 14 days during the 2019 season, treatment T7 was the most effective one followed by T6 and T4 (2.72, 3.24 and 3.62) respectively compared to control treatment (5.26).

Effect of SA and GB treatments on peel and arils color (chroma)

As shown in Fig. 4 and 5, the changes in color of pomegranate fruit peel and arils (chroma) were scored at 7, 14 and 21 days after treatment with SA and GB treatments during 2018 and 2019 seasons. Obtained results indicated that the most effective treatments on the chroma peels and arils were the treatment T7 (GB at 40 mM) followed by T4 (SA at 4 mM) at 7, 14 and 21 days of storage, compared to control. At 21 days of storage treatment T7 significantly exhibited the highest chroma peel units (54.23 and 55.14), followed by T4 (53.87 and 54.78) compared to the control treatment T1 (50.96 and 51.87), respectively during two seasons (Fig. 4). Furthermore, T7 also significantly exhibited the highest chroma aril units (34.88 and 35.85), followed by T4 (34.88 and 34.85) compared to the control treatment T1 (31.31 and 32.22), respectively during two seasons (Fig. 5).

Effect of SA and GB treatments on MDA content

As shown in Fig. (6 and 7), MDA contents in all treatments increased gradually with the storage time. "Wonderful" pomegranate fruits treated with SA and GB in different concentrations remarkably inhibited the increase of MDA content, in comparison with control during the storage period. At the end of storage, treatments T4 and T7 significantly inhibited the increase of MDA fruit content in peel (11.68 and 11.75) respectively, compared to the control treatment T1 (14.08) during 2018 season (Fig.6), and T4 recorded the least value (11.15) in the second season, followed by treatment T7 and T8 which also inhibited the increase of MDA fruit content (11.29 and 12.11) respectively. Similar results were obtained with treatment in arils (Fig. 7).

Effect of SA and GB treatments on anthocyanin, and vitamin C

The fruit application of SA and GB treatments had a significant effect on the anthocyanin and vitamin C content. As summarized in Fig. (8) and Table (3). Anthocyanin percentage in all treatments increased gradually with the storage time. Wonderful pomegranate fruits treated with SA and GB in different concentrations remarkably increased anthocyanin and vitamin C content, in comparison with control treatment during the storage period. Treatment T7 significantly increased the anthocyanin percentage at 7, 14 and 21 days (17.99, 18.50 and 18.62 mg/100 ml) compared to the control treatment T1 (17.34, 16.45 and 17.57 mg/100 ml), respectively during 2018 season (Fig. 8). Followed by treatment

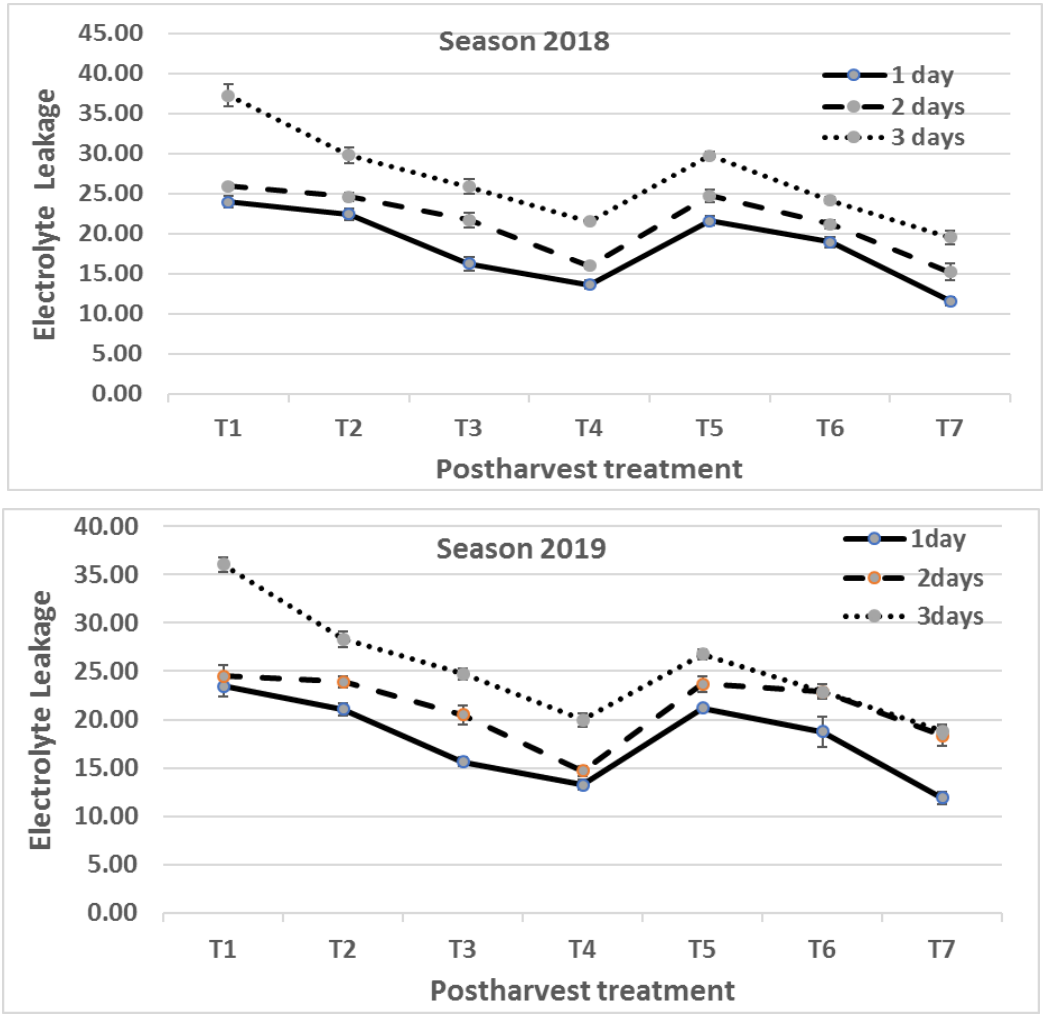
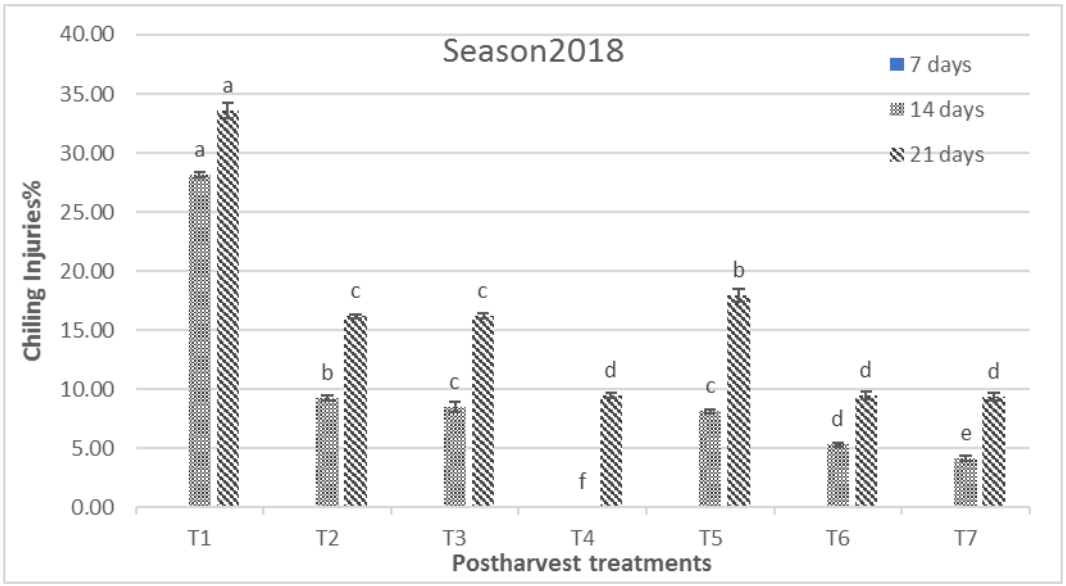


Fig. (1): Effect of postharvest treatments on Electrolyte Leakage of artificially inoculated "Wonderful" pomegranate fruits during 2018 and 2019 seasons. Treatments: (T1) control, (T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5)Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. ($p < 0.05$), day = days after inoculation



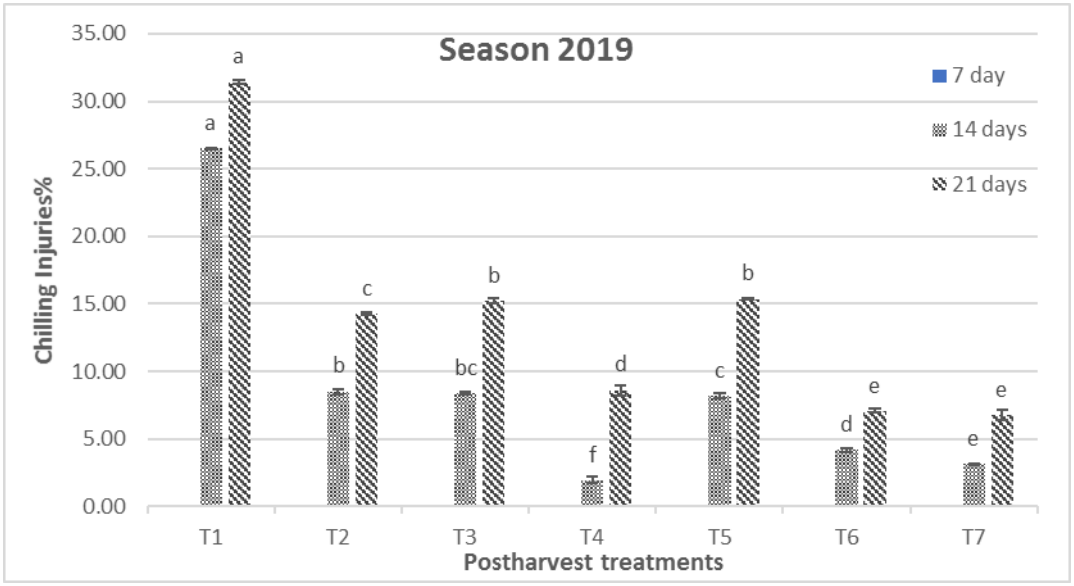


Fig. (2): Effect of postharvest treatments on chilling injury % of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control, (T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5)Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

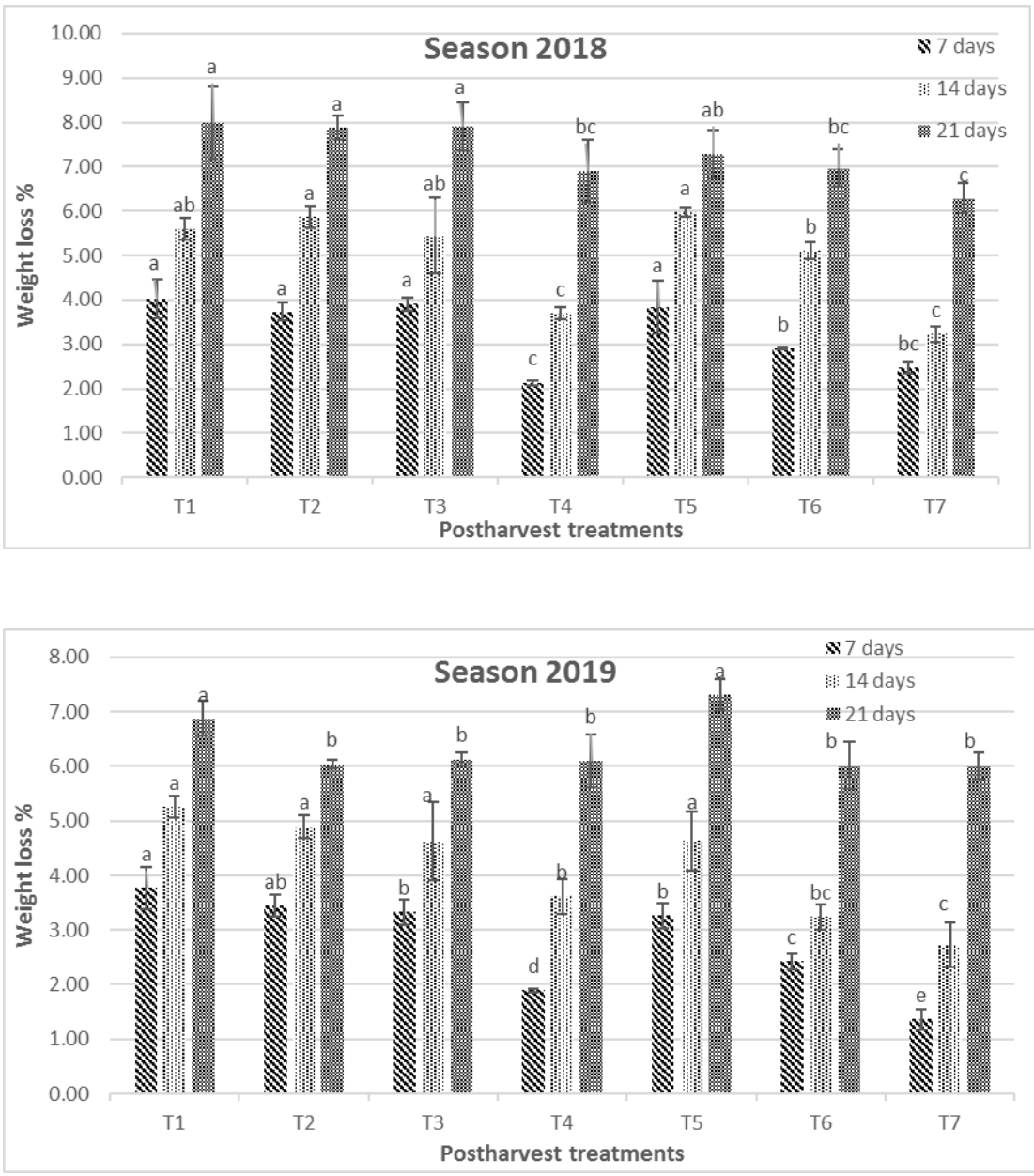


Fig. (3): Effect of postharvest treatments on weight loss % of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control,(T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5) Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

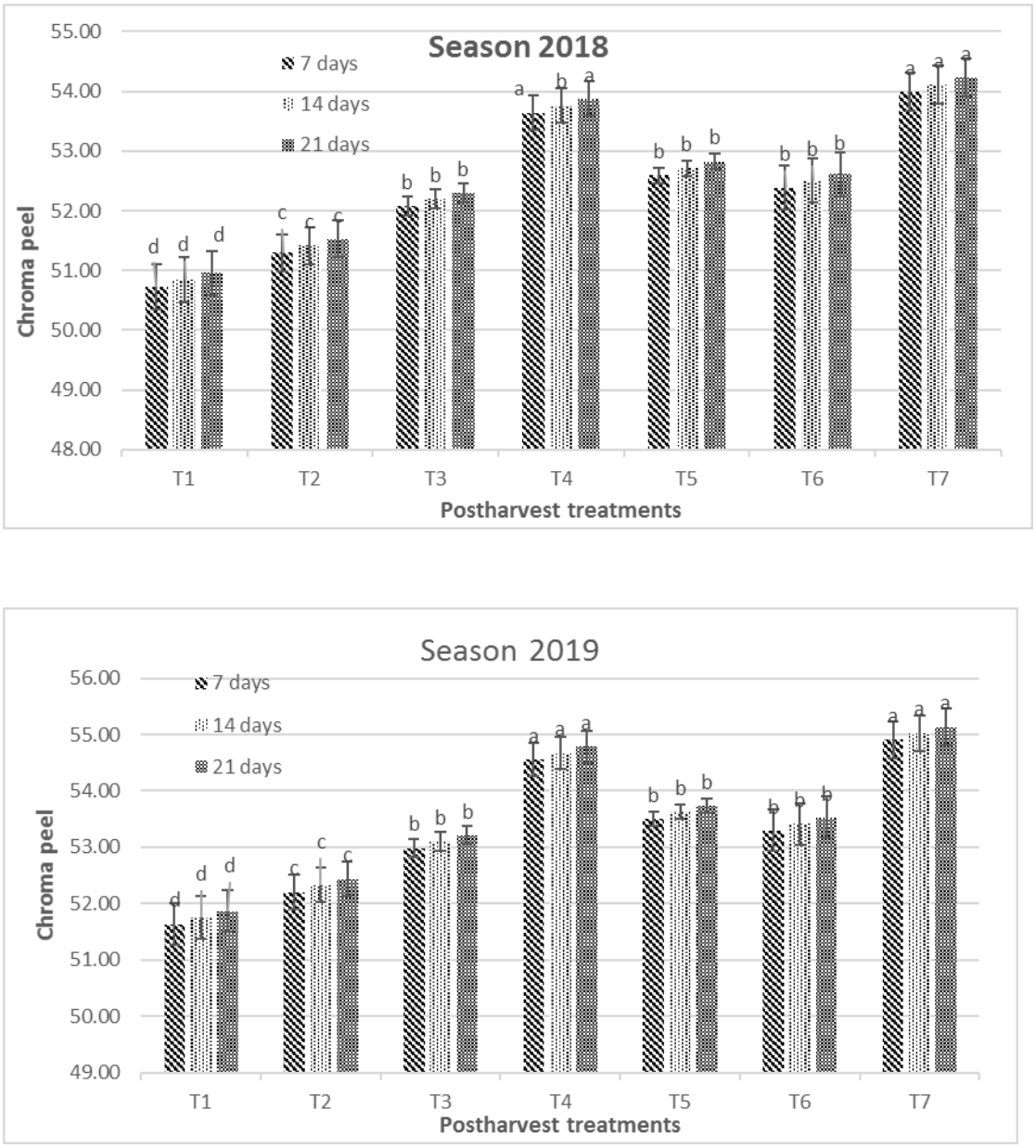


Fig. (4): Effect of postharvest treatments on chroma peel of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control, (T2) Salicylic acid at 2 mM, (T3) Salicylic acid at 3 mM (T4) Salicylic acid at 4 mM, (T5) Glycine Betaine at 20 mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40 mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

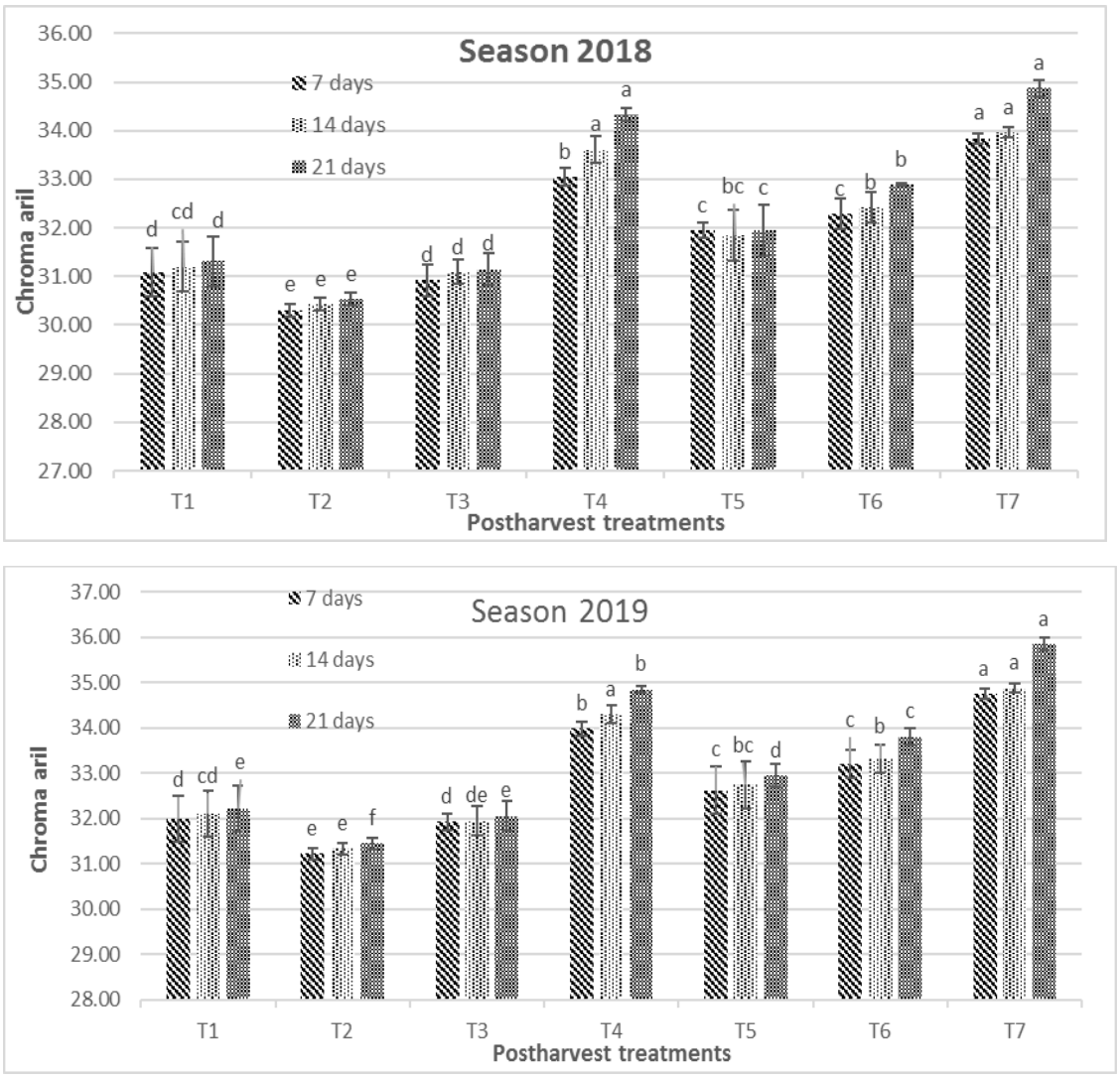


Fig. (5): Effect of postharvest treatments on chroma aril of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control, (T2) Salicylic acid at 2 mM, (T3) Salicylic acid at 3 mM (T4) Salicylic acid at 4mM, (T5)Glycine Betaine at 20 mM (T6) Glycine Betaine at 30 mM, (T7) Glycine Betaine at 40 mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

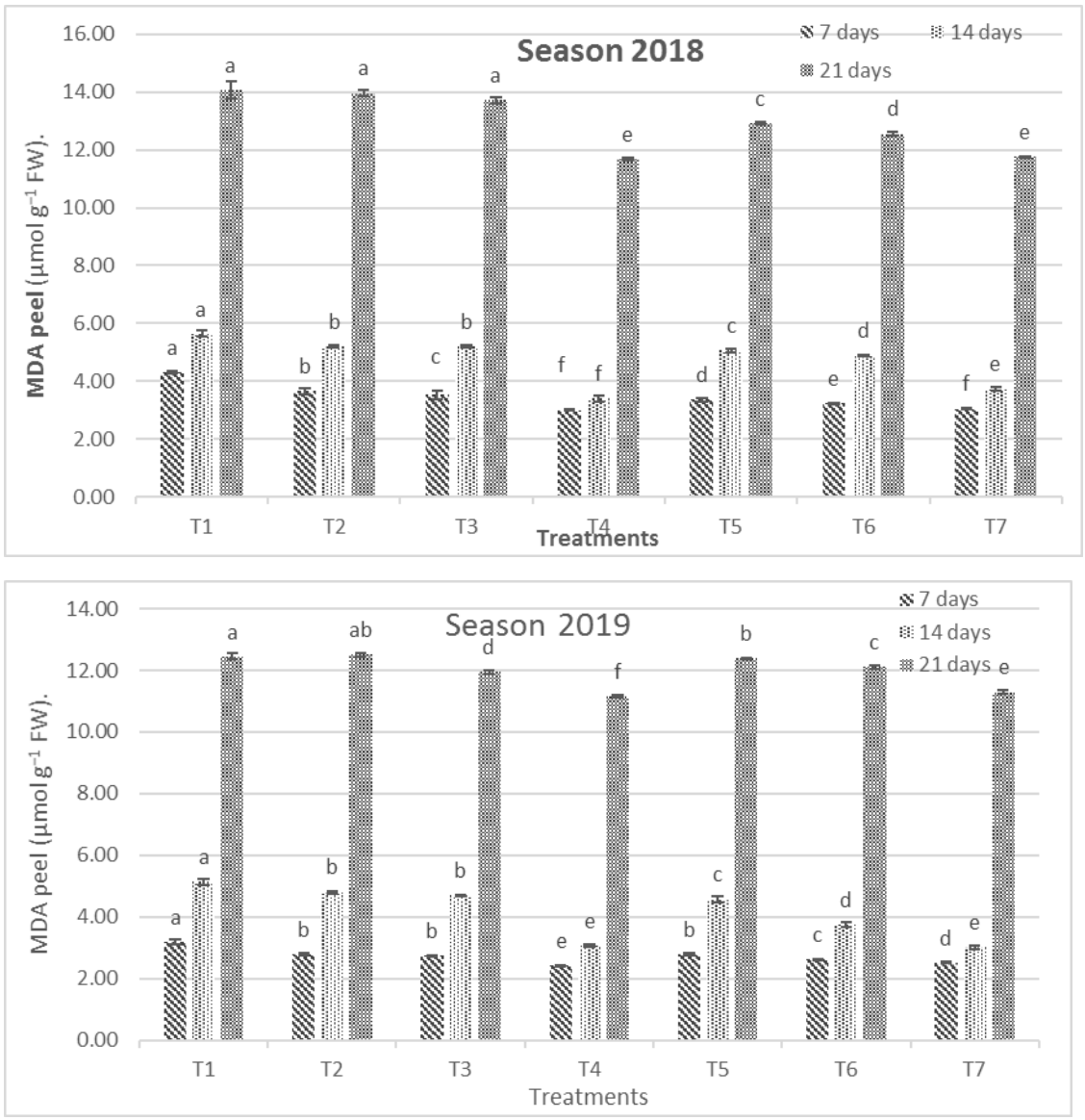


Fig. (6): Effect of postharvest treatments on MDA(µmolg^{-1}FW) of "Wonderful" pomegranate fruit peel during 2018 and 2019 seasons. Treatments: (T1) control,(T2) Salicylic acid at 2 mM, (T3) Salicylic acid at 3 mM (T4) Salicylic acid at 4 mM, (T5) Glycine Betaine at 20 mM (T6) Glycine Betaine at 30 mM, (T7) Glycine Betaine at 40 mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

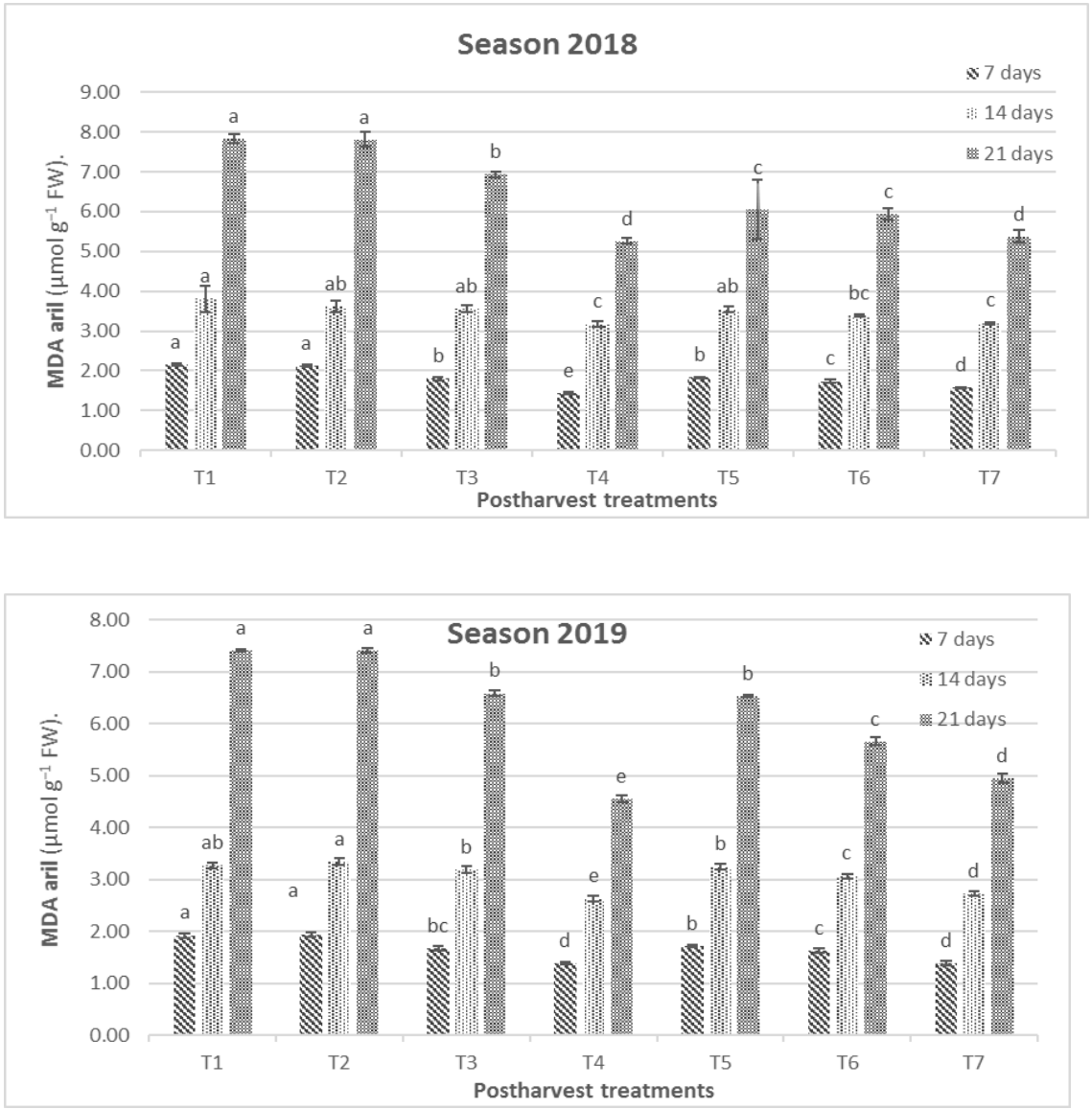


Fig. (7): Effect of postharvest treatments on MDA(µmolg^{-1}FW) of "Wonderful" pomegranate fruit arils during 2018 and 2019 seasons. Treatments: (T1) control, (T2) Salicylic acid at 2 mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4 mM, (T5)Glycine Betaine at 20 mM (T6) Glycine Betaine at 30 mM, (T7) Glycine Betaine at 40 mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

T4 which also increased the anthocyanin at the same period. Similar results were obtained with treatment T7 followed by T4 on vitamin C content (Table 3). Treatment T7 significantly increased the content of vitamin C at 7, 14 and 21 days (12.64, 12.59 and 12.48 mg 100 g^{-1}) compared to the control treatment T1 (12.28, 12.26 and 12.13 mg 100 g^{-1}), respectively (Table 3). During the next season, similar results were obtained with treatment T7 which significantly increased the anthocyanin at 7, 14 and 21 days (18.42, 18.65 and 18.64 mg/100 ml) compared to the control treatment T1 (17.63, 17.73 and 17.82 mg/100 ml), respectively (Fig. 8). Furthermore, similar results were obtained with treatment T7 followed by T4 on vitamin C content (Table 3).

Effect of SA and GB treatments on POD and PPO enzyme activities

Obtained results in Fig. (9) show that during the first season of 2018, POD activity was increased gradually to treatment application through the storage period. During the first seven days of application treatments, POD activity with treatment T7 significantly decreased (0.75 OD/min/g) compared with control treatment T1 (0.94 OD/min/g). Interestingly, POD activity with treatment T7 significantly increased at 14 and 21 days (1.34 and 1.45 OD/min/g) compared to control treatment T1 (0.86 and 0.80 OD/min/g), respectively. Similar results were obtained through the next growing season 2019 (Fig. 9). On the other hand, PPO was significantly decreased after application of treatments of GB and SA in comparison with control treatment (Fig.10). During 7, 14 and 21 days of treatment application, PPO activity with treatment T7 significantly decreased (0.05, 0.07 and 0.18 OD/min/g) compared to the control treatment T1 (0.23, 0.28 and 0.47 OD/min/g), respectively in the growing season 2018. Similar results were obtained with the next growing season 2019 (Fig. 10).

TABLE 3. Effect of postharvest treatments on Ascorbic acid content (mg 100 g^{-1}) of "Wonderful" pomegranate fruit during 2018 and 2019 seasons.

Treatment	Ascorbic acid (mg 100 g^{-1})											
	Season 2018						Season 2019					
	7 days		14 days		21 days		7 days		14 days		21 days	
T1	12.28±	0.03f	12.26±	0.02e	12.13±	0.02e	13.46±	0.03c	13.39±	0.05c	13.33±	0.02e
T2	12.34±	0.01e	12.30±	0.01d	12.23±	0.03d	13.45±	0.02c	13.42±	0.02c	13.32±	0.01e
T3	12.42±	0.03d	12.38±	0.04c	12.33±	0.03c	13.55±	0.03b	13.51±	0.02b	13.39±	0.03d
T4	12.57±	0.03b	12.49±	0.03b	12.44±	0.03a	13.74±	0.07a	13.69±	0.06a	13.62±	0.04b
T5	12.44±	0.02d	12.41±	0.02c	12.30±	0.01c	13.54±	0.02b	13.49±	0.03b	13.41±	0.03cd
T6	12.51±	0.02c	12.46±	0.01b	12.39±	0.04b	13.58±	0.02b	13.54±	0.04b	13.45±	0.03c
T7	12.64±	0.03a	12.59±	0.03a	12.48±	0.03a	13.77±	0.03a	13.73±	0.02a	13.69±	0.02a

Treatments: (T1) control,(T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5)Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

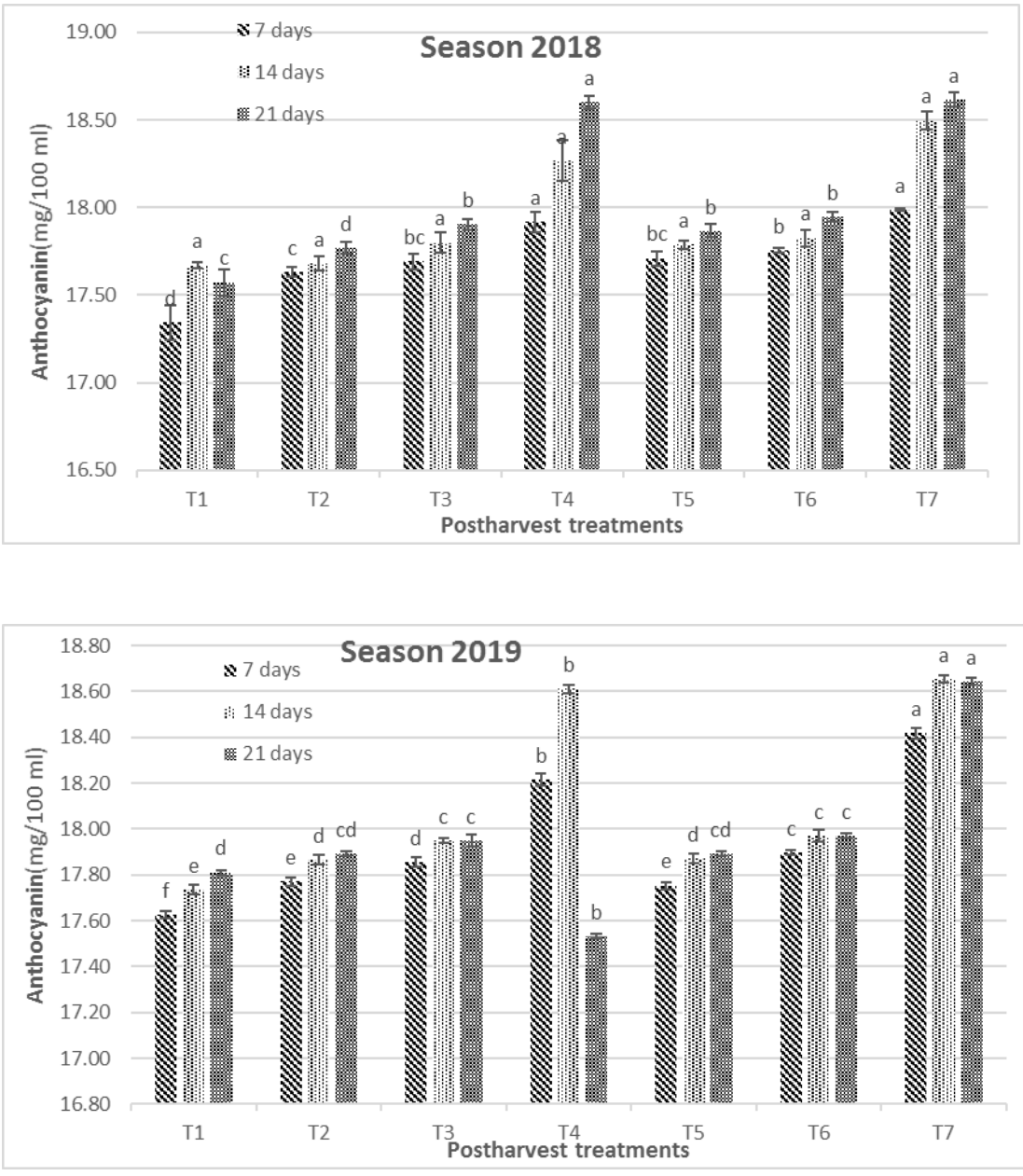


Fig. (8): Effect of postharvest treatments on Anthocyanin (mg/100 ml) of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control, (T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5)Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

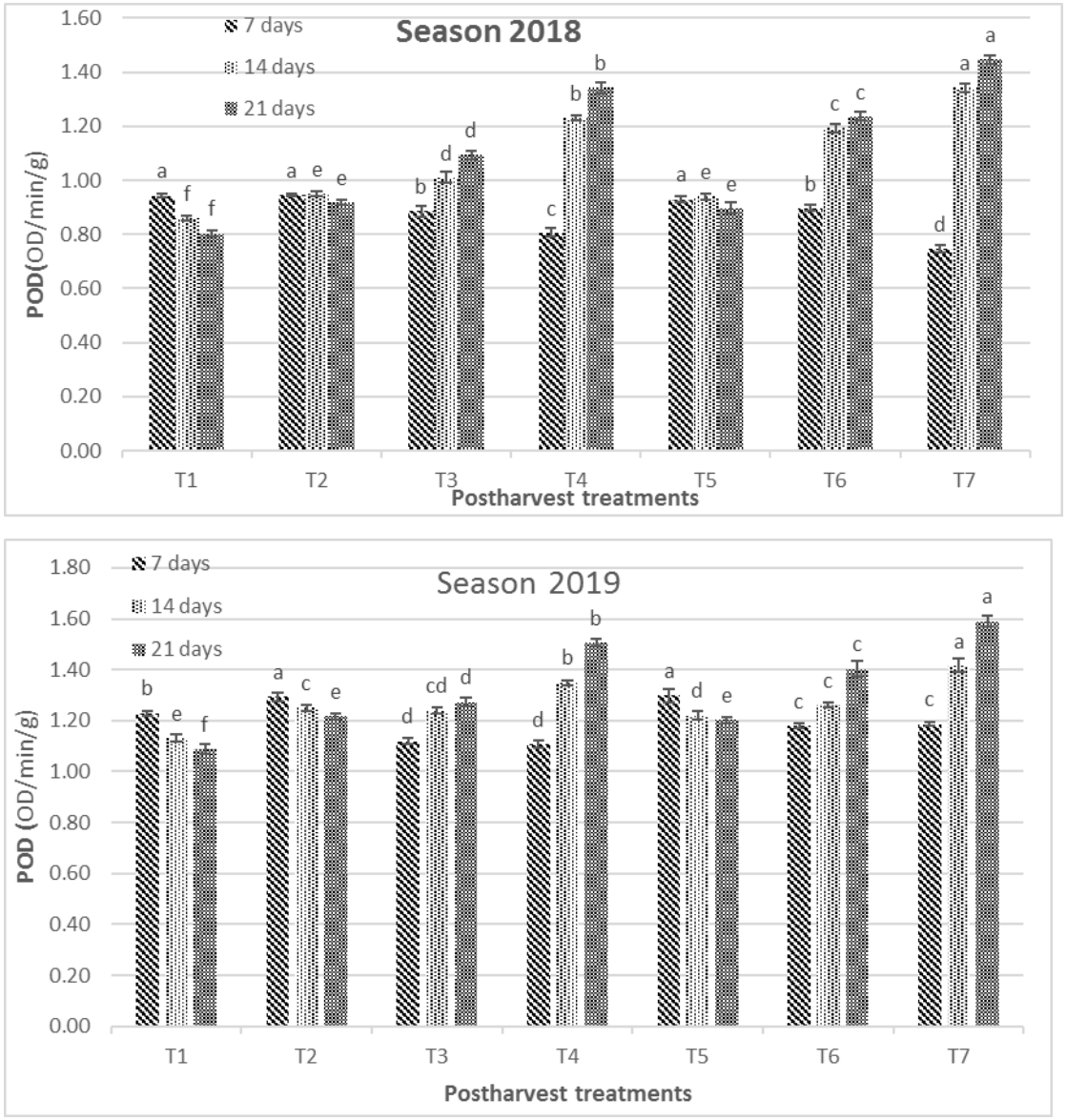


Fig. (9): Effect of postharvest treatments on POD(OD/min/g) of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control,(T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5) Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

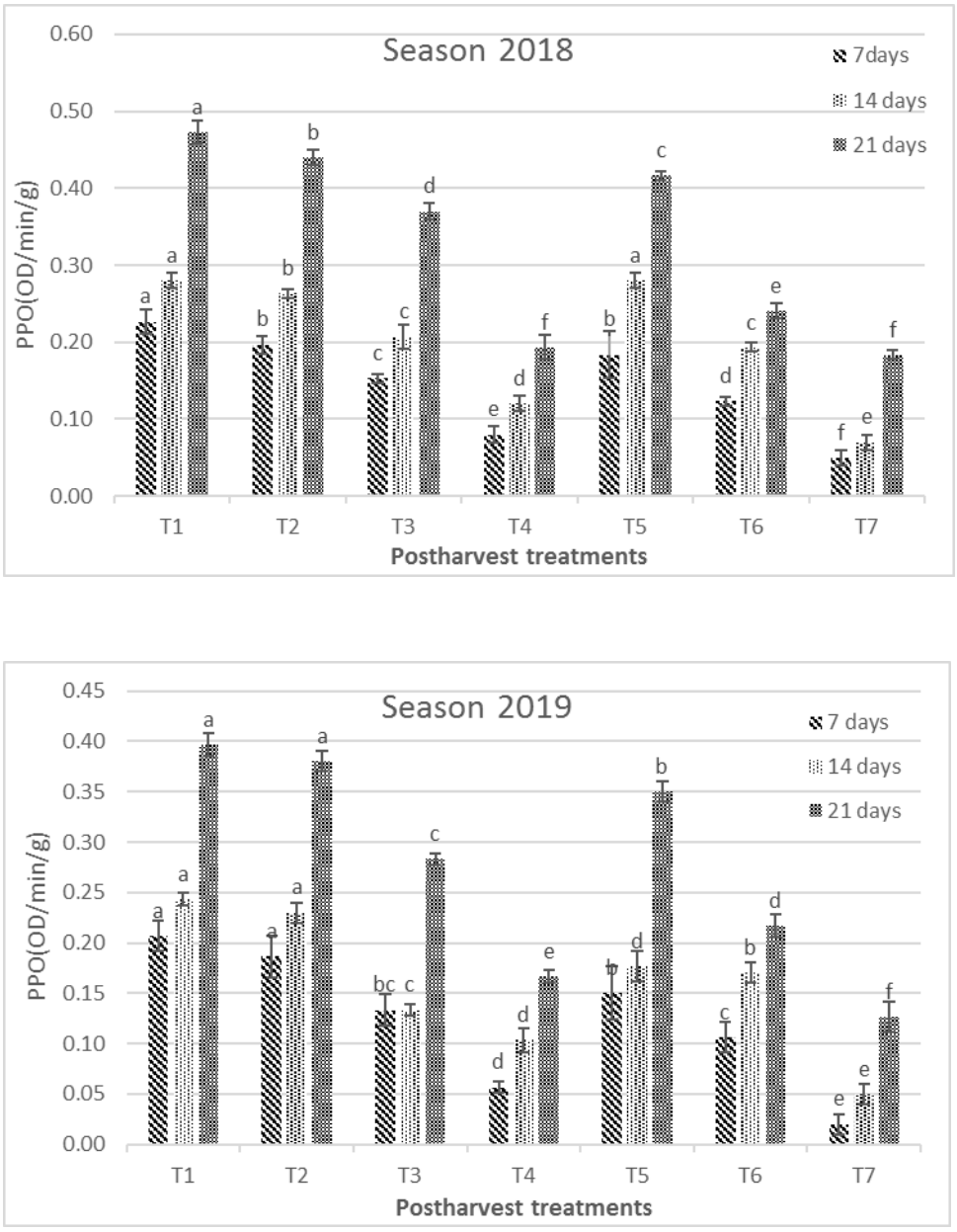


Fig. (10): Effect of postharvest treatments on PPO(OD/min/g) of "Wonderful" pomegranate fruit during 2018 and 2019 seasons. Treatments: (T1) control,(T2) Salicylic acid at 2mM, (T3) Salicylic acid at 3mM (T4) Salicylic acid at 4mM, (T5)Glycine Betaine at 20mM (T6) Glycine Betaine at 30mM, (T7) Glycine Betaine at 40mM. Values represent the mean ± SD for three replications. Different letters indicate significant differences among different treatments ($p < 0.05$).

Discussion

Admittedly, chilling injury symptoms are the strongest limit factor for the low-temperature application of pomegranate fruit especially under 1°C, leading to an increase in susceptibility of fruits to damage caused by various fungal pathogens such as *Aspergillus* spp., *Alternaria* spp., *Penicillium* spp., and *Botrytis cinerea*, as well as reducing marketability and the opportunity to export (Rasouli *et al*., 2019). Furthermore, the occurrence of chilling injury symptoms in pomegranate fruit during 21 days of storage at 0 °C was followed by decreasing membrane integrity represented by higher MDA accumulation, which may result from increased ROS accumulation in conjunction with increased membrane degrading enzymes phospholipase (PLD) and lipoxygenase (LOX) (Jannatizadeh, 2019). Consequently, successful ways to decrease CI and gray mold disease in pomegranate fruit have always been important to search for.

Salicylic acid improved chilling damage by strengthening antioxidant systems, which reduces the activity of cell membrane enzymes such as lipoxygenase (LOX) and phospholipase (PLD) led to diminish membrane damage thereby enhancing cell membrane integrity (Rasouli et al.,2019), that leakage of electrolytes was regarded as an accurate indicator of loss of membrane permeability through pathogen infection (Ketta, 2015) and it increased with storage advanced (Koushesh Saba and Zarei, 2019). Additionally, SA-applied playing a crucial task in the strengthening of disease resistance by attributing cell wall component accumulation such as sugars, organic acids and amino acids, which provide the cell with enough energy and strengthening to tackle pathogens and enhance defence-related metabolites (Zhu *et al*., 2016), and increasing the production of H_2O_2 which activates the plant systemic resistance against pathogens, and induction of the PR-proteins, ultimately extend the storability of fruits. In the present result the highest GB-treated, compared with the least concentration, led to an incidence of CI suppression, which Yao et al. (2018) document that GB treatment relieved fruit CI by suppressing LOX and PLD activities, lead to maintaining the level of unsaturated fatty acid, and enhancing variation antioxidant enzyme activities, including SOD, CAT, and APX as well as their gene expressions, thereby suppressed ROS that in turn upsurge in chilling resistance under storage status. As well as, proline accumulation in the cold-stored fruit may be the result of GB-treated, which helped to protect the membrane (Shan et al. 2016). Furthermore, GB improves fruit sugar metabolism and preserves the integrity of cell membranes and inhibits cell death to prevent cold stress (Shan et al., 2016; Wang et al., 2019). Simultaneously, with chilling injury, weight loss is indeed an obstacle factor during storage, leading to shrivelling of pomegranate fruit because of the porous structure of the fruit peel which enables unlimited immigration of the water vapor (Elyatem & Kader, 1984), and changes in antioxidant activity of the fruit-affected (Mukama *et al*., 2019). Being SA as an agent in mitochondrial electron transport, the catabolic reactions are presumably decreased which may be due to diminishing rates of respiration as well as stomata closer (Shafiee et al.,2010 and Haider et al., 2020)). thereby the weight of the fruit is preserved (da Rocha Neto et al., 2016).

As well as, GB–treated fruits were decreased in loss of fruit weight and maintained the structure of the membrane which help to prolong shelf life, that it could be associated with improvement in the cell membrane function or skin cuticular properties (Ramezanian and Rahemi, 2010), as well as accompanying lower in CI since affecting in skin integrity of the fruit. Decay by fungal pathogens especially *B. cinerea* is another significant factor cause of post-harvest losses that limits fruit storability. In response to pathogen infection, lipase enzyme activity increased leading to releasing free fatty acid from the membrane. These free fatty acids directly have pivotal roles in response to pathogen infection as free fatty acids or indirectly via biosynthesis of oxylipins (Walley *et al*., 2013). Furthermore, increasing pathogen-related protein (PRs) accumulation is critical in the preservation of membrane fluidity and cellular fortitude (Zhang *et al*., 2016) and improving fruit quality by delay the cell breakdown and deterioration.

Aghdam and Fard (2017) suggested that the lower decay in fruits treated with GB may be due to higher unsaturated fatty acids/saturated fatty acids, which results from higher ATP provided by GABA shunt pathway activity. Malondialdehyde is a product that can reflect lipid peroxidation of the cell membrane (Sevillano *et al*., 2009) which the starting point of the CI symptoms. Salicylic acid-treated fruits diminish the levels of MDA(

Aghdam and Bodbodak 2013), perhaps due to decrease the activity of LOX, as well as enhance the activity of antioxidant enzymes (Lo'ay and Taher, 2018; Rasouli *et al.*, 2019) which involved in the maintenance of unsaturated fatty acids during cold storage (Sayyari *et al.*, 2016). Treatments with GB markedly reduced the MDA content under cold storage. That reduction in MDA content by GB could be due to suppress the activity of the LOX and PLD genes expression, which can induce phospholipid and fatty acid deterioration) (Pinhero *et al*., 1998). Furthermore, GB treatment has shown a considerable influence on the gene-related expression of the antioxidant defence enzyme accumulation (Yao et al., 2018), which lead to maintaining the cell membrane integrity as well as unsaturated fatty acids content (Aghdam and Bodbodak, 2013), resulting in raising the chilling resistance (Sun *et al*., 2020). Previous results of Siboza *et al*., (2014) reported that SA-treatments inhibited the activities of PPO which recorded with higher POD activity as well as increasing of phenylalanine ammonia lyase (PAL) which involved in phenolic biosynthesis. SA-treatments were motivated the activity of POD due to its ability to induce antioxidant systems (Sayyari *et al*., 2016). As a response to GB treatment, the activities of POD (antioxidant enzyme) in ROS scavenging are upward tendency, that in turn reduction of ROS, and inhibition the activity PPO, as well as enhancement of phenolic compounds, which is accompanied by decreased contents of H_2O_2 and O_2^{-} as a radical oxygen species (Ketta *et al*., 2017) by regulating phenylpropanoid pathway in the fruit, which could play key roles in the protection of cellular membrane systems and the tolerance to cold stress under cold conditions (Sun *et al*., 2020).

Under cold storage anthocyanins biosynthesized by phenylpropanoid pathway through participate in PAL enzyme activity which has displayed powerful non-enzymatic antioxidant capacity (Sevillano *et al*., 2009). Furthermore, Salicylic acid may stimulate anthocyanin synthesis through phenylpropanoid pathway activation, and during storage (Giménez et al., 2017 and Sayyari et al., 2016) , as well as Koyuncu et al. (2019) represented the best color by SA-treated pomegranate fruit. Because of its scavenging effects on free radicals of the hydroxyl group, hydrogen peroxide, and superoxide anion through the ascorbate peroxidase reaction, ascorbic acid can protect fruits and vegetables from oxidative damage. (Nair et al., 2018). Salicylic acid treatments could increase the ascorbic acid content by inducing the activity of ascorbate peroxidase as well as motivation the ascorbate-glutathione pathway and inhibition ascorbic acid oxidase (AAO) as well as higher GR/APX system activity (Rao *et al*., 2011). Aswell as GB-treatments are maintaining high content of polyphenols and ascorbic acid (Zhang *et al*., 2016). These results are in agreement with (Awad et al., 2017)and (Babalar et al., 2018).

Conclusions

Effect of salicylic acid and glycine betaine under different concentrations on chilling injury and gray mold caused by *Botrytis cinerea*, of pomegranate cv. Wonderful fruits were investigated during 2018 and 2019 seasons. Chilling injury and gray mold disease were reduced significantly ($p<0.05$) when treated with glycine betaine (40 mM, treatment T7) followed by salicylic acid (4 mM, treatment T4). Results indicated that the most effective treatment in decreasing electrolyte leakage and malondialdehyde was the treatment T7 followed by T4. Therefore, SA and GB treatments could maintain normal cell membrane structure and function through down-regulation of MDA content and up-regulation of peroxidase enzyme activity to alleviate chilling injury and gray mold disease in pomegranate cv. Wonderful fruits. Furthermore, the content of vitamin C and anthocyanin was retained, resulting in the preservation of fruit quality.

Authors' contributions: HME created the idea of the manuscript. HAK and HME collected the literature, designed the experiments, started and followed the experiments, obtained results and discussed it and wrote the manuscript. HAk and HME revised the manuscript several times. HAK and HME formatted the manuscript according to the journal guidelines. All authors have read and approved the manuscript.

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