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Postharvest Treatments of Arginine, Asparagine, and Glycine Reduce Chilling Injury and Improve Okra Pod Quality

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ABSTRACT

Okra (*Abelmoschus esculentus* L.) belongs to the Malvaceae family and is susceptible to chilling injury. To evaluate the efficiency of amino acids in preventing okra pods from chilling injury and maintaining their quality during storage, an experiment was performed to evaluate the possible role of amino acids in maintaining okra quality during cold storage. In this regard, okra pods were dipped for 10 min in distilled water (as the control), and four solutions were prepared as treatments, containing 2mM arginine, asparagine, or glycine, which were applied either individually or in combination with the amino acids. The samples were then stored at 4°C for 14 days. The results showed that glycine and asparagine individually reduced the rate of pod weight loss by as much as 43% and 37%, respectively. Moreover, the pretreatment of okra with amino acids, particularly glycine, remarkably diminished chilling disorders because of their roles in inducing antioxidant activity and reducing lipid peroxidation in the pods. The results also revealed that the pretreatment of okra with amino acids significantly mitigated polyphenol oxidase activity, and this could delay the appearance of browning lesions in the pods during cold storage. In conclusion, the results suggested that the pretreatment of okra pods with amino acids, especially glycine, could reduce biochemical changes that occurred in the pods due to chilling in cold storage.

Abbreviations: Catalase (CAT), Chilling index (CI), Malondialdehyde (MDA), Peroxidase (POD), Polyphenoloxidase (PPO), Reactive oxygen species (ROS), Total antioxidant capacity (TAC), Total phenol (TP)

Introduction

Okra belongs to the Malvaceae family and originates in warm regions. The pods contain carbohydrates, protein, lipids, vitamins, and minerals (Gemede et al., 2016). Due to the high respiration rate, which is associated with high rates of dehydration and metabolism, and their harvest at an immature stage, okra is an extremely perishable fruit. Therefore, okra fruit must be stored in cold storage to maintain its quality (Finger et al., 2008). This requires protection from chilling and the consequences of chilling (Phornvillay et al., 2019). Chilling injury (CI) emerges initially on the surface of pods like pits, and then gradually becomes brown (Huang et al., 2012). At the postharvest stage, some disorders such as softening tissues, fading colors of the pericarp, puncturing pods in the pericarp, and browning lesions are often emerged in the fruits of okra due to CI incidence, and these disorders, unfortunately, affect fruit appeal to consumers (Huang et al., 2012; Phornvillay et al., 2019). For this reason, various postharvest approaches have been introduced to alleviate the harmful effects of CI in line with extending the shelf life of okra fruit in cold storage (Finger et al., 2008; Huang et al., 2012; Phornvillay et al., 2019).

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Nowadays, amino acids are notably being utilized in agricultural industries (Mohammadipour and Souri, 2019). In this context, amino acids are considered organic forms of nitrogen that are used as nitrogen-source substrates (Teixeira et al., 2018). After entering plant cells, amino acids are easily absorbed by cells without wasting energy, because of their high purity, and saving more energy under stressful conditions helps plants to tolerate environmental stresses (Pan et al., 2014). So far, previous studies have substantiated the role of amino acids in alleviating harmful effects of chilling injury, oxidative stress, and pathogens during storage (Rodríguez-Zapata et al., 2015; Zhang et al., 2017; Li et al., 2019a; Babalar et al., 2018; Pan et al., 2019). As a precursor, arginine plays a significant role in the synthesis of some hormones responsible for setting and developing fruits as well as stimulating endogenous-plant enzymes involved in tolerating stressful conditions (Zeid, 2009). In addition, glycine can facilitate plant growth and development, chlorophyll synthesis, and nutrient absorption (Mohammadipour and Souri, 2019). However, asparagine not only enhances plant growth and seed germination but also engages in saving nitrogen and contributing to its translocation in plants, while also reinforcing some metabolic cycles such as the carbon cycle and nitrogen metabolism cycle. These cycles result in generating sugars and proteins vital for plant growth and development (Lea et al., 2007). Broadly speaking, amino acids can play a direct role in maintaining some macromolecules such as protein, retaining cell membrane integrity, and inducing antioxidant activities under stressful conditions (Nasibi et al., 2011; Wang et al., 2017).

To the best of our knowledge, there is little evidence of the role of amino acids as postharvest treatments on okra pod quality in cold storage. The purpose of this research was to evaluate the effects of arginine, asparagine, and glycine, individually and in combination, on chilling injury while maintaining pod quality at the postharvest stage.

Materials and Methods

Plant materials

The pods of okra were collected in September 2019 from a village located in Sirvan, Ilam province, Iran. The healthy-immature pods of the same size were harvested, and then they were immediately transferred to a lab in the Horticultural Department, Agricultural Faculty, Ilam University, Ilam, Iran. The treatments included the control (distilled water) and experimental solutions containing arginine, asparagine, or glycine, either individually or in combination (Arg+Asp+Gly). The pods in the experimental group were dipped in 2 mM of each solution for 10 min. The pods of the control group were dipped in distilled water for 10 min (Li et al., 2019a). After leaving the pods for 2 h in a room, they were packed in special containers and kept for 14 days at 4 °C and 80-85% RH.

Weight loss and chilling injury

At the end of 14 days of storage, the weight loss of pods was calculated by subtracting their earlier weight from later ones. The magnitude of the browning, translucency, or pitting area on each okra pot was considered an indicator of CI. The scale was categorized as follows: 0, no visible symptoms; 1, less than 25% of fruit area affected; 2, less than 50% of fruit area affected; 3, less than 75 of fruit area affected; 4, 76-100% of fruit area affected. The index of chilling injury was calculated using the following formula (Huang et al., 2012):

$$Chilling\ injury = \Sigma\,(Chilling\ scale \times Percentage\ of\ corresponding\ fruit\ within\ each\ group)$$

Malondialdehyde

To measure malondialdehyde content (MDA), a method described by Heath and Parker (1968) was utilized. Here, 500 µl of fruit juice and 500 µl of 20% trichloroacetic acid containing 0.5% thiobarbituric acid were mixed. The mixture was left for 30 min at 95°C. After placing it in an ice bath to stop the reaction, the mixture was centrifuged at 10000 rpm for 5 min at 4°C, and subsequently, its supernatant was read at both 532 and 600 nm. Finally, MDA was measured based on an extinction coefficient of 115 mM-1 cm-1 and the results were reported as nanomole per gram of fresh weight.

Total phenol

Total phenol (TP) was measured using the Folin-Ciocalteu reagent (Singleton et al., 1999). In this regard, 0.05 g of fresh tissue was homogenized with 2 ml of methanol 80%. The homogenate was placed in bathwater for 15 min at 70°C, followed by centrifuging it at 5000 rpm for 15 min. Then, 1 mL of its supernatant was added to 1.8 mL of distilled water and 0.2 ml of Folin-Ciocalteu reagent. Keeping the mixture at 25°C for 5 min was followed by adding 1 ml sodium carbonate (12% W/V) to the resultant mixture

and reading its absorbance at 765 nm using a spectrophotometer. The rate of TP was expressed as mg gallic acid per fresh weight of tissue.

Total antioxidant capacity

To measure the total antioxidant capacity (TAC) of samples, 75 µl of the fruit juice and 2925 µl of DPPH were vortexed, and immediately the yielded mixture was read at 517 nm using a spectrophotometer (Specord 50, Analytik Jena). After 30 min, its absorbance was read again. According to Brand-Williams et al. (1995), the percentage of TAC in different samples was estimated by the following equation:

$$Total\ antioxidant\ capacity = \frac{At0 - At30}{At0} \times 100$$

Where At0 represents the absorbing solution at T0, and At30 is the absorption of the solution in 30 minutes.

Polyphenol oxidase activity

To measure the activity of polyphenol oxidase (PPO), a method proposed by Kar and Mishra (1976) was employed with slight modifications. In this context, 1.5 ml of tris buffer was added to 0.4 ml Pyrogallol and 0.1 ml of the enzymatic extract. The mixture was placed in bathwater for 25 min. The curve of absorbance change was plotted at 420 nm for 2 min. Finally, the activity of polyphenol oxidase was expressed as units (i.e. as the amount of enzyme that caused a change in the absorbance 0.001 min^{-1}) per mg^{-1} protein.

Catalase activity

For measuring catalase (CAT), as proposed by Kar and Mishra (1976), a mixture of 0.75 mL potassium phosphate buffer 100 mM (pH=7), 20 µl of H_2O_2, and 1500 µl distilled water was provided. Then, a change in the absorbance of the mixture was read at 240 nm for 120 s using a spectrophotometer. The activity of CAT was expressed as a change in the absorbance in minutes for each mg of protein.

Peroxidase activity

To assay peroxidase (POD), a method described by Plewa et al., (1991) was used. Accordingly, a mixture of 0.81 mL of potassium phosphate buffer 50 mM (pH=6.6), 20 µl of sample extracts, and 90 µl guaiacol 1% as the donor of the electron was prepared. The absorbance of the mixture was read at 470 nm for 120s using a spectrophotometer. Changes in the activity of peroxidase were expressed as the formation of 1 mM of tetraguaiacol per minute.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) with three replications. The data were analyzed using SAS software (9.1 version) and the comparison of means was made using Duncan's multiple range test ($p<0.05$).

Results

Weight loss and chilling injury index

The results showed that treating the pods with arginine, asparagine, and glycine mitigated their weight loss and CI, compared to the control group (Fig. 1 and 2). The lowest level of weight loss (8.19 and 9.52 %) was obtained when the pods were treated with glycine and asparagine, respectively, whereas the highest level (14.28%) was observed in the control group (Fig. 1). Furthermore, arginine decreased CI by as much as 15%, while asparagine and glycine reduced it by as much as 25% and 50%, respectively (Fig. 2).

MDA accumulation

According to the results of this research, amino acids reduced MDA accumulation in the treated pods. The highest MDA accumulation (4.66 and 3.12 nmol/g FW) was observed in the seeds of non-treated and arginine-treated pods, respectively, while the lowest amount (1.11 nmol/g FW) was found in the seeds of glycine-treated pods. In general, all pods that were treated with amino acids showed significant reductions in their accumulation of MDA levels in the pericarp (Fig. 3).

Total phenol content

Compared to the other amino acids, arginine and glycine significantly increased the total phenol content by 32.06 and 43.29 %, respectively, in the seeds of treated pods. Meanwhile, in the pericarp of fruits, the application of arginine and glycine, as well as their combination, enhanced total phenol content by 25.42, 29.51 and 44.21%, respectively, compared to the control. In this research, the highest total phenol content (11.97 mg/g FW) was obtained by a combination of arginine, glycine, and asparagine (Fig. 4).

Protein content

The comparison of mean values revealed that protein content in amino acid-treated pods was higher than that in the control group. In other words, the lowest protein content was found in

the seeds (0.76 mg g^{-1} FW) and pericarp (1.82 mg g-1 FW) of pods belonging to the control group, whereas the highest protein content was found in the seeds (5.3 mg g^{-1} FW) and pericarp (7.91 mg g^{-1} FW) of pods treated with glycine alone (Fig. 5).

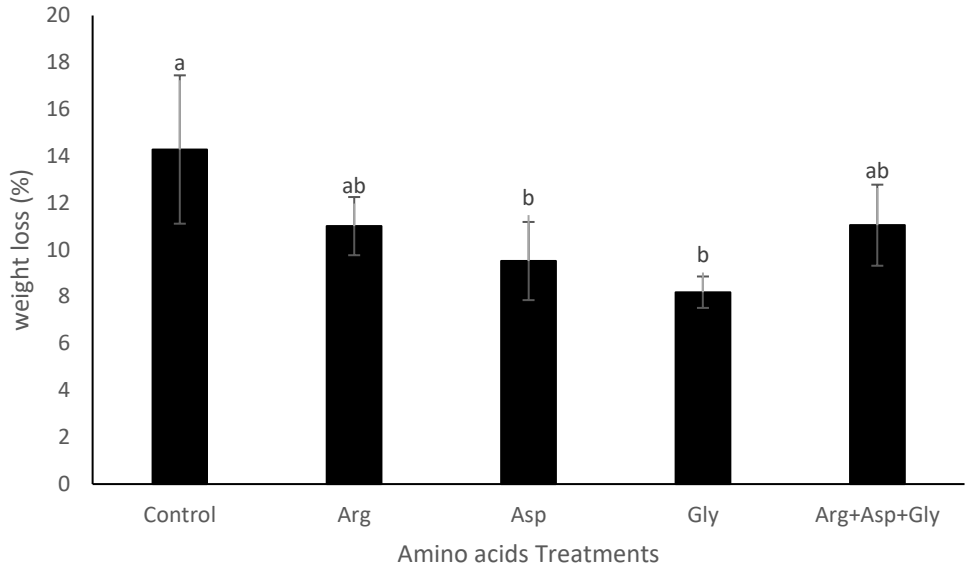

Fig. 1. Effects of 2mM arginine (Arg), asparagine (Asp), and glycine (Gly) on weight loss of okra pods after storage at 4oC for 14 days. Bars with different letters are significantly different according to Duncan's multiple range test ($p<0.05$).

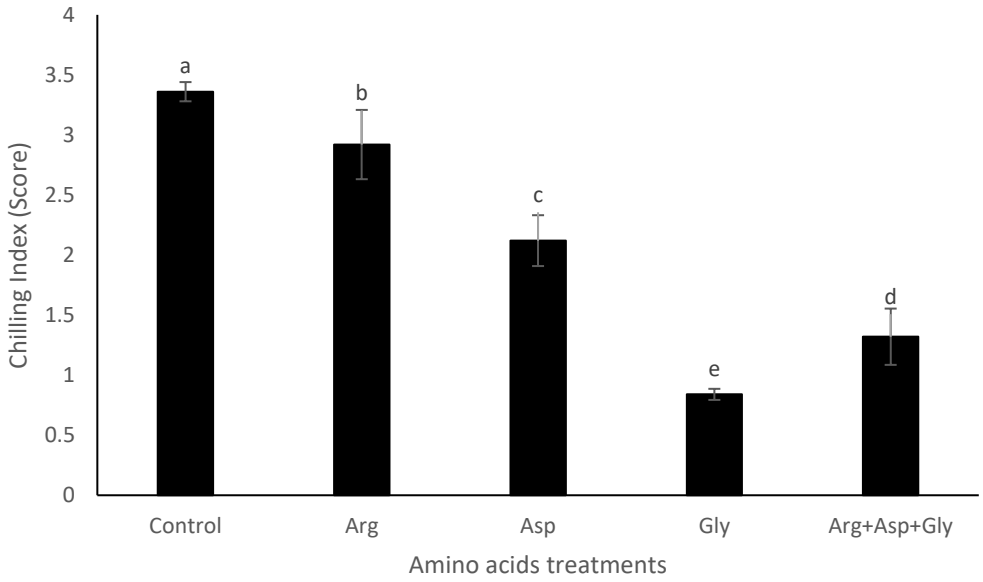

Fig. 2. Effects of 2mM arginine (Arg), asparagine (Asp), and glycine (Gly) on chilling injury of okra pods after storage at 4oC for 14 days. Bars with different letters are significantly different according to Duncan's multiple range test ($p<0.05$).

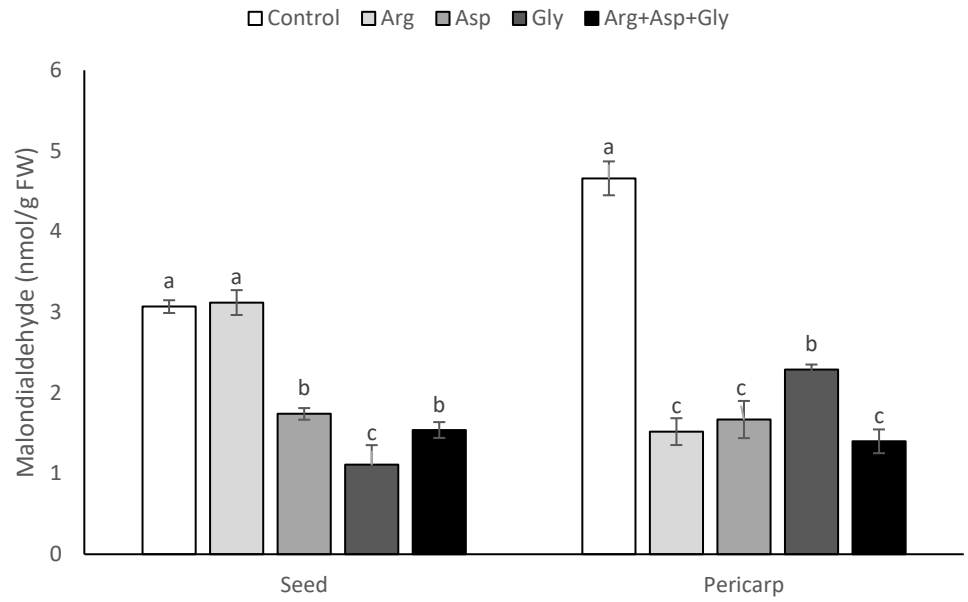

Fig. 3. Effects of 2mM arginine (Arg), asparagine (Asp), and glycine (Gly) on malondialdehyde accumulation in the seeds and pericarp of okra after storage at 4oC for 14 days. Bars with different letters are significantly different according to Duncan's multiple range test ($p<0.05$).

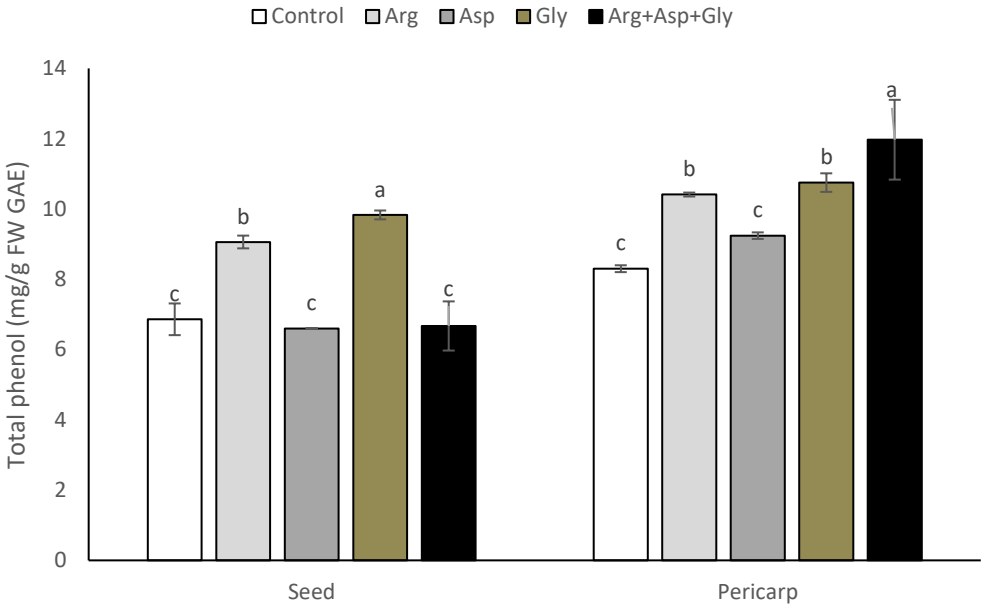

Fig. 4. Effects of 2mM arginine (Arg), asparagine (Asp), and glycine (Gly) on total phenol content in the seeds and pericarp of okra after storage at 4oC for 14 days. Bars with different letters are significantly different according to Duncan's multiple range test ($p<0.05$).

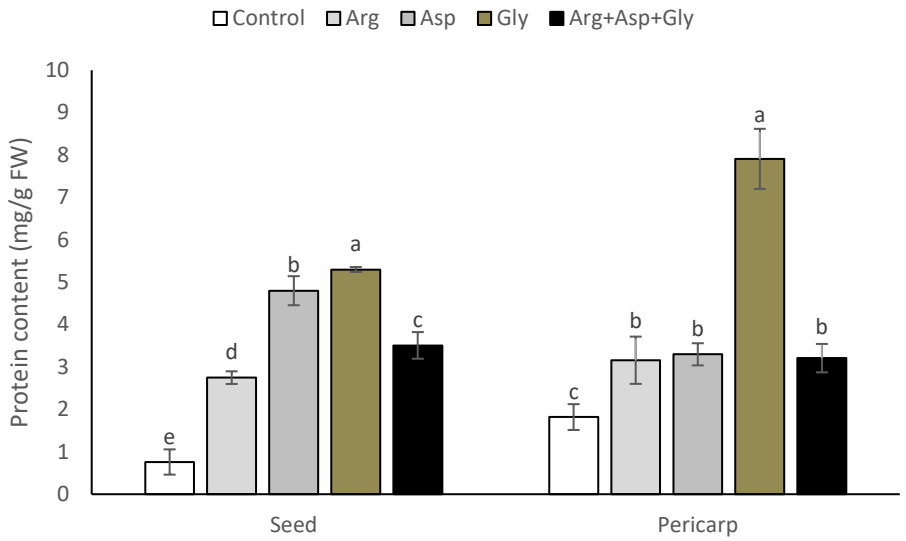

Fig. 5. Effects of 2mM arginine (Arg), asparagine (Asp), and glycine (Gly) on protein content in the seeds and pericarp of okra after storage at 4oC for 14 days. Bars with different letters are significantly different according to Duncan's multiple range test ($p<0.05$).

Total antioxidant capacity

According to the results of this research, individual applications of asparagine, glycine, and arginine had different effects on total antioxidant capacity (TAC) in the seeds and pericarp of pods. As compared to the control, all of the amino acids caused an increase in TAC (46-77%) in the pericarp of pods, although only asparagine (36.40 %) and Arg+Asp+Gly (21.08 %) increased the TAC in the seeds of the treated pods (Fig. 6).

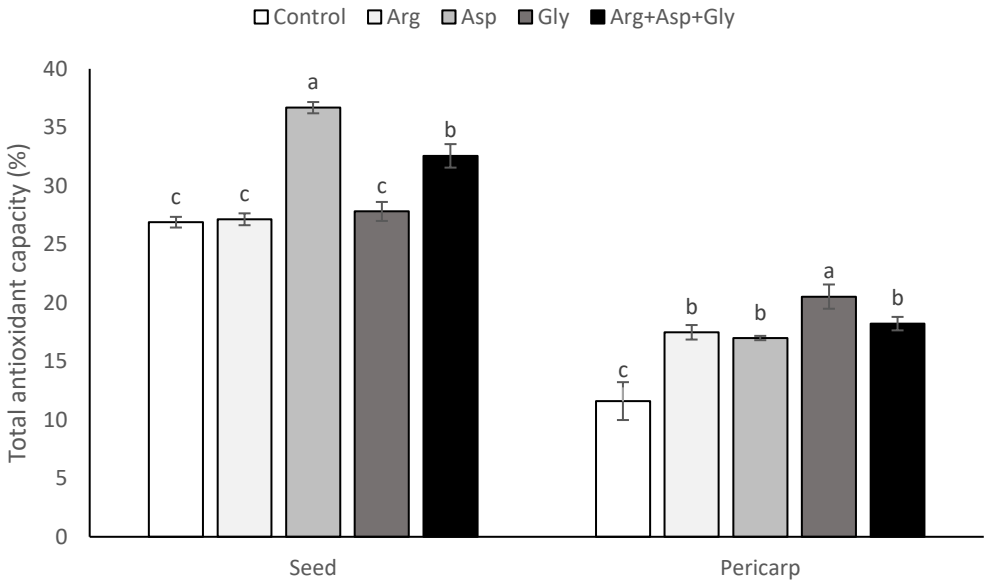

Fig. 6. Effects of 2mM arginine (Arg), asparagine (Asp), and glycine (Gly) on total antioxidant capacity in the seeds and pericarp of okra after storage at 4oC for 14 days. Bars with different letters are significantly different according to Duncan's multiple range test ($p<0.05$).

Antioxidant enzymes activities

In comparison with the control, okra pods treated with arginine, asparagine, and glycine bore seeds that showed significant inductions in POD activity (64.11, 85.88, and 115.78 %, respectively) (Table 1). Interestingly, okra pericarps treated with glycine showed improvements in POD activity by 13.71%, compared to the control (Table 1). In other words, glycine significantly raised CAT activity in the seeds of okra pods by 25.19%, compared to the control. Meanwhile, CAT activity in the pericarp of treated pods improved by 16.44, 33.33 and 18.66 % as a result of asparagine, glycine, and Arg+Asp+Gly treatments, respectively, compared to the control (Table 1).

Table 1. Effects of 2mM arginine, asparagine, and glycine on enzyme activities in the seeds and pericarp of okra after storage at 4oC for 14 days.

Enzyme activity (Units/ mg protein)	Okra tissue	Control	*Arginine*	*Asparagine*	*Glycine*	*Arginine+Asparagine+Glycine*
Peroxidase	Seed	4.18^{d}	6.86^{bc}	7.77^{ab}	9.02^{a}	5.58^{cd}
	Pericarp	17.21^{bc}	16.17^{bc}	18.33^{ab}	19.57^{a}	15.12^{c}
Catalase	Seed	1.27^{b}	1.36^{b}	1.21^{b}	1.59^{a}	1.38^{b}
	Pericarp	2.25^{c}	2.36^{bc}	2.62^{b}	3.00^{a}	2.67^{ab}
Polyphenol oxidase	Seed	19.64^{a}	17.48^{ab}	15.83^{b}	9.68^{c}	11.64^{c}
	Pericarp	21.03^{a}	20.87^{a}	11.60^{b}	12.21^{b}	11.12^{b}

Values with different letters in the same row are significantly different according to Duncan's multiple range test ($p<0.05$).

Polyphenol oxidase activity

According to the effects of amino acid treatments on the PPO enzyme activity of okra seeds and pericarp (Table 1), during the postharvest period, the PPO enzyme activity decreased by 19.39, 50.71 and 40.73 % in okra seeds treated with asparagine, glycine, and Arg+Asp+Gly, respectively. However, glycine-treated seeds had lower levels of PPO activity than the control group (Table 1). The applications of asparagine, glycine, and Arg+Asp+Gly caused remarkable reductions (44.84, 41.94, and 47.12%) in the PPO activity, respectively, compared to the control (Table. 1).

Discussion

Chilling stress usually generates reactive oxygen species (ROS) responsible for oxidative damage and the breakdown of cell membrane integrity, which ultimately results in water loss from crops (Maalekuu et al., 2006). In this context, storing tropical and subtropical crops such as okra at low temperatures often results in physiological disorders such as pitting, translucency, and browning lesions in their tissues (Finger et al., 2008; Huang et al., 2012). Under chilling, the browning of tissues often occurs because of poly phenoloxidase activity, by which phenols are oxidized into o-quinones (Queiroz et al., 2008). Browning lesions are symptoms of chilling injury that have been observed in okra fruits in cold storage (Huang et al., 2012). The results of this study showed that pretreating the fruits with arginine, asparagine, and glycine mitigated the harmful effects of CI in postharvest storage. Our findings are in agreement with Li et al. (2019b) who found that pre- and post-harvest applications of glycine betaine on cherry, stored at 0°C for 4 weeks, weakened the occurrence of different postharvest disorders such as weight loss, peduncle browning, pitting, and fruit decay. In papaya, glycine betaine also maintained fruit quality by alleviating the deleterious effects of CI in cold storage (Pan et al., 2019). By binding to the cell membrane, amino acids are believed to inhibit water loss from the pericarp, and this may reduce the extent of the CI in cold storage.

ROS are significantly accumulated under environmental stress, and they could inflict damage to the cellular membrane and peroxidase membrane lipids (Davey et al., 2005). The lipid peroxidation that results from chilling is measured using MDA accumulation, and the amount of MDA accumulation in stressed tissues significantly and positively correlates with the severity of chilling damage in cold storage (Babalar et al., 2018). In this context, Wang et al. (2017) demonstrated that arginine could modulate both MDA accumulation and senescence of green asparagus spears in cold storage. The positive effects of amino acids on decreasing MDA content have previously been reported, regarding the role of MDA in improving cell membrane integrity (Babalar et al., 2018; Li

et al., 2019a). Amino acids could stimulate antioxidant activity and decrease MDA accumulation to minimize damage to the cell membrane. As a result, these substrates could induce plant tolerance to CI (Wang et al., 2017; Zheng et al., 2017).

Phenolic compounds are plant-derived secondary metabolites biosynthesized through three different biosynthetic pathways, and their amounts in plant cells usually depend on different environmental stresses (Bhattacharya et al., 2010). It has been documented that phenols generally accumulate in chilling-stressed plants (Babalar et al., 2018). Phenols act as defensive compounds to induce tolerance to biotic and abiotic stresses (Minatel et al., 2017). Furthermore, phenolic compounds can restrict peroxidation due to their antioxidant property, which reduces membrane fluidity in favor of delaying the distribution of free radicals throughout the tissues of chilling-stressed plants (Blokhina et al., 2003; Minatel et al., 2017). Li et al. (2019a) demonstrated that arginine increased the total phenolic content in white mushrooms during storage. Furthermore, previous studies have shown that arginine could improve total phenol content and qualitative traits in asparagus (*Asparagus officinalis*) (Wang et al., 2017) and pomegranate (Babalar et al., 2018) in cold storage. This suggests the positive role of arginine in enhancing secondary metabolite production to enable plants to cope with CI.

At the postharvest stage, the synthesis of proteins in plants usually reduces as the period of their storage is extended, which is considered a senescence symptom in plants during stressful conditions (Meng, 2014). Li et al. (2019a) reported that arginine, during chilling, increased the protein content in white button mushrooms, which confirms the current findings. Also, it has been reported that glycine betaine could increase protein content in banana fruits during the postharvest stage in storage at low temperatures (Rodríguez-Zapata et al., 2017). Under chilling stress, an increase in the rate of proteins may be associated with enhanced protein synthesis in plant tissues to tolerate chilling conditions (Zhang et al., 2012). Thus, amino acids may decelerate the senescence process in plants and, consequently, may better maintain the quality of okra fruits in chilling conditions.

According to the results of this research, asparagine, glycine, and arginine significantly improved the TAC. Likewise, Wang et al. (2017) stated that the application of arginine enhanced TAC in asparagus to diminish ROS production and protect plants from stress. Also, arginine could augment antioxidant activity in pomegranate fruits to mitigate CI and extend their shelf life in cold storage (Babalar et al., 2018). This may be associated with accumulating phenolic compounds in the tissues with antioxidant features (Minatel et al., 2017). Therefore, a postharvest application of arginine, glycine, and asparagine may maintain pod quality in storage because of their impact on paving the way for antioxidant activity in the treated pods.

Exposing the sensitive-chilling plants to low-temperature conditions results in generating more free radicals and oxidative stress. To tolerate oxidative stress, plants often activate their defensive antioxidant systems (Ghanbari and Sayyari, 2018). Antioxidant enzymes such as catalase and peroxidase are essential components of defensive systems in plants responsible for alleviating damages that result from oxidative stress (Wang e al., 2016). During stressful conditions, these enzymes keep cellular hemostasis and protect cellular membranes from oxidative damage by neutralizing free radicals that result from chilling stress (Blokhina et al., 2003). As a result, an increase in the antioxidant activity of enzymes, as mentioned above, is considered an essential defensive response by plants to chilling stress. In this research, amino acids reinforced antioxidant activities in the seed and pericarp of pods. Previous studies demonstrated that amino acids can alleviate the harmful effects of environmental stress on tomatoes (Nasibi et al., 2011) and sweet pepper (Wang et al., 2016) by stimulating antioxidant activities in plants. It seems that amino acid-treated pods have a higher capacity for scavenging extra ROS that is generated during cold storage.

One of the most common symptoms of chilling in okra fruits, stored at low temperatures, is browning disorder (Phornvillay et al., 2019). It usually appears on fruit tissues due to poly phenoloxidase (PPO) enzyme activity, often compartmentalized in the plastids and with phenolic compounds located in vacuoles as a substrate (Queiroz et al., 2011). Previous studies have shown that PPO activity is usually amplified during low-temperature storage, resulting in plant sensitivity to chilling stress (Li et al., 2019). Therefore, a reduction in PPO activity could diminish the occurrence of browning disorder and, thus, contribute to the maintenance of fruit quality in cold storage (Queiroz et al., 2008). Babalar et al. (2018) found that arginine could reduce PPO activity and chilling injury in pomegranates during cold storage. Moreover, the activity of PPO on glycine betaine-treated fruits

and its relationship with emerging browning lesions have been documented in the literature (Rodríguez - Zapata et al., 2015). In the present study, we found a growing number of browning lesions in the seeds and pericarp of non-treated pods, which were accompanied by a rise in PPO activity. This indicated that amino acids can inhibit PPO activity in favor of controlling browning lesions and their rate of incidence at cold storage.

Conclusion

In the present study, arginine, asparagine, and glycine were able to maintain okra quality in cold storage (4°C) by hindering MDA accumulation and polyphenol oxidase activity and, conversely, enhancing TAC in the seeds and pericarp of the treated fruits. In many cases, a combination of arginine, asparagine, and glycine did not show a synergistic effect on improving pod quality, although only glycine individually acted better in this regard. In conclusion, amino acids, especially glycine, can alleviate chilling injury, suppress lipid peroxidation, and retain the green color of the pods to a certain extent under cold storage.

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Conflict of interest

The authors declare that they have no conflict of interest.

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