International Journal of Horticultural Science and Technology (2021) Vol. 8, No. 4, pp. 401-413

International Journal of Horticultural Science and Technology

Journal homepage: http://ijhst.ut.ac.ir

Improving of Winter Cold Hardiness by Glycine Betaine in Strawberry

Hassan Sarikhani* **and Mohammad-Sadegh Safariyan-Nejad**

Department of Horticultural Science, Bu-Ali Sina University, Hamedan, Iran

ARTICLE INFO

Article history:

Received: 14 October 2020
Received in revised form: 28 February 2021
Accepted: 6 March 2021

Article type:

Research paper

Keywords:

Cold stress,
Carbohydrate,
LT50,
Electrolyte leakage,
Proline,
Tetrazolium staining test.

ABSTRACT

One of the most important problems of strawberry cultivation in temperate regions is winter cold injuries. Current study investigated impacts of foliar application of glycine betaine (GB) at 0, 0.5, 1, 2 and 4 mM concentrations on the cold hardiness of strawberry. The plants were divided into two groups: one group for evaluation of cold hardiness at temperatures of -6, -9, -12, -15 and -18 ˚C; and the other for study of some biochemical characteristics. Results showed that GB treatment increased soluble carbohydrate and proline concentrations in both leaf and crown tissues, total protein concentration in leaf, and relative water content in leaf as compared to those in control. Based on LT_{50} calculated from electrolyte leakage and tetrazolium staining test, the GB application increased cold hardiness in strawberry plant based on its concentration. The highest cold hardiness was found in the 2 mM GB concentration based on electrolyte leakage and tetrazolium staining tests at -13.3 and -15.3 ˚C. Meanwhile, the lowest values of cold hardiness were observed in the control treatments based on electrolyte leakage and tetrazolium staining tests at -10.2 and -11.0 ˚C. Significant correlations were found between soluble carbohydrate and proline concentrations in leaf and crown, and total protein concentration with LT_{50} calculated from electrolyte leakage and tetrazolium staining test. We conclude that application of 2 mM GB has the capacity to increase the freezing tolerance of strawberry and could be used as a prophylactic tool to reduce winter cold injury.

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.), is one of the most important small fruits cultivated in many temperate regions of the world (Hancock, 1999). One of the main difficulties of strawberry cultivation in temperate regions is sporadic chilling injury at winter (Karami et al., 2018), which leads to huge economic loss during production season (Zhang et al., 2019; Hancock, 1999). Recently, due to global warming and instability of snow cover in the temperate regions, strawberry vulnerability to winter cold has increased (Lukoseviciute, 2014). Although hardiness of different cultivars varied, it was generally reported that, in unprotected conditions, the strawberry crowns were severely damaged below -9 ˚C; while acclimated plants injured

* Corresponding Author's Email: sarikhani@basu.ac.ir

below -12 to -15 ˚C (Galletta and Himmelrick, 1990; Nestby and Bjorgum, 1999; Maughan et al., 2015).

Cold tolerance in plants is a complex process that is influenced by several factors such as temperature, day length, plant maturity, tissues water content , nutritional status, physiological age, and dormancy progress (Hincha and Zuther, 2014; Ouellet and Charron, 2013). There are various strategies to deal with cold stress including selection of cold tolerant genotypes, breeding of new resistance genotypes, site selection, and use of proper agricultural practices (Sarikhani et al., 2014; Iba, 2002). In addition, practice of chemicals and plant growth regulators could also increase cold hardiness (Sarikhani et al., 2014; Khandan-Mirkohi et al., 2017; Nasibi et al., 2020). There have been reports of successful use of glycine betaine (GB) in combating environmental stresses such as water, salinity, and cold stresses in plants (Demiral and Türkan, 2004; Chen and Murata, 2008; Aras and Esitken, 2013; Adak, 2019). It is one of the four compounds of ammonium that is synthesized in response to stresses in many plant species (Ashraf and Foolad, 2007). Exogenous use of GB in plants that lack or contain very little of it facilitates response to adverse effects of environmental stress (Yang and Lu, 2005).

GB levels in the leaves of strawberry plants increased during cold treatment, and reported to be effective in inducing cold tolerance in response to ABA and natural cold in strawberry (Rajashekar et al., 1999). Due to the increased concentration of GB in stress conditions, it seems that increasing its concentration through foliar application also increases the resistance to stress in plants. Various studies have been conducted on the use of GB for cold tolerance in plants. Application of GB during growing season in maize (Chen et al., 2000), tomato (Park et al., 2006) and strawberry (Aras and Esitken, 2013) decreased chilling injury. Aras and Esitken (2013) reported the survival of most strawberry bushes at -2.3 ˚C during the growing season after GB foliar application. Given the positive effects of GB on reducing the adverse effects of cold during the growing season on many plants and the threat of winter cold problem, studies targeting cold tolerance are of vital importance for developing strawberry cultivation in cold regions.

In this study, the effect of application of GB on winter cold hardiness of strawberry cv. Paros, as a short day cultivar and suitable for field conditions, was investigated. Paros is one of the popular strawberry cultivars, and as a semi-tolerant cultivar to winter cold injuries, it is widely planted under open-filed condition in temperate regions.

Materials and Methods

Plant material and experiment site

The study was carried out in the research center of Department of Horticultural Science, Bu-Ali Sina University, Hamedan, Iran in 2018 and 2019. In late September, the daughter plants of strawberry cv. Paros were cultivated in five-liter plastic pots containing an equal proportion of crop soil, sand, and decayed manure.

Application of GB

To prepare GB solutions, the required amount of GB (MW = 153.6) was dissolved in distilled water and brought to the desired volume. GB at concentration of 0.5, 1.0, 2.0, and 4.0 mM was sprayed twice on the plants foliage containing 0.05% v/v Tween-20. The first stage of treatment was done on 5th of October and the next round was repeated on 20th of October. For the control plants, distilled water containing Tween-20 was used. After application of treatment, the plants were divided into two groups: one group for evaluation of biochemical traits and relative water content, and the other for evaluation of cold hardiness with simulated cold. Biochemical traits were obtained 7- 10 days after the second treatment of GB during which

the plants were kept under field condition (Fig. 1). The cold-hardiness of canes was evaluated using a programmable freezing room. The starting temperature was 4 ˚C, and the freezer was programmed to reduce the temperature gradually by 3 ˚C h^{-1} to -6 ˚C, -9 ˚C, -12 ˚C, -15 ˚C, -18 ºC, then hold each temperature for 3 h. At the end of each temperature treatment, pots were taken out and the injury rates were evaluated using electrolyte leakage measurement and the tetrazolium staining test.

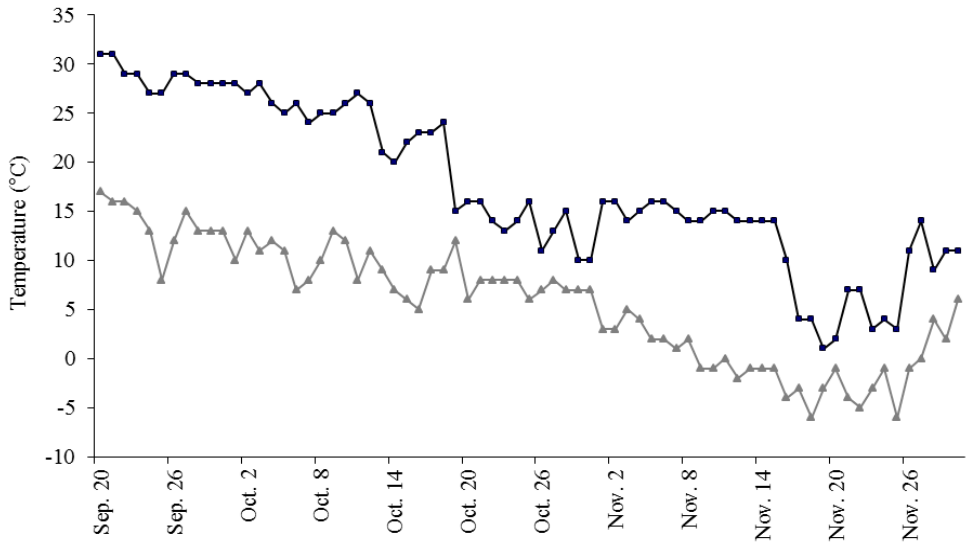

Fig. 1. Daily maximum (■) and minimum (▲) air temperatures at the research site during the experiment.

Chlorophyll concentration

The Porra et al. (1989) method was used to measure chlorophyll concentration in the leaf samples. Briefly, 0.2 g of fresh fully developed leaf tissue was homogenized in 5 mL of 80% acetone. The solution was centrifuged (Herolab model, Germany) for 5 min at 4000 rpm. After the supernatant was removed, the above steps were repeated twice with 80% acetone. The total supernatant volume brought to 20 mL. The absorbance of the samples was then measured by spectrophotometer (UV-1280, Shimadzu, Japan) at 645 and 664 nm. Chlorophyll concentration was calculated based on mg/g FW using the following equation:

$$\text{Total chlorophyll} = (17.76\ A_{645}) + (7.34\ A_{664})$$

Total soluble carbohydrate concentration

To measure the soluble carbohydrate content, the Paquin and Lechasseur (1979) method was used. For this purpose, 0.2 g of leaf tissue was carefully grounded using 5 mL of 95% ethanol in a mortar, and then the supernatant was separated. Later 5 mL of 70% ethanol was added to the previous residues. This procedure was repeated twice and the extract was centrifuged for 10 min at 4000 rpm. Next, 0.1 mL of the extract was mixed with 3 mL of freshly prepared anthrone (150 mg anthrone + 100 mL of 72% sulfuric acid). This solution was incubated in a hot water bath at 95 ˚C for 10 min, and its absorbance was read using a spectrophotometer (UV-1280, Shimadzu, Japan) at 625 nm. Finally, the concentration of soluble carbohydrate was determined by comparing with the standard curve of glucose based on mg/g FW.

Proline concentration

The Bates et al. (1973) method was used to measure proline concentration. First, 0.2 g of leaf tissue was grounded in liquid nitrogen in mortar, and then 10 mL of 3% sulfosalicylic acid (3 g of sulfosalicylic acid in 100 mL

distilled water) was added. The extract was later centrifuged at 4000 rpm for 10 min at 4 ˚C. Next, 2 mL of the supernatant was removed and poured into the test tube, giving it 2 mL of glacial acetic acid and 2 mL of freshly prepared ninhydrin (1.25 g ninhydrin + 30 mL of glacial acetic acid + 20 mL of 6 M phosphoric acid). Then, the samples were incubated in a hot water bath at 80 ˚C for one an hour. After that, the samples were kept on ice for 5 min, and then 4 mL of toluene was added to each sample before being shaken vigorously for 30 seconds. Finally, the absorbance of above phase was read by a spectrophotometer (UV-1280, Shimadzu, Japan) at 520 nm. To calculate the concentration, the absorbance of the samples was compared with the standard curve, and the final concentration of proline was calculated in mg/g FW.

Concentration of leaf soluble proteins

The Bradford (1976) method was used to measure soluble proteins in leaf sample. Briefly, 0.3 g of frozen leaf tissue was mixed with 6.25 mL of extraction buffer (for one liter, 121.14 g Trice dissolved in water and pH adjust 6.8 using 0.1 N HCl) and placed at 4 ˚C for 24 h. Then, the samples were grounded again in a mortar in buffer solution and centrifuged at 6,000 rpm for 20 min. Later, 100 µL of the supernatant was carefully removed and 5 mL of fresh Biuret reagent was added. To prepare the Biuret reagent, 100 mg of Coomassie Brilliant Blue G-250 was mixed with 50 mL of ethanol and brought to 800 mL volume with distilled water. Next, 100 mL of 85% phosphoric acid was added to it and final volume was reached 1000 mL with distilled water. Finally, the absorbance of the solution was read using a spectrophotometer (UV-1280, Shimadzu, Japan) at 595 nm, and the protein concentration was determined by bovine albumin standard curve.

Leaf relative water content (RWC)

This trait was measured according to Arndt et al., (2015) method. For this purpose, one square cm leaf pieces were selected from its healthy area (without the main veins) and weighed. Then, fragments were placed in a petri dish containing distilled water for 24 hours and stored at 4 ˚C in the refrigerator away from the illumination to allow for cell turgidity. They were later removed from distilled water, and let excess water drained using filter paper before being weighed. Next, the samples were placed in an oven at 72 ˚C for 48 hours and dry weights of specimens were measured. Finally, the RWC was calculated using the following equation:

RWC (%) = [(FW-DW)/ (TW-DW)] *100

Where FW = fresh weight, DW = dry weight and TW = turgid weight.

Electrolyte leakage

Electrolyte leakage of leaf and crown samples was performed according to the method of Bajji et al. (2002). According to this method, after washing the samples with distilled water, fresh and fully developed leaves/crowns were cut into one cm^2 pieces and placed in 25 mL distilled water. The specimens were then placed on a shaker at 100 rpm for 2 h, followed by storage at room temperature and dark for 24 h. Later, the primary electrolyte conductivity (EC_1) was read using an EC-meter (model 720, Inolab, Germany). Next, the samples were autoclaved at 120 ˚C for 20 min, cooled, and secondary electrolyte conductivity (EC_2) was measured. Finally, the electrolyte leakage of the samples was calculated using the following equation:

Electrolyte leakage (%) $= EC_1 * 100 / EC_2$

The lethal temperature (LT) at which 50% of total electrolyte leakage (EL) occurred in leaf and crown tissues ($EL\text{-}LT_{50}$) was calculated based on the method described by Andrews et al. (1984).

Tetrazolium staining test

First, 1% tetrazolium solution was prepared by dissolving 1 g of 2,3,5-triphenyltetrazolium

chloride in 100 mL distilled water. Cold temperature treated samples were analyzed for survival using tetrazolium staining test. The crown samples were immersed in the tetrazolium solution and kept at room temperature and dark for 24 h. Damage was assessed using Leica binocular (MS5, Heerbrugg, Switzerland) and based on the amount of tetrazolium staining of the tissues using a binominal scale as the followings: 1 = no damage, 2 = 100% phloem damage. The specimens that were red in the skin and phloem were considered alive and those lacking red in this area were considered as dead tissue, and then the damage was calculated using the following equation:

Damage rate (%) = damaged sample $\times$100/total no. of samples

The lethal temperature at which 50% of the crown tissues died estimated by tetrazolium staining test (T-LT_{50}) was calculated by fitting response curves.

Data analysis and experimental design

Data of electrolyte leakage and tetrazolium staining test were analyzed via a factorial experiment in completely randomized design with two factors including GB concentrations and low temperature treatment. In addition, data of T-LT_{50} and EL- LT_{50} was evaluated in a completely randomized design. The biochemical properties including total chlorophyll concentration, leaf soluble carbohydrate concentration, proline concentration of leaf and crown, leaf protein concentration and relative water content were also analyzed in a completely randomized design. Data were analyzed using SAS software (Version 9.1; SAS Institute, 2003) and the means were compared using Duncan's multiple range tests at the 5% level of significance. Correlation coefficients between biochemical properties and T-LT_{50} and EL- LT_{50} values were calculated using Pearson's correlation coefficient.

Results

Total chlorophyll concentration

According to the analysis of variance, the effect of GB treatment on total chlorophyll concentration was significant at 5% level (Table 1). The highest concentration of total chlorophyll was observed in 2 mM GB treated plants (3.12 mg/g FW). Also, the lowest total chlorophyll concentration was detected in 4 mM GB treatment with no significant difference with control and 0.5 and 1 mM GB treatments (Table 2).

Table 1. Variance analysis of glycine betaine effect on biochemical properties and RWC of strawberry

Sources of variance	DF	Mean of square					
		Total chlorophyll	Leaf soluble carbohydrate	Leaf proline	Crown proline	Leaf protein	Leaf RWC
GB concentration	4	0.506*	95.414**	4.940**	10.211**	1.591**	7.676**
Error	10	0.098	3.682	0.189	0.451	0.074	1.383
CV (%)	-	12.99	7.01	13.51	16.52	8.13	1.26

** and * significant at 1% and 5%, respectively.

Table 2. Effect of glycine betaine on biochemical properties and RWC of strawberry

Glycine betaeine concentration (mM)	Total chlorophyll (mg/g FW)	Leaf soluble carbohydrate (mg/g FW)	Leaf proline (mg/g FW)	Crown proline (mg/g FW)	Leaf soluble protein (mg/g FW)	Leaf RWC (%)
0 (control)	2.18b	21.73c	1.38c	1.59b	2.58c	94.8a
0.5	2.01b	23.54c	2.43b	2.63b	3.13b	94.7a
1	2.28b	31.27b	4.06a	5.19a	3.37b	93.7b
2	2.81a	35.14a	4.50a	5.81a	4.56a	92.1c
4	1.98b	25.12c	3.72a	5.12a	3.18b	91.1d

In each column, means with the same letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Leaf soluble carbohydrate concentration

According to the analysis of variance, the effect of GB treatment on leaf carbohydrate concentration was significant at 1% level (Table 1). The highest amount was observed in 2 mM GB (35.14 mg/g FW), and then in 1 mM GB (31.27 mg/g FW). The lowest leaf carbohydrate content was observed in control and there was no significant difference among control, 0.5 mM and 4 mM GB concentrations (Table 2).

Proline concentration of leaf and crown

The effect of GB treatment on leaf and crown proline concentration was significant at 1% level (Table 1). In both leaf and crown tissue, the highest proline concentration was observed in 2 mM GB treatment which showed no significant differences with 1 mM and 4 mM GB concentrations. The lowest leaf proline content was observed in control plants. The lowest amount of crown proline was observed in control plants with no significant difference from 0.5 mM GB (Table 2).

Leaf protein concentration

The effect of GB treatment on leaf protein concentration was significant at 1% level (Table 1). The highest amount was observed in 2 mM GB (4.55 mg/g FW) followed by 1, 4 and 0.5 mM GB, respectively, which showed no significant differences among them. The lowest leaf protein was also detected in control treatment (Table 2).

Relative content of leaf water

The effect of GB treatment on leaf RWC was significant at 5% level (Table 1). The highest amount of RWC was observed in control treatment (94.8%), which had no significant difference with 0.5 mM GB treatment. By increasing GB concentration from 1 mM to 4 mM, RWC reduced significantly (Table 2).

Electrolytic leakage of leaves and crowns

The effect of temperature, GB and their interaction on electrolytic leakage of leaf and crown were significant at 1% level (Table 3). In both leaf and crown samples, the lowest electrolytic leakage was observed in 2 mM GB treated plants under -6 ˚C; while the highest one was seen in control plants treated at -18 ˚C. In leaf sample, at -6 ˚C, with increasing GB concentration up to 2 mM, the electrolytic leakage decreased. Although no significant difference was observed at -9 ˚C between 1 and 2 mM GB treatments, at -12 ˚C the lowest electrolytic leakage was observed at 2 and 4 mM GB treatments. Moreover, at -15 ˚C the lowest electrolytic leakage was observed at 2 mM GB treatment (Fig. 2a). In crown samples at all treated temperatures, by increasing GB concentration up to 2 mM, electrolytic leakage decreased, while it increased subsequently at 4 mM GB (Fig. 2b).

The effect of GB treatment on leaf and crown EL-LT_{50} was significant at 1% level (Table 4). In both leaf and crown, the lowest EL-LT_{50} was observed in GB at 2 mM and then in 1, 4 and 0.5 mM GB, which showed significant differences. The highest EL-LT_{50} values were observed for leaf and crown tissues in control treatment (Table 5).

Crown injuries estimated from tetrazolium staining test

Analysis of variance revealed that the effect of temperature and GB, and their interaction on crown injury, estimated by tetrazolium test, were significant at the 1% level (Table 3). The highest percentage of crown survival was observed in the 2 mM GB treated plants under low temperature of -6 ˚C. However, the lowest percentage of crown survival was observed in control plants under -18 ˚C. In all treated temperatures, by increasing GB concentration up to 2 mM, crown survival rate was increased and then decreased in 4 mM GB treated plants (Fig. 2c).

According to the analysis of variance, the effect of GB treatment on T-LT_{50} value of crown was significant at 1% level (Table 4). Means comparisons showed the lowest T-LT_{50} value were: 2 mM GB followed by 1, 4 and 0.5

mM GB treated plants, with significant differences with each other. The highest T-LT_{50} value was observed in the control (Table 5).

Results showed a significant correlation between EL-LT_{50} and chlorophyll concentration at 5% level. Also, there were significant correlations between leaf EL-LT_{50} with leaf soluble carbohydrate, leaf proline, crown proline, leaf protein and relative water content. Also, significant correlations were found between EL-LT_{50} of crown with leaf soluble carbohydrate, leaf proline, crop proline, leaf protein, relative water content and leaf EL-LT_{50}. The results showed a significant correlation between T-LT_{50} and RWC at 5% level, as well. Meanwhile, significant correlations were found between T-LT50 and EL-LT_{50} of leaf sample with EL-LT_{50} of crown samples (Table 6).

Table 3. Variance analysis for the effects of glycine betaine and low temperature on strawberry leaf and crown electrolytic leakage and crown tetrazolium test

Sources of variance	DF	Mean of square		
		Leaf electrolytic leakage	Crown Electrolytic leakage	Crown tetrazolium test
GB concentration	4	1185.235**	1089.570**	2727.833**
Temperature	4	55.908**	59.335**	778.666**
temperature × GB concentration	16	34.345**	16.582**	113.75**
Error	50	0.446	1.679	10.333
CV (%)	-	1.42	2.54	6.44

** significant at 1%.

Table 4. Variance analysis of glycine betaine effect on leaf and crown EL-LT_{50} and T-LT_{50} of strawberry

Sources of variance	DF	Mean of square		
		Leaf EL-LT_{50}	Crown EL-LT50	T-LT_{50}
GB concentration	4	27.196**	22.346**	22.334**
Error	10	0.115	0.144	0.109
CV (%)	-	3.13	4.06	2.73

** significant at 1%

Table 5. Effect of glycine betaine effect on leaf and crown EL-LT_{50} and T-$LT_{50}$of strawberry

Glycine betaeine concentration (mM)	Leaf EL-LT_{50}	Crown EL-LT_{50}	T-LT_{50}
0 (control)	-9.6e	-10.2e	-11.0e
0.5	-10.3d	-10.8d	-12.8d
1	-13.2b	-12.1b	-14.2b
2	-15.8a	-13.3a	-15.3a
4	-11.3c	-11.4c	-13.6c

In each column, means with the same letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

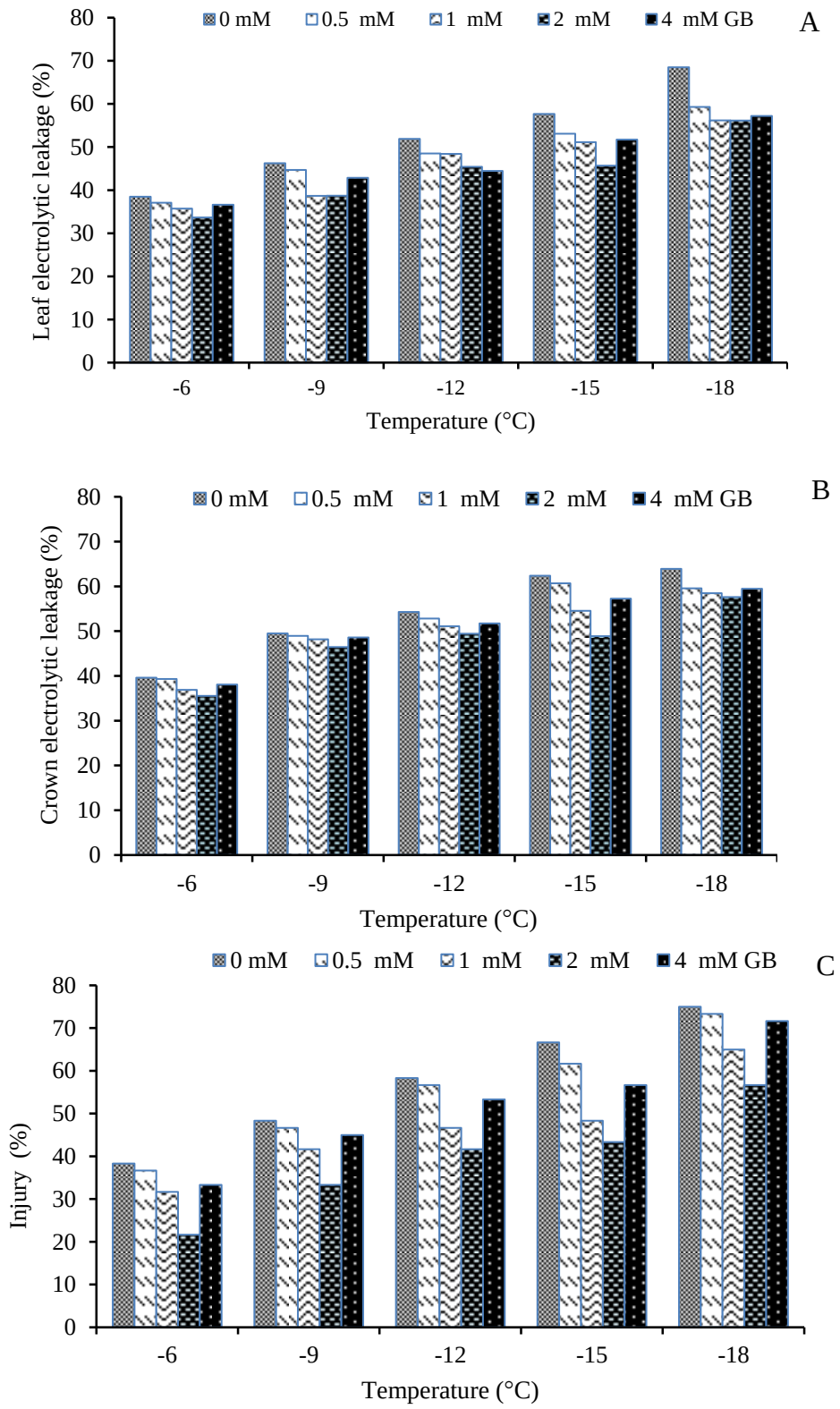

Fig. 2. Glycine betaine (GB) effect on leaf (A) and crown (B) electrolyte leakage and crown injuries (C) based on tetrazolium staining test under different low temperature in strawberry. In each part, mean followed by the same lower letter are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

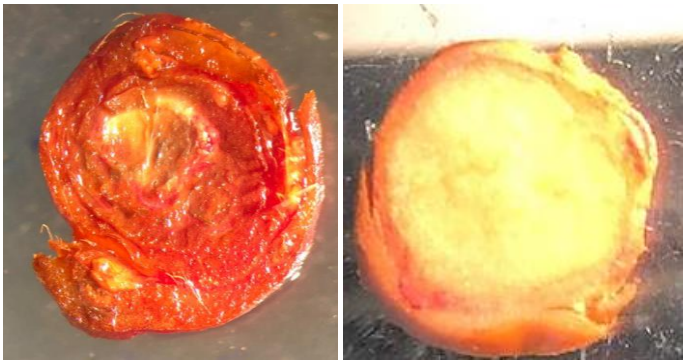

Fig. 3. Comparison of the viability of strawberry crown using tetrazolium staining test after cold treatment; Left: Crown staining belonged to the 2 mM glycine betaine without remarkable damage at -12 ˚C cold treatment, Right: the crown in this treatment showed defective staining indicating injuries.

Table 6. Pearson correlation coefficients between EL-LT_{50} and T-LT_{50}, and soluble carbohydrate, total protein, and proline concentrations and relative water content in strawberry following glycine betaine treatment

	Total chlorophyll	Soluble carbohydrate concentration	Leaf proline concentration	Crown proline concentration	Total protein concentration	RWC	Leaf EL-LT_{50}	Crown EL-LT_{50}
Leaf EL-LT_{50}	-0.563*	-0.933**	-0.916**	-0.855**	-0.790**	+0.657**	1	-
Crown EL-LT_{50}	-0.582*	-0.927**	-0.889**	-0.861**	0.866**	+0.675**	+0.974**	1
Crown T-LT_{50}	-0.673**	-0.952**	-0.816**	-0.817**	0.899**	+0.628*	+0.962**	+0.972**

**,* significant at $P \leq 0.01$ and $P \leq 0.05$, respectively.

T, tetrazolium stain test; EL, electrolyte leakage test.

Discussion

In the present study, the effect of GB on biochemical traits was significant. Increase in chlorophyll concentration after some GB treatments could occur for two reasons: increasing chlorophyll synthesis and preventing chlorophyll degradation. Exogenous GB treatment in strawberry (Aras and Esitken, 2013) and grapevine (Mickelbart et al., 2006) increased total chlorophyll concentration. Reduction of low temperature-degraded chlorophyll due to increased chlorophyllase activity (Meng et al., 2009) is another reason for the higher chlorophyll concentration in GB treated plants (Yildirim et al., 2015). In the present study, only the 2 mM GB increased total chlorophyll concentration and no significant difference was observed between the other treatments and those of control. Although high GB concentration increased chlorophyll content (Mickelbart et al., 2006), while no effect of 4 mM GB is odd. Moreover, low concentrations of GB had also no effect on increasing total chlorophyll.

There have been several reports of an increase in soluble carbohydrates in plants treated with GB (Ali and Ashraf, 2011; Weibing and Rajashekar, 2018). One of the main reasons for such increase seems to be related to the plant's need for carbohydrate intake for amino acid synthesis. Since GB contains nitrogen and its biosynthesis requires NADPH, it is costly for plants to consume soluble carbohydrates (Gorham, 1995). In this study, the concentration of soluble carbohydrate increased with rising concentration of GB from 0 to 2 mM but decreased in 4 mM treatment. Previous research reported increasing carbohydrate content under GB treatment in beans (Weibing and Rajashekar, 2018), tomato (Mickelbart et al., 2006) and maize (Ali and Ashraf, 2011).

The production and accumulation of osmotic preservatives occur in a wide range of plant species in response to environmental stresses such as drought, salinity, and cold (Fariduddin et al., 2011). Exogenous application of GB increases the accumulation of these molecules under stress conditions. Proline, as an amino acid, naturally accumulates in higher plants under stress conditions (Kavi Kishore et al., 2015; Hirt and Shinozaki, 2004). Proline is an osmotic potential regulator under stress conditions and stabilizes cellular structures (including cell membranes and proteins), eliminating free radicals, and neutralizing cellular redox potential (Irigoyen et al., 2010). GB synthesizes osmotic regulators such as proline and thereby induces resistance to stress (Chaum et al., 2006). In the present study, the proline content of leaf and crown elevated by increasing GB concentration, which was in agreement with the results of Farooq et al. (2008) and Chaum et al. (2006).

Foliar application of GB increased total protein concentrations in leaf. Protein accumulation as protective components of the cell can prevent the formation of ice crystals or slow down its formation (Cheng et al., 2004). The accumulation of soluble proteins (similar to the accumulation of soluble carbohydrates) is an adaptation factor reducing the damage caused by low temperatures and thereby increasing the freezing tolerance in plant tissue (Goldsmith, 2009; Guy, 2008). GB protects proteins and cell membranes in abiotic stresses. Sakamoto and Murata (2002) stated that the use of GB stabilized the quaternary structure of the protein complex under osmotic stress. Although GB improved the cold-stress tolerance, perhaps because of its cryoprotective or anti-freeze properties, the mechanism involved is still unknown (Wisniewski et al., 2003). Furthermore, the use of GB protects enzymes such as Rubisco (Makela et al. 1999), which is the most abundant protein of the leaf (Feller et al., 2008). In the present study, application of GB increased the total protein concentration compared to the control, but the highest amount was detected in 2 mM GB treatment. It is consistent with previous studies that used different concentrations of GB and reported elevated protein concentration (Sakamoto and Murata, 2002).

There are numerous reports of the effect of GB on maintaining relative water content under osmotic stress (Demiral and Türkan, 2004; Farooq et al. 2008; Weibing and Rajaskar, 2018). GB treatment increased the uptake or retention of water in leaves. Maintaining good water status is the most important factor in the usefulness of this amino acid in improving plant condition under osmotic stress. However, Demiral and Türkan (2004) reported a slight decrease in RWC in GB treated strawberry cultivar under normal condition. Increasing RWC by GB treatments was reported by Weibing and Rajaskar (2018) on green bean plant, which confirms findings of this study.

Studies have shown that the cell membrane is the first site of damage due to stress damage (Bajji et al., 2002). Cold stress changes the membrane state from crystal-liquid to solid-gel, thereby disrupts membrane activity (Park et al., 2006). In fact, ionic leakage shows cell membrane injuries (Bajji et al., 2002). Yang and Jian (2010) believed that cold stress increased the electrolyte leakage and the concentration of harmful oxygen compounds. The accumulation of these toxic compounds may lead to the peroxidation of cell membrane lipids and organelles, and may eventually cause physiological disturbance and cold stress injuries in plants. After low temperature damage to the membrane, the cell membrane permeability increases. Any factor that reduces the damage to the membrane and reduces electrolyte leakage can reduce the adverse effects of cold damage (Leul and Zhou, 1999; Bajji et al., 2002; Campos et al., 2003). There have been some reports on the application of GB on reduction of cold damage in plants. For instance, electrolyte leakage was significantly reduced in corn plants treated with GB under cold stress (Chen et al., 2000). The GB seems to protect and stabilize the membrane against intracellular material leaking out of the cell.

GB accumulates in different cellular segments to regulate osmotic pressure, thereby contributing to cell membrane stability.

High correlation between electrolyte leakage and tetrazolium staining was reported earlier by Sarikhani et al. (2014) and Okamoto et al. (2000). In his study, there was a close correlation between the cold resistance measured by electrolyte leakage and the tetrazolium method, which was consistent with the previous results. Results indicated that at lower temperatures, GB in 2 and 1 mM increased membrane viability and protection against cold damage. Tetrazolium staining is previously used to evaluate the cold resistance of various plants such as grapevine (Sarikhani et al., 2014). In this study, T-LT50 value of crown injuries was determined by tetrazolium test, the highest value of which was observed in 2 mM GB treatment with the highest 50% viability obtained at -15.8 ˚C. The lowest values were also observed in the control treatment at -9.6 ˚C. Among the concentrations of GB, the highest cold hardiness was observed in 2 mM treated plants followed by 1, 4 and 0.5 mM GB treatments.

The main activity of GB in relation to its effect on reducing the effects of cold stress can be related to its effect on antioxidant system of plant. Although GB by diverse mechanisms such as boosting antioxidant systems, accumulation of compatible solutes, improving preservatives and cell membrane stability can protect the cell and ultimately the plant, antioxidant systems was not investigated in the present study and can be recommended for further investigation.

Conclusion

Low temperature in winter severely restricts the strawberry survival. Application of GB augmented the synthesis of compatible solutes, improved the integrity of cellular membranes and enabled the strawberry plant to tolerate adverse effect of cold temperatures. Based on LT_{50} calculated from electrolyte leakage as well as tetrazolium staining test, GB especially at 2 mM concentration increased the cold hardiness in strawberry plant, which was correlated with applied GB concentration, while the lowest amount of cold hardiness was detected in control treatment.

Acknowledgements

This work was supported by the Bu-Ali Sina University (No. 970701).

Conflict of Interest

The authors declared no conflict of interest.

References

Adak N. 2019. Effects of glycine betaine concentrations on the agronomic characteristics of strawberry grown under deficit irrigation conditions. Applied Ecology and Environmental Research 17(2), 3753-3767.

Ali Q, Ashraf M. 2011. Exogenously applied glycine betaine enhances seed and seed oil quality of maize (*Zea mays* L.) under water deficit conditions. Environmental and Experimental Botany 71(2), 249-259.

Andrews P.L, Sandidge C.R, Toyama T.K. 1984. Deep supercooling of dormant and deacclimating *Vitis* buds. American Journal of Enology and Viticulture 35, 175-177.

Aras S, Esitken A. 2013. Effects of antifreeze proteins and glycine betaine on strawberry plants for resistance to cold temperature. International Conference on Agriculture and Biotechnology 60(21), 107-111.

Arndt S.K, Irawan A, Sanders GJ. 2015. Apoplastic water fraction and rehydration techniques introduce significant errors in measurements of relative water content and osmotic potential in plant leaves. Physiologia Plantarum 155 (4), 355-368.

Ashraf M.F, Foolad, M. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany 59(2), 206-216.

Bajji M, Kinet J.M, Lutts S. 2002. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regulation 36 (1), 61-70.

Bates L.S, Waldren R.P, Teare ID. 1973. Rapid determination of free proline for water-stress studies. Plant and Soil 39(1), 205-207.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of

protein utilizing the principle of protein-dye binding. Analytical biochemistry 72(1-2), 248-254.

Campos P.S, Quartin V, Chicho Ramalho J, Nunes MA. 2003. Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. Journal of Plant Physiology 160(3), 283-292.

Chaum S, Supaibulwatana K. Kirdmanee C. 2006. Water relation, photosynthetic ability and growth of Thai Jasmine rice (*Oryza sativa* L.) to salt stress by application of exogenous glycine betaine and choline. Journal of Agronomy and Crop Science 192(1), 25-36.

Chen T.H.H, Murata N. 2008. Glycine betaine: an effective protectant against abiotic stress in plants: review. Trends in Plant Science 13(9), 499-505.

Chen WP, Li PH, Chen THH. 2000. Glycinebetaine increases chilling tolerance and reduces chilling-induced lipid peroxidation in *Zea mays* L. plant. Cell and Environment, 23(6), 609-618.

Cheng L, Ma F, Ranwala D. 2004. Nitrogen storage and its interaction with carbohydrates of young apple trees in response to nitrogen supply. Tree Physiology 24(1), 91-98.

Demiral T. Türkan I. 2004. Does exogenous glycinebetaine affect antioxidative system of rice seedlings under NaCl treatment. Journal of Plant Physiology 161(10), 1089-1100.

FAO. 2018: FAOSTAT, Statistical Database. – Available: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor [25 December 2019].

Fariduddin Q, Yusuf M, Chalkoo S, Hayat S, Ahmad A. 2011. 28-homobrassinolide improves growth and photosynthesis in *Cucumis sativus* L. through an enhanced antioxidant system in the presence of chilling stress. Photosynthetica 49(1), 55-64.

Farooq M, Basra SMA, Wahid A, Cheema ZA, Cheema MA, Khaliq A. 2008. Physiological role of exogenously applied glycinebetaine to improve drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). Journal of Agronomy and Crop Science 194(5), 325-333.

Feller U, Anders I, Demirevska K. 2008. Degradation of rubisco and other chloroplast proteins under abiotic stress. Gen Applied Plant Physiology 34(1-2), 5-18.

Galletta G.J, Himmelrick DG, 1990. Strawberry management. In: Small Fruit Crop Management. Prentice Hall, Englewood Cliffs, NJ, pp. 83–156.

Goldsmith L.H.T. 2009. Freezing tolerance and dehydrin protein expression in Frontenac and Seyval blanc grapevine bark and xylem cane tissues during acclimation, midwinter, and deacclimation. Journal of Agronomy and Crop Science 187(2), 108-118.

Gorham J. 1995. Betaines in higher plants – biosynthesis and role in stress metabolism. In: Wallsgrove R. (Ed.), Amino Acids and their Derivatives in Higher Plants (Society for Experimental Biology Seminar Series, pp. 173-204). Cambridge: Cambridge University.

Guy C.L. 2008. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annual Review of Plant Biology 41(1), 187-223.

Hancock J.F. 1999. Strawberries. Wallingford, CABI. 237. p.

Hincha D.K, Zuther E. 2014. Introduction: Plant Cold Acclimation and Freezing Tolerance. In: Hincha D, Zuther E. (eds) Plant Cold Acclimation. Methods in Molecular Biology (Methods and Protocols), vol 1166. Humana Press, New York, NY

Hirt H, Shinozaki K. 2004. Plant responses to abiotic stress. Springer. Heidelberg. Germany. 300. P.

Iba K. 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annual Review of Plant Biology 53(1), 225-245.

Irigoyen J.J, Einerich D.W, Sánchez-Díaz M. 2010. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum 84(1), 55-60.

Karami F, Gholami M, Ershadi A, Sio-Se Mardeh A. 2018. Evaluation of winter cold tolerance and critical temperature (LT50) estimation in 21 strawberry cultivars. Iranian Journal of Horticultural Science, 49(1), 79-91. (in Farsi with abstract in English).

Kavi Kishor P.B, Hima Kumari P, Sunita M.S, Sreenivasulu N. 2015. Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. Frontier in Plant Science 6, 544.

Khandan-Mirkohi A, Khalili Halbi M, Salami S, Lesani H. 2017. Improving effects of mild cold stress and salicylic acid on growth and physiology of periwinkle (*Catharanthus roseus* Don.). International Journal of Horticultural Science and Technology 4(1), 67-78.

Leul M, Zhou W.J. 1999. Alleviation of waterlogging damage in winter rape by uniconazole application: effects on enzyme activity, lipid

peroxidation, and membrane integrity. Journal of Plant Growth Regulation 18(1), 9-14.

Lukoseviciute V, Rugienius R, Baniulis D, Savickiene N, Brazaityte A, Ruzgas V, Jariene E, Kupcinskiene E, Liobikas J, Slepetiene A. 2014. Characterization of cold acclimation and cold hardiness of strawberry *in vitro* and *in vivo*. Aleksandro Stulginskio Universitetas. 18(2), 182-199.

Makela P, Kontturi M, Pehu E, Somersalo S. 1999. Photosynthetic response of drought-and salt-stressed tomato and turnip rape plants to foliar-applied glycinebetaine. Physiologia Plantarum 105(1), 45-50.

Maughan T.L, Black B.L, Drost D. 2015. Critical temperature for sub-lethal cold injury of strawberry leaves. Scientia Horticulturae 183, 8-12.

Meng X, Han J, Wang Q, Tian S. 2009. Changes in physiology and quality of peach fruits treated by methyl jasmonate under low temperature stress. Food Chemistry, 114(3), 1028-1035.

Mickelbart M.V, Chapman P, Collier-Christian L. 2006. Endogenous levels and exogenous application of glycinebetaine to grapevines. Scientia Horticulturae 111(1), 7-16.

Nestby R, Bjorgum R., 1999. Freeze injury to strawberry plants as evaluated by crown tissue browning, regrowth and yield parameters. Scientia Horticulturae 81 (3), 321–329.

Okamoto G, Wang S, Hirano K. 2000. Cold resistance in root and cane of own-root Kyoho grapevines. Tree Physiology 89(1), 23-29.

Ouellet F, Charron J.B. 2013. Cold acclimation and freezing tolerance in plants. eLS, John Wiley and Sons, Ltd. Retrieved May 29, 2014, from http://onlinelibrary.wiley.com/doi/10.1002/9780470015902.a0020093.pub2/pdf/.

Paquin R, Lechasseur P. 1979. Observations sur une methode de dosage de la proline libre dans les extraits de plantes. Canadian Journal of Botany 57(18), 1851-1854.

Park E.J, Jeknic Z, Chen T.H. 2006. Exogenous application of glycinebetaine increases chilling tolerance in tomato plants. Plant and Cell Physiology 47(6), 706-714.

Porra R.J, Thompson W.A, Kriedemann P.E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta 975(3), 384-394.

Rajashekar C.B, Zhou H, Marcum KB, Prakash O. 1999. Glycine betaine accumulation and induction of cold tolerance in strawberry (*Fragaria* × *ananassa* Duch.) plants. Plant Science 148(2), 175-183.

Sakamoto A, Murata N. 2002. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant, Cell & Environment 25(2), 163-171.

Sarikhani H, Haghi H, Ershadi A, Esna-Ashari M, Pouya M. 2014. Foliar application of potassium sulphate enhances the cold-hardiness of grapevine (*Vitis vinifera* L.). Journal of Horticultural Science and Biotechnology 89 (2), 141-146.

Weibing X, Rajashekar C.B. 2018. Alleviation of water stress in beans by exogenous glycine betaine. Plant Science 148(2), 185-192.

Wisniewski M, Bassett C, Gusta LV. 2003. An overview of cold hardiness in woody plants: seeing the forest through the trees. HortScience 38(5), 952-959.

Xing W, Rajashekar CB. 2001. Glycine betaine involvement in freezing tolerance and water stress in *Arabidopsis thaliana*. Environmental and Experimental Botany 46(1), 21-28.

Yang X, Jian J.B. 2010. Study on cold hardiness testing of *Ginbian seris* almond by electrical conductivity. Journal of Shanxi Agriculture Science 3, 94-98.

Yang X, Lu C. 2005. Photosynthesis is improved by exogenous glycinebetaine in salt-stressed maize plants. Physiologia Plantarum 124(3), 343-352.

Yildirim E, Ekinci M, Turan M, Dursun A, Kul R, Parlakova F. 2015. Roles of glycine betaine in mitigating deleterious effect of salt stress on lettuce (*Lactuca sativa* L.). Archives of Agronomy and Soil Science 61, 1673-1689.

Zhang Y, Zhang Y, Lin Y, Luo Y, Wang X, Chen Q, Tang H. 2019. A transcriptomic analysis reveals diverse regulatory networks that respond to cold stress in strawberry (*Fragaria*×*ananassa*). International Journal of Genomics 61(1), 121-128.