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Foliar Application of Anti-frost Solution Influences Physiological and Biochemical Parameters in *Bougainvillea*, *Canna* and *Petunia*

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Abstract

Frost is the coating or deposit of ice that may form in cold conditions, usually overnight. If the temperature drops far enough for the plant cells to freeze, non-hardy plants will die. Therefore, a research was laid out to study the influence of an anti-frost solution on growth, flower quality and biochemical parameters of ornamental plants. The anti-frost solution was applied as foliar sprays in different concentrations of T0: 0 (mg L^{-1}) (only water) as control, T1: 250 (mg L^{-1}), T2: 500 (mg L^{-1}), T3: 1000 (mg L^{-1}), T4: 1500 (mg L^{-1}), T5: 2000 (mg L^{-1}) 'ferti-frost', on bougainvillea (*Bougainvillea glabra*), canna (*Canna indica*) and petunia (*Petunia axillaris*). Results showed that flowering percentage and flowering duration in the treated plants was higher than those in control. The highest flowering percentage and flowering duration were observed in T5 (81.22% and 13.86 days, respectively). By as anti-frost solution rate was increased, damaging percentage was decreased. The lowest damaging percentage of 69.77% was associated with T5. Total chlorophyll was enhanced with the rate of anti-frost solution. The highest total chlorophyll content belonged to T5 (50.80 mg g^{-1}). The comparison of the effect of different treatments on proline content revealed that control had highest proline content of 0.56 µM g-1. As anti-frost rate was increased, proline content was decreased so that it was minimized in T5 (0.08 µM g^{-1}). Electrical conductivity was affected by different treatments in all flowers. The highest electrical conductivity was recorded for control (9.0 mS g^{-1} cm^{-1}) and the lowest was related to T5 (2.26 mS g^{-1} cm^{-1}). All treatments differed significantly at the 0.1% level.

Keywords: Chlorophyll, Cold, Electrical conductivity, Proline.

INTRODUCTION

Beautiful flowers can increase the value of a landscape. It is important to plant flower bulbs and seeds in winter so that they can bloom in spring and summer. In this case, flowers such as begonia, calla, lily, tuberose, dahlia etc. could be used. Sudden freezing after prolonged hot weather, along with falling moisture, can inflict damage to young shoots and leaves which are not adapted to the cold.

Rapid changes in temperature are common in the deserts. Proper plant selection and crop management can prevent chilling injuries to plants. A major factor in the success of plantation in a new location might be its adaptability to frost, so injury or death resulting from frost conditions will be decreased. Most species show some degrees of low-temperature hardening and dehardening on an annual cycle (Larcher and Bauer, 1981).

Frost damage occurs when ice forms inside the plant tissue and injures the plant cells. It can occur in annuals (grasses, legumes, silage crops, cereals, horticultural and ornamental crops) and perennials (deciduous and evergreen trees). Frost damage may have a drastic effect on the entire plant or may affect only a small part of the plant tissue, which impairs the yield or merely product quality. The consequences of bud freezing injuries come in terms of quality and ornamental values. The objective of this study was to investigate the advantages of this new anti-frost solution and to introduce it to the society so that the frost tolerance of ornamental plants can be improved.

MATERIALS AND METHODS

The research was conducted at the Eram Botanical Garden Research Center in Shiraz, Iran to determine the influence of anti-frost solution on physiological and biochemical parameters of bougainvillea (*Bougainvillea glabra*) belonging to the Nyctaginaceae family, canna (*Canna indica*) belonging to the Cannaceae family and petunia (*Petunia axillaris*) belonging to the Solanaceae family.

Flowers were selected with the same size and conditions. Inter-row and within-plant spacing was 50 cm and 30 cm, respectively. The anti-frost solution was manufactured by Kimia Company, Shiraz. It was applied as foliar spray in October. Fifteen days later, the second foliar spray was replicated in November when the flowers were full bloom opened. A back-held spray pump was used for foliar application of the solution. After each treatment, the pump was washed thoroughly. Tap water was sprayed on the plants in the control treatment. All foliar spraying was carried out in the morning. The anti-frost solution was applied in different concentrations including T0: 0 (mg L^{-1}) only water as control, T1: 250 (mg L^{-1}), T2: 500 (mg L^{-1}), T3: 1000 (mg L^{-1}), T4: 1500(mg L^{-1}) and T5: 2000 (mg L^{-1}).

The anti-frost solution, commercially named "Ferti-Frost", consisted of salicylic acid, manganese, SLES (Sodium Lauryl Ether Sulfate), iron, antifoam ar30, copper, rusticyanin, zinc, amino acids, boron, potassium, and molybdenum.

Traits measurement

Flowering percentage

Ten days after the spray of the anti-frost solution on the plants, the number of flowers on each plant was counted and then calculated on the basis of percentage to show the effect of anti-frost solution on the flowers survival.

Flowering duration

After the anti-frost solution was sprayed on the plants, we monitored the longevity of flowers on each plant to show the effect of anti-frost solution on the flowering duration.

Damaging percentage

Plant health was another parameter that was assessed after the anti-frost solution was sprayed on the plants. The percentage of damaged part of each plant was recorded to show the

effect of the anti-frost solution on decreasing the damaging percentage.

Electrical conductivity

At the end of the experiment (10 days after spraying the anti-frost solution), the electrical conductivity was measured. Electrical conductivity was used to assess membrane permeability. Electrical conductivity was measured using an electrical conductivity meter (Swiss, Metrohm, 644 Conductometer) according to the standard procedure given by Blum and Ebercon (1981). In order to remove surface contamination, containers were rinsed with distilled water three times. Three plants per replicate were selected randomly and 10 discs were separated for each treatment. Leaf samples were placed in individual conical flasks containing 25 mL of distilled water. The samples were covered with aluminum foil and incubated at room temperature (25ºC) in a shaker (100 rpm) for 24 h. Electrical conductivity (EC1) of the bathing solution was determined after incubation. Then, samples were placed in an autoclave at 120ºC for 20 min and EC2 was determined after cooling the solutions to room temperature. Electrical conductivity was calculated as EC1/ EC2 and expressed as percent.

Proline

Amount of proline was measured by the standard procedure described by Troll and Lindsey (1955). 0.5 g of tissue was separated and homogenized in a pestle and mortar with 10 ml of 3% aqueous sulphosalicylic acid and filtered through Whatman No. 2 filter paper. The extraction was repeated and the filtrate was collected. Then, 2 ml of filtrate, 2 ml glacial acetic acid and 2 ml ninhydrin were added and mixed. Then, it was kept in boiling water bath for 1 hr and then, the reaction was terminated by placing in ice bath. 4 ml of toluene was added and mixed vigorously for 20-30 sec. The colored layer (toluene) was separated and warmed to room temperature. The absorbance of red color was measured at 520 nm against a reagent blank. Finally, the amount of proline was calculated in the sample using a standard curve prepared from pure proline and expressed on fresh weight basis of sample.

$$\text{Proline } (\mu\text{mol g}^{-1}) = (\mu\text{g proline/ml } \mu\text{g proline/ml} \times \text{ml toluene})/(115.5) \times 5/(\text{g sample})$$

where;

115.5 is the molecular weight of proline.

Chlorophyll

To estimate total chlorophyll, 1 g of finely cut and well mixed representative sample of leaf was weighed into a clean pestle and mortar. The tissue was ground to a fine pulp with the addition of 20 ml of 80% acetone and was centrifuged at 5000 rpm for 5 min. Then, the supernatant was transferred to a 100-ml volumetric flask. The residue was ground with 20 ml of 80% acetone and was centrifuged. The supernatant was transferred to the volumetric flask. This procedure was repeated until the residue was colorless. The mortar and pestle were washed thoroughly with 80% acetone and the clear washings were collected in the volumetric flask. The volume was made up to 100 ml with 80% acetone. The absorbance of the solution was read at 645, 663 and 652 nm against the solvent (80% acetone) blank.

Total chlorophyll in the extract was calculated using the following equation:

$$\text{Chlorophyll } (\text{mg g}^{-1}) = 20.2\,(A_{645}) + 8.02(A_{663}) \times V/(1000 \times W)$$

where;

A = Absorbance at specific wavelengths;

V = Final volume of extracted chlorophyll in 80% acetone;

W = Fresh weight of the plant tissue.

Experimental design

Laboratory tests were carried out at Agriculture University, Shiraz, Iran using a factorial

experiment based on a randomized complete block design with three replications. Analysis of variance (ANOVA) was carried out according to Panse and Sukhatme (1985). The data were analyzed using SAS software and means comparison was carried out by the Least Significant Difference (LSD) at the 0.01% level of probability (Steel and Torrie, 1960).

RESULTS

Proline, electrical conductivity and damaging percentage were decreased and total chlorophyll, flowering percentage and flowering duration were increased in all three plant species with the increase in anti-frost concentration. In our experiment, it was shown that the effect of varieties was significant on all parameters at the 0.1% level of significance, except for proline and electrical conductivity which showed non-significant differences. Effect of anti-frost concentration was significant on all parameters at the 0.1% level of significance. Interaction effect of plant species and anti-frost rate was not significant, except for the damaging percentage (Table 1).

Table 1. Analysis of variance for the studied traits

S.o.V	df	Proline	Electrical conductivity	Chlorophyll	Flowering percentage	Flowering duration	Damaging percentage
Block	2	0.02983 ^{ns}	9.82260***	77.23174 ^{ns}	1429.018***	1.692 ^{ns}	6.463 ^{ns}
P	2	0.00206^{ns}	1.47388 ^{ns}	1214.57861***	635.796***	433.896***	1898.685***
A.F.D.	5	0.25160***	54.24415***	1421.61113***	1425.363***	44.3296***	717.9296***
P × A.F.D.	10	0.00571^{ns}	0.39721^{ns}	46.09994^{ns}	32.730 ^{ns}	4.7206 ns	34.441***
Error	34	0.01164	0.49316	33.27054	67.156	4.900	34.365 ^{ns}
CV (%)		42.21174	12.30595	16.51588	12.49358	21.17576	7.255482

***, ^{ns} = Significance at 0.1 and non-significance, respectively. P: Plant, A.F.D: Anti-frost dose

Flowering percentage

Our experiment showed that canna had the highest amount of flowering percentage (71.99%) and petunia recorded the lowest amount (60.16%). Difference in the flowering percentage in all three varieties was significant at the 0.1% level of significance (Fig. 1).

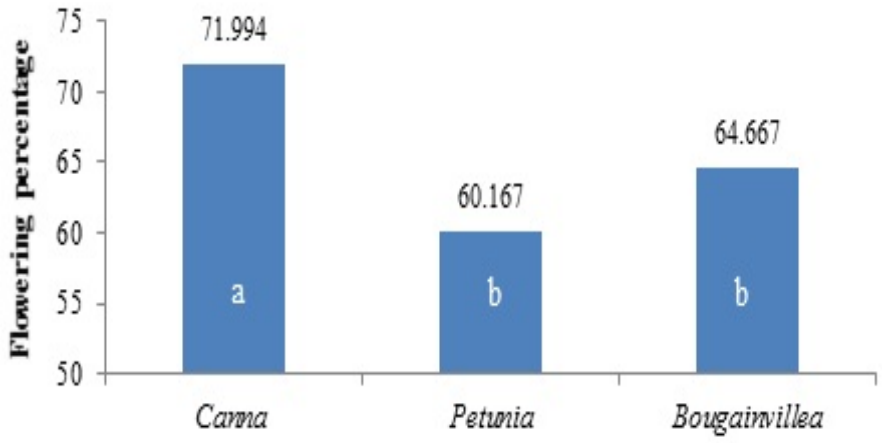
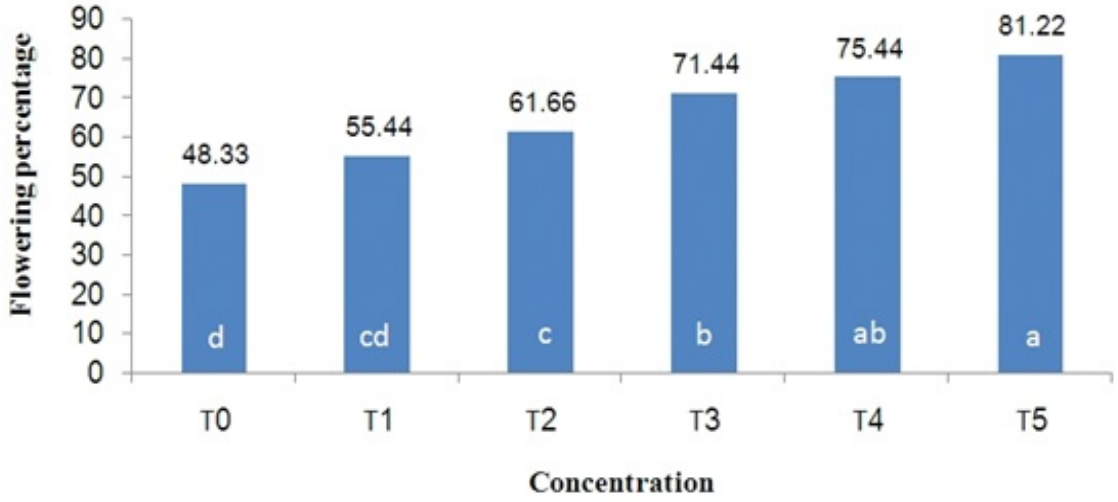


Fig.1. The effect of plant species on flowering percentage.

Flowering percentage was enhanced with the increase in the concentration of anti-frost solution. The highest flowering percentage was related to T5 (81.22%) and T4 (75.44%), respectively. The difference in all treatments was significant at the 0.1% level of significance (Fig. 2).



T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000 (mg L^{-1})

Fig.2. The effect of anti-frost concentration on flowering percentage.

Flowering duration

Results showed that canna had the longest flowering duration (14.0 days) and petunia had the shortest (4.85 days). The studied varieties significantly differed in the flowering duration at the 0.1 % level of significance (Fig. 3).

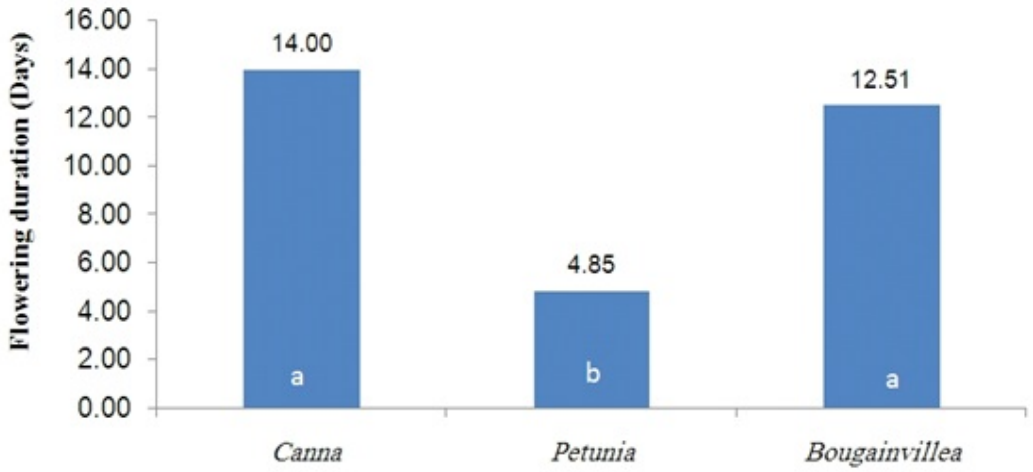
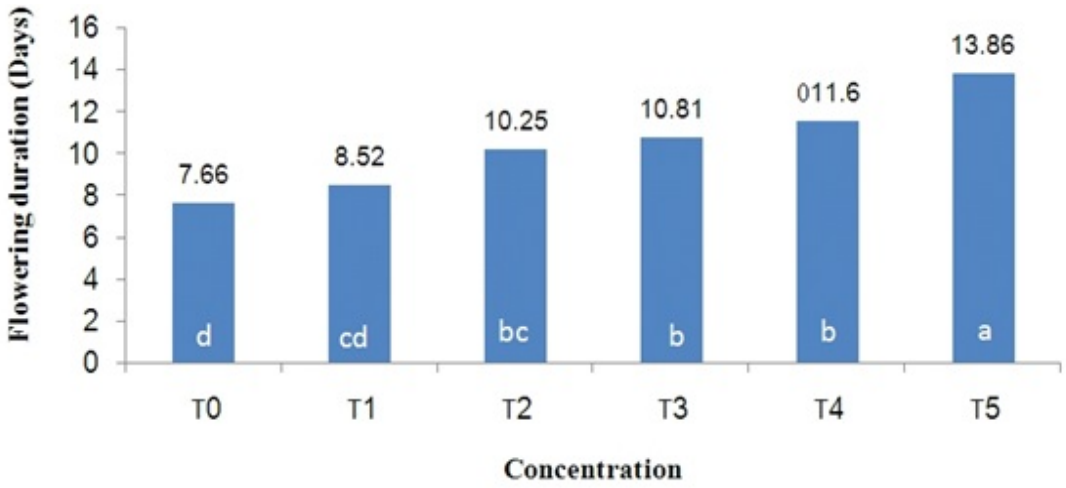


Fig.3. The effect of plant species on flowering duration.

Flowering duration was extended as the rate of anti-frost solution was increased. The highest flowering duration was associated with T5 (13.86 days) and T4 (11.6 days), respectively. The difference of all the treatments was significant at the 0.1% level of significance (Fig. 4).



T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000 (mg L^{-1})

Fig.4. The effect of anti-frost concentration on flowering duration.

Damaging percentage

Our experiment showed that canna had the lowest damaging percentage (74.0%) and petunia exhibited the highest amount (92.61%). Damaging percentage was significantly different among all three varieties at the 0.1% level of significance (Fig.5).

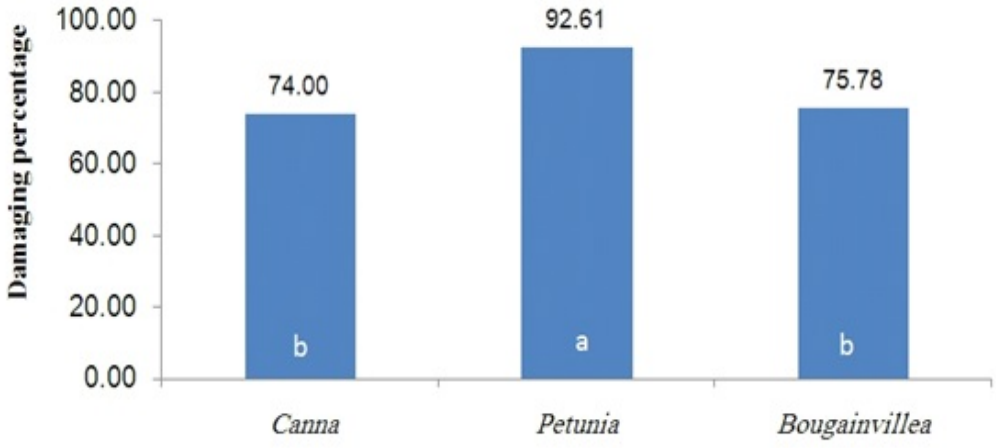
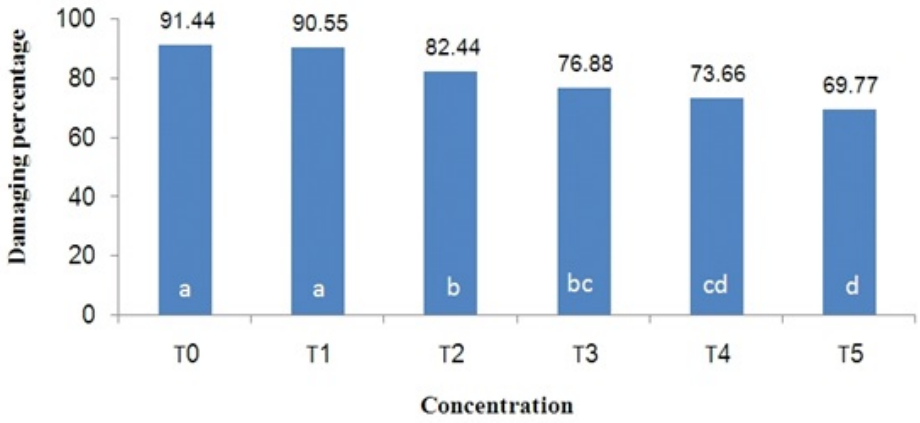


Fig. 5. The effect of plant species on damaging percentage.

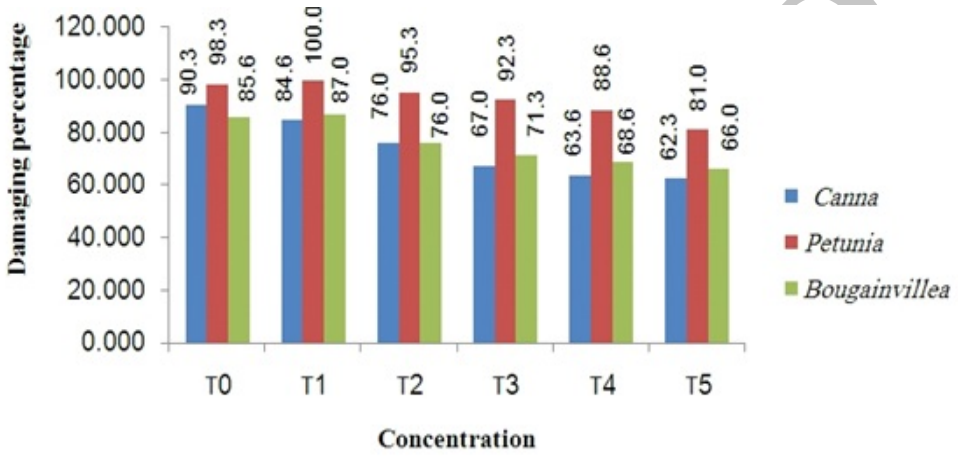
Damaging percentage was alleviated with the increase in anti-frost solution rate. The lowest damaging percentage was observed in plants treated with T5 (69.77%) and T4 (73.66%), respectively. The difference of all the treatments was significant at the 0.1% level of significance (Fig. 6).

Interaction effect of anti-frost concentration and plant species on damaging percentage showed significant differences at the 0.1% level of significance. We observed the lowest amount at T5 in canna (62.33%), but petunia recorded the highest amount in the same treatment (81.00%) (Fig. 7). With the increase in the concentration of anti-frost solution, damaging percentage was decreased (Fig. 6).



T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000 (mg L^{-1})

Fig.6. The effect of anti frost concentration on damaging percentage.



T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000 (mg L^{-1})

Fig.7. The interaction effect of plant species and anti-frost concentration on damaging percentage.

Chlorophyll

It was revealed that petunia had the highest total chlorophyll (43.60 mg g^{-1}) and canna recorded the lowest (27.27 mg g^{-1}). The difference in total chlorophyll in all three varieties was significant at the 0.1% level of significance (Fig. 8).

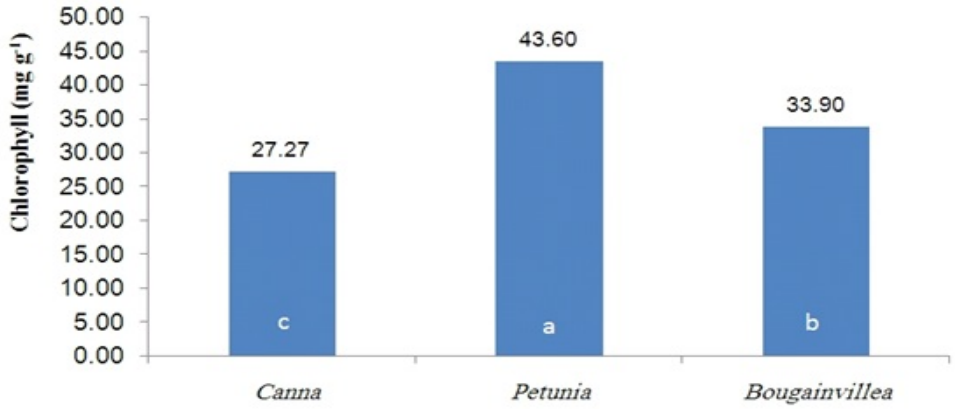
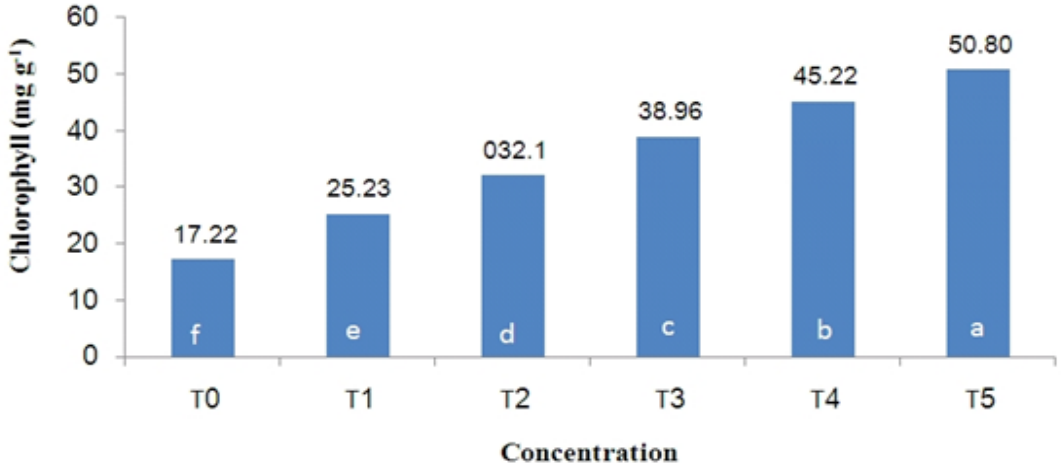


Fig.8. The effect of plant species on total chlorophyll.

Total chlorophyll showed an ascending trend with the increase in the concentration of anti-frost solution. The highest total chlorophyll was observed in plants treated with T5 (50.80 mg g^{-1}) and T4 (45.22 mg g^{-1}), respectively (Fig. 9).



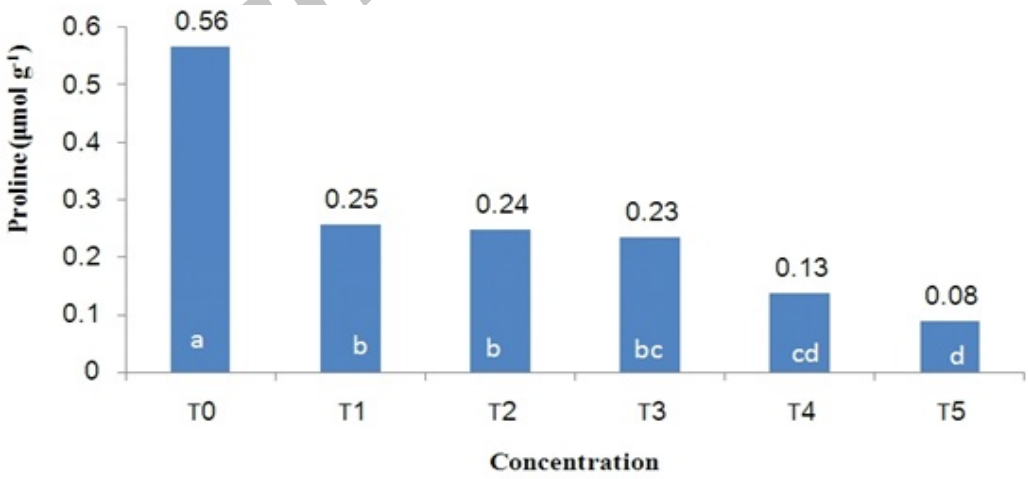
T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000(mg L^{-1})

Fig. 9. The effect of anti-frost concentration on total chlorophyll

Proline

Means comparison for all three plants showed that bougainvillea had the highest proline content (0.26 µmol g^{-1}) and canna showed the lowest one (0.24 µmol g^{-1}). The difference in proline content in all three varieties was non-significant at the 0.1% level of significance.

The comparison of the effect of different treatments on proline content showed that control (only water) had the highest proline content (0.56 µmol g^{-1}) followed by T1 (0.25 µmol g^{-1}). With the increase in anti-frost solution rate, proline content was decreased and T5 recorded the lowest amount (0.08 µmol g^{-1}) (Fig. 10).



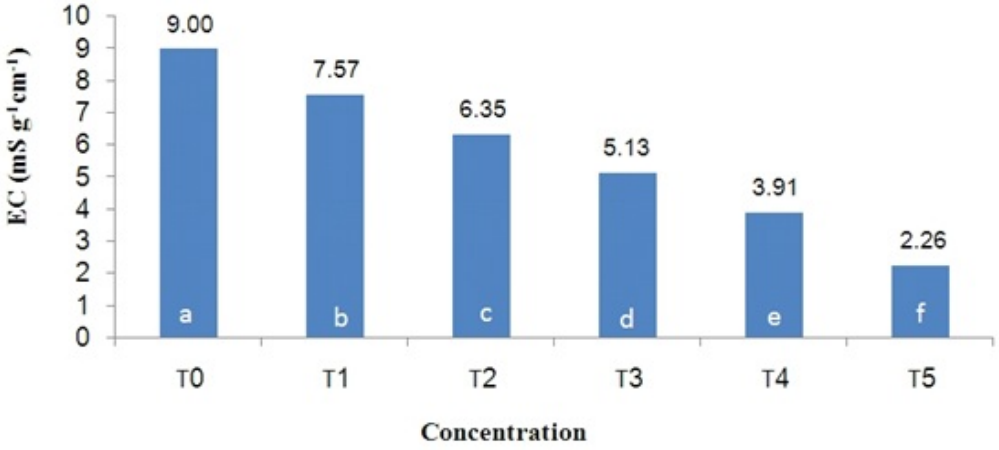
T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000 (mg L^{-1})

Fig.10. The effect of anti-frost concentration on proline content

Electrical conductivity

All three varieties were different in the amount of electrical conductivity. Petunia recorded the highest amount (5.91 mS g^{-1} cm^{-1}) and *bougainvillea* recorded the lowest amount of electrical conductivity (5.38 mS g^{-1} cm^{-1}).

The results revealed that electrical conductivity was affected by different concentrations of anti-frost solution and all treatments showed significant differences in the amount of electrical conductivity. The highest amount of electrical conductivity was recorded for control (9.0 mS g^{-1} cm^{-1}) and the lowest was 2.26 mS g^{-1} cm^{-1} associated with T5 (Fig. 11).



T0: 0, T1: 250, T2: 500, T3: 1000, T4: 1500, T5: 2000 (mg L^{-1})

Fig.11. The effect of anti-frost concentration on electrical conductivity

DISCUSSION

Johnson *et al*., (1988) reported that people can make a difference in the amount of winter damage and finally trees and shrubs sustain the cold. Gardeners can prevent winter injury to their perennial plants by some methods. For example, they can select cold-hardy trees and shrubs which are adapted to inorganic, alkaline soils and dry climate, break up caliche layers and hardpans prior to planting, amend light and heavy soils with organic matter before planting, do mulching around the base of plants, locate plants to avoid freezing, drying, winter winds and direct sun, provide winter protection, maintain plants in good vigor during the growing season, replenish soil moisture before the ground freezes, not excessive irrigation after the plants dormant, pruning or using fertilizer late in the season and remove damaged and diseased plant parts immediately to prevent invasion by insects and diseases. Anti-frost solution is a new method, which has not been used yet. It is a new invention that prevents winter damage to the plants.

A major factor in determining the success of a plant in a new location may be its ability to harden and withstand frost damage and hence avoid injury or death resulting from low-temperature frost conditions. Most species show some degrees of low-temperature hardening and dehardening on an annual cycle (Larcher and Bauer, 1981). Frost damage occurs when water freezing happens inside the cells. So, cells die and symptom of brown tissue occurs (Schuch *et al*., 2008). Evergreen broad-leaved species from northern zones are more tolerant than other species (Stanley and Warrington, 1988). Many plants are sensitive to coldness. For example, Pagter and Williams (2011) reported that the buds of *Hydrangea macrophyllaare* are more sensitive to frost injury than stems

and the vulnerability of stems to frost injuries, caused by an unstable temperature regime, changes during winter. Olson and Steeves (1983) reported that damaged regions of the opening flowers appeared brown and shriveled presumably resulting in the subsequent drop of the flower in *Amelanchier alnifulia*.

The exotic sample of that is named compo frost, which is produced in Germany. They reported that the advantages of this solution include preventing valuable crops from frost damage due to its physiologically active ingredients and saving the grower from significant yield losses.

Our results indicated that total chlorophyll enhanced with the increase in the concentration of anti-frost solution. The leaf chlorophyll index was lower in freeze-damaged pecan trees compared to non-damaged trees (Wells, 2008). Frost hardening in *Pinus sylvestris* L. decreased total chlorophyll–protein and increased the amount of free, solubilized chlorophyll (Öquist and Strand, 1986). In the frost-sensitive cultivar (pink oleander), non-photochemical quenching (NPQ) and the maximum photochemical efficiency of photosystem II (Fv/Fm) were decreased after the same freezing treatment. Measuring chlorophyll fluorescence may provide a rapid method to assess freezing injury in oleander (Miralles-Crespo *et al*., 2011(. Cryoprotectant is a substance used to protect biological tissue from freezing damage (i.e. that due to ice formation). Arctic and Antarctic insects, fish and amphibians create cryoprotectants (antifreeze compounds and antifreeze proteins) in their bodies to minimize freezing damage during winter. Pollock (1986) reported that the potential selective advantages of the possession of fructan metabolism in different species are assessed with reference to possible roles as cryoprotectants in osmotic control and as storage carbohydrates whose metabolism can continue at low temperatures (0 to 5°C). Also, it has been reported that mechanisms to cold adaptation could be the same among kingdoms. In a recent review, Janská and colleagues summarized all the important metabolic changes occurring in cold acclimation, reinforcing the idea that the synthesis of cryoprotectant molecules is of vital importance (Guy *et al*., 2008).

At low temperature, a reprogramming of carbon metabolism was shown to result in a shift in partitioning of fixed carbon into sucrose rather than starch (Strand *et al*., 1997, 1999). Additionally, the overexpression of sucrose phosphate synthase leads to an improved photosynthetic activity and an increased flux of fixed carbon into sucrose associated with an increase in cold tolerance as compared to wild-type plants (Strand *et al*., 2003). Besides the finding that sucrose may act as a cryoprotectant for membrane systems in plants (Hincha *et al*., 2003), it may also serve as a substrate for the synthesis of other cryoprotective compounds, such as raffinose. Analyzing the cold acclimation potential of two genetically distinct *Arabidopsis* accessions, C24 and Columbia (Col-0), the basic cold tolerance and the capacity for cold acclimation were found to correlate with tissue raffinose concentrations (Klotke *et al.*, 2004). Recently, it was demonstrated that raffinose specifically acts to protect the photosystems located in the thylakoid membranes of plastids from damage during freeze-thaw cycles (Knaupp *et al.*, 2011).

Shin *et al.* (2000) and Kadam *et al.* (2013) reported that the number of days from bud breaking to flowering was increased and flowering duration was decreased with the decrease in temperature. The number of days to flower was primarily influenced by the temperature after the visible bud, which is consistent with our findings. We showed that flowering percentage and flowering duration were increased and the damaging percentage was decreased with the increased cold tolerance of the plants. It might, thus, be speculated that cytosolic sucrose accumulates rapidly after cold exposure, serving as a transient cryoprotectant for cellular membranes at early stages of cold exposure, while later it becomes replaced by a metabolically less critical compound, for example, raffinose. At this stage, sucrose in the cytosol would serve as a substrate for the synthesis of other cryoprotectants or has a regulatory role in cold acclimation. In this context, it is interesting that raffinose accumulation follows the rise in cytosolic sucrose (Nägele and Heyer, 2013). As sucrose and galactinol are the substrates for raffinose synthesis taking place in the cytosol (Peterbauer and Richter, 2001), the increase in sucrose in the cytosol might be considered a trigger for raffinose production. Raffinose could, then, be transported

across the chloroplast envelope, probably by an active transport mechanism as proposed by Schneider and Keller (2009), serving in the plastids as a cryoprotectant of the thylakoids (Knaupp *et al.*, 2011). However, both cytosolic and plastidial sucrose content were increased significantly at a faster pace than raffinose content, suggesting an additional transitory protective effect of sucrose for the thylakoids. This is difficult to test because of the simultaneous role of sucrose as substrate for raffinose synthesis. A likely reason for a substitution of sucrose by raffinose might be the reduced metabolic reactivity of raffinose and its marginal regulatory influence on primary carbon metabolism.

We showed that by increasing the amount of anti-frost solution, plants became more tolerant and total chlorophyll content was increased which is in agreement with Sing *et al.*, (2012) and Liu *et al.*, (2013) who reported that total chlorophyll content was increased in tolerant plants in cold conditions. By comparing the effect of different treatments on proline content, it was shown that control had the highest amount of proline and with the increase in the amount of solution, proline content was decreased. Our results confirm the findings of Esra *et al.*, (2010), Nobari *et al.*, (2012) and Saghfi and Eivazi (2014) who reported that with the increase in cold condition, proline content was increased. Electrical conductivity was affected by different treatments in all flowers and all treatments showed significant differences in the amount of electrical conductivity. Chilling-induced irreversible membrane damage, so the highest amount of electrical conductivity was recorded for control and the lowest amount belonged to T5. Our results were in agreement with Marbach and Mayer (1985), Cottee *et al.* (2007) and Campos *et al.* (2003) who reported that electrical conductivity was decreased with the increase in cold conditions.

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