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Effects of drought stress on cold hardiness of non-acclimated viola (*Viola* × *wittrockiana* 'Iona Gold with Blotch') in controlled conditions

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ABSTRACT

Predicted increases in winter temperatures may negatively impact plant survival by preventing maximal cold acclimation prior to cold temperatures. Accordingly, research is needed to identify strategies that may help promote cold hardiness and increase freezing tolerance of cold season ornamental plants. Therefore, the objectives of this research were to study the effects of drought stress on freezing tolerance of *Viola* × *wittrockiana*' Iona Gold with Blotch' during cold season under non-cold acclimating (20 ± 1 °C) conditions and examine the physiological and biochemical changes in plants in response to freezing temperature. After being grown in the greenhouse, plants were first subjected to different levels of soil water availability including control (90%), 70% and 50% field capacity (FC). Then, some traits including proline, carbohydrate and chlorophyll were determined. Finally, plants were transferred to the Thermo Gradient Freezer with six freezing temperatures (0, −3, −6, −9, −12, and −15 °C), as well as 20 °C as the control treatment. Electrolyte leakage (EL) after temperature treatments, plant survival percentage (SU) and some traits related to regrowth after recovery period were determined. Result showed that drought stress increased some factors such as carbohydrate, proline and chlorophyll. Electrolyte leakage significantly increased by lowering temperature and increased by 46% at 15 °C compared to control. Plant survival was significantly affected by treatments so that lowering the temperature to −15 °C caused total mortality in all the plants of irrigation treatments. Plants under 70% FC at 0 °C had the highest increased reproductive component. Number of leaf and leaf area peaked under the moderate water deficit (70% FC) conditions at 0 °C. Plants under 70% FC at 0 °C had the highest increases (87, 134, 90 and 101%, respectively) in dry weights of vegetative, reproductive, root, and total dry weights compared to control. Drought stress can increase freezing tolerance of viola depending on temperature regime.

1. Introduction

Climate changes are expected to cause great impacts on ecosystems worldwide. During the last 50 years, the greatest warming trends have been observed in winter season, and significant increases in both the occurrence and duration of winter warming have already been predicted (IPCC, 2014). Generally, predicted future climate change scenarios will lead to less than optimal cold acclimation conditions, leading to reduction in freezing tolerance and predisposition of plants to winter injury. Nevertheless, it is clear that the impacts of climate change on ornamental plant will be greatly influenced by how climate affects the rate of crop development, and hence the timing of crop growth (Craufurd and Wheeler, 2009).

Viola is the most important ornamental plant, and it is therefore interesting to study how it will perform in a changing climate in winter situation. Pansy is a biennial grown as an annual for mid-fall to late-spring color. Plant developmental rate is influenced primarily by temperature. The root system can survive the cold weather (Kafi and Ghahsareh, 2009). Change in climate causes drought stress in the winter, which is one of the most important abiotic factors adversely affecting growth, metabolism and yield of plants (Wang et al., 2003).

Many plants increase their freezing tolerance when facing cold and short day conditions, a phenomenon known as cold acclimation which is the process allowing plants to develop essential tolerance for freezing survival through multiple levels of biochemical and morphological changes (Yadav, 2010). Growing plants at higher temperatures results in de-acclimation, which reduces their resistance to chilling, and if this period is adequately long or the temperature is adequately high, growth is resumed (Thorsen and Höglind, 2010).

Therefore, it is necessary to identify strategies that may help improve freezing tolerance in ornamental plants. A number of studies found that the acclimation of plant to water stress increased freezing

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tolerance (Li et al., 2002; Chen et al., 1975; Anisko and Lindstrom, 1996). Drought tolerance in plants is dependent upon different strategies: dehydration escape or avoidance, and drought tolerance. In escape scenario, plants complete their life cycle before soil dehydration. Drought avoidance contains different strategies such as development of larger root system, decrease of leaf growth, synthesis of osmotically active protective compounds, growth stop and early stomatal closure for preserving leaf water content (Farooq et al., 2009; Lopes et al., 2011; Marok et al., 2013).

Hoffman et al. (2012) reported that exposing two perennial ryegrass (*Lolium perenne* L.) cultivars ('Buccaneer' and 'Sunkissed') to moderate drought stress caused an improvement in cold tolerance for Buccaneer, but had no significant effect on freezing tolerance of Sunkissed. Furthermore, drought preconditioning (DP) resulted in an increase in carbohydrate and proline contents depending upon cultivar, tissue, and temperature regime. In a different study, Rajashekar and Panda (2014) reported that low temperature and water stress contributed significantly to the induction of freezing tolerance. Water stress is a dominant factor in inducing freezing tolerance, contributing roughly to 56% of freezing tolerance acquired by natural cold acclimation in strawberry. Typical cold acclimation treatment of plants for two weeks enhanced their freezing tolerance by about 14 °C to −20.7 °C while the same treatment, in the absence of the accompanying water stress, increased their freezing tolerance only by 5 °C, indicating the importance of water stress during cold acclimation. Increase in both drought and freezing tolerance has been associated with the capacity to accumulate similar protective compounds including carbohydrates and amino acids that minimize the negative effects of desiccation (Hoekstra et al., 2001).

It is generally acknowledged that the ideal method to assess the frost tolerance of cultivars is to freeze whole plants and determine electrolyte leakage after freezing treatment, survival percentage, and dry weight by regrowth for up to three weeks (Olien, 1967; Levitt, 1980; Gusta et al., 2003). Damage to plant tissues due to cold conditions inhibits the activity of cell walls and electrolyte leakage from inside to the outside of cell. Cold damage turns cell membrane from crystal-liquid form to solid-gel forms which inhibit cell membrane activity (Carapetian, 2001).

Low tolerance to freezing temperature results in the reduction of survival percentage. Zhang et al. (2008) showed in ryegrass that freezing temperature can cause a reduction in survival percentage. Iles and Howard Agnew et al., 1993 found that dry weight of Chatterbox (*Heuchera sanguinea*) was affected by freezing. Lowering the temperature to −8 °C caused a severe reduction in plant dry weight, and further decrease in temperature up to −10 °C resulted in 48% decrease in dry weight compared to 0 °C. In a different study, Rashed Mohassel et al. (2009) found that reduction in leaf area and height of fennel (*Foeniculum vulgare*) seedlings were associated to the reduction of temperature. Pansies are perfect for planting in containers of all types, hanging baskets, window boxes and green space in winter, but changing temperature in winter can be a significant problem. The objective of this study was to determine if water stress is an essential component of cold acclimation in winter-flowering pansies or it induces freezing tolerance. We hypothesized that drought stress may improve freezing tolerance of *Viola* × *wittrockiana* in the absence of cold acclimation by increasing the production of protective compounds. In addition, to further characterize the role of the components of cold acclimation, plant responses to low temperature and water stress were examined.

2. Materials and methods

2.1. Plant material and drought regimes

Viola (*Viola* × *wittrockiana* 'Iona Gold with Blotch') seeds were provided by the Takii seed company. The seeds were sown in trays with coco peat and perlite mix. Following a four-week germination period in October, plants watered three times per week and fertilized weekly with full strength Hoagland solution (Hoagland and Arnon, 1950). After four weeks (3-leaf stage), five plants transferred to each pot (18 cm high and 7 cm in diameter) a mixture of garden soil, sand and rotted mature (2:1:1) and plants were grown natural photoperiod. The soil of the experimental site had a pH of 7.7 and an EC of 1 mmos/cm. In order to determine the effect of irrigation treatments on freezing tolerance under non-cold acclimating conditions (20 ± 1 °C) and, after the emergence of first flower in February, water stress treatments applied as follows: well-watered (90% FC) and drought stress (70 and 50% FC) for two weeks by gravimetric method (Campbell and Mulla, 1990). The *experiment* was consisted of 84 pots containing five plants. Twenty one pots were selected for measurement of traits after irrigation treatments. For measurement of carbohydrate, proline, chlorophyll and carotenoid, the top leaf tissues were collected from a minimum of 10 independent plants. For biochemical traits, each composed of three replications, were used for carbohydrate, proline, chlorophyll and carotenoid measurements. The rest of the plants were sampled for assessment of freezing tolerance.

2.2. Examined traits before freezing temperatures

2.2.1. Proline and carbohydrate

Proline and carbohydrate content were determined using the methods described by Bates et al. (1973) and McCready et al. (1950), respectively.

2.2.2. Chlorophyll and carotenoid

Chlorophyll and carotenoid were measured based on the method described by Arnon (1949). Leaves pigments were extracted by 80% acetone and the absorption rate of control samples was measured at wavelengths of 663, 645, and 470 nm by using spectrophotometer. The amounts of chlorophyll and carotenoid were then calculated based on the following formulae.

$$\text{Chlorophyll a} = [12.7(A663) - 2.69(A\ 645)] \times V/W \times 1000$$

$$\text{Chlorophyll b} = [22.9(A645) - 4.68(A\ 663)] \times V/W \times 1000$$

$$\text{Total chlorophyll} = [20.2(A645) + 8.02\ (A\ 663)] \times V/W \times 1000$$

$$\text{Carotenoid} = [1000A470 - 1.82(\text{chlorophyll a}) - 85.02\ (\text{chlorophyll b}]/198$$

2.3. Controlled freezing test

Freezing tolerance was determined based on whole plant survival. After drought stress, plants were transferred to Thermo Gradient Freezer with six freezing temperatures (0, −3, −6, −9, −12, and −15 °C), as well as non-frozen temperature as control (20 °C) in greenhouse. For each testing temperature, there were three replicates containing five plants. In order to obtain the desired temperature, the freezer was cooled down in a stepwise trend at a rate of 2 °C h^{-1}, and each testing temperature was maintained for 1 h. After adjusting each target temperature, plants were removed from the freezer and thawed for at least 24 h at 5 °C (Nezami et al., 2012a,b). After thawing, plants were placed in greenhouse at 20 ± 1 °C for around four weeks under natural photoperiod.

2.4. Measured traits after freezing temperatures

2.4.1. Electrolyte leakage (EL)

After freezing temperature, five leaves were removed from 5 plants and placed in tubes containing 40 ml distilled deionized water. Freezing-induced electrical conductivity (EC) of leaf leachate was measured on the following day using a solution analyzer (Cole-Parmer Instrument Co., Chicago). To determine potential EC, the samples were then autoclaved for 20 min at 121 °C to release the total electrolytes from the samples. After maintaining the samples at 21 °C overnight, EC of the leachate was measured. Percentage of EL for each plant was determined at each treatment temperature according to the following formula:

EL (%) = freezing-induced EC/ potential EC × 100 (Rajashekar et al., 1982).

2.4.2. Morphological assay

The pots containing the other remained five plants were transferred to the greenhouse for 28 days and their regrowth was evaluated. Following regrowth period, whole plant survival (%) was calculated for each replicate as: (number of plants survived/total number of plants) × 100.

At the end of recovery period, the number of node on the main stem and the number of leaves and stolon per plant were counted. The plant height was evaluated in the laboratory using a measuring tape. Data on the number and diameter of flowers and buds have been measured throughout the experiment, but due to the large number of traits evaluated, only data on the end of the recovery period (full bloom) has been reported in this paper. All leaves were measured for leaf area and leaf area was determined by leaf area meter (LI-3000C). To determine dry matter content, harvested plants were dried in an oven for 72 h at 70 °C.

2.5. Statistical analysis

The first part of the study was performed in a completely randomized design with three replications. In the second part, data were arranged in 2-factor factorial experiment based on a completely randomized design, and analyzed using MSTAT-C software. Differences in means were compared by using the least significant difference (LSD) test at $p \leq 0.05$ level.

3. Results

3.1. Traits after irrigation regimes

Leaf proline content increased from 1.27 μmol g^{-1} FW in control to 2.02 μ mol g^{-1} FW in drought stress treatment (70% FC). Leaf proline content reached its highest concentration (2.41 μmol g^{-1} FW) in response to drought stress (50% FC) compared to all other treatments; this treatment resulted in 90% increase in leaf proline levels compared to control treatment (Table 1).

Carbohydrate content significantly increased in 50% FC compared to 90% FC (by 4%). Overall, drought stress resulted in an increase in carbohydrate accumulation compared to control treatment. Treatment of 70% FC did not exhibit significant difference from 50% FC treatment in terms of leaf carbohydrate accumulation (Table 1).

Drought stress also caused significant increase in chlorophyll and carotenoid (Table 1). Plants exhibited higher chlorophyll and carotenoid in response to 50% FC treatment compared to 90% FC. There were no differences in chlorophyll a in response to 70 and 50% FC treatments. In general, well-watering resulted in the reduction of chlorophyll a and b contents compared to 70 and 50% FC treatments. In contrast, plants exhibited significantly higher total chlorophyll in response to 50% compared to 90% FC. Carotenoid significantly increased (by 55%) in 50% FC compared to 90% FC (Table 1).

3.2. Traits after freezing test

3.2.1. Electrolyte leakage (EL %) and plant survival (SU %)

The tolerance to freezing treatments was also investigated by using the EL (%) assay. No differences were observed in EL (%) of plant in response to irrigation treatments. EL (%) significantly increased in −15 °C compared to 20 °C (by 464%), but it was not affected by interaction of irrigation and temperature (Table 2).

The SU (%) increased significantly by 13 and 11%, respectively, when plants were under deficit water (70 and 50% FC) compared to control. In the absence of cold treatment (20 °C), SU (%) decreased compared to 0 °C temperature. The non-acclimated plants survived completely from 0 to −6 °C temperature, and reducing temperature to −12 °C caused a significant reduction (by 26%) in the survival percentage compared to control. Lowering the temperature to −15 °C caused the total mortality of viola plants (Table 2).

The results showed that SU (%) was significantly increased by drought stress under freezing conditions. In all the irrigation treatments, SU (%) was constant in temperatures of 0 to −6 °C and decreased in temperatures below −6 °C. Based on Fig. 1, 90 and 70% FC treatments at -12 °C had the highest (47%) and lowest (20%) decreases in SU (%), respectively, compared to 0 °C temperature. There was a negative relationship between EL (%) and SU (%) as the survival percentage decreased with increased electrolyte leakage; however, increase in EL (%) in −15 °C compared to 20 °C caused 100% decrease in SU (%) under drought stress.

3.2.2. Morphological and physiological changes

3.2.2.1. Characteristics of vegetative growth. Although plant height was not affected by irrigation treatments, freezing temperature significantly reduced plant height. Decrease in the temperature negatively affected plant height, so that plant height showed 9% significant decrease when temperature reduced from 20 to −12 °C (Table 2).

Drought stress significantly increased plant height under different temperatures. Lowering the temperature to −12 °C resulted in 10, 14 and 21% reduction in plant height in 90, 70 and 50% FC treatments, respectively, as compared to control (Fig. 2).

No difference was seen in the number of node on the main stem in response to irrigation treatments, but the effects of temperature and interaction of irrigation and temperature on this parameter were significant (Table 2). In general, node number of all plants declined as the temperature decreased from 20 to −12 °C. In all irrigation treatments, the highest number of node was related to 0 °C temperature. No significant difference was observed in 90% FC treatment when temperature decreased from 20 to −9 °C (Fig. 2).

Irrigation, temperature treatments and their interaction had significant effects on the number of stolon. Table 2 shows that the number of stolon significantly increased under drought conditions, so that the

Table 1
Changes in proline, carbohydrate content, chlorophyll a, chlorophyll b, total chlorophyll and carotenoid of Viola. Treatments consisted of exposure to different irrigation regimes (90, 70 and 50% Field Capacity).

Treatments	Proline (μmolg^{-1}FW)	Carbohydrate (mg^{-1}DW)	Chlorophyll a (mg^{-1}FW)	Chlorophyll b (mg^{-1}FW)	Total chlorophyll (mg^{-1}FW)	Carotenoid (mg^{-1}FW)
Irrigation						
90% (FC)	1.27^{c}	78.3^{b}	0.510^{b}	0.289^{c}	0.795^{b}	0.117^{c}
70% (FC)	2.02^{b}	80.3^{ab}	0.725^{a}	0.373^{b}	1.09^{a}	0.151^{b}
50% (FC)	2.41^{a}	81.7^{a}	0.710^{a}	0.418^{a}	1.13^{a}	0.181^{a}
Significance						
Irrigation	**	*	**	**	**	**

Note: Means with the same letter in each column are not significantly different. * and ** are Significant at $p \leq 0.01$ and $p \leq 0.05$, respectively.

Table 2
Mean comparison of main effects and significance of irrigation regimes and freezing temperatures on EL, SU, plant height, number of node on the main stem, number of stolon, number of leaf and leaf area of viola.

Treatments	EL (%)	SU (%)	Plant height (cm)	Number of node on main stem	Number of stolon	Number of leaf	Leaf area (cm²)
Irrigation (FC)							
90%	28.4[a]	71.4[b]	6.19[a]	1.52[a]	0.669[b]	12.8[a]	39.1[a]
70%	28.4[a]	80.9[a]	5.95[a]	1.47[a]	1.162[a]	15[a]	48.8[a]
50%	29.6[a]	79.1[a]	5.63[a]	1.26[a]	0.843[ab]	13.9[a]	43[a]
Temperature (°C)							
20	9.93[f]	93.3[b]	6.99[bc]	1.66[c]	0.839[c]	16.2[bc]	49.6[c]
0	16.7[ef]	100[a]	7.46[a]	2[a]	1.933[a]	18.4[a]	78.8[a]
−3	20.9[de]	100[a]	7.29[ab]	1.99[ab]	1.24[b]	17.4[ab]	61.8[b]
−6	25.1[cd]	100[a]	6.85[c]	1.82[bc]	1.08[b]	16.8[cd]	56.5[b]
−9	31.9[c]	77.8[c]	6.67[cd]	1.44[d]	0.678[cd]	14.9[cd]	36.6[d]
−12	41[a]	68.9[c]	6.33[d]	1[e]	0.463[d]	13.7[d]	22.2[e]
−15	56[b]	0[e]	0[f]	0[f]	0[e]	0[e]	0[f]
Significance							
Irrigation	Ns	*	ns	ns	**	ns	ns
Temperature	**	**	**	**	**	**	**
Irrigation × Temperature	**	**	**	**	**	**	*

Means followed by the same letter within each column shows no significant differences among treatments at 0.05 level by LSD. (EL: Electrolyte Leakage; SU: Survival; FC: Field Capacity; ns: not significant. *: $P < 0.05$, and **: $P < 0.01$).

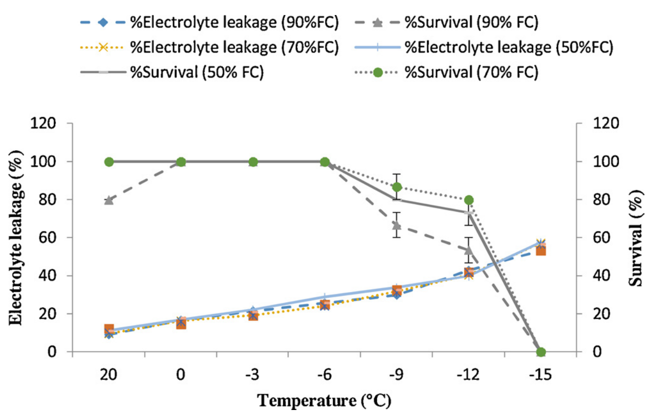

Fig. 1. Interaction effects of irrigation regimes (90%, 70% and 50% Field Capacity) under different temperature treatments on survival and electrolyte leakage in Viola. Data (means ± SD, n = 3) indicate a significant difference at P ≤ 0.01.

highest stolon number was recorded at 70% FC. Furthermore, the number of stolon declined significantly along with the decrease in temperature from 20 to −12 °C. In 70% FC treatment, the stolon number was constant in temperatures ranging from 0 to −6 °C, and decreased in temperatures below −6 °C. The results showed that the number of stolon in 70 and 50% FC at −12 °C temperature appeared to be unaffected by temperature compared to control and this parameter significantly decreased by 93% in 90% FC treatments, respectively, at −12 °C compared to control (Fig. 2).

3.2.2.2. Characteristics of leaves. The number of leaf and leaf area were not affected by the irrigation treatments but significantly affected by temperature (Table 2). In this study, these traits peaked at 0 °C temperature and then decreased under lower temperatures. The maximum reduction in leaf number (15%) and leaf area (55%) was observed in −12 °C temperature compared to control. All drought stress treatments caused significant increase in characteristics of leaves compared to well-watered plants under temperature treatments. Fig. 2 shows that, after lowering temperature to −12 °C, the maximum reduction in leaf number (20%) was due to 90% FC treatment, while the minimum reduction (9%) were found under drought conditions. Leaf area followed the same trend as the number of leaf. A slight increase (by 18%) in leaf area of pansy was observed at 70% FC compared to 90% FC under 0 °C. The maximum leaf area (84.7 cm²) was obtained in 70% FC irrigation treatment at 0 °C temperature and the minimum leaf area (16.3 cm²) was observed in 50% FC treatment at −12 °C temperature (Fig. 2).

3.2.2.3. Characteristics of reproductive growth. The effect of irrigation treatments on flower number and flower diameter were not significant, but on bud number were significant (Table 3). The number of bud increased by 65% in 70% FC compared to 50% FC.

The effect of temperature treatments on ornamental characteristics of viola including flower number, bud number and flower diameter were significant. Low temperature markedly decreased flower number compared to 20 °C temperature. By lowering the temperature to −12 °C, flower number decreased (by 26%) and bud number increased (by 33%) compared to control plants (Table 2). The numbers of flower and bud significantly increased in 70 and 50% FC compared to 90% FC treatment under freezing temperature. The maximum (6) and minimum (1.9) numbers of flower were found in 70% FC treatment at 0 °C and 90% FC at −21 °C, respectively. Although the flower and bud numbers in all irrigation treatments in 0 °C temperature increased compared to the 20 °C, the lowest and highest increase belonged to 90 and 70% FC, respectively. The number of bud increased by 120%, when plants were exposed to −9 °C temperature compared to 20 °C under 70% FC (Fig. 3).

The diameter of flower in control treatment (20 °C) was significantly smaller than that in 0 °C treatment (Table 2). The results showed a significant reduction in the flower size in all irrigation treatments as temperature decreased from 0 to −12 °C. After lowering temperature to −12 °C, diameter of flower decreased by 18%, in drought treatments compared to control (Fig. 3).

3.2.2.4. Dry weight. Dry weights were significantly affected by irrigation, temperature and their interaction. The response of 70% FC treatment compared to other irrigation treatments was better, and the highest dry weight of reproductive, vegetative growth, root and total dry weights (973, 715, 172 and 1861 mg) were assigned to this treatment (Table 4). Dry weights of different parts of plant (vegetative, reproductive and root) significantly decreased by 15, 31 and 41%, respectively, when plants were under −12 °C compared to control temperature (20 °C).

The results showed that dry weights were significantly increased by drought stress under freezing conditions. Dry weights of vegetative and reproductive parts were the highest (1560 and 1030 mg) when plants

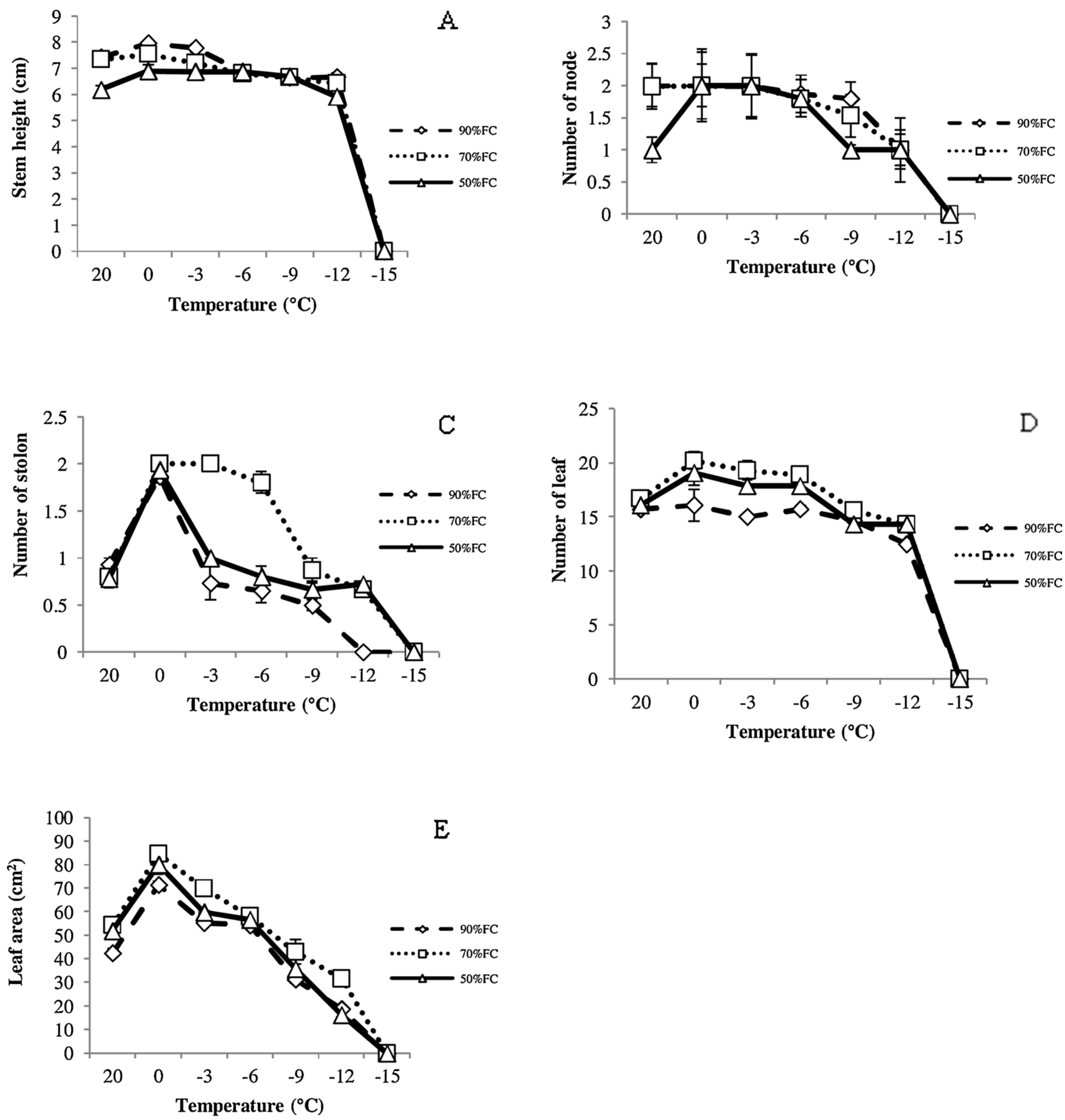

Fig. 2. Interaction effects of irrigation regimes under different freezing temperatures on stem height (A), number of node (B), number of node stolon (C), number of node leaf (D) and leaf area (E) in viola. Values are means ± SD of three replicates.

were exposed to 70% FC and 0 °C temperature. Dry weights in vegetative and reproductive parts of plants showed 31 and 5% reduction, respectively, in 50% FC compared to 70% FC at 0 °C. Plants under 70% FC at 0 °C showed the highest increase (90%) in dry weight of root compared to control. The maximum and minimum total dry weights were found at 0 °C and 70% FC treatment (2879 mg), and −12 °C and 90% FC (1128 mg), respectively. Drought treatments elevated (by 32 and 13%) the total dry weights compared to well-watering at 0 °C (Fig. 4).

4. Discussion

As a major ornamental plant, pansy yield is highly affected by freezing temperature, particularly when the weather gets warmer during winter. In a number of ornamental plants cultivated in green space, freezing injuries can be caused by insufficient mid-winter cold hardiness or de-acclimation, which are the result of covering of plants during winter or planting the winter plants in greenhouse and their transferring to outdoor. However, to avoid economic losses due to de-acclimation, it is desirable to manipulate freezing resistance in some bedding plants. Factors that increase tolerance including water deficit have frequently been used as a mean to increase hardiness.

One of the major objectives of our study was to determine changes in the accumulation of specific compounds in response to drought stress and their effect on the improvement of freezing tolerance. Increases in both drought and freezing tolerance have been associated with the capacity to accumulate protective compounds, including carbohydrates and amino acids (Bray, 1997; Praxedes et al., 2006; Mafakheri et al., 2011; Metwally et al., 2013). In general, we found that applying drought stress on non-acclimated plants can increase the amount of carbohydrate in leaf tissues. As a result, tolerance to both prolonged low temperature and drought has been associated with the accumulation of carbohydrate (Dionne et al., 2010). Carbohydrate has been reported to improve membrane stability in response to dehydration-related stresses (Valluru and Van den Ende, 2008) and to delay freezing by direct inhibition of ice crystal growth in the apoplast (Livingston et al., 2009), and is proposed to have roles in the oxidative stress response (Parvanova et al., 2004).

The results of the present study showed that viola acquired freezing tolerance by exposure to drought stress, and that this change was associated with the accumulation of proline. In addition to other components, proline has been associated with drought (Chen et al., 2018)

Table 3
Mean comparison of main effects and significance of irrigation regimes and freezing temperatures on number of flower, number of bud and flower diameter of viola.

Treatments	Number of flower	Number of bud	Flower diameter (cm)
Irrigation (FC)			
90%	2.94[a]	0.40[c]	3.60[a]
70%	3.81[a]	3.09[a]	3.79[a]
50%	3.46[a]	1.87[b]	3.67[a]
Temperature (°C)			
20	3.60[c]	1.20[c]	3.82[d]
0	5.60[a]	2.56[a]	4.74[a]
−3	4.29[b]	2.44[a]	4.53[a]
−6	4.11[bc]	2.31[a]	4.47[ab]
−9	3.53[c]	2.40[a]	4.20[bc]
−12	2.68[d]	1.60[b]	4.10[cd]
−15	0[e]	0[d]	0[e]
Significance			
Irrigation	ns	**	ns
Temperature	**	**	**
Irrigation × Temperature	**	**	**

Means followed by the same letter within each column shows no significant differences among treatments at 0.05 level by LSD. (FC: Field Capacity; ns: not significant; *: P < 0.05, and **: P < 0.01).

Table 4
Mean comparison of main effects and significance of irrigation regimes and freezing temperatures on dry weights of vegetative growth, reproductive growth, root and total dry weights of viola.

Treatments	Dry weights			
	Vegetative growth (mg)	Reproductive growth (mg)	Root (mg)	Total (mg)
Irrigation (FC)				
90%	738[b]	481[b]	124[a]	1344[b]
70%	973[a]	715[a]	172[a]	1861[a]
50%	745[b]	568[ab]	159[a]	1473[b]
Temperature(°C)				
20	914[bc]	645[c]	164[c]	1724[c]
0	1226[a]	983[a]	295[a]	2506[a]
−3	1012[b]	769[b]	217[b]	1999[b]
−6	963[b]	748[b]	169[c]	1881[bc]
−9	841[cd]	526[d]	121[d]	1489[d]
−12	776[d]	444[e]	95.9[d]	1316[e]
−15	0[e]	0[f]	0[e]	0[f]
Significance				
Irrigation	**	**	ns	**
Temperature	**	**	**	**
Irrigation × Temperature	**	**	**	**

Means followed by the same letter within each column shows no significant differences among treatments at 0.05 level by LSD. (FC: Field Capacity; ns: not significant, *: P < 0.05, and **: P < 0.01).

and cold tolerance. Proline has been reported to improve cell turgor, maintain cell osmotic adjustment and protect cell during dehydration (Chegah et al., 2013). We observed that proline concentration was higher in plants under drought treatments as compared with well-watered plants. These findings are in agreement with the results reported by other investigations, and support the importance of proline as a protective component in response to stress (Man et al., 2011; Baloglu et al., 2012; Aghaie et al., 2018).

Severe drought stress can also alter the concentrations of chlorophyll and carotenoid (Merwad et al., 2018). Reduction of chlorophyll content has been reported in *Catharanthus roseus* (Jaleel et al., 2008), *Helianthus annuus* (Kiani et al., 2008) and *Vaccinium myrtillus* (Tahkokorpi et al., 2007) under severe drought stress. In contrast to *other* studies*, our results* indicate that chlorophyll and carotenoid content increased under drought stress. The results showed that too much would be adverse for the accumulation of photosynthesis pigments. The highest chlorophyll and carotenoid contents were observed in 50% FC, and decreased along with the increase in irrigation. Plants under well-watered conditions cannot uptake some elements including magnesium and calcium resulted reduction of chlorophyll also, respiration and oxygen absorption become restrict (Hopkins and Huner, 2009).

The results also showed that electrolyte leakage was not affected by

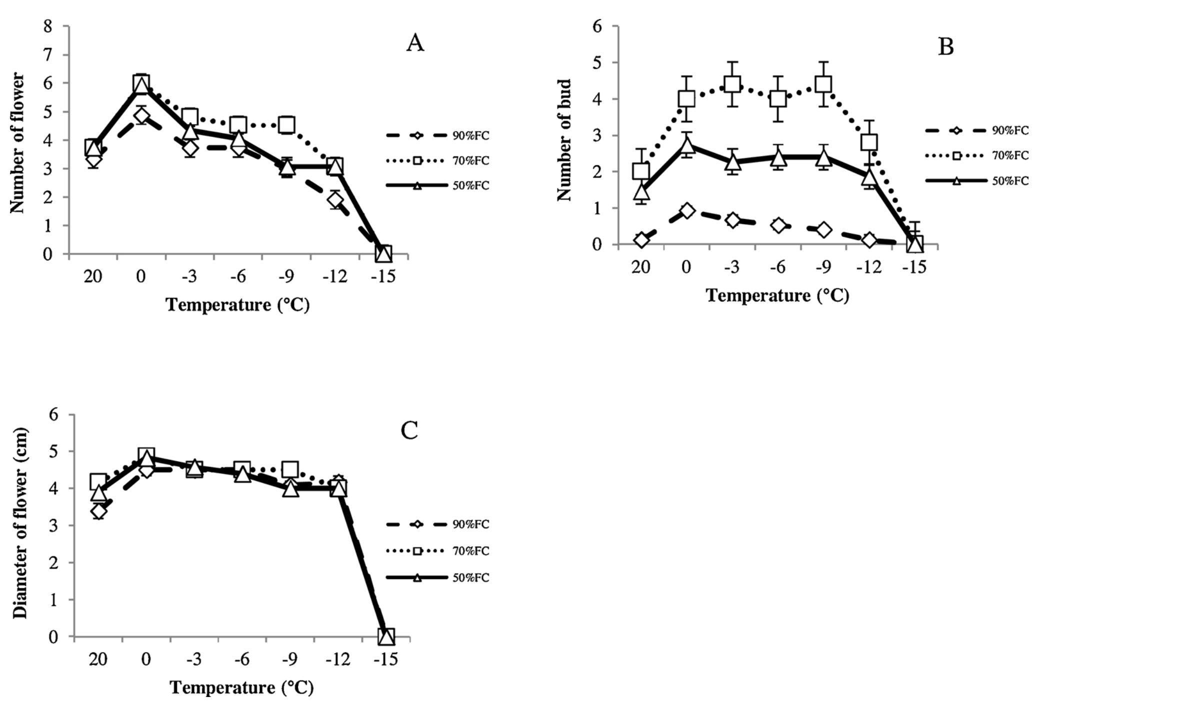

Fig. 3. Interaction effect of irrigation regimes under different freezing temperature on the number of flower (A), number of bud (B), and diameter of flower (C) in viola. Values are means ± SD of three replicates.

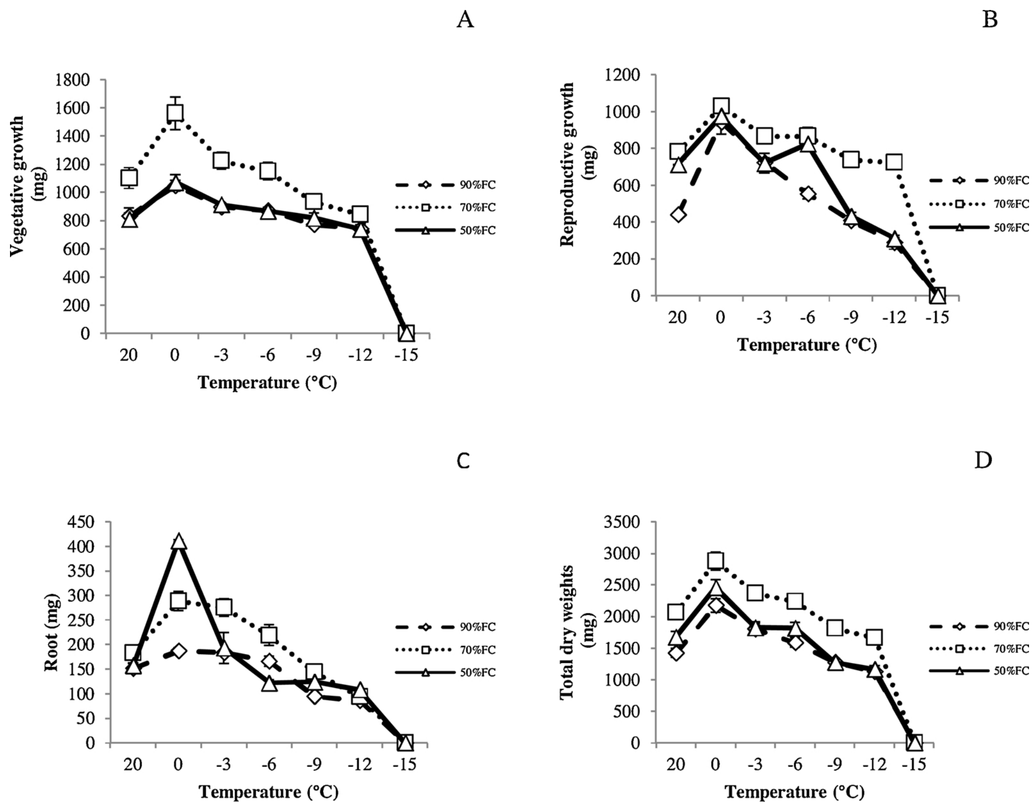

Fig. 4. Interaction effects of irrigation regimes and different freezing temperatures on dry weights of vegetative growth, reproductive growth, root and total dry weights in viola. Values are means ± S.D of three replicates.

irrigation treatments. These findings suggested that 70 and 50% FC treatments caused mild drought stress with no significant damage to plants, but there was a significant increase in the electrolyte leakage when the plants were exposed to low temperature. Cold hardiness was estimated by using the electrolyte leakage test, which determines frost damage to the plasma membrane by measuring electrolyte leakage from symplast to the apoplast (Zhang and Willison, 1986). Lowering the temperature to −12 °C showed the maximum rate of leakage as well as lower tolerance to freezing stress compared with other treatments.

The freezing tolerance of plants was evaluated by SU (%). Furthermore, there was a significant negative relationship between EL (%) and SU (%), so that increase in electrolyte leakage resulted in the reduction of plant survival. Survival percentage in tolerant plants was increased by maintaining the integrity of plasma membrane and decreasing electrolyte leakage percentage after freezing treatment. Drought has been shown to be effective in improving tolerance to different abiotic stresses, including low temperature. Cloutier and Siminovitch (1982) also reported a similar increase in freezing tolerance due to a two-week drought period in winter wheat and winter rye plants.

Kim and Anderson (2006) showed that freezing temperature can reduce survival (%) in (*Dendranthema- grandiflora* Tzvelv.). Gusta et al. (1980) also reported that gains in cold hardiness induced by drought were highly dependent on the degree of tissue dehydration in Kentucky bluegrass. In contrast, exposing of plants to well-watered conditions did not result in the improvement in freezing tolerance compared to plants treated with mild water stress, regardless of temperature regime. Nonetheless, both components of cold acclimation and drought stress are needed for plants to acquire maximum freezing tolerance, and it appears that they have an additional role in freezing tolerance. In our study, the SU (%) decreased when the plants were under well-watered conditions compared to drought treatments at 20 °C. We found that drought treatment resulted in an increase in freezing tolerance in the absence of cold acclimation (20 °C), and had a synergistic effect on the improvement of freezing tolerance.

At the level of whole plant, the effect of stress is usually perceived as a reduction in growth. Freezing tolerance tests based on the measurements of growth parameters could be of great help in the selection of correct plant for green space. To attain the required frost resistance, plants should cease their growth during the cold season (Rapacz, 1999). In the current study, some growth characteristics including, height, number of node, stolon number and leaf growth decreased under freezing temperature. According to the results, low temperature produced the shortest plants with the lowest number of node compared to control temperature. These results are in agreement with Thapa et al. (2008), who showed that freezing temperature had a negative effect on plant height of barrel clover (*Medicago. truncatula*). Similarly, the results of Jenabiyan et al. (2014) showed that number of node in main stem decreased under freezing stress. Izadi Darbandi et al. (2011) reported that number of branches and leaves decreased by cold treatments, while unstressed plants showed the highest number of branches and leaves.

Data presented in this study clearly showed that overall number of bud was affected by irrigation treatments. Our analysis demonstrated that the quantitative parameters including the number of flower, bud and diameter of flower after freezing temperature decreased, which is in *agreement* with the results reported by other studies (Nezami et al., 2012a,b, 2014). By lowering the temperature, the number of flower and bud were decreased but with two weeks drought treatments, plants showed the largest increase in reproductive growth during recovery period. In addition, it is becoming increasingly evident that altering the flowering by using drought stress is an evolutionary strategy adopted by plants to maximize the chances of reproduction under freezing stress conditions.

One of the first signs of stress is the decrease in turgor which causes a decrease in both growth and cell development, especially in the stem and leaves. The growth of cells is the most important process that is affected by stress, and decrease in the growth of cells leads to decrease in dry weight of plant. In our study, results showed that lowering the temperature decreased dry weights of plant. Growth reduction as a result of different stresses has been widely reported in many studies (El-Boraie et al., 2009; Soad et al., 2010; Ekren et al., 2012; Hassan et al., 2013). The well-watered treatment resulted in higher reproductive growth as compared to the other treatments before exposure to temperature stress, and regrowth following cold treatment resulted in a significant decrease in flowering, because reproductive growth (flower and bud) is more sensitive to freezing temperature than vegetative growth. Generally, control plants (90% FC) had lower dry weight under freezing temperature compared to other plants. Under well-watered conditions, the plants cannot uptake some elements, resulting in lower dry matter (Pirzad et al., 2015). Plants (at 0 °C) had immediate regrowth from frozen crown, while delayed regrowth was observed in lower temperatures up to −12 °C.

Hekneby et al. (2006) studied freezing hardiness of several annual legumes and observed that plant dry weight under different conditions of freezing decreased, but the acclimated plants had higher dry weight than non-acclimated plants. Our findings are in agreement with Chen et al. (1983), who reported that dry weight of wheat (*Triticum aestivum* L) decreased by freezing temperature. Similarly, Azizi et al. (2007) found that dry weight of wheat at −12 °C decreased by 81% compared to the control conditions. Drought treatments under non-cold acclimation were effective in the alleviation of the adverse effect of freezing temperature in viola. As water stress appears to be an integral part of cold acclimation, the physiological and morphological responses to cold and water stress show a great deal of similarities. In general, 70% FC provided more favorable conditions for plants than well-watered conditions; however, when plants faced with cold temperatures, an increase was observed in freezing tolerance.

5. Conclusion

Freezing frequently causes negative effects on growth and flowering of bedding plants. It is essential to improve cold tolerance of plant which can cope with cold without losing their ornamental and aesthetic values. Based on the results of the present study, drought stress resulted in an improvement in freezing tolerance of viola and induced the greatest accumulation of carbohydrate content and proline. We also observed changes in growth parameters in response to freezing that were dependent upon temperature regime. Among the different parameters evaluated, 70% FC treatment at 0 °C most consistently induced an increase reproductive and vegetative growth, suggesting a synergistic relationship between drought exposure and low temperature. Thus, it can be concluded that plants are unable to acquire full freezing tolerance during non-cold acclimation process without the accompanying water stress. In addition, future research should explore the effects of repeated mild drought events on freezing tolerance in acclimated plant by using strategies such as wilt-based irrigation scheduling and partial root zone drying or deficit irrigation.

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