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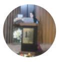

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ORIGINAL ARTICLE

# The effects of three levels of irrigation water on the improvement of cold tolerance of acclimated viola

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## Abstract

Due to global climate changes, the growth of ornamental plants will be influenced by warming episodes in winter. The purpose of this examination was to estimate the effect of three levels of irrigation regimes on the freezing tolerance of acclimated viola (*Viola × wittrockiana* 'Iona Gold with Blotch'). This experiments included three irrigation regimes (80, 60 and 40% FC) with different temperatures (20, 0, − 3, − 6, − 9, − 12, − 15, − 18, − 21 and − 24 °C). After irrigation regimes, proline and carbohydrate contents were recorded. Then, plants were moved to the thermo-gradient freezer and electrolyte leakage percentage (EL %), survival percentage (SU %), lethal temperature resulting in 50% mortality according to the electrolyte leakage percentage and survival percentage (LT50el and LT50su) were evaluated. The carbohydrate and proline content increased when plants were under drought stress conditions. Electrolyte leakage percentage across three levels of irrigation treatment increased by lowering the temperature; this increase at − 24 °C was fivefold more than the control. Survival percentage in all treatments was not affected in the thermal ranges of 0 to − 9 °C, but decreasing the temperature to − 24 °C resulted in the full loss in all treatments. Vegetative growth and reproductive components were significantly affected by drought and freezing temperature treatments. Dry weights of different parts of plants reached peak values when plants were under 60% FC at 0 °C condition. A negative correlation between LT50el with carbohydrate and proline levels was found and drought treatment significantly increased freezing tolerance (lower $LT_{50}$) in plants grown under 60% FC compared to other treatments.

**Keywords** Freezing tolerance · Drought · Hardening · *Viola × wittrockiana*

## Introduction

During the last decades, scientists have reported the global climate change-driven an increase in winter warming occurrence, and industrial of ornamental plants impacted to this phenomenon (Johnson et al. 2018). But, it is not obvious whether water deficiency during winter months is associated with the frost resistance process or it increases cold tolerance in ornamental plants. Moreover, there is a lot of evidence suggesting that water stress is solely able to induce cold tolerance in some plants (Cox and Levitt 1976; Cloutier and Andrews 1984; Thomas and James 1993). Alterations in plant physiological responses associated with improved cold endurance include the collections of protective components, the behavior of lipids and protein in membranes, and their structure (Barrero-Sicilia et al. 2017). Very small alterations are the results of the cold situation, but many others are needed for the promotion of frost tolerance (Tommasini et al. 2008). Cloutier and Siminovitch (1982) found in seedlings of wheat and rye that freezing resistance increased after drought stress, which was similar to the hardening influence following exposure to cold treatment. According to some investigations, acclimation of plants to drought stress has shown that there are several morpho-physiological changes in common, including decreased growth ratio, reduced osmotic potential, and assemblage of several high-molecular substances such as proline and carbohydrate (Ingram and Bartels 1996; Bray 1997). Imposing drought stress on *Spinacia oleracea* L. led to accumulation of some metabolites and maximal initiation of cold stress tolerance (Guy et al. 1992).

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Hoffman et al. (2012) showed that imposing water stress on *Lolium perenne* cv. Buccaneer resulted in an enhancement in cold tolerance (lower $LT_{50}$), but another cultivar ('Sunkissed') was not affected by water deficiency. Also, drought pre-treatment brought about an improvement in the accumulation of proline and carbohydrate, but this response was determined by species, plant part tested, and cold temperature regimes. Additional research is required to be conducted to know the essential items related to the drought-caused improvement of cold tolerance. It is generally suggested that the perfect method for assessment of cultivars tolerance to frost is freezing the plant and determining the survival percentage (SU %) and electrolyte leakage (EL) in the regrowth period (Olien 1967). Damage to plant tissue as a result of low-temperature situations prevents the operation of cell walls and increases the electrolyte leakage (Peixoto and Sage 2016). As a result of cold damage, cell membrane shifts in lipid and protein structures, and thus the action of the cell membrane is inhibited (Takashi et al. 2013).

Cold acclimation is a phenomenon that enhances the tolerance of frost stress in plants, but different mechanisms regulate cold acclimation and cold tolerance (Rahman et al. 2020). Pansies are used as bedding plants and are grown in gardens for their fall, winter, and spring blooms, and many regions in Iran grow pansies outdoors all year through. The plant needs some shade and lots of moisture in hot weather and is also suitable for cold weather. Most pansies wither when hot summer weather commences and heat or water stress occurs (Dole and Wilkins 2005). Lower water availability as a consequence of global climate change is one of the first and foremost vital items, which negatively affects growth ratios and survival of plants (Ramakrishna and Ravishankar 2011).

This paper aims to reveal whether water stress induced in the middle of winter due to temporary warm weather is an integral part of cold acclimation in the viola or not, It was also tried to find whether plants should be irrigated under these conditions or not and finally to further characterize the role of different irrigation regimes on freezing tolerance of bare-root viola in cold acclimation.

## Materials and methods

### Plant materials and irrigation treatments

The seeds of viola (*Viola* × *wittrockiana* 'Iona Gold with Blotch') were planted in the nursery in July. In October, seedlings were transferred to the main field, and 98 plants were planted in 1.5 $m^2$ of each plot with 10 cm space between them. In this experiment, each level of irrigation treatment included three replications (plot). In December, irrigation regime treatments were initiated. Table 1 shows the physical and chemical analysis of the soil. To evaluate the effect of pre-drought treatments on freezing tolerance of bare-root pansy under natural conditions, three levels of irrigation treatment were designed as control (80% FC) and water stress (60 and 40% FC) for 2 weeks. Three leaves of bare-root pansy were carefully removed from the plant, quickly washed clean using deionized water, and then the carbohydrate and proline contents were determined. The plants in the central plot served for data measurements, while the outer rows were used as borders. Besides, 50 bare-root plants per plot were sampled for freezing tolerance assessment.

### Examined indicators before freezing treatment

#### Carbohydrate and proline

Estimation of carbohydrate was carried out following McCready et al. (1950) and proline concentrations were determined by an accurate method based on the reaction of proline with acid ninhydrin (Bates et al. 1973).

#### Controlled freezing tests

Freezing tolerance was carried out as explained by Nezami et al. (2012). Root-bare plants were covered in a wet paper towel and transferred into bags. Following the preparation, bags containing five plants were moved in the thermo-gradient freezer with nine freezing temperatures (0, − 3, − 6, − 9, − 12, − 15, − 18, − 21 and − 24 °C); for control (20 ± 1 °C), plants were kept in the greenhouse. For all measurements, three replicates with five plants were used. After 1 h, plants were moved to a growth chamber at 4 ± 1 °C for 24 h to

**Table 1** The characteristics of soil samples were examined

| Size of particle (%) | | | Texture class | PH | EC | O.M (%) | Available (ppm) | | |
|---|---|---|---|---|---|---|---|---|---|
| Sand | Silt | Clay | | | | | N | P | K |
| 74.01 | 20.5 | 5.49 | Clay loam | 7.13 | 0.38 | 1.16 | 28 | 200 | 0.068 |

reduce the speed of ice melting and then five plants were planted in one pot and placed in the greenhouse at 21 ± 1 °C.

## Examined traits after freezing treatment

### Electrolyte leakage (EL)

Electrolyte leakage after freezing test was determine for freezing injury to pansy plants. Five leaves were moved to 250 mL flasks containing 50 mL of distilled, deionized water. The leaves were submerged for 8 h in room temperature under shaking condition at 85 rpm. The flasks were kept at − 80 °C, thawed, shaken again for 8 h, and the final conductivity of the leachate was measured.

EL (%) = freezing-induced EC/ potential EC × 100 (Gusta et al. 2003).

Lethal temperature resulting in 50% mortality according to electrolyte leakage ($\mathrm{LT}_{50}$) was evaluated using the following model developed for electrolyte leakage data:

$$\mathrm{ELp} = \mathrm{Ell} + [(\mathrm{Elm} - \mathrm{Ell})/(1 + e - B(T - \mathrm{Tm}))],$$

where ELp is the predicted EL value, Ell is the lower bound of EL value, Elm is the higher bound of EL value, $e$ is 2.714, $B$ is the rate of change in the slope of the curve, $T$ is the absolute value of the treatment temperature and Tm is the inflection point of the curve (Anderson et al. 1988).

### Morphological assay

Following the regrowth period (one month after freezing treatments), plant survival (%) was determined for each replicate as (no. of plants survived/total no. of plants) × 100. LT50su were estimated based on the survival percentage index by plotting these values against the freezing temperatures.

A potable leaf area meter (LI-3000C) was used to determine the leaf area. Dry weights of different parts of viola were calculated after oven drying. The temperature resulting in a 50% reduction of dry matter and leaf area (RDMT50 and RLAT50) was determined by drawing plant dry weights and leaf area graphs against chilling temperatures and determining the temperature that causes a 50% reduction in the studied traits compared to the control. The numbers of flowers, buds, leaves, and flower diameter per plant were also measured.

## Statistical analysis

This first part of the experiment was conducted in a completely randomized design and the second part of the study was performed as a split plot based on a completely randomized design. The data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by the LSD test ($p \leq 0.05$). Irrigation treatments served as main plots and temperature treatments as subplots. For all measurements, three replicates were used.

## Results

### Measured traits after irrigation treatments

The accumulation of carbohydrate increased more in the case of drought-stressed plants (40 and 60% FC) compared to well-watered plants and the lower content of carbohydrate was recorded when plants were grown under 80% FC condition (Fig. 1a). All drought stress treatments elevated proline content compared to well-watered treatment. Leaf proline content significantly increased by 22.8% and 37.8%, respectively, when plants were under moderate (60% FC) and 40% FC compared to control condition (Fig. 1b).

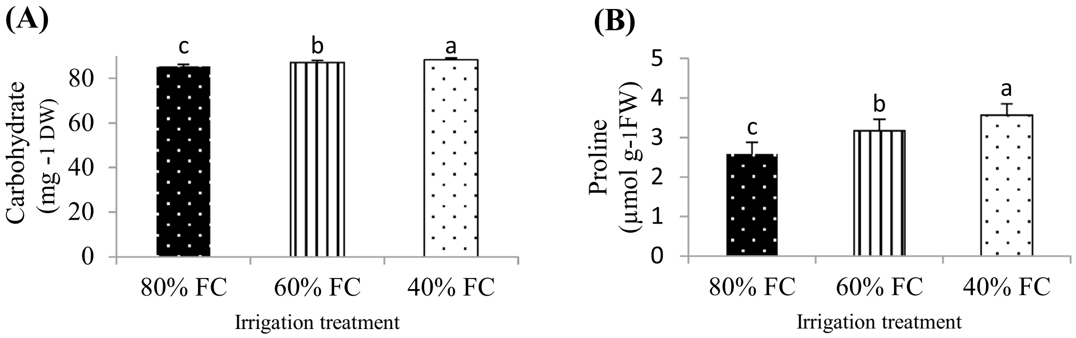

**Fig. 1** Effect of irrigation regime on carbohydrate (**a**) and proline (**b**) of the *Viola × wittrockiana*. Results are shown as means and standard deviation (± SD)

## Measured traits after freezing treatments

### Electrolyte leakage (EL %) and plant survival (SU %)

Figure 2 shows that well-watered conditions significantly ($p \leq 0.01$) elevated the EL % (by 42.4%) compared to plants under drought conditions (60% FC) at 20 °C. All well-watered treatments resulted in higher EL % in plant tissue. Lowering the temperature from 20 to – 24 °C increased the EL % in the three irrigation treatments. The highest EL % was observed at – 24 °C in all treatments. Table 2 shows that plants under 60% FC treatment showed more freezing tolerance (LT50el of – 19 °C) compared to plants grown under control condition (LT50el of – 15.3 °C).

Survival percentage had a negative relationship with freezing temperatures as the SU % decreased at lower temperatures (Fig. 2). Regrowth of plants under 0 °C started when plants were transferred to the greenhouse, but regrowth stop was recorded at lower temperatures on decreasing the temperature up to – 24 °C. In three irrigation treatments, decreasing the temperature to – 24 °C resulted in the death of the whole plants. Reducing temperature below – 9 °C caused a significant reduction in the SU % in 80% FC. Although the SU % in 60 and 40% FC treatments was constant up to the temperature ranges of 20 to – 12 °C, it showed a decreasing trend when the temperature reduced further. Plants treated with 40 and 60% FC (except for 80% FC) were able to tolerate the reduced temperature up to – 21 °C, but their survival percentage decreased (by 26.7% and 40%) compared to control. The temperature of – 18 °C and drought stress of 60% FC resulted in a 44.5% survival enhancement when compared to well-watered plants. Plant survival decreased by increasing the EL %, and in 60% FC treatment, an increase in EL % from 20 °C (by 4.37%) to – 24 °C (by 39.9%) caused plant SU % to decrease from 100 to 0% (Fig. 2). LT50su showed significant decrease (by 17%) in 60% FC compared to 80% FC treatment (Table 2). A negative correlation ($p \leq 0.01$) was recorded between EL % and SU % (Table 3).

**Table 2** Effect of irrigation treatments on LT50el, LT50su, RLAT50 and RDMT50 of viola under freezing stress under controlled conditions

| Irrigation treatment | LT50el | LT50su | RLAT50 | RDMT50 |
|---|---|---|---|---|
| 80% | – 15.3$^{b}$ | – 18.9$^{c}$ | – 12.9$^{b}$ | – 18.1$^{b}$ |
| 60% | – 19$^{a}$ | – 22.1$^{a}$ | – 15$^{a}$ | – 20$^{a}$ |
| 40% | – 18.4$^{a}$ | – 21.9$^{b}$ | – 13$^{b}$ | – 18.7$^{b}$ |
| Significance Irrigation regime | ** | ** | * | ** |

Means followed by the same letter within each column shows no significant differences among treatments at 0.05 level by LSD

* Significant at $p \leq 0.01$

** Significant at $p \leq 0.05$

### Morphological and physiological traits

Irrigation treatments and freezing temperatures had a significant ($p \leq 0.01$) effect on flower and bud numbers and flower diameter. Despite the fact the reproductive growth (the flower and bud numbers) increased in all irrigation regimes at 0 °C compared to 20 °C, there were no differences between the three irrigations at 0 °C (Fig. 3a, b). In 40% FC treatment, lowering the temperature to – 21 °C decreased flower number by 34.4% compared to 80% FC. The results revealed that the average flower size in three irrigation regimes decreased as freezing temperatures decreased from 0 to – 18 °C. In 60% FC, the diameter of the flower was significantly smaller at 20 °C than – 3 °C. Among the three irrigation treatments, the maximum percentage of increase

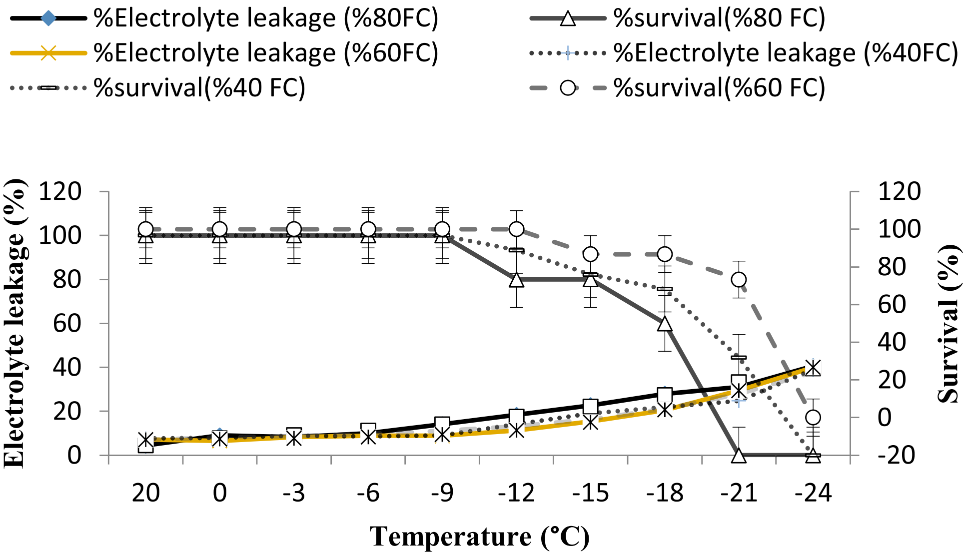

**Fig. 2** Survival percentage and electrolyte leakage percentage in *Viola × wittrockiana* as affected by irrigation treatments and temperature under controlled conditions. Results are shown as means and standard deviation (± SD)

**Table 3** Correlation coefficients between different characteristics in viola affected by drought and temperature stress, under controlled conditions

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Electrolyte leakage | 1 | | | | | | | | | | |
| 2. Survival | −0.90** | 1 | | | | | | | | | |
| 3. Number of flowers | −0.89 ** | 0.88** | 1 | | | | | | | | |
| 4. Number of buds | −0.84** | 0.90** | 0.91** | 1 | | | | | | | |
| 5. Diameter of flower | −0.92** | 0.94** | 0.91** | 0.92** | 1 | | | | | | |
| 6. Number of leaves | −0.91** | 0.94** | 0.91** | 0.92** | 0.95** | 1 | | | | | |
| 7. Leaf area | −0.90** | 0.80** | 0.88** | 0.84** | 0.88** | 0.86** | 1 | | | | |
| 8. Dry weight of vegetative parts | −0.89** | 0.86** | 0.88** | 0.87** | 0.91** | 0.89** | 0.92** | 1 | | | |
| 9. Dry weight of reproductive parts | −0.85** | 0.77** | 0.83** | 0.82** | 0.85** | 0.85** | 0.93** | 0.93** | 1 | | |
| 10. Dry weight of root | −0.80** | 0.76** | 0.84** | 0.86** | 0.85** | 0.83** | 0.92** | 0.91** | 0.91** | 1 | |
| 11. Total dry weights | −0.85** | 0.28** | 0.78** | 0.71** | 0.90** | 0.89** | 0.94** | 0.99** | 0.98** | 0.93** | 1 |

**, *and ns significant at 1 and 5% probability levels and not significant, respectively

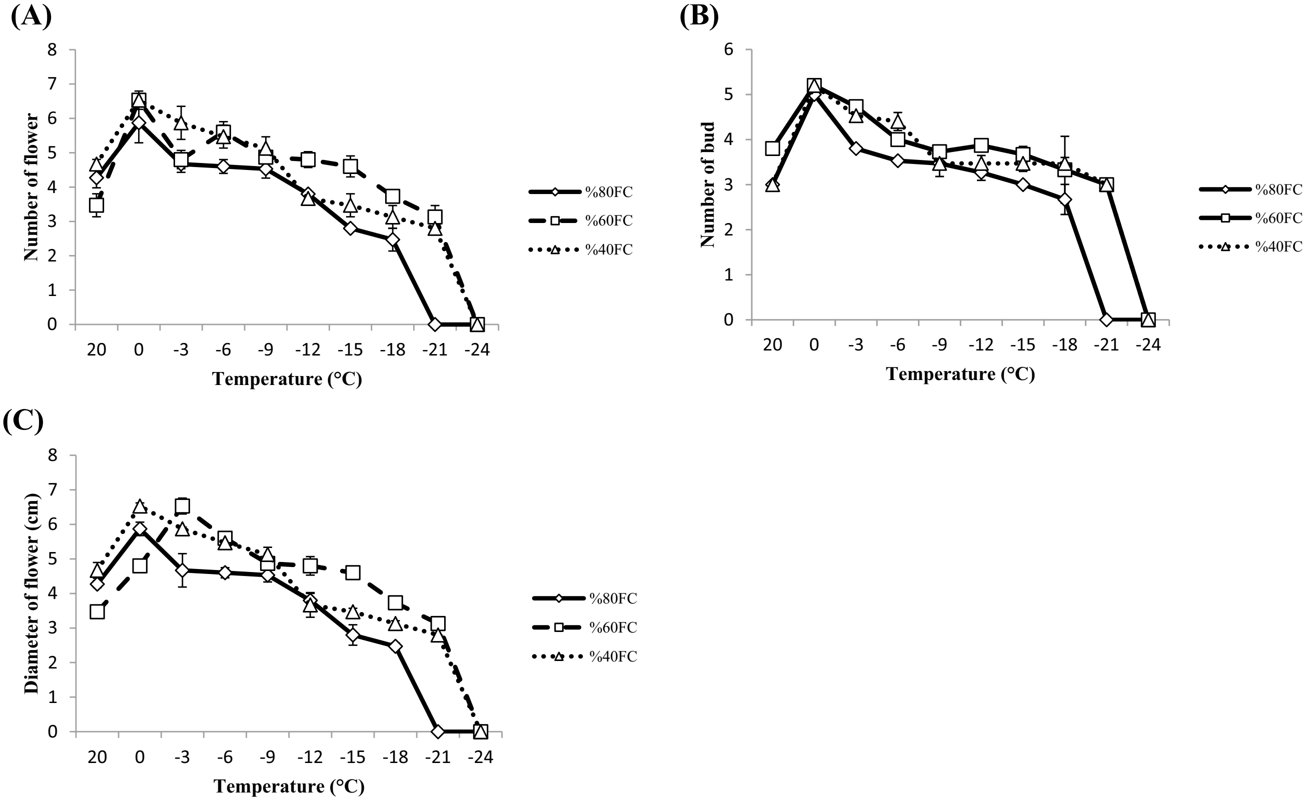

**Fig. 3** Interaction effects of irrigation and temperature treatments on the number of flower and bud and diameter of flower (**a**–**c**) in viola under controlled conditions. Results are shown as means and standard deviation (± SD)

in flower diameter, as compared to control treatment, was obtained in 60% FC (47.8) at 0 °C (Fig. 3c).

All pre-drought treatments significantly increased the number of leaf and leaf area under lower temperature. The maximum number of leaves (41.7) was observed in 60% FC at 0 °C, and the minimum number (20.3) was obtained in well-watered treatment at − 18 °C and 40% FC at − 21 °C. The number of leaves significantly decreased by 33% in 40% FC at − 18 °C (Fig. 4a). Figure 4b shows that the leaf area significantly increased by 98.8% and 88.2%, respectively, when plants were exposed to 60 and 40% FC treatments at 0 °C compared to control. The minimum (− 15 °C) and the maximum (− 12.9 °C) temperatures

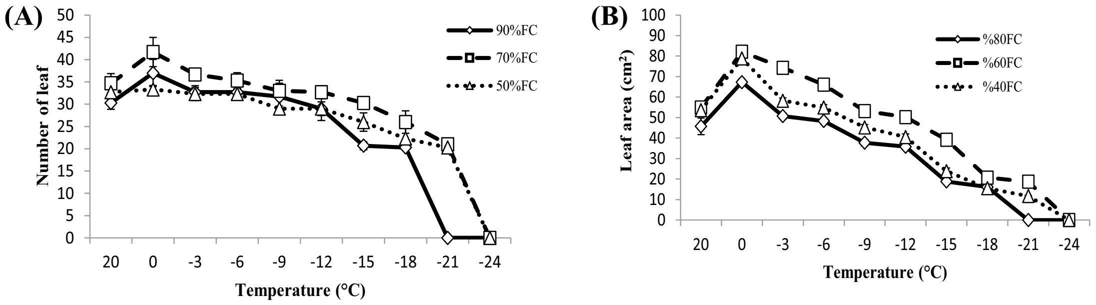

**Fig. 4** Interaction effects of irrigation and temperature treatments on the number of leaves and leaf area (**a**, **b**) in viola under controlled conditions. Results are shown as means and standard deviation (±SD)

resulting in a 50% reduction of leaf area (RLAT50) were observed in 60 and 80% FC, respectively.

Dry weight was significantly ($p \leq 0.01$) affected by irrigation regimes under cold conditions. Although the dry weight of vegetative growth in 60% FC treatment was constant up to the temperature ranges of 0 to − 9 °C, it showed a decreasing trend when the temperature was further reduced (Fig. 5a). The highest dry weight of reproductive components (2695 mg) was obtained in 60% FC at 0 °C, while this parameter decreased in plants under severe stress (40% FC) at − 21 °C. The dry weights of reproductive components and root, and total dry weights significantly increased by 23.7, 45.4 and 41%, respectively, when plants were exposed to moderate water deficit (60% FC) compared to well-watered conditions at 0 °C. Lowering the temperature to − 18 °C caused a decrease of the dry weight of all parts in irrigation treatments compared to control treatment. The highest total dry weights (6576 mg) were observed when moderate water stressed-plants (60% FC) were grown under 0 °C condition (Fig. 5b–d). A significant difference was observed between plants under different treatments in terms of RDMT50. The values of RDMT50 in 80, 60 and 40% FC treatments according to the dry weight were − 18, − 20 and − 18.7 °C, respectively (Table 2).

Survival percentage showed a significant and positive correlation with the characteristics of flower, leaf and dry weights. A positively significant ($p \leq 0.01$) correlation was observed between the dry weight of vegetative parts with leaf area and the dry weight of reproductive parts with the number of flowers (Table 3). Table 4 shows LT50el were

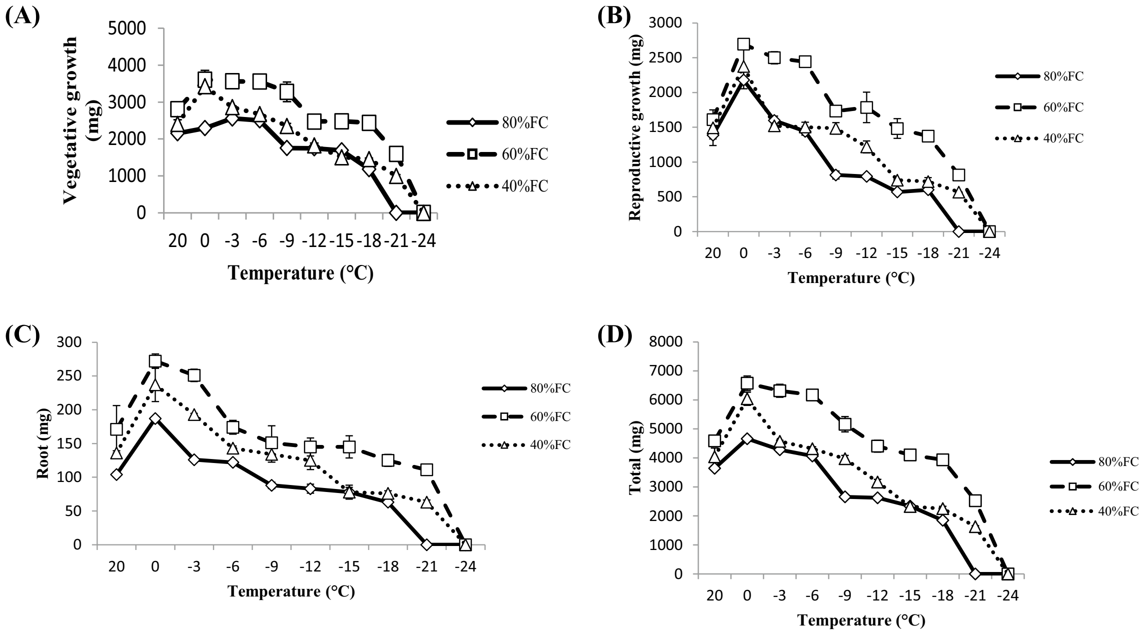

**Fig. 5** Interaction effects of irrigation and temperature treatments on dry weights of vegetative, reproductive and root, and total dry weight (**a**–**d**) in viola under controlled conditions. Results are shown as means and standard deviation (±SD)

**Table 4** Correlation coefficients between the measured parameters before freezing treatments and LT50el, LT50Su, RDMT50 and RLAT50 in viola plants

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Carbohydrate | 1 | | | | | |
| Proline | 0.97** | 1 | | | | |
| LT50el | −0.75** | −0.80** | 1 | | | |
| LT50su | 0.85** | 0.89** | −0.93** | 1 | | |
| RDMT50 | 0.41$^{\text{ns}}$ | 0.42$^{\text{ns}}$ | −0.73* | 0.75* | 1 | |
| RLAT50 | 0.09$^{\text{ns}}$ | 0.14$^{\text{ns}}$ | −0.63$^{\text{ns}}$ | 0.57$^{\text{ns}}$ | 0.84** | 1 |

**, *and ns significant at 1 and 5% probability levels and not significant, respectively

negatively correlated with LT50su ($r = -0.93$, $p \leq 0.01$) and RDMT50 ($r = -0.73$, $p \leq 0.01$) and a positive and significant correlation was observed between LT50su with carbohydrate ($r = 0.75$, $p \leq 0.01$) and proline content ($r = 0.85$, $p \leq 0.01$). The results show that proline and carbohydrate increased in pre-drought conditions and resulted in increased survival percentage after freezing temperature (Fig. 6).

## Discussion

The analysis of physiological and morphological traits presented in this study may help to clarify the different strategies of acclimated *Viola* × *wittrockiana* under freezing conditions. In general, the results obtained in this present study showed that pre-drought stress can increase the content of carbohydrate in acclimated viola. It has been reported recently that carbohydrate had multiple effects, namely improving membrane structure in response to water stresses (Zhang et al. 2016), functioning as osmoregulator (Hartmann and Trumbore 2016) and playing a part in the redox balance in the cells (Heiber et al. 2014). Frost hardiness and water deficit resistance depend on carbohydrate accumulations during stress situations (Spier et al. 2012; Dionne et al. 2010).

Proline acts as a highly beneficial function as a protection of membrane integrity and has multiple physiological and biochemical functions in plant and stress response (Forlani et al. 2019; Gomes et al. 2010). Researchers have shown that increased resistance to drought stress and frost is due to an increase in the accumulation of proline in plants (Moreno-Galvan et al. 2020; Hoffman et al. 2012). In the current study, drought stress (40% FC) caused the highest increase in proline accumulation of the viola. Different studies on the relationship between accumulation of carbohydrate and proline in plants has been stated, and these experiments indicate the important role of these two substances as supportive components in response to water deficit (Silva and Arrabaca 2004; Ghaffari et al. 2019).

The freezing tolerance of pansy plants that were exposed to freezing temperatures was estimated by electrolyte leakage (EL) and survival percentage (SU %). Under freezing temperature, water stress (60 and 40% FC) decreased the EL % compared to the plants under normal irrigation (80% FC). The degree of electrolyte leakage was negligible until −9 °C, and, along with the further reduction in temperature, 80% FC treatment recorded the highest EL % and these plants were susceptible to freezing temperatures. The improvement value of EL % in the plants under 80% FC immediately after the effect of freezing temperatures, compared to other drought stress conditions, occurred in all monitored plants. The increase of values of SU %, due to decreased EL %, is one of the strongest strategies of cold tolerance plant, leading to the protection of the structural integrity of cellular membranes. A similar response has been shown by Grace et al. (2009). Tomas and James (1993) also reported that drought stress-induced osmotic adjustment caused higher cold hardiness of *Lolium perenne*. Patton et al. (2007) stated that the carbohydrates and proline increased during cold acclimation and these parameters had a negative correlation with LT$_{50}$. In our study, plants under 80% FC condition did not follow the drought-stressed plant trend and freezing tolerance decreased as a consequence of freezing temperature. The highest concentrations of carbohydrate and proline coincided with the lowest LT$_{50}$. In contrast, the lowest carbohydrate and proline accumulation was related to the susceptibility to low temperatures. The results revealed that carbohydrate and proline may play important roles in freezing tolerance in pansy. In our experiment, the negative correlation between electrolyte leakage and survival percentage was recorded and a lower SU % was caused by high EL % in viola plants. The negative correlation between the Lt50el index with carbohydrate and proline levels indicated that LT50 decreased in drought stress, because the accumulation of osmotic adjustment compounds was increased. A negative and significant correlation between the LT50el with LT50su and RDMT50 indicated that the plants which had a lower electrolyte leakage showed higher LT50su. Intolerant plants (60 and 40% FC), with a decrease of electrolyte leakage, the LT50el index was decreased and plant dry weight increased. Such a significant correlation between the same indices was also observed in zoysia grass (Patton et al. 2007) and centipede grass (Cai et al. 2004). The reproductive growth significantly decreased when plants were under freezing temperatures, but through per-drought conditions, violas revealed the highest increase in the number of flowers and buds after the regrowth time. Flower and bud numbers were affected by freezing stress during the generative phase. A different study on the negative relationship between freezing stress and the reproductive component has been noted (Szymajda et al. 2013; Oraee et al. 2018).

Cold sensitivity was associated with many growth alterations developed after the stress period. Plants with higher RDMT50 (higher frost tolerance) had higher dry matter. Water deficit and freezing temperatures are responsible for the reduction in growth parameters (Jaleel et al. 2008; Cerekovik et al. 2013; Riaz et al. 2016). Well-watered condition induced an increase in cold sensitivity of viola plants by decreasing plant regrowth during the recovery period. Decreased growth during drought stress increases the amount of osmotic and protective substances in plants and increases the resistance of these plants to subsequent stresses (Feng et al. 2016).

The findings of Hekneby et al (2006) showed that cold-acclimated plants have higher regrowth capacity and dry matter partitions than non-acclimated ones. Freezing tolerance was associated with higher starch, soluble sugar, and proline and lower $LT_{50}$. Similarly, Chen et al. (1983) reported both root and shoot growths were reduced in cold-hardened wheat (*Triticum aestivum* L.) plants frozen to − 11 °C or lower.

The reduction of reproductive growth of plants under 80% FC after freezing temperature seems to be caused mainly by increase of flower and bud number as a result of fast growing before facing freezing temperature. As observed in our study, the incomplete recovery of reproductive growth affected by freezing temperature testifies to the persistent susceptibility of flower and bud to lower temperature. A negative and significant correlation between leakage percentage and all morphological traits (number of flowers, buds, leaf, leaf area, and dry weights) proved that a decrease in EL caused the successful growth of the viola in the recovery stage. A similar correlation coefficient was obtained in calendula (Perez et al. 2014).

As a result of our experiment regarding irrigation regimes and the freezing temperature of the investigated plants, it seems possible to distinguish the following types of responses induced by water stress before and after freezing temperature and the end of the regrowth period.

Temperature has the greatest influence on the rate of growth and development in pansy throughout the experiment sequences. The garden pansy tends to develop in cold weather because its development would be restricted by the hot weather (Dole and Wilkins 2005). For this reason, plants used to these situations have to be grown at lower temperatures to increase the growing season. The increase of frost resistance during cold weather seems to be caused mainly by a range of biological and physiological responses of plants (Xin and Browse 2000).

Many scientists reported that cold acclimation situations induced the activity of protective components in plants, suggesting that proline and carbohydrate play a vital role in plants and this component operates as a substrate for increasing cold tolerance (Palonen et al. 2008; Jankovska et al. 2019). When plants are subjected to non-cold acclimation (20 °C) treatment, it is unlikely that they experience low temperature, but water stress increased frost resistance and it was less operative than cold acclimation conditions. Similar to the results reported by Rajashekar and Panda (2014), our data indicated that water deficit is an essential element of the cold acclimation process and plays a crucial role in inducing freezing tolerance in the viola plants.

Among the different evaluated parameters, survival percentage, reproductive and vegetative growth parameters significantly increased in 60% FC treatment at 0 °C, implying the existence of a cooperative effect between water stress exposure and freezing conditions. The observed stimulation of violas growth by cold conditions could show efficiency adaptation of plants to water stress before the recovery period, but the amount of irrigation water required varies with the stage of plant growth, soil type and climate. These results showed that viola plants can survive more frostbite if they are not irrigated, because by increasing carbohydrate and proline, plants show greater resistance to freezing. Oraee et al. (2018) also reported a similar trend, indicating that the lowest survival of non-acclimated pansy was recorded when well-watered plants were grown under freezing temperature (− 12 °C), but in this study, acclimated pansy tolerated temperatures until − 18 °C. This improvement in freezing tolerance was found in the acclimated pansy. However, pre-drought conditioning allowed acclimated plants to handle better the adverse effects of freezing temperatures compared to non-acclimated plants. In our study, cold acclimation and water deficit caused higher freezing tolerance in pansy plants under stressed and non-stressed conditions. Similarly, the result of Hao and Aroral (2009) showed that guava plants exhibited a partly overlapping reaction to frost and water stress.

The carbohydrate and proline contents in pansy plants were correlated ($r = -0.75$ and $-0.80$) with LT50el. Also, changes in freezing tolerance were related to carbohydrate and proline contents of pansy plants. Determination of freezing tolerance induced by irrigation treatments revealed that drought treatments (60 and 40% FC) have a valuable mechanism to avoid freezing by maintaining osmotic adjustment. Besides, drought stress can be considered as a tool to maintain the winter survival of viola plants under freezing temperatures.

**Author contribution statement** AT designed the research, AO performed the experiment and collected the data, AN and AT analyzed the data and wrote the manuscript. AT, AN and MSH edited the manuscript and provided guidance during experimentation.

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