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

Atiyeh Oraee[a], Ali Tehranifar[a,*], Ahmad Nezami[b], Mahmoud Shoor[a]

[a] Department of Horticultural Science and Landscape, Ferdowsi University of Mashhad, Iran
[b] Department of Agronomy Faculty of Agriculture, Ferdowsi University of Mashhad, Iran

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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

* Corresponding author.
E-mail address: tehranifar@um.ac.ir (A. Tehranifar).

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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