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Evaluating the potential drought tolerance of pansy through its physiological and biochemical responses to drought and recovery periods

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ABSTRACT

Drought is one of the major factors limiting ornamental plant production. Exposure to long-term drought conditions inhibits plants growth and leads to their yield loss. In this study, various responses of pansy plants to drought stress and recovery period were comparatively studied at physiological and biochemical levels, after one, two and three week period of exposure to drought stress and one week of recovery period. This study results showed that prolonged drought stress dramatically decreased relative water content (RWC), chlorophyll *a*, b and total chlorophyll and antioxidants including ascorbate peroxidase (APX), superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR) activities of plants while increased the accumulation of proline, sucrose, glucose, and fructose content, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and glutathione peroxidase (GPX) activity. Under well-watered condition, plants exhibited an increase in electrolyte leakage after three weeks period of exposure to drought stress, followed by rapid recovery. Under moderate and severe drought stress, plants displayed relatively less adaptability to drought, with a slower recovery after re-watering and a greater increase in electrolyte leakage. This study findings highlighted that enhanced antioxidative protection and osmotic adjustment plays an important role in pansy tolerance against drought. It was concluded that even though plants recovered after re-watering, the final dry matter was affected by drought stress, and its extent was depend on the drought intensity and its duration.

1. Introduction

Winter months during the last 20 years have experienced the greatest warming trend, and it has been reported that winter warming episodes have increased in terms of occurrence and duration (IPCC, 2014). Drought stress occurring in winter as a result of climate change is one of the most important abiotic factors, which adversely affects growth, metabolism and yield of plants (Lamaoui et al., 2018). Drought is one of the most important environmental stress factors that has been considered a primary cause for decreases in flower production under water-limiting conditions (Khan et al., 2017; Tombesi et al., 2018; Caser et al., 2019). In order to respond and adapt to drought stress, plants have evolved various drought resistance strategies at physiological and morphological levels (Larkunthod et al., 2018; Cal et al., 2019). Plants drought resistance can be classified into four major mechanisms: drought escape, drought avoidance, drought tolerance, and drought recovery, among which drought avoidance, via reduced water loss and enhanced water uptake, and drought tolerance, via antioxidant defense system and osmoprotection, osmotic adjustment, are the two major mechanisms of drought resistance of plants (Mansuri et al., 2017; Zali and Ehsanzadeh, 2018; Ghaffari et al., 2019).

Overproduction of reactive oxygen species (ROS) under stress conditions is a hallmark, comprising both free radical, superoxide radicals, hydroxyl radical, perhydroxy radical, and alkoxy radicals and non-radical (molecular) forms, like hydrogen peroxide and singlet oxygen (Thorpe et al., 2013; Das and Roychoudhury, 2014; Kim et al., 2017). Production of excess ROS is very reactive in nature and interact with numerous biomolecules such as DNA, proteins, RNA, lipids, pigments, and other vital cellular molecules, leading to serious damages (Liu et al., 2007; Mishra et al., 2011). The antioxidant defense mechanism of plants protects them against damage caused by environmental stress. ROS also plays a key role in acclimation process of plants to several abiotic stresses (Sharma et al., 2019). Drought stress induces lipid peroxidation causing irreversible damage to membrane structural and functional integrity too (Fu and Huang, 2001). For this reason, cell membrane stability and malondialdehyde (MDA) accumulation in the cell are a widely used indicator of plant tolerance to drought stress (Bacelar et al., 2006; Dias et al., 2018).

The accumulation of compatible solutes (osmotic adjustment) is a biochemical mechanism that helps plants to mitigate the water stress

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(Marimuthu and Murali, 2018; Shamoon Sadak et al., 2019). Osmotic adjustment (OA) results in a net increase of the number of osmotically active substances in the cell (Kozlowski and Pallardy, 2002). This increase of solutes leads to a more negative osmotic potential, which in turn can improve the degree of cell hydration, maintaining turgor in leaf tissue and in other metabolically active cells (Wilson et al., 2014; Blum, 2017; Omena-Garcia et al., 2019). In other words, plants can survive longer and maintain metabolic processes in drying soil if OA occurs. A wide range of substances can contribute to OA, including inorganic cations and anions, organic acids, carbohydrates, and amino acids. OA is often associated with an accumulation of specific solutes with protective functions. These compatible solutes rich in hydroxyl (−OH) groups such as sugars (fructose, sucrose, glucose), sugar alcohols (mannitol), amino acids (proline) and glycine betaine, can accumulate in the cytoplasm and help to protect cellular proteins, enzymes, and cellular membranes against dehydration (Zivcak et al., 2016; Turner, 2017; Rajasheker et al., 2019).

Carbohydrates are among the most accumulated solutes in plant tissue under water deficit conditions (Ennajeh et al., 2006; Rejskova et al., 2007). In addition to improving water absorption capacity of the plant, these compounds are involved in the osmoprotection of membranes and macromolecules by replacing water molecules in their vicinity thus preventing the formation of intra-molecular hydrogen bonds that can cause irreversible structural disturbances (Zhu, 2001; Chaves et al., 2003). Still, it is important to recognize that individual solutes do not contribute greatly to OA in many species and that OA is mainly achieved by the accumulation of a multitude of solutes (Silva et al., 2010; Maatallah, 2010; Warren et al., 2007). As OA requires the metabolism or uptake of solutes it is generally a slow process, and is sensitive to the timing and intensity of stress (Sanders and Arndt, 2012).

Viola × Wittrockiana, known as pansy, is one of the most popular commercially important cool season garden crop for landscape, and one of the five best-selling bedding plants in both developed and undeveloped countries (Gandolfo et al., 2016). Pansy is a short-lived evergreen perennial that is grown as annual or biennial in cool weather and it is best grown in humus and well-drained soils in full sun to part shade. Pansy, planted in fall for bloom throughout the winter and early spring, is the top-selling winter bedding plant in the world. Temperature and irrigation dominate and control its rate of plant development (Dole and Wilkins, 1999; Henson et al., 2006).

Within an agricultural context, drought is a prolonged period of deficient precipitation which results in negative impacts on ornamental plant such as pansy. An increasingly warming climate is expected to intensify the frequency and severity of drought in the near future (Raza et al., 2019). Thus, identifying key physiological limitations to productivity under drought will be important for improving yield stability in a changing climate. Immediately after drought stress termination, the availability of even a small amount of rainfall can have a significant effect on plant physiological functions, ranging from whole-plant responses to biochemical responses. Therefore, it is of particular importance to investigate the underlying mechanisms contributing to drought tolerance of pansy. We hypothesized that final productivity in pansy would be dependent on the ability to maintain RWC and chlorophyll, under drought stress and to rapidly recover to pre-drought levels upon re-watering, and the ability to osmotically adjust and protect cellular components from oxidative stress will be critical factors influencing tolerance to episodic drought. The effects of drought stress have been well-documented in many crop species; however, reports addressing physiological responses to progressive drought and recovery upon re-watering in pansy are relatively limited, so the present experiment was carried out to quantify physiological and biochemical responses of pansy to episodic drought and re-watering when they were subjected to different intensities of drought.

2. Materials and methods

The experiment was carried out during 2018 growing season in the college of Agriculture, Ferdowsi University of Mashhad, Iran. Pansy (*Viola ×wittrockiana*' Swiss Giants Rhinegold') seeds were provided by the Takii seed company. The seeds were sown in trays filled with coco peat and perlite mix. Following a four-week germination period in August, plants watered three times per week and fertilized weekly with full strength Hoagland solution (Hoagland and Arnon, 1950). After four weeks (3-leaf stage), one plants transferred to each pot (18 cm high and 16 cm in diameter) containing a mixture of garden soil, sand and rotted mature (2:1:2) under natural photoperiod. The soil of the experimental site had a pH of 7.7 and an EC of 1 mmos/cm. Potted plants were irrigated regularly for 4 months to well-watered (90 % FC) level. By the end of the December, plants were divided into three uniform groups: control plants (80 % FC) and drought-stressed plants (60 and 40 % FC). After three weeks of exposure to drought, stressed plants were re-watered to 90 % FC and followed by a recovery for one week. Leaf materials were collected for physiological and biochemical analyses one week after starting drought stress (1WDS), the 2th and 3rd week of drought stress (2WDS, 3WDS), and one week after re-watering (1WRW). The temperature and rainfall data were recorded by a data logger at experiment site. The drought stress was carried during winter month, but no rainfall was recorded during the test period on 2018.

Water treatments applied for four weeks based on gravimetric method (Campbell and Mulla, 1990). For measurement of electrolyte leakage, relative water content, proline, chlorophyll, carbohydrate (sucrose, glucose, and fructose), malondialdehyde, hydrogen peroxide, and antioxidant activity (superoxide dismutase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione peroxidase) the top leaves tissues were collected from a minimum of 10 independent plants. To determine dry matter content, harvested plants were dried in an oven for 72 h at 70 °C.

2.1. Electrolyte leakage (EL) assay

During the growing season (1, 2, and 3 weeks of imposing drought stress and 1 week re-watering), discs with 8 mm diameter were removed from leaves and placed in tubes containing 40 ml distilled deionized water. Leaf electrical conductivity (EC) was measured on the following day using a solution analyzer (Cole-Parmer Instrument Co., Chicago). To determine potential EC_1, the samples were autoclaved for 20 min at 121 °C to release the total electrolytes from the samples. After maintaining the samples at 21 °C overnight, EC_2 was measured. Percentage of Electrical Leakage (EL) for each plant was determined for each treatment according to the following formula (Reddy et al., 2004):

$$EL\ (\%) = EC_1 / EC_2 \times 100.$$

2.2. Relative Water Content (RWC) assay

Relative water content was determined using the method described by Turner (1981). Relative water content was calculated based on the following formulae:

$$RWC\ (\%) = ((FW\text{-}DW))/((TW\text{-}DW)) \times 100$$

Where FW is fresh weight, DW is dry weight and TW is turgor weight of leaf samples.

2.3. Proline assay

Proline content were determined using the methods described by Bates et al. (1973).

2.4. Chlorophyll content assay

Chlorophyll content was 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, and 645 nm by using spectrophotometer (UV-160A UV–vis Recording Spectrophotometer; Shimadzu, Tokyo, Japan). The amounts of chlorophyll were then calculated using the following formulae.

Chlorophyll a = [12.7(A663) - 2.69(A 645)] × V/W × 1000

Chlorophyll b = [22.9(A645) - 4.68(A 663)] × V/W × 1000

Total chlorophyll = [20.2(A645) + 8.02 (A 663)] × V/W × 1000

2.5. Carbohydrates assay

The concentration of individual sugars was measured by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI) as described previously (Singh et al., 1994). Briefly, lyophilized leaf tissue (100 mg dry weight) was ground with mortar and pestle. The powder was homogenized with ethanol 80 % (v/v) and the mixture heated to 90 °C for 10 min. The mixture was centrifuged at 2,500 × g for 10 min, and the supernatant was dried via rotary evaporation and resuspended in 1.5 mL of acetonitrile:water (78:22 v/v). The solution was analyzed by HPLC using a Librocart 250-4 column linked to a pre-column Librocart 4-4 at a flow of 1.5 mL/min under isocratic conditions. The mobile phase was acetonitrile:water (78:22 v/v). The detector was a Merck Hitachi Refractive Index Detector LaChrom 7490. Calibration curves for each standard (Merck) were used to determine the concentration of each sugar within the samples.

2.6. MDA and H_2O_2 assays

To determine the malondialdehyde (MDA) content in the leaves, 0.5 g leaf tissue was homogenized in 5 ml 0.1 % (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged for 10 min. The supernatant (1 mL) was mixed with 4 mL of thiobarbituric acid (TBA) reagent (0.5 % of TBA in 20 % TCA). The reaction mixture was heated at 95 °C for 30 min in a water bath and was then quickly cooled in an ice bath and centrifuged at 11 500 × g for 15 min. The amount of MDA–TBA complex (red pigment) was measured by a spectrophotometer (UV-160A UV–vis Recording Spectrophotometer; Shimadzu, Tokyo, Japan) at 532 and 600 nm with the extinction coefficient of 155 $mM^{-1} cm^{-1}$ and was expressed as μmol MDA g^{-1} FW (Velikova et al., 2000). Hydrogen peroxide (H_2O_2) was assayed according to the method described by Velikova et al. (2000). The optical absorption of the supernatant was measured by a spectrophotometer at 390 nm to determine the H_2O_2 content (D = 0.28 $\mu M^{-1} cm^{-1}$) and was expressed as μmol g^{-1} FW.

2.7. Antioxidant enzyme extraction

Approximately 300 mg of leaf (fresh weight) were harvested, frozen in liquid nitrogen, and stored at −80 °C for determination of antioxidant enzyme activities. Extraction was based on the method previously described by Da Costa and Huang (2007). Tissues were homogenized in 4 mL of 150 mM cold phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was transferred to 15 mL tubes and used for enzyme activity determination. Activity of Ascorbate peroxidase (APX) was determined using the method of Nakano and Asada (1981). The reaction (50 mM K-phosphate buffer (pH 7.0), 0.5 mM ascorbate (AsA), 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 mL) was initiated by the addition of H_2O_2, and the activity was measured by observing the decrease in absorbance at 290 nm for 2 min using an extinction coefficient of 2.8 $mM^{-1} cm^{-1}$ using a spectrophotometer.

Superoxide dismutase (SOD) activity was determined using the method of Sairam et al. (2002) with modifications. A 100 L aliquot of extractant was added to a solution containing 50 mM phosphate buffer (pH 7.8), 60 M riboflavin, 195 mM methionine, 3 M EDTA, and 1.125 mM nitro blue tetrazolium (NBT) ditetrazolium chloride. A solution lacking enzyme extract was used as control. Test tubes were irradiated under fluorescent lights at approximately 300 $mol^{-2} m^{-2} s^{-1}$ for 30 min and then placed in the dark for 10 min to stop the reaction. The absorbance was measured at 560 nm and one unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of nitroblue tetrazolium (NBT) photoreduction. The SOD activity was expressed as enzyme activity per gram leaf fresh weight. DHAR activity was analyzed using the method of Ma and Cheng (2004) with some modifications. The glutathione peroxidase (GPX) activity was measured as described by Rao et al. (1996) using H_2O_2 as a substrate. The oxidation of NADPH was recorded at 470 nm for 2 min, and the activity was calculated using the extinction coefficient of 26.6 $mM^{-1} cm^{-1}$.

2.8. Statistical analysis

The experiment was set up in a factorial based on a complete randomized design with three replications. Data were subjected to repeated measures ANOVA with SAS statistical software (Version 9.2, SAS Institute, Cary, NC, USA). Data are presented as the mean ± SD of three replicates. LSD values were calculated in cases where significant variance was found at $P < 0.05$.

3. Result

Drought stress significantly increased the electrolyte leakage (EL) during the stress period (Fig. 1A). The magnitude of EL increment was greater for plants under 40 % FC than plants under 80 % FC and it was more pronounced after third week compared to first week measurement. EL increased by 33.8 % and 58.9 %, when plants were under moderate and severe water deficient respectively (60 and 40 % FC) compared to 90 % FC at 3WDS. After re-watering, plants recovered rapidly to same level as control plants at 1WDS, whereas the 60 % and 40 % FC plants showed incomplete recovery within the same re-watering time frame (Fig. 1A).

During stress period, the loss of leaf water levels was indicated by the progressive decline of RWC. RWC significantly decreased under drought stress. In relation to intensity of drought stress, lowest RWC was observed in severe drought stress than other treatments and this decline was highest at third week compared to the first week. After three weeks, RWC gradually dropped to 78.3, 76.8 and 69.7 % under 80, 60 and 40 % FC respectively. After re-watering, RWC tended to be restored for 80 % and 60 % FC treatments and recovered immediately almost to the level of those plants at one WDS however under severe drought stress, recovery of RWC was slower (Fig. 2B).

Drought stress resulted in a gradual increase of proline (Fig. 1C) under 80 % FC treatment. The amount of proline were very similar in 1WDS and 2WDS, but higher amounts were recorded at 1WDS (1.89 μmol g^{-1} FW). Higher increase of proline under 60 % FC treatment was observed at third week, but under severe drought stress (40 % FC), proline amounts showed largest increase, and was more pronounced at 3WDS. After re-watering, proline under 80 % FC treatment started gradually to recover, and it was immediately restored similar to the control level at 1 WDS, but a higher proline content was observed under moderate and severe drought stress (60 and 40 % FC) at re-watering condition compared to control plants (Fig. 1C).

Chlorophyll (a, b and total) amount progressively declined in all irrigation treatments at 3WDS compared to 1WDS (Figs. 2A.B.C). After three weeks imposing severe drought stress, chlorophyll a and b significantly decreased by 28.7 % and 28.9 % respectively, compared to

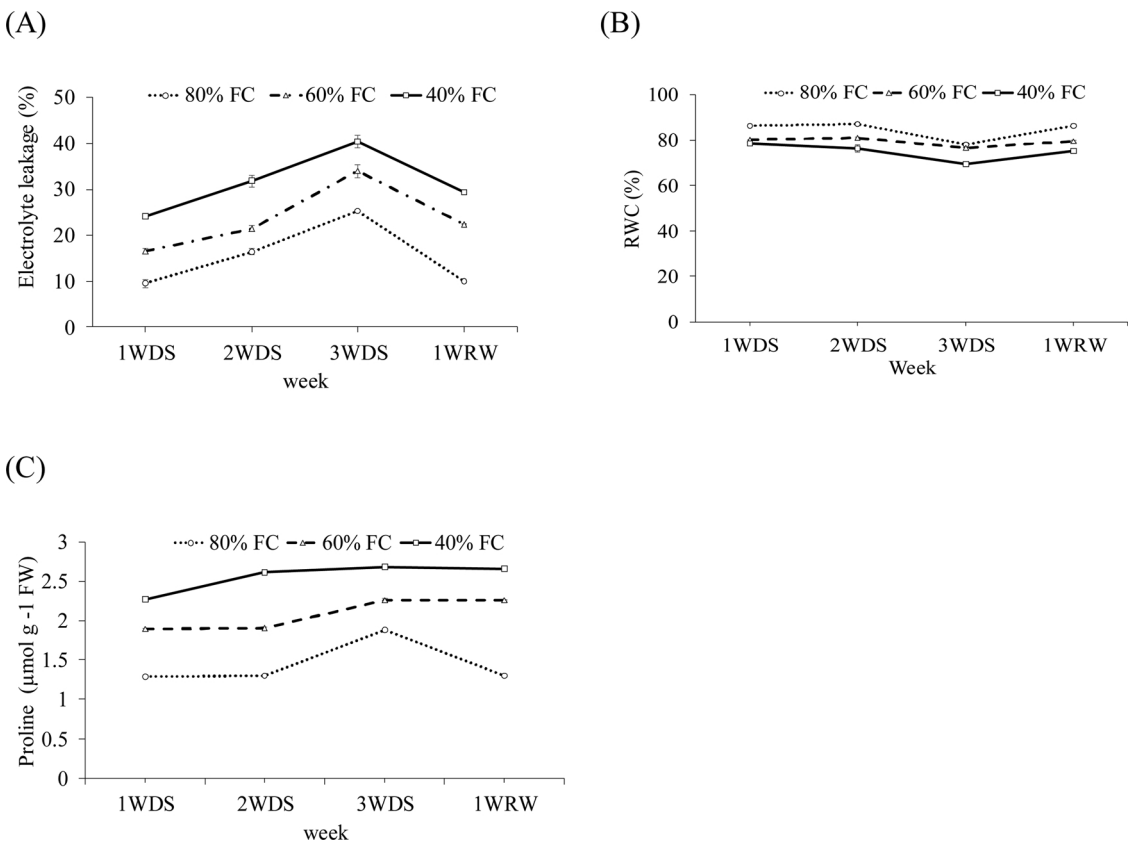

Fig. 1. Effect of drought stress and re-watering on electrolyte leakage (A), relative water stress (B), and proline content (C) of pansy. Values are means ± SD of three replicates.

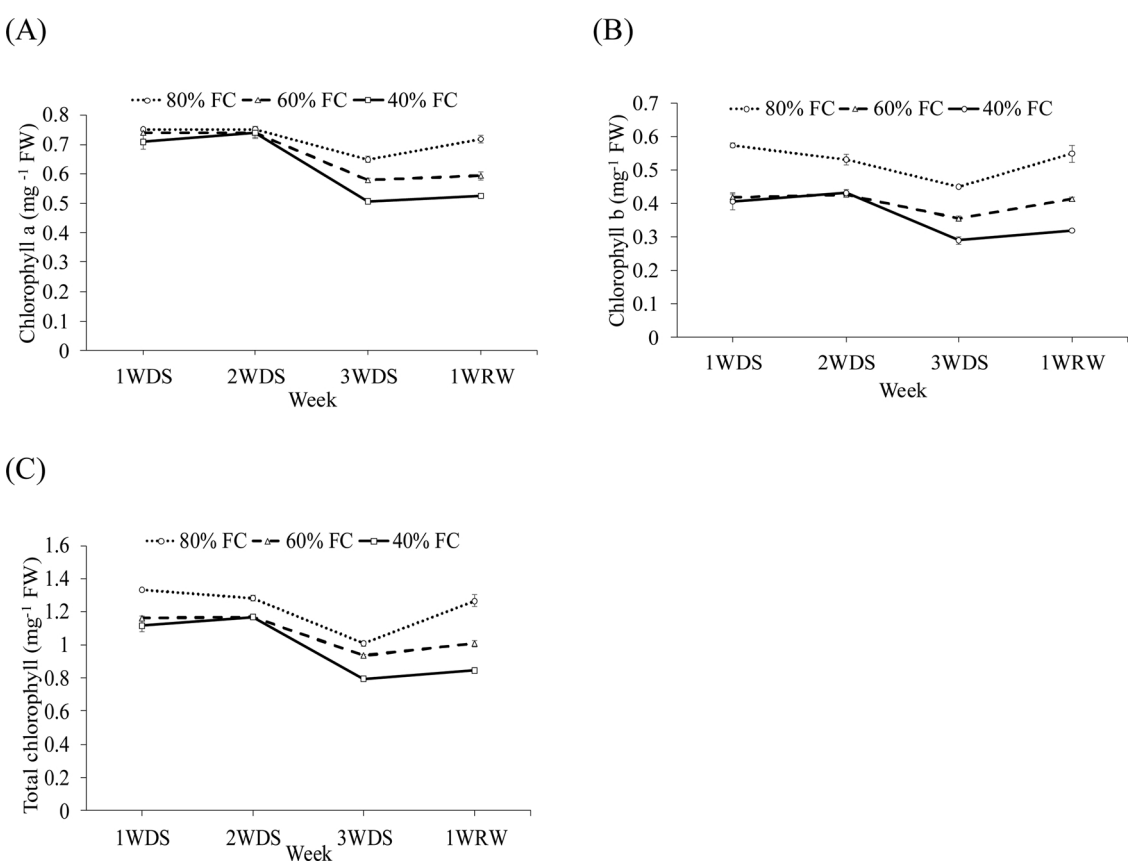

Fig. 2. Effect of drought stress and re-watering on chlorophyll *a* (A), chlorophyll b (B), and total chlorophyll (C) of pansy. Values are means ± SD of three replicates.

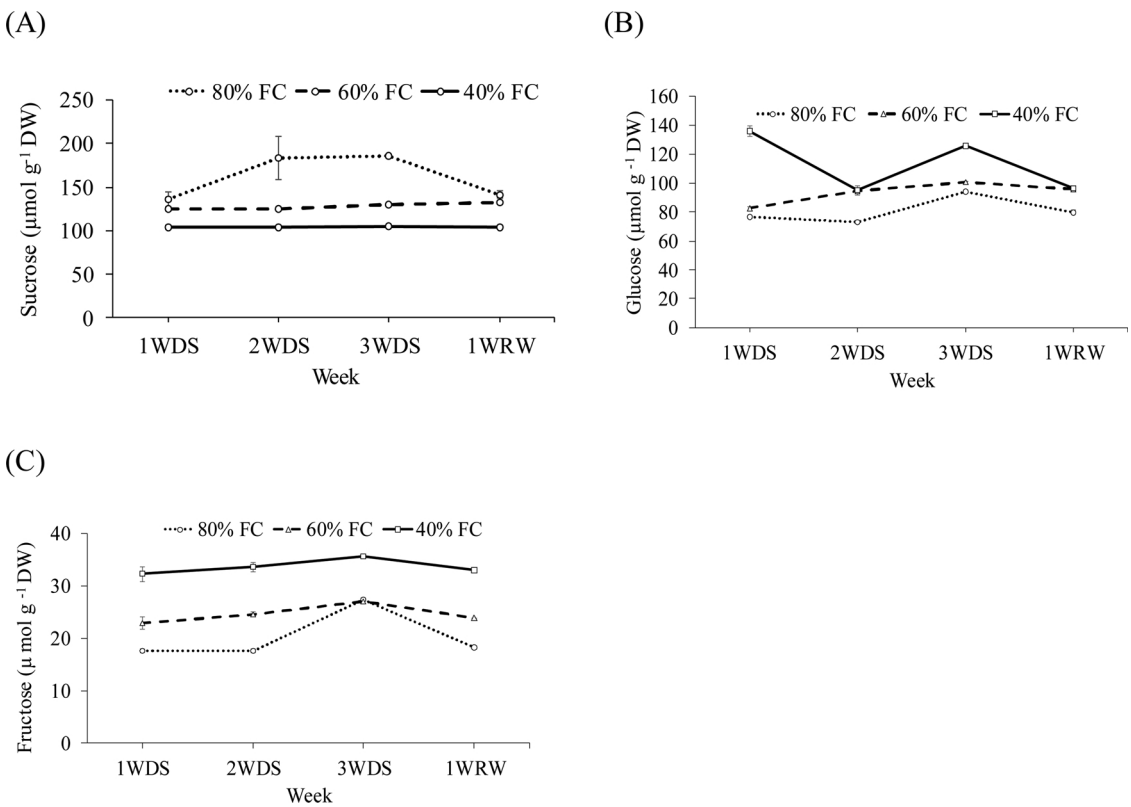

Fig. 3. Effect of drought stress and re-watering on sucrose (A), glucose (B), and fructose (C) of pansy. Values are means ± SD of three replicates.

only one week drought stress (1WDS). Total chlorophyll showed the same trend against water availability. At both levels of drought stress (60 % and 40 % FC), a rapid decrease in the total chlorophyll was observed during the early weeks of stress. As drought progressed, a decline in the pool of chlorophyll occurred in all irrigation treatments and reached the lowest value at 3WDS. After re-watering, chlorophyll increased but still control plants showed higher amount of chlorophyll compared to plants under severe stressed, even one week after re-watering. A gradual increase in chlorophyll *b* was observed in both control and drought-stressed plants, and at 1WDS chlorophyll values under 80 %, 60 % and 40 % FC treatment were not significantly different (Fig. 2B). Total chlorophyll followed the same trend as the others and significantly increased but all treatments showed incomplete recovery within the same re-watering time frame. The highest chlorophyll content was observed in plants under 80 % FC at 1DWS (Fig. 2C).

Moderate and severe drought stress significantly improved the plant carbohydrate content compared to control plants (Fig. 3A.B.C). Sucrose, fructose, and glucose content showed the same trend against water availability and they all reached to their peak amount when plants were under the severe water stress condition (40 % FC). Water deficit of 60 % and 40 % FC, compared to 80 % FC condition at 3WDS, significantly decreased sucrose by 29.8 % and 43.8 % respectively. After re-watering, sucrose under 80 %, 60 % and 40 % FC gradually recovered and were restored to the same level of control plants at 1WDS (Fig. 3A). Plants under severe drought condition, showed high values of glucose at the beginning of experiment. After three weeks, in comparison with 80 % FC plants, glucose increase were about 6.4 % and 26 % for moderate and severe drought stress respectively. One week after re-irrigation, glucose of 80 % FC plant, was recovered to the level similar to control plants at 1WDS, but under 60 % and 40 % FC conditions, glucose decreased to same values of plants at 2WDS (Fig. 3B). The leaf fructose of the control plants were between 18 and 27 µmol g^{-1} DW before re-watering and increased in all treatments from first week to third week. At the end the 3WDS, fructose increased by about 33.3 % for 40 % FC compared to control plants fructose content. One week after re-watering, fructose in the leaves of all plants (80, 60 and 40 % FC) recovered to values similar to those of control and stressed plants at 1WDS (Fig. 3C).

The contents of MDA rapidly increased under 60 % and 40 % FC at 3WDS compared to those plants at 1WDS, but when plants were under 80 % FC, no significant difference was observed between first and third week. Drought-stressed plants contained higher amount of MDA. After re-watering, the concentration of MDA rapidly decreased, especially in control and moderate stress plants and were similar to plants at 1WDS. For severe stress plants, contents of MDA never fully declined to the level of plants at 1WDS (Fig. 4A).

Fig. 4B shows that water stress (60 and 40 % FC) significantly elevated the H_2O_2 content (by 125 % and 166 %, respectively) compared to plants under the control condition (80 % FC) at 1WDS. The highest H_2O_2 content was accumulated under severe water stress (40 % FC) at 3WDS. During stress period, increasing of H_2O_2 was indicated by the progressive increase of the MDA content. After re-watering for 7 days, H_2O_2 content of control and stressed-plant were recovered to the value of those plants at 1WDS.

The enzymatic activities of APX, SOD, DHAR, and GPX significantly increased by 27.8 %, 24.6 %, 103 %, and 175 % respectively, when plants were under severe water stress (40 % FC) compared to 80 % FC at 1WDS (Fig. 5A.B.C.D). A rapid increase in the activities of APX, SOD, and DHAR was observed and reached to its maximum at second week of experiment. Fig. 5A shows that the APX activity dramatically decreased as a consequence of water stress conditions at 3WDS, and the lowest amount was recorded at 80 % FC. After re-watering the APX activity of plants under 80 and 60 % FC decreased to the levels equivalent to those of the plants at 1WDS, but under 40 % FC, the activity of APX never fully declined to the level of plants at 1WDS. At control (80 % FC), a slowly increase in the activity of SOD was observed at 2WDS. After 2WDS, a rapid decrease in APX activity was recorded under 80 and 40 % FC conditions, and reached its minimum at 3WDS. In general,

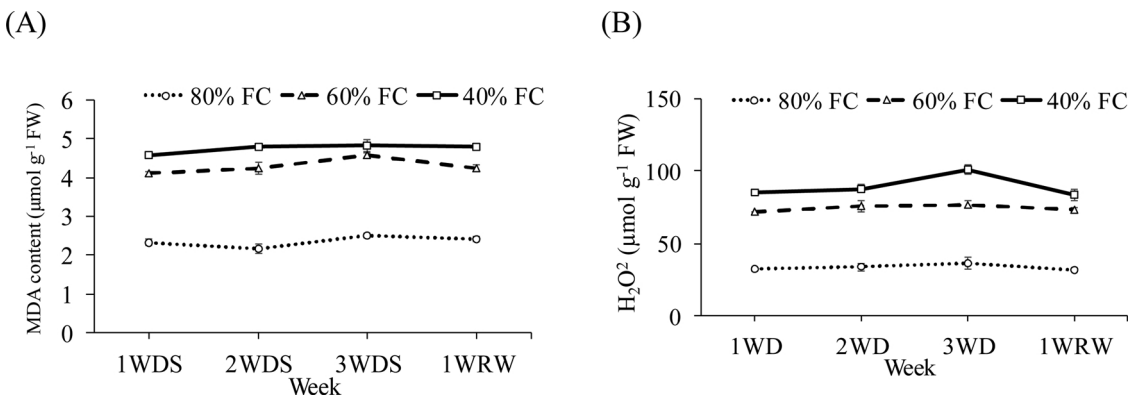

Fig. 4. Effect of drought stress and re-watering on MDA content (A), and H_2O_2 (B) of pansy. Values are means ± SD of three replicates.

antioxidant activities were higher under severe drought stress than medium drought stress. After re-watering, SOD activity tended to be restored in all treatments, and recovered immediately almost to the level of those plants at first week of treatments (Fig. 5B). Fig. 5C shows that drought stress induced a gradual increase in DHAR activity. As drought stress intensified, a decrease in the activity of DHAR occurred in 80, 60 and 40 % FC conditions, which was more prominent at 40 % FC. After re-watering, DHAR activity decreased but remained higher in severe drought stress plants compared to control plants, even after 3WRW however for those plants at 1WDS, its activity immediately restored to same value under all stress treatments. GPX activity did not follow the same trend as the other antioxidant enzymes. GPX activity increased in the 60 and 40 % FC compared to the 80 % FC. The highest GPX activity was observed under 40 % FC at 3WDS compared to other treatments and its activity increase were about 114 % and 178 % for moderate and severe drought stress respectively, as compared with 80 % FC plants at 3WDS. By comparison, in plants under 80, 60 and 40 % FC, GPX activities recovered to the level of those plants on first week of drought stress (Fig. 5D).

Drought stress decreased dry weight of pansy. The dry weight of above ground parts of pansy significantly decreased in 60 and 40 % FC (by 5.67 % and 44.9 %) compared to 80 % FC at 1DWS. As drought continued, a decrease in the dry weight of stem occurred in all treatments, which was more pronounce at 40 % FC after three weeks of drought stress. Water stress (60 and 40 % FC) significantly decreased dry weight (by 19.8 % and 43.5 %, respectively) compared to plants under the control condition at 3WDS. Plants showed root low dry weight of at the beginning of experiment under severe drought condition. After three weeks, root dry weight was about 14.2 %, and 30.2 % lower for moderate and severe drought stress respectively, compared to control plants. One week after re-watering, dry weight of above ground parts of plants started to recover gradually, and it was immediately restored to the levels of those plants under control and drought stress conditions at 1WDS (Fig. 6A). Fig. 6B shows that root dry weight

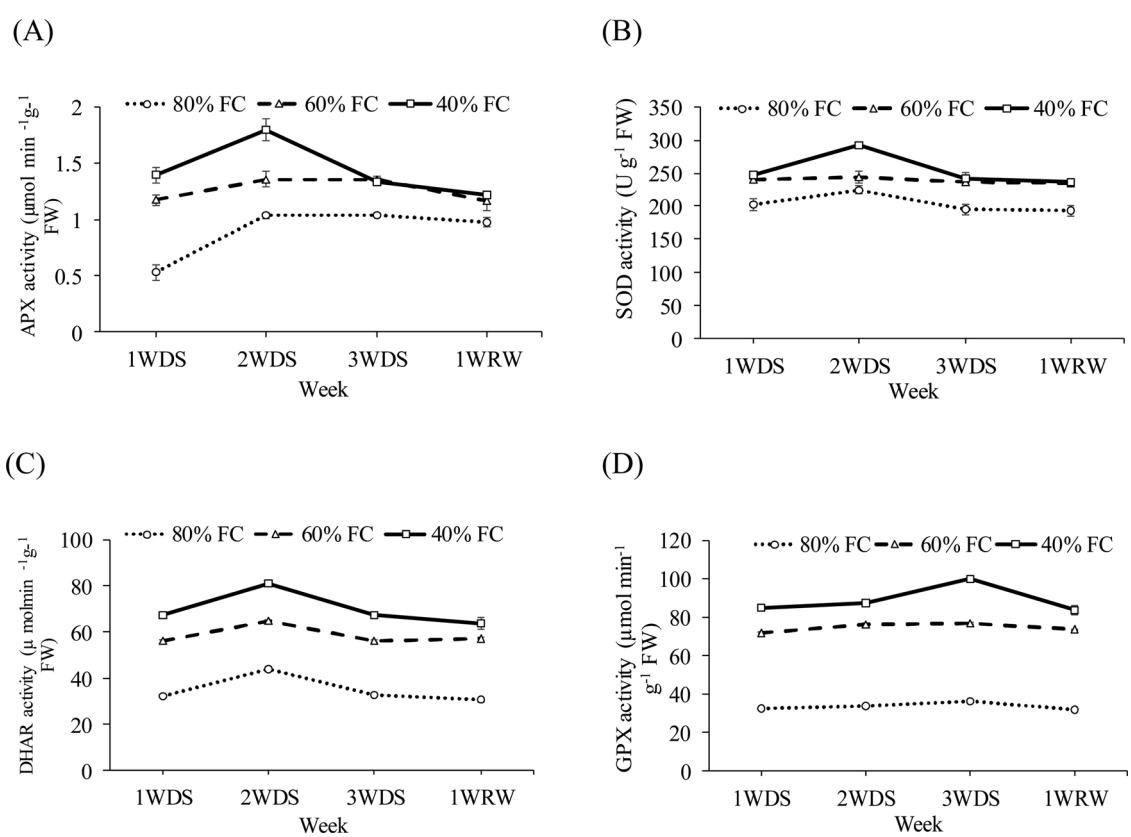

Fig. 5. Effect of drought stress and re-watering on APX (A), SOD (B), DHAR (C), and GPX (D) of pansy. Values are means ± SD of three replicates.

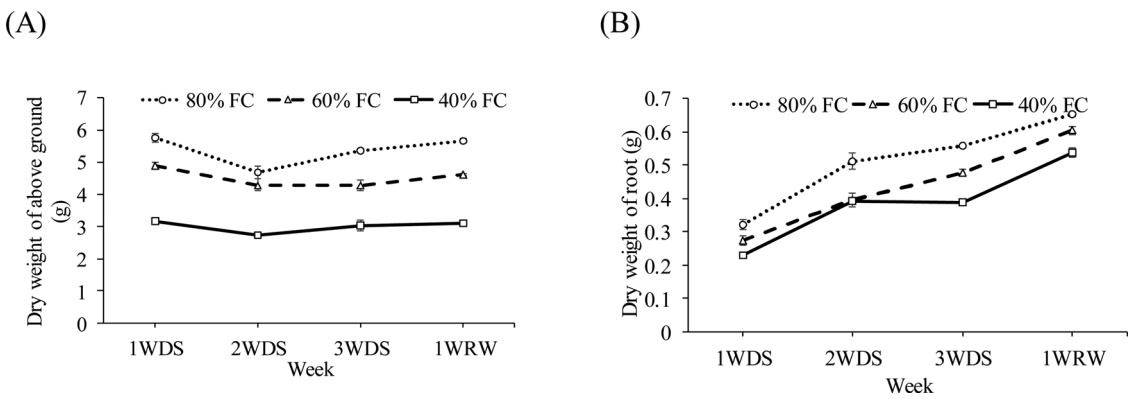

Fig. 6. Effect of drought stress and re-watering on dry weight of above ground parts (A) and root dry weight (B) of pansy. Values are means ± SD of three replicates.

significantly increased in 80 %, 60 % and 40 % FC conditions after re-watering compared to 1WDS (by 102 %, 120 % and 135 %, respectively).

4. Discussion

Several drought tolerance mechanisms were employed by the pansy plants and included ROS detoxification, maintenance of leaf water relations, and improved membrane stability. These mechanisms enabled the plants to avoid lasting drought-induced damage, thereby allowing tolerant plants to more readily recover their physiological functions after re-watering. It was observed that plants up regulated ROS scavenging processes through enhanced antioxidant enzyme activity and exhibited osmotic adjustment. However, the ability of pansy plants to maintain functioning during drought stress and recover after re-watering were dependent on the severity of drought stress.

Electrolyte leakage was affected by irrigation treatments and low value of EL was observed at the beginning of the study, but EL significantly increased after three weeks. Structural integrity of cellular membranes is also important for survival under severe dry periods, or in situations where random droughts occur (Martinez et al., 2004). It is generally accepted that the decrease of EL under water stress is a major component of drought tolerance in plants (Bajji et al., 2002; Kocheva et al., 2004). In the other hands, cell membrane injury might be a result of ROS accumulation leading to damages in membrane stability and integrity (Foyer and Noctor, 2005). This study results showed that there was a significant increase in the EL when the plants were exposed to severe drought stress, and accumulation of ROS in plants under severe water stress was higher than other treatments. Therefore, osmotic adjustment and maintaining cell turgor, and ROS scavengers are involved in decreasing damage to biological membranes. These different strategies depends on plant cultivar and the intensity of water stress (Pourghayoumi et al., 2017).

Relative water stress as a rapid and reliable index of stress level is very important in a number of plant species and closely related to physiological responses at the leaf and whole plant level (Ganji Arjenaki et al., 2012; Wang et al., 2019). It is an important practical index in irrigation management for ornamental plants. In our study the high difference of relative water content between control and water stressed plants confirms that sufficient water stress was imposed on plants. RWC significantly decreased in 40 % FC compared to other treatments. As water stress continued, RWC was significantly affected in all irrigation treatment, but plants maintained high RWC at 3WDS. High RWC during drought stress is very important to maintain metabolic activities in plants, and is a well-known mechanism in breeding toward drought tolerance (Soltys-Kalina et al., 2016). After re-watering, RWC tended to be restored in 80 and 60 % FC conditions, and recovered immediately almost to the level of those plants at 1WDS and a slower recovery of RWC was exhibited by severe drought stress (40 % FC). Recovery of RWC after re-watering is a very important factor in drought tolerant studies. Fast recovery of RWC in the areas with water scarcity is an important factor for ornamental planting (Slabbert and Kruger, 2014).

Severe drought stress can also alter the concentrations of chlorophyll. Reduction of chlorophyll content has been reported in Tagetes erecta (Asrar and Elhindi, 2011), *Nerium oleander* (Kumar et al., 2017) and *Periploca angustifolia* (Dghim et al., 2018) under severe drought stress. Chlorophyll (a, b and total) contents of plants showed a decrease in the present study, when they were deprived from water. Photosynthetic pigment is the material basis of plant photosynthesis, and its composition and content directly affect the photosynthesis of leaves and dry weight (Rahbarian et al., 2011; Li et al., 2019). The change of chlorophyll content under drought stress was related to the biological characteristics of plant and the maintenance of high chlorophyll content might be a physiological adaptation mechanism of plants under drought stress (Rosales-Serna et al., 2004). After re-watering, chlorophyll *a*, and b tended to be restored in 80 % FC conditions, and recovered immediately to the level of those plants at 1WDS. Increasing chlorophyll content in control and drought-stress plants is more effective way to avoid the damage of photo-oxidation and ROS induction by drought (Vilagrosa et al., 2010; Lu et al., 2019).

One of the major objectives of this study was to determine changes in the accumulation of specific compounds in response to drought stress and their effect on the improvement of drought tolerance. Increases in drought tolerance have been associated with the capacity to accumulate protective compounds, including amino acids and carbohydrates (Mafakheri et al., 2011; Metwally et al., 2013). Proline has been reported to improve cell turgor, maintain cell osmotic adjustment and protect cell during dehydration (Chegah et al., 2013). In this study it was observed that proline concentration was higher in plants under drought treatments as compared with control plants at 3WDS. These findings are in agreement with the results reported by other studies (Goodarzian Ghahfarokhi et al., 2015; Abid et al., 2018) and support the importance of proline as a protective component in response to stress (Man et al., 2011; Baloğlu et al., 2012; Aghaie et al., 2018). We observed that after re-watering, the proline concentration of plants under 80 % FC returned to the values of control plants at 1WDS. Under drought stress, the accumulation of proline was inversely proportional to the water status of plants (Fig. 1B), i.e., proline production were correlated with a decrease in RWC (Zegaoui et al., 2017), suggesting the contribution of these solutes in osmotic adjustment. In our study, proline content was associated with antioxidant activity ($r = 0.89$, $P < 0.01$). Recent researches have noted that proline may act also as an antioxidant and not only as an osmotic protectant. Thus, accumulated proline is assumed to play a protective role under drought stress as antioxidant or through stabilizing macromolecules during drought (Seki et al., 2007).

The glucose, fructose and sucrose contents of the pansy plant in control and water stress conditions, and following re-watering, were

compared, in relation to water stress. It is well established that high soluble carbohydrate accumulation contributes to the adaptive mechanism in response to drought stress in plants (Shamsi, 2010; Gupta et al., 2018). In this study, the long-term drought stress provoked a significant increase in glucose, fructose, and sucrose concentrations (Fig. 3). These results are in agreement with reports about Frankenia (Chegah et al., 2011), and Safflower (Mohammadi et al., 2016). Carbohydrate has been reported to improve membrane stability in response to dehydration-related stresses (Lei et al., 2014), and is proposed to have roles in the oxidative stress response (Keunen et al., 2013). The rapid increase in glucose and fructose under drought stress and the high rate at which it decreased after re-watering suggests that the prime role of soluble carbohydrate in these plants is the storage of carbon. By contrast, the behavior of sucrose during the drought phase suggests that it was more available than other carbohydrate.

The dramatic decrease in sucrose, the major carbohydrate reserve, detected in leaves of pansy, during exposure to drought conditions, may have contributed to the observed loss of dry weights of plants in 60 and 40 % FC conditions. However, the rate of breakdown of this carbon source may also provide evidence of other metabolic processes, accompanying glucose and fructose mobilization, and of important changes in carbon partitioning, relating to the initiation of biosynthetic pathways, leading to repair of stress-induced cell membrane damage during recovery processes, including the re-establishment of the photosynthetic system (Akinci and Losel, 2010). Under drought stress, chlorophyll content will be reduced, and translocations of carbohydrate will be slowed from source leaves to sink tissue because of reduced water movement and inhibited growth of active sink cells (Lemoine et al., 2013; Yang et al., 2019). In addition, synthesis capabilities of glucose and fructose would be increased under drought stress.

In this study, under drought stress, a higher accumulation of soluble sugars, and proline were recorded (Figs. 1.3). It is suggested that these compatible solutes may aid in stress tolerance in pansy plants by improving osmotic adjustment, ROS detoxification, and cell membrane protection (Hayat et al., 2012; Hajihashemi and Sofo, 2018; Singh and Bhardwaj, 2019). As a result of the higher accumulation of osmolytes, the osmotic potential of cells was decreased, which in turn facilitated diffusion of water into the cell, thereby maintained a higher turgor potential. The maintenance of favorable cellular turgor potential under water limited conditions allows the plants to maintain physiological and morphological functions such as CO_2 assimilation, cell expansion, development and growth (Serraj and Sinclair, 2002). Data presented in Fig. 3 indicated that pansy plants responded to drought stress with increased sugar accumulation, which might have increased the ROS scavenging potential of plants under drought condition (Wei et al., 2019). Additionally, after re-watering, a rapid reduction in sugar levels might be an indication of a quick breakdown of sugars upon relief from stress providing the plants with sufficient energy to repair damaged tissues (Abid et al., 2018). Therefore, it can be elucidated that the synergistic association of sugars with the cellular antioxidative system contributes to drought tolerance in pansy.

Because of chlorophyll degradation, CO_2 availability and carbon fixation are reduced, ROS formed due to saturation of the electron transport system but limited availability end electron acceptors (Najar et al., 2019). We observed an excessive accumulation of H_2O_2, leading to oxidative stress which caused an increase in MDA content (Fig. 4), an indicator of oxidative damage to the membranes of stressed plants (Chakraborty and Pradhan, 2012; Hussain et al., 2019). Higher ROS and MDA contents in the 40 % FC condition as compared to other treatment might be associated with greater electrolyte leakage under drought stress and an increased potential for ROS production. ROS capable of damaging the photosynthetic apparatus and cause oxidation of lipids and carbohydrates (Hussein and Safinaz, 2013; Kabiri et al., 2014).

Our results showed that higher MDA concentration in drought-stressed plants was associated with higher H_2O_2 content, especially in the severely drought-stressed plants. Pansy plants displayed a suite of drought resistance and recovery traits to overcome the effects of oxidative stress. Among the wide array of antioxidative enzymes that function in scavenging ROS species, ascorbate peroxidase (APX) has a special role as it act directly upon the H_2O_2 molecules, and reducing it to water. SOD carries out the dismutation reaction by reducing O^{-2} molecule to H_2O_2 whereas APX and GPX are involved in converting H_2O_2 to water thus ensuring its removal (Gupta et al., 2016). During water stress period, the levels of SOD, APX, and DHAR immediately increased, but as the stress period prolonged to 3DWS, their levels decreased (Fig. 5). This might be because these ROS scavengers are usually water soluble, and are destroyed during ROS detoxification or through self-oxidation. The cell has limited capability to re-synthesize the destroyed or oxidized scavengers during an extended period of stress (Khanna-Chopra and Selote, 2007). Consequently, tissues become extremely prone to ROS attack under prolonged stress (Tripathy and Oelmüller, 2012).

Severe drought stress displayed enhanced antioxidant enzyme activity (SOD, APX and DHAR) and higher MDA accumulation. Our results indicate that enhanced ROS detoxification promotes drought tolerance by decreasing oxidative damage to tissues, thereby facilitating greater recovery in more drought tolerant. An increase in GPX concentration in all irrigation treatments at 3WDS seems counterintuitive initially, but it is likely that upregulation of antioxidant enzymes is the dominant method of ROS detoxification in the plant, whereas the plants exploited GPX as an attempt to mitigate oxidative stress (Fig. 5D). A greater magnitude of increase in the concentration of GPX in plants under 40 % FC relative to pansy leaves during drought is in accordance with the previous report about rice crop given by Kamarudin et al. (2018), who reported that a similar increase in GPX due to drought stress.

The chlorophyll might have played an important role in restraining ROS accumulation in the chloroplasts via photoprotection of the photosystem (Abid et al., 2018). After re-watering, the return of H_2O_2, and MDA concentrations in plants under 80 % FC to the level of control plants at 1WDS indicated that pansy plants have the ability to tolerate and recover from water stress at the cellular level. Moreover, the activities of SOD, APX, HDAR and GPX remained higher under severe stress but returned to level of control plants at 1WDS under 80 % FC condition after re-watering (Fig. 5), indicating that enzymatic antioxidants of plants under 80 % FC recovered to levels comparable to control conditions at 1WDS, and that a steady-state level of ROS generation and scavenging rates was reached that minimized oxidative stress, which was also confirmed by lower MDA levels after re-watering (Fig. 4).

One of the first signs of stress is lower turgor which causes a decrease in both growth and cell development, especially in the stems. The growth of cells is the most important process that is affected by stress, and low cells growth leads to decrease in plant dry weight of. Growth reduction as a result of different stresses has been widely reported in many studies (Cirillo et al., 2017; Souza et al., 2018; Alvarez et al., 2018). In this study, specific metabolic responses were exhibited by pansy plants to cope with the effects of drought stress. These differences in metabolic response to drought also resulted in altered dry matter accumulation. This positive relationship revealed that dry weight is related to the plant water status. Our results showed that the reduction in dry weight in 40 % FC was larger than those in other treatments. Nevertheless, under this treatment high RWC was maintained during the stress period. After three weeks imposing water stress, plants showed high dry weight under 40 % FC with the lowest RWC and highest sugar accumulation. It seems that osmotic adjustment mechanism did not play a significant role against drought stress.

5. Conclusion

Pansy plants exhibited different metabolic features in terms of ROS

accumulation, oxidative damage, antioxidant capacity, and production of osmotically active solutes under moderate and severe drought levels in the present study. In the current study there were two tolerance mechanisms employed in response to drought stress; the first involved in accumulation of soluble sugars, and proline to facilitate osmotic; whereas the second involved adjustment the increase of antioxidant enzyme (APX, SOD, DHAR, and GPX) activity. These metabolites allow the pansy plant to withstand and survive water-stress conditions. These results revealed that plants ability to maintain physiological functions during drought and recover after re-watering during flowering period are important for determining final productivity of pansy.

CRediT authorship contribution statement

Atiyeh Oraee: Writing - original draft, Investigation, Software. **Ali Tehranifar:** Supervision, Writing - review & editing, Validation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2020.109225.

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