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**ABSTRACT**

It is necessary to identify the heat-tolerant cultivars of bedding plants in order to cope with the challenges of the global warming. The physiological and anatomical responses of four calendula cultivars (Candyman’, ‘Zen’, ‘Indian Prince’, and ‘Pacific Beauty’) to four duration levels (0, 7, 14 and 21 days) of heat stress (in a mean range of 35–42°C), were investigated after their establishment under greenhouse conditions. The results indicated that calendula cultivars displayed different responses to various heat duration levels in most parameters. Although the gas exchange of ‘Candyman’ was the highest in control treatment, it showed a considerable reduction in all levels of stress durations. In contrast, ‘Indian Prince’ maintained gas exchange and chlorophyll content and exhibited the most resistance to heat stress among others, especially for longer duration exposure, which might be resulted from the greater soluble sugar content, higher stomatal density, and thicker leaves. Experiment to assess cell membrane thermostability showed heat stress resulted in significant increase in electrolyte leakage percentage as the incubation temperature of all cultivars was increased. However, the least membrane stability was observed in ‘Zen’, indicating the higher sensitivity of this cultivar to heat-stress conditions in comparison to three other cultivars.

**Introduction**

Heat stress is a serious threat to agricultural production worldwide and it is projected to be increased at 1.5–5.8° by 2100 due to the global warming (Hemantaranjan et al., 2014). By definition, heat stress occurs when the temperature exceeds a critical threshold for a sufficient time to motivate irreversible damages to plant processes. The effect of high-temperature stress depends upon the duration, rate, and magnitude increase in temperature (Hall, 2001). At these elevated temperatures, a set of morphophysiological, anatomical, biochemical, and molecular modifications may occur, leading to reductions in vegetative and reproductive growth in plants (Wahid, Gelani, Ashraf, & Foolad, 2007).

Photosynthetic function is recognised as one of the most sensitive indicators of physiological processes to elevated temperatures (Crafts-Brandner & Salvucci, 2002). In general, stomatal and non-stomatal limitations reduce plant gas exchanges under heat stress (Pelletier, Pepin, Gallichand, & Caron, 2016). Stomata closure to reduce water loss, altering stomata and trichome density are the most common anatomical changes observed under high environmental temperatures in plants (Bañon Fernandez, Franco, Torrecillas, Alarcón, & Sánchez-Blanco, 2004). At the cellular level, high temperature also causes alterations, including membrane damage, reduction of enzymes activity, degradation, aggregation or denaturation of proteins, and inhibition of protein synthesis (Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013). Cell membrane thermostability (CMT) test is an accurate and fast laboratory procedure to assess heat tolerance of several genotypes in plant species. In this technique, percentage of membrane injury is determined by measuring the electrical conductivity of water around the leaf tissue exposed to a range of temperatures (Wang & Yeh, 2008).

Some of the specific organic compounds with low-molecular weight known as compatible osmolytes like proline, sugar alcohols (polyols) and sugars, glycine betaine, quaternary and tertiary ammonium cations are accumulated in the plant under abiotic stress including high temperature. This is referred as one of the most basic adaptive strategies for the surviving plant species under stressful conditions (Harsh et al., 2016; Wahid et al., 2007).

Production of bedding plants that create a range of different flower colours and designs in landscape arrangement continues to have great economic value in the floriculture industry (Mader, 2009). High-temperature stress during summer months severely reduces the marketable quality and landscape survival capacity of
cool-season bedding plants (Natarajan, 2005). However, cultivars within species may vary in heat sensitivity or tolerance (Jong-Yi & Nhu-Ai, 2016). For example, Warner and Erwin (2006) reported that among the 12 pansy studied cultivars, the cultivars ‘Delta Yellow’ and ‘Super Majestic Giants Canary’ indicated the most thermotolerance determined by the weighted base selection index, while ‘Majestic Giants Rose Shades’ and ‘Super Majestic Giants Ocean’ were introduced as the most sensitive cultivars to high temperatures.

Calendula (Calendula officinalis) is a popular cool-bedding plant of the Asteraceae originated from southern Europe and the Mediterranean Sea basin (Dole & Wilkins, 2004). Low to moderate day/night temperature regime of 14–17/13–14°C is preferred for its growth (Anderson, 2013). Reduction in the flower diameter, and flower number in calendula has been reported with an increase in temperature (Warner & Erwin, 2005). The results of our previously reported study also showed that morphological characteristics (plant height, leaf area, shoot and root weight, flower diameter, flower number and flower longevity) of calendula cultivars were reduced by high temperature stress, especially in prolonged exposure. However, the extent to which growth and flowering parameters were reduced differed among cultivars (Nazdar, Tehranifar, Nezami, Nemati, & Samiee, 2017). The study of physiological and anatomical changes of calendula, as an annual ornamental-medicinal cool-bedding plant under heat stress, may provide a better understanding of the traits pertaining to its heat tolerance.

This research is the first report on the screening of the physiological responses of calendula cultivars to high-temperature stress. The purposes of the present research were: (1) to evaluate the leaf membrane damage in four cultivars of calendula exposed to various temperatures employing the CMT test, and (2) to evaluate the impact of different high-temperature exposure durations on physiological (chlorophyll content, net photosynthesis, stomatal conductance, transpiration rates, leaf soluble sugar, and proline content) and anatomical (stomatal number, stomata size, and leaf thickness) responses of calendula cultivars.

Materials and methods

Cell membrane thermostability (CMT) test

This experiment was carried out to survey if CMT test is an efficient method of screening heat tolerant cultivars of calendula. A modified version of Blum (1988)’s technique was used to evaluate the percentage of membrane damage. Two-month-old plants of four cultivars which had been grown in a greenhouse at an average daily temperature of 25°C and natural sunlight were acclimated for 24 h at 35/30°C in a programmed growth chamber. Fully expanded leaf samples were collected from three plants tested per cultivar. Ten leaf discs per replicate on each side of the middle vein (each of 10 mm in diameter) were punched using a cork borer. Freshly collected leaf discs were carefully washed two to three times with deionised water in glass vials to eliminate any electrolytes both sticking to the leaf surface and leaching from the injured portion of the leaf tissues. After drainage of water, glass vials were covered with aluminium foil to prevent water evaporation and placed in thermostated water baths set at 30, 35, 40, 45, 50, 55 and 60°C for 1 h. For the control treatment, the vials were kept at the same time at room temperature (25°C) (Natarajan, 2005). There were three vials per cultivar. After that, the test vials were cooled to reach room temperature and 25 mL of distilled deionised water was added to all vials for treatment at about 10°C for 24 h. The initial measurement of conductance of water solution around the leaf discs was taken using an electrical conductivity metre (Jenway, UK). Vials containing the pieces of leaves were then autoclaved for 15 min at 121°C in order to measure the maximum membrane damage due to the release of all electrolytes. After autoclaving, the vials were equilibrated at room temperature and final conductivity was recorded again. Electrical conductivity records were utilised for determination of the lethal temperature (LT_{50}). By definition, LT_{50} is the lethal temperature resulting in 50% membrane injury, measured by the electric conductivity of the solution surrounding the leaf tissues incubated at various rising temperatures (Ortiz & Cardemil, 2001).

The percent membrane damage was estimated by the following equation:

\[
\text{Percentage of membrane damage} = \frac{C_x - C_c}{C_m - C_c} \times 100
\]

where \(C_x\) and \(C_m\) are the mean conductivities of water around the leaf tissue samples incubated at various temperatures before and after autoclaving, respectively, while \(C_c\) is the conductivity of the test vial kept at 25°C (control).

Effect of heat-stress duration on physiological and anatomical parameters

Plant material and high-temperature treatment

This experiment was carried out in the agricultural research glasshouse of Ferdowsi University of Mashhad in Iran during September to March 2015–16. Four calendula (Calendula officinalis L.) cultivar seeds, including ‘Candyman’, ‘Zen’, ‘Indian Prince’, and ‘Pacific Beauty’ were obtained from Hem Zaden BV (Netherlands), Takii (Kyoto, Japan) and Chiltern Seeds (Wallingham, England). The two studied cultivars of calendula, ‘Indian Prince’ and ‘Pacific Beauty’ were selected because they have been introduced empirically as more resistant cultivars to high temperature than ‘Candyman’ and ‘Zen’.

Materials and methods

Cell membrane thermostability (CMT) test

This experiment was carried out to survey if CMT test is an efficient method of screening heat tolerant cultivars of calendula. A modified version of Blum (1988)’s technique was used to evaluate the percentage of membrane damage. Two-month-old plants of four cultivars which had been grown in a greenhouse at an average daily temperature of 25°C and natural sunlight were acclimated for 24 h at 35/30°C in a programmed growth chamber. Fully expanded leaf samples were collected from three plants tested per cultivar. Ten
The seeds were germinated in plastic trays containing cocopeat kept at ~ 27/18°C (day/night regime) and 12-h photoperiod, and a PPFD (photosynthetically active radiation) of 400–600 μmol m⁻² s⁻¹ was supplied from natural sunlight. Forty-five days after germination, healthy and uniform size seedlings were transplanted into plastic pots (14 cm in diameter and 12 cm in length) filled with a mixture of loam soil, sand, and vermicompost (6:3:1 v/v). There were 32 pots of each cultivar with a total of 128 pots. Plants were established for 50 days in a greenhouse described above before being subjected to high-temperature treatments. To determine the effects of heat-stress duration on calendula cultivars, 24 pots of each established cultivar were transferred to a mini plastic greenhouse setting of 42/35°C (mean maximum and minimum temperature during the heat phase period of experiment), where they were maintained for 21, 14 and 7 days. Heat-stress conditions were provided by two electric fan heaters equipped with a thermostat. The control plants (eight pots of each cultivar) were maintained at 28/20°C until the end of heat-stress treatments (for 21 days) in a separate mini plastic greenhouse under identical conditions, except for the heat source. During the stress period, the position of the pots was changed randomly every day in plastic greenhouses to avoid positional effects. The relative humidity in both two mini plastic greenhouses was 75–80%, and the photoperiod was 12 h (from 06:00 to 18:00) with the PPFD of 200–400 μmol m⁻² s⁻¹ at the topmost part of the plant canopy which was supplemented with fluorescent and incandescent lamps. Plants were watered daily for non-heat phase and twice a day for heat phase throughout the experiment to avoid any water-deficit stress. The irrigation system was manual, and all pots received equal quantities of water.

After heat stress, all pots were returned to the original greenhouse (27/18°C) with conditions stated above. All physiological and anatomical measurements were made on the youngest fully expanded leaf after the plants had been returned to the greenhouse (2–4 days after the end of exposure to heat-stress conditions). Physiological traits were measured on the four plants of each cultivar at each of the four temperature durations, while anatomical traits were measured on three plants of two cultivars, i.e. 'Candyman' and 'Indian Prince'.

**Measurements**

**Chlorophyll content.** To estimate leaf chlorophyll (Chl) concentration, samples were collected from the recently fully developed leaves of four plants per treatment. One-hundred mg of fresh leaves from the middle part of each leaf was ground with 4 mL of 80% acetone for pigment extraction. The supernatant was then centrifuged at 3000 rpm for 5 min, and the absorbance of the extracts was measured using a spectrophotometer (2100) (SHIMADZU; AA670, JAPAN) at 647 and 664 nm. Chlorophyll content (a, b and total) was calculated using the Lichtenthaler equation (Lichtenthaler, 1987).

**Gas exchange parameters.** The net photosynthetic rate (Pn), transpiration rate (E) and stomatal conductance (gs) were determined on the youngest entirely expanded leaf using an LCI portable photosynthesis system (ADC Bioscientific Ltd., Hoddesdon, England) in a relative humidity of 50 ± 5%, 400–600 μmol m⁻² s⁻¹ PAR, leaf temperature of 25°C, and ambient CO₂ concentration.

**Proline content.** The proline concentration of the leaf tissue was measured as per Bates (1973)’s procedure. So, 0.5 g of fresh leaf samples was homogenised with 10 mL of 3% sulphosalicylic acid. After 5 minutes of centrifuging at 3000 rpm, 2mL of the supernatant was reacted with equal volumes of glacial acetic and ninhydrin reagent acid (1.25 mg ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) at 100°C for one hour. After incubating, the reaction was ended in an ice bath and then, the reaction mixture was extracted with 4 mL of toluene and was mixed vigorously with a test tube stirrer for 15–20 sec. The absorbance of the upper phase was read at 520 nm by a spectrophotometer (SHIMADZU; AA670, JAPAN) after 20 minutes. The proline content was calculated using the standard curve and expressed in μg g⁻¹ fresh weight.

**Total soluble sugar content.** Total soluble sugar (TSS) content was determined as described by Irigoyen, Emerich, and Sanches-Dias (1992). A sample of 0.5 g of freshly collected leaves was crushed in a mortar and 5 mL of 95% (v/v) ethanol was added to it. The insoluble fraction of the extract was washed with 5 mL of 70% ethanol twice. The mixture was centrifuged at 4500 rpm for 15 min. Then, 0.1 mL of the alcoholic extract was blended with 3 mL of freshly prepared anthrone (150 mg of anthrone, 100 mL of 72% sulphuric acid, W/W). The mixture was held in a boiling water bath for 10 min. After cooling, the light absorption of the samples was determined by a spectrophotometer (SHIMADZU; AA670, JAPAN) at 625 nm. Total content of soluble sugar was calculated by using glucose standard and was expressed in mg g⁻¹ fresh weight of leaves.

**Stomata features and leaf thickness.** Leaf anatomic characteristics, including stomatal number, stomata size, and leaf thickness, were determined in two cultivars ('Candyman' and 'Indian Prince' as heat sensitive and resistant cultivars, respectively). These two cultivars were selected based on visual differences in morphological resistance to heat stress before data
analysis. Three recently fully expanded leaf samples per treatment were collected from the plants during the middle of the day to take scanning electron micrograph images. The leaf samples were dried using a freeze dryer for 48 h. The dried samples were mounted on normal scanning electron microscope (SEM) stubs with a double-sided adhesive tape, coated with 25-nm gold palladium by using a Hummer II Sputter Coater and investigated with SEM (LEO1450VP) operated at 20 kV. The SEM images of abaxial surface and cross-section were magnified to a fixed resolution (1000 × for stomatal density, 5000 × for stomata size, and 1000 × for leaf cross-section). Leaf stomatal density was defined as the number of stomata per unit leaf area. SEM images were analysed to measure leaf thickness and the size of stomata by ImageJ imaging software (ver1.44).

In general, *C. officinalis* was reported to be amphistomatous (bearing their stomata on both surfaces of leaves). Stomata are present in about equal number on both leaf sides of calendula. However, at low irradiance, more stomata are found on the abaxial side (Groen, 1973; Kumar, Sharma, & Sharma, 2010), so we used the lower surface on calendula.

**Experimental design and statistical analysis**

Data were analysed using JMP8 software package (SAS Institute Inc., Cary, NC, USA) in a 2-factor factorial experiment based on a completely randomised design for heat-stress duration experiment and a single-factor completely randomised design for CMT test. Differences in means were compared by the Least Significant Difference (LSD) test at $p < 0.05$ level.

**Results**

**Cell membrane damage**

As the incubation temperature was raised, there was an increase in the percent membrane damage of all studied cultivars (Figure 1). Percent membrane damage was raised more quickly at the onset at 25°C for ‘Zen’, whereas the three other cultivars showed similar results, and a sigmoidal fashion in their membrane damage was found: a gradual rate at first from 25°C to 35°C, and as temperature was raised to 45°C, membrane damage was very rapid. Above 45°C, all cultivars reacted similarly to high temperatures. There was a difference of 14.4°C, 13.8°C, and 11.8°C among the LT50 of ’Zen’ with ’Candyman’, ‘Indian Prince’ and ‘Pacific Beauty’, respectively; it was 44.4°C for ’Candyman’, 43.8°C for ’Indian Prince’, 30°C for ’Zen’, and 41.8°C for ’Pacific Beauty’ (Figure 1).

**Chlorophyll content**

The results of chlorophyll a and total chlorophyll content are shown in Figure 2 (a, b). Both chlorophyll a and total chlorophyll contents were not affected significantly by various durations in ’Candyman’ and ’Indian Prince’. In contrast, compared with the control, ’Zen’ cultivar showed an average loss of 38.31%, 53.98% and 37.61% in the total chlorophyll concentration, and the content of chlorophyll a was reduced by 40.92%, 60.85%, and 44.83% at 7, 14 and 21 days heat stress, respectively. In ’Pacific Beauty’, the reduction was significant only for 21 day level.

**Leaf gas exchange parameters**

As can be seen in Figure 2(c), the photosynthesis of all genotypes was significantly decreased with extending

![Figure 1. Lethal temperature for 50% membrane damage (LT50) of leaves in four cultivars of calendula. The LT50 values are indicated with arrows for each cultivar.](image-url)
heat-stress duration except for ‘Indian Prince’. Heat stress for 7 days resulted in the greatest loss of photosynthesis rate in ‘Pacific Beauty’ and ‘Zen’ by 49.19% and 33.74%, respectively. However, after 21 days, ‘Candyman’ was most affected by heat stress and as indicated in Figure 2(c), compared with the control, the percent reduction of the photosynthesis was about 70.78%, 20.67%, 58.73% and 64.23% for ‘Candyman’, ‘Indian Prince’, ‘Zen’ and ‘Pacific Beauty’, respectively.

The stomatal conductance of ‘Candyman’ was significantly decreased in response to heat stress, while in three other cultivars, gs was not significantly affected. Extending stress duration from 0 to 7, 14 and 21 days caused a decline in ‘Candyman’ gs by 20.36%, 43.64% and 56%, respectively (Figure 2(d)).

Heat stress significantly reduced transpiration of calendula as compared to control. However, there was no significant interaction between cultivar and duration (data not shown).

**Osmoregulations compounds**

As compared to control, the proline content of ‘Candyman’ and ‘Indian Prince’ was not significantly affected by heat stress, while in ‘Pacific Beauty’, it was

![Figure 2. Effects of heat stress duration on (a) chlorophyll a, (b) total chlorophyll, (c) net photosynthesis (Pn), (d) stomatal conductance (gs), (e) proline, and (f) soluble sugar content in four cultivars of calendula. Bars indicate the mean standard Error (± SE), values with the same letter(s) are not significantly different at P ≤ 0.05 (LSD test).}
significantly decreased after 7 days of heat stress. In contrast, ‘Zen’ showed the highest accumulation under heat stress for 7 days which was up to 2.77 times greater than the control (Figure 2(e)).

The soluble sugar content values obtained from the ‘Candyman’ and ‘Zen’ across the various durations were not significantly different. The soluble sugar content in ‘Pacific Beauty’ was increased by 74.09% under 7 days of high temperature stress as compared to control, while it was decreased by longer heat-stress durations. In ‘Indian Prince’, the soluble sugar content was not significantly affected at 7 and 14 days, as compared with the control. However, when subjected to heat stress for 21 days, it was enhanced significantly by 73.76% (Figure 2(f)).

Stomatal characteristics and leaf thickness

The data reported in Table 1 show that ‘Indian Prince’ had a higher stomata density than ‘Candyman’. In addition, a loss of stomata number was observed with the extension of stress duration, whereas heat-stress duration and cultivar had no significant interaction effect on stomata density (Table 1, Figure 3).

Both genotypes showed an increase in stomatal size after heat stress for 7 days, though the differences were not significant. The percentage increase in stomatal size of ‘Candyman’ after 14 and 21 days of heat stress were about 95.86 and 104.25, respectively, as compared to control plants. However, the stomatal size of ‘Indian Prince’ was significantly affected only by 21-d duration (Table 1, Figure 4).

Though there was a significant effect on leaf thickness in 7 and 14 days levels for both cultivars, the increase was very small. The increase in leaf thickness was about 2.5 times greater under 21 days high temperature than the control temperature for ‘Indian Prince’, but for ‘Candyman’, this increase was about 38.73% (Table 1, Figure 5).

Discussion

Cell membrane integrity under stress conditions is vital to maintaining plant physiological functions such as respiration and photosynthesis (Blum, 1988). In this study, heat markedly affected the cell membrane damage in ‘Zen’ cultivar and a significant change in its membrane damage response curve was detected due to more severe injury to the membrane as compared to the other three cultivars at 30, 35 and 40°C (Figure 1). The greater values of LT50 in ‘Candyman’, ‘Indian Prince’ and ‘Pacific Beauty’ implies that foliar tissues in these cultivars are more heat tolerant than ‘Zen’, which was similar to the finding of Ortiz and Cardemil (2001), Natarajan (2005), and Natarajan, Joshi, Anand, Verma, and Pathak (2005) in various species.

It is observed that chlorophyll content (a and total) was significantly decreased under all durations of heat stress for ‘Zen’ and 21 d stress for ‘Pacific Beauty’. In the other words, the chlorophyll content was most affected by heat stress in ‘Zen’ that was found to be a sensitive cultivar in CMT test. Our observations on reduction in chlorophyll concentration confirm the findings of Guo, Zhou, and Zhang (2006) and Kaur, Bains, Bindumadhava, and Nayyar (2015), who have reported that the content of chlorophyll decreased considerably in heat-stressed species. Disruptions in chloroplast membranes and the inhibition of chlorophyll fluorescence might be resulting in degradation of chlorophyll and inhibition of its biosynthesis under heat stress (Kaur et al., 2015). The decrease in chlorophyll has also been noted to be caused by active oxygen species (ROS) (Djanaguiraman, Prasad, & Seppanen, 2010).

The decrease in gas exchange capacity due to stomatal and non-stomatal factors is considered as one of the major responses to heat stress in plants (Pelletier et al., 2016). It has also been suggested that the inhibition of photosynthesis in response to heat stress may be a consequence of the loss of chlorophyll concentration (Farooq, Bramley, Palta, & Siddique, 2011) or the decrease in Rubisco activation (Crafts-Brandner & Salvucci, 2002). Therefore, the sustained leaf gas exchange under high-temperature stress could be a good indicator of thermostolerance (Wahid et al., 2007). In this study, although gs was decreased in all four cultivars, it was significantly decreased under various durations of heat stress only for ‘Candyman’ (Figure 1(d)). A significant decline in photosynthesis at all durations in ‘Pacific Beauty’ and ‘Zen’ as well as at longer heat stress durations (14 and 21 days) in ‘Candyman’ indicates more sensitivity of these cultivars than ‘Indian Prince’ (Figure 1(c)). Similar conclusions were also reached by Camejo et al. (2005) and Natarajan (2005) where maintaining gas exchange in heat resistant cultivars was higher

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>D (day)</th>
<th>SD (num/mm²)</th>
<th>SS (µm²)</th>
<th>LT (µm)</th>
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<tbody>
<tr>
<td>Candyman</td>
<td>0</td>
<td>192.31 *</td>
<td>171.67 c</td>
<td>70.18 de</td>
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<tr>
<td></td>
<td>7</td>
<td>192.31 *</td>
<td>222.75 bc</td>
<td>83.15 c</td>
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<tr>
<td></td>
<td>14</td>
<td>169.87 a</td>
<td>136.24 a</td>
<td>80.88 cd</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>118.59 a</td>
<td>350.63 a</td>
<td>97.36 b</td>
</tr>
<tr>
<td>Indian Prince</td>
<td>0</td>
<td>230.77 a</td>
<td>170.29 c</td>
<td>65.29 *</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>192.31 *</td>
<td>226.93 bc</td>
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<td></td>
<td>14</td>
<td>166.67 a</td>
<td>226.93 bc</td>
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<td>21</td>
<td>160.26 a</td>
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<td>ANOVA</td>
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* * indicate significance at P < 0.01, 0.05, respectively; ns: not significant, by LSD test.

Superscript letters signify statistical differences among means.
than sensitive ones. The decrease in net photosynthesis observed in calendula plants could be partly attributed to stomatal limitation because of the decline in stomatal conductance under high-temperature stress as compared to control. Niu and Rodriguez (2006), Cui, Li, Fan, Xu, and Zhang (2006), Djanaguiraman, Prasad, Boyle, and Schapaugh (2011) and Chen, Zheng, Li, and Guo (2012) reported a similar loss in stomatal conductance and photosynthesis in other plant species. Stomatal closure which helps reducing the water loss resulted in lower cooling rate of leaf by transpiration (Pelletier et al., 2016). However, transpiration rate is dependent on several factors including environmental conditions (such as vapour pressure deficit, leaf and air temperature, light) and leaf characteristics (leaf size and shape, leaf orientation, leaf area, root to leaf ratio, leaf thickness, and leaf surface characteristics) in addition to the stomatal traits (size, distribution and stomatal openness) (MaBen-Asher, Garcia, Flitcroft, & Hoogenboom, 2013; Mahan, McMichael, & Wanjura, 1997).

Proline and soluble sugar are involved in osmotic adjustment helping plant survival under heat stress in many plant species. Protection of plant cellular structure by these osmolites is thought to maintain membrane stability and cell water balance, scavenge reactive oxygen species (ROS), buffer cellular redox potential, and increase protein stability (Harsh et al., 2016; Wahid & Close, 2007).

Figure 3. SEM images of *Calendula officinalis* stomata density at 1000x magnification (a) 'Candyman', control, (b) 'Candyman', stress for 7 d, (c) 'Candyman', stress for 14 d, (d) 'Candyman', stress for 21 d, (e) 'Indian Prince', control, (f) 'Indian Prince', stress for 7 d, (g) 'Indian Prince', stress for 14 d, and (h) 'Indian Prince', stress for 21 d.
Reducing sugars of soybean leaves (fructose and glucose) has been found to increase by 82.6% when leaf temperature is increased from 28°C to 38°C (Djanaguiraman et al., 2011). Other studies suggest that sucrose at high and low concentrations acts as a ROS scavenger and signalling molecules, respectively (Bita & Gerats, 2013). High carbohydrate has been considered as an important physiological trait associated with heat tolerance (Han, Fan, Zhang, & Wang, 2013; Liu & Huang, 2000). Results of this study indicated that the soluble sugar content of calendula was increased under high-temperature stress, depending on the cultivars. A difference in soluble sugar accumulation suggests that this trait is under genetic control, so high sugar accumulation could be considered a mechanism associated with the higher heat tolerance of the cultivars. Based on the results, the extending heat-stress duration to 21 days was not significantly effective in increasing the soluble sugar content of ‘Candyman’ and ‘Zen’. However, it increased dramatically in ‘Indian Prince’. Similar to the results reported by Han et al. (2013), our data confirmed that soluble sugar accumulation was more increased in high-temperature tolerant variety than high-temperature sensitive varieties.

Ashraf, Saeed, and Qureshi (1994) noted that proline is one of the most important indicators of heat-stress tolerance. The proline increment percentage ranged from 10.75% to 1075% in 28 genotypes of

Vigna aconitifolia as the temperature was increased to 42°C (Harsh et al., 2016). Similarly, the accumulation of proline under heat stress has been reported in sunflower (Amutha, Muthulaksmi, Baby Rani, Indira, & Mareeswari, 2007) and sorghum (Gosavi et al., 2014). Our results showed that although ‘Zen’ was more sensitive to heat stress in most studied traits, the accumulation of proline under heat stress in ‘Zen’ was larger than those in three other cultivars. These results are consistent with those found for other plant species (Han et al., 2013; Premachandra, Hahn, Rhodes, & Joly, 1995; Sundaresan & Sudhakaran, 1995). However, this is contrary to the results of Hien et al. (2003) who reported that proline accumulation in tolerant cultivars is higher than in sensitive cultivars. These differences may be due to test condition variability, plant growth stage, and leaf relative water content. So, it was concluded that there is not always a positive relationship between proline content with heat tolerance level and probably other mechanisms such as sugar accumulation, antioxidant, and heat-shock proteins involved in inducing heat tolerance. It has been stated that proline content can be a water status indicator of plant but not an indicator of its heat-stress tolerance (Harsh et al., 2016).

Gas exchange in plant leaves is usually optimised by altering stomatal frequency, stomatal size, and the degree of stomatal pore openness. In general, stomatal features are under genetic control, but they may be

**Figure 5.** SEM images of leaf cross section at 1000× magnification of *Calendula officinalis* (a) ‘Candyman’, control, (b) ‘Candyman’, stress for 7 d, (c) ‘Candyman’, stress for 14 d, (D) ‘Candyman’, stress for 21 d, (e) ‘Indian Prince’, control, (f) ‘Indian Prince’, stress for 7 d, (g) ‘Indian Prince’, stress for 14 d, and (h) ‘Indian Prince’, stress for 21 d.
affected by environmental variables (Salem-Fnayou et al. 2011; Zheng et al., 2013).

With regard to anatomic responses to heat stress, inconsistent results have been found. For example, as reported by Xu, Zhou, and Shimizu (2009) and Zhang, Niu, Wang, Li, and Zhao (2010), an increased stomatal density has been observed by exposure to high temperature, while the present study indicated that heat stress resulted in a decrease in stomata number which is consistent with the reports by Beerling and Chaloner (1993), Ferris, Niju, Behaeghe, and Impens (1996) and Bañón Fernandez et al. (2004). It is probably due to dissimilar methodological approaches, such as different warming sources and different stress intensity leading to unequal environmental conditions. Moreover, these stomatal traits are also affected by the species (Zheng et al., 2013). Ferris et al. (1996) and Zheng et al. (2013) found that high-temperature conditions significantly increased the stomatal length and width, respectively. In this study, our results showed that the size of stomata was increased especially in longer durations of heat stress. Some studies have reported that stomatal size was negatively correlated with stomatal frequency, and thermo-resistant cultivars had smaller stomata with higher stomatal density than thermo-sensitive cultivars (Han, Li, & Wang, 1997; Maghsoudi & Maghsoudi Moud, 2008), which is consistent with our observations.

The high-temperature stress in this study induced an increase in leaf thickness for both cultivars. However, ‘Indian Prince’ developed thicker leaves under 21 days high temperature as compared to ‘Candyman’. Natarajan and Kuehny (2008) also observed that Salvia splendens increased its leaf thickness to a much higher extent in heat-tolerant than heat-sensitive cultivar under heat stress. They noted that increased leaf thickness was originated from increasing mesophyll and spongy parenchyma layer as well as cuticular thickening. Salem-Fnayou et al. (2011) suggested that the increased leaf thickness in Vitis vinifera under high-temperature conditions is assumed to be an avoidance mechanism to decline evaporation. On the other hand, because transpiration is a cooling mechanism of the plant, the closure of stomata resulted in an increase in the leaf temperature and a reduction in net photosynthesis (Natarajan, 2005).

**Conclusion**

Exposure to heat stress, especially for longer periods, has negative effects on the physiological processes of calendula. This study showed that among four tested cultivars, ‘Indian Prince’ was identified as the most tolerant cultivar as this cultivar had higher stomatal density, thicker leaves, and showing higher soluble sugars, gas exchange maintenance, and chlorophyll content maintenance when exposed to heat stress compared to other cultivars. Therefore, it can be used in regions with short springs such as Mashhad, Iran or in plant breeding programs. According to the results of CMT response curves, ‘Zen’ suffered from more damage to its cell membrane in terms of percent electrolyte leakage when compared to the other three cultivars. So, this study and previous studies lead us to the conclusion that CMT test could probably be applied as an inexpensive and rapid method to screen for heat-sensitive cultivars of calendula.

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