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Effect of heat acclimation on thermotolerance of *in vitro* strawberry plantlets

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ABSTRACT

Strawberry production is facing a serious decline with the increase in global temperature as a result of climate change. Improving the heat tolerance is imperative for the strawberry plants to remain productive under high temperature conditions. The present work aimed to study the effect of heat acclimation on the thermotolerance of strawberry plants subjected to severe heat stress. Tissue cultured *Fragaria* 'Taoyuan No. 1' plantlets were subjected to four heat-acclimation treatments with gradual increase of temperatures from 30°C to 42°C for 1.25 hr to 10 hr before exposing them to the lethal temperature of 48°C for 4 hr. Survival, new leaf emergence and root growth, electrolyte leakage, chlorophyll and proline contents, as well as antioxidant enzyme activities were compared between the control, acclimated and non-acclimated plantlets. Results indicated that heat acclimation was required for the strawberry plantlets to survive under lethal temperature conditions. The acclimated plantlets registered a lower degree of electrolyte leakage and chlorophyll degradation, and a higher proline content compared to the non-acclimated plantlets. The activities of the antioxidant enzymes increased with the elevation of acclimation temperature and peaked at 42°C except for ascorbate peroxidase (APX) whose activity peaked at 39°C. Higher activities of superoxide dismutase (SOD), APX, glutathione reductase (GR), catalase (CAT) and peroxidase (POD) were observed in the acclimated plantlets compared to the non-acclimated plantlets. This study demonstrates that heat acclimation improved the thermotolerance of *in vitro* strawberry plantlets by reducing electrolyte leakage and chlorophyll degradation, as well as by enhancing proline content and antioxidant enzyme activities under severe heat stress.

Keywords: antioxidant enzyme activity, climate action, *Fragaria* × *ananassa*, heat adaptation, heat tolerance, lethal temperature

INTRODUCTION

Climate change is a severe threat to global crop production because it can alter light, water, nutrients, gases, temperature, and toxins in the environment that may lead to a multitude of abiotic stresses to the plants (Blumward and Mittler, 2018). High temperature is particularly concerning as the Earth surface temperature is predicted to rise between 1.4ºC and 5.8ºC by the end of this century (IPCC, 2014). Plants exposed to high temperatures may undergo heat stress. Temperatures beyond the threshold level for normal plant growth and development for a sufficient period of time can cause irreversible damage to the plant (Masouleh and Sassine,

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2020). Both severe heat stress and long-term exposure to moderately high temperatures can cause morphological, physiological, biochemical, and molecular changes in plants due to the uncoupling of enzymes and metabolic pathways (Ahuja et al., 2010; Fahad et al., 2017), and thus pose a serious threat to the sustainability of crop yield (Hall, 2001). The heat stress symptoms are more severe for those plants that originate from different climate zones and have low genetic diversity to thrive in the fluctuating environment.

Plants have evolved various mechanisms for thriving under high prevailing temperatures. These include short-term avoidance or acclimation mechanisms or long-term evolutionary adaptations. Some major tolerance mechanisms, including ion transporters, heat-shock proteins, osmoprotectants, antioxidant defence, and factors involved in signalling cascades and transcriptional control, are essential to counteract the stress effects (Wang et al., 2004; Rodriguez et al., 2005). All these mechanisms, which are regulated at the molecular level, allow plants to thrive under heat stress conditions (Wahid et al., 2007). Heat acclimation, a method of exposing plants to elevated and non-lethal temperatures, may provide protection against a subsequent heat stress event. Subjecting plants to higher-than-optimum growth temperatures but below their lethal temperature has been known to cause acquired thermotolerance in plants (Husaini and Neri, 2016). The benefit of heat acclimation may be attributed to signalling cascades and transcriptional control (Sairam et al., 2000; Almeselmani et al., 2006), synthesis of osmoprotectants and heat shock proteins (Hasanuzzaman et al., 2013), and the induction of antioxidative systems to prevent the overaccumulation of reactive oxidant species (ROS) and membrane lipid peroxidation during severe heat stress (Xu et al., 2006).

An acclimation experiment is commonly designed to include three treatments. The control treatment, where plants are maintained in the recovery conditions without any exposure to heat stress; the acclimation treatment, where the plants are subjected to gradually increasing temperatures before exposure to the lethal temperature and the non-acclimation treatment, where the plants are directly exposed to the lethal temperature without prior acclimation. After the acclimation and non-acclimation treatments, the plants are placed under normal growth conditions for recovery. Treatment conditions are developed and standardised based on plant species, cultivar and their growth stages. The lethal temperature is defined as the minimum temperature that causes less than 10% plant survival at the end of the set recovery period (Ange et al., 2016; Vidya et al., 2017; Sujatha et al., 2018). Commonly, the time that plants are exposed to the lethal temperatures ranges from 1 hr to 10 hr (Kheir et al., 2012; Gomathi et al., 2014). The acclimation temperatures are usually from 30°C to 54°C for a period of 2 hr to 10 hr (Gomathi et al., 2014; Partheeban et al., 2017). Recovery periods can be set from a few hours up to 10 days (Satbhai et al., 2014; Vidya et al., 2017).

Strawberry has been cultivated internationally for centuries as a popular fruit crop (Hancock et al., 2008). It grows best between 10°C and 26°C (Husaini and Neri, 2016). However, high temperatures in recent years have posed a major challenge to its production. The negative impacts of high temperature on strawberry plants include, for instance, inhibited fruit development (Twitchen et al., 2021), decreased pollen viability and germination rate (Ledesma and Sugiyama, 2005) and reduced number of flowers and fruits (Ledesma et al., 2008), all of which can severely affect its yield and productivity. To our knowledge, the effect of heat acclimation on thermotolerance of strawberry plants has not been reported to date. The present study therefore aimed at evaluating the heat-acclimation influence on heat-stressed strawberry plants at the morphological, physiological and biochemical levels. The entire study was conducted under the *in vitro* conditions to exclude temperature-unrelated stress factors.

MATERIALS AND METHODS

Plant materials

In vitro strawberry plantlets of *Fragaria* × *ananassa* 'Taoyuan No. 1' (selected from 'Toyonoka') were maintained on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg · L^{-1} 6-benzylaminopurine (BAP) for shoot multiplication. Individual shoots were excised and transferred to 40 mL of MS medium and incubated for 28 days under a 16/8 hr day/night photoperiod illuminated with ca. 72.9 µmol · m^{-2} · s^{-1} at 25 ± 2°C.

Heat-acclimation treatments

Strawberry plantlets with ca. five leaves and four roots were used for the heat-acclimation experiment. Four heat-acclimation treatments namely T1, T2, T3 and T4 were designed with gradually increasing temperatures from 30°C to 42°C, with an increase of 3°C at each temperature increment for a total duration of 1.25–10 hr (Figure 1). After heat acclimation, the plantlets were subjected to the lethal temperature of 48°C for 4 hr before being placed at normal growth conditions for recovery. In addition to the four heat-acclimation treatments, plantlets not subjected to heat acclimation and maintained constantly at normal growth conditions were used in the control treatment (T0), whereas plantlets subjected to lethal temperature without prior heat acclimation were regarded as the non-acclimation treatment (TN).

Analysis of the survival and morphological parameters

Following each treatment, the plantlets were monitored for survival every 7 days for up to 35 days. A plantlet was considered as survived when new leaf emergence was observed. The survival percentage was evaluated from 10 plantlets per treatment and calculated using Eq. (1). The experiment was conducted twice.

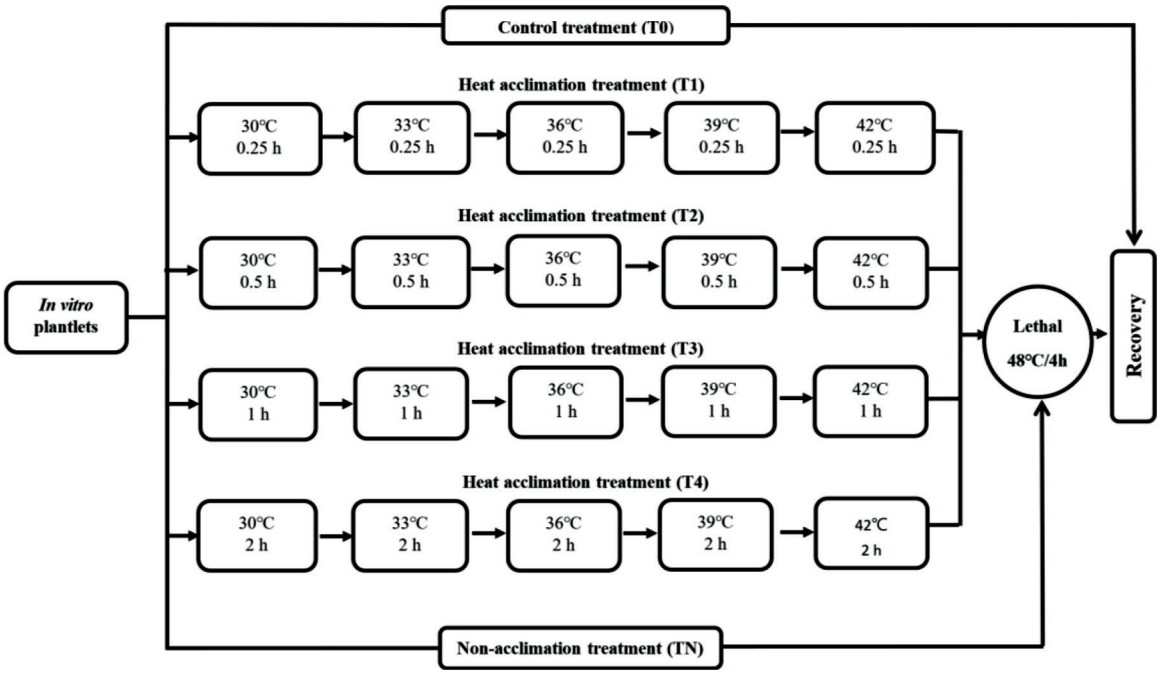

Figure 1. Overview of the control, heat acclimation and non-acclimation treatments used in this study.

Survival (%) = No. plantlets with new leaf emergence/ Total no. plantlets × 100 (1)

The number of new leaves, new adventitious roots and new lateral roots were counted every 7 days up to 56 days, based on 10 plantlets for each treatment.

Analysis of physiological parameters

Chlorophyll content

The chlorophyll contents of control (T0), acclimated (T3) and non-acclimated (TN) plantlets were evaluated based on the procedure of Lichtenthaler (1987). The analysis was conducted at 96 hr after the lethal temperature exposure, as the impact of heat stress on leaf colour was mild in the early recovery phase and became more noticeable at 4 days following stress. Three replications were used for each treatment. The contents of chlorophyll a, chlorophyll b and total chlorophyll were calculated based on Eqs (2)–(4) respectively. In these equations, $A_{663.2}$ and $A_{646.8}$ are the absorbances used to detect chlorophyll a and b, respectively, FW is the fresh weight (mg) of samples and V1 is the total volume (mL) of solution with 99.5% high-performance liquid chromatography (HPLC) grade acetone as solvent.

$$\text{Chlorophyll a} = 12.25 \times A_{663.2} - 2.79 \times A_{646.8}/(\text{FW/V 1}) \quad (2)$$

$$\text{Chlorophyll b} = 21.50 \times A_{646.8} - 5.10 \times A_{663.2}/(\text{FW/V 1}) \quad (3)$$

$$\text{Total chlorophyll} = \text{chlorophyll a} + \text{chlorophyll b} \quad (4)$$

Electrolyte leakage

The extent of electrolyte leakage was evaluated for control (T0), heat acclimated (T3) and non-acclimated non-acclimation treatment (TN) plantlets right after they were subjected to lethal temperature, using a procedure which was modified from Bajji et al. (2002). Three replications were used for each treatment. The initial electrolyte leakage (EC_i) and the final electrolyte leakage (EC_f) were recorded using the Thermo Scientific™ EutechTM CON 700 conductivity meter (Eutech Instruments, Pte Ltd Blk 55, Ayer Rajah Crescent, #04-16/24, Singapore 139949). The calculation for electrolyte leakage is featured in Eq. (5).

$$\text{Electrolyte leakage (\%)} = (EC_i/EC_f) \times 100 \quad (5)$$

Proline content

The control (T0), heat acclimated (T3) and non-acclimated (TN) plantlets after the lethal temperature stress were analysed for their proline content according to the procedure of Bates et al. (1973). Three replications were used for each treatment. The calculation of proline was based on the equation created from the standard curve that was developed using different concentrations of L-proline.

Analysis of biochemical parameters

Protein estimation

Protein was estimated by the modified Bradford (1976) methods. A standard curve was developed using different concentrations of bovine serum albumin (BSA). The protein concentration was calculated using the equation derived from the standard curve.

Enzyme extraction and analysis

The activity of the antioxidant enzymes was analysed for each temperature of the heat-acclimation process.

In addition, the enzyme activities of the control (T0), acclimated (T3) and non-acclimated (TN) plantlets were evaluated and compared after the lethal temperature exposure. Three replications were used for each temperature step and for each of the T0, T3 and TN groups.

The procedures of Garg and Kaur (2012) and Yin et al. (2009) were modified to carry out the analysis of superoxide dismutase (SOD) activity. The measurement of ascorbate peroxidase (APX) activity followed the methods described by Nakano and Asada (1981) and Dalal (2014). The activity of glutathione reductase (GR) was assessed using the methods of Schaedle and Bassham (1977) and Dalal (2014). The catalase (CAT) activity was determined using the protocol described by Aebi (1984). The methods of Chance and Maehly (1955) and Dalal (2014) were modified and used to evaluate the peroxidase (POD) activity.

Experimental design and data analysis

All the experiments were arranged in a completely randomised design. The survival percentages were arcsine transformed before the statistical analysis. Data were analysed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 26 (Chicago, USA), and mean separation was calculated with Duncan's multiple range test, where $p < 0.05$ was considered as significant.

RESULTS

Survival

Non-acclimated plantlets (TN) were unable to cope with lethal heat stress and died within 7 days of recovery as evidenced by leaf colour change from green to brown (Figure 2). Heat acclimation improved the heat tolerance of the plantlets as seen by the slower deterioration of the original leaves and emergence of new leaves (Figure 2). At 7 days of recovery, 55%–80% of heat-acclimated plantlets survived the lethal heat stress with no difference among the T1–T4 treatments (Table 1). At 14 days and 21 days of recovery, the survival rates of T3 and T4 plantlets reached 85%–95% and 95%–100%, respectively, which were non-significantly different than the T0 plantlets. At 28 days and 35 days of recovery, the survival rate of T2 plantlets was raised up to 95% that was comparable to the T3, T4 and T0 plantlets, which all exhibited 100% survival. On the other hand, the T1 treatment with its 75% survival rate was inferior to the T3, T4 and T0 treatments at the same observation points.

New leaf and root count

At 7 days of recovery, the T0 plantlets had the highest number of new leaves (0.8) followed by the T3 and T4 plantlets, which both produced 0.4 leaves, and T1 plantlets, which produced 0.1 leaves (Table 2). At 14 days of recovery, the T2, T3 and T4 plantlets regenerated 1.3–2.1 new leaves, which were comparable to the T0 plantlets with 1.3 leaves. Between 21 days and 42 days of recovery, the T2 treatment surpassed the T0 treatment in the number of new leaves produced. However, at 49 days of recovery, no difference in the new leaf number was observed between the T0, T2, T3 and T4 treatments. At the last observation time point of 56 days, all treatments showed comparable number of new leaves, which ranged between 6 and 7.4.

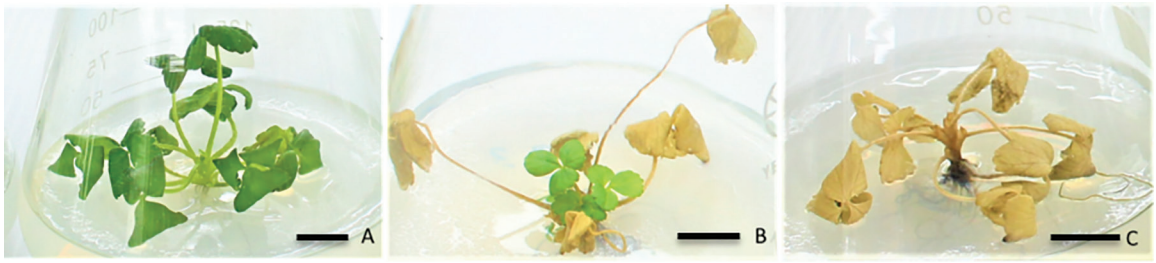

Figure 2. Morphology of strawberry plantlets 21 days after lethal temperature exposure. (A) T0 plantlet with healthy green leaves. (B) T3 plantlet with deterioration of original leaves and emergence of new leaves. (C) TN plantlet with deterioration of original leaves without new leaf growth. Bar = 1 cm.

Table 1. Survival rates of heat-acclimated strawberry plantlets at different days after lethal heat stress.

Treatment	7 days	14 days	21 days	28 days
T0	100 a	100 a	100 a	100 a
T1	55 ± 0.31 b	55 ± 0.10 b	75 ± 0.36 b	75 ± 0.36 b
T2	55 ± 0.10 b	60 ± 0.00 b	85 ± 0.36 b	95 ± 0.32 ab
T3	70 ± 0.00 b	85 ± 0.58 ab	95 ± 0.14 ab	100 a
T4	80 ± 0.26 b	95 ± 0.32 a	100 a	100 a
TN	0 c	0 c	0 c	0 c

Values are means ± SE. Values were arcsine transformed before analysis. Those followed by different superscript letters in the same column are significantly different at $p < 0.05$, separated by Duncan's multiple range test.

Table 2. Number of new leaves produced by heat-acclimated strawberry plantlets at different days after lethal heat stress.

Treatment	7 Days	14 Days	21 Days	28 Days	35 Days	42 Days	49 Days	56 Days
T0	0.80 ± 0.13 a	1.30 ± 0.21 ab	2.20 ± 0.20 b	3.10 ± 0.18 c	4.20 ± 0.20 b	5.10 ± 0.23 b	5.70 ± 0.15 a	6.00 ± 0.21 a
T1	0.10 ± 0.10 c	1.00 ± 0.33 b	2.10 ± 0.43 b	3.50 ± 0.45 c	4.40 ± 0.56 b	5.00 ± 0.65 b	5.80 ± 0.74 a	6.30 ± 0.83 a
T2	0	1.30 ± 0.42 ab	3.50 ± 0.43 a	4.80 ± 0.42 a	6.00 ± 0.54 a	6.50 ± 0.45 a	6.60 ± 0.43 a	6.60 ± 0.43 a
T3	0.40 ± 0.16 b	2.10 ± 0.31 a	3.50 ± 0.40 a	4.60 ± 0.54 ab	5.20 ± 0.51 ab	5.70 ± 0.45 ab	6.50 ± 0.40 a	7.40 ± 0.45 a
T4	0.40 ± 0.16 b	1.50 ± 0.22 ab	2.50 ± 0.22 b	3.70 ± 0.30 bc	4.60 ± 0.27 b	5.40 ± 0.27 ab	6.00 ± 0.26 a	6.70 ± 0.33 a

Values are means ± SE. Values followed by different superscript letters in the same column are significantly different at $p < 0.05$, separated by Duncan's multiple range test.

Table 3. Number of new adventitious roots produced by heat-acclimated strawberry plantlets at different days after lethal heat stress.

Treatment	7 Days	14 Days	21 Days	28 Days	35 Days	42 Days	49 Days	56 Days
T0	1.40 ± 0.40 a	2.70 ± 0.56 a	4.10 ± 0.72 a	5.00 ± 0.98 a	5.70 ± 1.09 a	6.40 ± 1.28 a	6.50 ± 1.32 a	6.80 ± 1.31 a
T1	0	0	0.40 ± 0.31 bc	1.10 ± 0.46 bc	1.10 ± 0.46 cd	1.80 ± 0.55 cd	2.90 ± 0.80 b	3.10 ± 0.77 b
T2	0	0.30 ± 0.21 bc	0.60 ± 0.31 bc	1.40 ± 0.69 bc	1.50 ± 0.67 cd	2.10 ± 0.64 c	2.60 ± 0.82 b	3.00 ± 0.84 b
T3	0.40 ± 0.27 b	0.90 ± 0.28 b	1.60 ± 0.37 b	2.60 ± 0.50 b	3.70 ± 0.50 b	4.40 ± 0.50 b	4.70 ± 0.50 ab	4.80 ± 0.49 ab
T4	0	0.40 ± 0.22 bc	1.30 ± 0.47 b	1.80 ± 0.44 bc	2.90 ± 0.43 bc	3.20 ± 0.51 bc	3.80 ± 0.61 b	3.90 ± 0.60 b

Values are means ± SE. Values followed by different superscript letters in the same column are significantly different at $p < 0.05$, separated by Duncan's multiple range test.

Table 4. Number of new lateral roots produced by heat-acclimated strawberry plantlets at different days after lethal heat stress.

Treatment	7 Days	14 Days	21 Days	28 Days	35 Days	42 Days	49 Days	56 Days
T0	0	0.80 ± 0.51bc	1.20 ± 0.76 cd	1.50 ± 0.82 c	1.50 ± 0.82 c	1.50 ± 0.82 cd	1.50 ± 0.82 cd	1.50 ± 0.82 c
T1	0	0.60 ± 0.50 bc	1.80 ± 0.94 bcd	2.40 ± 1.09 bc	2.40 ± 1.09 bc	2.90 ± 1.15 bc	3.50 ± 1.22 bc	4.60 ± 1.53 b
T2	0.50 ± 0.50 a	1.60 ± 1.08 abc	3.20 ± 1.44 abc	4.50 ± 1.27 ab	4.70 ± 1.21 ab	5.10 ± 1.22 ab	5.60 ± 1.14 ab	6.10 ± 1.22 ab
T3	0	2.20 ± 0.66 ab	4.80 ± 0.76 a	5.60 ± 0.81 a	5.70 ± 0.80 a	6.30 ± 0.78 a	7.00 ± 0.76 a	7.20 ± 0.79 ab
T4	0.20 ± 0.20 a	3.00 ± 0.67 a	4.20 ± 0.79 ab	4.50 ± 0.89 ab	5.10 ± 0.81 a	6.50 ± 0.64 a	7.40 ± 0.52 a	7.90 ± 0.38 a

Values are means ± SE. Values followed by different superscript letters in the same column are significantly different at $p < 0.05$, separated by Duncan's multiple range test.

Table 5. Chlorophylls, electrolyte leakage and free proline content of heat-acclimated strawberry plantlets after lethal heat stress.

Treatment	Chlorophyll (mg · g^{-1} FW)			Electrolyte leakage (%)	Proline (mg · g^{-1} FW)
	Total	Ch a	Ch b		
T0	3.92 ± 0.02 a	2.81 ± 0.02 a	1.11 ± 0.04 a	10.76 ± 0.04 c	1.38 ± 0.41 b
T3	1.81 ± 0.04 b	1.26 ± 0.02 b	0.54 ± 0.03 b	22.46 ± 0.70 b	3.34 ± 0.13 a
TN	1.11 ± 0.01 c	0.75 ± 0.01 c	0.36 ± 0.02 c	38.26 ± 0.62 a	1.69 ± 0.09 b

Values are means ± SE. Values followed by different superscript letters are significantly different at $p < 0.05$, separated by Duncan's multiple range test.
FW, fresh weight.

The T0 plantlets maintained at normal growth conditions had an intact root system, which produced 1.4 new roots at 7 days of recovery (Table 3). Plantlets from the heat-acclimation treatments had their roots stressed out following lethal heat stress and only the T3 plantlets were able to produce new adventitious roots at 7 days of recovery. The other treatments initiated new roots from 14 days to 21 days. During the recovery period of 21 days and 42 days, the number of new roots remained the highest in the T0 treatment, followed by the T3 and T4 treatments, with the lowest number in the T1 and T2 treatments. Only the T3 plantlets were able to produce a comparable number of adventitious roots to the T0 plantlets at 49 days and 56 days of recovery.

It was observed that the T2 and T4 plantlets were the first and the only ones to generate new lateral roots at 7 days of recovery (Table 4). The remaining treatments initiated their new lateral roots after this date. Between 14 days and 21 days of recovery, the T3 and T4 plantlets produced a higher number of new lateral roots compared to the T0 plantlets. From day 28 until day 49, the T2 treatment caught up with the T3 and T4 treatments as the three treatments recorded a higher number of new lateral roots than with the T0 treatment. At day 56 of recovery, all the four heat-acclimation treatments presented superior new lateral root number compared to the T0 treatment.

Chlorophyll, electrolyte leakage and proline content

At 96 hr of recovery, the total chlorophyll, chlorophyll a and chlorophyll b contents of T3 plantlets (1.81, 1.25, 0.54 mg) were all higher than the TN plantlets (1.11, 0.75, 0.36 mg) (Table 5). However, the T0 plantlets registered the highest amount of total chlorophyll, chlorophyll a and chlorophyll b (2.92, 2.81, 1.11 mg) among the three treatments.

The membrane damage was assessed indirectly by the conductometric measurements of ion leakage from leaf cells. The highest percentage of electrolyte leakage was observed in the TN plantlets (38.3%) followed by T3 (22.5%) and T0 plantlets (10.8%) (Table 5). The extent of electrolyte leakage of T3 plantlets was 2.08 folds higher than T0 plantlets, whereas TN plantlets had 3.5 folds higher of electrolyte leakage compared to T0 plantlets.

The T3 plantlets accumulated the highest amount of proline (3.3 mg) followed by T0 (1.4 mg) and TN plantlets (1.7 mg) (Table 5). The amount of proline accumulated in the T3 plantlets was 1.9 and 2.3 folds higher than the T0 and TN plantlets.

Antioxidant enzyme activities

The SOD activity remained stable from 25°C to 42°C (25–27 units) (Figure 3). A significant increase in the SOD activity was observed after exposure to 48°C (43.7 units). The APX activity gradually increased from 25°C (545.5 nmol) to 39°C (791.8 nmol) but declined afterwards reaching 368 nmol at 48°C. The GR activity remained stable from 25°C to 36°C (ca. 30 nmol), then gradually increased and reached 43 nmol at 42°C before decreasing to 23.8 nmol at 48°C. The CAT activity also increased from 19.2 nmol at 25°C to 34.1 nmol at 42°C before declining to 29 nmol at 48°C. The POD activity significantly increased from 1.38 nmol at 25°C to 13.9 nmol at 42°C, before declining to 3.62 nmol at 48°C. Maximum enzyme activity of GR, CAT and POD was recorded at 42°C except for APX whose activity peaked at 39°C.

For SOD and POD, it was observed that the acclimated plantlets recorded the highest enzyme activities followed by the non-acclimated and the control plantlets (Figure 4). For APX and GR, the opposite was observed where the control plantlets had the highest enzyme activities followed by the acclimated and the non-acclimated ones. Only in the case of CAT, the control and the acclimated plantlets recorded similar enzyme activities, which were both superior to the non-acclimated plantlets.

DISCUSSIONS

Heat stress is one of the most important abiotic stress factors reducing crop productivity and economic yield in many regions of the world. Heat acclimation is the process in which plants acquire increased thermotolerance following exposure to intermediate levels of heat stress. This process has been shown to improve the heat tolerance of diverse plants such as *Brassica* (Kaur et al., 2009), wheat (Végh et al., 2018), barley and oat (Darkó et al., 2019) and chickpea (Arslan, 2023). Its promotive effect is being investigated in this study on the strawberry plantlets growing under *in vitro* conditions. The *in vitro* environment offers the plants

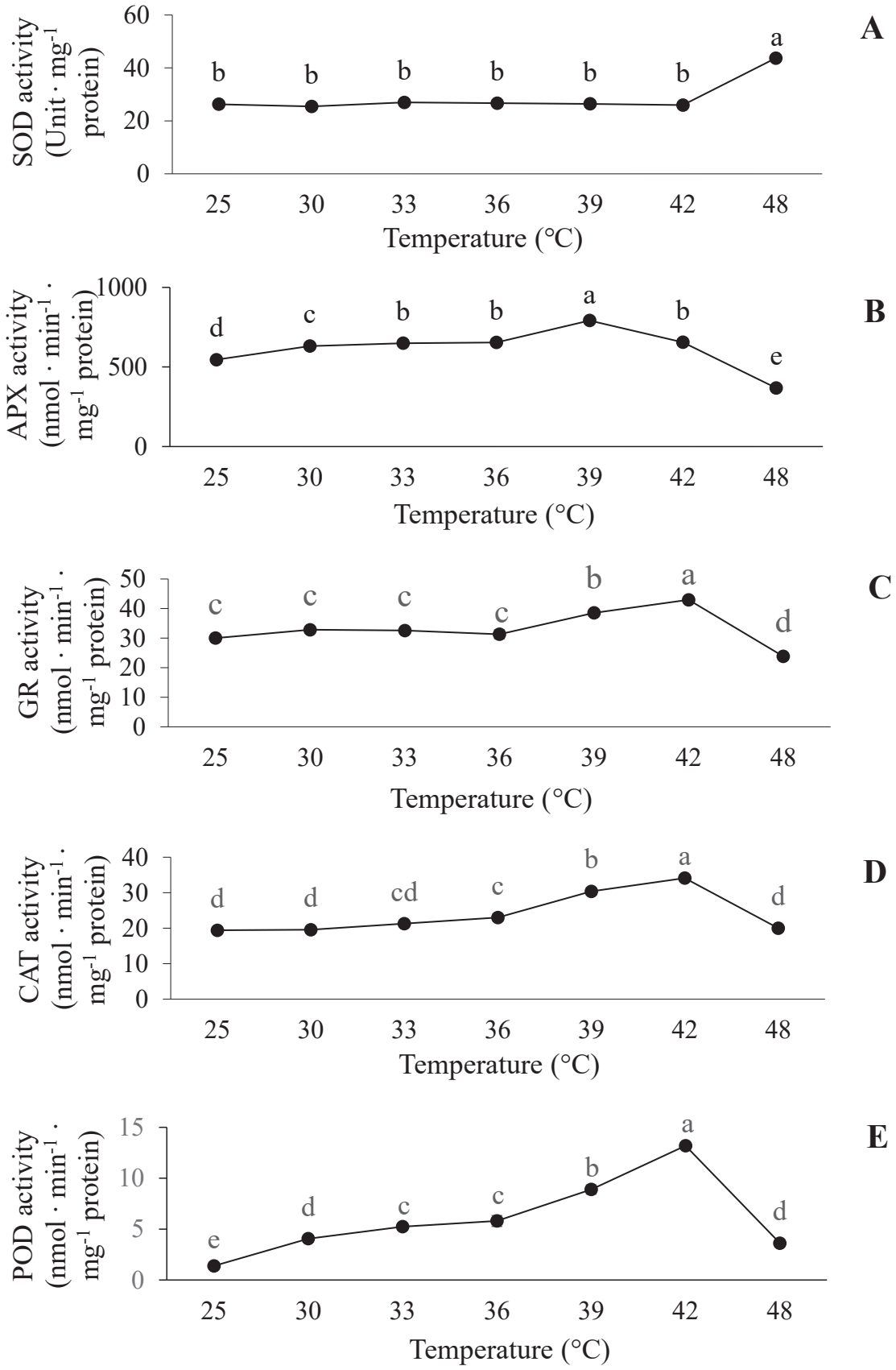

Figure 3. Activity of SOD (A), APX (B), GR (C), CAT (D) and POD (E) of strawberry plantlets following heat acclimation and lethal heat stress from 25°C to 48°C. Values are mean ± SE. Values followed by different letters are significantly different at $p < 0.05$, separated by Duncan's multiple range test. APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; POD, peroxidase; SOD, superoxide dismutase.

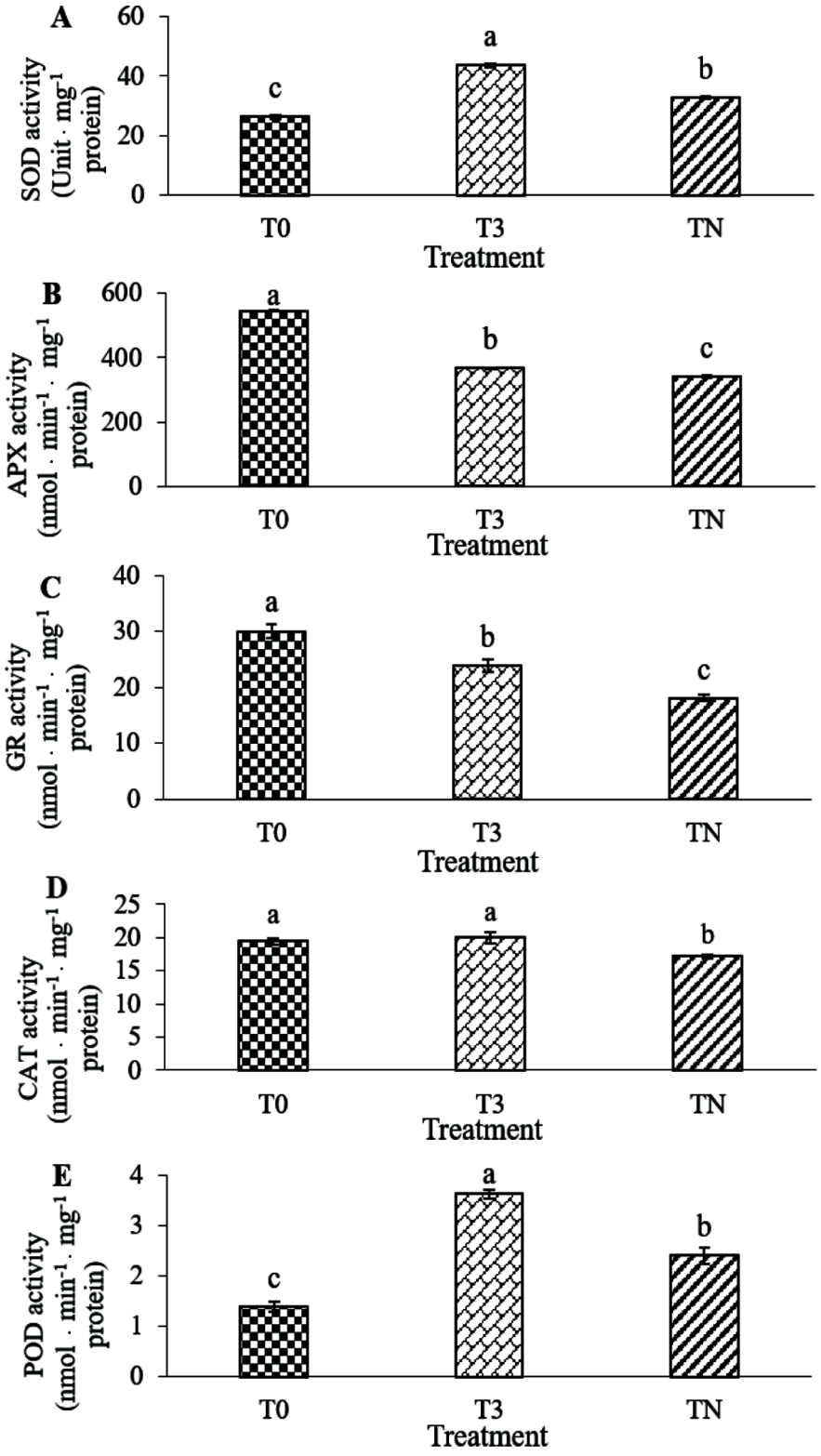

Figure 4. Antioxidant enzyme activities of the strawberry plantlets following the control (T0), heat-acclimation treatment (T3) and non-acclimation treatment (TN). (A) SOD, (B) APX, (C) GR, (D) CAT and (E) POD. Values are means ± SE. Values followed by different letters are significantly different at $p < 0.05$, separated by Duncan's multiple range test. APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; POD, peroxidase; SOD, superoxide dismutase.

with optimal growth conditions and therefore was adopted in this study to minimise any environmental non-treatment variations.

Four heat-acclimation treatments were applied to the strawberry plantlets, and their effectiveness was evaluated based on plantlet survival following lethal heat stress. Among the treatments, higher survival rates (85%–95%) were obtained for the T3 and T4 plantlets compared to T1 and T2 plantlets (55%–60%). Although all the treatments ranged from 30°C to 42°C with five temperature steps at 3°C intervals, the T3 and T4 treatments were characterised by longer acclimation durations (5 hr and 10 hr) and slower rates of temperature increase (ca. 1.2–2.4°C · hr^{-1}) compared to shorter acclimation durations (1.25 hr and 2.5 hr) and higher rates of temperature increase (ca. 4.8–9.6°C · hr^{-1}) of the T1 and T2 treatments. The rates at which the plants were exposed to the acclimation treatments may play an important role in plant-stress tolerance. For instance, soybean seedlings exposed to 34–42°C for 5 hr at the rate 2°C · hr^{-1} had the highest survival percentage after subjecting to a challenging temperature of 48°C for 3 hr (Ange et al., 2016). Ragi seedlings exposed to a gradual increase in the temperature range of 32–48°C for 5 hr at the rate of 3.2°C · hr^{-1} were able to recover after lethal temperature exposure of 54°C for 2 hr (Sujatha et al., 2018). The rate of 1.2–2.4°C · hr^{-1} at which the strawberry plantlets were exposed to during T3 and T4 heat-acclimation treatments in this study was comparable to the above-mentioned studies. It is speculated that higher rates of 4.8–9.6°C · hr^{-1} in the T1 and T2 treatments may be deleterious to the plantlets because of excessive temperature fluctuations in short time periods.

New leaf growth was delayed in the heat-acclimated strawberry plantlets at the early recovery phase due to high-temperature stress. However, all acclimated plantlets were able to resume growth from the centre of the plantlet at 14 days of recovery and produced a comparable number of new leaves (5.8–6.6) than the control plantlets (5.7) at 49 days of recovery. New adventitious root growth, however, was more treatment-dependent. Only T3 plantlets produced comparable amounts of new roots than the control plantlets at 49 days of recovery. The remaining treatments were all inferior to the control treatment for their new root production even at our last observation time point of 56 days. It was postulated that the optimal temperature for root growth tends to be lower than the optimal temperature for shoot growth (Calleja-Cabrera et al., 2020). So, roots may be more vulnerable compared to shoots during lethal temperature exposure, which resulted in their slower recovery compared to shoots in the heat-acclimated plantlets in this study. However, a different scenario was observed regarding the lateral root production. Throughout the entire recovery period, the number of new lateral roots in the control plantlets was inferior to the heat-acclimated plantlets. Among the heat-acclimation treatments, the T3 and T4 treatments produced lateral roots faster, followed by T2 and lastly T1 treatment. This may be attributed to the fact that the roots of the control plantlets were unstressed and so continued to elongate during the recovery period. Meanwhile, the roots of the heat-acclimated plantlets were heat stressed so lateral roots were produced as a result of root tip damage. According to Calleja-Cabrera et al. (2020), root exposure to higher-than-optimal temperatures can cause a decrease in the number of first-order lateral roots and increase in the number of second- and third-order lateral roots with a larger diameter. This was seen in cassava and sweet potato where the number of the first-order lateral roots was decreased when the root zone temperature was elevated (Pardales et al., 1999). Similarly in potato, the temperature increase inhibited adventitious and lateral root initiation and elongation (Sattelmacher et al., 1990). Our results differ from the above-mentioned studies where the number of first-order lateral roots was increased following heat exposure and the second- and third-order lateral roots were absent during the recovery period. The difference may be due to the plant species and *in vitro* conditions used in this study. Based on the above-mentioned findings, the T3 treatment was selected as the best acclimation treatment and subsequent physiological and biochemical analyses were conducted using T3-treated plantlets.

Stressful environments such as unfavourable temperatures can considerably hamper the photosynthetic process in plants by altering the organelle ultrastructure, pigment and metabolite concentrations and enzymes and stomatal regulation (Ashraf and Harris, 2013). Heat stress causes membrane disruption, particularly of thylakoid membranes, thereby inhibiting the activities of membrane-associated electron carriers and enzymes (Ristic et al., 2008; Rexroth et al., 2011), which ultimately results in a reduced rate of photosynthesis. Several studies indicated that plants exposed to high-temperature stress show reduced chlorophyll biosynthesis (Balouchi, 2010; Reda and Mandoura, 2011). Lesser accumulation of chlorophyll in high temperature-stressed plants may be attributed to impaired chlorophyll synthesis or its accelerated degradation or a combination of both. In this study, the contents of total chlorophyll, chlorophyll a and chlorophyll b were stable in the control plantlets not subjected to heat stress. In contrast, the acclimated and non-acclimated plantlets showed reduced chlorophyll contents following stress. Nevertheless, the degradation of chlorophylls was slower in the acclimated plantlets compared to the non-acclimated plantlets as evidenced by comparatively higher total chlorophyll, chlorophyll a and chlorophyll b contents. A similar finding was reported by Gosavi et al. (2014) where susceptible genotypes showed higher reduction in total chlorophyll content than tolerant genotypes in sorghum under heat stress. The benefit of heat acclimation was reported by Xu et al. (2006), who observed that the ultrastructure of chloroplasts had lower damage in heat-acclimated turfgrass leaves under heat stress than those without

heat-acclimation pretreatment. It was noted in this study that the chlorophyll b remained relatively more stable compared to the chlorophyll a, possibly because of its lower content in the plantlets and so the changes to its concentration were less noticeable.

The stability of the cell membrane has commonly been used to express the level of plant stress tolerance, and higher membrane stability could be correlated with higher stress tolerance (Premachandra et al., 1992). In this study, the percentage of electrolyte leakage was increased in both acclimated and non-acclimated strawberry plantlets compared to the controls, but with a higher increase in the non-acclimated plantlets. This result is consistent with another study in strawberry, where the electrolyte leakage was increased in both gradual heat stressed (GHS) and shock heat stressed (SHS) leaf tissues, but the SHS plants were injured more than the GHS plants as evidenced by a higher injury percentage and a lower LT50 (temperature causing half-maximal injury) (Gulen and Eris, 2004). Heat acclimation has been connected to an increase in the degree of saturation of fatty acids of membrane lipids, which in turn leads to increased rigidity of cell membranes and increase in their thermostability (Larkindale and Huang, 2004; Ilík et al., 2018).

Proline is a widely distributed compatible solute accumulating in plants exposed to abiotic stress, thus playing an important role in plant stress tolerance (Kaur and Asthir, 2015). Proline plays a major role as metal chelator, antioxidative defence molecule and signalling molecule during stress, and can directly scavenge hydroxyl radicals from plant cells, or indirectly scavenge ROS by enhancing the plant antioxidant defence system (Rejeb et al., 2014). In this study, the content of proline in the acclimated plantlets was between 1.9 and 2.3 folds higher than the control and non-acclimated plantlets. Similarly, higher proline accumulation was observed in wild sorghum genotypes, which presented higher heat stress tolerance compared to domesticated genotypes with lower tolerance level (Gosavi et al., 2014). The proline accumulated under heat stress conditions may act as an osmoprotectant, stabilising and protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS.

Exposure of plants to high temperatures usually increases the production of ROS. Living organisms, especially plants, where the avoidance mechanisms are less important than for animals, have evolved various enzymatic and non-enzymatic detoxification mechanisms to regulate ROS concentrations (Tripathy and Oelmüller, 2012). Tolerance to high-temperature stress in crop plants has been associated with an increase in the activity of enzymatic antioxidants (Babu and Devraj, 2008). The activation/deactivation and increase/decrease of these enzymes may occur at different times and may depend on the temperature treatments and plants tested. Chakraborty and Pradhan (2011) observed an increase in CAT, APX and SOD activities from 20°C to 50°C, while POD and GR activities declined at all temperatures ranging from 20°C to 50°C. In another study on wheat, it was observed that the activities of SOD, APX, CAT, GR and POX increased significantly at all stages of growth in heat-tolerant cultivars, while susceptible cultivars showed a significant reduction in CAT, GR and POX activities in response to high-temperature treatment of 35°C (Almeselmani et al., 2009). Rani et al. (2016) also documented higher activities of SOD, POX, CAT, APX and GR in heat-tolerant *Brassica juncea* genotype over the heat-susceptible genotype when exposed to high-temperature treatments of 45°C. In strawberry, the activities of APX, CAT and GR increased when leaf samples were subjected to gradual heat stress from 30°C to 60°C (Ergin et al., 2016). In this study, the SOD was the only enzyme whose activity remained constant throughout the heat-acclimation period and increased only following 48°C heat stress. SOD is the first line of defence in plants under high-temperature conditions and is activated in the presence of the superoxide radical (Szollosi, 2014). It is not clear why an increase in SOD activity was not detected at the different heat-acclimation temperatures. It may possibly be due to a higher temperature requirement (>42°C) of strawberry plantlets to activate SOD. In the exception of SOD, all the remaining enzymes (CAT, POD, GR and APX) had their activities increased following stepwise rise of temperatures during heat acclimation. The increase was moderate between 25°C and 36°C but more rapid after 36°C and up to 39°C for APX and 42°C for GR, CAT and POD. The enzyme activity decreased after 39°C for APX and after 48°C for GR, CAT and POD. The reason why APX peaked at 39°C rather than at 42°C may be attributed to the high affinity that APX has for H2O2 (Szollosi, 2014). It is speculated that APX was bound with most of the H_2O_2 in the early stages of heat acclimation and helped to bring the H_2O_2 level down from 25°C to 39°C. Thereafter, GR, CAT and POD continued to scavenge the remaining H_2O_2 present in the plantlets at 42°C after APX deactivation at 39°C. Therefore, the heat-acclimation procedure used in this study may improve the heat tolerance of strawberry plantlets *via* the gradual elevated activities of GR, CAT and POD. These three enzymes can possibly be used as indicators of heat tolerance in future studies.

It was observed that the activities of all the five enzymes (i.e. SOD, APX, GR, CAT and POD) were higher in the acclimated strawberry plantlets compared to the non-acclimated plantlets. It is possible that the non-acclimated plantlets had a higher level of ROS that they could not scavenge, leading to a more severe oxidative stress which in turn causing lower plantlet survival. Whereas the acclimated plantlets were able to cope with heat stress due to an enhanced antioxidative system. A similar phenomenon was observed in mustard seedlings where heat acclimation of 45°C for 1 hr induced 50% higher GR activity during the first 2 hr and 30% higher APX activity 1 hr after the treatment (Dat et al., 1998). According to these authors, the induced thermotolerance of mustard seedlings may be due to

changes in the antioxidant activities following heat acclimation.

CONCLUSIONS

In conclusion, the T3 heat-acclimation treatment consisted of a gradual exposure from 30°C to 42°C at 3°C intervals for a total duration of 5 hr, and it proved to be the most effective for improving the heat tolerance of the *in vitro* strawberry plantlets. Under lethal heat stress of 48°C for 4 hr, the heat-acclimated plantlets showed 100% survival and reduced extents of chlorophyll degradation and electrolyte leakage. The benefits of heat acclimation may be attributed to increased proline content and enhanced activities of the antioxidant enzymes including APX, GR, CAT and POD in the acclimated plantlets.

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AUTHOR CONTRIBUTIONS

J.Y.F. designed and supervised the experiments, and performed the critical revision of the figures and manuscript text. Z.J.S. and J.M.C. performed the experiments, figures plotting, statistical analysis of data and drafted the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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