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Adaptation of Cucurbit Species to Changes in Substrate Temperature: Root Growth, Antioxidants, and Peroxidation

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To investigate their response to changes in substrate temperatures, the roots from six species of cucurbit plants were exposed to 14°C, 24°C, or 34°C, while their aerial portions were maintained at natural ambient temperatures (23°C to 33°C). These species could be classified into three groups based on their stress response: Group A, *Cucurbita ficifolia* and *C. maxima*, heat-sensitive but cold-tolerant; Group B, *Cucumis sativus* and *C. melo*, heat- and cold-sensitive; and Group C, *Luffa cylindrica* and *Benincasa hispida*, heat-tolerant but cold-sensitive. The highest growth rates and lowest contents of malondialdehyde (MDA) for plants in Groups A, B, and C were achieved at temperatures of 14°C, 24°C, and 24°C to 34°C, respectively. Superoxide dismutase (SOD) activity was lowest in the roots exposed to optimal growth temperatures while activities of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (G-POD) operated coordinately in a complicated manner to prevent the accumulation of reactive oxygen species (ROS) in the root cells. Moreover, all plants, regardless of species, responded to unfavorable temperatures by increasing their synthesis of ascorbate and glutathione as well as by reducing the redox ratio of those two important antioxidants.

Keywords: antioxidant enzyme, cucumber, lipid peroxidation, non-enzymatic antioxidants, reactive oxygen species (ROS), stress

The geographic distribution and agronomic productivity of crop plants can be limited by their sensitivity to fluctuations in temperature. Exposure to sub-optimal conditions induces significant changes in physiological processes, such as photosynthesis, ion uptake, and mitochondrial respiration (Hendrickson et al., 2004; Munro et al., 2004). Although much information is available about the effects of sub- or supra-normal temperatures on the physiological metabolism and functioning in the aerial parts (Hendrickson et al., 2004; Munro et al., 2004; Xu and Zhou, 2006; Dwyer et al., 2007), relatively little is known about the adaptation by plant roots to changes in substrate temperature (Xu and Huang, 2000a, b; Rachmilevitch et al., 2006). In both warm and cool climate regions, roots are often exposed to prolonged high or low soil temperatures, which can significantly impact shoot growth and even whole-plant survival (Udomprasert et al., 1995; Xu and Huang, 2000a, b).

Soil temperatures can modify biomass allocation, photosynthesis, root growth, leaf water status, and nutrient uptake and translocation from roots to shoots (Legros and Smith, 1994; Lee et al. 2004). In cucumber, net photosynthesis and the photochemical efficiency of PSII are considerably decreased at root temperatures below 15°C, even if the aerial temperature remains optimal (Ahn et al., 1999). Lee et al. (2004) have shown that many stress-induced dysfunctions or disturbances in physiological metabolism arise from the oxidation of cellular components as a result of the increased generation of reactive oxygen species (ROS) in the cells (Lee et al., 2004). Plants possess a series of detoxification systems that break down highly toxic ROS via antioxidant enzymes, e.g., superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT), as well as by non-enzyme antioxidants, such as glutathione (GSH), ascorbate (AsA), á-tocopherol, and carotenoids, thereby limiting oxidative damage under stress conditions (Foyer et al., 1994). Accordingly, the response of antioxidants to changes in environmental factors is now an important research topic in modern plant biology and agricultural sciences (Ghanati et al., 2005; Gechev et al., 2006).

Cucurbit plants are valuable horticultural crops worldwide (Tindall, 1983). Normally cultivated under warm conditions, they exhibit great variations in their tolerance to sub-optimal environments. For example, cucumber plants may develop poorly at a relatively low soil temperature of around 15°C, whereas the figleaf gourd grows well under such conditions (Ahn et al., 1999). However, no extensive and systematic studies had, until now, been conducted to characterize the adaptation of cucurbit crops to different root temperatures in term of their physiological metabolism. Here, six economically important cucurbit species with different origins were compared by investigating their root growth, cell membrane lipid peroxidation, and antioxidant metabolism in order to elucidate how their ROS metabolism was related to their adjustment to low, moderate, or high substrate temperatures.

MATERIAL AND METHODS

Plant Materials and Growth Measurements

Six cucurbit species -- figleaf gourd (*Cucurbita ficifolia* Bouché), turban squash (*C. maxima* Duch., cv. Lutianbao), cucumber (*Cucumis sativus* L., cv. Jinyou No. 40 and *C. melo* L., cv. Xiangyu), sponge gourd (*Luffa cylindrica* Roem., cv. Zhongchangsigua), and wax gourd (*Benincasa hispida* Cogn., cv. Fenpidonggua) -- were evaluated. Seeds were sown in

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moist vermiculite in trays in a greenhouse. After 7 d, groups of eight seedlings per species were transplanted to a 13-L tank (39×27×13 cm) filled with half-strength Enshi nutrient solution (Yu and Matsui, 1997) that was continuously aerated with an air-pump. Mean daily maximum and minimum air temperatures were 33°C and 23°C, respectively. After 2 weeks of this pre-culturing, plants with three to five leaves were treated in solutions set at 14±1°C, 24±1°C, or 34±1°C. The solution temperatures were maintained by cooling and heating pipe systems connected to individual tanks. After 7 d, roots were sampled and stored at -80°C before they were oven-dried and weighed. Each treatment included 18 plants and 3 replicates.

Determination of Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA) Contents

H_2O_2 content was measured according to the method of Patterson et al. (1984). Roots (0.3 g) were homogenized in 2.5-fold cold acetone in a chilled pestle and mortar. The extract was centrifuged (3,000 × g), and 0.2 mL $TiCl_4$ in HCl (20% v/v $TiCl_4$ in concentrated HCl) was added to the supernatant. After shaking, 0.4 mL of one-fifth strength NH_4OH was added dropwise with thorough mixing. The samples were then centrifuged (3,000 × g) and the precipitates washed three times with 2 mL volumes of acetone. The precipitates were solubilized in 3 mL 2 N H_2SO_4 and were measured by monitoring the A_{410} of this titanium-peroxidase complex.

MDA concentration was estimated from the supernatant by the method of Buege and Aust (1984), with some modification. Samples were ground in a cold 2.5-fold buffer solution with 25 mM HEPES buffer (pH 7.8) that contained 0.2 mM EDTA and 2% (w/v) polyvinylpyrrolidone. After centrifugation (12,000 × g), 1 mL of the supernatant (homogenate containing 0.1 to 0.2 mg of protein) was mixed thoroughly with 2 mL of TCA-TBA-HCl (15% w/v TCA and 0.5% w/v TBA in 0.25 N HCl). Absorbance of the samples was determined at 535 and 600 nm in a double-beam spectrophotometer against a suitable blank.

Analysis of Antioxidant Enzyme Activity

Root samples were homogenized with 0.05 M sodium phosphate buffer (pH 7.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 × g for 20 min, and the resulting supernatant was used for determining enzyme activity. The extraction buffer for superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (G-POD) contained 0.2 mM EDTA and 2% (w/v) polyvinylpyrrolidone. To determine the activity of ascorbate peroxidase (APX), 1 mM reduced ascorbate (AsA) and 2 mM 2-mercaptoethanol were added to that buffer. All extractions were performed at 4°C.

SOD (EC 1.15.1.1) activity was measured by monitoring the inhibition of photochemical reduction of ρ-nitro tetrazolium, as described by Beyer and Fridovich (1987). One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition in the rate of ρ-nitro blue tetrazolium chloride reduction at 560 nm. CAT (EC 1.11.1.6) activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu, 1987). This decrease was followed by a decline in optical density at 240 nm, and activity was calculated using the extinction coefficient (40 mM cm^{-1} at 240 nm) for H_2O_2 (Kato and Shimizu, 1987). APX (EC 1.11.1.11) activity was determined by monitoring the decrease in AsA at A_{290} (ε=2.8 mM cm^{-1}), according to the protocol of Jiménez et al. (1997). G-POD (E.C.1.11.1.7) activity was measured by modifying the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient (22.6 mM cm^{-1} at 470 nm) for tetraguaiacol.

Measurements of Reduced Ascorbate (AsA), Dehydroascorbate (DHA), Reduced Glutathione (GSH), and Oxidized Glutathione (GSSG) Contents

The contents of AsA and DHA were determined according to the method of Sgherri et al. (2000). Each 0.3 g sample of root material was homogenized in ice-cold 5% TCA (w/v). After centrifugation at 20,000 × g for 15 min, AsA and total AsA (AsA + DHA) were determined in the supernatants. The assay was based on the reduction of Fe^{3+} by AsA, followed by the formation of a complex between Fe^{2+} and bipyridyl, which showed absorbance at 525 nm. Total AsA was determined through a reduction of DHA to AsA by dithiothreitol. DHA content was then estimated from the difference between total AsA and AsA. We used a standard curve covering the range of 0 to 25 nmol AsA, as described by Sgherri et al. (2000).

GSH and GSSG were determined according to the technique of Hissin and Hilf (1976), with some modifications. Each 0.3 g sample of root tissue was extracted with 3 mL 5% (w/w) meta-phosphoric acid. After centrifugation at 10,000 × g for 30 min, 49.5 mL of phosphate-EDTA (pH 8.0) buffer was added to 0.5 mL of the supernatant. The final assay mixture contained a 0.05% (w/w) o-phthaldialdehyde solution. After incubation at 30°C for 15 min, fluorescence at 420 nm was determined with activation at 350 nm.

RESULTS AND DISCUSSION

The six cucurbit species evaluated here exhibited quite different responses to changes in temperatures (Fig. 1). Root dry weights for *Cucurbita ficifolia* and *C. maxima* were highest at 14°C, followed by 24°C, and 34°C. In contrast, dry-root biomass was greatest from both *Cucumis sativus* and *C. melo* at 24°C. For *Luffa cylindrica* and *Benincasa hispida*, the lowest dry weight accumulations were measured when plants were treated at 14°C, and no differences in weights were seen between 24°C and 34°C (Fig. 1). Based on these results, we classified these six species into three groups: A, *Cucurbita ficifolia* and *C. maxima* being heat-sensitive; B, *Cucumis sativus* and *C. melo* being both heat- and cold-sensitive; and C, *Luffa cylindrica* and *Benincasa hispida* being heat-tolerant but cold-sensitive.

In plants, stress-induced injuries are associated with the generation and activity of reactive oxygen species (ROS) (Almeselmani et al., 2006). Here, the Group-A plants had the highest H_2O_2 contents at 34°C while those in Group C showed the highest amount of H_2O_2 at 14°C. In comparison, H_2O_2 contents in the B-group plants were greatest at

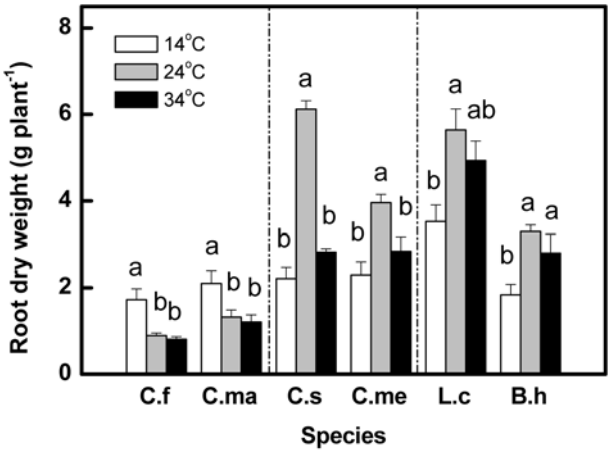

Figure 1. Changes in root dry weights as influenced by root temperature. C.f, *Cucurbita ficifolia*; C.ma, *Cucurbita maxima*; C.s, *Cucumis sativus*; C.me, *Cucumis melo*; L.c, *Luffa cylindrica*; B.h, *Benincasa hispida*. Data are means of four or five replicates, with standard deviations shown by vertical bars. Bars sharing same letters are not significantly different at $p < 0.05$.

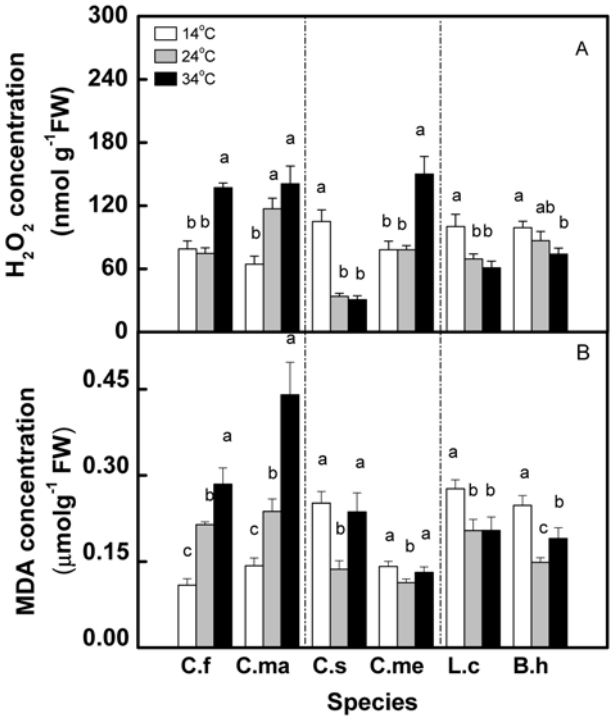

Figure 2. Changes in concentrations of H_2O_2 and malondialdehyde (MDA) as influenced by root temperature. C.f, *Cucurbita ficifolia*; C.ma, *Cucurbita maxima*; C.s, *Cucumis sativus*; C.me, *Cucumis melo*; L.c, *Luffa cylindrica*; B.h, *Benincasa hispida*. Data are means of four to six replicates, with standard deviations shown by vertical bars. Bars sharing same letters are not significantly different at $p < 0.05$.

14°C and 34°C (Fig. 2A). These increases in H_2O_2 may have been a result of growing our plants at sub-optimal root temperatures. Under stress conditions, the extent of ROS production exceeds the antioxidant defense capability of the cells, causing damage (Van Breusegem and Dat, 2006). We also examined the effect of membrane lipid peroxidation, expressed as the MDA content, and found, as with biomass,

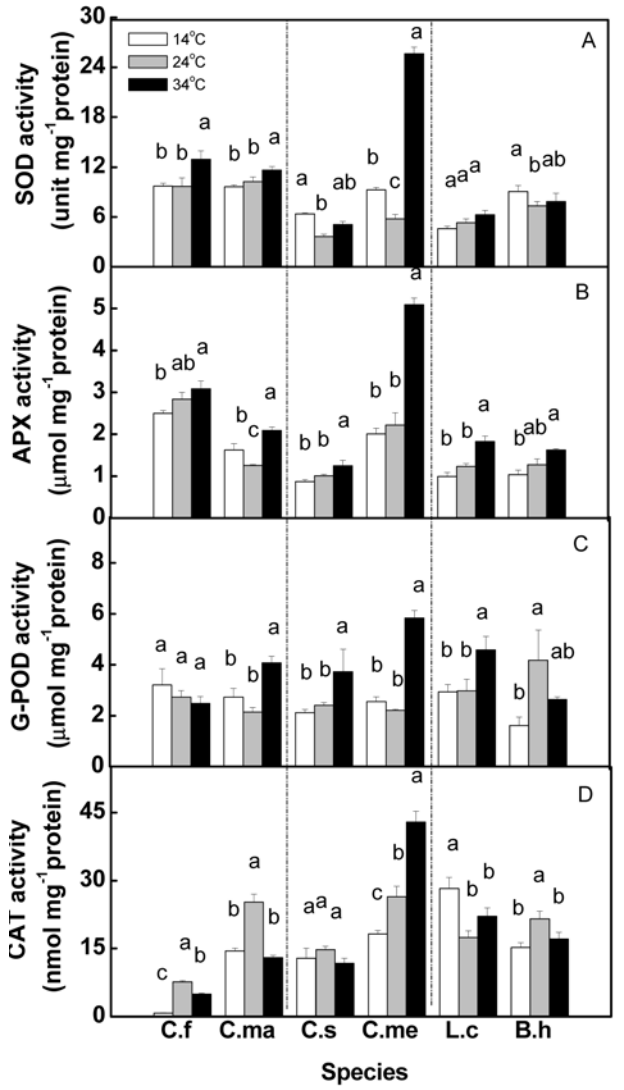

Figure 3. Changes in activities of superoxide dismutase (SOD; **A**), ascorbate peroxidase (APX; **B**), guaiacol peroxidase (G-POD; **C**), and catalase (CAT; **D**) as influenced by root temperature. C.f, *Cucurbita ficifolia*; C.ma, *Cucurbita maxima*; C.s, *Cucumis sativus*; C.me, *Cucumis melo*; L.c, *Luffa cylindrica*; B.h, *Benincasa hispida*. Data are means of four replicates, with standard deviations shown by vertical bars. Bars sharing same letters are not significantly different at $p < 0.05$.

that species varied in their amounts of malondialdehyde (Fig. 2B). MDA contents in Group-A species (*Cucurbita ficifolia* and *C. maxima*) increased as root temperature rose, whereas those for Group B (*Cucumis sativus* and *C. melo*) were all significantly higher when the temperature went up from 24°C to 34°C or down to 14°C. Finally, plants in Group C (*Luffa cylindrica* and *Benincasa hispida*) had greater MDA contents at 14°C than at either 24°C or 34°C. Oxidative stress can be induced when roots are exposed to high salt, aluminum, or chilling (Ghanati et al., 2005; Huang et al., 2005; Katsuhara et al., 2005). As with previous studies, our results clearly demonstrate that plants grown at sub-optimal root temperatures show more membrane lipid peroxidation than those at optimal temperatures.

To reduce oxidative damage under stress conditions,

plants utilize a series of detoxification systems (both enzymatic and non-enzymatic) that break down the highly toxic ROS and protect their cells (Foyer et al., 1994). Figure 3 presents the changes measured in the activities of four antioxidant enzymes (SOD, APX, G-POD, and CAT) after our cucurbit species were exposed to various root temperatures. For all three groups, plants had the lowest SOD activity at their own specific optimal temperatures whereas SOD activity was always highest at the most stressful temperature for a particular species. For example, the greatest SOD activity in chilling-tolerant *Cucurbita ficifolia* was recorded at 34°C while *Cucumis melo*, which is both heat- and cold-sensitive, had its lowest activity at 24°C but its highest at 34°C. SOD activity is closely related to the generation of $O_2^{\bullet -}$ under unfavorable growing conditions because this key enzyme dismutates $O_2^{\bullet -}$ to H_2O_2. Thus, production of $O_2^{\bullet -}$ was lowest at 14°C, 24°C, and 34°C for Groups A, B, and C, respectively. Our observation is in agreement with earlier findings that SOD activity increases after plants are exposed to elevated temperatures, high irradiation, drought, or chilling (Clare et al., 1984; Schoner and Krause, 1990; Pastori and Trippi, 1993).

APX, G-POD, and CAT are responsible for the scavenging of H_2O_2. Here, when the root temperature of any species rose from 24°C to 34°C, APX activity increased whereas a drop from 24°C to 14°C led to diminished activity, although that difference was not always significant. Meanwhile, boosting the root temperature from 24°C to 34°C increased G-POD activity while a decline from 24°C to 14°C had little effect in all species except *Benincasa hispida*. Peroxidase activity can be induced under a variety of stress conditions, e.g., drought, chilling, salinity, γ-radiation, and toxic contamination (Qadir et al., 2004; Kim et al., 2005; D'Arcy-Lameta et al., 2006). Catalase, a ubiquitous oxidoreductase that decomposes H_2O_2 to water and molecular oxygen, is a key enzyme involved in the removal of toxic peroxides. Lowering the root temperature from 24°C to 14°C decreased CAT activity in most plants, regardless of their group (Fig. 3B). However, increasing that temperature from 24°C to 34°C led to mixed results among our six species. Taken together, it is difficult to draw a general conclusion about the role of each enzyme in H_2O_2-scavenging, but it seems likely that APX, CAT, and G-POD work coordinately. In fact, multiple isoforms of these three isoenzymes have been reported in higher plants, being under the control of different genes. They respond differently to environmental stresses, e.g., low temperature, high light, pathogen infection, paraquat, and salinity (Kwon et al., 2002; Parida et al., 2004; Syros et al., 2005). Further study is needed to elucidate the response of different isoenzymes to temperature changes.

In plant cells, reduced ascorbate (AsA) is the most abundant low-molecular-weight non-enzymatic antioxidant, participating in ROS-scavenging via the AsA-GSH cycle. AsA is the primary cellular antioxidant (Alscher et al., 1997) because it provides a reservoir for regenerating *a*-tocopherol, a membrane-bound antioxidant (Foyer, 1993). Besides scavenging lipid peroxide radicals, AsA functions as a secondary signal in plant defenses (Fryer, 1992). In our root experiments, AsA, DHA, and the pool size of ascorbate (AsA+DHA) were higher at 34°C and at 14°C for both A-group species

Table 1. Concentrations of reduced ascorbate (AsA) and dehydroascorbate (DHA), the pool size of ascorbate (AsA+DHA), and the ascorbate redox state (AsA/(AsA+DHA)) in cucurbit roots exposed to different temperatures.

Species	Treatment	AsA	DHA	AsA+DHA	AsA/(AsA+DHA)
		(mmol g^{-1} FW)			
C.f	14°C	0.051(0.004)b	0.022(0.02)b	0.076(0.005)b	0.683(0.010)a
	24°C	0.049(0.004)b	0.026(0.004)b	0.078(0.007)b	0.642(0.018)b
	34°C	0.061(0.005)a	0.047(0.005)a	0.112(0.005)a	0.560(0.039)c
C.ma	14°C	0.047(0.005)a	0.025(0.004)c	0.076(0.007)b	0.643(0.030)a
	24°C	0.043(0.002)a	0.037(0.037)b	0.083(0.003)b	0.540(0.044)b
	34°C	0.047(0.002)a	0.075(0.001)a	0.126(0.002)a	0.393(0.010)c
C.s	14°C	0.080(0.009)a	0.034(0.003)b	0.117(0.006)a	0.690(0.042)b
	24°C	0.056(0.004)b	0.012(0.001)c	0.072(0.005)b	0.798(0.001)a
	34°C	0.079(0.003)a	0.043(0.004)a	0.125(0.002)a	0.639(0.029)b
C.me	14°C	0.087(0.007)a	0.100(0.007)a	0.190(0.007)a	0.465(0.032)b
	24°C	0.063(0.003)c	0.029(0.001)b	0.096(0.004)b	0.672(0.003)a
	34°C	0.074(0.001)b	0.093(0.009)a	0.170(0.009)a	0.444(0.022)b
L.c	14°C	0.084(0.002)a	0.064(0.004)a	0.151(0.002)a	0.566(0.019)b
	24°C	0.050(0.003)b	0.023(0.001)b	0.076(0.004)b	0.668(0.006)a
	34°C	0.045(0.003)b	0.020(0.002)b	0.068(0.002)b	0.674(0.033)a
B.h	14°C	0.067(0.002)a	0.046(0.005)a	0.117(0.006)a	0.588(0.024)c
	24°C	0.060(0.002)b	0.012(0.003)c	0.075(0.005)b	0.804(0.032)a
	34°C	0.062(0.002)ab	0.022(0.001)b	0.088(0.002)b	0.720(0.007)b

C.f, *Cucurbita ficifolia*; C.ma, *Cucurbita maxima*; C.s, *Cucumis sativus*; C.me, *Cucumis melo*; L.c, *Luffa cylindrica*; B.h, *Benincasa hispida*. Data are the means of three or four replicates with standard deviations. Values followed by the same letters within a column are not significantly different at $p<0.05$.

Table 2. Concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG), the pool size of glutathione (GSH+GSSG), and the glutathione redox state (GSH/(GSH+GSSG)) in cucurbit roots exposed to different temperatures.

Species	Treatment	GSH	GSSG	GSH+GSSG	GSH/(GSH+GSSG)
		(mmol g^{-1} FW)			
C.f	14°C	0.23(0.010)b	0.098(0.011)c	0.33(0.002)b	0.70(0.03)a
	24°C	0.22(0.002)b	0.115(0.007)b	0.33(0.007)b	0.66(0.02)b
	34°C	0.29(0.016)a	0.201(0.003)a	0.49(0.014)a	0.59(0.02)c
C.ma	14°C	0.19(0.008)b	0.172(0.011)a	0.36(0.009)b	0.52(0.02)c
	24°C	0.16(0.007)c	0.061(0.003)c	0.23(0.006)c	0.73(0.02)b
	34°C	0.31(0.003)a	0.082(0.003)b	0.39(0.001)a	0.79(0.01)a
C.s	14°C	0.33(0.010)a	0.071(0.005)a	0.40(0.005)a	0.82(0.02)c
	24°C	0.31(0.006)b	0.025(0.001)c	0.33(0.006)c	0.93(0.00)a
	34°C	0.32(0.006)b	0.048(0.009)b	0.36(0.004)b	0.87(0.02)b
C.me	14°C	0.32(0.006)a	0.047(0.006)a	0.36(0.005)a	0.87(0.02)b
	24°C	0.31(0.003)a	0.026(0.005)b	0.34(0.004)b	0.92(0.01)a
	34°C	0.32(0.001)a	0.033(0.005)ab	0.35(0.001)ab	0.90(0.01)ab
L.c	14°C	0.60(0.020)a	0.170(0.014)a	0.77(0.006)a	0.78(0.02)c
	24°C	0.32(0.019)b	0.048(0.004)b	0.37(0.023)b	0.87(0.00)b
	34°C	0.33(0.035)b	0.029(0.004)b	0.36(0.032)b	0.92(0.02)a
B.h	14°C	0.58(0.005)a	0.088(0.007)a	0.67(0.012)a	0.87(0.01)c
	24°C	0.43(0.045)b	0.010(0.001)c	0.44(0.046)b	0.98(0.00)a
	34°C	0.36(0.008)c	0.025(0.001)b	0.39(0.008)b	0.93(0.00)b

C.f, *Cucurbita ficifolia*; C.ma, *Cucurbita maxima*; C.s, *Cucumis sativus*; C.me, *Cucumis melo*; L.c, *Luffa cylindrica*; B.h, *Benincasa hispida*. Data are the means of three or four replicates with standard deviations. Values followed by the same letters within a column are not significantly different at $p<0.05$.

(heat-sensitive *Cucurbita ficifolia* and *C. maxima*) and C-group species (*Luffa cylindrica* and *Benincasa hispida*). Meanwhile, the roots of B-group species (heat- and cold-sensitive *Cucumis sativus* and *C. melo*) had the lowest contents of AsA, DHA, and AsA+DHA contents at 24°C. The opposite was observed for alterations in the ascorbate redox state (AsA/AsA+DHA). GSH is a versatile antioxidant that can directly scavenge ROS and participate in the AsA-GSH cycle. Here, GSH and GSSG contents underwent changes similar to those described for AsA and DHA after exposure to different temperatures (Table 1). *Cucurbita ficifolia* and *C. maxima* accumulated the greatest amounts of GSH, GSSG, and GSH+GSSG in roots at 34°C but the least at 14°C. In contrast, *Luffa cylindrica* and *Benincasa hispida* roots generally accumulated the highest contents of GSH, GSSG, and GSH+GSSG at 14°C and the lowest at 34°C. Meanwhile, *Cucumis sativus* and *C. melo* had their lowest contents at 24°C, although differences between temperatures were not always significant. Compared with these changes, a contrasting trend was observed with variations in the GSH/GSH+GSSG redox ratio (Table 2). GSH synthesis is driven by increased demand for GSH in response to oxidative stress, whereas GSSG can be formed in a reaction of GSH, with oxygen radicals being generated. Taken together, an increase in the total and reduced AsA and GSH pools could minimize the membrane damage caused by sub- (Groups B and C) or supra-optimal (Groups A and B) root temperature-mediated lipid peroxidation (Chaoui et al., 1997; Baryla et al., 2000). All of these changes are in strong agreement with those in biomass accumulation and MDA contents.

In conclusion, our six cucurbit species showed different adaptations to fluctuating root temperatures. Heat- or cold-sensitive plants growing at sub- or supra-optimal temperatures had increased ROS levels, which elevated the activity of antioxidant enzymes, as well as higher amounts of non-enzymatic antioxidants. Meanwhile, sub-optimal temperatures induced greater peroxidation in the root cells, leading to inhibited growth.

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