Effects of Cultural Temperatures on Antioxidant Levels in Leaves of *Cattleya* and *Cymbidium*

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Orchids of *Brassolaeliocattleya* Chian-Tzy Emperor (*Cattleya*) and *Cymbidium* Sazanami were cultured at 32/20°C, 30/15°C, and 28/8°C (maximum/minimum temperature) for 2 months. It was found in *Cattleya* leaves that superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities were lower than in *Cymbidium* leaves, while polyphenol oxidase (PPO) and ascorbate oxidase (AOX) activities were higher. The high cultural temperature enhanced activities in all enzymes in *Cattleya* and in four enzymes except CAT in *Cymbidium*, indicating that it greatly affects scavenging enzyme activities compared to the low temperatures which induced only an increase in APX activity in *Cattleya* and a decrease in CAT activity in *Cymbidium*. PPO activity increased and AOX decreased in both orchids at the low temperature. *Cattleya* leaves at both high and low cultural temperatures decreased ascorbic acid, while *Cymbidium* increased it. *Cattleya* at the low temperature increased markedly SH-containing substances but the latter at the high temperature decreased these substances. Lipid peroxides increased in *Cymbidium* leaves under both high and low temperatures, but for *Cattleya* neither temperature conditions affected lipid peroxides.

Keywords: antioxidant level, *Cattleya*, cultural temperature, *Cymbidium*

INTRODUCTION

Commercial orchids such as cattleyas and cymbidiums are cultivated in greenhouses in the winter season and outdoors in summer. In order to force *Cymbidium* culture, plants are transferred to mountain ranges in summer in order to ensure flower bud development and then brought back to greenhouses in autumn. To delay flowering, they are grown in non-heated greenhouses during winter and bloom in early spring. However, sudden frost damages distress growers. Unendurable heat stress induces physiological disorders like the burning, dropping of flower buds (Northen, 1970) and leaf bleaching (Powles, 1984). Thus, orchid production is accompanied by several cultural methods requiring adequate care for cultural temperatures and light intensity. Available reports indicate that very little work has been done on acclimation of orchids.

As oxygen levels are high in plant tissues due to photosynthesis, abiotic stresses such as temperature, light and gases as well as biotic stresses yield much reactive oxygen species (ROS) in leaf tissues. To detoxify such ROS, plants enhance activities in oxygen-scavenging enzymes

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and antioxidant levels in their leaves. Superoxide dismutases (SOD) play a primary role in defensive reactions of aerobic organisms to ROS (Fridovich, 1986) and detoxify superoxide (O_2^-) with NADPH at PSI to yield H_2O_2 (Scandalios, 1993). Ascorbate peroxidases (APX) located in chloroplasts and cytosols are known to decompose H_2O_2 (Asada, 1992; Alscher et al., 1997). It has also been reported that catalase (CAT) decomposes H_2O_2 in peroxisomes (Salin, 1988; Asada, 1992). Increases in SOD activity by induction of stresses were shown in a chilling tolerant *Zea diploperennis* (Jahnke et al., 1991) and in chilling-tolerant and sensitive cucumbers (Shen et al., 1999). Increased APX activities in corn plants grown in an elevated O_2 level (Foster and Hess, 1982), in a C_4 plant of a habitat in a cold climate (Hakam and Simon, 1996) and in a *Zea* plant (Jahnke et al., 1991) were also reported. Moreover, levels of antioxidants are correlated with growth irradiance and photosynthetic capacity (Grace and Logan, 1996).

Cattleyas and miniature cymbidiums are abundantly produced for cut flowers and potted plants. Parental species of the former are crassulacean acid metabolism (CAM) plants (Avadhani et al., 1982) that originate in areas of the tropic American Continent surrounded by an isothermal line of 27°C and relatively tolerant to hot weather. The latter hybrids are obtained by crossing oriental species which originate from areas showing an isothermal line of 21°C in East Asia and relatively tolerant to cold but not to heat (Karasawa, 1972). They are C_3 plants.

In this paper, an attempt was made to clarify the effect of cultural temperatures, one of the determinant factors for their habitations, on defensive reactions to ROS caused by thermal stress. Findings obtained may serve as acclimation methods and an indicator(s) to predict presumable disorders induced by cold and heat stresses.

MATERIALS AND METHODS

Plant materials. Twelve potted plants of *Brassolaeliocattleya* Chian-Tzy Emperor 'CT-Golden Sun' (referred to as *Cattleya*) and *Cymbidium* Sazanami 'Haru-no-umi' were grown in a 30% shaded greenhouse through summer and autumn. On 10th November, they were transferred to three rooms which were shaded at 60% of full sunlight intensity and in which temperatures were controlled at 32/20°C, 30/15°C and 28/8°C (maximum/minimum temperature). The plants were exposed to natural sunshine condition, irrigated every 3 d and dressed with aqueous Hyponex fertilizer every 20 d. Leaves of both orchids were collected for determination of ROS-scavenging enzymes and antioxidant concentrations from nine o'clock to nine thirty 60 and 75 d after the treatment, respectively. Collected leaves weighing 2 g were sliced into small pieces, and stored below -20°C after freezing with liquid nitrogen.

Enzyme extraction and assay.

SOD: Two grams of samples were ground in 8 ml of 0.1 mol phosphate-borate buffer (pH 7.8) with 1 mmol EDTA, 3 mmol DDT and 4% (w/v) PVP. After the macerate was filtered through coarse cheesecloth the filtrate was centrifuged at $10,000 \times g$ for 20 min. The supernatant was used for crude enzyme extract. The activity was determined using the Nitro-Blue Tetrazolium (NTB) reduction method (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NTB reduction measured at 560 nm.

APX: Crude enzyme extract was prepared by grinding 2 g samples in 8 ml of 50 mmol phosphate buffer (pH 7.0). The reaction was initiated by adding 1 ml of 5 mmol H_2O_2 to the reaction mixture composed of 0.4 ml of the crude enzyme extract, 1 ml of 0.2% ascorbic acid and 2 ml of water. After 2 min, 1 ml of metaphosphoric acid was added to terminate the reaction. Ascorbic acid consumption by ascorbate oxidase (AOX) activity was determined

by adding the same volume of the enzyme extract to the reaction mixture composed of 1 ml of 0.2% ascorbic acid and 3 ml of water. The remaining ascorbic acid was titrated with 0.005 N iodine reagent.

CAT: Samples weighing 2 g were ground with 4 ml of water and 0.4 g calcium carbonate. After centrifugation of the macerate, 1 ml of the supernatant was diluted with 9 ml of water. The diluted solution was used for a crude enzyme extract. Aliquot of enzyme extract (3 ml) was mixed with 2 ml of 3% H₂O₂, after which the O₂ evolved was estimated 6 min afterwards.

Polyphenol oxidase (PPO) and AOX: Crude enzyme extract was prepared by grinding 2 g samples in 50 ml of 1/15 mmol phosphate buffer (pH 6.0). The reaction for PPO activity was initiated by adding 2 ml of the crude enzyme extract to the reaction mixture composed of 2 ml of 0.2% ascorbic acid, 1 ml of 30 mmol *o*-catechol and 4 ml water. The catechol solution was replaced by water for determination of AOX activity. After 10 min, 1 ml of metaphosphoric acid was added and the remaining ascorbic acid was estimated.

Antioxidant, peroxide and organic acid extraction and determination.

Ascorbic acid: Two grams of plant materials were macerated with 5% metaphosphate. The assay was performed by the 2,4-dinitrophenyl hydrazine method (Roe et al., 1948).

SH containing substances (SHS): The 2 g samples were extracted with 8 ml of 0.05 M Tris-HCl buffer (pH 8.0) including 3 mmol EDTA, 5 mmol DTT, 1 μ mol leupeptin hemisulfate and 0.83% casein and the assay was performed using Ellman's reagent (Ellman, 1959). Absorbance of 1.2 ml of a sample extract mixed with 1.2 ml of 0.2 mol phosphate buffer (pH 8.0, containing 10 mmol EDTA) was determined at 412 nm. Then, the absorbance of a mixture of this sample and 0.1 ml of 10 mmol dithiobis (2-nitrobenzoic acid) was again determined at the same wavelength.

Lipid peroxides: Fifty grams of leaves were ground in hexane 3 times and then extracted with 150 ml of 80% alcohol 3 times. Lipid peroxides were determined according to the TBA (2-thiobarbituric acid) method (Tien and Aust, 1982). Red TBA oxidized with lipid peroxides was measured at 535 nm, and calculated in terms of malondialdehyde.

Organic acids: Acids in 20 ml water extracts prepared from 5 g leaves were titrated with 0.1 N NaOH. Their concentrations were calculated in terms of citric acid.

RESULTS

Oxygen-scavenging enzymes

SOD, APX and CAT activities in leaves of *Cattleya* and *Cymbidium* plants cultured at the different temperature are shown in Fig. 1. They were markedly lower in the former plants

Fig. 1 Activities in superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in leaves of *Cattleya* (▨) and *Cymbidium* (■) plants cultured at different temperatures.

H: 32/20°C (maximum and minimum temperature), M: 30/15°C, L: 28/8°C. Vertical bars indicate SE ($n=4$).

than in the latter. Besides, they were varied within the plants grown at different cultural temperatures. In both plants SOD activity was significantly higher at the 32/20°C temperature condition than at 30/15°C, but not different between at 28/8°C and 30/15°C. High APX activities were found in *Cattleya* plants at the high and low temperatures and in *Cymbidium* at the high temperature. However, CAT activities did not differ among *Cattleya* plants cultured at the different temperatures but decreased in *Cymbidium* cultured at either high or low temperatures. Foliar PPO and AOX activities were markedly higher in *Cattleya* than in *Cymbidium* (Fig. 2). The former enzyme in both *Cattleya* and *Cymbidium* was more activated at both high and low cultural temperatures, while AOX activities were positively dependent on a rising cultural temperature.

Antioxidant and peroxide levels and organic acids

Ascorbic acid concentration in *Cattleya* leaves cultured at 30/15°C was markedly higher than in *Cymbidium* ones at the same temperature. *Cattleya* at 32/20°C and at 28/8°C decreased ascorbic acid concentration, while at the same conditions *Cymbidium* increased it markedly. SHS in *Cattleya* leaves was highest at 28/8°C. Contrary to *Cattleya*, SHS was significantly lower in *Cymbidium* at 32/20°C than at 30/15°C. Lipid peroxide concentrations in leaves did not significantly vary with *Cattleya* plants cultured at different temperatures, while those of *Cymbidium* cultured at both high and low temperatures increased significantly. Organic acid concentrations in *Cattleya* leaves at any cultural temperature were markedly higher than in *Cymbidium*. They were lower in both *Cattleya* and *Cymbidium* plants at

Fig. 2 Activities in polyphenol oxidase (PPO) and ascorbate oxidase (AOX) in leaves of *Cattleya* (■) and *Cymbidium* (■) plants cultured at different temperatures.

H: 32/20°C (maximum and minimum temperature), M: 30/15°C, L: 28/8°C. Vertical bars indicate SE ($n=4$).

Fig. 3 Concentrations of ascorbic acid, SH containing substances, lipid peroxides and organic acid in leaves of *Cattleya* (■) and *Cymbidium* (■) plants cultured at different temperatures.

Lipid peroxides and organic acid are shown as malondialdehyde and citric acid, respectively. H: 32/20°C (maximum and minimum temperature), M: 30/15°C, L: 28/8°C. Vertical bars indicate SE ($n=4$).

either high and low cultural temperature than at 30/15°C (Fig. 3).

DISCUSSION

The ideal night temperature range for cattleyas is 12.7–15.6°C and the day temperature should ideally be about 5.6 degrees higher than it is at night, but favorable conditions remain up to 29.4°C. On the other hand, for cymbidiums the ideal night and daytime temperatures in winter are 10°C and 15.6–18.4°C, respectively. In summer temperatures above 29.4°C are not advantageous (Northen, 1970). Thus, the room temperature of 30/15°C imposed seems to be a moderate temperature range. Also, 32/20°C and 28/8°C are known to be respectively high and low cultural temperatures. No apparent disorders occurred at both high and low temperatures (data not shown). This finding is in agreement with indications that both orchids can tolerate 35°C for several hours (Northen, 1970) and with no disorders in cut flowers of *Cattleya* stored at 4.4°C and of *Cymbidium* stored at –0.56°C (Mastalerz, 1977). However, the high and low temperatures induced changes in scavenging enzyme activities and antioxidant and peroxide levels. They also affected maturation of new shoots and flowering (Li et al., unpublished data).

Defensive reactions of plants to ROS induced by various stresses vary with plant species (Foster and Hess, 1982; Feierabend et al., 1992) and factors of causal stresses (Alscher et al., 1997) as well. *Cattleya* showed lower SOD, APX and CAT activities and higher PPO and AOX activities than *Cymbidium*. This finding might be due to photosynthetic behaviors of both orchids. CAM plants have photorespiration as C_3 plants do (Osmond, 1978; Avadhani et al., 1982), but they mitigate it by increasing CO_2 levels in their cells by means of releasing CO_2 from C_4 acids (Osmond, 1978) and by closing their stomata in the daytime. Thus, H_2O_2 level in *Cattleya* leaves is formed by not only the oxidation of glycolate to glyoxylate (Osmond, 1978) but also the reduction of O_2^- by SOD and is predicted to be low. Hence high SOD, APX and CAT activities might not be necessary for ROS-scavenging systems.

Cultural temperatures different from the moderate temperature induced changes in the activities of the scavenging enzymes. The high temperature for *Cattleya* enhanced SOD, APX and CAT activities. This fact indicates that formation of ROS continues under the high temperature condition since high temperatures enhance photorespiration (Powles, 1984), induce SOD in the presence of O_2 in bacteria and mammals (Fridovich, 1974) and increase CAT in maize scutellum in H_2O_2 containing media (Scandalios, 1994). PPO, possibly mediating photoreduction of molecular O_2 by PSI in chloroplasts (Vaughn et al., 1988), and AOX also increased activity. Increased PPO activity may serve to cause leaf burning if leaf temperature increases beyond the critical temperature. These results seem to verify the ability of cattleyas to tolerate heat stress. The high cultural temperature for cymbidiums also enhanced activities in scavenging enzymes except for CAT which is reported to reduce at high temperatures and to be associated with induction of photoinhibition at PSII (Feierabend et al., 1992). However, increased lipid peroxides and decreased SHS suggest that the *Cymbidium* was still struggling against the hot climate to stabilize cell membranes. Besides, a decrease in CAT activity in *Cymbidium* suggests that APX functioned mainly to detoxify H_2O_2 by using increased ascorbic acid.

The low cultural temperature did not affect (or affected slightly) scavenging enzymes in both *Cattleya* and *Cymbidium*: APX increased in *Cattleya*, while CAT decreased in *Cymbidium*. Two possible explanations may be given: firstly the temperature subjected was not so much low. However, at this temperature PPO and AOX activities and antioxidant levels were influenced. Therefore, it is rational to predict that orchids activate or inactivate the scavenging enzymes during the early cultivation period and then reverted to their original

activities during further cultivation. The fact that CAT in *Cattleya* did not respond to the low temperature was contradictory to earlier reports that CAT activity in several crops decreases under this condition (Omran, 1980; Feierabend et al., 1992).

A marked increase in SHS in leaves of *Cattleya* cultured at the low temperature might not be responsible for ROS-scavenging activities but for the stabilization of proteins to enable this orchid to tolerate cold climate. Zhao and Blumwald (1998) reported that formation of -SS- bonds of proteins in Jack pine roots was dependent on reduced glutathione/oxidized glutathione mole ratios and concentrations of reduced glutathione which increased during cold-acclimation. Thus, lipid peroxides did not increase, though ascorbic acid decreased at the low cultural temperature. Increases in lipid peroxides in leaves of *Cymbidium* cultured at both low and high temperatures were inconsistent with increased ascorbic acid levels because ascorbic acid inhibits peroxidation of thylakoid membrane by α -tocopherol-mediated reduction (Alscher et al., 1997).

In conclusion, the scavenging enzyme activities as well as the antioxidant and superoxide levels in leaves of *Cattleya* were different from those of *Cymbidium* plants. They were influenced by cultural temperatures. Thus, it is clear that the high and low temperatures imposed acted as thermal stress to evoke ROS-scavenging systems. However, some inconsistent results, for example, no (or slight) changes in SOD, APX and CAT activities were found in leaves under the low cultural temperature. To clarify those changes in relation to thermal stress, further elaborate experiments on effects of short-term thermal acclimation are needed.

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〈和文抄録〉

栽培温度が *Cattleya* と *Cymbidium* 葉の抗酸化レベルに及ぼす影響

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ラン科植物の *Brassolaeliocattleya* Chian-Tzy Emperor (*Cattleya*) と *Cymbidium* Sazanami を最高/最低温度がそれぞれ 32/20°C, 30/15°C, 28/8°C の条件で 2 カ月栽培した。*Cattleya* の葉のスーパージンクシドジスムターゼ (SOD), アスコルビン酸ペルオキシダーゼ (APX) と、カタラーゼ (CAT) 活性は *Cymbidium* に比べ低く, ポリフェノールオキシダーゼ (PPO) とアスコルビン酸オキシダーゼ (AOX) 活性は高かった。高温栽培では *Cattleya* はこれらの酸素のすべて, *Cymbidium* は CAT を除く四つの酵素活性を高めた。低温栽培は *Cattleya* では APX 活性のみが上昇し, *Cymbidium* では CAT 活性のみが低下し, 高温栽培に比べて影響が少なかった。二つのランとも PPO 活性が上昇し, AOX 活性は低下した。抗酸化物質のレベルについて *Cattleya* では高, 低温栽培条件でアスコルビン酸含量は低下したが, *Cymbidium* では増加した。前者は低温栽培では SH 基含量が著しく増加し, 後者は高温栽培で著しく低下した。葉中の過酸化脂質含量は高, 低いずれの栽培温度でも *Cattleya* では変化が少なかったが, *Cymbidium* で増大した。