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Responses of two field-grown coffee species to drought and re-hydration

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Abstract

The gas exchange, parameters of chlorophyll fluorescence, contents of pigments, and activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), as well as lipid peroxidation were investigated in two field-grown coffee species, *Coffea arabica* and *C. liberica*, exposed to drought and re-hydration. Drought caused a more pronounced inhibition of net photosynthetic rate in *C. liberica* compared to *C. arabica*. The de-epoxidation of xanthophyll cycle pigments at midday estimated by leaf reflectance was much higher in *C. arabica* than in *C. liberica*, but no significant change was found in response to drought. Under moderate drought, the activities of SOD and APX increased significantly only in *C. arabica*. The maximum photochemical efficiency of photosystem 2, PS2 (F_v/F_m) at predawn did not change and there was no lipid peroxidation during this time. Under severe drought F_v/F_m decreased and initial fluorescence (F_0) increased for both species, and SOD activity increased, APX activity remained relatively high, and malondialdehyde (MDA) accumulated in *C. arabica*, while APX decreased in *C. liberica*. The photosynthetic apparatus of *C. arabica* was completely recovered after 5 d of re-irrigation as indicated by the restoration of F_v/F_m to the control values. A lack of recovery upon re-watering of *C. liberica* indicated irreversible damage to PS2. Hence compared to *C. liberica*, *C. arabica* possesses a higher desiccation-induced antioxidative protection and higher portion of the total pigment pool used in photoprotection, which might aid alleviating photoinhibitory damage during desiccation and photosynthesis recovery when favourable conditions are restored.

Additional key words: antioxidant system; ascorbate peroxidase; chlorophyll fluorescence; *Coffea* species; lipid peroxidation; malondialdehyde; photosynthesis; superoxide dismutase; water deficit.

Introduction

Coffee was originally classified as obligatory shade species and widely planted in the tropical area. Irradiation at midday is usually strong enough to over-saturate the photosynthetic apparatus of coffee leaves, and induce severe photoinhibition and photooxidative damage (Nunes *et al*. 1993, DaMatta and Maestri 1997), because irradiance in the tropical area is commonly over 2 000 μmol m^{-2} s^{-1}. A number of environmental stresses, including drought, may increase the sensitivity to photoinhibition and photodamage. To protect the photosynthetic apparatus from oxidative stress, plants must dissipate excessive photon energy. This can be achieved by down-regulation of the photochemical efficiency by way of the xanthophyll cycle (Demmig-Adams and Adams 1992) or by maintenance of electron flux involving alternative pathways such as photorespiration and the Mehler peroxidase reaction (Asada 1999, Ort and Baker 2002). However, both pathways lead to an increased production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and H_2O_2. To cope with ROS, plants are endowed with a complex enzymatic antioxidant system including superoxide dismutases (SOD), which catalyse the reaction from $O_2^{\cdot-}$ to H_2O_2, and ascorbate peroxidase (APX), which detoxifies the H_2O_2 produced (Asada 1999, Mittler 2002).

Coffee was introduced to China more than 100 years ago. *Coffea arabica* was the dominant planted coffee species in the mountainous areas and *C. liberica* was also widely planted in lowland areas in the southern China. Soil water deficit in the dry season is an important environmental factor that largely decreases the productivity of coffee (Long and Wang 1997). The ability of coffee to produce satisfactorily in the areas subjected to water deficit has been termed through a suite of morphological and physiological adaptations (leaf area reductions, stomatal closure, osmotic adjustment, *etc*.) that allow it to survive water stress (DaMatta *et al*. 2002, 2003) but the degree of adaptation to drought may vary considerably

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within species and also within clones (DaMatta and Maestri 1997, DaMatta *et al*. 1997, 2003, Lima *et al*. 2002). The objective of this study was to investigate possible mechanisms responsible in both mentioned coffee species. The effects of drought were studied to elucidate the mechanisms that confer protection from photoinhibition and oxidative stress and to analyze the recovery after re-hydration in these two species during the dry season in Xishuangbanna tropical area, southern China. For the purpose, we measured (*1*) photosynthetic characteristics and chlorophyll (Chl) *a* fluorescence, (*2*) pigment composition, and (*3*) activities of reactive oxygen-scavenging enzymes, in order to determine their possible relation to physiological changes in droughted plants.

Materials and methods

Plants and treatments: Seeds of two coffee species (*Coffea arabica* L and *C. liberica* Bull *ex* Hien) were collected from Yunnan Province, PR China. Seedlings were grown in six flat plots of 3×10 m^2 each in the Xishuangbanna Tropical Botanical Garden (21°56'N, 101°15'E, altitude 560 m), Chinese Academy of Sciences. The site received an average annual rainfall of 1 500 mm with a marked dry season from November to April.

During the first one and half a years after planting, all plants were fully irrigated and with an unlimited nutrient supply. Experiments were conducted from 15 March to early April 2003, when the plants were on average 45 cm tall. Two water treatments were applied, one group of plants was continuously irrigated daily to maintain the soil close to the field capacity (control) and the second group was subjected to drought by omitting irrigation for 10 d. Subsequently, the plants subjected to drought were irrigated daily for 5 d to reach the field capacity again and the recovery was studied. Plot soil surfaces were covered with rice mulch to minimize evaporation, thus allowing a slower establishment of the water stress. There was no appreciable rainfall during the experimental period. Environmental conditions were monitored with a weather station that was situated 30 m from the experimental plot. Vapour pressure deficit (VPD) was calculated from air temperature (T_a) and relative humidity according to Nobel (1991).

Plant pigment contents, enzymatic activities, and the extent of lipid peroxidation [malondialdehyde (MDA) content] were measured in fully developed young leaves collected on clear sunny days at midday.

Water status and total leaf area: The relative leaf water content (RWC) was determined as 100×(FM – DM)/(TM – DM), where FM is fresh mass, TM is turgid mass after re-hydrating the leaves for 24 h at 4 °C in darkness, and DM is dry mass after oven-drying the leaves for 24 h at 70 °C. Total leaf area per plant was measured using a leaf area meter (*Li-3000A*, *Li-Cor*, Lincoln, NE, USA) from four seedlings randomly selected per each treatment.

Pigment analysis: Five discs (diameter 20 mm) were randomly removed from each coffee species. Chlorophylls (Chl) and carotenoids (Car) of the control and stressed leaves were extracted in 80 % acetone and absorbances at 663, 645, and 470 nm were measured using an UV-B spectrophotometer (*UV-B 2501*, *Shimadzu*, Japan). Chl and Car contents were then calculated according to Arnon (1949).

Gas exchange and Chl *a* fluorescence: Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) were measured using a portable gas exchange system (*LiCor-6400*, *Li-COR*, Lincoln, NE, USA) on uppermost, fully expanded leaves at mid-morning, between 10:00 and 10:30 [26–27 °C, relative humidity *ca*. 70 %, photosynthetic photon flux density (PPFD) 1 100 µmol m^{-2} s^{-1}, and CO_2 concentration 380–390 µmol mol^{-1}].

Chl *a* fluorescence was measured at predawn *in situ* on attached leaves with a portable pulse-modulated fluorescence system (*FMS-2.02*, *Hansatech*, King's Lynn, UK). Initial (F_0) and maximal fluorescence (F_m) were measured in leaves dark-adapted for 10 min. Maximum fluorescence (F_m) was recorded after a 0.8 s pulse of saturating irradiance (4 000 µmol m^{-2} s^{-1}). The maximal photochemical efficiency of PS2 was estimated by the ratio $F_v/F_m = (F_m – F_0)/F_0$.

Leaf reflectance: A *UniSpec* spectral analysis system (*PP Systems*, Haverhill, MA, USA) was used to measure spectral reflectance at wavelengths from 306 to 1 138 nm. A spectral reflectance standard was regularly referenced and scans were corrected for the instrument's dark current. Each scan represented the mean of six passes and the instrument's integration time was set at 125 ms. The photochemical reflectance index, which was calculated as $PRI = (R_{531} – R_{570})/(R_{531} + R_{570})$ (Gamon and Surfus 1999), is correlated with the epoxidation state of the xanthophyll cycle pigments and photosynthetic radiation-use efficiency (net photosynthesis/incident PPFD) (Gamon *et al*. 1992, Peñuelas *et al.* 1995). Method for estimating the degree of de-epoxidation of xanthophyll cycle pigments was to sample PRI under predawn and midday on the same leaf to derive a ΔPRI (expressed as the predawn PRI *minus* the midday PRI values – Gamon and Surfus 1999).

Enzymatic activities and lipid peroxidation analysis: For the determination of total superoxide dismutase (SOD) activity, 0.4 g fresh leaf tissue from the leaves was ground in a chilled mortar in 50 mM phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 50 mg polyvinyl-

pyrrolidone (PVP), and 0.1 % *Triton X-100*. For the extraction of total ascorbate peroxidase (APX), leaf tissue was ground in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 5 mM ascorbate, 0.5 % (m/v) PVP, 0.1 % (v/v) *Triton X-100*, and 0.05 % (v/v) β-mercaptoethanol. After centrifuging at 12 000×*g* and 4 °C for 15 min, the supernatant of each extract was used as the crude enzyme extract for determination of soluble protein concentration and the activities of SOD and APX. SOD activity was assayed by determining the ability of the extracted enzymes to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). One unit of SOD [U] was defined as the amount of SOD that caused 50 % inhibition of the photo-reduction of NBT. APX activity was determined according to Nakano and Asada (1987) by monitoring the rate of ascorbate oxidation at 290 nm (coefficient of absorbance = 2.8 mM^{-1} cm^{-1}). The 3 cm^3 of reaction mixture contained 50 nM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.3 mM H_2O_2, and 0.04 cm^3 of enzyme extract. One unit of APX [U] was defined as the amount of enzyme that oxidized 1 µmol of ascorbate per min at room temperature. Soluble protein content was determined using a spectrophotometer at 595 nm according to Bradford (1976) with bovine serum albumin as a standard.

Lipoperoxidation was monitored by the spectrophotometric determination of MDA using thiobarbituric acid according to Popham and Novacky (1991). Plant material (1 g FM) was homogenized in 2 cm^3 of trichloroacetic acid, TCA (10 %, m/v) and centrifuged at 15 000×*g* for 20 min. To 250-mm^3 aliquot of crude extract 250 mm^3 of TCA (10 %, m/v) plus 1 cm^3 of thiobarbituric acid (0.2 %, m/v) in 10 % TCA was added. The mixture was boiled at 95 °C for 30 min and cooled on ice for 5 min. After centrifugation at 10 000×*g* for 10 min, absorbance of the supernatant was determined at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated from its extinction coefficient (155 mM^{-1} cm^{-1}).

Statistical analyses: Statistical differences between measurements on different treatments or on different times were analyzed following the Student's *t*-test using *SPSS11.0* (Chicago, IL, USA). Differences were considered significant at a probability level of $p \leq 0.05$.

Results

Water status and growth: During the dry season from March 15 to early April, the maximum PPFD varied from 1 643 to 1 823 µmol m^{-2} s^{-1} and the highest air temperature varied from 28.8 to 31.2 °C (Fig. 1). After 4 d of drought treatment, RWC in leaves of *C. arabica* and *C. liberica* attained 90 and 88 % (moderate water stress); RWC reached *ca.* 80 % after 10 d of drought (severe water stress), respectively. Drought induced a slightly larger decrease in RWC in *C. liberica* than in *C. arabica*.

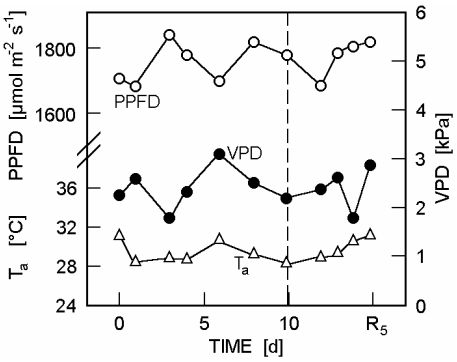

Fig. 1. Environmental conditions during the experiment. Air temperature (T_a) and vapour pressure deficit (VPD) were measured at midday (at maximum PPFD). R_5: re-hydrated for 5 d.

Moreover, an almost full recovery of leaf status was achieved only in *C. arabica* (99.2 % of the controlled value; p=0.87) after 5 d of re-hydration (Table 1).

New leaves of the two coffee species developed and expanded throughout the study, thereby increasing the total leaf area per plant of 15.2 % in *C. arabica* and 12.6 % in *C. liberica* under controlled conditions (data not shown). However, the total leaf area only increased by 11.9 % in *C. arabica* and 5.8 % in *C. liberica* after the 15-d experiment, respectively (Table 1).

Photosynthesis: *C. arabica* and *C. liberica* did not show significant differences in their P_N, E, and g_s for unstressed plants, but the former was clearly superior to the latter in its resistance to drought. Moderate water stress caused decreases in g_s (36.1 %), E (17.3 %), and P_N (22.6 %) in *C. arabica*. In *C. liberica*, such decreases were 37.8 % for g_s, 24.6 % for E, and 37.6 % for P_N (Table 1). The drop in P_N did not parallel changes in F_v/F_m of both species (Table 1), suggesting that the photosynthetic apparatuses were relatively resistant to the early stage of leaf desiccation.

When water deficit was severe (water deficit for 10 d), the decline in P_N was associated with a continuous decrease in g_s in either species. The predawn values of F_v/F_m, and F_0 were affected significantly by severe water stress. F_v/F_m decreased by 8.9 % in *C. arabica* and by 17.4 % in *C. liberica*. Moreover, a full recovery of F_v/F_m was found only in *C. arabica* (*ca.* 100 %) as the RWC progressively increased during irrigation (Table 1).

Photosynthetic pigments and the conversion of xanthophyll cycle pigments: Chl content did not decline during the moderate drought period, but was affected by severe drought in both species (25.1 % in *C. arabica* and 38.8 % in *C. liberica*) and recovery to the control values

after 5 d of re-hydration was observed only in *C. arabica*. The Car/Chl ratio increased during the drought period in *C. arabica*, whereas only a slight change was observed in *C. liberica*. On the other hand, the de-epoxidation of xanthophyll cycle pigments estimated by leaf reflectance at midday was not enhanced in either species during drought and recovery ($F = 2.34$; $p<0.05$), although it was higher in *C. arabica* than in *C. liberica* ($p<0.01$) (Fig. 2).

Table 1. Effects of dehydration (0, 4, and 10 d) and re-hydration (R_5 = 5 d) on relative water content (RWC), total leaf area per plant, net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), F_0, and F_v/F_m of two coffee species. Means ± SD (n = 4). Different letters indicate significantly different means.

		0	4	10	R_5
RWC [%]	*C. arabica*	94.6±2.1 a	89.7±3.2 b	80.4±1.7 c	92.8±1.1 ab
	C. liberica	95.1±3.4 a	88.3±0.6 b	79.1±1.2 c	88.7±0.4 b
Leaf area [m^2 $plant^{-1}$]	*C. arabica*	4.60±0.12 b	–	4.91±0.09 ab	5.15±0.11 a
	C. liberica	4.34±0.08 c	–	4.43±0.07 c	4.59±0.18 b
P_N [µmol m^{-2} s^{-1}]	*C. arabica*	6.45±0.25 a	5.02±0.11 b	1.28±0.01 d	3.89±0.23 c
	C. liberica	6.54±0.14 a	4.12±0.21 bc	0.86±0.15 d	1.56±0.23 cd
g_s [mmol m^{-2} s^{-1}]	*C. arabica*	108.5±5.2 a	68.7±5.4 b	36.7±9.1 c	92.6±5.4 ab
	C. liberica	112.3±4.5 a	70.2±3.1 b	23.4±1.9 c	70.3±3.9 b
E [µmol m^{-2} s^{-1}]	*C. arabica*	1.67±0.23 a	1.37±0.12 b	0.68±0.11 b	1.56±0.02 b
	C. liberica	1.70±0.11 a	1.28±0.08 bc	0.53±0.09 b	0.67±0.13 d
F_0	*C. arabica*	123±5.6 a	132±5.3 ab	143±4.3 b	134±2.9 ab
	C. liberica	126±5.7 a	142±4.7 b	158±4.2 c	148±7.8 b
F_v/F_m	*C. arabica*	0.832±0.015 a	0.827±0.023 a	0.76±0.018 b	0.823±0.013 a
	C. liberica	0.828±0.011 a	0.831±0.004 a	0.698±0.025 c	0.756±0.008 b

Antioxidative protection and lipid peroxidation during drought and recovery were evaluated (Fig. 3). Antioxidative enzymes (SOD and APX) differed in their responses to drought and recovery between *C. arabica* and *C. liberica*. In *C. arabica*, moderate drought stress increased the activities of SOD (44.5 %) and APX (18.2 %); moreover, the activities of these enzymes were relatively high under severe drought and after 5 d of re-hydration. However, even if in *C. liberica* moderate drought caused increases in the activities of SOD (18.2 %) and APX (8.5 %), APX activity decreased dramatically (41.7 %) during the severe drought and could recover only to 87.1 % of the controlled value after 5 d of irrigation. Under moderate drought stress, MDA was not accumulated in either species. However, severe drought-induced increases in lipid peroxidation were 16.6 % in *C. arabica* against 44.5 % in *C. liberica*. Almost full recovery of MDA content to the control value was observed in *C. arabica*, but it was still maintained high in *C. liberica* after the recovery period.

Discussion

Drought affected numerous physiological and metabolic processes in the two coffee species: g_s was more sensitive to early drought than E, as has also been shown by DaMatta *et al.* (1997, 2003). The marked decrease in g_s (Table 2) and a lesser increase in leaf area compared to the control plants of both species can be considered avoidance mechanisms that minimize water losses. Such behaviours were previously observed in other taxa (Martínez-Ferri *et al*. 2000, Sánchez-Blanco *et al*. 2002). Chl fluorescence parameter F_v/F_m remained unaffected by moderate drought treatment in both species (Table 3), which showed that no photodamage to PS2 reaction centres or development of slowly relaxing excitation energy quenching had been induced by drought (Foyer *et al.* 1994, Asada 1999). Thus the early P_N reduction might be through a mechanism dependent on the stomatal closure, not caused by the damage of PS2 during this period. In the later stage of drought, the reduction of P_N may be caused by both stomatal and non-stomatal mechanisms, because the F_v/F_m decreased and F_0 increased greatly (Table 1), indicating PS2 damage (Epron *et al.* 1992, Maxwell and Johnson 2000). After re-hydration, *C. arabica* plants showed a rapid recovery in F_v/F_m, which was in parallel with that of P_N. The fast recovery of Chl fluorescence parameters suggests that the decline in PS2 efficiency was reversible, serving as a photoprotective role. The failure of *C. liberica* to resume full photochemical activity when re-hydrated indicates that the photosynthetic apparatus had been adversely affected during desiccation.

Despite Chl being highly sensitive to soil drought (Castrillo and Trujillo 1994, Tuba *et al*. 1996) and drought-induced reductions in pigment contents were previously found in several crop species (Moran *et al*. 1994, Loggini *et al.* 1999), Chl contents did not change greatly under moderate water stress in either coffee species

(Fig. 2), as also noted in *C. arabica* and *C. canephora* by DaMatta and Maestri (1997). However, Chl contents declined significantly during the severe drought period, which may be related to membrane disintegration due to oxidative stress (Moran *et al*. 1994, Alonso *et al*. 2001). Although it is a negative consequence of stress that photosynthetic apparatus must be re-synthesized *de novo* upon re-watering, Chl loss has also been considered an adaptive feature, which reduces the possibility of further damage to the photosynthetic machinery by the formation of ROS under an excess of excitation energy (Munné-Bosch and Alegre 2000, Kranner *et al*. 2002). At the same time, relatively higher Car/Chl ratio in droughted *C. arabica* indicated that water-stressed plants were protected from damage by a relatively higher amount

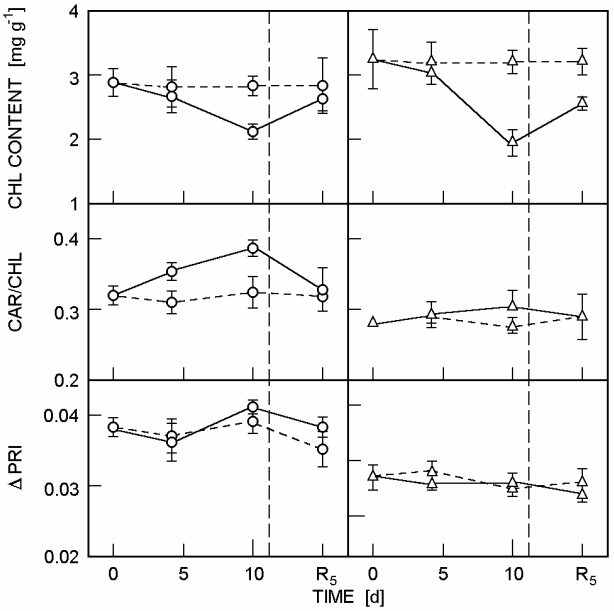

Fig. 2. Effects of dehydration and re-hydration (R_5 for 5 d) on chlorophyll (Chl) contents, carotenoid (Car) to Chl ratio, and the difference in photochemical reflectance index between predawn and midday (ΔPRI) in leaves of *C. arabica* (○) and *C. liberica* (Δ). *Error bars* indicate SD (*n* = 3–4). *Dash line*, control; *solid line*, drought-stressed.

of Car than in *C. liberica* (Logan *et al*. 1997). However, although the ΔPRI values in *C. arabica* were much higher than in *C. liberica*, photoprotection of PS2 was not achieved by an increase in non-radiative energy dissipation during drought and recovery as ΔPRI values of the treated plants were not significantly different from those of the controlled plants in both coffee species (Fig. 2). In this case, the amount of xanthophyll cycle pigments and their de-epoxidation (assessed by the difference in PRI between predawn and midday) may be mainly determined by irradiance as the midday PPFD (>1 600 µmol m^{-2} s^{-1}) is much higher than the saturation irradiance (*ca*. 850 µmol m^{-2} s^{-1}; DaMatta *et al*. 2002, Silver *et al*. 2004) of coffee species.

The drought stress brought about other biochemical responses in coffee plants in order to minimize its deleterious effects. The important components of protective systems are enzymatic defences such as SOD and APX,

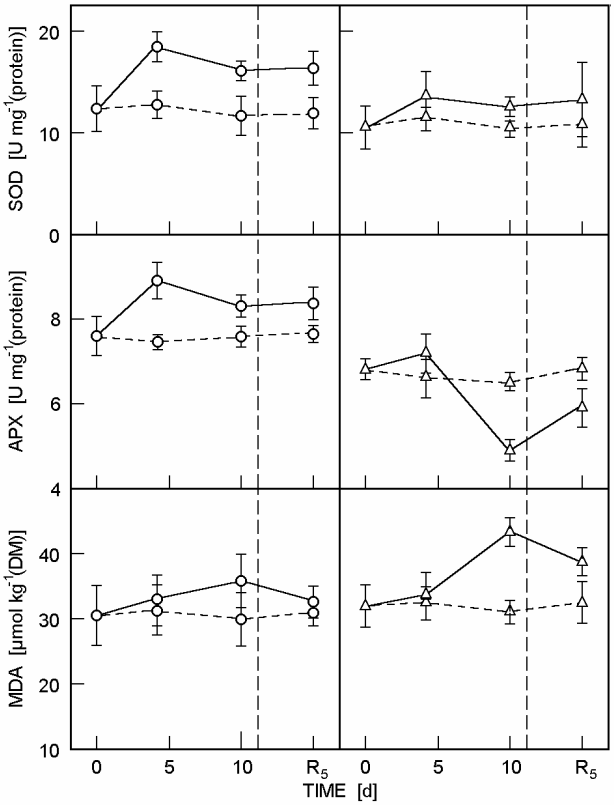

Fig. 3. Effects of dehydration and re-hydration (R_5 for 5 d) on enzymatic activities and malondialdehyde (MDA) accumulation in leaves of *C. arabica* (○) and *C. liberica* (Δ). *Error bars* indicate SD (*n* = 3–4). *Dash line*, control; *solid line*, drought-stressed.

which scavenge $O_2^{\cdot-}$ and H_2O_2, respectively (Sgherri *et al*. 1994, Sgherri and Navarri-Izzo 1995, Loggini *et al*. 1999). Drought-tolerant cultivars exposed to osmotic or oxidative stress show less oxidative damage and higher antioxidative enzyme activities than the sensitive cultivars in crop species, such as wheat (Pastori and Trippi 1993, Lascano *et al*. 2001) and maize (Pastori and Trippi 1993). In our experiments, total SOD and APX activities were higher in *C. arabica* than in *C. liberica* (Fig. 3). During the early drought period, the activities of SOD and APX increased and MDA did not accumulate (Fig. 3), indicating that SOD and APX counteracted the potentially harmful effects of H_2O_2 as well as $O_2^{\cdot-}$ similarly as has been reported for other crops (Lascano *et al*. 2001, Lima *et al*. 2002, Munné-Bosch *et al*. 2003). This result agreed with the reports on the dynamics of these enzymes in water-stressed leaves of barley (Acar *et al*. 2001), wheat (Sgherri *et al*. 2000, Lascano *et al*. 2001), and maize (Pastori and Trippi 1993). But with the

progress of drought, the depression of SOD and especially APX activities was observed in *C. liberica*, while they were still maintained relatively high in *C. arabica* (Fig. 3). In this study, photoinhibition in *C. liberica* was evident as shown in decreased F_v/F_m values and increased F_0 values (Table 1). When coffee plants were irrigated, it took more than 5 d for P_N and F_v/F_m of droughted *C. liberica* plants to fully recover (Table 1), whereas the enzymatic activities recovered faster (Fig. 3).

In conclusion, although the two coffee species studied have developed water conservation strategies involving changes in leaf areas and early stomata closure, the more drought-tolerant *C. arabica* showed higher resistance to drought stress than the more drought-sensitive *C. liberica* because of greater de-epoxidation of violaxanthin in the xanthophyll cycle and higher activities of antioxidant enzymes, which resulted in a less oxidative damage and lipid peroxidation.

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