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ORIGINAL PAPER

Inhibition of monoterpene biosynthesis accelerates oxidative stress and leads to enhancement of antioxidant defenses in leaves of rubber tree (*Hevea brasiliensis*)

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Abstract This paper mainly studies the possible antioxidant of monoterpene and effects of its absence on other antioxidant defense. The leaves of rubber tree (*Hevea brasiliensis*) were fed with fosmidomycin through transpiration stream, in the dark, at room temperature for 2 h, and were then exposed to bright illumination (1,500 μmol m^{-2} s^{-1}) and moderately high temperature (30°C) for 1 h. The results showed that monoterpene biosynthesis in leaves was considerably inhibited by fosmidomycin, and the elevated levels of both hydrogen peroxide and malondialdehyde were observed in the leaves fed with fosmidomycin (LFF). Compared to the control leaves (CK), $\Delta F/F_m'$ in the LFF was markedly lower during the first 20 min; however, there were no significant differences in non-photochemical quenching and photosynthetic pigments (chlorophylls and carotenoids). In contrast, the activities of antioxidant enzymes (superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase) were enhanced in the LFF. Meanwhile, the contents of antioxidant metabolites (ascorbate and glutathione) were also elevated in the LFF, when compared with the CK. The results obtained here suggest that monoterpene may be very effective molecule in protecting plants against oxidative stress, the absence of monoterpene leads to the increased responses of the enzymatic and non-enzymatic antioxidant defenses to oxidative stress, and the enhancement of the enzymatic and non-enzymatic antioxidant defenses may, in part, compensate for the loss of antioxidant conferred by monoterpene.

Keywords Monoterpene · Oxidative stress · Antioxidant enzyme · Antioxidant metabolite

Abbreviations

H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
Chl	Chlorophyll
Cars	Carotenoids
SOD	Superoxide dismutase (EC 1.15.1.1)
CAT	Catalase (EC 1.11.1.6)
GR	Glutathione reductase (EC 1.6.4.2)
POD	Guaiacol peroxidase (EC 1.11.1.7)
APX	Ascorbate peroxidase (EC 1.11.1.11)
AsA	Reduced ascorbate
DHA	Oxidized ascorbate
GSH	Reduced glutathione
GSSG	Oxidized glutathione
NPQ	Non-photochemical quenching
$\Delta F/F_m'$	Actual photochemical efficiency of photosystem II
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid

Introduction

The terpenoids, or isoprenoids, are the largest and the most diverse family of natural chemical products, ranging in

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structure from linear to polycyclic molecules, and in size from five-carbon hemiterpenes to natural rubber that is comprised of thousands of isoprene units (Mahmoud and Croteau 2002). It has been considered that some isoprenoids (including several carotenoids and tocopherols) play an effective role in photoprotection, whereas others such as isoprene and some monoterpene, which do not occur in some plant species, represent a way for increasing plasticity in photoprotection (Peñuelas and Munné-Bosch 2005). Peñuelas and Llusià (2002) have reported that the formation of monoterpene might depend on photorespiratory activity, and that under non-photorespiratory conditions monoterpene seem to replace photorespiration in providing protection against high temperature. Furthermore, it has been proved that monoterpene improved thermotolerance at elevated temperatures (Loreto et al. 1998), and that monoterpene had a protecting role against oxidative stress (Loreto et al. 2004). Therefore, two hypotheses concerning the physiological functions of both isoprene and monoterpenes have been proposed: (1) stabilization and protection of plant membranes against high temperatures (Sharkey and Singsaas 1995; Loreto et al. 1998; Singsaas 2000), and (2) antioxidation.

On the other hand, it has been shown that leaves producing isoprene and specific monoterpene (e.g. limonene, α- and β-pinene) withstand higher temperatures in the light than those in which isoprene or monoterpene production is inhibited (Peñuelas and Llusià 2002; Peñuelas et al. 2005; Sharkey and Singsaas 1995; Sharkey and Yeh 2001). Moreover, isoprene biosynthesis can be exclusively inhibited by fosmidoycin (Zeidler et al. 1998; Loreto and Velikova 2001; Sharkey et al. 2001). If the fosmidoycin-fed leaves were exposed to high light (1,500 μmol m^{-2} s^{-1}) and moderately high temperature (30°C), under such condition, the leaves would lead to photo-oxidative injury, including secondary oxidative stress. Oxidative stress arises from an imbalance in the generation and metabolism of reactive oxygen species. However, plants have evolved antioxidant mechanisms (enzymatic and non-enzymatic), by which reactive oxygen removes species from the cell (Noctor and Foyer 1998). Catalase and peroxidase are the two major systems for the enzymatic removal of H_2O_2 in plants (Willekens et al. 1995).

Our test plant was rubber trees (*Hevea brasiliensis*), which does not produce isoprene in detectable amount, but produces considerable monoterpene (mainly α- and β-Pinene and sabinene) in the leaves (Klinger et al. 2002). The leaves of rubber tree were fed with fosmidoycin through transpiration stream. We hypothesized that, like isoprene, monoterpene biosynthesis would be considerably inhibited by fosmidoycin, and that, without monoterpene, the enzymatic and non-enzymatic antioxidative mechanism might be more efficient. Thus, the objectives of the present study were to determine whether the inhibition of monoterpene biosynthesis induces the enhancement of the activities of other enzymatic and non-enzymatic antioxidants in leaves of rubber tree exposed to high illumination and moderate temperature, and further confirm that monoterpene may effectively confer antioxidant.

Materials and methods

Plant material and experimental treatment

The experimental plants were rubber trees, growing at the edge of a 30-year-old rubber/tea-mixed plantation, and therefore, receiving full light on the sampled branches. The leaves of sampled branch were cut, maintained with the petiole in water and fed with 30 μM fosmidomycin through transpiration stream in the dark at room temperature for 2 h. The control leaves were cut and maintained with the petiole in water but not fed with fosmidomycin. A fan was used to facilitate the uptake of chemicals by increasing the transpiration. After the feeding of chemicals, both the fosmidomycin-fed leaves and control leaves were exposed to the bright illumination (1,500 μmol m^{-2} s^{-1}) and the moderately high temperature (30°C) for 1 h. The actual photochemical efficiency of photosystem II ($\Delta F/F_m'$) was measured in every other 10 min. At the end of exposure, the leaves were rapidly frozen in liquid nitrogen and later analyzed for the contents of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), monoterpene, and antioxidant metabolites as well as the activities of antioxidant enzyme.

Measurement of chlorophyll fluorescence

Chlorophyll *a* fluorescence was analyzed using a portable fluorescence system (FMS-2.02, Hansatech, King's Lynn, UK). Non-photochemical quenching (*NPQ*) was calculated according to Schreiber et al. (1994). *NPQ* was estimated from the Stern-Volmer equation as: $(F_m–F_m')/F_m'$, where F_m' and F_m are maximum yield of fluorescence in light-acclimated or dark-adapted leaves, respectively. In this case, F_m was measured after 20 min dark adaptation at room temperature prior to the exposure of high light. The actual quantum efficiency of Photosystem II ($\Delta F/F_m'$) was calculated as: $(F_m'–F_s)/F_m'$, where F_s is the steady-state fluorescence yield.

Determinations of H_2O_2 and photosynthetic pigments

The level of H_2O_2 was determined according to the methods described by Velikova et al. (2000). The contents of chlorophyll (Chl) and carotenoids (Cars) were measured

according to the methods described by Lichtenthaler and Wellburn (1983).

Determination of MDA

The content of MDA was analyzed by the method described by Hodges et al. (1999) with slight modification, for taking into account, the possible influence of interfering compounds in the assay for thiobarbituric acid (TBA)-reactive substances. Leaf tissues were repeatedly extracted with 4 ml of 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000×*g* for 15 min and an aliquot of appropriately diluted sample was added to a test tube with an equal volume of either: (1) −TBA solution containing 20% (w/v) TCA and 0.01% butylated hydroxytoluene; or (2) +TBA solution containing the above solution plus 0.65% (w/v) TBA. Samples were heated at 95°C for 25 min, then after cooling, the absorbance was read at 440, 532 and 600 nm. MDA equivalents were calculated as $10^6 \times [(A - B)/157{,}000]$, where $A = [(\mathrm{Abs}_{532+\mathrm{TBA}}) - (\mathrm{Abs}_{600+\mathrm{TBA}}) - (\mathrm{Abs}_{532-\mathrm{TBA}} - \mathrm{Abs}_{600-\mathrm{TBA}})]$, and $B = [(\mathrm{Abs}_{440+\mathrm{TBA}} - \mathrm{Abs}_{600+\mathrm{TBA}}) \times 0.0571]$.

Determinations of ascorbate and glutathione

The fresh leaf material was homogenized in an ice bath with 4 ml of 5% (w/v) TCA. The homogenate was centrifuged at 15,000×*g* for 15 min and the supernatant was used for assays of contents of ascorbate and glutathione. The content of reduced ascorbate (AsA) was analyzed according to the methods described by Nakagawara and Sagisaka (1984), which were based on the reduction of ferric ion to ferrous ion with AsA in acid solution, followed by formation of the red chelate between ferrous ion and bathophenanthroline, which absorb at 534 nm. After the reduction of oxidized ascorbate (DHA) to AsA by dithiothreitol, the total AsA content was measured as described above, DHA content was determined by subtraction of AsA from the total AsA content. The contents of reduced and oxidized forms of glutathione were measured by the method described by Doulis et al. (1997) via the increase in absorbance at 412 nm following addition of GR for determination of reduced glutathione (GSH) or GR and NADPH for determination of oxidized glutathione (GSSG) to a solution containing extract and 5,5′-Dithiobis (2-nitrobenzoic acid).

Analysis of antioxidant enzymes activity

The extracts for the determination of antioxidant enzyme activities were prepared from 1.0 g of leaf materials homogenized under ice-cold conditions in 4 ml of 0.2 M phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 1% (w/v) polyvinylpyrrolidone (PVP) and 10 mM dithiothreitol. The homogenate was centrifuged at 15,000×*g* for 20 min and the supernatant was used for the assays, and all the assay steps were carried out at 0 ± 4°C. The protein was measured according to Lowry et al. (1951) using bovine serum albumin as a standard.

Catalase (CAT, EC 1.11.1.6) activity was measured in the presence of 10 mM H_2O_2 by monitoring the decrease in absorbance at 240 nm in 50 mM potassium phosphate buffer (pH 7.2). The activity was expressed as ΔA_{240} min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured in the presence of 0.5 mM ascorbate, 0.1 mM EDTA, and 1.0 mM H_2O_2 by monitoring the decrease in absorbance at 290 nm in 50 mM potassium phosphate buffer (pH 7.0). The activity was expressed as ΔA_{290} min^{-1} mg^{-1} protein.

Glutathione reductase (GR, EC 1.6.4.2) activity was measured in the presence of 0.5 mM oxidized glutathione, 1 mM EDTA, and 0.15 mM NADPH by monitoring the decrease in absorbance at 340 nm in 50 mM Tris-HCl buffer (pH 7.5). The activity was expressed as ΔA_{340} min^{-1} mg^{-1} protein.

Guaiacol peroxidase (POD, EC 1.11.1.7) activity was measured in the presence of 16 mM guaiacol and 10 mM H_2O_2 by monitoring the increase in absorbance at 470 nm in 50 mM potassium phosphate buffer (pH 7.0). The activity was expressed as ΔA_{470} min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the photochemical method as described by Giannopolitis and Ries (1977), and one unit of SOD activity was defined as the amount of enzyme, which produced a 50% inhibition of nitroblue tetrazolium reduction at 560 nm.

Analysis of monoterpene

The leaf fresh material was submerged in liquid nitrogen and then the sample was homogenized in ice-cold pentane under liquid nitrogen. A non-terpenoid volatile internal standard, dodecane was used to avoid interference of terpenes. It was added to the pentane extraction procedure before grinding in order to quantify the recovery. Detailed assays of monoterpene concentration were conducted as described by Llusià and Peñuelas (2000).

Statistical analysis

Statistical analysis was performed with software SPSS (Chicago, IL, USA) using Student's *t* test to evaluate differences in assayed parameters.

Results

Feeding the leaves with fosmidomycin drastically inhibited the biosynthesis of monoterpene (Fig. 1). Compared to the control leaves (CK), the concentrations of α-pinene, β-pinene, sabinene and total monoterpene in the leaves fed with fosmidomycin (LFF) were decreased by 87% ($P < 0.05$), 88% ($P < 0.05$), 83% ($P < 0.05$) and 88% ($P < 0.05$), respectively. However, feeding the leaves with fosmidomycin had no effects on photosynthetic pigments (Fig. 2). In brief, there were no significant differences in the contents of Chl and Cars between CK and LFF.

After the exposure to bright illumination and moderately high temperature, $\Delta F/F_m'$ in both LFF and CK were continuously decreased, but $\Delta F/F_m'$ of the LFF was lower than that of the CK during the first 20 min, thereafter this difference was disappeared (Fig. 3a). Nevertheless, *NPQ* did not exhibit a significant difference between LFF and CK.

The activities of antioxidant enzymes in the LFF were enhanced when compared to the CK (Fig. 4a–e). The activities of SOD, CAT, POD, APX, and GR were increased by 66% ($P < 0.05$), 100% ($P < 0.05$), 80% ($P < 0.05$), 51% ($P < 0.05$) and 21% ($P < 0.1$), respectively. There was no significant difference in the content of GSH between LFF and CK (Fig. 5a), but GSSG and glutathione pool (GSH + GSSG) were increased by 34% ($P < 0.05$) and 11% ($P < 0.1$) in the LFF (Fig. 5b, c). Meanwhile, the ratio of GSH to glutathione pool was decreased by 12% ($P < 0.1$) in the LFF (Fig. 5d). Correspondingly, the contents of AsA, DHA, and ascorbate pool (AsA + DHA) in the LFF were increased by 12% ($P < 0.1$), 134% ($P < 0.05$), and 63% ($P < 0.05$), respectively (Fig. 6a–c). In contrast, the ratio of AsA to ascorbate pool was decreased by 34% ($P < 0.05$) in the LFF (Fig. 6d).

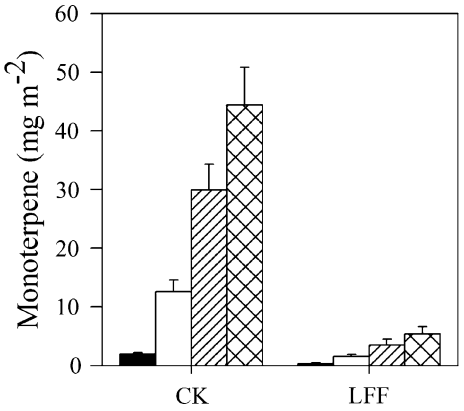

Fig. 1 Effects of fosmidomycin feedings on concentration of monoterpene. Mean ($n = 6$–8) ± SE are shown for controls (CK) and leaves fed with fosmidomycin (LFF). Sabinene (*shaded rectangle*), α-Pinene (*open rectangle*), β-Pinene (*striped rectangle*), Total monoterpene (*boxed rectangle*). Significant differences ($P < 0.05$) were confirmed by Student's *t* test

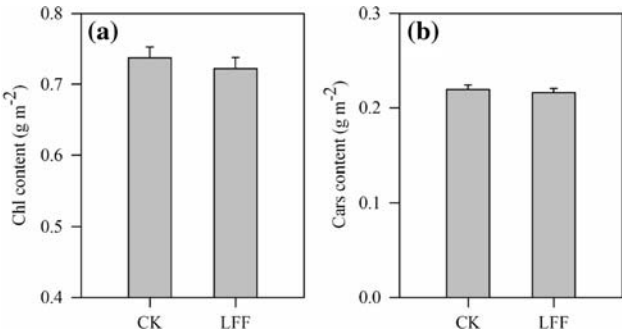

Fig. 2 Effects of fosmidomycin feedings on contents of chlorophylls (Chl, **a**) and carotenoids (Cars, **b**). Means ($n = 5$–7) ± SE are shown for controls (CK) and leaves fed with fosmidomycin (LFF). Significant differences ($P < 0.05$) were confirmed by Student's *t* test

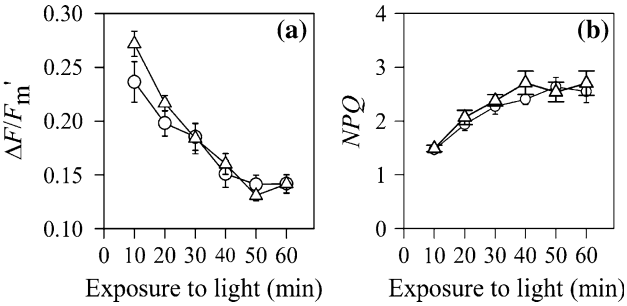

Fig. 3 Effects of fosmidomycin feedings on actual photochemical efficiency ($\Delta F/F_m'$, **a**) and non-photochemical quenching (*NPQ*, **b**) of photosystem II. Mean ($n = 5$–7) ± SE are shown for controls (CK, *Open triangles*) and leaves fed with fosmidomycin (LFF, *Open circles*). Significant differences ($P < 0.05$) were confirmed by Student's *t* test

Furthermore, the LFF showed significantly higher accumulations of H_2O_2 and MDA compared to the CK (Fig. 7). The contents of H_2O_2 and MDA were increased by 68% ($P < 0.05$) and 32% ($P < 0.05$) in the LFF, respectively.

Discussion

The considerable inhibition of monoterpene biosynthesis by fosmidomycin in our experiment (Fig. 1) is in accordance with the reported results that fosmidomycin caused rapid and complete inhibition of monoterpene biosynthesis (Loreto et al. 2004). Indeed, the effects of fosmidomycin on other isoprenoids, such as carotenoids, which may be involved in antioxidant protection directly or indirectly, need to be extensively elucidated, due to the facts that the biosynthesis of carotenoids is also inhibited by fosmidomycin (Laule et al. 2003). In our experiment, the effects of fosmidomycin on the Chl and Cars were examined, and the

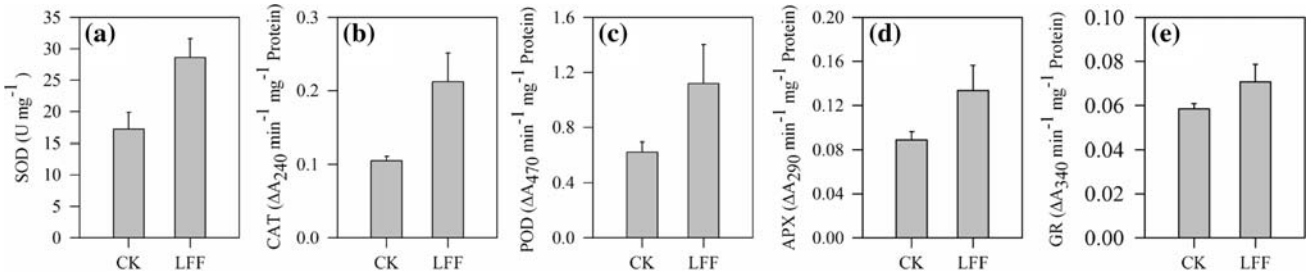

Fig. 4 Effects of fosmidomycin feedings on activities of antioxidant enzymes of superoxide dismutases (SOD, **a**), catalase (CAT, **b**), guaiacol peroxidase (POD, **c**), ascorbate peroxidase (APX, **d**), and glutathione reductase (GR, **e**). Mean ($n = 5$–7) ± SE are shown for controls (CK) and leaves fed with fosmidomycin (LFF). Significant differences ($P < 0.05$) were confirmed by Student's t test

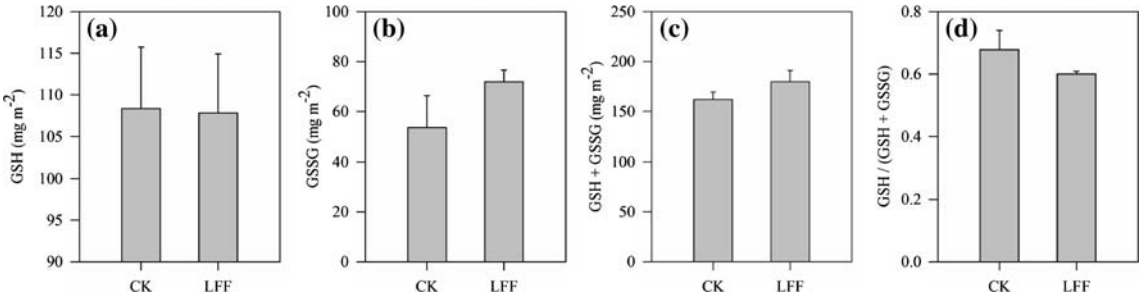

Fig. 5 Effects of fosmidomycin feedings on contents of reduced glutathione (GSH, **a**), oxidized glutathione (GSSG, **b**), and glutathione pool (GSH + GSSG) (**c**) as well as GSH/(GSH + GSSG) (**d**). Mean ($n = 5$–7) ± SE are shown for controls (CK) and leaves fed with fosmidomycin (LFF). Significant differences ($P < 0.05$) were confirmed by Student's t test

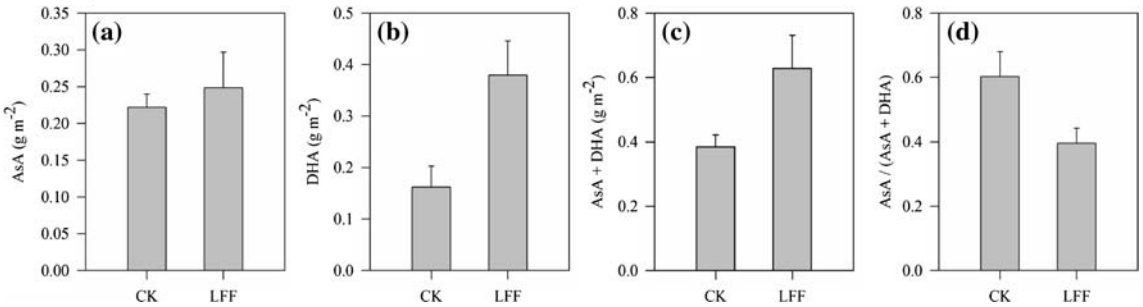

Fig. 6 Effects of fosmidomycin feedings on contents of reduced ascorbate (AsA, **a**), oxidized ascorbate (DHA, **b**), and ascorbate pool (AsA + DHA) (**c**) as well as AsA/(AsA + HAD) (**d**). Mean ($n = 5$–7) ± SE are shown for controls (CK) and leaves fed with fosmidomycin (LFF). Significant differences ($P < 0.05$) were confirmed by Student's t test

results, however, revealed that fosmidomycin had no significant effects on the contents of Chl and Cars (Fig. 2). Furthermore, *NPQ*, which depends on the de-epoxidation status of xanthophylls, did not significantly change between LFF and CK over the experimental period (Fig. 3b). These results agree with the reported results that both the de-epoxidation status of xanthophylls and the contents of carotenoids remained similar for hours after the inhibition of monoterpene by fosmidomycin (Loreto et al. 2004). It may be, thus speculated that fosmidomycin may eventually inhibit de novo synthesis of carotenoids, but not at the concentrations we used and not during experimental period we conducted, which probably indicates a much slower turnover of the more complex carotenoids with respect to monoterpene (Loreto et al. 2004; Laule et al. 2003). Therefore, we conclude that, in our experiment, fosmidomycin exclusively inhibit the biosynthesis of monoterpenes, and has no effects on the involvement of carotenoids in protective action.

Increased H_2O_2 and MDA levels and higher activities of antioxidative enzymes in the LFF as compared to the CK indicated that the inhibition of monoterpene biosynthesis in the leaves of rubber tree provoked an oxidative stress, which may be related to membrane damage, further confirming the powerful antioxidant role of monoterpene (Loreto et al. 2004). Membrane denaturation because of the attack of H_2O_2 and other active oxygen species results in the accumulation of end products of lipid peroxidation such

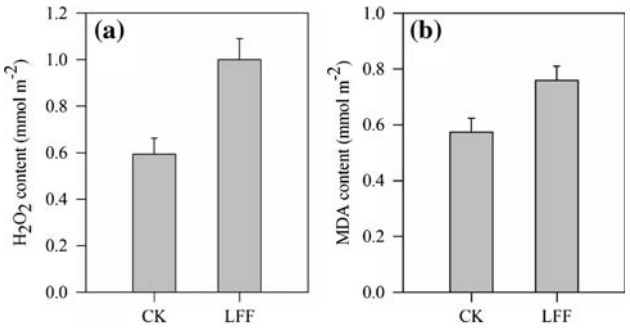

Fig. 7 Effects of fosmidomycin feedings on contents of hydrogen peroxide (H_2O_2, **a**) and malonyldiadehyde (MDA, **b**). Mean ($n = 5$–7) ± SE are shown for controls (CK) and leaves fed with fosmidomycin (LFF). Significant differences ($P < 0.05$) were confirmed by Student's t test

as MDA (Heath and Parker 1968). Higher levels of MDA were observed in the LFF (Fig. 7b). This demonstrates that lipid peroxidation is enhanced when monoterpene is absent and indicates that monoterpene may effectively protect membranes against denaturation. Therefore, the absence of monoterpene should be responsible for the higher accumulations of H_2O_2 and MDA in the LFF.

When the monoterpene biosynthesis was inhibited by fosmidomycin, the leaves became more susceptible to high illumination and moderately high temperature. It is well known that thylakoid membranes become leaky at moderately high temperature (Pastenes and Horton 1996; Bukhov et al. 1999). In our study, although no significant difference in $\Delta F/F_m'$ was observed between LFF and CK with prolonged exposure to high illumination, $\Delta F/F_m'$ in the LFF was markedly lower during the first 20 min exposure (Fig. 3a). Furthermore, the high levels of H_2O_2 and MDA were eventually accumulated in the LFF (Fig. 7). Increased H_2O_2 and MDA levels in the LFF effectively confirm that monoterpene may confer antioxidant action. It is consistent with the results reported by Loreto et al. (2004) that monoterpenes conferred isoprene-like antioxidant action. That is to say, the higher accumulation of H_2O_2 and MDA in the LFF may be primarily attributed to the absence of monoterpenes' antioxidant action.

H_2O_2 may act as a signal molecule to activate a variety of molecular, biochemical and physiological responses within cells and plants (Neill et al. 2002). The increased levels of H_2O_2 in the LFF as a result of monoterpene inhibition can serve as a signal for activating other antioxidant systems. Hence, the enhanced activities of antioxidant enzymes of SOD, CAT, POD, APX, and GR in LFF should be an indirect or direct response to the increased levels of H_2O_2; so do the enhanced antioxidant metabolites of ascorbate and glutathione. Tichy and Vermaas (1999) have found that a high activity of CAT and POD was not critical for normal growth but became critical when H_2O_2 was added at sublethal concentrations in the growth medium. Moreover, several studies have also suggested that H_2O_2 regulates gene expression during defense responses (Levine et al. 1994; Foyer et al. 1997; Etienne et al. 2000). Therefore, the dual role of H_2O_2 in plants is well known. At the low level, it acts as a messenger molecule involved in acclamatory signaling, triggering tolerance against various abiotic stresses, and at the high level, it destroys cell membranes and induces programmed cell death (Dat et al. 2000). The higher accumulation of H_2O_2 in the LFF may enhance gene expression, in turn, stimulating the activities of enzymatic and non-enzymatic antioxidant defenses and consequently compensating for the absence of monoterpenes' antioxidant action in these leaves. It is consistent with the reported results that exogenously fumigated isoprene suppressed the activities of the non-enzymatic antioxidants α-tocopherol and ascorbic acid in *Quercus ilex* leaves under high temperature (Peňuelas et al. 2005), and the inhibition of isoprene biosynthesis enhanced the activities of CAT and POD in *Phragmites australis* leaves exposed to high illumination (Velikova and Loreto 2005). Overall, the absence of monoterpene may lead to the increased responses of the other antioxidant defenses to photo-oxidative stress.

In conclusion, the results in our study suggest that endogenous monoterpene confer an antioxidant action and is able to protect leaves against photo-oxidative stress. Under high illumination, the inhibition of monoterpene biosynthesis accelerates oxidative stress resulting in the increased levels of H_2O_2 and MDA, and consequently leads to the enhancement of other antioxidant defense, which may compensate for the absence of monoterpene antioxidant.

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