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Effect of high irradiance and high temperature on chloroplast composition and structure of *Dioscorea zingiberensis*

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Abstract

High irradiance (HI) and high temperature (HT) increased in chloroplasts the content of monogalactosyldiacylglycerol (MGDG) and decreased the contents of digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylinositol (PI). HI and HT accelerated the transformation of DGDG to MGDG. The contents of unsaturated fatty acids in chloroplasts increased, while those of saturated fatty acids decreased. The contents of total carotenoids, neoxanthin, violaxanthin, lutein, and β-carotene increased first, then decreased. The content of chlorophyll decreased. HI caused the unfolding of thylakoids that was not resumed after a 72-h recovery.

Additional key words: carotenoids; chlorophylls; fatty acids; lipids; recovery.

Introduction

Dioscorea zingiberensis C.H. Wright belonging to the Dioscoreaceae is distributed only in the mountain areas of southern China (Ding 1983). The plant has a very high content of diosgenin (Tang *et al.* 1979), which is widely used in pharmaceutical industry to produce corticosteroids, such as cortisone, sexual hormones, and anabolic agents. The glycoside of diosgenin, dioscin, is also effective in improving cardiovascular function (Ding *et al.* 1983). Since the 1970s, the species has been over-exploited and its natural reserves are now greatly diminished. At present, the production of *D. zingiberensis* as raw material for pharmaceutical uses mainly depends on plantations in the mountain areas of southern China.

Mountain areas receive very strong sunlight. High photon flux densities cause photoinhibition or photodamage (Farage and Long 1991, Ögren and Rosenqvist 1992, Nishio *et al.* 1994, Andersson and Barber 1996, Ball *et al.* 1997, Sonoike 1998). Heat changes fatty acid contents and chloroplast ultrastructure in Andean legumes (Matos *et al.* 2002). *D. zingiberensis* plants growing in the wild are often found at the margins of forests and mainly exposed to scattered irradiation. Therefore, mountain areas are unsuitable for the optimal growth of *D. zingiberensis* as the yield of rhizomes is low, thus not meeting the demands of the pharmaceutical industry in China.

To date, most studies on cultivation techniques for *D. zingiberensis* have focused mainly on the effects of soil, water, and temperature (Institute of Biology in Sichuan Province 1974, Huai *et al.* 1989, Zhou 1989), but we studied the influence of irradiance on leaf lipids of *D. zingiberensis* (Li *et al.* 2002). Choice of an appropriate plant cultivar is undoubtedly one of the most important factors in successful cultivation in the mountainous areas. *D. zingiberensis* plants which are adapted to high irradiance (HI), are preferable (Li and Wang 1999). In this study, we determined the composition of chloroplasts and the ultrastructure of *D. zingiberensis* after stress by HI and high temperature (HT).

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Abbreviations: Chl: chlorophyll; DGDG: digalatosyldiacylglycerol; DZTH: *Dioscorea zingiberensis* type high irradiance; DZTL: *Dioscorea zingiberensis* type low iradiance; DZTM: *Dioscorea zingiberensis* type medium irradiance; HI: high irradiance; HT: high temperature; LHC1: light-harvesting complex 1; LHC2: light-harvesting complex 2; MGDG: monogalactosyldiacylglycerol; PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PPC: pigment-protein complex; PS: photosystem; RC: reaction centre; SQDG: sulfoquinovosyldiacylglycerol; TMSH: trimethylsulfonium hydroxide.
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Materials and methods

Plants: Three types of *D. zingiberensis* from the Institute of Ecology of Jishou University in P.R. China have been found in former studies (Zhu and Wang 2000, Zhu *et al.* 2001), which were named *D. zingiberensis* type low irradiance [DZTL; adapted to 39 (29–55) μmol m^{-2} s^{-1}], *D. zingiberensis* type high irradiance [DZTH; adapted to 227 (181–273) μmol m^{-2} s^{-1}], and *D. zingiberensis* type medium irradiance [DZTM; adapted to 700 (550–585) μmol m^{-2} s^{-1}]. In this study, we used the DZTL.

HI stress treatment: As the DZTL under 55 μmol m^{-2} s^{-1} grows well, we used it to test the effect of HT (32 °C) and HI. Healthy leaves were selected on plants treated with 1 000 μmol m^{-2} s^{-1} for 1.5 h. At the same time, a part of the leaf was covered with tiny paper. The temperature of the leaves was kept at 32 °C. After treatment, the leaves were returned to the original place.

Chloroplasts were prepared according to Schmid (1971). The Tris buffer solution (pH 7.4) contained 0.4 M saccharose, 0.01 M NaCl, 0.01 M $MgCl_2$, 0.05 M Tris-HCl, 0.005 M EDTA, and 0.6 % polyethyleneglycol 4 000 (m/v). The solution was filtrated through eight layers of gauze and centrifuged for 5 min at 250×*g*. The upper layer was centrifuged for 10 min at 2 000×*g*. The sediment contained chloroplasts. The chloroplasts were purified with silicon dioxide sol (*Percoll TM*, *Sigma*) by the same centrifugation.

Chlorophyll (Chl) determination: Chloroplast liquid suspension was shaken with methanol and then centrifuged at 800×*g* for 1–2 min. In the upper layer, the extinctions at 663 (E_{663}) and 645 (E_{645}) nm were determined with a *Zeiss* spectrophotometer. The content of Chl was calculated according to He *et al.* (1995).

The analysis of lipids: The purified chloroplasts were eluted with solvent mixture isopropyl alcohol : chloroform : methanol (1 : 1 : 1, v/v/v), concentrated, vaporized at 30 °C, and centrifuged at 5 000×*g*. The upper layer was dissolved using chloroform : water (4 : 1), the chloroform solution was separated and evaporated. The lipids were extracted in toluene and kept at –20 °C. Thin layer chromatography was used to analyse the lipids on silica gel plates in the solvent system chloroform : methanol : acetic acids : water (85 : 15 : 10 : 3.5, v/v/v/v) (He *et al.* 1995). For the development of glycolipids, the silica gel plates were sprayed with anthrone-sulphuric acid and heated to 95 °C. Galactolipids were detected as blue-violet spots. Phospholipids were marked by spraying with phosphatide reagent (He *et al.* 1995). For the labelling of all lipids the plates were sprayed with phosphomolybdic acid (0.5 % in ethanol) and heated for 20 min at 100 °C. Lipids appeared as dark-blue spots on yellow background.

Separation of carotenoids and fatty acids: The fatty acids were removed using 0.5 M sodium hydroxide. After heating, circumfluence, and cooling, the carotenoids were extracted with petrol ether (40–60 °C). The petrol ether solution (containing carotenoids) was washed with water and then concentrated. The carotenoids dissolved in toluene were kept at –20 °C. The water solution was acidified with muriatic acid (pH 1.0). The fatty acids were extracted with petrol ether. After washing with water and concentrating, the fatty acids were dissolved in 0.1 cm^3 of hexane and kept at –20 °C.

Carotenoids were dissolved in ethanol and analysed using the high pressure liquid chromatograph *Kontron*. The columns were *Li Chrospher 100 RP-18* (5 μm) and *NO419272* (*Merck*). For analysis of standard samples of carotenoids (*Sigma*) and carotenoid samples, methanol-water (4 : 1) (*A*) or (3 : 1) (*B*) were used. The flow speed was 25 mm^3 s^{-1}. The checking wavelength was 430 nm. The standard samples were neoxanthin, violaxanthin, lutein, and β-carotene (*Sigma* and *Hoffmann-La Roche*, Basel, Switzerland).

Gas chromatography of fatty acids: The alkaline sample was acidified with HCl in order to liberate fatty acids. Thereafter fatty acids were separated by shaking three times with petrol ether (40–60 °C). Fatty acids were then concentrated and dissolved in 100 mm^3 TMSH-reagent (trimethylsulphonium hydroxide; *Machery and Nagel*, Düren, Germany). Fatty acids were transformed into their methyl esters and analysed in a gas chromatograph *Hewlett Packard* model *5890*, series *II plus via* using a 10-m ethylene glycolsuccinate capillary column at 190 °C. The carrier gas was nitrogen. The temperature of the injection block and detector was 300 °C. Methyl esters (*Nu-Check Prep*., Elysian, MN, USA) were used as fatty acid standards.

Chloroplast ultrastructure: The leaves were chopped into sample of 1 mm width and 4–5 mm length after a 1-h dark period. Samples for electron microscope were fixed by fix slice solution (5.0 % glutaraldehyde and 4 % formaldehyde), washed by phosphate buffer solution of pH 7.0, fixed by 2 % osmic acid, dehydrated with acetone, penetrated, embedded, and polymerized by the *812* epoxy resin. The structure of chloroplasts was photographed by *Hitachi-600* penetrating electron microscope after the sample was located, sliced, and dyed with Pb-citrate.

Results and discussion

Contents of chloroplast lipids changed obviously after stress (Fig. 1). The change of MGDG after HI stress was opposite to that of DGDG: the content of MGDG increased, but that of DGDG decreased. The content of SQDG was small, but visible on the TLC board. During 48 h of recovery after stress the content of PG decreased by two-thirds. PI is a fatty acid seldom seen in the chloroplasts, but it exists in normal chloroplasts of *D. zingiberensis*. PI almost vanished after HI stress. The content of PG did not change much. Most studies showed that the distribution of lipids at inside and outside of membrane was anisomeric. 60 % of total glycolipids were found at the inside, but only 40 % at the outside. Most MGDG and SQDG were distributed at the inner membrane, but the content of PG was larger outside than inside. The distribution of DGDG was almost the same inside and outside of membrane (Yan 1999). The contents of MGDG and DGDG were large in thylakoids and stroma. Normally, the contents of MGDG and DGDG in thylakoid area were lower than those in stroma, the DGDG contents being even less (Anderson 1987). The content of PG at the folded grana area was higher than that at unfolded grana area (Yan 1999). The grana unfolded after HI stress and vanished at last, which could explain the change of PG. The vanishing of thylakoids resulted in the reduction of PG, but it was very difficult to find a relationship between the other fatty acids and the change of thylakoid membrane of chloroplast.

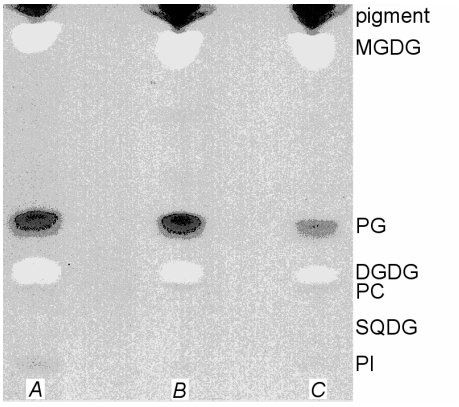

Fig. 1. Dynamics of lipid pattern of chloroplasts (A – normal; B – stressed after 24 h of recovery; C – stressed after 48 h of recovery) of *D. zingiberensis*.

Low temperature stress could cause the inter-conversion of MGDG and DGDG resulting in a decrease of chloroplast temperature T_c (Quinn and Williams 1983). In chloroplasts of *Dunaliella salina* the content of MGDG was higher at high temperature, while that of DGDG was higher at low temperature (Lynch and Thompson 1982) which is in agreement with our results. The growth temperature of *D. zingiberensis* was 22/27 °C (night/day) and the treatment temperature was 32°C. So the content of MGDG increased after stress, while that of DGDG continuously diminished. Thus short time HT affected the metabolism of fatty acids. Of course, the HI affected the course of change.

Kuang *et al.* (1988) summarize the main characteristics of membrane lipids. MGDG and DGDG are the glycolipids without electric change in chloroplasts and the main components of thylakoids. The contents of unsaturated fatty acids in MGDG and DGDG are very high, and the $C_{18:3}$ is the main unsaturated fatty acid. MGDG is not the double deck structure lipid, in normally inverted cylindrical micelles, and sometimes in inverted spherical micelles, which is different from the double deck structure of DGDG, SQDG, PG, and PC in water under physiological temperature. PG is a fatty acid with anion, and is the main phosphorous fatty acid in the chloroplasts. PG contains the $C_{16:1trans}$ only in chloroplasts and is synthesized in the light. SQDG is the sulphur fatty acid with anion. The outside of thylakoids contains more PG, which is confirmed by antibody marker study. So is the LHC2. The distribution of MGDG is different when different materials and methods are used. The content of DGDG at the inside of fatty double deck is very rich. Our determination showed that there were more PI in the leaves of *D. zingiberensis* and the purified chloroplasts.

The change of fatty acids of chloroplasts: The content of saturated fatty acids and $C_{18:0}$ first increased, and then decreased at 48 h after stopping stress, while the change of unsaturated fatty acids was opposite (Table 1). The contents of $C_{12:0}$, $C_{14:0}$, and $C_{16:1cis}$ increased continuously, but the content of $C_{16:0}$ continuously decreased. Hence the LHC2 was reduced and the grana were disaggregated. The content of $C_{20:0}$ first decreased, but then increased and exceeded that of control. The content of $C_{15:0}$ was constant. Hence the adjustment of fatty acids of chloroplasts was not finished 48 h after stopping stress, which was in agreement with the change of chloroplast ultrastructure.

The changes of fatty acids showed that: (*1*) Reaction of different fatty acids to HT and HI was different, which illustrated the differences in their function in different environments. Normally, the content of unsaturated fatty acid increased when growing under low temperature, but decreased when growing under HT (Williams *et al.* 1998). (*2*) The reactions of different fatty acids were different during the recovery after stopping stress. For example, the content of saturated fatty acids first decreased and then increased. The decrease in 24 h was the reaction to HT and HI, but the increase was the reaction to environment after stopping the stress. Fatty acids reacted quickly. The contents of $C_{12:0}$ and $C_{14:0}$ increased continuously in 48 h after stopping stress, which was the reaction to HT and HI induced by stress. The long time

increase illustrated that the adjustment was very slow. In the chloroplasts of leaves of *D. zingiberensis*, the $C_{18:0}$ and $C_{20:0}$ saturated fatty acids and $C_{18:3}$, $C_{16:1cis}$ unsaturated fatty acid reacted very quickly, while the $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1trans}$, $C_{18:1}$, and $C_{18:2}$ reacted very slowly.

Environment affects the composition of fatty acids in chloroplasts. Irradiance, photon quantity, and temperature affect the change of fatty acids (Harwood 1998). HI damages the unsaturated components, and HT changes the membrane state and metabolism. Our results on fatty acids differed from those of other researchers. $C_{16:1trans}$ was the only anti-form fatty acid and component of fatty acids combined with the anion phosphatidyl glycerol. The $C_{16:1trans}$ is the key substance in the structure of grana according to Tremolieres *et al.* (1982), not in our experiments with DZTL.

Table 1. Fatty acids [% of total] of chloroplasts of *D. zingiberensis* following high irradiance-high temperature stress.

Fatty acids	Before stress	Recovery time after stress	
		24 h	48 h
$C_{12:0}$	0.47±0.01	0.69±0.03	0.89±0.02
$C_{12:x}$	0.21±0.02	0.27±0.02	0.62±0.09
$C_{14:0}$	0.85±0.01	1.40±0.10	1.58±0.04
$C_{15:0}$	0.25±0.07	0.21±0.00	0.22±0.04
$C_{16:0}$	13.62±0.23	8.57±0.10	6.07±0.02
$C_{16:1cis}$	0.41±0.00	0.60±0.01	0.54±0.01
$C_{16:1trans}$	1.41±0.00	1.06±0.02	0.44±0.01
$C_{18:0}$	1.68±0.03	0.76±0.04	0.88±0.00
$C_{18:1}$	2.17±0.07	1.19±0.04	0.75±0.03
$C_{18:2}$	2.40±0.05	1.98±0.02	1.73±0.01
$C_{18:3}$	60.62±4.19	71.96±0.60	68.11±2.89
$C_{20:0}$	15.93±3.88	11.32±0.85	18.19±3.45
C_0	32.80	22.95	27.79
Cx	67.20	77.05	72.21
C_{16}	15.44	10.23	7.00
C_{18}	66.87	75.89	71.44

The fatty acid analysis of three eco-types showed that the content of PG in DZTH was less than those in DZTL and DZTM. The analysis of fatty acids showed that the content of saturated fatty acids decreased in the series DZTL>DZTM>DZTH. The change of $C_{16:1trans}$ agreed with that of PG, which could be explained as the decrease in thylakoid folding.

Long-term irradiation experiments showed that the content of PG in the leaves increased with the increasing irradiance. The content of $C_{16:1trans}$ increased from 0.3 % in the dark to 1.6 % in HI. The electron micrograph of chloroplasts showed a decrease in grana number under HI. So the content of $C_{16:1trans}$ of the leaves growing under HI should reduce. But our research showed that the content of $C_{16:1trans}$ increased with the reduction of grana structure. Therefore the $C_{16:1trans}$ may be related to adaptation to HI or stabilization of grana structure.

Short time HI and HT stress induced a gradual decrease in PG content after stress, *i.e.* a two-third reduction at 48 h after stress (Fig. 1). The content of $C_{16:1trans}$ decreased from 1.41 % before stress to 0.44 % after 48 h recovery (69 % decrease), which reflects the reduction of LHC2 and the disorganization of grana. The electron micrographs showed that the disorganization of grana began just after stress and vanished completely 18–24 h after stopping stress (Fig. 2*D*). The grana restored gradually for 48 h after stopping stress, and the content of $C_{16:1trans}$ should increase. So the form of grana was not necessarily related with the content of PG and $C_{16:1trans}$ or appeared only in some plants.

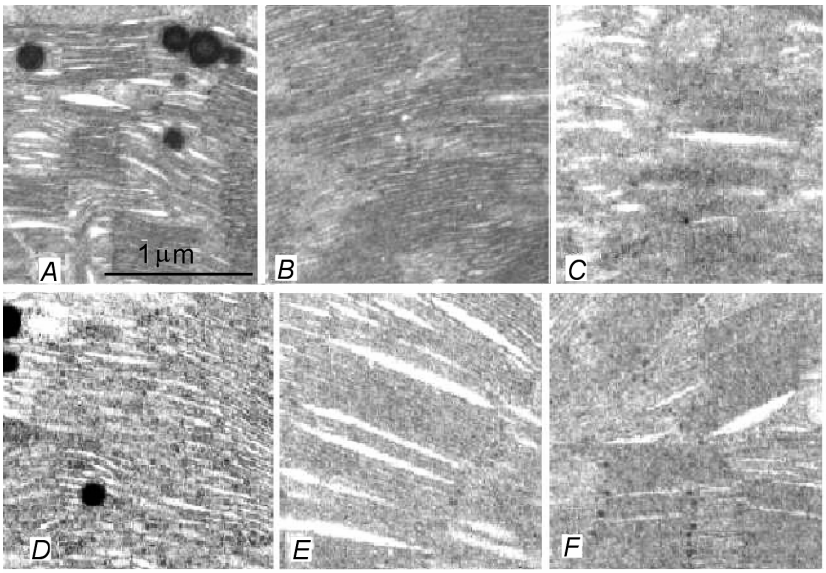

Fig. 2. Dynamics of chloroplast ultrastructure of *D. zingiberensis* before (*A*) and 10 min (*B*), 5 (*C*), 18 (*D*), 48 (*E*), and 72 (*F*) h after stress treatment of high irradiance. *Bar* = 1 μm (for all parts).

The dynamics of carotenoids: The change of the content of four carotenoids was the same after HI, *i.e.* first an increase and then a decrease (Table 2). The content of lutein increased by 38 % after 12-h recovery, but decreased after 48-h recovery by 17.3 %. The content of β-carotene increased by 38 % after 12 h recovery, but then was reduced by 19 % in comparison to that before stress. The changes of neoxanthin and violaxanthin were the same, but the scope was smaller. Those changes illustrated that the chloroplast pigments react quickly to the change of environment. We thought that the increase during the 24-h recovery was the reaction to stress, and the following reduction was a normal reaction to the environment. HI resulted in the oxidation of carotenoids. The plants would start the synthesis of these pigments in order to adopt the environment and compensate the loss. Because too much pigment was synthesized, the contents of pigments increased, which was an over-compensation.

Table 2. Contents of chloroplast carotenoids [μg] in total chloroplast lipids of *D. zingiberensis* following high irradiance.

	Normal chloroplast	Recovery time after stress 12 h	48 h
Neoxanthin	0.05±0.01	0.07±0.01	0.04±0.01
Violaxanthin	0.18±0.05	0.19±0.03	0.15±0.04
Lutein	0.58±0.08	0.93±0.12	0.48±0.06
β-carotene	0.21±0.00	0.29±0.05	0.17±0.01
Sum of carotenoids	1.02	1.48	0.84

When the stress stopped, the plant felt the normal environment and adjusted to it again, so stopped the synthesis of pigments or started their decomposition. Because too much pigments were decomposed, the content of pigment decreased in 48 h, which was an over-reduction.

The dynamics of Chl: HI stress resulted in destruction of Chl in leaves adapted to low irradiance (10 μmol m^{-2} s^{-1}) for a long time (Table 3). HI of 1 000 μmol m^{-2} s^{-1} applied for 1 h reduced 21 % of Chl. The content of Chl was restored in 48 h to 86.4 % of that before stress, but it was again reduced to only 81.5 % after 72 h. The ratio Chl *a/b* increased continuously from 2.06 before stress to 2.81 after 72 h, which is a typical adaptation to HI. Short time HI (1 h) could keep the adjustment for several days. The dynamics of Chl was similar to that of carotenoids.

The dynamics of chloroplast ultrastructure: The ultrastructure of chloroplasts changed after HI stress (Fig. 2). The normal chloroplast contained some small macula.

Table 3. Chlorophyll (Chl) content in leaves of *D. zingiberensis* following high irradiance-high temperature stress.

	Before stress	Recovery time after stress 0 h	24 h	48 h	72 h
Chl (*a*+*b*) [mg m^{-2}]	258.4	204.0	213.6	223.2	210.6
Chl *a/b*	2.06	2.28	2.45	2.56	2.81

But we do not know what the macula was. However, during the course of unfolding and folding of grana, the grana were illegible and difficult to distinguish.

The ultrastructure of chloroplasts did not change much immediately after the stress (Fig. 2*A*,*B*) although the reduction of quantum yield was the greatest at this time. At the same time, the small macula distributed regularly around the grana became disordered and were in the inside of grana, on the lamellae of thylakoids. The lamellae of grana began to separate at 5-h recovery after stress. The grana thylakoids almost became stroma thylakoids at 18-h recovery and no folds of thylakoids were seen (Fig. 2*D*). Many small macula surrounded some rings in Fig. 2*C*,*E*. Fig. 2*C* shows the midterm of unfolding, while Fig. 2*D* shows the resuming of original grana. Some grana folded into tight structure after the 48-h recovery (Fig. 2*E*) and some grana were formed after the 72-h recovery (Fig. 2*F*).

The functions of grana were summarized by Anderson (1999). Coincident results are: (*1*) When the irradiance is lower than saturating, the thylakoids fold to grana in order to improve the function of PS2. (*2*) The grana protect the PS2 under continuous irradiance. (*3*) The dynamics of grana is driven by transmitting the signal through the oxidation and de-oxidation state of plastoquinone and cytochrome b_6f. (*4*) The grana connection with the more fluid unfolded area helps to the adjustment of the assistant protein enzyme and the adjustment of oxidation and de-oxidation of genes coded by chloroplasts and nuclei.

Our conclusion is as follows: (*a*) After the thylakoids of chloroplasts were stressed, the unfolding of grana was still on after the stress was stopped. (*b*) The restoration of grana needed a long time. In our experiments, the grana did not restore to the original state (such as grana density) even after a 72-h recovery. (*c*) Although the recovery of grana needed a long time, the recovery of the function of leaves (the recovery of quantum yield) was very quick. The maximal quantum yield was restored to 91 % after 24-h recovery, but the structure of grana did not restore. So the grana structure did not have any relationship with the quantum yield during the course of fixing of thylakoids of chloroplasts.

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