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Activity of photosystem 2, lipid peroxidation, and the enzymatic antioxidant protective system in heat shocked barley seedlings

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Abstract

The impact of heat shock on minimising the activity of photosystem 2 (PS2) initiating high lipid peroxidation (POL) level and consequently changes in the enzymatic-antioxidant protective system was studied in seedlings of two Egyptian cultivars of barley (Giza 124 and 125). Heat doses (35 and 45 °C for 2, 4, 6, and 8 h) decreased chlorophyll (Chl) contents coupled with an increase in Chl *a/b* ratio, diminished Hill reaction activity, and quenched Chl *a* fluorescence emission spectra. These parameters reflect the disturbance of the structure, composition, and function of the photosynthetic apparatus as well as the activity of PS2. POL level, as dependent on the balance between pro- and anti-oxidant systems, was directly correlated with temperature, exposure time, and their interaction. Heat shock caused an increase in the electric conductivity of cell membrane, and malonyldialdehyde content (a peroxidation product) coupled with the disappearance of the polyunsaturated linolenic acid ($C_{18:3}$), reflecting the peroxidation of membrane lipids which led to the loss of membrane selective permeability. Moreover, it induced distinct and significant changes in activities of antioxidant enzymes. Superoxide dismutase and peroxidase activities have been progressively enhanced by moderate and elevated heat doses, but the most elevated one (45 °C for 8 h) showed a decrease in activities of both enzymes. In contrast, catalase activity was reduced with all heat shocks.

Additional key words: catalase; chlorophyll fluorescence; fatty acids; *Hordeum vulgare*; malonyldialdehyde; membrane leakage; peroxidase; superoxide dismutase.

Introduction

Aerobic organisms gain significant energetic advantages by using molecular oxygen as a terminal oxidant in respiration. However, the presence of oxygen in cellular environment poses a constant oxidative threat to cellular structures and processes (Alscher *et al*. 1997, Mallick and Mohn 2000). In plants, reactive oxygen species (ROS) are formed during electron transport activities of chloroplasts, mitochondria, peroxisomes, and glyoxisomes (Tolbert 1981, Fridovich 1995, Foyer 1997, Jiang and Zhang 2002). These species include singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxides (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). During the assimilation of CO_2 in actively photosynthesising chloroplasts, oxygen reduction takes place and consequently the reactive species, formation is initiated (Meloni *et al*. 2003). The O_2 formed is rapidly dismutated by superoxide dismutase to H_2O_2 or by non-enzymatic dismutation to yield H_2O_2 and $^1O_2$ (Mallick and Mohn 2000). H_2O_2, through Harber-Weiss reaction (with $O_2^{\cdot-}$), gives rise to $^{\cdot}OH$ which is the most reactive species and capable of initiating lipid peroxidation (Tsang *et al*. 1991).

Lipid peroxidation (POL) is considered the best criterion for damage caused by increasing ROS production (Halliwell 1991, Rao *et al*. 1995). It occurs when ($^{\cdot}OH$) radicals are generated, close to cell membranes, and attack the unsaturated fatty acid side chains of membrane lipids resulting in the formation of lipid hydroperoxides (Halliwell 1991, Rao *et al*. 1995, Bestwick *et al*. 2001). Accumulation of lipid hydroperoxides in membranes disrupts their function and can cause them to collapse, leading to their leakage and loss of selective permeability (Saelim and Zwiazek 2000).

Plant cells have evolved defence antioxidant mechanisms to combat the danger posed by the presence of ROS. These include enzymatic mechanisms involving antioxidant enzymes such as superoxide dismutases

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Abbreviations: CAT = catalase; EC = electrical conductivity; MDA = malonyldialdehyde; POL = lipid peroxidation; POX = peroxidase; PS2 = photosystem 2; ROS = reactive oxygen species; SOD = superoxide dismutase.

(SODs), peroxidases (POXs), and catalases (CATs) (Landberg and Greger 2002, Meloni *et al.* 2003). However, the delicate balance between ROS production and its processing by antioxidant enzymes, crucial for functioning and minimising oxidative damage to leaves, is compromised when the latter is overwhelmed, often in plants with limited acclimation (Alscher *et al*. 1997, Foyer 1997, Mallick and Mohn 2000). The importance of SOD in defence against oxidative stresses has been established by the demonstration that SOD-deficient mutants of *E. coli* (Carlioz and Touati 1986) and yeast (Van Loon *et al*. 1986) are hypersensitive to oxygen toxicity. In this concern, an increase in H_2O_2 content was observed under heat and salt stresses (Troitskaya *et al*. 1999, Meloni *et al*. 2003).

Under natural growth conditions, plants are adapted for minimising the damage induced by ROS. However, O_2 toxicity emerges when ROS production exceeds the quenching capacity of protective system (Dai *et al*. 1997). This occurs under various stress conditions due to dysfunction of the photosynthetic and respiratory electron flow (Doke *et al*. 1994, Mallick and Mohn 2000, Meloni *et al*. 2003, Rakwal *et al*. 2003). Accumulation of ROS limits the activity of several enzymes and causes disturbance to the membrane structure and function (Alscher *et al*. 1997, Mallick and Mohn 2000). This accumulation declines the generation of ATP and redox metabolites that are essential to cellular defence and repair (Foyer 1997). Also, it causes denaturation of proteins and mutation of DNA (Mallick and Mohn 2000). These detrimental effects of the overproduction of ROS could limit the plant tolerance to stresses resulting in growth retardation and early senescence (Biswal and Mohanty 1976, Doke *et al*. 1994).

Heat shock is a form of the oxidative stress, resulting in the formation of many toxic ROS in plants (Tsang *et al*. 1991, Gong *et al*. 2001). Therefore, the objective of this work was to study the response of two Egyptian barley cultivars (Giza 124 and 125) to elevated temperatures. This is essential prior to assessing both cultivars for thermo-tolerance and, hence, for cultivation in warmer periods or regions. For this purpose, photosynthetic performance was detected by determining chlorophyll (Chl) contents, Hill reaction activity, and Chl *a* fluorescence emission spectra. Also, ROS production was monitored in terms of changes in concentrations of linolenic acid and malonyldialdehyde, and membrane leakage and the enzymatic antioxidant protective system.

Materials and methods

Plants, growth conditions, and treatments: Grains of barley (*Hordeum vulgare* L., cultivars Giza 124 and Giza 125), obtained from the Agricultural Research Center (Giza, Egypt) were surface-sterilised in 5 % *clorox* for 5 min, and thoroughly rinsed and washed with sterile distilled water. Thereafter, grains were germinated in Petri dishes (10 cm diameter, half-filled with sand which was previously washed with concentrated HCl, then with distilled water). Grains (25 per dish) were irrigated with distilled water and grew for 6 d under a 14 h photoperiod (*i.e*. 14 h in light of 120–140 μmol m^{-2} s^{-1} and 10 h in dark) with day/night temperature of 25/16 °C and 65–75 % relative humidity.

The six-day old seedlings were subjected to heat shock treatments of 35 or 45 °C for 2, 4, 6, or 8 h. Another set of seedlings not exposed to any heat treatment represented the control. Seedling samples were immediately harvested after heat shock and prepared for measurements described below.

Chl was extracted from 0.5 g fresh mass of green leaves in 10 cm^3 of pure *N,N*-dimethyl formamide (Ebrahim *et al*. 1998). The extract was kept in dark for 2 d at 4 °C, and then centrifuged for 15 min at 66.7 rps. Chl concentration in the supernatant was spectrophotometrically determined according to the equations of Moran and Porath (1980).

Photosynthetic (Hill reaction) activity: Photosystem 2 (PS2) activity of chloroplasts isolated from seedling leaves expressed as electron-transport rate was determined by using 2,6-dichlorophenol indophenol (DCPIP) as electron acceptor (Biswal and Mohanty 1976). Chloroplasts were isolated in the cold as described by Osman and El-Shintinawy (1988). The concentration of Chl *a*+*b* in the supernatant was determined according to the equation of Arnon (1949). For measuring the PS2 activity, assay sample was prepared by mixing 1.6 cm^3 of 10 mM DCPIP (dissolved in 96 % ethanol) with 50 μg Chl, and then the volume was completed to 3 cm^3 by the reaction buffer. The sample was irradiated (at right angles) with red actinic radiation (300 W m^{-2}, 10 min) provided from a slide projector. The DCPIP photoreduction was assayed spectrophotometrically according to Ebrahim and Aly (2004).

Chl *a* fluorescence emission spectra were measured at room temperature (26±2 °C) according to Tripathy *et al*. (1981) with minor modifications. The blue actinic radiation was switched on and focused on the sample cuvette by a spectrofluorometer (model *510*, *Shimadzu*, Japan). Chloroplast isolation and Chl determination were carried out as described above. Isolated chloroplasts were suspended in the reaction buffer, then transferred to the sample cuvette. The assay volume was 3 cm^3 and the Chl concentration was 5 g m^{-3}. All samples were incubated in dark for 15 min prior to measurements. Thereafter, samples were excited under blue actinic radiation (460 nm). Produced emission kinetics (signals) were photomultiplied and recorded by means of a recorder.

Linolenic acid content: Lipid transmethylation and extraction of fatty acid methyl esters were carried out according to Garcés and Mancha (1993) with minor modifications of El-Shintinawy and Selim (1995). Frozen leaves (1 g) were placed in test tubes containing 10 cm^3 reagent mixture of methanol : heptane : toluene : H_2SO_4 (39 : 34 : 25 : 2 by volume). The test tubes were kept in water bath at 80 °C for 2 h, heated at 100 °C for 2–3 min, shaken vigorously to mix all components, then cooled at room temperature, and shaken again. After phase separation, the upper phase containing the fatty acid methyl esters was analysed using gas chromatography (*Hewlett-Packard*, *HP 6890* series).

The content of MDA, the peroxidation product of linolinic acid (18 : 3), was determined by the method of Heath and Packer (1968). A fresh sample of 0.5 g of leaves was extracted in 10 cm^3 of 5 % (m/v) trichloroacetic acid. The homogenate was then centrifuged at 66.7 rps for 10 min. The supernatent (2 cm^3) was mixed with 2 cm^3 of 0.67 % (m/v) thiobarbituric acid, then incubated in boiling water bath for 20 min, and cooled immediately. MDA content was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} by measuring the absorbances at 532 and 600 nm (Heath and Packer 1968).

Membrane leakage: Leaves were sliced into pieces (*ca*. 0.5 cm in length), then 0.5 g fresh sample was immersed in distilled water for 1 h and the electrical conductivity (EC) of the exudates was measured by EC meter (*Wiss.-Techn.-Werkstätten D 812*,Weilheim, Germany) at 26 °C.

Antioxidant enzyme assays: A sample of 0.5 g leaves was ground in liquid nitrogen and homogenised in 8 cm^3 of 50 mM cold phosphate buffer of pH 7 (modified from Beauchamp and Fridovich 1971). The homogenate was centrifuged at 66.7 rps for 20 min at 4 °C. The supernatant was purified removing low molecular mass compounds, and used as a raw extract for antioxidant enzyme activities.

Superoxide dismutase (SOD) was assayed on the basis of its ability to inhibit the photochemical reduction of nitroblue-tetrazolium (Beauchamp and Fridovich 1971). The reaction was initiated by switching on "white light" and allowing the reaction to run for 10 min before being stopped by switching the light off. Thereafter, the absorbance of the reaction mixture was measured at 560 nm. Log A_{560} was plotted as a function of the volume of enzyme extract (0–200 cm^3) in the reaction mixture. The volume of raw extract (enzyme) producing 50 % inhibition of the initial rate of the reaction, in absence of the enzyme, was calculated from the resultant curve and defined as one unit of SOD activity.

Peroxidase and catalase were assayed by measuring the initial rate of disappearance of H_2O_2 and tetraguaiacol, respectively (Kato and Shimizu 1987). For peroxidase (POX), the decrease in tetraguaiacol was followed as a decline in the absorbance at 470 nm and the activity was calculated using the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm, Meloni *et al*. 2003) for tetraquaiacol. For catalase, the change in the absorbance, induced by the decrease in H_2O_2, was measured at 420 nm and the activity was calculated using the extinction coefficient (40 mM^{-1} at 240 nm) for H_2O_2.

Statistical analysis: Data were averaged and statistically analysed by using three-way analysis of variance. The least significant difference (LSD) at 5 % level was used to compare means (Steel and Torrie 1980).

Results

The Chl *a* and *b* contents as well as the photosynthetic electron transport rate of the leaves of the two barley cultivars (Giza 124 and 125) were significantly decreased by all heat doses compared with the control, whereas a contrary trend was observed for the Chl *a/b* ratio (Table 1). This response was more pronounced at 45 °C than at 35 °C, especially in the cv. Giza 125. Hence, Giza 125 seemed to be more sensitive to heat shocks than Giza 124.

Comparing with the control, all heat treatments caused a slight decrease in Chl *a* fluorescence of chloroplasts isolated from leaves of both cultivars (Fig. 1). However, the degree of quenching depended on both temperature and exposure time.

A highly significant increase in the electrical conductivity was detected in cell membranes of both cultivars with all heat doses compared with the control (Table 2). At 35 °C/8 h, the increase in membrane leakage was 43 and 50 % in Giza 124 and 125, respectively, while at 45 °C/8 h the increase reached 187 % in each cultivar. Prolonged and increased heat shock caused a progressive and distinct increase in the MDA concentration (Table 2). The highest increase (*ca*. 177 and 192 % compared with the control) was obtained at 45 °C/8 h in seedlings of Giza 124 and 125, respectively. In addition, this increase was larger in Giza 125 than in Giza 124. Parallel to this increase, the polyunsaturated linolenic acid ($C_{18:3}$) completely disappeared upon the exposure to any heat dose.

The intracellular alterations under heat stress, induced by accumulating POL products, can evidently serve as triggers for appropriate protective mechanisms. In seedlings of Giza 124 and 125, activities of antioxidant enzymes (SOD, POX, and CAT) were highly significantly (p<1 %) influenced by temperature, exposure time, and their interactions (Table 2). Activities of SOD and POX were positively correlated with most prolonged and higher heat doses, whereas a contrary trend was observed in the case of CAT. In both cultivars, POX reached its maximal activity (>194 % of the control) at 45 °C/6 h. However, SOD recorded the highest activities (*ca*. 187

Table 1. Effect of heat shock on chlorophyll content [g kg^{-1}(DM)], Chl *a/b* ratio, and photosystem 2 (PS2) activity [μmol(DCPIP reduced) kg^{-1}(Chl) s^{-1}] of chloroplasts isolated from seedling leaves of two barley cultivars (Giza 124 and 125) previously exposed to 35 and 45 °C for 2, 4, 6, and 8 h. NS indicates non-significant difference.

Cv.	Treatment temperature [°C]	time [h]	Chl *a*	Chl *b*	Chl *a/b*	PS2 activity
Giza 124	25 (control)		10.62	5.20	2.04	0.41
	35	2	10.28	5.16	1.98	0.40
		4	9.19	4.29	2.14	0.37
		6	7.95	3.40	2.33	0.28
	45	2	8.94	4.39	2.03	0.34
		4	7.88	3.47	2.26	0.21
		6	6.95	3.06	2.27	0.14
		8	5.62	2.33	2.40	0.07
Giza 125	25 (control)		10.53	5.06	2.00	0.40
	35	2	10.36	4.92	2.10	0.39
		4	9.29	4.06	2.20	0.37
		6	7.70	3.29	2.40	0.25
		8	6.38	3.15	2.20	0.23
	45	2	8.58	4.03	2.12	0.32
		4	7.46	3.22	2.31	0.20
		6	6.42	2.47	2.32	0.14
		8	5.28	2.20	2.38	0.05
LSD	Cv.	5 %	0.118	0.073	0.138	0.015
		1 %	0.158	0.098	0.184	0.020
	Temperature	5 %	0.144	0.090	0.169	0.018
		1 %	0.193	0.120	0.226	0.024
	Time	5 %	0.167	0.104	0.195	0.021
		1 %	0.223	0.138	0.261	0.028
	Cv.×temperature	5 %	NS	NS	NS	NS
		1 %	NS	NS	NS	NS
	Cv.×time	5°%	NS	NS	NS	NS
		1°%	NS	NS	NS	NS
	Temperature×time	5°%	0.289	0.181	0.338	0.036
		1°%	0387	0.245	0.452	0.048
	Cv.×temp.× time	5°%	NS	NS	NS	NS
		1 %	NS	NS	NS	NS

and 173 % of the control) in Giza 124 and 125 when seedlings were subjected to 35 °C for 6 and 8 h, respectively. In contrast to SOD and POX, control seedlings of each cultivar showed the maximum activity of CAT, while the minimum activity (*ca*. 28 and 25 % of the control) was recorded at 45 °C/8 h in Giza 124 and 125, respectively (Table 2).

Discussion

The decrease in Chl *a* and *b* contents by all heat treatments is in accordance with results obtained by El-Shourbagy *et al.* (1992) and Ebrahim *et al.* (1998). They referred this response to the effect of temperature on the developmental processes leading to synthesis of the photosynthetic apparatus and Chl, and/or to effects on activities of the chloroplast enzymes. The increase in Chl *a/b* ratio in heat shocked seedlings could be mainly attributed to greater destruction of Chl *b* than Chl *a*. This lead to changes in composition and structure of the light-harvesting Chl *a/b* protein complex as reported by Ghoshroy and Nadakavukaren (1990). This affects the chloroplast development (El-Shintinawy 2000). Photosynthetic activity in Giza 125 was more suppressed than that in Giza 124. Hence the heat shock may directly or indirectly reduce the photochemical efficiency of PS2 which could be due to either the inefficient energy transfer from the light-harvesting complex to the reaction centre, or the inability of the reaction centre to accept photons as a result of the structural alterations in the PS2 complex (Krupa 1988, El-Shintinawy 2000). To examine these possibilities, Chl *a* fluorescence emission spectra were recorded (Fig. 1). The results obtained indicated that heat shock affects both the light-harvesting complex and the reaction centre of PS2.

Under normal physiological conditions, electron

Table 2. Effect of heat shock on contents of linolenic acid [%] and malonyldialdehyde, MDA [mmol kg^{-1}(FM)], electrical conductivity [μohm cm^{-1}], and activities of superoxide dismutase, SOD [unit per mg(FM) s^{-1}], and peroxidase (POX) and catalase (CAT) [μmol(substrate reacted) kg^{-1}(FM) s^{-1}] in seedling leaves of two barley cultivars (Giza 124 and 125) previously exposed to 35 and 45 °C for 2, 4, 6, and 8 h. NS indicates non-significant difference.

Cv.	Treatment temperature [°C]	time [h]	Linolenic acid ($C_{18:3}$)	MDA	Electrical conductivity	SOD	POX	CAT
Giza 124	25 (control)		2.45	18.1	30.4	1.43	18.49	18.67
	35	2	-	18.8	32.9	1.83	20.34	18.00
		4	-	20.5	37.9	2.23	25.34	16.34
		6	-	22.3	40.1	2.67	32.67	14.33
		8	-	23.9	43.6	2.54	19.34	12.00
	45	2	-	22.0	39.1	2.41	21.00	15.34
		4	-	24.3	54.1	2.32	35.67	13.67
		6	-	27.0	65.5	1.08	37.01	7.67
		8	-	32.1	87.2	0.91	30.84	5.33
Giza 125	25 (control)		14.67	18.2	30.7	1.44	18.00	17.50
	35	2	-	18.6	34.3	1.94	20.34	17.00
		4	-	20.8	38.7	2.32	22.50	15.34
		6	-	21.9	42.0	2.42	30.17	12.34
		8	-	24.1	46.0	2.49	32.17	9.84
	45	2	-	22.0	42.7	2.40	21.00	13.67
		4	-	24.9	56.1	2.19		9.67
		6	-	27.3	72.4	1.04	35.01	6.00
		8	-	35.0	88.0	0.64	27.84	4.50
LSD	Cv.	5 %	-	0.24	0.94	NS	2.100	1.667
		1 %	-	0.32	1.24	NS	2.801	2.234
	Temperature	5 %	-	0.29	1.14	0.061	2.567	2.050
		1 %	-	0.39	1.52	0.082	3.434	1.734
	Time	5 %	-	0.34	1.31	0.071	2.967	2.367
		1 %	-	0.45	1.75	0.091	3.967	2.151
	Cv. × temperature	5 %	-	0.41	1.61	NS	NS	NS
		1 %	-	0.55	2.15	NS	NS	NS
	Cv. × time	5 %	-	0.47	NS	NS	NS	NS
		1 %	-	0.63	NS	NS	NS	NS
	Temperature × time	5 %	-	0.58	2.27	0.122	3.151	2.101
		1 %	-	0.78	3.04	0.163	3.868	2.468
	Cv. temp. × time	5 %	-	0.24	NS	NS	NS	NS
		1 %	-	Ns	NS	NS	NS	NS

transport is directed toward the sequential reduction of intermediate electron acceptors, PS2 and PS1 operating in complete concordance. However, high temperature provokes the state of hyper-reduction in the electron transport chain, which in turn enhances the generation of superoxide radicals (Dash and Mohanty 2002). The disturbances in the functioning of the electron transport chain may enhance the electron transport to oxygen as shown in cotton (Meloni *et al*. 2003) and rice (Rakwal *et al*. 2003). In accordance with Halliwell (1991) and Bestwick *et al*. (2001), one of the possible causes of stress-induced POL enhancement is the activation of phospholipases, which results in the accumulation of free unsaturated fatty acids. These fatty acids serve as POL substrate (Rakwal *et al*. 2003). Heat shock induced activation of these processes causes the accumulation of intermediate and end products of POL, which provokes the modification of membranes and certain enzymes (Dash and Mohanty 2002).

The enhanced generation of MDA can be attributed to the oxidation of linoleic and linolenic acids as the components of phospho- and galacto-lipids, participating in the arrangement of the reaction centres of photosystems in chloroplast membranes (El-Shintinawy 1999). MDA is capable to interact with free amino groups of proteins and with phospholipid molecules (Rakwal *et al*. 2003). In addition, it can initiate ethylene generation in membranes (Jiang and Zhang 2002). All these alterations can modulate the properties of both inter- and intracellular membranes, resulting in increased leakage of various ions through membranes (Saelim and Zwiazek 2000, Dash and Mohanty 2002). As known from literature (Fridovich 1995), increasing the substrate level activates SOD, whereas the increase of SOD activity in our work could be ascribed to an increase in its biosynthesis and/or to an activation of its latent form or both (Doke *et al*.

1994). However, SOD does not provide complete protection of the cell against oxidative stresses, since H_2O_2 emerges as a product of its functioning (Meloni *et al*. 2003). POXs and CATs can destroy H_2O_2, and therefore our results in Table 2 can support this suggestion. Although the response of SOD and POX to heat shock was similar, a contrary trend was recorded in the case of CAT (Table 2). This finding is in line with several authors (*e.g*. Rady *et al*. 1994, El-Shintinawy 1999, Mallick and Mohn 2000).

In conclusion, heat shock progressively enhanced the POL level in seedlings of the two tested barley cultivars. The increase in membrane leakage and MDA content coupled with the disappearance of linolenic acid after heat treatment confirms this conclusion. This response is a well-known phenomenon (Bestwick *et al*. 2001, Jiang and Zhang 2002) and could be referred to the hyperthermia (elevated production of ROS) induced POL activation. This hyperthermia is primarily dependent on disturbances in the functioning of photosynthetic and oxidative electron transport (shown by our findings) where the generation of reactive oxygen derivatives proceeds with the highest efficiency (Heath and Packer 1968, Dash and Mohanty 2002). Therefore, heat shock can shift the prooxidant-antioxidant balance in leaf chloroplasts due to the acceleration of peroxidative processes. The products of POL, generated in higher amounts under these conditions, can be considered as the primary mediators of the stress effect at high temperature, which triggers the appropriate protective mechanisms such as antioxidant enzymes (SOD, POX, and CAT). The alterations in the membrane-structure and cell metabolism provoked by POL enhancement at heat shock seem to be among the initial and primary events in the sequence of reactions. These events finally lead to the development of the stress-state and mobilisation of antioxidant systems that can enhance the tolerance of the plant. In this concern, seedlings of Giza 124 showed lower POL level and higher activities of antioxidant enzymes compared with seedlings of Giza 125. Therefore, Giza 124 appeared to be more tolerant to heat stress than Giza 125.

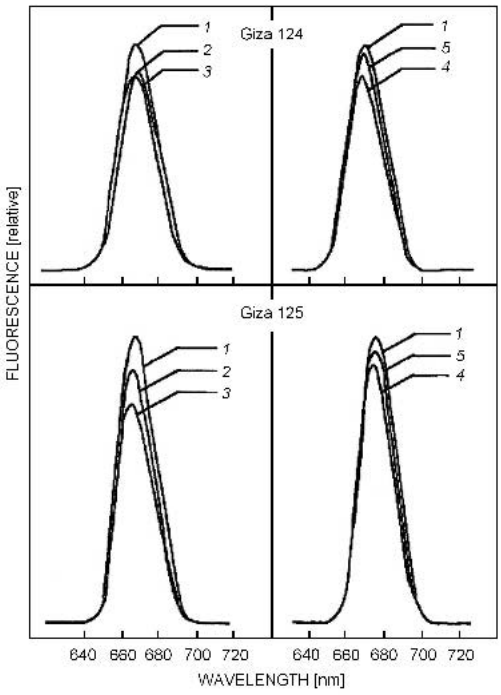

Fig. 1. Chlorophyll *a* fluorescence emission spectra of leaf extracts from seedlings of two barley cultivars (Giza 124 and 125) previously exposed to different heat shocks. *1*: control, *2*: 35 °C/4 h, *3*: 35 °C/8 h, *4*: 45 °C/4 h, and *5*: 45 °C/8 h.

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