CHANGES IN THE CHLOROPHYLL FLUORESCENCE PARAMETERS AND SUPEROXIDE DISMUTASE ACTIVITY DURING HEAT SHOCK TREATMENT AND THE RECOVERY PERIOD IN TOMATO PLANTS

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ABSTRACT. Plants of tomato cv *Amalia* were exposed to heat shock of 45ºC for two and three hours. After stress conditions, a group of plants was recovered under normal growth conditions of temperature of 25/20ºC day/night, relative humidity of 60 % and a photon flux density of 250 μmol photons.m^{-2}.s^{-1} PAR. Furthermore, they were recovered at 25ºC for 20 hours. An initial fluorescence increase and a variable fluorescence decrease were detected in stressed plants by both stress conditions. A photochemical efficiency decrease was noted during stress. After the recovery period (20 hours), fluorescence parameters showed a significant recovery, except the photochemical efficiency. SOD total activity was reduced by heat shock; however, several isoenzymes did not show a similar sensitivity to high temperature. Fe-SOD showed resistance to high temperature, whereas Cu/Zn-SOD was thermosensible. Thus, 20 hours of recovery were considered sufficient to restore SOD activity.

Key words: superoxide dismutase, chlorophylls, shock, tomato

RESUMEN. Plantas de tomate de la variedad Amalia se expusieron a un choque de calor de 45ºC durante dos y tres horas. Después de la condición de estrés, un grupo de plantas se recuperó en las condiciones de temperatura de 25/20ºC día/noche, humedad relativa de 60 % y una densidad de flujo de fotones de 250 μmol fotones.m^{-2}.s^{-1} PAR. Un incremento en la fluorescencia inicial y una disminución en los valores de fluorescencia variable se detectaron en las plantas estresadas por ambas condiciones de estrés. Una disminución en la eficiencia fotoquímica se observó durante el estrés. Después del período de recuperación (20 horas), los parámetros de fluorescencia mostraron una recuperación significativa, excepto en la eficiencia fotoquímica. La actividad total de la enzima SOD se redujo por el choque de calor; sin embargo, las distintas isoenzimas no mostraron igual sensibilidad a la alta temperatura. La isoenzima Fe-SOD mostró resistencia a la alta temperatura, mientras que Cu/Zn-SOD resultó termosensible. Se encontró que 20 horas de recuperación fueron suficientes para restaurar la actividad de la enzima SOD.

Palabras clave: superóxido dismutasa, clorofilas, shock, tomate

INTRODUCTION

High temperature influences plant photosynthetic functions, as it affects the rate of chemical reactions and structural organization (1). Physiological damages provoked by high temperatures appear at all levels of plant structural organisation. Water loss from chloroplasts (2), damage to primary photosynthetic processes (3), changes in phosphorylation and thylakoid structures (4) are examples of temperature effect at the sub-cellular level.

In plants, the photosynthetic process makes high intracellular concentrations of activated oxygen species (AOS) and reduces compounds such as NADPH (5). High sunlight effect together with extreme temperatures may lead to photoinhibition and oxidative stress in photochemical active tissue, because of an imbalance between light capture and its utilization for assimilation (6). The dissipation of excess light energy in PS_{II} core and antenna may cause O_2 generation, which is potentially dangerous (6). The photosynthetic electron transport also inevitably leads to the formation of superoxide, since molecular oxygen competes with $NADP^+$ for reduction at the acceptor side of PSI (7).

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Abbreviations:
F_o, initial chlorophyll fluorescence; F_m, maximum chlorophyll fluorescence; F_v, variable chlorophyll fluorescence; F_v/F_m, photochemical efficiency; PS_{II}, photosystem II; PSI, photosystem I; ΦPS_{II} light, efficiency of PS_{II} in light; AOS, activated oxygen species; SOD, superoxide dismutase

These processes must, therefore, be accompanied by a protective array of enzymes. Superoxide dismutase enzymes (SODs EC 1.15.1.1), which rapidly convert superoxide (O_2^-) into H_2O_2, are found on the thylakoid membranes and in the chloroplast stroma (8). Three types of SODs, defined by their component metal prosthetic groups (Cu/Zn, Fe and Mn) are found in leaves (9, 10). Chloroplasts contain Cu/Zn-SODs, encoded by nuclear sod Cp gene, and Fe-SODs, which are coded by nuclear sod B genes (10, 11). The presence of both Cu/Zn-SOD and Fe-SOD isoforms in the chloroplasts could be a physiological advantage under stress conditions, because of the different characteristics of enzymes (10).

It is known that high temperatures alter AOS levels of enzymatic and non-enzymatic antioxidants involved in the detoxification, which may result in oxidative damage (12). Thus, the ability of a plant to improve its AOS scavenging capacity may be a key element in heat stress tolerance.

Under tropical conditions, tomato crop is really hard, since high temperatures and relative humidity co-limit fruit production. Therefore, several investigations are performed to obtain new tolerant varieties; however, its selection is only based on morphoagronomic aspects, since physiological and biochemical parameters have not been considered. This work proves the thermotolerance of a photosynthetic apparatus in a new tomato variety obtained under tropical conditions and the superoxide dismutase activity induced by high temperatures as well as its relation with thermotolerance.

MATERIALS AND METHODS

Planting material and experimental conditions. Seeds from the tomato cv *Amalia* (13) (obtained at the National Institute of Agricultural Sciences) were germinated on moistened filter paper at 25°C in darkness. Seedlings were planted in plastic pots (14 x 12 cm) under the conditions of a growth chamber at 25/20°C day/night, relative humidity of 60 % and a photoperiod of 16 h, with a photon flux density of 250 mmol photons.$m^{-2}.s^{-1}$ PAR. Plants were daily watered to avoid water stress.

At the fourth-true-leaf stage, a group of 50 plants was transferred to a growth chamber with air temperature of 45°C and light intensity of 250 mmol photons.$m^{-2}.s^{-1}$. They were kept under these conditions for two and three hours (45°C-2 h, 45°C-3 h). Another group of 24 plants was maintained at 25°C as control treatment (0-hour exposure to stress conditions). Subsequent to stress, a group of 25 plants was chosen to record their physiological and biochemical measurements whereas the others were transferred to normal growth conditions of temperature of 25/20°C day/night, relative humidity of 60 % and a photon flux density of 250 mmol photons.$m^{-2}.s^{-1}$ PAR. Furthermore, plants were harvested to evaluate their recovery.

Both physiological and biochemical measurements were recorded immediately after stress exposure and recovery period. Physiological measurements were taken at 25°C.

Chlorophyll fluorescence measurements. These measurements were made on the third leaf, using a portable fluorometer model OS30 (Opti-Sciences Inc., Tyngsboro, MA, USA). Plants were dark-adapted for 20 min before starting to measure chlorophyll fluorescence.

The initial fluorescence level, *Fo*, was excited by a weak red light modulated. The maximum chlorophyll fluorescence level, *Fm*, was induced by the pulse of a strong white light (>2 600 mmol photons $m^{-2}.s^{-1}$ PAR) for 0.8 seconds. The *Fo* and *Fm* values were read in the fluorometer, whereas calculus of fluorescence parameters: *Fv*, variable fluorescence; *Fv*/*Fm*, photochemical efficiency and ΦPS_{II} light, PS_{II} efficiency in light followed Maxwell and Johnson (14).

Fraction of chloroplasts. The overall procedure was carried out at 4°C. Leaf tissue was carefully mashed in a mortar with an extraction medium containing Mops-KOH 30 mM pH 7.5; mannitol 350 mM; cysteine 1 mM and BSA (0.2 %). Homogenate was squeezed through two layers of muslin and centrifuged at 2 200 g for 30 seconds in a centrifuge Beckman model J2-21. The precipitate was carefully removed with a brush and resuspended in a medium containing Mops-KOH 30 mM pH 7.5; mannitol 350 mM; cysteine 1 mM and centrifuged at 2 200 g for 30 seconds. The fraction of precipitated chloroplasts was carefully removed and resuspended in a minimum volume of medium.

Proteins were determined from chloroplast-rich extract according to Bradford's method (15), using BSA as a standard.

Superoxide dismutase activity. The SOD activity was assayed as previously described by McCord and Fridovich (16). Total SOD activity was measured in 3 mL reaction mixture containing potassium phosphate 50 mM pH 7.8; $EDTANa_2$ 0.1 mM; cytochrome c 1 mM; xantine 1 mM and 25-100 mL sample. The reaction started by adding xantine-oxidase, and the reduction of cytochrome c was monitored at 550 nm. SOD resistant to CN activity was determined by the addition of CN^- (10^{-3}M) to the reaction mixture. The enzymatic activity was expressed as units of SOD/mg protein.

Total chlorophylls. Chlorophyll content was determined in its fraction. To do it, 10-30µL of the extract was incubated in darkness with acetone (80 %) all night long. The extract was centrifuged at 15 000 g for five minutes and the supernatant was collected to read at 652 nm. The chlorophyll content of its fraction was calculated as:

$$\text{Chlorophyll (mg x mL}^{-1}) = 27.8 \times Abs_{652} \times F$$

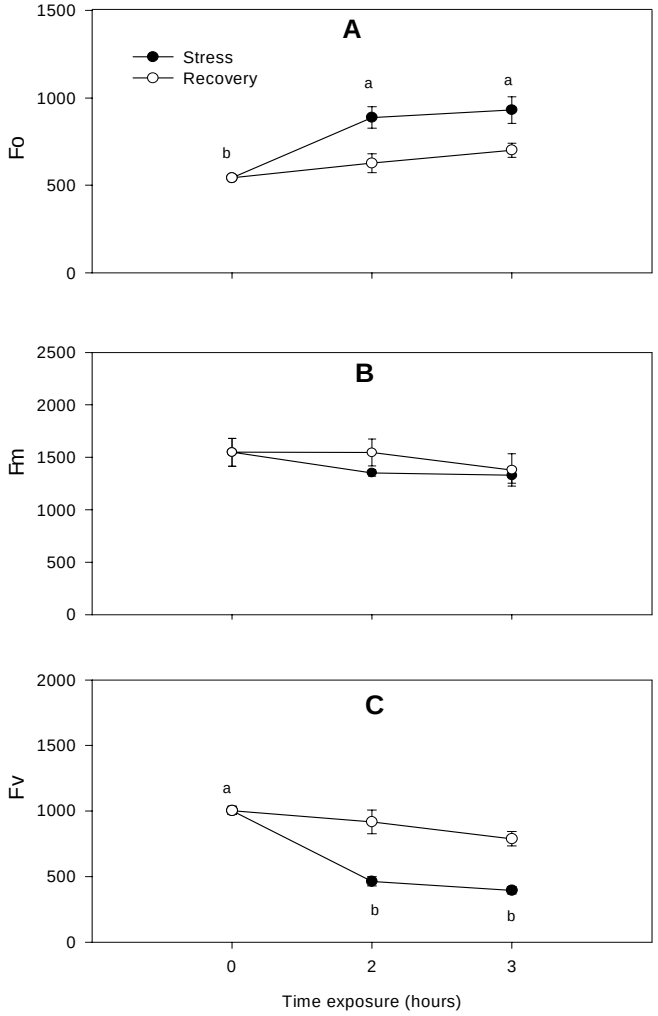
where Abs_{652} represents the absorbance to weak long 652 and F is the diluting factor.

Statistical analysis. The experiments were conducted in a randomized complete design. Fluorescence results are the means of four independent replicates in four plants of each treatment. Pigment content results are the means of six replicates in three plants per treatment, and SOD activity is the means of three replicates in four plants per treatment. The significance of differences between mean values was determined by one-way analysis of variance. Duncan's multiple range test was used to compare the means when necessary.

RESULTS

High temperature modified the reactions involved in the trap of light in PS_{II}. An increase in *Fo* levels was recorded in treated plants independently of the exposure time (Figure 1A).

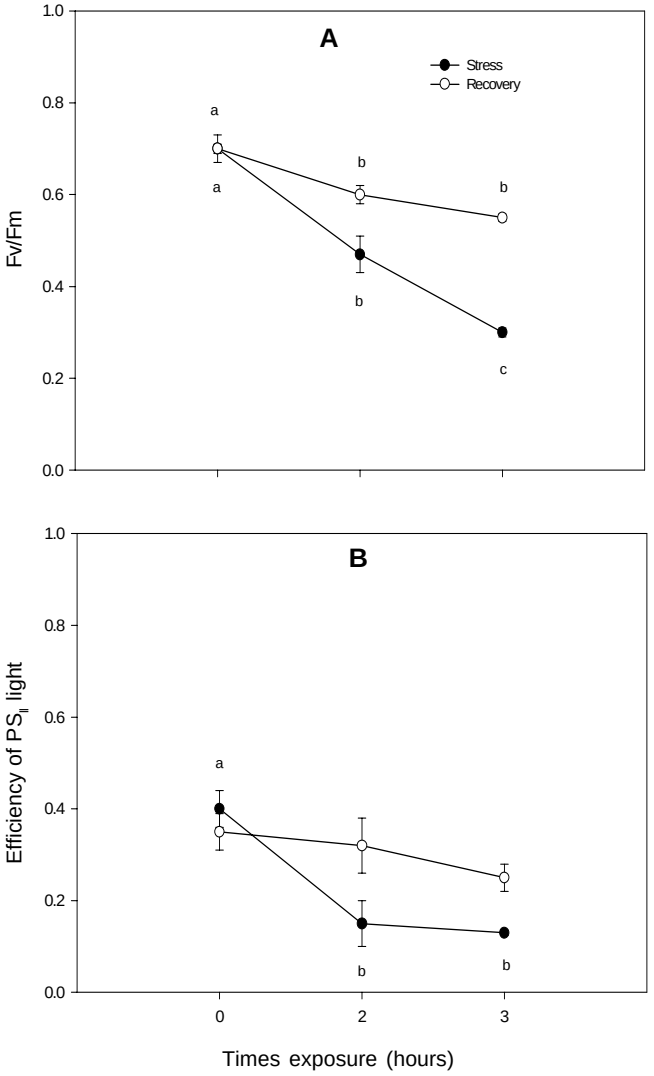
On the contrary, *Fm* levels were modified neither by high temperature nor exposure time (Figure 1B). However, the increase in *Fo* levels provoked a significant reduction in *Fv* levels in treated plants for two and three hours (Figure 1A and C).



Means for each parameter and time that do not have a common letter are significantly different by Duncan's test, p<0.05

Figure 1. Initial chlorophyll fluorescence (*Fo*) (A), maximum chlorophyll fluorescence (*Fm*) (B) and variable fluorescence (*Fv*) (C) values in leaves from *Amalia*. Dark circles represent the stress period and white circles represent recovery period. Columns represent the mean±SE of four replicates per treatment

The photochemical efficiency of PS_{II} estimated from *Fv*/*Fm* ratio was reduced by both stress conditions, indicating the presence of photoinhibitory processes (Figure 2A). Similarly, the efficiency of PS_{II} in light (ΦPS_{II} light) was reduced by high temperature (Figure 2B).



Means for each parameter and time that do not have a common letter are significantly different by Duncan's test, p<0.05

Figure 2. Photochemical efficiency of PS_{II} (F_v/F_m) (A) and efficiency of PS_{II} in light (ΦPS_{II} light) (B) values in leaves from *Amalia*. Dark circles represent the stress period and white circles represent recovery period. Columns represent the mean±SE of four replicates per treatment

Chlorophyll fluorescence variables (*Fo*, *Fv*, *Fv*/*Fm*, ΦPS_{II} light) were completely recovered 20 hours after removing stress conditions (Figures 1 and 2). The recovery of PS_{II} indicates that the imposed high temperatures provoked reversible damages on PS_{II}, presumably on light harvesting complex.

Total chlorophyll content was not altered by high temperatures; similar values were recorded in stressed and control plants. Thus, 20 hours after removing stress conditions, chlorophyll content remained unaltered (Figure 3). Increment of the initial fluorescence (*Fo*) values was not related to chlorophyll content modifications.

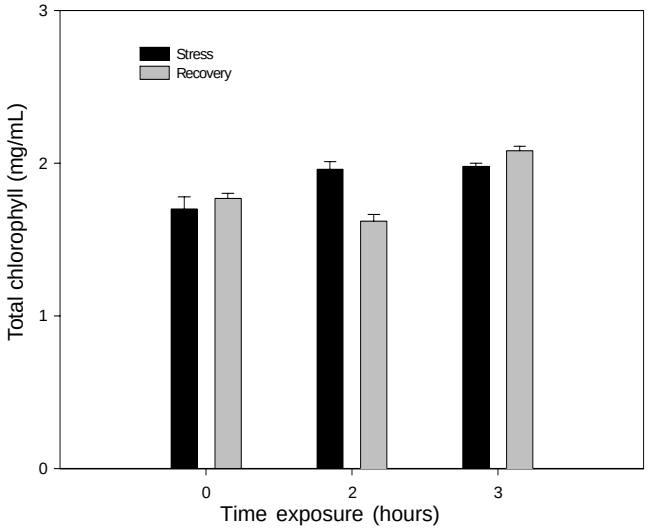
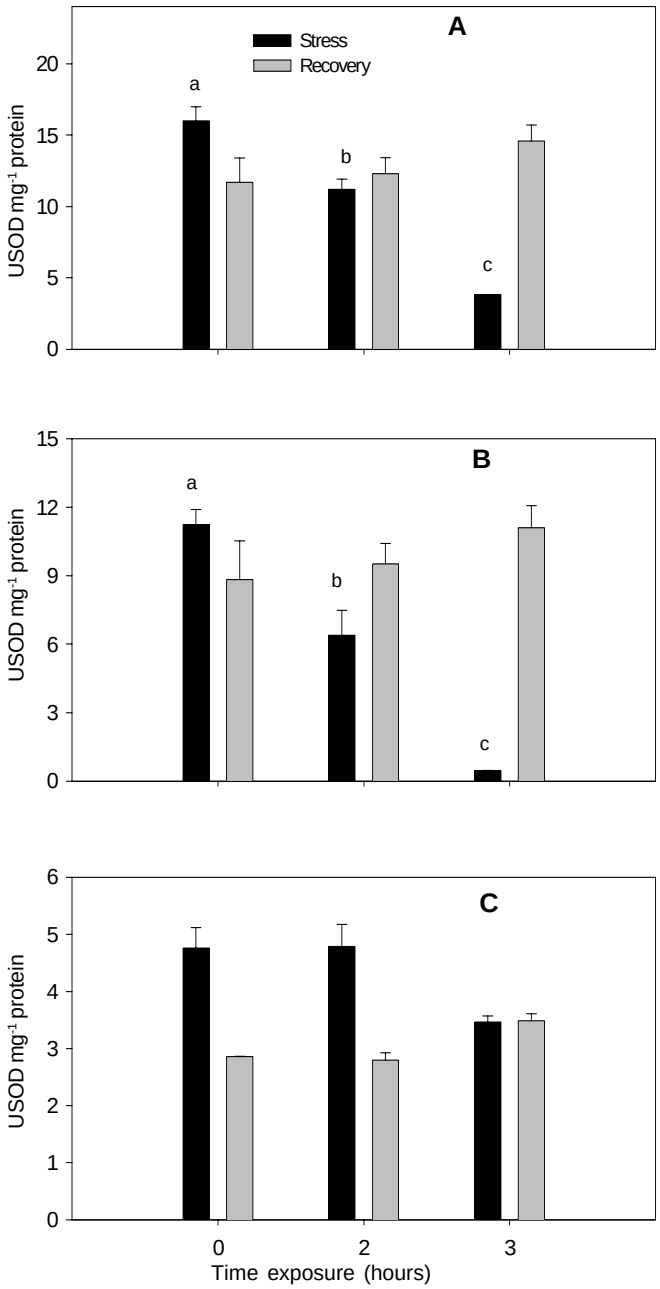


Figure 3. Total chlorophyll content in leaves from *Amalia* during heat-shock treatments (45ºC-2 hours and 45 ºC-3 hours) and recovery. Columns represent the mean±SE of six replicates per treatment

Total SOD activity was sensitive to heat and increased with the exposure time to high temperatures. A significant reduction in total SOD activity was detected in stressed plants. The three hour-exposure time was enough to provide a higher reduction than 50 % (Figure 4A). However, the SOD activity sensitive to CN^- (considered as Cu/Zn-SOD activity) was reduced by 44 and 97 % in stressed plants for two and three hours, respectively (Figure 4B). The SOD activity resistant to CN^- (considered as Fe-SOD activity) detected in stressed plants was similar to that in control plants (Figure 4C).

It is noted that Cu/Zn-SOD/total SOD and Fe-SOD/total SOD ratios present in chloroplast fraction changed with stress conditions (Figure 5). In control plants, the total SOD activity was represented by 70 % of Cu/Zn-SOD and 30 % of Fe-SOD. However, Cu/Zn-SOD activity represented 57 and 10 % of the total SOD activity in stressed plants for two and three hours, respectively, whereas Fe-SOD activity represented 43 and 90 % of the total SOD activity, respectively (Figure 5A and B). Different modifications recorded in the active isoenzymes of chloroplasts suggested that Fe-SOD is the most active isoform also responsible for its protection.

Thus, 20 hours after removing stress conditions, the total SOD activity detected in stressed plants was similar to the one recorded in control plants. Similarly, the SOD active isoenzymes/total SOD ratio was recovered in 20 hours, Cu/Zn-SOD activity representing the 75 % whereas Fe-SOD activity 25 % (Figure 5).



Means for each parameter and time that do not have a common letter are significantly different by Duncan's test, $p<0.05$

Figure 4. Total SOD activity (units.mg^{-1} protein) (A), SOD activity sensible to CN^- (considered as Cu/Zn-SOD) (B) and SOD activity resistant to CN (considered as Fe-SOD) (C) present in the extract of chloroplasts in leaves from *Amalia* during heat-shock treatments (45ºC-2 hours and 45ºC-3 hours) and recovery. Columns represent the mean±SE of three replicates per treatment

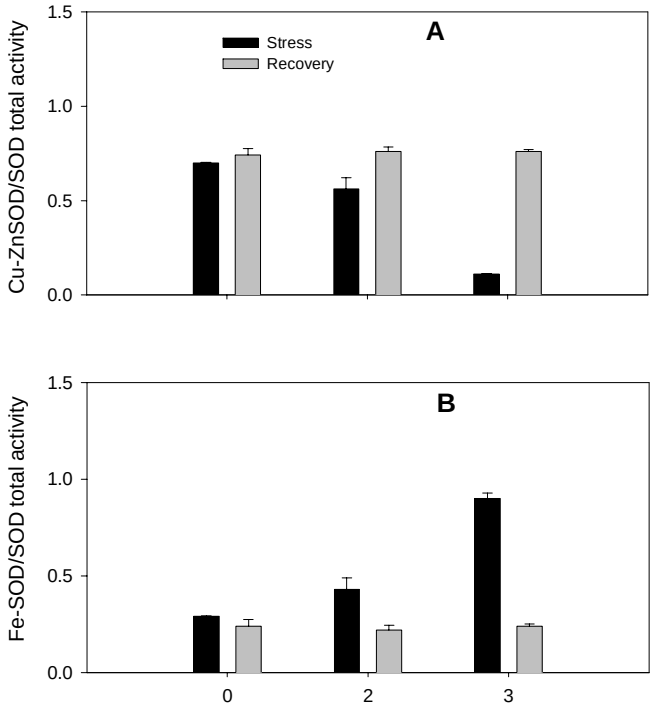


Figure 5. Cu/Zn-SOD/total SOD activity (A) and Fe-SOD/total SOD (B) ratio present in the extract of chloroplasts in leaves from *Amalia* during heat-shock treatments (45ºC-2 hours and 45ºC-3 hours) and recovery

DISCUSSION

In the present study, one high-yielding tomato variety obtained under tropical conditions was used to know if extreme temperature conditions and several exposure-times at high temperature induced changes on chlorophyll fluorescence parameters and if it was related to chloroplast SOD activity.

These results showed that both high temperature conditions enhanced photo inhibition as shown by *Fv*/*Fm* decrease. At all exposure-times, this decrease was greatly due to *Fo* increase while the decrease in *Fm* was not significant. Several authors have reported that environmental stresses, as low and high temperatures, limit plant ability to use sunlight and increase photo inhibitory response (17, 18, 19, 20, 21, 22). *Fo* increase in stressed plants indicates a decreased energy transfer to the reaction center with an increase of temperature, independently of exposure time, probably related to a non-association of the light-harvesting complex and the reaction center. It is well known that the increase in *Fo* values indicates structural-functional modifications of PS_{II}, presumably related with disorders in energy trap capacity (23). A *Fo* increase could be attributed to irreversible detachment of light-harvesting chlorophyll a\b protein complexes from reaction center complex of PS_{II}, to partly reversible inactivation of PS_{II} (24) and to dark reduction of QA (25).

Several authors have demonstrated the increase of *Fo* values with temperature (23, 26, 27, 28, 29). Two interpretations have been proposed to explain the increase of *Fo* values: the first is related with changes at antenna levels (23, 26), while the second is related with changes on reaction center of PS_{II} (27, 28, 29). In this case, we refused the fact that modifications in the total chlorophyll content are responsible for increasing *Fo* values under high temperature conditions. Similar contents of total chlorophyll were recorded in stressed and control plants. However, we did not refuse the fact that some variations in a/b chlorophyll ratio could determine the variations in *Fo* values.

Therefore, 20 hours after applying stress conditions, the effects observed in the chlorophyll fluorescence parameters (*Fo* and *Fv*) disappeared, indicating that high temperature provoked brief and reversible modifications on PS_{II}. However, photo inhibitory events limited the complete recovery of photochemical efficiency of PS_{II}, probably related with recovery D_1 proteins. A biphasic kinetic of PS_{II} efficiency recovery after photoinhibition had been reported (17, 30), which showed the presence of two phases on the photosynthetic apparatus recovery (fast and slow phases). Regarding the slow phase of recovery, the photo inhibited reaction center is repaired by replacing the damage D_1 protein with newly synthesized copy (31).

Even when the increase of *Fo* values indicates a reduction of energy absorption capacity, it can not reduce to zero the effect of high temperatures on the electron transport chain and the over-reduction of its intermediates. That situation favors the production of reactive species of oxygen, as O^{2-} in chloroplasts, which can be controlled and means an efficient antioxidant system catalyzed by SOD. It was found that SOD total activity was diminished with temperature increase. Studies carried out in several species of tomato have reported that SOD activity is reduced under high temperature conditions (32).

However, the action of active isoenzymes present in chloroplast extract showed a different stability to temperature increase. Under temperature conditions of 25ºC, Cu/Zn-SOD activity represented 70 % of the total activity of the extract. However, under high temperature conditions, it was severely reduced, representing the 57 and 30 % of SOD total activity in stressed plants for two and three hours respectively, which indicates the susceptibility of this isoenzyme. There is some evidence that cytoplasmic and chloroplast Cu/Zn-SOD activities are reduced with temperature increase in pea plants (33).

On the contrary, Fe-SOD activity showed great stability under high temperature conditions, suggesting that this isoenzyme in chloroplasts is the responsible for protecting the photosynthetic apparatus of dangerous effects of reactive species of oxygen. The activity of Fe/SOD isoenzyme represented 43 and 90 % of SOD total activity in stressed plants for two and three hours,

respectively. Results from this experiment recognized this isoenzyme as a key for protecting photosynthetic apparatus under these stress conditions, when the oxidative stress could be favored by the irregularity in transport electron chain.

Thus, 20 hours after applying heat stress, the total SOD activity as well as its isoforms were recovered; this fact was related to the recovery of the photo inhibition of PS_{II}, which reduced the occurrence of oxidative stress in chloroplasts. Previous studies suggested that Fe-SOD $_{chl}$ gene expression is controlled by the oxidative stress itself and not as part of a global response for the induction of genes involved in photosynthesis (34). At the molecular level, the negative effect of high temperature stress on leaves may be partly a consequence of the oxidative damage to important molecules, as a result of the imbalance between production of activated O_2 and antioxidant defenses (6).

These results enabled to demonstrate that the moderate thermotolerance of this variety under field conditions would not be related with tolerance of photochemical functions and SOD activity. However, the fast and reversible recovery of the event damage during high temperature can explain its tolerance, if it is considered that plant recovery capacity determines the possibility of plants to survive under unfavorable conditions.

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