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Quantification of OJIP Fluorescence Transient in Tomato Plants Under Acute Ozone Stress

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ABSTRACT

Ozone is one of the environmental stresses that limit crop photosynthesis. To determine structural and functional alterations of photosynthetic machinery, tomato plants aged 30 d were exposed to ozone (500 $\mu g.m^{-3}$) for 4 hr. The plants were grown in a net house at the Department of Horticulture, Kasetsart University, Thailand from May to June 2012. Crop responses to ozone were evaluated by a chlorophyll a fluorescence OJIP test using a FluorPen FP 100 fluorometer, where minimum fluorescence was measured at 50 μs when all PSII reaction centers are open and is defined as the O step, followed by the J step (at 2 ms), the I step (at 60 ms) and at maximum fluorescence (F_M) when all PSII reaction centers are closed, known as the P step. Measurements were done three times (before ozone exposure, 20 min and 20 hr after ozone treatment). Results indicated that the shape of the OJIP fluorescence transient alters under ozone stress. F_M significantly reduced after ozone exposure while other fluorescence levels (from O to J) did not change significantly. The percentage of relative variable fluorescence and the net rate of photosystem II closure following ozone exposure were greater in ozone-treated plants. However, specific energy fluxes per reaction center and the performance index were significantly decreased under ozone stress. Reduction of the maximum quantum yield of primary photochemistry (the maximal variable fluorescence divided by the maximal fluorescence intensity) and efficiency following ozone exposure depicted that ozone impaired photosynthetic systems by deactivating reaction centers. It can be concluded that the OJIP test can easily detect photosynthetic activity induced by ozone.

Keywords: fluorescence induction, ozone, photosynthetic activity, reaction center, tomato

INTRODUCTION

In agriculture, plant growth, development and specifically crop yields mainly depend on photosynthesis. Crop photosynthesis is limited by different environmental factors such as biotic and abiotic stresses (Sampol *et al.,* 2003; Munns *et al.,* 2006; Chaves *et al.,* 2009; Bilgin *et al.,* 2010) that lead to reduced crop yields. Ozone is considered as a secondary air pollutant and is one of the abiotic stresses in agricultural crop production as it is highly reactive and rapidly reacts with a range of compounds associated with the cell wall and membranes (Roshchina and Roshchina, 2003). Consequently, it directly reacts with internal cellular cell components (Heath, 1996). Ozone directly affects the functional processes of photosynthesis and decreases carbon fixation and consequently reduces plant biomass (Navakoudis *et al.,* 2003).

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Among horticultural crops, tomato stands as one of the important vegetables in the world for its high sources of vitamins and minerals (United States Department of Agriculture, 2014). Many articles have described tomato plants as being very sensitive to ozone and various physiological processes change under ozone stress (Iriti *et al.,* 2006; Calvo *et al.,* 2007; Thwe *et al.,* 2013). Since photosynthesis is known to be most affected by ozone and tomato is considered as an ozone-sensitive crop, the determination of the photosynthetic activity of tomato plants under ozone stress would provide a better approach to detect environmental stress.

Strasser *et al*. (2004) described the photosynthetic pathway and subsequent action stating that in the pathway of the photosynthetic process, light is absorbed by the antenna molecules within the photosynthetic membrane and the absorbed energy is transferred as excitation energy. The energy is then either trapped at a reaction centre and used in photochemistry, or dissipated mainly as heat and fluorescence. The features of the emitted fluorescence are basically determined by the absorbing pigments, the excitation energy transfer, and the nature and orientation of the fluorescing pigments. Chlorophyll (Chl) fluorescence is a non-invasive method to monitor the alterations of photosynthetic processes (Krause and Weis, 1991) by measuring the emitted radiation from the crop. It is a useful indicator since it gives important information about the state of health of a photosynthetic sample and it is a valuable tool for plant studies from leaf to ecosystem levels (Adams and Demming-Adams, 2004). Therefore, photosynthetic activities of crops under environmental stress are usually measured by chlorophyll a fluorescence.

Chl a fluorescence can be characterized by different phases: Chlorophyll Fluorescence Induction Kinetics (OJIP) is fluorescence transience corresponding to the redox states of photosystem PSII and PSI, and to the efficiencies of electron transfer through the intersystem chain to the end electron acceptors at the PSI acceptor side (Strasser *et al.,* 2000; Strasser *et al.,* 2004).

A plant is likely to be exposed to at least one environmental stress throughout its life. Understanding how plants respond to environmental stresses could help crop improvement strategies through better management practices under stress situations. In this research, functional alterations of ozone-treated tomato plants were determined to capture the state of photosynthetic activity. The structural and functional alterations of the photosynthetic machinery induced by ozone were evaluated with chlorophyll fluorescence being a useful parameter to detect the photosynthetic responses of tomato plants under ozone exposure.

MATERIALS AND METHODS

Experimental situations and crop management

This research was undertaken in an experimental field of the Plant Physiology Laboratory, Department of Horticulture, Kasetsart University, Thailand from May to June, 2012. Well-mixed soil (garden soil:compost:coconut husk at the ratio of 2:1:1 volume per volume) was filled into plastic pots with a capacity of approximately 10 L. Then, tomato (*Solanum lycopersicum* L.) seeds were sown directly into the plastic pots at the rate of three seeds per pot. When the seeds germinated, the seedlings were thinned leaving one seedling per pot. The plants were grown under a net house with 60 cm spacing. Manual watering occurred daily. Practices for crop nutrition and crop protection were followed according to the procedures reported by Thwe *et al.* (2013).

Practices for ozone exposure

Ozone was given when the plants were 30 days old. Two plastic closed-top chambers (120 cm × 120 cm × 200 cm) were used; one chamber for the ozone treatment and one chamber for

the control were constructed using transparent polyethylene plastic. Tomato plants aged 30 d were transferred to the plastic chambers for ozone exposure. Ozone (500 $\mu g.m^{-3}$) was exposed to the plants for 4 hr (0730 to 1130 hours). Ozone was supplied using an ozone generator (Model OZ 8010; Ozonic International Co. Ltd. Bangkok, Thailand) and the ozone concentration in the chamber was measured by a computerized ozone analyzer (Model 49i, Thermo Fisher Scientific Inc., Waltham, MA, USA) throughout the fumigation period. The ambient air passing through the cooling pad was blown to the control chamber throughout the fumigation period. The temperature and relative humidity inside and outside the chamber were measured throughout the fumigation period using a USB data logger (OM-EL-USB-2; Omega Engineering, Inc.; Stamford, CT, USA). After ozone exposure, the plants were returned to their place in the net house. Ten sample plants were used for the treatment and control.

Chlorophyll fluorescence measurements

The OJIP fluorescence transient was measured using a portable fluorometer (FluorPen FP 100; Photon Systems Instruments; Drasov, Czech Republic). Fully developed youngest leaves were selected for the measurements. The leaves were dark-adapted for 30 min before starting the measurements using leaf clips provided by the manufacturer. Measurements were done three times (before ozone exposure, 20 min and 20 hr after the end of ozone exposure) on the adaxial leaf surface. For each measurement, two places per leaf were selected in one plant. Data were analyzed from 10 measurements (five plants with two places per leaf, and one leaf per plant) for each treatment and control.

The OJIP fluorescence parameters were calculated based on the formulas shown in Table 1 and detailed explanations are expressed in Table 2. Minimum fluorescence (F_0) was measured at 50 μs when all PSII reaction centers are open and it is defined as the O step, followed by the J step (at 2 ms), the I step (at 60 ms) and at maximum fluorescence (F_M) when all PSII reaction centers are closed, known as the P step (Strasser *et al.,* 2000). The JIP test represents a translation of the original data to biophysical parameters that quantify the energy flow through PS II (Thach *et al.,* 2007). From each OJIP fluorescence induction, specific energy fluxes per reaction center were analyzed and compared.

The performance index (PI) was calculated based on the absorption basis (PI_{Abs}: the energy conservation from photons absorbed by PSII antenna, to the reduction of Q_B) and the total fluorescence basis (PI_{total}: the energy conservation from photons absorbed by PSII antenna, until the reduction of PSI acceptors) according to Strasser *et al.* (2000) as shown in Table 1.

All data were analyzed using SAS 9.2 (SAS Institute, 2007). Mean values of ozone-treated and non-treated plants were compared using a *t* test comparison at the significance level of $P < 0.05$ and the highly significant level of $P < 0.05$.

RESULTS

OJIP fluorescence transient

Ozone affects the shape of the Chl a fluorescence transient in tomato plants. Twenty minutes after the end of ozone exposure, the P level from the OJIP steps reduced in the ozone-treated plants in comparison with the control. Other fluorescence levels (OJI) did not show any significant differences (Figures 1A and 1B). Fluorescence reduction still continued up to 20 hr later (Figure 1C). Comparing the three evaluation periods (before ozone exposure, 20 min and 20 hr after ozone exposure), the strongest reduction of fluorescence was observed at 20 min after ozone treatment, followed by 20 hr after (Figure 1D) and they were significantly lower than those before ozone exposure. The area of fluorescence between the OJIP fluorescence curve and F_M significantly reduced in the ozone-treated plants at both 20 min

and 20 hr after the treatment (Table 3). F_M was reduced by 44% and 35% 20 min and 20 hr later, respectively, in comparison with the control.

Relative variable fluorescence at 2 ms

The relative variable fluorescence is the fraction of closed reaction centers (RCs) at 2 ms expressed as a proportion of the total number of RCs that can be closed. The relative variable fluorescence increased significantly following ozone exposure. It was increased by 38% 20 min after the end of the treatment and the increased percentage reduced to 28% 20 hr later (Figure 2a).

Net rate of photosystem II closure

The net rate of photosystem II closure (M_0) is a measure of the rate of the primary photochemistry. The results showed that ozone increased the net rate of photosystem II closure in both evaluation periods. At both 20 min and 20 hr after treatment, the ozone-treated plants showed increased percentages of M_0 about 26% relative to the control (Figure 2b).

Maximum quantum yield of primary photochemistry (F_V/F_M) and its efficiency (ψ_0)

A significant reduction of the maximum quantum yield of primary photochemistry (F_V/F_M)

Table 1 Parameters of the steps of fluorescence induction.

Parameter abbreviation	Formula explanation
F_0	$F_{50\mu s}$, fluorescence intensity at 50µs
F_j	Fluorescence intensity at J-step (at 2 ms)
F_i	Fluorescence intensity at i-step (at 60 ms)
F_M	Maximal fluorescence intensity
F_V	$F_V = F_M – F_0$ (Maximal variable fluorescence)
V_j	$V_j = (F_j – F_0)/ (F_M - F_0)$
V_i	$V_i = (F_i – F_0)/ (F_M - F_0)$
M_0 or $(dV/dt)_0$	$M_0 = TR_0/RC – ET_0/ RC = 4 (F_{300} – F_0)/ (F_M – F_0)$
Area	Area between fluorescence curve and F_M (background subtracted)
Fix Area	Area below the fluorescence curve between $F_{40\mu s}$ and F_{1s} (background subtracted)
φ_P0	$\varphi\_P0 = 1 – (F_0 / F_M)$ (or F_V / F_M)
ψ_0	$\psi\_0 = 1 – V_j$
φ_E0	$\varphi\_E0 = (1 – (F_0 / F_M))* \psi\_0$
φ_D0	$\varphi\_D0 = 1 – \varphi\_P0 – (F_0 / F_M)$
φ_Pav	$\varphi\_Pav = \varphi\_P0 (S_M / t_{FM})$ t_{FM} = time to reach F_M (in ms)
r_{RC2}	$r_{RC2} = Chl_{RC} / Chl_{total}$
ψ_ET2o	$= 1 – F_j / F_M$
ABS/RC	$ABS/RC = M_0 * (1 / V_j) * (1 / \varphi\_P0)$
TR_0/RC	$TR_0/RC = M_0 * (1 / V_j)$
ET_0/RC	$ET_0/RC = M_0* (1 / Vj)* \psi\_0$
DI_0/RC	$DI_0/RC = (ABS / RC) – (TR_0 / RC)$
PI_{Abs}	$PI_{Abs} = [r_{RC2} / (1–r_{RC2})] * [\varphi\_P0 / (1 – \varphi\_P0)] * [\psi\_ET2o / (1– \psi\_ET2o)]$
PI_{total}	$PI_{total} = PI_{Abs} * \{(1 – Vi) / (1 – Vj)\} / [1 – \{(1 – Vi) / (1 – Vj)\}]$

Source: Strasser *et al.* (2000); Stirbet and Govindjee (2011)

was observed in ozone-treated plants at both 20 min and 20 hr after the treatment in comparison with the control (Figure 2c). The maximum fluorescence parameter (F_M) strongly reduced in both evaluation periods; however the minimum fluorescence (F_0) did not change significantly. Between 20 min and 20 hr after, F_M from 20 min after was much lower than 20 hr after in the ozone-treated plants and they were significantly lower than the control (Table 3). A similar pattern was observed in the efficiency of primary photochemistry (Psi_0, ψ_0) which significantly reduced after ozone exposure (Figure 2d).

Specific energy flux per reaction center

The initial stage of photosynthetic activity of a reaction center (RC) complex is regulated by three functional steps—absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET)—(Thach *et al.,* 2007). The results indicated that the absorption of light energy per reaction center (ABS/RC) slightly increased after ozone exposure, but it did not show any significant alteration between the treatment and control. The trapping of excitation energy per reaction center (TR_0/RC) dropped significantly (–8%) 20 min

Table 2 Detailed explanation of the parameters described in Table 1

	Flux ratio of PS II
φ_{P0}	Maximum quantum yield of primary photochemistry
ψ_0	Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-
φ_{E0}	Quantum yield of electron transport
φ_{D0}	Probability that an absorbed photon is dissipated
	Flux ratio of PS I
δ_{R0}	The efficiency with which an electron can move from the reduced intersystem electron acceptors to the PS I end electron acceptors;
φ_{R0}	Quantum yield of electron transport from Q_A^- to PS I end electron acceptors;
ρ_{R0}	The efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to PS I end electron acceptors.
	Specific energy fluxes per RC
ABS/RC	Absorption (a measure of the apparent antenna size, i.e., average amount of absorbing antenna chlorophylls per fully active (Q_A reducing) reaction center)
ET_0/RC	Electron transport (electron transport flux from Q_A to Q_B)
TR_0/RC	Trapping (the initial rate of the closure of photoactive RCs per total number of photoactive RCs)
DI_0/RC	Dissipation (energy dissipation as heat and fluorescence)
RE_0/RC	Reduction of end acceptors at the PS I electron acceptor side
	Phenomenological energy fluxes per excited cross section (CS_M, subscript $_M$ refer to time F_M)
$ABS/CS_M = F_M$	Absorption
ET_0/CS_M	Electron transport
TR_0/CS_M	Trapping
DI_0/CS_M	Dissipation
RC/CS_M	Density of RCs
PI_{ABS}	Performance index on the absorption basis
PI (PI_{total})	Total performance index up to the PS I end electron acceptors

after the treatment. Similarly, the conversion of excitation energy to electron transport (ET_0/RC) was reduced –32% and –13% relative to the control at both 20 min and 20 hr after, respectively. However, the energy dissipation (DIo/RC) was increased up to +48% after the treatment (Table 4).

Performance index

The performance index (PI) describes the overall expression of the internal force of the sample to resist constraints from outside. Ozone reduced the performance index (PI_{Abs} and PI_{total}) significantly in both evaluation periods in comparison with the control. Twenty minutes after the treatment, ozone exposure reduced PI up to –67% and –71% in PI_{Abs} and PI_{total}, respectively, while they were –57% and –64%, respectively, 20 hr later (Table 4). The PI reduction percentage observed at 20 min after was stronger than those observed at 20 hr after.

DISCUSSION

In this research, 500 $\mu g.m^{-3}$ ozone was exposed to tomato plants aged 30 d and the

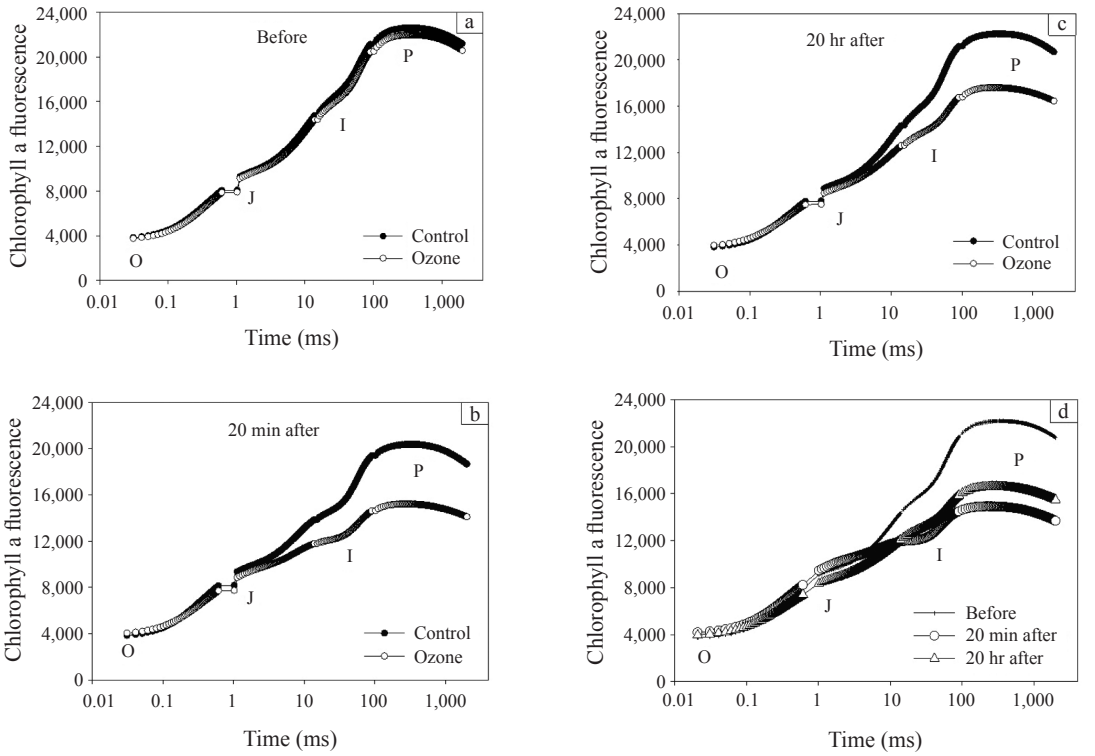

Figure 1 Comparison of OJIP steps of the fluorescence transient between ozone-treated and non-treated plants at different evaluation periods. The value of the X axis is expressed on a logarithmic scale. Mean values are the average of 10 measurements with measurements done three times (before ozone exposure, 20 min and 20 hr after ozone exposure). Comparisons were made between ozone-treated and non-treated plants at each evaluation period (a–c) and among the three evaluation periods (d). Comparing the three evaluation periods (d), all values from before measurement were averaged. O step = F_0, fluorescence intensity at 50 µs; J step = F_j, Fluorescence intensity at J-step (at 2 ms); I step = F_i, Fluorescence intensity at I step (at 60 ms); P step = F_M, Maximal fluorescence intensity.

fluorescence transient was evaluated using a portable fluorescence device. Measurements were done three times (before ozone exposure, 20 min and 20 hr after ozone treatment) to determine the ozone impacts on fluorescence induction of tomato plants. The results indicated that ozone stress (500 $\mu g.m^{-3}$ for 4 hr) altered the response of chlorophyll a fluorescence in comparison with the control. The OJIP fluorescence transient strongly reduced under ozone stress mainly at the P level. Other parameters such as energy fluxes per reaction center and the performance index were also affected by ozone exposure.

Ozone affects the shape of the OJIP fluorescence transient. Fluorescence reduction was more pronounced at the P level (F_M) which significantly reduced under ozone stress. A lower F_M value after ozone exposure corresponds to a reduction of the maximum quantum yield of primary photochemistry (FV/FM) since F0 does not change significantly. Layne and Flore (1992) stated that the decrease of FM is associated with the increase of the non-photochemical de-excitation constant and the decrease of F_0 indicates an enhancement of the overall de-excitation constants. The increase of F_0, on the other hand, is considered an expression of irreversible damage in PSII (Krause, 1988) and indicates that heat dissipation occurs in an uncontrolled manner, producing an excess of excitation within

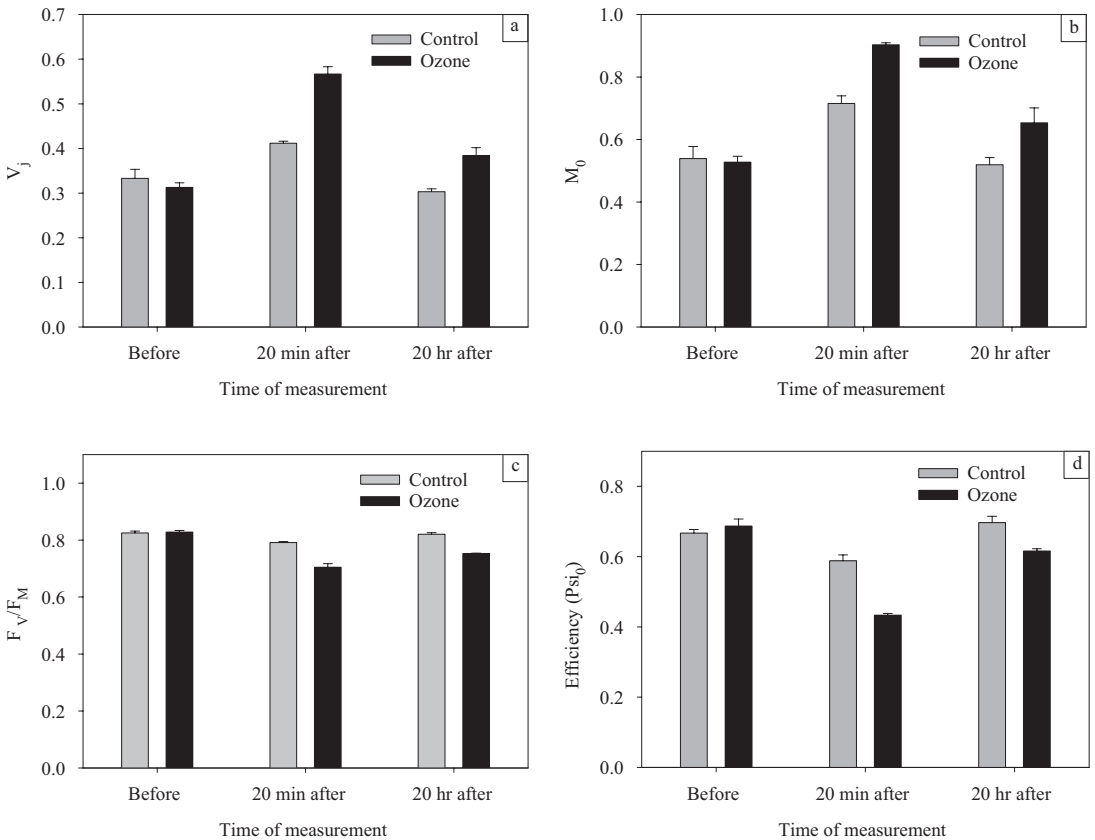

Figure 2 Comparison between the treatment and control of: (a) Relative variable fluorescence at 2 ms stage (V_j); (b) Net rate of PS II closure (M_0); (c) Maximum quantum yield of primary photochemistry (F_V/F_M); and (d) Efficiency (Psi_0, ψ_0). Measurements were done three times (before ozone exposure, 20 min and 20 hr after the end of ozone exposure). Comparisons were made between ozone-treated and non-treated plants at each evaluation period.

the leaves. In this research, exposure of ozone (500 $\mu g.m^{-3}$) for 4 hr caused the reduction of F_V/F_M by –11% and –8% at 20 min and 20 hr after, respectively, relative to the control without affecting minimum fluorescence (F_0). This result highlighted F_M was sensitive to ozone stress and F_0 was found to be insensitive. Low F_M indicates an increase of non-photochemical quenching when the plants are under ozone stress. The decreasing trend of F_M and low F_V/F_M was also observed in other plants which were under stress from heat, light and other pollutants (Lorenzini *et al.,* 1990; Calatayud and Barreno, 2004). Insensitivity of F_0 under ozone stress has been observed in *Plantago*

Table 3 Comparison of OJIP steps of fluorescence transient in response to ozone exposure.

	Before		20 min after		20 hr after	
	Treatment	Control	Treatment	Control	Treatment	Control
F_0	3,985.75	3,719.25	4,317.00 ^{ns}	4,091.67	4,098.00 ^{ns}	3,835.33
F_j	9,855.75	9,673.75	10,219.67 ^{ns}	10,482.00	8,940.33 ^{ns}	9,146.33
F_i	16,463.75	15,845.25	12,177.00 ^{ns}	14,453.33	13,187.33 ^{ns}	15,194.33
F_M	22,744.50	21,603.00	14,889.00**	19,588.33	16,584.00**	21,349.33
Area	6,605,513.75	6,539,231.50	2,910,016.00** (–44%)	5,164,440.33	4,305,916.67** (–35%)	6,644,069.67

See Tables 1 and 2 for complete definitions of terms used.

Fluorescence was measured after 30 min dark adaptation for all samples. Minimum fluorescence (F_0) was measured at 50 µs when all PSII reaction centers were open (O step) followed by the J step (at 2 ms), I step (at 60 ms) and maximum fluorescence (F_M) when all PSII reaction centers were closed (Strasser *et al.,* 2000). Area is the fluorescence between the OJIP curve and F_M. Mean values are the average of 10 measurements. Samples were compared between ozone-treated and non-treated plants using a *t* test. Value in parentheses is the decrease percentage compared to the control

^{ns} = No significant difference; ** = Significant difference at 1% level.

Table 4 Comparison of specific energy flux per reaction center and the performance index between ozone-treated and non-treated plants.

	Before		20 min after		20 hr after	
	Treatment	Control	Treatment	Control	Treatment	Control
ABS/RC	2.05	1.95	2.27^{ns} (+3%)	2.19	2.25^{ns} (+8%)	2.09
TRo/RC	1.69	1.62	1.60* (-8%)	1.73	1.70^{ns} (-1%)	1.71
ETo/RC	1.16	1.08	0.70** (-32%)	1.02	1.04* (-13%)	1.19
DIo/RC	0.36	0.34	0.67** (+46%)	0.46	0.56** (+48%)	0.38
PI_Abs	5.15	5.11	0.83** (-67%)	2.5	2.23** (-57%)	5.14
PI_Total	5.36	5.12	1.22** (-71%)	4.26	1.94** (-64%)	5.38

Detailed explanations of the symbols are provided in Table 1. Mean values are the average of 10 measurements. Samples were compared between ozone-treated and non-treated plants at each evaluation period using a *t* test comparison. Values in parentheses are the decreased or increased percentage of the treatment relative to the control.

^{ns} = No significant difference; *= Significant at 5% level; ** = Highly significant at 1% level.

major (Reiling and Davison, 1992) and *Triticum aestivum* (Grandjean Grimm and Fuhrer, 1992). Moreover, lowering the efficiency (ψ_0) in ozone-treated plants suggested that the probability of a trapped exciton that moves an electron into the electron transport chain beyond Q_A^- was reduced accordingly.

Moreover, the current results indicated that the specific energy fluxes per reaction center (ET_0/RC and TR_0/RC) were reduced under ozone stress. Reduction of the trapping excitation energy (TR_0/RC) and conversion of the excitation energy to electron transport (ET_0/RC) suggested that the plants cannot trap all the energy that was absorbed and reduce the power to convert excitation energy to electron transport due to the closure of reaction centers. These findings were supported by the data of relative variable fluorescence that were measured at 2 ms (V_j). Reductions of variable fluorescence up to 38% following ozone exposure highlighted that the number of closed RCs at 2 ms (the proportion of the total number of RCs that can be closed) was higher under ozone stress. Moreover, ozone also impacted the net rate of PSII closure (M_0) as indicated by the increase in (M_0) in the ozone-treated plants, suggesting that ozone increased the net rate of PSII closure. Since performance indices depend on the three functional steps of photosynthetic—activity by a PSII RC complex (light energy absorption, trapping of excitation energy, and conversion of this energy to electron transport occurring in PSII) (Strasser *et al.,* 2000)—alteration of these processes during the ozone stress significantly lowered the PIs ($PI_{\_Abs}$ and $PI_{\_Total}$) in both evaluation periods (20 min and 20 hr after the treatment) as shown in Table 4. A greater increase in the excitation energy dissipation per active reaction centre (DI_0/RC) revealed that RCs were inactivated under ozone stress because the RCs dissipated most of the energy that was absorbed as heat and fluorescence reaction. A similar finding was observed by Nussbaum *et al.* (2001) in which a large increase in energy dissipation per active reaction center (DI_0/RC) was observed in herbaceous species under ozone stress. In the above parameters studied, photosynthetic activity and fluorescence induction measured at 20 min after were much stronger than at 20 hr later, suggesting a recovery process of photosynthetic systems 20 hr later.

CONCLUSION

Ozone affects the shape of the OJIP fluorescence transient, being more pronounced at the P level (F_M) which was significantly reduced under ozone stress. The maximum quantum yield of primary photochemistry (F_V/F_M) was reduced by ozone in both evaluation periods (20 min and 20 hr after). The reduction of specific energy fluxes per reaction center (ET_0/RC and TR_0/RC) under ozone stress showed that the plants cannot trap all the energy that was absorbed and reduced the power to convert excitation energy to electron transport and thus the performance indices ($PI_{\_Abs}$ and $PI_{\_Total}$) were depressed. Decreased variable fluorescence of up to 38% following ozone exposure highlighted that the number of closed RCs at 2 ms was greater under ozone stress. A greater increase in the excitation energy dissipation per active reaction centre (DI_0/RC) revealed that the RCs were deactivated under ozone stress. It can be concluded that ozone altered the fluorescence induction and impaired photosynthetic systems in tomato plants. The OJIP test is a good indicator to detect fluorescence induction and photosynthetic activity of the PSII RC complex of ozone-treated tomato plants.

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