

Optimum Dark Adaptation Period for Evaluating the Maximum Quantum Efficiency of Photosystem II in Ozone-Exposed Rice Leaves

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ABSTRACT

Because the transient O₃ injury of leaves is lost with time, the evaluation of O₃ effect on the maximum quantum efficiency of PSII (F_v/F_m) is difficult. Thus, the authors examined F_v/F_m in rice leaves exposed to different O₃ concentrations (0, 0.1, and 0.3 cm³·m⁻³, expressed as O⁰, O^{0.1}, and O^{0.3}) under different dark adaptation periods (0, 1, 5, 10, 20, and 30 min, expressed as D⁰, D¹, D⁵, D¹⁰, D²⁰, and D³⁰) to ascertain its optimum time span. F_v/F_m was inhibited by O₃; however in the O⁰ and O^{0.1} plants, it recovered during dark adaptation. In the O^{0.3} plants, F_v/F_m decreased gradually with time. F_0 was found to be increased by O₃, and it increased further in the O^{0.3} plants during dark adaptation. Under a high light intensity, F_m was decreased by O₃, and the O₃-induced damage to F_v/F_m was therefore more pronounced. However, the sensitivity of F_m was lower than that of F_0 . Consequently, the damage to PSII was mainly attributed to the inhibition of electron transport from Q_A to Q_B. The F_v/F_m ratio in the O⁰ plants was fully recovered at D¹⁰, and in the O^{0.1} plants, F_v/F_m increased from D¹⁰ to D²⁰. The effects of O₃ on the xanthophyll cycle-dependent quenching (fast relaxation phase) of q₁ disappeared when the dark adaptation period was greater than 20 min. However, it was difficult to distinguish the effects of O₃ and other factors (e.g., light) before D⁵. The current results demonstrate that the optimum dark adaptation period in rice leaves is 10 min because the effect of O₃ remains maximal, while the effects of other factors on F_v/F_m disappear during this period. By accurate measurement of F_v/F_m , the physiology of O₃ effect on PSII in rice leaves is precisely evaluated.

Keywords: Dark-Adapted State; *Oryza sativa*; Ozone Stress; Photosystem II; Quantum Efficiency

1. Introduction

In the Kanto region of Japan, where rice is cultivated as a staple summer crop, 10 - 20 warnings regarding high photochemical oxidants ($\cong 0.12$ cm³·m⁻³) are received every growing season, and the hourly peak values of these substances are sometimes close to 0.2 cm³·m⁻³ [1]. Overall, ozone (O₃) accounts for 90% or more of the total photochemical oxidants [2,3]. When exposed to O₃, rice plants suffer damage caused by the inhibition of net photosynthetic rate (P_N) and photosystem II (PSII) [4,5] as well as decreases in the contents of ribulose 1,5-bisphosphate carboxylase/oxygenase [6], the contents of chlorophyll and carotenoids [7], and nitrite reductase activity [8], in addition to visible leaf-related symptoms [4] and

breakdown of the cellular ultrastructure [9]. Moreover, O₃ suppresses growth [10], alters photoassimilate partitioning [11], and decreases grain yield [10,12,13]. Detrimental effects of O₃ have also been reported in many other crops and trees [14,15].

Because PSII of plants is deteriorated immediately after O₃ exposure [5], the monitoring of PSII is useful as a tool to evaluate O₃ damage and recovery from it. Recently, the examination of chlorophyll fluorescence measurements has become established practice for the diagnosis of changes in PSII caused by environmental stresses such as excessive light and water stress. Many studies assessing the effects of environmental stress on PSII have been conducted [16,17]. By analyzing chlorophyll fluorescence measurements, Kobayakawa and Imai [5] showed that the maximum (F_v/F_m) and operating (F_q'/F_m') quan-

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tum efficiencies of PSII photochemistry were decreased by acute (5-h) O₃ exposure. In addition, PSII in rice leaves is adversely affected by chronic O₃ exposure [7]. Among the indicators obtained through performing chlorophyll fluorescence measurements, F_v/F_m is used most frequently. F_v/F_m is determined from the easiest and simplest type of chlorophyll fluorescence measurement. However, before such measurements can be obtained, knowledge regarding the dark adaptation period is required. When a leaf is kept in the dark, the primary quinone acceptor of PSII (Q_A) becomes maximally oxidized. The PSII reaction centers can then perform photochemical reduction of Q_A , and the minimal fluorescence in the dark-adapted state (F_0) can be determined. Thereafter, when a leaf is exposed to a short actinic pulse of high PPFD (typically of less than 1 s at several thousand $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$), Q_A reaches a maximally reduced state, and the maximal fluorescence in the dark-adapted state (F_m) can be determined. Baker [18] described variable fluorescence (F_v) as the difference between F_0 and F_m values. Generally, the dark adaptation period for the leaves subjected to this treatment is 30 min. However, Sonoike [19] observed that if the dark adaptation period is prolonged, the effects of short-term stress on PSII will disappear. In fact, many researchers [20-29] have measured the effect of O₃ on F_v/F_m based on qualification of chlorophyll fluorescence under various dark adaptation periods, the shortest of which was 5 min [27], and the longest was 60 min [23,28].

This study was conducted to establish the optimum dark adaptation period for measurement of the PSII F_v/F_m in rice leaves under O₃ stress or stress free conditions to clarify the inhibition and recovery of photosynthesis by O₃.

2. Materials and Methods

2.1. Plant Materials and Gas Exposure

Two independent experiments were conducted in April and July 2012 and were designated Exp. 1 (April) and Exp. 2 (July). Japonica rice (*Oryza sativa* L. cv. Koshihikari) seeds were sown directly in Wagner pots (1/5000a) filled with 2.2 kg of dry soil and 12.5 g of compound fertilizer (N, P₂O₅, K₂O = 8, 8, 8%). The seeds were grown in natural light, gas exposure chambers (width × depth × height = 2 m × 2 m × 1.9 m, S-2003A; Koito Industries, Ltd., Yokohama, Japan) at 28/23°C (12-h day/12-h night) under 70% RH and 400 $\text{cm}^3\cdot\text{m}^{-3}$ CO₂. Immediately after full expansion of the eighth leaves (Haun index = 8.0) [30], the plants were exposed to 0, 0.1, or 0.3 $\text{cm}^3\cdot\text{m}^{-3}$ O₃ (expressed as O⁰, O^{0.1}, and O^{0.3}, respectively) for 5 h during the day (8:00 - 13:00 h local

time). Ozone was supplied using a high voltage ozone generator with dry air (ED-OG-R6; Ecodesign Inc., Ogawa, Saitama, Japan). CO₂ was supplied from cylinders containing liquid CO₂. These gases were injected into air that had been charcoal-filtered. The O₃ and CO₂ concentrations were measured and computer controlled using an ultraviolet absorption-type O₃ analyzer (EG-2001F; Ebara Jitsugyo, Tokyo, Japan) and an infrared CO₂ analyzer (ZRH, Fuji Electric Systems, Tokyo, Japan), respectively.

2.2. Chlorophyll Fluorescence Measurements

A fluorometer (LI-6400-40; Li-Cor Inc., Lincoln, NE, USA) attached to a portable photosynthesis and transpiration measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA) and a portable fluorometer (MINI-PAM; Heinz Walz GmbH, Effeltrich, Germany) were used to measure the chlorophyll fluorescence of the eighth leaves from 0.1 - 1.1 h after O₃ exposure for each of five replicate plants. Chlorophyll fluorescence parameters were determined in these plants by applying 0.2 and 7000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of measuring light and a saturating pulse (0.8 s). Prior to the fluorescence measurements, the leaves were kept in the dark for 0, 1, 5, 10, 20, or 30 min (expressed as D⁰, D¹, D⁵, D¹⁰, D²⁰, and D³⁰, respectively). Subsequently, F_0 and F_m were determined by irradiating the measuring light and saturating pulses, respectively. In addition to F_v/F_m , $(1/F_0) - (1/F_m)$ was calculated as an indicator of PSII photoinactivation [18,19,31]. The dark adaptation treatments and fluorescence measurements were conducted at 28°C. In Exp. 2, chlorophyll fluorescence was measured using only a portable fluorometer (MINI-PAM; Heinz Walz GmbH, Effeltrich, Germany).

2.3. Statistical Analysis

All chlorophyll fluorescence-related data were subjected to a two-way analysis of variance (ANOVA). The data were further subjected to a multiple comparison by Tukey's test to clarify the effects of O₃ concentrations with elapsing dark adaptation period. Statistical analyses were performed using Excel Statistics 2010 for Windows software package (Social Survey Research Information Co. Ltd., Tokyo, Japan). Differences among treated samples were considered statistically significant at $P \leq 0.05$ or $P \leq 0.01$ compared with non-treated plant group at each time interval. Appropriate standard errors of the means (SE) were calculated, and the results are presented as line graphs.

3. Results

In Exp. 1, we were concerned about the different re-

sponses of the two applied fluorometers. The F_0 , F_m and F_v/F_m values measured using one fluorometer (MINI-PAM) were slightly lower than those measured using the other fluorometer (LI-6400-40); however, the trends were similar (**Figure 1**). Thus, we employed MINI-PAM fluorometer in the replicate experiment (Exp. 2) because it was easier to carry than the LI-6400-40 fluorometer. The F_v/F_m ratio was lowest at D^0 in all treatments, and F_v/F_m was found to be decreased in an O_3 -concentration-dependent manner. In the O^0 plants, F_v/F_m increased from D^0 to D^{10} and was then nearly constant from D^{10} to D^{30} . The F_v/F_m ratio in the $O^{0.1}$ plants increased from D^0 to D^{10} , as in the O^0 plants, but then increased further after

D^{10} . In the $O^{0.3}$ plants, F_v/F_m increased from D^0 to D^5 and then tended to decrease with the time in darkness (**Figures 1(a) and (b)**). F_0 , a component of F_v/F_m , decreased slightly with the period of darkness in the O^0 plants. In contrast, in the $O^{0.3}$ plants, F_0 increased with the period of darkness: the F_0 measured at D^{30} had increased to 135% (MINI-PAM) - 154% (LI-6400-40) of its value at D^0 (**Figures 1(c) and (d)**). F_m , a component of F_v/F_m , was decreased by $O^{0.3}$ though it substantially increased with the period of darkness (**Figures 1(e) and (f)**). $(1/F_0) - (1/F_m)$, which is an indicator of PSII inactivation, increased in the O^0 plants, was unchanged in the $O^{0.1}$ plants, and decreased in the $O^{0.3}$ plants with the period of dark-

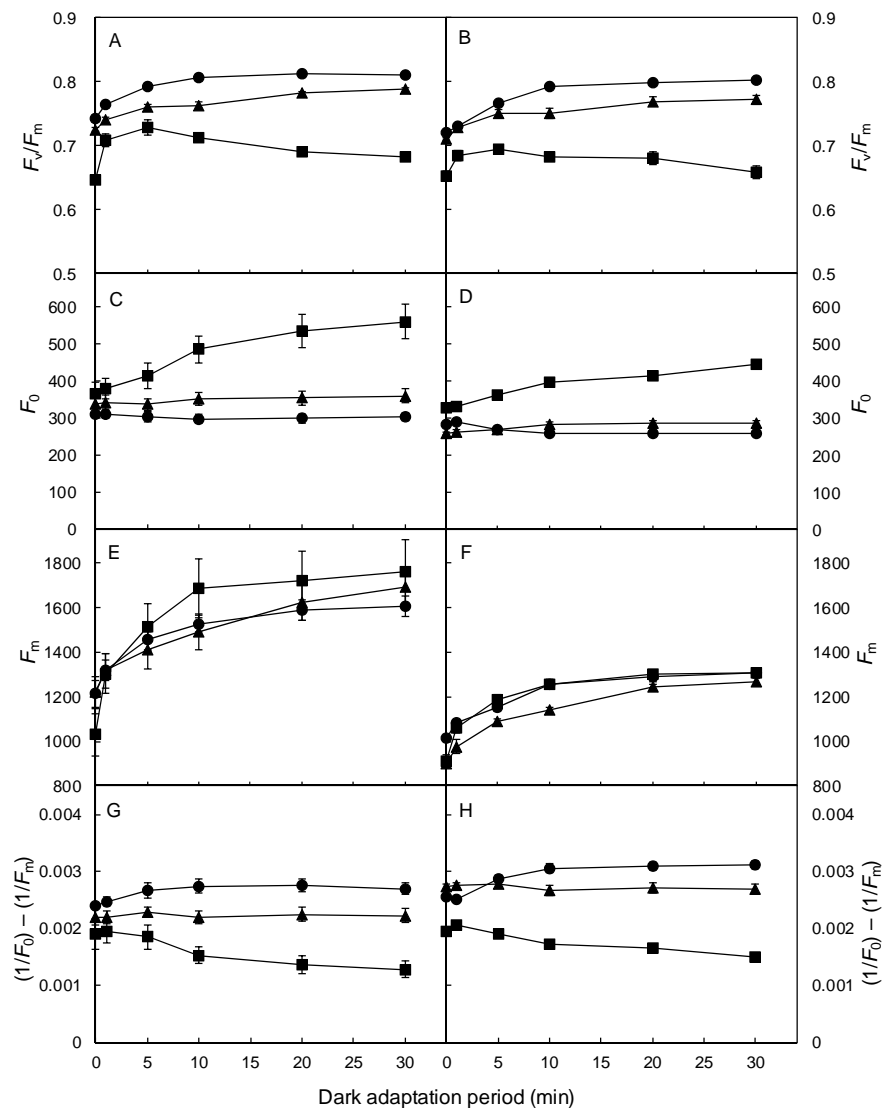


Figure 1. Effects of O_3 on the maximum quantum efficiency of PSII (F_v/F_m), minimum fluorescence (F_0), maximum fluorescence (F_m), and $(1/F_0) - (1/F_m)$ under different dark adaptation periods in rice leaves (Exp. 1). The fluorescence parameters for 1A, 1C, 1E, and 1G were obtained using LI-6400-40 fluorometer, whereas those for 1B, 1D, 1F, and 1H were obtained using MINI-PAM fluorometer. The vertical bars represent the standard errors of the mean ($n = 5$). ●, ▲, ■: 0, 0.1, and 0.3 $\text{cm}^{-3} \cdot \text{m}^{-3} \cdot O_3$.

Table 1. Statistical analyses of the effects of O₃ and dark adaptation period on chlorophyll fluorescence parameters in rice leaves. **p* < 0.05, *p* < 0.01, ****p* < 0.001. n.s., not significant by two-way ANOVA. *F*₀, minimal fluorescence in the dark-adapted state; *F*_m, maximal fluorescence in the dark adapted state; *F_v/F_m*, maximal quantum efficiency of PSII.**

Experiment	Factor	<i>F_v/F_m</i>	<i>F</i> ₀	<i>F</i> _m	(1/ <i>F</i> ₀) – (1/ <i>F</i> _m)
Exp. 1 (LI-6400XT)	O ₃	***	***	n.s.	***
	dark adaptation period	***	*	***	n.s.
	O ₃ × dark adaptation period	***	**	n.s.	n.s.
Exp. 1 (MINI-PAM)	O ₃	***	***	***	***
	dark adaptation period	***	***	***	n.s.
	O ₃ × dark adaptation period	***	***	n.s.	***
Exp. 2 (MINI-PAM)	O ₃	***	***	***	***
	dark adaptation period	***	**	***	***
	O ₃ × dark adaptation period	*	***	n.s.	***

Table 2. Statistical analyses of the chlorophyll fluorescence parameters in rice leaves between control plants (O⁰) and O₃-treated plants (O^{0.1} or O^{0.3}) under different dark adaptation periods. **p* < 0.05, *p* < 0.01, ****p* < 0.001. n.s., not significant by Turkey's test. *F*₀, minimal fluorescence in the dark-adapted state; *F*_m, maximal fluorescence in the dark adapted state; *F_v/F_m*, maximal quantum efficiency of PSII.**

Experiment	Parameter	O ₃ (cm ³ ·m ⁻³)	Dark adaptation period (min)					
			0	1	5	10	20	30
Exp. 1 (LI-6400XT)	<i>F_v/F_m</i>	O ^{0.1}	n.s.	n.s.	n.s.	***	**	*
		O ^{0.3}	***	**	***	***	***	***
	<i>F</i> ₀	O ^{0.1}	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		O ^{0.3}	n.s.	n.s.	*	**	***	***
	<i>F</i> _m	O ^{0.1}	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		O ^{0.3}	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
(1/ <i>F</i> ₀) – (1/ <i>F</i> _m)	O ^{0.1}	n.s.	n.s.	n.s.	*	n.s.	n.s.	
	O ^{0.3}	n.s.	n.s.	*	***	***	***	
Exp. 1 (MINI-PAM)	<i>F_v/F_m</i>	O ^{0.1}	n.s.	n.s.	n.s.	**	*	*
		O ^{0.3}	***	***	***	***	***	***
	<i>F</i> ₀	O ^{0.1}	**	**	n.s.	n.s.	n.s.	n.s.
		O ^{0.3}	***	***	***	***	***	***
	<i>F</i> _m	O ^{0.1}	*	*	*	**	n.s.	n.s.
		O ^{0.3}	*	n.s.	n.s.	n.s.	n.s.	n.s.
(1/ <i>F</i> ₀) – (1/ <i>F</i> _m)	O ^{0.1}	**	**	n.s.	**	*	**	
	O ^{0.3}	***	***	***	***	***	***	
Exp. 2 (MINI-PAM)	<i>F_v/F_m</i>	O ^{0.1}	n.s.	n.s.	*	***	**	**
		O ^{0.3}	***	**	***	***	***	***
	<i>F</i> ₀	O ^{0.1}	n.s.	n.s.	**	**	**	*
		O ^{0.3}	**	*	*	***	***	***
	<i>F</i> _m	O ^{0.1}	n.s.	n.s.	*	***	**	**
		O ^{0.3}	***	***	***	***	***	***
(1/ <i>F</i> ₀) – (1/ <i>F</i> _m)	O ^{0.1}	n.s.	n.s.	**	***	***	***	
	O ^{0.3}	n.s.	n.s.	***	***	***	***	

ness (**Figures 1(g)** and **(h)**). Statistical analyses (**Tables 1** and **2**) examining the O_3 inhibition of F_v/F_m , F_0 , F_m , and $(1/F_0) - (1/F_m)$ support the description provided above regarding the fluorometers (LI-6400-40 and MINI-PAM), with special reference to the dark adaptation period.

In Exp. 2, F_v/F_m was found to be decreased by O_3 treatment, while F_0 was increased (**Figures 2(a)** and **(b)**), as observed in Exp. 1. However, the trend found for F_m differed from that observed in Exp. 1. The value of F_m recovered dramatically with the period of darkness; however, because the decrease that occurred at D^0 was more pronounced than in Exp. 1, this recovery was insufficient (**Figure 2(c)**). $(1/F_0) - (1/F_m)$ was decreased by O_3 treatment, as in Exp. 1, and this value decreased gradually with the period of darkness in the $O^{0.3}$ plants (**Figure 2(d)**).

Statistical analyses (**Tables 1** and **2**) of the results obtained using a single fluorometer (MINI-PAM) supported the existence of differential responses to early dark adaptation periods in the two experiments. In Exp. 2, F_0 , F_m , and $(1/F_0) - (1/F_m)$ showed lower significant levels than those of Exp. 1 when compared to each of the control plants (O^0) at early dark adaptation periods.

4. Discussion

Consistent with the results of a previous study by our group [5], F_v/F_m was found to be adversely affected by O_3 in the present study. The F_v/F_m ratios in all plants

were lowest at D^0 . In the O^0 and $O^{0.1}$ plants, F_v/F_m recovered with the period of darkness, however, because F_v/F_m decreased gradually with the period of darkness in the $O^{0.3}$ plants, we inferred that the inhibition of PSII by O_3 was exacerbated (**Figures 1(a)**, **(b)** and **2(a)**). F_v/F_m decreases when F_0 increases and/or F_m decreases (*i.e.*, $F_v = F_m - F_0$) [18,19]. In the present study, the F_0 values recorded in the $O^{0.1}$ and $O^{0.3}$ plants were generally higher than in the O^0 plants at all measurement times, and the values increased gradually with the progression of dark adaptation (**Figures 1(c)**, **(d)** and **2(b)**). Previous studies in snap bean [20,28] and *Betula pendula* [24] support the current results that F_0 was increased by O_3 exposure. The increase in F_0 and decrease in F_m are induced by the inhibition of electron transport from Q_A to Q_B and the oxygen-evolving system from the manganese cluster to the tyrosine residue of the D1 protein, respectively [19]. Therefore, it appeared that the damage that occurred to PSII in the $O^{0.3}$ plants was induced mainly by the inhibition of electron transport from Q_A to Q_B . In addition, as $(1/F_0) - (1/F_m)$ was found to be decreased in the $O^{0.3}$ plants with the period of darkness (**Figures 1(g)**, **(h)** and **2(d)**), PSII was gradually inactivated even during dark adaptation. However, F_m was observed to be decreased by O_3 treatment at any measurement time in Exp. 2. The F_v/F_m obtained at D^0 in Exp. 2 was lower than in Exp. 1. As the O_3 -induced decrease in F_m observed in Exp. 2 (**Figure 2(c)**) was more pronounced than that in Exp. 1 (**Figures 1(e)** and **(f)**), it is possible that the detrimental

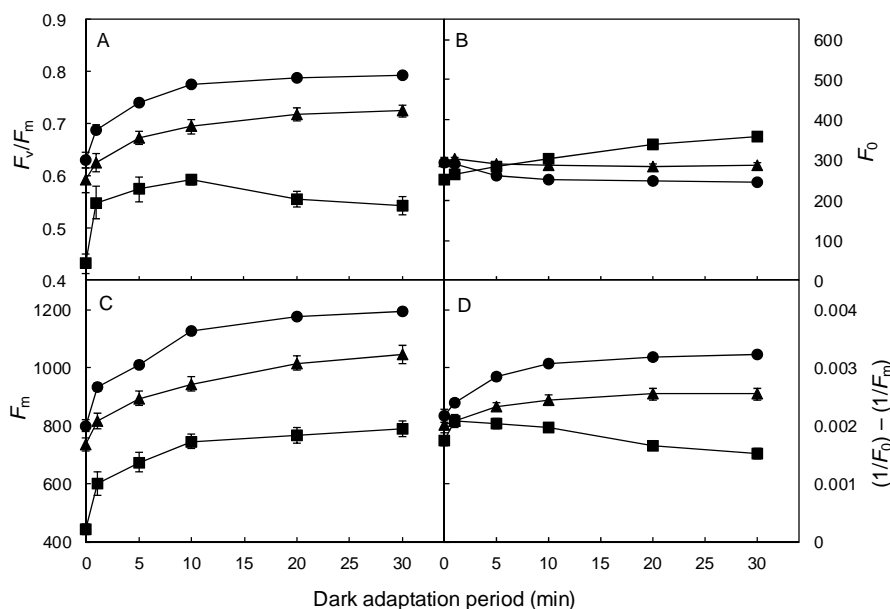


Figure 2. Effects of O_3 on the maximum quantum efficiency of PSII (F_v/F_m), minimum fluorescence (F_0), maximum fluorescence (F_m), and $(1/F_0) - (1/F_m)$ under different dark adaptation periods in rice leaves (Exp. 2). The fluorescence parameters were obtained using MINI-PAM fluorometer. Vertical bars represent the standard errors of the mean ($n = 5$). ●, ▲, ■: 0, 0.1, and 0.3 $\text{cm}^{-3} \cdot \text{m}^{-3} \cdot \text{O}_3$.

effect of O₃ was more pronounced in Exp. 2 due to the higher light intensities involved. In fact, the PPFD values recorded at 12:00 h in the natural light growth chamber were approximately 900 and 1100 μmol·m⁻²·s⁻¹ in Exp. 1 and Exp. 2, respectively. Guidi *et al.* [23] also observed that the inhibition of P_N and PSII activity by O₃ was greater under high light intensities (30 - 1000 μmol·m⁻²·s⁻¹ PPFD). Similarly, Kobayakawa and Imai [32] reported that the intercepted radiation is a major determining factor in O₃ inhibition. While the decrease in F_v/F_m was induced by an increase in F₀ in the present study, in previous studies, a decrease in F_v/F_m was found to be induced by a decrease in F_m in O₃-exposed lettuce [25] and tobacco [26]. Guidi *et al.* [22] measured the effect of O₃ on chlorophyll fluorescence in 14 bean cultivars and reported that the cause of the inhibition of F_v/F_m (e.g., increased F₀ and/or decreased F_m) depended on the cultivar. Interestingly, the F_v/F_m was found to be decreased by both an increase in F₀ and a decrease in F_m in chronically O₃-exposed rice leaves [7]. Therefore, the cause of the decrease in F_v/F_m depends on the species, cultivar, and environmental conditions involved.

F_v/F_m is determined without irradiating actinic light. Therefore, the obtained values can only be decreased by the non-photochemical quenching coefficient (q_N). Three major components of q_N have been identified in plant leaves *in vivo*: the energy (ΔpH)-dependent quenching coefficient (q_E), photoinhibitory quenching coefficient (q_I), and state-transition quenching coefficient (q_T). The relaxation kinetics of these components differ: the half time (t_{1/2}) is 1 min for q_E, 5 - 10 min for q_T, and >30 min for q_I [16,17]. Because q_T is suppressed under strong light, it should not be regarded as a photoprotective mechanism. In this study, the leaves of the plants were exposed to O₃ during sunny daytime hours. Therefore, F_v/F_m was decreased mainly by q_E and q_I. q_I includes the xanthophyll cycle-dependent component and inactivation of the D1 protein component. The relaxation time of these two components differs: the t_{1/2} of the former is shorter than 30 min, whereas that of the latter is longer than 1 h [16,17]. In all plants, F_v/F_m recovered dramatically from D⁰ to D¹ (**Figures 1(a), (b) and 2(a)**). This recovery was likely induced by the relaxation of q_E. Consequently, in addition to the O^{0.1} and O^{0.3} plants, a decrease in F_v/F_m caused by q_E also occurred in the O⁰ (control) plants. Furthermore, as the degree of recovery observed in the O^{0.3} plants from D⁰ to D¹ was higher than in the O⁰ and O^{0.1} plants, q_N was increased by a higher O₃ concentration (O^{0.3}). Additionally, in the O⁰ plants, F_v/F_m did not change from D¹⁰ onward (**Figures 1(a), (b) and 2(a)**). Consequently, it appeared that q_I disappeared in the O⁰ plants during a 10 min period of dark adaptation. Thus, because F_v/F_m decreased in the O⁰ plants prior to D⁵, it is

difficult to distinguish the effects of O₃ and other factors (e.g., light) prior to this time point. As F_v/F_m recovered in the O^{0.1} plants (increased) from D¹⁰ to D²⁰, the xanthophyll cycle-dependent quenching (fast relaxation phase) of q_I would also have been increased during that time. Consequently, if the dark adaptation period is greater than 20 min, the effects of O₃ on the fast relaxation phase of q_I will disappear. However, when only the effects on D1 protein inactivation are to be evaluated, leaves must be maintained for more than 20 min in the dark. In the O^{0.3} plants, F_v/F_m decreased as the dark adaptation period progressed from D⁵. Therefore, if the dark adaptation period is too long, F_v/F_m might differ from the value obtained immediately after O₃ exposure.

The results of these experiments imply that the optimum dark adaptation period for evaluating F_v/F_m of PSII in O₃-exposed rice leaves is 10 min because the effect of O₃ is maximal at this time, and the effects of other factors on F_v/F_m disappear.

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