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Assessment of the Antioxidative Function of Electrolyzed-Reduced Water in Relation to Photoinhibition of Photosynthesis

Keiko IWABUCHI[1], Kenji KURATA and Yasuomi IBARAKI*

Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan
** Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[1] *Present address : Department of Biology and Environment Sciences, National Agricultural Research Center for Tohoku Region, Morioka 020-0198, Japan*

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Electrolyzed-reduced water (reduced water) is produced during electrolysis of KCl solution near cathodes, and exhibits high pH, and extremely negative oxidation-reduction potential. Shirahata et al. (Biochem. Biophys. Res. Commun. **234** : 269-274, 1997) reported that reduced water could scavenge reactive oxygen species. The objective of this research was to determine whether application of reduced water could suppress photooxidative damage of photosynthetic apparatus induced under low temperature and high-intensity of photosynthetic photon flux density (PPFD) conditions. Hydroponically-grown plants of spinach and sweet potato were treated with reduced water, and exposed to low temperature and high light intensity conditions, and the chlorophyll fluorescence parameters, ϕ_{PSII} and F_v/F_m were measured. The photoinhibitory treatment caused decreases in ϕ_{PSII} for both spinach and sweet potato even when the plants were irrigated, sprayed or showered with reduced water. In addition, ϕ_{PSII} was measured for mesophyll cells of sweet potato suspended in reduced water. After the exposure to high-intensity of PPFD (12 000 μmol m^{-2} s^{-1}), the ϕ_{PSII} of the suspended cells in reduced water was nearly zero when pH of the suspensions was not adjusted. In the experimental conditions, the application of reduced water did not suppress photooxidative damage of photosynthesis induced by low temperatures and high light intensity.

Keywords : active oxygen species, antioxidative function, chlorophyll fluorescence, electrolyzed-reduced water, photoinhibition

INTRODUCTION

Electrolysis of low-concentration solutions of salts such as KCl and NaCl produces electrolyzed-reduced water (reduced water) and electrolyzed-oxidized (oxidized water) water near the cathode and the anode respectively. Oxidized water has been gradually recognized as having properties such as low pH and high free chlorine concentration which contribute to bactericidal activity, and its application has been evaluated in several fields such as the disinfection of medical instruments (Iwasawa and Nakamura, 1996), and crop disease control (Fujiwara et al., 1998). Reduced water, which exhibits high pH and extremely negative oxidation-reduction potential (ORP), is occasionally utilized for irrigation, however, the physiological effect of reduced water on plant growth is still unknown.

Corresponding author : Keiko Iwabuchi, fax : +81-19-641-7794, e-mail : iwabuchi@affrc.go.jp

Recently, it was demonstrated that reduced water could scavenge reactive oxygen species including $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and •OH, and could protect single strand DNA from oxidative damage in vitro (Shirahata et al., 1997). This result implies that reduced water may protect biological macromolecules from oxidative damage in living cells. Reactive oxygen species or free radicals are generated concurrently with metabolic processes in living cells, and have a highly destructive reactivity with biological molecules. In green plants, light-dependent depression of photosynthetic activity is often observed under conditions where plants are stressed. This phenomena, called photoinhibition, is attributed to photooxidative damage of photosynthetic reaction centers and several Calvin cycle enzymes caused by reactive oxygen species produced in chloroplasts (Asada 1994; Foyer et al., 1994). If reactive oxygen species generated in plant cells could be scavenged by reduced water then the photosynthetic apparatus might be protected from photooxidative damage.

In a previous study, it was shown that reduced water irrigation did not enhance the growth of potted lettuce seedlings grown under typical photoinhibitory conditions, mild drought, low temperature and high light intensity (Iwabuchi et al., 2000). Since the ORP property of reduced water was very variable, the antioxidative function might remain only for short term and could not enhance growth. Therefore, direct assessment of leaf photoinhibition may reveal whether the antioxidative function of reduced water affects to photoinhibition of photosynthesis.

Photoinhibition is often observed under conditions of plant stress such as chilling, high temperature and drought. Under stress conditions, the utilization of photon energy is limited due to inhibited carbon metabolism, resulting in excessive production of reactive oxygen that induces destructive oxidization of the photosynthetic apparatus. In particular, light-dependent, chill-induced decreases in photosynthesis have been attributed to photoinhibition of photosystem II (PSII) activity (Ortiz-Lopez et al., 1990). For many years, chlorophyll fluorescence has been used as a probe to estimate the efficiency of electron transport through PSII. The ratio of the level of variable to maximal fluorescence (F_v/F_m) of dark-adapted leaves estimates the maximum quantum efficiency of PSII photochemistry and has been used as an index of photoinhibition induced by environmental stress (Genty et al., 1989). The quantum efficiency of PSII photochemistry (ϕ_{PSII}) of leaves operating at steady-state photosynthesis at a selected photosynthetic photon flux density (PPFD) also provides a useful probe of the photosynthetic performance of leaves during chill-induced photoinhibition (Andrews et al., 1995).

The objective of this study is to examine the effect of reduced water on light-dependent, chill-induced photoinhibition of photosynthesis. Chlorophyll fluorescence parameters were determined for spinach and sweet potato leaves which were treated with reduced water and exposed to low temperature and high light intensity. Similarly, sweet potato mesophyll cells suspended in reduced water were also tested.

MATERIALS AND METHODS

Plant material. One-month-old plants of spinach (*Spinacia oleracea* L., cv. Try) and sweet potato (*Ipomoea batatas* Lam., cv. Beniazuma) with eight to ten unfolded leaves were used for the experiments. Seeds of spinach were sown on polyurethane cubes with distilled water, and 7 days after the germination, the seedlings were transplanted into containers filled with standard Enshi nutrient solution (Hori, 1966). Micropropagated plantlets of sweet potato were acclimatized and cuttings including two or three unfolded leaves were hydroponically grown as well. Both plants were grown under 200 ± 30 $\mu mol\ m^{-2}\ s^{-1}$ of PPFD provided by white fluorescent lamps in a controlled-environment room. Air temperature was set at 23°C during the 14-h photoperiod and 18°C during the 10-h dark period, and relative humidity was 50±10%.

Electrolyzed-reduced water. In this study, experiments using attached leaves and cell suspensions were carried out. In the experiment using attached leaves, deionized water (DW) was made by a water purification system (type: ERN-W, EYELA Co. Ltd., Japan), which is equipped with a carbon filter cartridge and ion exchange cartridges. In the cell suspension experiment, another water purification system (Aquarius GS-20R, Advantec Toyobo Kaisha Ltd.) equipped with water distillation and ion exchange devices was used to obtain DW. Electrical conductivity (EC) of obtained DW was less than 10 $\mu S\ cm^{-1}$ in both systems. KCl was added at a rate of 1 $g\ L^{-1}$ to the DW so that the EC was high enough for electrolysis, then the KCl solution was electrolyzed at a maximum voltage of 15 V for 15 min by using a water electrolysis device equipped with a platinum-coated titanium electrode (Super Oxseed Lab, JED-020, Aoi Engineering Co. Ltd., Japan). The treated water in the cathode side bath was used as reduced water.

Reduced water exhibits high EC, very low ORP and high pH (Fig. 1). However, the low value of ORP is not stable and will increase gradually, whereas the pH value is relatively stable. The change in the ORP value suggested that the antioxidative function of the reduced water also might be unstable, therefore the reduced water was used within 1 h of electrolysis.

Chlorophyll fluorescence measurement. Photochemical inactivation of PSII was assayed by measuring of chlorophyll fluorescence parameters using a pulse modulated portable chlorophyll fluorometer (MINI-PAM, Walz Co. Ltd., Germany). The frequency of modulated light was 0.6 kHz. The measurement system provided saturating pulses (0.8 s) of white light (12 000 $\mu mol\ m^{-2}\ s^{-1}$ on the leaf) for measurements and automatically calculated ϕ_{PSII} and F_v/F_m. Measurements of ϕ_{PSII} were made for attached leaves and leaf cell suspensions exposed to photoinhibitory conditions. F_v/F_m, which estimates photoinhibition of photosynthesis, was also determined in attached leaf experiments. For determination of F_v/F_m, whole plants were kept in a dark room for 15 min prior to the measurements.

Experiment I: photoinhibition of attached leaves. On the evening prior to an experiment, two or three plants randomly selected were transplanted into a container (24×14×9 cm) filled with 2 L of new nutrient solution and kept under the standard growing conditions until the next morning when measurement started. First, chlorophyll fluorescence parameters ϕ_{PSII} or F_v/F_m were measured under the standard growing conditions as an initial reference, and then various types of treated water without nutrients were applied. The types of treated water

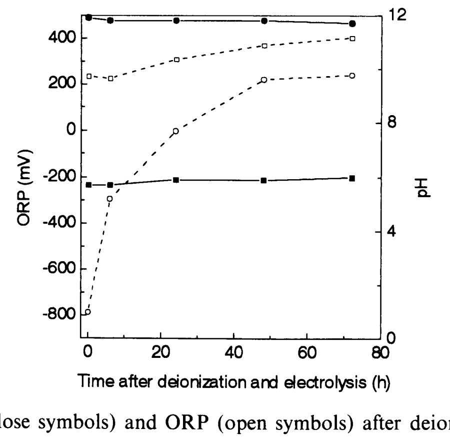

Fig. 1 Changes in pH (close symbols) and ORP (open symbols) after deionization and electrolysis of deionized water (squares) and electrolyzed-reduced water (circles), respectively.

tested in this experiment were reduced water, DW, KCl solution (1 g L^{-1}) without electrolysis, and 0.88 g L^{-1} KOH solution whose pH was the same as reduced water. Table 1 shows the properties of the treated water.

The following three methods to apply the treated water were tested : (1) irrigate (exchanging the nutrient solution with the treated water), (2) spray (for spinach) or (3) shower (for sweet potato) directly onto the leaves. After applying treated water, the plants were exposed to photoinhibitory conditions, 13°C air temperature and 600 μmol m^{-2} s^{-1} PPFD for 30 to 40 min. The measurements of ϕ_{PSII} were conducted before and after the photoinhibitory treatments. Also, during the photoinhibitory conditions, ϕ_{PSII} for leaves illuminated with the provided light intensity, or F_v/F_m for dark-adapted leaves were measured in every 15 or 30 min. In the irrigation test, the plants receiving treated water were kept under standard growing conditions for 30 min before exposing them to photoinhibitory conditions to allow them to uptake the newly exchanged water. Four leaves were randomly selected from plants in each treatment, and chlorophyll fluorescence parameters were measured at five different points on each leaf.

Experiment II : photoinhibition of cell suspensions. In Experiment II, sweet potato leaf mesophyll cells were suspended in reduced water directly so that reduced water could rapidly and easily permeate into each cell. Fully expanded leaves (approximately 7–8 cm in width, 8 FW g) were cut into small pieces and digested in 12 mL of 5% driserase (Kyowa Hakko Inc.) solution with 0.45 M of mannitol for 6.5 h. The cell suspension obtained was rinsed with Bicine Buffer (0.08 g L^{-1}, pH 8.0, Dojindo Laboratories, Japan) containing 0.45 M mannitol three times using centrifugation (1 500 rpm). The leaf mesophyll cells were resuspended in 1/4 strength MS media using the treated water with 0.4 M mannitol and 1.0 mM Bicine Buffer.

In this experiment, the effect of pH adjustment was also tested. Table 2 shows the description and properties of the treated water used in this experiment. The cell population density of the cell suspension was adjusted to 1.9 FW g mL^{-1}, and 0.25 mL of the cell suspension was put into a glass bottomed dish (14 mm in diameter, 0.08–0.12 mm in bottom thickness). White light illumination of 12 000 μmol m^{-2} s^{-1} PPFD was provided for 10 s from the

Table 1 The properties of the treated water in Exp. I.

Treatment	EC (μS cm^{-1})	pH	ORP (mV)
DW	9	5.5	220
RW	2 000	11.6	−810
KCl	1 200	5.5	220
High-pH	1 500	11.6	30

DW, deionized water ; RW, electrolyzed-reduced water ; KCl, KCl solution ; High-pH, high pH solution (KOH solution).

Table 2 The description and the properties of the treated water in Exp. II.

Treatment	Electrolysis[y]	KCl content (g L^{-1})	pH[z]	ORP (mV)
DW	—	0	8.0	80
RW-low-pH	+	1.0	8.0	−660
RW-high-pH	+	1.0	10.5	−840
KCl-low-pH	—	1.0	8.0	140
KCl-high-pH	—	1.0	10.5	160

[z] pH was adjusted by HCl and KOH except for RW-high-pH.
[y] Treated solution was electrolyzed (+) ; not electrolyzed (−).

bottom of the glass dish using a cool white glass fiber. The chlorophyll fluorescence parameter, ϕ_{PSII} was measured before and after the illumination at 60-s intervals. Chlorophyll fluorescence measurement was performed for eight samples before the illumination and four samples after illumination for each treated cell suspension. The fluorescence sensor was positioned 5 mm from the dish bottom. Air temperature and PPFD during the measurements were 23.0°C and 8.0 $\mu mol\ m^{-2}\ s^{-1}$, respectively.

RESULTS AND DISCUSSION

Experiment I

Figure 2 shows the changes in quantum efficiency of PSII measured at PPFD of 180 $\mu mol\ m^{-2}\ s^{-1}$ under the standard growing conditions, and at 600 $\mu mol\ m^{-2}\ s^{-1}$ under photoinhibitory conditions, for attached leaves of spinach and sweet potato irrigated with treated water. Irrigation with treated water did not affect ϕ_{PSII} values for neither spinach nor sweet potato, and the values remained constant during the initial 30 min under standard growing conditions. When the plants were exposed to photoinhibitory conditions (low temperature and high light intensity), ϕ_{PSII} decreased in all measured leaves regardless of the type of irrigation water. The decline in ϕ_{PSII} was particularly remarkable in sweet potato. After exposure to the photoinhibitory conditions, the plants were transferred back and kept under

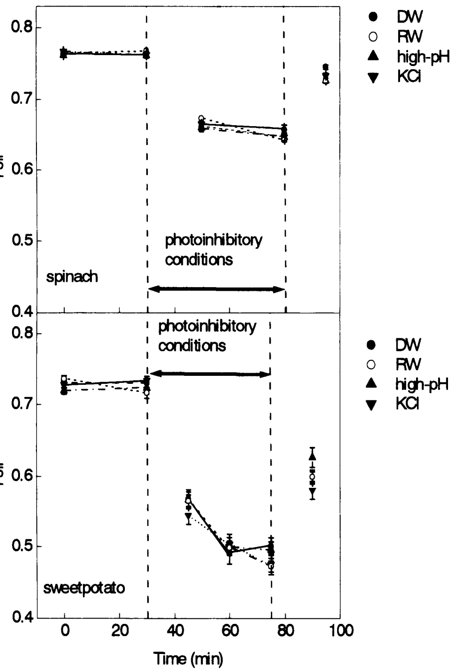

Fig. 2 Changes in ϕ_{PSII} of illuminated leaves of spinach and sweet potato irrigated with various types of treated water.

PPFD during the measurements was 180 $\mu mol\ m^{-2}\ s^{-1}$, or 600 $\mu mol\ m^{-2}\ s^{-1}$ under the photoinhibitory conditions. See Table 1 for treatment codes. Bars show standard errors.

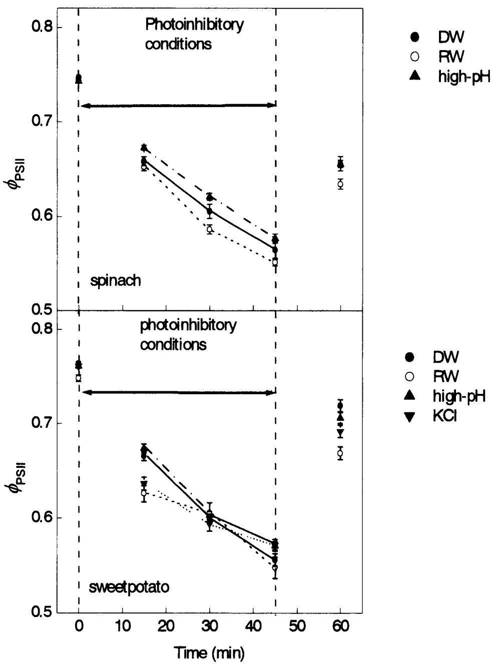

Fig. 3 Changes in ϕ_{PSII} of illuminated leaves of spinach, sprayed and sweet potato, showered with various types of treated water.

PPFD during the measurements was 180 $\mu mol\ m^{-2}\ s^{-1}$, or 600 $\mu mol\ m^{-2}\ s^{-1}$ under the photoinhibitory conditions. See Table 1 for treatment codes. Bars show standard errors.

the growing conditions for approximately 20 min. For spinach and sweet potato, ϕ_{PSII} measured after the photoinhibitory treatment was lower than the initial values, while there was no significant difference among leaves irrigated with the various types of water. The decline in ϕ_{PSII} for spinach was less than that for sweet potato. It could be reasonable because spinach is adapted to cool climate (Boese and Huner, 1992), while sweet potato is adapted to warm climate. Andrews et al. (1995) demonstrated that ϕ_{PSII} could be a useful probe of stress-induced changes in photosynthetic performance of leaves operating at steady-state photosynthesis at a selected light intensity. These results indicate that reduced water irrigation did not improve photosynthetic performance for leaves of two species with different tolerance to cool climate.

In the irrigation experiments, reduced water did not seem to protect plants from photooxidative damage. However, it is possible that the antioxidative function of the reduced water dissipated through the transpiration stream from root to leaf mesophyll cells. Therefore, we attempted to directly apply the treated water to leaves by spray (for spinach) or shower (for sweet potato). However, there was no significant difference in ϕ_{PSII} values among the leaves directly applied with the various types of water during exposure to photoinhibitory conditions (Fig. 3). In addition, the maximum quantum yield of PSII photochemistry, F_v/F_m, which can be used to estimate the degree of photoinhibition, was determined for spinach leaves sprayed with the various types of water (Fig. 4). Low temperature and high light intensity caused a decline in F_v/F_m for all leaves sprayed with the various types of water, indicating that photoinhibition was induced regardless of the type of sprayed water.

Declines in both F_v/F_m and ϕ_{PSII} of leaves were induced by low temperature and high light intensity conditions across all applied water treatments. These results indicated that applying reduced water to roots or leaves does not suppress photoinhibition of photosynthesis. This was consistent with the previous study showing that reduced water irrigation did not enhance plant growth grown under conditions of plant stress (Iwabuchi et al., 2000).

In this study, a high pH solution and a KCl solution were also compared with reduced water, however, no significant difference was observed in chlorophyll fluorescence parameters. In a previous study on the protective function for rice blast disease of electrolyzed water, it was reported that once the pathogen penetrated the host it could not be suppressed, indicating that the sprayed water hardly infiltrated into plant cells (Tamaki et al., 2001). Thus, even if the antioxidative function of reduced water could perform against photoinhibition, there is the

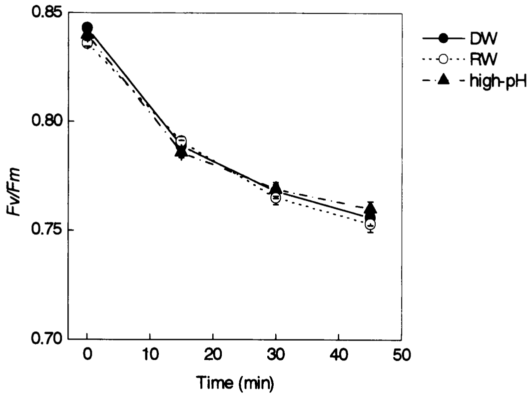

Fig. 4 Changes in F_v/F_m of dark-adapted leaves of spinach sprayed with various types of treated water during the photoinhibitory treatment.

See Table 1 for treatment codes.

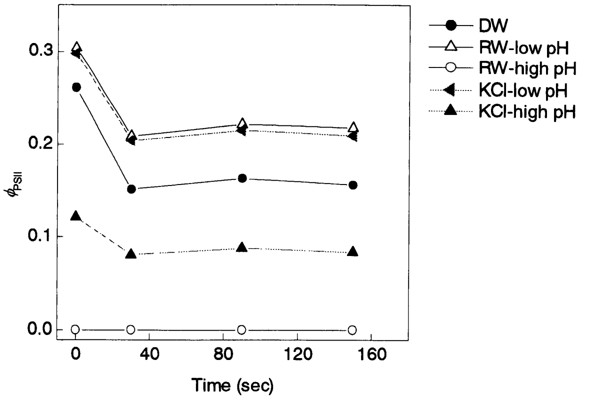

Fig. 5 Changes in ϕ_{PSII} of mesophyll cells suspended in various types of water shown in Table 2. Prior to the measurements, cell suspension were exposed to 12 000 μmol m^{-2} s^{-1} PPFD for 10 s. The PPFD during the measurements was 8 μmol m^{-2} s^{-1}.

practical problem of identifying an appropriate application method that arrows the reduced water to penetrate into the photosynthetic apparatus while retaining its antioxidative function.

Experiment II

Figure 5 shows the changes in ϕ_{PSII} values of mesophyll cells which were suspended in various types of treated water and exposed to the high light intensity. In all treatments, ϕ_{PSII} was dramatically reduced even before exposure to the high intensity light, however the values in RW-low-pH and KCl-low-pH were significantly higher than those in DW, KCl-high-pH, and RW-high-pH. After light exposure, the values in all treatments decreased especially during the first 30 s, except for the RW-high-pH treatment whose ϕ_{PSII} value was less than 0.01 over the experiments. The values of ϕ_{PSII} were seemed to remain constant in all treatments after 30 s. In RW-low-pH and KCl-low-pH, ϕ_{PSII} remained higher than in DW and KCl-high-pH over the measurements, and there was no significant difference in ϕ_{PSII} between RW-low-pH and KCl-low-pH. Obviously, the digesting of leaves to single cells decreased ϕ_{PSII}, however, additional KCl and an adjustment of pH to moderate level lead to moderate ϕ_{PSII} values regardless of water electrolysis, whereas high pH caused a significant decrease in ϕ_{PSII}. In addition, the depressed ϕ_{PSII} values in RW-high-pH suggests that reduced water without pH adjustment reduces the photosynthetic performance of plants considerably.

CONCLUSION

The application of reduced water to plants did not protect leaves or mesophyll cells from photooxidative damage in the experimental conditions. Furthermore, for cell suspension experiments, reduced water without pH adjustment appeared to depress photosynthetic performance.

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〈和文抄録〉

光合成の光阻害に対する電解陰極水の活性酸素消去能の評価

岩渕桂子[1]・蔵田憲次・荊木康臣*

東京大学大学院農学生命科学研究科・* 山口大学農学部
[1] 現在：東北農業研究センター

近年，機能水の一つである電解陰極水に，活性酸素除去能力があるという実験結果が発表された．そこで本研究では，活性酸素が介在して生じる光合成の光阻害が，電解陰極水によって抑制されうるかどうかを評価することを目的に実験を行った．水耕栽培したホウレンソウおよびサツマイモ苗に電解陰極水を与え，光阻害が発生しやすい強光および低温条件下に一定時間おいた後，光阻害の発生の指標となるクロロフィル蛍光のパラメータ，ϕ_{PSII} および F_v/F_mを測定した．電解陰極水を灌水，葉面へのスプレーまたはシャワーにより与えたホウレンソウとサツマイモの ϕ_{PSII} は，いずれもストレス条件下で低下した．また，電解陰極水を用いたサツマイモの遊離細胞けん濁液を強光に暴露したときの ϕ_{PSII} も同様に低下した．これらの結果から，電解陰極水は低温・強光ストレスによる光阻害の抑制の効果はないことが示唆された．