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Effects of Light Condition after Simulated Acid Snow Stress on Leaves of Winter Wheat

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Winter plants regrow after freeze-thawing in acidic meltwater from the acid-snow layer in early winter or early spring. In this study, the responses of cold-acclimated wheat seedlings to different light conditions during the regrowth period after simulated acid snow (SAS) stress were investigated. After freeze-thawing in sulfuric acid (SAS stress) of pH 2.0, dry weight and the maximal quantum yield of photosystem II (PSII) decreased more in mature leaves than in young leaves. In a subsequent regrowth period under light condition, dry weight, relative water content, and the maximal quantum yield of PSII were severely affected in mature leaves but were only slightly affected in SAS (pH 2.0)-stressed young leaves. The levels of membrane lipid peroxidation and hydrogen peroxide in mature leaves of SAS (pH 2.0)-stressed seedlings were significantly higher than those in young leaves during the regrowth period under light condition. The superoxide dismutase activity in young leaves was higher than that in mature leaves during the regrowth period. These results indicate that mature leaves of seedlings during the snow melt season are more sensitive than young leaves to photooxidative stress because of their low acid snow stress tolerance and low capacity for the detoxification of superoxide.

1. Introduction

Acid precipitation is a global environmental problem that adversely affects ecosystems.[1–3] In snowfall areas, the acidification of snow (acid snow) has been observed over the last decade.[4,5] Therefore, acid snow may become a winter stress. It has been verified under field conditions that the state of acid pollutants in snow is different from that in rain.[6] In the recrystallization process of granular snow crystals in a snow layer, acid pollutants may be locally concentrated, particularly on the surface and in inner parts of granular snow.[6–8] At the early and late stages of the snowmelt season, acid pollutants on the surfaces of snow crystals are dissolved in meltwater at relatively high concentrations. Concentrated acid pollutants in the meltwater are released from the snow cover into soils, streams and lakes, whose pH is temporally lowered significantly (i.e., acid shock).[4–9] Therefore, the shoots of wintering plants under an acid-snow cover may be exposed to concentrated acid pollutants or may be freeze-thawed in acidic meltwater in early spring or early winter in cold regions.

It has been shown that plant cells become dehydrated and deformed by the growth of extracellular ice when the leaves of herbaceous plants are subjected to a subzero temperature.[10,11] Severe freezing stress induces irreversible structural changes in the cellular membrane, resulting from the interaction by the close approach of endo-

membranes because of the freezing-induced deformation of cells.[11–13] In addition, chronic freezing leads to the concentration of intracellular and/or extracellular toxic solutes because of the extracellular freezing of cells, resulting in the injury of cellular membranes and the denaturation of intracellular macromolecules.[14] Also, several experimental studies have shown that herbaceous plants are more sensitive than woody plants to an acidic conditions.[1] However, the degree of damage may depend on experimental and physiological conditions such as the pH of simulated acid rain (SAR), the composition of the acid solution of SAR and the treatment procedure.[1,15–19] Contact with acid solution causes pH-dependent anatomical changes in the leaves of beans[20] and tomatoes.[17] The inhibition of photosynthetic activity and a change in the activity of antioxidant enzymes in cucumbers[16,19] and beans[15,21] were induced by treatment with SAR with a pH below 3.0. In addition, it has been reported that reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical and superoxide radical are associated with the injury caused by freezing stress[22,23] and SAR stress,[15,16,24] as well as other environmental stimuli.[25] There are several antioxidant enzymes involved in the scavenging of ROS in plants. It has been reported that the activities of some of them, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), are increased by oxidative stress.[25] SOD plays a key role, since the enzyme converts superoxide to hydrogen peroxide and oxygen. Hydrogen peroxide is then removed by the actions of APX and CAT. The activity of these antioxidative enzymes has been shown to differ depending on plant tissue[26] and leaf age.[27,28] Previous studies have shown that the CAT activity in the leaves of cold-acclimated plants, including winter wheat, is significantly lower than that in crown tissues.[26] When a senescent leaf was subjected to a high light condition, the activities of antioxidative enzymes such as SOD and CAT and the gene expression levels of some antioxidative enzymes decreased in comparison with those in a young leaf.[27,28] ROS that are endogenously overproduced by environmental stresses attack membrane lipids, proteins, nucleic acids and other biologically important molecules.[25] For example, it was reported that membrane lipid peroxidation induced by attacks by ROS led to membrane damage.[18,19,24,25]

We previously carried out an *in vitro* study on simulated acid snow (SAS) stress to determine the direct effects of freeze-thawing in acidic meltwater on leaf segments of winter wheat (*Triticum aestivum* L.).[29] We found that the survival rates of leaf segments significantly decreased after freeze-thawing in sulfuric acid solution (pH 2.0) used as the SAS stress. Also, the survival rates of leaf segments significantly decreased after chronic SAS stress at pH 2.0 for four weeks and slightly decreased after chronic SAS stress at pH 4.0 or 3.0 for four weeks. These findings suggest that freeze-thawing in acidic meltwater is a potential environmental stress factor for wintering plants during winter. Thus, we hypothesized that wintering plants are further damaged by photooxidative stress during the regrowth period after the melting of acid snow. Our preliminary experiment showed that SAS-stressed seedlings were further damaged during the regrowth period under a given light condition.[30] Therefore, we investigated the physiological responses of winter wheat leaves to different light conditions during the regrowth period after SAS stress. Since our preliminary data showed different responses to SAS stress for mature and young leaves , the differences in the physiological responses of mature and young leaves of wheat seedlings to different light conditions during the regrowth period after SAS stress were investigated.

2. Materials and Methods

2.1 *Plant materials*

Seeds of winter wheat (*T. aestivum* L. cv Chihokukomugi) were germinated for two days on wet paper at 18°C in the dark. The seedlings were planted in cultivating

soil and grown at 18°C (12-h light/12-h dark) for one week in a growth chamber under a photosynthetic photon flux density (PPFD) of approximately 120 μmol m^{-2} s^{-1} at the soil surface of the pot. The light source was a daylight-type white fluorescent tube. One-week-old seedlings were cold-acclimated at 4°C/2°C (12-h light/12-h dark) for four weeks in a growth chamber under a PPFD of approximately 200 μmol m^{-2} s^{-1} at the top of the seedlings.

2.2 *SAS stress and regrowth conditions*

After the cultivating soil had been carefully removed from the roots by washing with pure water and the water had been blotted with a paper towel, three seedlings were put in polyethylene bags. Five milliliters of pure water of pH 5.6 (control) or sulfuric acid solution of pH 4.0, 3.0 or 2.0 was added to each seedling bag as a SAS treatment (cf. 3 mM sulfuric acid solution shows pH 2.0). The samples were set in a modified programmable freezer (ES-100P, Tajiri Co., Ltd., Sapporo, Japan) precooled at –1°C, and then equilibrated at –1°C for 30 min. After seeding ice, the seedlings were equilibrated at –1°C for 1 h and then cooled at a rate of 2.4°C h^{-1} to –4°C. When the temperature had reached –4°C, the seedlings were kept at –4°C for seven days. Then the seedlings were warmed to 10°C at a rate of 5°C h^{-1} and withdrawn from the polyethylene bags. The seedlings were gently washed with pure water to remove the acid solution and blotted with a paper towel. Then, the seedlings were cultivated hydroponically using a 2,000-fold dilution of Hyponex (Hyponex Japan, Osaka, Japan) at 10°C for 48 h after SAS stress under two light conditions, continuous light (PPFD of approximately 60 μmol m^{-2} s^{-1} at the top of the seedlings: 48-h light) and dark/light (24-h dark prior to 24-h light) conditions. Cultivating pots were covered with aluminum foil to prevent underground tissues from being exposed to light. Plant materials harvested for biochemical analysis were immediately frozen in liquid nitrogen and stored at –80°C until use.

2.3 *Measurement of physiological parameters in cold-acclimated wheat seedlings before SAS treatment*

The freezing tolerance of mature (first leaf) and young (second leaf) leaves was estimated as described previously.[29] Freezing tolerance was represented as the temperature that induces 50% lethality (LT_{50}) in the population.

Osmotic concentration was determined by the following procedure. After leaf segments (1.0±0.02 g fresh weight (FW)) in a vial tube were boiled for 20 min and cooled to room temperature, they were homogenized using a glass stick. The homogenates were centrifuged at 15,000 *g* for 10 min at 4°C and the supernatants were recovered. The osmotic concentration of the supernatant was determined using a vapor pressure osmometer (Type 5520, Wescor, USA).

The content of total soluble sugars was determined by the method of Yemm and Willis.[31] Leaf segments (100±2 mg FW) in a vial tube were crushed in liquid nitrogen and homogenized in 1 ml of 0.2 N perchloric acid and 1% polyvinylpolypyrrolidone (PVPP) on ice. After incubation for 1 h on ice, the sample was centrifuged at 14,000 *g* for 5 min at 4°C, and the supernatant was neutralized to pH 7.0 with potassium bicarbonate and then centrifuged again at 14,000 *g* for 5 min at 4°C. A 0.02-ml aliquot of the supernatant was added to 0.23 ml of pure water and 0.75 ml of precooled 0.2% (w/v) anthrone dissolved in 98% concentrated sulfuric acid solution. After incubation in boiling water for 7 min, the absorbance of the reaction mixture at 625 nm was measured. The content of total soluble sugars was calculated using a calibration curve prepared with glucose as the standard.

2.4 *Measurements of physiological parameters in SAS-stressed wheat seedlings*

To measure chlorophyll absorption spectra, leaf segments (50 mg FW) in a vial tube were crushed in liquid nitrogen and homogenized with sodium carbonate (50 mg) to prevent the degradation of chlorophyll in the procedure of leaf extraction by the method of Shan.[32] The chlorophyll fraction was extracted from the leaves using 80% acetone and the soluble fraction was recovered after centrifugation at 14,000 *g* for 5 min. The absorption spectra of the extract were measured from 500 to 700 nm with a spectrophotometer (Ultrospec 3300 pro, GE Healthcare, UK).

Relative water content (RWC) was determined by the method of Sgherri *et al*.[33] After measuring the FW of an excised leaf, the leaf was put in water for 4 h at room temperature in the dark and then the turgid weight (TW) of the leaf was measured. Then dry weight (DW) was measured after drying the leaf at 120°C for 24 h. RWC was determined using the following formula: RWC (%) = [(FW–DW)/(TW–DW)]×100.

The maximal quantum yield of photosystem II (PSII; Fv/Fm) was measured at room temperature in the dark with a portable fluorometer (MINI-PAM, Walz, Germany). After adaptation in the dark at 10°C for 20 min, Fv/Fm was measured.

To determine the content of thiobarbituric acid-reactive substances (TBARSs), which are typical indicators of oxidative damage, and the content of H_2O_2, which is one of the ROS, leaf segments (50±2 mg FW) in a vial tube were crushed in liquid nitrogen and homogenized in 1.6 ml of 5% (w/v) trichloroacetic acid (TCA) containing 0.1% (w/v) butylhydroxytoluene using a glass stick. The homogenates were filtered through one layer of Miracloth (Calbiochem, USA), and the filtrate was poured into a tube on ice and centrifuged at 12,000 *g* for 20 min twice to remove cell debris. The supernatant was divided into two aliquots for a subsequent determination of TBARS and H_2O_2 contents.

TBARS content was determined by the method of Valenzuela.[34] A 0.2-ml aliquot of the supernatant was added to 0.1 ml of 5% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA; Nacalai Tesque, Kyoto, Japan). After the sample solution had been incubated in boiling water for 30 min, the reaction was stopped by cooling the tubes on ice for 10 min. The absorbance of the solution at 532 nm was measured. TBARS content was calculated using the extinction coefficient 156 mM^{-1} cm^{-1}.

H_2O_2 content was determined by the method of Alexieva *et al*.[35] A 0.2-ml aliquot of the supernatant was added to 0.1 ml of 10 mM potassium phosphate buffer (pH 7.0). The absorbance of the solution at 390 nm was measured for 1 min after adding 0.2 ml of 1 M KI. H_2O_2 content was calculated using a calibration curve with various concentrations of H_2O_2.

To measure the activities of SOD, APX and CAT, which are typical antioxidant enzymes, a crude extract of the leaf was prepared as described below. Leaf segments (50±2 mg FW) in a vial tube were crushed in liquid nitrogen and homogenized in 2 ml of 0.2 M potassium phosphate buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) PVPP and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. The homogenates were filtered through one layer of Miracloth, and the filtrate was poured into a tube on ice and centrifuged at 12,000 *g* for 30 min twice to remove cell debris. The supernatant was used as the crude extract. The crude extract was divided into three aliquots for a subsequent determination of SOD, APX and CAT activities. Protein concentration was determined using a protein assay kit according to the Bradford dye-binding procedure (Bio-Rad Laboratories, USA) and γ-globulin as the standard.[36]

The SOD activity in the crude extract was determined by the method of McCord and Fridovich.[37] A 0.02-ml aliquot of the supernatant was added to 0.68 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 ml of 1 mM xanthine (Sigma-Aldrich) and 0.1 mM ferricytochrome *c* (horse

heart, Sigma-Aldrich). After incubation at 37°C for 5 min, 0.1 ml of a 0.125-unit xanthine oxidase solution (grade I, Sigma-Aldrich) diluted with 1 M ammonium sulfate solution was added to the reaction mixture. The absorbance of the solution at 550 nm was measured for 5 min. In this study, one unit of activity was defined as the amount of enzyme required to inhibit ferricytochrome *c* reduction by 50%.

The APX activity in the crude extract was determined by the method of Nakano and Asada.[38] A 0.05-ml aliquot of the supernatant was added to 0.75 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1 ml of 5 mM ascorbic acid. After incubation at 37°C for 5 min, 0.1 ml of 10 mM H_2O_2 diluted with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA was added to the reaction mixture. The absorbance of the sample solution at 290 nm was measured for 3 min. APX activity was calculated using the extinction coefficient 13.7 mM^{-1} cm^{-1}. The enzyme activity was expressed in μmol of ascorbic acid min^{-1} mg^{-1} DW.

The CAT activity in the crude extract was determined by the method of Dhindsa *et al*.[28] A 0.05-ml aliquot of the supernatant was added to 0.75 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. After incubation at 37°C for 5 min, 0.2 ml of 75 mM H_2O_2 diluted with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA was added to the reaction mixture. The absorbance of the solution at 240 nm was measured for 3 min. CAT activity was calculated using the extinction coefficient 39.8 mM^{-1} cm^{-1}. The enzyme activity was expressed in μmol of H_2O_2 min^{-1} mg^{-1} DW.

In order to determine the effect of SAS stress on specific physiological parameters, the values of SAS-stressed seedlings are expressed in percent (%) compared with those of seedlings before SAS stress (untreated seedlings) in the text. Also, in order to determine the effect of the regrowth condition on the physiological parameters, the values of SAS-stressed seedlings during the regrowth period are expressed in percent compared with those of control seedlings that were freeze-thawed in water in the same regrowth period.

2.5 *Statistical analyses*

Statistical analyses were performed with the SPSS® statistical package. Analysis of variance (ANOVA) was used to test all parameters. When the significance of a treatment was detected, Tukey's HSD test was performed to identify significant differences among the treatments.

3. Results

3.1 *Differences in physiological parameters of mature and young leaves of cold-acclimated wheat seedlings*

Table 1 shows the freezing tolerance (LT_{50}) and parameters of solute accumulation that are involved with freezing tolerance in cold-acclimated mature and young leaves. The LT_{50} values of the cold-acclimated mature and young leaves were about –8.4 and –12.1°C, respectively. The osmotic concentration and the content of total soluble sugars of young leaves were 17 and 30% higher than those of mature leaves, respectively, in cold-acclimated wheat seedlings.

3.2 *Effects of SAS stress on mature and young leaves of cold-acclimated wheat seedlings*

Table 2 shows the effects of SAS stress on the DW and RWC of mature and young leaves. The DWs of mature and young leaves decreased after freeze-thawing in water (pH 5.6, control), compared with those before SAS stress (untreated). The DW of mature leaves further decreased after SAS stress (pH 2.0). The DW of young

leaves was only slightly decreased by acidification induced by sulfuric acid in the process of freeze-thawing. The RWCs of mature and young leaves slightly decreased in the process of SAS stress (pH 2.0).

Figure 1 shows the effects of SAS stress on the absorption spectra of chlorophyll extracts of mature and young leaves. A major peak at 663 nm and small peaks at 616 and 538 nm were detected in the chlorophyll extracts of mature and young leaves after freeze-thawing in water (pH 5.6). The major peak of chlorophyll at 663 nm was slightly shifted to 665 nm, and small peaks at 606, 535 and 507 nm that are distinct from those of chlorophyll were detected in the extracts of mature and young leaves after SAS stress (pH 2.0). Chlorophyll content, on a fresh weight basis, decreased significantly in mature leaves after SAS stress (pH 2.0) (Fig. 1(A)).

Table 1
Freezing tolerance (LT_{50}) values, osmotic concentrations and contents of soluble sugars in leaves of cold-acclimated wheat seedlings.

	LT_{50} (°C)	Osmotic concentration (mol kg^{-1})	Sugar (mmol g^{-1} DW)
Mature	–8.4±1.1	0.75±0.03	0.57±0.02
Young	–12.1±1.8	0.88±0.03	0.74±0.05

Data are means±S.D. from 3–10 different plants.

Table 2
Effects of SAS stress on DW and RWC of wheat leaves.

		DW (mg)	RWC (%)
Mature	Untreated	17.3±2.9	100.8±1.5
	pH 5.6	12.9±4.5	100.0±0.0
	pH 2.0	10.5±0.1	99.3±0.9
Young	Untreated	31.5±3.5	100.4±1.0
	pH 5.6	18.6±2.4	100.4±0.0
	pH 2.0	18.2±2.8	98.6±0.5

Data are means±S.D. from 3–6 different plants.

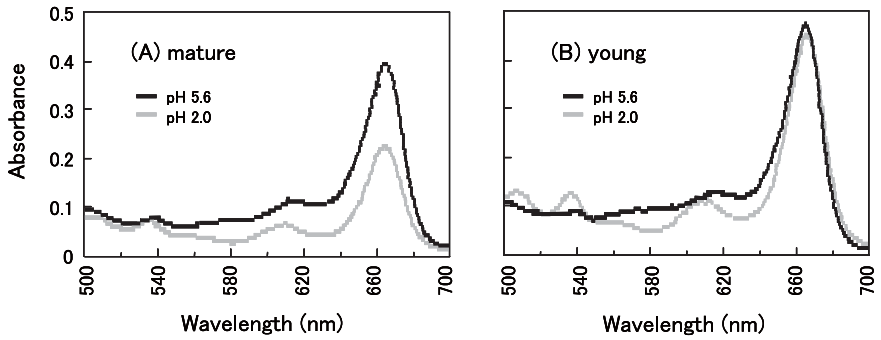

Fig. 1. Absorption spectra of chlorophyll extracts of mature and young leaves of SAS (pH 2.0)-stressed seedlings. Chlorophyll extracts were prepared from mature (A) and young (B) leaves of wheat seedlings that had been subjected to freeze-thawing in pure water of pH 5.6 (black line) or SAS stress using sulfuric acid solutions of pH 2.0 (gray line). The absorption spectra of the chlorophyll extracts were analyzed as described in Materials and Methods.

Table 3 shows the effects of SAS stress on the maximal quantum yield of PSII (Fv/Fm), and the membrane lipid peroxidation (TBARS contents) levels and H_2O_2 contents of mature and young leaves. The Fv/Fm values of the mature and young leaves decreased to 84 and 85% of that of the untreated seedlings, respectively, after freeze-thawing in water (pH 5.6). The Fv/Fm of the mature and young leaves further decreased to 62 and 71% of that of the untreated seedlings, respectively, after SAS stress (pH 2.0). The TBARS contents of the mature and young leaves increased to 457 and 416% of that of the untreated seedlings, respectively, after SAS stress (pH 2.0). The H_2O_2 contents of mature and young leaves increased to 266 and 316% of that of the untreated seedlings, respectively, after SAS stress (pH 2.0).

Table 4 shows the effects of SAS stress on the activities of antioxidant enzymes (SOD, APX and CAT) of the mature and young leaves. The SOD activities of the mature and young leaves increased to 266 and 1,667% of that of the untreated seedlings, respectively, by SAS stress (pH 2.0). The APX activities of mature and young leaves were not significantly changed by SAS stress (pH 2.0). The CAT activity of mature leaves was not significantly changed by SAS stress (pH 2.0), but that of young leaves slightly increased to 234% of that of the untreated seedlings.

These results show that mature and young leaves differentially responded to SAS stress (pH 2.0) and that the mature leaves were more affected by SAS stress (pH 2.0) than the young leaves.

Table 3
Effects of SAS stress on maximal quantum yield of PSII (Fv/Fm), membrane lipid preoxidation (TBARS contents) levels and H_2O_2 content of wheat leaves.

		Fv/Fm	TBARS (μmol g^{-1} DW)	H_2O_2 (μmol g^{-1} DW)
Mature	Untreated	0.79±0.02	0.28±0.03	36.5±6.0
	pH 5.6	0.66±0.06	1.07±0.27	54.7±9.8
	pH 2.0	0.49±0.08	1.28±0.03	97.0±24.4
Young	Untreated	0.80±0.01	0.32±0.03	35.9±1.0
	pH 5.6	0.68±0.03	1.31±0.18	114.6±11.8
	pH 2.0	0.57±0.05	1.33±0.20	113.3±5.1

Data are means±S.D. from 3–9 different plants.

Table 4
Effects of SAS stress on activities of SOD, APX and CAT of wheat leaves.

		SOD (U μg^{-1} DW)	APX (μmol min^{-1} mg^{-1} DW)	CAT (μmol min^{-1} mg^{-1} DW)
Mature	Untreated	0.70±0.18	0.031±0.006	3.71±0.84
	pH 5.6	4.06±0.18	0.041±0.012	4.45±0.34
	pH 2.0	1.86±0.63	0.028±0.014	4.00±0.79
Young	Untreated	0.75±0.32	0.025±0.006	1.51±0.16
	pH 5.6	13.4±2.25	0.065±0.013	5.33±0.19
	pH 2.0	12.5±2.98	0.045±0.005	3.54±0.71

Data are means±S.D. from 3–4 different plants.

3.3 *Changes in DW, RWC, maximal quantum yield of PSII, membrane lipid peroxidation, H_2O_2 contents, and antioxidant enzyme activities of wheat leaves during regrowth period after SAS stress*

Figure 2 shows the effects of light condition during the regrowth period after SAS stress on the DWs of the mature and young leaves. The DWs of SAS (pH 2.0)-stressed mature and young leaves decreased during the regrowth period compared with those of the control leaves that were freeze-thawed in water. The DWs of the control and SAS (pH 4.0 and 3.0)-stressed mature leaves gradually increased during the 48-h regrowth period after SAS stress under the continuous light condition (Fig. 2(A)). However, the DW of the SAS (pH 2.0)-stressed mature leaf slightly decreased. The change in the DW of mature leaves under the dark/light condition after SAS stress (pH 2.0) was similar to that in the case of continuous light, but the magnitudes of the DW changes in the control and SAS (pH 4.0 and 3.0)-stressed mature leaves were relatively small (Fig. 2(B)). In young leaves, even the DW of SAS (pH 2.0)-stressed leaves gradually increased over the 48-h period under both light conditions. However, the magnitude of increase in DW for SAS (pH 2.0)-stressed young leaves was relatively small (Figs. 2(C) and 2(D)).

Figure 3 shows the effects of light condition during the regrowth period after SAS stress on the water statuses of mature and young leaves. The RWC of an SAS (pH 2.0)-stressed mature leaf significantly decreased to 77% at 12 h of regrowth under the continuous light condition (Fig. 3(A)) but only slightly decreased at 12 h of regrowth under the dark condition (Fig. 3(B)). On the other hand, the RWCs of SAS (pH 2.0)-stressed young leaves slightly decreased to 84 and 90% at 12 h of regrowth

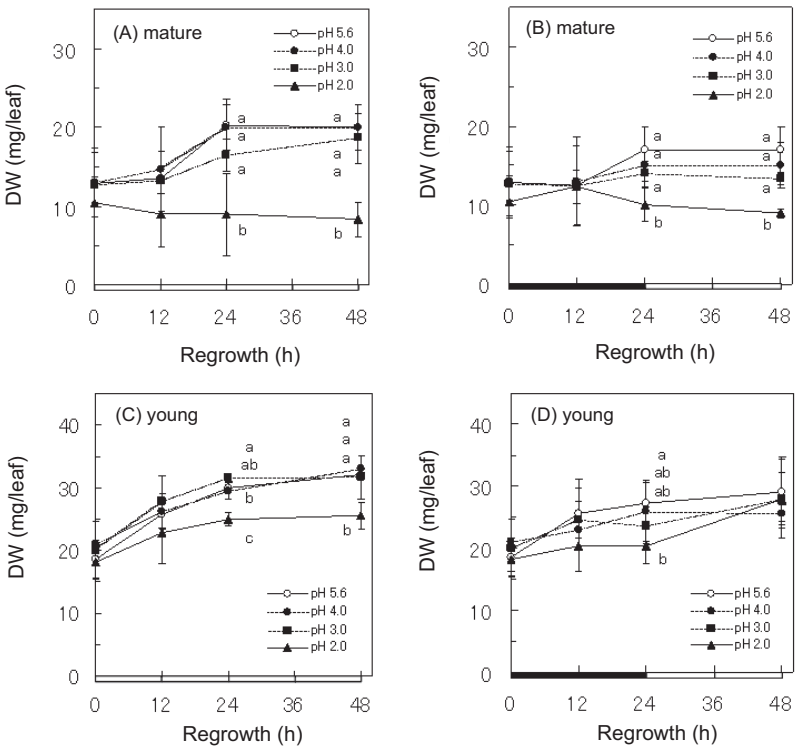

Fig. 2. Changes in DW of wheat leaves during the regrowth period after SAS stress. Wheat seedlings that had been subjected to SAS stress using sulfuric acid solutions of pH 4.0 (closed circles), 3.0 (closed squares) or 2.0 (closed triangles) or pretreated using pure water of pH 5.6 (open circles) were regrown under a continuous light condition for 48 h (A, C) or in a dark condition for 24 h followed by a light condition for 24 h (B, D: dark/light condition). A and B, mature leaves; C and D, young leaves. The closed and open bars on the X-axis represent the dark and light periods of regrowth, respectively. Data are means±S.D. from 3–6 different plants. Within a column, the values with different letters are significantly different at $p < 0.05$.

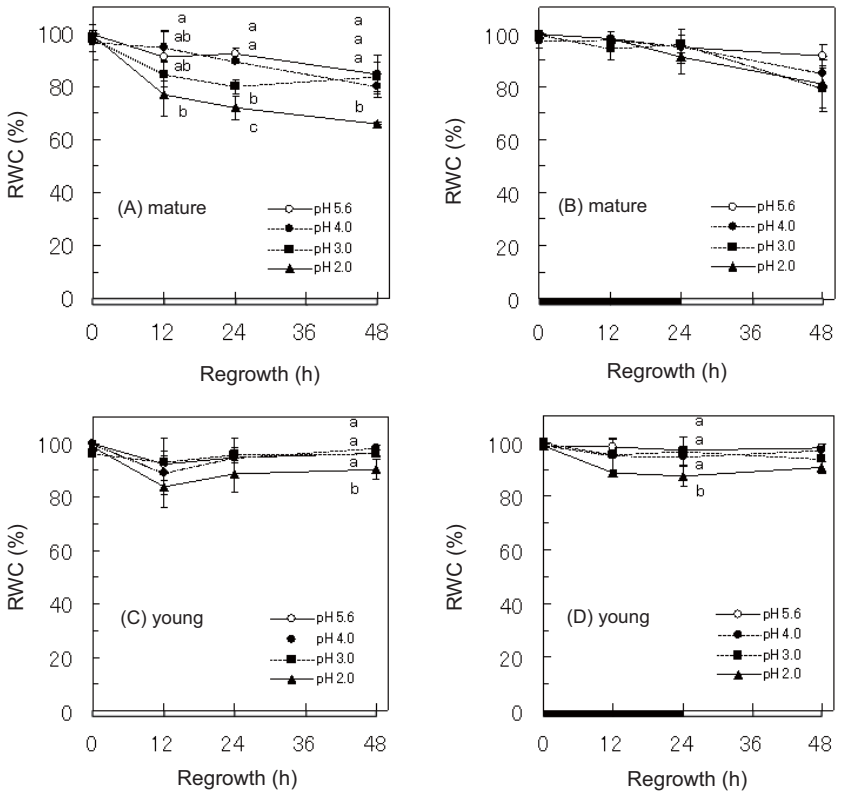

Fig. 3. Changes in RWC of wheat leaves during regrowth period after SAS stress. Experimental conditions of SAS stress and regrowth are the same as those described in the legend to Fig. 2. The closed and open bars on the X-axis represent the dark and light periods of regrowth, respectively. Data are means±S.D. from 3–6 different plants. Within a column, values with different letters are significantly different at $p < 0.05$.

under the continuous light and dark/light conditions, respectively, and then recovered gradually under both light conditions (Figs. 3(C) and 3(D)).

Figure 4 shows the effects of light condition during the regrowth period after SAS stress on the maximal quantum yield of PSII (Fv/Fm) in the mature and young leaves. The Fv/Fm of SAS (pH 2.0)-stressed mature leaves significantly decreased within 12 h of the regrowth period and further decreased over the course of the regrowth period under the continuous light condition (Fig. 4(A)). The Fv/Fm of SAS (pH 2.0)-stressed mature leaves remained constant in the dark period and significantly decreased in the subsequent light period (24–48 h) of the dark/light condition (Fig. 4(B)). On the other hand, the Fv/Fm of SAS (pH 2.0)-stressed young leaves decreased within 12 h of the regrowth period but recovered during the 24-h regrowth period to the initial level at 0 h of regrowth under the continuous light condition (Fig. 4(C)). However, the Fv/Fm of SAS (pH 2.0)-stressed young leaves did not significantly change under the dark/light condition (Fig. 4(D)).

Figure 5 shows the TBARS contents of the mature and young leaves during the regrowth period after SAS stress. The TBARS content of SAS (pH 2.0)-stressed mature leaves markedly increased to 153% of that of the control leaves within 12 h and remained at a relatively high level during the regrowth period under the continuous light condition (Fig. 5(A)). The TBARS content of an SAS (pH 2.0)-stressed mature leaves remained at a relatively low level during the 24-h dark period but significantly increased to 225% of that of the control leaves during the light period (24–48 h) of the dark/light condition (Fig. 5(B)). On the other hand, the TBARS content of an SAS (pH 2.0)-stressed young leaves increased to 134% of that of the control leaves within 12 h of the regrowth period under the continuous light condition (Fig. 5(C)). The TBARS content of an SAS (pH 2.0)-stressed young leaves remained at a relatively low level in the dark period (0–24 h) but increased to 144% of that of the control leaves during the subsequent light period (24–48 h) of the dark/light condition (Fig. 5(D)).

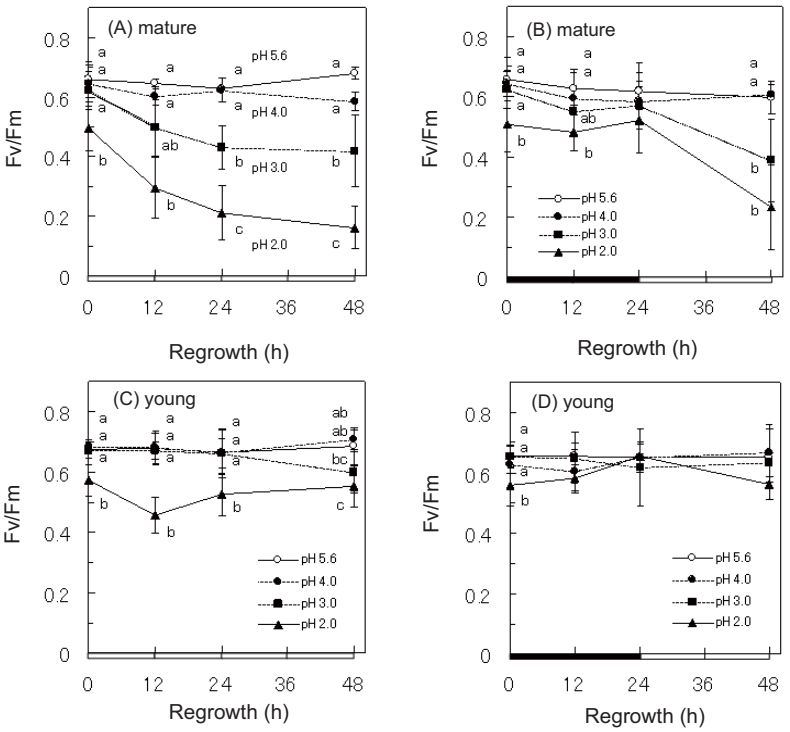

Fig. 4. Changes in maximal quantum yield of PSII of wheat leaves during regrowth period after SAS stress. The maximal quantum yield of PSII is represented as Fv/Fm. The experimental conditions of SAS stress and regrowth are the same as those described in the legend to Fig. 2. The black bar on the X-axis (B and D) represents the dark period of regrowth. Data are means±S.D. from 5–9 different plants. Within a column, values with different letters are significantly different at $p < 0.05$.

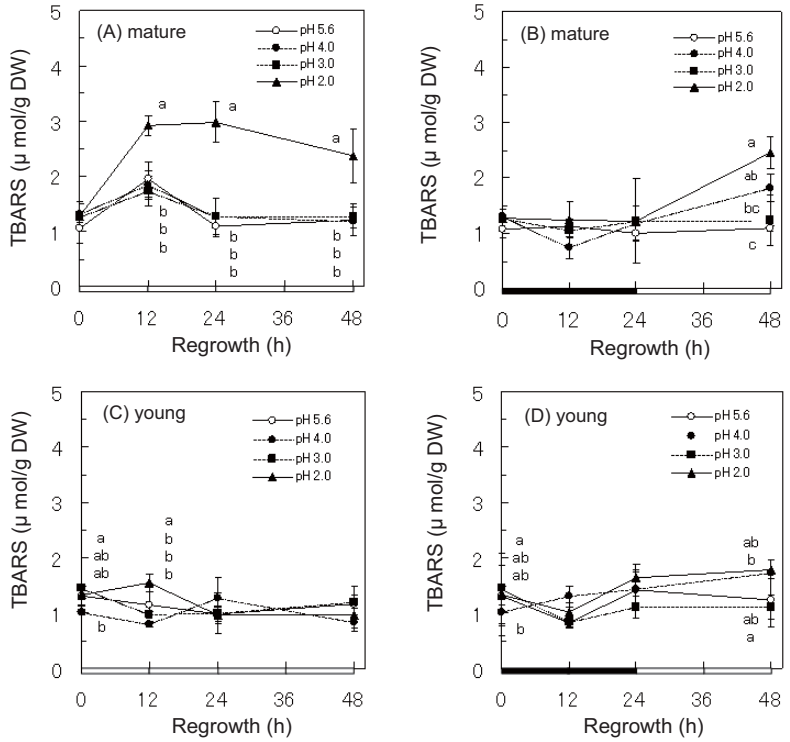

Fig. 5. Membrane lipid peroxidation levels of wheat leaves during regrowth period after SAS stress. The experimental conditions of SAS stress and regrowth are the same as those described in the legend to Fig. 2. The lipid peroxidation level of wheat leaves was measured using the TBA test as described in Materials and Methods. Lipid peroxidation is represented as production of TBARSs. The closed and open bars on the X-axis represent the dark and light periods of regrowth, respectively. Data are means±S.D. from 3–4 different plants. Within a column, values with different letters are significantly different at $p < 0.05$.

Figure 6 shows the H_2O_2 contents of the mature and young leaves during the regrowth period after SAS stress. The H_2O_2 content of the SAS (pH 2.0)-stressed mature leaves increased markedly to 269% of that of the control leaves within 24 h and remained at a high level during the regrowth period under the continuous light condition (Fig. 6(A)). The H_2O_2 content of the SAS (pH 2.0)-stressed mature leaves was low during the 24-h dark period, but it significantly increased to 215% of that of the control leaves during the light period (24–48 h) of the dark/light condition (Fig. 6(B)). On the other hand, the H_2O_2 contents of the control and SAS (pH 2.0)-stressed young leaves did not significantly change compared with that of the mature leaves over the 48-h regrowth period under either the continuous light or dark/light condition.

Figure 7 shows the activities of antioxidant enzymes in the wheat leaves during the regrowth period after SAS stress. The SOD activities of the mature leaves of the control and the SAS (pH 2.0)-stressed seedlings were maintained at relatively low levels over the 48-h regrowth period under both light conditions. On the other hand, the SOD activities of the young leaves of the control and SAS (pH 2.0)-stressed seedlings were maintained at levels about three-times higher than the levels of the mature leaves under both light conditions.

The APX activities of both the mature and young leaves of the control and SAS (pH 2.0)-stressed seedlings were low over the 48-h regrowth period under the continuous light condition (Fig. 7(C)). However, the APX activities of the SAS (pH 2.0)-stressed mature and young leaves within 24 h of the regrowth period under the dark condition were significantly higher than those of the control leaves but decreased during the light period (24–48 h) under the dark/light condition (Fig. 7(D)).

The CAT activity in the young leaves under the continuous light condition (Fig. 7(E)) was similar to that under the dark/light condition (Fig. 7(F)). However, the CAT activity of the SAS (pH 2.0)-stressed mature leaves decreased within 12 h of the regrowth period and then increased within 48 h of the regrowth period under the continuous light condition (Fig. 7(E)). The CAT activity of the SAS (pH 2.0)-stressed mature leaves during the dark period (0–24 h) was higher than that during the light period (24–48 h) of the dark/light condition (Fig. 7(F)).

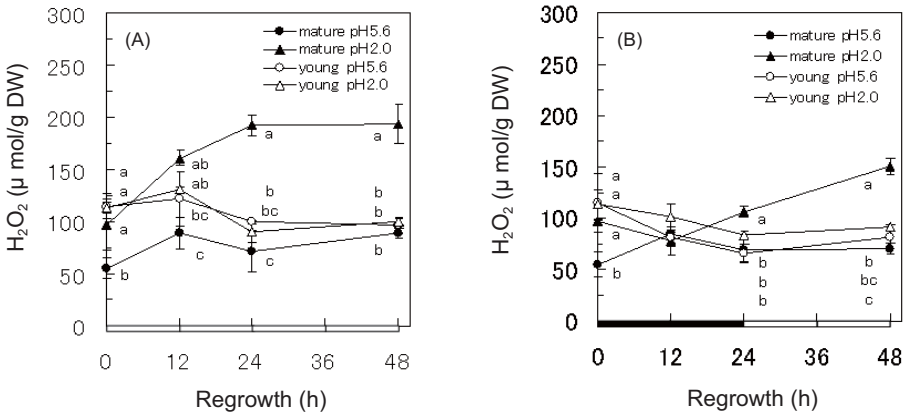

Fig. 6. Hydrogen peroxide content of wheat leaves during regrowth period after SAS stress. Wheat seedlings that had been subjected to SAS stress using sulfuric acid solutions of pH 2.0 (triangles) or freeze-thawed in pure water of pH 5.6 (circles) were regrown in a continuous light (A) or dark/light condition (B). The hydrogen peroxide contents of wheat leaves were spectrophotometrically measured using reaction with potassium iodide as described in Materials and Methods. Closed symbols, mature leaves; open symbols, young leaves. The closed and open bars on the X-axis represent the dark and light periods of regrowth, respectively. Data are means±S.D. from 3–4 different plants. Within a column, values with different letters are significantly different at $p < 0.05$.

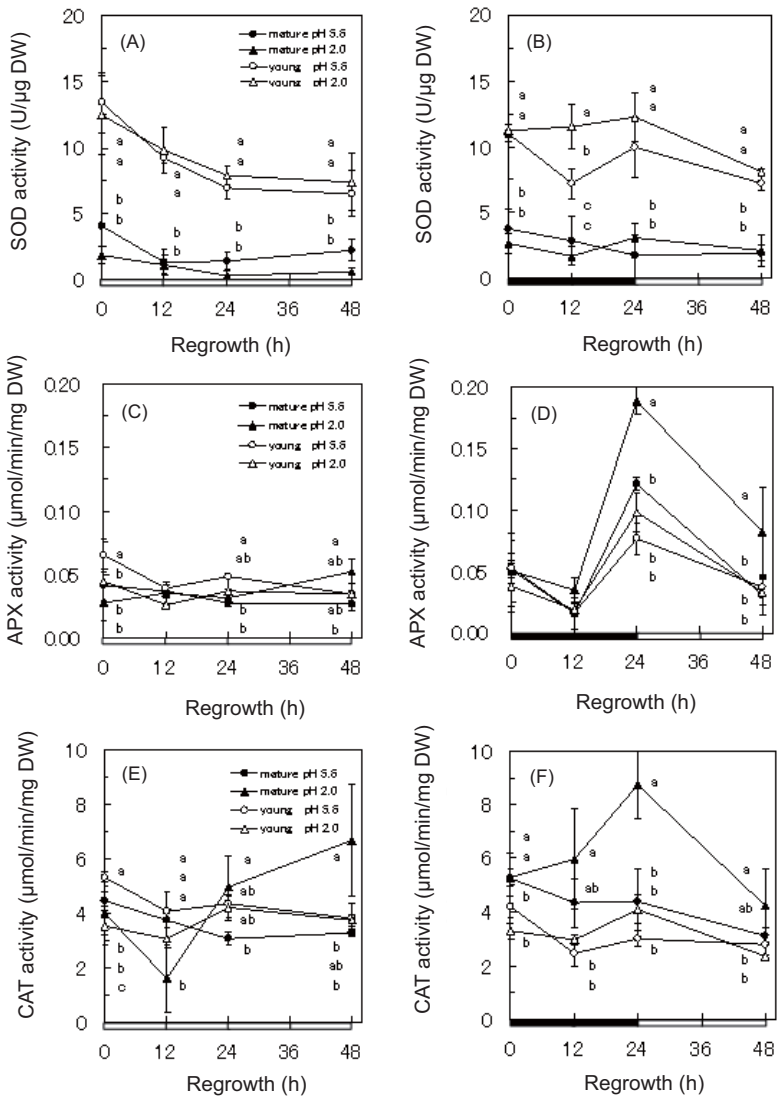

Fig. 7. Antioxidative enzyme activities of wheat leaves during regrowth period after SAS stress. Wheat seedlings that had been subjected to SAS stress using sulfuric acid solutions of pH 2.0 (triangles) or freeze-thawed in pure water of pH 5.6 (circles) were regrown in a continuous light (A, C and E) or dark/light condition (B, D and F). Activities of SOD, APX and CAT as antioxidative enzymes in the leaf extracts were measured as described in Materials and Methods. A and B, SOD; C and D, APX; E and F, CAT. Closed symbols, mature leaves; open symbols, young leaves. The closed and open bars on the X-axis represent the dark and light periods of regrowth, respectively. Data are means±S.D. from 3–4 different plants. Within a column, values with different letters are significantly different at $p < 0.05$.

4. Discussion

In this study, we investigated the effects of light conditions on SAS-stressed winter wheat seedlings during the regrowth period. When wheat seedlings were regrown after freeze-thawing at –4°C for seven days in pure water (pH 5.6) as the control or in sulfuric acid solutions of pHs 4.0, 3.0 and 2.0 (SAS stress), no visible injury was observed in young leaves under a continuous light condition over the 48-h regrowth period, but injury was clearly observed at the tip of SAS (pH 2.0)-stressed mature leaves under a continuous light condition over the 48-h regrowth period (data not shown). Considering the harmful effect of SAS stress (pH 2.0), as shown in our previous study, the damage caused by SAS stress (pH 2.0) might be enhanced by

the following light condition in the regrowth period. In this study, the effects of the SAS stress (Tables 2–4, Fig. 1) and the following regrowth period with light (Figs. 2–7) on mature and young leaves of wheat seedlings were assessed separately, because the cause of injury due to SAS stress should be different from that of injury due to regrowth with light.

4.1 *Effect of SAS stress on wheat leaves*

This study showed how the wheat leaves are affected by extracellular acidification in the process of SAS stress, i.e., freeze-thawing in sulfuric acid solution, which was estimated by evaluating the differences between untreated leaves and SAS-stressed leaves (Tables 2–4). This study also clearly showed that the tolerance level of young leaves to SAS stress is higher than that of mature leaves, suggesting that cold-acclimated young leaves might have higher levels of freezing and/or acid tolerance than those of mature leaves. In fact, the freezing tolerance (LT_{50}) of cold-acclimated young leaves was higher than that of mature leaves (Table 1). This difference has also been observed in *Arabidopsis*, and the concentrations of compatible solutes in young leaves were higher than those in mature leaves.[39,40] It is generally accepted that the accumulation of compatible solutes such as sugars, proline and glycinebetaine during cold acclimation is involved in the development of freezing tolerance due to the amelioration of cell dehydration, the freezing point depression of cells, and the increase in the cryostability of cellular membranes[39–45] such as the plasma membrane, which is known to be the primary site of freezing injury in plants.[25,46] Also, the accumulation of some compatible solutes or osmolytes, such as proline, mannitol and fructan, has been suggested to reduce oxidative damage.[23,47,48] Therefore, the accumulation of compatible solutes at high concentrations in young leaves will contribute to the reduction of injury caused by freezing stress in the process of SAS stress (Table 1). It is also possible that other cellular components induced during cold acclimation, such as some cold-inducible stress proteins,[45] may contribute to the tolerance to SAS stress in wheat leaves.

The molecular mechanism of acid tolerance was not clarified in this study. However, the acid tolerance of leaves seemed to develop to some degree during cold acclimation, because the survival rates of cold-acclimated leaf segments after cooling at 4°C with sulfuric acid (pH 2.0) were significantly higher than those of nonacclimated leaf segments in our previous study.[29] It is thought that the mechanism of acid tolerance may be related to the intracellular pH stat, which involves the proton pump activity and cytoplasmic buffering action and stabilization and/or restoration of macromolecules against strong acid,[49–52] in wheat seedlings. Further study is needed to elucidate the molecular mechanism of acid tolerance in relation to the SAS-stress tolerance of wheat seedlings.

It is known that cells of winter herbaceous plants are dehydrated and deformed by the growth of extracellular ice in a severe extracellular freezing process,[10,11] resulting in ultrastructural changes in plasma membranes and endomembranes.[11,13] In addition to the freezing-induced mechanical stress acting on the cells, the increase in the concentrations of intracellular and/or extracellular toxic ions due to extracellular freezing results in the denaturation of cellular membranes and intracellular macromolecules.[14] It is thought that the concentrations of solutes in the residual unfrozen solution of a freezing solution depend on the subzero temperature in the process of freezing point depression.[14] Therefore, sulfuric acid should be concentrated to the same concentration in the residual unfrozen solutions at given subzero temperatures in all SAS stress treatments regardless of whether the initial acid solution for SAS stress is of mild or strong acidity. By simple arithmetic, sulfuric acid should be concentrated to about 717 mM in a residual unfrozen solution at –4°C. In this study, because the initial volume of sulfuric acid solutions and freezing temperatures were the same in the SAS stress treatments, the volumes of residual unfrozen solution at

a subzero temperature is dependent on the initial concentration of sulfuric acid and, consequently, the more acidic the initial pH of acid snow becomes, the greater the volume of residual unfrozen solution will be at a subzero temperature. Thus, wheat leaves might be locally subjected to the strong acidification caused by the freezing-induced concentration of acid pollutants at subzero temperatures during SAS stress. Gout *et al*.[49] reported that intracellular pH decreases with the extracellular acidification of the culture medium in cambial sycamore cells. Velikova *et al*.[21] also suggested that H^+ ions of an exogenous acid solution penetrate through the plasma membrane into the cytosol in cells of the bean leaf after SAR treatment. Therefore, the freezing-induced concentration of acid pollutants might cause intracellular acidification during SAS stress. This may be one of the causes of the direct damage of wheat leaves caused by SAS stress.

As shown in Fig. 1, the absorption spectra of chlorophyll extracts were changed by SAS stress (pH 2.0) in mature and young leaves, showing a slight shift of the major peak to 665 nm and the development of small peaks of 606, 535 and 507 nm. Although the origin of the peak of 507 nm is unknown, the peaks of 665, 606 and 535 nm correspond to the characteristic peaks of pheophytin *a*, indicating the degradation of chlorophyll to pheophytin.[32] However, these peaks were hardly detected in the chlorophyll extracts of mature and young leaves after freeze-thawing in pure water (pH 5.6). Since it has been shown that the pheophytinization of chlorophyll is induced under an acid condition,[53] intracellular acidification might occur in SAS-stressed wheat leaves. Furthermore, Shan[32] hypothesized that the influx of H^+ ions into leaf cells of a conifer needle after SAR treatment results in the degradation of chlorophyll to pheophytin, leading to reduction of the efficiency of the use of chlorophyll in photosynthesis. Therefore, it is thought that the degradation of chlorophyll may lead to the decrease in the Fv/Fm of wheat leaves after SAS stress in this study. The presence of pheophytin *a* in the SAS (pH 2.0)-stressed leaf may cause further oxidative damage under light condition during the regrowth period because the intermediate products of chlorophyll catabolism potentially generate free radicals.[54,55]

It is thought that the combination of mechanical stresses such as deformation and dehydration caused by extracellular chronic freezing and chemical stress caused by a strong acidification of the residual unfrozen solution with concentrated sulfuric acid may promote cell injury through irreversible ultrastructural changes in cell membranes such as plasma membranes and thylakoid membranes and through a disturbance of cell metabolism induced by intracellular acidification in the process of SAS treatment. Further experiments are needed to clarify these mechanisms.

4.2 *Effects of regrowth conditions on wheat leaves after SAS stress*

When wheat seedlings after SAS treatment were regrown under light conditions, mature leaves were damaged more severely than young leaves. This means a promotion of the injury by photooxidative stress in mature leaves that have been damaged more severly by SAS treatment than young leaves (Figs. 2–7). The difference between the extents of damage after the regrowth period in mature leaves and young leaves may be caused by the difference in the extents of damage caused by SAS stress prior to regrowth (Tables 1–4, Fig. 1) and the difference in the levels of antioxidative stress tolerance during the regrowth period (Table 4 and Fig. 7). Young leaves of cold-acclimated wheat seedlings may have high levels of tolerance to SAS and photooxidative stresses compared with mature leaves.

It has been reported that the photosynthetic ability and capacity for detoxification of ROS of young leaves are greater than those of mature leaves.[27,56,57] It has also been shown that the development of freezing tolerance is correlated with the activities and gene expression of antioxidant enzymes during cold acclimation.[26,58] Our preliminary data showed that the SOD activity of young leaves doubled during cold acclimation (data not shown). As shown in Figs. 2–6, the extent of damage to young

leaves was less than that of mature leaves even under the light condition during the regrowth period. Therefore, the photooxidative damage of young leaves during the regrowth period after SAS stress may be reduced by the induction of antioxidant defense systems such as a marked increase in the level of SOD activity and the accumulation of compatible solutes possessing ROS detoxification capacity during cold acclimation. It is also possible that other antioxidant enzymes and components may contribute to the reduction of photooxidative damage to young leaves during the regrowth period after SAS.

The results of this study show a low level of SOD activity and high TBARS and H_2O_2 contents of mature leaves under the continuous light condition during the regrowth period. On the other hand, a high level of SOD activity, a high Fv/Fm and low TBARS and H_2O_2 contents were found in young leaves. Therefore, it is thought that one of the causes of the photooxidative damage in a mature leaf is superoxide radicals. Superoxide radicals, which are ROS, accelerate freezing injury on cell membranes.[22] Therefore, levels of superoxide radicals during the regrowth period after SAS stress in mature and young leaves should be analyzed in the future.

The results of this study show a significant decrease in the Fv/Fm and significant increases in the TBARS and H_2O_2 contents of SAS-stressed mature leaves during the regrowth period under the light condition. The overproduction of ROS such as H_2O_2 and superoxide radical promotes the peroxidation of membrane lipids, which leads to membrane injury.[15,16,18] Superoxide radical causes disruptive changes in the molecular organization of membrane lipid bilayers, an increase in the lipid phase transition temperature and the accumulation of free fatty acids.[18,22] Since H_2O_2 is a strong oxidant that can initiate oxidative damage, an increase in the level of H_2O_2 may lead to a disruption of metabolic function due to the perturbation of the redox status of surrounding cells and tissues at sites where H_2O_2 accumulates.[59] Therefore, damage to the SAS-stressed mature leaves was promoted by photoinhibition during the regrowth period with light.

Furthermore, H_2O_2 and superoxide radical inactivate antioxidant enzymes such as CAT and SOD.[15,60–62] In bean plants, a reduction of CAT activity was caused by an increase in H_2O_2 content during the early period after SAR treatment with a pH of 1.8.[15] Therefore, it is possible that the accumulation of H_2O_2 in the early stage (within 12 h) of regrowth under the continuous light condition might temporarily reduce the level of CAT activity induced in SAS (pH 2.0)-stressed mature leaves (Figs. 6(A) and 7(E)). After the temporary decrease in the level of CAT activity, the activity started to increase during the regrowth period under the continuous light condition (12–24 h), although the H_2O_2 level gradually increased (Figs. 6(A) and 7(E)). The mechanism by which CAT activity is induced even in the situation of H_2O_2 accumulation under light condition is not known. On the other hand, it seemed that the CAT activity in the SAS (pH 2.0)-stressed mature leaves was temporarily induced in the dark period of regrowth and might function because of low photooxidative stress in the dark period of regrowth (Fig. 7(F)). However, CAT activity decreased and H_2O_2 level increased in SAS (pH 2.0)-stressed mature leaves in the light period (24–48 h) of the dark/light condition in parallel with the decrease in Fv/Fm (Figs. 4(B), 6(B), and 7(F)). This might have also resulted from the inactivation of CAT by ROS such as superoxide and H_2O_2.[15,60,62]

The APX activities of SAS-stressed mature and young leaves were low during the regrowth period under the continuous light condition, but the activities were temporarily increased during the 24-h dark period in both mature and young leaves (Figs. 7(C) and 7(D)). Although APX isozymes are also responsible for scavenging H_2O_2, they are sensitive to photooxidative stress.[63] It is possible that APX activity might be reduced by the accumulation of ROS such as H_2O_2 and superoxide radical during the regrowth period under the light condition. Velikova *et al*.[24] suggested that ROS reduce the fluidity of the thylakoid membrane because of a decrease in the

level of unsaturated fatty acids, resulting in a reduction of the functional activity of the photosynthetic apparatus. Also, the photosynthetic electron transport activities in chloroplasts are inhibited when thylakoid lipids are peroxidized.[64] In addition, chlorophyll degradation, which was detected in mature leaves after SAS stress, might promote further membrane lipid peroxidation in the regrowth period with light (Fig. 5). Therefore, in this study, the photoinhibition in mature leaves that had been injured by SAS stress (pH 2.0) was induced during the regrowth period under the light condition.

Because the RWC of SAS (pH 2.0)-stressed young leaves recovered over the 48-h regrowth period, the regrowth conditions did not greatly affect the water uptake and transport in the underground tissues of roots and crown. Mature leaves of wheat seedlings may be more susceptible than young leaves, crowns and roots during the regrowth period after SAS stress. The deterioration of mature leaves, which act as source organs, caused by SAS stress may delay or inhibit the development of young leaves during the regrowth period of winter plants in the field. The survival rate of winter crops under an acid-snow cover might be affected by pH, the amount of acid snow and the duration of the acid-snow season. Furthermore, the production of winter crops after acid-snow stress might be affected by light and/or a combination of other environmental stimuli such as UV, ozone and water deficit.

In conclusion, leaves of winter wheat seedlings are directly damaged to some extent by acid snow stress and are further damaged by photooxidative stress during the regrowth period in the snow melt season. The results obtained in this study indicate that the extent of damage is greater in mature leaves of winter wheat seedlings, which have less freezing tolerance and less capacity for the detoxification of ROS than young leaves.

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