

Full Length Research Article

Promotive effect of 5-aminolevulinic acid on the antioxidant system in *Ginkgo biloba* leaves

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The effect of 5-aminolevulinic acid (ALA), a key precursor in the biosynthesis of porphyrins, at low levels (10 and 100 mg l⁻¹) on the antioxidant system in *Ginkgo biloba* leaves were investigated. The results showed that ALA-treatment accelerated hydrogen peroxide accumulation and temporarily increased the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) at day 4 and 8. The activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR) and the contents of ascorbic acid (AsA) and glutathione (GSH) were all significantly increased by ALA, while dehydroascorbate (DHA) content was decreased and no variety was observed in oxidized glutathione (GSSG) content after ALA treatment. The ALA-treated leaves had higher values of AsA/DHA and GSH/GSSG than those in the control leaves. These results suggested that foliar treatment with a low concentration of ALA might provide a useful means of improving beneficial antioxidant activity in *G. biloba* leaves.

Key words: Antioxidant system, flavonoids, 5-aminolevulinic acid, *Ginkgo biloba*.

INTRODUCTION

In recent years, there has been an increasing awareness of the benefit of functional foods. A major thrust of current research has been the aim of developing new food products, which have functional properties. With *Ginkgo biloba* achieving unprecedented popularity over the past decade and the recognition of the important therapeutic effects shown by this plant, there is a growing market for phytomedicines based on its extracts.

One current example of such a phytomedicine is a yellow-green tea infusion based on *G. biloba* leaves which has a smooth, light bamboo taste. Available commercially, it is postulated to be useful in the treatment of arteriosclerosis, varicose veins and haemorrhoids (Tenney, 1996; Zuess, 1998). Antioxidants are increasingly being recognized as important health promoters in conditions such as cardiovascular problems, many forms of cancer and even aging (Packer, 1999).

The antioxidants are able to reduce the effect of free radicals formed in the body either due to exposure to environmental pollutants or because the bodies' own defense mechanisms are reduced in dealing with the natural production of these compounds. Pharmacologically, there is one significant group of compounds found in *Ginkgo* leaves, the flavonoids, which give *Ginkgo* its antioxidant activity and possible protection against the damage caused by free radicals. In addition, *Ginkgo* leaves possessing antioxidant property are suspected to be owing to other antioxidant constituents, such as free-radical-scavenging enzymes, e.g. superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD), ascorbate-glutathione cycle enzymes; e.g. ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) and contents of antioxidant substances, mainly ascorbic acid (AsA) and glutathione (GSH) (Ellnain-Wojtaszek et al., 2002; Goh et al., 2003).

Reactive oxygen species (ROS) are generated enzymatically as by-products of normal metabolism through mitochondrial respiration and the cytochrome P450 system and are stimulated by enzyme activity and

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as a result of non-enzymatic reactions of oxygen with organic compounds (Bowler and Fluhr, 2000). Levels of ROS *in vivo* depend upon the balance between their generation and the capacity to remove them. Major ROS-scavenging activity of plants includes SOD, CAT, APX and GR. The balance of these enzyme activities in cells is crucial for determining the steady-state levels of superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). SOD converts $O_2^{\cdot-}$ to H_2O_2 , which is then decomposed by CAT, a process widely present in both plants and animals (Fridovich, 1986). In addition, ROS can also be removed through a series of oxidation-reductions involving AsA and GSH (Gullner and Dodge, 2000). AsA is regenerated in a reduced GSH-dependent reaction catalyzed by DHAR and is then utilized by plants through an APX reaction and converted to dehydroascorbate (DHA). The AsA regeneration and utilization recycling system protects plants from possible injury from ROS. The generation of ROS and the cellular antioxidant defense systems in fresh leaf has been widely studied (Cakmak and Marschner, 1992; Bowler and Fluhr, 2000; Yang et al., 2007).

5-aminolevulinic acid (ALA) is an essential precursor in the biosynthesis of porphyrins such as chlorophyll and heme (Zhang et al., 2006). Low concentrations of ALA could enhance the antioxidant level in spinach and pakchoi (Nishihara et al., 2003; Memon et al., 2009) and improve plant's tolerance to cold (Hotta et al., 1998; Wang et al., 2004) and salinity stresses (Watanabe et al., 2000; Nishihara et al., 2003). Although ALA has a great application potential in promoting crop antioxidant level (Wang et al., 2003), there is limited information of application of ALA to *Ginkgo* trees. No studies have been conducted on the effect of exogenous ALA on antioxidant system of *Ginkgo* leaves. The objectives of the present study are to evaluate the physiological action of foliar treatment of ALA and to investigate the effects of ALA on the active oxygen-scavenging system of *Ginkgo* leaves at low concentrations.

MATERIALS AND METHODS

Plant materials and ALA treatment

G. biloba cv. *Jiafoshou* seeds were sown in pots (50 × 30 × 7 cm) filled with sandy soil on 20 March 2007. At the 2-3 leaf stage, 300 seedlings of uniform-size per pot were selected and cultivated in a greenhouse until reaching the 7 to 8-leaf stage, irrigated daily with 1/2 Hoagland solution. At the 7 to 8-leaf stage, all pots were transferred to a growth chamber under a 12 h photoperiod (25°C, 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 75% RH. After 8 days, the seedlings were simultaneously treated by foliar spray with an aqueous solution of ALA (20 ml pot^{-1}) at 3 concentrations of 0 (the control) 10 and 100 mg l^{-1} . The fourth and fifth leaves were harvested from *Ginkgo* seedlings at days 0, 4, 8

and 16 for all tested indices. Experiments were performed in triplicate, with 60 leaves per replicate sample pooled for each determination. Results represent the means \pm standard error with each experiment performed in triplicate of 3 samples.

Extraction and determination of antioxidant enzymes

1 g of fresh leaves were homogenized in 10 ml of 50 mM Tris-HCl (pH 7.0) containing 20% (w/v) glycerol, 1 mM AsA, 1 mM GSH and 5 mM MgCl_2 with a chilled pestle and mortar. After the tow-step centrifugation at 12000 × g for 5 min and 26900 × g for 15 min, the supernatant was prepared for determination of the activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Catalase (CAT) was extracted in 0.1 mM potassium phosphate buffer (pH 7.0) including 1% (w/v) PVPP using the same procedure as for the previous three enzymes. To measure superoxide dismutase (SOD) activity, enzyme was extracted in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% PVPP with centrifugation at 12000 × g for 20 min.

APX (E.C. 1.11.1.11) activity was measured by estimating the decreasing rate of ascorbate oxidation at 290 nm, according to Nakano and Asada (1981). DHAR (E.C. 1.8.5.1) activity was determined by monitoring the increase in absorbance at 265 nm (Nakano and Asada, 1981). Activity of GR (E.C. 1.6.4.2) was measured by the method of Kocsy et al. (2001). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 2.5 mM EDTA, 0.75 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 1 mM oxidized glutathione (GSSG), enzyme extract and 0.1 mM NADPH in a final volume of 1 ml. The activity of GR, expressed as nanomoles of NADPH oxidized per mg of protein per min. SOD (E.C. 1.15.1.1) activity was based on the method described by Cakmak and Marschner (1992). One unit of SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of nitro blue tetrazolium (NBT) reduction at 560 nm. CAT (E.C. 1.11.1.6) activity was determined directly by the decomposition of H_2O_2 at 240 nm (Yang et al., 2007). Protein was estimated according to Bradford method using BSA as a standard (Bradford, 1976).

Determination of ascorbate and glutathione

500 milligrammes of fresh leaves were homogenized in 2.5 ml 5% (w/v) metaphosphoric acid with a chilled pestle and mortar. After centrifugation at 20000 × g for 15 min, the supernatant was stored at 20°C and used for determination of ascorbate and glutathione.

Ascorbate was measured according to Cakmak and Marschner (1992) with some modifications. To determine

total ascorbate, 0.2 ml supernatant was initially reacted with 0.1 ml of 10 mM dithiothreitol (DTT), which convert DHA into its reduced form AsA. Then 0.5 ml of 150 mM phosphate buffer (pH 7.5) containing 5 mM EDTA and 0.1 ml of 0.5 M N-ethylmaleimide (NEM) was added. After adding 0.4 ml of 10% (w/v) trichloroacetic acid (TCA), 0.4 ml of 44% (v/v) orthophosphoric acid, 0.4 ml of 4% (w/v) 2,2'-dipyridyl in 70% ethanol and 0.2 ml of 3% (w/v) FeCl_3 , the mixture was incubated at 37°C for 60 min and absorbance at 525 nm was read. The AsA was measured with the same procedure as total ascorbate except that DTT and NEM were replaced with 0.2 ml of H_2O . The standard curve was produced with AsA. DHA level was obtained as the difference between total ascorbate and AsA.

Glutathione was assayed by the method for Kocsy et al. (2001) with minor modification. Total glutathione was measured in a mixture containing 0.4 ml of reagent 1: 110 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 15 mM EDTA, 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 0.04% BSA; 0.32 ml of reagent 2: 1 mM EDTA, 50 mM imidazole, 0.02% BSA, 1.5 unit GR (baker's yeast, Type III, Sigma) ml^{-1} ; and 0.4 ml of a 1:50 dilution of supernatant in 5% Na_2HPO_4 (pH 7.5). The reaction was initiated with 80 μl of 3 mM NADPH. The change in absorbance at 412 nm was recorded. GSSG was determined by first incubating the mixture with 1 ml of the 1:50 dilution of acid extract and 40 μl of 2-vinylpyridine for 60 min at 25°C.

Standard curves, prepared with authentic GSH and GSSG, were used in the calculation of the amounts of total glutathione and GSSG in tissue respectively. Reduced glutathione was obtained as the difference between total glutathione and GSSG.

Statistics analysis

Data were analyzed with 1-way ANOVA using SPSS 11.0 for Windows and means were compared with Duncan's multiple range test at $P < 0.05$. For each treatment, the measurement unit was an average of 3 samples. Figures were drawn using microsoft excel 2003 and bars in the Figures show standard errors (SE) of the means with 3 independent assays.

RESULTS

Effect of ALA on H_2O_2 content

During *Ginkgo* leaf growth, the H_2O_2 content of the control was maintained with only small fluctuations, but the H_2O_2 contents of 10 and 100 mg l^{-1} ALA underwent dramatic increase (Figure 1). Treatment with ALA significantly ($P < 0.05$) enhanced the H_2O_2 content in *Ginkgo* leaves during day 4 to 16 (Figure 1). The increases for 100 mg l^{-1} ALA were higher and started

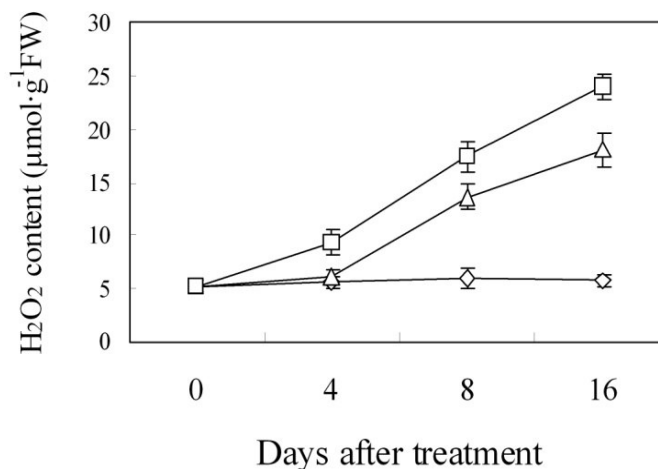


Figure 1. Time-course changes of H_2O_2 content in *Ginkgo* leaves after foliar application of 5-aminolevulinic acid (ALA). ALA concentrations: ◇ 0 mg l^{-1} , △ 10 mg l^{-1} , □ 100 mg l^{-1} . Vertical bars indicate SE values ($n = 3$).

earlier than those for 10 mg l^{-1} ALA compared with those of the control. At day 16, the increments in H_2O_2 contents of 10 and 100 mg l^{-1} ALA reached the maximum values and were 3.1-fold and 4.1-fold respectively, than that of the control.

Effect of ALA on antioxidant enzymes activities

The effects of ALA on the time course changes of SOD activity are shown in Figure 2A. After the treatment, the SOD activities of activity of 10 and 100 mg l^{-1} ALA were both markedly ($P < 0.05$) higher than that of control except for no significant difference in SOD activity between 10 mg l^{-1} ALA and the control at day 16. The increments of activities of 100 mg l^{-1} ALA were notably higher than those of 10 mg l^{-1} ALA during day 4 to 8. The highest increased values of 10 mg l^{-1} ALA (17.5%) and 100 mg l^{-1} ALA (80.7%) respectively, presented at day 8 and 4.

The CAT activities of *Ginkgo* leaf after ALA treatments were temporarily increased at day 4 (Figure 2B). That is, the activities of 10 and 100 mg l^{-1} ALA were strongly enhanced 1.4-fold and 1.9-fold, respectively, compared to the control at day 4. At day 8, the CAT activities of each ALA were decreased. Furthermore, the CAT activities of 10 and 100 mg l^{-1} were significantly ($P < 0.05$) higher than that of the control and the CAT activity of 100 mg l^{-1} ALA was slightly lower than that of 10 mg l^{-1} ALA at day 8. However, the CAT activities of 0 and 10 mg l^{-1} ALA, which values both similar to those observed at day 0, were significantly ($P < 0.05$) lower than that of 100 mg l^{-1} ALA at day 16.

The time course changes of APX activity showed a similar tendency to those of CAT activity (Figure 2B and

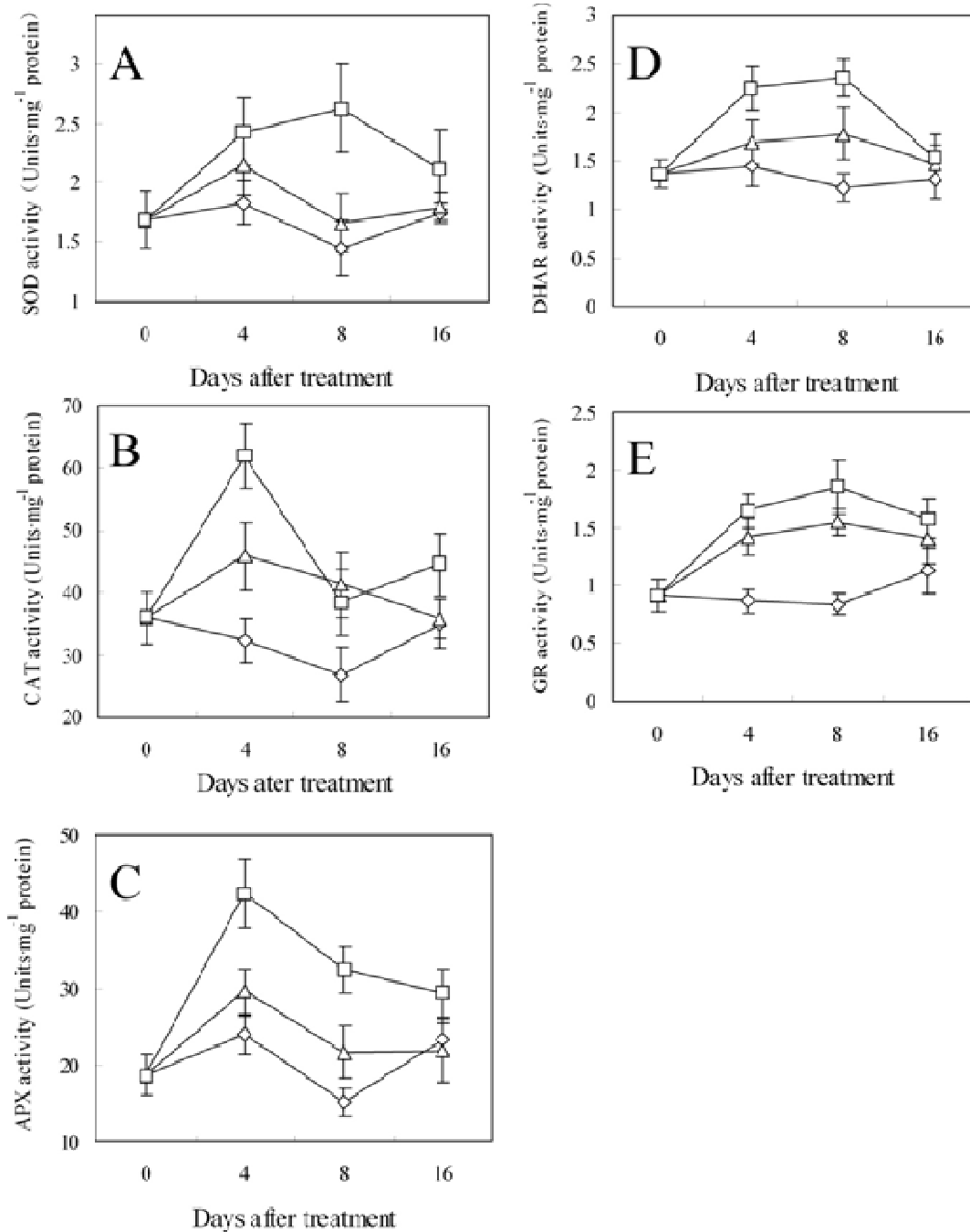


Figure 2. Time-course changes of SOD activity (A), CAT activity (B), APX activity (C), DHAR activity (D) and GR activity (E) in *Ginkgo* leaves after foliar application of 5-aminolevulinic acid (ALA). ALA concentrations: \diamond 0 mg l⁻¹, \triangle 10 mg l⁻¹, \square 100 mg l⁻¹. Vertical bars indicate SE values (n = 3).

with the control at day 16 (Figure 2C). Specifically, compared with the APX activity of the control, these activities of 10 and 100 mg l⁻¹ ALA were enhanced by 23.6 and 77.3% at day 4 and were enhanced by 112.6 and 42.6% at day 8 respectively.

The DHAR activities of 10 and 100 mg l⁻¹ ALA increased by 16.6 and 55.2% in comparison to the activity of the control at day 4 (Figure 2D). The differences were significant and these increases were enhanced to 45.9 and 92.6% at day 8, afterwards show-

ing decreases from day 8 and day 16. APX activities of both 10 and 100 mg l⁻¹ ALA had no significant change compared with that of the control at the day 16.

A similar behavior was found between the time course changes of GR activity and DHAR activity (Figure 2D and E). The GR activities of 10 and 100 mg l⁻¹ ALA both increased to about twice as that of the control at day 4. The increases of GR activities due to ALA treatment were maintained until day 8, after then showed a decline tendency. Altogether, the GR activities of 10 and 100 mg l⁻¹ ALA always maintained a significant ($P < 0.05$) increase compared with that of the control throughout the experimental period.

Effect of ALA on non-enzyme components in the AsA-GSH cycle

Non-enzyme components, such as AsA and DHA, are important for reducing toxicity of ROS during leaf growth. Figure 3 showed the results of the effect of ALA on the time course changes of AsA and DHA contents. AsA contents of each ALA treatment increased at day 4 and then declined except that slight increase was observed in 100 mg l⁻¹ ALA-treatment during day 4 to 8, showing a decrease after day 8 (Figure 3A). The AsA contents of 10 and 100 mg l⁻¹ ALA were significantly ($P < 0.05$) enhanced compared with that of the control and 100 mg l⁻¹ ALA had greater effect than 10 mg l⁻¹ ALA, while the DHA content of control was higher than those of 10 and 100 mg l⁻¹ ALA treatments (Figure 3B). Based on the results of both AsA and DHA contents in all ALA treatments, 100 mg l⁻¹ ALA showed the highest AsA/DHA ratio, while the control showed the lowest during the experimental period (Figure 3C).

As shown in Figure 4A, the GSH content of the control maintained with only small fluctuations during the whole experiment, whereas, the GSH contents of 10 and 100 mg l⁻¹ ALA increased gradually and reached the maximum values at day 16. Figure 4B showed there was no significant difference in GSSG contents among whatever concentrations or times of ALA treatment. Changes in ratios of GSH to GSSG presented a similar trend to that of GSH content during the whole experiment. Accordingly, ALA application maintained significantly higher ($P < 0.05$) GSH/GSSG ratio than that of the control during the whole experiment and the 100 mg l⁻¹ ALA had a more positive effect than 10 mg l⁻¹ ALA on GSH/GSSG ratio (Figure 4C).

DISCUSSION

Oxidative processes during leaf development were measured based on the content of H₂O₂ and lipid peroxidation. Bowler and Fluhr (2000) reported that H₂O₂ produced in response to a variety of stimuli acts as

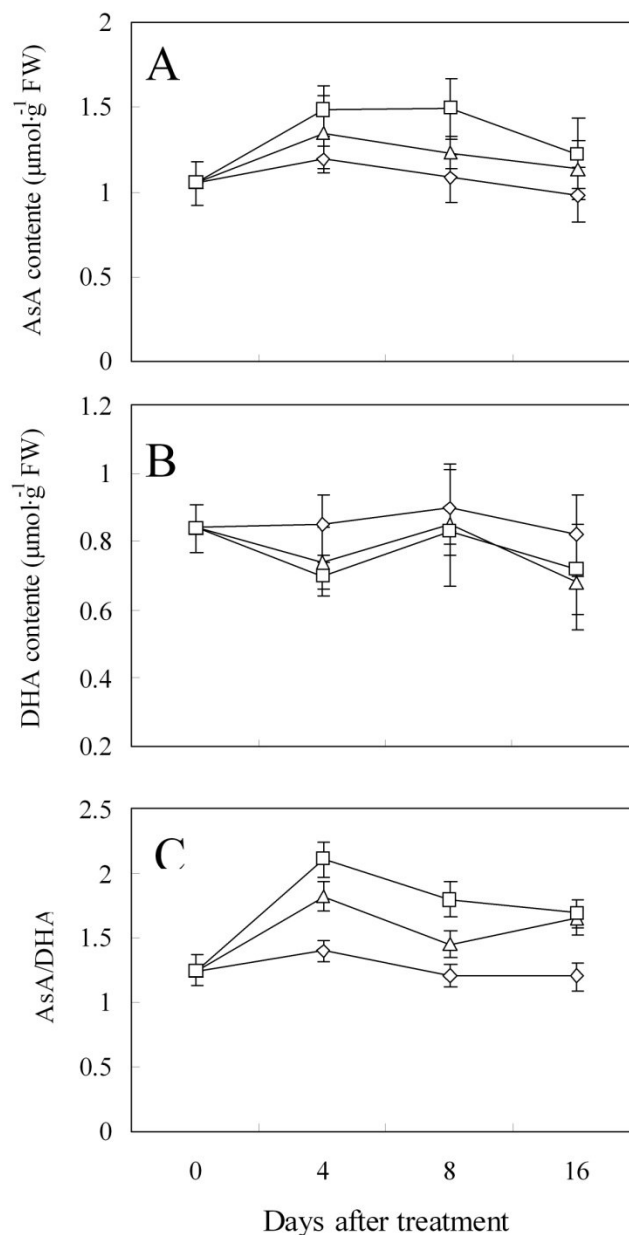


Figure 3. Time-course changes of the values of AsA (A), DHA (B) and AsA/DHA (C) in Ginkgo leaves after foliar application of 5-aminolevulinic acid (ALA). ALA concentrations: \diamond 0 mg l⁻¹, \triangle 10 mg l⁻¹, \square 100 mg l⁻¹. Vertical bars indicate SE values ($n = 3$).

signaling molecule/second messenger and contributed to the phenomenon of cross-tolerance. In this study, treatment with ALA significantly increased the H₂O₂ contents in *Ginkgo* leaves, as has been reported in spinach (Nishihara et al., 2001) and potato (Zhang et al., 2006). The mechanism underlying this increase remained to be clarified. One reason was assumed to be the increasing photosynthetic rate by ALA treatment (Nishihara et al., 2001). Another major possibility was that ALA played

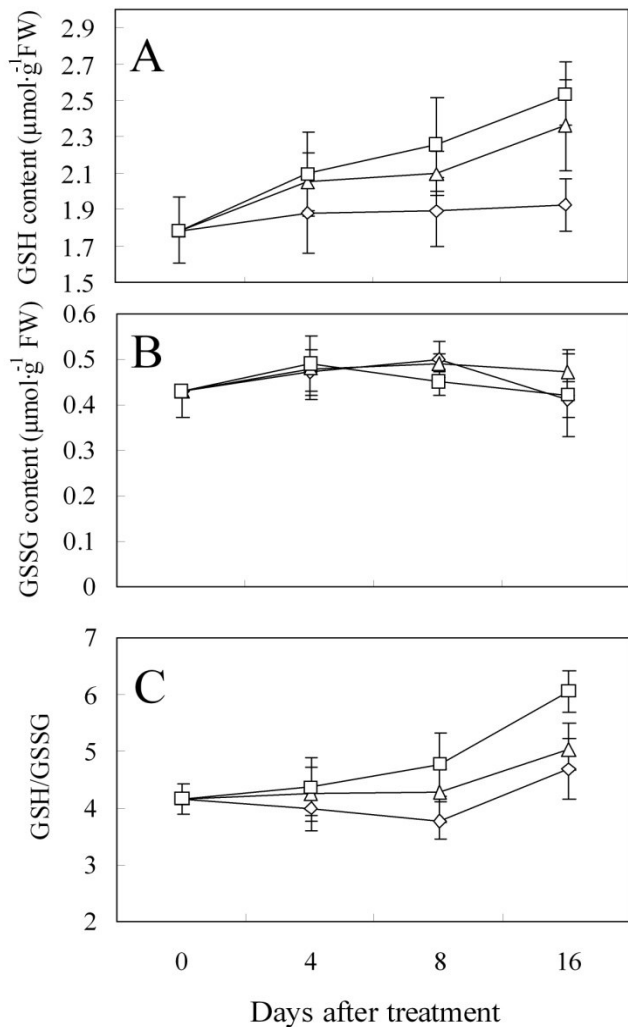


Figure 4. Time-course changes of the values of GSH (A), GSSG (B) and GSH/GSSG (C) in *Ginkgo* leaves after foliar application of 5-aminolevulinic acid (ALA). ALA concentrations: \diamond 0 mg l⁻¹, \triangle 10 mg l⁻¹, \square 100 mg l⁻¹. Vertical bars indicate SE values (n = 3).

played an important role in the alteration of H₂O₂ source of electron transport. In detail, either electrons or energy flow to the oxygen and the activated oxygen is subsequently metabolized to form H₂O₂. It was supposed to be induced by the generation of ¹O₂, which causes photodynamic damage, from higher concentration of ALA application (Nishihara et al., 2003). That is, the generation of ¹O₂ could induce looseness of structure of the thylakoid membrane and the NADH-dependent production of O₂⁻ might be increased by ALA. Then the enzymatic action of SOD, which is a major scavenger of O₂⁻ and was induced by ALA in the present research, results in the generation of H₂O₂. This H₂O₂ is metabolized in a Halliwell-Asada pathway by APX, originally described in the chloroplast (Nakano and Asada, 1981).

The results from the present study showed that APX and CAT activities of 100 and 10 mg l⁻¹ ALA were significantly increased in comparison to that of the control at day 4, which was consistent with the results of Nishihara et al. (2001, 2003), who detected increases of APX and CAT activities by ALA treatment in spinach leaves under both non-NaCl and NaCl stress. The additional heme proteins, which was derived from exogenous-ALA-stimulated heme turnover, was incorporated into the peroxidase molecule, resulting in the increase of APX and CAT activities (Nishihara et al., 2003). However, the content of H₂O₂ of ALA treatment also increased in *Ginkgo* leaves compared with that of control in the present study, which might be ascribed to the fact that production of H₂O₂ predominated over APX and CAT scavenging activity.

SOD has been implicated as an essential component for defense against the potential toxicity of ROS (Cakmak and Marschner, 1992) and this defensive action was affected by ALA in the present research. Higher SOD activity was measured in *Ginkgo* leaves with ALA treatment in comparison with that of the control, in line with results from pakchoi (Memon et al., 2009). The increase might be related to the promotion of activity of SOD with different isoforms such as Fe-SOD and Mn-SOD, but the particular mechanism by which it does this are still plausible. Nevertheless, no variety in SOD activity was observed between ALA treatment and the control in spinach leaves (Nishihara et al., 2001). It remains elusive for occurrence of contradictory change in SOD activities under ALA treatment, which may be dependent upon plant species, type and development stage of tissues employed for experiment.

It has been reported that stress tolerance of plants may be associated with their ability to remove ROS (Bowler and Fluhr, 2000), in which enzymes responsible for the ascorbate-glutathione cycle, such as APX, DHAR and GR, may play key roles. In the presence of NADPH, GR contributes to the regeneration of ascorbate (Yang et al., 2007), which, along with APX, decomposes H₂O₂. In addition, GR catalyzes the NADPH-dependent reduction of GSSG to GSH, keeping the ratio of GSH/GSSG high. During day 0 to 16, the activities of GR and DHAR of 10 and 100 mg l⁻¹ ALA maintained distinctly higher levels compared to those of the control. Also, the increment of GR and DHAR activities by ALA showed increase when the period of treatment was extended from day 0 to 8 and then displayed a decrease at day 16, suggesting 16 days possibly have been the supra-optimal application timing of ALA. Furthermore, 100 mg l⁻¹ ALA had a significantly greater effect on GR and DHAR activities in *Ginkgo* leaves than 10 mg l⁻¹ ALA at day 4 and 8, suggesting that the influence of ALA on the antioxidant enzyme may be related to concentration. Nishihara et al. (2001) also showed that the increment of GR activity by treatment with ALA linearly increase from day 0 to 6 and afterward declined at day 9, exhibiting higher increase in 1.8 mM

ALA compared to 0.6 mM ALA. Yet, to our knowledge, there is no literature related to the effect of ALA on DHAR activity in plant. Further studies are needed to confirm the role of ALA in influencing the activity of the enzymes involved in ascorbate-glutathione cycle and elucidate its physiological mechanism.

AsA, essential metabolites and powerful regulators for regulating cell functions, play a pivotal role in antioxidant defense (Smimoff, 1995). In our investigation, AsA content in the control leaves were lower than that treated with ALA from day 0 to 16, in agreement with the reports on spinach (Nishihara et al., 2003). AsA functions as a co-substrate of plant POD, for production of DHA (Halliwell, 1982). On the other hand, there was significant decrease in DHA content by ALA condition during the whole experiment, totally opposite to the change in AsA content. This demonstrated that ALA was instrumental in mobilizing the antioxidant defense of AsA and DHA in *Ginkgo* leaves. High AsA content in the *Ginkgo* leaves treated with ALA may result from an acceleration of biosynthetic pathways or a decrease in catabolism through an accumulation of DHA. Both changes lead to a shift from the oxidized form to the reduced form and as observed, to an increase in the AsA/DHA ratios, indicating that more oxidized ascorbate needed to be converted into the reduced form to scavenge ROS in *Ginkgo* leaves.

Like AsA, glutathione is a low-molecular-mass compound with recognized antioxidant functions. It exists in cells in two forms, reduced (GSH) and oxidized forms (glutathione disulfide, GSSG), the former dominating. In this study, GSH content was higher in ALA - treated *Ginkgo* leaves than that of the control leaves, while negligible differences were observed in GSSG among all ALA treatment, in accordance with other reports on pea (Gullner and Dodg, 2000) and spinach (Nishihara et al., 2001, 2003). These results may be relative to changes of GR and DHAR activities. Apart from GSH, the ratio of reduced to oxidized glutathione (GSH/GSSH) has to be taken into consideration. It has been suggested that a high GSH/GSSG ratio is necessary for several physiological functions including activation and inactivation of redox-dependent enzymes systems (Gullner and Dodge, 2000). Kocsy et al. (2001) suggested that a change in GSH/GSSG ratio was more important than GSH content in cell resistance to oxidative stress. The present study showed that ALA - treated leaves had higher GSH/GSSG ratio than the control, in agreement with Nishihara et al. (2001) who reported that ALA increased salt tolerance of spinach by promoting the ascorbate-glutathione cycle.

In conclusion, the work presented here showed that application of ALA at low concentrations of 10 and 100 mg l⁻¹ could increase antioxidant enzyme activity (SOD, CAT, GR, APX, DHAR) and improve levels of antioxidant components (AsA and GSH) as well. It can be supposed that ALA foliar applications to *Ginkgo* leaves might protect it from cell damage. Therefore, we suggest that treatment with ALA at low concentration may be a useful strategy to

improve antioxidant-condition of *Ginkgo* leaves. However, ALA treatment of *Ginkgo* leaves for this purpose is probably too expensive for commercial use due to high price in current market (over 80 US Dollars per g). Selecting a sort of substance, which possesses similar physiological effects and inexpensive market price, instead of ALA, make such treatment profitable in the future.

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