ENVIRONMENTAL SCIENCES Vol. 1, No. 2, 81~86
Bulletin of the Korean Environmental Sciences Society

Effects of Paraquat on Ascorbic Acid and Malondialdehyde Contents, and Superoxide Dismutase Activity in Spinach Chloroplasts under Light and Dark

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(Manuscript received on 5 September, 1997)

The amounts of ascorbic acid in chloroplasts treated with light and light+paraquat (PQ) were reduced by 81 and 82% of initial level, respectively at 24 hr at incubation. And those treated with dark and dark+PQ were decreased by 46 and 55% of the original level, respectively.

Malondialdehyde (MDA) contents at 24 hr of dark and dark+PQ treatment were increased by 6 and 31% of the initial level, respectively. When chloroplasts were treated with light and light+PQ, MDA contents after 24 hr were increased by 88 and 146% of the initial level, respectively.

SOD activities treated with light and light+PQ were increased by 10 and 20% of the initial level, respectively for 3 hr and thereafter reduced by 46 and 49% of the original level, respectively at 24 hr. However, the SOD activities treated with dark and dark+PQ were decreased by 37 and 30% of the initial level, respectively.

It is considered that PQ triggers the oxidation of ascorbic acid, the induction of lipid peroxidation and the inactivation of SOD under light so that PQ has inhibitory effect on the pathway of plant metabolism.

Key word: ascorbic acid, malondialdehyde, superoxide dismutase, paraquat, lipid peroxidation.

1. Introduction

Some of the herbicides have been found to be very efficient in killing noxious weeds (Harvey and Fraser, 1980), but most herbicides have contaminated water and soil. And the repeated use of these chemicals may even induce the resistance to them in the target plants (Shaaltiel and Gressel, 1987).

Paraquat (1,1′-dimethyl-4,4′-bipyridylium) is a member of the methyl viologens and well known as non-selective contact herbicide and total-kill compounds (Ashton and Crafts, 1981). And it acts in the chloroplast in the light through the generation of superoxide in a chain reaction, producing more reactive oxygen species such as singlet oxygen (Dodge, 1994). These oxygen radicals may directly or indirectly cause peroxidation of membrane lipid and start the syndrome of effects typical of paraquat toxicity (Bus, 1976).

The stroma of spinach chloroplasts contains ascorbic acid (Law, 1983). The ascorbic acid redox system is a powerful part of the anti-oxidative system in plant leaves. Ascorbic acid reacts rapidly with most of the reactive oxygen species such as superoxide, hydroxyl radical, hydrogen peroxide and singlet oxygen to give rise to dehydroascorbic acid (Halliwell and Gutteridge, 1985). Law *et al.* (1983) reported that

addition of paraquat to illuminated chloroplasts caused a rapid oxidation of ascorbic acid. Also, paraquat led to a rapid loss of ascorbic acid in sapling of *Picea abie* L. (Westphal, 1992). It is concluded that the amount of ascorbic acid is reduced in the presence of toxic oxygen species induced by paraquat.

The oxidative degradation of unsaturated fatty acids can be followed by determining the amount of a product of lipid peroxidation (Placer, 1966). Paraquat damaged cellular membrane systems as a result of lipid destruction and caused the formation of MDA, an indicator of lipid peroxidation in plant cells (Dalton, 1992). Isolated chloroplasts should readily undergo peroxidation since the fatty acids of the lamellar system are about 75% unsaturated (Heath and Packer, 1968). Lipids present in the chloroplasts contain a high percentage of polyunsaturated fatty acids and are very susceptible to peroxidation. Schimazaki *et al.* (1980) reported that MDA was formed because of singlet oxygen generated from superoxide produced by paraquat. It was reported that active oxygen, especially superoxide radical participated in the formation of MDA (Sakaki, 1983). These reports suggest that toxic oxygen species produced by paraquat induce the lipid peroxidation and lead to membrane damage.

The superoxide dismutase (SOD. EC 1.15.1.1) constitutes a primary defense of cells against oxygen free radicals. The isozymes of SOD are classified according to the metal at the active site : cooper and zinc (Cu Zn-SOD), manganese (Mn-SOD) or iron (Fe-SOD) (Tandy, 1989). The plant SOD enzyme was found to be associated with chloroplasts (Salin, 1988). Most abundant enzyme in plants is Cu Zn-SOD, which is inhibited by cyanide and hydrogen peroxide (Forman and Fridovich, 1973). The major part of the activity of this enzyme was found in chloroplast with a distinct portion associated with the thylakoid membranes (Salin, 1988). Paraquat treatment of maize (*Zea mays*) and pea (*Pisum sativum*) plants resulted in 40-100% increase in SOD activity at the lower concentration of paraquat (Matters and Scandalios, 1986; Peleg, 1992). Dalton (1992) reported that higher activity of SOD was observed after treatment with 1mM paraquat. It seems likely that the increase in SOD activity by the lower concentration of paraquat may be due to superoxide radicals.

The purpose of this paper is to investigate the physiological effects of paraquat on ascorbic acid and MDA contents, and SOD activity of spinach chloroplasts under light and dark.

2. Materials and Methods

2.1. Plant Materials

Fresh leaves of spinach (*Spinacia oleracea* L.) were obtained from Bu-Joun Market, Pusan. All procedures were performed under a dim light at 4℃ and enzyme assay was run at 25℃.

2.2. Chloroplast Isolation

100 g of leaves were washed several times with distilled water and then were deveined. They were homogenized in a home blender at full speed for 10 sec in 380 ml of grinding medium containing 0.33 M sorbitol, 25 mM HEPES, 2 mM EDTA adjusted to pH 7.6. The resulting homogenate was filtered through 8 layers of cheesecloth, and the filtrate centrifuged at 350g for 10 min to remove the debris. The supernatant was centrifuged at 2,000g for 15 min and discarded to obtain chloroplast pellets. The green chloroplast pellets were washed with resuspension medium (pH 7.6) containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM

$MnCl_2$ and 50 mM HEPES.

2.3. Paraquat Treatment and Incubation

Chloroplast pellets were suspended in resuspension medium to give a chlorophyll concentration of about 80 μg per ml. The chloroplast preparation was divided into 4 of test tubes, and 2 of them were treated with PQ solution, yielding final PQ concentration of 50 ppm for experimental group. Others without PQ solution were used for control group. One of the control groups was designed to keep in the complete darkness. The same method was used for experimental group. And then all test tubes were incubated in the growth chamber under 5,500 lux at 25℃ for 24 hr.

2.4. Measurement of Ascorbic Acid Contents

30 ml aliquot of each chloroplast suspension was centrifuged at 2,000g for 15 min to sediment chloroplasts. Green pellets were ground with the prechilled mortar and pestle in 3 ml of ice-cold 5% metaphosphoric acid. The extracts were then centrifuged at 18,000g for 20 min. 2 ml aliquot of the supernatant was removed and added to 1 ml of citrate/acetate buffer (pH 4.15) and 1 ml of 0.2 mM 2,6-dichlorophenolindophenol.

After 30 sec, the absorbance was determined at 520 nm.

2.5. Measurement of MDA Contents

MDA contents were assayed according to Heath and Packer (1968) to determine the amount of lipid peroxidation. 1 ml aliquot of each chloroplast suspension was added to 2 ml of 0.38% thiobarbituric acid in 15% trichloroacetic acid, and then incubated in a boiling water for 30 min. The mixture was rapidly cooled in an ice bath and then centrifuged at 18,000g for 8 min to clarify the solution.. The amount of MDA was calculated by using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.6. Measurement of SOD Activities

50 $\mu\ell$ aliquot of each chloroplast suspension was homogenized in 0.1 M potassium phosphate (pH 7.8) with prechilled mortar and pestle at 4℃. The homogenate was centrifuged at 10,000g for 30 min at the same temperature. The supernatant was dialyzed overnight against 10 mM phosphate buffer (pH 7.8). After centrifugation of the dialyzed solutions at 15,000g for 30 min, the supernatant was used for SOD assay. SOD was assayed by the inhibition of cytochrome C reduction by superoxide. 3 ml of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM cytochrome C, 0.1 mM xanthine, enzyme preparation and xanthine oxidase. The reaction was started by the addition of 50 $\mu\ell$ of xanthine oxidase. One unit of SOD was defined as the amount which inhibited the reduction rate of cytochrome C by 50% under the assay conditions.

3. Results and Discussion

The first and characteristic effect of the paraquat is the disorganization of membranes. The effect of membrane destructoin is based on the ability of this compound to accept electrons from photosystem I to produce radicals and in complex series of reactions to generate reactive oxygen species such as superoxide (Dodge, 1994). Superoxide and hydroxyl radical can directly damage membrane lipid (Szigeti and Sarvari, 1992)

The stroma of spinach chloroplasts contains ascorbic acid (Law, 1983). The reduction-oxidation system of ascorbic acid is a powerful part of the antioxidative system in plant leaves. Addition of PQ to illuminated chloroplasts caused a great loss of ascorbic acid (Law, 1983). And ascorbic acid is particularly effective antioxidant against toxic superoxide and is present in the chloroplast stroma (Halliwell, 1982).

In this experiment, chloroplasts were prepared from each group and determined for their amounts of ascorbic acid. Fig. 1 shows the changes in amounts of ascorbic acid in the control groups and the experimental groups.

The amounts of ascorbic acid in light-treated groups were decreased drastically during 3 hr after beginning and thereafter gradually decreased for about 9 hr. After 12 hr the contents of ascorbic acid treated with light did not change nearly. The ascorbic acid contents treated with light and light+PQ were reduced by 81 and 82% of the initial level, respectively at 24 hr after incubation. And the ascorbic acid contents treated with dark, and dark+PQ were reduced by 46% and 55%, respectively at 24 hr after incubation. This results suggest that light decrease ascorbic acid contents more than darkness does. It is concluded that ascorbic acid is oxidized by reactive oxygens during illumination.

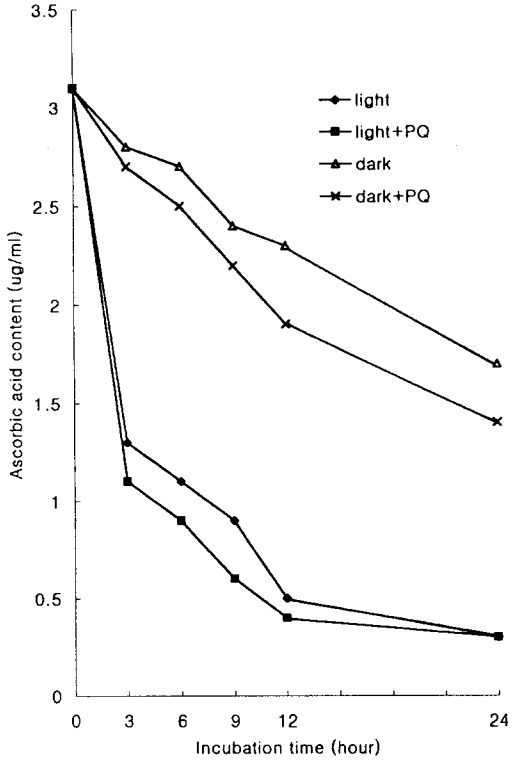

Fig. 1. Changes in ascorbic acid contents in spinach chloroplasts by light, light+PQ, dark and dark+PQ treatment.

MDA, a product of lipid peroxidation was formed because of singlet oxygen produced from superoxide radical (Peter, 1992). Heath and Packer (1968) reported that little MDA was produced in the dark but MDA was increased in the presence of light. Sakaki *et al,* (1983) reported that the contents of MDA were considerably increased in spinach leaves by toxic oxygen species.

MDA contents were measured to determine the amount of lipid peroxidation. Fig. 2 shows the accumulation of MDA in chloroplasts during the treatments.

As shown in Fig. 2 the amounts of MDA were a little increased in dark and dark+PQ-treated groups. MDA contents at 24hr of dark and dark+PQ treatments were increased by 6 and 31% of initial level, respectively. When chloroplasts were treated with PQ under illumination, however, MDA contents slowly increased for the initial 3 hr of treatments, followed by a subsequent drastic rise. MDA content at 24 hr of light+PQ treatment was increased by 146% of the initial level. The amounts of MDA in chloroplasts under light were gradually increased by 88% for 24 hr. These results suggest that light and PQ damage membrane lipid as a result of lipid peroxidation, and cause the formation of MDA.

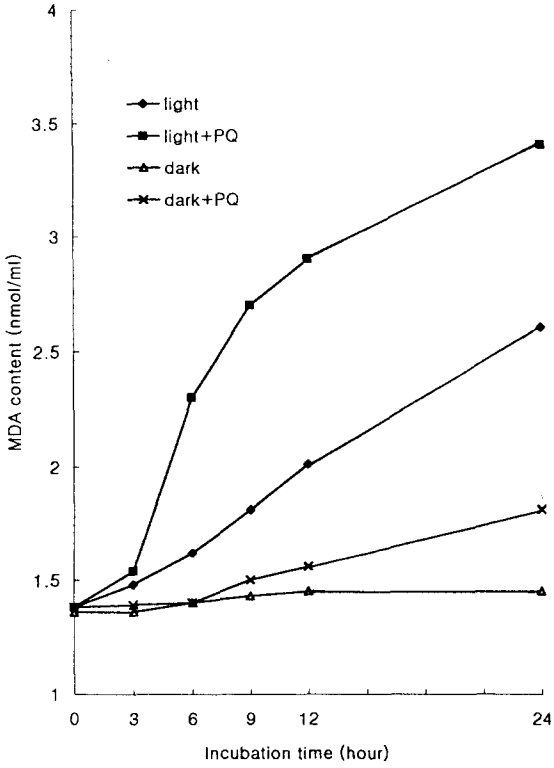

Fig. 2. Changes in MDA contents in spinach chloroplasts by light, light+PQ, dark and dark+PQ treatment.

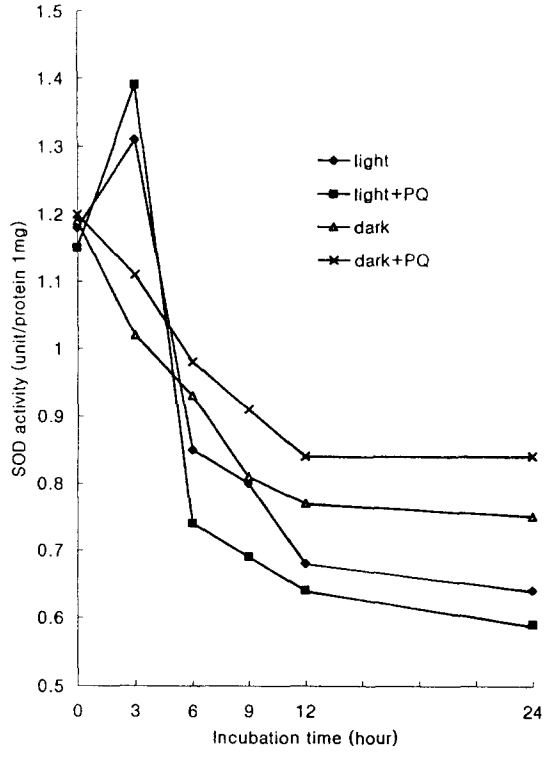

Fig. 3. Changes in SOD activities in spinach chloroplasts by light, light+PQ, dark and dark+PQ treatment.

Chloroplasts produce superoxide radical under light, but most of the superoxide radical formed in chloroplasts is scavenged by SOD (Dalton, 1992). One unit of SOD is defined as the amount of enzyme which inhibits the rate of cytochrome c reduction by 50%. Fig. 3 presents the effect of PQ and light on the activities of SOD in chloroplasts.

SOD activities treated with light and light+PQ were increased by 10 and 20% of the initial level, respectively, for 3 hr and thereafter reduced by 46 and 49% of the original level, respectively at 24 hr. However, the SOD activities treated with dark and dark+PQ were gradually decreased by 37 and 30% of the initial level, respectively. It seems likely that the increase in SOD activities by light and light+PQ treatments for 3 hr may be due to reactive oxygens generated in chloroplasts. When the amounts of reactive oxygen species produced by light and PQ exceed the SOD capacity to dismutate them, peroxidative damage to membrane lipids may occur and SOD activities may decrease.

In the results obtained in this experiment, it is considered that PQ triggers the oxidation of ascorbic acid, the induction of lipid peroxidation and the inactivation of SOD under light so that PQ has inhibitory effect on the pathway of plant metabolism.

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