Effect of singlet oxygen generating substances on the ascorbic acid and glutathione content in pea leaves

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Abstract

Ascorbate and glutathione levels were investigated in pea leaf discs exposed to various singlet oxygen generating compounds: eosin, rose bengal, monuron, acifluorfen and 5-amino-levulinic acid (ALA). The cellular level of the major antioxidant ascorbate was markedly decreased by the herbicides monuron, acifluorfen and ALA (in light-dependent reactions), as well as by the xanthene dyes eosin and rose bengal (independently of light). No significant accumulation of dehydroascorbate could be observed in any treatments. In contrast to ascorbate, the foliar glutathione levels were considerably increased by subtoxic or slightly toxic concentrations of eosin, rose bengal, acifluorfen and ALA in a light-dependent manner. Monuron treatments led to unchanged or decreasing glutathione contents. The activities of three antioxidative enzymes (ascorbate peroxidase, glutathione reductase and glutathione *S*-transferase) were also induced by eosin in light-dependent reactions. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Acifluorfen; 5-Aminolevulinic acid; Ascorbate; Glutathione; Monuron; Xanthene dye

1. Introduction

The accumulation of active oxygen species (AOSs) has been observed in various plants exposed to biotic and abiotic stress effects [1]. Among other AOSs, singlet oxygen ($^1\text{O}_2$), the excited form of molecular oxygen is also capable of damaging important plant cell constituents [2–4]. Singlet oxygen sources in biological systems include light-dependent reactions (photosensitization) and chemical generations in dark reactions [5].

A large variety of dyes act as photosensitizers. The cell damaging effects of the xanthene dyes

eosin and rose bengal are well known in plant leaves [2,6,7]. The light excitation energy of these dyes is transferred directly to dioxygen, leading to the generation of singlet oxygen.

Chlorophyll itself is capable of photodynamic reactions involving $^1\text{O}_2$ when its excited states are insufficiently quenched by the photosystems or by carotenoids [3,6]. Phenylurea herbicides, such as monuron (3-[*p*-chlorophenyl]-1,1-dimethylurea), promote these reactions by blocking the electron flow between the photosystems [6]. Other porphyrin derivatives are also effective photosensitizers [8]. These intermediates are normally present in small amounts, but an excess might act as photosensitizer because they are unable to pass on light excitation energy to light harvesting accessory proteins. Instead, excitation energy results in triplet state sensitizers, which excite oxygen to the singlet state. The herbicide acifluorfen (5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoic acid), which exerts its effect in the light, is known to induce the accumulation of protopor-

Abbreviations: ALA, 5-aminolevulinic acid; AOS, active oxygen species; AP, ascorbate peroxidase; DHA, dehydroascorbate; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase.

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phyrin IX and other tetrapyrrole derivatives in plant leaves [9,10]. The chlorophyll precursor 5-aminolevulinic acid (ALA) can also cause accumulation of abnormally high levels of tetrapyrrole intermediates in darkness if it is added exogenously. As a consequence, ALA treatments led to marked photodynamic herbicidal effects [4,8,11].

Plants are protected by various mechanisms against the oxidative stress caused by singlet oxygen. Membrane bound antioxidants, carotenoids and α -tocopherols are well-known quenchers of $^1\text{O}_2$ [2,3]. However, much less information, mostly from in vitro studies, is available about the role of water-soluble antioxidants in the detoxification of singlet oxygen. Ascorbate is a major primary antioxidant, which reacts directly with singlet oxygen [1,12]. Glutathione (GSH) is also known to scavenge $^1\text{O}_2$ [1,5]. Ascorbate and GSH are closely related as both are constituents of the antioxidative ascorbate-glutathione cycle which detoxifies hydrogen peroxide in the chloroplasts [1].

In order to gain more information about the role of ascorbate and GSH in the detoxification of singlet oxygen in plant tissues, we have studied their cellular levels in pea leaf discs exposed to various $^1\text{O}_2$ generating substances (eosin, rose bengal, monuron, acifluorfen and ALA). In addition, the activities of the antioxidative enzymes ascorbate peroxidase (AP; E.C. 1.11.1.11.), glutathione reductase (GR; E.C. 1.6.4.2.) and glutathione *S*-transferase (GST; E.C. 2.5.1.18.) were also investigated in pea leaf discs exposed to eosin. AP and GR are constituents of the ascorbate-glutathione cycle [1]. GST, beside other detoxification reactions, can catalyze the breakdown of fatty-acid hydroperoxides and contributes to the protection of plant cells against oxidative membrane damage [13,14].

2. Materials and methods

2.1. Plant material

Pea (*Pisum sativum* L. var. Meteor) seedlings were grown in Levington Universal compost in a greenhouse with a 14 h photoperiod (natural daylight extended by 400 W Thorn mercury vapour lamps) and a mean air temperature of 22°C. Leaf discs (12 mm in diameter) were excised from sub-apical leaves of 2–3 week-old pea plants. Discs

were floated on the aqueous solutions of singlet oxygen generators (or on tap water) in Petri dishes for 24 h in darkness, then they were exposed to continuous light for 72 h in a light cabinet maintained at 25°C. Illumination ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$) was provided by warm fluorescent tubes (65/85-W; Thorn). After varying time periods the leaf discs were washed and used for biochemical assays. Parallel incubations were carried out in darkness. Dry leaf weights were measured after the incubation of leaf discs at 110°C for 2 h.

ALA, eosin Y (2',4',5',7'-tetrabromofluorescein), and rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) were obtained from Sigma (St. Louis, MO, USA). Acifluorfen and monuron were purchased from Riedel-de Haen (Seelze, Germany).

2.2. Enzyme assays

For enzyme assays cell-free homogenates were prepared at 0–4°C. Leaf material (0.5 g) was frozen in liquid nitrogen, pulverized with a mortar and a pestle, and suspended in 3 ml of ice-cold 0.2 M Tris–HCl buffer (pH 7.8) containing 3% soluble polyvinylpyrrolidone and 0.1 mM $\text{Na}_2\text{-EDTA}$. The homogenate was strained through muslin and centrifuged at $8000 \times g$ for 20 min. The supernatants were used as enzyme source.

The enzyme activities were determined by spectrophotometric methods. The activity of AP was determined as described in Ref. [13], except that in our experiments 0.25 mM ascorbate and 0.5 mM hydrogen peroxide were used. GR and GST activities were measured by following the reduction of oxidized glutathione (GSSG) at 340 nm, and by measuring the formation of the GSH-conjugate using 1-chloro-2,4-dinitrobenzene as substrate at 340 nm, respectively, as described earlier [13].

2.3. Other assays

The level of acid-soluble, non-protein thiols was measured spectrophotometrically with 5,5'-dithiobis-(2-nitrobenzoic acid) according to De Kok and Graham [15]. Parallel with the thiol measurements, total glutathione (GSH + GSSG) contents were also determined in the plant extracts with a specific enzymatic method using GR as described by Smith [16].

Ascorbate and dehydroascorbate levels were measured by enzymatic methods using ascorbate oxidase enzyme as described by Wise and Naylor [17], and Foyer et al. [18], respectively.

2.4. Statistics

At least three independent parallel experiments were carried out in each case. The significant differences between mean values were evaluated by Student's *t*-test. Differences were considered to be significant at $P = 0.05$.

Fig. 1. Changes in the ascorbate content of pea leaf discs exposed to xanthene dyes (eosin or rose bengal) at various concentrations. All discs were preincubated for 24 h in darkness then illuminated ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$). Parallel experiments were carried out in darkness. Means of three independent experiments $\pm$ S.D. are shown. Symbols, illuminated discs: $\circ$, control; $\square$, 10 μM dye; $\triangle$, 1 mM dye. In darkness: $\bullet$, control; $\blacksquare$, 10 μM dye; and $\blacktriangle$, 1 mM dye.

3. Results and discussion

3.1. Xanthene dyes

The application of xanthene dyes to pea leaf discs resulted in phytotoxic symptoms (bleaching) in the light, but not in darkness. The exposure of illuminated pea leaf tissue to 10 μM eosin led to only a very weak bleaching effect after 24 h, but 1 mM eosin was markedly phytotoxic. Rose bengal was much less toxic than eosin: only as high as 1 mM rose bengal concentration caused visible symptoms (moderate bleaching) in pea leaf tissue.

Eosin, and to a lesser extent also rose bengal decreased the ascorbate content in a concentration-dependent manner. Xanthene dye treatments led to ascorbate degradation in both illuminated and dark-incubated leaf discs (Fig. 1). Since singlet oxygen can not be produced by xanthene dyes without the excitation energy of light, these results proved that the ascorbate degradation was not related to singlet oxygen quenching. The dehydroascorbate (DHA) levels also markedly decreased in the leaf discs exposed to xanthene dyes (data not shown). In untreated pea leaves DHA accounted for $26 \pm 6\%$ ($n = 5$) of the total ascorbic acid content. The light-independent reactions leading to the depletion of ascorbate and dehydroascorbate pools in the presence of xanthene dyes are not known. In darkness, ascorbate levels markedly declined also in untreated pea leaf discs as it had been observed earlier [19] showing that light was necessary for its synthesis or recycling.

In contrast to ascorbate, both the non-protein thiol and the total glutathione contents increased in pea leaf discs exposed to subtoxic or slightly toxic concentrations of the xanthene dyes under illumination. The maximal increases were found after 24 h (Fig. 2), later the thiol and GSH levels slowly returned to control levels. However, at the very toxic 1 mM eosin concentration a dramatic GSH loss was found. Similar results were obtained when GSH levels were calculated on dry weight basis (data not shown). In darkness, the GSH content of untreated leaf discs slightly decreased as observed earlier in other plants [20]. The xanthene dyes did not alter the total glutathione levels in darkness as compared to dark controls.

Increases in GSH levels were often observed in plants in response to oxidative stimuli [14,21,22]. The rate-limiting step of GSH biosynthesis is cata-

Fig. 2. Changes in the total glutathione level of pea leaf discs incubated with eosin or rose bengal at various concentrations for 24 h under illumination ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$) preceded by 24 h preincubation in darkness. The values presented are percentages of the glutathione level in illuminated control leaf discs ($0.46 \pm 0.04 \mu\text{mol glutathione g FW}^{-1}$). Means of three independent experiments $\pm$ S.D. are shown. Symbols: $\circ$, illuminated leaf discs; and $\bullet$, dark incubations.

Table 1

Activities of ascorbate peroxidase (AP), glutathione reductase (GR), and glutathione *S*-transferase (GST) in pea leaf discs exposed to various concentrations of eosin^a

Eosin concentration	Enzyme activities (% of illuminated control) ^b		
	AP	GR	GST
<i>In the light</i>			
1 μM	116 $\pm$ 15	118 $\pm$ 17	103 $\pm$ 7
10 μM	159 $\pm$ 9	151 $\pm$ 16	121 $\pm$ 13
100 μM	135 $\pm$ 14	156 $\pm$ 10	123 $\pm$ 5
1 mM	39 $\pm$ 5	34 $\pm$ 2	20 $\pm$ 2
<i>In darkness</i>			
10 μM	75 $\pm$ 6	127 $\pm$ 11	82 $\pm$ 8
1 mM	67 $\pm$ 9	95 $\pm$ 4	80 $\pm$ 9

^a Leaf discs were preincubated in darkness for 24 h then illuminated ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h. Parallel incubations were carried out in darkness. Means of three different experiments $\pm$ S.D. are shown.

^b Enzyme activities in illuminated control leaves were $2.6 \pm 0.3 \mu\text{mol ascorbate g FW}^{-1} \text{min}^{-1}$, $1.5 \pm 0.1 \mu\text{mol NADPH g FW}^{-1} \text{min}^{-1}$, and $0.61 \pm 0.7 \mu\text{mol conjugate g FW}^{-1} \text{min}^{-1}$ for AP, GR and GST, respectively, ($n = 5$).

Fig. 3. Ascorbate levels in pea leaf discs exposed to various concentrations of monuron. All discs were preincubated for 24 h in darkness then illuminated ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$), except in the parallel dark incubations. Means of three independent experiments $\pm$ S.D. are shown. Symbols, illuminated discs: $\circ$, control; $\square$, 10 μM monuron; and $\diamond$, 100 μM monuron. In darkness: $\bullet$, control; $\blacksquare$, 10 μM monuron; and $\blacklozenge$, 100 μM monuron.

lyzed by the enzyme γ -glutamylcysteine synthetase [21]. A recent report demonstrated the rapid post-transcriptional activation of this enzyme during various plant stress responses in *Arabidopsis* suspension cultures. Discrete signal transduction pathways permit discrimination between different stress effects [22]. To meet the increased demand for the intermediate cysteine the assimilatory sul-

fate reduction is also activated in plants exposed to stress effects [21]. The effects of singlet oxygen generators on these biosynthetic pathways are not known.

In our experiments, the total glutathione content accounted for $92 \pm 5\%$ ($n = 4$) of the non-protein thiol level in the untreated leaves. Alterations of the non-protein thiol level after dye treatments were nearly identical to those of the total glutathione (GSH + GSSG) level (data not shown). These results showed that no substantial increase in the ratio of GSSG to GSH was brought about by the xanthene dyes.

Eosin significantly induced also the activities of AP, GR and GST after 24 h in the light at 10–100 μM concentrations, but at 1 mM it strongly inhibited each enzyme activity (Table 1). After 48 and 72 h incubations the activities shifted toward control values (data not shown). The induction of the enzymes was light-dependent (Table 1). The induction of AP, GR and GST was already observed earlier in various plants exposed to oxidative stress [1,13,23]. It is interesting to note that rose bengal treatments led to the accumulation of salicylic acid and the development of systemic induced resistance in tobacco leaves [7].

3.2. Monuron

In contrast to xanthene dyes, monuron treatments substantially decreased the ascorbate level in pea leaf tissue in a light-dependent manner. After 24 h of illumination, 100 μM monuron, which caused weak visible bleaching, reduced the ascorbate level by 88% (Fig. 3). In the darkness, the ascorbate level decreased only by 23%. These results show that the ascorbate pool was depleted by a photodynamic reaction, but further studies are necessary to confirm the role of singlet oxygen in this process. It is conceivable that the decrease of ascorbate level by monuron was mediated not only by degradation but also by changes in the ascorbate biosynthesis. A substantial amount of new information has been published recently on the biosynthetic pathways of ascorbate [24,25], but no information is available on the effects of singlet-oxygen generating compounds on these pathways. No significant increase of DHA level was found neither in dark nor in light experiments (data not shown).

In contrast to the xanthene dyes, monuron treatments led to a slow decrease of GSH content in pea leaf tissues incubated in the light. At 100 μM concentration monuron caused a significant decrease of GSH only after 48 h of illumination (to 62% of control), but after 72 h the GSH levels shifted back toward control values. At 10 μM monuron concentration no effect was found. In dark incubations 100 μM monuron did not change significantly the GSH levels as compared to dark controls. Changes of non-protein thiol levels were similar to those of total glutathione levels after monuron treatments (data not shown).

3.3. Tetrapyrrole accumulation

Applications of both acifluorfen (at 10 μM or higher concentrations) and ALA (at 1 mM and higher concentrations) led to visible bleaching symptoms on pea leaf discs after 24 h of illumination. The photodynamic herbicides did not cause any visible symptoms in dark incubations during a 96 h experimental period.

In the light, the exposure of leaf discs to 100 μM acifluorfen strongly decreased the ascorbate level (to 31% of control after 48 h of illumination) in accordance with earlier results obtained with cucumber [26]. ALA (1 mM) decreased the ascorbate level only by 18% after 72 h of incubation. The herbicides did not modify the ascorbate level of pea leaf tissues in dark incubations. Acifluorfen treatments (10–100 μM) decreased slightly the DHA levels in the light (data not shown). In contrast, DHA levels were not influenced significantly by ALA treatments.

Acifluorfen (10–100 μM) markedly increased the GSH level in illuminated pea leaf discs. In darkness only 100 μM acifluorfen elevated the GSH level (Fig. 4). Changes of non-protein thiol levels were similar to those of total glutathione levels after acifluorfen treatments (data not shown). GSH accumulation was already observed in acifluorfen-treated plant tissues [23,27]. Our results showed that the elevation of GSH level by acifluorfen in pea leaf discs was only partially light-dependent. It is known that during the degradation of acifluorfen in plants, the first cleavage product forms conjugates with GSH or homoglutathione [28]. It is possible that the plant tissue synthesize more GSH also in the darkness in order to meet greater GSH demand as a result of detoxification reactions. Our results confirm those of Knörzer et al. [13] who also found different responses of ascorbate and GSH in soybean cells exposed to the peroxidizing herbicide oxyfluorfen.

GSH levels were considerably elevated only by high ALA concentrations (1–3 mM). After 48 h of illumination the GSH content rose to 265% of the control in the presence of 3 mM ALA (Fig. 5). In darkness, ALA (0.1–3 mM) exerted no effect on GSH levels. Changes of non-protein thiol levels were similar to those of total glutathione levels after ALA treatments (data not shown). The effects of ALA on the enzymes of GSH biosynthesis are not known yet.

Fig. 4. Effect of acifluorfen on the total glutathione level in pea leaf discs. All discs were preincubated for 24 h in darkness then illuminated ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$), except in the parallel dark incubations. Means of three independent experiments $\pm$ S.D. are shown. Symbols: ○, control; ▼, 1 μM acifluorfen; ■, 10 μM acifluorfen; and ◆, 100 μM acifluorfen.

Fig. 5. Effect of 5-aminolevulinic acid (ALA) on the total glutathione level in pea leaf discs. All discs were preincubated for 24 h in darkness then illuminated ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$), except in parallel dark incubations. Means of three independent experiments $\pm$ S.D. are shown. Symbols, illuminated discs: ○, control; ◇, 100 μM ALA; △, 1 mM ALA; and ▽, 3 mM ALA. In darkness: ●, control.

In summary, both ascorbate and GSH levels were strongly altered in pea leaf discs by very different singlet oxygen generating reactions. Ascorbate levels markedly decreased in all treatments independently of the site (or mode of action) of singlet oxygen generation. The xanthene dyes are known to act predominantly in the cytoplasm of plant cells [6]. Monuron exerts its oxidative effect in the chloroplasts [6]. Tetrapyrrole derivatives initially accumulate in the plastids (predominantly in the chloroplasts) after acifluorfen and ALA treatments [4,9,29], but the accumulated tetrapyrroles may subsequently leak out of the plastids and photosensitize other cellular compartments [29]. In contrast to ascorbate, the foliar GSH levels were considerably increased by singlet oxygen generators (except by monuron). Singlet oxygen production in both the cytoplasm (by xanthene dyes) and in the plastids (by acifluorfen and ALA) led to marked GSH accumulations. Further studies are necessary to elucidate the effects of singlet oxygen generators on the biosynthetic pathways of both ascorbate and GSH.

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