**ANTIOXIDANT RESPONSES OF PEA (*PISUM SATIVUM* L.) PROTOPLASTS**

by

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## ABSTRACT

Freshly isolated protoplasts from pea leaves were used to investigate the responses of antioxidant enzymes to oxidative stress. Two cultivars, Progress (tolerant) and Nugget (sensitive), that have differing resistance with respect to oxidative stress at the whole plant level were used. Sulfite and the superoxide generating herbicide, paraquat, were used as the oxidants. Final sulfite concentrations during photosynthetic incubations ranged from 1.5 mM to 30.0 mM. During the polarographic estimation of photosynthesis, $CO_2$-dependent $O_2$ evolution did not decrease. At sulfite concentrations of 3.0 mM or less, light-dependent $O_2$ evolution increased and was probably due to a concomitant $SO_2$-dependent $O_2$ evolution. Photosynthesis determined as $^{14}CO_2$ fixation was not increased at these low concentrations of sulfite. Concentrations greater than 7 mM sulfite inhibited photosynthetic $^{14}CO_2$ fixation. No difference in these responses was found between the two cultivars.

At 0.1 $\mu$M paraquat, the relative resistance to oxidative

stress was reversed compared to previous studies at the whole plant level. With the tolerant cultivar, activity of the plastid antioxidant enzyme, glutathione reductase, increased after a three-hour exposure. Changes in the steady state level of glutathione reductase protein, as judged by immunoblots, did not correlate with the observed changes in enzyme activity. No change in the *de novo* synthesis of glutathione reductase occurred over the same period as a consequence of paraquat application. A mechanism, unrelated to oxygen free radical scavenging, may contribute to the relative tolerance to low concentrations of paraquat. On the other hand, after an eight-hour exposure to 0.1 mM PQ in the presence of Gamborg's basal salts, superoxide dismutase activity of Progress protoplasts was enhanced 288% above the preexposure levels while glutathione reductase activity decreased 70% and ascorbate peroxidase activity decreased 90%. The relationship of these changes to oxidative damage to the photosynthetic machinery remains to be assessed.

## DEDICATION

This dissertation is dedicated to my wife Vassiliki and to our son Georgio.

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| | |
|---|---|
| Ab | antibody |
| AFR | ascorbate free radical |
| ASC | ascorbate |
| AP | ascorbate peroxidase |
| BSA | bovine serum albumin |
| Chl | chlorophyll |
| cv | cultivar |
| DHA | dehydroascorbate |
| DHAR | dehydroascorbate reductase |
| EDTA | ethylenediaminetetraacetic acid |
| HEPES | N-2-hydroxyethylpiperazine N'-2-ethane sulfonic acid |
| hpi | hours post isolation |
| GR | glutathione reductase |
| GSH | glutathione, reduced form |
| GSSG | glutathione, oxidized form |
| KDa | KiloDalton |
| $\mu$E | microEinstein |
| MDA | monodehydroascorbate |
| MES | 2-(N-morhpolino)ethene sulfonic acid |
| MW | molecular weight |
| NADPH | nicotinamide dinucleotide phosphate, reduced form |
| NBT | nitro blue tetrazolium |
| PQ | paraquat |
| PS I | photosystem I |
| PVPP | polyvinylpolypyrrolidone |
| SDS-PAGE | sodium dodecyl sulfate - polyacrylamide gel electrophoresis |
| SOD | superoxide dismutase |

# INTRODUCTION

Reductively activated species of oxygen that can form in biological systems are far more reactive than ground state oxygen. These species include the superoxide radical $O_2^-$, hydrogen peroxide ($H_2O_2$), and hydroxyl radical $HO^{\cdot}$. In addition to those short-lived "free" oxygen species, there are also organic derivatives corresponding to the reduced species of oxygen. These latter species are regarded as the "long-life" species of active oxygen (Asada and Takahashi, 1987). Oxygen-activating reactions have to be controlled in the living cell in order to avoid detrimental effects since the products of these reactions can oxidize many cellular components.

## Oxygen activation and sequestration in different plant cell compartments

Oxygen activation occurs in different compartments and organelles of plant cells. Cell wall bound or associated peroxidases catalyze the hydrogen peroxide-mediated polymerization of phenylpropanoid precursors to lignin. Hydrogen peroxide, in this case, is directly produced in the cell walls (Gross *et al.*, 1977). In peroxisomes hydrogen peroxide is generated during glycolate oxidation (Selitch, 1972). Catalase degrades $H_2O_2$ rapidly and prevents any $H_2O_2$

dependent oxidations. In mitochondria during cytochrome *c* oxidase-mediated oxygen uptake, some $O_2^-$ is produced via the interaction of dioxygen with NADH dehydrogenase and ubiquinone-cytochrome *b* (Rich and Bonner, 1978; Boveris *et al.*, 1978).

Chloroplasts are organelles with high rates of oxygen turnover. Dioxygen, in normally functioning chloroplasts, is photoreduced to superoxide radical in the chloroplasts through the Mehler reaction. The most probable site is at the reducing site of PS I of the thylakoids, at the expense of reduced ferredoxin (Elstner, 1982). When NADP (derived from the oxidation of NADPH during the operation of the Calvin cycle) is not available in adequate amounts, dioxygen is reduced instead. The superoxide anion may be produced at either side of the thylakoid membranes and/or in the aprotic interior of the thylakoid membranes (Asada and Takahashi, 1987; Takahashi and Asada, 1988) and diffuses to both the lumen and the stroma sides of the thylakoid. Its production increases when the integrity of the thylakoids is damaged (Takahashi and Asada, 1982). Alternatively, superoxide can be produced by the PS I non-heme Fe-S center (Elstner, 1982). The superoxide is disproportionated to hydrogen peroxide in a reaction mainly catalyzed by superoxide dismutase (SOD). Cu- and Zn- containing SODs are localized both in the lumen and the stroma (Jackson *et al.*, 1978; Hayakawa *et al.*, 1984).

Hydrogen peroxide is itself mildly reactive. One mode of its toxicity in the chloroplast is the oxidation of -SH groups of enzymes with their subsequent inactivation (Charles and Halliwell, 1980). It also initiates a chain reaction that generates highly reactive free radical species. It acts as a Hill oxidant (Anderson *et al.*, 1983) with its subsequent reduction to water during the pseudo-cyclic photosynthetic electron flow. Consequently it diverts photoreductant from carbon fixation (Nakano and Asada, 1980). Tanaka *et al.* (1982b) showed that hydrogen peroxide produced in leaves during sulfur dioxide ($SO_2$) fumigations of plants oxidizes and inactivates -SH containing light-modulated enzymes of the Calvin cycle (e. g., fructose 1,6 bis-phosphatase). The hydrogen peroxide produced in the chloroplast is reduced to water through the ascorbate-glutathione cycle (Foyer and Halliwell, 1976) as shown in Fig. 1.

**Antioxidants can protect against environmentally imposed oxidative stress**

Aerobic organisms constantly cope with the potential of attack by oxyradicals. The toxicity of an externally imposed biotic or abiotic oxidative stress can be partly attributed to the overriding of existing resistance mechanisms. Only when those mechanisms are overwhelmed would injury occur. High activities of the ascorbate-glutathione cycle enzymes

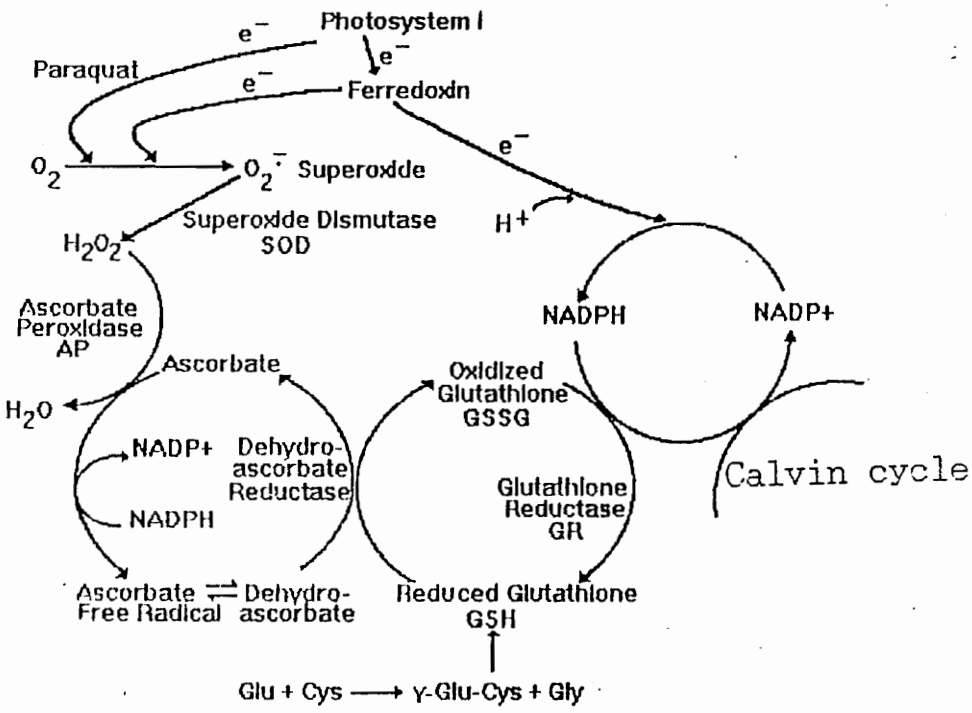
Photosystem I
e⁻
Paraquat
e⁻
Ferredoxin
e⁻
O2
O2⁻ Superoxide
Superoxide Dismutase
SOD
H+
H2O2
Ascorbate
Peroxidase
AP
NADPH
NADP+
Ascorbate
Oxidized
Glutathione
GSSG
H2O
NADP+
Dehydro-
ascorbate
Reductase
Glutathione
Reductase
GR
Calvin cycle
NADPH
Ascorbate
Free Radical
Dehydro-
ascorbate
Reduced Glutathione
GSH
Glu + Cys → γ-Glu-Cys + Gly

Fig. 1 Production and scavenging of superoxide and hydrogen peroxide in illuminated chloroplasts. At the reducing side of photosystem I (PSI) dioxygen is univalently reduced to the superoxide anion radical. This radical disproportionates to form hydrogen peroxide and dioxygen. The reaction is catalyzed by superoxide dismutase. The hydrogen peroxide is reduced to water by AP. Ascorbate is regenerated from the primary oxidation product (ascorbate free radical, AFR) of AP reaction and from its disproportionation product (dehydroascorbate, DHA) by MDA and DHA reductases, respectively. The necessary electrons are derived from PSI via glutathione (GSH/GSSG) re-reduction which is catalyzed by GR (adapted from figure 10.12 of Asada and Takahashi, 1987).

ascorbate peroxidase (AP), glutathione reductase (GR) and superoxide dismutase (SOD) have been correlated with increased resistance to different stresses (Jansen *et al.*, 1990; Wise and Naylor, 1987). Leaves of peas grown at high light intensities contained high levels of ascorbate (ASC), ascorbate preoxidase (AP), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) compared to those grown at low intensities (Gillham and Dodge, 1987). Increased antioxidants and antioxidant enzyme activity may serve to counteract the increased photodynamic damage potential at high light levels.

**Sulfite as an oxidant and its effect on chloroplast metabolism**

The gaseous pollutant $SO_2$ has been shown to produce a series of activated oxygen species. The predominant, hydrated form of gaseous $SO_2$ in the stroma is sulfite ($SO_2^-$). Approximately 1 ppm of $SO_2$ concentration in the air is considered to be in equilibrium with 1 mM of sulfite in the plastid (Alscher *et al.*, 1987b). The subsequent reactions and effects on metabolism are reviewed by Alscher *et al.* (1987b) and Asada (1980). Damage is mostly light-dependent. Asada and Kiso (1973) showed an oxidative chain reaction of sulfite to be initiated by the superoxide produced in chloroplasts. Since the photooxidation rate of the sulfite in the thylakoids is higher than the rate of superoxide production (as

determined by photoreduction of cytochrome *c*, it was concluded that the former proceeds as a chain reaction.

Proposed reactions:

initiation reaction

$SO_3^{-} + O_2^{-} + 3\ H^+ \rightarrow HSO_3^{\cdot} + 2\ HO^{\cdot}$

chain propagation reactions:

$SO_3^{-} + HO^{\cdot} + 2\ H^+ \rightarrow HSO_3^{\cdot} + H_2O$

$HSO_3^{\cdot} + O_2 \rightarrow SO_3 + O_2^{-} + H^+$

$HSO_3^{\cdot} + HO^{\cdot} \rightarrow SO_3 + H_2O$

hydration / sulfate production

$SO_3 + H_2O \rightarrow SO_4^{2-} + 2\ H^+$

chain termination

$2HSO_3^{\cdot} \rightarrow SO_3 + HSO_3^{-} + H^+$

$O_2^{-} + O_2^{-} + 2\ H^+ \rightarrow H_2O_2 + O_2$

$HO^{\cdot} + HO^{\cdot} \rightarrow H_2O_2$

The superoxide, hydroxyl, and sulfur trioxide radicals produced during the chain-propagation reactions are the chain carriers. The disproportionations of sulfur trioxide and of superoxide radicals and the recombination of the hydroxyl radicals are the chain termination reactions (Mottley *et al.*,

1982). The increased production of superoxide as well as of the other radicals impose an additional oxidative stress on the chloroplast and may cause phooxidative damage when the chain oxidation of sulfite is not adequately suppressed. Destruction of cellular components such as chlorophyll and carotenoids can occur (Peiser and Yang, 1979). Whole-chain electron-transport (water to NADP) is reduced when leaves are exposed to $SO_2$ (Shimazaki *et al.*, 1984) possibly via an interaction of sulfite with a component of PS II. Tanaka (1982a) showed that hydrogen peroxide accumulates in the chloroplast of $SO_2$-fumigated spinach leaves. In turn hydrogen peroxide oxidizes -SH containing-light modulated enzymes of the Calvin cycle (Tanaka *et al.*, 1982b).

**PQ as a pro-oxidant in the green cell plastid**

The bipyridylium herbicide, paraquat (PQ), or methyl viologen (1,1'-dimethyl-4,4'-bipyridilium dicloride) is the active ingredient of many, commercially available, broad-spectrum herbicides (Hassan, 1984). It is water soluble, ceases photosynthesis rapidly, and induces bleaching and desiccation in all plant green material with which it comes in contact (Summers, 1980).

Reduction and oxidation of PQ in the illuminated chloroplast

The mode of action of PQ is summarized by Dodge (1989).

Zweig *et. al.*, (1965) working with isolated chloroplasts showed that PQ competes for electron flow with ferredoxin. As a consequence $NADP^+$ is not reduced and this leads to a rapid cessation of carbon dioxide incorporation (Harris and Dodge, 1972b). PQ possesses a redox potential of - 446 mV which is very close to that of soluble ferredoxin ($E_o$ = -420 Mv) (Summers, 1980). Experimental data suggested that PQ diverts electrons from the iron sulfur centers A and B of PSI (Warden and Bolton, 1974). Those centers have redox potentials around -590 and -530 mV respectively.

For the rapid action of PQ, light and oxygen are needed (Mees, 1960). Light is required for photosynthetic electron flow and oxygen for the production of toxic oxygen radicals.

The PQ divalent cation during its one electron reduction becomes a radical monovalent cation. This radical in the presence of oxygen will react with $O_2$ with a reaction rate constant of around $7.7 * 10^8$ $M^{-1}$ $s^{-1}$ and can produce a $\mu M$ concentration of superoxide anion ($O_2^-$) within the chloroplast grana (1973). This is a PQ autoxidation reaction. During this process of oxygen reduction, PQ is oxidized back to its divalent cationic ground state and is ready again to capture an electron from PS I. Hence, the herbicide acts as a cyclic catalyst. In the absence of oxygen, reduction of PQ by one electron yields a stable colored cation (Zweig *et al.*, 1965).

Production of oxygen free radicals during PQ action

Superoxide anion production occurs to a limited extent under normal unstressed circumstances (the Mehler reaction) and can account for up to 10-20% of the total photosynthetic electron flow (Furbank, 1984). Under these conditions, rates of superoxide production are determined by the ratio of reduced to oxidized ferredoxin (Asada and Nakano, 1978). The Mehler reaction also involves the production of hydrogen peroxide ($H_2O_2$) by a two step process (Allen, 1975).

Superoxide and hydrogen peroxide can interact in a Fenton reaction which involves cyclic interconversion between the two redox forms of iron ($Fe^{3+}$ / $Fe^{2+}$). During this reaction the highly reactive (and damaging) hydroxyl radical ($OH^{\cdot}$) is produced. Under normal circumstances plant cell iron is stored as phytoferritin. Superoxide may promote the release of iron from this storage protein (Saito *et al.*, 1985) thus exacerbating hydroxyl radical production via the Fenton reaction.

Overall, the interaction of PQ with chloroplast electron flow, results in the elevation of superoxide by as much as 10-20 fold (Asada *et al.*, 1977).

Non-catalytic and catalytic scavenging of oxygen free radicals

Superoxide can be directly scavenged by ascorbate at a rate constant of $2.7 * 10^5\ M^{-1}\ s^{-1}$. Ascorbate is present within

the chloroplast stroma in a concentration of around 20 mM. Dehydroascorbate thus produced will be rereduced through the action of dehydroascorbate reductase which uses reduced glutathione as an electron donor.

Additionally, superoxide can dismutate without catalysis at a relatively high rate. The end products of this reaction are hydrogen peroxide and ground state oxygen. This spontaneous dismutation is highly pH-dependent, since it involves the protonation of $O_2^-$ to $HO_2\cdot$ with a $pK_a$ of 4.8 (Halliwell and Gutteridge, 1985). Under the alkaline conditions of the stroma of illuminated chloroplasts (pH=8), it is likely that the majority of the physiologically relevant dismutation takes place under the catalytic influence of the enzyme superoxide dismutase. Under these conditions the rate of the uncatalyzed dismutation of superoxide is at least four orders of magnitude lower than the $V_{max}$ of SOD ($2 * 10^9\ M^{-1}\ s^{-1}$) (Getzoff *et al.*, 1992) and is of little biological importance. Indeed, the catalytic efficiency of SOD approaches that of diffusion limitation. In the absence of PQ, SOD sufficiently maintains the superoxide concentration at a steady state level of around $6.0 * 10^{-9}$ M. It seems that during the addition of PQ, SOD and the rest of the glutathione ascorbate cycle enzymes are overtaxed and the generated reactive oxygen radicals are not effectively scavenged.

Broader physiological effects of PQ

PQ can indirectly cause the inhibition of photosynthetic carbon dioxide incorporation but above all it promotes the generation of massive amounts of oxygen free radicals (mostly $OH^{\cdot}$). The hydroxyl radical reacts indiscriminately and oxidatively destroys membrane unsaturated fatty acids, a number of protein amino acids including methionine and histidine, deoxyribose sugar and thymine of DNA, and a number of aromatic compounds. The most pronounced effect at the whole cell level involves membrane damage. This damage involves the plasmalemma (Baur *et al.*, 1969), the tonoplast (Harris and Dodge, 1972a), the chloroplast envelope (Dodge and Law, 1974), and the thylakoid membrane (Harvey and Fraser, 1980). The consequence of these events is that cellular disruption follows, involving damage due to osmotic and pH changes and the release of toxic compounds from the cell vacuole. The rapid bleaching of green plant tissues exposed to PQ involves the oxidative destruction of chlorophyll (Harvey and Harper, 1982) to pheophytin.

Crude cell-free extracts from plants, animals, and bacterial origins have been shown to reduce the bication PQ to the monocation paraquat in the presence of NADPH or an NADPH-generating system (i.e., glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^{+}$) (Hassan, 1984). The enzyme that reduces paraquat in the cytosol is a diaphorase-

type enzyme that is specific for NADPH (Baldwin *et al.*, 1975; Hassan and Fridovich, 1978). The mechanism of action of PQ at high concentrations in the dark is thought to involve the reoxidation of free radicals arising from reduction of the PQ by the respiratory electron transport chain (Harris and Dodge, 1972b).

**Inducibility and regulatory aspects of antioxidants under increased oxidative stress**

Exposure of maize seedlings to an atmosphere containing 75% $O_2$ resulted in a three-fold increase in GR activity but not in increased SOD activity (Foster and Hess, 1982) nor in the peroxisomal enzymes glycolate oxidase and peroxidase (Foster and Hess, 1980). An increase in AP activity in spinach leaves was detected during fumigation with $O_3$ (Tanaka *et al.*, 1985). In another study, total glutathione accumulated more rapidly followed by an increase in GR activity in a tolerant variety of pea "Progress" as compared to a more sensitive variety "Nugget" (Madamanchi and Alscher, 1991). Increased amounts of the protein and an increase in the population of SOD mRNAs was found in the tolerant variety (Madamanchi, Alscher and Cramer, manuscript in preparation). Tanaka *et al.* (1988b) found increases in GR activity and *de novo* synthesis of protein in ozone-fumigated spinach leaves.

Gillham and Dodge (1987) showed that pretreatment of pea

leaves with high light intensities resulted in higher activities of AP, dehydroascorbate reductase, GR and ascorbate compared with leaves grown at lower light intensities. These elevated levels of antioxidants were linked to an increased resistance to PQ.

A PQ-resistant *Conyza bonariensis* biotype showed cross resistance to $SO_2$ and to herbicides which generate oxidants (Shaaltiel *et al.*, 1988b). $SO_2$ resistant *Lolium perenne* strain was 10 times more tolerant to PQ compared to a sensitive strain. The resistant strain had 70% more SOD and 20% more GR constitutive activities compared to the sensitive strain (Shaaltiel *et al.*, 1988b). In another study Tanaka *et al.* (1988a) found that tobacco plants, regenerated from a PQ tolerant callus, had constitutive SOD activity significantly higher compared to non tolerant ones and were more resistant to absorbed $SO_2$. Activities of other antioxidant enzymes were not elevated, however. On the other hand, activities of stromal AP, SOD and GR highly correlate with oxidative stress resistance of different origins (Shaaltiel and Gressel, 1986; Jansen *et al.*, 1990). Crosses between the two biotypes of *Conyza bonariensis* established previously to have differential resistance to PQ were carried out. Elevated activities of the three scavenging stromal enzymes co-segregated with resistance in both the $F_1$ and $F_2$ generations. Malan *et al.* (1990) working with different maize inbreds, found that drought and

photooxidative herbicide tolerances were both significantly correlated with high levels of Cu,Zn SOD and with GR activities. Additionally, high levels of SOD or GR alone did not correlate with any of the tolerances. Indirect evidence that the activity of more than one antioxidant enzyme has to increase in order for increased resistance to be manifested comes from the study of Tepperman and Dunsmuir (1990). They transformed plants with plastidic SOD (more copies of SOD gene resulting in increased expression of the protein). Transgenic plants were not more resistant to superoxide toxicity. Jansen *et al.* (1990) propose that either a very tight linkage exists or one dominant nuclear gene controls resistance by pleiotropically controlling the levels of all three aformentioned stromal enzymes.

In *Escherichia coli* a regulon, which is coordinately controlled by a single protein factor (the oxyR protein), has been described and is responsive to oxidative stress induced by $H_2O_2$. Another similar regulon (soxR) seems to be responsive to superoxide (Storz *et al.*, 1990). These regulons code for proteins such as SOD and GR *inter alia*. The resistance circuitry in a plant cell may be more complex compared to a bacterial one.

<u>Participation of glutathione in induction of plant genes during oxidative stress</u>

Increased levels of GSH were detected during $SO_2$ exposure of a tolerant pea variety (Progress) while no increase was found in a susceptible one (Nugget), (Alscher *et al.*, 1987a). An increase in whole leaf glutathione preceded an increase in GR activity of whole leaf extracts from peas exposed to $SO_2$ (Madamanchi and Alscher, 1991). The time course of these responses suggests that events that take place during the biosynthesis of glutathione, may be associated with the signal needed for the increased expression of genes and induction of antioxidant enzymes (Alscher *et al.*, 1991). Indeed, it has been shown that GSH interacts with a *cis*-acting element involved in regulating the quantitative expression of a bean chalcone synthase promoter (Dron *et al.*, 1988). Additionally, GSH causes a massive and selective induction of plant defense genes (Wingate *et al.*, 1988). On the other hand, Herouart *et al.* (Hérouart *et al.*, 1993) reported that the transcription of a gene encoding a cytosolic Cu,Zn SOD from *Nicotiana plumbaginifolia* is activated by GSH (as well as by the reducing thiol compounds cysteine and dithiothreitol). So it is plausible to propose that GSH at some point participates in redox stress perception, signal transduction and induction of antioxidant enzymes. Whether the increase in glutathione is a primary or a secondary event cannot be determined at this moment.

**Reduction and *de novo* synthesis of glutathione**

Glutathione synthetase participates in glutathione biosynthesis and catalyses the addition of glycine to the C-terminal site of γ-glutamylcysteine. Its activity is equally distributed between the chloroplast and the cytoplasmic fractions of the pea leaf cell (Klapheck *et al.*, 1987). A 3-fold increase in synthesis of glutathione was demonstrated in cases of catalase deficiency during oxidative stress (Smith *et al.*, 1985). This increase was accounted for by a rise in both the chloroplast and the non-chloroplast fractions of oxidized glutathione with the respective levels of GSH at the same levels. The newly synthesized glutathione appeared initially in the oxidized form (GSSG) and was converted slowly to GSH. The product of glutathione biosynthesis is GSH and not GSSG (Rennenberg, 1982). It was concluded that the accumulation of GSSG must have been caused by oxidation of newly synthesized GSH, and the appearance of GSH by the action of GR. Increased biosynthesis of antioxidants during oxidative stress precedes increase of GR activity (Schmidt and Kunert, 1986; Madamanchi and Alscher, 1991). Artificial elevation of ascorbate or glutathione, in the absence of oxidative stress, did not increase the activities of the antioxidant enzymes ascorbate free radical reductase, dehydroascorbate reductase and GR (Hausladen and Kunert, 1990).

Hell and Bergmann (1990) showed that partially purified

γ-glutamylcysteine synthetase (γ-GCS) (another enzyme participating in glutathione biosynthesis) is effectively inhibited by physiological concentrations of GSH which suggests a feedback inhibition of the enzyme. It can, thus, be proposed that a mechanism for increased levels of GSH can be the release of the feedback inhibition of γ-GCS when GSH becomes oxidized to GSSG under oxidative stress.

In suspention cultures of *Arabidopsis thaliana*, the development of aminotriazole-induced mild oxidative stress (via inactivation of catalase) was monitored by the oxidative inactivation of aconitase (May and Leaver, 1993). Addition of 2 mM aminotriazole to cells preincubated with 50 μM L-buthionine sulfoximine (BSO, a specific GSH biosynthesis inhibitor) for 15 hours lead to a rapid loss of aconitase activity and significant accumulation of products of lipid peroxidation (malondialdehyde). Then BSO was removed and cells were allowed to recover. During this recovery period a strong correlation was demonstrated between the level of reduced GSH and the degree of protection against oxidative injury. May and Leaver concluded that induction of GSH synthesis by an oxidative stimulus plays a crucial role in determining the susceptibility of cells to oxidative injury.

**Preconditioning and induction of antioxidant enzymes**

It has been shown in bacteria that antioxidant enzymes

may be induced and that increased resistance to oxidative stress can be conferred to higher subsequent oxidative stress when they initially experience a low oxidative stress (Christman *et al.*, 1985). Similarly, it was shown that poplar plants increased the SOD content in their young leaves when grown for several days in a low concentration of $SO_2$ (0.1 ppm). Those preconditioned plants showed a higher tolerance to a subsequent, high $SO_2$ concentration (2.0 ppm), (Tanaka and Sugahara, 1980).

**Protoplasts as a system for study of stress-induced responses**

Intact tissue constitutes a complex system where cuticle and cell cohesion hamper the study of solute transport as well as homogeneous incorporation of metabolic precursors and stressor molecules. Protoplasts appear to be a convenient and more homogeneous system (March and Tremolieres, 1985). Protoplast isolation efficacy and viability depends on growth conditions of the initial plant material, such as leaf age and position, light regime and nutrition (Rees *et al.*, 1985). However, the question arises as to what extent protoplasts, which are free of tissue restrictions, deprived of the cell wall, and subjected to an unusual osmotic stress, are metabolically similar to the original material. Rees *et al.* (1985) found that isolated soybean protoplasts had photosynthetic rates ($CO_2$-dependent $O_2$ evolution) which

compared favorably with rates of cells from similarly aged plants at the same saturating conditions of light and bicarbonate. Nevertheless protoplast photosynthetic rate was approximately one third of intact tissue. Wheat leaf protoplasts had half the photosynthetic rate of the tissue from which they were derived (McCloskey and Bayer, 1990). The above researchers determined protoplast variable chl a fluorescence decay and proposed that a decrease of the electron transport rate at the $Q_A$ / $Q_B$ level is one of the factors diminishing the photosynthetic potential of protoplasts. Mieth *et al.* (1986) found that the isolation procedure alone was adequate to induce a stress response to isolated protoplasts. This is not true for parsley protoplasts (Dangl *et al.*, 1987). Cassels *et al.* (1980) showed that tobacco protoplasts, which produced ethylene in excess of 30 nL/g fresh weight, had less than 60% survival. Ishii (1990) showed that rice cells, treated with xylanase and pectin lyase, produced superoxide radical and suggested that oxidative membrane damage in plasma membranes may be involved in protoplast damage. This stress may represent an additional burden to the antioxidant mechanism of the cells which are going to be treated with oxidative compounds.

Our search for a stress response signal started within the plastid. It was the overall objective of this study to

establish an experimental system to address specifically the questions of inducibility and regulation of the plastidic and cytosolic oxidative stress responses, as well as the coordination of those responses between those two subcellular compartments. Two different inducers of oxidative stress were used. They were: 1) sulfite, which is known to generate a series of oxygen free radicals in the chloroplast in the light, and 2) PQ, which promotes the Mehler reaction. Freshly isolated photosynthesizing protoplasts of the resistant and the tolerant pea cultivars [Progress, Nugget respectively; Alscher *et al.*, (1987a)] were used as an experimental system.

## OBJECTIVES

The following specific objectives were formalized in order to resolve questions about the induction and regulation of antioxidant metabolism in plant cells:

1. Standardize the protoplast isolation and purification scheme for both pea cultivars (Progress and Nugget). Establish the time course over which protoplasts show optimal metabolic behaviour and determine control photosynthetic rates under saturating light conditions.

2. Determine; the appropriate photosynthetic assay applicable to the incubation conditions; the particular manifestation of oxidative stress caused by sulfite; the dose response of photosynthesis of both cultivars to increasing concentrations of this oxidant. Determine a sulfite concentration to be used for subsequent applications for the study of antioxidant responses of protoplasts.

3. Determine the appropriate photosynthetic assay applicable to the incubation conditions and oxidative stress caused by PQ and determine the dose response of photosynthesis of both cultivars to increasing concentrations of PQ. Determine a PQ concentration to be used for subsequent

applications for the study of antioxidant responses of protoplasts.

4. Study the response of the activities of the antioxidant enzymes AP, GR and superoxide dismutase to a range of PQ concentrations at the whole cell (protoplast) level.

5. Use the PQ concentration selected in objective 3 to study the time response of the activities of the antioxidant enzymes in protoplasts.

6. Use the PQ concentration selected in objective 3 to study the time response of the activities of the antioxidant enzymes in isolated plastids.

7. Design a representative set of experiments under conditions where changes in activity at the whole cell level of AP and GR were detected (upon addition of PQ) and determine changes at the steady state level of the respective proteins.

8. At a PQ concentration which causes enhanced GR activity, determine changes in *de novo* GR protein synthesis upon addition of PQ.

## MATERIALS AND METHODS

### Plant Material

Seedling growth conditions

Peas (Pisum sativum) cv Progress and cv Nugget were grown in a Conviron or Control Environment Chambers growth chamber according to Walker *et al.* (1987). Growth conditions were: 16 h photoperiod, light rack was set to yield light intensity at plant level of 350 $\mu E\ m^{-2}\ s^{-1}$, temperature was 21 °C during the dark and 24°C during the light periods, respectively. Relative humidity was 70% and 80% during the dark and light periods. Seedlings were grown in synthetic soil (Pro-mix, BX) in fiber pots (6 5/8 X 5 3/8 X 3 inches). 12-14 seeds per pot were sown. Seeds (Wetsel Seed Company, Inc.) were stored in the refrigerator. Plants were watered every other day. Maintenance of the same conditions throughout the experiment was important for uniformity of protoplast preparations (Cassells and Barlass, 1976; Gillham and Dodge, 1987).

Protoplast isolation

Protoplasts were isolated from the leaves of 9-11 day old seedlings by the method of Rothermel and Alscher (1985) and Alscher and Strick (1984) with the following modifications. First node leaves were used. The abaxial surface of the leaves was gently abraded with 320 grit carborundum using

cotton swabs. Leaves were floated in 10 ml of plasmolysis buffer (0.45 M sorbitol, 0.1% BSA, 0.01 M $MgCl_2$, 1 mM $KH_2PO_4$, 0.02 M MES, NaOH/pH=5.5) in a 10 cm diameter shallow Petri dish. When all leaves were abraded, plasmolysis buffer was aspirated out of the dish and replaced with 10 ml digestion buffer (same as plasmolysis plus 5% w/v Driselase - *Basidiomycetes* fungal extract, Sigma, St Louis, MO). Digestion took place on a shaking water bath (30 rpm, 25°C) for 1 h. The digestion buffer was then aspirated off and replaced with "wash" buffer (0.45 M sorbitol, 0.01 M $MgCl_2$, 1 mM $KH_2PO_4$, 5 mM $CaCl_2$, 0.01 M HEPES, NaOH/pH=7.6). Caution was taken not to release the protoplasts during the wash step. The wash buffer was aspirated off and replaced with new wash buffer. Protoplasts were released by swirling the dish or gently shaking individual leaves until the majority of the green tissue floated away from the leaf mass. The protoplast suspension was passed through a steel mesh (Spectra, 55 $\mu$m). All following steps were completed on ice. The filtrate was transferred to a centrifuge tube and spun in a benchtop centrifuge (International Clinical Centrifuge, ICC) with swinging buckets for 4 min., at 800 rpm or ca. 100X*g*. Supernatant was decanted and the remaining fluid was drained by inverting the tubes on a paper towel. A small aliquot (2-3 drops) of resuspension buffer (0.45 M sorbitol, 1 mM $MgCl_2$, 0.1 % (w/v) BSA, 5 mM $CaCl_2$, 0.02 M MES NaOH/pH=6.5 and 28 or

30% (v/v) of 100% Percoll) was added and the pellet was gently resuspended. Then the volume was increased to 10 ml with buffer. On top of the 28 or 30% Percoll buffer, a second 3 ml solution (0.45 M sorbitol, 1 mM $MgCl_2$, 0.1 % (w/v) BSA, 5 mM $CaCl_2$, 0.02 M MES NaOH/pH=6.5) was overlaid taking care not to disrupt the interface. Tubes were spun for 4 min. in the ICC at 1100 rpm (800Xg). A 3 ml sample including most of the intact protoplasts was collected from the interface and transferred to new tubes to which 9 ml of wash buffer was added. Protoplasts were spun for 4 min. at 800 rpm (100 g) in the ICC. Washed protoplasts were resuspended in 1.5 ml of wash buffer and comprised the intact protoplast stock solution.

**Photosynthetic incubation and stress application**

After isolation, intact protoplasts were stored in wash buffer at 4 °C for 1-3 h. Their photosynthetic capacity ($CO_2$ dependent oxygen evolution) was determined. For this and all subsequent oxygen evolution measurements, a Clark-type oxygen electrode (Hansatech Instrument Ltd) was used. Light intensity at the reaction cuvette was set at 600 $\mu E\ m^{-2}\ s^{-1}$ and temperature of the water jacket at 25°C. Light was provided from a fiber optic equipped quartz lamp (Bausch and Lomb, Fiber-Lite). After rates of photosynthesis were determined, aliquots were placed in 2-5 Erlenmayer flasks and incubated

under 420 $\mu E\ m^{-2}\ s^{-1}$ at 25°C and 60 rpm. A 10 cm deep plexiglass water tank, seated just below the light source, served as a heat filter. The light source, during incubation, was a single floodlamp of 150 W. The photosynthetic incubation medium included 10 mM $NaHCO_3$, 330 mM sorbitol, 10 mM $CaCl_2$, 1 mM EDTA, 50 mM HEPES NaOH/pH=7.6 (simple incubation medium). In the incubation flasks chlorophyll concentration was 25-35 $\mu g/ml$. Protoplasts were incubated for an initial period of 20-35 min during which photosynthesis lag time was abolished (for Progress). The first photosynthetic measurement ($CO_2$-dependent oxygen evolution) was taken on samples from all flasks and then the stressor was added at the appropriate concentration. Each single photosynthetic measurement was completed in approximately 10 min. After protoplasts were transferred to the oxygen electrode, they were diluted 1:2 with an equal volume of fresh photosynthetic medium. This dilution minimized the additional effect of PQ on the oxygen evolution rate during the actual period of measurement. Subsequent photosynthetic measurements were taken at 1, 2, and 3 h after the addition of the stressor (Fig. 2).

**Light intensity calibration**

Light intensities at the level of the oxygen electrode reaction vessel were determined by placing a LI-185 (Li-Cor

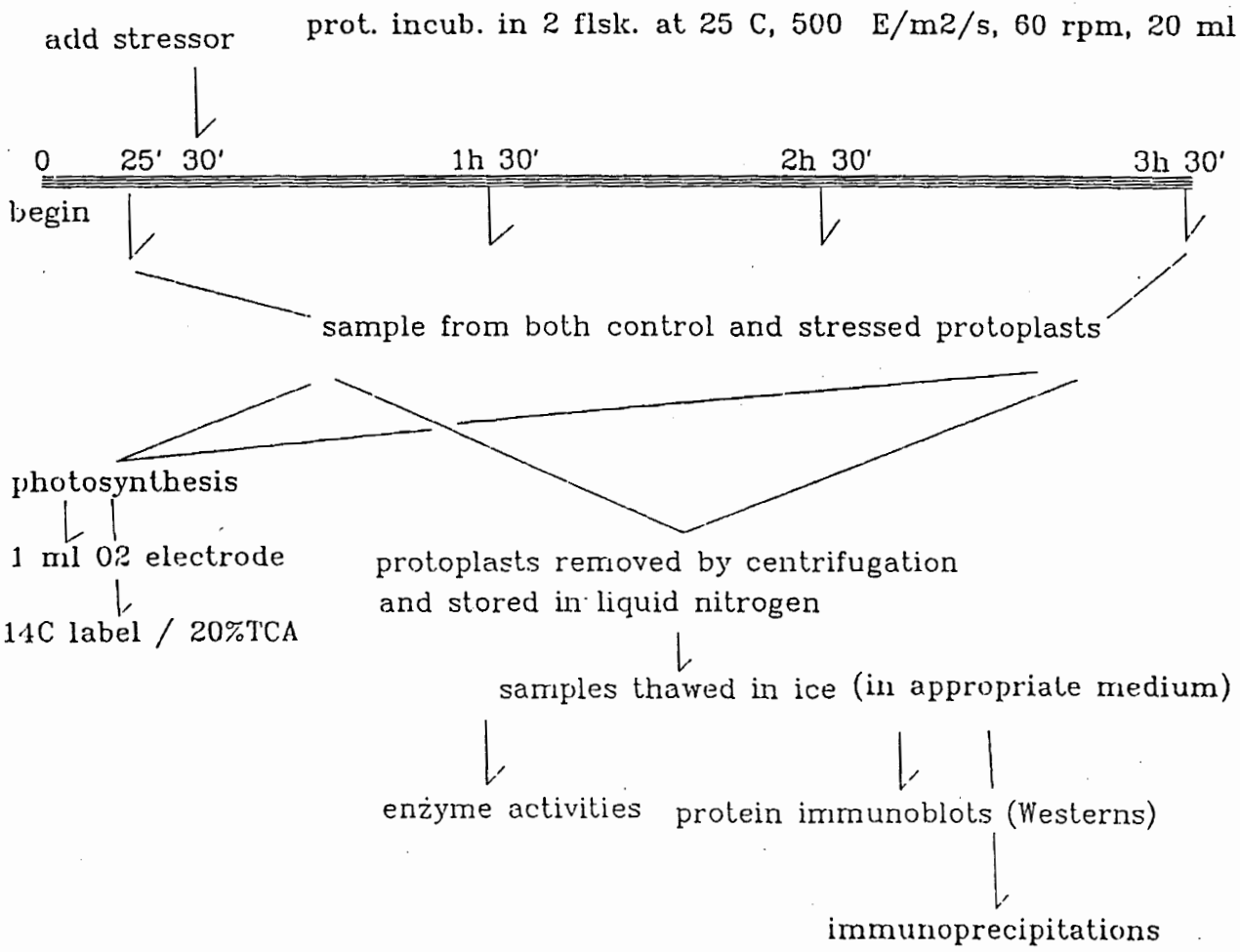
add stressor
prot. incub. in 2 flsk. at 25 C, 500 E/m2/s, 60 rpm, 20 ml
0
25' 30'
1h 30'
2h 30'
3h 30'
begin
sample from both control and stressed protoplasts
photosynthesis
1 ml 02 electrode
14C label / 20%TCA
protoplasts removed by centrifugation
and stored in liquid nitrogen
samples thawed in ice (in appropriate medium)
enzyme activities
protein immunoblots (Westerns)
immunoprecipitations

Figure 2. Time schedule of samplings during photosynthetic incubations of pea protoplasts in the presence or absence of stressor. A three-hour incubation time shedule is shown. Nevertheless, the same approach was used for longer incubations 10-14 hours).

corporation, Lincoln, NE) quantum meter immediately behind the transparent cuvette that houses the electrode on the opposite side to the light guide.

**Gamborg's basal salts**

Gamborg's B-5 basal salts (Sigma, St Louis, MO) were included for the protoplast incubations that were longer than 4 h. Those salts were first introduced for suspension culture from soybean root by Gamborg *et al.* (1968). For composition of B-5 salts see Appendix A.

**Stressors**

Paraquat (methyl viologen, Sigma, St Louis, MO) was prepared at stock solutions of 0.01 mM, 0.1 mM, and 1 mM in distilled water. The range of final concentrations in the incubation reaction flasks was from 0.01 mM to 0.1 $\mu$M .

Sulfite was prepared fresh every day from sodium sulfite and diluted into protoplast photosynthesis buffer. This buffer was selected as one in which sulfite autoxidation was prevented (Peiser *et al.*, 1982). The inhibition of sulfite autoxidation was probably due to sorbitol present in the buffer (0.45 M). This concentration was more than double the concentration needed for half-inhibition of sulfite autoxidation (Asada and Kiso, 1973). Additionally, the presence of EDTA in this buffer ensures removal of metal

traces which are known to promote free radical formation (which would induce the sulfite autoxidation). For the determination of sulfite autoxidation and its inhibition, the oxygen electrode was used (data not shown).

**Protoplast sampling**

Simultaneous with the photosynthetic determinations, and at selected time points, 1.5-3.0 ml (40-80 μg chlorophyll) aliquots of protoplasts were removed and centrifuged gently (150 g at an Eppendorf 5415 centrifuge for 4 min.). The pellet was dipped in liquid $N_2$ and stored at -80 °C for later whole cell enzyme activities or specific protein determination.

**Chloroplast isolation from protoplasts**

Chloroplasts were isolated according to the method of Edwards *et al.*, (1978) as modified by Mills and Joy (1980). Briefly, protoplasts were spun down in the ICC for 4 min. at 100Xg. They were then resuspended to half their initial volume with the "protoplast disruption" buffer (0.33 M sorbitol, 0.1% w/v BSA, 1 mM EDTA, 0.01 MES, NaOH/pH=6.5). Protoplasts were broken by one passage through a 15 μm nylon mesh (Spectra) fit to a 25 mm filter holder that was attached to a 10 ml syringe. The mixture was carefully pipetted on top of a solution containing 0.33 M sorbitol, 0.1% w/v BSA, 1 mM

EDTA, 0.01 M MES, NaOH pH=6.5 and 40 % (v/v) Percoll. The tubes were spun at 4,500 g (6000 rpm in a Sorvall fixed angle SS-34 rotor) for 1 min. at full speed. Intact chloroplasts sedimented to the bottom of the tube. The upper layer was decanted first, followed by the 40 % Percoll solution. A Pasteur pipette was used to remove the pellet because it was very loosely bound to the tube. Pellets were frozen in liquid $N_2$ and stored at -80°C for later enzyme activity determinations. Chloroplast isolation took approximately 30 min (per two samples).

**Enzyme activity**

Extraction

Pellets were taken out of the freezer and placed on ice. 200-400 $\mu$l of the enzyme extraction buffer (50 mM phosphate / ph=7.0, 2% PVPP, 0.5% Triton X-100, 1 mM ascorbate) was added before pellets thawed. Tubes were vortexed for 1-2 sec to resuspend pellet. Pellets were ground for 20 sec using a conical Teflon grinder (specifically designed to fit the 1.5 ml Eppendorf tubes) which was attached to a hand held tooth polisher. Homogenates were centrifuged for 20 min., full speed in an Eppendorf 5415 centrifuge. Supernatant was used for enzyme activities, chlorophyll and protein concentration determinations.

Quantification

All determinations were performed with a Beckman DU-65 spectrophotometer equipped with a Peltier temperature controller For all enzyme assays temperature was set at 25 °C.

Glutathione reductase - Activity was determined by monitoring the oxidized glutathione dependent oxidation of NADPH at 340 nm according to the method of Foyer and Halliwell (1976). Reaction mixtures consisted of 0.5 mM GSSG, 0.05 mM NADPH and 1 mM EDTA in total 1 ml of 100 mM Tris (HCl / pH=7.8). 50-100 µl enzyme extract was used. NADPH was added last to start the reaction.

Superoxide dismutase - The basis of this assay was the ability of enzyme extracts to inhibit the light mediated reduction of NBT (Beauchamp and Fridovich, 1973). The reaction medium consisted of 13 mM methionine, 75 µM NBT, 2 mM riboflavin, and 0.1 mM EDTA in a total of 3 ml of 50 mM potassium phosphate buffer (pH=7.8). 30-100 µl of enzyme extract were used with each assay tube. Ri [illegible] was added last. Glass tubes containing the reactant [illegible] below 2 fluorescent lamps of 15 W each f [illegible] were turned off and absorbance at 560 nm w [illegible] kept in the dark for 10 min served as d [illegible] with no enzyme extract served as light cor [illegible]

enzyme extract producing 50% inhibition of color development was taken to represent one unit of SOD activity (Dhindsa *et al.*, 1981).

Ascorbate peroxidase - Activity was determined by a modification of the method of Nakano and Asada (1987) by following the oxidation of ascorbate to dehydroascorbate (decrease of absorbance at 290 nm). Rates were corrected for the non-enzymatic oxidation of ascorbate. The reaction medium consisted of 0.5 mM ascorbate, 0.1 mM $H_2O_2$ in a total of 1 ml of 50 mM potassium phosphate buffer (pH=7.0). 10-40 µl of enzyme extract was used.

**Protein concentration**

Protein concentrations were determined by the Bradford method using the BioRad protein determination kit (semi-micro method - BioRad Inc. Richmond, CA). Sample absorbances were read against a 20-100 µg/ml BSA standard curve.

**Chlorophyll concentration**

Chlorophyll was determined according to the method of Arnon (1949).

**Immunoblotting and antibodies**

Proteins were extracted from protoplasts as for enzyme

activity determinations with the difference that as grinding/extraction buffer 50 mM potassium phosphate (pH=7.0) containing 1 mM EDTA, 0.1 % (v/v) Triton X-100, 10 mM ß-mercaptoethanol was used.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), (12% and 10%) was carried out according to Laemmli (1970) and according to manufacturers instructions in a Mini-PROTEAN II Dual slab cell (BioRad, Richmond, CA). Proteins were transferred from the gels to nitrocellulose filer paper (Schleicher and Schuell, Keene, NH) in a semidry blotting apparatus (Millipore, Bedford, MA) according to manufacturer's instructions. Immunodetection (visualization) of the transferred proteins was performed according to Ausubel *et al.* (1987). As secondary Ab, horseradish peroxidase conjugated goat-anti-rabbit IgG from Bio-Rad at a 1:1000 dilution was used. Color development was with 4-chloro-1 naphthol from BioRad. Quantification of immunostained proteins was performed directly on the nitrocellulose membrane with a Shimadzu CS 9000 U (Japan) scanner set at 595 nm.

Polyclonal antibodies raised against an isoform of GR found in the plastids of pea leaves were used (Madamanchi *et al.*, 1993). Polyclonal antibodies raised against the chloroplastic isoform of tea AP were kindly provided by Dr Kozi Asada (Kyoto University, Japan) and were prepared

according to Chen and Asada (1989). Both Ab were used with a 1:2000 dilution factor.

**$NaH[^{14}C]O_3^-$ fixation**

The procedure of Hatzios (1986) with the following modifications was used. Incubation conditions were identical to those used for $CO_2$-dependent oxygen evolution studies with the exception that no water filter below the light source was used. Temperature was maintained at 25°C. A 2.4 ml stock solution of 200 mM of cold $NaHCO_3$ was initially prepared. To this 24 µl of stock $NaH[^{14}C]O_3^-$ (specific activity 2 µCi/µl) were added resulting in a specific activity of ca. 1 mCi/mmole $CO_2$ in the stock vial. Protoplasts were diluted with photosynthetic reaction buffer at a chlorophyll concentration of *ca.* 5-10 µg/ml. 1.9 ml of protoplast suspension was transferred to the 25 ml Erlenmeyer flasks to which 100 µl of the bicarbonate stock was added resulting in 2 µCi/Erlenmeyer flask. Light was turned on, and protoplasts were allowed to photosynthesize for 30 min. whereupon the first sampling took place and stressors were added at the appropriate concentrations. Sampling involved spotting of 100 µl of protoplast suspension on to Whatman no 1 paper filters. They were dried under the hood and wetted with 120 µl of ice cold 20% tricarboxylic acid to release any unfixed bicarbonate.

Filters were put into glass scintillation vials where 10 ml of ScintiVerse E scintillation fluid was added and counted in a Beckman scintillation counter.

**Metabolic labelling of *de novo* synthesized protoplast proteins with [$^{35}$S]-L-methionine**

Protoplast incubation and protein *in vivo* labelling (Day 1)

Two Erlenmeyer 50 ml flasks with 60 $\mu$g chl/ml protoplasts were prepared according to the conditions used for the $CO_2$ dependent $O_2$ evolution experiments. Incubation under light proceeded for up to 3.25 h.

At times 0, 1.5, 3 h (time elapsed from turning on light) 3 ml were sampled from both stress and control flasks and placed in new glass scintillation vials[1] that contained 2 $\mu$l [$^{35}$S]-L-met stock solution of specific activity 10.4 $\mu$C/$\mu$l and swirled well[2]. From the mixture in the scintillation flasks,

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[1] Glass scintillation vials were secured and contained in an acrylic tray (dimensions 5 1/2 X 4 1/2 X 2) and are incubated under the same light as the flasks that contain the protoplasts. Tray has a bag with activated charcoal pellets constructed with Miracloth. The glass scintillation vials were chosen for the photosynthtic incubation of the protoplasts in the presence of [$^{35}$S]-L-methionine because they provide good closure (so methionine radioactive degradation products will not escape) while allowing for complete light transmission (needed for photosynthesis) through their glass walls.

[2] The [$^{35}$S]-L-met was handled according to the Nov 22, 1988 memorandum concerning volatile breakdown products during use of [$^{35}$S]-amino acids (Radiation Safety Officer, VPI and SU, VA).

2 X 5$\mu$l were sampled and spotted on glass fiber filters. The filters were then dried in the hood and placed in scintillation vials (polypropylene, with 4 ml scintillant). The Beckman automated liquid scintillation counter (LCS 5000 TA, Beckman Instruments Inc., Fullerton, CA) was used to determine radioactivity present in each vial during incubation. After incubation under light for 30 min, the scintillation vials were transferred to ice and 150 $\mu$l cold met (4 mM final concentration) were added. Suspension was decanted to 15 ml screw cap centrifuge tubes and centrifuged in International Clinical Centrifuge (ICC) at 100 g for 4 min. Supernatant was discarded to $^{35}S$ liquid waste. To the pellet, 3 ml of protoplast photosynthesis buffer containing 4 mM L-met was added and the protoplasts were gently resuspended. Following centrifigation at 100Xg, the supernatant was discarded and the pellets were transferred to new 1.5 ml screw-cap Eppendorf tubes which were frozen in liquid $N_2$ and stored at -70°C

Protein extraction and immunoprecipitation (Day 2)

Eppendorf tubes were put on ice, and 200 $\mu$l protein extraction buffer[3] was added. The tubes were sealed with

[3] 50 Mm tris/acetate pH=7.8, 0.15 M NaCl, 2% triton X-100, 4 mM L-methionine

Parafilm and the contents were ground with plastic disposable grinding tips for 20 sec. Tubes were stored on ice and spun in an Eppendorf centrifuge (17,000, 20 min.). Supernatant (ca. 200 µl) was transferred to fresh tubes and old tubes were discarded to the [$^{35}$S] dry waste. From the supernatant 2 X 5 µl were sampled, spotted on glass filter papers, dried, placed in scintillation vials, and counts were determined (a measure of total radioactivity taken up by the protoplasts). From the same supernatant 2 X 5 µl were sampled, added to new tubes containing 10 µl of 0.1 M NaOH and mixed. After 10 min. incubation at room temerature, 0.5 ml cold 5% TCA was added, tubes were vortexed and left on ice for 15 min. Glass fiber filters were placed into a suitable filtration device, a gentle vacuum was applied and the filters were prewet with a few ml of 10% TCA. Samples from the tubes were then added dropwise to the filters that were washed twice with 10% TCA using a few ml for each wash. The filters were removed, allowed to dry, and placed in scintillation vials. Scintillant was added and counts were determined (a measure of radioactivity incorporated into protein). Equivalent amounts of radioactivity were added to each well on SDS-PAGE.

Aliquots of 50 to 100 µl of the supernatant were placed (to achieve same DPM for all extracts) in fresh Eppendorf tubes and all tubes were brought to the same volume by adding protein extraction buffer.

To appr. 100 µl of supernatant, 5 µl GR antibody were added and shaken at room temperature for 30 min. and incubated overnight at 4°C while shaking (in a Lab. Quake).

Processing of the protein - antibody complex, SDS-PAGE, and autoradiography (Day 3)

The following morning the suspension was transferred carefully to new Eppendorf tubes containing a cushion of 0.4 ml of 1 M sucrose, tubes were spun at full speed for 10 min. in an Eppendorf centrifuge. Only protein - antibody complexes were heavy enough to sediment through sucrose.

The supernatant was removed with a drawn pipette, washed twice with "protein extraction buffer" and repelleted. Pellets were resuspended in 20 µl 1X Laemmli sample buffer[4]. $^{14}C$ methylated protein standards, MW range 14,300 - 200,000 (BRL, Inc, Gaithersburg, MD), were used. To the samples an equal volume of 2X strength of sample buffer was added. Samples were heated at 90°C for 10 min, and spun for 5 min. 10 to 20 µl sample volumes were loaded (equal counts were loaded per well) and SDS-PAGE was run according to established protocols. 1.5 and 3.0 µl of standards were run on different lanes. Gel was impregnated with ENHANCE (DuPont-New England Nuclear, Wilmington, DE) dried and exposed to X-ray film

[4] 50 Mm Tris/acetate, pH=8.0, 2% SDS, 10% glycerol, 0.002% bromophenol blue; 1 ml of above buffer + 25 µl ß-mercaptoethanol

(Kodak XAR-5 for 3 months in order to visualize the newly synthesized protein bands.

**Data report and statistical analysis**

Measurements were made at 30 min after light was turned on and the stressor was added. Parameters were also determined at specific time points after the addition of the stressor. Rates were shown as percent change over this elapsed period of time ($(RATE_{\text{at a specific time after addition of stress}}/RATE_{\text{before addition of stress}}) * 100$).

T-tests were performed using SAS (Lentner and Bishop, 1993).

## RESULTS

### Establishing the protoplast system

#### Protoplast light curves

Protoplasts were illuminated in the $O_2$ electrode in a total volume of 2 ml. Increasing light intensities were used for the different incubations in order to optimize protoplast illumination. Results are summarized in Fig. 3. Data for Progress and Nugget were plotted separately since $CO_2$-dependent $O_2$ evolution was quite different for the two cultivars. $CO_2$-dependent $O_2$ evolution rates in cv. Progress were found to be approximately 2/3 that of Nugget's at all light intensities. Eventually 420 $\mu E\ m^{-2}\ s^{-1}$ was chosen as the light intensity to be used for all subsequent $O_2$ evolution determinations. This light intensity was well within the range of photosynthetic optimum. At this light intensity Progress and Nugget photosynthesize at rates 70 and 100 $\mu$moles oxygen $mg^{-1}$ chl $h^{-1}$ respectively.

#### Percent intactness of chloroplasts derived from protoplasts

In three separate experiments, chloroplasts were extracted from protoplasts of both pea cultivars immediately after protoplast isolation and before any incubation. Percent intactness was $82.2 \pm 1.4$ (n=3).

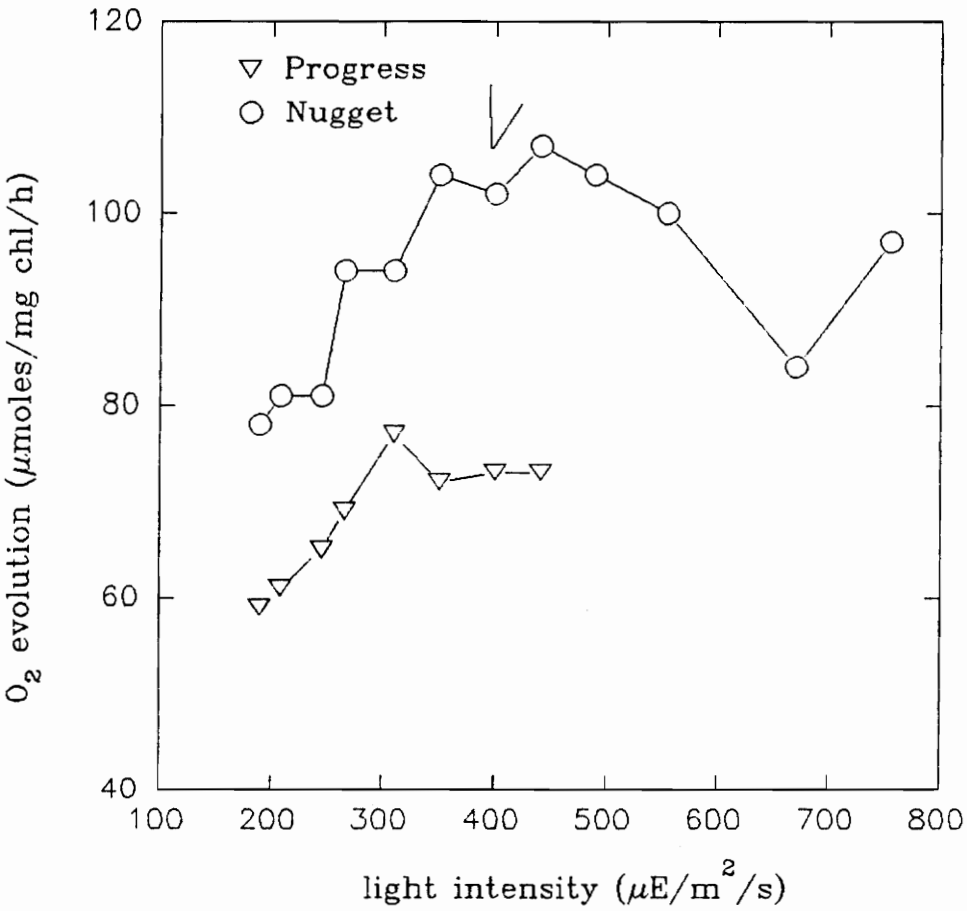


Fig. 3. Effects of increasing light intensity on $CO_2$-dependent $O_2$ evolution by Progress and Nugget protoplasts incubated under photosynthetic conditions. Different protoplast aliquots were used for every point measurement. They were derived from the same batch for each cultivar. Arrow indicates the light intensity that was chosen for all subsequent experiments.

**Exposure of protoplasts to oxidants**

Sulfite

Sulfite (1.5 and 3.0 mM) added during polarographic estimation of photosynthesis - Progress and Nugget protoplasts were incubated in the oxygen electrode in the absence or presence of 1.5 and 3.0 mM $SO_3^{2-}$. The protoplasts were allowed to recover (lag phase - 3 min) and $CO_2$-dependent $O_2$ evolution was recorded. $SO_3^{2-}$ to a final concentration of 1.5 mM was added to the $O_2$ electrode and after 5 min the $O_2$ rate was recorded once more. An additional (1.5 mM) $SO_3^{2-}$ was added again, thus bringing the final sulfite concentration to 3 mM. Five minutes later the $O_2$ rate was recorded. Results for the two varieties are indicated in Table 1. Evolution rates declined with increasing [$SO_3^{2-}$] in the electrode (down to *ca.* 60% at 3.0 mM). No differences in sulfite tolerance were observed between Progress and Nugget.

At this point it seemed important to assess the effect of longer sulfite exposures of protoplast preparations (or any other source of oxidative stress). Additionally, the incubations had to be designed in such a way that enough material for later analyses could be obtained. Accordingly, the incubations/exposures scheme described above where incubation/exposure and photosynthetic measurements took place simultaneously inside the $O_2$ electrode was abandoned. It was replaced with incubations/exposures in Erlenmeyer flasks and

Table 1. $O_2$ evolution rate[1] of pea protoplasts in the presence of 10 mM $NaHCO_3$ and different amounts of sulfite[2].

| $[SO_3]$, mM | CULTIVAR | | | |
|---|---|---|---|---|
| | Progress | | Nugget | |
| | $O_2$ evol. | % of control | $O_2$ evol. | % of control |
| 0.0 | 65 | 100 | 60 | 100 |
| 1.5 | 62 | 95 | 56 | 93 |
| 3.0 | 41 | 63 | 38 | 61 |
| 3.0 | -- | -- | 31[3] | 51 |

[1] $\mu$moles $O_2$ evolved $mg^{-1}$ chl $h^{-1}$

[2] 30 and 24 $\mu$g of chl of Progress and Nugget protoplasts were incubated in the $O_2$ electrode (reaction volume 2 ml) under 800 $\mu E\ m^{-2}\ s^{-1}$ of actinic light; 10 min after a linear rate was obtained, $SO_3$ to a final concentration of 1.5 mM was added and the rate was recorded; 5 min later 1.5 mM $SO_3$ were added (bringing $SO_3$ final concentration to 3.0 mM) and the rate was recorded again

[3] with Nugget protoplasts, the rate at 3.0 mM $SO_3$ was recorded twice: first after 5 min and then after 5 more minutes

separate photosynthetic determinations in the $O_2$ electrode (see Materials and Methods and brief description below).

Subsequent polarographic estimation of photosynthesis; -

Nugget; 1.5 mM

In all subsequent experiments, sulfite was not added during the measurement of photosynthesis. Protoplasts were incubated in Erlenmeyer flasks in a shaking water bath (60 RPM) maintained at 25°C, and illuminated with water-filtered light which was provided by a flood lamp. At specific time intervals aliquots were taken from the flasks, diluted two- to three-fold with fresh incubation buffer in the $O_2$ electrode and the rate of oxygen evolution was determined. In two separate experiments, Nugget protoplasts were exposed to 1.5 mM $SO_3^{2-}$ (Figs. 4 and 5). In the experiment shown in Fig. 4, $SO_3^{2-}$ was added as soon as the light was turned on while in the experiment shown in Fig. 5 $SO_3^{2-}$ was added just before the first aliquot was taken (20 min after light was turned on). In the latter case, the treatment was initiated after the lag phase of photosynthesis was over. Rates of protoplasts incubated without $SO_3^{2-}$ (controls) are also included in the figures.

For the first experiment (Fig. 4) the photosynthetic behavior of controls followed a sigmoidal pattern while in the second experiment (Fig. 5) controls increased slightly over

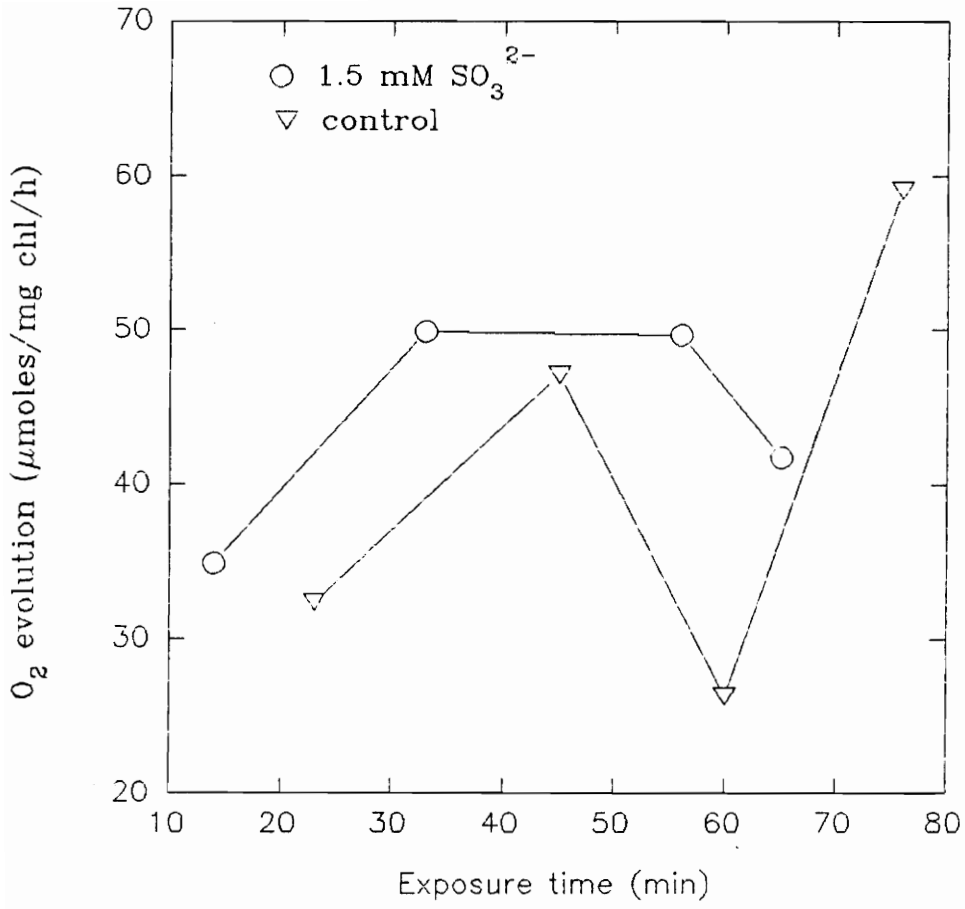


Fig. 4. $CO_2$–dependent $O_2$ evolution of Nugget protoplasts derived from 12–day–old plants. Sulfite, (1.5 mM) was administered at the begining of light incubation. Incubation was begun 4.75 h. post isolation.

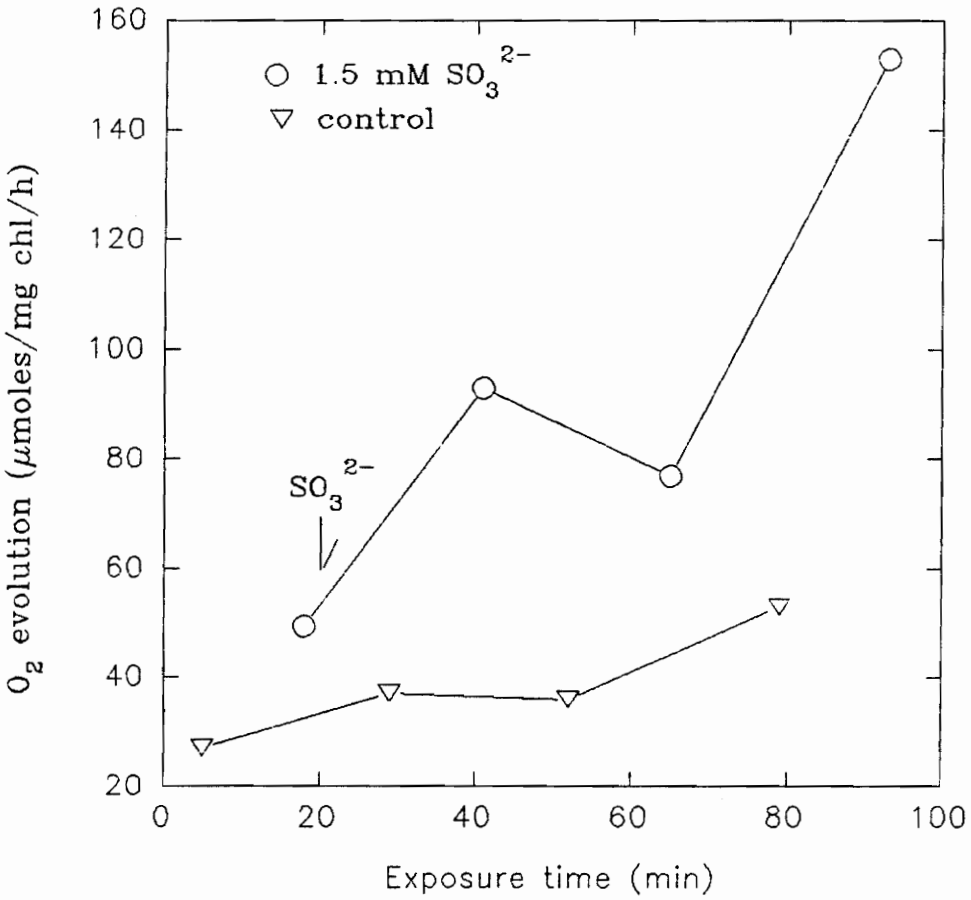


Fig. 5. $CO_2$–dependent $O_2$ evolution of Nugget protoplasts derived from 15–day–old plants. Sulfite, (1.5 mM) was administered 20 min after light was turned on. Incubation begun 6.5 h isolation.

time. Nevertheless in both experiments $SO_3^{2-}$-treated protoplasts exhibited higher $O_2$ evolution rates for all time points.

Progress; 1.5 mM

Protoplasts were exposed to 1.5 mM $SO_3^{2-}$ for 1 h in an experiment repeated twice in one day (Figs. 6 and 7). The same batch of protoplasts was used for both incubation series. In the first experiment (Fig. 6) photosynthesis increased continuously throughout the whole incubation period. Treated protoplasts behaved very similar to controls. In the experiment that began 7 h later (Fig. 7) photosynthesis in the controls initially seemed to decline and eventually increased as incubation continued. Treated protoplasts photosynthesized less compared to controls. Obviously, storing protoplasts on ice for 10 h increased their lag phase and decreased their tolerance to $SO_3^{2-}$.

Both cultivars; 3.0 mM

When the [$SO_3^{2-}$] was increased to 3.0 mM, the stimulation of photosynthesis by sulfite was not apparent until control rates started to decline for both varieties (Figs. 8; Nugget and 9; Progress). This decline occured after ca. 50 min in light. Protoplasts used in the experiment the results of which are shown in Figs. 9 and 10 were derived from the same

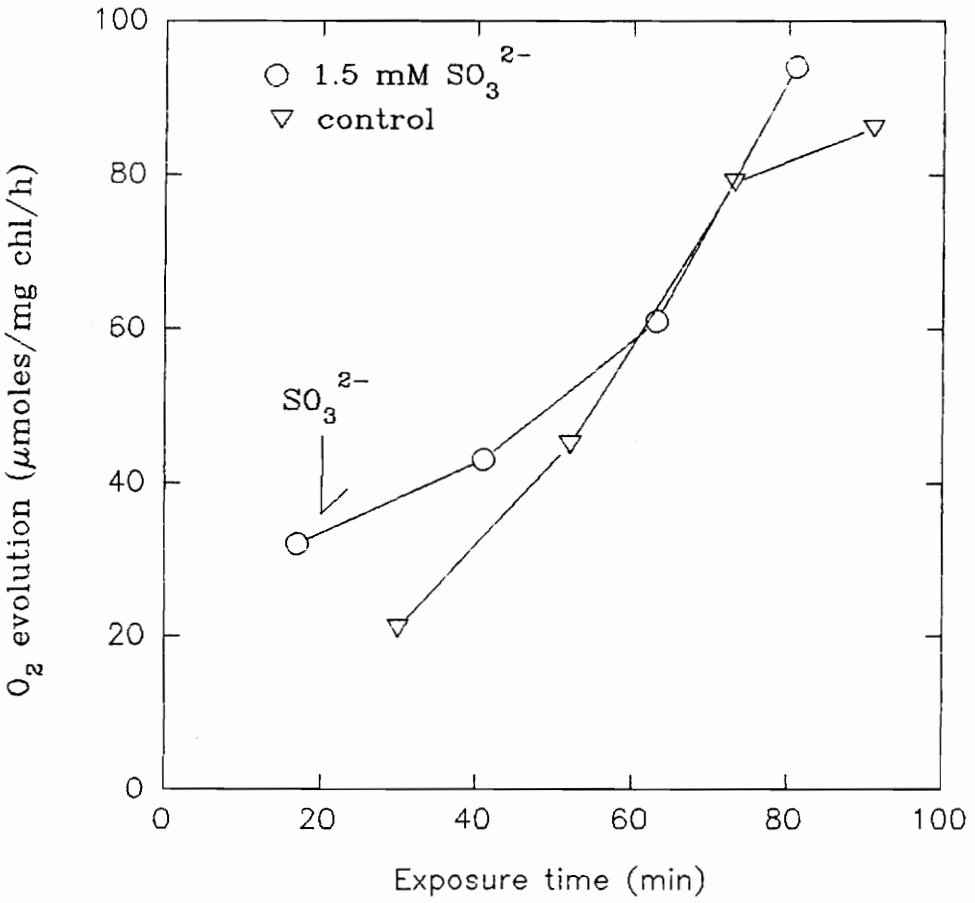


Fig. 6. $CO_2$–dependent $O_2$ evolution of Progress protoplasts derived from 14–day–old plants. Sulfite (1.5 mM) was administered 20 min after light was turned on. Incubation was begun 3.5 h post isolation.

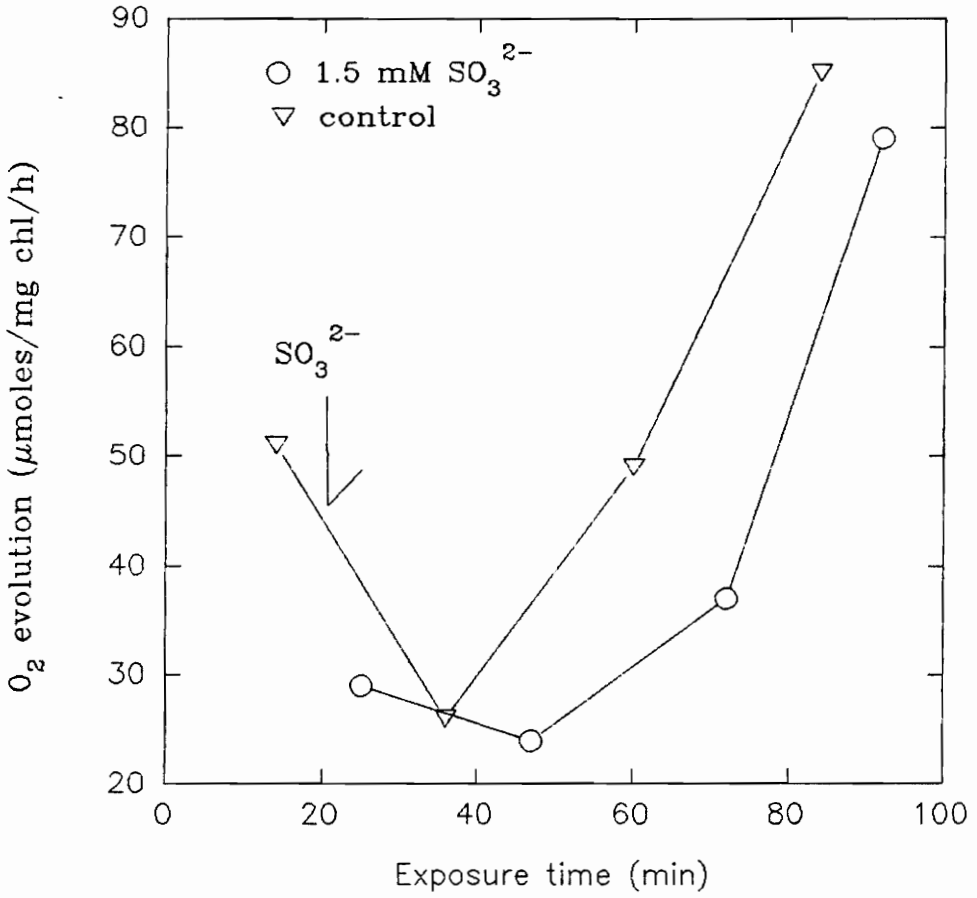


Fig. 7. $CO_2$-dependent $O_2$ evolution of Progress protoplasts derived from 14-day-old plants. Sulfite (1.5 mM) was administered 20 min after light was turned on. Same protoplast material as used for Fig. 6 but that incubation was begun 10.25 h post isolation.

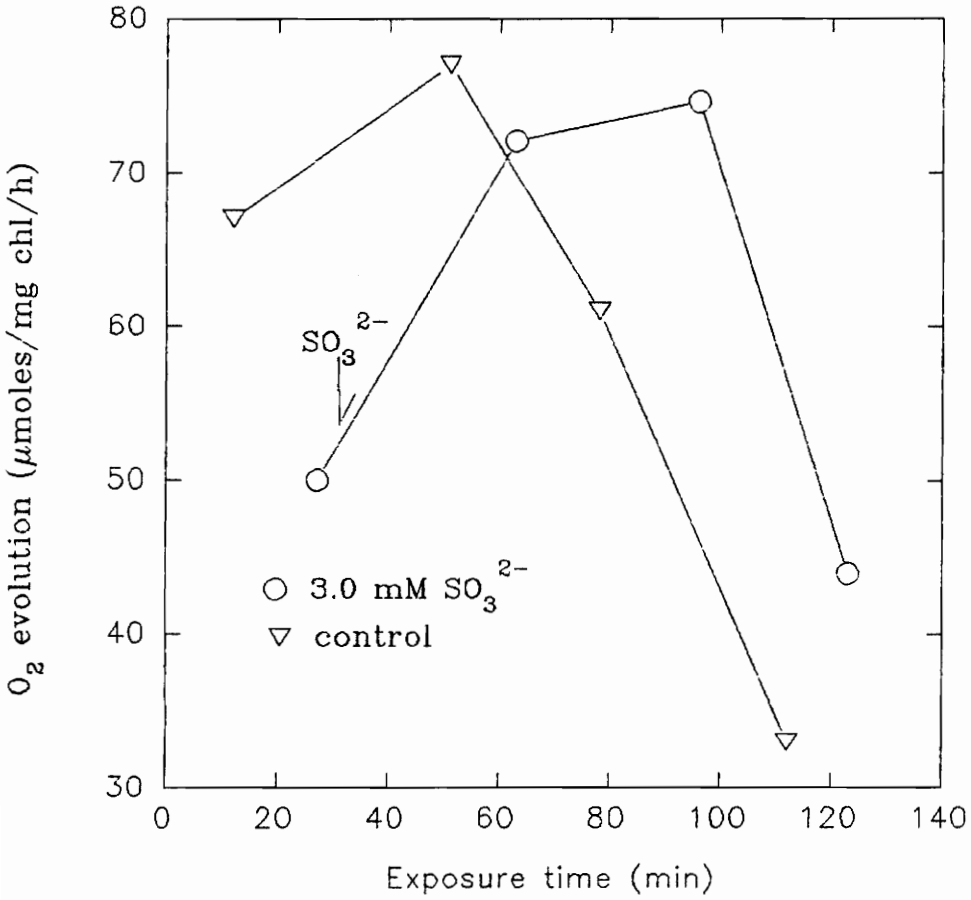


Fig. 8. $CO_2$-dependent $O_2$ evolution of Nugget protoplasts derived from 13-day-old plants. Sulfite (3.0 mM) was added 35 min after the light was turned on. Incubation was begun 9 h h post isolation.

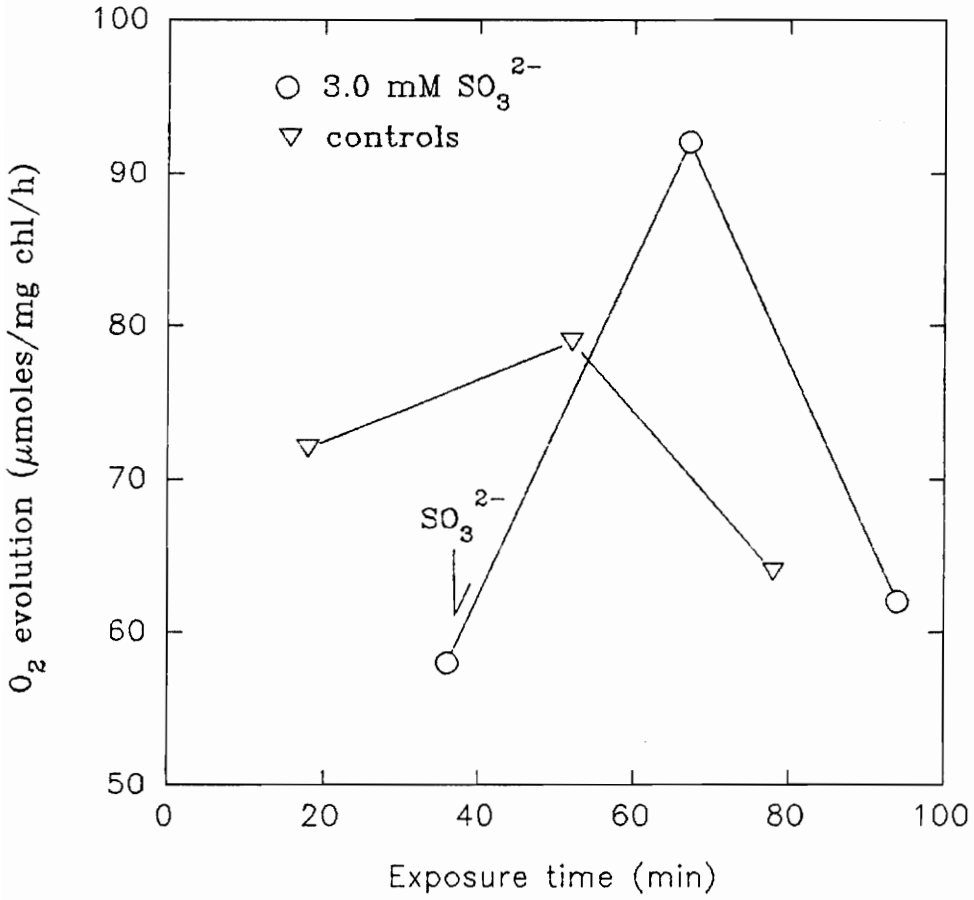


Fig. 9 $CO_2$–dependent $O_2$ evolution of Progress protoplasts derived from 15–day–old plants. Sulfite (3.0 mM) was added 35 min after light was turned on. Incubation was begun 1.5 h post isolation.

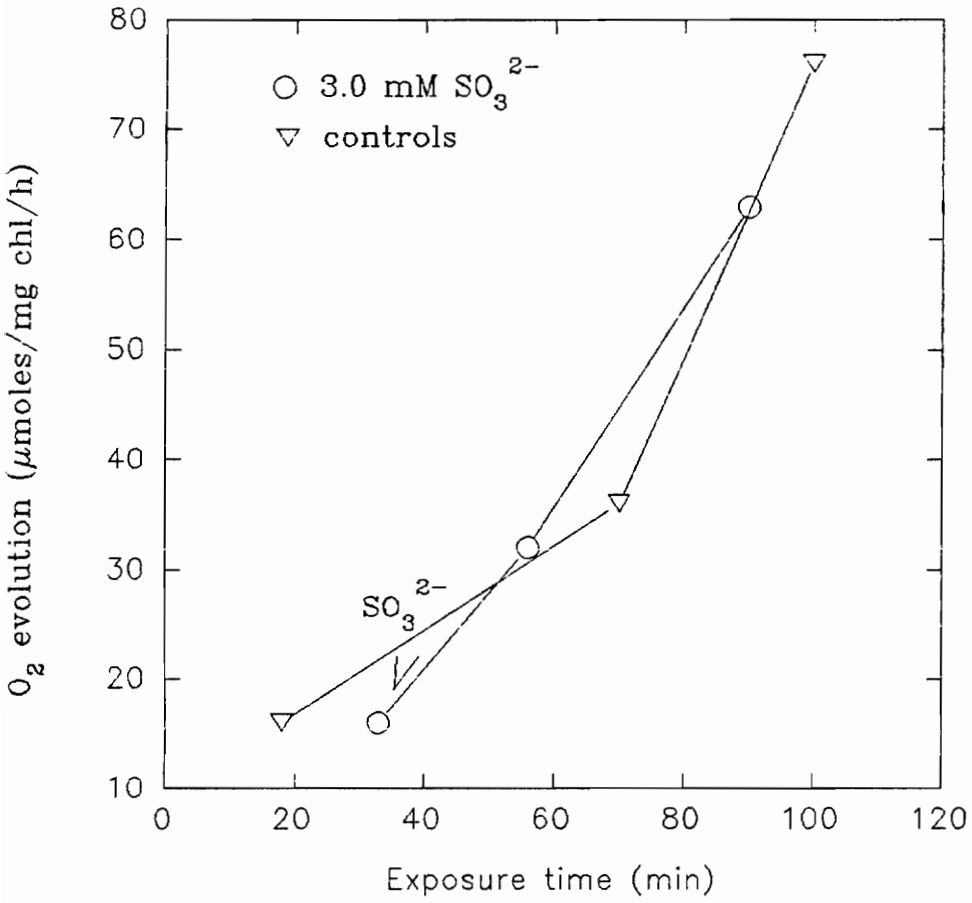


Fig. 10. $CO_2$-dependent $O_2$ evolution of Progress protoplasts derived from 15-day-old plants. Sulfite (3.0 mM) was added 35 min after the light was turned on. Same protoplast material as used for Fig. 9 but incubation was begun 6 h post isolation.

batch of protoplasts. Incubations for the experiment indicated in Fig. 9 began 1.5 h post isolation (hpi) while those indicated in Fig. 10 began 6 hpi. In this second incubation the oxygen evolution enhancement observed with the addition of sulfite was abolished. In agreement with the conclusion drawn from the experiments shown in Figs. 6 and 7, storing protoplasts on ice for 5-10 h seemed to decrease their tolerance to sulfite.

Progress; 4.5 mM

[$SO_3^{2-}$] was further increased to 4.5 mM. Two replicate incubations were conducted in the presence of this concentration with protoplasts derived from the same batch. The first incubation began 2.5 hpi (Fig. 11) and the second 9 hpi (Fig. 12). In the first incubation, $SO_3^{2-}$ addition did not seem to affect the difference in oxygen evolution rates observed (between control and treated) before the addition of sulfite. In the second one addition of 4.5 mM $SO_3^{2-}$ seemed to enhance oxygen evolution after 1 h total incubation time.

Progress; 5 to 30 mM sulfite - In order to minimize the variability reported in Figs. 3-11 a purification step was included in the protoplast isolation procedure for all subsequent experiments. Purified protoplasts represent a more homogeneous plant cell population compared to non-purified

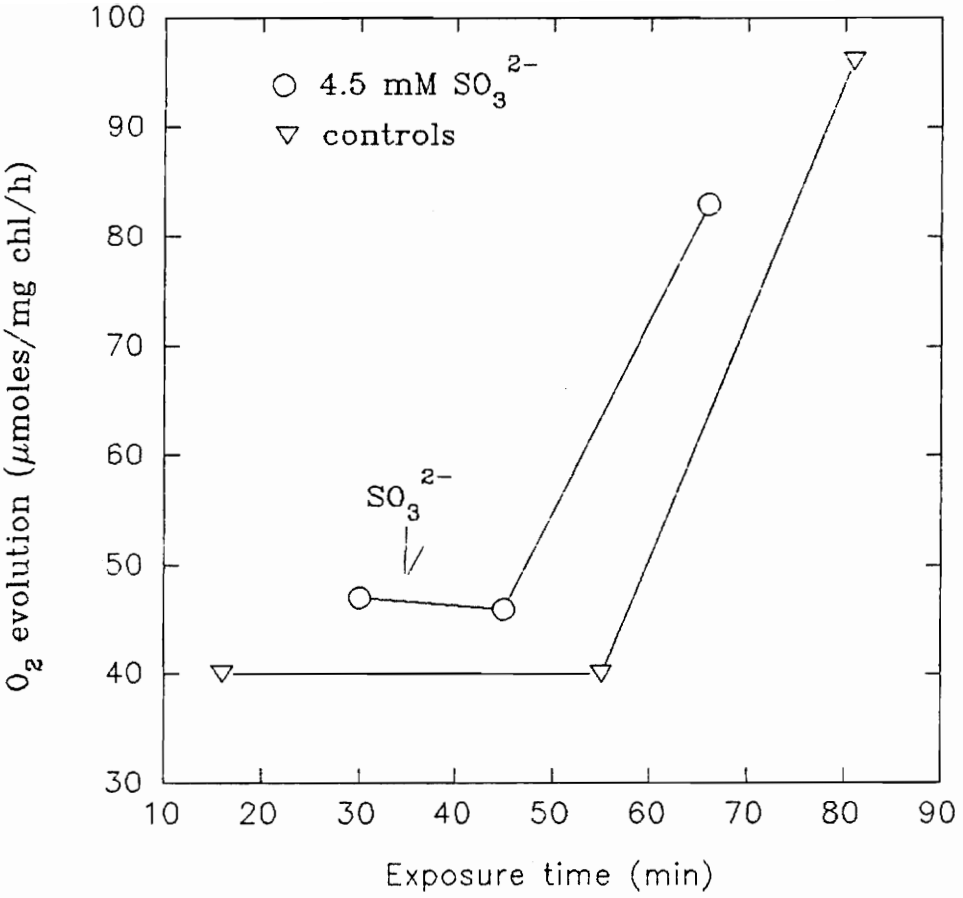


Fig. 11. $CO_2$-dependent $O_2$ evolution of Progress protoplasts derived from 13-day-old plants. Sulfite (4.5 mM) was added 35 min after the light was turned on. Incubation begun 2.5 h post isolation.

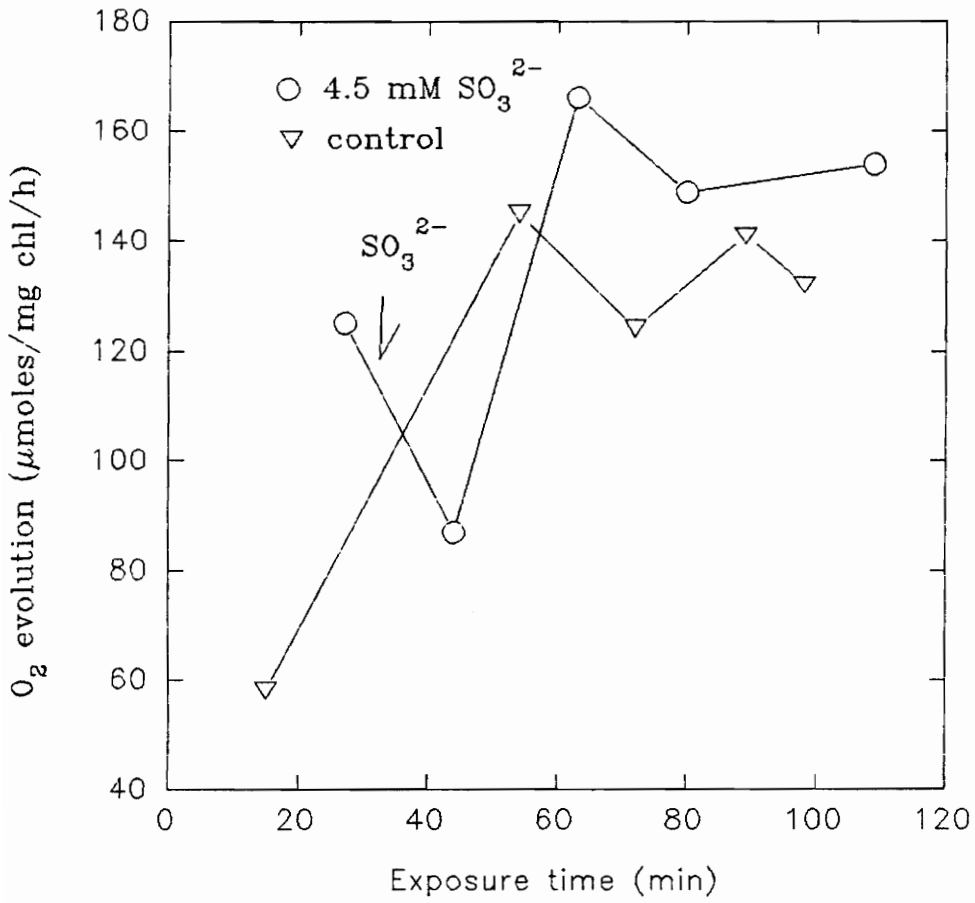


Fig. 12. $CO_2$-dependent $O_2$ evolution of Progress protoplasts derived from 13-day-old plants. Sulfite (4.5 mM) was added 35 min after the light was turned on. Same protoplast material as used for Fig. 11 but that incubation was begun 9 h post isolation.

protoplasts and might be expected to give less experimental variation.

In the two experiments described in this section, increasing concentrations of sulfite (5-30 mM) were administered in a series of separate incubation flasks containing purified protoplasts which were derived from 13- and 14- day old Progress peas. Results are summarized in Table 2. $O_2$ evolution rates were recorded immediately before and one hour after the administration of sulfite and the percent change calculated. Controls received no sulfite. In the first experiment, $O_2$ evolution of treated protoplasts was less than controls. In the second experiment, treated protoplasts showed enhanced $O_2$ evolution. Addition of a range of $SO_3^{2-}$ concentrations (spanning both experiments) does not affect $O_2$ evolution.

Summary of responses to low concentrations of sulfite (1.5 to 4.5 mM) - Results obtained by incubating Progress and Nugget protoplasts with low amounts of sulfite were summarized in Tables 3a and 3b. In every experiment the shape of the oxygen evolution curve of treated protoplasts closely resembled that of controls. Inclusion of sulfite in the incubation mixture either does not alter or partly stimulated oxygen evolution.

Table 2. Percent change, after one hour incubation, of $O_2$ evolution rates of Progress protoplasts in the presence of 10 mM $NaHCO_3$ and different amounts of sulfite[1].

| Experiment 1[2] | | | | Experiment 2[3] | | | |
|---|---|---|---|---|---|---|---|
| $[SO_3]$, mM | | | | | | | |
| 0 | 10 | 5 (n=2) | 7 (n=2) | 0 | 10 | 20 (n=2) | 30 (n=2) |
| 132 | 119 | 113±25 | 122±0.5 | 88 | 102 | 114±11 | 93±3 |

[1] protoplasts were allowed to photosynthesize 30 minutes in 25 ml Erlenmeyer flasks and then sulfite was added to the different final concentrations indicated

[2] protoplasts were derived from 14-day-old plants

[3] protoplasts were derived from 13-day-old plants

Table 3a. Summary of qualitative results from incubations of pea protoplasts (cv Progress) in the presence of low amounts of sulfite (1.5 to 4.5 mM).

| Exp.[1] # | | hpi[2] | [$SO_3$][3] | Controls[4] | Effect of $SO_3$ on photosynthesis[5] |
|---|---|---|---|---|---|
| 1 | (6) | 3.5 | 1.5 | increasing | identical to controls |
| | (7) | 10.2 | 1.5 | increase after 1 h incubation | slight stimulation |
| 2 | (9) | 1.5 | 3.0 | relatively stable | slight stimulation |
| | (10) | 6.0 | 3.0 | increasing | identical to controls |
| 3 | (11) | 2.5 | 4.5 | increase after 1 h incubation | slight stimulation |
| | (12) | 9.0 | 4.5 | increase up to 1 h | initial decline but after 1 h showstimulation |

[1] each experiment includes results from two separate incubations which were performed on one day (one incubation after the other) with protoplasts derived from the same batch. That batch was prepared earlier that day. Experiment number was assigned according to the order in which they were performed. Numbers in parentheses indicate number of figure discussed.

[2] hours post isolation; time elapsed between protoplast isolation and initiation of incubation

[3] final concentration of sulfite in the incubation flask; 1/2 of that concentration was present in the oxygen electrode during the oxygen evolution measurement. In experiment # 1 sulfite was administered as soon as light was turned on.

[4] pattern of oxygen evolution of non-treated protoplasts over the course of the incubation

[5] oxygen evolution of protoplast treated with sulfite compared with that of control protoplasts

Table 3b. Summary of qualitative results from incubations of pea protoplasts (cv Nugget) in the presence of low amounts of sulfite (1.5 and 3.0 mM).

| Exp.[1] # | hpi[2] | $[SO_3]$[3] | Controls[4] | Effect of $SO_3$ on photosynthesis[5] |
|---|---|---|---|---|
| 1 (4) | 4.8 | 1.5 | not stable | slight stimulation |
| 2 (5) | 6.5 | 1.5 | low Pn, stable | stimulation |
| 3 (8) | 9.0 | 3.0 | initial increase, decline after 1 h | slight stimulation |

[1] Experiment number was assigned according to the order in which they were performed. Numbers in parentheses indicate number of figure discussed

[2] hours post isolation; time elapsed between protoplast isolation and initiation of incubation

[3] final concentration of sulfite in the incubation flask; 1/2 of that concentration was present in the oxygen electrode during the oxygen evolution measurement. In experiment # 1 sulfite was administered as soon as light was turned on.

[4] pattern of oxygen evolution of non-treated protoplasts over the course of the incubation

[5] oxygen evolution of protoplast treated with sulfite compared with that of control protoplasts

From all the data presented it cannot be concluded whether or not $SO_3^{2-}$ induces oxidative stress in pea protoplasts under the particular exposure/measurement conditions.

Response of the $^{14}CO_2$ fixation of Progress protoplasts - $^{14}CO_2$ incorporation into 10% TCA stable radioactivity was used to determine how $SO_3^{2-}$ concentrations, used for the photosynthetic incubations discussed above, would affect photosynthetic carbon fixation. Acid stable label for each time point was normalized to preexposure levels. The formula used was: $(label_{t=i\ hours}/label_{t=0.5\ hours})*100$ (this formula was used for all subsequent calculations where data were presented as % of t=0.5 min). Consequently, $^{14}CO_2$ fixation was presented as percent increase over the label determined at time 0.5 h (just before the administration of sulfite). Data from three separate experiments performed with purified Progress protoplasts are summarized in Figs. 13 to 14. In all figures, treated protoplasts show the same fixation pattern as their respective controls but at a diminished capacity. The extent of inhibition is greater at higher $[SO_3^{2-}]$. In one experiment (Fig. 14) administration of 1.5 mM $SO_3^{2-}$ enhanced carbon fixation. Controls continue to accumulate label up to 3 / 3.5 h. At the highest $[SO_3^{2-}]$ used (15 mM) the decline in photosynthesis ranged from 20 to 60% of controls.

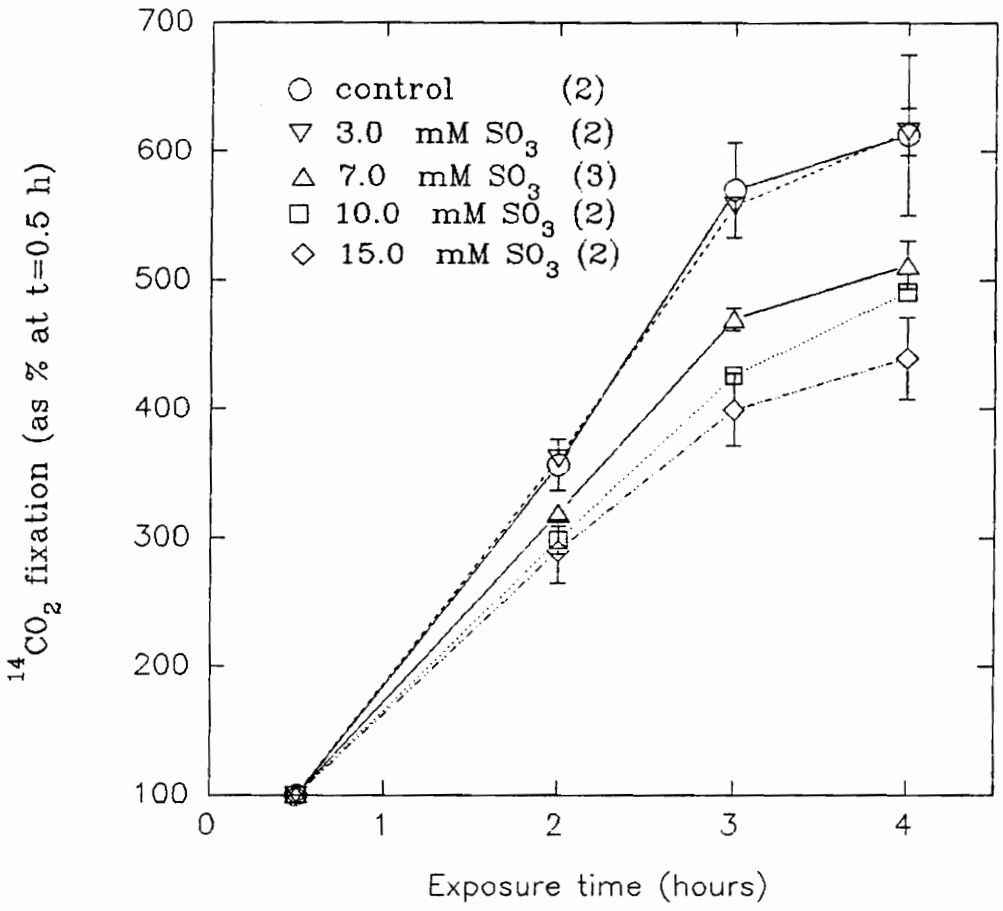


Fig. 13. $^{14}CO_2$ fixation of protoplasts derived from 10-day-old Progress plants incubated in the presence of different concentrations of sulfite. Numbers in parentheses indicate sample size.

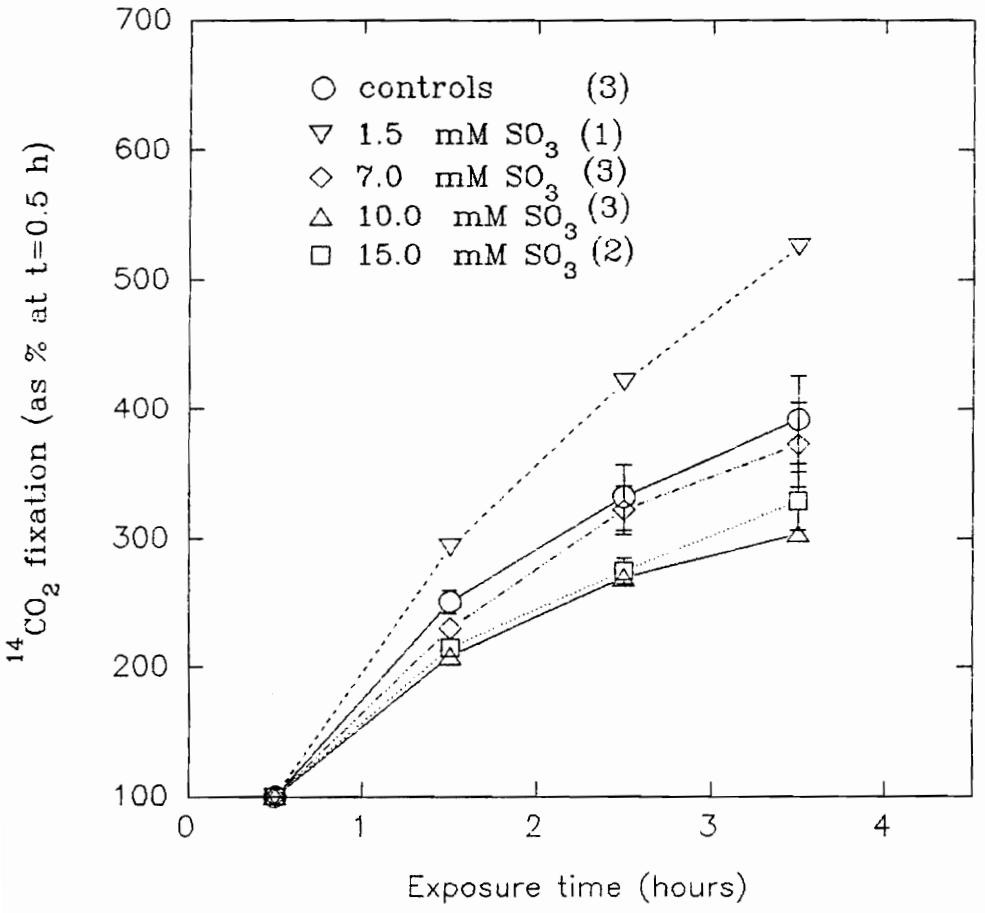


Fig. 14. $^{14}CO_2$ fixation of Progress protoplasts derived from 12-day-old plants incubated with different concentrations of sulfite. Numbers in parenthese indicate sample size.

Response of the $^{14}CO_2$ fixation of Nugget protoplasts - The effect of a range of sulfite concentrations (1.5, 7.0, 10.0, and 15.0 mM) was tested on the carbon fixation capacity of purified protoplasts derived from Nugget. Data are summarized in Fig. 15. Nugget protoplasts showed a similar response to Progress protoplasts. Low [$SO_3^{2-}$] (1.5 mM) seemed to enhance carbon fixation. The effect of all other concentrations was to diminish carbon fixation.

Summary of photosynthetic responses to sulfite - In both cultivars photosynthesis (as judged by $^{14}CO_2$ fixation) declined to approximately the same extent. In sharp contrast to the $CO_2$ fixation response to sulfite, the $CO_2$-dependent $O_2$ evolution observed with protoplasts derived from both cultivars exposed to the same range of $SO_3^{2-}$ concentrations was either enhanced or not changed. During the process of sulfite reduction reducing equivalents are needed. In photosynthesis electrons are derived from water. The photolysis of water (in the oxygen evolving center in photosystem II) liberates oxygen. Consequently, $SO_2$-dependent oxygen evolution may mask the ability to observe any change in $CO_2$-dependent $O_2$ evolution caused by sulfite. On the other hand, if sulfite competes for electrons with the Calvin-Benson cycle it will operate in a lower pace in the presence of sulfite (see Discussion).

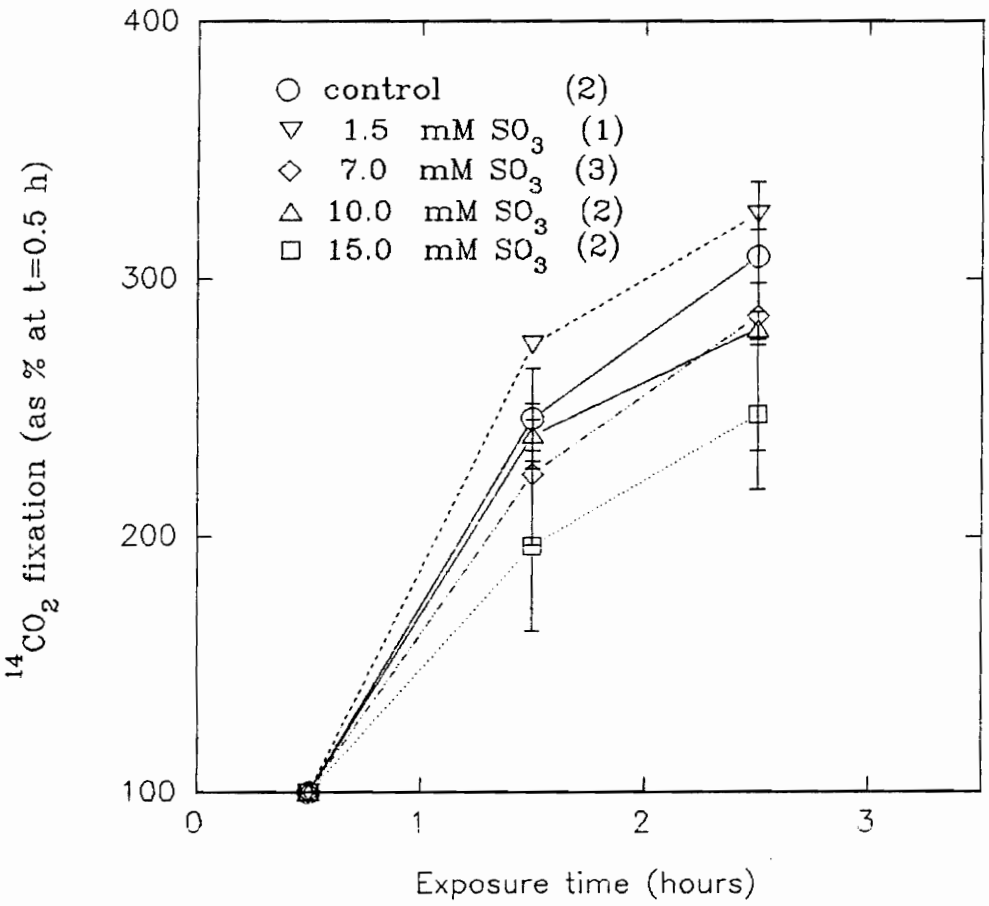


Fig. 15. $^{14}CO_2$ fixation of Nugget protoplasts derived from 10-day-old plants incubated with different concentrations of sulfite. Numbers in parentheses indicate sample size.

PQ

$CO_2$-dependent $O_2$ evolution - Protoplasts from both cultivars were exposed to different PQ concentrations. Just before administration of PQ and one hour after, a 1 ml aliquot of protoplasts was diluted 1:1 (i.e., 1 ml of fresh incubation mixture was added) and transferred in the $O_2$ electrode where the $CO_2$-dependent $O_2$ evolution rate was recorded. In separate preliminary experiments, it was determined that the presence up to 1 $\mu$M pq in the $O_2$ electrode does not affect the short term (up to 15 min) $CO_2$-dependent $O_2$ evolution of freshly incubated protoplasts (data not shown). Results were summarized in Fig. 16 with the concentrations axis in the common logarithmic scale. Both cultivars seemed to behave similarly in the region up to 0.1 $\mu$M. As PQ concentrations increased Progress photosynthesis declined and was completely abolished at 1 $\mu$M. Up to 2 $\mu$M, photosynthesis in Nugget was only partially inhibited (ca. 40%).

$^{14}CO_2$ fixation - Purified protoplasts from Progress and Nugget were incubated in the customary photosynthesis incubation medium in the presence of $^{14}CO_2$. Percent change in 10% TCA stable radioactivity was determined immediately before and three times after the addition of PQ (up to 3.5 h). In Fig. 17 it can be seen that, in the case of Progress, 0.1 $\mu$M PQ inhibited photosynthesis by 20% and 40% after 1.5 h and 3.5

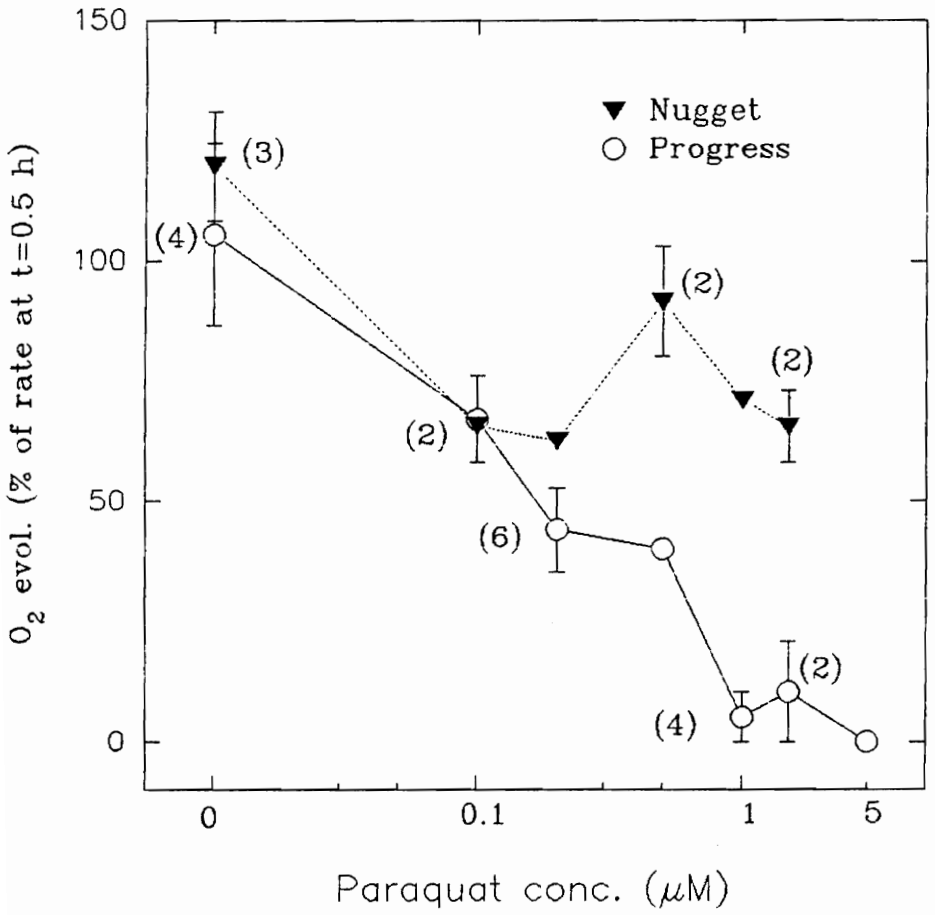


Fig. 16. $CO_2$–dependent $O_2$ evolution of pea protoplasts from cvs. Progress and Nugget after 1 h incubation in the presence of increasing concentrations of paraquat. Values are expressed as % of pre–exposure rates. Numbers in parentheses indicate numbers of separate experiments.

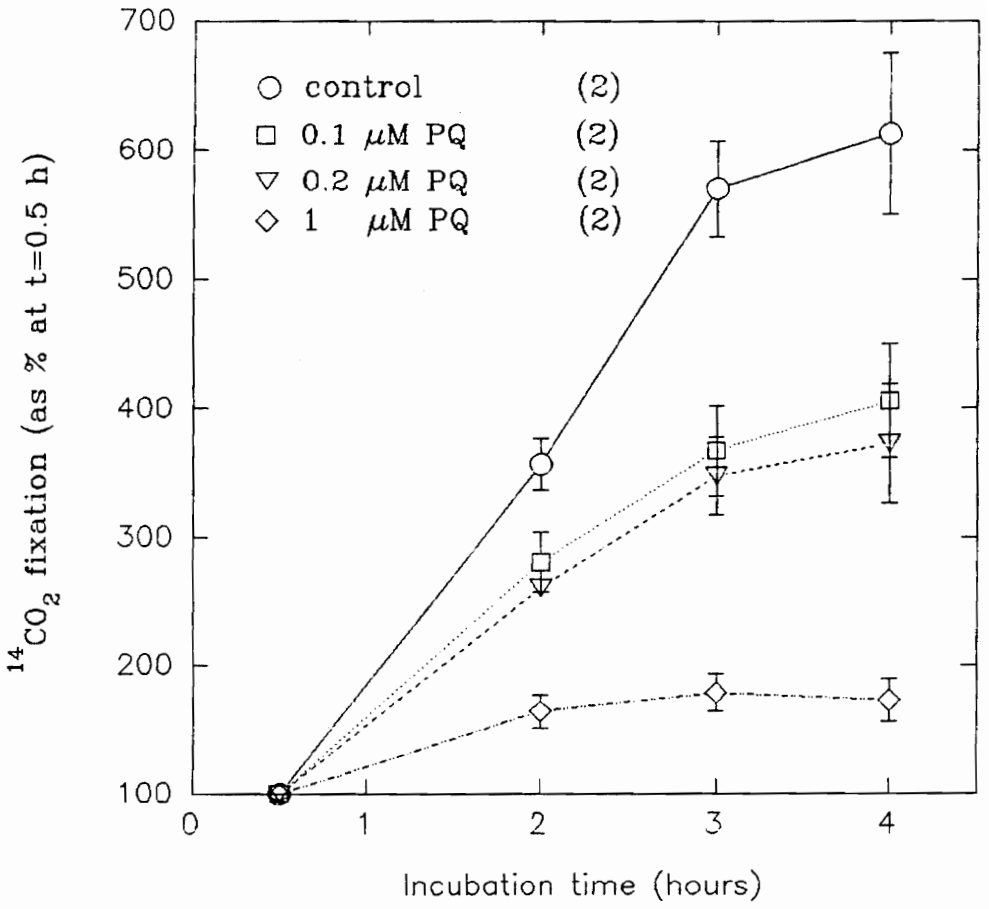


Fig. 17. $^{14}CO_2$ fixation of Progress protoplasts derived from 10-day-old plants incubated with different paraquat concentrations.
Numbers in parentheses indicate sample size.

h of exposure time respectively. 1 μM was needed to completely inhibit carbon fixation after 1.5 h of exposure. Instead, in Fig. 18 it can be seen that 0.1 μM PQ had no effect on carbon fixation by protoplasts prepared from Nugget. One μM inhibited photosynthesis by 50% after 1.5 h of exposure and 10 μM was needed to inhibit photosynthesis completely after the 1.5 h point. These data confirmed the conclusions drawn from the $O_2$ evolution curves (Fig. 16) that 1 μM PQ completely inhibited Progress photosynthesis while with Nugget it was only 40% diminished.

Antioxidant enzyme activities and steady state protein levels - Ascorbate peroxidase (AP) activity from Progress leaf homogenates was assayed at two pH levels chosen from the literature (Table 4). AP activities were considerably higher at pH 7.0 compared to pH 7.6. Possibly the majority of the AP activity resides in the cytosol. The more acidic pH assay buffer was adopted for all subsequent AP assays.

In Fig. 19 oxygen evolution rates shown initially in Fig. 16 were overlaid with the percent change of GR (A) and ascorbate peroxidase (B) activities detected after one h incubation of Progress protoplasts in the presence of various amounts of PQ.

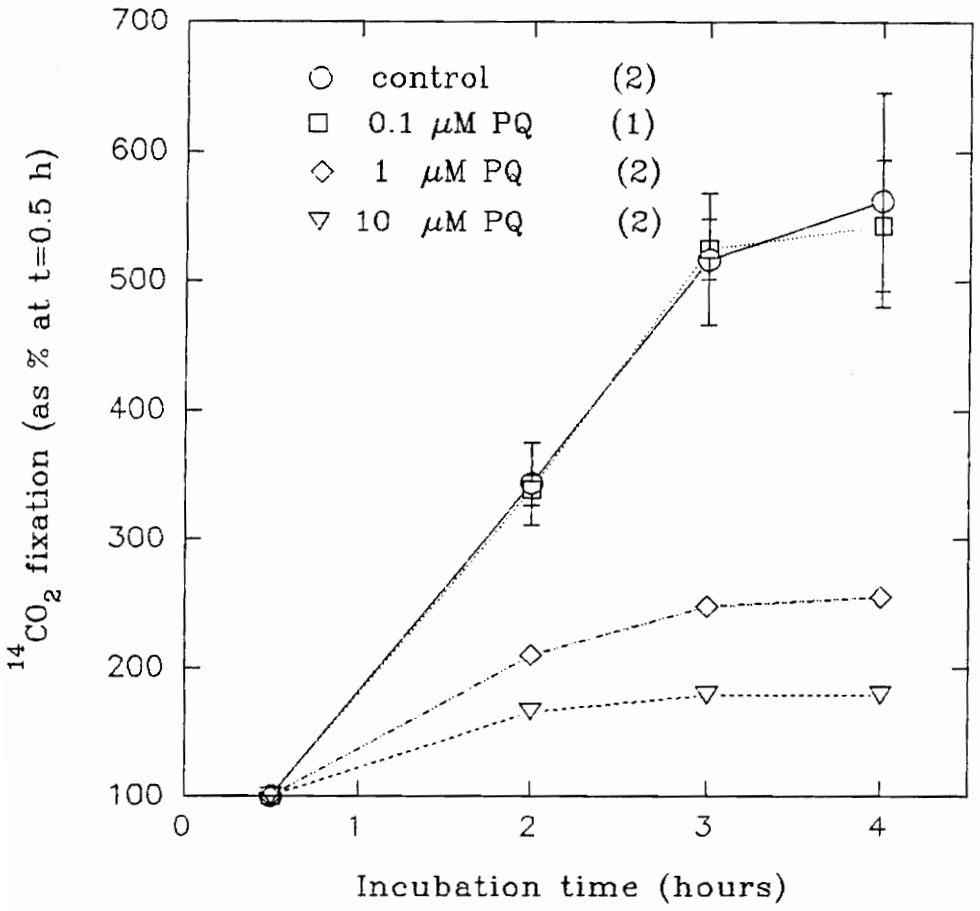


Fig. 18. $^{4}CO_2$ fixation of protoplasts derived from 10-day-old Nugget seedlings incubated with different paraquat concentrations. Numbers in parentheses indicate sample size.

Table 4. Ascorbate peroxidase activity of pea leaves (cv. Progress) determined at two different pH.

| pH | Rate[1] (n=3) |
|---|---|
| 7.6 | 3.9±0.7 |
| 7.0 | 6.0±0.35 |

[1] rates were calculated as μmoles ascorbate oxidized/100 μl extract/min.

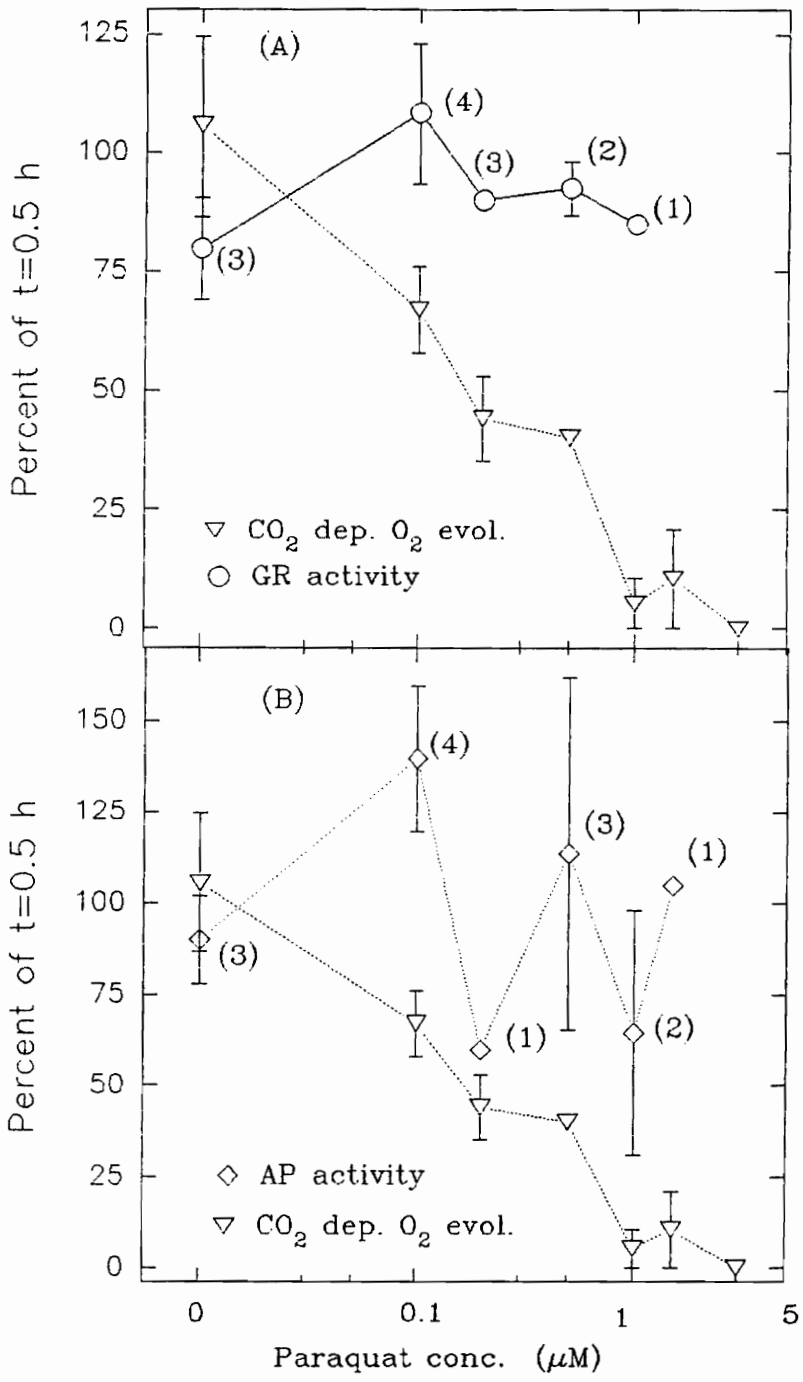


Fig. 19. Effect of increasing concentrations of pq on the activities of GR (A) and AP (B) of Progress protoplasts after 1 h incubation. $CO_2$ dependent $O_2$ evolution rates from Fig. 16 are included for comparison purposes. Numbers in parentheses indicate sample size.

GR activity of controls (protoplasts incubated in the absence of PQ) declined to 77% after the one h incubation period. GR activity of protoplasts, incubated with 0.2 μM PQ on the other hand, did not change at the end of the one h incubation period (an approximately 30% stimulation compared to controls). When protoplasts were incubated at higher PQ concentrations (for 1 h) their extractable GR activity declined to about 80% of the preexposure levels.

AP activities were stimulated by 50% at 0.1 μM PQ (when compared to untreated protoplasts; $p=0.11$). At higher PQ concentrations, activities did not exhibit any discernible pattern.

Samples from one representative experiment were analyzed by protein fractionation, transfer and immunodetection (Western technique). Steady state protein levels of AP and GR were estimated before and one h after exposure to a series of PQ concentrations. Percent change of protein levels was indicated in Table 5 along with the corresponding activities. Activities from that experiment (Fig. 19) were also included in this table for comparison purposes. A representative immunoblot (Western blot) is indicated in Fig. 20. In this figure it can be seen that an unknown epitope cross reacts with the GR antibody. This epitope has a molecular weight (MW) of 36 KDa ((Madamanchi *et al.*, 1993)). In these experiments, it appears to have a MW of 32 KDa. Preimmune

Table 5. Comparison of changes[1] of activity[2] and protein[3] of ascorbate peroxidase and glutathione reductase in Progress protoplasts incubated to different levels of PQ for one hour[4].

| Treatment | ascorbate peroxidase | | glutathione reductase | |
|---|---|---|---|---|
| | activity (%) | protein (%) | activity (%) | protein (%) |
| Control | 192 | 114 | 101 | 84 |
| 0.2 μM | 222 | 64 | 101 | 84 |
| 0.5 μM | 110 | N/A | 57 | N/A |
| 1.0 μM | 66 | 109±3 (n=2) | 158 | 62±17 (n=2 |
| 1.5 μM | 175 | 99 | 98 | 106 |

[1] parameters were determined immediately before and 1 h after administration of PQ; values are reported as percent of preexposure levels

[2] activity data are included in figure 19; they are indicated in this table for comparison purposes

[3] changes in protein levels were determined by fractionating proteins on SDS-PAGE, subsequent semi-dry transfer on nitrocellulose filter paper and immunodecoration of transferred proteins with respective antibodies (see materials and methods)

[4] one representative experiment which showed a relatively wide range of activity changes was chosen for a subsequent protein analysis

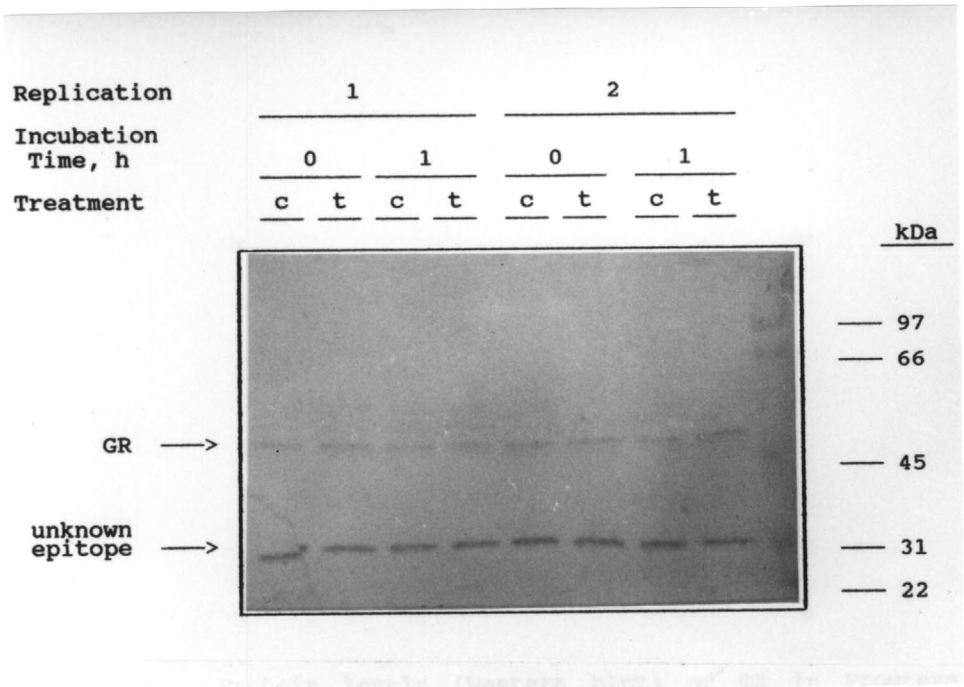


Figure 20. Protein levels (Western blot) of GR in Progress protoplasts (derived from 12-day-old plants) exposed to 1 μM PQ. Protoplasts were incubated under customary photosynthetic conditions and were sampled immediatelly before (t=0 h) and 1 h after (t=1 h) the addition of PQ; (t-PQ treated, c-controls). Crude extracts (10 μg protein) were subjected to 10% SDS-PAGE and then transferred onto nitrocellulose filter paper. GR on the filter was first probed with pea anti-GR (from rabit antiserum; diluted 1:2,000) and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. Color was developed with 4-chloro-1-naphthol. Pre-stained low molecular weight markers (kDa) are shown at the right; phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalabumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa). The blot was scanned and the data were included in Table 5.

serum, from the animal used to produce the GR antibody, did not cross react with the GR protein from pea (Madamanchi, personal communication). In Table 5 it can be seen that in the cases where activity was enhanced, protein was not changed. Possibly enhanced catalytic properties occurred upon imposition of oxidative stress (enzyme activation). GR activity and protein levels were determined immediately before and three h after administration of 0.2 μM PQ (Table 6). Activity decreased in both control and treated protoplasts over the course of incubation. Steady state protein level (as determined by immunoblots) decreased in the control but remained unchanged with treated protoplasts.

Antioxidant enzyme activities of chloroplasts - In a series of four experiments, Progress protoplasts were exposed to 0.1 μM PQ up to 3 h. Protoplast AP, GR and SOD activities at 1 and 3 h were determined (Table 7, part (A)). Chloroplasts were isolated from protoplasts after 1 or 3 h of incubation. Specific activities of the enzymes in the chloroplasts are reported in Table 7 part (B). Statistical comparisons (t-tests) were performed between controls and treated samples separately at each time point. Control $O_2$ evolution data indicated that photosynthesis continued to increase during the first hour of incubation but after three h it had returned to its "original" (0.5 h after the onset of

Table 6. Comparison of changes[1] of activity and protein[2] of glutathione reductase of Progress protoplasts incubated to 0.2 μM PQ for three hours.

| Treatment | activity (%) | protein (%) |
|---|---|---|
| control | 65 | 88 |
| paraquat | 73 | 100±2 |

[1] parameters were determined immediately before and 3 h after administration of PQ; values are reported as percent of preexposure levels

[2] changes in protein levels were determined by fractionating proteins on SDS-PAGE, subsequent semi-dry transfer on nitrocellulose filter paper and immunodecoration of transferred proteins with respective antibodies (see materials and methods)

Table 7. Effect of exposure of protoplasts to 0.1 μM PQ on activities[1] of antioxidant enzymes glutathione reductase (GR), ascorbate peroxidase (AP) and superoxide dismutase (SOD) present in B) purified intact chloroplasts isolated from protoplasts and A) protoplast extracts. Photosynthetic rates are indicated in A)

(A) PROTOPLASTS

| HOURS | TREATMENT | AP | GR | SOD | $O_2$ |
|---|---|---|---|---|---|
| 1 | CONTROL | 130±42 | 102±7 | 101±7 | 181±39 |
| | PQ | 112±77 | 95±0.5 | 80±7(b)[2] | 67±15**[3] |
| 3 | CONTROL | 166±115 | 90±8 | 114±5 | 107±35 |
| | PQ | 428±526 | 76±16 | 99±5(b) | 34±7(a) |

(B) CHLOROPLASTS

| HOURS | TREATMENT | AP | GR | SOD |
|---|---|---|---|---|
| 1 | CONTROL | 189±151 | 124±14 | 122±11 |
| | PQ | 193±7 | 142±4 | 104±1 |
| 3 | CONTROL | 145±25 | 150±8 | 130±40 |
| | PQ | 107±94 | 288±70* | 38±20(c) |

[1] mean±standard error from 2-4 separate experiments; data are indicated as percent change over the indicated time period (in hours) after the first sample was taken (30 min after turning on lights), PQ was administered (to the treated) immediately after the first sample was taken

[2] (a), (b), (c) significantly different means between treatments within the same time point at the 0.12, 0.13, and 0.14 probability levels respectively as judged by T-test

[3] **, * significantly different means between different treatments at the same time point at p=0.05 and p=0.10 levels respectively as judged by T-test

incubation) rate. PQ (0.1 μM) diminished protoplast photosynthesis over time. No conclusions were drawn from the highly variable AP activities. Control GR and SOD activities did not change either after 1 or 3 h of incubation. Photosynthesis of treated protoplasts was lower when compared to that of the respective controls. Overall, the decline of photosynthesis due to PQ was accompanied by decline of AP and GR activities at the whole cell level.

Control chloroplast SOD activity increased over the course of the incubation. After exposure for 3 h to 0.1 μM PQ on the other hand, plastid SOD activity was diminished ($p=0.14$). GR of control plastids increased at both time points. This increased activity in chloroplasts, isolated from treated protoplasts, was more pronounced especially with the 3 h point (statistically significant at $p<0.10$). This increase in GR activity was accompanied by a 70% decrease in photosynthesis compared to preexposure levels. However, when GR activity was not enhanced upon exposure of protoplasts to 10 times higher [PQ] (Table 8), photosynthesis also decreased.

An identical experiment tested the effect of elevating PQ concentration 10-fold (up to 1 μM) on whole cell and plastid antioxidant enzymes. Results from two replicate experiments were summarized in Table 8. Enzyme activities extracted from control protoplasts increased at both 1 h and 3. With treated protoplasts no changes were detected.

Table 8. Effect of exposure of protoplasts to 1 μM PQ on activities[1] of antioxidant enzymes glutathione reductase (GR), ascorbate peroxidase (AP) and superoxide dismutase (SOD) present in B) purified intact chloroplasts isolated from protoplasts and A) protoplast extracts. Photosynthetic rates are indicated in A)

(A) PROTOPLASTS

| HOURS | TREATMENT | AP | GR | SOD | $O_2$ |
|---|---|---|---|---|---|
| 1 | CONTROL | 121±14 | 130±23 | 120±6 | 123±23 |
| | PQ | 112±37 | 102±9 | 93±2 **[2] | 80±28 |
| 3 | CONTROL | 111±2 | 108±13 | 114±2 | 77±31 |
| | PQ | 79±6 ** | 95±9 | 106±1 * | 18±18 |

(B) CHLOROPLASTS

| HOURS | TREATMENT | AP | GR | SOD |
|---|---|---|---|---|
| 1 | CONTROL | 242±177 | 98±7 | 114±0.5 |
| | PQ | 171±153 | 109±22 | 97±8 |
| 3 | CONTROL | 302±174 | 119±5 | 126±5 |
| | PQ | 86±24 | 89±3 ** | 89±6 ** |

[1] mean±standard error from two separate experiments; data are indicated as percent change over the indicated time period (in hours) after the first sample was taken (30 min. after turning on lights), PQ was administered (to the treated) immediately after the first sample was taken

[2] *, ** significantly different means between different treatments of the same time point at the 0.10 and 0.05 probability levels respectively as judged by T-test

Control plastid SOD activities increased at both time points. No increase occurred in plastids isolated from treated protoplasts. In fact, at 3 h, treated plastids contained significantly less ($p<0.05$) SOD activity compared to controls.

GR activities in plastids from control protoplasts increased at 3 h. At this time, GR activities of treated plastids were significantly less than controls. Changes in AP activities were not consistent between the two experiments. No stimulation of enzyme activity was observed at the whole cell or plastid level upon addition of PQ. Under the exposure conditions of the experiment presented in Table 8, activities of antioxidant enzymes were not detectably enhanced and no protection to photosynthesis was conferred.

Generally, it can be said that changes in SOD activities show the greater reproducibility in trends and magnitude (Tables 7 and 8).

Effects on *de novo* GR synthesis - Since PQ-mediated increases in plastid GR activities (0.1 $\mu$M, 3 h incubation) were detected, it was of interest to determine whether or not *de novo* synthesis of GR protein was affected by exposure to PQ. Table 9 shows *de novo* synthesis of total protein expressed as the percentage of TCA precipitable radioactivity over the radioactivity taken up by the protoplasts. The first

Table 9. *De novo* protein synthesis of photosynthesizing protoplasts (cv. Progress)[1], expressed as percentage of acid precipitable radioactivity over the total radioactivity taken up by protoplasts[2].

| Treatment | | Time in light (min.) | | |
|---|---|---|---|---|
| | | 30 | 120 | 195 |
| | activity | | | |
| controls | | | | |
| | taken up | 5061 | 5594 | 7235 |
| | 10% TCA precip. | 410 | 337 | 680 |
| | % | 8.1 | 6.0 | 9.3 |
| 0.1 μM PQ[3] | | | | |
| | taken up | 4127 | 4195 | 5644 |
| | 10% TCA precip. | 391 | 525 | 651 |
| | % | 9.4 | 12.5 | 11.5 |

[1] Protoplasts were incubated under customary photosynthetic conditions. 30 minutes before the indicated time points, protoplasts were transferred to vials containing $^{35}$S-met and allowed to photosynthesize for 30 more minutes. They were then taken from the light, the $^{35}$S-met incorporation reaction was stopped by placing vials on ice and adding an exess amount of met. Protoplasts were then spun, washed and stored at -80°C. Protoplasts were ground and centrifuged and the radioactivity present in the supernatant was assigned to total taken up radioactive methionine. Additionally, the acid precipitable radioactivity of the supernatant was determined and assigned to the whole population of the newly synthesized protein for the duration of the pulse.

[2] means from two duplicate measurements are indicated

[3] PQ was added after 50 minutes light incubation

sample was taken 30 min after illumination was begun; PQ was administered 20 min after this sampling. Protoplasts to be treated incorporated into acid precipitable material slightly more radioactivity (1.3%), compared to controls, (20 min before the administration of PQ; 30 total incubation time). After 70 min in the presence of PQ (120 min total incubation time) the difference between controls and treated increased to 6.5%. This difference declined to 2.2% after 75 additional minutes in the presence of PQ (195 min incubation time). Under the current incubation conditions incorporation of label into TCA precipitable material (as determined by the applied protocol) represents a 6 to 12% of the total activity taken up by the protoplasts.

Proteins from this experiment were immunoprecipitated with polyclonal antibodies raised against plastid GR, washed, and fractionated on SDS-PAGE. The fixed gel was exposed to an X-ray film for 3 months in - 80° C and developed (Fig. 21). TCA-precipitable counts were determined after the immunoprecipitation step but before the immune complex wash step. Equal counts were loaded in all six lanes. Bands corresponding to 55 KDa protein, the expected size of a GR monomer, appeared in lanes 1, 2, 3 and 4. Band in lane 2 (20 min before addition of PQ) appears much more intense compared to the same time control lane (#1). This variability could be due to any type of experimental variation. The difference, in

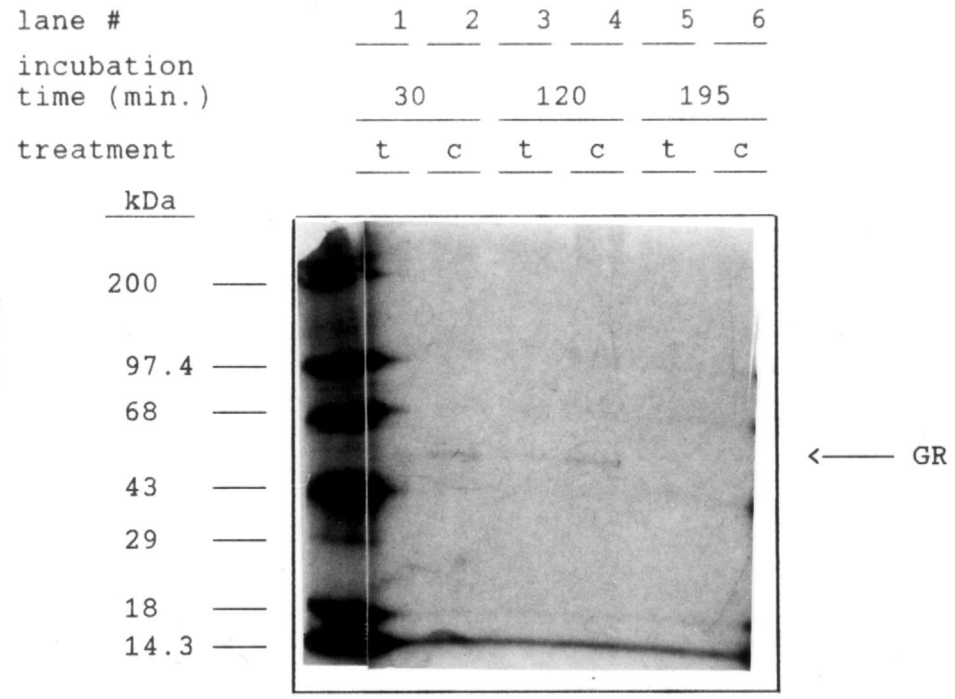


Figure 21. Fluorograph of immunoprecipitated GR protein labeled in vivo with $^{35}S$- met of Progress protoplasts incubated under customary photosynthetic conditions. Paraquat (0.1 $\mu$M) was added after 50 min. of light incubation to treated protoplasts (t- treated; c-controls). 30 minutes before the indicated time points, protoplasts were transferred to vials containing $^{35}S$-met and allowed to photosynthesize for 30 more minutes. They were then taken from the light, the $^{35}S$-met incorporation reaction was stopped by placing vials on ice and adding an excess amount of met. Protoplasts were then spun, washed and stored at -80°C. GR antiboby was added at a ratio of 1:20 to protoplast homogenates and incubated overnight. Immunoprecipitated proteins were spun down washed and resuspended in equal volumes of electrophoresis sample buffer and run on a 12% SDS-PAGE. Acid precipitable radioactivity was determined before the immunoprecipitation step and used to load equal counts to the SDS-PAGE wells. Molecular weight markers (kDa) are shown at the left; myosin (H-chain) (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalabumin (43 kDa), carbonic anhydrase (29 kDa), ß-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa).

GR synthesis between controls and "treated" samples, was maintained 70 min after the addition of PQ (120 min total incubation time) - lane 3: controls vs lane 4: treated. No difference in the intensities of the bands in lanes #2 and #4 was obvious. Exposure to PQ did not apparently affect *de novo* GR protein synthesis under the present conditions. Apparently, no new GR protein was synthesized at 3.25 h incubation either in the presence or absence of PQ. Increases in GR activity were not accompanied by increases in total protein or enhancement of new protein synthesis. These preliminary results suggested that a mechanism of enzyme activation may bring about increase of activity during oxidative stress.

PQ plus Gamborg's salts

Longer time incubation - Since short term exposure to 0.1 $\mu$M PQ appeared to affect GR activity, altered incubation conditions were used to allow a longer exposure periods. A more complex incubation medium was chosen that involved addition of basal Gamborg's salts (1X strength) to the standard medium (see Materials and Methods). At the three h point $CO_2$-dependent $O_2$ evolution in the presence of PQ was 20% of the pre-exposure rates (see above). For the longer incubations, parameters of performance should be chlorophyll and protein concentrations (and not $O_2$ evolution). Figs. 22

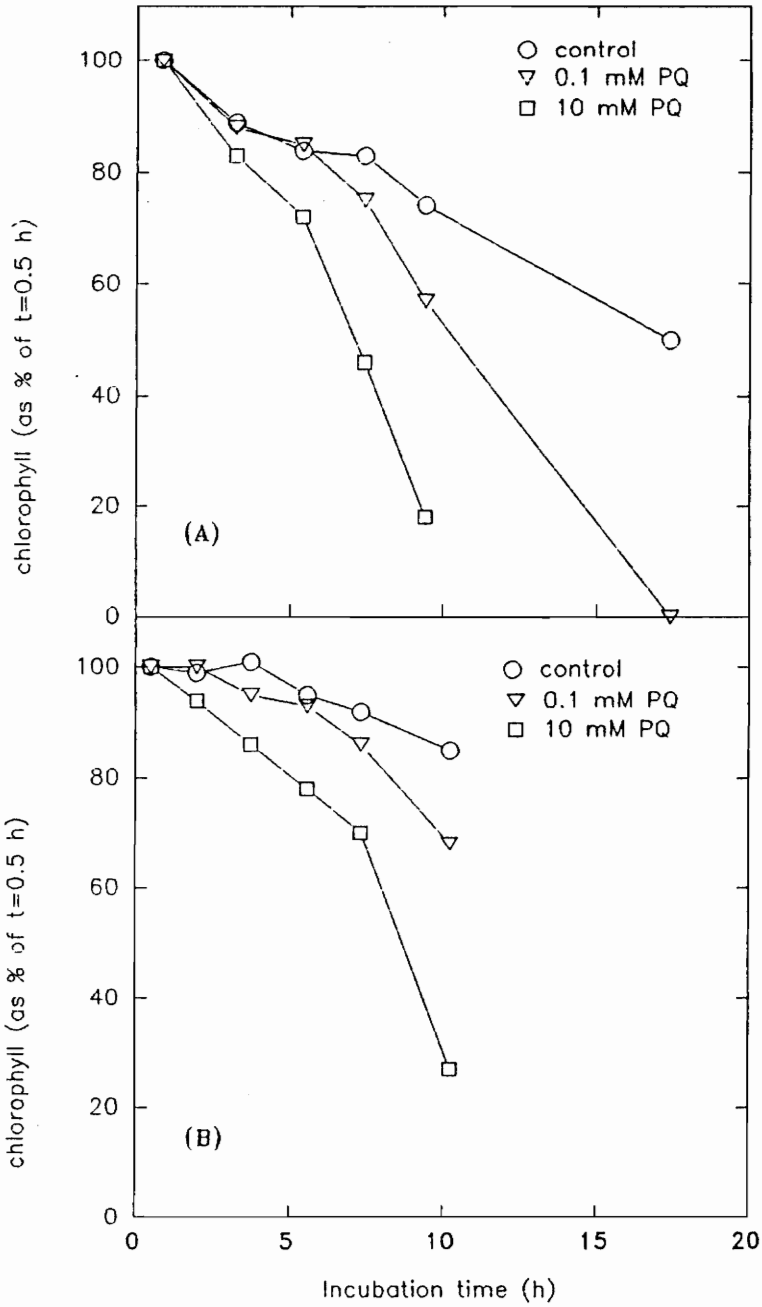


Fig. 22. Effect of exposure to PQ (0.1 and 10 mM) on chlorophyll content of Progress protoplasts derived from 10-day-old plants in the absence (A) and in the presence (B) of Gamborg's basal salt mixture.

(A), 23 (A), and 24 (A) indicated percent change of chlorophyll, protein content, and GR activity respectively from a long incubation (up to 19 h) of Progress protoplasts incubated in the standard medium. Figs. 22 (B), 23 (B), 24 (B) show the behavior of chlorophyll, protein, and GR in the presence of Gamborg's basal salt mixture.

From Fig. 22 (A) it can be seen that in the absence of Gamborg salts control chlorophyll declined from the onset of the experiment while in the presence of those salts the decline begins only after the fourth h. Chlorophyll was maintained by 20-35% more at all respective time points in the presence of Gamborg salts and 0.1 or 10 mM PQ.

In control samples, protein was maintained in the 90% region for up to 7 h in the presence of Gamborg salts (Fig. 23 (B)) while it had declined to 60% in their absence during the same time period (Fig. 23 (A)). In the presence of the two concentrations of PQ used, protein declined less in the presence of Gamborg's salts.

Control GR activity remained above 90% in the presence of the salts for up to 10 h [Fig. 24 (B)]. By 10 h GR was 60% of initial levels in the absence of the salts (Fig. 24 (A)). In the presence of 0.1 mM PQ-treated protoplasts GR activity behaved the same in the two media.

Since inclusion of the Gamborg's basal salts had a

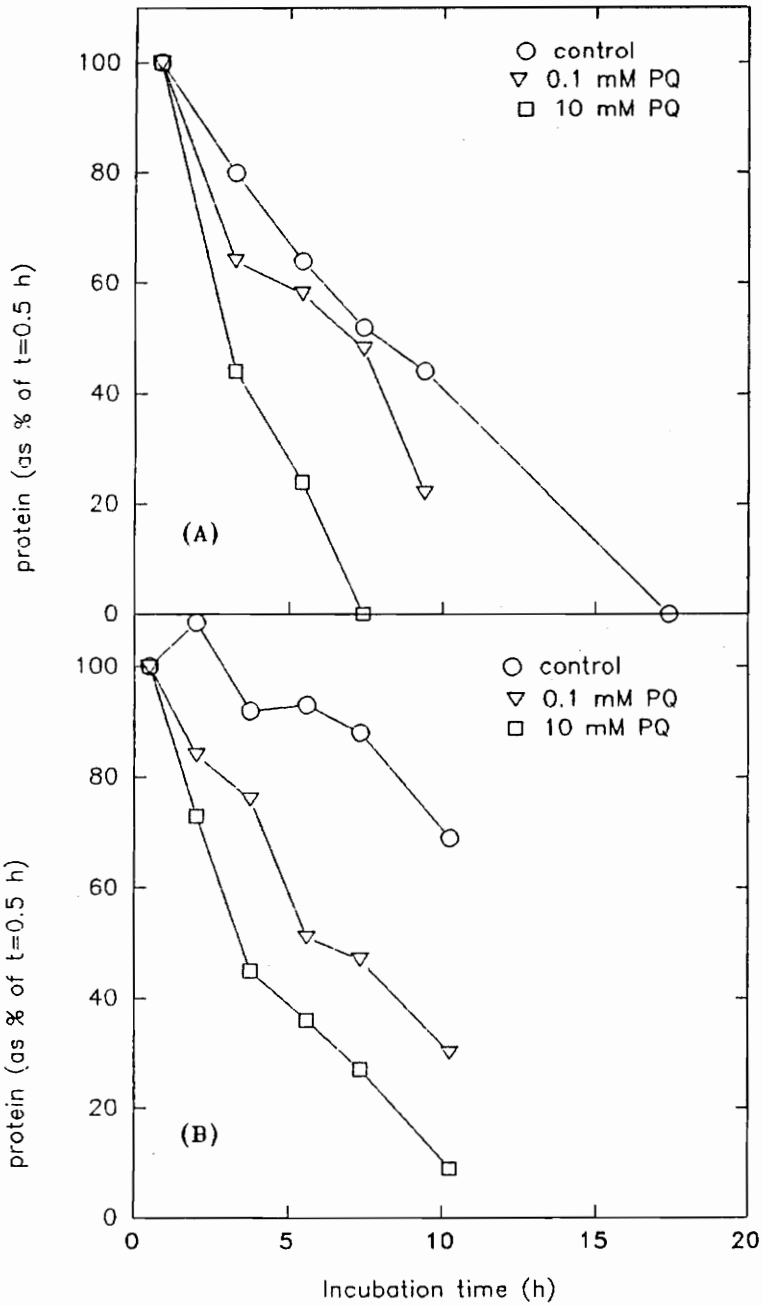


Fig. 23. Effect of exposure to PQ (0.1 and 10 mM) on protein content of Progress protoplasts derived from 10-day-old plants in the absence (A) and in the presence (B) of Gamborg's basal salt mixture.

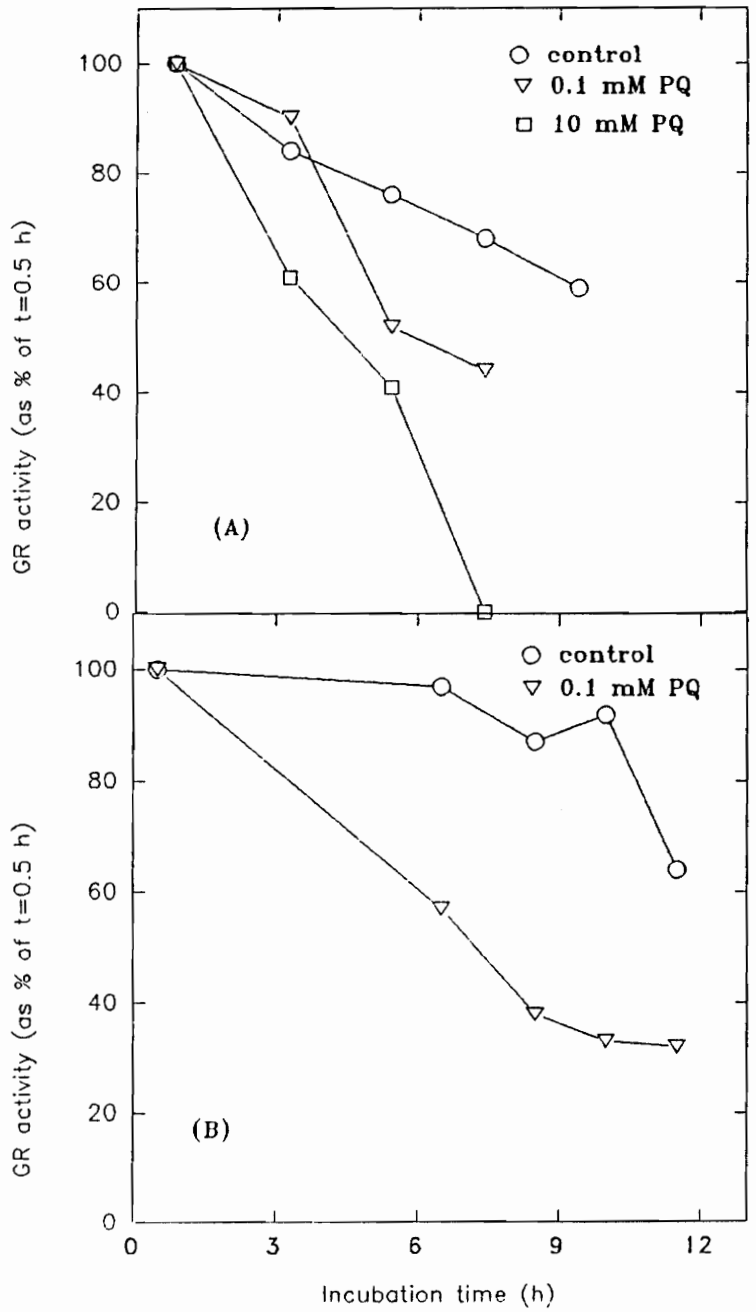


Fig. 24. Effect of exposure to PQ (0.1 and 10 mM) on GR activity of Progress protoplasts derived from 10-day-old plants in the absence (A) or presence (B) of Gamborg's basal salt mixture.

beneficial effect on protoplast stability, they were subsequently included in all 12-14 h incubations.

Long-term exposure at 0.1 mM PQ - Protein and chlorophyll of controls did not degrade appreciably over 8 h (see above). Changes in GR activity (Fig. 24 (B)) followed the pattern of change of total protein levels. GR of treated protoplasts declined to 30% of its original value at the end of the 10 h incubation period. At that time point, control protoplast GR activity was down to 70%. AP activity (Fig. 25) of both control and treated protoplasts declined at a much faster rate compared to GR activities. In the treated protoplasts, AP activity was down to 10% of its pre-exposure levels at about 6.5 h. At the same point, control AP activity was at 70% of its initial value. (The enhancement of AP activity of treated protoplasts after the $10^{th}$ h point was not seen with other incubations). The changes observed in superoxide dismutase activities were quite different (Fig. 26). Control activities did not change throughout the course of the whole incubation and did not drop below 80% of pre-exposure levels. Treated protoplast activities showed an increase after eight to ten h of incubation. The exact onset of this increase varied among experiments by 1 to 2 h. The magnitude of this increase also varied. Means of chlorophyll and protein concentration and AP, GR, and SOD activity by the end of the ten-hour incubation

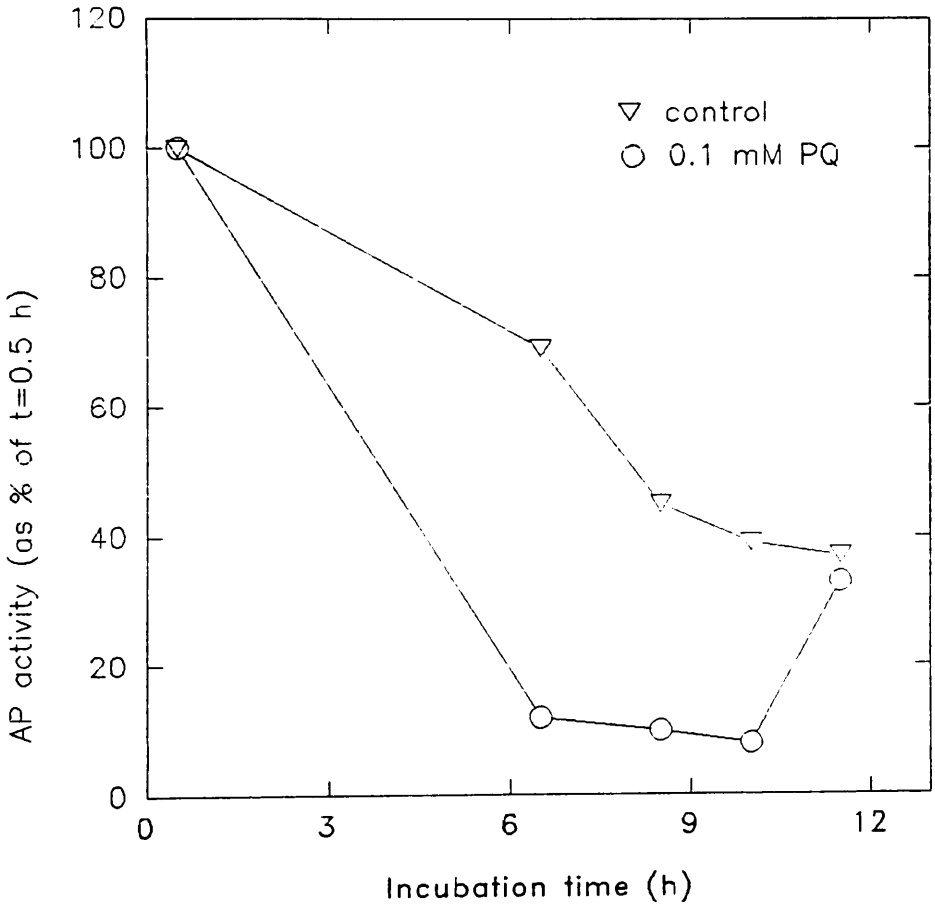


Fig. 25. Percent AP activity of protoplasts derived from 10-day-old Progress plants incubated with 0.1 mM PQ in Gamborg's basal salts.

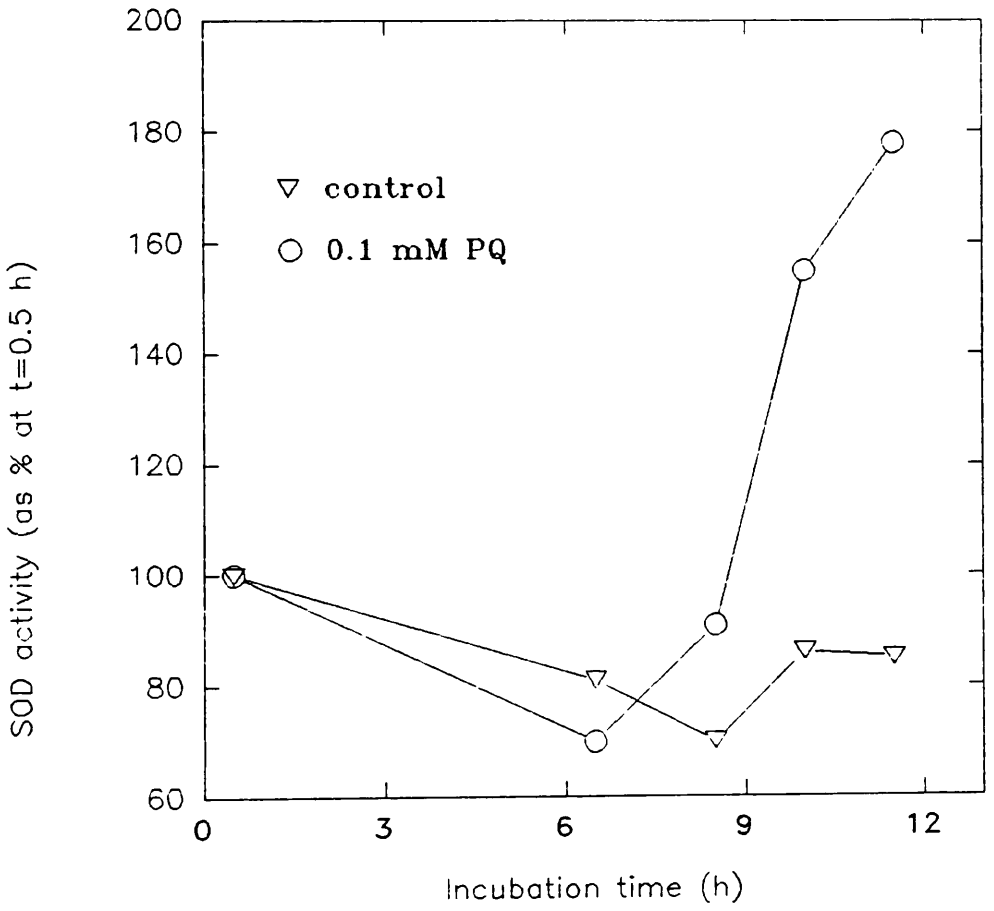


Fig. 26. Percent SOD activity of protoplasts derived from 10-day-old Progress plants incubated with 0.1 mM PQ in Gamborg's basal salts.

period from all experiments were summarized separately for control and treated protoplasts in Table 10. PQ-treated protoplast SOD activity increased significantly over the ten-hour period by 126% compared to untreated protoplasts. Total protein, however, decreased 39% in treated protoplasts over this same time interval. The levels of all other parameters (chlorophyll, protein, GR and AP activities) of treated protoplasts declined 20-40% compared to the levels of control protoplasts (Table 10).

Table 10. Effect[1] of exposure for 10 hours to 0.1 mM PQ of Progress protoplasts incubated in basal Gamborg's medium on chlorophyll, protein and activities of GR, AP and SOD.

| Treatment | Chl (n=2) | Protein (n=2) | GR (n=3) | AP (n=2) | SOD (n=3) |
|---|---|---|---|---|---|
| Control | 79±7 | 63±8 | 77±18 | 35±3 | 113±41 |
| PQ | 49±27 | 24±8 | 28±12 | 16±23 | 238±59 |

[1] Levels of each parameter immediately before and ten hours after administration of PQ were determined. Parameters are reported as percent of preexposure values.

## DISCUSSION

### Higher plant protoplasts in the study of plant responses

Photosynthesizing protoplasts isolated from plant leaf tissue have been used to study stress responses. Inferences may then be made about *in vivo* plant cellular behavior (for a technical review see Hatzios, 1986 and references therein). Different herbicides, among them the pro-oxidants PQ and acidfluorfen, have been used as stressors. Protoplasts are usually incubated under photosynthetic conditions for up to 4 h and their photosynthetic responses to the stressors followed as well as broader metabolic responses (protein and nucleic acid synthesis). Photosynthesizing protoplasts are also used to study solute permeability of the plasmalemma (Kaiser and Heber, 1983), absorption (McCloskey and Bayer, 1990), the relationship between respiration and photosynthesis (Krömer *et al.*, 1993), and enzyme localization (Ignatova *et al.*, 1993). Protoplasts or cells from non-photosynthesizing cultures have also been used to study stress responses (Mieth *et al.*, 1986; Dangl *et al.*, 1987).

The uniqueness of the present study lies in the simultaneous quantification of different components of antioxidant metabolism and the response of photosynthesis to oxidative stress imposed on actively photosynthesizing protoplasts. Imposition of sulfite-mediated oxidative stress

on photosynthesizing protoplasts is reported for the first time. Initially, responses were followed for 1 or 3-4 h. The addition of Gamborg's basal salt mixture in the incubation medium enabled the photosynthetic and antioxidant responses to be further followed up to 14 h.

**Effect of $SO_2$ (sulfite) on photosynthesis**

A protoplast system was developed in order to resolve questions pertaining to cellular antioxidant behavior and inducibility of antioxidant mechanisms. Protoplasts from two pea varieties (cv. Progress and Nugget) were used. An illumination of 400 $\mu E\ m^{-1}\ s^{-1}$ resulted in optimum photosynthesis. This intensity corresponds very well with the environmental conditions in the growth chamber where light intensity at the plant level was 350 $\mu E\ m^{-2}\ s^{-1}$. In a similar comparison Saradadevi and Ragavendra (1992) determined that the optimal light intensity required for maximal rates of $CO_2$-dependent $O_2$ evolution by mesophyl protoplasts of pea (cv. Arkel) was 1250 $\mu E\ m^{-2}\ s^{-1}$. In their case, however, pea plants were grown outdoors where midday light intensity was still somewhat greater. Additionally, the $CO_2$-dependent $O_2$ evolution of control Arkel protoplasts was 170 $\mu mol\ mg^{-1}$ Chl $h^{-1}$ while those of Nugget and Progress was ca 100 and 70 respectively. This difference in performance may be attributed to the high light of the outdoors environment of Arkel and possibly

intercultivar differences.

In the past, protoplasts from Nugget and Progress fixed carbon at almost identical rates (20.5 and 23.5 μmol carbon $mg^{-1}$ chl $h^{-1}$ for Progress and Nugget respectively, (Alscher *et al.*, 1987b)). Under the growth and exposure conditions of the present study protoplasts from both cultivars fixed $^{14}CO_2$ at double that rate (data not shown).

These two cultivars were found to be differentially sensitive to externally imposed abiotic oxidative stress at the whole plant level (Alscher *et al.*, 1987a). In that experiment, the authors fumigated whole plants with 0.8 ppm $SO_2$ and determined whole leaf photosynthetic attributes. At the onset of fumigation the two cultivars had identical photosynthetic potential. During fumigation, Nugget photosynthesis initially declined at twice the rate of Progress. When fumigation was arrested after 80 minutes, Progress recovered to pre-exposure levels in 50 minutes while Nugget had only half recovered at that time. The authors determined that more sulfite (the major hydrated form of $SO_2$ at the pH range of 7 to 8; (Schroeter, 1966), (Ziegler, 1975)) had accumulated in the leaves of the sensitive cultivar while the tolerant showed an increase in reduced glutathione (GSH).

In this study photosynthesis was determined both as $CO_2$-dependent $O_2$ evolution and incorporation of acid-stable $^{14}CO2$-

dependent counts ($CO_2$ fixation). During exposure of protoplasts to all levels of [$SO_3$] employed, no significant decline in $O_2$ evolution was detected (Tables 1-3, Figs. 4-12). In comparison, application of $SO_3^{2-}$, at concentrations higher than 3 mM, to photosynthesizing protoplasts, reduced $CO_2$ fixation in a concentration- and incubation-time-dependent fashion (Figs. 13-15). In order to explain the apparent disagreement between the two ways of estimating photosynthesis, some consideration has to be given to the different metabolic fates of sulfite inside the protoplasts under photosynthetic conditions. Sulfite is rapidly oxidized to sulfate in an autocatalytic process that is initiated by the superoxide anion produced by PS I during photosynthetic electron transport (Asada, 1980). Sulfate is eventually transported to the vacuole in an ATP-dependent process (Kaiser *et al.*, 1989). In the process of sulfite oxidation, more superoxide and additional hydroxyl radicals are produced (see Introduction). If the radicals are not scavenged, they can have a detrimental effect on thylakoid membrane integrity and Calvin-Benson enzymes activation state (reviewed by Alscher *et al.*, 1987). In addition to its oxidative fate, sulfite can be reductively detoxified by sulfite reductase which uses ferredoxin as the electron donor (Rothermel and Alscher, 1985; von Arb and Brunold, 1983). In the presence of adequate amounts of *O*-acetylserine and SOD activity intact chloroplasts

have been shown to reduce sulfite to cysteine and to exhibite sulfite-dependent oxygen evolution (Ghisi *et al.*, 1990). In that experiment chloroplast sulfite-dependent $O_2$ evolution became visible only when sulfite oxidation was largely suppressed. Hence, it is conceivable that under my experimental conditions oxidative damage caused by sulfur oxide and oxygen radicals produced during the aerobic oxidation of sulfite (which would have been manifested by diminution of $CO_2$-dependent $O_2$ evolution) is masked by a concomitant sulfite-dependent oxygen evolution (during its reduction to cysteine and probably eventually to glutathione). This latter conclusion can be supported by the fast and sustained increase of reduced glutathione upon fumigation of the tolerant cultivar (Progress) with $SO_2$ (Madamanchi and Alscher, 1991). Cardinal in the plausibility of this last contention is the issue of the ratio of reductive vs oxidative detoxification of sulfite. If the oxidative route is preferred *in vivo*, then little sulfite will be metabolized to form glutathione. It has been shown that only 18% of the exogenously provided $^{35}SO_4$ (the oxidation product of $SO_3$) was metabolized to glutathione during oxidative conditions (Smith *et al.*, 1985). If, on the other hand, the reductive route is preferred (yielding as intermediate product hydrogen sulfide), then it is expected that more sulfite will end up as glutathione.

Exposure of Progress and Nugget protoplasts to 1.5 mM sulfite lead in enhancement of $CO_2$ fixation under the present conditions (Figs. 14 and 15 respectively). When, in a similar experiment, Paul and Bassham (1978) incubated photosynthesizing cells of *Papaver somniferum* with 10 or 20 mM sulfite they saw a stimulation of $CO_2$ incorporation up to 30% (compared to untreated controls). Based on metabolite profile analysis the authors proposed that a glyoxylate-$HSO_3^-$ adduct (an $\alpha$-hydroxysulfonate) inhibits glycolate oxidase. Their conclusion was supported by an earlier finding of Zelitch (1957). In turn glycolate will accumulate and possibly inhibit the oxygenation reaction catalyzed by ribulose bisphosphate carboxylase oxygenase. Such an efflux may favor the carboxylation reaction. In another study it was shown that both the sulfite and the sulfate anions, at saturating concentrations of $CO_2$, enhanced the catalytic activity of RUBISCO above that achieved with $CO_2$ and $Mg^{2+}$ alone (Parry and Gutteridge, 1984). Additionally, the activation by $CO_2$ and $Mg^{2+}$ of a slow activating[5] form of the carboxylase in the presence of the two anions produced high specific activities with significant lower concentrations of $CO_2$ than normally required.

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[5]This terminology is abondoned today. It has been shown that the enzyme RUBISCO activase is involved in the catalytic activation of RUBISCO. Nevertheless, there exists a requirement of $CO_2$ and $Mg^{2+}$ for full RUBISCO activation.

Contrary to the cases where enhancement was observed, Marques and Anderson (1986) working with isolated pea (cv Little Marvel) chloroplasts found that as little as 0.4 mM sulfite was adequate to inhibit the reaction catalyzed by ribulose bisphosphate carboxylase.

Carbon fixation of pea protoplasts was decreased in the presence of 7, 10, and 15 mM sulfite (Figs. 13-15). This decrease can be brought about either by reductant competition between the sulfur assimilation pathway and the "dark reactions" of the Calvin-Benson cycle (Ghisi *et al.*, 1990) or by oxidative deactivation of the reductively activated enzymes of the latter cycle (Tanaka *et al.*, 1982b).

Additionally, another possibility may explain sulfite sequestration and a concomitant decrease of its effective concentration. Their formation (of sulfite addition compounds) is light-dependent and is considered to alleviate partly the sulfite toxicity problem (Dittrich *et al.*, 1992).

Eventually, with the present system, the mild oxidative potential of sulfite and the possible enhancement of antioxidant defenses (after exposure to $SO_2$ or sulfite) were not further pursued. No measurable effect of sulfite on photosynthesis could be detected with the initial photosynthetic measurements ($CO_2$-dependent $O_2$ evolution). The enhancement of antioxidant defenses, upon imposition of oxidative stress caused by sulfite, is well documented and has

been observed in leaves of Progress (Madamanchi and Alscher, 1991) of poplar (Tanaka and Sugahara, 1980; Gupta *et al.*, 1991), and in *Chlorella sorokiniana* cells (Rabinowitch and Fridovich, 1985). Instead, a more potent pro-oxidant (PQ) was chosen for the subsequent oxidative treatment of protoplasts.

**Effect of PQ on protoplast photosynthesis**

One of the advantages of using a protoplast system lies with the quick equilibration of administered compounds between the incubating medium and the cell. Additionally, the compound is in direct contact with the cell and no amount is sequestered in any other extracellular structure of the plant. For those reasons, a series of low PQ concentrations (0.1 to 5 $\mu$M) were chosen to generate oxidative stress to the protoplasts.

Photosynthesis was determined both as $CO_2$-dependent $O_2$ evolution and $^{14}CO_2$ fixation. A good agreement was found between the two indices. In this case, unlike that of sulfite treated protoplasts, reduction in oxygen evolution (Fig. 16) parallels reduction in carbon fixation (Figs. 17 and 18).

Nevertheless, the observed relative tolerance to PQ in the two varieties is opposite of what has been previously reported. Nugget is relatively more tolerant at the non-bleaching PQ concentrations used in the first part of this study. Alscher et al., (1987a) established that Progress was

relatively insensitive and Nugget relatively sensitive to oxidative stress caused by 80 min. exposure to 0.8 $\mu$l $l^{-1}$ $SO_2$. Additionally, Progress was relatively more tolerant to bleaching concentrations (10 $\mu$M) of the oxidative herbicides PQ and acidfluorfen (Madamanchi *et al.*, 1994). Chlorophyll destruction monitored as leaf decoloration was used to estimate tolerance.

Activities of GR and AP are stimulated by 30 and 50% respectively in Progress protoplasts incubated with 0.1 $\mu$M PQ for 1 hour (figure 19). At that point photosynthesis had declined to 70% of the pre-exposure rates. Despite the increases of antioxidant enzyme activities, photosynthesis was not maintained at the same levels as before addition of PQ. At higher PQ concentrations this stimulation was abolished (although GR activity stays at higher levels compared to controls) while photosynthesis continued to decline (no photosynthesis at 1 $\mu$M PQ or higher). Perhaps, PQ may decrease GR activity by directly interacting with the protein (Liochev and Fridovich, 1993).

Whole cell steady state protein levels are not stimulated in the presence of the low PQ concentrations (0.2, 1.0, 1.5 $\mu$M) after one hour (for AP and GR; Table 5) or after three h (0.2 $\mu$M PQ, for GR; Table 6). Total protein levels decline to 60-80%. Comparable GR protein changes have not been reported.

Overall, at the whole cell level and at non-bleaching PQ concentrations, the response of photosynthesis to this herbicide can only minimally be attributed to antioxidant mechanisms. Possibly some mechanism unrelated to antioxidant protection is relevant under the particular experimental conditions.

There appear to be a plethora of different mechanisms contributing to PQ tolerance which cannot be tested in a protoplast system (like the one used in the current study). Intracultivar differential resistance of soybean to bleaching concentrations of PQ was attributed to reduced mobility or a delayed release of PQ in the mesophyll cells of the tolerant cultivar (Kim and Hatzios, 1993a). In a follow up study it was shown that antioxidant substrates and GR activities were similar between the two soybean cultivars (Kim and Hatzios, 1993b).

In roots of hydroponically grown intact maize seedlings, PQ uptake was shown to occur via a carrier-mediated system similar to the one proposed for animal tissues (Hart *et al.*, 1992). Further work with protoplasts from maize suspension cells indicated that putrescine (a polyamine with the same charge distribution as PQ) competitively inhibited PQ uptake. Along with additional kinetic analysis, it was proposed that PQ uptake is mediated by a polyamine transport mechanism which is localized in the plasmalemma (Hart *et al.*, 1993). Whether

this putrescine-related mechanism constitutes a locus of differential resistance to PQ is a possibility which merits investigation.

Results, obtained with leaf slices from resistant and tolerant varieties of *Hordeum glaucum* and *Hordeum leporinum*, indicated that inhibition of photosynthetic $O_2$ evolution was delayed in resistant biotypes compared to susceptible biotypes (Preston *et al.*, 1992). This delay correlated with a 50 % reduction in the amount of herbicide transported in the case of the resistant biotype (a process which can not be acounted for in a protoplast system). In a previous study activities from crude leaf homogenates of superoxide dismutase, guaiacol peroxidase, and catalase were not increased in the resistant biotype (Powles and Cornic, 1987).

Regarding the relationship of steady state protein levels of the antioxidant enzymes and their activity levels, it has been shown that PQ induced a 50% increase in total SOD protein after 12 h of a 10 $\mu$M application on maize leaves (Matters and Scandalios, 1986). This increase was accompanied by stimulation of SOD activity (Matters and Scandalios, 1986) and was due to increased *de novo* synthesis of SOD protein as was judged by *in vivo* labelling with $^{35}$S-methionine. In that experiment, neither GR activity nor rate of GR protein synthesis were determined.

No information could be found in the literature regarding

*de novo* protein synthesis of photosynthesizing protoplasts under stress.

**Antioxidant responses in whole cells and plastids**

Activities of antioxidant enzymes extracted from total cell homogenates were decreased after 1 and 3 h of protoplast incubation with 0.1 μM PQ (Table 7). Those activities tended to be approximately 15% lower compared to untreated protoplasts at similar time points.

GR, AP, and SOD activities from plastids isolated from protoplasts were also determined. After three h incubation with 0.1 μM PQ, plastid GR activity almost tripled (a 288% increase; table 7). At this time photosynthesis was 30% of the preexposure rate. Without further study it can not be concluded whether this increase in GR activity was beneficial for photosynthesis. The increase of plastid GR activity was not observed when 10 times more PQ was used (1 μM; Table 8).

*Conyza bonariensis*, after repeated agronomic applications of PQ, has developed a biotype with 100 fold greater resistance to PQ compared to the original native population (susceptible biotype) (Shaaltiel and Gressel, 1986). Contrary to what it was proposed for *Hordeum sp.* (at the whole leaf level - see discussion above), the *Conyza b.* tolerant biotype was found to contain 60, 150 and 200% relative higher constitutive activities of superoxide dismutase, AP and GR

respectively in chloroplast stromal extracts compared to the sensitive biotype. Those constitutively high activities were concluded to be under the pleiotropic control of a single nuclear dominant gene (Shaaltiel *et al.*, 1988a).

In the Gressel study, differences were determined between the two biotypes with respect to steady state protein levels of SOD, AP, and GR as well as constitutive activities from plastids. Differences in protein levels (between biotypes) were found to agree very well with the differences in activity levels. Nevertheless, activity and protein levels were not compared after application of PQ (as is done in our current study) and no conclusions were, therefore, drawn regarding the inducibility of activities or total protein levels.

The two *Conyza* biotypes exhibited the same severe inhibition of whole leaf photosynthesis at the differentiating PQ dose (100 $\mu$M) within 30 min. Upon removal of the stressor the resistant biotype recovered (while the susceptible did not) and the authors concluded that the high constitutive levels of the enzymes of the active oxygen detoxification pathway keep the resistant plants alive while PQ is being actively sequestered (Shaaltiel and Gressel, 1987).

In addition to the possibility of higher levels of steady state protein levels, activity can also be enhanced by activation of a preexisting enzyme population. In the case of GR, a preexisting protein population can be activated upon

altering the redox potential of the enzyme milieu towards the oxidizing side. Alleviation of the inhibitory effect of NADPH can be achieved by redox interconversion (Mata *et al.*, 1985) and may involve the reduction of an intramolecular disulfide bridge (Schirmer and Krauth-Siegel, 1989; Peinado *et al.*, 1991). My data show considerable variability; neither activity stimulation nor activity maintenance to preexposure levels was accompanied by increase in more protein. Hence, it is possible that enzyme activation is taking place under the conditions of the experiment summarized in Table 5.

Chloroplasts extracted from untreated protoplasts (Table 7) did not show any time- and light-dependent stimulation of GR activity. It is possible that the 288% increase detected in GR chloroplast activity from treated protoplasts was a specific response to PQ treatment.

Due to its variability no conclusion can be drawn regarding the behavior of AP while SOD activity increased in controls and decreased in chloroplasts extracted from treated protoplasts. The optimum of the pea plastidic AP is sharply pH 8.0 while the cytosolic isoform has a broad pH optimum (5-8) (Mittler and Zilinskas, 1991). The AP assay buffer used in the present study had a pH 7.0. Hence, the AP activities reported here may reflect the cytosolic rather the plastidic isoform.

Overall, it can be concluded that, under the specific experimental conditions, coordinate stimulation of the antioxidant enzymes in pea protoplasts does not take place. Thus, the proposal derived from Gressel's group conclusions regarding the coordinate activity enhancement of antioxidant enzymes (Jansen *et al.*, 1990; Shaaltiel and Gressel, 1986; Malan *et al.*, 1990) does not appear to apply here. Gressel's group formulated their conclusions (regarding the coordination of antioxidant protein activity and induction) on the basis of the relationship of constitutive enzyme levels of a population which is resistant by a factor of 100 (Jansen *et al.*, 1990). In my system instead, the potential for activity and/or protein stimulation within 1 to 4 h of oxidative insult was tested. Regarding the unchallenged (constitutive) activity of GR and SOD the two cultivars contained similar levels (Madamanchi and Alscher, 1991).

**Long-term responses of Progress protoplasts to higher concentrations**

Induction by oxidative stress of the different enzymic components of the ascorbate - glutathione antioxidant cycle has been demonstrated in previous studies (Foster and Hess, 1982; Tanaka and Sugahara, 1980). Consequently, an alteration of incubation conditions allowing for longer exposure time was sought in order to maximize the possibility of observing

effects of oxidative insult on antioxidant metabolism. This took place as a two step process. First, a more complex incubation medium was based on addition of Gamborg's basal salts to the simple medium already in use. Second, higher PQ levels were used which fall in the same range as those used for whole leaf PQ applications. So, the 1 μM range was replaced with 0.1 mM and 10 mM.

In the presence of Gamborg's salts, stability of protoplasts increased. These salts allowed for longer maintenance of control chlorophyll and protein content at above 90% of the initial levels (up to 8 h; Figs. 22 and 23 respectively). The salts were eventually used for all subsequent incubations which took place in the presence of high PQ concentration (0.1 mM).

After 8 h in the medium, senescence processes seem to overcome the maintenance processes. Chlorophyll breakdown and proteolysis closely relate to other components of the senescence process such as cell permeability and hydroperoxide content (Trippi and De Luca d'Oro, 1985). Hence chlorophyll and protein contents were chosen as parameters with which to monitor protopast stability with 0.1 mM PQ.

GR activity, present in protoplast homogenates, incubated with 0.1 mM PQ under these conditions declines to 28% of preinsult levels at *ca.* 10 h while control activity stays approximately at 77% for the same time period (Table 10). AP

activities of both control and treated protoplasts decline with the latter declining to 10% of preinsult levels by the 7$^{th}$ hour (Table 25). Control SOD activity does not change but treated protoplasts have activity 238% higher compared to preexposure levels after 10 h (Fig 26; Table 10).

In part, water stress is similar to oxidative stress. Consequently water stress-resistant lines can also be used for probing antioxidant responses. Decline of GR activity has been seen with a maize strain sensitive to water stress (LG11) but not in the tolerant line (LIZA). Application of 1 mM PQ for 12 h, either in the light or in the dark, to disks from senescing leaves of the sensitive strain led to decline in activity (Pastori and Trippi, 1993). On the other hand, GR activity increased when disks, treated with PQ, from non-senescing leaves of the tolerant as well the sensitive strains were used. This enhancement was many-fold greater in the tolerant strain. It is interesting to note that this enhancement was not due to any perceived photooxidative stress since in a parallel study it was shown that in the dark (Pastori and Trippi, 1992) the GR activity increase was positively correlated with increasing PQ concentrations.

Fumigation of spinach leaves with 0.07 ppm $O_3$ also led to significant increases in extractable GR activities (Tanaka *et al.*, 1988b). These increases were accompanied by increases in the protein levels of GR. Hence, in this case ozone exposure

lead to inductive biosynthesis of GR.

In agreement with the results reported in Figs. 19 and 25, AP activity also declined over 72 h in pea leaf discs floated on a 10 $\mu$M PQ solution and illuminated with 160 $\mu E\ m^{-2}\ s^{-1}$ of continuous light (Gillham and Dodge, 1984). However, under the same conditions on 0.1 $\mu$M PQ, AP activity in the leaf disks increased (Gillham and Dodge, 1984). In another experiment, 14-day-old pea plants (cv. Progress) were sprayed with PQ and left for 5 h under 300 $\mu E\ m^{-2}\ s^{-1}$. AP activity increased following the application of a lower PQ concentration (1 $\mu$M) but decreased following application of a higher concentration (10 $\mu$M) (Mittler and Zilinskas, 1992). Both concentrations caused no visible injury and produced a 3-fold increase in the transcript encoding the cytoplasmic isoform of AP. However, application of 100 $\mu$M PQ, under the same conditions, resulted in severe necrosis in plant tissue and caused a decrease in both cytosolic AP transcript and whole cell activity. During all these treatments, cytosolic AP protein levels (measured with monospecific polyclonal antibody) did not change. Probably the control on AP activity was exerted independently at two different levels (translationally and post-translationally). In that study the behavior of the plastid AP isoform was not followed. In mung beans (cv. Feltham First) and peas (cv. Alaska) ethylene-promoted AP activity protected the plants against subsequent

application of hydrogen peroxide and 50 μM PQ (Mehlhorn, 1990).

It has been found that levels of both extraplastidic and plastidic AP isoforms are regulated in light via phytochrome (Thomsen *et al.*, 1992). The control, over the appearance of the cytosolic isoform, may be exerted by photooxidative processes in the plastid. This response is obligatorily oxygen-dependent and is abolished by quenchers of singlet oxygen.

In agreement with results presented in the current study enhancement of SOD activity occurred after PQ application. Treating 10-day-old maize leaves with $10^{-5}$ M PQ for 12 h resulted in a 40% increase in SOD activity (Matters and Scandalios, 1986). This increase was accompanied by higher levels of specific chloroplastic as well as cytosolic isozymes that resulted from newly synthesized protein. Messenger RNA that codes for those isozymes, also increased suggesting that the response of SOD to PQ might be due to enhanced transcription of these genes. In the same experiment, application of juglone (another superoxide generating compound) stimulated SOD activity. The authors concluded that enhanced expression of SOD was due to the higher levels of $O_2^{\cdot}$. PQ stimulated the activities of SOD, catalase and peroxidase in a photomixotrophic culture line of *Chenopodium rubrum* tolerant to PQ (Bhargava, 1993). In the absence of PQ insult

the tolerant and susceptible lines had similar constitutive activities of these enzymes. Total SOD activity increased in the green alga *Chlorella* upon administration of 25 μM PQ and this made the cells resistant to a subsequent higher PQ administration (Rabinowitch *et al.*, 1983). Also, a new form of Mn SOD appeared. In another study Perl-Treves and Galun (1991) working with leaves from intact tomato plants determined that increases in chloroplastic and cytosolic Cu,Zn SOD transcripts were followed by increases in both isoforms. On the other hand, in the present system (Fig. 26), it was determined that the stimulation of total SOD activity (upon application of PQ) was preceded by 3 h by a proportional increase in the cytoplasmic isoform (Janet Donahue; personal communication). Hence, the increases in whole cell SOD activity, seen with Progress protoplasts, can be mostly attributed to increases of the cytoplasmic Cu/Zn isozyme. The isozyme ratio did not change in the absence of PQ. Similarly, Pitcher *et al.* (1992) found that in bean leaves the percent increase in Cu/Zn-$SOD_{cyt}$ activity was strongly and significantly associated with percent leaf surface injury caused by ozone. The increases in cytosolic SOD activities in protoplasts may indicate also injury rather than a defense response and bears little if any relationship to resistance. Pitcher *et al.* (1992) did not observe any change in Cu/Zn-$SOD_{chl}$ during ozone exposure.

*Nicotiana plumbaginifolia* plants were sprayed with 50 μM PQ and the time- and light-dependent steady-state level of different SOD transcripts was followed (Hérouart *et al.*, 1993). Fe-$SOD_{chl}$ transcript abundance increased approximately 40-fold after 27-41 h in light. Mn-$SOD_{mit}$ and Cu/Zn-$SOD_{cyt}$ transcript levels were increased by 30- and 15-fold respectively in the above time period. In the dark only Cu/Zn-$SOD_{cyt}$ increased by 12-fold. No measurements of protein levels or enzyme activities were made.

In contrast to GR and AP, it appears that SOD activities (both plastid and cytosolic) withstood the PQ-induced oxidative destruction up to six hours of exposure. Since the chloroplast is the known main site of injury in the light, it is not surprising that the plastid form of the enzyme was relatively more affected than was the cytosolic form. Conversly, the majority of GR activity (70%) lies in the chloroplast and, in the injured plastid, accounts for the decline in GR activity observed in treated protoplasts.

**Enhancement of antioxidant enzymes by gene addition**

Indirect evidence for the importance of the different antioxidant enzymes in conferring resistance to oxidative stress has recently arisen from results with transformed plants. Aono *et al.* (1993) achieved expression of a GR

transgene in leaf cells of transgenic *Nicotiana tabacum* and confirmed the accumulation of the product of its translation in chloroplasts immunochemically. Leaves of some transgenic plants had GR activities that were about 3-fold higher than those of the untransformed plants. Those transgenic plants exhibited lower susceptibility to both PQ and $SO_2$ in the light than untransformed plants in terms of the extent of visible foliar damage. The authors concluded that the chloroplastic GR plays a role in the resistance of plants to photooxidative stress caused by PQ or $SO_2$.

Perl *et al.* (1993) succeeded in introducing copies of either the Cu,Zn-$SOD_{chl}$ or Cu,Zn-$SOD_{cyt}$ from tomato in potato. Transgenic potato lines harboring either of the transgenes showed elevated tolerance to PQ. Performance was determined either by scoring symptoms visually or by estimating photosynthetic evolution based on photoacoustic measurements. More specifically, when *in vitro* culture shoots of untransformed plants were exposed to $3*10^{-5}$ M PQ for 4 h it was found that the relative quantum yield of oxygen evolution was drastically reduced to about 5% of the control level. In the presence of either of the SOD genes, this reduction in oxygen evolution was 70-90% of controls. The leaves of the transgenic plants that harbored the *chl* Cu,Zn SOD gene were significantly more resistant to PQ than leaves of the transgenic plants harboring the *cyt* Cu,Zn SOD transgene. The

enhancement of either the chloroplastic or the cytosolic SOD activities in the transgenes alleviated a plastid originating photooxidative stress. Hence, communication exists between the plastid and the cytosol. In a previous study with non-transformed plants, it was determined that upon exposure of plants to PQ the transcripts of the *cyt* or the *chl* Cu,Zn SOD genes were increased several fold within 1-4 h (Perl-Treves and Galun, 1991). Contrary to this fast response, the respective increase of SOD activities was much delayed; little or no increase was detected within 4 h of PQ treatment. The authors, summarizing the results from both papers, concluded that this lag in the defense response (of the non-transformed) could be critical to plants under acute oxidative stress. Instead, a constitutively expressed SOD activity, imposed by transgenes, would provide interim protection against superoxide radicals until the endogenous SOD genes are induced and a higher SOD activity is reached. This latter activity would provide the long-range protection.

In a third case transgenic tobacco plants that overexpress the Cu/Zn $SOD_{chl}$ from pea were produced (Sen Gupta *et al.*, 1993a). The transgenic protein was localized in the chloroplast of the transformed plants. Chloroplast localized SOD activity was enhanced by 2-fold compared to the endogenous chloroplast levels. Leaf disks from untransformed and transgenic plants were subjected to photoinhitory treatment.

Photosynthesis declined in both cases but only in the transformed plants recovered once the treatment was removed. The authors contrasted their results with those of Tepperman and Dunsmuir (1990) and proposed that the moderate increases in activity provided the protection in their case. Tepperman and Dunsmuir (1990) had produced some plants with 30-fold increases in chloroplast localized SOD activity that were not more resistant to oxidative stress caused by PQ. The same authors had also produced some transgenics with moderate (2- to 4-fold) increases in SOD activity which, similarly to transgenics with high SOD activity, were not more tolerant to oxidative stress. Hence the difference between Sen Gupta *et al.* (1993a) and Tepperman and Dunsmuir (1990) has to be sought in a reason other than the fold activity enhancement of transgenics. The transgenic plants produced by Tepperman and Dunsmuir (1990) were also used by Pitcher *et al.*, (1991) who found that those plants were not protected against ozone toxicity. Based on foliar injury measurements the authors concluded that an increase in chloroplastic Cu, Zn SOD alone is not sufficient to reduce ozone toxicity.

It seems that an additional factor is needed in order for the enhanced SOD activity to participate effectively in an antioxidant defense. In a subsequent study to the one discussed above (Sen Gupta *et al.*, 1993a), Sen Gupta *et al.*, (1993b) determined that transgenic plants over-expressing the

chloroplast-localized Cu,Zn SOD also exhibited a similar moderate (3-4 fold) increase in AP specific activity and an increase in levels of AP mRNA. The authors suggested that the increased levels of hydrogen peroxide generated through the activity of additional SOD present within the plastids of the transformed cells were responsible for the increase in transcript and activity abundance. It is also interesting to note that in both their transformed and untransformed plants, GR increased in response to stress. These data are compatible with the existence of an independent mechanism for the modulation of GR in stressed plants. This latter notion would contrast the proposal of the coordination of activity of antioxidant enzymes on which the current study was based.

Apparently, enhanced levels of SOD alone may protect cells against treatment with PQ in the short term. However, damaged cells result from long-term exposure to PQ because they have insufficient ability to detoxify $H_2O_2$. Particularly, in the case of SOD of up to 6-fold enhancement of human Cu/Zn-SOD in transfected mouse L-cells and human HeLa cells both PQ mediated toxicity was extenuated and the lipid peroxidation enhanced (Elroy-Stein *et al.*, 1986). On the other hand, Tanaka *et al.* (1988a) regenerated tobacco plants from a PQ-tolerant callus. They assayed activities of AP, GR, SOD, and guaiacol peroxidase. They found that only SOD was higher (2-3 times) in the PQ-tolerant plants. These plants were also more

resistant to $SO_2$.

The photosynthesizing protoplast system that was partially characterized in this study can be used to examine antioxidant responses. Inclusion of Gamborg's basal salt mixture allowed valid observations of photosynthetic incubations up to 14 hours. After 10 hours in the presence of 0.1 mM PQ, Progress protoplasts had significantly higher SOD activity while AP and GR activities were significantly decreased. Further work at the molecular level is needed to elucidate the response to oxidative stressors of the different components of the antioxidant metabolism.

## SUMMARY AND CONCLUSIONS

1. Pea plants (cv. Progress and Nugget) were grown in synthetic soil under a 16 hour photoperiod, 21°/24° C night/day temperature, 70/80 % relative humidity, and 350 $\mu E\ m^{-2}\ s^{-1}$ light intensity in a growth chamber. Protoplasts, derived from 11-day-old plants, had a photosynthetic light optimum around 420 $\mu E\ m^{-2}\ s^{-1}$. At that light intensity Nugget and Progress protoplasts had a photosynthetic rate of $CO_2$-dependent $O_2$ evolution of $100\pm12$ and $70\pm8$ $\mu$moles $mg^{-1}$ chl $h^{-1}$ respectively. Both cultivars incorporated $^{14}CO_2$ at a rate of $40\pm6$ $\mu$mol $mg^{-1}$ chl $h^{-1}$ for up to 3 hours.

2. The presence of sulfite during the polarographic measurement of photosynthesis ($CO_2$-dependent $O_2$ evolution) greatly confounds results perhaps due to a concomitant $SO_2$-dependent $O_2$ evolution. Alternatively, the effect of sulfite on photosynthesis was more directly assessed with the assaying of photosynthesis that involved $^{14}CO_2$ incorporation into acid stable products. It was, thus, determined that protoplasts from both cultivars had decreased rates of photosynthesis by 10% in 7 mM sulfite and 30% in 15 mM sulfite at all time points.

3. PQ (0.1 $\mu$M) causes a 50 % decrease in photosynthesis

in protoplasts from both cultivars. However, at higher PQ concentrations Progress photosynthesis continues to decline while Nugget photosynthesis remains unaffected. These results are in contrast to the whole plant responses to PQ exposure where Progress was the tolerant cultivar and Nugget the more sensitive. A mechanism involving more than antioxidants may be involved in tolerance to low concentrations of PQ.

4. During a 1 hour incubation in the presence of 0.1 μM PQ, activities of AP and GR were enhanced by 50 and 30 % when compared to controls.

5. Progress protoplasts incubated for 3 hours in the presence of 0.1 μM PQ contained chloroplasts with significantly higher GR activity. This enhancement was abolished at 10 μM PQ. *De novo* synthesis of GR protein did not appear to be involved in the response of Progress protoplasts to 0.1 μM PQ.

6. Changes of AP and GR activities upon incubation of Progress protoplasts for 1 hour in the presence of a range of PQ concentrations (0.2 to 1.5 μM) can not be explained by changes in the steady state level of the respective proteins. Increases in enzyme activities in this case could be due to enzyme activation.

7. Inclusion of Gamborg's B-5 basal salt mixture in the incubation medium resulted in increased stability of isolated protoplasts for up to 10 hours in light.

8. In the presence of Gamborg's salts and after 10 hours in light protoplasts treated with 0.1 mM PQ showed a 126% enhancement in whole cell SOD activity.

Overall conclusions

A protoplast system from pea leaves was developed that allowed the application of oxidative stress and the subsequent evaluation of responses of some of the cytosolic and plastid components of antioxidant metabolism. These responses could be assayed for a period up to 14 hours.

SOD activity in protoplasts was significantly enhanced after 8-10 hours in the presence of PQ (0.1mM) when photosynthesis was inhibited. On the other hand, plastidic GR activity increased during the initial 3 h treatment with 0.1 $\mu$M PQ.

Further work at the molecular level is required in order to characterize the responses of the protoplast system to oxidative stress.

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## Appendix A
## GAMBORG'S B-5
## BASAL SALT MIXTURE (Strength 1 X)

| Components | mg/L |
|---|---|
| Ammonium Sulfate | 134.0 |
| Boric Acid | 3.0 |
| Calcium Chloride Anhydrous | 113.24 |
| Cobalt Chloride Hexahydrate | 0.025 |
| Cupric Sulfate Pentahydrate | 0.025 |
| Disodium EDTA Dihydrate | 37.25 |
| Ferrous Sulfate Heptahydrate | 27.85 |
| Magnesium Sulfate Anhydrous | 122.09 |
| Manganese Sulfate Monohydrate | 10.0 |
| Potassium Iodide | 0.75 |
| Potassium Nitrate | 2500.0 |
| Sodium Molybdate Dihydrate | 0.25 |
| Sodium Phosphate Monobasic Anhydrous | 130.5 |
| Zinc Sulfate Heptahydrate | 2.0 |

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Source: Sigma plant culture 1993 catalogue

## VITA

Andreas G. Doulis was born in Thessaloniki, Greece on August 15, 1960 to Maria A. Douli and Georgios A. Doulis. He received his B.S. in Forestry from the Aristotelian University of Thessaloniki, Greece in 1985. He received his M.S. from the Virginia Polytechnic Institute and State University, Department of Forestry in May 1990.

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