Counteraction of Paraquat Toxicity at the Chloroplast Level*

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Six pyridyl derivatives [benzylviologen, 2-anilinopyridine, 1,2-bis(4-pyridyl)ethane, 1,2-bis(4-pyridyl)ethylene, 2-benzoylpyridine, and 2-benzylaminopyridine] and five heme-iron derivatives [hemoglobin, hemin, hematin, ferritin, and ferrocene] were screened for their potential to counteract paraquat (1,1′-dimethyl-4,4′-bipyridinium ion) toxicity on pea (*Pisum sativum* L.) isolated chloroplasts. The $H_2O \rightarrow$ methylviologen(MV)/O_2 and $H_2O \rightarrow$ ferredoxin(Fd)/$NADP^+$ were two Hill reactions assayed with these compounds. Antagonists of paraquat toxicity should inhibit the first Hill reaction but not the latter. All pyridyl derivatives examined did not inhibit the reaction $H_2O \rightarrow MV/O_2$. Ferritin and ferrocene were also ineffective as inhibitors of this reaction. Hemoglobin inhibited the reaction $H_2O \rightarrow MV/O_2$ without inhibiting the reaction $H_2O \rightarrow Fd/NADP^+$, providing protection to pea chloroplasts against paraquat. Hemin and hematin inhibited both Hill reactions examined. They also inhibited $H_2O \rightarrow$ diaminodurene$(DAD)_{ox}$ and durohydroquinone $\rightarrow MV/O_2$ Hill reactions but not the dichlorophenol indophenol$_{red} \rightarrow MV/O_2$ and $DAD_{red} \rightarrow MV/O_2$ Hill reactions. These results suggest that hemin and hematin are inhibiting the photosynthetic electron transport in the plastoquinone-pool region.

Introduction

Chemical and genetic manipulation of crop tolerance to herbicides has challenged herbicide technologists for many years [1–3]. Recent advances in agricultural biotechnology offer new options and alternative approaches to meet this challenge. Plant tolerance to nonselective herbicides can be a result of one or more of the following mechanisms [1, 4]: a) altered uptake and translocation or compartmentation of the herbicide in tolerant plants; b) extensive metabolic detoxification of the herbicide in tolerant plants; c) modification of the target site of the herbicide in tolerant plants; d) overproduction of a target enzyme in tolerant plants; and e) oversynthesis of substrates able to reverse the herbicide-induced inhibition of growth in tolerant plants. Specific examples for the involvement of these mechanisms in the development of plant tolerance to herbicides have been reported [1, 3]. Many of these mechanisms may be manipulated by genetic or chemical means.

Paraquat is a nonselective herbicide used extensively for total weed control in no-till crop production and as a harvest aid [5]. The active ingredient of paraquat is methylviologen (MV) which is routinely used in studying selected photochemical reactions mediated by plant chloroplasts. Paraquat exerts its phytotoxicity by accepting photosynthetic electrons from PSI and transferring these electrons to oxygen producing toxic oxygen species which cause lipid peroxidation and membrane breakdown [6]. During this process, paraquat is reduced to a cationic radical which is quickly reoxidized by molecular oxygen. Superoxide radical (O_2^-) is the product of this reaction [7]. Disproportionation of superoxide radicals to H_2O_2 is catalyzed enzymatically by superoxide dismutase (SOD) (EC 1.15.1.1), which is located in plant chloroplasts [8]. Paraquat-mediated peroxide production is cytochemically located along the stroma lamellae and on the ends of the grana stack [9]. Hydrogen peroxide readily accepts electrons from reduced transition metals [10–12] or from reduced paraquat [13] to form hydroxal radicals

Abbreviations: DCMU, 3,4-dichlorophenyl-N,N′-dimethylurea; KFeCN, potassium ferricyanide; DCIP, dichlorophenol indolphenol; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene); DHQ, tetramethyl-*p*-hydroquinone (durohydroquinone); MES, [2-(N-morpholino)-ethanesulfonic acid]; HEPES, [N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid]; DBMIB, dibromothymoquinone; PET, photosynthetic electron transport; PS I and PS II, photosystem I and II; Fd, ferredoxin; Chl, chlorophyll; Asc, ascorbate; SOD, superoxide dismutase; MV, methylviologen (1,1′-dimethyl-4,4′-bipyridinium dichloride).

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($OH^{\cdot}$). Lipid peroxidation is most likely initiated by $OH^{\cdot}$ [10, 14, 15]. The superoxide radical is neither a strong oxidant nor reductant [16] and is probably not directly involved in lipid peroxidation [17]. However, superoxide is an efficient reductant of transition metals [11, 14, 18] and can play an indirect role in oxygen toxicity by maintaining transition metals in a reduced state [10, 12, 19]. The plant is normally protected from the harmful effects of O_2^-, H_2O_2 and $OH^{\cdot}$ through the action of SOD, ascorbate peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6), and α-tocopherol, respectively [8, 20]. Treatment of plants with paraquat enhances the production of toxic oxygen species to levels far exceeding those that can be protected by the natural defense systems of plants.

Interest in manipulating crop tolerance to paraquat has been renewed recently, following the discovery of several weed biotypes that are tolerant to this herbicide. Biotypes of horseweed (*Conyza linefolia* L. Cronq.) survive treatments with paraquat by excluding the herbicide from its site of action. Autoradiography and cytochemical, and biochemical studies showed that translocation of paraquat in tolerant *Conyza* biotypes was limited to the major leaf veins and insufficient amounts of the herbicide reached the mesophyll cells containing the target site (chloroplast) of this herbicide [21, 22]. The tolerance of perennial ryegrass (*Lolium perenne* L.) biotypes to paraquat, however, is not due to differential uptake, translocation, or metabolism of paraquat [23]. It is the result of increased activity of the endogenous protectants of toxic oxygen species such as the enzymes catalase, SOD, and peroxidase [24]. Tobacco (*Nicotiana tabacum* L.) cell lines, tolerant to the herbicide paraquat, have been selected through tissue culture procedures [25]. Increased levels of peroxidase and catalase activities in these cell lines were identified as the causes for the tolerance of these lines to paraquat [26, 27].

Attempts to chemically regulate plant tolerance to paraquat have also been reported. D-penicilamine, a copper chelate with superoxide dismutating activity, offered partial protection to flax (*Linum ussitatisimum* L.) cotyledons against paraquat [28]. Preconditioning of plants to sublethal doses of stresses which increase oxygen toxicity, such as ferrous sulfate [29] and sulfite [30] tends to raise the levels of protective mechanisms toward toxic oxygen species and protects plants from paraquat injury. Ferrous sulfate applied as a spray with paraquat could protect wheat and oats from paraquat injury but the antidote to herbicide ratio (100:1) was very uneconomical [30].

An alternative approach for the chemical manipulation of plant tolerance to paraquat is the counteraction of its activity at the chloroplast level. Until recently, this approach was not apparently feasible since it is traditionally accepted that paraquat is reduced by the primary electron acceptor of PSI which is also responsible for the reduction of $NADP^+$ [6]. Current work on the primary electron acceptor of PSI, however, shows that a series of electron acceptors are involved before the reduction of ferredoxin (Fd) [31]. The primary electron acceptor from P700 may be a monomeric chlorophyll anion designated Ao^-. Electron transfer probably proceeds to a semiquinone (A_1^-) followed by three iron-sulfur (Fe-S) centers denoted as Fx, Fa, and Fb. It is still unresolved whether these Fe-S centers act in series or in parallel. The experimental evidence tends to favor the concept that electron transfer to the Fe-S centers, particularly Fa and Fb, is in parallel under physiological conditions [31]. It is not known which one of these electron acceptors (Fx, Fa, and Fb) interacts with Fd and paraquat in the reduction of O_2. An unidentified inhibitor from hemolyzed rabbit sera is capable of inhibiting paraquat- or Fd-mediated O_2 uptake without inhibiting $NADP^+$-dependent O_2 evolution [32]. The physiological implication of this finding is that electron flow after PSI is branched (parallel). One branch is involved in $NADP^+$ reduction whereas another branch is involved in the paraquat- or Fd-mediated O_2 reduction. The branch for reduction of O_2 offers a potential target site for regulating paraquat toxicity since electron flow to paraquat could be inhibited without interfering with $NADP^+$-reduction.

In this study, we report the results of our attempts to counteract paraquat toxicity at the chloroplast level with several chemical compounds that could potentially inhibit electron flow to paraquat but not to $NADP^+$. Two major groups of compounds, nonherbicidal pyridyl analogues of paraquat and heme/iron derivatives were screened.

Materials and Methods

Peas (*Pisum sativum* cv. Little Marvel) were grown in a soilless mixture of 1:2:2 of peat:vermiculite:weblite®, plus a slow release fertilizer (Osmocote®). Plants were maintained in a growth chamber

with a 12 h light/dark cycle at temperatures of 20 °C day/17 °C night. The light intensity was varied during the day cycle, starting with 40 $\mu E \cdot m^{-2} \cdot sec^{-1}$ and gradually increasing for 3 h to a maximum intensity of 800 $\mu E \cdot m^{-2} \cdot sec^{-1}$, which was held constant for 1 h, after which the process was reversed. After 4 weeks of growth in this environment, pea plants were used for chloroplast isolation.

Chloroplasts were isolated as follows: 6–7 leaves were gathered from 5–7 plants and macerated for 5 sec in a partially frozen extraction medium (75 ml) containing 330 mM sorbitol, 5 mM $MgCl_2$, 20 mM MES-NAOH/pH 6.5. The homogenate was filtered through a single layer of Miracloth and centrifuged for 1 min at $2,000 \times g$ at 0 °C. The pellet was resuspended in a 1:20 dilution of the extraction medium (10 ml) then centrifuged for 1 min at $4,000 \times g$ at 0 °C. The pellet was resuspended in an assay medium containing 330 mM sorbitol, 50 mM HEPES-NAOH/pH 7.6, 2 mM $MgCl_2$, 1 mM NH_4Cl, and 2 mM EDTA.

Hill reaction rates were determined by monitoring changes in O_2 concentration as a function of time using a Gilson-Oxygraph Clark-type oxygen electrode. Assay volumes, light intensities at the surface of the reaction vessel, and the assay temperature were 1.5 ml, 2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$, and 20 °C, respectively. All chemicals were from Sigma Chemical Company, except for sorbitol and MES which were from Calbiochem. Dibromothymoquinone (DBMIB) was a gift from Dr. Draber, Bayer AG, Wuppertal, West Germany. Chlorophyll concentrations (3 replicates) were determined according to the methods of Arnon [33] and usually ranged from 35–50 μg Chl per assay.

Results and Discussion

Several compounds were screened for their ability to counteract paraquat toxicity. Each compound was added to a $H_2O \rightarrow MV/O_2$ Mehler reaction. The compounds that inhibited this reaction were then added to a NADP Hill reaction. Successful compounds should not inhibit the latter reaction. It has been established that the only pyridyl compounds exhibiting herbicidal action are those which contain coplanar rings [34]. Examples of such compounds are the 4,4′- and 2,2′-bipyridine series, such as paraquat (methylviologen), diquat and triquat. Other pyridyl compounds do not exhibit herbicidal activity. It is possible that pyridyl compounds which do not exhibit herbicidal activity could act as paraquat antagonists inhibiting the reduction of oxygen by binding to the active site of paraquat reduction. The pyridyl compounds tested were: benzylviologen; 2-anilinopyridine; 1,2-bis(4-pyridyl)ethane; 1,2-bis(4-pyridyl)-ethylene; 2-benzoylpyridine and 2-benzylaminopyridine (Table I). Unfortunately, none of these compounds inhibited paraquat mediated O_2 uptake and most likely they do not act as paraquat antagonists. These results are in agreement with Lewinsohn and Gressel [35]. They found that benzylviologen protected *Spirodela oligorrhiza* (Kurz) Hegelm. colonies from damaging levels of diquat. However, the protection was not found to be a direct interaction with thylakoids but was explained in part as an increase in SOD levels. Other paraquat analogues were also tested but these compounds did not offer any protection to *S. oligorrhiza* colonies [35].

In recent studies, Upham *et al.* [32] showed that an unidentified compound from rabbit sera inhibits O_2 reduction but not $NADP^+$ reduction in thylakoid preparations. This inhibitor is probably a product of red blood cell hemolysis [32]. Therefore, compounds that could be a product or resemble products of hemolysis were screened as potential paraquat antagonists. One such compound was hemoglobin which inhibited paraquat mediated O_2 uptake by 66% and had no effect on $NADP^+$-dependent O_2 evolution (Table I). However, to obtain reproducible results, hemoglobin had to be added three times with approximately 4 sec or greater time between each addition. The final concentrations after each addition were 10, 20, and 30 μM. No inhibition was observed when hemoglobin was added once with a final concentration of 30 μM. No explanation could be offered for these results which appear to be concentration independent. Further, it should be noted that hemoglobin cannot be used on a live plant since it cannot traverse cell walls and membranes.

Another iron-containing protein used was ferritin. This compound did not inhibit O_2 uptake (Table I). Other compounds chosen were those that may resemble breakdown products of hemoglobin. Benzylimidazole was chosen for its pyrrole nitrogen, which exists in the tetrapyrole ring of heme-structures. This compound also did not inhibit O_2 uptake (Table I). Hemin and hematin, two heme-structures containing reduced and oxidized iron, respectively, did inhibit O_2 uptake (Table I) but also inhibited NADP reduc-

Table I. Efficacy of various compounds in counteracting paraquat toxicity in isolated pea thylakoids[a].

Experimental compounds	% Inhibition by experimental compounds	
	$H_2O \rightarrow PQ/O_2$	$H_2O \rightarrow Fd/NADP$
Pyridyl compounds		
benzylviologen	0 ± 0	ND
2-anilinopyridine	0 ± 0	ND
1,2-bis(4-pyridyl)ethane	0 ± 0	ND
1,2-bis(4-pyridyl)ethylene	0 ± 0	ND
2-bezoylpyridine	0 ± 0	ND
2-benzylaminopyridine	0 ± 0	ND
1-benzylimidazole	0 ± 0	ND
Heme/iron compounds		
rabbit sera[b]	100 ± 0	0 ± 0
hemoglobin	66 ± 3	0 ± 0
ferritin	0 ± 0	ND
hemin	100 ± 0	100 ± 0
hematin	100 ± 0	100 ± 0
ferrocene	0 ± 0	ND

[a] Assay medium was as described in Materials and Methods. The concentrations of PQ, Fd, NADP, hemoglobin, ferritin and the remaining experimental compounds were 10 μM, 100 μg/ml, 10 μM, 10 μM, and 1 mM, respectively. Each value ± standard deviation represents an average of 3 replicates. ND = not determined. All rates were greater than 2.00 μmol $O_2 \cdot min^{-1} \cdot mg\ Chl^{-1}$.

[b] Data for rabbit serum is from experiments with spinach thylakoids reported by Upham *et al.* [32].

tion. These data show hemin and hematin are unsuitable for paraquat safening of isolated plant chloroplasts.

Ferrocene was also tested. This compound contains an iron molecule coordinated with two aromatic benzene rings in a non-planar configuration. This is in contrast to heme-structures in which iron is coordinated to aromatic rings in a planar configuration. Ferrocene did not inhibit paraquat-mediated oxygen uptake (Table I).

The inhibitory effects of hemin and hematin on PET were further characterized to determine their approximate site of inhibition on PET. The electron donors: DCIP/ascorbate and DAD/ascorbate, donate electrons to P700 [36, 37] and the cytochrome *b*-Rieske(Fe-S)-cytochrome *f*/plastocyanin region [38], respectively. Hemin and hematin did not inhibit the above Hill reactions (Table II). This indicates that inhibition by hemin and hematin is not on the reducing side of PSI. The inhibitor DCMU was used to block electron flow from PS II. Durohydroquinone is known to donate electrons to the plastoquinone region [39]. The electron acceptor DAD_{ox} is probably reduced in the same region [40]. Hemin and hematin inhibited these two Hill reactions (Table II). Thus, it appears hemin and hematin are inhibiting PET in the region of the plastoquinone pool. The inhibitor DCMU was used to block electron flow from PS II for the DHQ reaction. Ferricyanide was added to DAD to keep it oxidized and DBMIB was used to inhibit electron flow to FeCN.

Table II. Localization of the PET site of inhibition by hemin and hematin.

Hill reaction	% Inhibition by	
	Hemin	Hematin
$H_2O \rightarrow PQ/O_2$	100	100
DCIP/Asc/DCMU/SOD → PQ/O_2	0	0
DAD/Asc/DCMU/SOD → PQ/O_2	0	0
DHQ/SOD → PQ/O_2	100	100
$H_2O \rightarrow$ DAD/FeCN/DBMIB	100	100

Assay medium was as described in Materials and Methods. The concentrations of hemin, hematin, PQ, DCIP, DAD, Asc, DCMU, DHQ, FeCN, DBMIB, and SOD were 100 μM, 100 μM, 10 μM, 1 mM, 1 mM, 1 mM, 5 μM, 1 mM, 1 mM, 10 μM, and 150 units, respectively. Each value represents an average of 3 replicates with standard deviation of 0. All rates were greater than 2.00 μmol $O_2 \cdot min^{-1} \cdot mg\ Chl^{-1}$.

The results of our research on the chemical manipulation of plant tolerance to paraquat are not very optimistic. However, the use of rabbit sera [32] demonstrates the potential for counteracting paraquat toxicity at the chloroplast level. The pyridyl and heme/iron compounds tested in this study were not suitable for counteracting paraquat toxicity. Hemin and hematin were effective inhibitors of PET at or near the plastoquinone pool. These heme compounds could be useful in studying the photosynthetic electron transport. Further screening of additional compounds may be successful. Also, continued research on the characterization of the O_2 reduction pathway may provide useful information for the development of a paraquat safener.

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