On the Mechanism of Resistance to Paraquat in
Hordeum glaucum and H. leporinum

Delayed Inhibition of Photosynthetic O₂ Evolution after Paraquat Application

Christopher Preston, Joseph A. M. Holtum, and Stephen B. Powles

Department of Crop Protection, Waite Agricultural Research Institute, The University of Adelaide,
PMB 1, Glen Osmond S.A. 5064, Australia

ABSTRACT

The mechanism of resistance to paraquat was investigated in biotypes of Hordeum glaucum Steud. and H. leporinum Link. with high levels of resistance. Inhibition of photosynthetic O₂ evolution after herbicide application was used to monitor the presence of paraquat at the active site. Inhibition of photosynthetic O₂ evolution after paraquat application was delayed in both resistant biotypes compared with the susceptible biotypes; however, this differential was more pronounced in the case of H. glaucum than in H. leporinum. Similar results could be obtained with the related herbicide diquat. Examination of the concentration dependence of paraquat-induced inhibition of O₂ evolution showed that the resistant H. glaucum biotype was less affected by herbicide compared with the susceptible biotype 3 h after treatment at most rates. The resistant H. leporinum biotype, in contrast, was as inhibited as the susceptible biotype except at the higher rates. In all cases photosynthetic O₂ evolution was dramatically inhibited 24 h after treatment. Measurement of the amount of paraquat transported to the young tissue of these plants 24 h after treatment showed 57% and 53% reductions in the amount of herbicide transported in the case of the resistant H. glaucum and H. leporinum biotypes, respectively, compared with the susceptible biotypes. This was associated with 62% and 66% decreases in photosynthetic O₂ evolution of young leaves in the susceptible H. glaucum and H. leporinum biotypes, respectively, a 39% decrease in activity for the resistant H. leporinum biotype, but no change in the resistant H. glaucum biotype. Photosynthetic O₂ evolution of leaf slices from resistant H. glaucum was not as inhibited by paraquat compared with the susceptible biotype; however, those of resistant and susceptible biotypes of H. leporinum were equally inhibited by paraquat. Paraquat resistance in these two biotypes appears to be a consequence of reduced movement of the herbicide in the resistant plants; however, the mechanism involved is not the same in H. glaucum as in H. leporinum.

The bipyridylium herbicides paraquat and diquat are non-selective contact herbicides that act by intercepting electrons from the photosynthetic electron transport chain at PSI. This reaction results in the production of bipyridyl radicals that readily react with O₂ to produce superoxide and then, through a series of reactions, produce H₂O₂ and the hydroxyl radical. These toxic oxygen species cause extensive lipid peroxidation leading to loss of cell membrane integrity and rapid desiccation (2, 12).

Resistance to these herbicides has appeared in at least 10 weedy species after repeated exposure to the herbicides (see 8 for review). The mechanism of paraquat resistance has been investigated in six of these species (6, 7, 9, 13, 16, 19); however, the mechanism of resistance has not been determined unambiguously for any biotype. In two resistant biotypes, Conyza bonariensis (16) and Lolium perenne (9), it has been proposed that the mechanism of resistance is a result of increased levels of enzymes that detoxify active O₂ species. In other cases where the mechanism of paraquat resistance has been investigated, evidence has been presented purporting to show reduced movement of the herbicide in the leaf of the resistant plants (3, 7, 19). These studies mainly have been performed by feeding [¹⁴C]paraquat through the petiole or leaf bases of detached leaves and observing its spread by autoradiography. In a paraquat-resistant biotype of Hordeum glaucum Steud., it has been shown that there is no difference in the susceptibility of protoplasts isolated from resistant and susceptible individuals to inhibition by paraquat, suggesting that, in this biotype, resistance might be conferred by the exclusion of herbicide from the cell (15).

Here we have reinvestigated the mechanism of paraquat resistance in a biotype of H. glaucum (3, 15) previously examined and compared it with a paraquat-resistant biotype of H. leporinum Link. We have used photosynthetic O₂ evolution as an indicator of the appearance of paraquat at the active site and have confirmed reduced movement of paraquat within the leaves of the resistant biotypes of H. glaucum and H. leporinum. In both cases there is reduced translocation of paraquat to the young tissue in the resistant biotypes that remains photosynthetically active and continues to grow, whereas that of the susceptible biotypes is killed.

MATERIALS AND METHODS

Plant Material and Herbicide Application

Seeds of paraquat-resistant Hordeum glaucum Steud. and H. leporinum Link. were obtained from plants originally collected from two separate alfalfa fields in Victoria, each with

1 Partially supported by an Australian Research Council Postdoctoral Fellowship (C.P.).
a long history of paraquat use. The susceptible biotypes were collected from pastures that had no history of herbicide use. The response to paraquat of the paraquat-resistant and paraquat-susceptible biotypes has been described elsewhere (14, 20). Plants were grown in soil in 18-cm pots and kept in a growth room with a 14 h/10 h, 20°C/15°C light/dark cycle with a light intensity of 300 μE m⁻² s⁻¹. The plants were used between 4 and 6 weeks after sowing, at which stage they were actively tillering.

Plants were sprayed with herbicide using a custom-built laboratory spray cabinet (20). This was a moving-belt (1 m s⁻¹), twin-nozzle unit that applied a total volume of 118 L ha⁻¹ at 250 kPa pressure. The herbicide products applied were Gramoxone (a.i.,² 200 g L⁻¹ paraquat) and Reglone (a.i., 200 g L⁻¹ diquat). To all spray treatments 0.2% nonionic surfactant (Agral 600) was added. Plants were sprayed in the morning and placed in the dark immediately after treatment.

O₂ Evolution Measurements

O₂ evolution of leaf segments was measured with a Hansatech leaf disc O₂ electrode at 25°C. Leaf segments (2.5 cm long) were cut from young, expanded leaves under water and placed in an irrigated brass clip (to counter desiccation) in the chamber. The leaves were illuminated at 700 μE m⁻² s⁻¹ in an atmosphere of 21% (v/v) O₂, 5% (v/v) CO₂, 74% (v/v) N₂ for 10 min before measurements. The leaf segments used for measurements were taken from the central third of the leaf. The leaf area of the segments was determined after the O₂ evolution measurements by placing the leaf segments on photographic paper and exposing them to light. The leaf area was then calculated from the weight of a known area of photographic paper. For paraquat concentration dependence experiments, one plant in a 10-cm pot with at least three tillers was used for each treatment.

Paraquat Determinations

Paraquat in the leaf tissue was determined spectrophotometrically by the method described by Calderbank and Yuen (5). Plants that had been grown in the growth room were sprayed with 200 g of paraquat per hectare then placed in the dark. Twenty-four hours after spraying, the plants were harvested, and the parts that had not been directly exposed to herbicide, young leaves not yet emerged, and the bases of older leaves were selected. These were pooled for each pot (five plants) and were boiled in 1 N H₂SO₄ for 4 h. After cooling, the solution was filtered and the paraquat separated using a Duolite 225 column (BDH Pharmaceuticals, London). The recovery of paraquat in the extraction process was estimated from control experiments, where known amounts of paraquat were added immediately before boiling, at greater than 85% for all samples, and there were no differences between resistant and susceptible biotypes.

² Abbreviation: a.i., active ingredient; I₅₀, concentration required for 50% inhibition.

Photosynthesis by Leaf Slices

Leaves were sliced into approximately 0.5- to 1.0-mm-wide strips under buffer consisting of 50 mM Hepes-KOH, pH 7.6, 500 mM sorbitol, and 2 mM CaCl₂. The leaf slices were then transferred to a Clark-type O₂ electrode in 3 mL of the same buffer with 10 mM NaHCO₃ added. The leaf slices were incubated in the presence of paraquat (supplied as the dichloride salt) for 10 min in the dark and then illuminated for 5 min at 1000 μE m⁻² s⁻¹ when the rate of O₂ evolution was determined. Chl was extracted from the leaf slices by grinding in 80% acetone and the Chl concentration was determined as described by Arnon (1).

RESULTS

Paraquat Inhibition of O₂ Evolution

The presence of paraquat in the chloroplast in the light has three important consequences. First, paraquat will accept electrons from PSI and inhibit CO₂ fixation. Second, the paraquat radical thus formed will react with O₂ to produce superoxide and will, therefore, reduce net O₂ evolution. Third, the toxic oxygen species produced as a result of paraquat action, in particular the hydroxyl radical, will rapidly destroy chloroplast membranes, rendering the chloroplast inactive. We have used measurements of net O₂ evolution to estimate the time taken for paraquat to appear in the chloroplasts of leaves. Paraquat-susceptible and paraquat-resistant plants of H. glaucum and H. leporinum were sprayed in the laboratory sprayer and placed in the dark; photosynthetic O₂ evolution was then measured up to 4 h after spraying. Paraquat, when applied at 100 g a.i. ha⁻¹, rapidly enters the chloroplast of the susceptible biotypes, and appreciable inhibition of photosynthetic O₂ evolution of leaf segments can be observed 40 min after spraying the plants (Figs. 1C and 2C). Reducing the herbicide concentration delayed the onset of inhibition of O₂ evolution such that at 20 g of a.i. ha⁻¹, inhibition of O₂ evolution was not observed until 2 h after spraying of the susceptible biotypes (Figs. 1A and 2A). At each paraquat concentration, the inhibition profiles of the two paraquat-susceptible biotypes were similar.

The paraquat-resistant H. glaucum biotype showed a delay in the onset of inhibition of O₂ evolution after paraquat application, compared with the susceptible biotype at all rates of herbicide application (Fig. 1). The biggest difference between the two biotypes was observed at 50 g of a.i. ha⁻¹, where inhibition of O₂ evolution of the resistant biotype was delayed 1.5 h compared with the susceptible biotype (Fig. 1B). In contrast to the situation observed for paraquat-resistant H. glaucum, the differences in onset of inhibition of O₂ evolution between paraquat-susceptible and paraquat-resistant H. leporinum were small (Fig. 2). The largest difference in time of onset of inhibition of O₂ evolution was observed at 50 g of a.i. ha⁻¹, where inhibition of O₂ evolution of the resistant H. leporinum biotype was delayed by 0.5 h compared with the susceptible biotype (Fig. 2B).

The inhibition of O₂ evolution in response to paraquat dose was examined in a series of experiments in which plants were sprayed with herbicide and then placed in the dark. Photosynthetic activity of leaf segments from each plant was meas-
concentrations, where the resistant biotype had a net positive O₂ evolution rate, whereas the susceptible biotype had a net negative rate.

The same experiment performed on paraquat-susceptible and paraquat-resistant *H. leporinum* biotypes produced a slightly different result. Photosynthetic O₂ evolution was equally inhibited in the two biotypes 3 h after paraquat treatment except at the highest two rates (100 and 200 g of a.i. ha⁻¹) (Fig. 4A). By 24 h after treatment, photosynthetic O₂ evolution had been greatly inhibited for both biotypes at all herbicide concentrations (Fig. 4B). No recovery of photosynthetic activity occurred in the 24 h after paraquat treatment for any of the biotypes examined here.

**Diquat Inhibition of O₂ Evolution**

The paraquat-resistant biotypes of *H. glaucum* and *H. leporinum* show considerably greater tolerance to the related bipyridyl herbicide diquat than do the susceptible biotypes (14, 20). Diquat inhibition of photosynthetic O₂ evolution was examined in the same way as for paraquat and, like

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**Figure 1.** Time course of inhibition of photosynthetic O₂ evolution in paraquat-susceptible (○) and paraquat-resistant (●) biotypes of *H. glaucum* after application of paraquat. Paraquat was applied at 20 (A), 50 (B), or 100 (C) g of a.i. ha⁻¹. Each point is the mean (±SE) of four experiments.

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**Figure 2.** Time course of inhibition of photosynthetic O₂ evolution in paraquat-susceptible (○) and paraquat-resistant (●) biotypes of *H. leporinum* after application of paraquat. Paraquat was applied at 20 (A), 50 (B), or 100 (C) g of a.i. ha⁻¹. Each point is the mean (±SE) of four experiments.
MECHANISM OF PARAQUAT RESISTANCE IN *Hordeum* SP.

Figure 3. Concentration dependence of paraquat inhibition of O$_2$ evolution in paraquat-susceptible (○) and paraquat-resistant (●) biotypes of *H. glaucum* 3 h (A) and 24 h (B) after herbicide application. Each measurement is the mean (±se) of four experiments. Values below 0 denote net uptake of O$_2$.

Figure 4. Concentration dependence of paraquat inhibition of O$_2$ evolution in paraquat-susceptible (○) and paraquat-resistant (●) biotypes of *H. leporinum* 3 h (A) and 24 h (B) after herbicide application. Each measurement is the mean (±se) of four experiments. Values below 0 denote net uptake of O$_2$.

Figure 5. Time course of inhibition of photosynthetic O$_2$ evolution in paraquat-susceptible (○) and paraquat-resistant (●) biotypes of *H. glaucum* (A) and *H. leporinum* (B) after application of diquat. Diquat was applied at 200 g of a.i. ha$^-1$. Each point is the mean (±se) of four experiments.

Paraquat, diquat rapidly inhibited O$_2$ evolution in both susceptible biotypes (Fig. 5). The concentration of diquat required to elicit the same response was greater than for paraquat, consistent with the reduced efficacy of diquat on grass species (4). For both paraquat-resistant biotypes there was a delay in the inhibition of photosynthetic O$_2$ evolution of the resistant compared with the susceptible biotypes; however, this delay was greater for *H. glaucum* than for *H. leporinum* (Fig. 5). The responses of the two paraquat-resistant biotypes to paraquat and diquat are similar, which suggests that the mechanisms of resistance to the two herbicides are likely to be the same within each biotype.

Paraquat Transport

The O$_2$ evolution data presented above suggest that there is reduced movement of paraquat in the leaves of the paraquat-resistant individuals. Field observations are that the paraquat-resistant individuals of *H. glaucum* and *H. leporinum* suffer injury from the herbicide, but then grow back from undamaged tissue, whereas the susceptible biotype does not regrow. We tested the hypothesis that paraquat resistance is a consequence of reduced translocation of herbicide to the growing points by directly measuring the amount of paraquat in the young tissue 24 h after the application of 200 g of a.i. ha$^-1$ paraquat to intact potted plants. Using a spectrophotometric method for detecting paraquat residues in plant tissue (5), a technique that does not detect the photochemical degradation products of paraquat (18), we observed that the
amount of paraquat translocated to the young tissue was reduced in the paraquat-resistant biotypes of H. glaucum and H. leporinum by 57% and 53%, respectively (Table I). More paraquat was detected in the young tissue of H. glaucum than in H. leporinum, but the reason for this is not known.

In a separate experiment, resistant and susceptible plants were treated with paraquat and placed in the dark for 24 h. The young, unsprayed leaves were dissected out of the leaf sheath, and photosynthetic O₂ evolution was measured using the leaf disc O₂ electrode. The results presented in Table I show that photosynthetic O₂ evolution of these leaves from the resistant H. glaucum biotype was largely unaffected by the application of paraquat, and that of the resistant H. leporinum biotype was inhibited by 39%. Young leaves from the susceptible biotypes showed 62% and 66% decreases in photosynthetic O₂ evolution for H. glaucum and H. leporinum, respectively.

**Leaf Slice Photosynthesis**

No difference in susceptibility of photosynthetic O₂ evolution to inhibition by paraquat between the resistant and susceptible biotypes of H. glaucum was observed at the isolated protoplast level (15), yet there is a difference when leaf segments are used (Fig. 1). Powles and Cornic (15) suggested that the cell walls of H. glaucum may be involved in resistance to paraquat. After unsuccessful attempts to isolate intact cells from these species, this hypothesis was tested using thin (0.5–1 mm) slices of leaf tissue exposed to different concentrations of paraquat. Paraquat was readily able to inhibit O₂ evolution activity of the susceptible H. glaucum biotype with an I₅₀ of about 100 μμ (Fig. 6A). Leaf slices from the resistant H. glaucum biotype proved to be less susceptible to inhibition by paraquat compared with the susceptible biotype and had an I₅₀ of >500 μμ. Photosynthetic O₂ evolution of leaf slices from the susceptible H. leporinum biotype was as sensitive to paraquat as that from the susceptible H. glaucum biotype (Fig. 6B). In contrast to the situation in H. glaucum, the photosynthetic O₂ evolution of leaf slices from the resistant H. leporinum biotype was equally sensitive to paraquat compared with the susceptible biotype. These data suggest that there is a barrier to movement of the herbicide from outside the cell to the chloroplast in the paraquat-resistant biotype of H. glaucum, a barrier that is not present in any of the other biotypes.

**DISCUSSION**

Previous investigations on the mechanism of resistance to paraquat in a biotype of H. glaucum have shown that resistance is not due any change at the active site or to changes in permeability of the chloroplastic or plasma membranes to the herbicide (15). In addition, levels of the enzymes that detoxify active O₂ species were not increased in the resistant biotype compared with the susceptible biotype (15). Herbicide penetration of the cuticle was also not different between the resistant and susceptible biotypes (3). It was suggested that paraquat resistance in this biotype was due to exclusion of the herbicide from the cytoplasm through sequestration in the apoplast (3, 15).

Paraquat is a photosynthetic herbicide that subverts electron transport. This property of paraquat was used to track movement of the herbicide in the leaves of the resistant and susceptible biotypes of H. glaucum and H. leporinum. The results of these experiments demonstrate that paraquat takes longer to reach an inhibitory concentration in the chloroplasts of the resistant biotypes compared with the susceptible biotypes. The time differential for paraquat to inhibit photosynthetic O₂ evolution between the resistant and susceptible

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Table I. Paraquat Content and Photosynthetic O₂ Evolution of the Young Tissue of Paraquat-Resistant and Paraquat-Susceptible Biotypes of H. glaucum and H. leporinum 24 h after Treatment with 200 g of a.i. ha⁻¹ Paraquat

<table>
<thead>
<tr>
<th>Species</th>
<th>Biotype</th>
<th>Paraquat Content</th>
<th>Photosynthetic O₂ Evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg (g fresh weight)⁻¹</td>
<td>μmol (mg Chl)⁻¹ h⁻¹</td>
</tr>
<tr>
<td>H. glaucum</td>
<td>Susceptible</td>
<td>3.45 ± 0.20</td>
<td>125 ± 22 (38)</td>
</tr>
<tr>
<td></td>
<td>Resistant</td>
<td>1.54 ± 0.19</td>
<td>329 ± 14 (93)</td>
</tr>
<tr>
<td>H. leporinum</td>
<td>Susceptible</td>
<td>1.78 ± 0.05</td>
<td>132 ± 28 (34)</td>
</tr>
<tr>
<td></td>
<td>Resistant</td>
<td>0.84 ± 0.08</td>
<td>214 ± 20 (61)</td>
</tr>
</tbody>
</table>

* Values in parentheses are percentage of those obtained from control plants treated with wetting agent only.

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Figure 6. Paraquat inhibition of O₂ evolution of leaf slices from paraquat-susceptible (○) and paraquat-resistant (●) biotypes of H. glaucum (A) and H. leporinum (B). Each point is the mean (±SE) of four experiments. One hundred percent activity was 75 ± 4, 69 ± 2, 45 ± 6, and 47 ± 3 μmol of O₂ evolved (mg Chl)⁻¹ h⁻¹ for the paraquat-susceptible and paraquat-resistant H. glaucum biotypes, and paraquat-susceptible and paraquat-resistant H. leporinum biotypes, respectively.
biotypes was greater in the case of *H. glaucum* than *H. leporinum*. Photosynthetic O\(_2\) evolution, once inhibited, stayed inhibited for at least 24 h after treatment. The similarity of the results obtained with paraquat and diquat suggests that a single mechanism might provide resistance to both herbicides in each species. The response of photosynthetic O\(_2\) evolution of the resistant biotypes of *H. glaucum* and *H. leporinum* to paraquat application (Figs. 1–4) is in contrast to that reported for other paraquat-resistant weed biotypes. Paraquat-resistant biotypes of *C. canadiensis* (13) and *Lolium perenne* (10) showed little or no inhibition of photosynthetic activity, measured as CO\(_2\) fixation, up to 4 h after treatment with paraquat, whereas photosynthetic activity of the paraquat-susceptible biotypes was rapidly and permanently inhibited. In contrast, photosynthetic activity, measured as \(^{14}\)CO\(_2\) fixation, was reported to be transiently inhibited and then to recover in a paraquat-resistant biotype of *C. bonariensis* (11, 17). Increased levels of the enzymes that detoxify active O\(_2\) species have been implicated in paraquat resistance in *C. bonariensis* (16) and *L. perenne* (9), but not in *C. canadiensis* (13) or in other studies on *C. bonariensis* (21). On the basis of differences in response of photosynthetic activity to herbicide application, we suggest that the mechanism of resistance to paraquat in *H. glaucum* and *H. leporinum* must differ from that in *C. bonariensis*, *C. canadiensis*, and *L. perenne*.

The delay in inhibition of photosynthetic O\(_2\) evolution of the resistant biotypes of *H. glaucum* and *H. leporinum* after paraquat or diquat application suggests that reduced herbicide movement may be a mechanism of resistance. Any reduced movement observed within leaf segments should, on a larger scale, result in reduced movement of paraquat to the mesistematic tissue. This hypothesis was confirmed by measuring the amount of herbicide in the young tissue 24 h after treatment. Paraquat contents of this tissue were found to be reduced by 53% to 57% in the paraquat-resistant biotypes compared with the paraquat-susceptible biotypes (Table I). Reduced herbicide movement already has been reported in paraquat-resistant biotypes of *C. bonariensis* (7), *Erigeron philadelphicus* (19), and *H. glaucum* (3) where \(^{14}\)Cparaquat has been fed through the petiole or leaf bases of detached leaves. Here, in contrast, we have demonstrated a significantly reduced movement of paraquat in a basipetal direction in paraquat-resistant plants treated and grown under conditions and herbicide dosages relevant to the field situation. This reduced translocation of paraquat is correlated with a reduced inhibition of photosynthetic activity of the young leaves in the resistant biotypes. Photosynthetic activity of the young leaves 24 h after spraying of both susceptible biotypes was inhibited by more than 60%, whereas that of the resistant *H. glaucum* biotype was unaffected by paraquat application and that of the resistant *H. leporinum* biotype was inhibited by 39%.

The paraquat that was observed in the young leaves of the resistant *H. glaucum* biotype had not reached the active site because no inhibition of O\(_2\) evolution was observed. In contrast, a proportion of the translocated herbicide in the susceptible biotypes and the resistant *H. leporinum* biotype had reached the active site, as indicated by the reduced levels of O\(_2\) evolution. The differences in photosynthetic O\(_2\) evo-

**LITERATURE CITED**


