Evolution of resistance to HPPD-inhibiting herbicides in a wild radish population via enhanced herbicide metabolism

Huan Lu, Qin Yu, Heping Han, Mechelle J Owen and Stephen B Powles

Abstract

BACKGROUND: Relatively new herbicides that target 4-hydroxyphenylpyruvate dioxygenase (HPPD) are now available for use on the world's great grain crops (rice, wheat, corn and soybean) and for other uses. With widespread and persistent use of HPPD-inhibiting herbicides, the evolution of HPPD-inhibiting herbicide resistant weeds is inevitable. Currently, resistance to HPPD-inhibiting herbicides is known in two weed species, waterhemp and Palmer amaranth. Here, we report a HPPD-inhibiting herbicide resistant wild radish population from the Western Australia grain belt. This population was not selected with HPPD-inhibiting herbicides, rather it evolved resistance to earlier used herbicides with different modes of action and exhibits cross-resistance to HPPD-inhibiting herbicides.

RESULTS: Dose–response experiments showed the resistant (R) population exhibits 4 to 6.5-fold resistance to the HPPD-inhibiting herbicides mesotrione, tembotrione and isoxaflutole, compared to the susceptible (S) population. This resistance is not target-site based as cloning of full coding sequences of the HPPD genes from S and R plants did not reveal resistance-endowing single nucleotide polymorphisms. The HPPD gene expression levels are similar in S and R plants. In addition, no differences in [14C]-mesotrione uptake and translocation were observed in the S and R plants. However, the time required for R plants to metabolise 50% [14C]-mesotrione is 7.7-fold faster than for the S plants.

CONCLUSION: We confirm resistance to HPPD-inhibiting herbicides exists in a population of the economically damaging global weed wild radish. The resistance in this population is due to a non-target-site based enhanced rate of herbicide metabolism.

Keywords: wild radish; HPPD-inhibiting herbicides; herbicide resistance; HPPD gene; enhanced metabolism

1 INTRODUCTION

Synthetic herbicides have made a great contribution to global crop yield and quality since being introduced in the 1950s. However, overreliance on herbicides has led to herbicide resistance evolution in many weed species. New commercial herbicide sites of action are rare, with the 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides, including three chemical groups of isoxazoles, triketones and pyrazolones, being relatively new herbicides. HPPD-inhibiting herbicides have become very widely and globally used due to their broad weed control spectrum, low application rates, crop selectivity and low toxicity. HPPD is a key enzyme in the biosynthesis of tocopherol and plastoquinone (PQ). Tocopherol is an antioxidant protecting membranes and PQ is not only an electron transporter in photosynthesis but a cofactor in carotenoid biosynthesis. When HPPD is inhibited, the levels of tocopherol and PQ decrease, leading to carotenoid depletion, then leaf bleaching and plant death. Many HPPD-inhibiting herbicides are now introduced globally for use in the naturally tolerant major grain crops corn (Zea mays), rice (Oryza sativa) and wheat (Triticum aestivum), and in genetically modified (GM) soybean (Glycine max).

Resistance to HPPD-inhibiting herbicides has thus far only been reported in North America in waterhemp (Amaranthus tuberculatus) and Palmer amaranth (Amaranthus palmeri). Among these resistant populations, most of them have been selected by HPPD-inhibiting herbicides, with the exception of a Palmer amaranth population. In the resistant populations studied to date, the resistance is conferred by enhanced rates of metabolism of HPPD-inhibiting herbicides. In addition, increased target-site HPPD gene expression also contributes to resistance to mesotrione in Palmer amaranth.

Wild radish (Raphanus raphanistrum) is an economically damaging annual dicot weed infesting crops in many parts of the world. Wild radish is genetically diverse, cross-pollinated and resistance-prone. Wild radish has evolved resistance to multiple herbicide modes of action, including acetolactate synthase...
with a speed of 3.6 km h⁻¹.

Table 1. List of herbicides applied for herbicide dose–response assays in S and R wild radish populations

<table>
<thead>
<tr>
<th>Herbicide^a</th>
<th>Commercial name</th>
<th>Dose applied (g ha⁻¹)</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesotrione</td>
<td>Callisto</td>
<td>0, 3, 6, 12, 24, 48, 96</td>
<td>Syngenta Canada Inc, Guelph, Ontario, Canada</td>
</tr>
<tr>
<td>Tembotrione</td>
<td>Laudis</td>
<td>0, 2, 5, 10, 20, 40, 80</td>
<td>Bayer Crop Science Pty Ltd, Durham, NC, USA</td>
</tr>
<tr>
<td>Isoxaflutole</td>
<td>Balance</td>
<td>0, 3, 6, 12, 25, 50, 100</td>
<td>Bayer Crop Science Pty Ltd, Kwinana, WA, Australia</td>
</tr>
</tbody>
</table>

^a Mesotrione and tembotrione are not yet registered in Australia, and the recommended rate for isoxaflutole is 100 g ha⁻¹. 1% Hasten (crop oil concentrate) was included in herbicide treatment solution.

(ALS), photosystem II (PSII), phytoene desaturase (PDS) and synthetic-auxin.25–30 A wild radish population (H2/10), collected in 2015 from the northern grain growing region of the Western Australia (WA) grain belt, exhibits resistance to multiple herbicides, including atrazine, chlorsulfuron, diflufenican and 2,4-Dichlorophenoxyacetic acid (Owen, unpublished data). This population, although never selected with HPPD-inhibiting herbicides, was suspected to be resistant to HPPD-inhibiting herbicides. We hypothesised that the resistance to HPPD-inhibiting herbicides in this wild radish population is endowed by a metabolic resistance mechanism that evolved under selection from previously used herbicides and which serendipitously endows resistance to HPPD-inhibiting herbicides. The objectives of this study were to (i) confirm and quantify HPPD-inhibiting herbicide resistance and (ii) to reveal the underlying resistance mechanism(s).

2 MATERIALS AND METHODS

2.1 Materials

A wild radish population (H2/10) from the WA grain belt survived herbicides of four different modes of action, with evidence of resistance to the PSII inhibiting atrazine, ALS inhibiting chlorsulfuron, PDS inhibiting diflufenican and synthetic-auxinic disrupting 2,4-D (Owen, unpublished data). Plants at the two- to three-leaf stage (6–8 cm) were treated with the HPPD-inhibiting herbicide mesotrione (Callisto, Syngenta Canada Inc, Guelph, Ontario, Canada) at 24 g ha⁻¹. Eleven surviving plants were grown in pots in a glasshouse during the normal growing season (May to September) and hand-crossed for producing seeds. The progeny population generated is hereinafter referred to as the HPPD-inhibiting herbicide resistant (R) population. A standard herbicide susceptible (S) wild radish population WARR731 was used as a control. In addition, another herbicide susceptible wild radish population (WARR36) was also included.

2.2 Dose-responses to HPPD-inhibiting herbicides

Seeds were germinated on moist filter paper (Double Rings) for 2 days in the dark. Germinated seeds were transplanted into pots (18 cm diameter, 4.5 L) containing potting mix (50% peatmoss, 25% sand and 25% pine bark). Seedlings were grown in a glasshouse and when seedlings reached the two- to three-leaf stage (6–8 cm), a range of doses (Table 1) of three HPPD-inhibiting herbicides (mesotrione, tembotrione and isoxaflutole) were applied to S and R seedlings. Three replicates each with 12 seedlings were used for each treatment. Herbicides were applied by a cabinet sprayer (TeeJet XR11001 flat fan, Spraying System Co, Wheaton, IL, USA) delivering a volume of 118 L ha⁻¹ at 200 kPa with a speed of 3.6 km h⁻¹. The plant percentage survival of each pot was assessed at 21 days after treatment (DAT), and treated plants with new growth were considered as alive. The experiments were performed twice during the normal growing season in glasshouses.

2.3 Malathion effects on resistance to HPPD-inhibiting herbicides

The two- to three-leaf stage R plants were treated with the P450 inhibitor malathion (1000 g ha⁻¹), Malathion Garden Spray, David Grays, O’Connor, WA, Australia) 1 h prior to the treatment of 12 g mesotrione ha⁻¹, 10 g tembotrione ha⁻¹ and 12 g isoxaflutole ha⁻¹, respectively. The experiment design contained four treatments: untreated control, malathion treatment alone, herbicide treatment alone and malathion followed by herbicide treatment. Three replicates each with 12 seedlings were used for each treatment. The experiment was conducted in the glasshouse during the normal growing season. Plant survival percentage was assessed at 21 DAT.

2.4 Cloning and sequencing of HPPD gene

Total RNA of leaf tissue from an S wild radish plant was extracted using the ISOLATE II RNA Plant Kit (BIOLINE, Alexandria, NSW, Australia). The genomic DNA (gDNA) was removed using the TURBO-DNA Free Kit (Ambion, Thermo Fisher Scientific, Carlsbad, CA, USA). Reverse transcription of gDNA-free RNA was conducted using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). A pair of primers (HPPD-PF1/HPPD-PR, Table 2) was designed for amplification of the partial HPPD gene (including the 5′-end) based on the HPPD gene sequence of wild radish from the radish genome database (http://radish.plantbiology.msu.edu/index.php?title=RadishDB). The polymerase chain reaction (PCR) was conducted in 25 μL volume, consisting of 1 μL cDNA, 10 μM of each primer and 12.5 μL of GoTaq Green Master mix (Promega, Madison, WI, USA) and run in a Thermal Cycler (Thermo Fisher Scientific) with the following steps: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, followed by 7 min at 72 °C. The PCR product was purified from agarose gel (1%) using the WizardSV Gel and PCR Clean-up System (Promega). The fragment was then cloned into pGEM-T vector (Promega) and transformed to Escherichia coli. White colonies were selected for PCR re-amplification and the recombinant plasmid was isolated for gene sequencing. 3′-RACE System for Rapid Amplification of CDNA Ends (Thermo Fisher Scientific) was used to clone the 3′-end of the HPPD gene. The specific primers for the first and second round of nested PCR (HPPD-SP1, HPPD-SP2) are listed in Table 2. The primer pair (HPPD-FL-F/HPPD-FL-R, Table 2) was designed to amplify the full-length HPPD coding sequences in both S and R plants.

PCR amplification of the HPPD gene using the primer pair HPPD-FL-F/HPPD-FL-R yielded multiple peaks in sequencing chromatography, thus further HPPD gene cloning was performed using three S plants from the S population (referred to as S-1), and three S plants (referred to as S-2) and three R plants isolated.
from within the R population. Briefly, the first true leaf of each plant from the R population was pre-harvested, and the remaining plants were treated with mesotrione (12 g ha\(^{-1}\)) the following day. The dead plants were designated as S-2 and the survivors as R plants (assessed at 21 DAT). HPPD genes from S-1, S-2 and R plants were amplified and five colonies from each individual sample were selected for sequencing. The results were analysed using DNAMAN (version 7.0, Lynnon Corp., Quebec, Canada).

### 2.5 HPPD gene expression

The first fully expanded leaves of S and R wild radish plants (five individual plants per population) were harvested and snap-frozen in liquid nitrogen for total RNA extraction using the ISOLATE II RNA Plant Kit. The total RNA was qualified by agarose gel (1%) electrophoresis and quantified using a Nanodrop spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific). Reverse transcription was conducted using the SuperScript III Reverse Transcriptase. Real-time PCR (qPCR) reaction was conducted in a 20 μL volume, consisting of 10 μL of SYBR Green mastermix (BIOLINE), 125 nM each of forward and reverse primers (HPPD-q-F/R and TEF2-q-F/R, Table 2) and 50 ng of cDNA. The qPCR was performed on the 7500 Real-time PCR System (Thermo Fisher Scientific) with 20 s at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 30 s at 60 °C. The translation elongation factor 2 (TEF2) gene previously used as a housekeeping gene in wild radish was employed in this work. A melting curve was performed and the results demonstrated that the primer pairs were specific. Amplification efficiencies of the primer pairs were tested to be 90–110%. Relative HPPD gene expression was expressed using the \(2^{-\Delta \Delta CT}\) method.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPPD-PF</td>
<td>GAGTCTAGTTCAAGCACGAG</td>
<td>Amplification of partial HPPD gene</td>
</tr>
<tr>
<td>HPPD-PR</td>
<td>CCACCATCCTCTCCTGTC</td>
<td>Nested PCR for 3’-RACE</td>
</tr>
<tr>
<td>HPPD-SP1</td>
<td>CGCTTCAACATCATCGGTAGT</td>
<td></td>
</tr>
<tr>
<td>HPPD-SP2</td>
<td>CCAATGCACTTCTCACAC</td>
<td></td>
</tr>
<tr>
<td>HPPD-FL-F</td>
<td>ATGGGCGACGAAAGACCGGTGC</td>
<td>Amplification of full-length HPPD gene</td>
</tr>
<tr>
<td>HPPD-FL-R</td>
<td>TCAACCACAAGGCTGTTGCC</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.6 \[^{14}\text{C}]-mesotrione uptake and translocation

The S and R wild radish seeds were germinated and transplanted to plastic cups (60 × 60 × 60 mm\(^3\)) with potting mix. The seedlings were grown in a controlled environment room (CER) with 20/15 °C day/night temperature, 12 h light period, 300 μmol m\(^{-2}\) s\(^{-1}\) and 70% relative humidity. At the two- to three-leaf stage, \[^{14}\text{C}]-mesotrione (Institute of Isotope Co Ltd, Budapest, Hungary) solution mixed with mesotrione commercial formulation plus 1% (v/v) fasten plus 0.25% (v/v) BS1000 (referred to as treatment solution) was applied to the first leaf at a 1 μL droplet. Total mesotrione concentration applied per plant was equal to 3 g ha\(^{-1}\) with radioactivity of 0.55 kBq. Plant roots were gently washed out of the soil using 100 mL water at 24, 48, 72 and 96 h after treatment (HAT). The treated leaf of each plant was rinsed in 20 mL 20% (v/v) methanol plus 0.2% (v/v) Triton X-100 to remove unabsorbed radioactivity as described in Lu et al.\(^{30}\). Seven replicate individual plants per population per time point were harvested. The \[^{14}\text{C}]-mesotrione uptake was calculated from applied radioactivity minus that in leaf wash, and \[^{14}\text{C}]-mesotrione translocation was visualised using a Typhoon Phosphor Imager (GE Healthcare, Little Chalfont, UK) and quantified using Image Quant TL software (version 8.2, GE Healthcare). Each plant sample was separated to treated leaf (TL), untreated aboveground part (UAP) and root, and four replicate individual plants per population per time point were used to analyse the translocation pattern.

#### 2.7 \[^{14}\text{C}]-mesotrionemetabolism

\[^{14}\text{C}]-mesotrione treatment solution was applied to the first and second true leaves of the S and R plants with 3.5 μL in each leaf. Total mesotrione concentration applied per plant was equal to 3 g ha\(^{-1}\) with radioactivity of 4.30 kBq. The aboveground plant material was harvested at 24, 48, 72, 120 and 168 hAT. The treated leaves were washed, blotted-dry, snap-frozen in liquid nitrogen and stored at −80 °C until use. Three replicate samples per population per time point with two plants per sample were used. Extraction of \[^{14}\text{C}]-mesotrione and its metabolites was according to Lu et al.\(^{30}\). Corn (Zea mays), a naturally mesotrione tolerant crop, was used as a positive control (harvested at 48 hAT).

Reverse-phase HPLC analysis was performed with a 600E dual-head pump with 717 plus autosampler (Waters, Milford, MA, USA) according to Ma et al.\(^{19}\). Separation was achieved with a 250 × 4.6 mm\(^2\) Apollo C\(_{18}\), 5 μm particle column (Grace Davison Discovery Sciences, Deerfield, IL, USA) with a mobile phase flow rate of 1.0 mL min\(^{-1}\) at 24 °C. Eluent A was 0.1% (v/v) formic acid in Milli-Q water and eluent B was acetonitrile. The mobile phase was a linear gradient as follows: 20% to 40% eluent B linear change over 12 min, then linear change from 40% to 70% eluent B over 5 min, followed by linear change from 70% to 90% eluent B over 2 min and subsequently held at 90% eluent B for 5 min before immediate change back to starting conditions of 20% eluent B and re-equilibration for 15 min prior to next injection. A β-RAM model 28 detector (IN/US Systems Inc., Pine Brook, NJ, USA) was used to monitor \(^{14}\text{C}\) eluting from the column. Ultima Gold XR (PerkinElmer Inc., Melbourne, VIC, Australia) scintillator at 1.0 mL min\(^{-1}\) was mixed post column. Injection volumes were adjusted to provide the same sample loading with respect to total radioactivity for all samples. The proportions of the herbicide and metabolites were expressed as a percentage peak area of total radioactivity in the sample injection.

#### 2.8 Data analysis

The data from repeated herbicide dose–response experiments were pooled together as no significant difference between the two replicate experiments was observed (analysed by ANOVA and \(P > 0.05\)). Herbicide rate causing 50% plants mortality \((LD_{50})\), time required for 50% mesotrione to be degraded \((M_{50})\) and time required for 50% major metabolites to be detected \((MM_{50})\) were estimated using a three-parameter Sigmoidal-logistic model (SigmaPlot 13.0, Systat Software, San Jose, CA, USA): \(y = a/(1 + (x/b)^c)\), where \(a\) is the upper asymptote, \(x_0\) equals the LD\(_{50}\), M\(_{50}\) or MM\(_{50}\), and \(b\) is the slope at \(x_0\). Significant differences in LD\(_{50}\), MM\(_{50}\) values and treatment means from malathion effect, HPPD gene expression, herbicide uptake and translocation experiments between S and R populations were analysed by the t-test using Prism 5.0 (GraphPad Software, La Jolla, CA, USA).
(a) Wild radish plants in response to treatment with the HPPD-inhibiting herbicides mesotrione (a), tembotrione (b) and isoxaflutole (c) assessed at 21 DAT.

Figure 1. The percentage survival of S (●) and R (○) wild radish plants in response to treatment with the HPPD-inhibiting herbicides mesotrione (a), tembotrione (b) and isoxaflutole (c) assessed at 21 DAT.

Table 3. LD<sub>50</sub> values and relative parameter estimates of dose responses to mesotrione, tembotrione and isoxaflutole of S and R wild radish populations using the non-linear sigmoidal-logistic regression analysis (a is the upper asymptote, b is the slope at x<sub>0</sub> equivalent to LD<sub>50</sub>)

<table>
<thead>
<tr>
<th>Population</th>
<th>a</th>
<th>b</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (g ha&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>R/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesotrione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>100 (0.4)</td>
<td>2.2 (0.1)</td>
<td>2.0 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td>R</td>
<td>103 (5.9)</td>
<td>2.6 (0.6)</td>
<td>10.9&lt;sup&gt;a&lt;/sup&gt;(1.2)</td>
<td>5.5</td>
</tr>
<tr>
<td>Tembotrione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>100 (0.2)</td>
<td>3.2 (0.1)</td>
<td>1.3 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td>R</td>
<td>102 (5.0)</td>
<td>2.9 (0.6)</td>
<td>8.4&lt;sup&gt;b&lt;/sup&gt;(0.7)</td>
<td>6.5</td>
</tr>
<tr>
<td>Isoxaflutole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>99.7 (0.6)</td>
<td>3.4 (0.1)</td>
<td>5.3 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td>R</td>
<td>98.9 (3.8)</td>
<td>3.5 (0.8)</td>
<td>21.2&lt;sup&gt;a&lt;/sup&gt;(1.7)</td>
<td>4</td>
</tr>
</tbody>
</table>

Standard errors are in parentheses.
<sup>a</sup> The LD<sub>50</sub> values of S and R wild radish populations are significantly different by the t-test, P < 0.001.

3 RESULTS

3.1 Dose responses to HPPD-inhibiting herbicides

As expected, the standard S wild radish population (WARR7) was well-controlled (more than 80% mortality) by 6 g mesotrione ha<sup>-1</sup>, with less than 30% survival at 3 g ha<sup>-1</sup> (Fig. 1). To exclude possible variations among susceptible populations, an additional susceptible population (WARR36) was tested for susceptibility to mesotrione in comparison to WARR7. Results showed that WARR36 had a similar susceptibility to WARR7, with 20% survival at 3 g mesotrione ha<sup>-1</sup> and 0% survival at 6 g ha<sup>-1</sup>, and thereafter only WARR7 was used as the S control. The R population showed more than 90% survival at 6 g mesotrione ha<sup>-1</sup>, exhibiting an LD<sub>50</sub> value of 10.9 g ha<sup>-1</sup> (Fig. 1(a) and Table 3). Thus, the R population was 5.5-fold more resistant to mesotrione than the S population based on the LD<sub>50</sub> R/S ratio (Table 3).

The S wild radish plants were normally susceptible to the HPPD-inhibiting herbicides tembotrione and isoxaflutole, with control (more than 80% mortality) at 2.5 and 10 g ha<sup>-1</sup>, respectively, resulting in low LD<sub>50</sub> values to these two herbicides (Fig. 1(b),(c) and Table 3). The R population needed higher rates for control, with LD<sub>50</sub> values being 6.5-fold (tembotrione) and 4-fold (isoxaflutole) greater than their respective S population (Table 3, P < 0.001). These results establish that the R population is resistant to HPPD-inhibiting herbicides mesotrione, tembotrione and isoxaflutole.

3.2 The effects of malathion on resistance to HPPD-inhibiting herbicides

Pre-treatment with the known cytochrome P450 inhibitor malathion followed by mesotrione or isoxaflutole did not significantly affect plant mortality, compared to the herbicide treatment alone (P = 0.67 for malathion + mesotrione vs mesotrione, P = 1.00 for malathion + isoxaflutole vs isoxaflutole, Table 4). In contrast, malathion followed by tembotrione greatly enhanced plant mortality as compared to tembotrione treatment alone (Table 4). Hence the known cytochrome P450 inhibitor malathion could reverse tembotrione resistance in the R population.

3.3 Alignment of HPPD genes

Two full lengths of HPPD coding sequences were cloned from the S-1 plants with lengths of 1326 (HPPD-1, Genbank ID: MN244457) and 1317 bp (HPPD-2, Genbank ID: MN244458), encoding 441 and 438 amino acids, respectively. These two transcripts show 96.38% identity. In addition, a three amino acid deletion (Ala-Arg-Pro) in position 110–112 (amino acid numbering starts with the Met in the HPPD-1 protein of the S-1 plants) was found in the HPPD-2 amino acid sequence. Only HPPD-2 was detected and cloned from the R plants. Alignment of HPPD-2 transcripts from S-1 and R plants revealed two amino acid substitutions (Pro-20-Leu and Asp-348-Gly). In order to confirm if these substitutions are related to mesotrione resistance, the HPPD gene was cloned from the S plants isolated within the R population (S-2). Likewise, only HPPD-2 was detected and cloned from the S-2 plants. Alignment of HPPD-2 sequences from S-2 and R plants revealed the presence of the two amino acid substitutions (Pro-20-Leu and Asp-348-Gly) in the
Enhanced metabolism confers resistance to HPPD herbicides

**3.4 HPPD gene expression**

The HPPD gene expression levels of S and R plants were compared and no significant difference was found ($P = 0.48$, Fig. 2), thus there is not any HPPD gene differential expression that could explain the resistance to HPPD-inhibiting herbicides.

**3.5 $[^{14}C]$-mesotrione uptake and translocation**

Both S and R plants absorbed $[^{14}C]$-mesotrione rapidly with more than 90% mesotrione absorption by 24 HAT. No significant differences were observed in $[^{14}C]$-mesotrione uptake between S and R wild radish plants up to 72 HAT, except for a lower absorption in the R plants at 96 HAT (Fig. 3). Translocation of $[^{14}C]$-mesotrione was similar in S and R plants with about 25% of total absorbed radioactivity translocating to untreated leaves and stems and 15% to roots by 72 HAT (Figs 3(c),(d) and 4). Based on the results, it is clear that mesotrione uptake and translocation are similar between S and R plants, and therefore differential uptake or translocation is not responsible for the resistance to HPPD-inhibiting herbicides.

**3.6 $[^{14}C]$-mesotrione metabolism**

Under our HPLC conditions, the $[^{14}C]$-mesotrione was resolved at a retention time (RT) of about 22.5 min (Fig. 5). As expected, mesotrione-tolerant corn metabolised more than 90% mesotrione by 48 HAT with four major metabolites eluted at RTs of 4.5 min (M1), 5.2 min (M2), 9.9 min (M3) and 15.2 min (M4), respectively (Fig. 5). Both S and R wild radish plants metabolised mesotrione, with two major metabolites (M3 and M4) in the S plants.
and three (M2, M3 and M4) in the R plants (Fig. 5). By 24 HAT, the S plants had metabolised only 5% mesotrione, while the R plants had metabolised 30% of the mesotrione. More substantial differences were observed at 168 HAT with 30% mesotrione being metabolised in the S plants compared to 70% in the R plants (Fig. 6(a)). Correspondingly, much a greater percentage of major mesotrione metabolites was present in the R plants (65%) at 168 HAT, compared to only 20% in the S plants (Fig. 6(b)). Nonlinear regression analysis of remnant mesotrione in plant tissue (Table 5) showed that the R plants were 7.7-fold faster than the S plants in metabolising mesotrione. Correspondingly, the accumulation rate of the major metabolites was at least 5.4-fold higher in the R than the S plants (Table 5). These results demonstrate that an enhanced rate of HPPD-inhibiting herbicide metabolism confers resistance to HPPD-inhibiting herbicides in the R population.

4 DISCUSSION

We confirm that a wild radish population exhibits resistance to at least three different HPPD-inhibiting herbicides mesotrione, tembotrione and isoxaflutole, relative to the S population. The level of resistance is low comparing the LD50 values of the R population (Fig. 1 and Table 3) to the field rates of HPPD-inhibiting herbicides (e.g. 105 g ha$^{-1}$ for mesotrione in the USA). However, this glasshouse measured low-level resistance will aggravate in the field as field conditions (weather, temperature, plant stage and size, etc.) often result in reduced herbicide application rate and efficacy. In addition, for determination of low-level herbicide resistance, more than one susceptible population should be used (e.g. in our case, the susceptible populations of WARR7 and WARR36). As resistance to HPPD-inhibiting herbicides has thus far only been reported in waterhemp and Palmer amaranth in North America, wild radish has become the third weed species exhibiting resistance to the HPPD-inhibiting herbicide group.

HPPD-inhibiting herbicides have become widely used in global crop production, and resistance evolution has been very limited thus far compared to other major herbicide modes of action (e.g. ALS and acetyl CoA carboxylase). HPPD-inhibiting herbicide resistant waterhemp was first reported in Illinois in 2009 after 6 years of HPPD-inhibiting herbicide use.10 Shortly afterwards, resistance was documented in a Palmer amaranth population from Kansas.11 Noticeably, this Palmer amaranth population had never been treated with HPPD inhibitors, but had a long history of ALS and PSII herbicide selection. HPPD-inhibiting herbicide resistant *Amaranthus* populations have been reported in Iowa, Nebraska and Arkansas.12–15 Mechanistic studies have revealed that the predominant mechanism for HPPD-inhibiting herbicide resistance in both *Amaranthus* species is enhanced herbicide metabolism.34 It is envisaged that target-site HPPD gene mutations are rare in field-evolved resistant populations, as HPPD-inhibiting herbicides are competitive inhibitors with respect to the substrate 4-hydroxyphenyl pyruvate.35 HPPD gene mutations that endow resistance may incur plant fitness cost and hence are selected against. In the present study, we demonstrate that resistance to HPPD-inhibiting herbicides in a wild radish population from Australia is non-target-site, enhanced herbicide metabolism based. This is envisaged as this particular wild radish field population (H2/10) has resistance to multiple herbicide modes of action, with no exposure to HPPD inhibitor herbicides at the farm site prior to 2015 (when seeds were collected). Hence, this non-target-site resistance to HPPD-inhibiting herbicides was very likely selected by other herbicides, which is similar to a North American case of HPPD-inhibiting herbicide resistant Palmer amaranth.11,21

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Figure 4. Visualisation of $^{14}$C-mesotrione translocation in S and R wild radish plants at 72 HAT with camera and phosphor images.
Enhanced metabolism confers resistance to HPPD herbicides

Corn, as a naturally mesotrione-tolerant crop, predominantly metabolises mesotrione to 4-hydroxy-mesotrione, which is also the major metabolite in the resistant waterhemp. In our study, two major mesotrione metabolites M2 and M4 in R wild radish plants significantly increased with time (Fig. 5), contributing to mesotrione metabolic resistance. The metabolite M4 (likely 4-hydroxy-mesotrione) is chromatographically similar in wild radish plants and corn, but the more polar metabolite M2 (likely a conjugate of 4-hydroxy-mesotrione) is not. In R wild radish plants, M2 was eluted as a single peak and corn had two poorly separated peaks M1 and M2. This indicates that R wild radish plants share with corn similar but not identical metabolic pathways for HPPD-inhibiting herbicides. In addition, our study also showed that mesotrione resistance in the R population cannot be reversed by malathion, but tembotrione resistance can be malathion-reversed, which is similar to HPPD-inhibiting herbicide resistant waterhemp from Nebraska. These P450 inhibitor studies suggest that multiple metabolic genes/enzymes (including P450s) may be involved in weed resistance to HPPD-inhibiting herbicides. A recent study has identified that a gene encoding an Fe(II)/2-oxoglutarate-dependent oxygenase endows resistance to HPPD-inhibiting herbicides in rice. However, genes conferring metabolic resistance to HPPD-inhibiting herbicides have not been identified in weeds.

Wild radish is a damaging weed in Australia cropping regions and has evolved resistance to multiple herbicide groups.
HPPD-inhibiting herbicides are now widely used for weed control in the world. Therefore, this first case of resistance to HPPD-inhibiting herbicides in wild radish is a significant negative development, especially as newer HPPD-inhibiting herbicide products are still under development and overall use of HPPD-inhibiting herbicides is increasing. Moreover, the recent market introduction of GM isoxaflutole tolerant soybeans (LibertyLink GT27) might also augment the use of HPPD-inhibiting herbicides, thus increasing the selection pressure on weeds. Diversity in herbicide usage and adoption of non-chemical weed control strategies must occur to sustain these herbicides. For instance, in Australia, harvest weed seed control (HWSC, e.g. harvest weed seed destructor) has been shown to be efficient in reducing seed bank replenishment, removing a high proportion (more than 90%) of retained wild radish seeds.\(^6\)\(^-\)\(^8\) HWSC combined with effective herbicide application (e.g. full herbicide label rates plus appropriate adjuvants) can keep weed densities at very low levels,\(^10\)\(^-\)\(^16\) which is vital to sustainable agriculture.

ACKNOWLEDGEMENTS

This work was supported by the China Scholarship Council (CSC, No. 201606750002) and the University of Western Australia and partially funded by the Australian Grains Research and Development Corporation.

REFERENCES


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