# Powles Plain English

*Making crops and weeds research interesting, understandable, and accessible to all.*

**Colleagues,**

Since the late 1980's it has been evident to us that herbicide resistance in Lolium populations involves non-target site enhanced rates of herbicide metabolism as a very widespread and important resistance mechanism. Of course, Lolium exhibits multiple resistance mechanisms, embracing non-target site resistance mechanisms and target site mutations. As knowledge and technology developed it was possible to identify target site ALS, ACCase mutations, EPSPS gene mutations in resistant Lolium. However, we have been far less successful in identifying non-target site resistance genes.

Over the next few years we hope to identify the genes/enzymes responsible for enhanced rates of herbicide metabolism based resistance in resistant Lolium populations. This is being accomplished through a deep sequencing approach in a fruitful collaboration with Bayer Frankfurt. This paper will help us in this endeavour. We knew that 2,4-D could induce P450 activity in Lolium (unpublished). In this latest AHRI paper, by Han et al we establish in vivo that in herbicide susceptible Lolium that 2,4-D can induce resistance against diclofop-methyl due to enhanced rates of diclofop metabolism.

In this work, with herbicide susceptible Lolium pre-treated with 2,4-D the plants show faster rates of diclofop metabolism and show transient resistance to diclofop (not inherited to the next generation). In this way the 2,4-D pre-treated herbicide susceptible Lolium respond identically to Lolium biotypes resistant to diclofop by enhanced rates of herbicide metabolism. Importantly, in both cases there is cross resistance to the chemically and mode of action dissimilar chlorsulfuron.

Our hypothesis remains that this is due to enhanced activity of cytochrome P450 enzymes (plus potentially GSTs or other mechanisms). This effect of 2,4-D in inducing transient resistance in Lolium is helping us in our deep sequencing results aimed to identify the specific genes responsible for enhanced metabolism based resistance in Lolium. Over the coming period we hope to make major progress in identifying the specific genes responsible for non-target site enhanced metabolism resistance in Lolium.

Thank you,

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Rapid Report

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# Enhanced herbicide metabolism induced by 2,4-D in herbicide susceptible *Lolium rigidum* provides protection against diclofop-methyl

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## Abstract

**BACKGROUND: The auxinic herbicide 2,4-D amine is known, *in vitro*, as a cytochrome P450 inducer. The current study uses 2,4-D pre-treatment, at the whole plant level, to study mechanism(s) of non-target site based herbicide resistance to the ACCase-inhibiting herbicide diclofop-methyl in *Lolium rigidum*.**

**RESULTS: The 2,4-D pre-treatment caused up to 10-fold shift in $LD_{50}$ and $GR_{50}$ in dose–response to subsequently applied diclofop-methyl in a herbicide susceptible *L. rigidum* population. Foliar uptake and translocation of $^{14}$C-diclofop-methyl did not differ in 2,4-D pre-treated versus untreated plants. HPLC analysis revealed that de-esterification of diclofop-methyl to toxic diclofop acid was similar, but further metabolism of diclofop acid to non-toxic metabolites was significantly (1.8-fold) faster in 2,4-D pre-treated than untreated plants. HPLC profile of major polar metabolites was similar when *L. rigidum* and diclofop-methyl tolerant wheat were compared, but wheat metabolised diclofop acid three-fold faster than *L. rigidum*. In addition, 2,4-D pre-treatment also induced cross-protection against the ALS-inhibiting herbicide chlorsulfuron, and the known P450 inhibitor malathion can reverse this effect.**

**CONCLUSIONS: Protection against diclofop-methyl provided by 2,4-D pre-treatment in susceptible *L. rigidum* is associated with higher rates of herbicide metabolism, mirroring that identified in field-evolved, non-target site-based diclofop-methyl resistant populations. 2,4-D may induce higher level expression of herbicide-metabolising genes hence providing protection, and therefore, this 2,4-D induction system can be used, in combination with other genomic approaches, to assist isolating cytochrome P450 and other genes that are involved in herbicide metabolism and endow herbicide resistance in *L. rigidum*.**


**Keywords:** 2,4-D amine; antagonism; chlorsulfuron; cytochrome P450; diclofop-methyl; herbicide metabolism; *Lolium rigidum*

## 1 INTRODUCTION

The annual diploid grass *Lolium rigidum* is genetically diverse, present at high densities in Australia, where since the first report[1] it has evolved herbicide resistance across vast areas, especially to the ACCase- and ALS-inhibiting herbicides (hereafter referred to ACCase or ALS herbicides).[2] It is a feature of these *L. rigidum* populations that multiple herbicide resistance mechanisms are accumulated within individual resistant plants.[3,4] While target site resistance can be precisely determined,[5] non-target site herbicide resistance (NTSR) is relatively difficult to study. For many years our laboratory has been investigating NTSR in *L. rigidum* and has established that NTSR to ACCase and ALS herbicides in *L. rigidum* populations involves enhanced rates of herbicide metabolism.[6,7] Additionally, we have compelling indirect evidence using known cytochrome P450 inhibitors that P450s are involved in the enhanced rates of metabolism of ACCase and ALS herbicides.[7,8] However, progress has been slow at the molecular level in understanding P450 genes involved in herbicide resistance. Plant P450s have wide diversity and substrate specificity, and so far only a small number of herbicide-metabolising plant P450s have been identified and characterised.[9]

The auxinic herbicide 2,4-D is known, *in vitro*, as a P450 inducer,[10] but its relevance to *in vivo* herbicide metabolism and hence herbicide resistance is unknown in *L. rigidum*. Here, in whole plant studies we show that 2,4-D pre-treatment can protect a herbicide susceptible *L. rigidum* population against diclofop-methyl and the dissimilar herbicide chlorsulfuron. We demonstrate that this 2,4-D induced protection against diclofop-methyl is due to enhanced rates of diclofop-methyl metabolism, mimicking that which occurs in field-evolved herbicide resistant *L. rigidum* populations. Application of this 2,4-D induced protection system is discussed in assisting the discovery of genes responsible for NTSR in *L. rigidum*.

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## 2 MATERIALS AND METHODS

### 2.1 Plant material and herbicide treatment

The herbicide susceptible *L. rigidum* population VLR1 (hereafter referred to as 'susceptible'), subjected to recurrent herbicide selection with low rates of the ACCase herbicide diclofop-methyl, is uniformly susceptible to ACCase and ALS herbicides.[11]

Seeds were germinated in plastic trays containing potting soil and maintained in a controlled environmental room (CER) or glasshouse, as specified below. Uniform two-leaf stage germinating seedlings were herbicide treated. Herbicides were applied as commercial formulations. To obtain maximum effects and to avoid any direct herbicide physical/chemical interactions, the 2,4-D (amine, 4000 g a.i. $ha^{-1}$ commercial formulation plus 0.2% surfactant BS 1000) was applied 24 h prior to the subsequent herbicide treatment. Herbicides were applied using a laboratory spray cabinet equipped with two flat fan nozzles delivering 118 L $ha^{-1}$ water at 200 kPa pressure. Mortality (no new growth or active tillering) was determined 21 days after herbicide application. Plant above-ground material was harvested, oven dried (70 °C) for 2 days and dry biomass obtained.

### 2.2 Diclofop-methyl dose response with and without 2,4-D pre-treatment

Experiments were conducted in a CER under day/night temperatures of 15/10 °C. *L. rigidum* seedlings (about 30–40 per pot) were treated with diclofop-methyl, that had or had not been previously treated with 2,4-D (4000 g a.i. $ha^{-1}$). Pots were randomised every 2 d to minimise variation and there were 2 replicate pots per treatment.

### 2.3 Effect of 2,4-D on efficacy of the ALS herbicide chlorsulfuron in the absence and presence of P450-inhibitor malathion

Seedlings (about 50 per tray) were grown in the glasshouse (20–25 °C under natural light) and treated with chlorsulfuron alone (100, 200 g $ha^{-1}$), 2,4-D plus chlorsulfuron, and 2,4-D plus malathion (at 1000 g $ha^{-1}$) plus chlorsulfuron. The 2,4-D was applied 24 h prior to the malathion application then immediately followed by chlorsulfuron treatment. There were three replicates for each treatment.

### 2.4 Foliar uptake and translocation of $^{14}$C-diclofop-methyl

Seedlings were grown in trays in a CER with 20/15 °C day/night temperatures, 12 h photoperiod with 650 µmol photons $m^{-2}$ $s^{-1}$ and relative humidity of 75%. Two-leaf stage seedlings were treated with 2,4-D followed 24 h later by a low rate of diclofop-methyl (50 g $ha^{-1}$) to induce metabolic activity. Treated plants were returned to the CER for 2 h before application of a droplet of $^{14}$C-diclofop-methyl (uniformly labelled with $^{14}$C in the phenyl ring adjacent to the oxypropanoid acid moiety, with a specific radioactivity of 179 µCi $mg^{-1}$; Bayer CropScience, Frankfurt, Germany). The $^{14}$C-diclofop-methyl was dissolved in acetonitrile and diluted in aqueous solution containing 0.25% BS1000, giving a final radioactivity of 0.76 kBq $\mu L^{-1}$ and diclofop-methyl concentration of 0.45 mM (equivalent to 17 g $ha^{-1}$). A 1 µL droplet of the treatment solution was applied with a micropipette on the middle of the first leaf. Individual plants (including roots) were harvested 24, 48 and 72 h after treatment, and the treated leaf of each plant rinsed in 20 mL washing buffer containing 20% (v/v) methanol and 0.2% (v/v) Triton X-100. The radioactivity in the leaf wash solution was determined by liquid scintillation spectrometry to determine unabsorbed radioactivity. Root tissue for each plant was rinsed in another 20 mL washing buffer and the radioactivity in root wash was found to be negligible. Plants were blot dried with tissue paper and oven dried (70 °C) for 2 days and $^{14}$C translocation was visualised using a BioRad Personal Molecular Imager (BioRad PMI, Sydney, Australia) and quantified using the QuantityOne software (version 4.6.7; Bio-Rad PMI). There were five replicate plants per harvest per treatment.

### 2.5 Metabolism of $^{14}$C-diclofop-methyl

Experimental conditions, plant growth and herbicide treatment was the same as described for the leaf uptake and translocation experiments, except that $^{14}$C-radioactivity contained in 1 µL droplet treatment solution was 4.75 kBq with a final concentration of 2.81 mM (equivalent to 105 g $ha^{-1}$ as compared to the field recommended rate of 375 g $ha^{-1}$). The treatment solution was spread along the adaxial surface (close to the leaf base) of the first leaf to facilitate diclofop-methyl translocation and to avoid local phytotoxicity. The shoots of treated seedlings were harvested 48 and 96 h after treatment. The treated leaf of each plant was rinsed as described above, blotted dry, snap frozen in liquid $N_2$ and stored at −80 °C. Six plants per treatment were bulked as a replicate and two replicates per treatment were analysed for each time point.

Extraction, separation and HPLC identification of the parent diclofop-methyl herbicide and its metabolites were according to Holtum *et al*.[3] Parent herbicide and its metabolites were separated by gradient reverse-phase HPLC equipped with a 600E dual-head pump with 717 plus autosampler (Waters, Milford, MA, USA). Separation was done on a Waters Spherisorb 5 µm ODS2 (250 mm long × 4.6 mm i.d.) column. Radioactivity was detected with an in-line Beta-RAM model 2B (IN/US Systems Inc., Pine Brook, NJ, USA) detector. Injection volumes were adjusted to provide the same sample loading with respect to total radioactivity in all samples. The proportion of the herbicide and metabolites were expressed as a percentage peak area of total radioactivity in the sample injection. This experiment was conducted twice. Wheat seedlings, known to rapidly metabolise diclofop-methyl,[12] were included in the metabolism study as a positive control.

### 2.6 Statistics

Data were expressed as percentage of untreated controls and subjected to non-liner regression analysis using SigmaPlot 12.0. The $LD_{50}$ or $GR_{50}$ (herbicide rate causing 50% plant mortality or dry mass reduction) were estimated using the four-parameter logistic equation: $y = C + (D - C)/[1 + (x/I_{50})^b]$, where $C$ is the lower limit at the indefinitely large doses, $D$ is the upper limit close to untreated controls, $I_{50}$ is the herbicide dose giving 50% response, and $b$ is the slope of the best fitting curve through the $LD_{50}$ or $GR_{50}$. The treatment means were subjected to analysis of variance and compared using Tukey's HSD ($\alpha = 0.05$).

## 3 RESULTS

### 3.1 2,4-D induces protection against diclofop-methyl in *L. rigidum*

As expected, the susceptible population was susceptible to diclofop-methyl with no survivors at the field rate of 375 g $ha^{-1}$ (Fig. 1). The estimated $LD_{50}$ and $GR_{50}$ were 64 ± 2.1 and 63 ± 7.2 g diclofop-methyl $ha^{-1}$, respectively, for the susceptible plants. However, when there was 2.4-D pre-treatment of the susceptible plants there was 71% survival at the field diclofop-methyl rate and the

  

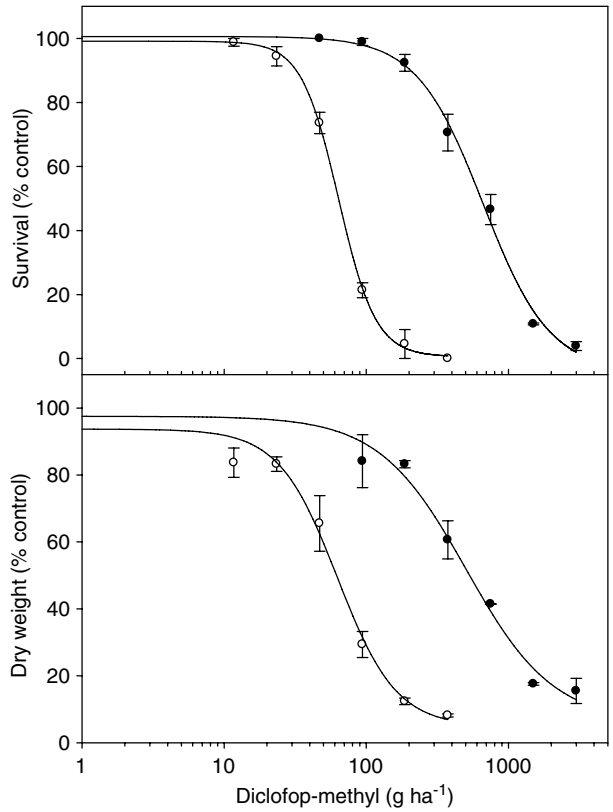


**Figure 1.** Diclofop-methyl dose response of the susceptible *L. rigidum* population with (closed circle) or without (open circle) 2,4-D (4000 g a.i. $ha^{-1}$) pre-treatment. The experiment was conducted in a controlled environmental room under day/night temperature of 15/10 °C. Each data point is the mean ± SE ($n = 2$).

estimated $LD_{50}$ and $GR_{50}$ were 664 ± 56 and 504 ± 100 g $ha^{-1}$, respectively, which is eight- to 10-fold greater than that without 2,4-D pre-treatment (Fig. 1). The similar $LD_{50}$ and $GR_{50}$ ratios were obtained when the experiment was conducted at 30/25 °C (data not shown). Clearly, in this susceptible *L. rigidum* population the 2,4-D pre-treatment induced high level protection against diclofop-methyl.

### 3.2 2,4-D induces cross-protection against chlorsulfuron and the protection can be reversed by the P450 inhibitor malathion

In addition to 2,4-D inducing protection against diclofop-methyl, there was concomitant protection against the dissimilar ALS herbicide chlorsulfuron (Table 1). As expected, 2,4-D or malathion alone did not affect plant mortality. When chlorsulfuron was used alone at 100 or 200 g $ha^{-1}$, there was only maximum 5% survival. However, when plants were pre-treated with 2,4-D, there was 15–32% survival. Chlorsulfuron was evaluated because both diclofop-methyl and chlorsulfuron are known to be readily metabolised by cytochrome P450 enzymes in wheat[6,12] and in herbicide resistant *L. rigidum*.[3,6] Malathion, a known P450 inhibitor, is able to reverse metabolism-based chlorsulfuron resistance, especially in *Lolium*.[7,8] When the susceptible plants were pre-treated with 2,4-D and malathion, chlorsulfuron regained efficacy (Table 1). In contrast, malathion could not reverse 2,4-D induced protection against diclofop-methyl (data not shown), suggesting that different P450s are induced by 2,4-D to metabolise chlorsulfuron and diclofop-methyl, respectively.

**Table 1.** Antagonistic effect of 2,4-D on the ALS herbicide chlorsulfuron and reversal by the P450 inhibitor malathion in the susceptible *Lolium rigidum* population

| Chlorsulfuron (g $ha^{-1}$) | 2,4-D (g $ha^{-1}$) | Malathion (g $ha^{-1}$) | Survival (%) |
|---|---|---|---|
| 0 | 4000 | 0 | $100 \pm 0^{a}$ |
| 0 | 0 | 1000 | $100 \pm 0^{a}$ |
| 100 | 0 | 0 | $5.3 \pm 1.3^{d}$ |
| 100 | 4000 | 0 | $32.3 \pm 2.3^{b}$ |
| 100 | 4000 | 1000 | $6.7 \pm 1.3^{d}$ |
| 200 | 0 | 0 | $2.0 \pm 1.2^{d}$ |
| 200 | 4000 | 0 | $14.7 \pm 0.7^{c}$ |
| 200 | 4000 | 1000 | $0.7 \pm 0.7^{d}$ |

Each value is the mean ± SE ($n = 3$).
Means with different letters in a column are significantly different according to Tukey's HSD ($\alpha = 0.05$)

### 3.3 2,4-D pre-treatment does not change leaf uptake or translocation of $^{14}$C- diclofop-methyl

Foliar uptake of $^{14}$C-diclofop-methyl was rapid such that 92% of applied $^{14}$C was within the leaf by 24 h after treatment. There was no difference in leaf diclofop-methyl uptake in 2,4-D pre-treated versus untreated plants (24 to 72 h after treatment, data not shown). Movement of radioactivity away from the application site increased with time, with a maximum of 21% basipetal translocation. Overall there was no major difference in the $^{14}$C-translocation pattern, between 2,4-D pre-treated and untreated plants (data not shown).

### 3.4 2,4-D pre-treatment induced enhanced rates of diclofop acid metabolism

The HPLC profile of $^{14}$C-diclofop-methyl metabolism *in vivo* was found to be qualitatively similar but quantitatively different in 2,4-D pre-treated versus untreated plants. As shown in Fig. 2, diclofop-methyl, diclofop acid and its metabolites were clearly resolved at 31, 29, and between 2–6 and 10–13 min, respectively, under our HPLC conditions and compared to authentic standards (diclofop-methyl, diclofop acid). As expected, the conversion of diclofop-methyl to phytoxic diclofop acid was rapid, with only 3–7% of total radioactivity recovered as diclofop-methyl 24 h after treatment. There were no significant differences in rates of diclofop-methyl de-esterification observed in 2,4-D pre-treated versus untreated plants, although a relatively higher de-esterification rate was consistently observed in 2,4-D pre-treated samples (Table 2, Fig. 2). However, metabolism of diclofop acid to non-toxic metabolites was faster in the 2,4-D treated versus untreated plants such that the diclofop acid level was about 1.8-fold lower in 2,4-D pre-treated versus untreated plants, 48 h or 96 h after treatment. This lower diclofop acid level in 2,4-D pre-treated plants was well correlated with a higher level of total metabolites, and especially the percentage of the major metabolites (retention time 2–4 min) was increased nearly two-fold by 2,4-D treatment. As wheat is known to tolerate diclofop-methyl due to rapid P450-mediated herbicide metabolism,[12] it was used as the positive control in this study. We confirmed that diclofop acid metabolism in wheat is much faster than in *L. rigidum* (Table 2, Fig. 2). The major metabolites resolved at retention time 2–6 min were similar in wheat and *L. rigidum*, but wheat did not have the less polar

  

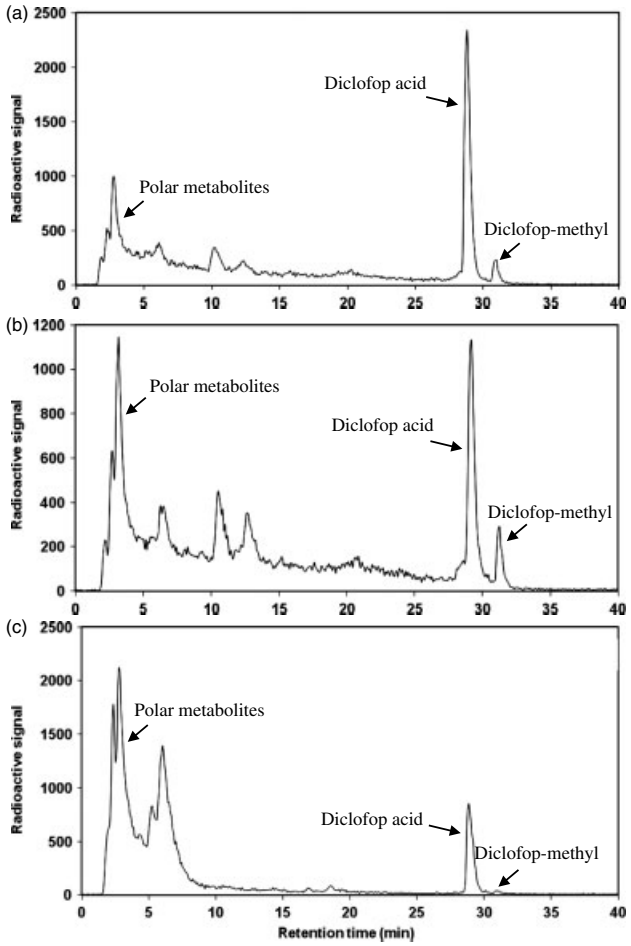


**Figure 2.** HPLC chromatograms of $^{14}$C-diclofop-methyl and its metabolites in extracts of susceptible *L. rigidum* 96 h after treatment with diclofop-methyl alone (a) or pre-treated with 2,4-D (4000 g a.i. ha$^{-1}$) (b). Wheat 48 h after treatment without 2,4-D was included as a positive control (c).

(relative to the major metabolite) metabolite peaks at retention time 10–14 min (Fig. 2).

## 4 DISCUSSION

The herbicide susceptible *L. rigidum* population used in this study is susceptible to the ACCase herbicide diclofop-methyl[11] (Fig. 1). However, it can be protected against diclofop-methyl by pre-treatment with the auxinic herbicide 2,4-D. In addition, the 2,4-D pre-treatment also induced a level of protection to the dissimilar ALS herbicide chlorsulfuron (Table 1). It is striking that this 2,4-D induced protection pattern in susceptible *L. rigidum* against both ACCase and ALS herbicides is very similar to that exhibited in field-evolved herbicide resistant *L. rigidum* biotypes, where the resistance is non-target site, metabolism based.[6–8] The obvious question is how 2,4-D pre-treatment can provide protection in susceptible *L. rigidum* against diclofop-methyl and chlorsulfuron? Our hypothesis is that 2,4-D pre-treatment enhances the existing capacity for metabolism of diclofop-methyl and chlorsulfuron, hence endowing a level of protection against these subsequently applied herbicides.

It is known that ACCase herbicide efficacy is reduced by 2,4-D (and MCPA) in *Avena fatua* and other grass species, and various mechanisms have been proposed including recovery from membrane depolarisation,[13] reduction in herbicide uptake or translocation[14,15] and altered herbicide metabolism including reduced diclofop-methyl de-esterification.[15,16] Here, we demonstrate that 2,4-D induced protection against diclofop-methyl in susceptible *L. rigidum* is not caused by altered herbicide uptake or translocation. Rather, the 2,4-D pre-treatment induces enhanced rates of herbicide metabolism (Table 2, Fig. 2). This is similar to the NTSR mechanism determined for field-evolved ACCase herbicide resistant *L. rigidum* populations.[3,7] In addition, the HPLC chromatograph showed similar number of metabolite peaks in 2,4-D pre-treated versus untreated samples (Fig. 2), indicating 2,4-D mainly enhances (up-regulates) pre-existing herbicide metabolic capacity, rather than inducing new pathways. The major metabolite peaks with the retention time at 2–3 min were resolved in HPLC chromatographs of both wheat and 2,4-D pre-treated *L. rigidum*, suggesting similar (but not identical) metabolic pathways for diclofop acid. In wheat, the major detoxification reactions are P450-mediated diclofop ring-hydroxylation followed by conjugation to an acidic aryl glycoside.[12] Therefore, 2,4-D induced protection against diclofop-methyl in *L. rigidum* likely involves wheat-like enhanced metabolic activities of (unknown) P450 and glucosyl transferase (GT). There is evidence showing 2,4-D induces P450 activities and P450 gene expression,[10,17] and 2,4-D antagonises the inhibitory effect of terbufos (a P450 inhibitor) on ALS herbicide metabolism.[18] In the current study, we also demonstrated that 2,4-D induces cross-protection against the ALS herbicide chlorsulfuron and this cross-protection can be reversed by the known P450 inhibitor malathion (Table 1), further implicating P450 involvement. This is similar to field-evolved resistance to diclofop-methyl and chlorsulfuron

**Table 2.** Metabolism of $^{14}$C-diclofop-methyl by the susceptible *Lolium rigidum* plants*

| | | | Radiolabel (% of radioactivity recovered) | | |
|---|---|---|---|---|---|
| Time point | Population | Treatment | Total metabolites | Diclofop acid | Diclofop-methyl |
| 48 h | Susceptible | − 2,4-D | $30.44 \pm 0.61^{c}$ | $64.86 \pm 0.22^{a}$ | $4.71 \pm 0.40^{ab}$ |
| | | + 2,4-D | $56.27 \pm 6.60^{b}$ | $36.87 \pm 3.05^{b}$ | $6.86 \pm 3.55^{a}$ |
| | Wheat | − 2,4-D | $87.67 \pm 0.65^{a}$ | $12.15 \pm 0.63^{c}$ | $0.19 \pm 0.12^{b}$ |
| 96 h | Susceptible | − 2,4-D | $36.61 \pm 0.30^{c}$ | $60.15 \pm 1.63^{a}$ | $3.25 \pm 1.34^{ab}$ |
| | | + 2,4-D | $57.85 \pm 1.26^{b}$ | $35.81 \pm 0.42^{b}$ | $6.35 \pm 0.84^{a}$ |

*The herbicide susceptible *L. rigidum* population VLR1.
The proportion of the parent herbicide and metabolites were expressed as a percentage peak area of total radioactivities in the sample injection.
Six plants per treatment were bulked as a replicate and two replicates per treatment were analysed for each time point.
Means with different letters in a column are significantly different by the Tukey's HSD test ($\alpha = 0.05$)

in resistant *L. rigidum* biotypes where our previous work established the resistance is due to enhanced rates of herbicide metabolism involving P450s.[6–8] This is also similar to rapidly evolved resistance to diclofop-methyl and chlorsulfuron in initially herbicide susceptible *L. rigidum* populations under recurrent low-herbicide-dose selection where we recently established that the resistance is due to enhanced capacity of herbicide metabolism.[19] Based on this evidence, 2,4-D induced enhanced herbicide metabolism may involve enhanced expression of a number of P450s, and these genes are likely also responsible for field-evolved resistance to diclofop-methyl and chlorsulfuron in *L. rigidum*. Therefore, 2,4-D induction system can be explored in *L. rigidum* to assist identification and cloning of specific P450 and other genes that are involved in herbicide metabolism and resistance evolution.

## ACKNOWLEDGEMENTS

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