Research Article

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Enhanced production of water-soluble cinmethylin metabolites by *Lolium rigidum* populations with reduced cinmethylin sensitivity

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Abstract

Background: Cinmethylin, a pre-emergence herbicide inhibiting fatty acid thioesterase activity, has recently been introduced to Australian cereal cropping for the control of *Lolium rigidum* Gaud. (annual ryegrass). To date, there have been no confirmed cases of cinmethylin resistance identified in this species, but some populations exhibit reduced sensitivity to this herbicide. To explore the mechanism which contributes to reduced sensitivity of annual ryegrass to cinmethylin, the extent and nature of cinmethylin metabolism, using carbon-14 (^{14}C)-labelled herbicide, were analysed in three reduced-sensitivity annual ryegrass populations, alongside a susceptible population and cinmethylin-tolerant wheat as controls.

Results: All samples showed the same metabolite profile, with the extent of production of a specific water-soluble metabolite being correlated to the level of herbicide sensitivity. Application of the cytochrome P450 inhibitor phorate caused a decrease in water-soluble metabolite production as well as seedling growth in the presence of cinmethylin, indicating that reduced cinmethylin sensitivity in annual ryegrass could be wholly or partially due to oxidative modification of cinmethylin.

Conclusion: Because annual ryegrass has the potential to metabolize cinmethylin in the same way as wheat, careful stewardship is required to ensure the longevity of this herbicide.

Supporting information may be found in the online version of this article.

Keywords: annual ryegrass; cinmethylin; metabolites; resistance; wheat

1 INTRODUCTION

Annual ryegrass (*Lolium rigidum* Gaud.) is the most economically damaging weed of southern Australian cropping systems and is also a major problem in parts of Africa, Europe and the Middle East.[1,2] In response to repeated and intensive use of selective post-emergence herbicides inhibiting acetyl-CoA carboxylase and acetolactate synthase, annual ryegrass populations have developed widespread resistance to these modes of action, and growers are returning to soil-applied pre-emergence herbicides to maintain adequate levels of weed control.[3] Inevitably, this has led to an increasing frequency of resistance to pre-emergence herbicides such as trifluralin (an inhibitor of microtubule assembly, mode of action group 3), and prosulfocarb, triallate and pyroxasulfone (inhibitors of very-long-chain fatty acid biosynthesis, group 15),[4–6] with most resistant populations exhibiting resistance to two or more of these herbicides.

Metabolic detoxification of herbicides, which has been experimentally demonstrated in some annual ryegrass populations resistant to the pre-emergence herbicides listed earlier,[7,8] is usually mediated by cytochrome P450 monooxygenases (P450), glutathione transferases (GST) and glucosyltransferases (GT), all of which are enzymes belonging to large superfamilies involved in diverse pathways of secondary metabolism and detoxification.[9] Cytochrome P450s can oxidize, dealkylate or deaminate some herbicide molecules to a more polar or reactive form, permitting their subsequent conjugation to glutathione (catalysed by GSTs) and sugars (catalysed by GTs), thus rendering them relatively non-herbicidal.[9] Conversely, the action of P450s is also

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hypothesized to increase the phytotoxicity of certain herbicides: the thiocarbamates (e.g. prosulfocarb, triallate) and isoxazolidinones (e.g. clomazone) require oxidation to their sulphoxide and 5-keto forms, respectively, in order to become herbicidally active.[10–12] Pharmacological studies with the organophosphate insecticide phorate, a known inhibitor of plant P450s,[13] have indeed demonstrated a decrease in efficacy of prosulfocarb and clomazone when applied in combination with this inhibitor.[13,14] Phorate can increase the potency of other herbicides, such as trifluralin,[15] which is metabolized by the plant P450 CYP706A3.[16]

Cinmethylin is a newly (re-)introduced herbicide for the pre-emergence control of grass weeds, including those that are resistant to other pre-emergence herbicides. Originally commercialized in 1982 and quickly overshadowed by selective post-emergence herbicides belonging to the acetolactate synthase-inhibiting and acetyl CoA carboxylase-inhibiting modes of action,[17] cinmethylin was registered due to high levels of weed resistance to the more widely used herbicides.[18] Like prosulfocarb and pyroxasulfone, cinmethylin (group 30) affects fatty acid biosynthesis; however, its target is the thioesterases that release fatty acids from their carrier proteins in the plastids,[18] rather than the elongases that extend the fatty acid carbon chains in the endoplasmic reticulum.[19] Due to the recent introduction and relatively limited use of cinmethylin, no confirmed cases of resistance have thus far been detected in weeds,[1,6] and little information has been published about its metabolism in plants. Wheat, which is relatively tolerant to cinmethylin, was killed by a combination of cinmethylin and phorate in pot experiments, suggesting the involvement of P450s in cinmethylin detoxification.[20] Jung *et al.*[21] demonstrated that certain plant GTs are capable of conjugating glucose to synthetic 15-hydroxy cinmethylin (hydroxylated at the 2-methylbenzyl moiety), and earlier studies on animals revealed that cinmethylin (Fig. 1(A)) is hydroxylated at the 2-methylbenzyl position and on the cineole portion of the molecule (Fig. 1(B)), and then conjugated to glucuronic acid.[22,23] More recent unpublished work, available in the regulatory documents of various countries, has confirmed that cinmethylin is also hydroxylated in mature wheat plants (flag leaf stage) and then conjugated to glucose and malonyl glucose (Fig. 1(C,D)).[24,25] As is the case in animals, the major metabolite in wheat leaves is the sugar conjugate(s) of the cinmethylin derivative hydroxylated at the 2-methylbenzyl moiety, but hydroxylation and sugar conjugation also occurs on the cineole moiety and on the phenyl ring to yield mono- and multiple-hydroxylated derivatives[24] (Fig. 1(B)). Taken together, these results strongly suggest that cinmethylin is detoxified by P450-mediated hydroxylation followed by conjugation to large polar molecules.

The aim of this study was to assess the level and nature of cinmethylin metabolism in annual ryegrass in the presence and absence of phorate, comparing putative (low-level) resistant field populations to fully-susceptible annual ryegrass and to cinmethylin-tolerant wheat. The outcome of this study will provide an indication of the potential for metabolic resistance to evolve in annual ryegrass populations exposed to cinmethylin.

2 MATERIALS AND METHODS

2.1 Plant material

The annual ryegrass population VLR1 was used as the cinmethylin-susceptible control,[26] and is hereafter referred to as 'S'. A population from Wickepin, Western Australia (32 °47′ S, 117° 30′ E), identified in a 2019 survey of herbicide-resistant annual ryegrass as putatively cinmethylin-resistant,[6] was subjected to two rounds of selection at a sublethal dose of cinmethylin (250 g ha^{-1}; recommended field rate: 375 g ha^{-1}) using the method of Busi *et al.*[27] and is referred to as 'R1'. Two additional populations from near Tammin, Western Australia (31° 38′ S, 117° 29′ E), identified as putatively resistant to cinmethylin in a 2021 herbicide resistance screening (R Busi, unpublished) and designated as 'R2' and 'R3', were used in the current study without further selection. Wheat cv. Mace was included in the study as a cinmethylin-tolerant control. Although the R1, R2 and R3 populations used in this study have not been officially confirmed as cinmethylin-resistant,[28] the term 'resistant' will be used hereafter to denote reduced herbicide sensitivity.

2.2 Dose–response assays

To quantify the relative level of cinmethylin resistance of each population, dose–response assays in potting mix and on cinmethylin-containing agar were performed. Seeds of each population (25 seeds per population per treatment) were sown onto the surface of moist potting mix (50% washed river sand, 25% peat moss, 25% composted pine bark) in seedling trays (30 cm × 20 cm × 5 cm) and sprayed with formulated cinmethylin (Luximax, 750 g cinmethylin L^{-1}; BASF, Melbourne, Australia) at rates of 25, 50, 125, 250, 375 or 500 g cinmethylin ha^{-1} using a custom-built cabinet sprayer equipped with dual Teejet XR11001 flat-fan nozzles delivering 106 L water ha^{-1} at 210 kPa.[6] Seeds were covered with a 1 cm layer of fresh potting mix immediately after spraying, watered well, and placed on benches in a naturally-lit glasshouse at the University of Western Australia. Unsprayed controls were included to calculate percent seedling emergence from the treated potting mix. The mean temperature in the glasshouse during the experiment (performed in June, 2021) was 17 °C, with a day length of approximately 10 h. Trays were kept moist throughout the experiment and the number of healthy (two-leaf) seedlings was counted at 28 days after spraying and compared to the untreated controls. There were three replicates of each treatment for each population.

For the agar-based experiment, seeds were sown on 0.6% (*w/v*) agar containing increasing concentrations of formulated cinmethylin in 90-mm diameter Petri dishes. Annual ryegrass populations were sown on agar with concentrations of 0, 0.5, 1, 5, 10, 20, 40, 60 or 120 nmol L^{-1} cinmethylin, whereas wheat was sown on agar with 0, 1, 10, 40, 60, 120, 240, 480 or 960 nmol L^{-1} cinmethylin. Dishes were sealed with Parafilm, placed in sealed plastic bags containing moist paper towel and incubated at 25 °C/15 °C day/night with a 12 h photoperiod of cool white light-emitting diode (LED) light (90 µmol m^{-2} s^{-1}). After 7 days, the coleoptile and radicle lengths of the seedlings were measured to the nearest millimetre using a ruler. There were three replicates of each cinmethylin concentration with ten seeds per replicate. Two independent experiments were performed and the data were pooled.

2.3 Pharmacological assays

The effect of phorate on seedling growth in the presence of cinmethylin was assessed by incubating seedlings on agar containing a cinmethylin concentration, determined from the earlier dose–response study, which inhibited coleoptile elongation by approximately 50% (20 nmol L^{-1} for annual ryegrass, 240 nmol L^{-1} for wheat) in the presence or absence of phorate. Based on preliminary studies to determine the concentration of phorate that did not greatly affect seedling growth when applied on its own, 1 mg phorate (applied as 10 mg of Thimet 100G granules; Amgrow, Sydney, Australia) per dish was used. There were four

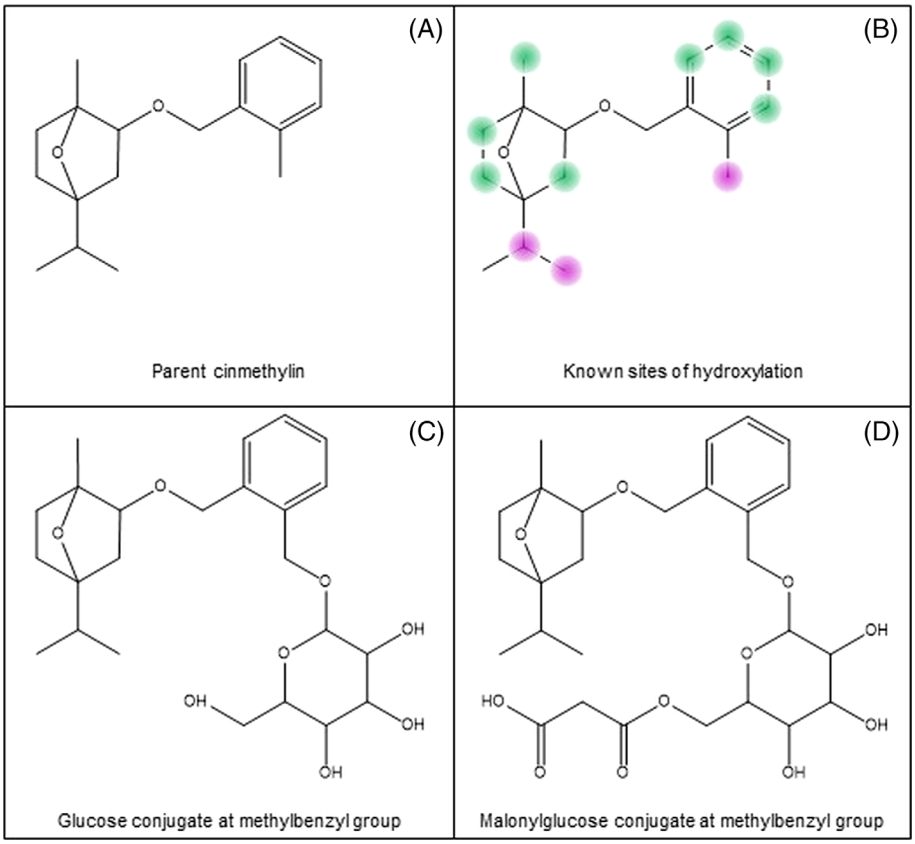

Figure 1. Structure of the parent cinmethylin molecule (A), the known sites of hydroxylation identified in wheat (green shading) and both wheat and animals (pink shading) (B),[22–24] and the major cinmethylin metabolites identified in mature wheat plants (C, D).[24]

treatments in total: controls containing neither cinmethylin nor phorate; cinmethylin alone; phorate alone; and cinmethylin plus phorate. There were three replicates of each treatment, with ten seeds per population per treatment. Seedling growth was measured after 7 days as described earlier.

2.4 Analysis of cinmethylin metabolism

Seeds were germinated in the dark on agar containing a sublethal concentration (7.5 nmol L^{-1}) of formulated cinmethylin and incubated at 25 °C/15 °C for 3 days (wheat; 16 seedlings per extraction) or 5 days (annual ryegrass; 70 seedlings per extraction). A 1 µL droplet of carbon-14 (^{14}C)-labelled cinmethylin (phenyl ^{14}C [U]-cinmethylin; 2.3 GBq $mmol^{-1}$; BASF, Frankfurt, Germany) representing 180 Bq and 43 µmol L^{-1} cinmethylin, was applied to the coleoptile base of each seedling and in some cases 100 mg Thimet 100G granules (10 mg phorate) were distributed over the surface of the agar immediately after [^{14}C]-cinmethylin application. A higher amount of phorate was used in the metabolism studies than in the pharmacological studies due to the shorter incubation time of the former, and the need to ensure that sufficient phorate had diffused into the agar. Seedlings were incubated at 25 °C/15 °C for a further 24 or 72 h. All subsequent steps were performed at 4 °C unless otherwise indicated. Whole seedlings were removed from the agar and homogenized in 2–3 mL ice-cold 100% methanol. The homogenate was filtered through pre-wetted Miracloth and the residue re-extracted in 0.5–1 mL water, filtered, and combined with the first filtrate. This was clarified by centrifugation at 12 000 × *g* for 3 min and used for analysis of [^{14}C]-labelled cinmethylin metabolites. To account for any potential non-enzymatic modification of cinmethylin during the extraction process, untreated seedlings were spiked with [^{14}C]-labelled cinmethylin immediately before homogenization and included in the earlier extraction process.

Preliminary studies showed that drying of methanol/water extracts in a rotary vacuum concentrator (RVC 2-25 CDplus; Martin Christ, Osterode, Germany) prior to high-performance liquid chromatography (HPLC) caused almost total loss of parent [^{14}C]-cinmethylin, due to the volatility of this compound. Therefore, in order to quantify the relative amount of parent cinmethylin in each extract, methanol/water extracts were partitioned three times against an equal volume of hexane,[7] and aliquots of the unconcentrated organic and aqueous fractions were subjected to liquid scintillation counting using a Packard TriCarb 1500 Liquid Scintillation Analyzer and Ultima Gold scintillation cocktail (Perkin-Elmer, Melbourne, Australia). The remainder of the hexane fractions were rapidly concentrated under a stream of air and qualitatively analysed by HPLC to confirm that parent cinmethylin was the only [^{14}C]-labelled compound partitioning into the hexane phase. The aqueous fractions were concentrated to 100–400 µL in the rotary vacuum concentrator and used for HPLC. There were three independent replicates of each treatment for each population.

Aqueous fractions from wheat (16 seedlings) and pooled annual ryegrass populations R2 and R3 (100 seedlings; 50 of each population) were also dissolved in 0.1 mol L^{-1} sodium acetate (pH 5.2) and digested overnight at 37 °C with 7.5 U β-glucosidase from almond (Sigma-Aldrich, Sydney, Australia). Standard [^{14}C]-cinmethylin was also included, as was wheat extract incubated

in the absence of β-glucosidase. Following incubation, reactions were adjusted to pH 3 with hydrochloric acid (HCl) and partitioned three times against chloroform.[22] Both the organic and aqueous fractions were concentrated as described earlier and analysed by HPLC.

Partitioned plant extracts were separated by reversed phase HPLC performed with a 600E dual-head pump, 717 plus autosampler and 2998 photodiode array detector (Waters, Milford, MA, USA) coupled to a β-RAM model 2B radioactive detector (IN/US Systems Inc., Pine Brook, NJ, USA). Separation was achieved using a 250 mm × 4.6 mm Apollo C_{18}, 5 µm particle column with a 7.5 mm × 4.6 mm, 5 µm guard column (Grace Davison Discovery Sciences, Deerfield, IL, USA) with a mobile phase flow rate of 1.0 mL min^{-1} at 40 °C. Samples in the autosampler were kept at 15 °C. Where possible, injection volumes were adjusted to provide similar total radioactivity loading in all samples.

The mobile phase consisted of eluent A [0.1% (*v/v*) formic acid in nanopure water] and eluent B [acetonitrile with 0.1% (*v/v*) formic acid]. Elution was achieved with a gradient of 10% eluent B for 5 min, then a linear change to 100% eluent B over 15 min, subsequently held at 100% eluent B for 5 min before an immediate change back to the starting condition of 10% eluent B to re-equilibrate the column for 13 min prior to the next injection.

2.5 Additional translocation experiments on wheat

Wheat seedlings were grown and treated as described earlier for the metabolism experiments. For visualization of ^{14}C translocation following application of [^{14}C]-cinmethylin to the coleoptile base, seedlings were briefly rinsed in 50% (*v/v*) methanol containing 0.1% (*v/v*) Tween 20, pressed between pieces of paper towel, and dried at 60 °C for 3 days before being exposed to a BAS-IP MS2040 storage phosphor plate (GE Healthcare, Sydney, Australia) for 24 h. Plates were scanned using an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare, Chicago, IL, USA). Samples of eight to ten seedlings were collected at 2, 8, 24 and 72 h after application of [^{14}C]-cinmethylin in the presence or absence of phorate. To quantify translocation and assess cinmethylin metabolism in different seedling tissues, seedlings (72 h post-treatment, plus or minus phorate) were separated into coleoptiles (minus the basal 5 mm), coleoptile bases (including a thin layer of seed tissue), seeds (uncontaminated by coleoptile-base tissue) and radicles, and extracted in methanol and water in the same way as for analysis of metabolites. The proportion of recovered ^{14}C in each seedling part was measured in aliquots of the methanol/water extracts by liquid scintillation counting. The remainder of each extract was partitioned three times against hexane, and the organic and aqueous fractions were counted separately. There were four seedlings per replicate, with four replicates per treatment. The aqueous fractions were dried in the rotary vacuum concentrator and used for radio-HPLC, but it was necessary to pool the replicates before injection in order to obtain a reliable signal from the β-RAM. The same translocation/metabolism experiment was not performed with annual ryegrass due to its very narrow coleoptiles and the difficulty in ensuring that none of the [^{14}C]-cinmethylin droplet applied to the coleoptile bases trickled down to the seeds and radicles.

2.6 Data analysis

Dose–response data were fitted to a three-parameter log-logistic model using the drc package in R[29,30] to estimate the cinmethylin rate or concentration causing a 50% decrease in survival (pot experiment) or coleoptile and radicle elongation (agar experiment) (ED_{50}):

$$y = \frac{d}{1+\exp(b(\log x - \log e))}$$

where d is the upper limit of survival or length of coleoptile or radicle (expressed as a percentage of the untreated control, i.e. 100%), b is the slope of the curve, x is the concentration of cinmethylin, and e is the cinmethylin concentration at which survival or coleoptile and radicle growth was inhibited by 50% (the ED_{50}). Resistance index (RI) is the ratio of ED_{50} values of the resistant *versus* susceptible populations.

Differences in seedling growth between samples treated with single doses of cinmethylin, phorate or both were analysed using Welch's *t*-test in a pairwise comparison of samples. Potential synergistic interactions between cinmethylin and phorate were assessed using a modified Colby analysis as described by Flint *et al.*[31]:

$$I_{ij} = \log(T_{ij}) - \log(A_{i0}) - \log(B_{0j}) + \log(AB_{00})$$

where I_{ij} is the expected interaction between chemicals, T_{ij} is the observed growth when the chemicals are applied together, A_{i0} is the growth when chemical A is applied alone, B_{0j} is the growth when chemical B is applied alone, and AB_{00} is the growth of the untreated control. If $I_{ij} = 0$, the interaction between the chemicals is additive; if $I_{ij} < 0$, synergistic; and if $I_{ij} > 0$, antagonistic.[30] The I_{ij} values were calculated for each replicate of each population, and Welch's *t*-test was used to determine if the mean I_{ij} was significantly different from zero.[32]

HPLC data were analysed by integrating the area under each radiolabelled peak detected by the β-RAM. Each peak was expressed as a percentage of the total peak area of the chromatogram, which represents the aqueous phase of the extract. The peak areas were then corrected to account for the proportion of parent cinmethylin in the total recovered radioactivity from the sample. For example, a metabolite peak representing 40% of the total aqueous chromatogram area in a sample with 60% of the ^{14}C signal in the aqueous phase would correspond to 24% of the total recovered radioactivity. Relative metabolite abundances in pairs of samples were compared using Welch's *t*-test. The correlation between the ED_{50} of each population and the relative abundance of each ^{14}C-labelled metabolite was determined using weighted least squares regression in R.[33]

3 RESULTS

3.1 Cinmethylin sensitivity levels and interaction with phorate

The cinmethylin dose causing 50% mortality in soil-grown seedlings (ED_{50}) was three- to eight-fold higher in annual ryegrass populations R1, R2 and R3 compared to population S, but this was not statistically significant (Table 1). The ED_{50} values of all ryegrass populations were far lower than the recommended application rate of 375 g ha^{-1}, whereas that of wheat was > 400 g ha^{-1} (Table 1).

In all populations, the cinmethylin concentration required to inhibit seedling growth on agar by 50% (ED_{50}) was an order of magnitude higher in the coleoptiles than in the radicles (Table 1). On the basis of coleoptile elongation, annual ryegrass populations R2 and R3 were significantly less sensitive to

Table 1. Cinmethylin dose–response analyses of seedling survival in potting mix and coleoptile and radicle growth on agar

Survival	ED_{50} (g ha^{-1})	RI	*P*-Value
S	7.9 ± 5.4	1.0	n/a
R1	26.5 ± 12.2	3.4 ± 2.7	0.393
R2	57.1 ± 33.1	7.2 ± 6.4	0.335
R3	63.1 ± 35.8	8.0 ± 7.0	0.324
Wheat	422 ± 80	53 ± 37	<0.001
Coleoptiles	ED_{50} (nmol L^{-1})	RI	*P*-Value
S	11.8 ± 2.0	1.0	n/a
R1	15.9 ± 3.4	1.4 ± 0.4	0.340
R2	31.7 ± 3.7	2.7 ± 0.6	0.003
R3	37.5 ± 4.5	3.2 ± 0.7	0.001
Wheat	221 ± 17	18 ± 2	<0.001
Radicles	ED_{50} (nmol L^{-1})	RI	*P*-Value
S	1.1 ± 0.3	1.0	n/a
R1	2.0 ± 0.6	1.7 ± 0.7	0.305
R2	4.1 ± 1.5	3.7 ± 1.7	0.118
R3	5.0 ± 1.2	4.5 ± 1.7	0.043
Wheat	13.6 ± 1.6	12.6 ± 1.9	<0.001

The cinmethylin rate or concentration resulting in 50% inhibition of seedling survival or growth (ED_{50}) was calculated for each annual ryegrass population (S, R1, R2 and R3) as well as for wheat. The resistance index (RI) represents the ratio of ED_{50} values between each resistant population and the S population. Values ± standard error are shown ($n = 3$; data for the agar experiment were pooled from two independent experiments). Not applicable (n/a).

cinmethylin than population S (approximately three-fold), whilst population R1 was not different to S (Table 1). On a radicle elongation basis, only population R3 had a significantly higher ED_{50} than the S population. Wheat seedlings had a higher overall level of tolerance to cinmethylin on agar, being > 13-fold more tolerant than annual ryegrass population S (Table 1).

Phorate alone had little or no effect on seedling growth (Fig. 2). In combination with cinmethylin, phorate generally caused a greater decrease in coleoptile elongation than did cinmethylin alone, but it did not enhance the inhibitory effects of cinmethylin on radicle elongation (Fig. 2). A Colby analysis indicated that the interaction between cinmethylin and phorate was synergistic in the coleoptiles of all populations except S, where it was antagonistic, and additive in the radicles of all populations except R2, where it was synergistic (Fig. 2).

3.2 Metabolism of [^{14}C]-labelled cinmethylin in annual ryegrass and wheat seedlings

In extracts of untreated seedlings spiked with labelled cinmethylin, there were no [^{14}C]-labelled peaks with a retention time different to that of parent cinmethylin (28.5 min), indicating no non-enzymatic modification of cinmethylin during the extraction process (data not shown). Chromatograms of [^{14}C]-labelled compounds in the organic (hexane) phase of treated-seedling extracts revealed a single peak with a retention time of 28.5 min, the same as standard [^{14}C]-labelled cinmethylin (Fig. 3 (A,B)), confirming that no metabolites partitioned into the organic phase. The aqueous phase of seedling extracts revealed four major peaks at average retention times of 17.8 min (designated metabolite 1), 18.8 min (metabolite 2), 19.5 min (metabolite 3) and 21.2 min (metabolite 4), with a representative chromatogram shown in Fig. 3(C). Treatment of the seedlings with phorate caused changes in the relative peak area of each metabolite, but did not affect the retention times, and thus, by inference, the identity of the metabolites (Fig. 3(D)). Quantification of the area under each peak in the aqueous phase, accounting also for the relative abundance of parent cinmethylin measured in the organic phase of seedling extracts prior to concentration, revealed that there were very few changes between the 24 and 72 h seedling incubation times. Therefore, the data for both time points were pooled.

Metabolite 1 showed little to no difference in relative abundance between populations or phorate treatments, except that it was around three-fold lower in wheat (3% of total recovered ^{14}C) compared to annual ryegrass (9%) (Fig. 4(A,F)). Metabolite 2 also showed little response to phorate, but was present at around 15% of total recovered ^{14}C in both annual ryegrass and wheat (Fig. 4(B,F)). Metabolite 3 was of similar abundance to metabolite 2, but was significantly (three-fold) decreased by phorate treatment in annual ryegrass (Fig. 4(C,F)). In the absence of phorate, metabolite 4 was the most abundant aqueous metabolite in both annual ryegrass and wheat, although it comprised a significantly higher proportion of total recovered ^{14}C in wheat (44%) compared to annual ryegrass (27% averaged across all populations) (Fig. 4(F)). Among annual ryegrass populations, metabolite 4 was > 1.5-fold higher in populations R2 and R3 compared to S and R1 (Fig. 4(D)). Phorate caused a dramatic decrease in metabolite 4 abundance in all annual ryegrass populations (75% on average) and a significant but smaller decrease in wheat (40%) (Fig. 4(F)). Parent cinmethylin was four-fold higher in annual ryegrass than in wheat (33% of total recovered ^{14}C *versus* 8%) in the absence of phorate, but the relative abundance in annual ryegrass population R3 was almost half of that in population S (Fig. 4 (E,F)). Application of phorate resulted in a significant increase in parent cinmethylin abundance to around 60% of total recovered ^{14}C in all annual ryegrass populations, and a smaller increase (to around 30%) in wheat (Fig. 4(E,F)). On average, the cinmethylin metabolite profile of wheat treated with phorate was very similar to that of annual ryegrass in the absence of phorate (Fig. 4(F)).

Regression of relative metabolite abundance against population ED_{50} (as a measure of resistance) revealed that with respect to seedling survival in soil and coleoptile elongation on agar, there were positive correlations between cinmethylin resistance and metabolites 3 and 4, and negative correlations between resistance and both metabolite 1 and parent cinmethylin (Table 2). The results were very similar when the ED_{50} for radicle elongation was used as the measure of resistance, except that the positive correlation with metabolite 3 abundance just missed statistical significance (Table 2).

β-Glucosidase digestion of aqueous fractions from both annual ryegrass and wheat gave inconclusive results, as most of the ^{14}C remained in the aqueous phase following digestion and eluted from the HPLC column at retention times corresponding to metabolites 1, 2, 3 and/or 4 (Supporting Information Fig. S1). However, the low proportion of ^{14}C (< 25%) in the post-digestion organic phase was more polar than parent cinmethylin (eluting at 20.8 min rather than 28.5 min), and could potentially represent a hydroxylated aglycone[24] (Fig. S1).

3.3 Translocation of [^{14}C]-cinmethylin in wheat seedlings

Phosphor images of [^{14}C]-cinmethylin-treated wheat seedlings showed that between 2 and 72 h post-treatment, the ^{14}C signal spread from the point of application at the coleoptile base to

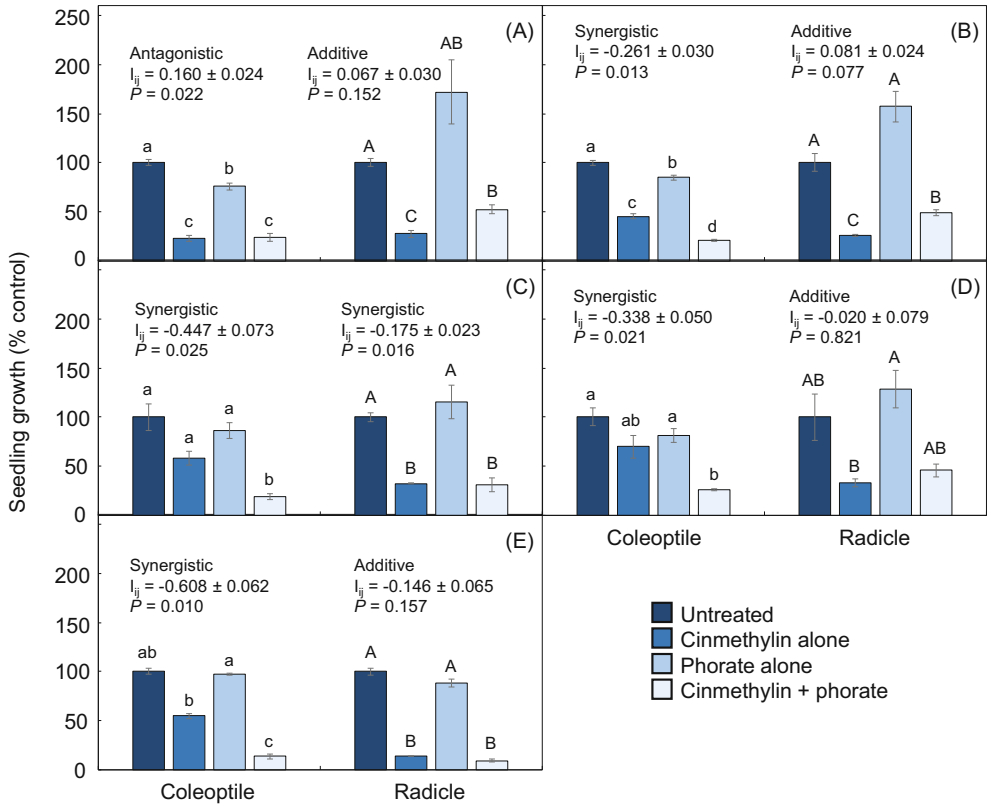

Figure 2. Interaction between cinmethylin and phorate. Seeds of annual ryegrass populations S (A), R1 (B), R2 (C), R3 (D) and of wheat (E) were germinated on agar containing cinmethylin alone, phorate alone or a combination of the two, and coleoptile and radicle lengths were recorded after 7 days of growth. Values are means ± standard error ($n = 3$); different letters above bars indicate significant ($P < 0.05$) differences between means within each tissue type (lower-case letters for coleoptiles, upper-case for radicles). The results of the Colby analysis for coleoptiles and radicles are given above the bars (I_{ij}, the expected interaction between cinmethylin and phorate).

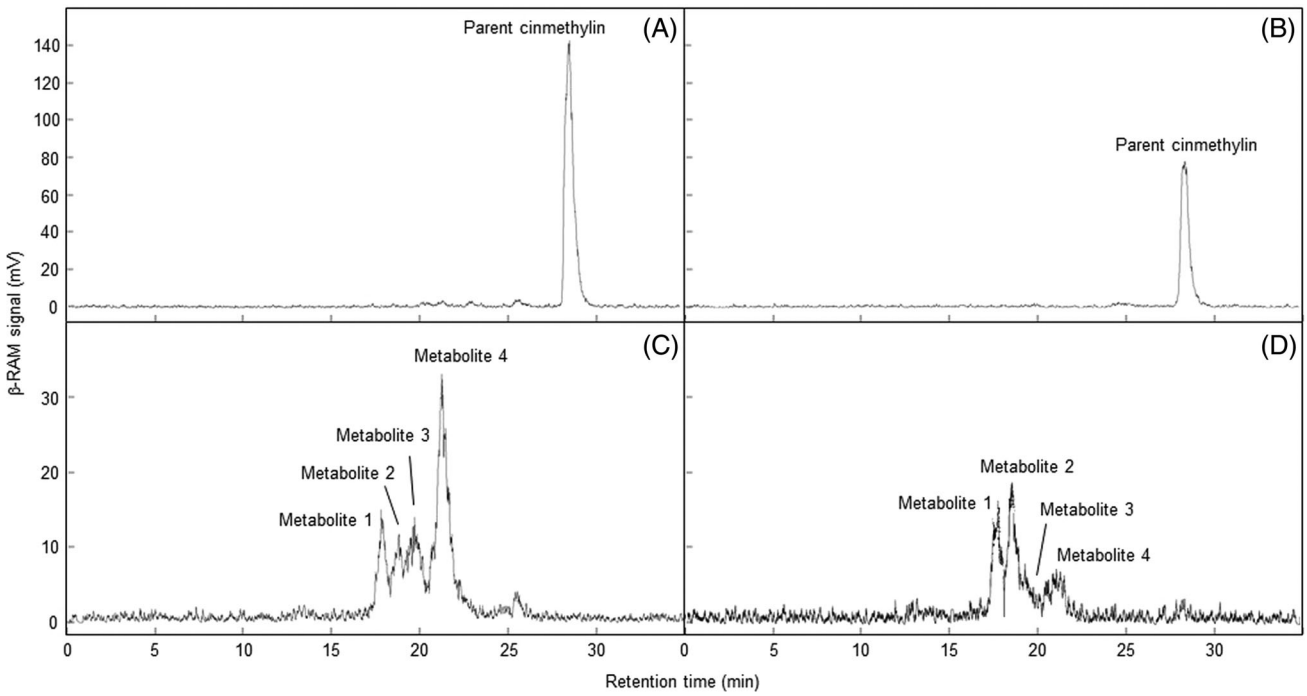

Figure 3. Representative chromatograms of [^{14}C]-labelled cinmethylin and its metabolites. (A) Standard [^{14}C]-cinmethylin; (B) the hexane fraction of an extract from annual ryegrass seedlings treated with [^{14}C]-cinmethylin; (C,D) the aqueous fraction of extracts from annual ryegrass seedlings treated with [^{14}C]-cinmethylin in the absence and presence, respectively, of phorate. Peaks represent the [^{14}C]-labelled compounds present in each extract as detected by the β-RAM.

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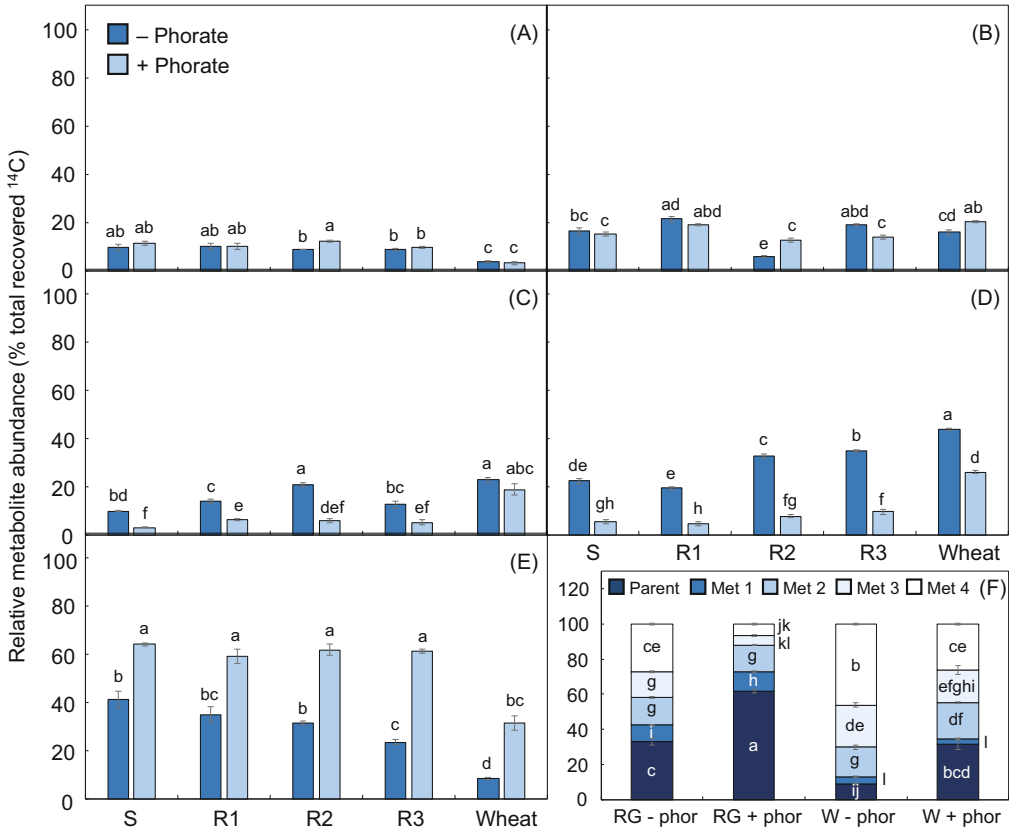

Figure 4. Relative abundance of metabolite 1 (A), metabolite 2 (B), metabolite 3 (C), and metabolite 4 (D) and parent cinmethylin (E) in seedlings of wheat and annual ryegrass populations S, R1, R2 and R3 treated with [^{14}C]-labelled cinmethylin in the presence or absence of phorate. Stacked columns comprising all [^{14}C]-labelled metabolites in wheat (W) and pooled annual ryegrass populations (RG) in the presence or absence of phorate (phor) are shown in (F). Seedlings were incubated for 24 or 72 h and the data pooled. Values are means ± standard error ($n = 3$); different letters above or within bars denote significant ($P < 0.05$) differences between means.

Table 2. Association between relative metabolite abundance and cinmethylin resistance level

	Survival ED_{50}		Coleoptile ED_{50}		Radicle ED_{50}	
	Adjusted R^2	*P*-Value	Adjusted R^2	*P*-Value	Adjusted R^2	*P*-Value
Parent cinmethylin	(−)0.925	0.006	(−)0.921	0.006	(−)0.942	0.004
Metabolite 1	(−)0.994	0.000	(−)0.994	0.000	(−)0.987	0.000
Metabolite 2	(−)0.332	0.956	(−)0.331	0.952	(−)0.333	0.989
Metabolite 3	0.747	0.038	0.752	0.036	0.681	0.054
Metabolite 4	0.822	0.022	0.823	0.021	0.806	0.025

Weighted least squares regression was performed on the abundance of each carbon-14 (^{14}C)-labelled metabolite against the effective dose causing 50% mortality (ED_{50}) values for seedling survival in the potting mix experiment, and coleoptile and radicle elongation in the agar experiment. A negative sign preceding the R^2 value indicates a negative correlation.

the seed, the remainder of the coleoptile, and finally the radicles (Fig. 5(A–D)). Phorate did not affect the overall distribution of ^{14}C, with most of the ^{14}C signal being detected in the seeds and coleoptile bases in both the presence and absence of phorate (Figs S2 and 5 (E)). In the absence of phorate, almost all (≥ 85%) of the ^{14}C recovered from each tissue type partitioned into the aqueous phase, with metabolites 3 and 4 being most abundant in the coleoptile bases and seeds, metabolite 2 most abundant in the seeds and metabolite 1 predominant in the radicles (Fig. 5(E)). Phorate caused a statistically significant increase in the proportion of parent cinmethylin in each tissue, but the effect was most dramatic in the seeds, so that 22% of the total ^{14}C recovered from phorate-treated seedlings consisted of seed-localized parent cinmethylin, compared to 6% in seeds of untreated seedlings (Fig. 5(E)). The relative proportion of metabolite 4 was decreased by around two-fold in all phorate-treated tissues, whereas changes in metabolites 1, 2 and 3 were small or negligible, except that metabolite 1 was present in phorate-treated but not phorate-untreated seeds (Fig. 5(E)).

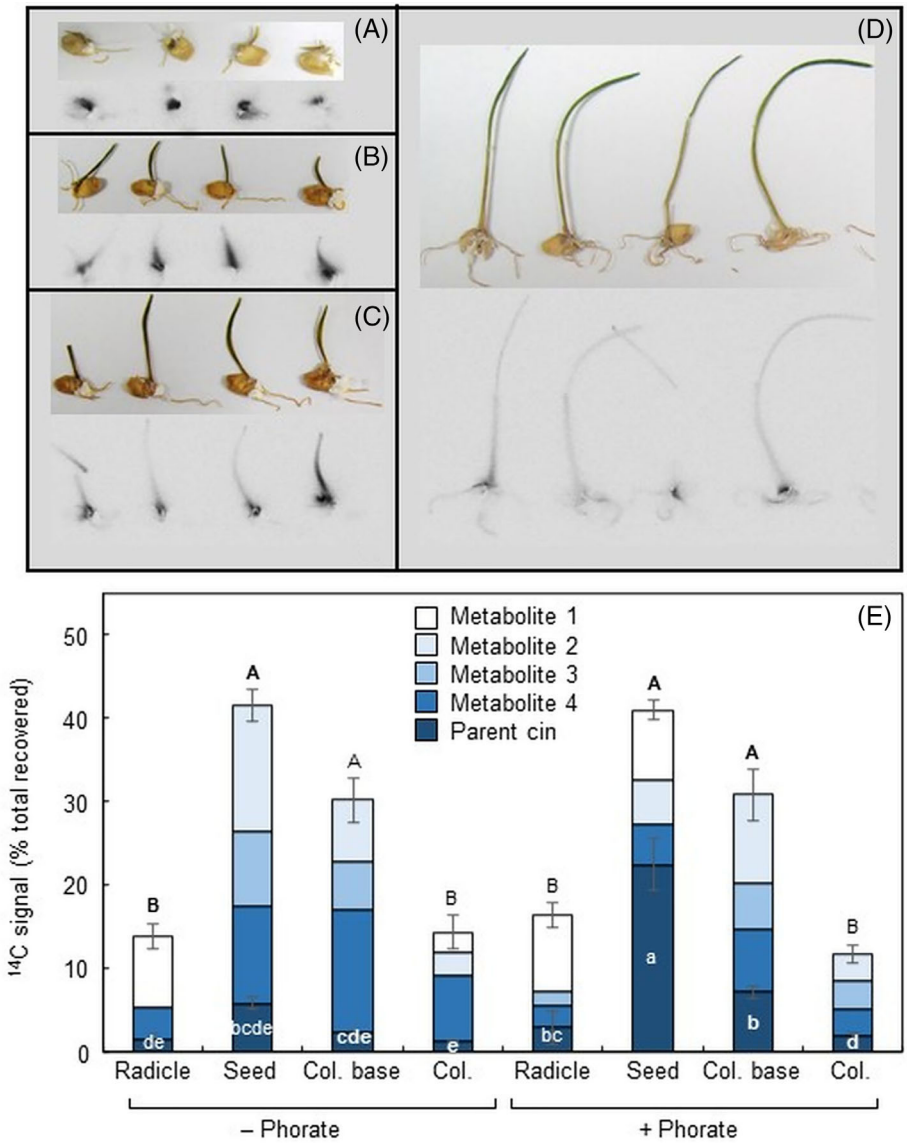

Figure 5. Distribution of [^{14}C]-labelled compounds in wheat seedlings after application of [^{14}C]-cinmethylin to the coleoptile base. Treated seedlings were exposed to storage phosphor plates to trace the movement of ^{14}C at (A) 2 h, (B) 8 h, (C) 24 h or (D) 72 h after application (top panel in A–D: seedling photograph; bottom panel: phosphor image). Separated parts of seedlings incubated for 72 h after [^{14}C]-cinmethylin application, in the presence or absence of phorate, were extracted and partitioned against hexane in order to quantify the extent of cinmethylin metabolism in each seedling part; the aqueous metabolites 1–4 were separated and quantified by HPLC (E). Standard error bars at the top of each column refer to the proportion of total ^{14}C recovered from each seedling part, and different uppercase letters signify significant differences ($P < 0.05$) between means ($n = 4$). Standard error bars on the parent cinmethylin segments refer to the organic-phase ^{14}C (parent cinmethylin) recovered from each seedling part, and different lowercase letters signify significant differences ($P < 0.05$) between means ($n = 4$). The replicate aqueous extracts used for scintillation counting had to be pooled for HPLC, so no statistical analysis on the individual aqueous metabolites could be performed.

4 DISCUSSION

Although cinmethylin resistance is not yet a visible problem in Australian cropping fields, the presence of two populations with a slightly enhanced ability (compared to a fully susceptible population) to metabolize cinmethylin, as identified in the current study, provides a warning that a potential pathway for development of resistance already exists in annual ryegrass. This species is well known for its ability to rapidly evolve resistance under recurrent herbicide selection.[34–36] As an obligate outcrosser, there are high levels of standing genetic variation within populations that allows them to rapidly adapt to environmental stresses, especially when the stress is repeated.[2] Metabolic resistance to different herbicide chemistries can evolve in outcrossing species by accumulation of different resistance genes over generations,[37] and/or through promiscuity of certain xenobiotic-metabolizing enzymes.[38] Clearly, careful stewardship of cinmethylin is vital to minimize the evolution of metabolic resistance in field populations of annual ryegrass.

There is little published information on the fate of cinmethylin in plants, although the major metabolic pathway in crop species (wheat, canola, carrot, soybean, peanut) begins with hydroxylation at various positions followed by conjugation to glucose and malonyl glucose.[24,25] In the current study, four major [^{14}C]-labelled metabolites were detected in extracts from both wheat

and annual ryegrass plants treated with [^{14}C]-cinmethylin. These metabolites have not yet been identified, and so their relative phytotoxicities to annual ryegrass are unknown. The high abundance (production) of metabolite 4 is positively correlated with cinmethylin resistance level; this metabolite likely represents one of the major metabolites identified in mature wheat plants, i.e. the glucose or malonylglucose conjugate of the 2-methylbenzyl-hydroxylated derivative of cinmethylin[24] (see Fig. 1), despite the low efficiency of β-glucosidase digestion observed in the current study. Similarly, the negative correlation of metabolite 1 abundance with cinmethylin resistance (and its near-absence in cinmethylin-tolerant wheat) could indicate that this metabolite retains phytotoxicity. The identification and laboratory synthesis of metabolites 1–4, and their use in bioassays to quantify the level of phytotoxicity of each, is required to fully understand the pathway of cinmethylin detoxification in young annual ryegrass and wheat seedlings. Ideally, a greater number of cinmethylin-resistant populations (once they are discovered or artificially selected) would also need to be assessed to confirm whether the observed association between metabolite 4 abundance and cinmethylin resistance holds across a larger sample size.

The involvement of cytochrome P450(s) is inferred in the conversion of cinmethylin to metabolite 4 and in phenotypic resistance to this herbicide, because the known plant P450 inhibitor phorate dramatically lowered the ability of wheat and annual ryegrass seedlings to grow on agar in the presence of cinmethylin as well as to produce metabolite 4. Because phorate was much more effective in inhibiting cinmethylin metabolism in annual ryegrass compared to wheat, the putative P450(s) involved in cinmethylin metabolism in annual ryegrass could be more sensitive to phorate, and/or wheat may possess a greater number of P450s with the ability to metabolize cinmethylin, some of which are insensitive to phorate. In both species, however, radicle elongation was far more sensitive to cinmethylin than was coleoptile growth, and cinmethylin was not synergized by phorate in the radicles, suggesting that the coleoptiles are more efficient metabolizers of cinmethylin (see later).

Although the main focus of this study was to detect cinmethylin metabolism in resistant annual ryegrass, it has also highlighted the need for further understanding of how cinmethylin translocates and is metabolized in very young seedlings. Based on its uptake and translocation in mature soybean roots, Hsu *et al.*[39] proposed that the highly hydrophobic cinmethylin molecule (log K_{ow} calculated as 4.6), would be partitioned into the root tissues as it translocated through the xylem, and thus take longer to reach the shoot than would a more hydrophilic herbicide. With its pre-emergence application timing, however, cinmethylin is expected to come into contact with all tissues of just-germinated seedlings, and it is reported to be absorbed by both the roots and shoots (and seeds to a lesser extent) via diffusion.[40] In the current study, a droplet of [^{14}C]-labelled cinmethylin applied to the coleoptile base of 3-day-old wheat seedlings resulted in a large proportion of ^{14}C being detected in the seed, probably indicating diffusion through the symplast of the coleoptile base and into the seed tissue,[39] followed by slower diffusion into the coleoptiles and radicles. The different metabolite profile and phorate sensitivity in each tissue suggests that parent cinmethylin is the translocated compound and it is metabolized in each tissue. This supposition is supported by comparing the seeds, which showed greatly decreased production of metabolites 2, 3 and 4 and increased production of metabolite 1 in the presence of phorate, with the radicles that showed only a minimal phorate response. The difference in metabolite profile between the radicles (mainly producing metabolite 1) and the coleoptiles and bases (mainly producing metabolite 4) may also help to explain the greater cinmethylin tolerance of coleoptiles compared to radicles.

In summary, the potential of annual ryegrass to evolve P450-based metabolic resistance to cinmethylin has been highlighted by the greater ability of two populations with low-level resistance to produce the putatively low-toxicity metabolite 4, in a phorate-inhibitable manner. The relative ability of different tissues to translocate and metabolize cinmethylin in both annual ryegrass and wheat seedlings needs further investigation, as this could have a bearing on the depth and timing of crop sowing (currently recommended as ≥ 3 cm and within 3 days of herbicide application for Luximax in Australia). A full understanding of how cinmethylin works and how resistance develops is important for maximizing the efficacy of cinmethylin within an integrated weed management system.

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CONFLICT OF INTEREST

Aimone Porri is an employee of BASF Germany, which manufactures and sells cinmethylin products.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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