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Bixlozone Metabolism in Crop and Weed Species: A Basis for Selectivity and Evolved Resistance

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ABSTRACT: Bixlozone, a proherbicide that requires in planta activation to its phytotoxic form, 5-ketobixlozone, is used to selectively control annual ryegrass (*Lolium rigidum*) in Australian cereal and canola crops. Bixlozone resistance has been detected in southern Australian annual ryegrass populations, and this can be increased with recurrent selection. The metabolic fate of bixlozone in young weed and crop seedlings was investigated by liquid chromatography−mass spectrometry to determine if differential metabolism can explain differences in bixlozone response. The observed tolerance of canola and wild radish (*Raphanus raphanistrum*) was due to the reduced activation of bixlozone to 5-ketobixlozone. In contrast, a resistant annual ryegrass population and tolerant wheat and barley showed preferential conversion of bixlozone to hydroxylated derivatives, whereas susceptible annual ryegrass populations produced more 5-ketobixlozone. Direct application of 5-ketobixlozone to seedlings resulted in an unexpected conversion to hydroxylated metabolites, potentially implicating plant reductases in 5-ketobixlozone metabolism.

KEYWORDS: *annual ryegrass (Lolium rigidum), cytochrome P450, herbicide metabolism, isoxazolidinones, reductases, wild radish (Raphanus raphanistrum)*

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INTRODUCTION

The 2021 introduction of the isoxazolidinone herbicide bixlozone (2-[(2,4-dichlorophenyl)methyl]-4,4-dimethyl-1,2-oxazolidin-3-one) to the suite of pre-emergence herbicides available for weed control in Australian cropping has provided another option for growers confronting increasing herbicide resistance in annual ryegrass (*Lolium rigidum*) populations.[1] Bixlozone and the closely related molecule clomazone (introduced in Australia in 1999) are the only members of the 1-deoxy-d-xylulose-5-phosphate synthase (DXS)-inhibiting group of herbicides, which inhibit isoprenoid production at an early stage of the methylerythritol phosphate pathway.[2] This leads to characteristic plant bleaching as a result of the lack of isoprenoid precursors for the synthesis of the phytol tail of chlorophyll, UV-protective carotenoids, and antioxidant tocopherols.[2] Studies on clomazone have shown that it is taken up by the roots of seedlings and transported in the xylem to the shoots and leaves,[3] and it is also absorbed by cultured cells derived from leaf material.[4] Differential uptake has been proposed to be a basis of selectivity between some tolerant and sensitive species,[3] but in other cases, tolerant or resistant plants take up as much, or more, clomazone than their sensitive counterparts.[4−6]

So far, there is little information on whether the differential sensitivity of DXS contributes to clomazone (or bixlozone) sensitivity among plant species and cultivars, and no cases of resistance due to target-site DXS mutations have yet been identified in weeds.[7] The main determining factor of differential tolerance and evolved resistance, therefore, appears to be herbicide metabolism. Clomazone is a pro-herbicide that needs to be bioactivated in planta to its phytotoxic form, 5-ketoclomazone, via 5-hydroxyclomazone (note that neither parent clomazone nor 5-hydroxyclomazone can inhibit DXS).[2] Therefore, differences in clomazone efficacy can potentially be due to a decreased capacity for bioactivation to 5-ketoclomazone, and/or increased ability to divert clomazone and 5-hydroxyclomazone away from 5-ketoclomazone and toward low-toxicity hydroxylated and conjugated metabolites. The difference in clomazone response between tolerant rice and sensitive *Echinochloa oryzoides*, a weed of rice crops, is thought to be due to the greater bioactivation of clomazone in the latter.[8] In contrast, the evolved clomazone resistance observed in populations of *E. phyllopogon* and *Leptochloa fusca* ssp. *fasicularis*, also weeds of rice fields, is due to enhanced metabolism of parent clomazone to hydroxylated derivatives, particularly hydroxymethylclomazone.[6,9]

The oxidative reactions involved in the bioactivation of clomazone are likely to be catalyzed by cytochrome P450 monooxygenases (P450s). P450 inhibitors such as phorate, disulfoton, and 1-aminobenzo-triazole (ABT) can antagonize clomazone in crop species and in susceptible and resistant *E. phyllopogon* by preventing oxidative metabolism of clomazone to its phytotoxic metabolites.[5,10] In a more recent study, *Arabidopsis thaliana* plants transformed with the P450 gene *CYP709C9* from *E. phyllopogon* were shown to be more sensitive to clomazone, indicating that this P450 is likely to activate clomazone to 5-ketoclomazone.[11] On the detoxifica-

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tion side, the P450 inhibitor malathion greatly increased the sensitivity of resistant *L. fusca* and *Alopecurus aequalis* plants to clomazone[6] and bixlozone,[12] respectively, implying P450 involvement in the formation of low-toxicity hydroxylated derivatives. When treated directly with 5-ketoclomazone, only the clomazone-susceptible, and not the resistant, *E. phyllopogon* population responded to disulfoton (which antagonized 5-ketoclomazone) and ABT (which greatly enhanced 5-ketoclomazone toxicity).[5] It was hypothesized that susceptible plants may synthesize further toxic compounds from 5-ketoclomazone in P450-mediated reactions, while resistant plants may employ 5-ketoclomazone detoxification pathways that do not involve P450 activity.[5] However, recent genetic studies have identified that the *E. phyllopogon* P450 isoform CYP81A15 preferentially acts on 5-ketoclomazone,[13] conferring high-level resistance in transgenic *Arabidopsis thaliana*,[14] while CYP81A12, A21 and A24 can metabolize parent clomazone.[13]

Given that bixlozone has only recently been introduced, reported cases of evolved resistance in weeds are rare.[12,15] However, recurrent selection studies have demonstrated that three populations of South Australian annual ryegrass with cross-resistance to various fatty acid biosynthesis inhibitors not only possessed resistance to bixlozone, but the level of resistance increased with each round of selection.[16] The mechanism of resistance was unlikely to be reduced activation of bixlozone, as phorate antagonized bixlozone in both the susceptible and resistant populations.[16] Although a study on the fate of bixlozone in food products has identified the residues present in mature wheat grains and some fruits,[17] there is no published information on differential bixlozone metabolism in young seedlings of crop species or susceptible and resistant weeds. Therefore, this study aimed to determine the basis of bixlozone resistance in annual ryegrass and to compare the metabolic fate of bixlozone in young seedlings of the major crop and weed species of southwestern Australia.

MATERIALS AND METHODS

Plant Materials. Two populations of annual ryegrass (*Lolium rigidum* Gaud.), VLR1 and AFLR2, hereafter termed S1 and S2, respectively, were used as bixlozone-susceptible controls. A herbicide-resistant annual ryegrass population collected from South Bodallin, Western Australia (31.516°S, 118.839°E) which was included in a 2019 screening of annual ryegrass responses to bixlozone (before the release of bixlozone in Australia), was identified as potentially having resistance to bixlozone (R Busi, unpublished). This was used as the germplasm for recurrent selection with bixlozone, using the methods of Busi et al.[18] Briefly, 50 seeds were sown onto the surface of moist, low organic matter potting mix (15% composted pine bark, 5% peat moss, 80% washed river sand) and sprayed with the recommended rate (500 g ai ha^{-1}) of Overwatch (400 g bixlozone L^{-1}; FMC, Sydney, Australia) using a custom-built cabinet sprayer equipped with dual TeeJet XR11001 flat-fan nozzles (Spraying Systems Co., Wheaton, IL, USA) delivering herbicide solutions in 106 L ha^{-1} at 200 kPa and a boom speed of 3.6 km h^{-1}.[19] Seeds were immediately covered with a 5 mm fresh potting mix and watered well. Seedlings were maintained in a naturally lit glasshouse (temperature range: 20−25 °C) and were fertilized with commercial soluble fertilizer (Diamond Red, 27% N, 5.7% P, 10.9% K plus trace elements: Campbells Fertilizers, Laverton North, Australia) once a week. Surviving plants (those that showed minimal bleaching and produced healthy new leaves) were transplanted into a high organic matter potting mix and grown to maturity. The cohort of surviving plants was allowed to cross-pollinate while other pollen was excluded, with seeds collected at the end of the growing season, in November. The population resulting from three rounds of selection with bixlozone, named pr6-P3, was used in the current study and also in a separate study on prosulfocarb and trifluralin metabolism.[20] Commonly used cultivars of the three major crop species for which bixlozone is registered in Australia (wheat *cv* Mace, barley *cv* Maximus CL and canola *cv* Hyola 410XX), as well as the major dicot weed of Western Australian agriculture [wild radish (*Raphanus raphanistrum* L.), represented by herbicide-susceptible population WARR7], were also included in the study. Anecdotally, wheat and canola are more tolerant to bixlozone than barley. There have so far been no cases of bixlozone resistance reported in wild radish, but it should be noted that the label for Overwatch herbicide (www.fmccrop.com.au) states that wild radish is only suppressed by bixlozone, not fully controlled. For simplicity, the seven plant samples used in this study will hereafter be referred to as "populations", and natural crop tolerance and evolved weed resistance to bixlozone will come under the blanket term of "resistance".

Synthesis of 5-Ketobixlozone. 2-[(2,4-Dichlorophenyl)-methyl]-4,4-dimethyl-3,5-isoxazolidinedione (5-ketobixlozone) was synthesized by Epichem (Perth, Australia) using the method for the synthesis of 5-ketoclomazone[21] from the oxime of 2-chlorobenzaldehyde,[22] except that 2,4-dichlorobenzaldehyde was used as the starting material in order to obtain 5-ketobixlozone. The final compound had a purity of 93% and a molecular mass of 288.13 g mol^{-1} (confirmed by LC-MS), and its structure was confirmed by ^{1}H NMR.

Quantification of Resistance to Bixlozone and 5-Ketobixlozone. Dose−response experiments using bixlozone and 5-ketobixlozone incorporated into agar were used to quantify resistance levels. Seeds of each population were sown on 0.6% (w/v) agar (25 mL agar in 70 mL capacity round plastic containers of 60 mm diameter) containing formulated bixlozone or 5-ketobixlozone at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, or 20 μM, as well as 40 μM for 5-ketobixlozone only, and incubated under controlled conditions (25/15 °C day/night with a 12 h photoperiod of white LED light at a fluence rate of 200 μmol m^{-2} s^{-1}) for 10 d. The shoot tissue was then harvested, weighed, and used to extract chlorophyll in 80% (v/v) acetone/water according to the method of Arnon.[23] Shoot chlorophyll concentration was used to quantify the bixlozone resistance level because bleaching is a characteristic symptom of bixlozone action and takes place much earlier than seedling growth inhibition. Clarified extracts were diluted 1:1 with 80% acetone and the absorbance at 663 and 647 nm recorded; chlorophyll concentration was calculated using the formula of Porra and Scheer.[24] There were three replicates of each herbicide concentration for each population with 10 seedlings per replicate for the annual ryegrass populations and five seedlings per replicate for the other species.

To assess the effect of phorate on the response to bixlozone and 5-ketobixlozone, dose−response experiments were also performed in the presence of 1 mg of phorate (applied to the surface of the agar as 10 mg of formulated Thimet 100G granules: AgNova Technologies, Brisbane, Australia) for populations S1, pr6-P3, wheat, and barley. Single-dose experiments in the presence or absence of 1 mg of phorate were done for populations S2 (0.1 μM bixlozone, 2.5 μM 5-ketobixlozone), canola (20 μM bixlozone, 1 μM 5-ketobixlozone) and wild radish (10 μM bixlozone, 1 μM 5-ketobixlozone), using discriminating herbicide concentrations determined from the minus-phorate dose−response assays described above. Seedlings in the single-dose experiment were incubated and extracted as for the dose−response study, with three replicates of each treatment.

Analysis of Bixlozone and 5-Ketobixlozone Metabolites by LC−MS. As bixlozone is used as a pre-emergence herbicide which is taken up by both the root and shoot tissue of germinating seedlings, the metabolism of bixlozone and 5-ketobixlozone in the radicles (plus seeds for the Poaceae populations) was analyzed separately to that in the shoots (coleoptiles plus enclosed leaf tissue for the Poaceae; hypocotyls, cotyledons and shoot apical meristems for the Brassicaceae) to determine if the amount and type of bixlozone metabolites in the shoot could potentially be affected by its metabolism in the root tissue. Seeds were sown on top of herbicide-containing agar and allowed to imbibe and germinate in

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Table 1. Peaks with m/z Values and Fragmentation Patterns Corresponding to Bixlozone and Its Metabolites Detected in Extracts of Bixlozone- or 5-Ketobixlozone-Treated Seedlings Analyzed by High-Resolution LC-MS/MS and Selected Reaction Monitoring[a]

peak name	retention time (min)	precursor ion $[M + H]^+$ m/z	product ion(s) $[M + H]^+$ m/z	tentative identity
bixlozone	9.98	274.0395	158.9763	bixlozone (coelutes with standard)
KB	10.30	288.0188	158.9763	5-ketobixlozone closed ring (coelutes with standard)
KBO	8.03	306.0293	158.9763	5-ketobixlozone open ring (coelutes with standard)
KM1	7.27	468.0823	306.0293	glucosylated 5-ketobixlozone open ring
			158.9763	
HM1	6.48	452.0873	290.0345	glucosylated hydroxymethylbixlozone
			158.9763	
HM2	7.12	290.0345	158.9763	5-hydroxybixlozone with unknown conjugate
HM3	7.17	452.0873	290.0345	glucosylated 5-hydroxybixlozone
			158.9763	
HM4	7.45	290.0345	158.9763	5-hydroxybixlozone with unknown conjugate
HM5	8.40	290.0345	158.9763	5-hydroxybixlozone with unknown conjugate
HM6	8.53	290.0345	158.9763	5-hydroxybixlozone aglycone
DM1	5.47	468.0823	306.0293	glucosylated 5-hydroxy, aryl-hydroxybixlozone
			174.9712	
DM2	5.78	468.0823	306.0293	glucosylated hydroxymethyl, aryl-hydroxybixlozone
			174.9712	
DM3	7.65	306.0293	190.9669	aryl-dihydroxybixlozone

[a]The precursor ion is either the mass based on the presence of a glucose conjugate (for metabolites KM1, HM1, HM3, DM1, and DM2), or the unconjugated form of the metabolite (for the standards and metabolites HM2, HM4-6, and DM3). The product ions are those formed via fragmentation of the precursor ion in MS/MS. Tentative identities were assigned based on m/z values of precursor and product ions, relative retention times as compared with those in Yasuor et al.,[9] and the presence or absence of peaks in 5-ketobixlozone-treated seedlings (see Figure 2).

the presence or absence of 1 mg of phorate, as described above for the dose−response study. Concentrations of 2.5 μM bixlozone and 10 μM 5-ketobixlozone were used for all populations. Ten-day-old seedlings were separated into belowground parts (hereafter referred to generically as radicles + seeds) and shoots, and the fresh tissues were immediately weighed and extracted using a method based on that of Yasuor et al.[9] Tissues were homogenized in 2.5 vol extraction solvent (water:acetonitrile:isopropanol, 4:3:1) using a mortar and pestle, and the extracts were centrifuged at 12,000 *g* for 5 min. The pellets were re-extracted in another 2.5 vol solvent and centrifuged, and the two supernatants were combined. Supernatant volume was recorded to obtain an exact tissue:solvent ratio (approximately 200 mg tissue mL^{-1} solvent). Matrix blanks, using untreated tissue samples, were also produced for each population germinated in the presence and absence of phorate. For all treatments and matrix blanks, there were three replicates of 50 (annual ryegrass populations) or 10 (wheat, barley, canola, and wild radish) seedlings.

LC-MS was performed on a Vanquish UHPLC system coupled to an Orbitrap Exploris 120 high-resolution mass spectrometer using a heated electrospray ionization source (Thermo Fisher Scientific, Waltham, MA). The system was controlled by using Xcalibur 4.7 software (Thermo Scientific) for method setup and data acquisition. Extracts (1−5 μL), held in the autosampler at 10 °C, were injected onto a Hypersil Gold C18 column (1.9 μm, 100 × 2.1 mm id) (Thermo Fisher Scientific, Melbourne, Australia) and separated using a mobile phase of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile containing 0.1% (v/v) formic acid (solvent B) at a flow rate of 0.3 mL min^{-1} with a column temperature of 25 °C. Gradient elution was performed with an initial 5% solvent B held for 1 min, then a linear gradient of 5−95% solvent B over 11 min, and then held at 95% solvent B for 4 min. The column was re-equilibrated at 5% solvent B for 6 min before the next injection.

The mass spectrometer was operated in positive ionization mode, with a capillary voltage of 3500 V, sheath gas flow rate of 50 (arb); auxiliary gas flow rate of 10 (arb); sweep gas flow rate of 1 (arb); ion transfer tube temperature 325 °C; and vaporizer temperature 350 °C. The MS analysis was performed in full scan with data-dependent MS2 (ddMS2) mode over a mass range of m/z 100−1000, with an Orbitrap resolution of 60,000 full width at half-maximum (FWHM) and radio frequency lens of 70%. The automatic gain control (AGC) was set to 200% with a maximum ion injection time of 100 ms. For ddMS2 scans at 15,000 FWMH resolution using dynamic exclusion (4 s), four ddMS2 scans were performed with stepped collision energies of 10, 35, and 80% with UHP nitrogen as the collision gas. High-resolution mass measurements were made with Pierce FlexMix (Thermo Fisher Scientific) as the external calibration solution while EASY-IC was used for internal calibration. Both HP and UHP nitrogen were generated from a Castore XL iQ 70 nitrogen generator (LNI Swissgas, Torreglia, Italy).

For selected reaction monitoring (SRM) experiments, potential glucose conjugates of hydroxylated bixlozone metabolites and 5-ketobixlozone (closed and open rings) were targeted. The mass list included the expected $[M + H]^+$ for all three potential conjugates with stepped collision energies as per the ddMS2 experiments above. The Q1 resolution was 2 m/z and SRM scans were conducted in an unscheduled manner looking for transitions to both 158.9763 m/z ($C_7H_5Cl_2$) and 174.9712 m/z ($C_7H_5OCl_2$). The $[M + H]^+$ for the $^{35}Cl^{37}Cl$ isotope with transitions to 160.9733 m/z ($C_7H_5{}^{35}Cl^{37}Cl$) and 176.9683 m/z ($C_7H_5O^{35}Cl^{37}Cl$) was also included in the mass list.

Data Analysis. Using the drc package in R,[25,26] dose−response data were fitted to a three-parameter log−logistic model with the following equation:

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

where y is the chlorophyll concentration as a percentage of the untreated control, d is the upper limit of relative chlorophyll concentration, b is the slope of the curve, x is the concentration of bixlozone or 5-ketobixlozone, and e is the herbicide concentration at which chlorophyll was at 50% of control levels (ED_{50}). The resistance index was calculated as the ratio of resistant:susceptible ED_{50} values. Differences between treatments in the single-dose experiments were assessed using Welch's *t*-test in Excel.

Raw mass spectral data were analyzed using FreeStyle 1.8 SP2 QF1 (v 1.8.65.0) (Thermo Fisher Scientific, Waltham, MA, USA). Extracted ion chromatograms were produced for the expected metabolites of bixlozone, based on previous studies on clomazone

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Table 2. Effect of Bixlozone and Ketobixlozone on Shoot Chlorophyll Concentration in Seedlings Grown on Herbicide-Containing Agar in the Presence and Absence of Phorate[c]

(A) dose−response in the absence of phorate

population	bix ED_{50} (μM)[a]	RI (against S1)[b]	keto ED_{50} (μM)[a]	RI (against S1)[b]	ratio keto:bix[b]
S1	0.08 ± 0.01 f		0.41 ± 0.16 cd		5.3 ± 2.2***
S2	0.17 ± 0.03 e	2.2 ± 0.5*	2.91 ± 0.36 b	7.1 ± 2.8*	16.9 ± 3.4***
pr6-P3	1.13 ± 0.17 d	14.5 ± 3.4***	39.4 ± 8.3 a	96.1 ± 41.6*	34.9 ± 9.0***
wheat	2.55 ± 0.18 c	32.8 ± 6.4***	34.3 ± 6.4 a	83.7 ± 35.4***	13.5 ± 2.7***
barley	1.29 ± 0.14 d	16.6 ± 3.5***	34.2 ± 7.3 a	83.4 ± 36.2*	26.5 ± 6.3***
canola	11.60 ± 1.26 a	149 ± 31***	1.22 ± 0.16 c	3.0 ± 1.2 ns	0.1 ± 0.0***
wild radish	6.51 ± 0.59 b	83.7 ± 16.9***	0.43 ± 0.03 d	1.1 ± 0.4 ns	0.1 ± 0.0***

(B) dose−response in the presence of phorate

population	Bix + P ED_{50} (μM)	ratio bix + P:bix-P[b]	Keto + P ED_{50} (μM)	ratio keto-P:keto + P[b]
S1	1.99 ± 0.49	25.65 ± 9.90 ***	0.22 ± 0.07	1.83 ± 0.90 ns
pr6-P3	15.25 ± 4.77	13.50 ± 4.40 ***	23.13 ± 5.43	1.70 ± 0.40 ns
wheat	3.54 ± 0.68	1.39 ± 0.29 ns	7.42 ± 2.10	4.62 ± 1.97 ns
barley	1.93 ± 0.20	1.50 ± 0.19 ***	2.75 ± 0.29	12.43 ± 13.26 ns

(C) chlorophyll concentration (% control) in the presence or absence of phorate

population	Bix − P	Bix + P[b]	Keto − P	Keto + P[b]
S2	67 ± 5	77 ± 8 ns	55 ± 2	64 ± 7 ns
canola	29 ± 5	36 ± 5 ns	59 ± 2	49 ± 4 ns
wild radish	32 ± 7	52 ± 13 ns	22 ± 1	15 ± 1*

[a]Significant ($P < 0.05$) differences among population ED_{50} values are denoted by different letters down columns. [b]Resistance indices and differences between bixlozone and 5-ketobixlozone treatments (A) or differences between phorate treatments (B,C) are indicated as ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. [c](A) Herbicide concentrations causing a 50% decrease in chlorophyll (ED_{50}) were estimated from dose-response curves, and the resistance index (RI) was calculated as the ratio of ED_{50} values of each population and S1. For each population, the ratio of ED_{50} values for ketobixlozone and bixlozone (keto:bix) was also calculated. (B) Results from dose-response experiments in the presence of phorate, performed at the same time as the minus-phorate experiments in (A), on populations S1, pr6-P3, wheat, and barley. The ratio of ED_{50} values for each herbicide in the presence (+P) or absence (-P) of phorate was calculated to quantify the effect of phorate. (C) Response of shoot chlorophyll concentration to a single discriminating dose of bixlozone and 5-ketobixlozone applied to populations S2 (0.1 and 2.5 μM), canola (20 and 1 μM) and wild radish (10 and 1 μM) in the presence or absence of phorate.

metabolism in plants.[8,9,27] Where possible, ddMS2 spectra were used to distinguish between compounds with the same mass but different structures, giving product ions of different masses upon fragmentation (Table 1). Peaks were integrated manually and their spectra were inspected to ensure that the accurate m/z and deduced chemical formula of the chromatographed compound matched that of bixlozone or its metabolites and that the signal-to-noise ratio of the mass spectrum was >3. Further confirmation was obtained by the isotopic signature of the two chlorine atoms present in bixlozone and all metabolite molecules, giving signals at $[M + H]^+ + 2$ for the $^{35}Cl^{37}Cl$ compound and, for the more intense peaks, also at $[M + H]^+ + 4$ for the $^{37}Cl_2$ compound. It was not possible to obtain standards for the likely bixlozone metabolites, and therefore retention times of their unconjugated forms could not be confirmed absolutely. Peak areas were normalized by tissue:solvent ratio and injection volume, and the areas of coeluting nontarget compounds from the matrix blanks were subtracted from those of the treated samples. Peaks that were present in only one replicate of each sample were excluded from analysis, as were peaks that were <10% of the area of the major peak (excluding parent bixlozone) in the sample. To correct for potential differences in recovery from different tissue types and plant species, all metabolite abundances were expressed as a percentage of the total metabolite peak area. Pairwise comparisons between relative peak areas were performed using Welch's t-test in Excel.

Relationships between shoot chlorophyll concentration (representing bixlozone resistance status) and 5-ketobixlozone production in the radicles + seeds or shoots of 2.5 μM bixlozone-treated seedlings were assessed using weighted least-squares regression (WLS) in R.[26,28]

■ RESULTS

Quantification of Resistance to Bixlozone and 5-Ketobixlozone. The concentrations of bixlozone and 5-ketobixlozone required to decrease the shoot chlorophyll concentration by 50% (ED_{50}) in the absence of phorate were calculated for each population. All populations had a significantly higher ED_{50} than S1, with the resistance index ranging from 2 (S2) to 149 (canola) and the Brassicaceae being significantly more resistant than the Poaceae (Table 2A). In contrast, the two Brassicaceae populations were equally as susceptible to 5-ketobixlozone as population S1, while the other Poaceae populations had resistance indices of 7 (S2) to 96 (pr6-P3) (Table 2A). All the Poaceae were 5- to 35-fold more tolerant to 5-ketobixlozone than to bixlozone, whereas the Brassicaceae were around 10-fold more tolerant to bixlozone than to 5-ketobixlozone (Table 2A).

The interaction between phorate and bixlozone or 5-ketobixlozone was assessed by measuring chlorophyll in the coleoptiles of seedlings germinated on each compound alone, or on herbicide plus phorate. In the dose−response study performed on populations S1, pr6-P3, wheat, and barley, phorate caused a significant (>10-fold; $P < 0.05$) increase in the ED_{50} of bixlozone in S1 and pr6-P3, but a less than 2-fold increase in wheat and barley (Table 2B). The single-dose study performed on S2, canola, and wild radish showed that phorate did not have a significant effect on the chlorophyll concentration of any bixlozone-treated population (Table 2C). The response to 5-ketobixlozone was unaffected by phorate in all populations except wild radish, where there was a slight decrease in chlorophyll concentration (1.4-fold; $P = 0.02$) in the presence of phorate (Table 2B,C).

Biotransformation of Bixlozone. Across all populations, high-resolution mass spectrometric analysis of metabolites

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produced by seedlings incubated with bixlozone or 5-ketobixlozone in the presence or absence of phorate revealed the presence of bixlozone (in bixlozone-treated seedlings only and predominantly in the closed-ring form) and 5-ketobixlozone (predominantly in the open-ring form) along with several metabolites (Table 1). Hereafter, closed-ring 5-ketobixlozone will be referred to as KB, and the open-ring form as KBO.

In most seedlings treated with bixlozone, a very high proportion of the total metabolite peak area comprised unmodified parent bixlozone (Figure 1A). The shoots of the Poaceae showed a significantly ($P < 0.05$) lower proportion of parent bixlozone (average, 77%) than the radicles + seeds (98%) in the absence of phorate (Figure 1A). In contrast, biotransformation of parent bixlozone was minimal in both tissues of the Brassicaceae, comprising around 98% and 99% of total metabolites in the shoots and radicles + seeds, respectively (Figure 1A). Treatment with phorate inhibited bixlozone biotransformation in Poaceae shoots so that 99% of the total metabolite peak was in the form of parent bixlozone (Figure 1A). Among Poaceae populations, S2 had the lowest proportion of parent bixlozone in its shoots in the absence of phorate (<40%), followed by S1, pr6-P3, wheat (all ∼70%) and barley (88%) (Figure 1A).

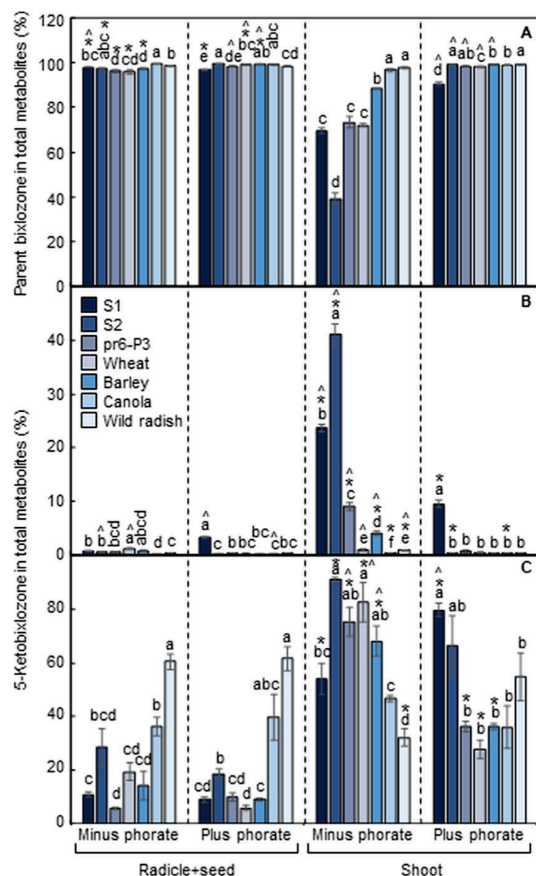

Figure 1. Proportion of recovered metabolites present as (A) parent bixlozone or (B) 5-ketobixlozone in extracts of radicles + seeds and shoots of seedlings incubated on bixlozone in the presence or absence of phorate, and (C) 5-ketobixlozone in seedlings incubated on 5-ketobixlozone in the presence or absence of phorate. Values are means ± SE (n = 3). Within each tissue type and phorate treatment, different letters above bars denote significant ($P < 0.05$) differences among populations. Asterisks (*) denote a significant difference between radicles + seeds and shoots within a population and phorate treatment. Carets (^) denote a significant difference between phorate treatments within a population and tissue type.

Abundance of 5-Ketobixlozone and Its Relationship with Resistance. The LC-MS peaks corresponding to KB (retention time 10.3 min, m/z 288.0188) and KBO (retention time 8.03 min, m/z 306.0293) were summed and expressed as a percentage of the total metabolite peak area in order to compare populations, treatments, and tissues. In the radicles + seeds of bixlozone-treated seedlings in the absence of phorate, the proportion of KB + KBO was <1.5% in all populations (Figure 1B). In the shoots (the target tissue of bixlozone), the proportion of KB + KBO in the absence of phorate ranged from 0.4 to 40% across populations (Figure 1B). Susceptible populations S1 and S2 had a >3-fold ($P < 0.05$) higher proportion of KB + KBO than pr6-P3 and barley, which were in turn >4-fold higher than wheat, canola, and wild radish (Figure 1B). Phorate had variable but minimal effects on KB + KBO in the radicles + seeds of bixlozone-treated seedlings, but in the shoots, it decreased KB + KBO abundance by >2-fold ($P < 0.05$) in all populations except canola (Figure 1B).

In seedlings treated directly with 5-ketobixlozone, the proportion of KB + KBO was, as would be expected, much higher than in seedlings treated with bixlozone, so there were fewer differences among populations (Figure 1C). Phorate had no significant effect on KB + KBO in the radicles + seeds of any population but caused a 2-fold decrease in the shoots of pr6-P3, wheat, and barley (Figure 1C). A notable feature of the direct 5-ketobixlozone treatment was that although KB + KBO was the predominant metabolite in the shoot tissue of the Poaceae populations, comprising 50−90% of the total metabolites, it was present at much lower proportions ($P < 0.05$) in the radicles + seeds (5−30%) (Figure 1C). In contrast, KB + KBO made up on average 40−60% of total metabolites in both tissues of the Brassicaceae (Figure 1C).

Analysis of absolute normalized peak areas for KB + KBO gave similar results as the analysis of percentages described above, in terms of differences among populations, tissues, and treatments. Among bixlozone-treated populations, S1 had a significantly higher ($P < 0.05$; ∼9-fold) amount of KB + KBO in both tissue types than the other populations, while wheat had 3−81 times less KB + KBO in its shoots (Figure S1A). In the direct 5-ketobixlozone treatment, the Brassicaceae had significantly higher amounts of KB + KBO in both tissues than most of the Poaceae populations, and phorate had relatively little effect on the amount of KB + KBO in either tissue type (Figure S1B).

Weighted least-squares regression analysis was used to quantify the correlation between shoot chlorophyll concentration and relative KB + KBO abundance in seedlings treated with bixlozone in the absence of phorate. There were significant negative correlations between shoot chlorophyll concentration and the relative abundance of KB + KBO in both the radicles + seeds (adjusted r^2 = 0.152; P = 0.046) and the shoots (adjusted r^2 = 0.473; $P < 0.001$). The same relationships between chlorophyll and KB + KBO were observed using the absolute peak areas for KB + KBO reported in Figure S1A (for radicles + seeds and shoots, respectively, adjusted r^2 values were 0.602 and 0.199; P values were <0.001 and 0.025).

Tentative Identification of Bixlozone Metabolites. With the lack of availability of bixlozone metabolite standards

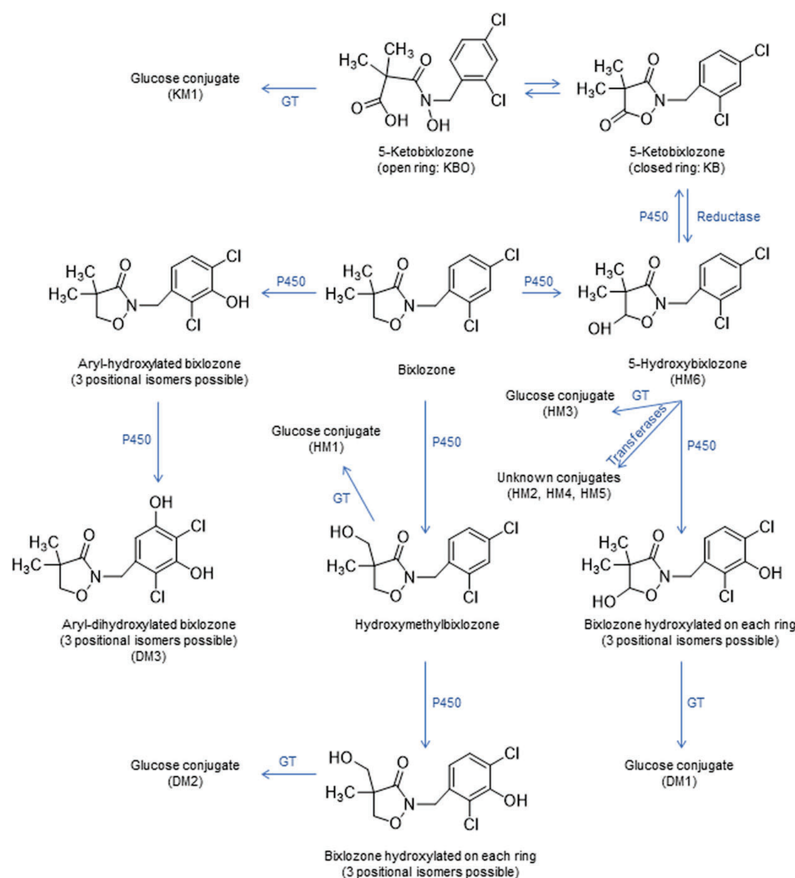

Figure 2. Proposed pathway of bixlozone metabolism in plants, based on the clomazone pathway of Yasuor et al.[9] and the LC-MS analysis of bixlozone and 5-ketobixlozone-treated plants. The tentative identities of the 10 quantified bixlozone metabolites (KM1, HM1-6, DM1-3: Table 1) are shown, along with the enzyme families that may be involved in their formation. GT, glucosyltransferase; P450, cytochrome P450 monooxygenase.

for the current study, it was not possible to assign metabolites based on coelution with specific standards. However, using the relative retention times of clomazone metabolites reported in Yasuor et al.,[9] the observed MS/MS transitions in the current study, and the presence of metabolites in bixlozone- vs 5-ketobixlozone-treated samples, tentative identifications of the 10 metabolites present at >10% of the area of the dominant metabolite peak were made (Table 1) and placed into a proposed scheme for the metabolism of bixlozone in Poaceae shoots (Figure 2).

As the only monohydroxylated bixlozone metabolite not detected in 5-ketobixlozone-treated seedlings and based on its precursor and product ions (m/z 452 → 290 → 159), HM1 can be tentatively identified as the glucose conjugate of hydroxymethylbixlozone. Metabolite DM2, which was also conjugated to glucose (m/z 468 → 306 → 175) and detected only in bixlozone-treated samples, could represent HM1 with additional hydroxylation on the aryl ring (Table 1 and Figure 2). Another dihydroxylated metabolite detected only in bixlozone-treated samples, DM3, had a transition corresponding to bixlozone dihydroxylated on the aryl ring (m/z 306 → 191) (Table 1).

The unexpected presence of HM2, HM3, and HM6 as the dominant metabolites in seedlings treated directly with 5-ketobixlozone indicates that these isoxazolidinone ring-hydroxylated compounds are likely to be derived from 5-ketobixlozone via reduction to 5-hydroxybixlozone (Figure 2).

The transition (m/z 290 → 159) and retention time (8.53 min) of metabolite HM6 suggest that it is the unconjugated form of 5-hydroxybixlozone, as it is less polar than open-ring ketobixlozone (retention time 8.03 min) (Table 1), a pattern which corresponds closely to that of 5-hydroxyclomazone (9.13 min) and open-ring 5-ketoclomazone (8.62 min).[9] With transitions of m/z 452 → 290 → 159 and a shorter retention time (7.17 min), HM3 is likely to be the glucose conjugate of HM6 (Table 1). DM1, also present in the 5-ketobixlozone treatment, could potentially be HM3 with an additional hydroxyl group on the aryl ring (m/z = 468 → 306 → 175). The precursor ions for HM2, HM4, or HM5 are unknown, but their shorter retention times compared to HM6 indicate that they are conjugated to polar moieties (Table 1).

A glucose conjugate of KBO (KM1: m/z 468 → 306 → 159) eluting at 7.25 min was also detected (Table 1), predominantly in the bixlozone plus phorate treatment (Figure 3B,D). Of the dihydroxylated metabolites, DM1 was only detected in wheat and barley, DM2 only in the shoots of bixlozone-treated pr6-P3 and wheat, and DM3 in pr6-P3, wheat, barley, and wild radish (Figure 3). Except for trace amounts of DM3 in barley radicles + seeds (Figure 3B), the three dihydroxylated metabolites were not detected in phorate-treated tissues.

Among the monohydroxylated metabolites, wheat was the only population to produce non-negligible levels of HM1, and indeed this was the predominant metabolite in bixlozone-

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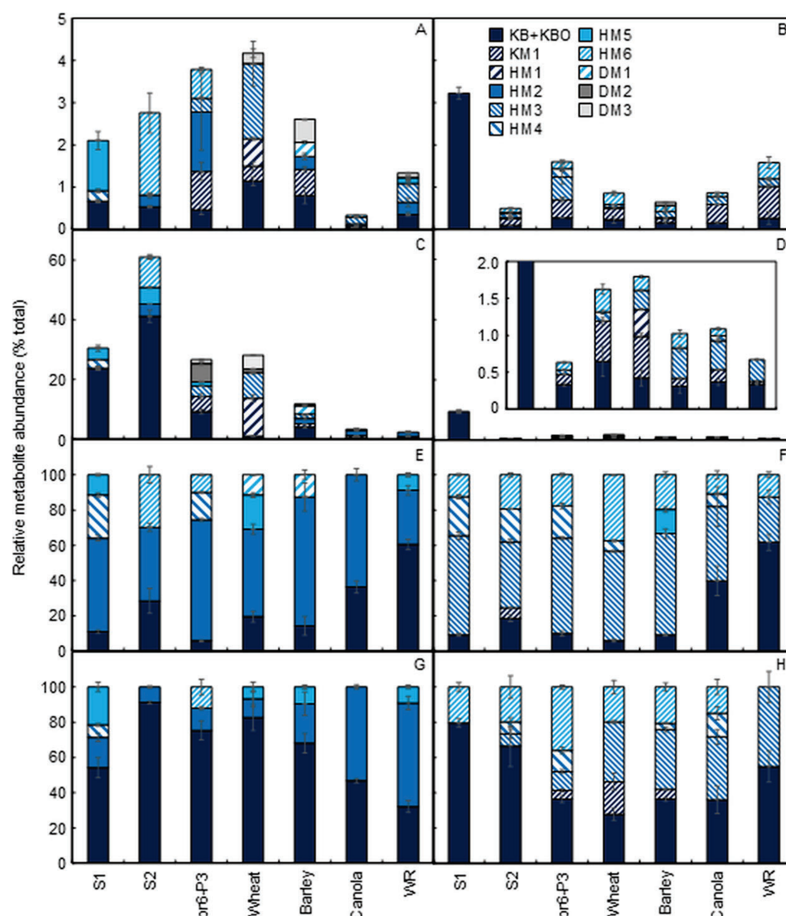

Figure 3. Relative abundance of bixlozone metabolites in radicles + seeds (A,B,E,F) and shoots (C,D,G,H) of seedlings treated with bixlozone (A–D) or 5-ketobixlozone (E–H) in the absence (A,C,E,G) or presence (B,D,F,H) of phorate. The inset in (D) shows the data with an expanded *y*-axis scale. Parent bixlozone is not shown. Values are means ± SE ($n = 3$). WR, wild radish.

treated wheat shoots in the absence of phorate (Figure 3A,C,D). S1 was the only population in which phorate completely abolished the production of detectable levels of monohydroxylated metabolites from applied bixlozone, although they were decreased by phorate in all populations (Figure 3B,D). In 5-ketobixlozone-treated seedlings, the predominant metabolite in the absence of phorate was HM2, and the radicles + seeds were overall more effective at metabolizing 5-ketobixlozone than the shoots (Figure 3E,G). In the presence of phorate, HM2 was effectively replaced by HM3 as the major metabolite of applied 5-ketobixlozone, and HM6 was also present in relatively high proportions (Figure 3F,H).

■ DISCUSSION

Herbicide metabolism-based mechanisms of crop selectivity and weed resistance are intrinsically complex, involving large superfamilies of enzymes that can catalyze a wide range of chemical transformations, rearrangements, and conjugations.[29,30] In the case of pro-herbicides that require bioactivation in planta, such as the isoxazolidinones (clomazone, bixlozone) and the thiocarbamates (e.g., prosulfocarb, triallate), an added layer of complexity comes from the fact that the activating enzymes may or may not belong to the same superfamily as the metabolizing enzymes and may have different expression levels, inhibitor sensitivity, and substrate specificity. Clomazone has been used for weed control since the 1990s, but it is only in the past few years that cytochrome P450 isoforms with proven ability to metabolize this herbicide have been identified.[11,13] Bixlozone, which differs from clomazone by the addition of a second chlorine atom and has different profiles of crop selectivity and efficacy on weeds,[31] has only recently been released as a herbicide and little is known about its metabolism in major Australian weed species. Therefore, an analysis of bixlozone responses and metabolism in crop and weed species commonly found in Australian agriculture was performed to identify the basis of crop tolerance and weed resistance.

Activation of Bixlozone to 5-Ketobixlozone Is Restricted in Canola and Wild Radish. The two Brassicaceae species studied, canola and wild radish, were highly resistant to parent bixlozone but very susceptible to direct application of its activation product, 5-ketobixlozone, suggesting that the bixlozone tolerance in these species is due to a reduced capacity for bioactivation. In accordance with this conclusion, LC-MS analysis showed that the Brassicaceae populations converted only negligible proportions of parent bixlozone to 5-ketobixlozone or other metabolites in both the shoots and the radicles + seeds.

Although directly applied 5-ketobixlozone was a highly potent inhibitor of chlorophyll accumulation in the Brassicaceae populations, it was unexpectedly less effective than parent bixlozone when applied to the Poaceae populations (suscep-

tible and resistant annual ryegrass, wheat, and barley). This can potentially be explained by the restricted uptake of 5-ketobixlozone by the Poaceae shoots and/or an inability of the radicles to transport this compound to the shoots via the xylem. Uptake and translocation were not investigated in the current study, but a previous study using the accumulation of phytoene in barley leaf segments to measure clomazone response also reported that applied 5-ketoclomazone was less potent than parent clomazone.[21] As 5-ketoclomazone was, however, very highly effective at inhibiting purified DXS, it was concluded that restricted uptake of 5-ketoclomazone was the reason for its lesser effect in intact barley tissue.[21]

Relative Production of 5-Ketobixlozone and Hydroxylated Bixlozone Metabolites in Poaceae Shoots Is Related to the Resistance Level. In contrast to the observed lack of bixlozone metabolism in the Brassicaceae populations, there were significant levels of bixlozone biotransformation observed in the shoots of all of the Poaceae populations studied. The inhibition of this biotransformation by phorate points to the involvement of oxygenases, most likely P450s, in the metabolism of bixlozone, as has previously been proposed for the metabolism of clomazone by grass weeds.[6,9,13] However, the biotransformation of bixlozone in the radicles and seeds of the Poaceae was negligible and thus minimally affected by phorate. This is in line with the previously observed inability of Poaceae radicles + seeds to efficiently convert prosulfocarb to its phytotoxic sulfoxide,[20] and suggests that the below-ground tissues lack the capacity to oxidatively activate pro-herbicides. Therefore, the ability of a pre-emergence pro-herbicide to translocate from the root to the shoot tissue, where it can be activated, is likely to be important for optimal herbicide performance.

Accumulation of phytotoxic 5-ketobixlozone in the shoots of bixlozone-treated seedlings was significantly higher in bixlozone-susceptible annual ryegrass populations S1 and S2 compared with the other Poaceae populations. On the other hand, the production of dihydroxylated bixlozone metabolites was largely restricted to tolerant wheat and barley and the resistant annual ryegrass population pr6-P3. This, along with the high proportion of the monohydroxylated metabolite HM1 measured in wheat shoots, is in accordance with previous studies on clomazone metabolism in grass weed populations, where hydroxylated metabolites were preferentially produced by the resistant populations, and 5-ketoclomazone by the susceptible populations.[6,9]

The relationship between relative amounts of tissue 5-ketobixlozone and resistance to directly applied 5-ketobixlozone was less clear. In spite of the higher resistance of pr6-P3, wheat, and barley compared to S1 and S2, there were comparatively minor differences in relative 5-ketobixlozone abundance or metabolite profile among the 5-ketobixlozone-treated Poaceae populations. Therefore, it is possible that the DXS enzyme in the resistant populations is less sensitive to 5-ketobixlozone than that in the susceptible populations, although this was not investigated in the current study.

Potential Pathways of Bixlozone Metabolism. Apart from the diversion of bixlozone to HM1 and dihydroxylated metabolites by wheat, barley, and pr6-P3 shoots, implicating cytochrome P450 involvement in bixlozone resistance, there was the intriguing presence of a range of other monohydroxylated metabolites in seedlings treated directly with 5-ketobixlozone, whose overall production (predominantly in the radicles + seeds) was essentially insensitive to phorate. The most likely candidate for a 5-ketobixlozone-derived, hydroxylated metabolite is its reduction product, 5-hydroxybixlozone, and this was supported by the MS/MS fragmentation patterns of the metabolites. A recent study of the metabolism of a noncommercial 4-hydroxyphenylpyruvate dioxygenase-inhibiting herbicide, syncarpic acid-3, demonstrated that the ketone group on carbon-1 is reduced to a hydroxyl group,[32] a reaction analogous to that proposed here for the reduction of 5-ketobixlozone to 5-hydroxybixlozone. Enzymes potentially capable of this reduction reaction are present in the aldo-keto reductase (AKR) superfamily, some members of which show broad substrate specificity toward both endogenous and exogenous compounds.[33] Although HM2−HM6 are all putatively derived from 5-ketobixlozone reduction to 5-hydroxybixlozone, the identities of their conjugates are unknown, except for HM3 (glucose) and HM6 (likely none). Given that HM3 essentially replaces the more-polar HM2 in the presence of a phorate, it is tempting to speculate that HM2 is derived from HM3 in a phorate-inhibitable conjugation reaction. There is limited information on the global effect of phorate on plant tissue, but a transcriptomic study on peanut leaves showed that a range of genes involved in cellular metabolism, stress response, and plant defense are either up- or down-regulated by phorate.[34] Early studies on clomazone metabolism also raised the possibility of glutathione conjugation,[8,35] but there is no recent literature describing glutathione-derived metabolites of clomazone or bixlozone.

Practical Implications. In summary, this study has demonstrated that crop selectivity of bixlozone can be due to either a lack of capacity for bixlozone activation to phytotoxic 5-ketobixlozone (canola) or enhanced production of low-toxicity hydroxylated metabolites (wheat and barley). Evolved bixlozone resistance in an annual ryegrass population recurrently selected with this herbicide mirrors the tolerance mechanism of the cereal crops, with greater conversion of bixlozone to (di)hydroxylated metabolites than in susceptible annual ryegrass. Therefore, repeated, intensive use of bixlozone on annual ryegrass populations should be avoided in order to slow the spread of metabolic resistance. There is a strong possibility that in the near future, bixlozone will be formulated as a postemergence herbicide for application to established annual ryegrass seedlings. Given the apparent restricted uptake of applied 5-ketobixlozone by Poaceae shoots, it would be worth investigating whether 5-ketobixlozone could also be formulated as a postemergence herbicide for control of dicot weeds (such as wild radish) in cereal crops. Similarly, if the putative 5-ketobixlozone reductase activity could be highly expressed in cereal shoots, with their existing high hydroxylating activity, the 5-hydroxybixlozone formed from 5-ketobixlozone reduction could be rapidly transformed to low-toxicity dihydroxylated metabolites, further increasing the selectivity of 5-ketobixlozone.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c00162.

Absolute amount of 5-ketobixlozone recovered from seedlings treated with bixlozone or 5-ketobixlozone in the presence or absence of phorate (PDF)

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Notes

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