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Original Article

Mechanistic basis for synergism of 2,4-D amine and metribuzin in *Avena sterilis*

Heping Han,[1] Gilmar José Picoli Jr.,[2] Haibin Guo,[3] Qin Yu[1,*] and Stephen Bruce Powles[1]

[1] *Australian Herbicide Resistance Initiative, School of Agriculture & Environment, University of Western Australia, Australia*
[2] *Bayer Crop Science, Brazil*
[3] *State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bio-Resources, South China Agricultural University, China*

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The combination of herbicides with different modes of action has been adopted not only to improve weed control but also to increase the environmental sustainability of plant-protection products. In this study, we showed a synergistic effect of the auxin herbicide 2,4-D amine with the PSII-inhibiting herbicide metribuzin to control the global grass weed wild oat (*Avena sterilis*) population and investigated the underlying mechanisms. Pretreatment with 2,4-D amine did not change the foliar absorption of metribuzin but did increase metribuzin translocation to the roots and new leaves, although enhancement of the metribuzin metabolism was also observed. Considering that the expression level of the target site *psbA* gene is significantly higher in leaves than in roots, increased metribuzin translocation to new leaves is likely the major cause of the observed synergism, even though enhanced metribuzin metabolism may offset the metribuzin efficacy. This is the first report on the synergistic mechanism between 2,4-D amine and metribuzin in weed control.

Keywords: 2,4-D amine, *Avena sterilis*, metabolism, metribuzin, translocation, weed control.

Introduction

Using two or more herbicides, *via* tank mixing or sequential application, is a frequent practice in weed control.[1)] Herbicide mixtures can lead to additive, synergistic, or antagonistic effects.[1,2)] Concurrent use of herbicides with synergistic or additive effects can reduce application frequency, slow the evolution of herbicide resistance, broaden the weed control spectrum and efficacy, and contribute to the environmental sustainability of herbicides.[1,2)] Since the auxin herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) entered commercial use in 1950, it has been widely used as a selective dicot-active herbicide.[3)] Frequently, 2,4-D is used in a mixture with a grass-active herbicide to provide one-pass control of both dicot and grass weed species.[4–6)] However, the antagonism of auxin herbicides (including 2,4-D) in mixtures with certain herbicides is often observed with grass weeds, including wild oats (*Avena* spp.), annual ryegrass (*Lolium rigidum*), and Johnson grass (*Sorghum halepense*).[2,7)] For example, the antagonism of mixtures of auxin herbicides with acetyl-CoA carboxylase (ACCase)-inhibiting herbicides (*e.g.*, diclofop-methyl, haloxyfop-methyl, or fenoxaprop-methyl), glyphosate, or paraquat has led to weed-control failure.[8–16)] Such antagonism can be due to increased metabolism or reduced uptake and translocation induced by 2,4-D.[14,17)]

Photosystem II (PSII)-inhibiting herbicides (PSII herbicides) inhibit the photosynthetic electron flow through photosystem II. PSII herbicides include *S*-triazines, triazinones, uracils, ureas, phenylcarbamates, anilides, cyanophenols, and dinitrophenols.[18)] Since the introduction of the triazine herbicide atrazine in the late 1950s, PSII herbicides have been widely used for weed control in many crop species. In Australia, atrazine and simazine control weeds in canola (triazine tolerant), and the triazinone metribuzin controls weeds in lupin crops.[19,20)] PSII herbicides are sometimes mixed with auxin herbicides 2,4-D and dicamba for weed control in crop fields.[21)] Synergism between 2,4-D amine and the PSII herbicide atrazine was reported earlier in *Polygonum aviculare*.[22)] In Australia, farmers often observe better weed control when 2,4-D amine and PSII herbicides are used in mixture or in sequence. However, the physiological basis of PSII herbicide synergism with 2,4-D amine is unknown.

Wild oats (*Avena* spp.) are among the most economically damaging global weeds in cropping systems with evolved resistance to multiple herbicides.[23)] In Australia, *A. fatua* and *A. sterilis* wild oats are major weeds infesting grain crops, and aceto-

* To whom correspondence should be addressed.
E-mail: qin.yu@uwa.edu.au
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lactate synthase (ALS)- and ACCase-inhibiting herbicides have been widely used for their control, with evident ensuring resistance.[24] Integrated weed management options are important for controlling these weed species. Avena, as with most grass species, is little affected by 2,4-D amine but is susceptible to the PSII herbicide metribuzin. In this study, we confirmed the synergistic interaction of 2,4-D amine and the PSII herbicide metribuzin in *A. sterilis* and investigated the underlying mechanisms.

Materials and Methods

1. Plant material and growth conditions

An herbicide-susceptible *A. sterilis* population (A252-1) collected from Queensland, Australia, was used in this study. Seeds of *A. sterilis* were dehusked and germinated on 0.6% agar-solidified water in plastic containers. Uniform seedlings were transplanted into plastic pots 18 cm in diameter with a commercial potting mix (50% peatmoss, 25% sand, and 25% pine bark). Seedlings were grown in a naturally lit greenhouse at the University of Western Australia with regular watering and fertilization, with an average day/night temperature of about 20/15°C unless otherwise stated.

2. Herbicide treatment

To focus on the interaction of 2,4-D and metribuzin in *A. sterilis*, these herbicides were sequentially applied in this study. Two-leaf-stage seedlings were treated with 2,4-D amine (Amicide Advance 700, Nufarm, Australia) 24 hr prior to treatment with metribuzin (Mentor 750 WG, Adama, Australia) using a cabinet sprayer with a spray volume of 118 L/ha at a pressure of 200 kPa. The herbicide rates were 0, 1,000, or 2,000 g/ha for 2,4-D amine and 0, 188, 375, or 750 g/ha for metribuzin. There were 20 plants per pot and 3 replicates per treatment. After treatment, the plants were returned to the greenhouse and assessed for survival three weeks after treatment. The above-ground material was harvested and oven dried for two days at 60°C for dry-weight measurement. The expected value (E) of mortality or growth inhibition was determined using Colby's equation $E=(X+Y)-(XY)/100$ for each herbicide combination.[25] X represents the mortality or growth inhibition by a rate of herbicide A, and Y represents that by a rate of herbicide B. The observed and expected values were compared using chi-squared (X^2) test with a degree of freedom of 1. The synergistic or antagonistic effect of an herbicide combination is determined when the observed value is significantly greater or lesser than the expected value ($p<0.05$), respectively, and the additive effect is indicated if $p>0.05$. The means of the observed mortality among different treatments were analyzed using Tukey's HSD test at $\alpha=0.05$. The experiments were repeated twice with similar results.

3. Foliar uptake and translocation of ^{14}C-metribuzin

Metribuzin uptake and translocation in herbicide-susceptible *A. sterilis* were evaluated with ^{14}C-labeled metribuzin (Institute of Isotopes Co., Ltd., Hungary) in the presence or absence of 2,4-D amine. Seedlings were grown in a controlled environment room (CER) at a day/night temperature of 20/15°C with a 12 hr photoperiod at a photosynthetically active radiation (PAR) of 650 photons/m^2/s and a relative humidity of 70%. The two-leaf-stage seedlings were pretreated with and without 2,4-D amine (2000 g/ha) 24 hr before application of ^{14}C-metribuzin with a specific radioactivity of 4.874 MBq/mg. ^{14}C-metribuzin was dissolved in acetonitrile and diluted in aqueous solution containing a 15.8 mM (equal to 400 g/ha) commercial metribuzin formulation plus 0.25% (v/v) nonionic surfactant BS1000, giving a final radioactivity of 0.94 kBq/μL. The treatment solution (1 μL) was applied to the center of the first leaf. Root and shoot materials of individual plant were harvested 24, 48, and 72 hr after treatment, and the treated leaf of each plant was rinsed in 20 mL of washing buffer containing 20% (v/v) methanol and 0.2% (v/v) Triton X-100. The root tissue of each plant was rinsed in 50 mL of deionized water. Plants were blotted dry with tissue paper and oven dried for two days at 60°C for the visualization of ^{14}C-metribuzin using a BioRad Personal Molecular Imager (BioRad PMI, Australia). After visualization, the plants were divided into two parts: treated leaves and untreated parts, including the roots, stem, and new growth. Each section was combusted in a biological oxidizer (RJ Harvey Instrument Corporation, USA). The sample radioactivity recovery (radioactivity recovered from oxidized samples and from the leaf and root wash was divided by the total applied radioactivity) averaged 90%. Quantification of the ^{14}C-metribuzin uptake and translocation was according to Han *et al.*[26] There were six plants for each treatment at each time point, and the experiment was repeated twice with similar results.

4. Metabolism of ^{14}C-metribuzin

Experiments, plant growth, and 2,4-D treatment were conducted as described above except that the ^{14}C-radioactivity applied to each plant in a 1 μL droplet was 4.2 kBq to increase the signal-to-noise ratio in the HPLC chromatograph. The shoot material of treated plants was harvested 24, 48, and 72 hr 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 until use. Four plants per treatment were bulked as a replicate, and three replicates per treatment were analyzed for each time point. As narrowleaf lupin (*Lupinus angustifolius*) is known to metabolize metribuzin,[27] it was included as a positive control (only harvested at 48 hr).

Extraction, separation, and identification of the parent metribuzin and its metabolites using HPLC were essentially according to Lu *et al.*[27] Briefly, metribuzin and its metabolites were extracted from plant tissue with 80% (v/v) cold methanol. The crude homogenate was centrifuged, the supernatant was decanted, and the residue was re-extracted with 80% cold methanol, followed by 50% (v/v) cold methanol. The supernatants were pooled, and radioactivity recovery was determined by LSS. The pooled supernatant was evaporated to dryness under vacuum, resuspended in 50% (v/v) methanol, and centrifuged at 14,000 *g* for 5 min for HPLC injection.

Table 1. Observed and expected control of *Avena sterilis* with 2,4-D amine and metribuzin applied alone or in mixtures based on mortality and dry weight three weeks after herbicide application.[a)]

2,4-D amine (g/ha)	Metribuzin (g/ha)	Mortality					Growth inhibition				
		Observed %	Expected %	χ^2	p	Interaction effect	Observed %	Expected %	χ^2	p	Interaction effect
0	0	0.0±0.0 b					0.0±1.3 d				
	375	7.5±2.0 b					73.0±4.0 b				
1000	0	0.0±0.0 b					27.8±2.3 c				
	375	55.0±4.1 a	7.5	325	0.00	Synergism	96.3±2.7 a	80.5	16.0	0.00	Synergism
2000	0	0.0±0.0 b					40.1±0.6 c				
	375	42.5±2.0 a	7.5	177	0.00	Synergism	91.8±1.1 d	83.8	4.7	0.03	Synergism

[a)] The expected values of mortality and growth inhibition were determined by the Colby's equation E=(X+Y)−(XY)/100 for the herbicide combination. The observed and expected control were compared using the chi-square (χ^2) test (p=0.05). Data are means±S.E. (n=2), and different letters (a to d) in a column indicate significant difference by Tukey's HSD test (α=0.05).

Metribuzin and its metabolites were separated using a gradient reversed-phase HPLC equipped with a 600E dual-head pump with a 717 plus autosampler (Waters, Milford, MA, USA). A 4.6×250 mm Apollo C18 5 μm particle column (Grace Davison Discovery Sciences, USA) was used with a mobile phase flow rate of 1.0 mL min^{-1} at 24°C. The mobile phase consisted of a linear gradient of 15–30% acetonitrile in water (with 1% acetic acid) for 20 min, then held at 30% acetonitrile for 10 min before an immediate change to 100% acetonitrile, then held at 100% acetonitrile for 10 min, followed by re-equilibration with 15% acetonitrile for 15 min. A β-RAM model 2B detector (IN/US Systems Inc., USA) was used to monitor ^{14}C eluting from the column. Injection volumes were adjusted to provide similar sample loading with respect to total radioactivity for all samples. The proportion of metribuzin and its major metabolites was expressed as a percentage peak area of total radioactivity in the sample injection.

5. *The* psbA *gene expression in the leaf and root tissues of* A. sterilis

Quantitative RT-PCR (RT-qPCR) was conducted to measure the *psbA* gene expression in *A. sterilis*. Uniform three-leaf-stage plants were treated with and without 2,4-D amine (2,000 g/ha). Leaf and root samples were harvested separately from control and treated plants 24 hr after treatment and snap-frozen in liquid nitrogen. Total RNA was extracted using the ISOLATE II Plant RNA kit (Bioline, UK), and genomic DNA was removed using the Turbo DNA-free kit (Invitrogen, USA). DNA contamination was further checked with the exon–intron junction primers TublinF (5′-GTA CCT TGA ACT ATT CGA CTT TGC-3′) and TublinR (5′-GCA GTT TCC AGC TCC TGT TC-3′) prior to cDNA synthesis using the SuperScript III reverse transcriptase (Invitrogen, USA). The RT-qPCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with the SensiFAST SYBR Lo-ROX kit (Bioline, UK) with 50 ng cDNA for each reaction. The GAPDH gene was used as the reference gene with the primer pair of AsGAPDHF (5′-CGT CAG GAA CCC TGA AGA AA-3′) and AsGAPDHR (5′-CTT TGC TAG GGG CTG AAA TG-3′).[28)] A 149 bp PCR fragment of the *psbA* gene was amplified with the primer pair of AspsbAF (5′-GTT ATA TGG GTC GTG AGT GGG-3′) and AspsbAR (5′-TTC TCT GAT GGT ATG CCT TTA-3′). The am-

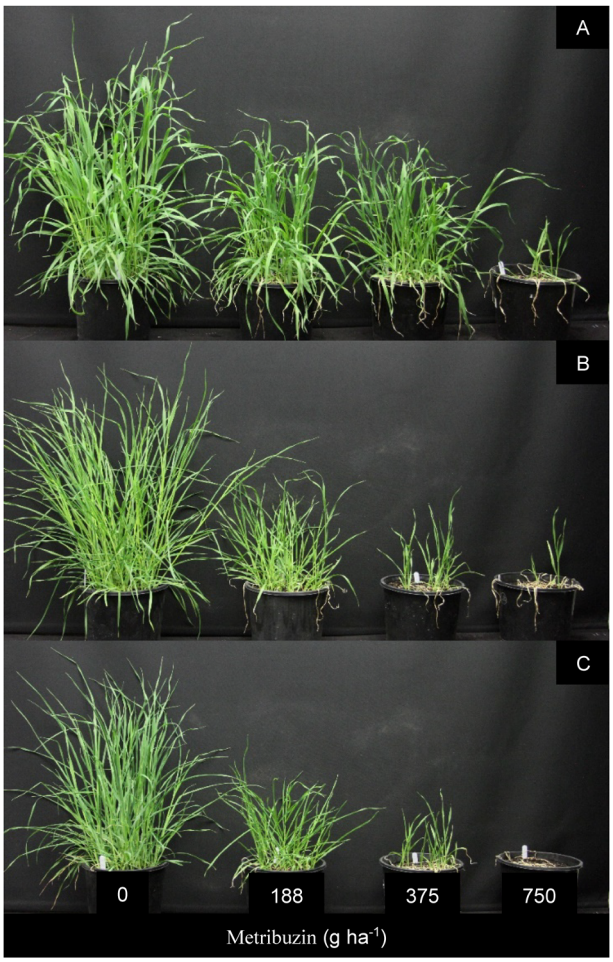

Fig. 1. Effect of metribuzin on the control of susceptible *Avena sterilis* in the absence (A) and presence of 2,4-D amine pretreatment at 1,000 g/ha (B) or 2,000 g/ha (C). 2,4-D amine was applied 24 hr prior to metribuzin applications at 0, 188, 375, and 750 g/ha. Photos were taken three weeks after metribuzin treatment.

Table 2. Foliar uptake and translocation of ^{14}C-metribuzin in *Avena sterilis* plants pre-treated without and with 2,4-D amine at 2,000 g/ha.[a)]

Time point	2,4-D (g/ha)	Foliar uptake (% applied)	Translocation (% absorbed)	
			Treated leaf	Root and new leaf
24 hr	0	41.6±2.5 c	95.4±0.9 a, b	4.6±0.9 b, c
	2000	42.3±1.9 c	92.7±1.3 b, c	7.3±1.3 a, b
48 hr	0	72.7±2.6 a, b	98.6±0.3 a	1.4±0.3 c
	2000	65.3±3.5 b	92.9±0.9 b, c	7.1±0.9 a, b
72 hr	0	81.4±2.0 a	97.8±0.3 a	2.2±0.3 c
	2000	74.7±1.9 a, b	91.8±0.6 c	8.2±0.6 a

[a)] Data are means±S.E. (n=6), and different letters (a to c) in a column indicate significant difference by Tukey's HSD test (α=0.05).

plification efficiency for the two primer pairs was determined to be 87% and 90%, respectively. The relative expression level induced by 2,4-D was calculated as $2^{-(\Delta CT\ treatment-\Delta CT\ control)}$, and the relative expression level in the leaf *versus* root tissue was calculated as $2^{-(\Delta CT\ leaf-\Delta CT\ root)}$. There were five plants per sample and three replicate samples per treatment. The standard error (SE) was calculated from $2^{-\Delta CT}$ and the data analyzed using Student's t-test at p=0.05.[29)]

Results

1. *2,4-D amine and the PSII herbicide metribuzin have a synergistic effect on the control of* A. sterilis

As expected, there was no mortality of *A. sterilis* when treated with 2,4-D alone at 1,000 and 2,000 g/ha, although there was a dry-weight reduction of 28% and 40%, respectively, compared with the untreated control (Table 1, Fig. 1). However, when plants were pretreated with 2,4-D amine and then metribuzin, there was substantially more mortality and biomass reduction than with metribuzin treatment alone (Table 1, Fig. 1). For example, metribuzin at 375 g/ha caused 7.5% mortality, whereas there was 43–55% mortality with 2,4-D amine pretreatment at 1,000 and 2,000 g/ha followed by metribuzin, significantly greater than the expected value of 7.5% (p<0.05). Similarly, metribuzin treatment (375 g/ha) alone caused a 73% plant biomass reduction, whereas treatment with 2,4-D plus metribuzin resulted in up to 96% biomass reduction, significantly greater than the expected value of 81% (p<0.05). Clearly, 2,4-D amine interacted synergistically with the PSII herbicide metribuzin in *A. sterilis* (Table 1, Fig. 1).

2. *Pretreatment of 2,4-D amine enhanced total ^{14}C-metribuzin translocation in* A. sterilis

The rates of foliar uptake of ^{14}C-metribuzin in *A. sterilis* were initially slow, with only 42% of applied metribuzin absorbed 24 hr after application. However, by 72 hr after treatment, up to 81% of the applied ^{14}C-metribuzin was absorbed by the leaves. 2,4-D amine pretreatment did not change the rates of metribuzin uptake, as there was no significant difference in the leaf and root wash-off between plants treated with and without 2,4-D amine at each time point (data not shown); consequently, no difference in ^{14}C-metribuzin absorption by *A. sterilis* plants with and without 2,4-D amine treatments was observed (Table 2).

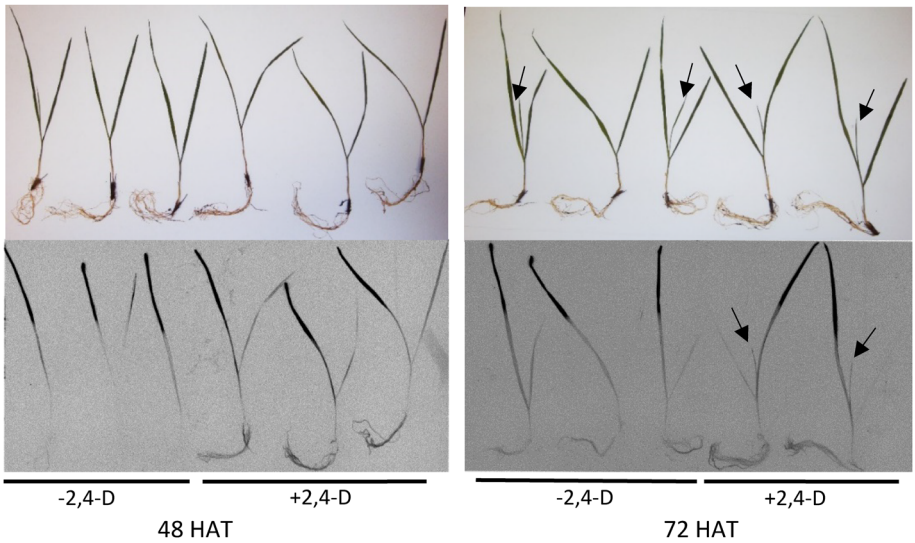

Fig. 2. Visualization of the ^{14}C-metribuzin translocation pattern with a camera (top panel) and Phosphor imager (bottom panel) in *Avena sterilis*. The two-leaf-stage seedlings were pretreated with and without 2,4-D amine 24 hr before the application of ^{14}C-metribuzin on the first fully expanded leaf. Plants were imaged 48 hr and 72 hr after treatment (HAT).

The translocation of metribuzin (and its metabolites) was limited, with more than 90% of ^{14}C radioactivity retained in the treated leaves throughout the experiment period. However, pretreatment with 2,4-D amine significantly enhanced ^{14}C translocation from the treated leaf relative to untreated plants over the experiment period (Table 2, Fig. 2). In ^{14}C imaging, the translocation of metribuzin (and its metabolites) from treated leaves to roots and the second leaves of 2,4-D amine–pretreated plants became evident 48 hr after treatment, but such was not the case for plants treated with metribuzin alone (Fig. 2). Metribuzin translocation to the new growth (the third leaf) of 2,4-D amine-pretreated plants was evident 72 hr after treatment (Fig. 2). Quantitative analysis revealed that 48 hr after treatment, ^{14}C translocation out of the treated leaves in 2,4-D amine-pretreated plants was 5 times higher than that in plants without 2,4-D amine pretreatment (Table 2, Fig. 2). By 72 hr after metribuzin treatment, ^{14}C translocation out of the treated leaves remained 3.7 times higher in 2,4-D-pretreated plants, resulting in ^{14}C appearance in the new leaf growth (Table 2, Fig. 2).

3. *Pretreatment with 2,4-D amine increases metribuzin metabolism in* A. sterilis

Under our HPLC experimental conditions, ^{14}C-metribuzin was eluted at 34.2 min (Fig. 3A). Only one major metribuzin metabolite peak was eluted at 13.6 min without pretreatment with 2,4-D amine (Fig. 3B). However, with 2,4-D amine pretreatment, there were at least four polar (9.6, 11.1, 13.4, and 16.7 min) metabolites and one nonpolar (37.8 min) metribuzin metabolite (Fig. 3C). The metribuzin metabolism increased with time in both 2,4-D amine-pretreated and untreated plants. However, the metribuzin metabolism in 2,4-D amine-pretreated plants was significantly enhanced compared to the control plants at all time points. For example, 72 hr after treatment, 58% of ^{14}C was recovered as major metabolites in plants pretreated with 2,4-D amine, compared to 32% in plants without 2,4-D amine pretreatment (Table 3). The metribuzin metabolism in metribuzin-tolerant lupin plants differed from that in *A. sterilis*, with the three major metabolites being eluted at 11.1, 13.4, and 20.5 min (Fig. 3D).

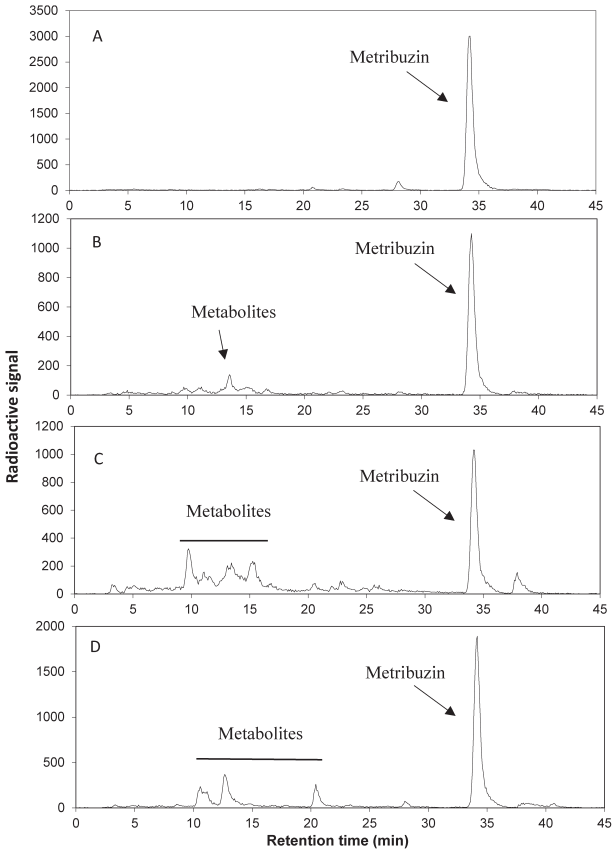

Fig. 3. HPLC chromatogram of ^{14}C-metribuzin and its metabolites in extracts of susceptible *Avena sterilis* 48 hr after treatment with ^{14}C-metribuzin alone (B) or pretreated with 2,000 g 2,4-D amine/ha (C). Lupin plants were included as the positive control without 2,4-D amine treatment (D) together with the standard ^{14}C-metribuzin (A).

4. *The* psbA *gene was specifically overexpressed in leaf tissue*

The expression of the PSII herbicide target site *psbA* gene in both leaves and roots was investigated with and without 2,4-D pretreatment in susceptible *A. sterilis*. The *psbA* gene expression in both leaves and roots was unaffected by pretreatment with 2,4-D amine. However, as expected, the expression level of the *psbA* gene is 4144 and 5971 times greater in leaves than in roots

Table 3. Metabolism of ^{14}C-metribuzin by *Avena sterilis* plants.[a)]

Time point	Plant species	2,4-D (g/ha)	Radiolabel (% of radioactivity recovered)	
			Metribuzin	Total metabolites
24 hr	*A.sterilis*	0	91.1±0.5 a	8.9±0.5 c
		2000	71.7±1.2 c	28.3±1.2 c
48 hr	*A.sterilis*	0	80.1±1.9 b	19.9±2.0 d
		2000	50.4±0.5 d	49.6±2.0 b
72 hr	*A.sterilis*	0	67.9±1.9 c	32.1±1.7 c
		2000	41.8±1.4 e	58.2±1.4 a
48 hr	Lupin	0	69.5±0.1 c	30.5±0.1 c

[a)] The proportion of the parent herbicide metribuzin and its major metabolites were expressed as a percentage peak area of total radioactivities in the sample injection. Data are means±S.E. (n=3). Means with different letters (a to e) in a column are significantly different by Tukey's HSD test (α=0.05).

Table 4. Expression levels of *psbA* gene in response to 2,4-D amine in leaves and roots of the susceptible *Avena sterilis* plants.[a)]

	Treatment	Fold change	*p*
Leaf/Root	Control	5972±660	0.012
	Treated	4144±621	0.022
Treated/Control	Leaf	1.3±0.2	0.249
	Root	1.8±0.3	0.209

[a)] The samples were collected 24 hr after 2,4-D amine treatment at 2,000 g/ha. Data are means±S.E. (n=3) and are subject to the student's t-test (p=0.05).

in the presence and absence of 2,4-D treatment, respectively (Table 4).

Discussion

Herbicides are often mixed to achieve a wider weed-control spectrum (*e.g.*, dicot plus grass-weed control) or to attain greater efficacy. Complex interactions may occur between dissimilar herbicides. Here, at least in the widespread grass weed *Avena* (wild oat), we report synergism between 2,4-D amine and metribuzin, increasing the efficacy of metribuzin on wild oat (Table 1, Fig. 1). Similar synergistic results were obtained in *Polygonum aviculare*.[22)] Therefore, a synergistic interaction between 2,4-D amine and a PSII herbicide improved *Avena* weed control. We demonstrate that this synergism of 2,4-D amine and metribuzin is likely due to enhanced metribuzin translocation in plants (Table 2, Fig. 2). The mechanism by which 2,4-D enhances metribuzin translocation is unknown. At the applied level, field experiments should be conducted to examine this synergism.

As an auxinic herbicide, 2,4-D amine acts similarly to the plant endogenous auxin hormone indole-3-acetic acid (IAA) and can regulate the expression of auxin-responsive genes, particularly those involved in agrochemical metabolism and transportation, leading to synergetic, additive, or antagonistic effects.[30)] For example, the antagonism of 2,4-D and ACCase-inhibiting herbicides, paraquat and glyphosate, has been reported in several grass-weed species.[8–15,31)] We showed that 2,4-D amine antagonizes the ACCase-inhibiting herbicide diclofop-methyl in *L. rigidum* due to enhanced diclofop metabolism.[17)] Recently, the 2,4-D antagonism of glyphosate was reported to be due to reduced glyphosate uptake and translocation in *Echinochloa colona*[32)] and *S. halepense*[14)] or between dicamba and glyphosate in *E. crus-galli* and *Amaranthus palmeri*[33)] and in *Kochia scoparia*.[34)] In our current research, the synergism between 2,4-D and metribuzin in *A. sterilis* is likely due to the enhanced translocation of metribuzin (and its intermediate metabolites) (Table 2, Fig. 2). The contrasting effect of 2,4-D amine as compared with glyphosate and metribuzin is interesting and invites further investigation.

Metribuzin is a selective herbicide in certain crops due to the different rates of metabolism between crop and weed.[35–37)] The metribuzin metabolism has been demonstrated in these crops *via* nonconjugative and conjugative metabolic pathways. For the nonconjugative pathway, metribuzin is metabolized *via* deamination and dethiomethylation to deaminated metribuzin (DA), diketo metribuzin (DK), and deaminated diketo metribuzin (DADK).[35–37)] The *N*-glucoside and homoglutathione conjugations of metribuzin were reported to be the major detoxification pathway in tomato and soybean, respectively.[38–40)] Our experiments demonstrated that metribuzin can be metabolized to some extent in susceptible *A. sterilis*, and 2,4-D amine pretreatment clearly enhanced metribuzin metabolism (Table 3, Fig. 3). Our previous transcriptome work revealed that 2,4-D can increase the expression of an array of metabolic enzymes, including, but not limited to, P450, glutathione *S*-transferase (GST), and glucosyltransferase (GT).[41)] These and other enzymes, very likely to be GT,[38)] may be involved in metribuzin metabolism, although it is not clear how.

However, we consider the stimulation of metribuzin metabolism by 2,4-D unlikely to be the cause of increased metribuzin activity. A similar HPLC protocol was used for metribuzin metabolism studies in the metribuzin-resistant crop lupin[27)] and weed species of *Raphanus raphanistrum*[27)] and *L. rigidum*.[42)] Although it is not possible to draw a clear conclusion due to the lack of peak identifications, the results in this paper showed an increase in polar metabolites (Fig. 3), similar to the HPLC profile in the resistant species studied (*e.g.*, major polar metabolites eluted during 10–15 min). These results indicate that the major or intermediate metabolites of metribuzin are likely less potent than the parent metribuzin. Meanwhile, 2,4-D amine pretreatment enhanced ^{14}C translocation (Table 2, Fig. 2). As enhanced metribuzin metabolism tends to antagonize but increase metribuzin translocation to synergize metribuzin, we hypothesize that 2,4-D amine/metribuzin synergism is the balance of the two events. As the *psbA* gene is specifically expressed in leaves (Table 4), metribuzin has to be translocated to new leaves to be effective on the whole plant. However, we observed that metribuzin itself is poorly translocated in the absence of 2,4-D amine; therefore, any changes in the translocation pattern will increase metribuzin efficacy. This is consistent with the result showing that more ^{14}C was accumulated in the new growth (meristem) 72 hr after treatment with 2,4-D amine (Table 2, Fig. 2).

The mechanism of enhanced metribuzin translocation by 2,4-D is not clear but may involve the 2,4-D-induced expression of transporters capable of moving metribuzin or the 2,4-D-induced modification of metribuzin mobility *via* metabolism. Indeed, ABC transporters have been reported to be inducible by 2,4-D treatment.[43,44)]

In conclusion, our work revealed a synergistic effect of the auxin herbicide 2,4-D amine and the PSII herbicide metribuzin in *A. sterilis*, and enhanced PSII herbicide translocation to new leaves is likely responsible for the synergism. This is in contrast to our previous work demonstrating antagonism of 2,4-D with herbicides of different modes of action.[17,32)] Collectively, these results are very informative for correctly using herbicide mixtures to achieve maximum efficacy and sustainability. Field work is required to assess any commercial benefit of this synergy.

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