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# Exploring chemical control of 2,4-D–resistant wild radish (*Raphanus raphanistrum*) with auxin-related compounds

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## Research Article

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## Abstract

Synthetic auxin herbicides were developed and commercialized 60 yr before their mode of action was definitively elucidated. Although evolution of resistance to auxinic herbicides proceeded more slowly than for some other herbicide chemistries, it has become a major problem in the dicotyledonous weeds of many cropping areas of the world. With the molecular characterization of the auxin perception and signaling pathway in the mid-2000s came a greater understanding of how auxinic herbicides work, and how resistance may develop in weeds subjected to repeated selection with these herbicides. In wild radish (*Raphanus raphanistrum* L.) populations in southern Australia, resistance to multiple herbicides, including synthetic auxins such as 2,4-D, has reduced the number of chemical control options available. The aim of this study was to determine whether compounds involved in auxin biosynthesis, transport, and signaling are able to synergize with 2,4-D and increase its ability to control 2,4-D–resistant *R. raphanistrum* populations. Although some mild synergism was observed with a few compounds (abscisic acid, cyclanilide, tryptamine), the response was not large or consistent enough to warrant further study. Similarly, alternative auxinic herbicides applied pre- or postemergence were no more effective than 2,4-D. Therefore, while use of auxinic herbicides continues to increase due to the adoption of transgenic resistant crops, nonchemical control techniques will become more important, and chemical control of 2,4-D–resistant *R. raphanistrum* should be undertaken with alternative modes of action, using mixtures and good stewardship to delay the development of resistance for as long as possible.

## Introduction

Wild radish (*Raphanus raphanistrum* L.) is a highly competitive outcrossing species that has become naturalized across most temperate regions of the world and is often a problematic weed of crops and pastures (Warwick and Francis 2005). It arrived in Australia in the 1860s as a contaminant of grain products and is now widespread across the southern cropping region (Donaldson 1986), with infestations in Australian wheat (*Triticum aestivum* L.) crops being responsible for a $50 million loss in revenue annually (Llewellyn et al. 2016). In the Western Australian grain belt, *R. raphanistrum* has formed genotypically distinct populations adapted to local conditions (Bhatti et al. 2016). The adaptability of *R. raphanistrum* has also resulted in its development of resistance to most of the herbicides used for its control in Australia, Brazil, and South Africa (Heap 2023). In a random herbicide-resistance survey of the Western Australian grain belt performed in 2015, almost 90% of the collected *R. raphanistrum* populations contained plants resistant to the acetolactate synthase (ALS)-inhibiting herbicide chlorsulfuron, and 61% of populations showed resistance to the synthetic auxin herbicide 2,4-D (Owen and Powles 2018). Mutations in the target *ALS* gene are potentially responsible for chlorsulfuron resistance (Yu et al. 2012), but the mechanism(s) of 2,4-D resistance in *R. raphanistrum* remains unclear. The mode of action of synthetic auxin herbicides is complex, reflective of the fact that endogenous auxin (indole-3-acetic acid [IAA]) synthesis, metabolism, transport, and signaling are very finely tuned in order to maintain the correct pattern and timing of plant growth,

development, and environmental response. Therefore, it is possible that auxin precursors, compounds affecting auxin signaling and transport, or molecules synthesized in response to high auxin levels, could act as synergists for synthetic auxin herbicides and help to ameliorate resistance (Ang and Østergaard 2023).

There are two pathways of IAA biosynthesis: the tryptophan-independent pathway, about which little is known, and the tryptophan-dependent pathway, which comprises four alternative routes stemming from tryptophan (Gomes and Scortecci 2021). Concentrations of IAA in the cell can also be modulated by reversible or irreversible conjugation to amino acids (Böttcher et al. 2011). The means by which auxin is able to influence almost all aspects of the plant life cycle was finally elucidated in the mid-2000s (Tan et al. 2007) and has been extensively studied and reviewed since then (e.g., Calderón Villalobos et al. 2012; Chandler 2016; Kubeš and Napier 2019). Briefly, as summarized in Caumon and Vernoux (2023), the ARF family of transcription factors bind to DNA and activate or repress expression of auxin-responsive genes. The Aux/IAA family of transcriptional repressors in turn bind to the ARF proteins, preventing them from regulating the expression of their bound genes. However, in the presence of high levels of auxin, perceived by the TIR1/AFB family of auxin co-receptors, Aux/IAA repressors are recruited to the TIR1/AFB auxin-binding site and degraded by the ubiquitin ligase complex, of which TIR1/AFB is a subunit. Upon degradation of the Aux/IAA proteins, the auxin-responsive genes regulated by ARF transcriptional activators are expressed.

The fine control of auxin responses is mediated not only by IAA synthesis and metabolism, but also by the presence of local concentration maxima and minima within and between plant tissues, achieved by cell-to-cell auxin transport (Geisler et al. 2017). Three major families of transporters interact to achieve polar transport of auxins: (1) the AUX1/LAX influx transporters, (2) the dynamically localized PIN efflux transporters, and (3) the ABCB efflux transporters that use ATP to move auxin against steep concentration gradients (Geisler et al. 2017) and prevent membrane insertion and re-uptake of IAA by the cells adjoining the vascular tissue during long-distance phloem transport (Reemmer and Murphy 2014).

Auxin action is influenced by calcium acting as a second messenger (e.g., calmodulin physically interacts with Aux/IAA proteins and promotes derepression of the ARFs: Zhang et al. 2022) and by crosstalk between auxin and other hormones (e.g., cytokinin response factors transcriptionally control expression of the PIN-type auxin transporters and may contribute to the control of auxin transport; Šimášková et al. 2015). With particular relevance to synthetic auxin herbicides, a major outcome of auxin-responsive gene expression is the enhanced production of two other plant hormones, abscisic acid (ABA) and ethylene (Grossmann 2010). Ethylene synthesis begins when methionine is converted to *S*-adenosylmethionine (SAM), but the first committed step is the further conversion of SAM to 1-amino-cyclopropane-1-carboxylic acid (ACC, the immediate precursor of ethylene) by ACC synthase, which is the product of an auxin-responsive gene (Wang et al. 2002b). In turn, ethylene can mediate auxin synthesis and transport (Liu et al. 2017), and expression of certain *Aux/IAA* genes can be repressed by ethylene-responsive transcription factors, ultimately resulting in the promotion of leaf senescence (Koyama 2014).

The critical regulatory step of ABA synthesis, cleavage of 9-*cis*-epoxycarotenoids to xanthoxin, is also catalyzed by the product of an auxin-upregulated gene, *9-cis-epoxycarotenoid dioxygenase* (*NCED*) (Han et al. 2004). McCauley et al. (2020) proposed that the sustained accumulation of ABA in plants treated with auxinic herbicides contributes to the observed global downregulation of photosynthesis that is likely to be the actual cause of plant death. ABA is well known for its role in seed dormancy and inhibition of germination, but crosstalk between auxin and ABA during germination and emergence, which affects plant sensitivity to ABA, is also critical for seedling establishment (Liu et al. 2017). This could potentially have implications for the use of auxinic herbicides in a preemergence context, and so one of the aims of this study was to assess whether 2,4-D–resistant *R. raphanistrum* populations could be more efficiently controlled by auxinic herbicides applied to the seeds rather than young plants.

It was also hypothesized that compounds sitting upstream of IAA in its biosynthetic pathway could potentially influence the performance of 2,4–D applied in the field, as could compounds synthesized by the plant in response to high IAA levels, or those involved in downstream auxin signaling and hormone crosstalk. A number of chemicals associated with, or inhibiting, aspects of auxin biology (Figure 1; Table 1) were therefore assessed for their potential to synergize 2,4-D in susceptible and resistant *R. raphanistrum* populations using agar-based and pot studies. Anthranilate is the product of the rate-limiting step in tryptophan synthesis (Wang et al. 2022a), while tryptamine and indole-3-acetamide (IAM) are the first intermediates in two of the tryptophan-dependent IAA biosynthesis pathways (Gomes and Scortecci 2021). Tryptamine itself possesses a similar activity to IAA in terms of plant growth regulation (Di et al. 2016). Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) prevents the binding of calmodulin to Aux/IAA repressors (Zhang et al. 2022), 6–benzylamino purine (BAP) is a synthetic cytokinin, methionine and ACC are precursors of ethylene (Wang et al. 2022b), and ABA is a major product of the plant response to high auxin levels (McCauley et al. 2020). Cyclanilide not only inhibits polar auxin transport (Burton et al. 2008), but also synergizes ethephon (a synthetic ethylene precursor) (Pedersen et al. 2006) and downregulates cytokinin catabolism and ABA-responsive genes (Ma et al. 2022). 1-Butanol is an inhibitor of phospholipase D that prevents the formation of phosphatidic acid, a signaling lipid required for the normal phosphorylation, membrane localization, and hence function of PIN transporters (Gao et al. 2013). Adenosine is an inhibitor of type II phosphatidylinositol-4-kinases, which, among other functions, are involved in the regulation of IAA biosynthesis (Tang et al. 2016) and conjugation (Zhao and Xue 2020) via their protein kinase activity and direct interaction with E3 ligases that modulate transcription factor stability.

A final hypothesis, based on the observed (unpublished) differential responses to different auxinic herbicides in tolerant crops, was that 2,4-D–resistant *R. raphanistrum* populations may be more sensitive to other synthetic auxin herbicides such as MCPA, mecoprop, and halauxifen. MCPA and mecoprop are anecdotally reputed to be effective on 2,4-D–resistant weed populations, especially when applied as a mixture, and halauxifen was found to be more effective than 2,4-D in the control of horseweed [*Conyza canadensis* (L.) Cronquist] (McCauley and Young 2019). Picloram belongs to the same chemical class as halauxifen and was included in the study as a comparison, because our previous unpublished data showed that wild-type *R. raphanistrum* is relatively insensitive to picloram.

The complexity of crosstalk and feedback loops associated with auxin has made it difficult to determine whether endogenous IAA

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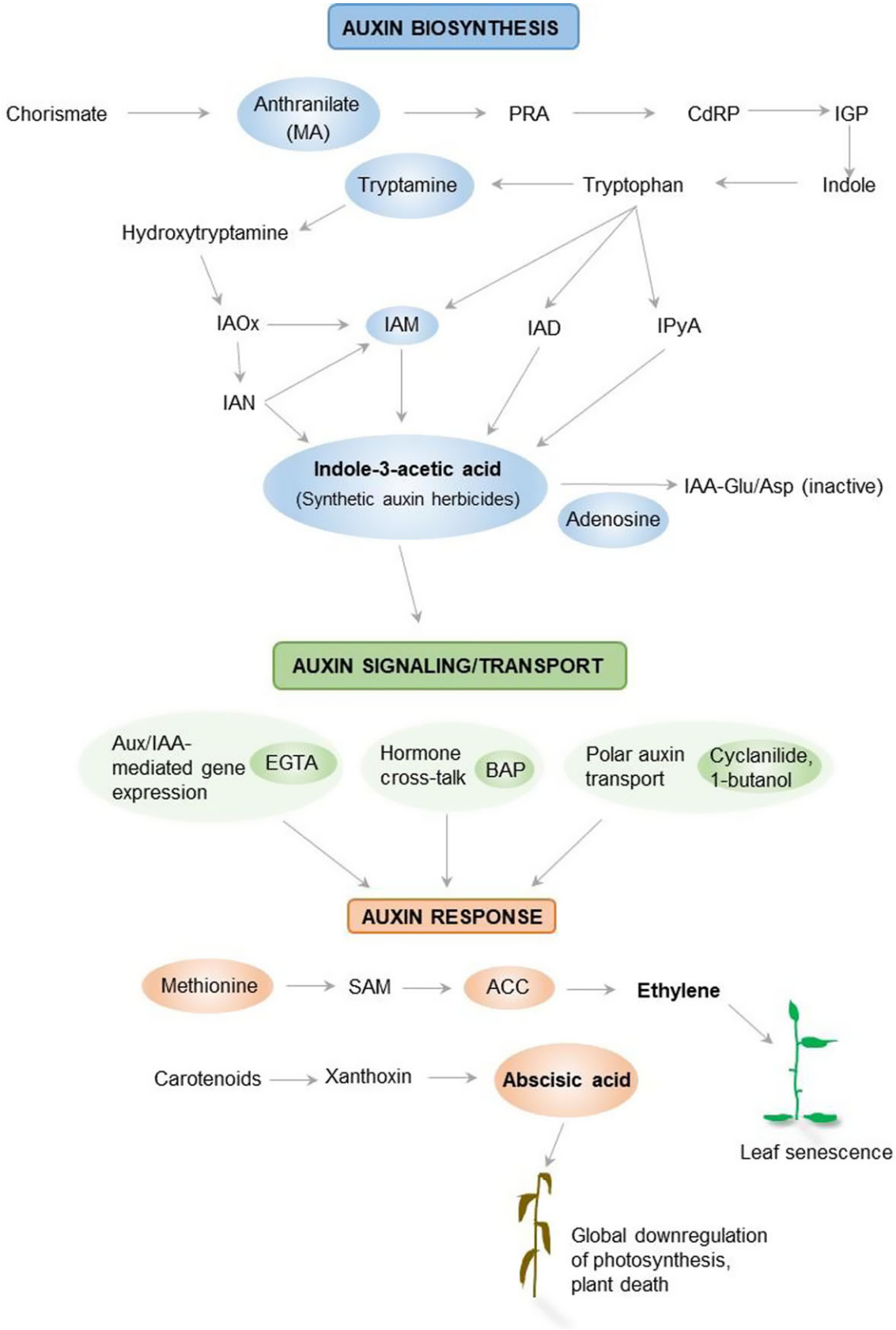


**Figure 1.** Compounds used as potential 2,4-D synergists or substitutes, and how they fit into the schemes of auxin biology. The compounds used in the current study are shown in shaded ovals, with blue representing the auxin biosynthesis pathway, green representing auxin signaling and transport, and orange representing auxin response. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; BAP, 6-benzylaminopurine; CdRP, 1-(*O*-carboxylphenylamino)1-deoxyribulose-5-phosphate; EGTA, ethylene glycol bis(2-aminoethyl)tetraacetic acid; IAD, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IGP, indole glycerol phosphate; IPyA, indole-3-pyruvic acid; MA, methylanthranilate; PRA, phosphoribosylanthranilate; SAM, *S*-adenosylmethionine.

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**Table 1.** Compounds added to agar for synergism assays.

| Additive[a] | *Raphanus raphanistrum* populations | | |
|---|---|---|---|
| | S1 | R2 | R3 |
| Auxin biosynthesis | | | |
| 2,4-D | 0.02 µM | 0.1 µM | 0.1 µM |
| Adenosine | 200 µM | 200 µM | — |
| IAM | 0.35 µM | — | 0.35 µM |
| Methyl anthranilate | 10 µM | — | 10 µM |
| Tryptamine | 0.1 µM | — | 0.3 µM |
| Auxin signaling/transport | | | |
| BAP | 0.01 µM | 0.1 µM | — |
| 1-Butanol | 0.15% (v/v) | 0.15% (v/v) | — |
| Cyclanilide | 5 µM | — | 0.5 µM |
| EGTA | 600 µM | — | 600 µM |
| Auxin response | | | |
| ABA | 5 µM | 10 µM | — |
| ACC | 0.1 µM | 0.35 µM | — |
| DL-methionine | 0.1 mM | 2.5 mM | — |

[a]Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; BAP, 6-benzylaminopurine; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IAM, indole-3-acetamide.

promotes cell survival or cell death (Kacprzyk et al. 2022). Similarly, the effects of supplementation of an auxinic herbicide with auxin-related compounds cannot be readily predicted until various combinations are tested.

## Materials and Methods

### Chemicals

Methyl anthranilate, tryptamine, IAM, cyclanilide, 2,4-D acid, and the formulated herbicides Amicide Advance 700 (2,4-D amine), Polo 570 LVE (MCPA 2-ethylhexyl ester), mecoprop, and Kamba 750 (dicamba amine) were kindly provided by Nufarm Australia (Victoria, Australia). All other chemicals listed in Table 1 were sourced from Sigma-Aldrich (Sydney, Australia).

### Plant Material

The 13 *R. raphanistrum* populations characterized in previous studies on 2,4-D resistance, namely S1, S2 (susceptible), and R1 to R11 (resistant; selected twice with 500 g $ha^{-1}$ 2,4-D following collection of the original populations from the field) (Goggin et al. 2018) were used, in order to (1) identify potential 2,4-D synergists effective on resistant populations and (2) ensure that there were no unexpected antagonistic effects in susceptible populations. The various root elongation experiments used populations S1, S2, R1, R2, and R3, while the pot experiments assessing efficacy of MCPA and mecoprop used all populations except R9 (glasshouse) or used populations S2, R2, R4, R7, and R8 (outdoors). The study on preemergence use of auxinic herbicides used all 13 characterized populations as well as 11 populations collected from the field in 2020 and identified as potentially 2,4–D–resistant during screening by the UWA herbicide-resistance testing service.

### Synergism Assays with Auxin-related Compounds

Seeds were surface sterilized for 5 min in 0.8% sodium hypochlorite containing 0.1% Tween-20 and rinsed well in deionized water before being placed on 0.6% agar (Ajax Finechem, Sydney, Australia) dissolved in deionized water and imbibed in the dark at room temperature for 3 d. Radicle lengths were recorded, and the seedlings were then placed on agar containing the appropriate additives. Seedlings were incubated at 20 C with a 12-h photoperiod of 90 µmol $m^{-2}$ $s^{-1}$ white light for a further 7 d, and the radicle lengths were recorded again in order to calculate the rate of radicle elongation (mm $d^{-1}$).

Pilot dose–response studies were performed to identify the concentration of each additive causing an ~50% inhibition of radicle elongation in each population (Supplementary Table S1). Concentrations similar to this value were then used in the main study, which consisted of four treatments: untreated control, 2,4-D alone, additive alone, and 2,4-D plus additive. Table 1 summarizes the concentrations of each additive used for each population. There were 5 to 10 seedlings per replicate, and three replicates of each treatment.

In response to a potential synergism observed between tryptamine and 2,4-D, a small pilot experiment was performed in which soil-grown seedlings (2- to 3-leaf stage) of the R3 population were foliar-sprayed with tryptamine and 2,4-D, either stand-alone or in a mixture. Formulated 2,4-D amine (Amicide Advance 700, Nufarm Australia) was applied at 250 g $ha^{-1}$, corresponding to half the recommended field rate. Tryptamine, which was dissolved in dimethylsulfoxide (DMSO) and then diluted for spraying in 0.2% (v/v) Tween 20 (final DMSO concentration: 5%), was applied at 178 g $ha^{-1}$. This rate was selected as matching the molar concentration of 2,4-D in the spray solution (5.2 mM). Control plants were sprayed with 0.2% Tween 20 and 5% DMSO, and these additives were also included in the stand-alone 2,4-D treatment. Plants were grown and sprayed as described later for the large-scale dose–response assays and were monitored for symptoms over 21 d.

### Dose–Response Assays with Auxinic Herbicides

To assess the efficacy of mecoprop and MCPA, as well as 1:1 mixtures of these herbicides, seeds were sown into moist potting mix (50% composted pine bark, 25% river sand, 25% peat moss) in plastic seedling trays and placed either in a naturally lit glasshouse or outdoors at the University of Western Australia during autumn and winter. When seedlings had reached the 2- to 3-leaf stage, they were sprayed with the appropriate herbicide or mixture, as specified in Table 2, using a custom-built cabinet sprayer equipped with a TeeJet® XR11001 flat-fan dual nozzle (Spraying Systems, Wheaton, IL, USA) delivering herbicide in 106 L of water $ha^{-1}$ at a pressure of 200 kPa, moving at 3.6 km $h^{-1}$ (Owen et al. 2014). Plants were then returned to their original location and grown for another 21 d, with the plants watered from above as required and fertilized weekly with commercial liquid fertilizer (Diamond Red, Campbells Fertilisers, Victoria, Australia). The number of surviving plants (classified as those with asymptomatic new growth) was counted and expressed as a percentage of the number of plants that were treated. There were three replicates of 10 seedlings per treatment. The glasshouse experiment was performed in 2021 and the outdoor experiment in 2022.

To assess the efficacy of halauxifen in comparison with 2,4-D and picloram, agar-based root elongation assays were performed as described earlier for the synergism study, using populations S1, S2, R1, and R3. Individual herbicides were incorporated into the agar at concentrations of 0, 0.01, 0.1, 0.5, 1, 10, or 50 µM. There were three replicates of five seedlings for each population and herbicide treatment.

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**Table 2.** Herbicides and doses used in pot-based dose–response experiments conducted for *Raphanus raphanistrum*.

| | Glasshouse experiment (2021) | Outdoor experiment (2022) |
|---|---|---|
| Treatment 1 | Untreated control | Untreated control |
| Treatment 2 | Mecoprop 300 g ha$^{-1}$ | Mecoprop 172 g ha$^{-1}$ |
| Treatment 3 | Mecoprop 600 g ha$^{-1}$ | Mecoprop 344 g ha$^{-1}$ |
| Treatment 4 | Mecoprop 900 g ha$^{-1}$ | Mecoprop 688 g ha$^{-1}$ |
| Treatment 5 | Mecoprop 1,200 g ha$^{-1}$ | Mecoprop 1,376 g ha$^{-1}$ |
| Treatment 6 | MCPA 300 g ha$^{-1}$ | Mecoprop 2,752 g ha$^{-1}$ |
| Treatment 7 | MCPA 600 g ha$^{-1}$ | Mecoprop 5,504 g ha$^{-1}$ |
| Treatment 8 | MCPA 900 g ha$^{-1}$ | MCPA 172 g ha$^{-1}$ |
| Treatment 9 | MCPA 1,200 g ha$^{-1}$ | MCPA 344 g ha$^{-1}$ |
| Treatment 10 | Mecoprop + MCPA 150 + 150 g ha$^{-1}$ | MCPA 688 g ha$^{-1}$ |
| Treatment 11 | Mecoprop + MCPA 300 + 300 g ha$^{-1}$ | MCPA 1,376 g ha$^{-1}$ |
| Treatment 12 | Mecoprop + MCPA 450 + 450 g ha$^{-1}$ | MCPA 2,752 g ha$^{-1}$ |
| Treatment 13 | Mecoprop + MCPA 600 + 600 g ha$^{-1}$ | MCPA 5,504 g ha$^{-1}$ |
| Treatment 14 | — | Mecoprop + MCPA 86 + 86 g ha$^{-1}$ |
| Treatment 15 | — | Mecoprop + MCPA 172 + 172 g ha$^{-1}$ |
| Treatment 16 | — | Mecoprop + MCPA 344 + 344 g ha$^{-1}$ |
| Treatment 17 | — | Mecoprop + MCPA 688 + 688 g ha$^{-1}$ |
| Treatment 18 | — | Mecoprop + MCPA 1,376 + 1,376 g ha$^{-1}$ |
| Treatment 19 | — | Mecoprop + MCPA 2,752 + 2,752 g ha$^{-1}$ |

### *Preemergence Herbicide Treatments*

Seeds were sown onto the surface of moist potting mix in 20-cell trays and sprayed with one of the following herbicides: 2,4-D at 560 g ha$^{-1}$, MCPA at 570 g ha$^{-1}$, or dicamba at 750 g ha$^{-1}$. The higher rate of dicamba was based on its lower postemergence efficacy on *R. raphanistrum* (Goggin et al. 2018). Seeds were then immediately covered with moist potting mix and briefly watered to remove air pockets. Seedling emergence was assessed at 28 d after spraying and expressed as a percentage of the emergence of the untreated controls. A parallel experiment wherein the same herbicides and rates were applied postemergence was also performed on the field-collected populations, with plant survival assessed at 28 d after spraying. There were 10 to 15 seeds/seedlings per treatment, with one replicate for each of the two susceptible populations (S1 and S2: used as controls for herbicide efficacy); the 11 putative resistant, field-collected populations; and the 11 confirmed resistant, 2,4-D–selected populations (R1 to R11).

### *Data Analysis*

Root elongation data were analyzed for potential synergism or antagonism between 2,4-D and the various additives by employing the statistical treatment of the Colby method as described by Flint et al. (1988):

$$I_{ij} = \log(T_{ij}) - \log(A_{i0}) - \log(B_{0j}) + \log(AB_{00}) \quad [1]$$

where $I_{ij}$ is the expected interaction if the two chemicals are additive, $T_{ij}$ is the observed root elongation in the presence of both chemicals, $A_{i0}$ is the observed root elongation when 2,4-D is applied alone, $B_{0j}$ is the observed root elongation when the additive is applied alone, and $AB_{00}$ is the observed root elongation of the untreated control. The interaction $I_{ij}$ was calculated for each replicate, and Welch's *t*-test was used to determine whether the mean $I_{ij}$ was different from 0, with $I_{ij} < 0$ indicating synergism and $I_{ij} > 0$ indicating antagonism (Flint et al. 1988).

The dose–response data from the pot experiments on MCPA and mecoprop and the agar-based experiments on halauxifen, 2,4-D, and picloram were used to estimate the herbicide dose required to kill 50% of the population or inhibit root elongation by 50% ($ED_{50}$). Using the DRC package in R (R Core Team 2019; Ritz et al. 2015), survival and root elongation data were fit to a three-parameter log-logistic model:

$$y = \frac{d}{1 + \exp(b(\log x - \log e))} \quad [2]$$

where $y$ is plant survival (expressed as a percentage of the total plants treated) or root elongation (expressed as a percentage of the root elongation of untreated seedlings), $d$ is the upper limit of survival or root elongation, $b$ is the slope of the curve, $x$ is the herbicide dose, and $e$ is the dose at which survival or root elongation was 50% ($ED_{50}$).

For the preemergence experiment, the mean survival of the 11 field-collected populations was compared with that of the eleven 2,4-D–selected populations using Welch's *t*-test. The same test was used to compare the mean survival of the field-collected populations under preemergence versus postemergence auxinic herbicide treatment.

## Results and Discussion

### *Synergism Assays with Auxin-related Compounds*

In agar-based root elongation assays, adenosine, 1-butanol, EGTA, indole-3-acetamide, and methyl anthranilate antagonized 2,4-D in either or both of the susceptible and resistant populations tested (Figure 2). ABA and cyclanilide synergized 2,4-D in the susceptible population, and tryptamine synergized 2,4-D in the resistant population (Figure 2). Due to the fact that ABA and cyclanilide did not synergize 2,4-D in the resistant population, they were not investigated further. Soil-grown 2,4–D–resistant seedlings sprayed with tryptamine alone showed very mild leaf curling, while those sprayed with tryptamine plus 2,4-D appeared similar to those sprayed with 2,4-D alone, with extensive curling and epinasty of the treated leaves, but relatively healthy new growth with only mild symptoms (Supplementary Figure S1).

The observed possible synergism between 2,4-D and ABA in the susceptible population is in line with the synergistic interaction observed between endogenous IAA and ABA during seed germination (Emenecker and Strader 2020) and lateral root formation and embryonic axis elongation (reviewed in Asghar et al. 2019). However, there is not enough evidence in the current study that ABA mixed with an auxinic herbicide would lead to improved control of 2,4-D–resistant weed populations. The lack of effect of tryptamine in the pot experiment, in contrast to the observed synergism in the agar experiment, could be due to inefficient uptake through the leaf cuticle and/or rapid metabolism of tryptamine, which is an IAA precursor occurring endogenously in plants and therefore subject to the strict controls placed on auxin biosynthesis (Quittenden et al. 2009). Further work is required to determine whether the rate of tryptamine metabolism in

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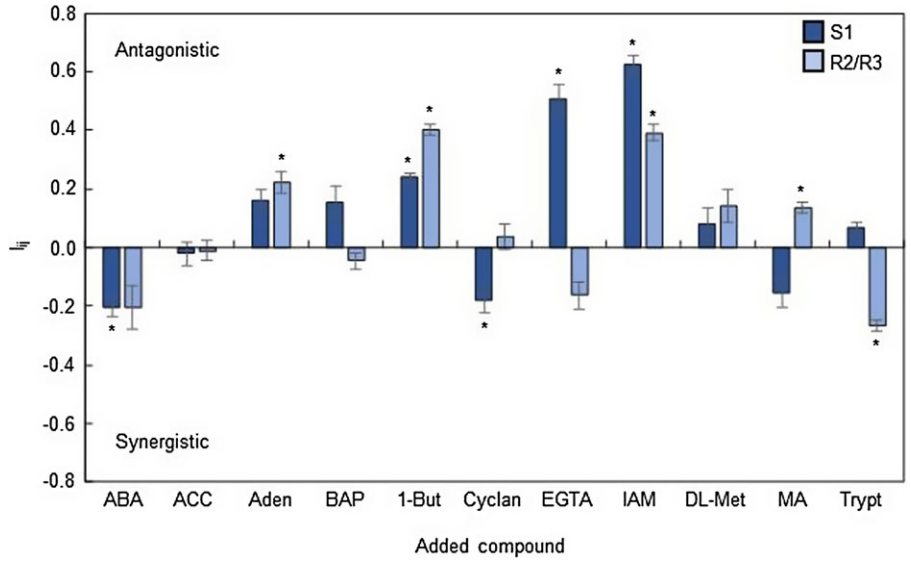


**Figure 2.** Interaction between 2,4-D and other auxin-related compounds in 2,4-D–susceptible (S1) or 2,4-D–resistant (R2 or R3) *Raphanus raphanistrum* populations. Seedling radicle elongation on agar in the presence of 2,4-D, a potential synergist, or both, was measured, and the interaction between chemicals was assessed using a Colby analysis. Values are means ± SE of three replicates. Asterisks denote an interaction ($I_{ij}$) significantly different from an additive interaction, with negative values indicating synergism and positive values indicating antagonism. Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; Aden, adenosine; BAP, 6-benzylaminopurine; 1-But, 1-butanol; Cyclan, cyclanilide; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IAM, indole-3-acetamide; DL-Met, DL-methionine; MA, methyl anthranilate; Trypt, tryptamine.

*R. raphanistrum* is too high for it to be used as an efficient synergist, and whether a combination of tryptamine and auxinic herbicide would be detrimental to the surrounding cereal crop.

### Dose–Response Assays with Auxinic Herbicides

It is known that different synthetic auxins preferentially bind to different members of the TIR/AFB receptor family (Walsh et al. 2006), leading to varying herbicide efficacies in different plant species. In a study on three representative auxin receptors (TIR1, AFB2, and AFB5), Prusinska et al. (2022) demonstrated that among the phenoxy acid class of synthetic auxin herbicides, mecoprop binds more strongly to TIR1 than do 2,4-D and MCPA, while dicamba (benzoic acid class) binds weakly to all three receptors, and halauxifen (picolinic acid class) binds extremely strongly to AFB5. It is likely that in weedy plant species, the relative efficacy of various auxinic herbicides will be determined by the levels of receptor sequence similarity and expression. For example, halauxifen is markedly more effective on *C. canadensis* than either 2,4-D or dicamba (McCauley and Young 2019), and 2,4-D is more effective than dicamba on *R. raphanistrum* (Goggin et al. 2018).

The pot experiments investigating the efficacy of MCPA, mecoprop, and a 1:1 mixture on 2,4–D–resistant *R. raphanistrum* showed that overall, these herbicides are not effective at controlling these populations. In the glasshouse experiment on 12 populations, there were no significant differences between herbicide treatments in any of the populations. Reliable $ED_{50}$ values could not be calculated (see Supplementary Table S1), because only four doses of each herbicide or mixture were used rather than the recommended minimum of six to eight (Keshtkar et al. 2021), but at the highest herbicide rate (1,200 g $ha^{-1}$) the 2,4-D–resistant populations, with the exception of population R2, had significantly higher survival than populations S1 and S2, ranging from 6% to 70% (Figure 3A). In the outdoor dose–response experiment on five populations, there were also few significant differences between herbicide treatments, except that R2 showed a 4-fold higher $ED_{50}$ to mecoprop stand-alone compared with the MCPA or MCPA plus mecoprop treatments (Figure 3B). Again, the 2,4-D–resistant populations showed higher survival than the S2 population (with the exception of R2 treated with MCPA alone or in a mix; Figure 3B).

Overall, in spite of anecdotal reports that a mixture of MCPA and mecoprop is effective on 2,4-D–resistant *R. raphanistrum*, the current study demonstrated that although the resistance indices of these herbicides (average 10 for MCPA, 9 for mecoprop, and 8 for a 1:1 mixture) were lower than those of 2,4-D (average 30) and dicamba (average 23) measured in a previous study (Goggin et al. 2018), there was a significant lack of weed control, and it is likely that the 2,4-D–resistance mechanism(s) in these populations is also effective against the other phenoxy acids.

The agar-based experiments on halauxifen, 2,4-D, and picloram showed that the $ED_{50}$ values obtained for 2,4-D (Table 3) were similar to those in a previous study on the same populations (Goggin et al. 2018). Based on the ratios of $ED_{50}$ values, there was less resistance to halauxifen and picloram than to 2,4-D (Table 3). On average, 2,4-D was >50 times more effective than picloram and 3 times more effective than halauxifen on *R. raphanistrum*, although the difference between 2,4-D and halauxifen was only statistically significant in the susceptible populations (Table 3). The fact that halauxifen was on average 25 times more potent than another AFB5-binding herbicide, picloram, again indicates the complexity and species specificity of the auxin response in weeds.

### Preemergence Herbicide Treatments

There are indications that reduced translocation of 2,4-D from the treated leaf may contribute to either resistance itself or to the recovery of resistant plants in the 2,4-D–selected *R. raphanistrum* populations under study (Goggin et al. 2020). Therefore, a preemergence application of auxinic herbicides could potentially circumvent this resistance mechanism. However, the 2,4–D–selected populations, when treated preemergence, showed high

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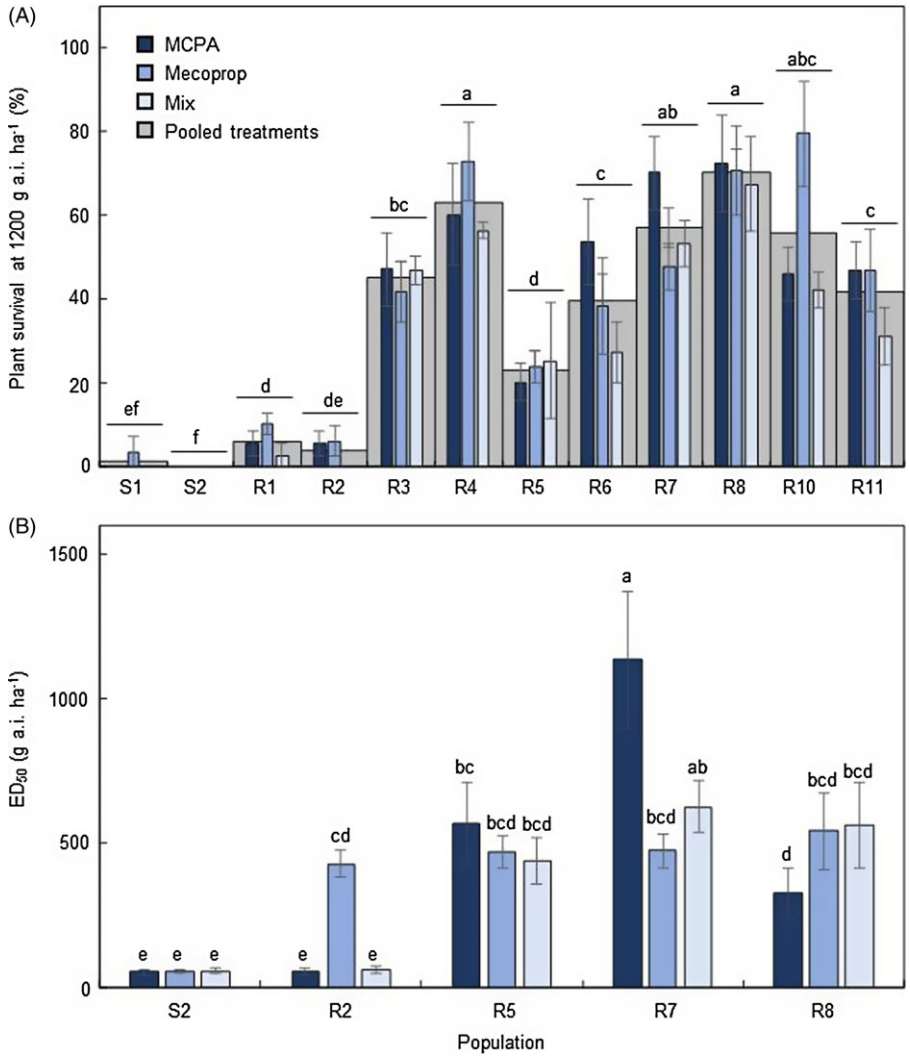


**Figure 3.** Response of 2,4-D–resistant *Raphanus raphanistrum* populations to MCPA and mecoprop. Populations at the 2-leaf stage were sprayed with MCPA, mecoprop, or a 1:1 mix of each herbicide, and their survival was assessed after 21 d. (A) Survival in the glasshouse following treatment with 1,200 g ai $ha^{-1}$ MCPA or mecoprop standalone, or 600 + 600 g $ha^{-1}$ MCPA + mecoprop. As there were no significant differences among treatments within each population, the data were pooled, and means are shown as wide gray bars behind the blue bars that represent each individual herbicide treatment. Different letters above bars denote significant ($P < 0.05$) differences among populations in response to the pooled treatments (values are means ± SE; $n = 3$). (B) Dose of MCPA or mecoprop or a 1:1 mix required to kill 50% of individuals ($ED_{50}$) in an outdoor dose–response experiment. Different letters above bars denote significant differences in $ED_{50}$ values within and among populations.

survival to all three herbicides (Figure 4). Survival of the field-collected populations with suspected 2,4-D resistance was lower than for the 2,4-D–selected populations, but was still above 20%. There was no significant difference in the effect of 2,4-D, MCPA or dicamba applied preemergence compared with postemergence in the field-collected populations, and no difference among the three herbicides (Figure 4). Although not compared statistically, the percent survival of the 2,4-D–selected populations to auxinic herbicides applied preemergence was similar to their survival under postemergence regimes used previously (Goggin et al. 2018). The observed lack of improvement in auxinic herbicide efficacy in this experiment not only rules out preemergence application as a potential weed control method, but also indicates that the major resistance mechanism in these *R. raphanistrum* populations is likely present in germinating seedlings as well as in older plants.

In conclusion, there are no immediately promising candidates to synergize with 2,4-D within the bounds of the auxin-related compounds used in this study. Replacing 2,4-D with an alternative auxinic herbicide (MCPA, mecoprop, halauxifen) was also ineffective, and in any case would likely lead to a resistance problem even if the treatments were initially successful (Vencill et al. 2012). Chemical control of 2,4-D–resistant *R. raphanistrum* is likely to be more successful when two (or more) different modes of action are mixed, as demonstrated in Busi et al. (2022). Nonchemical *R. raphanistrum* control tactics that have shown promise in field-based studies are centered around depletion of the soil seedbank by (1) using minimal tillage, leaving the seeds exposed to harsh conditions and ant predation over summer; (2) incorporating a pasture phase in which slashing and cutting are timed to minimize flowering and seed set; (3) collecting weed seeds at harvest; and (4) avoiding introduction of weed seeds from contaminated farm equipment, stock feed, or grain (Cheam et al. 2008). Interestingly, treatment of *R. raphanistrum* with auxinic herbicides makes it more palatable to livestock (Cheam et al. 2008), so a cropping field heavily infested with 2,4-D survivors could potentially be grazed to prevent the problem becoming worse in subsequent years.

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**Table 3.** Response of 2,4-D–resistant and 2,4-D–susceptible *Raphanus raphanistrum* populations to halauxifen and picloram: seedlings were grown on agar in the presence of various concentrations of 2,4-D, halauxifen, or picloram, and their rate of root elongation was measured.

| A. Herbicide concentrations resulting in 50% inhibition of root elongation ($ED_{50}$) were estimated from dose–response curves.[a] | | | |
|---|---|---|---|
| | $ED_{50}$ (μM) ± SE | | |
| Population | 2,4-D | Halauxifen | Picloram |
| S1 | 0.022 ± 0.008 hi | 0.110 ± 0.030 fg | 1.110 ± 0.566 abc |
| S2 | 0.016 ± 0.005 i | 0.064 ± 0.028 egi | 1.621 ± 0.660 bfh |
| R1 | 0.697 ± 0.094 cd | 0.414 ± 0.072 de | 4.908 ± 1.239 ab |
| R3 | 0.193 ± 0.043 ef | 0.295 ± 0.057 ef | 13.483 ± 2.252 a |
| B. The ratios of $ED_{50}$ values for pairs of populations were calculated to indicate the relative level of resistance to each herbicide.[b] | | | |
| | Population comparison within herbicides ($ED_{50}$ ratio ± SE) | | |
| Comparison | 2,4-D | Halauxifen | Picloram |
| S2:S1 | $0.7 \pm 0.4^{ns}$ | $0.6 \pm 0.3^{ns}$ | $1.5 \pm 1.0^{ns}$ |
| R1:S1 | 32 ± 12* | 3.8 ± 1.2* | $4.4 \pm 2.5^{ns}$ |
| R3:S1 | 8.8 ± 3.8* | $2.7 \pm 0.9^{ns}$ | $12 \pm 6.5^{ns}$ |
| R1:S2 | 44 ± 16** | $6.5 \pm 3.0^{ns}$ | $3.0 \pm 1.5^{ns}$ |
| R3:S2 | 12 ± 5.0* | $4.6 \pm 2.2^{ns}$ | 8.3 ± 3.7* |
| R3:R1 | 0.3 ± 0.1** | $0.7 \pm 0.2^{ns}$ | 2.7 ± 0.8*** |
| C. The ratios of $ED_{50}$ values for pairs of herbicides within each population were calculated to indicate the relative effectiveness of each herbicide.[b] | | | |
| | Herbicide comparison within populations ($ED_{50}$ ratio ± SE) | | |
| Population | Halauxifen:2,4-D | Picloram:2,4-D | Picloram:halauxifen |
| S1 | 5.1 ± 2.3*** | 51 ± 32*** | 10 ± 5.8*** |
| S2 | 4.0 ± 2.2*** | 102 ± 55*** | 30 ± 18*** |
| R1 | $0.6 \pm 0.1^{ns}$ | 7.0 ± 2.0*** | 12 ± 3.6*** |
| R3 | $1.5 \pm 0.5^{ns}$ | 70 ± 19*** | 46 ± 12*** |

[a]Different letters denote significant ($P < 0.05$) differences between $ED_{50}$ values.
[b]Significance levels: ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

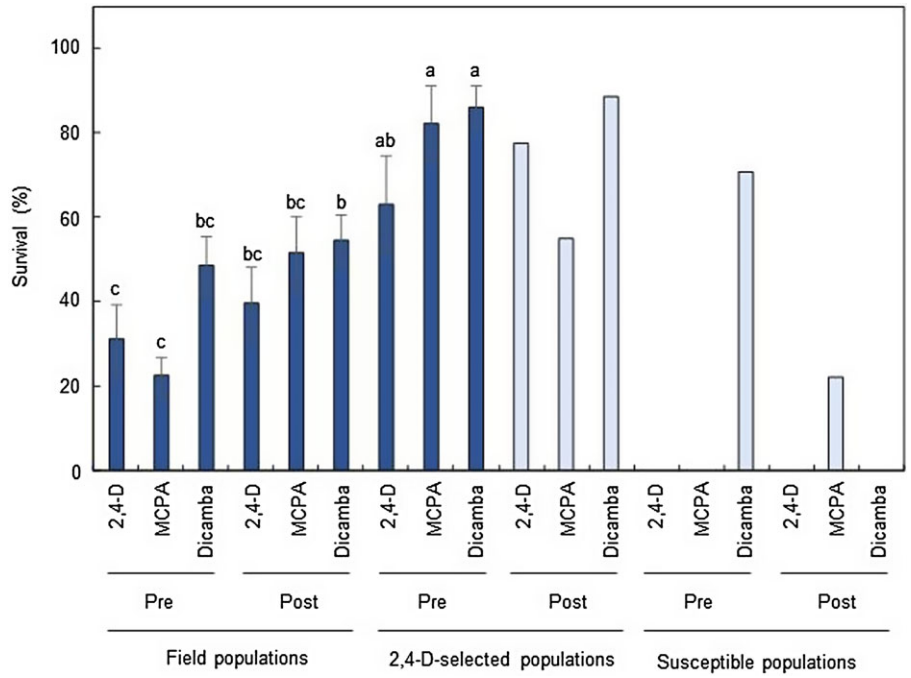


**Figure 4.** Assessment of auxinic herbicides applied preemergence to suspected and confirmed 2,4-D–resistant *Raphanus raphanistrum* populations. Field-collected populations with suspected resistance to 2,4-D (11 populations) and the confirmed resistant, 2,4-D–selected populations (populations R1–R11) were sprayed preemergence with 560 g $ha^{-1}$ 2,4-D, 570 g $ha^{-1}$ MCPA, or 750 g $ha^{-1}$ dicamba, and the field-collected populations were also sprayed postemergence as part of the same experiment (dark blue bars). Values are means ± SE ($n = 11$, with each population representing one replicate), and different letters above bars denote significant ($P < 0.05$) differences between means. For visual comparison, the averaged data for populations R1–R11 sprayed postemergence with 500 g $ha^{-1}$ 2,4-D or dicamba or 600 g $ha^{-1}$ MCPA were also included, along with the pre- and postemergence data for the pooled susceptible (S1 and S2) populations (light blue bars). Data for the postemergence 2,4-D and dicamba treatments of populations R1–R11 were taken from Goggin et al. (2018: supplementary table S3) and from the current glasshouse study for the postemergence MCPA treatment.

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**Supplementary material.** To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2023.54

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