Weed Science

www.cambridge.org/wsc

Rapid necrosis: a novel plant resistance mechanism to 2,4-D

Andrew R. S. de Queiroz[1], Carla A. Delatorre[2], Felipe R. Lucio[3], Caio V. S. Rossi[3], Luiz H. S. Zobiole[3] and Aldo Merotto Jr[2]

[1]Graduate Student, Department of Crop Science, Federal University of Rio Grande do Sul–UFRGS, Porto Alegre, RS, Brazil; [2]Professor, Department of Crop Science, Federal University of Rio Grande do Sul–UFRGS, Porto Alegre, RS, Brazil and [3]Integrated Field Science, Dow AgroSciences Industrial LTDA, São Paulo, Brazil

Research Article

Cite this article: Queiroz ARS de, Delatorre CA, Lucio FR, Rossi CVS, Zobiole LHS, Merotto A Jr (2020) Rapid necrosis: a novel plant resistance mechanism to 2,4-D. Weed Sci. **68**: 6–18. doi: 10.1017/wsc.2019.65

Received: 6 March 2019
Revised: 19 May 2019
Accepted: 24 October 2019
First published online: 5 November 2019

Associate Editor:
William Vencill, University of Georgia

Keywords:
Auxin; herbicide resistance; hypersensitive response; rapid response; reactive oxygen species; Sumatran fleabane; tall fleabane

Author for correspondence:
Aldo Merotto Jr, Department of Crop Science, Federal University of Rio Grande do Sul–UFRGS, 7712 Bento Goncalves Avenue, Porto Alegre, RS, Brazil 91501-970. (Email: merotto@ufrgs.br)

Abstract

Plants of Sumatran fleabane [*Conyza sumatrensis* (Retz.) E. Walker] were identified in a field with an unusual rapid leaf-injury herbicide symptoms after application of 2,4-D in mixture with glyphosate. The objectives of this study were to confirm the occurrence of resistance to 2,4-D herbicide and to characterize the occurrence of rapid necrosis as the mechanism associated with the herbicide resistance in *C. sumatrensis*. The studies performed were an initial screening, effect of 2,4-D alone and associated with glyphosate, cross- and multiple-resistance evaluation, effect of commercial formulation and analytical product, and rate of H_2O_2 evolution. The Marpr9-rn accession was identified with rapid necrosis symptoms and survival to 804 g ae ha^{-1} of 2,4-D. The resistance factor to the herbicide 2,4-D was 18.6 at 49 d after spraying. The analytical product 2,4-D and the commercial formulation resulted in similar symptoms of rapid necrosis. This symptom did not occur for the six other auxinic herbicides (dicamba, florpyrauxifen-benzyl, fluroxypyr, halauxifen-methyl, picloram, and triclopyr), indicating absence of cross-resistance. Multiple resistance to the herbicides paraquat, saflufenacil, and ammonium glufosinate was not identified in the Marpr9-rn population. However, survival following treatment with the herbicides glyphosate and chlorimuron-ethyl occurred. The evolution of H_2O_2 began at 15 min after application and was less pronounced in low light. These results indicate the first case of resistance to 2,4-D and occurrence of rapid necrosis in *C. sumatrensis*.

Introduction

Recently, biotypes of Sumatran fleabane [*Conyza sumatrensis* (Retz.) E. Walker] exhibiting an unusual contact-type herbicide symptom after application of 2,4-D in mixture with glyphosate were detected in Paraná State, Brazil, after burndown application before soybean [*Glycine max* (L.) Merr.] sowing. In those plants, the classical epinasty symptoms in response to 2,4-D were not observed, and the plants exhibited, within a few hours, rapid leaf-cell death, visually similar to necrosis. After approximately 10 d, regrowth occurred, resulting in plant survival and seed production.

Herbicide resistance in the genus *Conyza* has been documented to the following modes of action: inhibitors of photosystem I and II, enolpyruvylshikimate phosphate synthase (EPSPS), and acetolactate synthase (ALS) (Fuerst et al. 1985; Kruger et al. 2009; Lehoczki et al. 1984; VanGessel 2001). Multiple resistance has been reported to glyphosate (EPSPS inhibitor) and chlorimuron-ethyl (ALS inhibitor) (Santos et al. 2014) and to glyphosate, chlorimuron-ethyl, and paraquat (Heap 2019). Chemical alternatives in use involve inhibitors of glutamine synthetase, as glufosinate-ammonium, and mainly the synthetic auxins dicamba and 2,4-D (Flessner et al. 2015; Soares et al. 2012). The evolution of resistance to glyphosate and ALS inhibitors possibly resulted in the increase of the selection pressure of 2,4-D in *C. sumatrensis* in southern Brazil.

Auxinic herbicides have been used as effective weed control agents since the introduction of 2,4-D herbicides in 1945 (Smith 1989). Despite resistance to 2,4-D being reported as early as 1957 in wild carrot (*Daucus carota* L.) and spreading dayflower (*Commelina diffusa* Burm. f.) (Hilton 1957; Switzer 1957), the number of auxin-resistant weed species has been considered low based on long worldwide use of 2,4-D (Heap 2019; Peterson et al. 2016). Resistance to auxinic herbicides has been studied in several weeds such as prickly lettuce (*Lactuca serriola* L.), wild mustard (*Sinapis arvensis* L.), wild radish (*Raphanus raphanistrum* L.), and yellow starthistle (*Centaurea solstitialis* L.) (Burke et al. 2009; Hall et al. 1993; Jugulam et al. 2013; Valenzuela-Valenzuela et al. 2001; Wang et al. 2001). Different mechanisms have been implicated in resistance or tolerance to 2,4-D, such as decreased absorption in ground ivy (*Glechoma hederacea* L.) (Kohler et al. 2004), reduced translocation in *R. raphanistrum* and common hempnettle (*Galeopsis tetrahit* L.) (Goggin et al. 2016; Weinberg et al. 2006),

increased metabolism of 2,4-D in waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] (Figueiredo et al. 2018), and differential binding to auxin-binding proteins (Mockaitis and Estelle 2008). To date, resistance to 2,4-D has been reported in 26 weed species, as well as seven cases of dicamba resistance, four cases of fluroxypyr resistance, and three cases of picloram resistance (Heap 2019). The specific mechanisms of resistance are unknown for the majority of cases.

Two main types of auxin receptors are known, ABP1 and the complex $SCF^{TIR1/AFB}$ (Grones and Friml 2015). ABP1 is believed to be involved in fast responses to auxin (Dahlke et al. 2010), and the complex $SCF^{TIR1/AFB}$ acts in combination with the auxin regulators Aux/IAAs in the transcriptional response to auxins (Mockaitis and Estelle 2008). The herbicide 2,4-D is thought to bind to the auxin receptor TIR1 or some analogue, causing higher expression of auxin-responsive genes, leading to increased synthesis of ethylene and abscisic acid (ABA) (Grossmann 2010; Song 2014). Inability of plants to modulate the concentration of 2,4-D results in continuous auxin signaling, leading to the classical symptoms of epinasty, followed by growth inhibition and death (Grossmann 2010). The resistance to auxinic herbicides related to lower translocation (Mithila et al. 2011; Riar et al. 2011; Weinberg et al. 2006) might involve changes in influx auxin transporters AUX1/LAX (AUXIN RESISTANT 1/LIKE AUX1), efflux-transporters PIN (PIN-FORMED), and ABC transporters (Cho and Cho 2013; Titapiwatanakun and Murphy 2009). In *Arabidopsis thaliana*, mutations in genes coding for auxin receptors and transporters can generate plants resistant to auxinic herbicides (Kubes et al. 2012; Roux and Reboud 2005; Walsh et al. 2006; Yu and Wen 2013). However, the role of these proteins in causing resistance to auxinic herbicides in weeds has not yet been characterized.

About 33 dicotyledonous weed species have been reported as having evolved resistance to auxinic herbicides (Heap 2019). In general, resistant individuals are able to produce asymptomatic new growth within a week of herbicide application. Variation of 2,4-D effects in the genus *Conyza* has been described by studies developed by Kruger et al. (2008, 2010), in which an accession was able to survive and produce seeds after treatment with 560 g ae ha^{-1} of 2,4-D. In these studies, the resistance mechanism was not described, but abnormal symptomology has not been reported.

The symptoms described here in biotypes of *C. sumatrensis* are similar to those defined as rapid response or hypersensitive response, identified in giant ragweed (*Ambrosia trifida* L.) resistant to glyphosate (Brabham et al. 2011; Jeffery 2014; Lespérance 2015; Van Horn et al. 2017). Rapid cell death and H_2O_2 accumulation were found in leaves of this accession of *A. trifida* after glyphosate application, symptoms similar to those seen with photosystem I inhibitors (Jeffery 2014). Alterations in chloroplast volume and starch accumulation in photosynthetic tissues were also found (Lespérance 2015). These symptoms were inhibited in the absence of light (Jeffery 2014) and appeared to be related to programmed cell death (Lespérance 2015). Recently, Moretti et al. (2017) suggested that a source of carbon might be required to induce rapid necrosis symptoms. Higher concentrations of [^{14}C]glyphosate in mature leaves and lower concentrations in apical meristems of plants showing rapid necrosis symptoms compared with susceptible plants at 24 h after spraying suggest reduced translocation is a possible resistance mechanism (Lespérance 2015). The nomenclature of this new mechanism of resistance is not a consensus. The denomination of "apoptosis" is described as inappropriate in plants (Doorn et al. 2011), and "hypersensitivity" is more adequate for response to pathogens (Roden and Ingle 2009). "Rapid response" is also used, but the response could be related to several other plant reactions not associated with cell and tissue death. The term "rapid necrosis" is associated with the acute cell death response that develops rapidly in plants (Proskuryakov et al. 2003), which is more closely related to the first observed symptom in the 2,4-D–resistant plants. It will be useful to rename this process once the actual cause of this mechanism of resistance is identified.

In glyphosate-resistant *A. trifida* with rapid necrosis symptoms, it was observed that the rapid increase of H_2O_2 (a reactive oxygen species, or ROS) predates leaf necrosis (Moretti et al. 2017). The rise of ROS in response to auxins has also been demonstrated (Morré et al. 1995; Pazmiño et al. 2012; Peer et al. 2013). Auxin-induced ROS production seems to be mediated, at least in part, by the NAD(P)H oxidase RbohD (Mangano et al. 2017; Peer et al. 2013), and it is a downstream signal for auxin (Schopfer et al. 2002). However, ROS are also involved in auxin oxidation, an irreversible mechanism of auxin elimination (Peer et al. 2013). Therefore, resistance to 2,4-D might be obtained by changing redox status. Consequences of ROS include lipid peroxidation followed by leaf necrosis. The rapid and deregulated production of ROS could be the mechanism causing the occurrence of rapid necrosis in plants that have resistance to herbicides.

The objectives of this study were to evaluate the occurrence of resistance to 2,4-D herbicide and to characterize the rapid necrosis as the mechanism of resistance in *C. sumatrensis*.

Materials and Methods

Plant Material, Screening and Symptom Characterization

Seeds of 10 *C. sumatrensis* plants were individually collected in the spring of 2016 in a crop field located in the city of Maripá, PR, Brazil (24.55°S, 53.72°W), where the herbicides 2,4-D and glyphosate were used in the burndown application. Plants in that area showed an atypical symptomatology of 2,4-D herbicide associated with the occurrence of rapid necrosis and resumption of plant growth similar to the effect of contact herbicides (Figure 1A). The susceptible control populations used in this study consisted of the accessions Londs4-s (23.33°S, 51.21°W) and SM (29.71°S, 53.73°W) collected in Londrina, PR, and Santa Maria, RS, Brazil, respectively. The procedure for seed germination started by soaking the seeds in water at 4 ± 2 C for 4 d. After that, the seeds were transferred to plastic trays (15 cm by 10 cm) containing substrate based on charcoal rice husks and vermiculite in a ratio of 1:1. Pots were maintained in a growth chamber (Percival, Boone, IA, USA) at a temperature of 23 ± 3 C and a photoperiod of 13 h (300 µmol m^{-2} s^{-1}). Plants with one developed leaf were transplanted into 200-ml plastic pots with drainage holes containing a mixture (3:1) of the substrate described earlier and soil (classified as Hapludult, sandy clay loam, with 34% clay). The fertilizer NPK (5-20-10) was added at 5 g kg^{-1} of substrate and soil mixture. Six individual plants derived from each of the field-sampled plants were used for a total of 60 plants. The plants were maintained in a greenhouse at 25 ± 5 C and irrigated daily.

The initial screening was performed with the herbicide 2,4-D (DMA® 806 BR SL, Dow AgroSciences Industrial, São Paulo, SP, Brazil) at a dose of 804 g ae ha^{-1}. The herbicide application was performed when the plants were between 7- and 12-cm tall (7 to 10 leaves) in a spray chamber (Generation III Research Sprayer, DeVries Manufacturing, Hollandale, MN, USA) calibrated at 262 kPa delivered by an 80.02E nozzle, resulting in an output volume equivalent to 200 L ha^{-1}. The symptoms of rapid

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

Figure 1. *Conyza sumatrensis* plants (A) under field conditions showing the rapid necrosis symptoms and (B) in the greenhouse after 2 h. The susceptible Londs4-s (left) and Marpr9-rn (right) accessions at 1 (C), 2 (D), and 21 (E) d after treatment of 806 g ha^{-1} of 2,4-D.

necrosis were monitored at 15, 30, 60, 120, 240, and 420 min and 1, 2, 4, 8, 12, 15, 21, and 28 d after treatment (DAT). After the last evaluation (28 DAT), the surviving plants with rapid necrosis were selected and transplanted to 5-kg pots. Before flowering, the plants were protected with a soft and fine tulle fabric to avoid pollen contamination and seed movement. The following studies were conducted using seeds from a single rapid necrosis surviving plant named Marpr9-rn.

Additional characterization of the rapid necrosis symptoms was performed regarding the effect of glyphosate and of the compounds present in the commercial 2,4-D formulation. The evaluation of the effect of glyphosate (Glizmax Prime® SL, Dow AgroSciences Industrial) was performed by application of the herbicide 2,4-D at doses of 40.2 and 402 g ae ha^{-1}, glyphosate at 864 g ae ha^{-1}, and 2,4-D at 402 g ae ha^{-1} in a tank mixture with glyphosate at 864 g ae ha^{-1}. The evaluation of the effect of the 2,4-D formulation consisted of commercial 2,4-D herbicide (DMA® 806 BR), 2,4-D analytical product (ACS standard) with 98% purity (Sigma Aldrich, St Louis, MO, USA) diluted in a solution of water and ethanol 50/50%, and an untreated check. Both 2,4-D treatments were applied at a dose of 804 g ae ha^{-1}. The experiment was conducted once, and the number of replicates was five. Plant growth and herbicide spraying conditions were similar to those described earlier. The plant injury evaluation was performed at 2, 4, and 8 h and 1, 3, 7, 14, and 21 DAT. Data analyses were performed using ANOVA followed by a Tukey's test at 5%.

Dose–Response Curve to 2,4-D

The experiments were carried out with completely randomized design, in a bifactorial arrangement, with four replicates. The experiments were repeated, and results were also confirmed in plants from the second generation (seeds from the second autocross derived from single-seed descendants of the plants identified in the field). Factor A consisted of two 2,4-D–susceptible (Londs4-s and SM) and Marpr9-rn accessions. Factor B was the 2,4-D dosage: 0, 50.25, 100.5, 201, 402, 804, and 1,608 g ae ha^{-1}. Herbicide spraying was performed as described earlier. The occurrence of symptoms of rapid necrosis was evaluated at 2 and 4 h and 1, 7, 14, 21, 28, 35, 42, and 49 DAT by scoring the visual injury at percentage scale, where 0% corresponded to absence of symptoms and 100% to total plant necrosis. The injury was converted to percent tolerance (green leaves) by calculating 100 − injury. Fresh and dry aboveground weight were evaluated at 49 DAT. Data were analyzed by ANOVA followed by a three-parameter nonlinear logistic model:

$$[y = a/1 + (x/x_0)^b] \quad [1]$$

where y is the response expressed as percentage of the untreated control, a is the asymptotic value of y at the upper limit, b is the slope of the curve around the point of inflection, and x_0 is the dose required to reduce the y value by 50% (LD_{50}) when $a = 100$.

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

Table 1. Herbicide and doses used in cross-resistance, dose–response, and multiple-resistance experiments.

Herbicide	Dose	Commercial product
	—g ae or ai ha^{-1}—	
—Cross-resistance experiment—		
2,4-D	804	DMA® 806 BR SL[a]
Florpyrauxifen	7.5	Loyant™SL[b]
Fluroxypyr	299.7	Prestige® EC[a]
Halauxifen	7	Arylex™ SC[b]
Picloram	480	Padron® SL[a]
—Dose–response experiments—		
2,4-D	0, 50.25, 201, 402, 804, 1,608, and 3,216	DMA® 806 BR SL[a]
Dicamba	30, 120, 240, 480, 960, and 1,920	Clarity® SL[c]
Triclopyr	45, 180, 360, 720, 1,440, and 2,880	Garlon 480 BR® EC[a]
—Multiple-resistance experiment—		
2,4-D	804 and 1,608	DMA® 806 BR SL[a]
Ammonium glufosinate	600 and 1,200	Finale® SL[d]
Chlorimuron	20 and 40	Clorim WG[e]
Glyphosate	1,440 and 2,880	Glizmax Prime® SL[a]
Paraquat	400 and 800	Gramoxone® SL[f]
Saflufenacil	49 and 98	Heat® WG[c]

[a]Dow AgroSciences Industrial Ltda., São Paulo, SP, Brazil; https://www.corteva.br.
[b]Dow AgroSciences LCC, Indianapolis, IN, USA; https://www.corteva.us.
[c]BASF Corporation, Durham, NC, USA; www.basf.com/usa.
[d]Bayer AG, Belford Roxo, RJ, Brazil; https://www.bayer.com.br.
[e]UPL do Brasil, Ituverava, SP, Brazil; https://br.uplonline.com.
[f]Syngenta, Paulínia, SP, Brazil; https://www.syngenta.com.br.

The software SigmaPlot v. 11 was used to perform the regressions. The resistance factor (RF) was obtained by the ratio of the LD_{50} values of the resistant and susceptible accessions.

Analyses of Cross-Resistance and Multiple Resistance

The experiments were carried out in a completely randomized design in a factorial arrangement with four independent biological replicates and were repeated twice. The doses used are described in Table 1. In all experiments, Factor A consisted of the known susceptible Londs4-s and the putative resistant Marpr9-rn. The cross-resistance experiment consisted of the herbicides 2,4-D, fluroxypyr (Prestige® EC, Dow AgroSciences Industrial), picloram (Padron® SL, Dow AgroSciences Industrial), florpyrauxifen-benzyl (Loyant™SL, Dow AgroSciences, Indianapolis, IN, USA), and halauxifen-methyl (Arylex™ SC, Dow AgroSciences).

An additional cross-resistance dose–response experiment was carried out with the herbicides 2,4-D, dicamba (Clarity® SL, BASF, Durham, NC, USA), and triclopyr (Garlon 480 BR® EC, Dow AgroSciences Industrial). The visual injury was evaluated at 2, 14, and 49 DAT. Data were analyzed as described earlier for the dose–response curve for 2,4-D.

For the multiple-resistance experiment, Factor B was the herbicides 2,4-D, saflufenacil (Heat® WG, BASF, Guaratinguetá, SP, Brazil), chlorimuron-ethyl (Clorim WG, DVA Agro do Brasil, Barueri, SP, Brazil), paraquat (Gramoxone® SL, Syngenta, Paulínia, SP, Brazil), ammonium glufosinate (Finale® SL, Bayer, Belford Roxo, RJ, Brazil), and glyphosate (Glizmax Prime® SL, Dow AgroSciences Industrial). Factor C was the doses as described in Table 1. The herbicides were applied when the plants presented 10 to 15 leaves (10 to 14 cm). The spraying conditions and plant injury evaluations were the same as described earlier. The visual injury was evaluated at 2, 14, and 49 DAT. Fresh and dry aboveground weights were evaluated at 49 DAT. Data analyses for the cross- and multiple resistance were evaluated using ANOVA followed by a Tukey test at 5%.

Evaluation of Presence of H_2O_2 in Function of Light

The evaluation of the presence of H_2O_2 was performed using the 3,3′-diaminobenzidine-hydrochloride (DAB) staining method (Jeffery 2014; Moretti et al. 2017). In the first experiment, the treatments consisted of the susceptible Londs4-s and Marpr9-rn accessions at the 8- to 10-leaf stage treated with 804 g ae ha^{-1} of 2,4-D, with paraquat at 600 g ai ha^{-1} as the positive control and a negative untreated control. The application of the herbicides was performed as already described. Mature leaves were sampled by cutting the petiole base with a scalpel at 7 h after treatment (HAT).

In the second experiment, two 12-mm-diameter leaf disks were collected from the fifth and sixth leaves using a metal hole puncher after 804 g ae ha^{-1} of 2,4-D spraying at time intervals of 0, 15, 30, 60, 120 240, 420, and 1440 min after treatment. Plants were continuously exposed to high (848 µmol m^{-2} s^{-1}) and low light (29 µmol m^{-2} s^{-1}) in a greenhouse with a temperature of 25 ± 5 C. The low-light treatment was obtained with two layers of black mesh with 80% shading. The radiation measure was evaluated at 1200 hours on a sunny day, and the light sensor was positioned at plant level. Each experiment was conducted once, with four replicates.

Leaf and leaf disks were infiltrated with DAB (1 mg ml^{-1}, pH 3.8) and incubated in DAB solution at room temperature overnight. After incubation, samples were decolorized by being boiled in 90% ethanol for 10 min to remove chlorophyll before being photographed. The presence of H_2O_2 was visualized by color change (brown) where DAB polymerized with this compound. The analysis of the amount of area with DAB was carried out using the color deconvolution tool in Image J (Ruifrok and Johnston 2001).

Results and Discussion

Screening and Symptom Characterization

Different mechanisms have been associated with resistance to the herbicide 2,4-D; however, to date, none have been connected with the symptoms of rapid necrosis. Therefore, the characterization of this new phenotype's response to 2,4-D, as determined here, is the first report of the resistance mechanism. The symptoms of rapid necrosis were observed in all six replicates of nine out of the 10 field-sampled plants evaluated after application at 804 g ha^{-1} of 2,4-D in the Marpr9-rn population. The seeds were collected from plants under field conditions approximately 2 mo after spraying, and because of that, seeds from a susceptible plant may also have been sampled, resulting in absence of the rapid necrosis symptoms in one individual. The atypical symptoms of the rapid necrosis in the population Marpr9-rn were noticeable at approximately 2 h after 2,4-D application, with partial leaf wilt and light necrotic spots (Figure 1B), and after 4 h, leaf necrosis appeared mainly in the borders and tips (Figure 1C). At 1 DAT, the leaves presented total necrosis in the blade region (Figure 1D). The progression of the rapid necrosis symptoms was continuously observed, and at 21 DAT, plant growth resumed via development of axillary buds (Figure 1E). The symptoms were observed in the mature leaves and the meristems; rapid necrosis symptoms were absent from young leaves. The symptoms were more evident in the upper leaves, and the lower leaves presented only necrotic spots, probably due to lower herbicide interception. In plants of

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

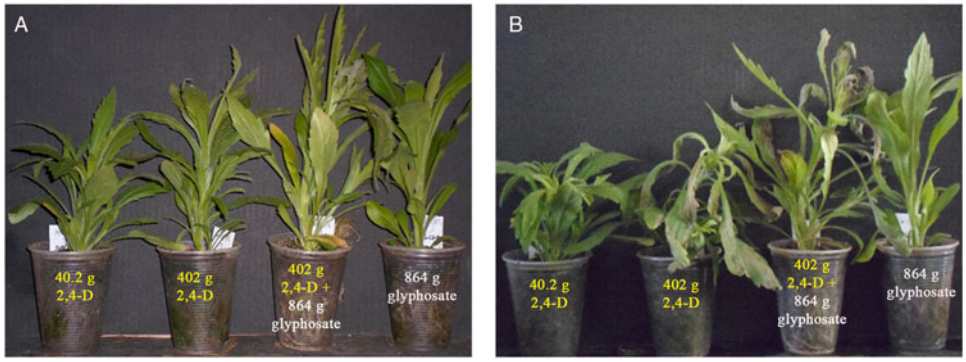

Figure 2. Plants of the *Conyza sumatrensis* Marpr9-rn accession at 4 h (A) and 1 d (B) after treatment of 2,4-D and glyphosate herbicides alone and in combination.

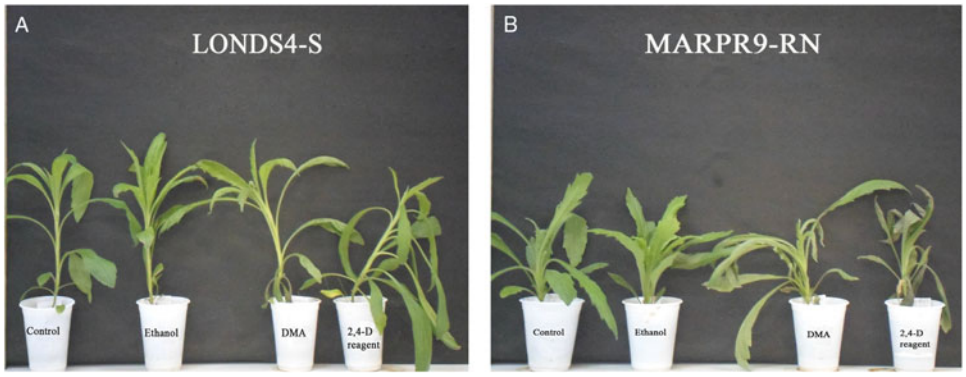

Figure 3. Plants of the *Conyza sumatrensis* Londs4-s (susceptible) (A) and Marpr9-rn (B) accessions at 8 h after treatment of 804 g ha^{-1} of 2,4-D analytical product (reagent) diluted in ethanol 50% and of the commercial DMA formulation.

the susceptible accession, only the usual epinasty symptoms were observed. The symptoms observed in the Marpr9-rn plants are consistent to those of hypersensitivity to pathogens (Bestwick et al. 1998) as well as those described by Brabham et al. (2011) and Van Horn et al. (2017) in glyphosate-resistant *A. trifida*. The time required in the present study for the necrosis symptoms to appear in *C. sumatrensis* treated with the herbicide 2,4-D differed slightly from the time reported for *A. trifida* in response to glyphosate. The onset of necrosis occurred before 4 h after 2,4-D treatment. Van Horn et al. (2017) described necrosis occurring within 6 h after glyphosate application in *A. trifida*. However, a significant delay in the occurrence of the rapid necrosis occurs for plants growing at low radiation (Jeffery 2014). In addition, the rapid necrosis symptoms caused by glyphosate in *A. trifida* is more pronounced in the older leaves, similar to what was observed in the present study. The apical meristem of the treated plants was not affected with rapid necrosis symptom.

The occurrence of rapid necrosis associated with 2,4-D has not been previously described. Therefore, to confirm 2,4-D as the causal agent of this symptomatology, additional evaluations were performed. Considering the similarities between rapid necrosis caused by 2,4-D and the effects of glyphosate in *A. trifida*, response to glyphosate (864 g ae ha^{-1}) was evaluated; rapid necrosis was not observed (Figure 2), despite the occurrence of glyphosate resistance in *C. sumatrensis* in the region where seeds were sampled. Glyphosate has been extensively used at burndown and for POST application for *Conyza* spp. control. However, the symptoms of rapid necrosis identified in *C. sumatrensis* were caused only by 2,4-D, and this symptom also occurred when this herbicide was sprayed in mixture with glyphosate (Figure 2). The intensity and the time required for the onset of the rapid necrosis symptoms depended on the 2,4-D dose.

To confirm that rapid necrosis was a response to 2,4-D and not to any other component of the commercial herbicide formulation, a solution equivalent to 804 g ae ha^{-1} of the analytical product 2,4-D (ACS grade) diluted in ethanol 50% v/v was prepared. The rapid necrosis symptoms occurred similarly for applications of both the commercial and laboratory 2,4-D products (Figure 3B; Table 2). That indicates that other compounds used in the formulation of the herbicide 2,4-D herbicide (DMA™ 806 BR) are not associated with the rapid necrosis and that the 2,4-D molecule is the cause of this symptom.

Dose–Response Curve to 2,4-D

The role of 2,4-D in the rapid necrosis symptoms having been confirmed, the increased tolerance obtained from rapid necrosis was determined. In the evaluation at 2 HAT, only drooping and initial slight necrotic spots symptoms were observed in the Marpr9-rn accession, even for the highest dose of 2,4-D (1,608 g ha^{-1}). No visual symptoms were present in the susceptible Londs4-s accession (Figure 4A and B). At 4 HAT, rapid necrosis began to occur at doses equal to or higher than 100.5 g ha^{-1} of 2,4-D in the Marpr9-rn accession, and mild epinasty appeared in the susceptible Londs4-s (Figure 4C and D). In the evaluation performed at 1 DAT, the typical 2,4-D symptom of epinasty was clearly observed in the susceptible Londs4-s accession, but the more pronounced symptoms of rapid necrosis occurred in the Marpr9-rn

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

Table 2. Plant injury (%) in susceptible (Londs4-s) and resistant to 2,4-D with rapid necrosis (Marpr9-rn) accessions of *Conyza sumatrensis* in response to the application of the 2,4-D analytical product and commercial DMA formulation at 2, 14, and 21 d after treatment (DAT).

Treatment	2 DAT		14 DAT		21 DAT	
	Londs4-s	Marpr9-rn	Londs4-s	Marpr9-rn	Londs4-s	Marpr9-rn
	Injury[a] %					
Control (0)	0.0 aB	0.0 aC	0.0 aC	0.0 aC	0.0 aB	0.0 aC
Ethanol 50%	0.0 aB	0.0 aC	0.0 aC	0.0 aC	0.0 aB	0.0 aC
DMA formulation	23.7 bA	55.0 aB	61.2 aA	51.2 bB	92.5 aA	50.0 bB
2,4-D + ethanol	25.0 bA	65.0 aA	56.2 bB	61.2 aA	86.2 aA	60.0 bA

[a]Lowercase letters compare accessions in the same time (rows), and uppercase letters compare herbicide treatments (columns); $P > 0.05$ using Tukey's test.

accession (Figure 4E and F). Even in the lower leaves, where rapid necrosis was not present, epinasty symptoms were not observed, an indication that the 2,4-D herbicide may not have been translocated from the treated leaves. In the following evaluations, the resumption of growth of the Marpr9-rn plants was observed in contrast to the mortality of the susceptible accession Londs4-s (Figure 4G and H). The dose–response curves of the evaluations at 1, 14, and 49 DAT are shown in Supplementary Figure S1. The result of the rapid necrosis symptoms is the resumption of plant growth in the Marpr9-rn accession, indicated by the increasing RF. In the evaluation at 7 DAT, the RF was 1.16, and this value was 18.62 in the evaluation at 49 DAT (Table 3). In this evaluation, the LD_{50} was 1,133 g ae ha^{-1} of 2,4-D for the Marpr9-rn accession and 60.8 and 56.2 g ha^{-1} for the susceptible Londs4-s and SM accessions, respectively (Table 3).

There have been no scientific reports of resistance to 2,4-D herbicide in the *Conyza* genus. However, in a study carried out with horseweed (*Erigeron canadensis* L.), tolerance differences between populations were evidenced, with an LD_{50} amplitude of 12.3 to 19.7 g ae ha^{-1} and LD_{90} of 42 g to 107 g ae ha^{-1} of 2,4-D among the most susceptible and tolerant populations, respectively (Kruger et al. 2008). The LD values obtained in that study are low and possibly do not characterize herbicide resistance. In addition, the occurrence of rapid necrosis in response to 2,4-D was not observed. In 2,4-D–resistant *A. tuberculatus* plants, the dose required to reduce growth by 50% (GR_{50}) was 176 g ha^{-1}, and the resistance factor was 8-fold in relation to the susceptible accession (Figueiredo et al. 2018). In the same study, previous application of cytochrome P450 inhibitor reduced the GR_{50} of the resistant accession 7-fold, indicating that 2,4-D detoxification was likely to be the mechanism responsible for resistance. However, the occurrence of rapid necrosis has not been reported in 2,4-D–resistant *A. tuberculatus* accessions. The occurrence of rapid necrosis has been reported only in *A. trifida* in response to glyphosate, but the mechanism of resistance is unknown (Moretti et al. 2017).

Cross-Resistance to Auxinic Herbicides

Mechanisms related to defects in the general auxin response pathway are likely to cause resistance to other auxinic herbicides (LeClere et al. 2018). Therefore, studies were conducted to characterize the response of this accession to several additional auxinic herbicides. At 1 d after treatment, the rapid necrosis symptoms were observed in response to 2,4-D but not the other four auxinic herbicides tested (Table 4). The symptom caused by these other auxin herbicides was consistent with the typical auxin-induced epinasty (Figure 5A). At 21 DAT, regrowth was evident only for 2,4-D treatment (Figure 5B). The plants of the Marpr9-rn accession were also susceptible to the herbicides dicamba and triclopyr, as indicated in the dose–response study, in which the RF values at 49 DAT were 1.2 and 0.8, respectively (Table 5; Supplementary Figure S2). Therefore, Marpr9-rn is resistant to 2,4-D, but cross-resistance to the other auxinic herbicides—fluroxypyr, triclopyr and picloram (pyridine carboxylic acid), dicamba (benzoic acid), and florpyrauxifen-benzyl and halauxifen-methyl (arykpicolin acid)—was not identified.

To date, resistance to 2,4-D has been reported in 21 weed species, in some cases coupled with resistance to other auxinic herbicides (Heap 2019). Cross-resistance to 2,4-D, MCPA, and dicamba herbicides was identified in *L. serriola* (Burke et al. 2009) and oriental mustard (*Sisymbrium orientale* L.) (Preston et al. 2013). A complete understanding of the mechanism of resistance to auxinic herbicides has yet to be determined for most resistant species. However, there are several reports about the process possibly involved in the resistance. One possibility is related to defects in the downstream response from auxins, such as the induction of ethylene synthesis. In *S. arvensis*, the resistance to picloram was associated with the activity of the acetyl-CoA synthase enzyme that is a precursor of ethylene (Hall et al. 1993). Similarly, 20-fold lower ethylene levels were identified in a biotype of *C. solstitialis* resistant to picloram (Fuerst et al. 1996). Reduction of ethylene might be associated with the induction of de novo synthesis of a 19-kDa protein found in those biotypes. Reduced translocation was also reported as the mechanism of resistance to 2,4-D in *S. orientale* (Dang et al. 2018) and *L. serriola* (Riar et al. 2011). It was recently observed in plants of *R. raphanistrum* susceptible to 2,4-D that the application of inhibitors of auxin efflux or ABCB transporters mimicked the reduced translocation of the resistant biotype (Goggin et al. 2016).

Changes in the ability to sense the auxin may also cause resistance. Variation of the modulation of calcium transport in the cell was associated with *S. arvensis* resistance to 2,4-D, dicamba, and picloram (Wang et al. 2001). A comparative study with tobacco (*Nicotiana tabacum* L.) cells with antisense ABP1 suggested that resistance to auxinic herbicides would occur due to the lower concentration of ABP (Mithila and Hall 2005). In kochia [*Bassia scoparia* (L.) A. J. Scott], cross-resistance was endowed by mutation of two base pairs (GGT_s – AAT_R) in the gene *IAA16* (LeClere et al. 2018). IAA16 is a member of AUX/IAA family that inhibits the action of auxin-responsive factors in the absence of auxin and is labeled to degradation by 26S proteasome upon auxin interaction with the $CF^{TIR1/AFB}$ complex (Song 2014). The complete cross-resistance pattern to herbicides from the different chemical families was not evaluated in these studies. In addition, the phenotype of rapid necrosis was not reported in any of the studies cited.

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

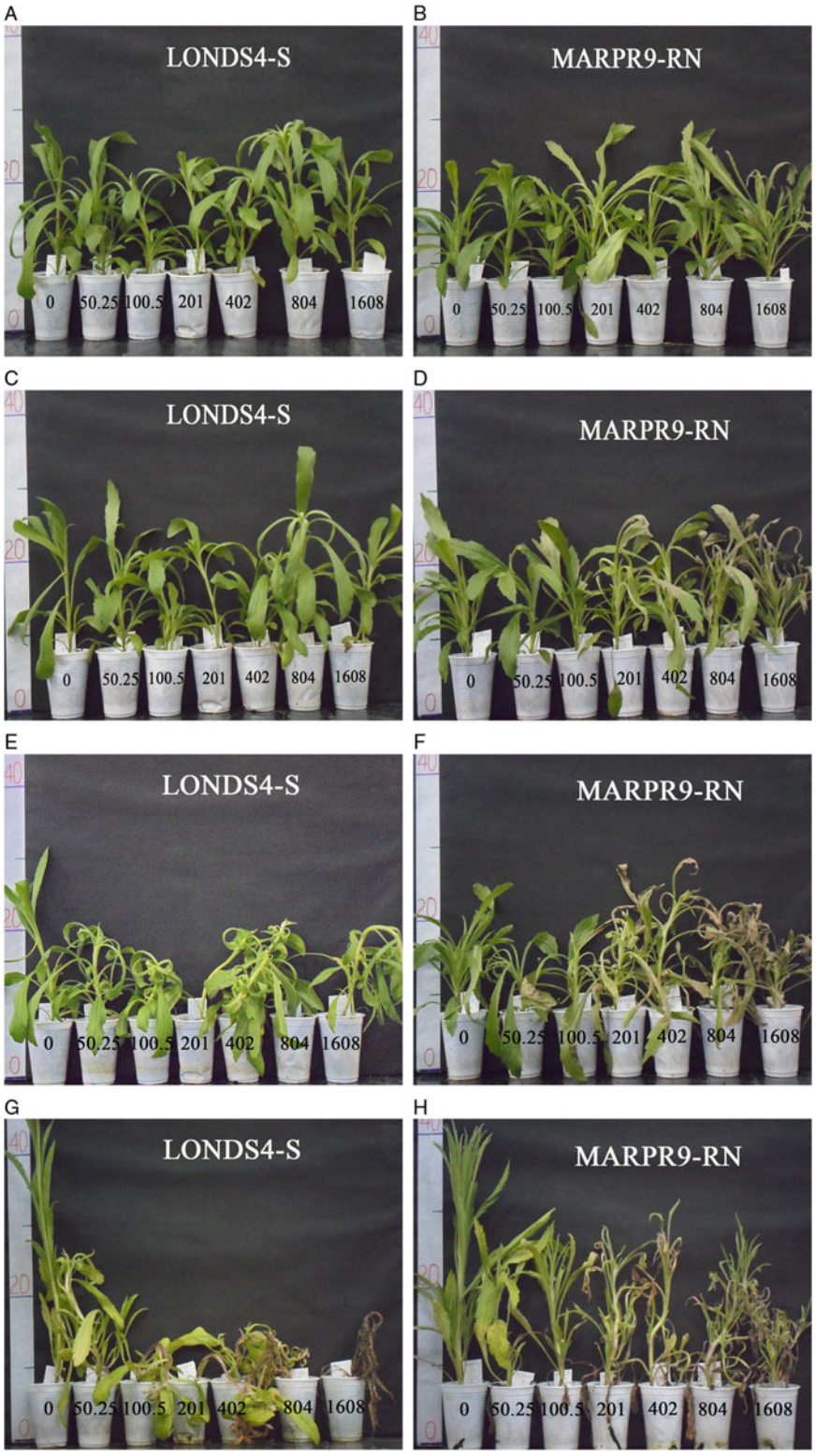

Figure 4. Plants of the *Conyza sumatrensis* Londs4-s (susceptible) and Marpr9-rn accessions treated with 0, 50.25, 100.5, 201, 402, 804, and 1,608 g ae ha^{-1} of 2,4-D at 2 (A, B) and 4 h (C, D) and 1 (E, F) and 28 d (G, H) after treatment.

Multiple-Resistance Analysis

Resistance to other herbicides caused by the same mechanism involved in rapid necrosis in response to 2,4-D was not expected, considering that cross-resistance was not found. However, taking into account that *C. sumatrensis* has been reported as resistant to other herbicides, resistance to several other herbicide modes of action applied at recommended doses was assessed in addition to 2,4-D. The evaluation of the herbicide plant injury effects at 14 and 49 DAT indicated that the herbicides paraquat, ammonium glufosinate, and saflufenacil were efficient for controlling the

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

Table 3. Parameters of the logistic equation,[a] concentrations of the herbicide 2,4-D required for 50% reduction of 100 − visual injury (LD_{50}) of *Conyza sumatrensis*, and resistance factor (RF) in the whole-plant dose–response studies.

Accessions[b]	*a*	*b*	x_0	LD_{50}		RF[e]
				g ha^{-1}[c]	95% CI[d]	
100 − injury (%) at 1 DAT						
Londs4-s	99.62	0.33	12,335.14			
Marpr9-rn	100.81	0.67	554.87	568	(469.5; 640.2)	
SM	100.42	0.25	27,492.22			
100 − injury (%) at 7 DAT						
Londs4-s	99.98	0.57	560.14	559.7	(491.7; 628.5)	
Marpr9-rn	100.86	0.59	629.39	647.8	(518.7; 740.0)	1.16
SM	100.52	0.6	895.79	911	(693.0; 1,098.6)	
100 − injury (%) at 14 DAT						
Londs4-s	99.12	0.61	283.86	275	(189.6; 378.1)	
Marpr9-rn	100.37	0.51	669.40	679	(606.3; 732.5)	2.47
SM	100.15	0.72	505.30	507.4	(415.3; 595.4)	
100 − injury (%) at 21 DAT						
Londs4-s	98.31	0.94	151.60	146	(109.9; 193.2)	
Marpr9-rn	100.86	0.45	641.44	666	(457.6; 825.3)	4.56
SM	98.91	0.88	271.94	265.2	(214.9; 328.9)	
100 − injury (%) at 28 DAT						
Londs4-s	97.71	1.10	126.77	121.4	(94.5; 158.9)	
Marpr9-rn	100.63	0.41	703.93	725	(502.6; 905.3)	5.97
SM	96.73	1.18	181.72	171.5	(139.8; 223.6)	
100 − injury (%) at 35 DAT						
Londs4-s	99.09	1.18	104.32	102.7	(88.00; 120.7)	
Marpr9-rn	100.61	0.45	749.07	769	(568.3; 929.8)	7.49
SM	98.39	1.45	105.12	102.7	(79.5; 130.7)	
100 − injury (%) at 42 DAT						
Londs4-s	99.19	1.24	88.59	87.4	(77.3; 99.9)	
Marpr9-rn	100.60	0.38	1,006.51	1,038	(626.7; 1,386.32)	11.88
SM	100.21	3.29	60.04	60.1	(56.9; 63.5)	
100 − injury (%) at 49 DAT						
Londs4-s	100.23	1.38	60.64	60.8	(55.0; 66.3)	
Marpr9-rn	100.56	0.36	1,098.67	1,133	(664.6; 1532.8)	18.63
SM	100.03	4.85	56.20	56.2	(53.7; 58.7)	

[a]$y = a/1 + (x/x_0)^b$, where *y* is the response expressed as percentage of the untreated control, *a* is the asymptotic value of *y* at the upper limit, *b* is the slope of the curve around the point of inflection, x_0 is the dose required to reduce the *y* values by 50% (LD_{50}) when *a* equals 100. $P < 0.05$ for all log-logistic equations.
[b]DAT, days after treatment.
[c]Concentrations required for 50% reduction of 100 − injury (%) (LD_{50}); $P < 0.05$.
[d]Values in this column are 95% confidence intervals.
[e]RF: LD_{50} ratio between the corresponding biotype and the susceptible Londs4-s accession.

Table 4. Plant injury (%) in susceptible (Londs4-s) and resistant to 2,4-D with rapid necrosis (Marpr9-rn) accessions of *Conyza sumatrensis* in response to the application of five auxinic herbicides at 1, 14, and 49 d after treatment (DAT) and fresh weight at 49 DAT.

		Accessions[a]							
		Londs4-s	Marpr9-rn	Londs4-s	Marpr9-rn	Londs4-s	Marpr9-rn		
Herbicides		1 DAT		14 DAT		49 DAT		Londs4-s	Marpr9-rn
	Dose g ha^{-1}	Injury %						Fresh weight g	
Control (0)	0	0.0 aD	0.0 aD	0.0 aD	0.0 aD	0.0 aB	0.0 aD	17.4 bA	21.8 aA
2,4-D	804	27.5 bB	43.7 aA	65.0 aC	58.7 bC	100.0 aA	60.0 bC	0.5 bB	4.5 aB
Picloram	480	36.2 aA	27.5 bB	85.0 aA	82.5 aA	100.0 aA	100.0 aA	0.8 aB	0.5 aB
Fluroxypyr	299.7	35.0 aAB	28.7 bB	72.5 aBC	65.0 bC	100.0 aA	90.0 bB	0.5 aB	0.7 aB
Halauxifen	7	33.7 aAB	17.5 bC	81.2 bA	87.5 aA	100.0 aA	100.0 aA	0.8 aB	0.5 aB
Florpyrauxifen	7.5	17.5 aC	16.2 aC	77.5 aAB	73.7 aB	100.0 aA	95.0 aAB	0.8aB	0.9 aB

[a]Lowercase letters compare means between accessions in the same time (rows), and uppercase letters compare means within accessions (columns); $P > 0.05$ using Tukey's test.

2,4-D–resistant Marpr9-rn accession (Table 6; Figure 6). However, low herbicide efficacy was observed for glyphosate at 1,440 and 2,880 g ha^{-1} and chlorimuron-ethyl at 20 and 40 g ha^{-1}, indicating that resistance to these herbicides could also occur in the 2,4-D–resistant Marpr9-rn accession. Resistance of *Conyza* spp. to glyphosate and ALS inhibitors is one the main problems related to weed control in several regions of the world (Byker et al. 2013; Urbano et al. 2007; VanGessel 2001). The occurrence of multiple resistance to glyphosate and chlorimuron-ethyl herbicides in *C. sumatrensis* was previously identified in the same region where Marpr9-rn was collected by Santos et al. (2014). In this study, it was observed the occurrence of accessions with LD_{50} of 449.60 g ae ha^{-1} for glyphosate and 26.12 g ai ha^{-1} for chlorimuron-ethyl, resulting in a RF of 6.74 and 26.09, respectively. More recently, *C. sumatrensis* accessions with multiple resistance to the herbicides glyphosate, chlorimuron-ethyl, and paraquat have been reported (Heap 2019). However, in the

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

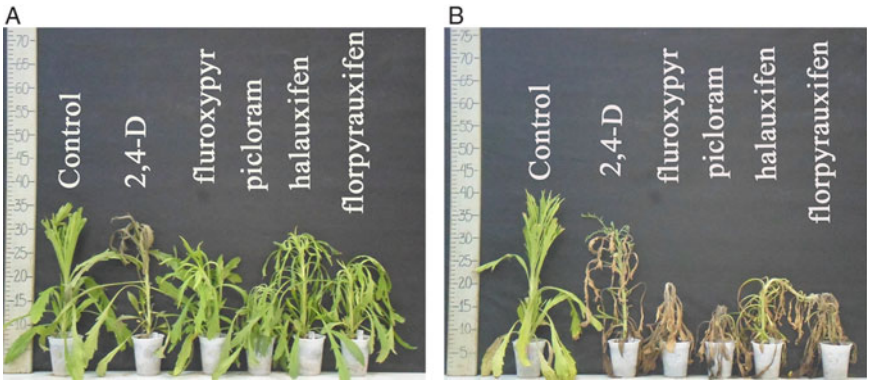

Figure 5. Plants of the *Conyza sumatrensis* Marpr9-rn accession treated with the auxinic herbicides 2,4-D, fluroxypyr, picloram, halauxifen-methyl, and florpyrauxifen benzyl ester at 1 (A) and 21 d (B) after spraying.

present study, the survival of plants treated with the herbicide paraquat at the doses of 400 and 800 g ai ha^{-1} was not evidenced.

The high frequency of resistance to glyphosate and ALS inhibitors and the absence of rapid necrosis symptoms in the resistant accessions of *Conyza* identified previously (Santos et al. 2014; Vargas et al. 2007) indicate that multiple resistance of the Marpr9-rn occurred independently to 2,4-D resistance. Therefore, the evolution of resistance to 2,4-D associated with rapid necrosis in this accession likely occurred in plants that previously had developed resistance to glyphosate and ALS inhibitors. The broad expansion of no-till systems was initially based on the use of glyphosate alone in burndown applications, despite recommendations for use of multiple herbicides. The problem of glyphosate resistance was not evident before the utilization of transgenic soybean resistant to glyphosate. Consequently, *Conyza* resistance to this herbicide evolved rapidly, and this problem was solved with ALS inhibitors, more specifically chlorimuron-ethyl. Once again, the continuous use of this herbicide in mixture with glyphosate in burndown and PRE applications, and sometimes POST, resulted in multiple resistance to both products.

The herbicide 2,4-D has been used in burndown applications in no-till for several decades. However, the use of this herbicide was always in combination with glyphosate or followed by other contact herbicides, mainly paraquat. Due to the already present resistance of *Conyza* spp. to glyphosate and ALS inhibitors, the selection pressure for 2,4-D increased, and these species were initially controlled by 2,4-D alone. The consequence of that was the selection of 2,4-D resistance, probably caused by mechanisms independent from those of resistance to glyphosate and ALS inhibitors. Rapid necrosis was also identified in *A. trifida* in response to glyphosate (Brabham et al. 2011; Moretti et al. 2017; Van Horn et al. 2017). However, the effect of glyphosate on the Marpr9-rn accession observed in the present study was not associated with rapid necrosis (Figures 2 and 6). Therefore, this new mechanism of resistance appears to be elicited by two different herbicides, glyphosate and 2,4-D, in two different species, *A. trifida* and *C. sumatrensis*, respectively. The origin and regulation of the rapid necrosis mechanism of resistance and the similarities between these two cases of herbicide resistance remain to be discovered.

Evaluation of Presence of H_2O_2 in Function of Light

ROS production is essential for the development of the epinasty symptom in plants treated with 2,4-D (Pazmiño et al. 2014). In these studies, H_2O_2 production was identified after the occurrence of epinasty symptoms within 3 to 4 DAT of the herbicide to sensitive plants. It is possible that enzymes involved in oxidative stress relief may act on sensitive plants within the first hours after 2,4-D application. Preliminary studies with susceptible and resistant *C. sumatrensis* accessions indicated formation of H_2O_2 in the leaves of the resistant Marpr9-rn accession caused by 2,4-D at 7 HAT (Supplementary Figure S3). The herbicide paraquat caused similar symptoms in the Marpr9-rn and susceptible Londs4-s accessions, indicating that the method was effective for identification of H_2O_2 formation (Supplementary Figure S3). A subsequent study based on earlier evaluations indicated that H_2O_2 had already increased at 15 min after the application of 2,4-D in both accessions (Figure 7; Supplementary Figure S4). However, in the Marpr9-rn accession, H_2O_2 production increased substantially at 30 min after application and remained high until 1 DAT. For the susceptible Londs4-s accession, H_2O_2 remained constant until 420 min (7 h) after application (Figure 7). The presence of peroxide was equal in both accessions 1 d after the 2,4-D herbicide application. Several studies report the effect of 2,4-D on the production of H_2O_2 and other ROS in the plant death process (McCarthy-Suárez 2017; Pazmiño et al. 2012; Rodríguez-Serrano et al. 2014; Romero-Puertas et al. 2004).

The results obtained in the present study indicated the production of H_2O_2 at 15 min after application in both accessions, but only in the resistant Marpr9-rn did the production increase greatly after that (Figure 7; Supplementary Figure S4). Production of H_2O_2 is associated with the first appearance of the rapid necrosis visual symptoms, which occurred at 2 h after herbicide spraying, as described earlier (Figure 1). The increase of H_2O_2 in the susceptible Londs4-s at 1 d after 2,4-D application was associated with the beginning of the epinastic response.

Light intensity influenced the H_2O_2 production in the Marpr9-rn accession. In the low-light condition (29 µmol m^{-2} s^{-1}), the amount of leaf area labeled by DAB was lower in relation to the high-light condition (848 µmol m^{-2} s^{-1}) (Figure 7; Supplementary Figure S4). Atypical dehydration symptoms also showed late onset in the low-light condition, 4 HAT, in contrast to 1 HAT in high-light condition. A similar delay was also observed in *A. trifida* resistant to glyphosate (Moretti et al. 2017) and in the hypersensitivity response to plant pathogens (Roden and Ingle 2009). The light effect appears to modulate the velocity of the beginning of the rapid necrosis and certainly is related to the variability of the occurrence of this symptom in experimental and field conditions.

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

Table 5. Parameters of the logistic equation,[a] concentrations of the herbicides 2,4-D, dicamba, and triclopyr required for 50% reduction of 100 − visual injury (LD_{50}) and shoot fresh weight (GR_{50}) of *Conyza sumatrensis*, and resistance factor (RF) in the whole-plant dose–response studies.

Accessions, herbicides, and evaluations	*a*	*b*	x_0	LD_{50} or GR_{50}		RF[e]
				g ha^{-1}[c]	95% CI[d]	
2,4-D						
100 − injury (%) at 49 DAT[b]						
Londs4-s	99.96	1.02	45.81	45.7	(37.4; 54.2)	
Marpr9-rn	99.89	0.26	3129.20	3102	(2,435.5; 3,822.8)	67.88
Dicamba						
100 − injury (%) at 49 DAT						
Londs4-s	99.81	0.76	43.10	42.8	(27.3; 58.9)	
Marpr9-rn	100.25	0.78	59.61	59.9	(46.3; 72.9)	1.39
Triclopyr						
100 − injury (%) at 49 DAT						
Londs4-s	99.47	0.90	48.15	47.5	(32.5; 63.8)	
Marpr9-rn	99.71	1.09	84.39	83.9	(72.1; 96.7)	1.77
2,4-D						
Shoot fresh weight (% of untreated control)						
Londs4-s	102.14	0.52	383.81			
Marpr9-rn	99.91	0.24	3727.40			
Dicamba						
Shoot fresh weight (% of untreated control)						
Londs4-s	99.12	0.44	418.79	402	(276.3; 561.3)	
Marpr9-rn	99.63	0.49	503.24	495.6	(292.2; 714.3)	1.23
Triclopyr						
Shoot fresh weight (% of untreated control)						
Londs4-s	99.15	0.63	101.67	98.8	(59.8; 143.6)	
Marpr9-rn	99.94	0.63	79.66	79.5	(68.8; 90.5)	0.80

[a] $y = a/1 + (x/x_0)^b$, where *y* is the response expressed as percentage of the untreated control, *a* is the asymptotic value of *y* at the upper limit, *b* is the slope of the curve around the point of inflection, x_0 is the dose required to reduce the *y* values by 50% (LD_{50}) when *a* equals 100. $P < 0.05$ for all log-logistic equations.
[b] DAT, days after treatment.
[c] Concentrations required for 50% reduction of fresh weight (GR_{50}) or 100 − injury (%) (LD_{50}); $P < 0.05$.
[d] Values in this column are 95% confidence intervals.
[e] RF: LD_{50} ratio between the correspondent biotype and the susceptible Londs4-s accession.

Table 6. Plant injury (%) at 2, 14, and 49 d after treatment (DAT) and fresh weight at 49 DAT in susceptible (Londs4-s) and resistant to 2,4-D with rapid necrosis (Marpr9-rn) accessions of *Conyza sumatrensis* in response to the application of six herbicides with different mechanisms of action.

		Accessions[a]							
		Londs4-s	Marpr9-rn	Londs4-s	Marpr9-rn	Londs4-s	Marpr9-rn		
Herbicides		2 DAT		14 DAT		49 DAT		Londs4-s	Marpr9-rn
	Dose g ha^{-1}	Injury %						Fresh weight g	
Control (0)	0	0.0 aF	0.0 aE	0.0 aD	0.0 aF	0,0 aC	0.0 aD	26.3 aA	27.5 aA
2,4-D	804	31.7 bE	50.0 aCD	93.3 aA	48.3 bCD	100.0 aA	55.0 bC	1.0 bB	17.2 aB
	1608	31.7 bE	63.3 aB	100.0 aA	61.7 bB	100.0 aA	58.7 bC	0.7 bB	11.8 aB
Paraquat	400	88.3 bA	98.3 aA	100.0 aA	100.0 aA	100.0 aA	100.0 aA	0.7 aB	0.6 aC
	800	93.3 bA	98.3 aA	100.0 aA	100.0 aA	100.0 aA	100.0 aA	0.5 aB	0.8 aC
Saflufenacil	49	58.3 aC	58.3 aBC	100.0 aA	100.0 aA	100.0 aA	100.0 aA	1.0 aB	0.7 aC
	98	68.3 aB	58.3 bBC	100.0 aA	100.0 aA	100.0 aA	100.0 aA	0.7 aB	0.5 aC
Glufosinate	600	41.7 aD	41.7 aD	81.7 bB	96.7 aA	100.0 aA	100.0 aA	0.9 aB	0.4 aC
	1200	51.7 aC	45.0 bD	90.0 bAB	96.7 aA	100.0 aA	100.0 aA	0.7 aB	0.6 aC
Glyphosate	1440	0.0 aF	0.0 aE	90.0 aAB	48.3 bCD	93.7 aA	65.0 bB	0.6 aB	4.3 aC
	2880	0.0 aF	0.0 aE	91.7 aAB	58.3 bBC	100.0 aA	68.7 bB	1.1 aB	3.5 aC
Chlorimuron	20	0.0 aF	0.0 aE	45.0 aC	31.7 bE	73.7 aB	48.7 bC	1.2 bB	12.8 aB
	40	0.0 aF	0.0 aE	50.0 aC	45.0 aD	75.0 aB	63.7 bBC	1.2 bB	10.4 aB

[a] Lowercase letters compare means between accessions in the same time (rows), and uppercase letters compare means within accessions (columns); $P > 0.05$ using Tukey's test.

The causes of the rapid increase in H_2O_2 (ROS) production in herbicide hypersensitivity in weeds have not yet been elucidated (Ghanizadeh and Harrington 2017). Also, it is not yet known whether the reduced herbicide translocation occurs due to the rapid foliar necrosis or if this necrosis occurs due to the absence of translocation of the herbicide. In the studies examining rapid necrosis of *A. trifida* in response to glyphosate, it was verified that the joint supply of the amino acids phenylalanine and tryptophan inhibited the occurrence of the rapid necrosis phenotype (Moretti et al. 2017). The biosynthesis of these amino acids is inhibited by the action of glyphosate. Based on these results, the authors concluded that the phenotype of rapid necrosis was not caused by the

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

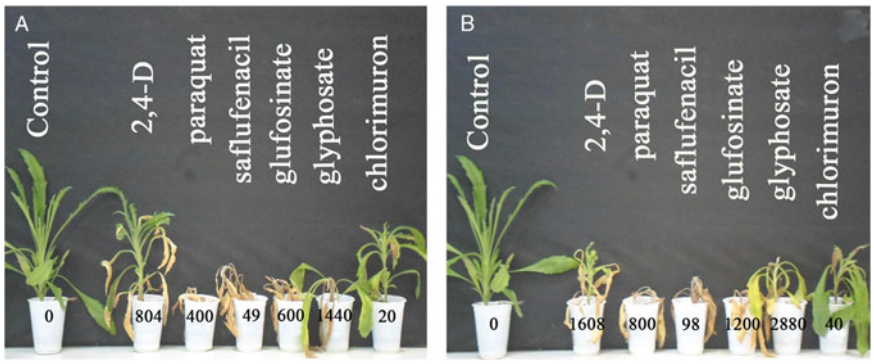

Figure 6. Plants of the *Conyza sumatrensis* Marpr9-rn accession treated with 1X (dose 1) (A) and 2X (dose 2) (B) of 2,4-D, paraquat, saflufenacil, ammonium glufosinate, glyphosate, and chlorimuron-ethyl at 14 d after treatment.

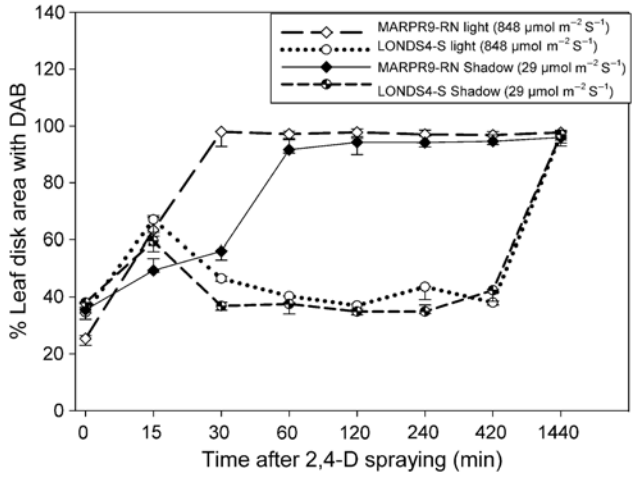

Figure 7. Percentage of leaf disk area with 3,3′-diaminobenzidine-hydrochloride (DAB) dye in *Conyza sumatrensis* susceptible (Londs4-s) and resistant to 2,4-D (Marpr9-rn) accessions in two light conditions. Average of three replicates ± SE.

herbicide molecule but by the effects of the herbicide mechanism. To date, rapid necrosis in *C. sumatrensis* has been found to be specific for 2,4-D herbicide and does not occur in response to glyphosate, which indicates that in this species, this symptom is not possibly associated with amino acid biosynthesis as described for glyphosate effect in *A. trifida*.

In conclusion, the biotype Marpr9-rn is resistant to 2,4-D and the rapid necrosis symptoms begin at approximately 2 h after herbicide application with partial leaf wilt and light necrotic spots. A build-up of ROS precedes the visual occurrence of the rapid necrosis in the resistant biotype. This symptom occurs faster under light conditions, which are conditions that favor the herbicidal effects of 2,4-D. Cross-resistance to other auxinic herbicides was not identified in the Marpr9-rn biotype, but multiple resistance to ALS and EPSPS inhibitors occurs, apparently caused by independent mechanisms. This is the first report of resistance to the herbicide 2,4-D caused by rapid necrosis. This rapid response phenotype had been previously reported only for glyphosate in *A. trifida* (Brabham et al. 2011). Knowledge about the origin and regulation of rapid necrosis resulting in resistance to these herbicides is not available. Rapid necrosis is a new mechanism of herbicide resistance and characterizes the increasing complexity of weed control based only on herbicide strategies.

Acknowledgments. The authors thank Dow AgroSciences Industrial LTDA, especially the Integrated Field Scientist Team, for assistance in seed collection and shipment. The authors are grateful to the National Council for Scientific and Technological Development (CNPq) for a scholarship granted to ARSdQ and fellowships awarded to CAD and the sixth author. Funding was provided by CNPq and Dow AgroSciences Industrial LTDA. The authors declare that there is no conflict of interest.

Supplementary Material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2019.65

References

Bestwick CS, Brown IR, Mansfield JW (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol 118:1067–1078

Brabham CB, Gerber CK, Johnson WG (2011) Fate of glyphosate-resistant giant ragweed (*Ambrosia trifida*) in the presence and absence of glyphosate. Weed Sci 59:506–511

Burke IC, Yenish JP, Pittmann D, Gallagher RS (2009) Resistance of a prickly lettuce (*Lactuca serriola*) biotype to 2,4-D. Weed Technol 23:586–591

Byker HP, Soltani N, Robinson DE, Tardif J, Lawton MB, Sikkema PH (2013) Occurrence of glyphosate and cloransulam resistant Canada fleabane (*Conyza canadensis* L. Cronq.) in Ontario. Can J Plant Sci 93:851–855

Cho M, Cho HT (2013) The function of ABCB transporters in auxin transport. Plant Signal Behav 8:e22990

Dahlke RI, Luethen H, Steffens B (2010) ABP1: an auxin receptor for fast responses at the plasma membrane. Plant Signal Behav 5:1–3

Dang HT, Malone JM, Boutsalis P, Krishnan M, Gill G, Preston C (2018) Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia. Pest Manag Sci 74:1524–32

Doorn WG van, Beers EP, Dangl JL, Franklin-Tong VE, Gallois P, Hara-Nishimura I, Jones AM, Kawai-Yamada M, Lam E, Mundy J, Mur LAJ, Petersen M, Smertenko A, Taliansky M, Van Breusegem F, *et al.* (2011) Morphological classification of plant cell deaths. Cell Death Differ 18:1241–1246

Figueiredo MRA, Leibhart LJ, Reicher ZJ, Tranel PJ, Nissen SJ, Westra P, Bernards ML, Kruger GR, Gaines TA, Jugulam M (2018) Metabolism of 2,4-dichlorophenoxyacetic acid contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population. Pest Manag Sci 74: 2356–2362

Flessner ML, McElroy JS, Mccurdy JD, Toombs JM, Wehtje GR, Burmester CH, Price AJ, Ducar JT (2015) Glyphosate-resistant horseweed (*Conyza canadensis*) control with dicamba in Alabama. Weed Technol 29:633–640

Fuerst EP, Nakatani HY, Dodge AD, Penner D, Arntzen (1985) Paraquat resistance in *Conyza*. Plant Physiol 77:984–989

Fuerst EP, Sterling TM, Norman MA, Prather TS, Irzyk GP, Wu Y, Lownds NK, Callihan RH (1996) Physiological characterization of picloram resistance in yellow starthistle. Pestic Biochem Physiol 56:149–161

Ghanizadeh H, Harrington KC (2017) Non-target site mechanisms of resistance to herbicides. Crit Rev Plant Sci 36:24–34

Goggin DE, Cawthray GR, Powles SB (2016) 2,4-D resistance in wild radish: reduced herbicide translocation via inhibition of cellular transport. J Exp Bot 67:3223–3235

Grones P, Friml J (2015) Auxin transporters and binding proteins at a glance. J Cell Sci 128:1–7

Grossmann K (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manag Sci 66:113–120

Hall JC, Alam SMM, Murr DP (1993) Ethylene biosynthesis following foliar application of picloram to biotypes of wild mustard (*Sinapis arvensis* L.) susceptible or resistant to auxinic herbicides. Pestic Biochem and Physiol 47:36–43

Heap I (2019) The International Survey of Herbicide Resistant Weeds. http://www.weedscience.org. Accessed: May 13, 2019

Hilton HW (1957) Herbicide Tolerant Strains of Weeds. Hawaiian Sugar Planters Association Annual Report. Honolulu, HI: University of Hawaii, Manoa Library. Pp 69–72

Jeffery T (2014) Investigation into the Molecular and Biochemical Mechanisms of Resistance in Two Biotypes of Glyphosate Resistant Giant Ragweed. MS dissertation. Guelph, Ontario: University of Guelph. 77 p

Jugulam M, Dimeo N, Veldhuis LJ, Walsh M, Hall JC (2013) Investigation of MCPA (4-chloro-2-ethylphenoxyacetate) resistance in wild radish (*Raphanus raphanistrum* L.). J Agric Food Chem 61:12516−12521

Kohler EA, Throssell CS, Reicher ZJ (2004) 2,4-D rate response, absorption, and translocation of two ground ivy (*Glechoma hederacea*) populations. Weed Technol 18:917–923

Kruger GR, Davis VM, Weller SC, Johnson WG (2008) Response and survival of rosette-stage horseweed (*Conyza canadensis*) after exposure to 2,4-D. Weed Sci 56:748–752

Kruger GR, Davis VM, Weller SC, Johnson WG (2010) Growth and seed production of horseweed (*Conyza canadensis*) populations after exposure to postemergence 2,4-D. Weed Sci 58:413–419

Kruger GR, Davis VM, Weller SC, Stachler JM, Loux MM, Johnson WG (2009) Frequency, distribution, and characterization of horseweed (*Conyza canadensis*) biotypes with resistance to glyphosate and ALS-inhibiting herbicides. Weed Sci 57:652–659

Kubes M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA, Murphy AS (2012) The *Arabidopsis* concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis. Plant Journal 69:640–654

LeClere S, Wu C, Westra P, Sammons RD (2018) Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an *AUX/IAA* gene. Proc Natl Acad Sci USA 115:E2911–E2920

Lehoczki E, Laskay G, Polos E, Mikulás J (1984) Resistance to triazine herbicides in horseweed (*Conyza canadensis*). Weed Sci 32:669–674

Lespérance M (2015) Programmed Cell Death and Altered Translocation Cause Glyphosate Resistance in Giant Ragweed (*Ambrosia trifida* L.). MS dissertation. Guelph, Ontário: University of Guelph. 95 p

Mangano S, Denita-Juarez SP, Choi H-S, Marzol E, Hwang Y, Ranocha P, Velasquez SM, Borassi C, Barberini ML, Aptekmann AA, Muschietti JP, Nadra AD, Dunand C, Cho H-T, Estevez JM (2017) Molecular link between auxin and ROS-mediated polar growth. Proc Natl Acad Sci USA 114: 5289–5294

McCarthy-Suárez I (2017) Role of reactive oxygen species in auxin herbicide phytotoxicity: current information and hormonal implications—are gibberellins, cytokinins, and polyamines involved? Botany 95: 369–385

Mithila J, Hall JC (2005) Comparison of ABP1 over-expressing *Arabidopsis* and under-expressing tobacco with an auxinic herbicide-resistant wild mustard (*Brassica kaber*) biotype. Plant Sci 169:21–28

Mithila J, Hall JC, Johnson WG, Kelley KB, Riechers DE (2011) Evolution of resistance to auxinic herbicides: historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed Science 59:445–457

Mockaitis K, Estelle M (2008) Auxin receptors and plant development: a new signaling paradigm. Annu Rev Cell Dev Biol 24:55–80

Moretti ML, Van Horn CR, Robertson R, Segobye K, Weller SC, Young BG, Johnson WG, Douglas Sammons R, Wang D, Ge X, d'Avignon A, Gaines TA, Westra P, Green AC, Jeffery T, *et al.* (2017) Glyphosate resistance in *Ambrosia trifida*. Part 2. Rapid response physiology and non-target-site resistance. Pest Manag Sci 74:1079–1088

Morré DJ, Brightman AO, Hidalgo A, Navas P (1995) Selective inhibition of auxin-stimulated NADH oxidase activity and elongation growth of soybean hypocotyls by thiol reagents. Plant Physiol 107:1285–1291

Pazmiño DM, Rodríguez-Serrano M, Sanz M, Romero-Puertas MC, Sandalio LM (2014) Regulation of epinasty induced by 2,4-dichlorophenoxyacetic acid in pea and *Arabidopsis* plants. Plant Biol 16:809–818

Pazmiño DM, Romero-Puertas MC, Sandalio LM (2012) Insights into the toxicity mechanism of and cell response to the herbicide 2,4-D in plants. Plant Signal Behav 7:425–427

Peer WA, Cheng Y, Murphy AS (2013) Evidence of oxidative attenuation of auxin signalling. J Exp Bot 64:2629–2639

Peterson MA, McMaster SA, Riechers DE, Skelton J, Stahlman PW (2016) 2,4-D past, present, and future: a review. Weed Technol 30:303–345

Preston C, Dolman FC, Boutsalis P (2013) Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). Weed Sci 61:185–192

Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1–16

Riar DS, Burke IC, Yenish JP, Bell J, Gill K (2011) Inheritance and physiological basis for 2,4-D resistance in prickly lettuce (*Lactuca serriola* L.). J Agric Food Chem 59:9417–23

Roden LC, Ingle RA (2009) Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant–pathogen interactions. Plant Cell Online 21:2546–2552

Rodríguez-Serrano M, Pazmiño DM, Sparkes I, Rochetti A, Hawes C, Romero-Puertas MC, Sandalio LM (2014) 2,4-Dichlorophenoxyacetic acid promotes *S*-nitrosylation and oxidation of actin affecting cytoskeleton and peroxisomal dynamics. J Exp Bot 65:4783–4793

Romero-Puertas MC, Mccarthy I, Gómez M, Sandalio LM, Corpas FJ, Del Río LA, Palma JM (2004) Reactive oxygen species-mediated enzymatic systems involved in the oxidative action of 2,4-dichlorophenoxyacetic acid. Plant Cell Environ 27:1135–1148

Roux F, Reboud X (2005) Is the cost of herbicide resistance expressed in the breakdown of the relationships between characters? A case study using synthetic-auxin-resistant *Arabidopsis thaliana* mutants. Genet Research 85:101–110

Ruifrok AC, Johnston DA (2001) Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol 23:291–299

Santos G, Oliveira JR RS, Constantin, J., Francischini AC, Osipe JB (2014) Multiple resistance of *Conyza sumatrensis* to chlorimuron-ethyl and glyphosate. Planta Daninha 32:409–416

Schopfer P, Liszkay A, Bechtold M, Frahry G, Wagner A (2002) Evidence that hydroxyl radicals mediate auxin-induced extension growth. Planta 214:821–828

Smith AE (1989) Degradation, fate, and persistence of phenoxyalkanoic acid herbicides in soil. Rev Weed Sci 4:1–24

Soares DJ, Oliveira WS, López-Ovejero RF, Christoffoleti P (2012) Control of glyphosate resistant hairy fleabane (*Conyza bonariensis*) with dicamba and 2,4-D. Planta Daninha 30:401–406

Song Y (2014) Insight into the mode of action of 2, 4 -dichlorophenoxyacetic acid (2, 4 -D) as an herbicide. J Int Plant Biol 56:106–113

Switzer CM (1957) The existence of 2,4-D-resistant strains of wild carrot. Proceedings of the Northeastern Weed Control Conference 11:315–318

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press

Titapiwatanakun B, Murphy AS (2009) Post-transcriptional regulation of auxin transport proteins: cellular trafficking, protein phosphorylation, protein maturation, ubiquitination, and membrane composition. J Exp Bot 60:1093–1107

Urbano JM, Borrego A, Torres V, Leon JM, Jimenez C, Dinelli G, Barnes J (2007) Glyphosate-resistant HAIRY FLEABANE (*Conyza Bonariensis*) in Spain. Weed Technol 21:396–401

Valenzuela-Valenzuela JM, Lownds NK, Sterling TM (2001) Clopyralid uptake, translocation, metabolism, and ethylene induction in picloram-resistant yellow starthistle (*Centaurea solstitialis* L.). Pestic Biochem Physiol 71:11–19

VanGessel MJ (2001) Glyphosate-resistant horseweed from Delaware. Weed Sci 49:703–705

Van Horn CR, Moretti ML, Robertson RR, Segobye K, Weller SC, Young BG, Johnson WG, Schulz B, Green AC, Jeffery T, Lespérance MA, Tardif FJ, Sikkema PH, Hall JC, McLean MD, *et al.* (2017) Glyphosate resistance in *Ambrosia trifida*: Part 1. Novel rapid cell death response to glyphosate. Pest Manag Sci 74:1071–1078

Vargas L, Bianchi M, Rizzardi M, Agostinetto D, Dal Magro T (2007) *Conyza bonariensis* biotypes resistant to the glyphosate in southern Brazil. Planta Daninha 25:573–578

Walsh TA, Neal R, Merlo AO, Honma M, Hicks GR, Wolff K, Matsumura W, Davies JP (2006) Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in *Arabidopsis*. Plant Physiol 142:542–552

Wang Y, Deshpande S, Hall JC (2001) Calcium may mediate auxinic herbicide resistance in wild mustard. Weed Sci 49:2–7

Weinberg T, Stephenson GR, McLean MD, Hall JC (2006) MCPA (4-chloro-2-ethylphenoxyacetate) resistance in hemp-nettle (*Galeopsis tetrahit* L.). J Agric Food Chem 54:9126–9134

Yu J, Wen CK (2013) *Arabidopsis aux1^{rcr1}* mutation alters AUXIN RESISTANT1 targeting and prevents expression of the auxin reporter *DR5:GUS* in the root apex. J Exp Bot 64:921–933

https://doi.org/10.1017/wsc.2019.65 Published online by Cambridge University Press