

Article

First Report of *Amaranthus hybridus* with Multiple Resistance to 2,4-D, Dicamba, and Glyphosate

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Abstract: In many countries, *Amaranthus hybridus* is a widespread weed in agricultural systems. The high prolificacy and invasive capacity as well as the resistance of some biotypes to herbicides are among the complications of handling this weed. This paper reports on the first *A. hybridus* biotypes with resistance to auxinic herbicides and multiple resistance to auxinic herbicides and the EPSPs inhibitor, glyphosate. Several dose response assays were carried out to determine and compare sensitivity of six population of *A. hybridus* to glyphosate, 2,4-D, and dicamba. In addition, shikimic acid accumulation and piperonil butoxide effects on 2,4-D and dicamba metabolism were tested in the same populations. The results showed four populations were resistant to dicamba and three of these were also resistant to 2,4-D, while only one population was resistant to glyphosate. The glyphosate-resistant population also showed multiple resistance to auxinic herbicides. Pretreatment with piperonil butoxide (PBO) followed by 2,4-D or dicamba resulted in the death of all individual weeds independent of herbicide or population.

Keywords: herbicide resistance; amarantaceae; smooth pigweed

1. Introduction

The genus *Amaranthus* contains several species that are main weeds in crops [1], with *Amaranthus hybridus* L. subsp. *hybridus* (smooth pigweed) particularly cited as a major weed in summer crops [2]. This species is a widespread weed in agricultural systems in many countries. In Argentina, it is a common weed in current cropping systems that has biotypes known to be susceptible to glyphosate [3]. However, the weed has been cited as being resistant to ALS inhibitors [4] and to glyphosate [5]. Recently, lack of control with auxinic herbicides was also observed by technicians and farmers in Argentina.

Amaranthus hybridus (syn. of *A. quitensis*) is an annual erect herb that is up to 1 m tall and characterized by glabrous stems and petiolate leaves, ovate shape, pointed apices, terminal panicle form inflorescence, and hermaphrodite flowers [6]. Its presence as a weed is partially explained by the fecundity and longevity of its seeds [2].

Biotypes of nine species of the *Amaranthus* genera have evolved resistance to different herbicides around the world [7]. Of particular concern are a biotype of *Amaranthus palmeri* S. Watson, which has

multiple resistance to herbicides of three different action mechanisms simultaneously and a biotype of *Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif, which is resistant to five groups of herbicides, including synthetic auxins. Some mechanisms of resistance to herbicides have been studied in *A. hybridus*. In the biotypes cited in Argentina, ALS inhibitor herbicides resistance is due to site-specific mutation [8]. Glyphosate resistance mechanism is still unknown, but in a biotype of *A. palmeri* in USA, gene amplification was proven to be the cause [9,10]. However, a rapid quantification of shikimic acid accumulation can be measured in glyphosate-resistant biotypes [11].

Auxinic herbicides include the most commonly used 2,4-D and dicamba. Selectivity in crops is primarily due to auxinic herbicide metabolism [12], and metabolism also plays a key role in conferring resistance to these herbicides in weed species [13]. This metabolism is primarily conferred by cytochrome P450 and can be inhibited by pretreatment of malathion or piperonyl butoxide conferring sensibility in plants with enhanced metabolism [14]. The development and commercialization of 2,4-D resistant crop varieties and the rapid emergence of resistant weeds could increase the use of 2,4-D and its selection pressure over weed flora [15]. Despite many years since its introduction, only 34 weed species are known to have evolved resistance to auxinic herbicides [7] compared to other herbicide modes of action.

Currently, no reports of resistance and no confirmation of any process interrupted has been reported for *A. hybridus* in Argentina. For this reason, the objectives of this paper is to determine the sensitivity and resistance level to auxinic herbicides and glyphosate in six *A. hybridus* populations and to investigate the mechanism of resistance to the herbicides.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

Seeds of *A. hybridus* were collected in 2016 (total of six populations) in the cities of Esperanza (ESP2), San Justo (SJT1 and SJT2), and Colonia Marina (CMA1 and CMA2) in Argentina. An extra population from a seed bank collected in 2009 in Esperanza was added as a sensitive control population (ESP1). Seeds were taken from ten mature plants in soybean crop areas except for ESP1, where the sample consisted of seeds of three plants. The seeds of all the populations were harvested from soybean fields during 2015 and 2016, except for the one that was collected in 2009. The selected fields had been treated in previous years with at least two applications per year of glyphosate and at least one application per year of dicamba.

Germination was conducted on a moistened filter paper in petri dishes. The seedlings were planted in pots containing peat and a sandy loam potting mixture (1:2 v/v) in a growth chamber at 28 °C/18 °C (day/night) in a 16 h photoperiod under 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density and 80% relative humidity.

2.2. Dose Response Assays

To compare the differential susceptibility on all populations, a dose response assay was conducted in each population when plants reached between 6 and 8 cm high and growth stage of 1.3 to 1.4 according the BBCH phenological scale (three to four nodes stage [16]).

The doses used for 2,4-D (Zamba dimethylamine salt 48.5%, p/v) were 0, 30, 60, 120, 240, 480, 960, 1920, and 3840 g ai ha⁻¹; the doses used for dicamba (Banvel dimethylamine salt 57.8%, p/v) were 0, 22.5, 45, 90, 180, 360, and 720 g ai ha⁻¹; and the doses used for glyphosate (Roundup full II, potassium salt 66.2%, p/v) were 0, 25, 50, 100, 200, 400, 800, 1500, 3200, 6400, 12,800, and 25,600 g ai ha⁻¹. The recommended field dose is 480, 90, and 800 g ai ha⁻¹ for 2,4-D, dicamba, and glifosato, respectively. All applications were made with a laboratory spray chamber, with flat fan nozzles calibrated to deliver 175 L ha⁻¹ at 275 kPa pressure.

The shoot fresh weight per plant was determined at 21 days after spraying and expressed as % of untreated control (GR). The data were pooled and fitted to a nonlinear, three parameter logistic

regression model with the upper asymptote fixed at 100%, Equation (1), “R” with “drc plug-in” statistical software [17,18].

$$f(x) = c + \frac{d - c}{1 + \exp b(\log(x) - \log(GR_{50}))} \quad (1)$$

The parameter GR_{50} is the dose producing a response halfway between the upper limit, d (fixed at 100), and the lower limit (fixed at 0), c . The parameter b denotes the relative slope around GR_{50} [19].

From each model, the herbicide rates needed to inhibit plant growth by 50% and 80% with respect to the nontreated control (GR_{50} and GR_{80}) were determined for each population.

Resistance factor was calculated by comparing regression parameter among resistant and sensitive biotypes for each herbicide ($GR_{50 \text{ resistant}} / GR_{50 \text{ sensitive}}$) [20].

The complete experiment was repeated three times with ten replications per dose, herbicide, and biotype.

2.3. Shikimic Acid Accumulation

All populations were sprayed, as previously described, with commercially formulated glyphosate at 400 g ai ha^{−1}. Last expanded leaves of all biotypes (same stage as described before) were harvested for shikimic acid extraction at 24, 48, 72, and 96 HAT (hours after treatment). Leaf tissues were homogenized (50 mg fresh weight) and frozen at −80 °C following the protocol of Singh and Shaner [21]. Shikimic acid accumulation was optically determined at 382 nm using a Biotraza 722 spectrophotometer. The standard curve was determined using untreated plants and a scale of known concentrations of shikimic acid. The complete experiment was repeated three times with five replications per harvest time, per species.

2.4. Piperonil Butoxide (PBO) Effects on 2,4-D and Dicamba Metabolism

All plant population were grown under controlled conditions as described above. The treatments were arranged in a factorial design (6 × 2 × 2). The first factor was the six population of *A. hibrydus*; the second factor was the treatment or not with PBO (an inhibitor of cyt-P450 monooxygenase) at 2100 g ha^{−1}, 1 h before herbicide treatment; and the third factor was the application of herbicides 2,4-D (300 g ai ha^{−1}) and dicamba (240 g ai ha^{−1}). Applications were made as described before.

Twenty one days after application, plants of all treatments were harvested and weighted. Fresh weights were compared independently for the herbicide and population used; a *t*-test was used to evaluate differences ($\alpha = 0.05$) with or without PBO application. The complete experiment was repeated three times with five replications per species, pretreatment, and herbicide.

3. Results and Discussion

Dose response analysis showed a reduction in fresh weight as the dose increased; however, there were differences depending on the biotype and the herbicide (Table 1). Three populations—CMA1, SJT1, and CMA2—were resistant to 2,4-D (Figure 1), with resistance factors between 2.6 and 5.3. Four populations—SJT1, CMA1, SJT2, and CMA2—showed resistance to dicamba, with factors between 4.6 and 7.9 (Figure 2). Only one population—SJT1—was resistant to glyphosate (Figure 3).

Table 1. Regression parameters of dose response based on fresh weight of six *Amaranthus hybridus* biotypes. Lack of Fit test indicated that the regression accurately described the data ($p = 0.58$ for 2,4-D, $p = 0.87$ for dicamba and $p = 0.99$ for glyphosate).

Population	Herbicide	b	d	GR ₅₀	se	Res. Factor	p-Value	GR80	se
CMA1	2,4-D	1.161	1.066	272.78	102.86	2.64	0.001	900.30	425.07
CMA2	2,4-D	5.563	0.075	547.03	128.11	5.31	0.012	704.33	335.80
ESP1	2,4-D	1.849	0.148	123.13	35.45	1.20	0.591	256.19	70.46
ESP2	2,4-D	1.686	0.114	103.10	22.41	-	-	225.23	67.69
SJT1	2,4-D	2.229	0.089	336.73	69.43	3.27	0.022	627.17	209.17
SJT2	2,4-D	1.008	0.143	309.84	137.07	3.01	0.178	1224.61	472.87
CMA1	Dicamba	1.791	1.021	299.29	83.37	5.50	0.001	648.90	279.50
CMA2	Dicamba	2.971	1.077	428.28	105.77	7.87	0.001	682.90	289.50
ESP1	Dicamba	2.657	1.089	54.40	8.86	-	-	91.72	21.35
ESP2	Dicamba	1.593	1.058	83.87	24.36	1.54	0.106	200.27	77.41
SJT1	Dicamba	4.240	1.077	248.12	44.44	4.56	0.001	344.07	135.50
SJT2	Dicamba	1.192	1.157	326.36	86.78	6.00	0.001	1043.88	533.02
CMA1	Glyphosate	2.538	1.017	63.51	8.16	0.76	0.251	109.66	18.11
CMA2	Glyphosate	1.142	0.976	82.47	19.20	0.98	0.955	277.74	79.47
ESP1	Glyphosate	2.875	0.926	83.81	14.00	-	-	135.76	32.63
ESP2	Glyphosate	0.819	0.987	119.86	70.33	1.43	0.482	652.00	336.91
SJT1	Glyphosate	1.959	0.875	5018.2	1315.2	167.0	0.001	10,181.8	3434.2
SJT2	Glyphosate	1.867	1.130	66.85	14.25	0.79	0.456	140.47	38.62

The parameter GR₅₀ is the dose producing a response halfway between the upper limit, d (fixed at 100), and the lower limit (fixed at 0). The parameter b denotes the relative slope around GR₅₀; se = standard error; Res. factor (Resistance Factor) is calculated as (GR₅₀ resistant / GR₅₀ sensitive); and p-value is the probability level of significance of the resistance factor. GR₈₀ is the herbicide dose that reduces 80% of the fresh weight compared with untreated plant.

Field studies were conducted in 2015 and 2016 at the sites where seeds of CMA1 and CMA2 population were collected. Plants treated with 2000 mL of 2,4-D showed control between 20% and 30% if compared with untreated control (data not shown); the surviving plants continued to grow and produce seeds, according to the criteria of Burgos et al. [22]. These can be classified as resistant biotypes.

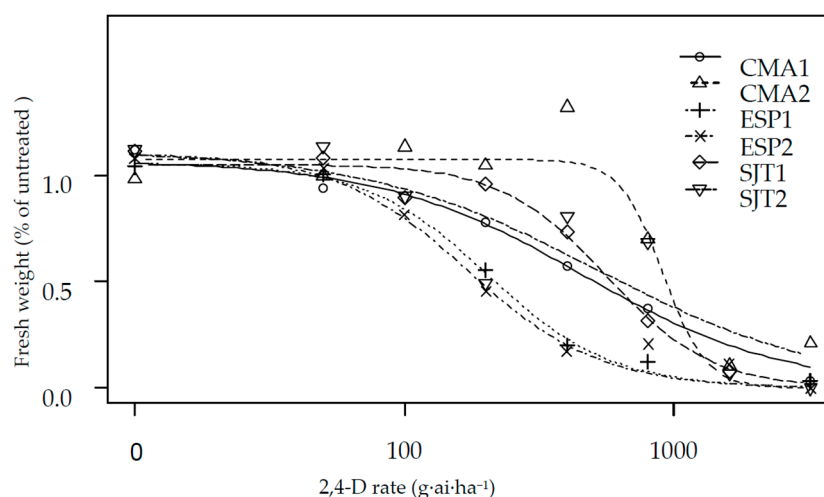


Figure 1. Weight response of six biotypes of *Amaranthus hybridus* to 2,4-D.

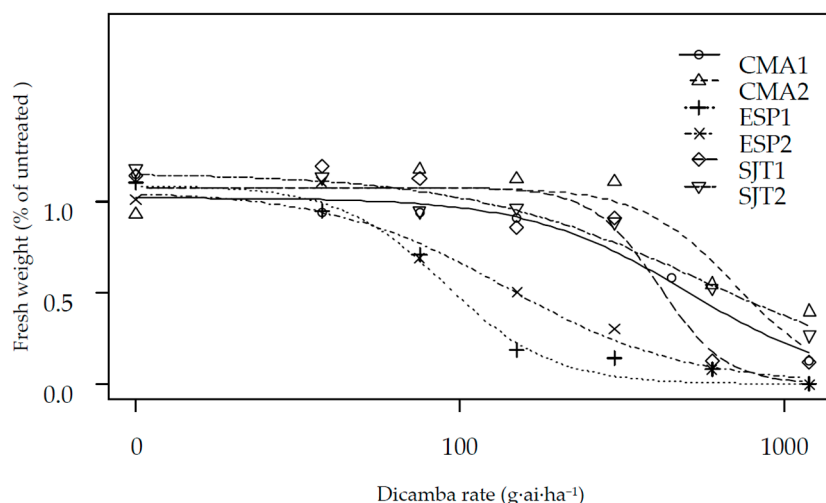


Figure 2. Weight response of six biotypes of *Amaranthus hybridus* to dicamba.

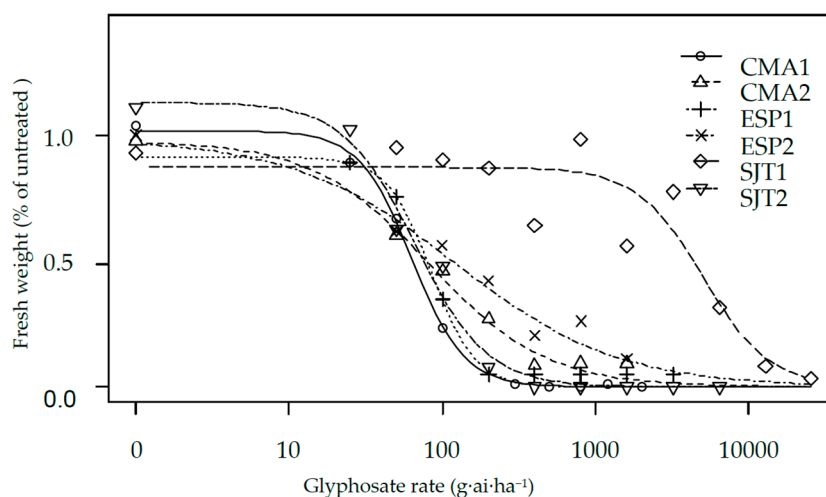


Figure 3. Weight response of six biotypes of *Amaranthus hybridus* to glyphosate.

A study of *A. tuberculatus* 2,4-D-resistant population with larger plants of 8–12 cm tall [23] showed that similar doses of 2,4-D and dicamba to those found in this work were needed to reduce dry weight by 50% in susceptible plants. The highest dose of 2,4-D (6400 mL ha^{-1}) was not enough to kill the CMA2 population completely, while for dicamba, the highest doses reduced fresh weight but did not kill populations CMA2 and SJT2. Plants of the biotypes treated with 2,4-D and dicamba at 480 and 180 g ai ha^{-1} , respectively, showed epinasty symptoms but kept growing in growth chamber and produced flowers and seeds at a similar time compared to untreated plants.

Resistance factors in 2,4-D are between 2.5 fold for *Raphanus raphanistrum* [24] to 29 fold in *Fimbristylis miliacea* [25]. Resistance in the biotypes studies in this work were in the lower range (between 2.6 and 5.3), perhaps due to the initial stage of selection. For dicamba, the range of resistance in this work was between 1.57 and 7.87 above that cited by Bernards et al. [23] for *A. tuberculatus*.

The major 2,4-D resistance mechanism identified in wild radish is their inability to translocate 2,4-D away from its site of foliar application [26]. However, in some cases, enhanced metabolism in addition to reduced translocation of 2,4-D was cited as the resistance mechanism in *Papaver rhoeas* [13]. To test the hypothesis that enhanced 2,4-D and dicamba metabolism is conferred by cyt-P450, the known cyt-P450-inhibitor PBO was tested. Pretreatment with PBO followed by 2,4-D or dicamba resulted in the death of all plants independent of herbicide or population (Table 2). Symptoms

in PBO pretreated plants were similar but occurred several days earlier compared with non-pretreated plants. Most pretreated plants in dicamba or 2,4-D treatments showed general necrosis followed by death. Therefore, detoxification or metabolism of auxinic herbicides via cyt-P450 can be considered as the possible mechanism involved in the resistance for the studied biotypes.

Table 2. Fresh weight of six biotypes of *Amaranthus hybridus* with treatments of 2,4-D; 2,4-D + piperonyl butoxide (PBO); dicamba; and dicamba + PBO.

Population/Active	2,4-D	2,4-D + PBO	Dicamba	Dicamba + PBO
CMA1	0.79 ± 0.28	0.06 ± 0.06 *	0.68 ± 0.25	0.09 ± 0.13 *
CMA2	0.89 ± 0.33	0.06 ± 0.09 *	0.97 ± 0.25	0.03 ± 0.06 *
ESP1	0.08 ± 0.14	0.07 ± 0.15	0.57 ± 0.06	0.05 ± 0.09 *
ESP2	0.22 ± 0.08	0.07 ± 0.09 *	0.30 ± 0.15	0.10 ± 0.15 *
SJT1	0.45 ± 0.20	0.07 ± 0.06 *	0.95 ± 0.25	0.04 ± 0.04 *
SJT2	0.81 ± 0.26	0.06 ± 0.09 *	0.78 ± 0.39	0.11 ± 0.14 *

* mean significant differences (*t*-test, $\alpha = 0.05$) due to the addition of PBO within the same population and active.

Shikimate accumulation is a known marker of EPSPS inhibition in populations with sensitivity to glyphosate [27]. The inhibition of the shikimic acid metabolic pathway were confirmed in all biotypes of *A. hybridus* except in SJT1 biotype. The absence of modification in the pathway of the last biotype confirms glyphosate resistance (Figure 4). After 96 h of application, accumulation of shikimic acid over test plants were between 1.92 mg sk g⁻¹ and 2.84 mg sk g⁻¹ fresh tissue in sensitive biotypes against 0.01 mg sk g⁻¹ fresh tissue in resistant SJT1 biotype.

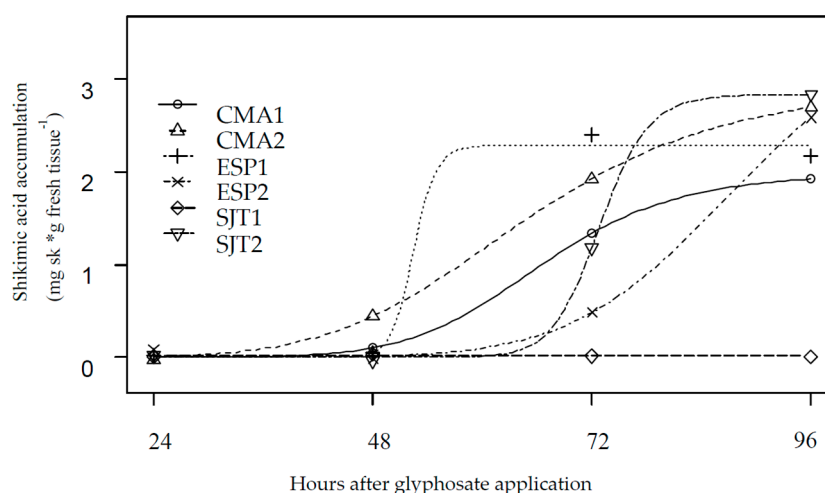


Figure 4. Shikimic acid accumulation over untreated plants from 24 h to 96 h after glyphosate application (mg/g fresh tissue).

With respect to the behavior of biotypes, ESP1 and ESP2 was susceptible to all herbicides tested, while SJT1 biotype shows multiple resistance to 2,4-D, dicamba, and glyphosate.

The accumulation of several resistance mechanisms within resistant individuals is now the normal situation for *L. rigidum* [14]. It is characteristic of metabolic herbicide resistance that the responsible enzymes can confer cross-resistance [28] to herbicides of different chemical groups and sites of action.

It is interesting to note what happens with the populations studied and the resistance to 2,4-D and dicamba. Resistance to 2,4-D was first reported in 1957 in *Daucus carota* and *Commelina diffusa* [29,30], while the cases of resistance to dicamba are more recent. The latest citations have corresponded to cross-resistance in dicamba, 2,4-D, and others auxinic herbicides like picloram, MCPA, and others.

4. Conclusions

In conclusion, the biotypes ESP1 and ESP2 can be considered susceptible to 2,4-D, dicamba, and glyphosate, while SJT2 is resistant to dicamba only. The biotypes CMA1 and CMA2 are resistant to both 2,4-D and dicamba, while SJT1 biotype have multiple resistance to 2,4-D, dicamba, and glyphosate. Lack of inhibition of shikimic acid pathway was confirmed in biotype SJT1 and the pretreatment with piperonyl butoxide made resistant plants sensitive to dicamba and 2,4-D herbicides.

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