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First Report of Resistance to Glyphosate in Several Species of the Genus *Echinochloa* in Argentina

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Abstract: The genus *Echinochloa* consists of about 33 species worldwide; some of these are weeds that are very difficult to control in Argentina, and only *E. colona* was reported as resistant to glyphosate. The objective of this work is to determine if one or more populations of *E. colona*, *E. crus-galli*, *E. oryzoides*, and *E. chacoensis* are resistant to or less susceptible to glyphosate. Between 2015 and 2017, seeds of different *Echinochloa* populations were collected from the provinces of Córdoba, Santa Fe, Buenos Aires, and Entre Ríos, all from fields with a history of at least 10 consecutive years of glyphosate application and complaints from farmers due to failures in control. With these populations, survival, dose–response, and shikimic acid quantification tests were carried out to determine their level of susceptibility to glyphosate. The results obtained allow us to report the first worldwide case of resistance to glyphosate in populations of *E. crus-galli*, *E. oryzoides*, and *E. chacoensis* and expand the information on *E. colona*.

Keywords: dose–response curves; shikimate accumulation; herbicide efficacy; herbicide resistance; *Echinochloa*; "capín"; junglerice; barnyardgrass; early watergrass

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1. Introduction

The genus *Echinochloa* consists of about 33 species worldwide, including subspecies and varieties [1], while in Argentina, seven species are present [2], but few of these are reported as weeds. Instead, and also as weeds, the two most widespread species that are found both globally and in Argentina are *Echinochloa colona* (L.) Link and *Echinochloa crus-galli* (L.) P. Beauv. Additionally, *Echinochloa chacoensis* Michael ex Renvoize and *Echinochloa oryzoides* (Ard.) Fritsch are present in Argentina as weeds in various crops [3], particularly in soybean and corn crops [4].

Worldwide, they are found in crops such as rice, sorghum, sugarcane, cotton, peanuts, etc. [5]. There four *Echinochloa* species behave similar to weeds and compete strongly with many crops. *E. colona* reduces soybean yield by 12 [kg/ha] to 27 [kg/ha] per percent of cover by this weed [4,6] and *E. crus-galli* can reduce rice yield by about 25% in heavy infestations [7].

In Argentina, from 2010 to the present, the genus *Echinochloa* recorded a sustained development in the occupied area, reaching 35% of the agricultural area in 2021 and positioning itself as the fourth most important weed [8]. In particular, *E. colona* has increased its presence and abundance as a result of the continuous use of glyphosate [9].

All species of the genus *Echinochloa* are C4 [10] annuals, with emergence in spring and summer. They reproduce by seed and coexist in agricultural areas. The plants in this genus are very diverse, and their taxonomy is confusing [11,12]. Only *E. chacoensis* has ligules; the other three species do not have ligules or auricles. The inflorescence of *E. crus-galli* is an erect pyramidal panicle consisting of 15 to 30 pseudo-spikes. The panicle of *E. colona* is erect and has a greater number of reproductive organs arranged on lateral branches, between 5 and 15 of them. *E. oryzoides* has contracted pointed inflorescences with 6–15 short first-order branches, and *E. chacoensis* has a contracted panicle with densely packed spikelets on the branches [13–15]. Various ecotypes, high seed production, short seed dormancy, rapid growth, competitive potential, allelopathic interaction, and resistance to various herbicides make these weeds a more adaptable and persistent challenge in various agroecosystems [5,6].

Regarding their chemical control, worldwide failures are registered in different populations of the *Echinochloa* genus. The first resistant population of *E. colona* was confirmed in 2007 in Australia [7]. The Weed Science Society of America declares cases of glyphosate resistance in the population of *Echinochloa* sp. for the United States as of 2008 and in Argentina as of 2009 [7,8].

Herbicide resistance is an evolutionary and ecological process. The dynamics of this process are influenced by (1) weed characteristics, which further include the morphological and genetic factors associated with the weed; (2) herbicide characteristics, which further include the mode or site of action of herbicide; and (3) finally the operational factors, i.e., the prevalent farm practices [16].

A total of 83 cases of eight species and cultivars of the genus *Echinochloa* have been reported worldwide with resistance to various groups of herbicides, including *E. colona*; *Echinochloa crus-galli* (L.) P. Beauv. var. crus-galli; *Echinochloa crus-galli* var. formosensis Ohwi; *Echinochloa crus-galli* var. zelayensis (Humb., Bonpl. & Kunth) Hitchc.; Echinochloa crus-pavonis (Kunth) Schult; *Echinochloa erecta* (Pollacci) Pignatti; *E. oryzoides* and *Echinochloa phyllopogon* (Stapf) Stapf ex Kossenko (=E. oryzicola) [17]. *E. crus-galli* var. crus-galli presents the highest number of cases of resistance and together with *E. colona*, they are the two most widespread species in extensive crops. In all species, resistance occurs mainly in rice crops, but there are also some cases in maize, soybeans, sorghum, cotton, grapes, orchards, watermelons, fallow land, fences, and roadsides [18].

For these various species, there are no previous publications that confirm glyphosate herbicide resistance worldwide [18]. Additionally, there has not been a comparative study of the susceptibility of the different species of *Echinochloa* in Argentina [19].

For this reason, this study aims to determine whether populations of *E. colona*, *E. crus-galli*, *E. oryzoides*, and *E. chacoensis* that currently infest different annual crops in Argentina are resistant or less susceptible to glyphosate.

2. Materials and Methods

Plant Material:

To determine the susceptibility to glyphosate, an initial of nine populations of *E. colona*, seven populations of *E. crus-galli,* six populations of *E. oryzoides*, and three populations of *E. chacoensis* were examined for possible resistance.

Between 2015 and 2017, seeds of the genus *Echinochloa* were collected from the provinces of Córdoba, Santa Fe, Buenos Aires, and Entre Ríos; all populations proceed from cultivated fields with a history of at least 10 consecutive years of glyphosate application and complaints from farmers of control failures.

Seeds were collected from 25 fields. In each place, only one species is observed dominating the community of gramineous weeds. One hundred whole plants were taken and bulked into a single sample from each field, for the determination of glyphosate resistance. In all these fields, it was known that *Echinochloa* sp. plants had survived multiple glyphosate applications with reduced phytotoxic effects. Samples were stored at room temperature (~l8 °C) in the laboratory until sowing. The determination of the species was made using the keys of Flora Argentina [11]. Table 1 details the origin of each population along with the abbreviated name of each one.

Table 1. Populations of the genus *Echinochloa* used.

Population	Species	Crop in Which it Was Found	Location (Town)	Province	GPS Location	Years of Agriculture	Date of Harvest
Ec02	*E. colona*	Soybean-Corn	Manfredi	Córdoba	−63.76988 −31.84076	20	2015
Ec03	*E. colona*	Soybean-Corn	Colonia Marina	Córdoba	−62.41800 −31.21785	20	2017
Ec04	*E. colona*	Soybean-Corn	Freyre	Córdoba	−62.0710 −31.17283	20	2015
Ec05	*E. colona*	Soybean-Corn	Luxardo	Córdoba	−62.25967 −31.27033	10	2015
Ec06	*E. colona*	Soybean-Corn	Estación Luxardo	Córdoba	62.09588 −31.31240	20	2017
Ec07	*E. colona*	Soybean-Corn	Colonia Castelar	Santa Fe	62.13270 −31.65483	20	2015
Ec12	*E. colona*	Soybean-Corn	San Francisco	Córdoba	−62.18506 −31.39578	20	2016
Ec14	*E. colona*	Soybean-Corn	Arias	Córdoba	−62.45239 −33.62968	15	2017
Ec16	*E. colona*	Soybean-Corn	El Paraíso	Buenos Aires	−59.96448 −33.58911	15	2017
Ecg06	*E. crus-galli*	Soybean-Corn	Colonia Mascias	Santa Fe	−60.00472 −30.78352	10	2016
Ecg07	*E. crus-galli*	Soybean-Corn	Saladero Cabal	Santa Fe	−60.05598 −30.88797	10	2016
Ecg12	*E. crus-galli*	Soybean	Santa Anita	Entre Ríos	−58.86091 −32.15184	10	2016
Ecg13	*E. crus-galli*	Rice	Villa Elisa	Entre Ríos	−58.38793 −32.17088	15	2016
Ecg14	*E. crus-galli*	Corn	Chajarí	Entre Ríos	−57.95368 −30.76185	15	2016
Ecg15	*E. crus-galli*	Soybean	San Salvador	Entre Ríos	−58.54736 −31.64929	15	2016
Ecg16	*E. crus-galli*	Not agricultural	C. del Uruguay	Entre Ríos	−58.24531 −32.42552	—-	2016
Eo02	*E. oryzoides*	Soybean-Corn	Colonia Mascías	Santa Fe	−60.02201 −30.83483	15	2015
Eo03	*E. oryzoides*	Not agricultural	Colonia Mascías	Santa Fe	−59.99370 −30.83492	—–	2015
Eo04	*E. oryzoides*	Soybean-Corn	Colonia Mascías	Santa Fe	−60.10391 −30.75559	15	2016
Eo05	*E. oryzoides*	Rice	Colonia Mascías	Santa Fe	−60.1063 −30.79600	15	2016
Eo06	*E. oryzoides*	Soybean-Corn	Colonia Mascías	Santa Fe	−60.1063 −30.67708	15	2016
Eo07	*E. oryzoides*	Soybean-Corn	Colonia Mascías	Santa Fe	−60.20002 −30.81921	15	2016
Ech01	*E. chacoensis*	Soybean-Corn	Colonia Mascías	Santa Fe	−59.99849 −30.83808	10	2016
Ech02	*E. chacoensis*	Soybean-Corn	Colonia Mascías	Santa Fe	−60.12071 −30.8313	10	2016
Ech03	*E. chacoensis*	Not agricultural	Colonia Mascías	Santa Fe	−60.08638 −30.7233	10	2016

Screening (survival):

A hundred seeds of each specie and population were cleaned and then sown in 500 [cm^3] pots, which contained approximately 720 [g] of typical Esperanza Series Argiudol soil (loam-clay-silty texture; 66.7-28.7-4.7). Then they were placed in a growth room at a temperature of 28/18 [°C] (day and night), with a photoperiod of 16 h, a light intensity of 850 [μmol m^{-2} s^{-1}], and 60–80% relative humidity (as suggested for summer spring cycle species [20]).

When the seedlings had four leaves a discriminant dose of 600 [g a.i./ha] of glyphosate (36 [% p/v a.i.]) were applied. All applications were carried out with a laboratory spray chamber equipped with a Magnojet 8001 flat fan nozzles, calibrated to spray 175 [L/ha] at a pressure of 275 [kPa].

Before application (at plant emergence) and 21 days after application of glyphosate (plant survived), the number of plants present per pot was determined. A plant was considered alive if a meristem was green [21]. The % survival is determined as (plant survival)/(plants emergence) × 100.

The populations were classified into three groups according to their survival rate: (i) resistant, if more than 20% of the plants survived the herbicide treatment, (ii) in development of resistance if 6–19% of the plants survived, and (iii) susceptible if less than 5% survived to herbicide treatment [22].

Dose Response (vegetal material):

Populations of all *Echinochloa* species (Table 1) with suspected low sensitivity to glyphosate were cultivated; in addition, populations of known high sensitivity were also cultivated for comparison.

They were germinated on a moistened filter paper in Petri dishes, and then transplanted to one-liter pots and placed in a growth room under the same conditions mentioned in the previous trial.

For each combination of dose and population, 10 replicates were used, and the test was performed in a completely randomized design. To compare the susceptibilities of the different populations, three dose–response trials were conducted, for which increasing doses of glyphosate 0 X, $\frac{1}{4}$ X, $\frac{1}{2}$ X, X, 2 X, 4 X, and 8 X will be applied, with X (1080 g a.i./ha). All applications will be carried out with the laboratory spray chamber previously described.

The fresh weight was determined per pot 21 days after application. These weights were expressed as a percentage of the untreated control, and statistical analysis was performed using the drc package [23] in R. The data by species and population are adjusted to a logistic model represented by the function of 4 parameters (1), where GR50 is the dose that produces the average response between the upper limit d and the lower limit c. Parameter b marks the inflection around GR50 [24,25].

$$(Fresh\ weight) = c + \frac{d - c}{1 + exp(b[log(herbicide\ dose) - log(GR_{50})])} \quad (1)$$

The dose of herbicide that inhibits growth by 50 and 80% concerning the untreated control (GR50 and GR80) was also determined for each population. The susceptibility differences were also evaluated using the resistance factor (RF) to be determined as GR50 (resistant)/GR50 (susceptible).

Shikimic Acid Accumulation:

Individuals from all populations of the before-mentioned species, under the same growth conditions described above, were sprayed with a glyphosate solution containing 600 [g a.i./ha] under the same specific conditions as the previous test. The last expanded leaves of plants of all species were harvested for shikimic acid extraction 24, 48, 72, and 96 h after application. The foliar tissues (50 [mg] fresh weight) were homogenized, and the samples were frozen at −40 [°C].

Subsequently, the protocol described by Singh and Shaner [26] was followed. The absorption of shikimic acid was optically measured at 382 [nm] using a Biotraza 722 spectrophotometer (Biotraza, China). Standard curves are determined using untreated plants and known shikimic acid concentration solutions. Both experiments were repeated 3 times with 10 replicates per treatment.

3. Results

3.1. Survival

All the plants used in the survival test showed different symptoms, but all produced shoots and viable seeds.

In *E. colona*, of the total populations, five of them (Ec02, Ec03, Ec06, Ec12, and Ec16) did not show resistance; only one presented resistance in evolution (Ec05), and the rest of the populations (Ec07, Ec14, and Ec04) show survival between 25 and 94% of individuals at the use dose. In *E. crus-galli*, only Ecg13 did not show resistance (susceptible), Ecg12 presented resistance in evolution, and the remaining populations (Ecg06, Ecg07, Ecg14, Ecg15, and Ecg16) exceeded 20% survival, with values ranging between 25 and 96%.

For *E. oryzoides*, four of the six populations (Eo02, Eo03, Eo07, and Eo08) had very high survival values between 75 and 87%, qualifying them as resistant. On the other hand, two populations (Eo04 and Eo06) did not survive the application of glyphosate. In addition, finally, the survival test for *E. chacoensis* shows that only one population (Ech02) has a survival rate higher than 20%, the other two were populations proved to be susceptible.

3.2. Dose Response Trials

In the four species, it was observed that the biomass of the aerial part of the populations decreases as the herbicide dose increases, but the GR_{50} differs significantly between the different populations of each species.

3.2.1. *Echinochloa colona*

As can be seen in Table 2 and Figure 1, the highest GR_{50} is in the Ec04 and Ec14 populations. The dose–response experiments confirmed that the Ec12 and Ec16 populations were not significantly different from the Ec03 population, while the rest of the populations were different from the Ec03 population.

Table 2. Logistic adjusted model parameters (3 parameters, GR_{50} as parameter and GR_{80} estimated); the doses required a reduction of 50% and 80% of the fresh weight, respectively, parameter e corresponds to the GR_{50} parameters c, d, and b that were defined in formula (1); fixed lower limit at 0.

Population	b	d	GR_{50}	se	RF [1]	*p*-Value *	GR_{80}	se
Ec02	2.585585	1.065524	224.276	23.825	1.35	0.0218	383.39	61.27
Ec03	3.726338	1.008724	165.993	18.160	1.00	-	240.80	26.87
Ec04	10.74425	0.746329	610.123	30.627	3.68	<0.0001	694.15	78.72
Ec05	2.569705	0.957845	292.053	40.473	1.76	<0.0001	500.90	76.89
Ec06	5.147392	0.933955	300.983	19.148	1.81	<0.0001	394.01	38.05
Ec07	24.22164	0.871925	385.122	39.361	2.32	<0.0001	407.81	22.27
Ec12	3.294016	0.984736	175.349	23.188	1.06	0.7428	267.10	29.37
Ec14	5.708362	0.926718	608.017	24.187	3.66	<0.0001	775.15	47.18
Ec16	1.635326	0.985857	187.777	37.313	1.13	0.5632	438.33	84.83

[1] RF: resistance factor. * The value of *p* is the level of probability of significance for each factor for the model. The Lack of Fit test indicates that the regression accurately describes the data in the table (p = 0.0177).

The regression parameters also indicate that the Ec04 and Ec07 populations have most expressed this susceptibility variation, while the rest are less susceptible to dose variation; this is quantified by the inflection point around e (regression parameter b) (Table 2).

The Ec04 and Ec14 populations presented a RF of 3.66 to 3.68 compared to the Ec03, although their GR_{80} was between 30 and 35% lower than the dose taken as field use (1080 [g a.i./ha]). In addition, populations Ec07, Ec06, Ec05, and Ec02 presented an RF of 1.35 to 2.32 with respect to Ec03, and their GR_{80} was between 2 and 3 times below the use dose previously considered.

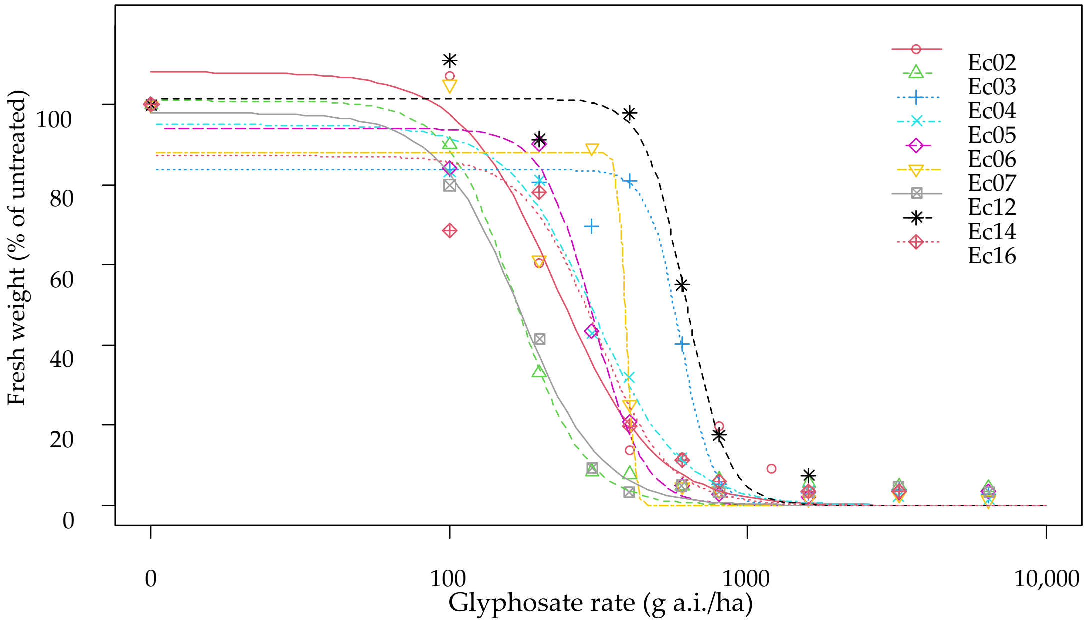

Figure 1. Dose response curves corresponding to the different populations of *Echinochloa colona*. Each point is the average of three experiments. These curves were calculated using the model proposed by Streibig and Kudsk [27].

3.2.2. *Echinochloa crus-galli*

The populations Ecg06, Ecg07, Ecg14, and Ecg15 have GR_{50} values higher than the rest (Table 3 and Figure 2), and it was determined that these populations were significantly different from the Ecg12 population (considered susceptible). On the other hand, the Ecg13 and Ecg16 populations were not significantly different from the Ecg12.

Table 3. Parameters of the logistic adjusted model (3 parameters, GR_{50} as parameter and GR_{80} estimated); the doses required for a reduction of 50% and 80% of the fresh weight, respectively, parameter e corresponds to the GR_{50} parameters c, d, and b that were defined in formula (1); fixed lower limit at 0.

Population	b	d	GR_{50}	se	RF [1]	*p*-Value *	GR_{80}	se
Ecg06	2.657	91.137	**884.881**	138.486	**6.91**	<0.0001	1491.045	359.492
Ecg07	1.595	93.014	**984.309**	241.805	**7.72**	<0.0001	2347.048	600.342
Ecg12	2.405	97.351	127.408	20.451	1.00	-	226.77	44.102
Ecg13	17.329	87.430	185.437	127.837	1.45	0.62128	200.881	11.082
Ecg14	1.776	98.269	**1039.178**	172.755	**8.15**	<0.0001	2267.936	524.427
Ecg15	4.070	97.405	**1667.596**	190.313	**13.09**	<0.0001	2344.365	615.555
Ecg16	4.145	102.580	160.410	17.114	1.26	0.18881	224.119	29.17

[1] RF: resistance factor. * The value of *p* is the level of probability of significance for each factor for the model. The Lack of Fit test indicates that the regression accurately describes the data in the table ($p = 0.586$).

In addition, the regression parameters indicate that the Ecg07, Ecg15, and Ecg14 populations are the ones that have expressed this susceptibility variation the most, while the Ecg13 population was the least susceptible to the dose variation; this is quantified by the inflection point around GR50 (parameter b of the regression) (Table 3).

The Ecg06, Ecg07, Ecg14, and Ecg15 populations presented an RF of 6.91 to 13.08 compared to Ecg12, and their GR80 was above the dose of use (1080 [g a.i./ha]) between 1.5 and 2.5 times. Ecg13 and Ecg16 populations presented an RF of 1.25 to 1.45 with respect to Ecg12, in agreement with their GR80, which was well below the previously considered use dose.

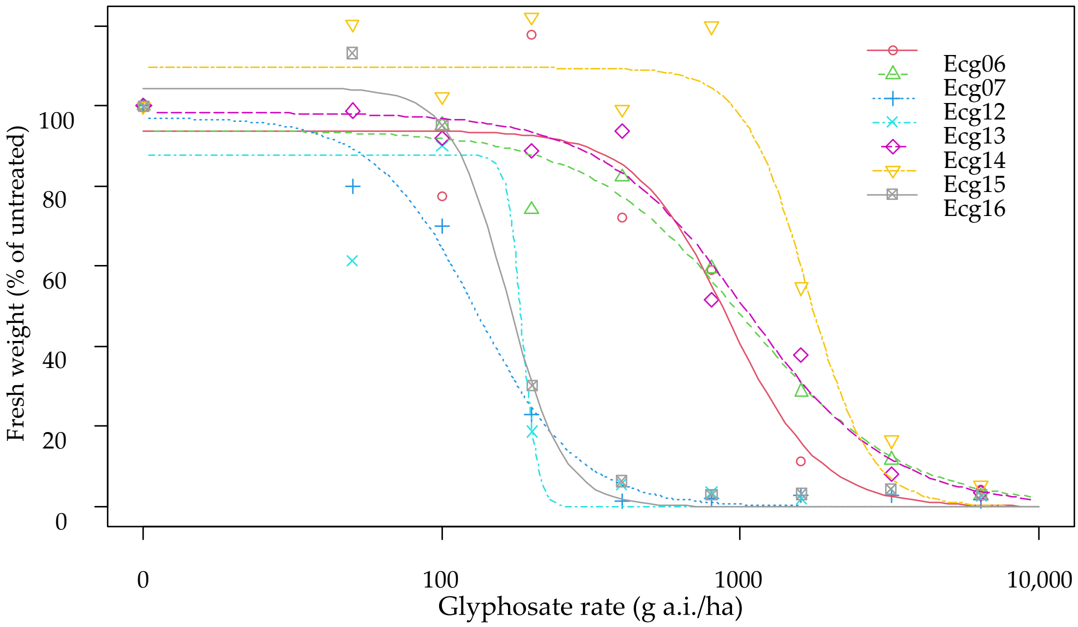

Figure 2. Dose–response curves corresponding to the different populations of *Echinochloa crus-galli*. Each point is the average of three experiments. These curves were calculated using the model proposed by Streibig and Kudsk [27].

3.2.3. *Echinochloa oryzoides*

GR50 was the highest in the populations Eo02, Eo03, Eo07, and Eo08 (Table 4 and Figure 3), while it was intermediate in the Eo04 population. Evaluating the susceptibility to the herbicide glyphosate in the dose–response trials in the different populations, it was determined that Eo02, Eo03, Eo07, and Eo08 were significantly different from population Eo06 (considered susceptible) (Table 4).

Table 4. Parameters of the logistic adjusted model for *Echinochloa oryzoides* (3 parameters, GR_{50} as parameter and GR_{80} estimated); the doses required for a reduction of 50% and 80% of the fresh weight, respectively, parameter e corresponds to the GR_{50} parameters c, d, and b that were defined in formula (1); fixed lower limit at 0.

Population	b	d	GR_{50}	se	RF [1]	*p*-Value *	GR_{80}	se
Eo02	1.400	95.449	**3.006.63**	615.244	**8.53**	0.0003	8090.58	1.798.65
Eo03	0.988	98.007	**3.312.02**	856.805	**9.39**	0.0023	13463.87	3.820.10
Eo04	1.984	112.915	804.93	117.447	**2.28**	0.0045	1619.15	450.23
Eo06	4.295	104.756	352.54	45.943	1	-	486.83	83.65
Eo07	1.282	96.955	**3.070.23**	981.319	**8.71**	0.00001	9055.23	3.455.12
Eo08	1.488	96.658	**5.107.50**	1.050.115	**14.49**	0.00001	12969.33	3.078.01

[1] RF: resistance factor. * The value of *p* is the level of probability of significance for each factor for the model. The Lack of Fit test indicates that the regression accurately describes the data in the table ($p = 0.586$).

The regression parameters also indicate that the populations Eo02, Eo03, Eo07, and Eo08 are the populations that have most expressed this variation in susceptibility, while Eo06 was less susceptible to the variation in dose, as quantified by the point of inflection around e (parameter b of the regression) (Table 4).

Unlike the other species evaluated, in *E. oryzoides*, all populations presented an RF from 2.28 to 14.49 with respect to Eo06, and their GR80 was above the dose of use (1080 [g a.i./ha]) between 1.5 and 12 times. The RF values in this species are much higher compared to all other species.

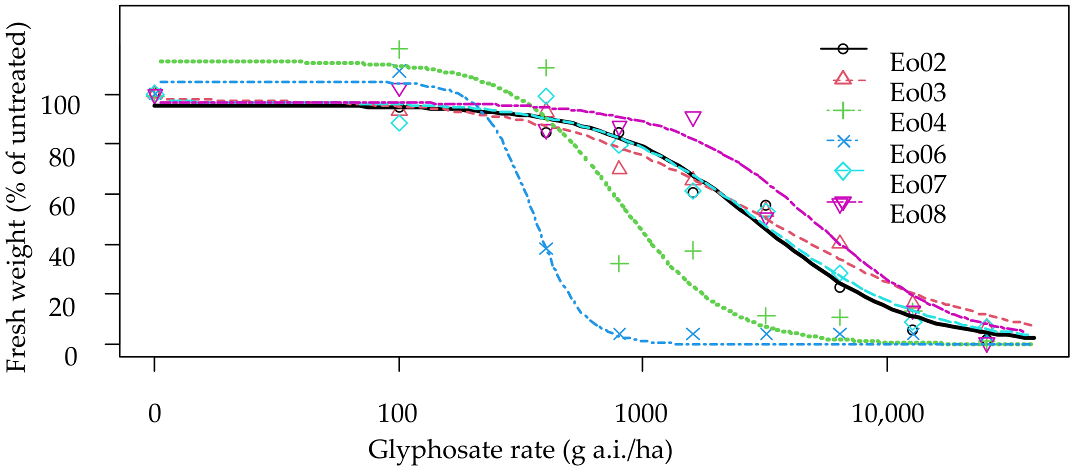

Figure 3. Dose–response curves corresponding to the different populations of *Echinochloa oryzoides*. Each point is the average of three experiments. These curves were calculated using the model proposed by Streibig and Kudsk [27].

3.2.4. *Echinochloa chacoensis*

The GR_{50} differs significantly between the populations, being the highest in the population Ech02 (Table 5 and Figure 4), while it was intermediate in the Ech03 population.

Table 5. Parameters of the logistic adjusted model for *Echinochloa chacoensis* (3 parameters, GR_{50} as parameter and GR_{80} estimated); the doses required for a reduction of 50% and 80% of the fresh weight, respectively, parameter e corresponds to the GR50 parameters c, d, and b that were defined in formula (1); fixed lower limit at 0.

Population	b	d	GR_{50}	se	RF [1]	*p*-Value *	GR_{80}	se
Ech01	1.708	99.45	99.67	13.13	1.00	-	224.420	31.045
Ech02	4.004	86.91	**269.36**	21.08	**2.70**	<0.0001	380.785	39.791
Ech03	2.622	99.93	152.44	12.63	1.53	0.00101	258.623	27.930

[1] RF: resistance factor. * The value of p is the level of probability of significance for each factor for the model. The Lack of Fit test indicates that the regression accurately describes the data in the table.

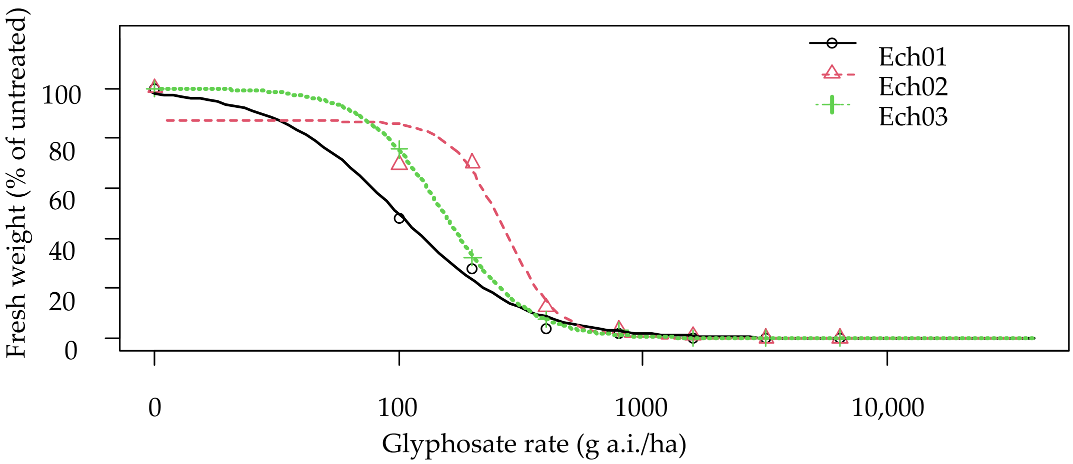

Figure 4. Dose response curves corresponding to the different populations of *Echinochloa chacoensis*. Each point is the average of three experiments. These curves were calculated using the model proposed by Streibig and Kudsk [27].

Evaluating the susceptibility to glyphosate in the dose–response trials in the different populations, it was determined that Ech02 and Ech03 were significantly different from the population Ech01 (considered susceptible). This specie presented an RF of 1.53 to 2.70 compared to Ech01, but its GR_{80} was well below the dose of use (1080 [g a.i./ha]), between three and four times the same.

3.3. Shikimic Acid

The accumulation of shikimic acid in the nine populations of *E. colona* in leaves after the application of 600 [g a.i./ha] of glyphosate was quantified in [mg] of shikimic acid per gram of leaf. Additionally, 33% of them increased the level of shikimic acid as time went by, as can be seen in Figure 5. Populations Ec03, Ec12, and Ec16 showed an increase at 48 h post application, respectively, reaching maximum values of 2.50 and 2.60 [mg] shikimic acid/[g] leaf at 96 h post application.

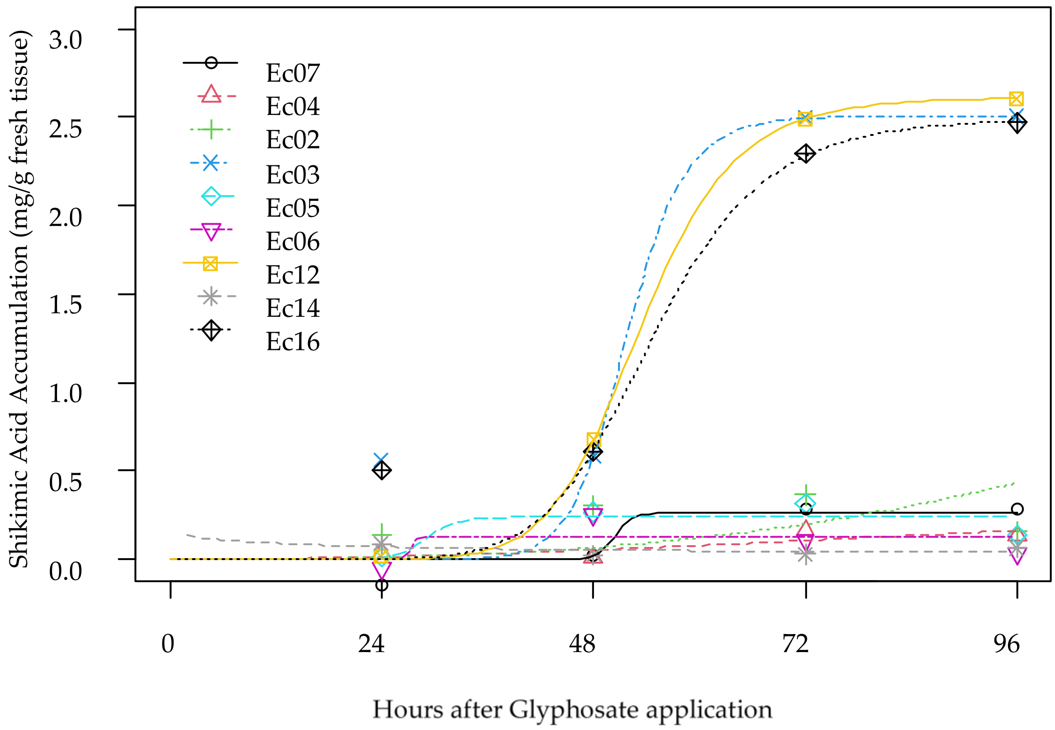

Figure 5. Shikimic acid accumulation from 24 to 96 h after glyphosate application on *E. colona* plants.

Carrying out particular contrasts, it was found that the populations Ec03, Ec12, and Ec16 accumulate values significantly different from the rest of the times after 48 h ($p = 0.0003$ for 48 h and $p > 0.0001$ for 72 and 96 h).

In populations Ec02, Ec04, Ec05, Ec06, and Ec07, these increases were not significant and did not exceed 0.50 [mg] of shikimic acid per gram of leaf.

In the populations of *E. crus-galli*, 33% of them (Ecg12, Ecg13, and Ecg16) increased the levels of shikimic acid after 48 h, as can be seen in Figure 6. In the populations Ecg06, Ecg07, Ecg14, and Ecg15, these increases were not significant and did not exceed 0.50 [mg] of shikimic acid per gram of leaf. While Ecg12, Ecg13, and Ecg16 inhibited their metabolic pathway and reached maximum values of 1.59, 2.30, and 2.53 [mg] shikimic acid/[g] leaf, respectively, at 96 h post application.

Carrying out particular contrasts, it was found that the Ecg13 and Ecg16 populations accumulate shikimic acid after 48 h, presenting values significantly different from the rest of the time ($p = 0.0037$; $p = 0.0009$; and $p > 0.0001$ for 48, 72, and 96 h, respectively).

E. oryzoides presents only one population (Eo06) that increases shikimic acid levels above 0.50 [mg/g] of leaf after 96 h (Figure 7), while populations Eo02, Eo03, Eo04, Eo07, and Eo08; these increases were not significant. The metabolic pathway of the Eo06 population reaches its maximum value of 2.12 [mg] shikimic acid/[g] leaf at 96 h post application.

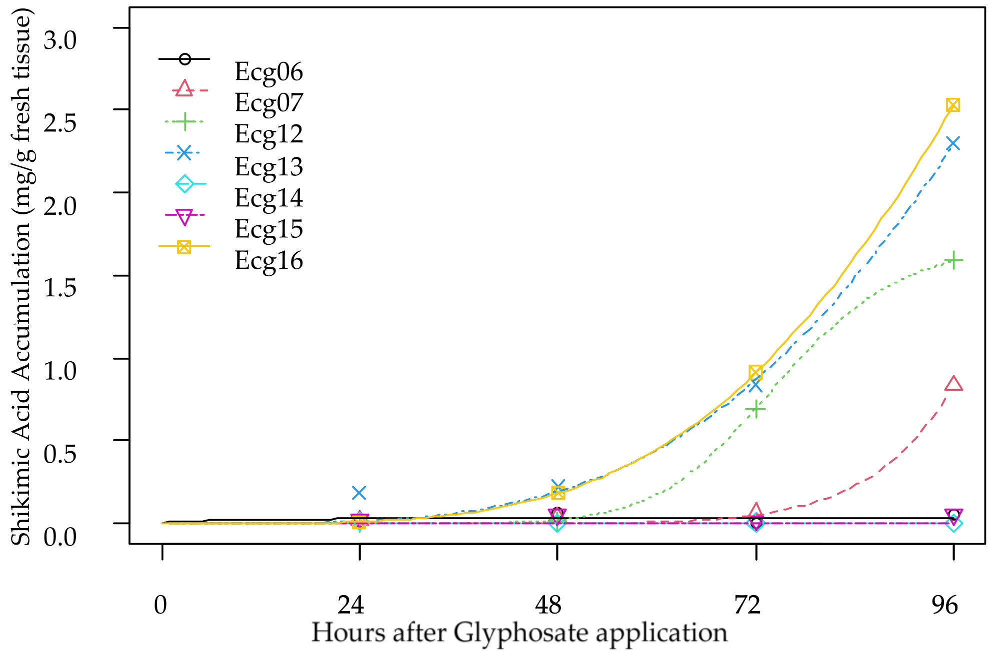

Figure 6. Shikimic acid accumulation from 24 to 96 h after glyphosate application on E. crus-galli plants.

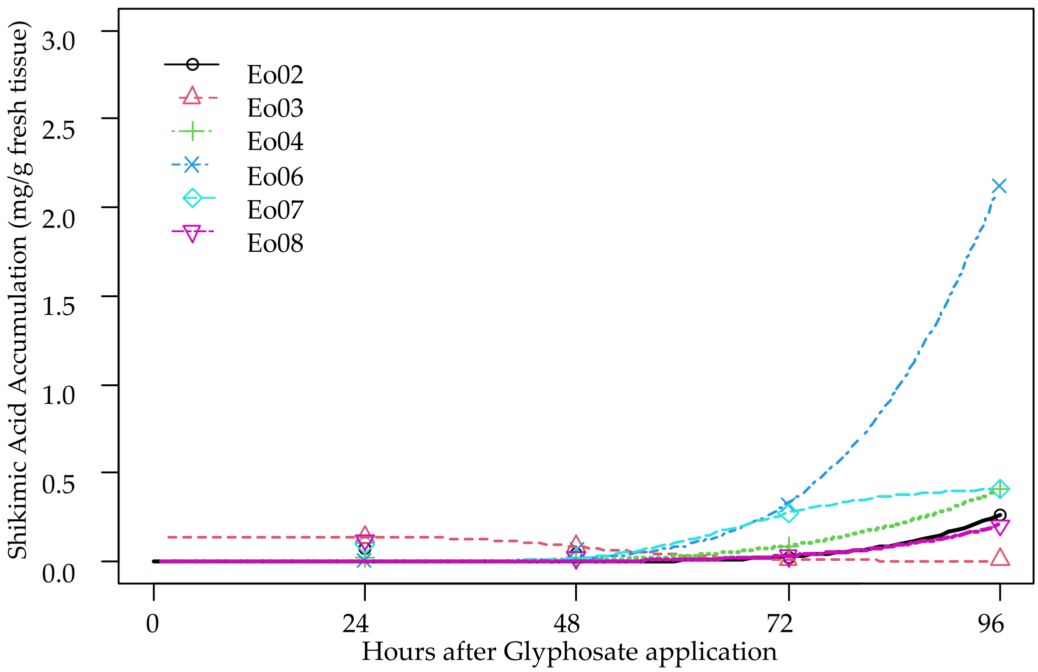

Figure 7. Shikimic acid accumulation from 24 to 96 h after glyphosate application on *E. oryzoides* plants.

The contrasts confirm that the Eo06 population accumulates shikimic acid only after 96 h, presenting values significantly different from the rest of the times ($p > 0.0001$).

In *E. chacoensis*, the 3 evaluated populations increase the levels of shikimic acid above 0.50 [mg/g] of leaf after 72 h (Figure 8), but the Ech02 population decreases at 96 h below the value considered as a limit. The Ech01 and Ech03 populations and their metabolic pathways reach their maximum value of [mg] shikimic acid/[g] leaf at 96 h post application.

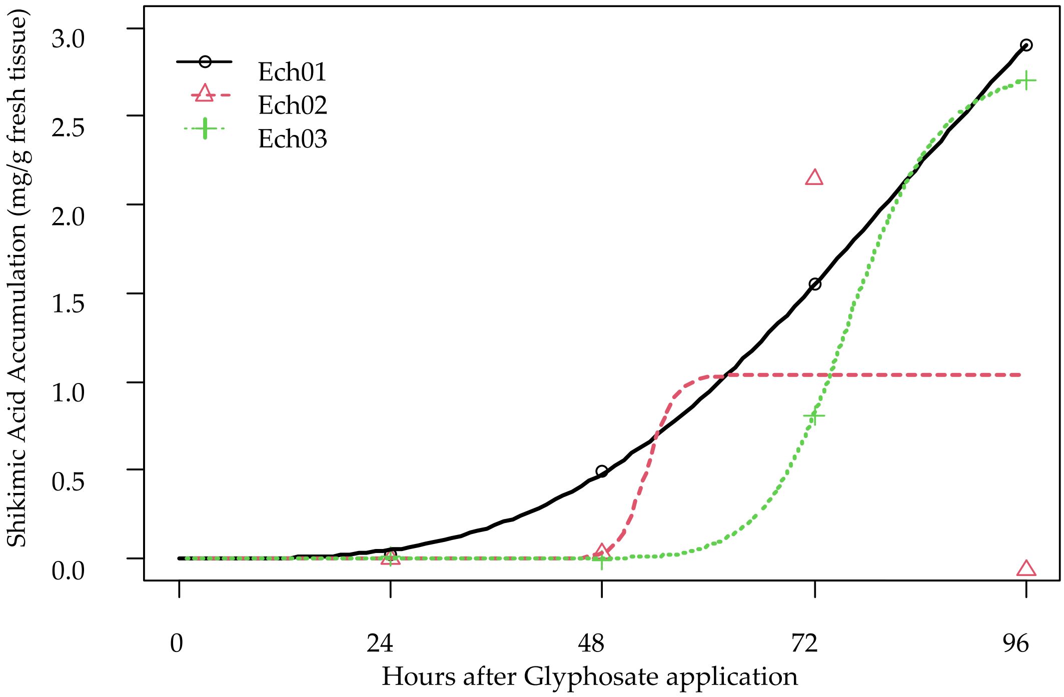

Figure 8. Shikimic acid accumulation from 24 to 96 h after glyphosate application on *E. chacoensis* plants.

The contrasts prove that the populations Ech01 and Ech03 accumulate shikimic acid only after 96 h, presenting values that are significantly different from the rest of the time ($p > 0.0001$).

4. Discussion

The survival tests carried out with the different populations of *Echinochloa* analyzed show differences between them. The analyzed samples were divided into three categories according to the percentage of survival observed [22] with the discriminant dose of 600 [g a.i./ha].

In the evaluated populations of the genus *Echinochloa*, thirteen of them presented high survival and can be considered resistant; another group made up of two populations presented with developing resistance; and the remaining ten populations presented survival values of less than 5%, which makes them susceptible.

The survival values found in this work for *E. colona* are similar to those of Alarcón-Reverte et al. [28] or Alarcón-Reverte et al. [29], with values ranging from 4 to 80% in populations of *E. colona* from California, although in this experiment the discriminating dose was 620 [g a.i./ha]. In populations from southern and southwestern Australia, Asaduzzaman et al. [30] found that in eighteen populations of *E. colona* exposed to a glyphosate dose of 540 [g a.i./ha], two of them survived at greater than 80% and 87%, respectively.

Regarding the dose–response experiments, differences in susceptibility to the herbicide glyphosate can be seen in all populations of the four species studied.

Based on the estimated resistance factor (RF), which is taken as a reference value to discriminate between resistant and susceptible populations [31], in the populations of *E. colona* Ec04, Ec07, and Ec14, they presented an RF greater than 2; therefore, they are considered populations resistant to glyphosate and the rest of the populations (Ec02, Ec03, Ec05, Ec12, and Ec16) as susceptible.

Studies carried out on *E. colona* by various authors in other countries [28,32,33] confirm that the population used has differences in susceptibility to this herbicide; however, the values of RF and GR50 calculated vary between the different authors.

Gaines et al. [32] in Australia, reported an RF 5.6 times higher than the susceptible population. Nguyen et al. [34], in another region of Australia, determined an RF of 2 to

12 times higher compared to the susceptible biotype. In Greece, Travlos et al. [35] obtained populations with an RF that was between 3.6 and 12.4. Closer to the values found in this work in *E. colona* are those found in the populations of California (USA), analyzed by Morran et al. [33], who found RF between 2.1 and 6.3. In other populations of southwestern and southern Australia, Asaduzzaman et al. [30] found populations that were 2.5 to 5 times more resistant than their corresponding susceptible phenotypes. In other populations of northern Australia, Mahajan et al. [36] evaluated populations that showed RF values between 6.7 and 15.1 in relation to susceptible populations.

The *E. colona* population with the highest RF in this study required 610 [g a.i./ha], similar to California populations that required 736 [g a.i./ha], but far from the 1116, 1289, 1187, and 3064 [g a.i./ha] required by populations in southern and southeastern Australia to reduce GR50. They were also very different from populations from northern Australia, with 1086 to 2339 [g a.i./ha] or 1220 [g a.i./ha] in the case of *E. colona* populations from Greece.

In *E. crus-galli*, the resistant populations (whose RF is greater than 2) are Ecg06, Ecg07, Ecg14, and Ecg15, whereas the susceptible populations are Ecg12, Ecg13, and Ecg16. In the populations declared resistant, the GR50 values of *E. crus-galli* are about three times those of *E. colona*, so it is important to recognize the species and act accordingly.

Experiences carried out in other countries show considerable evidence that these two species have the propensity to develop resistance to most groups of herbicides. Therefore, efforts to manage and control herbicide resistance in this species must be diligently continued [37].

In *E. oryzoides*, Eo06 is a population susceptible to glyphosate, and the rest (Eo02, Eo03, Eo04, Eo07, and Eo08) are resistant since their RFs are in a range of 2.28 to 14.49. In *E. chacoensis*, only the Ech02 population is resistant, while the others (Ech01 and Ech03) are susceptible.

In the work of Vázquez-García et al. [38], they classified susceptibility to glyphosate based on the RF value obtained, where populations with an RF value of less than 4 are susceptible, between 4 and 10 are resistant, and above 11 are very resistant. Using this classification, it is only of interest to determine the populations that meet the "very resistant" criterion, namely the Ecg15 population (RF of 13.089) in *E. crus-galli* and the Eo08 population (RF of 14.49) in *E. oryzoides*.

Shikimic acid accumulated after exposure to glyphosate (at a dose of 600 [gr a.i./ha]) in all susceptible populations of the different *Echinochloa* species. This accumulation suggests that glyphosate is successfully reaching the chloroplast and that at least part of the EPSPS enzyme present is sensitive to the herbicide. Here, differences were also found between the 4 species analyzed regarding the accumulation of shikimic acid over the time evaluated. In addition, it allows discrimination between the different levels of susceptibility to glyphosate in different populations.

In *E. colona* and *E. chacoensis*, 66% of the populations had their metabolic pathways inhibited (Ec2, Ec3, Ec5, Ec6, Ec12, Ec16, Ech01, and Ech03, respectively). In *E. crus-galli*, this percentage was 40% (Ecg12, Ecg13, and Ecg16), and in *E. oryzoides*, it was only 17% (Eo06). While in the rest of the populations, there was no inhibition of the metabolic pathway, which is consistent with what was explained by Singh and Shaner [26], who explained that the lack of accumulation of shikimate in plants treated with glyphosate can be taken as an indication that the plant is resistant to this herbicide.

The results obtained agree with what was expressed in the work of Alarcon-Reverte et al. [28] and Morran et al. [33], which also determined in *E. colona* that the accumulation of shikimic acid is greater in susceptible populations than in resistant ones. Such accumulation over time (24 h after application) in susceptible populations can lead to more than twice the concentration of shikimic acid than in resistant populations. At 96 h, these shikimic acid values can be up to fivefold.

Finally, if we take into account the classification between resistant and susceptible weeds that we described in the different experiments, we consider that a population is a biotype resistant to a herbicide when it has more than 20% survival, a resistance factor (RF) greater than 2, and does not have an accumulation of shikimic acid greater than 0.5 [mg/g] of a leaf.

The proportion of populations resistant to glyphosate within the genus *Echinochloa* clarifies the situation initially raised in the first reports of resistance [9]. Both the resistant populations and the less susceptible ones explain the current position as the fourth most important weed species in Argentina. A change in the strategy for the control of the species of the genus will be necessary for good agronomic management.

5. Conclusions

Summarizing the three tests carried out in this work, the following populations are considered resistant to glyphosate: Ec04, Ec07, and Ec14 of *E. colona*, Ecg06, Ecg07, Ecg14, and Ecg15 of *E. crus-galli*, Eo02, Eo03, Eo04, Eo07, and Eo08 of *E. oryzoides,* and Ech02 of *E. chacoensis*.

The populations susceptible to glyphosate are Ec02, Ec03, Ec05, Ec06, Ec12, and Ec16 for *E. colona*, Ecg12, Ecg13, and Ecg16 for *E. crus-galli*, only Eo06 for *E. oryzoides,* and Ech01, and Ech03 for *E. chacoensis*.

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References

1. Soreng, R.J.; Peterson, P.M.; Romaschenko, K.; Davidse, G.; Teisher, J.K.; Clark, L.G.; Barberá, P.; Gillespie, L.J.; Zuloaga, F.O. A Worldwide Phylogenetic Classification of the Poaceae (Gramineae) II: An Update and a Comparison of Two 2015 Classifications: Phylogenetic Classification of the Grasses II. *Jnl. Sytematics Evol.* **2017**, *55*, 259–290. [CrossRef]
2. Belgrano, M.J.; Morrone, O.; Zuloaga, F.O. *Catálogo de Las Plantas Vasculares del Cono Sur: (Argentina, Sur de Brasil, Chile, Paraguay y Uruguay)*; Catalog of the Vascular Plants of the Southern Cone; Missouri Botanical Garden Press: St. Louis, MO, USA, 2008; ISBN 978-1-930723-76-4.
3. Zuloaga, F.O.; Belgrano, M.J.; Zanotti, C.A. Actualización del Catálogo de las Plantas Vasculares del Cono Sur. *Darwiniana Nueva Ser.* **2019**, *7*, 208–278. [CrossRef]
4. Picapietra, G.; Ponsa, J.C. Competencia y Manejo de Capín de Arroz En El Cultivo de Soja | Instituto Nacional de Tecnología Agropecuaria. Informe Técnico INTA. Ediciones INTA. 2015.
5. Holm, L.G. *The World's Worst Weeds: Distribution and Biology*; Krieger: Malabar, FL, USA, 1991; ISBN 978-0-89464-415-3.
6. Pautasso, L. *Pérdida del Rendimiento del Cultivo de Soja por la Presencia de Rama Negra y Capín*; Serie Extensión No 76 Actualización Técnica Soja; Instituto Nacional de Tecnología Agropecuaria: Buenos Aires, Argentina, 2015; pp. 87–91.
7. Chin, D.V. Biology and Management of Barnyardgrass, Red Sprangletop and Weedy Rice. *Weed Biol. Manag.* **2001**, *1*, 37–41. [CrossRef]
8. REM Malezas: Continúa El Avance de Las Resistencias. Available online: https://www.aapresid.org.ar (accessed on 14 October 2022).
9. Papa, J.C.; Tuesca, D.; Bacigaluppo, D. *Detección Reciente en la Provincia de Santa Fe de Biotipos de Echinochloa colona Sospechosos de Presentar Resistencia a Glifosato. Para Mejorar la Producción 45. Revista Cultivos Estivales. Inta Oliveros*; Instituto Nacional de Tecnología Agropecuaria: Buenos Aires, Argentina, 2010; pp. 91–94.
10. Rao, A.N.; Johnson, D.E.; Sivaprasad, B.; Ladha, J.K.; Mortimer, A.M. Weed Management in Direct-Seeded Rice. In *Advances in Agronomy*; Sparks, D.L., Ed.; Academic Press: Cambridge, MA, USA, 2007; Volume 93, pp. 153–255.
11. Damalas, C.A.; Dhima, K.V.; Eleftherohorinos, I.G. Morphological and Physiological Variation among Species of the Genus *Echinochloa* in Northern Greece. *Weed Sci.* **2008**, *56*, 416–423. [CrossRef]
12. Yabuno, T. Biology of Echinochloa Species. In Proceedings of the Conference on Weed Control in Rice, Los Baños, Philippines, 31 August–4 September 1981; International Rice Research Institute: Los Baños, Philippines, 1983; pp. 307–318.
13. Pfitscher, E.M.; Barreto, I.L. Species of the genus Echinochloa (Gramineae) occurring in Rio Grande do Sul (Brazil). *Anu. Tec. Do Inst. De Pesqui. Zootec. Fr. Osorio* **1976**, *3*, 245–289.
14. Martínez Crovetto, R. Las Gramíneas Argentinas Del Género Echinochloa. *Rev. Argent. Agron.* **1942**, *9*, 310–342.
15. Renvoize, S. A new species of Echinochloa (Poaceae) from Bolivia and Northern Argentina. *Kurtziana* **1995**, *24*, 161–163.

16. Sudershan, M.; Guru, S.K.; Nitin, K.; Prinsa, R.; Babita, J. Herbicide Resistance: A Threat to Herbicide Utility and Sustainability. In *Phytoremediation of Heavy Metals: A Green Technology to Clean Environment*; Volume Advances in Agriculture Sciences; Anik Publication: New Delhi, India, 2020; pp. 115–129; ISBN 978-93-88112-21-5.
17. WFO. World Flora Online. Available online: http://www.worldfloraonline.org (accessed on 20 December 2022).
18. Heap, I.M. The International Survey of Herbicide Resistant Weeds. Available online: weedscience.org (accessed on 14 October 2022).
19. Leguizamón, E. *Rama Negra Conyza bonariensis (L. Cronquist) Bases para su Manejo y Control en Sistemas de Producción*; Manejo de Malezas Problema; REM Aapresid: Rosario, Argentina, 2011.
20. Panigo, E.S.; Dellaferrera, I.M.; Acosta, J.M.; Bender, A.G.; Garetto, J.I.; Perreta, M.G. Glyphosate-Induced Structural Variations in Commelina Erecta, L. (Commelinaceae). *Ecotoxicol. Environ. Saf.* **2012**, *76*, 135–142. [CrossRef]
21. González-Torralva, F.; Norsworthy, J.K.; Piveta, L.B.; Varanasi, V.K.; Barber, T.; Brabham, C. Susceptibility of Arkansas Palmer Amaranth Accessions to Common Herbicide Sites of Action. *Weed Technol.* **2020**, *34*, 770–775. [CrossRef]
22. Busi, R.; Beckie, H.J. Are Herbicide Mixtures Unaffected by Resistance? A Case Study with Lolium Rigidum. *Weed Res.* **2021**, *61*, 92–99. [CrossRef]
23. Ritz, C.; Baty, F.; Streibig, J.C.; Gerhard, D. Dose-Response Analysis Using R. *PLoS ONE* **2015**, *10*, e0146021. [CrossRef]
24. Ritz, C.; Streibig, J.C. Bioassay Analysis Using R. *J. Stat. Softw.* **2005**, *12*, 1–22. [CrossRef]
25. Knezevic, S.Z.; Streibig, J.C.; Ritz, C. Utilizing R Software Package for Dose-Response Studies: The Concept and Data Analysis. *Weed Technol.* **2007**, *21*, 840–848. [CrossRef]
26. Singh, B.; Shaner, D. Rapid Determination of Glyphosate Injury to Plants and Identification of Glyphosate-Resistant Plants. *Weed Technol.* **1998**, *12*, 527–530. [CrossRef]
27. Streibig, J.C.; Kudsk, P. *Herbicide Bioassays*; CRC Press: Boca Raton, FL, USA, 1993.
28. Alarcón-Reverte, R.; García, A.; Urzúa, J.; Fischer, A.J. Resistance to Glyphosate in Junglerice (Echinochloa Colona) from California. *Weed Sci.* **2013**, *61*, 48–54. [CrossRef]
29. Alarcón-Reverte, R.; García, A.; Watson, S.B.; Abdallah, I.; Sabaté, S.; Hernández, M.J.; Dayan, F.E.; Fischer, A.J. Concerted Action of Target-Site Mutations and High EPSPS Activity in Glyphosate-Resistant Junglerice (Echinochloa Colona) from California. *Pest Manag. Sci.* **2015**, *71*, 996–1007. [CrossRef]
30. Asaduzzaman, M.; Koetz, E.; Wu, H.; Hopwood, M.; Shephard, A. Fate and Adaptive Plasticity of Heterogeneous Resistant Population of Echinochloa Colona in Response to Glyphosate. *Sci. Rep.* **2021**, *11*, 14858. [CrossRef]
31. Burgos, N.R.; Tranel, P.J.; Streibig, J.C.; Davis, V.M.; Shaner, D.; Norsworthy, J.K.; Ritz, C. Review: Confirmation of Resistance to Herbicides and Evaluation of Resistance Levels. *Weed Sci.* **2013**, *61*, 4–20. [CrossRef]
32. Gaines, T.A.; Cripps, A.; Powles, S.B. Evolved Resistance to Glyphosate in Junglerice (Echinochloa Colona) from the Tropical Ord River Region in Australia. *Weed Technol.* **2012**, *26*, 480–484. [CrossRef]
33. Morran, S.; Moretti, M.L.; Brunharo, C.A.; Fischer, A.J.; Hanson, B.D. Multiple Target Site Resistance to Glyphosate in Junglerice (Echinochloa Colona) Lines from California Orchards. *Pest Manag. Sci.* **2018**, *74*, 2747–2753. [CrossRef]
34. Nguyen, T.H. *Evolution and Spread of Glyphosate Resistant Barnyard Grass (Echinochloa Colona (L.) Link) from Australia*; School of Agriculture, Food and Wine Faculty of Sciences, The University of Adelaide: Adelaide, Australia, 2015.
35. Travlos, I.; Kanatas, P.; Tsekoura, A.; Gazoulis, I.; Papastylianou, P.; Kakabouki, I.; Antonopoulos, N. Efficacy of Different Herbicides on Echinochloa Colona (L.) Link Control and the First Case of Its Glyphosate Resistance in Greece. *Agronomy* **2020**, *10*, 1056. [CrossRef]
36. Mahajan, G.; Kaur, V.; Thompson, M.; Chauhan, B.S. Growth Behavior and Glyphosate Resistance Level in 10 Populations of Echinochloa Colona in Australia. *PLoS ONE* **2020**, *15*, e0221382. [CrossRef] [PubMed]
37. Talbert, R.E.; Burgos, N.R. History and Management of Herbicide-Resistant Barnyardgrass (Echinochloa Crus-Galli) in Arkansas Rice. *Weed Technol.* **2007**, *21*, 324–331. [CrossRef]
38. Vázquez-García, J.G.; Rojano-Delgado, A.M.; Alcántara-de la Cruz, R.; Torra, J.; Dellaferrera, I.; Portugal, J.; De Prado, R. Distribution of Glyphosate-Resistance in *Echinochloa crus-galli* across Agriculture Areas in the Iberian Peninsula. *Front. Plant Sci.* **2021**, *12*, 33. [CrossRef]