Evolved Resistance to Glyphosate in Junglerice (*Echinochloa colona*) from the Tropical Ord River Region in Australia

Todd A. Gaines, Andrew Cripps, and Stephen B. Powles*

The objective of this study was to determine whether a junglerice population from the tropical Ord River region of northwest Australia was glyphosate resistant, and whether alternative herbicides labeled for junglerice control were still effective. Seed samples collected from the field site were initially screened with glyphosate in the glasshouse, and surviving individuals were self-pollinated for subsequent glyphosate dose-response studies. Glyphosate resistance was confirmed, as the suspected resistant population was found to be 8.6-fold more resistant to glyphosate than a susceptible population based on survival (LD_{50} of 3.72 kg ha^{-1}), and 5.6-fold more resistant based on biomass reduction (GR_{50} of 1.16 kg ha^{-1}). The glyphosate-resistant population was susceptible to label-recommended doses of all other herbicides assessed, including three acetyl-CoA carboxylase (ACC) –inhibiting herbicides (fluazifop-P, haloxyfop, and sethoxydim), two acetolactate synthase (ALS) –inhibiting herbicides (imazamox and sulfometuron), paraquat, and glufosinate. Glyphosate resistance has previously evolved in numerous species found in glyphosate-resistant cropping systems, no-till chemical fallow, fence line, and perennial crop situations. Here we report the evolution of glyphosate resistance in a cropping system that included annual tillage. The evolution of glyphosate resistance in junglerice from a tropical cropping system further demonstrates the need for improved glyphosate stewardship practices globally.

Nomenclature: Fluazifop-P; glufosinate; glyphosate; haloxyfop; imazamox; paraquat, sethoxydim; sulfometuron; junglerice, *Echinochloa colona* (L.) Link.

Key words: Evolution, glyphosate resistance, herbicide resistance.

El objetivo de este estudio fue determinar si una población de *Echinochloa colona* de la región tropical de Ord River en el noroeste de Australia era resistente a glyphosate, y si herbicidas alternativos con etiqueta para el control de *E. colona* eran todavía efectivos. Muestras de semillas colectadas en el campo fueron inicialmente tratadas con glyphosate en una invernadero, y los individuos sobrevivientes fueron autopolinizados para estudios posteriores de respuesta a dosis con glyphosate. La resistencia a glyphosate fue confirmada, al determinar que la población que se sospechaba resistente fue 8.6 veces más resistente a glyphosate que la población susceptible según la sobrevivencia (LD_{50} de 3.72 kg ha^{-1}) y 5.6 veces más resistentes según la reducción de biomasa (GR_{50} de 1.16 kg ha^{-1}). La población resistente a glyphosate fue susceptible a las dosis recomendadas en etiqueta de todos los demás herbicidas evaluados, incluyendo tres herbicidas inhibidores de acetyl-CoA carboxylase (ACC) (fluazifop-P, haloxyfop y sethoxydim), dos herbicidas inhibidores de acetolactate synthase (ALS) (imazamox y sulfometuron), paraquat y glufosinate. La resistencia a glyphosate ha evolucionado previamente en numerosas especies que se encuentran en sistemas con cultivos resistentes a glyphosate, en barbechos químicos con cero labranza, en cercas y en situaciones con cultivos perennes. Aquí reportamos la evolución de resistencia a glyphosate en un sistema de cultivos que incluía labranza anual. La evolución de resistencia a glyphosate en *E. colona* en un sistema de cultivos tropical demuestra aún más la necesidad de mejorar las prácticas de buen manejo de glyphosate globalmente.

A major annual challenge to global food and fiber production is posed by weed species that infest crops and reduce yield and quality. For the past half century, the weed challenge has been successfully managed with herbicides to remove unwanted weed species. The most widely used and successful herbicide in global agriculture is glyphosate (Duke and Powles 2008). However, continued glyphosate sustainability as the world's most important herbicide is threatened by overreliance and lack of weed control diversity producing intense selection pressure for the evolution of glyphosate-resistant weed populations (Powles 2008). Since the first reports (Powles et al. 1998; Pratley et al. 1999), weed populations from 21 species globally have now evolved glyphosate resistance (Heap 2011). One shared feature evident in most cases is the overreliance on glyphosate for weed control without diversity in herbicides or in nonherbicide weed control tools. Here, we report on a case of glyphosate resistance evolution in a rather unique tropical region with annual tillage and irrigation, and glyphosate usage consisting of at least three applications per year in most situations.

The Ord River region in northwestern Australia was established in 1967 with the creation of Lake Argyle, and thus the Ord River Irrigation Scheme. This tropical region has a hot, wet season (791 mm average wet season rainfall) from mid-November through March, and a warm, dry season from April to October (Smith et al. 2007). The abundance of irrigation water enables irrigated cropping during the dry season, including watermelons (*Citrullus lanatus* Thunb.), rice (*Oryza sativa* L.), and cotton (*Gossypium hirsutum* L.). Fields are generally inaccessible to farm equipment during the rainy season, so weed control typically consists of aerial glyphosate applications. The repeated use of glyphosate on weeds with

large population sizes and multiple generations per year presents a high-risk situation for resistance evolution.

Junglerice is a species that is common in the tropical Ord River region, and has been managed with glyphosate for over 20 yr. Glyphosate resistance in junglerice has previously been reported in northeast Australia (Cook et al. 2008; Dolman et al. 2009; Storrie et al. 2008; Thornby and Walker 2009), Argentina (Heap 2011), and California (Alarcón-Reverte et al. 2012). Following reports of problems controlling junglerice with glyphosate in the Ord River region, a problem site was investigated and seed samples were collected. The objective of this study was to determine whether a suspected junglerice population was glyphosate-resistant and whether alternative herbicides labeled for control of the weed were still effective.

Materials and Methods

Plant Material. Following reports of glyphosate control failures on junglerice, seed samples were collected from a 32-ha field in the tropical Ord River region of northern Western Australia that had been in production for 10 yr with no prior herbicide use. Over the 10-yr period, the field had been devoted to watermelon production during the dry season, and burndown weed control during the rainy season fallow phase (November to March) each year consisted of three aerial applications of glyphosate at 1.0 kg ae ha^{-1}. Three seed samples were collected, each consisting of multiple suspected glyphosate-resistant junglerice individuals that had survived glyphosate applications during the preceding rainy season. One seed sample of junglerice was also collected from a nearby roadside area for use as a glyphosate-susceptible population. Because of the known difficulty in correctly identifying *Echinochloa* species (Tabacchi et al. 2006), samples were verified as junglerice by the Western Australia herbarium.

Initial Evaluation of Seed Collections. Seeds were scarified in concentrated sulfuric acid for 8 min, and then triple rinsed with distilled water and dried. Scarified seeds were placed on 0.8% water-solidified agarose in petri dishes, and placed in an incubator set to 25/15 C day/night temperatures, with 12 h light for 4 d. Germinated seeds of each seed sample were transplanted to 2-L pots (10 plants per pot) containing potting mix (50% composted pine bark, 25% peat, 25% river sand) and transferred to a glasshouse. Plants were kept well watered and fertilized. Suspected resistant samples and the susceptible population were initially screened in the glasshouse with two replications of 10 individuals each at glyphosate doses of 0.4 and 0.8 kg ae ha^{-1} (Roundup PowerMax®, 540 g L^{-1} potassium salt, Nufarm Australia Ltd., 103-105 Pipe Road, Laverton North, Victoria 3026, Australia). Plants were treated at the three- to four-leaf stage with the use of a custom-built, dual-nozzle (TeeJet XR11001 flat fan, TeeJet Australasia Pty Ltd., 65 West Fyans Street, Newtown, Victoria 3220, Australia) track sprayer calibrated to deliver 110 L ha^{-1} water volume at 200 kPa, at a speed of 3.6 km h^{-1} and from a height of 0.5 m. Plant survival was assessed 21 d after treatment, and seed was produced for progeny dose-response tests by allowing three selected surviving individuals from the suspected glyphosate-resistant samples to self-pollinate, and allowing four nontreated individuals from the glyphosate-susceptible population to self-pollinate.

Glyphosate Dose Response. Progeny from three self-pollinated surviving individuals were tested with increasing doses of glyphosate: 0, 0.4, 0.8, 1.6, 3.2, and 9 kg ha^{-1}. Progeny of the susceptible population were tested at 0, 0.2, 0.4, 0.8, and 1.6 kg ha^{-1}. The completely randomized experimental design consisted of three replications with 10 plants each. The experiment was conducted in pots outdoors during the normal summer growing season for this species, with average day maximum temperature of 30 C. Plants were kept well-watered and fertilized. Plant survival was assessed 21 d after treatment, and plant aboveground biomass was harvested, dried for 7 d at 60 C, and weighed. The experiment was conducted twice.

Assessment of Resistance to Other Herbicides. Plants of the suspected glyphosate-resistant and -susceptible population were grown to the three- to four-leaf stage, as previously described. Treatments consisted of three replications with six plants each. Commercial formulations of herbicides were applied, as previously described, at label-recommended rates for junglerice. Herbicides evaluated included fluazifop-P at 105 g ai ha^{-1} (Fusilade Forte®, 128 g L^{-1}, Syngenta Crop Protection Pty Ltd., 2-4 Lyonpark Road, Macquarie Park, New South Wales 2113, Australia), glufosinate at 400 g ai ha^{-1} (Basta®, 200 g L^{-1}, Bayer CropScience Pty Ltd., 391-393 Tooronga Road, East Hawthorn, Victoria 3123, Australia), haloxyfop at 52 g ai ha^{-1} (Verdict®, 520 g L^{-1}, Dow AgroSciences Australia Ltd., Locked Bag 502, Frenchs Forest, New South Wales 2086, Australia), imazamox at 35 g ai ha^{-1} (Raptor®, 700 g kg^{-1}, Crop Care Australasia Pty Ltd., Unit 15, 16 Metroplex Avenue, Murarrie, Queensland 4172, Australia), paraquat at 300 g ai ha^{-1} (Gramoxone®, 250 g L^{-1}, Syngenta Crop Protection Pty Ltd., 2-4 Lyonpark Road, Macquarie Park, New South Wales 2113, Australia), sethoxydim at 186 g ai ha^{-1} (Sertin®, 186 g L^{-1}, Bayer CropScience Pty Ltd., 391-393 Tooronga Road, East Hawthorn, Victoria 3123, Australia), and sulfometuron at 150 g ai ha^{-1} (Oust®, 750 g kg^{-1}, Du Pont Australia Ltd., 7 Eden Park Drive, Macquarie Park, New South Wales 2113, Australia). All treatments included 0.25% v/v nonionic surfactant (BS1000, Crop Care Australasia Pty Ltd., Unit 15, 16 Metroplex Avenue, Murarrie, Queensland 4172, Australia), except haloxyfop, with 1% v/v spraying oil adjuvant (Uptake Spraying Oil, Dow AgroSciences Australia Ltd., Locked Bag 502, Frenchs Forest, New South Wales 2086, Australia) and paraquat with no additional surfactant. Plant mortality was visually assessed 21 d after treatment by scoring each individual as alive or dead.

Data Analysis. Glyphosate dose-response data of plant survival and biomass were analyzed with nonlinear logistic regression to determine the glyphosate dose causing 50% mortality (LD$_{50}$) or 50% reduction in biomass (GR$_{50}$). Regression analysis was performed in R2.11.1 (R Development Core Team 2010) with the use of a three-parameter log-logistic model (Knezevic et al. 2007):

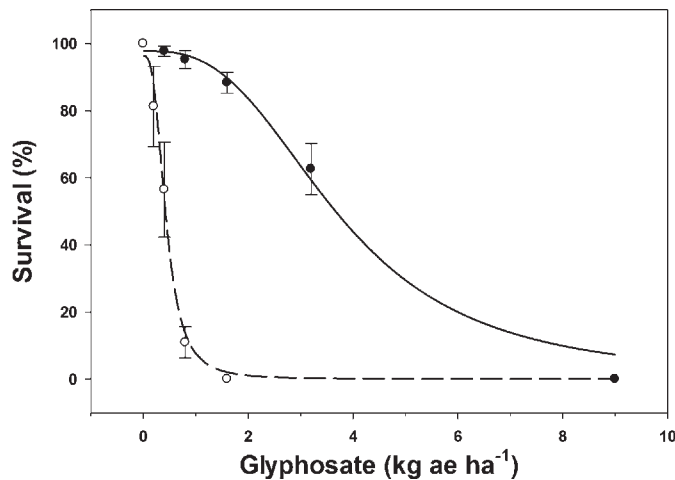$$Y = \frac{d}{1 + \exp[b(\log[(x - \log)(e)])]},\qquad [1]$$

Figure 1. Plant survival (%) of a glyphosate-resistant (black circles, solid line) and -susceptible (open circles, dashed line) junglerice population from Australia as affected by increasing dose of glyphosate. Data points are means and standard errors from two experimental repetitions, and lines were fit with the use of Equation 1 (see text).
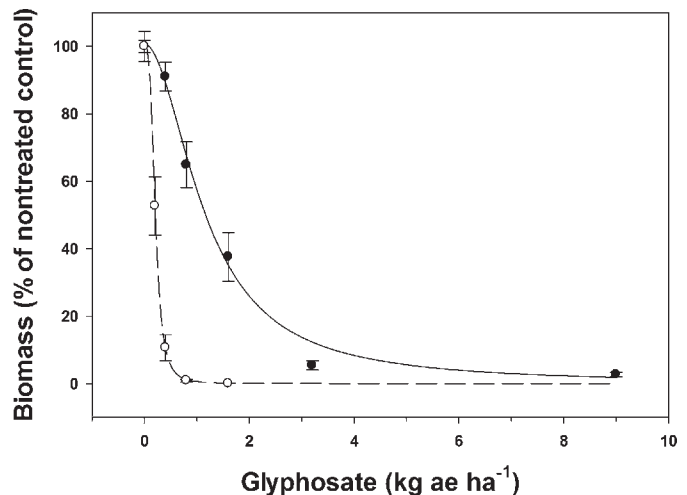

Figure 2. Biomass response (% of nontreated control) of a glyphosate-resistant (black circles, solid line) and -susceptible (open circles, dashed line) junglerice population from Australia to increasing dose of glyphosate. Data points are means and standard errors from two experimental repetitions, and lines were fit with the use of Equation 1 (see text).

where the parameter d is the upper limit, b is the slope of the curve, x is the herbicide dose, e is the dose producing a 50% reduction in response, and the lower limit set at 0. The *drc* package was used to compare LD_{50} and GR_{50} estimates between suspected glyphosate-resistant and -susceptible junglerice populations statistically.

Results and Discussion

Initial Evaluation of Seed Collections. The initial seed collections from the Ord River region field site were heterogeneous for glyphosate response. During initial glasshouse screening, 6, 0, and 25% of plants of the three suspected glyphosate-resistant samples survived glyphosate at 0.4 kg ha^{-1}, and 0, 0, and 20% survived 0.8 kg ha^{-1} glyphosate. Averaged across all samples and both treatments, 12% of the screened individuals survived. Three surviving individuals were self-pollinated for further evaluation, two from the 0.4 kg ha^{-1} dose and one from the 0.8 kg ha^{-1} dose, and progeny seed was harvested individually from each of the three surviving individuals for subsequent evaluation. No individuals in the putative susceptible population survived either glyphosate dose.

Glyphosate Dose Response. Dose-response studies were conducted outdoors during the summer to evaluate glyphosate

resistance in the progeny of three glyphosate-resistant junglerice individuals identified from field collections. Results from one progeny set, the individual surviving 0.8 kg ha^{-1}, are presented here; statistically similar LD_{50} and GR_{50} values were obtained from the other two progeny sets. Glyphosate resistance was confirmed in both plant survival (Figure 1, Table 1) and biomass response (Figure 2, Table 1). This glyphosate-resistant junglerice progeny was found to be 8.6-fold more resistant to glyphosate than the susceptible population based on percent survival, and 5.6-fold more resistant based on biomass reduction (Table 1). Therefore, the population was classified as moderately resistant to glyphosate.

Assessment of Resistance to Other Herbicides. The glyphosate-resistant plants were susceptible to label-recommended doses of all other herbicides assessed, including three ACC-inhibiting herbicides, two ALS-inhibiting herbicides, and the nonselective herbicides paraquat and glufosinate (Table 2). Glufosinate and paraquat are two of the primary alternative herbicides available for nonselective fallow and preplant applications. These results indicate herbicide diversity can be used to manage this glyphosate-resistant junglerice population.

Junglerice seed was collected from a field where glyphosate had been used as the sole means of weed control during the rainy season, a practice typical in the tropical Ord River

Table 1. Regression parameters (see Equation 1 in text) of the response of a glyphosate-resistant (R) and -susceptible (S) junglerice population from Australia to increasing dose of glyphosate, and resistance factor (R/S) for survival and biomass.[a]

	Survival					Biomass				
	b	d	LD_{50}	R/S	P	b	d	GR_{50}	R/S	P
			———— kg ha^{-1} ————					———— kg ha^{-1} ————		
R	2.9	97.8	3.72	8.6	<0.0001	1.9	100.7	1.16	5.6	<0.0001
S	2.9	96.4	0.43			3.2	99.9	0.21		

[a] GR_{50}, glyphosate dose resulting in a 50% reduction in biomass; LD_{50}, glyphosate dose resulting in 50% mortality. Resistance factor = GR_{50} or LD_{50} of R/S.

Table 2. Response of glyphosate-resistant and -susceptible junglerice to alternative herbicides.

Herbicide	Rate	Site of action[a]	Resistant	Susceptible
	(g ai or ae ha^{-1})		——% Mortality ——	
Paraquat	300	Photosystem I	100	100
Glufosinate	400	Glutamine synthetase	100	100
Fluazifop-P	105	ACC	100	100
Haloxyfop	52	ACC	100	100
Sethoxydim	186	ACC	100	100
Sulfometuron	150	ALS	100	100
Imazamox	35	ALS	100	100

[a] Abbreviations: ACC, acetyl-CoA carboxylase; ALS, acetolactate synthase.

region of Western Australia, and the junglerice population had been exposed to at least 30 glyphosate applications over 10 yr with no diversity in other burndown control methods. Resistant individuals were identified in the seed collections, these individuals were self-pollinated, and their progeny were highly homogeneous in their response to glyphosate. The level of glyphosate resistance was relatively high, as the LD$_{50}$ of the resistant progeny was 3.72 kg ha^{-1}, 8.6-fold greater than the susceptible population—more than 6.5 times the Australian-labeled glyphosate dose for junglerice control in fallow, and 3.7 times the aerial application rate used in the Ord River region. A different junglerice population from northeast Australia was only threefold resistant compared to a susceptible population (Werth et al. 2008) and had an LD$_{50}$ of 0.3 kg ha^{-1}, suggesting that this population and the Ord River population may have evolved a different glyphosate resistance mechanism(s).

Previous glyphosate-resistant weed species have occurred in glyphosate-resistant cropping systems, no-till chemical fallow, fence line, and perennial crop (vineyards, orchards) situations (reviewed by Powles and Yu 2010). Additional resistant populations continue to be reported frequently. The reporting of seven new cases of glyphosate resistance in *Weed Science* and *Weed Technology* in 2011 (Binkholder et al. 2011; de Carvalho et al. 2011; Dickson et al. 2011; Light et al. 2011; Mueller et al. 2011; Norsworthy et al. 2011; Riar et al. 2011) highlights that the reliance on glyphosate for weed control continues to exert substantial selection pressure for resistance to this herbicide. Here, we report the evolution of glyphosate resistance in a cropping system that also includes intensive tillage and land preparation for furrow irrigation prior to crop planting. However, glyphosate is the sole control measure for junglerice during the rainy season; the exclusive reliance on glyphosate for weed control for 10 yr during the rainy season has selected for glyphosate-resistant individuals. Alternative sites of action including ALS- and ACC-inhibiting herbicides, paraquat, and glufosinate were found to be effective against this glyphosate-resistant junglerice population, and represent potential options for alternative herbicide control during the rainy season and for preplant applications.

The mechanism(s) endowing glyphosate resistance in this junglerice population is presently unknown and will be the topic of future research. Comparisons of glyphosate resistance mechanisms found in junglerice populations from other regions in Australia, Argentina, and California may be useful

to understand the evolution of glyphosate resistance in this species. Due to the diversity of *Echinochloa* species that are problematic weeds in different regions around the world, another area of research should be the potential for glyphosate-resistant junglerice to hybridize with related species in the genus and transfer glyphosate resistance, a process previously shown in both *Conyza* and *Amaranthus* (Gaines et al. 2012; Zelaya et al. 2007). The evolution of glyphosate resistance in junglerice from a tropical cropping system further demonstrates the need for improved glyphosate stewardship practices globally.

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