Weed Science 2014 62:441–450

Characterization of Glyphosate-Resistant Tropical Sprangletop (*Leptochloa virgata*) and Its Alternative Chemical Control in Persian Lime Orchards in Mexico

Macrina Pérez-López, Fidel González-Torralva, Hugo Cruz-Hipólito, Francisco Santos, José A. Domínguez-Valenzuela, and Rafael De Prado*

Field, greenhouse, and laboratory experiments were conducted to investigate resistance to glyphosate in tropical sprangletop biotypes (Lv8 and Lv9) collected in Persian lime from Veracruz, Mexico. Assays to determine the dose required to reduce seedling fresh weight by 50% indicated a resistance factor (RF) of 4.9 and 3.2 for biotypes Lv8 and Lv9, respectively; whereas the LD_{50} showed a RF of 4.4 and 3.3 for biotypes Lv8 and Lv9, respectively. On the other hand, the RFs using whole plant dose–response assays were lower (RF of 3 for Lv8 and 2.3 for Lv9). The susceptible biotype (LvS) accumulated 5.5 and 11.8 times more shikimate than biotypes Lv8 and Lv9, respectively, at 96 h after treatment (HAT). In field experiments, alternatives to glyphosate-resistant tropical sprangletop management were identified. Indaziflam + glufosinate and paraquat + diuron provided over 80% control of in-field populations of tropical sprangletop at 60 d after treatment (DAT). These results confirmed the first reported case of glyphosate-resistant tropical sprangletop.
Nomenclature: Diuron; indaziflam; paraquat; glufosinate; glyphosate; tropical sprangletop, *Leptochloa virgata* (L.) P. Beauv.; Persian lime, *Citrus latifolia* Tan.
Key words: Alternative herbicides, field experiments, resistance, shikimic acid.

Glyphosate is one of the most widely used nonselective herbicides due to its broad spectrum of weed control and its low toxicity to mammals, birds, and fish (Velini et al. 2009). Glyphosate has been used in perennial crops, uncultivated areas, and no-tillage systems since development in the 1970s (Baylis 2000). Since 1996, glyphosate use has increased with the development of resistant crops (Duke et al. 2012).

Glyphosate targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an important enzyme in the shikimate pathway (Steinruecken and Amrhein 1980). Inhibition of this enzyme affects the biosynthesis of aromatic amino acids, disrupts the generation of many secondary products, and results in the deregulation of the shikimate pathway (Nandula 2010; Nandula et al. 2008).

The overreliance on glyphosate together with the lack of an integrated weed management in the different cropping systems has led to the selection for resistant biotypes. Herbicide resistance is an important issue in agriculture and the evolution of glyphosate-resistant weeds has become a very significant problem in different parts of the world (Heap 2012; Nandula 2010; Powles and Yu 2010). To date, biotypes of 28 species have been identified as being resistant to glyphosate in 24 countries worldwide, and almost 50% are monocot weeds. Reported monocot biotypes correspond to the species: ripgut brome (*Bromus diandrus* Roth), windmill grass (*Chloris truncata* R. Br.), gramilla mansa (*Cynodon hirsutus* Stent), sourgrass [*Digitaria insularis* (L.) Mez ex Ekman), junglerice [*Echinochloa colona* (L.) Link], goosegrass [*Eleusine indica* (L.) Gaertn.], tropical sprangletop, perennial ryegrass (*Lolium perenne* L.), Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam.) Husnot], rigid ryegrass (*Lolium rigidum* Gaudin), annual bluegrass (*Poa annua* L.), johnsongrass [*Sorghum halepense* (L.) Pers.], and liverseedgrass (*Urochloa panicoides* Beauv.) (Heap 2014). Glyphosate resistance information in different species (i.e., rigid ryegrass, johnsongrass) can be found elsewhere, however in tropical sprangletop this information is lacking.

Tropical sprangletop is a perennial grass that is widely distributed across the areas of Persian lime production in Veracruz, Mexico. The species, which is native to Asia, is an erect grass, with short rhizomes and glabrous subcompressed stems that are 40 to 60 cm tall. Tropical sprangletop spreads primarily by seed and is found infesting citrus orchards, coffee plantations, wastelands, roadsides,

DOI: 10.1614/WS-D-13-00177.1
* First, second, and sixth authors: Ph.D Student, Researcher and Professor, Agricultural Chemistry and Soil Sciences, University of Cordoba, 14071 Cordoba, Spain; third and fourth authors: Researchers, Bayer Company, 11520, Mexico, D.F.; fifth author: Professor, Agricultural Parasitology, Chapingo Autonomous University, 56230 Chapingo, Mexico. Corresponding author's E-mail: z82gotof@uco.es

and highways in tropical regions (Lorenzi 2008). Information regarding yield losses in citrus orchards is lacking but, in other crops such as rice (*Oryza sativa* L.) the species bearded sprangletop [*Leptochloa fascicularis* (Lam.) Gray] can reduce the rice production by up to 36% if density of plants reaches 108 plants m^{-2} (Smith 1983). The efficacy of herbicides on Chinese sprangletop [*Leptochloa chinensis* (L.) Nees] decreases if the plants are treated at later growth stages, i.e. the eight-leaf stage instead of the four-leaf stage (Chauhan and Abugho 2012).

The state of Veracruz is the largest producer of citrus fruit in Mexico, mainly orange (*Citrus sinensis* Osbeck) and Persian lime (USDA 2012). Herbicides, particularly glyphosate, are the predominant method for weed control in these production systems. The Persian lime orchards in the municipalities of Cuitlahuac and Martinez de la Torre, Veracruz, have used glyphosate, sometimes as many as three to four applications per year, as the only tool for controlling weeds for over 15 yr. As a consequence, in 2009 weed control failures following glyphosate applications were reported.

There are clearly major agro-economic disadvantages to the presence of glyphosate-resistant tropical sprangletop, and hundreds of hectares of Persian lime in Veracruz, Mexico, are infested. Therefore, the goals of this study were to (1) assess the efficacy of glyphosate in two tropical sprangletop biotypes (collected in Cuitlahuac and Martinez de la Torre), (2) characterize the accumulation of shikimic acid and retention of herbicide in tropical sprangletop biotypes, and (3) evaluate alternative herbicides for the control of glyphosate-resistant tropical sprangletop.

Material and Methods

Field Screening. Due to the complaints of local farmers about a decrease in control of tropical sprangletop with glyphosate, a field screening in the municipalities of Cuitlahuac (18.79° N, 96.68° W) and Martinez de la Torre (20.16° N, 97.09° W) was carried out. Treatments were applied when the plants were 15 to 20 cm tall using a pneumatic backpack sprayer equipped with TeeJet 11002 VS flat fan-nozzle tips (TeeJet Mexico, Qro, Mexico) and calibrated to deliver 325 L ha^{-1} at 276 kPa. Treatments included an untreated control and glyphosate at 1× field rate corresponding to 720 g ai ha^{-1}. The experiment was designed as a completely randomized design with three replications. Each replication consisted of an experimental unit of 42 m^2 in size. Three weeks after treatment, a visual evaluation was made (0: no injury; 100: plants dead) and compared to untreated plots. Seeds from the plants surviving glyphosate were harvested, and placed in a paper bag at room temperature.

Plant Material and Growing Conditions. Seeds of tropical sprangletop collected in the field screening were considered suspect glyphosate-resistant biotypes. Suspected biotypes were collected in Cuitlahuac and Martinez de la Torre and designated Lv8 and Lv9, respectively. The susceptible biotype (named LvS) was collected in a sugarcane field in Cuitlahuac (18.76° N, 96.52° W).

Seeds were sown in containers with sterilized substrate (peat) and used in all the experiments described below (except field assays). Seedlings were then transplanted into individual pots (7 by 7 by 7 cm) with a substrate mixture of sand: peat (2 : 1, v/v) and placed in a growth chamber at 28/18 C day/night under a 16-h photoperiod with 200 µmol m^{-2} s^{-1} photon flux density, and 80% relative humidity.

Petri Dish Assay. The methodology described by De Carvalho et al. (2011) was followed. Experiments were performed in petri dishes (9-cm diam) containing two sheets of filter paper moistened with 5 ml of different glyphosate (Roundup Energy® 45% SL w/v Monsanto Agricultura España, Madrid, Spain) concentrations, and 100 seeds per biotype. Previous assays showed a germination of 96 ± 3% (data not shown). The glyphosate concentrations were 0, 7.4, 14.8, 22.2, 29.6, 59.2, and 118.4 mg ae L^{-1} for the susceptible biotype, and 0, 29.6, 59.2, 118.4, 148, 236.8, and 296 mg L^{-1} for both resistant biotypes. The petri dishes were transferred to the growth chamber under the same conditions as described before. Nine days after initiation, the seedling mortality (dead coleoptile) and fresh weight were determined, and expressed as a percentage with respect to an untreated control. The experiment was arranged in a completely randomized design, with three replications per glyphosate concentration for each biotype and the experiment was performed twice.

Whole Plant Dose–Response Assay. Treatments were applied on plants in the three- to four-leaf stage in a laboratory spray chamber equipped with a flat fan nozzle (TeeJet 8002 EVS) calibrated to deliver 200 L ha^{-1} at a pressure of 200 kPa. Concentrations of 0, 66.6, 133.2, 266.4, 532.8, and 1,065.6 g ha^{-1} for the resistant biotypes, and 0,

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

Table 1. Herbicide treatments applied to tropical sprangletop in field experiments in Martinez de la Torre and Cuitlahuac (Veracruz, Mexico). Assays conducted 2009 to 2011.

	Treatment	HRAC[a] group	Formulated product[b]	Rate
				g ai ha^{-1}
1	Untreated	—		—
2	Glyphosate	G	Coloso Total 360®	720
3	Glyphosate	G	Faena Ultra®	720
4	Indaziflam + glufosinate	L + H	Alion 500® + Finale Ultra®	50 + 455
5	Indaziflam + glufosinate	L + H	Alion 500® + Finale Ultra®	50 + 682
6	Indaziflam + glufosinate	L + H	Alion 500® + Finale Ultra®	75 + 455
7	Indaziflam + glufosinate	L + H	Alion 500® + Finale Ultra®	75 + 682
8	Glufosinate	H	Finale Ultra®	682
9	Paraquat + diuron	D + C2	Gramocil®	400 + 200

[a] Abbreviations: HRAC, Herbicide Resistance Action Committee; G, inhibition of EPSP synthase; L, inhibition of cell wall (cellulose) synthesis; H, inhibition of glutamine synthetase; D, photosystem-I-electron diversion; C2, inhibition of photosynthesis at photosystem II.

[b] Herbicide manufacturers: Coloso Total 360 and Gramocil, Syngenta Crop Protection, Mexico City, Mexico, http://www.syngentacropprotection.com/cropmain.aspx; Faena Ultra, Monsanto Comercial, Mexico City, Mexico, http://www.monsanto.com/pages/default.aspx; Alion 500 and Finale Ultra, Bayer de Mexico, Mexico City, Mexico, http://www.monsanto.com/pages/default.aspx.

18.5, 37, 74, 111, 148, 185, 222, 370, 592, and 740 g ae ha^{-1} for the susceptible biotype were applied. At 21 d after herbicide application, the plants were harvested and weighed to evaluate the reduction in fresh weight (%) with respect to an untreated control. The experiments were arranged in a completely randomized design with five replications per treatment per biotype and they were performed twice.

Shikimic Acid Assay. Plants at the three- to four-leaf stage were treated with glyphosate at 265 g ha^{-1}. Fresh tissues (50 mg) were harvested at 24, 48, 72, 96, and 168 HAT, and immediately placed in vials containing 1 ml of 0.25 N HCl, and frozen in liquid nitrogen. Shikimic acid extraction was performed according to Cromartie and Polge (2000) and the modifications of Perez-Jones et al. (2007). The absorbance at 380 nm was measured using a spectrophotometer (Beckman Coulter DU 640, Brea, CA, USA). The amount of shikimic acid was determined based on a calibration curve made with different known concentrations, and the results were expressed as micrograms of shikimic acid per gram fresh weight. The experiment was conducted in a completely randomized design, with five replicates per biotype and repeated in time.

Spray Retention Assay. We followed the methodology described by Gauvrit (2003). The application was performed on plants at the three- to four-leaf stage in the treatment chamber described before. The application rate of glyphosate was 266 g ha^{-1} + 100 mg L^{-1} Na-fluorescein-5 mM NaOH, with an application volume of 200 L ha^{-1}. After the impregnated solution dried on the foliage (≅ 20 min) the plants were cut at ground level, and immersed for 30 s in 50 ml 5 mM NaOH. The tissues were dried in an oven at 80 C for 72 h. The absorbance of fluorescein was measured using a spectrofluorometer at 490/510 nm. The retention was expressed in microliters of glyphosate solution per gram dry matter. The experiment was conducted in a completely randomized design, using five plants of each biotype and repeated.

Field Assays. Field assays were conducted in two Persian lime orchards (the same described in the Field Screening section) during the seasons 2009 to 2010 and 2010 to 2011 (once per year). A total of eight herbicide treatments and one untreated control (Table 1) were arranged in a randomized complete block design with four replications; the plot dimensions were 7 by 6 m (42 m^2), with a young tree in the center of each experimental unit. The glufosinate doses were applied based on the volume of water applied, that is, 455 or 682 g ai per 100 L ha^{-1} of water. Treatments were applied when the plants were 15 to 20 cm tall using a pneumatic backpack sprayer equipped with TeeJet 11002 VS flat fan-nozzle tips and calibrated to deliver 325 L ha^{-1} at 276 kPa. The pH of the water was adjusted to 5.5 using DAP-Plus conditioner (Química SAGAL, S. A. Monterrey, Mexico). Visual evaluations of the percentage of tropical sprangletop control were performed at 15, 30, and 60 DAT. The control ratings were expressed on a 0 (no injury) to 100 (dead plant) scale.

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

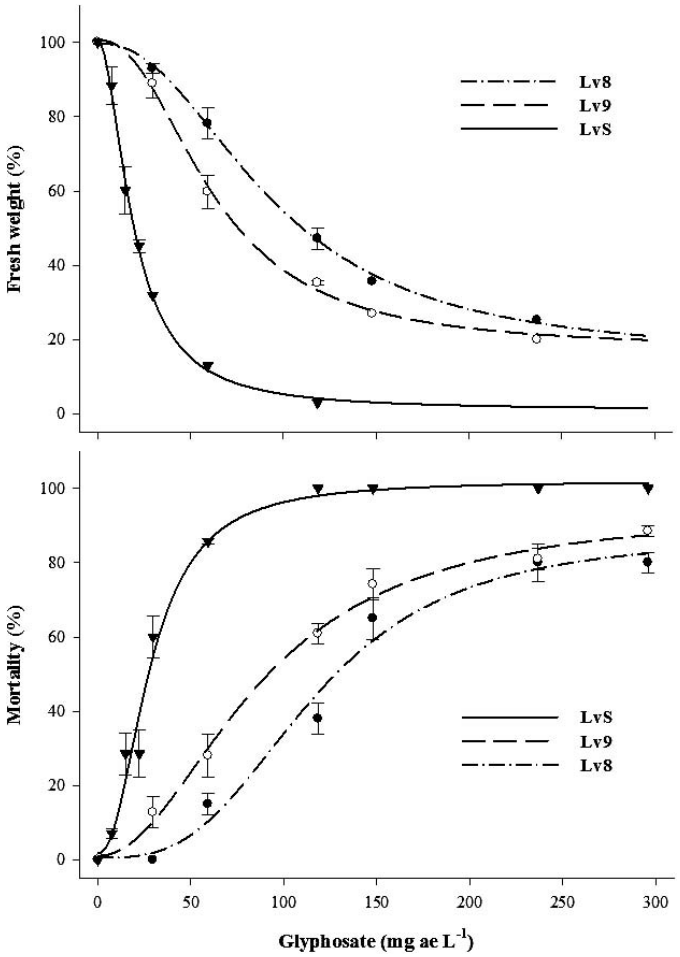

Figure 1. Fresh weight (top) and mortality (bottom) percentage of tropical sprangletop biotypes subjected to different glyphosate concentrations. Vertical bars represent ± standard errors of the mean.

Statistical Analysis. For the petri dish assay we determined the dose required to reduce seedling fresh weight by 50% (EC_{50}) and the LD_{50}, whereas for the whole plant's dose–response assay the herbicide rate that reduces fresh weight by 50% (ED_{50}) was calculated. Parameters were estimated with SigmaPlot 10.0 software (Systat Software Inc. San Jose, CA, USA) by fitting the data to a nonlinear log-logistic regression model, as follows:

$$Y = c + \left\{ (d-c) / \left[1 + (x/g)^{b} \right] \right\} \quad [1]$$

where Y is the percentage of fresh weight compared to the untreated control, c and d are coefficients corresponding to the lower (minimum growth), and upper (maximum growth) asymptotic limits, b is the slope of the curve, g is the herbicide dose (EC_{50}, ED_{50}, or LD_{50}) at the point of inflection midway between the upper and lower asymptotes, and x is the dose of herbicide. The RF was calculated as the ratio of the EC_{50}, ED_{50}, and LD_{50} values of resistant plants with respect to those in the susceptible biotype.

Because variance stability tests showed no differences for the percentage of control between the years, the field data for both years were averaged within the locations and subjected to ANOVA. Analyses were performed using Statistix (version 9.0) (Analytical Software, Tallahassee, FL) software. Means were separated using Tukey's (honest significant difference) test at a 5% probability when necessary.

Results and Discussion

Field Screening. Control of tropical sprangletop plants was ineffective with glyphosate. Efficacy did not exceed 20% at 3 wk after treatment in either location. Visually, at the beginning, the plants were chlorotic, but at the end of the experiment the plants recovered completely without showing any damage from the herbicide. This screening confirmed the low glyphosate efficacy in tropical sprangletop plants.

Petri Dish Assay. Mortality and fresh weight of the LvS, Lv8, and Lv9 seedlings decreased with increasing concentrations of glyphosate (Figure 1), and both EC_{50} and LD_{50} were useful for quantifying sensitivity or resistance in the tropical sprangletop biotypes. The EC_{50} value corresponding to the fresh weight of the seedlings for the LvS biotype was 19.1 mg ae L^{-1}, whereas Lv8 and Lv9 were 94.3 and 62.2 mg ae L^{-1}, respectively (Table 2). LD_{50} values ranged from 26 mg ae L^{-1} in the LvS biotype to 116.5 mg ae L^{-1} in the Lv8 biotype, and showed the Lv8 biotype as being the most resistant one, with an RF of over 4 (Table 2).

Similar RF values have been obtained in other glyphosate-resistant monocot species. In perennial ryegrass, petri dish tests showed 3.2 times more glyphosate was needed in the resistant plants to achieve the same level of germination inhibition as the susceptible plants; also, when measuring the plumule length, the susceptible plants needed 4.4 times less glyphosate than their resistant counterparts to obtained a similar response (Yanniccari et al. 2012). In Italian ryegrass, dose–response petri dish assays exhibited an increase of 2.3 and 5 times more glyphosate to inhibit the germination in two resistant biotypes with respect to a susceptible check (Perez-Jones et al. 2007). In sourgrass, the petri dish experiments showed that resistant biotypes required

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

Table 2. Parameters of the log-logistic equation[a] used to calculate the EC_{50}[b] and LD_{50} values of susceptible (LvS) and resistant (Lv8 and Lv9) biotypes of tropical sprangletop using the petri dish assay.

Biotype	*c*	*d*	*b*	R^2 adjusted	EC_{50} (mg ae L^{-1})	RF[c]
LvS	1.07	100.50	1.87	0.98	19.1 ± 0.7	—
Lv8	15.02	99.66	2.24	0.99	94.3 ± 3.2	4.9
Lv9	17.53	100.57	2.25	0.99	62.2 ± 3.2	3.2
					LD_{50} (mg ae L^{-1})	
LvS	2.72	93.92	−2.50	0.97	26.0 ± 2.6	—
Lv8	0.66	87.30	−3.04	0.96	116.5 ± 12.8	4.4
Lv9	1.16	94.49	−2.04	0.99	87.5 ± 7.9	3.3

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the seedling fresh weight or mortality, *c* and *d* are lower and upper asymptotes, *b* is the slope of the curve, *g* is the herbicide rate required for 50% reduction of the seedling fresh weight or mortality, *x* is the concentration of herbicide.

[b] Abbreviations: EC_{50}, LD_{50}, dose required to reduce seedling fresh weight and mortality by 50%, respectively; LvS, susceptible biotype; Lv8 and Lv9, resistant biotypes; RF, resistance factor.

[c] RF = EC_{50}, LD_{50} (resistant)/EC_{50}, LD_{50} (susceptible).

between 3.5 and 5.6 times more glyphosate than the susceptible biotype to achieve a 50% fresh weight inhibition (De Carvalho et al. 2011). Although the petri dish assay is simple, rapid, and inexpensive, and has been used to quantify herbicide resistance, it is necessary to complement this experiment with a whole-plant dose–response assay because it provides more realistic values that are comparable to results under field conditions (De Carvalho et al. 2011; González-Torralva et al. 2012a; Perez and Kogan 2003; Perez-Jones et al. 2007).

Whole Plant Dose-Response Assay. Biomass reduction in the susceptible biotype was observed at low doses of herbicide (Figure 2), whereas higher doses were required to observe this effect in the resistant biotypes. The ED_{50} results obtained for biotypes LvS, Lv8, and Lv9 were 126.7, 381.2, and 304 g ae ha^{-1}, respectively (Table 3). The Lv8 and Lv9 biotypes showed RF values of 3 and 2.3, respectively. Therefore, Lv8 showed a slightly higher resistance level than Lv9, which is in agreement with the results obtained in the petri dish assay.

Our values found here are similar to those reported in the glyphosate-resistant sourgrass and Italian ryegrass biotypes. In sourgrass, it was reported in Brazil that the herbicide rates to reduce fresh weight by 50% were in the range of 63.9 to 249 g ae ha^{-1}, with resistance factors between 2.3 and 3.9 (De Carvalho et al. 2011), whereas in an Italian ryegrass biotype described in Spain, the resistance factors were around 4.8, with values of 78.1 and 380.8 g ai ha^{-1} for susceptible and resistant plants, respectively (González-Torralva et al. 2012a). Nonetheless, the tropical sprangletop values are low compared with other glyphosate-resistant species such as junglerice, rigid ryegrass, and johnsongrass. In junglerice reported in Australia, the growth reduction by 50% (GR_{50}) value in the resistant biotype was 1.16 kg ha^{-1}, whereas in the susceptible population this value was 5.6 times lower; in other biotypes reported in the United States this resistance factor found was of 6.6, with the biomass reduction values of 0.14 and 0.89 kg ha^{-1} for susceptible and resistant biotypes, respectively (Alarcón-Reverte et al. 2013; Gaines et al. 2012). In rigid ryegrass, biotypes have been reported with different resistance indexes; for instance in southern Australia the reported values were in the range of 2.5 to 15.2, whereas in Italy they were from 4.7 to 37.1 (Bostamam et al. 2012; Collavo and Sattin 2012). Finally, in johnsongrass, the GR_{50} reported values were in the range of 62 to 76 g ha^{-1} for the tested susceptible biotypes and 543 to 653 g ha^{-1} for the

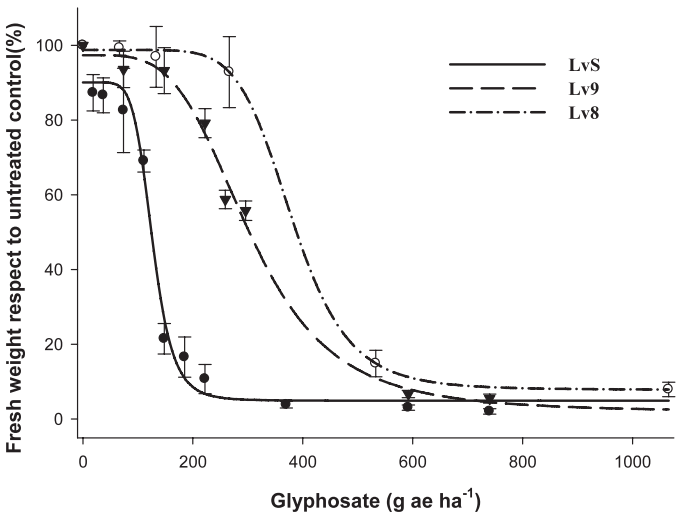

Figure 2. Dose–response curves of susceptible and resistant biotypes of tropical sprangletop. Vertical bars represent ± standard error of the means.

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

Table 3. Parameters of the log-logistic equation[a] used to calculate the ED_{50}[b] of resistant (Lv8 and Lv9) and susceptible (LvS) biotypes of tropical sprangletop.

Biotype	*c*	*d*	*b*	R^2 adjusted	ED_{50}	RF[c]
LvS	4.94	90.09	6.81	0.97	126.7 ± 2.0	—
Lv8	7.88	98.75	7.42	0.98	381.2 ± 5.1	3.0
Lv9	1.97	97.36	4.04	0.98	304.0 ± 4.4	2.3

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the fresh weight, *c* and *d* are lower and upper asymptotes, *b* is the slope of the curve, *g* is the herbicide rate required for 50% reduction of the fresh weight (ED_{50}), *x* is the dose of herbicide.

[b] Abbreviations: ED_{50}, herbicide rate that reduces fresh weight by 50%; LvS, susceptible biotype; Lv8 and Lv9, resistant biotypes; RF, resistance factor.

[c] RF= ED_{50}(resistant)/ED_{50}(susceptible).

resistant, with RF values ranging from 7.1 to 10.5 (Vila-Aiub et al. 2007).

Shikimic Acid Assay. The susceptible biotype accumulated the highest amount of shikimic acid, and was significant with respect to both resistant biotypes. The ANOVA test performed on the data for the different biotypes for each time point indicated no difference in shikimate accumulation in the resistant biotypes at 24 HAT ($P = 0.499$). However, at 72 and 96 HAT, there were significant differences between biotypes; the Lv8 biotype showed the lowest shikimic acid accumulation. At the end of the experiment (168 HAT), both resistant biotypes accumulated similar amounts of shikimic acid, with no significant differences between them (Table 4).

Shikimic acid accumulation in glyphosate-resistant and -susceptible plants of rigid ryegrass treated at 225 g ha^{-1} was similar to those found in this work; at 168 HAT accumulation in susceptible plants was of ≅ 3 mg g^{-1} fresh weight whereas in the resistant plants this value was lower (< 1 mg g^{-1} fresh weight) (Wakelin and Preston 2006). In the glyphosate-resistant and -susceptible biotypes of sourgrass treated at 157.5 g ha^{-1}, shikimic acid accumulation followed a similar trend. At the end of the assay (168 HAT), the susceptible biotype had accumulated on the order of 400 µg g^{-1} fresh weight; in contrast, resistant biotypes accumulated four times less shikimic acid (De Carvalho et al. 2011). In biotypes of Italian ryegrass described in Spain, the susceptible plants accumulated approximately two times more shikimic acid at 96 HAT at 200 g ai ha^{-1} with respect to those found for tropical sprangletop; in comparison, the resistant plants of both species accumulated similar amounts at this time (González-Torralva et al. 2012a). On the other hand, in junglerice biotypes treated at 0.42 kg ae ha^{-1}, shikimic acid accumulation at 7 DAT was higher than that found for tropical sprangletop; resistant and susceptible biotypes accumulated ≅ 3 and ≅ 10 mg g^{-1} fresh weight, respectively (Alarcón-Reverte et al. 2013).

A high level of shikimic acid in plants treated with glyphosate indicates susceptibility to glyphosate due to inhibition of the enzyme EPSPS (Shaner 2009). Therefore, shikimate accumulation in the treated plants is accepted as a biomarker to determine resistance or sensitivity to glyphosate (González-Torralva et al. 2010). In our experiments, the shikimate accumulation in tropical sprangletop decreased at 168 HAT in both resistant biotypes, which could be due to the resistance mechanism (or mechanisms) involved which avoid the glyphosate damage. Sequestration of glyphosate into the vacuole, the metabolism of glyphosate to nontoxic forms such as AMPA and glyoxylate, gene amplification of *EPSPS* gene, or target site mutation could be taking part in the tropical sprangletop resistance mechanism (or mechanisms) (Baerson et al. 2002; Cruz-Hipolito et al. 2011; De Carvalho

Table 4. Shikimic acid accumulation (µg g^{-1} fresh weight) in the leaves of resistant (Lv8 and Lv9) and susceptible (LvS) tropical sprangletop biotypes. Plants were treated with glyphosate at 265 g ae ha^{-1} when they reached the three- to four-leaf stage.

	Hours after treatment[a]				
	24	48	72	96	168
LvS	31.1 ± 2.6 B	122.5 ± 5.8 A	756.6 ± 20.4 A	935.8 ± 10.1 A	3280.2 ± 72.8 A
Lv8	46.9 ± 5.4 A	48.9 ± 6.5 B	49.1 ± 7.2 C	78.8 ± 9.7 C	46.2 ± 5.8 B
Lv9	44.8 ± 4.9 A	113.1 ± 7.0 A	118.9 ± 8.1 B	167.8 ± 7.7 B	68.8 ± 5.9 B

[a] Columns followed by the same letter are not significantly different at the 5% level as determined by the Tukey honest significant difference test. Values ± standard errors of the mean.

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

Table 5. Spray retention of glyphosate solution for susceptible (LvS) and resistant (Lv8 and Lv9) tropical sprangletop biotypes. Plants were treated at the three- to four-leaf stage.

Biotype	Retention[a]
	µl g^{-1} dry matter
LvS	250.6 ± 16.2 A
Lv8	266.7 ± 16.9 A
Lv9	273.1 ± 7.6 A

[a] Means followed by the same letter are not significantly different at the 5% level as determined by the Tukey honest significant difference test. Values ± standard error of the mean.

et al. 2011; Ge et al. 2010; González-Torralva et al. 2012b; Rojano-Delgado et al. 2012; Salas et al. 2012).

Spray Retention Assay. The biotypes studied showed similar results for glyphosate spray retention; according to ANOVA, no significant differences between the three biotypes were found ($P = 0.071$) (Table 5). Therefore, this physical parameter is not responsible for the observed resistance in tropical sprangletop. The effectiveness of a herbicide may be determined by the maximum amount of herbicide that can be captured by the target site. In some cases, differences in spray retention have been found between species (Chachalis et al. 2001; González-Torralva et al. 2010) and biotypes of the same species resistant to herbicides (Michitte et al. 2007; Rosario et al. 2011). However, differences in spray retention were not found in other cases, such as in glyphosate-resistant sourgrass (De Carvalho et al. 2011). Although in this research the leaf area was not measured among biotypes, this parameter could take part in the resistance depending on the species studied. For instance, in a Powell amaranth (*Amaranthus powellii* S. Wats) biotype resistant to acetohydroxyacid synthase herbicides, plants produced 58% less leaf area than susceptible plants (Tardif et al. 2006), whereas in an acetolactate synthase inhibitor–resistant downy brome (*Bromus tectorum* L.) biotype, the resistant biotype produced less shoot dry weight, leaf area, and seed with respect to that produced by the susceptible biotype (Park and Mallory-Smith 2005). Nonetheless, in noncompetitive conditions biotypes of sulfonylurea-resistant and -susceptible kochia [*Kochia scoparia* (L.) Schrad.] produced similar dry weight and leaf area (Christoffoleti 1997).

Field Assays. Results obtained in field assays corroborated the farmers' complaints, with a low glyphosate efficacy in the control of tropical sprangletop. Glyphosate alone at 720 g ha^{-1} isopropylamine salt resulted in only 22.9 to 27% control in the Cuitlahuac orchard, and 31.5 to 47.1% for Martinez de la Torre at 15 DAT (Tables 6 and 7). With the exception of the glyphosate (treatment 3) in Cuitlahuac, the control percentage decreased at 30 DAT. The efficacy of glyphosate alone was better in Martinez de la Torre than in Cuitlahuac, which is most likely related to the lower RF observed in Martinez de la Torre. However, tropical sprangletop control was reduced at 60 DAT in both locations. On the basis of visual evaluation, the levels of control were higher with indaziflam 50 g ai ha^{-1} + glufosinate 455 g ha^{-1} in Cuitlahuac (85.2%) and Martinez de la Torre (99.2%) at 15 DAT. The highest levels of control were achieved with indaziflam 50 g ha^{-1} +

Table 6. Percentage of control of tropical sprangletop in a Persian lime orchard in Cuitlahuac, Veracruz, Mexico.

	Treatment	Rate	% Control[a]		
			15 DAT[b]	30 DAT	60 DAT
		g ai ha^{-1}			
2	Glyphosate[c]	720	27.0 C[d]	11.6 D	4.7 C
3	Glyphosate	720	22.9 C	28.3 D	1.9 C
4	Indaziflam + glufosinate	50 + 455	85.2 A	55.0 C	55.1 B
5	Indaziflam + glufosinate	50 + 682	96.6 A	89.1 AB	88.4 A
6	Indaziflam + glufosinate	75 + 455	72.0 A	65.4 BC	72.1 AB
7	Indaziflam + glufosinate	75 + 682	96.6 A	91.3 AB	88.2 A
8	Glufosinate	682	96.2 A	85.3 AB	78.0 AB
9	Paraquat + diuron	400 + 200	97.3 A	93.2 A	93.0 A

[a] Average of 2 yr of cultivation.
[b] Abbreviation: DAT, d after treatment.
[c] See Table 1 for herbicide details.
[d] Columns with the same letter are not significantly different at the 5% level as determined by the Tukey honest significant difference test.

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

Table 7. Percentage of control of tropical sprangletop in a Persian lime orchard in Martinez de la Torre, Veracruz, Mexico.

	Treatment	Rate	% Control[a]		
			15 DAT[b]	30 DAT	60 DAT
		g ai ha^{-1}			
2	Glyphosate[c]	720	47.1 B[d]	39.9 B	21.7 B
3	Glyphosate	720	31.5 B	20.1 BC	8.3 B
4	Indaziflam + glufosinate	50 + 455	99.2 A	96.6 A	67.6 A
5	Indaziflam + glufosinate	50 + 682	99.6 A	99.3 A	84.3 A
6	Indaziflam + glufosinate	75 + 455	98.5 A	91.9 A	66.5 A
7	Indaziflam + glufosinate	75 + 682	99.9 A	99.7 A	94.3 A
8	Glufosinate	682	99.7 A	99.6 A	88.4 A
9	Paraquat + diuron	400 + 200	99.7 A	97.8 A	85.7 A

[a] Average of 2 yr of cultivation.
[b] Abbreviation: DAT, d after treatment.
[c] See Table 1 for herbicide details.
[d] Columns followed by the same letter are not significantly different at the 5% level as determined by the Tukey honest significant difference test.

glufosinate 682 g ha^{-1}, indaziflam 75 g ha^{-1} + glufosinate 682 g ha^{-1}, glufosinate alone, and paraquat + diuron in both locations. As detailed in Tables 6 and 7, weed control decreased with time (30 to 60 DAT). Indaziflam 50 g ha^{-1} + glufosinate 682 g ha^{-1}, indaziflam 75 g ha^{-1} + glufosinate 682 g ha^{-1}, and paraquat + diuron were the best treatments at 60 DAT for the Cuitlahuac field, whereas the best treatment over time in terms of time period and percentage of control in the Martinez de la Torre field was indaziflam 75 g ha^{-1} + glufosinate 682 g ha^{-1} (Table 7). Glufosinate alone showed a great efficacy in both locations at the beginning of the experiment, but this efficacy was not maintained through the time, most likely due to plant's regrowth and null residual activity, whereas indaziflam + glufosinate at the highest rate and the formulated mixture of paraquat + diuron (except for Martínez de la Torre field) showed a slightly better efficacy at the end of the experiment, most likely due to the residual effect and a possible mixture synergism. Based on the efficiency of the mixture of glufosinate with indaziflam, and the formulated mixture of paraquat + diuron, these herbicides are suggested as an alternative management for tropical sprangletop in Persian lime orchards, and can be used in rotating in order to avoid or delay (as long as possible) the selection of additional resistant biotypes in the nearest fields.

The repeated use of glyphosate has led to the selection of resistant individuals each year in the treated fields. The evolution of resistant tropical sprangletop is similar to a population of rigid ryegrass in an orchard in Australia following two or three applications of glyphosate for 15 yr (Powles et al. 1998). In Malaysia, goosegrass evolved resistance to glyphosate in only 4 yr, with resistant factors between 8 and 12 times that of a susceptible biotype (Lee and Ngim 2000). The mixture of glufosinate with other herbicides with different modes of action may be necessary for the complete control of tropical sprangletop in citrus orchards, particularly at the beginning and end of the rainy season. Glyphosate, however, continues to be effective against other weed species present in these Persian lime orchards, suggesting that it can be used together with PRE herbicides in a well-designed management program for sustainable agriculture.

Summarizing, the two biotypes of tropical sprangletop harvested from different locations of citrus fruit in Mexico were assayed for resistance to EPSPS-inhibiting herbicides, both in vivo and in vitro, with all results showing the evolved resistance of tropical sprangletop to glyphosate herbicide. This was the first case of glyphosate resistance in this species worldwide, and the first case described of a glyphosate-resistant weed in Mexico. Under field conditions, alternative chemical options were tested and showed their efficacy in controlling these biotypes. The selection pressure exerted by glyphosate in this species is still low, which implies that the rotation of herbicides will help to avoid the dispersion of the glyphosate-resistant biotypes. Integrated weed management should be implemented to effectively control and limit the dissemination of resistant biotypes.

Future research efforts include further assays to determine the molecular mechanism for the observed glyphosate resistance. A molecular analysis of both resistant biotypes and other tropical sprangle-

https://doi.org/10.1614/WS-D-13-00177.1 Published online by Cambridge University Press

top populations from different locations with varying glyphosate susceptibilities will be conducted. These results will be useful in generating potential solutions and the best recommendations for management practices to control glyphosate-resistant tropical sprangletop while maintaining no-tillage production practices.

Acknowledgments

The authors are grateful for technical help from Rafael A. Roldan-Gómez and Ricardo Alcántara de la Cruz. This work has been cofinanced by MICINN (Spanish Ministry of Science and Innovation) (AGL2010-16774) and Bayer Company, Mexico.

Literature Cited

Alarcón-Reverte R, García A, Urzúa J, Fischer AJ (2013) Resistance to glyphosate in junglerice (*Echinochloa colona*) from California. Weed Sci 61:48–54

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA, Dill GM (2002) Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol 129:1265–1275

Baylis AD (2000) Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pest Manag Sci 56:299–308

Bostamam Y, Malone JM, Dolman FC, Boutsalis P, Preston C (2012) Rigid ryegrass (*Lolium rigidum*) populations containing a target site mutation in EPSPS and reduced glyphosate translocation are more resistant to glyphosate. Weed Sci 60:474–479

Chachalis D, Reddy KN, Elmore CD, Steele ML (2001) Herbicide efficacy, leaf structure, and spray droplet contact angle among *Ipomoea* species and smallflower morningglory. Weed Sci 49:628–634

Chauhan BS, Abugho SB (2012) Effect of growth stage on the efficacy of postemergence herbicides on four weed species of direct-seeded rice. Sci World J 2012:1–7

Christoffoleti PJ (1997) Growth analysis of sulfonylurea-resistant and -susceptible kochia (*Kochia scoparia*). Weed Sci 45:691–695

Collavo A, Sattin M (2012) Resistance to glyphosate in *Lolium rigidum* selected in Italian perennial crops: bioevaluation, management and molecular bases of target-site resistance. Weed Res 52:16–24

Cromartie TH, Polge ND (2000) An improved assay for shikimic acid and its use as a monitor for the activity of sulfosate. Proc Weed Sci Soc Am 40:291

Cruz-Hipolito H, Rojano-Delgado A, Domínguez-Valenzuela JA, Heredia A, De Castro MDL, De Prado R (2011) Glyphosate tolerance by *Clitoria ternatea* and *Neonotonia wightii* plants involves differential absorption and translocation of the herbicide. Plant Soil 347:221–230

De Carvalho LB, Cruz-Hipolito H, González-Torralva F, Da Costa Aguiar Alves PL, Christoffoleti PJ, De Prado R (2011) Detection of sourgrass (*Digitaria insularis*) biotypes resistant to glyphosate in Brazil. Weed Sci 59:171–176

Duke SO, Lydon J, Koskinen WC, Moorman TB, Chaney RL, Hammerschmidt R (2012) Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. J Agric Food Chem 60:10375–10397

Gaines TA, Cripps A, Powles SB (2012) Evolved resistance to glyphosate in junglerice (*Echinochloa colona*) from the tropical Ord River region in Australia. Weed Technol 26: 480–484

Gauvrit C (2003) Glyphosate response to calcium, ethoxylated amine surfactant, and ammonium sulfate. Weed Technol 17:799–804

Ge X, André d'Avignon D, Ackerman JJH, Sammons RD (2010) Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. Pest Manag Sci 66:345–348

González-Torralva F, Cruz-Hipolito H, Bastida F, Mülleder N, Smeda RJ, De Prado R (2010) Differential susceptibility to glyphosate among the *Conyza* weed species in Spain. J Agric Food Chem 58:4361–4366

González-Torralva F, Gil-Humanes J, Barro F, Brants I, De Prado R (2012a) Target site mutation and reduced translocation are present in a glyphosate-resistant *Lolium multiflorum* Lam. biotype from Spain. Plant Physiol Biochem 58:16–22

González-Torralva F, Rojano-Delgado AM, Luque de Castro MD, Mülleder N, De Prado R (2012b) Two non-target mechanisms are involved in glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes. J Plant Physiol 169:1673–1679

Heap I (2012) Current global status of glyphosate-resistant weeds and the criterion required for confirming resistance. Pages 15–17 *in* De Prado R, ed. Glyphosate Weed Resistance: European Status and Solutions. Cordoba, Spain: Printalias Servicios De Impresion Digital S.L.

Heap I (2014) International Survey of Herbicide Resistant Weeds. http://www.weedscience.org. Accessed February 19, 2014

Lee LJ, Ngim J (2000) A first report of glyphosate-resistant goosegrass (*Eleusine indica* (L.) Gaertn) in Malaysia. Pest Manag Sci 56:336–339

Lorenzi H (2008) Plantas Daninhas do Brasil—Terrestres, Aquáticas, Parasitas e Tóxicas. São Paulo, Brazil: Instituto Plantarum de Estudos da Flora. 520 p

Michitte P, De Prado R, Espinoza N, Ruiz-Santaella JP, Gauvrit C (2007) Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile. Weed Sci 55:435–440

Nandula VK (2010) Glyphosate Resistance in Crops and Weeds. History, Development, and Management. Hoboken, NJ: J. Wiley. 321 p

Nandula VK, Reddy KN, Poston DH, Rimando AM, Duke SO (2008) Glyphosate tolerance mechanism in Italian ryegrass (*Lolium multiflorum*) from Mississippi. Weed Sci 56:344–349

Park KW, Mallory-Smith CA (2005) Multiple herbicide resistance in downy brome (*Bromus tectorum*) and its impact on fitness. Weed Sci 53:780–786

Perez A, Kogan M (2003) Glyphosate-resistant *Lolium multiflorum* in Chilean orchards. Weed Res 43:12–19

Perez-Jones A, Park KW, Polge N, Colquhoun J, Mallory-Smith CA (2007) Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. Planta 226:395–404

Powles SB, Lorraine-Colwill DF, Dellow JJ, Preston C (1998) Evolved resistance to glyphosate in rigid ryegrass (*Lolium rigidum*) in Australia. Weed Sci 46:604–607

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317–347

Rojano-Delgado AM, Cruz-Hipolito H, De Prado R, Luque De Castro MD, Franco AR (2012) Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *utilis* plants. Phytochemistry 73:34–41

Rosario JM, Cruz-Hipolito H, Smeda RJ, De Prado R (2011) White mustard (*Sinapis alba*) resistance to ALS-inhibiting herbicides and alternative herbicides for control in Spain. Eur J Agron 35:57–62

Salas RA, Dayan FE, Pan Z, Watson SB, Dickson JW, Scott RC, Burgos NR (2012) EPSPS gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. Pest Manag Sci 68:1223–1230

Shaner DL (2009) Role of translocation as a mechanism of resistance to glyphosate. Weed Sci 57:118–123

Smith JRJ (1983) Competition of bearded sprangletop (*Leptochloa fascicularis*) with rice (*Oryza sativa*). Weed Sci 31:120–123

Steinruecken HC, Amrhein N (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. Biochem Biophys Res Commun 94:1207–1212

Tardif FJ, Rajcan I, Costea M (2006) A mutation in the herbicide target site acetohydroxyacid synthase produces morphological and structural alterations and reduces fitness in *Amaranthus powellii*. New Phytol 169:251–264

[USDA] U.S. Department of Agricultre (2012) Foreign Agricultural Service. http://www.fas.usda.gov. Accessed December 10, 2012

Velini ED, Duke SO, Trindade MLB, Meschede DK, Carbonari CA (2009) Modo de ação do glyphosate. Pages 113–133 *in* Velini ED, Trindade MLB, Meschede DK, Carbonari CA, eds. Glyphosate. Botucatu, São Paulo, Brasil: FEPAF

Vila-Aiub MM, Balbi MC, Gundel PE, Ghersa CM, Powles SB (2007) Evolution of glyphosate-resistant johnsongrass (*Sorghum halepense*) in glyphosate-resistant soybean. Weed Sci 55:566–571

Wakelin AM, Preston C (2006) A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Res 46:432–440

Yanniccari M, Istilart C, Giménez DO, Castro AM (2012) Glyphosate resistance in perennial ryegrass (*Lolium perenne* L.) from Argentina. Crop Prot 32:12–16

Received December 3, 2013, and approved March 13, 2014.