Weed Science 2016 64:579–587

Genetic Relationships between Tropical Sprangletop (*Leptochloa virgata*) Populations from Mexico: Understanding Glyphosate Resistance Spread

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The susceptibility to glyphosate and genetic diversity based on intersimple sequence repeat markers were characterized for 17 tropical sprangletop populations collected from two separate regions mainly in Persian lime groves in Veracruz, Mexico. The whole-plant dose response together with shikimic acid assays indicated different levels of glyphosate resistance in those populations. Genetic diversity values (h) estimated using POPGENE ranged from 0.119 to 0.198 and 0.117 to 0.214 within susceptible and resistant populations, respectively. The average genetic diversity (H_S) within the susceptible populations was 0.157, and the total genetic diversity (H_T) was 0.218. The H_S of the resistant populations was 0.144, and the H_T was 0.186. The analysis of molecular variance based on the response to glyphosate indicated that most of the genetic variation was found within groups of susceptible and resistant populations (90% of the genetic variation), whereas 10% or less was among groups. The high level of genetic diversity between glyphosate-resistant tropical sprangletop populations from distant and adjacent locations is likely due to both short- and long-distance seed dispersal and independent evolutionary events in tropical sprangletop populations among Persian lime groves in Veracruz.

Nomenclature: Glyphosate; tropical sprangletop, *Leptochloa virgata* (L.) P. Beauv.; Persian lime, *Citrus latifolia* Tan.

Key words: Diversity within/among populations, genetic diversity, glyphosate resistance spread, ISSR markers, tropical sprangletop populations, resistance index.

The genus *Leptochloa* (Poaceae, Chloridoideae) comprises approximately 40 species of C_4 plants of tropical and subtropical origin. The diversity of taxa in the tropics for *Leptochloa* spp. is high on all continents (Snow 1997). Plants of this genus are prolific seed producers; for example, 100 plants of *Leptochloa obtusiflora* (Hochst.) can produce up to 3.3 million seeds (Bogdan and Pratt 1967). The germination and emergence varies between species depending on intrinsic factors, such as the presence or absence of latency, and extrinsic factors such as temperature, light, depth, flood, and salinity (Snow et al. 2008). Some species, such as Chinese sprangletop [*Leptochloa chinensis* (L.) Nees], can exhibit vegetative propagation (Häfliger and Scholz, 1981), although they reproduce primarily by sexual means (Benvenuti et al. 2004). These factors can promote staggered germination, emergence, and reproduction, which make control of these plants very difficult.

Tropical sprangletop is a perennial grass native to Asia that reproduces predominantly by cross-pollination (Peterson et al. 2012; Snow 1997; Snow et al. 2008), and it is found to be affecting the main tropical crops or cropping systems in Mexico, such as sugarcane, citrus orchards, and coffee plantations (Pérez-López et al. 2014; Snow et al. 2008).

Citrus production is one of the most important agricultural activities in the state of Veracruz, mainly producing Persian lime and orange (*Citrus sinensis* (L.) Osbeck). In the last 15 yr, the use of herbicides has been adopted as the only tool to control weeds in the municipalities of Cuitláhuac and Martínez de la Torre, where glyphosate has been predominantly applied three to four times per year to control a wide range of weeds (Pérez-López et al. 2014). Unfortunately, resistant populations of tropical sprangletop have appeared mainly due to the selection pressure exerted by glyphosate, and the lack of other weed management practices.

Glyphosate is the most widely used herbicide in the world (Duke and Powles 2008). Glyphosate is the only herbicide that inhibits 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), an en-

DOI: 10.1614/WS-D-15-00183.1

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https://doi.org/10.1614/WS-D-15-00183.1 Published online by Cambridge University Press

zyme required in the shikimic acid pathway for the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan (Haney et al. 2002; Siehl 1997; Wiersema et al. 2013). This is the only known site of action of glyphosate. However, there is indirect evidence that another site may play a role in the herbicide mechanism of action in higher plants (Saes et al. 2010). The most significant dysfunction caused by inhibition of EPSPS enzyme is the inhibition of the shikimic acid pathway, which leads to the accumulation of high levels of shikimate-3-phosphate (Haney et al. 2002; Wiersema et al. 2013; Duke et al. 2003), causing disruption of the carbon flow for other essential pathways (Orcaray et al. 2012).

The main glyphosate resistance mechanisms described are: (1) in the target-site, represented by amino acid substitutions, or overexpression of EPSPS; and (2) exclusion or nontarget site mechanisms through biochemical or physiological characteristics such as reduced absorption and translocation, vacuolar sequestration, and enhanced metabolism, an ability to handle the toxic agent produced by the pesticide and thereby avoid a toxic result (Alcántara-de la Cruz et al. 2016; De Corvalho et al. 2011; Duke 2011; Ge et al. 2012). Currently there are 35 confirmed weed species resistant to glyphosate in 27 countries (Heap 2016). Tropical sprangletop was reported glyphosate resistant in Mexico in 2010 (Heap 2016), and has been confirmed in 2014 by this research group (Pérez-López et al. 2014). Different levels of response to glyphosate were detected in the tropical sprangletop populations evaluated in the laboratory tests. This remains the only case of herbicide resistance in this species worldwide (Heap 2016).

To understand the evolution of herbicide resistance, it is necessary to understand the genetic processes involved (Jasieniuk et al. 1996). Genetic diversity analysis within and among weed populations and geographical regions often provides information on the pathways and mechanisms of resistance spread (Délye et al. 2010; Menchari et al. 2007). The genetic diversity of herbicide-resistant weed populations has often been determined using intersimple sequence repeat (ISSR) markers (Huanfu et al. 2009; Imaizumi et al. 2013; Osuna et al. 2011). Polymerase chain reaction (PCR) amplification of ISSR can show interesting results on the study of the distribution and genetic variability of the species, and differentiate between closely related individuals (Osuna et al. 2011). ISSR amplification does not require genome sequence information but produces highly polymorphic patterns (Bornet et al. 2001).

Because of the agronomic and economic problems caused by selection pressure exerted by glyphosate on tropical sprangletop, it is of great importance to gain a better understanding of the status and distribution of glyphosate resistance in this species. A better knowledge of the genetic diversity of tropical sprangletop would provide a much-informed basis for understanding broad-scale patterns of glyphosate resistance for its appropriate management in Persian lime groves of Veracruz, Mexico. Therefore, the objectives of this study were: (1) to evaluate the susceptibility to glyphosate of 16 putative resistant tropical sprangletop populations and a susceptible population; (2) to quantify shikimic acid accumulation to confirm glyphosate resistance, and (3) to elucidate the genetic processes contributing to the spread of glyphosate resistance in tropical sprangletop by characterizing the genetic diversity among different populations.

Material and Methods

Plant Material and General Experimental Conditions. Sixteen populations of tropical sprangletop with suspected resistance to glyphosate were collected from Persian lime groves from Cuitláhuac and Martínez de la Torre municipalities, Veracruz, Mexico that had been treated with glyphosate (720 g ae ha^{-1}) for several years. A susceptible population (LvS) was collected on the edge of a sugarcane field that had never been treated with glyphosate. In each treated glyphosate grove sampled, 20 plants were selected haphazardly and approximately 1 g of seed was collected from each tropical sprangletop plant. All seeds collected from a field were bulked and subsampled to test for resistance or susceptibility to glyphosate and considered to constitute a sample from a single population. The location of each of the collection sites was recorded using a global positioning system (Table 1, Figure 1). The samples were labeled and named with respect to the order of collection.

The seeds were germinated in peat, and the seedlings were transplanted individually in pots with a substrate mixture of sand and peat (1 : 1, v/v). The plants were placed in a greenhouse at a temperature regime of 26/18 C day/night.

Whole-Plant Dose–Response Experiments. Glyphosate (Roundup EnergyPro 45% p/v [potassium salt]; Monsanto, Spain) treatments were

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Table 1. Geographical locations of 17 tropical sprangletop populations used in this study collected in Persian lime groves from Cuitháhuac (site A) and Martínez de la Torre (site B) municipalities, Veracruz, Mexico.

		Location		
Population	Collection site	Latitude	Longitude	Altitude[a]
				masl
LvS[b]	A	18.79°	96.69°	328
Lv1	A	18.80°	96.70°	354
Lv2	A	18.80°	96.70°	342
Lv3	A	18.80°	96.69°	333
Lv4	A	18.79°	96.69°	326
Lv5	A	18.79°	96.69°	327
Lv6	A	18.79°	96.68°	321
Lv7	A	18.79°	96.68°	315
Lv8	B	20.16°	97.09°	101
Lv9	B	20.17°	97.10°	124
Lv10	B	20.17°	97.10°	130
Lv11	A	18.76°	96.52°	230
Lv12	A	18.73°	96.52°	145
Lv13	A	18.73°	96.52°	145
Lv14	A	18.75°	96.53°	134
Lv15	A	18.75°	96.53°	131
Lv16	A	18.74°	96.61°	209

[a] Abbreviations: masl, meters above sea level; LvS, susceptible population.

[b] LvS population was collected on the edge of a sugarcane field that had never been treated with glyphosate.

applied at the three- to four-leaf seedling stage with a laboratory spray chamber equipped with a flat-fan nozzle (Tee Jet 8002EVS) calibrated to deliver 200 L ha^{-1} at a pressure of 200 kPa. The glyphosate application rates were: 0, 66.6, 133.2, 266.4, 532.8, and 1,065.6 g ae ha^{-1} for the resistant populations and 0, 18.5, 37.0, 74.0, 111.0, 148.0, 185.0, 222.0, 370.0, 592.0, and 740.0 g ha^{-1} for the susceptible one. The plants were harvested and weighed at 21 d after treatment (DAT) to evaluate the reduction in fresh weight (%) with respect to an untreated control. The experiments were repeated twice, arranged in a completely randomized design with 10 replicates per rate.

Shikimic Acid Accumulation Assay. Five plants from each population, in a completely random design, were treated in the three- to four-leaf stage with glyphosate at 265 g ae ha^{-1} under conditions described in the previous assay. Fresh tissues (50 mg) were harvested at 96 and 168 h after treatment (HAT) and immediately placed in vials containing 1 ml of 0.25 N HCl and frozen in liquid nitrogen. The experiment included an untreated control at each time evaluated. Shikimic acid accumulation was determined by the method described by Cromartie and Polge (2002). Sample absorbance was measured with a Beckman DU-640 spectrophotometer (Fullerton, CA) at 380 nm. Shikimic acid accumulation was determined on the basis of a calibration curve made with known shikimic acid concentrations, and the results were expressed as micrograms of shikimic acid per gram fresh weight.

DNA Extraction and ISSR Amplifications. Approximately 100 mg of leaf tissue from 10 individual plants within each population was ground to a fine powder in liquid nitrogen. DNA was extracted using Speedtools plant DNA extraction kit (BIOTOOLS, Madrid, Spain). In all cases, the DNA was quantified using a NANODROP-1000 (Thermo-Scientific), diluted to a final concentration of 10 ng μl^{-1} and used for PCR or stored at –21 C until use.

A total of eight ISSR primers from primer set #9 from the University of British Columbia Biotechnology Laboratory (Vancouver, Canada) was selected for DNA amplification. Table 2 lists the sequences of ISSR primers that exhibited polymorphism in tropical sprangletop. DNA amplifications were carried out in a reaction mix containing 1 µl (10 ng μl^{-1}) of DNA; 2.5 µl of buffer 10× (with 15 mM $MgCl_2$); 2 µl (2.5 mM) of deoxynucleotide triphosphate mix; 1 µl (1 µM) of primer, and 0.2 µl (5 U/µl) of Taq DNA polymerase (BIOTOOLS) per 25 µl of reaction mix. PCR amplification was performed in a Bio-Rad thermocycler programmed for 35 cycles as: 1 cycle of 7 min at 94 C, 35 cycles of 30 s at 94 C, 30 s at 52 to 55 C, and 1 min at 72 C, and a final extension cycle of 10 min at 72 C. The amplified product was visualized on a 1% agarose gel by staining with gel red 10,000× (Biotium) and photographed under UV light (transilluminator ALPHA-INNOTECH). The molecular size of the fragments was estimated with reference to a 100-base pair DNA ladder (Fisher; 100 to 2,000 range). At least two PCR amplifications were performed for each sample to ensure consistency of the fragment sizes during gel electrophoresis.

Statistical Analysis. In whole dose–response experiments, the herbicide that causes a 50% fresh weight reduction (GR_{50}) was calculated (Menéndez et al. 2006). SigmaPlot 10.0 software (Systat Sofware Inc. San Jose, CA) was used to obtain those parameters by fitting the data to a log-logistic regression curve (Seefeldt at el. 1995):

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

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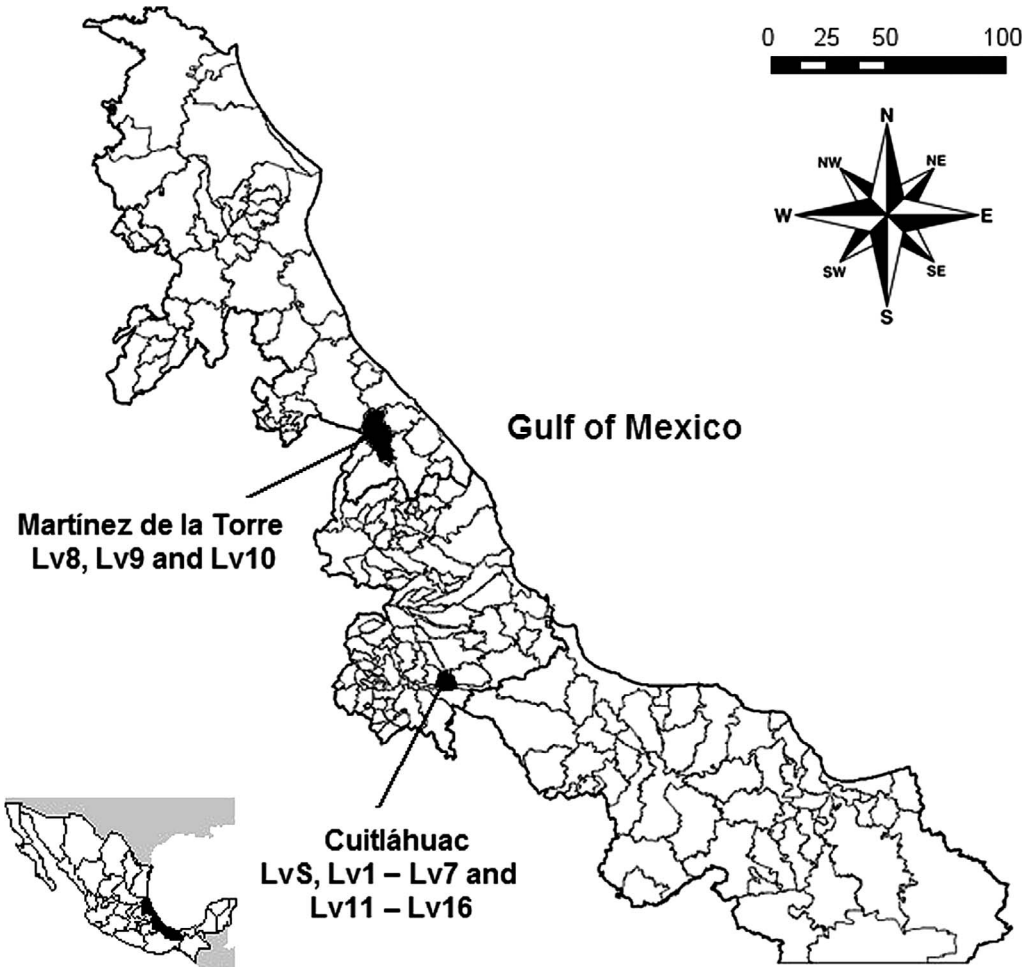

Figure 1. Geographical locations of the Martínez de la Torre and Cuitháhuac municipalities, Veracruz, Mexico, where 17 populations of tropical sprangletop were collected. The susceptible population (LvS) was collected on the edge of a sugarcane field.

where Y is the percentage of fresh weight compared with 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 that yields GR_{50} at the point of inflection midway between the upper and the lower asymptotes, and x is the glyphosate rate. Resistance index (RI) was calculated as GR_{50} of the resistant population (R) / GR_{50} of the susceptible population (S).

The data pertaining to shikimic acid accumulation were subjected to ANOVA and the means were compared using Tukey's honestly significant difference test at 95% probability when necessary. Statistix 9.0 software (Analytical Software, Tallahassee, FL) was used to perform the statistical analysis.

The ISSR bands were interpreted as being dominant markers and were scored as diallelic characters either as 1 (present) or 0 (absent) at a particular locus. A pair-wise similarity matrix was calculated using the simple matching coefficient. This similarity matrix was used to construct a dendrogram by the unweighted pair group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 2.2 package (Exeter Software,

Table 2. Intersimple sequence repeat (ISSR) primers used in tropical sprangletop populations from primer set #9 from the University of British Columbia Biotechnology Laboratory (Vancouver, Canada).

Primers	Primer sequence	T_m [a]
		C
ISSR 808	AGAGAGAGAGAGAGAGC	55
ISSR 810	GAGAGAGAGAGAGAGAT	55
ISSR 827	ACACACACACACACACG	52
ISSR 836	AGAGAGAGAGAGAGAGYA	55
ISSR 857	ACACACACACACACACYG	52
ISSR 880	GGAGAGGAGAGGAGA	55
ISSR 889	DBDACACACACACACAC	52
ISSR 890	VHVGTGTGTGTGTGTGT	52

[a] Abbreviation: T_m, melting temperature.

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Table 3. Characterization of 17 tropical sprangletop populations with respect to resistance or sensitivity to glyphosate.

Population	GR_{50}[a]	95% CI		RI	Shikimic acid[b] (SE)	
		Lower	Upper		96 HAT	168 HAT
LvS	145.8	140.0	151.6	—	3,412 (276) a	3,886 (193) a
Lv1	315.0	293.5	336.5	2.2	135 (24) c	156 (19) cd
Lv2	407.7	352.9	462.5	2.8	91 (28) c	115 (46) cd
Lv3	326.0	310.4	341.6	2.2	102 (37) c	136 (7) cd
Lv4	367.9	313.1	422.7	2.5	227 (54) c	325 (93) c
Lv5	343.9	324.3	363.5	2.4	37 (3) c	344 (35) c
Lv6	325.7	265.0	386.4	2.2	46 (11) c	232 (34) cd
Lv7	515.8	470.8	560.8	3.5	166 (22) c	72 (22) cd
Lv8	430.0	388.9	471.1	2.9	68 (21) c	41 (8) d
Lv9	177.3	161.7	192.9	1.2	971 (48) b	3,199 (108) b
Lv10	351.7	341.9	361.5	2.4	117 (54) c	38 (6) d
Lv11	173.1	161.4	184.8	1.2	935 (26) b	3,280 (271) b
Lv12	467.0	412.2	521.8	3.2	20 (6) c	19 (2) d
Lv13	507.2	415.1	599.3	3.5	32 (3) c	25 (24) d
Lv14	385.4	367.8	403.0	2.6	18 (11) c	16 (5) d
Lv15	700.9	540.2	861.6	4.8	16 (1) c	15 (7) d
Lv16	400.8	387.1	414.5	2.7	24 (10) c	126 (32) cd

[a] Abbreviations: GR_{50}, amount of herbicide resulting in 50% fresh weight reduction (given in g ae of glyphosate ha^{-1}); CI, 95% confidence intervals ($n = 10$); RI, resistance index (GR_{50}resistant [R]/GR_{50}susceptible [S]); HAT, hours after treatment.

[b] Given in $\mu g\ g^{-1}$ fresh weight. SE represents the standard error of the mean ($n = 5$). Values in the same column with the same letter are not significantly different at 95% probability determined by Tukey's honestly significant difference test.

NY). The levels of genetic diversity within populations were assessed using Nei's gene diversity value (h) (Nei 1973). Calculations were based on data from 17 tropical sprangletop populations using POPGENE 32 software (Yeh et al. 2001). POPGENE was also used to estimate Shannon's information index (H_{pop}), total genetic diversity for all individuals (H_T), and the average genetic diversity within populations (H_S). These values were used to estimate the coefficient of genetic differentiation ($G_{ST} = [H_T - H_S]/H_T$), which indicates the genetic diversity among populations (Excoffier et al. 1992). The genetic diversity among populations was also assessed with an analysis of molecular variance (AMOVA) using the GeneAlEx 6.5 software (Peakall and Smouse 2006).

Results and Discussion

Whole-Plant Dose–Response Experiments. The whole-plant dose–response assay showed different levels of glyphosate resistance in the populations tested. The GR_{50} values ranged from 146 (LvS) to 701 (Lv15) g ae of glyphosate ha^{-1} (Table 3). RI ($RI = GR_{50}R/GR_{50}S$) values ranged from 1.2 to 4.8, with a vast majority of the populations falling in the range of 2.2 to 3.5. Glyphosate resistance was confirmed in 14 of the 16 (except for Lv9 and Lv11) putative resistant tropical sprangletop populations on the basis of a RI value > 2. The population with a RI value of 4.8 (Lv15) was identified as highly resistant (Table 3).

Shikimic Acid Assay. EPSPS enzyme inhibition generates the changes in shikimic acid levels in plants (Amrhein et al. 1980). Shikimic acid test in treated plants is accepted as an appropriate measure for determining resistance or susceptibility to glyphosate (Henry et al. 2007).

The level of shikimic acid accumulation was significantly different between the resistant and susceptible populations. The values of shikimic acid accumulation ranged from 16 (Lv15) to 3,412 (LvS) $\mu g\ g^{-1}$ fresh weight at 96 HAT; and 15 (Lv15) to 3,886 (LvS) $\mu g\ g^{-1}$ fresh weight at 168 HAT. Thus, LvS population accumulated more shikimic acid at 96 and 168 HAT than the other populations. The populations Lv 9 and Lv11 also showed high accumulation of shikimic acid at 96 and 168 HAT (Table 3). High accumulation of shikimic acid indicates susceptibility to glyphosate (De Corvalho et al. 2011) and species with a low shikimic acid accumulation require a greater amount of glyphosate to be lethal (Alcántara-de la Cruz et al. 2016).

The populations LvS, Lv9, and Lv11 also had the lowest GR_{50} values, confirming the association between high shikimic acid accumulation and susceptibility to glyphosate. Results suggest that

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Table 4. Parameters of genetic variability of 17 tropical sprangletop populations.

	H_{pop}[a]	P (%)	N_e	h
Susceptible populations				
$H_T = 0.218$ $H_S = 0.157$ $G_{ST} = 0.279$				
LvS	0.166	26.72	1.224	0.119
Lv9	0.292	51.31	1.336	0.198
Lv11	0.245	45.64	1.266	0.159
Resistant populations				
$H_T = 0.186$ $H_S = 0.144$ $G_{ST} = 0.225$				
Lv1	0.196	35.85	1.222	0.134
Lv2	0.224	41.51	1.247	0.147
Lv3	0.307	58.49	1.364	0.214
Lv4	0.192	35.85	1.215	0.131
Lv5	0.251	41.51	1.307	0.175
Lv6	0.188	32.08	1.221	0.128
Lv7	0.189	32.08	1.223	0.129
Lv8	0.195	33.96	1.228	0.131
Lv10	0.195	33.96	1.228	0.131
Lv12	0.148	26.42	1.156	0.117
Lv13	0.209	37.74	1.237	0.143
Lv14	0.252	43.40	1.302	0.169
Lv15	0.248	41.51	1.281	0.162
Lv16	0.171	30.19	1.192	0.121

[a] Abbreviations: H_{pop}, Shannon's information index; P, percentage of polymorphic loci; N_e, number of effective alleles; h, Nei's (1973) gene diversity.

shikimic acid detection can be used as a rapid diagnostic test for confirming glyphosate resistance in tropical sprangletop. Rapid screening tests based on shikimic acid detection have been previously used for different species/populations with encouraging results. For instance, in species such as junglerice [*Echinochloa colona* (L.) Link], sourgrass [*Digitaria insularis* (L.) Mez ex Ekman], and Italian ryegrass (*Lolium multiflorum* Lam.), the susceptible population accumulated three to seven times more shikimic acid with respect to the resistant population (De Corvalho et al. 2011; González-Torralva et al. 2012; Alarcón-Reverte et al. 2013).

Genetic Diversity within and among the Tropical Sprangletop Populations. The genetic diversity within the populations (h) ranged from 0.119 to 0.198 for the susceptible group, and from 0.117 to 0.214 for the resistant group. The averages of Shannon's information index (H_{pop}) were 0.234 and 0.211; the averages of genetic diversity within the populations (H_S) were 0.157 and 0.144; and the total genetic diversity averages (H_T) were 0.218 and 0.186 for the susceptible and resistant groups, respectively (Table 4). These data indicated that about 22 and 27% of the diversity in the resistant and susceptible populations, respectively, were due to diversity among populations.

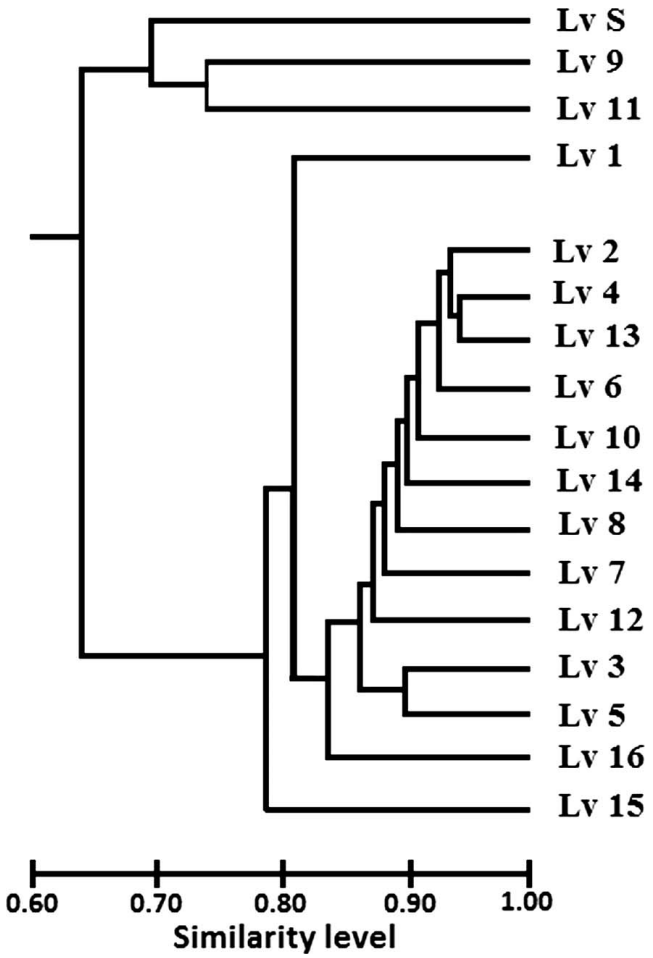

Figure 2. Dendrogram of tropical sprangletop populations based on Nei's (Nei 1973) genetic distance for the unweighted pair group method with arithmetic averages (UPGMA) modified from the NEIGHBOR procedure of PHYLIP. Distance metrics among populations were based on Nei's unbiased measures of genetic identity and genetic distance.

The number of effective alleles (N_e) ranged from 1.156 (Lv12) to 1.364 (Lv3), with the average of 1.177 by population description statistics. The higher value of N_e indicates that the population Lv3 has a greater number of heterozygotes than the theoretical value of an equilibrium population.

On the basis of the dendrogram obtained from the UPGMA algorithm, the 17 tropical sprangletop populations were structured as two groups: susceptible and resistant. The dendrogram provides evidence for a degree of genetic differentiation between the susceptible- and resistant-populations (Figure 2).

The genetic diversity values among populations (G_{ST}) estimated using POPGENE were 0.279 and 0.225 for the susceptible and resistant populations, respectively (Table 4); and F_{ST} estimated using AMOVA was 0.1636 (Table 5). The diversity

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Table 5. Analysis of molecular variance (AMOVA) of 17 tropical sprangletop populations analyzed as two groups.

Source of variation	df	Variance component		Fixation index[a]	P
		Absolute	%		
Among groups	1	0.169	9.87	$F_{CT} = 0.0987$	0.0001
Among populations within groups	15	0.096	5.36	$F_{SC} = 0.0417$	0.0001
Within populations	154	1.586	84.7	$F_{ST} = 0.1636$	0.0001
Total	170	1.851			

[a] Fixation indices are *F*-statistics correlating molecular diversity among groups: F_{CT}, correlation among random intersimple sequence repeat (ISSR) haplotypes within groups relative to the correlation of random pairs drawn from the whole sample; F_{SC}, correlation among random ISSR haplotypes within populations relative to the correlation of random pairs drawn from the group; F_{ST}, correlation among random ISSR haplotypes within populations relative to the correlation of random pairs drawn from the whole sample.

between the two groups was 9.87%, and among populations within each group was 5.36%, whereas the remaining 84.74% of the diversity was found within populations. Both analyses indicate that approximately 90% of the diversity in the structured populations was within populations, whereas 10% or less was among populations. The diversity among groups ($F_{CT} = 0.0987$) was highly significant ($P < 0.0001$). The heterogeneity among tropical sprangletop populations could be sustained via artificial selection (e.g., herbicide selection), and naturally by breeding system and life form (Nybom and Bartish 2000).

At the molecular level, the populations of tropical sprangletop (3 glyphosate susceptible and 14 resistant) presented a wide genetic variation. This variation could be due to the cross-pollination of the species and the agronomic practices in each crop (high selection pressure) (Imaizumi et al. 2013; Menchari et al. 2007).

The overall G_{ST} values among the susceptible tropical sprangletop populations were slightly higher (0.279) compared with those among the resistant populations (0.225) (Table 3). However, most of the susceptible and resistant populations showed high genetic diversity values. Only the populations LvS, Lv12, and Lv16 had much lower genetic diversity values than the rest of the populations (Table 4). These results are consistent for other cross-pollinating weed species such as blackgrass (*Alopecurus myosuroides* Huds.) (Menchari et al. 2007) and rock bulrush [*Schoenoplectus juncoides* (Roxb.) Palla] (Imaizumi et al. 2013), where some herbicide-resistant populations showed higher genetic diversity values similar to susceptible populations. In other cases, reductions in genetic diversity in herbicide-resistant populations have often been observed in predominantly selfing weeds (Osuna et al. 2011).

The repeated use of the same herbicide group could select for resistant genotypes every season. Therefore, resistant plants may add seeds to the soil seed bank. This could reduce genetic diversity among resistant populations (Aagaard et al. 1998). Of course, genetic differentiation among tropical sprangletop populations that are susceptible or unexposed to herbicides can also occur without herbicide selection. This could be due to local ecological factors, such as a variation in soil type, tillage practices, types of crops, fertilizers, etc. (Imaizumi et al. 2013; Nymbom and Bartish 2000).

Herbicide resistance can occur across a cropping region as a consequence of two processes: gene flow via pollen or seed dispersal, or both, and by independent evolution within weed populations, which is generally due to local selection for existing mutations rather than de novo mutation events (Burgos et al. 2013; Maxwell et al. 1990). Tropical sprangletop populations can be separated on the basis of the acquisition of resistance. The populations with acquired resistance were treated repeatedly only with glyphosate under different selection pressure levels (Pérez-López et al. 2014). In our work, according to the dendrogram obtained from the UPGMA algorithm (Figure 2), there was a great similarity between adjacent glyphosate-resistant populations. For example, the Lv2, Lv3, Lv4, and Lv5 populations from Cuitláhuac and the Lv8 and Lv10 populations from Martínez de la Torre were genetically and geographically very similar (Lv2 with Lv4; Lv3 with Lv5; and Lv8 with Lv10 populations) (Table 1, Figure 2). This could be due to gene flow mainly by pollen transfer or seed dispersal among these populations. However, there was also similarity between tropical sprangletop populations from distant locations, such as the glyphosate-resistant Lv8 and Lv10 populations from Martinez de la Torre, which were genetically similar to the Lv14 population from Cuitláhuac (Figure 1),

https://doi.org/10.1614/WS-D-15-00183.1 Published online by Cambridge University Press

as well as the glyphosate-susceptible Lv9 population from Martínez de la Torre being similar to the LvS and Lv11 populations from Cuitláhuac. This could be explained by long-distance seed dispersal. Populations were also found to be geographically close but genetically different, such as the Lv14 and Lv15 populations (Table 1, Figure 1) whose RI values were 2.6 and 4.8, respectively, higher than the LvS population. This indicates that the glyphosate resistance developed could also be due to independent evolutionary events. Similar results were reported by Osuna et al. (2011), who described both processes in causing spread of thiobencarb-resistant early watergrass [*Echinochloa oryzoides* (Ard.) Fritsch] populations collected in rice (*Oryza sativa* L.) fields from the California Central Valley.

Special attention should be paid in locations where the Lv7, Lv12, Lv13, and Lv15 populations were collected, and any further seed dispersal of these populations should be avoided. The prevention and control of seed dispersal should be a very important component in the integrated management of glyphosate resistance in this species. However, the environmental conditions of the citrus region from Veracruz makes weed control difficult; mechanical control facilitates dispersal of tropical sprangletop seed, and in large groves this is not viable. A proper herbicide rotation would reduce the glyphosate resistance of tropical sprangletop. Mixtures of herbicides glufosinate + indaziflam ($50 + 682$ g ai ha^{-1}) and paraquat + diuron ($400 + 200$ g ai ha^{-1}) were used, and only glufosinate (682 g ai ha^{-1}) reached up to 90% of control of tropical sprangletop at 15 DAT in field experiments carried out in Persian lime groves from Cuitláhuac and Martínez de la Torre, and a control higher than 85 and 80% at 30 and 60 DAT, respectively (Pérez-López et al. 2014).

In conclusion, the characterization of the efficacy of glyphosate by whole-plant dose–response experiments and shikimic acid accumulation assays corroborated glyphosate resistance in tropical sprangletop. Glyphosate resistance spread across Persian lime groves from Veracruz between tropical sprangletop populations from distant and adjacent locations is likely due to both short- and long-distance seed dispersal, as well as independent evolutionary events.

Results from this research will be useful for better glyphosate resistance management of tropical sprangletop by adapting diverse management programs and appropriate strategies to prevent the dispersal of resistant seeds.

Acknowledgments

This research has been funded by Monsanto Company, Spain's Ministry of Science and Innovation, and Mexico's National Council for Science and Technology through projects AGL2010-16774, AGL2011-27714, and CONACYT-231972. The authors are grateful for the assistance of the MSc. Macrina Pérez-López and the technician Rafael A. Roldán-Gómez for their technical help. We thank Dr. Muthukumar V. Bagavathiannan and anonymous reviewers for their valuable comments and critical suggestions to improve this manuscript.

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Received October 26, 2015, and approved May 9, 2016.

Associate Editor for this paper: Muthukumar V. Bagavathiannan, Texas A&M.

https://doi.org/10.1614/WS-D-15-00183.1 Published online by Cambridge University Press