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TESIS DOCTORAL

MECANISMOS FISIOLÓGICOS, BIOQUÍMICOS Y MOLECULARES DE TOLERANCIA/RESISTENCIA A GLIFOSATO EN ESPECIES DE MÉXICO

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TITULO: *MECANISMOS FISIOLÓGICOS, BIOQUÍMICOS Y MOLECULARES DE TOLERANCIA/RESISTENCIA A GLIFOSATO EN ESPECIES DE MÉXICO*

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Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de Junio de 2016

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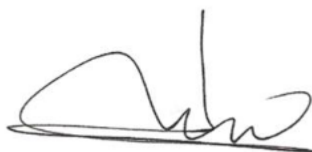
Responsable de línea de investigación

Dr. Rafael De Prado



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todo el mundo fracasa en algo.

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Lista de Abreviaturas

^{14}C-	carbono 14
ACCasa	acetil coenzina A carboxilasa
ae	ácido equivalente
ALS	acetolactato sintasa
AMPA	ácido aminometilfosfónico
AMOVA	análisis de varianza molecular
ANOVA	análisis de la varianza
BGE	electrolito fondo
bp	par (es) de base (s)
cDNA	ADN complementario de la primera cadena
CE	electroforesis capilar
CI	intervalos de confianza
CTAB	bromuro de hexadeciltrimetilamonio
2,4-D	ácido 2,4-diclorofenoxiacético
DAD	detector de diodos en fila
DAT/DDT	días después del tratamiento
DNA	ácido desoxirribonucleico
dNTP	desoxirribonucleótidos trifosfato
ED_{50}	dosis de herbicida capaz de reducir en un 50% el peso fresco de las plantas frente al control
EPSPS	enol piruvato shiquímato fosfato sintasa
FCT	diversidad entre grupos
g	gramo (s)
GPS	sistema de posicionamiento global
G_{ST}	coeficiente de diferenciación genética
h	valor de diversidad genética
ha	hectárea
HAT/HDT	hora (s) después del tratamiento
H_{pop}	índice de información de Shannon
H_S	Promedio de diversidad genética dentro de las poblaciones
H_T	diversidad genética total
I_{50}	dosis de herbicida capaz de reducir en un 50% la actividad

	enzimática
ISSR	repeticiones de secuencias internas simples
kBq	kilobequerelio
LD_{50}	dosis letal media
LSS	espectrometría de centelleo líquido
masl	metros sobre nivel del mar
MBq	megabequerelio
MIM	manejo integrado de malezas
mmol	mili molar
n	tamaño de muestra
NF	factor de normalizacion
ng	nanogramo (s)
nd	no detectado
Ne	número de alelos efectivos
nm	nanómetro (s)
NTSR	resistencia fuera del sitio de acción herbicida
PCR	reacción en cadena de la polimerasa
pH	Potencial de hidrogeno
qPCR	PCR cuantitativa
RNA	ácido ribonucleico
RI	índice de resistencia
RT	índice de tolerancia
SE	error estándar de la media
SIAP	Servicio de Información Agroalimentaria y Pesquera, México
SNP	polimorfismo de nucleótido único
TSR	resistencia en el sitio de acción herbicida
UPGMA	método del grupo de pares no ponderados con medias aritméticas
USDA	Departamento de Agricultura de los Estados Unidos
UV	ultravioleta

CAPITULO I

Introducción General

Abstract

Chemical weed is widespread in Mexico, mainly with glyphosate. Continued use of herbicides has resulted to the appearance of weed resistant species. However, in Mexico only a few cases have been reported. This is due to the lack of prospection works allowing the identification of new cases, as well as species identification with acceptable levels of herbicide tolerance, which can be used as a cover crop in efficient programs of Integrated Weed Management. Is evident the lack of scientific-technical publications, to guide for further works with the vision to improve agricultural extension services. So far there is no source that includes all resistant weed biotypes in Mexico. According to *The International Survey of Herbicide Resistant Weeds*, there are seven weed species resistant confirmed in the country to ACCase, ALS and EPSPS inhibiting herbicides. Glyphosate is an important herbicide for weed control yet. It is necessary to extend its life by the lack of new active substances. In this work the mechanisms of resistance to glyphosate were characterized in *Bidnes pilosa* and *Leptoclhoa virgata,* weed species that continues being unique glyphosate resistance cases worldwide. Furthermore, the natural glyphosate tolerance of *Cologania broussonetii* was characterized, a species with high potential as a cover crop in temperate regions of the country.

Keywords: EPSPS enzyme, glyphosate resistance, glyphosate tolerance, herbicide resistance, Mexico, resistance mechanisms, weed resistance

Resumen

El control de malas hierbas con herbicidas está ampliamente extendido en México, principalmente con glifosato. El uso continuo de herbicidas ha causado la aparición de especies resistentes a herbicidas. Sin embargo, en México existen pocos casos registrados debido a la falta de trabajos de prospección que permitan su identificación, así como de la identificación de especies con niveles aceptables de tolerancia a herbicidas, que puedan usarse como cultivo de cobertura en programas eficientes de Manejo Integrado de Malas Hierbas. Es evidente la carencia de publicaciones científico-técnicas, que sirvan de guía para la realización de nuevos trabajos con la visión de mejorar los servicios de extensión agrícola. Hasta el momento, no existe una fuente en la que se incluya a todos los biotipos resistentes de malezas en México. De acuerdo con "The International Survey of Herbicide Resistant Weeds", actualmente existen solo siete especies de malas hierbas resistentes a herbicidas inhibidores de la ACCasa, ALS y EPSPS. Dada la importancia que continúa teniendo el glifosato como herbicida para el control de malas hierbas, y la necesidad de alargar su vida útil por la falta de nuevas materias activas, en este trabajo se han caracterizado los mecanismos de resistencia a glifosato de *Leptochloa virgata* y *Bidens pilosa,* especies de malas hierbas que siguen siendo casos únicos en el mundo. Además, se ha caracterizado la tolerancia natural a glifosato que presenta *Cologania broussonetti,* una especie con alto potencial como cultivo de cobertura en regiones de clima templado del país.

Palabras clave: enzima EPSPS, glifosato, mecanismos de resistencia, malas hierbas resistentes, México, resistencia a herbicidas, tolerancia a glifosato

Importancia de las Malas Hierbas

La agricultura es una actividad de gran importancia para el desarrollo y riqueza de las naciones, y su práctica se ve afectada por diversos factores bióticos y abióticos que causan grandes pérdidas en el rendimiento de los cultivos (Oerke, 2006). Uno de estos factores bióticos más importantes en la producción agrícola son las malas hierbas.

Desde el punto de vista antropogénico, *una mala hierba es aquella planta que crece de forma predominante en situaciones marcadamente alteradas por el hombre y que resulta no deseable por él en un lugar y momento determinado* (Pujadas y Hernández, 1988). Las malas hierbas ocasionan pérdidas directas cuando compiten por luz, agua y nutrientes con los cultivos con variaciones regionales muy grandes, pudiendo causar pérdidas que oscilan entre 34 hasta el 60 % (Pimentel et al. 2001). Además, causan daños indirectos ya que dificultan las labores de recolección de los cultivos, también pueden ser reservorio de plagas y enfermedades que afectan a los cultivos (Liebman et al. 2001).

Desde el primer tercio del siglo pasado, los herbicidas se empezaron a utilizar para el control de malas hierbas (Ross y Lembi, 2009). En la agricultura moderna, los herbicidas se han convertido en la principal herramienta para el control de malas hierbas. Su uso excesivo ha dado lugar a la rápida evolución de malas hierbas resistentes a herbicidas (Powles y Yu 2010).

Resistencia a Herbicidas

La resistencia a herbicidas es la capacidad natural y heredable que un individuo de una especie de mala hierba posee para sobrevivir, completar su ciclo de vida y reproducirse sexualmente cuando el herbicida es aplicado a las dosis recomendadas de campo (Beckie, 2006). Desde entonces, se han registrado 470 casos únicos de resistencia (especie x herbicida) en 250 especies (145 dicotiledóneas y 105 monocotiledóneas) en 66 países (Heap, 2016).

Los grupos de herbicidas más importantes que se ven afectados por la problemática de la resistencia son los inhibidores de la ALS, inhibidores del

fotosistema I y II, Inhibidores ACCasa, inhibidores de la EPSPS y las auxinas sintéticas (Heap, 2014). Además, la resistencia a herbicidas múltiples dentro de biotipos individuales está muy extendida (Harker y O´Donovan, 2013).

Mecanismos de Resistencia a Herbicidas

La resistencia a herbicidas involucra mecanismos dentro del sitio de acción (target-site) y fuera del sitio de acción (non target-site).

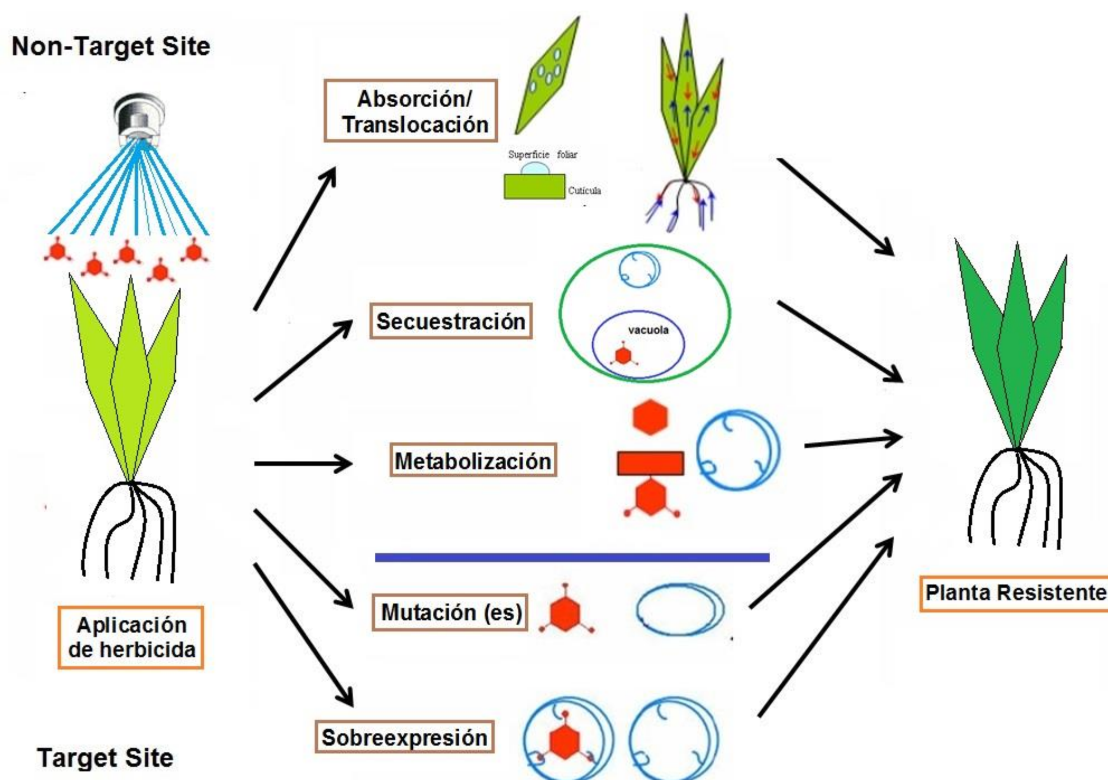

Figura 1. Mecanismos de resistencia a herbicidas dentro del sitio de acción (target-site) y fuera del sitio de acción (non target-site). Las plantas resistentes pueden expresar uno o múltiples mecanismos de resistencia al mismo o distintos herbicidas.

De acuerdo con Heap (2014), la resistencia a herbicidas es debida a cinco mecanismos primarios (**Figura 1**).

1. Disminución de la absorción y/o translocación es la restricción de movimiento de un herbicida en cantidad suficiente al sitio de acción que permite la planta para sobrevivir.
2. El secuestro de un herbicida sobre las paredes celulares o en vacuolas reduce la concentración de herbicida que alcanza el sitio de acción y puede resultar en resistencia.

3. Metabolismo mejorado es el aumento de la capacidad de una planta para metabolizar (degradar) a sustancias no toxicas un herbicida antes de que la mate.
4. La resistencia en el target-site (a menudo una enzima) resulta por sustitución (es) de nucleótidos en la secuencia de ADN de la enzima target causando mutaciones alterando su traducción a proteínas, pero sin que esta pierda su función, limitando o reduciendo la capacidad de un herbicida de unirse al sitio de acción.
5. La amplificación génica/sobreexpresión aumenta la producción de la enzima diana (target-site), por lo que se requiere una concentración más alta de herbicida para para inhibir la enzima diana y causar la muerte.

Además de la resistencia monoherbicida, las malas hierbas pueden desarrollar resistencia cruzada o resistencia múltiple. La resistencia cruzada ocurre cuando un único mecanismo de resistencia confiere resistencia a más de un herbicida. La resistencia cruzada de target-site es el tipo más común, y es el resultado de un sitio diana alterada que confiere resistencia a otros herbicidas que inhiben la misma enzima (Heap, 2014). La resistencia múltiple se presenta cuando una planta expresa más de un mecanismo de resistencia, y es generalmente el resultado de la selección secuencial de mecanismos de resistencia a herbicidas con diferentes sitios de acción o por medio de la acumulación de genes de resistencia a través de flujo de polen (Heap y LeBarron, 2001).

Resistencia a Glifosato

El glifosato [N-(phosphonomethyl) glycine] es un herbicida pos emergente de amplio espectro (Duke y Powles, 2008). Actúa inhibiendo la 5-enolpiruvil-shiquimato-3-fosfato sintasa (EPSPS), responsable de la biosíntesis del corismato, un intermediario en la vía del ácido shiquímico que guía a la síntesis de aminoácidos aromáticos esenciales (triptófano, fenilalanina y tirosina) de las plantas (**Figura 2**) (Franz et al. 1997).

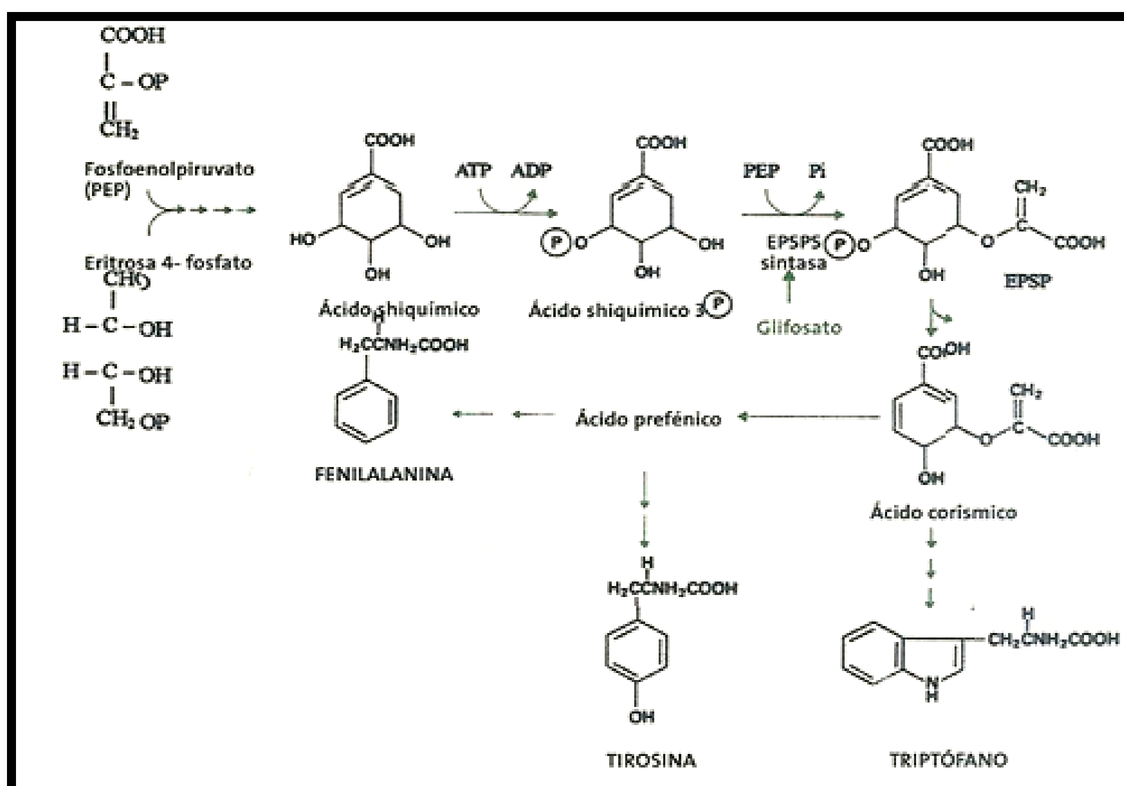

Figura 2. Biosíntesis de aminoácidos aromáticos vía del ácido shiquímico. El glifosato actúa como inhibidor competitivo de la enzima. EPSPS (5-enolpiruvil shiquimato 3-fosfato sintasa), interactuando con el sitio activo de la enzima que se uniría al grupo fosfato del PEP.

Desde su introducción al mercado en 1974, el glifosato se ha convertido en el herbicida más popular e importante globalmente para el control de malas hierbas (Orcaray et al. 2012, Székács y Darvas, 2012). Por más de 20 años no se registró ningún caso de resistencia a glifosato (Duke y Powles, 2008). En 1996 se reportó el primer caso de resistencia en Australia (Powles et al. 1998), año en que también se introdujeron las primeras variedades de cultivos transgénicos resistentes a este herbicida (Duke y Powles, 2008).

La dependencia casi exclusiva del glifosato para el control de malezas, al igual que con otros herbicidas, ha llevado a la evolución de las poblaciones de malas hierbas resistentes, principalmente influenciado, pero no exclusivamente, por la adopción de estos cultivos transgénicos (Sammons y Gaines, 2014; Yanniccari et al. 2016). Actualmente existen 35 especies resistentes a glifosato en el mundo con 258 casos reportados (**Tabla 1**) (Heap, 2016).

Tabla 1. Aparición cronológica de especies resistentes a glifosato en el mundo (Heap, 2016).

1er año	Especie	País[a] (no. de casos registrados)
1996	*Lolium rigidum*	AUS (8), ZAF (2), ESP (1), FRA (1), ITA (1), ISR (1), USA (1)
1997	*Eleusine indica*	MAS (2), USA (2), ARG (1), BOL (1), BRA (1), CHN (1), COL (1), CRC (1), INA (1), JPN (1)
2000	*Conyza canadensis*	USA (29), CAN (2), BRA (1), CHN (1), CZE (1), ESP (1), GRE (1), JPN (1), ITA (1), POL (1), POR (1)
2001	*Lolium perenne* ssp. *multiflorum*	USA (8), CHI (4), ARG (2), BRA (2), ITA (2), ESP (1), JPN (1), NZL (1), SUI (1)
2003	*Plantago lanceolata*	ZAF (1)
	Conyza bonariensis	AUS (3), USA (2), BRA (1), COL (1), ESP (1), GRE (1), ISR (1), POR (1), ZAF (1)
2004	*Ambrosia artemisiifolia*	USA (17), CAN (1)
	Ambrosia trifida	USA (15), CAN (2)
	Parthenium hysterophorus	COL (1), USA (1)
2005	*Hedyotis verticillata*	MAS (1)
	Sorghum halepense	USA (3), ARG (2)
	Amaranthus palmeri	USA (33), ARG (1), BRA (1)
	Amaranthus tuberculatus (=*A. rudis*)	USA (22), CAN (1)
	Digitaria insularis	BRA (1), PAR (1)
2007	*Echinochloa colona*	AUS (3), ARG (1), USA (1),VEN (1)
	Kochia scoparia	USA (13), CAN (3)
2008	*Cynodon hirsutus*	ARG (1)
	Lolium perenne	ARG (1), NZL (1), POR (1)
	Urochloa panicoides	AUS (1)
2009	*Conyza sumatrensis*	BR (2), ESP (1), FRA (1), GRE (1)
2010	*Chloris truncata*	AUS (2)
	Poa annua	USA (3)

	Raphanus raphanistrum	AUS (1)
	Leptochloa virgata	MEX (1)
2011	*Bromus diandrus*	AUS (1)
2012	*Amaranthus spinosus*	USA (1)
2013	*Amaranthus hybridus* (syn: *quitensis*)	ARG (2)
2014	*Bidens pilosa*	MEX (1)
	Brachiaria eruciformis	AUS (1)
	Bromus rubens	AUS (1)
	Chloris elata	BRA (1)
	Sonchus oleraceus	AUS (1)
2015	*Chloris virgata*	AUS (3)
	Lactuca serriola	AUS (1)
	Salsola tragus	USA (1)

[a] Códigos ISO de 3 letras asignado a ese país según la norma ISO 3166-1 por Organización Internacional de Normalización.

Manejo de la Resistencia a Herbicidas

El manejo de la resistencia a herbicidas se refiere al manejo integrado de malas hierbas (MIM). El MIM se puede definir como un enfoque que integra los diferentes métodos de control de malas hierbas para poner al cultivo en ventaja frente a las estas. Se práctica en casi todo el mundo en diferentes niveles de una finca a otra. El MIM tiene el potencial de restringir las poblaciones de malas hierbas a niveles manejables, reducir el impacto ambiental de las prácticas de manejo de malas hierbas individuales, aumentar la sostenibilidad de los sistemas de cultivo, y reducir la presión de selección ejercido por los herbicidas para evitar la selección de biotipos de malas hierbas resistentes a herbicidas (Harker y O´Donovan, 2013).

Para desarrollar un buen programa de MIM que prevenga o retrase la aparición de la resistencia es importante conocer cómo se desarrolla la resistencia. Los factores a considerar incluyen las características de la especie de mala hierba, de los herbicidas y prácticas culturales (Zita, 2012).

El factor principal en la evolución de la resistencia es la presión de selección ejercida por el herbicida que depende de la dosis, eficacia y frecuencia de aplicación del producto. Por lo tanto, para evitar o retrasar la evolución de la resistencia, es necesario disminuir la presión de selección (Valverde et al. 2007). Por desgracia, la "integración" que consta sólo de componentes de control químico es común en los sistemas modernos de cultivo (Harker y O´Donovan, 2013).

De acuerdo con el Comité de Acción de Resistencia a Herbicidas (HRAC, por sus siglas en inglés), los principios generales del manejo de la resistencia a herbicidas recomendados por el son (HRAC, 2016):

- Utilizar herbicidas solo cuando sea necesario.
- El seguimiento de las malas hierbas permite conocer cuales afectan las distintas etapas del proceso productivo, su situación histórica, identificar los sectores más problemáticos y los escapes evitando su propagación.
- Rotación de herbicidas con diferente sitio de acción en secuencia o mezcla en combinación con la superposición de los espectros de malas hierbas.
- La aplicación correcta de los herbicidas, no sólo incluye a la tecnología de aplicación apropiada sino también la dosis correcta y el momento oportuno para lograr el máximo impacto con la mínima dosis de exposición del medio al herbicida.
- Explorar los campos después de la aplicación para asegurar el control de las malas hierbas.
- Evitar que las malas hierbas que se reproducen por semilla o vegetativamente proliferen.
- Limpiar los equipos de laboreo entre diferentes sitios con el fin de no diseminar semillas procedentes de individuos resistentes.
- Comenzar con un campo limpio y controlar las malas hierbas tempranas mediante prácticas culturales de quemado o labranza, en combinación con un herbicida residual preemergente según sea apropiado.

- Rotación de cultivos para mejorar el aprovechamiento de los recursos (agua, luz y nutrientes), favoreciendo el efecto de la competencia del cultivo sobre las malas hierbas.
- Uso de cultivos de cobertura.

Uso de cultivos de cobertura en el MIM

Un cultivo de cobertura es una cubierta vegetal temporal o perenne que cubre el suelo creciendo específicamente para mantenerlo cubierto protegiéndolo de la erosión, evitando la pérdida de nutrientes por lavado y escurrimiento, en caso de ser leguminosa incorporando nitrógeno al suelo (Hartwig y Amón, 2002). Los cultivos de cobertura también están involucrados en la supresión de poblaciones de malas hierbas (Renard y Franqueville, 1991).

Sin embargo, el empleo de cultivos de cobertura para el manejo de malezas presenta limitaciones. Generalmente las especies utilizadas como cultivos de cobertura son altamente susceptibles a la interferencia con las poblaciones de malas hierbas en las primeras etapas de crecimiento establecimiento (Skerman et al. 1991). En estas etapas sería aceptable el uso de herbicidas con bajo perfil toxicológico y ambiental que faciliten el establecimiento del cultivo de cobertura.

Adecuados niveles de tolerancia a herbicidas en especies que se puedan usar como cultivos de cobertura serían deseables para facilitar su establecimiento (Lal et al. 1991). De acuerdo con la Sociedad de Malas Hierbas de Estados Unidos, *la tolerancia a herbicidas es la capacidad inherente de una especie para sobrevivir y reproducirse después del tratamiento herbicida sin que haya sufrido previamente algún proceso de selección o manipulación genética, es decir la tolerancia es fenómeno natural* (WSSA, 1998). De este modo, algunas especies utilizadas como cultivo de cobertura permiten la aplicación de herbicidas en menores dosis y con menor frecuencia.

Especies de leguminosas que presentan tolerancia natural a glifosato como *Canavalia ensiformis, Clitoria ternatea, Neonotonia wightii* y *Mucuna pruriens*, se han utilizado de manera exitosa como cultivos de cobertura en

huertos de frutales de regiones tropicales de México, principalmente en el estado de Veracruz (Cruz-Hipolito et al. 2009, 2011; Rojano-Delgado et al. 2012). Los mecanismos de tolerancia a glifosato están relacionados con los mecanismos de resistencia descritos en biotipos resistentes de malas hierbas (Shaner, 2009). En México, se sospecha de especies que presentan niveles de tolerancia a herbicidas aceptable. Sin embargo, no se han realizado los esfuerzos necesarios para confirmar esta tolerancia e implementar dichas especies como cultivos de cobertura (Domínguez-Valenzuela, comunicación personal).

Situación de la Resistencia a Herbicidas en México

En México, los estudios que se refieren a la distribución de malas hierbas resistentes a herbicidas y sus implicaciones, ya sean introducidas o nativas, son escasos (Domínguez-Valenzuela, comunicación personal). Desde el primer caso confirmado de *Phalaris minor* en 1996 que mostró resistencia cruzada a herbicidas inhibidores de la ACCasa a la fecha, y de acuerdo con "*The International Survey of Herbicide Resistant Weeds*" (Heap, 2016), en México existen siete casos de malas hierbas resistentes a herbicidas registrados oficialmente (**Tabla 2**), mientras países como Estados Unidos reportan más de 500 casos (Heap, 2016).

Tabla 2. Especies de malas hierbas resistentes a herbicidas en México (Heap, 2016).

No.	Especie	Año	Sitio de acción
1	*Phalaris minor* Retz.	1996	Inhibidores de la ACCasa
2	*Phalaris paradoxa* L.	1996	Inhibidores de la ACCasa
3	*Avena fatua* L.	1998	Inhibidores de la ACCasa
4	*Sorghum halepense* (L.) Pers.	2009	Inhibidores de la ALS
5	*Leptochloa virgata* (L.) P. Beauv.	2010	Inhibidores de la EPSPS
6	*Bidens pilosa* L.	2014	Inhibidores de la EPSPS
7	*Ixophorus unisetus* (J.Presl) Schltdl.	2014	Inhibidores de la ALS

Otras fuentes informan que puede haber más de 20 casos de resistencia en México (Zita, 2012). Sin embargo, no existe una fuente oficial en la que se incluya a todos los biotipos resistentes de malas hierbas en México, ya que

muchas veces solamente se da a conocer en congresos y simposios. La mayoría de los casos son caracterizaciones a base de ensayos de dosis-respuesta, por lo se desconocen los mecanismos de resistencia involucrados.

El grupo de investigación Herbicidas "Acción de los Pesticidas sobre el Ecosistema" del Departamento de Química Agrícola y Edafología, Universidad de Córdoba, España, en colaboración con el equipo de investigación de "Manejo Integrado de Malas Hierbas" del Departamento de Parasitología Agrícola, Universidad Autónoma Chapingo, México, tienen conocimiento de otras especies con sospecha de resistencia como *Echinochloa colona* y *Euphorbia heterophylla* a herbicidas inhibidores del fotosistema II, *Phalaris brachystachys* a herbicidas inhibidores de la ACCasa, *Amaranthus tuberculatus, Chloris elata, Eleusine indica* y *Parthenium hysterophorus* con resistencia a glifosato, entre otras especies, pero hasta el momento no ha sido posible llevar a cabo la caracterización de los mecanismos involucrados en dicha resistencia de todas estas especies.

Dada la importancia que continúa teniendo el glifosato como herbicida, y la necesidad de alargar su vida útil, así como de otros herbicidas que son muy efectivos, debido a la falta de aparición de nuevas materias activas (Heap, 2014), en este trabajo se han caracterizado los mecanismos de resistencia a glifosato de *Leptochloa virgata* y *Bidens pilosa,* especies de malas hierbas que siguen siendo casos únicos de resistencia a glifosato en el mundo (Heap, 2016). Además, se ha caracterizado la tolerancia natural a glifosato que presenta *Cologania broussonetti,* una especie con alto potencial como cultivo de cobertura en regiones de clima templado del país.

Los objetivos específicos que se han planteado en esta investigación son:

1. Caracterizar la eficacia del glifosato mediante ensayos de dosis-respuesta en condiciones de invernadero en: poblaciones con sospecha de resistencia a glifosato de *B. pilosa* y *L. virgata*; y poblaciones tolerantes de *C. broussonetii* comparada con una población susceptible de *Conyza bonariensis.*
    a) Estimar la dosis media efectiva que reduce el peso al 50% (ED_{50})

en las poblaciones de *B. pilosa, C. broussonetii, C. bonariensis* y *L. virgata.*

b) Estimar la dosis media efectiva que produce la mortalidad de una población al 50% (LD_{50}) en las poblaciones de *B. pilosa* y *L. virgata.*

2. Estudiar parámetros que indican resistencia/tolerancia a glifosato.
   a) Determinar los niveles de acumulación de ácido shiquímico en las poblaciones de *B. pilosa, C. broussonetii, C. bonariensis* y *L. virgata.*
   b) Determinar los niveles de actividad basal enzimática de la EPSPS, y la concentración de glifosato para inhibirla la actividad enzimática al 50% (I_{50}) en las poblaciones *B. pilosa* y *L. virgata.*
3. Identificar los posibles mecanismos fuera del sitio de acción (NTSR) involucrados en la resistencia/tolerancia a glifosato
   a) Determinar la capacidad de retención foliar de solución de herbicida en poblaciones de *B. pilosa, C. broussonetii* y *C. bonariensis.*
   b) Cuantificar las posibles diferencias de absorción y traslocación de ^{14}C-glifosato en las poblaciones de *B. pilosa, C. broussonetii, C. bonariensis* y *L. virgata*.
   c) Visualizar la translocación de ^{14}C-glifosato con autoradiografías de fosforo en las poblaciones de *B. pilosa, C. broussonetii, C. bonariensis* y *L. virgata*.
   d) Determinar si *C. broussonetii* es capaz de metabolizar el glifosato en sustancias no toxicas (ácido amino métil fosfonico, glioxilato, sarcosina y formaldehído) en comparación con *C. bonariensis*
4. Estudiar posibles mecanismos de resistencia en el sitio de acción (TSR)
   a) Identificar posibles mutaciones del gen de la EPSPS en las poblaciones de *B. pilosa* y *L. virgata.*
   b) Cuantificar los niveles de expresión del gen de la EPSPS en poblaciones de *L. virgata*.
5. Estimar la diversidad genética de 17 poblaciones resistentes y susceptibles a glifosato de *L. virgata* mediante marcadores ISSR, para comprender la dispersión de la resistencia en esta especie.

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CAPITULO II

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

Abstract

The susceptibility to glyphosate and genetic diversity based on inter simple sequence repeat (ISSR) 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 (AMOVA) 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.

Keywords: diversity within/among populations; genetic diversity; glyphosate resistance spread; ISSR markers; tropical sprangletop populations; resistance index.

Resumen

La susceptibilidad a glifosato y la diversidad genética 17 poblaciones de zacate carricillo (*Leptoclhoa virgata*) fueron caracterizadas utilizando marcadores *inter simple sequence repeat* (ISSR). Las semillas de las poblaciones fueron colectadas en dos regiones separadas geográficamente, principalmente en huertos de limón persa en Veracruz, México. Los resultados de los ensayos de dosis-respuesta junto con los de acumulación de ácido shiquímico revelaron diferentes niveles de resistencia a glifosato es estas poblaciones. Los valores de diversidad genética (*h*) estimados usando POPGENE oscilaron de 0.119 a 0.198, y de 0.117 a 0.214 dentro de las poblaciones susceptibles y resistentes, respectivamente. La diversidad genética promedio (H_S) dentro de las poblaciones susceptibles a glifosato fue 0.157. y la diversidad genética total (H_T) fue 0.218. Para las poblaciones resistentes, los valores de estos parámetros fueron H_S = 0.144, y H_T = 0.186. El análisis de varianza molecular (AMOVA), basado en la respuesta a glifosato de las poblaciones, indico que la mayor variación de diversidad genética fue encontrada dentro de los grupos de poblaciones susceptibles y resistentes (90% de la variación genética), mientras solo el 10% o menos entre los grupos. El alto nivel de diversidad genética entre las poblaciones de zacate carricillo, de lugares distantes o adyacentes, es probable que se deba a la dispersión de semillas, tanto a corta como larga distancia, y eventos evolutivos independientes en las poblaciones de zacate carricillo entre huertos de limón persa in Veracruz.

Palabras clave: diversidad dentro/entre poblaciones, diversidad genética, dispersión de la resistencia a glifosato, índice de resistencia, marcadores ISSR, zacate carricillo.

Introduction

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 *L. obtusiflora* 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, *L. chinensis,* 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 (Snow, 1997; Snow et al. 2008; Peterson et al. 2012), and it is found to be affecting the main tropical crops or cropping systems in Mexico, such as sugarcane, citrus orchards, and coffee plantations (Snow et al. 2008; Pérez-López et al. 2014).

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 years, 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 (N-phosphonomethyl glycine) 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 enzyme 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, and/or overexpression of EPSPS; and (2) exclusion or non-target 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. 2012; 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 inter simple sequence repeat (ISSR) markers (Imaizumi et al. 2013; Osuna et al. 2011; Huanfu et al. 2009). 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 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 3, Figure 3**). 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 ºC day/night.

Table 3. 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.

Population	Collection site	Location		Altitude
		Latitude	Longitude	(masl)[a]
LvS[b]	A	18°47′41.70″	96°41′26.58″	328
Lv1	A	18°48′01.92″	96°42′04.50″	354
Lv2	A	18°47′53.52″	96°41′46.44″	342
Lv3	A	18°47′44.16″	96°41′24.48″	333
Lv4	A	18°47′38.94″	96°41′07.98″	326
Lv5	A	18°47′37.50″	96°41′09.42″	327
Lv6	A	18°47′37.68″	96°41′05.46″	321
Lv7	A	18°47′33.96″	96°40′56.64″	315
Lv8	B	20°09′37.26″	97°05′40.08″	101
Lv9	B	20°10′28.68″	97°05′48.12″	124
Lv10	B	20°10′26.97″	97°05′46.31″	130
Lv11	A	18°45′48.30″	96°30′58.68″	230
Lv12	A	18°43′46.02″	96°30′59.94″	145
Lv13	A	18°43′46.68″	96°30′58.80″	145
Lv14	A	18°45′00.23″	96°31′35.12″	134
Lv15	A	18°45′00.00″	96°31′35.40″	131
Lv16	A	18°44′38.40″	96°36′23.28″	209

[a] masl: meters above sea level. [b] The susceptible population (LvS) was collected on the edge of a sugarcane field that had never been treated with glyphosate.

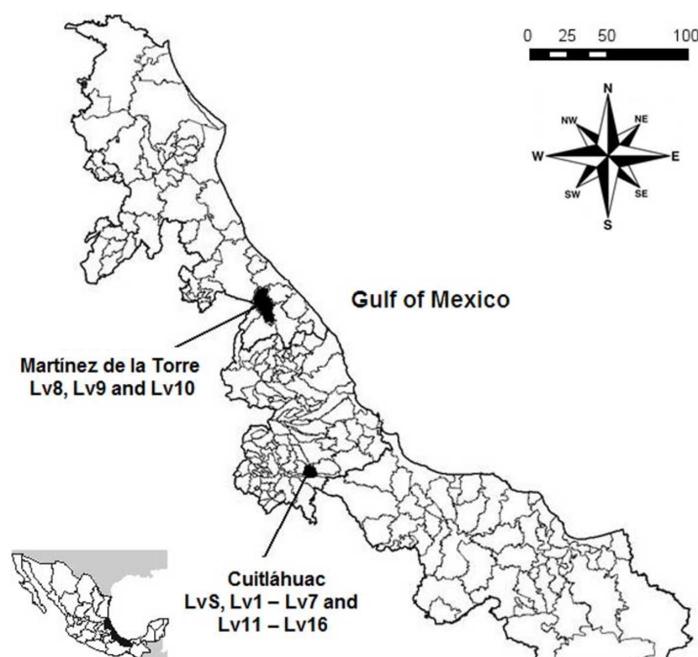

Figure 3. Geographical locations of the Martínez de la Torre and Cuitháhuac municipalities, Veracruz, Mexico, where 17 populations of tropical sprangletop were collected. The susceptible population (LvS) was collected on the edge of a sugarcane field.

Whole plant dose response experiments

Glyphosate (Roundup EnergyPro 45% w/v (potassium salt); Monsanto, Spain) treatments were 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 ae 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 0.25N 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 basis on a calibration curve made with known shikimic acid concentrations, and the results were expressed as µg of shikimic acid g^{-1} 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 ºC until use.

A total of eigth ISSR primers from primer set #9 from the University of British Columbia Biotechnology Laboratory (Vancouver, Canada) was selected for DNA amplification. **Table 4** 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 10x (with 15 mM $MgCl_2$); 2 µl (2.5 mM) of dNTP 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 ºC; 35 cycles of 30 s at 94 ºC, 30 s at 52 to 55 ºC, and 1 min at 72 ºC; 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 10000x (Biotium) and photographed under UV light (transilluminator ALPHA-INNOTECH). The molecular size of the fragments was estimated with reference to a 100-base pais 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.

Table 4. Inter simple 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] (ºC)
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] T_m= melting temperature

Statistical analysis

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

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

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 ED_{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 ED_{50} of the resistant population (R)/ED_{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 test (HSD) 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, 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 ED_{50} values ranged from 146 (LvS) to 701 (Lv15) g ae of glyphosate ha^{-1} (**Table 5**). RI (RI = $ED_{50}R/ED_{50}S$) 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 in14 of the 16 (except for Lv9 and Lv11) putative resistant tropical sprangletop populations, on the basis on a RI value > 2. The population with a RI value of 4.8 (Lv15) was identified as highly resistant (**Table 5**).

Table 5. Characterization of 17 tropical sprangletop populations with respect to resistance or sensitivity to glyphosate.

Population	ED_{50}[a]	95% CI[b]		RI[c]	Shikimic acid[d] (SE)	
		Lower	Upper		96 HAT	168 HAT
LvS	145.8	140.0	151.6	----	3412 (276) a	3886 (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	3199 (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	3280 (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] Given in g ae of glyphosate ha^{-1}; [b] CI values are the 95% confidence intervals (n=10).[c] Resistance Index $RI=ED_{50}R/ED_{50}S$. [d] Given in µg 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 (HSD) test.

Shikimic acid assay

EPSPS enzyme inhibition generates the changes in shikimic acid levels in plants (Amrhein et al. 1980). Shikimic acid tests 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 3412 (LvS) µg g^{-1} fresh weight at 96

HAT; and 15 (Lv15) to 3886 (LvS) µg of shikimic acid 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 5**). 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 ED_{50} values, confirming the association between high shikimic acid accumulation and susceptibility to glyphosate. Results suggest that 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 *Echinocloa colona*, *Digitaria insularis* and *Lolium multiflorum,* 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 (Hs) 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 6**). These data indicated that about 22 and 27% of the diversity in the resistant and susceptible populations, respectively were due to diversity among populations.

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

Table 6. Parameters of genetic variability of 17 tropical sprangletop populations.

	H_{pop}	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

h= Nei´s (1973) gene diversity; H_{pop}=Shannon´s information index; P=percentage of polymorphic loci; N_e=number of effective alleles.

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 4**).

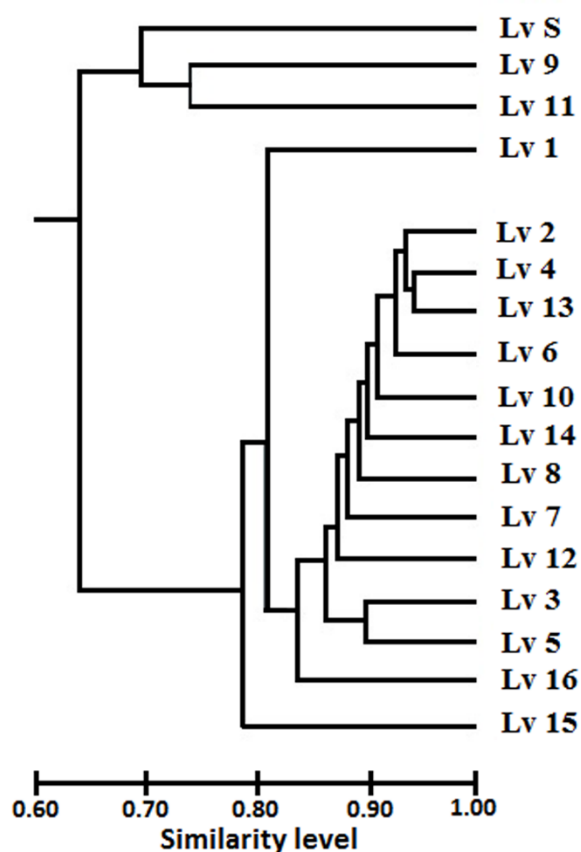

Figure 4. Dendrogram of tropical sprangletop populations based on Nei´s (Nei, 1973) genetic distance for the unweighted pair group with arithmetic averages (UPGMA) modified from the NEIGHBOR procedure of PHYLIP. Distance metrics among populations were based on Nei´s unbiased measures of genetic identity and genetic distance.

The genetic diversity values among populations (G_{ST}) estimated using POPGENE were 0.279 and 0.225 for the susceptible and resistant populations, respectively (**Table 6**); and F_{ST} estimated using AMOVA was 0.1636 (**Table 7**). The diversity 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).

Table 7. 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 inter simple 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.

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 5**). 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 6**). These results are consistent for other cross-pollinating weed species such as *Alopecurus myosuroides* (Menchari et al. 2007) and *Schoenoplectus juncoides* (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 (Maxwell et al. 1990; Burgos et al. 2013). 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 4**), 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 3, Figure 3**). 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 3**), as well as the glyphosate-susceptible Lv9 population from Martínez de la Torre was 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 3, Figure 3**) 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 *Echinochloa oryzoides* populations collected in rice 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).

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CAPITULO III

Resistance mechanisms characterization in glyphosate-resistant *Leptochloa virgata* populations collected in citrus orchards from Mexico

Abstract

Leptochloa virgata is an annual weed common in citrus groves in the states of Puebla and Veracruz, Mexico limiting their production. Three glyphosate-resistant populations (R8, R14 and R15) were used to study their resistance mechanisms comparing them to one susceptible population (S). Dose-response and shikimic acid accumulation assays confirmed the glyphosate resistance of the three resistant populations. Higher doses of up to 720 g ae ha^{-1} (field dose) were needed to control by 50% plants of resistant populations. The S population absorbed between 7 and 13% more ^{14}C-glyphosate than resistant ones, and translocated up to 32.2% of ^{14}C-glyphosate to the roots at 96 h after treatment (HAT). The R8, R14 and R15 populations translocated only 24.5, 26.5 and 21.9%, respectively. The EPSPS enzyme activity was not different in the S, R8 and R14 populations. The R15 Population exhibited 165.9 times greater EPSPS activity. Additionally, this population showed a higher EPSPS basal activity and a substitution in the codon 106 from Proline to Serine in the EPSPS protein sequence. EPSPS gene expression in the R15 population was similar to that of S population. In conclusion, the three resistant *L. virgata* populations presented reduced absorption and translocation of ^{14}C-glyphosate. Moreover, a mutation in the gene target-site conferred higher resistance to glyphosate to the R15 population.

Keywords: *Leptochloa virgata,* EPSPS gene, glyphosate, Pro-106 substitution, reduced translocation, glyphosate resistance mechanisms.

Resumen

Leptochloa virgata es una mala hierba anual común en huertos de cítricos en los estados de Puebla y Veracruz (México), que limita su producción. En este trabajo se utilizaron tres poblaciones (R8, R14 y R15) resistentes a glifosato de *L. virgata* para estudiar sus mecanismos de resistencia en comparación con una población susceptible (S). Los ensayos de dosis-respuesta y acumulación de ácido shiquímico confirmaron la resistencia de las tres poblaciones resistentes. Se requirieron dosis superiores a 720 g ae ha^{-1} (dosis de campo) para controlar el 50% de las plantas de las poblaciones resistentes. La población S absorbió entre 7 y 13% más de ^{14}C-glifosato que las poblaciones resistentes, y transloco hasta 32.2% de ^{14}C-glifosato a las raíces a las 96 horas después del tratamiento (HDT). Las poblaciones R8, R14 y R15 translocaron solo 24.5, 26.5 y 21.9%, respectivamente. La actividad enzimática de la EPSPS no fue significativa en las poblaciones S, R8 y R14. La población R15 exhibió 165.9 veces mayor actividad de la EPSPS. Adicionalmente, esta población mostro una mayor actividad basal de la EPSPS y una sustitución en el codón 106 de Prolina a Serina en la secuencia de la proteína de la EPSPS. La expresión del gen de la EPSPS de la población R15 fue similar a la expresión del mismo gen de la población S. En conclusión, las tres poblaciones resistentes de *L. virgata* presentaron reducida absorción y translocación de ^{14}C-glifosato. Además, una mutación en el target-site (gen de la EPSPS) confiere mayor resistencia a glifosato a la población R15.

Palabras clave: gen de la EPSPS, glifosato, *Leptochloa virgata,* mecanismos de resistencia a glifosato, reducida absorción, reducida translotión, sustitución Pro-106.

Introduction

Glyphosate is a systemic herbicide of broad spectrum (Duke and Powles, 2008) with a low toxicological profile (Giesy et al. 2010), and it has been used for over 40 years (since 1974) to control weeds (Székács and Darvas, 2012). The target-site of glyphosate is the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) (Amrhein et al. 1980). Its inhibition by glyphosate does not allow the synthesis of phenylalanine, tyrosine and tryptophan (Franz et al. 1997).

For over 20 years there was no exist evidence of glyphosate resistance (Duke and Powles, 2008). The first case of it was identified in a biotype of *Lolium rigidum* in 1996 (Powles et al. 1998), coinciding with glyphosate-resistant crops (Powles and Duke, 2008). The introduction and rapid adoption of these crops, and the generalized dependence on glyphosate for weed control, has made it the most used herbicide in the world, causing a rapid evolution of glyphosate-resistant weeds (Malone et al. 2016; Yanniccari et al. 2016). To date 35 weed species have been reported as being resistant to glyphosate with more than 256 cases registered (Heap, 2016).

Resistance to glyphosate could be caused by different mechanisms either in the target or non-target site (Salas et al. 2015). The major mechanisms of glyphosate resistance characterized in weed species are: amino acid substitutions in the EPSPS gene, multiple EPSPS copy numbers, increased EPSPS expression, reduced absorption, altered translocation, vacuolar sequestration and metabolism (Shaner, 2009; Duke, 2011; Ge et al. 2012; Sammons and Gaines, 2014; Yu et al. 2015; Malone et al. 2016), limiting the amount of glyphosate which reaches the EPSPS, or causing a loss of affinity between the EPSPS and the glyphosate.

Leptochloa virgata (L.) P. Beauv.) is an annual or perennial grass native to Asia, growing in numerous vegetation and soil types, but mostly in more mesic climates (Snow et al. 2008). In 2010, *L. virgata* was reported as being resistant to glyphosate (Heap, 2016) in citrus orchards from Veracruz, Mexico by this research group. Since then, glyphosate resistance studies and genetic characterization of different resistant populations, and field trials to provide

alternative herbicides for their control have been carried out (Pérez-López et al. 2014; Alcántara-de la Cruz et al. 2016). However, no studies on the glyphosate resistance mechanisms developed by this species have conducted. The objective of this work was to determine whether the glyphosate resistance developed by resistant *L. virgata* populations was due either to alterations in the target site, or by reduced glyphosate absorption and translocation.

Material and Methods

Biological material and growing conditions

Seeds of four *L. virgata* populations (S, R8, R14 and R15) collected in Persian lime groves from Veracruz, Mexico, were used. The populations were previously characterized with different levels of susceptibility to glyphosate (Alcántara-de la Cruz et al. 2016). Populations R14 and R15 were used in this study and were selected for being geographically close (Cuitláhuac municipality), but genetically different. Also, the most resistant population (R8) from Martínez de la Torre municipality was included in this study.

The seeds were sown on trays with peat saturated at field capacity. The trays were covered with a layer of plastic until emergence and placed in a growth chamber at 26/18 °C (day/night) with a photoperiod of 16 h at 850 mmol m^{-2} s^{-1} of light density and 60% relative humidity.

Germinated seedlings were individually transplanted into plastic pots in 250 mL of substrate (sand and peat 1:1). Subsequently, the pots were placed in a growth chamber under the conditions described above and watered daily.

Glyphosate dose-response

Plants of the four populations with 3-4 true leaves were treated with the following glyphosate rates: 0, 45, 90, 180, 360, 720, 1040, 1440 and 1800 g ae ha^{-1}. Glyphosate (Roundup Energy 45% w/v, Monsanto, Spain) applications were carried out in a treatment chamber (Devries Manufacturing, Hollandale, Minnesota) equipped with a TeeJet 8002EVS flat fan nozzle calibrated at 200 kPa, a height of 50 cm and 200 L ha^{-1} of application volume.

The plants were harvested at ground level 21 days after treatment (DAT) and stored separately in paper envelopes. The samples were dried in an oven (JP Selecta S.A., Barcelona, Spain) at 60 ºC for 4 days and weighed to determine the dry weight. Data were expressed as percentage of dry weight compared to the untreated control plants according to González-Torralva et al. (2012). The experiment was repeated twice in a completely randomized design with 10 replicates per dose.

***In vivo* shikimic acid accumulation**

Samples of 50 mg (leaf discs 4 mm in diameter) of plant tissue were taken from young leaves of 3 three plants with 3-4 true leaves of each *L. virgata* population according to Dayan et al. (2015). The discs were placed in 2 mL-Eppendorf tubes containing 999 µl of monoammonium phosphate ($NH_4H_2PO_4$ 10 mM, pH 4.4). Volumes of 1 µl of glyphosate solutions at different concentrations were added (0, 0.1, 1, 10, 50, 100, 200, 400, 600 and 1000 µM). The samples were incubated for 24 h in the growth chamber under growing conditions described above. Next, they were incubated at 60 ºC for 30 min. Volumes of 250 µl of HCl 1.25 N were added and incubated at 60 ºC for 15 min. Aliquots of 250 µl were transferred to new tubes adding 500 µl of periodic acid (0.25 % w/v) and sodium metaperiodate (0.25% w/v) in a 1:1 ratio. The samples were incubated at room temperature (25ºC) during 90 minutes, and next, 500 µl of a mix of sodium hydroxide (NaOH 0.6 N) plus sodium sulfite (Na_2SO_3 0.22 N) in a 1:1 ratio was added and mixed.

The experiment was arranged in a completely randomized design with three replications per population for each glyphosate concentration, and the study was repeated twice. A standard curve was done using known concentrations of shikimate. The absorbance of samples was measured at 382 nm in a spectrophotometer (DU-640, Beckman Coulter Inc. Fullerton, USA) following the methodology described by Cromartie and Polge (2002). The absorbance values were converted into mg of shikimic acid per g of fresh weight.

^{14}C-Glyphosate absorption and translocation

Plants with 3-4 true leaves from the four *L. virgata* populations were treated with a solution of ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq mmol^{-1}, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) + commercial glyphosate. The solution applied contained a specific activity of 0.834 kBq µl^{-1} and a glyphosate concentration of 1.8 g ea L^{-1} (360 g ea ha^{-1} in 200 L). One drop of 1 µl plant^{-1} of solution was applied with a micropipette (Lab Mate HTL, Matosinhos, Portugal) on the adaxial surface of the first-second leaf. After treatment, the plants were maintained in the growth chamber at the growing conditions described above.

At 24, 48, 72 and 96 HAT, the treated leaves were washed three times separately with one mL of water-acetone (1:1 v/v) to recover the non-absorbed ^{14}C-glyphosate. The washing solution was mixed with two mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard), and analyzed by liquid scintillation spectrometry (LSS) in a scintillation counter (LS 6500, Beckman Coulter Inc. Fullerton, USA) during 10 min per sample.

The whole plants were carefully removed from the pot and washed, mainly the roots. The plants were individually divided into treated leaf, remainder of the plant and root. The samples were stored in flexible combustion cones (Perkin-Elmer, BV BioScience Packard), dried in an oven at 60ºC for 4 days. Next, the samples were combusted in a biological oxidizer (Packard Tri Carb 307, Packard Instrument Co., IL, USA). The CO_2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of each individual sample was quantified by LSS during 10 min per sample. The experiment was arranged in a completely random design with five replicates per population at each time evaluated.

The radioactive values were used to calculate recovery percentage as: [(kBq in treated leaf + kBq in plant + kBq in roots + kBq from washes) / kBq total applied] x 100. The average total recovery of ^{14}C-glyphosate applied was > 96 % to the S, R8 and R14 populations, and < 93% from the R15 population.

Visualization of ^{14}C-glyphosate translocation

Three whole plants per population at each visualization time (24, 48, 72 and 96 HAT) were treated under the same conditions as in the previous assay. The plants were washed individually, fixed on filter paper (25 x 12.5 cm) and dried at room temperature for one week. The samples were placed for 4 h beside a phosphor storage film (Storage Phosphor System: Cyclone, Perkin–Elmer Packard BioScience BV). A phosphor imager (Cyclon, Perkin-Elmer, Packard BioScience BV) was used to reveal the translocation.

Enzyme activity of the EPSPS

Plants of the four *L. virgata* populations were grown in pots (25 cm in diameter × 15 cm high: 4 plants per pot) under greenhouse conditions, in temperatures ranging from 17 to 31ºC, and a photoperiod of 16 h. The natural light was complemented by 900 $\mu mol^{-2}\ s^{-1}$ photosynthetic photon flux density delivered by incandescent and fluorescent lights. Samples of 5 g of foliar tissue from each population were obtained from the second and third youngest totally expanded leaves.

The methodology described by Sammons et al. (2007) was used for EPSPS extraction. The total content of proteins in the extract was measured according to the method of Bradford (1976) using a Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain).

The specific EPSPS activity in plants from *L. virgata* populations was studied in the presence and absence (basal activity) of glyphosate. The EPSPS activity was determined using a EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA). The glyphosate concentrations used were: 0, 1, 10, 100, 1000, 10000 µM. Three replicates at each glyphosate concentration were analyzed. The release of phosphate on the bottom level was measured during 10 minutes at 360 nm in a spectrophotometer (DU-640, Beckman Coulter Inc. Fullerton, USA).

Amplification and sequencing of the EPSPS gene

Samples of young leaf tissue (≈ 100 mg) from 5 glyphosate-susceptible (S) and 15 -resistant (R8, R14 and R15) *L. virgata* individuals were taken, and

stored at -80 ºC for RNA extraction. Total mRNA was isolated following the methodology described by Pistón (2013). Integrity of RNA was verified in 0.8% agarose gel and quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). First strand complementary DNA (cDNA) synthesis was carried out using one µg of RNA in all the samples. An iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA) was employed following the manufacturer´s instructions.

Lv-F3 and Lv-R2 primers (**Table 8**) were designed using the software Primers3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), based on conserved regions of the EPSPS gene sequences from *Eleusine indica* (GenBank Accession: AY157642.1, HQ403647.1) and *Lolium multiflorum* (GenBank Accession: DQ153168.2). Individual PCR reactions were carried out using cDNA from each sample of the S and R15 populations.

Each PCR reaction was performed using 50 ng of cDNA, 0.2 µM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) in a final volume of 25 µl. The PCR conditions were: 1 cycle of 94 ºC for 5 min; followed by35 cycles at 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 1 min; and a final extension at 72ºC for 10 min. PCR products (10 µl) were checked by 1% agarose gel to corroborate amplification.

The PCR products were ligated using the pGEM-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) following the manufacturer`s instructions, and cloned into competent cells of *E. coli* DH5α. Positive transformants were selected and fragment insertion confirmed through PCR using the M13F and M13R primers (**Table 8**). A total volume of 15 µl per sample containing 0.2 µM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of non-proofreading (*Thermus thermophilus*) polymerase (BIOTOOLS, Madrid, Spain). The PCR conditions were: 1 cycle of 94 ºC for 5

min; followed by 28 cycles of 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 1 min; and a final extension at 72ºC for 7 min.

The plasmids were purified with the ilustra plasmidPrep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), following the manufacturer`s instructions. Sanger sequencing was carried out by the STABVIDA sequencing service (Caparica, Portugal). A total of 30 clones from each population were sequenced. The assembly of the sequences was carried out by SeqMan Pro (Version 11, DNASTAR; Wisconsin, USA) and Geneious (Version 8.1.8, Biomatters Ltd, Auckland, New Zealand) software´s.

The *L. virgata* EPSPS cDNA sequences information can be found in GenBank with accession numbers KX425854 and KX425855.

Table 8. Names and sequences of primers used in EPSPS gene sequencing and expression analysis in *L. virgata* populations.

Name	Sequence (5´ to 3´)
EPSPS gene sequencing and cloning	
Lv-F3[a]	AAGAGCTGTWGTYGTTGGCTG
Lv-R2[a]	AATAGCACCTCGCACTTGAG
M13F	CGCCAGGGTTTTCCCAGTCACGAC
M13R	TCACACAGGAAACAGCTATGAC
qPCR	
qLv-For1[a]	GGCAGGTTCCCGATTGARAA
qLv-Rev1[a]	YGCATTTCCACCAGCAGCTA
ADP-RF(a) For	TCTCATGGTTGGTCTCGATG
ADP-RF(a) Rev	GGATGGTGGTGACGATCTCT
β-Act F1[a]	ATGGTAGGGATGGGACAGAA
β-Act R1[a]	TCCATGTCATCCCAGTTGCT

[a] The primers were designed with the software Primers3Plus online.

EPSPS gene expression

Young leaf samples (≈ 100 mg) from six untreated plants of both S and R15 populations were taken before treatment. Plants were then treated with 360 g ae ha^{-1} of glyphosate in the conditions used in the dose-response assays, and

new samples were collected at 24 HAT. In both cases, the samples were stored at -80 ºC for RNA extraction and cDNA synthesis in the same conditions described in the previous section.

The qLvFor1 and qLv-Rev1 primers (**Table 8**) to amplify a fragment of 114 bp were designed from the EPSPS gene sequences obtained in the previous section. The β-Actin and ADP-ribosylation factor genes were used as reference genes. Pairs of β-Actin primers (**Table 8**) were designed on the basis of the *Agrostis stolonifera* (JX644005.1), *Avena sativa* (KP257585.1), *Lolium multiflorum* (AJ585201.1), *Triticum monococcum* (AF326781.1) and *Zea mays* (U60510.1) sequences from GenBank. β-Actin and qLv primers were designed using Primers3Plus. The ADP-RF(a) primers designed by Giménez et al. (2011) were used to amplify the ADP-ribosylation factor gene (**Table 8**).

For each quantitative RT-PCR reaction 40 ng of cDNA, 10 µl PerfeCTa SYBR Green FastMix ROX (Quanta Bioscience), and 0.2 µM of both forward and reverse primers in a 15 µl final reaction volume were used. The PCR conditions were: initial cycle at 94 ºC for 5min; 40 cycles of 94 ºC 30 s and 62 ºC 1 min. The PCR reactions were carried out using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Three to four technical replications per plant were carried out in a factorial design of two glyphosate treatments and two populations.

The PCR efficiency for each pair of primer and sample was determined by the program qPCR data analysis LinRegPCR (version 11) according to Ruijuter et al. (2009) using raw fluorescence as input data. Expression level of both the reference and target genes for each sample was determined with the follow equation:

$$N_0 = 0.2 / E^{Cq}$$

Where N_0 is expression of the gene, E is the PCR efficiency for each primer, Cq is the number of cycles needed to reach 0.2 arbitrary units of fluorescence. The mean PCR efficiency for each gene, population and treatment was determined according to Giménez et al. (2011).

The stability of the expression of the reference genes (β-Actin and ADP-ribosylation factor) and Normalization Factor (NF) were determined using geNorm software for each sample according to Vandesompele et al. (2002).

Statistical analysis

The percentages data of dry weight reduction, survival and EPSPS enzyme activity were submitted to a non-linear regression analysis. The dose of glyphosate needed to reduce the weight of a population (ED_{50}), mortality (LD_{50}), and to inhibit EPSPS activity (I_{50}) by 50% were calculated. A log-logistic model of four parameters was conducted using the *drc* statistical package (Ritz et al. 2015) in the program R version 3.2.5. The statistical model is:

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

Where *Y* is the percentage of dry weight, survival and/or EPSPS-inhibiting with respect to the control, *c* and *d* are coefficients corresponding to the upper and lower asymptotic limits, *b* is the Hill slope, *g* is the glyphosate dose (ED_{50}, LD_{50} or I_{50}) at the mean point of inflexion between the upper and lower asymptote and *x* (independent variable) corresponds to the glyphosate dose. The data were plotted using SigmaPlot (Version 11.0, Systat Software, Inc., USA) software.

EPSPS normalized expression level was calculated for each qPCR reaction, and the average and standard error from technical replicates was recorded for each plant and population.

Absorption, translocation and EPSPS expression results were subjected to ANOVA using Statistix version 9.0 from Analytical Software (Tallahassee, FL, USA). When necessary, the means were compared using Tukey’s test´s at the 95% probability level.

Results

Glyphosate dose-response

Glyphosate resistance was confirmed in three *L. virgata* populations (R8, R14 and R15) collected in Persian lime groves from Veracruz. These glyphosate resistant populations were less sensitive to glyphosate than the S population (**Figure 5**). R8 and R14 populations had similar response to glyphosate (**Figure 5A**). The ED_{50} values of the R8, R14 and R15 populations were 447.6, 402.0 and 713.8 g ae of glyphosate ha^{-1}, respectively (**Table 9**). The resistant index (RI) of the glyphosate resistant populations ranged between 2.9 to 5.2 with respect to S population.

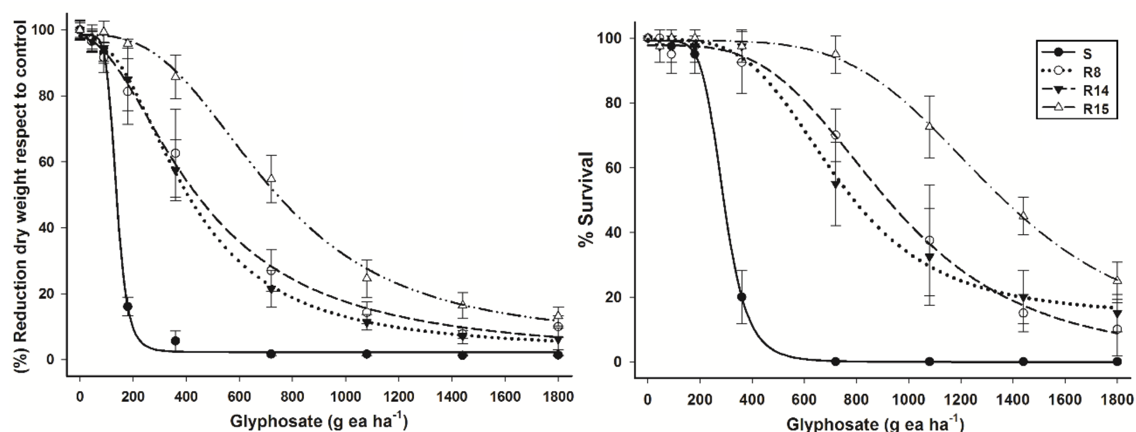

Figure 5. Log–logistic curves of glyphosate-susceptible and -resistant *L. virgata* populations evaluated at 21 DAT. A) Dose-response curve with respect to percentage of dry mass reduction. B) Dose-response curve with respect to percentage of survival. Vertical bars represent the standard error of the mean (n = 10).

Based on 50% mortality (LD_{50}), the R8, R14 and R15 populations were, respectively, 2.5, 2.3 and 4.6 times more resistant than the S population (**Figure 5B, Table 9**). A field dose of glyphosate (720 g ae ha^{-1}), used in Persian lime groves of Veracruz, was enough to achieve full control in the S population. However, with this dose of glyphosate, only 50% mortality was observed in R8 and R14 populations, and to the R15 for population, a dose 1.8 times more glyphosate than the field dose was required to obtain the same level of control. The ED_{50} and LD_{50} parameters showed the different resistance levels to glyphosate acquired by resistant *L. virgata* populations (**Table 9**).

Table 9. Parameters of the sigmoidal equation used to estimate values of Dose-Response curves of the glyphosate-susceptible and resistant *L. virgata* populations.

Population	c	d	b	R^2aj	g ae ha^{-1} (CI95%)[a]	RI[b]
Parameters of ED_{50} value						
S	2.28	98.34	6.62	0.99	137.7 (129.2, 146.1)	
R8	0.70	97.69	1.94	0.97	447.6 (403.5, 491.8)	**3.3**
R14	2.80	98.17	2.33	0.98	402.0 (371.2, 433.0)	**2.9**
R15	5.67	98.49	2.90	0.98	719.8 (668.2, 771.4)	**5.2**
Parameters of LD_{50} value						
S	-0.07	99.19	6.47	0.99	291.3 (263.7, 318.7)	
R8	0.10	97.80	3.52	0.95	932.3 (777.0, 1087.6)	**3.2**
R14	13.94	99.51	3.79	0.95	724.4 (624.9, 823.0)	**2.5**
R15	6.97	99.18	4.57	0.96	1330.3 (1084.2, 1576.4)	**4.6**

c = lower limit, d = upper limit, b = Hill´s slope, R^2aj = 1 − (sums of squares of the regression / corrected total sums of squares). ED_{50} = effective dose required for 50% reduction in plant biomass. LD_{50} = effective dose needed to kill 50% plants of a population. [a] CI values are the 95% confidence intervals (n=10). [b] RI = Resistance index (R/S) calculated using the corresponding ED_{50} or LD_{50} value of the resistant populations respect to the susceptible one.

Shikimic acid accumulation

The amounts of shikimic acid accumulated after glyphosate treatment is highly variable between species and populations. In this work, the shikimic accumulation was different in each *L. virgata* population. The S population presented the highest level of shikimic acid accumulation (**Figure 6**). This accumulation was exhibited at lower glyphosate concentrations (between 0.1 to 100 µM) reaching an average of 12.35 mg shikimic g^{-1} fresh weight from 200 to 1000 µM of glyphosate. The resistant populations only presented an appreciable accumulation from 100 µM of glyphosate. The averages of shikimic acid accumulated were 8.4, 7.9 and 2.6 mg shikimic g^{-1} fresh weight for the R8, R14 and R15 populations, respectively, at the glyphosate concentration of 1000 µM.

The response patterns to glyphosate in shikimate accumulation assays were similar to that observed in the dose-response study (**Figure 5**). The lowest

shikimate accumulation observed in the R15 population compared to the R8 and R14 populations at the different glyphosate concentrations was consistent with the lower growth reduction and mortality observed in the plants (**Figures 5** and **6**).

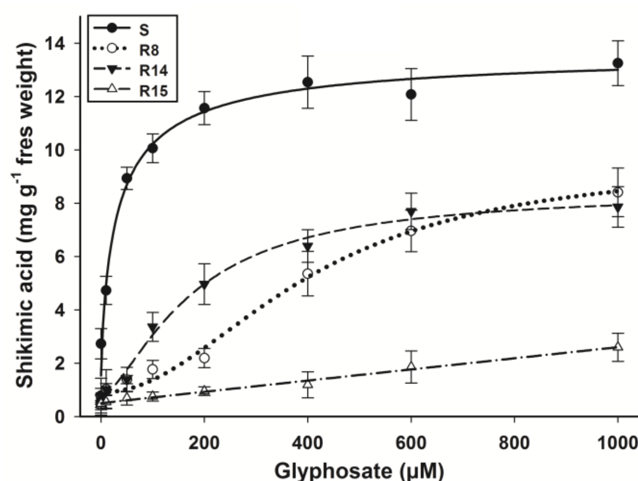

Figure 6. Shikimic acid accumulation of glyphosate-susceptible and -resistant *L. virgata* populations at different glyphosate concentrations. Vertical bars represent the standard error of the mean (n = 3).

^{14}C-Glyphosate absorption and translocation

The four *L. virgata* populations presented a high absorption index of ^{14}C-glyphosate, absorbing amounts of over 50% from recovered herbicide at 96 HAT (**Figure 7**). However, the resistant *L. virgata* populations showed a clear reduced ^{14}C-glyphosate absorption, mainly comparing the S population to the R15 population. At 24 HAT, the S population presented a high absorption rate of ^{14}C-glyphosate (42.2%) of at least 15% greater than the resistant populations (**Figure 7**). At the same evaluation time, the ^{14}C-glyphosate absorption ranged from 20.7 to 27.7% among the resistant populations. Between 48 to 96 HAT, glyphosate absorption was similar (**Figure 7**), ranging from 63.8 to 67.7% for the S population, whereas in resistant populations the herbicide absorption ranged between 47.7 and 60.3%.

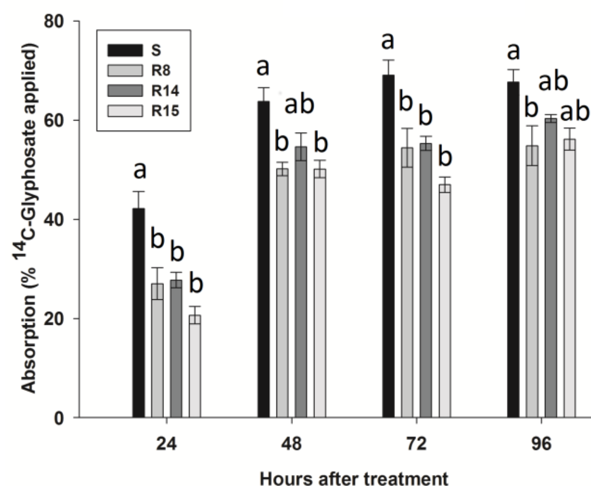

Figure 7. ^{14}C-glyphosate absorption in glyphosate-susceptible and -resistant plants of the *L. virgata* populations. Different letter at each evaluation time is statistically different at 95% probability determined by the Tukey´s test. Vertical bars represent the standard error of the mean (n = 5).

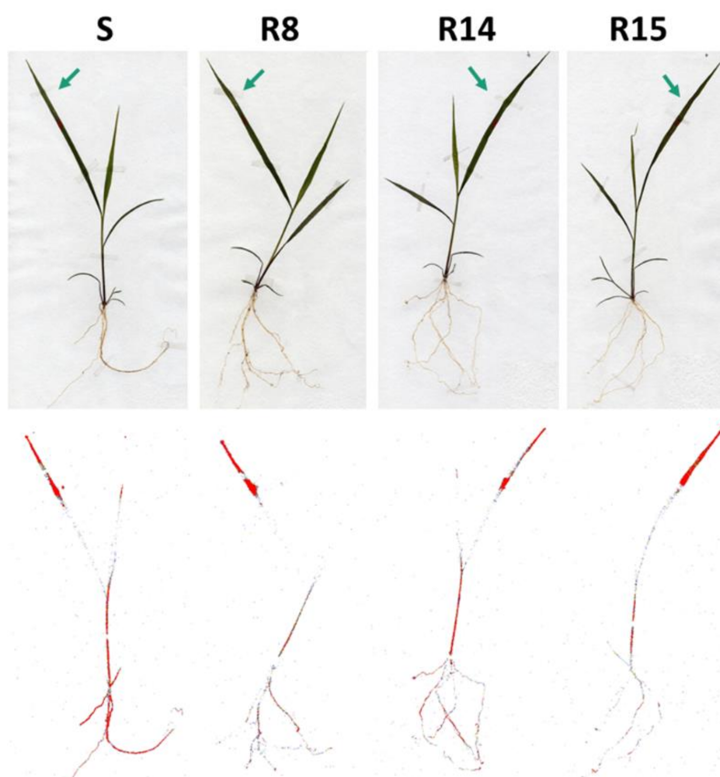

Figure 8. Digital images (top row) and autoradiograph images (bottom row) of ^{14}C-glyphosate translocation in glyphosate-susceptible and -resistant plants of *L. virgata* populations. The autoradiograph images were obtained from treated plants at 96 HAT. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

Table 10. Translocation percentage of ^{14}C-glyphosate in plants of glyphosate-susceptible and -resistant *L. virgata* populations.

Population	HAT	Traslocation (% from absorbed)[a]		
		Treated leaf	Rest of plant	Root
S	24	75.1±2.0 ab	16.0±0.8 h	8.9±2.6 j
	48	55.7±2.6 c	24.2±2.0 ef	20.2±1.3 defg
	72	44.7±1.0 de	30.3±1.4 bcd	25.0±1.1 bc
	96	33.9±1.9 g	33.9±1.0 ab	32.2±1.2 a
R8	24	74.5±2.1 ab	16.8±1.5 gh	8.7±1.2 j
	48	55.1±1.9 c	27.9±1.8 cde	17.0±2.0 fgh
	72	45.3±2.4 de	33.8±2.6 ab	20.9±1.5 cdef
	96	38.2±1.1 fg	37.3±3.8 a	24.5±2.6 bcd
R14	24	70.3±0.9 b	18.8±1.1 fg	10.9±0.4 ij
	48	56.4±1.9 c	27.5±1.0 cde	16.0±2.0 gh
	72	43.5±2.3 def	36.2±1.2 a	20.3±2.1 cdefg
	96	39.8±2.8 ef	33.7±3.5 ab	26.5±1.0 b
R15	24	78.0±2.2 a	12.3±1.1 gh	9.6±1.3 j
	48	58.7±1.7 c	26.4±1.7 de	14.9±0.8 hi
	72	48.7±2.2 d	32.3±1.0 abc	19.0±1.6 efgh
	96	43.2±1.5 def	34.9±1.5 ab	21.9±2.2 cdef

[a] Means with different letter within a column are statistically different at 95% probability determined by the Tukey´s test. ± Standard error of the mean (n = 5).

L. virgata populations showed similar ^{14}C-glyphosate translocation patterns (**Table 10**). The amount of herbicide quantified in the treated leaf ranged between 70.3 to 78.0% at 24 HAT, and from 33.9 to 43.2% at 96 HAT. In the aerial part of the plant (rest of plant), the amounts of ^{14}C-glyphosate translocated ranged from 12.3 to 18.8%, and from 33.7 to 37.3% at 24 and 96 HAT, respectively. However, the greatest differences of ^{14}C-glyphosate translocation were found in the roots. The R15 population had the lowest rate of translocation to the root (21.9% of the absorbed herbicide) at 96 HAT (**Table 10**), translocating ±10% less herbicide than the S population. The R8 and R14 populations translocated an average of 24.5 and 26.5%, respectively, of herbicide translocated to the roots at 96 HAT. At this time, the S population had

already reached a balanced distribution of herbicide between treated leaf, rest of plant and roots (**Table 10**).

The Phosphor Imager images confirmed the previous results of ^{14}C-glyphosate translocation (**Figure 8**). At 96 HAT, the plants of resistant populations, mainly the R15 population ones, translocated smaller amounts of ^{14}C-glyphosate from treated leaf to the root than the S population plants (**Figure 8**).

Enzyme activity

In the absence of glyphosate, significant differences (P = 0.0035; DF = 3; n = 12) in the basal EPSPS activity between plants of *L. virgata* populations were found. R15 population presented an average of 0.41 μmol μg total soluble protein $(TSP)^{-1}$ min^{-1}, while the S, R8 and R14 populations an average of 0.29 μmol μg TPS^{-1} min^{-1}. The EPSPS enzyme activity was inhibited by glyphosate in plants from susceptible and resistant populations while the concentrations increased (**Figure 9**). To inhibit EPSPS activity by 50% (I_{50}) for the S population was required 0.32 μM of glyphosate (**Table 11**). Plants from the R15 population showed an RI of 165.9 higher with respect to the S population plants (**Figure 9**). According to the confidence intervals (CI95%), S, R8 and R14 population plants showed no significant differences in their EPSPS enzyme activity (**Table 11**).

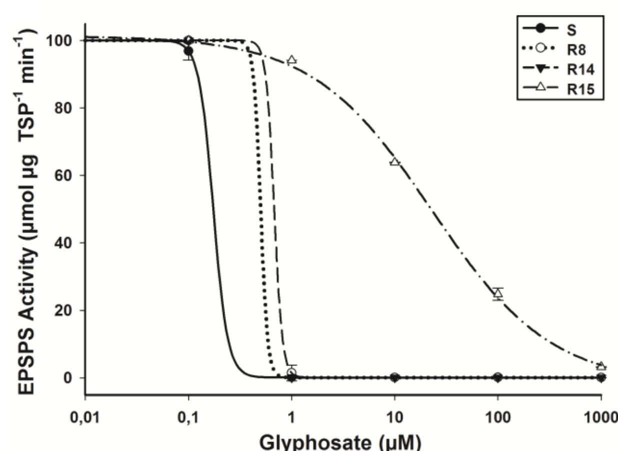

Figure 9. EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible and resistant *L. virgata* populations. Vertical bars represent the standard error of the mean (n = 3).

Table 11. Parameters of the sigmoidal equation used to estimate the amount (µM) of glyphosate needed to reduce the EPSPS enzyme activity by 50% (I_{50}) in plants of the glyphosate-susceptible and resistant *L. virgata* populations.

Population	C	D	b	R^2aj	I_{50} (µM) (CI95%)[a]	RI[b]
S	0.12	100.0	2.9	0.99	0.32 (0.04, 0.59)	
R8	0.10	100.0	11.0	0.99	0.67 (0.12, 1.22)	2.1
R14	0.11	100.0	13.0	0.99	0.50 (0.16, 0.84)	1.6
R15	1.64	100.2	1.2	0.99	53.09 (30.39, 75.78)	165.9

c = lower limit, d = upper limit, b = Hill´s slope, R^2aj = 1 − (sums of squares of the regression / corrected total sums of squares). [a] CI values are the 95% confidence intervals (n=3). [b] RI = Resistance index ($ID_{50}R/ID_{50}S$).

EPSPS sequencing and gene expression

A fragment of 559 bp in length were amplified included the Thr-102 and Pro-106 positions in the protein sequence. These positions correspond to point mutations associated with conferring glyphosate resistance in weeds (Sammons and Gaines, 2014; Chen et al. 2015; Yu et al. 2015). The predicted amino acid sequence of S population presented as the same consensus of *Lolium rigidum* (GenBank: AF349754) (**Figure 10**), and others grass weed species susceptible to glyphosate. In the thr-102 position no mutation was found. Only the R15 population showed a codon change from CCA to TCA resulting in an amino acid substitution from Proline to Serine at 106 position (**Figure 10**).

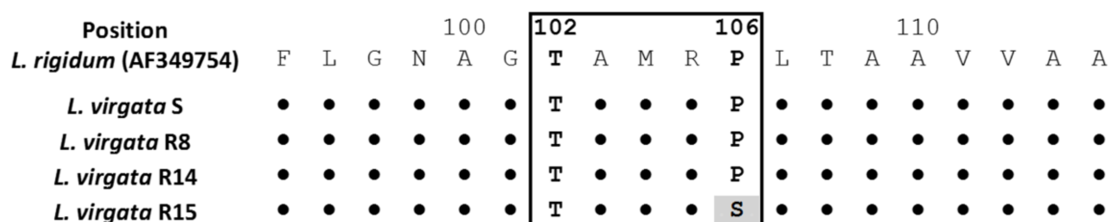

Figure 10. Partial alignment of protein sequences of EPSPS gene in glyphosate-susceptible and -resistant *L. virgata* populations. The highlighted color indicates a change at 106 position from CCA (Proline) to TCA (Serine) in the consensus nucleotide sequence. Box includes from the 102 to 106 positions (amino acid number based on the start codon of *L. rigidum* [GenBank: AF349754] EPSPS sequence), corresponding to point mutations associated for conferring glyphosate resistance.

No significant differences ($P = 0.6924$; DF = 3; n = 12) were found in the EPSPS expression in untreated (0 HAT) plants used as a control between populations (**Table 12**). The S and R15 populations showed an increased in the EPSPS expression level after glyphosate application, but it was similar in both populations, with an average of 3.84 times higher at 24 HAT with respect to expression level at 0 HAT (**Table 12**).

Table 12. EPSPS expression level in treated and untreated plants of the glyphosate-susceptible and resistant *L. virgata* populations.

Population	Expression level[a] (EPSPS:NF[b])	Expression Index[c]	EPSPS expression level (0 HAT:24 HAT)[d]
S	4.67 ± 0.50		4.02 ± 0.61
R15	4.50 ± 0.53	0.96	3.67 ± 0.44
Mean	**4.58**		**3.84**

[a]Values (x 10^3) obtained from untreated (0 HAT) plants. [b]NF = Normalization Factor. [c]Expression index = Expression level R/Expression level S. [c]The coefficients of expression were estimated as: Expression level at 24 HAT/Expression level at 0 HAT of each population. ± Standard error of the mean (n = 6).

Discussion

The IR of resistant *L. virgata* populations ranged from 2.9 to 5.2 times more than the S population. Previously, *L. virgata* was effectively controlled with glyphosate in citrus orchards of Veracruz with 720 g ae ha^{-1} (field dose), until the identification of the first resistant populations (Pérez-López et al. 2014). The LD_{50} values estimated in the resistant populations studied in this work were higher than those at the field glyphosate dose (**Table 9, Figure 5**). Other glyphosate-resistant grass weeds such as: *Bromus diandrus, Digitaria insularis, Echinochloa colona, Eleusine indica, Lolium perenne* spp. *multiflorum, L. rigidum, Poa annua,* among others (de Carvalho et al. 2011; González-Torralva et al. 2012; Alarcón-Reverte et al. 2015; Chen et al. 20015; Cross et al. 2015; Salas et al. 2015; Yu et al. 2015; Malone et al. 2016), exhibited RI values that ranged between 3 to 19, and between 4 to < 182 based on in their ED_{50} or LD_{50}, respectively. Differences in the level of glyphosate resistance between these species were due to various resistance mechanisms.

Both shikimic acid accumulation and dose-response results indicated that the R8 and R14 populations of *L. virgata* possessed similar levels of glyphosate resistance, to despite coming from different geographical places, and the R15 population was the most resistant one (**Table 9, Figure 6**).

Different glyphosate concentrations in the tissue are related to differences in glyphosate efficacy (Alarcón-Reverte et al. 2015). The resistant *L. virgata* populations showed low glyphosate translocation rates, mainly to the roots (**Table 10, Figure 7**), the lowest one being exhibited by the R15 population. Reduced translocation has been reported as being a mechanism responsible for endow glyphosate resistance in different grass weed species such as *D. insularis, L. multiflorum, L. perenne*, *L. rigidum, Sorghum halepense* (Bostman et al. 2012; de Carvalho et al. 2012; González-Torralva et al. 2012; Vila-Aiub et al. 2012; Adu-Yeboah et al. 2014; Granizadeh et al. 2015), among others. In some cases, it was reported as a major resistance mechanism (Adu-Yeboah et al. 2014). On other hand, reduced absorption is a mechanism not usually involved in the glyphosate resistance. It has been described in a few species such as *D. insularis*, *L. multiflorum* and *S. halepense* (Michitte et al. 2007; de Carvalho et al. 2012; Vila-Aiub et al. 2012). However, here played an important role in the resistant *L. virgata* populations, mainly in the first 24 HAT, due to a clear reduction being shown compared to respect to the S population, producing a significant reduction in the amount of glyphosate reaching the target-site. This indicates that *L. virgata* developed absorption and reduced translocation as resistance mechanisms against to glyphosate first, independently of their geographic or genetic relationship.

The different shikimic acid accumulation, reduced absorption and translocation patterns presented by the R15 population, suggest that their glyphosate resistance mechanism may be differ to that of the R8 and R14 populations. In addition, reduced glyphosate absorption into plants and higher EPSPS activity, have been associated with a decreased sensitivity to glyphosate (Kaundun et al. 2008; Salas et al. 2012).

EPSPS enzyme activity tests, in addition to any other appropriate parameter to determine glyphosate resistance (Dayan et al. 2015), also allows

us to suspect the possible mechanisms that may be involved in the target-site. The R8 and R14 populations of *L. virgata*, showed no significant differences in their inhibition of the EPSPS enzyme by glyphosate with respect to the S population (**Table 11**). This suggests that their resistance mechanism did not involve the target-site. Moreover, these populations showed no mutation in the EPSPS gene (**Figure 9**). Similar results were reported in resistant populations of *E. indica* (population R3) and *E. colona* (population RLB2) (Chen et al. 2015; Alarcón-Reverte et al. 2015), in which no important differences in their inhibition of the EPSPS activity with respect to their sensitive populations were reported.

On the other hand, a greater basal activity is often associated with a larger number of copies or overexpression of the EPSPS gene (Salas et al. 2012). Due to only the R15 population showed a greater basal activity, the EPSPS expression was studied in this resistant population in comparison to the S population. However, our qPCR analysis (**Table 12**) indicated that the greater EPSPS basal activity of the R15 population, cannot be ascribed to an overexpression of the EPSPS gene. Alarcón-Reverte et al. (2015) suggested that it could have been an enhanced basal EPSPS activity as an additional target-site resistance mechanism. The number of copies were not studied because, according to Salas et al. (2015), the manifestation of a larger EPSPS gene copies does not always result in differences in the protein product. Glyphosate-resistant populations of *A. palmeri, A. tuberculatus. B. diandrus, E. colona, E. indica, K. skoparia, L. perenne* spp. *multiflorum* (Salas et al. 2012, Ribeiro et al. 2014, Alarcón-Reverte et al. 2015; Chatham et al. 2015; Chen et al. 2015; Wiersma et al. 2015; Malone et al. 2016), are examples of species which presented differences in the EPSPS copy numbers and/or overexpression of the EPSPS gene as glyphosate resistance mechanisms, in some cases as major ones.

Therefore, the scant inhibition of EPSPS enzyme activity by glyphosate in the R15 population, suggests that the mutation found in the 106 position of EPSPS gene (**Figure 11**), provides the resistance to glyphosate. This indicates that the R15 population has three mechanisms of resistance to glyphosate: reduced absorption, reduced translocation and target-site mutation.

Amino acid substitution at 106 position in the EPSPS gene from Proline to Serine, Alanine, Threonine and/or Leucine have been widely reported to partially confer resistance to glyphosate, and often are accompanied by another mechanism (Sammons and Gaines, 2014). Some species which presented a mutation in combination with other resistance mechanisms (either reduced absorption, reduced translocation, multiple EPSPS gene copy number, overexpression of the EPSPS gene, and even metabolism) are: *A. palmeri. A. tuberculatus, B. diandrus, D. insularis, E. colona, E. indica, L. perenne* sp. *multiflorum, L. rigidum, K. scoparia* (Bostman et al. 2012; de Carvalho et al. 2012; González-Torralva et al. 2012; Alarcón-Reverte et al. 2015; Chatham et al. 2015; Chen et al. 2015; Salas et al. 2015; Wiersema et al. 2015; Malone et al. 2016).

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CAPITULO IV

Target and Non-Target Site Mechanisms Involved in the First Case of Glyphosate-Resistant Hairy beggarticks (*Bidens pilosa*)

Abstract

In 2014 hairy beggarticks (*Bidens pilosa* L.) has been identified as being glyphosate-resistant in citrus orchards from Mexico. The mechanisms involved in the resistance of two populations (R1 and R2) and one susceptible (S) of this species were studied. Experiments of dose-response, shikimic acid accumulation, uptake-translocation, enzyme activity and EPSPS gene sequencing were carried out in each population. The R1 and R2 populations were 20.4 and 2.8-fold less glyphosate sensitive, respectively, than the S population. The resistant populations showed a lesser shikimic acid accumulation and enzyme activity than the S population. In the latter one, 24.9% of ^{14}C-glyphosate was translocated to the roots at 96 h after treatment (HAT); in the R1 and R2 populations only 12.9 and 15.5%, respectively, was translocated. Qualitative results confirmed the reduced ^{14}C-glyphosate translocation in the resistant populations. A single (Pro-106-Ser), and a double (Thr-102-Ile followed by Pro-106-Ser) mutations were identified in the EPSPS2 gene conferred high resistance in R1 population. Target-site mutations associated with a reduced translocation were responsible for the higher glyphosate resistance in the R1 population. The low-intermediate resistance of the R2 population was mediated by reduced translocation. This is the first glyphosate resistance case confirmed in hairy beggarticks in the world.

Keywords: 5-enolpyruvyl shikimate-3-phosphate synthase, *Bidens pilosa,* EPSPS2, glyphosate resistance mechanisms, Pro-106 substitution, reduced glyphosate translocation, TIPS mutation

Resumen

La aceitilla o romerillo (*Bidens pilosa* L.), ha sido identificada como resistente a glifosato en huertos de cítricos de México. Los mecanismos de resistencia involucrados fueron estudiados en dos poblaciones (R1 y R2) y una población susceptible (S). Se realizaron experimentos de dosis-respuesta, acumulación de ácido shiquímico, absorción-trnaslocación, actividad enzimática y secuenciación del gen de la EPSPS en cada población. Las poblaciones R1 y R2 fueron 20.4 y 2.8 veces, respectivamente menos sensibles a glifosato que la población S. Las poblaciones resistentes mostraron una menor acumulación de ácido shiquímico y actividad enzimática que la población S. Esta última población translocó 24.9% de ^{14}C-glifosato a las raíces 96 h después del tratamiento (HDT). Las poblaciones R1 y R2 solamente translocaron 12.9 y 15.5%, respectivamente. Los resultados cualitativos confirmaron la reducida absorción de ^{14}C-glifosato que presentaron las poblaciones resistentes. Se identificaron mutaciones únicas (Pro-106-Ser) y dobles (Thr-102-Ile seguida por Pro-106-Ser) en el gen de la EPSPS confiriendo alta resistencia en la población R1. Mutaciones en el target-site asociadas con una reducida translocación de ^{14}C-glifosato fueron las responsables de la mayor resistencia a glifosato que mostro la población R1. La resistencia intermedia de la población R2 es debido solo a la reducida absorción de ^{14}C-glifosato. Este es el primer caso confirmado de resistencia a glifosato de la aceitilla o romerillo en el mundo.

Palabras clave: 5-enolpiruvil siquimato-3-fosfato sintasa, *Bidens pilosa,* EPSPS2, mecanismos de resistencia a glifosato, mutación TIPS, reducida translocación de glifosato, sustitución Pro-106.

Introduction

Mexico is the top producer and exporter of limes and lemons worldwide (USDA, 2016). Persian lime (*Citrus latifolia* Tan.) is the most economically important crop (SIAP, 2016), because of its large volume of exports. Weeds are the main limiting factor in lime production, and the use of herbicides has been adopted as the main tool for weed control in this crop, mainly glyphosate, which is applied up to 4 applications per year (Pérez-López et al. 2014). Glyphosate use has induced great changes in weed flora in Persian lime (*Citrus latifolia* Tan.) groves, where two cases of glyphosate resistance have been reported in Mexico: tropical sprangletop (*Leptoclhoa virgata* (L.) P. Beauv.), and hairy beggarticks (*Bidens pilosa* L.) (Pérez-López et al. 2014; Heap, 2016).

Hairy beggarticks is a native asteraceae from Mexico, widely spread over the country`s tropical and subtropical regions and in the world (Vibrans 1995). It is an annual weed that reproduces itself by seeds, affecting annual and perennial crops (Rzedowski and Rzedowski, 2008). In 1991 it was reported as being resistant to paraquat in coffee plantations from Kenya, and in 1993 to ALS-inhibiting herbicides in soybean crops in Brazil (Heap, 2016). Hairy beggarticks is susceptible to glyphosate, but field prospections made by this research group in citrus orchard areas of Mexico have allowed the identification of glyphosate-resistant populations of this weed (Heap, 2016).

Glyphosate is a systemic non-selective herbicide that has been used globally for over 40 years in weed management (Duke and Powles, 2008; Székács and Darvas, 2012). When it is properly used, i.e. following label recommendations, it does not have any adverse effects on wildlife (Giesey et al. 2010). Glyphosate acts rapidly in reducing photosynthesis activity (Duke et al. 2003), and it is translocated with photosynthates from the leaves to the meristematic tissue to reach the target-site, achieving maximum uptake at 96 hours after treatment (Cruz-Hipolito et al. 2011; González-Torralva et al. 2012b). Glyphosate is a phosphonomethyl derivative of the amino acid glycine (Székács and Darvas, 2012), and kills plants by preventing the synthesis of three essential amino acids (phenylalanine, tyrosine and tryptophan) (Franz et al. 1997), inhibiting the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS;

EC 2.5.1.19) (Duke and Powles, 2008; Székács and Darvas, 2012). Thereby, the biosynthesis of chorismate, an intermediate in the shikimate pathway, is blocked causing the accumulation of high levels of shikimate-3-phosphate (Amrhein et al. 1980; Franz et al. 1997), and deregulation of the carbon flow to other essential pathways (Orcaray et al. 2012).

Depending almost exclusively on the use of glyphosate for weed management has led to the evolution of resistant populations (Alcántara-de la Cruz et al. 2016). During 20 years there was no evidence of any glyphosate-resistant weed populations (Duke and Powles, 2008). The first case reported was *Lolium rigidum* in 1996 (Powles et al. 1998). Since then, 254 glyphosate resistance cases have been reported in 35 weed species (Heap, 2016), mainly, but not only, influenced by adoption of transgenic glyphosate-resistant crops (Duke and Powles, 2008).

Glyphosate resistance in weeds is due to different mechanisms (Salas et al. 2015), grouped and commonly known as Non-Target Site Resistance (NTSR) and Target Site Resistance (TSR) mechanisms (Sammons and Gaines, 2014). The NTSR mechanisms limit glyphosate reaching its site of action (EPSPS) (Alcántara-de la Cruz et al. 2016). This group includes: reduced uptake (Michitte et al. 2007; de Carvalho et al. 2012), altered translocation (Pérez-Jones et al. 2007), increased vacuolar sequestration (Ge et al. 2012), and metabolism to non-toxic compounds (de Carvalho et al. 2012; González-Torralva et al. 2012b), causing less glyphosate transport via phloem to the EPSPS. These mechanisms are influenced by enhanced physiological and biochemical characteristics (Alcántara-de la Cruz et al. 2016), and generally, each of these mechanisms confers moderate levels of glyphosate resistance (Yu et al. 2015).

The TSR mechanisms are those related to the EPSPS, either by a loss of affinity between the linking protein and glyphosate caused by mutations, or by the EPSPS overexpression (Sammons and Gaines, 2014). Different single mutations in the Pro-106 position (to Ala, Thr and Leu) of EPSPS gene have been identified as conferring low-intermediate glyphosate resistance in weeds (de Carvalho et al. 2012; González-Torralva et al. 2012a, 2014; Alarcón-

Reverte et al. 2015; Salas et al. 2015). Moreover, a double mutation was found in the Thr-102-Ile position followed by Pro-106-Ser conferring higher resistance in *Eleusine indica* (Chen et al. 2015; Yu et al. 2015). This double mutation is used in transgenic crops (Sammons and Gaines, 2014). Multiple EPSPS copy numbers and/or increased EPSPS expression are also involved in glyphosate resistance. These mechanisms have been described in mono and dicotyledonous weed species (Alarcón-Reverte et al. 2015; Chatham et al. 2015; Salas et al. 2015; Wiersma et al. 2015; Malone et al. 2016).

In this paper, the target and non-target site mechanisms involved in glyphosate resistance of two resistant populations (R1 and R2) of hairy beggarticks in comparison to one susceptible (S) (as control), were studied by physiological, biochemical and molecular methods.

Material and Methods

Biological material and experiment conditions

Seeds of resistant populations (R1 and R2) were harvested directly in two Persian lime groves of the San Manuel farm, Puebla, Mexico, (20º 06' 28" N, 97º 09' 34" W) from at least 20 plants that had been survived to the last glyphosate treatment at the recommended field rate (720 g acid equivalent (ae) ha^{-1}). Persian lemon groves had a history of 6 (R2) and 13 (R1) years of continuous use of glyphosate (3-4 application per year). Seeds of a susceptible population (S) never treated were collected near the Persian lime groves. Seeds collected from a grove were bulked and constitute a sample from a single population.

Seeds were seeded on trays (15 x 15 x 8 cm) with peat saturated at a field capacity. The trays were covered with plastic layer until germination and placed in a growth chamber under controlled conditions (day/night temperature of 26/18ºC, photoperiod of 16 h at 850 $\mu mol^{-2}s^{-1}$ of light intensity, and 60% relative humidity).

The seedlings were transplanted individually into 250 mL pots containing a mixture of sand/peat (1:1 v/v) + 0.4 gr of fertilizer (NPK 17-09-11 + 2% MgO).

The pots were placed in the growth chamber under the conditions described above and watered daily.

The glyphosate applications (Roundup Energy 45% w/v, Monsanto, Madrid, Spain) for the dose-response, foliar retention and shikimic acid assays were made with a Generation III Research Track Sprayer (DeVries Manufacturing Inc., Minnesota, USA) equipped with an 8002EVS nozzle (TeeJet, Spraying System Spain, S.L., Madrid, Spain) delivering 200 L ha^{-1}.

Dose-response assays

Plants from the S, R1 and R2 populations with four true leaves were treated with the following doses of glyphosate: 0, 31.25, 62.5, 125, 250, 500, 1000 and 2000 g ae ha^{-1}. At 21 days after treatment (DAT), the plants were cut off at ground level and wrapped in filter paper envelopes. Later, the plants were dried in a stove (JP Selecta S.A., Barcelona, Spain) at 60 ºC for one week and weighed to determine their dry weight. Data were expressed as a percentage of dry weight, compared to untreated control plants (Cruz-Hipolito et al. 2011). The experiment was arranged in a completely random design with 10 replications per dose. The assays were repeated twice.

Foliar retention assays

The methodology adapted by González-Torralva et al. (2010) was employed. Seven plants from each population with four true leaves, in a completely random design, were treated with a solution containing 360 g ae ha^{-1} of glyphosate (0.5 of field rate) + 100 mg L^{-1} Na-fluorescein. When the solution applied on the plant´s foliage dried (20-25 min after application), the treated plants were cut off at ground level and washed with 50 mL of NaOH 5 mM in a test tube shaking it vigorously for 30 s. The washing solution was recovered in glass flasks and the absorbance of fluorescein was immediately measured at $490_{exc}/510_{em}$ nm (Hitachi F-2500 spectrofluorimeter). The plants were wrapped in filter paper envelopes and dried in a stove at 60 ºC for one week, and weighed. The retention was expressed in µl of glyphosate solution g^{-1} dry matter.

Shikimic acid accumulation

An assay at different intervals of time was carried out. Plants with four true leaves from S, R1 and R2 populations, were treated with glyphosate at 360 g ae ha^{-1}. Samples of 50 mg of tissue corresponding to the first and second leaf of treated and untreated plants (the latter used as a control) were cut at 24, 48, 72 and 96 h after treatment (HAT). The samples were placed in an Eppendorf with 1 mL of HCl 1 M, immediately frozen in liquid nitrogen and stored at -40 º C up to their analysis. The shikimic acid accumulation was determined by the methodology described by Cromartie and Polge (2002). Sample absorbance was measured with a spectrophotometer (Beckman DU-640, Fullerton, CA, USA) at 380 nm. The shikimic acid accumulation was obtained from the difference between treated and untreated plants, its rate was measured at between 24 and 96 HAT and the results were expressed in mg of shikimic acid g^{-1} fresh tissue. Five treated and untreated plants from each population at each time evaluated were used in a completely random design.

In an *in vivo* bioassay leaf discs 4 mm in diameter were taken until completing 50 mg of plant tissue from plants of hairy beggarticks populations S, R1 and R2 with four true leaves (Dayan et al. 2015). The disks were placed in Eppendorfs containing 999 µl of monoammonium phosphate ($NH_4H_2PO_4$ 10 mM, pH 4.4). Next, 1 µl of glyphosate at different concentrations were added (0, 1, 10, 50, 100, 200, 400, 600, 1000 and 10000 µM). The samples were incubated for 24 hours in the growth chamber under controlled conditions described above. Then the samples were frozen at -20 º C until their analysis. After thawing the samples at room temperature, they were incubated at 60 ºC for 30 min. Volumes of 250 µl of HCl 1.25 N were added and incubated again at 60 ºC for 15 min. Aliquots of 250 µl were transferred to new Eppendorfs adding 500 µl of periodic acid (0.25 % w/v) and sodium metaperiodate (0.25 % w/v) at a ratio of 1:1. The samples were incubated at room temperature (25ºC) for 90 minutes, and next, 500 µl of a mix of sodium hydroxide (NaOH 0.6 N) + sodium sulfite (Na_2SO_3 0.22 N) was added at a ratio of 1:1, and mixed. Absorbance was measured at 380 nm in a spectrophotometer (Beckman DU-640). The experiment was arranged in a completely random design with 3 replications for

each glyphosate concentration. The absorbance values were converted into mg of shikimic acid g^{-1} fresh weight.

Uptake and translocation of ^{14}C-glyphosate

Plants with four true leaves from S, R1 and R2 populations were treated with a solution of ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq $mmol^{-1}$, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) + commercial glyphosate. The solution applied contained a specific activity of 0.834 kBq^{-1} µl and a glyphosate concentration of 1.8 g ea L^{-1} (360 g ea ha^{-1} in 200 L). One drop of 1 µl $plant^{-1}$ of solution was applied with a micropipette (Lab Mate HTL, Matosinhos, Portugal) on the adaxial surface of the first-second leaf. The treated leaf was washed 3 times separately with 1 mL of water-acetone (1:1 v/v) to recover the non-absorbed ^{14}C-glyphosate at 24, 48, 72 and 96 HAT. The washing solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard), and analyzed by liquid scintillation spectrometry (LSS) in a scintillation counter (LS 6500, Beckman Coulter Inc. Fullerton, USA). Complete plants were carefully removed from the pot and washed. They were divided into treated leaf, remainder of the plant and root, and stored individually in flexible combustion cones (Perkin-Elmer, BV BioScience Packard). The samples were dried in a stove at 60ºC for one week and combusted in a biological oxidizer (Packard Tri Carb 307, Packard Instrument Co., Downers Grove, IL, USA). The CO_2 released from the combustion was captured in 18 mL of a mix of Carbo-Sorb E and Permafluor (9:9 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of induvial sample was quantified by LSS. The experiment was arranged in a completely random design with five replications per population at each time evaluated. The radioactive values were used to calculate recovery as: (kBq in treated leaf + kBq in plant + kBq in roots + kBq from washes / kBq total applied) x 100. The average total recovery of ^{14}C-glyphosate applied was > 94 %.

The glyphosate translocation was visualized in plants from S, R1 and R2 populations. At 24, 48, 72 and 96 HAT, whole plants were washed, fixed on filter paper (25 x 12.5 cm) and dried at room temperature for one week. The samples were placed for 6 h beside a phosphor storage film (Storage Phosphor

System: Cyclone, Perkin–Elmer Packard BioScience BV). A phosphor imager (Cyclon, Perkin-Elmer, Packard BioScience BV) was used to reveal the translocation. The experiment was carried out using three plants per population at each evaluation time.

Basal and enzyme activity of the EPSPS

Plants S, R1 and R2 populations were grown in pots (25 cm in diameter × 15 cm high: 4 plants per pot) under greenhouse conditions, in temperatures ranging from 17 to 31ºC, and a photoperiod of 16 h. The natural light was complemented by 900 $\mu mol^{-2}s^{-1}$ photosynthetic photon flux density delivered by incandescent and fluorescent lights. The two youngest totally expanded leaves of plants with four true leaves were harvested until completing 5 g of foliar tissue for each population. Samples were frozen and stored at -40 ºC up to the protein extraction. The EPSPS extraction assays were conducted following the methodology described by Sammons et al. (2007). The total content of proteins in the raw extract was measured using the colorimetric method of Bradford (1976) following the manufacturer`s instructions with a Modified Lowry Kit for Protein Determination (Sigma-Aldrich, Madrid, Spain) following the manufacturer´s instructions.

The specific EPSPS activity in plants from S, R1 and R2 populations was studied in the presence and absence of glyphosate. In order to determine the EPSPS activity, a continuous assay of the release of inorganic phosphate was made with EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer´s instructions. The glyphosate concentrations used were: 0, 1, 10, 100, 1000, 10000 µM. Three replicates at each glyphosate concentration were analyzed. The release of phosphate above background level was measured during 10 minutes at 360 nm in a spectrophotometer (Beckman DU-640). The EPSPS activity was calculated to determine the amount of phosphate (µmol) released µg of total soluble protein $(TSP)^{-1}$ min^{-1}.

Amplification and sequencing of the EPSPS gene

Samples (100-200 mg) of young leaf tissue were collected of plants from S, R1 and R2 populations and stored at -80 ºC for RNA extraction. The frozen samples were milled with liquid nitrogen in a STAR-BEATER 412-0167 mill

(VWR International Eurolab S.L., Barcelona, Spain). Total RNA was isolated following the methodology described by Pistón (2013). Integrity of RNA was verified in 0.8% agarose gel and it was quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Walthman, MA, USA). First strand complementary DNA (cDNA) synthesis was carried out using 1 µg from the total RNA in all the samples. An iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA) was employed following the manufacturer´s instructions.

The PCR reactions were carried out with cDNA samples from each populations (R1, R2, S) using the following primers: Bidens-F13 (5'-TTGCCYGGRTCMAAGTCTTT-3') and Bidens-R11 (5'-GTCCCAASTATCACTRTGTTC-3') designed with software Primers3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on EPSPS gene sequences of *Amaranthus tuberculatus* (Accesion FJ869880.1, FJ869881.1), *A. palmeri* (FJ861242.1), *A. spinosus* (KF569213.1), *Conyza bonariensis* (EF200074.1), *C. canadensis* (AY545666.1, AY545667.1, FR872821.1), *C. sumatrensis* (AY834207.1), *Helianthus salicifolius* (AY545662.1) from the GenBank. A total volume of 25 µl which contained 50 ng of cDNA, 0.2 µM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of a 100:1 enzyme mixture of non-proofreading (*Thermus thermophilus*) and proofreading (*Pyrococcus furiosus*) polymerases (BIOTOOLS, Madrid, Spain) per reaction using a thermocycler (Gene Amp PCR System 9700; Applied Biosystems, CA, USA). The PCR conditions were: 94 ºC for 5 minutes, 35 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 1 min; and a final extension at 72ºC for 10 min. PCR products were checked by 1% agarose gel. The amplified fragments of 639 bp in length included the Thr-102 and Pro-106 positions, which corresponds to the sequence of the EPSPS gene of *Arabidopsis thaliana* (GenBank: CAA29828.1), point mutations associated with glyphosate resistance in weeds (Sammons and Gaines, 2014; Chen et al. 2015; Yu et al. 2015).

The PCR products were ligated using the pGEM-T Easy Vector System (Promega Biotech Ibérica, SL, Madrid, Spain) following the manufacturer`s instructions, and cloned into competent cells of *E. coli* DH5α. Positive

transformants were selected. The fragment insertion was confirmed through a PCR using the M13F (5´-CGCCAGGGTTTTCCCAGTCACGAC-3´) and M13R (5-TCACACAGGAAACAGCTATGAC-3´) primers at a total volume of 15 µl containing 0.2 µM of each primer, 0.2 mM dNTP mix (PE Applied Biosystems; Life Technologies S.A., Madrid, Spain), 2 mM $MgCl_2$, 1X buffer, and 0.625 units of non-proofreading (*Thermus thermophilus*) polymerase (BIOTOOLS, Madrid, Spain) per reaction. The PCR conditions were as follows: 94ºC for 5 minutes, 28 cycles of 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 1 min; and a final extension at 72ºC for 7 min. The plasmids were purified with the ilustra plasmidPrep Mini Spin kit (GE Healthcare, Buckinghamshire, UK), following the manufacturer`s instructions. Sanger sequencing was carried out by the STABVIDA sequencing service (Caparica, Portugal). Five biological samples were used per population. A total of 15 clones from each population were sequenced. The assembly of the sequences was carried out by SeqMan Pro (Version 11, DNASTAR; Wisconsin, USA) and Geneious (Version 8.1.8, Biomatters Ltd, Auckland, New Zealand) software´s.

A second EPSPS sequencing with 15 new individuals from R1 population to confirm mutations was carried out. A total of 45 clones were sequenced.

The hairy beggarticks EPSPS cDNA sequences information can be found in GenBank with accession numbers KU984452-KU984458.

Statistical analysis

The dry weight and survival percentage data were submitted to a non-linear regression analysis. The dose needed to reduce the growth of a population by 50% (ED_{50}), the mortality by 50% (LD_{50}), and to inhibit EPSPS activity by 50% (I_{50}) were calculated. The log-logistic model was conducted using SigmaPlot (Version 11.0, Systat Software, Inc, USA) software. The statistical model is:

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

Where *Y* is the dry weight, survival and/or EPSPS inhibiting percentage with respect to the untreated control, *c* and *d* are coefficients corresponding to the upper (maximum growth) and lower (minimum growth) asymptotic limits), *b*

is the Hill slope, *g* is the herbicide dose (ED_{50}, LD_{50} or I_{50}) at the mean point of inflexion between the upper and lower asymptote and *x* (independent variable) corresponds to the herbicide dose.

Statistical analyses between the hairy beggarticks populations were performed using Statistix version 8.0 Analytical Software. The experimental results were subjected to analysis of variance, and means were compared using Tukey's or LSD test´s at the 95% probability level.

Results

Dose-response

This experiment confirmed the resistance of R1 and R2 hairy beggarticks populations to glyphosate. A large reduction of biomass in population S was observed at low glyphosate doses in comparison to that of the resistant populations (**Figure 11A**). The ED_{50} value for the S population was 51.7 g ae ha^{-1}, whereas the R1 and R2 populations exhibited a higher ED_{50}, with resistance index (RI) values ($ED_{50}R/ED_{50}S$) of 20.4- and 2.7-fold more resistant, respectively (**Table 13**). The R1 population showed an ED_{50} value 1.46-fold higher than the glyphosate field rate recommended (720 g ae ha^{-1}).

According to LD_{50} values, R1 and R2 populations were 9.5- and 3.4-fold more resistant than the S population (**Figure 11B, Table 13**). A field rate of glyphosate showed total control for the S population. A glyphosate field rate > 2.7-fold was needed to kill 50% R1 population plants. Even though the R2 population presented 5-fold less ED_{50} than the field rate, just 50% of the population was eradicated (LD_{50} =774.4 g ae ha^{-1}) with this rate. The chlorosis symptoms caused by glyphosate application in resistant populations became evident as the glyphosate doses increased, although they were not sufficient to control the R1 population, in which plants survived treatment at 21 DAT, and continued growing up to the reproductive phase.

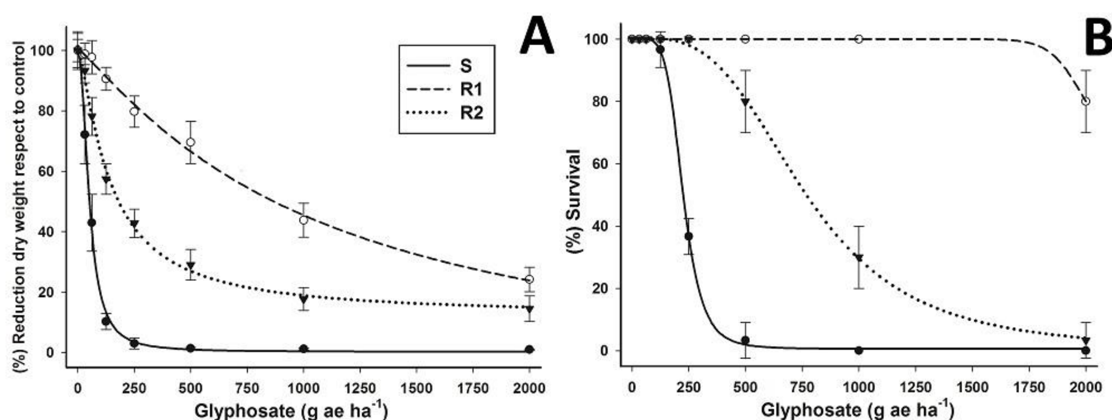

Figure 11. Log–logistic curves of glyphosate-susceptible and -resistant *Bidens pilosa* populations evaluated at 21 DAT. A) Dose-response curve with respect to percentage of dry mass reduction. The equations of log–logistic curves to estimates the ED_{50} values are: S: $Y = 0.178 + \{(99.354 - 0.178) / [1 + (dose/ED_{50})^{2.134}]\}$; R1: $Y = -3.295 + \{(100.56 + 3.295) / [1 + (dose/ED_{50})^{1.140}]\}$; R2: $Y = 12.121 + \{(101.39 - 12.121) / [1 + (dose/ED_{50})^{1.288}]\}$. B) Dose-response curve with respect to percentage of survival. The equations of log–logistic curves to estimates the LD_{50} values are: S: $Y = 0.623 + \{(100.17 - 0.623) / [1 + (dose/LD_{50})^{5.3963}]\}$; R1: rates used did not permit to estimate LD_{50} value; R2: $Y = -1.156 + \{(101.47 + 1.156) / [1 + (dose/LD_{50})^{3.238}]\}$. Vertical bars represent the standard error of the mean (n = 10).

Table 13. ED_{50}, LD_{50} and ID_{50} values of glyphosate-susceptible and -resistant *Bidens pilosa* populations.

Population	ED_{50} (g ae ha^{-1})	RI [a]	LD_{50} (g ae ha^{-1})	RI [a]	I_{50} (μM)	RI [a]
S	51.7 ± 2.3		225.4 ± 4.4		0.95 ± 0.0	
R1	1055.8 ± 34.9	20.4	> 2000	> 8.7	122.6 ± 2.1	128.4
R2	142.7 ± 10.8	2.8	774.4 ± 79.5	3.4	8.2 ± 1.1	8.5

[a] RI = Resistance indexes (R/S) calculated using the ED_{50}, LD_{50} or I_{50} values of the resistant populations respect to the susceptible population. ± Standard error of the mean.

Foliar retention

There were significant differences in foliar retention between hairy beggarticks populations (P = 0.0045; DF = 2; n = 21). The R2 (A) population retained the highest amount of glyphosate solution (392 ± 39 μl g^{-1} of dry weight), followed by the S (B) population with a mean value of 343 ± 34 μl g^{-1} of dry weight, whereas the R1 (B) population reached a mean of 328 ± 32 μl.

Shikimic acid accumulation

In the assay at different time intervals with whole plants, the S population presented an accumulation of 0.76 ± 0.13 mg shikimic acid g^{-1} of fresh weight at 24 HAT, reaching up to 4.5 ± 0.52 mg g^{-1} of fresh weight at 96 HAT (**Figure 12A**). The S and R2 populations showed an accumulation of shikimic acid since 24 HAT, while R1 population alone presented a considerable accumulation as from 72 HAT. Thus, S population was 7.7-fold more susceptible than population R1, and 1.6-fold in comparison to R2 population. The *in vivo* results obtained from different glyphosate concentrations were consistent with the results obtained in the assays with the whole plants. The hairy beggarticks populations accumulated shikimic acid as the glyphosate concentrations increased (**Figure 12B**). The greater accumulation of shikimic acid exhibited by population S was consistent with the greater reduction in growth observed in plants of these populations at these low rates (**Figure 11A**). Populations R1 and R2 were 3.3- and 1.9-fold more resistant, respectively, than S population.

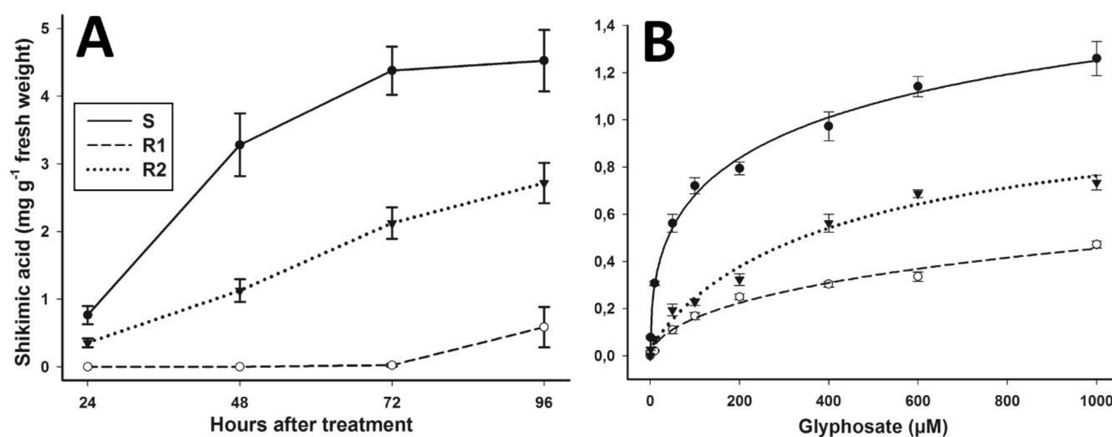

Figure 12. Shikimic acid accumulation of glyphosate-susceptible and -resistant *Bidens pilosa* populations. A) Shikimic acid accumulation after a glyphosate application at 360 g ae ha^{-1} at different intervals of time. B) Shikimic acid accumulation at different glyphosate concentrations. Vertical bars represent the standard error of the mean (n = 6 technical replicates).

^{14}C-glyphosate uptake and translocation assays

The differences in foliar uptake of ^{14}C-glyphosate between the resistant hairy beggarticks populations compared to the S population were highly significant ($P < 0.0001$; DF = 2; n = 60) (**Figure 13A**). The amount of ^{14}C-glyphosate absorbed ranged between 29.7 and 47.8%, 13.9 and 38.5%, 15.2

and 41.6%, for populations S, R1 and R2, respectively, between 24 and 96 HAT. At 24 and 96 HAT, the S population showed a greater uptake compared to R1 and R2 populations. However, after 48 and 72 HAT, the values were similar in the three populations.

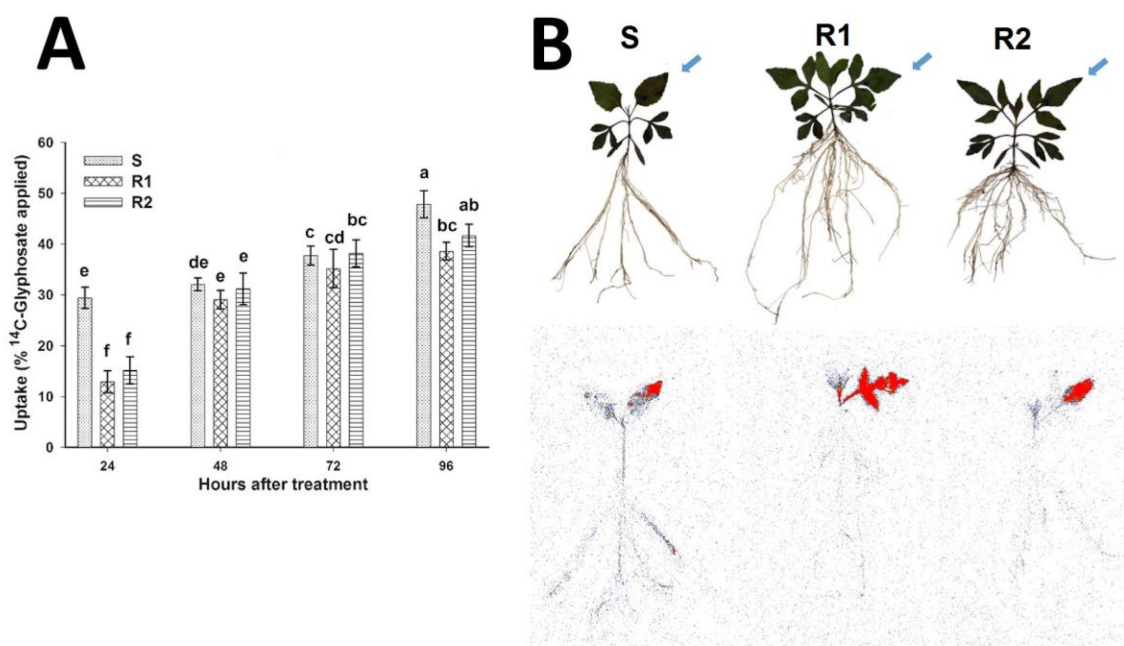

Figure 13. ^{14}C-Glyphosate uptake and translocation in glyphosate-susceptible and -resistant plants of *Bidens pilosa* populations. A) ^{14}C-glyphosate uptake in glyphosate-susceptible and -resistant *B. pilosa* plants. Different letters are statistically different at 95% probability determined by LSD test. Vertical bars represent the standard error of the mean (n = 5). B) Digital images (upper plants) and autoradiograph images (lower plants) that show the distribution of ^{14}C-glyphosate translocation in glyphosate-susceptible and -resistant *B. pilosa* plants at 96 HAT. The highest concentration of ^{14}C-glyphosate is highlighted in red. Arrows indicate the treated leaf.

With respect to the ^{14}C-glyphosate translocation, the initial amount quantified from 68.2% at 24 HAT in the treated leaf diminished to 42.6% at 96 HAT in S population. Conversely, the larger amount of herbicide applied was retained in the leaf treated in the resistant populations, dropping from 79.6% to 64.6% in R1 population, and from 73.3% to 59.7% in R2 population at 24 and 96 HAT, respectively. In the S population, an average of 24.9% of the glyphosate translocated reached the root at 96 HAT, whereas in R1 and R2 populations it was only of 12.9 and 15.5%, respectively. **Table 14** shows the results of the percentage of ^{14}C-glyphosate translocated to the remainder of the plant and root in hairy beggarticks plants.

Table 14. Translocation percentage of ^{14}C-glyphosate in plants of glyphosate-susceptible and -resistant *Bidens pilosa* populations.

Population	HAT	Traslocation (% from uptake)[a]		
		Treated leaf	Remainder of plant	Root
S	24	68.2 ± 2.0 c	18.3 ± 1.1 ef	13.5 ± 1.0 cde
	48	59.7 ± 1.6 d	23.0 ± 1.6 c	18.5 ± 1.2 b
	72	52.3 ± 2.2 e	29.1 ± 1.7 b	19.6 ± 0.6 b
	96	42.6 ± 2.0 f	33.5 ± 0.6 a	24.9 ± 1.5 a
R1	24	79.6 ± 2.1 a	13.0 ± 0.5 g	7.4 ± 2.3 g
	48	73.6 ± 2.3 b	16.9 ± 3.2 ef	10.5 ± 0.9 f
	72	67.6 ± 3.3 c	19.1 ± 2.4 e	13.3 ± 1.3 cde
	96	64.6 ± 2.0 c	22.5 ± 2.5 cd	12.9 ± 1.0 de
R2	24	73.3 ± 2.3 b	15.2 ± 0.8 fg	11.5 ± 1.3 ef
	48	68.5 ± 1.9 b	17.8 ± 2.2 ef	13.7 ± 1.6 cde
	72	66.2 ± 4.1 c	19.4 ± 1.9 de	14.4 ± 1.2 cd
	96	59.7 ± 2.8 d	24.8 ± 2.3 c	15.5 ± 1.3 c

[a] Means with different letter within a column are statistically different at 95% probability determined by the Tukey test. ± Standard error of the mean (n = 5).

The images obtained in the Phosphor Imager confirmed the previous results obtained for translocation. At 96 HAT it was seen how, in the plants of resistant populations, the glyphosate was retained mainly in the treated leaf, and only small amounts were translocated across the remainder of the plant in comparison to the S population (**Figure 13B**).

Enzyme activity

There were no significant differences (P = 0.3416; DF = 2; n = 9) in the basal EPSPS activity (average = 0.3911 μmol of glyphosate μg TSP^{-1} min^{-1}) in plants of glyphosate-susceptible and -resistant hairy beggarticks populations in the absence of glyphosate (**Figure 14A**). The EPSPS enzyme was inhibited by glyphosate in plants of susceptible and resistant populations. For the S population, only 0.95 μM of glyphosate was necessary to inhibit EPSPS activity by 50% (I_{50}). The resistant plants of R2 and R1 populations, on average, were

8.5- and 128.4-fold, respectively, less sensitive to glyphosate than the susceptible plants (**Table 13, Figure 14B**).

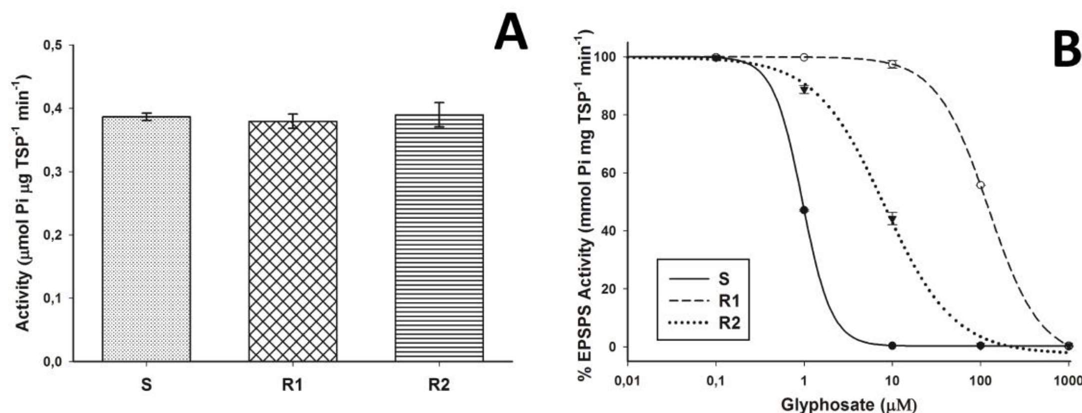

Figure 14. EPSPS activity in glyphosate-susceptible and -resistant plants of *Bidens pilosa* populations. A) Basal EPSPS activity for glyphosate-susceptible and -resistant *B. pilosa* plants. Histograms represent treatment means and vertical bars SE of the mean (n = 3). B) EPSPS enzyme activity expressed as percentage of the untreated control in leaf extracts of plants from glyphosate-susceptible and resistant *B. pilosa* populations. The equations of log–logistic curves to estimates the I_{50} values are: S: $Y = 0.227 + \{(99.98 - 0.227) / [1 + (dose/I_{50})^{2.619}]\}$; R1: $Y = -4.065 + \{(99.92 + 4.065) / [1 + (dose/I_{50})^{1.477}]\}$; R2: $Y = -2.471 + \{(99.79 + 2.471) / [1 + (dose/I_{50})^{1.108}]\}$. Vertical bars represent the standard error of the mean (n = 3).

Sequencing of the EPSPS gene

The sequencing from cDNA revealing the presence of two different EPSPS genes that are expressed in the three hairy beggarticks populations (**Table 15, Figure 15**), showed one homology above 92% between EPSPS1 and EPSPS2 genes based on their predicted proteins, and above 80% with respect to *Arabidopsis thaliana* (GenBank: CAA29828.1) (**Figure 15**). In the three populations, some individuals only showed the EPSPS1 gene, others the EPSPS2 gene, and others showed both genes.

Some R1 population plants were identified with a single mutation in Pro-106 position alone, and other plants presented a double mutation in the Thr-102 and Pro-106 positions in the EPSPS2 gene (**Figure 15**). The amino acid substitutions consisted of Threonine (ACC) to Isoleucine (ATC) in Thr-102 position, and from Proline (CCA) to Serine (TCA) in Pro-106 positon (**Figure 15**). Mutations were not found in the EPSPS1, and the R2 population did not show any mutation. Of the 20 individuals sequenced from R1 population, only 2

had the I102-S106 (TIPS) allele; 11 individuals the T102-S106 allele, and 7 had the wild type allele T102-P106 corresponding to 10, 55 and 35% of the sample size analyzed. Percentage frequency of EPSPS genes and alleles implicated in glyphosate resistance are shown in the **Table 15**.

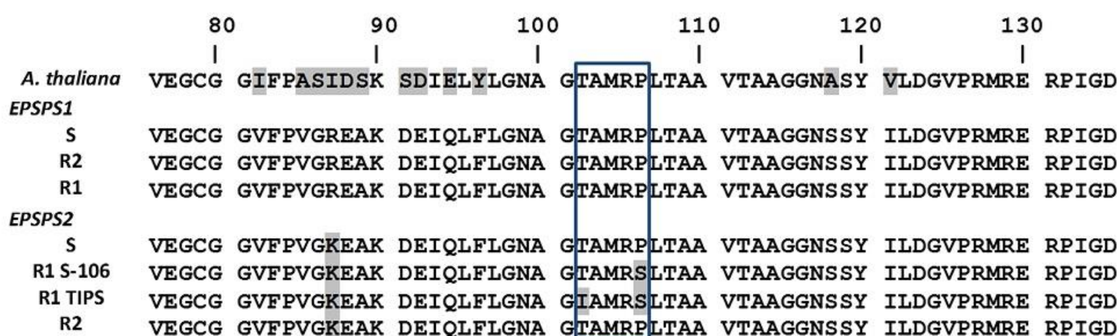

Figure 15. Partial alignment of protein sequences of EPSPS1 and EPSPS2 genes in glyphosate-susceptible and -resistant *Bidens pilosa* populations. The highlighted color indicates changes to codons from the consensus nucleotide sequence. Box includes from the 102 to 106 positions (amino acid number based on the start codon (ATG) of *A. thaliana* [GenBank: CAA29828.1] EPSPS sequence).

Table 15. Frequency percentage of EPSPS genes, and polymorphisms at 102 and 106 positions in glyphosate-susceptible and -resistant plants of *Bidens pilosa* populations.

Population	Number of individuals/ clones	Gene	Gene Frequency (%)	Alleles[a]	Allele frequency (%)
S	5/15	EPSPS1	60.0	T102-P106	60.0
		EPSPS2	40.0	T102-P106	40.0
R1	20/60	EPSPS1	56.7	T102-P106	56.7
		EPSPS2	43.3	T102-P106	14.3
				T102-S106	23.3
				I102-S106	6.6
R2	5/15	EPSPS1	53.3	T102-P106	53.3
		EPSPS2	46.7	T102-P106	46.7

[a] T102-P106 = wild type or glyphosate susceptible; T102-S106 = low-intermediate glyphosate resistance; and I102-S106 = high glyphosate resistance.

Discussion

The ED_{50}, LD_{50} and I_{50} parameters (**Table 13**) showed the highest level of resistance developed by resistant hairy beggarticks populations. Our results of dose-response results are according with other glyphosate-resistant species. The RI in resistant *Conyza bonariensis, C. canadensis,* and *C. sumatrensis* populations, asteraceae species like hairy beggarticks, ranged between 7- to 17-fold more compared to their respective susceptible population (Koger et al. 2004; González-Torralva et al. 2010, 2012b). Other dicotyledonous species such as *A. palmeri* and *Kokia scoparia* presented similar variation of resistance level showed RI between 3- to 18-fold (Whitaker et al. 2013; Ribeiro et al. 2014; Wiersema et al. 2015). A resistant *A. palmeri* population showed an RI 18-fold higher than its S population with an ED_{50} of 2565 g ae ha^{-1} (Whitaker et al. 2013).

On the basis of LD_{50}, the RI ranged between 3- to 15-fold in weed species, such as *L. perenne* spp. *multiflorum* and *K. scoparia* (Salas et al. 2015; Wiersema et al. 2015). To achieve a total control in resistant hairy beggarticks populations, one needs to apply at least double the rate of glyphosate of that of their corresponding LD_{50}. However, higher doses increase selection pressure and will accelerate the evolution of resistant populations (Salas et al. 2015).

Shikimic acid and enzyme EPSPS activity tests are accepted as appropriate parameters to determine susceptibility level to glyphosate (Dayan et al. 2015). With respect to EPSPS activity, some resistant *L. perenne* spp. *multiflorum* and *E. colona* populations showed differences in basal EPSPS activity compared to their respective susceptible populations (Salas et al. 2012; Alarcón-Reverte et al. 2015), but these differences were associated with a greater number of copies of the EPSPS gene. Therefore, the similar basal EPSPS activity between hairy beggarticks populations suggests that resistant populations could not have any differences in the number of copies of the EPSPS gene respect to susceptible populations. Multiple EPSPS copy numbers and/or increased EPSPS expression have been described as glyphosate resistance mechanisms in dicotyledonous species such as *A. palmeri, A. tuberculatus*, *K. scoparia* (Ribeiro et al. 2014; Chatham et al. 2015; Wiersma et

al. 2015), among others species. However, similar gene copy numbers may not necessarily show the same level of resistance to glyphosate (Salas et al. 2012). As in resistant hairy beggarticks populations, higher glyphosate concentrations were necessary in resistant *L. perenne* spp. *multiflorum* and *E. colona* populations to inhibit EPSPS activity by 50% (I_{50}) (Salas et al. 2012; Alarcón-Reverte et al. 2015).

In both assays of shikimic acid, the resistant hairy beggarticks populations showed a lesser shikimic acid accumulation than the S population (**Figure 12**). This evidenced the high susceptibility to glyphosate of the S population, and a different resistance level between R1 and R2 populations. Similar results have been reported in other species of glyphosate-resistant weeds, for instance, resistant *L. rigidum*, *E. colona* and *P. annua*) populations (Pérez-Jones et al. 2007; Alarcón-Reverte et al. 2013; Cross et al. 2015. Any species with a low accumulation of shikimic acid requires a larger amount of glyphosate in order for it to be lethal (Cruz-Hipolito et al. 2011; Alcántara-de la Cruz et al. 2016). This can happen when, in the differential accumulation of shikimic acid, glyphosate does not reach the target site in sufficient amounts due to altered translocation patterns (Alarcón-Reverte et al. 2015; Cross et al. 2015). In this work, both susceptible and resistant populations accumulated shikimic acid. This indicated that the glyphosate arrived at the target site inhibiting the EPSPS, but that that inhibition was at different levels (Duke and Powles, 2008; Alarcón-Reverte et al. 2015), this being significantly greater in the S population.

Herbicide foliar retention and uptake are influenced by physiological and morphological traits (Cruz-Hipolito et al. 2011; Alcántara-de la Cruz et al. 2016), and are not major mechanisms conferring glyphosate resistance. Foliar retention capacity depends on the phenology of the plants. Studies on *Conyza* spp. showed that foliar retention was greater during the elongation of the stem than during flowering (González-Torralva et al. 2010). Only few weed species have presented differences in reduced glyphosate uptake and foliar retention as mechanisms involved in their resistance. For instance, resistant *A. tuberculatus*, *L. multiflorum* and *D. insularis* populations showed a reduced uptake (Michitte et al. 2007; de Carvalho et al. 2012; Nandula et al. 2013); and only *L.*

multiflorum presented a lower foliar retention. These traits play an important role in innate glyphosate-tolerant species (Cruz-Hipolito et al. 2011; Alcántara-de la Cruz et al. 2016). In hairy beggarticks populations neither foliar retention nor ^{14}C-glyphosate uptake were not mechanisms involved in the resistance. However, the ^{14}C-glyphosate translocation results suggest that a reduced translocation could be the main mechanism involved in resistance (**Table 14, Figure 13B**), but the different resistance levels and shikimic acid accumulation between resistant hairy beggarticks populations suggests that their resistance mechanisms may differ between each other. Sammons and Gaines (2014) pointed out several cases with at least two resistance mechanisms. These cases involved TSR and NTSR mechanisms.

Hairy beggarticks presented two different EPSPS genes. Species such as *C. sumatrensis* and *E. colona* also presented two genomes of the EPSPS gene, one of them showing a mutation in Pro-106 position conferring glyphosate resistance (Alarcón-Reverte et al. 2015; González-Torralva et al. 2014).

A single Pro-106 mutation has been widely described in several resistant weed species to glyphosate (Cross et al. 2015; Salas et al. 2015; Alarcón-Reverte et al. 2015; González-Torralva et al. 2012a, 2014; between others). The levels of resistance conferred by single target-site mutation to date tend to vary at between 2- and 10-fold, depending on whether a target site mutation or reduced translocation is the mechanism (Bostamam et al. 2012), and up to 4 to 15-fold when two different mechanisms are involved (Sammons and Gaines, 2014), for instance, resistant *A. tuberculatus* (Nandula et al. 2013), *L. rigidum* (Yu et al. 2007; Bostamam et al. 2012) and *L. multiflorum* (González-Torralva et al. 2012a) populations, exhibited a mutation in Pro-106 position, and a reduced translocation with an RI 5-fold for the resistant *A. tuberculatus* and *L. multiflorum* populations, whereas for those of *L. rigidum*, the RI was 6-8-fold (Bostamam et al. 2012), and 14-fold (Yu et al. 2007) higher than their susceptible population.

The Thr-102-Ile mutation would be unlikely to occur first or independently (Weinreich et al. 2006), it has usually been associated with the Pro-106-Ser mutation, commonly known as TIPS. Such mutation has been reported to

provide high resistance/tolerance to glyphosate in studies with *E. coli* (Healy-Fried et al. 2007), and it is used in transgenic glyphosate-resistant crops (see Table 3, review by Sammons and Gaines, 2014). Recently, the TIPS mutation was identified in two resistant *E. indica* populations. The latter was one population from Malaysia, as the first TIPS mutation naturally identified in weed species (Yu et al. 2015), and one from China (Chen et al. 2015). In both cases, the frequency of I102-S106 (high resistance) allele corresponding to TIPS mutation was lower (26% of frequency in the population from the Malaysia (3 from 193 individuals), and 16.7% in the population from China (8 from 30 individuals) than T102-S106 (low-intermediate resistance) and/or T102-P106 (wild type or susceptible) alleles. The hairy beggarticks R1 population also presented a low frequency (6.6 %) of I102-S106 allele in 2 from 20 individuals studied. This is another example of naturally evolved of TIPS mutation. Due to the insect pollination that shows hairy beggarticks, the allele of TIPS mutation could be easily spread to other Persian lime groves by insects. For this reason, future studies will focus on characterizing glyphosate-resistant genotypes using dCAPS markers, as well as the fitness cost of glyphosate resistance in this species.

These results revealed the first case of a double mutation (TIPS) evolved on the target site in a wild dicotyledonous weed, due to high selection pressure exerted by repeated glyphosate applications (3-4 times per year) in citrus groves from Mexico (Pérez-López et al. 2014).

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CAPITULO V

Physiological, Morphological and Biochemical Studies of Glyphosate Tolerance in Mexican Cologania (*Cologania broussonetii* (Balb.) DC.).

Abstract

In recent years, glyphosate-tolerant legumes have been used as cover crops for weed management in tropical areas of Mexico. Mexican cologania (*Cologania broussonetii* (Balb.) DC.) is an innate glyphosate-tolerant legume with a potential as a cover crop in temperate areas of the country. In this work, glyphosate tolerance was characterized in two Mexican cologania (a treated (T) and an untreated (UT)) populations as being representatives of the species, compared in turn to a glyphosate-susceptible hairy fleabane (S) (*Conyza bonariensis* (L.) Cronq.) population. Experiments revealed that T and UT Mexican cologania populations had a higher tolerance index (TI), and a lower shikimic acid accumulation and foliar retention than the hairy fleabane S population. Absorption and translocation, leaf morphology and metabolism studies were only carried out in the Mexican cologania T population and the hairy fleabane S population. The latter absorbed 37% more ^{14}C-glyphosate compared to the Mexican cologania T at 96 hours after treatment (HAT). Mexican cologania T translocated less herbicide from the treated leaf to the remainder of the plant than hairy fleabane S. The Mexican cologania T presented a greater epicuticular wax coverage percentage than the hairy fleabane S. This morphological characteristic contributed to the low glyphosate absorption observed in the Mexican cologania. In addition, the Mexican cologania T metabolized glyphosate mainly into AMPA, formaldehyde and sarcosine. These results indicate that the high glyphosate tolerance observed in Mexican cologania is mainly due to the poor penetration and translocation of glyphosate into the active site, and the high glyphosate degradation into non-toxic substances.

Keywords: absorption/translocation, cover crop, glyphosate tolerance, glyphosate metabolism, morphological characteristics, shikimic acid

Resumen

En años recientes, se ha implementado el uso de leguminosas tolerantes a glifosato como cultivo de cobertura, para el manejo de malas hierbas en áreas tropicales de México. Cologania (*Cologania broussonetii* (Balb.) DC.) es una leguminosa con tolerancia natural a glifosato, y posee un gran potencial como cultivo de cobertura en regiones templadas del país. En este trabajo se caracterizó el nivel de tolerancia de dos poblaciones de cologania (una población tratada con glifosato (T), y una población nunca tratada (NT), como poblaciones representativas de la especie. A su vez, estas poblaciones fueron comparadas con una población sensible (S) a glifosato de coniza (*Conyza bonariensis* (L.) Cronq.). Los experimentos revelaron que las poblaciones T y NT de cologania tuvieron un alto índice de tolerancia (IT), una baja acumulación de ácido shiquímico y menor retención foliar que la población S de coniza. Estudios de absorción-translocación, morfología foliar y metabolismo solo se llevaron a cabo en la población S de coniza y en la población T de cologania. A 96 horas después del tratamiento (HDT), coniza absorbió 37% más ^{14}C-glifosato que la población T de cologania. Esta última población transloco menos herbicida desde la hoja tratada al resto de planta y raíces que coniza. Además, cologania también presento una mayor cobertura de ceras epicuticulares. Esta característica morfológica contribuye en la baja absorción observada en cologania, y el poco glifosato absorbido fue metabolizado principalmente a ácido aminometil fosfonico (AMPA), formaldehido y sarcosina. Estos resultados demuestran que la alta tolerancia a glifosato observada en cologania es, principalmente, debido a la pobre penetración y translocación de ^{14}C-glifosato a su sitio de acción, y la alta degradación en sustancias no toxicas.

Palabras clave: absorción/translocación, ácido shiquímico, características morfológicas, cultivo de cobertura, metabolismo de glifosato, tolerancia a glifosato.

Introduction

Weeds reduce crop productivity, displace native species, contribute to land degradation and increase production costs. A cover crop is constituted by a living vegetal that keeps the soil covered, protects it from erosion, and prevents nutrient loss (Hartwig and Ammon, 2002). Cover crops are also involved in the elimination of weeds and they reduce the spread of numerous pathogens (Renard and Franqueville, 1991). Cover crops allow for the selective use of herbicides within integrated weed management programs in some agricultural systems, drastically reducing germination, emergence and/or growth of weed populations (Lal et al. 1991; Buhler et al. 2001). The use of cover crops for weed displacement and soil protection against erosion presents some limitations, such as the interference of weeds at the early stages of cover crop growth (Cameron et al. 1991). At that point, the use of herbicides with good toxicological and environmental profiles, such as glyphosate, could facilitate crop establishment (Domínguez-Valenzuela, personal observation).

Herbicide tolerance is a natural, hereditary mechanism that allows a plant species to survive and reproduce after herbicide application; this survival is due to inherent morphological and/or physiological characteristics. Therefore, herbicide-tolerant populations have never been susceptible (Cruz-Hipolito et al. 2009). Resistant populations of weed species are naturally present and occur at low densities. Herbicide resistance happens when resistant weeds are able to survive and complete their life cycles when the herbicide is applied at recommended field doses. Repeated selection pressure through herbicide applications selects and promotes the expansion of the resistant population (Beckie, 2006). Herbicide tolerance and resistance are phenomena of interest although weed resistance to herbicides is a serious problem due to the loss of active ingredients. Therefore, herbicide tolerance in a cover crop would be desirable (Cameron et al. 1991).

Glyphosate (N-phosphonomethyl glycine) is currently the most important herbicide (Duke and Powles, 2008), and it is used for broad-spectrum weed control in glyphosate-resistant crop. Glyphosate is systemic, non-residual, non-selective, and also has an acceptable toxicity profile. It is easily transported

from the leaves to meristematic tissues, rapidly reducing photosynthesis and increasing shikimate levels (Duke et al. 2003). Glyphosate acts by inhibiting 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), responsible for the biosynthesis of chorismate, an intermediate in the shikimic acid pathway that leads to the synthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan) (Amrhein et al. 1980; Siehl, 1997; Shaner et al. 2005; Wiersema et al. 2013).

Like many herbicides, the continuous use of glyphosate has led to the evolution of resistant weed biotypes. However, many other species have some natural tolerance degree, which could be related to the same resistance mechanisms found in resistant weed biotypes (Duke, 2011). New weed management technologies are a priority in sustainable agriculture (Hartwig and Ammon, 2002). In Mexico, some suspected glyphosate-tolerant species could be being used as cover crops. Nevertheless, this tolerance has still not been confirmed (Dominguez-Valenzuela, personal observation).

Mexican cologania (*Cologania broussonetii* (Balb.) DC.), is a perennial legume from America, distributed in Central and South America. It grows in temperate regions of Mexico (Vibrans, 2009a), and has morphological and physiological characteristics that could be useful in weed management. In previous unpublished studies by this research group, some level of tolerance to glyphosate has been observed in Mexican cologania. Beneficial morphological and physiological characteristics and glyphosate tolerance permit its use as a cover crop to control weeds such as hairy fleabane in fruit orchards with temperate climates. Glyphosate applications may favor the establishment of Mexican cologania and the selective suppression of weeds.

Hairy fleabane (*Conyza bonariensis* (L.) Cronq.) is an annual weed that originated in South America, which is commonly found along roadsides, pastures and abandoned farm fields (Vibrans, 2009b). This weed has a prolific seed production, producing more than 50,000 seeds per plant (Holm et al. 1997). Seed dormancy up to 3 years has been documented in the soil of some crops (Wu et al. 2007). In addition, hairy fleabane is a very common weed

species hard to control in perennial crops of Mexican temperate regions with high population densities.

Glyphosate tolerance/resistance in triggered through two major mechanisms, Non-Target Site Resistance (NTSR) and Target Site Resistance (TSR). The NTSR mechanisms include the reduced absorption and/or translocation of the herbicide, vacuolar sequestration, glyphosate metabolism and others (Cruz-Hipolito et al. 2009; Sammons and Gaines, 2014), so that less herbicide reaches the action site. Enhanced metabolism can also cause tolerance or resistance to herbicides, but whether this happens with glyphosate is unclear (Duke, 2011). Glyphosate is enzymatically metabolized to other non-toxic compounds such as glyoxylate, sarcosine, formaldehyde and aminomethyl phosphonate (AMPA) (Sammons and Gaines, 2014). Glyphosate degradation to non-toxic compounds has been documented in several cases (Cruz-Hipolito et al. 2011; Alarcón-Reverte et al. 2013; Sammons and Gaines, 2014). The TSR mechanisms are due to a loss of affinity between the binding protein (EPSPS) and the herbicide, overexpression of this protein and spontaneous mutations that occur randomly, mainly in the Pro106 amino acid position (González-Torralva et al. 2014; Sammons and Gaines, 2014), and recently the Thr102 position was described (Yu et al; 2015).

Glyphosate tolerance can involve inherent morphological and histological characteristics. The main barrier to herbicide absorption is the cuticle (Heredia-Guerrero et al. 2014). The main entry points for herbicides are the guard cells of the stomata, trichomes and leaf veins in broadleaf species. A thicker leaf cuticle, some trichomes and/or stomata limit herbicide absorption in an aqueous solution such as glyphosate (Devine et al. 1992; Heredia-Guerrero et al. 2014).

Mexican cologania is used as a cover crop in Mexican temperate areas to suppress weeds. During cover crop establishment, weed control is needed and this can be implemented by applying a certain dose of glyphosate that is toxic to weeds like hairy fleabane and others species, but not to Mexican cologania in fruit orchards with temperate climates. Therefore, we to understand why this differential glyphosate sensitivity occurs among these species.

The purpose of this study was to determine the efficacy of glyphosate in two Mexican cologania populations and a hairy fleabane susceptible population by dose-response assays under growth chamber conditions. Physical (foliar retention), physiological (accumulation of shikimic acid, absorption and translocation of ^{14}C-glyphosate), morphological (leaf morphology) and biochemical (metabolism) characteristics of glyphosate tolerance were examined in Mexican cologania and hairy fleabane plants.

Material and Methods

Plant material and experimental conditions

Mexican cologania T population seeds were collected in the plots of experimental fields from University of Chapingo, Mexico (19º 29' 43'' N, 98º 52' 44'' W), that had survived sporadic applications of glyphosate at the recommended field dose, 720 g acid equivalent (ae) ha^{-1}. Mexican cologania UT population seeds were also collected from glyphosate untreated experimental fields. Mexican cologania was considered as being a representative of the entire species. The objective of comparing the UT population with respect to T population was to identify possible differences in the innate glyphosate tolerance of this species. Hairy fleabane S population seeds never treated with herbicide were collected in a public lands from Tequila, Mexico (20º 53' 03'' N, 103º 48' 18'' W). The seeds of both species were collected in 2009 and 2010, respectively.

Mexican cologania T and UT populations seeds were germinated in 15 cm Petri dishes containing two layers of filter paper moistened with distilled water and sealed with parafilm. The hairy fleabane S population was planted in 663 cm^3 trays with peat saturated at field capacity and covered with a plastic layer until emergence. Both species were placed in a growth chamber at 28/18ºC (day/night) with a photoperiod of 16 h, a light density of 850 mmol m^{-2} s^{-1}, and 60% relative humidity.

Once germinated, the seedlings of both species were transplanted into 250 mL pots containing (one plant per pot) a mixture of peat/sand (1:1 v/v). Pots

were watered to saturation and placed in a growth chamber under the conditions described above. Irrigation was suspended for one day after transplanting to stimulate root growth. Subsequently, pots were watered daily.

All glyphosate (Roundup® Energy 45% w/v, Monsanto, Spain) applications, except for absorption and translocation, were performed in a spray chamber (Devries Manufacturing, Hollandale, Minnesota) equipped with a Tee Jet 8002EVS flat fan nozzle with a pressure of 200 kPa, a height of 50 cm and an application volume of 200 L ha^{-1}.

Dose-response assays

Plants of the Mexican cologania T and UT populations were treated at the growth stage BBCH 13-14, and the hairy fleabane S population plants at BBCH 16-18 (Hess et al. 1997) with the following doses of glyphosate: 0, 50, 100, 200, 300, 400, 600, 800 and 1000 g ae ha^{-1} for the Mexican cologania T and UT populations; and 0, 11.25, 22.5, 45, 90, 180, 360, 720 g ae ha^{-1} for the hairy fleabane S population. The plants were cut at ground level 21 days after treatment (DAT) and immediately weighed to determine the fresh weight. Different doses for each species were used because in previous assays hairy fleabane showed high glyphosate sensitivity. Data were expressed as a percentage of fresh weight compared to untreated control plants according to Cruz-Hipolito et al. (2011). The experiment was arranged in a completely randomized design with 10 replicates per dose. The assays were conducted twice.

Spray retention assays

To carry out these assays, the methodology described by Gauvrit (2003) was followed. Plants of Mexican cologania T and UT, and hairy fleabane S populations were sprayed with a solution that contained 720 g ae ha^{-1} of glyphosate and 100 mg L^{-1} Na-fluorescein. Once the solution had dried on the foliage, the treated plants were cut at ground level. The tissue was washed by immersion in 50 mL of 5 mM NaOH. Subsequently, the plants were wrapped in cellulose envelopes and dried in a heater (JP Selecta SA) at 60ºC for 72 hours. The fluorescein absorbance of the wash was determined using a Hitachi F-2500 spectrofluorimeter at 490exc/510em nm. The retention was expressed as mL of

glyphosate solution g^{-1} dry matter. The experiment was arranged in a completely randomized design with 10 plants per species.

Shikimic acid accumulation

The plants from the two species were treated with glyphosate at 360 g ae ha^{-1} (0.5 from the recommended field dose). At 24, 48, 72 and 96 HAT, 50 mg of fresh tissue (from the second and third leaf) from treated and non-treated plants was harvested and placed in an Eppendorf tube with 1 mL of HCl 0.25 N and immediately frozen in liquid nitrogen. The vials were stored at -40º C until use. The shikimic acid extraction was performed according to the methodology described by Perez-Jones et al. (2005).

The shikimic acid accumulation was determined according to the methodology described by Singh and Shaner (1998). The absorbance of the samples was measured with a Beckman DU-640 spectrophotometer at 380 nm. The net accumulation of shikimic acid was deduced as the difference between the treated and untreated plants in each population. The experiment was arranged in a completely randomized design. Three treated plants and three untreated plants from each species were used per collection time point. The results were expressed as mg shikimic acid g^{-1} fresh weight.

^{14}C-glyphosate absorption and translocation

The ^{14}C-glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq $mmol^{-1}$, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) was mixed with a commercially formulated glyphosate to prepare a solution with a specific activity of 0.834 KBq^{-1} µl and a glyphosate concentration of 1 g ae L^{-1} (200 g ae ha^{-1}). Previous tests determined that the best results were obtained from the dose and specific activity used in this work. A drop of 1 µl of the radiolabeled solution was applied with a micropipette (Lab Mate HTL) onto the adaxial surface of the second leaf of each plant in both species. To analyze the percentage of absorption and translocation, the treated plants were carefully removed from the pots at 24, 48, 72 and 96 HAT and washed. The treated leaves were washed 3 times with 1 mL of a water-acetone solution (1:1 v/v) to recover the unabsorbed ^{14}C-glyphosate. The rinse solution was mixed with 4 mL of scintillation fluid and analyzed by spectrometry (LSS) (Scintillation Counter,

Beckman LS 6500). Subsequently, the plants were divided into the treated leaf, the remainder of the plant, and the roots. The plant tissues were placed in an oven at 60°C for 72 h and then combusted in a biological oxidizer (Packard Tri Carb 307). The $^{14}CO_2$ corresponding to all possible glyphosate metabolites was trapped in 18 mL of a mixture of Carbo-Sorb E and Permafluor E (9:9 v/v) (Perkin-Elmer, Packard Bioscience BV). Radioactivity was quantified by LSS, and the percentage of absorbed herbicide was expressed as [(KBq in combusted tissue) / (KBq in combusted tissue + KBq in leaf washes)] x 100. The experiment was arranged in a completely randomized design with five plants per species. The average total recovery of ^{14}C-glyphosate only was > 89% for Mexican cologania, and > 96% for hairy fleabane.

The translocation of the herbicide was visualized using a phosphor imager (Cyclone, Perkin-Elmer, Packard Bioscience BV). At 24, 48, 72 and 96 HAT, the whole plant was washed, pressed on filter paper (25 x 12.5 cm) and dried to room temperature for 4 days. The plants were placed with a phosphor storage film for 14 h and scanned for radiolabel dispersion. The experiment was carried out using three plants per species.

Leaf morphology

Pieces (0.5 x 0.5 cm) of fresh leaves from plants of Mexican cologania T and hairy fleabane S populations were cut with a scalpel and fixed in 2% glutaraldehyde (v/v) in 0.2 M phosphate buffer at pH 7 overnight at 4°C. Samples were then washed in fresh phosphate buffer. The samples were dehydrated with a series of progressively increasing concentrations of ethanol (20, 40, 60, 80 and 100% (v/v) alcohol) as incubation times increased from 15 to 90 min. The leaf pieces were fixed in a metal support with double sided tape and coated with a 0.05-micron gold film. To examine the samples, a scanning electron microscope (SEM; JEOL JSM-840) was used at 10-20 kV. Wax coverage was digitally captured, enhanced by using Adobe Photoshop software, and quantified (expressed as μm^{-2}) using ImageJ software (version 1.31) from National Institutes of Health, USA.

Metabolism

Plants of both species were treated with glyphosate at a dose of 100 g ae ha^{-1}. Untreated plants were used as controls. A low dose was used because in the dose- response assays, the plants of the hairy fleabane S population died from 90 g ae ha^{-1} at 21 DAT. Glyphosate and its metabolites (aminomethyl phosphanete (AMPA), glyoxylate, sarcosine and formaldehyde) were determined at 4, 8, 12, 16 and 20 DAT according to the methodology described by Rojano-Delgado et al. (2010). Reversed-polarity capillary-electrophoresis was performed using a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD) (190-600 nm). Metabolites used as standards (Sigma-Aldrich, Spain) were as follows: glyphosate, AMPA, sarcosine, formaldehyde and glyoxylate. The plants were washed with distilled water, immediately frozen in liquid N_2 and stored at -40°C until use. The background electrolyte (BGE) consisted of 10 mM potassium phthalate, hexadecyltrimethylammonium bromide (CTAB) 0.5 mM and 10% acetonitrile at pH 7.5. The calibration curve was made using untreated plants and known concentrations of the different standards, which were determined in the electropherogram through the enclosed area under the respective peaks. The average value of the glyoxylate naturally produced by the plant was subtracted from the average content in each population according to Rojano-Delgado et al. (2012). The experiment was repeated twice for each population with three plants per collection time point.

Statistical analyzes

In the dose-response assay, the fresh weight as a percentage of the non-treated control data was subjected to a non-linear regression analysis to determine the Effective Dose 50 (ED_{50}) values and curves according to Cruz-Hipolito. The ED_{50} indicates the amount of herbicide required to reduce the growth of a population by 50%. A log-logistic model was conducted using Sigma Plot software (version 11.0) from Systat Software, Inc (USA). The data were adjusted to a non-linear regression curve whose statistical model is (Seefeldt et al. 1995):

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

Where *Y* is expressed as a percentage of the value for untreated plants; *c* and *d* are the lower and upper asymptotes, respectively; *b* is the slope of the curve; *g* denotes ED_{50} (which coincided with the point of inflection halfway between the upper and lower asymptotes); and x is an independent variable representing the herbicide dose.

The data obtained in the spray retention were subjected to ANOVA. Effects of the population and time (HAT), and their interaction on shikimic acid accumulation, ^{14}C-glyphosate absorption and translocation, and glyphosate metabolism were tested using ANOVA. The population was treated as a fixed factor and time was considered a random factor. Means and standard errors (of the mean) of ^{14}C-glyphosate absorption and translocation were computed for all parts of plants, and means were tested for group differences and compared. For each analysis, assumptions such as equality of variance and normal distribution were evaluated. When required, the Tukey HSD test at 5% probability was used to separate means. A statistical analysis was performed using Statistix software (version 9.0) from Analytical Software (USA).

Results

Dose-response

Under growth chamber conditions, the two Mexican cologania populations exhibited a similar response to glyphosate, but hairy fleabane a different one (**Figure 16**). The tolerance index ($ED_{50}T$ / $ED_{50}UT$) between the Mexican cologania populations was only 1.05. The ED_{50} values for the tolerant Mexican cologania populations ranged from 166.4 (UT) to 175.3 (T) g ae ha^{-1}, whereas for the hairy fleabane S population it was only 47.3 g ae ha^{-1}. **Table 16** includes the parameters of the model used to calculate the ED_{50} of glyphosate in Mexican cologania T and UT, and hairy fleabane S populations. These results show that the Mexican cologania T and UT populations were, on average, 3.6 times more glyphosate-tolerant than the hairy fleabane S population.

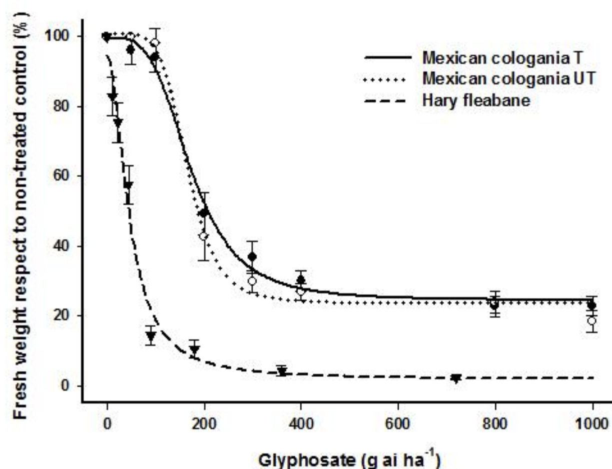

Figure 16. Dose-response curves of the Mexican cologania T and UT populations and one hairy fleabane S population evaluated at 21 DAT. Vertical bars represent the standard deviation of the mean (*n* = 10).

Table 16. Parameters of the sigmoidal equation used to estimate ED_{50} values of two Mexican cologania populations and one hairy fleabane S population.

Species	c	d	b	R^2aj	p^b	ED_{50} (g ae ha^{-1})	TI
Mexican cologania T	23.4	99.7	3.5	0.98	<0.0001	175.3 ± 3.3	
Mexican cologania UT	23.2	100.9	5.4	0.98	<0.0001	166.4 ± 2.9	**3.7**
Hairy fleabane S	1.7	94.6	1.9	0.97	<0.0001	47.3 ± 1.9	**3.5**

c = lower limit, d = upper limit, b = Hill´s slope, R^2aj = 1 − (sums of squares of the regression / corrected total sums of squares), p^b value = probability level of significance of the non-linear model, ED_{50} = effective dose required for 50% reduction in plant biomass, and TI = Tolerance Index (ED_{50}T / ED_{50}S). ± Standard error of the mean (*n* = 10).

Spray retention assays

The ANOVA analysis of spray retention suggests that the two species are significantly different (P < 0.0001, *n* = 10, DF = 1). The hairy fleabane S population retained a large amount of herbicide solution (1.06 ± 0.07 mL g^{-1} dry weight) compared to the Mexican cologania populations (0.65 ± 0.04 (UT) and 0.62 ± 0.02 (T) mL g^{-1} dry weight, respectively). Leaf retention depends on plant phenology. After spraying, hairy fleabane retained a larger amount of glyphosate during stem elongation (BBCH 32) than during flowering (BBCH 55) (González-Torralva et al. 2012).

Shikimic acid accumulation

Shikimic acid concentrations in the absence of glyphosate (non-treated control) were low and increased in the leaf tissues of both species after a glyphosate application. The hairy fleabane S population accumulated a larger amount of shikimic acid that ranged from 2.7 to 3.5 times with respect to UT and T Mexican cologania populations, respectively. The hairy fleabane S population accumulated 0.9 mg shikimic acid g^{-1} fresh weight at 24 HAT reaching 3.9 mg at 96 HAT. The Mexican cologania populations only reached 1.1 mg shikimic acid g^{-1} fresh weight at 96 HAT for the T population, and 1.4 mg shikimic acid for the NT population (**Figure 17**). A significant increase in the shikimic acid accumulation was observed in Mexican cologania T population between 48 and 72 HAT from 0.1 to 0.9 mg shikimic acid g^{-1} fresh weight, respectively.

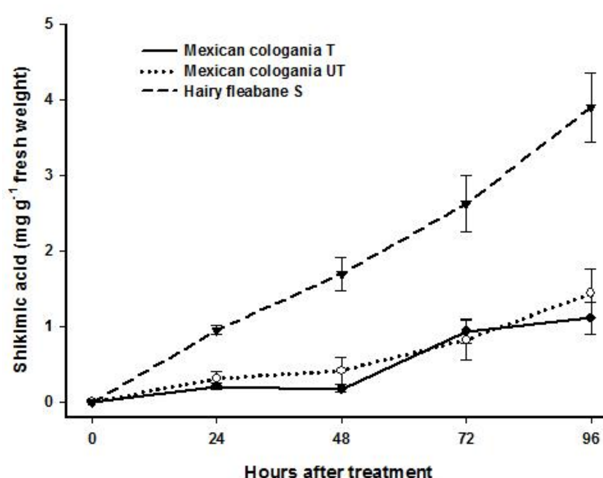

Figure 17. Accumulation of shikimic acid in two Mexican cologania populations and one hairy fleabane S population after application of glyphosate. Vertical bars represent the standard deviation of the mean (*n* = 9 technical replicates).

^{14}C-glyphosate absorption and translocation

Mexican cologania T plants showed significant differences (interaction population × time, $P < 0.0001$) in their foliar absorption (**Figure 18A**), and ^{14}C-glyphosate translocation compared to hairy fleabane plants (**Table 17**). The penetration of the herbicide was greater in hairy fleabane; 8.7% of the applied herbicide was absorbed at 24 HAT, and 57.2% at 96 HAT. ^{14}C-Glyphosate absorption in Mexican cologania T plants ranged from 0.9 to 19.5% between 24 and 96 HAT, respectively (**Figure 18A, Table 17**).

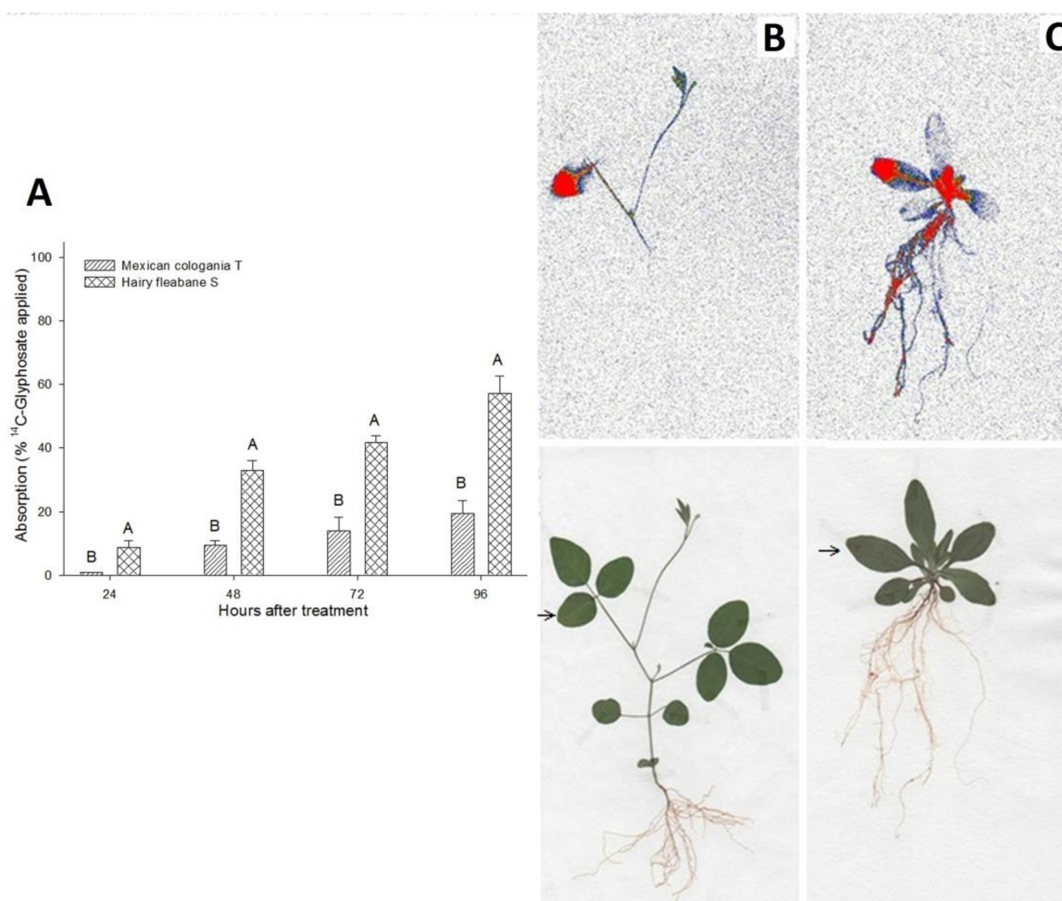

Figure 18. ^{14}C-glyphosate absorption and translocation in Mexican cologania T population and the hairy fleabane S population. A) Percentage of foliar absorption from ^{14}C-glyphosate applied in plants of the Mexican cologania T population and the hairy fleabane S population. Vertical bars represent the standard deviation of the mean (*n* = 5). B) Representative images that show the distribution of ^{14}C-glyphosate in the Mexican cologania T population (B) and the hairy fleabane S population (C) plants at 96 HAT. The highest concentration of ^{14}C-glyphosate is in indicated in red. Arrows indicate the treated leaf.

Thus, the Mexican cologania T population absorbed 37% less ^{14}C-glyphosate applied at 96 HAT compared to the hairy fleabane S population. A significant difference exists between the two species in the amount of herbicide translocated from the treated leaf to the rest of the plant (treated leaf, $P < 0.0001$; rest of plant, $P = 0.0012$; and roots, $P < 0.0001$). From the average percentage of ^{14}C-glyphosate absorbed in hairy fleabane S population, the percentage of ^{14}C-glyphosate in the treated leaves dropped from 73.8% to 34.4% between 24 and 96 HAT, respectively (**Table 17**). The percentage in the Mexican cologania T population ranged from 72.8% to 89% in treated leaves at 24 and 96 HAT, respectively (**Table 17**).

Table 17. Translocation of ^{14}C-glyphosate in plants of the Mexican cologania T population and hairy fleabane S population.

Species	HAT	Translocation (from % absorbed)		
		Treated leaf	Rest of plant	Root
Mexican cologania T	24	72.8 ± 2.1 c	21.9 ± 1.3 bc	5.3 ± 0.8 d
	48	78.3 ± 2.5 bc	19.4 ± 2.7 cd	2.3 ± 0.7 d
	72	82.9 ± 2.5 b	15.3 ± 2.3 d	1.9 ± 0.5 d
	96	89.0 ± 2.4 a	9.3 ± 2.2 e	1.7 ± 0.3 d
Hairy fleabane S	24	73.8 ± 2.6 c	15.7 ± 2.0 cd	10.5 ± 1.9 c
	48	64.2 ± 3.7 d	21.5 ± 2.4 cd	14.3 ± 1.8 c
	72	48.3 ± 2.8 e	28.3 ± 2.2 b	23.4 ± 2.1 b
	96	34.5 ± 2.7 f	35.4 ± 1.6 a	30.1 ± 1.6 a

Means with different letter within a column are statistically different at 5% probability determined by the Tukey test. ± Standard error of the mean (*n* = 5).

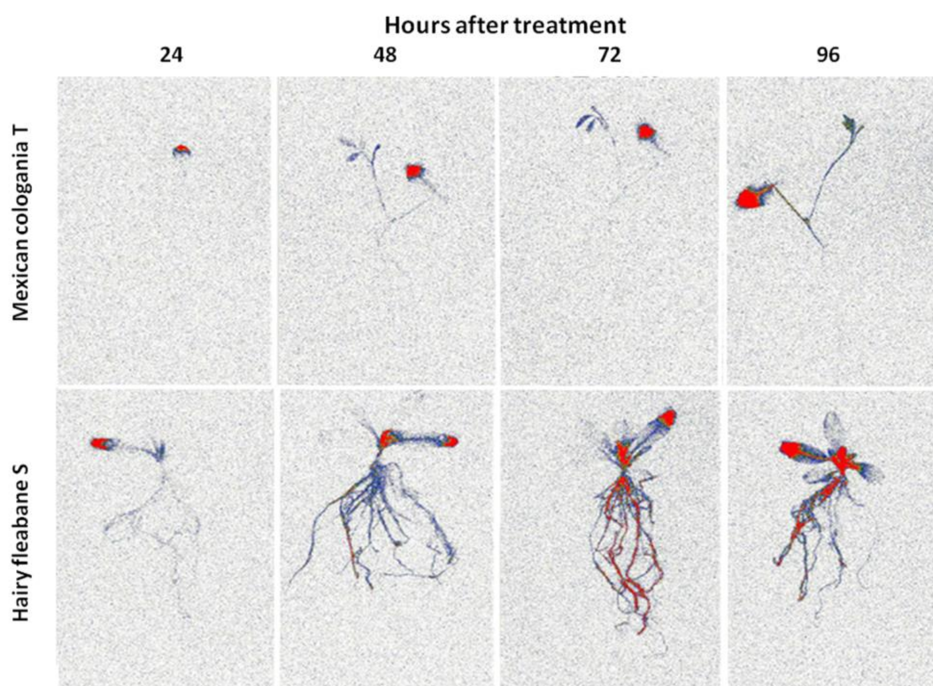

Figure 19. ^{14}C-Glyphosate translocation in plants of the Mexican cologania T populations and the hairy fleabane S population from 24 to 96 HAT. The ^{14}C-glyphosate distribution increased progressively from the treated leaves to the roots in the hairy fleabane S population as the exposure time increased. In the Mexican cologania T population, the herbicide mainly remained in the treated leaf and only small amounts reached the young shoots. The highest concentration of ^{14}C-glyphosate is indicated in red.

The translocation of ^{14}C-glyphosate in the Mexican Cologania T and hairy fleabane S populations was visualized using a phosphor imager, and it was clearly different (**Figure 18B** and **18C**). The hairy fleabane S population translocated a larger amount of ^{14}C-glyphosate from the treated leaf to young leaves 96 HAT. In roots, the combustion indicated that small amounts of ^{14}C-glyphosate were detected in the roots of the Mexican cologania T population (**Table 17**). However, in general, this amount was not detected at any time in the autoradiographies obtained from the phosphor imager compared to hairy fleabane S population (**Figure 18B** and **18C, Figure 19**).

Leaf morphology

The foliar histological traits of Mexican cologania T and hairy fleabane S, which play important roles in tolerance and susceptibility to glyphosate in both species, was observed by SEM. Wax coverage differed significantly between the two species (**Figure 20**).

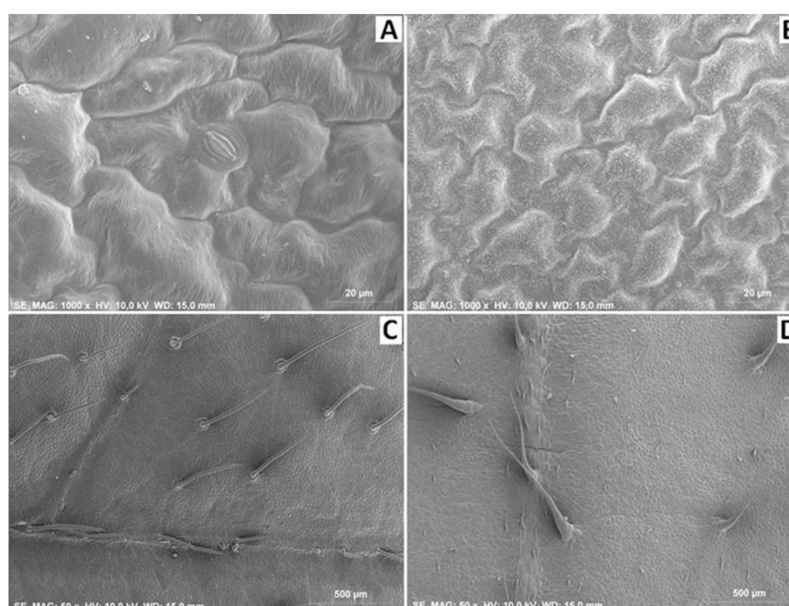

Figure 20. Micrographs of the wax coverage and foliar trichome distribution in leaves of the Mexican cologania T population and the hairy fleabane S population. Wax coverage on the outer surface from hairy fleabane S (A) and Mexican cologania T (B) leaves (bar = 20 µm). View of the foliar trichome distibution in hairy fleabane S (C) and Mexican cologania T (D) leaves (bar = 500 µm). The samples were fixed in glutaraldehyde.

The wax coverage percentage was not higher than 4.3% in hairy fleabane plants; Mexican cologania plants had an average wax coverage of

27.2%, exhibiting a greater coverage and small wax crystals forming a nonuniform 3D cover. The cuticular surface of hairy fleabane had a low wax coverage. **Figure 20** (**A** and **B**), shows an image of the wax coverage on leaves with an identical magnification. In addition, a larger number of trichomes per leaf can be seen (parameter not estimated) in the hairy fleabane S population compared to the Mexican cologania T population (**Figure 20C** and **20D**).

Metabolism

The glyphosate metabolism study revealed significant differences between the Mexican cologania T population and the hairy fleabane S population. Smaller glyphosate amounts were detected in Mexican cologania compared to hairy fleabane. This latter species translocated a larger amount of herbicide to the root due to greater penetration, as evidenced by the ^{14}C-glyphosate absorption and translocation assays. The gradual metabolism of glyphosate in Mexican cologania reached the lowest level in leaves at 20 DAT (13.21 nmol g^{-1} fresh weight). At this point in time, the concentration of herbicide was reduced very little in hairy fleabane leaves, and larger amounts of herbicide were detected in the roots of this species (17.32 nmol g^{-1} fresh weight 20 DAT). The major metabolite found in hairy fleabane was glyoxylate. The levels of this compound were far higher in hairy fleabane than in Mexican cologania from 4 to 20 DAT, and glyoxylate was concentrated mainly in the aerial parts of the plant. AMPA was also found in hairy fleabane (0.19 nmol g^{-1} fresh weight). However, this compound was only detected at 20 DAT (**Table 18**).

Mexican cologania was capable of metabolizing glyphosate to other compounds. In this species, the glyoxylate levels in the leaves and roots were very low and constant at all times. However, AMPA was detected at 8 DAT in the leaves and at 16 DAT in the roots. At 20 DAT, AMPA content reached 15.60 and 0.48 nmol g^{-1} fresh weight in the leaves and roots, respectively. Sarcosine was detected only in Mexican cologania in the leaves at 16 DAT and in the roots at 12 DAT; formaldehyde was detected in the leaves at 20 DAT and in the roots at 16 DAT (**Table 18**). The fact that metabolites were first detected in Mexican cologania suggests that it metabolizes glyphosate faster than hairy fleabane.

Table 18. Glyphosate metabolites found in treated plants of the Mexican cologania T and hairy fleabane S populations.

Part	Species	DAT	Metabolites (nmol g^{-1} fresh weight) Glyphosate	Glyoxylate	AMPA	Sarcosine	Formaldehyde
Leaves	Mexican cologania T	4	35.73 ± 3.61 d	-0.41 ± 0.05 b	nd	nd	nd
		8	36.89 ± 2.21 d	-0.88 ± 0.02 b	0.04 ± 0.01 d	nd	nd
		12	33.70 ± 2.28 d	-0.22 ± 0.08 b	2.98 ± 0.10 c	nd	nd
		16	28.43 ± 1.60 d	0.05 ± 0.01 b	6.44 ± 0.45 b	0.06 ± 0.01 c	nd
		20	13.22 ± 2.71 ef	0.07 ± 0.01 b	15.60 ± 1.56 a	0.14 ± 0.01 c	0.03 ± 0.01 b
	Hairy fleabane S	4	66.24 ± 6.07 a	6.59 ± 0.59 a	nd	nd	nd
		8	76.56 ± 4.30 a	5.10 ± 0.44 a	nd	nd	nd
		12	63.53 ± 2.64 b	6.22 ± 0.76 a	nd	nd	nd
		16	47.29 ± 5.03 c	5.19 ± 0.13 a	nd	nd	nd
		20	49.04 ± 0.95 c	6.01 ± 0.21 a	0.20 ± 0.03 d	nd	nd
Root	Mexican cologania T	4	0.04 ± 0.01 h	0.09 ± 0.01 b	nd	nd	nd
		8	0.07 ± 0.01 h	-0.05 ± 0.01 b	nd	nd	nd
		12	0.18 ± 0.04 h	-0.01 ± 0.01 b	nd	0.29 ± 0.05 b	nd
		16	0.29 ± 0.01 h	0.02 ± 0.01 b	0.27 ± 0.05 d	0.32 ± 0.04 b	0.06 ± 0.01 b
		20	0.65 ± 0.04 h	-0.01 ± 0.01 b	0.48 ± 0.02 d	0.48 ± 0.02 a	0.17 ± 0.01 a
	Hairy Fleabane S	4	0.48 ± 0.05 h	0.08 ± 0.01 b	nd	nd	nd
		8	1.79 ± 0.67 gh	0.13 ± 0.06 b	nd	nd	nd
		12	3.59 ± 0.96 fgh	0.14 ± 0.01 b	nd	nd	nd
		16	10.68 ± 1.20 efg	0.09 ± 0.01 b	nd	nd	nd
		20	17.32 ± 2.30 e	0.13 ± 0.01 b	nd	nd	nd

Means with different letter within a column are statistically different at 5% probability determined by the Tukey test. ± Standard error of the mean (*n* = 9 technical replicates). nd: Not detected.

Discussion

Due to the fact that in previous tests the Mexican cologania T and UT populations had not shown any significant differences, the hairy fleabane S population was used in this work as a sensitive type reference. Furthermore, other works on glyphosate tolerance have been documented comparing taxonomically different species: e.g. *Canavalia ensiformis, Clitoria ternatea, Commelina benghalensis, Ipomoea grandifolia* and *Neonotonia wightii* were compared to *Amaranthus hybridus* (Cruz-Hipolito et al. 2009, 2011; Mantero et al. 2004), and *Mucuna pruriens* compared to *A. retroflexus* (Rojano-Delgado et al. 2012).

Dose-response results strongly suggest that Mexican cologania is naturally glyphosate-tolerant. Ribeiro et al. (2015), found significant differences between accessions of *Ipomea lacunosa*, a species with possible glyphosate tolerance which had been treated or not with glyphosate. The ED_{50} values ranged from 58 to 151 g ae of glyphosate ha^{-1} between the least tolerant accession and the most tolerant one of *I. lacunosa*. In addition, in a field assay conducted in Chapingo, Mexico, Matínez-Aguilar (2009), reported an ED_{50} of 784g ae ha^{-1} in Mexican cologania. This result differs from those obtained in our experiments, because the plants respond differently when exposed to different glyphosate concentrations under controlled conditions (Henry et al. 2007), which vary widely with respect to field conditions as indicated by Matínez-Aguilar (2009), under wich Mexican cologania tolerated applications of glyphosate of above 720 g ae ha^{-1}. This is the recommended field dose sufficient to control a large number of susceptible annual weed species such as *Amaranthus hybridus* (ED_{50} = 42.2 g active ingredient ha^{-1}) (Cruz-Hipolito et al. 2011), *Conyza canadensis* (ED_{50} = 34.9 g ae ha^{-1}) (Gonzalez-Torralva et al. 2010), among others. Cruz-Hipolito et al (2009, 2011) and Rojano-Delgado et al. (2012), reported in other legumes with a high tolerance to glyphosate, an ED_{50} of 315 (g ai ha^{-1}), 600, 403 and 362 g ae ha^{-1} in *Canavalia ensiformis, Clitoria ternatea*, *Mucuna pruriens* and *Neonotonia wightii*, respectively.

Due to the low ED_{50} value estimated for Mexican cologania populations (166.4 (UT) and 175.3 (T) g ae ha^{-1}), this species could be considered as a

non-tolerant species (**Table 16**). However, a gradual reduction in growth and the appearance of chlorosis symptoms were observed when treatment doses were increased. However, the treated plants of Mexican cologania T and UT populations did not die at a dose of 1000 g ae ha^{-1} at 21 DAT, and regained strenuousness, while hairy fleabane plants died after an application with only 90 g ae ha^{-1} (**Figure 21**). The growth inhibition caused by glyphosate is almost immediate. It is followed by chlorosis in growth buds and necrosis throughout the plant, which occurs within 1-2 weeks (Shaner et al. 2005; Henry et al. 2007).

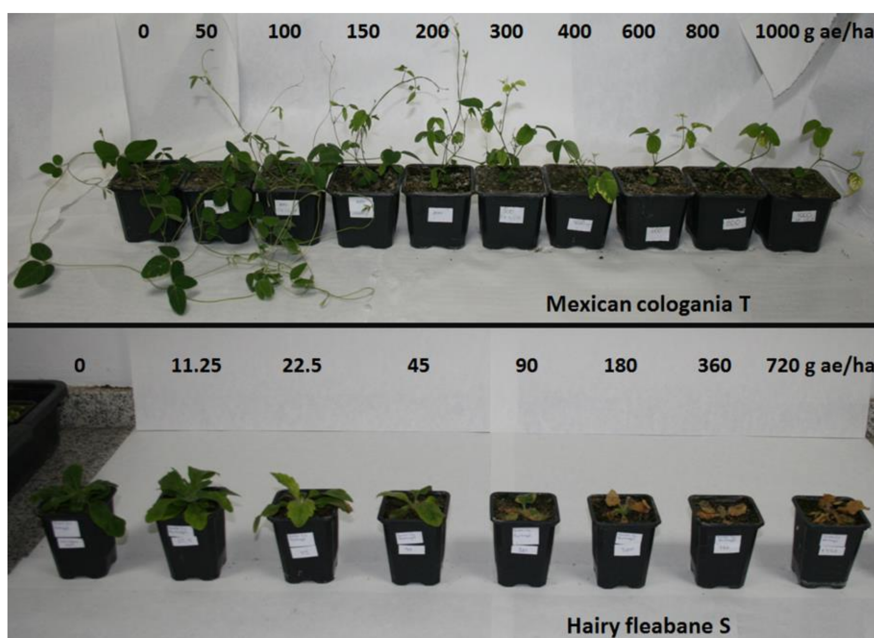

Figure 21. Plants of the Mexican cologania T population and the hairy fleabane S population from dose-response assay before evaluation at 21 DAT. Mexican cologania T population showed a progressive growth reduction and chlorosis symptoms as the glyphosate dose increased, but the plants did not die. Plants of the hairy fleabane S population died from 90 g ae ha^{-1}.

Changes in shikimic acid levels in plants are the result of the specific inhibition of EPSPS enzyme (Amrhein et al. 1980). The accumulation of shikimic acid in treated plants is accepted as an appropriate parameter for determining resistance or susceptibility to glyphosate (Henry et al. 2007). A species with a low shikimic acid accumulation requires a larger amount of glyphosate to be lethal (i.e., a higher ED_{50}).

The hairy fleabane S population accumulated a larger amount of shikimic acid that ranged from 2.7 to 3.5 times with respect to UT and T Mexican cologania populations, respectively. Similar results were previously obtained by

Cruz-Hipolito et al. (2009, 2011) and Rojano-Delgado et al. (2012) in other glyphosate tolerant legumes (*Canavalia ensiformis, Clitoria ternatea, Mucuna pruriens* and *Neonotonia wightii*) from Mexico when they were compared to *Amaranthus* spp. This demonstrates the high susceptibility to glyphosate observed in hairy fleabane S population compared to the populations of Mexican cologania.

According to the dose-response, leaf retention and shikimic acid assays there was no significant differences between T and UT Mexican cologania populations; the ^{14}C-glyphosate absorption and translocation, leaf micromorphology and metabolism studies were only carried out in Mexican cologania T and hairy fleabane S populations.

For hairy fleabane S population, the ^{14}C-glyphosate absorbed was distributed throughout the plant. In Mexican cologania T population, the greatest accumulation of ^{14}C-glyphosate was retained mainly in the treated leaf (**Figure 19**). This was due to reduced glyphosate translocation presented by the Mexican cologania T population. These results are consistent with Cruz-Hipolito et al. (2009, 2011) and Rojano-Delgado et al (2012), where reported similar behavior in other glyphosate-tolerant legumes (*Canavalia ensiformis, Clitoria ternatea, Mucuna pruriens* and *Neonotonia wightii*). The Mexican cologania T population only showed a small amount of translocated glyphosate both acropetally and basipetally at 96 HAT. Changes in the transport of glyphosate in the symplast have been previously reported in several resistant biotypes with a decreased translocation to the roots, shoots and meristematic zones of untreated young leaves (Shaner, 2009; Feng et al. 1999, 2004).

The abundant presence of wax on Mexican cologania leaves may have prevented a direct contact of the glyphosate solution with leaf surfaces, thus limiting penetration. The cuticular waxes are non-polar, similar to oil in their ability to repel water (Heredia-Guerrero et al. 2014). Herbicides in aqueous solutions, such as glyphosate, are absorbed less when cuticular waxes are abundant (Devine et al. 1992). According to the literature, some trichomes can facilitate herbicide absorption (Devine et al. 1992). Higher spray retention and glyphosate absorption in hairy fleabane plants may be attributed to less wax coverage and a larger number of trichomes compared to Mexican cologania

plants. These results partially explain the difference in susceptibility observed between the two species.

The total summation of glyphosate metabolites was not equal 100% of the glyphosate applied due to translocation and physical, chemical and biological degradation. Glyphosate is converted into AMPA, glyoxylate, sarcosine and formaldehyde reducing the amount of glyphosate that can reach the action site. Glyphosate degradation and metabolism has been examined as a potential resistance mechanism by several researchers (Sammons and Gaines, 2014; Alarcón-Reverte et al. 2013; Feng et al. 1999, 2004; Lorraine-Colwill et al. 2002; Dinelli et al. 2006). Glyoxylate was found in larger amounts in both treated populations. This compound is involved in photorespiration and is derived from the degradation of glyphosate and the glyoxylate cycle (Pedotti et al. 2006; Weber, 2006). In Mexican cologania, the glyoxylate concentration decreased with treatment. These results indicate that glyphosate metabolism is a mechanism of glyphosate tolerance in Mexican cologania. Other authors who have worked with glyphosate-tolerant plants (*Clitoria ternatea, Convolvulus arvensis, Ipomea lacunosa, I. purpurea, Mucuna pruriens* and *Neonotonia wightii*) have also demonstrated the ability of these plants to metabolize glyphosate mainly to AMPA (Mantero et al. 2004; Cruz-Hipolito et al. 2009, 2011; Rojano-Delgado et al. 2012; Ribeiro et al. 2015). This compound is moderately toxic (González-Torralva et al. 2012). Similarly, glyphosate-resistant *Digitaria insularis* biotypes metabolize glyphosate to the same metabolites as legumes (de Carvalho et al. 2012). However, glyphosate degradation to non-toxic compounds has not been found to be the mechanism of resistance in other species such as *Conyza canadensis* and *Lolium rigidum* from USA (Feng et al. 1999, 2004; Lorraine-Colwill et al. 2002; Dinelli et al. 2006).

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CAPITULO VI

Conclusions

Conclusions

The results of research conducted allowed to conclude the following:

- The characterization of the efficacy of glyphosate by whole plant dose response experiments and shikimic acid accumulation assays corroborated glyphosate resistance in *L. virgata*.

- Glyphosate resistance spread, across Persian lime groves from Veracruz between *L. virgata* populations from distant and adjacent locations, is likely due to both short- and long-distance seed dispersal, as well as independent evolutionary events.

- The resistant *L. virgata* populations presented a reduced absorption and translocation as main mechanisms to against to glyphosate, and one mutation in the target-site endowed of a higher resistance to the R15 population.

- The reduced translocation and the relationship between the parameters of ED_{50}, LD_{50} and I_{50} confirmed glyphosate resistance of *B. pilosa*.

- The R2 *B. pilosa* population used reduced translocation of ^{14}C-glyphosate, to its target site (EPSPS) as major mechanism, to resist against glyphosate presenting a low-intermediate resistance.

- The Ser-106 and TIPS mutations found in the EPSPS2 gene, in association with a reduced glyphosate translocation, those were responsible for conferring high resistance to the R1 *B. pilosa* population.

- The confirmation of *L. virgata* and *B. pilosa* resistance suggests the need to make an appropriate rotation of herbicides to weed manage in citrus groves. In addition, to adapt diverse management programs and appropriate strategies to prevent the dispersal of resistant seeds.

- The main glyphosate tolerance mechanism involved in *C. broussonetti* was the reduced absorption, and poor translocation to its action site.

- Glyphosate absorption of *C. broussonetti* is influenced by morphological features, which play an important role in herbicide penetration.

- Furthermore, *C. broussonetti* have the ability to metabolize the glyphosate to different non-toxic compounds such as AMPA, glyoxylate, sarcosine and formaldehyde.

- The use of *C. broussonetti* as a cover crop in fruit orchards of temperate climate, where glyphosate can be applied selectively for weed management, is highly feasible.

Conclusiones

Los resultados de las investigaciones realizadas permitieron llegar a las siguientes conclusiones:

- La caracterización de la eficacia del glifosato mediante experimentos de dosis-respuesta y los ensayos de acumulación de ácido shiquímico corroboraron la resistencia al glifosato en *L. virgata*.

- La dispersión de la resistencia a glifosato, en las plantaciones de limón persa de Veracruz entre las poblaciones de *L. virgata* desde lugares distantes y adyacentes, es probablemente debido a la dispersión de semillas, tanto a corta como a larga distancia, así como a eventos evolutivos independientes.

- Las poblaciones resistentes (R8, R14 y R15) de *L. virgata* presentaron una reducida absorción y translocación de glifosato como principales mecanismos de resistencia al glifosato. Además, una mutación (Pro-106-Ser) en el target-site confiere de una mayor resistencia a la población R15.

- La reducida translocación y la relación entre los parámetros de ED_{50}, LD_{50} e I_{50} confirmaron la resistencia a glifosato de *B. pilosa*.

- La población R2 de *B. pilosa* utiliza la reducida translocación de ^{14}C-glifosato si target-site (EPSPS), como principal mecanismo para resistir al glifosato presentado una resistencia intermedia.

- Las mutaciones Ser-106 y TIPS encontradas en el gen de la EPSPS2, en asociación con una reducida translocación de glifosato, son los responsables de conferir alta resistencia a glifosato en la población R1 de *B. pilosa*.

- La confirmación de la resistencia a glifosato de *L. virgata* y *B. pilosa* sugiere la necesidad de hacer una adecuada rotación de herbicidas para el manejo de malas hierbas en plantaciones de cítricos de los estados de Puebla y Veracruz (México). Además, adaptar diversos programas de

gestión y estrategias apropiadas para evitar la dispersión de semillas resistentes.

- El principal mecanismo de tolerancia a glifosato implicado en *C. broussonetii* fue la reducida absorción y translocación a su sitio de acción.

- La reducida absorción de glifosato que presento *C. broussonetii* está influenciado por rasgos morfológicos, que desempeñan un importante papel en la penetración del herbicida.

- Además, *C. broussonetii* tiene la capacidad de metabolizar el glifosato a compuestos no tóxicos tales como AMPA, glioxilato, sarcosina y formaldehído.

- El uso de *C. broussonetii* como cultivo de cobertura en los huertos de frutales de clima templado, en los que el glifosato se puede aplicar de forma selectiva para el control de malezas, es altamente factible.

CAPITULO VII

Publications

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 inter simple sequence repeat (ISSR) markers were characterized for seventeen 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 (*Hs*) 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 (AMOVA) 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.

Keywords: diversity within/among populations; genetic diversity; glyphosate resistance spread; ISSR markers; tropical sprangletop populations; resistance index.

Physiological, Morphological and Biochemical Studies of Glyphosate Tolerance in Mexican Cologania (*Cologania broussonetii* (Balb.) DC.).

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Research article

Physiological, morphological and biochemical studies of glyphosate tolerance in Mexican Cologania (*Cologania broussonetii* (Balb.) DC.)

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In recent years, glyphosate-tolerant legumes have been used as cover crops for weed management in tropical areas of Mexico. Mexican cologania (*Cologania broussonetii* (Balb.) DC.) is an innate glyphosate-tolerant legume with a potential as a cover crop in temperate areas of the country. In this work, glyphosate tolerance was characterized in two Mexican cologania (a treated (T) and an untreated (UT)) populations as being representatives of the species, compared in turn to a glyphosate-susceptible hairy fleabane (S) (*Conyza bonariensis* (L.) Cronq.) population. Experiments revealed that T and UT Mexican cologania populations had a higher tolerance index (TI), and a lower shikimic acid accumulation and foliar retention than the hairy fleabane S population. Absorption and translocation, leaf morphology and metabolism studies were only carried out in the Mexican cologania T population and the hairy fleabane S population. The latter absorbed 37% more ^{14}C-glyphosate compared to the Mexican cologania T at 96 h after treatment (HAT). Mexican cologania T translocated less herbicide from the treated leaf to the remainder of the plant than hairy fleabane S. The Mexican cologania T presented a greater epicuticular wax coverage percentage than the hairy fleabane S. This morphological characteristic contributed to the low glyphosate absorption observed in the Mexican cologania. In addition, the Mexican cologania T metabolized glyphosate mainly into AMPA, formaldehyde and sarcosine. These results indicate that the high glyphosate tolerance observed in Mexican cologania is mainly due to the poor penetration and translocation of glyphosate into the active site, and the high glyphosate degradation into non-toxic substances.

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1. Introduction

Weeds reduce crop productivity, displace native species, contribute to land degradation and increase production costs. A cover crop is constituted by a living vegetal that keeps the soil covered, protects it from erosion, and prevents nutrient loss (Hartwig and Ammon, 2002). Cover crops are also involved in the elimination of weeds and they reduce the spread of numerous pathogens (Renard and Franqueville, 1991). Cover crops allow for the selective use of herbicides within integrated weed management programs in some agricultural systems, drastically reducing germination, emergence and/or growth of weed populations (Lal et al., 1991; Buhler et al., 2001). The use of cover crops for weed displacement and soil protection against erosion presents some limitations, such as the interference of weeds at the early stages of cover crop growth (Cameron et al., 1991). At that point, the use of herbicides with good toxicological and environmental profiles, such as glyphosate, could facilitate crop establishment (Domínguez-Valenzuela, personal observation).

Herbicide tolerance is a natural, hereditary mechanism that allows a plant species to survive and reproduce after herbicide application; this survival is due to inherent morphological and/or physiological characteristics. Therefore, herbicide-tolerant populations have never been susceptible (Cruz-Hipolito et al., 2009). Resistant populations of weed species are naturally present and occur at low densities. Herbicide resistance happens when resistant weeds are able to survive and complete their life cycles when the herbicide is applied at recommended field doses. Repeated

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Response of *Eleusine indica* and *Paspalum distichum* to Glyphosate Following Repeated Use in Perennial Field Crops.

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Response of *Eleusine indica* and *Paspalum distichum* to glyphosate following repeated use in citrus groves

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ABSTRACT

Eleusine indica L. Gaertn. and *Paspalum distichum* L. are annual and perennial grasses, respectively that are widely distributed in turf and perennial cropping systems throughout Spain. Often, glyphosate is used between rows of perennial crops for control of these grasses, but variable responses have been observed. Sensitivity to glyphosate in each species was examined under greenhouse, laboratory and field conditions. *In vitro* tests on whole plants of both *P. distichum* and *E. indica* revealed no differences in sensitivity to glyphosate for areas with long-term use (treated; T) and no history of use (not treated; NT). The NT population of *P. distichum* (ED_{50} 73.1 g ae ha^{-1}) was 11.6% more sensitive to glyphosate than NT *E. indica* (ED_{50} 81.6 g ae ha^{-1}). No differences between T and NT populations of both species were observed for foliar retention of glyphosate as well as accumulation of shikimate. However, glyphosate retention and shikimate accumulation were up to 64 and 24.4% greater, respectively in *P. distichum* compared to *E. indica*. Within 96 h after treatment (HAT), foliar absorption of ^{14}C-glyphosate was similar among T and NT populations, but 8.8% higher for *P. distichum* compared to *E. indica*. Retention of ^{14}C-glyphosate in treated leaves of *P. distichum* was approximately 55% lower compared to *E. indica*. Translocation from the treated leaf into other shoot tissue (2.8-fold) and roots (8.5-fold) was higher for *P. distichum* versus *E. indica*. This would suggest that differences in *E. indica* versus *P. distichum* response to glyphosate are based upon differential retention in treated leaves and reduced movement out of treated tissue in other shoot and root tissue. In separate field experiments in citrus orchards, glyphosate and other herbicides were applied to assess control of *E. indica* and *P. distichum* over two years. Flazasulfuron and cycloxidim resulted in 90% or greater control of both species by 60 days after treatment (DAT). Only glufosinate, oxyfluorfen, paraquat and iodosulfuron resulted in >85% control of *E. indica*. These corresponding treatments ranged in effectiveness from 73 to 92% on *P. distichum*. Integration of effective herbicides with modes of action different than glyphosate should be used for management of *E. indica* and *P. distichum* and may delay the selection for resistance to glyphosate.

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1. Introduction

In the Mediterranean region, no-till practices are adopted commonly to conserve soil resources in perennial cropping systems such as olive (*Olea europaea* L.) groves, *Citrus* spp. orchards and grape (*Vitis vinifera* L.) vineyards (Cerda et al., 2015). In the absence of tillage, living cover crops consisting of barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and legumes such as vetch (*Vicia* spp.) and lupins (*Lupinus* spp.) are established to deter weed establishment, build soil organic matter, and reduce soil erosion (Gomez et al., 2011; Hartwig and Ammon, 2002). In some cases, grass weeds are allowed to develop in open canopy areas to conserve soil. Growth of cover crops or naturally established weeds is controlled by mowing, non-selective herbicides or animal grazing.

The herbicide glyphosate is frequently applied beneath perennial crops in Spain to manage cover crops or other vegetation (Costa, 1997). Lacking residual activity, glyphosate is non-selective and controls a broad-spectrum of annual and perennial plant

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Underlying Resistance Mechanisms in the *Cynosurus echinatus* Biotype to Acetyl CoA Carboxylase-Inhibiting Herbicides.

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Underlying Resistance Mechanisms in the *Cynosurus echinatus* Biotype to Acetyl CoA Carboxylase-Inhibiting Herbicides

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Hedgehog dogtail (*Cynosurus echinatus*) is an annual grass, native to Europe, but also widely distributed in North and South America, South Africa, and Australia. Two hedgehog dogtail biotypes, one diclofop-methyl (DM)-resistant and one DM-susceptible were studied in detail for experimental dose-response resistance mechanisms. Herbicide rates that inhibited shoot growth by 50% (GR_{50}) were determined for DM, being the resistance factor ($GR_{50}R/GR_{50}S$) of 43.81. When amitrole (Cyt. P_{450} inhibitor) was applied before treatment with DM, the R biotype growth was significantly inhibited (GR_{50} of 1019.9 g ai ha^{-1}) compared with the GR_{50} (1484.6 g ai ha^{-1}) found for the R biotype without pretreatment with amitrole. However, GR_{50} values for S biotype do not vary with or without amitrole pretreatment. Dose-response experiments carried out to evaluate cross-resistance, showed resistance to aryloxyphenoxypropionate (APP), cyclohexanedione (CHD) and phenylpyrazoline (PPZ) inhibiting herbicides. Both R and S biotypes had a similar ^{14}C-DM uptake and translocation. The herbicide was poorly distributed among leaves, the rest of the shoot and roots with unappreciable acropetal and/or basipetal DM translocation at 96 h after treatment (HAT). The metabolism of ^{14}C-DM, D-acid and D-conjugate metabolites were identified by thin-layer chromatography. The results showed that DM resistance in *C. echinatus* is likely due to enhanced herbicide metabolism, involving Cyt. P_{450} as was demonstrated by indirect assays (amitrole pretreatment). The ACCase *in vitro* assays showed that the target site was very sensitive to APP, CHD and PPZ herbicides in the *C. echinatus* S biotype, while the R biotype was insensitive to the previously mentioned herbicides. DNA sequencing studies confirmed that *C. echinatus* cross-resistance to ACCase inhibitors has been conferred by specific ACCase double point mutations Ile-2041-Asn and Cys-2088-Arg.

Keywords: ***Cynosurus echinatus*, ^{14}C-DM, metabolism, ACCase activity, ACCase point mutations**

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HIGHLIGHT

Hedgehog dogtail (*Cynosurus echinatus*) is an annual grass that is native to Europe, but is also widely distributed in North and South America, as well as South Africa and Australia. Plants are sensitive to herbicides that selectively inhibit the plastid enzyme ACCase, including diclofop-methyl (DM). Following many years of effective control, some reports have surfaced recently

Unravelling the Resistance Mechanisms to 2,4-D (2,4-Dichlorophenoxyacetic Acid) in Corn Poppy (*Papaver rhoeas*).

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Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*)

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In southern Europe, the intensive use of 2,4-D (2,4-dichlorophenoxyacetic acid) and tribenuron-methyl in cereal crop systems has resulted in the evolution of resistant (R) corn poppy (*Papaver rhoeas* L.) biotypes. Experiments were conducted to elucidate (1) the resistance response to these two herbicides, (2) the cross-resistant pattern to other synthetic auxins and (3) the physiological basis of the auxin resistance in two R (F-R213 and D-R703) populations. R plants were resistant to both 2,4-D and tribenuron-methyl (F-R213) or just to 2,4-D (D-R703) and both R populations were also resistant to dicamba and aminopyralid. Results from absorption and translocation experiment revealed that R plants translocated less [14C]-2,4-D than S plants at all evaluation times. There was between four and eight-fold greater ethylene production in S plants treated with 2,4-D, than in R plants. Overall, these results suggest that reduced 2,4-D translocation is the resistance mechanism in synthetic auxins R corn poppy populations and this likely leads to less ethylene production and greater survival in R plants.

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1. Introduction

Agricultural weeds cause major crop losses by competing for nutrients, water or light. Even though non-chemical methods have been used for controlling weeds, herbicides are considered the most effective means of weed control [1]. 2,4-D (2,4-dichlorophenoxyacetic acid), an auxinic herbicide, was commercially released in 1946 becoming the first successful selective herbicide to specifically target dicotyledonous weeds. 2,4-D still remains as one of the most commonly used herbicides in the world as a consequence of its low cost, selectivity, efficacy and wide spectrum of weed control [2]. The auxinic herbicide family (group O according to the Herbicide Resistance Action Committee, HRAC; and group 4 according to the Weed Science Society of America, WSSA) contains four chemical groups, including pyridine-carboxylic acids (i.e. aminopyralid), quinolinecarboxylic acids (i.e. quinclorac), benzoic acids (i.e. dicamba), and phenoxy-carboxylic acids (i.e. 2,4-D).

After 60 years of widespread and repeated usage, few examples of resistance to this mode of action have been reported. Generally, the selection of synthetic auxin resistant biotypes requires more generations than for other modes of action herbicides, particularly acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACCase) inhibitors [3]. Several reasons have been proposed to explain this phenomenon, including low mutation rates, fitness penalties and redundancy in auxin receptors within the plant [2,4]. Nowadays, there are 32 auxinic herbicide resistance species, 15 of those being resistant to 2,4-D [5]. The precise mode of action for these herbicides, and consequently, the resistance mechanisms in weeds are, however, still poorly understood [2,6]. Nonetheless, new discoveries including nuclear auxin receptors (F-box proteins), influx and efflux carriers and plasma membrane bound receptors have provided basic clues as to the molecular mode of action of these herbicides [6–10].

The characterization of resistance mechanisms has been investigated in few auxinic herbicide-resistant weeds. Differential absorption, translocation, or metabolism were not the basis for resistance in the majority of the assessed species [11–15]. Only in a few weeds these non-target-site mechanisms (NTSM) have been related with the resistance response [3,16,17]. Additionally, it has been reported that the application of auxinic herbicides stimulates ethylene biosynthesis in sensitive, but not in resistant plants [13,15,18]. This unregulated auxin response and the resulting hyperaccumulation of ethylene, abscisic acid (ABA) and reactive oxygen species (ROS) in auxinic herbicide sensitive plants may be involved in the induction of tissue damage and cell death after synthetic auxins application [19].

Corn poppy (*Papaver rhoeas* L.) is a major weed of cereal crops in Southern Europe [20]. Its extended germination period, high seed production, and seed bank persistence makes it especially difficult to manage. It has been estimated that corn poppy can decrease wheat yields up

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Resistance to Imazamox in Clearfield Soft Wheat (*Triticum aestivum* L.).

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ABSTRACT

Imidazolinone (IMI)-resistant crops exhibit relative tolerance to herbicides that inhibit the enzyme acetolactate synthase (ALS). The principal objective of this work was to evaluate the different resistance levels to imazamox between five Clearfield wheat cultivars. The IMI-resistant wheat cultivars (Bicentenario, Dollinco, Impulso, Invento, and Ikaro), widely planted in large areas in Latin America, were compared to a sensitive cultivar (Pandora) using several *in vivo* and *in vitro* experiments. The imazamox dose, expressed as g ai ha^{-1} that reduced the wheat fresh biomass by 50% (ED_{50}), ranged from 151.0 (Ikaro) to 1.6 (Pandora). The herbicide concentrations (μM) that inhibited the ALS activity by 50% (I_{50}) were in agreement with the ED_{50} values, suggesting that imazamox resistance could be due to a mutation in the ALS enzyme. The order of Clearfield wheat cultivars by the level of resistance to imazamox was: Ikaro > Impulso > Invento > Bicentenario = Dollinco >>> Pandora.

The finding that IMI-resistant wheat cultivars regained their photosynthetic activity with time and the fact that Ikaro plants showed an increased level of resistance over time suggest that other resistance mechanisms might be involved in the differential tolerance to imazamox in these wheat cultivars.
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1. Introduction

The wheat is one of the major world crop with a production exceeding 650 million tons. Weeds are included among the major biotic limitations to crop productivity (Shennan, 2008). Unfortunately, weeds have been and are a barrier which reduce the quality and quantity of this important food. The weeds establish a relationship of competition with the main crop to obtain water and nutrients, essential for growth and development of plants. This competition can reduce more than 50% of the yield (Munsif et al., 2014).

The control of several weeds harmful to wheat has improved since the 1980's with the release of herbicides that inhibit the enzyme acetolactate synthase (ALS). This group of herbicides comprises five chemical families: imidazolinones (IMIs), sulfonylureas, triazolopyrimidines, pyrimidinylthiobenzoates, and sulfanilamide-carbonyl-thiazolidinones (Devine et al., 1993; Pang et al., 2003; McCourt et al., 2006; Yu and Powles, 2014).

In partnership with the BASF (Badische Anilin und Soda Fabrik) (BASF, 2010) company, agricultural research institutes in Chile have developed, through classical plant breeding methods (mutagenesis, plant selection, and back crosses with elite cultivars), several wheat cultivars resistant (R) to IMI herbicides, commercialised under the trade name "Clearfield crops" (Newhouse et al., 1992). Some of these are Bicentenario, Dollinco, Ikaro, Impulso, and Invento.

In general, the possible herbicide resistance mechanisms in plants comprise altered herbicide retention on the crop leaf surface, impaired uptake, impaired translocation and/or vacuolar sequestration, herbicide detoxification by the plant, as well as insensitivity of the target enzyme to the herbicide (Park and Mallory-Smith, 2004; Tan et al., 2005; Ge et al., 2010; Powles and Yu, 2010; Rosario et al., 2011; Yu and Powles, 2014).

The development of Clearfield wheat cultivars that are resistant to IMI herbicides is one approach to increasing wheat grain yields. A resistant crop to imazamox is normally attributed to a mutation on the target site enzyme acetolactate synthase (ALS or EC 2.2.1.6) (Shaner et al., 1996; Anderson et al., 2004). However, other resistance mechanisms cannot be excluded (Tan et al., 2005).

Due to the ALS enzyme functions at the chloroplast level, it has also been hypothesised that variables such as photosynthesis

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