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Departamento de Química Agrícola, Edafología y Microbiología

Programa de Doctorado

BIOCIENCIAS Y CIENCIAS AGROALIMENTARIAS

TESIS DOCTORAL

RESISTENCIA A IMITADORES DE AUXINAS Y HERBICIDAS INHIBIDORES DE LA EPSPS Y ALS EN MALAS HIERBAS DICOTILEDÓNEAS. MECANISMOS DE RESISTENCIA

RESISTANCE TO AUXIN MIMICS AND EPSPS AND ALS INHIBITOR HERBICIDES IN DICOTYLEDONOUS WEEDS. RESISTANCE MECHANISMS

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TÍTULO DE LA TESIS:

Resistencia a imitadores de auxinas y herbicidas inhibidores de la EPSPS y ALS en malas hierbas dicotiledóneas. Mecanismos de Resistencia

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INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El **Dr. Rafael A. De Prado Amián**, catedrático emérito de la Universidad de Córdoba (España) y el **Dr. Hugo E. Cruz Hipólito**, Gerente de Desarrollo en FMC Agroquímica (México) como directores del presente trabajo de investigación titulado "**Resistencia a imitadores de auxinas y herbicidas inhibidores de la EPSPS y ALS en malas hierbas dicotiledóneas. Mecanismos de Resistencia"**, el cual constituye la memoria que presenta el D. Candelario Palma Bautista para aspirar al grado de **Doctor en Biociencias y Ciencias Agroalimentarias**.

INFORMAN:

Que habiendo realizado en el laboratorio del Departamento de Química Agrícola, Edafología y Microbiología de la Universidad de Córdoba bajo nuestra dirección y supervisión. Consideramos que el doctorando cumple con los requisitos legales para optar al grado de **Doctor en Biociencias y Ciencias Agroalimentarias**. Los resultados obtenidos por el trabajo realizado son de gran relevancia para el avance en la confirmación de resistencia de malas hierbas a herbicidas que permiten implementar estrategias sostenibles en los sistemas de producción. El doctorando, a lo largo de su formación doctoral ha colaborado en varios trabajos de investigación que ahora están publicados en revistas internacionales con alto índice de impacto (Q1-JCR). A continuación, se presenta una relación de los trabajos publicados a los que ha dado lugar la investigación realizada y que, a su vez, tres de estos forman parte del cuerpo de la Tesis.

Publicaciones:

García, M. J., **Palma-Bautista, C.**, Rojano-Delgado, A. M., Bracamonte, E., Portugal, J., Alcántara-De la Cruz, R., & de Prado, R. (2019). The triple amino acid substitution TAP-IVS in the *EPSPS* gene confers high glyphosate resistance to the superweed *Amaranthus hybridus*. In *International Journal of Molecular Sciences* (Vol. 20, Issue 10). MDPI AG. https://doi.org/10.3390/ijms20102396

García, M. J., **Palma-Bautista, C.**, Vazquez-Garcia, J. G., Rojano-Delgado, A. M., Osuna, M. D., Torra, J., & de Prado, R. (2020). Multiple mutations in the *EPSPS* and *ALS* genes of *Amaranthus hybridus*

underlie resistance to glyphosate and ALS inhibitors. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-74430-0

Palma-Bautista, C., Alcántara-de la Cruz, R., Rojano-Delgado, A. M., Dellaferrera, I., Domínguez-Martínez, P. A., & de Prado, R. (2019). Low temperatures enhance the absorption and translocation of 14C-glyphosate in glyphosate-resistant *Conyza sumatrensis*. *Journal of Plant Physiology*, *240*. https://doi.org/10.1016/j.jplph.2019.153009

Palma-Bautista, C., Belluccini, P., Gentiletti, V., Vázquez-García, J. G., Cruz-Hipolito, H. E., & de Prado, R. (2020). Multiple resistance to glyphosate and 2,4-D in *Carduus acanthoides* L. from Argentina and alternative control solutions. *Agronomy*, *10*(11). https://doi.org/10.3390/agronomy10111735

Palma-Bautista, C., Cruz-Hipólito, H. E., Alcántara-de la Cruz, R., Vázquez-García, J. G., Yanniccari, M., & de Prado, R. (2021). Comparison of premix glyphosate and 2,4-D formulation and direct tank mixture for control of *Conyza canadensis* and *Epilobium ciliatum*. *Environmental Pollution*, *281*. https://doi.org/10.1016/j.envpol.2021.117013

Palma-Bautista, C., Hoyos, V., Plaza, G., Vázquez-García, J. G., Rosario, J., Rojano-Delgado, A. M., & de Prado, R. (2020). Evolving multiple resistance to EPSPS, GS, ALS, PSI, PPO, and synthetic auxin herbicides in Dominican Republic *Parthenium hysterophorus* populations. A physiological and biochemical study. *Agronomy*, *10*(4). https://doi.org/10.3390/agronomy10040554

Palma-Bautista, C., Rojano-Delgado, A. M., Vázquez-García, J. G., Yanniccari, M., & de Prado, R. (2020). Resistance to Fomesafen, Imazamox and Glyphosate in *Euphorbia heterophylla* from Brazil. *Agronomy*, *10*(10 October). https://doi.org/10.3390/agronomy10101573

Palma-Bautista, C., Tataridas, A., Kanatas, P., Travlos, I. S., Bastida, F., Domínguez-Valenzuela, J. A., & de Prado, R. (2020). Can control of glyphosate susceptible and resistant *Conyza sumatrensis* populations be dependent on the herbicide formulation or adjuvants? *Agronomy*, *10*(10 October). https://doi.org/10.3390/agronomy10101599

Palma-Bautista, C., Vazquez-Garcia, J. G., Travlos, I., Tataridas, A., Kanatas, P., Domínguez-Valenzuela, J. A., & de Prado, R. (2020). Effect of adjuvant on glyphosate effectiveness, retention, absorption and translocation in *Lolium rigidum* and *Conyza canadensis*. *Plants*, *9*(3). https://doi.org/10.3390/plants9030297

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

Córdoba, 12 de mayo de 2022

Firma de los directores

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Cande

PUBLICACIONES DE LA TESIS DOCTORAL

A continuación, se presentan los parámetros de calidad de las **publicaciones de la tesis doctoral,** obtenidos del **Journal Citations Reports** para la categoría correspondiente.

Capítulo	Revista	Año de publicación	Factor de impacto	Cuartil	Categoría
II	Journal of Agricultural and Food Chemestry	2019	3.571	Q1	Agriculture, Multidisciplinary
III	Agronomy	2021	3.417	Q1	Agronomy
IV	Journal of Agricultural and Food Chemestry	2022	5.758	Q1	Agriculture, Multidisciplinary

Nota: Con el fin de establecer una coherencia formal a lo largo de todo el trabajo, el formato de las referencias se ha uniformado (usando el formato de American Psychological Association 7th edition) y se han editado los trabajos originales, eliminando de ellos el formato propio de la revista donde fue publicado.

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Resumen

Actualmente el control químico de las malas hierbas es la principal herramienta utilizada en el mundo dentro y fuera de los cultivos. Desde la introducción de los herbicidas se ha sometido a una gran cantidad de malas hierbas a una selección constate que ha dado como resultado la rápida evolución de la resistencia a los herbicidas. La ciencia de las malas hierbas ha establecido y sigue desarrollando protocolos para conocer y dilucidar los mecanismos que confieren resistencia a uno o varios herbicidas y especies de malas hierbas. La resistencia a herbicidas se divide en: resistencia dentro del sitio de acción (TSR) y resistencia fuera del sitio de acción (NTSR). La resistencia dentro del sitio de acción se atribuye generalmente a mutaciones en el gen que codifica la enzima objetivo del herbicida que provoca una disminución de su afinidad con el herbicida. La resistencia fuera del sitio de acción es el producto de la evolución más avanzada de las malas hierbas ya este impide que el herbicida llegue adecuadamente a su sitio de acción. Dentro de este grupo encontramos la absorción y translocación reducida, el secuestro vacuolar y el metabolismo de los herbicidas. Las malas hierbas pueden tener un solo mecanismo de resistencia o acumular varios mecanismos que se traduce en resistencia múltiple a varios herbicidas y que representan un gran desafío para la sostenibilidad de los herbicidas en la agricultura. En este trabajo de investigación se da a conocer el primer caso de resistencia a glifosato en *Amaranthus palmeri* que involucra exclusivamente mecanismos NTSR como la absorción y translocación diferenciada entre poblaciones. También se encontró resistencia a 2,4-D en seis especies dicotiledóneas donde el metabolismo mejorado y la translocación reducida son los responsables de la resistencia a este imitador de auxinas. Por otro lado, se estudió una población de *Conyza bonariensis*, donde por primera vez en el mundo se informa de la aparición de resistencia múltiple a herbicidas inhibidores de la aceto lactato sintasa (ALS) y la 5-enolpiruvylshikimato-3-fosfato sintasa (EPSPS), desviadores de electrones del fotosistema I (PSI), inhibidores del fotosistema II (PSII) y herbicidas imitadores de auxinas. Conocer y estudiar los mecanismos de resistencia a fondo es imprescindible para comprender la resistencia y construir soluciones que permitan desarrollar sistemas agrícolas sostenibles. El manejo integrado de malas hierbas (IWM) debe jugar un papel

importante para disminuir esa presión de selección al que las malas hierban se sometido.

Palabras clave: Mecanismos TSR, mecanismos NTSR, absorción y translocación reducida, metabolismo mejorado, resistencia simple y múltiple.

Abstract

Chemical control of weeds is currently the main tool used in the world inside and outside of crops. Since the introduction of herbicides, large number of weeds have been subjected to constant selection pressureresulting to the rapid evolution of herbicide resistance. Weed science has established and continues to develop protocols to test and know the mechanisms that confer resistance for various herbicides and weed species. Herbicide resistance is classified into target site resistance (TSR) and non-target site resistance (NTSR). Target site resistance is generally attributed to mutations in the gene encoding the herbicide target enzyme that cause a decrease in its affinity for the herbicide. Non target site resistance is the product of the more advanced evolution and prevents the herbicide from reaching its site of action correctly. Within this group are reduced absorption and translocation, vacuolar sequestration and metabolism of herbicides. Weeds have a single resistance mechanism or accumulate several mechanisms that result in multiple resistance to several herbicides and that represent a major challenge to the sustainability of herbicides in agriculture. In this research work we report the first case of glyphosate resistance in *Amaranthus palmeri* involving exclusively NTSR mechanisms such as differential absorption and translocation between populations. Resistance to 2,4-D was also found in six dicot species in which enhanced metabolism and reduced translocation are responsible for resistance to this auxin mimic. On the other hand, a population of *Conyza bonariensis* was studied, where for the first time in the world multiple resistance to acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor herbicides, photosystem I electron diverters, photosystem II inhibitors and auxin mimic herbicides. Knowledge and study of resistance mechanisms is essential to understand resistance and build solutions for the development of sustainable agricultural systems. Integrated weed management (IWM) has an important role to play in reducing the selection pressure to which weeds have been subjected.

Keywords: TSR mechanisms, NTSR mechanisms, reduced absorption and translocation, enhanced metabolism, single and multiple resistance.

CAPITULO I

Introducción general

1 Introducción General

1.1 Las malezas en la agricultura

La agricultura forma parte del sector primario en la economía de cualquier país, convirtiéndose en un proceso de producción indispensable que contribuye al desarrollo y la economía de las naciones (Loizou et al., 2019). A nivel mundial la agricultura debe satisfacer la creciente demanda de alimentos, piensos, fibras, biocombustibles, entre otros productos de base biológica (Popp et al., 2012). Sin embargo, el desarrollo de su práctica se ve afectada por diferentes factores clasificados como bióticos y abióticos que causan una potencial disminución en el rendimiento de los cultivos. Los factores bióticos son los responsables de las principales perdidas de los cultivos donde las malas hierbas ocupan el primer lugar con una estimación del 34%, seguida de las plagas y enfermedades con un 16 a 18% (Oerke, 2006).

1.2 Definición e importancia de las malas hierbas

La Malherbología es la ciencia que estudia la biología, ecología y manejo de malas hierbas o también llamadas malezas en varias regiones del mundo. Son muchos los conceptos que existen sobre las malas hierbas, sin embargo, todas comparten que es un concepto antropogénico y que son plantas que aparecen en la agricultura como resultado de las actividades humanas y que se adaptan a las condiciones de alteración continua de los agroecosistemas.

Las malas hierbas representan un problema constante en la producción agrícola. Los niveles de infestación de las malas hierbas en los cultivos dependen de las practicas agronómica utilizadas, por ejemplo, el tipo de cultivo y su capacidad competitiva, la rotación de cultivos, el tipo de labranza, el método y el momento de la fertilización, la densidad de población del cultivo y los herbicidas, las infestaciones también dependen del tipo de suelo y su fertilidad, así como las condiciones climáticas del lugar (Chauhan et al., 2012; Swanton et al., 2015).

Las mala hierbas son consideradas una plaga y estan en el mismo nivel trófico que las plantas cultivadas, y ejercen competencia con los cultivos por luz, agua, nutrientes, espacio, etc. que se reflejan en pérdidas de rendimiento y la calidad de los cultivos (Ramesh et al., 2017; Swanton et al., 2015).

Las malas hierbas ocasionan pérdidas directas a la producción agrícola con variaciones regionales muy grandes. Se estima que las pérdidas económicas causadas por las malas hierbas ascienden a poco más de 120 mil millones de dólares (Müller and Appleby 2010). La Sociedad Americana de la Ciencia de la Maleza (WSSA) ha estimado la perdida potencial en valor económico causados por las malas hierbas para algunos cultivos con datos obtenidos entre 2007 y 2012. Para el maíz (*Zea mays*) es de 27 mil millones de dólares y para la soja (*Gycine max*) de 16 mil millones de acuerdos con datos obtenidos entre 2007-2013. La misma WSSA plantea que en las perdidas promedio en rendimiento sin control de malas hierbas es de un 52% ara maíz y de para la soja del 49.5% (WSSA, 2020).

Para los agricultores una reducción en el rendimiento debido a la presencia y competencia de las malas hierbas en sus cultivos representa un problema constante, indeseable e inevitable. Desde el inicio de la agricultura esta amenaza y sus impactos negativos han jugado un papel importante en los cultivos.

1.3 Control de malas hierbas

Existen diferentes métodos de control de las malas hierbas. Sin embargo, la clasificación de estos varía dependiendo del autor consultado. Resumiendo, estos se pueden dividir en dos grandes grupos: los métodos no químicos y químicos que han evolucionado con el paso del tiempo y que son utilizados en la agricultura con mayor o menor intensidad dependiendo de la región, sus condiciones orográficas, clima y tipos de suelo, así como la condición económica de los agricultores (Abouziena and Haggag, 2016; Harker and O'Donovan, 2013; Rask and Kristoffersen, 2007; Tu et al., 2001). Dentro de los métodos de control no químicos se pueden encontrar los siguientes:

- Métodos preventivos: son todos aquellos que se encargan de prevenir la entrada o movimiento de una región a otra de las malas hierbas. Consiste en utilizar material se siembra libres de semillas de malas hierbas, limpieza de la maquinaria y implementos agrícolas, limpieza de orillas de carreteras y canales de riego, etc.
- Métodos culturales: Dentro de este se utiliza la rotación de cultivos, preparación del terreno, uso de variedades competitivas, densidades de población optima,

rotación de cultivos, fechas de siembra, cultivos de cobertura, acolchado y el manejo de agua.

- Métodos físicos: son todos los métodos que cortan, entierran, cubren y queman la vegetación de no interés agrícola.
- Métodos biológicos: incluye el uso de organismos vivos que mantienen las densidades de población por debajo del nivel de daño permitidos de los cultivos. Las plantas, animales o microorganismos pueden ser utilizados para este fin y que son liberados en diversas ocasiones, se establecen y se monitorean.
- Métodos químicos: se basa en el uso de herbicidas para controlar las malas hierbas y que es actualmente el más utilizado en la agricultura por sus ventajas insustituibles por los otros métodos de control como son su eficacia, rapidez, selectividad y bajos costos.

Depender de un solo método de control puede causar efectos negativos en el manejo de las malas hierbas. El control químico fue más utilizado en las últimas décadas para solucionar el problema sin considerar su biología que permite la integración de programas de manejo que permiten incluir otros métodos de control y que ocasiono que se presentaran casos de tolerancia o resistencia a herbicidas (Buhler, 2002). El manejo integrado de malezas (MIM) puede ser definido como la implementación y integración de los diferentes métodos de control que permitan a los cultivos mantener todas las ventajas posibles sobre las malas hierbas. El MIM tiene el potencial de restringir las poblaciones de malezas a niveles manejables, reducir el impacto ambiental de prácticas individuales de manejo de malezas, incrementar la sostenibilidad de los sistemas de cultivos y reducir la presión de selección sobre la resistencia a herbicidas de las malezas (Buhler, 2002; Harker and O'Donovan, 2013).

1.4 Control químico de malas hierbas

Un herbicida es un producto químico utilizado para inhibir o interrumpir gravemente el crecimiento y desarrollo de una planta. Los herbicidas desde su introducción han sido extensivamente en la agricultura, la industria y en zonas urbanas. Cuando estos son utilizados correctamente, proporcionan un control eficiente de las malas hierbas a un bajo costo comparado con otros métodos de control (Holt, 2013; Todd and Suter, 2010).

Sin embargo, si los herbicidas no son bien aplicados pueden causar daños en los cultivos, medio ambiente y la salud de los aplicadores (Holt, 2013; Tu et al., 2001).

Desde la obtención de la patente de un herbicida hasta que sale a la venta en el mercado requiere de inversiones de alrededor de 285 millones de dólares y toma aproximadamente 11,3 años en promedio (Sparks and Lorsbach, 2017). Los herbicidas se comercializan en formulaciones liquidas o solidas que están relacionados con la solubilidad del ingrediente activo y su forma de aplicación.

1.5 Clasificación de los herbicidas

Los herbicidas pueden clasificarse de deferentes formas y considerando varios criterios. Los más utilizados son considerando la época de aplicación (pre y post-emergentes), la selectividad (selectivos a cultivos o no selectivos), movilidad en la planta (sistémicos o de contacto), por la familia química y por su modo de acción. La clasificación de aceptada por la comunidad científica es por su modo de acción que se divide en 27 grupos (HRAC, 2020).

1.5.1 Modo de acción

El modo de acción es la secuencia de eventos que ocurren desde la absorción del herbicida hasta la muerte de la planta. Aquellos herbicidas con el mismo modo de acción son similares en cuanto a la absorción, transporte e incluso producen síntomas similares en las plantas tratadas. Por otro lado, esta clasificación permite conocer el espectro de control de las malas hierbas, las épocas de aplicación, su selectividad a los cultivos y la residualidad en el suelo (HRAC, 2021; Sherwani et al., 2015). Dentro del modo de acción se encuentra el mecanismo de acción que forma parte de la secuencia de eventos que causan la muerte de la planta y que son conceptos que a menudo son confundidos.

1.5.2 Mecanismo o sitio de acción

El mecanismo o sitio de acción es la principal reacción bioquímica o biofísica que es afectada por el herbicida para causar la muerte de la planta. Los herbicidas dentro de la planta y dependiendo del modo de acción del herbicida pude bloquear algún proceso enzimático o un sistema biológico vital para continuar con su funcionamiento regular y causando la eventual muerte de la planta (Sherwani et al., 2015). Los herbicidas también se pueden ser clasificados por su mecanismo o sitio de acción el cual implica tener

conocimientos muy amplios de los síntomas en las plantas tratadas. En la tabla 1.1 se muestran los diferentes mecanismos o sitios de acción de los herbicidas de acuerdo con la nueva clasificación realizada por la HRAC (HRAC, 2020).

Tabla 1.1 Clasificación por mecanismo o sitio de acción de los herbicidas aceptadas por el HRAC.

Grupo	Mecanismo o sitio de acción
1	Inhibición de la Acetil CoA Carboxilasa (ACCasa)
2	Inhibición de Acetolactato Sintasa (ALS)
3	Inhibición del conjunto de microtúbulos
4	Imitadores de auxinas
5	Inhibidores de fotosistema II (PSII)-Aglutinante de serina 264
6	Inhibidores de (PSII): Aglutinantes de histidina 215
9	Inhibidor de la enolpiruvil shikimato fosfato sintasa (EPSPS)
10	Inhibidor de la Glutamina sintetasa (GS)
12	Inhibidores de la fitoeno desaturasa (PDS)
13	Inhibidor de la desoxi-D-xilulosa fosfato sintasa
14	Inhibidor de la protoporfirinógeno oxidasa (PPO)
15	Inhibidores de la síntesis de ácidos grasos de cadena muy larga
18	Inhibidor de la dihidropteroato sintasa
19	Inhibidores del transporte de auxinas
22	Inhibidores del fotosistema I (PSI): Desviación de electrones
23	Inhibidores de la organización de los microtúbulos
24	Desacopladores
27	Inhibición de hidroxifenil piruvato dioxigenasa (4-HPPD)
28	Inhibidor de la dihidroorotato deshidrogenasa vegetal
29	Inhibición de la síntesis de celulosa
30	Inhibición de la tioesterasa de ácidos grasos
31	Inhibición de la proteína fosfatasa de serina-treonina
32	Inhibición de la solanesil difosfato sintasa
33	Inhibición de homogentisato solanesiltransferasa
34	Inhibición de la licopeno ciclasa
Ø	Desconocido

En el presente trabajo de tesis se estudió la resistencia a herbicidas que involucran a dos mecanismos de acción muy importantes para el manejo de las malas hierbas. Estos modos de acción son los inhibidores de la biosíntesis de aminoácidos y los reguladores de crecimiento.

1.6 Mecanismos de acción estudiados

1.6.1 Grupo 2: Inhibidores de la Acetolactato Sintasa (ALS)

Este grupo de herbicidas también son conocidos como inhibidores de la síntesis de aminoácidos por su modo de acción, estos herbicidas inhiben la acción de la enzima Acetolactato Sintasa (ALS). También conocida como acetohidroxiácido sintasa (AHAS), la ALS cataliza el primer paso en la síntesis de los aminoácidos de cadena ramificada, como leucina, isoleucina y valina (Stidham, 1991; Whitcomb, 1999). Estos también se denominan inhibidores de AHAS o inhibidores de aminoácidos de cadena ramificada. Los inhibidores de la ALS, que comprenden la familia química de imidazolinonas, pirimidiniltiobenzoatos, sulfonilaminocarboniltriazolinonas, sulfonilureas y triazolopirimidinas, que forman parte del grupo más grande de herbicidas comerciales. La mayoría de estos herbicidas actúan a dosis bajas, obteniéndose buenos resultados con pocos gramos por hectárea (Bellinder et al., 1994; Fletcher et al., 2002). Las plantas tratadas con estos inhibidores detienen su crecimiento, se marchitan y adquieren una coloración rojiza debido a la acumulación de antocianinas inducidas por el estrés provocando el marchitamiento de la planta y en última instancia su muerte.

1.6.2 Grupo 4: Reguladores del crecimiento

Este grupo actualmente conocido como imitadores de la auxina (HRAC, 2020). Este grupo de herbicidas es utilizado principalmente para controlar malezas de hoja ancha en cultivos de hoja estrecha a excepción de los ácidos quinolincarboxílicos (Grossmann, 2010). El modo de acción de este herbicida se encarga de imitar la auxina principal en las plantas que es el ácido indol-3-acético (IAA), aumentando así las actividades de transcripción, traducción y biosíntesis de proteínas dentro de las células, lo que se traduce en un crecimiento vascular descontrolado, provocando destrucciones celulares y la muerte final de las células y plantas. (Lopez-Lauri, 2016). En los herbicidas comerciales que se encuentran en esta familia química se puede encontrar a ácidos fenoxicarboxílicos, ácidos benzoicos, ácidos carboxilquinólicos y los ácidos piridincarboxílicos. Todas estas sustancias químicas alteran el metabolismo de los ácidos nucleicos y la integridad de la pared celular al activar la bomba de protones de adenosina trifosfato (ATP) que aumenta la actividad enzimática en la pared celular (Grossmann, 2010; Lopez-Lauri, 2016; Sherwani et al., 2015). El mecanismos o sitio de acción

específico responsable de la activación del IAA aún no se ha descrito y por lo tanto sigue siendo desconocido (HRAC, 2022).

1.6.3 Grupo 9: Inhibidores de aminoácidos aromáticos

Su modo de acción actúa al inhibir la síntesis de aminoácidos aromáticos esenciales. En este grupo encontramos al herbicida glifosato, que es el más utilizado en todo el mundo (Benbrook, 2016). El glifosato es un herbicida total, es decir que matan o controlan a cualquier planta con el que hayan tenido algún tipo de contacto (amplio espectro de control), excepto con los cultivos resistentes a este herbicida como el algodón, la soja, el maíz y canola principalmente (Duke, 2018). El glifosato inhibe a la enzima la 5-enolpiruvil shikimato-3-fosfato sintasa (EPSPS), enzima requerida en la ruta del ácido shikímico para la biosíntesis de los aminoácidos aromáticos esenciales fenilalanina, tirosina y triptófano (Funke et al., 2006). Los diferentes productos formulados de glifosato se encuentran disponibles como sales de amonio, sales de diamonio, sales de dimetilamonio, sales de isopropilamina y sales de potasio. Debido a su amplio espectro de control y la facilidad de translocación (a través del xilema y el floema) y la dificultad de las malezas para superar sus efectos hacen que los herbicidas de este grupo sean candidatos ideales para el control de las malas hierbas.

1.7 Resistencia a herbicidas

De acuerdo con la Sociedad Americana de la Ciencia de la Maleza (WSSA) la resistencia a herbicidas se define como la capacidad evolutiva de una población de malezas para sobrevivir a la aplicación de un herbicida que se sabe previamente era controlada (WSSA, 2011). Bajo una presión de selección continua, es decir, el uso repetido de herbicidas con el mismo modo de acción, las plantas resistentes aumentan en frecuencia con el tiempo, dando como resultado la prevalencia de individuos resistentes a ese herbicida. Además de la presión de selección de los herbicidas, los factores biológicos y genéticos de las especies de malezas, las propiedades de los herbicidas y las prácticas agronómicas también desempeñan un papel importante en la evolución y propagación de la resistencia a los herbicidas (Powles and Yu, 2010). El concepto de resistencia puede ser confundido con la tolerancia a herbicidas de las malas hierbas. La tolerancia a herbicidas es la capacidad inherente de una especie para sobrevivir y reproducirse después de un tratamiento herbicida. No ha existido una selección sobre la especie de

maleza tolerante y no hay cambios en la falta de respuesta de la especie de maleza al herbicida a través del tiempo (WSSA, 2011). Por lo tanto, en la tolerancia a herbicidas , no hay un cambio a través del tiempo debido a que la población siempre ha sido tolerante al herbicida.

Existen tres tipos de resistencia a los herbicidas: resistencia simple, resistencia cruzada y resistencia múltiple. La resistencia simple es cuando una maleza es resistente a una sola familia de herbicidas y/o un modo de acción. Mientras que en la resistencia cruzada un mecanismo de resistencia confiere a la mala hierba resistir a una o más familias de herbicidas que pertenecen al mismo modo de acción. Por otro lado, la resistencia múltiple es cuando una mala hierba es resistente al menos a dos familias de herbicidas que pertenecen a dos o más modos de acción (Knezevic et al., 2016; Powles & Preston, 2020; Sherwani et al., 2015).

1.8 Mecanismos de resistencia a herbicidas

Un aspecto importante para de comprender la evolución de cada una de las características de la resistencia a herbicidas es comprender el mecanismo(s) involucrado en resistencia a herbicidas. Los mecanismos de resistencia a los herbicidas en las malas hierbas se pueden clasificar en términos generales en resistencia en el sitio objetivo (TSR) y resistencia en el sitio no objetivo (NTSR) (figura 1.1).

Los mecanismos de TSR implican en gran medida mutaciones en el sitio objetivo de acción de un herbicida que confiere un cambio de aminoácidos en un enzima objetivo que impide la unión a herbicida (Murphy and Tranel, 2019). Los mecanismos TSR están determinados principalmente por rasgos monogénicos (Délye et al., 2013). Además, dentro de los TSR también puede ocurrir como resultado de la amplificación o sobreexpresión del gen, desarrollándose una mayor cantidad de enzimas en la proteína objetivo, por lo que se requiere una mayor concentración de herbicida para inhibir la proteína (Sammons and Gaines, 2014).

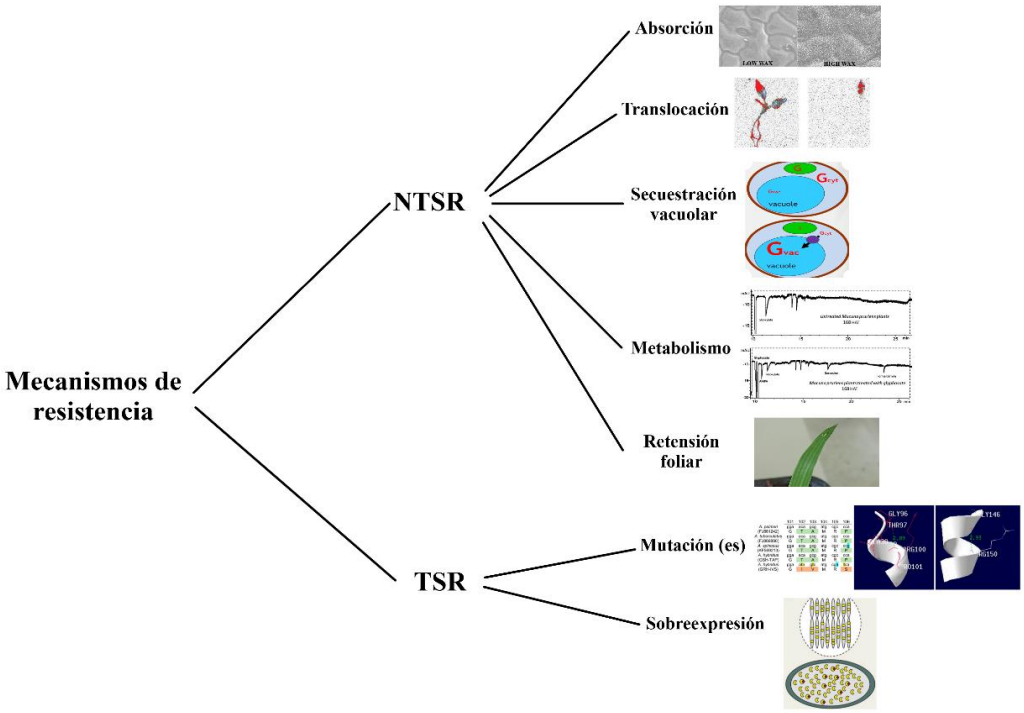

Figure 1.1 Mecanismos de resistencia a herbicidas dentro del sitio de acción (target-site) y fuera del sitio de acción (non target-site). Las plantas resistentes pueden expresar uno o múltiples mecanismos de resistencia al mismo o distintos herbicidas.

Dentro de los mecanismos de NTSR se encuentra una reducción de la absorción/translocación, un aumento del metabolismo, una disminución de la tasa de activación y/o secuestro de los herbicidas (Gaines et al., 2020). Los NTSR donde se encuentra el metabolismo implica el aumento de la actividad de complejos enzimáticos como las esterasas, el citocromo P450 (CYP450), glutatión S-transferasas (GST) y/o uridina 5'-difosfo (UDP)-glucosiltransferasas (Powles & Yu, 2010). La desintoxicación por metabolismo de herbicidas por estas enzimas suele estar reguladas por muchos genes (poligénicos) y puede conferir resistencia a herbicidas con modos de acción completamente diferentes (Délye et al., 2013; Sammons and Gaines, 2014). Sin embargo, la resistencia por herencia monogénica de los NTSR también se ha informado en algunas malas hierbas resistentes a herbicidas (Gion et al., 2014; Huffman et al., 2015; Yamada et al., 2000). La evolución de los NTSR a través del metabolismo de herbicidas es una seria amenaza para el manejo de malezas, ya que puede otorgar resistencia a múltiples herbicidas, dejando pocas opciones de herbicidas alternativos para el control de malezas, así como resistencias potenciales a herbicidas que aún no están disponibles comercialmente (Huffman et al., 2015).

1.9 Resistencia a los herbicidas Inhibidores de la Acetolactato Sintasa (ALS)

Los herbicidas inhibidores de la acetolactato sintasa (ALS) tambien conocida como la acetohidroxiácido sintasa (AHAS) constituyen actualmente el mayor grupo de modo de acción (con 54 ingredientes activos en cinco grupos químicos) y se han utilizado ampliamente en la agricultura mundial desde que se introdujeron por primera vez en 1982. La enzima ALS es la primera enzima en la ruta de síntesis de los aminoácidos de cadena ramificada de la valina, leucina e isoleucina, y el agotamiento de estos aminoácidos es el modo de acción de los herbicidas inhibidores de la ALS (Yu & Powles, 2014). Actualmente existen 169 informes de malezas resistente a este grupo de herbicidas. Este es el grupo con más número de casos reportados en el mundo (Heap, 2022.) Pueden mostrar resistencia cruzada a otros herbicidas y actuar reduciendo la producción de aminoácidos ramificados en presencia de la enzima ALS (Sherwani et al., 2015).

Las malas hierbas desarrollan resistencia a los inhibidores de ALS con mucha facilidad y este es el talón de Aquiles de estos herbicidas y la resistencia a menudo se debe a las sustituciones del sitio objetivo. La resistencia evolucionada en malas hierbas se deba a sustituciones en cada uno de los siguientes ocho aminoácidos: Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653 y Gly- 654 (Figura 1.2). A menudo, varias sustituciones diferentes en cada uno de estos ocho sitios pueden conferir resistencia (Murphy & Tranel, 2019; Yu & Powles, 2014). La resistencia cruzada proporcionada por una mutación de resistencia ALS se nombra por su posición en el gen ALS. Por ejemplo, las mutaciones en Ala-122 confieren resistencia a los herbicidas de la familia imidazolinonas, pero no a los sulfonilurea, las mutaciones en Pro-197 confieren resistencia a sulfonilurea pero no a imidazolinonas y las mutaciones en Trp-574 proporcionan resistencia tanto a sulfonilurea como a imidazolinonas. Con el descubrimiento de múltiples mutaciones en la ALS, se ha descubierto que la resistencia cruzada también depende de mutaciones específicas, grupos químicos inhibidores de la ALS y herbicidas específicos dentro de un grupo determinado, y a veces especies de malezas (Murphy & Tranel, 2019; Tranel & Wright, 2002; Yu & Powles, 2014). Sin embargo, la identificación de plantas que carecen de mutaciones en el dominio ALS y

que sobreviven a la aplicación de herbicidas ha llevado a los investigar los mecanismos NTSR.

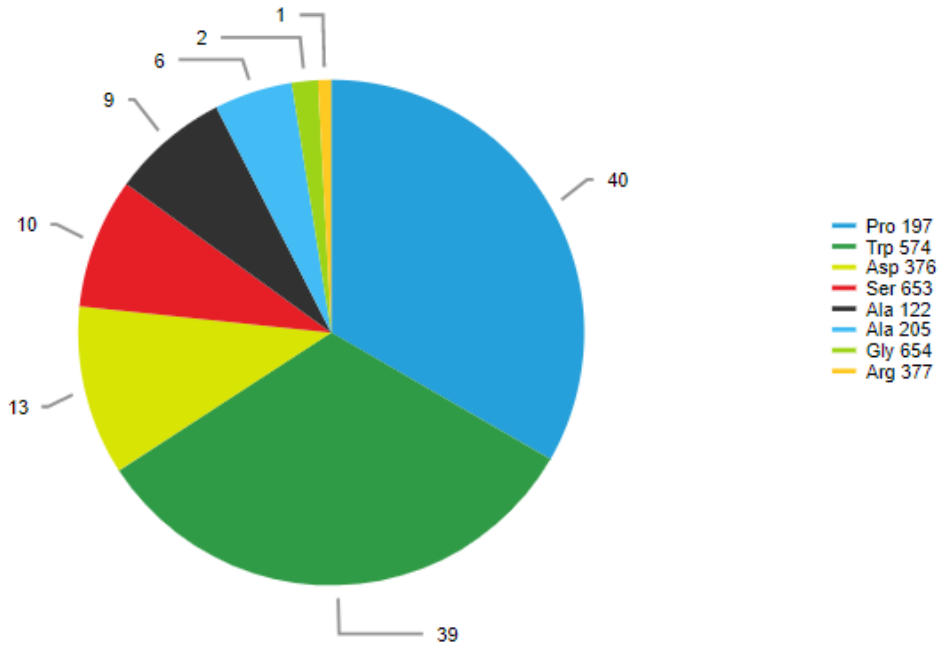

Figura 1.2 Número de especies con mutaciones en la ALS y los números de residuos de aminoácidos (Heap, 2022).

Las investigaciones realizadas han documentado el metabolismo mejorado que confiere resistencia a los inhibidores de ALS en malas hierbas mono y dicotiledóneas (Hada et al., 2021; Riar et al., 2012). En su mayoría estos estudios han identificado predominantemente múltiples genes CYP450 que se expresan de forma constitutiva o se regulan al alza después de la aplicación de los herbicidas inhibidores de la ALS (Iwakami et al., 2014; Liu et al., 2018; Zhao et al., 2019). Además de los CYP450, también se ha reportado la presencia de los transportadores GST, GT y sitio de unión del ATP (ABC). (Duhoux et al., 2015; Liu et al., 2018; Yang et al., 2016; Zhao et al., 2017). para Para probar la resistencia metabólica por CYP450 a los inhibidores de ALS se ha observado el aumento de la sensibilidad tras la aplicación con inhibidores de CYP450, como Butóxido de piperonio (PBO), forato y malatión.

1.10 Resistencia a los herbicidas Imitadores de auxinas

Desde la introducción de los herbicidas imitadores de auxinas en el año de 1945, la evolución de la resistencia ha sido lenta, se han informado 41 casos de malas hierbas resistentes [figura 1.3] (Heap, 2022). Con respecto a otros grupos de herbicidas son dirigidos a una proteína especifica, los imitadores de auxinas tienen un número muy amplio de proteínas dentro de las cuales se encuentras las siguientes familias: TIR1 y Auxin F-Box, la proteína AUX/IAA, el transportador de entrada AUX1/LAX, el transportador de salida PIN y ABCB (Dharmasiri et al., 2005; Vieten et al., 2007). Sin embargo, la evolución de la resistencia a los imitadores de auxina solo se ha informado en las familias AFB y AUX/IAA. No se ha establecido una numeración convencional de aminoácidos debido a que esta investigación es muy reciente.

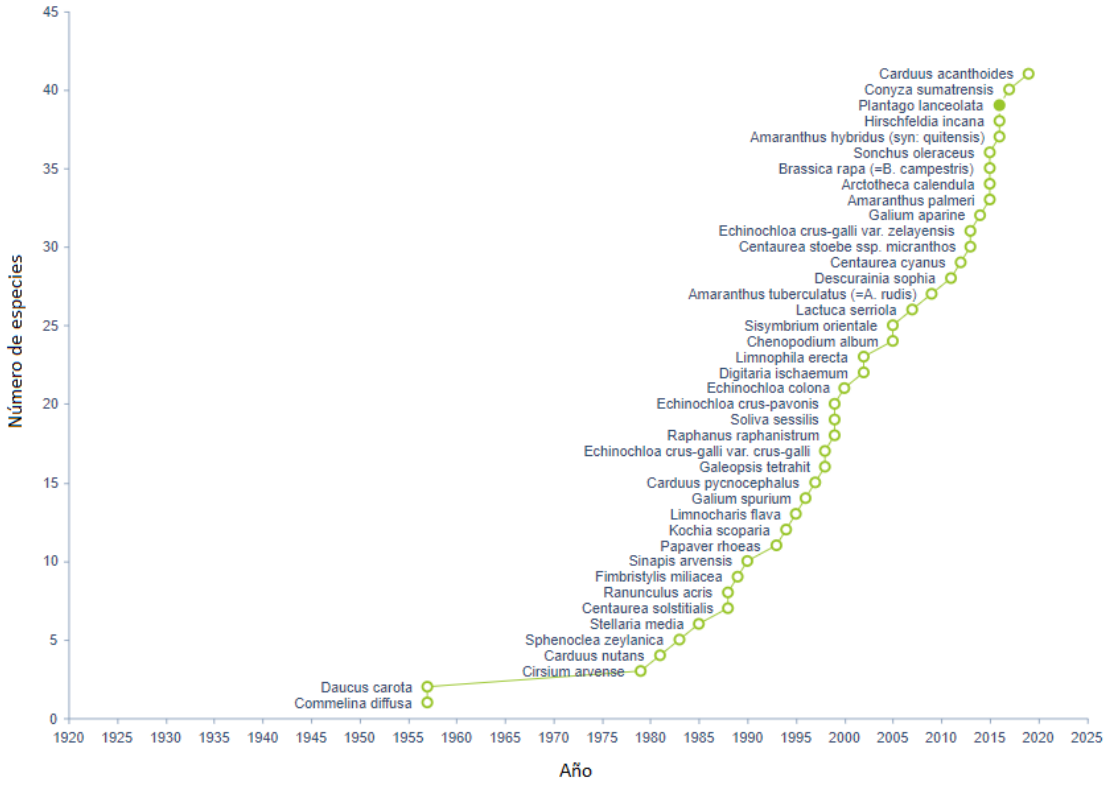

Figura 1.3 Especies de malas hierbas resistentes a herbicidas imitadores de auxina (Heap, 2022).

Fue en la mala hierba *Kochia scoparia* donde se identificó un mecanismo de resistencia en el sitio objetivo que representa a tres clases de imitadores de auxinas: benzoatos, fenoxi-carboxilatos y piridina-carboxilatos. La secuenciación del transcriptoma y el análisis de secuencia identificaron una sustitución de dos nucleótidos que da como

resultado una sustitución de un solo aminoácido en la región GWPPV/I altamente conservada (GWPPV/I→NWPPV/I) de KsIAA16. Los ensayos desarrollados en dos levaduras hibridas demostraron una pérdida de interacción entre KsIAA16 y KsTIR1 en presencia de benzoatos, fenoxicarboxilatos y piridina-carboxilatos dando como resultado de la sustitución de Gly-73-Asn (numeración basada en la secuencia de *Arabidopsis thaliana* IAA16) (LeClere et al., 2018).

En la mayoría de las malas hierbas se han documentado mecanismos NTSR por medio de falta de absorción y translocación, así como el aumento del metabolismo mejorado. La falta de absorción se ve afectada por las propiedades de la cutícula en la hoja u otras barreras estructurales que impiden la absorción del herbicida después de la aplicación del herbicida (Kohler et al., 2004). Sin embargo, la falta de translocación se traduce en la disminución del movimiento de los imitadores de auxinas al sitio de acción. Tal reducción en la translocación se ha informado en varias especies de malas hierbas (Dang et al., 2018; Goggin et al., 2016; Mora et al., 2019; Palma-Bautista et al., 2021). El metabolismo de los imitadores de auxinas es otro mecanismo importante NTSR reportado en varias especies de malas hierbas dicotiledóneas, donde de manera similar a las especies monocotiledóneas naturalmente tolerantes, la desintoxicación de los herbicidas se produce a través de la hidroxilación en anillo seguida de la conjugación causada por el CYP450 (Mora et al., 2019; Palma-Bautista et al., 2020, 2021; Torra et al., 2021).

1.11 Resistencia a herbicidas Inhibidores de aminoácidos aromáticos

El glifosato, es el único herbicida químico dentro del grupo. Se conoce la estructura cristalina de la EPSPS, obtenida de *Escherichia coli* en complejo con glifosato (Schönbrunn et al., 2001). La numeración de aminoácidos se toma como referencia la enzima EPSPS de las plantas adultas la secuencia de *A. thaliana*, AT2G45300 (Sammons & Gaines, 2014). Actualmente hay 55 malas hierbas reportadas como resistentes a glifosato [figura 1.4] (Heap, 2022).

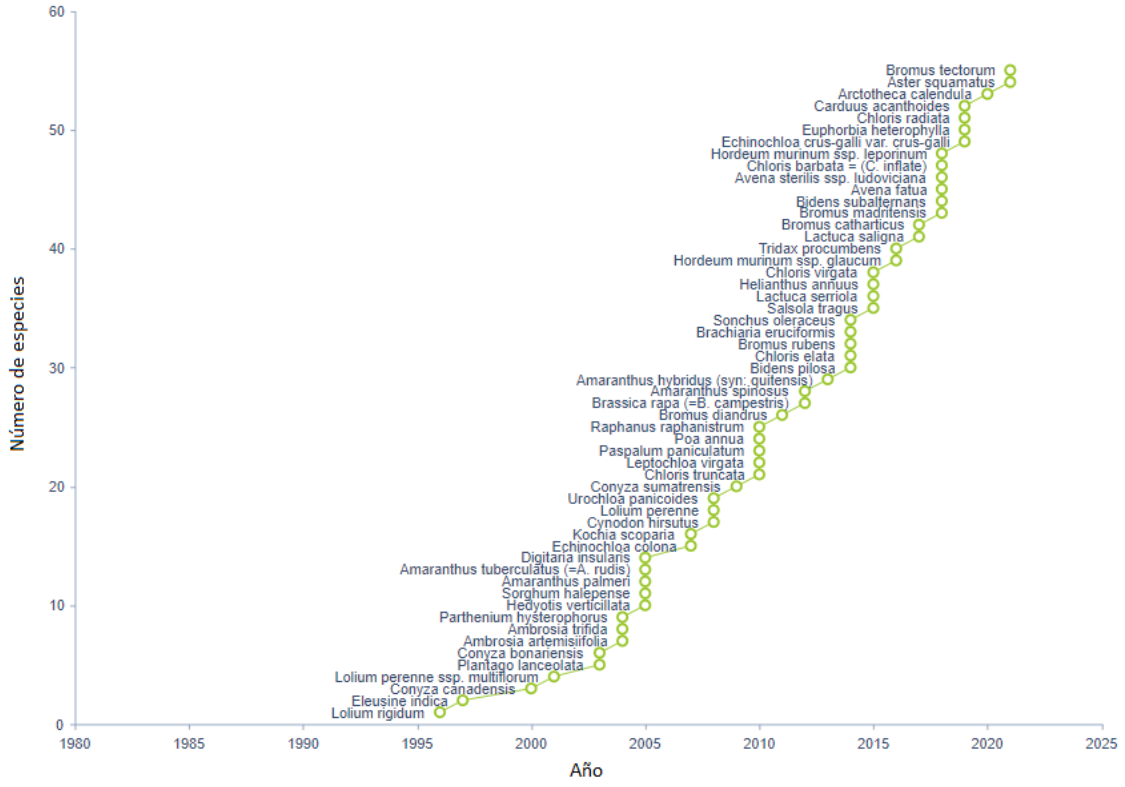

Figura 1.4 Especies de malas hierbas resistentes a glifosato (Heap, 2022).

La resistencia del sitio objetivo es única debido a múltiples sustituciones de aminoácidos para conferir una fuerte respuesta fenotípica. Las sustituciones en Pro-106 se han observado de forma aislada; sin embargo, la sustitución thr-102-Ile sólo se ha observado en combinación con Pro-106-Ser (García et al., 2019; Takano et al., 2020). La amplificación o duplicación de genes aumenta el número de copias de genes y en consecuencia aumenta la producción del objetivo molecular o las enzimas involucradas en la desintoxicación (Bass & Field, 2011).

Se ha informado que la translocación reducida de glifosato es el mecanismo NTSR más común. La reducción de la translocación se ha atribuido a la evolución de un transportador que secuestra el glifosato dentro de la vacuola de la planta, evitando así que llegue al cloroplasto (Ge et al., 2010; Shaner, 2009). Un metabolismo mejorado del glifosato es otro mecanismo responsable de la alta tolerancia al glifosato (Bianco de Carvalho et al., 2013; González-Torralva et al., 2012; Pan et al., 2019).

Hipótesis y objetivos

Los herbicidas son la herramienta de control de malas hierbas más utilizadas en las principales áreas de cultivo del mundo, esto junto con su uso inadecuado ha derivado en una fuerte selección, que ha permitido a las poblaciones de malas hierbas desarrollar mecanismos que les permitan sobrevivir y reproducirse en presencia del herbicida, como resultado de la adaptación evolutiva. Diferentes características biológicas y morfológicas han permitido que especies de malezas dicotiledóneas sean un grave problema nivel global y difícil de controlar. En la resistencia a herbicidas son dos los mecanismos de resistencia involucrados; la resistencia dentro del sitio de acción (TSR) y fuera del sitio de acción (NTSR). El estudio y conocimiento de la resistencia en las especies de malezas nos permitirá determinar de forma objetiva cual (es) son los mecanismos involucrados de conferir la resistencia a dichas especies, ya que si no conocemos cuales son éstos difícilmente podremos plantear alternativas de control eficaces para estas malezas que hoy amenazan la sustentabilidad de la agricultura a nivel global.

Este trabajo persigue estudiar a fondo los mecanismos de resistencia que confieren un alto grado de resistencia en malezas dicotiledóneas para los principales herbicidas como son las auxinas sintéticas y los inhibidores de la EPSPS y ALS, que se han sido utilizado desde hace décadas para el control de estas, y que hoy su efectividad se ve minimizada y desgastada ante estas malas hierbas.

El objetivo de estudio de la presente investigación es caracterizar los mecanismos de resistencia que confieren la resistencia a los herbicidas imitadores de auxinas y a los inhibidores de la EPSPS y ALS en malas hierbas dicotiledóneas.

Los objetivos específicos planteados son:

1. Confirmar la resistencia de *Amaranthus* sp. *Conyza* sp., *Hirschfeldia incana*; *Papaver rhoeas*, *Parthenium hysterophorus* a los herbicidas glifosato, tribenuron-metil y 2,4-D mediante:
    - Ensayos de dosis-respuesta para estudiar la dosis media necesaria para reducir el peso fresco al 50% (GR_{50}) y/o la dosis letal media que ocasiona la mortalidad al 50% (LD_{50}) de cada población.
    - Acumulación de ácido shikímico o producción de etileno.

2. Caracterizar los mecanismos de resistencia dentro del sitio de acción (TSR) según corresponda para cada herbicida mediante:
    - Actividad enzimática.
    - Sustituciones de aminoácidos en la secuencia de ADN.
    - Número de copias en el gen de interés.
    - Amplificación en el gen de interés.
3. Caracterizar los posibles mecanismos de resistencia fuera del sitio de acción (NTSR) correspondiente a cada grupo de herbicida por medio de:
    - Absorción y translocación con ^{14}C.
    - Metabolismo de los herbicidas a sustancias no toxicas.

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CAPITULO II

Reduced Absorption and Impaired Translocation Endows Glyphosate Resistance in *Amaranthus palmeri* Harvested in Glyphosate-Resistant Soybean from Argentina

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Reduced Absorption and Impaired Translocation Endows Glyphosate Resistance in *Amaranthus palmeri* Harvested in Glyphosate-Resistant Soybean from Argentina

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ABSTRACT: *Amaranthus palmeri* S. Watson is probably the worst glyphosate-resistant (GR) weed worldwide. The EPSPS (5-enolpyruvylshikimate-3-phosphate-synthase) gene amplification has been reported as the major target-site-resistance (TSR) mechanism conferring resistance to glyphosate in this species. In this study, TSR and non-target-site-resistance (NTSR) mechanisms to glyphosate were characterized in a putative resistant *A. palmeri* population (GRP), harvested in a GR soybean crop from Argentina. Glyphosate resistance was confirmed for the GRP population by dose−response assays. No evidence of TSR mechanisms, as well as glyphosate metabolism, was found in this population. Moreover, a susceptible population (GSP) that absorbed about 10% more herbicide than the GRP population was evaluated at different periods after treatment. The GSP population translocated about 20% more glyphosate to the remainder of the shoots and roots at 96 h after treatment than the control, while the GRP population retained 62% of herbicide in the treated leaves. This is the first case of glyphosate resistance in *A. palmeri* involving exclusively NTSR mechanisms.

KEYWORDS: *EPSPS gene amplification, glyphosate resistance crops, nontarget-site-resistance, Palmer amaranth, yuyo colorado*

1. INTRODUCTION

Several attributes confer to *Amaranthus* species the capacity to become major global weeds that are very difficult to control.[1] Among those traits that must be highlighted include the C4 photosynthetic pathway, high growth rate, reproduction capacity, genetic variability, and stress tolerance.[2] The occurrence of *Amaranthus* species becomes even more concerning due to the evolution of multiple herbicide-resistant biotypes.[3] Among them, *Amaranthus palmeri* S. Watson is unique because it is a dioecious species. Compared with other common *Amaranthus* species, *A. palmeri* is the most competitive, largest sized (height and weight), and prolific weed.[4,5] Under ideal conditions, a single *A. palmeri* plant can shed more than 600 thousand seeds and surpass 2 m in height.[6]

A. palmeri is native to the Sonoran Desert in North America, where it could be found from Southern California to Northern Mexico.[7] In about 20 years, it has extended its range from Ontario (Canada) to Brazil and Argentina.[1] Its prone to evolve resistance to herbicides, particularly to glyphosate, which in part explains this rapid spreading together with the commercialization of glyphosate-resistant (GR) crops.[8,9] This species is the most troublesome weed in row crops, especially for cotton and soybean producers on much of the American continent.[9] In Argentina, this species was first reported in 1966,[9] but it was not found again as an alien species in the country until 2004. From this year, *A. palmeri* started to be detected in summer cropping systems in southern parts of Cordoba and San Luis provinces, and from 2012 it was also found in maize, mani, soybean, and sorghum in the country.[9]

Resistance to glyphosate governed by target-site-resistance (TSR) mechanisms in *A. palmeri* has received special attention. Since the first report of GR *A. palmeri* in 2006,[10] most of populations, collected mainly from across the United States, have shown target-site mediated resistance to this herbicide.[11,12] The EPSPS (5-enolypyruvylshikimate-3-phosphate synthase) gene amplification has been the most common TSR mechanism to glyphosate described in this species,[11,12] though the point-mutation Pro-106-Ser has also been found in some populations from Mexico.[13] Most recently it has been proposed that amplified EPSPS gene copies of GR *A. palmeri* are present in the form of extrachromosal circular DNA molecules which are transferred to the next generation by tethering to mitotic and meiotic chromosomes.[14]

Non-target-site-resistance (NTSR) mechanisms to glyphosate seem not to be relevant in *A. palmeri*. However, some glyphosate-resistant populations of this species, collected in GR cotton from Mexico in 2015, showed NTSR and TSR mechanisms.[13] The characterization of NTSR mechanisms demonstrated that the restricted absorption and impaired

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Abstract

Amaranthus palmeri S. Watson is probably the worst glyphosate-resistant (GR) weed worldwide. The *EPSPS* (5-enolpyruvylshikimate-3-phosphate-synthase) gene amplification has been reported as the major target-site-resistance (TSR) mechanism conferring resistance to glyphosate in this species. In this study, TSR and non-target-site-resistance (NTSR) mechanisms to glyphosate were characterized in a putative resistant *A. palmeri* population (GRP), harvested in a GR soybean crop from Argentina. Glyphosate resistance was confirmed for the GRP population by dose–response assays. No evidence of TSR mechanisms, as well as glyphosate metabolism, was found in this population. Moreover, a susceptible population (GSP) that absorbed about 10% more herbicide than the GRP population was evaluated at different periods after treatment. The GSP population translocated about 20% more glyphosate to the remainder of the shoots and roots at 96 h after treatment than the control, while the GRP population retained 62% of herbicide in the treated leaves. This is the first case of glyphosate resistance in *A. palmeri* involving exclusively NTSR mechanism.

Keywords: EPSPS gene amplification, glyphosate resistance crops, nontarget-site-resistance, Palmer amaranth, yuyo colorado

Resumen

Amaranthus palmeri S. Watson es probablemente la peor maleza resistente a glifosato (GR) en todo el mundo. La amplificación del gen *EPSPS* (5-enolpiruvilshikimato-3-fosfato-sintasa) ha sido informada como el principal mecanismo de resistencia en el sitio de acción (TSR) que confiere resistencia a glifosato en esta especie. En este estudio, se caracterizaron los mecanismos TSR y los mecanismos de resistencia al glifosato en sitio fuera del sitio de acción (NTSR) en una población de *A. palmeri* (GRP) con sospechas de resistencia, colectada en un cultivo de soja GR (resistente a glifosato) de Argentina. La resistencia al glifosato se confirmó en la población de GRP mediante ensayos de dosis-respuesta . No se encontró evidencia de mecanismos TSR, así como del metabolismo del glifosato en esta población. Además, se evaluó una población susceptible (GSP) que absorbió aproximadamente un 10 % más del herbicida que la población GRP en diferentes períodos de tiempo después del tratamiento. La población de GSP translocó alrededor de un 20 % más de glifosato al resto de los brotes y raíces a las 96 h después del tratamiento comparado con el control, mientras que la población de GRP retuvo un 62 % del herbicida en las hojas tratadas. Este es el primer caso de resistencia a glifosato en *A. palmeri* que involucra exclusivamente los mecanismos NTSR.

Palabras clave: Amplificación del gen EPSPS, cultivos resistentes a glifosato, resistencia fuera del sitio de acción, amaranto palmera, yuyo colorado

1. Introduction

Several attributes confer to *Amaranthus* species the capacity to become major global weeds that are very difficult to control (Küpper et al., 2017). Among those traits that must be highlighted include the C4 photosynthetic pathway, high growth rate, reproduction capacity, genetic variability, and stress tolerance (Chaudhari et al., 2017). The occurrence of Amaranthus species becomes even more concerning due to the evolution of multiple herbicide-resistant biotypes (Heap, 2019). Among them, *Amaranthus palmeri* S. Watson is unique because it is a dioecious species. Compared with other common Amaranthus species, *A. palmeri* is the most competitive, largest sized (height and weight), and prolific weed (Horak & Loughin, 2000; Sellers et al., 2003). Under ideal conditions, a single *A. palmeri* plant can shed more than 600 thousand seeds and surpass 2 m in height (Keeley et al., 1987).

A. palmeri is native to the Sonoran Desert in North America, where it could be found from Southern California to Northern Mexico (Ehleringer, 1983). In about 20 years, it has extended its range from Ontario (Canada) to Brazil and Argentina (Küpper et al., 2017). Its prone to evolve resistance to herbicides, particularly to glyphosate, which in part explains this rapid spreading together with the commercialization of glyphosate-resistant (GR) crops (Morichetti et al., 2013; Shergill et al., 2018). This species is the most troublesome weed in row crops, especially for cotton and soybean producers on much of the American continent (Morichetti et al., 2013). In Argentina, this species was first reported in 1966 (Morichetti et al., 2013), but it was not found again as an alien species in the country until 2004. From this year, *A. palmeri* started to be detected in summer cropping systems in southern parts of Cordoba and San Luis provinces, and from 2012 it was also found in maize, mani, soybean, and sorghum in the country (Morichetti et al., 2013).

Resistance to glyphosate governed by target-site-resistance (TSR) mechanisms in *A. palmeri* has received special attention. Since the first report of GR *A. palmeri* in 2006 (Culpepper et al., 2006), most of populations, collected mainly from across the United States, have shown target-site mediated resistance to this herbicide (Gaines et al., 2010; Patterson et al., 2018). The *EPSPS* (5-enolypyruvylshikimate-3-phosphate synthase) gene amplification has been the most common TSR mechanism to glyphosate described

in this species (Gaines et al., 2010; Patterson et al., 2018), though the point-mutation Pro-106-Ser has also been found in some populations from Mexico (Dominguez-Valenzuela et al., 2017). Most recently it has been proposed that amplified *EPSPS* gene copies of GR *A. palmeri* are present in the form of extrachromosal circular DNA molecules which are transferred to the next generation by tethering to mitotic and meiotic chromosomes (Koo et al., 2018).

Non-target-site-resistance (NTSR) mechanisms to glyphosate seem not to be relevant in *A. palmeri*. However, some glyphosate-resistant populations of this species, collected in GR cotton from Mexico in 2015, showed NTSR and TSR mechanisms (Dominguez-Valenzuela et al., 2017). The characterization of NTSR mechanisms demonstrated that the restricted absorption and impaired translocation of glyphosate contributed to the resistance in those *A. palmeri* populations (Dominguez-Valenzuela et al., 2017). In other weed species, the most widespread NTSR mechanism to glyphosate was also the impaired translocation of glyphosate by sequestering the herbicide into the vacuoles (Ge et al., 2012; Ghanizadeh & Harrington, 2017; Sammons & Gaines, 2014). Moreover, a novel NTSR mechanism was described in *Ambrosia trifida*, the rapid cell death in response to the glyphosate application (Moretti et al., 2018; Van Horn et al., 2018). Finally, metabolism of glyphosate has been studied as a potential NTSR mechanism conferring resistance to this herbicide (De Carvalho et al., 2012; Duke, 2011).

The diversity of resistance mechanisms to glyphosate highlights the dangers of extrapolating knowledge obtained from one resistant population to others. Considering that for *A. palmeri* almost all reported cases of glyphosate resistance were governed by TSR (Gaines et al., 2010; Patterson et al., 2018; Sammons & Gaines, 2014), this should encourage one to reinvestigate the long-overlooked NTSR mechanisms, because they may contribute to resistance in selected populations. In this work, molecular experiments were carried out to confirm if two populations of *Amaranthus* from Cordoba, Argentina, one putative glyphosate resistant (GRP) collected in a GR-soybean field that survived glyphosate applications and one susceptible (GSP) without a history of glyphosate applications, belonged to *A. palmeri*. In addition, resistance levels to glyphosate and the different NTSR and TSR mechanisms that could be present in the GRP population were also characterized.

2. Materials and Methods

2.1 Plant Material

Matured seeds of a putative glyphosate resistant *A. palmeri* population (GRP) used in this research were harvested from GR soybean fields in Cordoba province (Argentina) in 2016. Seeds of a glyphosate susceptible population (GSP) were also collected in 2016 from an area near the Campus of the University of Cordoba (Argentina), without a history of glyphosate applications. GRP and GSP seeds were sown in pots containing peat wetted at field capacity and maintained under controlled conditions (28/18 °C day/night, photoperiod of 16 h, light density of 850 µmol m^{-2} s^{-1}, and 80% relative humidity) in a growth chamber. Seedlings with both cotyledons were transplanted into 250 mL pots containing sand/peat (1:2 v/v) and brought to a greenhouse with a temperature and photoperiod similar to that in the growth chamber.

2.2 Species Identification

Because *A. palmeri* and *Amaranthus hybridus* are difficult to distinguish by Argentinean farmers, since both species have the common name of yuyo Colorado (Morichetti et al., 2013), genetic analyses were needed to confirm and distinguish *A. palmeri* from other related species found in Argentina. Foliar tissues from 10 plants of each putative *A. palmeri* population were taken for genomic DNA (gDNA) isolation using the DNeasy Plant mini kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. The species identification was done by PCR using the specific primers AW90/AW155 developed by Wright et al., 2016. The length of amplicons was verified by gel electrophoresis. Individuals of *A. hybridus* and *Amaranthus viridis* were also included for distinction using the respective specific primers [AW473/AW483 (1623 bp) and AW477/AW493 (1215 bp), respectively] (Wright et al., 2016).

2.3 Shikimic Acid Accumulation Fast Screening

Ten plants per population were used for a fast screening using shikimic acid accumulation as a parameter to categorize plants as resistant or susceptible within a population, as well as to know the homogeneity or heterogeneity in and between *A. palmeri* populations. For individuals, three foliar disks (4 mm in diameter) were placed into 2 mL tubes, and then 1 µL of glyphosate at a concentration of 1000 µM was added to each tube (Dayan et al., 2015). Four replications per individual were obtained, and

the assay was repeated three times. A low shikimic acid accumulation implied a high resistance level (GRP), while a high shikimic acid accumulation implied high susceptibility (GSP). The plants were separated and transplanted into different pots (30 × 60 cm), and after 3–4 months, new seeds (F1) were collected for all future experiments.

2.4 Dose–Response Assays

The F1 seeds of GRP and GSP populations were germinated as described above in the Plant Material section. Young plants of *A. palmeri* with four true leaves were treated with the following increasing doses of glyphosate: 0, 31.25, 62.50, 125, 250, 500, 1000, 2000, and 4000 g ae (acid equivalent) ha^{-1}. The trade formulation of glyphosate used was Roundup Energy SL (450 g ae L^{-1} as isopropylamine salt, Monsanto). The herbicide treatments were performed using a spray chamber (SBS-060 De Vries Manufacturing, Hollandale, MN) equipped with a Tee Jet 8002EVS nozzle pressurized with 200 kPa to deliver 200 L ha^{-1} 50 cm above the plant level. The experiment was conducted using 10 plants from each population per glyphosate dose. Percentages of plant mortality (LD) and reduction of fresh weight (GR) were determined 28 days after treatment (DAT)

2.5 EPSPS Enzyme Activity Assays

According to Dayan et al., 2015 5 g of finely powered leaf tissue from each population were transferred to tubes with 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine), 70 µL of β-mercaptoethanol, and 1% polyvinylpolypyrrolidone (PVPP). After an agitation process and subsequent centrifugation, $(NH_4)_2SO_4$ was added in a proportion of 45% (w/v) to the supernatant. The mixture was stirred and then centrifuged twice to precipitate the total soluble protein (TPS). All pellets were dissolved in 3 mL of extraction buffer and dialyzed in 2 L of dialysis buffer (30 mm, 1000-MWC dialysis tubing at 4 °C on a stir plate) over 12 h. The TPS concentrations in the raw extract were determined using the colorimetric method of Bradford (Bradford, 1976).

The specific EPSPS activities of the GRP and GSP *A. palmeri* populations were determined using the EnzCheck phosphate assay kit (Invitrogen, Carlsbad, CA). The glyphosate concentrations tested to estimate inhibition of EPSPS activity by 50% (I_{50}) ranged from 0.1 to 1000 µM. The experiments were conducted with five replications of each population per glyphosate concentration and repeated three times. The EPSPS activity

was expressed as a percentage of the phosphate (µmol) released µg of TSP^{-1} min^{-1} in comparison to the controls (EPSPS basal)

2.6 EPSPS Gene Sequencing

Two samples of leaf tissue, one for total RNA extraction and the other for DNA extraction, were collected from 10 individuals from each *A. palmeri* population and stored at −80 °C. The total RNA was extracted using the Tri Reagent solution (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. The cDNA synthesis was carried out from 1 µg of the total RNA in all samples using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA). A fragment of the EPSPS gene, including the Thr102 and Pro106 positions, was amplified by using the EPSF1 (5'-ATGTTGGACGCTCTCAGAACTCTTGGT-3') and EPSR1 (5'-GTCATAAGTTTCAATGGCGGTGG-3') primers and PCR conditions described by Gaines et al., 2010. The EPSPS fragments were inserted in the pGEM-T Easy Vector System (Promega Biotech Iberica, SL, Madrid, Spain) to clone them into competent cells of *Escherichia coli* DH5a (Promega). Sanger sequencing of positive clones was carried out by STABVIDA (Caparica, Portugal).

2.7 *EPSPS* Gene Copy Number and Amplification

The leaf tissue samples taken for DNA extraction in the previous section were used to determine the *EPSPS* gene copy number and expression. The gDNA was isolated using the same media as for the identification of species. *EPSPS* gene copy number (from gDNA) and gene amplification (from cDNA used for EPSPS gene sequencing) assays were performed using the EPSPS and acetolactato synthase (ALS) primers developed by Gaines et al., 2010. Reactions were performed using a qRT-PCR Bio-Rad CFX connect thermal cycler and the following amplification profile: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and 95 °C for 15 s. PCR reactions were set up in 20 µL of SYBR Green PCR Master Mix (Bio-Rad), following the manufacturer's instructions. Controls containing water were included to check for contamination in the qPCR reactions. The *ALS* gene was used as a reference gene to normalize qRT-PCR results. The relative amplification levels were calculated from the threshold cycle (Ct) values and the primer efficiencies by the Pfaffl method (Pfaffl, 2001). The *EPSPS* gene copy number in the *A. palmeri* gDNA was determined as described by Gaines et al., 2010. Results were expressed as relative *EPSPS* gene copy number in relation to the *ALS* gene

by the Pfaffl method (Pfaffl, 2001). Triplicate technical replications were used to calculate the mean and standard error of the increase in *EPSPS* gene amplification or copy number relative to ALS. Standard curves were performed for each primer pair to confirm appropriate efficiency of amplification (E = 100 ± 10%)

2.8 ^{14}C-Glyphosate Absorption and Translocation

^{14}C-Glyphosate (American Radiolabeled Chemicals, Inc., Saint Louis, MO) and the trade glyphosate formulation were mixed to prepare a solution with 0.834 KBq μL^{-1} specific activity. The final concentration of the glyphosate solution was 300 g ae ha^{-1} in 200 L ha^{-1}. Twenty-three *A. palmeri* plants per population with four true leaves (three plants were reserved for the visualization of ^{14}C-glyphosate) received a 1 µL drop (0.834 KBq $plant^{-1}$) onto the adaxial surface of the second leaf. The plants and subsequent samples [rinse solution, treated leaf (TL), remaining shoot tissue (ST), and roots system (RS)] were handled at 24, 48, 72, and 96 h after treatment (HAT) (five plants per population at each time evaluated) according to the method of Dominguez-Valenzuela et al., 2017. The experiment had a completely randomized design, it was repeated twice, and the results of absorption and translocation of ^{14}C-glyphosate were expressed as percentages of the total herbicide recovered and absorbed, respectively (Rojano-Delgado et al., 2012).

The distribution of the ^{14}C (in form of ^{14}C-glyphosate or ^{14}C-metabolites) within *A. palmeri* plants was visualized by using a phosphor imager (Cyclone, PerkinElmer Packard Bioscience BV) at 96 HAT. The three whole plants of each population reserved were handled as described by Rojano-Delgado et al., 2012.

2.9 Glyphosate Metabolism

A. palmeri plants with six true leaves were treated with 300 g ae ha^{-1} glyphosate as in the dose–response assays. The same numbers of plants, without glyphosate treatment, were used as the control. Treated and untreated plants were cut and divided into the aboveground part (aerial part) and roots at 48 and 96 HAT, washed with distilled water, rapidly frozen in N2 liquid, and stored at −40 °C before being used. Glyphosate and the metabolites aminomethyl phosphonate (AMPA), formaldehyde, glyoxylate, and sarcosine were quantified according to the method of Rojano-Delgado et al., 2010. Calibration equations were obtained using known concentrations of standards of glyphosate and its metabolites (Sigma–Aldrich, St. Louis, MO). Five plants per population

were used in a completely randomized design, and the experiment was repeated three tim

2.10 Data Analysis

The parameters GR_{50}, LD_{50}, and I_{50} were determined using a three-parameter log–logistic equation, $Y = d/1 + (x/g)^b$, where Y is the response by 50% in relation to the control; *x* is the herbicide rate; *d* is the upper limit; g is the GR_{50}, LD_{50}, or I_{50}; and *b* is curve slope in *g*. Nonlinear regression analyses were conducted in the R program using the drc package (Ritz et al., 2015). The R/S ratios of GR_{50}, LD_{50}, or I_{50} were calculated to indicate the indices of resistance (RI).

The data of ^{14}C-glyphosate absorption and translocation, glyphosate metabolism, shikimic acid accumulation at 1000 µM glyphosate, and basal enzyme activity were analyzed using the software Statistix, version 9.0 (Analytical Software). Percentage data were transformed into arcsine before the ANOVA, and the model assumptions of normal distribution of errors and homogeneous variance were inspected graphically. Values of $P<0.05$ from the ANOVA were considered significant, and the means were separated using the Tukey HSD test ($\alpha = 0.05$).

3. Results

3.1 Species Identification

Using specific primers for the EPSPS intron 1 of *A. palmeri*, the resulting gel images revealed a band of 697 kb in length, confirming that all plants did belong to this species, both for GRP and GSP populations (Figure 2.1).

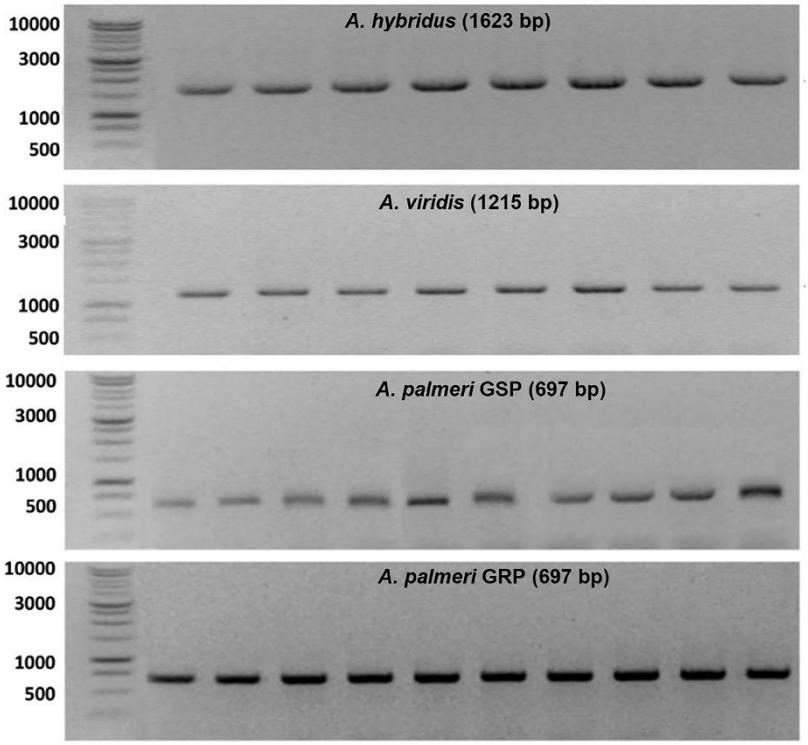

Figure 2.1 Gel images of PCR to distinguish between *Amaranthus* species by sequencing the intron 1 of the 5-enolpyruvylshikimate-3-phosphate synthase gene with specific primers. The first lane is a 1 kb ladder ranging from 10 to 0.3 k.

3.2 Accumulation of Shikimic Acid as a Biomarker for Glyphosate Resistance

The highest shikimic acid accumulation was observed in plants from the GSP population, ranging from 0.4 to 0.55 mg mL^{-1}, while for the GRP population values ranged from 0.17 to 0.32 mg mL^{-1} (Figure 2.2).

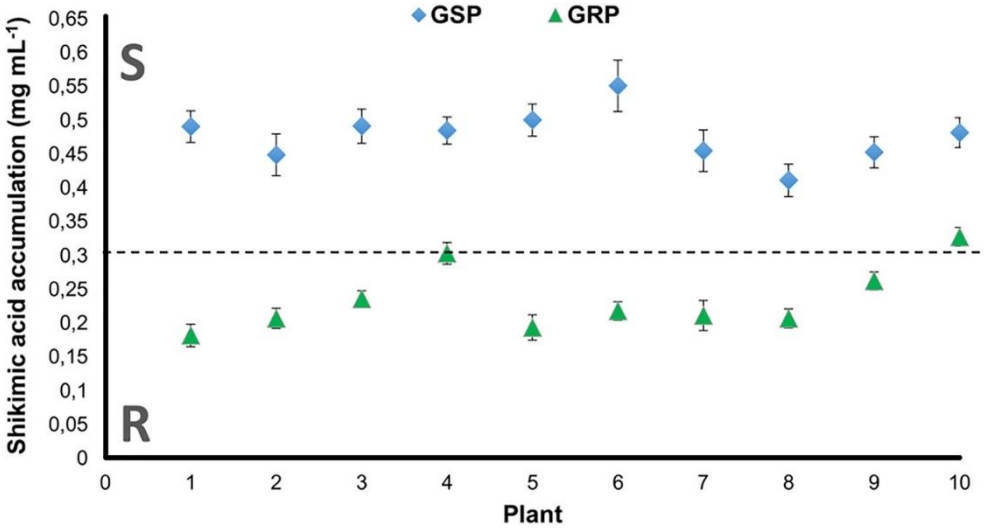

Figure 2.2. Shikimic acid accumulation (mg mL^{-1} HCl) at 1000 µM glyphosate in 10 GRP and GSP plants of *A. palmeri* populations from Cordoba, Argentina.

3.3 Assays of Dose–Response to Glyphosate

Resistance to glyphosate was confirmed for the GRP *A. palmeri* population. The GR_{50} and LD_{50} values estimated for the GSP population were 15.9 and 32.4 g ae ha^{-1}, respectively. According to these values, the GRP population was 34.6 (based on GR_{50}) and 59.7 (based on LD_{50}) times more resistant in comparison to the GSP population (Figure 2.3).

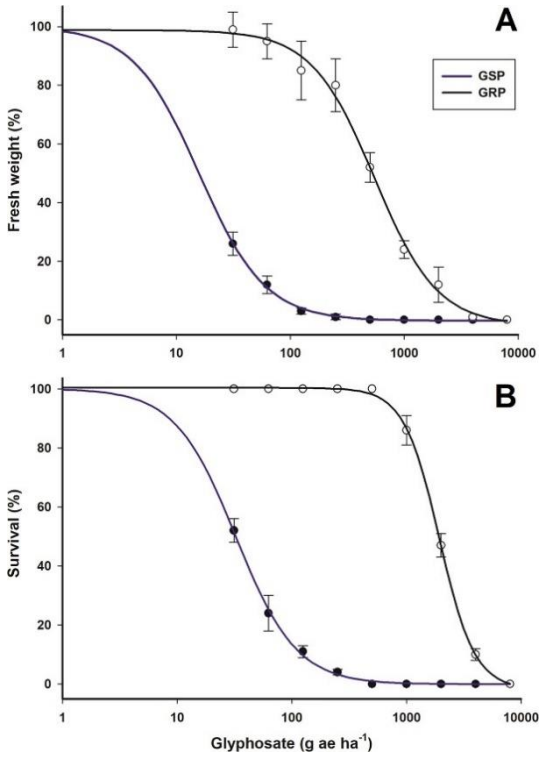

Figure 2.3 Dose–response curves relative to percentages of fresh weight reduction (A) and plant survival (B) in two populations (GRP and GSP) of *A. palmeri* from Cordoba, Argentina, treated with different glyphosate doses evaluated at 28 d after treatment. The log–logistic equations to estimate the GR_{50} values are $y = 100.0/[1 + (dose/GR_{50})^{0.90}]$ for GSP and $y = 100.3/[1 + (dose/GR_{50})^{1.73}]$ for GRP. The log–logistic equations to estimate the LD_{50} values are $y = 99.9/[1 + (dose/GR_{50})^{0.63}]$ for GSP and $y = 98.8/[1 + (dose/GR_{50})^{1.89}]$ for GRP. Vertical bars represent the standard error of the mean (n = 10).

3.4 EPSPS Enzyme Activity

Both basal and enzyme activities of the EPSPS showed no differences between *A. palmeri* populations. The basal activities were 0.062 and 0.063 µmol Pi μg^{-1} TSP min^{-1} for the GRP and GSP populations, respectively. The I_{50} values of each population were 5.6 and 5.2 µM, respectively (Figure 2.4).

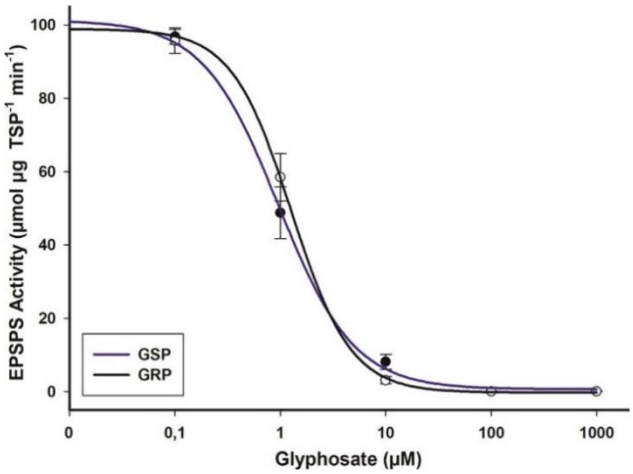

Figure 2.4 5-Enolypyruvylshikimate-3-phosphate synthase enzyme activity in leaf extracts from two populations (GRP and GSP) of *A. palmeri* from Cordoba, Argentina. The log–logistic equations to estimate the I50 values are y = 99.6/[1 + (concentration/I_{50})0.82] for GSP and y = 99.0/[1 + (concentration/I_{50})1.06] for GRP. Vertical bars represent the standard error of the mean (n = 3).

3.5 EPSPS Gene Sequencing, Copy Number, and Gene Amplification

The sequenced fragment of the EPSPS of 462 bp did not reveal mutations in either the Pro106 position or the Thr102 for any *A. palmeri* population (Figure 2.5A). The EPSPS copy number relative to ALS and also the gene amplification showed no differences between GSP and GRP populations. The averages of copy number and gene amplification were 0.97 and 1.41, respectively, for the GSP population and 1.03 and 1.32 for the GRP population (Figure 2.5B).

A

Amino acid position	101	**102**	103	104	105	**106**	107
Nucleotide consensus	gga	**aca**	gcg	atg	cgc	**cca**	ttg
A. palmeri S (FJ861242.1)	G	**T**	A	M	R	**P**	L
A. palmeri R (FJ861243.1)	G	**T**	A	M	R	**P**	L
A. palmeri GSP	G	**T**	A	M	R	**P**	L
A. palmeri GRP	G	**T**	A	M	R	**P**	L

B

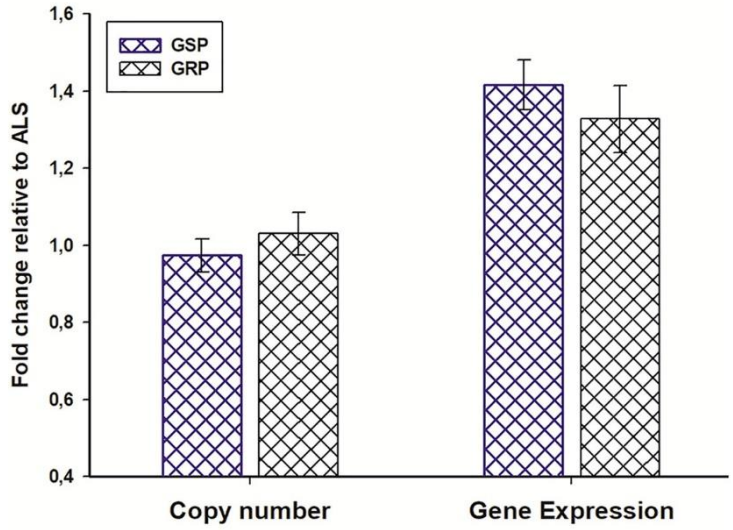

Figure 2.5 (A) Partial alignment of predicted amino acids of 5-enolpyruvylshikimate-3-phosphatesynthase (EPSPS) genes of two populations (GRP and GSP) of *A. palmeri* from Cordoba, Argentina. Blue boxes include positions 102 and 106, corresponding to point mutation sites confirmed to confer glyphosate resistance. (B) EPSPS copy numbers relative to the acetolactate synthase gene and EPSPS amplification levels. Vertical bars represent the standard error of the mean (n = 10).

3.6 Absorption, Translocation, and Distribution of ^{14}C-Glyphosate

The absorption of ^{14}C-glyphosate differed between *A. palmeri* populations, which increased steadily from 12 to 96 HAT in both populations. The percentage of absorbed herbicide at 12 HAT was 18 and 25% for the GRP and GSP, respectively, and up to 70 and 80% at 96 HAT (Figure 2.6A). Similar amounts of ^{14}C-glyphosate were found in the treated leaves in both GRP and GSP plants at 12 HAT (91.4 and 86.4%, respectively); however, GSP plants moved on average 20% more ^{14}C-glyphosate to the remainder of shoot and roots than the GRP plants at 96 HAT. In this evaluated period, in the GRP plants 17.2 and 20.8% of the herbicide were found in shoot and roots, respectively, while in the GSP plants 24.0 and 31.2% (Table 1) were found. Phosphor images, obtained at 96 HAT, corroborated these results, showing that GRP plants retained most of the ^{14}C-glyphosate within the treated leaf, while GSP plants moved more herbicide to the rest of the plant (Figure 2.6B).

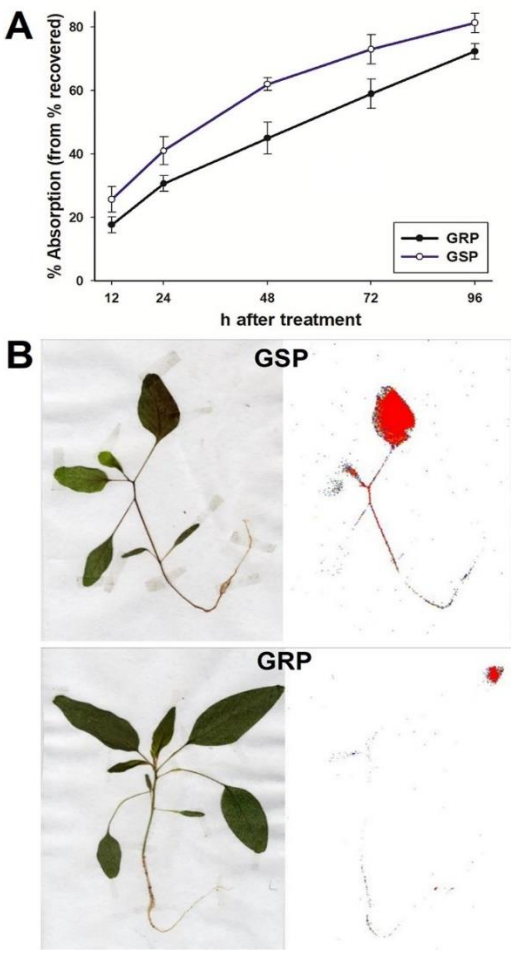

Figure 2.6 ^{14}C-Glyphosate absorption and translocation in plants of two populations (GRP and GSP) of *A. palmeri* from Cordoba, Argentina. (A) ^{14}C-Glyphosate absorption from 12 to 96 h after treatment. Vertical bars represent the standard error of the mean (n = 5). (B) Digital (left plants) and autoradiograph (right plants) images that show the distribution of ^{14}C within A. palmeri plants at 96 h after treatment. The highest concentration of ^{14}C is highlighted in red.

Table 2.1 ^{14}C-Glyphosate Translocation (%) in Two Populations (GRP and GSP) of A. palmeri from Cordoba, Argentina, at Different Hours after Treatment (HAT).

		^{14}C distribution (% of absorbed) [a]		
population	**HAT**	**treated leaf**	**remainder of shoot**	**root**
GRP	12	91.4 ± 3.5 ns	4.4 ± 0.2 ns	4.2 ± 0.2 b
GSP		86.4 ± 3.3 ns	6.4 ± 0.4 ns	7.2 ± 0.6 a
GRP	24	87.4 ± 3.0 a	6.0 ± 0.7 b	6.6 ± 0.6 b
GSP		76.8 ± 4.6 b	11.4 ±1.2 a	11.8 ± 1.2 a
GRP	48	80.4 ± 3.7 a	8.6 ± 0.4 b	11.0 ± 0.8 b
GSP		69.0 ± 3.7 b	15.8 ± 1.9 a	15.2 ± 1.6 a
GRP	72	73.8 ± 3.2 a	11.2 ± 0.8 b	15.0 ± 2.8 b
GSP		60.6 ± 4.8 b	18.4 ± 1.8 a	21.0 ± 2.4 a
GRP	96	62.0 ± 6.5 a	17.2 ± 2.7 b	20.8 ± 3.3 c
GSP		41.8 ± 5.3 b	24.0 ± 2.3 a	31.2 ± 3.9 a

[a]Means with different letters per plant section in a certain evaluation period are statistically different at $P < 0.05$ according to the Tukey test. ns = not significant. Values reflect the mean ± standard error of the mean (n = 5).

3.7 Glyphosate Metabolism

Glyphosate metabolites, such as AMPA or glyoxylate, were not detected in both populations, reinforcing that *A. palmeri* plants translocated only the herbicide. The amounts of glyphosate found in the roots were close to those quantified in the assays of absorption and translocation with ^{14}C-glyphosate. In the GRP plants, 58.8 and 171.6 nmol glyphosate (g fresh weight)$^{-1}$ were found at 48 and 96 HAT, respectively, corresponded to 12.2 and 21.8% of total glyphosate quantified, while for GSP plants, 14.7 and 29.1% [627.2 and 258.4 nmol glyphosate (g fresh weight)$^{-1}$, respectively] were found (Table 2.2).

Table 2.2 Glyphosate Metabolism [in nmol glyphosate/metabolites (g fresh weight)$^{-1}$] in Two Populations (GRP and GSP) of *A. palmeri* at 48 and 96 h after Treatment (HAT) with Glyphosate at 300 g ae ha^{-1}.

population	HAT	leaf area		roots	
		Glyphosate	Metabolites	Glyphosate	Metabolites
GRP	48	421.7 ± 10.6	ND[a]	58.8 ± 8.3	ND
	96	614.9± 9.1	ND	171.6 ± 9.4	ND
GSP	48	538.6 ± 9.5	ND	93.2 ± 13.3	ND
	96	627.2 ± 15.2	ND	258.4 ±15.8	ND

[a]ND = not detected.

2. Discussion

3.8 Species Identification and Resistance Confirmation

Species identification following genetic analysis confirmed that both populations, GPS and GRP, belong to A. *palmeri*. The great phenotypic plasticity of *A. palmeri* can give an erroneous identification of this species, because it is also an obligate outcrosser that can hybridize with other *Amaranthus* species (Gaines et al., 2012). The two main species found in soybean production areas of Cordoba province, Argentina, are *A. palmeri* and *A. hybridus* (Morichetti et al., 2013); therefore, ensuring species identification of the *Amaranthus* genus is important since Argentinian farmers have difficulty identifying them morphologically (Morichetti et al., 2013; Wright et al., 2016).

Once the GPS and GRP populations were distinguished as being *A. palmeri*, the glyphosate resistance was confirmed in the putative GRP population by its low shikimic acid accumulation, an unequivocal biochemical indicator of glyphosate resistance (Kleinman & Rubin, 2017), as well as its higher GR_{50} and LD_{50} values in comparison to the

GSP population. Comparing these values with those registered in other glyphosate-resistant *A. palmeri* populations from New Mexico in the USA (Mohseni-Moghadam et al., 2013) and other countries such as Brazil or Mexico, they were similar (Dominguez-Valenzuela et al., 2017; Küpper et al., 2017). The low shikimate accumulation in the GRP population was congruent with the lower impact on growth reduction and plant mortality as glyphosate doses increased in comparison to the GSP population. The low GR_{50} and LD_{50} values estimated for the GSP population resulted from the high inhibition of EPSPS enzyme that produced a high and rapid accumulation of shikimic acid (Kleinman & Rubin, 2017).

3.9 TRS Mechanisms Characterization

The increase in the enzymatic activity of the EPSPS is indicating that TSR mechanisms are contributing to the resistance to glyphosate. However, the EPSPS basal activity or its inhibition by glyphosate were similar between the GRP and GSP *A. palmeri* populations. The selection of glyphosate resistance in *Amaranthus* species is apparently well-described. *EPSPS* gene amplification has been reported as the major TSR mechanism reported for A. *palmeri* (Gaines et al., 2010; Vila-Aiub et al., 2014), as well as other *Amaranthus* species (Chatham et al., 2015; Lorentz et al., 2014; Nandula et al., 2014), noting that most of these resistance cases were documented in the USA. The lack of associated fitness cost makes the *EPSPS* gene duplication an important and widespread glyphosate resistance mechanism (Vila-Aiub et al., 2014), which presumably had a common origin of selection and spread rapidly across the USA (Molin et al., 2018). Interestingly, our qPCR results showed the unlikelihood of a greater EPSPS gene copy number and/or its amplification in the GRP population. The distant geographic origin (Argentina) of the GSP and GRP populations compared to the populations of *A. palmeri* from the USA showed that the *EPSPS* gene amplification cannot be considered as the main mechanism of glyphosate resistance in this species in a generalized way. In addition, *EPSPS* gene sequencing did not reveal any amino acid substitution at positions Thr102 and Pro106, point mutation sites that can promote changes reducing the binding of glyphosate with EPSPS; i.e., these substitutions can endow resistance to this herbicide, as confirmed for *A. palmeri* from Chihuahua, Mexico (Dominguez-Valenzuela et al., 2017), and *Amaranthus tuberculatus* from Missisippi, USA (Nandula et al., 2013),

as well as other weed species (Sammons & Gaines, 2014). Consequently, results indicated that TSR mechanisms were not involved in the resistance to glyphosate of the *A. palmeri* GRP. However, it cannot be ruled out that other South American populations of *Amaranthus* species may select for glyphosate resistance by TSR mechanisms

3.10 NTSR Mechanisms Characterization

NTSR are important evolutionary mechanisms of herbicide resistance (Ghanizadeh & Harrington, 2017). However, relatively few cases in which the cause of glyphosate resistance is at least a NTSR mechanism have been described. The main NTSR mechanism reported to endow glyphosate resistance is the alteration of translocation patterns of the herbicide; thus, in resistant plants, less glyphosate is translocated to meristematic growing points and it is retained in the treated leaves (Preston & Wakelin, 2008). In this research, both quantitative and qualitative results revealed altered patterns of ^{14}C-glyphosate absorption and translocation as the NTSR mechanisms in the GRP *A. palmeri* population from Argentina. Accordingly, the low accumulation of shikimate in GR plants evidenced the occurrence of NTSR mechanisms, since this pattern is due to the reduced foliar absorption or the modified subcellular distribution (Kleinman & Rubin, 2017), reducing the glyphosate amounts that reach the EPSPS. Therefore, it is congruent to infer that low absorption and impaired translocation of glyphosate were the primary and major mechanisms of resistance in the *A. palmeri* GRP population. These results are consistent with those previously observed in this species (Dominguez-Valenzuela et al., 2017), other *Amaranthus* species (Nandula et al., 2013), or other weed species (Adu-Yeboah et al., 2014; Alcántara de la Cruz et al., 2016; De Carvalho et al., 2012; Kleinman & Rubin, 2017; Palma-Bautista et al., 2019). The low glyphosate absorption observed in the GRP was likely due to differences in the external leaf surfaces between populations (Alcántara de la Cruz et al., 2016; D'Avignon & Ge, 2018; Délye, 2013), while impaired translocation resulted from the greater retention of herbicide near to the treated area (Adu-Yeboah et al., 2014; De Carvalho et al., 2012). The strongest evidence has shown that the sequestration of glyphosate into the vacuole is the main NTSR mechanism responsible for altering the translocation patterns of this herbicide (D'Avignon & Ge, 2018; Ge et al., 2012, 2014), which is regulated by tonoplast-active transporters (Ge et al., 2014).

Enhanced metabolism as a NTSR mechanism has been reported in plants at most herbicide action sites (Ghanizadeh & Harrington, 2017), but never for glyphosate. In this study, glyphosate was not metabolized in treated leaves of either GR or GS *A. palmeri* plants. Glyphosate metabolism is not frequent in plants, and so far, it seems not to play an important role as an NTSR mechanism for glyphosate resistance (Duke, 2011). Therefore, these results allow one to conclude that glyphosate metabolism did not contribute to resistance of the GRP population but also confirmed that *A. palmeri* plants translocated only the parent herbicide, demonstrating that GRP plants selected for similar mechanisms of resistance to glyphosate like other weeds, reinforcing the remarkable repeated evolution of herbicide resistance (Baucom, 2016).

Molecular characterization confirmed that both GSP and GRP populations were *A. palmeri*, and the glyphosate resistance of the second population was confirmed. This research is the first study unraveling the resistance mechanisms in *A. palmeri* from Argentina, revealing the noninvolvement of TSR mechanisms, i.e., neither mutations nor *EPSPS* gene amplification was found in this population. By contrast, the GRP population exhibited a low absorption and impaired translocation of glyphosate as the main resistance mechanisms. This is the first case worldwide of glyphosate resistance in *A. palmeri* based only on NTSR mechanisms. Future experiments are required to unravel the physiological and biochemical basis of the reduced absorption and translocation found in this research, including gene amplification and regulation, which could drive the evolution of NTSR to glyphosate in this and other weed species.

4. References

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CAPITULO III

Resistance Mechanisms to 2,4-D in Six Different Dicotyledonous Weeds Around the World

agronomy

MDPI

Article

Resistance Mechanisms to 2,4-D in Six Different Dicotyledonous Weeds Around the World

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Abstract: 2,4-D resistance is increasing around the world due to both transgenic crops and resistance to other herbicides. The objective of the this study was to characterize the currently unknown mechanisms of 2,4-D resistance in five weed species from around the globe: *Amaranthus hybridus* (Argentina), *Conyza canadensis* (Hungary), *Conyza sumatrensis* (France), *Hirschfeldia incana* (Argentina) and *Parthenium hysterophorus* (Dominican Republic), using *Papaver rhoeas* (Spain) as a standard resistant (R) species. Dose-response trials using malathion and absorption, translocation and metabolism experiments were performed to unravel the resistance mechanisms. R plants produced at least 3-folds less ethylene than susceptible plants, confirming the resistance to 2,4-D, together with resistance factors >4. *A. hybridus*, *P. hysterophorus* and *P. rhoeas* showed both reduced translocation and enhanced metabolism. In the two *Conyza* sps., the only resistance mechanism found was enhanced metabolism. Malathion synergized with 2,4-D in all these species, indicating the role of cytochrome P450 in the herbicide degradation. In *H. incana*, reduced translocation was the only contributing mechanism to resistance. Among the six dicotyledonous weed species investigated, there was a differential contribution to 2,4-D resistance of enhanced metabolism and reduced translocation. Thus, extrapolating 2,4-D resistance mechanisms from one weed species to another is very risky, if even related.

Keywords: *Amaranthus hybridus*; *Conyza* sp.; cytochrome P450; enhanced metabolism; *Hirschfeldia incana*; *Papaver rhoeas*; *Parthenium hysterophorus*; reduced translocation

1. Introduction

Commercially released in 1946, 2,4-D was used principally to control a wide spectrum of dicotyledonous weeds [1]. It is one of the oldest herbicides and its appearance revolutionized the discipline of weed science by introducing a new control method [2]. 2,4-D still remains 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 [1,2]. According to its site of action (SoA), this molecule belongs to the group of synthetic auxin herbicides (SAH) (HRAC group O; WSSA Group 4) along with dicamba, picloram and others, which mimic the naturally occurring plant hormone indole-3-acetic acid [3]. 2,4-D is

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Keywords: *Amaranthus hybridus*; *Conyza* sp.; cytochrome P450; enhanced metabolism; *Hirschfeldia incana*; *Papaver rhoeas*; *Parthenium hysterophorus*; reduced translocation.

Resumen

La resistencia al 2,4-D está aumentando en todo el mundo debido al uso de los cultivos transgénicos como a la resistencia a otros herbicidas. El objetivo de este estudio fue caracterizar los mecanismos de resistencia al 2,4-D desconocidos hasta ahora en cinco especies de malas hierbas de todo el mundo: *Amaranthus hybridus* (Argentina), *Conyza canadensis* (Hungría), *Conyza sumatrensis* (Francia), *Hirschfeldia incana* (Argentina) y *Parthenium hysterophorus* (República Dominicana), utilizando *Papaver rhoeas* (España) como especie resistente (R). Se realizaron ensayos dosis-respuesta con malatión y experimentos de absorción, translocación y metabolismo para conocer los mecanismos de resistencia. Las plantas R produjeron al menos 3 veces menos etileno que las plantas susceptibles, confirmando la resistencia a 2,4-D, con factores de resistencia >4. *A. hybridus*, *P. hysterophorus* y *P. rhoeas* mostraron una translocación reducida y un metabolismo mejorado. En las dos *Conyza* spp., el único mecanismo de resistencia encontrado fue un incremento en el metabolismo. El malatión hizo sinergia con el 2,4-D en todas especies estudiadas, indicando el papel del citocromo P450 en la degradación del herbicida. En *H. incana*, la reducción de la translocación fue el único mecanismo que contribuyó a la resistencia. Entre las seis especies de malas hierbas dicotiledóneas estudiadas, hubo una participación diferencial en la resistencia al 2,4-D entre el metabolismo mejorado y la translocación reducida. Por lo tanto, extrapolar los mecanismos de resistencia a 2,4-D de una especie de mala hierba a otra es muy arriesgado, si es que está relacionado.

Palabras clave: *Amaranthus hybridus*, *Conyza* sp., citocromo P450, metabolimo mejorado, *Hirschfeldia incana*, *Papaver rhoeas*, *Parthenium hysterophorus*, translocación reducida.

Introduction

Commercially released in 1946, 2,4-D was used principally to control a wide spectrum of dicotyledonous weeds (Mithila et al., 2011). It is one of the oldest herbicides and its appearance revolutionized the discipline of weed science by introducing a new control method (Peterson et al., 2016). 2,4-D still remains 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 (Mithila et al., 2011; Peterson et al., 2016). According to its site of action (SoA), this molecule belongs to the group of synthetic auxin herbicides (SAH) (HRAC group O; WSSA Group 4) along with dicamba, picloram and others, which mimic the naturally occurring plant hormone indole-3-acetic acid (Grossmann, 2010). 2,4-D is a phenoxy-carboxylate among the five chemical families within SAH. It is available in several different formulations of the ester, amine salt and acid. The dimethylamine salt and the ester account for around 95% of all formulations sold globally (Gervais et al., 2008).

SAH action is given by three ways: 1, altering the plasticity of the cell walls; 2, influencing the amount of protein production; and 3, increasing ethylene production (Song, 2014). All these effects can cause the plant death. The efficacy of 2,4-D as herbicide depends on several factors that involve uptake and translocation to the plant meristems (Song, 2014; Weintraub et al., 1954). The complexity surrounding SAH to understand their SoA is due to the involvement of large protein families in auxin perception, signaling, transport and metabolism (Matthes et al., 2019). Because its precise SoA is not well established to date, the mechanisms involved in this resistance still remain poorly understood (Busi et al., 2018). The first cases of 2,4-D resistance were reported in *Daucus carota* and *Commelina diffusa* in 1957(Hilton, 1957; Switzer, 1957). Besides is extended use after >70 years, few cases of resistant (R) weeds to this SoA have been reported compared to others (Heap, 2019). To date, 41 species have developed resistance to auxinic herbicides in 22 countries (Heap, 2019).

2,4-D can be absorbed through roots, stems and leaves, but due to its foliar mode of application, its uptake is mainly by leaves (Song, 2014). 2,4-D uptake and translocation is representative of the active phloem loading pathway (Grossmann, 2010). In this sense, reduced SAH absorption and/or translocation has been related to the resistance

response in several dicotyledonous weeds species (Schulz & Segobye, 2016). More recently, the lack of 2,4-D translocation has also been reported in R biotypes of *Papaver rhoeas* (Rey-Caballero et al., 2016), *Sisymbrium orientale* (Dang et al., 2018 and *Raphanus raphanistrum* (Goggin et al., 2020).

2,4-D metabolism in plants can follow three paths: first, primarily through direct conjugation, leading to phytotoxic metabolites usually in susceptible (S) dicotyledonous; second, ring hydroxylation, mediated by cytochrome P450 (Hatzios, 2005), which leads to a non- or partially phytotoxic metabolite usually in tolerant monocots; and as a third option, but less common, it can be metabolized by side-chain cleavage (Peterson et al., 2016). In this regards, potential detoxification of SAH, as a non-target-site resistance (NTSR) mechanism, can contribute to resistance in several dicotyledonous weeds species, such as *Amaranthus tuberculatus*, *Carduus nutans*, *Kochia scoparia*, *Stellaria media* and *Papaver rhoeas* for 2,4-D, or *Sinapis arvensis* for dicamba. In the case of *A. tuberculatus* and *P. rhoeas*, malathion synergized with 2,4-D, indicating the potential role of P450 (Figueiredo et al., 2018; Torra et al., 2017)

SAH have several target sites within plants: a nuclear auxin receptor proteins family (TIR1 and Auxin F-Box proteins, AFB1–5), an auxin cell influx carrier (AUX1) and two auxin efflux carrier proteins families (PIN and ABCB proteins) (Busi et al., 2018). These potential multiple sites of action of SAH could explain the rarity of target-site resistance (TSR) mechanisms described so far in weeds, among other factors (Mithila et al., 2011). For the first time, the presence a mutation of the *AUX1* gene in *K. scoparia* has only very recently been fully demonstrated as a TSR mechanism conferring cross-resistance to 2,4-D and dicamba (LeClere et al., 2018).

When 2,4-D binds with its nuclear receptor TIR1, it promotes a cascade of processes, resulting in derepression of auxin-regulated genes that in turn leads to the physiological and morphological events associated with its action (Gray et al., 2001). Among those, the enhanced expression of the ACC synthase, which stimulates ethylene production, usually in S but not in R plants, is well acknowledged (Goggin et al., 2018). Though ethylene biosynthesis it is not necessarily related to the nature of the resistance mechanism involved, it can be used as a fast screening to detect resistance to 2,4-D and other SAH (Howatt et al., 2006; Rey-Caballero et al., 2016).

Between 1980 and 2009, 0.8 resistance cases per year to SAH were reported, while from 2010 until today, the rate has increased to 1.4 cases per year (Heap, 2019). It is expected that the number of cases will increase trough the next years due to both new uses of 2,4-D enabled by the introduction of 2,4-D R crops, and also its incremental use due to greater dependence on SAH in response to the emergence of resistance to other herbicides (Nandula, 2019). Among many countries, very important new weeds R to 2,4-D have been cited: *Amaranthus hybridus* from Argentina (Dellaferrera et al., 2018) and *Parthenium hysterophorus* from Cuba (Mora et al., 2019), in which enhanced metabolism seems to be involved, and *Papaver rhoeas* is now spreading in France and Italy (Kati et al., 2019). Other cases have been reported but not yet investigated, including *Conyza canadensis* from Hungary and *C. sumatrensis* from France, both found in vineyards, and *Hirschfeldia incana* from Argentina in winter cereals, reported by the co-author Dr. Vigna (Heap, 2019).

The aim of the current study was to characterize the currently unknown mechanisms of evolved 2,4-D resistance in the above-mentioned species *Amaranthus hybridus* from Argentina, *Conyza canadensis* from Hungary, *Conyza sumatrensis* from France, *Hirschfeldia incana* from Argentina and *Parthenium hysterophorus* from the Dominican Republic, using *Papaver rhoeas* from Spain as a standard R species. This knowledge should be useful to develop strategies to ameliorate 2,4-D resistance in these weed species.

1. Materials and Methods

1.1. Chemicals

A 2,4-D of formulated product (ester, Esteron 60® (EC, 60% w/v), Dow Agrosciences Iberica, Pozuelo de Alarcón, Spain) was used in greenhouse tests and laboratory studies. The analytical grade (>99.5%) was used to determine the herbicide effects on physiological and biochemical studies in plants. ^{14}C-2,4-D acid (specific activity 2.035 GBq mmol^{-1}) with 95% radio-chemical purity was obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA).

1.2. Plant Material

The seeds of original field evolved 2,4-D R populations were harvested from six different countries from Latin America and Europe in 2017 (Table 3.1). These populations

displayed a survival of >90% at the recommended field dose (480 g ai ha^{-1}) for *Amaranthus hybridus*, *Hirschfeldia incana* and *Parthenium hysterophorus*, or at 600 g ai ha^{-1} for *Conyza canadensis* and *Papaver rhoeas*, while it was ~25% for *Conyza sumatrensis* (data not shown). 2,4-D S populations belonging to these species were originally harvested from areas close to the fields evolving resistance to this herbicide, except for *P. rhoeas* in which a known S standard was used (Rey-Caballero et al., 2016, 2017). Seeds were maintained at 4 °C in a cold chamber and they had more than an 80% germination rate during this study.

Table 3.1. Most important characteristics of the dicotyledonous species resistant (R) to 2,4-D studied.

Species	Famlly	Country/region	Crop	Survived Field Dose (g ai ha^{-1})
Amaranthus hybridus	Amaranthaceae	Argentina/Colonia Marina	Soybean	480
Conyza canadensis	Asteraceae	Hungary/Badacsony	Vineyard	600
C. sumatrensis	Asteraceae	France/Jonquieres	Vineyard	600
Hirschfeldia incata	Brassicaceae	Argentina/Buenos Aires	Wheat	480
Parthenium hysterophorus	Asteraceae	R. Domican/Basima	*Citrus sinensis*	480
Papaver roheas	Papaveraceae	Spain/Baldomar	Winter cereal	600

1.3. Ethylene Production Fast Screening

The ethylene biosynthesis stimulation could be considered to be a fast screening to detect resistance to 2,4-D and other SAH (Howatt et al., 2006; Rey-Caballero et al., 2016). 480 g ai ha^{-1} of 2,4-D was applied on R and S plants of each dicotyledonous species tested (Table 3.1). The experiment was performed using 10 replicates (one plant per pot) at the growth stage of four leaves. The herbicides were applied in a laboratory chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 250 L ha^{-1} at the height of 50 cm from plant level. Twenty-four h after treatment (HAT), seedlings were excised and 400 g shoot fresh weight was placed into 10 mL syringe with 1 mL distilled water and sealed (Tahmasebi et al., 2018). The syringes were placed in a dark incubator at 27 °C for 4 h and 1 mL of the headspace gas was analyzed for ethylene (C_2H_4) by gas chromatography (Tahmasebi et al., 2018). The

C_2H_4 was expressed as a µL per gram of fresh weight by h. The experiment was repeated twice.

1.4. Response of Dicotyledonous Weed Species to 2,4-D Pre-Treated with or without Malathion

Seeds of R and S populations of the six dicotyledonous weed species were germinated on moistened filter paper in Petri dishes, and one week later, seedlings were planted in 8 × 8 × 10 cm pots (one plant per pot). After two weeks when R and S plant populations were at the 4–6 leaf stage, they were sprayed with 2,4-D at different doses (0, 45, 90, 180, 360, 720 and 1440 g ai ha^{-1}) as shown above. This dosage was determined in a preliminary test carried out at doses of 50, 300 and 600 g ai ha^{-1} in the R and S populations of the six dicotyledonous species. Above-ground dry weight per plant was determined 28 days after treatment (DAT), and data were expressed as the percentage of the untreated control.

Malathion has previously been shown to inhibit 2,4-D metabolism in R *P. rhoeas* and *A. tuberculatus* by inhibiting P450 (Figueiredo et al., 2018; Tahmasebi et al., 2018). Seedlings of R and S populations for the six weed species were treated with malathion at 2000 g ai ha^{-1} at the 4–6 leaf stage and then they were dried for 2 h at laboratory temperature (24 °C). After that, 2,4-D was applied at doses as shown above. The experiments were repeated twice with three replicates (ten technical replications for each population).

1.5. ^{14}C-2,4-D Absorption and Translocation in Dicotyledonous Weeds

The ^{14}C-2,4-D absorption and translocation assays were carried out as described by Rey-Caballero et al., 2016 and Bracamonte et al., 2016, with some modifications. ^{14}C-herbicide was mixed with commercial formulations of 2,4-D at different field doses for each species (Table 3.1). The final 2,4-D concentrations were 480 g ai ha^{-1} (*A. hybridus*, *H. incana* and *P. hysterophorus*) and 600 g ai ha^{-1} (*C. canadensis*, *C. sumatrensis* and *P. rhoeas*) in 250 L ha^{-1} with a specific activity of 0.834 kBq μL^{-1}. Plants of the R and S populations at the four-leaf stage were treated with a drop (1 µL $plant^{-1}$) of radiolabeled solution on the adaxial surface of the second youngest leaf. The herbicides were applied in separate experiments. There were five repeats and each experiment was arranged in a completely randomized design.

The plants were harvested at 96 HAT. Previous experiments with different dicotyledonous weeds, such as *P. rhoeas*, established this time point to be the most suitable for finding maximum differences between S and R populations to SAH (Goggin et al., 2016; Rey-Caballero et al., 2016, 2017; Riar et al., 2011). The unabsorbed ^{14}C-herbicide was removed from the treated leaf with a water-acetone solution (1:1 v/v) by washing the plants three times separately with 1 mL of that solution. The rinse solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard, Groningen, The Netherlands) and measured by liquid scintillation spectrophotometry (LSS) using a Beckman LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA). The whole washed plants were removed from the pot and sectioned into treated leaves, the rest of the plant and roots. The plant sections were individually stored in cellulose cones for combustion, dried at 60 °C for 96 h and burned using a biological oxidant (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA, USA). The CO_2 released from combustion was captured in 18 mL of a mixture of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of each sample was measured for 10 min by LSS. The radioactive values of absorption and translocation of ^{14}C were expressed as a percentage of the total ^{14}C-herbicide applied and absorbed, respectively.

1.6. 2,4-D Metabolism in Dicotyledonous Weeds

Seedlings from S and R populations at six true leaves of development (4–6 cm) were treated with 2,4-D doses, at 0 and 480 g ai ha^{-1} and 600 g ai ha^{-1}, which are the field recommended rates in Latin America and Europe, respectively (Table 3.1), as described above for the dose-response experiments. Five plants from each population and dose were harvested at 96 HAT. As abovementioned in the preceding experiment, this time point was established according to previous research (Rey-Caballero et al., 2017; Torra et al., 2017). Plants were separated into aerial part (leaves and shoots) and root system, and were rinsed using distilled water to remove unabsorbed herbicide. They were rapidly frozen in liquid nitrogen and then stored at −80 °C until use. For these assays, the methodology described by Torra et al., 2017 and Mora et al., 2019 was followed using a Gold LC System from Beckman Coulter (Fullerton, CA, USA) equipped with a diode array detector (wavelength range 190–600 nm). The chromatographic separation

was carried out using a Kinetex® EVO C18 column (150 mm, 4.6 mm id, 2.6µm particle size) from Phenomenex Inc. (Torrance, CA, USA), furnished with a 4.6 mm SecurityGuardTM ULTRA cartridges. Quantification of 2,4-D and its metabolites was based on the calibration curve of 2,4-D. The results were expressed as percentage of 2,4-D and its metabolites. The experiment was performed for each dicotyledonous species (R and S populations) and was repeated twice at different times in the laboratory.

1.7. Data Analysis

The amount of herbicide causing a 50% reduction in dry weight compared to the untreated control (GR_{50}) was calculated by submitting the percentage data to a non-linear regression analysis using a log-logistic model of three parameters (four parameters ($Y = c + \{(d-c)/[1 + (x/g)^b]$), where *c* and *d* are the upper and lower asymptotic limits, *b* is the slope, *g* is the GR_{50} and *x* is the herbicide concentration) (Seefeldt et al., 1995). The three-parameter model assumes that the lower limit is zero. Regression analyses were performed in SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA, USA) with the R program. The resistance factor (RF) was computed as GR_{50} (R)/GR_{50} (S) (Bracamonte et al., 2016).

Data from ethylene production, absorption and translocation, as well as metabolism experiments, were submitted to an analysis of variance (ANOVA) in a completely randomized design for each species, comparing R and S populations. Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected for all tests. Values of $P < 0.05$ were considered statistically significant and mean comparisons were performed using the Tukey's test at the 5%. Statistical analyses were conducted with the Statistix 9.0 software (Analytical Software, Tallahassee, FL, USA).

2. Results

2.1. Ethylene Production Fast Screening

Foliar application of 2,4-D stimulated ethylene production in all plants studied (both R and S for each species), but the comparison between R and S populations showed always significant differences in all species. S plants produced between three- and nine-folds more ethylene than R plants (Figure 3.1). When comparing the S populations across the species, there were no differences in the production of ethylene in S plants of *A.*

hybridus, *C. canadensis*, *C. sumatrensis*, *P. hysterophorus* and *P. rhoeas*, while *H. incana* produced roughly two-folds less ethylene. On the other hand, *C. sumatrensis* R plants produced more ethylene than the rest of the species.

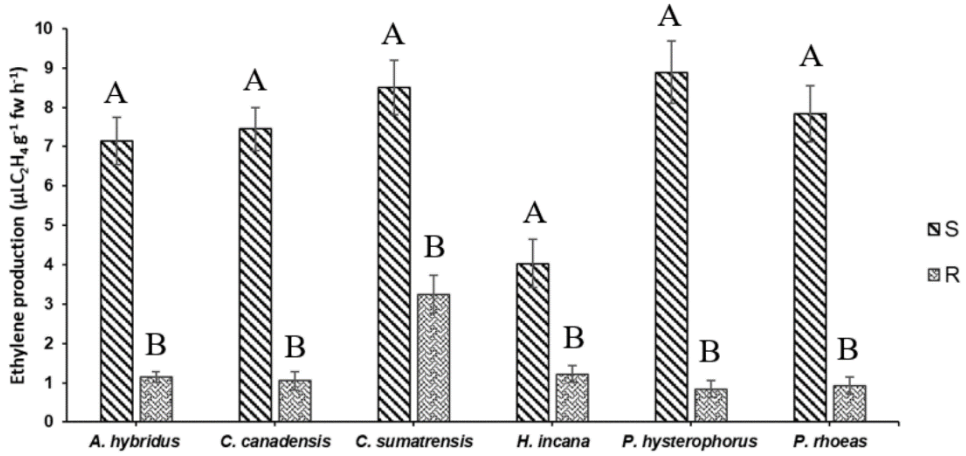

Figure 3.1 Ethylene production induced by 2,4-D at 480 g ai ha^{-1} in different dicotyledonous weed species resistant (R) and susceptible (S) populations. Means followed by the same letter within each species do not differ by the Tukey test ($P < 0.05$). Vertical bars are means ± the standard errors of the mean (n = 10).

2.2. Response of Dicotyledonous Weed Species to 2,4-D Pre-Treated with or without Malathion

All R and S populations of the six species had a general common behavior after the first days of 2,4-D application in the preliminary test: appearance of epinasty, growth reduction and morphological damage at high doses (600 g ai ha^{-1}) in the five R populations and at low doses (50 g ai ha^{-1}) in the six S populations. The R-*C. sumatrensis* population suffered these deformations at doses of 300 g ai ha^{-1}. While all S populations died 21 DAT at the dose of 300 g ai ha^{-1}, the R populations survived at the dose of 600 g ai ha^{-1} except for *C. sumatrensis* that died. The GR_{50} values clearly showed 2,4-D resistance of the species *A. hybridus*, *C. canadensis*, *H. incana*, *P. hysterophorus* and *P. rhoeas*, while the case of *C. sumatrensis* was special because although it had an RF greater than 4, the plants did not survived the field dose (Table 3.2).

Table 3.2 Parameters of the log-logistic used to estimate the dose-response regression curves (% dry weight) in dicotyledonous weeds susceptible (S) and R to 2,4-D with (+malathion) or without (-malathion) previous application of malathion at 2000 g ai ha^{-1}.

Species	Population/ ±malathion	d	b	GR_{50} (g ai ha^{-1}) ± SE	RF	*P*-value
A. hybridus	R-malathion	99.09	2.72	572.62 ± 34.17	6.06	<0.001
	R+malathion	99.97	2.25	294.10 ± 8.77	3.26	<0.001
	S-malathion	100.25	2.35	94.41± 2.49	-	<0.001
	S+malathion	100.49	2.51	90.12± 6.11	0.95	<0.001
C. canadensis	R-malathion	100.61	3.12	797.25 ±31.21	16.36	0.018
	R+malathion	100.21	2.67	55.91 ± 3.52	1.14	<0.001
	S-malathion	100.02	3.49	48.71 ± 4.82	-	<0.001
	S+malathion	100.01	3.42	44.95 ± 4.85	0.92	<0.001
H. incana	R-malathion	101.11	3.56	563.06 ± 15.55	4.17	<0.001
	R+malathion	100.94	3.43	553.46 ± 13.65	4.10	<0.001
	S-malathion	99.11	2.36	134.74 ± 4.66	-	<0.001
	S+malathion	98.69	2.84	140.91± 7.23	1.04	0.019
P. hysterophorus	R-malathion	100.53	3.10	847.65 ± 30.46	9.95	<0.001
	R+malathion	98.95	2.03	386.03 ± 12.97	4.53	<0.001
	S-malathion	100.39	2.47	85.15 ± 3.61	-	<0.001
	S+malathion	100.52	2.46	86.85 ± 2.65	1.01	<0.001
P. roheas	R-malathion	100.71	2.71	875.36 ± 22.06	11.03	<0.001
	R+malathion	100.50	2.08	399.14 ± 12.92	5.03	<0.001
	S-malathion	99.75	1.76	79.34 ± 2.33	-	<0.001
	S+malathion	99.88	2.73	83.26 ± 3.43	1.04	<0.001
C. sumatrensis	R-malathion	100.07	3.21	112.28 ± 4.15	4.37	0.017
	R+malathion	100.74	1.96	27.05 ± 2.16	1.05	<0.001
	S-malathion	99.99	1.93	25.64 ± 2.29	-	0.023
	S+malathion	100.01	2.26	22.43 ± 3.06	0.87	<0.001

d is the upper limit and *b* is the slope of the curve; GR_{50} is the herbicide rate inhibiting plant growth by 50% with respect to the untreated control. RF = Resistance factor = GR_{50} of the R population/GR_{50} of the S population. ± Standard error of the mean (*n* = 10).

To test the hypothesis that enhanced 2,4-D metabolism is conferred by P450, its known inhibitor malathion was tested. Pretreatment with malathion followed by 2,4-D resulted in partial reduction of RF (around 50%) in *A. hybridus*, *P. hysterophorus* and *P. rhoeas* compared to plants only treated with the herbicide. In the cases of *C. canadensis* and *C. sumatrensis*, malathion fully synergized with 2,4-D, and all R plants became S. Finally, this pretreatment had no effect on *H. incana*.

2.3. ^{14}C-2,4-D Absorption and Translocation in Dicotyledonous Weeds

The absorption remained similar between R and S populations of all species at 96 HAT, suggesting no differences could be attributed to this factor. However, there were several

differences in translocation between R and S plants of most species except for *C. canadiensis* and *C. sumatrensis*, which showed similar amounts of radiolabeled herbicide retained in treated leaves (Table 3.3). Comparison between R and S populations of the same species showed around 25% less herbicide moved from treated leaves to the rest of the plants in *P. rhoeas* and 40% for *P. hysterophorus*, while *A. hybridus* and *H. incana* showed intermediate values around 32%.

Table 3.3 ^{14}C-2,4-D absorption (% of from total applied) and translocation (% from uptake) in different dicotyledonous weeds R and S populations at 96 h after treatment (HAT).

Species	Populations	Absorption	Translocation		
			Treated leaf	Rest of Plant	Root
A. hybridus	R	59.1±2.48 A	97.6±2.54 A	1.5±1.54 B	0.9±0.25 A
	S	60.1±2.02 A	65.3±2.18 B	24.1± 3.04 A	10.6±0.31A
C. canadensis	R	69.4 ± 5.7 A	97.2±2.1 A	1.8± 1.1 A	1.0± 0.3 A
	S	73.8±6.3 A	98.1±4.3 A	1.2± 0.9 A	0.7± 0.4 A
H. incana	R	64.7± 6.2 A	96.6± 2.6 A	2.3±0.8 B	1.1± 0.5 B
	S	70.5± 6.9A	64.9± 5.6 B	25.7± 2.1 A	9.4± 0.7 A
P. hysterophorus	R	62.4± 3.2 A	98.2± 2.3 B	1.2± 0.2 B	0.6±0.2 B
	S	61.9± 5.6 A	58.3± 5.4 A	25.6 ± 2.6 A	16.1± 3.4 A
P. roheas	R	66.73± 4.2 A	95.6± 4.4 A	2.6± 0.9 B	1.8± 0.7 B
	S	65.8± 3.2 A	70.3±3.2 B	23.2± 1.2 A	6.5± 0.4 A
C. sumatrensis	R	78.9±3.2 A	96.8±3.6 A	3.1± 1.5 A	0.1± 0.1 A
	S	80.1±2.4 A	97.6± 1.8 A	1.8± 0.9 A	0.6± 0.5 A

Same letter within a column is not different by the Tukey test at 95%. ± standard error of the mean (n = 5).

2.4. 2,4-D Plant Metabolism

After 96 HAT of application, 2,4-D remained unmetabolized in all S populations. Conversely, R plants of *A. hybridus*, *C. canadiensis*, *P. hysterophorus*, *P. rhoeas* and *C. sumatrensis* had transformed between 40 and 62% of 2,4-D applied to nontoxic metabolites in foliar tissues (Table 3.4). In those species, greater proportions of metabolites were found in foliar tissues; however, between 0.8 and 1.9 percent of nontoxic metabolites were also detected in roots. An exception was *C. sumatrensis* where no metabolites were detected in roots. No metabolites were detected in the foliar tissues or roots of *H. incana*.

Table 3.4 2,4-D metabolism (expressed as %) in different dicotyledonous weeds R and S populations at 96 h after treatment (HAT).

Populations	Populations	Foliar		Root	
		2,4-D	Metabolites non-toxic	2,4-D	Metabolites non-toxic
A. hybridus	R	46.5± 4.5 B	51.8 ± 3.3	ND[a]	1,7 ± 0.4
	S	87.4 ± 3.6 A	ND	12,58 ± 1.8	ND
	P-value	0.0001	-	-	-
C. canadensis	R	36.2 ± 5.1 B	62.2 ± 2.9	ND	1.6 ± 0.3
	S	97.6 ± 1.8 A	ND	2.4 ± 0.9	ND
	P-value	0.0001	-	-	-
H. incana	R	98.4 ± 1.2 A	ND	1.6 ± 0.5 B	ND
	S	89.9 ± 2.1 B	ND	10.1 ± 1.2 A	ND
	P-value	0.0037	-	0.0143	-
P. hysterophorus	R	38.8 ± 6.1 B	59.3 ± 3.5	ND	0.8 ± 0.2
	S	86.2 ± 5.6 A	ND	13.8 ± 2.9	ND
	P-value	0.0010	-	-	-
P. roheas	R	38.2 ± 2.7 B	58.6 ± 2.1	1.3± 0.6 B	1.9 ± 0.7
	S	91.2 ± 1.4 A	ND	8.8 ± 0.8 A	ND
	P-value	0.0001	-	0.0004	-
C. sumatrensis	R	59.4 ± 4.3 B	39.7 ± 2.7	0.9 ± 0.2 B	ND
	S	96.9 ± 2.4 A	ND	3.1 ± 1.0 A	ND
	P-value	0.0005	-	0.0109	-

[a]ND = not detected. Same letter within a column is not different by the Tukey test at 95%. ± standard error of the mean (*n* = 5).

3. Discussion

Resistance to 2,4-D was confirmed in all the R populations studied for the six dicotyledonous weed species, both thanks to ethylene production and dose-response experiments (Figure 3.1 and Table 3.2). The RF (always ≥4) were in the range reported in previous research for *A. hybridus* (Dellaferrera et al., 2018), *P. rhoeas* (Rey-Caballero et al., 2016, 2017; Torra et al., 2017), *P. hysterophorus* (Mora et al., 2019) or close relative species to *H. incana* such as *R. raphanistrum* (Goggin et al., 2018). Also, in this work, ethylene biosynthesis supported susceptibility and resistance of all populations; all R plants tested accumulated less ethylene than S plants for each species, as in previous studies (Rey-Caballero et al., 2016; Tahmasebi et al., 2018). In this respect, S plants of *H. incana* accumulated less ethylene than the other species, which was correlated with the highest GR_{50} (135 g ai ha^{-1}), suggesting that this species would be

less S and more 2,4-D tolerant, than the rest. Overall, these results suggested a clear 2,4-D resistance in the studied species, except in the case of *C. sumatrensis* in which a slow evolution of the resistance to 2,4-D was observed, because R plants did not survive the field dose and showed the lowest reduction in ethylene production (3-folds) compared to the rest of the species.

Absorption was not related to 2,4-D resistance in the six weed species of this work, and globally, the results were comparable with other studies on these and other plant species where this NTSR mechanism played a negligible role in explaining 2,4-D resistance (Busi et al., 2018). In very few cases, differential ^{14}C-2,4-D absorption between S and R plants contributed to resistance, like *Glechoma hederacea* (Kohler et al., 2004) and *Lactuca serriola* (Riar et al., 2011).

Altered 2,4-D translocation and increased metabolism were clearly related to resistance in this work, although, the weight of each mechanism was different among the species studied. Three species showed both mechanisms: *A. hybridus* (54% of 2,4-D was degraded), *P. hysterophorus* (61% degraded) and *P. rhoeas* (62% degraded). In all these three species, non-toxic metabolites were also found in the roots at 96 HAT. In the two *Conyza* sps., only enhanced metabolism was found: while *C. canadensis* degraded 64% of the herbicide and metabolites were detected in the roots, *C. sumatrensis* degraded only 41% of 2,4-D and metabolites were not found in the roots, again suggesting a lower degree of 2,4-D resistance evolution in this species. In all of the above-mentioned five species, malathion synergized with 2,4-D, indicating that P450 is involved in the herbicide degradation. Interestingly, in *Conyza* sps., without reduced translocation, the sensitization of R plants was full. Accordingly, 2,4-D enhanced metabolism was reported in an *A. tuberculatus* population with similar behavior, with a full phenotype reversion of the R plants with malathion, and with no differences in translocation between biotypes (Figueiredo et al., 2018). In *A. hybridus*, *P. hysterophorus* and *P. rhoeas*, malathion partially reversed the phenotype from R to S. Similarly, in a multiple 2,4-D and glyphosate R *P. hysterophorus* population, the same reduced translocation and partial reversion of the phenotype with malathion occurred (Mora et al., 2019). No metabolism was detected in *H. incana*.

Two scenarios are plausible when impaired translocation and enhanced metabolism both occur, particularly when full synergism is not accomplished with a P450 inhibitor. If reduced transport was a secondary effect of metabolism and only P450 was involved in the degradation, it would be expected that malathion should fully reverse the phenotype. If not, it could be hypothesized that other degrading enzymes could be involved, as different metabolism routes for 2,4-D can be found in plants (Peterson et al., 2016). On the other hand, a second mechanism responsible of the reduced transport could be present, such as SAH influx or efflux transporters (Busi et al., 2018). The necessity of unraveling the relationship between these two physiological processes has already been stated for *P. rhoeas* (Torra et al., 2017). In this sense, the presence of two 2,4-D resistance mechanisms, though not metabolism, has already been appointed for other species (Goggin et al., 2020). Also, inheritance studies of SAH resistance have shown that two or several minor genes can be involved in different weeds species (Ashworth et al., 2016; Busi & Powles, 2017; Weinberg et al., 2006). Applying the parsimony principle, reduced translocation might be a secondary effect of enhanced metabolism, but it should be further investigated together with the possible role of other enzyme families.

Metabolic resistance places a serious challenge for potential cross-resistance to other herbicides, especially to other SAH families. For example, among the species studied, cross-resistance to dicamba (benzoate) in 2,4-D R populations has already been cited for *P. rhoeas* (Rey-Caballero et al., 2016), or for *A. hybridus* and *P. hysterophorus* by means of enhanced metabolism (Dellaferrera et al., 2018; Mora et al., 2019). For other species not studied here, like *A. tuberculatus* (Bernards et al., 2012) or *R. raphanistrum* (Goggin et al., 2018), cross-resistance to dicamba has also been demonstrated.

In this research, *H. incana* was the only species in which the only contributing mechanism to 2,4-D resistance was the reduced translocation. The absence of metabolism detected in R plants was supported by lack of effect in the inhibition of P450 by malathion. Similarly, in R biotypes of *L. serriola* and *R. raphanistrum* 2,4-D metabolism was not different compared with S populations, and showed reduced uptake (Goggin et al., 2016; Riar et al., 2011). Also, lack of 2,4-D translocation was the likely mechanism of resistance in *Sisymbrium orientale* from Australia, though metabolism was not

investigated (Dang et al., 2018). Some studies point out that influx or efflux carriers could be responsible or contribute to this impaired transport (Goggin et al., 2016, 2018, 2020; LeClere et al., 2018). It is known that these transporters can be SAH targets, and thus, can confer both TSR and NTSR in weeds (Busi et al., 2018), which will depend on the species and SAH. If these carriers might be involved, it should unraveled if they are the target of the SAH, in order to understand if the mechanism is TSR or NTSR based, as might be the case for the *H. incana* population in this study.

This research represents the first report worldwide of 2,4-D resistance in the important weed species *C. canadensis*. Also, resistance mechanisms were investigated for the first time in *H. incana* and *A. hybridus*. Moreover, for *C. sumatrensis*, this is first case reporting 2,4-D resistance in Europe, while there is a single case in the rest of the world citing a multiple R biotype to fives SoAs, including 2,4-D, from Brazil in 2017 (Heap, 2019); however, this research represents the first one to unravel the resistance mechanisms in the species. In addition, this is the second study worldwide reporting resistance to 2,4-D in *P. hysterophorus* (Mora et al., 2019), and the first case for Dominican Republic. For *P. rhoeas*, results confirmed previous research with other populations from the same country (Rey-Caballero et al., 2016; Torra et al., 2017).

4. Conclusions

Overall, across the six dicotyledonous weed species investigated, there was a differential contribution to 2,4-D resistance of the two resistance mechanisms analyzed, enhanced metabolism involving P450 and reduced translocation, while in some species both contributed, and in others, only one of them seemed to be responsible. This is in agreement with previous research in which a variety of resistance mechanisms have been described depending on the species, maybe as a result of the multi target nature of SAH (Busi et al., 2018). The latest studies have stated the likelihood of different resistance-conferring mechanisms even within populations of the same species (Goggin et al., 2018, 2020). Therefore, this study also emphasizes the dangers of extrapolating 2,4-D resistance mechanisms from a few weed species to others, even if they are close relatives. Future research is required to identify the P450 genes and other enzymes potentially responsible to understand the routes of 2,4-D degradation on a species basis. Also, it is important to unravel the relationship between enhanced metabolism and

reduced transport when both occur. Could impaired translocation be a secondary effect of metabolism? Or, is there another resistance mechanism also contributing to resistance? If so, or if reduced transport is the only mechanism present, like in *H. incana* in the current study, the precise mechanism should be found and its TSR or NTSR nature deciphered.

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CAPITULO IV

Non-Target-Site Resistance Mechanisms Endow Multiple Herbicide Resistance to Five Mechanisms of Action in *Conyza bonariensis*

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Non-Target-Site Resistance Mechanisms Endow Multiple Herbicide Resistance to Five Mechanisms of Action in *Conyza bonariensis*

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ABSTRACT: The repeated use of herbicides can lead to the selection of multiple resistance weeds. Some populations of *Conyza bonariensis* occurring in olive groves from southern Spain have developed resistance to various herbicides. This study determined the resistance levels to 2,4-D, glyphosate, diflufenican, paraquat, and tribenuron-methyl in a putative resistant (R) *C. bonariensis* population, and the possible non-target-site resistance (NTSR) mechanisms involved were characterized. Resistance factors varied as follows: glyphosate (8.9), 2,4-D (4.8), diflufenican (5.0), tribenuron-methyl (19.6), and paraquat (85.5). Absorption of ^{14}C-glyphosate was up to 25% higher in the susceptible (S) population compared to the R one, but ^{14}C-paraquat absorption was similar (up to 70%) in both populations. S plants translocated more than 60% of both ^{14}C-glyphosate and ^{14}C-paraquat toward shoots and roots, while R plants translocated less than 10%. The R population was able to metabolize 57% of the 2,4-D into nontoxic metabolites and 68% of the tribenuron-methyl into metsulfuron-methyl (10%), metsulfuron-methyl-hydroxylate (18%), and conjugate-metsulfuron-methyl (40%). Among the NTSR mechanisms investigated, absorption and translocation could be involved in glyphosate resistance, but only translocation for paraquat. Proofs of the presence of enhanced metabolism as a resistance mechanism were found for tribenuron-methyl and 2,4-D, but not for diflufenican. This research informs the first occurrence of multiple resistance to five herbicide classes (acetolactate synthase inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase inhibitors, photosystem I electron diverters, photosystem II inhibitors, and synthetic auxin herbicides) in *C. bonariensis*.

KEYWORDS: *^{14}C-herbicides, cytochrome P450, hairy fleabane, herbicide metabolism, malathion*

1. INTRODUCTION

Weed management is one of the prime concerns for sustainable crop production. *Conyza bonariensis* (L.) Cronq., commonly called hairy fleabane, is one of the most problematic, invasive, and widespread weeds in modern-day agriculture, being one of the most troublesome weed species across the world.[1,2] This weed may cause 28−68% yield loss in many annual and perennial crops every year, and it is more prevalent in nontill systems, becoming a major issue in conservation agriculture.[1,3] Its stem is erect and originates as sole or in clusters from the basal rosette. This Asteraceae weed species is native to South America and has invaded a large number of countries in Africa, Asia-Pacific, and Europe.[3,4] *C. bonariensis* is widely distributed in some European countries, including the Czech Republic, France, Greece, Hungary, Italy, Portugal, and Spain.[5,6]

Perennial crops occupy about 4 830 000 ha in Spain; among them olive, vineyard, citrus, and stone and pip fruit trees are the most important.[7] Farmers invest to keep crops free of pests, diseases, and weeds to obtain high yields and high-quality products.[8] Herbicides are the most widely used weed control method, with glyphosate being the main herbicide used in perennial crops for this purpose since its introduction in 1974.[9,10] For more than four decades (1980−2020), herbicides have been repeatedly applied year after year, alone or as a tank mix, to control *Conyza* spp.: among them simazine (banned in 2000); terbutylazine (restricted use since 2021), glyphosate, MCPA, 2,4-D, fluroxypyr, and glufosinate-ammonium (banned in 2018); paraquat (banned in 2003); diquat, diflufenican, oxyfluorfen, pyraflufen-ethyl, flazasulfuron, and tribenuron-methyl. The evolutionary response of weeds to selection pressure imposed by the herbicide applications is a major concern in agriculture, as it may trigger resistance, in some cases to herbicides with different mechanisms of action.[11,12]

Herbicide resistance, in addition to being a great phytosanitary control challenge, is a concern since the management of herbicide resistant weeds often increases control costs, because it involves one or more herbicides with a mode of action (MoA) different from the one that selected the resistance.[13]

Since the first detected cases to 2,4-D back in 1957, the characterization of herbicide resistance mechanisms in weeds has been a great scientific challenge.[14] It was not until the end of the 1980s with the help of molecular biology that resistance mechanisms began to be characterized in both monocots and

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Abstract

The repeated use of herbicides can lead to the selection of multiple resistance weeds. Some populations of *Conyza bonariensis* occurring in olive groves from southern Spain have developed resistance to various herbicides. This study determined the resistance levels to 2,4-D, glyphosate, diflufenican, paraquat, and tribenuron-methyl in a putative resistant (R) *C. bonariensis* population, and the possible non-target-site resistance (NTSR) mechanisms involved were characterized. Resistance factors varied as follows: glyphosate (8.9), 2,4-D (4.8), diflufenican (5.0), tribenuron-methyl (19.6), and paraquat (85.5). Absorption of ^{14}C-glyphosate was up to 25% higher in the susceptible (S) population compared to the R one, but ^{14}C-paraquat absorption was similar (up to 70%) in both populations. S plants translocated more than 60% of both ^{14}C-glyphosate and ^{14}C-paraquat toward shoots and roots, while R plants translocated less than 10%. The R population was able to metabolize 57% of the 2,4-D into nontoxic metabolites and 68% of the tribenuron-methyl into metsulfuron-methyl (10%), metsulfuron-methyl-hydroxylate (18%), and conjugate-metsulfuron-methyl (40%). Among the NTSR mechanisms investigated, absorption and translocation could be involved in glyphosate resistance, but only translocation for paraquat. Proofs of the presence of enhanced metabolism as a resistance mechanism were found for tribenuron-methyl and 2,4-D, but not for diflufenican. This research informs the first occurrence of multiple resistance to five herbicide classes (acetolactate synthase inhibitors, 5-enolpyruvylshikimate-3-phosphate synthase inhibitors, photosystem I electron diverters, photosystem II inhibitors, and synthetic auxin herbicides) in *C. bonariensis*.

Keywords: ^{14}C-herbicides, cytochrome P450, hairy fleabane, herbicide metabolism, malathion

Resumen

El uso repetido de herbicidas puede conducir a la selección de malas hierbas con resistencia múltiple. Algunas poblaciones de *Conyza bonariensis* presentes en olivares del sur de España han desarrollado resistencia a varios herbicidas. En este estudio se determinaron los niveles de resistencia a 2,4-D, glifosato, diflufenican, paraquat y tribenuron-metil en una población resistente (R) de *C. bonariensis* y se caracterizaron los posibles mecanismos de resistencia fuera del sitio de acción (NTSR). Los factores de resistencia variaron de la siguiente manera: para glifosato (8,9), 2,4-D (4,8), diflufenican (5,0), tribenuron-metilo (19,6) y paraquat (85,5). La absorción de ^{14}C-glifosato fue hasta un 25% mayor en la población susceptible (S) en comparación con la R, pero la absorción de ^{14}C-paraquat fue similar (hasta un 70%) en ambas poblaciones. Las plantas S translocaron más del 60% tanto del ^{14}C-gifosato como del ^{14}C-paraquat por la parte aérea y las raíces, mientras que las plantas R translocaron menos del 10%. La población R fue capaz de metabolizar el 57% del 2,4-D en metabolitos no tóxicos y el 68% del tribenuron-metil en metsulfuron-metil (10%), metsulfuron-metil-hidroxilato (18%) y conjugado-metsulfuron-metil (40%). Entre los mecanismos NTSR investigados, la absorción y la translocación podrían ser las responsables de la resistencia al glifosato, pero sólo la translocación estaría involucrada para el paraquat. Se encontraron pruebas de la presencia de un metabolismo potenciado como mecanismo de resistencia para el tribenuron-metil y el 2,4-D, pero no para el diflufenican. Esta investigación informa la primera aparición de resistencia múltiple a cinco diferentes herbicidas (inhibidores de la acetolactato sintasa, inhibidores de la 5-enolpiruvilsiquimato-3-fosfato sintasa, desviadores de electrones del fotosistema I, inhibidores del fotosistema II y herbicidas de auxinas sintéticas) en *C. bonariensis*.

Palabras clave: ^{14}C-herbicidas, citocromo P450, cola de caballo, metabolismo de herbicidas, malatión.

Introduction

Weed management is one of the prime concerns for sustainable crop production. *Conyza bonariensis* (L.) Cronq., commonly called hairy fleabane, is one of the most problematic, invasive, and widespread weeds in modern-day agriculture, being one of the most troublesome weed species across the world (Matzrafi et al., 2015; Trezzi et al., 2015). This weed may cause 28–68% yield loss in many annual and perennial crops every year, and it is more prevalent in nontill systems, becoming a major issue in conservation agriculture (Bajwa et al., 2016; Trezzi et al., 2015). Its stem is erect and originates as sole or in clusters from the basal rosette. This Asteraceae weed species is native to South America and has invaded a large number of countries in Africa, Asia-Pacific, and Europe (Bajwa et al., 2016; Heap, 2019). *C. bonariensis* is widely distributed in some European countries, including the Czech Republic, France, Greece, Hungary, Italy, Portugal, and Spain (Bastida et al., 2021; Travlos & Chachalis, 2010).

Perennial crops occupy about 4 830 000 ha in Spain; among them olive, vineyard, citrus, and stone and pip fruit trees are the most important (Eurostat, 2020). Farmers invest to keep crops free of pests, diseases, and weeds to obtain high yields and high-quality products (Möhring et al., 2020). Herbicides are the most widely used weed control method, with glyphosate being the main herbicide used in perennial crops for this purpose since its introduction in 1974 (Duke, 2018; Kudsk & Mathiassen, 2020). For more than four decades (1980–2020), herbicides have been repeatedly applied year after year, alone or as a tank mix, to control *Conyza* spp.: among them simazine (banned in 2000); terbutylazine (restricted use since 2021), glyphosate, MCPA, 2,4-D, fluroxypyr, and glufosinate-ammonium (banned in 2018); paraquat (banned in 2003); diquat, diflufenican, oxyfluorfen, pyraflufen-ethyl, flazasulfuron, and tribenuron-methyl. The evolutionary response of weeds to selection pressure imposed by the herbicide applications is a major concern in agriculture, as it may trigger resistance, in some cases to herbicides with different mechanisms of action (Gaines et al., 2020; Neve et al., 2009).

Herbicide resistance, in addition to being a great phytosanitary control challenge, is a concern since the management of herbicide resistant weeds often increases control costs, because it involves one or more herbicides with a mode of action (MoA) different from the one that selected the resistance (Hicks et al., 2018).

Since the first detected cases to 2,4-D back in 1957, the characterization of herbicide resistance mechanisms in weeds has been a great scientific challenge (Busi et al., 2018). It was not until the end of the 1980s with the help of molecular biology that resistance mechanisms began to be characterized in both monocots and dicots (Gaines et al., 2019, 2020). These studies are essential to design and establish successful integrated weed management (IWM) strategies for herbicide resistant weeds, in both the short term and long term (Gage et al., 2019). These IWM strategies are an explicit measure to not only control and minimize herbicide resistance, but also to reduce the environmental impact of herbicides by using only those that will be effective in the field (Menalled et al., 2016), (17) or even produce herbicide-free crops (Dominschek et al., 2021; Palma-Bautista et al., 2021).

Mechanisms that confer resistance to herbicides in plants have been broadly grouped into target-site resistance (TSR) and non-target-site resistance (NTSR) (Dominschek et al., 2021; Palma-Bautista et al., 2021). The TSR mechanisms involve alterations in the target site, such as amino acid substitutions that modify the structural makeup of the target enzyme and/or its overproduction by gene amplification or overexpression. NTSR is a collection of mechanisms that result from reduced uptake and/or translocation, increased vacuolar sequestration, or metabolism to nontoxic compounds, which decrease the amount of herbicide interacting with its target site (Amaro-Blanco et al., 2018; Rigon et al., 2020). In general, spraying herbicides at higher rates favors the evolution of TSR mechanisms, whereas lower than recommended rates favor NTSR metabolic mechanisms (Busi et al., 2012). (23) Multiple herbicide resistance and NTSR can be polygenetic, while a single gene is involved in TSR (Heap, 2014). Many plants, particularly weeds, can reach this condition, because they contain a great amount of genetic variation that allows them to survive under different biotic and abiotic conditions (Kreiner et al., 2018; Neve et al., 2009).

Altered translocation patterns and enhanced herbicide metabolism are the NTSR mechanism more often described in herbicide resistant weeds (Gaines et al., 2020). Differential movement has been described mainly for paraquat and glyphosate, and does not confer cross-resistance to other MoAs (Gaines et al., 2019, 2020). On the other hand, enhanced metabolism can affect almost all herbicide chemistries, often providing,

sometimes unpredicted, cross-resistance between different MoAs. That is why it is acknowledged as the most threatening herbicide resistance mechanism (Rigon et al., 2020). The enzyme family most frequently involved in enhanced herbicide metabolism in resistant plants is the cytochrome P450 (Cyt-P450). The role of Cyt-P450 in resistant (R) weeds can be indirectly assessed in in vivo plant experiments using known inhibitors of this enzyme family in plants, such as the organophosphate insecticide malathion. When there is a synergistic effect between the herbicide and the Cyt-P450 inhibitor, the phenotype is reversed from resistant to (more) susceptible (Gaines et al., 2020; Rigon et al., 2020).

In view of the above-stated facts, this study had the following aims: (a) to confirm resistance to glyphosate, 2,4-D, diflufenican, tribenuron-methyl, and paraquat in a *C. bonariensis* population sampled in southern Spain and (b) to investigate the NTSR mechanisms involved in the multiple resistance. The results will support the development of IWM strategies to cope with herbicide resistant *C. bonariensis* in perennial crops.

2. Materials and Methods

2.1 Chemicals

Table 4.1 lists the trade herbicides used in this study to determine the physiological effects on the populations resistant (R) and susceptible (S) to *C. bonariensis*. The following radiolabeled herbicides were used to perform the absorption, translocation (glyphosate and paraquat), and metabolism (tribenuron-methyl) assays: ^{14}C-glyphosate (glycine-2-^{14}C, specific activity 273.8 MBq mmol^{-1}, Institute of Isotopes Co., Ltd., Budapest, Hungary); ^{14}C-paraquat (specific activity 6.2789 MBq/mg, American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA); and ^{14}C-tribenuron-methyl, which together with its metabolites (MM, metsulfuron-methyl; OH-MM, hydroxylated metsulfuron-methyl) was supplied by Dupont de Nemours & Co. (Nambsheim, France).

Table 4.1 Active Ingredients, Trade Products, Mode of Action (MoA), Field Dose (g ai ha^{-1}) and Doses of the Herbicides Used in Dose–Response Assays on *C. bonariensis* Populations Collected in an Olive Grove from Southern Spain

Herbicide	Trade product	MoA[a]	Field dose[b]	Dose-response (g ai ha^{-1})
2,4-D	U26 D Complet®, 60% w/v	Auxin mimic	400	0, 50, 100, 200, 400, 800, 1600
Diflufenican	Urbole®, 50% w/v	PDS inhibitor	100	0, 20, 40, 60, 120, 240, 480, 960
Glyphosate	36% w/v SL, Roundup®	EPSPS inhibitor	1080	0, 62.5, 125, 250, 500, 1000, 2000, 3000
Paraquat	Gramoxone® SL, 25% w/v	PS I inhibitor	500	0, 5, 25, 50, 200, 500, 1000, 2000, 3000
Tribenuron-methyl	Granstar®, 50% w/v	ALS inhibitor	20	0, 1.25, 2.5, 5, 10, 20, 40, 80, 160

[a]MoA: auxin mimic, inhibitor of phytoene desaturase (PDS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), photosystem I (PSI), and acetolactate synthase (ALS).
[b]Glyphosate in grams of acid equivalent per hectare (g ae ha^{-1}).

2.2 Plant Material

Seeds of a *C. bonariensis* population with suspected multiple resistance (R) to herbicides were collected from olive groves located in La Carlota, southern Spain, which have a history of herbicide use several times a year since 1980. Mature seeds of one herbicide S population from a location close to the R population were collected as well.

All mature seeds harvested from the field were germinated in Petri dishes with filter paper moistened with distilled water and placed in a growth chamber at 28/18 °C (day/night) with a photoperiod of 16 h, 850 µmol m^{-2} s^{-1} photosynthetic photon flux, and 80% relative humidity. Seedlings were transplanted into pots containing sand/peat in a 1:2 (v/v) ratio and placed in a greenhouse at 28/18 °C (day/night) with a 16 h photoperiod.

2.3 Rapid Resistance Test

A greenhouse rapid resistance screening was carried out to ascertain whether these R and S *C. bonariensis* populations survive the application of herbicides. Herbicides were applied postemergence on plants with four to six true leaves, except diflufenican, which was applied at two true leaves. The experiments were arranged in a completely randomized design using 50 plants (one plant per pot) per treatment. Herbicides were applied at field doses (Table 4.1) in a treatment chamber (De Vries Manufacturing,

Hollandale, MN, USA) equipped with a Tee Jet 8002 EVS flat fan nozzle calibrated to deliver 200 L ha^{-1} at 200 kPa at a height of 50 cm. At 4 weeks after application (WAA), the number of surviving plants was counted. The experiments were conducted twice at different times (spring and fall), and the average of the two was obtained. Surviving R individuals (>80% survivors) were grown to maturity, bulked, and allowed to produce seeds.

2.4 Dose–Response Curve

R seeds obtained in screening tests were used to obtain plants for the dose–response assays. R and S plants were grown and treated with the different herbicides under the same conditions as in the screening test. The applied doses of each herbicide are detailed in Table 4.1. For each dose of herbicide, five plants of each population were treated, which were taken randomly. After treatments, plants were taken to a greenhouse, held under a temperature regime of 28/18 °C day/night, and watered as necessary. At 4 WAA, the number of dead plants was recorded and they were cut at ground level, stored individually in paper bags, dried at 60 °C for 4 days, and weighed. The data of dry weight and plant mortality were transformed into a percentage relative to the untreated control to estimate the LD_{50} (herbicide dose required to kill by 50% a weed population) and GR_{50} (dose required to reduce shoot weight by 50% relative to the control) values.

2.4.1 Malathion Effects on Resistance to 2,4-D, Diflufenican, and Tribenuron-methyl

R and S *C. bonariensis* plants at the four-leaf stage were treated with the doses (10 plants dose–1) of diflufenican, 2,4-D, and tribenuron-methyl used in the dose–response assays (Table 4.1). However, prior to the application of the herbicides, plants were treated with malathion (Malation 90% w/v, BASF, Spain) at 1000 g ha^{-1}. The plants were brought into the greenhouse and kept under the conditions described above (section 2.4) until evaluation. Preliminary tests showed that malathion applied alone did not have phytotoxic effects on the R and S *C. bonariensis* plants, showing similar growth in untreated control plants. At 4 WAA, the dry weight of the plants was determined to calculate the GR_{50} values. The dose–response curves of this experiment were compared with those of the dose–response assays of the respective herbicides without the application of malathion.

2.5 Absorption and Translocation of ^{14}C-Glyphosate and ^{14}C-Paraquat

R and S *C. bonariensis* plants were treated with glyphosate or paraquat solution prepared with its corresponding ^{14}C-herbicide plus trade formulation. The final herbicide application rate of the solutions was 450 g ae ha^{-1} in 200 L ha^{-1} for glyphosate and 100 g ai ha^{-1} in 300 L ha^{-1} for paraquat. In both cases, the specific activity of the ^{14}C-herbicide solution was 0.834 kBq µL^{-1}. Before the application of the ^{14}C-herbicides, the second youngest expanded leaf of a plant was covered with aluminum foil and the whole plants were sprayed with nonradiolabeled glyphosate or paraquat. After 30 min, the aluminum foil was removed and one drop (1 µL plant^{-1}) of the corresponding ^{14}C-herbicide solution was applied to the adaxial surface of the leaf by using a microapplicator PB-600 (Hamilton Co., Reno NV, USA). Plants were maintained in the growth chamber under the growing conditions described above for seed germination (section 2.2) until evaluation; however, those treated with ^{14}C-paraquat were maintained in the dark for 12 h before being exposed to light (Brunharo & Hanson, 2017).

Plants treated with ^{14}C-glyphosate were evaluated at 12, 24, 48, 72, or 96 h after treatment (HAT), while those treated with ^{14}C-paraquat were evaluated at 3, 6, 12, 24, and 48 HAT. For each evaluation time, five plants of each population were evaluated per herbicide (n = 5). The unabsorbed ^{14}C-herbicide was recovered by washing the treated leaf three times with 1 mL of distilled water/acetone (1:1, v/v) for ^{14}C-glyphosate and 1 mL of distilled water for ^{14}C-paraquat. Each ^{14}C-herbicide treated leaf was washed three times by dripping 1 mL of the corresponding washing solution each time. Rinsate solution of each wash was recovered in 5 mL vials of liquid scintillation, where 2 mL of liquid scintillation cocktail (Ultima Gold, PerkinElmer, Netherlands, MA) was subsequently added. The radioactivity of the nonabsorbed ^{14}C-herbicides was analyzed for 5 min by liquid scintillation spectrometry (LSS), and the measurements were repeated 24 h later.

After the treated leaf was washed, the plant was immediately and carefully removed from the pot, the roots were washed with distilled water, and the plant tissue was cut into the treated leaf, the remaining shoot tissue, and the roots. The plant tissue section was stocked in combustion cones, dried at 60 °C for 96 h, and combusted for 3 min in a Packard Tri Carb 307 biological sample oxidizer (Packard Instruments, Meriden, CT,

USA). Evolved $^{14}CO_2$ was trapped and counted by LSS in an 18 mL mixture of Carbo-Sorb E and Permafluor E+ (1:1 v/v) (PerkinElmer, Packard Bioscience BV). The radioactivity of the combustions was also analyzed by LSS for 5 min (reanalyzed at 24 HAT). Radioactive values were used to calculate the rates of recovery, absorption, and translocation of both ^{14}C-herbicides with eqs 1, 2, and 3, respectively (Alcántara-de la Cruz et al., 2021).

$$\%\ recovery = \left(\frac{Bq\ from\ washes + Bq\ in\ treated\ leaf + Bq\ in\ plant + Bq\ in\ roots}{Bq\ total\ applied}\right) \times 100 \quad (1)$$

$$\%\ absorption = \left(\frac{Bq\ in\ treated\ leaf + Bq\ in\ plant + Bq\ in\ roots}{Bq\ total\ applied}\right) \times 100 \quad (2)$$

$$\%\ translocation = \left(\frac{Bq\ in\ plant + Bq\ in\ roots}{Bq\ in\ treated\ leaf + Bq\ in\ plant + Bq\ in\ roots}\right) \times 100 \quad (3)$$

Absorption and translocation experiments were not performed with 2,4-D because previous studies indicated that these NTSR mechanisms were not present in Conyza species (Palma-Bautista et al., 2020). For diflufenican, they have never been described for any weed R biotype worldwide (Gaines et al., 2020).

2.6 Herbicide Metabolism

2.7 Metabolism of 14C-Tribenuron-methyl

Young plants of R and S *C. bonariensis* populations were treated with a radiolabeled ^{14}C-tribenuron-methyl solution (20 g ai ha^{-1} (grams of active ingredient per hectare) in 200 L with a specific activity of 0.834 kBq µL^{-1}). The application and recovery of the unabsorbed ^{14}C-tribenuron-methyl was made by using the same technique and washing solution as those used for glyphosate (section 2.5). The collection of samples to evaluate the metabolism of ^{14}C-tribenuron-methyl was carried out at 96 HAT. The metabolism of ^{14}C-tribenuron-methyl was determined following the methodology described by Cruz-Hipolito et al., 2013. After the treated leaf was washed, the aerial parts were sectioned and stored at −40 °C until analysis. For extraction, the samples were macerated in a porcelain mortar, recovering the ^{14}C with two washes of 3 and 4 mL, respectively, of methanol:water (4:1 v/v). Samples were transferred to 15 mL tubes to be centrifuged at 20000*g* (Avanti J-25, Beckman Coulter) for 20 min at 4 °C. Supernatants were decanted, and the pellets were washed with 2 mL of the above methanol solution, oven-dried, and combusted. To quantify the total radioactivity of the supernatants by LSS, aliquots of 100 µL (three per sample) were taken and mixed in 3 mL of liquid scintillation cocktail.

Moreover, to determine the metabolites of this herbicide, the supernatants were mixed and dried at room temperature under a flow of liquid nitrogen at 0.25 atm. The dried extract was suspended in 200 µL of isopropanol, and ^{14}C-tribenuron-methyl and its metabolites were separated by thin layer chromatography (TLC) on a 400 cm^2 silica gel plate (silica gel 60, Merck, Darmstadt, Germany) with a solution of isopropanol, ethyl acetate, ammonia, and distilled water (10:6:3:1 v:v:v:v). Chromatograms of the radioactive areas were obtained with a radioscanner. Each product was determined by comparison with known standards [tribenuron-methyl, metsulfuron-methyl (MM), and metsulfuron-methyl-hydroxylate (OH-MM)]. The radioactivity of an individual product was quantified with a linear plate analyzer (Berthold LB 2821, Wildbald, Germany). In this assay, three plants from each population (R and S) were used and the experiment was repeated twice.

2.6.2 Metabolism of 2,4-D

Three plants of each *C. bonariensis* population were treated with 2,4-D (400 g ai ha^{-1}). Another group of untreated plants (dose 0) was used as a control. At 48 HAT, each plant was cut and washed with distilled water to remove unabsorbed herbicide and divided into aerial and foliar parts. They were flash frozen in liquid nitrogen and stored at −80 °C until use. For these assays, the methodology described by Torra et al., 2017 was followed, in which three partitions were made to obtain the different metabolites. A first step of sample-metabolite cleanup, separation and preconcentration, was done using TLC, where 2,4-D and its metabolites were separate from the rest of the sample in butanol–ammonium hydroxide–water (5:1:4). The metabolites were scratched from the TLC plates and dissolved in 0.5 mL of acetone. The reconstituted sample was filtered through a nylon filter syringe before chromatographic analysis. A high performance liquid chromatograph (HPLC; Beckman Coulter Gold LC system, Fullerton, CA, USA) equipped with a diode array detector (wavelength range 190–600 nm) was used with a Kinetex EVO C18 column (150 mm, 4.6 mm i.d., 2.6 µm particle size) from Phenomenex Inc. (Torrance, CA, USA), fitted with 4.6 mm Security Guard TM ULTRA cartridges. Quantification of 2,4-D and its metabolites was based on the 2,4-D calibration curve according to retention times. The results were expressed as percentages of 2,4-D and its nontoxic metabolites (hydroxylated and conjugate compounds). The experiment was

repeated twice at different times in the laboratory with three replicates (dose and population) in each repetition.

Glyphosate, paraquat, and diflufenican metabolisms as resistance mechanisms were not investigated in this study, since there are very few or no reported cases worldwide (Gaines et al., 2020), and there were no preliminary proofs of their presence in the R population.

2.8 Statistical Analysis

Percentage data of the dose–response assays with or without malathion were used to determine the GR_{50} and LD_{50} values using nonlinear regression analysis with the three-parameter log-logistic equation:

$$y = d/[1+b(\log(x)-\log(e))],$$

where *d* is the upper asymptote, *b* is the slope at the inflection point of the curve, and *e* is the herbicide rate at the inflection point; *y* = 50% of plant response (GR_{50} or LD_{50}) and *x* is the herbicide dose. Regression analyses were conducted using the package *drc* for the statistical environment R. Resistance factors (RF = R/S) were computed as R-to-S GR_{50} and LD_{50} ratios.

Analysis of variance (ANOVA) was conducted to test for differences between the R and S populations in the herbicide absorption, translocation, and metabolism assays. For the cases of replicated metabolism assays, ANOVA was conducted according to a generalized randomized block design, with each repetition of the experiment representing a block. In neither case were interaction terms including blocks significant, and thus they were not included in the final ANOVA models. Percentage data were previously transformed (arcsine of the square root) to meet model assumptions of normality of error distribution and homogeneity of variance. The requirement of homogeneity of variance was verified with the Bartlett test, and the normality of the data was analyzed with the Shapiro–Wilk test. When needed, differences between means were separated by using the Tukey HSD test at $P < 0.05$. ANOVAs were conducted with Statistix (ver. 9.0) software (Analytical Software, USA).

3. Results

3.1 Screening Resistance Test

Most of the applied herbicides evaluated in the rapid resistance screening test using the field dose controlled 100% of the individuals in the S *C. bonariensis* population, except for paraquat since 15% of the S plants survived. By contrast, the plant mortality observed in the R population was poor. 2,4-D, diflufenican, and paraquat only killed 20, 15, and 10% of the plants, respectively, while all plants treated with glyphosate and tribenuron-methyl survived.

3.2 Dose–Response Curves

The herbicide doses required to kill the R C. bonariensis population by 50% (LD50) were higher than the field doses used in southern Spain for all applied herbicides, while for the S population these doses were below the field doses (Table 4.2, Figure 4.1), corroborating the results of the rapid resistance screening test. On the basis of the GR_{50} and LD_{50} values, the R population was between 4.8 and 85.5 times more R than the S population depending on the herbicide. The resistance order to the five tested herbicides was: paraquat > tribenuron > glyphosate > diflufenican ≥ 2,4-D (Table 4.2).

Table 4.2. Parameters of the Log-Logistic Model[a] Used to Estimate the Growth (GR_{50}) and Plant Survival (LD_{50}) Reductions by 50% in Multiple Herbicide Resistant (R) and Susceptible (S) Populations of *C. bonariensis* Collected in an Olive Grove from Southern Spain

Herbicide	Pop.	Growth reduction				Plant survival			
		d	b	GR_{50}	RF	d	b	LD_{50}	RF
Glyphosate[b]	R	99.2	1.7	869.0 ± 52.1	8.8	99.3	3.0	2455.6 ± 65.5	13.4
	S	100.5	1.3	98.7 ± 6.9		101.2	5.3	181.9 ± 9.3	
Paraquat	R	99.7	2.1	385.1 ± 24.8	85.5	99.9	3.4	2077.1 ± 58.1	84.4
	S	99.9	0.7	4.5 ± 0.5		100.3	3.4	24.6 ± 0.9	
2,4-D	R	98.9	2.9	529.1±28.8	4.8	100.4	3.7	1252.9 ± 50.1	8.6
	S	100.7	2.3	110.6±8.2		100.2	3.0	145.5 ± 6.3	
Diflufenican	R	99.5	2.0	201.6±12.6	5.0	100.0	3.6	398.3 ± 15.9	4.8
	S	100.4	2.3	40.3 ± 5.7		101.1	3.2	81.3 ± 4.1	
Tribenuron-methyl	R	101.2	1.7	29.5± 1.9	19.6	100.3	3.2	122.9 ± 4.7	21.1
	S	100.1	1.8	1.5±0.1		99.6	2.5	5.1± 0.2	

[a]Y = d/[1+*b*(log(*x*)-log(*e*))]; where *d* is the upper asymptote, *b* is the slope at the inflection point of the curve, *e* is the plant response at 50% (GR50 or LD50), and *x* the herbicide dose. Resistance factors (RF)= R/S.
[b]g ae ha^{-1}.

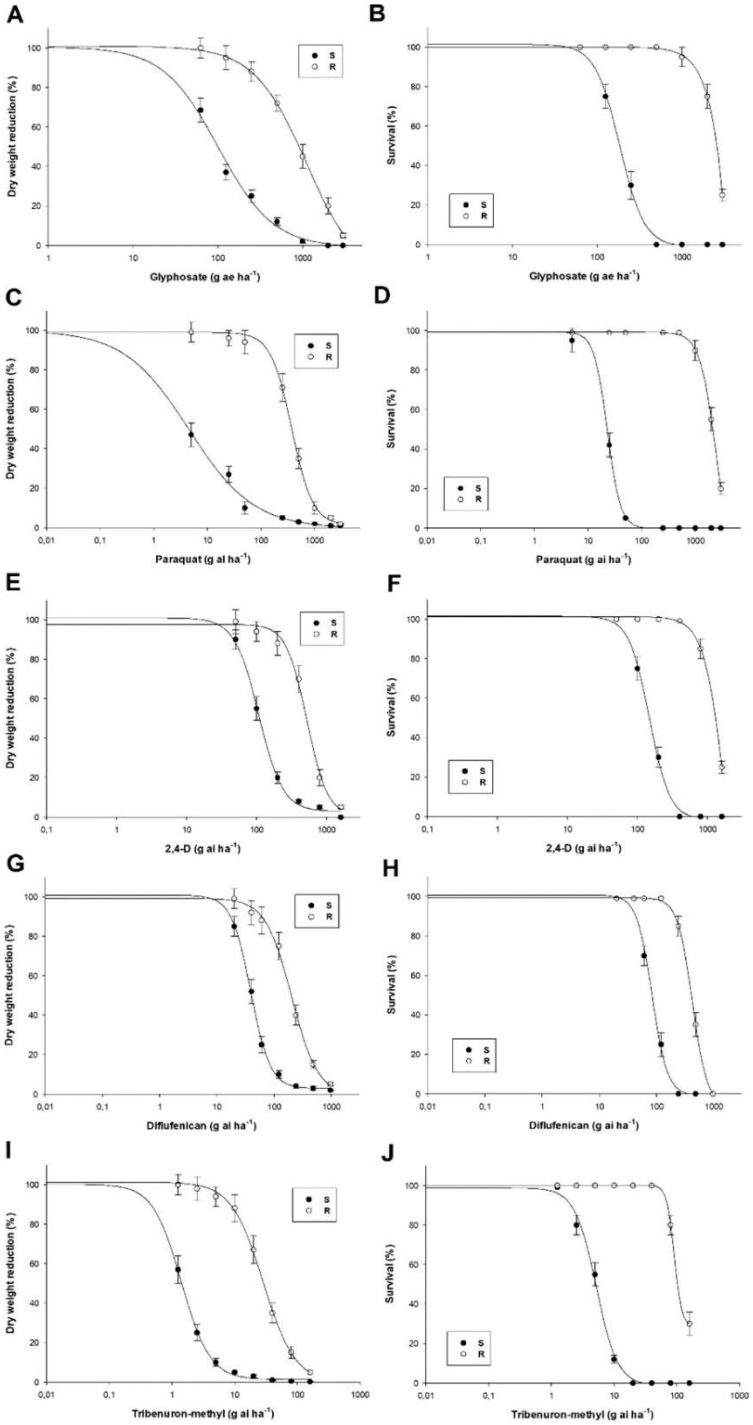

Figure 4.1 Dose–response curves of dry weight reduction (A, C, E, G, I) and plant survival (B, D, F, H, J) in susceptible (S) and resistant (R) *C. bonariensis* populations treated with different glyphosate, paraquat, 2,4-D, diflufenican, and tribenuron-methyl doses.

3.2.1 Malathion Effects on Resistance to 2,4-D, Diflufenican, and Tribenuron-methyl

The effect of malathion as an inhibitor of Cyt-P450 was null on S *C. bonariensis* plants treated with 2,4-D, diflufenican, and tribenuron-methyl, and the GR_{50} values (110.6, 40.3, and 1.5, respectively) were similar to those observed when malathion was not applied (101.3, 44.9, and 2.1) (Table 4.2). However, the observations for the R population suggest that Cyt-P450 participates in resistance depending on the herbicide applied. The application of malathion fully reversed the resistance to 2,4-D (RF down from 4.8 to 1.0) and partially reversed the resistance to tribenuron-methyl (RF from 19.6 to 8.5); however, the response to diflufenican was not affected by the Cyt-P450 inhibition in R plants (Table 4.3, Figure 4.2).

Table 4.3. Herbicide Dose (g ai ha^{-1}) Required to Reduce the Dry Weight to 50% (GR_{50}) in the Multiple Herbicide Resistant (R) and Susceptible (S) Populations of *Conyza bonariensis* Collected in an Olive Grove from Southern Spain, Pretreated with Malathion[a] and Resistance Factors (RFs)

Herbicide	Population	b	d	GR_{50}	RF[b]
2,4-D	R	2.8	102.8	100.2±5.1	1.01
	S	4.7	101.3	98.5±3.2	
Diflufenican	R	1.8	100.1	211.1±9.8	4.7
	S	2.4	103.1	44.9±1.6	
Tribenuron-methyl	R	1.6	101.6	17.9±0.9	8.5
	S	3.5	100.6	2.1±0.1	

[a]The R and S populations were treated with the cytochrome P450 inhibitor malathion.
[b]RF = GR_{50} R/GR_{50} S.

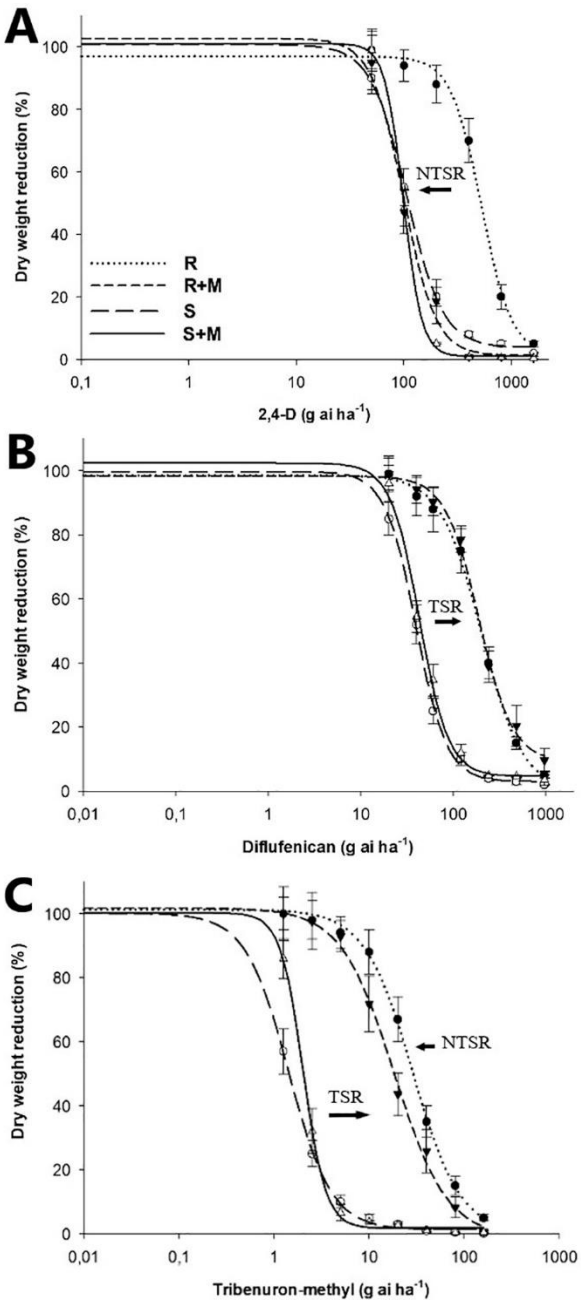

Figure 4.2. Effect of cytochrome P450 inhibitor malathion (+M) in multiple herbicide resistant (R) and susceptible (S) populations of *C. bonariensis*, collected in an olive grove from southern Spain, treated with 2,4-D (A), diflufenican (B), and tribenuron-methyl (C) compared to dose–response curves without malathion pretreatment.

3.3 Absorption and Translocation of 14C-Herbicides

The ^{14}C-glyphosate recovered in *C. bonariensis* ranged from 93.8 to 95.5%. Glyphosate absorption was rapid, starting from ~20% in both populations at 12 HAT, to 80% in the S population and 60% in the R population at 72 HAT. Between 72 and 96 HAT, 14C-glyphosate absorption did not increase significantly; however, the greatest difference was observed between populations (S population exhibited 25% more absorption than

the R population) (Figure 4.3A). ^{14}C-Glyphosate translocation differed in all evaluated times, being higher in the S population. At 96 HAT, S plants moved out of the treated leaf 53% more 14C-glyphosate than R plants (Figure 4.3B). The movement of glyphosate to shoots (34%) and roots (29%) was also greater in the S population than that which occurred in the R population at 96 HAT (Figure 4.3C,D).

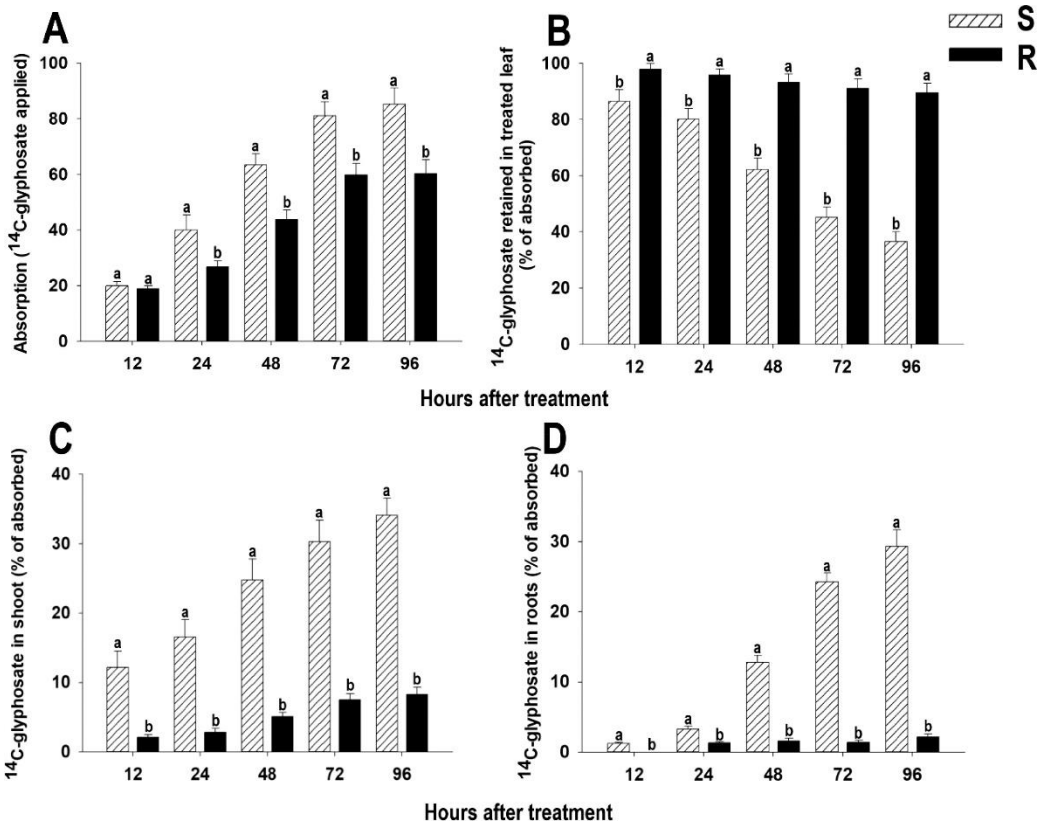

Figure 4.3. Percentage of ^{14}C-glyphosate absorption (A) and translocation (B–D) in multiple herbicide resistant (R) and susceptible (S) populations of *C. bonariensis*, collected in an olive grove from southern Spain. The vertical bars represent ± the standard errors of the mean (n = 5). Different letters denote significant differences between S and R populations at each sampling time.

The ^{14}C-paraquat recovered in the *C. bonariensis* populations was greater than 90%. The absorption of ^{14}C-paraquat was similar between populations throughout the evaluation period, increasing over time from ~10% at 3 HAT up to 70% at 48 HAT (Figure 4.4A). However, the ^{14}C-paraquat translocation from the treated leaf to shoots and roots was greater and faster in S plants than in R ones. At 48 HAT, the S plants translocated up to 30% of the ^{14}C-herbicide to shoots and 35% to roots, while the R plants did not transfer even 10% of the ^{14}C-paraquat out of the treated leaf (Figure 4.4B,C).

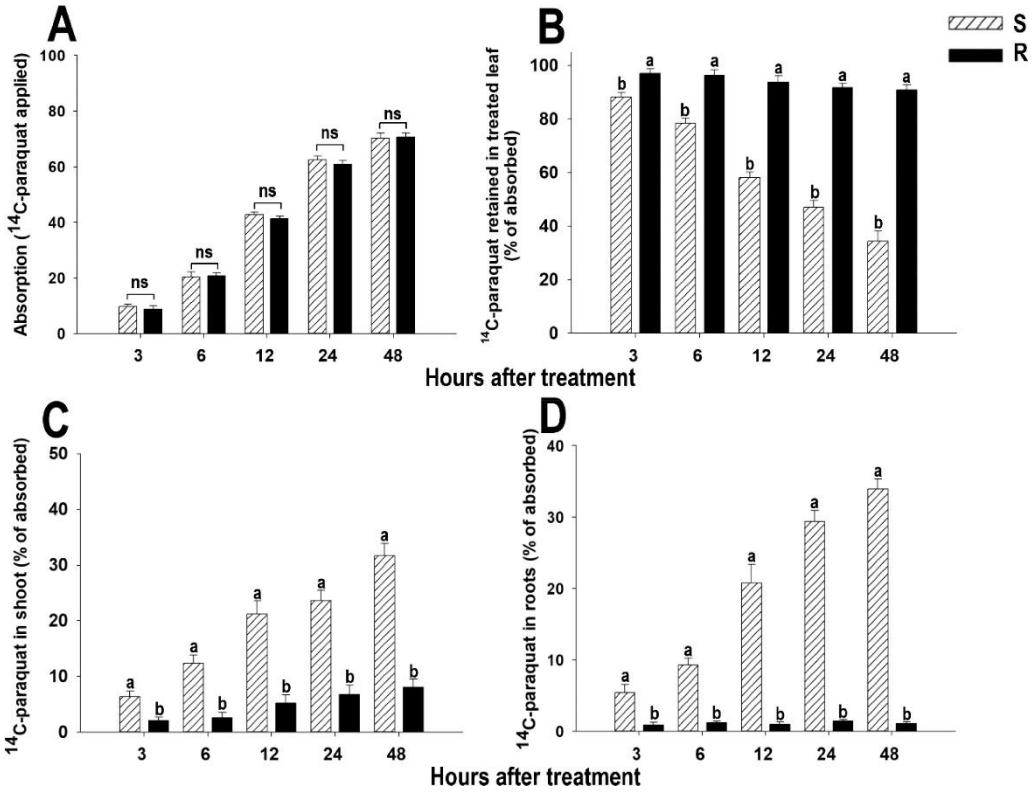

Figure 4.4. Percentage of ^{14}C-paraquat absorption (A) and translocation (B–D) in multiple herbicide resistant (R) and susceptible (S) populations of *C. bonariensis*, collected in an olive grove from southern Spain. The vertical bars represent ± the standard errors of the mean (n = 5). Different letters denote significant differences between S and R populations at each sampling time; ns, not significant.

3.4 Herbicide Metabolism

Most of the ^{14}C-tribenuron-methyl applied on S *C. bonariensis* plants remained as active herbicide (91%) at 96 HAT, while the other 9% corresponded to MM (another active sulfonylurea herbicide) obtained by N-demethylation. However, the metabolism patterns of tribenuron-methyl detected for the R population were different, and only 32% of tribenuron-methyl remained so. The toxic metabolite MM (10%) was further degraded to OH-MM (18%), mainly through hydroxylation of the phenyl ring. Conjugate-MM was the third and major metabolite (40%), which was formed by conjugation of OH-MM to carbohydrates. The sum of these two nontoxic metabolites, OH-MM and conjugate-MM, in plants of the R population corresponded to 58% of the total tribenuron-methyl applied (Figure 4.5).

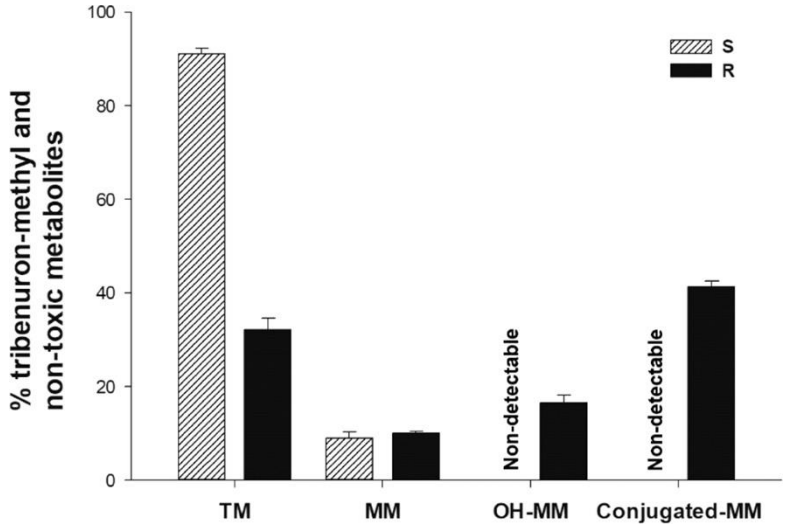

Figure 4.5. Percentage of tribenuron-methyl (TM) and metabolites (metsulfuron-methyl (MM), metsulfuron-methyl-hydroxylate (OH-MM), and conjugate-MM) in multiple herbicide resistant (R) and susceptible (S) populations of *C. bonariensis*, collected in an olive grove from southern Spain, at 96 HAT.

The metabolism of 2,4-D differed between S and R *C. bonariensis* populations. At 48 HAT, the S population metabolized into nontoxic metabolites only 2.5% of the absorbed herbicide. In contrast, the R population was able to transform 57% of 2,4-D into nontoxic metabolites. In both populations, no traces of the herbicide or metabolites were found in the roots of the plants (data not shown).

4. Discussion

C. bonariensis is considered one of the most problematic weeds in perennial crops in the Iberian Peninsula. This species has developed resistance to herbicides of four different MoAs globally, including 5-enolpyruvylshikimate-3-phosphate synthase inhibitors (EPSPS), photosystem I electron diverters (PSI), photosystem II inhibitors (PSII), and acetolactate synthase inhibitors (ALS) (Heap, 2019). In this study, multiple resistance to 2,4-D (auxin mimic), paraquat (PSI), glyphosate (EPSPS), tribenuron (ALS), and diflufenican (phytoene desaturase, PDS), to five MoAs, is recorded for the first time in *C. bonariensis* worldwide, characterizing resistance levels by in vivo and in vitro assays.

The high levels of control achieved for the S *C. bonariensis* population with the five herbicides evaluated showed that they continue to be useful weed control tools when used as part of an IWM scheme (Amaro-Blanco et al., 2018). Combining multiple tools and techniques in an IWM program is a step forward, but many management programs

are still integrated by chemical tools only (MacLaren et al., 2020). However, applications at advanced growing stages or ignoring environmental factors during herbicide use could lead to the evolution of resistance within a few years (Powles & Yu, 2010). The population characterized as R could be due to these events, numerous applications in successive years, increase in recommended field doses, and no alternative applications with other herbicides with different MoAs (Aves et al., 2020; Kleinman & Rubin, 2017; Moretti et al., 2013). The continuous and inadequate use of herbicides, in addition to contributing to the selection of multiple resistance, can lead to environmental pollution damage to nontarget organisms, change in soil biological biodiversity, and negative effects on human health (Mendes et al., 2020; Rose et al., 2016). Therefore, farmers should use weed management strategies that contribute to the sustainability of agroecosystems, such as reducing the intensity and frequency of herbicide use, reducing tillage to strips or the inter-row plow, and live or dead plant covers (MacLaren et al., 2020). Although the adoption of nonchemical weed management practices delays the evolution of herbicide resistance in the long term, in most cases, the costs of implementing these practices are not economically attractive for farmers in the short term (Norsworthy et al., 2012).

The R *C. bonariensis* population examined in this study showed that there is resistance to multiple herbicide MoAs in southern Spain. Resistance to paraquat was high (R/S > 85), resistance to tribenuron-methyl (R/S > 19) and glyphosate (R/S > 8) was moderate, and resistance to 2,4-D and diflufenican (R/S between 4 and 5) low. There are only three cases of C. bonariensis with multiple resistance in the world: to glyphosate and paraquat; (Moretti et al., 2013) to atrazine, sulfometuron-methyl, and pyrithiobac-sodium; (Matzrafi et al., 2015) and to glyphosate and chlorsulfuron (Aves et al., 2020).

A reduction in absorption and/or translocation has been reported to confer glyphosate resistance in *C. bonariensis* (Amaro-Blanco et al., 2018; Kleinman & Rubin, 2017). The R *C. bonariensis* population tested in this study also showed reduced ^{14}C-glyphosate absorption and translocation, proving that these two NTSR mechanisms were responsible for resistance to this herbicide. The cuticular wax layer provides a protective barrier for a wide range of abiotic stresses (herbicides), so limited glyphosate absorption by the R plant population could be due to differences in outer leaf surfaces (Cruz-

Hipolito et al., 2011; Shepherd & Wynne Griffiths, 2006). Differences in translocation could be explained by the accumulation of the 14C-glyphosate near to the treated region in R plants, based on modified subcellular distribution of the herbicide (Kleinman & Rubin, 2017). Both of these NTSR mechanisms have also been reported in other dicot weeds, including *Bidens pilosa*, *Conyza canadensis*, *Conyza sumatrensis*, and *Parthenium hysterophorus* (Alcántara-de la Cruz et al., 2016; Amaro-Blanco et al., 2018; Bracamonte et al., 2016). Recently, an ABCC-type transporter endowing glyphosate resistance has been identified for the first time in a R weed (Pan et al., 2021).

In ^{14}C-paraquat-treated plants exposed to light after application, the translocation of the herbicide from the treated leaf to other foliar and/or root tissues is limited, since the mechanism of action of paraquat is based on light-driven electron transport producing reactive oxygen species that rapidly kill cells (Hawkes, 2014). To allow ^{14}C-paraquat to translocate before causing plant tissue damages, *C. bonariensis* plants were incubated in the dark. The S and R populations showed similar ^{14}C-paraquat absorption patterns during the first 48 HAT, reinforcing that absorption of this herbicide through the plant cuticle is not light dependent (Brunharo & Hanson, 2017). However, there was less and slower translocation of paraquat from the treated leaf to the rest of the plant and roots in the R population. This reduced translocation may be a result of a higher transport of the paraquat into the vacuole as the main resistance mechanism (Moretti et al., 2017). Reduced translocation was characterized as being the mechanism responsible for resistance to paraquat in other Conyza species (Hawkes, 2014; Moretti et al., 2017). Therefore, the reduction in translocation found was likely the mechanism responsible for paraquat resistance in the R *C. bonariensis* population.

Some Cyt-P450 enzymes are involved in the metabolism of various herbicides, which are able to confer selectivity to certain herbicides in cultivated plants and resistance in weeds (Pandian et al., 2020). In the dose–response assays, malathion reversed resistance to 2,4-D and partially reversed resistance to tribenuron-methyl in the R *C. bonariensis* population, but did not reverse resistance to diflufenican. Resistance to diflufenican in *Raphanus raphanistrum* can be due to enhanced metabolism mediated by Cyt-P450 enzymes (Lu et al., 2020), representing one of the few cases of putative metabolic resistance to this MoA. In this study, malathion application ruled out Cyt-P450

metabolic-based resistance to diflufenican in the R *C. bonariensis* population. Most cases of resistance to PDS inhibitors have involved target site mutations in the PDS gene (Dang et al., 2019; Liu et al., 2013), suggesting that the diflufenican resistance of the R *C. bonariensis* population might be based on TSR mechanisms.

Because the R *C. bonariensis* population showed a response to 2,4-D similar to that of the S population after treatment with malathion, it was clear that metabolic resistance mediated by Cyt-P450 was the only mechanism against the phytotoxic action of 2,4-D. In the 2,4-D metabolism assay, the R population was able to metabolize more than 55% of the herbicide into nonphytotoxic metabolites in the foliar systems of plants. However, in the root system 2,4-D or other types of metabolites were not found, which could be due to the null translocation of the herbicide (Goggin et al., 2016). Malathion completely reversed resistance based on 2,4-D metabolism in *Amaranthus tuberculatus*; (Figueiredo et al., 2018) however, in *Amaranthus hybridus*, *Parthenium hysterophorus*, and *Papaver rhoeas*, the reversal of the resistance to 2,4-D was only partial (Palma-Bautista, Hoyos, et al., 2020; Palma-Bautista, Rojano-Delgado, et al., 2020; Torra et al., 2017)). When the R phenotype is completely reversed with a Cyt-P450 inhibitor, only this enzyme family would be involved in the resistance response, while when it is partial other enzyme families or NTSR mechanisms could also be participating (Gaines et al., 2020; Palma-Bautista, Rojano-Delgado, et al., 2020).

Participation of Cyt-P450 in the metabolism of tribenuron-methyl was only partial, allowing that R *C. bonariensis* plants transformed 58% of the herbicide applied into OH-MM and the conjugate-MM. Metabolism-based resistance to ALS inhibiting herbicides have long been reported and are increasing in both monocot and dicot weed species (Gaines et al., 2020; Hada et al., 2021). However, there are a few cases in which the metabolism of sulfonylureas has been described as the dominant resistance mechanism in dicot weeds (Bai et al., 2018; Cruz-Hipolito et al., 2013; Mora et al., 2019). Because the susceptibility to tribenuron-methyl of the R population did not decrease at the level of the S population with the application of malathion, it is possible that a target site mutation, other metabolic enzyme families, or NTSR mechanisms also participate in the resistance to this herbicide; i.e., resistance to tribenuron-m ethyl involves TSR and NTSR mechanisms.

This study characterized for the first time in *C. bonariensis* one multiple R case to 2,4-D (auxin mimic), paraquat (PSI), glyphosate (EPSPS), tribenuron (ALS), and diflufenican (PDS) developed in the field in any plant species. Considering that only some NTSR mechanisms were investigated for each herbicide, proofs of impaired transport for paraquat and glyphosate, and of enhanced metabolism by Cyt-P450 for 2,4-D and tribenuron, were provided as putative resistance mechanisms. Given that TSR was not researched, their presence cannot be ruled out, particularly for ALS and PDS inhibitors. Given the high level of resistance to these herbicides associated with multiple resistance mechanisms, increasing the dose of herbicide is unlikely to improve the control of *C. bonariensis* populations. Some adverse effects on the environment associated with herbicide application have emerged in the form of an increase in the populations of R weeds; decline in beneficial organisms such as predators, pollinators, and earthworms; change in soil microbial diversity; and contamination of agroecosystems. Therefore, sustainable strategies based on the integration of herbicides with nonchemical methods are needed to achieve an effective level of weed control and to slow down the evolution of herbicide resistance in this weed species while conserving the agroecosystem and biodiversity.

5. References

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CAPITULO V

Conclusiones

En las poblaciones caracterizadas como *Amaranthus palmeri*, se confirmó la resistencia al glifosato. Esta investigación es el primer estudio de los mecanismos de resistencia en *A. palmeri* de Argentina, donde se muestra que los mecanismos de TSR no estan involucrados, es decir, no se encontraron mutaciones ni amplificación del gen *EPSPS*. Por el contrario, la población de GRP exhibió una baja absorción y una translocación reducida del glifosato como los principales mecanismos de resistencia. Este es el primer infrome mundial de resistencia a glifosato en *A. palmeri* basado solo en mecanismos NTSR. No obstante, requieren experimentos futuros para conocer la base fisiológica y bioquímica de la absorción y translocación reducida que se ha descrito en esta investigación, incluida la amplificación y regulación de genes, lo que podría impulsar la evolución de mecanismos NTSR a glifosato en esta y otras especies de malas hierbas.

1. Por otro lado, entre las seis especies de malas hierbas dicotiledóneas estudiadas, hubo una repuesta diferenciada en la resistencia a 2,4-D. Los dos mecanismos de resistencia analizados el metabolismo mejorado que involucraba al citocromo P450 y una translocación reducida, en algunas especies ambos contribuyeron y en otras solo uno a la resistencia. Tal vez como resultado del sitio de acción multiobjetivo de los imitadores de auxinas. Por lo tanto, este estudio también enfatiza los peligros de extrapolar los mecanismos de resistencia a 2,4-D de unas pocas especies de malezas a otras, incluso si son parientes cercanos.
2. *Conyza bonariensis* mostró resistencia múltiple a 2,4-D (imitador de auxina), paraquat (PSI), glifosato (EPSPS), tribenuron (ALS) y diflufenican (PDS) desarrollada en el campo. se proporcionaron pruebas de translocación reducida para el paraquat y glifosato y de metabolismo mejorado por citocromo P450 para 2,4-D y tribenuron como mecanismos reponsables de la resistencia. Dado que los TSR no fueron investigados, no se puede descartar su presencia, particularmente para los inhibidores de ALS y PDS. Dado el alto nivel de resistencia a estos herbicidas asociado con múltiples mecanismos de resistencia, es poco probable que aumentar la dosis de herbicida mejore el control de *C. bonariensis*.

Finalmente, la sostenibilidad de los herbicidas juegan un papel importante en la agricultura y que deben utilizarce concientemente para ayudar a garantizar el suministro de alimentos. La aparicion de resistencia simple o multiple con uno o muchos mecanismos de resistencia es el principal desafío para la sostenibilidad de los herbicidas, actualmente cuestionada por la rapida evolución de las malas hierbas resistentes a los herbicidas. Los resultados de las investigaciones donde se conocen y estudian los mecanismos involucrados en dicha resistencia ayudaran a plantear estrategias adecuadas para una producción sustentable basadas en la integración de herbicidas con métodos no químicos y lograr un nivel efectivo de control de las malas hierbas y desacelerar la evolución de la resistencia a los herbicidas, mientras se conserva el agroecosistema y la biodiversidad.

CAPITULO VI

Otras publicaciones

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The Triple Amino Acid Substitution TAP-IVS in the *EPSPS* Gene Confers High Glyphosate Resistance to the Superweed *Amaranthus hybridus*

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Abstract: The introduction of glyphosate-resistant (GR) crops revolutionized weed management; however, the improper use of this technology has selected for a wide range of weeds resistant to glyphosate, referred to as superweeds. We characterized the high glyphosate resistance level of an *Amaranthus hybridus* population (GRH)—a superweed collected in a GR-soybean field from Cordoba, Argentina—as well as the resistance mechanisms that govern it in comparison to a susceptible population (GSH). The GRH population was 100.6 times more resistant than the GSH population. Reduced absorption and metabolism of glyphosate, as well as gene duplication of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) or its overexpression did not contribute to this resistance. However, GSH plants translocated at least 10% more ^{14}C-glyphosate to the rest of the plant and roots than GRH plants at 9 h after treatment. In addition, a novel triple amino acid substitution from TAP (wild type, GSH) to IVS (triple mutant, GRH) was identified in the EPSPS gene of the GRH. The nucleotide substitutions consisted of ATA^{102}, GTC^{103} and TCA^{106} instead of ACA^{102}, GCG^{103}, and CCA^{106}, respectively. The hydrogen bond distances between Gly-101 and Arg-105 positions increased from 2.89 Å (wild type) to 2.93 Å (triple-mutant) according to the EPSPS structural modeling. These results support that the high level of glyphosate resistance of the GRH *A. hybridus* population was mainly governed by the triple mutation TAP-IVS found of the EPSPS target site, but the impaired translocation of herbicide also contributed in this resistance.

Keywords: 5-enolpyruvylshikimate-3-phosphate synthase; EPSPS gene mutation; glyphosate-resistant crops; nontarget site; smooth pigweed; target site resistance

1. Introduction

Plant breeding methods have delivered herbicide resistant crops that offer advantages for weed control [1]. The introduction of glyphosate-resistant (GR) crops in 1996 revolutionized weed management practices [2]. Agricultural areas occupied by these crops, mainly GR-soybean and GR-corn, increased considerably in Argentina, Brazil and USA [2,3]. Inadequate adoption of GR-crops, i.e., higher doses and more applications of glyphosate than recommended by the manufacturer, has

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Low temperatures enhance the absorption and translocation of ^{14}C-glyphosate in glyphosate-resistant *Conyza sumatrensis*

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ABSTRACT

Influence of low temperatures on the glyphosate efficacy was studied in glyphosate-resistant (R) and -susceptible (S) *Conyza sumatrensis* biotypes. For this purpose, the physiological and enzymatic aspects involved were characterized under two growing temperature regimes [high (30/20 °C) and low 15/5 °C temperatures day/night]. The R biotype was 5.5 times more resistant than the S biotype at high temperatures; however, this R-to-S ratio decreased to 1.6 at low temperatures. At 96 h after treatment (HAT), the shikimic acid accumulation was higher in the S biotype in both temperature regimes (4.6 and 1.9 more shikimic acid at high and low temperatures, respectively), but the accumulation of the R biotype increased 2.6 times at low temperatures compared to high ones. From 24 to 96 HAT, the ^{14}C-glyphosate absorption ranged from 28 to 65% (percentage reached from 48 HAT) at low temperatures, and from 20 to 50% at high temperatures (gradual increase), but there were no differences between *C. sumatrensis* biotypes within each temperature regime. At high temperatures, the ^{14}C-glyphosate translocation was different between biotypes, where the R one retained at least 10% more herbicide in the treated leaves than the S biotype at 96 HAT. So, the S biotype translocated 40% of ^{14}C-glyphosate absorbed to roots, and the R biotype translocated only 28% of herbicide at the same period. At low temperatures, there were no differences between biotypes, and at 96 HAT, the ^{14}C-glyphosate found in treated leaves was ~47% and up to ~42% reached the roots, i.e., the resistance mechanism was suppressed. The basal and enzymatic activities of the 5-enolpyruvyishikimate 3-phosphate synthase were different between temperature regimes, but there was no differences between biotypes within each temperature regime, showing that target-site resistance mechanisms did not contribute in the glyphosate resistance of the R biotype. Low temperatures enhanced the absorption and translocation of glyphosate by suppressing the resistance mechanisms improving its efficacy on resistant plants. This is the first characterization about the role of temperatures in the glyphosate efficacy on *C. sumatrensis*.

1. Introduction

The use of herbicides is increasing in worldwide crop production each year due, among other factors, to the reduction of workers for hand weeding (Gianessi, 2013). Indiscriminate use of herbicides, together with a lack of an integrated weed management have led to the appearance of weed biotypes resistant to herbicides in different cropping systems. A weed biotype resistant to a given herbicide is able to survive, complete its life cycle and reproduce by seed after application of the herbicide at a dose normally lethal for a wild biotype of the same species (Beffa et al., 2019). Herbicide resistance is one of the major concerns in the modern agriculture (Burgos et al., 2013), and worldwide, there are 255 species (148 dicots and 107 monocots) resistant to herbicides (Heap, 2019).

Glyphosate is a full spectrum herbicide that acts by inhibiting the enzyme 5-enolpyruvyishikimate 3-phosphate synthase (EPSPS), an important enzyme in the shikimate pathway for the biosynthesis of aromatic amino acids (Steinruecken and Amrhein, 1980). This herbicide has been used to control weeds in different crop situations such as citrus orchards, olive groves, and vineyards in southern Spain. However, biotypes of *Conyza* species (*C. bonariensis, C. canadensis* and *C. sumatrensis*) has been reported to be glyphosate resistant in this country

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Article

Effect of Adjuvant on Glyphosate Effectiveness, Retention, Absorption and Translocation in *Lolium rigidum* and *Conyza canadensis*

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Abstract: Glyphosate retention, absorption and translocation with and without adjuvant were examined in *Lolium rigidum* and *Conyza canadensis* in greenhouse and laboratory settings to develop an understanding of the influence of the selected adjuvant on glyphosate activity. Tests on whole plants show that the dose of herbicide needed to reduce dry weight by 50% (GR_{50}) or plant survival (LD_{50}) decreases by mixing glyphosate and adjuvant to 22%–24% and 42%–44% for both populations of *L. rigidum* and *C. canadensis*, respectively. This improvement in efficacy could be attributed to the higher herbicide retention and lower contact angle of the glyphosate + adjuvant drops on the leaf surface compared to the glyphosate solution alone. Plants of both species treated with ^{14}C-glyphosate + adjuvant absorbed more glyphosate compared to non-adjuvant addition. Furthermore, the movement of the herbicide through the plant was faster and greater with the adjuvant. Our results reveal that the use of adjuvants improves the effectiveness of glyphosate in two of the most important weeds in agricultural crops in Mediterranean countries.

Keywords: rigid ryegrass; horseweed; efficacy; retention; absorption and translocation

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine] has been used worldwide for several decades in a wide range of agricultural and non-agricultural situations, with glyphosate-based herbicides serving as broad-spectrum, water-soluble, non-selective, systemic, post-emergence herbicides [1–4]. Its mode of action is the inhibition of the shikimic acid pathway, blocking the synthesis of the aromatic amino acids (AAA) phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) [2,3].

Glyphosate is considered as the most important herbicide globally [2], which has led to overreliance on it. Repeated application of glyphosate has contributed to the widespread occurrence of glyphosate resistance in several weed species, with 48 glyphosate-resistant (GR) weed species reported [5]. Currently, there are more than five hundred cases worldwide of weeds which have evolved herbicide resistance [5].

A proactive approach to preventing GR weeds could be achieved by including alternative herbicides with different mechanisms of action, by improving herbicide efficacy and through the

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Evolving Multiple Resistance to EPSPS, GS, ALS, PSI, PPO, and Synthetic Auxin Herbicides in Dominican Republic *Parthenium hysterophorus* Populations. A Physiological and Biochemical Study

Candelario Palma-Bautista [1], Verónica Hoyos [2], Guido Plaza [3], José G. Vázquez-García [1], Jesús Rosario [1], Antonia M. Rojano-Delgado [1] and Rafael De Prado [1,*]

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Abstract: Two *Parthenium hysterophorus* populations resistant (R) and susceptible (S) harvested in banana crop from the Dominican Republic were studied. All S plants died when the herbicides were applied at field dose, except with paraquat. For the R population, the order of plant survival was as follows: glyphosate and paraquat > flazasulfuron > glufosinate > fomesafen > 2,4-D. The resistance factors obtained in the dose–response assays showed a high resistance to glyphosate, flazasulfuron, and fomesafen, medium resistance to glufosinate and 2,4-D, and a natural tolerance to paraquat (resistance factor (RF) = 1.0). The I_{50} values obtained in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), acetolactate synthase (ALS), and glutamine synthetase (GS) activity studies with glyphosate, flazasulfuron, and glufosinate, respectively, were greater in R than in S. The effect of fomesafen was measured by the Proto IX levels, obtaining five times more Proto IX in the S than in the R population. The resistance to 2,4-D in the R was determined by the lower accumulation of ethylene compared to the S population. The studies with ^{14}C-paraquat conclude that the lower absorption and translocation in both the R and S populations would explain the natural tolerance of *P. hysterophorus*. This is the first case of multiple resistance to herbicides with different mechanisms of action confirmed in *P. hysterophorus*.

Keywords: *Parthenium hysterophorus*; Dominican Republic; multiple resistance

1. Introduction

Parthenium hysterophorus is a weed that is widely distributed in Africa, Asia, and Oceania and is native to tropical America. For example, in Cuba, it is considered as one of the most noxious species [1,2]. The International Union for Conservation of Nature (IUCN) considers it to be one of the 100 most invasive species in the world [3].

This weed has a high seed production capacity (130,000–200,000 seeds m^{-2}), as well as persisting in the soil and germinating in a wide range of temperatures at any time of the year. These characteristics have contributed to the propagation via flowing water, the movement of vehicles, animals, and machinery, or it can be blown by wind (presence of achenes), facilitating its dissemination

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Resistance to Fomesafen, Imazamox and Glyphosate in *Euphorbia heterophylla* from Brazil

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Abstract: *Euphorbia heterophylla* is a species of weed that was previously controlled by fomesafen, imazamox and glyphosate, but continued use of these herbicides has selected resistant populations from the Rio Grande do Sul (Brazil). One resistant (R) strain and one susceptible (S) strain to fomesafen, imazamox and glyphosate were compared, the latter by recurrent selection. Dose-response tests showed multiple resistance to these herbicides. The required imazamox concentration to inhibit ALS by 50% was approximately 16 times greater in the R population than in the S population. Based on the EPSPS activity results, the R population was 10 fold less sensitive to glyphosate than the S counterpart. In addition, basal EPSPS activity from R plants was 3.3 fold higher than the level detected on S plants. The Proto IX assays showed high resistance to fomesafen in the R population that accumulated less Proto IX than the S population. Malathion assays showed the participation of CytP450 in fomesafen resistance, but a molecular mechanism could also be involved. To our knowledge, this is the first characterisation of multiple resistance to these three groups of herbicides in *E. heterophylla* in the world.

Keywords: *Euphorbia heterophylla*; fomesafen; imazamox; glyphosate; multiple resistance; NTSR mechanisms; TSR mechanisms

1. Introduction

Euphorbia heterophylla (Wild poinsettia) is a summer annual plant with a short life cycle and with two or more generations per year. This species is a very common weed in South America that was later extended to Mexico and the Southern United States [1]. In soybean [Glycine max (L.) Merr.], *E. heterophylla* can cause a daily yield loss of up to 5.1 kg ha^{-1}, depending on the weed density [2]. Due to a high level of cross-pollination and elevated genetic recombination, there is high variability among populations of *E. heterophylla* which contributes to a rapid evolution of herbicide resistance [3,4].

Soybean is the most important crop in Brazil [5], and in 2018, occupied more than 30 million hectares corresponding to 45% of the cultivated area (~35 million ha) in the country [6]. In the 1980s, the control of *E. heterophylla* was achieved with protoporphyrinogen oxidase (PPO) inhibitor herbicides (fomesafen) and later in the mid-1980s, acetolactate synthase (ALS) inhibitor herbicides (mainly imidazolinones) began to be used [6]. However, in the early 2000s, biotypes of this species had already been selected for multiple resistance to fomesafen and imazamox [7]. On the other hand, the rapid adoption of glyphosate-resistant (GR) soybeans led to the exclusive use of glyphosate for the control *E. heterophylla* in the first years after the introduction of this technology. GR soybean varieties were officially introduced in Brazil in 2005; however, they began to be cultivated irregularly since 2000

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Multiple mutations in the *EPSPS* and *ALS* genes of *Amaranthus hybridus* underlie resistance to glyphosate and ALS inhibitors

Maria J. García[1]✉, Candelario Palma-Bautista[2], José G. Vazquez-Garcia[2], Antonia M. Rojano-Delgado[2], María D. Osuna[3], Joel Torra[4] & Rafael De Prado[2]

Amaranthus hybridus **is one of the main weed species in Córdoba, Argentina. Until recently, this weed was effectively controlled with recurrent use of glyphosate. However, a population exhibiting multiple resistance (MR2) to glyphosate and imazamox appeared in a glyphosate resistant (GR) soybean field, with levels of resistance up to 93 and 38-fold higher to glyphosate and imazamox, respectively compared to the susceptible (S) population. In addition to imidazolinones, MR2 plants showed high resistance levels to sulfonylamino-carbonyl (thio) benzoates and moderate resistance to sulfonylureas and triazolopyrimidines. Multiple amino acid substitutions were found in both target genes, acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), responsible for conferring high herbicides resistance levels in this *A. hybridus* population. In the case of *EPSPS*, the triple amino acid substitution TAP-IVS was found. In addition, MR2 plants also showed increased *EPSPS* gene expression compared to susceptible plants. A Ser653Asn substitution was found in the *ALS* sequence of MR2, explaining the pattern of cross-resistance to the ALS-inhibitor herbicide families found at the ALS enzyme activity level. No other mutations were found in other conserved domains of the *ALS* gene. This is the first report worldwide of the target site resistance mechanisms to glyphosate and ALS inhibitors in multiple herbicide resistance *Amaranthus hybridus*.**

Triazine- and dinitroaniline-resistant weeds that evolved in the 1970s and 1980s were controlled by addition or replacement with acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACCase) inhibitors in the 1980s and 1990s. In fact, with the introduction of soybean in South America, farmers used IMI herbicides (imidazolinones) as their first chemical option in pre-planting and post-emergence of soybean. This high selection pressure led to the emergence of ALS-resistant populations in the late 1990s. Between 1993 and 2004, resistance to ALS was reported for *Bidens pilosa, Bidens subalternans, Euphorbia heterophylla* and *Amaranthus hybridus* in countries such as Brazil, Paraguay and Argentina[1,2], and these biotypes are now widespread. ALS- and ACCase inhibitor-resistant weeds were controlled by the addition of protoporphyrinogen oxidase (PPO) inhibitors or glyphosate in glyphosate-resistant (GR) crops[3].

Farmers in South America quickly adopted technology packages for GR (glyphosate resistant) crops, mainly soybeans and corn. Ninety percent of these two main crops in this region are transgenic GR crops[4,5]. The low costs of technology packages and the limited use of herbicides, specifically in plants affected by glyphosate, allowed them to be very competitive in the world market. The basis of this new tool was the use of residual herbicide imazethapyr (imidazolinone) and subsequently glyphosate in post-emergence of weeds. For a decade, glyphosate was efficiently used to control *Amaranthus* species, but since 2016, an *A. hybridus* population multiple resistant to synthetic auxins and glyphosate has appeared in GR soybean fields south of Cordoba, Argentina[8]. Abuse of herbicides in GR crops has led to the appearance of a wide range of superweeds resistant mainly to the herbicide glyphosate[5].

Various attributes such as its high growth rate, high fertility, high genetic variability, stress tolerance, and the ability to evolve herbicide resistance confer to *Amaranthus* species the ability to become a dominant weeds that are difficult to control in summer crops[6,7]. Herbicide resistance in *Amaranthus* species becomes worrying due to

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Can Control of Glyphosate Susceptible and Resistant *Conyza sumatrensis* Populations Be Dependent on the Herbicide Formulation or Adjuvants?

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Abstract: In this work, we studied the effect of three glyphosate formulations (isopropylamine, ammonium and potassium salts) and two non-ionic adjuvants on the resistance response of two resistant (R1, R2) and one susceptible population of the highly invasive Asteraceae, *Conyza sumatrensis*, from Southern France vineyards. Only in R1, an amino acid substitution (Pro106Thr) was found in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). The two adjuvants, in a similar fashion, significantly reduced GR_{50} values for every population and glyphosate formulation. Without adjuvants, glyphosate as potassium salt was the only formulation able to significantly reduce the GR_{50} values of every population. For every population, the two adjuvants improved, indistinguishably, leaf retention of the herbicidal solution and the potassium salt formulation led to the highest retention, both with and without the adjuvant added. Uptake responses paralleled those of retention and adjuvant addition was more effective in increasing foliar uptake of the lower performing formulations (isopropylamine and ammonium salts). The allocation pattern of glyphosate among plant compartments was only dependent on population, with R2 retaining most glyphosate in the treated leaf, clearly suggesting the occurrence of a Non-Target Site Resistance (NTSR) mechanism. Results indicate that control of weed populations possessing NTSR mechanisms of resistance to glyphosate may be improved through adequate selection of formulation and adjuvant use.

Keywords: TSR and NTSR mechanisms; foliar retention; ^{14}C-glyphosate; absorption; translocation

1. Introduction

Glyphosate is a broad-spectrum, foliar, non-selective and systemic herbicide acting as an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [1–3]. EPSPS is an enzyme that plays an important role in the shikimate pathway for the biosynthesis of aromatic amino acids: phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) [4,5]. Following foliar application, this herbicide is absorbed and translocated via the phloem to the apical meristematic tissues [4,6]. This mode of action leads to lethal injuries to susceptible plants [2]. Thus, this herbicide has been successfully and extensively used in a broad range of agricultural and non-agricultural areas both against monocot and dicot weeds [7]. Glyphosate in its various formulations has been continuously applied, especially in recent

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Multiple Resistance to Glyphosate and 2,4-D in *Carduus acanthoides* L. from Argentina and Alternative Control Solutions

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Abstract: *Carduus acanthoides* L. is an invasive species native to Europe and distributed in other parts of the world, including North and South America. In Cordoba, Argentina, control failures of this species have been reported in Roundup Ready (RR) soybean crops where glyphosate and 2,4-D have frequently been applied, although there are no confirmed reports worldwide of resistance to glyphosate and 2,4-D in this species. Dose–response tests showed multiple-resistance to both active principles. The resistant population (R) had LD_{50} values of 1854.27 and 1577.18 g ae ha^{-1} (grams of acid equivalent per hectare), while the susceptible (S) population had LD_{50} values of 195.56 and 111.78 g ae ha^{-1} for glyphosate and 2,4-D, respectively. Low accumulations of shikimic acid (glyphosate) and ethylene (2,4-D) at different doses in the R population compared to the S population support the results observed in the dose–response curves. No significant differences in leaf retention were observed for glyphosate and 2,4-D in the R and S populations. However, the use of adjuvants increased the retention capacity of herbicides in both populations. Ten alternative herbicides with seven different action mechanisms (MOAs) were evaluated and the most effective active principles were dicamba, bromoxynil, atrazine, tembotrione, flazasulfuron, glufosinate, and paraquat. These findings are the first evidence of glyphosate and 2,4 D resistance in *C. acanthoides*.

Keywords: dose–response; shikimate accumulation; ethylene accumulation; adjuvants; efficacy of herbicides; alternative chemical control

1. Introduction

Glyphosate [n-(phosphonomethyl) glycine], has been the most widely used herbicide in the world due to its physicochemical characteristics [1–3]. Poor implementation of intensively cultivated glyphosate-resistant crops and poor management of herbicide application programs have generated significant dependence on glyphosate, resulting in the evolution of weed resistance to this herbicide [4–6]. Glyphosate was introduced in 1974 and weed resistance was not reported until 1995, when a population of resistant *Lolium rigidum* was detected in Australia [7]. Currently in Argentina, more than 90% of the soybean fields are planted with glyphosate-resistant soybeans. The intense use of glyphosate has contributed to the spread of weeds with resistance to this herbicide in Argentina, including species such as *Sorghum halepense*, *Lolium multiflorum*, *Lolium perenne*, *Cynodon hirsutus*, *Echinochloa colona*, *Eleusine indica*, *Conyza bonariensis*, *Brassica rapa*, *Amaranthus quitensis*, *Amaranthus palmeri*, *Bromus catharticus*, *Urochloa panicoides*, *Echinochloa crus-galli*, and recently, *Carduus acanthoides* [8,9]. The greatest problem

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Comparison of premix glyphosate and 2,4-D formulation and direct tank mixture for control of *Conyza canadensis* and *Epilobium ciliatum*☆

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ABSTRACT

Premix or tank mix of glyphosate and 2,4-D are a good alternative to control glyphosate-resistant and -tolerant weeds; however, the combination of herbicides may increase the environmental impacts, since mixtures often have higher toxicity than a single herbicide. In addition, antagonism between these herbicides has also been reported. We compared the efficacy of a premix glyphosate+2,4-D formulation with respect to the tank mix of both herbicides on glyphosate-resistant *Conyza canadensis* and -tolerant *Epilobium ciliatum* populations in laboratory and field experiments. 2,4-D suppressed the glyphosate-resistance/tolerance of both species, whose populations presented similar responses to their susceptible counterparts ($LD_{50} \geq 480+320$ g ha^{-1} glyphosate + 2,4-D, respectively). Plants of both species treated with the premix formulations retained ~100-μL more herbicide solution, accumulated 20–25% and 28–38% more shikimate and ethylene, respectively, and presented greater ^{14}C-glyphosate absorption and translocation, depending on the species, compared to plants treated with the tank mix treatment. Although doubling the field dose (720 + 480 g ha^{-1}) improved (5–22%) the control of these weeds in the field, split applications of both premix and tank mix provided the best control levels (≤70%), but premix treatments maintained control levels above 85% for longer (120-d). No antagonism between glyphosate and 2,4-D was found. The addition of 2,4-D controlled both broadleaf species. For all parameters evaluated on the *C. canadensis* and *E. ciliatum* populations in the laboratory and in the field, the premix treatments showed better performance than the tank mix treatments. Premix formulations could reduce the environmental impact of herbicides used to control glyphosate resistant/tolerant weeds by decreasing the herbicide amount needed to achieve an acceptable weed control level.

1. Introduction

Perennial crops in European Mediterranean areas include mainly olive, vineyard, citrus and stone and pip fruit trees. According to Eurostat data (Eurostat, 2020), around 6% of the European agricultural area was covered with perennial crops, which correspond to 11 million ha in 2016. Spain (4,830,000 ha) and Italy (2,372,910 ha) have been the most important member countries of the EU-28 Mediterranean Region in terms of perennial crops (Eurostat, 2020).

Farmers invest keep crops free of pests, diseases and weeds to obtain high yields and high-quality products (Möhring et al., 2020). The most widely used weed control method is the application of herbicides at different times of the crop cycle (Kudsk and Mathiassen, 2020), with glyphosate being the main herbicide used in perennial crops for this purpose (Franz et al., 1997; Duke et al., 2018). Glyphosate is a foliar, systemic and broad-spectrum herbicide that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) provoking the shikimate accumulation (Steinrücken and Amrhein, 1980). However, the continuous use of this herbicide, sometimes more than two applications a year in the same crop, has exerted a high selection pressure on the flora, causing the appearance of glyphosate-resistant and/or -tolerant weeds (Heap, 2020).

Acquired resistance to glyphosate is provided by target-site

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