Universitat de Lleida

Unravelling herbicide resistance in corn poppy (Papaver rhoeas L.) to improve integrated weed management strategies

Jordi Rey Caballero

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Unravelling herbicide resistance in corn poppy (*Papaver rhoeas* L.) to improve integrated weed management strategies.

Desxifrant la resistència a herbicides en rosella (*Papaver rhoeas* L.) per millorar les estratègies de maneig integrat.

DISSERTATION

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Jordi Rey Caballero

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A Paz Portilla y Mercedes Caballero

de Torrejoncillo del Rey

Presentació

Aquesta tesis s'ha desenvolupat sota la direcció del professor Dr. Jordi Recasens Guinjuan i el Dr. Joel Torra Farré dins del grup de recerca consolidat de Malherbologia i Ecologia Vegetal del Departament d'Hortofructicultura, Botànica i Jardineria de l'Escola Tècnica Superior d'Enginyeria Agrària (ETSEA) de la Universitat de Lleida. El present treball s'emmarca dins de dos convenis de recerca (projectes C10060 i C14048) finançats per les empreses Dow AgroScience i DuPont de Nemours i desenvolupats entre els anys 2012 i 2015.

L'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) de la Generalitat de Catalunya em va concedir una beca de doctorat des del febrer de 2013 al gener de 2016. Durant la realització d'aquesta tesis s'han dut a terme dos estades en altres centres d'investigació nacionals:

-En la Universidad de Huelva, Huelva amb el Dr. Julio Menéndez Calle (octubre 2014-desembre 2014).

-En la Finca de la Orden, CICYTEX, Badajoz amb la Dra. María Dolores Osuna Ruíz (abril 2015).

A partir dels resultats obtinguts durant el desenvolupament d'aquesta tesis, s'han elaborat diferents articles que ressenyem a continuació:

- **Article 1**: Resistance mechanisms to ALS inhibiting herbicides in Spanish *Papaver rhoeas* populations: molecular basis and cross resistance patterns. Rey-Caballero J., Menéndez J., Osuna M.D., Salas M. & Torra J. Enviat a la revista *Pest Management Science* a principis de març de 2016.

- **Article 2**: Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). Rey-Caballero J., Menéndez J., Giné-Bordonaba J., Salas M., Alcántara R. & Torra J. Publicat en *Pesticide Biochemistry and Physiology* en març de 2016.

- **Article 3**: New management options for herbicide resistant *Papaver rhoeas* populations in Europe. Rey-Caballero J., Royo-Esnal A., Recasens J., González I. & Torra J. Enviat a la revista *Pest Management Science* a principis d'octubre de 2015.

A més, durant el transcurs del present treball s'ha dut a terme la difusió dels resultats obtinguts a diversos congressos i grups de treball d'àmbit nacional e internacional:

- Rey-Caballero J., Torra J., Royo-Esnal A., Gonzalez I., Ferrer R. & Recasens J. Nuevas opciones de manejo integrado de poblaciones de *Papaver rhoeas*

resistentes a herbicidas. XIV Congreso de la Sociedad Española de Malherbología, Valencia, octubre 2013. (Comunicació oral).

- Rey-Caballero J., Menéndez J., Osuna M.D., Alcántara R., Salas M. & Torra J. Resistencia a inhibidores de la ALS en biotipos de *Papaver rhoeas* (L.) resistente. Comité para la prevención de resistencias a herbicidas, Madrid, gener 2014. (Comunicació oral).
- Rey-Caballero J., Montull J.M., Taberner A. & Torra J. Resistance study of (Spanish) *Papaver rhoeas* (biotypes) to bromoxynil. 17th European Weed Research Society Symposium, Montpellier, abril 2015. (Pòster).
- Rey-Caballero J., Menéndez J., Salas M. & Torra J. Resistance mechanisms to 2,4-D (2,4-dichlorophenoxy acetic acid) in Spanish biotypes of *Papaver rhoeas.* Resistance Event, Rothamsted Research, Harpenden, setembre 2015. (Pòster).
- Rey-Caballero J., Giné-Bordonaba J., Edo-Tena E. & Torra J. Análisis de la producción de etileno en biotipos de *Papaver rhoeas* L. resistentes y sensibles a 2,4-D. XV Congreso Soc. Española de Malherbología, Sevilla, octubre 2015. (Pòster).
- Rey-Caballero J., Montull J.M., Taberner A. & Torra J. Estudio de sensibilidad al bromoxinil de un biotipo de *Papaver rhoeas* L. XV Congreso Soc. Española de Malherbología, Sevilla, octubre 2015. (Pòster).
- Rey-Caballero J., Menéndez J., Salas M. & Torra J. Estudio de mecanismos de resistencia "Non-Target-Site"en biotipos de *Papaver rhoeas* L. con resistencia múltiple. XV Congreso Soc. Española de Malherbología, Sevilla, octubre 2015. (Comunicació oral).
- Rey-Caballero J., Menéndez J., Osuna M.D., Salas M. & Torra J. Bases moleculares de la resistencia a inhibidores de la ALS en *Papaver rhoeas* (L.). Comité para la prevención de resistències a herbicidas, Lleida, gener 2016. (Comunicació oral). Premi Phytoma al millor treball presentat per un jove investigador predoctoral.
- Torra J., Royo-Esnal A., Rey-Caballero J., Recasens J. & Salas M. Opciones de manejo de *Papaver rhoeas* con resistencia múltiple a herbicidas. XV Congreso Soc. Española de Malherbología, Sevilla, octubre 2015. (Comunicació oral). Premi Actas SEMh a la millor comunicació.

Al llarg d'aquest període també he publicat articles en revistes de divulgació:

- Rey-Caballero J., Pallares LL. & Rodríguez G. (2012) Mecanismos de acción de los herbicidas en plantas. *Phytoma*.
- Rey-Caballero J., Torra J. & Recasens J. (2014) Opciones de manejo integrado de amapola resistente a herbicidas en cereales de invierno. *Vida Rural*: 373.
- Rey-Caballero J., Torra J. & Recasens J. (2014) Manejo integrado de amapola (*Papaver rhoeas*) resistente. Situación actual y nuevas opciones de manejo integrado. *Tierras:*220.
- Rey-Caballero J. & Montull J.M. (2014) Valoración Económica del Manejo de Resistencias en Amapola y Vallico. *Tierras*: 220.
- Rey-Caballero J., Menéndez J., Salas M. & Torra J. (2016) Mecanismos de resistencia Non-Target-Site en biotipos de amapola (*Papaver rhoeas*) con resistencia múltiple. *Phytoma*: 269.

A finals de 2015 vaig publicar un treball de la meva fase investigadora prèvia a la realitzada en el grup de Malherbologia i Ecologia Vegetal de la Universitat de Lleida:

- Aguilar-Fenollosa E., Rey-Caballero J., Blasco J.M., Segarra-Moragues J., Hurtadi M.A., & Jacas J.A. (2015) Patterns of ambulatory dispersal in *Tetranychus urticae* can be associated with host specialization. *Experimental and Applied Acarology*.

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Resumen

La presencia de biotipos de amapola (*Papaver rhoeas* L.) resistentes a herbicidas constituye uno de los principales problemas de muchas áreas cerealistas de secano. La solución del mismo pasa por una correcta caracterización de la resistencia y por el establecimiento de una adecuada estrategia de manejo integrado. El presente trabajo se ha planteado precisamente con este fin. Para ello a) se han seleccionado biotipos con resistencia múltiple (a inhibidores de la acetolactato sintasa -ALS- y a herbicidas auxínicos) y biotipos únicamente resistentes a 2,4-D originarios de las zonas cerealistas del noreste peninsular de los que se han estudiado las bases moleculares y fisiológicas de estas resistencias; b) se ha querido discernir bajo condiciones controladas (invernadero), si los fallos de control observados en campo, mediante herbicidas inhibidores del fotosistema II (bromoxinil) son debidos al estadio fenológico de la mala hierba en el momento de aplicación o a la presencia de una posible resistencia incipiente, y c) se han establecido diferentes estrategias de manejo integrado de amapola en campos comerciales de cereales de secano del noreste peninsular.

Respecto a la caracterización molecular de la resistencia a inhibidores de la ALS se ha verificado que las diferentes mutaciones encontradas en la posición Prolina 197 del gen que codifica la enzima ALS (6 para un total de 13 genotipos diferentes) son las responsables de la fuerte resistencia a tribenurón-metil, y que ni la absorción ni la translocación de este herbicida han resultado tener una implicación directa en dicha resistencia. Por el contrario, sólo la substitución por Serina o por Triptófano parece tener, respectivamente, alguna significación en la resistencia a la triazolopirimidina florasulam y a la imidazolinona imazamox. Aparte de estas mutaciones, unas pocas plantas han presentado mutaciones (ácido Glutámico 427 y Leucina 648) fuera de las regiones conservadas del gen, no descritas anteriormente. Una falta de correlación entre el genotipo y el fenotipo de aquellas plantas tratadas con imazamox y florasulam junto a la aparición de plantas no mutadas capaces de resistir las aplicaciones de imazamox, son indicios que nos hacen pensar en la presencia de mecanismos de resistencia "non-target-site" para aquellos inhibidores de la ALS no sulfonilureas.

Los estudios llevados a cabo con 2,4-D han revelado aspectos clave acerca del mecanismo de resistencia de la amapola a herbicidas auxínicos. Las poblaciones con resistencia a 2,4-D también presentaron resistencia cruzada a otras auxinas sintéticas (dicamba y aminopiralid). Se han observado también diferencias significativas en la

síntesis de etileno entre plantas resistentes y sensibles tras la aplicación de 2,4-D. El empleo de 2,4-D marcado ha permitido verificar, en biotipos resistentes, tanto a nivel cuantitativo como cualitativo, la falta de movilidad de este herbicida. Estos resultados podrían *per se,* explicar la resistencia de estos biotipos. En este sentido, la falta de movilidad podría explicarse por una distorsión de las principales proteínas implicadas en el transporte de las auxinas. Pero esta falta de translocación podría ser también consecuencia de otro mecanismo de resistencia, dado que la metabolización del herbicida auxínico podría alterar su posterior transporte.

El deficiente control de amapola con bromoxinil observado en campo se atribuye, a priori, a la presencia de plantas con una fenología avanzada en el momento de la aplicación. No obstante, los resultados observados en uno de los biotipos, establecieron una respuesta diferencial respecto al homólogo sensible cuando el bromoxinil se aplicó en una fenología avanzada.

Los ensayos de manejo integrado han demostrado que las rotaciones cereal-girasol y cereal-guisante son capaces de reducir la infestación de amapola notablemente ya que rompen el ciclo de la mala hierba, además de permitir la integración de herbicidas que pertenecen a familias químicas distintas a la de las ALS o a la de los herbicidas auxínicos. Sin embargo, la rotación con colza no ha resultado una estrategia recomendable para el manejo de esta mala hierba. La necesidad de una siembra precoz de este cultivo no constituye una herramienta cultural adecuada para interferir en el ciclo de la amapola. Además, existen pocas materias activas capaces de controlar esta mala hierba en colza. En estos ensayos se han obtenido mejores resultados en los tratamientos precoces (pre y post-emergencia precoz) que en los tratamientos en post-emergencia, probablemente gracias a que los primeros han reducido la variabilidad fenológica de la amapola en el momento de la intervención y, por lo tanto, los posibles escapes.

Los resultados obtenidos han permitido avanzar considerablemente en el estado de la ciencia de los mecanismos que confieren resistencia a herbicidas inhibidores de la ALS y herbicidas auxínicos en *Papaver rhoeas*, conocer la existencia de una respuesta diferencial de biotipos a bromoxinil así como explicar los fallos de control detectados en campo y establecer un programa de manejo integrado de amapola en cereales de secano.

Resum

La presència de biotips de rosella (*Papaver rhoeas* L.) resistents a herbicides constitueix un dels principals problemes de moltes àrees cerealistes de secà. La solució del mateix passa per una correcta caracterització de la resistència així com en l'establiment d'una adequada estratègia de maneig integrat. El present treball s'ha plantejat precisament amb aquesta finalitat. Per això a) s'han seleccionat biotips amb resistència múltiple (a inhibidors de la acetolactato sintasa -ALS- i a herbicides auxínics) i biotips únicament resistents a 2,4-D originaris de les zones cerealistes del nord-est peninsular i s'han estudiat les bases moleculars i fisiològiques d'aquestes resistències; b) s'ha volgut discernir sota condicions controlades (hivernacle), si la manca de control observada en camp, mitjançant herbicides inhibidors del fotosistema II (bromoxinil), és deguda a l'estadi fenològic de la mala herba en el moment de l'aplicació o a la presència d' una possible resistència incipient i c) s'han establert diferents estratègies de maneig integrat de rosella en camps comercials de cereals de secà del nord-est peninsular.

Pel que fa a la caracterització molecular de la resistència a inhibidors de l'ALS s'ha verificat que les diferents mutacions trobades en la posició Prolina 197 del gen que codifica l'enzim ALS (6 per a un total de 13 genotips diferents) són les responsables de la forta resistència a tribenuron-metil, i que ni l'absorció ni la translocació d'aquest herbicida han resultat tenir una implicació directa en aquesta resistència. Per contra, només la substitució per Serina o per Triptòfan sembla tenir, respectivament, alguna significació en la resistència a la triazolopirimidina florasulam i a la imidazolinona imazamox. A banda d'aquestes mutacions, unes poques plantes han presentat mutacions (àcid Glutàmic 427 i Leucina 648) fora de les regions conservades del gen, no descrites anteriorment. Una manca de correlació entre el genotip i el fenotip d'aquelles plantes tractades amb imazamox i florasulam conjuntament amb l'aparició de plantes no mutades que van resistir les aplicacions d'imazamox, semblen ser indicis que ens fan pensar en la presència de mecanismes de resistència "non target-site" per a aquells inhibidors de l'ALS no sulfonilurees.

Els estudis duts a terme amb 2,4-D han revelat aspectes clau sobre el mecanisme de resistència de la rosella a herbicides auxínics. Les poblacions amb resistència a 2,4-D també van presentar resistència creuada a altres auxines sintètiques (dicamba i aminopiralid). També s'han observat diferències significatives en la síntesi d'etilè entre plantes resistents i sensibles després de l'aplicació de 2,4-D. Emprant 2,4-D marcat s'ha

pogut verificar en biotips resistents, tant a nivell quantitatiu com qualitatiu, la manca de mobilitat d'aquest herbicida. Aquests resultats podrien *per se* explicar la resistència d'aquests biotips. En aquest sentit, la manca de mobilitat podria explicar-se per una distorsió de les principals proteïnes implicades en el transport de les auxines. Però aquesta manca de translocació podria ser també conseqüència d'un altre mecanisme de resistència, atès que la metabolització de l'herbicida auxínic podria alterar el seu posterior transport.

El deficient control de rosella amb bromoxinil observat en camp s'atribueix, a priori, a la presència de plantes amb una fenologia avançada en el moment de l'aplicació. No obstant, els resultats observats en un dels biotips, han establert una resposta diferencial respecte a l'homòleg sensible quan el bromoxinil es va aplicar en una fenologia avançada.

Els assajos de maneig integrat han demostrat que les rotacions cereal-gira-sol i cereal-pèsol poden reduir la infestació de rosella de manera notable ja que trenquen el cicle de la mala herba, a més de permetre integrar herbicides que pertanyen a famílies químiques diferents a la de les ALS o la dels herbicides auxínics. Per contra, la rotació amb colza no resulta una estratègia recomanable per al maneig d'aquesta mala herba. La necessitat d'una sembra precoç d'aquest cultiu no constitueix una eina cultural adequada per interferir en el cicle de la rosella. A més a més, en colza existeixen poques matèries actives que controlin aquesta mala herba. En aquests assajos es van obtenir millors resultats en els tractaments precoços (pre i post-emergència precoç) que en els tractaments en post-emergència, probablement gràcies a que els primers redueixen la variabilitat fenològica de la rosella en el moment de la intervenció, i per tant, possibles fuites.

Els resultats obtinguts han permès avançar considerablement en l'estat de la ciència dels mecanismes que confereixen resistència a herbicides inhibidors de l'ALS i herbicides auxínics en rosella, conèixer l'existència d'una resposta diferencial de biotips a bromoxinil així com explicar la manca de control detectada en camp, i establir un programa de maneig integrat de rosella en cereals de secà.

Summary

The persistence of resistant corn poppy (*Papaver rhoeas* L.) biotypes is one of the most pressing problems in rainfed Spanish cereal crops. Resolution to this problem begins with the proper characterization of the resistant profile, followed by the establishment of an appropriate integrated management system. The study herein has been conducted precisely towards this end, while maintaining the following bases as a general framework: a) corn poppy biotypes with multiple resistance (acetolactate synthase - ALS- inhibiting herbicides and auxinic herbicides) and only 2,4-D resistance from north-eastern Spain were selected for analysis of molecular and physiological resistance matrices; b) we have tried to investigate under controlled conditions, if failures observed in the field, by photosystem II inhibiting herbicides (bromoxynil), could be directly attributed to the phenological stage of the weed at application time or inherent resistance and c) the creation of integrated management strategies for corn poppy and rainfed cereal fields in North-Eastern Spain.

In the molecular characterization of ALS resistant inhibitors, our study confirms that the different mutations found at the Proline 197 position of the gene encoding ALS enzyme (6 for a total of 13 different genotypes) are responsible for resistance to tribenuron-methyl, and that neither the absorption nor translocation of this herbicide have proven to be directly involved in resistance. By contrast, only the substitution of Serine or Tryptophan seems to have some significance in resistance to triazolopyrimidine florasulam and imidazolinone imazamox, respectively. Aside from these mutations, select plants have introduced mutations (i.e. Glutamic acid, 427; and Leucine, 648) outside the conserved regions of the gene. A lack of correlation between genotype and phenotype in some plants treated with imazamox and florasulam together with the presence of non-mutated plants able to resist imazamox applications, is evidence suggesting the presence of "non-target-site" resistance mechanisms for non-sulfonylureas ALS inhibitors.

Studies carried out with 2,4-D have revealed important aspects of the resistance mechanism of corn poppy against these auxinic herbicides. 2,4-D resistant populations also showed cross-resistance to other auxinic herbicides (dicamba and aminopyralid). Furthermore, significant differences between resistant and susceptible plants in ethylene biosynthesis after 2,4-D application were observed. In both quantitative and qualitative studies, bio-marked 2,4-D was observed to have a lack of mobility in resistant biotypes.

In the case that these results explain the resistance of the biotypes, the lack of mobility may be explained by a distortion of the main proteins involved in auxin transport. On the other hand, lack of translocation may, be the result of another mechanism of resistance, for example altered metabolism and subsequent transport of the auxinic herbicide.

In the field, lack of corn poppy control with bromoxynil observed in field is attributed to the presence of plants with advanced phenology at the time of application. Analysis of the results for one of the biotypes did however reveal a differential response between these plants and susceptible ones when bromoxynil was applied during advanced phenology.

The integrated management experiments have revealed that cereal-sunflower and cereal-pea crop field rotations are able to significantly reduce corn poppy infestation because they break the lifecycle of this weed and permit the integration of herbicides belonging to the same chemical families as ALS or auxinic herbicides. In contrast, the cereal-oilseed rape rotation has proven to be an unsuitable strategy for corn poppy management. The need for early sowing of this crop is not an appropriate cultural tool for interfering with the corn poppy lifecycle; moreover, in the case of oilseed rape, there are few active ingredients able to control this weed. Trials showed that early treatment (pre- and early post-emergence) have led to better results than post-emergence treatments, most likely because earlier treatment reduces the phenological variability of corn poppy at the time of intervention, therefore reducing the possibility of treatment failure.

Results obtained in this study have been instrumental in catalysing the progress required to elucidate the mechanisms in *Papaver rhoeas* that confer resistance to ALS-inhibiting and auxinic herbicides. We now know that there is a differential response of biotypes to bromoxynil, explaining the lack of control detected in fields; which has led to the establishment of an integrated corn poppy programme in rainfed cereal.

CHAPTER 1

Introduction and Research Objectives

Managing resistant weeds, from molecular bases to field solutions

Weeds can produce potential crop losses of over the 34%, but weed control practices reduce these overall potential penalties to actual losses of about 10% (Oerke 2005). Herbicides are the basis of weed control in commercial agricultural systems. However, herbicide resistant weed biotypes are evolving rapidly as a natural response to selection pressure imposed by this modern agricultural weed management techniques (Norsworthy et al., 2012). The added cost associated with the management of herbicide resistance has been quantified and it goes from 26 to 60 €/ha in some of the most sophisticated agricultural systems (Mueller et al., 2005; Norsworthy et al., 2012). In this scenario, finding sustainable and highly effective weed management strategies is mandatory. But to achieve this goal the process is long and needs to recruit essential information in several phases and at different scales, from gen to farm (Figure 1.1). This information will assist farmers and advisers (or steakholders) in the development of effective weed control systems for the field in addition to assist herbicide manufacturers in the development of appropriate stewardship programmes for their products (Heap 2005). Checking if the lack of control observed in fields is due to a natural variation in the response of weed populations or to a resistance mechanism is the first step in this process. Greenhouse experiments (dose-response experiments) will assist in this aim, moreover, these experiments are convenient to quantify the level of resistance, when it is present. Clarify the precise details of the physiologic and molecular means by which weeds evolve herbicide resistance will contribute to wiser use of precious herbicide resources, new innovations, and more sustainable strategies for weed management (Powles and Yu 2010). Besides, knowledge of herbicide resistant weeds biology (emergence patterns, fecundity, dispersal mechanisms, etc.) allows practitioners to develop strategies that target the most sensitive life stages to management (Norsworthy et al., 2012). Finally, in order to provide farmers with an effective weed management strategy, all proposals generated through all this information need to be validated under field conditions.

Corn poppy (*Papaver rhoeas*) in Spanish winter cereal areas

Corn poppy (*Papaver rhoeas* L.) is amongst the most important broad-leaf weeds infesting cereals across Europe; mostly in southern areas with a Mediterranean climate (Kaloumenos 2014).

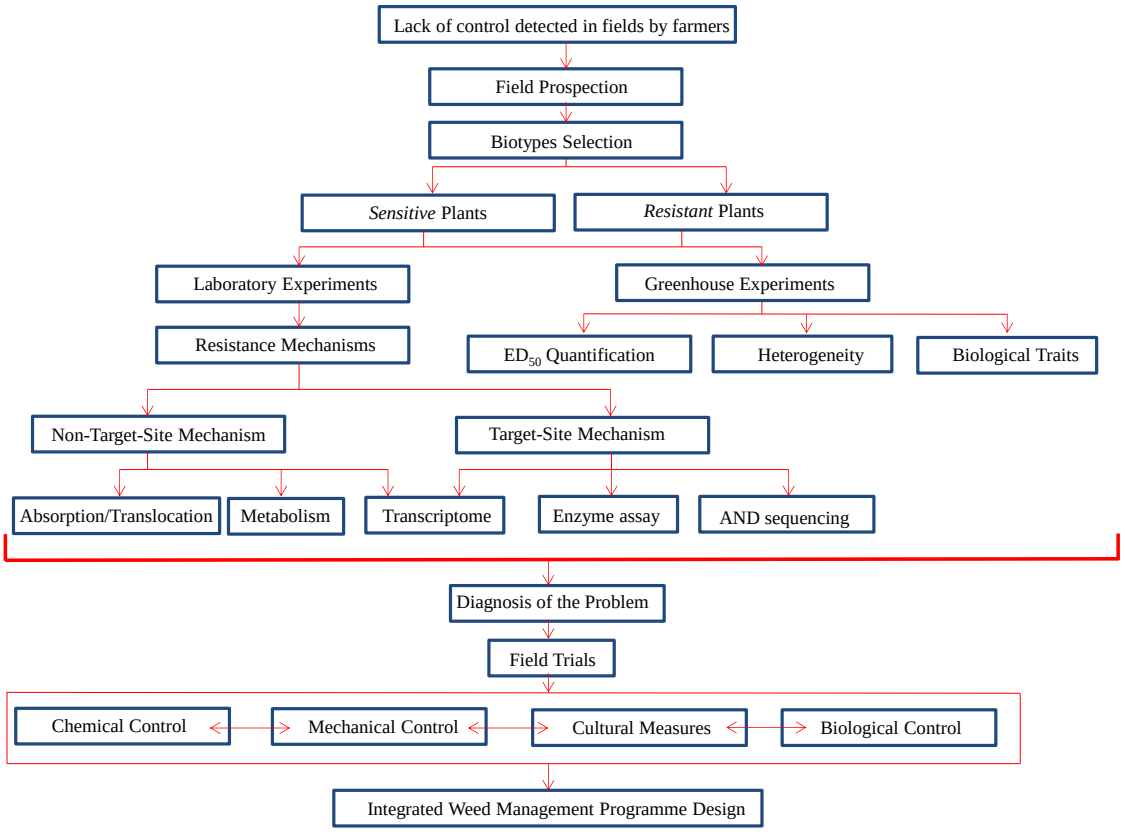

Figure 1.1. Information recruitment process previous to designing a suitable Integrated Weed Management programme for herbicide resistant weeds

In North-Eastern Spain, corn poppy, together with other grasses such as *Lolium rigidum* Gaudin, *Avena sterilis* L. and *Bromus diandrus* Roth are the main problematic weeds infesting winter cereals (Cirujeda 2001). Corn poppy is an insect-pollinated, diploid hermaphrodite species (2n = 14) with very high self-incompatibility (Délye et al., 2011). Outcrossing contributes to high levels of genetic variation and heterozygosity (Aguinagalde et al., 2005). It is a competitive species and, depending on its density causes significant yield reductions (up to 32% of the yield) (Torra and Recasens 2008). The ability of this species to invade, grow, and remain in arable fields can be attributed to several factors as: the development of a persistent seed bank, viability of their seeds up to 70% then of 77 months after burial (Cirujeda et al., 2006); an extended germination period that goes from early autumn to early spring (Cirujeda et al., 2008); and a high seed production, up to 40,000 seeds per plant in competition with wheat (Torra and Recasens 2008). Probably due to both, its above mentioned high genetic variability and the overuse of 2,4-D and tribenuron-methyl in its control, corn poppy has become an increasing problem in the last decades due to the appearance of herbicide resistant biotypes to synthetic auxins and/or to acetolactate synthase (ALS) inhibitors

(Claude et al., 1998). A survey conducted in North-Eastern Spain between 1990 and 2001 in those fields where local farmers reported poor corn poppy control following tribenuron-methyl applications, indicated that 85% and 72% of sampled *P. rhoeas* populations were resistant to 2,4-D and tribenuron-methyl, respectively (Cirujeda 2001). Moreover, in the last years, lack of control of corn poppy with some post-emergence mixtures containing both synthetic auxins and photosystem II (PS II) inhibitor herbicides (ioxynil + bromoxynil + MCPP p) has been reported in a few cases (Cirujeda 2001; Kaloumenos 2014).

Resistance towards 2,4-D

2,4-Dichlorophenoxyacetic acid (2,4-D), an auxinic herbicide (group O according to the Herbicide Resistance Action Committee, HRAC), was the first synthetic herbicide to be commercially developed (Song 2014). The introduction of this herbicide in the Spanish agriculture started in the 50's and due to its high efficacy and the lack of alternative products the use of 2,4-D for cereal broad-leaf weeds became very frequent (Cirujeda 2001). Poor control of corn poppy in Spain with 2,4-D was first reported in 1992 (Taberner et al., 1992). Nowadays, there are 32 auxinic herbicide resistance species worldwide, 15 of those are resistant to 2,4-D (Heap 2015). Usually, 2,4-D, or other similar auxinic herbicides are applied in cereal fields in post-emergence and mainly mixed with ALS inhibitors, PS II inhibitors or inhibitors of carotenoid biosynthesis. Despite their extensive use, the precise mode of action and consecutively the resistance mechanisms to auxinic herbicides in weeds is not completely understood (Mithila et al., 2011). However, new discoveries of nuclear auxin receptors, influx and efflux carriers and research in the metabolism of auxinic herbicides (2,4-D) have provided basic information which could help in the description of those resistance mechanisms (Peterson et al., 2015).

Resistance towards tribenuron-methyl

The sulfonylurea tribenuron-methyl is an ALS inhibitor (group B according to the HRAC). This herbicide binds within the substrate-access channel of ALS enzyme in plants and blocks the substrate access to the active site (Duggleby et al., 2008). ALS is a key plant enzyme responsible for the biosynthesis of branched-chain, essential amino acids valine, leucine, and isoleucine. Consequently, plants affected by this herbicide die due to the lack of those branch-chain amino acids. ALS inhibitors have been

revolutionary to the herbicide market because they are highly effective at low rates and environmentally safe (Tranel and Wright 2002). However, ALS inhibiting herbicides are the most prone mode of action worldwide in evolving herbicide resistant weeds due to very high selection pressures (Yu and Powles 2014). To date, 155 species all over the word have evolved resistance to ALS inhibitors (Heap 2015). Tribenuron-methyl has been sold in Spain since 1986 (Cirujeda 2001) and in 1998 the first case of a well-studied corn poppy biotype resistant to both 2,4-D and tribenuron-methyl was published (Claude et al., 1998). Nowadays tribenuron-methyl resistant corn poppy has evolved in other numerous countries across Europe. In Spain the resistance to tribenuron-methyl is conferred by Pro197 to Ser substitutions in the ALS gene (Durán-Prado et al., 2004). This target site mutation makes that increased doses of tribenuron-methyl do not have any effect in tribenuron-methyl resistant corn poppy plants and confers some degree of cross resistance to other ALS inhibitors.

Integrated management of herbicide resistant corn poppy biotypes. Current situation. Limitations and future prospects.

As mentioned above, problems with corn poppy in Spain are mainly located in rainfed cereal areas of Northern Spain. It is precisely in these areas where fewer rotations with spring or summer crops are practised (CPRH 2013). Since decades zero or minimum tillage is the most frequent soil tillage practice in North-Eastern Spain (Álvaro-Fuentes et al., 2007) and further West, in Huesca, Navarra and Burgos ploughing is still conducted more frequently (Cirujeda 2001). Additionally, in all these zones weed control is based largely on herbicides. Frequently, one single post-emergence tank mix of herbicides controlling both grasses and broad-leaf weeds is applied between November and March. A second application with the same or different herbicides is less frequent, and it is practised in order to control surviving or new emerged weeds. Moreover, rotations of herbicides with different modes of action (MOA onward) from year to year is not very common (Cirujeda 2001). In this scenario, the development of Integrated Weed Management (IWM) programmes are mandatory because multiple resistant corn poppy biotypes have been selected as a result of the overuse of few molecules and the reduction of cultural management techniques in the last decades. IWM is a component of integrated pest management (IPM) that focuses primarily on weeds (Buchanan 1976). It is described as the integration of various control strategies and application of ecological principles to control pests in agricultural systems (Smith

and Van den Bosch 1967). Several tools have been proposed to be introduced in an integrated resistant corn poppy management programme. Mechanical control of this species through post-emergence harrowing was found to be an effective method when corn poppy density was not extremely high and under dry conditions. Likewise, ploughing was considered an effective method for placing a proportion of corn poppy seeds in non-optimal germination situations, but this method should not be repeated for a few years (Cirujeda et al., 2003). Other cultural management practices such as delayed sowing or fallows showed good results in reducing corn poppy densities, but only when these cultural methods were combined with chemical or cultivation methods (Torra et al., 2011). Although in some dry Spanish cereal areas crop rotations are limited, it is interesting to check the effect of these few options (oil-seed rape, peas and sunflower) on corn poppy infestations. It is well known that crop rotations provide farmers with opportunities to employ variable crop life cycles, sowing dates, harvest dates, tillage and weed management practices to restrict the evolution of weeds adapted to cereal monocrop. In addition, crop rotation allows the introduction of herbicides having different MOA's.

Research objectives

The main objectives of this thesis are:

1- Deepen into the corn poppy molecular bases of ALS inhibitors resistance.

2- Unravelling and characterize the resistance mechanisms involving 2,4-D in corn poppy.

3- Understand which causes may be involve in the lack of control with bromoxynil detected in some fields.

4- Design new options for an integrated management of multiple resistant corn poppy populations.

Each of these objectives is presented as a chapter in this thesis taking the shape of a scientific paper (with the corresponding sections: abstract, introduction, material and methods, results, discussion, conclusions and references), which allows readers to understand each one independently of the others. Finally and to address the main goal of the thesis, results from the different chapters are jointly discussed, leading to the main conclusions.

Methodology and outline of the thesis

To achieve Objective 1, different corn poppy populations were picked up in fields where problems with florasulam had been reported. Dose-response experiments were conducted with ALS inhibitors herbicides belonging to three different families: sulfonylureas, imidazolinones and triazolopyrimidines. From there, ALS gen was sequenced and comparisons of the genotype with the phenotype were performed in order to determine the cross resistant patterns among ALS inhibitors families. Finally, penetration and translocation of [^{14}C]-tribenuron-methyl in S and R plants were also examined. Results of this work are reported in Chapter 2.

To answer Objective 2, both greenhouse and lab experiments were conducted. First steps were to characterize the 2,4-D response of some resistant (R) and susceptible (S) populations through dose-response experiments. In order to explore some non-target-site resistant mechanisms, penetration and translocation of 2,4-D among S and R plants was studied with [^{14}C]-2,4-D. In addition, ethylene production in R and S populations was analyzed after spraying 2,4-D. Finally in those 2,4-D resistant plants, cross resistant patterns among other synthetic auxins were also checked. Results of all these studies are collected in Chapter 3.

To meet Objective 3, corn poppy seeds from fields where the post-emergence mixture of ioxynil + bromoxynil + MCPP p did not achieve a good control of this species were collected. Dose-response experiments at different phenological stages were done in order to establish if lack of control was due to an incipient resistant process or to a unsuitable phenological stage when this product was sprayed. Results and recommendations are exposed in Chapter 4.

To evaluate the effect of different integrated weed management strategies (Objective 4), experiments in two commercial fields with multiple resistant corn poppy populations (to 2,4-D and tribenuron-methyl) were conducted during three years. Different chemical and cultural tools (different crop rotations, delayed sowing, different herbicide programmes) were assessed. Results and recommendations are exposed in Chapter 5.

In Chapter 6 results for the preceding chapters are jointly discussed and general conclusions are exposed.

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CHAPTER 2

Resistance mechanisms to ALS inhibiting herbicides in Spanish *Papaver rhoeas* populations: molecular bases and cross resistance patterns

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Abstract

In the present study target-site and non-target-site resistance mechanisms to ALS inhibitors have been investigated in multiple resistant (tribenuron-methyl and 2,4-D) and only 2,4-D resistant, corn poppy populations. Six amino-acid replacements at the Pro197 position (Ala197, Arg197, His197, Leu197, Thr197 and Ser197) have been found in three multiple resistant populations. These replacements were responsible for the high tribenuron-methyl resistance response, and some of them, especially Thr197 and Ser197, elucidated the cross-resistant pattern for imazamox and florasulam, respectively. Mutations outside of the conserved regions of the ALS gene (Gly427 and Leu648) were identified, but their implication(s) in resistance remains uncertain. Moreover, non-mutated plants were found to survive imazamox applications. Lack of [^{14}C]-tribenuron-methyl translocation in the sensitive population, compared to the resistant populations, was attributed to the process of phytotoxicity. Mobility of labelled tribenuron-methyl in only 2,4-D resistant plants was, however, similar to plants with multiple resistance. Lack of correlation between phenotype and genotype in plants treated with florasulam or imazamox revealed signs of the presence of non-target-site resistance mechanisms to non-sulfonylureas. On this basis, selection pressure with ALS inhibitors bears the risk of promoting the evolution of non-target-site resistance mechanisms in corn poppy.

Keywords: Target-site resistance, non-target-site resistance, mutation, amino-acid residue, synthetic auxins and translocation pattern.

Introduction

Acetohydroxy acid synthase (AHAS, EC 4.1.3.18), also referred to as acetolactate synthase (ALS, EC2.2.1.6), is the first enzyme involved in the biosynthesis of branched chain amino-acids valine, leucine and isoleucine (Duggleby et al., 2008; Singh et al., 1991). This enzyme is the target site of five herbicide chemical groups: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl-thiobenzoates (PTB) and sulfonyl-aminocarbonyl-triazolinones (SCT). These herbicides, commonly referred to as ALS inhibiting herbicides, are highly effective at a low rate and environmentally safe (Duggleby et al., 2008). Only five years after the introduction of the first SU, resistant biotypes of *Lactuca serriola* L. (Mallory-Smith et al., 1990) and *Kochia scoparia* L. (Primiani et al., 1990) were reported. To date, 155 species in locations all over the world (94 dicots and 61 monocots) have evolved resistance to ALS inhibitors (Heap 2015).

The SU and IMI herbicides are not competitive inhibitors of ALS because they do not directly bind to the substrate's active site. Instead, these herbicides bind within the substrate-access channel of the ALS enzyme in plants. In this way, both herbicides inhibit ALS by blocking substrate access to the active site. It is well documented that SU are better ALS inhibitors than IMI because SU fit better (more hydrogen bonds are involved) and deeper into the channel (closer to the active site) (Duggleby et al., 2008). In most cases, resistance to ALS inhibitors is caused by mutation of the ALS gene, which results in the change of a single amino-acid residue in the herbicide-binding site (Target-site resistance, TSR) (Tranel and Wright 2002). Thus far, 28 amino-acid substitutions endowing ALS inhibitors resistance have been reported, mainly at the Pro197 site (Ala, Arg, Asn, Gln, His, Ile, Leu, Lys, Met, Ser, Thr, Trp and Tyr), and also at Ala122 (Thr, Tyr and Val), Ala205 (Val), Asp376 (Glu), Trp574 (Arg, Leu, Gly and Met), Ser653 (Asn, Ile and Thr) and Gly654 (Glu and Asp) in resistant weed species (Beckie and Tardif 2012; Heap 2015). There is a wide variation in the resistant response among species with a given substitution (Beckie and Tardif 2012), as ALS inhibitors cross-resistance is also dependent on specific mutations, ALS inhibitor chemical groups, specific herbicides within a given group, and sometimes even weed species (Yu and Powles 2014). Generally, a high level of resistance is conferred by Pro197 substitutions to SU and by Trp574 substitutions to all classes of ALS inhibitors. A second mechanism of resistance to ALS inhibitors is to reduce the amount of

herbicide reaching ALS to be below the lethal level (Non-target-site resistance, NTSR). Reduced absorption and translocation rarely underlay resistance to ALS inhibitors (Cruz-Hipolito et al., 2009; 2013; Poston and Wilson 2001; Veldhuis et al., 2000), and in only a few cases they have been reported as a partial resistance mechanism (Riar et al., 2013; White et al., 2002). On the other hand, an enhanced herbicide metabolism rate has been well documented in *Lolium rigidum* L., (Christopher et al., 1991) *Sinapis arvensis* L. (Veldhuis et al., 2000) and *Echinochloa phyllopogon* L. (Yasuor et al., 2009).

An amalgam of different factors has been proposed to contribute to the number of ALS inhibitor-resistant cases. Additionally, the repeated use of these herbicides is an most important aspect (Tranel and Wright 2002), though genetic, molecular and physiological biology of this resistance must be considered. High mutation rates in ALS genes of some species account for the relatively high frequency of resistant alleles to ALS inhibitors in natural populations (Harms et al., 1991; Preston and Powles 2002). Moreover, resistant ALS alleles are dominant over susceptible alleles and because ALS is a nuclear gene, resistant alleles are disseminated by both pollen and seed (Tranel and Wright 2002). Studied resistant species have not shown any fitness cost associated to the most common mutations of the ALS gene (Pro197 and Trp574) (Légère et al., 2013; Li et al., 2013; Yu et al., 2010). For this reason, it has been considered that these resistant characteristics will persist in the populations and not decline with time (Yu and Powles 2014).

Papaver rhoeas L. (corn poppy) is the most common dicotyledonous weed in winter cereals in southern Europe (Torra et al., 2011), it is an annual, diploid species that is insect-pollinated and self-incompatible (Délye et al., 2011). In recent years, corn poppy with multiple resistance to 2,4-D and tribenuron-methyl has been reported in Spain (Claude et al., 1998) and Italy, and independent resistance to ALS inhibitors has evolved in a number of other countries across Europe (Belgium, Denmark, France, Germany, Greece, Poland, Sweden and United Kingdom) (Heap 2015). In Spain, the resistance to tribenuron-methyl is conferred by Pro197 to Ser substitutions (Duran-Prado et al., 2004). In addition, irregular responses to other ALS inhibitors (mainly IMI and TP) have been reported in post-emergence field applications. Recently, the presence of NTSR mechanisms in Italian corn poppy has been suggested because plants resistant to imazamox, but not carrying mutant ALS alleles, were identified (Scarabel et al.,

2015). These resistance mechanisms and how they affect the different ALS inhibitor chemistries still needs to be uncovered.

The objectives of this study were to (1) to characterise, the cross resistance patterns of four Spanish corn poppy populations primarily to ALS inhibitors with dose-response experiments, and secondarily to 2,4-D; (2) to sequence the ALS gene from these corn poppy populations in order to identify potential mutations; (3) to compare the genotype with the phenotype of individual plants in order to establish a relationship between the molecular results and the ALS inhibitors response; and (4) to determine if absorption or translocation are NTSR mechanisms contributing to the resistance response of these corn poppy populations.

Materials and Methods

Plant material

Before winter cereal harvest, mature corn poppy capsules were collected from four fields in North-Eastern Spain where corn poppy control with ALS inhibitors and/or 2,4-D had been reported as a failure. In addition, seeds from two susceptible (S) populations were obtained; one was provided by a seed dealer (Herbiseed, Twyford, UK) and the other was collected from the same region where suspicious resistant populations were collected. Further details regarding these populations are summarized in Table 2.1. Corn poppy seeds previously sterilized in a 30% hypochlorite solution were sown in Petri dishes with 1.4% agar supplemented with 0.2% KNO_3 and 0.02% gibberellin GA_3. Seeds were placed in a growth chamber at 20/10 °C day/night, a 16 h photoperiod under 350 µmol photosynthetic photon-flux density m^{-2} s^{-1}. After 14 days, seedlings were transplanted in pots filled with a silty loam:sand:peat (40:20:40, w/v) potting mix. Pots were placed in a greenhouse (41°37’43.1”N - 0°35’52.6”E) and were watered as needed. All plants produced in this manner were employed in the subsequent experiments.

Dose-response assays

Five seedlings were sown per pot and afterwards thinned to three per pot. At the six leaf stage (a 5-6 cm rosette), plants were sprayed with tribenuron-methyl, florasulam, imazamox and 2,4-D at a range of herbicide rates (rates are detailed in Table 2.2). Four replicates (pots) were applied with each herbicide rate. Herbicides were applied using a precision bench sprayer delivering 200 l ha^{-1}, at a pressure of 215 kPa. Four weeks after

treatment, plants were harvested (above ground). Samples were dried at 65 °C for 48h, and the dry weights were measured. Finally, weight reduction was calculated as a percentage of the untreated control for each population.

DNA extraction, ALS gene sequencing and restriction analysis

At the six leaf stage, a total of fifty-one plants per population were sprayed with tribenuron-methyl, florasulam and imazamox (seventeen plants for each product) at the recommended field rate. Plants from the S-113 population were not included in this experiment, but results from unpublished work did not detect any mutation among thirty plants.

Table 2.1. Location and date of collection of corn poppy (*Papaver rhoeas*) populations used in the experiments.

Code	Location	Sampling location Latitude	Longitude	Year collected	Herbicide management in the field during preceding years.
S-013	--	--	--	2008	Susceptible standard population obtained from Herbiseed (Herbiseed,Twyford, UK).
S-113	Belorado (Burgos)	42º24'57.8"N	3º10'49.3"W	2013	Susceptible standard population collected in a non-treated zone, far from fields.
R-213	Baldomar (Lleida)	41°54'39.0"N	1°00'21.2"E	2013	Florasulam plus 2,4-D in post-emergence.
R-313	Tosantos (Burgos)	42°24'43.7"N	3°14'39.9"W	2013	Aminopiralid plus florasulam, bifenox plus isoproturon and bromoxinil plus ioxinl plus MCPP in post and early post-emergence.
R-114	Sant Antolí (Lleida)	41°37'58.4"N	1°19'44.6"E	2014	Iodosulfuron-methyl plus mesosulfuron-methyl and florasulam plus 2,4-D in post-emergence.
R-703	Almacelles (Lleida)	41°43'39.6"N	0°27'29.5"E	2003	--

Table 2.2. Herbicide used in dose-response experiments.

Herbicide active ingredient	Commercial product	Field rate (g a.i.·ha^{-1})	Manufacture		Dose rate used (g a.i.·ha^{-1})
Tribenuron-methyl	Granstar 50 SX	18.7	DuPont	*R*	1200, 600, 150, 75, 37.5, 18.7, 9.3, 4.6 and 0
				S	18.7, 9.3, 4.6, 2.3, 1.1, 0.5, 0.25 and 0
Florasulam	Nikos	7.5	Dow AgrosciencesIberica	*R*	480, 240, 60, 15, 7.5, 3.7, 1.8, 0.9 and 0
				S	7.5, 3.7, 1.8, 0.9, 0.4, 0.2, 0.1 and 0
Imazamox	Pulsar 40	50	BASF España	*R*	3200, 1600, 400, 100, 50, 25, 12.5, 6,2 and 0
				S	50, 25, 12.5, 6.2, 3.1, 1.5, 0.7 and 0
2,4-D	Esteron 60	600	Dow AgrosciencesIberica	*R*	4800, 1200, 600, 300, 150, 75 and 0
				S	600, 300, 150, 75, 37.5, 18.7, 9.3 and 0

The herbicide was applied as described above. One week before application, a leaf fragment (~100 mg) from each plant was taken and frozen for subsequent molecular analyses. Four weeks after treatment, individual plant responses were evaluated. Dead plants were classified as susceptible (S). Plants re-growing from the centre of the rosette were classified as moderately resistant (r) and plants that were unaffected by herbicide were classified as resistant (R) (Figure 2.1). DNA from the leaf fragment was extracted using the Speed tools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Valle de Tobalina, Madrid, Spain) and the DNA sample concentration was measured in a NANODROP Thermoscientific spectrophotometer (ThermoFisher, Nano-Drop Products, Wilmington, DE). Each DNA sample was diluted to a final concentration of 10 ng/µl, which was immediately used for the polymerase chain reaction (PCR) test or stored at -20ºC until use.

Figure 2.1. Corn poppy (*Papaver rhoeas*) phenotype characterisation four weeks after ALS inhibitor treatments. Resistant plants (R), plants with re-growth from the centre rosette (r), and dead plants (S).

All mutations conferring ALS resistance in corn poppy have been detected in the C, A, D domains of the gene (Pro197) (Durán-Prado et al., 2004; Kaloumenos et al., 2009; Marshall et al., 2010; Scarabel et al., 2004), only one corn poppy plant out of 729 tested was classified as resistant because of a mutation in the B, E domains (Trp574) (Délye et al., 2011). Based on this, C, A, D domains were analysed first for all the samples. B, E domains were checked in 153 samples out of the 255, and analyses on this region are still ongoing. To date no mutations at the B, E domains have been found. Fragments of the ALS gene that included the regions of domains C, A, D were amplified using corn poppy primers described in a previous work (Kaloumenos et al., 2009). The amplification was accomplished following the procedures described in the above mentioned work (Kaloumenos et al., 2009). PCR amplification products were separated

in a 1.5% agarose gel. Gels were then observed under ultraviolet light (320 nm; ALPHA DIGI DOC Pro instrument, Alpha Innotec Corporation, Johannesburg, South Africa) and images recorded with gel photography. Amplified DNA fragments were purified using the Speed tools PCR Clean-Up Kit (Biotools, B&M Labs, Madrid, Spain), then sequenced. Restriction analyses were conducted to define double-peaks detected in the sequence chromatograms. For this analysis, primers and procedures were utilized as described by Kaloumenos et al. (2009). The resulting electrophoresis bands were visualized under UV light after being stained with GelRed (Biptium, California, USA). The digestion profile for each population was compared with its respective, non-digested control profile as well as the S-control digestion profile. Haplotype inference was determined by comparing sequences obtained from the other samples within the same population. In some specific cases where the same genotype at C, A, D domain expressed different responses to the same herbicide, other possible positions of the ALS gene were examined. Methodology for this part was conducted as described in a previous work (Délye et al., 2011).

Tribenuron-methyl absorption and translocation experiment

[^{14}C]-tribenuron-methyl ([^{14}C]-Tri) with a specific activity of 1.422 MBq/mmol (Institute of Isotopes Co. Ltd. Budapest, Hungary) was mixed with commercial formulated tribenuron-methyl in distilled water up to a final concentration of 0.093 g L^{-1} (18.7 g a.i.·ha^{-1} dissolved into 200 L ha^{-1} of distilled water). Four 0.5 µL droplets of this mixture were applied per plant to the adaxial surface of the fourth leaf at the six true leaf stage of development (a 5-6 cm rosette). Every plant received a radioactivity of 166.5 $Bqmmol^{-1}$. Five repetitions (considering every plant as a repetition) from each population were harvested at 12, 24, 48, and 96 h after treatment (HAT). Unabsorbed [^{14}C]-Tri was rinsed from the treated leaves of each plant using 2 ml of an acetone and water (1:1 v/v) solution. The rinse of each replication was mixed with 15 mL scintillation fluid (UltimaGold^{TM}, Perkin-Elmer, Packard Bioscience BV), and analyzed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA scintillation counter; Beckman Instruments, CA, USA). Washed plants were separated into treated leaf, shoots and root, dried at 70ºC for 48 h and parts were combusted in a sample oxidizer (OX 500; R. J. Harvey Instrument, Tappan, NY, USA). The radioactivity of the resulting [^{14}C]-CO_2 was determined by LSS. Foliar absorption (%) was calculated as (1)

$$\text{Foliar absorption (\%)} = \frac{\text{[Radioactivity recovered from plant parts]}}{\text{[Total radioactivity recovered]}} X\ 100 \quad (1)$$

and translocation (%) was calculated as (2). Percentage or recovery was always greater than 80%.

$$\text{Translocation(\%)} = \frac{\text{[Taken-up radioactivity in treated leaf,shoot or root]}}{\text{[Taken-up radioactivity in all tissues]}} X\ 100 \quad (2)$$

Statistical analysis

For the dose-response experiment, statistical analyses were carried out with a nonlinear regression model with the *drc* (Knezevic et al. 2007) package in R (R Development Core Team 2013). The herbicide rate causing 50% of plant growth reduction (GR_{50}) was calculated via four type (3) parameter logistic curves:

$$y = \mathrm{c} + \frac{(d-c)}{1+\mathrm{EXP}[b(\log(x)-\log(GR50)]} \quad (3)$$

Where c = the lower limit, d = the upper limit and b = the slope at the GR_{50}. In this regression equation, the herbicide rate (g a.i.·ha^{-1}) was the independent variable (x) and the dry weight (percentage of the untreated control for each population) was the dependent variable (y). The resistance index (RI) was computed as GR_{50}(R)/GR_{50}(S). Analysis of variance (ANOVA) was conducted with [^{14}C]-Tri percentages. Data were transformed as needed (arcs[√(x+0.5)]) when normal assumptions were not met. Population means from each evaluation time were compared using a post-hoc Tukey´s pairwise test (Hothorn et al. 2008), at P = 0.05. Data were then back transformed for their presentation.

Results

Dose-response experiments

R-213, R-313 and R-114 plants were 286-, 695- and 351-fold more resistant to tribenuron-methyl than susceptible plants (Table 2.3). The R-703 population displayed a very small resistant index (RI) to tribenuron-methyl (2 times more resistant than the S-013 plants). Results for the TP florasulam revealed some degree of cross-resistance to this herbicide. Florasulam GR_{50} was 0.16 g a.i. ha^{-1} in S-013 plants; this parameter was increased 24-fold in R-213 plants (3.90 g a.i. ha^{-1}) and 18-fold in both R-313 and R-114 populations (2.90 and 2.92 g a.i. ha^{-1} respectively). R-703 plants were two times more resistant to florasulam than H-S013 plants (Table 2.3). Cross-resistance to the IMI

imazamox was also observed in these populations. The GR_{50} value for imazamox in the susceptible population (S-013), was 0.61 g a.i. ha^{-1}. This parameter was 30 (18.08 g a.i. ha^{-1}), 40 (24.37 g a.i. ha^{-1}) and 24 (14.73 g a.i.ha^{-1}) times greater in R-213, R-313 and R-114 populations, respectively. R-703 plant results exposed them to be 6 times more resistant (a GR_{50} value of 4.05 g a.i. ha^{-1}) to imazamox than the susceptible biotype (Table 2.3). Dose-response experiments conducted with 2,4-D revealed that all populations were resistant to 2,4-D, their RI's ranging from 12 to 18 (Table 2.3). Minimal differences in the two S population responses were observed for the different tested herbicides.

Table 2.3. Equation parameters of the log-logistic model used to estimate the GR_{50} of tribenuron-methyl, florasulam, imazamox and 2,4-D in S-013, S-012, R-213, R-313, R-114 and R-703 populations of corn poppy (*Papaver rhoeas*).

Biotype	GR_{50} ± SE (g a.i.·ha^{-1})[a]	*b*± SE[b]	Res SS[c]	RI[d]
Tribenuron-metil				
S-013	0.08 ± 0.02	0.49 ± 0.08	5171	--
S-113	0.05 ± 0.04	0.57 ± 0.17	2689	0.6
R-213	25.22 ± 6.38	0.58 ± 0.09	10084	286
R-313	61.27 ± 12.00	0.63 ± 0.07	22189	695
R-114	30.92 ± 8.06	0.61 ± 0.09	10609	351
R-703	0.17 ± 0.04	0.52 ± 0.11	328	2
Florasulam				
S-013	0.16 ± 0.03	0.69 ± 0.12	21738	--
S-113	0.37 ± 0.08	0.88 ± 0.16	8530	2
R-213	3.90 ± 0.38	2.01 ± 0.36	3899	24
R-313	2.90 ± 0.68	0.60 ± 0.11	2311	18
R-114	2.92 ± 0.30	0.87 ± 0.08	1529	18
R-703	0.41 ± 0.08	1.27 ± 0.41	1704	2
Imazamox				
S-013	0.61 ± 0.10	0.76 ± 0.16	8917	--
S-113	0.22 ± 0.08	0.41 ± 0.15	2428	0.5
R-213	18.08 ± 1.00	4.26 ± 1.23	4534	30
R-313	24.37 ± 3.50	1.76 ± 0.43	6544	40
R-114	14.73 ± 1.00	1.15 ± 0.10	966	24
R-703	4.05 ± 0.55	1.50 ± 0.32	1098	6
2,4-D				
S-013	68.60 ± 10.20	1.15 ± 0.16	23693	--
S-113	71.37 ± 24.01	0.81 ± 0.21	10303	1
R-213	816.60 ± 96.00	1.27 ± 0.16	2872	12
R-313	1238.40 ± 436.20	0.80 ± 0.27	18435	18
R-114	925.80 ± 156.01	1.02 ± 0.28	5038	13
R-703	1039.70 ± 402.00	0.74 ± 0.18	8399	15

[a]GR_{50}, herbicide concentration for 50% reduction of corn poppy dry weight.
[b]Slope at the GR_{50}
[c]Res SS, residual sum of square.
[d] RI (resistance index) = GR_{50}(Population) ÷ GR_{50}(S-013).

Table 2.4. Herbicide sensitivity to three ALS inhibitors applied at the field rate and ALS alleles identified in five different corn poppy (*Papaver rhoeas*) populations (three multiple resistant: R-213, R-313 and R-114; one synthetic auxin resistant: R-703; and one susceptible: S-013).

	[a]R-213						R-313						R-114							R-703				S-013			
	[b]Tri	Flo			Ima		Tri	Flo		Ima			Tri	Flo			Ima			Tri	Flo	Ima		Tri	Flo	Ima	
	[c]R	R	r	S	R	r	R	r	S	R	r	S	R	S	R	r	S	R	r	S	S	r	S	S	S	r	S
[d]Pro/Pro									1			1		3			3			17	17	4	12	17	17	1	16
Ala/Pro							1																				
Leu/Leu	2		3		2	1		1																			
Leu/Pro			1		1																						
Ser/Ser	3	3			2	2	2						2		1	1			1								
Ser/Pro	8	1		1			2				2		3			1			1								
Thr/Thr							3	5		1			5			1	1	3				1					
Thr/Pro							2		1	2	1		1			1	2	3	1								
Ser/Thr							4	6	1	5	3		2			3	1	1	2								
Ser/Leu	4	1	7		6	2	1									1											
Thr/His							1																				
Thr/Leu													1				1	3	2								
Thr/Arg									2	1	1																
Leu/Arg						1																					

[a] Corn poppy population.

[b] Herbicide applied, tribenuron-methyl (Tri), florasulam (Flo) and imazamox (Ima).

[c] Herbicide response to ALS inhibitors. R, resistance; r, moderately resistance (re-growth) and S, susceptible. For every product, only reported responses have been represented.

[d]Genotype at codon 197.

Table 2.5. Correlation between observed individual response to imazamox or florasulam and individual nucleotide and amino-acid sequence of corn poppy (*Papaver rhoeas)* acetolactate synthase. Only positions related with ALS resistance (Ala122, Pro197, Ala205, Asp376, Trp574, Ala653 and Gly654) and those positions where mutations were found (Glu427 and Leu648) have been represented. All sequences were compared with the wild type corn poppy ALS gene (GenBank: AJ577316). In sensitive ALS corn poppy, codon 653 encodes an alanine and not a serine residue as in other species.

Code	Ala122	Pro197	Ala205	Asp376	Trp574	Glu427	Leu648	Ala653	Gly654	Response
Wild type ALS	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	--
Imazamox										
S-013[a] (21)[b]	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
S-013 (22)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	S
R-703 (24)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
R-703 (29)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
R-703(30)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	r
R-703 (21)	GCA	CCT	GCA	GAT	TGG	GAA	TTG	GCT	GGT	S
R-114 (26)	GCA	*MYT*	GCA	GAT	TGG	*RAA*	*TYG*	GCT	GGT	R
R-114 (25)	GCA	*MYT*	GCA	GAT	TGG	*RAA*	TTG	GCT	GGT	r
Florasulam										
R-114 (20)	GCA	*ACT*	GCA	GAT	TGG	*AAA*	TTG	GCT	GGT	r
R-114 (17)	GCA	*ACT*	GCA	GAT	TGG	*AAA*	TTG	GCT	GGT	S

[a] Population code.

[b] Code of the sample within the population

* indicate mutated residue nucleotides

ALS sequencing

No substitutions at codon Pro197 were found in S-013 plants. However, six amino-acid replacements were identified at this position (Ala197, Arg197, His197, Leu197, Ser197 and Thr197) in populations R-213, R-313 and R-114. Only one plant out of fifty-one in R-703 population presented a substitution (Thr197). Six different genotypes were identified in R-213 (Leu/Leu; Leu/Pro; Ser/Ser; Ser/Pro; Ser/Leu and Leu/Arg), with 76% of the plants being classified as resistant homozygous (RR), 24% resistant heterozygous (RS), and 0% susceptible homozygous (SS). In R-313, twelve different genotypes were detected (Pro/Pro; Ala/Pro; Leu/Leu; Ser/Ser; Ser/Pro; Thr/Thr; Thr/Pro; Ser/Thr; Ser/Arg; Ser/Leu; Thr/His and Thr/Arg) 76%, 20% and 4% of these plans were RR, RS and SS, respectively. Finally, eight different genotypes were observed in R-114 plants (Pro/Pro; Ser/Ser; Ser/Pro; Thr/Thr; Thr/Pro; Ser/Thr; Ser/Leu and Thr/Leu), 61% of the plants belonging to this population were characterised as RR, 25% as RS and 14% as SS (Table 2.4). Results obtained by the multiple resistant populations (R-213, R-313 and R-114), revealed that all plants carrying at least one mutant ALS allele showed a R response to tribenuron-methyl treatments. Those few plants which were classified as S (3 out of 51) did not show any mutation at position 197. Opposite, the majority of the plants treated with imazamox were classified as R or r and most of the imazamox R plants carried at least one Thr197 or Leu197 allele. The response to florasulam was also different and few plants treated with this herbicide were R, most of them were r or S. The majority of the plants which did not survive florasulam application had a Thr197 substitution (Pro/Pro genotype not included), and all plants with R response to this herbicide carried at least one Ser197 allele (Table 2.4). No mutant plants were found in population S-013 and no survivors were found for either to tribenuron-methyl nor to florasulam, nevertheless only one plant survived the imazamox (r). All plants in population R-703 died when they were sprayed with tribenuron-methyl or florasulam, however, five plants survived to imazamox (r) and only one plant presented a substitution (Thr/Thr) at position 197 (Table 2.4). Some plants identically genotyped at C, A, and D domains displayed different responses to florasulam or imazamox (Table 2.5). Moderate imazamox resistance (r) and susceptible (S) responses from populations S-013 and R-703 did not show any difference at the other studied positions (Table 2.5). Samples 25 and 26 from population R-114 were r and R (respectively) to imazamox and both plants carried a Leu/Thr substitution at

position 197. Additionally, these plants displayed a heterozygous mutation at position 427 (Glu/Lys) and only sample 26 also carried a heterozygous mutation at position 648 (Leu/Ser). Samples 20 and 17 from population R-114 showed a Thr homozygous mutation at position 197, but r (20) and S (17) responses to florasulam were observed. Apart from this, both plants also carried a homozygous mutation at position 427 (Lys/Lys) (Table 2.5).

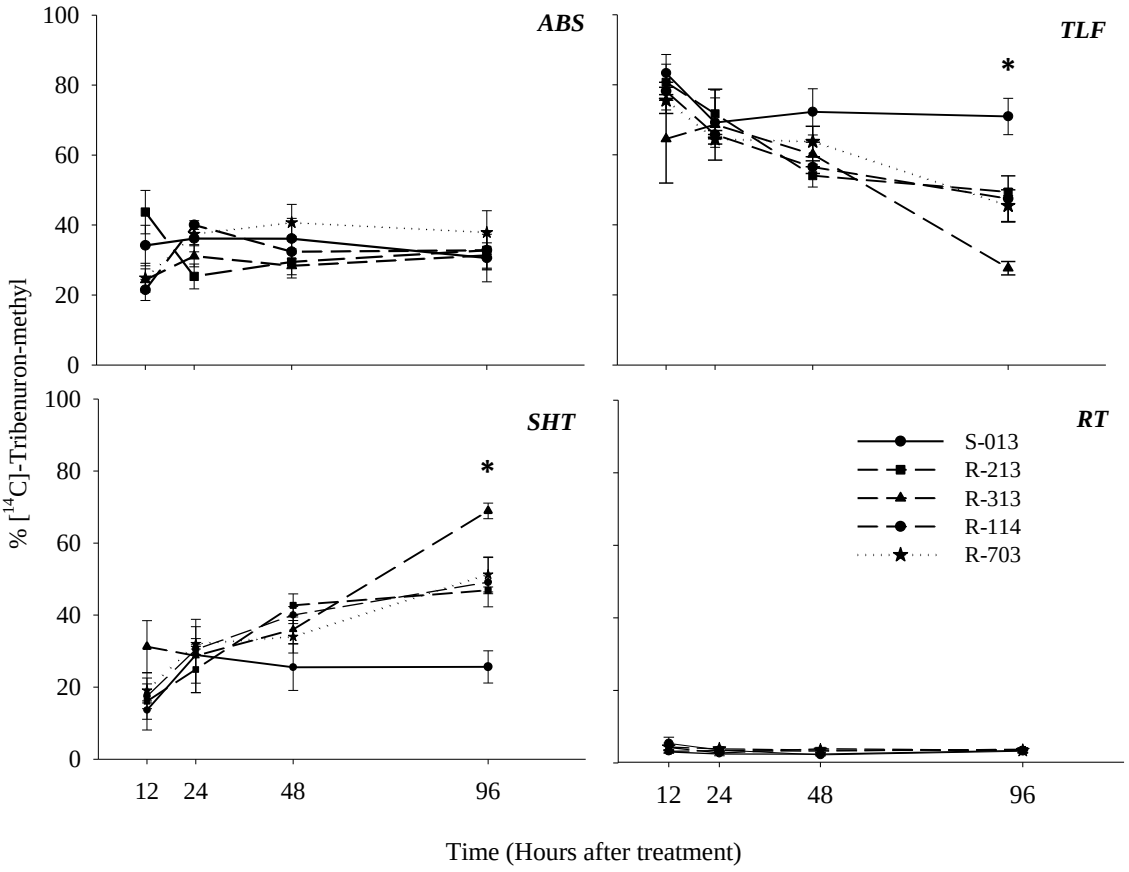

Figure 2.2. [^{14}C]-tribenuron-methyl absorption (ABS, expressed as % recovered radioactivity), remained in the treated leaf (TFL, % penetrated radioactivity), translocation to the shoot (SHT, % penetrated radioactivity) and translocation to the root (RT, % penetrated radioactivity) in corn poppy (*Papaver rhoeas*) populations S-013, R-213, R-313, R-114 and R-703. Bars represent standard error of the means. * indicates significant differences between S-013 and the rest of populations ($p<0.05$) at a given time

[^{14}C]-tribenuron-methyl experiments

There were no differences in [^{14}C]-Tri absorption patterns between corn poppy populations (Figure 2.2). In addition, there were no significant time-related differences

in terms of [^{14}C]-Tri absorption among all the tested populations, with percentages ranging from 24.3% (at 12 HAT) to 37.8 % (at 96 HAT) of the recovered radioactivity (Figure 2.2). Different behaviours in the translocation of the [^{14}C]-Tri were detected between populations. These differences started 48 HAT, with a maximum at 96 HAT, being statistically significant (Figure 2.2). While radioactivity in susceptible plants remained asymptotic, radioactivity evaluated in the treated leaf of R-213, R-313, R-114 and R-703 decreased. Therefore, at 96 HAT the percentage of [^{14}C]-Tri found in the treated leaves of the S plants was 70.9%, which was statistically different from the rates obtained for the rest of populations. These data were consistent with those observed in the shoot, where significant differences were only detected at 96 HAT. R-313 plants translocated almost 3-fold more [^{14}C]-Tri to the shoots (68.9% of the penetrated radioactivity) than S-013 plants (25.6%). Radioactivity detected in R-213, R-114 and R-703 shoots at the same evaluation time was 46.8, 49.1 and 51.2%, respectively (Figure 2.2). No differences between populations in terms of herbicide translocation to roots were detected at any evaluation time, thus radioactivity evaluated in this part was negligible (Figure 2.2). Percentages of recovered radioactivity ranged from 80 to 88% in the H-S013 population, from 85 to 99%, from 80 to 85%, from 77 to 97% and from 80 to 86% in R-213, R-313, R-114 and R-703 populations, respectively (data not show).

Discussion

Multiple resistance to tribenuron-methyl and 2,4-D was detected in R-213, R-313 and R-114 corn poppy populations. GR_{50} values for these products were consistent with those reported in Greek ALS resistant and multiple resistant corn poppy populations (Kati et al., 2014). As observed in previous studies (Kaloumenos et al., 2011), the degree of resistance varied among ALS inhibitors, resistant factors being much lower for florasulam and imazamox than for tribenuron-methyl.

In our study, six amino-acid replacements at the Pro197 position have been found (Ala197, Arg197, His197, Leu197, Thr197 and Ser197); the first five replacements being new for Spanish corn poppy populations, and consistent with previously published European works (Délye et al., 2011; Kaloumenos et al., 2009; Marshall et al., 2010). The strong resistance to tribenuron-methyl showed by any kind of substitution at Pro197 is because Pro197 amino-acid residue is directly involved in anchoring the aromatic ring of SU. Any replacement in this position will affect SU binding, resulting in strong resistance to this herbicide (Duggleby et al., 2008; Shane-Friesen 2007).

Cross-resistance patterns between ALS inhibitors depend on both the codon mutated and the specific amino-acid replaced at the codon (Han et al., 2012). Due to this, different substitutions at Pro197 can give strong, moderate, or no resistance among IMI and TP. In concordance with results in another study (Délye et al., 2011), corn poppy plants carrying the Thr197 substitution were resistant or moderately resistant to imazamox. Although Pro197 is not involved in binding IMI (Duggleby et al., 2003), certain substitutions of these amino-acid residues may result in IMI resistance because the replacement of Pro by a bulky amino-acid obstructs the entry of IMI into the ALS tunnel (Duggleby et al., 2008). Additionally, it has been suggested that Thr197 substitution can confer strong negative interactions with Arg199, Met200 and Asp257 amino-acid residues, which have been proposed to play a relevant role in the binding of some IMI (Shane-Friesen 2007). Regarding TP, our results show that the substitution of Pro197 by Ser lead to plants that were moderately cross-resistant to florasulam, as observed by Délye et al. (2011) To date, the florasulam crystal structure is unknown, and no data is available regarding the behaviour and binding's sites of this molecule in the ALS tunnel. The florasulam response of Ser197 mutated plants is hypothesized to occur in the same terms as the Thr197 substitution for IMI.

The overuse of tribenuron-methyl during the early 80's in Spanish fields probably selected a wide variety of Pro197 substitutions in corn poppy. Consecutive ALS herbicide management practices in each field contributed to the reduction, or not, of ALS genotype diversity, depending on which ALS herbicide family were predominantly used. This case is clearly apparent in R-213 plants continuously treated with florasulam + 2,4-D in recent years, as this population has the highest florasulam resistant index, together with the highest Ser allele frequency reported.

Plants carrying a double mutation at positions Pro197 and Gly427 (by Lys), and a triple mutation at positions Pro197, Gly427 and Leu648 (by Ser), were detected in this study. Results from a previous work conducted with ALS resistant corn poppy from Spain also detected a point mutation located outside the conserved domains: a replacement of Gly281 by Glu (Durán-Prado et al., 2004). It was difficult to attribute any direct implication of these two new mutations in the observed herbicide response, especially when plants carrying these mutations displayed different responses to the same herbicide. Nevertheless, position Leu648 is near other important positions involved in IMI anchoring (Ser653) (Duggleby et al., 2008), and a similar interaction could be occurring as that above described between Pro197 and Arg199, Met200 and Asp257.

In this research, plants with the same genotype at ALS did not always show the same phenotype when they were treated with florasulam or imazamox. Analogous results were reported in ALS inhibitors resistant *Raphanus raphanistrum* L. and *P. rhoeas* (Shane-Friesen 2007; Scarabel et al., 2015). Délye et al. (2011) and Scarabel et al. (2015) indicated that a NTSR mechanism to ALS inhibitors, yet to be determined, could be behind the mismatch between the genotype and phenotype. Moreover, five plants without any mutation were able to survive imazamox application among all populations. In other weeds, NTSR mechanisms (metabolism related) were assumed to be present by identifying sensitive ALS in plants with resistant phenotypes (Scarabel et al., 2015; Yu and Powles 2014; Yu et al., 2009). Neither the present nor previously mentioned studies were able to find evidence of NTSR mechanisms conferring resistance to SU. In terms of the unique Pro197 mutated plant found in the R-703 population, gene flow via pollen or seed from other fields is presumed to explain this result.

As observed in corn poppy, no differences in absorption between resistant and susceptible biotypes were also reported in other studies conducted with ALS inhibitors (Cruz-Hipolito et al., 2009; 2013; Dimeo et al., 2013; Riar et al., 2013). Results with *Sinapis arvensis* L. and [^{14}C]-ethametsulfuron-methyl were similar to the present ones, detecting more translocation in resistant than in susceptible plants (Veldhuis et al., 2000). Hyper-accumulation of carbohydrates in susceptible *Pisum sativum* L. leaves treated with ALS inhibitors has been reported (Zabalza et al., 2004), suggesting that ALS inhibitors affect the transport of assimilates into the phloem (Bestman et al., 1990). On these bases, perhaps this is the explanation for lower [^{14}C]-tribenuron-methyl translocation in susceptible plants. In agreement with previous studies (Cruz-Hipolito et al., 2013), minimum [^{14}C]-tribenuron-methyl root translocation was detected in the corn poppy roots of all populations.

The [^{14}C]-tribenuron-methyl translocation pattern in R-703 plants resulted controversial because it was similar to those observed for ALS resistant populations (R-213 and R-114). What marked R-703 plants different from the other populations was that these plants were only resistant to 2,4-D (only one plant out of 51 presented a mutation in the Pro197 position). Data suggested that tribenuron-methyl phytotoxicity in R-703 plants, which all died at the end, was not evolving as in susceptible plants (S-013), almost during the 96 hours following the herbicide application. Moreover, four plants without any mutation that survived the imazamox application had baffling results for R-703. It must be stressed that this study lacked a population that was both SU resistant and 2,4-

D susceptible so that, it was impossible to disentangle, if possible, NTSR mechanisms from ALS inhibitors are directly related to 2,4-D resistance or vice versa. Divergences above mentioned in the [^{14}C]-tribenuron-methyl translocation pattern between multiple resistant, synthetic auxin resistant and susceptible populations, could suggest that the 2,4-D resistance mechanism interferes with the normal phytotoxic processes triggered by tribenuron-methyl a few hours after its application.

Conclusions

In the present study, three populations were multiple resistant while one population was only resistant to synthetic auxins. Substitutions at Pro197 took charge of the tribenuron-methyl resistance response, although it resulted difficult to extrapolate this conclusion the other tested ALS inhibitors. Non-target-site resistance mechanisms affecting sulfonylurea herbicides, if any, did not become evident under the strong resistance conferred by any amino-acid substitution at Pro197 to this chemical group. Nevertheless, for non-SU ALS inhibitors, the presence of these NTSR mechanisms may become more evident, as plants with the same genotype did not express the same phenotype. This was especially true for the IMI imazamox, where non-mutated plants were able to survive its application. Therefore, selection pressure with ALS inhibitors has the risk to promote the evolution of NTSR mechanisms in corn poppy, such as in grasses. It is unknown if those mechanisms affect other modes of action, which are crucial for the management of herbicide resistance. The results exposed in this work will help in the development of future experiments aimed at disentangling the relationship between the ALS inhibitors and the synthetic auxins resistant response, and to deepen in the NTSR mechanisms to non- sulfonylurea ALS inhibitors.

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CHAPTER 3

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

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Abstract

In southern Europe, the intensive use of 2,4-D (2,4-dichlorophenoxyacetic acid) and tribenuron-methyl in cereal crop systems has resulted in the evolution of resistant (R) corn poppy (*Papaver rhoeas* L.) biotypes. Experiments were conducted to elucidate (1) the resistance response to these two herbicides, (2) the cross-resistant pattern to other synthetic auxins and (3) the physiological bases of the auxin resistance in two R (R-213 and R-703) populations. R plants were resistant to both 2,4-D and tribenuron-methyl (R-213) or just to 2,4-D (R-703) and both R populations were also resistant to dicamba and aminopyralid. Results from absorption and translocation experiment revealed that R plants translocated less [^{14}C]-2,4-D than S plants at all evaluation times. There was between four and eight-fold greater ethylene production in S plants treated with 2,4-D, than in R plants. Overall, these results suggest that 2,4-D does not promote the signaling pathway in the R plants because does not activate the nuclear receptor, either due to its alteration or as a consequence of reduced translocation.

Keywords: Auxinic herbicide, cross resistance, ethylene production, herbicide resistance, radioactivity, translocation.

Introduction

Agricultural weeds cause major crop losses by competing for nutrients, water or light. Even though a lot of non-chemical methods have been used for controlling weeds, herbicides are considered the most effective (Deng et al., 2015). 2,4-D (2,4-dichlorophenoxyacetic acid), an auxinic herbicide, was commercially released in 1946 becoming the first successful selective herbicide to specifically target dicotyledonous weeds. 2,4-D still remains as one of the most commonly used herbicides in the world as a consequence of its low cost, selectivity, efficacy and wide spectrum of weed control (Mithila et al., 2011). The auxinic herbicide family (group O according to the Herbicide Resistance Action Committee, HRAC; and group 4 according to the Weed Science Society of America, WSSA) contains four chemical groups, including pyridine-carboxylic acids (i.e. aminopyralid), quinolinecarboxylic acids (i.e. quinclorac), benzoic acids (i.e. dicamba), and phenoxy-carboxylic acids (i.e. 2,4-D).

After 60 years of widespread and repeated usage, few examples of resistance to this herbicide's mode of action have been reported. Generally, the selection of synthetic auxin resistant biotypes requires more generations than for other modes of action herbicides, particularly acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACCase) inhibitors (Riar et al., 2011). Several reasons have been proposed to explain this phenomenon, including low mutation rates, fitness penalties and redundancy in auxin receptors within the plant (Mithila et al., 2011; Preston and Malone 2014). Nowadays, there are 32 species resistant to auxinic herbicides, 15 of those being resistant to 2,4-D (Heap 2015). The precise mode of action for these herbicides, and consequently, the resistance mechanisms in weeds are, however, still poorly understood (Mithila et al., 2011; Song 2014). Nonetheless, new discoveries and frontiers involving nuclear auxin receptors (F-box proteins), influx and efflux carriers and plasma membrane bound receptors have provided basic clues as to the molecular mode of action of these herbicides (Guilfoyle and Janvier 2007; Krecek et al., 2009; Song 2014; Tan et al., 2007; Tromas et al., 2010).

The characterization of resistance mechanisms has been investigated in few auxinic herbicide-resistant weeds. Differential absorption, translocation, or metabolism were not the bases for resistance in the majority of the assessed species (Cranston et al., 2001; Van Eerd et al., 2005; Kern et al., 2005; Peniuk et al., 1993; Valenzuela-Valenzuela et al., 2001). Only in a few weeds these non-target-site mechanisms (NTSM) have been related with the resistance response (Jugulam et al., 2013; Riar et al., 2011; Weinberg et

al., 2006). Additionally, it has been reported that the application of auxinic herbicides stimulates ethylene biosynthesis in sensitive, but not in resistant plants (Abdallah et al., 2006; Van Eerd et al., 2005; Valenzuela-Valenzuela et al., 2001). This unregulated auxin response and the resulting hyperaccumulation of ethylene, abscisic acid (ABA) and reactive oxygen species (ROS) in auxinic herbicide sensitive plants may be involved in the induction of tissue damage and cell death after synthetic auxins application (Romero-Puertas et al., 2004).

Corn poppy (*Papaver rhoeas* L.) is a major weed of cereal crops in Southern Europe (Délye et al., 2011). Its extended germination period, high seed production, and seed bank persistence makes it especially difficult to manage. It has been estimated that corn poppy can decrease wheat yields up to 32% (Torra et al., 2011). Moreover, the increase in both monoculture farming and overuse of 2,4-D since the 60s, followed by tribenuron-methyl application in the early 80s, have selected ALS and/or 2,4-D herbicide-resistant biotypes. The International Survey of Herbicide Resistant Weeds records ALS inhibitors herbicide-resistant biotypes of corn poppy in ten different European countries. Furthermore, 2,4-D resistant biotypes have been detected in Italy (Heap 2015). While it is well known that resistance to ALS inhibitors in corn poppy is caused by a single point mutation in the ALS gene (target-site mechanisms, TSM) (Délye et al., 2011; Durán-Prado et al., 2004; Kaloumenos et al., 2009; Marshall et al., 2010), no studies have attempted to understand the resistance mechanisms to synthetic auxins in this specie. A better understanding of the 2,4-D resistant mechanisms in corn poppy may also improve resistance management by better defining herbicide use patterns to delay or avoid resistance to this herbicide's mode of action (Preston and Malone 2014).

This study was thus conducted in order (1) to determine the herbicide rate causing 50% mortality (GR_{50}) and the resistance index (RI) of a resistant (R) and a susceptible (S) population to 2,4-D and tribenuron-methyl, (2) to characterize the cross-resistance response of R and S plants to other synthetic auxins chemical groups used in cereals systems, (3) to compare the physical and physiological features by means of contact angle and absorption and translocation of [^{14}C]-2,4-Dtyhj between R and S plants and (4) to examine the ability of 2,4-D to induce ethylene biosynthesis in R and S corn poppy plants.

Material and Methods

Plant material

Before winter cereal harvest, corn poppy mature capsules form at least twenty different plants were collected in two fields where failure of corn poppy control with ALS inhibitors and/or 2,4-D had been reported. R-213 population, suspected to be multiple resistant, was collected from a field located in Baldomar, north of Spain (41º54'39.0"N and 1º00'21.2"W) in 2013. R-703 population, with suspected resistance to 2,4-D, was collected from a field located in Almacelles (41°43'39.6"N and 0°27'29.5"E) in 2003. Two susceptible populations (S-013 and S-012) were included in this study. S-013 was obtained from a seed dealer (Herbiseed, Twyford, UK) in 2008, and S-012 was collected in 2012 from a cereal field in Almenar (41º47'30.5"N and 0°27'29.5"E) where no resistance problems had been reported. Corn poppy seeds were sterilized in a 30% hypochlorite solution. Sterilized seeds were sown in Petri dishes with 1.4% agar supplemented with 0.2% KNO_3 and 0.02% gibberellin GA_3. Seeds were placed in a growth chamber at 20/10 °C day/night, 16 h photoperiod under 350 µmol photosynthetic photon-flux density m^{-2} s^{-1}. After 14 days, seedlings were transplanted in 7 x 7 x 7 cm plastic pots filled with the following soil mixture: silty loam soil 40% (w/v), sand 30% (w/v), peat 30% (w/v). Pots were placed in a greenhouse in Lleida, north-eastern Spain (41° 37'N, 0° 38'W) and were watered regularly to field capacity.

Dose-response experiments

Five seedlings were sown per pot and after establishing, were thinned to three per pot. At the six leaf stage (5-6 cm), all populations were tested with tribenuron-methyl and 2,4-D. Tribenuron-methyl (Granstar 50 SX, DuPont, 50%) was applied at 0, 4.6, 9.3, 18.7 (field dose), 37.5, 75, 150, 600 and 1200 g a.i.·ha^{-1} to R plants and at 0, 0.25, 0.5, 1.1, 2.3, 4.6, 9.3, and 18.7 g a.i.·ha^{-1} to S plants. 2,4-D (Esteron 60, Dow AgroSciences, 60%) was applied at 0, 75, 150, 300, 600 (field dose), 1200 and 4800 g a.i.·ha^{-1} to R populations and at 0, 9.3, 18.75, 37.5, 75, 150, 300 and 600 g a.i.·ha^{-1} to S plants. Non-treated plants were used as controls. A total of four replicates (three plants per pot) were included at each dose. Herbicides were applied using a precision bench sprayer delivering 200 L·ha^{-1}, at a pressure of 215 kPa. Four weeks after treatment, plants were harvested (above ground) and the dry weight (65 °C for 48 h) was measured.

Cross-resistance patterns of synthetic auxins

Both R populations (R-703 and F-R213) and S-013 plants were sprayed with dicamba (Benzoic acid) and aminopyralid (Pyridine-carboxylic acid) in order to study the effects of other synthetic auxins. Dicamba (Banvel D, Syngenta, 48%) and aminopyralid (Dow AgroSciences, 3.9%) were sprayed at their field rates (144 and 9.9 g a.i.·ha^{-1}, respectively) as well as two times their field rates. Five replicates (three plants per pot) and five control pots (non-treated plants) were included at each dose. Applications and evaluations were done as described above.

[^{14}C]-2,4-D uptake and translocation experiments

Ring labeled [^{14}C]-2,4-D with specific activity of 1576 MBq·mmol^{-1} was provided by Dow AgroSciences (Dow AgroSciences, Indianapolis, USA). Seedlings from S-013 and both R populations at six true leaves of development (5-6 cm), were treated with four droplets of 0.5 µL (2 µL per plant) of radio labeled herbicide solution containing [^{14}C]-2,4-D and commercial 2,4-D mixed to a final herbicide concentration of 3 g·L^{-1} (equivalent to a 600 g a.i.·ha^{-1} delivered at 200 L·ha^{-1} spraying volume). Every plant received a total activity of 18.4 MBq mmol^{-1}. Five plants from each population were harvested at 12, 24, 48, and 96 h after treatment (HAT). Unabsorbed herbicide was rinsed from the treated leaves using 2 ml of an acetone/water (1:1 v/v) solution. The rinse solution was mixed with 15 mL of scintillation fluid (Ultima Gold^{TM}, Perkin-Elmer, Packard Bioscience BV). Washes were analyzed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA scintillation counter; Beckman Instruments, CA, USA). Plants were separated into three parts; treated leaf, shoot and root, each of which was dried at 70 °C for 48 h and combusted in a sample oxidizer (OX 500; R. J. Harvey Instrument, Tappan, NY, USA). The trapped [^{14}C]-CO_2 was determined by LSS. Foliar absorption (%) was calculated as () and translocation (%) was calculated as () x 100.

$$\text{Foliar absorption (\%)} = \frac{\text{[Radioactivity recovered from plant parts]}}{\text{[Total radioactivity recovered]}} X\ 100 \quad (1)$$

$$\text{Translocation(\%)} = \frac{\text{[Taken-up radioactivity in treated leaf,shoot or root]}}{\text{[Taken-up radioactivity in all tissues]}} X\ 100 \quad (2)$$

To assess translocation of 2,4-D, two treated plants for H-S013, D-R703 and F-R213 populations were removed from pots 48 HAT. Roots were rinsed and whole plants were

dried (65 °C for 48 h) and pressed against a 25 by 12.5–cm phosphor storage film (PerkinElmer Life and Analytical Sciences, Shelton, CT) for 6 h, and scanned using a phosphor imager (Cyclone, Perkin-Elmer, Packard Bioscience BV).

Contact angle and microroughness assays

To assess any effects of leaf surface, 2,4-D was applied as one drop of 0.5 µL in the adaxial surface of the fourth leaf. Immediately after, individual droplets were photographed using a laboratory-built device consisting of a dissection microscope (Leica MZ6; Leica Microsystems Ltd., Heerbrugg, Switzerland) plus a high-definition digital camera with macro objective (Leica Dililux 4.6; Leica Camera AG, D35606 Solms, Germany). Thirty drops for each population (from different plants) were photographed and contact angle of the drops were analyzed using image processing software (Image J 1.31v; US National Institutes of Health, Bethesda, MD, USA). The same procedure was followed for the microroughness determination, where an acetone/water (1:1 v/v) solution was used instead of the herbicide.

Ehylene production

Experiments were conducted to evaluate the amounts of endogenous ethylene produced by R (R-213 and R-703) and S (S-013 and S-012) plants in response to 2,4-D treatment. Two seedlings were sown in a 145 ml pot (BeltaLab, Barcelon, Spain) and once established, were reduced to one per pot. Plants were sprayed, as described above, with commercial 2,4-D at 0, 150, 300 and 600 g a.i.·ha^{-1}. Treatments were replicated six times. Prior to each treatment, the soil mixture was covered with a layer of perlite to avoid deposition of the herbicide on the substrate. Immediately following treatments, the pots were closed with a specific hermetic top and the two holes beneath the pot were sealed with vaseline and Parafilm. Ethylene was measured by withdrawing a 1 ml gas sample from the head-space with a syringe and injecting it into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) equipped with an alumina column F1 80/100 (2m x 1/8 x 2.1, Teknokroma, Barcelona, Spain) and a flame ionization detector (FID) (Giné Bordonaba et al., 2014). This experiment was repeated twice; in October 2014 and again in February 2015 (the later only with S-012 as a S population).

Statistical analysis

Data from dose-response experiments were analyzed using a non-linear regression model (1). The herbicide rate required for 50% growth reduction of plants (GR_{50}) was calculated with the use of a four parameter logistic curve of the type:

$$y = \mathrm{c} + \frac{(d-c)}{1+\mathrm{EXP}[b(\log(x)-\log(GR50)]} \quad (3)$$

where c = the lower limit, d = the upper limit and b = the slope at the GR_{50}. In this regression equation, the herbicide rate (g a.i.·ha^{-1}) was the independent variable (x) and the plants' dry weight expressed as percentage of the untreated control was the dependent variable (y). The resistance index (RI) was computed as $GR_{50}(R)/GR_{50}(S)$. Data from [^{14}C]-2,4-D uptake and translocation experiments were subjected to analysis of variance (ANOVA). The requirement of homogeneity of variance was checked by visual inspection of the residual plots and residuals were analyzed using Shapiro–Wilk Test. Where variances were not homogeneous, Generalized Linear Models (GLM's) were used. The binomial distribution (Logit-link) was used in all GLM, because this distribution resulted in normally distributed residues. Populations' means were compared using a post-hoc Tukey´s pairwise procedure at P = 0.05. Data from the cross resistant experiment (efficacy) and ethylene production assay ($\mu L C_2H_4 \cdot g^{-1} \cdot h^{-1}$) were subjected to analysis of variance (ANOVA) and means were separated using Tukey´s pairwise comparison at 0.05 probability level. Repetitions from the ethylene experiment (October and February) were not pooled due to statistical differences found between experiments. All statistical analyses were carried out with the use of the R programming language (R Development Core Team 2013). *drc* packag (Knezevic et al., 2007) for the non-linear regression and *multcom* (Hothorn et al., 2008) for the post hoc Tukey´s test were employed

Results

Both R and S plants showed morphological damage after 2,4-D application. Plant growth was reduced, and leaves were curled. R plant produced new growth within a few days of herbicide application. S and R plants treated with 600 g a.i.·ha^{-1} and 4800 g a.i.·ha^{-1} of 2,4-D, respectively, died 14 days after application. Regarding to GR_{50} comparisons, no differences were observed between S populations treated with 2,4-D (66.3 vs 68.6 g of a.i.·ha^{-1}). R-213 and R-703 plants were 12-fold (816.6 vs 68.6 g of

a.i.·ha^{-1}) and 15-fold (1039.7 vs 68.6 g of a.i.·ha^{-1}) more resistant to 2,4-D than S-013 plants, respectively. There was very little control of R-213 plants with tribenuron-methyl at 600 g a.i.·ha^{-1} (thirty-two times the field rate), and GR_{50} was 25.2 g a.i.·ha^{-1}, 286-fold more resistant than S-013 plants. Tribenuron-methyl at 18.7 g a.i.·ha^{-1} (field rate) controlled the population R-703 (Figure 3.1), and it showed a very low RI (Table 3.1). Differences between S populations in the response to tribenuron-methyl were minimal (Figure 3.1).

Table 3.1. Estimated GR_{50} and resistance index (RI) values to tribenuron-methyl and 2,4-D for S-013, S-012, R-703 and R-213 corn poppy (*Papaver rhoeas*) populations.

Herbicide	Field dose	Population	GR_{50} (g a.i.·ha^{-1}) ± SE	RI
Tribenuron-methyl	18.75 g a.i.·ha^{-1}	S-013	0.08 ± 0.02	--
		S-012	0.10 ± 0.02	1.1
		R-703	0.17 ± 0.04	2
		R-213	25.22 ± 6.4	286
2,4-D	600 g a.i.·ha^{-1}	S-013	68.60 ± 10.2	--
		S-012	66.36 ± 20.4	0.9
		R-703	1039.70 ± 402.0	15
		R-213	816.60 ± 96.0	12

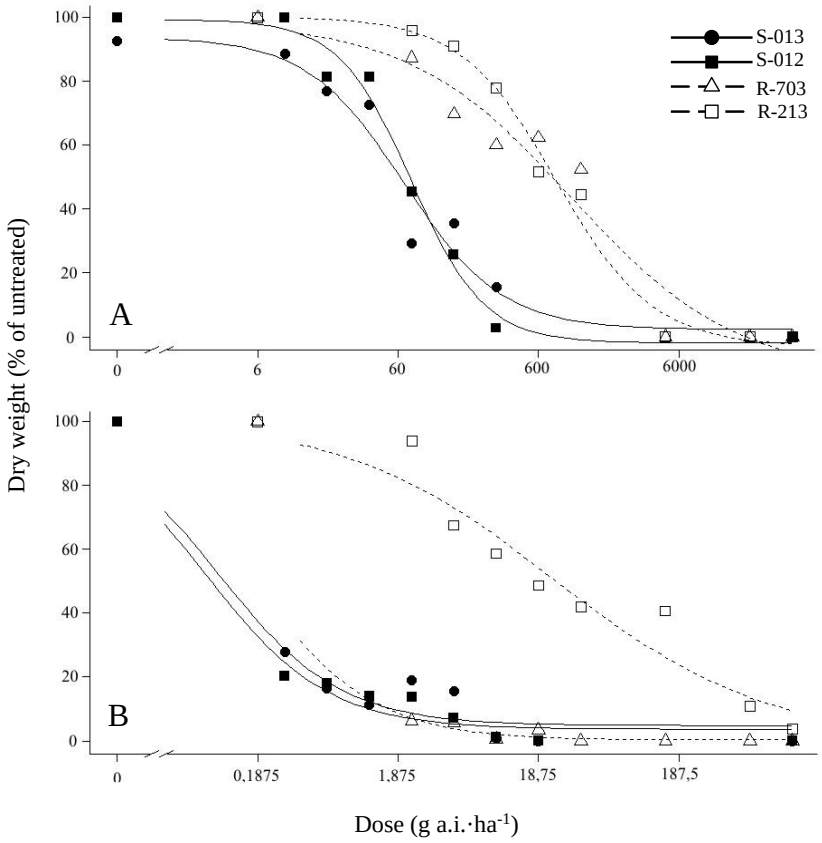

Figure 3.1. Dose-response regression curves of susceptible (S-013 and S-012), and resistant (R-703 and R-213) corn poppy (*Papaver rhoeas*) populations to 2,4-D (A) and tribenuron-methyl

(B) (log scale). Data were expressed as percentage of the mean dry weight of untreated control plants.

The R-703 and R-213 populations were also resistant to dicamba and aminopyralid at field rate (144 and 9.9 g a.i.·ha^{-1}, respectively; Figure 3.2). The effectiveness of auxinic herbicides on the R population increased when they were applied at two times the field rate, but other than dicamba on R-213, they failed to control the populations (Figure 3.2).

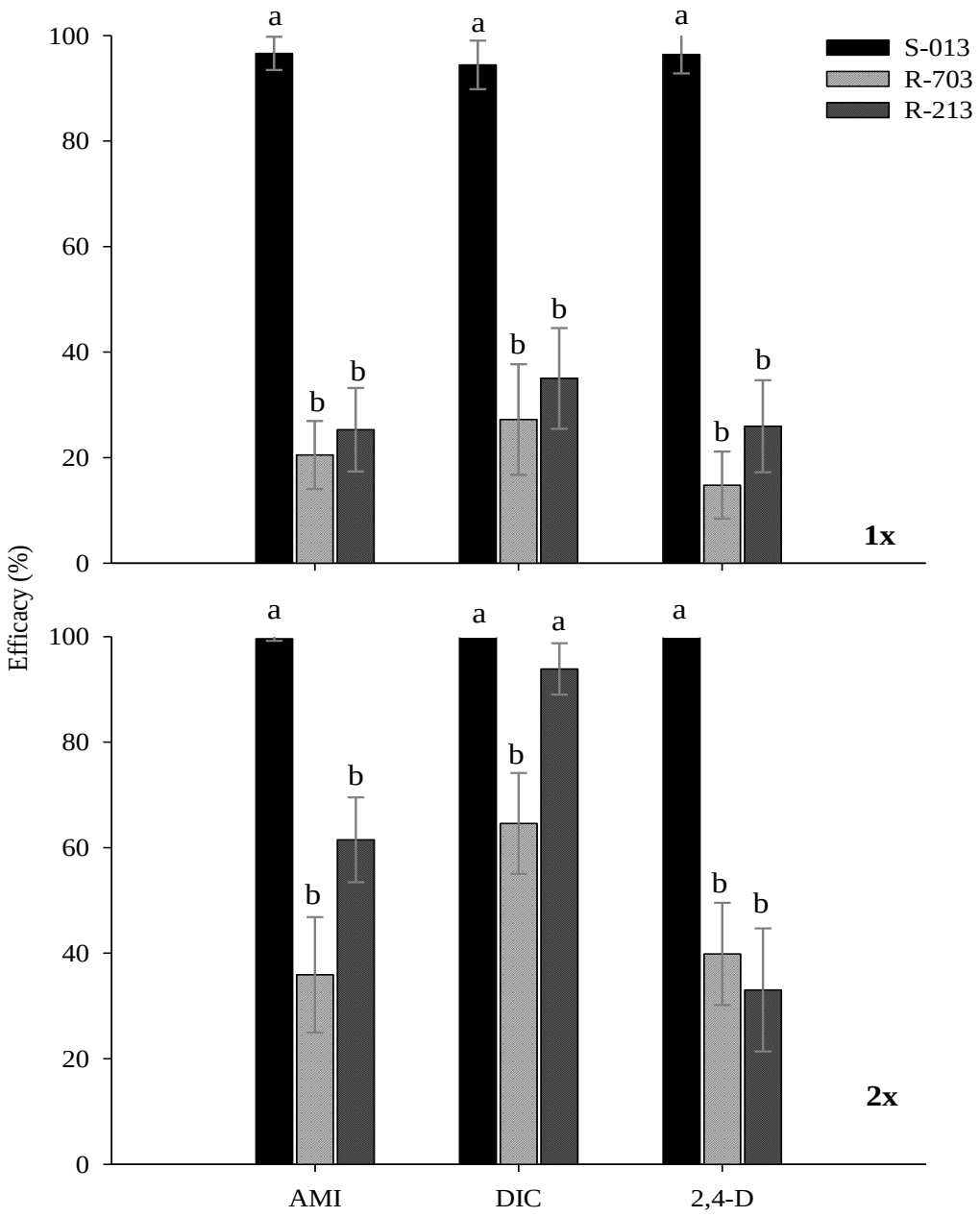

Figure 3.2. Efficacy of aminopyralid (AMI), dicamba (DIC) and 2,4-D at the field rate: 9.9, 144 and 600 g a.i.·ha^{-1} (1x) and two-fold the field rate: 19.8, 288 and 1200 g a.i.·ha^{-1} (2x) on S-013 (black), R-703 (dark grey) and R-213 (grey) corn poppy (*Papaver rhoeas*) populations. Vertical

bars represent the standard error. Columns with different letters indicate significant differences (P<0.05) for each product and dose.

There were no significant differences between R (R-703 and R-213) and S (S-013) plants in the quantity of [^{14}C]-2,4-D absorbed, with between 65 to 70% of the herbicide applied absorbed at 12 HAT. R-213 and R-703 plants translocated much less [^{14}C]-2,4-D than S-013 plants with, significantly less translocation to the shoots and roots compared to the susceptible population (Table 3.2). Percentages of recovered radioactivity ranged from 89 to 96% in S-013 plants and from 85 to 98% in the R plants. Images obtained from the qualitative studies at 48 HAT confirmed the above results (Figure 3.3). Data from the contact angle and microroughness assays did not reveal any kind of differences between R and S plants (data not shown).

Table 3.2. Absorption (percentage of recovered radioactivity) and translocation (percentage of penetrated radioactivity) of [^{14}C]-2,4-D in S-013, R-213 and R-703 populations of corn poppy (*Papaver rhoeas*) at different times. Data are means with standard error and means followed by different letters indicate significant differences in each time and location (Absorption, treated leaf, shoots and roots) (P<0.05).

Population	12 h	24 h	48 h	96 h
Foliar absorption (% recovered radioactivity)				
S-013	70.98 ± 3.3 a	78.06 ± 5.7 a	62.71 ± 5.7 a	65.81 ± 5.5 a
R-703	65.67 ± 5.3 a	69.55 ± 5.5 a	69.26 ± 8.7 a	71.98 ± 5.6 a
R-213	65.83 ± 9.1 a	78.22 ± 8.7 a	70.54 ± 7.5 a	76.98 ± 7.8 a
Remainder in the treated leaf (% penetrated radioactivity)				
S-013	93.79 ± 1.21 a	83.60 ± 3.1 a	78.36 ± 3.8 a	70.04 ± 6.5 a
R-703	97.34 ± 0.71 b	96.45 ± 1.1 b	98.56 ± 0.2 b	96.87 ± 1.2 b
R-213	99.08 ± 0.06 b	96.26 ± 1.5 b	98.29 ± 0.4 b	97.49 ± 0.3 b
Translocation to the shoots (% penetrated radioactivity)				
S-013	4.25 ± 0.97 a	12.77 ± 2.5 a	15.05 ± 3.3 a	22.22 ± 6.1 a
R-703	2.23 ± 0.76 ab	2.27 ± 0.5 b	0.77 ± 0.2 b	2.44 ± 1.1 b
R-213	0.32 ± 0.04 b	2.69 ± 1.3 b	0.55 ± 0.1 b	1.04 ± 0.3 b
Translocation to the roots (% penetrated radioactivity)				
S-013	1.95 ± 0.49 a	3.61 ± 0.6 a	6.57 ± 0.5 a	7.73 ± 1.0 a
R-703	0.41 ± 0.15 b	1.26 ± 0.8 ab	0.65 ± 0.2 b	0.34 ± 0.1 c
R-213	0.58 ± 0.06 b	1.04 ± 0.3 b	1.14 ± 0.3 b	1.46 ± 0.1 b

No differences in ethylene production among populations were detected in untreated (0 g a.i·ha^{-1}) or plants sprayed at 150 g a.i·ha^{-1} of 2,4-D. There were differences between R and S populations starting at 300 g a.i.·ha^{-1} of 2,4-D, with maximum differences at the field rate (600 g a.i.·ha^{-1}), when S plants produced between five and eight times more ethylene than R plants (Figure 4.4). Even though statistical differences in ethylene

production were determined between repeated trials (October and February), similar patterns between R and S populations were confirmed in both experiments (Figure 4.4).

Figure 3.3. Digital image (upper panel) and autoradiographic image (lower panel) depicting [^{14}C]-2,4-D translocation throughout plants tissues of S-013, R-703 and R-213 populations of corn poppy (*Papaver rhoeas*), 48 HAT. Arrows in the upper image indicate the leaf where [^{14}C]-2,4-D droplets were applied.

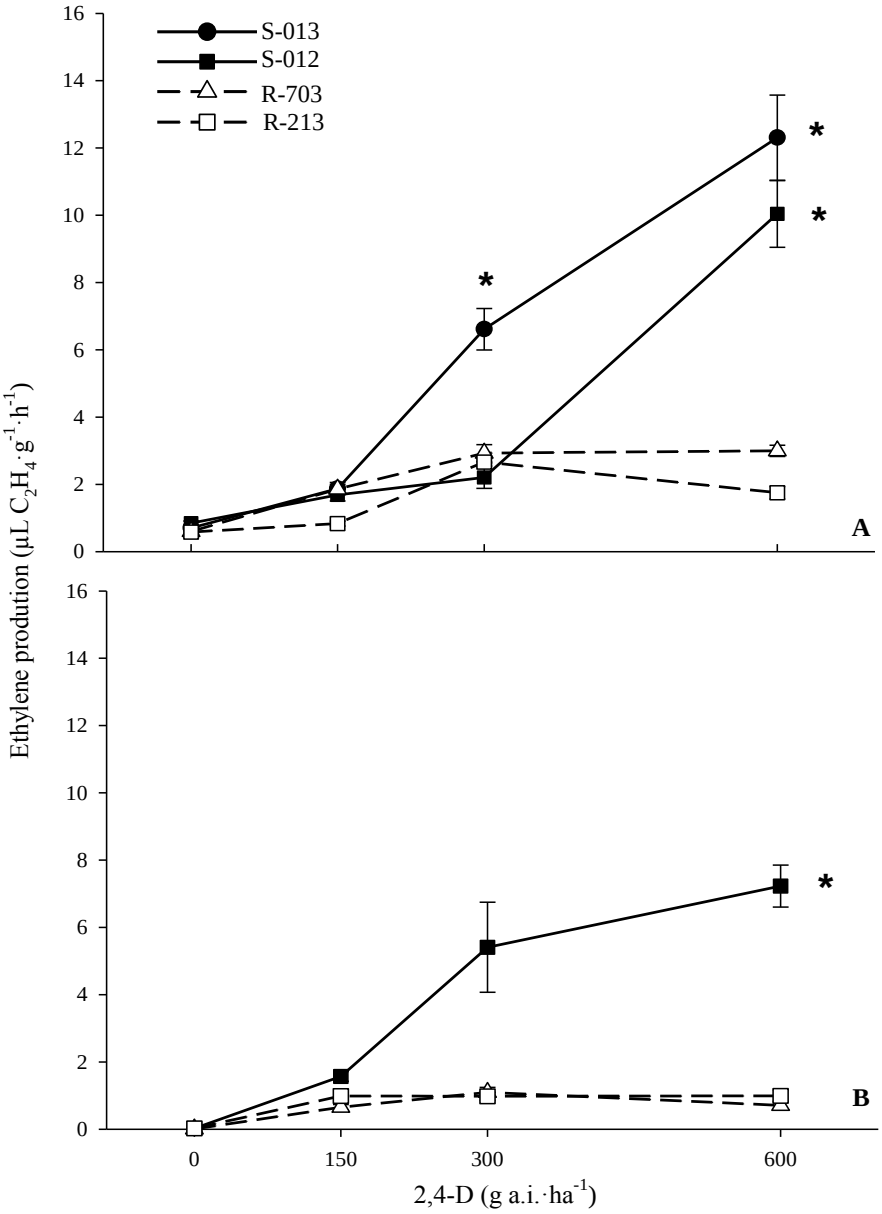

Figure 3.4. Ethylene production (µL $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) in susceptible (S-013 and S-012), and resistant (R-703 and R-213) corn poppy (*Papaver rhoeas*) populations after foliar application of 2,4-D at different concentrations. The experiment was repeated twice, in October 2014 (A) and February 2015 (B). Ethylene was measured 16 h after treatment (HAT). Vertical bars represent the standard error. * indicate significant differences (P<0.05) between R and S plants for each application dose.

Discussion

Resistance to both tribenuron-methyl and 2,4-D in R-213 plants was confirmed in our study. Multiple resistant corn poppy populations have also been previously detected in Italy and Greece (Heap 2015; Kati et al., 2014). Resistance to both auxinic and ALS

inhibitor herbicides have been reported in others dicots weed as: *Gallium spurium* L. (Van Eerd et al., 2005), *Sisymbrium orientale* L. (Preston and Malone 2014), *Kochia scoparia* L. (Kern et al., 2005), *Limnocharis flava* L. and *Raphanus raphanistrum* L. (Heap 2015). Resistant factors obtained to tribenuron-methyl and 2,4-D were similar to those observed in other studies (Kaloumenos et al., 2011; Kati et al., 2014; Evangelia Chatzaki 2014).

The resistant plants were also resistant to dicamba and aminopyralid. Resistance to multiple synthetic auxins was also observed in *Lactuca serriola* L. (Burke et al., 2009), *Sinapis arvensis* L. (Peniuk et al., 1993), and *K. scoparia* (Kern et al., 2005). New discoveries of proteins involved in auxins' mode of action have indicated that specific alterations in nuclear receptors might contribute as a potential resistance mechanisms in auxinic herbicide resistant dicotyledonous weeds (Mithila et al., 2011). Similar to the results presented in this study, cross-resistance between 2,4-D and dicamba was also found in a F-box receptor mutant of *Arabidopsis thaliana* L. (Gleason et al., 2011)

There was no difference in absorption of 2,4-D, however, reduced [^{14}C]-2,4-D translocation was observed in 2,4-D resistant corn poppy populations. Reduced synthetic auxin translocation has previously been reported for resistant populations of *Galeopsis tetrahit* L. (Weinberg et al., 2006) and *L. serriola* (Riar et al., 2011). Alteration to the auxin efflux carriers (PIN-FORMD, PIN; ATP-binding cassette, ABC) could explain the lack of translocation observed in 2,4-D resistant corn poppy plants. Members of the PIN and ABC efflux carrier families have been considered the main mechanism involved in active and long-distance auxin transport (Zazímalová et al., 2010). Recent studies conducted with *A. thaliana* suggested that ABCB4 transporter (ABC family) is the target of 2,4-D (Kubeš et al., 2012). In addition, a mutation in *A. thaliana* in another efflux carrier of ABC family, ABCG9, has been reported to provide increased tolerance to 2,4-D without affecting endogenous auxin Indole-3-acetic-acid (IAA) transport (Ito and Gray 2006).

Results from the ethylene experiments are consistent with previous studies conducted with other species. A three-fold increase in ethylene was induced in quinclorac-sensitive *G. spurium* plants compared with quinclorac-resistant plants (Van Eerd et al., 2005). Sensitive and resistant *K. scoparia* plants demonstrated greater than four-fold difference in ethylene production when they were treated with dicamba and sampled 24 HAT (Howatt et al., 2006). The stimulation of ethylene biosynthesis through the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase has been described as one of

the first phases after 2,4-D and F-box proteins binding (Grossmann 2010). Therefore, our results suggest that in R plants 2,4-D may not be binding this nuclear receptor.

Overall, these results suggest that 2,4-D does not promote the signaling pathway in R plants because its receptor is not activated, either due to its alteration or as a consequence of reduced translocation involving any of the known auxin transporter families. The first step toward uncovering this mechanism could be seeking an alteration in these specific proteins affecting the auxinic nuclear reception or auxin efflux carriers (a specific transporter belonging to PIN or ABC families). In addition, 2,4-D metabolism studies should be considered since this resistance mechanism could be present in the studied populations together with those proposed above as it has been suggested in 2,4-D resistant *R. raphanistrum* (Goggin and Powles 2014). A comprehensive understanding of the resistance mechanisms in corn poppy biotypes, especially in those with multiple resistance to auxinic and ALS inhibitor herbicides, is needed to further understand the risk of resistance evolution to others modes of action. This information will be crucial to assist in the design of integrated weed management strategies.

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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to 32% [21]. Moreover, the increase in both monoculture farming and overuse of 2,4-D (since the 60s) followed by tribenuron-methyl application (early 80s) have selected ALS and/or 2,4-D herbicide-resistant biotypes. The International Survey of Herbicide Resistant Weeds records ALS inhibiting herbicide-resistant biotypes of corn poppy in ten different European countries. Furthermore, 2,4-D resistant biotypes have been detected in Italy [5]. While it is well known that resistance to ALS inhibitors in corn poppy is caused by a single point mutation in the ALS gene (target-site mechanisms, TSM) [20,22–24], no studies have attempted to understand the resistance mechanisms to synthetic auxins in this species. A better understanding of the 2,4-D resistant mechanisms in corn poppy may also improve resistance management by better defining herbicide use patterns to delay or avoid resistance to this mode of action [4].

This study was thus conducted in order to (1) determine the herbicide rate causing 50% mortality (GR_{50}) and the resistance index (RI) of resistant (R) and a susceptible (S) populations to 2,4-D and tribenuron-methyl, (2) characterize the cross-resistance response of R and S plants to other synthetic auxins chemical groups used in cereals systems, (3) compare the physical (contact angle) and physiological features (absorption and translocation of [14C]-2,4-D) between R and S plants and (4) to examine the ability of 2,4-D to induce ethylene biosynthesis in R and S corn poppy plants.

2. Material and methods

2.1. Plant material

Before winter cereal harvest, mature capsules form at least twenty different corn poppy plants were collected in two fields where failure of corn poppy control with ALS inhibitors and/or 2,4-D had been reported. F-R213 population, suspected to be multiple resistant, was collected from a field located in Baldomar, north of Spain (41°54′39.0″N and 1°00′21.2″W) in 2013. D-R703 population, with suspected resistance to 2,4-D, was collected from a field located in Almacelles (41°43′39.6″N and 0°27′29.5″E) in 2003. Two susceptible populations (H-S013 and S-S012) were included in this study. H-S013 was obtained from a seed dealer (Herbiseed, Twyford, UK) in 2008, and S-S012 was collected in 2012 from a cereal field in Almenar (41°47′30.5″N and 0°27′29.5″E) where no resistance problems had been reported. Corn poppy seeds were sterilized in a 30% hypochlorite solution. Sterilized seeds were sown in Petri dishes with 1.4% agar supplemented with 0.2% KNO_3 and 0.02% gibberellin GA_3. Seeds were placed in a growth chamber at 20/10 °C day/night, 16 h photoperiod under 350 μmol photosynthetic photon-flux density m^{-2} s^{-1}. After 14 days, seedlings were transplanted in 7 × 7 × 7 cm plastic pots filled with the following soil mixture: silty loam soil 40% (*w*/*v*), sand 30% (*w*/*v*), peat 30% (*w*/*v*). Pots were placed in a greenhouse in Lleida, north-eastern Spain (41°37′N, 0°38′W) and were watered regularly to field capacity.

2.2. Dose-response experiments

Five seedlings were sown per pot and after establishing, were thinned to three per pot. At the six leaf stage (5–6 cm), all populations were tested with tribenuron-methyl and 2,4-D. Tribenuron-methyl (Granstar 50 SX, DuPont, 50%) was applied at 0, 4.6, 9.3, 18.7 (field dose), 37.5, 75, 150, 600 and 1200 g a.i.·ha^{-1} to R plants and at 0, 0.25, 0.5, 1.1, 2.3, 4.6, 9.3, and 18.7 g a.i.·ha^{-1} to S plants. 2,4-D (Esteron 60, Dow AgroSciences, 60%) was applied at 0, 75, 150, 300, 600 (field dose), 1200 and 4800 g a.i.·ha^{-1} to R populations and at 0, 9.3, 18.75, 37.5, 75, 150, 300 and 600 g a.i.·ha^{-1} to S plants. Non-treated plants were used as controls. A total of four replicates (three plants per pot) were included at each dose. Herbicides were applied using a precision bench sprayer delivering 200 L·ha^{-1}, at a pressure of 215 kPa. Four weeks after treatment, plants were harvested (above ground) and the dry weight (65 °C for 48 h) was measured.

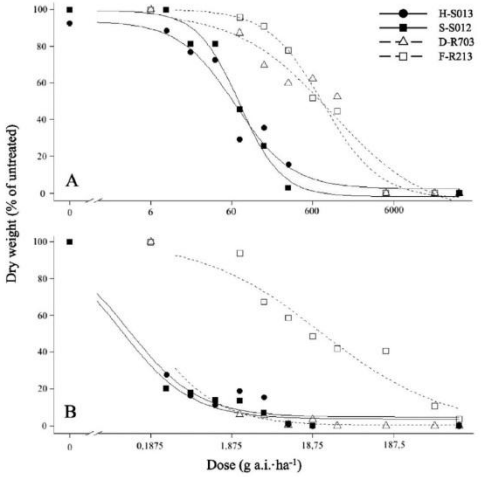

Fig. 1. Dose-response regression curves of susceptible (H-S013 and S-S012), and resistant (D-R703 and F-R213) corn poppy (*Papaver rhoeas*) populations to 2,4-D (A) and tribenuron-methyl (B) (log scale). Data were expressed as percentage of the mean dry weight of untreated control plants.

2.3. Cross-resistance patterns of synthetic auxins

Both R populations (D-R703 and F-R213) and H-S013 plants were sprayed with dicamba (Benzoic acid) and aminopyralid (Pyridine-carboxylic acid) in order to study the effects of other synthetic auxins. Dicamba (Banvel D, Syngenta, 48%) and aminopyralid (Dow AgroSciences, 3.9%) were sprayed at their field rates (144 and 9.9 g a.i.·ha^{-1}, respectively) as well as two times their field rates. Five replicates (three plants per pot) and five control pots (non-treated plants) were included at each dose. Applications and evaluations were done as described above.

2.4. [14C]-2,4-D uptake and translocation experiments

Ring labeled [14C]-2,4-D with specific activity of 1576 MBq $mmol^{-1}$ was provided by Dow AgroSciences (Dow AgroSciences, Indianapolis, USA). Seedlings from H-S013 and both R populations at six true leaves of development (5–6 cm), were treated with four droplets of 0.5 μL (2 μL per plant) of radio labeled herbicide solution containing [14C]-2,4-D and commercial 2,4-D mixed to a final herbicide concentration of 3 g L^{-1} (equivalent to a 600 g a.i.·ha^{-1} delivered at 200 L·ha^{-1} spraying volume). Every plant received a total activity of 18.4 MBq $mmol^{-1}$. Five plants from each population were harvested at 12, 24, 48, and 96 h after treatment (HAT). Unabsorbed herbicide was rinsed from the treated

Table 1
Estimated GR_{50} and resistance index (RI) values to tribenuron-methyl and 2,4-D for H-S013, S-S012, D-R703 and F-R213 corn poppy (*Papaver rhoeas*) populations.

Herbicide	Field dose	Population	GR_{50} (g a.i.·ha^{-1}) ± SE	RI
Tribenuron-methyl	18.75 g a.i.·ha^{-1}	H-S013	0.08 ± 0.02	–
		S-S012	0.10 ± 0.02	1.1
		D-R703	0.17 ± 0.04	2
		F-R213	25.22 ± 6.4	286
2,4-D	600 g a.i.·ha^{-1}	H-S013	68.60 ± 10.2	–
		S-S012	66.36 ± 20.4	0.9
		D-R703	1039.70 ± 402.0	15
		F-R213	816.60 ± 96.0	12

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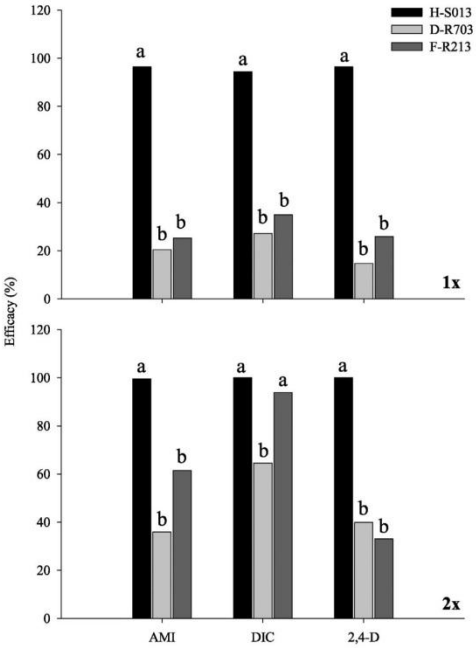

Fig. 2. Efficacy of aminopyralid (AMI), dicamba (DIC) and 2,4-D at the field rate: 9.9, 144 and 600 g a.i.·ha^{-1} (1×) and two-fold the field rate: 19.8, 288 and 1200 g a.i.·ha^{-1} (2×) on H-S013 (black), D-R703 (dark grey) and F-R213 (grey) corn poppy (*Papaver rhoeas*) populations. Columns with different letters indicate significant differences ($P < 0.05$) for each product and dose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

leaves using 2 mL of an acetone/water (1:1 *v*/*v*) solution. The rinse solution was mixed with 15 mL of scintillation fluid (Ultima Gold™, Perkin-Elmer, Packard Bioscience BV). Washes were analyzed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA scintillation counter; Beckman Instruments, CA, USA). Plants were separated into three parts; treated leaf, shoot and root, each of which was dried at 70 °C for 48 h and combusted in a sample oxidizer (OX 500; R. J. Harvey Instrument, Tappan, NY, USA). The trapped [14C]-CO_2 was determined by LSS. Foliar absorption (%) was calculated as: (radioactivity recovered from plant parts) / (total radioactivity recovered) × 100. Translocation (%) was calculated as: (total radioactivity in treated leaf, shoot or root) / (total radioactivity in all tissues) × 100.

To assess translocation of 2,4-D, two treated plants for H-S013, D-R703 and F-R213 populations were removed from pots 48 HAT. Roots were rinsed and whole plants were dried (65 °C for 48 h) and pressed against a 25 by 12.5–cm phosphor storage film (PerkinElmer Life and Analytical Sciences, Shelton, CT) for 6 h, and scanned using a phosphor imager (Cyclone, Perkin-Elmer, Packard Bioscience BV).

Table 2
Absorption (percentage of recovered radioactivity) and translocation (percentage of penetrated radioactivity) of [14C]-2,4-D in H-S013, F-R213 and D-R703 populations of corn poppy (*Papaver rhoeas*) at different times. Data are means.

Population	12 h	24 h	48 h	96 h
Foliar absorption (% recovered radioactivity)				
H-S013	70.98 a*	78.06 a	62.71 a	65.81 a
D-R703	65.67 a	69.55 a	69.26 a	71.98 a
F-R213	65.83 a	78.22 a	70.54 a	76.98 a
Remained in the treated leaf (% penetrated radioactivity)				
H-S013	93.79 a	83.60 a	78.36 a	70.04 a
D-R703	97.34 b	96.45 b	98.56 b	96.87 b
F-R213	99.08 b	96.26 b	98.29 b	97.49 b
Translocation to the shoots (% penetrated radioactivity)				
H-S013	4.25 a	12.77 a	15.05 a	22.22 a
D-R703	2.23 ab	2.27 b	0.77 b	2.44 b
F-R213	0.32 b	2.69 b	0.55 b	1.04 b
Translocation to the roots (% penetrated radioactivity)				
H-S013	1.95 a	3.61 a	6.57 a	7.73 a
D-R703	0.41 b	1.26 ab	0.65 b	0.34 c
F-R213	0.58 b	1.04 b	1.14 b	1.46 b

* Means followed by different letters indicate significant differences in each time and location (absorption, treated leaf, shoots and roots) between populations ($P < 0.05$).

2.5. Contact angle and microroughness assays

To assess any effects of leaf surface on herbicide deposition, 2,4-D was applied as one drop of 0.5 μL in the adaxial surface of the fourth leaf. Immediately after, individual droplets were photographed using a laboratory-built device consisting of a dissection microscope (Leica MZ6; Leica Microsystems Ltd., Heerbrugg, Switzerland) plus a high-definition digital camera with macro objective (Leica Dililux 4.6; Leica Camera AG, D35606 Solms, Germany). Thirty drops for each population (from different plants) were photographed and contact angle of the drops were analyzed using image processing software (Image J 1.31v; US National Institutes of Health, Bethesda, MD, USA). The same procedure was followed for the microroughness determination, where an acetone/water (1:1 *v*/*v*) solution was used instead of the herbicide.

2.6. Ethylene production

Experiments were conducted to evaluate the amount of ethylene produced by R (F-R213 and D-R703) and S (H-S013 and S-S012) plants in response to 2,4-D treatment. Two seedlings were sown in a 145 mL pot (BeltaLab, Barcelon, Spain) and once established, were reduced to one per pot. Plants were sprayed, as described above, with commercial 2,4-D at 0, 150, 300 and 600 g a.i.·ha^{-1}. Treatments were replicated six times. Prior to each treatment, the soil mixture was covered with a layer of perlite to avoid deposition of the herbicide on the substrate. Immediately following treatments, the pots were closed with a specific hermetic top and the two holes beneath the pot were sealed with vaseline and Parafilm. Ethylene was measured by withdrawing a 1 mL gas sample from the head-space with a syringe and injecting it into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) equipped with an alumina column F1 80/100 (2 m × 1/8 × 2.1, Teknokroma, Barcelona, Spain) and a flame ionization detector (FID) [25]. This experiment was repeated twice; in October 2014 and again in February 2015 (the later only with S-S012 as a S population).

2.7. Statistical analysis

Data from dose-response experiments were analyzed using a non-linear regression model (1). The herbicide rate required for 50% growth reduction of plants (GR_{50}) was calculated with the use of a four parameter logistic curve of the type:

$$y = c + \frac{(d - c)}{1 + \mathrm{EXP}[b(\log(x) - \log(GR_{50})]} \quad (1)$$

where c = the lower limit, d = the upper limit and b = the slope at the GR_{50}. In this regression equation, the herbicide rate (g a.i.·ha^{-1}) was the independent variable (x) and the plants' dry weight expressed as percentage of the untreated control was the dependent variable (y). The resistance index (RI) was computed as GR_{50}(R) / GR_{50}(S). Data

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Fig. 3. Digital image (upper panel) and autoradiographic image (lower panel) depicting [14C]-2,4-D translocation throughout plants tissues of H-S013, D-R703 and F-R213 populations of corn poppy (*Papaver rhoeas*), 48 HAT. Arrows in the upper image indicate the leaf where [14C]-2,4-D droplets were applied.

from [14C]-2,4-D uptake and translocation experiments were subjected to analysis of variance (ANOVA). The requirement of homogeneity of variance was checked by visual inspection of the residual plots and residuals were analyzed using Shapiro–Wilk Test. Where variances were not homogeneous, generalized linear models (GLM) were used. The binomial distribution (Logit-link) was used in all GLM, because this distribution resulted in normally distributed residues. Population means were compared using a post-hoc Tukey's pairwise procedure at $P = 0.05$. Data from the cross resistant experiment (efficacy) and ethylene production assay ($\mu LC_2H_4 \cdot g^{-1} \cdot h^{-1}$) were subjected to analysis of variance (ANOVA) and means were separated using Tukey's pairwise comparison at 0.05 probability level. Repetitions from the ethylene experiment (October and February) were not pooled due to statistical differences between experiments.

All statistical analyses were carried out with the use of the R programming language [26], *drc* package [27] for the non-linear regression and *multcom* [28] for the post hoc Tukey's test were employed.

3. Results

Both R and S plants showed morphological damage after 2,4-D application. Plant growth was reduced, and leaves were curled. R plants produced new growth within a few days of herbicide application. S and R plants treated with 600 g a.i.·ha^{-1} and 4800 g a.i.·ha^{-1} of 2,4-D, respectively, died 14 days after application. The GR_{50} for 2,4-D were the same for the two S populations (66.3 vs 68.6 g of a.i.·ha^{-1}). F-R213 and D-R703 plants were 12-fold and 15-fold more resistant to 2,4-D than H-S013 plants, respectively. There was very little control of F-R213 plants with tribenuron-methyl at 600 g a.i.·ha^{-1} (thirty-two times the field rate), and GR_{50} was 25.2 g a.i.·ha^{-1}, 286-fold more resistant than H-S013 plants. Tribenuron-methyl at 18.7 g a.i.·ha^{-1} (field rate) controlled the population D-R703 (Fig. 1), and it showed a very low RI (Table 1). Differences between S populations in the response to tribenuron-methyl were minimal (Fig. 1).

The D-R703 and F-R213 populations were also resistant to dicamba and aminopyralid at the field rate (144 and 9.9 g a.i.·ha^{-1}, respectively; Fig. 2). The effectiveness of auxinic herbicides on the R population increased when they were applied at two times the field rate, but other than dicamba on F-R213, they failed to control the populations (Fig. 2).

There were no significant differences between R (D-R703 and F-R213) and S (H-S013) plants in the quantity of [14C]-2,4-D absorbed, with between 65 and 70% of the herbicide applied absorbed at 12 HAT. F-R213 and D-R703 plants translocated much less [14C]-2,4-D than H-S013 plants with, significantly less translocation to the shoots and roots compared to the susceptible population (Table 2). Percentages of recovered radioactivity ranged from 89 to 96% in H-S013 plants and from 85 to 98% in the R plants. Images obtained from the qualitative studies at 48 HAT confirmed the above results (Fig. 3). Data from the contact angle and microroughness assays did not reveal any kind of differences between R and S plants (data not shown).

No differences in ethylene production among populations were detected in untreated (0 g a.i·ha^{-1}) or plants sprayed at 150 g a.i·ha^{-1} of 2,4-D. There were differences between R and S populations starting at 300 g a.i.·ha^{-1} of 2,4-D, with maximum differences at the field rate

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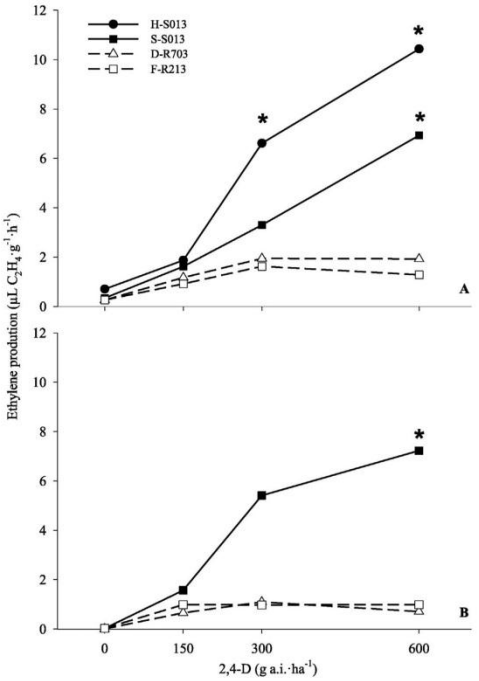

Fig. 4. Ethylene production (µL $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) in susceptible (H-S013 and S-S012), and resistant (D-R703 and F-R213) corn poppy (*Papaver rhoeas*) populations after foliar application of 2,4-D at different concentrations. The experiment was repeated twice, in October 2014 (A) and February 2015 (B). Ethylene was measured 16 h after treatment (HAT). * indicate significant differences ($P < 0.05$) between R and S plants for each application dose.

(600 g a.i.·ha^{-1}), when S plants produced between five and eight times more ethylene than R plants (Fig. 4). Even though statistical differences in ethylene production occurred between repeated trials (October and February), similar patterns between R and S populations were confirmed in both experiments (Fig. 4).

4. Discussion

Resistance to both tribenuron-methyl and 2,4-D in F-R213 plants was confirmed in our study. Multiple resistant corn poppy populations have also been previously detected in Italy and Greece [5,29]. Resistance to both auxinic and ALS inhibitor herbicides have been reported in other dicot weeds such as: *Gallium spurium L.* [13], *Sisymbrium orientale L.* [4], *Kochia scoparia L.* [14], *Limnocharis flava L.* and *Raphanus raphanistrum L.* [5]. Resistant factors obtained to tribenuron-methyl and 2,4-D were similar to those observed in other studies [29,30].

The resistant plants were also resistant to dicamba and aminopyralid. Resistance to multiple synthetic auxins was also observed in *Lactuca serriola L.* [31], *Sinapis arvensis L.* [11], and *K. scoparia* [14]. New discoveries of proteins involved in auxin mode of action have indicated that specific alterations in nuclear receptors might contribute as a potential resistance mechanisms in auxinic herbicide resistant dicotyledonous weeds [2]. Similar to the results presented in this study, cross-resistance between 2,4-D and dicamba was also found in an F-box receptor mutant of *Arabidopsis thaliana L.* [32].

There was no difference in absorption of 2,4-D, however, reduced [14C]-2,4-D translocation was observed in 2,4-D resistant corn poppy populations. Reduced synthetic auxin translocation has previously been reported for resistant populations of *Galeopsis tetrahit L.* [16] and *L. serriola* [3]. Alteration to the auxin efflux carriers (PIN-FORMED, PIN; ATP-binding cassette, ABC) could explain the lack of translocation observed in 2,4-D resistant corn poppy plants. Members of the PIN and ABC efflux carrier families have been considered the main mechanism involved in active and long-distance auxin transport [33]. Recent studies conducted with *A. thaliana* suggested that ABCB4 transporter (ABC family) is the target of 2,4-D [34]. In addition, a mutation in *A. thaliana* in another efflux carrier of ABC family, ABCG9, has been reported to provide increased tolerance to 2,4-D without affecting endogenous auxin Indole-3-acetic-acid (IAA) transport [35].

Results from the ethylene experiments are consistent with previous studies conducted with other species. A three-fold increase in ethylene was induced in quinclorac-sensitive *Galium spurium* plants compared with quinclorac-resistant plants [13]. Sensitive and resistant *K. scoparia* plants demonstrated greater than four-fold difference in ethylene production when they were treated with dicamba and sampled 24 HAT [36]. The stimulation of ethylene biosynthesis through the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase has been described as one of the first phases after 2,4-D and F-box proteins binding [37]. Therefore, our results suggest that in R plants 2,4-D may not be binding this nuclear receptor.

Overall, these results suggest that 2,4-D does not promote the signaling pathway in R plants because its receptor is not activated, either due to its alteration or as a consequence of reduced translocation involving any of the known auxin transporter families. The first step toward uncovering this mechanism could be seeking an alteration in these specific proteins affecting the auxinic nuclear reception or auxin efflux carriers (a specific transporter belonging to PIN or ABC families). A comprehensive understanding of the resistance mechanisms in corn poppy biotypes, especially in those with multiple resistance to auxinic and ALS inhibitor herbicides, is needed to further understand the risk of resistance evolution to others modes of action. This information will be crucial to assist in the design of integrated weed management strategies.

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ABC	ATP-binding cassette
ACCase	Acetyl-coenzyme A carboxylase
(ACC) synthase	1-Aminocyclopropane-1-carboxylic acid synthase
ALS	Acetolactate synthase
GR_{50}	Herbicide rate causing 50% mortality
HAT	Hours after treatment
MCPA	4-Chloro-2-ethylphenoxyacetate
NTSM	Non-target-site mechanisms
PIN	PIN-FORMED proteins
R	Resistant
RI	Resistant index
ROS	Reactive oxygen species
S	Sensitive
TSM	Target-site mechanisms
WAT	Weeks after treatment

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CHAPTER 4

Assessing resistance to bromoxynil in Spanish corn poppy (*Papaver rhoeas)* populations

Abstract

In the last decades *Papaver rhoeas* L. (corn poppy) has evolved resistant towards tribenuron-methyl and/or 2,4-D herbicides, both an ALS inhibitor and a synthetic auxin, respectively. In the wake of those resistances other post-emergence herbicides have been extensively used. One of these herbicides is the photosystem II (PS II) inhibitor bromoxynil. Recently, lack of proper control following applications with mixtures containing bromoxynil has been reported in some specific fields in North-Eastern Spain. Seeds from these fields were collected and dose-responses experiments at different phenological stages were conducted in greenhouse in order to determine if a bromoxynil resistant process is behind the responses observed at the fields. Populations studied in this work (R-313, R-413 and R-213) were multiple resistant to 2,4-D and tribenuron-methyl. However, minimum differences between susceptible plants and those collected from fields were detected when bomoxynil was sprayed at the recommended growth stage (5-6 cm of rosette). Applications on larger plants (phenological stage 10-11 cm of rosette) caused a slight shift in the dose-response curve of population R-313. This shift was less marked for populations R-413 and R-213. Based on these results, lack of control detected on the fields could be attributed to the presence of larger corn poppy plants at post-emergence application.

Keywords: corn poppy, photosystem II inhibitor, phenological stage, mixture, tribenuron-methyl and 2,4-D.

Introduction

Photosystem II (PS II), which is the first protein complex involved in the photosynthetic process, catalyzes the oxidation of water and the reduction of plastoquinone (Q_A and Q_B) using energy derived from light (Minagawa and Takahashi 2004). This complex is the target site of herbicides commonly named as PS II inhibitors. These herbicides inhibit electron flow by competing with Q_B for anchoring to the binding pocket of D1 protein of the PS II (Hess 2000). As a result of this process, susceptible plants treated with the PS II inhibitors produce large amounts of ^{3}Chl, $^1O_2$, H_2O_2 and O_2^- that destroy the integrity of membranes and lead to the plant death (Fuerst and Norman 2011). PS II inhibitors are generally classified in three separated herbicide groups, according to how they bind to the D1 binding pocket (Sobolev and Edelman 1995). These are named C_1: Triazines, Triazolinone, Triazinones, Pyridazinone, Phenylcarbamates and Uracils; C_2: Ureas and Amides; and C_3: Nitriles, Phenyl-pyridazines and Benzothiadiazinone, according to the Herbicide Resistance Action Committee, HRAC (homologous of groups 5, 6 and 7 designed by the Weed Science Society of America, WSSA) (Mallory-Smith and Retzinger 2003). First case of resistance to PS II inhibitors was reported in 1970 in a *Senecio vulgaris* L. population that evolved resistant to triazine (Ryan 1970). To date, 105 biotypes all over the world have evolved resistance to PS II inhibitors. Of these, 73 biotypes are resistant to C_1, 28 to C_2 and only 4 to C_3 (Heap 2015). Resistance to these herbicides is mainly due to an alteration of the D1 protein (target-site resistance, TSR), which is encoded by the chloroplast psbA gene (Gronwald 1994). In higher plants, mutations at five positions (Val219, Ala251, Phe255, Ser264 and Asn266) have been related with resistance cases to PS II inhibitors. Substitution of Ser by Gly at position 264 is, by far, the most frequently evolved mutation in weeds and causes resistance of 100-fold the field atrazine dose (Gronwald 1994). However, mutations in psbA gene are not the unique mechanism that confer resistance to PS II inhibitors. Some non-target-site resistance (NTSR) mechanisms have also been identified. For example a simazine resistant biotype of *Sonchus oleraceus* L. showed enhanced glutathione S-transferase (GST) activity (Fraga and Tasende 2003) and increased detoxification with cytochrome P450 was the main resistance mechanism to different triazine herbicides in a *Lolium rigidum* L. resistant population (Powles 1993).

Corn poppy *(Papaver rhoeas* L.) is the most common dicotyledonous weed in winter cereals in southern Europe (Torra et al., 2011). It is a competitive species and,

depending on its density, its presence within the crop results in significant yield reduction (Wilson et al., 1995). In Spain multiple resistant 2,4-D and tribenuron-methyl corn poppy populations have been reported in the last decades (Heap 2015). As occur in other European regions (Cruz-Hipolito et al., 2013; Kaloumenos et al., 2009), a single nucleotide substitution at position Pro197 of the ALS gene is responsible for the resistant response (Durán-Prado et al., 2004). With regard to 2,4-D resistance, a new study conducted with Spanish 2,4-D resistant corn poppy populations suggested that lack of translocation in resistant plants could be the main resistant mechanism against 2,4-D (Rey-Caballero et al., 2016a). Recently lack of control of corn poppy has been described in a few fields in Spain where post-emergence mixtures containing bromoxynil were applied (Kaloumenos 2014). Recommendations about the use of herbicide rates are based on weed growth stages found at the application moment. However, it is highly unlikely to find uniform phenological stages within weed populations, especially in species like corn poppy which have an extended germination period (Cirujeda et al., 2008). This source of variability leads to a variation in the active ingredient quantity that arrives by unit weight or leaf area of the target plants. This can produce a "diluting effect" of the herbicide in those firstly emerged weeds (largest plants), promoting sub-lethal conditions (Vila-Aiub and Ghersa 2005). Generally, low doses of herbicides (sub-lethal doses) have the potential of accelerating resistance evolution and leading to more cross-resistance by metabolic resistance (Neve et al., 2014). Recent studies conducted with different species have demonstrated that bromoxynil efficacy depends on the phenological stage (Corbett et al., 2004; Forcella et al., 2015). For all this, the objectives of this work were (1) to characterise the resistance patterns to ALS inhibiting herbicides and 2,4-D of some Spanish corn poppy populations which were not controlled with post-emergence mixtures containing bromoxynil (bromoxynil + ioxynil + MCPP-p) with dose-response experiments; and (2) to study the effect of bromoxynil on these problematic biotypes at different phenological stages in order to confirm or discard a resistance process.

Material and Methods

Plant material and dose-response experiments

Seeds were collected from a few fields where post-emergence mixtures containing bromoxynil (bromoxynil + ioxynil + MCPP-p) did not reach a good control of corn poppy. R-313, R-413 and R-213 seeds were picked up during summer 2013. Only R-

313 population was resampled during 2014. Additionally, seeds from two susceptible (S) populations were obtained from a seed dealer (Herbiseed, Twyford, UK) and collected in the same region where problematic populations came from (Table 4.1).

Table 4.1. Location and date of collection of corn poppy (*Papaver rhoeas*) populations used in the experiments.

Code		Sampling location			Year collected
		Town	Latitude	Longitude	
S*	S-013	--	--	--	2008
	S-113	Belorado (Burgos)	42º24'57.8"N	3º10'49.3"W	2013
R-313		Tosantos (Burgos)	42°24'43.7"N	3°14'39.9"W	2013 and 2014
R-413		Belorado (Burgos)	42°24'55.8"N	3°11'55.1"E	2013
R-213		Baldomar (Lleida)	41°54'39.0"N	1°00'21.2"E	2013

* susceptible populations

All populations were sown in 23 x 15 x 4 cm aluminum trays with 1 cm of peat and were watered regularly. Trays were placed in a growth chamber at 20/10 °C day/night, 16-h photoperiod under 350 µmol photosynthetic photon-flux density m^{-2} s^{-1}. After 14 days, seedlings were transplanted in pots (three seedlings per pot) and filled with the following substrate: silty loam soil 40% (w/v), sand 20% (w/v), peat 40% (w/v). Bromoxynil was applied at two different phenological stages: at 5-6 cm of rosette (the recommended growth stage) and at 10-11 cm of rosette (Figure 4.1).

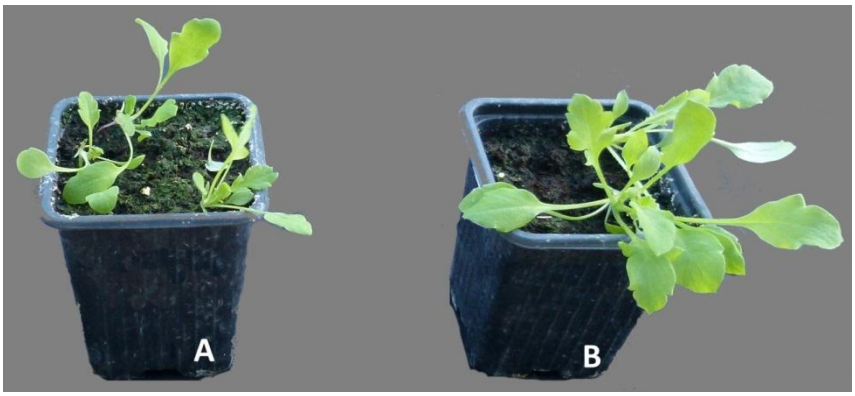

Figure 4.1. (A) First phenological stage, 5-6 cm of rosette, and (B) second phenological stage, 10-11 cm of rosette of corn poppy (*Papaver rhoeas*), used in the bromoxynil dose-response assays.

The PS II inhibitor bromoxynil (Buctril, Bayer, 24%) was applied at 0, 49.5, 99, 198, 396 (maximum field dose), 792 and 1584 g a.i.·ha^{-1} on the suspicious populations and at 0, 24.7, 49.5, 99, 198, 396, 792 and 1584 g a.i.·ha^{-1} on the susceptible populations.

Eight replicates at each dose were included and the experiment was conducted twice (March 2014 and February 2015). Moreover, tribenuron-methyl and 2,4-D characterisation of these populations was also performed at field recommended growth stage. Tribenuron-methyl (Granstar 50 SX, DuPont, 50%) was applied at 0, 4.6, 9.3, 18.7 (field dose), 37.5, 75, 150, 600 and 1200 g a.i.·ha^{-1} in suspicious plants. Rates applied for this herbicide in the susceptible populations were 0, 0.25, 0.5, 1.1, 2.3, 4.6, 9.3, and 18.7 g a.i.·ha^{-1}. The 2,4-D (Esteron 60, Dow AgroSciences, 60%) was applied at 0, 75, 150, 300, 600 (field dose), 1200 and 4800 g a.i.·ha^{-1} in suspicious populations and at 0, 9.3, 18.75, 37.5, 75, 150, 300 and 600 g a.i.·ha^{-1} in susceptible plants. A total of four replicates (three plants per pot) were included at each dose. All herbicides were applied using a precision bench sprayer delivering 200 l·ha^{-1}, at a pressure of 215 kPa. Four weeks after all treatments, dry aboveground biomass was measured after drying the plant at 65 °C during 48h.

Statistical analysis

For the dose-response experiments, statistical analysis was carried out with a non-linear regression model with *drc* package in R. The herbicide rate causing 50% of growth reduction (GR_{50}) of plants was calculated with the use of four parameter logistic curves of the type (1):

$$y = \mathrm{c} + \frac{(d-c)}{1+\mathrm{EXP}[b(\log(x)-\log(GR50)]} \quad (1)$$

Where c = the lower limit, d = the upper limit and b = the slope at the GR_{50}. In this regression equation, the herbicide rate (g a.i.·ha^{-1}) was the independent variable (x) and the efficacy of the treatment (100 - percentage of weight reduction referred to the untreated control) was the dependent variable (y). The resistance index (RI) was computed as GR_{50} (suspicious population)/GR_{50} (susceptible). Analysis of variance (ANOVA) for the bromoxynil dose-response experiments showed no significant interaction between experiments conducted in March 2014 and February 2015, so data from the two experiments were pooled prior to non-linear regression analysis. Also both susceptible populations (S-013 and S-113) were considered as a unique population (S) because ANOVA did not established differences in the responses between both populations and experiments.

Results and Discussion

Multiple resistance to 2,4-D and tribenuron-methyl was confirmed for populations R-313, R-413 and R-213. On the bases of the resistance index (RI), these populations were 612, 373 and 252 fold more resistant to tribenuron-methyl than susceptible plants. In a previous work, molecular studies conducted with R-313 and R-213 plants, identified different mutant ALS alleles: Ala197, Arg197, His197, Leu197, Ser197 and Thr197 (Rey-Caballero et al., 2016b). When all these populations were sprayed with 2,4-D, R-313 and R-213 plants showed a RI of 19 and 12 respectively, while R-413 plants obtained a much more reduced RI, just 4 times more resistant to 2,4-D than the susceptible plants (Table 4.2). Experiments conducted with [^{14}C]-2,4-D had revelled abnormal translocation rates in some resistant corn poppy populations (R-213 plants included) (Rey-Caballero et al., 2016a). Additionally, in a recent review, lack of 2,4-D translocation in those resistant plants has been attributed to the 2,4-D metabolism process (Peterson et al., 2015).

Table 4.2. GR_{50} and Resistant Index (RI) obtained from the log-logistic model for B-R313, B-R413, F-R213 and susceptible (S) corn poppy (*Papaver rhoeas*) populations (two pooled populations) when they were sprayed with tribenuron-methyl and 2,4-D at 5-6 cm of rosette.

Population	GR_{50} ± SE (g a.i.·ha^{-1})	Res SS	RI
Tribenuron-methyl			
S	0.10 ± 0.02	9140	--
R-313	61.27 ± 12.00	22189	612.7
R-413	37.33 ± 6.28	27571	373.3
R-213	25.22 ± 6.38	10084	252.2
2,4-D			
S	64.16 ± 22.40	10990	--
R-313	1238.40 ± 436.20	18435	19.3
R-413	253.20 ± 49.80	9744	3.9
R-213	816.60 ± 96.00	2872	12.7

No resistance was detected for bromoxynil when this herbicide was applied at 5-6 cm of rosette which corresponds to the field recommended growth stage. GR_{50} for bromoxynil in susceptible plants was 41.67 (± 5.54) g a.i. ha^{-1} and minor differences were detected in the equation parameters with suspicious populations. RI's for those populations were 1.7, 1.2 and 1.7 for R-313, R-413 and R-213, respectively (Table 4.3). No susceptible plant survived neither full (396 g a.i. ha^{-1}) nor half (198 g a.i. ha^{-1}) of the maximum bromoxynil field dose and efficacies at these dosages ranged from 100 to 97 and from 100 to 93 in the suspicious populations, respectively. Bromoxynil efficacies of

susceptible and R-313 and R-213 plants were significantly different at 99 g a.i. ha^{-1}. At this dose, 92 (± 2) % of the susceptible plants were controlled, however efficacy in R-313 and R-213 was 72 (± 5) and 67 (± 4) %, respectively (Figure 4.2).

Applications at the second phenological stage showed a slight shift in the dose-response curves. Thus, GR_{50} in the susceptible populations was 54.92 (± 6.73) g a.i. ha^{-1}, while this parameter was 2.2 (120.47 ± 18.61 g a.i. ha^{-1}) times higher in population R-313 (Table 4.3). GR_{50} for R-413 and R-213 populations increased slightly but these values were similar to the GR_{50} observed in susceptible plants (83.95 ± 8.3 and 89.16 ± 8.3, respectively), therefore RI's for these populations were 1.5 and 1.6, also similar to those obtained in the first phenology (Table 4.3). This herbicide reached efficacies of 91 (± 3), 94 (± 3) and 95 (± 3) % at 198 g a.i. ha^{-1} in susceptible, R-413 and R-213 plants respectively, but efficacy in R-313 population at this dose was significantly lower (67% ± 6) (Figure 4.2).

Table 4.3. Equation parameters obtained from the log-logistic model for B-R313, B-R413, F-R213 and susceptible (S) corn poppy (*Papaver rhoeas*) populations (two pooled populations) when they were sprayed with bromoxynil at two different phenological stages (5-6 cm and 10-11 cm of rosette).

Population	GR_{50} ± SE (g a.i.·ha^{-1})[a]	b ± SE[b]	c ± SE[c]	d ± SE[d]	Res SS[e]	RI[f]
First phenological stage (5-6 cm of rosette)						
S	41.67 ± 5.54	2.11 ± 0.15	3.74 ± 1.80	102.01 ± 1.23	43854	--
R-313	70.38 ± 2.77	3.25 ± 0.33	3.13 ± 0.10	99.31 ± 1.49	28484	1.7
R-413	52.99 ± 2.38	3.33 ± 0.57	3.70 ± 0.10	100.01 ± 1.78	18790	1.2
R-213	72.41 ± 4.24	2.44 ± 0.29	3.24 ± 0.01	99.98 ± 1.95	4388	1.7
Second phenological stage (10-11 cm of rosette)						
S	54.92 ± 6.73	1.08 ± 0.10	2.43 ± 2.12	107.01 ± 3.17	33979	--
R-313	120.47 ± 18.61	1.38 ± 0.26	5.27 ± 0.11	106.15 ± 6.42	35631	2.2
R-413	83.95 ± 8.36	2.66 ± 0.39	4.52 ± 0.09	101.14 ± 2.50	13513	1.5
R-213	89.16 ± 8.32	3.45 ± 1.04	6.25 ± 0.64	98.13 ± 4.23	3129	1.6

[a] GR_{50}, herbicide concentration for 50% reduction of corn poppy dray weight.

[b] Slope at the GR_{50}.

[c] Lower limit.

[d] Upper limit.

[e] Res SS, residual sum of square.

[f] RI (resistance index) = GR_{50} (Population) / GR_{50} (S).

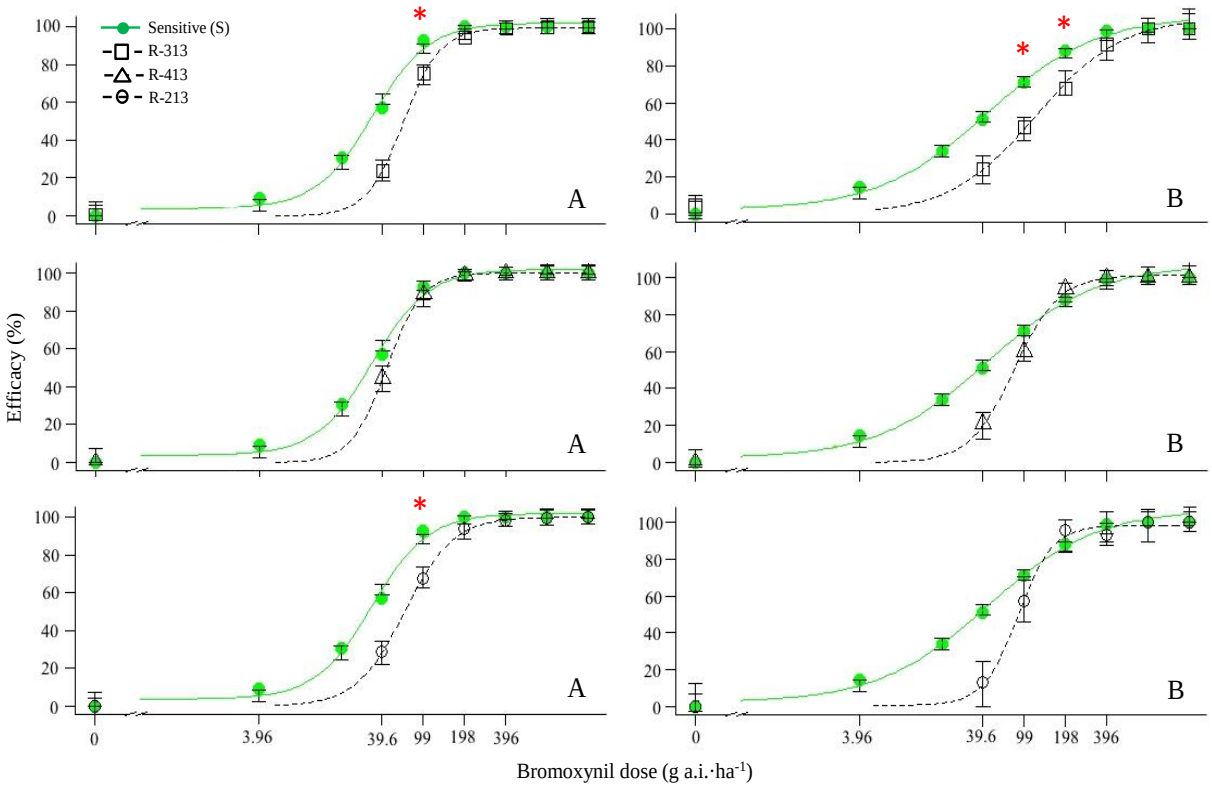

Figure 4.2. Dose-response regression curves of R-313, R-413, R-213 and susceptible (S) corn poppy (*Papaver rhoeas*) populations (two pooled populations) treated with bromoxynil at two different phenological stages: 5-6 cm (A) and 10-11 cm (B) of rosette. Efficacy was expressed as percentage of the mean dry weight of untreated control plants. Red asterisks indicate doses (those similar to field rates, from 120 to 210 g a.i. ha^{-1}) where significant differences in efficacy (P<0.05) were detected between suspicious (R-313, R-413, R-213) and S plants

At the moment, few resistance cases to nitriles have been described (Heap 2014) and in even fewer cases bromoxynil resistance has been studied. A *Senecio vulgaris* L. biotype collected from peppermint fields in Oregon, was reported to be 10 times more resistant to bromoxynil than its susceptible homologous plants (Mallory-Smith 1998; Park and Mallory-Smith 2006). GR_{50} of those susceptible and resistant biotypes were 2.5 and 26 g a.i. ha^{-1} (Mallory-Smith 1998) and 9 and 91 g a.i. ha^{-1} (Park and Mallory-Smith 2006), respectively. It has to be noted that GR_{50} values described for susceptible *S. vulgaris* biotypes were much lower than GR_{50} values obtained by susceptible corn poppy plants in this study (41.6 g a.i. ha^{-1}). Moreover, GR_{50} obtained by R-313 plants in the first phenological stage (70.43 g a.i. ha^{-1}) was comprehended between GR_{50} values obtained in those bromoxynil resistant *S. vulgaris* biotypes (Mallory-Smith 1998, Park and Mallory-Smith 2006). Those bromoxynil resistant *S. vulgaris* biotypes showed a

substitution at codon Asn266 (Thr) of the *psbA* gene. Plants carrying Thr266 *psbA* allele displayed low-level of resistance to triazinones metribuzin and hexazinone (RI's of 4.2 and 2.6 respectively) and no resistance to triazine herbicides atrazine and simazine or to the urea herbicide diuron (Park and Mallory-Smith 2006).

In those fields where bromoxynil did not achieve a good corn poppy control, this product was sprayed in mixture with ioxynil and MCPP-p (Image, Nufarm, 12%, 12%, 36%). Recommended field dose of this mixture goes from 1 to 1.75 l/ha, so that commercial bromoxynil rate ranges between 120 to 210 g a.i. ha^{-1}. As it has been described above R-313 plants are not controlled with bromoxynil when they are spayed at the larger phenology and at 198 g a.i. ha^{-1} of this herbicide. Because corn poppy has an extended period of germination (Holm et al., 1997) different phenological stages can be found at herbicide application time, especially in those post-emergence herbicides. Thus, lack of control with mixtures containing bromoxynil could be explained because large corn poppy plants are present at herbicide application timing in those highly-infested fields. As it has been demonstrated in this and in other experiments (Cirujeda 2001; Forcella et al., 2015), bromoxynil efficacy decreases as phenological growth stage advances. Larger plants receive less effective dosage compared to smaller plants; these sub-lethal rates can be the conditions to select non-target site resistances mechanisms (Cirujeda et al. 2008, Vila-Aiub and Ghersa 2005), like for bromoxynil. On the other hand, and contrary to other cases, target site resistance to bromoxynil (substitutions in position 266 of the *psbA* gene) has displayed moderate RI (Park and Mallory-Smith 2006). Low rates of this type of mutations could also explain why some plants were able to survive this herbicide in this study.

Further researches should focus on obtaining a second generation from those surviving plants and test again through dose-response experiments, if exist differences in the RI's of those resistant and susceptible populations. In addition, it could be interesting to study the most commonly mutated positions of the *psbA* gene. In this research, the population which could be bromoxynil resistant was also 2,4-D resistant, and therefore, it should be investigated if there is a relationship between those non-target site resistance mechanisms that confer resistance to synthetic auxines and the response of those few corn poppy plants able to survive nitriles.

Finally, it is important to underline that post-emergence applications of mixtures containing bromoxynil should be done in early-post-emergence at full rates, and that further field escapes should be monitored.

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CHAPTER 5

New management options for herbicide resistant *Papaver rhoeas* populations in Spain

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Abstract

Papaver rhoeas (L.) is the most widespread broadleaved weed infesting winter cereals in Southern Europe. Resistant (R) biotypes to both 2,4-D and tribenuron-methyl have evolved in recent decades, thus complicating its control. In this study, the effects of different strategies on *P. rhoeas* management, including crop rotations, delayed sowing, and different herbicide programmes were tested in field experiments at two locations over three seasons. R profiles for 2,4-D and tribenuron-methyl were characterized with dose-response experiments and both biotypes were confirmed to be R to both herbicides. After three years, all integrated management strategies reduced the initial density of *P. rhoeas*. The most successful systems were those which either included a suitable crop rotation (sunflower or field peas), or had a variation in the herbicide application timing (early post-emergence or combining pre-emergence and post-emergence). The efficacy of the tested management systems differed between both locations, possibly due to a different cross R pattern to ALS inhibitors between biotypes. Integrated management of multiple herbicide R *P. rhoeas* is necessary in order to reduce selection pressure by herbicides, mitigate the evolution of new resistant biotypes and reduce the weed density in highly infested fields. Moreover, a deeper understanding of biotypes is necessary to improve the design of chemical strategies.

Keywords: corn poppy, integrated weed management strategy, 2,4-D, tribenuron-methyl, crop rotation, herbicide management.

Introduction

Weeds are the major cause of yield losses because they compete with the crop for nutrients, water and light (Oerke 2005). Herbicides are the principal tool used for weed control in modern agriculture and they are highly effective on most weeds, but are not a complete solution to the complex challenge that weeds represent (Harker et al., 2013). The overuse of herbicides has imposed a strong selection for any trait that enables plant populations to survive and reproduce under recurrent herbicide pressure. This has contributed to the evolution of weed resistance to herbicides worldwide. Herbicide resistance (HR) in weeds must be avoided because it is a major limiting factor to food security in global agriculture (Busi et al., 2013). Also, HR causes higher short-term costs on weed management, as well as crop yield loss, weed-seed contamination, reduced land values, increase of mechanical and cultural management costs, and additional expense of eventual alternative herbicides or cropping systems (or both) for managing these populations (Norsworthy et al., 2006). The best way to prevent the evolution of HR weeds is to implement diversified cropping systems with less frequent herbicide use by employing non-chemical weed management practices (Beckie 2006).

Papaver rhoeas L. is a major weed of arable crops in southern Europe (Délye et al., 2011). Its competitive nature, which can decrease cereal yields up to 32%, makes it especially noxious in winter cereals (Torra et al., 2011). The ability of this species to invade, grow, and remain in arable fields can be attributed to several factors: the development of a persistent seed bank, an extended germination period and high seed production (Torra and Recasens 2008). *Papaver rhoeas* is becoming an increasing problem due to the appearance of HR biotypes to synthetic auxins and/or to acetolactate synthase (ALS) inhibitors. Poor control of *P. rhoeas* in Spain with the synthetic auxin 2,4-D was first reported in 1992 (Taberner et al., 1992), and a biotype resistant to both 2,4-D and tribenuron-methyl (ALS inhibitor) was first reported in 1998 (Claude et al., 1998). A survey conducted in North-Eastern Spain between 1990 and 2001 was intended to identify fields containing HR *P. rhoeas* populations. The majority of the samples were collected in fields where local farmers reported poor weed control following tribenuron-methyl applications. Results indicated that 85% and 72% of sampled *P. rhoeas* populations were resistant to 2,4-D and tribenuron-methyl, respectively (Cirujeda 2001). Resistance to ALS inhibitors in *P. rhoeas* has also evolved in numerous other countries across Europe (from Sweden to Italy and from the

UK to Poland) (Heap 2015). In all studied cases, resistance was attributed exclusively to mutant ALS alleles (Délye et al., 2011; Kaloumenos et al., 2009; Marshall et al., 2010). In Spain, the resistance to tribenuron-methyl is reported to be due to a single point substitution in domain A of the ALS gene (Duran-Prado et al., 2004). Multiple resistance of *P. rhoeas* to 2,4-D and tribenuron-methyl has also been reported in Italy (Heap 2015), but the resistance mechanism to synthetic auxins in this weed still remains unknown.

Because herbicides alone are not always enough to control HR *P. rhoeas* populations, and in order to prevent the appearance of new resistant biotypes to other modes of action (MOA's), the development of new management tools is required. Chemical control strategies should be combined with non-chemical ones in an integrated weed management (IWM) programme. This programme should be specifically designed and tested for each region (Powles and Bowran 2000), taking into account climatic and socioeconomic factors. For example, in Spanish dry land areas where cereal yields are low and possibilities of crop rotations are limited (Cantero-Martínez et al., 2007). Various chemical and non-chemical tools have been analyzed to control many HR weeds species. Crop rotations provide farmers with opportunities to employ variable crop life cycles, sowing dates, harvest dates, tillage and weed management practices to restrict the evolution of weeds adapted to monocultures (Liebman and Staver 2001). Crop rotations have been proposed to manage several HR weeds like *Alopecurus myosuroides* L. (Moss et al., 2007), *Lolium rigidum* Gaud. (Busi and Powles 2013) or *Avena fatua* L. (Harker et al., 2009). In addition, for HR management, crop rotation allows for the introduction of herbicides having different MOA's (Vencill et al., 2012). Mechanical control of *P. rhoeas* by ploughing was considered to be an effective method for placing a proportion of the seeds in non-optimal germination situations, but this method should not be repeated for a few years because new seeds would move upwards in the soil strata due to their high survival capacity (Cirujeda et al., 2003). Harrowing is a good technique for *P. rhoeas* management, but is highly dependent on the initial *P. rhoeas* densities (Cirujeda et al., 2003; Torra et al., 2011). Delayed sowing (three months) and different types of fallow (physical and chemical) conducted in Spain showed their effectiveness in reducing *P. rhoeas* densities, but only combined with other control methods, like chemical control or cultivation (Torra et al., 2011). The results observed in Spanish winter cereals indicate that 2,4-D and/or tribenuron-methyl resistant *P. rhoeas* populations can be controlled by application of pre-emergence

(PRE) or post-emergence (POST) herbicides with alternative MOA's (Torra et al., 2010). Up to now, the effects of several available cultural methods on the management of HR *P. rhoeas* have not been studied, including crop rotation or variation of herbicide application timings between years in a chemical programme. This knowledge is necessary in order to implement and design effective integrated weed management programmes, particularly in context of the present scenario where no new MOA has been discovered in recent decades and some of the herbicides which currently provide good results controlling *P. rhoeas* will probably not be available in the future. The aims of this study are: first, to characterize the herbicide resistance patterns of the *P. rhoeas* populations that are object of this study; and secondly, to analyze the integrated effect of some management strategies (different crop rotations, delayed sowing, different herbicide programmes) over three years on their capacity to control *P. rhoeas* populations in winter cereals whilst providing new data on the effect of individual methods, which are later combined in IWM programmes.

Materials and Methods

Sites description

Experiments were conducted on two commercial winter cereal fields in the Lleida province, North-Eastern Spain, with high *P. rhoeas* infestations. At Baldomar (Location 1, L-1) (41° 54'N, 1° 0'W), at 334 m height, the soil was silty–clay loam (48.2% sand, 15% clay, and 36.8% silt), pH was 8.2, and organic matter content was 2.5%. At Sant Antolí (Location 2, L-2) (41° 37'N, 1° 19'W), at 581 m height, the soil was silty–clay loam (25.2% sand, 23.4% clay, and 51.4% silt), pH was 8.1, and organic matter content was 2.8%. In the years preceding the trials, the fields had been under a monocrop of continuous winter cereals, managed with minimum tillage. Selective POST herbicides (florasulam + 2,4-D in L-1 and iodosulfuron-methyl + mesosulfuron-methyl alternating with florasulam + 2,4-D in L-2) were employed for weed control during recent years at both sites.

Resistance profile of the Papaver rhoeas *populations*

Seeds from the two field experiments were collected and stored during summer 2012. In autumn, dose response experiments were conducted with L-1 and L-2 populations together with one susceptible (SC) population purchased from a seed dealer (Herbiseed, Twyford, UK). Seeds were sterilized in a 30% hypochlorite solution and sown in Petri

dishes with 1.4% agar supplemented with 0.2% KNO_3 and 0.02% gibberellin. Petri dishes were placed in a growth chamber at 20/10°C day/night, and a 16-h photoperiod under 350 µmol photosynthetic photon-flux density $m^{-2}s^{-1}$. After 14 days, seedlings were transplanted to 8 x 8 x 8cm plastic pots filled with a mixture of silty loam soil 40% (w/v), sand 30% (w/v), and peat 30% (w/v). Five seedlings were transplanted per pot, which were later thinned to three per pot. In the suspected resistance populations, at the 5- to 6-leaf stage (5-6 cm) the ALS inhibitors tribenuron-methyl (tribenuron-methyl 500 g a.i. Kg^{-1}, SG) and florasulam (florasulam 22.8 g a.i. l^{-1}, WG) were applied at 1200, 600, 150, 75, 37.5, 18.7, 9.3, 4.6 and 0 g a.i. ha^{-1}, and 480, 240, 60, 15, 7.5, 3.7, 1.8, 0.9 and 0 g a.i. ha^{-1}, respectively. 2,4-D (2,4-D ethyl-hexyl 600 g a.i. L^{-1}, EC) was applied at 4800, 1200, 600, 300, 150, 75 and 0 g a.i. ha^{-1}. Susceptible plants were sprayed at the same phenological stage at 18.7, 9.3, 4.6, 2.3, 1.1, 0.5, 0.25 and 0 g a.i. ha^{-1} of tribenuron-methyl; 7.5, 3.7, 1.8, 0.9, 0.4, 0.2, 0.1 and 0 g a.i. ha^{-1} of florasulam and 600, 300, 150, 125, 75, 37.5, 18.7, 9.3 and 0 g a.i. ha^{-1} of 2,4-D. A total of four replicates were included at each dose. Herbicides were applied using a precision bench sprayer delivering 200 L ha^{-1}, at a pressure of 215 kPa. Pots were placed in a greenhouse in Lleida, Spain (41°37'43.1"N - 0°35'52.6"E) and were watered regularly to field capacity. Four weeks after treatment, (WAT) above ground biomass of the plants from each dose were harvested. Samples were dried at 65°C for 48h, and the dry weights were measured.

Field experimental design

The experiments were carried out during three consecutive cropping seasons (2011–12, 2012–13 and 2013–14) to evaluate the effect of eight different weed management strategies on two HR *P. rhoeas* populations.

The eight management systems were: 1-Traditional (TRA), wheat monocrop with POST chemical control; 2-Herbicide Rotation (HROT), wheat monocrop with POST chemical control (active ingredient rotation); 3-Early Post (EAPOST), wheat monocrop with chemical control (active ingredient rotation and application timing rotation); 4-PRE plus POST (PRE+POST), wheat monocrop with chemical control (active ingredient rotation and application timing rotation); 5-Oilseed rape rotation (OSR), wheat–Oilseed rape–wheat with chemical control; 6-Field pea rotation (FPR), wheat–field pea–wheat with chemical control; 7-Sunflower rotation (SFLR), wheat–sunflower–wheat with chemical control; 8-Seed delay (DLY), wheat monocrop with seed delay in the first and third

seasons (almost one month) and chemical control (active ingredient rotation). The experimental design was a complete randomized block with three replicates and eight plots (10 × 10 m). A 4 m corridor was left between plots. Sowing doses for wheat cv. 'Berdún' was 200 kg ha^{-1}, 260 kg ha^{-1} for oilseed reap cv. 'Arsenal', 180 kg ha^{-1} for field peas cv. 'Enduro', and 9 kg ha^{-1} for sunflower cv. 'Limasun'. Sowing dates for each management system are specified in Table 5.1. The applications were performed with a backpack plot sprayer using a 2-m-wide boom calibrated to deliver 300 l ha^{-1} of water at 253 kPa pressure. All details about the herbicide applications are summarized in Table 5.2. Agronomic practices were the constant in the areas of study.

Table 5.1. Sowing dates for each management system in 2011-12, 2012-13 and 2013-14 seasons at Baldomar (L-1) and Sant Antolí (L-2).

Management strategy	2011-12		2012-13		2013-14	
	(L-1)	*(L-2)*	*(L-1)*	*(L-2)*	*(L-1)*	*(L-2)*
1-TRAD	Wheat		Wheat		Wheat	
	26/10	30/10	25/10	30/10	22/10	04/11
2-HROT	Wheat		Wheat		Wheat	
	26/10	30/10	25/10	30/10	22/10	04/11
3-EAPOST	Wheat		Wheat		Wheat	
	26/10	30/10	25/10	30/10	22/10	04/11
4-PRE+POST	Wheat		Wheat		Wheat	
	26/10	30/10	25/10	30/10	22/10	04/11
5-OSR	Wheat		Oilseed rape		Wheat	
	26/10	30/10	01/10	01/10	22/10	04/11
6- FPR	Wheat		Field peas		Wheat	
	26/10	30/10	15/11	15/11	22/10	04/11
7- SFLR	Wheat		Sunflower		Wheat	
	26/10	30/10	29/04	29/04	22/10	04/11
8- DLY	Wheat		Wheat		Wheat	
	30/11	28/11	25/10	30/10	26/11	26/12

Papaver rhoeas density was counted monthly, from sowing to harvest, by randomly throwing ten frames of 0.10 m^2 into each plot. Initial densities were estimated between December and February in each season. These estimations were proxies of the management effects of the preceding season in the *P. rhoeas* seed bank. These values were considered more appropriate and less time consuming for estimating the *P. rhoeas* seed bank than other methodologies, which are of limited predictive efficacy due to extended seed longevity (i.e. germination in greenhouse) and/or tiny seed size (i.e. cleaning of samples). The experiment ended in June 2014 (2013-14 season), but *P. rhoeas* densities were also counted at the beginning of the 2014-15 season in January

2015. This sampling was considered as a proxy of the overall cumulative effect of the three years of management strategy application on the *P. rhoeas* seed bank.

Table 5.2. Herbicide application date, herbicide hanagement, active ingredient and rate (g a.i. ha^{-1}) used for different management systems in 2011-12, 2012-13 and 2013-14 seasons at Baldomar (L-1) and Sant Antolí (L-2).

MANAGEMENT STRATEGY	**2011-12**		**2012-13**		**2013-14**	
	(L-1)	*(L-2)*	*(L-1)*	*(L-2)*	*(L-1)*	*(L-2)*
1-Traditional (TRAD)	05/01 [a] Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630[d]	09/01	05/02 Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630	20/02	18/02 Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630	19/02
2-Herbicide Rotation (HROT)	05/01 Post Application Aminopyralid + Florasulam 10 + 4.5	09/01	05/02 Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630	20/02	18/02 Post Application Aminopyralid + Florasulam 10 + 4.5	19/02
3-Early Post (EAPOST)	05/12 [b] Early Post Application [e] Hormonal mixture --	20/12	05/02 Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630	20/02	21/01 Early Post Application [e] Hormonal mixture --	01/02
4-Pre + Post (PRE+POST)	02/11 [c] Pre Aplication Isoxaben 125 05/12 Post Application Aminopyralid + Florasulam 10 + 4.5	01/11 20/12	05/02 Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630	20/02	18/12 Pre Application Isoxaben 125 18/02 Post Application Aminopyralid + Florasulam 10 + 4.5	18/12 19/02
5- Oilseed rape rotation (OSR)	05/12 Post Application Aminopyralid + Florasulam 10 + 4.5	20/12	05/11 Pre Application Propyzamide 700 01/02 Post Application Aminopyralid + Clopyralid 6.25 + 127	25/10 01/02	18/02 Post Application Aminopyralid + Florasulam 10 + 4.5	19/02
6- Field peas rotation (FPR)	05/12 Post Application Aminopyralid + Florasulam 10 + 4.5	20/12	15/11 Pre Application Pendimenthalin 1,365	15/11	18/02 Post Application Aminopyralid + Florasulam 10 + 4.5	19/02
7- Sunflower rotation (SFLR)	05/12 Post Application Aminopyralid + Florasulam 10 + 4.5	20/12	29/04 Pre Application Benfluralin 990	29/04	18/02 Post Application Aminopyralid + Florasulam 10 + 4.5	19/02
8- Seed dealy (DLY)	05/01 Post Application Aminopyralid + Florasulam 10 + 4.5	09/01	05/02 Post Application Bromoxynil + Ioxynil + MCPP 210 + 210 + 630	20/02	18/02 Post Application Aminopyralid + Florasulam 10 + 4.5	19/02

[a] Post: Post-emergence application.

[b] Early-Post: Early Post-emergence application.

[c] Pre: Pre-emergence application.

[d] g a.i. ha^{-1}.

[e] Hormonal mixture. Hormonal mixture containing a new synthetic auxin was employed in the early post-emergence applications.

Statistical analysis

Data from dose-response experiments were analyzed using a non-linear regression model. The GR_{50} of plants was calculated using a four parameter logistic curve of type 1:

$$y = c + \frac{(d-c)}{1+\mathrm{EXP}[b(\log(x)-\log(GR50)]} \quad (1)$$

where *c* is the lower limit, *d* is the upper limit, GR_{50} is the herbicide rate required for 50% growth reduction and *b*, the slope at GR_{50}. In this equation, the herbicide rate (g a.i. ha^{-1}) was the independent variable (*x*) and the dry weight (percentage of the untreated control for each population) was the dependent variable (*y*).

For the field experiment, the effect of treatments on both initial and final *P. rhoeas* densities in each season was tested with Linear Mixed-effects Models (LMM). Densities for each location were analyzed separately because different *P. rhoeas* biotypes were considered at each site. The treatments were established as fixed factors, whereas replicates blocks were used as random factors. *Papaver rhoeas* density data were transformed as needed (log (x+1) or √ (x+0.5)) prior to the analysis because exploratory analysis revealed some non-normal data distributions and heterogeneity of variances (Zuur et al., 2010). Only in two cases (2011/12 and 2012/13 final densities of L-2) where these assumptions were not met, a non-parametrical tests (Kruskal-Wallis) was employed. Finally, a post-hoc Tukey´s pairwise comparison was used to test differences between treatment means (at $P<0.05$). Data was back-transformed to the original scale for presentation. Data from management involving PRE treatments or a seed delay were not included in initial *P. rhoeas* density analysis because these interventions disturbed the natural germination pattern of *P. rhoeas* seedlings.

The reduction in initial *P. rhoeas* densities (seedlings m^{-2}) between 2011 and 2015 (DR) was calculated as (2):

$$\mathrm{DR} = 100 - \left[\frac{(\text{Initial Density in 2015}*100)}{\text{Initial Density in 2011}}\right] \quad (2)$$

Analysis of variance (ANOVA) was conducted with DR values. Data were transformed as needed with (arcs[√(x+0.5)]) when normal assumptions were not met. DR means of the different managements were separated using Tukey´s pairwise comparison at a 0.05 probability level. Data were back-transformed for presentation.

All statistical analyses were carried out with the use of the R programming language (R Development Core Team 2013). *drc* package (Knezevic et al., 2007) for the non-linear regression and *LME4* (Bates et al., 2014) together with *nlme* (Pinheiro et al., 2014) packages for the LMM were employed.

Results and Discussion

Resistance profile of the Papaver rhoeas *populations, greenhouse experiment*

The presence of multiple HR resistant biotypes was confirmed at both localities. No mortality of populations from L-1 and L-2 at the herbicides' commercial label rates was found (data not shown). In contrast not a single SC plant survived at field rates of tribenuron-methyl (18.7 g a.i. ha^{-1}), florasulam (7.5 g a.i. ha^{-1}) or 2,4-D (600 g a.e. ha^{-1}). In contrast, there was. The GR_{50} for tribenuron-methyl were 320 and 392 times higher in plants from L-1 and L-2 than in the SC population (Table 5.3).

Table 5.3. Estimated GR_{50}, slope at GR_{50} and resistance factor (RF) values for Baldomar (L-1), Sant Antolí (L-2) and susceptible (SC) corn poppy (*Papaver rhoeas*) populations when sprayed with tribenuron-methyl, florasulam and 2,4-D.

Population	GR_{50} ± SE (g a.i. ha^{-1})[a]	Slope ± SE[b]	Res SS[c]	RF[d]
tribenuron-methyl				
L-1	25.22 ± 6.38	0.58 ± 0.09	10084	286
L-2	30.92 ± 8.06	0.61 ± 0.09	10609	351
SC	0.08 ± 0.02	0.43 ± 0.08	4894	1
florasulam				
L-1	3.90 ± 0.38	2.01 ± 0.36	3899	24
L-2	2.92 ± 0.30	0.87 ± 0.08	1529	18
SC	0.16 ± 0.03	0.69 ± 0.12	21738	1
2,4-D				
L-1	816.60 ± 96.00	1.27 ± 0.16	2872	12
L-2	925.80 ± 156.01	1.02 ± 0.28	5038	13
SC	68.60 ± 10.20	1.15 ± 0.16	23693	1

[a]GR_{50}, ALS inhibitor concentration for 50% reduction of *P. rhoeas* dray weight biomass.

[b]The slope at $GR_{50.}$

[c]Res SS, residual sum of square.

[d]RF (resistance factor) = GR_{50}(Population L-1 or L-2) / GR_{50}(SC).

Resistance factors obtained for tribenuron-methyl in this study were similar to those observed in resistant *P. rhoeas* biotypes in Greece (Kaloumenos et al., 2011). In that study multiple substitutions in Pro_{197} were also determined. Resistance to tribenuron-methyl established in L-1 and L-2 biotypes could also be the result of different

substitutions of this amino acid. In addition, cross resistance between sulfonylureas and triazolopyrimidines was observed in plants at both locations, and L-1 and L-2 biotypes were 24 and 18 times more resistant to florasulam than SC plants (Table 5.3). Similar experiments conducted with tribenuron-methyl-resistant *P. rhoeas* confirmed cross resistance between these two different ALS-inhibiting herbicide classes (RF for florasulam went from 5 to 25) (Kaloumenos et al., 2011). Resistance to 2,4-D was also confirmed and plants from L-1 and L-2 were 12 and 13 times more resistant to this herbicide than the SC plants (Table 5.3). Results obtained for a multiple HR resistant Greek biotype established a GR_{50} for 2,4-D of 1127 g a.i. ha^{-1} (Kati et al., 2014). In our experiment, these values were 816 and 925 g a.i. ha^{-1} for L-1 and L-2, respectively.

Papaver rhoeas *density evolution*

At the beginning of the first season (2011-12), the densities within each location were homogenous, and no statistical differences were detected between plots. Initial *P. rhoeas* density at L-1 reached on average 326 seedlings m^{-2}, being lower than at L-2, where 740 seedlings m^{-2} were counted on average (Figure 5.1 and Table 5.4). In this first season, three herbicide management strategies were used (PRE, Early POST and POST application) and only one cultural management (DLY) was performed. All these treatments significantly reduced the *P. rhoeas* density at the end of this season, but the strategy that achieved best results in both locations was PRE+POST, with 3 and less than 1 plants m^{-2} at L-1 and L-2, respectively (Table 5.4).

Overall, initial density in the second season (2012-13) was lower than those initial densities observed in the preceding season (Figure 5.1). At L-2 the system PRE+POST obtained statistically less initial density (36 seedlings m^{-2}) than the other management systems (from 83 to 119 seedlings m^{-2}) (Table 5.4). Similarly, at L-1 the strategy PRE+POST also obtained the lower initial density (49 seedlings m^{-2}), but it was not different from densities obtained by other strategies such as DLY, EAPOST and HROT (54, 66 and 77 seedlings m^{-2}, respectively) (Table 5.4). In the second season, one herbicide management strategy was used in cereals (POST), reducing the *P. rhoeas* density at the end of the season to on average of 11 plants m^{-2} at L-1 and less than 1 plants m^{-2} at L-2.

Figure 5.1. Corn poppy (*Papaver rhoeas*) density (plants m^{-2}) during four seasons for each management strategy at Baldomar, L-1 (a) and Sant Antolí, L-2 (b). *TRAD*, wheat monocrop with chemical control; *HROT*, wheat monocrop with active ingredient rotation; *EAPOST*, wheat monocrop with active ingredient rotation and application timing rotation; *PRE+POST*, wheat monocrop with active ingredient rotation and application timing rotation; *OSR*, wheat–Oilseed reap–wheat rotation; *FPR*, wheat–field pea–wheat rotation; *SFLR*, wheat–sunflower–wheat rotation; *DLY*, wheat monocrop with seed delay in the first and third seasons.

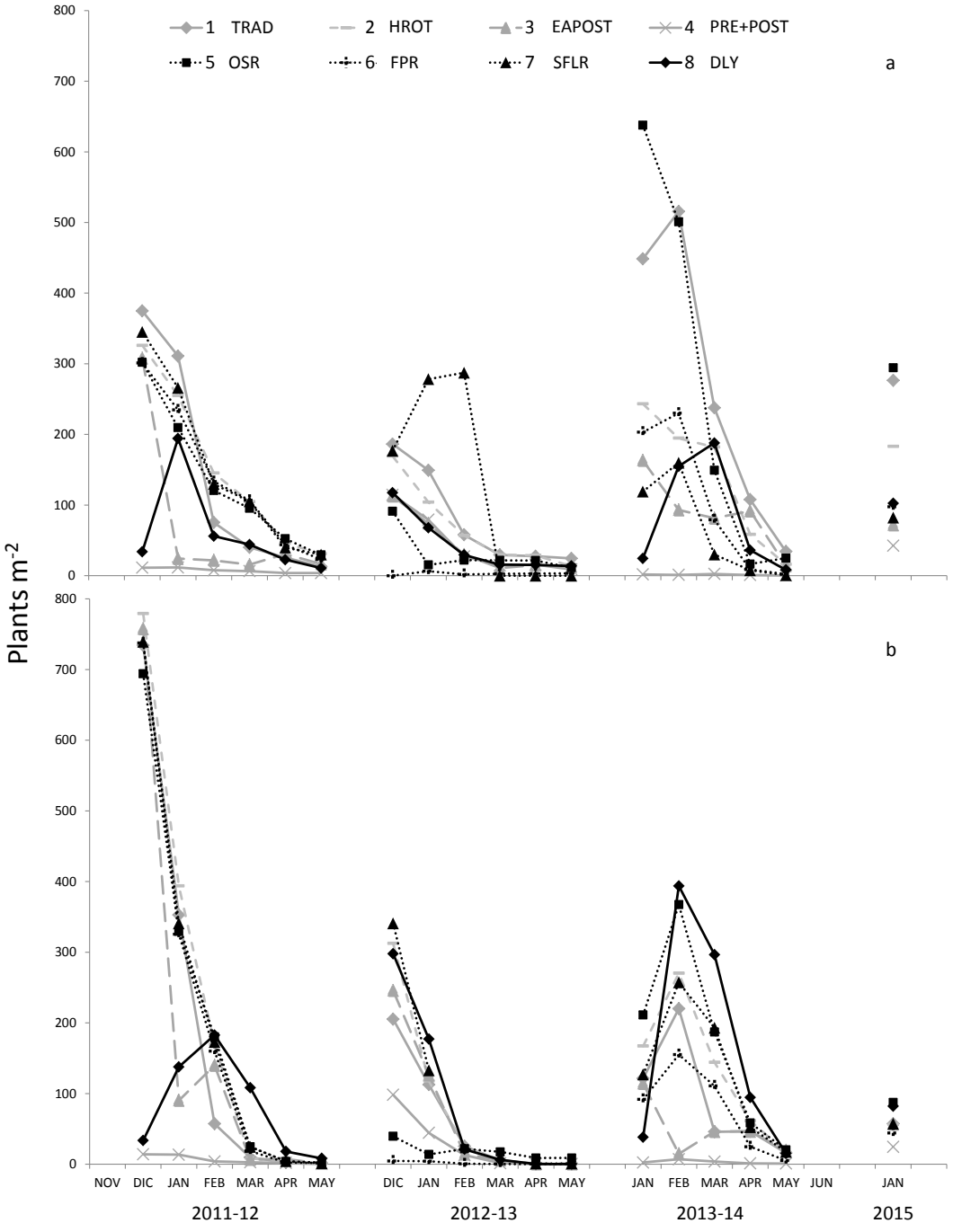

Table 5.4. Mean corn poppy (*Papaver rhoeas*) densities (plants m^{-2}) in different management systems in 2011-12, 2012-13, 2013-14 and 2015 for data collected at Baldomar (L-1) and Sant Antolí (L-2). Data are back-transformed means used for the LMM. *TRAD*, wheat monocrop with chemical control; *HROT*, wheat monocrop with active ingredient rotation; *EAPOST*, wheat monocrop with active ingredient rotation and application timing rotation; *PRE+POST*, wheat monocrop with active ingredient rotation and application timing rotation; *OSR*, wheat–Oilseed reap–wheat rotation; *FPR*, wheat–field pea–wheat rotation; *SFLR*, wheat–sunflower–wheat rotation; *DLY*, wheat monocrop with seed delay in the first and third seasons.

	[a]**2011-12**				**2012-13**				**2013-14**				**2015**	
	L-1		*L-2*		*L-1*		*L-2*		*L-1*		*L-2*		*L-1*	*L-2*
	Initial Density	*Final Density*	*Initial Density*	*[d]Final Density*	*Initial Density*	*Final Density*	*Initial Density*	*[d]Final Density*	*Initial Density*	*Final Density*	*Initial Density*	*Final Density*	*Initial Density*	*Initial Density*
1-TRAD	320.27 ([b]*A*)	26.87 (*CB*)	616.62 (*A*)	1.53 (*BA*)	144.44 (*A*)	17.70 (*A*)	83.65 (*A*)	0.30 (*A*)	366.80 (*A*)	29.31 (*A*)	200.81 (*A*)	11.48 (*BA*)	276.41 (*AB*)	57.24 (*AB*)
2-HROT	274.79 (*A*)	28.97 (*A*)	666.35 (*A*)	1.22 (*BA*)	77.42 (*BC*)	10.20 (*CBA*)	99.12 (*A*)	0.91 (*A*)	206.63 (*B*)	11.76 (*B*)	229.48 (*AB*)	11.12 (*BA*)	182.93 (*B*)	56.22 (*AB*)
3-EAPOST	284.71 (*A*)	19.79 (*CB*)	616.53 (*A*)	0.91 (*B*)	66.87 (*B*)	9.92 (*CBA*)	93.93 (*A*)	0.30 (*A*)	141.10 (*BC*)	10.92 (*B*)	117.80 (*C*)	12.07 (*BA*)	71.89 (*DC*)	56.51 (*AB*)
4-PRE+POST	[c] --	2.99 (*D*)	--	0.30 (*B*)	49.25 (*B*)	7.00 (*DB*)	36.74 (*B*)	0.30 (*A*)	--	0.23 (*D*)	--	0.78 (*C*)	42.36 (*D*)	24.41 (*C*)
5-OSR	266.53 (*A*)	26.72 (*A*)	627.06 (*A*)	2.14 (*BA*)	--	12.50 (*BA*)	--	8.87 (*B*)	611.67 (D)	3.79 (*DC*)	320.46 (*B*)	14.33 (*A*)	294.43 (*A*)	87.39 (*A*)
6-FPR	280.71 (*A*)	19.82 (*BA*)	620.92 (*A*)	1.83 (*BA*)	--	2.83 (*DC*)	--	0.30 (*A*)	174.76 (*BC*)	1.74 (*DC*)	128.08 (*C*)	3.89 (*CB*)	97.70 (*DC*)	44.19 (*BC*)
7-SFLR	294.61 (*A*)	38.43 (*A*)	588.29 (*A*)	1.53 (*BA*)	114.53 (*AC*)	1.05 (*D*)	92.33 (*A*)	0.30 (*A*)	101.99 (*C*)	0.47 (*D*)	233.22 (*AB*)	11.87 (*BA*)	82.25 (*DC*)	57.24 (*AB*)
8-DLY	--	9.10 (*DC*)	--	8.26 (*A*)	54.93 (*B*)	11.07 (*BA*)	119.84 (*A*)	0.30 (*A*)	--	6.11 (*CB*)	--	12.47 (*A*)	102.45 (*C*)	81.98 (*A*)

[a]Sampling dates included in the statistical analysis. Initial density: Season 2011-12: 20/12/2011 Season 2012-13: 09/01/2013; Season 2013-14: 21/01/2014 in Baldomar and 10/02/2014 in Sant Antoli and in 2015: 15/01/2015. Final density: Season 2011-12: 03/05/2012; Season 2012-13: 08/05/2013; Season 2013-14: 27/05/2014.

[b]Means within a column followed by the same letter indicate that no significant difference ($P < 0.05$) was detected by means of the Tukey (HSD) test at the 5% level of probability

[c]Initial density data from those managements with any intervention that avoid the natural germination pattern of *P. rhoeas* seedlings (seed delay and PRE treatments) were not included in the analysis.

[d]Due to the abundance of zeros non parametric test were conducted with 2011-12 and 2012-13 final density data in L-2.

The results for the crop rotations at the end of this second season were unequal, FPR (3 and less than 1 plants m^{-2} at L-1 and L-2, respectively) and SFLR (1 and less than 1 plants m^{-2} at L-1 and L-2, respectively) also significantly reduced the number of plants, while OSR was the management system that achieved more density in May 2013 (12 and 8 plants m^{-2} at L-1 and L-2, respectively) (Table 5.4).

The analysis of the initial *P. rhoeas* density in the third season (2013-14), revealed that in both locations the OSR rotation obtained the highest density. These results highlight the importance of avoiding the incorporation of new seeds into the soil in order to achieve an effective management strategy in the mid to long term (Norsworthy et al., 2012), especially for weeds like *P. rhoeas*, with persistent and abundant seedbanks (Cirujeda et al., 2008). On the contrary, at L-1 the SFLR system was the management strategy that obtained the lowest initial *P. rhoeas* density (101 seedlings m^{-2}), but this was not statistically different from that observed in other managements (FPR and EAPOST) (Table 5.4). At L-2, those strategies that reached a lower initial density were EAPOST and FPR with mean values of 117 and 128 seedlings m^{-2}, respectively (Table 5.4).

With less than one plant per square meter in both locations, the PRE+POST strategy was the alternative with the least amount of plants at the end of the third season. TRAD at L-1 and OSR at L-2 were the managements where the most plants were counted in May 2014: 29 and 14 plants m^{-2} (Table 5.4).

Three-year assessment of management systems

The initial density evaluated in 2015 reflects the cumulative effect of the three preceding seasons for the different evaluated management systems. Data collected in both locations (before any POST herbicide applications) showed that out of all the different management strategies, those which included sunflower or field peas, or those that introduced a modification to herbicide timing (PRE+POST and EAPOST) registered the lowest initial *P. rhoeas* densities after three years (Table 5.4). The favorable results observed for SFLR management can be explained by the agronomic practices used in sunflower which contributed to the elimination of emerged seedlings of a great number of *P. rhoeas* plants. Sunflower sowing begins in April, and *P. rhoeas* emergence in semi-arid Mediterranean conditions occurs mainly in autumn and winter (Cirujeda et al., 2008). For this reason, seedbed preparation and crop sowing in winter break the weed life-cycle, thus eliminating almost all *P. rhoeas* plants. Despite the

significant reduction in *P. rhoeas* density achieved by this system, lack of rainfall in spring and summer in North-Eastern Spain hinders the integration of this type of crop rotation. In other areas of Spain with higher rainfall, and where herbicide resistant *P. rhoeas* is present, this crop rotation is a real option for resistant population control. *P. rhoeas* reduction obtained by the FPR strategy was achieved mainly due to the use of pendimethalin in PRE. This herbicide has been proposed as one of the best chemical options for HR *P. rhoeas* control in Spanish dry land areas (Torra et al., 2010). The use of a FPR could be improved using spring varieties of field peas, which again would allow eliminating seedlings in winter and, thus, breaking the life cycle of *P. rhoeas*. Regarding the management strategies that introduced an herbicide timing modification (PRE+POST and EAPOST), it is hypothesized that early applications (both PRE and early POST) achieve higher efficacy because variability in weed phenology at application time is avoided compared to POST treatments. Finally, it was proposed that drastic tools could be necessary in those highly infested fields with herbicide resistant weeds (Cirujeda and Taberner 2009). As PRE+POST showed, this strategy could be seriously considered in cases where *P. rhoeas* densities are high and its control is tough.

A sowing delay of one month did not improve *P. rhoeas* control within a season when compared to the other systems with normal sowing dates (Table 5.4). An extended sowing delay is most likely necessary to improve the management of this weed due to its broad emergence, which can last from December to March (Cirujeda et al., 2008). The use of cereal varieties with short life cycles and delaying the sowing three months was proposed as a management option that can improve the *P. rhoeas* seed bank depletion (Torra et al., 2011). OSR was also inefficient in the management of *P. rhoeas* in this study. This strategy obtained more initial density in 2015, as in 2013-14, especially at L-1 where an average of 294 seedlings m^{-2} were counted (Table 5.4). Contrary to the situation with SFLR, the agronomic practices required by oilseed rape (the majority of them conducted in September when sown), extend the emergence period of *P. rhoeas* within a crop situation, and thus do not break its life cycle. Moreover, oilseed rape is not a competitive crop in its early life stages, and a small number of active ingredients are available for dicotyledonous weed control in POST. Finally, TRAD management system did not achieve good results, (276 and 57 seedlings m^{-2} at L-1 and L-2, respectively) especially at L-1 (Table 5.4). At high densities, even if the timing of POST application is optimal, some overgrown *P. rhoeas* individuals will

escape treatments. Few of those uncontrolled plants can be enough to replenish the seedbank for the subsequent seasons due to their high fecundity (Torra et al., 2008).

Over three years of management (from the end 2011 until early 2015), it was possible to reduce *P. rhoeas* infestations levels at both locations. It is striking that reduction was much higher for L-2 than for L-1 being on average 57% at L-1 and 90% at L-2 (Table 5.5). At L-1, PRE+POST (81%) and SFLR (72%) were the strategies which led to a more drastic reduction of the initial *P. rhoeas* densities, but these percentages were not significantly different from those obtained by other management strategies like EAPOST or FPR. However, OSR, TRAD and HROT were the alternatives that less density reduction (DR) reached (20, 33 and 41%, respectively). At L-2, PRE+POST and FPR managements, followed by HROT and TRAD, obtained the highest percentages of initial *P. rhoeas* DR after three years (95, 92, 91 and 90 %, respectively), while OSR, SFLR and DLY obtained lower DR values (84, 89 and 87%, respectively) without statistical differences among them (Table 5.5).

Table 5.5. Reduction of the initial corn poppy (*Papaver rhoeas*) density between initial density in December 2011 and initial density in January 2015 (DR) at Baldomar (L-1) and Sant Antolí (L-2). *TRAD*, wheat monocrop with chemical control; *HROT*, wheat monocrop with active ingredient rotation; *EAPOST*, wheat monocrop with active ingredient rotation and application timing rotation; *PRE+POST*, wheat monocrop with active ingredient rotation and application timing rotation; *OSR*, wheat–Oilseed reap–wheat rotation; *FPR*, wheat–field pea–wheat rotation; *SFLR*, wheat–sunflower–wheat rotation; *DLY*, wheat monocrop with seed delay in the first and third seasons.

	L-1	*L-2*
1-TRAD	33.28 ([a]*A*)	90.45 (*AB*)
2-HROT	41.71 (*A*)	91.67 (*AB*)
3-EAPOST	74.58 (*B*)	90.17 (*B*)
4-PRE+POST	81.87 (*B*)	95.65 (*A*)
5-OSR	20.62 (*A*)	84.87 (*C*)
6-FPR	65.74 (*B*)	92.56 (*AB*)
7-SFLR	72.14 (*B*)	89.44 (*BC*)
8-DLY	65.84 (*B*)	87.88 (*BC*)

[a]Means within a column followed by the same letter indicate that no significant difference ($P < 0.05$) was detected by DR means of the Tukey (HSD) test at the 5% level of probability.

As noted above, all strategies achieved much more density reduction at L-2 than at L-1. The differences could be due to the different efficacy levels achieved to florasulam (ALS inhibitor) plus aminopyralid (synthetic auxin) in the first and third year, which

were applied in all management strategies except TRAD (Table 5.2). *P. rhoeas* population from L-2 was more susceptible than population from L-1 which could be explained by differences in the ALS cross resistant patterns between biotypes (L-1 and L-2). Recent work has shown that only plants carrying a Ser_{197} ALS allele were moderately resistant to florasulam compared to plants carrying ALS alleles with other substitutions, which were susceptible (Délye et al., 2011). In this study, the RF for florasulam was six points higher in L-1 compared to L-2, highlighting higher frequencies of Pro_{197} to Ser mutants in the first location (L-1). More complete knowledge on the genetic basis of resistance and cross resistance patterns for ALS-inhibiting herbicides has been described as important for formulating adequate chemical control strategies of local *P. rhoeas* populations (Torra et al., 2010).

Conclusions

To summarize, the integration of different control tools, both chemical and cultural, were useful for the management of multiple HR *P. rhoeas* populations in winter cereals. Crop rotation with (spring) field peas is an interesting option, and in those areas where rainfall is not restrictive summer crops, such as sunflower, are very promising alternatives. Rotation and combination of herbicides with different application timings can also be effective in managing HR *P. rhoeas*. PRE plus POST interventions can provoke a significant depletion of the soil seedbank and could be an option in highly infested fields. This study also highlights that complete knowledge of the genetic basis of resistance and cross-resistance patterns for ALS inhibitors could be important in designing better chemical programmes adapted to local biotypes. Therefore, successful integrated management strategies of multiple HR *P. rhoeas* populations is necessary for reducing herbicide selection pressure and slowing down the evolution of new resistant biotypes.

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CHAPTER 6

General discussion

The mechanisms conferring resistance to ALS inhibitors as well as to synthetic auxin were investigated in 2,4-D and 2,4-D and tribenuron-methyl multiple resistant Spanish corn poppy (*Papaver rhoeas)* populations. The basis of lack of control of some populations with the PS II inhibitor bromoxynil were also explored. To finalize, several integrated corn poppy resistance management strategies were designed and tested at two fields with high densities of multiple resistant corn poppy populations.

The study of resistance to ALS inhibitors in multiple resistant (2,4-D and tribenuron-methyl), 2,4-D resistant and susceptible corn poppy populations has shown six amino-acid replacements at position Pro197 (Ala197, Arg197, His197, Leu197, Thr197 and Ser197). As expected, all these replacements conferred strong resistance with resistance indexes higher than 300 to SU's (tribenuron-methyl), but this assumption could not be held for others non-SU ALS inhibitors as TP's (florasulam) or IMI's (imazamox). Only substitutions in Pro197 by Thr for imazamox and Ser for florasulam showed moderate to strong resistant responses to these herbicides. Perplexing results were reached when genotype and phenotype of some plants treated with florasulam or imazamox were compared between each other. Plants identically genotyped showed different responses to the same herbicide (florasulam or imazamox). As it has been recently published (Scarabel et al., 2015), non-target-site resistant (NTSR) mechanisms may explain these results. So, in this point we hypothesize that those NTSR mechanisms affecting SU herbicides, if any, do not become evident under the strong resistance conferred by any amino-acid replacement at Pro197. On the contrary, because Pro197 is not involved in anchoring those non-SU herbicides, mutation at this position does not confer a high level of resistance and under these conditions are when suspected NTSR mechanisms can co-exist and become evident together with target-site resistance (TSR) mechanisms. Moreover, no mutated plants from only the 2,4-D resistant population, together with one no mutated plant from the control susceptible population were able to survive the imazamox applications.The presence of metabolic resistance can be confirmed by identification of resistance phenotypes with a lack of ALS resistant mutations (Scarabel et al., 2015; Yu and Powles 2014; Yu et al., 2009). Therefore, the presence of NTSR mechanisms in Spanish corn poppy populations to non-SU ALS inhibitors was confirmed in this research. Our results suggest that this weed species may be armed with low-level defences against herbicides, at least for non-SU herbicides, prior to the imposition of any selection. Evolution of resistance simply requires them to evolve

enhancements of physiological capacities that this species already possess (Neve et al., 2014). Metabolism-based resistance pre-exist in weed populations at sufficient levels that allow individuals in previously unselected populations to survive (Neve and Powles 2005). In this line, a recent work reveals that metabolic resistance to ALS inhibitor herbicides in weeds usually mimics herbicide-tolerant crops via enhanced rates of herbicide metabolism, often involving cytochrome P450 (Yu and Powles 2014). Resistance to ALS inhibitors in corn poppy appears far more complex than previously thought from studies having only identified TSR in this species. Besides, in this study two mutations outside of the conserved regions of the ALS gene (Gly427 and Leu648) were also detected, but they did not seem to play a relevant role in ALS inhibitors resistant response. Finally, results obtained in the experiment with labelled ^{14}C-tribenuron-methyl by plants only 2,4-D resistant suggested that the 2,4-D resistance mechanism could interfere with the normal phytotoxical process triggered by tribenuron-methyl few hours after its application. Hypothesis to explain these results are addressed below.

Until now, no other studies on the mechanistic basis of 2,4-D resistance in corn poppy have been carried out. Although leaf absorption of the herbicide was almost total and similar between multiple resistant, 2,4-D resistant and susceptible plants, the labelled 2,4-D did not move out of the treated leaf in those multiple resistant and 2,4-D resistant plants. In contrast ^{14}C-2,4-D was rapidly translocated in a susceptible biotype. Similar results were obtained in other dicot weeds with multiple resistance (2,4-D and ALS inhibitors) (Goggin and Powles 2014; Shane-Friesen 2007). An alteration in some specific transporters belonging to PIN-FORMD (PIN) or ATP-binding cassette (ABC), families, may explain why resistant plants do not transport the 2,4-D. Moreover, ABC transporters not only have been reported to mediate cellular transport of auxin, but these proteins have also been speculated to be involved in the detoxification of xenobiotics (Cho and Cho 2013). On these bases, these are candidate genes to be researched in future studies. However, it remains also possible that rapid production of polar metabolites, less phloem mobile than parent compounds (due to permanent sequestration in the vacuole and detoxification reactions), could have been decreasing the 2,4-D translocation (Peterson et al., 2015). 2,4-D metabolism occurs primarily through direct conjugation or ring hydroxylation. Direct glucose conjugation of 2,4-D occurs with glucosyl transferase enzymes to form glucose esters and generally; these conjugates are more prevalent in susceptible dicots, inducing auxin-related activity

similar to 2,4-D and are readily hydrolyzed back to 2,4-D acid (Hatzios and Hock 2005). On the other hand, ring hydroxylation occurs through a reaction with cytochrome P450 and metabolites from this pathway cannot be hydrolyzed back to 2,4-D (Cobb and Reade 2010).Taking into account all this information, the lack of 2,4-D mobility in resistant corn poppy plants could be consequence of the above mentioned metabolic pathways. But an alteration in some specific transporters and metabolic processes may coexist in resistant plants, as it has been proposed in a similar case (Goggin and Powles 2014). Returning to the results exhibited by plants only 2,4-D resistant in experiments conducted with ALS inhibitors. The above mentioned alterations of ABC transporters, also involved in detoxifying xenobiotics, or metabolic pathways associated to 2,4-D may explain the abnormal phytotoxical process triggered by tribenuron-methyl for the biotype only auxin resistant.

As it has been reported in many other synthetic auxins resistant weeds (Howatt et al., 2006), more ethylene production was detected in multiple and 2,4-D resistant corn poppy plants. 2,4-D spraying initiates a cascade of physiological responses within the plant, ultimately leading to plant death in susceptible dicots. In the first phase of this process ethylene production is stimulated (Grossmann 2010). Probably any of the previously mentioned deregulations could have a downstream effect, hence reducing the ethylene production in resistant plants. Our multiple and 2,4-D resistant corn poppy populations appeared to be also resistant to dicamba and aminopyralid, what means that those mechanisms conferring resistance to 2,4-D are also playing a relevant role against other auxinic herbicides. A new different hypothesis can explain these results: cross resistance between 2,4-D and dicamba in *Arabidopsis thaliana* was attributed to a mutation in the TIR-1 protein (F-box family of a nuclear auxin receptors) (Gleason et al., 2011). Nevertheless suspected metabolic process (P450s or glutathione S-transferases) have been pointed to lead to cross-resistance between synthetic auxin chemistries (Peterson et al., 2015). All this work is just the first steps, because the physiological bases of lack of translocation in those resistant plants needs further research and the effect of these mechanisms on ALS inhibitors resistance need to be elucidated. The study of the mechanisms conferring resistance to synthetic auxins is not an easy issue; few articles addressing resistance to these herbicides have been published compared to other modes of action. Moreover, an important factor to consider when resistance to synthetic auxin herbicides matter is addressed is that it may be extremely difficult to disentangle the strong links between auxin receptors/binding

proteins, auxin transport and metabolism between natural (IAA) and synthetic auxin herbicides. The introduction of new transgenic 2,4-D and dicamba resistant crops may result in increased use of these synthetic auxin herbicides. On these bases, increased emphasis on mechanisms of auxin resistance and their potential to arise in weed species is crucial.

Lack of control with mixtures containing bromoxynil in some corn poppy infested fields was attributed to an inappropriate phenological stage at herbicide application timing. Nevertheless, it has been described that these above mentioned circumstances, leading to sub-lethal dose conditions, have the potential to accelerate resistance evolution and lead to more cross-resistance by NTSR (Neve et al., 2014). Few plants from one population, however not enough to produce a significant shift in the dose-response curves, were able to survive recommended rates of bromoxynil. Further studies are being conducted with these plants in order to check the presence of mutations at *psbA* gene, as a possible TSR mechanism. If there were no mutations in these samples, NTSR has to be considered. Because all the populations considered in this study were multiple resistant, and if resistance to bromoxynil is confirmed in the future, it would remain to be elucidated which is the relationship between resistance mechanisms among these three different modes of action (ALS inhibitors, synthetic auxins and PS II inhibitors), if any. It could be possible that those mechanisms conferring resistance to 2,4-D were linked with NTSR to non-SU herbicides above mentioned and unspecific responses observed in bromoxynil, likely detoxification or altered transport.

To conclude, it has been demonstrated that effective control of multiple resistant corn poppy populations is possible using integrated weed management strategies. Results from our field studies suggest that not all crop rotations are helpful in a resistant corn poppy management programme. This is the case of cereal-oilseed rape rotation. As it was suggested previously (Cirujeda 2001), the agronomic practices associated with the oilseed rape did not break the life cycle of corn poppy. Moreover few useful herbicides are available in this crop to control it. On the contrary, incorporating field peas and sunflower in the cereal monocrop showed good results. Both rotations were able to affect significantly the life cycle of corn poppy and allowed also to introduce other herbicides with different modes of action (MOA's). Regarding herbicide management strategies, the most successful systems were those which included a variation in the herbicide application timing, early post-emergence or combining pre-emergence and post-emergence. These early applications allow avoiding largest corn poppy

phenologies that may reduce the treatment efficacy. Taking into account these results, we consider that diversification at all levels has to be the major objective in weed control. Crop rotations, with crops that really disturb the life cycle of weeds, must be the first step. Over this, modifications of the sowing date and variations on ploughing practices also have to be implemented. Moreover, making cropping systems more technical through the management of sowing densities and orientation together with the introduction of the most suitable irrigation practices (where it is possible) should be considered. Classical recipes in chemical control must be avoided and moreover, no single herbicide treatment will be definitive. Diversification is also necessary when using chemical and non-chemical methods, different MOA's and timing of applications have to be alternated every season. Finally, new tools derived from new technologies as decision support systems or precision agriculture must be explored and used in the most profit way.

In this study the complete knowledge of the genetic basis of the resistance together with the cross-resistance patterns between herbicides have been proposed as an important issue in designing better chemical control programmes. Moreover, reducing the reliance on herbicides for weed management through integration of cultural and mechanical methods must be the most important goal in the future.

Future research to be considered in this are:

- Bases of 2,4-D metabolism in corn poppy. The main metabolites formed from ring hydroxylation of 2,4-D are 4-hydroxy-2,5- dichlorophenoxyacetic acid and 4-hydroxy-2,3-dicholorophenoxyacetic acid.
- Gene sequencing of auxinic receptors and auxinic transporters of those 2,4-D resistant corn poppy plants: best candidate protein families are PIN and ABC for auxinic transport, and F-box (TIR-1) for nuclear receptors.
- Gene transcription analysis (Real Time-PCR or whole transcription), both for 2,4-D and ALS inhibitors employing ALS resistant, 2,4-D resistant, multiple resistant and susceptible biotypes. This would provide an insight in possible relationships between NTSR mechanisms, especially cytochrome P450 or glutation-S-tranferase, to synthetic auxins and ALS inhibitors.
- Check the most common mutations in the *psbA* gene (Val219, Ala251, Phe255, Ser264 and Asn266) that have been described in those PS II resistant weeds as TSR mechanisms.

- Study of dose-response to bromoxynil of progeny populations coming from studied populations to confirm heritability and shift of resistance factors.
- If bromoxynil resistance is confirmed and no mutations at *psbA* gene have been found, NTSR mechanisms to PS II inhibitors, absorption, translocation, and metabolism, need to be elucidated.

Some of these experiments have already been initiated this year.

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Conclusions/Conclusiones

The main conclusions of this thesis are:

Papaver rhoeas *resistance to ALS inhibiting herbicides.*

1. The different substitutions which have been found at position Pro197 (Ala197, Arg197, His197, Leu197, Thr197 and Ser197) of the ALS gene are responsible for the strong resistance shown to tribenuron-methyl by the treated resistant *P. rhoeas* biotypes.
2. The mutations at 197 amino acid provide certain degree of cross resistance to other ALS inhibiting herbicides. The substitutions of Pro by Ser give a moderate resistance to the triazolopyrimidine florasulam. Likewise, the appearance of a Thr at the same position shows moderate to strong resistance to the imidazolinone imazamox.
3. The experiments conducted with ^{14}C-tribenuron-metil demonstrated that absorption and translocation are not resistance mechanisms to sulfonylureas. Moreover differences in translocation patterns observed between resistant and susceptible plants, seem to be an indirect consequence of the above mentioned target-site resistant mechanism.
4. The biotype resistant only to 2,4-D showed a similar translocation pattern to that observed in the 2,4-D and tribenuron-methyl multiple resistant biotypes. In this biotype, phytotoxic processes triggered after tribenuron-methyl application seem to happen differently to those observed in the susceptible biotype.
5. The presence of non-target-site resistance mechanisms has been confirmed for imazamox and florasulam herbicides. Firstly because plants having the same ALS genotype showed different responses to these two herbicides. Secondly, because non-mutated plants in the ALS gene were able to survive imazamox.

Investigating 2,4-D resistance in P. rhoeas

6. Ethylene levels recorded in susceptible plants were between four and eight-fold greater than those levels reached in 2,4-D resistant and multiple resistant plants.
7. The 2,4-D and multiple resistant biotypes were not controlled satisfactorily with other auxinic herbicides as dicamba and aminopyralid.
8. Studies carried out with ^{14}C-2,4-D did not detect differences in absorption between resistant and susceptible biotypes. Conversely, a lower translocation of ^{14}C-2,4-D was detected in both only 2-4-D and multiple resistant biotypes compared with those

susceptible plants. This finding is, so far, the first resistance mechanism described in 2,4-D resistant *P. rhoeas*.

Assessing bromoxynil failures observed in the field

9. Bromoxynil controlled *P. rhoeas* populations that had been problematic in the field when this herbicide was applied at maximum field dose and at recommended phenological stage (rosette 5-6 cm). Efficacies ranged from 97 to 100%.
10. When bromoxynil was applied at a later phenological stages (11-12 cm rosette) one of the studied biotypes showed a shift of the dose-response curve compared to the susceptible biotype. This biotype presented a resistant index of 2.2.

Integrated management of multiple resistant P. rhoeas

12. Cereal-field peas and cereal-sunflower were crop rotations that achieved satisfactory results in *P. rhoeas* control. Percentages of *P. rhoeas* density reductions over three years of experiments ranged from 65 to 92% in the field peas rotation and from 72 to 89% in the sunflower rotation. The application of pendimenthalin in the field peas cycle was decisive for the good results obtained by this rotation. The cereal-sunflower rotation allows the incorporation of a spring sowing crop, so practices associated with sowing can eliminate most of the *P. rhoeas* plants emerged during autumn and winter.
13. Cereal-oilseed rape rotation did not reduce the densities of *P. rhoeas* in the highly infested fields with resistant biotypes. Lack of herbicides able to control *P. rhoeas* in post-emergence in oilseed rape, the low competitiveness of this crop in its early stages and planting dates (early September) are the main factors that hinder control of *P. rhoeas* in this crop.
14. A one-month delay in the cereal sowing date did not improve control of *P. rhoeas* respect to those managements in which a conventional sowing was done.
15. Those integrated management strategies which incorporated early chemical interventions such as pre-emergence and early post-emergence were able to reduce satisfactorily *P. rhoeas* infestation levels. Density reductions achieved by this kind of interventions ranged, on average, from 65 to 92% over the three years of experiments. Early treatments were more effective compared to post-emergence treatments because phenological heterogeneity is reduced at application time.

Las principales conclusiones que se extraen de esta tesis son las siguientes:

La resistencia de Papaver rhoeas *a herbicidas inhibidores de la enzima ALS*

1. Las diferentes substituciones encontradas en la posición Pro197 (Ala197, Arg197, His197, Leu197, Thr197 and Ser197) del gen de la ALS son las responsables de la fuerte resistencia que muestran los biotipos de *Papaver rhoeas* a la sulfonilurea tribenurón-metil.
2. Las mutaciones descritas en el aminoácido 197 otorgan cierto grado de resistencia cruzada a otros herbicidas inhibidores de la ALS. La substitución de la Pro197 por una Ser da una resistencia moderada a la triazolopirimidina florasulam. De la misma forma, la aparición de una Thr en esta misma posición otorga una resistencia de moderada a fuerte a la imidazolinona imazamox.
3. Los ensayos realizados con ^{14}C-tribenurón-metil establecieron que la absorción y la translocación no son mecanismos de resistencia fuera del lugar de acción para sulfonilureas. Además las diferencias observadas a nivel de translocación entre plantas resistentes y sensibles, parece ser una consecuencia indirecta de los mecanismos de resistencia en el lugar de acción anteriormente mencionados.
4. El biotipo únicamente resistente a 2,4-D presentó un patrón de translocación similar al observado en aquellos biotipos con resistencia múltiple (2,4-D y tribenuron-metil). En este biotipo, los procesos fitotóxicos desencadenados tras la aplicación del tribenurón-metil parecen acontecer de manera distinta a los observados en el biotipo sensible.
5. La presencia de mecanismos de resistencia fuera del lugar de acción ha sido confirmada para los herbicidas imazamox y florasulam. Primero, porque plantas que presentan el mismo genotipo en la ALS, manifiestan diferentes respuestas a estos dos herbicidas. Segundo, porque plantas sin mutaciones en el gen ALS son capaces de sobrevivir al imazamox.

Investigando la resistencia de P. rhoeas *al 2,4-D*

6. Los niveles de etileno registrados en planta viva fueron significativamente mayores (entre cuatro y ocho veces superiores) en el biotipo sensible en comparación con los valores establecidos por los biotipos con resistencia a 2,4-D y resistencia múltiple.

7. Las poblaciones de *P. rhoeas* con resistencia múltiple y únicamente resistentes a 2,4-D, no fueron controladas de forma satisfactoria con otros herbicidas auxínicos, dicamba y aminopiralid.
8. Los estudios llevados a cabo con ^{14}C-2,4-D no detectaron diferencias en términos de absorción entre biotipos resistentes y sensibles. Por el contrario, una menor translocación del ^{14}C-2,4-D fue detectada, tanto en el biotipo con resistencia múltiple como en el biotipo únicamente resistente a 2-4-D, respecto al biotipo sensible. Esto supone, hasta el momento, el primer mecanismo de resistencia descrito en poblaciones de *P. rhoeas* resistentes a 2,4-D.

Evaluando los fallos del Bromoxinil observados en campo

9. El bromoxinil controló de forma aceptable aquellas poblaciones de *P. rhoeas* que habían presentado problemas en campo, cuando este herbicida se aplicó a la dosis máxima de registro y a la fenología recomendada (5-6 cm de roseta). Las eficacias de este producto a la máxima dosis de campo fue del 97% y 100% en aquellas poblaciones sospechosas.
10. Cuando el bromoxinil se aplicó en una fenología más avanzada (11-12 cm de roseta) uno de los biotipos estudiados presentó cierto desplazamiento de la curva dosis respuesta respecto a su homólogo sensible. Este biotipo presentó un índice de resistencia de 2.2 puntos.

Manejo Integrado de P. rhoeas *con resistencia múltiple.*

11. Las rotaciones cereal-guisante y cereal-girasol consiguieron resultados muy satisfactorios. El porcentaje de reducción de densidad inicial de *P. rhoeas* a lo largo de los tres años de experimento, osciló entre el 65 y el 92% en el caso de la rotación con guisante y entre el 72 y el 89% en la alternativa con girasol. La incorporación de la pendimentalina en pre-emergencia durante el ciclo del guisante resultó ser determinante en los buenos resultados obtenidos por esta rotación. La rotación cereal-girasol permite incorporar un cultivo que se siembra en primavera, de esta manera las labores asociadas a la preparación y siembra del girasol permiten eliminar todas las plantas de *P. rhoeas* emergidas durante otoño e invierno.
12. La rotación cereal-colza no redujo los niveles de *P. rhoeas* en aquellos campos altamente infestados. La falta de materias activas en colza para el control de *P. rhoeas* en post-emergencia, la baja competitividad de este cultivo en sus estadios

iniciales y la fechas de siembra recomendadas (principios de septiembre) son los principales factores que dificultan el control del de esta mala hierba en esta rotación.

13. Un retraso de un mes en la fecha de siembra del cereal no mejoró el control de *P. rhoeas* respecto a aquellos manejos en los que se practicó una siembra en una fecha convencional.
14. Aquellas estrategias de manejo integrado que incorporaron tratamientos químicos precoces como son los de pre-emergencia y post-emergencia temprana consiguieron reducir la infestación de *P. rhoeas*. La reducción de la densidad inicial tras tres años de ensayos conseguida por este tipo de intervenciones, obtuvo en promedio, valores que oscilaron entre un 65 y un 92%. Este tipo de intervenciones resultan más eficaces respecto a los tratamientos en post-emergencia ya que consiguen minimizar la heterogeneidad fenológica en el momento de la aplicación.

Abbreviations used

2,4-D [2,4-dichlorophenoxyacetic acid]; ABA[Abscisic acid]; ABC [ATP-binding cassette]; ACCase [Acetyl-coenzyme A carboxylase]; ACCsynthase [1-aminocyclopropane-1-carboxylic acid synthase]; ALS [Acetolactate synthase]; GLM [Generalized linear models]; GR_{50} [Herbicide rate causing 50% mortality]; HAT [Hours after treatment]; HR [Herbicide resistance]; IMI [Imidazolinones]; LMM [Linear mixed-effects models]; MCPA [4-Chloro-2-ethylphenoxyacetate]; MOA [Modes of action]; NTSM [Non-target-site mechanisms]; PCR [polymerase chain reaction]; PIN [PIN-FORMD proteins]; POST [Post-emergence]; PRE [Pre-emergence]; PTB [pyrimidinyl-thiobenzoates]; R [Resistant]; RI [Resistant Index]; ROS [Reactive oxygen species]; RR [Resistant homozygous]; RS [Resistant heterozygous]; S [Susceptible]; SCT [sulfonyl-aminocarbonyl-triazolinones]; SS [Susceptible homozygous]; SU [Sulfonylureas]; TP[Triazolopyrimidines]; TSM [Target-site mechanisms]; TSR [Target-site resistance]; WAT [Weeks After Treatment]; [^{14}C]-Tri [[^{14}C]-tribenuron-methyl].