Universitat de Lleida

Escola Tècnica Superior d'Enginyeria Agrària de Lleida (ETSEA)
Departament d'Hortofructicultura, Botànica i Jardineria
Programa de Doctorat en Producció Fructícola i Botànica Aplicada

Integrated Management of Herbicide Resistant *Papaver rhoeas* L. Populations

Control Integrado de Poblaciones de *Papaver rhoeas* L. Resistentes a Herbicidas

Doctoral Thesis
Proposed to obtain the degree of European Dr.

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Octubre 2001

Integrated Management of Herbicide Resistant *Papaver rhoeas* L.

Doctoral Thesis Memory presented by Alicia Cirujeda Ranzenberger to opt to the Doctor degree by the Universitat de Lleida

This research has been performed in the Departament de Hortofructicultura, Botànica i Jardineria, Universitat de Lleida under the direction and tutorage of Dr. Andreu Taberner Palou and Dr. Jordi Recasens Guinjuan, professors of the Universitat de Lleida.

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Andreu Taberner: gràcies per TOT.

A Antonio Roque. Gracias a tu constante trabajo he podido descansar fines de semana, veranos y he podido redactar este texto con más tranquilidad. Los buenos ratos pasados ahí están para siempre, esto no se olvida!!
A en Ramon Anguera. Gràcies pel teu companyerisme i amistat, ajut, ànims i suport.
A en Ramon Tarragó. Em vas ajudar a veure que no tot són moments fàcils i que amb ganes, tot es supera.
A en Jordi Recasens. Pel teu suport, ànims i amistat. I per l'espenta final llegint, corregint i fent estadística.
A en Jordi Voltas. Per la teva disponibilitat en l'ajut amb l'estadística.

A en Francesc Paris i Ramon Alet i a d'altres pagesos per deixar-nos els vostres camps un cop, un altre cop i un altre cop més! A en Lluís Roqué per estar sempre disponible a passar la grada tot fent malabarismes d'agenda.

A tots i totes les persones que treballen o que han passat pel Servei de Protecció dels Vegetals de Lleida durant aquest temps: Àngels Fondevila, Jaume Almacelles, Ramon Torà, Joaquín García de Otazo, Victoria, Núria, Sandra, Vanessa, Marta, Josep, Jordi i "Antonio's", Jordi i Anna, prospectors i prospectores, tècnics d'ADV's etc. También a nuestros "vecinos" Armando, Rafa, Joan, Susana. I a en Josep Soldevila, per la teva paciència. La vostra simpatia fan de la feina un lloc agradable i més ple de sentit.

A la Núria Llop i la Carme Castillo per la vostra paciència amb el paperam….

To Peter Boutsalis, for encouraging me to start, to continue and to finish this work. Marco Quadranti, Ruth Sorg, Jutta Glock und alle anderen für Euer Interesse in mir. Schließlich begann ich dann doch mit der Doktorarbeit…

Gertrud, José, Christine, Hans, Eulàlia, Pol, Bernat und Paul, weil ihr immer da seid.

A mi familia de Huesa del Común, pues mi vocación por la agricultura posiblemente empezó con vosotros. Me habéis transmitido, entre otras cosas, el respeto que las culturas rurales sentís por la naturaleza. ¡Intentaré heredarlo!

A tots els amics, especialment Txell Pastor, Àngels Fondevila, Rosa Vaquera, Anna Pallí; David Moret, Manolo Ibáñez, Chelo Pla, Raquel Banyeres, Remei Soler. I companys de pis: Mª José Lis, Sergi Martinez, Núria Casas, Mario Nuño. Què hagués sigut aquest temps sense vosaltres!

To Ilse Ramussen, Henrik Grøndål, Viebeke Anderssen, Ida-Marie, Karsten Rasmussen, Bo Melander and the others for your help with mechanical weed control and for showing me how people live in Denmark. Maget tak!

Quiero expresar mi agradecimiento por haber disfrutado de la beca FPU concedida por el Ministerio de Educación y Ciencia desde 1998 que ha posibilitado la realización de este trabajo.

The protagonist, *Papaver rhoeas* IS:

Papavero, rosolaccio, bambagelle in Italian
Poppy in English
Coquelicot, pavot, ponceau, poinceau, pavot coq in French
Klatschmohn, Klapperrose, Klatschrose in German
Kornvalmue in Danish
Blauwmaanzaad, klapproos in Dutch
Klaproes, kollebloem in Flemish
Silkkiunikko in Finish
Papoula, papoila-das-searas, papoila in Potuguese
P a p a r**o?**n a in Greek
Kornvallmo in Swedish

Gelincik in Turkish
Mak polny in Polish
Mak vlcí in Tchechian
Pipacs in Hungarian
Ma? ?a? oce??a in Russian
Li chun hua in Chinese
Deydahaan, zaghleel in arabic

En España se la llama

Amapola, ababol, gamapola, rosillas, loraguillo

Papola, papoila, papoileira, mapoula

Rosella, roella, gallarets, kikerikis, peperepets, pepiripips, cascall bord, puputs, badabadocs, paparota, gall, gallordet

Mitxoleta, bobelarra, urdamuturra, kukubelarra, lobedarra, kali-kola, mikelete

etc.

Summary

Herbicide resistance towards the herbicides tribenuron-methyl and 2.4-D was detected in most of the 148 analysed *Papaver rhoeas* L. populations collected in a semi-directed field survey in North-eastern Spain. The most frequent situations found were populations slightly resistant to 2.4-D and with a high frequency of resistant plants towards tribenuron-methyl.

One seed-based quick-test for each herbicide on agar medium was developed and found valuable for detecting herbicide resistance after validating with greenhouse and field trials. In the case of tribenuron-methyl, the test allowed to quantify the frequency of resistant plants, while in the case of 2.4-D the resistance degree was expressed with a hipocotyl length ratio. Resistance towards tribenuron-methyl seems to be caused by a target-site mutation, while resistance towards 2.4-D is probably metabolism-based.

In the study area, *P. rhoeas* germinated mainly in autumn and early winter, especially between October and December but was detected since September until April, depending on the locations and on the year's characteristics. No differences in germination habits were found between susceptible and resistant populations. Depending on the climatic conditions, germination was found between 12 and 70% during the first season for freshly sown seeds. Seeds, which did not germinate after the first season probably entered in a secondary dormancy stage. Cultivation clearly stimulated weed emergence and could be used in field to favour germination.

A generally strong natural weed mortality was detected in most of the years and fields. In spite of this, high initial weed populations reached often still 300 plants m^{-2} at the end of the cropping cycle.

Ploughing was found to be an effective method for placing *P. rhoeas* seeds in a non-favourable position for germination. An average reduction of around 40% emergence was observed. Regardless if the *P. rhoeas* population was susceptible or resistant, between 63 and 99% *P. rhoeas* seeds were still viable 31 months after burial in soil at 20 cm depth. Therefore, ploughing should be conducted occasionally only as the seeds moved upwards again were able to germinate.

Harrowing can be an effective control method of herbicide resistant *P. rhoeas* but require an accurate observation of the weed and crop size. The effect of pre-emergence harrowing were very little or inappreciable. In post-emergence harrowing should better be conducted at early growth stages of *P. rhoeas*. As this species has a tap root, efficacy decreased if plants were too big. In dry years efficacy was higher as damaged plants could not recover. In better moisture conditions, *P. rhoeas* plants often recovered after being buried so that initial high efficacy decreased in time. In few occasions harrowing stimulated germination. Mechanical control of *P. rhoeas* by harrowing was insufficient in some trials but reduced weed plant number in all cases.

Chemical *P. rhoeas* control was possible with herbicides of different classification groups defined by the Herbicide Resistance Action Commitee (HRAC). In pre-emergence, pendimethaline, trifluraline + linuron and trifluraline + chlortoluron were the most effective and most regular herbicides. In early post-emergence a less constant but quite high control efficacy was achieved with MCPA + diflufenican, isoproturon + diflufenican and tribenuron-methyl + metribuzine. In late post-emergence, mixtures containing ioxinil or bromoxinil, especially bromoxinil + MCPP, ioxinil + bromoxinil + MCPP as well as florasulam + 2.4-D often controlled the herbicide resistance populations well.

In order to prevent and to manage the herbicide resistance in *P. rhoeas* these different control methods should be used together, defining an integrated weed control strategy.

Resumen

En la mayoría de las 148 poblaciones de *Papaver rhoeas* L. analizadas, se detectó resistencia frente a los herbicidas tribenurón-metil y 2.4-D. Las poblaciones fueron recogidas en una prospección de campo semidirigida en el Nordeste de España. La situación más frecuente fue la de poblaciones ligeramente resistentes a 2.4-D y con una proporción de plantas resistentes a tribenurón-metil elevada.

Se desarrolló un método rápido de detección a la resistencia para cada herbicida basado en semillas, los cuales fueron considerados válidos después de que los resultados fueron comprobados con ensayos de invernadero y de campo. Para el caso de tribenurón-metil, el test permitió cuantificar la frecuencia de plantas resistentes, mientras que para el caso del 2.4-D el grado de resistencia fue expresado con un ratio calculado a partir de la longitud del hipocótilo. La resistencia frente a tribenurón-metil parece estar causada por una mutación mientras que la resistencia frente a 2.4-D está probablemente basada en un cambio metabólico de las plantas resistentes.

En la zona de estudio, *P. rhoeas* germinó principalmente en otoño y principios de invierno, mayoritariamente entre octubre y diciembre, aunque fue detectada desde septiembre hasta abril, dependiendo de la localidad y del año. No se encontraron diferencias en los hábitos de germinación entre poblaciones sensibles o resistentes a los herbicidas. En función de las condiciones climáticas la germinación fue de 12 hasta 70% después del primer otoño e invierno para las semillas recogidas en el verano anterior. Semillas que no germinaron tras el primer invierno probablemente entraron en un estadio de dormición secundaria. El laboreo del suelo estimuló claramente la emergencia de *P. rhoeas* y puede ser una técnica apropiada en campo para favorecer la germinación.

Se detectó una mortalidad natural de *P. rhoeas* en la mayoría de los años y en la mayoría de ensayos. A pesar de ello, a menudo densidades iniciales de las poblaciones en campo alcanzaban todavía 300 plantas m^{-2} al final del ciclo de cultivo.

El uso de un arado de vertedera previo a la siembra del cereal fue un método efectivo para colocar las semillas de *P. rhoeas* en una posición no favorable para a germinar. Se observó una reducción media de 40% en la emergencia. Independientemente de si la población de *P. rhoeas* fue sensible o resistente, entre un 63 y 99% de las semillas fueron todavía viables tras 31 meses de enterrado en el suelo en 20 cm de profundidad. Por ello, el uso del arado de vertedera se debería restringir a un uso ocasional ya que las semillas devueltas a capas superficiales del suelo estarán capacitadas para germinar si ha transcurrido poco tiempo después de su enterrado.

El uso de la grada de púas flexibles puede ser un método efectivo para el control de *P. rhoeas* resistente a los herbicidas pero requiere una observación detallada del tamaño de la hierba y del cultivo. El efecto del uso de la grada en pre-emergencia fue muy pequeño o inapreciable. En post-emergencia la grada fue más eficaz sobre plantas pequeñas de *P. rhoeas*, ya que esta especie desarrolla una raíz pivotante muy fuerte. En años secos, la eficacia fue mayor, ya que las plantas dañadas no pudieron recuperarse. En condiciones de humedad más idóneas, las plantas de *P. rhoeas* a menudo se recuperaron después del tratamiento así que la eficacia inicial disminuyó con el tiempo. En pocas ocasiones el uso de la grada estimuló la germinación de nuevas plantas. El control de *P. rhoeas* fue insuficiente en algunos campos pero se observó una disminución de la densidad en todos los casos.

El control químico de *P. rhoeas* resistente a tribenurón-metil y a 2.4-D es posible con herbicidas pertenecientes a grupos distintos de la clasificación propuesta por el Comité de Acción de Resistencia a los Herbicidas (HRAC). En pre-emergencia, pendimetalina, trifluralina + linuron y trifluralina + clortolurón fueron los herbicidas más efectivos y de acción más regular. En post-emergencia precoz se alcanzó un control menos constante pero también elevado con MCPA + diflufenicán, isoproturón + diflufenicán y tribenurón-metil + metribuzina. En post-emergencia tardía, las mezclas conteniendo ioxinil o bromoxinil así como florasulam + 2.4-D controlaron las poblaciones resistentes en muchos casos. Con el objetivo de prevenir y manejar la resistencia a herbicidas en *P. rhoeas* estos diferentes métodos de control deben ser usados conjuntamente, definiendo una estrategia de control integrado.

Resum

A la majoria de les 148 poblacions de *Papaver rhoeas* L. analitzades es va detectar resistència front als herbicides tribenuró-metil i 2.4-D. Les poblacions van ser recollides a una prospecció de camp semi-dirigida en camps de cereal d'hivern del Nord-est d'Espanya. La situació més freqüent va ser la de poblacions lleugerament resistents al 2.4-D i amb una proporció de plantes resistents a tribenuró-metil elevada.

Es va desenvolupar un mètode de detecció a la resistència per a cada herbicida basats en llavors, els quals van ser considerats com a vàlids després de que els resultats van ser comprovats amb assaigs en hivernacle i en camp. Per a tribenuró-metil, el test permetia quantificar la freqüència de plantes resistents, mentre que pel 2.4-D el grau de resistència va ser expressat mitjançant un ratio basat en la longitud del hipocotil. La resistència enfront al tribenuró-metil sembla estar causada per una mutació mentre que la resistència front a 2.4-D està probablement basada en un canvi metabòlic de les plantes resistents.

A la zona d'estudi, *P. rhoeas* va germinar a la tardor i principis d'hivern, principalment entre octubre i desembre, tot i que es va detectar germinació entre setembre i abril, depenent de la localitat i de l'any. No es van trobar diferències als hàbits de germinació entre poblacions sensibles o resistents als herbicides. En funció de les condicions climàtiques la germinació va ser d'entre 12 i 70% després de la primera tardor i hivern per a llavors recollides l'estiu anterior. Llavors que no van germinar després del primer hivern probablement van entrar en un estat de dormició secundària. Cultivar el sòl va estimular clarament la emergència de *P. rhoeas* i pot ser una tècnica apropiada a camp per a afavorir la germinació.

Es va detectar una mortalitat natural de *P. rhoeas* a la majoria dels anys i a la majoria dels assaigs. Tot i així, densitats inicials elevades conduïen sovint a densitats finals de fins a 300 plantes m^{-2} al final del cicle de cultiu.

La utilització de les arreus prèviament a la sembra del cultiu va ser un mètode efectiu per a col·locar les llavors de *P. rhoeas* en una posició no favorable per a germinar. Es va observar una reducció mitja del 40% en la emergència. Independentment de si la població de *P. rhoeas* va ser sensible o resistent, entre un 63 i 99% de les llavors van ser encara viables després de 31 mesos d'enterrat al sòl a 20 cm de profunditat. Per això, l'ús de les arreus hauria de ser restringit a un ús ocasional, ja que les llavors que tornen a ser col·locades en superfície poden germinar si ha passat poc temps després d' haver estat enterrades.

La utilització de la grada de pues flexibles pot ser un mètode efectiu per al control de *P. rhoeas* resistent als herbicides, però requereix una observació detallada de la mida de la herba i del cultiu. L'efecte de la grada en pre-emergència va ser molt petit o inapreciable. En post-emergència es va observar que la grada va ser més eficaç sobre plantes petites de *P. rhoeas*, ja que aquesta espècie desenvolupa una arrel pivotant molt forta. En anys secs la eficàcia va ser més gran ja que les plantes danyades no van poder recuperar-se. En condicions més humides les plantes de *P. rhoeas* es van recuperar sovint després del tractament així que la eficàcia inicial va disminuir amb el temps. En poques ocasions la grada va estimular la germinació de noves plantes. El control de *P. rhoeas* va ser insuficient en alguns camps, però es va observar una disminució de la densitat a tots els casos.

El control químic de *P. rhoeas* resistent a tribenuró-metil i a 2.4-D és possible amb herbicides pertanyent a diversos grups de la classificació proposada pel Comité d'Acció de Resistència als Herbicides (HRAC). En pre-emergència, pendimetalina, trifluralina + linuró i trifluralina + clortoluró van ser els herbicides més efectius i d'acció més regular. En post-emergència precoç es va obtenir un control menys constant, però també elevat amb MCPA + diflufenican, isoproturé + diflufenican i tribenuró-metil + metribuzina. A post-emergència tardana les barreges amb ioxinil o bromoxinil així com florasulam + 2.4-D van controlar les poblacions resistents a molts casos. Amb l'objectiu de prevenir i controlar la resistència a herbicides a *P. rhoeas* aquests diferents mètodes de control han de ser utilitzats conjuntament, definint una estratègia de control integrat.

INDEX OF CONTENTS

Accepted and submitted papers

Preliminary chapter: Optimisation of *Papaver rhopeas* L. germination on agar medium.

has been exposed and discussed as a scientific poster in the 11th European Weed Research Society Symposium (EWRS) in 1999 in Basel (Switzerland).

Chapter 1: A qualitative quick-test for herbicide resistance detection to tribenuron-methyl in *Papaver rhoeas* L. grown on agar medium

has been adapted from the paper accepted in revised form in the Journal "Weed Research" on April 24th 2001.

Chapter 4: Germination and survival habits of a susceptible and a herbicide resistant *Papaver rhoeas* L. population in North-eastern Spain (Catalonia)

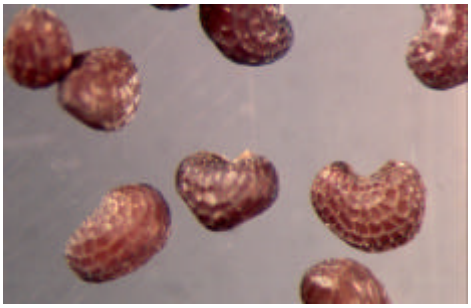
has been adapted from the manuscript sent for acceptance to the Journal "Weed Research" on August 17th 2001.

Chapter 5: : Seasonal changes in the dormancy and evolution of viabiliy of buried seeds of herbicide resistant and susceptible *Papaver rhoeas* L. in North-eastern Spain

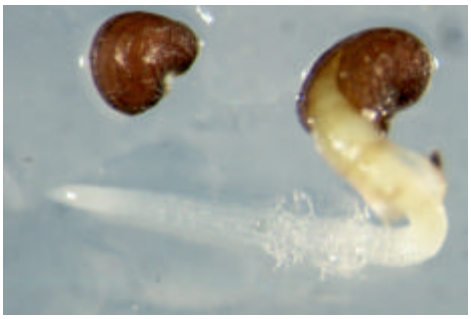
has been adapted from the manuscript sent to the Journal "Seed Science Research" on August 17th 2001.

Chapter 6: Ploughing and harrowing effect on a herbicide resistant *Papaver rhoeas* L. population in North-eastern Spain (Catalonia)

has been adapted form the manuscript sent for acceptance to the Journal "Weed Technology" on August 17th 2001.



I. General Introduction



1. Setting the scene

1.1. The weed populations and Papaver rhoeas *in winter cereals in the study area*

Before the appearance of herbicides a common practice in the dryer areas of North-eastern Spain was one alternative year of crop and one with fallow. During the fallow period, cultivation allowed to reduce the weed populations and it was aimed to let the soil recover. Other crops, which were occasionally used in rotation with cereal in these areas, were vetch (*Vicia villosa*) and sainfoin (*Onobrychis sativa*).

In the moister areas, crop rotation within wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), alfalfa (*Medicago sativa*), potatoes (*Solanum tuberosum*) and vetch (*Vicia villosa*) was common in North-eastern Spain including some of these crops in the areas with higher moisture (Taberner *et al.*, 1992).

These cropping systems resulted in much more diverse weed populations than the barley monocrop grown in large areas at present. Crop rotation clearly reduced the weed problem and some older practices like under-sowing of alfalfa in the cereal contributed to decrease the weed competition.

Intensification of crop production has led to severe changes in weed communities during the last five decades (Bischoff & Mahn, 2000). Not only species composition changed but also alterations in whole weed communities have been observed (Mahn 1984). This author states that one of the main sources of structural changes of weed communities is the application of herbicides under long-term conditions. Previous to the introduction of 2.4-D broad-leaved (dicotiledoneous) weeds dominated in North-eastern Spain and grass weeds were unknown in many areas. The use of this first herbicide shifted the weed populations towards the dominance of grass species like *Lolium rigidum*, *Avena sterilis* and *Bromus* spp. and also towards *Galium aparine*, not controlled by 2.4-D either (Taberner *et al*, 1992).

The posterior appearance of herbicides of the urea group and other graminicides controlled these weeds effectively so that, enhanced by the growing herbicide resistance towards 2.4-D the broadleaf weeds were again protagonists. The appearance of the sulfonylureas in the 80's solved this question again. But herbicide resistance towards this new group started already five to six years afterwards and placed the *Papaver rhoeas* control again into a problematic situation.

Moreover, herbicide resistance towards *L. rigidum* and also starting with *Avena* sp. is a growing problem at the moment (Taberner, pers. comm.). Depending on the management strategies of the field both problems with almost monospecific grass- or broadleaf weeds can be found in the area. The combination of one of each, as e.g. *P. rhoeas* and *A. sterilis* or *L. rigidum* is also found sometimes, but in very few cases more than two weed species are dominant in the cereal fields of the study area.

Within the broadleaf weeds, *P. rhoeas* is still the most important species infesting winter cereals in North-eastern Spain. In a survey conducted in 1990, this species occurred in 39% of the surveyed fields (Riba *et al*., 1990). This weed is present in every continent but is most abundant in Europe where it originated (Holm *et al.*,

1997). *P. rhoeas* is found especially in winter cereals and existed in Great Britain since the Bronze Age (reviewed by Holm *et al*., 1997) where this species has been extensively studied (McNaughton & Harper; 1964, Froud-Wiliams *et al.*; 1984, Roberts & Feast, 1973; Wilson *et al.*, 1988). In Tasmania, control problems are quoted in the crop *P. somniferum*, due to its similarity competing vigorously and causing yield decreases (Bishop & Pemberton, 1996).

The extreme prolificity and gregariousness of this species guarantees the survival of individual plants. The important yield decreases due to an abundant *P. rhoeas* population is well known by the farmers. Probably its strength is due to the huge densities as well as to its high seed production capacity, because an only individual plant is described to have week competition ability (McNaughton & Harper, 1964). Under favourable conditions, plants can produce several hundred capsules containing more than 1000 seeds each (McNaughton & Harper, 1964). In a favourable year, germination can be very high causing plant densities of 1000 plants m^{-2} (A. Cirujeda and A. Taberner, non-published data). On the other hand survival through unfavourable periods is achieved through seed dormancy. Even if plant growth is reduced by poor soil fertility and other factors, plants survive in fairly dry conditions.

P. rhoeas shows also a great genetic variability. The species is almost entirely self-sterile (McNaughton & Harper, 1964). Cross-polinization is, thus, the main strategy enhancing genetic diversity inside the populations.

All these characteristics suggest that for facing *P. rhoeas* control at least more than one different strategy will be necessary, as adaptation of the weed towards control methods is very probable.

1.2. Herbicide resistance

The appearance of herbicide weed resistance was first described in 1964. Currently, 153 resistant grass and broadleaf weed biotypes in about 50 countries world-wide have been recorded. The frequency of resistant biotypes is different from one herbicide family to the other. The first herbicide group with resistance problems was the synthetic auxins, which were also the first herbicides appearing in the market. After them, resistance was found in the triazines. Nowadays, most number of resistant species are in the acetolactate synthase (ALS) inhibiting herbicides followed by the triazines. The sulfonylureas appear in the fifth place of the ranking (Heap, online, 2001).

Herbicide resistance is defined as the natural occurring inheritable ability of some weed biotypes within a given weed population to survive a herbicide treatment that would, under normal use conditions, effectively control that weed population (HRAC, 2001a). The process is normally due to a selection of herbicide resistant individuals by submitting them to a continuous selection pressure. Figure I.1. illustrates the selection process of herbicide resistant individuals.

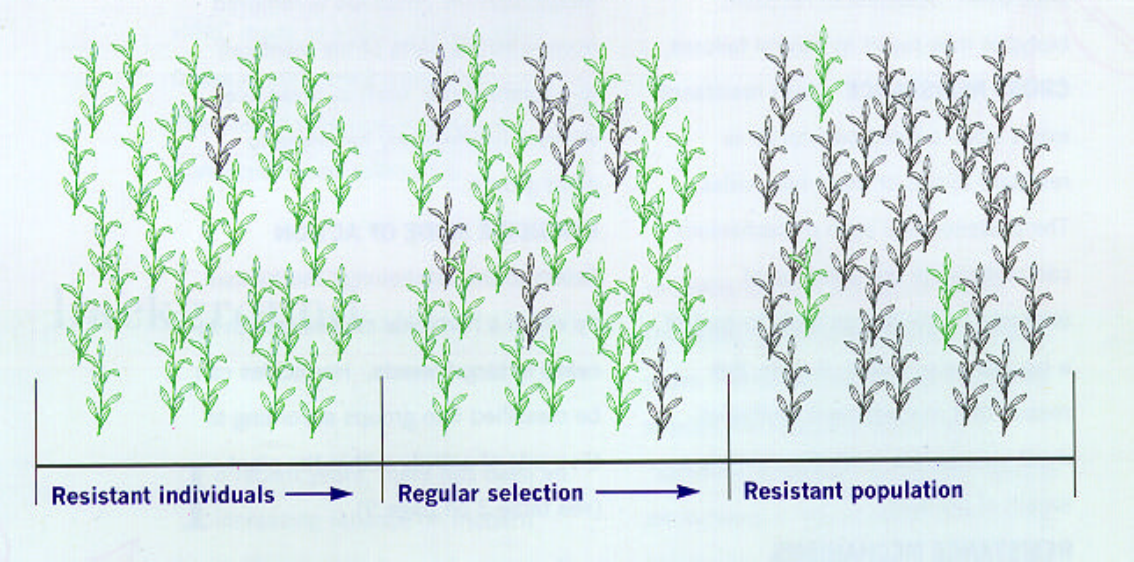


Figure I.1. Selection process of resistant weed plants (in black) caused by the continuous use of a herbicide with the same active ingredient (HRAC, 2001a).

Many parameters play a role for herbicide resistance evolution. Some important ones following Saari *et al*. (1994) are the selection pressure imposed by the herbicide, the absolute and relative fitness of the resistant biotypes, the initial frequency of resistant genes, the average lifetime in the soil seed bank and the gene flow. In bigger weed populations the resistance gene will also be more frequent. Thus, depending on the herbicides' and weeds' characteristics, appearance of herbicide resistance can be much faster or slower.

Three main mechanisms are frequent in weed herbicide resistance, although others have also been quoted for some species.

Enhanced metabolism confers the plants the ability of metabolising faster than normally the initially harming herbicide (HRAC, 2001a). Increasing herbicide rates can still control the weeds, but selects for more resistant plants at the same time. In many cases this resistance is due to an additive dominant gen, so that crossings between individual plants result in higher resistance levels.

The second mechanism is an **altered target-site** and refers to a mutation in the point of action of the herbicide. The herbicide does not fit exactly in this point any more, so that no blocking of the enzyme is possible (HRAC, 2001a). In most of the cases this mutation is caused by a single nuclear gene with incomplete dominance and is in most of the cases not related to fitness penalty (including seed germination, production and longevity, plant growth and competition, etc.) (reviewed by Saari *et al.*, 1994).

The third main resistance mechanism is **compartmentalism sequestration**. The herbicide is removed to parts of the plant cell where the herbicide can not be active, as e.g. to the vacuoles (HRAC, 2001a). Other mechanisms include reduced uptake due to a thicker cuticula or to other reasons.

Other important concepts are cross- and multiple resistance. **Cross-resistance** is defined as the expression of a genetically-endowed mechanism conferring the ability to withstand herbicides from different chemical classes (Powles & Preston, 1995). In some cases, resistance exists to just one herbicide group whereas in other populations resistance extends across many herbicide groups and modes of action.

Multiple resistance to herbicides is defined as the expression of more than one resistance mechanism. Simple cases have been reported for a small number of weed species though the majority of cases and the most complicated situations for control have been reported for *Lolium rigidum* biotypes. The most difficult control situations are given when a number of resistance mechanisms involving both target site and non-target site resistance mechanisms are present within the same individual (Powles & Preston, 1995).

1.2.1. Herbicide resistance in Papaver rhoeas

Problems in controlling *P. rhoeas* in winter cereal with herbicides have been quoted since 1992 in Spain (Taberner *et al.*, 1992). In this first publication, herbicide distributors, applicators and farmers complained on resistance towards 2.4-D. In 1995, resistant biotypes of *Amaranthus hybridus, A. retrofleus* and *Chenopodium album* in towards triazine in maize and again *P. rhoeas* resistant to 2.4-D are quoted (Taberner *et al.*, 1995). Claude et al. (1998) reported the first analysis on a *P. rhoeas* population found in North-eastern Spain resistant to 2.4-D and to tribenuron-methyl.

1.2.1.1. Herbicide resistance towards 2.4-D

The auxin analog herbicides including 2.4-D were discovered during the 1940s and were the first selective organic herbicides to be developed (Coupland, 1994). 2.4-D belongs to the Herbicide Resistance Action Committee (HRAC) classification group O4 (HRAC, 2001b). Its molecular formula is $C_8H_6Cl_2O_3$ and its chemical structure is shown in Figure I.2. The chemical name following the IUPAC is (2,4-dichlorophenoxy)acetic acid (Tomlin, 1994).

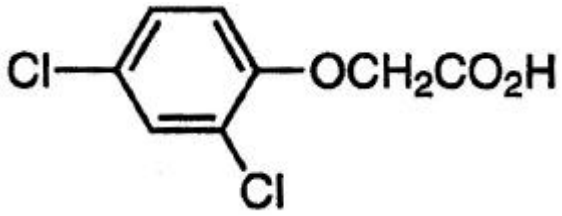


Figure I.2. Chemical structure of the auxin analog herbicide 2.4-D.

The introduction of this herbicide in the Spanish agriculture started in the same decade. No alternative products existed and the use of 2.4-D for broad-leaf weed control in cereals became very popular due to its high efficacy. Nevertheless, complains of bad controlled populations started around 1990 (A. Taberner pers. comm.). Farmers had to spray three- or more fold the recommended herbicide rate in order to control the weeds, which recovered after a lower dose application. The first quoted case of herbicide resistance towards 2.4-D is *Carduus nutans* in New Zealand since the 1970s.

Currently, 20 weed species are known to be resistant to synthetic auxins worldwide. Active ingredients with deficient control in any species are 2.4-D, MCPA, quinclorac, dicamba, dichlorprop, mecoprop and picloram. In 1998, Claude *et al*. published the first case of a well-studied *P. rhoeas* population in Spain resistant to 2.4-D and to tribenuron-methyl. Besides this case and the populations studied in the present work, no other cases on *P. rhoeas* resistance towards 2.4-D had been quoted in another country until the moment (Heap, 2001).

2.4-D is applied in spring on grown weed plants in rosette stage in late tillering stage of the cereal crop, when temperature ranges between 7 and 20ºC and the plants are in full development. Also resistant plants show initial leaf deformations, which disappear afterwards. Due to this, farmers notice lack of efficacy late, when no other control method is generally possible. So, when 2.4-D fails, nothing can be done until the next cropping season.

1.2.1.2. Herbicide resistance towards tribenuron-methyl

Herbicide resistance towards ALS-inhibitors was first quoted for an Australian *L. rigidum* biotype in 1982. The first dicotyledoneous weed species resistant to ALS-inhibitors was quoted in 1987 in USA: *Kochia scoparia, Lactuca serriola* and *Salsola iberica* infesting in all cases wheat fields. The updated International Survey of Herbicide Resistance lists at present 69 different weed species resistant to ALS-inhibitors. 48 are dicotiledoneous species (Heap, 2001).

The sulfonylurea tribenuron-methyl belongs to the B2 group of the HRAC classification (HRAC, 2001b) and has been sold in Spain since 1986. Its molecular formula is $C_{14}H_{15}N_5O_6S$ and its chemical name following the IUPAC nomenclature is 2-[4-methoxy-6-methyl-1,3,5-triazin-2-yl(methyl)carbamoxylsulfamoyl]=benzoic acid.
The chemical structure is shown in Figure I.3. (Tomlin, 1994).

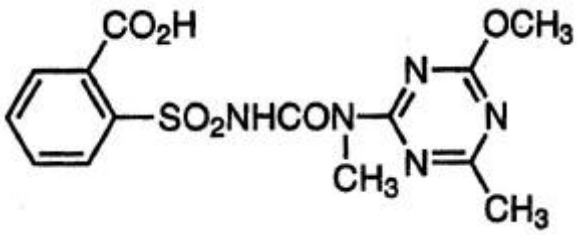


Figure I.3. Chemical structure of the sulfonylurea herbicide tribenuron-methyl.

Due to the high efficacy of tribenuron-methyl at low rates, low mammalian toxicity and wide crop selectivity (Saari *et al.*, 1994) it quickly achieved a wide use for broad-leaf weed control in cereal fields. Herbicide resistance towards tribenuron-methyl on *P. rhoeas* in winter cereals are also spreading in Italy (Sattin, 2001) and in Greece (Kotoula-Syka, 2001). In the second case, the selection pressure had been conducted with chlorsulfuron, so that cross-resistance towards tribenuron-methyl evolved.

In Spain, in many cases this herbicide was sprayed on fields containing 2.4-D resistant *P. rhoeas*. Therefore, when tribenuron-methyl herbicide resistance evolved after some years of continuous use, many of these populations showed multiple resistance to both herbicides. In 1998, Claude *et al*. published the first case of a well-studied *P. rhoeas* population resistant to 2.4-D and to tribenuron-methyl (Claude *et al.*, 1998).

Evolution of herbicide resistance was much faster for tribenuron-methyl than for 2.4-D. Probably this was due to different causes including different selection pressures between the two herbicides, different initial resistant plant number for each of them and different resistance mechanisms for the two herbicides, which are, as commented previously, some of the main parameters influencing herbicide resistance evolution.

Saari et al. (1994) reviewed that most species are resistant to ALS inhibitors due to the presence of a single nuclear gene with incomplete dominance. No cases of a fitness penalty has been documented suggesting resistant plants should coexist with susceptible plants even in the absence of herbicides.

But apart from these reasons, 2.4-D resistant populations, which are probably metabolism-based, die with increased field rates, so that farmers hesitated in complaining seriously. In the case of tribenuron-methyl, increased doses do not kill any more target-site resistant plants, so that complains arrived much faster.

Following the farmers and applicators complains as well as the company's comments, in 1998, when the present PhD thesis started, the affected area in North-eastern Spain was increasing.

1.3. The winter cereal cropping system in North-eastern Spain

264.000 ha of rainfed winter cereals were sown in Catalonia in 1998 (DARP, 1998). In North-eastern Spain the use of chemical products in agriculture started in the 1950's together with mechanisation and practices like ploughing, mechanical weed control with a harrow as well as fallow started to disappear.

In the North-western parts of Catalonia (Osona region), where more rainfall and lower temperatures are recorded, some crops are included in the crop rotation apart from barley (*H. vulgare*) as it is wheat (*T. aestivum*), vetches (*V. villosa*) or alfalfa (*M. sativa*). In this area, farmers combine the crop production often with pig breeding. This leads farmers to spend less time on plant production, as animal breeding is often a high-productive activity. Herbicide use is the most frequent weed control method, complemented with ploughing in some cases.

In the drier areas of Catalonia (Lleida and Tarragona provinces), crop rotation is very difficult, as rainfall is sparse and irregular and is mainly concentrated in the autumn and winter months. At the moment, barley monocrop is the most frequent situation found in the drier rainfed cereal areas of Catalonia. Only in some moister areas oil-seed rape (*Brassica napus*), peas (*Pisum sativum*), hemp (*Cannabis sativa*), flax (*Linum usitatissimum*) and vetch (*Vicia villosa*) are sown. Other crops as potatoes or

alfalfa are not grown any more in these areas (Taberner *et al.*, 1992). Since no subsidies are paid for hemp and flax, much less surface of these crops is sown.

In these dryer and poorer areas of Catalonia, minimum tillage is the most frequent soil tillage practice since decades. In some areas even direct drilling is being adopted, so that very few farmers plough the soil. The almost only weed control measure in these areas is the herbicide use. Frequently, one single post-emergence tank mix of herbicides controlling broadleaf and graminea weeds is sprayed between November and March depending on the weed infestation and on the weather conditions. In order to control surviving or new emerged weeds, a second application with the same or different herbicides may be realised in some cases. Moreover, alternation of herbicides from year to year is not very common. Also in these areas, pig breeding is a very common complementary activity to barley production.

Further east, in Huesca and in Zaragoza, climatic conditions are similar to those of Lleida. The differences towards Lleida are that farmers are usually only plant producers and that ploughing is still conducted more frequently.

Even more east, finally, in Navarra and especially in Burgos, ploughing is a normal practice. Soils are very fertile and farmers are usually exclusively plant producers. More wheat than barley is grown in these cooler and moister areas. Herbicides were introduced some years later in these areas compared to Catalonia, but are, at present, very widespread.

Following the HRAC (HRAC, 2001a) the described situation is of moderate to high-risk for herbicide resistance development (Table I.1.) depending on the concrete location.

Table I.1.: Assessment of the risk of herbicide resistance development for target weed species depending on the management practices. The highlighted cells correspond to the general situation in the study area towards tribenuron-methyl and 2.4-D (Adapted from Herbicide Resistance Action Commitee, 2001a).

	Risk of herbicide resistance occurrence		
Management strategy	**LOW**	**MODERATE**	**HIGH**
Herbicides used in the cropping system	> 2 modes of action	2 modes of action	1 mode of action
Weed control in the cropping system	Cultural, mechanical and chemical	Cultural and mechanical	Chemical only
Use of the same mode of action per season	Once	More than once	Many times
Cropping system	Full rotation	Limited rotation	No rotation
Resistance status to the mode of action	Unknown	Limited	Common
Weed infestation	Low	Moderate	High
Control in the last 3 years	Good	Declining	Poor

2. Objectives

The main objectives of this work were:

2.1. To describe the situation of *P. rhoeas* herbicide resistance towards 2.4-D and tribenuron-methyl in winter cereals in North-eastern Spain.

Therefor, it was aimed to develop a seed-based quick-test for herbicide resistance detection for each herbicide (**Chapters preliminary, 1 and 2**). Before use, the tests should be validated with whole plant experiments.

To describe all the collected populations behaviour towards 2.4-D and tribenuron-methyl with these methods (**Chapter 3**).

2.2. To analyse different control strategies in order to offer a wide range of management possibilities to farmers with herbicide resistance.

To study biological aspects of *P. rhoeas* important for control methods optimisation. These included the description of the germination cycles, the viability after burial in different depths and survival habits in field for susceptible and for herbicide resistant populations (**Chapters 4, 5**).

To study the effect of ploughing as a weed control method by describing the evolution of the weed emergence and of the seed bank (**Chapter 6**).

To optimise the use of the tined weed harrow in order to contribute in designing a management strategy for mechanical *P. rhoeas* control (**Chapter 7)**.

To use different herbicides, which are allowed for weed control in cereals, in order to test their efficacy on herbicide resistant *P. rhoeas* (**Chapter 8)**.

Therefor, to provide the basis for the development of an Integrated Weed Management strategy for *P. rhoeas* control in the study area.

3. Experimental work

3.1. Characterisation of the herbicide resistance problem towards *Papaver rhoeas* in North-eastern Spain

3.1.1.Survey of herbicide resistant Papaver rhoeas *populations* (Chapters preliminary, 1, 2 and 3)

3.1.1.1. Methods used for the characterisation of the collected populations

In order to determine the behaviour of the collected *P. rhoeas* populations this study included different analysis techniques.

As a **first step**, whole plant assays were conducted in order to analyse the populations' behaviour towards 2.4-D and towards tribenuron-methyl. These kind of tests may provide information about cross- or multiple-resistance and alternative herbicides to be used (HRAC, 1999). An initial opinion on the resistance status could be given this way. Many studies on herbicide resistant weeds have used whole plant tests: Purba *et al.* (1993) in Australia on *Vulpia bromoides*, Boutsalis & Powles (1995) in Australia on *Sonchus oleraceus* and *Sisymbrium orientale* and Bravin *et al*. (2001) in Italy on *Lolium rigidum.*

The experiment was conducted in a plastic tunnel. Seeds were sown in 0.2 m x 0.15 m aluminium trays containing a 1:1 peat sand mixture. Plants were sprayed with a quarter, a half, single and double field rate of 2.4-D and of tribenuron-methyl. Unfortunately, in both trials conducted in 1998-99 and in 1999-00, in which 68 and 70 populations were tested, respectively, the climatic conditions caused high natural mortality making distinction of death causes difficult. In the first trial, a long freezing period followed by too high temperatures stressed plants very much. In the second trial, many plants didn't survive the heat in spring. Concluding, those populations, which survived despite the bad conditions, were probably really resistant. Data of these trials were thus only considered as additional information for the following tests.

In order to overcome the described handling problems the **second step** was to develop quick-tests for both herbicides, which are frequently seed-based (**Chapters preliminary, 1 and 2**). By these methods it was searched to be able to provide the farmer with a diagnostic of the sample as fast as possible. Another advantage is that these methods are less expensive and require less labour than the whole plant trials. They are also less dependent on the weather conditions, as they are conducted in growth chambers or in laboratory. These kind of tests are useful for routine screening of large number of susceptible or resistant populations (Heap, 1994) as it was the case of the present work. A disadvantage is that samples for analysing possible herbicide resistance can not be taken during the cropping season. It has to be waited that the plants develop mature seeds.

The "SYNGENTA test" had been tested successfully with different dicotiledoneous weed species such as *Amaranthus retroflexus, Chenopodium album, Raphanus raphanistrum* and *Polygonum aviculare* (Boutsalis, 2001). It consists in

cutting down grown plants allowing fresh tissues to develop, which would be sprayed with herbicides afterwards.

After testing, however, it has been found that *P. rhoeas* is very susceptible to transplantation and to any handling, so that this species could not be analysed with this method (data not shown). This characteristic of *P. rhoeas* is consistent with the description of Jones & Blair (1996) who found that this species is very susceptible to uprooting, regardless of the tested moisture conditions and on the shading after uprooting (Jones *et al.*, 1999).

Other methods described in the literature by Heap (1994), Letouzé *et al.* (1997) and Moss *et al.* (1999) are seed-based quick-tests on filter paper for graminea like *L. rigidum, Alopecurus myosuroides* and *Avena* sp.. In contrast to these species, *P. rhoeas* did not germinate properly when the seeds were surrounded by water, so that a different method had to be developed.

A preliminary study included the optimisation of *P. rhoeas* germination on agar medium in order to avoid the dormancy problems (Cirujeda *et al*., 1999) (**preliminary chapter**). This study was based on previous germination tests contained in a Master Project of Javier Nievas (Nievas, 1998) at the University of Lleida. He found the best germination results at intermittent temperatures between 20 and 10ºC at day and at night, respectively and with additions of gibbereline independently of the light conditions.

Seeds were placed on agar medium containing KNO_3, gibbereline and the herbicide (**Chapters 1 and 2**). The first studies for designing these methods lead to a Master Project at the University of Lleida by Lourdes Ruiz (Ruiz, 2000).

The **third step** was to obtain dose-response curves for 2.4-D and for tribenuron-methyl conducted on whole plants for some representative populations. This way, for the case of 2.4-D, the classification resulting from the quick-tests could be related to field rates.

3.1.1.2. Field survey

Seeds of 116 different *P. rhoeas* populations were collected in June and July 1998, 1999 and 2000. The regions of Catalonia, Aragón and part of Burgos and Navarra in North-eastern Spain were surveyed. 56 populations, which had been collected from 1990 to 1997, were included in the study.

Possible susceptible standard populations were gathered as well as samples from fields with unknown spraying history. Unsprayed fields were avoided in order to collect as many suspicious populations as possible.

Most of the populations came from fields, in which herbicide effect had been less than expected. These fields were found after asking farmers, herbicide distributors, applicators and technicians for efficacy complains.

One of the aims of the survey was to confirm the generalised suspicion of herbicide resistance in *P. rhoeas* in the surveyed area, as very few cases had been characterised previously.

The levels of resistance for the two herbicides were also investigated.

Another aim was to find out, which was the most frequent situation in the study area: resistance to 2.4-D, to tribenuron-methyl or to both herbicides.

The results of the studies on the randomly collected seed samples aimed to describe the generalised situation of fields infested with *P. rhoeas* in North-eastern Spain.

After validating the quick-tests with whole plant trials in greenhouse and field trials, all the populations were analysed with these quick-tests (**Chapter 3**).

3.2. Control strategies of herbicide resistant *Papaver rhoeas*

3.2.1. Integrated Weed Management (IWM)

Following Regehr (1993) IWM is characterised by processes and practices that complement and reinforce each other, in order to exploit weaknesses in weed species.

In the study area, herbicide resistance on *L. rigidum* has already forced some farmers to adopt very different management strategies besides the use of herbicides. Crop rotation, sowing delay, ploughing and even fallow are some measures they have to use again in order to get rid of the high grass populations. The crop rotation mainly allows these farmers to include other active ingredients to reduce the weed populations besides breaking the weed life cycle, but at least they do not rely exclusively on herbicides.

Currently, due to the socio-cultural background of the farmers, a weed management strategy for any weed species excluding herbicides in the study area is difficult to propose. On the other hand, it is considered possible to introduce some new aspects and to approach IWM strategies.

Numerous examples of effective IWM in resistant weed species have been reported, e.g. Powles & Matthews (1996) for multiple resistant Australian *L. rigidum*; Recasens *et al.* (2000, 2001) for Spanish *L. rigidum* resistant to diclofop-methyl and to chlortoluron; Thill et al. (1994) for *A. fatua* resistant to several graminicides; Read *et al.* (1997) for *Alopecurus myosuroides* resistant to aryloxyphenoxypropanoates (“fops”) and cyclohexanediones (“dims”); Curran (1999) for atrazine-resistant *Amaranthus* sp. and *C. album*; Thill & Mallory-Smith (1996) for ALS-inhibitor herbicide resistant *Kochia scoparia*.

Thill *et al*. (1994) think that the likelihood of selecting for herbicide resistant weed biotypes should be minimal if an effective IWM plan is part of a crop production program. IWM is, thus, both, a preventive and a curative strategy. Liebmann & Gallandt (1997) state that IWM strategies should include tactics that affect crop-weed competition, weed seed production, seed dispersal and seed survival. The same authors

discuss the numerous control tactics for subjecting weeds to multiple, temporary variable stresses. This philosophy of using "many little hammers" to manage weeds does not exclude the use of direct controls as the herbicides but focuses on many indirect interactions that can lead to a successful management (Liebmann & Gallandt 1997).

Bhowmik (1997) has a similar opinion. She thinks that currently, the research emphasis is no more studying the effects of crop sowing dates, row spacing, use of cover crops, cultivating etc. but trying to face the need of understanding basic weed biology. This leads to study weed seed bank dynamics and modelling weed seedling emergence. Knowledge on these basic aspects will help designing the correct management strategies. Following the review of Thill *et al.* (1994) these strategies should include a consideration of the tillage system, of the critical period of weed interference, alternative methods of weed control, enhanced crop competitiveness, crop rotation, weed seed bank dynamics and modelling of crop-weed interference. Most of these aspects have been studied in the present work.

After detecting and characterising the *P. rhoeas* weed control problem in the present case, three main biological aspects were studied: seed bank dynamics, seed germination and emergence habits and seedling survival. All the studies were conducted on susceptible and on herbicide resistant populations. This was done in order to find out if any fitness penalty on the resistant populations was observed and to find out if different management strategies should thus be considered depending on the population.

3.2.2. Study of biological aspects of **Papaver rhoeas** *focusing on its control*

3.2.2.1. Study of the weed seed germination habits in field conditions **(Chapter 4)**

Little data has been found on *P. rhoeas* germination patterns in North-eastern Spain. Izquierdo & Recasens (1992) describe main germination in this area between September and December based on one-year observations. As germination is weather-dependent, it was considered useful to follow germination of different populations at different locations.

The knowledge of *P. rhoeas* germination habits throughout the different years and locations is needed for the correct timing of any control strategy, which normally should be conducted after the main germination flushes. Knowledge of germination patterns can be useful for other control strategies, also. A high germination rate may be induced by false seedbed preparation in the correct moment and weed destruction conducted later but previous to crop sowing. Thanks to these studies it can also be known since when germination can be expected and thus since when cereal sowing date should be delayed to reduce weed germination inside the crop.

The effect of cultivation on germination contributes to find out if e.g. untilled fallow is a possible control strategy or if cultivation in an appropriate moment is more effective reducing the *P. rhoeas* seed bank. Previous observations (data not shown) demonstrated that late autumn soil cultivation can be followed by abundant *P. rhoeas* germination while post-harvest cultivation of cereal fields in early summer provokes the

rise of very few or no *P. rhoeas* seedlings. In the experiments, cultivation was simulated in autumn and early winter, simulating normal farmers' practices.

Germination habits were described in two locations with different climate taking into account the influence of the sowing year, of the seed age, of the population's origin, of the susceptibility or herbicide resistance of the population and of the influence of cultivation on germination. Monthly germination from a known number of freshly produced seed lots was recorded. The same observations were conducted on mixed-aged seed lots contained in the field soil.

3.2.2.2. Study of the seedling survival in field conditions **(Chapter 4)**

Survival patterns of a weed species may help deciding if a control method is justified in a concrete case. If e.g. high natural mortality is expected to occur, initial low weed density may perhaps not justify a control method. Additionally, if natural mortality is very high, a control method and even a herbicide, which achieve a certain efficacy but does not arrive to control the population completely, could take benefit of this mortality and end in a almost weed-free situation.

P. rhoeas plant number m^{-2} evolution was described in the untreated plots of all field trials during the cropping cycle. The diversity of climatic conditions throughout the study area and throughout the years as well as the soil diversity guarantees a broad picture of the possible emergence, establishment and survival patterns of this weed in North-eastern Spain. The influence of inter- and intra-specific concurrence is recorded this way. The practical information of these data is to know the possible *P. rhoeas* infestation levels expected in cereal fields as well as its possible evolution in time.

3.2.2.3. Study of the weed seed bank dynamics: dormancy cycles and seed viability in time **(Chapter 5)**

Germination capacity of *P. rhoeas* seeds exhumed after burial in different depths have been analysed for herbicide susceptible populations by diverse authors in Northern and Central European countries (Kjær, 1940; Roberts and Feast, 1973; Froud-Williams *et al.*, 1984; Barralis *et al.*, 1988). Results of these authors, however, are quite diverse although the general observations suggest that this weed species has a persistent seed bank. However, no data have been found for *P. rhoeas* dormancy and longevity behaviour in Mediterranean conditions.

This knowledge has different practical applications for weed management. On one hand, knowing the dormancy cycle it can be predicted when germination is supposed to occur and when it is not probable. This way, critical germination periods can either be avoided during a cropping cycle or could be exploited enhancing germination with later destruction in a no-crop period. On the other hand, knowing about the viability of the seeds in time after burial will be crucial to design a control program using the seed burial strategy by ploughing.

In the present study, the dormancy cycles and the persistence of the seed bank of one susceptible and two herbicide resistant populations was analysed.

3.2.3. Direct weed control measures

Mainly caused by the increased incidence of herbicide resistance and by environmental concerns, a shift from chemical technologies towards ecologically based strategies is desirable (Liebmann & Gallandt 1997). Based on this idea, ploughing, mechanical weed control using a tine harrow and also chemical weed control were the tested direct control methods in this work. The same authors think that combining indirect and direct control methods environmental and economic objectives of weed management may be more readily met. A combination of all these methods for *P. rhoeas* control is, thus, desirable.

3.2.3.1. The effect of ploughing on Papaver rhoeas *emergence and on the seed bank* **(Chapter 6*)***

Numerous authors have described the influence of ploughing versus the use of other tools as chisel or cultivator in the vertical seed distribution of weed seeds. Ball (1992) found 53% of the total weed seeds between 10 and 15 cm depth while only 17% were located in that depth in the chiseled plots. After six growing seasons Clements *et al*. (1996) observed 38% of the total weed seeds in the same depth in front of the 16% when a chisel plough was used. Maximum depth of *P. rhoeas* emergence was found to be 2 cm depth (Froud-Williams *et al*. 1984) so that germination after ploughing in 20cm depth would be very unlikely to happen in this weed species. McCloskey *et al.* (1991) found ploughing capable to control *Galium aparine, Bromus sterilis* and *P. rhoeas*, although little information could be given on this last species as it was an uncommon species in their trial.

The use of the plough is, thus, considered as one more possible density reduction method useful also in cases of very high *P. rhoeas* infestations. The soil inversion is presumed to bury seeds into depths, which would not allow germination unless the soil was inverted again.

Another important aspect is to know how *P. rhoeas* seeds would behave after being moved again upwards after one, two or more cropping seasons i.e. to establish the approximate adequate ploughing frequency. Previous studies conducted in North and Central European countries as France, United Kingdom and Denmark are mainly coincident with a long survival capacity of this species (Kjær, 1940; Roberts and Feast, 1973; Froud-Williams *et al.*, 1984; Barralis *et al*., 1988). Data on the rates of decrease as well as survival after some years of burial are disperse between these authors mainly finding high rates of seed survival. Nevertheless, Roberts & Feast (1972) found low values of recovered seeds from the upper layer of 2.5 cm of soil compared to other weed species. It is, then, not very clear how *P. rhoeas* seeds would react in front of a repeated ploughing strategy.

This burial strategy has been effectively tested in wild oats. After reviewing different tillage experiments in this species, Thill *et al*. (1994) concluded that occasional deep ploughing as once every 4 years could reduce populations of wild oats. If ploughing was conducted every year, wild oat populations increased because buried seeds were brought to surface. In this case, the authors suggest this control strategy in order to prevent the appearance of herbicide resistance.

Studies on seed burial have also been conducted in other herbicide resistant species. One example is a sulfonylurea herbicide resistant *Sisymbrium orientale* whose behaviour after burial was studied by Boutsalis & Powles (1998). Short seed-bank longevity was described for this species so that in this case, seed burial would probably be useful for population reduction if new seed rain was prevented at the same time.

To test this strategy, which is effective in other weed species with *P. rhoeas* was one of the aims of this study.

A field trial was kept from autumn 1998 since summer 2001, in which ploughing was conducted in winter 1998, winter 2000 and in some plots, both times. The alone effect of ploughing on the weed population was observed as well as in combination with harrowing. This study was complemented with the seed bank dynamic experiment described before in order to determine the long-term viability of the seeds.

3.2.3.2. Mechanical weed control **(Chapter 7***)*

As commented previously, *P. rhoeas* is very susceptible to transplantation. Regardless of the moisture and shade after uprooting, survival was very little in pot experiments conducted by Jones & Blair (1996) and Jones *et al.* (1999). Taking into account that uprooting is probably the main mortality factor in weed harrowing (Kurstjens & Kropff, 2001), this behaviour is a good starting point for mechanical weed control in this weed species.

The use of tine harrows was widespread in some areas of Catalonia before the appearance of herbicides in the 50's. The actual commercial models are much more sophisticated than the ancient harrows, but the system is similar. The use of the harrow was studied alone, without herbicide combination. In case of small weed infestations it was thought that the harrow use could be enough for maintaining the weed populations at low density levels but the present work wanted to test how effective this method could be controlling huge densities of *P. rhoeas* by its own.

Harrowing has been tested in Northern Europe since many years. Habel (1954), Koch (1964a, b) and Kees (1962) published some of the first results commenting already the need of at least combining herbicides with other control methods. A second flush of work focused on the correct timing, the need of repetitions in time, the best speed and other aspects of the correct use of the implement (Böhrnsen, 1993; Rasmussen, 1990, 1991, 1992, 1993, 1996; Rasmussen & Svenningsen, 1995; Rydberg, 1993, 1994; Welsh *et al.*, 1997; Wilson *et al.*, 1993). It was found, however, that the effect of the implement is very dependent on many factors, including the weed species and the environmental factors. Additionally, most of the work has been done in northern

countries, where the cold winter and the often wet autumn and winter are limiting factors for the harrow use.

The present local conditions, however, are so different that probably an adaptation of the strategies should be designed. Soils are often dry during long periods of time and much stonier. The winter periods can also be moist but normally rainfall is concentrated in few days during winter and spring, allowing usually harrowing in these periods. Also the winter cereal life cycle is much shorter than in the Northern countries starting at ends of October and finishing at ends of May or beginnings of June. All these factors affect the possible optimal harrowing moment, the need or not of repeating the treatment more than one time during the cropping season, the position of the harrow, etc.

In Spain, Lacasta *et al*. (1997), Lezaún *et al.* (2001), Moyano *et al*. (1998), Pardo *et al*. (2001) and Zaragoza *et al*. (1999), between others, have been recently testing harrowing in cereal. Very few data in these studies, however, refer to *P. rhoeas*.

In the present work, the intention of defining a mechanical weed control strategy led to try to answer some of the following questions:

- Which is the optimal harrowing moment taking into account *P. rhoeas* emergence and growth?
- This means: how long can a farmer wait if the soil and climatic conditions are not suitable for harrowing?
- On the other hand: if the control is done too early, can new *P. rhoeas* germination be stimulated?
- Can pre-emergence harrowing in the conditions of North-eastern Spain be useful for *P. rhoeas* control?
- Is there a big difference in efficacy depending on the soil moisture?
- Is it useful to conduct more than one harrowing during the same season, despite the shorter cropping seasons compared to northern countries?
- Is the presence of stones a big inconvenient?
- Which speed is the best one? Is there a gradual increase in efficacy related to speed?

Therefore, 10 field trials have been conducted trying to design a mechanical control strategy for the local climatic, soil and cropping conditions.

Rasmussen, (1996) considered that mechanical weed management will probably never be as effective as herbicides. Therefore, the challenge of herbicide cut-back is not a simple question of replacing herbicides by mechanical control. In order to take the maximum benefit out of it, the use of these methods should be studied as well as possible and, if possible, other measures should be taken into account. In the present work, also the combination of ploughing and weed harrowing was studied (**Chapter 6**).

Moreover, some authors also propose the use of sub-lethal herbicide doses previous to mechanical weed control in order to debilitate weeds and to enhance the mechanical weed control. Caseley *et al.* (1993) found that this technique held the weeds at a growth stage vulnerable to cultivation and inhibited moreover shoot and root growth following harrowing. Blair & Green (1993) used 20% of label recommended rate of mecoprop-P

followed by cultivation at growth stage 30 (following BBCH code) in spring wheat achieving better *P. rhoeas* control than using the herbicide alone at full rate.

These combinations of chemical and mechanical weed control, however, are a bit risky because in some cases the low rate can be too low to affect the weeds. This occurred with tribenuron-methyl in *P. rhoeas* in an experiment of Cashmore & Caseley (1995). It can also happen that the herbicide is very effective at low rates and harrowing is not necessary any more. In Italy, Ferrero & Vidotto (2000) used sequences of chemical and mechanical weed control successfully, reducing the need of chemicals.

Combinations of mechanical and chemical weed control were not studied in the present work but are an interesting topic to work on. Regarding herbicide resistance prevention , any method diversifying the use of herbicides is welcome.

3.2.3.3. Herbicide control **(Chapter 8)**

The most used control strategy in the studied areas is the exclusive rely on herbicide application, which is at the same time the responsible for the existence of the resistance problem. The appearance of herbicide resistance forces to change the weed management strategy. The smallest possible change in this strategy is the alternation of herbicides belonging to groups of different modes of action. Other alternatives, which do not require a big change in the farmers' attitude are offered by spraying at other application moments (which normally leads to a change in the mode of action): pre-emergence, early post-emergence or late post-emergence referring to crop and weeds.

One first aim towards an IWM of herbicide resistant *P. rhoeas* was to test the efficacy of several authorised herbicides for broadleaf control in winter cereals. The results should offer a range of herbicides giving different options both for herbicide resistance appearance prevention and for the control of already herbicide resistant weed populations.

Following the official herbicide recommendations of the Plant Protection Service of the Catalan Government (Generalitat de Catalunya) (Taberner *et al.*, 2000) a wide range of herbicides and herbicide mixtures active on *P. rhoeas* are allowed in winter cereals. Table I.2. summarises the main characteristics of these products.

Table I.2. a: Herbicides authorised in winter cereal in Spain (adapted from Taberner *et al.*, 2000). MS = medium susceptible for *Papaver rhoeas*, S = susceptible for *Papaver rhoeas*. Chlorsulfuron and Isoproturon + diflufenican are authorised in wheat fields, only. Application rate in L or kg ha^{-1}. E = efficacy. HRAC = Herbicide Resistance Action Committee.

Herbicides recommended for the control of *Lolium rigidum* and broad-leaved weeds **Herbicides to be used in post-sowing of the cereal and in pre-emergence of the weeds**				
Composition	**Rate**	**Commercial name**	**E**	**HRAC group**
Chlorsulfuron 75%	0.02	Glean / Belure	S	B
Triasulfuron 0.6% + terbutryne 59.4%	0.50-0.60	Logran extra	S	B + C1
Triasulfuron 0.25% + terbutryne 10.75% + chlortoluron 53%	1.00-1.50	Tricuran 64 WG	S	B + C1 + C2
Trifluraline 24% + linuron 12%	3.00-4.00	Several	MS	K1 + C2
Chlortoluron 50%	3.00-5.50	Several	S	C2
Chlortoluron 43% + terbutryne 7%	4.00-5.50	Several	S	C2 + C1
Isoproturon 50%	3.00-4.00	Several	MS	C2
Isoproturon 45% + diflufenican 4.2%	3.00	Javelo	S	C2 + F1
Isoaxaben 50%	0.20-2.50	Rokenyl 50	S	L
Metribuzin 2.8% + isoproturon 50%	2.00-2.50	Sencor IP	S	C1 + C2
Pendimethaline 33%	5.00	Several	S	K1
Triasulfuron 20%	0.015-0.0375	Logran 20	S	B

Table I.2. b: Herbicides authorised in winter cereal in Spain (adapted from Taberner *et al.*, 2000). MS = medium susceptible for *P. rhoeas*, S = susceptible for *Papaver rhoeas*. Application rate in L or kg ha^{-1}. E = efficacy. HRAC = Herbicide Resistance Action Committee.

Herbicides recommended for the control of broad-leaved weeds only **Herbicides to be used in post-emergence of the cereal on seedlings and young plants**				
Composition	**Rate**	**Commercial name**	**E**	**HRAC group**
Amidosulfuron 75%	0.02-0.04	Gratil 75 WG	MS	B
Bromoxinil octanoic ester 24%	1-2	Several	MS	C3
Bromoxinil octanoic ester 12% + 2.4-D 36%	1.5-2	Asitel	MS	C3 + O
Bromoxinil 22% + MCPA 30%	2-2.5	Primma BX	MS	C3 + O
Bromoxinil 12% + MCPP 36%	2-3	Bromoxan super	MS	C3 + O
Ioxinil 24%	1.5-2.5	Several	S	C3
Ioxinil 12% + MCPP 36%	2-3	Certrol-H	S	C3 + O
Ioxinil 7.5% + bromoxinil 7.5% + MCPP 37.5%	2-3	Brioxil / Oxytril	S	C3 + C3 + O
Triasulfuron 0.6% + terbutryne 59.4%	0.4	Logran extra	S	B + C1
Tribenuron-methyl 75%	0.015-0.025	Granstar	S	B
Tribenuron-methyl 25% + thifensulfuron-methyl 50%	0.030-0.045	Posta	S	B + B
Isoproturon 50% + metribuzine 2.8%	2.5	Sencor IP	S	C2 + C1
Metribuzine 70%	0.1	Several	MS	C1
Terbutrine 49%	0.3	Several	MS	C1

Table I.2. c: Herbicides authorised in winter cereal in Spain (adapted from Taberner *et al.*, 2000). MS = medium susceptible for *Papaver rhoeas*, S = susceptible for *Papaver rhoeas*. Application rate in L or kg ha^{-1}. E = efficacy. HRAC = Herbicide Resistance Action Committee.

Herbicides to be used in post-emergence of the cereal on young and already developed weeds				
Composition	**Rate**	**Commercial name**	**E**	**HRAC group**
2.4-D (several formulations)	0.5-2	Several	MS, S	O
Dichlorprop-p 60%	2	Duplosan	S	O
Dichlorprop-p 23.3% + bentazon 33.3%	2-3	Basagran DP	S	0 + C3
Dichlorprop-p 31% + MCPP 13% + MCPA 16%	2.5	Duplosan super	S	O + O + O
Dicamba 48%	0.3-0.6	Banvel D	S	O
Dicamba 3% + MCPA 36%	1.5-2.5	Magarzel	S	O + O
Dicamba 10% + MCPA 26.5% + 2.4-D 29.6%	0.8-1.5	Herbicruz Magapol / Banvel Triple	S	O + O + O
Clorpyralid 3.5% + 2.4-D 36%	1.5-2	Primmatrel	S	O + O
MCPA (several formulations)	0.7-3	Several	MS, S	O
MCPA + 2.4-D (several formulations)	0,7-1.5	Several	MS, S	O + O
MCPP 57.5%	2-4	Primma Galium / Herbimur Forte	MS	O
MCPP 40% + MCPA 10%	3-4	Primma Combi	MS	O + O

Table I.2. d: Herbicides authorised in winter cereal in Spain (adapted from Taberner *et al.*, 2000). MS = medium susceptible for *Papaver rhoeas*, S = susceptible for *Papaver rhoeas*. Chlorsulfuron is authorised in wheat fields, only. Application rate in L or kg ha^{-1}. E = efficacy. HRAC = Herbicide Resistance Action Committee.

Herbicides to be used in post-emergence of the cereal controlling grass weeds and broad-leaved weeds.				
Composition	**Rate**	**Commercial name**	**E**	**HRAC group**
Chlorsulfuron 75%	0.01-0.02	Glean / Belure	MS	B
Chlortoluron 50%	2.5-4	Several	MS	C2
Chlortoluron 40% + diflufenican 2.5%	1.75-3	Harpo-Z	S	C2 + F1
Chlortoluron 43% + terbutrine 7%	2.5-4.5	Several	S	C2 + C1
Chlortoluron 53% + terbutrine 10.75% + triasulfuron 0.25%	1.5-2	Tricuran 64 WG	S	C2 + C1 + B
Imazamethabenz-methyl 12.5% + pendimethaline 20%	5	Chacal	S	B + K1
Isoproturon 50%	2.5-3.5	Several	MS	C2
Isoproturon 45% + diflufenican 4.2%	3	Javelo	S	C2 + F1
Isoproturon 50% + metribuzine 2.8%	2.5	Sencor IP	S	C2 + C1
Metribuzine 70%	0.1	Several	MS	C1
Terbutrine 49%	0.3	Several	MS	C1

Out of this big range of possibilities, the most common, the most active and the most known herbicides from each of the groups were chosen and tested on herbicide resistant *P. rhoeas*. 16 field trials were conducted during the cropping season 1997-98 since 2000-01.

The different *P. rhoeas* populations were resistant to tribenuron-methyl only or resistant to tribenuron-methyl and 2.4-D. Unfortunately, it was not possible to conduct any trial on a field resistant to tribenuron-methyl, only. Two fields were kept during two and one field during three cropping seasons.

The sites repetition was done with the aim of observing if the herbicides act in a constant way under changing climatic conditions. In fact, rainfall and temperatures were very different during the four cropping seasons reflecting the climatic variability of the Mediterranean climate. Very dry towards very moist winters, very mild towards strong freezing winters as well as warm and dry towards rainy springs were probably the more contrasted situations between years.

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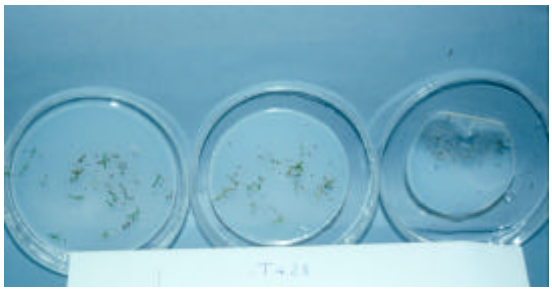
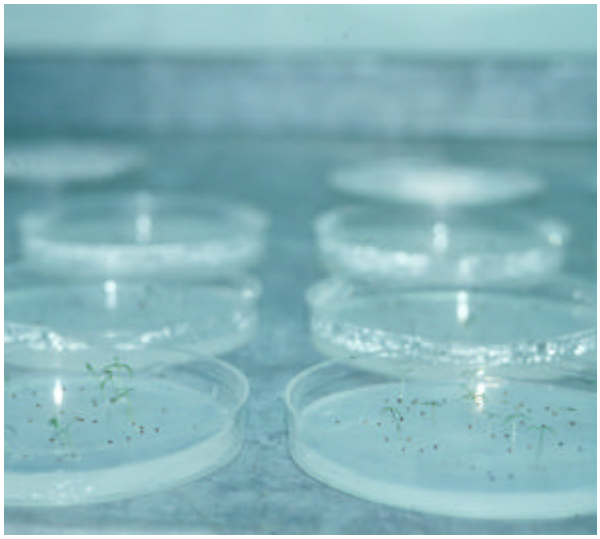
II. Results

Part A

Characterisation of herbicide resistance in *Papaver rhoeas*





Preliminary chapter

Optimisation of *Papaver rhoeas* L. germination on agar medium

11th EWRS (European Weed Research Society) Symposium 1999 Basel

Optimization of *Papaver rhoeas* (L.) germination on agar medium

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INTRODUCTION

Freshly harvested *Papaver hoeas* (L.) seeds show strong dormancy which decreases gradually in time Holm et al. (1997). Different authors describe diverse methods used for enhancing germination. Bishop et al. (1996) stratify the seeds during 14 days at 4ºC, Barralis et al. (1988) add 1g GA_3/l to the water placed under the Petri dishes. Quick-tests are necessary for herbicide resistance detection (Letouzé et al., 1998) and there is an interest in finding dormancy breaking techniques. The aim of this work is to find the best germination conditions on agar medium.

MATERIALS AND METHODS

Seeds of three different populations collected in July 1998 were sown in Petri dishes on agar medium (13 g/l). The three replications per treatment were placed randomly in a growth chamber. Different seed pretreatments were tested during five months. Following the IBPGR recommendations the *FIRST STEP* was to test the germination at different temperature and light regimes. This was analysed in previous experiments. The chosen regime was 18h of illumination at 25ºC and 6 hours of darkness at 10ºC, although the germination rate was only high for older seeds (Nievas et al., 1998).

The *SECOND STEP* was to test the effect of the addition of KNO_3. As there was no germination in the control dishes the addition of 2 g KNO_3/l was taken as standard in all further experiments.

Due to the frequent fungus contamination observed the *THIRD STEP* was to test the effect of fungicide use. Benzimidazole and TCMTB were compared and afterwards 15, 20 and 25 ppm of benzimidazole were used.

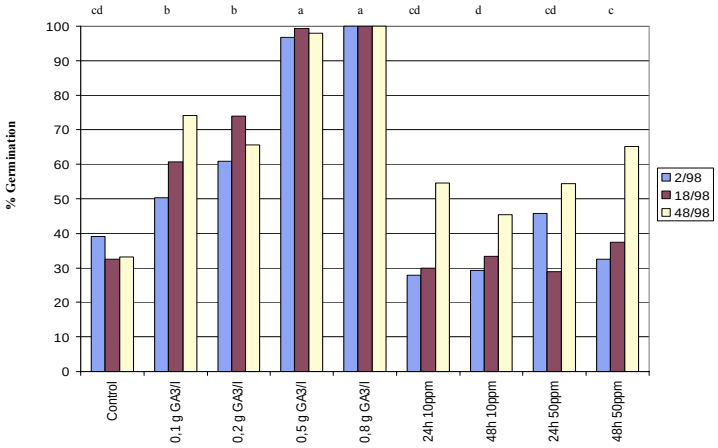

Fig. 3: Germination rate of seeds of three different populations of *Papaver rhoeas* L. after 20 days. Seeds were placed on agar medium containing 0.1, 0.2, 0.5 or 0.8 g GA_3 or were inmersed in 10 or 20 ppm of GA_3 during 24 or 48 hours before sown. 2 g KNO_3/l were added.

The *FOURTH STEP* was to test different dry and wet storage of the seeds. The seeds were kept dry at 4ºC during 5 days or in the growth chamber under the alternating conditions with and without light during 27 days. The wet storage included stratification at 4ºC during 15 and 40 days without light or keeping the seeds in deionized water during one, three or eleven days at 20ºC with alternating light.

The *FIFTH STEP* consisted in adding 0.1, 0.2, 0.5 and 0.8 g GA_3/l to the medium. It was also tested to leave the seeds in a 10 and 50 ppm solution of GA_3 during 24 and 48 hours at 20ºC.
Moreover, germination of seeds buried in July 1998 was compared with the warehouse stored ones.

The results were analysed with the SAS statistical system and submitted to an arcsinus transformation.

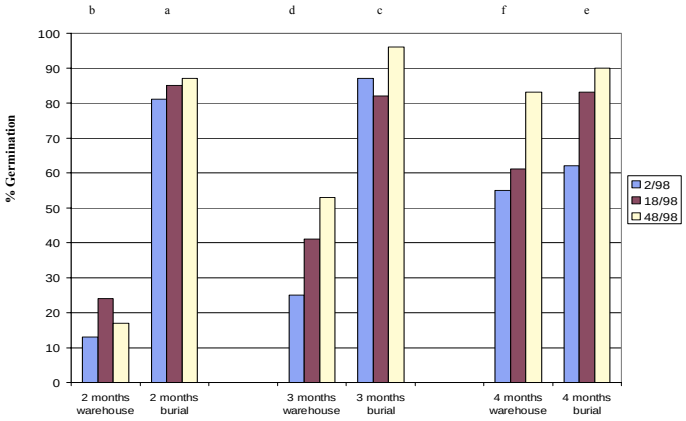

Fig 2: Germination rate of seeds of three populations of *Papaver rhoeas* L. on agar medium after 20 days having been stored during two, three and four months in warehouse or buried in 2 cm depth during also two, three and four months (2 g KNO_3/l and 50ppm of benimidazole were added to the agar medium).

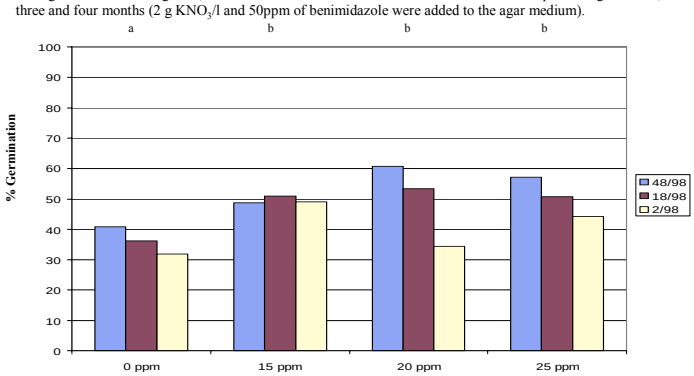

Fig. 3: Germination rate of seeds of three populations of *Papaver rhoeas* L. on Petri dishes after 20 days adding 15, 20 and 25 ppm of benzimidazole to the agar medium.

RESULTS

The germination of the warehouse stored seeds increased substantially during the testing period (Fig. 2). The burial in 2 cm depth increased the germination rate significantly in all three months. *STEP TWO* showed that the addition of 1-3 g KNO_3/l significantly increased germination compared to no addition. Best values were achieved for 2 g KNO_3/l (data not shown).

The addition of fungicide (*STEP THREE*) reduced fungus contamination, especially benzimidazole. In addition, germination was increased. All three concentrations (15, 20 and 25 ppm) gave significantly greater germination rates than the control dishes (Fig.3).

The different dry seed pretreatments (*STEP FOUR*) resulted in little differences in the germination rate (data not shown). The stratification during 15 or 40 days resulted in a significant reduction of the germination rate in comparison with the seeds kept in the warehouse. Keeping the seeds in water at 20ºC during 1, 3 or 11 days didn't increase significantly the germination at none of the populations. Strange growth was detected after keeping the seeds in water prior to sowing.

The addition of gibbereline (*STEP FIVE)* to the medium gave significant differences: 0.5 and 0.8 g GA_3/l gave very high germination rates, higher than 0.2 and 0.1 g/l (Fig. 1) but showed abnormal root growth in some experiments. All four concentrations resulted in significantly higher germination rates than the control dishes. In all four cases the germination was also faster and not so gradually. Keeping the seeds in gibbereline solution prior to sowing increased germination significantly in one population.

CONCLUSIONS

1. **The seeds of *Papaver rhoeas* showed a natural loss of dormancy.**
2. **The addition of 0.5 and 0.8 g GA_3/l gave the highest germination rates and enhanced growth but showed sometimes growth problems.**
3. **The burial of the seeds in 2 cm depth promoted also germination.**
4. **The use of 2 g KNO_3/l enhanced germination**
5. **All the other tested treatments resulted in small differences on the germination rate.**

Chapter 1

A qualitative quick-test for herbicide resistance detection to tribenuron-methyl in *Papaver rhoeas* L. grown on agar medium

A qualitative quick-test for herbicide resistance detection to tribenuron-methyl in *Papaver rhoeas* L. grown on agar medium

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Summary

A qualitative seed-based method useful for the detection of herbicide resistance to tribenuron-methyl in *Papaver rhoeas* L. is described. Seeds were germinated on 35 ml of a 1.3 % agar medium containing 2 g KNO_3 L^{-1} in 8.5 cm Petri dishes in a growth chamber under 20 μmol S^{-1} m^{-2} of fluorescent light. When 0.24 μM tribenuron-methyl or more was added, growth in susceptible plants stopped after the cotyledon stage and they turned chlorotic. The resistant plants continued developing new leaves. The same effect was achieved when 0.2 g GA_3 L^{-1} and 7.68 μM tribenuron-methyl or 0.5 g GA_3 L^{-1} and 61.44 μM tribenuron-methyl were added.

Germination percentage rose with gibbereline in the presence or absence of the herbicide. Plants developed rapidly with only about 14 days needed to finish the test but sometimes root growth was reduced due to the addition of gibbereline. In the absence of gibbereline but in the presence of the herbicide, plants grew slower and developed smaller leaves with a 17-day evaluation period requirement.

The test was validated with pot experiments in a greenhouse and also with field trials. The best combination was found to be 0.2 g GA_3 L^{-1} and 7.68 μM tribenuron-methyl assuring homogenous germination and testing of dormant seeds but avoiding root inhibition associated to too much gibbereline.

Keywords: herbicide resistance, quick-test, tribenuron-methyl, *Papaver rhoeas* L., dormancy, gibbereline.

Introduction

The first case of herbicide weed resistance was identified in 1964. Currently, more than 150 resistant grass and broadleaf weed biotypes in about 50 countries world-wide have been recorded (Heap, 2000). One of the most significant occurrences in herbicide resistance has been the advent of weeds resistant to herbicides that inhibit acetolactate synthase (ALS) (Saari *et al.*, 1994). *Papaver rhoeas* L. is the most important broadleaf weed infesting winter cereals in North-eastern Spain occurring in 39% of surveyed fields (Riba *et al.*, 1990). Resistance in this weed to the sulfonylurea herbicide tribenuron-methyl has been reported recently (Taberner *et al.*, 1995; Claude *et al.*, 1998). Some cases of sulfonylurea resistance in *P. rhoeas* have also been recently cited in Italy (Sattin, pers. comm.) and in Greece (Kotoula-Syka, pers. comm.).

In Spain, the highly-active tribenuron-methyl has been available since 1986. In many situations this herbicide has been used annually in winter cereals imposing a high selection pressure on *P. rhoeas.* Imposition of a high selection pressure coupled with biological characteristics of *Papaver rhoeas* L. such as its high genetic variability (McNaughton & Harper, 1964), its outcrossing habit, self-incompatibility and the high seed production (Holm *et al.*, 1997) can favour the appearance of herbicide resistance.

Moreover, Saari *et al.* (1994) have reviewed that most of the reported species are resistant to ALS inhibitors due to the presence of a single nuclear gene with incomplete dominance. No cases of a fitness penalty have been documented suggesting resistant plants could co-exist with susceptible plants even in the absence of herbicides.

P. rhoeas with multiple resistance to sulfonylureas (tribenuron-methyl) and to 2.4-D has been documented (Claude *et al.*, 1998). For this reason, there is a need for a quick-test to detect tribenuron-methyl and 2.4-D resistant biotypes. Currently, the period of time for collecting *P. rhoeas* seeds from suspected resistant fields and conducting outdoor pot trials to confirm resistance is too short to provide feedback to farmers. This is often the case of tribenuron-methyl which is applied as an early post-emergence treatment. In the case of 2.4-D, which is applied later in the growing season, the results of the outdoor pot trials on resistance responses of *P. rhoeas* field samples can be obtained in time for the next cropping season.

Various tests have been discussed for screening seeds collected from suspect resistant plants for herbicide resistance. Tests referred to as Petri dish assays involve germinating seeds on agar or filter paper impregnated with herbicides (Heap, 1994). Such bioassays are comparatively quick and inexpensive, are reliable and are particularly useful for routine screening of a large number of susceptible or resistant populations (Heap, 1994). Examples of these techniques are the Rothamsted rapid resistance test described by Moss *et al.* (1999) and the method described by Letouzé *et al.* (1997).

Other resistance tests include germinating seeds in pots and spraying the plants with herbicides (HRAC, 1999). Pot tests may provide information about cross- or multiple-resistance and alternative herbicides to be used. Other tests such as germinating pollen on agar (Richter & Powles, 1993, Letouzé & Gasquez, 1998) or growing tillers in herbicide media (Letouzé *et al.*, 1997) have been reported. Recently, quick-tests which test plants collected from the field by growing in agar (Salas & Claude, 1999) or regenerating cuttings by growing in soil (Boutsalis, 1999) and spraying have shown the potential for a rapid feedback to farmers within the same cropping season. These tests are reliable because they test for all potential mechanisms of resistance. To date, all these tests have concentrated on grass weed resistance with the exception of the SYNGENTA test, which has also been used successfully with broad-leaved weed species such as *Amaranthus retroflexus, Chenopodium album, Raphanus raphanistrum* and *Polygonum aviculare* (Boutsalis, personal communication).

Physiological tests such as a chlorophyll fluorescence test (Cadahia *et al.*, 1982) for measuring resistance to Photosystem II herbicides are used in the field. Biochemical tests investigating the activity of various target enzymes such as acetolactate synthase (ALS) inhibited by sulfonylurea, imidazolinone, triazolopyrimidine and pyrimidinylthiobenzoate herbicides (Singh *et al.*, 1988) or ACC-ase inhibited by aryloxyphenoxypropionate and cyclohexanedione herbicides (Matthews *et al.*, 1990) have been documented. These tests are laboratory based and not currently offered as routine tests.

Freshly-collected *P. rhoeas* seed samples have a strong primary dormancy (Holm *et al.*, 1997). When collected in July and stored in plastic pots kept in a warehouse, this dormancy diminishes gradually during the first months reaching a certain stability after approximately seven months (A. Cirujeda & A. Taberner, unpublished data). The addition of gibbereline (GA_3) at 0.2 or 0.5 g L^{-1} can increase the germination rate considerably.

Dormancy of field collected seeds has been found to vary greatly between populations. Our studies have characterised germination of populations collected in different locations during the same summer (data not shown). The obtained germination rates were in some cases superior to 50% without adding any germination stimulant in some samples, while other populations require the addition of even 0.5 g GA_3 L^{-1} to achieve similar germination percentages (A. Cirujeda & A. Taberner, unpublished data).

GA_3 was investigated as a potential method of relieving dormancy of freshly harvested seeds to enable immediate testing after collecting *P. rhoeas* seeds from farmer fields.

The objective of this work was to develop a quick-test for the detection of herbicide resistance to tribenuron-methyl for *P. rhoeas.* Specific objectives were:

1) finding the optimal herbicide medium composition that distinguishes resistant from susceptible populations on agar medium (percentage of germination, root length or phenology/development).

2) After defining the methodology, screening different populations of different ages, from different locations, and with different herbicide sensitivity with the quick-test.

3) comparing the quick-test results with the results of field and greenhouse pot experiments.

Materials and methods

Plant material

Seeds of the poppy populations were collected in rainfed winter cereal fields in June and July of 1995 to 1999 in North-eastern Spain. Most of the fields were chosen because farmers complained about herbicide control problems and some were taken randomly without knowing their spraying history. The field history of most of the fields was a continuous or little-interrupted application of three to ten years of tribenuron-methyl.

Most of the populations were collected from fields with a barley monoculture due basically to the semiarid climatic conditions which allow little crop rotation; some had barley-wheat rotation and only a few populations were collected in fields in which crop rotation included canola (*Brassica napus* L.), peas (*Pisum sativum* L.) or maize (*Zea mays* L.).

The populations chosen for finding the optimal medium conditions were two susceptible populations (from 1995 referred to as S1 and from 1998 referred to as S2) and two resistant ones (from 1996 referred to as R1 and from 1998 referred to as R2). Their origins were North-eastern Spain (Catalonia). The susceptible standards came from a rainfed (S1) and irrigated (S2) region respectively where no herbicides had been used for at least ten years. The R1 is the population described by Claude *et al.* (1998). The R2 population came from a field in which resistance to tribenuron-methyl had been confirmed in a field trial in 1998/99. All seed samples had been stored in closed plastic pots kept in a warehouse.

Seeds were placed to germinate in Petri dishes on agar medium and then 10 seedlings were transplanted in an aluminium tray (0.20 m x 0.15 m) containing a 1:1 peat sand mixture. After reaching the four to five leaf stage, the plants were sprayed with tribenuron-methyl (Granstar, 750 g a.i. kg^{-1}, DuPont) at 0, 4.7, 9.4, 14.1, 18.8, 23.5, 28.1, 32.9 and 37.5 g a.i. ha^{-1}. Two replicates were done per rate and population.

Data were submitted to a Student's t-test at P = 0.01. The dose-response curves of these populations are shown in Figure 1.1. Both susceptible reference populations

died at a quarter of field dose (4.7 g a.i. ha^{-1}). The resistant standard populations survived a double field rate (37.5 g a.i. ha^{-1}). Within the susceptible and the resistant populations the response was not statistically different.

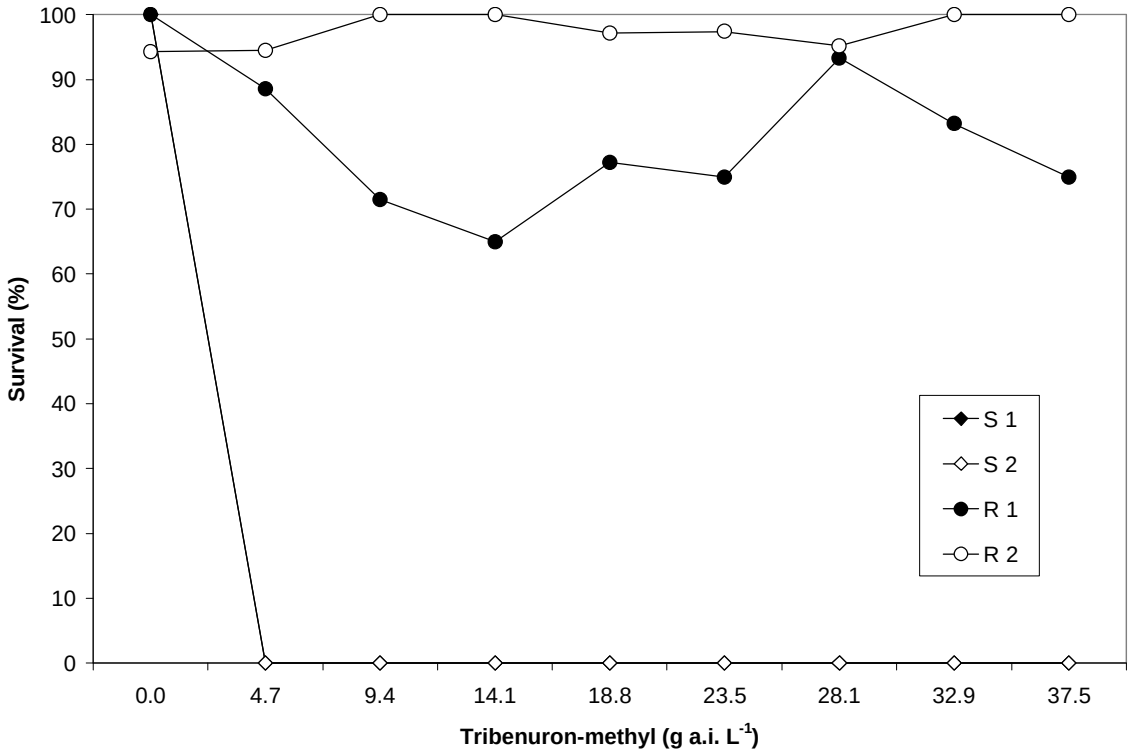

Figure 1.1. Dose-response curve of the four *Papaver rhoeas* reference populations (susceptible: S1, S2; resistant: R1 and R2). Differences between R and S populations were significant at P = 0.01 (Student's t-test) at all applied rates of tribenuron-methyl, but there were no significant differences between the R1 and R2, or S1 and S2 populations at any date. S1 and S2 symbols are overlapped.

Developing the test

The filter paper method used by Moss *et al.* (1999) for grass weeds was not useful in this case because the small poppy seeds failed to germinate when immersed in water. Consequently, solidified agar in Petri dishes was used as a germination medium. The standard composition of the agar medium was 1.3 % agar and 2 g KNO_3 L^{-1}, as described by Cirujeda *et al.* (1999).

In order to decide if it was necessary to include the dormancy breaking substance gibbereline, germination with selected combinations of GA_3 and tribenuron-methyl doses was studied on different aged populations.

Germination on agar medium containing 1.3 % agar, 2 g KNO_3 L^{-1} and 0.24 μM tribenuron-methyl (i.e. 0.908 x 10^{-4} g a.i. L^{-1}) was tested on 69 populations: six populations were from 1995, four from 1996, 15 from 1997 (more than 20 months old) and 44 from 1998 (8 to 17 months old). Germination on a medium containing 1.3 % agar, 2 g KNO_3 L^{-1}, 0.2 g GA_3 L^{-1} and 7.68 μM tribenuron-methyl was tested with 36

populations: two populations were from 1995, three from 1996, one from 1997, four from 1998 (17 months old) and 26 from 1999 (4 months old). Germination on 1.3 % agar, 2 g KNO_3 L^{-1}, 0.5 g GA_3 L^{-1} and 61.44 μM tribenuron-methyl was tested on 17 populations: one population from 1995, one from 1996, four from 1998 (16 months old) and 11 from 1999 (4 months old).

Figure 1.2. shows the results of these studies, classifying between populations, which showed a low or high germination capacity under the different doses.

Most of the older populations from 1995, 1996 and 1997 showed germination rates higher than 50% when no gibbereline was added. Younger populations (from 17 months) needed the addition of at least 0.2 g GA_3 L^{-1} to reach these germination rates. Younger populations even needed 0.5 g GA_3 L^{-1} to achieve 50% germination. These results suggested the importance of adding gibbereline to the medium especially if freshly harvested samples are analysed.

The samples contained a seed mixture of different plants growing in the same field. As dormancy can vary highly between plants depending e.g. on the light the plant received during growth (Salisbury & Ross, 1992), in order to obtain representative data as much germination as possible on the dishes was targeted and therefore, gibbereline was included in the study.

Different concentrations of tribenuron-methyl were added to the medium before solidifying as well as several concentrations of gibbereline in order to evaluate the effect of the herbicide on plant growth and development, and to find the least GA_3 and tribenuron-methyl rates to distinguish susceptible from resistant plants.

The studied combinations were: 0 and 0.2 g GA_3 L^{-1} with 0.24, 0.48, 0.96, 1.92, 3.84 and 7.68 μM tribenuron-methyl and 0.5 g GA_3 L^{-1} with 7.68, 15.36, 30.72, 61.44 and 122.88 μM tribenuron-methyl. 35 ml of this medium were poured into each dish. Fifty seeds of the S1, S2, R1 and R2 populations were sown on the medium surface and the dish closed. Three replications were made of each treatment. The dishes were placed randomly in a growth chamber with 20 μmol S^{-1} m^{-2} of fluorescent light under the optimal germination conditions (20ºC during 16 hours with light and 10ºC during 8 hours in darkness) (Taberner, unpublished data).

All the screening experiments were conducted in the same growing chamber under the same light and temperature conditions.

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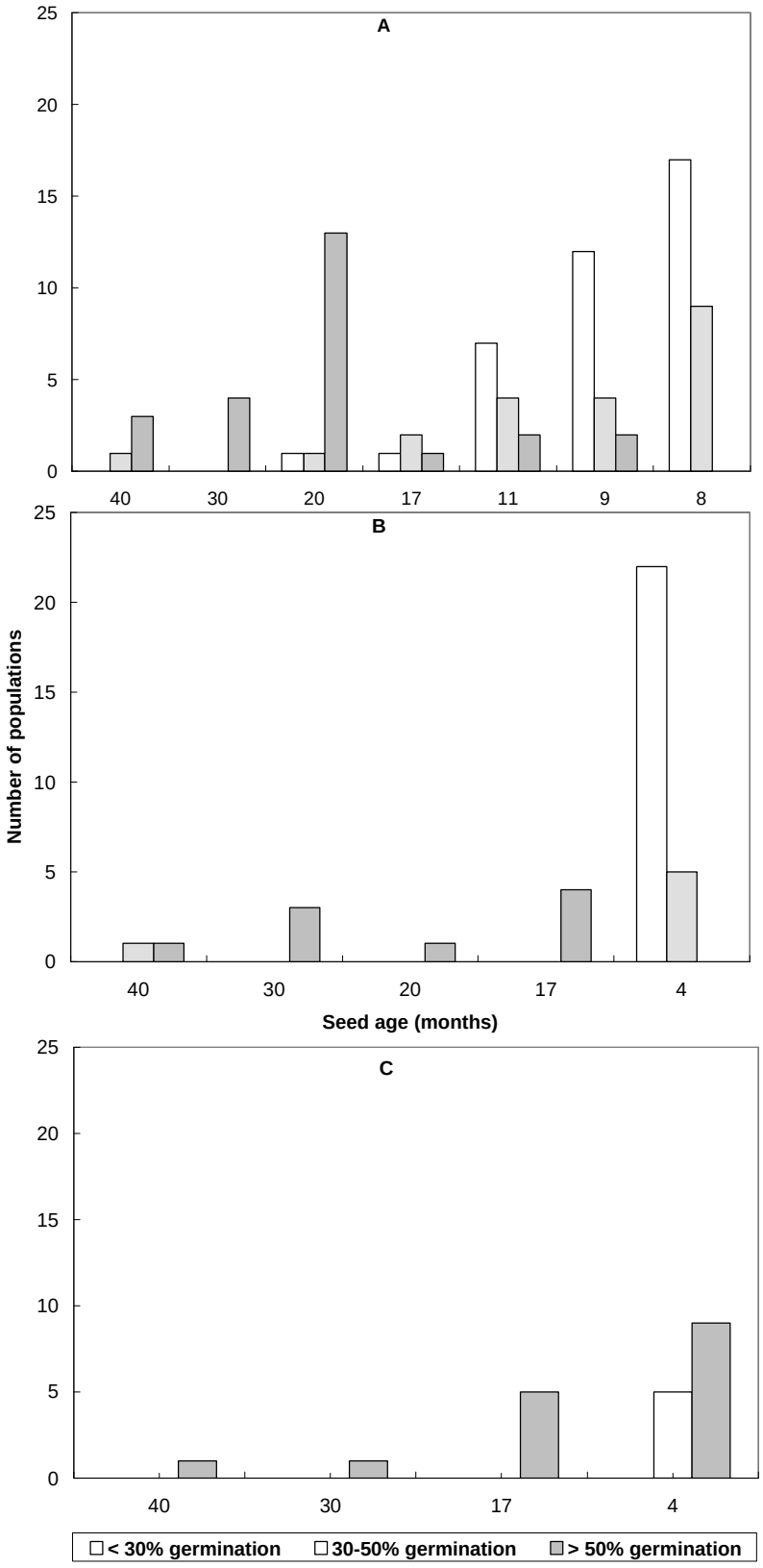

Figure 1.2. Absolute frequency of *Papaver rhoeas* populations in relation to seed age (months) and germination rate (<30%, 30-50%, >50%) determined on agar medium (1.3 % agar, 2 g KNO_3 L^{-1}) containing selected combinations of GA_3 L^{-1} and tribenuron-methyl doses. A) no GA_3 + 0.24 µM tribenuron-methyl (assessments made 17 days after sowing); B) 0.2 g GA_3 L^{-1} + 7.68 µM tribenuron-methyl (assessments made 14 days after sowing); C) 0.5 g GA_3 L^{-1} + 61.44 µM tribenuron-methyl (assessments made 14 days after sowing).

All the screening experiments were conducted in the same growing chamber under the same light and temperature conditions.

Verifying the methodology with other populations

After having determined the best GA_3 / tribenuron-methyl combinations, the methodology was tested on three further populations (codified as 18/98, 48/98 and 51/99). The chosen combination of 7.68 µM tribenuron-methyl with 0.2 g GA_3 L^{-1} was tested on four other populations (codified as 36/98, 11/99, 12/99 and 52/99). All these populations were also tested by some other methods as described below.

Validation of the results in the Petri dishes in greenhouse and field experiments

The results were validated by a greenhouse experiment. Seeds were placed to germinate in Petri dishes on agar medium containing 0.2 g GA_3 L^{-1} and 2 g KNO_3 L^{-1} and then 20 seedlings were transplanted in an aluminium tray (0.20 m x 0.15 m) containing a 1:1 peat-sand mixture. The plants were kept growing until a 4 to 5 leaves stage and then 0, 6.27, 12.5, 18.8, 25.0 and 37.5 g a.i. ha^{-1} of tribenuron-methyl were applied with a constant pressure sprayer. Three replicates of each treatment were placed randomly in a greenhouse with temperatures ranging from 10ºC to 25ºC. Final survival evaluation was performed 20 days after treatment.

The field experiments were conducted in different years (1997-2000) on three resistant populations (including the reference population R2) and on one susceptible population. The other reference populations were not available for treatment. A randomised block design with three replicates was used. Plots measured 2m x 5m. Application of 18.8 g a.i. tribenuron-methyl ha^{-1} was made by a constant pressure sprayer at a 4 to 5 leaves *P. rhoeas* stage. Untreated checks were included in the experiments. Live *P. rhoeas* plants were counted within three 0.1 m^2 frames per plot after 15, 30, 45 and 60 days.

Efficacy was calculated following Abbott's formula:

[% efficacy = (1-*Ta* / *Ca*) 100], where

Ta is the infestation in the treated plot after application and
Ca is the infestation in the check plot after application (Ciba-Geigy, 1992).

Weed infestations were very homogenous in all the fields and *P. rhoeas* density ranged from 131 plants m^{-2} (population 12/99 in 1999-2000) to 752 plants m^{-2} (population 18/98 in 1997-98) at the beginning of February, when the early post-emergence herbicides were applied. Winter barley was the most frequent crop, although winter wheat was sown in the experiments of population 12/99 in 1999-00 and of population 48/98 in 1997-98.

Results and discussion

Defining the optimum methodology of the test with the reference populations

When tribenuron-methyl was added, the development of the plants in the Petri dishes was found to be inhibited in the susceptible populations and not in the resistant ones. It was observed that whilst the resistant individuals continued to develop new leaves, the susceptible plants stopped developing after the cotyledons stage. Moreover, the cotyledons turned light-green in contrast to the dark-green colour of the plants in the control dishes and of the resistant populations on the herbicide medium.

When no gibbereline was added, 0.24 μM tribenuron-methyl per dish added to a standard medium was found to be sufficient to clearly distinguish the resistant from the susceptible populations on the basis of development cessation (data not shown). Differences in colour started to be visible after seven days; clear results could be observed after a maximum of 17 days in most of the populations. If the dishes were kept longer and assessment was made later, confusion started due to the mortality of some well- or undeveloped plants caused by humidity or the presence of fungi.

The addition of gibbereline to the medium caused higher germination rates and a stronger growth and development. Seed germination was also more homogeneous in both the resistant and the susceptible populations. The mortality of plants due to humidity or fungi was delayed for several days. Gibbereline had an antagonistic effect on the herbicide efficacy and higher concentrations were needed.

Adding 0.2 g GA_3 L^{-1} the desired effects started at 7.68 μM tribenuron-methyl while when 0.5 g GA_3 L^{-1} were added, the desired herbicide effect was visible again with 61.44 μM tribenuron-methyl. Susceptible and resistant plants could be distinguished 14 days after sowing.

The gibbereline affected root growth of all the populations irrespective of the addition of herbicide. This effect was greater with an increased gibbereline dose and greatest when herbicide was also added to susceptible populations. Nevertheless, in most of the experiments this did not affect the possibility of distinguishing well developed from undeveloped plants.

No differences were found between the three replicates in any case. When the agar test was repeated for the same populations some time later the results were always consistent (data not shown).

Percentages of germination, well-developed (resistant) and undeveloped (susceptible) plants were recorded. Table 1.1. a shows the results on seven populations using the three best medium combinations.

Table 1.1. a: Results of the quick test in seven populations of *Papaver rhoeas* sown on agar medium in Petri dishes using the three best GA_3/tribenuron-methyl combinations.
Data show percentage (means ± SD) of germination, well-developed (resistant) plants, yellow plants in cotyledon stage (susceptible) and plants which could not be classified at the evaluation date (too young or dead). When no gibbereline was added, counts were performed 17 days after sowing; in the other cases, assessment was done 14 days after sowing.

Population R/S	GA_3 (g L^{-1})	Tribenuron-methyl (mM)	Germination (%)	Well-developed plants (%)	yellow plants in cotyledons (%)	too young or dead plants (%)
1/95 S1	0	0.24	53.3 ± 3.06	0	100	0
	0.2	7.68	60.7 ± 4.62	0	100	0
	0.5	61.44	85.3 ± 1.15	0	91.4 ± 2.64	8.6 ± 2.64
2/98 S2	0	0.24	40.7 ± 13.32	0	100	0
	0.2	7.68	64.0 ± 8.72	0	100	0
	0.5	61.44	78.0 ± 2.00	0	94.9 ± 0.13	5.1 ± 0.13
9/96 R1	0	0.24	72.0 ± 3.46	80.5 ± 2.87	12.2 ± 4.92	7.3 ± 5.53
	0.2	7.68	76.7 ± 4.16	85.3 ± 3.43	7.9 ± 3.06	6.8 ± 5.85
	0.5	61.44	85.3 ± 7.57	90.7 ± 1.62	0	9.3 ± 1.62
1/98 R2	0	0.24	24.0 ± 8.00	63.2 ± 10.28	8.3 ± 14.43	28.5 ± 24.68
	0.2	7.68	50.0 ± 5.29	77.7 ± 9.66	0	22.3 ± 9.66
	0.5	61.44	73.3 ± 3.06	84.4 ± 6.70	0	15.6 ± 6.70
18/98 R	0	0.24	31.3 ± 6.11	61.6 ± 16.97	8.8 ± 4.14	29.7 ± 20.46
	0.2	7.68	66.7 ± 1.15	88.0 ± 3.16	4.0 ± 1.78	8.0 ± 1.85
	0.5	61.44	82.0 ± 5.29	89.4 ± 1.05	0	10.6 ± 1.05
48/98 R	0	0.24	51.3 ± 6.43	50.7 ± 8.22	1.2 ± 2.14	48.1 ± 10.19
	0.2	7.68	73.3 ± 7.02	91.0 ± 3.95	1.8 ± 3.12	7.2 ± 1.05
	0.5	61.44	84.0 ± 5.29	93.6 ± 1.71	0	6.4 ± 1.71
51/99 S/R	0	0.24	62.7±10.26	58.4±9.11	40.7±7.68	0.9±1.56
	0.2	7.68	81.3±2.31	44.3±3.56	49.3±6.10	6.4±8.99
	0.5	61.44	86.7±3.06	27.7±12.36	40.0±6.45	32.3±6.32

Most of the populations had a response with clear majority of resistant or susceptible individuals although some populations showed a mixed sensitivity. The proportion of resistant and susceptible plants for each population was very similar on all three medium combinations. In the resistant populations 9/96, 1/98, 18/98 and 48/98 the proportion of susceptible plants decreased with the addition of gibbereline and tribenuron-methyl. So, distinction seemed to be clearest with 0.5 g GA_3 L^{-1} and 61.44 µM tribenuron-methyl. Nevertheless, the proportion of these possible susceptible plants was small (less than 10%) with the combination 0.2 g GA_3 L^{-1} + 7.68 µM tribenuron-methyl.

When no gibbereline was added, germination in the dishes was staggered and not simultaneous. So, at the evaluation moment, some plants had not yet developed new leaves whilst other plants were already affected by fungus before reaching the one-leaf stage. These plants could not be classified. This explains the higher percentage of too young or dead plants especially in resistant populations in dishes without gibbereline (Table 1.1. a). When 0.2 g GA_3 L^{-1} gibbereline was added, this percentage decreased due to more homogenous germination. The addition of 0.5 g GA_3 L^{-1} inhibited in some cases the root growth making classification impossible reaching important percentages in some cases (Table 1.1. a and other not shown data).

Testing the different combinations of gibbereline and tribenuron-methyl with other populations

The other three populations tested behaved similarly to the reference populations (Table 1a). Percentage of too young or dead plants was highest when no gibbereline or 0.5 g GA_3 L^{-1} was used. The proportion of resistant and susceptible plants was very similar for all three medium combinations. Germination rose with gibbereline addition in all three cases.

Table 1b shows the results of four populations tested using the chosen agar medium containing 0.2 g GA_3 L^{-1} with 7.68 µM a.i. tribenuron-methyl. In all four cases, it was possible to distinguish resistant from susceptible plants and the percentage of too young or dead plants was acceptable. Population 11/99 had only resistant plants, 12/99 only susceptible plants. Populations 52/99 and 36/98 were mixed populations with both resistant and susceptible plants (higher than 10%).

Validation of the results in the Petri dishes with the greenhouse and field experiments

In all the 11 populations tested the results of the Petri dish experiments were consistent with the greenhouse and field experiments (Table 1.1. a, 1.1. b and 1.2.).

Table 1.1. b: Results of the quick test in four *Papaver rhoeas* populations in Petri dishes on agar medium containing 1.3 % agar, 2 g KNO_3 L^{-1}, 0.2 g GA_3 g L^{-1} and 7.68 µM tribenuron-methyl. Data show percentage (mean ± SD) of germination, well-developed (resistant) plants, yellow plants in cotyledon stage (susceptible) and plants which could not be classified at the evaluation date (too young or dead). Assessment was done 14 days after sowing.

Population R/S	Germination (%)	well-developed plants (%)	yellow plants in cotyledons (%)	too young or dead plants (%)
11/99 R	38.0 ± 8.00	76.3 ± 17.13	0	23.7 ± 17.13
12/99 S	69.3±4.62	0	92.4±1.20	7.6±1.20
52/99 R/S	70.0±2.00	65.8±4.20	25.7±5.45	8.5±2.71
36/98 R/S	74.0±2.00	82.8±8.51	11.8±4.41	5.4±4.69

Both uniform seed lots and mixed populations were detected. Therefore, there were resistant biotypes found from fields with a smaller or higher resistance proportion of the plants to tribenuron-methyl in *P. rhoeas*.

Both the pure resistant or susceptible and the mixed populations showed similar proportions of survival and death in the dishes and the greenhouse and field experiments. This shows that all three methods are capable of giving similar information.

Field trials are expensive, time-consuming and show a strong dependence on other external factors which cause irregular seedling distribution as well as other mortality factors on the weeds irrespective of the herbicide. It is difficult to describe exactly how many individual plants are resistant or susceptible.

Greenhouse tests also require more space facilities compared to the Petri dish test as well as watering of the plants. The duration is of at least one month compared to the 14 days necessary for the Petri dish trials. Herbicide effects on plants can be observed individually better than in the field trials.

The Petri dish test has many advantages: it is quick, no special facilities are requested apart from the growth chamber, and the distinction between resistant and susceptible plants in the Petri dishes is very clear.

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The Petri dish test has many advantages: it is quick, no special facilities are requested apart from the growth chamber, and the distinction between resistant and susceptible plants in the Petri dishes is very clear.

Conclusions

After repeating the method with 11 populations and confirming the results of greenhouse pot experiments and with six field experiments, the method seems valid. The advantages of this method are the rapid results, the independence of climatic factors like temperature and water and the little need for working material and human effort. Another advantage is the possibility of enhancing germination with gibbereline. This means that the test can be done on *P. rhoeas* immediately after harvest.

Under the described temperature and light conditions the best medium combination found was 0.2 g GA_3 L^{-1} with 7.68 µM a.i. tribenuron-methyl. This medium ensured a high and uniform germination proportion, root damage due to gibbereline use was not important and plants were vigorous and healthy ensuring individual plant classification.

It has been observed that in growth chambers with more light higher herbicide doses can be required when 0.2 g GA_3 L^{-1} is used. More light also increases the toxic effect of gibbereline.

Table 1.2.: Percentage (mean ± SD) of *Papaver rhoeas* survival treated with tribenuron-methyl in the greenhouse (assessment at 20 days after treatment) and field experiments (assessment at 60 days after treatment)

	Greenhouse experiment					Field experiments		
	% survival at tribenuron-methyl rates (g a.i. ha^{-1})					% survival at 18.8 g a.i. ha^{-1}		
Population	**6.27 g**	**12.5 g**	**18.8 g**	**25.0 g**	**37.5 g**	**1997-98**	**1998-99**	**1999-00**
1/95 S1	0	0	0	0	0	-	-	-
2/98 S2	0	0	0	0	0	-	-	-
12/99 S	1.9±3.21	0	0	0	0	-	-	2.3±3.98
9/96 R1	85.5±2.09	64.0±19.35	83.6±19.35	66.1±16.16	77.8±12.82	-	-	-
1/98 R2	100	100	100	100	100	-	100	93.6±11.06
18/98 R	83.2±6.05	90.3±10.65	93.5±6.67	94.4±4.90	70.7±17.79	67.8±15.38	-	78.6±37.11
48/98 R	92.5±1.18	87.3±16.64	93.2±7.16	75.6±8.41	72.9±11.45	80.6±33.67	-	
11/99 R	97.6±3.37	94.2±5.57	92.0±7.03	87.0±3.21	97.2±4.81	-	-	-
51/99 R/S	35.7±7.41	55.7±6.77	48.8±6.81	55.4±3.97	23.4±13.60	-	-	-
52/99 R/S	70.7±1.01	67.5±4.35	73.8±9.21	58.1±3.68	64.2±15.02	-	-	-
36/98 R/S	95.8±5.89	75.1±26.10	86.1±11.15	87.8±10.72	78.5±12.03	-	-	-

Acknowledgements

The authors thank Lourdes Ruiz Luján for her contributions in the laboratory trials. The authors also thank Peter Boutsalis, and two anonymous referees for their useful comments on the manuscript. Alicia Cirujeda Ranzenberger thanks the Spanish Ministry of Education for her FPI fellowship.

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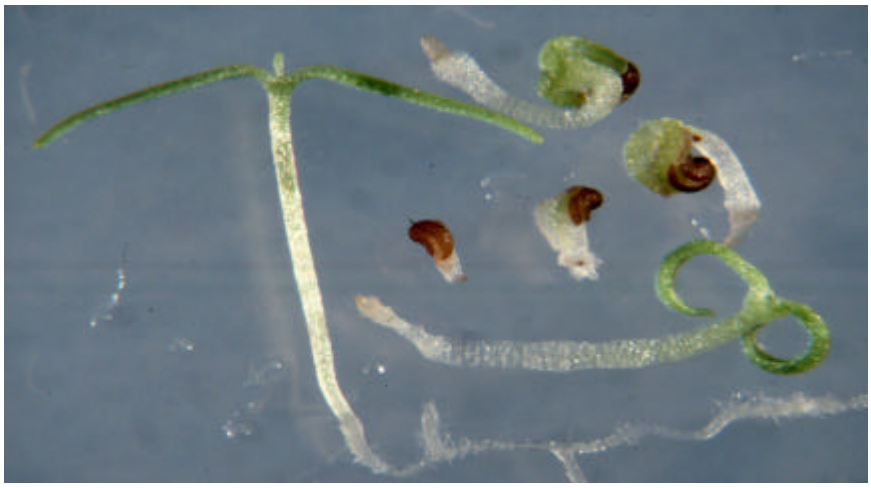

Chapter 2

A seed-based quick-test for herbicide resistance detection to 2.4-D in *Papaver rhoeas* L. grown on agar medium

Summary

A seed-based quick-test on agar medium for herbicide resistance detection towards 2.4-D was developed for *Papaver rhoeas* L. The results were validated in whole-plant experiments conducted in a greenhouse and with field trials.

Different combinations of gibbereline and of 2.4-D were added to the standard medium containing 1.3 % agar and 2 g KNO_3 L^{-1}. Gibbereline was added with the aim of overcoming the dormancy problem of the *P. rhoeas* seeds.

Germination rose with the addition of gibbereline (GA_3) and decreased with increasing 2.4-D doses. All the plants including the resistant ones grew deformed. The mean hipocotyl length of the seedlings, however, was found to be an appropriate parameter for distinction between susceptible and resistant plants.

The combinations of 0.2 g GA with 3.49, 5.24, 8.73 and 17.5 µmol L^{-1} 2.4-D and of 0.5 g GA_3 L^{-1} with 3.49 and 5.24 µmol L^{-1} 2.4-D allowed the distinction between susceptible and resistant populations by measuring the hipocotyl length. A length ratio was calculated dividing the obtained mean hipocotyl length with the mean length of the susceptible standard population included in each experiment. Only the combination of 0.2 g GA_3 L^{-1} with 3.49 µmol L^{-1} 2.4-D allowed a gradual classification within the resistant populations and was chosen as the best medium combination.

The quick-test was validated with greenhouse and field trials, confirming the usefulness of this test.

Herbicide resistance towards 2.4-D was found to be gradual between the different resistant populations and within the seed samples. Also in the greenhouse tests a response towards increasing 2.4-D rates was observed suggesting metabolism-based herbicide resistance.

A four-group classification was established based on the quick-test ratio and on the greenhouse trial results. Populations were classified as susceptible if the length ratio was ≤ 1, corresponding to high plant mortality with 0.6 L 2.4-D ha^{-1} in the greenhouse tests. Populations were classified as one-star resistant if the length ratio was comprised between 1 and 1.5, corresponding to plant survival with 0.9 and 1.2 L 2.4-D ha^{-1} in the greenhouse tests. Populations were classified as two-star resistant if the length ratio was comprised between 1.5 and 2, corresponding already to some plant survival at 1.8 L 2.4-D ha^{-1}. The populations classified as three-star resistant had length ratios bigger than 2 and a remarkable plant survival was found in the greenhouse tests at 1.8 L 2.4-D ha^{-1}.

The described quick-test can be used for screening *P. rhoeas* populations towards 2.4-D providing comparable information than the more expensive and laborious whole plant tests in greenhouses.

Keywords: herbicide resistance, 2.4-D, *Papaver rhoeas* L., hipocotyl length, gibbereline.

Introduction

Problems in controlling *Papaver rhoeas* L. with 2.4-D have been quoted since 1992 in Spain (Taberner *et al.*, 1992). This herbicide was commonly used in winter cereals since the 1950's in Spain. Since 1987, the highly active ingredient tribenuron-methyl has been sold in Spain replacing in many cases the use of 2.4-D. Resistance appeared also towards this herbicide. The first published case in North-eastern Spain was in 1998 by Claude *et al*. (1998) of a *P. rhoeas* population resistant to 2.4-D and to tribenuron-methyl.

Evolution of herbicide resistance was much faster for tribenuron-methyl than for 2.4-D. Probably this was due to different causes as different selection pressures, different initial resistant plant number and different resistance mechanisms, which are some of the parameters, which play a role in herbicide resistance evolution (Saari *et al.* 1994). The increasing complains from farmers, applicators and solders lead to a field survey conducted in North-eastern Spain. Main affected and surveyed areas were winter cereal monoculture in rainfed cropping systems.

Despite being used and studied for several decades, the mode of action of auxinic herbicides and the molecular basis of auxinic herbicide resistance remain unknown (Zheng & Hall, 2001). In the cases review compiled by Coupland (1994) differential metabolism of 2.4-D was probably the resistance mechanism found in *Carduus nutans*. In the cases reviewed by Holt *et al.* (1993) enhanced metabolism is also thought to be the basis of resistance of 2.4-D resistant *Stellaria media*.

The natural tolerance of *Silene vulgaris* towards 2.4-D, however, was not due to metabolism, translocation or absortion differences (Wall *et al.*, 1991). Also in *Brassica kaber* no differences in uptake, transport and metabolism was found between susceptible and resistant biotypes suggesting that the resistance mechanism is due to an altered target site (Zheng & Hall, 2001). In *Sinapis arvensis* no differences in uptake, translocation or metabolism were found either (reviewed by Holt *et al.*, 1993).

Thus, at least two different herbicide resistance mechanisms towards 2.4-D are possible depending on the weed species. No other case of resistant *P. rhoeas* to 2.4-D are quoted besides the Spanish one (Heap, 2001) so that the possible mechanism in this species is unknown.

Following the literature, whole plant essays are the most frequently used methods for herbicide resistance detection, mainly in order to obtain dose-response curves. This method has been used by Cranston *et al.* (2001) and Hall *et al.* (1998) in resistance towards other auxinic herbicides namely dicamba and quinclorac. In the case of *P. rhoeas*, a very susceptible weed species to drought high and cold temperatures, it is difficult to obtain very accurate results without a high natural mortality even in the untreated plants.

Other techniques commonly used for herbicide resistance screenings are seed-based quick-tests as the Rothamsted rapid resistance test described by Moss *et al.* (1999) and the method described by Letouzé *et al.* (1997). In the case of *P. rhoeas* a seed-based quick test on agar medium has been developed for tribenuron-methyl (Cirujeda *et al.*, in press).

The aim of the present work was to find a seed-based quick-test for herbicide resistance detection of *P. rhoeas* towards 2.4-D. This test was validated with whole plant experiments in greenhouse and with field trials.

Material and methods

Characterisation of the resistance towards 2.4-D in Petri dishes on agar medium

First experiments were conducted with four *P. rhoeas* populations in Catalonia (North-eastern Spain). Three populations were collected in July 1998 (codified as 2/98 S, 43/98 R and 51/98 R) and one population in July 1997 (codified as 31/97 R). The population 2/98 was collected from a small field set-aside during several years where no herbicide had ever been sprayed. Population 43/98 was collected after complains in control problems with tribenuron-methyl and chlorsulfuron. The spraying history of the populations 31/97 and 51/98 is unknown. The populations had been tested previously in greenhouse trials and resistance towards 2.4-D confirmed in the last three of them. 2/98 was included as a susceptible standard.

First experiments were conducted on a 1.3 % agar medium containing 2 g KNO_3 L^{-1}. This basic medium had already been used for determining herbicide resistance of tribenuron-methyl in Petri dishes with *P. rhoeas* (Cirujeda *et al.*, in press). The addition of 2.4-D affected the germination rate and the development of the seedlings, which grew twisted, did not develop roots and in the best cases developed cotyledons.

As some populations germinated very little, the addition of 0.2 and 0.5 g GA_3 L^{-1} in combination with rates of 3.49, 5.24, 8.73 and 17.5 µmol L^{-1} 2.4-D was tested on these four populations. Dishes were placed in a growth chamber under 40 µmol S^{-1} m^2 fluorescent light at the optimal germination conditions (20ºC during 16 hours with light and 10ºC during 8 hours in darkness) (Taberner, unpublished data).

A growth response towards 2.4-D of the resistant plants was observed with this medium composition, so that length of the *P. rhoeas* hipocotyl was measured 13 days after sowing using a 0.05 cm precision ruler under a binocular at a 6.5-fold enlargement. The average hipocotyl length of the germinated seeds was determined and a length ratio was calculated dividing the average length of the tested sample with the average length of the susceptible standard population included in each test. This standard was population 2/98, chosen after analysing several possible susceptible populations. Populations with a positive ratio bigger than 1 were considered resistant.

Characterisation of the sensibility towards 2.4-D in greenhouse conditions and in field trials

Nine populations were tested in the greenhouse trials. Four were the populations tested in the first Petri dish experiments. The other populations were 2/95 R, 17/96 R, 57/98 R, 46/98 R and 42/99 R collected in 1995, 1996, 1998 and 1999, respectively. 2/95 came from a field, where the farmer complained about lack of control with 2.4-D. The population 17/96 comes from a neighbour field of the populations described by Claude *et al.* (1998) and is very resistant to 2.4-D and to tribenuron-methyl. The populations 46/98 and 57/98 come from fields, where the farmers complain about lack of efficacy of tribenuron-methyl but do not use any more 2.4-D. Finally, population 42/99 was randomly collected without knowing the spraying history of the field, where it came from.

Seeds were placed in Petri dishes for germination on agar medium containing 2 g KNO_3 L^{-1}. 15 seedlings were transplanted per aluminium tray (0.20 m x 0.15 m) containing a 1:1 peat-sand mixture. The plants were kept growing in the greenhouse approximately 25 days until reaching a diameter of 5 cm and sprayed afterwards with 0, 0.6, 0.9, 1.2, 1.5 and 1.8 L a.i. ha^{-1} of 2.4-D isooctilic ester with a constant pressure sprayer at 2 bar. The commercial field rate suggested for *P. rhoeas* control is 0.6 L a.i. ha^{-1} of 2.4-D. Three replicates of each treatment and of each population were placed randomly in a greenhouse with temperatures ranging from 15ºC to 25ºC using the fog cooling system. Final survival evaluation was performed 15 days after treatment, when distinction between alive and dead *P. rhoeas* plants was clear enough.

The field experiments were conducted in three different years (1997-2000) on one susceptible and on five resistant populations towards 2.4-D. These populations were 1/98 R, 18/98 R, 11/99 S, 12/99 R, 35/99 R and 5/00 R. The populations 1/98, 18/98, 12/99 and 35/99 were resistant to 2.4-D and to tribenuron-methyl. 11/99 was resistant to tribenuron-methyl, only and 5/00 was resistant to 2.4-D, only. The fields were found after complains on lack of efficacy.

A randomised block design with three replicates was used. Plots measured 2 m x 5 m. Application of 0.6 L a.i. ha^{-1} of 2.4-D was made using a constant pressure sprayer at 2 bar at the middle of March. *P. rhoeas* had reached the rosette stadium and measured five to 15 cm diameter and the winter cereal was in full tillering. Untreated checks were included in each block of the experiments. Live *P. rhoeas* plants were counted within three 0.1 m^2 frames per plot 30 to 50 days after application. Final evaluation was done visually in all the field trials besides on the population 18/98 in the years 1997-98 and 2000-01, where the last assessment was also done by counting.

Efficacy was calculated following Abbott's formula

[% efficacy = (1-*Ta* / *Ca*) 100], where

Ta is the infestation in the treated plot after application and
Ca is the infestation in the check plot after application (Ciba-Geigy, 1992).

Depending on the climatic characteristics of each year and of each location, spraying date as well as initial plant number varied from site to site. In 2000-01 plant growth was faster than normally due to a mild winter and spraying was conducted already around the 12th March. The latest applications were conducted around the 16th April in the cropping season 1999-00 characterised by a cold winter and a dry spring delaying plant development.

Minimum initial plant number was recorded in the experiment on population 35/99 reaching 50 plants m^{-2}; maximum initial plant number found was 258 plants m^{-2} on the population 18/98.

Winter barley was the major crop with the exception of the trial conducted on the population 12/99 and on the population 48/98, in which winter wheat was grown.

Data was subjected to an ANOVA-analysis (SAS, 1991).

Results and discussion

Results of the effect of 2.4-D on the germination of Papaver rhoeas *in Petri dishes on agar medium*

The percentage of germination as affected by the addition of gibbereline and 2.4-D on the agar medium containing 1.3 % agar and 2 g KNO_3 L^{-1} is shown in Table 2.1.

Table 2.1.: Germination (%) of four *Papaver rhoeas* populations sown in Petri dishes on a 1.3% agar medium containing 2 g KNO_3 L^{-1} and different combinations of GA_3 and 2.4-D.

Population	**2.4-D rate (µmol L^{-1})**				
	0	**3.49**	**5.24**	**8.73**	**17.5**
			0 g GA_3 L^{-1}		
2/98	27.3±3.06				
43/98	21.3±1.15				
51/98	30.0±2.00				
31/97	48.7±9.02				
			0.2 g GA_3 L^{-1}		
2/98	48.7±4.16	24.0±5.29	19.3±9.02	31.3±4.62	18.0±2.00
43/98	70.0±8.72	52.7±9.02	49.3±4.16	38.7±6.11	34.7±13.32
51/98	66.0±10.58	44.0±4.00	38.0±3.46	36.7±4.62	40.7±7.57
31/97	41.3±3.06	38.0±7.21	38.7±9.02	37.3±8.08	32.0±6.00
			0.5 g GA_3 L^{-1}		
2/98	76.0±12.49	53.3±8.33	58.0±9.17	34.7±5.77	48.0±10.58
43/98	77.3±10.07	53.3±11.72	62.0±5.29	52.0±4.00	52.0±3.46
51/98	76.0±5.29	48.0±6.93	56.7±12.06	49.3±4.16	42.0±12.00
31/97	81.3±4.16	69.3±4.62	67.3±7.02	55.3±11.37	35.0±9.90

Germination rose with the addition of GA_3 (Table 2.1.), as described by Cirujeda *et al.* (1999). Initial seed dormancy was especially high with populations collected more recently, while without addition of GA_3 the population from 1997 showed higher germination rates than the other populations.

Germination was faster and non-staggered compared to the dishes without GA_3 and stopped after around 14 days (data not shown). When no gibbereline was added, still some germination was recorded 21 days after sowing, so that also more fungal contamination occurred interfering in any kind of assessments. Moreover, higher germination was considered to represent better each population and to give more reliable results.

Germination generally decreased with increasing 2.4-D rates (Table 2.1.) in both the resistant and the susceptible populations. When 0.5 g GA_3 L^{-1} was added this decrease was less important, so that still quite high germination rates were observed in some cases (Table 2.1.).

Results of the effect of 2.4-D on the seedling hipocotyl length of Papaver rhoeas in Petri dishes on agar medium

In the tested medium combinations all the plants were deformed and did not develop properly. In spite of this, a response in the hipocotyl length was observed, which was supposed to be related to the resistance degree of the population. The resistant plants grew longer than the susceptible plants, which sometimes did not even reach 1 mm length. Even in the resistant populations only some few individual plants developed almost normal roots. Plants grew shorter with the addition of more gibbereline and differences between populations were more difficult to detect (Figures 2.1. a, b). Higher 2.4-D rates also clearly reduced plant length.

In any of the tested combinations of 2.4-D with 0.2 g GA_3 L^{-1} it was possible to distinguish the susceptible from the resistant populations (Figure 2.1. a). When 0.5 g GA_3 L^{-1} were used, adding low 2.4-D rates namely 3.49 and 5.24 µmol L^{-1} 2.4-D, the distinction between resistant and susceptible populations was still clear despite the generally lower hipocotyl lengths. Combinations of higher rates of 2.4-D with 0.5 g GA_3 L^{-1} resulted in wrong results (Figure 2.1. b).

It was only possible to establish a ranking within the resistant populations with the combination 0.2 g GA_3 L^{-1} with 3.49 µmol L^{-1} 2.4-D. This combination was, thus, considered the best out of the tested ones. Moreover, an advantage of this medium combination was, as described previously, high germination rates, guaranteeing representative data for the analysed populations.

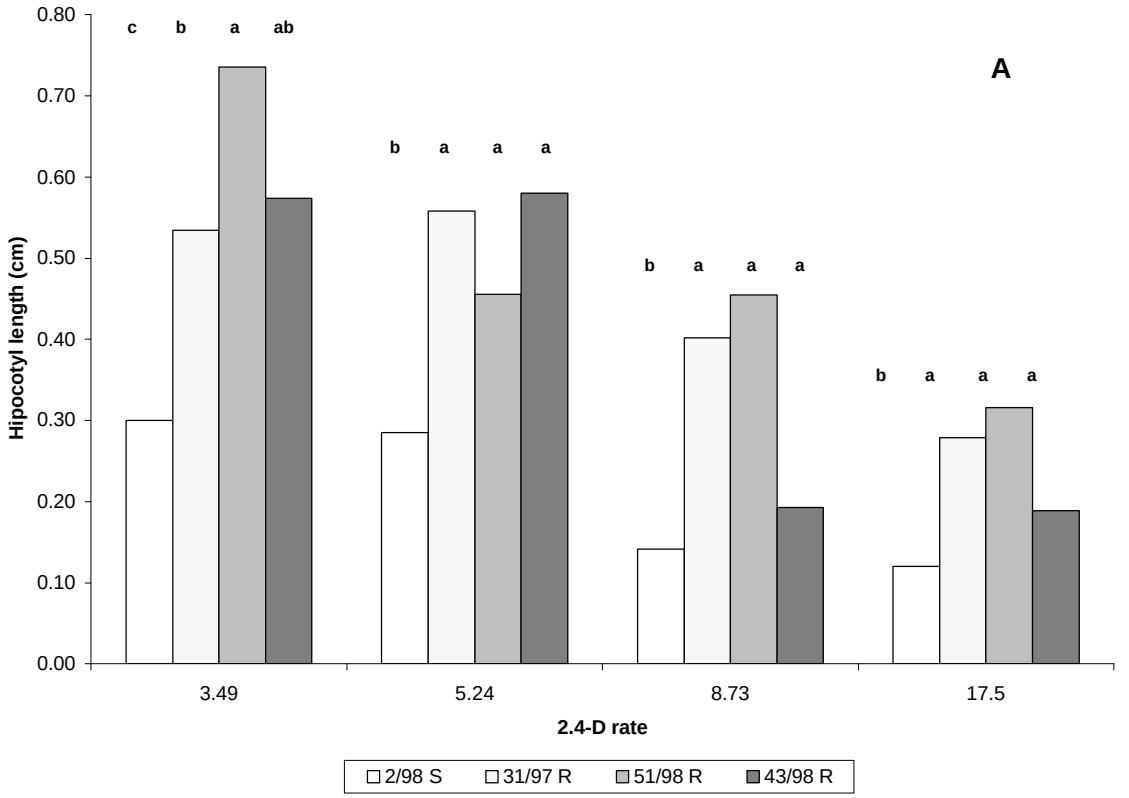

Figure 2.1. a. Average *Papaver rhoeas* hipocotyl length in the quick-test on agar medium containing 0.2 g GA_3 L^{-1} and different rates of 2.4-D (expressed in µmol 2.4-D L^{-1}).

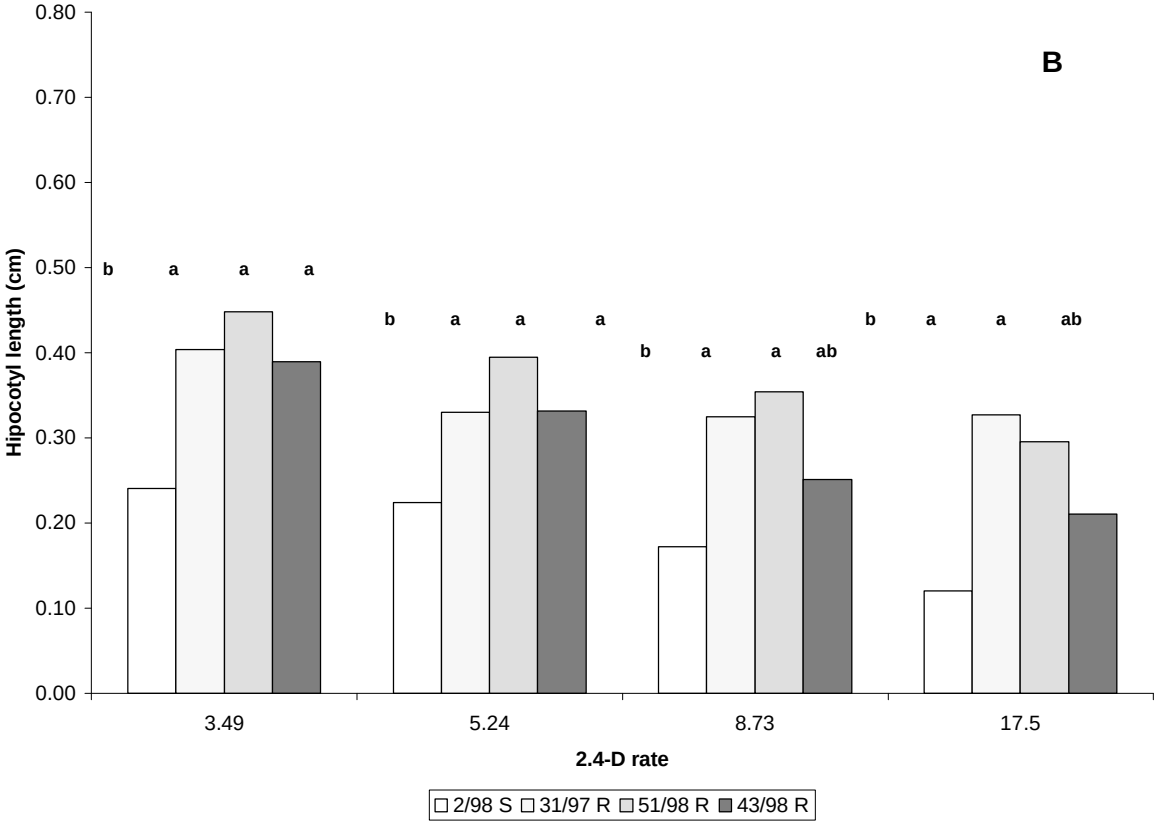

Figure 2.1. b. Average *Papaver rhoeas* hipocotyl length in the quick-test on agar medium containing 0.5 g GA_3 L^{-1} and different rates of 2.4-D (expressed in µmol 2.4-D L^{-1}).

Also inside each Petri dish a range of shorter and longer hipocotyls was found, indicating that even plants of the same population had different degrees of response towards 2.4-D.

Different degrees of resistance between populations reflected in the hipocotyl length gradation suggest that the resistance of *P. rhoeas* to 2.4-D is gradual. This indicates that a possible herbicide resistance mechanism is enhanced metabolism. This hypothesis is supported by other cases as *Carduus nutans* also resistant to 2.4-D (reviewed by Coupland, 1994).

Validation of the Petri dish method with greenhouse and field experiments

Already few hours after treatment in the **greenhouse**, both susceptible and resistant *P. rhoeas* plants reacted to the herbicide by leaf deformations. Resistant plants recovered afterwards, in some cases keeping the deformed leaves but producing new healthy ones. Otherwise, susceptible plants stopped growing and their growth point became hard and round-shaped.

Excluding the susceptible populations, it was very difficult to observe constant dose-response behaviour of the populations (Table 2.3., 2.4.) despite of including three the replicates and of treating many plants per dish. One explanation of the irregular behaviour could be that the populations' seed samples were not homogenous containing plants with different gradual response towards the herbicide. As commented previously, this had also been observed in the quick-test. On the other hand, *P. rhoeas* is a very sensitive plant towards drought, towards high and cold temperatures as well as towards too much moisture. In these long one-month duration experiments some problems in keeping plants in a perfect stage even in the untreated dishes (data not shown) were also observed, affecting probably the results.

A clear relationship between the quick-test result and the survival in the pot experiment could be observed for the 15 tested populations (Tables 2.3., 2.4.).

Table 2.3.: Ratio obtained in the quick-test and survival (%) (mean of three replicates ± SD) of 2.4-D on several *Papaver rhoeas* populations in a pot experiment in greenhouse conditions. The stars indicate the quick-test classification. No data = n. d.

Population	**Quick-test ratio**	**L 2.4-D ha^{-1}**				
		0.6	**0.9**	**1.2**	**1.5**	**1.8**
57/98 S	0.79	0	0	0	0	0
2/98 S	1.00	0	0	0	0	0
46/98 R *	1.06	0	4±7.7	0	0	0
2/95 R *	1.35	63±24.6	44±20.6	24±25.1	0	0
31/97 R **	1.18 / 1.57	n.d.	0	14±10.5	8±2.4	0
43/98 R **	1.61 / 1.64	n.d.	n.d.	4±5.1	6±8.3	41±57.9
51/98 R **	1.74 / 1.98	32±10.6	12±11.3	25±4.3	5±8.2	3±5.2
42/99 R ***	2.28	n.d.	12±2.7	20±16.9	18±31.1	24±16.3
17/96 R ***	2.99	76±34.7	44±23.5	12±15.5	14±24.7	28±13.2

Susceptible populations in the Petri dishes like the populations 2/98 and 57/98 died already at the rate of 0.6 L 2.4-D ha^{-1}, which corresponds to the commercial field rate (Table 2.3.). The susceptible population 11/99 showed very slight survival at superior rates (Table 2.4.). This could be explained with a low presence of plants with low-degree resistance.

In order to classify all the tested populations depending on their resistance degree, the star-system defined by Moss (1999) for *Alopecurus myosuroides* was adapted.

Populations were classified into susceptible, into low-degree resistance codified by one star (*), into middle-degree resistance codified by two stars (**) or into high resistance codified by three stars (***). The resistance degrees were defined depending on the response obtained in the dose-response experiments conducted in the greenhouse, which correlated with the ratio found in the quick-test.

Populations with a ratio between 1 and 1.5 survived 0.9 or 1.2 L 2.4-D ha^{-1} with the exception of population 1/98, which had some very little survival at 1.5 and 1.8 L 2.4-D ha^{-1} (Tables 2.3., 2.4.) and were classified as one-star resistance. When the ratio was bigger than 1.5 but under 2, similar survival rates were recorded for lower 2.4-D rates but always some survival was recorded at 1.8 L 2.4-D $ha^{-1}$and populations were classified as two-star-resistance. When the ratio exceeded 2, high survival rates in lower 2.4-D were recorded as well as quite remarkable survival at 1.8 L 2.4-D ha^{-1} (Tables 2.3., 2.4.) and populations were classified as three-star-resistant. Table 2.5. summarises the proposed classification.

Table 2.4.: Resistance evaluation on six *Papaver rhoeas* populations. Results of the Petri dish quick test in ratios. Values >1 were classified as resistant. The stars refer to the degree of resistance classification as explained in Table 2.2. Survival (%) of *Papaver rhoeas* in whole plant greenhouse trials, survival (%) of *P. rhoeas* in field trials. DAT = days after treatment. Mean of three replicates ± SD.

	Quick test Ratio Repetition			**Greenhouse trial at L 2.4-D ha^{-1}**					**Field trials at 0.6 L^{-1} a.i. ha^{-1}**			
Population	**1**	**2**	**3**	**0.6**	**0.9**	**1.2**	**1.5**	**1.8**	**1997-98**	**1998-99**	**1999-00**	**2000-01**
11/99 S	1.05	-	-	0	2±3.2	2±3.9	4±7.2	0	-	9±10.0 (DAT=51)	-	-
5/00 R *	1.11	-	-	6	8	0	0	0	-	-	59±35.3 (DAT=60)	-
1/98 R *	1.37	1.20	1.29	0	7±7.2	2±3.4	6±5.9	2±3.0	-	35±4.5 (DAT=38)	-	-
35/99 R **	1.71	-	-	34±18.3	31±13.8	22±10.4	31±5.4	12±6.8	-	88±10.4 (DAT=51)	-	-
12/99 R **	1.77	-	-	56±22.2	37±11.8	47±21.0	32±27.0	6±6.3	-	-	53±25.2 (DAT=34)	70±0.0 (DAT=38)
18/98 R *** / **	2.52	1.42	1.51	43	24±22.2	25±25.0	0	3±4.4	37±13.9 (DAT=75)	-	67±31.5 (DAT=38)	63±26.9 (DAT=21)

Table 2.5.: Classification criteria for the *Papaver rhoeas* populations resistant to 2.4-D. 50 plants were grown on a Petri dish on agar medium containing 3.49 µmol L^{-1} 2.4-D and the mean length of the hipocotyls found. Length ratio was calculated by dividing this value with the value of the susceptible standard population.

Length ratio	Classification
Ratio ≤ 1	S
1 < Ratio ≤1.5	*
1.5 <Ratio ≤2.0	**
Ratio >2.0	***

Following these results, the ratio found in the quick-test was coincident with the survival of the different populations. The classification established in Table 2.5. had a clear relationship with the dose-response of the populations towards 2.4-D.

The results of the **field trials** are shown in Table 2.4. The results were consistent with the data found with the quick-test and with the greenhouse trials. Resistance was confirmed in all the fields with the exception of the field with population 11/99 (Table 2.4.). Nevertheless, in this kind of experiments it was more difficult to observe the different degree of resistance described with the quick-test and confirmed with the dose-response experiments in the greenhouse. Many environmental parameters influenced the results. Following the previous classification, the survival of population 35/99 and especially 5/00 would be expected to be inferior (Table 2.4.). Also survival of population 18/98 could have been higher. Anyway, the trials confirmed the observed resistance towards 2.4-D.

Different than in the qualitative quick-test on agar medium for tribenuron-methyl resistance detection described by Cirujeda *et al.* (in press) the information deriving from the present test is related to the used herbicide doses in the greenhouse tests. Thus, as this quick-test on 2.4-D gives information on the approximate expected plant survival, it replaces the greenhouse trials, when too many populations want to be screened. Nevertheless, a validation of the method with whole plant trials of some of the tested populations is always recommended.

Conclusions

A quick-test for herbicide resistance towards 2.4-D in *P. rhoeas* validated with field and greenhouse experiments was found. The combination of 0.2 g GA_3 L^{-1} with 3.49 µmol L^{-1} 2.4-D overcame the dormancy problem and allowed distinction between susceptible and resistant populations and establishing a ranking within the resistant population in a period of 13 days, only.

The measure of the hipocotyl length was found to be the best discriminating characteristic. The length ratio resulting from dividing the average value of the population with the average value of the susceptible standard population allowed classifying the populations.

A classification into three groups of resistant populations depending on their degree of resistance was defined.

Variable hipocotyl length was observed in the quick-test between populations and also inside the same population. Additionally, little survival was found at three-fold field rate in the greenhouse trials even for the most resistant *P. rhoeas* tested populations, suggesting metabolism-based herbicide resistance towards 2.4-D.

The described quick-test allows screening many populations, arrives to similar results and is less costly than greenhouse trials.

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