SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 5
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

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Abogado(a)/Señor(a)
LUZ CLEMENCIA SUAREZ DE PAEZ

Asunto: Radicación: 12-36557- -11
Trámite: 11
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Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/US2010/04571				
Fecha de Presentación Internacional	01/09/2010				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	12-036557-00000-0000	01/marzo/2013
Reivindicaciones:	12-036557-00000-0000	01/marzo/2013
Dibujos:	12-036557-00000-0000	01/marzo/2013
Secuencias:	12-036557-00000-0000	01/marzo/2013
Prioridad:	US 61/238.906 US 61/365.298	01/septiembre/2009 16/julio/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En la presente solicitud se estudiaran las 102 reivindicaciones del readicado 12-036557-00000-0000.

Título de la solicitud: “Plantas tolerantes a herbicidas”

El problema planteado en esta solicitud consiste en la necesidad de desarrollar cultivos de arroz resistentes a herbicidas, de manera que la aplicación de los mismos no perjudique dichos cultivos.

Para resolver este problema técnico la solicitud en estudio proporciona plantas con ACCasa modificada la cual provee resistencia a herbicidas específicos.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 01 H 5/00; C 12 N 15/52; C 12 N 15/82

4. Excepción a la Patentabilidad (Art 20 D 486 literales (c))

El objeto de las reivindicaciones 1 a 27, 31 a 33, 42 a 66, 70 a 72, 81 a 84, 88 a 90 y 102 no se considera una invención, de acuerdo con el Art citado.

El objeto de las reivindicaciones 30, 34, 69, 73, 87, 73, 91, 100 y 101 no se considera una invención, de acuerdo con el Art citado, ya que corresponde a métodos que comprende etapas esencialmente biológicas correspondientes a la obtención de plantas mediante la cruza de plantas con características deseadas.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 40, 79 y 97 no es patentable, de acuerdo con el Art 14.

6. De la solicitud de patente

Titulo

Se sugiere al solicitante modificar el titulo de manera que abarque la materia patentable de la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 28, 29, 41, 67, 68, 80, 85, 86 y 98 reclaman un método para controlar malezas de la vecindad de una planta de arroz, caracterizando este método a partir de propiedades exclusivas de la planta de arroz, esto es porque expresa una proteína ACCasa modificada. Sin embargo, esta característica no tiene relación directa con las malezas puesto que las malezas no presentan esta proteína característica y la ubicación de ellas alrededor de la planta de arroz no puede reconocerse como una característica esencial del método. Por otra parte, la aplicación del herbicida, que sería el paso fundamental para controlar las malezas, corresponde a un uso de dicho herbicida y no es una característica fundamental propia para un método de control de malezas alrededor de una planta de arroz transgénica, sino que sería un uso común para cualquier control de malezas, independientemente si están o no cerca a una planta de arroz transgénica.

Las reivindicaciones 35 a 38, 74 a 77 y 92 a 95 no tienen sustento en la descripción de la presente solicitud, puesto que el problema técnico planteado por la solicitud corresponde a la necesidad de desarrollar cultivos de arroz resistentes a herbicidas, de manera que la aplicación de los mismos no perjudique el cultivo, y no a el diseño y obtención de un producto a partir de una planta modificada. De hecho los ejemplos de la descripción divulgan metodologías para la selección y producción de plantas que expresan una proteína que les confiere resistencia a herbicidas, así como la identificación de la secuencia de dicha proteína. Por otra parte, se recuerda al solicitante que un producto debe definirse a partir de sus componentes, en el presente caso no puede considerarse una característica esencial de los productos reclamados el hecho de ser obtenidos a partir de una planta transformada,

puesto que el efecto técnico de la transformación de la planta es la resistencia a la aplicación de herbicidas, y dicho efecto no proporciona ninguna característica esencial a un producto obtenido a partir de una planta modificada frente al obtenido de una planta no modificada.

Las reivindicaciones 78 y 96 no son claras porque no proporcionan la SEQ ID NO correspondiente, se recuerda al solicitante que un acido nucleíco debe ser caracterizado por la secuencia nucleótida completa, indicando las modificaciones de la misma frente al estado de la técnica, y que no se aceptan definiciones a partir del peptido codificado o de porcentajes de homología puesto que dicha definición no permite establecer la estructura exacta de la materia reclamada. Por otra parte se aclara al solicitante que si desea reclamar la proteina ACCasa, debe hacerlo indicacando la secuencia de aminoácidos correspondientes, asi como las modificaciones hechas sobre la misma.

El método de la reivindicación 99 no es claro porque no proporciona todas las etapas esenciales para que un experto en la materia pueda reproducirlo, dicha reivindicación se refiere a un método de selección de una célula vegetal transformada, pero no indica los parámetros de identificación y selección de las células transformadas.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 01 de septiembre de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	US5290696	Method for imparting cyclohexanedione and/or aryloxyphenoxypropanioc acid herbicide tolerance to maize plants	24/Enero/1984
D2	Pest Manag Sci 64:1179–1186 (2008)	Cross-resistance patterns to ACCase-inhibiting herbicides conferred by mutant ACCase isoforms in Alopecurus myosuroides Huds. (black-grass), re-examined at the recommended herbicide field rate	2008

El documento D1[1] divulga una planta de maíz en el que el crecimiento y el desarrollo de dicha planta es tolerante a la inhibición por un herbicida de ciclohexanodiona, un herbicida de ácido aryloxyphenoxypropanoic, o mezclas de los mismos, a niveles que normalmente inhiben el crecimiento y el desarrollo de maíz, en el que dicha tolerancia se confiere por una alteración de acetil coenzima A carboxilasa (ACCasa) tolerante de la inhibición por dicho herbicida a niveles que normalmente inhiben la actividad de una ACCasa inalterada (Resumen Pág. 1)

El documento D2[2] divulga péptidos mutantes de ACCasa con mayor resistencia a herbicidas, donde las mutaciones propuestas ocurren en las posiciones 2027, 2041, 2078 de la proteína nativa (Pág. 1180 Col. Derecha párr [02]).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 28 consiste en "*Un método para controlar el crecimiento de malezas cerca de una planta de cualquiera de las reivindicaciones 1 a 25 caracterizado porque comprende aplicar a las malezas y las plantas una cantidad de un herbicida inhibidor de la Acetil-Coenzima A carboxilasa que inhibe el crecimiento de una planta de tipo salvaje*"

Comparación de las características técnicas esenciales, con las del estado de la técnica:

1URL: http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=19940301&CC=US&NR=5290696A&KC=A
2URL: http://www.ncbi.nlm.nih.gov/pubmed/18537107

Características esenciales	D1	D2
Método para controlar el crecimiento de malezas cerca de una planta de cualquiera de las reivindicaciones 1 a 25	Metodo para controlar el crecimiento de malezas (Col. 1 ln 56-63) cerca de una planta que comprende un gen ACCasa que confiere resistencia a herbicidas (Col 6 ln 32-49)	Identificación de péptidos mutantes de ACCasa con mayor resistencia a herbicidas, donde las mutaciones propuestas ocurren en las posiciones 2027, 2041, 2078 de la proteína nativa (Pág. 1180 Col. Derecha párr [02]).
Aplicar a las malezas y las plantas una cantidad de un herbicida inhibidor de la Acetil-Coenzima A carboxilasa que inhibe el crecimiento de una planta de tipo salvaje	Aplicar a las malezas y las plantas una cantidad de un herbicida inhibidor de la Acetil-Coenzima A carboxilasa que inhibe el crecimiento de una planta de tipo salvaje (Col. 16 ln 20 a 47)	

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

El documento D1 divulga un método para controlar el crecimiento de malezas (Col. 1 ln 56-63) cerca de una planta que comprende un gen ACCasa que confiere resistencia a herbicidas (Col 6 ln 32-49), donde dicho metodo comprende aplicar a las malezas y las plantas una cantidad de un herbicida inhibidor de la Acetil-Coenzima A carboxilasa que inhibe el crecimiento de una planta de tipo salvaje (Col. 16 ln 20 a 47). Dicho documento además divulga métodos de selección de plantas que presenten la resistencia, que comprenden la aplicación de un herbicida (Col 14).

La diferencia entre la invención, definida en la reivindicación 1 y el procedimiento de D1 consiste en que la solicitud hace referencia a plantas modificadas con ACCasa que comprende mutaciones en posiciones especificas del péptido.

El efecto que logra el incluir las mutaciones en posiciones especificas de la secuencia de ACCasa es que provee una mayor resistencia a herbicidas aplicados, haciendo más eficiente el método de control de malezas ya que es posible aplicar una mayor cantidad del herbicida sin perjuicio del cultivo.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo modificar las plantas utilizadas en el método conocido en el D1 para que presenten una mayor resistencia a herbicidas.

Sin embargo, la utilización de mutaciones en la secuencia de ACCasa para aumentar la resistencia a herbicidas, ya está divulgada en el documento D2 que identifica péptidos mutantes de ACCasa con mayor resistencia a herbicidas, donde las mutaciones propuestas ocurren en las posiciones 2027, 2041, 2078 de la proteína nativa (Pág. 1180 Col. Derecha párr [02]).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría mutaciones en la secuencia de ACCasa en posiciones específicas como las divulgadas en el documento D2 en el método divulgado en el documento D1 para llegar así al objeto de la reivindicación 28 de la solicitud.

Las reivindicaciones 29, 41, 67, 68, 80, 85, 86, 98, 78, 96 y 99 no proporcionan características técnicas adicionales, por lo cual se considera tampoco cumplen con el requisito de nivel inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US5290696	28, 29, 41, 67, 68, 80, 85, 86, 98, 78, 96 y 99	Nivel inventivo

D2	Pest Manag Sci 64:1179–1186 (2008)	28, 29, 41, 67, 68, 80, 85, 86, 98, 78, 96 y 99	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-08-13

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Cross-resistance patterns to ACCase-inhibiting herbicides conferred by mutant ACCase isoforms in *Alopecurus myosuroides* Huds. (black-grass), re-examined at the recommended herbicide field rate

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Abstract

BACKGROUND: Target-site-based resistance to acetyl-CoA carboxylase (ACCase) inhibitors in *Alopecurus myosuroides* Huds. is essentially due to five substitutions (Isoleucine-1781-Leucine, Tryptophan-2027-Cysteine, Isoleucine-2041-Asparagine, Aspartate-2078-Glycine, Glycine-2096-Alanine). Recent studies suggested that cross-resistance patterns associated with each mutation using a seed-based bioassay may not accurately reflect field resistance. The authors aimed to connect the presence of mutant ACCase isoform(s) in *A. myosuroides* with resistance to five ACCase inhibitors (fenoxaprop, clodinafop, haloxyfop, cycloxydim, clethodim) sprayed at the recommended field rate.

RESULTS: Results from spraying experiments and from seed-based bioassays were consistent for all mutant isoforms except the most widespread, Leucine-1781. In spraying experiments, Leucine-1781 ACCase conferred resistance to clodinafop and haloxyfop. Some plants containing Leucine-1781 or Alanine-2096 ACCase, but not all, were also resistant to clethodim.

CONCLUSION: Leucine-1781, Cysteine-2027, Asparagine-2041 and Alanine-2096 ACCases confer resistance to fenoxaprop, clodinafop and haloxyfop at field rates. Leucine-1781 ACCase also confers resistance to cycloxydim at field rate. Glycine-2078 ACCase confers resistance to all five herbicides at field rates. Only Glycine-2078 ACCase confers clethodim resistance under optimal application conditions. It may be that Leucine-1781 and Alanine-2096 ACCases may also confer resistance to clethodim in the field if the conditions are not optimal for herbicide efficacy, or at reduced clethodim field rates.

Keywords: ACCase; ALOMY; bioassay; field rate; herbicide; target-site resistance

1 INTRODUCTION

Herbicides are currently the easiest way of controlling weeds in crops. In developed countries they are the most frequently used weed control tool, and in intensive cropping systems they are often the only weed control method used by growers. However, intensive and systematic herbicide use has selected for, and favoured the spread of, resistant weed genotypes in 183 species to date.[1] The number of reported resistance cases is still increasing.[1] Herbicide resistance must therefore be considered when designing herbicide spraying programmes. For this purpose, assays unambiguously confirming and/or detecting resistance are required. Such assays must be able reliably to assess resistance in the field. That is, plants characterised as herbicide resistant or herbicide sensitive by these assays must actually be herbicide resistant or herbicide sensitive in the field.

'Classical' herbicide sensitivity assays are bioassays. The history of their use in monitoring herbicide resistance has been reviewed elsewhere.[2] They involve exposing viable individuals from weed populations where resistance is suspected to one or several discriminating herbicide dose(s) and observing herbicide effects.[2–4] When a gene endowing resistance is identified and its cross-resistance pattern is established, molecular assays can be developed for resistance diagnosis.[5–7] Molecular assays are fast and can be adapted to high-throughput diagnosis procedures. However, they are only relevant if the cross-resistance pattern established for the targeted resistance gene reflects field reality. Connection of the occurrence of a resistance gene in

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weed individuals with resistance of these individuals in the field can be directly obtained by carefully performing treatments to ensure optimal herbicide efficacy, and by subsequently genotyping the resistant and sensitive plants obtained. This implies treating plants at a sensitive growth stage under adequate temperature, moisture and wind conditions using herbicide at the field rate. However, such a procedure is time consuming and subject to environmental variation.[8] The connection is therefore generally obtained indirectly by genotyping weed individuals characterised as resistant or sensitive using bioassays. As a consequence, if the bioassay used to establish a cross-resistance pattern does not accurately reflect resistance in the field, then the molecular assay cannot be reliably used to monitor resistance in the field.

Alopecurus myosuroides Huds. (black-grass) is a major, troublesome grass weed of winter crops in northern Europe that has evolved resistance to herbicides inhibiting acetyl-coenzyme A carboxylase (ACCase, EC 6.4.1.2). A total of seven point mutations in the gene encoding ACCase, causing a total of five amino-acid replacements, have been demonstrated to confer dominant resistance to ACCase-inhibiting herbicides in this species.[9] These substitutions are Isoleucine-1781-Leucine (two mutations), Tryptophan-2027-Cysteine (two mutations), Isoleucine-2041-Asparagine, Aspartate-2078-Glycine and Glycine-2096-Alanine. Molecular tools have been developed for the detection of these seven mutations in *A. myosuroides*.[5,6,10,11] The altered sensitivity of the five ACCase isoforms resulting from the seven mutations has been demonstrated in *A. myosuroides* using ACCase enzyme assays.[10–12] Cross-resistance patterns associated with each of the five mutant ACCase isoforms have been established using a seed-based bioassay in which germinating *A. myosuroides* seeds grow on a filter paper soaked with herbicide solution.[3] However, recent results have suggested that *A. myosuroides* individuals containing Leucine-1781 ACCase isoform(s), which were characterised as sensitive to the ACCase-inhibiting herbicide clodinafop using this seed-based bioassay, could withstand clodinafop application in the field.[13] Similarly, *A. myosuroides* individuals containing Cysteine-2027, Asparagine-2041 or Alanine-2096 ACCase isoforms were characterised as sensitive to the ACCase-inhibiting herbicides cycloxydim and clethodim using a seed-based bioassay.[10,11] However, these isoforms displayed a reduced sensitivity to cycloxydim and clethodim in ACCase enzyme assays.[10,11] The purpose of this study was therefore to determine the accuracy of the cross-resistance patterns established using seed-based herbicide sensitivity bioassay for five ACCase-inhibiting herbicides, and to try to connect unambiguously the presence of mutant ACCase isoform(s) in *A. myosuroides* individuals with resistance or sensitivity to these herbicides applied at field rate.

2 MATERIALS AND METHODS

In the following, the mutant ACCase isoforms will be referred to as L1781 (Leucine-1781), C2027 (Cystein-2027), N2041 (Asparagine-2041), G2078 (Glycine-2078) and A2096 (Alanine-2096). Plant genotype at a given codon will be abbreviated as ABXXXX, where A and B stand for the amino acid encoded at codon XXXX by each of the two copies of the gene encoding ACCase in *A. myosuroides* (e.g. IL1781 indicates a heterozygous plant containing one Isoleucine-1781 and one Leucine-1781 ACCase isoform).

2.1 Plant materials

In previous work, the authors investigated the occurrence of mutant ACCase alleles in 243 *A. myosuroides* seed samples collected across France.[13] All samples were collected in different fields. Here, a total of nine of these samples that contained plants with mutant ACCase isoforms and two sensitive samples that did not contain any mutant ACCase isoform or any resistant plants[13] were used. Four 'purified' populations respectively containing 100% NN2041,[10] CC2027, GG2078 or AA2096[11] individuals were also analysed. For simplicity, all samples and populations will be referred to as 'populations' in this paper. The characteristics of the populations studied are summarised in Table 1.

Seeds from each population were placed into petri dishes onto blotting paper soaked with a solution of potassium nitrate (20 mM) and allowed to germinate for one week in controlled environment chambers (20 °C, 12 h light). Germinated seeds were then planted into plastic trays (1 L volume, $17 \times 12.5 \times 5.5$ cm in dimensions) filled with soil from an *A. myosuroides*-free wheat field from Dijon. Thirty germinated seeds were planted in each tray and thinned down to 25 before herbicide spraying.

The efficacy of ACCase-inhibiting herbicides against grass weeds can vary substantially, depending on the growth stage of the targeted weed.[14] Herbicide efficacy also depends on weed physiology during and after herbicide application, which in turn depends on environmental factors.[8] To ensure herbicide application and herbicide symptom observation in conditions that were both optimal for herbicide efficacy at field rate and as close as possible to field conditions, the trays were placed in a greenhouse mimicking spring temperatures in Dijon (20 °C day, 15 °C night). Seedlings were watered as needed and allowed to grow until they reached the 3–4-leaf stage, which is the growth stage at which ACCase-inhibiting herbicide application is recommended.

2.2 Seed-based bioassay

The sensitivity of plants containing C2027, N2041, G2078 and A2096 ACCase isoforms to five ACCase-inhibiting herbicides was previously assessed[10,11] using a seed-based bioassay described elsewhere.[3] These five herbicides belonged to two chemical families:

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Table 1. *Alopecurus myosuroides* populations studied

Code	Origin	Isoform[a]	Genotype(s) present[b]
00-017	Field	Reference population[c]	WT
00-029	Field	Reference population	WT
V00-009	Field	L1781	LL1781
V00-008	Field	L1781	WT, IL1781, LL1781
00-015	Field	L1781	WT, IL1781, LL1781
Cys2027F1	Controlled pairings	C2027	CC2027
00-045	Field	C2027	WT, WC2027, CC2027
00-081	Field	C2027	WT, WC2027, CC2027
00-092	Field	C2027	WT, WC2027, CC2027, IL1781, LL1781, IL1781/WC2027[d]
02-F1	Controlled pairings	N2041	NN2041
V01-007	Field	N2041	WT, IN2041, NN2041
Gly2078F1	Controlled pairings	G2078	GG2078
V00-005	Field	G2078	WT, DG2078, GG2078, IL1781, LL1781, IL1781/DG2078[d]
Ala2096F1	Controlled pairings	A2096	AA2096
00-094	Field	A2096	WT, GA2096, AA2096

[a] Isoform for which the population is used to check the cross-resistance pattern.
[b] Genotype(s) detected in the population in previous studies.[10,11,13] WT: individual(s) not containing any of the five known mutant ACCase isoform(s).
[c] Reference, sensitive population where no plant resistant to any ACCase inhibitor tested was detected in seed-based bioassays.
[d] Double-heterozygous plants containing two types of mutant ACCase isoform (e.g. one C2027 and one L1781 isoform).

aryloxyphenoxypropionates (fenoxaprop, clodinafop and haloxyfop) and cyclohexanediones (cycloxydim and clethodim). The sensitivity of plants containing L1781 ACCase isoform(s) to all these herbicides apart from clethodim was previously assessed.[5] Previously published seed-based bioassay results are summarised in Table 2. Sensitivity of seedlings containing L1781 ACCase isoform(s) to clethodim was assessed using 100 seedlings from each of the populations V00-009, V00-008 and 00-015 (Table 1), as previously described.[11]

2.3 Herbicide treatments

For each of the five ACCase-inhibiting herbicides studied, the commercial formulation most widely used in France was applied without extra adjuvant at the recommended field rate. Formulation and field rates were: Puma LS (Bayer CropScience France), 69 g fenoxaprop ha^{-1}; Célio (Syngenta Agro), 60 g clodinafop ha^{-1}; Éloge (Dow Agroscience), 52 g haloxyfop ha^{-1}; Stratos Ultra (BASF Agro), 200 g cycloxydim ha^{-1}; Centurion 240 EC (Arysta Lifescience), 120 g clethodim ha^{-1}.

For each of the 15 populations studied, 75 individuals per population at the 3–4-leaf stage were sprayed with one of the five herbicides studied. One extra batch of 50 individuals per population was sprayed with water to serve as an untreated control. Herbicides were applied using a custom-built, single-nozzle (nozzle 110-04; Albuz, France) sprayer delivering herbicide in 300 L ha^{-1} water at 400 kPa, at a speed of 6.6 km h^{-1}. Seedling trays were transferred from the greenhouse to the sprayer in the morning, immediately sprayed and transferred back to the greenhouse about 20 min later, when herbicide droplets deposited on their leaves were no longer visible.

One day after treatment, a 1 cm leaf fragment was collected from each individually identified herbicide-treated plant and stored at −20 °C until subsequent DNA extraction. Herbicide sensitivity was assessed 2–3 weeks after treatment, when plants from the sensitive populations were all clearly dead. At that time, plants from the other populations were dead, producing a last chlorotic leaf or producing new green leaves. Chlorotic plants died 1–2 weeks after the plants from the sensitive populations and were therefore classified as sensitive, together with plants that died at the same time as plants from the sensitive populations. Plants alive and producing new green leaves at the time herbicide sensitivity was assessed subsequently grew vigorously and were classified as resistant.

The experiment was performed twice independently, starting on 5 September 2005 and on 20 March 2006.

2.4 Genotyping mutations within the gene encoding ACCase

All plants sprayed with herbicide were genotyped following a double-blind procedure, i.e. genotype at ACCase and herbicide sensitivity were independently assessed for each plant. DNA was extracted using a previously described rapid protocol[13] and stored at −20 °C or immediately used for genotyping. Genotyping was performed on all plants using previously described allele-specific PCR assays.[5,10,11]

3 RESULTS

Characterisation of resistant or sensitive phenotypes associated with all mutant ACCase isoforms using the seed-based bioassay is from previously published work (Table 2), except for the assessment of the sensitivity to clethodim of plants containing L1781 ACCase

Table 2. Altered sensitivity of the five known mutant ACCase isoforms to five ACCase-inhibiting herbicides and cross-resistance patterns observed in whole *Alopecurus myosuroides* plants

			Whole-plant sensitivity[c]			
			Seed-based bioassay		Herbicide field rate	
Mutation	Herbicide[a]	Mutant isoform resistance factor[b]	Heteroz[d]	Homoz[d]	Heteroz[d]	Homoz[d]
I1781L (two alleles)	Fenoxaprop	>27.5[12]	R[5]	R[5]	R	R
	Clodinafop	NA[e]	S[5]	S[5]	R	R
	Haloxyfop	NA	S[5]	S[5]	R	R
	Cycloxydim	NA	R[5]	R[5]	R	R
	Clethodim	NA	S[f]	S[f]	r	r
W2027C (two alleles)	Fenoxaprop	53.0[11]	R[11]	R[11]	R	R
	Clodinafop	90.5[11]	R[11]	R[11]	R	R
	Haloxyfop	19.0[11]	R[11]	R[11]	R	R
	Cycloxydim	5.0[11]	S[11]	S[11]	S	S
	Clethodim	3.0[11]	S[11]	S[11]	S	S
I2041N	Fenoxaprop	37.0[10]	R[10]	R[10]	R	R
	Clodinafop	202.5[10]	R[10]	R[10]	R	R
	Haloxyfop	77.5[10]	R[10]	R[10]	R	R
	Cycloxydim	4.5[10]	S[10]	S[10]	S	S
	Clethodim	2.5[10]	S[10]	S[10]	S	S
D2078G	Fenoxaprop	53.0[11]	R[11]	R[11]	R	R
	Clodinafop	125.5[11]	R[11]	R[11]	R	R
	Haloxyfop	35.0[11]	R[11]	R[11]	R	R
	Cycloxydim	83.5[11]	R[11]	R[11]	R	R
	Clethodim	36.0[11]	R[11]	R[11]	R	R
G2096A	Fenoxaprop	20.5[11]	R[11]	R[11]	R	R
	Clodinafop	57.5[11]	R[11]	R[11]	R	R
	Haloxyfop	16.0[11]	R[11]	R[11]	R	R
	Cycloxydim	9.0[11]	S[11]	S[11]	S	S
	Clethodim	6.5[11]	S[11]	S[11]	r	r

[a] Aryloxyphenoxypropionate herbicides: fenoxaprop, clodinafop, haloxyfop. Cyclohexanedione herbicides: cycloxydim, clethodim.
[b] Computed for each herbicide as the ratio between herbicide concentrations inhibiting 50% of mutant and wild-type ACCase isoform activity. Numbers in the superscript to the resistance factor values are the references for resistance factor determination.
[c] S = sensitive; R = resistant; r = moderately resistant (see the discussion in Section 4). Numbers in the superscript are the references for seed-based assay results.
[d] Heteroz = plants heterozygous at ACCase, i.e. plants containing one mutant ACCase allele (e.g. IL1781); Homoz = plants homozygous at ACCase, i.e. plants containing two mutant ACCase alleles (e.g. LL1781).
[e] NA = not assayed.
[f] Seed-based bioassay results obtained in the present work.

isoforms, which was completed in the present work. All seedlings from the three populations containing only L1781 ACCase isoforms (Table 1) were found to be sensitive to clethodim in seed-based bioassays (Table 2).

A total of 11 250 *A. myosuroides* plants (5625 from each experiment) from 15 populations were analysed in this work. At least two distinct populations were analysed per mutant ACCase isoform (Table 1) to minimise the risk of resistant phenotypes observed being due to non-ACCase-based resistance mechanisms. In both spraying experiments, all control plants sprayed with water subsequently grew vigorously. Each plant treated with one of the five ACCase-inhibiting herbicides used was genotyped using allele-specific PCR following a double-blind procedure. Association of phenotype (i.e. resistant or sensitive) with genotype [i.e. presence of no, one or two mutant ACCase allele(s)] was subsequently performed for each individual plant. The phenotype observed for plants carrying a given mutant ACCase allele was consistent across the two spraying experiments for all ACCase alleles. For instance, all plants containing N2041 ACCase allele(s) survived fenoxaprop application, but were killed by clethodim in both experiments. The results from both experiments were therefore pooled (Table 3).

All IL1781 plants and all LL1781 plants survived fenoxaprop, clodinafop, haloxyfop or cycloxydim application. A total of 17 IL1781 plants and 86 LL1781 plants from these three populations survived clethodim application, but most of these plants (56 IL1781 plants and 280 LL1781 plants) were killed, although they expanded a last chlorotic leaf. Similar results were obtained for IL1781 and LL1781 plants in population 00-092 which predominantly contained C2027 ACCase and population V00-005 which predominantly contained G2078 ACCase (Table 3).

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Table 3. Herbicide sensitivity at field rate and ACCase alleles detected in *Alopecurus myosuroides* plants in two spraying-and-genotyping experiments

		Herbicides									
		Fenoxaprop		Clodinafop		Haloxyfop		Cycloxydim		Clethodim	
Populations	Genotypes[a]	R[b]	S	R	S	R	S	R	S	R	S
00-017	WT	0[c]	150	0	150	0	150	0	150	0	150
00-029	WT	0	150	0	150	0	150	0	150	0	150
V00-009	WT	0	3	0	0	0	2	0	2	0	0
	IL1781	6	0	4	0	5	0	3	0	0	7[d]
	LL1781	141	0	146	0	143	0	145	0	23	120[d]
V00-008	WT	1	3	0	2	0	6	0	4	0	0
	IL1781	32	0	28	0	31	0	36	0	11	17[d]
	LL1781	114	0	120	0	113	0	110	0	44	78[d]
00-015	WT	4	9	0	5	0	10	0	12	0	11
	IL1781	36	0	43	0	42	0	37	0	6	32[d]
	LL1781	101	0	102	0	98	0	101	0	19	82[d]
	P(IL1781)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*0.11*	
	P(LL1781)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*0.08*	
Cys2027F1	CC2027	150	0	150	0	150	0	0	150	0	150
00-045	WT	3	9	0	8	0	6	0	8	0	7
	WC2027	80	0	88	0	82	0	0	87	0	84
	CC2027	58	0	54	0	62	0	0	55	0	59
00-081	WT	0	4	0	3	0	5	0	4	0	4
	WC2027	50	0	54	0	49	0	0	52	0	53
	CC2027	96	0	93	0	96	0	0	94	0	93
00-092	WT	2	2	0	3	0	0	0	3	0	2
	WC2027	43	0	44	0	42	0	0	46	0	44
	CC2027	28	0	33	0	34	0	0	35	0	30
	IL1781	29	0	29	0	27	0	25	0	9	19
	LL1781	10	0	9	0	13	0	10	0	5	7
	IL1781/WC2027	36	0	32	0	34	0	31	0	6	28
	P(WC2027)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*1.00*		*1.00*	
	P(CC2027)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*1.00*		*1.00*	
02-F1	NN2041	150	0	150	0	150	0	0	150	0	150
V01-007	WT	2	6	0	4	0	5	0	7	0	6
	IN2041	68	0	74	0	72	0	0	71	0	68
	NN2041	74	0	72	0	73	0	0	72	0	76
	P(IN2041)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*1.00*		*1.00*	
	P(NN2041)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*1.00*		*1.00*	
Gly2078F1	GG2078	150	0	150	0	150	0	150	0	150	0
V00-005	WT	0	4	0	2	0	3	0	0	0	5
	DG2078	122	0	125	0	123	0	124	0	120	0
	GG2078	12	0	11	0	13	0	14	0	13	0
	IL1781	7	0	6	0	5	0	7	0	0	5
	IL1781/DG2078	5	0	6	0	6	0	5	0	7	0
	P(DG2078)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		$<10^{-3}$	
	P(GG2078)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		$<10^{-3}$	
Ala2096F1	AA2096	150	0	150	0	150	0	0	150	54	96[d]
00-094	WT	3	17	0	18	0	22	0	17	0	20
	GA2096	93	0	88	0	91	0	0	91	16	71[d]
	AA2096	37	0	44	0	37	0	0	42	10	33[d]
	P(GA2096)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*1.00*		*0.04*	
	P(AA2096)[e]	$<10^{-3}$		$<10^{-3}$		$<10^{-3}$		*1.00*		$<10^{-3}$	

[a] Genotypes present in each population. WT = plant(s) not containing any of the five known mutant ACCase isoform(s). Plants containing mutant ACCase alleles are referred to after their genotype, e.g. IL1781 = heterozygous plants containing one wild-type ACCase and one mutant L1781 ACCase allele; LL1781 = homozygous plants containing two mutant L1781 ACCase alleles; IL1781/WC2027 and IL1781/DG2078 = double-heterozygous plants containing two types of mutant ACCase isoform (e.g. one C2027 and one L1781 isoform).

[b] S = sensitive; R = resistant.

[c] Number of plants in a given category of genotypes (e.g. WT) and of herbicide sensitivity (e.g. resistant). The total number of plants assayed with one herbicide in one population is 150.

[d] Plants classified as sensitive that produced a last, yellowing leaf before dying.

[e] *P*-value of Fisher's exact test for the association of the genotype in parentheses with resistance to a herbicide.

WC2027 and CC2027 plants (four populations), IN2041 and NN2041 plants (two populations) and GA2096 and AA2096 plants (two populations) all survived fenoxaprop, clodinafop or haloxyfop application. All those genotypes were killed by cycloxydim and died like plants from the sensitive populations. Plants containing only C2027 or N2041 allele(s) were killed by clethodim and died like plants from the sensitive populations. A total of 16 GA2096 plants and 64 AA2096 plants survived clethodim application, but the majority of these plants (71 GA2096 plants and 129 AA2096 plants) were killed, although they expanded a last chlorotic leaf. In population 00-092, all 31 double-heterozygous mutant plants (i.e. WC2027/IL1781 plants) survived cycloxydim application, while plants only containing C2027 allele(s) were killed and died like plants from the sensitive populations (Table 3). In the two populations containing G2078 ACCase alleles, all DG2078 plants and all GG2078 plants survived application of each of the five herbicides used, as did all double-heterozygous mutant plants (i.e. DG2078/IL1781 plants) in population V00-005 (Table 3). The cross-resistance patterns conferred by each of the five mutant ACCase isoforms under optimal herbicide application conditions were established from these results and are given in Table 2.

4 DISCUSSION

The purpose of this work was to check whether cross-resistance patterns conferred by the five mutant resistant ACCase alleles known in *A. myosuroides* that were established using a seed-based bioassay were consistent with plant sensitivity at the herbicide field doses. The procedures used in seed-based bioassays and in herbicide spraying experiments are dissimilar. ACCase-inhibiting herbicides are leaf-applied herbicides. In seed-based bioassays, germinating seeds are deposited onto blotting paper soaked with a herbicide solution.[2,3] This means that plants are subjected to herbicide doses different from, and probably higher than, those in the field, at a much earlier growth stage, and that herbicide absorption is mostly via seedling roots. In spite of these differences, the cross-resistance patterns obtained from seed-based herbicide sensitivity bioassay and from herbicide spraying experiments were consistent for isoforms C2027, N2041 and G2078 (Table 2). Isoforms C2027 and N2041 conferred resistance in seed-based bioassays and at field rate to the three aryloxyphenoxypropionate herbicides used, but not to the two cyclohexanedione herbicides (Tables 2 and 3). Isoform G2078 conferred resistance in seed-based bioassays and at field rate to all five herbicides assayed (Tables 2 and 3). Isoform A2096 conferred resistance in seed-based bioassays and at field rate to the three aryloxyphenoxypropionate herbicides used, but not to cycloxydim (Tables 2 and 3). Results for clethodim sensitivity of plants containing isoform A2096 are discussed below.

In seed-based bioassays, isoform L1781 conferred resistance to the aryloxyphenoxypropionate herbicide fenoxaprop and to the cyclohexanedione herbicide cycloxydim, but not to the aryloxyphenoxypropionate herbicides clodinafop and haloxyfop, nor to the cyclohexanedione herbicide clethodim (Table 2). However, isoform L1781 conferred resistance to all three aryloxyphenoxypropionate herbicides and to cycloxydim when herbicides were applied to plants at field rate rather than to seeds in bioassays (Tables 2 and 3). Although the sensitivity at the enzyme level of this mutant ACCase isoform has been characterised with fenoxaprop only in *A. myosuroides* (Table 2), the present results were consistent with L1781 ACCase displaying a resistance factor of 14.0 to haloxyfop in another grass weed, *Lolium* sp. (rye-grass).[15] The present results also confirmed a previous hypothesis that plants containing L1781 ACCase allele(s) could withstand clodinafop application in the field.[13] This result is of importance, since clodinafop, together with fenoxaprop, is the most widely used ACCase-inhibiting herbicide in France.[16] Furthermore, isoform L1781 is the most widespread mutant resistant ACCase isoform reported to date, not only within *A. myosuroides*[13,16] but also among other grass weed species.[9,17] The limited characterisation of the sensitivity of *A. myosuroides* L1781 ACCase to herbicides is unfortunate, because the results of the herbicide spraying experiment obtained with clethodim for plants containing this isoform were not clear cut. Some plants containing L1781 allele(s) survived clethodim application. The majority were killed but, unlike plants in the sensitive populations, they expanded a last chlorotic leaf (Table 3). Similar results were also observed for ACCase isoform A2096 (Table 3). Two hypotheses can be proposed to explain these results. The first one is the occurrence of additional mechanisms conferring resistance to clethodim in the resistant plants. Because *A. myosuroides* is a diploid species, homozygous AA2096 and LL1781 plants resistant to clethodim cannot contain an extra, yet unknown, mutant ACCase allele conferring resistance to clethodim. Therefore, these putative additional resistance mechanisms must be non-ACCase based. However, metabolism of most cyclohexanedione herbicides, including clethodim, has never been reported in plants. Besides, this hypothesis would imply that such a mechanism specifically occurred in all five *A. myosuroides* populations containing L1781 or A2096 ACCase isoforms, and not in any of the ten other populations investigated. The second hypothesis is that isoforms L1781 and A2096 display a reduced sensitivity to clethodim at the enzyme level that is insufficient reliably to endow survival at clethodim field rates. Such plants can be termed 'moderately resistant'. In *A. myosuroides*, the G2078 ACCase isoform that confers resistance to clethodim at the whole-plant level (Tables 2 and 3) displays a high resistance factor to

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this herbicide (36.0) (Table 2). The A2096 ACCase isoform displays the second highest resistance factor to clethodim (6.5) (Table 2), which is in favour of this hypothesis. In *Lolium* sp., L1781 ACCase displays a resistance factor to clethodim of 18.0.[15] The present authors thus expect the resistance factor of the L1781 ACCase isoform to clethodim to be of a similar value in *A. myosuroides*. This needs to be checked by appropriate ACCase enzyme activity experiments.

The occurrence of mutant ACCase isoforms with an altered sensitivity to ACCase-inhibiting herbicides, such that plants are moderately resistant to these herbicides at the field rate, is a result of practical significance. Indeed, optimal herbicide efficacy is rarely attained in the field, and cyclohexanedione herbicides such as clethodim are subjected to photolysis.[8,14] As a consequence, some moderately resistant plants could withstand clethodim application in the field and consequently lead to clethodim not only selecting for G2078 ACCase alleles but also for L1781 and A2096 alleles. Survival of plants containing these alleles is also likely if growers reduce herbicide application rates below the recommended values. In this respect, it must be noted that all mutant ACCase isoforms characterised so far display a reduced affinity for all herbicides assayed (Table 2). Thus, cutting herbicide rates could also lead to the survival in the field of plants that contain mutant ACCase alleles but are sensitive to ACCase-inhibiting herbicides sprayed at full rate in optimal conditions. Additionally, the results reported here were obtained at French recommended field rates, which are relatively high (see Section 2.3). They may not be valid in countries where the same herbicides are used at lower rates. In this respect, it is noteworthy that survival of LL1781 *Lolium* sp. plants was found to be 100% at the Australian clethodim field rate (60 g clethodim ha^{-1}), which is half the French field rate, while survival of these plants was reduced to 50% at a rate of 98 g clethodim ha^{-1}, and to about 20% at 120 g clethodim ha^{-1}.[15]

At the herbicide field rate, resistance is dominant over sensitivity for all combinations of mutant isoforms and herbicides where resistance was observed (Table 3). That is, plants containing a single mutant ACCase isoform are resistant to all herbicides to which this isoform confers resistance (Table 3). This confirms literature data,[9,15] implying that resistant ACCase isoforms are selected for as soon as they appear or arrive in an *A. myosuroides* population sprayed with ACCase-inhibiting herbicides.

5 CONCLUSION

This work identified the cross-resistance patterns conferred by five mutant ACCase isoforms in *A. myosuroides* to herbicides used at the French field rate. Some of these isoforms have also been identified in other species and appeared to confer cross-resistance patterns similar to those observed in *A. myosuroides*.[9,15,18] Therefore, the present results may extend to other grass weed species. However, this needs to be carefully checked, because the intrinsic sensitivity to ACCase inhibitors varies among species.[9] Also, given that the reduction in sensitivity observed for each mutant ACCase isoform depends on the herbicide molecule (Table 2), the present results cannot directly be extended to ACCase inhibitors not studied here.

For most herbicide–isoform combinations studied, the present results confirmed those obtained using seed-based bioassays. The major discrepancy observed was the dominant resistance to clodinafop and haloxyfop that was conferred by L1781 ACCase isoforms at doses used in the field, in contrast to the sensitivity observed in seed-based bioassay (Tables 2 and 3). This illustrates the need for a dose of herbicide both reliably discriminating resistant and sensitive plants in bioassays and consistent with results from the field. The moderate resistance conferred by L1781 and A2096 ACCase isoforms to clethodim illustrates the difficulty in determining such a dose. Applying herbicides at field rates to weeds at the appropriate growth stage is clearly the most reliable way of diagnosing resistance, especially when no resistance gene has been identified, or when multiple genes are involved as in metabolism-based resistance.[9] However, the use of miniaturised rapid tests such as molecular resistance detection assays should permit a first and fast analysis of weed samples. Cross-resistance patterns associated with known mutant ACCase isoforms have been verified. Molecular assays proved adaptable to quantitative genotyping,[19] which will significantly reduce their cost. They are therefore of particular interest for routine resistance surveys analysing large numbers of plants. From the results obtained, a focused series of spraying experiments can subsequently be performed to achieve a full characterisation of resistance in the corresponding fields.

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