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Lolium rigidum, a Pool of Resistance Mechanisms to ACCase Inhibitor Herbicides

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Three diclofop-methyl (DM) resistant biotypes of *Lolium rigidum* (R1, R2, and R3) were found in different winter wheat fields in Spain, continuously treated with DM, DM + chlortoluron, or DM + isoproturon. Herbicide rates that inhibited shoot growth by 50% (ED_{50}) were determined for DM. There were found that the different biotypes exhibited different ranges of resistance to this herbicide; the resistant factors were 7.2, 13, and 36.6, respectively. DM absorption, metabolism, and effects on ACCase isoforms were examined in these biotypes of *L. rigidum*. The most highly resistant, biotype R3, contained an altered isoform of ACCase. In biotype R2, which exhibited a medium level of resistance, there was an increased rate of oxidation of the aryl ring of diclofop, a reaction most likely catalyzed by a cytochrome P450 enzyme. In the other biotype, R1, DM penetration was significantly less than that observed in the resistant (R2 and R3) and susceptible (S) biotypes. Analysis of the leaf cuticle surface by scanning electron microscopy showed a greater epicuticular wax density in the leaf cuticles of biotype R1 than in the other biotypes.

KEYWORDS: **Diclofop-methyl; *Lolium rigidum*; ACCase isoforms; cytochrome P450; leaf cuticle**

INTRODUCTION

Lolium rigidum Gaud (ryegrass) is a mayor annual grass weed of arable crops in Spain and other western European countries. Diclofop-methyl (DM) resistant populations of *Lolium multiflorum* were first recorded in the United Kingdom in 1990, and later in 1992 in Spain appeared different *L. rigidum* populations with cross-resistance to acetyl-CoA carboxylase (ACCase), ALS, PSII, and tubuline inhibitor herbicides (*1*). In Spain since 1992, there has been an increase in the resistance to the ACCase inhibiting aryloxyphenoxypropionate (AOPP) and cyclohexanodione (CHD), which have been used increasingly since the introduction of a wide number of graminicides (*2*). The AOPP and CHD herbicides are used to control grass weed in broadleaf crops and cereals such as wheat (*3*, *4*). In general, its selectivity is based on their effects at the herbicide target site, the enzyme ACCase (EC 6.4.1.2), which is located in both cytosol and chloroplasts and catalyzes the first commited step in de novo fatty acid biosynthesis (*5*). The selectivity of AOPP and CHD herbicides is expressed at the level of the plastic-localized ACCase (*3*, *5*, *6*). This difference in susceptibility at the target site is due to the presence of two different types of ACCases in the plastids of grasses and dicots. The plastids of grasses contain a graminicide sensitive, eukaryotic type, multifunctional ACCase with a biotinylated subunit of approximately 36 kDa (*6*−*9*). The plastidic ACCase in dicots is the prokaryote form of the enzyme, a multisubunit protein. Both grasses and dicots contain cytosolic multifunctional ACCases (*6*, *7*, *9*, *10*). These cytosolic, multifunctional ACCases exhibit a level of graminicide tolerance that is significantly higher than that of the plastidic, multifunctional ACCases of grasses but lower than that of plastidic, multisubunit ACCases of dicots.

Evolved resistance in *Lolium* spp. biotypes can be due to (i) an enhanced ability to metabolize herbicides such as phenylurea (*11*, *12*), acetolactate syntase inhibiting herbicides (*13*), and ACCase inhibiting herbicides (*14*) [in all cases, two types of enzyme have been implicated, cytochrome P450 monooxygenase (*15*) and glutathione S-transferases (*15*)] and (ii) an altered isoform of ACCase having an altered affinity for herbicide, which was associated with the substitution of an isoleucine susceptible to a leucine in a 3300 bp DNA fragment encoding the carboxyl-transferase domain of the multidomain, chloroplastic ACCase in resistant biotypes (*16*).

The specific objectives of the present study were to (i) confirm the resistance of *L. rigidum* populations found in Spain to DM, (ii) determine the mechanism of resistance by evaluating the effects of DM on penetration and metabolism and ACCase activity in resistant and susceptible populations of *L. rigidum*, and (iii) examinate whether both multifunctional and multisubunit ACCases are present in *L. rigidum* leaves and characterized by their sensitivity to DM.

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MATERIALS AND METHODS

Chemicals. [^{14}C]DM (specific activity, 95.5 kBq μmol^{-1}) was provided by Bayer CropScience (Germany). A commercial formulation (Iloxan; 36% w/v EC) of this herbicide used for growth assays was supplied by Bayer CropScience (Spain).

Plant Material. *L. rigidum* seeds were collected from wheat fields where recommended rates of DM, either alone or in combination with chlorotoluron or isoproturon, had failed to control weeds. Seeds were collected between 1995 and 2000 from three winter wheat fields in Spain. *L. rigidum* resistant biotype (R2) was collected from a winter wheat fields that had been treated annually with a mixture of DM plus chlortoluron or DM plus isoproturon for at least 10 years. The other resistant biotypes (R1 and R3) were collected from fields treated annually with DM alone for at least 10 years. Control seeds of a susceptible biotype were collected from nearby olive tree groves that had never been treated with herbicides.

Seeds of R and S biotypes were germinated in Petri dishes with a blotter moistened with distilled water. The Petri dish cover was sealed with Parafilm, and seeds were germinated in a growth chamber at 23/18 °C (day/night) in a 16 h photoperiod at 80 °C relative humidity. Four or three pregerminated seeds (for growth and absorption/translocation assays, respectively) were planted per pot (7 cm diameter, 7 cm high plastic pots) in a peat/soil mixture (1/2, v/v). Plants were grown in a growth chamber under the same conditions as for germination.

Growth Assays with DM. At the 2−3 leaf stage, the R and S biotypes of *L.rigidum* were sprayed with a commercial formulation of DM at several concentrations (S: 0.1, 0.2, 0.4, and 0.8 kg a.i. ha^{-1}; R1, R2, and R3: 1, 2, 4, 6, and 12 kg a.i. ha^{-1}) using a laboratory track sprayer (Tee-Jet 8001 flat-fan nozzle) delivering 200 L ha^{-1} at 200 kPa. Treatments were replicated three times, and the shoot fresh weight was evaluated after 21 days for each treatment. The concentration of herbicide that caused a 50% decreased in growth with respect to the control (ED_{50}) was determined for each biotype (*17*).

[^{14}C]DM Penetration. [^{14}C]DM was mixed with commercially formulated DM to prepare an emulsion with a specific activity of 37.9 Bq mg^{-1} and a DM concentration of 6.6 g L^{-1} (corresponding to 1.0 kg ha^{-1} DM at 150 l ha^{-1}). This formulation of labeled herbicide was applied to the adaxial of the second leaf of each plant in four 0.5 μL droplets using a microapplicator (Hamilton PB-600). A total of 833.33 Bq were applied on each plant.

Plants were harvested in batches of three plants at several time intervals after herbicide application (0, 3, 6, 12, 24, and 48 h) and separated into treated leaves and the remainder of the shoots. Roots were discarded, as herbicide translocation from leaves to roots was reported as being undetectable in wheat (*18*). Unabsorbed [^{14}C]DM was removed from the leaf surface by washing the treated area with 1.5 mL of acetone. Washes from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA). The plant tissue was dried at 60 °C for 48 h and combusted in a sample oxidizer (Packard 307). The $^{14}CO_2$ evolved was trapped and counted in 10 mL of Carbosob/Permafluor E$^+$ (3/7 V/V) (Packard Instruments Co.). The radioactivity was quantified by LSS and expressed as a percentage of the recovered radioactivity, according to the following formula:

$$\%\ \text{absorption} = [^{14}\text{C in combusted tissue}/(^{14}\text{C in combusted tissue} + {}^{14}\text{C in leaf washes})] \times 100$$

The experiment was repeated three times.

Scanning Electron Microscopy (SEM). Small pieces of fresh *L. rigidum* leaves were cut off with a sharp razor blade and fixed in 2% glutaraldehyde (v/v) in 0.2 M phosphate buffer, pH 7, overnight at 4 °C. According to ref *19*, the samples were thoroughly rinsed in fresh phosphate buffer and dehydrated through an ethanol solution series: 20, 40, 60, 80, and 100% (v/v) and increasing times, from 15 min to 1 h and 30 min. The pieces were placed on a metallic holder using a double-faced adhesive and coated with a 0.05 μm thin film of gold. A JEOL JSM-840 scanning electron microscope operated at 10−20 kV was used for the examination of the samples.

Metabolism Studies Using [^{14}C]DM. The metabolism of [^{14}C]DM was examined in leaf tissue of R2, R3, and S plants at the two leaf stage as has been developed for penetration studies. The labeled herbicide was applied to the adaxial surface of the second leaf in 10 0.5 μL droplets using a microaplicator. A total of 5000 Bq was applied on each plant. Plants of R2, R3, and S were sampled 6, 12, 24, and 48 h after treatment. Treated leaves were washed as above. An aliquot of the leaf wash solution was assayed for radioactivity, and the remaining solution was stored at −20 °C until analysis. The treated plants were separeted into roots and shoots. The shoots from each plant were pulverized in liquid nitrogen using a mortar and pestle. The powder was extracted at 4 °C with 4 mL of 80% methanol, and the homogenate was centrifuged at 20000*g* for 20 min. The pellet was washed with 80% methanol until ^{14}C was no longer extracted. The pellets were oven dried and combusted as above. The supernatants were combined, evaporated to dryness at 40 °C under a stream of N_2 at 10 kPa, and redissolved in 500 μL of 80% methanol. The DM and its metabolites in the supernatant were identified by thin-layer chromatography on 20 cm × 20 cm, 250 μm silica gel plates (Merck; silica gel 60) and a toluene/ethanol/acetic acid (150/7/7, v/v/v) mobile phase. The radioactive zones were detected with a radiochromatogram scanner (Berthold LB 2821), and their chemical nature was identified by comparing their R_f values with those of standards (DM, 0.70; diclofop acid, 0.44; hydroxy-diclofop, 0.34; polar conjugates, 0.00). For quantitative determinations, the radioactive spots were scraped off, extracted with 80% methanol, and counted by LSS. The experiment was repeated three times.

Effect of a Cytochrome P450 Inhibitor on DM Metabolism. ABT (1- aminobenzotriazole) has previously been shown to inhibit DM metabolism in *L. rigidum* (*20*). In the ABT experiments, plants with two fully expanded leaves were removed from pots and the roots were carefully washed. Individual plants were placed in 50 mL containers filled with nutrient solution containing 7.5 mg L^{-1} ABT. The nutrient solution was aerated with filtered air. After 8 days of ABT incubation, the plants were treated with [^{14}C]DM as previously described and harvested 48 h after herbicide application. Parent DM and metabolites were extracted, chromatographed, and identified as described above.

Enzyme Purification. Leaves (10 g fresh weight) of R3 and S biotypes were harvested from plants in the 3−4 leaf stage and ground in liquid N_2 with a mortar and then added to 40 mL of extraction buffer [0.1 M Hepes−KOH (pH 7.5), 0.5 M glycerol, 2 mM EDTA, and 0.32 mM phenylmethyl sulfonyl fluoride]. The homogenate was mixed for 3 min with a magnetic stirrer and filtered sequentially through four layers of cheesecloth and two layers of Miracloth (Calbiochem). The crude extract was centrifuged (24000*g*, 30 min). The supernatant was fractionated with ammonium sulfate. Material precipitating between 35 and 45% $(NH_4)_2SO_4$ saturation was resuspended in 2 mL of S400 buffer [0.1 M Tricine−KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, and 0.5 mM dithiothreitol (DTT) and centrifuged (17000*g*, 10 min)]. The supernatant was applied to a Sephacryl S400 column (2.5 cm × 46 cm) that had been equilibrated with S400 buffer. Fractions were eluted with S400 buffer at a flow rate of 15 mL h^{-1}. Fractions from the S400 column containing maximum ACCase activity were pooled and applied to a FPLC Fractogel EMD-TMAE 650 (S) anion exchange column (1 cm × 15 cm) equilibrated with TMAE buffer [0.67 M Tricine−KOH (pH 8.3), 0.5 M glycerol, 1.3 mM EDTA, and 0.67 mM DTT]. ACCase activity peaks were eluted with a linear KCl gradient (0−375 mM) in TMAE buffer, and 1 mL fractions were collected.

ACCase Assays. The enzyme activity was assayed by measuring the ATP-dependent incorporation of NaH(^{14}C)O_3 into an acid-stable ^{14}C product. The reaction product was previously shown to be [^{14}C]-malonyl-CoA (*21*). Assays were conducted in 7 mL scintillation vials containing 0.1 M Tricine−KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, 0.5 mM DTT, 1.5 mM ATP, 5 mM $MgCl_2$, 15 mM NaH(^{14}C)O_3 (33 μCi μmol^{-1}), 90 μL of enzyme fraction, and 5 mM acetyl-CoA in a final volume of 0.2 mL. The assay temperature was 36 °C. The reaction was stopped after 5 min by adding 30 μL of 4 N HCl. The acidified reaction medium was dried at 40 °C under a stream of air. The acid-stable radioactivity was determined by redissolving the dried samples in 0.5 mL of 50% (v/v) ethanol followed by

Table 1. Effect of DM on Growth of Different *L. rigidum* Biotypes[a]

biotype	ED_{50} (kg a.i./ha)	$ED_{50}R/ED_{50}S$
S	0.25 ± 0.02	
R1	1.80 ± 0.06	7.20
R2	3.25 ± 0.3	13.00
R3	9.15 ± 0.7	36.60

[a] Note: Data are means of three experiments ± SE.

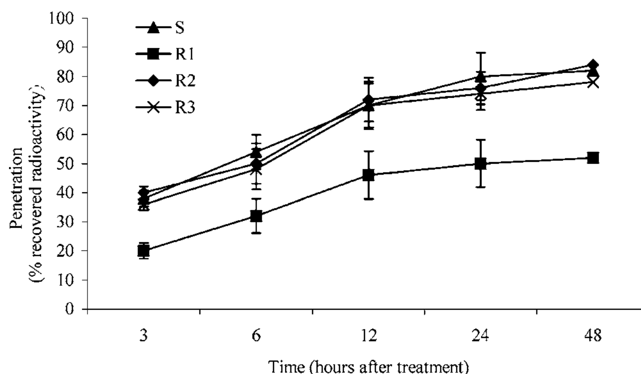

Figure 1. Penetration of DM in different biotypes of *L. rigidum*.

scintillation counting in 5 mL. The radioactivity was determined by LSS. Herbicide concentrations resulting in a 50% inhibition of enzyme activity (I_{50} values) were determined for diclofop acid.

Western Blotting. Crude and purified ACCase fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) in 4−15% (w/v) gradient PhastGels and PhastGels System. Prior to the electrophoresis, samples were boiled for 5 min. The migration distances were determined by regression analyses against the migration distances on high molecular mass biotinylated proteins standards (Bio Rad) plus horse spleen ferritin (220 kDa). Proteins on PhastGels were transferred to Inmobilon-P by capillary action. Blocks were incubated for 18 h in TBST [80 mM Tris HCl (pH 7.6), 0.5 M Na Cl, and 0.1% (v/v) Twen-20] at room temperature with shaking. Biotinylated proteins were probed using avidin-alkaline phosphatase (*22*).

Protein Determination. Protein was estimated by the Bradford assay using bovine serum albumin as a standard (*23*).

RESULTS AND DISCUSSION

Growth Assays with DM. The growth response to DM revealed a marked difference between *L. rigidum* biotypes. Treatment made at a field rate of DM (Iloxan 2.5 L ha^{-1}) showed that the S biotype was killed 15 days after treatment. Although R1, R2, and R3 biotypes were resistant to DM, the latter showed a higher resistance level than the former. The R1, R2, and R3 biotypes were 7.20, 13.00, and 36.60, respectively, less sensitive to DM than the S biotype (**Table 1**). These results suggest the involvement of different mechanisms of resistance. Thus, a mutation in the ACCase will give a higher level of resistance than an enhancement of the metabolism and this is also higher than the resistance due to a lack of herbicide penetration.

[^{14}C]DM Penetration. There were no significant differences in the penetration of [^{14}C]DM into the R2, R3, and S *L. rigidum* biotypes (data not shown); however, the R1 biotype showed a lower penetration rate than the other biotypes (**Figure 1**). After 24 h of application, about 75% of the recovered radioactivity had penetrated into the leaf tissue of the R2, R3, and S *L. rigidum* biotypes, while only 50% had penetrated into the R1 biotype. After 24 h, the herbicide penetration was less than 4% in all cases (**Figure 1**). This is the first report in *L. rigidum* of resistance being endowed by a lack of DM penetration as has been reported in other grass weeds (*22*, *24*−*27*). Other studies made on the R1 biotype such as the metabolism of [^{14}C]DM and the ACCase activity did not reveal any differences between this biotype and the susceptible one (data not shown).

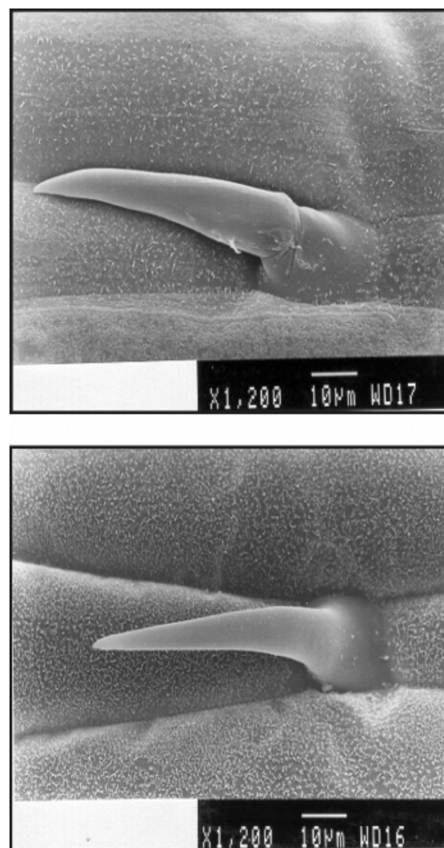

Figure 2. Scanning electron micrographs of the adaxial outer surface of the S (top) and R1 (bottom) biotypes of *L. rigidum*.

SEM. The outer surface of the cuticle of *L. rigidum* leaves corresponding to the different biotypes was studied by SEM. Significant morphological differences were found in the adaxial side of the leaves. **Figure 2** shows the scanning electron micrographs of the outer surface of the S and R1 biotypes. The cuticle appears as a thin, continuous, and smooth layer with a noticeable amount of waxes in the form of isolated platelets thickly distributed over the outer surface of the cuticle. This epicuticular wax morphology and ultrastructure has been observed in a wide variety of plant species (*28*). **Figure 2** also shows a significant difference between both biotypes: the amount of wax platelets per unit of cuticle surface area is higher in the case of the R1 biotype. This fact gives the cuticle of this biotype an additional and hydrophobic molecular barrier to further chemical diffusion. A qualitative chromatography analysis, made using thin-layer chromatography, of the isolated cuticular waxes of these two biotypes showed a similar qualitative composition indicating that wax esters were the main component of these waxes (data not shown). Plant wax esters, together with wax alkanes, are the most hydrophobic compounds that can be found in plant waxes (*28*). These results permit the hypothesis that this waxy barrier is responsible for a lesser penetration of the herbicide in biotype R1 and a lesser sensitivity to DM than the S biotype, which led to the supposition that this waxy barrier was responsible for a lesser penetration of the herbicide in biotype R1.

Metabolism Studies of [^{14}C]DM. Qualitatively, the pattern of DM metabolism was similar in the resistant (R2 and R3)

Table 2. DM Metabolism in Resistant (R2) and Susceptible (S) Biotypes of *L. rigidum* 6, 12, 24, and 48 h after Treatment

	% extracted radioactivity							
	6 h		12 h		24 h		48 h	
metabolites	R2	S	R2	S	R2	S	R2	S
DM	28.21 (3.2)	20.37 (7.4)	20.47 (1.3)	16.61 (3.2)	15.92 (1.1)	14.35 (6.9)	10.92 (1.1)	9.35 (3.2)
diclofop	64.48 (2.1)	75.23 (5.2)	60.00 (3.2)	78.31 (2.3)	36.92 (3.3)	76.73 (4.4)	20.32 (3.2)	77.37 (6.7)
conjugates	10.31 (0.1)	4.40 (1.2)	19.53 (0.4)	5.08 (0.1)	47.38 (2.4)	8.93 (2.2)	68.76 (4.1)	12.01 (4.2)

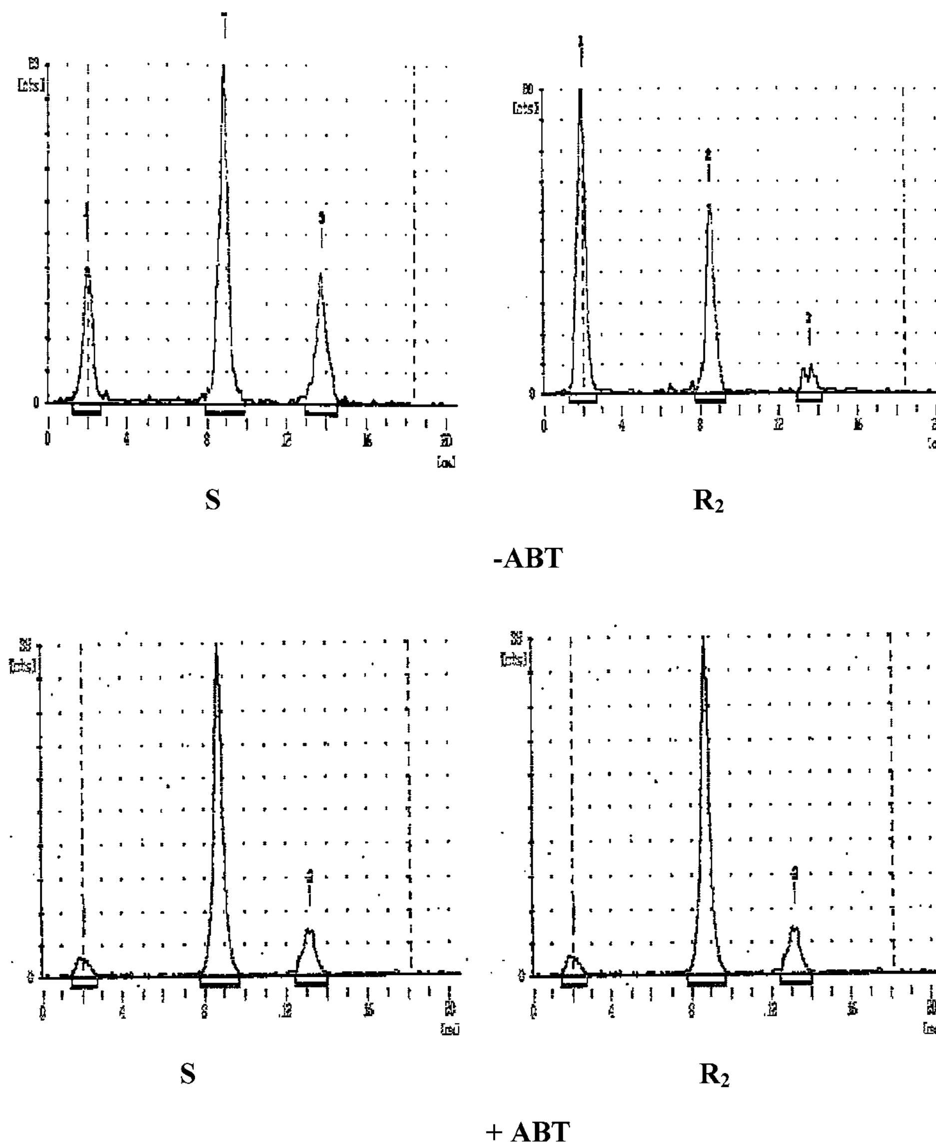

Figure 3. Distribution of ^{14}C-DM and its metabolites in *L. rigidum* biotypes preincubated (+ABT) or nonpreincubated (–ABT) in 7.5 mg L^{-1} of 1. Data for a typical experiment are shown.

and susceptible (S) biotypes of *L. rigidum*. DM is deesterified to the herbicidal diclofop acid, and this compound is then further metabolized to other compounds that are more polar than diclofop acid. These compounds are principally sugar ester conjugates of diclofop acid and sugar conjugates of hydroxy-diclofop. All of these metabolites (DM, diclofop acid, and polar conjugates) were found in all biotypes assays. However, DM was metabolized to a nontoxic compound significantly faster in the R2 than in S and R3, which quantitatively showed similar metabolisms during all times of the assay (data not shown).

The R2 and S biotypes rapidly hydrolyzed the penetrated DM to the acid form via esterase activity remaining less than 11% of DM after 48 h of treatment (**Table 2** and **Figure 3**). The concentration of phytotoxic diclofop acid was 3.8 times greater in the S biotype than in the R2 biotype after 48 h. This correlates with the kinetics of conjugate formation shown by S and R2 biotypes, where after 48 h of DM application, diclofop conjugate appeared more than five times higher in R2 than in the S biotype.

Preincubation in ABT solution significantly inhibited the metabolism of DM to polar conjugates in all biotypes assayed (**Figure 3**). In both R2 and S, the amount of nontoxic conjugates formed 48 h after application was reduced more than one-fourth of the amount observed in nonpreincubated plants. As the amount of polar metabolites decreased, a significant accumulation of diclofop acid was observed in R2, in agreement with other experiments using cyt P450 inhibitors (*29*).

Table 3. Purification of ACCase from S Biotype

step	protein (mg)	activity (U)	specific activity (U mg^{-1})	purification (fold)	recovery (%)
crude extract	119	1.13	0.01	1	100
$(NH_4)_2SO_4$ fractionation	4.71	0.51	0.11	12	46
S 400	1.34	0.42	0.31	34	37
ACCase I	0.24	0.22	0.91	98	20
ACCase II	0.02	0.02	0.78	84	2

Table 4. Purification of ACCase from R3 Biotype

step	protein (mg)	activity (U)	specific activity (U mg^{-1})	purification (fold)	recovery (%)
crude extract	112	1.21	0.01	1	100
$(NH_4)_2SO_4$ fractionation	5.98	0.50	0.08	8	41
S 400	1.26	0.40	0.32	32	33
ACCase I	0.22	0.18	0.84	84	15
ACCase II	0.03	0.02	0.62	62	2

Similar pathways were also involved in the detoxification of DM in *L. rigidum* (*14*) from Australia and some *Alopecurus myosuroides* biotypes fron Spain and the United Kingdom (*26*, *30*). The metabolic detoxification was catalyzed in both the R2 and the S biotypes of *L. rigidum* by cyt P450 monooxygenases since the inhibition of these enzymes by ABT resulted in a strong reduction in the detoxification rate by inhibiting diclofop hydroxylation (**Figure 3**). On the other hand, our data suggest the aryl-O-glucoside was the main component of the conjugate fraction, as the amount of polar conjugates was strongly affected by the inhibition of diclofop hydroxylation. In most gramineous susceptible species, diclofop acid is conjugated to give a glucose ester conjugate (*26*, *31*), which did not effectively detoxify the herbicide because of the reversible nature of the glycosyl bond formed (*32*). In most gramineous resistant species, ring-hydroxylated diclofop is conjugated to give an ary-O-glicoside of the irreversible nature (*27*).

Enzyme Purification. Partial purification and separation of ACCase activity did not reveal any differences between DM resistant and susceptible biotypes of *L. rigidum*. ACCase from DM resistant and susceptible biotypes was purified 84-fold by ammonium sulfate fractionation, gel filtration, and anion exchange chromatography. The three step purification procedure typically yielded about 250 μg of purified ACCase from 10 g (fresh weight) of R3 or S *L. rigidum* leaves, which represented over 25% of the initial ACCase activity in crude extract. These fractions contained about 3% of the soluble protein in crude extract (**Tables 3** and **4**).

TMAE anion exchange chromatography resolved two ACCase peaks, which eluted at 250 (ACCase I) and 210 mM KCl (ACCase II) (**Figure 4**). ACCase activity I represented about 90% of the total activity recovered from the TMAE column (15% of the original crude extract activity), having the highest specific activity. ACCase II showed lesser activity, with only 2% of the original crude extract activity and a specific activity of 75% of that shown by ACCase I.

Avidin-AP-probed Western blots of purification fractions separated by SDS−PAGE showed that both ACCase I and ACCase II fractions contained a single biotinylated polypeptide of 200 kDa. Therefore, both fractions contained other biotinylated polypeptides, the predominant one being a protein with a molecular mass of about 75 kDa, coeluting mainly with ACCase II during the TMAE purification step (data not sown).

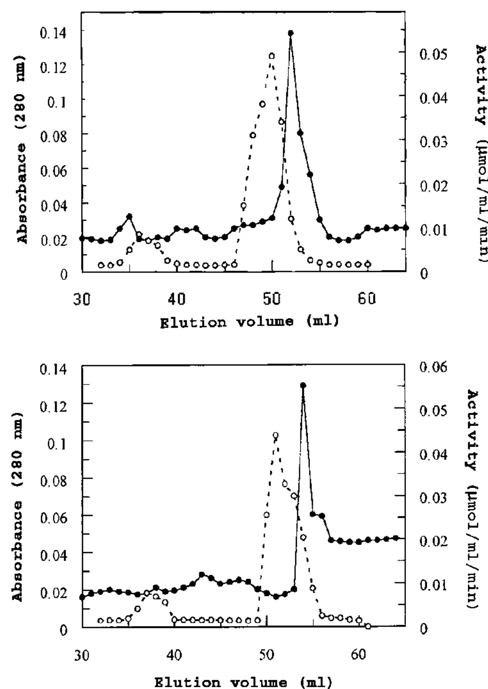

Figure 4. TMAE anion exchange chromatography of ACCase activities from leaves of diclofop-S (top) and -R3 (bottom) *L. rigidum.*

Table 5. Effect of Diclofop on ACCase Fractions Obtained by Sephacryl S400 Filtration and TMAE Anion Exchange Chromatography from Diclofop Susceptible and Resistant *L. rigidum*

	I_{50} (μM) S	I_{50} (μM) R3	resistance factor [I_{50}(R)/I_{50}(S)]
crude extract	0.43 ± 0.06	15.0 ± 0.82	34.9
S400	0.51 ± 0.08	16.0 ± 1.31	31.4
ACCase II	110 ± 7.26	110 ± 10.03	1.0
ACCase I	0.47 ± 0.06	17.5 ± 0.96	37.2

ACCase Assays. The I_{50} values were calculated for the different fractions in the purification. After the S-400 gel filtration, the value in both biotypes was similar to the one for the crude extract, about 0.5 μM in the S biotype and 15 and 16 μM in the R3 biotype. The resistance factor was about 30. The ACCase I and ACCase II fractions differed significantly in their inhibition by DM. The I_{50} value for diclofop inhibition for the predominant form (ACCase I) was 37-fold greater for the R3 biotype than for the S one. The minor isoform (ACCase II) was relatively insensitive to this herbicide in both biotypes with I_{50} values higher than those observed for the ACCase I (**Table 5**).

The results of this report demonstrate that *L. rigidum* leaves contain two different ACCase activities that differ in diclofop tolerance. The resistance to diclofop in R3 biotype is due to the presence of a resistant form of ACCase.

In this report, we have shown that *L. rigidum* leaves contain two ACCase activities: a predominant, herbicide sensitive, plastid-localized enzyme (ACCase I) and a minor enzyme (ACCase II), which differs in anion exchange properties, subunit size, and herbicide inhibition. The protocol involving ammonium sulfate precipitation, gel filtration (Sephacryl-S400), and TMAE anion exchange chromatography succeeded in both separating the ACCase I and II activities and improving their specific activity as was previously described in other species. The ACCase I and II have qualitatively and quantitatively similar

molecular masses to those found for other grass species (*33*−*35*). The 75 kDa polypeptide may represent the biotinylated subunit of methylcrotonyl-CoA carboxylase (MCCase), as this enzyme has a biotinylated subunit with a molecular mass in the range of 75−85 kDa depending on the species (*36*−*39*). The presence of MCCase in the fractions assayed would not interfere with ACCase assays since MCCase does not exhibit any cross-reactivity with acetyl-CoA (*36*).

As in the other grasses studied, where chloroplastic ACCase represents 80−95% of the total ACCase activity (*10*, *22*, *40*, *41*), this isoform was predominant in both resistant and susceptible *L. rigidum* leaves and eluted from a TMAE column at a higher salt concentration, as compared with the minor ACCase isoform (ACCase II) (*10*, *22*). In addition, this isoform in the resistant biotype was tolerant to diclofop while ACCase from the susceptible was sensitive. This result agrees with that previously found in maize (*10*) and *L. multiflorum* (*22*). However, the multifunctional ACCase II isoform shows a smaller fraction of total ACCase activity and exhibits a high level of diclofop resistance in grasses. Usually, I_{50} values for ACCase II for various graminicides are close to 2 orders of magnitude greater than those for wild-type ACCase I (*22*, *34*).

The simple monogenic nature of target site resistance, and the high degree of herbicide insensitivity conferred, helps explain why this type of resistance can build up rapidly, in contrast to the most polygenically based enhanced metabolism (*15*). Up to day, the resistance in four grass species, *Avena fatua* (*42*), *Setaria viridis* (*43*), *L. rigidum* (*17*), and *Alopecurus myosuroides* (*44*), has been shown to be associated with a mutation in the ACCase gene, resulting in an isoleucine to leucine substitution in the ACCase I isoform. In our case, future studies concerning graminicide resistance need to focus on cloning the ACCase I gene and identifying the sequence encoding the graminicide binding region in R and S biotypes.

It is clear from the results presented here and from previous studies (*16*) that the resistance in *L. rigidum* populations to herbicides which inhibit ACCase was due to multiple mechanisms. As was discussed in ref *45*, herbicide treatment of huge populations results in the survival of individuals that express any resistance mechanism conferring the ability to withstand herbicide at the rate used. In cross-pollinated species such *L. rigidum*, there is a gene flow among the survivors resulting in an exchange of different resistance genes and their accumulation in the next generation. Thus, depending upon genetic variation, the size of a population subjected to herbicide selection and the ability and efficacy of cross-pollination, there can be an enrichment of a number of resistance mechanisms. The likelihood and dynamics of gene exchange by pollen will influence whether cross-pollinated individuals will have one or several resistance mechanisms.

Thus, the key to combating resistance in *L. rigidum* is to adopt resistance prevention and management strategies at an early stage and to reduce reliance on herbicides by integrating herbicide use with nonchemical methods of weed control.

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