Characterization of the cross-resistance mechanism to herbicides inhibiting acetyl coenzyme-A carboxylase in itchgrass (*Rottboellia cochinchinensis*) biotypes from Bolivia

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Abstract

Whole-plant greenhouse bioassays and absorption, translocation, and metabolism experiments were conducted to investigate the resistance and cross resistance patterns and resistance mechanism of itchgrass (*Rottboellia cochinchinensis*) biotypes from Bolivia to herbicides that inhibit the enzyme acetyl coenzyme-A carboxylase. Varying levels of resistance to haloxyfop-R-methyl and sethoxydim were found in the biotypes designated as San Pedro and Yapacaní and cross-resistance among graminicides was confirmed. The resistance indices (RI) for haloxyfop-R-methyl based on the GR\textsubscript{50} values (herbicide dose required to inhibit growth by 50%) for these biotypes were 33.4 and 44.3, respectively. For the sethoxydim, the RI values were 9.3 and 4.7, respectively. The rate of \([^{14}\text{C}]\text{sethoxydim}\) absorption was similar in the resistant and susceptible biotypes at 6, 12, 48 and 96 h after treatment (HAT); there was no significant difference in the amount of \([^{14}\text{C}]\text{sethoxydim}\) uptake among biotypes. No differences in the translocation or metabolism of \([^{14}\text{C}]\text{sethoxydim}\) were observed between resistant and susceptible biotypes at any interval after application. In in vitro ACCase assays, the concentrations of sethoxydim required to inhibit ACCase activity by 50% (I\textsubscript{50}) were substantially higher (about 11 times) for the two resistant biotypes compared to the reference biotype, indicating that the resistant itchgrass biotypes have an ACCase that is relatively insensitive to the graminicides. These results suggest that cross resistance in itchgrass biotypes is conferred by a reduced sensitivity of the target enzyme.

Keywords: Herbicide resistance; Acetyl coenzyme-A carboxylase; Itchgrass

1. Introduction

Herbicide resistance is defined as the inherited ability of a weed population to survive a herbicide application that is normally lethal to the vast majority of individuals of that species (Powles et al., 1997). Weed populations evolve herbicide resistance through selection pressure imposed by frequent use of one or more herbicides with the same mode of action or metabolic degradation pathway at a location over an extended period of time (Valverde et al., 2000).

*Rottboellia cochinchinensis* is an aggressive and competitive annual grass weed, mainly in maize, rice, sugarcane, and soybean crops in many Central and South American countries. Herbicide resistance in this species was first reported in USA to the herbicide fluazifop-p-butyl but the resistance mechanism in this biotype remains unknown (Heap, 2005). To date, two weedy grass species (*Eriochloa punctata* and *Sorghum sudanense*) have evolved resistance to herbicides that inhibit acetyl-CoA carboxylase (ACCase) in soybean fields in the northern zone of Santa Cruz, Bolivia (CIAT, 2000).

Resistance to the ACCase herbicides, aryloxyphenoxypyridiones (APP) and cyclohexanediones (CHD) in grass weeds has become widespread, being the third most important group in resistance cases worldwide (Heap, 2005). Recent work on ACCase, partly stimulated by interest in the mechanism of action of graminicides, has
shown that this enzyme exists in two different structures in higher plants. Dicotyledonous species, except some Geraniaeae (Christopher and Holtum, 2000) contain a multi-subunit form of ACCase in the plastid and a high-molecular mass multifunctional-form outside the plastid, presumed to be cytosolic (Harwood, 1996; Konishi et al., 1996; Sasaki and Nagano, 2004). In contrast, grasses have two different multifunctional ACCase isoforms. Only the (plastid) isoform of grasses is significantly sensitive to the APPs and CHDs and therefore the graminicides kill such plants by preventing the de novo synthesis of fatty acids in chloroplasts (Walker et al., 1989; Harwood, 1996). In the majority of weed biotypes, resistance to ACCase herbicides is commonly conferred by reduced sensitivity of the target enzyme, and this is normally demonstrated by extraction and partial purification of ACCase from young tissue, followed by an enzyme activity assay in the presence of a range of herbicide concentrations (Devine, 1997). Resistance is conferred by target-site mutations thus an Ile–Leu substitution confers resistance to both haloxyfop and sethoxydim and an Ile–Asn mutation does the same for APPs but not for CDHs (Délye et al., 2003). Based on the sethoxydim and an Ile–Asn mutation does the same for resistance evaluating their absorption, translocation, and metabolism of \[^{14}C\]sethoxydim was mixed with commercially formulated sethoxydim (to prepare a dilution with a specific activity of 1.66 Bq µl\(^{-1}\)) to study both absorption and translocation. This preparation of labeled and commercial herbicide was applied on the adaxial surface of the second leaf of each plant in four 0.5 µl droplets, using a micro applicator. For the absorption studies, two plants growing in each pot were harvested at 6, 12, 48 and 96 h after herbicide application, and separated into treated leaf, upper leaf, lower leaf, and root. Unabsorbed \[^{14}C\]sethoxydim, was removed from the surface of the treated leaf by washing with 3 ml of 80% methanol. Washes were pooled and analyzed by liquid scintillation spectrophotometry (LSS). Plant tissue was dried separately at 50 °C for 72 h, and combusted in a sample oxidizer. The \(^{14}\)CO\(_2\) evolved was trapped and counted in 10 ml of a mixture of Carbosorb and Permafluor E (3:7 v/v). Radioactivity was quantified by LSS. Percent herbicide absorbed was expressed as (dpm in combusted tissue/(dpm in combusted tissue + dpm in leaf washes)) × 100 and the percentage translocated was expressed as: (dpm in plant tissue/dpm total in plant) × 100. Both experiments were repeated three times.

2. Materials and methods

2.1. Plant material

Seed of three itchgrass biotypes designated according to their collection site as San José, San Pedro and Yapa cani was obtained in 2002 from soybean fields in the northern area of Santa Cruz, Bolivia. San José was the sensitive reference population (control). The other two biotypes were selected because of their inadequate response to commercial applications of APP and CHD herbicides in fields with a history of ACCase-herbicide use for the past few years.

2.2. Dose–response bioassays

Greenhouse bioassays were conducted from May 2003 to October 2003 at Universidad Autonóma de Chapingo, Mexico. Plants were grown in plastic pots (five per pot) and ACCase-herbicides were sprayed at the three- to four-leaf growth stage (approximately 20 days after sowing) at several doses, using an experimental sprayer delivering 1851 ha\(^{-1}\) at 200 kPa with a Teejet 8002 flat-fan nozzle. Dose ranges (g a.i. ha\(^{-1}\)) were 0, 6, 12, 24, 48, 96, 192, 384 for haloxyfop-R-methyl (Galant 12 EC) and 0, 18, 36, 72, 144, 288, 576, 1152 for sethoxydim (Poast EC).

The treatments were replicated four times and fourteen days after spraying plants were weighed and GR\(_{50}\) values (herbicide dose required to reduce above-ground fresh weight by 50%) were calculated. Data from all experiments were fitted to a non-linear regression model, corresponding to a dose response log-logistic analysis. To estimate the parameters of the log-logistic response curve, a non-linear regression routine (procedure NLIN) was used with the SAS software (Streibig et al., 1993; Valverde et al., 2000). For each population, a resistance index (RI) was calculated as the ratio GR\(_{50}\) of the biotype of interest/GR\(_{50}\) of the reference biotype. When RI’s were higher than 2, the population was designated as resistant.

2.3. Absorption and translocation

The metabolism of \[^{14}C\]sethoxydim was examined in leaf tissue of the biotypes evaluated at the three- to four-leaf stage. \[^{14}C\]sethoxydim was diluted in commercial herbicide as in the absorption and translocation studies, to a final concentration 50 g a.i. ha\(^{-1}\), and 1.66 Bq µl\(^{-1}\) of specific activity. The labeled herbicide mixture was applied to the adaxial surface of the second leaf in four 0.5 µl droplets using a microapplicator. For the absorption studies, two plants growing in each pot were harvested at 6, 12, 48 and 96 h after herbicide application, and separated into treated leaf, upper leaf, lower leaf, and root. Unabsorbed \[^{14}C\]sethoxydim, was removed from the surface of the treated leaf by washing with 3 ml of 80% methanol. Washes were pooled and analyzed by liquid scintillation spectrophotometry (LSS). Plant tissue was dried separately at 50 °C for 72 h, and combusted in a sample oxidizer. The \(^{14}\)CO\(_2\) evolved was trapped and counted in 10 ml of a mixture of Carbosorb and Permafluor E (3:7 v/v). Radioactivity was quantified by LSS. Percent herbicide absorbed was expressed as (dpm in combusted tissue/(dpm in combusted tissue + dpm in leaf washes)) × 100 and the percentage translocated was expressed as: (dpm in plant tissue/dpm total in plant) × 100. Both experiments were repeated three times.
in a mortar with 4 ml of 80% methanol and the extract was centrifuged (20,000g/10 min, 4°C). Pellets were washed with 80% methanol until 14C was no longer extracted. The pellets were oven dried at 60°C for 48 h and combusted in the sample oxidizer. The supernatants were combined, evaporated to dryness at 40°C under a stream of N2 at 10 kPa and re-dissolved in 500 µl 80% methanol. Sethoxydym and its metabolites in the supernatant were separated by TLC on 20 x 20 cm, 250 µm silica gel plates (Merck; silica gel 60) and a chloroform/isopropanol (9:1 v/v) mobile phase. The radioactive zones were detected with a radiochromatogram scanner (Berthold LB 2821) and their chemical nature was identified by comparing with known polar conjugate metabolites. The experiment was repeated three times.

2.5 Enzyme purification and ACCase in vitro assay

ACCase isoforms were isolated using the protocol of Bradley et al. (2001) and De Prado et al. (2000) with slight modifications. Leaves (6 g fresh weight) were harvested from plants in the five to six-leaf stage and ground in liquid N 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, 0.32 mM PMSF]. The homogenate was mixed for 30 min). The supernatant was fractionated with ammonium sulfate. Material precipitating between 35% and 45% saturation was resuspended in 4.5 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 DTT] and centrifuged (17,000 g, 10 min). The clarified supernatant was applied to a Sephacryl S400 column (2.5 x 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 x 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 and ACCase activity was assayed by measuring the ATP-dependent incorporation of NaH [14CO3] into an acid stable 14C-product. The reaction product was previously shown to be [14C] malonyl-CoA (Gronwald et al., 1992). 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, and 0.5 mM DTT, 1.5 mM ATP, 5 mM MgCl2, 15 mM NaH [14C] O, (ICN, 33 µCi µmol⁻¹), 90 µl enzyme fraction and 5 mM acetyl-CoA in a final volume of 0.2 ml. Activity was assayed for 5 min at 34°C and the reaction was stopped by adding 30 µl of 4N HCl. A piece of filter paper was added to the reaction vial and the sample was dried at 40°C under a stream of air. After the sample was dried, ethanol:water (1:1, v/v, 0.5 ml) was added to the vial, followed by addition of 10 ml of scintillation cocktail (Ecolume, ICN). Radioactivity was determined by LSS. Background radioactivity, measured as acid-stable counts (dpm) in the absence of acetyl-CoA, was subtracted from each treatment. Herbicide concentrations resulting in a 50% inhibition of enzyme activity (I50 values) were determined in crude extracts for sethoxydym. The I50 values were calculated from linear plots of percent enzyme inhibition versus the logarithm of herbicide concentrations. Inhibitory effect of sethoxydym on ACCase fractions obtained by Sephacryl S400 filtration and TMAE-anion-exchange chromatography were determined as before. The experiment was repeated three times.

3. Results and discussion

3.1. Dose–response bioassays

The response, based on the fresh weight reduction of itchgrass plants, to APP (haloxyfop) and CHD (sethoxydym) herbicides and corresponding RI’s is presented in Table 1. Putative resistant biotypes (San Pedro and Yapacani) were indeed confirmed resistant to haloxyfop and cross-resistant to sethoxydym. Resistance levels of both biotypes to haloxyfop were considerably higher than those to sethoxydym. Both biotypes were collected in fields under soybean monoculture exposed to ACCase herbicides during the previous few years. The San Pedro biotype received five applications of fluazifop-p-butyl, two of butroxydym and one of haloxyfop during the eight cropping seasons before seeds were collected. The biotype

Table 1: Parameters describing the dose response of R. cochinichinensis biotypes to haloxyfop-R-methyl and sethoxydym

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Haloxyfop-R-methyl</th>
<th>Sethoxydym</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>San José</td>
<td>9.62</td>
<td>0.14</td>
</tr>
<tr>
<td>San Pedro</td>
<td>11.03</td>
<td>0.36</td>
</tr>
<tr>
<td>Yapacani</td>
<td>9.30</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*The parameters describe the logistic function: GR50 is the herbicide dose producing a response half-way between the upper limit, D, and lower limit, C. The parameter b denotes the relative slope around GR50 (Streibig et al., 1993).*
from Yapacaní was exposed to the same number of applications of fluazifop-p-butyl and butroxydim but received once an application of clethodim and none of haloxyfop. Thus selection pressure for ACCase-herbicide resistance was imposed mostly by fluazifop-p-butyl. Indeed both biotypes were also resistant to the latter herbicide as well as to clethodim (data not shown).

### 3.2. Absorption and translocation

Resistant and susceptible itchgrass biotypes absorbed [14C]sethoxydim similarly at 6, 12, 48 and 96 HAT (Fig. 1). [14C]sethoxydim absorption increased with time but without differentiation among biotypes. Most of the herbicide remained in the treated leaf and the amount of [14C]sethoxydim moving out of the treated leaf did not differ among biotypes (Fig. 2). Therefore it is unlikely that differential herbicide absorption and translocation contributes to resistance to sethoxydim in these Bolivian itchgrass biotypes.

### 3.3. Metabolism

In addition to the parent herbicide (P), three sethoxydim metabolites (M-1, M-2, and M-3) were observed in plant extracts. Resistant and susceptible biotypes did not differ in the percent [14C] recovered as sethoxydim at any time interval after application (Fig. 3). At 6 HAT, the amount of [14C] recovered as sethoxydim in the resistant biotypes San Pedro and Yapacaní was 37% and 34%, respectively, of the total [14C] absorbed, which decreased to 10% by 96 HAT in both populations. Similarly, the amount of [14C] recovered as sethoxydim in the susceptible biotype San José was 46% of the total [14C] absorbed, and this decreased to 17% by 96 HAT.

These results indicate that the rate of sethoxydim hydrolysis to sethoxydim acid is similar in the resistant and susceptible itchgrass biotypes. For example, the amount of [14C] recovered as sethoxydim acid at 48 HAT was 24% and 30% of the total [14C] absorbed in the resistant biotypes San Pedro and Yapacaní and 29% of the total [14C] absorbed in the susceptible biotype San José. Similarly, at
96 HAT, 34% and 44% of the total 14C absorbed was recovered as sethoxydim acid in the resistant and susceptible biotypes, respectively. Finally, resistant and susceptible biotypes did not differ in the percentage of 14C recovered as sethoxydim conjugates at any time interval after treatment.

The results from the metabolism experiments reveal a slightly higher rate of sethoxydim acid metabolism in the resistant itchgrass biotypes at 6 and 96 HAT, but no qualitative differences between the metabolites formed in either biotype. However these differences are relatively minor, when compared to other weed biotypes that are resistant due to enhanced metabolism of the APP and/or CHD herbicides.

3.4. ACCase in vitro assay

The ACCase from the resistant biotypes was less sensitive to sethoxydim than that of the reference biotype, San José. In the resistant biotypes, the concentration of sethoxydim that provided 50% inhibition of ACCase (I50) could not be obtained within the concentration range used in the experiment. The I50 for the susceptible-biotype ACCase was 260 μM for sethoxydim. In the absence of ACCase-inhibiting herbicides, the three biotypes had similar levels of ACCase specific activity (Fig. 4). These results suggest that an alteration of target site enzyme is likely the mechanism that confers resistance to the CHD and APP herbicides in the resistant itchgrass biotypes.

In ACCase-herbicide resistant biotypes of Lolium multiflorum resistance is conferred by a tolerant form of ACCase. ACCase activity measured in extracts from etiolated shoots of the resistant biotype was 28 fold more tolerant to diclofop than that from susceptible biotypes (Gronwald et al., 1992). Target-site based resistance is associated with a mutation of the nuclear gene encoding the ACCase I isoform (Délye et al., 2002a). In grasses two isoforms of dimeric multifunctional ACCase are present, ACCase-I and ACCase-II. Of these, ACCase-I is the predominant isoform. It is plastid localized and is highly susceptible to graminicides. In contrast, the multifunctional ACCase-II isoform represents a smaller fraction of total ACCase; it is extra plastidic and is resistant to graminicides. Based on I50 values, ACCase of resistant Setaria faberi was 5, 11 and 320-fold resistant to clethodim, fluazifop and sethoxydim and that of Digitaria sanguinalis was 6, 10 and 66-fold resistant to the respective herbicides compared to susceptible accessions. This clearly indicated that the resistance to ACCase inhibitors in these accessions resulted from an altered ACCase enzyme that confers a very high level of resistance to sethoxydim (Volenberg and Stoltenberg, 2002). Similarly, the relatively high tolerance of our R. cochinchinensis biotypes to the ACCase-inhibiting herbicides observed in the greenhouse dose-response experiments provides additional support to this proposed mechanism of resistance. The similarity in [14C]sethoxydim absorption and translocation between the resistant and susceptible itchgrass biotypes suggests that differential absorption and translocation do not contribute to graminicide-herbicide resistance. All biotypes showed the same qualitative pattern for the metabolism of [14C]sethoxydim. Quantitatively, in both resistant and susceptible biotypes sethoxydim was rapidly metabolized to less polar metabolites and remained stable during the span of the experiment. It is unlikely; therefore, that metabolism contributes to the high degree of resistance observed at the whole plant level.

Fig. 3. Percent-absorbed radioactivity found as sethoxydim (parent compound, P) and sethoxydim conjugates (M-1, M-2 and M-3) in leaf extracts of resistant (San Pedro and Yapacani) and susceptible (San José) biotypes of R. cochinchinensis. During a 96-h period after treatment with [14C]sethoxydim. Values are means of three experiments; vertical bars represent standard errors of the means.
The in vitro response of ACCase from the resistant biotypes of itchgrass was consistent with the results obtained in whole-plant bioassays (Avila et al., 2004). Resistance patterns to CHDs and APPs vary among species probably in relation to different target site mutations making it difficult to predict if a particular graminicide will remain effective against a particular resistant biotype (Marles et al. 1993; Leach et al. 1995; Tardif et al. 1996; Volenberg and Stoltenberg, 2002).

ACCase I is the predominant isoform in grass leaves representing more than 90% of total ACCase activity and is very susceptible to graminicides (Egli et al., 1993; Evenson et al., 1997). 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 nearly two orders of magnitude greater than those for wild-type ACCase I (Evenson et al., 1997). To date, resistance for four grass species, *Avena fatua* (Christoffers and Messersmith, 1999) and *S. viridis* (Zagnitko et al., 2001) from Canada, *Lolium rigidum* (Zagt et al., 2000) from USA, and *A. myosuroides* (Brown et al., 2002) from UK, 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, definite proof that graminicide resistance is target-site based awaits cloning the ACCase I gene and identifying the sequence encoding the graminicide-binding region in resistant and susceptible itchgrass biotypes. Thus, we would identify the various point mutations and the pattern of cross-resistance conferred by each. Results from such future studies could provide additional information on the topology of the graminicide-binding site and the structural determinants that regulate graminicide binding.

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**References**


Konishi, K., Shinozaka, K., Yamada, K., Sasaki, Y., 1996. Acetyl-CoA carboxylase in higher plants: most plants other than Gramineae have both the prokaryotic and the eukaryotic forms of this enzyme. Plant Cell Physiol. 37, 117–122.


