Influence of Safeners on the in vivo and in vitro Metabolism of Bentazon and Metolachlor by Grain Sorghum Shoots: a Preliminary Report*

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Metabolism of bentazon and metolachlor by excised shoots and a microsomal fraction isolated from the shoots, of 3-day-old, dark-grown, grain sorghum (Sorghum bicolor cv. Funk G 522 DR) seedlings was studied. The effects of seed treatments, on the subsequent metabolism of the herbicides, with the safeners naphthalic anhydride, oxabetrinil, and CGA 133205 were compared against surface-sterilization and Captan-treatments. Bentazon was aryl hydroxylated in both in vivo and in vitro studies with the hydroxylated derivative undergoing glycosylation only under in vivo conditions. Both shoots and microsomes isolated from shoots of safener-treated seed showed enhanced metabolism of bentazon relative to the controls. Inhibition by tetcyclacis, a potent inhibitor of plant cytochrome P-450 monoxygenases, in both the in vivo and in vitro studies, and a requirement for NADPH in the in vitro studies suggested that the formation of hydroxybentazon was mediated by a cytochrome P-450 monoxygenase. Metolachlor was metabolized to polar material and O-desmethylmetolachlor under in vivo conditions. Only the demethylated product was formed in vitro. Shoots isolated from safener-treated seed showed enhanced formation of polar compounds which were assumed to have arisen from conjugation with glutathione. Tetcyclacis did not affect the formation of the polar components. However, the formation of O-desmethylmetolachlor was depressed in the shoots excised from safener-treated seed under both in vivo and in vitro conditions. Tetcyclacis completely prevented formation of the demethylated metabolite. Hence, formation of this metabolite is considered to be P-450 mediated. The differential response obtained with the safeners, i.e., stimulation of aryl hydroxylation of bentazon and depression of metolachlor demethylation, suggests that the reactions are probably catalyzed by different cytochrome P-450 monoxygenases.

Introduction

The rate and extent of metabolism are considered to be the primary factors that determine susceptibility or tolerance of plants to most herbicides. The two main detoxication pathways in plants, as in animals, are the GSH and the microsomal cytochrome P-450 monoxygenase systems. The relative contribution of the two pathways in the metabolism of herbicides to terminal products remains to be elucidated for plants. For some herbicides, metabolism may predominantly involve one pathway with the reverse accounting for the metabolism of other herbicides. However, for some herbicides, both systems may play major metabolic roles. Herbicide safeners may confer protection by augmenting one or both systems.

In plants, bentazon undergoes aryl hydroxylation followed by glycosylation [1, 2]. Hydroxylation occurs at position 6 or 8 of the aromatic ring and the position of hydroxylation appears to be species-related. The participation of the P-450 system in the hydroxylation reaction has been documented with microsomal preparations isolated from corn [3] and sorghum shoots [4–6].

Metabolism of metolachlor, in plants, is mediated by both the GSH and monoxygenase systems. Alteration of the chloroacetyl side chain initially
involves conjugation with GSH followed by breakdown of GSH to the cysteine moiety [7]. Induction of GSTs by safener-treated grain sorghum seed and an enhanced metabolism of metolachlor have been documented [8, 9]. The ether linkage of the methoxypropyl side chain of metolachlor undergoes cleavage followed by glycosylation [7]. This demethylation reaction was shown to be mediated by a cytochrome P-450 preparation isolated from grain sorghum shoots [10].

Enhancement of P-450 monooxygenase-mediated metabolism in in vitro studies following treatment with safeners has been reported for bentazon [3] and other herbicides including some sulfonylureas [11, 12], diclofop [13], and chlorotoluron [14].

The objectives of the studies being reported here were (a) to compare the effects of selected safeners on the metabolism of metolachlor and bentazon by excised sorghum shoots and a microsomal fraction obtained from the shoots, and (b) to identify the relative contributions of the GSH and monooxygenase systems for the metabolism of the two herbicides. The safeners were applied as seed treatments and included NA, oxabetrinil, and CGA 133205. Captan- and Clorox-treated seed were included as controls.

Materials and Methods

Grain sorghum seed (Sorghum bicolor cv. Funk G 522 DR) were sown on folded sheets of germination paper and placed upright in 1000 ml beakers that contained 400 ml of 0.5 strength Hoagland’s solution. Seed were germinated in the dark at 28 °C for approximately 72 h. Prior to sowing, untreated seed were surface sterilized with a 10% solution of a commercial sodium hypochlorite solution (Clorox) for 10 min followed by washing in water for 10 min. The remainder of the seed were treated with Captan (0.2%, w/w). Safeners were added to the Captan-treated seed: NA (0.5%, w/w), oxabetrinil (approximately 0.1%, w/w), and CGA 133205 (approximately 0.04% w/w).

In vivo studies

Excised shoots (approximately 0.5 g) were incubated in 0.5 ml of a medium that contained 50 mM potassium phosphate buffer (pH 7.1), Hoagland’s solution (0.5 strength), tetcyclacis (100 μM) where indicated, and either [14C]bentazon (18 μM) or [14C]metolachlor (9 μM). After incubation for 0, 0.5, 1, 2, and 4 h, the treatment solution was decanted. The tissue was rinsed with 1 ml distilled water to remove surface-absorbed material and homogenized in 1 ml methanol. Cellular debris and precipitated protein were removed by centrifugation in a Beckman Microfuge (5 min at 10,000 × g).

In vitro studies

Microsomes were isolated from the excised shoots as detailed by Moreland et al. [10]. Dithionite-reduced, carbon monoxide difference spectra were obtained following standard procedures [15, 16] with an SLM/Aminco DW-2000 Spectrophotometer. Cytochrome P-450 and P-420 were estimated from millimolar extinction coefficients of 91 and 111, respectfully [17]. Microsomal protein was estimated spectrophotometrically by the method of Waddell [18] with crystalline bovine serum albumin as a standard.

The microsomal reaction mixture contained 0.5 mM NADPH, an NADPH-generating system (2.5 mM G 6 P, and 1.0 U G 6 P dehydrogenase), 50 mM potassium phosphate buffer (pH 7.1), tetcyclacis (100 μM) where indicated, 0.1μCi [14C]metolachlor (9 μM) or [14C]bentazon (18 μM), and freshly isolated microsomes (approximately 1.0 to 2.0 mg protein) in a total volume of 0.5 ml. Reactions were incubated at 25 °C for 45 min. The reaction was arrested with 0.5 ml acetone or methanol. Precipitated protein was removed by centrifugation in a Beckman Microfuge (5 min at 10,000 × g).

Analytical procedures

Components in the supernatants obtained from the in vivo and in vitro assays were analyzed by thin layer chromatography. For separation of bentazon and its metabolites, 250 μm reversed phase, C18 plates were developed with methanol/75 mM sodium acetate (pH 6.0), 50:50 (v/v). Separation of metolachlor and its metabolites was achieved on 250 μm silica gel plates that were developed in benzene/aceton (2:1, v/v). Following development, the plates were radiochromatographically scanned and areas under the radioactive peaks were integrated with a Bioscan System 400 Imaging Scan-
Quantification of the metabolites was based on the distribution of radioactivity in the TLC profiles and verified by scraping of the zones from the plates followed by liquid scintillation spectrometry.

All experiments were replicated at least two times with separate plantings of seed. The tissue obtained from a given planting of seed was used both for the excised tissue assays and for isolation of microsomes. Incubations with bentazon and metolachlor were conducted in parallel experiments.

Results

In vivo studies

Bentazon

Excised sorghum shoots readily metabolized bentazon. Shoots from safener-treated seed showed an enhanced capacity to metabolize bentazon. An example of the enhancement is shown in the radiographic traces of reversed phase chromatograms of shoot extracts from Captan- and oxabetrinil-treated seed that had been incubated with $[^{14}C]$bentazon for 4 h (Fig. 1A and 1C). Addition of 100 μM tetcyclacis prevented the metabolism of bentazon in shoots from both types of seed (Fig. 1B and 1D). Cochromatography with authentic standards suggested that the peak around 7 cm corresponded to bentazon. The more polar shoulder around 9 to 10 cm contained a mixture of free and glycosylated 6-hydroxybentazon. Free and conjugated 6-hydroxybentazon comigrate in the solvent system used and will be referred to as the 6-hydroxybentazon complex in this report. Small amounts of a less polar metabolite also were formed that migrated to around 4 cm. The identity of this minor metabolite has not been established. Treatment of the excised tissue with tetcyclacis essentially arrested formation of 6-hydroxybentazon and greatly reduced the amount of minor metabolite that was formed (Fig. 1B and 1D). Inhibition by tetcyclacis, which is a potent inhibitor of plant cytochrome P-450 monoxygenases [19], suggested that the metabolism of bentazon being measured was probably P-450-mediated.

Responses obtained with Clorox-treated seedlings were similar to those of the Captan-treated seed, and the responses with tissue from CGA 133205- and NA-treated seed were qualitatively similar to the results presented for the oxabetrinil treatment.

An example of the general trends that occurred over the 4 h incubation period is shown in Fig. 2 for the metabolism of $[^{14}C]$bentazon by tissue excised from oxabetrinil-treated seed. Qualitatively similar trends were obtained with shoots from all of the seed treatments, however, quantitative variations were associated with the treatments. Bentazon associated with the tissue generally increased rapidly during the first 30 to 60 min and then leveled-off or decreased slightly until the end of the
experiment at 4 h (Fig. 2, triangles). The amount of bentazon associated with the tissue always was higher in the tetcyclacis-treated tissue than in the non-tetcyclacis treated tissue. The 6-hydroxybentazon complex increased curvilinearly with time (Fig. 2, circles). The less-polar secondary metabolite (Fig. 2, squares), was formed much more slowly than 6-hydroxybentazon and the concentration leveled-off after about 2 h. Tetcyclacis greatly suppressed the formation of 6-hydroxybentazon and had a weaker effect on the secondary metabolite.

In the conduction of the in vivo experiments, both bentazon and tetcyclacis were present in the incubation mixture before the tissue was added. Hence, the small amounts of metabolites formed in the presence of tetcyclacis (Fig. 2) may have resulted from a delay in absorption and transport of tetcyclacis to the metabolic site.

The concentrations of the 6-hydroxybentazon complex and secondary metabolite extracted from the sorghum shoots, in the absence and presence of tetcyclacis, at 4 h for all of the seed treatments are compared in Table I. Formation of 6-hydroxybentazon was enhanced from about 3.5- to 5.0-fold by the three safeners (oxabetrinil, CGA 133205, and NA) relative to the Clorox control (Table I). Formation of the secondary metabolite was enhanced approximately 2-fold by the safeners relative to the Clorox control. Tetcyclacis inhibited the formation of both metabolites, but had

![Fig. 2. Uptake and metabolism of [14C]bentazon, in the absence and presence of tetcyclacis, as a function of time, by shoots excised from oxabetrinil-treated sorghum seed. Legend: open symbols and solid lines obtained in the absence of tetcyclacis; solid symbols and dashed lines obtained with tetcyclacis (100 μM) added to the incubation medium; triangles, bentazon; circles, 6-hydroxybentazon complex; squares, less polar metabolite. The data plotted represent arithmetic averages of two replications.](image)

Table I. Metabolism of bentazon and metolachlor by sorghum shoots excised from safener-treated seed after incubation for 4 h, in the presence and absence of tetcyclacis*.

<table>
<thead>
<tr>
<th>Seed treatment</th>
<th>Tetcyclacis [100 μM]</th>
<th>Bentazon</th>
<th>Metolachlor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Secondary component</td>
<td>6-Hydroxy complex</td>
<td>Polar components</td>
</tr>
<tr>
<td>Clorox</td>
<td>–</td>
<td>0.62</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.46</td>
<td>0.32</td>
</tr>
<tr>
<td>Captan</td>
<td>–</td>
<td>0.67</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.37</td>
<td>0.13</td>
</tr>
<tr>
<td>Oxabetrinil</td>
<td>–</td>
<td>1.16</td>
<td>4.38</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.52</td>
<td>0.27</td>
</tr>
<tr>
<td>CGA 133205</td>
<td>–</td>
<td>1.23</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td>Naphthalic anhydride</td>
<td>–</td>
<td>1.37</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.51</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* Data are presented as the arithmetic averages of two replications.
a slightly stronger effect on the depression of the 6-hydroxybentazon complex. The secondary metabolite cochromatographed with a trace amount of a radioactive contaminant in the [14C]bentazon sample used in this study. However, the quantity increased with time and its formation was suppressed by tetcyclacis (Fig. 1, 2 and Table I).

Metolachlor

The capacity of shoots excised from unsafened and safened sorghum seed to metabolize metolachlor also was determined. Shoots from safened seed showed an enhanced capacity to metabolize metolachlor. An example of the enhancement is shown in the radiographic traces of chromatograms of shoot extracts from Captan- and oxabetrinil-treated seed that had been incubated with [14C]metolachlor for 1 h (Fig. 3A and 3C). Cochromatography with synthetic standards indicated that the peak at about 12 cm corresponded with metolachlor and the one at 8 cm corresponded to O-desmethylmetolachlor. The peak near the origin will be referred to as the polar fraction, the components of which have not been identified. The shoots from oxabetrinil-treated seed showed a large enhancement of the polar fraction, a reduction in the amount of O-desmethylmetolachlor formed, and a large reduction in the amount of unmetabolized metolachlor (Fig. 3C versus 3A).

Tetcyclacis prevented the formation of O-desmethylmetolachlor, but did not have a strong effect on the formation of polar materials (Fig. 3B and 3D). Based on previously published reports, O-desmethylmetolachlor is formed by a cytochrome P-450 monoxygenase [10], whereas the polar fraction probably consists of one or more GSH-derived metabolites [9]. Enhancement in the formation of the polar compounds by seed treatment with safeners such as oxabetrinil is in agreement with observations reported by others [9]. The benzene/acetone solvent system used in the routine analyses did not separate constituents of the polar fraction. However, development in another solvent system such as chloroform/methanol/water (65:25:4, v/v) partially separated the fraction into 3 or 4 products (data not shown).

An example of the trends that occurred in the metabolism of metolachlor over the 4 h treatment is shown in Fig. 4 for tissue excised from oxabetrinil-treated seed. The concentration of metolachlor associated with the tissue peaked between 0.5 and 1 h (Fig. 4, triangles) and was rapidly metabolized to the polar fraction (Fig. 4, squares), both in the absence and presence of tetcyclacis. The amounts of unmetabolized metolachlor and polar compounds associated with the tissue was consistently greater when tetcyclacis was present. O-desmethylmetolachlor (Fig. 4, circles) reached a maximum
Fig. 4. Uptake and metabolism of [14C]metolachlor, in the absence and presence of tetcyclacis, as a function of time, by shoots excised from oxabetrinil-treated sorghum seed. Legend: open symbols and solid lines obtained in the absence of tetcyclacis; solid symbols and dashed lines obtained with tetcyclacis (100 μM) added to the incubation medium; triangles, metolachlor; circles, O-desmethylmetolachlor; squares, polar metabolites. The data plotted represent arithmetic averages of two replications.

concentration at around 1 h and decreased slightly in concentration with time until the end of the experiment. Tetcyclacis completely inhibited formation of this metabolite. O-desmethylmetolachlor has been reported to be glycosylated to form a terminal product [7] in plants. If this had occurred in our experiments, the conjugate would be one of the components in the polar fraction, and could account for the decrease in the amount of the metabolite that was present in the tissue after 1 h (Fig. 4).

The concentration of the polar fraction and O-desmethylmetolachlor extracted from sorghum shoots, in the absence and presence of tetcyclacis at 4 h for all seed treatments, are compared in Table I. A 2-fold enhancement of the polar fraction was evident in the Captan treatment relative to the Clorox control. However, there was between a 7.5- and 10.5-fold enhancement of the polar fraction in the safener treatments relative to the Clorox control. Tetcyclacis had only a limited, if any, effect on the magnitude of the polar fraction. The situation was reversed with respect to the amount of O-desmethylmetolachlor present at the end of 4 h. Captan and the safeners depressed the formation of this metabolite with the concentration being lowest in the CGA 133205 treatment, i.e., a 6-fold decrease relative to the Clorox control.

In vitro studies

In different extractions, the concentration of cytochrome P-450 in microsomal preparations isolated from the grain sorghum shoots ranged from around 90 to 150 pmol/mg protein. The amount of cytochrome P-420 ranged from 0 to 3% relative to the P-450 content. There was a tendency for the concentration of P-420 to reach approximately 6% in the shoots from oxabetrinil- and CGA 133205-treated seed. However, the safeners did not increase the total concentration of cytochromes measured (on a per mg protein basis).

Bentazon

Microsomes isolated from the same tissue used in the in vivo studies also metabolized bentazon. An example of responses measured for the oxidation of bentazon with microsomes is shown in Fig. 5 for an isolation from oxabetrinil-treated seed. The microsomes had been incubated with [14C]bentazon for 45 min. Metabolites formed by a complete control reaction mixture are shown in Fig. 4A. The identity of the constituents has been previously detailed herein. The major difference between the in vivo and in vitro studies is that all of the 6-hydroxybentazon is present in an unconjugated form in the in vitro studies. Considerable smearing of both the 6-hydroxybentazon and the secondary metabolite occurred. However, when the corresponding zones on the plates were scraped and components respotted, discrete peaks were obtained. Neither of the metabolites was formed when tetcyclacis was added to the reaction mixture (Fig. 5B) or in the absence of NADPH (Fig. 5C). Inhibition by tetcyclacis and a requirement for NADPH support the suggestion that the metabolism of bentazon that was measured, was P-450 mediated.
Effects of the different seed treatments on the formation of 6-hydroxybentazon by isolated microsomes are compared in Table II. Results are expressed on both a per mg protein/h and a per nmol P-450/h basis. As in the in vivo studies, microsomes isolated from safener-treated tissue also reflected an enhanced ability to metabolize bentazon. The safeners enhanced metabolism by a factor of around 2.5 when compared on a per mg protein basis and about 1.6 when expressed on a nmol P-450 basis relative to the Clorox control. Microsomes from the Captan treatment were slightly less active than those from the Clorox control.

Metolachlor

Microsomes isolated from the tissue used in the in vivo studies also metabolized metolachlor. An example of the response measured with microsomes is shown in Fig. 6 for an isolation from NA-treated seed. As shown in Fig. 6A, only O-desmethylmetolachlor was formed in the complete control reaction mixture. This held true for all of the treatments. Formation of the metabolite was prevented by tetcyclacis (Fig. 6B) and required NADPH (Fig. 6C). These observations implicate a cytochrome P-450 monooxygenase in the formation of the demethylated metabolite.

Effects of the different seed treatments on the formation of O-desmethylmetolachlor by isolated microsomes are compared in Table II. The amount of metabolite formed, expressed on a protein basis, was greatest in the microsomes from the Clorox, Captan, and NA-treated seed and was depressed in the oxabetrinil and CGA 133205-

<table>
<thead>
<tr>
<th>Seed treatment</th>
<th>6-Hydroxybentazon</th>
<th>O-Desmethylmetolachlor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg prot/h</td>
<td>nmol/nmol P-450/h</td>
</tr>
<tr>
<td>Clorox</td>
<td>0.39</td>
<td>4.12</td>
</tr>
<tr>
<td>Captan</td>
<td>0.36</td>
<td>3.20</td>
</tr>
<tr>
<td>Oxabetrinil</td>
<td>0.94</td>
<td>8.22</td>
</tr>
<tr>
<td>CGA 133205</td>
<td>1.00</td>
<td>6.61</td>
</tr>
<tr>
<td>Naphthalic anhydride</td>
<td>1.01</td>
<td>7.31</td>
</tr>
</tbody>
</table>

* Data are presented as the arithmetic averages of two replications.
treated seed. In the P-450 comparison, microsomes from control seedlings had the highest activity and microsomes from oxabetrinil- and CGA 133205-treated seed had the lowest activity. Microsomes from the NA treatment were intermediate in activity. Suppression of the formation of O-desmethylmetolachlor by safener treatments was reported previously herein in the in vivo studies.

Discussion

Safeners applied to sorghum seed enhanced the activity of the GSH system, but depressed the activity of the monooxygenase system for the metabolism of metolachlor, under both in vivo and in vitro conditions. The depression of the in vivo system could be explained by competition between the two pathways for the same substrate (metolachlor). However, this explanation would not hold for the in vitro experiments where only one degradative pathway was operative.

The metabolism of bentazon, at least under the experimental conditions, seemed to be entirely monooxygenase dependent. Safeners enhanced the monooxygenase activity under both in vivo and in vitro conditions. Results obtained to date suggest that the side-chain O-demethylation of metolachlor and the aryl hydroxylation of bentazon may be catalyzed by different forms of cytochrome P-450. The isozyme involved in the aryl hydroxylation of bentazon was induced by safeners, whereas the isozyme associated with the demethylation of metolachlor was not induced, but seemed to be suppressed.

Not only safeners, as documented previously, but other xenobiotics readily induce components of the plant monooxygenase system. Induction of different substrate-specific isozymes has been measured with clofibrate [20, 21]; phenobarbital [13, 21, 22]; 2,4-dichlorophenoxyacetic acid [20, 23]; and other herbicides [22].

In experiments reported herein, safener treatments did not appear to increase the size of the bulk cytochrome P-450 pool as measured by difference spectrophotometry. Other investigators also have not detected an increased pool size following induction by safeners and other xenobiotics [3, 20], whereas some have observed increases [11, 13, 14, 21, 23]. The bulk cytochrome P-450 probably consists of a number of catalytically active isoforms which remain to be precisely identified. Hence, the induction of a specific highly active isoform of P-450 may not be detectable within the bulk P-450 pool. The suggestion also has been made that regulatory mechanisms may offset the increase of one form of P-450 by a decrease of another so that the P-450 pool is maintained at a constant level [20].

Acknowledgements


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