Physiological Responses of *Cyperus serotinus* Rotb.
to Imazosulfuron in Combination with Valine,
Isoleucine and Leucine

Yasushi Tanaka* and Harutoshi Yoshikawa*

**Abstract:** Various physiological responses were studied in *Cyperus serotinus* Rotb. following treatment with imazosulfuron. RNA synthesis measured by [3H] uridine incorporation and DNA synthesis measured by [3H] thymidine incorporation were inhibited to 82% and 51%, respectively, 4 hr after treatment of 10 ppb of imazosulfuron, while protein synthesis measured by [3H] leucine incorporation was not inhibited. Inhibition of DNA synthesis began within 1 hr after treatment and reached 28% of the zero time value of radioactivity after 6 hr. The amount of reversal from reduction in plant height induced by 3 ppb or more of imazosulfuron increased further when valine, isoleucine and leucine were added at higher concentrations. Three amino acids at 100 ppm or above alleviated DNA synthesis inhibited by 10 ppb of imazosulfuron 4 hr after treatment more than the control. Supplement of amino acids at 300 ppm greatly alleviated the inhibition of DNA synthesis within 2 hr and did the same to the control to almost the same degree 6 hr following pretreatment of imazosulfuron for 24 hr. The level of the soluble proteins decreased to 47% of the control 11 days after treatment with a concentration of 100 ppb of imazosulfuron, while the free amino acid level increased to 354% of the control. We suggest that starvation of valine, isoleucine and leucine induced by imazosulfuron cause the inhibition of DNA synthesis in a short period of treatment and lead to protein turnover and increase in the level of amino acids over a longer period.

**Key Words:** imazosulfuron, DNA synthesis, soluble protein, free amino acid, branched-chain amino acid

**Introduction**

A sulfonylurea herbicide, imazosulfuron, kills annual and perennial broadleaf and sedge weeds, and has a strong and broad herbicidal activity on paddy field weeds, while its safety to rice plants was previously reported17,18,19). This herbicide inhibited the growth of plant roots and shoots with further symptoms slowly developing after treatment, leading to death of the plant. The inhibitory growth effect in rice plants induced by imazosulfuron was blocked or reversed by adding branched-chain amino acids, valine, isoleucine and leucine16,20). Imazosulfuron inhibited acetolactate synthase (ALS), a key enzyme in the biosynthesis of valine, isoleucine and leucine17,20) in plants as did other sulfonylurea4) and imidazolinone herbicides15).

Branched-chain amino acids reversed the inhibition of growth and cell division induced by these herbicides5,9). Although chlorsulfuron and imazapyr did not affect protein synthesis,
they were associated with a decrease in DNA and RNA synthesis\(^4,9,14\). Rost\(^9\) showed that sulfonylureas blocked the cell cycle of pea root meristem cell in G1 and G2 phase and hypothesized that G2 was the primary block and G1 the secondary block. Other studies have also indicated that these chemicals indirectly affect cell division. Ray\(^5\) demonstrated that sulfonylureas did not directly inhibit DNA synthesis by inhibiting DNA polymerase. Likewise, DNA synthesis in isolated nuclei was unaffected by herbicide treatments and supplementing sulfonylurea-treated tissue with DNA precursors, such as deoxyribonucleosides or nucleotides, did not alleviate cell division inhibition in corn roots\(^5\).

The general hypothesis for the mode of action was that these herbicides work by depleting the branched-chain amino acid pool size and thereby perhaps causing the depletion of cell-cycle-specific proteins or nucleic acids\(^9,14\).

Although the biochemical site of these ALS inhibitors has been identified, the connection among inhibition of ALS, the rapid cessation of plant cell division, changes in amino acid profiles and general growth inhibition remains an area of active research.

The objectives of this work were to evaluate the effect of branched-chain amino acids on the growth inhibition induced by imazosulfuron and the inhibition of this herbicide on soluble protein levels, free amino acid levels and other metabolic responses using *Cyperus serotinus* Rottb.

**Materials and Methods**

**Plant growth measurements**

Tubers of *Cyperus serotinus* Rottb. were placed on a chemical cloth saturated with water in a stainless steel case and grown in the dark at 28°C for 3 days. Sprouted tubers were transferred to a 2000-ml plastic case containing 1000 ml of Kimura B nutrient solution at pH 5.5, and grown in a controlled growth chamber at 28°C with a 16-hr photoperiod and a 20,000-lux illuminance for 4 days.

*C. serotinus* grown as described above was placed in a 500-ml plastic beaker containing 250 ml of Kimura B nutrient solution. Imazosulfuron was added to the nutrient solution to give various final concentrations with or without valine, isoleucine and leucine. Plant height was measured after 14 days, and the experiment was repeated three times.

**Protein, RNA and DNA synthesis**

Measurements of the effects of imazosulfuron on protein, RNA and DNA synthesis were done by determining the incorporation of tritium \(^3\)H\)-labeled precursors into the appropriate metabolic product. The radiolabeled material consisted of \[^{3}\text{H}\]leucine (sp. act. 1.48–2.22 TBq/mmol, 37 kBq/ml) for protein synthesis, \[^{3}\text{H}\]uridine (sp. act. 1.29–1.85 TBq/mmol, 37 kBq/ml) for RNA synthesis and \[^{3}\text{H}\]thymidine (sp. act. 740 GBq/mmol, 37 kBq/ml) for DNA synthesis. All measurements were done on 2-cm root tips excised from *C. serotinus*. The plants grown as described above were treated with imazosulfuron contained in the nutrient solution. At the indicated time, root tips were excised just before they were used to measure the incorporation of the radiolabeled precursors into their respective metabolic products. The incubation medium contained 10 mM potassium phosphate buffer, pH 6, 1% sucrose, 2 μg streptomycin sulfate and 37 kBq of radiolabeled precursor per 5 ml. The roots were incubated for 1 hr at 28°C with constant shaking. The incorporation of radiolabeled leucine, uridine and thymidine into protein, RNA and DNA, respectively, was measured by the procedures of Rost and
The root tips were washed three times with cold, unlabeled incubation medium and then ground in 5 ml of cold (4°C) 80% ethanol. This extract was filtered through a GF/C grass fiber. The filter was washed successively with 15 ml each of 80% ethanol, 5% trichloroacetic acid, ethanol: diethyl ether (1:1, v:v) and diethyl ether to measure its incorporation in TCA-precipitable material. The filter was dried at room temperature and then placed in a scintillation vial with 10 ml of a scintillation cocktail so that the amount of radiolabeled material on the filter could be determined. The amount of radiolabeled precursor that was absorbed by the excised root tips. The results were expressed as the mean of 3 replications.

**Amino acid studies**

*C. serotinus* grown as described above was placed in the nutrient solution containing 10 ppb of imazosulfuron simultaneously with 300 ppm each of valine, isoleucine and leucine. Roots were excised at the terminal 2-cm of root tips 4 hr after treatment. DNA synthesis was measured as described above.

In time course studies of the effects of these amino acids, *C. serotinus* grown as described was placed in nutrient solution containing 10 ppb of imazosulfuron for 24 hr and retreated with either 10 ppb imazosulfuron or 10 ppb imazosulfuron plus 300 ppm each of valine, isoleucine and leucine. At the indicated time, the roots were excised and then DNA synthesis was measured as described above. The results were expressed as the mean of 3 replications.

**Soluble protein and amino acid levels**

*C. serotinus* grown as above was placed in nutrient solution containing 3, 10, 30 or 100 ppb of imazosulfuron and then harvested 1, 4, 8 or 11 days after treatment. Roots were excised at the terminal 2-cm of root tips. The level of soluble proteins was determined by grinding 100 mg of root tips in 5 ml of 10 mM potassium phosphate buffer (pH 6.0). The extract was centrifuged at 20,000g for 15 min and the content of soluble protein in the supernatant was determined by the method of Bradford.

Total free amino acid content was determined by grinding 100 mg of root tips in 5 ml of 5% trichloroacetic acid, centrifuging the extract at 20,000g for 15 min and measuring the content of the amino acids in the supernatant fraction by ninhydrin reaction. These experiments were replicated three times.

**Results**

**Effects of valine, isoleucine and leucine on inhibitory action of imazosulfuron**

Elongation of plant height of *C. serotinus* was significantly reduced by treatment with...
a concentration of 1 ppb or more of imazosulfuron and with 10 ppb was reduced to 33% of control (Fig. 1). The concentration causing 50% reduction in elongation of plant height was 3.4 ppb.

The reduction in the elongation of the plant height was largely alleviated by adding valine, isoleucine and leucine to the nutrient solution containing the herbicide. The amount of the reversal from the reduction in plant height induced by 3 ppb or more of imazosulfuron increased further but that induced by 1 ppb of the herbicide slightly decreased when amino acids were simultaneously added at higher concentrations.

The elongation of plant height was slightly reduced by treatment with 300 ppm of each of the amino acids, however, this was not significant. Similar reduction was also observed when 300 ppm of amino acids was added to the solution containing 0.3 ppb or less of imazosulfuron. The elongation alleviated by the treatment of 300 ppm of each of the amino acids simultaneously with the herbicide did not exceed that by the treatment of this amount of each amino acid alone.

Effect of imazosulfuron on protein, RNA and DNA synthesis

Uptake of [³H] thymidine, [³H] uridine and [³H] leucine was not inhibited 4 hr after treatment with 10 or 100 ppb of imazosulfuron (Table 1). Protein synthesis measured by leucine incorporation was also not inhibited by 10 or 100 ppb of the herbicide after 4 hr, while RNA and DNA synthesis measured by uridine and thymidine incorporation were significantly inhibited compared with the control. One way to separate the effect of imazosulfuron on uptake of a radiolabeled precursor from the effect on the incorporation of the precursor into its metabolic product is to compare the ratio of the amount of radiolabeled material incorporated into a metabolic product to the amount of radiolabeled material absorbed by the tissue. Imazosulfuron significantly reduced the proportion of thymidine and uridine incorporated into DNA and RNA, respectively, but had no significant effect on the amount of leucine incorporated into the protein. Treatment with 10 and 100 ppb of imazosulfuron reduced RNA synthesis measured by [³H] uridine incorporation to 82% and 77% of the

<table>
<thead>
<tr>
<th>Treatment [ppb]</th>
<th>Uptake [Bq]</th>
<th>Incorporation [Bq]</th>
<th>Ratio**</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0</td>
<td>1710</td>
<td>252</td>
<td>0.15</td>
</tr>
<tr>
<td>[³H]Thy</td>
<td>10</td>
<td>1988</td>
<td>152a</td>
<td>0.08a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1885</td>
<td>145a</td>
<td>0.08a</td>
</tr>
<tr>
<td>RNA</td>
<td>0</td>
<td>4988</td>
<td>599</td>
<td>0.13</td>
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<tr>
<td>[³H]Uri</td>
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<td>4811</td>
<td>494a</td>
<td>0.1 a</td>
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<td></td>
<td>100</td>
<td>4434</td>
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<td>Protein</td>
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<td>18068</td>
<td>8735</td>
<td>0.48</td>
</tr>
<tr>
<td>[³H]Leu</td>
<td>10</td>
<td>16495</td>
<td>8130</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>19402</td>
<td>8735</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* : Bq/100 mg fresh weight of root tips
** : Incorporation/Uptake
a : Significantly different from the control at P=0.05
control, respectively, and DNA synthesis measured by $[^3H]$thymidine incorporation to 51 and 52% after 4 hr.

Treatment with 10 ppb of imazosulfuron significantly reduced the amount of thymidine incorporated into DNA to 65% of the zero time value of radioactivity 1 hr after treatment and to 28% after 6 hr. Imazosulfuron also reduced the proportion of thymidine uptake with increasing length of imazosulfuron treatment and reduced that of thymidine incorporation into DNA to 63% after 6 hr (Fig. 2).

**Effects of valine, isoleucine and leucine on inhibitory action of imazosulfuron on DNA synthesis**

All three amino acids increasingly alleviated DNA synthesis inhibited by 10 ppb of imazosulfuron 4 hr after treatment with higher concentration of amino acids (Table 2). An exogenous supply of these amino acids at a concentration of 100 ppm or above reversed the amount of thymidine incorporation into DNA synthesis more than that of the control.

The recovery of thymidine incorporation rates began within 2 hr after addition of the three amino acids to roots that had been pretreated for 24 hr with imazosulfuron, and reached almost the same degree as the control within 6 hr (Fig. 3).

**Effect of imazosulfuron on soluble protein and free amino acid levels**

The level of soluble protein in root tips treated with 3 ppb and 10 ppb of imazosulfuron decreased to respectively 85% and 79% of that of control 8 days after treatment, but recovered to 94% and 90% during the following 11 days, while those treated with the herbicide at 30 ppb and 100 ppb continued to decrease for 11 days after the treatment (Fig. 4). The level treated with 100 ppb of the herbicide reached 47% of the control in the following 11 days.

The level of free amino acids measured in root tips treated with imazosulfuron increased with higher concentration of the herbicide (Fig. 5). The level treated with 3

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**Figure 2.** Effect of imazosulfuron (10 ppb) on $[^3H]$thymidine incorporation into DNA in 7-day-old *Cyperus serotinus* root tips. The zero-time values are 2.81 kBq/100 mg fresh weight of root tips for uptake and 252 Bq/100 mg fresh weight of root tips for incorporation into DNA.

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**Table 2.** Effects of valine, isoleucine and leucine on the inhibitory action of imazosulfuron (10 ppb) on DNA synthesis in 7-day-old *Cyperus serotinus* root tips.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (ppm)</th>
<th>$[^3H]$Thymidine incorporation (Bq*)</th>
<th>(% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>230c</td>
<td>55</td>
</tr>
<tr>
<td>Val+Ile+Leu</td>
<td>30</td>
<td>127e</td>
<td>85</td>
</tr>
<tr>
<td>Val+Ile+Leu</td>
<td>100</td>
<td>195d</td>
<td>107</td>
</tr>
<tr>
<td>Val+Ile+Leu</td>
<td>300</td>
<td>245b</td>
<td>114</td>
</tr>
</tbody>
</table>

*: Numbers followed by the same letter are not significantly different at the 0.05 level by Tukey's Multiple Range Test.

Bq : Bq/100 mg fresh weight of root tips
ppb, 10 ppb and 30 ppb of the herbicide was 175%, 225% and 343% of the control 4, 4 and 8 days after treatment, respectively, and thereafter slightly decreased, while amino acids treated with 100 ppb of the herbicide increased to 354% of the control in the following 11 days without subsequent decrease.

**Discussion**

The branched-chain amino acids, valine, isoleucine and leucine, alleviated growth inhibition in *C. serotinus*, which is sensitive to imazosulfuron, as their concentrations and treatment duration increased (Fig. 1). These amino acids also reversed growth inhibition in *Oryza sativa* which is tolerant to the herbicide. This result and information indicate that the inhibitory action of imazosulfuron is alleviated by the addition of these amino acids.
acids irrespective of the sensitivity of the plant to the herbicide. Imazosulfuron inhibited ALS in both the above plant. We also suggest that inhibition of ALS by imazosulfuron caused the depletion of the branched-chain amino acids, valine, isoleucine and leucine.

Inhibition of DNA synthesis appeared within a few hours after a treatment with imazosulfuron and then was reversed within a few hours by adding branched-chain amino acids (Table 1, Figs. 2, 3). Moreover, a concentration of 10 ppb (approximately 24 nM) of imazosulfuron, which is nearly identical to I₅₀ for ALS, suppressed thymidine incorporation to 51%. These results suggest that the primary action of imazosulfuron may involve DNA synthesis, although the concentration of 100 ppb did not increase the inhibition of DNA synthesis. The slope of the inhibition curve (Fig. 2) during 4 hr and 6 hr after treatment was more gradual than that observed during the first 2 hr (Fig. 2), and this was partly due to the uptake inhibition (Fig. 2). The action of chlorsulfuron on plant DNA synthesis appeared to be indirect, which has been suggested to be mediated by a lack of induction of ribonucleoside diphosphate reductase following a depletion of the branched-chain amino acid pool. We therefore suggest that imazosulfuron inhibits DNA synthesis indirectly by lowering valine, isoleucine and leucine levels leading to reduced DNA precursor synthesis as do other ALS inhibitors.

Some reports have showed inhibition of RNA synthesis by chlorsulfuron in both isolated and intact plants. In this study on C. serotinus roots, however, a slight inhibition in RNA synthesis was observed (Table 1). This effect may also be due to amino acid starvation induced by imazosulfuron treatment.

The starvation for branched-chain amino acids as a result of chlorsulfuron treatment was apparently a more specific type of metabolism effect on cell progression than was the injury caused carbohydrate starvation. However, sucrose starvation in pea roots shut down the macromolecular synthesis necessary for cycle progression. Inhibition of ALS also caused a rapid decrease in the translocation of photosynthate to the growing points of the plant. Therefore, starvation of carbon induced by imazosulfuron as a second action may be partially related to the inhibition of DNA synthesis.

There was no detectable decrease in the rate of radiolabeled leucine incorporated into the protein by briefer imazosulfuron application (Table 1). The level of soluble protein in the root tips decreased with time and concentration of imazosulfuron treatment (Fig. 4), while increasing free amino acid levels were measured with higher concentration of the herbicide (Fig. 5). Royuela et al. measured the accumulation of free amino acids and the increase in relative proportion of some of them in wheat and maize with longer treatments by chlorsulfuron. Rhodes et al. showed that the increase in amino acids in Lemna minor following chlorsulfuron treatment was due to protein hydrolysis. A high correlation between the pool sizes of valine and leucine and the amount of growth inhibition caused by imazaquin treatment suggested that growth inhibition was the result of depletion of these two amino acids. Therefore, depletion of valine, isoleucine and leucine caused by imazosulfuron treatment could lead to protein turnover and increase the level of amino acids after a longer period of treatment.

By increasing the rate of protein turnover, the pool sizes of the limiting amino acids could be maintained at a critical level without new synthesis of those amino acids, while...
the levels of other amino acids would increase. It is possible that although imazosulfuron decreases the pool sizes of valine, isoleucine and leucine, there are still enough reverses available to the plant to support protein synthesis for a rather extended period of time. This could partially explain why imazosulfuron does not kill plants quickly as do other ALS inhibitors.

We suggest that starvation of valine, isoleucine and leucine induced by imazosulfuron cause the inhibition of DNA synthesis in a short period of treatment, and lead to protein turnover and an increase in the level of amino acids in a longer period.

References


21) Van't Hof, J. 1968. Control of cell progression through the mitotic cycle by carbohydrate provision. I. Regulation of cell division in excised...
イマソルフロンによるミズガヤツリの生理的変化およびその変化に対するバリン、イソロイシンおよびロイシンの影響

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摘 要

イマソルフロンが卓効を示すミズガヤツリを供試植物として用い、イマソルフロン処理後の植物の生理的変化およびその変化が3種の分枝酸アミノ酸、バリン、イソロイシンおよびロイシンの添加によりどのように影響されるか検討した。

10ppbのイマソルフロンの4時間処理で、[3H]ウリシのRNA分画への取り込みおよび[3H]チミジンのDNA分画への取り込みはそれぞれ無処理の82％および51％となった（Table 1）。一方、[3H]ロイシンのタンパク質分画への取り込みは阻害されなかった（Table 1）。DNA生合成の減少はイマソルフロン処理後1時間以内に始まり、処理5時間後で処理0時間後の28％になった（Fig. 2）。

3ppb以上のイマソルフロン処理により抑制されたミズガヤツリの草丈では、添加された分枝酸アミノ酸、バリン、イソロイシンおよびロイシンの濃度の高まりとともに回復程度は大きくなった（Fig. 1）。10ppbのイマソルフロンにより阻害されたDNA生合成は、100ppm以上のこれらアミノ酸の添加により、無処理以上に回復した（Table 2）。また、イマソルフロンの24時間の処理により阻害されたDNA生合成は、これらアミノ酸（300ppm）の添加後2時間以内に急激に回復し、添加6時間後にはほぼ無処理並に回復した（Fig. 3）。

100ppbのイマソルフロン処理11日後の可溶性タンパク質は無処理の47％に減少した（Fig. 4）。一方、遊離アミノ酸量は354％に増大した（Fig. 5）。

以上より、イマソルフロンはミズガヤツリにおいて、バリン、イソロイシンおよびロイシンの生合成系を阻害することにより、短時間の処理ではDNA生合成を阻害し、長時間の処理ではタンパク質の代謝回転およびアミノ酸の増加を2次的に引き起こすと考えられた。

キーワード：イマソルフロン、DNA生合成、可溶性タンパク質、遊離アミノ酸、分枝酸アミノ酸

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