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Summary of Imazapic and Imazapyr Metabolism in GM and non-GM Crops

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Imazapyr

Plant metabolism studies are available for imazapyr in Bermuda grass, clover and imidazolione-resistant corn.

Bermuda grass. Radiolabeled imazapyr was applied to established Bermuda grass (variety, Bermuda Hulled), 69 days after seeding. The predominant residue, accounting for 78 to 97% TRR, was the parent compound, imazapyr. Metabolites CL 240000, a carboxylic methyl ester derivative and CL 247087, a cyclised product, each accounted for ≤ 10%TRR, while CL 9140, a pyridine dicarboxylic acid compound was present at a maximum of 12% TRR. The study demonstrated that imazapyr is metabolized slowly in Bermuda grass and the major residue in the foliage is parent imazapyr.

Clover. Radiolabeled imazapyr was applied to established clover. As seen in the Bermuda grass, imazapyr was the major residue in clover plants, accounting for 68 to 99% TRR. Metabolites CL 9140, CL 240000 and CL 247087 each accounted for insignificant proportions of the residue below 10% TRR. Metabolites CL 240000 (carboxymethyl ester of AC 243,997) and CL 247,087 (cyclized product of AC 243997) together, CL 252,974 (a dicarbamoyl substituted nicotinic acid), and CL 9140 (2,3-pyridinedicarboxylic acid) accounted for <0.1 to 18.4% (<0.01 to 9.05 ppm) of TRR, respectively.

Imidazolinone-Resistant Corn. Radiolabeled imazapyr was applied once to maize plants. As seen in the non-resistant grass and clover, parent imazapyr was the only major residue found in maize grains or in plants of different growth stages. Any metabolite observed was only present at insignificant concentrations in samples taken after the day of application, i.e. <0.01 mg/kg in grains or <0.05 mg/kg in whole plants. Additionally, extraction of maize oil with hexane showed that residues of imazapyr did not accumulate in that lipophilic matrix.

Summary of imazapyr metabolism in plant. Based on the identified minor metabolites, it appears that carboxylic acid and the imidazolinyl ring are the sites for metabolic transformation of imazapyr in plants. The carboxylic acid is subject to esterification, i.e. methylation to form the carboxymethyl ester (CL 240000) and/or internal cyclisation with the imidazolinyl ring yielding CL 247087. Further hydrolysis and oxidation lead to 2,3-pyridinecarboxylic acid (CL 9140).

Table 1. Summary of %Total Radioactive Residues in Plants after Treatment with Radiolabeled Imazapyr

	Bermuda Grass		Clover		Imidazolinone-Resistant Corn			
	10 DAT	21 DAT	10 DAT	21 DAT	Green Plant 0 DAT	Green Plant 14 DAT	Forage 30 DAT	Grain 114 DAT
Imazapyr	90.8	80.0	86.1	67.5	80.8	53.4-55.0	55.0	40.5-68.5
CL 240000 + CL 247087	0.2	3.0	7.9	18.4	-	-	-	-
CL 9140	4.1	12.8	0.5	2.4	<0.1	0.7-2.5	1.1	0.4-1.9
CL 252974	-	-	0.2	3.6	1.1	2.3-2.9	3.0	2.9-3.3
CL 252663	-	-	-	-	3.2	1.7-2.7	2.5	5.0-7.2
Unkowns	ND-1.5	ND-1.6	0.1-3.5	0.3-3.3	<0.1-2.7	0.2-8.2	0.5-6.3	0.0-6.9
PES	0.06	1.5	0.17	0.6	3.7	18.1-21.4	14.2-15.7	10.3-18.5

An overall proposed metabolic pathway for imazapyr in plants (gm and non-gm) is summarized below:

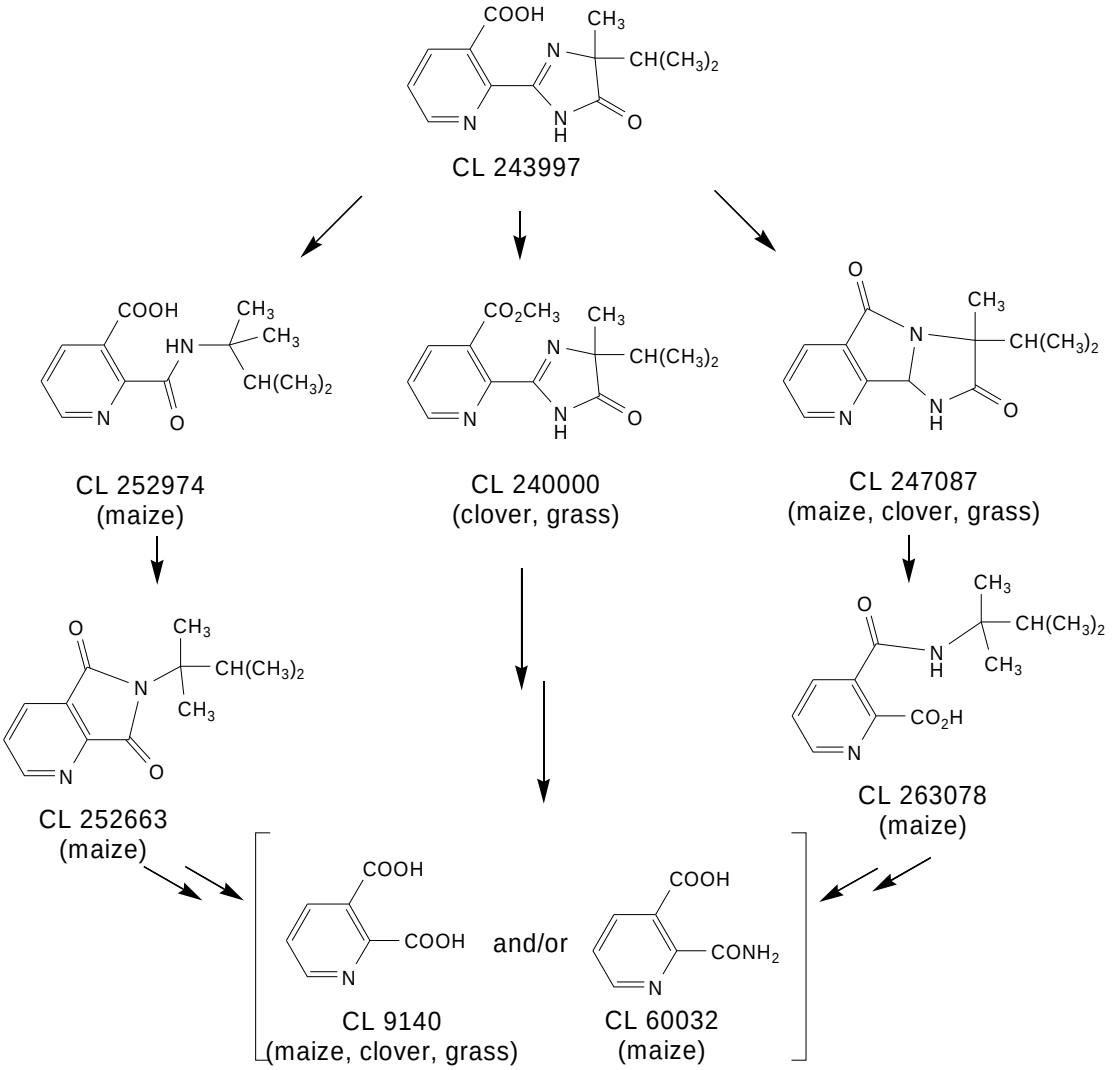

Figure 1. Proposed Metabolic Pathway of Imazapyr in Plants

Imazapic

Plant metabolism studies are available for imazapic in Bermuda grass, peanuts and sugarcane.

Bermuda grass. Bermuda grass plants were treated with radiolabeled imazapic. Parent imazapic and the 5-hydroxy metabolite M715H001 (CL 263,284, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-hydroxymethyl-3-pyridinecaroxylic acid), and its glucose conjugate M715H002 (CL 189,215) were identified as major components of the total residue at levels of 0.02-0.08 mg/kg in harvest samples. In both forage and hay, a large number of low level components comprised the remaining radio-components, all present at <0.01 mg/kg or <10% TRR.

Peanuts. Radiolabeled imazapic was applied to peanut plants at 30 days post-emergence. Imazapic was extensively metabolized. The hydroxy metabolite M715H001 (CL 263,284) and its glucose conjugate M715H002 (CL 189,215) were identified as major components of the total residue. Minor unidentified but characterized components in immature and harvest samples were all <10% TRR.

Sugarcane. Radiolabeled imazapic was applied to sugar cane (*Saccharum officinarium*). Parent imazapic and the hydroxy metabolite M715H001 (CL 263,284) were identified as major components of the total residue. The glucoside metabolite M715H002 (CL 189,215) was present in leaves, but at levels not exceeding 10% TRR and was not found in sugar cane stalks. Concentrations of other individual components (unidentified but characterized) in all samples were $\leq$ 0.0015 mg/kg ($\leq$ 8.5% TRR).

Soybean. A metabolism study with imazapic was not performed in soybeans due to the low tolerance of non-modified soybeans to imazapic. However the metabolism in soybeans of the structural analogue, imazethapyr, is known. Imazethapyr differs from imazapic in replacement of the methyl group with an ethyl group at the 5-position of the nicotinic acid ring. The results of the metabolism of imazethapyr in soybeans are included to confirm the metabolic pathway is consistent with that found for imazapic in peanuts, sugar cane and grass.

Radiolabeled imazethapyr was applied by either pre-plant incorporation in the soil or by post-emergence spray at the fourth-trifoliate stage. Due to the overall low residue levels and the low amount of residue extracted from seed for both pre-plant and post-emergent treatment (0.006-0.007 mg/kg), no characterization of the ^{14}C-residues in seed was done. Imazethapyr was extensively metabolized in green plant and straw samples for both pre-plant or post-emergence treatment. After post-emergent treatment, parent imazethapyr was present in green plants at levels of 1.4 – 15% TRR (0.009 - 0.785 mg/kg) and was not found in straw. The hydroxyethyl metabolite CL 288,511 was present at 4.6% TRR (0.239 mg/kg) to 8.0% TRR (0.050 mg/kg) in green plant and 42.2% TRR (0.101 mg/kg) in straw. The glucoside of the hydroxyethyl metabolite (CL 182,704) was present at 23.5% TRR (1.222 mg/kg) to 36.4% TRR (0.225 mg/kg) in green plant and was not seen in straw. A total of 26% TRR to 32% TRR was characterized as seven to ten minor components. After pre-plant treatment, parent Imazethapyr was present in green plants at levels of 0.2 – 3.4% TRR (0.001 - 0.009 mg/kg) and was not found in straw. The hydroxyethyl metabolite CL 288,511 was present at 22.3% TRR (0.060 mg/kg) to 13.4% TRR (0.044 mg/kg) in green plant and 37.4% TRR (0.075 mg/kg) in straw. The glucoside (CL 182,704) was present at 34.5% TRR (0.093 mg/kg) to 51.5% TRR (0.170 mg/kg) in green plant and was not seen in straw. Seven to ten minor components comprised 13.1 % TRR to 32.6% TRR.

Summary of imazapic metabolism in plant. The metabolic pathway of imazapic is similar in a range of different crops grass, sugarcane and peanuts (a legume), and is comparable to the metabolic pathway of a structural analogue, imazethapyr, in soybean. The methyl group at position 5 of the pyridine ring, or in the case of imazethapyr the 5-ethyl group, is hydroxylated, and the hydroxyl group is conjugated to form a glucoside.

Table 2. Summary of %Total Radioactive Residues in Plants after Treatment with Radiolabeled Imazapic

	Bermuda Grass			Peanuts				
	0 DAT	15 DAT	49 DAT	Green Plant 0 DAT	Green Plant 31 DAT	Hay 131 DAT	Hull 131 DAT	Nutmeat 131 DAT
Imazapic	89.3	5.9	2.6	76	2	3	2	1
CL 189215	1.0	3.9	9.2	2	32	16	36	35
CL 263284	0.7	30.2	20.5	1	12	28	28	8
Unknowns	0.4-1.5	1.6-6.4	2.3-7.1	<1	6	7	2	1

	Sugarcane				
	Forage (leaves + stalk) 63 DAT	Forage (leaves + stalk) 96 DAT	Forage (leaves + stalk) 151 DAT	Leaves 263 DAT	Stalk 263 DAT
Imazapic	16.4	31.4	18.0	5.7	11.2
CL 189215	-	-	5.1	2.7	-
CL 263284	38.7	37.6	42.5	26.3	10.1
Unknowns	0.7-6.3	1.5-3.6	0.4-4.6	1.2-8.5	1.6-8.4

An overall proposed metabolic pathway for imazapic in plants (gm and non-gm) is summarized below:

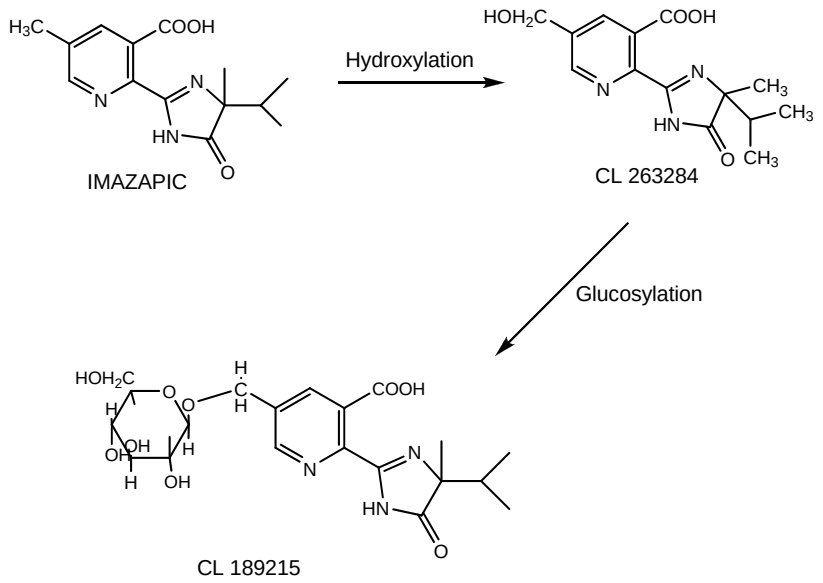

Figure 2. Proposed Metabolic Pathway of Imazapic in Plants

Impact of Modified AHAS Protein on the Metabolism of Imazapyr and Imazapic in Plants

Detailed molecular characterization of the genetically modified Cultivance soybean, CV127, an understanding of the biochemistry of the genetic modification in Cultivance soybean and the similarity of compositional analysis of the genetically modified soybean to a range of non-modified soybean cultivars support the thesis that the presence of a modified AHAS protein does not affect the metabolism of imidazolinone compounds, such as imazapyr and imazapic, in Cultivance soybean compared to the

metabolism of these compounds in a range of soybean cultivars. In addition, comparison of the rate and route of metabolism performed with ^{14}C-labeled material in a commercially available rice variety and in AHAS mutant rice verified no difference in the metabolism.

The mechanism of action of imidazolinone herbicides on weeds and non-tolerant plants is by inhibition of the AHAS enzyme and branched-chain amino acid biosynthesis. The AHAS enzyme is ubiquitous in plants and microbes and catalyzes the first step in the biosynthesis of the essential branched-chain amino acids valine, leucine, and isoleucine (Stidham and Singh, 1991). The AHAS enzyme of eukaryotes is composed of a large catalytic subunit (AHASL) and a small regulatory subunit (AHASS). The enzyme catalyzes the condensation of two molecules of pyruvate to form acetolactate, the precursor of valine and leucine, or the condensation of a molecule of pyruvate with a molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate, an intermediate in isoleucine biosynthesis (Delfourne *et al*., 1994; Singh and Shaner, 1995; Duggleby and Pang, 2000). AHAS is the key control enzyme within the branched-chain amino acid biosynthetic pathway and is regulated by feedback inhibition by the end product amino acids, valine, leucine and isoleucine. Regulation is mediated through binding of these amino acids to the AHASS.

Imidazolinone herbicides readily inhibit the activity of AHAS by binding to the active site of the protein resulting in plant death (Duggleby and Pang, 2000; Tan *et al*., 2005). Molecular modeling of the AHAS–imidazolinone interaction suggests that the herbicide binding pocket is at the entry site for the substrate of the AHAS enzyme, and imidazolinones may inhibit the enzyme by impeding substrate binding to AHAS (Tan *et al*., 2005). Studies have shown that specific single nucleotide mutations in the *ahasl* genes result in single amino acid substitutions in the AHAS protein, and these amino acid substitutions confer tolerance to imidazolinone herbicides by altering the binding site for these herbicides on the mutant AHAS enzymes (Tan *et al*., 2005).

The detailed molecular characterization of CV127 soybean confirmed that CV127 contains a single functional *csr1-2* gene cassette integrated in the soybean genome (Sathasivan *et al*., 1990).
The AtAHASL protein encoded by the *csr1-2* gene in CV127 soybean is structurally and functionally identical to the native AtAHASL, except for its tolerance to imidazolinone herbicides. Biochemical characterization of the imidazolinone-tolerant AtAHAS protein expressed in CV127 soybean showed that the AtAHAS protein is typical of other AHAS proteins in this diverse protein family as well as most dietary proteins. The mutation in the *csr1-2* gene responsible for imidazolinone herbicide tolerance is a single nucleotide change of guanine to adenine, which results in a codon change from AGT to AAT and a single amino acid substitution of serine to asparagine at position 653 of the AtAHAS protein. This amino acid change in plant AHAS proteins is known to confer imidazolinone herbicide tolerance, but have no effect on feedback regulation by branched chain amino acids or normal biosynthetic function of the enzyme (Tan *et al*., 2005; Newhouse *et al*., 1992). A second mutation was discovered in the *csr1-2* gene integrated in the genome of CV127 soybean, but this mutation, in which an arginine residue at position 272 is replaced by lysine, does not impact the enzymatic function of the AHAS enzyme or herbicide tolerance properties. A study was conducted to compare the AtAHASL subunit protein expressed in CV127 that contains both the S653N and R272K mutations with the AtAHASL encoded by the *csr1-2* gene that contains only the S653N mutation. In this study it was demonstrated that both AtAHAS enzymes had equivalent levels of catalytic activity and tolerance to imidazolinone herbicides.

Feedback regulation of AHAS activity by the branched chain amino acids is effected through the AHAS small subunit. The AtAHASL enzyme encoded by the *csr1-2* gene in CV127 interacts with the endogenous soybean AHAS small subunit protein (AHASS) to achieve this regulation. Therefore, the feedback regulation of the AtAHASL encoded by the *csr1-2* gene in CV127 was expected to be identical to that of the endogenous soybean AHAS. This was confirmed in enzyme activity studies with the CV127-produced AtAHAS protein. Also, this conclusion was supported by results of grain compositional analyses that showed levels of branched-chain amino acids in CV127 soybean are

comparable to levels in the control soybean, indicating that regulation of this biosynthetic pathway is equivalent between the two treatments.

The composition of CV127 soybean compared to conventional soybeans was investigated by analysis of key nutrients and anti-nutrients. Samples of grain or forage were harvested from CV127, the control, and two conventional soybean varieties from multi-location replicated field trials conducted in Brazil. Grain samples were collected from trials conducted in two separate growing seasons and analyzed for a comprehensive range of important nutrients and antinutrients of soybean. Forage samples were collected from trials in one growing season and were analyzed for proximates and fiber content. Statistical analysis of composition data from grain and forage of CV127 soybean treated with imidazolinone herbicide and from the control and two other conventional soybean varieties, demonstrated that the composition of grain and forage from CV127 soybeans is comparable to that of the control and conventional soybean varieties.

Several herbicide-tolerant crops that through mutation produce AHASL enzymes with the same serine to asparagine substitution at residue 653 as CV127 have been commercialized and marketed under the Clearfield® brand name for many years and include corn (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and oilseed rape (*Brassica napus* L.). A study compared the rate and route of metabolism of ^{14}C-labeled imidazolinone herbicides in Tebonnet (a non-tolerant commercially available rice line) and a mutant rice line (tolerant to imidazolinones). The rate of metabolism was compared for three herbicides using application of the formulated product to rice leaves (Figure). Pursuit (imazethapyr, CL 263,499) was rapidly metabolized in both Tebonnet and the mutant with a half-life of less than 24 hours in the treated leaf. Cadre (imazapic, CL 263,222) was metabolized somewhat more slowly with a half-life of just over 24 hours in the treated leaf. Arsenal (imazapyr, CL 243,997) was metabolized very slowly by both Tebonnet rice and the mutant with a half-life of over 48 hours in the treated leaf. No significant differences in the rates of metabolism were observed between the two types of rice for any of the herbicides (Figure 2). The rates of translocation from the leaf to the plant as determined by autoradiograms were also similar for Tebonnet and the mutant rice for the three herbicides.

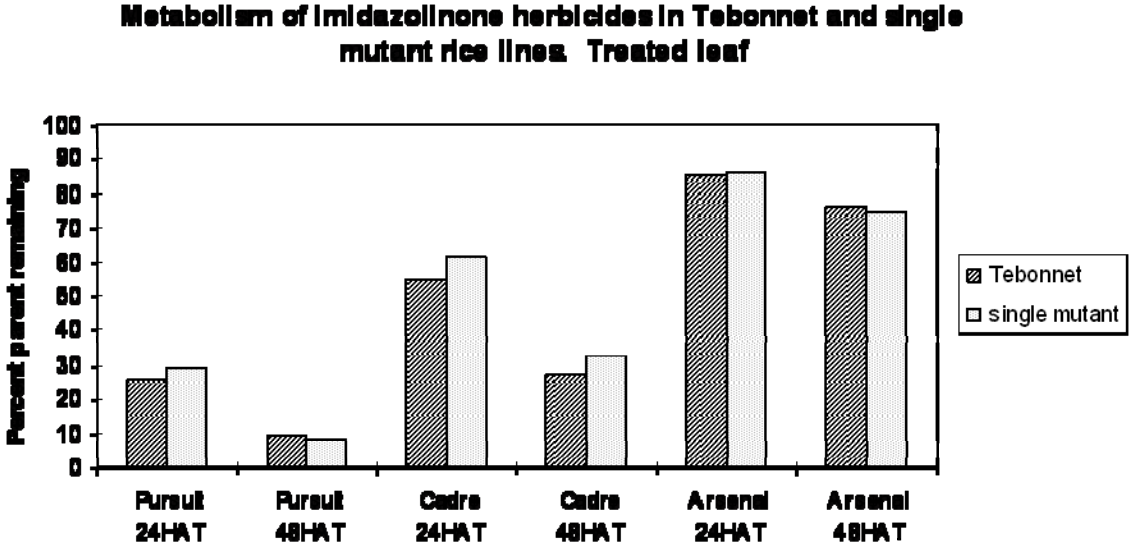

Figure 3. Comparison of Rate of Metabolism for Imidazolinone Herbicides in Non-tolerant and AHAS-mutant rice

The comparative rates and routes of metabolism of imazethapyr (CL 263,499) in Tebonnet and mutant rice were compared after application of formulated herbicide to rice seedlings at about 25% the typical use rate. The results indicated the herbicide was metabolized to the same hydroxylated metabolite (CL 288,511) at similar rates (Figure 3). The primary hydroxylated metabolite (CL 288,511) did not undergo further glycosidation as observed in the more tolerant plants such as legumes, possibly due to the short duration of the experiment.

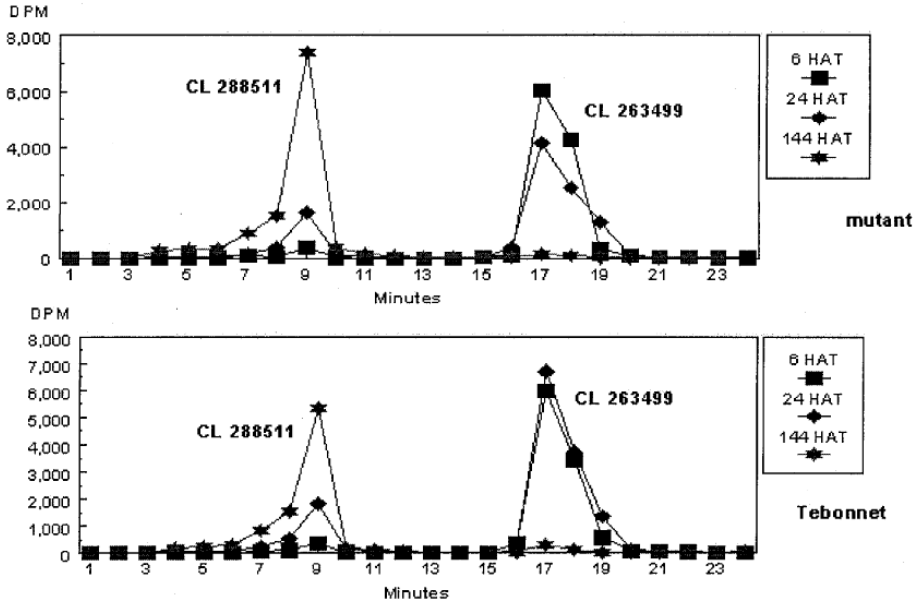

Figure 4. Comparison of Rate of Conversion of Parent to Metabolite in Tebonnet and AHAS-mutant Rice after Treatment with Imazethapyr

Conclusion

Several *ahasl* genes encoding AHASL enzymes that are tolerant to imidazolinone herbicides have been discovered in plants as naturally occurring mutations and through the process of chemically-induced mutagenesis. There are five single point mutations in *ahasl* genes that result in tolerance to imidazolinone herbicides in plants (Tan *et al*., 2005), including the S653N mutation in the *csr1-2* gene. For example, imidazolinone-tolerant corn (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oilseed rape (*Brassica napus* and *B. juncea* L.), and sunflower (*Helianthus annuus* L.) were developed through mutagenesis, selection, and conventional breeding technologies and have been commercialized under the Clearfield® brand name since 1993. Therefore, there has been a long history of safe production of crops containing an imidazolinone-tolerant AHAS with the same S653N amino acid substitution as that in the AtAHAS encoded by the *csr1-2* gene present in CV127 soybeans. The similarity in uptake, translocation, and metabolism demonstrated in Tebonnet and AHAS-mutant rice further substantiates that the modification of the AHAS protein has no impact on the plant metabolism of the imidazolinone herbicides. Thus, the studies in AHAS-mutant rice support the thesis that the metabolic pathways of imazapyr and imazapic in Cultivance soybean seed is the same as that found for imazapyr and imazapic in other tolerant and non-tolerant plants.