Ion Chromatographic Analysis of Selected Free Amino Acids and Cations To Investigate the Change of Nitrogen Metabolism by Herbicide Stress in Soybean (*Glycine max*)

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A simple and reliable method for the determination of NH_4^+, K^+, Na^+, aspartic acid, asparagine, glutamine, and alanine by ion chromatography has been developed. It is suitable for monitoring changes of nitrogen metabolism in soybean because it can accurately measure concentrations of asparagine and NH_4^+, two key substances for nitrogen storage and transport in this plant species. Analysis of asparagine distribution in soybean indicated that higher levels (up to 18.4 μmol g^{-1} of fresh mass) occur in stems and lower levels in roots (2.0 μmol g^{-1} of fresh mass) and leaves (1.6 μmol g^{-1} of fresh mass). When the herbicide metsulfuron-methyl (0.5, 5, and 50 ppb) was applied via the nutrient solution to the root system, asparagine concentrations increased 3−6 times in stems, roots, and leaves. Metsulfuron-methyl is known to impair the synthesis of branched amino acids and, in consequence, protein synthesis. Thus, nitrogen consumption was limited, leading to an accumulation of asparagine. The possible use of this physiological response in agricultural practice to identify herbicide stress in soybean and to detect low-level residues of sulfonylurea herbicides in the soil is discussed.

Keywords: *Asparagine; Glycine max; ion chromatography; metsulfuron-methyl; nitrogen metabolism*

INTRODUCTION

For soybean plants, Lam et al. (1995) proposed a model to explain the regulation of nitrogen metabolism, which has since been supported by the results of several investigations [e.g., Bacanamwo and Harper (1997), Delhon et al. (1995a,b), and Mizukoshi et al. (1995)]. These authors have suggested that asparagine (Asn) gene products are able to regulate the flow of nitrogen into Asn, which acts as a shunt for nitrogen storage and/or long distance nitrogen transport. Asn has a high N/C ratio and is stable in mature shoots (Bacanamwo and Harper, 1997). Hence, it represents an economical compound for nitrogen transport and storage. A preferential assimilation of excess nitrogen into Asn has frequently been reported for soybean [e.g., Layzell and LaRue (1982) and Parson et al. (1995)]. An impairment of nitrogen metabolism, which is not related to the uptake of nitrogen and the formation of Asn but affects, for examp;e, amino acid synthesis, should thus result in an increase of the Asn level in soybean. A reliable and quick analysis for determination of the key amino acids and cations, therefore, is useful for an investigation of the impairment of nitrogen metabolism in soybean.

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Acetohydroxyacid synthase, also known as acetolactate synthase (ALS; EC 4.1.3.18), catalyzes the first reaction common to the biosynthesis of the branched-chain amino acids L-valine, L-leucine, and L-isoleucine and is the target of the sulfonylurea herbicide group (Southan and Copeland, 1996; Hattori et al., 1995). Since the late 1980s, these herbicides have been studied and applied in agriculture. Chlorsulfuron [2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl] benzenesulfonamide], metsulfuron-methyl [MS; methyl 2-[[(4methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]aminosulfonyl] benzoate], and chlorimuron [ethyl 2-[[(4-chloro-6-methoxy-2-pyrimidinyl)aminocarbonyl]-aminosulfonyl] benzoate] are among the most effective weed killers, with very low application rates, for example, 8 g/ha for MS (Ackerson and Davis, 1987; Drobny, 1984). Unfortunately, residues of these herbicides, even at low levels, represent a potential risk to certain crops such as soybean, maize, or sorghum, due to their slow degradation in certain agroecosystems (Vega et al., 1992). Therefore, reliable methods are needed to detect low levels of sulfonylurea herbicides, especially in the soil, and to identify the herbicide-induced stress in sensitive crops such as soybean. Bioassays, using whole plants or cell tissues (Al-Khatip et al., 1992; Berger, 1993; Rahman et al., 1993; Olofsdotter et al., 1994), are commonly used to detect pesticide residues, frequently in combination with high-performance liquid chromatography (HPLC) and gas chromatography (GC) methods (Hershberger and Brennan, 1988; Cotterill, 1992; Galletti et al., 1995). In some

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cases, in vivo AHAS assays (Simpson et al., 1995) and immunoassays (Kelley et al., 1985; Jia, 1998) are available.

The objective of the present study was to develop a reliable ion chromatographic (IC) method for the determination of the key amino acids Asn, aspartic acid (Asp), glutamine (Gln), and α-alanine (α-Ala), as well as NH_4^+, K^+, and Na^+ cations in soybean seedlings. Evidence will be presented that the IC method is suitable for the investigation of changes of nitrogen metabolism in plants. Soybean was chosen as a model plant because it is sensitive to low levels of sulfonylurea herbicide residues in the root medium. Asn concentrations in roots, stems, and leaves of young soybean plants were analyzed and compared to identify the plant organ that is most suitable for the detection of herbicide effects. A possible practical application of the method as a coupled biological−chemical test system is discussed.

MATERIALS AND METHODS

Plant Culture. Fifty soybean seeds (*Glycine max* L. cv. Enrei) were sterilized by immersion in 70% ethanol solution (v/v) and 0.5% sodium hypochlorite solution (w/v) for 5 min and then thoroughly washed with deionized water. The seedlings were germinated and grown for 2 weeks in vermiculite in a controlled-environment chamber. After removal of the cotyledons, they were individually transferred into plastic cups, which contained 100 mL of nutrient solution. The solution was exchanged every morning, and its concentration was as follows ($mg \cdot L^{-1}$): K_2SO_4, 78.0; K_2HPO_4, 68.0; $MgSO_4 \cdot 7H_2O$, 123.0; $CaCl_2$, 69.0; NH_4NO_3, 80.0; Fe(III)-EDTA, 4.0. The controlled-environment chamber provided a 16 h photoperiod, a 26/24 °C day/night temperature regime, and a relative humidity of ∼70%. Light intensity was ∼500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation at plant height.

Metsulfuron-methyl Treatments and Sampling. After a 4 day adaptation period to hydroculture conditions, 16 plants were selected for uniformity and four plants each received nutrient solution with either 50, 5, and 0.5 ppb metsulfuron-methyl (MS) standard (Wako Pure Chemical Industries, Tokyo, Japan) or nutrient solution without MS as a control. Nutrient solution was not exchanged during the next 4 days. The plants were then harvested, blotted dry on paper tissue, and separated into primary leaves, stems, and roots. After determination of their fresh weight, the fractions were immediately frozen in liquid nitrogen and kept at −20 °C.

Measurement of Asparagine Distribution. For analysis of Asn within different plant organs, four soybean plants (23 days old), the cotyledons of which were not removed, were selected. The distribution of Asn in the root tips, middle part of the root, and upper part of the root was determined. The stem was fractionated into its lower 2 cm, middle part (∼1−3 cm below the insertion of the cotyledons), and its upper 3 cm below the primary leaves. Cotyledons (i.e., the beans) as well as primary leaves were separated into three fractions (leaf base, middle part, and leaf tip). A further four plants were harvested to provide leaf center (diameter = ∼1 cm), middle leaf, and margin of the leaf (outer 0.5 cm) samples of the primary leaves. Additionally, extra plants (23-day-old soybean) were used for ion chromatography analysis of the recovery of amino acids and cations in plant. For all experiments, the plants were neither incubated with *Rhizobium* nor nodulated.

IC. The frozen plant samples were cut into small pieces and transferred into 2 mL Ultrafree-MC tubes (the exclusion limit for globular protein = 1×10^4 Da, Millipore, MA). After thawing, the tubes were centrifuged for 60 min at 300*g* at 4 °C. The mass of the plant residue was determined with a mass balance. The filtrate was collected and appropriately diluted with deionized water (pH 6.0). A 100 μL of sample was analyzed, and cations and amino acids were detected by conductivity. The ion chromatograph had a precolumn (Shim-pack IC-GC1, ∅ 4 mm × 10 mm; Shimadzu, Kyoto, Japan) and a separation column (Shim-pack IC-C1, ∅ 5 mm × 150 mm; Shimadzu, Kyoto, Japan). The columns are filled with a cation-exchange resin of a polystyrene−divinylbenzene as a support incorporating a sulfonic acid base as a functional group. The column temperature was kept at 30 °C. An HNO_3 solution (5 mM) was used as mobile phase (flow rate was 0.8 $mL \cdot min^{-1}$). The detector signals were recorded with a desktop recorder (Ohkura Electric, Tokyo, Japan). The linearity of response of amino acids and cation concentrations were measured. The recoveries from the different plant organs were recorded by adding 0.2, 0.5, and 1.0 mg of the amino acids or cations per gram of fresh mass of roots, stems, or leaves.

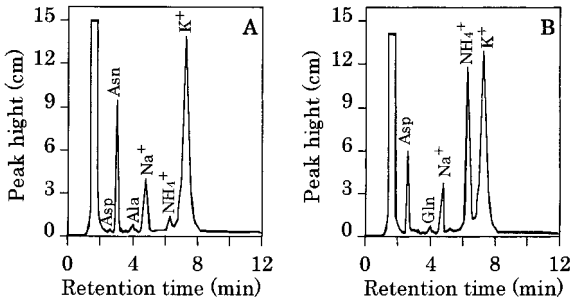

Figure 1. IC patterns of amino acids and further cations in soybean filtrates: (A) typical chromatogram of a stem section; (B) the same sample as in (A) but treated with 0.1 mL of 35% HCl to convert Asn into Asp.

Data Analysis. For statistical analysis of the metsulfuron-methyl application on amino acids and cation contents in soybean, the data were tested for normal distribution and variance homogeneity and were compared with Duncan tests using the SPSS statistical package (SPSS, Chicago, IL). A 5% probability level was accepted to indicate significant differences. In some instances data transformation, in all but one instances a logarithmic transformation, was necessary to stabilize the variances. If the variances were not homogeneously distributed, the nonparametric Wilcoxon test was used and the probability level corrected according to Bonferroni.

RESULTS AND DISCUSSION

Amino Acid and Cation Analysis. Separate injection of various amino acids revealed that the analytical system responded to aspartic acid (Asp), serine (Ser), threonine (Thr), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), cysteine (Cys), α-alanine (α-Ala), *β*-alanine (*β*-Ala), proline (Pro), valine (Val), *γ*-aminobutyric acid (*γ*-ABA), methionine (Met), tyrosine (Tyr), isoleucine (Ile), and leucine (Leu). Glycine (Gly), histidine (His), arginine (Arg), phenylalanine (Phe), tryptophan (Trp), lysine (Lys), and ornithine (Orn) could not be detected. Additionally, the system showed high sensitivity for Na^+, NH_4^+, and K^+. Some of the peaks in the chromatograms consisted of more than one component. For soybean samples, the first detectable peak consisted of Asp (t_R = 2.6 min) and the second was a single peak but consisted of Ser, Thr, Asn, and Glu (t_R = 3.0 min), followed by peaks for Gln (t_R = 3.3 min), α-Ala (t_R = 4.0 min), Na^+ (t_R = 4.8 min), NH_4^+ (t_R = 6.3 min), and K^+ (t_R = 7.3 min), and several broad peaks for Met, Tyr, Ile, and Leu (their t_R values were around 11.2, 11.6, 12.1, and 13.8 min, respectively) (Figure 1). The concentration of Cys (3.6 min) in the filtrate was too low to be detected. High concentration of K^+ in the filtrate impaired the reliable determination of Val and *γ*-ABA due to overlapping of peaks. The peaks for Na^+ and NH_4^+ also contained Pro and *β*-Ala, respectively, because of identical retention times. However, the concentrations of these amino acids are negligible (Bacanamwo and Harper, 1997; Delhon et al., 1995a; Mizukoshi et al., 1995).

Table 1. Linearity of Concentration Responses (Correlation Coefficient R^2) and Recoveries (Rec) of Aspartic Acid (Asp), Asparagine (Asn), Glutamine (Gln), α-Alanine (α-Ala), Na^+, NH_4^+, and K^+ in Soybean Primary Leaves, Stems, and Roots by IC

	standard			leaf sample				stem sample				root sample			
AA and ions[a]	slope (μmol/mL)	R^2	added (μmol/g)	found (μmol/g)	rec (μmol/g)	rec (%)	CV (%)	found (μmol/g)	rec (μmol/g)	rec (%)	CV (%)	found (μmol/g)	rec (μmol/g)	rec (%)	CV (%)
Asp	511.3	0.9997	0	1.777				1.356				2.225			
			0.2	1.969	0.192	95.95	15.75	1.561	0.205	102.3	17.65	2.414	0.189	94.37	20.69
			0.5	2.249	0.471	94.26	6.413	1.826	0.469	93.85	4.441	2.641	0.417	83.31	16.46
			1.0	2.650	0.873	87.27	3.667	2.427	1.071	107.1	8.918	3.025	0.801	80.05	10.15
Asn	784.4	0.9999	0	1.373				16.18				1.542			
			0.2	1.546	0.173	86.61	9.623	16.40	0.219	109.5	9.091	1.735	0.193	96.44	5.587
			0.5	1.854	0.481	96.14	3.744	16.65	0.471	94.25	13.58	2.046	0.504	100.8	3.704
			1.0	2.421	1.048	104.8	1.928	17.08	0.896	89.60	11.11	2.475	0.933	93.33	8.000
Gln	738.6	0.9995	0	0.340				0.181				0.169			
			0.2	0.514	0.174	86.83	3.816	0.353	0.172	86.08	13.04	0.387	0.218	108.8	6.818
			0.5	0.786	0.445	89.06	7.317	0.660	0.479	95.81	7.160	0.585	0.416	83.11	5.455
			1.0	1.329	0.988	98.83	5.036	1.034	0.853	85.33	8.459	1.025	0.856	85.59	9.176
α-Ala	764.7	0.9967	0	1.818				1.558				1.196			
			0.2	1.998	0.181	90.31	9.623	1.724	0.166	83.05	7.217	1.397	0.201	100.6	11.17
			0.5	2.249	0.432	86.31	6.977	2.015	0.458	91.35	9.462	1.624	0.428	85.64	12.03
			1.0	2.711	0.893	89.33	1.946	2.444	0.886	88.58	3.580	2.046	0.850	84.99	3.498
Na^+	1843.1	0.9996	0	0.975				1.912				2.383			
			0.2	1.141	0.166	82.84	10.02	2.081	0.169	84.64	15.00	2.557	0.175	87.29	20.83
			0.5	1.410	0.434	86.89	6.473	2.361	0.449	89.72	14.98	2.938	0.555	111.1	17.32
			1.0	1.833	0.858	85.79	5.4	2.894	0.982	98.18	11.27	3.192	0.809	80.94	7.402
NH_4^+	1378.4	0.9986	0	1.100				2.740				3.164			
			0.2	1.340	0.240	120.1	18.75	2.920	0.186	93.15	9.623	3.368	0.204	101.9	8.248
			0.5	1.670	0.570	114.1	4.558	2.180	0.440	87.97	3.529	3.591	0.427	85.39	6.818
			1.0	2.330	1.231	123.1	3.659	3.680	0.942	94.18	1.903	4.190	1.029	102.9	8.170
K^+	900.2	0.9993	0	190.7				89.32				147.8			
			0.2	190.9	0.241	120.5	30.55	89.57	0.244	121.9	27.27	148.0	0.166	83.10	21.65
			0.5	191.2	0.546	109.3	26.47	89.99	0.665	133.0	5.774	148.3	0.499	99.73	21.60
			1.0	192.1	1.366	136.6	17.65	90.48	1.152	115.2	23.32	148.9	1.080	108.0	21.84

[a] Selected amino acids and cations.

To summarize, in soybean, reliable determinations were obtained for Asp, Glu, α-Ala, Na^+, NH_4^+, and K^+. The concentration of the key amino acid Asn could be identified by quantitative hydrolysis to Asp according to the reaction

$$\text{Asn} + \text{HCl} \rightarrow \text{Asp} + \text{NH}_4^+ + \text{Cl}^- \qquad (1)$$

by adding 0.1 μL of 35% HCl to 0.4 μL of soybean filtrate and incubating the mixture at 80 °C for 15 min. Thus, two measurements were necessary to identify the concentrations of free Asp, Asn, Gln, α-Ala, Na^+, NH_4^+, and K^+ in soybean filtrates. In accordance with Delhon et al. (1995b), many samples of stem filtrate were characterized by high Asn contents with barely detectable concentrations of Ser, Thr, and Glu occurring at the same retention time in the chromatograms.

The relationship of peak area to amino acid or cation concentration was found to be linear between 0.2 and 500 ppm. The slope of the regression lines indicated that the analysis was most sensitive to changes of Na^+, followed by NH_4^+, K^+, Asn, α-Ala, Gln, and Asp (Table 1). To assess the accuracy of the IC method, leaf, stem, and root samples of soybean were spiked with 0.2, 0.5, and 1.0 μmol·g^{-1} of each substance, frozen overnight, centrifuged, and analyzed as mentioned above. The results are summarized in Table 1. They indicate that the recoveries were between 80 and 137%. Mean values are 93% for Asp, 97% for Asn, 91% for Gln, 89% for α-Ala, 90% for Na^+, 102% for NH_4^+, and 114% for K^+, respectively.

Distribution of Asn in Soybean Seedlings. The relative distribution of Asn per gram of fresh mass in soybean seedlings indicated distinct differences even within plant organs (Figure 2). The amount of Asn was comparatively low at the tip of the roots and their middle part (1.5 and 3.0 μmol·g^{-1} of fresh mass) but increased to ∼5.7 μmol·g^{-1} of fresh mass close to the stem. The largest amount of Asn (18.4 μmol·g^{-1} of fresh mass) was found in the lower part of the stem between root and cotyledons. In the upper part of the stem, up to 3 cm below the primary leaves, the Asn concentration was considerably lower, 9.2 μmol·g^{-1} of fresh mass. A similar concentration was found in cotyledons (∼8 μmol·g^{-1} of fresh mass), irrespective of the leaf position

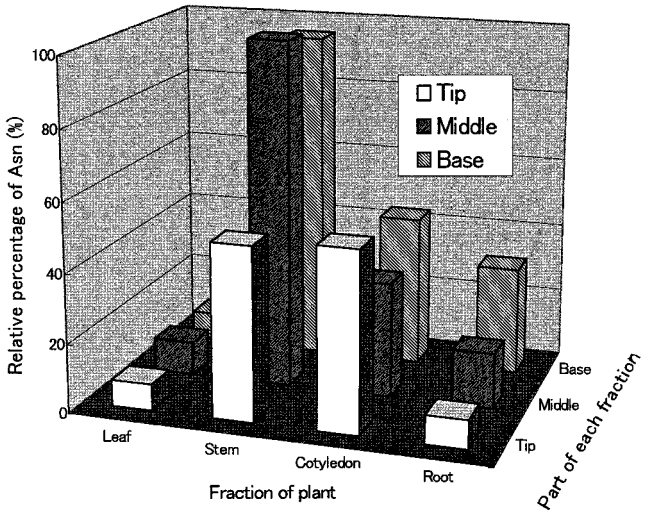

Figure 2. Relative distribution of asparagine contents per gram of fresh mass in several fractions of 23-day-old soybean plants. The highest concentration in the middle of the stem was set at 100%.

Table 2. Changes of Amino Acid Concentrations in Soybean Due to 0.5, 5, and 50 ppb Metsulfuron-methyl in the Nutrient Solution

MS (ppb)	Asp (μmol/g)	Asn (μmol/g)	Gln (μmol/g)	Ala (μmol/g)	Na^+ (μmol/g)	NH_4^+ (μmol/g)	K^+ (μmol/g)
				Leaf			
0	0.955 ± 0.200 a	0.975 ± 0.218 b	0.166 ± 0.060 b	1.197 ± 0.333 b	1.097 ± 0.663 a	0.316 ± 0.119 a	64.84 ± 11.53 a
0.5	1.293 ± 0.153 a	3.153 ± 0.823 a	1.358 ± 0.500 a	2.633 ± 0.535 a	1.132 ± 0.459 a	0.281 ± 0.106 a	63.48 ± 6.528 a
5.0	1.828 ± 1.059 a	5.795 ± 3.080 a	1.790 ± 0.778 a	3.176 ± 1.224 a	1.679 ± 0.880 a	0.535 ± 0.271 a	57.74 ± 12.90 a
50.0	1.190 ± 0.214 a	5.188 ± 1.891 a	1.860 ± 0.869 a	2.200 ± 0.453 ab	0.959 ± 0.405 a	0.416 ± 0.055 a	41.12 ± 6.285 b
				Stem			
0	1.190 ± 0.671 b	13.12 ± 2.431 b	1.025 ± 0.206 b	2.030 ± 0.465 a	1.737 ± 0.806 a	1.251 ± 0.828 bc	58.62 ± 4.171 a
0.5	2.195 ± 0.143 a	57.24 ± 15.16 a	4.037 ± 1.179 a	2.0.095 ± 0.530 a	1.573 ± 0.480 a	0.485 ± 0.157 c	72.53 ± 9.866 a
5.0	2.635 ± 0.335 a	86.69 ± 44.53 a	4.668 ± 2.870 a	2.290 ± 0.908 a	2.230 ± 0.594 a	2.382 ± 1.035 ab	69.32 ± 16.97 a
50.0	2.529 ± 0.437 a	73.82 ± 18.98 a	4.758 ± 1.143 a	2.064 ± 0.643 a	2.403 ± 0.566 a	3.386 ± 0.833 a	75.96 ± 22.15 a
				Root			
0	2.440 ± 1.408 a	1.773 ± 0.636 b	0.163 ± 0.085 b	1.137 ± 0.292 a	1.700 ± 0.532 a	3.101 ± 0.649 b	98.00 ± 22.28 a
0.5	4.881 ± 1.404 a	7.940 ± 2.775 a	0.781 ± 0.126 a	0.627 ± 0.150 b	1.962 ± 0.421 a	4.302 ± 1.692 ab	96.82 ± 16.75 a
5.0	4.852 ± 1.892 a	10.30 ± 2.601 a	0.663 ± 0.398 a	0.745 ± 0.317 ab	1.975 ± 0.970 a	5.334 ± 1.506 ab	99.49 ± 8.657 a
50.0	4.295 ± 2.510 a	9.426 ± 4.382 a	0.607 ± 0.343 a	0.831 ± 0.193 ab	1.545 ± 0.273 a	6.301 ± 1.671 a	78.12 ± 17.32 a

[a] Values are means and standard deviations of four replicates. Means of leaves, stems, and roots within a column were compared by Duncan or Wilcoxon tests. Different letters indicate significant differences at the 5% level.

(base, middle, or tip). Low Asn levels, ~1.6 μmol·g^{-1} of fresh mass, were found in the primary leaves. As for cotyledons, differences among leaf base, middle, and tip were not observed (Figure 2), but, when separated into leaf center, middle, and margin, distinct differences became evident. Asn concentrations were highest in the center (3.0 μmol·g^{-1} of fresh mass), intermediate in the middle part (1.8 μmol·g^{-1} of fresh mass), and lowest at the margin (0.7 μmol·g^{-1} of fresh mass).

The distribution of Asn corresponded well with earlier observations that Asn may be involved in nitrogen storage or long-distance transport (Lam et al., 1995; Bacanamwo and Harper, 1997). The trend is it is the lowest in growing plant parts, for example, leaf margin and root tips, and elevated in older plant tissues, which have storage capacity, such as the lower stem.

Response of Soybean Seedling to Herbicide-Induced Stress. Soybean seedlings were exposed to 0, 0.5, 5, or 50 ppb of metsulfuron-methyl (MS) in the nutrient solution for 4 days. Distinct effects on plant growth (fresh mass increment) could be observed only at the highest treatment. However, a concentration of MS as low as 0.5 ppb had a clear effect on amino acid content. Asn levels were elevated 3−6 times in leaves, stems, and roots of the plants treated by 0.5, 5, and 50 ppb of MS (Table 2). However, statistically significant differences among these MS concentrations could not be found. Gln and Asp responded similarly to MS stress.

Bacanamwo and Harper (1997) presented evidence that Asn may be a signal molecule involved in sensing the nitrogen status in soybean. It may regulate the assimilation of nitrogen by the soybean plant. A sensitive response of soybean to MS in the root medium could indirectly lead to an impairment of nitrogen uptake by the root and thus prevent or at least reduce a further accumulation of Asn. As a consequence, monitoring the Asn level of soybean seedlings may indicate whether MS or further ALS inhibitors are present in the root medium, even though it may not be possible to correlate changes of the Asn level with herbicide concentrations, at least for a short-term treatment. Alternatively, the nonlinear response of the Asn concentrations in roots, stems, and leaves to MS may be due to an increased accumulation of nitrogen in other components, for example, other amino acids. Determination of Asn levels in soybean seedlings may be used in rotational farming systems to test soil samples for the presence of sulfonylurea herbicides. This group of herbicides, such as MS, in the root medium causes distinct changes in the Asn level of leaves, stems, and roots, even at concentrations as low as 0.5 ppb. However, the fact that a dose−responce curve could not be determined during the short-term treatment may limit the applicability of this biological−chemical test system.

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