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A Biological-chemical Test System to Investigate the Influence of Sulfonylurea Herbicides such as Metsulfuron-methyl on Nitrogen Metabolism in Soybean

A.J. Keutgen[1)], N. Keutgen[1)], M. Jia[1)], S. Matsuhashi[1)], C. Mizuniwa[1)], T. Ito[1)], T. Fujimura[1)], T. Sekine[2)] and S. Hashimoto[1)]

([1)]Department of Radiation Research for Environment and Resources and [2)]Department of Materials Science Takasaki Radiation Chemistry Research Establishment, JAERI, Takasaki, Japan)

Summary

A simple and reliable method for the identification of residues of sulfonylurea herbicides in rotational farming systems has been developed using metsulfuron-methyl as a test substance. Metsulfuron-methyl such as other sulfonylurea herbicides impairs the synthesis of branched-chain amino acids and, in consequence, protein synthesis. Thus, nitrogen consumption is reduced, leading to an accumulation of amino acids and a reduction of nitrogen uptake. The biological-chemical test system is based on the determination of aspartic acid, asparagine, glutamine, and NH_4^+ by ion chromatography and is suitable for monitoring changes of nitrogen metabolism in soybean seedlings. Of the measured free amino acids, glutamine and aspartic acid represent key substances that reflect changes in nitrogen uptake, assimilation, transport, and storage. Their concentrations increased due to metsulfuron-methyl 2–15 times in roots, stems, and leaves. The asparagine content is a measure of the nitrogen status of soybean. However, the sensitivity of asparagine content to metsulfuron-methyl depended on the nutritious status of the plant. In case the asparagine level was already high, the herbicide did not induce a significant increase in asparagine. The NH_4^+ concentration may also add valuable information in case NH_4^+ is present in the root medium. With respect to the test system, the lower stem section of soybean seedlings represents the optimal plant section for free amino acid analyses, because samples can reproducibly be taken, the concentration of the cell sap is, compared to roots, less influenced by the water status, and this section is, compared to leaves, rather sensitive to sulfonylurea herbicides in the root medium. A reduction of leaf transpiration rate is a further effect of metsulfuron-methyl, so that its measurement may represent a valuable additional parameter to the described biological-chemical test system.

Key words. asparagine – aspartic acid – glutamine – ion chromatography – leaf transpiration – nitrate uptake – PETIS – $[^{13}N]NO_3^-$

Zusammenfassung

Ein biologisch-chemisches Testsystem zur Untersuchung des Einflusses von Sulfonylurea-Herbiziden wie Metsulfuron-methyl auf den Stickstoffmetabolismus von Soja. Eine einfache und verlässliche Methode für die Rückstandsanalyse von Sulfonylurea-Herbiziden im Fruchtfolgeanbau wurde entwickelt und am Beispiel von Metsulfuron-methyl getestet. Metsulfuron-methyl beeinträchtigt wie andere Sulfonylurea-Herbizide die Synthese von verzweigten Aminosäuren und, als Konsequenz, die Proteinsynthese. In Folge dessen wird der Stickstoffverbrauch reduziert, was zu einer Akkumulation von Aminosäuren und zu einer Reduktion der Stickstoffaufnahme führt. Das biologisch-chemische Testsystem basiert auf der Bestimmung von Asparginsäure, Asparagin, Glutamin und NH_4^+ mittels Ionenchromatographie und ermöglicht, Veränderungen im Stickstoffmetabolismus von Soja zu erfassen. Von den gemessenen freien Aminosäuren stellen Glutamin und Asparaginsäure Schlüsselsubstanzen dar, die Veränderungen in der Aufnahme, der Assimilation, dem Transport und der Speicherung von Stickstoff anzeigen. Ihre Konzentrationen in Wurzeln, Stängeln und Blättern steigen durch Metsulfuron-methyl um das 2–15-fache an. Der Asparagingehalt ist ein Maß für den Stickstoffstatus von Soja. Die Empfindlichkeit der Asparaginkonzentration gegenüber Metsulfuron-methyl hängt jedoch vom Ernährungszustand der Pflanze ab. Im Falle eines schon erhöhten Asparaginniveaus verursachte das Herbizid keine signifikante Steigerung des Asparagingehaltes. Die NH_4^+-Konzentration kann ebenfalls wertvolle Hinweise geben, wenn NH_4^+ im Wurzelraum vorhanden ist. Im Bezug auf das Testsystem stellt der untere Stängelbereich von Soja das optimale Pflanzenteil für die Analyse von freien Aminosäuren dar, weil Proben reproduzierbar gezogen werden können, die Konzentration des Zellsaftes im Vergleich zur Wurzel in einem geringeren Maß durch den Wasserstatus beeinflusst wird, und weil der untere Stängelbereich im Vergleich zu Blättern sehr empfindlich auf Sulfonylurea-Herbizide im Wurzelraum reagiert. Eine Abnahme der Blatttranspirationsrate ist ein weiterer Effekt von Metsulfuron-methyl, so dass deren Messung einen wertvollen zusätzlichen Parameter für das beschriebene biologisch-chemische Testsystem darstellt.

Introduction

The sulfonylurea herbicide metsulfuron-methyl (MS) interacts with acetohydroxyacid synthase, which is also known as acetolactate synthase. The enzyme catalyses the first reaction, that is common to the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (SOUTHAN and COPELAND 1996). MS is rapidly absorbed via the roots and leaves and is distributed throughout the entire plant within a few hours. Since the 1980s, sulfonylurea herbicides have been studied and applied in agriculture of many countries and are among the most effective weed killers with low application rates, e.g. 8 g/ha (DROBNY 1984; ACKERSON and DAVIES 1987). Unfortunately, residues of these herbicides, even at low levels, represent a potential risk to certain following crops such as soybean, maize, or sorghum due to their slow degradation in certain agroecosystems (VEGA et al. 1992). Nowadays, the use of sulfonylurea-tolerant varieties of these crops instead of traditional varieties has promise to minimise this problem (SALISBURY and BEAN 1998).

In soybean, asparagine (Asn) acts as a shunt for nitrogen storage and/or long distance nitrogen transport. It is said to reflect the nitrogen status of the plant (BACANAMWO and HARPER 1997) and together with glutamine (Gln) is considered to represent a potential candidate to regulate nitrogen uptake in non-nodulated soybean roots (RUFTY et al. 1993). Therefore, the present authors intended to develop a simple ion chromatographic method for the determination of these key amino acids. The hypothesis was tested, whether changes of their concentrations are able to reflect an influence of the sulfonylurea herbicide MS on nitrogen metabolism. Soybean was chosen as a model plant, because it is sensitive to low levels of sulfonylurea herbicides in the root medium.

The ion chromatographic method for the detection of Asn and Gln was developed by separately injection of various amino acids onto the system described below and evaluation of the system responses. Aspartic acid (Asp), Asn, Gln, α-alanine (α-Ala) as well as NH_4^+ could be determined. The contents of the first three of these amino acids in addition to NH_4^+ and NO_3^- were determined for roots, stems, and leaves of soybean exposed to MS. In the first experiment, soybean seedlings supplied with NH_4NO_3 in the nutrient solution were treated with different concentrations of MS in the root medium in order to evaluate the sensitivity of the test system. In a second experiment, only NO_3^- was the nitrogen source to investigate the influence of the nutritious status on the response of the key amino acids in soybean. For the third experiment, MS was supplied onto a side leaflet of a trifoliate to study, whether the application of MS into the root medium or onto the leaf influenced changes of the key amino acid concentrations. Moreover, we tried to investigate the spreading of MS effects in a soybean trifoliate from the MS-treated leaflet to the non-treated ones. To distinguish between effects of MS on nitrogen metabolism and nitrogen translocation, the short-lived ^{13}N radioisotope was used. The partitioning of $[^{13}N]NO_3^-$ within the trifoliate was chosen as an example and its distribution was followed using a positron-emitting tracer imaging system (PETIS).

Materials and Methods

Plant material and growth conditions

Soybean seeds [*Glycine max* (L.) Merr. cv. 'Enrei'] were surface-sterilised by soaking in 70 % ethanol solution (v/v) for one minute, then in 0.5 % sodium hypochlorite solution (w/v) for three minutes. After a thorough rinsing in sterile deionised water, seeds were germinated on deionised water in the dark at 25 °C. Five days later, the seedlings were transferred to 5 l plastic containers (10 seedlings per container) filled with vermiculite and supplied with a basal nutrient solution containing either 0.45 mM K_2SO_4, 0.40 mM K_2HPO_4, 0.50 mM $MgSO_4$ x $7H_2O$, 0.62 mM $CaCl_2$, 1.00 mM NH_4NO_3, 0.01 mM Fe(III)-EDTA (nutrient solution 1, NS1) or 0.04 mM K_2HPO_4, 0.50 mM $MgSO_4$ x $7H_2O$, 0.62 mM $CaCl_2$, 1.00 mM KNO_3, 0.01 mM Fe(III)-EDTA (nutrient solution 2, NS2). Noteworthy, in NS2 the phosphorus content was by a factor of 10 smaller than in NS1. However, based on the phosphorus concentration used by RUFTY et al. (1993) the phosphorus level in NS2 was considered sufficient to avoid deficiency symptoms in case the nutrient solution was exchanged daily (cf. experiment 2).

The soybean seedlings were grown in a controlled environment chamber under a 16 h photoperiod, a 26/24 °C day/night temperature regime, and a relative humidity of about 70 %. Light intensity was about 300 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at plant height. For all experiments, the plants were neither incubated with *Bradyrhizobium japonicum* nor nodulated.

For the first set of experiments with metsulfuron-methyl [MS, methyl 2-[[(4methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]aminosulfonyl]benzoate, Wako Pure Chemical Industries, Tokyo, Japan], soybean seedlings remained in the plastic containers with vermiculite for three weeks. Experiments were conducted with the first trifoliate leaf. After removal of the cotyledons, plants were individually transferred into plastic containers filled with 100 ml of nutrient solution NS1. The solution was continuously aerated and exchanged every morning. After a four-day adaptation period to hydroculture conditions, 16 plants were selected for uniformity and four plants each received nutrient solution with 0.5, 5 or 50 ppb MS or nutrient solution without MS as a control. The nutrient solution was not exchanged during the next four days. After these four days plants were harvested and separated into leaves, stems, and roots. Fresh mass of the samples was determined, the samples were frozen at –25 °C, and stored for final amino acid and NH_4^+ analyses.

In the second experiment, thirty two-week old seedlings were transplanted to a hydroponic culture with nutrient solution NS2. These seedlings had just expanded their primary leaves. Three seedlings were situated in one 100 ml plastic container. The nutrient solution was continuously aerated and exchanged every morning. Cotyledons were removed and soybean seedlings acclimatised for three days before the MS treatment. To half of the plants MS was supplied to 90 ml of a modified nutrient solution NS2 without KNO_3 so that the MS concentration reached 5 ppb after addition of further 10 ml KNO_3-solution (10 mM). KNO_3 was

added four hours after MS application and 1 ml samples of the nutrient solution were taken 10 min, 6 h and 7 h after the NO_3^- application to measure NO_3^- uptake of soybean seedlings. The differences in the NO_3^- concentrations at 10 min and 6 h as well as at 10 min and 7 h were calculated and expressed in μmol h^{-1} g^{-1} fresh weight. The mean of both values was calculated and regarded as a measure of NO_3^- uptake. One set of control and MS-treated plants was harvested at the end of each experimental day and separated into leaves, stems, and roots. After weighing, the samples were frozen at –25 °C and stored for final amino acid and NO_3^- analyses. These trials were repeated nine times. Additional measurements on leaves of these plants were performed to study MS-effects on leaf gas exchange.

For the third experiment, plants were grown as for the first (NS1), but 100 ppm MS was supplied to a side leaflet of a young, not completely expanded trifoliate overnight. Dipping a tissue paper in the MS solution and attaching it onto the leaflet surfaces applied the herbicide. The leaflet and tissue were placed into a plastic bag. The high MS concentration was chosen, because preliminary experiments revealed that lower concentrations frequently did not affect the trifoliate, possibly because the herbicide could not easily enter the leaf (but cf. DROBNY 1984). The remaining leaflets of the trifoliate served as controls. Contemporaneous to the MS application, the second side leaflet was treated with deionised water and the middle leaflet remained untreated. Some of the MS-treated and untreated soybean seedlings were transferred to the radioisotope laboratory and labelled with $[^{13}N]NO_3^-$ for 30 min. Further seedlings were harvested before and 1–3 days after MS application, separated into the three leaflets of the trifoliate and the upper petiole, and, after fresh mass determination, frozen at –25 °C for final amino acid and NO_3^- analyses. Additional measurements on single leaflets of the trifoliates were performed to investigate the effects of the MS application on leaf gas exchange.

Amino acid and NH_4^+ analyses

The frozen plant samples were cut into small pieces and transferred into 2 ml Ultrafree-MC tubes (the exclusion limit for globular protein = 1 x 10^4 Da, Millipore, Milford, MA, USA). The samples were centrifuged for 60 min at 3000 g at 4 °C and the mass of the plant residue was determined with a balance. The filtrate was stored at –25 °C. For the analyses of NH_4^+ and the amino acids aspartic acid (Asp), asparagine (Asn), α-alanine (α-Ala), and glutamine (Gln) a modified ion-chromatographic method was chosen (BLACKBURN 1968). The filtrate was appropriately diluted with deionised water (pH 6.0) and injected onto a precolumn (Shim-pack IC-GC1, Ø 4 mm x 10 mm, Shimadzu, Kyoto, Japan) connected to a separation column (Shim-pack IC-C1, Ø 5 mm x 150 mm, Shimadzu, Kyoto, Japan) filled with a cation exchange resin of polystyrene-divinylbenzene, which incorporates a sulfonic acid base as a functional group. Free amino acids and NH_4^+ were separated on the column using 5 mM HNO_3 (flow rate: 0.8 ml min^{-1}) as mobile phase. The column temperature was set at 30 °C and a conductivity detector was used for amino acid and NH_4^+ detection. The peaks were recorded with a desktop recorder (Ohkura Electric, Tokyo, Japan).

Analysis of plant NO_3^- content and NO_3^- uptake

The NO_3^- content of the soybean fractions and the nutrient solutions were also analysed by ion chromatography. The analytical system consisted of a precolumn and a separation column (TSKgel, IC-Anion-PW, Ø 4.6 mm x 50 mm, Tosoh Corporation, Tokyo, Japan) with TSK IC-Amino-A as mobile phase (1.2 ml min^{-1}). Column temperature was set at 30 °C and a conductivity detector was used for NO_3^- detection.

Visualisation of $[^{13}N]NO_3^-$ by PETIS

An aqueous solution of $[^{13}N]NO_3^-$ was produced according to the reaction ^{16}O (p, α) ^{13}N by bombarding water with 1 μA of 20 MeV H^+-particles from the TIARA AVF cyclotron. A cation exchange column (Accell Plus CM, Waters, Eschborn, Germany) was used to purify the $[^{13}N]NO_3^-$ solution from traces of other labelled ^{13}N compounds (ISHIOKA et al. 1999).

The PETIS (Positron-Emitting Tracer Imaging System) consists of a pair of block detectors (5 x 5 cm^2), which are composed of a $Bi_4Ge_3O_{12}$ scintillator array coupled with a position sensitive photomultiplier tube (PS-PMT, Hamamatsu R3941-2, Hamamatsu Photonics Co., Hamakita, Shizuoka, Japan). They produce a time-series of two-dimensional images with a spatial resolution of 2 mm. The position of tracer activity in the soybean trifoliate was determined by coincidence measurements of antiparallel γ-ray pairs produced from the annihilation of positrons emitted by the tracer (Fig. 1). The use of a 2 mm thick absorbing acrylic plate placed about 2 mm from the plant surface is necessary to quantify images of leaf tissues, because annihilation of positrons does not provide an accurate tracer position in the plant if the positron escapes the tissue (SATO et al. 1999; KUME et al. 1999).

At the day of the tracer experiment, soybean petioles were cut under deionised water and the young trifoliates were arranged in front of the PETIS detectors. Imme-

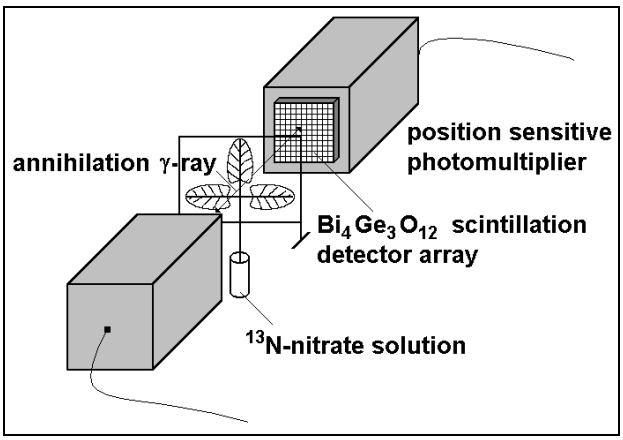


Fig. 1. Sketch of the experimental set-up for the measurement of the distribution of $[^{13}N]NO_3^-$ radioactivity in soybean trifoliates with the Positron-Emitting Tracer Imaging System (PETIS).

diately before the experiment, the water was replaced by the tracer solution. [^{13}N]NO_3^- (5 to 10 Mbq/ml) was supplied to soybean petioles for 30 min. During the tracer applications, data were recorded continuously with a sampling interval of 15 s. The radioactivity that accumulated within the soybean trifoliate is given in order to visualise [^{13}N]NO_3^- quantity and distribution.

Leaf gas exchange

Net photosynthesis rate (A), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) of single soybean leaflets were measured with the LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, Nebraska, USA). Gas exchange parameters were recorded at 500 µmol m^{-2} s^{-1} PAR, at about 25 °C leaf temperature, and about 60 % relative humidity. The ambient CO_2 concentration was about 350 ppm in the leaf cuvette. Gas exchange measurements were conducted during the first six hours of the photoperiod.

Data analysis

The experimental data were analysed with the SPSS-statistical package (SPSS, Chicago, USA). Concentrations of free amino acids, NH_4^+, NO_3^-, NO_3^- uptake by roots and leaf gas exchange measurements were in most cases compared by t-tests. In some instances an ANOVA was performed, but sometimes transformation of data was necessary to stabilise the variances. In case of variance homogeneity, Duncan tests were used. In case the variances were not homogeneously distributed, the non-parametric Wilcoxon test was used and the 5 % probability level was corrected according to Bonferroni.

Results

Amino acid analysis

The response of the analytical system was evaluated by separate injection of various amino acids. 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) could be detected, whereas glycine (Gly), histidine (His), arginine (Arg), phenylalanine (Phe), tryptophan (Trp), lysine (Lys), and ornithine (Orn) could not. The system showed also high sensitivity for Na^+, NH_4^+, and K^+. Unfortunately, some of the peaks consisted of more than one component. For soybean samples, the first detectable peak consisted of Asp (t_R=2.6 min). The second single peak contained four amino acids, viz. Asn, Glu, Ser, and Thr (t_R=3.0 min). This peak was 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 ranged between 11.2 min and 13.8 min). The concentration of Cys (t_R=6.3 min) was too low to be detected. The determination of Val and γ-ABA turned out to be impossible due to overlapping with the K^+ peak. The peaks of Na^+ and NH_4^+ impaired the detection of Pro and β-Ala, respectively. To summarise, in soybean, reliable determinations were measured for Asp, Glu, α-Ala, and NH_4^+ (Fig. 2). The concentration of the key amino acid Asn could be identified by quantitative hydrolysis to Asp by adding 0.1 µl of 35 % HCl p.a. to 0.4 µl of soybean filtrate (48 h incubation at 80 °C) in a separate run.

The relationship of peak height to amino acid and NH_4^+ concentration was linear. Judging from the slope of the regression lines, the ion chromatographic analysis was most sensitive to NH_4^+, followed by Asn, α-Ala, Gln, and Asp (Table 1). To assess the accuracy of the ion chromatographic method, primary leaf, stem, and root samples of soybean were spiked with 0.2, 0.5, and 1.0 µmol g^{-1} of each substance, frozen over night, centrifuged, and analysed. The results, which are summarised in Table 1, indicate that the recoveries were between 80 % and 123 %. Mean values are 88 % for α-Ala, 91 % for Gln, 93 % for Asp, 98 % for Asn, and 102 % for NH_4^+, respectively.

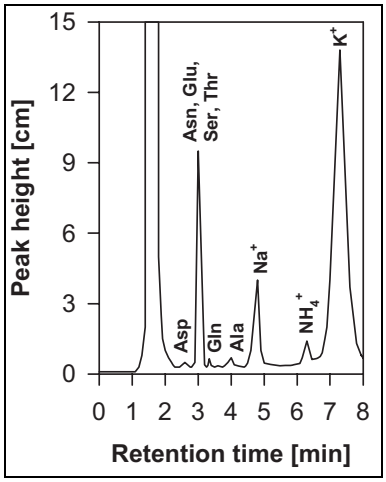


Fig. 2. Ion chromatography pattern of selected free amino acids and further cations in a filtrate from a stem section of soybean.

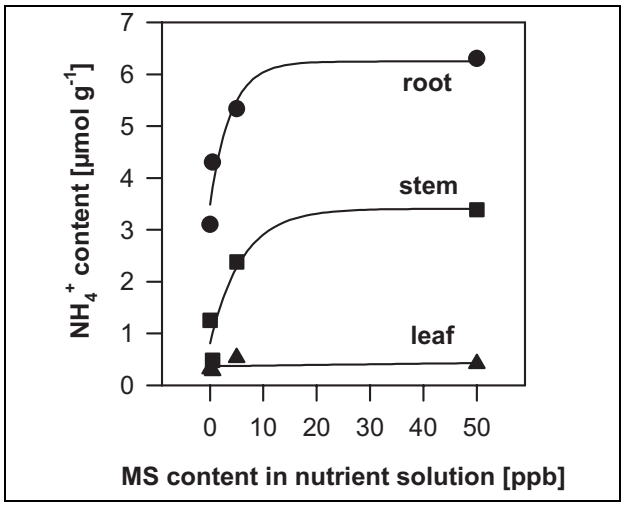


Fig. 3. NH_4^+ contents per g fresh weight in leaves, stems, and roots of soybean plants as affected by different concentrations of MS in the nutrient solution. Plants received nutrient solution NS1 containing 1 mM NH_4NO_3. Values represent means of four replicates.

Table 1. Linearity of concentration responses (correlation coefficient r^2) and recoveries (Rec) of aspartic acid (Asp), asparagine (Asn), glutamine (Glu), α-Ala (Ala), and NH_4^+ in soybean primary leaves, stems, and roots by ion chromatography.

Standard			Leaf sample					Stem sample				Root sample			
	slope [µmol ml^{-1}]	r^2	Added [µmol g^{-1}]	found [µmol g^{-1}]	Rec [µmol g^{-1}]	Rec [%]	CV (%)	found [µmol g^{-1}]	Rec [µmol g^{-1}]	Rec [%]	CV (%)	found [µmol g^{-1}]	Rec [µmol g^{-1}]	Rec [%]	CV (%)
Asp	511.3	0.9997	0	1.78				1.36				2.22			
			0.2	1.97	0.19	95	15.8	1.56	0.20	100	17.6	2.41	0.19	95	20.7
			0.5	2.25	0.47	94	6.4	1.83	0.47	94	4.4	2.64	0.42	84	16.5
			1.0	2.65	0.87	87	3.7	2.43	1.07	107	8.9	3.02	0.80	80	10.2
Asn	784.4	0.9999	0	1.37				16.18				1.54			
			0.2	1.55	0.18	90	9.6	16.40	0.22	110	9.1	1.74	0.20	100	5.6
			0.5	1.85	0.48	96	3.7	16.65	0.47	94	13.6	2.05	0.51	102	3.7
			1.0	2.42	1.05	105	1.9	17.08	0.90	90	11.1	2.48	0.94	94	8.0
Gln	738.6	0.9995	0	0.34				0.18				0.17			
			0.2	0.51	0.17	85	3.8	0.35	0.17	85	13.0	0.39	0.22	110	6.8
			0.5	0.79	0.45	90	7.3	0.66	0.48	96	7.2	0.58	0.41	82	5.4
			1.0	1.33	0.99	99	5.0	1.03	0.85	85	8.5	1.02	0.85	85	9.2
Ala	764.7	0.9967	0	1.82				1.56				1.20			
			0.2	2.00	0.18	90	9.6	1.72	0.16	80	7.2	1.40	0.20	100	11.2
			0.5	2.25	0.43	86	7.0	2.02	0.46	92	9.5	1.62	0.42	84	12.0
			1.0	2.71	0.89	89	1.9	2.44	0.88	88	3.6	2.05	0.85	85	3.5
NH_4^+	1378.4	0.9986	0	1.10				2.74				3.16			
			0.2	1.34	0.24	120	18.8	2.92	0.18	90	9.6	3.37	0.21	105	8.2
			0.5	1.67	0.57	114	4.6	3.18	0.44	88	3.5	3.59	0.43	86	6.8
			1.0	2.33	1.23	123	3.6	3.68	0.94	94	1.9	4.19	1.03	103	8.2

Table 2. Changes of amino acid concentrations per g fresh weight in soybean due to 0, 0.5, 5 and 50 ppb metsulfuron-methyl in the nutrient solution.

MS (ppb)	Gln (μmol g^{-1})	Asp (μmol g^{-1})	Asn (μmol g^{-1})
Leaf			
0	0.17 ± 0.06 b	0.96 ± 0.20 a	0.98 ± 0.22 b
0.5	1.36 ± 0.50 a	1.29 ± 0.15 a	3.15 ± 0.82 a
5.0	1.79 ± 0.78 a	1.83 ± 1.06 a	5.80 ± 3.08 a
50.0	1.86 ± 0.87 a	1.19 ± 0.21 a	5.19 ± 1.89 a
Stem			
0	1.02 ± 0.21 b	1.19 ± 0.67 b	13.1 ± 2.4 b
0.5	4.04 ± 1.18 a	2.20 ± 0.14 a	57.2 ± 15.2 a
5.0	4.67 ± 2.87 a	2.64 ± 0.34 a	86.7 ± 44.5 a
50.0	4.76 ± 1.14 a	2.53 ± 0.44 a	73.8 ± 19.0 a
Root			
0	0.16 ± 0.08 b	2.44 ± 1.41 a	1.77 ± 0.64 b
0.5	0.78 ± 0.13 a	4.88 ± 1.40 a	7.94 ± 2.78 a
5.0	0.66 ± 0.40 a	4.85 ± 1.89 a	10.3 ± 2.6 a
50.0	0.61 ± 0.34 a	4.30 ± 2.51 a	9.43 ± 4.38 a

Values represent means ± 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.

Root application of different MS concentrations with NH_4NO_3 as nitrogen source

Soybean seedlings exposed to 0, 0.5, 5 or 50 ppb MS in nutrient solution NS1 for four days were characterised by significant concentration changes of Gln and Asn in roots, stems, and leaves (Table 2). With respect to Asp, a significant herbicide effect was detectable for the stem. In roots, however, the effect was distinct, but not detectable for leaves. The results clearly indicate that a concentration of MS as low as 0.5 ppb affected amino acid content. In cases of a MS effect, however, statistically significant differences among the three MS concentrations could not be found. By contrast, NH_4^+ concentrations rose in roots and stems in a manner that is typical of a saturation curve (Fig. 3).

Root application of 5 ppb MS with only KNO_3 as nitrogen source

This experiment was performed to investigate the influence of nitrogen source on amino acid response and also the time dependency of amino acid concentration changes due to MS. First effects of the treatment with 5 ppb MS in the root medium were observed two days after MS application. NO_3^- content of roots rose significantly and concentrations of Gln and Asp in tendency. The increase of these amino acids became significant at the third day. NO_3^- uptake from the nutrient solution was significantly reduced three days after MS application (Fig. 4).

In the lower stem section, the concentration changes of amino acids were similar to those in roots, with the exception that a MS effect was already significant two days after MS application in case of Gln. Differences in NO_3^- concentrations of stems, however, could not be detected (Fig. 5).

In the leaf section, a MS effect was barely detectable. Only Asp content started to rise three days after MS application, which is one day later than in the root and lower stem section (Fig. 6).

Leaf gas exchange was significantly impaired by the application of 5 ppb MS to the nutrient solution. Stomatal conductance (g_s) and transpiration rate (E) were significantly lower at the third and fourth day after MS application. Net photosynthesis rate (A), however, was not affected, which corresponded well with the reduction of leaf intercellular CO_2 concentration (C_i) during the last two days of the experiment (Fig. 7).

Leaf application of metsulfuron-methyl

The application of a solution containing 100 ppm MS onto a trifoliate resulted in a time-dependent response of Gln, Asp, Asn, and NO_3^- concentrations (Fig. 8). In the MS-treated leaflet Gln, Asp, and Asn concentrations were somewhat elevated by the end of the first day after MS application, whereas the concentrations of these amino acids rose with a delay of one day in the remaining leaflets of the herbicide-treated trifoliate and also the petiole. However, in the petiole the Asn concentration was not affected by the herbicide treatment. NO_3^- concentrations were distinctly reduced at the third day after the MS application in all leaflets and the petiole. The decrease was most pronounced in the petiole and middle leaflet, but least in the MS-treated one (Fig. 8). Measurements of leaf gas exchange indicated a significant reduction of transpiration rate three days after MS application (data not shown). Noteworthy, all leaflets of the treated trifoliate responded similarly at

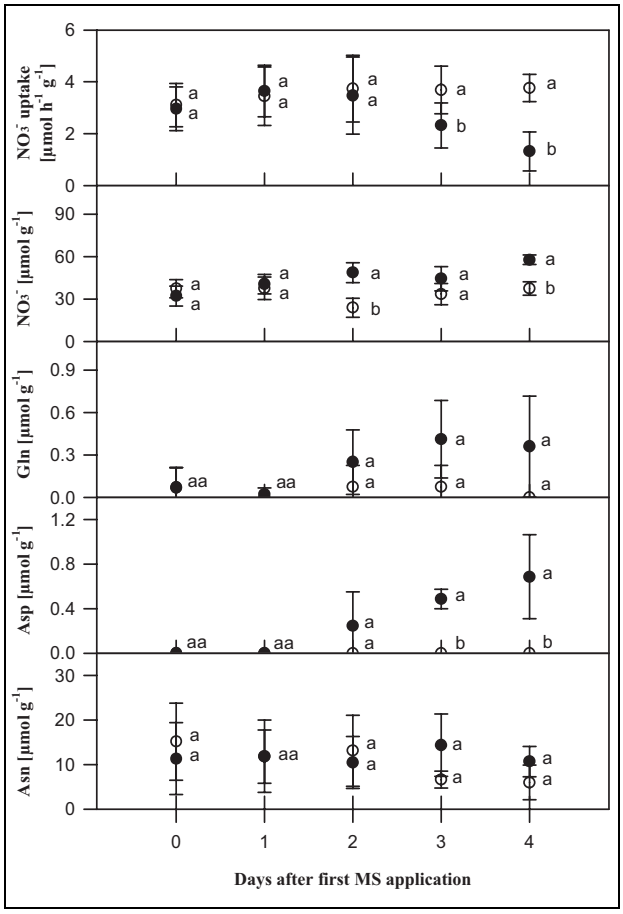


Fig. 4. NO_3^- uptake by roots as well as changes of NO_3^-, glutamine (Gln), aspartic acid (Asp), and asparagine (Asn) concentrations per g fresh weight in roots after application of 5 ppb MS to the nutrient solution containing 1 mM KNO_3 as nitrogen source. Means and standard deviations are given for controls (o) and MS-treated (●) soybean seedlings. Mean values of each day were compared by t-tests (P=0.05).

the third day of the experiment despite the different time response of amino acids.

The hypothesis was tested, whether the differences in leaflet NO_3^- contents at the last day of the experiment, resulted from different rates of NO_3^- uptake by the leaflets (cf. Fig. 8). The experiment with $[^{13}N]NO_3^-$ indicated that a comparable relative amount of tracer, entering the view of the PETIS detector, was translocated into each leaflet of a trifoliate before and during the three days after MS application, which was evident from the comparable distribution pattern of pixels in the leaflets of the trifoliates shown in each of the Figures 9 and 10. Nevertheless, the absolute amount of tracer that was translocated into the trifoliates was different in these figures. Whereas the shape of the trifoliate was well reflected by the pixels in Fig. 9, it was hardly visible in Fig. 10, where fewer radioactivity entered the trifoliate. In both figures, the highest amount of radioactivity appeared in the petiole, which was situated in the lower right (Fig. 9) or lower left side (Fig. 10) of the images. The comparison of tracer intensity in Fig. 9, which is representative of days before and during the first two days of MS application, and of Fig. 10, which represents the situation at the third day after MS application, thus proved that distinctly less $[^{13}N]NO_3^-$ entered the entire trifoliate at the last day of the experiment. This result corresponded well with the measured reduction of leaflet transpiration rates. If follows that the differences in leaflet NO_3^- contents at the last day of the experiment cannot be explained by differences in NO_3^- uptake by the leaflets.

Discussion

In rotational farming systems residues of sulfonylurea herbicides may represent a potential risk to sensitive crops. Therefore, reliable methods are needed to detect low levels of sulfonylurea herbicides in the soil. Bioassays, using whole plant or cell tissues (BERGER 1992; OLOFSDOTTER et al. 1994), are commonly used to detect pesticide residues, frequently in combination with high-performance liquid chromatography and gas chromatography methods (HERSHBERGER and BRENNAN 1988; COTTERILL 1992; GALLETTI et al. 1995). In some cases, in vivo AHAS assays (SIMPSON et al. 1995) and immunoassays (KELLEY et al. 1985; JIA 1998) are available. However, a biological-chemical test system based on the ion chromatographic analysis of the free key amino acids Gln and Asn in sulfonylurea herbicide sensitive soybean seedlings may represent an easy to handle, cost-effective, quick, and reliable alternative to

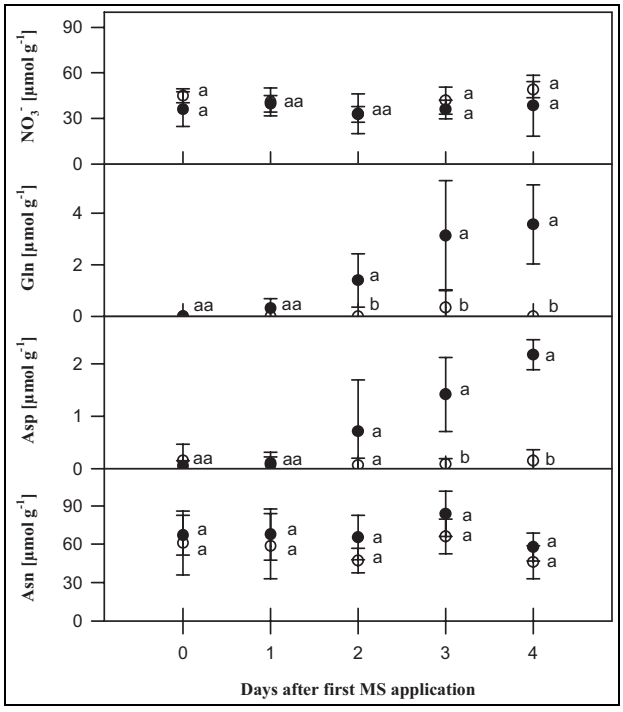


Fig. 5. Changes of NO_3^-, glutamine (Gln), aspartic acid (Asp), and asparagine (Asn) concentrations per g fresh weight in the lower stem section after application of 5 ppb MS to the nutrient solution containing 1 mM KNO_3 as nitrogen source. Means and standard deviations are given for controls (o) and MS-treated (●) soybean seedlings. Mean values of each day were compared by t-tests (P=0.05).

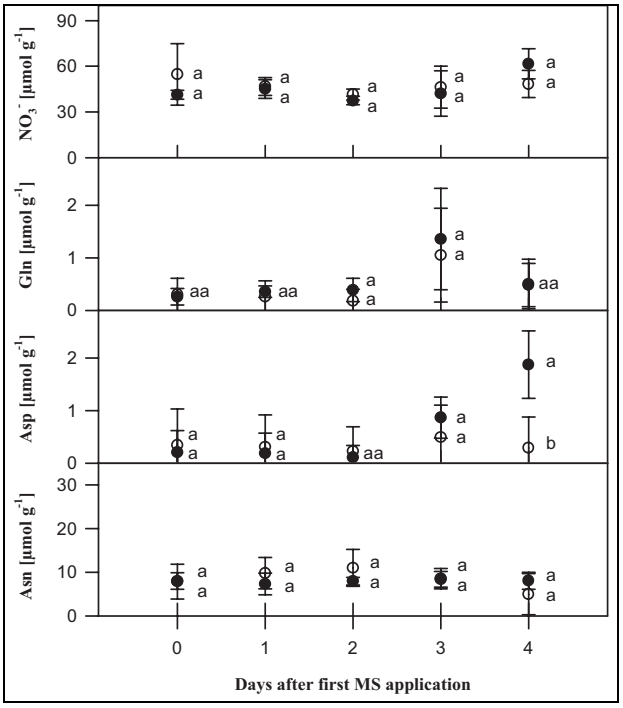


Fig. 6. Changes of NO_3^-, glutamine (Gln), aspartic acid (Asp), and asparagine (Asn) concentrations per g fresh weight in leafs after application of 5 ppb MS to the nutrient solution containing 1 mM KNO_3 as nitrogen source. Means and standard deviations are given for controls (o) and MS-treated (●) soybean seedlings. Mean values of each day were compared by t-tests (P=0.05).

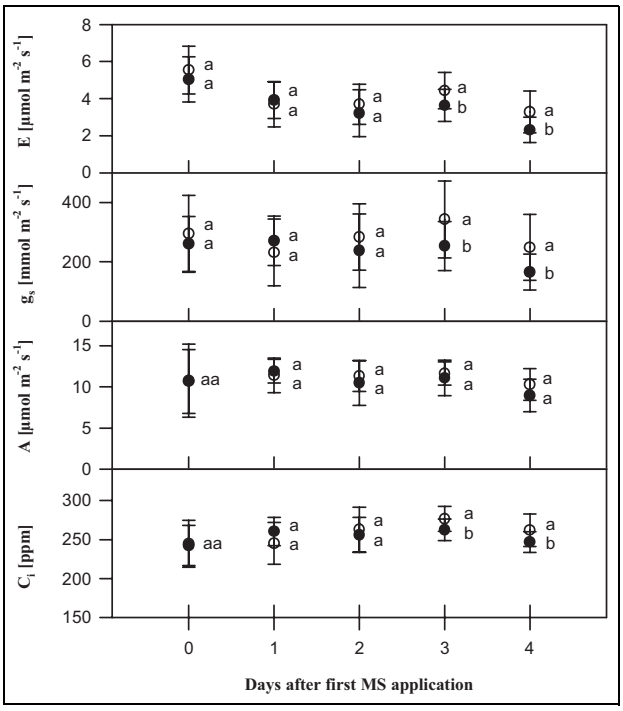


Fig. 7. Changes of transpiration rate (E), stomatal conductance (g_s), net photosynthesis rate (A), and leaf intercellular CO_2 concentration (C_i) after application of 5 ppb MS to the nutrient solution containing 1 mM KNO_3 as nitrogen source. Means and standard deviations are given for controls (o) and MS-treated (●) soybean seedlings. Mean values of each day were compared by t-tests (P=0.05).

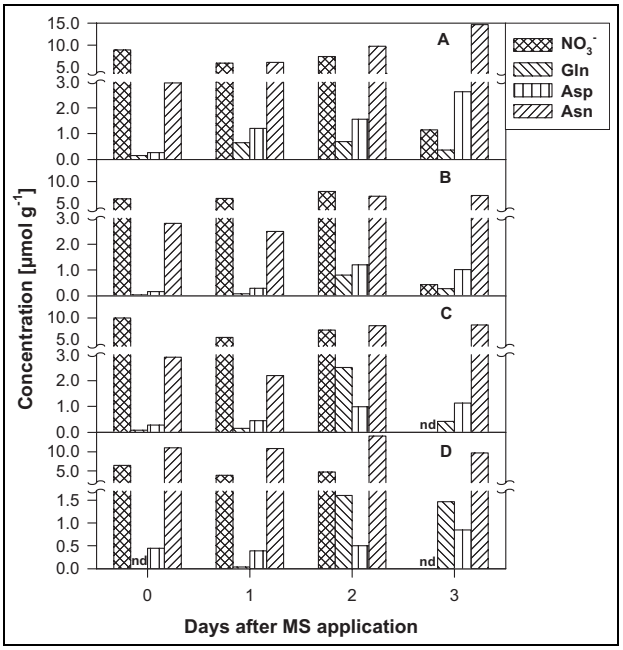


Fig. 8. Changes of NO_3^-, glutamine (Gln), aspartic acid (Asp), and asparagine (Asn) concentrations per g fresh weight in a soybean trifoliate and upper petiole after application of 100 ppm MS to a single leaflet overnight between days 0 and 1. A = MS-treated side leaflet, B = H_2O-treated side leaflet, C = untreated middle leaflet, D = upper petiole. Statistics and standard deviations are not given, because means were calculated from two measurements, only.

test for residues in the soil, especially in developing countries.

Soybean seedlings exposed to different amounts of MS in the root medium in the first experiment responded quite sensitive with respect to amino acid concentration changes. Contents of Gln, Asp, and Asn rose significantly or distinctly with the exception of Asp in leaves (Table 2). However, when only KNO_3 was the nitrogen source in the root medium, the response was slightly different. Gln and Asp rose significantly in roots and stems, whereas Asn concentrations did not change. In leaves, only Asp content rose significantly. The latter difference could be explained by the fact that a MS effect might not have been established completely. For example, a later increase of Gln content in the second experiment cannot be ruled out. Nevertheless, with respect to Asn levels especially in roots and stems this interpretation is not satisfying. In case only NO_3^- was the nitrogen source, it was converted via nitrate reductase and nitrite reductase into NH_4^+, which was converted by glutamine synthase and GOGAT to glutamic acid in plastids. Glutamic acid was the first amino acid to appear in the cytosol and was the nitrogen donor for the conversion of oxaloacetate to Asp, the immediate precursor of Asn (MENGEL 1991). Thus, the increase of Asp clearly showed a MS effect in the root and stem section, but not Asn levels. A comparison of the Asn concentrations in roots, stems and leaves of control plants indicated that in the second experiment with only NO_3^- as nitrogen source

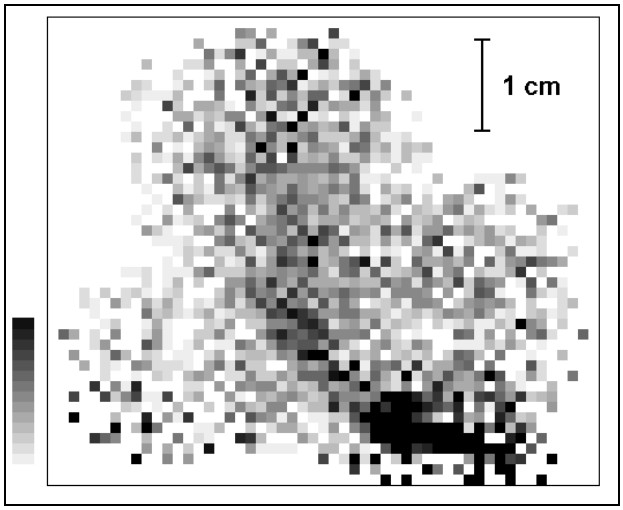


Fig. 9. PETIS image of the [^{13}N]NO_3^- radioactivity accumulated during 30 min in an untreated soybean trifoliate. The radioactivity is expressed in relation to the largest amount, which was observed in the petiole (black pixels). The brighter the pixel, the less is the relative radioactivity.

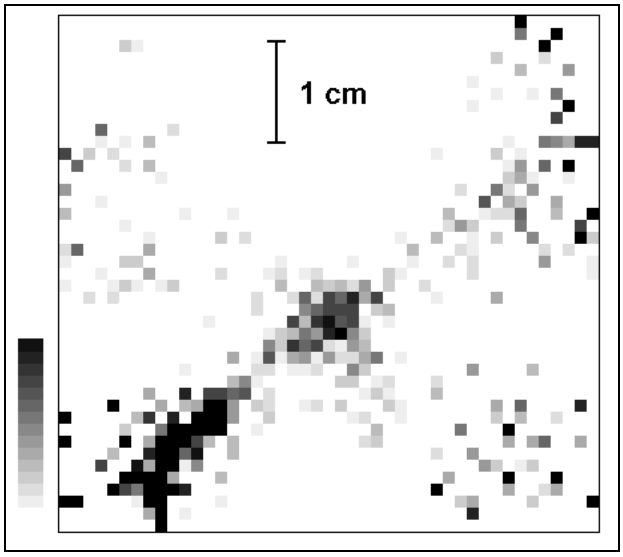


Fig. 10. PETIS image of the [^{13}N]NO_3^- radioactivity accumulated during 30 min in a MS-treated soybean trifoliate three days after MS application. The radioactivity is expressed in relation to the largest amount, which was observed in the petiole (black pixels). The brighter the pixel, the less is the relative radioactivity.

the Asn level was distinctly higher (Figs 4–6), being already at the level found in the MS-treated plants of the first experiment (Table 2). As Asn is considered to reflect the nitrogen status of soybean (BACANAMWO and HARPER 1997), the differences may simply indicate that the nitrogen supply was somewhat limited in the first experiment, when the nutrient solution was not replaced for four days, whereas it was exchanged every day in the second. Alternatively, we may assume that in the second experiment the soybean seedlings consumed less nitrogen. Another explanation could be the lower phosphorus level in the nutrient solution NS2, which might have caused an increase of Asn content in the second experiment due to phosphorus deficiency (RUFTY et al. 1993). To summarise, the nutritious status of the plant influenced the changes of Asn concentrations in response to the herbicide treatment in soybean.

The location of the MS application, for instance onto a soybean trifoliate in the third experiment, also affected the response of the amino acid concentrations. Gln, Asp, and Asn contents increased distinctly in leaves, whereas in the petiole only concentrations of Gln and Asp rose. A comparison of the first and third experiment, when the supplied nutrient solutions were similar but the locations of MS application different, revealed that Asp content of leaves rose significantly in the third but not in the first experiment. An explanation of this result is yet difficult. Nevertheless, in the second experiment the increase of Asp content due to MS was the only detectable response for leaves. Hence, its increase in the third experiment seems reliable. With respect to petioles, it has to be considered that the main transport forms of nitrogen absorbed by non-nodulated roots are NO_3^- and Asn. Both are transported to the leaves via the xylem (DELHON et al. 1995; SATO et al. 1999). The in spite of MS treatment unchanged Asn content in the petiole may thus indicate that Asn translocation had not been affected considerably by MS during the experiment. Consequently, the results of the third experiment showed that the location of the MS application also influenced the concentration responses of Asn.

Although nutrient supply and location of MS application affect amino acid concentration changes, the described ion chromatographic method for the determination of key amino acids within soybean seedlings may be used as a biological-chemical test system to identify the presence of sulfonylurea herbicides such as MS in the soil in rotational farming systems. In this test system, the herbicide enters the plant via the root; hence the effect of the location of herbicide application may be neglected. According to the present results, the optimal plant section for amino acid analyses represents the lower stem section of soybean seedlings, because here significant effects were first detectable (cf. Figs 4–6). Moreover, stem samples can reproducibly be taken and the concentration of the cell sap is, when compared to roots, less influenced by the water status. Independent from the nitrogen source Gln and Asp contents rose significantly in stems. In case of the presence of NH_4^+ in the root medium, an increase of its concentration in stem samples may also be detectable (Fig. 3). The amino acid Asn represents a less suitable indicator, because its concentration is obviously influenced by the nutritious status of the plant. Asp is to be used instead of Asn, which is advantageous, because the second ion chromatographic analysis to determine Asn by quantitative hydrolysis to Asp can be omitted.

The described test system has been investigated only for the sulfonylurea-sensitive soybean cv. 'Enrei', which was not incubated with *Bradyrhizobium japonicum*. In consequence, the influence of the occurence of *Bradyrhizobium* in the soil on the results of the test system is unclear. It has been proved that in soybean the major transport forms of nitrogen assimilated by root nodules are ureides, allantoic acid, and allantoin, whereas the main transport forms of nitrogen ab-

sorbed from roots are nitrate and asparagine (SATO et al. 1999). In consequence, differences in responses of Gln, Asp, and NH_4^+ contents to sulfonylureas in the soil may be expected when *Bradyrhizobium* established on soybean roots. However, the appearance of *Bradyrhizobium* can easily been checked by looking for nodules on the root system.

The nitrogen status of a plant should also influence its nitrogen assimilation. Recent models derived from molecular biological studies on *Arabidopsis thaliana* (L.) propose that the transcript abundances for NO_3^- and NH_4^+ transporters are regulated by Gln, whereas root NO_3^- and NH_4^+ contents may influence transporter activity (GLASS et al. 2001, 2002). This model corresponds well with the results of the second experiment with KNO_3 as nitrogen source. NO_3^- uptake was reduced three days after MS application, one day after Gln content started to rise and NO_3^- content of MS-exposed roots was elevated (Fig. 4). A regulation of NO_3^- uptake by Asn can be ruled out, because its concentration did not change significantly. The present results indicate that NO_3^- uptake was also impaired by MS due to its influence on root Gln level and NO_3^- assimilation (GODON et al. 1996).

NO_3^-, which has not been assimilated in the roots, is translocated in the xylem and may reach the leaves, where it is assimilated into amino acids. In the third experiment, when MS was applied onto a leaflet, NO_3^- concentrations decreased in the soybean trifoliate three days after MS application, with the highest NO_3^- content remaining in the MS-treated leaflet (Fig. 8). The $[^{13}N]NO_3^-$ tracer experiment showed that the import of NO_3^- into the leaflets did not differ within a single trifoliate (Fig. 10). It follows that the different NO_3^- concentrations in the leaflets of a trifoliate cannot be explained by different rates of NO_3^- import, but by a more impaired NO_3^- assimilation in the MS-treated leaflet. This response could be expected, because the MS effect was detectable in the MS-treated leaflet one day earlier (Fig. 8). However, the low leaflet $[^{13}N]NO_3^-$ levels at the last day of the experiment also indicated that MS impaired NO_3^- transport (cf. Figs 9–10), probably due to an impairment of leaf transpiration (E). This effect of sulfonylurea herbicides, which has yet rarely been focussed in literature (MASHHADI et al. 1986), was proved by gas exchange measurements (cf. Fig. 7) and may represent an additional parameter that could easily be checked in the mentioned biological-chemical test system for the identification of sulfonylurea herbicide residues in the soil in rotational farming systems. Noteworthy, the reduction of E is not closely related to the amino acid metabolism in the leaflets, as the differences in nitrogen metabolism in the time response of single leaflets were not reflected in the modification of E and stomatal conductance (g_s). According to LEONHARDT et al (1997), sulfonylurea herbicides can modulate the stomatal movements by their effect on guard cell potassium channels. For example, glibenclamide and tolbutamide resulted in an increase of g_s. Thus, the immediate effect of these sulfonylurea herbicides differed from the observed reduction of g_s. Presumably, the observed narrowing of stomatal aperture did not represent an immediate MS effect. Future investigations of changes in the abscisic acid content may help to understand the observed response.

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References

ACKERSON, R.C. and L.A. DAVIS 1987: Metsulfuron methyl – new herbicide for use in rice and plantation crops. Proceedings, 11th Asian Pacific Weed Science Society Conference, Asian Pacific Weed Science Society, Taipei, Taiwan, pp.137–143.

BACANAMWO, M. and J.E. HARPER 1997: The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. Physiologia Plantarum **100**, 371–377.

BERGER, B. 1993: Bioassay for the determination of the degradation of metsulfuron-methyl in soil. Gesunde Pflanzen **45**, 259–263.

BLACKBURN, S. 1968: The chromatographic separation of amino acids. In: BLACKBURN, S. (ed.): Amino acid determination. Methods and techniques. Marcel Dekker, Inc., New York, USA, pp. 31–38.

COTTERILL, E.G. 1992: Determination of the sulfonylurea herbicides chlorsulfuron and metsulfuron-methyl in soil, water and plant material by gas chromatography of their pentafluorbenzyl derivates. Pestic. Sci. **34**, 291–296.

DELHON, P., A. GOJON, P. TILLARD and L. PASSAMA 1995: Diurnal regulation of NO_3^- uptake in soybean plants. I. Changes in NO_3^- influx, efflux, and N utilization in the plant during the day/night cycle. Journal of Experimental Botany **46**, 1585–1594.

DROBNY, H.G. 1984: DPX-T 6376 – a new herbicide for cereals. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, Sonderheft **10**, 305–309.

GALLETTI, G.C., A. BONETTI and G. DINELLI 1995: High-performance liquid chromatographic determination of sulfonylureas in soil and water. J. Chromatogr. **692**, 27–37.

GLASS, A.D.M., D.T. BRITTO, B.N. KAISER, H.J. KRONZUCKER, A. KUMAR, M. OKAMOTO, S.R. RAWAT, M.Y. SIDDIQI, S.M. SILIM, J.J. VIDMAR and D. ZHUO 2001: Nitrogen transport in plants, with an emphasis on the regulation of fluxes to match plant demand. Journal of Plant Nutrition and Soil Science **164**, 199–207.

GLASS, A.D.M., D.T. BRITTO, B.N. KAISER, J.R. KINGHORN, H.J. KRONZUCKER, A. KUMAR, M. OKAMOTO, S. RAWAT, M.Y. SIDDIQI, S.E. UNKLES and J.J. VIDMAR 2002: The regulation of nitrate and ammonium transport systems in plants. Journal of Experimental Botany **53**, 855–864.

GODON, C., A. KRAPP, M.-T. LEYDECKER, F. DANILE-VEDELE and M. CABOCHE 1996: Methylammonium-resistant mutants of *Nicotiana plumbaginifolia* are affected in nitrate transport. Molecular and General Genetics **250**, 357–366.

HERSHBERGER, L.W. and D.E. BRENNAN 1988: Metsulfuron methyl. Anal. Methods Pestic. Plant Growth Regul. **16**, 83–103.

ISHIOKA, N.S., H. MATSUOKA, S. WATANABE, A. OSA, M. KOIZUMI, T. KUME, S. MATSUHASHI, T. FUJIMURA, A. TSUJI, H. UCHIDA and T. SEKINE 1999: Production of positron emitters and application of their labelled compounds to plant studies. Journal of Radioanalytical and Nuclear Chemistry **239**, 417–421.

JIA, M.H. 1998: Development of immunoassay for sulfonylurea herbicide in China. Proceedings of an international workshop on seeking agricultural products free of pesticide residue, Feb. 17–19, 1998, Australian Center of International Agricultural Research, Yogyakarta, Indonesia, pp. 278–289.

KELLEY, M.M., E.W. ZAHNOW, W.C. PETERSEN and S.T. TOY 1985: Chlorsulfuron determination in soil extracts by enzyme immunoassay. J. Agric. Food Chem. **33**, 962–965.

KUME, T. 1999: Application of positron emitting tracer imaging system (PETIS) for plant research. Radioisotopes **48**, 37–47.

LEONHARDT, N., E. MARIN, A. VAVASSEUR and C. FORESTIER 1997: Evidence for the existence of a sulfonylurea-receptor-like protein in plants: Modulation of stomatal movements and guard cell potassium channels by sulfonylureas and potassium channel openers. Proceedings of the National Academy of Science of the United States of America **94**, 14156–14161.

MASHHADI, H., B. BUGBEE and J.O. EVANS 1986: The use of direct photosynthetic measurement in herbicide research. Proceedings of the Western Society of Weed Science 1986, 44–50.

MENGEL, K. 1991: Ernährung und Stoffwechsel der Pflanze. 7. überarbeitete Auflage, Gustav Fischer Verlag Jena, Germany, pp. 141–151.

OLOFSDOTTER, M., A. OLESEN, S.B. ANDERSEN and J.C. STREIBIG 1994: A comparison of herbicide bioassays in cell cultures and whole plants. Weed Research **34**, 387–394.

RUFTY, T.W.Jr., D.W. ISRAEL, R.J. VOLK, J. QIU and T. SA 1993: Phosphate regulation of nitrate assimilation in soybean. Journal of Experimental Botany **44**, 879–891.

SALISBURY, C.D. and B.W. BEAN 1998: Tolerance of imidazolinone resistant corn and sulfonylurea tolerant soybean to sulfonylurea herbicide residues after wheat. In: Preparing for the New Millenium. 51st Annual Meeting January 26–28, 1998, Birmingham, Alabama. Southern Weed Science Society (USA) **51**, p.19.

SATO, T., N. OHTAKE, T. OHYAMA, N.S. ISHIOKA, S. WATANABE, A. OSA, T. SEKINE, H. UCHIDA, A. TSUJI, S. MATSUHASHI, T. ITO and T. KUME 1999: Analysis of nitrate absorption and transport in non-nodulated and nodulated soybean plants with $^{13}NO_3^-$ and $^{15}NO_3^-$. Radioisotopes **48**, 450–458.

SIMPSON, D.M., E.W. STOLLER and L.M. WAX 1995: An in vivo acetolactate synthase assay. Weed Technol. **9**, 17–22.

SOUTHAN, M.D. and L. COPELAND 1996: Physical and kinetic properties of acetohydroxyacid synthase from wheat leaves. Physiologia Plantarum **98**, 824–832.

VEGA, D., J. BASTIDE and C. POULAIN 1992: Chemical or microbial degradation of sulfonylureas in the soil. II. Metsulfuron methyl. Weed Research **32**, 149–155.

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Addresses of authors: A. J. Keutgen (corresponding author), Institut für Agrikulturchemie (Agricultural Chemistry), Carl-Sprengel-Weg 1, D 37075 Göttingen, Germany, Tel. 0551/395565, Fax 0551/395570, Email: AKeutge@gwdg.de, N. Keutgen, M. Jia, S. Matsuhashi, C. Mizuniwa, T. Ito, T. Fujimura and S. Hashimoto, Department of Radiation Research for Environment and Resources, Takasaki Radiation Chemistry Research Establishment, JAERI, Takasaki, Gunma 370–1292, Japan and T. Sekine, Department of Materials Science, Takasaki Radiation Chemistry Research Establishment, JAERI, Takasaki, Gunma 370–1292, Japan