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# AMMONIUM ASSIMILATION IN RICE BASED ON THE OCCURRENCE OF $^{15}N$ AND INHIBITION OF GLUTAMINE SYNTHETASE ACTIVITY[1]

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***ABSTRACT***: Assimilation of ammonium ($NH_4$) into free amino acids and total reduced nitrogen (N) was monitored in both roots and shoots of two-week old rice seedlings supplied with 5 mM 99% $(^{15}NH_4)_2SO_4$ in aerated hydroponic culture with or without a 2 h preincubation with 1 mM methionine sulfoximine (MSX), an inhibitor of glutamine synthetase (GS) activity. $^{15}NH_4$ was not assimilated into amino acids when the GS/GOGAT (glutamate synthase) cycle was inhibited by MSX. Inhibition of glutamine synthetase (GS) activity in roots with MSX increased both the amount of $NH_4$ and the abundance of $^{15}N$ labeled $NH_4$. In contrast, the amount of Gln and Glu, and their proportions as $^{15}N$, decreased in roots when GS activity was inhibited. This research confirms the importance of GS/GOGAT in $NH_4$ assimilation in rice roots.

$^{15}N$-labeled studies indicate that $NH_4$ ions incorporated by roots of rice are transformed primarily into glutamine (Gln) and glutamic acid (Glu) before being

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converted to other amino acids through transamination (15). The formation of amino acids such as aspartic acid (Asp) and alanine (Ala) directly from free $NH_4$ in roots also has been reported (4,15). Translocation of free $NH_4$ to plant shoots, based on the concentration of free $NH_4$ in xylem exudate, has been reported in tomato (13), although $NH_4$ in shoots primarily originates from nitrate reduction in the shoot. Photorespiration also can contribute to the accumulation of $NH_4$ in leaves (7).

The GS/GOGAT cycle appears to be primarily responsible for the assimilation of exogenously supplied $NH_4$ and $NH_4$ derived from nitrate reduction in leaves, as well as $NH_4$ derived from photorespiration (2,3,6,8). Genetic evidence cited to support this conclusion includes the lethal effect of photorespiratory conditions on plant mutants deficient in chloroplast-localized GS and GOGAT activities (2,3,9), and the rapid accumulation of free $NH_4$ in GS-deficient mutants under photorespiratory conditions (2,3,5).

The present study was initiated to quantify the *in vivo* amino acid synthesis in rice roots and shoots by analysis of $^{15}N$ labeling, and should provide a more complete understanding of this important system for $NH_4$ utilization.

## MATERIALS AND METHODS

### Plant Growth Conditions

Rice (*Oryza sativa* L) cv "LaMont" was germinated in silica sand and irrigated twice a day with a modified Hoagland solution containing 5 mM N as $NH_4NO_3$. One-week- old seedlings were transplanted to aerated Hoagland solution containing 5 mM N as $NH_4NO_3$, 2.0 mM $K_2SO_4$, 2.0 mM $MgSO_4$, 1.0 mM $KH_2PO_4$, 25 μM $H_3BO_3$, 2.0 μM $MnSO_4$, 4.0 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $NaMoO_4$, 50 μM KCl, and 100 μM Fe-DTPA, where they were grown for one more week prior to $^{15}N$ labeling. The pH was adjusted to pH 5.7 with $CaCO_3$. All experiments were carried out in growth chambers at 26°C by growing plants with a 16-h photoperiod before treatment with the L-methionine sulfoximine (MSX) and $^{15}N$, and at continuous light during treatment at a photon flux rate of 450 μE $sec^{-1}m^{-2}$ at plant level.

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**Administration of $^{15}N$-labeled Ammonium and *Methionine Sulfoximine***

Rice seedlings were incubated in minus N nutrient solution with or without 1 mM MSX two hours prior to treatment with 5 mM 99% ($^{15}N$) $(NH_4)_2SO_4$. The pH of the solution was adjusted to pH 5.7 with $CaCO_3$ before incubation and immediately after adding the $(^{15}NH_4)_2SO_4$.

**Harvest and Extraction of Free Amino Acids**

Twenty seedlings of each treatment were harvested at 1, 8, and 24 h after $^{15}N$ treatment; washed with $H_2O$, blotted dry, separated into shoot and root tissues, cut in small pieces, mixed, and weighed. A 1 g sample of each plant part was homogenized in 10 ml methanol and incubated for 48 h at 4°C. The methanol extracts were phase separated by adding 5 ml chloroform and 6 ml $H_2O$, shaking vigorously and letting them stand 1-2 h at 4°C until phase separation. The upper aqueous layer was transferred to 6 dram vials with a disposable Pasteur pipette, concentrated to dryness under an air stream and dissolved in 2 ml $H_2O$. γ-Amino-n-butyric acid (250 nM) and α-aminoadipic acid (250 nM) were added as internal standards.

**Determination and Isolation of Ammonium**

Ammonium was isolated from the aqueous extracts by applying a 1 mL sample of the aqueous extracts to a 1.0 x 4.5 cm column of Dowex-HCRW 2 $Na^+$ ion exchange chromatography resin (20-50 mesh) equilibrated with $H_2O$. Neutral and acidic amino acids were eluted with 6 mL $H_2O$, and $NH_4$ was recovered by eluting with 40 mL 0.2 M $Na_2HPO_4/NaH_2PO_4$ buffer (pH 7.5). The $NH_4$ was subsequently distilled in a LABCONCO steam distillation apparatus after the addition of 5 mL 6 N NaOH and collected as 20 mL of distillate in 2 mL of 2.5 N HCl. The $NH_4Cl$ recovered was rotatory evaporated to dryness at 35°C and dissolved in 1 mL $H_2O$. $NH_4$ was determined in a 25 μL sample of the aqueous extracts by the phenol-hypochlorite reaction where $NH_4$ reagent "A" contained 10 g phenol and 50 mg sodium nitroprusside in 1 L $H_2O$; and reagent "B" contained 5 g NaOH, 2.3 g $Na_2HPO_4$, and 10 mL NaOCl (10-14%). The reaction tubes were incubated at 37°C for 35 minutes and the absorbency was read at 625 nm (12).

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**Determination of Total Nitrogen in Plant Material**

Samples of 50 to 100 mg dry weight of tissue were digested in Folin-Wu tubes containing 2 mL concentrated $H_2SO_4$ in a heating block at 120°C. The digested material was diluted to 50 mL with $H_2O$, and 5 mL aliquots were subjected to steam distillation after the addition of 6 mL of 6 N NaOH. $NH_4$ was collected as 20 mL of distillate in 2 mL 2.5 N HCl. The $NH_4Cl$ recovered was evaporated to dryness in a rotary evaporator and dissolved in 1 mL $H_2O$. Total N as $NH_4$ was determined by the phenol-hypochlorite reaction (12).

**Isolation and Purification of Amino Acids**

A 1-mL sample of the aqueous methanol extract was applied to a 1 x 2 cm column of Dowex 50-$H^+$, washed with excess $H_2O$ (10 mL), and the amino acids eluted with 6 mL of 6 M $NH_4OH$. The amino acid fractions were concentrated to dryness, dissolved in 2 mL $H_2O$, and applied to 1 x 2 cm columns of Dowex-1-acetate equilibrated with water. Neutral and basic amino acids were eluted with 6 mL $H_2O$, and the acidic amino acids with 6 mL of 2 M acetic acid. Both fractions were evaporated to dryness and dissolved in 0.4 mL of 60% methanol.

**Derivatization of Amino Acids**

Aliquots (0.4 mL) of the amino acids in 60% methanol were placed in 1 mL microreaction vessels and evaporated to dryness under a stream of air at room temperature. The samples were dried further with 0.1 mL of methylene chloride, and 0.2 mL of a freshly prepared solution of isobutanol-AcCl (1 mL of acetyl chloride mixed with 5 mL ice-cold isobutanol in a sealed vial at 4°C with continuous stirring) was added to each vial. The vials were sealed with a cap and Teflon coated septum, vigorously mixed, and then heated under reflux in a heating block at 120°C for 20 min. After cooling, the excess isobutanol-AcCl was evaporated under a stream of dry air and 0.1 mL heptafluorobutyric anhydride (N-HFBI) was added. The vials were again sealed and heated at 120°C for 10 min. After cooling, the samples were evaporated to incipient dryness under a stream of dry air and then dissolved in 50 mL of ethyl acetate : acetic anhydride (1:1 v/v). Routinely, 1 μL injections were utilized

for determining the concentration of individual amino acids by GC and for GC-MS analysis (10).

**Quantification of Amino Acids by Gas Chromatography**

One μL of the amino acid derivatives was subjected to GC analysis using an external standard amino acid mixture (Sigma AA-S-18) to derive the response factors required for quantification of amino acids (10). The concentration of individual amino acids in the acidic and neutral amino acid/amide fractions of plant extracts were quantified by coderivatization of 250 nM α-amino-n-butyric acid and 250 nM α-aminoadipic acid as internal standards. Separate analyses were performed on the acidic and neutral amino acid fractions to distinguish between glutamate and glutamine-amino N and between aspartate and asparagine-amino N.

Peak areas were determined by an interface of the gas chromatograph with "Data System" (Hewlett Packard). Peak areas were related to the area of the internal standard, and the response factors for each amino acid derivative were determined from GC of N-HFBI esters of an amino acid standard mixture (Sigma). Amino acid levels are expressed as *n*M $g^{-1}$ fresh weight (fwt).

**Gas Chromatography-Mass Spectrometry of N-HFBI Esters of Amino Acids**

A 1 μL aliquot of the amino acid derivatives diluted with ethyl acetate: acetic anhydride (1:1, v/v) was analyzed by GC-MS (GC-MS model HP5996, Hewlett Packard, Palo Alto, CA, equipped with a HP9876A graphic printer and HP1000 computer system) (10). All analyses were performed on a 0.22 mm x 30 m fused silica DB5 capillary column with helium carrier gas at 40 cm/s linear velocity, a source temperature of 200°C, an injector temperature of 250°C and an interface temperature of 280°C. The oven temperature program was 100°C to 270°C at 10°C $min^{-1}$ Analyses were performed in electron impact mode scanning over the mass range 200 to 400 atomic mass units throughout the chromatograms. The $^{15}N$ abundance of various amino acids were determined by plotting extracted ion current profiles and calculating current ratios as described previously (10). The ions monitored were Ala (240:241), Gly (226:227), β-Ala (268:269), Val (268:269), Thr (253:254), Ser (239:240), Leu and Ile (282:283), GABA (282:283 or 226:227), Pro (266:267), Asp

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or Asn-amino N (284:285), Glu or Gln (280:281 or 298:299), Phe (316:317), and Lys (280:281). The results are expressed both as nM $g^{-1}$ fresh weight (fwt) and as % of labeled $^{15}N$ in each of the various amino acids.

## RESULTS AND DISCUSSION

The free amino-N pool in roots was rapidly labeled by $^{15}NH_4$ assimilated from nutrient solution. Gln, Glu, Asp and GABA were the most heavily labeled amino acids in roots at the 1 h sampling time (Tables 1 and 2). These results are consistent with reports that Glu and Gln were the first products of $NH_4$ assimilation in rice, and that Glu, Gln, Asp and GABA were the main forms of newly absorbed N translocated from roots to shoots in rice (14). Glu and Asp were the most abundant amino-N in roots (Table 3). Serine was heavily labeled 8 h after $NH_4$ application and was quantitatively important in N assimilation by MSX treated plants, although Yoneyama (14) did not consider Ser an important amino acid for N-transport in rice. Asn and Ala were labeled by 8 h and Ala was as strongly labeled as Asp, Glu and Gln after 24 h (Table 1). Most other amino acids also were labeled by 24 h.

$^{15}N$ labeling of soluble amino-N fractions in roots was very low in MSX-treated plants (Table 2). Only GABA showed significant labeling during the first hour of treatment. At 8 and 24 h, some Glu and Gln also were labeled; however, the labeling of amino N in the soluble N pool of plants treated with MSX was essentially negligible, whereas the free $NH_4$ pool was heavily labeled and was about the same size as the free amino-N pool in untreated plants (Tables 1 and 2). These results indicate N-assimilation via the GS/GOGAT system. The absence of rapid labeling of Glu and Gln in roots of MSX treated plants indicates that glutamine dehydrogenase (GDH) plays only a minor role in $NH_4$ assimilation in rice plants, and is consistent with previous observations in corn (7). The labeling of a small amount of the Glu and Gln fractions which was observed in roots of MSX-treated seedlings could be attributable to GDH activity or through incomplete inhibition of GS by MSX. Labeling observed in the total-N fraction indicates that protein synthesis is going on with amino-N from newly absorbed N (Tables 1, 2) in both MSX treated and

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**TABLE 1**

Percent $^{15}N$ in amino acids, free $NH_4$ and total N in tissues of rice in the absence of MSX.

| Amino Acid | Hours in $^{15}NH_4$ (no MSX added) | | | | | |
|---|---|---|---|---|---|---|
| | Root | | | Shoot | | |
| | 1 | 8 | 24 | 1 | 8 | 24 |
| Ala | 0.0* | 55.6 | 67.0 | 0.0 | 22.3 | 40.6 |
| Gly | 4.1 | 10.8 | 29.0 | 0.0 | 1.8 | 15.7 |
| β-Ala | 0.0 | 0.0 | 27.6 | 2.1 | 5.4 | 11.2 |
| Val | 3.1 | 14.7 | 29.4 | 0.0 | 7.7 | 16.1 |
| Thr | 7.3 | 25.8 | 34.2 | 0.9 | 13.1 | 29.0 |
| Ser | 7.5 | 32.0 | 47.5 | 0.4 | 32.6 | 47.6 |
| Leu | 7.8 | 17.1 | 38.6 | 0.0 | 6.1 | 20.2 |
| Ile | 7.5 | 14.7 | 37.3 | 0.0 | 6.4 | 15.6 |
| Gaba | 36.0 | 63.6 | 72.5 | 5.0 | 41.8 | 48.1 |
| Pro | 6.7 | 25.5 | 28.1 | 0.7 | 10.6 | 20.1 |
| Met | 13.6 | 19.6 | 29.6 | 3.1 | 7.8 | 12.7 |
| Asn | 13.0 | 37.0 | 63.5 | 0.0 | 4.7 | 39.3 |
| Asp | 35.7 | 59.5 | 67.5 | 0.6 | 34.5 | 52.7 |
| Gln | 32.6 | 52.9 | 64.6 | 0.1 | 39.0 | 47.0 |
| Glu | 36.3 | 60.4 | 68.0 | 1.5 | 35.6 | 50.7 |
| Phe | 2.9 | 20.2 | 23.0 | 1.7 | 12.2 | 25.4 |
| Lys | 0.0 | 0.0 | 18.2 | 0.0 | 1.8 | 11.4 |
| Tyr | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 |
| $NH_4$ | 29.8 | 40.0 | 46.3 | 29.3 | 29.6 | 30.5 |
| Total N | 2.7 | 13.9 | 19.2 | 1.6 | 3.2 | 8.9 |

*All values are the average of duplicate analyses.

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**TABLE 2**

Percent $^{15}N$ in amino acids, free $NH_4$ and total N in tissues of $^{15}NH_4$-grown rice in the presence of 1 mM MSX.

| | Hours in $^{15}NH_4$ + MSX | | | | | |
|---|---|---|---|---|---|---|
| Amino | Root | | | Shoot | | |
| Acid | 1 | 8 | 24 | 1 | 8 | 24 |
| Ala | 0.0* | 5.1 | 4.0 | 0.0 | 0.0 | 0.0 |
| β-Ala | 0.6 | 0.7 | 1.9 | 1.3 | 0.2 | 0.0 |
| Val | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| Thr | 1.6 | 1.3 | 2.6 | 0.6 | 0.8 | 0.0 |
| Ser | 0.0 | 0.6 | 2.5 | 0.1 | 0.0 | 1.4 |
| Leu | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 | 2.7 |
| Ile | 0.3 | 0.2 | 1.7 | 0.0 | 0.0 | 0.0 |
| GABA | 2.6 | 10.6 | 8.2 | 3.6 | 1.5 | 3.1 |
| Pro | 0.5 | 0.3 | 0.9 | 0.4 | 0.5 | 0.5 |
| Met | 0.9 | 0.0 | 1.9 | 0.7 | 0.6 | 1.3 |
| Asn | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Asp | 0.0 | 0.0 | 1.2 | 0.0 | 0.0 | 0.0 |
| Gln | 0.8 | 3.8 | 6.3 | 0.0 | 0.8 | 3.7 |
| Glu | 0.0 | 4.5 | 5.9 | 0.0 | 0.0 | 0.2 |
| Phe | 0.0 | 0.0 | 2.1 | 1.7 | 3.3 | 3.9 |
| Lys | 1.1 | 1.5 | 1.3 | 0.7 | 0.4 | 2.1 |
| Tyr | 0.0 | 0.0 | 0.0 | 1.3 | 0.8 | 0.0 |
| $NH_4$ | 33.2 | 60.0 | 63.5 | 2.2 | 8.6 | 13.8 |
| Total N | 1.3 | 5.8 | 5.3 | 1.5 | 1.5 | 2.2 |

*All values are the average of duplicate analyses.

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nontreated seedlings. Therefore, the possibility that some Glu was formed through reductive amination via GDH can not be ruled out, but it appeared to play a minor role in N assimilation by rice seedlings. Concentrations of Glu and Gln in the absence of MSX averaged 2 to 3 fold higher than those in MSX-treated plants; although the concentration of these amino acids in both treatments decreased with time (0 to 24 h) after starting the experiment (Tables 3 and 4).

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**TABLE 3**

Total free amino acids in roots of rice treated with and without MSX.

| Amino | Hours in $^{15}NH_4$ + MSX | | | Hours in $^{15}NH_4$ (- MSX) | | |
|---|---|---|---|---|---|---|
| Acid | 1 | 8 | 24 | 1 | 8 | 24 |
| | nM $g^{-1}$ fwt | | | | | |
| Ala | 193 * | 123 | 151 | 196 | 253 | 650 |
| Gly | 70 | 77 | 82 | 33 | 39 | 48 |
| β-Ala | 44 | 37 | 27 | 0 | 0 | 19 |
| Val | 187 | 357 | 375 | 93 | 74 | 105 |
| Thr | 187 | 227 | 177 | 86 | 65 | 70 |
| Ser | 440 | 447 | 566 | 286 | 257 | 239 |
| Leu | 101 | 112 | 19 | 45 | 26 | 58 |
| Ile | 109 | 204 | 205 | 46 | 27 | 46 |
| Bca | 0 | 0 | 0 | 0 | 0 | 0 |
| Gaba | 122 | 42 | 0 | 85 | 106 | 185 |
| Pro | 68 | 40 | 61 | 36 | 48 | 81 |
| Met | 42 | 81 | 20 | 0 | 11 | 26 |
| Asn | 108 | 56 | 61 | 253 | 228 | 268 |
| Asp | 226 | 189 | 227 | 632 | 472 | 430 |
| Phe | 49 | 75 | 67 | 41 | 20 | 34 |
| Gln | 98 | 84 | 57 | 282 | 703 | 613 |
| Glu | 507 | 342 | 317 | 1204 | 1068 | 958 |
| Lys | 46 | 43 | 34 | 16 | 0 | 31 |
| Tyr | 10 | 22 | 27 | 0 | 12 | 16 |
| Total AA | 2607 | 2558 | 2473 | 3334 | 3409 | 3877 |
| $NH_4$ | 1722 | 3277 | 3992 | 252 | 315 | 336 |
| MSX | 1574 | 1654 | 1980 | - | - | - |

*All values are the average of duplicate analyses.

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**TABLE 4**

Total free amino acids in shoots of rice treated with and without MSX.

| Amino | Hours in $^{15}NH_4$ + MSX | | | Hours in $^{15}NH_4$ (without MSX) | | |
|---|---|---|---|---|---|---|
| Acid | 1 | 8 | 24 | 1 | 8 | 24 |
| | nM $g^{-1}$ fwt | | | | | |
| Ala | 1360 * | 1115 | 1312 | 1640 | 1807 | 1997 |
| Gly | 724 | 615 | 678 | 793 | 667 | 377 |
| B-Ala | 0 | 55 | 38 | 37 | 22 | 13 |
| Val | 370 | 1475 | 2681 | 208 | 204 | 182 |
| Thr | 495 | 925 | 922 | 566 | 297 | 289 |
| Ser | 319 | 355 | 1464 | 1243 | 1083 | 1364 |
| Leu | 183 | 848 | 1359 | 93 | 77 | 71 |
| Ile | 163 | 826 | 1620 | 72 | 66 | 51 |
| Bca | 8 | 0 | 13 | 24 | 8 | 0 |
| Gaba | 91 | 90 | 207 | 175 | 165 | 112 |
| Pro | 227 | 695 | 388 | 102 | 75 | 96 |
| Met | 30 | 111 | 213 | 25 | 14 | 5 |
| Asn | 177 | 224 | 329 | 780 | 308 | 266 |
| Asp | 489 | 431 | 404 | 1458 | 1565 | 706 |
| Phe | 160 | 629 | 900 | 73 | 72 | 55 |
| Gln | 191 | 130 | 307 | 413 | 581 | 362 |
| Glu | 1606 | 1567 | 1494 | 3858 | 3484 | 2804 |
| Lys | 29 | 193 | 149 | 18 | 13 | 14 |
| Tyr | 48 | 134 | 206 | 39 | 34 | 24 |
| Total AA | 6670 | 10418 | 14684 | 11617 | 10542 | 8788 |
| $NH_4$ | 4002 | 10947 | 25781 | 504 | 934 | 777 |
| MSX | 17 | 84 | 177 | - | - | - |

*All values are the average of duplicate analyses.

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Labeling patterns of free amino N pools in shoots in the absence of MSX showed the same trends as those in roots although some differences were noted. Glu, Gln, Asp and GABA were heavily labeled in roots at the 1 h sampling while $^{15}N$ labeling in shoots increased at 8 h (Table 1). These results confirm the role of these amino-N compounds in the transport of N from roots to shoots of rice seedlings as suggested by Yoneyama (14), and are consistent with the hypothesis that $NH_4$ assimilation occurs primarily in the roots of plants. The labeling of free $NH_4$ in shoots before most amino-N is labeled (Table 1) clearly shows the direct translocation of some free $NH_4$ from roots to shoots before incorporation into amino acids in the absence of MSX. Direct transport of free $NH_4$ from roots to shoots in guinea grass varied from 7-15% of the N in xylem exudates (11).

$^{15}N$ labeling in shoots of MSX-treated plants appears first in GABA fractions and, most intensively, in the $NH_4$ fraction (Table 2). Gln was labeled only at 8 and 24 h after beginning the experiment, whereas Phe, β-Ala, Tyr, Lys, Met, Pro, Thr and GABA were labeled during the first hour of treatment (Table 2). Labeling of free $NH_4$ begins during the first hour and is higher than the total concentration of amino compounds in the presence of MSX (Table 2).

Analysis of the free amino-N pool in shoots clearly shows the effectiveness of MSX in inhibiting GS activity and its consequent effect on the composition of the free amino-N pool (Tables 3 and 4). Composition of part of this pool was due to N metabolism in the cells before $NH_4$ application to the nutrient solution. There was a reduction in levels of Glu, Gln and Asp in plants treated with MSX, and a large increase in the free $NH_4$ pool as compared to the minus MSX treatment (Table 4). The accumulation of $NH_4$ in the MSX-treated shoot apparently was not all the result of direct transport from roots since the % $^{15}N$ in the shoot remained relatively low (Table 2). It is possible that the accumulation might be attributable to a high rate of protein turn-over and photorespiration due to treatment with MSX; however, SDS polyacrylamide gel electrophoresis analysis showed no evidence of protein degradation (data not shown).

Based on this research, we conclude that the GS/GOGAT system is the primary means of N assimilation in rice as shown for other plants (1,8,6). These data also show the translocation of some free $NH_4$ from roots to shoots of rice seedlings although the importance of that transport to N assimilation in shoots could not be ascertained from this experiment. Glu, Gln, Asp and GABA are the main amino acids translocated from roots to shoots of rice, and GABA may play a special role in the assimilation of N in rice seedlings.

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