



Anaerobic nitrate and ammonium metabolism in flood-tolerant rice coleoptiles

Teresa W-M. Fan^{1,5}, Richard M. Higashi², Thomas A. Frenkiel³ and Andrew N. Lane⁴

¹ Department of Land, Air and Water Resources, University of California, Davis, CA 95616, USA

² Crocker Nuclear Laboratory, University of California, Davis, CA 95616, USA

³ MRC Biomedical NMR Centre, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

⁴ Division of Molecular Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Received 12 February 1997; Accepted 14 May 1997

Abstract

The tolerance of germinating rice seedlings to anaerobiosis cannot be fully accounted for by ethanolic fermentation alone. Nitrate metabolism (nitrate reduction to NH_4^+ plus its subsequent assimilation) may provide an additional sink mechanism for excess protons and NADH produced during anaerobiosis. To follow the fate of nitrate, ^{15}N -labelled nitrate and ammonium incorporation in aerobic and anaerobic rice coleoptiles was examined using ^{15}N -edited ^1H NMR and gas chromatography-mass spectrometry methods. After 22 h of treatments, protein-free Ala, Glu, Gln, and γ -aminobutyrate were the main ^{15}N -labelled products for both nitrate and ammonium-treated anaerobic rice coleoptiles, with Gln, Glu, and Ala being the most enriched. The total amount of ^{15}N label incorporation into Ala and GAB increased significantly in response to anaerobiosis. The ^{15}N -labelling pattern of Glu and Gln suggests that the GS/GOGAT system was primarily involved in ammonium assimilation whereas Glu dehydrogenase may play a role in nitrate assimilation. ^{15}N incorporation into protein-derived amino acids was also significant and was more substantial in anaerobic than in aerobic rice coleoptiles, which indicate that protein biosynthesis remained active in anaerobic rice coleoptiles. Thus, anaerobic assimilation of inorganic N into amino acids, particularly Ala and Glu/GAB, may serve to supplement ethanolic fermentation in

sustaining glycolysis and energy production in rice coleoptiles.

Key words: ^{15}N tracer, nitrate metabolism, ammonium assimilation, anaerobiosis, rice.

Introduction

When aerobic organisms are deprived of O_2 , mitochondrial oxidative phosphorylation is inhibited while anaerobic glycolysis to fermentation pathways become activated, leading to the accumulation of products such as ethanol, lactate, Ala, succinate, γ -aminobutyrate (GAB), glycerol, and malate (Crawford, 1978; Davies, 1980). The concerted activity of glycolysis and fermentation allows limited ATP synthesis to continue by substrate-level phosphorylation while regenerating NAD^+ and removing excess protons. The importance of ethanolic fermentation is that it is more efficient on a molar basis than lactic fermentation in regenerating NAD^+ and consuming protons. Hence, organisms capable of ethanolic fermentation (e.g. plants, microbes, invertebrates, some fishes) are more tolerant of prolonged O_2 deficiency than those dependent on lactic fermentation (e.g. mammals). Nevertheless, ethanolic fermentation alone is inadequate to meet the normal metabolic demands of aerobic organisms. Eventually, NADH build-up, cytoplasmic acidosis,

⁵ To whom correspondence should be addressed. Fax: +1 916 752 1552. E-mail: twfan@ucdavis.edu.

Abbreviations: GAB, γ -aminobutyrate; GC-MS, gas chromatography-mass spectrometry; PCA, perchloric acid; MTBSTFA, *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetamide; TOCSY, total shift correlation spectroscopy; HSQC, heteronuclear single quantum coherence; DSS, 2,2'-dimethylsilapentane-5-sulphonate; GS/GOGAT, glutamine synthetase/glutamate synthase; GDH, glutamate dehydrogenase.

and ATP depletion lead to decreased metabolic rates and cell injury (Hochachka and Mommsen, 1983; Garlick *et al.*, 1979).

Rice seedlings are among the few terrestrial plants that are able to survive under prolonged O₂ deficiency resulting from flooding. In particular, rice coleoptiles have the unusual ability to expand under anaerobic conditions (Fan *et al.*, 1992; Öpik, 1973). Several fermentation reactions are operative in anaerobic rice coleoptiles, resulting in the accumulation of ethanol, Ala, succinate, GAB, and lactate (Menegus *et al.*, 1988, 1989; Fan *et al.*, 1986a). Clearly, these reactions account for an important part of the anaerobic tolerance of rice coleoptiles. However, this picture of the anaerobic response in rice is by no means complete. For example, externally supplied nitrate promotes rice seed germination and seedling emergence (Hagiwara and Imura, 1991) as well as improves hypoxic growth of rice and several other seedlings (Prioul and Guyot, 1985; Trought and Drew, 1981; Malavolta, 1954; Arnon, 1937). In addition, nitrate is stored in appreciable amounts in rice seeds, and mobilized and reduced in the coleoptiles during anaerobic germination (Reggiani *et al.*, 1993a).

Despite the interesting effects of nitrate on the germination and development of anaerobic rice seeds, the physiological and biochemical basis of these effects is still unclear. It has been recently demonstrated that ¹⁵N-nitrate was incorporated into protein-free amino acids in the coleoptile of anaerobically germinating seeds (Reggiani *et al.*, 1995), which suggests that reductive nitrate assimilation pathways are operative in rice seeds under anaerobiosis. Nitrate reduction to ammonium consumes four NAD(P)H (8 electrons) and six protons per reaction cycle (Kamin and Privalle, 1987; Guerrero *et al.*, 1981), which is much more efficient in regenerating NAD⁺ and consuming protons than other known fermentation reactions, including ethanol production. If sufficiently active, anaerobic nitrate metabolism can facilitate energy production and alleviate cytoplasmic acidosis. However, whether the extent of nitrate metabolism does contribute significantly to the overall fermentation capacity requires a detailed characterization of the nitrate assimilation pathway.

Here, ¹⁵N-edited ¹H-detected NMR spectroscopy and gas chromatography–mass spectrometry (GC–MS) was used to follow the metabolic fate of ¹⁵N-nitrate as compared to that of ¹⁵N-ammonium ion in rice coleoptiles under both aerobic and anaerobic conditions. Incorporation of ¹⁵N into both free and protein-derived amino acids was measured to estimate the amount of total label originated from ¹⁵N-nitrate or -ammonium. This, in turn, allowed the contribution of nitrate or ammonium metabolism to fermentation capacity in anaerobic rice coleoptiles to be assessed.

Materials and methods

Plant growth and treatments

Rice (*Oryza sativa* M201) seeds were obtained from the University of California Rice Research Station, Biggs, California. Sodium ¹⁵N-nitrate and ¹⁵N-ammonium sulphate (99% enriched) were purchased from Cambridge Isotopes Laboratory (Andover, MA). Rice seeds were sterilized, imbibed, and germinated according to our previous procedure (Fan *et al.*, 1992). Apical 3–5 mm shoots were excised from 3-d-old dark-grown rice seedlings and 1.7–3.5 g tissues (wet weight) were incubated in the dark at 25 °C in 200 ml each of the appropriate treatment buffer aerated with 0.2 μm-filtered CO₂-free air or He for 22–28 h. Treatment buffers contained 0.5 mM CaSO₄, 5 mM glucose, 5 mM NaHCO₃, 10 mM MES (2-[*N*-morpholino]ethanesulphonate), and either 5 mM Na¹⁵NO₃ or (¹⁵NH₄)₂SO₄ at pH 6.0, which was sterilized by passing through 0.22 μm cellulosic filters. After incubation, aerobic and anaerobic tissues were rinsed extensively with oxygenated and He-purged deionized water, respectively, before lyophilization. Each treatment was repeated separately at least twice using similar conditions with treatment durations of 22–28 h.

PCA and protein extractions

The lyophilized tissues were pulverized in a micro ball mill (B. Braun Instruments, Melsungen, Germany) to <3 μm particles and extracted with 5% ice-cold perchloric acid (PCA) as described previously (Fan *et al.*, 1986b). An aliquot of the PCA extract was lyophilized and derivatized with *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetamide (MTBSTFA, Regis Technologies, Morton Grove, IL) for analysis by GC-flame ionization detection (FID) (Fan *et al.*, 1993a) and GC–MS, and the rest was lyophilized for analysis by NMR. The same tissues were also extracted for total proteins with a buffer containing 62.5 mM TRIS-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 12.5 ppm bromophenol blue, pH 6.8 (BioRad sample buffer) and boiled for 10 min. After centrifugation, the protein extract was dialysed extensively against deionized water using a Spectrapor 1 kDa molecular weight cutoff membrane (Spectrum Medical Industries, Inc., Los Angeles, CA). An aliquot of the dialysed protein extract was then lyophilized, weighed (approximately 1 mg each), and digested in 6 N HCl plus 10 mM phenol and 2.5% thioacetic acid at 110 °C under vacuum for 24 h (Moore, 1972). The final digest was lyophilized and redissolved in 0.9 M HCl in 10% D₂O for NMR analysis, followed by lyophilization and derivatization of an aliquot with MTBSTFA for GC–MS analysis. The digestion conditions were optimized based on the recovery of individual amino acids from a digestion time-course (12, 24, and 36 h) of one of the protein extracts. The digestion conditions chosen yielded some free Gln which is usually reported to be lost from the digest (Moore, 1972). However, no Asn was observed, which is expected to be lost through digestion.

NMR and GC–MS analysis

Lyophilized tissue extracts were first dissolved in 100% D₂O for analysis by ¹H NMR including two-dimensional (2-D) TOCSY (total shift-correlation spectroscopy) for metabolite identification as described previously (Fan *et al.*, 1993b).

Tissue extracts were then re-lyophilized and redissolved in 0.9 M HCl in D₂O:H₂O (1:9) for analysis of the exchangeable protons. The protein digests were prepared similarly. Under these conditions, the exchange of ammonium protons was sufficiently slow to be observed with water suppression (Preece

and Cerdan, 1993). NMR spectra were recorded at 11.75 T and 14.1 T on Varian (Palo Alto, CA) UnityPlus and Unity NMR spectrometers, respectively. Simple one-dimensional (1-D) spectra were recorded using the pulsed gradient (Watergate) method for water suppression (Piotto *et al.*, 1992) to avoid effects of saturation transfer from the water protons. The acquisition time was 2 s and the relaxation delay was 3 s.

For selectively observing and identifying ^{15}N -labelled metabolites, both 1-D and 2-D ^1H - ^{15}N HSQC (heteronuclear single quantum coherence) and HSQC-TOCSY spectra were recorded on either spectrometer using inverse detection of ^{15}N , with and without ^{15}N decoupling at 10 °C to further reduce the NH exchange rate. In all cases, ^{15}N decoupling was produced using a GARP sequence (Shaka *et al.*, 1985) while the spin-lock field was generated with a MLEV-17 sequence (Bax and Davis, 1985) with a typical strength of 5 kHz and mixing times of 36–58 ms. With ^{15}N decoupling, the acquisition time was restricted by hardware to 0.15 s which limited the spectral resolution. However, with the ^{15}N -coupled spectra, the acquisition time was 2 s, which resolved the fine splitting due to ^1H - ^1H coupling and provided additional information on the identity of NH (see below). The recycle time for 1-D experiments was 4 s. For 2-D HSQC-TOCSY (Bax and Pochapsky, 1992; Wider and Wüthrich, 1993), phase-sensitive data were recorded using the method of States *et al.* (1982) with Watergate (Piotto *et al.*, 1992) for water suppression. Proton chemical shifts were referenced to the C-1H of glucose at 5.24 ppm (as relative to that of DSS (2,2'-dimethylsilapentane-5-sulphonate)). Nitrogen chemical shifts were referenced indirectly to liquid ammonia using the proton shifts and the ratio of the gyromagnetic ratios of N and H (Live *et al.*, 1984).

Derivatized tissue extracts were analysed on a Varian 3400 gas chromatograph fitted with a 0.18 mm i.d. \times 40 m open-tubular column of 0.25 μm DB-1 stationary phase (permethylsiloxane) coat (J&W Scientific, Rancho Cordova, CA), that was fed through a line-of-site interface to a Finnegan (San Jose, CA, USA) ITD-806 mass spectrometer. GC temperatures were: injector = 260 °C, interface = 290 °C, column = 60 °C initial, held for 2.0 min, ramped to 150 °C at 20 °C min^{-1} , then to 290 °C at 6 °C min^{-1} , splitless valve closed for 1.5 min, then open for the duration of run. Hydrogen carrier gas, at 40 cm s^{-1} , was used in conjunction with the EI function of the ITD-806; this resulted in classical electron ionization mass spectra with hydrogen gas. MS parameters were: manifold temperature = 220 °C, electron energy = 70 eV, filament current = 10 μA with beam restrictor removed, automatic gain control = 99 amu, scan range = m/z 150–500, 4 full scans s^{-1} averaged into 1 spectrum s^{-1} , mass calibration and tune performed using perfluorobutylamine. A mass defect of 1.0 mmu/amu was applied to all spectra.

Isotope enrichment calculation

NMR analysis: The 1-D water-suppressed ^1H NMR spectra of tissue extracts (Fig. 3A) and protein digests in 0.9 M HCl were obtained for estimating the $\%^{15}\text{N}$ of GAB and NH_4^+ . This was achieved by comparing the peak area of the ^{15}N coupled proton satellites with that of the ^{14}N coupled proton resonances. The ratio of the area of the satellites to that of the total NH resonance ($^{15}\text{N} + ^{14}\text{N}$) represents the % enrichment in ^{15}N . Overlap of the NH resonance of Ala, Glu, and Gln precluded the use of this method for these amino acids. However, the three-bond coupling from ^{15}N to the methyl protons of Ala in the tissue extract spectra was sufficient to apply a similar procedure to measure the $\%^{15}\text{N}$ enrichment of Ala in D_2O (data not shown). The relative amount of ^{15}N label incorpora-

tion into Ala and GAB was calculated based on their NH peak areas in the 1-D HSQC spectra (Fig. 3B, C). The amount of ^{15}N label in ammonium ion of the protein digests was estimated by multiplying the $\%^{15}\text{N}$ enrichment by the content. The ammonium content ($[\text{NH}_4]$) of the digests was derived from the peak intensities of ammonium ion and Ala in the water-suppressed spectra:

$$[\text{NH}_4] = [\text{Ala}] \times 0.25 \times I(\text{NH}_4) / I(\text{Ala}) \times 3 \quad (1)$$

where $I(\text{NH}_4)$ is the sum of the intensities of the ammonium resonances, and $I(\text{Ala})$ is the intensity of the alanine methyl resonance and Ala content ($[\text{Ala}]$) was obtained from GC-MS analysis.

GC-MS analysis: ^{15}N enrichment analyses by GC-MS were performed as follows, using instrument conditions identical to that described above, and 20 scans were averaged in each case to obtain the mass spectrum. First, natural abundance isotope ratios were obtained for the 'pseudomolecular ion' (pM) cluster of a given analyte, using authentic standards derivatized by MTBSTFA. This pM is the M-57 ion, representing the highly-favoured loss of the *tert*-butyl group from the molecular ion (cf. Mawhinney and Madsen, 1982; Anderson *et al.*, 1987). Thus, in all cases, a high yield of pM was obtained, which contains the entire target analyte. Since at natural abundance the predominant isotope of N is ^{14}N (99.63%), this pM cluster effectively represents the ^{14}N amino acid. Then, samples from the ^{15}N -enriched experiments were analysed in identical fashion. Subtraction of natural abundance (standard) from enriched (sample) pM cluster (each normalized to the pM) yielded a spectrum representing only the ^{15}N -labelled amino acid (Fig. 4). From these two spectra, $\%^{15}\text{N}$ enrichment was then:

$$\%^{15}\text{N} = \text{height of } pM^* / (\text{height of } pM + \text{height of } pM^*)$$

where pM = pseudomolecular ion from the ^{14}N spectrum, pM^* = pseudomolecular ion from the ^{15}N (difference) spectrum. For double- ^{15}N -labelled compounds such as Gln**, the above process was repeated on isotope peaks of one mass unit larger. Linearity of isotope ratio response was verified by successively diluting the sample containing the highest ^{15}N enrichment (that from the $^{15}\text{NH}_4^+$ /anaerobic experiment) with natural abundance standards, followed by analysis in identical fashion (data not shown). ^{15}N label incorporation in $\mu\text{mol g}^{-1}$ dry wt was calculated from the product of $\%^{15}\text{N}$ and content of a given amino acid (with the multiplication factor of 2 for doubly labelled Gln and Asn). The total $\%^{15}\text{N}$ enrichment was calculated from the ratio of total ^{15}N label incorporation and content of total N in amino acids from both PCA and protein fractions.

For the determination of $\%^{15}\text{N}$ enrichment at the N-2 (α -amino) and N-5 (δ -amido) positions of Gln, the m/z 198 ion cluster which was fragmented from the tri-silylated Gln was also analysed. This cluster exhibited an enrichment in the m/z 199 ion, but not in the m/z 200 ion, in both singly and doubly ^{15}N labelled Gln, which showed that only one of either N-2 or N-5 nitrogens were contained in this fragment ion. More importantly, ^{15}N labelled Glu also resulted in the enrichment of the m/z 199 ion (data not shown), which is consistent with the m/z 198 ion cluster containing the N-2 instead of the N-5 nitrogen. Additionally, non-labelled tri-silylated Gln gave m/z 198–200 ion ratios very similar to the theoretical ion ratios of the expected fragment ion structure ($\text{C}_{10}\text{H}_{21}\text{NOSi}$) containing only the N-2 nitrogen (data not shown). Since the ion trap MS instrument gave very stable ion intensities and ratios under full-scan conditions for the fragments of the tri-silylated Gln, the m/z 198 ion cluster was employed instead of the more typically used fragment ions from the tetra-silylated Gln and selected

ion monitoring (Anderson *et al.*, 1987; Gibbs, 1992; Williams and Wolfe, 1994).

The m/z 198 ion cluster was analysed in identical manner described above for the pM cluster. The %Gln* at the N-2 (%Gln*^{N-2}) and the N-5 (%Gln*^{N-5}) positions was computed by (Gibbs, 1992):

$$\%Gln^{*N-2} = \%Gln^{total\ N-2} - \%Gln^{**}$$

$$\%Gln^{*N-5} = \%Gln^{*} - \%Gln^{*N-2}$$

$$\%Gln^{total\ N-5} = \%Gln^{*N-5} + \%Gln^{**}$$

where %Gln* is the % singly-labelled Gln in the N-2 plus the N-5 positions, %Gln** is the % doubly-labelled Gln, %Gln^{total N-2} is the %Gln* + Gln** at the N-2 position, and %Gln^{total N-5} is the %Gln* + Gln** at the N-5 position.

Results

Effect of anaerobiosis and N treatments on selected metabolite content

The effect of anaerobiosis on the content of major rice coleoptile metabolites was measured in the presence of either nitrate or ammonium ions. These metabolites were identified using the ¹H TOCSY spectra and quantified by GC-FID as described previously (Fan *et al.*, 1993, 1986b). Under both nitrate and ammonium treatments, ethanol, Ala, succinate, GAB, and lactate accumulated in anaerobic rice coleoptiles (Table 1), with the first four being the major products. A large quantity of ethanol was also observed in the medium, which was presumably excreted from the tissues. These results were qualitatively similar to those obtained in the absence of exogenous nitrogen

sources (Menegus *et al.*, 1988, 1989; Fan *et al.*, 1986a). The anaerobic accumulation of ethanol, GAB, and especially Ala was greater under ammonium than under nitrate treatments. In addition, the levels of Gln and Asn were substantially higher with ammonium than with nitrate treatments under both aerobic and anaerobic conditions (Table 1). This enhancement has also been reported in other studies (Pilbeam and Kirkby, 1992). Similar results were observed in three separate experiments (data not shown).

The amino acid content of rice coleoptile protein digests was also analysed for both nitrate and ammonium treatments as shown in Table 2. A similar content of major protein-derived amino acids were observed for the two N treatments under both aerobic and anaerobic conditions. Interestingly, anaerobiosis induced a comparable increase (at least 2-fold) in the content of protein-derived amino acids for both N treatments. These results were in contrast with the differences in free amino acid content (Table 1).

Identification of ¹⁵N-labelled metabolites by NMR and GC-MS

To determine the fate of exogenous nitrate and ammonium in rice coleoptiles, we supplied the tissues with either ¹⁵N-labelled nitrate or ammonium and analysed the tissue extracts and protein digests for ¹⁵N-labelled products by NMR and GC-MS. Instead of the conventional, insensitive ¹⁵N-detected NMR method (Menegus *et al.*, 1993), the ¹⁵N-edited HSQC method was used,

Table 1. Effects of anaerobiosis on the content of free amino acids and fermentation products in rice coleoptiles under two N treatments

Treatment conditions were as described in Materials and methods. Tissue metabolite content ($\mu\text{mol g}^{-1}$ dry wt) were determined by GC as described previously (Fan *et al.*, 1993a), nd denotes not-detected; values in parenthesis represent ethanol in the medium. Similar results were obtained from three separate experiments.

Compound	Treatments			
	Air/ ¹⁵ N-nitrate	He/ ¹⁵ N-nitrate	Air/ ¹⁵ NH ₄ ⁺	He/ ¹⁵ NH ₄ ⁺
Fermentation products				
ethanol	nd (nd)	126.6 (10321.7)	nd (nd)	189.0 (15728.6)
lactate	11.7	17.5	7.8	12.5
succinate	6.8	134.3	3.4	111.0
Ala	52.1	166.5	66.9	491.7
GAB	0.3	36.1	1.2	70.8
Glu	41.5	48.8	19.5	16.0
Other metabolites				
Asn	6.7	16.0	99.9	30.5
Asp	15.0	11.7	26.5	11.3
Gln	13.7	9.0	85.1	32.8
Gly	5.3	15.1	5.2	17.4
Ile	3.2	10.7	2.0	8.6
Leu	7.1	15.2	8.8	12.7
Met	11.7	18.7	13.4	20.3
Phe	2.4	6.5	2.8	5.5
Pro	4.5	10.0	4.6	8.7
Ser	16.2	21.4	15.2	26.2
Thr	5.6	22.5	17.4	6.4
Val	6.9	21.8	12.9	24.2

Table 2. Effects of anaerobiosis on the content of protein-derived amino acids in rice coleoptiles under two N treatments

Treatment conditions and protein digestion were as described in Materials and methods. Amino acid content ($\mu\text{mol g}^{-1}$ dry wt) of protein digests was determined by GC-MS as described in Materials and methods; NH_4^+ content was estimated from NMR analysis.

Compound	Treatments			
	Air/ ^{15}N -nitrate	He/ ^{15}N -nitrate	Air/ $^{15}\text{NH}_4^+$	He/ $^{15}\text{NH}_4^+$
Ala	71.1	140.6	88.6	167.1
Asp	46.6	104.3	55.2	133.7
Gln	6.6	20.7	8.2	29.3
Glu	12.3	43.5	17.5	69.6
Gly	71.4	136.1	94.1	162.9
Ile	29.4	60.7	36.7	71.6
Leu	178.0	421.6	209.5	389.7
Pro	31.3	68.8	37.4	80.7
Ser	40.7	80.2	45.3	95.9
Tyr	2.1	7.1	2.8	8.5
Val	48.0	91.6	56.7	110.7
NH_4^+	126.7	262.1	151.8	280.0

which selected only those protons directly bonded to ^{15}N and provides up to a 1000-fold enhancement in sensitivity. Also, when combined with ^1H TOCSY (Bodenhausen and Ruben, 1980), this method allows structure identification of both known and unknown ^{15}N -labelled compounds directly in crude extracts.

Figure 1 shows a representative 2-D ^{15}N - ^1H HSQC-TOCSY spectrum obtained from a crude extract of ^{15}N -ammonium-treated aerobic rice coleoptiles. Also shown in Fig. 1 is the corresponding 1-D ^{15}N - ^1H HSQC-TOCSY spectrum. One major cross-peak at 7.62 ppm (k, in the ^1H dimension) of the extract spectrum was connected to three other prominent cross-peaks at 3.15 (f), 2.62 (d), and 2.06 ppm (b), which by comparison with the standard spectrum corresponded to the γ -NH, γ -CH₂, α -CH₂, and β -CH₂ of GAB, respectively. Similarly, the three cross-peaks at 8.19 (l), 4.25 (h), and 1.60 (a) ppm were assigned to the α -NH, α -CH, and β -CH₃ of Ala, respectively while the four cross-peaks at 8.29 (n), 4.18 (g), 2.58 (e), and 2.24 (c) ppm were assigned to the α -NH, α -CH, γ -CH₂, and β -CH₂ of Gln (the γ -NH signal of Gln was not detected). Moreover, two more peaks at 7.16 (j) and 8.24 ppm (m) were noted. Peak j was assigned to ammonium based on the ^1H chemical shift (22) and lack of ^1H - ^1H correlations, and the other (peak m), to the α -NH of Glu based on the ^1H chemical shift (Fig. 1). The lack of ^1H connectivity of the m proton to the rest of the Glu protons was presumably due to its fast exchange in water. Additional confirmation of these assignments was obtained from the ^{15}N chemical shifts (Martin, 1985), one-bond NH coupling constants, and fine splitting structure from proton-proton coupling (Fig. 3A). For example, the doublet of the NH resonance of Ala is consistent with its coupling to the α -CH whereas the triplet of the NH resonance of GAB represents coupling to the γ -CH₂. Except for the ammonium ion, the identity of these ^{15}N -labelled metabolites was confirmed by GC-MS analyses of the same extract samples.

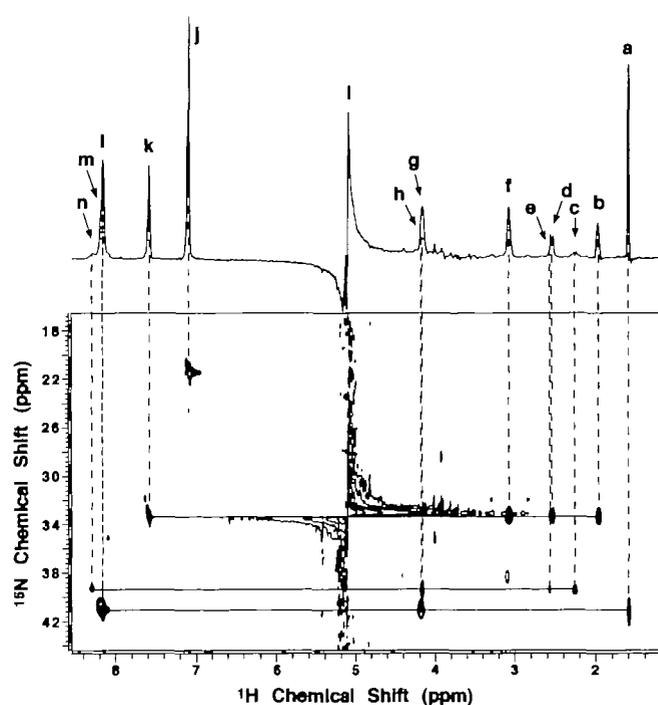


Fig. 1. 2-D ^{15}N - ^1H HSQC-TOCSY spectrum of the PCA extract of ^{15}N -ammonium-treated rice coleoptiles. Both 1-D and 2-D HSQC-TOCSY spectra were recorded at 10°C and at 11.75 T using a spin-lock field strength of 5.3 kHz for a duration of 58 ms as described in Materials and methods. Additional parameters for the 2-D spectrum included a spectral width of 2 kHz in the N dimension and 6 kHz in the H dimension, 128 increments each with 96 acquisitions, and a relaxation delay of 3 s. The final data matrix was zero-filled to 8192 by 1024 complex points, and apodized using a mild Gaussian function in both dimensions. Respectively, peaks a, h, and l denote β -CH₃, α -CH, and α -NH₃⁺ of Ala; b, d, f, and k, β -CH₂, α -CH₂, γ -CH₂, and γ -NH₃⁺ of GAB; c, e, g, and n, β -CH₂, γ -CH₂, α -CH, and α -NH₃⁺ of Gln; m, α -NH₃⁺ of Glu; j, NH_4^+ ; i, residual H₂O.

The protein digests were analysed by 2-D ^{15}N - ^1H HSQC spectroscopy for ^{15}N labelled amino acids and a representative spectrum for the ammonium/air treatment is shown in Fig. 2. A total of ten cross peaks were resolved

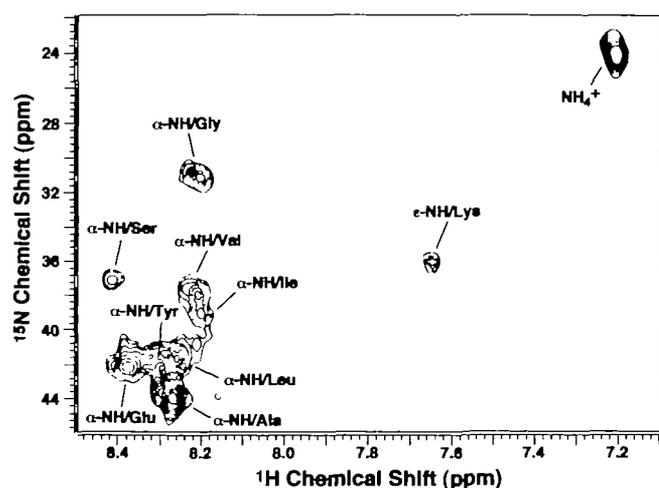


Fig. 2. 2-D ^{15}N - ^1H HSQC spectrum of protein digest from ^{15}N -ammonium-treated aerobic rice coleoptiles. The spectrum was recorded at 11.7 T and 10°C using Watergate (Piotto *et al.*, 1992) for water suppression, and GARP for ^{15}N decoupling during the proton acquisition. The spectral widths were 5000 Hz in F2 and 2500 Hz in F1. 128 complex points were collected in t_1 and the acquisition time in t_2 was 0.147 s. The data matrix was zero-filled to 4096 by 2048 complex points, and apodized using a Gaussian function in both dimensions, prior to Fourier transformation.

in the spectrum, which were assigned to NH_4^+ , ϵ -NH of Lys, and α -NH of Gly, Leu, Ile, Val, Ala, Glu, Ser, and Tyr, based on their chemical shifts, ^{15}N - ^1H connectivity as shown, and ^1H - ^1H TOCSY connectivity (data not shown). NH_4^+ was presumably the hydrolytic product of Asn and Gln during acid digestion. Except for Lys, the presence of ^{15}N label in all of the amino acids were confirmed by GC-MS analysis. Lys yielded no discernible GC-MS peaks, possibly due to a poor derivatization under the conditions used.

Quantification of ^{15}N -enrichment in amino acids of rice coleoptiles

Figure 3A illustrates two examples of 1-D water-suppressed ^1H NMR spectra of anaerobic rice coleoptile PCA extracts, which were acquired without ^{15}N editing. Consequently, all ^{15}N and ^{14}N -coupled protons were observed. The two relatively intense resonances at 7.62 ppm and 8.19 ppm were assigned to GAB and Ala, respectively (see above). These resonances consisted of a broad central component and two satellites spaced by 74 Hz due to the one-bond ^{15}N - ^1H coupling. The areas of these satellites compared with the total peak integral provided an estimate of the % ^{15}N -enrichment (see Materials and methods).

In comparison, the ^{15}N -edited 1-D HSQC spectra (Fig. 3B, C) were greatly simplified because only those protons that are bonded to ^{15}N were observed. Using this method, the pattern of ^{15}N -labelled amino acids in ^{15}N -ammonium- and -nitrate-treated rice coleoptiles was compared. By acquiring spectra at 10°C with a long recycle

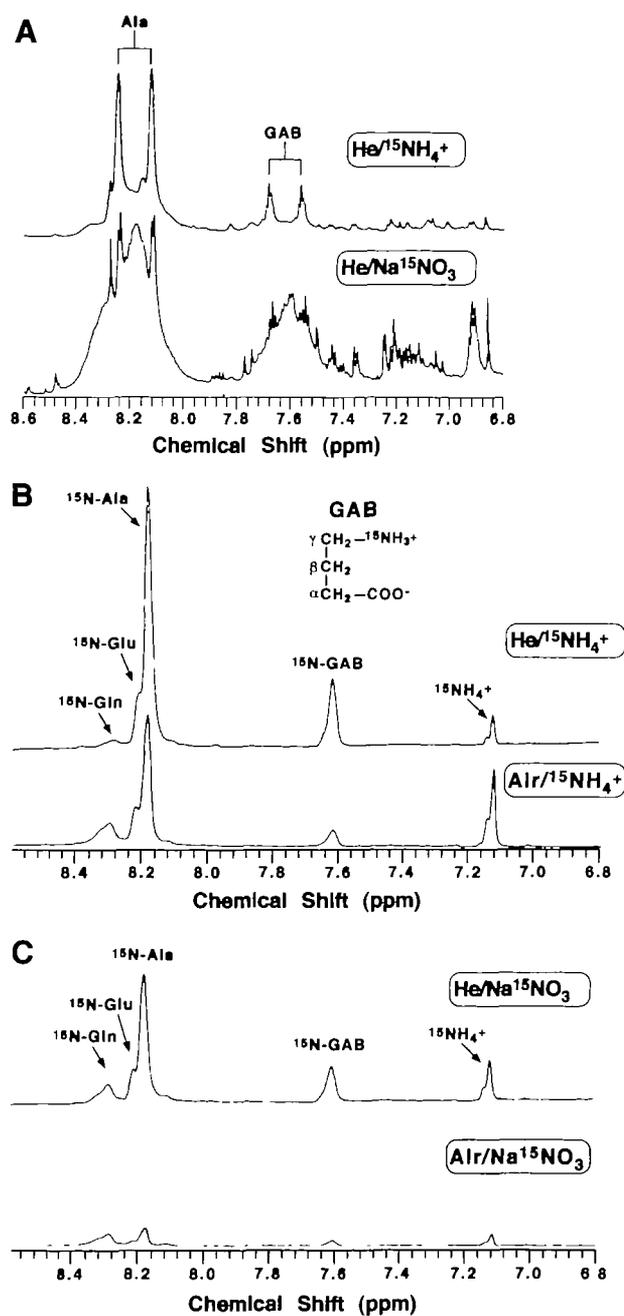


Fig. 3. 1-D water-suppressed ^1H and HSQC NMR spectra of PCA extracts of rice coleoptiles grown on different nitrogen and O_2 supplies. All spectra were recorded at 10°C in 90% $\text{HCl}/10\%$ D_2O at 14.1 T with a spectral width of 6 kHz, using Watergate for water suppression (Piotto *et al.*, 1992). Additional parameters for the HSQC spectra included a delay of $1/J$ (6.7 ms), acquisition time of 0.15 s, and relaxation delay of 2.5 s. The free induction decays were apodized using a 0.5 Hz line-broadening exponential function. (A) Water-suppressed spectra (without ^{15}N -editing) from anaerobic ^{15}N -ammonium and nitrate treatments. (B) ^{15}N -decoupled HSQC spectra from ^{15}N -ammonium/aerobic (lower) and ^{15}N -ammonium/anaerobic (upper) treatments. (C) ^{15}N -decoupled HSQC spectra from ^{15}N -nitrate/aerobic (lower) and ^{15}N -nitrate/anaerobic (upper) treatments, Y-scale expanded 10-fold relative to that in (B). Spectra (B) and (C) were directly comparable by normalizing to spectral parameters and tissue mass.

time (4 s) and using the Watergate method for water suppression (Piotto *et al.*, 1992), signal attenuation due to incomplete relaxation and saturation transfer from water to the NH resonances was minimized. Thus, the signal intensity for Ala and GAB in the 1-D HSQC spectra (Fig. 3B, C) was proportional to their total ^{15}N label, i.e. the product of $\%^{15}\text{N}$ enrichment and content. However, for Gln and Glu, their α -NH signal intensities were distorted somewhat due to a faster exchange. In addition, the γ -NH of Gln was presumably in such a fast exchange at low pH that the signal was not observed. It should be noted that a significant fraction of the GAB peak from aerobic nitrate treatment in Fig. 3C was attributed to the natural abundance of ^{15}N (0.37%) since negligible selective enrichment was detected using the GC-MS method (cf. Fig. 5).

Figure 3B also compares the ^{15}N decoupled-HSQC spectra for aerobic and anaerobic rice coleoptiles after 22 h of ammonium treatments. In both cases, signals arising from Ala, Glu, and GAB dominated the spectra, indicating a substantial incorporation of ^{15}N label into these amino acids. The presence of $^{15}\text{NH}_4^+$ signal was expected and indicates the uptake of ammonium ion from the medium. Moreover, the three amino acid signals increased in response to anaerobiosis. In contrast, with nitrate as the N source, the aerobic incorporation of ^{15}N into Ala, Glu, and GAB was low (Fig. 3C). However, under anaerobic conditions, the amount of ^{15}N -enrichment in these amino acids increased substantially. The presence of $^{15}\text{NH}_4^+$ signal indicates that $^{15}\text{NO}_3^-$ was taken up and reduced to ammonium ion in rice coleoptiles.

A similar set of 1-D ^1H spectra were also acquired for the protein digests from all treatments (data not shown). Due to a significant overlap, it was difficult to discern the α -NH signals arising from individual amino acids. However, regardless of the oxygen status, the signal intensities of the 1-D HSQC spectra were generally greater for the ammonium than for the nitrate treatments, indicating a higher amount of ^{15}N label incorporation into proteins for the former.

Except for the ammonium ion, quantification of ^{15}N -label ($\%^{15}\text{N}$ and total ^{15}N) in free and protein-derived amino acids was obtained from the GC-MS data, where all component peaks were well-resolved. A typical GC-MS chromatogram of the PCA extract of anaerobic rice coleoptiles is shown in Fig. 4A. Also illustrated (Fig. 4B) are the mass regions of the pseudo molecular ion of Ala with natural abundance isotope distribution and enriched in ^{15}N ; the $\%^{15}\text{N}$ -enrichment was calculated from such a data set. The $\%^{15}\text{N}$ -enrichment and ratio of total ^{15}N label for Ala and GAB in the PCA extracts were independently verified by the analysis of 1-D water-suppressed (Fig. 3A) and HSQC NMR spectra (Fig. 3B, C) since the pertinent NMR signals were adequately resolved.

Figures 5A and B summarize the $\%^{15}\text{N}$ enrichment in various N-metabolites from ^{15}N -ammonium and -nitrate treated coleoptiles, respectively. For the free amino acids, Gln and Glu were among the most labelled (up to 80%) under all treatments. Ala, GAB, Asp, and Pro were also significantly enriched, particularly for Ala and GAB in air/ammonium-treated tissues (c. 70–80%) and for Pro in the nitrate-treated tissues (up to 70%). The $\%^{15}\text{N}$ -enrichment patterns responded differently to anaerobiosis between nitrate and ammonium treatments. For example, the % doubly labelled Gln (Gln**), Glu*, Asn*, Asn**, and Asp* increased in nitrate-treated tissues but decreased in ammonium-treated tissues. Similar results were obtained for Gly, Ile, and Ser. The $\%^{15}\text{N}$ -enrichment of amino acids was generally lower in nitrate (Fig. 5B) than ammonium-treated tissues (Fig. 5A). Interestingly, while Gln was present mainly in the Gln** form in ammonium-treated tissues, the singly-labelled species (Gln*) became much more significant in anaerobic nitrate-treated tissues (up to 57% enriched). Except for the aerobic nitrate treatment, the ^{15}N label of Gln* was largely in the N-2 (60–100% of the Gln* label) instead of the N-5 position. These enrichment results were confirmed with the NMR data wherever feasible (Fig. 3) and were consistent in three separate experiments (data not shown).

In comparison, the $\%^{15}\text{N}$ enrichment was generally lower in protein-derived than in free amino acids for all treatments. The enrichment pattern in the free amino acids was not reflected in that of the protein-derived amino acids, most notably for Gln**, Gly*, Val*, Ser*, and Pro* under the nitrate treatment. Oxygen deficiency caused a decrease in the $\%^{15}\text{N}$ enrichment of most protein-derived amino acids with the ammonium treatment. However, with the nitrate treatment, the %Gln*, Asp*, and Pro* increased in response to anaerobiosis. Thus, the distribution of $\%^{15}\text{N}$ enrichment in protein-derived amino acids also differed between the two N treatments, as for the case of the free amino acids (see above).

By summing all of the ^{15}N -labelled products normalized against the total amino acid content, the total $\%^{15}\text{N}$ enrichment for the tissues and the two amino acid fractions of all treatments were estimated as shown in Table 3. It is clear that anaerobiosis led to a substantial decrease in the overall $\%^{15}\text{N}$ enrichment in free amino acids from the ammonium treatment while a small increase was observed for the nitrate treatment. In addition, the overall $\%^{15}\text{N}$ in protein-derived amino acids decreased in response to anaerobiosis under both N treatments.

Table 3 also lists the total amount of ^{15}N label in tissues and the two amino acid fractions. Sixty-four to 72% of the label resided in the free amino acid fraction under the ammonium treatment, while that under the nitrate treatment was lower (17–49%). A substantial increase in the total amount of label in tissues and both amino acid fractions was observed for the nitrate treatment in

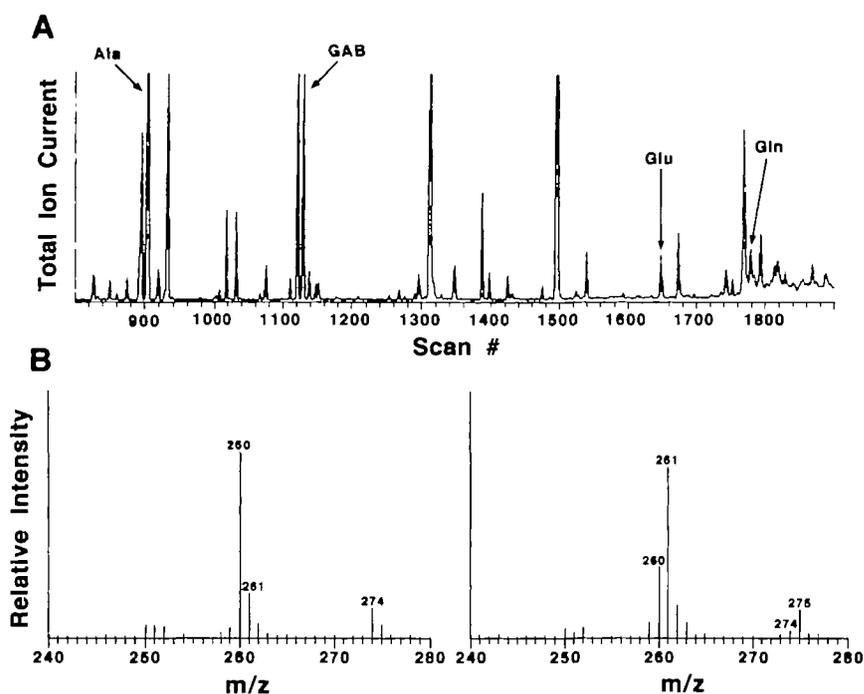


Fig. 4. GC-MS chromatogram of silylated extract of rice coleoptiles and mass spectra of silylated natural abundance and ¹⁵N-enriched Ala. The tissue extraction and GC-MS conditions were as described in Materials and methods. The chromatogram shown in (A) was acquired from anaerobic rice coleoptiles under ¹⁵N-ammonium treatments and illustrates the N sinks discussed in this paper. (B) shows mass spectra of silylated Ala standard (natural abundance) (left), and of the Ala peak from the chromatogram above (right).

response to anaerobiosis while the ammonium treatment exhibited an opposite trend. The elevated content of protein-derived amino acids (Table 2) primarily accounted for the increase in the total amount of ¹⁵N label in the protein fraction of nitrate-treated tissues, despite the reduction in the overall %¹⁵N enrichment.

Discussion

As with previous findings (Menegus *et al.*, 1988, 1989; Fan *et al.*, 1986a; Gibbs, 1992), the large accumulation of free ethanol, Ala, succinate, and GAB reported here (Table 1) indicated that these are the major fermentation products in anaerobic rice coleoptiles, regardless of the exogenous N source. However, the extent of anaerobic accumulation of ethanol, Ala, and GAB was greater in ammonium- than in nitrate-treated tissues. This could simply reflect a higher glycolytic rate for energy production in ammonium- than in nitrate-treated tissues during anaerobiosis. On the other hand, nitrate reduction in nitrate-treated tissues should have consumed some of the NADH and protons generated by glycolysis, thereby reducing the demand for the synthesis of fermentation products.

Protein-free Gln, Glu, and Ala were the most enriched amino acids (in terms of the %¹⁵N) (Fig. 5), which is consistent with their role as the primary N assimilation products, regardless of the oxygen status (Gibbs, 1992;

Lea *et al.*, 1990; Rhodes *et al.*, 1989). GAB was also among the most enriched metabolites under the aerobic/ammonium treatment, which suggests a channeled pathway from ammonium to GAB in rice coleoptiles. A similar conclusion can be made for Pro under the aerobic/nitrate treatment. Although the free Glu content was higher with the nitrate than ammonium treatment, its %¹⁵N enrichment showed the opposite, which indicates that endogenous N sources (e.g. protein degradation) may have contributed more to its production under nitrate treatments (see below).

The %¹⁵N enrichment pattern in Glu and Gln suggests that the enzymes involved in their synthesis (Fig. 6) exhibited different activities between the nitrate and ammonium treatments, particularly under anaerobic conditions. Under ammonium treatments, the much higher %Gln** over %Gln* (Fig. 5) indicates that Gln was primarily synthesized via the Gln synthetase/Glu synthase (GS/GOGAT) system (Lea *et al.*, 1990; Rhodes *et al.*, 1989) by utilizing exogenous ¹⁵NH₄⁺ (Fig. 6) and that Glu* was readily available for Gln** synthesis. This is also consistent with the observation that the %Gln** and Glu* were similar (Fig. 5).

Under the anaerobic/nitrate treatment, the large increase in %Gln*, the dominance of ¹⁵N label in the N-2 (α -amino) position of Gln*, and the maintenance of high %Glu* (Fig. 5) requires a different interpretation. One possibility is that catabolic deamination (e.g. from protein

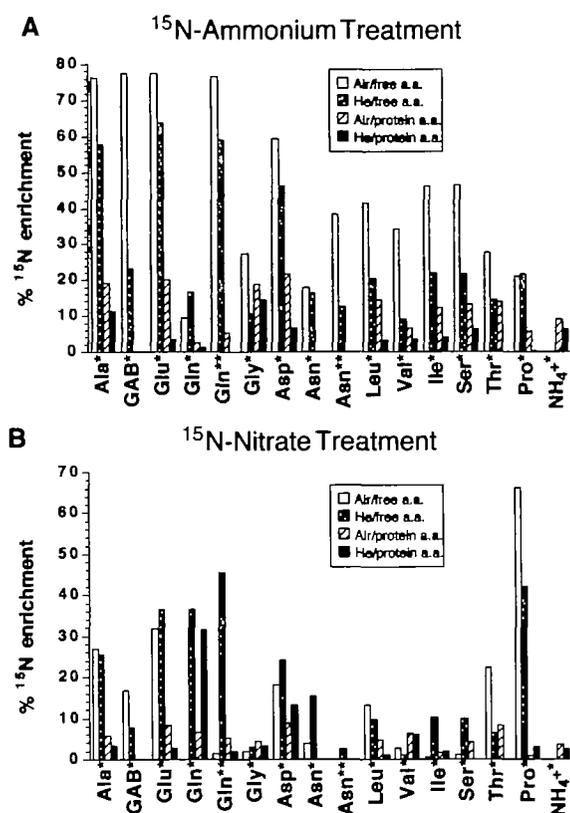


Fig. 5. ^{15}N -enrichment of free and protein-derived amino acids in rice coleoptiles under different N and oxygenation treatments. Treatments and ^{15}N isotope analysis of the GC-MS data were as described in Materials and methods. Total ^{15}N ($\mu\text{mol g}^{-1}$ dry wt) is expressed as the product of $\%^{15}\text{N}$ and absolute concentration of a given amino acid from Table 1. * and ** indicate singly and doubly ^{15}N -labelled, respectively. The Gln content for the aerobic/nitrate treatment was low, which made it difficult to determine its $\%^{15}\text{N}$ enrichment. (A) ^{15}N -ammonium treatments. (B) ^{15}N -nitrate treatments.

degradation) (Menegus *et al.*, 1993) added a significant amount of endogenous N (^{14}N) to the internal ammonium pool, in addition to that derived from the reduction of exogenous $^{15}\text{NO}_3^-$. This would have led to a lower $\%^{15}\text{N}$ in this pool, which would in turn favour Gln* over Gln** production, as observed. However, the label of Gln* would have been at the N-5 (δ -amido) position, if Gln*

were synthesized via the GS/GOGAT system. The dominance of the label at the N-2 position of Gln* suggests the existence of a distinct pool of Glu* which is synthesized by the Glu dehydrogenase (GDH) reaction (Rhodes *et al.*, 1989) (Fig. 6), followed by Gln* (at N-2 position) synthesis from Glu* and unlabelled ammonium ion. In addition, this Glu* pool is not accessible to the plastid GS/GOGAT system, which is consistent with the occurrence of mitochondrial GDH isolated from plastid GS (Yamaya *et al.*, 1986) and an enhancement of the GDH activity in anaerobic rice coleoptiles (Reggiani *et al.*, 1993b).

The overall pattern of $\%^{15}\text{N}$ enrichment (Fig. 5) is consistent with the transfer of the label from the primary products to other amino acids, which were then incorporated into proteins. The presence of ^{15}N label in proteins is evidence for *de novo* protein synthesis utilizing externally supplied nitrate or ammonium ion. In fact, protein synthesis appeared to remain active under anaerobic conditions in rice coleoptiles (Tables 2, 3), which is in contrast to the reduced activity observed for flood-sensitive tissues such as maize root (Sachs *et al.*, 1980). Maintenance of a high protein turnover rate was reported in anaerobic rice embryos previously (Mocquot *et al.*, 1981). However, a significant fraction of the protein synthesis in anaerobic rice coleoptiles utilized endogenous amino acid sources since the $\%^{15}\text{N}$ in all protein-derived amino acids was lower than that in free amino acids (Fig. 5). Whether the observed incorporation of exogenous nitrogen into proteins is relatively specific to a subset of proteins required for anaerobic metabolism remains to be determined.

For the ammonium treatment, the overall lower % and total ^{15}N enrichment in response to anaerobiosis (Fig. 5A; Table 3) is to be expected since ammonium assimilation into amino acids and proteins requires ATP, which is in short supply. A similar argument should also apply to the nitrate treatment. However, the nitrate treatment resulted in the increase in $\%^{15}\text{N}$ of several free and protein-derived amino acids in response to anaerobiosis (Fig. 5B). It is possible that the pathways from nitrate to

Table 3. Effects of anaerobiosis on total $\%^{15}\text{N}$ enrichment and amount of ^{15}N label in rice coleoptiles under two N treatments

	Treatment ^a			
	Air/ ^{15}N -nitrate	He/ ^{15}N -nitrate	Air/ $^{15}\text{NH}_4^+$	He/ $^{15}\text{NH}_4^+$
$\%^{15}\text{N}$				
Free AA's	15.9	19.3	68.6	46.1
Protein AA's	4.7	3.3	13.5	5.6
Total	5.4	5.6	32.7	13.0
^{15}N content				
Free AA's	7.1	48.9	318.8	178.8
Protein AA's	33.6	51.8	117.3	98.0
Total	40.7	100.7	436.1	276.8

^a ^{15}N label content is in $\mu\text{mol g}^{-1}$ dry wt, the total amount of ^{15}N label was summed from that of individual components including doubly labelled Gln and Asn.

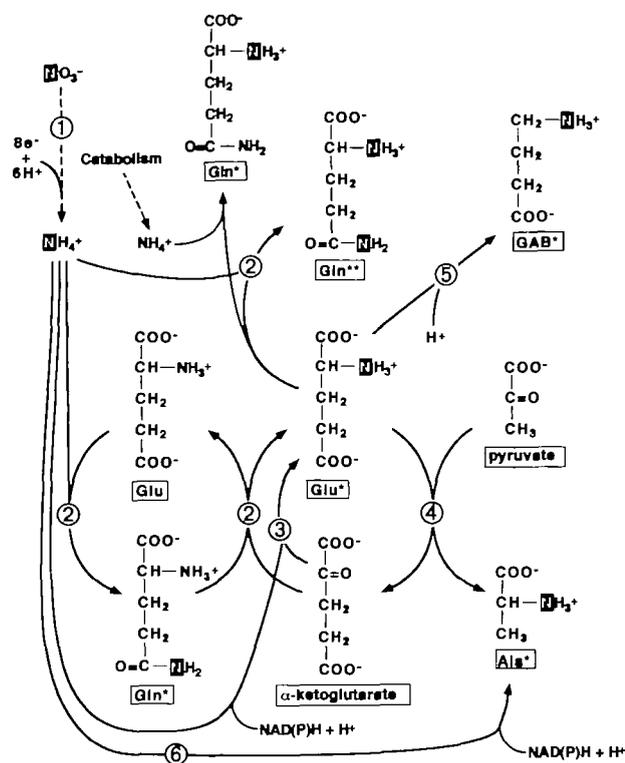


Fig. 6. $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ assimilation pathways in anaerobic rice coleoptiles. The proposed pathways are based on both the present findings and the literature. Process (1) converts $^{15}\text{NO}_3^-$ to $^{15}\text{NH}_4^+$ via nitrate and nitrite reductases (Reggiani *et al.*, 1993); processes (2) and (3) assimilate $^{15}\text{NH}_4^+$ into Gln and Glu via GS/GOGAT and GDH (Lea *et al.*, 1990; Rhodes *et al.*, 1989), respectively; process (4) incorporates $^{15}\text{NH}_4^+$ into Ala via Glu:pyruvate aminotransferase (Menegus *et al.*, 1993); process (5) produces GAB via Glu decarboxylase (Narayan and Nair, 1990); process (6) generates Ala via Ala dehydrogenase (Brunhuber and Blanchard, 1994). Also shown is the synthesis of Gln^* at N-2 position from Glu^* and unlabelled ammonium ion (e.g. derived from catabolism). The reversed N represents ^{15}N while * and ** denote singly and doubly ^{15}N -labelled species, respectively. It should be noted that the GDH reaction may occur in a distinct compartment (such as the mitochondria) separate from the GS/GOGAT system.

these amino acids are selectively activated and that their incorporation into proteins is favoured in anaerobic rice coleoptiles.

Under the anaerobic/nitrate treatment, the major labelled products, Ala, GAB, and Glu which also accumulated to a high content, may be significant in several aspects. Besides the 6 electrons and 8 protons required in nitrate reduction, Glu synthesis via GDH additionally consumes two electrons and one proton. The synthesis of GAB from Glu, via the action of Glu decarboxylase (GDC, Streeter and Thompson, 1972; Narayan and Nair, 1990; Carroll *et al.*, 1994) removes one more proton. Thus, the nitrate-Glu-GAB pathway may contribute towards the ability of anaerobic rice coleoptiles in maintaining the cytoplasmic pH (Fan *et al.*, 1992). Although consuming fewer protons and electrons, the ammonium-Glu-GAB pathway can also serve the same function. A

similar argument can be made for the anaerobic nitrate or ammonium to Ala pathway. Ala synthesis can involve Ala aminotransferase (Menegus *et al.*, 1993) or dehydrogenase (Magalhaes, 1991; Brunhuber and Blanchard, 1994). The latter reaction is analogous to the GDH reaction in consuming additional electrons and protons.

The additional sink for electrons and protons provided by nitrate reduction may have, in part, contributed to the reduced production of ethanol by rice coleoptiles under anaerobic conditions as compared with the ammonium treatments (Table 1). In addition, the large accumulation of Ala and GAB may help maintain the turgor pressure of the coleoptile (Menegus *et al.*, 1993). Moreover, GAB accumulation has been associated with the induction of mammalian hibernation (Nilsson and Lutz, 1993) which shares a common trait of low oxygen status as anaerobiosis. It is plausible that GAB may also be involved in some form of metabolic stasis in anaerobic rice coleoptiles. The wide occurrence of GAB in plants (Robinson, 1980), particularly in response to stresses (Narayan and Nair, 1990; Mayer *et al.*, 1990; Crawford *et al.*, 1994; Thompson *et al.*, 1966; Wallace *et al.*, 1984) warrants further investigations on the physiological role(s) of this amino acid.

In conclusion, the reductive assimilation of nitrate into Ala, GAB, Glu, and other amino acids may have important implications for the flood tolerance of germinating rice seedlings, and perhaps plants in general, as a means for sustaining glycolysis and energy production while minimizing cytoplasmic acidosis and conserving N.

Acknowledgements

The authors wish to thank Mr Jim Webster of UC Davis, for the generous gift of rice seeds and Dr Hank Greenway for his critical comments. This research was supported, in part, by the California Rice Research Board (project no. RB-5). The NMR instrumentation was made available by the Biomedical NMR Centre of the Medical Research Council, UK and the GC-MS instrumentation was supported in part by US EPA (grant No. R819658) Center for Ecological Health Research at UC Davis. Although the information in this document has been funded partly by the United States Environmental Protection Agency, it may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

References

- Anderson LW, Zaharevitz DW, Strong JM. 1987. Glutamine and glutamate: automated quantification and isotopic enrichments by gas chromatography/mass spectrometry. *Analytical Biochemistry* **163**, 358–68.
- Arnon DI. 1937. Ammonium and nitrate nitrogen nutrition of barley and rice at different seasons in relation to hydrogen-ion concentrations, manganese, copper, and oxygen supplied. *Soil Science* **44**, 91–121.
- Bax A, Davis DG. 1985. MLEV-17-based two-dimensional

- homonuclear magnetisation transfer spectroscopy. *Journal of Magnetic Resonance* **65**, 355–60.
- Bax A, Pochapsky SS.** 1992. Optimized recording of heteronuclear multidimensional NMR spectra using pulsed field gradients. *Journal of Magnetic Resonance* **99**, 638–43.
- Bodenhausen G, Ruben DJ.** 1980. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chemistry and Physics Letters* **69**, 185–9.
- Brunhuber NMW, Blanchard JS.** 1994 The biochemistry and enzymology of amino-acid dehydrogenases. *Critical Review in Biochemistry and Molecular Biology* **29**, 415–67.
- Carroll AD, Fox GG, Laurie S, Phillips R, Ratcliffe RG, Stewart GR.** 1994. Ammonium assimilation and the role of γ -aminobutyric acid in pH homeostasis in carrot cell suspension. *Plant Physiology* **106**, 513–20.
- Crawford RMM.** 1978. Metabolic adaptations to anoxia. In: Hook DD, Crawford RMM, eds. *Plant life in anaerobic environments*. Ann Arbor: Ann Arbor Science, 119–36.
- Crawford LA, Bown AW, Breitreuz KE, Guinel FC.** 1994. The synthesis of γ -aminobutyric acid in response to treatments reducing cytosolic pH. *Plant Physiology* **104**, 865–71.
- Davies DD.** 1980. Anaerobic metabolism and the production of organic acids. In: Davies DD, ed. *The biochemistry of plants*, Vol. 2. New York: Academic Press, 581–611.
- Fan TW-M, Higashi RM, Lane AN.** 1986a. Monitoring of hypoxic metabolism in superfused plant tissues by *in vivo* ^1H NMR. *Archives of Biochemistry and Biophysics* **251**, 674–87.
- Fan TW-M, Higashi RM, Lane AN, Jardetzky O.** 1986b. Combined use of ^1H NMR and GC-MS for monitoring metabolites and *in vivo* ^1H NMR. *Biochimica et Biophysica Acta* **882**, 154–67.
- Fan TW-M, Lane AN, Higashi RM.** 1992. Hypoxia does not affect rate of ATP synthesis and energy metabolism in rice shoot tips as measured by ^{31}P NMR *in vivo*. *Archives of Biochemistry and Biophysics* **294**, 314–18.
- Fan TW-M, Colmer TD, Lane AN, Higashi RM.** 1993a. Determination of metabolites by ^1H NMR and GC: Analysis for organic osmolytes in crude tissue extracts. *Analytical Biochemistry* **214**, 260–71.
- Fan TW-M, Lane AN, Higashi RM.** 1993b. Energy and fermentation metabolism in hypoxic rice coleoptiles—a multinuclear NMR approach. In: Jackson MB, Black CR, eds. *Interacting stresses on plants in a changing climate, NATO ASI Series*, Vol. 16. Berlin: Springer-Verlag, 333–52.
- Garlick PB, Radda GK, Seeley PJ.** 1979. Studies of acidosis in the ischaemic heart by phosphorus nuclear magnetic resonance. *Biochemical Journal* **184**, 547–54.
- Gibbs DJ.** 1992. Alanine accumulation in anaerobic rice coleoptiles. PhD thesis, the University of Western Australia.
- Guerrero MG, Vega JM, Losada M.** 1981. The assimilatory nitrate-reducing system and its regulation. *Annual Review of Plant Physiology* **32**, 169–204.
- Hagiwara M, Imura M.** 1991. Promotion of seedling emergence of paddy rice from flooded soil by coating seed with potassium nitrate. *Japanese Journal of Crop Science* **60**, 441–6.
- Hochachka PW, Mommsen TP.** 1983. Protons and anaerobiosis. *Science* **219**, 1391–7.
- Kamin H, Privalle LS.** 1987. Nitrite reductase. In: Ullrich WR, Aparicio PJ, Syrett PJ, Castillo F, eds. *Inorganic nitrogen metabolism*. Berlin: Springer Verlag, 112–17.
- Lea PJ, Robinson SA, Stewart GR.** 1990. In: Miflin BJ, Lea PJ, eds. *The biochemistry of plants*, Vol. 16. Ch. 4. San Diego: Academic Press.
- Live DH, Davis DG, Agosta WC, Cowburn D.** 1984. Long range hydrogen bond mediated effects in peptides: ^{15}N NMR study of Gramicidin S in water and organic solvents. *Journal of American Chemical Society* **106**, 1939–41.
- Magalhaes JR.** 1991. Kinetics of (NH_4^+) -N-15 assimilation in tomato plants—evidence for (NH_4^+) -N-15 assimilation via GDH in tomato roots. *Journal of Plant Nutrition* **14**, 1341–53.
- Malavolta E.** 1954. Studies on the nitrogenous nutrition of rice. *Plant Physiology* **29**, 98–9.
- Martin F.** 1985. ^{15}N -NMR studies of nitrogen assimilation and amino acid biosynthesis in the ectomycorrhizal fungus *Cenococcum graniforme*. *FEBS Letters* **182**, 350–4.
- Mawhinney TP, Madsen MA.** 1982. *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide and related *N*-*tert*-butyldimethylsilylamides as protective silyl donors. *Journal of Organic Chemistry* **47**, 3336–9.
- Mayer RR, Cherry JH, Rhodes D.** 1990. Effects of heat shock on amino acid metabolism of cowpea cells. *Plant Physiology* **94**, 796–810.
- Menegus F, Cattaruzza L, Chersi A, Fronza G.** 1989. Differences in the anaerobic lactate-succinate production and in the changes of cell sap for plants with high and low resistance to anoxia. *Plant Physiology* **90**, 29–32.
- Menegus F, Cattaruzza L, Chersi A, Serva A, Fronza G.** 1988. Production and organ distribution of succinate in rice seedlings during anoxia. *Physiologia Plantarum* **74**, 444–9.
- Menegus F, Cattaruzza L, Molinari H, Ragg E.** 1993. Rice and wheat seedlings as plant models of high and low tolerance to anoxia. In: *Surviving hypoxia: mechanisms of adaptation and control*. Boca Raton: CRC Press, 53–64.
- Mocquot B, Prat C, Mouches C, Pradet A.** 1981. Effect of anoxia on energy charge and protein synthesis in rice embryo. *Plant Physiology* **68**, 636–40.
- Moore S.** 1972. The precision and sensitivity of amino acid analysis. In: *Chemistry and biology of peptides*. Ann Arbor: Science Publishers, Ann Arbor, 629–53.
- Narayan VS, Nair PM.** 1990. Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochemistry* **29**, 367–75.
- Nilsson GE, Lutz PL.** 1993. Role of GABA in hypoxia tolerance, metabolic depression and hibernation—possible links to neurotransmitter evolution. *Comparative Biochemistry and Physiology* **105C**, 329–36.
- Öpik H.** 1973. Effect of anaerobiosis on respiratory rate, cytochrome oxidase activity, and mitochondrial structures in coleoptiles of rice (*Oryza sativa* L). *Journal of Cell Science* **12**, 725–59.
- Pilbeam DJ, Kirkby EA.** 1992. Some aspects of the utilization of nitrate and ammonium by plants. In: Mengel K, Pilbeam DJ, eds. *Nitrogen metabolism of plants, Proceedings of the Phytochemical Society of Europe*, Vol. 33, Oxford: Clarendon Press, 55–70.
- Piotto M, Saudek V, Sklenar V.** 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *Journal of Biomolecular NMR* **2**, 661–5.
- Preece NE, Cerdan S.** 1993. Determining ^{15}N to ^{14}N ratios in biofluids by single-pulse ^1H Nuclear Magnetic Resonance. *Analytical Biochemistry* **215**, 180–3.
- Prioul J-L, Guyot C.** 1985. Role of oxygen transport and nitrate metabolism in the adaptation of wheat plants to root anaerobiosis. *Physiologie Vegetale* **23**, 175–85.
- Reggiani R, Mattana M, Aurisano N, Bertani A.** 1993a. Utilization of stored nitrate during the anaerobic germination of rice seeds. *Plant Cell Physiology* **34**, 379–83.
- Reggiani R, Mattana M, Aurisano N, Bertani A.** 1993b. The rice coleoptile—an example of anaerobic nitrate assimilation. *Physiologia Plantarum* **89**, 640–3.

- Reggiani R, Bertini F, Mattana M.** 1995. Incorporation of nitrate nitrogen into amino acids during the anaerobic germination of rice. *Amino Acids* **9**, 385–90.
- Rhodes D, Brunk DG, Magalhaes JR.** 1989. Assimilation of ammonia by glutamate dehydrogenase. In: Poulton JE, Romeo JT, Conn EE, eds. *Plant nitrogen metabolism—recent advances in phytochemistry series*, Vol. 23. New York: Plenum Press, 191–226.
- Robinson T.** 1980. *The organic constituents of higher plants*. North Amherst: Cordus Press, 230.
- Sachs MM, Freeling M, Okimoto R.** 1980. The anaerobic proteins of maize. *Cell* **20**, 761–7.
- Shaka AJ, Barker PB, Freeman R.** 1985. Computer-optimized decoupling scheme for wideband applications and low-level operation. *Journal of Magnetic Resonance* **64**, 547–52.
- States DJ, Haberkorn RA, Ruben DJ.** 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *Journal of Magnetic Resonance* **48**, 286–92.
- Streeter JG, Thompson JF.** 1972. Anaerobic accumulation of γ -amino butyric acid and alanine in radish leaves (*Raphanus sativus* L.). *Plant Physiology* **49**, 572–8.
- Thompson JF, Stewart CR, Morris CJ.** 1966. Changes in amino acid content of excised leaves during incubation. I. The effect of water content of leaves and atmospheric oxygen level. *Plant Physiology* **41**, 1578–4.
- Trought MCT, Drew MC.** 1981. Alleviation of injury to young wheat plants in anaerobic solution cultures in relation to the supply of nitrate and other inorganic nutrients. *Journal of Experimental Botany* **32**, 509–22.
- Wallace W, Secor J, Schrader L.** 1984. Rapid accumulation of γ -aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiology* **75**, 170–5.
- Wider G, Wüthrich K.** 1993. A simple experimental scheme using pulsed field gradients for coherence-pathway rejection and solvent suppression in phase-sensitive heteronuclear correlation spectra. *Journal of Magnetic Resonance* **102**, 239–41.
- Williams BD, Wolfe RR.** 1994. Determination of amino-N-15 and amide-N-15 glutamine enrichment with tertiary butyldimethylsilyl derivatives. *Biological Mass Spectrometry* **23**, 682–8.
- Yamaya T, Oaks A, Rodes D, Matsumoto H.** 1986. Mitochondrial GDH? *Plant Physiology* **81**, 754–7.