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ORIGINAL ARTICLE

Effect of oxygen deficiency on nitrogen assimilation and amino acid metabolism of soybean root segments

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Abstract Plants submitted to O_2 deficiency present a series of biochemical modifications, affecting overall root metabolism. Here, the effect of hypoxia on the metabolic fate of ^{15}N derived from $^{15}NO_3^-$, $^{15}NO_2^-$ and $^{15}NH_4^+$ in isolated soybean root segments was followed by gas chromatography–mass spectrometry, to provide a detailed analysis of nitrogen assimilation and amino acid biosynthesis under hypoxia. O_2 deficiency decreased the uptake of the nitrogen sources from the solution, as ratified by the lower $^{15}NO_3^-$ and $^{15}NH_4^+$ enrichment in the root segments. Moreover, analysis of endogenous NO_2^- and $^{15}NH_4^+$ levels suggested a slower metabolism of these ions under hypoxia. Accordingly, regardless of the nitrogen source, hypoxia reduced total ^{15}N incorporation into amino acids. Analysis of ^{15}N enrichment patterns and amino acid levels suggest a redirecting of amino acid metabolism to alanine and γ-aminobutyric acid synthesis under hypoxia and a differential sensitivity of individual amino acid pathways to this stress. Moreover, the role of glutamine synthetase in nitrogen assimilation both under normoxia and hypoxia was ratified. In comparison with $^{15}NH_4^+$, $^{15}NO_2^-$ assimilation into amino acids was more strongly affected by hypoxia and NO_2^- accumulated in root segments during this stress, indicating that nitrite reductase may be an additional limiting step. NO_2^- accumulation was associated with a higher nitric oxide emission. $^{15}NO_3^-$ led to much lower ^{15}N incorporation in both O_2 conditions, probably due to the limited nitrate reductase activity of the root segments. Overall, the present work shows that profound alterations of root nitrogen metabolism occur during hypoxic stress.

Keywords Amino acid · Ammonium · Hypoxia · Nitrate · Nitrite · Nitrogen

Abbreviations

DAF-2	4,5-Diaminofluorescein
GABA	γ-Aminobutyric acid
GC–MS	Gas chromatography–mass spectrometry
GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
GS–GOGAT	Glutamine synthetase–glutamine-2-oxoglutarate aminotransferase
NH_4^+	Ammonium
NiR	Nitrite reductase
NO	Nitric oxide
NO_3^-	Nitrate
NO_2^-	Nitrite
NR	Nitrate reductase
O_2	Molecular oxygen

Introduction

Molecular oxygen (O_2) acts as the final electron acceptor of the mitochondrial respiratory chain and is essential for the activity of diverse enzymes, having an indispensable role in plant growth and metabolism (Licausi and Perata 2009). However, during their life cycle, plants and particularly their root systems can often be submitted to conditions of

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O_2 deficiency (Irfan et al. 2010). This stress occurs, for instance, after heavy rainfalls or excessive irrigation, situations in which the soil, depending on its drainage capacity, may become waterlogged, affecting O_2 supply to submerged tissues due to the low diffusion rate of this gas in aqueous medium (Licausi and Perata 2009).

Plants submitted to O_2 deficiency respond through several biochemical modifications, including the decrease of ATP synthesis by oxidative phosphorylation and a consequent stimulus of fermentation processes (Bailey-Serres and Voesenek 2008). In addition to primary carbon metabolism, nitrogen assimilation is also affected in hypoxic roots (Rocha et al. 2010a; Narsai et al. 2011). Under normal conditions, nitrate (NO_3^-) absorbed by roots is reduced by nitrate reductase (NR) to nitrite (NO_2^-), in turn reduced to ammonium (NH_4^+) by nitrite reductase (NiR). NH_4^+ is then incorporated into amino acids by the system glutamine synthetase-glutamine-2-oxoglutarate aminotransferase (GS–GOGAT) (Lea 1993). Many studies have shown that NO_3^- utilization and reduction occur even under hypoxia (Reggiani et al. 1995; Botrel et al. 1996). However, it is still controversial if there is an increase or decrease of NR activity under O_2 deficiency (Botrel et al. 1996; Allegre et al. 2004; Morard et al. 2004; Brandão and Sodek 2009).

On the other hand, it is well established that NiR activity is more strongly inhibited by O_2 shortage, resulting in NO_2^- accumulation and its release to external medium, especially when NO_3^- is supplied exogenously (Lee 1978; Botrel et al. 1996; Morard et al. 2004; Brandão and Sodek 2009). In a study with ^{15}N-labeled nitrogen sources, Libourel et al. (2006) observed limited $^{15}NO_2^-$ metabolism by maize root segments kept under anoxia. However, in previous studies, using gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance analysis, considerable incorporation of $^{15}NO_3^-$ into amino acids was detected in coleoptiles of rice seeds germinating anaerobically (Reggiani et al. 1995, 1997; Fan et al. 1997). NH_4^+ assimilation is also affected by O_2 deficiency, since the reaction catalyzed by GS is inhibited under this condition, probably due to its ATP dependence (Limami et al. 2008). Indeed, a lower incorporation of $^{15}NH_4^+$ into amino acids during hypoxia was demonstrated for rice coleoptiles and *Medicago truncatula* seedlings (Fan et al. 1997; Limami et al. 2008). Moreover, a possible role of glutamate dehydrogenase in nitrogen assimilation under anaerobic conditions has been considered (Fan et al. 1997).

Irrespective of the nitrogen source, hypoxia induces many alterations in amino acid metabolism (revised by Reggiani and Bertani 2003), including an increase in total content of free amino acids in roots and xylem sap (Puiatti and Sodek 1999; Thomas and Sodek 2006; van Dongen et al. 2009; Rocha et al. 2010a). Principally, O_2 deficiency is associated with a rise in alanine and γ-aminobutyric acid (GABA) levels (Rocha et al. 2010a; Narsai et al. 2011). In all studies with labeled nitrogen sources, it has been possible to detect ^{15}N incorporation into alanine during hypoxia (Reggiani et al. 1995, 1997; Fan et al. 1997; Libourel et al. 2006; Limami et al. 2008).

It is well established that NO_3^-, but not NH_4^+, exerts a beneficial effect on growth and metabolism of plants submitted to hypoxia (Trought and Drew 1981; Allegre et al. 2004; Thomas and Sodek 2005; Horchani et al. 2010). The role of NO_2^- in reducing acidosis induced by O_2 deficiency has been also demonstrated (Libourel et al. 2006). In spite of this, NO_3^- and NO_2^- metabolism by roots under hypoxia is not clearly understood. Particularly, most studies with ^{15}N-labeled nitrogen sources have been carried out with seedlings or germinating seeds (Reggiani et al. 1995, 1997; Fan et al. 1997; Limami et al. 2008), whereas studies with roots from plants in more advanced vegetative states are scarce. Moreover, many of these studies used rice (Reggiani et al. 1995, 1997; Fan et al. 1997), a plant that presents very high tolerance to flooding, which may not, therefore, be strictly comparable to less tolerant species.

In this scenario, the present study aimed to verify the effect of hypoxic stress on nitrogen assimilation and amino acid metabolism of soybean root segments, following the metabolic fate of ^{15}N derived from $^{15}NO_3^-$, $^{15}NO_2^-$ or $^{15}NH_4^+$.

Materials and methods

Plant cultivation and in vitro assays with isolated root segments

Non-nodulated soybean plants [*Glycine max* (L.) Merril cv. IAC-23] were grown in a greenhouse under natural conditions of light and temperature. Seeds were germinated in vermiculite and, after reaching the V1 stage (Fehr et al. 1971), the seedlings were transferred to a hydroponic system, using Hoagland and Arnon's (1950) N-free nutrient solution at 1/3 strength supplemented with KNO_3 (5 mM) as the nitrogen source. Four days before in the vitro assays, plants at the V3 stage were placed in N-free nutrient solution, to promote the consumption of endogenous NO_3^-. Segments from the median region of the roots were harvested, discarding the inferior and superior quarters. From each pot with three plants, four lots of 1 g root segments were obtained, paying careful attention to achieve a homogenous distribution of the different root tissues for each sample. After washing with distilled water, each lot of root segments was incubated in flasks containing 15 mL of sterile N-free nutrient solution supplemented with chloramphenicol (50 μM). One hour after

imposition of normoxia (with aeration of nutrient solution) or hypoxia (sealed flasks completely filled with nutrient solution without aeration), 150 µL of previously de-aerated 100× stock solution of the nitrogen sources, all with an enrichment of 30 % ^{15}N, were added to the solution, leading to a final concentration of 5 mM KNO_3, 1 mM $NaNO_2$ or 1 mM NH_4Cl. In control experiments, the roots were kept without nitrogen in the medium. Flasks were maintained in a growth chamber in the dark at 28 °C for 0, 1, 4 and 8 h. On harvest, root segments were washed for 2–3 min in a solution of $CaSO_4$ (0.5 mM) and KCl (1 mM), and then with distilled water, to eliminate any ^{15}N adsorbed on the root surface (Ashton et al. 2010) and then frozen in liquid N_2. A sample of the nutrient solution was also taken for analysis.

Extraction of primary metabolites and biochemical analysis

Each lot of root segments (1 g) was macerated in liquid N_2 and the metabolites extracted for 24 h with 10 mL of a methanol:chloroform:water (12:5:3, v:v:v) solution (Sousa and Sodek 2002). After centrifugation of 2,000 rpm for 30 min, one volume of chloroform and 1.5 volume of water were added to each 4 volumes of the obtained supernatant. After phase separation over 24 h, the aqueous phase was collected and the resulting extract concentrated for 15 h at 37 °C and then used for biochemical analysis. NO_3^- was determined spectrophotometrically at 410 nm by the salicylic acid method as described by Cataldo et al. (1975). NO_2^- and NH_4^+ were analyzed, respectively, by the Griess (Hageman and Reed 1980) and Berthelot reactions (McCullough 1967). NO_3^-, NO_2^- and NH_4^+ were determined both in root extracts and in samples of the nutrient solution.

Determination of NR activity and nitric oxide (NO) emission by root segments

NR activity was determined by the in vivo method (Jaworski 1971) following vacuum infiltration of the tissue with the reaction medium (K_2HPO_4 0.05 M, pH 7.5; propanol 1 %; KNO_3 0.05 M), and incubation at 30 °C in the dark. Aliquots were removed at 0 and 30 min for the determination of NO_2^-. NO emitted by root segments under hypoxia was detected using the free form of the fluorescent probe 4,5-diaminofluorescein (DAF-2), following the method described by Oliveira et al. (2009), with modifications. Briefly, root segments (40 mg) were incubated in the dark in phosphate buffer (50 mM, pH 7.2) containing DAF-2 (5 µM), in the presence of the nitrogen sources. After 1 h, the segments were discarded and the solution diluted fivefold in the buffer, before the analysis of the fluorescent emission at 515 nm (excitation at 495 nm), using a Hitachi F-2500 spectrofluorometer (Hitachi Ltd, Tokyo, Japan).

Gas chromatography–mass spectrometry (GC–MS) analysis

GC–MS analysis was carried out in a Shimadzu QP2010 plus system (Shimadzu Corporation, Tokyo, Japan), equipped with a DB-5 column (30 m, 0.32 mm I.D., 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). For amino acid determination, an aliquot of the root extract was acidified with an equal volume of 2 M acetic acid before being passed through a Dowex 50 W H^+ column, washed with water and the retained amino acids eluted with NH_4OH (4 M). The eluent was dried at 40 °C in a CentriVap (Labconco, Kansas City, USA) and derivatized using a mixture of 30 µL pyridine with 30 µL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (Sigma, Saint Louis, USA) at 70 °C for 30 min. Amino acids were analyzed in SIM mode following the conditions described by Chaves das Neves and Vasconcelos (1987). For NH_4^+ determination, samples were concentrated to obtain at least 50 nmol of NH_4^+ in a 100-µL volume. NH_4^+ was then derivatized to pentafluorobenzamine, through its reaction with pentafluorobenzoyl chloride (PFB-Cl) in $NaHCO_3$ 5 %, as described by Masclaux-Daubresse et al. (2006). After drying, the samples were derivatized and analyzed by GC–MS as described for the amino acids. Dried NO_3^- samples (100 nmol) were derivatized with 500 µL of toluene in 100 µL of trifluoroacetic anhydride acid at 70 °C for 30 min. After washing procedures, GC–MS analysis was carried out as in Smythe et al. (1999).

Analysis of ^{15}N enrichment

To calculate the ^{15}N enrichment in amino acids the following formula was used (Silvester et al. 1996):

$$\%^{15}N = 100 \times (R_e - R_c)/[1 + (R_e - R_c)]$$

in which $R_e = (M + 1)/M$ of the sample exposed to ^{15}N, and R_c is the same $M + 1$ and M ratio of the ^{14}N standards. The values of M and $M + 1$ represent the relative intensities of two forms of the same fragment, one with ^{14}N (M) and other with ^{15}N ($M + 1$). For amino acids, the main fragment is usually that whose m/z value is equivalent to molecular mass minus 57 (Mawhinney et al. 1986). ^{15}N enrichment at the amino-*N* and amide-*N* atoms of glutamine and asparagine was estimated after the isolation of the amides free of aspartate and glutamate using a Dowex-1x8 Ac^- column followed by their hydrolysis to glutamate and aspartate with 6 N HCl at 110 °C for 3 h. Label in the amino-*N* was then determined directly as described above

for amino acids and label in the amide-N group calculated by difference from the total label in glutamine and asparagine before hydrolysis. The enrichment of NH_4^+ in ^{15}N was calculated applying the same formula described above, using the molecular masses 268 (*M*) and 269 (*M* + 1). For NO_3^- analysis, the masses of the isomer *o* were considered (120 and 121) (Smythe et al. 1999).

Results

Uptake and initial metabolism of $^{15}NO_3^-$, $^{15}NO_2^-$ and $^{15}NH_4^+$ by root segments

The aim of the present study was to verify the metabolic fate of ^{15}N derived from $^{15}NO_3^-$, $^{15}NO_2^-$ or $^{15}NH_4^+$ in soybean root segments submitted to hypoxia, in comparison with those maintained under normoxic conditions. Initially, uptake of the nitrogen sources from the solution by the root segments was analyzed. Data from assays with $^{15}NO_3^-$-incubated segments are shown in Fig. 1a. As can be observed in the upper graph, NO_3^- from the nutrient solution was consumed, and at a much higher rate under normoxia (a total of 11.7 μmol NO_3^- consumed per 8 h, considering the 15-mL volume of the solution) than under hypoxia (3.3 μmol NO_3^- per 8 h). Concomitantly, an increase in endogenous NO_3^- content was detected in normoxia-incubated root segments (rising from 2.47 ± 0.13 to 5.71 ± 0.21 μmol/g), while no significant alteration was observed in hypoxia (Fig. 1a, middle graph). However, the analysis of ^{15}N enrichment for endogenous NO_3^- clearly showed that NO_3^- was absorbed by the

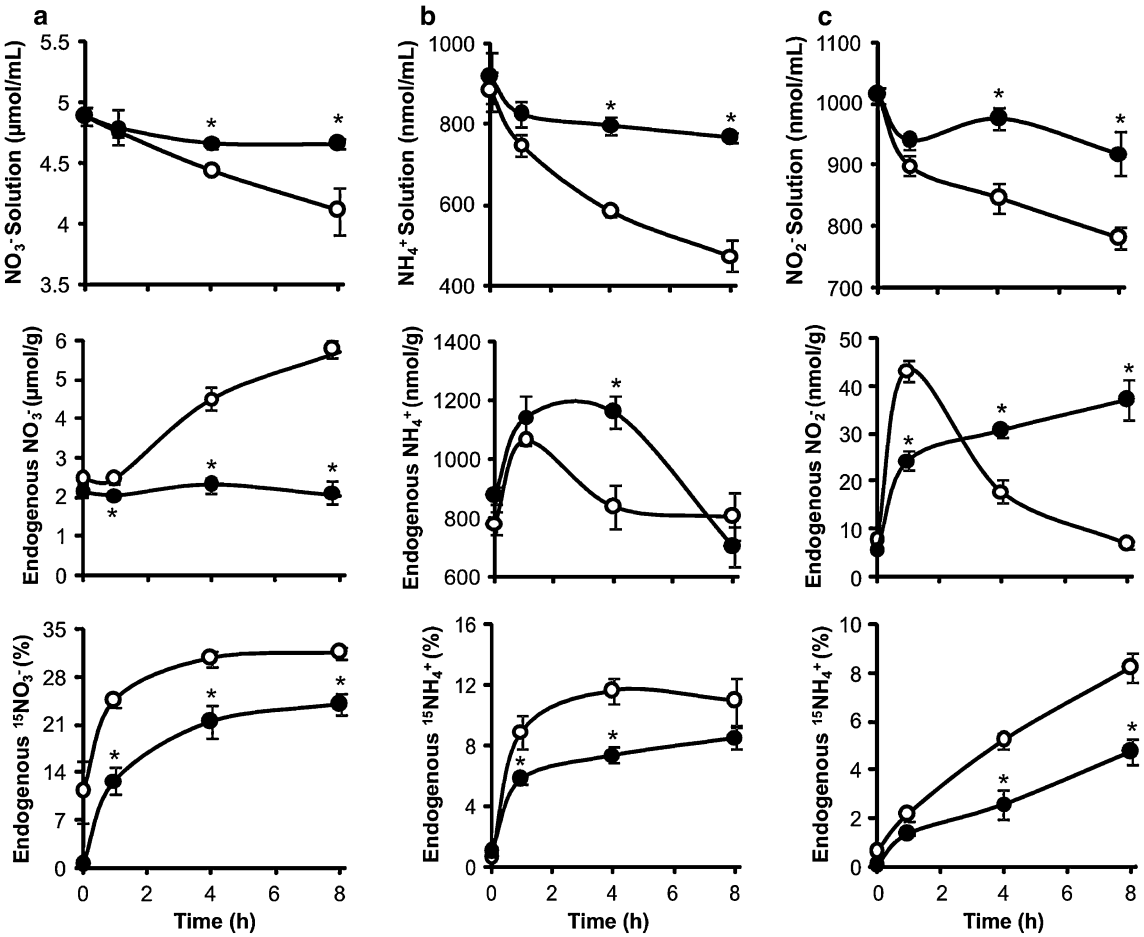


Fig. 1 Analysis of nitrogen uptake by soybean root segments. **a** NO_3^- (5 mM), **b** NH_4^+ (1 mM) or **c** NO_2^- (1 mM), all enriched with 30 % ^{15}N, were added to nutrient solution in which root segments were maintained under normoxia (*open circles*) or hypoxia (*closed circles*). The normoxia and hypoxia treatments were initiated 1 h before the addition of the N sources. The following parameters were evaluated 0, 1, 4 and 8 h after labeled nitrogen addition: **a** NO_3^- levels in the solution, endogenous NO_3^- content and its ^{15}N enrichment in root segments; **b** NH_4^+ levels in the solution, endogenous NH_4^+ content and its ^{15}N enrichment in root segments; **c** NO_2^- levels in the solution, endogenous NO_2^- content and $^{15}NH_4^+$ enrichment in root segments. Data represent the mean ± SE ($n = 3$). Similar results were obtained in repeat experiments (for all figures). *$P < 0.05$ compared to the respective normoxia control, according to Student's *t* test

tissues under both O_2 conditions (Fig. 1a, lower graph). As might be predicted from these data, $^{15}NO_3^-$ enrichment was higher in root segments maintained under normoxia compared with hypoxia.

When the segments were incubated with $^{15}NH_4^+$, a faster rate of consumption of this cation in the medium was observed under normoxia (a total of 6.1 μmol NH_4^+ per 8 h) compared with hypoxia (2.2 μmol per 8 h) (Fig. 1b, upper graph). NH_4^+ uptake by the roots for both O_2 conditions was confirmed by the rise in endogenous NH_4^+ content (Fig. 1b, middle graph) and by the $^{15}NH_4^+$ enrichment observed inside the segments (lower graph). Interestingly, the increase of NH_4^+ levels in the roots was transient, given that, in contrast to NO_3^-, large amounts of NH_4^+ do not accumulate in plant cells. Under normoxia, endogenous NH_4^+ increased from 783.9 ± 40.5 to 1062.5 ± 12.7 nmol/g after 1 h of incubation, but returned to basal levels after 4 h. In hypoxic segments, where a lower level of NH_4^+ uptake was observed, the endogenous levels of this cation returned to basal values only after 8 h of treatment, suggesting slower metabolism of NH_4^+ by the roots during O_2 deficiency.

Again, when the roots were incubated with $^{15}NO_2^-$, faster consumption of NO_2^- from the solution was detected under normoxia (a total of 3.5 μmol NO_2^- per 8 h) (Fig. 1c, upper graph), as observed in the experiments with NO_3^- and NH_4^+. NO_2^- consumption from the solution resulted in a transient increase in NO_2^- levels in the roots maintained under aeration (Fig. 1c, middle graph), which preceded the enrichment of the endogenous $^{15}NH_4^+$ pool (Fig. 1c, lower graph), demonstrating that after uptake by the roots NO_2^- was rapidly metabolized to NH_4^+. Under O_2 deficiency, less NO_2^- was consumed from the solution by the root segments (1.4 μmol NO_2^- per 8 h) (Fig. 1c, upper graph), but the higher endogenous levels of NO_2^- resulting from this uptake were maintained until the end of the treatment (middle graph), suggesting that NO_2^- metabolism was compromised under this condition, as observed for NH_4^+. Nevertheless, it was possible to detect ^{15}N enrichment of the NH_4^+ pool from $^{15}NO_2^-$ in root segments (lower graph). As might be expected, NH_4^+ enrichment was nevertheless lower than that observed under normoxia.

Besides its metabolism to NH_4^+, endogenous NO_2^- accumulation observed in hypoxia also stimulated the production of nitric oxide (NO) (Fig. 2). Root segments incubated with NO_2^- emitted NO at a rate of 46.7 ± 2.9 nmol h^{-1} g^{-1} that was some 48 % higher than that observed with the control N-free segments. On the other hand, incubation with NH_4^+ or NO_3^- did not have any significant effect on NO emission. Nor did exogenous addition of NH_4^+ or NO_3^- affect NO_2^- release in the nutrient solution, or cause changes in the endogenous levels of this anion in root segments during hypoxia (data not shown).

Effect of hypoxia on amino acid levels of roots segments

The effect of O_2 deficiency on amino acid metabolism was initially analyzed using soybean root segments maintained without any exogenous nitrogen source (–N). The scheme in Fig. 3 showed that hypoxia led to many alterations in amino acid metabolism of root segments. For most amino acids, there was an increase in their endogenous levels during hypoxia. Particularly, the most outstanding change was the induction of a very expressive rise in alanine and GABA contents by O_2 deficiency. On the other hand, under the same conditions, there was a decrease in glutamate and especially aspartate levels, amino acids directly related to alanine and GABA synthesis through transamination reactions. Interestingly, amino acids whose synthesis is derived from aspartate presented a tendency to decrease (asparagine and methionine) or remain stable (isoleucine) in their pool size under hypoxia.

Generally, amino acid metabolism of root segments treated with NO_3^-, NO_2^- or NH_4^+ responded to hypoxia similarly to those kept on N-free medium, differing mainly in the intensity of their response. This can be observed in Table 1, showing the effect of the incubation of root segments with NO_3^-, NO_2^- or NH_4^+ on the levels of some amino acids under normoxic and hypoxic conditions. With aeration, segments incubated for 8 h with NH_4^+ or NO_2^- presented significant increases in the levels of most of the

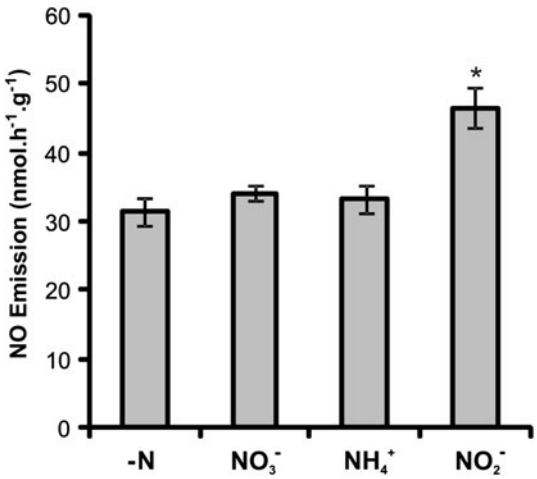
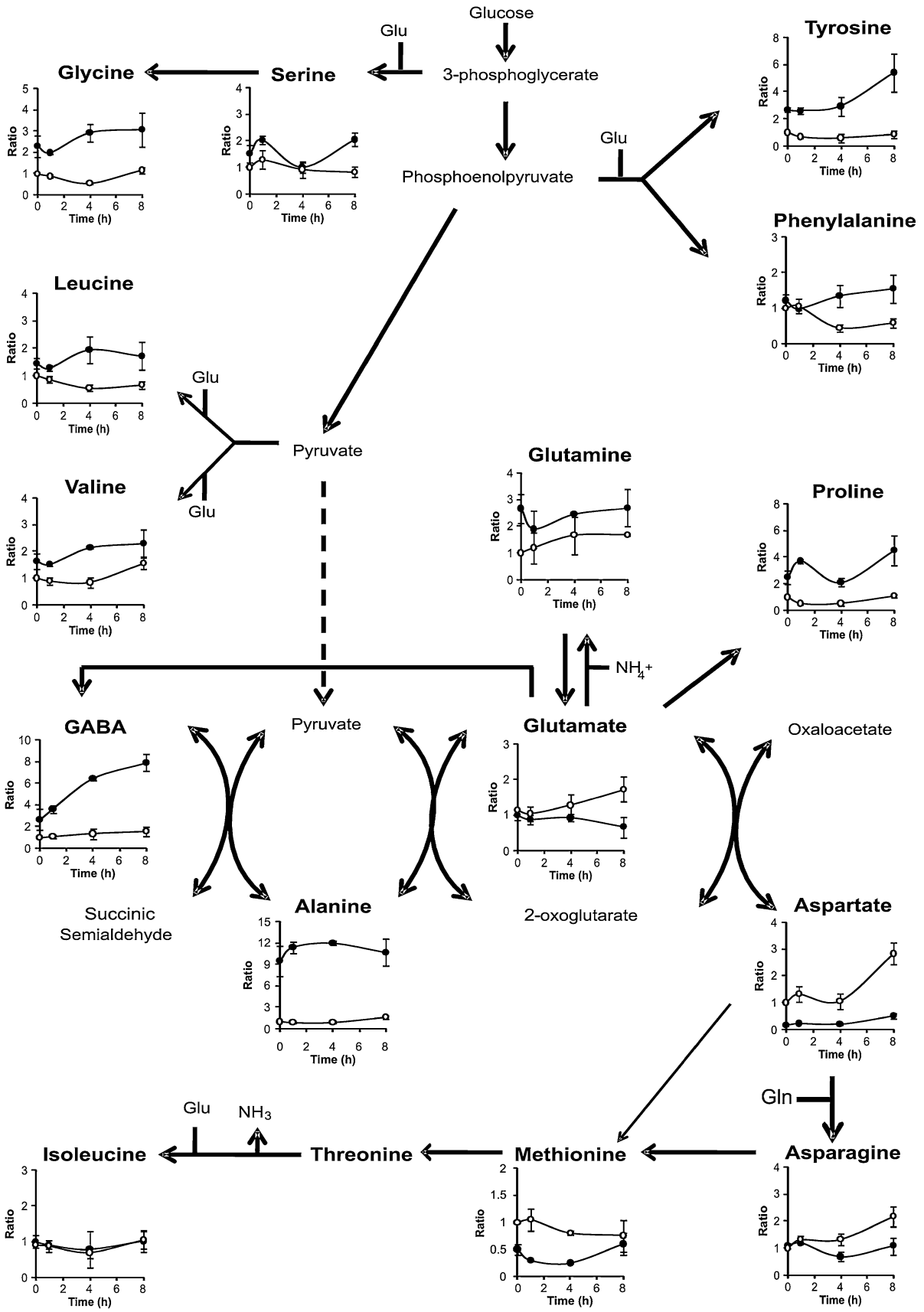


Fig. 2 Nitric oxide (NO) emission by root segments submitted to hypoxia. Root segments were incubated for 1 h under hypoxic conditions in a phosphate buffer (50 mM, pH 7.2) solution containing NO_3^- (5 mM), NH_4^+ (1 mM), NO_2^- (1 mM) or no nitrogen source (−N). NO emission was estimated using the fluorescent probe DAF-2. Data represent the mean ± SE ($n = 4$). *$P < 0.05$ compared to –N control, according to one-way ANOVA followed by the Tukey test


Glucose
Glu
3-phosphoglycerate
Serine
Glycine
Phosphoenolpyruvate
Glu
Tyrosine
Phenylalanine
Leucine
Glu
Pyruvate
Glu
Valine
Glutamine
Proline
NH4+
GABA
Pyruvate
Glutamate
Oxaloacetate
Succinic
Semialdehyde
Alanine
2-oxoglutarate
Aspartate
Gln
Glu
NH3
Isoleucine
Threonine
Methionine
Asparagine
Ratio
Time (h)

◀ **Fig. 3** Effect of hypoxia on amino acid metabolism of root segments. Root segments were incubated under normoxia (*open circles*) or hypoxia (*closed circles*) in a nutrient solution without exogenous nitrogen. In keeping with other experiments, the normoxia and hypoxia treatments were initiated 1 h before the first harvest of segments at 0 h (corresponding to the time point at which N sources were added in other experiments). Endogenous free amino acid levels were determined at 0, 1, 4 and 8 h by GC–MS. The amount of each amino acid was normalized to its respective level at 0 h of normoxia. Data represent the mean ± SE ($n = 3$)

analyzed amino acids in comparison with control N-free segments under normoxia, indicating the assimilation of the nitrogen from these sources in the presence of O_2. This effect was, however, greatly reduced during hypoxia. In the absence of aeration, NH_4^+ treatment induced a prominent increase of only alanine and GABA, while the rise in levels of other amino acids such as glutamine and asparagine was much lower than those detected under normoxia. At the same time, NO_2^- did not have any significant effect on endogenous amino acid content during hypoxia. NO_3^--incubated segments did not present any significant differences in the amino acid levels in relation to control −N segments in both O_2 conditions.

^{15}N incorporation into amino acids by root segments

In order to obtain a clearer understanding of the effect of hypoxia on nitrogen metabolism, ^{15}N incorporation into amino acids was verified for the different treatments. Figure 4 shows the incorporation into amino acids of ^{15}N derived from $^{15}NH_4^+$, $^{15}NO_2^-$ or $^{15}NO_3^-$, after 8 h of incubation under normoxia or hypoxia. $^{15}NH_4^+$ led to the highest incorporation of ^{15}N into amino acids (Fig. 4a), which is coherent with its more prominent effect in increasing the levels of these compounds (Table 1). Under normoxia, a total of 541.8 nmol of ^{15}N-labeled amino acids was detected, with a greater predominance of ^{15}N-glutamine and ^{15}N-asparagine, followed by ^{15}N-aspartate and ^{15}N-glutamate. O_2 deficiency reduced the total content of ^{15}N-amino acids to 94.1 nmol. Only alanine and GABA presented higher ^{15}N incorporation under hypoxia, consistent with the accumulation of these metabolites during this O_2 condition.

Similar results were observed when the root segments were incubated with $^{15}NO_2^-$ (Fig. 4b). In the presence of this ^{15}N source, there was a total of 264.9 nmol of ^{15}N-amino acids during normoxia. Under hypoxia an even greater relative reduction in ^{15}N incorporation (89 %) was observed in comparison with that seen in the $^{15}NH_4^+$ assays (83 %).

Although $^{15}NO_3^-$-incubated segments presented much lower ^{15}N incorporation into amino acids, it was possible to detect higher levels of ^{15}N-alanine and ^{15}N-GABA and a lower total amount of ^{15}N-amino acids under hypoxia in comparison with normoxia (Fig. 4c). This reduced ^{15}N incorporation may be related to the limited NR activity of the isolated root segments observed in our experimental conditions (Table 2). A strong dilution of external $^{15}NO_3^-$ in endogenous pools and no ^{15}N enrichment in amino acids were observed when plants were pretreated with NO_3^- until segment collection (data not shown). Even in this case, a drastic reduction of root NR activity was observed 1 h after incubation of the isolated segments both in normoxia and hypoxia treatments, leading to values similar to those of segments harvested from plants previously transferred to N-free solution (Table 2).

In addition to the determination of the total amount of ^{15}N-amino acids, the analysis of ^{15}N enrichment of each

Table 1 Effect of nitrogen treatments on individual amino acid levels (in nmol/g) of root segments under normoxic and hypoxic conditions

	Normoxia[a]				Hypoxia[a]			
	–N	NO_3^-	NH_4^+	NO_2^-	–N	NO_3^-	NH_4^+	NO_2^-
Ala[b]	28.3	26.5	55.9*	46.8*	184.1	150.1	389.5*	201.5
GABA	19.8	15.4	61.9*	26.1	101.2	82.5	165.4*	85.6
Asp	81.9	75.6	371.7*	304.2*	15.1	13.3	11.9	11.5
Glu	84.9	85.8	240.5*	245.0*	54.2	46.6	48.0	34.8
Asn	39.7	33.3	697.1*	436.9*	16.1	22.7	34.6*	21.8
Gln	2.9	7.3	417.0*	94.1*	10.6	6.5	49.7*	14.4
Others	100.4	69.5	156.0	135.7	218.7	226.1	209.9	159.3

NO_3^- (5 mM), NO_2^- (1 mM), NH_4^+ (1 mM) or no nitrogen source (–N) was added to nutrient solution in which root segments were maintained under normoxia or hypoxia. Amino acid levels were determined by GC–MS 8 h after nitrogen addition

* Difference significant ($P < 0.05$) when compared with respective –N control, according to one-way ANOVA analysis followed by the Tukey test

[a] Data represent mean ($n = 3$)

[b] "Others" includes serine, glycine, valine, leucine, isoleucine, phenylalanine, methionine and tyrosine

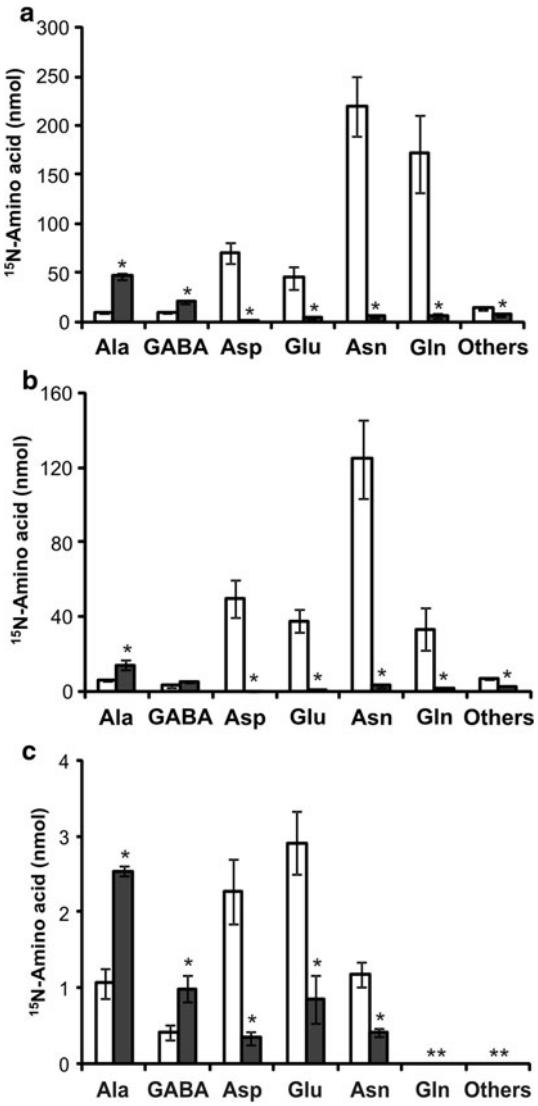


Fig. 4 Effect of hypoxia on ^{15}N incorporation into amino acids. **a** NH_4^+ (1 mM), **b** NO_2^- (1 mM) or **c** NO_3^- (5 mM), all enriched with 30 % ^{15}N, were added to nutrient solution in which root segments were maintained under normoxia (*open bars*) or hypoxia (*closed bars*). The normoxia and hypoxia treatments were initiated 1 h before the addition of the N sources. The amount of amino acids that incorporated ^{15}N was determined by GC–MS 8 h after labeled nitrogen addition. The "Others" column includes serine, glycine, valine, leucine, isoleucine, phenylalanine and tyrosine. Data represent the mean ± SE ($n = 3$). *$P < 0.05$ compared to the respective normoxia control, according to Student's *t* test. ** The quantification of these ^{15}N-amino acids was not possible

amino acid also revealed important information about the nitrogen metabolism in hypoxic condition. The scheme of Fig. 5 shows the evolution of ^{15}N percentage in free amino acids during the 8 h of incubation of the root segments with $^{15}NH_4^+$ under normoxia or hypoxia. In both situations, glutamine was strongly and rapidly labeled. More specifically, ^{15}N labeling occurred first in the amide group of this

Table 2 Nitrate reductase activity (nmol NO_2^- h^{-1} g^{-1}) of root segments collected from plants grown continuously on NO_3^- (NO_3^-) or from plants after being transferred to N-free solution for the last 4 days of cultivation (–N)

	Control	Normoxia	Hypoxia
–N	90.5 ± 9.1	64.7 ± 4.5	52.8 ± 7.7*
NO_3^-	696.3 ± 160.7	47.1 ± 9.5*	71.9 ± 14.1*

NR activity was determined just before (control) and after incubation of root segments in NO_3^--containing or N-free solution for 1 h under normoxia or hypoxia

Data represent mean ± SE ($n = 3$)

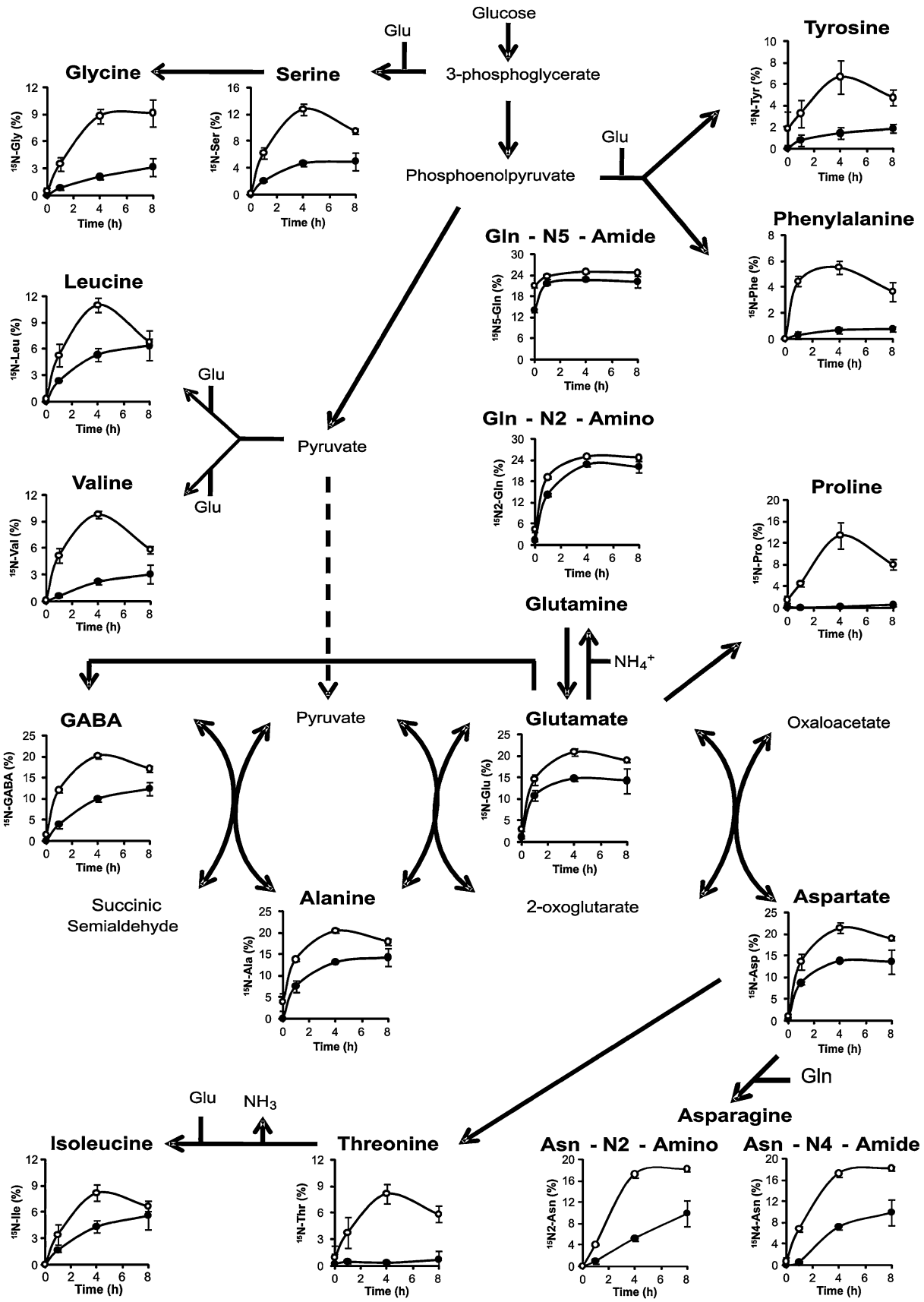
* Difference significant ($P < 0.05$) when compared with respective control, according to one-way ANOVA analysis followed by the Tukey test

amino acid, indicative of GS activity. After glutamine, similar ^{15}N enrichment was observed in glutamate, aspartate, alanine and GABA, amino acids closely related to primary nitrogen assimilation. On the other hand, the ^{15}N enrichment of asparagine was slower (especially under hypoxia), suggesting a slower turnover rate for this amino acid, which is consistent with its function in nitrogen storage and transport (Lea et al. 2007). ^{15}N incorporation into many other amino acids was also detected, but to a lesser extent. It is noteworthy that amino acids whose biosynthetic pathways are more closely related (such as phenylalanine and tyrosine; leucine and valine) presented similar patterns of ^{15}N enrichment.

Hypoxia reduced ^{15}N enrichment in all analyzed amino acids (Fig. 5), ratifying the lower ^{15}N assimilation under this condition. Specifically in the case of the amino acids proline and threonine, there was practically no ^{15}N incorporation during O_2 deficiency. Phenylalanine and tyrosine ^{15}N enrichment was also greatly affected by hypoxia treatment. It is noteworthy that the biosynthetic pathways of these four amino acids are ATP dependent (Morot-Gaudry et al. 2001), which may underlie their greater sensitivity to O_2 privation.

Root segments incubated with $^{15}NO_2^-$ or $^{15}NO_3^-$, although presenting generally lower ^{15}N percentages, did present a pattern of ^{15}N enrichment in amino acids very similar to that obtained for the $^{15}NH_4^+$ treatment, with hypoxia showing the same negative effect (see Online Resources 1 and 2).

Fig. 5 ^{15}N enrichment in amino acids of root segments treated with $^{15}NH_4^+$. NH_4^+ (1 mM, 30 % ^{15}N) was added to nutrient solution in which root segments were maintained under normoxia (*open circles*) or hypoxia (*closed circles*). The normoxia and hypoxia treatments were initiated 1 h before the addition of the N source. ^{15}N enrichment in the different amino acids was determined by GC–MS 0, 1, 4 and 8 h after labeled nitrogen addition. Data represent mean ± SE ($n = 3$) ▶


Glucose
Glu
3-phosphoglycerate
Phosphoenolpyruvate
Glycine
Serine
Tyrosine
Leucine
Phenylalanine
Gln - N5 - Amide
Glu
Pyruvate
Glu
Valine
Gln - N2 - Amino
Proline
Glutamine
NH4+
GABA
Pyruvate
Glutamate
Oxaloacetate
Succinic Semialdehyde
Alanine
2-oxoglutarate
Aspartate
Gln
Asparagine
Glu
NH3
Isoleucine
Threonine
Asn - N2 - Amino
Asn - N4 - Amide
15N-Gly (%)
15N-Ser (%)
15N-Tyr (%)
15N-Leu (%)
15N-Phe (%)
15N5-Gln (%)
15N-Val (%)
15N2-Gln (%)
15N-Pro (%)
15N-GABA (%)
15N-Glu (%)
15N-Ala (%)
15N-Asp (%)
15N-Ile (%)
15N-Thr (%)
15N2-Asn (%)
15N4-Asn (%)
Time (h)

Discussion

The present study provides a detailed analysis of the effect of O_2 deficiency on nitrogen assimilation and amino acid biosynthesis by isolated soybean root segments. In addition to verifying the exogenous and endogenous levels of nitrogen-related metabolites by conventional biochemical methods, the metabolic fate of ^{15}N derived from $^{15}NO_3^-$, $^{15}NO_2^-$ and $^{15}NH_4^+$ was followed by GC–MS, allowing straightforward conclusions to be reached. The relevance of this work is increased given the fact that previous studies with labeled nitrogen have been carried out with seedlings or germinating seeds, using mostly rice as a model and conditions quite different from those adopted here (Reggiani et al. 1995, 1997; Fan et al. 1997; Limami et al. 2008).

Consistent with these ^{15}N studies, a clear effect of hypoxia in reducing ^{15}N incorporation into amino acids was observed, regardless of the nitrogen source (Fig. 4). This result can be explained, at least in part, by the lower uptake of NO_3^-, NO_2^- and NH_4^+ from the solution by the root segments during hypoxia (Fig. 1). Specifically, the data with labeled NO_3^- (Fig. 1a, lower graph) provide direct evidence that NO_3^- is taken up by roots under hypoxia although at a much reduced rate compared with normoxia (Fig 1a, upper and middle graphs). In the experiments with $^{15}NO_2^-$ or $^{15}NH_4^+$, O_2 deficiency reduced the uptake of these metabolites by approximately 60 % (Fig. 1). At the same time, the amount of ^{15}N incorporated into amino acids declined by more than 80 % (Fig. 4), suggesting that hypoxia might be affecting other processes in addition to uptake. Consistent with this hypothesis, when exogenously provided, a more persistent accumulation of NO_2^- and NH_4^+ inside the root segments was observed under hypoxia, in comparison with normoxia assays (Fig. 1), suggesting that NO_2^- and NH_4^+ are metabolized more slowly during O_2 deficiency.

The increment of glutamine levels in NH_4^+-incubated segments was much lower in the hypoxia treatment in comparison with normoxia (Table 1). In the case of NO_2^- incubation, the increase of this amino acid was induced only under aeration. These data are, therefore, consistent with reduced GS activity during hypoxia. Moreover, the rapid initial ^{15}N enrichment in glutamine, in detriment to glutamate, and the detection of ^{15}N labeling first in the amide group of glutamine (Fig. 5) ratified that ^{15}N assimilation occurred by the GS-GOGAT system in both O_2 tensions. It is known that glutamine synthetase (GS) activity is decreased under conditions of O_2 privation, which may result from its dependence on ATP (Limami et al. 2008) as well as the repression of *GS* gene expression under hypoxia (Limami et al. 2008; Rocha et al. 2010a). The involvement of glutamate dehydrogenase (GDH) in nitrogen assimilation under stress conditions has also been suggested (Skopelitis et al. 2006). However, nitrogen incorporation via GDH has been detected only at high NH_4^+ concentrations, leading some authors to question this hypothesis (Limami et al. 2008). In this scenario, the present results, together with data from previous studies (Reggiani et al. 2000; Limami et al. 2008), support the role of GS in nitrogen assimilation even under O_2 deficiency, in detriment to GDH.

In addition to NH_4^+ assimilation into glutamine, the reduction of NO_2^- to NH_4^+ seemed to be negatively affected by hypoxia. In this situation, there was a drop of more than 42 % in the enrichment of endogenous $^{15}NH_4^+$ derived from $^{15}NO_2^-$ in relation to that detected under normoxia (Fig. 1c). At the same time, $^{15}NH_4^+$-incubated segments presented a reduction under hypoxia of only 22 % in endogenous $^{15}NH_4^+$ enrichment (Fig. 1b). This limitation in NH_4^+ formation from NO_2^- may be related to the more prominent effect of hypoxia in reducing the incorporation into amino acids of the ^{15}N derived from $^{15}NO_2^-$, in relation to $^{15}NH_4^+$ (Fig. 4). Consistent with this low assimilation into amino acids during hypoxia, NO_2^--incubated root segments presented amino acid levels similar to those of segments maintained without exogenous nitrogen, while NH_4^+ treatment induced a higher increase in alanine, GABA, glutamine and asparagine (Table 1). All these results are in accordance with the sensitivity of nitrite reductase (NiR) to O_2 deficiency, as previously verified in the literature (Lee 1978; Botrel et al. 1996). NO_2^- release in the external medium observed under O_2 deficiency situations is a well-described consequence of NiR inhibition (Morard et al. 2004; Brandão and Sodek 2009). In the present study, when NO_2^- was added exogenously, it was possible to detect accumulation of endogenous NO_2^- in root segments under hypoxia (Fig. 1c). In this situation, NO production by the root segments was stimulated (Fig. 2), consistent with growing evidence that NO_2^- is an important precursor for NO synthesis in plants (Moreau et al. 2010). Since NO acts as a signaling molecule in plants (Neill et al. 2008), this partial deviation of NO_2^- to NO production may be relevant in the response of roots to O_2 deficiency. Particularly, an involvement of NO derived from NO_2^- in anoxic pH regulation (Libourel et al. 2006) and modulation of mitochondrial oxygen consumption during hypoxia (Borisjuk and Rolletschek 2009) have been hypothesized.

In comparison with $^{15}NO_2^-$ and $^{15}NH_4^+$, $^{15}NO_3^-$ led to a much lower ^{15}N incorporation into amino acids, both under normoxic and hypoxic conditions (Fig. 4), associated with amino acid levels similar to those of –N control segments (Table 1). Nevertheless, for the $^{15}NO_3^-$ treatment ^{15}N incorporation into amino acids followed the same pattern observed in the assays with other nitrogen sources

(Figs. 4, 5; Online Resources 1 and 2). The limited NO_3^- metabolism observed in the present assays is probably a consequence of the sharp decline in NR activity presented by roots following the isolation of the segments, as pointed out earlier. Nevertheless, the data do show that uptake and metabolism of NO_3^- do take place under hypoxia, although at a reduced rate compared with normoxia. Further studies using intact roots rather than isolated segments, where normal levels of NR can be expected, are necessary for a better understanding of hypoxic NO_3^- metabolism.

Independent of the nitrogen source, the overall analysis of endogenous amino acid content and its ^{15}N enrichment indicate that hypoxia led to the redirection of amino acid metabolism, particularly towards alanine and GABA synthesis, amino acids that presented the highest accumulation in this situation (Fig. 3 and Table 1). Indeed, after 8 h of hypoxia, more than 50 % of ^{15}N incorporated into amino acids was found in ^{15}N-alanine and ^{15}N-GABA, reaching 72 % when $^{15}NH_4^+$ was used (Fig. 4). Moreover, a high ^{15}N percentage was detected in aspartate and glutamate (Fig. 5), while these amino acids always presented low levels during hypoxia (Table 1), consistent with the role of these amino acids as precursors to alanine and GABA synthesis. Alanine accumulation may be a consequence, at least in part, of increased expression and activity of the alanine aminotransferase enzyme, as described in soybean (de Sousa and Sodek 2003; Rocha et al. 2010b) and other plant species (Muench and Good 1994; Ricoult et al. 2006). It has been suggested that alanine acts as a non-toxic form of carbon and nitrogen storage during hypoxia, since it is able to form pyruvate and to participate in the synthesis of other amino acids by transamination during the recovery period on return to normoxia (de Sousa and Sodek 2003; Miyashita et al. 2007; Rocha et al. 2010b). Recently, the importance of GABA metabolism for alanine synthesis during O_2 deficiency was suggested in a study with mutants defective in genes related to the GABA shunt (Miyashita and Good 2008). Moreover, a role of GABA turnover in the tolerance to O_2 deficiency has been proposed, since its catabolism to γ-hydroxybutyrate contributes to NAD^+ regeneration under hypoxia (Breitkreuz et al. 2003) while synthesis of GABA via glutamate decarboxylase consumes H^+, thereby counterbalancing the detrimental effects of cytosolic acidification during hypoxia (Crawford et al. 1994). The involvement of alanine in regulating cytosolic pH under hypoxia has been also suggested (Shingaki-Wells et al. 2011).

In addition to alanine and GABA, many other amino acids presented increased levels during hypoxia, though to a lesser extent (Fig. 3; Table 1). This increase in total amino acid content has already been reported for many plant species submitted to O_2 deficiency (Fan et al. 1997; Sousa and Sodek 2002; van Dongen et al. 2009; Rocha et al. 2010a), and recently this was associated with a protective effect of some amino acids on cell viability (Shingaki-Wells et al. 2011). The modifications in amino acid metabolism induced by hypoxia were also observed in N-free root segments (Fig. 3), indicating that they do not necessarily depend on the assimilation of exogenous nitrogen, as previously suggested by Puiatti and Sodek (1999) and Sousa and Sodek (2002). Here, this hypothesis was ratified by the ^{15}N-enrichment analysis, given that this increase of free amino acid content (with exception of alanine and GABA) occurred in spite of a lower amount of ^{15}N incorporated in these compounds. As good examples, the amino acids proline and tyrosine practically did not present incorporation of ^{15}N during hypoxia (Fig. 5), yet their content increased by almost fivefold in comparison with normoxia (Fig. 3). Therefore, it is probable that these amino acids are provided exclusively by endogenous sources, such as the mobilization of proteins through the action of proteases (Reggiani et al. 1988). Furthermore, the hypoxia-induced decrease in the overall rate of protein synthesis would reduce the demand for amino acids and thereby also contribute to the accumulation of free amino acids during O_2 deficiency (Reggiani and Bertani 2003).

Overall, the present work demonstrated a clear effect of hypoxia in reducing the uptake and the assimilation of inorganic nitrogen sources by isolated soybean root segments. Nevertheless, all sources including nitrate were taken up and metabolized by the root segments under hypoxia. As verified by the pattern of ^{15}N enrichment, individual amino acids responded in a diverse way to O_2 privation, suggesting a differential sensitivity of each biosynthetic pathway to this stress. Further studies are necessary to determine the specific enzymes of each pathway that are more strongly affected by hypoxia, as well as the involvement of these processes in the metabolic adaptation to O_2 deficiency. Moreover, analysis of nitrogen assimilation by intact plants will be essential for determining the physiological relevance of the data provided by the present in vitro study.

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References

Allegre A, Silvestre J, Morard P, Kallerhoff J, Pinelli E (2004) Nitrate reductase regulation in tomato roots by exogenous nitrate: a possible role in tolerance to long-term root anoxia. J Exp Bot 55:2625–2634

Ashton IW, Miller AE, Bowman WD, Suding KN (2010) Niche complementarity due to plasticity in resource use: plant partitioning of chemical *N* forms. Ecology 91:3252–3260

Bailey-Serres J, Voesenek LACJ (2008) Flooding stress: acclimations and genetic diversity. Annu Rev Plant Biol 59:313–339

Borisjuk L, Rolletschek H (2009) The oxygen status of the developing seed. New Phytol 182:17–30

Botrel A, Magne C, Kaiser WM (1996) Nitrate reduction, nitrite reduction and ammonium assimilation in barley roots in response to anoxia. Plant Physiol Biochem 34:645–652

Brandão AD, Sodek L (2009) Nitrate uptake and metabolism by roots of soybean under oxygen deficiency. Braz J Plant Physiol 21:13–23

Breitkreuz KE, Allan WL, Van Cauwenberghe OR, Jakobs C et al (2003) A novel γ-hydroxybutyrate dehydrogenase: identification and expression of an *Arabidopsis* cDNA and potential role under oxygen deficiency. J Biol Chem 17(278):41552–41556

Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissues by nitration of salicylic acid. Commun Soil Sci Plant Anal 6:71–80

Chaves das Neves HJ, Vasconcelos AM (1987) Capillary gas chromatography of amino acids, including asparagine and glutamine: sensitive gas chromatographic-mass spectrometric and selected ion monitoring gas chromatographic-mass spectrometric detection of the N, O(S)-tert.-butyldimethylsilyl derivatives. J Chromatogr 392:249–258

Crawford LA, Bown AW, Breitkreuz KE, Guinel FC (1994) The synthesis of γ-aminobutyric acid in response to treatments reducing cytosolic pH. Plant Physiol 104:865–871

De Sousa CAF, Sodek L (2003) Alanine metabolism and alanine aminotransferase activity in soybean (*Glycine max*) during hypoxia of the root system and subsequent return to normoxia. Environ Exp Bot 50:1–8

Fan TM-W, Higashi RM, Frenkiel TA, Lane AN (1997) Anaerobic nitrate and ammonium metabolism in flood-tolerant rice coleoptiles. J Exp Bot 48:1655–1666

Fehr WR, Caviness DT, Burmood JS, Pennington JS (1971) Stage development descriptions for soybeans *Glycine max* (L.) Merril. Crop Sci 11:929–931

Hageman RH, Reed AJ (1980) Nitrate reductase from higher plants. Methods Enzymol 69:270–280

Hoagland DR, Arnon DI (1950) The water culture method of growing plants without soil. California Agricultural Experiment Station, Bulletin 347:1–32

Horchani F, Aschi-Smiti S, Brouquisse R (2010) Involvement of nitrate reductase in the tolerance of tomato (*Solanum lycopersicum* L.) plants to prolonged root hypoxia. Acta Physiol Plant 32:1113–1123

Irfan M, Hayat S, Hayat Q, Afroz S, Ahmad A (2010) Physiological and biochemical changes in plants under waterlogging. Protoplasma 241:3–17

Jaworski EG (1971) Nitrate reductase assay in intact plant tissues. Biochem Biophys Res Commun 43:1274–1279

Lea PJ (1993) Nitrogen metabolism. In: Lea PJ, Leegood RC (eds) Plant biochemistry and molecular biology. Wiley, New York, pp 155–180

Lea PJ, Sodek L, Parry MAJ, Shewry PR, Halford NG (2007) Asparagine in plants. Ann Appl Biol 150:1–26

Lee RB (1978) Inorganic nitrogen metabolism in barley roots under poorly aerated conditions. J Exp Bot 29:693–708

Libourel IG, van Bodegom PM, Fricker MD, Ratcliffe RG (2006) Nitrite reduces cytoplasmic acidosis under anoxia. Plant Physiol 142:1710–1717

Licausi F, Perata P (2009) Low oxygen signaling and tolerance in plants. Adv Bot Res 50:139–198

Limami AM, Ricoult C, Cliquet J, Planchet E (2008) Concerted modulation of alanine and glutamate metabolism in young *Medicago truncatula* seedlings under hypoxic stress. J Exp Bot 59:2325–2335

Masclaux-Daubresse C, Reisdorf-Cren M, Pageau K et al (2006) Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. Plant Physiol 140:444–456

Mawhinney TP, Robinett RSR, Atalay A, Madson M (1986) Analysis of amino acids as their tert.-butyldimethylsilyl derivatives by gas-liquid chromatography and mass spectrometry. J Chromatogr 358:231–242

McCullough H (1967) Determination of ammonia in whole blood by a direct colorimetric method. Clin Chim Acta 17:297–304

Miyashita Y, Good AG (2008) Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. Plant Cell Physiol 49:92–102

Miyashita Y, Dolferus R, Ismond KP, Good AG (2007) Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. Plant J 49:1108–1121

Morard P, Silvestre J, Lacoste L, Caumes E, Lamaze T (2004) Nitrate uptake and nitrite release by tomato roots in response to anoxia. J Plant Physiol 161:855–866

Moreau M, Lindermayr C, Durner J, Klessig DF (2010) NO synthesis and signaling in plants—where do we stand? Physiol Plant 138:372–383

Morot-Gaudry J-F, Job D, Lea PJ (2001) Amino acid metabolism. In: Lea PJ, Morot-Gaudry J-F (eds) Plant Nitrogen. Springer, Berlin, pp 167–211

Muench DG, Good AG (1994) Hypoxically inducible barley alanine aminotransferase: cDNA cloning and expression analysis. Plant Mol Biol 24:417–427

Narsai R, Rocha M, Geigenberger P, Whelan J, van Dongen JT (2011) Comparative analysis between plant species of transcriptional and metabolic responses to hypoxia. New Phytol 190:472–487

Neill S, Bright J, Desikan R, Hancock J, Harrison J, Wilson I (2008) Nitric oxide evolution and perception. J Exp Bot 59:25–35

Oliveira HC, Justino GC, Sodek L, Salgado I (2009) Amino acid recovery does not prevent susceptibility to *Pseudomonas syringae* in nitrate reductase double-deficient *Arabidopsis thaliana* plants. Plant Sci 176:105–111

Puiatti M, Sodek L (1999) Waterlogging affects nitrogen transport in the xylem of soybean. Plant Physiol Biochem 37:767–773

Reggiani R, Bertani A (2003) Anaerobic amino acid metabolism. Russ J Plant Physiol 50:733–736

Reggiani R, Cantu CA, Brambilla I, Bertani A (1988) Accumulation and interconversion of amino acids in rice roots under anoxia. Plant Cell Physiol 29:981–987

Reggiani R, Bertini F, Mattana M (1995) Incorporation of nitrate nitrogen into amino acids during the anaerobic germination of rice. Amino Acids 9:385–390

Reggiani R, Bertini F, Mattana M (1997) Incorporation of nitrate nitrogen in rice seedlings transferred to anaerobic conditions. Amino Acids 13:183–188

Reggiani R, Nebuloni M, Mattana M, Brambilla I (2000) Anaerobic accumulation of amino acids in rice roots: role of glutamine synthetase/glutamate synthase cycle. Amino Acids 18:207–217

Ricoult C, Echeverria LO, Cliquet JB, Limami AM (2006) Characterization of alanine aminotransferase (AlaAT) multigene family and hypoxic response in young seedlings of the model legume *Medicago truncatula*. J Exp Bot 57:3079–3089

Rocha M, Licausi L, Araújo WL et al (2010a) Glycolysis and the TCA-cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. Plant Physiol 152:1501–1513

Rocha M, Sodek L, Licausi F, Hameed MW et al (2010b) Analysis of alanine aminotransferase in various organs of soybean (*Glycine max*) and in dependence of different nitrogen fertilizers during hypoxic stress. Amino Acids 39:1043–1053

Shingaki-Wells RN, Huang S, Taylor NL, Carroll AJ et al (2011) Differential molecular responses of rice and wheat coleoptiles to anoxia reveal novel metabolic adaptations in amino acid metabolism for tissue tolerance. Plant Physiol 156:1706–1724

Silvester WB, Parsons R, Watt PW (1996) Direct measurement of release and assimilation of ammonia in the *Gunnera–Nostoc* symbiosis. New Phytol 132:617–625

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED et al (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell 18:2767–2781

Smythe GA, Matanovic G, Yi D, Duncan MW (1999) Trifluoroacetic anhydride-catalyzed nitration of toluene as an approach to the specific analysis of nitrate by gas chromatography–mass spectrometry. Nitric Oxide 3:67–74

Sousa CAF, Sodek L (2002) Metabolic changes in soybean plants in response to waterlogging in the presence of nitrate. Physiol Mol Biol Plant 8:97–104

Thomas AL, Sodek L (2005) Development of nodulated soybean plant after flooding of the root system with different sources of nitrogen. Braz J Plant Physiol 17:291–297

Thomas AL, Sodek L (2006) Amino acid and ureide transport in the xylem of symbiotic soybean plants during short-term flooding of the root system in the presence of different sources of nitrogen. Braz J Plant Physiol 18:333–339

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. J Exp Bot 32:509–522

van Dongen JT, Frohlich A, Ramirez-Aguilar SJ et al (2009) Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of *Arabidopsis* plants. Ann Bot 103:269–280