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Anaerobic accumulation of amino acids in rice roots: role of the glutamine synthetase/glutamate synthase cycle

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Summary. Accumulation of amino acids was studied in rice roots of 3-day-old seedlings subjected for 48 h to anaerobic conditions. Alanine and Gaba were the main amino acids accumulated under anoxia. Their synthesis was strongly inhibited by MSX and AZA, inhibitors of glutamine synthetase and glutamate synthase. These activities increased after 8 h of anaerobic treatment and, by immunoprecipitation of ^{35}S-labeled proteins, it was shown that glutamine synthetase and ferredoxin-dependent glutamate synthase were synthesized during the treatment. These findings indicate that the glutamine synthetase/glutamate synthase cycle play an important role in anaerobic amino acid accumulation.

Keywords: Amino acids – Anoxia – Glutamate synthase – Glutamine synthetase – Rice – Root

Introduction

In anaerobiosis, several compounds like alanine, γ-aminobutyric acid (Gaba), proline and putrescine are accumulated in plant tissues (Streeter and Thompson, 1971; Reggiani et al., 1988; Reggiani et al., 1989). Alanine and Gaba may represent about 50% of the amino acid pool in anoxic tissues (Reggiani et al., 1988). Alanine is anaerobically synthesized by the increase in alanine aminotransferase activity that involves the expression of the AlaAT-2 mRNA (Good and Crosby, 1989; Muench and Good, 1989). The synthesis of Gaba through glutamate decarboxylase in reduced oxygen supply occurred by effect of decreasing cytoplasmic pH (Carroll et al., 1994). Accumulation of this amino acid took place without changes in the quantity of glutamate decarboxylase protein (Serraj et al., 1998), but by an activation of glutamate decarboxylase, probably via a signal transduction pathway involving calcium and calmodulin (Arazi et al., 1995). Alanine, Gaba, proline and putrescine are synthesized, directly or indirectly, from glutamic acid. In plant tissues, this amino acid may derive: i) from proteolytic processes (Aurisano et al.,

1995); ii) by transamination reactions (from other amino acids); iii) by the operation of the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle (Miflin and Lea, 1976).

In rice coleoptile, a tissue highly tolerant to anaerobic stress, it was shown that GS, ferredoxin (Fd)-dependent GOGAT and Fd-$NADP^+$ reductase are newly synthezised during the stress for the re-assimilation of ammonia (Mattana et al., 1994; Mattana et al., 1996; Mattana et al., 1997). Recently, it has been established that nitrogen nitrate is incorporated in anaerobic rice roots into glutamine, glutamate, aspartate, alanine and Gaba (Reggiani et al., 1997). This paper suggested a role for the GS/GOGAT cycle in root under anaerobic conditions. Some studies have shown that the GS/GOGAT cycle is involved in the response to different abiotic stresses. Cordovilla et al. (1996) showed that the tolerance to salinity in bean may depend on the increase in activity and expression of GS and NADH-GOGAT. *E. coli* mutants lacking the GOGAT enzyme exhibited an altered tolerance to osmotic stress (Saroja and Gowrishankar, 1996). In tomato plants subjected to water stress, it was shown that cytosolic GS plays an important role in nitrogen mobilization from leaves to other tissues (Bauer et al., 1997).

In the present study, we investigated the role of the GS/GOGAT cycle in anaerobic amino acid accumulation in rice roots. This was assessed by the use of 2 inhibitors of the GS and GOGAT reactions, methionine sulfoximine and azaserine (MSX and AZA, respectively). These inhibitors were amply used in studying the role of GS and GOGAT in plant metabolism (Lea and Ireland, 1999). The importance of the GS/GOGAT cycle was also established by studying the anaerobic synthesis of GS and GOGAT proteins.

Materials and methods

Plant material and anaerobic treatments

Rice seeds were sterilized as previously described (Reggiani et al., 1993). The seeds were germinated in Petri dishes (100 seeds/Petri) in the presence of 1 mM MES (pH 6.0) and 0.5 mM $CaSO_4$ (MES-Ca solution). The germination occurred at 29°C in the dark. The seedlings were used 3 days after germination.

Rice seedlings were then anaerobically treated for 8, 24, 48 h. In every experiment, some seedlings were allowed to regrow in air to check viability of the root. The Petri dishes were put in an anaerobic jar and anoxic conditions were generated by the use of BBL GasPack Plus purchased from Becton Dickinson. Inside the jar, an indicator of anaerobiosis was included in order to control the anoxic condition. At the end of the anaerobic treatment, cell metabolism was immediately stopped by transferring the seedlings to iced water.

Amino acid analysis

Perchloric acid extracts were obtained as previously described (Reggiani et al., 1993). During the extraction, an aliquot of cysteic acid was added to evaluate the recovery efficiency that resulted of 86.8% ± 3.8.

To some treatments, MSX or AZA were added to a final concentration of 1 mM. The amino acid analysis by HPLC of the o-pthalaldehyde (OPA) derivatives was performed according to the method of Jarrett et al. (1986) modified by Reggiani et al. (1995). The

fluorescence of OPA-amino acids was monitored by a Jasco FP-920 fluorescence detector and continuous online quantification of chromatographic peaks was carried out by a D-2000 computing integrator (Hitachi-Merck). The amount of every amino acid was determined by comparison with a standard solution containing all the amino acids.

Since MSX co-eluted with glutamine by HPLC, the level of glutamine was differently quantitated. Perchloric acid extracts were subjected to acid hydrolysis (in 6N HCl at 110°C for 16 h) to convert glutamine to glutamic acid. The increase in glutamate concentration into samples was considered as glutamine. Ammonia was determined by Ammonia diagnostic kit (Sigma).

Extraction and assay of GS and GOGAT

To some treatments, cycloheximide (CHX) was added to a final concentration of 2 μg/ml. Extract preparation and assay for GS, Fd-GOGAT and NADH-GOGAT were performed as previously described (Lea et al., 1990). The assay was carried out immediately after extraction. Glutamine and glutamate formed during the GS and GOGAT reactions were estimated by HPLC by the method of Unnithan et al. (1984). OPA-derivatives of both amino acids were separated on a RP18 column (Merck) through isocratic elution with the following buffer: 50 mM sodium acetate (pH 5.9)/methanol (3:1). The separation was performed at a flow rate of 0.8 ml min^{-1}. The amount of glutamine and glutamate was determined by comparison with a solution containing a known amount of each amino acid. Protein content in the extracts was measured by Bio-Rad Protein Assay using bovine serum albumin as a standard.

Protein labeling and immunoprecipitation

After 3 days of germination, rice seedlings were transferred to special bottles filled with MES-Ca solution. To each bottle was added 129.5 MBq of a mixture of ^{35}S-methionine and ^{35}S-cysteine obtained from ICN Biomedicals. Anoxia in the bottles was obtained by flushing nitrogen gas at 40 L h^{-1} (Mattana et al., 1996). After 24 h of stress, the roots were removed and frozen in liquid nitrogen. The extraction was that appropriate for GS and GOGAT enzymes. The extracts were centrifuged at 4°C for 15 min at 13,000 g and the supernatant was used for immunoprecipitation. This occurred with anti-GS, anti-Fd-GOGAT and anti-NADH-GOGAT antibodies using the method of Gething et al. (1986). The final pellet was resuspended in sample buffer, denaturated and loaded on an SDS-PAGE gel for electrophoresis.

Results

The level of total amino acids in roots of rice seedlings subjected to anaerobic stress is shown in Fig. 1. Accumulation of amino acids was observed after 8 h of anoxic treatment and proceeded almost linearly for 40 h. After 48 h of anaerobiosis, the amino acid concentration was 3 times the level in air. In non-stress conditions, the amount of amino acids in rice roots remained relatively constant. MSX or AZA (inhibitors of GS and GOGAT, respectively) were added to the anaerobic treatments in order to evaluate the contribution of the GS/GOGAT cycle in amino acid accumulation. The presence of both inhibitors reduced the amino acids accumulation by 31% after 48 h of anoxia (Fig. 1). This reduction was underestimated because the two inhibitors, being themselves amino acids, increased the level of total amino acids in those treatments.

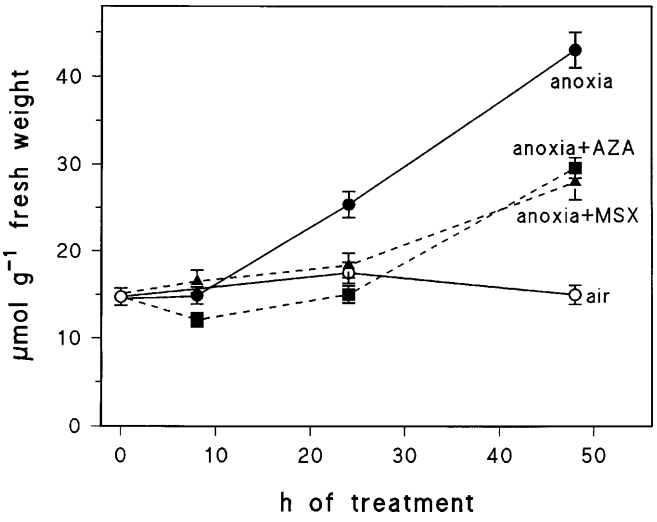

Fig. 1. Level of total amino acids in rice roots in air or during 48 h of anaerobic treatment in the absence or presence of 1 mM MSX or AZA. Vertical bars = SE; N = 3

The levels of alanine and Gaba in rice roots increased under anaerobic conditions, while they remained constant in air (Fig. 2). It is interesting to note that the alanine and Gaba accumulation started from the beginning of the anaerobic treatment, before the total amino acid increase (Fig. 1). After 48 h of stress, the concentration of alanine was increased of 6.6 times in comparison with the control in air while in AZA-treated roots was just 2 times. The roots treated with MSX showed, after 48 h of anaerobiosis, a 2.5-fold increase in alanine concentration respect to the aerobic value. The Gaba concentration increased faster in the first 8 h of stress (2.7 times) and then its level constantly grew in the further 40 h of treatment, though with a lower slope (Fig. 2). After 2 days of anaerobiosis, the level of Gaba was 5.3 times higher than in air. In the presence of the GS/GOGAT inhibitors, such accumulation was significantly reduced after 48 h of anaerobiosis (2.9 times higher than the aerobic control).

During the first 8 h of anoxic treatment, the concentration of glutamate and glutamine in roots was reduced 71% and 73% in comparison with the aerobic level, respectively (Fig. 3). Under aerobic conditions, the concentration of glutamine did not change appreciably while that of glutamate decreased by 29% in 48 h. In the presence of MSX, the glutamine level decreased more than in anaerobic roots, becoming close to zero after 48 h of stress. In the presence of AZA, the glutamine concentration was constantly higher than its anaerobic level in the absence of inhibitor. As expected, the inhibition of GS by MSX led to an accumulation of ammonia in root (about 7 times the aerobic concentration, Fig. 3). A higher ammonia concentration was also observed in AZA-treated roots in comparison with the anoxic roots. AZA decreased the glutamate concentration in the first 8 h of anaerobic treatment but, after this initial phase, its concentration began to grow. In the

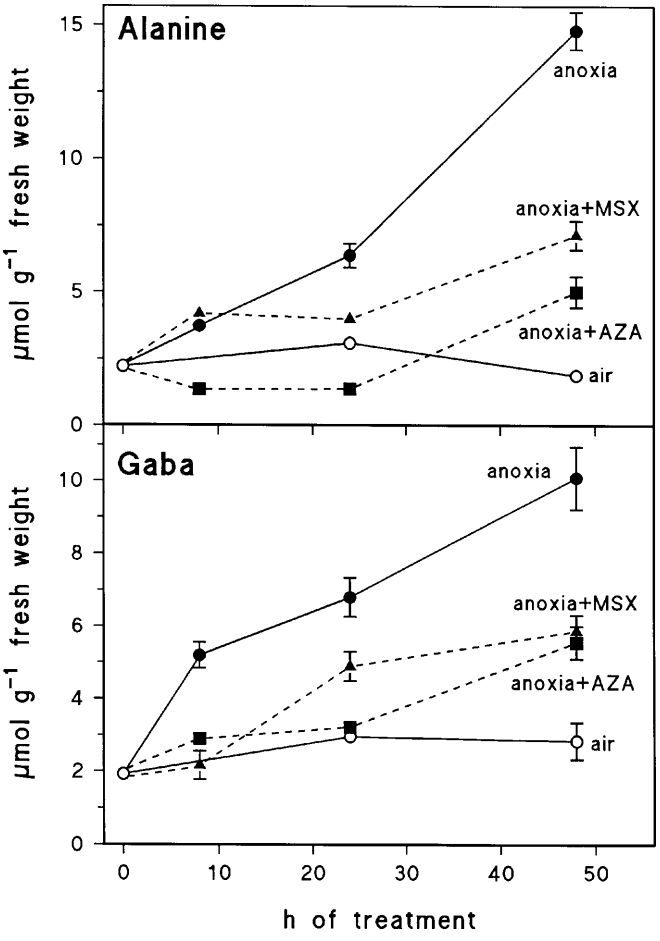

Fig. 2. Alanine and Gaba levels in rice roots in air or during 48 h of anaerobic treatment in the absence or presence of 1 mM MSX or AZA. Vertical bars = SE; N = 3

presence of MSX, the level of glutamate decreased during the anaerobic treatment and, after 48 h, was close to zero.

In Fig. 3 is shown the level of all the other amino acids not considered before. As can be seen, after a decrease in the first 8 h of anaerobiosis, the level of such amino acids constantly grew during the following 40 h up to reach a value 2.6 times higher than that before the stress. The level of these amino acids after 1 or 2 days of anaerobic treatment was not appreciably influenced by the presence of the two inhibitors. In air, the level of all the other amino acids remained constant.

GS, Fd-GOGAT and NADH-GOGAT activities

The specific activity of GS, Fd-GOGAT and NADH-GOGAT in rice roots subjected to anaerobiosis was determined during 24 h of treatment. To get information on the involvement of the protein synthesis in maintaining the

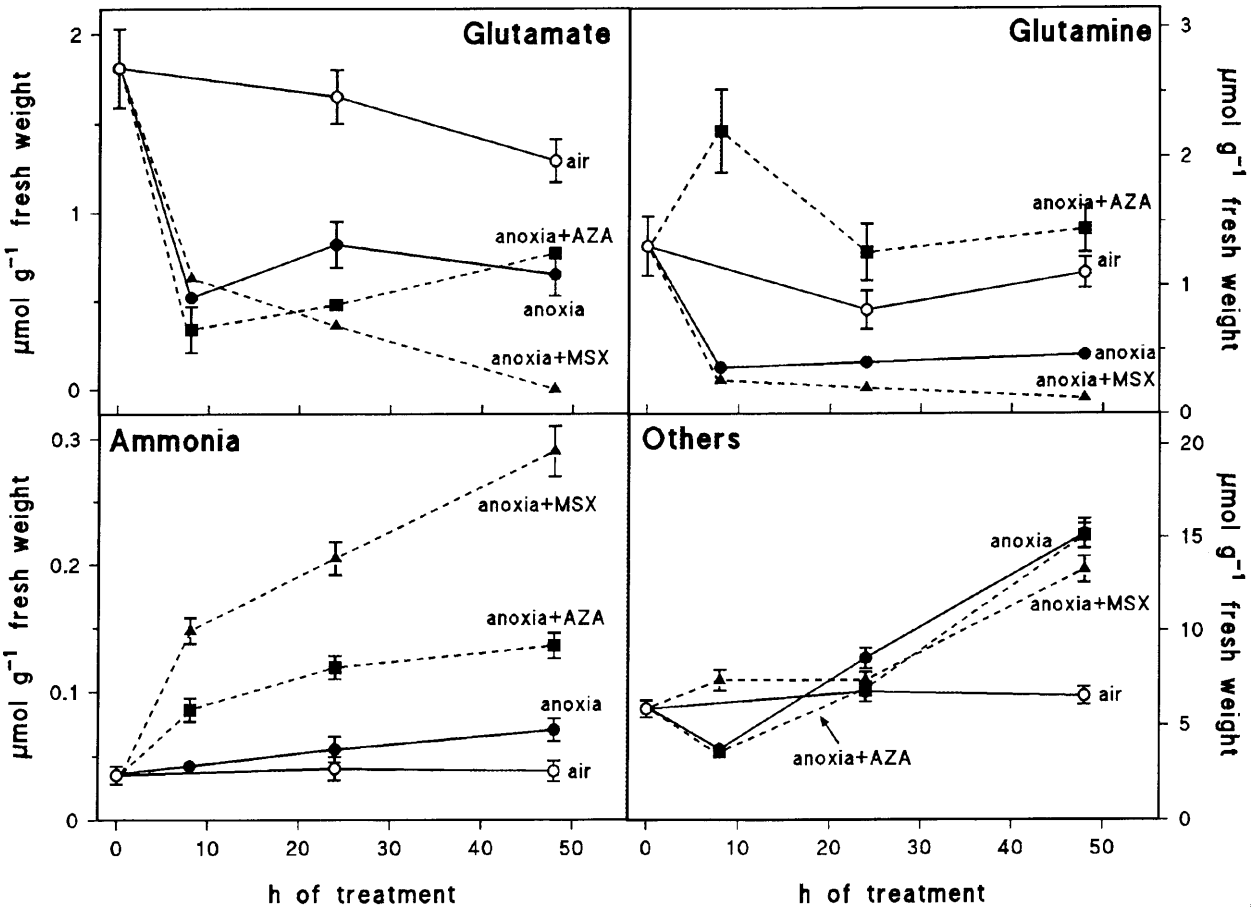

Fig. 3. Level of glutamate, glutamine, ammonia and other amino acids in rice roots in air or during 48 h of anaerobic treatment in the absence or presence of 1 mM MSX or AZA. Vertical bars = SE; N = 3

enzyme activity under anoxia, CHX was added to 24 h treatments. GS activity remained constant in the first 8 h of anaerobic treatment and then increased becoming 1.4 times higher than in air after 24 h (Fig. 4). Fd-GOGAT activity followed the same profile observed for GS activity. After 24 h of stress, this activity was 36% higher than in air. The presence of CHX reduced both activities after 24 h of anoxia and they resulted similar to the level before the imposition of the stress. The NADH-GOGAT activity in rice resulted 4.4 times lower than that of Fd-GOGAT (Fig. 4). It increased during the anoxic treatment and, after 24 h of stress, it was 64% higher than the activity in air. The presence of CHX did not significantly modify NADH-GOGAT activity.

Anaerobic expression of GS, Fd-GOGAT and NADH-GOGAT

To verify if GS, Fd-GOGAT and NADH-GOGAT proteins were *ex novo* synthesized in anaerobic rice roots, an *in vivo* experiment was carried out in

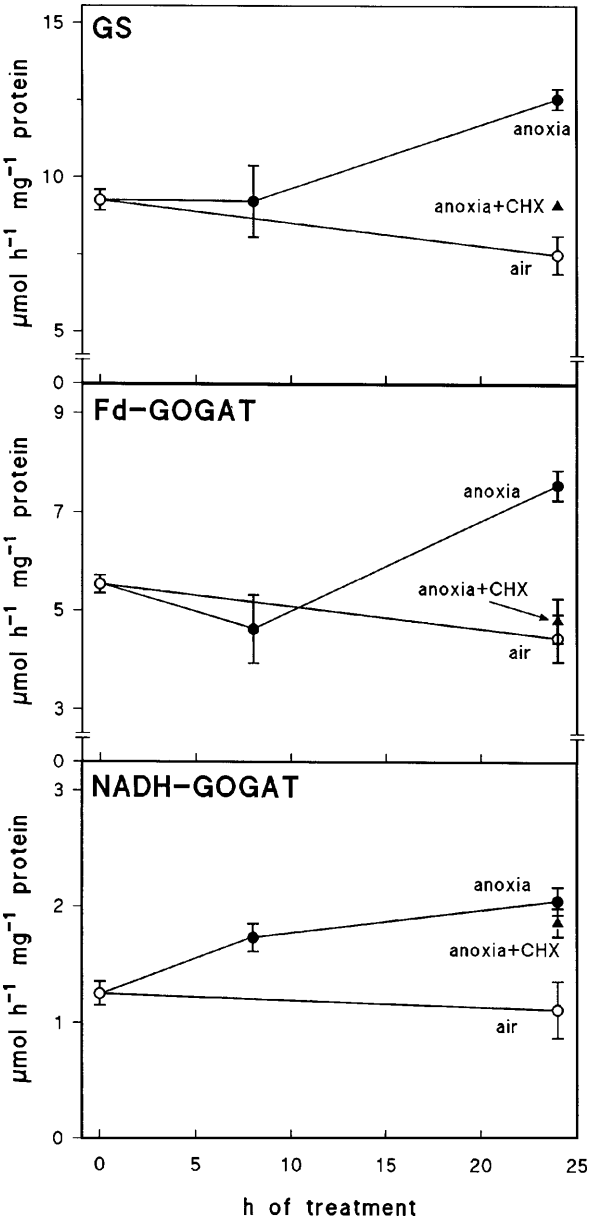

Fig. 4. Specific activity of GS, Fd-GOGAT and NADH-GOGAT in rice roots in air or during 24 h of anaerobic treatment. Two $\mu g\,ml^{-1}$ CHX was added to the 24 h-anaerobic treatment. Vertical bars = SE; N = 5

which 3-day-old seedlings were supplied with a mixture of ^{35}S-methionine and ^{35}S-cysteine during a 24 h anaerobic treatment. Root extracts were then used for immunoprecipitation with specific antibodies.

The Fig. 5 shows that anti-GS antibody recognizes a strong labeled 41 kDa band which corresponds to the cytosolic GS (Mattana et al., 1994). The plastidial GS (44 kDa) was not evident but its presence might be masked by

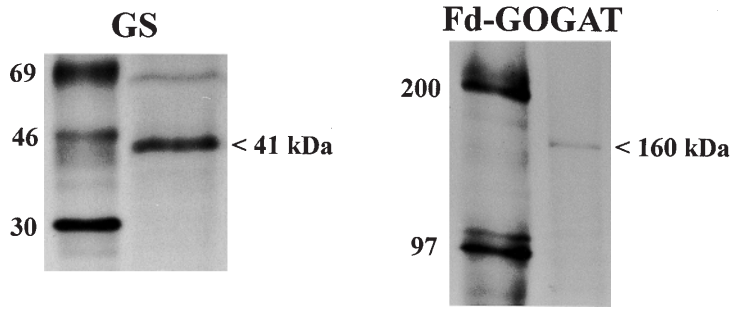

Fig. 5. Immunoprecipitation of *in vivo*-^{35}S-labeled GS and Fd-GOGAT extracted from anaerobic rice roots. Molecular masses of labeled markers are given in the left lane

the abundance of cytosolic GS. Anti-Fd-GOGAT antibody recognized a 160 kDa band; this mass corresponds to that described for the Fd-GOGAT of rice coleoptile (Mattana et al., 1996). On the contrary, antibodies against NADH-GOGAT did not recognize any band in the region corresponding to the expected molecular weight of this protein (193 kDa; Hayakawa et al., 1992).

Discussion

The aim of this work was to investigate the role of the GS/GOGAT cycle in anaerobic amino acid accumulation in rice roots. The accumulation of amino acids in plant tissues subjected to anaerobic stress has been described by numerous authors (Streeter and Thompson, 1971; Bertani and Brambilla, 1982; Reggiani et al., 1985). In rice roots, the concentration of amino acids increased progressively after the first 8 h of stress. The presence of MSX or AZA reduced the anaerobic concentration of total amino acids suggesting a role of the GS/GOGAT cycle in amino acid synthesis. The inhibition of amino acid synthesis by inhibitors may be imputed to a depletion in glutamate and glutamine pools as described by different authors (Rhodes et al., 1986; Lee et al., 1992; Magalhaes et al., 1995). This was evident in MSX-treated roots but not in the presence of AZA. The recovery of glutamate level in AZA-treated roots might indicate that, when severe inhibition of glutamate synthesis occurred, alternative pathway are activated (transamination, proteolysis).

The level of alanine and Gaba started to grow after the imposition of the stress while the level of total amino acids grew after 8 h of anoxia. This points out that the accumulation of these two amino acids is an early event in the anaerobic response. Alanine and Gaba are bio-compatible solutes and their accumulation may be a response to the decrease of osmotic potential and cytoplasmic pH (Reggiani et al., 1988; Carroll et al., 1994; Aurisano et al., 1995). In fact, the anaerobic sugar level strongly decreased as a consequence of a faster carbohydrate consumption (data not shown), and it is likely that amino acid synthesis serves to counteract a decrease in osmotic potential. The

activation of the process of alanine and Gaba accumulation induced an abrupt decrease in the glutamate and glutamine concentrations in the first 8 h of anaerobic treatment (71% and 73%, respectively). After this period, the concentration of these amino acids remained lower than that in aerobic condition. On the contrary, the level of alanine and Gaba in rice roots increased with the proceeding of the anoxic treatment. This datum suggests that a system had to be activated to regenerate glutamate for these synthesis.

In higher plants, glutamate can be produced under stress by transamination reactions from other amino acids (deriving from proteolysis) or through the GS/GOGAT cycle (every cycle produces a molecule of glutamate from ammonium and α-ketoglutarate). In this work, we evaluated the role of this last pathway in amino acid accumulation by the use of MSX and AZA. Both the inhibitors significantly reduced the accumulation of alanina and Gaba indicating that the GS/GOGAT cycle is important under anoxia in regenerating glutamate for the synthesis of these amino acids. Moreover, the effect of MSX and AZA on glutamate, glutamine and ammonia concentrations were consistent with a positive inhibition of GS and GOGAT enzymes. The importance of the GS/GOGAT cycle was strengthened by the evidence that cytosolic GS and Fd-GOGAT proteins are newly synthesized under anoxia, condition in which only few proteins are synthesized (mainly enzymes of carbohydrate metabolism). The cytosolic isoenzyme of GS would be very important in re-assimilation of ammonia deriving from deamination reactions (Aurisano et al., 1995). Moreover, GS and GOGAT activities resulted higher than the aerobic control after 24 h of stress and, with the exception of NADH-GOGAT, the activities were sensible to CHX, indicating that the protein synthesis is involved.

As demonstrated from the data here shown, the GS/GOGAT cycle is involved in glutamate synthesis required for the anaerobic accumulation of alanine and Gaba. Although, this cycle consumes energy (ATP) in the GS reaction and, energy saving is of primarily importance under anoxia, there are undoubted advantages. The regeneration of glutamate through the GOGAT reaction and the decarboxylation of glutamate to produce Gaba are H^+-consuming processes, thus contributing to H^+ homeostasis. Moreover, alanine and Gaba accumulation, besides to save carbon and nitrogen under stress, would contribute to the osmotic potential of the root.

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