Effect of High Levels of Ammonium or Nitrate on Growth and Nitrogen Metabolism in Roots and Leaves of Sorghum (Sorghum sudangrass) Plants

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Abstract: Sorghum (Sorghum sudangrass) plants were treated with 5, 20 and 50 mM of Nitrogen (Nitrate or Ammonium). Growth parameters (length, dry weight and fresh weight), biochemistry parameters (proteins, amino acids and chlorophyll content) and activities of enzymes involved in nitrogen metabolism such as: Nitrate reductase (NR), Glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were studied in the leaves and roots after 20 days of N exposure. Results showed that sorghum plants exhibited enhanced biomass production under high levels of inorganic nitrogen supply. Biochemistry parameters increased with increasing nitrogen concentration in roots and leaves especially in ammonium-treated plants. Exogenous nitrate but not ammonium induced significant difference in the leaves and roots nitrate reductase activity when compared with control plants (0.5 mM nitrate). While Glutamine synthetase 1 activity and protein accumulated in roots at increasing concentration of either nitrate or ammonium, particularly in ammonium-treated plants, glutamine synthetase 2 protein was more accumulated in leaves of nitrate-treated plants. Glutamate dehydrogenase activity was enhanced in both organs by increasing N source in the culture medium this increase is more pronounced in presence of ammonium. This data showed that sorghum plants exhibited higher adaptive potential under excessive concentration of ammonium by ability to detoxify this ion via nitrogen assimilation in roots.

Key words: Glutamine synthetase • Nitrate reductase and Glutamate dehydrogenase

INTRODUCTION

Most plant species can utilize nitrogen sources as NH₄⁺ or NO₃⁻. Although inorganic nitrogen is predominantly available to plants as nitrate in most soils, in certain soil and in hydroponic cultures, ammonium can be the majority nitrogen ion [1]. High concentrations of ammonium nitrogen in the soil or in nutrient solution may lead to accumulation of toxic amounts of ammonium ions in the plants [2, 3]. Depending on the nitrogen source, the response of plant species or cultivars varies widely. For example, free ammonium can be potentially cause a decrease in net photosynthesis and therefore in the growth of the plants [2]. Nitrate ions can accumulate in the vacuoles; thus most plant species can tolerate high nitrate concentrations without any sign of toxicity. However, preference for nitrate or ammonium varies according to the plant species, which is generally related to the physiological adaptations of plants to natural ecosystems [4].

Nitrate is taken up by roots moves in part, via the vascular bundle, to leaves for reduction. In leaves, nitrate is reduced to ammonium by cytosolic nitrate reductase (NR; EC 1.6.6.1) and then by plastidial ferredoxin-nitrite reductase (Fd-NiR, EC 1.6.6.4) [5]. Ammonium originated from direct absorption, NR activity, photorespiration, dinitrogen fixation or deamination of nitrogenous compounds, is assimilated by glutamine synthetase (cytosolic GS1 and plastidial GS2, EC 6.3.1.2) and then by glutamate synthase (Fd- GOGAT, EC 1.4.7.1; NADH-GOGAT, EC 1.4.1.14) in vegetative organs [6]. Alternatively, mitochondrial NADH-glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2) can incorporate high levels of ammonium into glutamate under stress [7].
Sorghum is an important staple food crop in developing countries and was ranked the seventh most important crop worldwide in terms of harvested area. We have used *Sorghum sudangrass* as experimental model, which is extensively used as a crop for their favourable characteristics such as ability to assimilate and stock excess of nitrogen, quick growth, nutritional quality and tolerance to heat and drought. Intensive growth of *S. sudangrass* hybrids in short periods of water shortage (summer) requires the supply of high levels of inorganic nitrogen that is provided usually in the form of nitrate. In previous work, we have reported that *S. sudangrass* hybrids exhibited enhanced biomass production under high levels of inorganic nitrogen supply as well as increased capacity for ammonium assimilation in roots by accumulation of GS1 polypeptide with low-affinity [8].

In this paper, we study in roots and leaves of sorghum plants, the influence of high level of nitrogen (ammonium or nitrate) on (i) activities of other enzymes involved in nitrogen metabolism such as: Nitrate reductase (NR), Glutamine synthetase (GS) and Glutamate dehydrogenase (GDH) (ii) concentrations of free amino acids, proteins and chlorophyll. Therefore, the aim of this study was to analyze the partitioning of N assimilation between roots and leaves which could help to elucidate the mechanism of ammonium tolerance in *S. sudangrass* plants.

**MATERIALS AND METHODS**

**Plant Material and Growth:** Sorghum seeds (*Sorghum sudangrass*) were sterilized with 5% of NaOCl for 15 minutes and washed thoroughly with sterile water and germinated on filter paper (Whatmann paper) in a petri dish soaked in distilled water for 4 days under dark condition at 26°C and planted in a pot filled with vermiculite, then grown in a growth chamber. The environmental conditions in the growth chamber were 70% humidity, 25°C and light intensity of 1000 Lux with a 14 h photoperiod. The plants received (twice a week (100 ml/pot)) a nutrient solution of 0.5 mM KNO₃, 0.375 mM KH₂PO₄, 0.125 mM K₂HPO₄, 0.375 mM MgSO₄, 0.1 mM NaCl, 1.25 mM CaSO₄, 10 mg/L Fe-ethylene-diamine tetraacetate (Fe-EDTA) and micronutrients [9], pH 6.4 (±0.1). For the treated cultures, the nutrient solution was supplemented with different concentrations of KNO₃ or (NH₄)₂SO₄ (5, 20, 50 mM).

Plants were harvested 20 days after initiating N treatments, then immediately sorted into leaves and roots. Roots were quickly and gently washed with deionized water to remove residual vermiculite. All experiments and enzyme preparations were performed with freshly harvested plants. For free amino acids (AA) estimation was determined in dried leaves or roots.

**Growth Parameters:** After 20 days of N treatment, 10 plants from each group were divided into separate leaves and roots fractions. Fresh weights of leaves and roots were weighed and lengths were measured. The samples then were dried in oven at 70°C for 72 h and dry weights were determined.

**Estimation of Protein and Free Amino Acids:** Protein was estimated by the method of Bradford *et al.* [10] using BSA as standard.

Free amino acids (AA) were measured in roots and leaves extracts with ninhydrin reagent according to Magné and Larher [11]. A standard curve was prepared with glycine.

**Estimation of Chlorophyll:** The extraction of leaf chlorophyll was performed with 80% acetone. The chlorophyll a, chlorophyll b and total chlorophyll quantities were calculated according to the method of Arnon [12].

**Extraction and Essay of Nitrate Reductase:** Leaves or roots were homogenized in chilled mortar and pestle with 100 mM potassium phosphate buffer (pH 7.4) containing 7.5 mM cysteine, 1 mM EDTA, 1 mM PMSF and 1.5% (w/v) casein. The homogenate was centrifuged at 30, 000g for 15 min at 4°C. Nitrate reductase activity (NRA) was determined according to the method described by Robin [13].

**Extraction and Assay of Glutamine Synthetase:** Leaves or roots were extracted in a cold mortar and pestle with grinding medium containing 50 mM Tris–HCl pH 8.0, 5 mM MgSO₄, 12 mM Glutamate, 2 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 13 000g for 30 min at 4°C. Glutamine synthetase activity was measured using the transferase assay as described by Shapiro and Stadtman [14].

**Extraction and Assay of Glutamate Dehydrogenase:** Leaves or roots were extracted in a cold mortar and pestle with grinding medium containing 50 mM Tris–HCl pH 8.2, 5 mM 2-mercaptoethanol, 1 mM CaCl₂ and 5% PVP. The homogenate was centrifuged at 13000g for 20 min at 4°C. The amination reaction was measured at 30 °C in...
100 mM Tris-HCl, pH 8.2, containing 100 mM NH₄Cl, 10 mM 2-oxoglutarate, 0.16 mM NADH and 4 mM CaC₁₂. The 2-oxoglutarate-dependent oxidation of NADH followed at 340 nm as described by Loyola-vargas and De Jimenez [15].

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blot Analysis: Crude enzymatic extracts were separated on 12.5% SDS–polyacrylamide gels, the resolved polypeptides were electrotransferred onto nitrocellulose membranes and the presence of GS polypeptides immunorevealed with specific antibodies. Immunolabelling was carried out essentially as described by Cánovas et al. [16] using the antisemir raised against recombinant pine GS [17]. Subsequent detection of immunocomplexes was carried out by a peroxidise assay.

Statistical Analysis: The data shown mean values ±S.E. Results were subjected to a one-way analysis of variance with a least significant difference (LSD) test between means using a Statgraphics 5.0. Levels of significance were represented by a at P<0.05, b at P<0.01 and c at P<0.001.

RESULTS AND DISCUSSION

Growth Parameters: Sorghum plants were grown for 20 days under low and high levels of inorganic nitrogen supply (Table 1). Similar biomass production was found at moderate level of nitrate (5 mM) when compared with control plants (0.5M nitrate) (Table 1). However, enhanced biomass (dry weight, fresh weight and height) were observed at high levels of nitrate (20 and 50 mM). The utilisation of ammonium as sole N source in the nutrient solution lead to enhanced values of dry matter production, fresh weight and height when compared with those observed in control plants (Table 1). Maximal biomass accumulation was observed at 5 mM of ammonium supply (2 fold of DW and 2.6 fold of FW). Paradoxically, plants growing under extremely high level of ammonium (50 mM) exhibited similar biomass production than control plants (Table 1).

Generally most plant species show reduced growth, smaller leaves and a stunted root system when exposed to high nitrogen concentrations and in severe cases this leads to the death of the plant [18]. The ability of S. sudangrass hybrids to growth and yield enhanced levels of plant biomass under high levels of either nitrate or ammonium nutrition and did not show apparent signs of toxicity is well correlated with the increased GS activity and the specific regulation of GS1 isoforms in roots [8].

Amino Acids: The presence of ammonium as a sole nitrogen source leads to a significant increase in content of free amino acids in leaves and roots, this increase is important that the ammonium concentration is high (Table 2). Nitrate also induces an increase in free amino acids in both organs. However, this increase is very modest compared to that induced by ammonium (Table 2).

In present study, the amino acid concentration (Table 2) was higher in the roots than in the leaves in presence of high nitrogen concentrations, particularly the ammonium. This accumulation is related to the rapid assimilation of ammonium absorbed, without which the accumulation of NH₄⁺ ions may reach toxic levels [19]. This requires keto acids especially 2-oxoglutarate and oxaloacetate for synthesis of glutamique acid and aspartique acid, as well as their amides; glutamine and asparagine. Our results shown that phosphoenolpyruvate carboxylase (PEPC) leads to an increase production of citric acid cycle carbon skeletons needed for ammonium assimilation by anaplerotic fixation of CO₂ (data not shown). Barneix and Causin [20] reported that the ammonia-grown plants often have higher free amino acids concentration in their tissues than nitrate fed plants.

Proteins: The presence of different concentrations of ammonium in nutrient medium leads to a significant increase of the protein content in the leaves and the roots when compared with control plants; being approximately 2 fold in 20 mM and 3 fold in 50 mM (Table 2). Whereas the nitrate did not affect the protein content that from 20 mM in the leaves, but in the roots induce a significant increase in protein content at different concentrations used compared to the control plants (Table 2).

Our results shown that the protein content was higher in plants treated with high levels of ammonium especially in leaves (Table 2). This shows that a part of amino acids synthesized in the roots, transported to leaves via xylem and used for protein synthesis necessary for tissue development in particular ; enzymes.

Chlorophyll: In this study, chlorophyll content increased in leaves sorghum plants treated with different concentrations of both nitrogen sources with a remarkable increase in presence of ammonium (Table 3). Smolov et al. [21] have shown that the presence of ammonium in the culture medium of Hetero- and Mixotrophic Glycine max induces a substantial increase in chlorophyll molecules involved in protein-pigment complexes of chloroplasts. Smolov and Semenova [22] suggested that ammonium exerts an indirect influence on the pigment synthesis in
Table 1: Effect of nitrogen supply (nitrate or ammonium) on length, fresh weight (FW) and dry weight (DW) in sorghum plants

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nitrate (mM)</th>
<th>Ammonium (mM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>48±7</td>
<td>59±5</td>
<td>64±3*</td>
</tr>
<tr>
<td>FW (mg)</td>
<td>265±31</td>
<td>260±42</td>
<td>47±13*</td>
</tr>
<tr>
<td>DW (mg)</td>
<td>60±3</td>
<td>55±8</td>
<td>85±6*</td>
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</table>

Each value represents the mean of ten leaves or roots with S.D. a, b and c indicate significance at P<0.05, P<0.01 and P<0.001 respectively when compared to control.

Table 2: Effect of nitrogen (ammonium or nitrate) supply on protein content (mg g⁻¹ FW) and amino acid content (AA) (mol g⁻¹ DW) in leaves and roots of sorghum plants

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nitrate (mM)</th>
<th>Ammonium (mM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>4.5±0.3</td>
<td>6.7±0.2⁷</td>
<td>6.9±0.4⁷</td>
</tr>
<tr>
<td>AA</td>
<td>0.43±0.04</td>
<td>0.5±0.06⁶</td>
<td>0.56±0.08⁶</td>
</tr>
<tr>
<td>Leaves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>12.9±0.4</td>
<td>13.8±0.6</td>
<td>16.7±0.3⁸</td>
</tr>
<tr>
<td>AA</td>
<td>0.38±0.05</td>
<td>0.44±0.06</td>
<td>0.41±0.01</td>
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Each value represents the mean of three or four independent observations with S.D. a, b and c indicate significance at P<0.05, P<0.01 and P<0.001 respectively when compared to control.

Table 3: Effect of nitrogen (ammonium or nitrate) supply on total chlorophyll (mg/gFW), chlorophyll a (mg/gFW) and chlorophyll b (mg/gFW) in leaves of sorghum plants

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nitrate (mM)</th>
<th>Ammonium (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Chl (a)</td>
<td>1.6±0.1</td>
<td>2±0.5</td>
<td>2.4±0.4⁵</td>
</tr>
<tr>
<td>Chl (b)</td>
<td>0.47±0.09</td>
<td>0.92±0.07⁶</td>
<td>1±0.06⁵</td>
</tr>
<tr>
<td>Chl (tot)</td>
<td>2.35±0.3</td>
<td>2.92±0.4⁵</td>
<td>3.4±0.4⁵</td>
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</table>

Each value represents the mean of three or four independent observations with S.D. a, b and c indicate significance at P<0.05, P<0.01 and P<0.001 respectively when compared to control.

the Cells of Soybean Mixotrophic Callus, for example, due to the activated synthesis of free amino acids triggering the chain of chlorophyll biosynthesis or due to the creation of additional sites for pigment incorporation into the newly formed membranes because of accelerated synthesis of protein components. Raab and Terry [23] reported that higher chlorophyll content in ammonium-fed Beta vulgaris L plant may even result in higher net photosynthetic rates as compared to nitrate-fed plants.

**Nitrate Reductase:** In most plants, nitrate assimilation takes place predominantly in leaves. In accordance, our results shown that NR activity (Fig. 1) was higher in leaves than in roots of sorghum plants. An elevated nitrate reduction in leaves compared to roots was related to the higher NR protein contents [24] and a sufficient availability of light and reducing power [25]. Figure 1 shows that nitrate reductase activity appears unaffected by the presence of ammonium as nitrogen source. However, in the presence of high levels of nitrate (20 and 50 mM), nitrate reductase activity increased significantly in both organs.

Our study shows that in sorghum–sudangrass hybrids, nitrate assimilation is distributed between roots and leaves in the presence of high levels of nitrate. These results are in agreement with those obtained by Andrews (1986) [26] who found that in Ricinus communis, Oryza sativa and Solanum tuberosum, the shoot is the major site of nitrate assimilation when given 1 mol m⁻³ of nitrate and the partitioning of nitrate assimilation between root and shoot remains constant as external nitrate concentration is increased. However, the presence of ammonium in the feeding medium has not affected the NRA of leaves or roots. Oaks et al. [27] reported that maize root NRA was depressed by ammonium at a feeding medium pH of 7.5, but stimulated by ammonium at pH 5.8. Thus, feeding medium pH could be an important factor in determining the effect of nutrient ammonium on NRA. The feeding medium pH in this experiment was maintained at the 6.4.
Fig. 1: Nitrate reductase activity (NR) in the leaves and the roots of sorghum–sudangrass hybrids grown under increased levels of nitrate or ammonium supply (Ammonium: , Nitrate: ). Each value represents the mean of three or four independent observations with S.D. a, b and c indicate significance at P<0.05, P<0.01 and P<0.001 respectively when compared to control (0.5 mM KNO$_3$).

Fig. 2: GS activity and GS protein abundance in the leaves and the roots of sorghum–sudangrass hybrids grown under increased levels of nitrate or ammonium supply (ammonium: , Nitrate: ). Each value represents the mean of three or four independent observations with S.D. a, b and c indicate significance at P<0.05, P<0.01 and P<0.001 respectively when compared to control (0.5 mM KNO$_3$). Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM). The size of the GS polypeptide (40 kDa) is indicated on the left. The same amount of protein was loaded per lane in the gels.

**Glutamine Synthetase:** In the present study, we have found that high levels of nitrate or ammonium lead to accumulation of GS activity in sorghum roots. However, this effect was more pronounced with ammonium (Fig. 2). Accumulation of GS activity in sorghum roots in presence of nitrate might be explained by an indirect effect of nitrate after its reduction to ammonium in roots. In fact, we have detected increased levels of nitrate reductase activity in the roots of nitrate-fed sorghum plants (Fig. 1). The increased GS activity in roots in presence of ammonium nutrition was due to accumulation of GS1 polypeptide with low-affinity who would provide a sustained glutamine biosynthesis at high levels of ammonium supply and may represent at the same time an efficient system of ammonium detoxification [8].

The increase of leaf GS activity observed in presence of nitrate or ammonium is due to an accumulation of chloroplastic GS (GS2), particularly in the presence of
Fig. 3: Glutamate dehydrogenase activity (GDH) in the leaves and the roots of sorghum–sudangrass hybrids grown under increased levels of nitrate or ammonium supply (Ammonium, : Nitrate). Each value represents the mean of three or four independent observations with S.D. a, b and c indicate significance at P<0.05, P<0.01 and P<0.001 respectively when compared to control (0.5 mM KNO₃) Ammonium nutrition stimulated GS activity but Müller [28] was reported that ammonium-fed plants (Fig. 3) in previous study; effect of NH₄⁺ on GS chloroplastic; in majority of plant species, ammonium does not seem to have any effect on chloroplastic GS activity but Müller [28] was reported that ammonium nutrition stimulated GS2 activity in barley leaves. Brechlin et al. [29] suggested that the NH₄⁺ was rapidly assimilated by root GS1 and presumably also by leaf GS2. Wallsgrove et al. [30] confirm the assumption that, besides its role in reassimilation of ammonium contents and suggest that high GDH activity may be related to a high incorporation of ammonium into amino acids to prevent toxicity.

CONCLUSION

The results showed that growth, proteins, amino acids, pigment levels and activities of enzymes involved in nitrogen metabolism such as glutamine synthetase (GS) and glutamate dehydrogenase (GDH), were several fold higher in leaves and roots of sorghum plants under high nitrogen levels especially in presence of ammonium. Thus, Sorghum (S. sudangrass) plants exhibited higher adaptive potential under excessive concentration of ammonium by ability to detoxify this ion via nitrogen assimilation in roots.

REFERENCES


