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Effect of Nitrogen Source and Concentration on Growth and Activity of Nitrogen Assimilation Enzymes in Roots of a Moroccan Sorghum Ecotype

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Abstract: Carbon and nitrogen metabolism pathways are regulated by complex mechanisms in order to optimize growth and development of plants. This study was conducted to contribute to the determination of roles of some key enzymes of carbon and nitrogen metabolism in sorghum roots. Sorghum were grown under nitrate (NO_3^-), ammonium (NH_4^+) or nitrate combined to ammonium at different concentrations. Growth parameters and protein content were evaluated. In addition, we analyzed key enzymes activities involved in nitrogen and carbon metabolism such as: nitrate reductase (NR), glutamine synthetase (GS), aspartate aminotransferase (AAT) and phosphoenolpyruvate carboxylase (PEPC). At high nitrogen levels (ammonium or nitrate), the sorghum roots showed an increase in biomass accompanied by an increase in protein content. Likewise, high concentrations induced accumulation of the non-photosynthetic isoform of phosphoenolpyruvate carboxylase. Glutamine synthetase and aspartate aminotransferase activities were also increased, especially at high levels of ammonium. These results showed that sorghum plants have an efficient system for nitrogen assimilation in their roots, allowing this plant to tolerate excessive concentration of this nutrient.

Keywords: Sorghum, Nitrogen Stress, Nitrogen Metabolism, Ammonium, Nitrate

1. Introduction

Fertilisation is a prerequisite to meet high yields and optimum quality of crops. Unlike most of mineral elements essential to plant development and which are needed in trace amounts, some key elements like nitrogen (N), phosphorus (P) and potassium (K) are needed in large amounts, these elements are frequently in short supply in the soil to allow optimal growth of crops. Of all mineral elements, nitrogen is the one that organisms require in greatest quantity [1], adequate nitrogen fertilization is therefore of importance for better growth.

Nitrate (NO_3^--N) and ammonium (NH_4^+-N) are the major forms of nitrogen that plants use for growth and development. These two forms of inorganic nitrogen are absorbed by specific transport systems in roots. NO_3^--N is converted to NO_2^--N and then to NH_4^+-N by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR),

Whereas, NH_4^+-N is directly assimilated into amino acids via the concerted activities of glutamine synthetase (GS) and glutamate synthase (GOGAT) [2]. Nitrogen incorporated into glutamine by glutamine synthetase and glutamate by glutamate dehydrogenase can be redistributed to other amino acids, mainly by the action of transaminase or aminotransferase as aspartate aminotransferase (AAT) which catalyzes the reversible transfer of an amino group from glutamate to oxaloacetate to form aspartate and α-ketoglutarate.

Carbon skeletons needed for the incorporation of ammonium into amino acids are provided by photosynthesis. Photosynthesis and nitrogen assimilation are two different metabolism pathways but which are tightly linked. Photosynthesis generates NADPH and ATP which are used in the synthesis of carbon and nitrogen assimilates, particularly, amino acids and carbohydrates which are the essential building blocks of growing plants.

PEPC participates in the primary CO_2 fixation by C_4 and CAM plants leaves [3, 4]. The enzyme also fulfils essential non-photosynthetic functions particularly the anaplerotic replenishment of tricarboxylic acid cycle intermediates for biosynthetic purposes and nitrogen assimilation [5]. This anaplerotic role makes this enzyme an ideal choice to study interaction between carbon and nitrogen metabolism.

Sorghum bicolor (L.) Monech is ranked the 5th most important cereal crop in the world. In most of the countries it is used primarily as animal feed, but in Africa and India it is used as human food, where it is a staple food for millions of people [6]. Sorghum is also a well established model for molecular and physiological studies in photosynthesis and C_4 metabolism [7]. A recent study demonstrate that sorghum-sudangrass exhibited enhanced biomass production under high levels of inorganic nitrogen supply as well as increased capacity for nitrogen assimilation in roots [8].

In this study we tried to contribute to the understanding of this interaction between carbon and nitrogen metabolism using a Moroccan sorghum ecotype and focusing on some enzymes activities like PEPC, NR, GS, and AAT.

2. Materials and Methods

2.1. Plant Material and Growth

Sorghum seeds (*sorghum bicolor*) 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 the vermiculite, and then grown in a growth chamber. The environmental conditions in the growth chamber were 28°C and 16 h photoperiod. the plants received (twice a week (100 ml/pot)) a nutrient solution of 0.5 mM KNO_3, 0.375 mM KH_2PO_4, 0.125 mM K_2HPO_4, 0.375 mM $MgSO_4$, 1.25 mM $CaSO_4$, 10 mg/L Fe-ethylene-diamine tetraacetate (EDTA) and micronutrients [9], pH 6.2±0.2. For the treated cultures, the nutrient solution was supplemented by different concentrations of KNO_3 or $(NH_4)_2SO_4$ or both at concentration of 5, 20 and 50 mM. Roots were harvested 21 days after the start of nutritional treatment. Roots were quickly and gently washed with deionized water to remove residual vermiculite. All enzyme preparations were performed with freshly harvested plants.

2.2. Growth Parameters

After 21 days of Nitrogen treatment, 10 plants from each group were used to measure fresh weights and lengths of roots.

2.3. Extraction and Assay of PEPC

Roots were extracted in a prechilled mortar and pestle in an extraction buffer containing 100 mM Hepes–KOH, pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 14 mM b-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride (PMSF) and sand. The homogenates were centrifuged at 15 000g for 15 min at 4°C. Ammonium sulphate was added at 60% to the supernatant. The solution was centrifuged at 12,000 g for 15 min at 4°C. The pellet containing the PEPC was taken up in the same extraction buffer and used for enzyme assays and protein determination. The PEPC enzymatic activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm for 5 min at 30°C. The assay medium (1 mL) contained 100 mM Hepes-KOH, pH 7.3, 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH, 5 U malic dehydrogenase (MDH) and 100 μL of the enzyme solution. The reaction was initiated by adding 1 mM phosphoenolpyruvate and the change in A_{340} was monitored.

2.4. Glutamine Synthetase

Roots were extracted in a cold mortar and pestle with grinding medium containing 50 mM Tris–HCl pH 8.0, 5 mM $MgSO_2$, 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 [10]. The assay mixture consisted of 90 mM imidazole-HCl (pH 7.0), 120 mM L-glutamine, 3 mM $MnCl_2$, 0.4 mM ADP, 20 mM sodium arsenate, 60 mM NH_2OH and the enzyme solution in a final volume of 2.25 ml. The L-glutamine was omitted in the blank test. The reaction was started by adding NH_2OH (prepared freshly, and neutralized to pH 7.0 with NaOH) and incubated at 37°C. The reaction was stopped by adding 0.75 ml of a mixture (1:1:1) of 10% $FeCl_3•6H_2O$ (in 0.2 N HCl), 24% TCA and 5% HCl after 15 min. The appearance of γ-glutamyl hydroxamate was measured at 540 nm.

2.5. Nitrate Reductase

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 20,000g for 20 min at 4°C. Ammonium sulphate was added at 60% to the supernatant. The solution was centrifuged at 20,000 g for 20 min at 4°C. The pellet was taken up in 500 ul of 100 mM hepes-KOH pH 7.55. Nitrate reductase activity (NRA) was determined according to the method described by Kaiser et Lewis [11]. The reaction mixture contained 100 mM Tris-HCl, pH 7.55, 5 mM KNO_3, 0.2 to 0.3 mM NADH. The reaction is initiated by addition of 0.1 ml of the protein extract. As there is, in the extract, endogenous NADH oxidase activity, control is performed without NO_3^--N. After 20 minutes of incubation at 30°C, 1 ml of 1% sulfanilamide (w / v) were added to this medium. After stirring, 1 ml naphthylethylenediamine dihydrochloride (NED) 0.02% (w / v) were added, and the mixture was stirred again. Nitrite formed will then be determined by colorimetry at 540 nm using a standard curve elaborated with a nitrite solution 0.1 mg / ml. NR activity was expressed as nmol NO_2^--N formed

per minute and per mg protein.

2.6. Aspartate Aminotransferase

Aspartate aminotransferase activity was measured by coupling oxalacetate production with malate dehydrogenase and NADH and measuring the decrease in absorbance at 340 nm at 30°C in a 1 ml assay mixture containing: Tris-HCl 50 mM, pH 7.8, L-aspartate 50 mM, 2-oxoglutarate 10 mM, NADH 0.1 mM, 2U of MDH and 20 μl of roots extract. The reaction was initiated by adding 2-oxoglutarate.

2.7. Estimation of Protein

Protein concentration was estimated by the method of Bradford & al. [12] using BSA as standard.

### 2.8. Non-denaturing PAGE and In-gel PEPC and AAT Activity

Samples were submitted to nondenaturing polyacrylamide gels (7% separating gel and 5% stacking gel) at 4°C (80V for 3 hours). After protein migration, the gels were incubated in a reaction medium containing for the PEPC activity, 100 mM Tris-HCl, pH 8, 40 mM NaHCO$_3$, 30 mM MgSO$_4$ and 0.05% (w / v) PEP. For the AAT activity, gel was stained by incubation in a solution composed of 100 mM Tris-HCl buffer pH 7.5, 40 mM L-aspartate and 5 mM 2-oxoglutaric acid. After 15 min of incubation, the gels were removed from the medium, rinsed with distilled water and then the band corresponding to the PEPC and AAT were detected by the staining method using 0.3% (w / v) Fast Violet B Salt solubilized in 100 mM Tris-HCl, pH 8.

2.9. Statistical Analysis

The data shown mean values ± S.D. Results were subjected to a one-way analysis of variance with a least significant difference (LSD) test between means using PASW Statistics 8.0. Levels of significance were represented by *, ** and *** at $P<0.05$, $P<0.01$ and at $P<0.001$ respectively.

3. Results and Discussion

Several plant species treated with low concentrations of ammonium as sole nitrogen source did not grow as well as when they are supplied with the same concentration of nitrate or ammonium combined to nitrate [13, 14]. The results presented in Fig. 1 show that the most significant growth was observed in plants treated with 20 mM ammonium or 'ammonium + nitrate'. However, similar biomass production was observed at very high concentration (50 mM nitrogen) in leaves (data not shown) and roots compared to plants treated with 0.5 and 5 mM nitrogen. High concentrations of nitrogen has also an effect on protein content (Fig. 2), in fact, 20 mM of ammonium and 50 mM of 'ammonium + nitrate' supply allow a greater accumulation of protein content compared with control plants. One of the most conspicuous characteristics of sorghum is its tolerance to high levels of ammonium. Sorghum tolerance was suggested to be due to the presence of a glutamine synthetase in its roots, involved in ammonium detoxification [8]. The increase in sorghum biomass under nitrogen-rich conditions, particularly under ammonium supply [8], would partly explain our experimental results reported in this study.
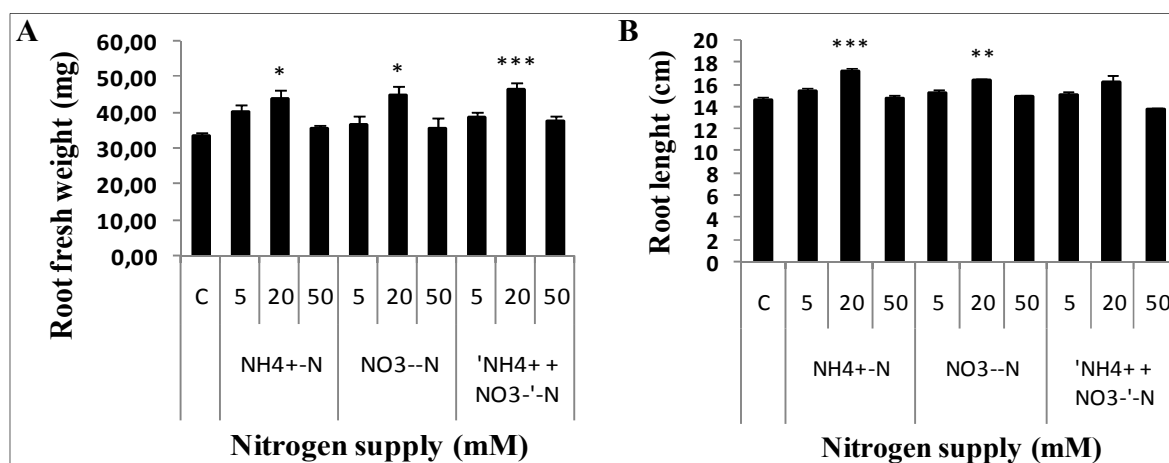

Figure 1. *Biomass accumulation of sorghum roots growing at increased levels of inorganic nitrogen supply. Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM). Length (A) and fresh weight (B) values are the mean of 10 or 8 plants per treatment ± standard error. Means were compared by Tukey's test (P ≤ 0,05).*

Fig. 3A shows that PEPC activity was induced by high concentrations of nitrogen. Plants supplied with ammonium showed a very significant increase in PEPC activity compared to the control plants especially at 50 mM ammonium. Plants treated with 'ammonium + nitrate' showed an intermediate increase in PEPC activity compared to plants treated with ammonium or nitrate as sole nitrogen source. To determine whether this activity is due to covalent modification of the enzyme by phosphorylation and / or accumulation of polypeptides after nitrogen treatment, proteins from each condition were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) followed by in-gel PEPC activity staining. The results obtained in Fig. 3B showed that the intensity of PEPC band

was greater when plants were treated with ammonium and 'nitrate + ammonium' compared to plants supplied with nitrate as sole nitrogen source. These results are consistent with the work reported by Lasa & al., [15] who showed that PEPC activity in pea roots is three times greater in plants treated with ammonium compared to nitrate [15]. Other authors have assessed the action of ammonium in stimulating root PEPC activity [16, 17 and 18]. Viktor and Cramer [19] argued that tomato cultivar F144 showed higher PEPC activity under ammonium nutrition. It has been also shown, in several studies, an accumulation of amino acids mainly in roots of nitrogen-fed plants and particularly in ammonium-fed plants [18, 20 and 21]. The accumulation of amino acids in the roots suggested that much of the ammonium ion is not substantially transported into the xylem sap and is mainly assimilated in the roots [22], thereby requiring a sufficient supply of keto acids, especially 2-oxoglutarate and oxaloacetate for the synthesis of glutamic acid and aspartic acid and their amides; glutamine and asparagine. Thus, the increase in PEPC activity in roots could be essential for the supply of carbon compound required for the synthesis of these amino acids (Fig. 3).
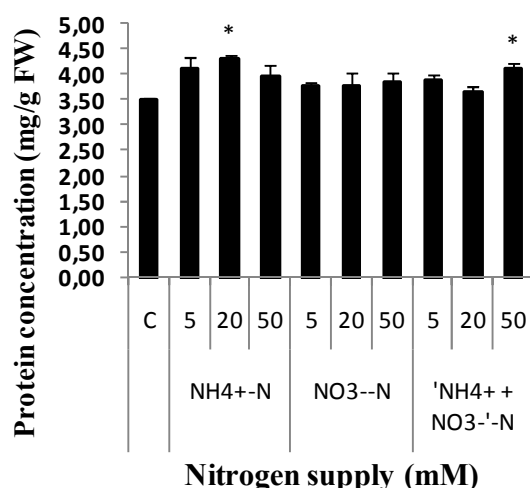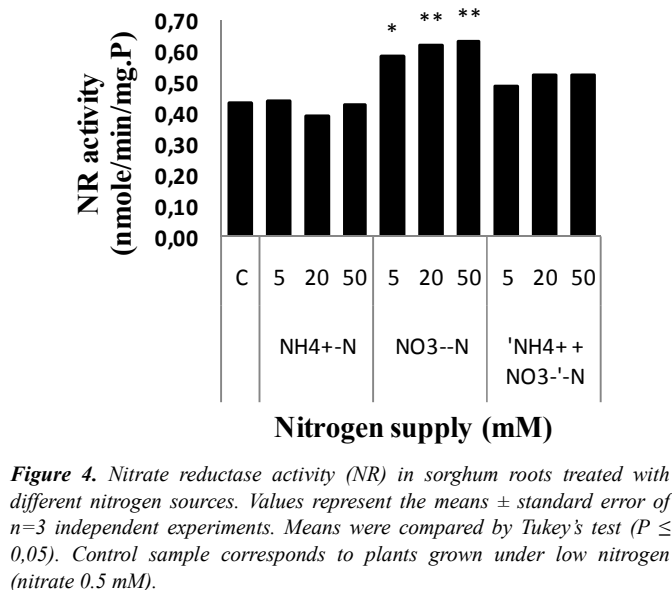

Figure 2. *Protein content of sorghum roots. Plants were treated with different nitrogen sources (ammonium, nitrate or 'ammonium + nitrate'). Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM). Proteins were assayed according to the Bradford method at 595 nm and expressed as mg / g fresh weight. Values represent the means ± standard error of n=3 independent experiments. Means were compared by Tukey's test (P ≤ 0,05).*

Results reported in Fig. 4 illustrate the nitrate reductase activity in roots of plants treated with different sources and concentrations of nitrogen. Nitrate as sole nitrogen source induce an increase in the NR activity, mainly at 20 and 50 mM nitrate. This activity is slightly induced by 'nitrate + ammonium' supply. Our results are in agreement with the results obtained by El Omari et al., and Robin et al., [21, 23], who found a clear relationship between the concentration of nitrate and nitrate reductase activity in sorghum roots. Atkins et al., [24] and Fan et al., [25] reported that NR activity of Phaseolus vulgaris roots and five legumes roots increased linearly with the increasing concentration of nitrate. There

was no significant increase in NR activity in sorghum roots of ammonium treated plants (Fig. 4). This activity was slightly lower at 20 mM ammonium compared to the control plants. Munzarova et al., [26] found that nitrate reductase activity was low in Phragmites australis and Glyceria maxima roots treated with only ammonium. Oaks et al., [27] reported a decrease in NR activity of maize roots in the presence of ammonium as nitrogen source at pH 7.5, however, the NR activity is stimulated at pH 5.8. Therefore, pH of the culture medium is an important factor determining the effect of ammonium on NR activity. In our experimental conditions, pH of the culture medium was 6.2 ± 0.2. From these results it could be assumed that the increase in NR activity observed in plants treated with 'nitrate + ammonium' would be due to the proportion of the nitrate (Fig. 4).
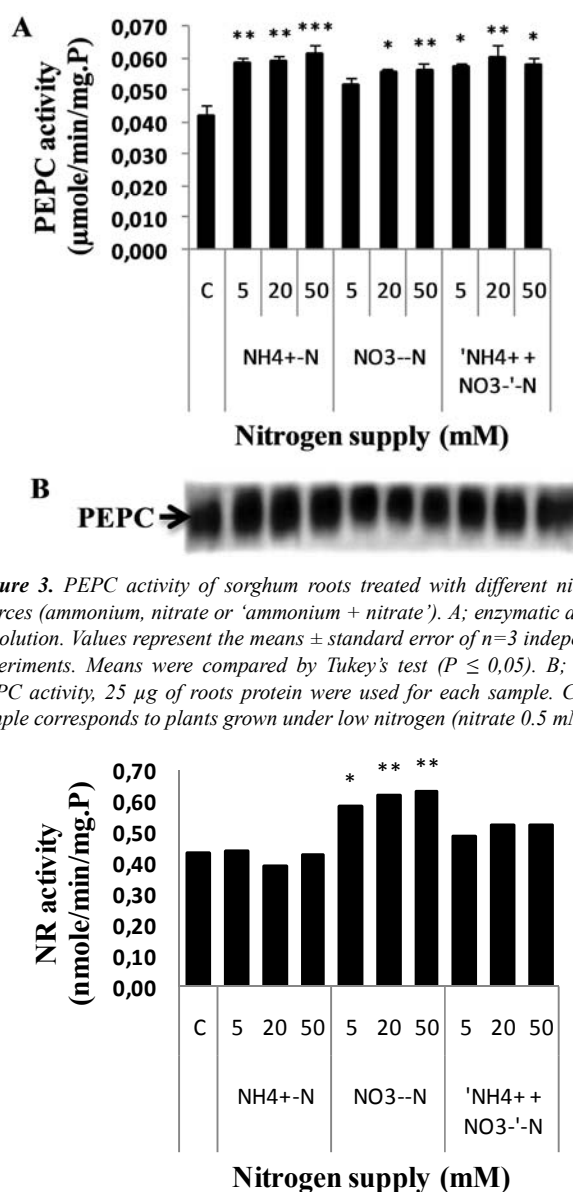
Figure 3. *PEPC activity of sorghum roots treated with different nitrogen sources (ammonium, nitrate or 'ammonium + nitrate'). A; enzymatic activity in solution. Values represent the means ± standard error of n=3 independent experiments. Means were compared by Tukey's test (P ≤ 0,05). B; in gel PEPC activity, 25 μg of roots protein were used for each sample. Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM).*

Figure 4. *Nitrate reductase activity (NR) in sorghum roots treated with different nitrogen sources. Values represent the means ± standard error of n=3 independent experiments. Means were compared by Tukey's test (P ≤ 0,05). Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM).*
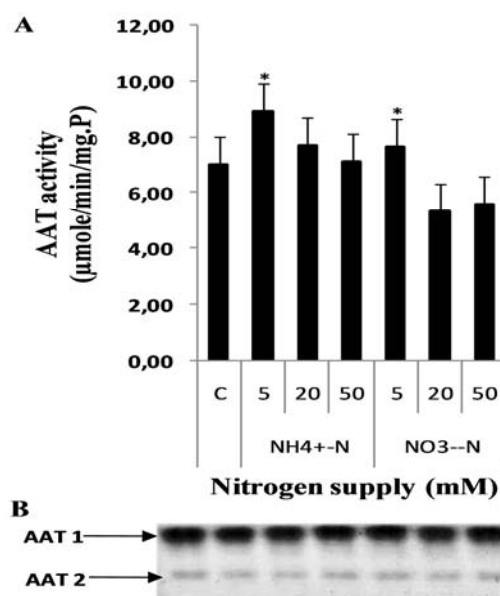
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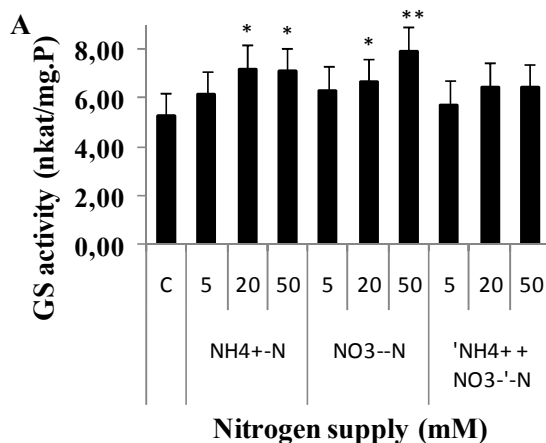
Figure 5. GS activity at increasing concentrations of different sources of nitrogen (ammonium, nitrate or 'ammonium + nitrate'). Values represent the means ± standard error of n=3 independent experiments, and samples treated by nitrate or ammonium. Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM).

Results showed in Fig. 5 indicate that, regardless of the nitrogen source (ammonium or nitrate), there was a significant increase in glutamine synthetase activity in sorghum roots at 20 and 50 mM concentration. This increase was the same for all the three sources of nitrogen at 5 mM concentration. However, the highest activity was observed in plants treated with 50 mM nitrate. The nitrogen source (ammonium + nitrate) has a lower activity compared to nitrate or ammonium used as sole nitrogen source. The increase in GS activity in the presence of ammonium was already reported in a study on soybean nodules and roots [8, 28]. Sugiyama and Sugiharto [20] showed that ammonium increased the GS and Fd-GOGAT activities, while nitrate leads to a relative increase of Fd-GOGAT activity compared to GS activity. The increase in GS activity on medium containing large amounts of ammonium is necessary for assimilation of this toxic compound. The reduction of nitrate to ammonium in the roots could be an explanation for the induction of GS activity in the presence of nitrate. As we noted in the introduction part, high concentration ammonium is toxic to many species of plants. In the light of a previous work [8], our present data confirm sorghum tolerance to large amounts of ammonium. This tolerance might be the result of a root GS activity that will support the excess of ammonium since its uptake by the root and its conversion into organic acids, which would save the sheet of ammonia syndrome [8]. The GS in this direction allow detoxification of the plant in soils containing high amounts of ammonium.

Until recently, it was thought that the aspartate was synthesized in plants by the action of an aspartate aminotransferase (AAT) similar to AAT existing in mammals; but it has recently been discovered a novel enzyme in plants, very similar to the enzyme from cyanobacterium (p-AAT), which has a high affinity for the glutamate [29]. These authors suggested that the physiological role of this AAT resides in the conversion of glutamate to aspartate for the biosynthesis of essential amino acids. In this study, we showed that the concentration of 5

mM ammonium induced a remarkable increase in aspartate aminotransferase activity (Fig. 6A). Nitrate induced a small increase in AAT activity, 20 and 50 mM nitrogen lead to a lower activity compared to the 5 mM concentration. Indeed, most plants use nitrogen concentrations at the range of 5 to 10 mM, therefore, in our conditions; nitrogen excess inhibits the AAT activity. In fact, this enzyme generates glutamate, which is sought by the GS/GOGAT cycle for the consumption of ammonium to yield glutamine. Glutamine is used for the synthesis of amino acids through the transamination reaction of the α-ketogularic acid which is a cosubstrate of AAT. According to this hypothesis we suggest that the depletion of substrate and product of AAT was the cause of the decrease in root AAT activity. Aspartate aminotransferase activity gel assay (Fig. 6B), reveals the presence of two isoforms (AAT1 and AAT2). The figure shows that isoform 1 is more abundant than isoform 2, contrary to what we obtained with sorghum leaves, where the isoform 2 were more abundant than isoform 1 (results not shown). In plants, there are various isoenzymes of aspartate aminotransferase localized in different tissues and cell organelles such as cytosol, mitochondria, chloroplasts, peroxisomes and glyoxysomes [30, 31]. This suggests that these different forms play different roles in the plant metabolism according to their cellular location. It is also important to note that these isoenzymes respond differently to environmental conditions such as light and nitrogen deficiency [32].

Figure 6. A; AAT activity at increasing concentrations of nitrate or ammonium. Values represent the means ± standard error of n=3 independent experiments. Means were compared by Tukey's test (P ≤ 0,05). B; in gel AAT activity, 30 µg of roots protein were used for each sample. Control sample corresponds to plants grown under low nitrogen (nitrate 0.5 mM).

4. Conclusion

Our results showed that growth parameters, proteins levels and activities of enzymes involved in nitrogen and carbon

metabolisms such as; glutamine synthetase, aspartate aminotransferase and phosphoenolpyruvate carboxylase were higher in roots of sorghum plants especially at high levels of ammonium. These results showed that sorghum have an efficient system for nitrogen assimilation in their roots, allowing this plant to tolerate excessive concentration of this nutrient.

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