

# Growth And Nitrate Assimilation In Pearl Millet Exposed To Aluminum Stress

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**Abstract.** Pearl millet (*Pennisetum typhoides* L) seedlings grown in solution culture were exposed to various aluminum (Al) concentrations (0, 10, 30, 60 and 100  $\mu\text{M}$ ) for 20 days. The effect of Al on nitrate ( $\text{NO}_3$ ) assimilation in roots and shoots was studied. Presence of Al in the growth medium lowered roots and shoots biomass. The nitrate reductase (NR) and nitrite reductase (NiR) activities in the root and shoot declined in Al-treated plants. Concurrently, tissue  $\text{NO}_3$  content was also decreased under Al-stress. Soluble proteins, and chlorophyll contents were slightly decreased at high Al level (100  $\mu\text{M}$ ). These results suggested that Al had an inhibitory effect on the assimilatory  $\text{NO}_3$  reducing system through restricting  $\text{NO}_3$  availability in the tissues.

**Key words:** aluminum stress, nitrate assimilation, nitrate uptake, nitrate reductase, nitrite reductase, pearl millet.

## Introduction

Aluminum (Al) toxicity is the primary factor limiting crop productivity on acid soils, which comprise large areas of the world's lands, particularly in the tropics and subtropics (Foy and Chaney, 1978; Foy, 1988). This element is potentially toxic to plants and, when taken up in sufficient amounts, can be harmful to the animals that feed on these plants.

Cultivated plants differ significantly in their response to Al-toxicity. Among cereals, rye is considered to be the most Al-tolerant, whereas wheat is regarded as very Al-sensitive (Taylor and Foy, 1985; Foy, 1988; Kochain, 1995). Numerous studies on the effect of Al on the physiological and biochemical processes in plants have been described, including Al accumulation in plant cell walls, nutrient

uptake and transport, photosynthesis and protein synthesis (Sarkunan *et al.*, 1984; Gomes *et al.*, 1985; Keltjen, 1987; Joslin and Wolf, 1989; Ryan *et al.*, 1993; Durieux *et al.*, 1993). However, the fundamental mechanism by which Al affects growth of crop plants is not yet clear and still an open question.

Nitrate ( $\text{NO}_3^-$ ) is the predominant source of nitrogen for crops in most agricultural soils (Feil *et al.*, 1993). Once nitrate is absorbed by the root, it can be assimilated in the root itself or translocated in xylem to the shoot. Nitrate reductase (EC 1.6.6.1), catalyzing reduction of nitrate to nitrite, is believed to be the rate-limiting step in the process of nitrate assimilation. Most of the absorbed nitrate is stored in the vacuole of either root or shoot tissues, and becomes available to the nitrate reductase (NR) enzyme under external  $\text{NO}_3^-$  deprivation. Although

**Table 1.** Effect of different Aluminum treatments on Chlorophyll content in pearl millet fresh leaves (mg/g). SE is given in parentheses.

| Al ( $\mu\text{M}$ ) | Chl. (a)    | Chl. (b)    | Total Chl. |
|----------------------|-------------|-------------|------------|
| 0                    | 2.11 (0.23) | 1.69 (0.14) | 3.8 (0.34) |
| 10                   | 1.99 (0.24) | 1.65 (0.21) | 3.6 (0.32) |
| 30                   | 2.21 (0.30) | 1.68 (0.18) | 3.7 (0.34) |
| 60                   | 1.86 (0.22) | 1.51 (0.16) | 3.4 (0.28) |
| 100                  | 1.65 (0.16) | 0.72 (0.12) | 2.3 (0.24) |

nitrogen plays a central role in plant metabolism, little information is available on the influence of Al on the enzymes of nitrogen metabolism. The present investigations describe the impact of this metal on the growth and enzymes of nitrate reduction in pearl millet seedlings.

## Materials and Methods

### Plant material and growth conditions

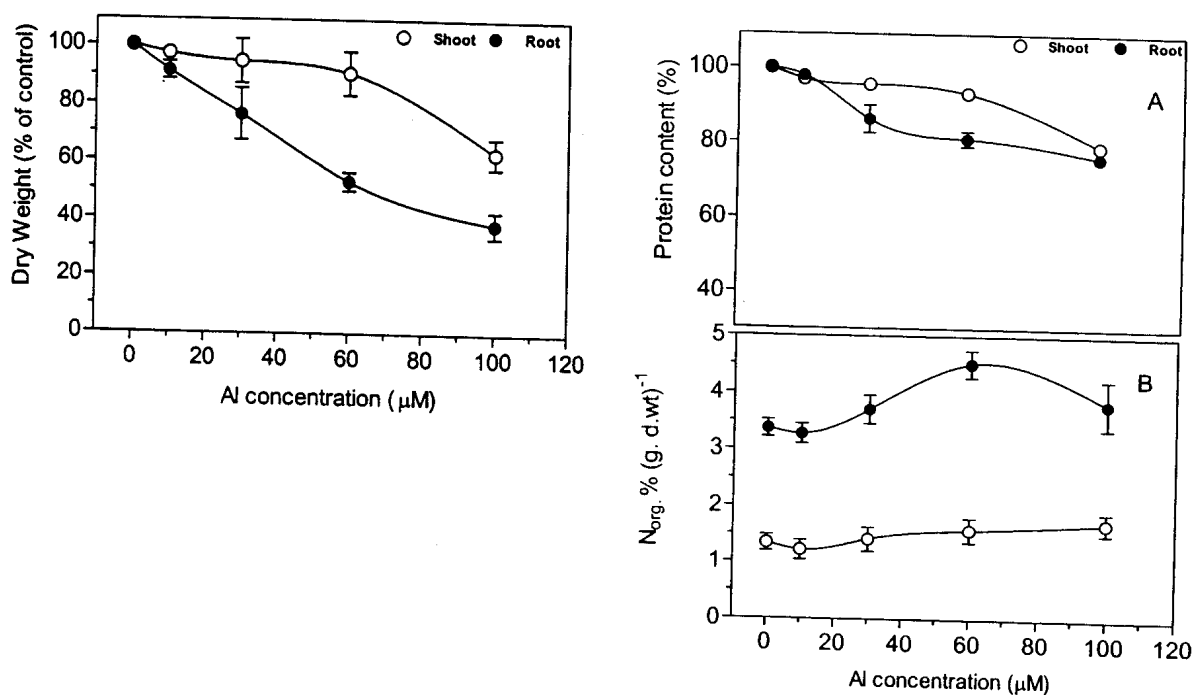
Pearl millet (*Pennisetum typhoides* L) seeds were surface sterilized in 1% (w/v) NaOCl for 10 min, rinsed thoroughly with tap water and germinated in the dark at 23°C in perlite. Selected seedlings were grown hydroponically in 3 liters of nutrient solution in polyethylene buckets with continuous aeration. The composition of the basal nutrient solution was as follows ( $\mu\text{M}$ ): 450  $\text{KNO}_3$ , 325  $\text{MgNO}_3$ , 10  $\text{KHPO}_4$ , 250  $\text{CaSO}_4$ , 40  $\text{FeEDTA}$ , 8  $\text{H}_3\text{BO}_4$ , 0.1  $\text{CuSO}_4$ , 0.2  $\text{ZnSO}_4$ , 0.2  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$  and 0.2  $\text{MnSO}_4$ . After an initial growth period of 6 days, Al treatments were imposed by adding  $\text{KAl}(\text{SO}_4)_3 \cdot 12\text{H}_2\text{O}$  in 0.5%  $\text{HNO}_3$  to the nutrient solution at different concentrations (0, 10, 30, 60, and 100

$\mu\text{M}$ ). The pH of the solution was maintained at  $4.2 \pm 0.2$  by additions of 2 N  $\text{H}_2\text{SO}_4$ . The plants were grown under controlled climatic conditions at 30/25°C day/night temperature, 12-h day length and 50  $\mu\text{Es}^{-1}\text{m}^{-2}$  light intensity. The nutrient medium was replaced every other day. The plants were harvested after 20 days, and roots and shoots were rinsed with distilled water, blotted in a paper towel and frozen in liquid nitrogen immediately. Dry weight of leaves and roots was determined after drying a part of the samples at 70°C for 48 hours.

### Tissue Extraction and Enzyme Assays

Liquid-nitrogen-frozen samples were ground in a chilled mortar with acid-washed sand and extracted with a buffer containing 50 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 3% (w/v) polyvinylpyrrolidone. The homogenate was filtered through Miracloth and centrifuged at 12,000 g at 4°C for 15 min. The supernatant was used for enzyme assays.

Nitrate reductase (NR, EC 1.6.6.1) and nitrite reductase (NiR, EC 1.7.7.1)



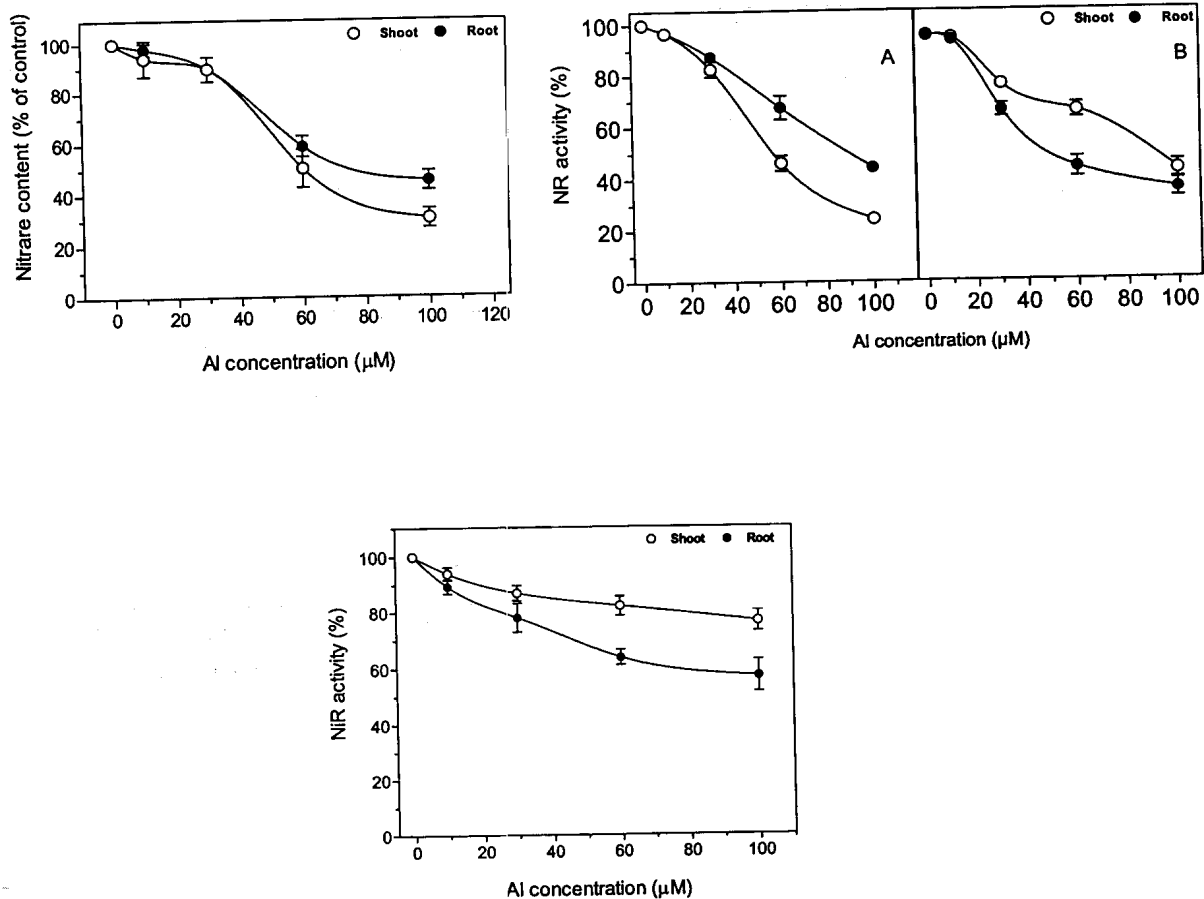
**Fig 1.** Relative growth (expressed as % of control dry weight) of pearl millet shoots and roots as a function of external Al concentration. Dry weight (mg) of control plant was  $623 \pm 13.3$  (shoots) and  $144 \pm 6.4$  (roots). Vertical bars represent  $\pm$  SE. **Fig 2.** Soluble protein (A) and organic nitrogen concentration (B) in shoots and roots of pearl millet seedlings after 20 days exposure to increasing concentration of Al. 100% protein content (mg/g f.wt.) was  $14.2 \pm 0.21$  (shoots) and  $0.23 \pm 0.14$  (roots). Vertical bars represent  $\pm$  SE.

activities were determined *in vitro* by measuring the formed nitrite calorimetrically (Cramer *et al.*, 1996). *In vivo* NR activity was measured according to the method of Scott and Neyra, (1979).

#### Analytical Methods

Protein concentration was determined according to the procedure of Bradford (1976), using bovine serum albumin as a standard. Chlorophyll content was estimated following the method of Withan *et al.* (1971). Free  $\text{NO}_3^-$  in the crude extract was reduced

to  $\text{NO}_2^-$  by the dissimilatory NR of *Escherichia coli* strain B (McNamara *et al.*, 1971) and determined calorimetrically (Snell and Snell 1949). Organic nitrogen ( $N_{\text{org}}$ ) content was determined according to the method of Solorzano (1969). Dried plant material of each treatment was digested in 13.5 M sulphuric acid containing 3.4% (w/v) salicylic acid and 0.2 g of selenium.  $N_{\text{org}}$  was calculated by subtracting the amount of nitrogen present as nitrate from the total amount of nitrogen obtained in this assay.



**Fig 3.** Changes in nitrate content in shoots and roots of pearl seedlings exposed to various Al levels for 20 days. 100% nitrate content ( $\mu\text{mol/g.f.wt.}$ ) was  $64.7 \pm 3.44$  (shoots) and  $52.4 \pm 4.63$  (roots). Vertical bars represent SE. **Fig. 4** Effect of Aluminum toxicity on the activity of NR in shoots and roots of pearl millet seedlings. (A) *In vitro* NR activity. The control rates (100%) were  $3.46 \pm 0.87$  and  $1.48 \pm 0.45$   $\mu\text{mol NO}_2^-$  formed /hr/g fr.wt. for shoot and root respectively. (B) *In vivo* NR activity. The control rates (100%) were  $3.21 \pm 0.67$  and  $2.56 \pm 0.36$   $\mu\text{mol NO}_2^-$  formed /hr/g fr.wt. for shoots and roots respectively. Vertical bars represent SE. **Fig. 5.** Effect of Aluminum toxicity on the activity of NiR in pearl millet shoots and roots. The control rates (100%) were respectively  $46.2 \pm 2.87$  and  $19 \pm 2.33$   $\mu\text{mol NO}_2^-$  reduced /hr/g fr.wt. Vertical bars represent  $\pm$  SE.

The data presented are the average of at least four replicates per treatment  $\pm$  standard error (SE). Each experiment was conducted in duplicate.

## Results

Supply of 10 - 100  $\mu\text{M}$  Al to pearl

millet seedlings showed reduced roots dry weight at 30  $\mu\text{M}$ , whereas, shoots dry weight was affected at >60  $\mu\text{M}$  Al (Fig. 1). The amount of total soluble protein per gram fresh weight of roots and shoots remained unaffected by the various Al treatments (10- 60  $\mu\text{M}$ ). At

100  $\mu\text{M}$  Al, soluble proteins was about 19% and 13% lower in shoots and roots, respectively (Fig.2A). In contrast, addition of 10 - 100  $\mu\text{M}$  Al to the nutrient medium increased total organic nitrogen of the roots, but considerable increase in shoots nitrogen was observed only in plants fed with 100  $\mu\text{M}$  Al (Fig.2B).

A marked decrease in the internal  $\text{NO}_3^-$  concentration was observed in the shoots and roots of Al-stressed plants (Fig. 3), indicating a limited supply of  $\text{NO}_3^-$ . Chlorophyll accumulation measured on the 20th day was slightly increased at the 10 - 30  $\mu\text{M}$  Al concentrations. A substantial decrease in chlorophyll content was however noted only at 100  $\mu\text{M}$  Al (Table 1).

The effect of Al on NR, the key enzyme of nitrate assimilation, is given in Figure 4. *In vivo* and *in vitro* NR activity in the shoots and roots decreased progressively with increasing Al concentration. The *in vitro* NR activity depressed to a greater extent in shoots than in the roots (Fig. 4A). On the other hand, *in vivo* estimation of NR activity in the root tissues was significantly decreased above 20  $\mu\text{M}$  Al concentration (Fig. 4B). NiR activity was also decreased in pearl millet shoots and roots under different levels (10 - 100  $\mu\text{M}$ ) of Al stress. A decrease in NiR activity was 6 - 24% in shoot and 11-43% in root tissues (Fig. 5). Reduction in NiR activity was more pronounced in the roots at high levels of Al (< 60  $\mu\text{M}$ ).

## Discussion

Growth of pearl millet was affected by increasing Al concentration in the nutrient medium. Dry matter yields and nitrate uptake were reduced to a greater extent. Retarded root growth is recognized as a major symptom of Al toxicity (Ryan *et al.*, 1993). Effects of Al on the shoot may be a secondary response, occurred as a result of the impaired root system to absorb and transport water and nutrients (Joslin and Wolf, 1989). Under comparable experimental conditions *Allium ursinum* L. (Andersson, 1993) and *Melica ciliata* L. (Tyler, 1993) showed highly retarded growth at 20 and 37  $\mu\text{M}$  Al respectively. The availability of  $\text{NO}_3^-$  both in shoots and roots is mainly controlled by  $\text{NO}_3^-$  uptake, which is the major determinant of the extent of  $\text{NO}_3^-$  assimilation within higher plants (Redinbaugh and Campbell, 1991). Inhibition of  $\text{NO}_3^-$  uptake by Al was reported in soybean genotypes differing in Al tolerance (Lazof *et al.*, 1994). Short time exposure of sorghum and maize to Al resulted in 25 to 40% reduction in  $\text{NO}_3^-$  uptake (Keltjen, 1988; Durieux *et al.*, 1993).

The reduced growth in pearl millet was accompanied by a substantial decrease in shoot and root NR activity. The considerable decline in the activity of NR, observed in pearl millet seedlings after 20 days exposure to Al, could be attributed to a reduced rate of the enzyme protein synthesis or inactivation because of impaired  $\text{NO}_3^-$  uptake from the nutrient solution. Oaks *et al.*, (1988) reported the availability of  $\text{NO}_3^-$  is necessary to

convert inactive NR protein to an active form.

In acid soils molybdenum (Mo) uptake by plants decreases as reported by Marschner (1995). NR is one of two plant enzymes known to require molybdenum (Mo) for activity (Campbell and Smarrelli, 1986). Therefore, it is conceivable that NR activity alteration by Al may also result from a Mo deficiency in plants and/or substitution of Al for Mo in NR complex.

*In vivo* NR activity in the roots showed more response to Al stress compared to the shoots. We noted a further decline for the *in vivo* NR activity in roots especially above 20  $\mu$ M Al. The possible explanation is that shoots restricted translocation of the heavy metal to the site of enzyme action and/or synthesis of metal-chelating proteins in the leaves induced by Al (Basu *et al.*, 1994a; Cruz-Ortega and Ownby 1993).

The adverse effect of Al on NR activity of other plant species have been documented previously (Foy and Felming 1982; Keltjens and Ulden 1987).

The second step in  $\text{NO}_3^-$  assimilation is the conversion of  $\text{NO}_2^-$  to  $\text{NH}_4^+$  by the action of, the NiR (Sivasankar and Oaks, 1996). In this study, NiR activity decreases in both shoots and roots of pearl millet seedlings exposed to increasing Al concentration in the culture medium. The two enzymes, NR and NiR, are co-regulated on the induction side (Redinbaugh and Campbell, 1991; Migge *et al.*, 1997), explaining their comparable response to Al toxicity.

The decrease of  $\text{NO}_3^-$  content in root and shoot tissues associated with a drastic inhibition of NR activity provides indirect evidence that Al

depresses the  $\text{NO}_3^-$  uptake in pearl millet plants. Therefore, restriction of  $\text{NO}_3^-$  uptake by Al should be considered as one of the possible control point of  $\text{NO}_3^-$  assimilation by plants subjected to Al stress. The present investigation revealed that the effect of Al on pearl millet is likely to cause marked perturbation in nitrogen metabolism by influencing  $\text{NO}_3^-$  uptake and reduction in plant tissue.

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## تأثير الألمنيوم على النمو وتمثيل النترات في نبات الدخن

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الملخص : تمت زراعة نبات الدخن في بيئة مائية تحت تراكيز مختلفة من عنصر الألمونيوم (٠ - ١٠٠ ميكرومولر) لمدة ٢٠ يوما لدراسة أثره على عملية تمثيل النترات في الجذور والأوراق. وجود عنصر الألمونيوم في بيئة النمو أدى إلى تقليل امتصاص النترات ومن ثم نقصان نمو الأوراق والجذور. أما محتوى النبات من البروتين والنيتروجين الكلي والكلوروفيل فقد تأثر قليلا وذلك عند أعلى تركيز من عنصر الألمونيوم (١٠٠ ميكرومولر). كذلك قل نشاط إنزيمي تمثيل النترات (محتزل النترات ومحتزل النيتريت) في الجذور والأوراق بسبب وجود عنصر الألمونيوم في المحلول الغذائي. هذه النتائج تدل على أن الأثر التثبيطي لعنصر الألمونيوم على عملية تمثيل النترات يحدث من خلال دوره المباشر في محدودية تركيز النترات في أنسجة النبات.