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# GROWTH AND GDH AND AAT ISOENZYME PATTERNS IN TERRESTRIAL AND EPIPHYTIC BROMELIADS AS INFLUENCED BY NITROGEN SOURCE

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ABSTRACT. Seedlings of three bromeliad species, *Pitcairnia flammea, Vriesea philippo-coburgii* and *Tillandsia pohliana,* with different growth habits were *in vitro* cultured for 6 months in a modified knudson medium containing 8mM nitrogen, such as $NO_3^-$, $NH_4^+$, $NH_4NO_3$, glutamine or urea. Growth analysis showed that all species presented a very efficient uptake and utilization of glutamine and $NH_4NO_3$. The presence of $NO_3^-$ substantially stimulated root development. To examine some possible effects of different nitrogen sources on nitrogen assimilation, protein was extracted from leaf shoots of three species and glutamate dehydrogenase (GDH) and aspartate aminotransferase (AAT) activities were determined by gel electrophoresis. Effects of nitrogen source were shown most clearly by the different mobility of GDH observed in samples which had been supplied with $Ca(NO_3)_2$ compared to those found in samples supplied with $(NH_4)_2SO_4$. Urea as a nitrogen source depressed AAT activity in the terrestrial species *Pitcairnia flammea* but enhanced it in the epiphyte *Tillandsia pohliana.* The latter species and the other epiphyte examined, *Vriesea philippo-coburgii,* were distinguishable from *P. flammea* through their AAT mobility, while the two epiphytes differed between themselves principally in the level of their AAT activity.

## INTRODUCTION

Plants depend upon the availability of light, $CO_2$, water, nutrients and favorable temperatures for growth, maintenance and reproduction. Physiological adaptations to alterations in nutrient availability in the environment may include improvements in root nutrient acquisition and assimilation systems (Redinbaugh and Campbell 1991). Regarding N availability much research has focused on crops, but there are few studies on non-agronomic species. Nitrogen is taken up by most higher plants in the form of nitrate or ammonium; the N form may be important in the habitat preference of a species (Falkengren-Grerup 1995). Organic forms of N, such as amino acids, are also utilized by some species (Chapin *et al.* 1993).

Very little is known about physiological and biochemical responses of bromeliads to nitrogen availability (Mercier 1993). Terrestrial bromeliads use nitrogen taken up from the soil through their roots. In contrast, epiphytic bromeliads absorb nutrients from airborne inputs and canopy throughfall, through absorbing foliar trichomes. The tank-forming epiphytic species can accumulate significant amounts of essential mineral elements, including organic nitrogen, in their leaf bases (Benzing 1980, 1990).

Nitrogen is an important regulator of gene expression of some proteins in higher plants, including Chl a/b light-harvesting complex apoproteins, nitrate reductase and phosphoenolpyruvate carboxylase (Sugiharto and Sugiyama 1992). The nitrogen assimilatory pathway involves various enzymes, including glutamate dehydrogenase (GDH) which catalyzes the amination of alpha-ketoglutarate as well as the deamination of glutamate (Oaks and Yamaya 1990), and aspartate aminotransferase (AAT) which plays an important role in the assimilation of reduced nitrogen into aspartic acid and asparagine from glutamate (Gantt *et al.* 1992). The existence of several isoenzymes of GDH has been demonstrated in a number of plants; different isoenzymes have been associated with physiological function, cellular differentiation or intracellular compartmentation (Gonzalez-Bravo and Maeso 1987). ATT has been studied in various plants, and different isoenzymes have been documented for this enzyme (Turano *et al.* 1990).

In the present paper, the effect of five nitrogen sources on *in vitro* growth of seedlings of three bromeliad species with different growth habits was examined. The alterations in N metabolism were followed by changes in total-N, ammonium-N and GDH and AAT isoenzyme patterns.

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Abbreviations: AAT, aspartate aminotransferase; GDH, glutamate dehidrogenase; NaEDTA, sodium ethylene-diamine-tetraacetic acid; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsuphonyl fluoride; SDS, sodium dodecyl sulfate; TRIS, trishydroxymethylaminomethane

## Materials and Methods

### Plant species and culture conditions

Seedlings of *Pitcairnia flammea* Lindley, a terrestrial species, *Vriesea philippo-coburgii* Wawra, a tank epiphyte bromeliad and *Tillandsia pohliana* Mez, an atmospheric tank-less epiphyte were grown in aseptic Knudson (Knudson 1946) basal medium modified by substituting the original nitrogen salts by equivalent concentrations (8 mM) of $Ca(NO_3)_2$, $(NH_4)_2SO_4$, $NH_4NO_3$, urea or glutamine. Concentrations of all other nutrients were unchanged. The micronutrients of the Murashige and Skoog (1962) formulation were added to these media. $CaSO_4(0.64gl^{-1})$ was used to replace $SO_4^{-2}$ ions, except for the $NH_4^+$ treatment. The culture media were buffered with $1gl^{-1}$ $CaCO_3$ to maintain the pH near 5.0 (Salama and Wareing 1979). The seedlings were obtained from seeds inoculated into 125ml Erlenmeyer flasks containing 50ml each of gel medium (0.7% agar) and kept under a 16h photoperiod with fluorescent light at $10Wm^{-2}$ and temperature of 26 ± 2°C.

After 6 months of incubation, the seedlings were harvested and evaluated for shoot length and number of roots and leaves per plant. The treatments were repeated 3 times with 60 seedlings per replication.

### Total-nitrogen

The amount of total nitrogen was assayed by the Kjeldahl method as described in Loomis and Shull (1937). Samples (500 mg dry weight) of tissue were digested with concentrated $H_2SO_4$; aliquots of the digested samples were alkaline steam distilled with 6N NaOH; $NH_3$ was trapped in 0.02N HCl. From the HCl volume used and the dry weight of tissue digested, the total reduced N content was calculated. Two independent samplings were made for each data point and their values averaged.

### Free-$NH_4^+$

Samples (200mg fresh weight) of tissue were ground in a mortar with 5ml distilled water at 60°C; 5ml chloroform was added, and the resulting extract was then filtered with Whatman #1 filter paper. The free ammonium was determined in aliquots of the upper aqueous phase by the phenol-hypochlorite reaction (Weatherburn 1967). Two independent samplings were made for each data point and their values averaged. The results were expressed on a dry weight basis for comparison with total-N determinations as described above.

### Electrophoresis of GDH and AAT

Leafy shoot samples (200mg fresh weight/ 200µl buffer) were homogenized in 0.05M TRIS-base buffer (pH 8.2) containing 6mM NaEDTA, 250mM sucrose, 50mM NaCl, 2mM PMSF and 2mM NEM. The homogenate was centrifuged at 15,000 xg for 10 min, and the supernatant was used for GDH and AAT electrophoresis assays.

Non-denaturing PAGE in 7% acrylamine was performed using TRIS-glycine buffer pH 8.3, according to the method used by Davies (1964). Volumes of 50µl, corresponding to approximately 150µg of protein, were introduced into the slots of each gel; gels were run at a constant current of 30mA for 5h with constant cooling. Proteins with GDH or AAT activity were detected by the staining procedures described by Shaw and Prasad (1970).

## Results and Discussion

### *In vitro* growth

*In vitro* germination and aseptic seedling culture were necessary in this nitrogen nutrition study, because contamination by nitrifying organisms and the conversion of ammonium to nitrate is a significant problem in ammonium-based solutions used for aeroponic, hydroponic, and sand culture (Padgett and Leonard 1993). Furthermore, for some tank bromeliads the phytotelmata is the site of $N_2$-fixation by cyanobacteria (Bermudes and Benzing 1991). Another advantage of using a sterile gellified medium is that it allows for both root nutrition, as is the case of *P. flammea,* as well as the foliar nutrition of the epiphytic species. The latter possibility occurs in the form of a surface film of nutritious solution which covers the leaves, making it possible for the nutrients to reach the leaf cells through diffusion.

The Knudson medium has been successfully used in this laboratory for the *in vitro* germination and culture of bromeliads (Mercier and Kerbauy 1992). Based on these results, a pilot project was begun in our laboratory about *in vitro* nitrogen nutrition in the Bromeliaceae, using the same concentration of total nitrogen present in basal Knudson medium (8 mM). Very little is known on nitrogen availability in native Bromeliaceae habitats. Benzing (1980) showed some results for *Guzmania monostachia,* a litter impounding bromeliad found in southern Florida, which contained 197.8 $mgl^{-1}$ (or 14 mM) of total nitrogen in the tank.

In the three studied species, $NO_3^-$ as the sole nitrogen source had significantly less effect on the shoot length, while in contrast, $NO_3^-$ stimulated

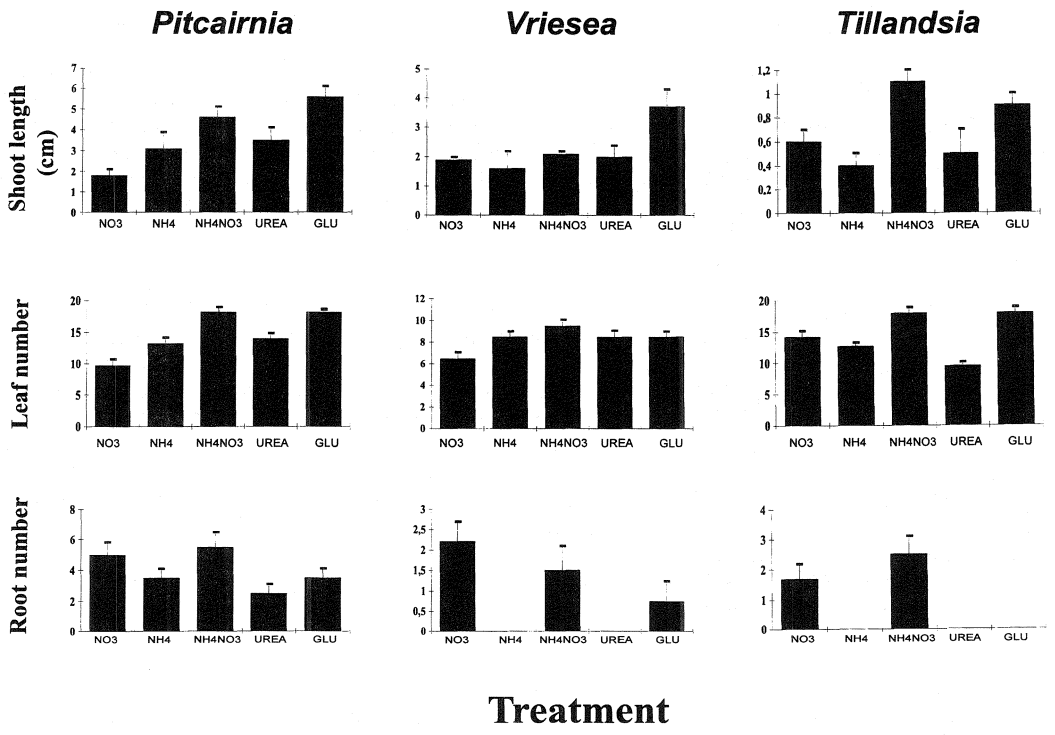


FIGURE 1. *In vitro* growth expressed as shoot length and leaf and root numbers per seedling of *P. flammea, V. philippo-coburgii* and *T. pohliana* cultivated for 6 months with five different nitrogen sources. Mean ± S.E.M.

root formation (FIGURE 1). In all three bromeliads, a decrease of shoot growth accompanied the $NH_4^+$ treatment while root development was inhibited in the two epiphytic species (FIGURE 1). Many seedlings died in this treatment. In the case of *P. flammea,* the leaves of surviving plants showed a dark green color parallel with curled laminae, suggesting an apparent $NH_4^+$ toxicity. High total-N concentrations were detected in the shoots of all three bromeliads, and, at least for *P. flammea* and *V. philippo-coburgii,* the accumulation of free $NH_4^+$ reached very high levels (FIGURE 2). $NH_4^+$-toxicity might be related to detoxification stress, resulting from the requirement for carbohydrates needed to assimilate a supra-optimum amount of $NH_4^+$ (Givan 1979). The increased demand on the carbon supply could become limiting for bromeliad growth. In this sense, GDH enzyme could fulfill an important function linking carbon and nitrogen metabolism (Robinson *et al.* 1992). For *P. flammea* and *T. pohliana* seedlings, the effect of $NH_4^+$ nutrition had a positive response in GDH activity (FIGURE 3).

All three bromeliads studied were characterized by an efficient utilization of nitrogen as $NH_4NO_3$, evident from the results of leaf and root growth (FIGURE 1). Especially low concentrations of free-$NH_4^+$ were detected, indicating that nitrate has perhaps inhibited the $NH_4^+$ uptake. Similar results were also obtained in *Picea abies* seedlings (Aarnes *et al.* 1995). In relation to the effect of glutamine as the sole nitrogen source, it was noted that this amide stimulated the shoot growth of all three bromeliad species (FIGURE 1). For *T. pohliana* no roots were detected. Organic nitrogen was preferentially accumulated in the epiphytes and free-$NH4^+$ content was very high in the atmospheric bromeliad (FIGURE 2). In nature, organic nitrogen in the form of amino acids is present in the precipitation that has previously contacted host tissues (Tuckey 1970). These organic nutrients, dissolved in the canopy fluids, can be absorbed through foliar trichomes. Nyman *et al.* (1987) showed net uptake of dissolved free amino acids on an intact epidermal surface of *Tillandsia paucifolia,* indicating their active transport and accumulation by specialized trichomes present on the leaves. Benzing (1970) showed that glutamine induced the best growth result in five species of bromeliads, including terrestrial and epiphytic species. They were *in vitro* cultured in a media containing 14 amino acids (2mM) added one by one. It was also reported that alanine and glutamic acid presented opposite effects, inhibiting the growth of them all.

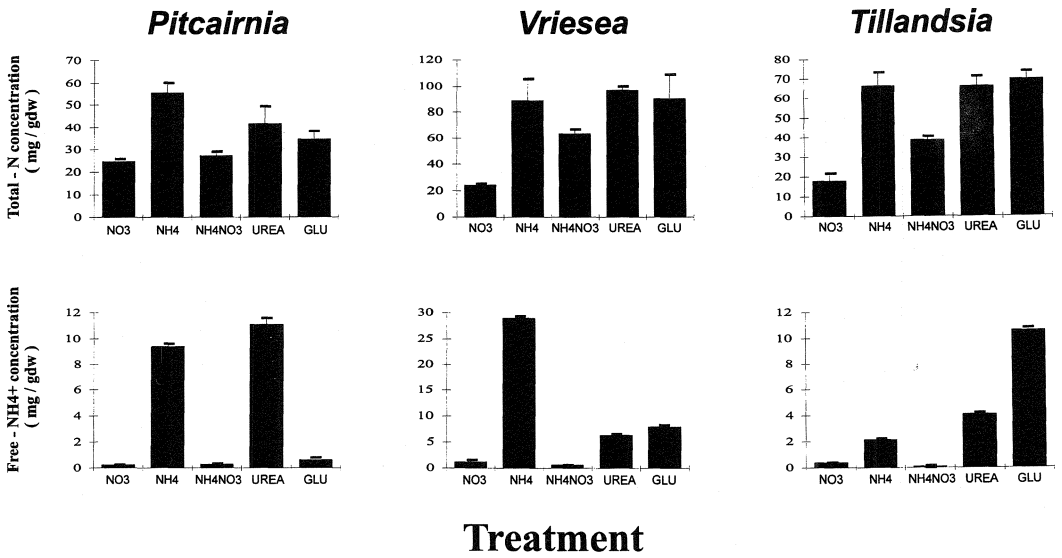


FIGURE 2. Total nitrogen contents and free-$NH_4^+$ levels found in shoots of 6 months seedlings of *P. flammea, V. philippo-coburgii* and *T. pohliana* grown with five different nitrogen sources. Mean ± S.E.M.

When urea was used, it was observed that for *P. flammea* the majority of the seedlings died after 1 month of incubation. So we decided to decrease the urea concentration to a half (4mM) for this species. Even with this reduction, the organic nitrogen source did not produce good development of the surviving seedlings, as shown in FIGURE 1. Apparently the high level of free-$NH_4^+$ detected in *P. flammea* shoot tissues (FIGURE 2) caused ammonium toxicity. On the contrary, the tank epiphyte *V. philippo-coburgii* showed good shoot growth results, even though root development was inhibited (FIGURE 1). No roots were detected also in *T. pohliana* seedlings grown in urea medium and shoot appearance was pale green in color. High levels of organic nitrogen were accumulated in the shoots of epiphytic bromeliads, especially in *V. philippo-coburgii,* while free-$NH_4^+$ was present to a lesser extent (FIGURE 2), suggesting different pathways of urea metabolism between terrestrial and epiphytic bromeliads. Urea can be hydrolyzed by urease, which is widespread in the plant kingdom, producing two moles of ammonia, or urea may be utilized as an intact molecule by condensation with ornithine to form arginine (Reinbothe and Mothes 1962). On the other hand, either the uptake of urea or the formation of ammonium from urea was perhaps slower relative to growth demands of *V. philippo-coburgii* and/or *T. pohliana,* without the accumulation of toxic free-$NH_4^+$ in shoot tissues. For *V. philippo-coburgii* the natural occurrence of urea as a form of tank-dissolved nitrogen may be considered, since it is common to find amphibian organisms living among the overlapping leaf bases.

### GDH and AAT enzymes

GDH activity was detected in all the samples regardless of the nitrogen source used, and in each sample it was restricted to a single zone in the gel (FIGURE 3). The mobility of the enzyme was not identical in all the samples and varied from Rm 0.17 to Rm 0.19. At least in *P. flammea* and *T. pohliana* the enzyme was less mobile (Rm 0.17) in treatments with $(NH_4)_2SO_4$ than in, for example, the treatments with $Ca(NO_3)_2$. *T. pohliana* supplied with urea gave a result similar to that obtained from the $(NH_4)_2SO_4$ treatment. Quantitatively, the slow form of the enzyme was more active than the fast form, showing $NH_4^+$ as a positive effector of GDH gene. Many authors have already detected a dependence of the banding pattern of GDH activity on nitrogen nutrition. In rice plant roots, the addition of $NH_4Cl$ to a basal nitrogen deficient medium caused the development of a new GDH isoform (Kanamori *et al.* 1972). In maize seedlings, the GDH enzyme could be resolved into only one band. In the seedling root extract, the effect of $NO_3^-$ or $NH_4^+$ nutrition showed that $NH_4^+$ had a positive response in GDH activity. In contrast, for the seedling leaf extract, the GDH activity was not altered in response to nitrogen sources (Oaks 1994).

In relation to AAT, all the samples of *P. flammea* and *V. philippo-coburgii* showed AAT ac-

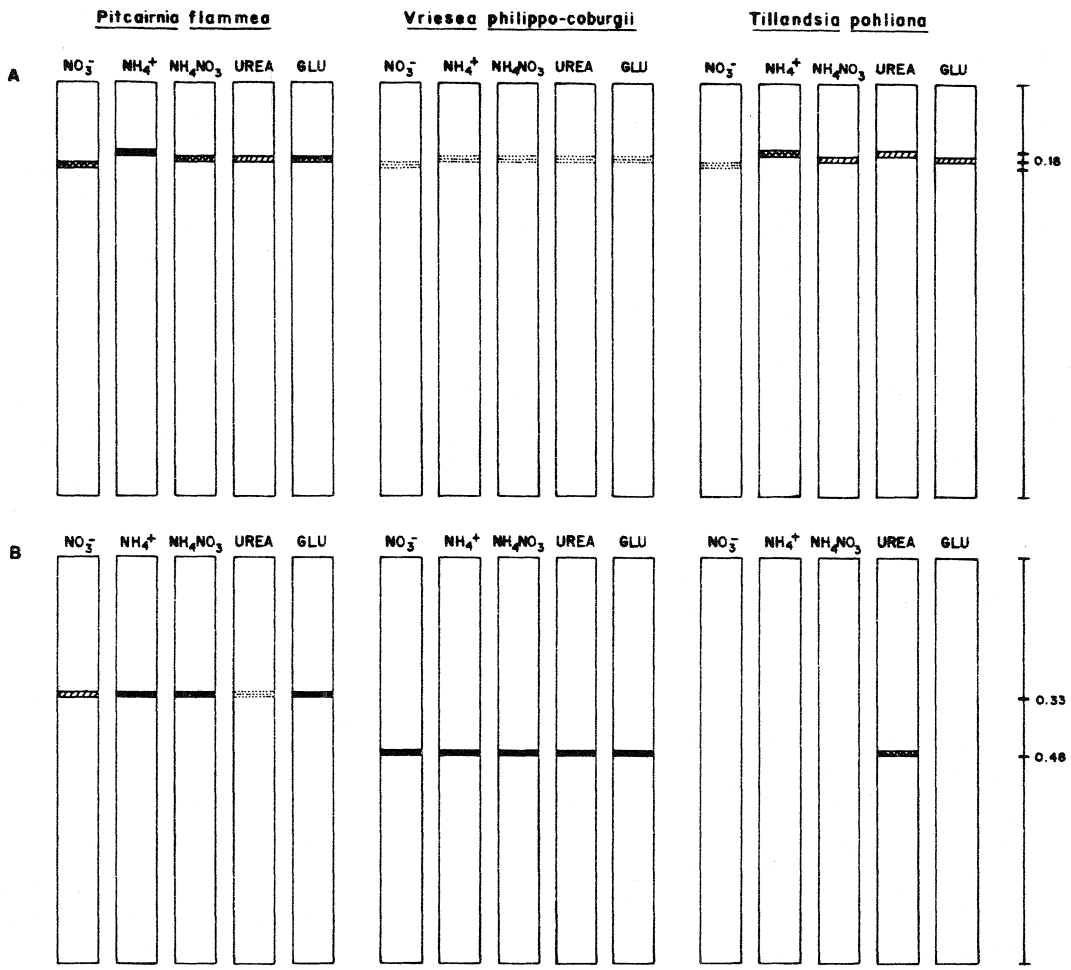


FIGURE 3. Effect of nitrogen sources on the GDH and AAT isoenzyme patterns in *P. flammea* (terrestrial), *V. philippo-coburgii* (tank epiphyte) and *T. pohliana* (atmospheric epiphyte). The isoenzymes were resolved in 7% polyacrylamide gel and all lanes were loaded with 150μg of protein. Staining intensity of the bands is in accordance to the relative abundance of the isoenzyme forms. GLU = glutamine.

tivity and in both species it was confined to a single band (FIGURE 3). However the mobility of the enzyme differed between the species with the terrestrial *P. flammea* presenting Rm 0.33 and *V. philippo-coburgii* Rm 0.48. Qualitatively no effect of nitrogen supply was observed. In contrast to these two species, *T. pohliana* showed activity only in the sample grown in the presence of urea. The mobility of the enzyme in this sample (Rm 0.48) was the same as that observed in the other epiphyte *V. philippo-coburgii.* With the addition of $NO_3^-$ or urea to *P. flammea,* AAT decreased in activity (FIGURE 3). Both treatments reduced seedling development, suggesting that aspartate was not sufficiently available to support growth. However our results from *T. pohliana* may reflect a role of nitrogen nutrition on the mechanism that regulates the expression of AAT isoenzymes, which might be specific for each genotype. Although AAT can be considered one of the most important aminotransferase, the physiological role of each AAT gene/isoenzyme in aspartate metabolism remains elusive (Lam *et al.* 1995).

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