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Nitrogen assimilation in the bromeliad *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal grown *in vitro* with different sources of inorganic nitrogen

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ABSTRACT – (Nitrogen assimilation in the bromeliad *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal grown *in vitro* with different sources of inorganic nitrogen). *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal is a native ornamental bromeliad of the endangered biome Cerrado. Therefore, approaches aimed at the preservation of this species, such as *in vitro* cultivation and micropropagation are needed. Nitrogen (N) is absorbed by plants, mainly as NO_3^- and/or NH_4^+, and assimilated into amino acids. The aim of this work was to evaluate the N assimilation in this bromeliad. Plants were grown *in vitro* for seven months in modified MS medium with 15, 30, 60, and 90 mM of N as NO_3^-, NH_4^+ or NH_4NO_3, and then transferred to *ex vitro* conditions for acclimatization. Plants grown with NH_4^+ had high mortality. During acclimatization plants cultivated with 30, 60, and 90 mM of N as NH_4NO_3 showed higher biomass. With regard to N assimilation, GS and NR showed the highest activity in plants cultivated with NH_4NO_3, whereas plants cultivated with NH_4^+ had the highest GDH activity. Consequently, *in vitro* and *ex vitro* cultivation of this species with 60 mM N as NH_4NO_3 is recommended.
Keywords: acclimatization, Bromeliaceae, *in vitro* culture, micropropagation, pineapple

RESUMO – (Assimilação de nitrogênio na bromélia *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultivada *in vitro* com diferentes fontes de nitrogênio inorgânico). *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal é uma bromélia ornamental nativa do Cerrado, um bioma ameaçado de extinção, sendo necessários estudos para sua preservação, como o uso de micropropagação. O nutriente nitrogênio (N) é absorvido pelas plantas, principalmente, como NO_3^- e/ou NH_4^+ e é assimilado em aminoácidos. O objetivo deste trabalho foi avaliar a assimilação de N nesta bromélia. As plantas foram cultivadas *in vitro* por sete meses em meio MS modificado com 15, 30, 60 e 90 mM de N como NO_3^-, NH_4^+ ou NH_4NO_3, depois as plantas foram aclimatizadas *ex vitro*. Plantas cultivadas com NH_4^+ tiveram alta mortalidade. Durante a aclimatização, plantas cultivadas com 30, 60 e 90 mM de N como NH_4NO_3 apresentaram maior biomassa. Em relação à assimilação do N, GS e NR apresentaram as maiores atividades em plantas cultivadas com NH_4NO_3, e as adubadas apenas com NH_4^+ tiveram alta atividade da GDH. Consequentemente, recomenda-se cultivar esta espécie *in vitro* e *ex vitro* com 60 mM de N como NH_4NO_3.
Palavras-chave: abacaxi, aclimatização, Bromeliaceae, cultivo *in vitro*, micropropagação

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Introduction

Ananas comosus var. *ananassoides* (Baker) Coppens & F.Leal, also known as field pineapple (Silva 2006, Crestani *et al.* 2010), is a terricolous bromeliad, native to Cerrado (Proença & Sajo 2007). This species, like other bromeliads from this biome, has ornamental and landscape potential (Zucchi *et al.* 2019) but is still little explored economically (Silva 2006, Souza *et al.* 2007, Crestani *et al.* 2010). It is characterized by the presence of leaves covered with trichomes, well-developed absorbent roots as it is terricolous, a globose inflorescence bearing bracts with complete and small flowers, and an 8-10 cm and creamy to pink-yellow infructescence located in the apical part of the stem which can measure up to 80 cm (Paula & Silva 2004, Souza *et al.* 2007). This ornamental bromeliad has been traditionally cultivated for export as a cut flower (Souza *et al.* 2012). Besides its economic importance as an ornamental plant, this species is native to a biome that belongs to the 34 global biodiversity hotspots, which are areas of high biodiversity and endemism (Myer *et al.* 2000, Orme *et al.* 2005). Therefore, conservation measures of their species are important and necessary. One way of contributing to the biome preservation, could be through the development of micropropagation studies of plants.

A key factor for *in vitro* cultivation is the selection of a suitable culture medium for the growth and development of the species of interest (Bunn *et al.* 2011). Most culture mediums are basic formulations such as the MS medium formulated by Murashige and Skoog (1962), which is the most commonly used for *in vitro* culture (Aranda-Peres *et al.* 2009, Werner *et al.* 2010, Lima *et al.* 2020). Among the nutrients, that compose the culture medium, the nitrogen (N) is required in a major amount by most plants (Marschner & Marschner 2012, Buchanan *et al.* 2015, Esteban *et al.* 2016).

Ammonium (NH_4^+) and nitrate (NO_3^-) ions are the most available N sources in the soil solution to be absorbed by plant roots (Epstein & Bloom 2006, Marschner & Marschner 2012, Esteban *et al.* 2016, Hachiya & Sakakibara, 2017). The roots absorb these two ions by high- (HATS) and low- (LATS) affinity transporters that are specific for each N form (Miller *et al.* 2007, Kraiser *et al.* 2011, Xu *et al.* 2012). Once absorbed, the N can be either readily assimilated in the roots or stored in the vacuoles, and also transported *via* the xylem to be assimilated in the shoot (Rennenberg *et al.* 2010, Braun *et al.* 2013). To enter in the assimilation pathway, the N in the form of NO_3^- is reduced to nitrite (NO_2^-) in the cell cytosol by the nitrate reductase (NR), and then transported inside the plastid, where it is reduced to NH_4^+ by the nitrite reductase (NiR) (Marschner & Marschner 2012, Taiz & Zeiger 2013). The NH_4^+ originated is assimilated into glutamine (GLN) by the glutamine synthetase (GS), and then transformed into glutamate (GLU) by the glutamine oxoglutarate aminotransferase (GOGAT) (Marschner & Marschner 2012, Taiz & Zeiger 2013, Buchanan *et al.* 2015, Liu & Wirén 2019). In addition, the glutamate dehydrogenase (GDH), which normally catalyzes the reversible deamination of glutamate to 2-oxoglutarate, it might be collaborating in the assimilation of N into GLU under certain conditions (Skopelitis *et al.* 2006, Andrews *et al.* 2013, Luo *et al.* 2015). Subsequently, GLU and GLN will be used for the biosynthesis of other organic N forms essential for cellular metabolism, promoting plant growth and development (Marschner & Marschner 2012, Andrews *et al.* 2013). Although the process of N assimilation in plants is well-known, much information is still lacking to better understand this metabolic activity (Krapp 2015).

After *in vitro* production, plants need to be acclimatized before their transfer to the field (Grattapaglia & Machado 1998, Berilli *et al.* 2011). Transfer to *ex vitro* conditions implies morphological and physiological changes that confer plants resistance to external environmental factors, such as to increased transpiration and luminous flux, temperature fluctuations, interaction with microorganisms, among others (Calvete *et al.* 2002, Hartmann *et al.* 2002, Albert 2004). Sudden changes in these conditions might be limiting for the propagation of some species (Grattapaglia & Machado 1998, Bregonci *et al.* 2008, Berilli *et al.* 2011), and therefore, highlight the importance of acclimatization studies.

Some studies showed that the joint use of NH_4^+ and NO_3^- promotes plant growth (Hachiya & Sakakibara, 2017), including some bromeliad species, such as *Ananas comosus* (L.) Merr.*, Vriesea fosteriana* L.B.Sm.*, Tillandsia pohliana* Mez, and *Nidularium minutum* Mez (Endres & Mercier 2001, Nievola *et al.* 2001, Tamaki & Mercier 2007, Silva *et al.* 2010, Andrade & Tamaki 2016). Some studies that cultivated *A. comosus* var. *ananassoides in vitro* in MS medium with modified concentrations of NO_3^- and $NH4^+$ proved that $NH4^+$ is not appropriate for the growth of this species (Dias *et al.* 2008, Dias *et al.* 2010, Dias *et al.* 2011a, b, Carvalho *et al.* 2012, Silva & Tamaki 2012, Dias *et al.* 2013, Silva *et al.* 2017). However, there are no studies published hitherto that evaluate the influence of different sources of inorganic nitrogen on the growth, nitrogen assimilation, and *ex vitro* acclimatization of this bromeliad. Therefore, this study aimed at evaluating the growth and nitrogen assimilation in the bromeliad *A. comosus* var. *ananassoides* cultivated *in vitro* and acclimatized *ex vitro* with different sources and concentrations of inorganic N.

Material and methods

Plant material - Seeds of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F. Leal, whose fruits were harvested at the Biological Reserve of Mogi Guaçu, São Paulo State, Brazil, were used in the experiments.

Obtaining nodal segments - Approximately 1,000 nodal segments were obtained from *in vitro* germination of approximately 400 seeds. These seeds were previously

disinfested and subsequently deposited in Petri dishes for germination according to Araujo *et al.* (2012). Posteriorly, the seedlings were transferred to 250 mL glass flasks containing 30 mL of modified Murashige & Skoog (1962) (MS) culture medium with only 50% of macronutrient concentration (MS/2), 30% of sucrose and 5% of agar. Seedlings were maintained in a culture room with a 12 h photoperiod, 30 μmol $m^{-2}.s^{-1}$ of photosynthetically active radiation, and a temperature of 26 ± 2 °C for two months. Leaves of plants grown in aseptic conditions were cut leaving the shoot with only 1 cm long, and transferred to 500 ml glass flasks containing 40 ml of MS/2 culture medium, in which they remained in the absence of light for five months to obtain nodal segments.

Plants obtained from nodal segments and grown *in vitro* with different nitrogen sources and concentrations - The nodal segments obtained were isolated and maintained in 350 mL glass flasks containing 30 mL MS/2 culture medium for two months in a culture room under the same conditions described previously. After this period, the grown plants were transferred to 350 mL glass flasks (five plants per flask) containing 30 mL of modified MS medium with different N sources (NH_4^+, NO_3^- and NH_4NO_3) and concentrations (15, 30, 60 and 90 mM) and were maintained in a culture room for seven months. After three months, the plants were transferred to a new culture medium with the same concentrations to avoid nutritional deficiencies and/or pH changes. After seven months, 24 hours before the harvest of the plant material, the plants were transferred to 350 mL glass flasks containing 30 mL modified MS medium with the different N sources and concentrations, to guarantee the N supply. Afterward, the shoot and root fresh and dry weights, the endogenous content of ammonium, nitrate and photosynthetic pigments, and the activity of nitrate reductase (NR), glutamine synthetase (GS), and NADH-dependent glutamate dehydrogenase (NADH-GDH) were evaluated. For the enzyme analysis, plants were harvested three hours after the beginning of the light period in the culture room. The dry weights of the shoots and roots were determined by drying the samples in an oven at 60 °C to constant weight.

Plant acclimatization- After seven months of *in vitro* culture with different N sources and concentrations, the plants were acclimatized in 600 mL plastic pots (10 plants per pot) containing washed sand as substrate. Leaves and roots were irrigated every 15 days with 40 mL of nutrient solution consisting of a modified MS formulation without sucrose and agar and different N sources (NH_4^+, NO_3^- and NH_4NO_3) and concentrations (15, 30, 60 and 90 mM). Plants were maintained in culture room for three months under the same controlled conditions described above. After this period, the shoot and root fresh and dry weight and the content of photosynthetic pigments were analysed. The shoot and root dry weights were determined by drying the samples at 60 °C in the oven to constant weight.

Determination of the photosynthetic pigments content - The analysis of photosynthetic pigments followed the method described by Munné-Bosch & Lalueza and (2007). Triplicates of the samples were analyzed in a spectrophotometer. The wavelengths for spectrophotometer reading, as well as the equations used for calculating the content of photosynthetic pigments, were described in Lichtenthaler & Buschmann (2001) and expressed in milligram of pigment per gram of dry weight (mg g^{-1} DW).

Determination of endogenous ammonium and nitrate content - Aliquots of ca. 100 mg of frozen material obtained from 10 plants were ground to a powder with liquid nitrogen and homogenized with 1,000 μL of ultrapure water. Samples were then centrifuged at 22,000 *g* and 4 ºC for 10 min, and the supernatants recovered. The endogenous ammonium content was determined with the colorimetric method based on the phenol hypochlorite assay (Weatherburn 1967, MCcullough 1967) and ammonium concentration was expressed as μmol of NH_4^+ produced per gram of dry weight (μmol NH_4^+ g^{-1} MS). The endogenous nitrate content followed the method described by Cataldo *et al.* (1975) and the nitrate concentration was expressed as μmol of NO_3^- produced per gram of dry weight (μmol NO_3^- g^{-1} DW).

Analysis of the NR activity - Nitrate reduction was measured using an *in vivo* assay (Jaworski 1971 modified by Nievola *et al.* 2001). Aliquots of ca. 350 mg of fresh material were weighed and incubated in 5 mL of 0.1 M phosphate buffer (pH 7.5) containing 3% propanol and 0.1 M KNO_3. Tubes containing the samples were vacuum infiltrated three times for 1 min and incubated in the dark at 30 ºC for 30 min. Aliquots of 1 mL were taken from the incubating medium at both the beginning and the end of the assay. The nitrite produced was determined by adding 0.3 mL of 1% sulphanilamide, which was prepared with 3 M HCl and 0.3 mL of 0.2% N-(1-naphthyl) ethylene-diamine. All assays were performed in triplicate. Absorbance was read at 540 nm after 30 min. Nitrate reductase activity was expressed as μmol nitrite produced per hour per gram of dry weight (μmol NO_2^- h^{-1} g^{-1} DW).

Extraction and analysis of GS and NADH-GDH activity - Aliquots of ca. 250 mg of frozen material obtained from 10 plants were ground to a powder with liquid nitrogen and homogenized with 1.5 mL of an extraction medium that consisted of 0.05 M imidazole buffer (pH 7.9) and 5 mM DTT. These samples were centrifuged at 21,000 g and 4 ºC for 60 min, and the supernatant was used to measure the activity of both enzymes. All assays were performed in triplicate.

Analysis of the GS activity - The GS activity was determined based on the *in vitro* method proposed by Elliott (1955) *apud* Farnden & Robertson (1980). The total volume of the enzymatic reaction was 0.5 mL and consisted of 0.1 M imidazole buffer, pH 7.5, 49 mM hydroxylamine, 40 mM

$MgCl_2$, 160 mM glutamate, and 35 mM ATP (pH 7.5). The reaction started with the addition of 50 µL of the plant extract followed by incubation of the tubes at 35 °C. After 60 min, the reaction was interrupted with the addition of 1mL of a solution containing 0.123 M ferric chloride, 0.25 M HCl, and 0.1225 M trichloroacetic acid. The absorbance was read in a spectrophotometer at 540 nm. The GS activity was expressed in mmol ɣ-glutamyl-hydroxamate produced per hour per gram of dry weight (mmol ɣGH h^{-1} g^{-1} DW).

Analysis of the NADH-GDH activity - The method described by Bullen (1956) was used for the determination of the NADH-GDH activity. Firstly, an aliquot of the plant extract was desalted through in the Sephadex PD10/G-25 column (Amersham Pharmacia Biotech) and used in the GDH assay. The NADH-GDH activity was quantified through the amount of NADH consumed in the reaction. The total volume of the reaction was 1 mL and was composed of 0.1 M Tris buffer (pH 8.2), 13.3 mM 2-oxoglutarate, 0.1 M $(NH_4)_2SO_4$, and 0.16 mM NADH. All reagents were maintained at 30 °C for 10 minutes before starting the enzymatic reaction. The reaction started with the addition of 0.25 mL of plant extract and instantly read in a spectrophotometer at 340 nm. The slope was calculated using the linear absorbance range recorded within a period of five minutes. The enzymatic activity was calculated using the molar extinction coefficient (ε 340) of 6.22×10^6 $mmol^{-1}.cm^{-1}$ and expressed as µmol of NADH consumed per minute per gram of dry weight (µmol NADH $min^{-1}.g^{-1}$ DW).

Statistical analyses and experimental design - The experiments followed a completely randomized design in a factorial arrangement (3×4). The experiment had 12 treatments and two harvesting times (seven months of *in vitro* growth and three months of *ex vitro* acclimatization) with three independent replicates. For *in vitro* growth, each experimental unit consisted of three glass flasks with five plants, whereas for the *ex vitro* acclimatization, each experimental unit had three pots with 10 plants. Data were subjected to a two-way analysis of variance (two-way ANOVA), and the differences among means were compared by Tukey´s test ($P < 0.05$).

Results and Discussion

Plants cultivated for seven months with NO_3^- and NH_4NO_3 presented 100% survival with all concentrations used and showed a healthier appearance compared to plants grown with NH_4^+, which had chlorotic leaves and atrophied appearance (figure 1). In addition, plants cultivated at 15, 30, 60, and 90 mM NH_4^+ showed a 55, 56, 57, and 65% survival, respectively. Furthermore, shoots of plants grown with different N sources and concentrations showed a biomass decrease when comparing the values of fresh and dry weights (figure 2 a and b). Several studies have described the toxicity of NH_4^+ for most higher plants when supplied as a single N source (Roosta & Schjoerring 2007, Marschner & Marschner 2012, Esteban *et al.* 2016, Ji *et al.* 2019). However, some aspects of the mechanism of the NH_4^+ toxicity are yet not fully understood (Britto & Kronzucker 2002, Li *et al.* 2014). Nevertheless, the tissue toxicity of the NH_4^+ excess can be attributed to uncoupling of photophosphorylation, disturbance in pH balance (Li *et al.* 2011, Li *et al.* 2014), high carbon consumption and accumulation of abscisic acid and free putrescine in the plant tissues (Garnica *et al.* 2010). Furthermore, Andrews *et al.* (2013) observed that NO_3^- is required to maintain the biosynthesis and flux of cytokinin at a level enough to mediate normal morphogenesis.

Plants cultivated with NO_3^- showed the highest means of fresh and dry weights of shoots and roots (figure 2 a and b). This increase in dry weight of plants cultivated with NO_3^- was also observed for other species, such as *Cucumis sativus* L. (Roosta & Schjoerring 2007) and *Triticum aestivum* L.cv Amarok (Garnica *et al.* 2010). The NO_3^- and NH_4^+ ions are the most available sources of N for root uptake in most soils, however, the absorption of one particular ion or both of them will depend on the plant species and its physiological characteristics, as well as the ion availability in the soil or substrate (Marschners & Marschners 2012, Liu & Wirén 2019). It is well-known that NO_3^- plays a key role in the cell signalling that activates the gene expression related to uptake, transport and N assimilation (Crawford 1995, Miller *et al.* 2007, Krapp 2015), and the cytokinin synthesis, which is responsible for regulating cell growth (division and multiplication) and development (expansion) in higher plants (Garnica *et al.* 2010, Andrews *et al.* 2013, Buchanan *et al.* 2015). This could be the reason why we found a greater accumulation of biomass in plants grown with $NO3^-$.

With regard to the dry and fresh weight of roots, the lowest values were also obtained for plants grown with NH_4^+ (figure 2 c and d). This reduction can be attributed to several biochemical and physiological factors previously mentioned for shoots, but which could also affect roots. In addition, Barth *et al.* (2010) reported that NH_4^+ inhibit the activity of the enzymes GDP-mannose pyrophosphorylase and GDP-mannose, which are required for the biosynthesis of L-ascorbic acid and N glycoproteins. Failure in the N-glycosylation process causes membrane and cell wall deformations, compromising the cell cycle and leading to cell death, consequently inhibiting root growth (Qin *et al.* 2008, Kempinski *et al.* 2013). Furthermore, it has been observed that the NH_4^+ excess decreases root gravitropism in *Arabidopsis* sp. by the redistribution of auxin, which implies a decrease in the root length (Zou *et al.* 2012). Thus, this disturbance negatively influenced root growth in plants cultivated with NH_4^+.

Moreover, both root fresh and dry weights gradually increased in plants cultivated with 15, 30, and 60 mM of N as NO_3^- compared to the other two nitrogen sources (figure

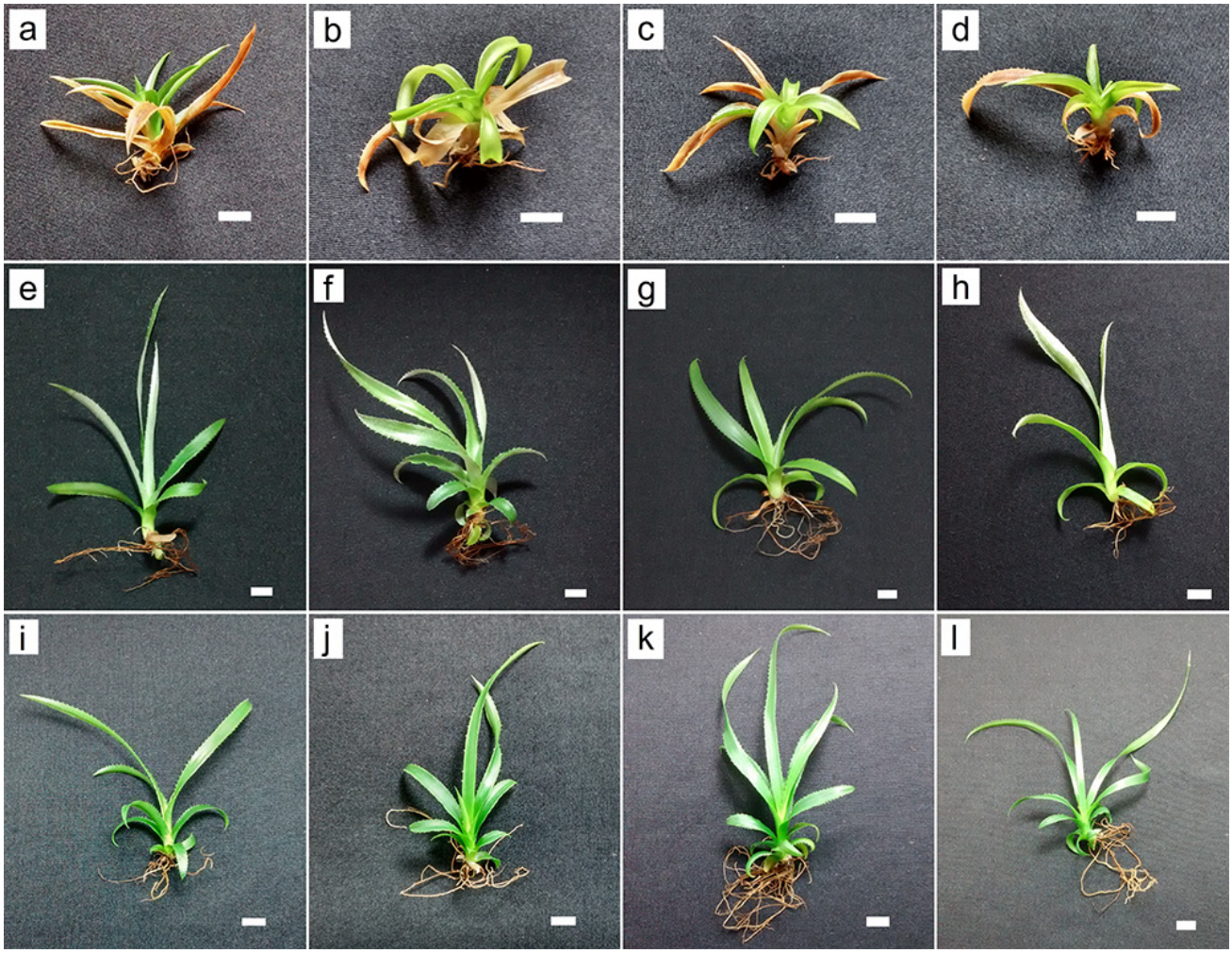

Figure 1. General view of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultivated *in vitro* with MS-1962 modified medium with different sources (NH_4^+, NO_3^-, and NH_4NO_3) and N concentrations (15, 30, 60, and 90 mM) for seven months. a-d. Plants grown with 15 mM, 30 mM, 60 mM, and 90 mM of N as NH_4^+, respectively. e-h. Plants grown with 15 mM, 30 mM, 60 mM, and 90 mM of N as NO_3^-, respectively. i-l. Plants grown with 15 mM, 30 mM, 60 mM, and 90 mM of N as NH_4NO_3, respectively. Bars = 1 cm. Photos from Priscila P. A. Silva.

2 c and d). As previously mentioned, NO_3^- is a signalling molecule for several cell functions, including cytokinin biosynthesis (Garnica *et al.* 2010, Andrews *et al.* 2013). Some authors have observed that the cytokinin regulates auxin distribution in the root apical meristem and promotes cell division in the quiescent centre of the root apical meristem, which is usually mitotically inactive (Ruzicka *et al.* 2009, Zhang *et al.* 2011, Zhang *et al.* 2013). This increase in cell division induced by the cytokinin might explain the increase in root biomass in plants grown with NO_3^-.

With regard to the photosynthetic pigments in plants cultivated *in vitro*, the highest values were obtained in plants grown at all NH_4NO_3 concentrations, with the highest mean achieved in those grown with 60 mM of N as NH_4NO_3 (figure 3). The content of chlorophyll *a* was more than twice of the chlorophyll *b* content and three times higher than of carotenoids (figure 3). This increase in photosynthetic pigments and the same differences in the quantities of each pigment were observed for the bromeliad *Alcantarea imperialis* (Carrière) Harms cultivated with the same N sources and concentrations (Kurita 2015). Studies have reported that plants supplemented with non-toxic concentrations of NH_4^+ present higher chlorophyll content per leaf unit area (Raab & Tarry 1994, Claussen & Lenz 1999). This explains the higher amount of pigments in plants grown with different NH_4NO_3 concentrations, compared to plants cultivated with NO_3^- concentrations. However, there are no published studies that clarify this increase in photosynthetic pigments associated with the supplementation of different sources of inorganic N. According to Raab & Tarry (1994), the carbohydrate requirement for NH_4^+ assimilation might influence the NH_4^+ toxicity in plants, since the faster the NH_4^+ absorbed is assimilated, the less is the damage caused by toxicity. Therefore, the increase

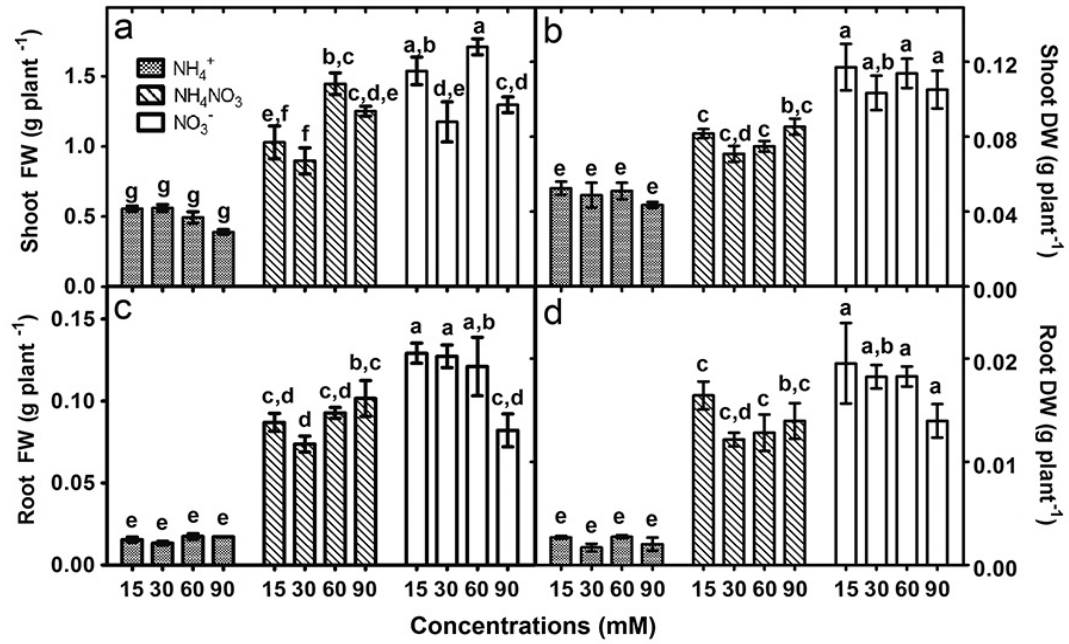

Figure 2. Fresh (FW) and dry weight (DW) of shoot and root of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultured *in vitro* for seven months with 15, 30, 60, and 90 mM of N in the forms of NH_4^+, NO_3^- or NH_4NO_3. Different letters indicate significant statistical differences ($p \leq 0.05$).

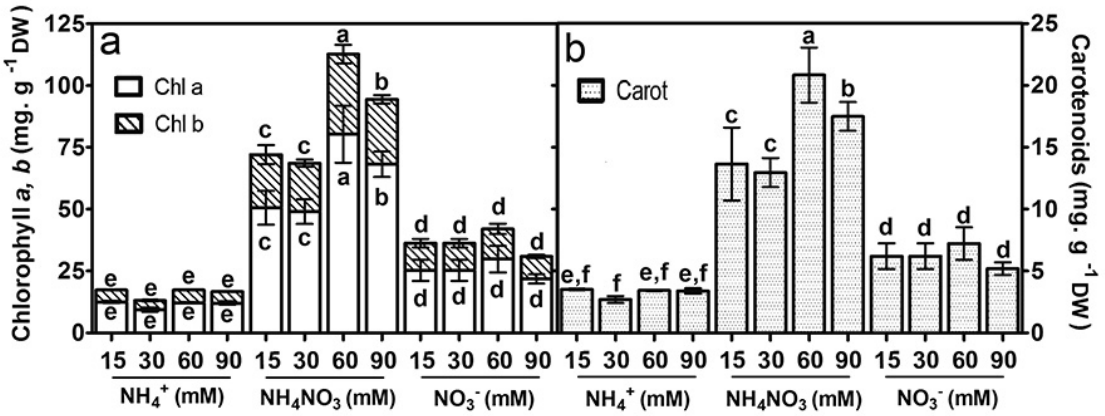

Figure 3. Photosynthetic pigments content in leaves of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultured *in vitro* for seven months with 15, 30, 60, and 90 mM of N in the forms of NH_4^+, NO_3^- or NH_4NO_3. a. Chlorophyll *a* and *b*. b. Carotenoids. Different letters indicate significant statistical differences ($p \leq 0.05$).

in photosynthetic pigments in plants of *A. comosus* var. *ananassoides* grown with different NH_4NO_3 concentrations seems to be related to the need for a supply of carbon skeletons for the assimilation of the $NH4^+$ absorbed in the roots.

With regard to nitrogen assimilation, both NR activity (NR) and endogenous NO_3^- content (ENC) were below the detection levels of the method in plants grown with different NH_4^+ concentrations, and therefore, it was not possible to quantify them. In these treatments, little growth was observed.

With regard to the NR activity, the highest means were obtained in plants cultivated with 90 mM of N as NH_4NO_3 compared to those cultivated with the same concentrations of NO_3^- (figure 4 a), which might be related to higher ENC in plants grown with 90 mM of N as NO_3^- (figure 4 b). This is because in angiosperms, the NO_3^- absorbed can be either reduced immediately in the roots or transported *via* the xylem to the shoot, where it is stored in the vacuoles for later reduction according to the plant necessity (Cárdenas-Navarro *et al.* 1999, Krapp 2015). The NO_3^- stored is important for the cellular osmotic balance, besides being a nitrogen reserve source for N-shortage periods (Chen *et al.* 2004, Miller *et al.* 2007, Krapp 2015).

As shown in figure 5 a, plants cultivated with 15, 30, 60, and 90 mM of N as NH_4NO_3 showed the highest glutamine

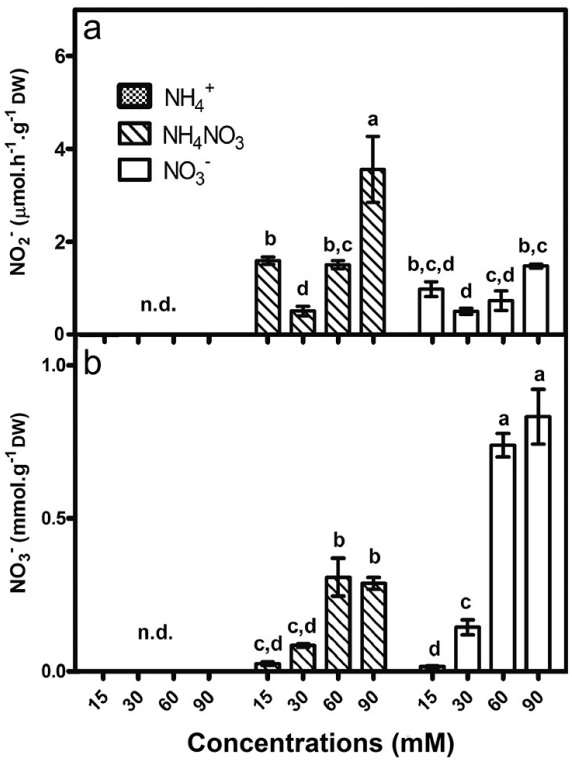

Figure 4. Nitrate reductase activity (a) and endogenous NO_3^- content (b) in leaves of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultured *in vitro* for seven months with 15, 30, 60, and 90 mM of N in the forms of NH_4^+, NO_3^- or NH_4NO_3. Different letters indicate significant statistical differences ($p \leq 0.05$).

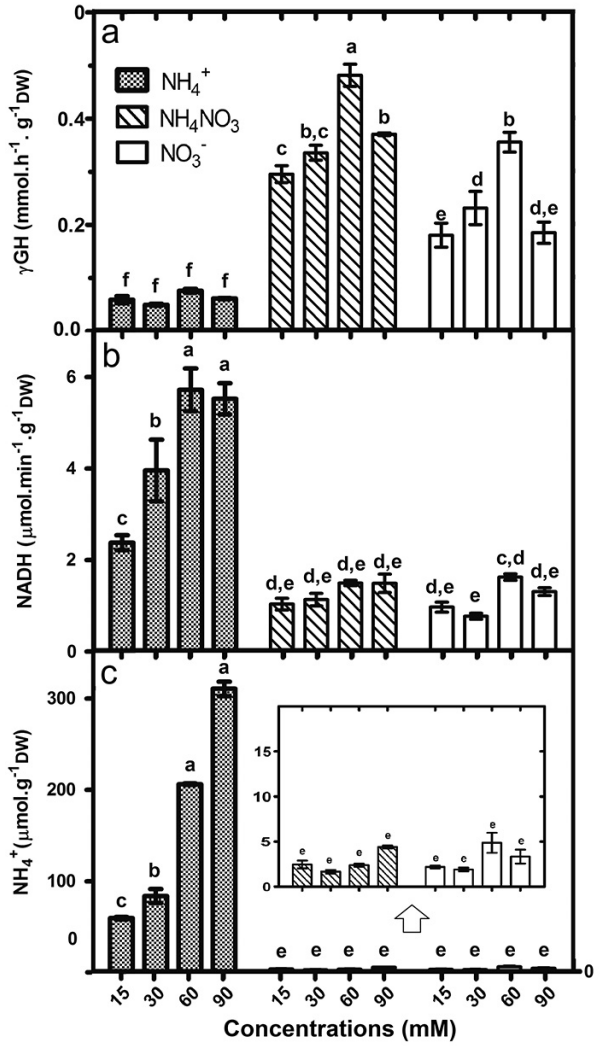

Figure 5. Glutamine synthetase activity (a), glutamate dehydrogenase activity (b) and endogenous NH_4^+ content (c) in leaves of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultured *in vitro* for seven months with 15, 30, 60, and 90 mM of N in the forms of NH_4^+, NO_3^- or NH_4NO_3. Different letters indicate significant statistical differences ($p \leq 0.05$).

synthetase activity (GS) when compared to plants grown with the other two nitrogen sources (NH_4^+ and NO_3^-). Some studies have reported that GS is not affected by different N supplies in *Solanum lycopersicum* L. and *Zea mays* L. (Magalhães & Huber 1991), *Vaccinium corymbosum* L., *Rubus idaeus* L., and *Fragaria ananassa* Duch. (Claussen & Lenz 1999). However, plants of *Catasetum fimbriatum* (Morren) Lindl. (Majerowicz *et al.* 2000) and *Solanum lycopersicum* L. cv. Rio Grande (Horchani *et al.* 2010) grown with NH_4NO_3 or NH_4^+ showed a higher GS activity than those grown with NO_3^-. Therefore, the GS response to the nitrogen source provided seems to vary according to the plant species.

The NH_4^+ assimilation into glutamine and glutamate performed by the enzymes glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) is considered the main pathway of N assimilation (Miflin & Habash 2002, Bernard & Habash 2009, Rennenberg *et al.* 2010, Xu *et al.* 2012, Krapp 2015), and NH_4^+ detoxification under normal conditions of plant growth. Thus, the higher GS activity observed in *A. comosus* var. *ananassoides* cultivated with NH_4NO_3 might be a mechanism to avoid the toxicity of the NH_4^+ absorbed.

The lower GS activity in plants grown with different NH_4^+ concentrations (figure 5 a), associated with the other parameters previously analysed (fresh and dry weights, and content of photosynthetic pigments) and the appearance of the plants, indicated that they were going through the senescence process. Some authors have verified that during the senescence process, leaves show low GS activity (Peeters & Van Laere 1992, Chen *et al.* 1997) due to the high degradation of proteins such as ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo), plastid GS and plastid GOGAT in the chloroplast stroma (Palatnik *et al.* 1999, Parry *et al.* 2003, Kato *et al.* 2005, Masclaux-Daubresse *et al.* 2010).

In addition, plants cultivated with NH_4^+ had the highest glutamate dehydrogenase activity (GDH) and endogenous NH_4^+ content (EAC) (figure 5 b and c). The NH_4^+ accumulation is due to the high proteolysis that occurs during the senescence process, which releases large amounts of amino acids that are catabolized by GDH and

asparaginases (Bernad & Habash 2009). The GDH can catalyse both the amination of 2-oxoglutarate (2-OG) and deamination of glutamate (Glu) (Lea & Miflin 2003, Forde & Lea 2007). These processes occur as a deviation to ensure that N-assimilation does not excessively decrease the 2-OG amount in the mitochondria since this is an intermediate in the tricarboxylic acid cycle (Robison *et al.* 1991, Miflin & Habash 2002, Lea & Miflin 2003). Therefore, the higher GDH in plants of *A. comosus* var. *ananassoides* cultivated with NH_4^+ can be related to the Glu catabolism to generate both 2-OG for the tricarboxylic acid cycle and NADH for other metabolic cell processes.

Three months after being transferred to *ex vitro* conditions, plants acclimatized with different NO_3^- and NH_4NO_3 concentrations showed no mortality and presented a healthy appearance in all concentrations used. On the contrary, plants grown with different NH_4^+ concentrations had 100% mortality. Changes in some factors, such as increases in transpiration rate and luminosity, replacement of a heterotrophic condition to autotrophic condition, among others, during the transition from *in vitro* culture to *ex vitro* conditions, might be limiting for the propagation of some species (Grattapaglia & Machado 1998, Hartmann *et al.* 2002, Bregonci *et al.* 2008, Berilli *et al.* 2011). Consequently, changes in these factors might have induced the death of the plants cultivated *in vitro* with NH_4^+.

Plants grown *ex vitro* with 30, 60, and 90 mM of N as NH_4NO_3 showed higher means in the fresh and dry weights of the shoots compared to the other treatments (figure 6 a and b). Up to date, there are no published studies that evaluate the acclimatization of plants cultivated *in vitro* with different N sources and concentrations. Nevertheless, Kurita (2015) verified that the bromeliad *A. imperialis* cultivated under controlled environmental conditions showed higher growth when cultivated with NO_3^- than when fertilized with NH_4^+ or NH_4NO_3. In the present study, opposite results were obtained for *A. comosus* var. *ananassoides*. In the case of roots, both the fresh and dry weights increased with 15 mM of N as NO_3^- (figure 6 c and d). Kiba *et al.* (2011) observed that high N supplementation suppressed root growth in most plants, while N limitation accelerated their growth. This modulation in the root growth related to low or high N availability is mediated mainly by the signalling of the phytohormones auxin and abscisic acid (Kiba *et al.* 2011, Vidal *et al.* 2013). Some studies have shown that a decrease in N availability might promote auxin accumulation in the roots and, consequently, lead to root elongation (Walch-Liu *et al.* 2006, Tamaki & Mercier 2007, Tian *et al.* 2008). Furthermore, it was observed that auxins were also responsible for the induction of the lateral root development in response to low N supplementation (Fukaki & Tasaka 2009, Ma *et al.* 2014).

The content of photosynthetic pigments in plants acclimatized *ex vitro* was the highest when cultivated with different NH_4NO_3 concentrations, with the highest mean in those plants grown with 60 mM of N as NH_4NO_3 (figure 7 a and b). In all treatments, the content of chlorophyll *a* was more than twice the content of chlorophyll *b* and three times greater than the carotenoid content (figure 7), showing a similar tendency to plants grown *in vitro*.

Both *in vitro* and *ex vitro* results indicated that the cultivation of *A. comosus* var. *ananassoides* with NH_4^+ as the only N source is not feasible. Our results indicated that the growth of plants with NO_3^- as the only N source is possible, but it is not favourable for photosynthetic pigments.

In addition, the enzymes NR and GS assimilated less N as NO_3^- than as NH_4NO_3. Therefore, the use of NH_4NO_3 for both *in vitro* and *ex vitro* cultivation seems to be the best option,

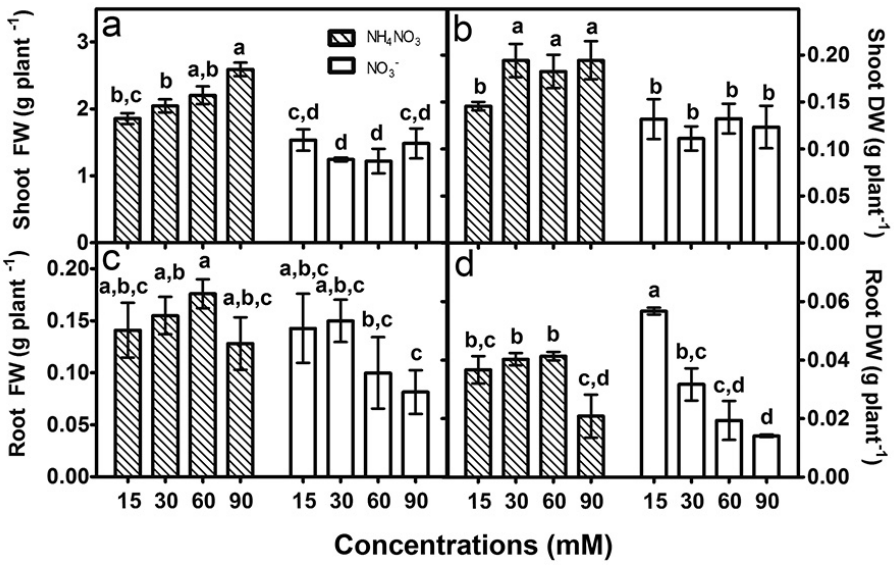

Figure 6. Fresh (FW) and dry weight (DW) of shoot and root of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal ex *vitro* acclimatized for three months with 15, 30, 60, and 90 mM of N in the forms of NH_4^+, NO_3^- or NH_4NO_3. Different letters indicate significant statistical differences ($p \leq 0.05$).

since plants presented higher amounts of photosynthetic pigments in all analysed concentrations, higher biomass in both *in vitro* and *ex vitro* cultivation, and higher N uptake *in vitro*. In general, 60 mM of N as NH_4NO_3 seems to be the best NH_4NO_3 concentration, since it leads to the highest content of photosynthetic pigments in both *in vitro* and *ex vitro* cultivated plants. Furthermore, plants grown with this concentration showed higher biomass and GS activity, and assimilated more N than plants cultivated with the other NH_4NO_3 concentrations.

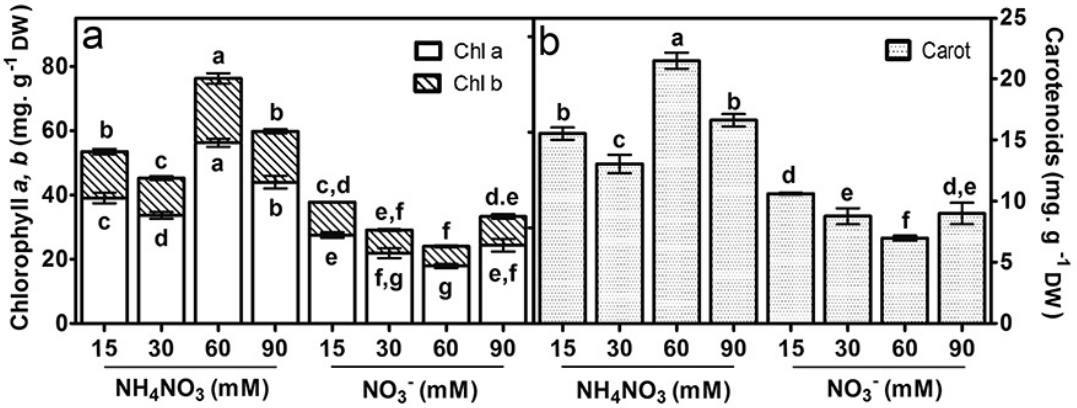

Figure 7. Photosynthetic pigments content in leaves of *Ananas comosus* var. *ananassoides* (Baker) Coppens & F.Leal cultured *ex vitro* for seven months with 15, 30, 60, and 90 mM of N in the forms of NH_4^+, NO_3^- or NH_4NO_3. (A) Chlorophyll *a* and *b*; (B) Carotenoids. Different letters indicate significant statistical differences ($p \leq 0.05$)

Conclusion

Our study showed that plants of *A. comosus* var. *ananassoides* are not able to grow with NH_4^+ as the sole source of N and that it is recommended to use 60 mM of N as NH_4NO_3 for both *in vitro* and *ex vitro* cultivation of this bromeliad.

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