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Nitrate Reductase Activity in *Eucalyptus urophylla* and *Khaya senegalensis* Seedlings: Optimization of the *in vivo* Assay

Mateus Pires Barbosa[1], Raul Antonio Araújo do Bonfim[1], Leandro Dias da Silva[1], Mikaela Oliveira Souza[1], Poliana Prates de Souza Soares[1], Milton Carriço Sá[1], Paulo Araquém Ramos Cairo[1*]

[1] Departament of Crop and Animal Sciences, State University of Southwestern Bahia (UESB), P.O. Box 95, Vitória da Conquista, 45031-900, Bahia, Brazil

* Corresponding author's e-mail: pcairo@uol.com.br

ABSTRACT

Nitrate assimilation in the plant cell is mainly regulated by the enzyme nitrate reductase (EC 1.6.6.1), which catalyzes the nitrate to nitrite reduction. Nitrate reductase activity (NRA) is measured at the major nitrate reduction site, which can be the root or shoot, depending on the species. The *in vivo* assay has often been used for NRA measurement, and protocol also usually varies with the species. The goals of this study were: (1) to identify the major nitrate reduction site in seedlings of two tree species, *Eucalyptus urophylla* and *Khaya senegalensis*, and (2) to optimize the *in vivo* nitrate reductase assay at the major nitrate reduction site in these species. Healthy seedlings aged 180 and 160 days, respectively, were selected for NRA measurement in fully expanded leaves and main root. After identifying the main nitrate reduction site of each species, the effects of variations in temperature, nitrate concentration and pH in the incubation medium were assessed. The results showed that the leaf and the root are the major nitrate reduction site of *Eucalyptus urophylla* and *Khaya senegalensis*, respectively. The optimal conditions for the *in vivo* assay in the leaf were 35 °C, KNO_3 100 mM, and pH 7.0, whereas for the root they were 30 °C, KNO_3 100 mM, and pH 7.5.

Keywords: nitrogen assimilation, enzymatic activity, *in vivo* assay, woody plants.

INTRODUCTION

Plant nutritional status is one of the determining factors for tree seedlings growth and establishment after planting in the field, and nitrogen (N) is the most required nutrient. Inorganic nitrogen is absorbed as nitrate (NO_3^-) and ammonium (NH_4^+) ionic forms [Hawkesford et al., 2012] although it is now acknowledged that organic N forms (e.g., amino acids) are also used by plants [Warren, 2006, 2009]. Ammonium is the major ionic form used by several forest species for N acquisition from the soil. However, after forest disturbances, soil conditions can change radically, and NH_4^+ is converted to NO_3^-, with soil nitrate concentration increasing [Yao et al., 2011]. Due to the nitrification, nitrate is often the majority N ionic form in soil water solution, especially in aerobic soils [Subba et al., 2017].

Compared to ammonium absorption, nitrate absorption and subsequent reduction to ammonium has a higher energy cost. Even though, some authors see nitrate absorption as advantageous, either by reducing the risk of ammonium toxicity [Liu and von Wirén, 2017], or by synergistically promoting the K^+, Ca^{2+}, and Mg^{2+} absorption, unlike ammonium, which competes for absorption with these cations [Wallace and Mueller, 2008; Delaire et al., 2014; Mantovani et al., 2018].

Nitrate absorption is followed by a reduction to ammonium or storage in the vacuole, and this can mostly occur in the root or shoot [Tischner, 2006]. In the cytosol, nitrate is reduced to nitrite by nitrate reductase (EC 1.6.6.1), whose reaction requires nicotinamide adenine dinucleotide

(reduced) (NADH). Nitrite then enters the plastid (chloroplast in the shoot) and is further reduced to ammonium by nitrite reductase (EC 1.7.7.1). Ammonium is further fixed by the GS/GOGAT pathway into amino acids (glutamine/glutamate) which serve as substrates for transamination reactions to produce all other protein amino acids.

The regulation of nitrate reductase activity (NRA) appears to differ from species to species as well as different plant parts [Hewitt et al., 1978; Lee, 1980; Schräder and Thomas, 1981]. The root is the main site for nitrate reduction in some species [Cairo et al., 1994; Delú-Filho et al., 1998; Nievola and Mercier, 2001], while in other species NRA is prominent in the leaves [Oliveira et al., 2005; Dovis et al., 2014; Arora et al., 2018]. Lee and Stewart [1979] and Pate [1983] observed that most of the woody species reduce nitrate only in the roots, except at very high nitrate supply, whereas Al Gharbi and Hipkin [1984] and Smirnoff et al. [1984] reported that a number of woody species possess considerable *in vivo* NRA in the leaves.

The major site for nitrate reduction greatly influences the carbon requirement for ammonium assimilation. Species with leaves as the major nitrate reduction site have the advantage to use excess reductant produced in photosynthesis [Pate, 1983]. By contrast, species with root as a major site must obtain their reductant from glycolysis and the oxidative pentose phosphate pathway [Bowsher et al., 1989]. Therefore, it is assumed that the carbohydrate metabolism is affected by the presence of nitrate since it shifts the relation between starch synthesis and sucrose synthesis in favor of the later [Tischner, 2006].

Measurement of nitrate reduction rate is usually performed using either *in vitro* or *in vivo* methods. The *in vitro* assay is based on the extraction of the enzyme from plant tissue, whereas *in vivo* assay involves the incubation of the original plant tissue in a reactive mixture. Difficulties encountered in measuring NRA with *in vitro* assay have led researchers to become interested in the *in vivo* assay since the pioneering article by Mulder et al. [1959], where a relationship between the two types of testing was reported.

Refined adjustments for the *in vivo* assay were further proposed by Jaworsky [1971], but studies on the optimization of this protocol have been frequently required, in order to meet the physiological peculiarities of different species. However, as each plant species has anatomical and physiological particularities, it is necessary to adjust methods previously developed for other species, so that it is possible to advance in the establishment of new protocols with appropriate conditions for the NRA estimation. Therefore, the goals of this study were: (1) to identify the major nitrate reduction site in *Eucalyptus urophylla* and *Khaya senegalensis* seedlings, based on *in vivo* nitrate reductase assay in leaf and root; and (2) to establish the optimum conditions for the *in vivo* nitrate reductase assay using the original plant tissue where NRA is prominent in these two species.

MATERIALS AND METHODS

Site description, seedlings cultivation and experimental design

The study site (14º51’08” S, 40º48’02” W; 881 m asl) was an experimental field located at the State University of Southwestern Bahia, in Vitória da Conquista, Bahia State, Brazil. The local climate is *Cwb* type (dry-winter subtropical highland climate), according to the Köppen-Geiger classification. During the experimental period, the maximum, minimum, and average values were 23.2, 21.9, and 22.5 °C for temperature, and 84.7, 78.9, and 81.8% for relative humidity respectively.

The *Eucalyptus urophylla* and *Khaya senegalensis* seedlings were grown in little conical tubes, and transplanting into experimental 15 dm^3 pots when they were 120 and 100-day-old, respectively. The pot substrate was a yellow dystrophic Tb latosol, with a sandy-clay texture, pH 5.6, 3.0 mg P dm^{-3}, and the following contents (in $cmol_c$ dm^{-3}): K^+ = 0.22, Ca^{2+} = 2.5, Mg^{2+} = 0.7, Al^{3+} = 0.1, H^+ = 1.9, S.B. = 3.4, t = 3.5, T = 5.4, V = 63, and m = 3. The water supply was sufficient to maintain the substrate moisture at 90% of the pot capacity, which was measured by the gravimetric method.

The *Eucalyptus urophylla* and *Khaya senegalensis* seedlings were removed from the pots when they were 180 and 160-day-old, respectively, and were selected observing a homogeneous vegetative appearance, for further use in enzymatic assays.

Plant material preparing and enzymatic assays

Fully expanded leaves and main roots were taken from each species, from 9:00 to 10:00 a.m., washed with clean water and then cut into small fragments (± 1–2 mm). First, enzymatic assays were performed on fresh leaves and roots, separately, in order to verify the major nitrate reduction site of each species. The protocols described by Jaworsky [1971] and Cairo et al. [1994] were used as guidance in setting the protocol for these preliminary nitrate reductase *in vivo* assays.

A sample with 500 mg of fresh leaves or 500 mg of fresh roots was added to a test tube containing 5 mL of potassium nitrate 100 mmol L^{-1}, potassium phosphate buffer 100 mmol L^{-1} (pH 7.4) and n-propanol 5% (v/v). The assay was kept in a water bath in the dark, at 30 °C, under constant agitation.

For nitrite determination after 60 min of reaction, an aliquot of each assay was added to a medium containing 1.0 mL of 1% (w/v) sulfanilamide solution in 1.5 N HCl, 1.0 mL of n-2-naphthyl ethylenediamine dihydrochloride 0.02% (w/v), and deionized water (sufficient to reach 4.0 mL total volume, depending on the aliquot). The aliquot volume (two aliquots per sample) was established based on the nitrate to nitrite reduction capacity of each species. The absorbance was measured using a spectrophotometer at 540 nm, and the data were compared to a standard curve for sodium nitrite. The NRA was expressed in μmol NO_2^- g^{-1} h^{-1}.

After detection of the major nitrate reduction site, the optimization of protocol for the *in vivo* enzymatic assay was performed by testing the effects of the following factors: temperature of 25, 30, 35, and 40 °C; KNO_3 concentration at 0, 100, 200, 300, and 400 mM; and pH at 5.8, 6.2, 6.6, 7.0, 7.4, and 7.8. The assays were conducted by testing one factor at a time.

Experimental design and statistical analysis

The experimental design was completely randomized, with four replications per treatment. Effects of each variable on NRA were tested with ANOVA, followed by a Tukey's test when significance ($p < 0.05$) was detected. For quantitative variables, data were submitted to a regression analysis. Data analysis was performed using the Sisvar statistical software [Ferreira, 2011].

RESULTS

Checking the major nitrate reduction site

The species showed a contrasting behavior regarding the major nitrate reduction site. For *Eucalyptus urophylla*, NRA was markedly greater in the leaf than in the root, while for *Khaya senegalensis* there was an inverse response, i.e., the root is the major site. Furthermore, the NRA at the major nitrate reduction site was greater in *Eucalyptus urophylla* (~1.65 μmol NO_2^- gFW^{-1} h^{-1}) than in *Khaya senegalensis* (~1.16 μmol NO_2^- gFW^{-1} h^{-1}) (Fig. 1).

Eucalyptus urophylla – optimization of the leaf in vivo assay

The highest leaf NRA was observed at 35 °C. Temperatures below or above this value caused a slight decrease of enzymatic activity (Fig. 2A). A considerable leaf NRA was detected when no substrate was added to the reaction medium. Even so, the NRA increased ~109%, reaching a maximum with KNO_3 100 mM in the incubation medium. However, higher concentrations of external nitrate decreased enzymatic activity, which reached values even lower than with no substrate, with of KNO_3 300-400 mM (Fig. 2B). Regarding pH, NRA reached a peak with 7.0. The pH values below or above this optimum caused a decrease of enzymatic activity. At pH values taken as too acidic (5.5–6.0) or too alkaline (8.0), NRA decreased to only ~29% of its maximum (Fig. 2C).

Khaya senegalensis – optimization of the root in vivo assay

The highest root NRA was observed at 30 °C. Temperatures below or above this value caused

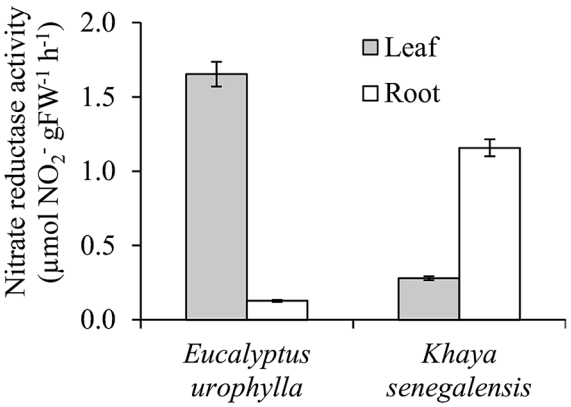

Figure 1. *In vivo* NRA in leaf and root of *Eucalyptus urophylla* and *Khaya senegalensis* seedlings

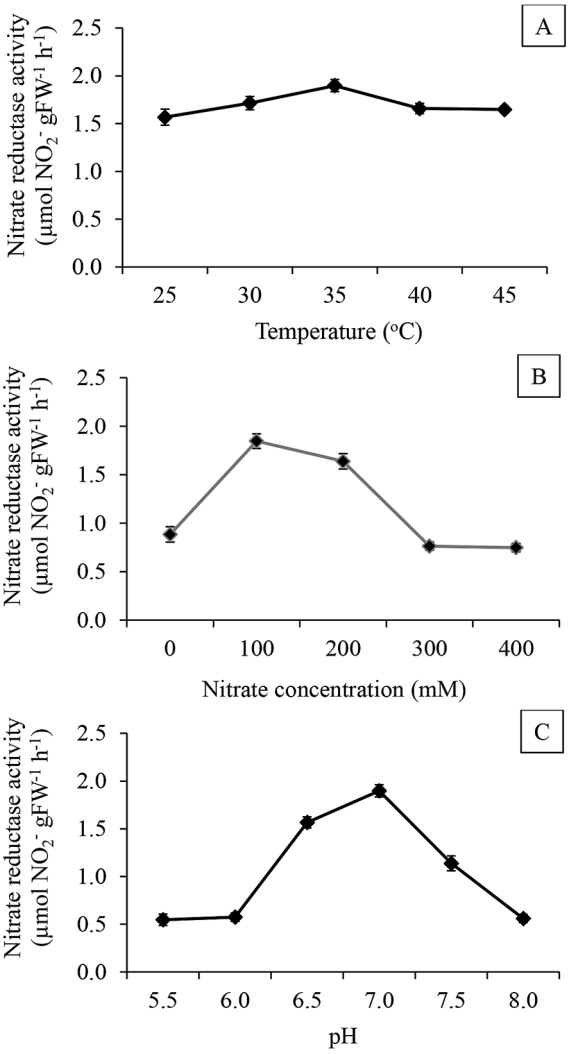

Figure 2. Leaf *in vivo* NRA in *Eucalyptus urophylla* seedlings, in response to different temperature [A], KNO_3 concentration [B], and pH [C]

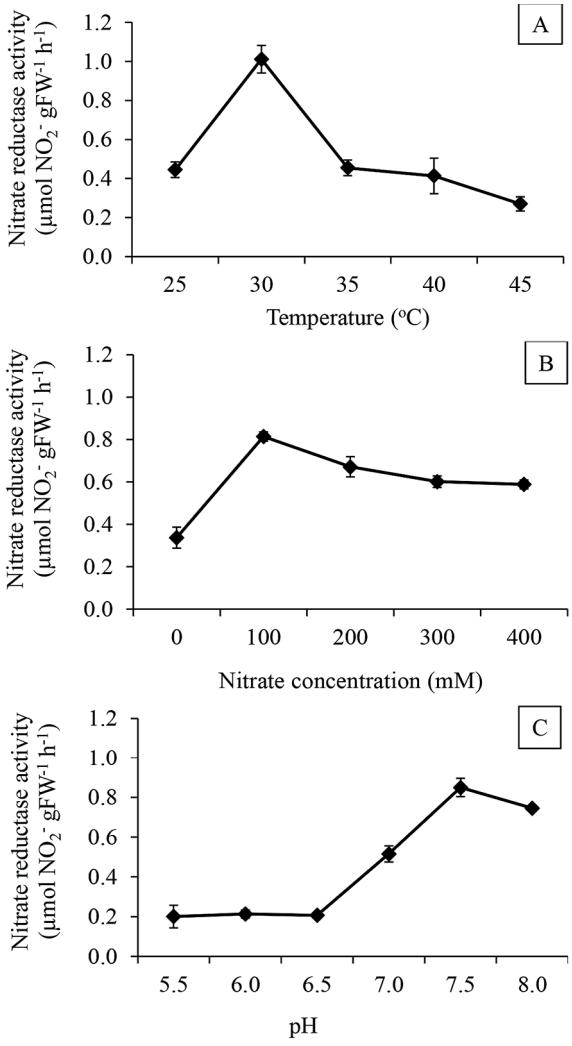

Figure 3. Root *in vivo* NRA in *Khaya senegalensis* seedlings, in response to different temperature [A], KNO_3 concentration [B], and pH [C]

a significant decrease of enzymatic activity (Fig. 3A). The addition of KNO_3 100 mM to the incubation medium increased NRA by ~141%, reaching a peak. Higher concentrations of external nitrate caused a slight decrease of enzymatic activity until it became stable (Fig. 3B). Regarding pH, NRA reached a peak with 7.5. At pH values below this optimum, there was a greater decrease in enzyme activity than at pH 8.0 (Fig. 3C).

DISCUSSION

The results show that the major nitrate reduction site is the leaf for *Eucalyptus urophylla* and the root for *Khaya senegalensis* (Fig. 1). The NRA values, both for leaf *Eucalyptus urophylla* and for root *Khaya senegalensis*, can be taken as low, when compared to those commonly observed in herbaceous species. However, this is not surprising since a very low NRA is often reported in studies on *Eucalyptus* [Granger et al., 1994; Pokhriyal et al., 1995; Stewart and Schmidt, 2002; Guimarães et al., 2014]. Reference values of NRA for *Khaya senegalensis*, in turn, remain scarce in the literature. Previous studies on the partitioning of nitrate assimilation in plants report that nitrate reduction occurs in varying proportions between shoot and root. The NRA is commonly found in the leaves of woody tropical and subtropical plant species, regardless of soil nitrate content [Andrews, 1986]. However, there are some exceptions such as *Calophyllum brasiliense* [Smirnoff et al., 1984] and *Hevea brasiliensis* [Delú Filho et al., 1998], in which the major nitrate reduction site is the root.

Considering the energy cost, nitrate assimilation in the leaf rather than in the root may be an advantage, due to the benefits of nitrate reductase having access to photosynthetic reductant in the shoot. When the root is the major nitrate reduction site, as for *Khaya senegalensis*, the nitrate assimilation depends on a reductant derived from glycolysis and the oxidative pentose phosphate pathway [Andrews, 1986; Miller and Cramer, 2005]. However, when the light limits photosynthesis or during the night, no advantage will be gained in leaf nitrate reduction, because this process and CO_2 fixation will directly compete for ATP and reductant generated by photosynthetic electron transport [Rubio-Asensio et al., 2014], leading to a decrease in CO_2 fixation. Another disadvantage of nitrate assimilation in the leaf, irrespectively the light level, is that hydroxyl ions generated during this process must be neutralized by the synthesis of organic acids (in the root, the pH balance may be maintained via decreased proton excretion or increased bicarbonate excretion) [Smirnoff and Stewart, 1985].

The nitrate reduction in the leaves requires the transport of considerable amounts of this anion through the xylem sap. For *Khaya senegalensis*, which has the major nitrate reduction site in the root, this may be associated both to insufficient root-to-shoot nitrate transport and to a low capacity to produce reductants in the leaf [Fernandes and Rossiello, 1995]. As nitrate translocation through the xylem sap also depends on the external concentration of this anion, the reduction in the root may be advantageous for plants subjected to low soil nitrate supply [Miller and Cramer, 2005].

Regarding the optimization of the *in vivo* assay, the results show that the influence of temperature on the NRA varied between species, reaching an optimum at 35 °C for the leaf of *Eucalyptus urophylla* (Fig. 2A) and at 30 °C for the root of *Khaya senegalensis* (Fig. 3A). Lower NRA with decreasing temperature may be a consequence of changes in membrane permeability, which reduces the absorption and transport of nitrate to the reduction sites within cells. Conversely, temperatures above the optimum can also decrease NRA, due to conformational changes in the molecular structure of the enzyme, impairing its catalytic function [Oliveira and Magalhães, 1989; Queiroz et al., 1991]. Furthermore, it was observed that variations in temperature were less influential on NRA in *Eucalyptus urophylla* than in *Khaya senegalensis*. The slight variation of NRA in *Eucalyptus urophylla* in response to temperature variation suggests that nitrate assimilation may remain satisfactory even over a wider temperature range.

A considerable NRA in the leaf of *Eucalyptus urophylla* was detected when no substrate was added to the reaction medium, suggesting a likely presence of a readily available pool of NO_3^- previously allocated inside the cell vacuoles [Oliveira et al., 2005; Dovis et al., 2014]. Even so, the addition of nitrate to the incubation medium is essential for checking NRA, since the nitrate reductase is an enzyme with synthesis and activity induced by substrate [Beevers and Hageman, 1969; Solomonson and Barber, 1990]. In our study, the results show that the addition of KNO_3 100 mM to the incubation medium was sufficient for NRA to reach a maximum in both species, with an increase of ~209% in the leaf of *Eucalyptus urophylla* (Figure 2B) and of ~242% in the root of *Khaya senegalensis* (Fig. 3B). However, very high concentrations of this substrate can negatively affect the estimate. For KNO_3 concentrations higher than 100 mM, there was a more consistent decrease in NRA in *Eucalyptus urophylla* than in *Khaya senegalensis*. A high ionic strength resulting from the increase in the nitrate concentration in the cytosol can destabilize the complex of subunits that make up the nitrate reductase molecule, causing a decrease in its catalytic activity [Oliveira and Magalhães, 1989; Cairo et al., 1994]. Therefore, for the *in vivo* method, it is recommended that the nitrate concentration in the incubation medium be sufficiently high, but not limiting, so as not to harm the enzymatic activity [Santos et al., 2014].

Regarding the influence of pH in the incubation medium, the results show that NRA reached a peak at 7.0 for the leaf of *Eucalyptus urophylla* and 7.5 for the root of *Khaya senegalensis* (Fig. 3C). Optimization of NRA at a pH close to neutrality tending to a slight alkalinity has been demonstrated for several species, such as wheat [Brunetti and Hageman, 1976], coffee [Meguro and Magalhães, 1982], soybean [Crafts-Brandner and Harper, 1982], barley [Lillo, 1983], apple tree [Lee and Titus, 1992], pineapple [Nievola and Mercier, 2001], *Brachiaria* [Cazetta and Villela, 2004], peach palm [Oliveira et al., 2005], sugar cane [Santos et al., 2014], and *Physalis angulata* L. [Tanan et al., 2019], among others. The effect of pH on NRA has been associated with its influence

on the distribution of charges at an active site, which changes the structural conformation of the enzyme [Nelson and Cox, 2018], or changes the rate of nitrate and nitrite translocation across the cell membrane [Prakashi and Nair, 1982; Carelli and Fahl, 1991]. In our study, pH values above or below the maximum resulted in a decrease in NRA for both species. However, the decrease in NRA in the root of *Khaya senegalensis* was less consistent with increased than decreased pH, suggesting a higher tolerance of nitrate reductase to alkalinity than to acidity. This effect may be associated with slightly alkaline cytosolic pH where nitrate reductase acts, due to a continuous H^+ exclusion from cytosol to apoplast through the proton pump [Hawkesford et al., 2012].

CONCLUSION

The leaf and the root are the major nitrate reduction site of *Eucalyptus urophylla* and *Khaya senegalensis*, respectively. The optimal conditions for the *in vivo* assay in the leaf were 35 °C, KNO_3 100 mM, and pH 7.0, whereas for the root they were 30 °C, KNO_3 100 mM, and pH 7.5.

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