PRIMARY METABOLITE MOBILIZATION DURING GERMINATION IN ROSEWOOD (*Aniba rosaeodora* Ducke) SEEDS[1]

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ABSTRACT – This study aimed to characterize protein, oil, starch and soluble sugar mobilization as well as the activity of α-amylase during rosewood seed germination. Germination test was carried out at 25°C and the following parameters were analyzed: percentage of germination, initial, average, and final germination time. Seed reserve quantification was monitored in quiescent seeds and during different stages of radicle growth. Starch mobilization was studied in function of α-amylase activity. Germination reached 87.5% at the initial, average, and final time of 16, 21 and 30 days, respectively. Oil mobilization showed a negative linear behavior, decreasing 40% between the first and the last stage analyzed, whereas protein levels increased 34.7% during the initial period of germination. Starch content (46.4%) was the highest among those of the metabolites analyzed and starch mobilization occurred inversely to the observed for soluble sugars; α-amylase activity increased until the 15th day, a period before radicle emission and corresponding to the highest starch mobilization. The high percentage of rosewood seed germination may be related to the controlled condition used in the germination chamber as well as to high seed reserve mobilization, in special oil and starch.

Keywords: α-amylase, carbohydrates and oil.

MOBILIZAÇÃO DE METABÓLITOS PRIMÁRIOS DURANTE A GERMINAÇÃO DE SEMENTES DE PAU-ROSA (Aniba rosaeodora Ducke)

RESUMO – Este estudo teve por objetivo caracterizar a mobilização de metabólitos primários (proteínas, óleos, amido e açúcares solúveis) e determinar a atividade da enzima α-amilase em sementes de pau-rosa durante diferentes períodos da germinação. Sementes quiescentes, após a assepsia, foram postas para germinar a 25 °C, analisando-se as seguintes variáveis: porcentagem de germinação e tempos inicial, médio e final de germinação. A quantificação das reservas orgânicas foi monitorada nas sementes quiescentes e nos diferentes estágios de crescimento da radícula. A mobilização do amido foi monitorada mediante a atividade da enzima α-amilase. A germinação atingiu 87,5% com os tempos inicial, médio e final iguais a 16, 21 e 30 dias, respectivamente. A mobilização de óleos apresentou comportamento linear negativo, diminuindo 40% entre o primeiro e o último estágio analisado, enquanto o teor das proteínas aumentou 34,7% nos estágios iniciais de germinação. O conteúdo de amido (46,4%) foi o mais elevado entre os metabólitos estudados, e sua mobilização ocorreu de forma inversa à dos açúcares solúveis. A atividade da α-amilase foi mais intensa até o décimo quinto dia, período que antecedeu a emissão da radícula e que correspondeu à maior mobilização do amido. A alta porcentagem de germinação das sementes de pau-rosa pode estar associada às condições controladas na câmara de germinação, e à elevada mobilização das reservas da semente, especialmente óleos e amido.

Palavras-chave: α-amilase, carboidratos e óleos.

1. INTRODUCTION

Seeds play a vital role as dispersal units, as well as sources of food reserve to sustain the development and establishment of seedlings, maintaining the diversity of plant species (BORGHETTI and FERREIRA, 2000; BUCKERIDGE et al., 2000). Germination of seeds is a multi-stage process requiring the coordinated expression of numerous genes in different tissues (POTONIKA et al., 2002). Under favorable conditions, seeds begins to germinate and the embryonic tissues resume growth, developing into seedlings, with germination being completed when a part of the embryo, the radicle, extends to penetrate the structures surrounding it (BEWLEY, 1997). Upon inhibition, the quiescent dry seeds rapidly resume metabolic activity (BEWLEY, 1997).

Proteins, lipids, and carbohydrates are the main components stored during the late stages of seed development. During germination, these reserves are hydrolyzed and mobilized by the embryo (KIDSON and WESTBOY, 2000; PONTES et al., 2002). The mobilization of storage food reserves is associated with seedling growth (BREDEMEIER et al., 2001). Lipids and carbohydrates are used as source of energy during germination and post-germination events, with the latter also contributing to water distribution into the seed (CARVALHO et al., 2001). Large amounts of storage proteins are accumulated for use as an initial source of reduced nitrogen by the germinating seedling (BECKERT et al., 2000). Mobilization of seed food reserves may be related with the changes in seed vigor (CRANE et al., 2006). Therefore, information concerning how an embryo mobilizes its internal reserves during the early stage of germination can provide insights into the metabolic process of germination and consequently into the ability to use such seeds as planting material (GONÇALVES et al., 2003).

Rosewood (*Aniba rosaeodora*) is a native tree species of the Amazon rainforest. Its trunk wood contains an essential oil rich in linalool, a chemical that can be transformed into a number of derivatives of aggregate value for the fragrance industry. Over the past decades, intensive harvest and high demand for the oil have threatened with extinction the remaining rosewood trees (CLAY et al., 1999). Despite its economic importance, almost no information is available on seed germination of this tropical forest species and on thebiochemical routes involved in this process (PONTES et al., 2002;

GONÇALVES et al., 2002). This investigation can help us to understand the biochemical aspects of rosewood seed germination that can be used to predict seed vigor. The aim of this work is to evaluate the mobilization of rosewood seed food reserves during germination at least up to radicle emergence.

2. MATERIAL AND METHODS

2.1. Plant material and germination test

Seeds of rosewood were collected in natural populations growing in the Adolpho Ducke Forest Reserve (3° 00' 02"S and 59° 58' 00"W), Manaus-AM. Seeds were transported to the Plant Physiology and Biochemistry Laboratory (MCT-INPA)) and their surfaces were disinfested using 0.5% (v/v) solution of commercial bleach (6% sodium hypochlorite) for 10 min. After tegument excision, the seeds were placed in plastic bags containing vermiculite as substrate and maintained in a germination chamber at 25°C (12h photoperiod). The seeds were considered germinated after radicle emission and the following variables were analyzed: germination percentage, initial, average, and final germination time (MAGUIRE, 1962).

2.2. Seed reserve extraction and quantification

Plant material was dried at 75°C and seed reserves (carbohydrates, lipids, and proteins) were extracted from quiescent seeds as well as from germinating seeds at different radicle emission stages (0cm, 2cm, 2-5cm, and 5-7cm). Oil extraction was performed using the Soxhlet apparatus with petroleum ether. Seeds and seedling oil contents were determined following the A.O.A.C method (AOAC, 1990). Proteins were extracted according to Passos (1996) using the hot alcohol method, and protein content was determined according to Bradford (1976), using bovine serum albumin as standard. For soluble sugar determination, seeds were crushed in 95% (v/v) ethanol, following centrifugation at 10.000rpm, for 10min at 25°C. The supernatant was stored and the pellet was submitted to a second round of extraction in 80% (v/v) ethanol (60°C) and centrifuged as mentioned before. The supernatants were combined for determination of soluble sugars, and the pellet was re-suspended in 10mL of 35% (v/v) perchloric acid. After centrifugation, the supernatant was collected for starch quantification. Soluble sugars were determined by the anthrone method (625nm) using glucose as standard (Sigma®) (MORRIS, 1948).

2.3. Extraction and determination of α-amylase (E.C 3.2.1.1) activity

Determination of α-amylase activity was performed in seeds after 6, 9, 12, 15, 18 and 21 days of germination according to Doman et al. (1982). Plant material was homogenized in 60mM phosphate buffer (pH 6.8), filtered and then centrifuged at 12.000 x *g*, 4°C, for 15min. I*n vitro* activity was determined in a reaction medium containing 60mM phosphate buffer (pH 6.8), 400μg/ml calcium chloride and 500 μg/ml starch. Plant protein extract (1ml) was added to the reaction medium following incubation in a water bath (25°C, 20min). After that, 100μl of lugol was added to stop the reaction; α-amylase activity was monitored at 620nm using starch (Sigma®) as control and enzyme activity was expressed in relation to starch consumption.

2.4. Statistical analysis

The experiments with quantitative values for seed reserve mobilization and α-amylase activity were carried out in triplicate and submitted to regression analysis. The computer program used to conduct the statistical analyses was SAEG 5.0 (Viçosa, MG).

3. RESULTS AND DISCUSSION

Seeds of rosewood tree showed a high germination percentage (87.5%) at 25°C (12h photoperiod), with an initial, average and final time of germination of 16, 21, and 30 days respectively (Figure 1) Similar results were found by Sampaio et al. (2003), with a germination percentage of 84% (seeds without tegument) and 94% (seeds with tegument) being obtained. However, when rosewood seeds were germinated in nurseries, germination percentage varied between 35 and 75.3% (MARQUES et al., 1999) and radicle emergence between 60 and 120 days (ALENCAR and FERNANDES, 1987). For rosewood, high percentage of seed germination is obtained from immediate plantation after seed collection (SAMPAIO, 1999). This seems to be the most common behavior for tropical wood plants, although, in many cases, germination is hampered by a hard coat or endogenous dormancy mechanisms (VÁSQUEZ-YANES and OROZCO-SEGOVIA, 1993).

Seed germination comprises two different metabolic processes: enzymatic hydrolysis of seed storage providing essential energy to fuel growth and formation of new cell structures until the seedling becomes photoautotrophic (PRITCHARD et al., 2002; SOLTANI et al., 2006). Breakdown of seed oil reserves is known to occur after radicle emergence, being converted in starch or soluble sugars, which become the primary nutrient source during heterotrophic growth (NYKIFORUK and JOHNSON-FLANAGAN, 1999; PONTES et al., 2002; RODRIGUES et al., 2005). During rosewood seed germination, it was observed a decrease in the oil content of 40% between the first stage (0 cm) and the last stage of radicle emergence analyzed (5-7 cm) (Figure 2A). A similar behavior was observed in the germinating seeds of *Pinus edulis* (HAMMER and MURPHY, 1994), *Brassica napus* L. (BALERONI et al., 1997) and *Euphorbia heterophylla* (SUDA and GIORGINI, 2000). Yaniv et al. (1998) also observed a decrease in the oil content in the species *Sinapis alba* and *Crambe abyssinica* in both dark and light-grown seedlings. However, the decrease was less pronounced in the seedlings developed in the dark. Several studies have shown that, when the glyoxylate cycle is operative in germinating oilseeds, the activity of the decarboxylative steps of the TCA cycle are suppressed, favoring the synthesis of carbohydrate over respiration (EASTMOND and GRAHAM, 2001).
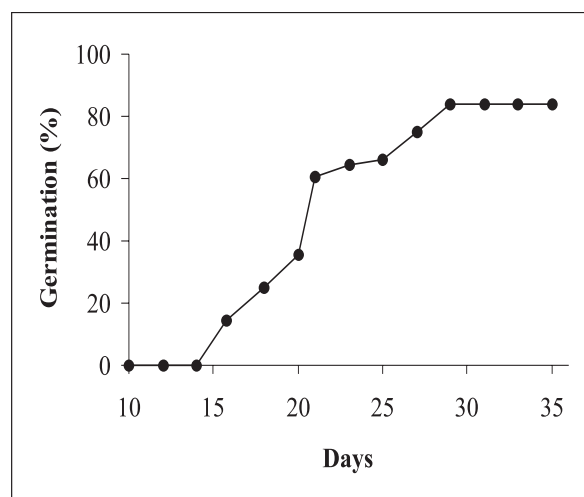

Figure 1 – Germination percentage at 25 °C during 36 days in rosewood seeds submitted to 12h photoperiod.
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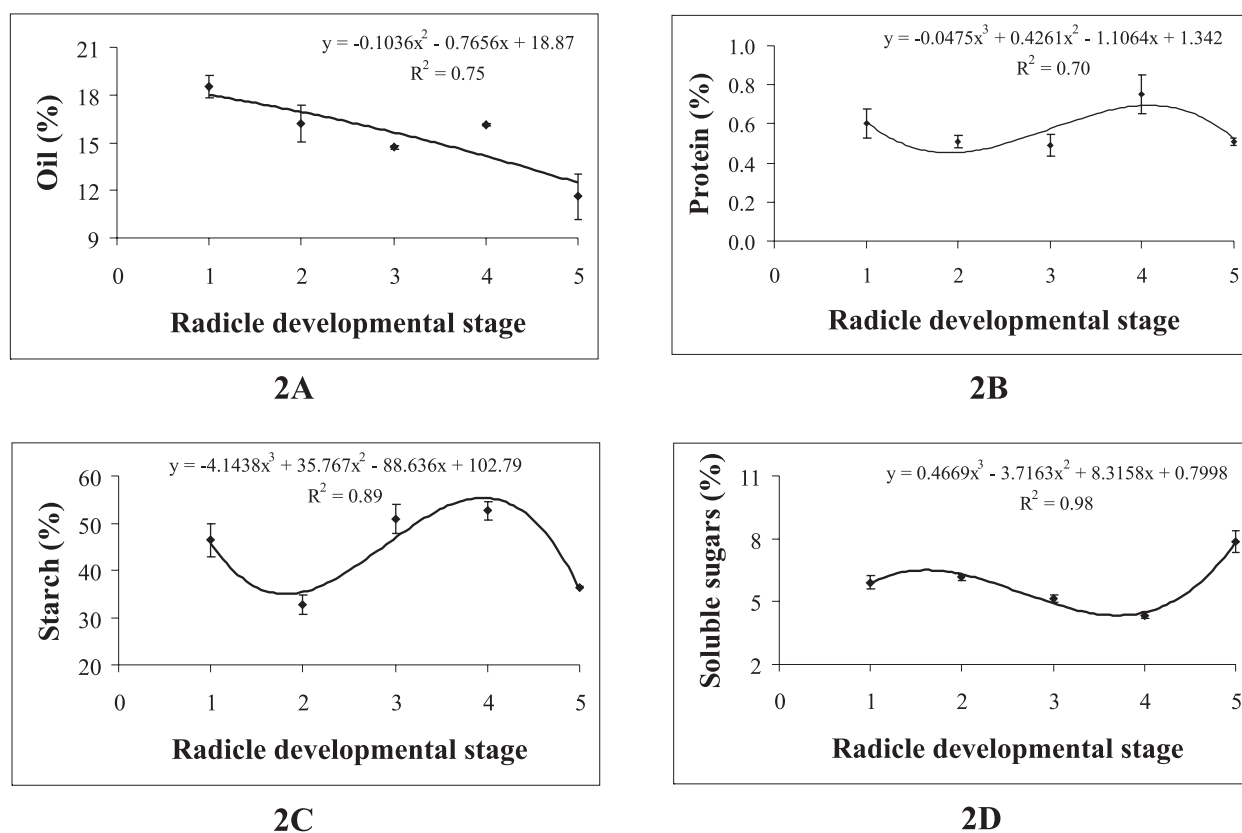
The equations shown in the figures are:

2A: $y = -0.1036x^2 - 0.7656x + 18.87$, $R^2 = 0.75$

2B: $y = -0.0475x^3 + 0.4261x^2 - 1.1064x + 1.342$, $R^2 = 0.70$

2C: $y = -4.1438x^3 + 35.767x^2 - 88.636x + 102.79$, $R^2 = 0.89$

2D: $y = 0.4669x^3 - 3.7163x^2 + 8.3158x + 0.7998$, $R^2 = 0.98$

Figure 2 – Compositional changes in quiescent seeds and seedlings of rosewood during different stages of radicle development. **(A)** Oil contents;**(B)** Protein contents; **(C)** Starch contents ; **(D)** soluble sugars. Treatments:**1 -**quiescent seeds, **2 -** 0 cm (5 days), **3 -** 2 cm, **4 -** 2 to 5 cm and **5 -** 5 to 7 cm radicle length. The bars indicate the standard deviation from mean values derived from three measurements.

Figura 2 – Compositional changes in quiescent seeds and seedlings of rosewood during different stages of radicle development. (A) Oil contents;(B) Protein contents; (C) Starch contents ; (D) soluble sugars. Treatments:1 -quiescent seeds, 2 - 0 cm (5 days), 3 - 2 cm, 4 - 2 to 5 cm and 5 - 5 to 7 cm radicle length. The bars indicate the standard deviation from mean values derived from three measurements.

During germination, enzymatic hydrolysis of storage proteins in the endosperm forms a reservoir of small peptides and amino acids, which are translocated to supply organic nitrogen to the growing seedling. Proteins involved in carbohydrate, energy and amino acid metabolism constituted to about ¼ of total proteins extracted in the germinating seeds (FU et al., 2005). In rosewood, protein levels were slightly modified during seed germination, being the highest content recorded in 2-5 cm radicles (Figure 2B). It is likely that proteins played a minor role during rosewood seed germination. Silva et al. (1998) observed a protein level increase in the beginning of *Dalbergia miscolobium* seed imbibition. In *Apuleia leiocarpa*, protein contents increased with prolonged times of seed imbibition (PONTES et al., 2002). However, in seeds of *Qualea grandiflora* protein levels were constant during nine weeks of seed germination (PAULILO and FELIPPE, 1994).

Starch was the main reserve accumulated in mature seeds of rosewood (46.4% dry weight basis). This reserve suffers a reduction of 29.5% until the beginning of the radicle emergence, a posterior increase of ca. 55% in 2-5 cm radicle, following an accentuated decrease to 35% in 5-7 cm radicles (Figure 2C). At the same period, seed soluble sugar content increased up to 82% (Figure 2D). In *Senna macranthera* seeds, starch content decreased within 72 hours (LIMA et al., 2002). According

to Pontes et al. (2002), levels of starch and soluble sugar mobilization are dependent on the plant species considered, being more expressive during the early or late stages of seed germination. Soluble sugar levels must be closely regulated in germinating seeds to ensure an adequate supply of energy and building materials for the developing seedling (TO et al., 2002). Under our conditions, seed soluble sugars were mobilized 30% in 2-5 cm radicle, and this mobilization was associated with the first leaf formation. In *Theobroma grandiflorum*, seed soluble sugar levels were reduced 25% during the first 90 days of germination (FIGUEIREDO et al. 2001).

During seedling germination, α-amylase activity plays a critical role in the transformation of starch hydrolysis into metabolizable sugars. The α-amylase expression is severely influenced *in vivo* and *in vitro* by the augmented levels of soluble sugars (YU et al., 1991; KARRER et al., 1992). In rosewood, α-amylase activity slightly changed up to the 15th day of germination, which is a period characterized by high starch mobilization and preceding radicle emergence (Figure 3). After this period, enzyme activity increased. During rosewood's early-seed germination, enzymes other than α-amylase, such as β-amylase, debranching enzyme or α-glucosidase might be responsible for the initial starch breakdown.

Our results suggest that despite of being considered a starchy seed, considerable oil levels are mobilized during early-seedling germination. Protein levels oscillated slightly during the germination period analyzed, with initial starch breakdown being likely performed by enzymes other than a-amylase. Analysis of primary metabolite mobilization can provide insights into the mechanisms involved during seed germination of tropical wood species like rosewood.

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The plot shows the equation $y = 1{,}29 + 0{,}0002x^3$ with $R^2 = 0{,}88$. The y-axis is labeled "α-amylase activity (μg of starch min^{-1} g MF)" ranging from 0 to 4, and the x-axis is labeled "Time after seed germination (d)" ranging from 3 to 24.

Figure 3 – α-amylase activity in germinating seeds of rosewood. The bars indicate the standard deviation from the mean values derived from three measurements.
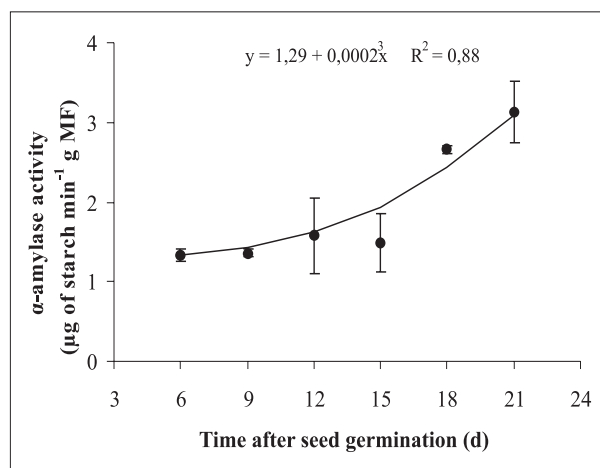*Figura 3* – *α-amylase activity in germinating seeds of rosewood. The bars indicate the standard deviation from the mean values derived from three measurements.*

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