



Research Journal of **Botany**

ISSN 1816-4919



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Accumulation of Raphides Crystals in *Euterpe oleracea* Mart. Embryo

¹M.A.M. Neto, ²A.C. Conceição, ¹A.S. Mendes, ²R.C.L. Costa and ³A.K.S. Lobato

¹Centro de Ciências Biológicas, Universidade Federal do Pará, Belém, Brazil

²Laboratório de Fisiologia Vegetal Avançada,
Universidade Federal Rural da Amazônia, Belém, Brazil

³Núcleo de Pesquisa Aplicada a Agricultura,
Universidade Estadual de Maringá, Maringá, Brazil

Abstract: The aim of this study was to determine if the crystals of the calcium oxalate present in the *Euterpe oleracea* embryo has function of calcium reserve during the germination process and if the accumulation is dependent of fruit production. In the experiment 1 the design was entirely randomized, with 3 evaluation periods (September, November and December). In the experiment 2 the design was entirely randomized, with 3 treatments (without cultivation, MS-CaCl₂ after 30 cultivation days and MS + CaCl₂ after 30 cultivation days). The results not confirm the function of calcium reserve of the raphides in *Euterpe oleracea* embryos, however was determined that the calcium oxalate crystals of the embryo are monohydrated and that the production and accumulation is dependent of the season, in which the accumulation is higher in the period of smaller fruit production.

Key words: *Euterpe oleracea*, calcium oxalate, seed

INTRODUCTION

The species *Euterpe oleracea* Mart. has great economical importance to the Amazon region, because with the fruit pericarp are produced several food products and energy drinks (Oliveira *et al.*, 2002). The palm and its derivatives might be utilized in the pulp production, animal ration, forest, medicine, natural colorants, besides producing heart of palm, commercialized mainly for the export, in which 95% of all hearts of palm produced in the Brazil is extracted of this palm (Oliveira and Muller, 1998).

The calcium oxalate crystals are very common in plants, in which it can be distributed in all the taxonomic levels with capacity of carry out photosynthesis. The accumulation these crystals are reported in organisms as small algae until angiosperm and gymnosperm (Franceschi and Nakata, 2005). The calcium oxalate is a product derived of the combination of the calcium coming from environment and oxalic acid synthesized by the plant (Horner and Wagner, 1995; Ilarslan *et al.*, 1997).

The crystal distribution by several tissues and organs might be in tissues that not carried photosynthesis (Franceschi and Nakata, 2005), as well as in vascular tissue (Paiva and Machado, 2005; Hudgins *et al.*, 2003), besides reproductive organs (Iwano *et al.*, 2004; Zona, 2004). These crystals can be distributed to random within of the cells or it might be found in idioblasts, in which it are specific cells, besides it be different of the others cells in size, content and function (Neto *et al.*, 1997).

The formation process is a basic physiologic process in several plants (Li *et al.*, 2003), in which it is carried out specifically within of the vacuole these specific cells (Arnott and Webb, 2000; Cutter, 1986; Mazen *et al.*, 2004; Neto *et al.*, 1997; Raven *et al.*, 2001; Webb, 1999).

Morphologically the types more found are druses, estiloids, raphides (Prychid and Rudall, 1999). The druses are structures generally spherical of crystals, with edge extended by all surface which it

occur in dicotyledonous and is relatively rare in monocotyledonous (Cutter, 1986). The estiloids are also described as prismatic crystals or pseudoraphides, in which they are more thick than the raphides and usually alone within of the cell. The raphides are long with aspect similar as needle and generally presented in parallel. Besides of this, these are the three main crystal types that occur in monocotyledons, as well as the raphides are present in all the palms (Prychid and Rudall, 1999; Zona, 2004).

The raphides presence in palm embryos can be used as taxonomic criterion, in which this researcher proposed two groups, when the embryo presents until ten parallel raphides is shortage and when it presents more of 25 parallel raphides is abundant. The species *Euterpe oleracea* Mart., which belong to Arecaceae family, it presented more of 25 parallel raphides in embryo and was classified as abundant (Zona, 2004).

Several investigations present the probable function of the raphides in the plants, in which it suggest that the function is dependent of the amount, form, size and position in the plant (Mazen *et al.*, 2004). The functions described are protection against herbivores animals (Finley, 1999; Hudgins *et al.*, 2003), besides regulation of the calcium levels in plant tissues and organs (Franceschi and Nakata, 2005; Paiva and Machado, 2005; Ruiz and Mansfield, 1994).

The function these crystals not are defined in the *Euterpe oleracea* embryo, however studies suggested that the crystals might reserve the calcium to future utilization. This way, the aim of this study was to determine if the crystals of the calcium oxalate present in the *Euterpe oleracea* embryo has function of calcium reserve during the germination process and if the accumulation of raphides crystals are dependent of fruit production.

MATERIALS AND METHODS

Plant Material and Evaluation Period

The study was carried out in the Laboratório de Cultura de Tecidos Vegetais of the Universidade Federal do Pará (UFPA). The seeds of *Euterpe oleracea* Mart. were harvested of mature fruits during three periods (September, November and December of 2007).

Seed Treatment

The seeds were washed with distilled water, after the mesocarp was immediately removed and again the seeds were washed distilled water and neutral liquid detergent. Subsequently the seeds were submitted to ultraviolet radiation in laminar flux chamber during 5 min and immerse in ethanol a 70%. After these proceedings, in each seed was makes longitudinal cut and the embryo was removed.

Experimental Designs

In the experiment 1 the design was entirely randomized, with 3 evaluation periods (September, November and December), in which each period had 30 embryos. In the experiment 2 the design was entirely randomized, with 3 treatments (without cultivation, MS-CaCl₂ after 30 cultivation days and MS + CaCl₂ after 30 cultivation days), in which the seeds were harvested in the month of December and each treatment had 20 embryos.

Medium Preparation

The embryos were inoculated in containers with MS medium (Murashige and Skoog, 1962) modified, in which the changes were total absence of cobalt chloride (CoCl₂.6H₂O) and potassium iodide (KI), as well as only the half of the salt amounts were used in the MS medium preparation, according method developed in the Laboratório de Cultura de Tecidos Vegetais. The pH of the medium was adjusted to 5.9.

The embryos were inoculated in the medium and the containers with the embryos were placed in growth chamber under constant light. After the evaluation period, the embryos were removed of the containers and fixed in presence of ethanol 70%, which was carried out the chemistry analysis.

Embryo Anatomy

The light microscopy was carried out in longitudinal cut of the embryo, in which it was previously stained with safrine at 1%. The blades were visualized with microscope (model Olympus BX41) and the images were captured with photographic chamber (model Canon PowerShot A430).

The scanning electron microscopy was carried out in longitudinal cuts of the embryo, in which the embryos were placed in oven at 60°C by 30 sec. The samples were conducted to the Laboratório de Microscopia Eletrônica de Varredura (LABMEV) do Instituto de Geociências da Universidade Federal do Pará (UFPA), in which it were prepared in aluminum support and were used a carbon cassette with duple face to the metallization and placed in the equipment (model LEO-1430). The analysis conditions to the secondary electron images were with current of electron parallel at 90 µA, voltage of the acceleration constant of 10 kV and the work distance of 15 mm.

In the analysis of X-rays diffraction, the embryos were removed of the seeds and placed in oven at 60°C by 5 min, after the embryos were triturated. The samples were pulverized and transferred to monosilicon plates. The standard of the rays-X diffraction was determined by rays-X diffractometer (model Xpert-pro) of the Laboratório de Difração de Raios X do Instituto de Geociências of the Universidade Federal do Pará (UFPA). In which was used Cu radiation (Copper) at 40 kV and 40 mA, as well as the scanning velocity of 0.5 a 5 sec under angle 2θ fluctuating of 5-75°.

Calcium Oxalate Quantification

The embryos were immersing in ethanol at 70% and after washing with distilled water, subsequently it were placed in oven at 60°C by 72 h and triturated. The samples were weighted and transferred to containers, in which it were dissolved in 5 mL sulfuric acid (H₂SO₄) and diluted in 20 mL of ultrapure water. The solution resultants were placed in boiler at 90°C by 20 min and after were add up potassium permanganate (KMnO₄) in the solutions until the solution present pink coloration by 15 sec. The determination of the calcium oxalate (CaC₂O₄) presents in the embryo was according with the methodology described by Ruiz and Mansfield (1994).

$$\text{CaC}_2\text{O}_4 (\%) = \frac{V(L) \text{ KMnO}_4 \times N \text{ KMnO}_4 \times \text{meq} \times 100 \text{ g}}{\text{Sample matter}}$$

Data Analysis

The test used to statistical analysis was the variance analysis (ANOVA), in which was utilized one criterion and the software used was the BIOESTAT 3.0.

RESULTS

The longitudinal cut of the embryo central region before of the *in vitro* cultivation reveals great amount of idioblasts with raphides in parallel (Fig. 1).

Figure 2 is showed that the raphides crystals has needle aspect, with fine extremity, however it not are in parallel, because probably were expel the cell contents and the raphides were scatter.

The seed harvested in different period presents variation in the calcium oxalate level of embryo, in which the months of September, November and December were showed 33, 55 and 84% of calcium oxalate, respectively (Fig. 3).

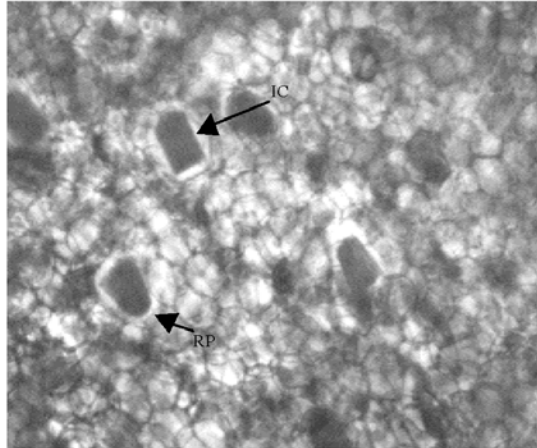


Fig. 1: Longitudinal cut of the *Euterpe oleracea* embryo before of cultivation, in which it reveals the idioblasts with raphides in parallel. IC: Idioblasts cells, RP: Raphides in parallel

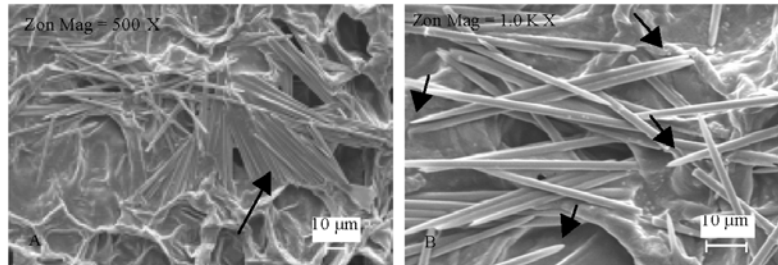


Fig. 2: Eletromicrographs of *Euterpe oleracea* embryo reveals the structure of the raphides crystals. (A) The arrow indicates the raphides in semi parallel. (B) The arrows indicate the fine extremities of the raphides

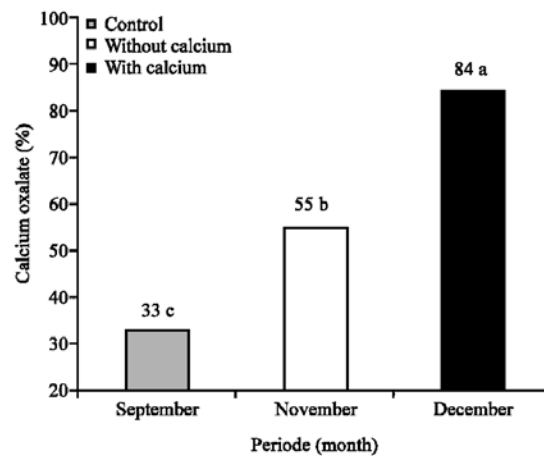


Fig. 3: Calcium oxalate level in the seed embryo before of the *in vitro* cultivation in different periods

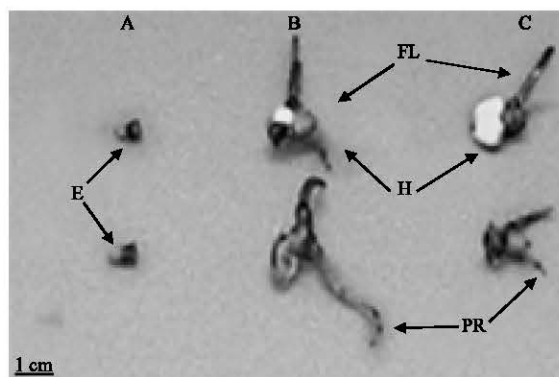


Fig. 4: Embryos with 0 and 30 cultivation days in MS medium. (A) Embryos with 0 cultivation days, (B) Embryos cultivated by 30 days in MS medium with addition of calcium and (C) Embryos cultivated by 30 days in MS medium without addition of calcium. E: Embryo, FL: First leaf, PR: Primary root and H: Haustorium

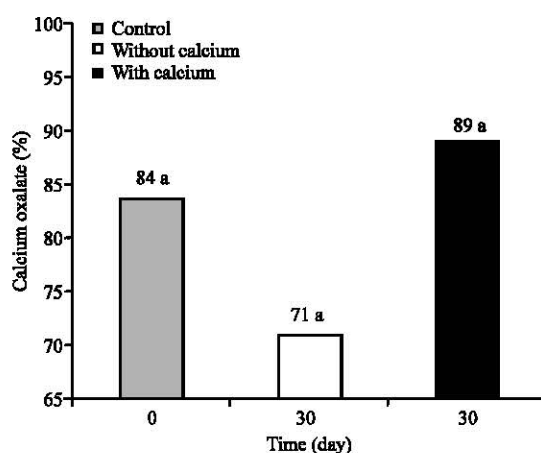


Fig. 5: Calcium oxalate level in seed embryos in the 0 day and after 30 cultivation days in MS medium with and without addition of calcium

The embryos submitted to MS medium with and without addition of CaCl_2 present normal development after 30 cultivation days, in which can be showed in the Fig. 4 that the embryos of both treatments present primary root, first leaf and small haustorium development.

The calcium oxalate level found in embryo after 30 cultivation days not presents significant difference, in agreement with ANOVA, in which the treatment submitted to MS medium with calcium was higher and treatment without addition of calcium was smaller, when compared with the treatment control in the 0 cultivation day (Fig. 5).

DISCUSSION

The embryos not present others crystal types as druses or estiloids, besides of this, this study corroborates the results found by Zona (2004) that also not showed the presence of others crystals in *Euterpe oleracea* embryos. In agreement with Prychid and Rudall (1999) the calcium oxalate crystals

are present in monocotyledonous and the crystal type more common found in the Arecaceae family are the raphides. Several studies describe the others crystal forms as showed by Arnott and Webb (2000) in plants of *Vitis* genus, as well as DeBolt (2007), Franceschi and Nakata (2005), Ilarslan *et al.* (1997), Mazen *et al.* (2004), Prychid and Rudall (1999), Volk *et al.* (2004) and Webb (1999).

The analysis of rays-X diffraction reveals that the calcium oxalate crystal found in the embryo is mono-hydrated, in which it is denominated as whewellite ($\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$). According to Franceschi and Nakata (2005), Horner and Wagner (1995), Monje and Baran (2002), Pennisi *et al.* (2001a, b), Prychid and Rudall (1999), Volk *et al.* (2002), Webb (1999), as well as Zona (2004) in the plants there be two forms of hydration of the calcium oxalate crystals, it are the mono-hydrated (whewellita/ $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$) and the di-hydrated (wheddellite/ $\text{CaC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$).

The druses and others crystal types can be found under two hydration forms, however in the raphides only are showed the form mono-hydrated (Prychid and Rudall, 1999; Zona, 2004). Studies conducted by Webb (1999) suggest that the mono form is more stable and common in the plants. Besides of this, the hydration state might be affected by the presence of others organic acids (Franceschi and Nakata, 2005).

The amount of calcium oxalate showed between the months of September and December can be linkage with the fruit amount produced, because in the period of September occur higher number fruits per plant and consequently smaller concentration of oxalate in the embryos, when compared with the month of December. The oxalate presents in plants can fluctuate in agreement with environment, soil, season and plant age (Çalışkan, 2000). Besides of this, the environment conditions might influence in the oxalate amount and crystal number produced (Prychid and Rudall, 1999).

The quantification of the calcium oxalate level demonstrates that the embryo in study can accumulate until 67% of calcium oxalate in relation to total dry matter, beside of this, when the this calculation is carried out in relation to total fresh matter the oxalate level had only 7.5%. Studies conducted by Ilarslan *et al.* (1997) with soybean reveal that this species had until 24% of oxalate in the seed, which the oxalate accumulation is dependent of the period after of the fertilization. The oxalate level and the harvest time is inversely proportional in *Xanthosoma sagittifolium*, in which the level fluctuates among 18 and 38 mg g^{-1} , besides occur great variation of the calcium oxalate level in the several plant parts (Seganfredo *et al.*, 2001).

The similar levels of calcium oxalate between the treatments with and without calcium, in relation with normal development of embryos, it suggest that the raphides not are work as calcium reserve during the time evaluated in this study. In which futures studies with higher cultivation time can explore this question.

Several studies reveal that when the endogenous calcium level is high or under abnormal conditions occur synthesis of calcium oxalate, in which Volk *et al.* (2002) working with *Pistia stratiotes* showed an increase in the synthesis of druses and raphides when submitted to high calcium concentration, as well as Franceschi (1989) investigating *Lemna minor* found that the formation process of the crystals is quick.

Results showed by Volk *et al.* (2002) indicated that the calcium deficiency provoked the significant reduction of the druses number, as well as the synthesis of raphides was paralyzed and that the young druses are partially dissolved. These responses in this species indicating that the crystals have the function of calcium reserve to future necessity.

The *Euterpe oleracea* embryo has higher level of free Ca in the cells and probably not needs of the calcium presents in the raphides during the initial development. As well as the formation of oxalate crystal in the embryo occurs due the regulation mechanism of the free calcium level in the cell, in agreement with Franceschi and Nakata (2005), Keates *et al.* (2000), Prychid *et al.* (2003) and Sugimura *et al.* (1999).

The results showed in this study not were concluded to the function of the calcium oxalate crystals in the embryos, however, it not rule out the possibility of that the raphides work as calcium reserve during the seed germination, because evaluation time of this study was short. Besides of this, the determination of the free Ca^{++} level present in the embryo before and during the cultivation is important to shown, if the maintenance of the calcium oxalate levels is coming from uses of the free calcium presents in the embryos before of the *in vitro* cultivated.

The variation of the calcium oxalate level presents in the *Euterpe oleracea* embryo in the different periods is a strong indicative that the crystal accumulation is dependents of the season. Besides of this, the similar levels of calcium oxalate in the treatments suggest that the raphides formation in the embryos not work as calcium reserve during the initial phases of the germination process. A hypothesis to increase of calcium level is that the formation calcium oxalate crystals can work as control mechanism of the calcium endogenous level in the cell or tissue to avoid toxicity of embryo, because the soils where this species is found are rich in calcium.

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