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Storage lipids and proteins of *Euterpe edulis* seeds

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ABSTRACT: Comparative studies on fatty acid and protein composition of the endosperm and embryo of palmito (*Euterpe edulis* Martius) were conducted using gas-liquid chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. On a dry weight basis, the embryo contained extremely lower amounts of lipids and proteins than did the endosperm, which was associated with the scarce lipid and protein bodies previously reported in axis and cotyledon. The fatty acid composition also exhibited differences between both tissues: (I) the fatty acid diversity was greater in embryo than in endosperm; (II) embryo and endosperm contained predominantly linoleic, palmitic, oleic and stearic acids even though the relative values were different for each tissue. As compared to other palm species, the higher fatty acid unsaturation in *Euterpe edulis* seed could be involved in the previously reported short longevity and recalcitrant behavior during storage. Proteins of both tissues were heterogeneous in molecular mass. Some proteins were tissue-specific, but other were common, among them a highly glycosylated protein which migrated at about 55 kDa. We hypothesize that the latter, also reported in all previously studied palm species, is one of the proteins characterizing the Arecaceae family.

Introduction

Seed studies carried out in *Phoenix dactylifera* L., *Washingtonia filifera* (Lindl.) Wendl. and *Elaeis guineensis* Jacq. show that the two areas of food reserves in a palm seed are the massive, hard endosperm and the small embryo (Alang *et al*., 1988; DeMason 1985; 1986; 1988; DeMason *et al*., 1983; 1989a, b; DeMason and Thomson, 1981; Meier, 1958; Meier and Reid, 1982). The stored reserves in palm endosperm are mannans, in the thickened cell walls, and lipid, protein, and mineral nutrients, in the cytoplasm. Lipids and proteins are in the form of lipid and protein bodies, and minerals are stored as phytin in the form of globoid crystal inside protein bodies. In the palm embryo, stored reserves also consist of lipids, proteins in the forms of lipid and protein bodies, the latter including phytin globoid crystals.

Differing from those three palm species, Panza *et al*. (2004) report that *Euterpe edulis* embryo cells have scarce storage reserves and exhibit an active state, with numerous mitochondria, rough endosperm reticulum cisternae, and Golgi apparatus, indicating a strategy of continuous development without the interposition, at maturity, of a dry state. In addition, that study indicates

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that whole embryo with 85% water content constitutes only 0.54% of the seed fresh weight, while that of the endosperm with 48.2% water content constitutes approximately 99% of the seed fresh weight.

Euterpe edulis seeds are short-lived and have recalcitrant storage behavior (Andrade, 2001; De León, 1961; Graziano, 1982; Martins *et al*., 2000; Reis *et al*., 1999), the latter feature differentiating this species from *Phoenix dactylifera* and *Washingtonia filifera*, both with orthodox seeds (Carpenter and Ream, 1976; Dickie *et al*., 1992; Krigman, 1974; Nixon, 1964; Sento, 1972) and also from *Elaeis guineensis*, in which seeds have an intermediate behavior (Ellis *et al*., 1991).

A difficulty in the interpretation of the experimental results on recalcitrant seeds is the differential water content between the embryo axis (and embryo) and the storage tissues (Berjak *et al*., 1989). In this respect Panza *et al*. (2007) demonstrate the need to study in separated form the embryo of the endosperma in *E. edulis* seeds.

To date, triacylglicerol seed composition has been studied only in two Arecaceae species, *Elaeis guinensis* (Salunkhe and Desai, 1986) and *Cocos nucifera* (Satyabalan, 1989). In both of them, the triacylglicerol composition is quite comparable, containing predominantly lauric (12:0), myristic (14:0), and palmitic (16:0) acids. Up to date, triacylglicerol composition of embryo and endosperm separately has not been reported for any palm species of Arecaceae.

Although palm endosperms are approximately 20% protein by weight, there is very little work on the characteristics of palm seed storage proteins. Previous studies, carried out by Chandra Sekhar and DeMason (1988a, b; 1989; 1990), DeMason and Chandra Sekhar (1990), and DeMason *et al*. (1985, 1989a) in *Phoenix, Washingtonia,* and *Cocos*, show that proteins are heterogeneous in molecular mass and charge ranging from 12 to 67 kDa and 3 to 10 in pI values. A number of common proteins exist in those genera which include both 7S and 11S globulins. In the three genera, the same authors have detected a highly glycosylated protein which migrates at about 55 kDa. They have also detected some individual differences between the three genera studied in relation to the relative quantities of the 67 kDa -7S globulin and 35 kDa -11S globulin. More recently García *et al*. (2005) determines in coconut that the basic polypeptide of the 11S globulin, which migrates at approximately 24 KDa, is glycosylated.

We have carried out a chemical study of the *Euterpe edulis* endosperm and embryo in which fatty acid and protein composition were analysed. This is the first chemical study in an embryo of the Arecaceae family. *E. edulis* is a tropical species occurring in a narrow range of rain forest in the Southern and Southeastern Brazil, Northeastern Argentina and Paraguay (Silva Matos and Watkinson, 1998). Its economical value is related to the production of "heart of palm", i.e. the growing apical bud surrounded by young leaves (Nodari and Guerra, 1986). Most of the time the plants are harvested before they reach maturity and produce seeds, the only propagation method of this species. The economical management has been conducted in an essentially predatory way and that represents a threat to the survival of this species. This study constitutes part of a monographic treatment on conservation of *Euterpe edulis*.

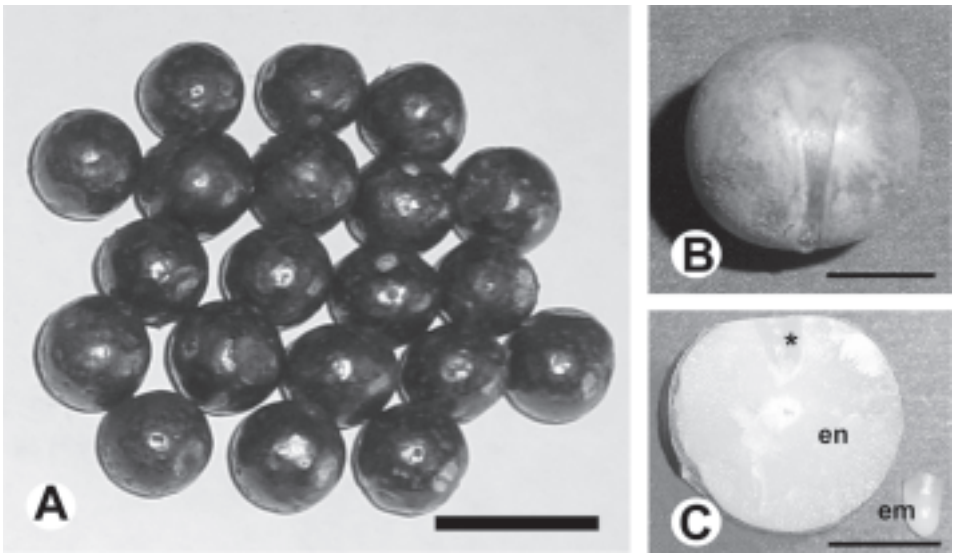

FIGURE 1. *Euterpe edulis* fruits and seeds: **A**, mature fruits; scale bar = 2 cm. **B**, seed cleaned of pericarp, as seen from the raphe: scale bar = 0.2 cm. **C**, longitudinal medial section trough a seed showing endosperm (en) and detached embryo (em). Asterisk indicates embryo position within the endosperm; scale bar = 0.2 cm.

Material and methods

Plant material

Mature fruits of *Euterpe edulis* Martius (Fig. 1A) were harvested from trees growing in Parque Nacional Iguazú, Provincia de Misiones Argentina, during the month of August for three consecutive years (2001 to 2003), and shipped by expedited post to the Bank of Germplasm of INTA, Castelar, Buenos Aires, Argentina, where studies were conducted.

Lipids and proteins were determined for embryo and endosperm tissues separately. For this purpose, both embryos and endosperms were removed from the seeds (Fig. 1C) and lyophilized.

Lipid extraction and fatty acid analysis

For fatty acid analysis, lyophilized and ground endosperm and embryo tissues were transferred into 1.5 ml eppendorf tubes and total lipids were extracted with chloroform – methanol mix using the procedure described by Folch *et al.* (1957). Total lipid extracts were dried and weighted, suspended in 2 ml of a fresh solution of 10% KOH in ethanol and saponified during 60 min at 80ºC using stopped glass tubes. Two ml hexane was added and fatty acids were extracted by shaking. The upper organic phase (non-saponifiable) was discarded. The aqueous layer was acidified with 1.5 ml of concentrated HCl and fatty acids were extracted twice with 1.5 ml hexane. Extracts containing free fatty acids were dried under a nitrogen stream, dissolved in 1.5 ml BF_3 (10% in methanol) and 1.5 ml benzene and esterified by heating and shaking at 100ºC for 1 h. Fatty acid methyl esters were extracted twice with hexane and washed with distilled water. After washing, the organic phase was evaporated under a nitrogen stream, re-dissolved in hexane, and analyzed by gas-liquid chromatography. One µl of the fatty acid methyl esters solution was injected into an Omegawax X250 (Supelco Inc., Bellefonte, Pennsylvania) capillary column (30 m x 0.25 mm, 0.25 µm film) in a Hewlett Packard HP-6890 chromatograph equipped with a flame ionization detector. The column temperature was programmed for a linear increase of 3ºC/min from 175 to 230ºC. The chromatographic peaks of fatty acid methyl esters were identified by comparison of their retention times with standards, under similar conditions.

Protein extraction and protein analysis

Total protein content was determined in triplicate for each year's collection (three years 2001-2003) by the Kjeldahl's method, using the value of 5.7 as conversion factor. After lipid extraction, the dried pellet was used to obtain different fractions of the major storage proteins for quantification using bovine serum albumin as standard (Bradford, 1976), as follows:

(I) To obtain water-soluble albumins, pellets were re-suspended in 50 mM Tris-HCl, pH 8.3, 1 µM benzamidine, 1 µM phenyl-methylsulfonyl fluoride in the ratio of 10 µl/mg of sample. Samples were stirred for 30 minutes, centrifuged for 10 minutes at 10,000 g and the supernatant collected as soluble fractions.

(II) To obtain salt-soluble globulins, pellets after low-salt extraction were re-suspended in 50 mM Tris-HCl, 1.0 M NaCl, 1 µM benzamidine, 1 µM phenyl-methylsulfonyl fluoride. After stirring for 60 minutes, the samples were centrifuged for 10 minutes at 10.000 g and the supernatant collected as a high salt fraction.

(III) To obtain prolamins, pellets were re-suspended in ethanol 70% at 65ºC, 1 µM benzamidine, 1 µM phenyl-methylsulfonyl fluoride. After stirring for 30 minutes, the samples were centrifuged for 10 minutes at 10.000 g and the supernatant collected as prolamins fraction.

(IV) To obtain glutelins, pellets were re-suspended in NaOH 0.1 M, 1 µM benzamidine, 1 µM phenyl-methylsulfonyl fluoride. After stirring for 30 minutes, the samples were centrifuged for 10 minutes at 10,000 g and the supernatant collected as glutelins fraction.

(V) The remaining proteins were obtained as follows: pellets after NaOH extraction were re-suspended in 50 mM Tris-HCl, 2% sodium dodecyl sulfate. The samples were stirred for 60 minutes, and then centrifuged for 10 minutes at 10,000 g. The supernatant was collected as remainder fractions. This procedure was repeated twice to ensure that all proteins were removed from the ground material.

Polyacrylamide gel electrophoresis under denaturation conditions was conducted according to Laemmli (1970) with some modifications. Electrophoresis was run in a linear gradient of acrylamide concentration (8-15%) in a Mini Protean III system (Bio-Rad), at constant voltage (130 V) for 90 min. Glycerol (5% v/v) was added to both stacking and resolving gel solutions. For analytical purposes, 40 µg proteins were loaded onto each well. Low Range Molecular Weight calibration stained kit (Bio-Rad) was used to estimate the molecular weight (MW) of the different proteins. Gels were

stained with 0.1% (w/v) Coomasie Brilliant Blue R-250 solution, and then analyzed with a Bio-Rad GS-800 Imaging Calibrated Densitometer. Images were captured and processed by Quantity One 1-D Analysis software. For glycoprotein detection, gels were stained with the periodate-Schiff reaction (Segrest and Jackson, 1972).

Results and Discussion

This is the first study on triacylglicerol and proteins composition in an embryo of the Arecaceae family. Previous studies were made on whole seeds. On this respect, Grout *et al*. (1983) and Berjak *et al.* (1989) warn on the difficulty of comparing groups of data in which there are marked differences on size and water content between both tissues. The difficulty is exacerbated in *Euterpe edulis* where the whole embryo is minute (Fig. 1C) and constitutes only 0.54% of the seed's fresh weight (Panza *et al*., 2004).

Fatty acid analysis

In *E. edulis* endosperm, total lipids represented around 0.45% of the total endosperm weight (dry weight basis). In the embryo, lipids were only present in extremely low quantity. Unsaturated fatty acids were predominant, the sum of which was around 65% of total fatty acids of endosperm and 60% of embryo. Endosperm and embryo contained predominantly linoleic (18:2ω6), palmitic (C16:0), and oleic (C18:1ω9) acids even though for each tissue the relative values were different (Fig. 2; Table 1). In embryo, α-linolenic (18:3 ω3) and palmitoleic (16:1 ω7) acid and stearic (C18:0) acid concentrations were almost similar. In endosperm, the α-linolenic and palmitoleic were minor fatty acids. The oleic isomer 18:1 ω7 (vacenic), biosynthetically derived from the palmitoleic acid, was also quantitatively important in embryo, but it is a minor fatty acid in endosperm. Traces of other fatty acids of 16, 20, 22, and 24 carbons were also detected in both tissues (Fig. 2; Table 1).

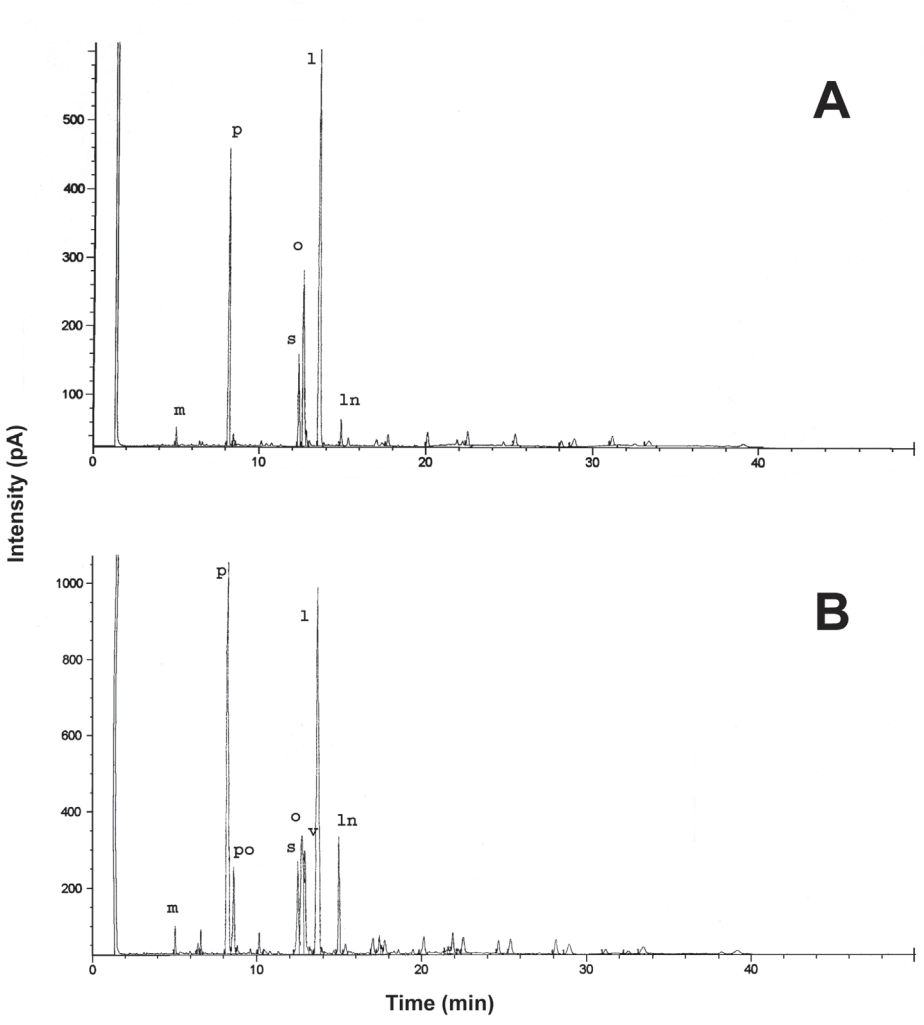

FIGURE 2. Fatty acid composition of *Euterpe edulis* endosperm and embryo as analyzed by gas-liquid chromatography. The chromatographic peaks of fatty acid methyl esters were identified by comparison of their retention times with those of standards chromatographed under the same conditions. A, endosperm; B, embryo. Abbreviations: *l*, linoleic acid; *ln*, linolenic acid; *m*, myristic acid; *o*, oleic acid; *p*, palmitic acid; *po*, palmitoleic acid; *s*, stearic acid; *v*, vacenic acid.

Gurr (1980) reports that, even though fatty acids tend to be characteristic of particular plant families, they could vary widely among species. To date, the seed fatty acid composition in palms is known for two species, *Elaeis guinensis* and *Cocos nucifera* (Salunkhe and Desai, 1986; Satyabalan, 1989). In those two species, lauric, myristic, and palmitic (saturated) acids are predominant. Differing from those species both tissues in *E. edulis* predominantly contained (as mentioned above) linoleic, palmitic, and oleic acids. According to Miquel and Browse (1995) 18-carbon unsaturated and polyunsaturated fatty acids are generally predominant in Angiosperms. In endosperm and embryo of *Euterpe edulis* they represented around 55 and 48%, respectively. On this respect, Staehelin and Newcomb (2000) identify linoleic and linolenic acids (in addition to palmitic acid) as the main fatty acids in plants frequently associated with membrane construction.

Additionally, *Euterpe edulis* seeds are short-lived (De León, 1961; Graziano, 1982) and have recalcitrant storage behavior (Andrade, 2001; Martins *et al*., 2000; Reis *et al*., 1999), differentiating this species from *Phoenix dactylifera* and *Washingtonia filifera*, both with orthodox seeds (Carpenter and Ream, 1976; Dickie *et al*., 1992; Krigman, 1974; Nixon, 1964; Sento, 1972) and also from *Elaeis guineensis*, in which seeds have an intermediate behavior (Ellis *et al*., 1991). At present we cannot establish in the *E. edulis* embryo the biochemical events responsible for its short longevity and recalcitrant behavior, but it is known that polyunsaturated fatty acids, the most oxygen sensitive molecules encountered in nature, are present in membranes (Spiteller, 2003). The high fatty acid unsaturation evokes changes in membrane structures, commonly enhancing their fluidities. In addition, unsaturation also increases fatty acid susceptibility to degradation as a consequence of the double bond peroxidation. The short longevity found in *E. edulis* seed (De León, 1961; Graziano, 1982), which is only capable of initiating germination within a short time following shedding, could be reflecting the relative high content of unsaturated acids found in *E. edulis* seed (which represented around 65% and 60% of total endosperm and embryo fatty acid, respectively). These facts or some other property conferred by the higher fatty acid unsaturation in *Euterpe edulis* seeds, as compared to other palm species, would also contribute to explain its behavior during storage.

TABLE 1.

Percentages of the *Euterpe edulis* fatty acids.

Fatty acid	Endosperm	Embryo
14:0	3.4	0.8
15:0		0.4
16:0	23.1 *	24.0 *
16:1 ω7	0.8	4.1 *
16:2		0.9
16:3		0.4
18:0	6.3 *	4.9 *
18:1 ω 9	15.6 *	10.8 *
18:1 ω 7	1.5	5.3 *
18:2 ω 6	36.4 *	27.6 *
18:3 ω 3	1.5	4.8 *
20:1 ω 9	2.5	1.1
20:3 ω 3	2.3	1.3
22:1	2.0	1.3
22:5	0.7	1.1
24:1	1.2	0.4
Others	2.7	10.8

The sum of the most abundant fatty acids (*) represents 81.4% and 81.5% of total endosperm and embryo fatty acids, respectively. The "other" category corresponds to unidentifiable acids, present in very small quantities. Values are the average of triplicate determinations.

The fatty acid spectrum was wider in embryo than in endosperm, i.e. several 15- and 16-carbon fatty acids were detected only in embryos, but were absent in endosperm. Such diversity could be associated with the more active biosynthetic mechanisms to elongate and to desaturate fatty acids which are needed during germination.

Protein analysis

Up to now, very little is known about seed proteins from palm seeds. In fact, Chandra Sekhar and DeMason (1988a, b; 1989; 1990), DeMason and Chandra Sekhar (1990), and DeMason *et al.* (1985; 1989a) electrophoretically characterize seed proteins in *Phoenix, Washingtonia,* and *Cocos*, showing that they are heterogeneous in molecular mass and charge ranging from 12 to 67 kDa. A number of common proteins exist in all those genera which include both 7S and 11S globulins. The same authors detect a highly glycosylated protein which migrates at about 55 kDa in the three above mentioned genera. They also detect some differences between the three genera studied in relation to the relative quantities of the 67 kDa - 7S globulin and 35 kDa -11S globulin.

In *Euterpe edulis* total protein content, quantified by the Bradford's method, represented 5.39% and 2.46% of the endosperm and embryo dry weight, respectively (Table 2). Total protein endosperm content quantified by the Kjeldahl's method represented 5.4% of the endosperm dry weight. Total soluble proteins were also quantified for different fractions of both endosperm and embryo, showing that the two major fractions were albumins, globulins and sodium dodecyl sulphate-soluble proteins for endosperm, and albumins and glutelins for embryo (Table 2). The difference in protein quantity correlates well with very small quantities of protein bodies present in the embryo tissues (Panza *et al*., 2004), consequently, we infer that the source of proteins in embryos may also be various organelles and the organelle-free cytoplasm.

TABLE 2.

Protein fractions and total proteins of endosperm and embryo of *E. edulis*.

Proteins	Endosperm	Embryo
Albumins	1.94	1.53
Globulins	1.03	0.34
Prolamins	0.00	0.04
Glutelins	0.42	0.55
Sodium dodecyl sulphate soluble proteins	1.99	0.00
Total proteins (Bradford)	**5.39**	**2.46**
Total proteins (Kjeldahl)	**5.40**	

Values are given as percentage of protein / total endosperm dry weigth and protein / total embryo dry weigth, respectively.

The electrophoretic study of total soluble proteins in *Euterpe edulis* showed a different profile for both endosperm and embryo tissues (Fig. 3, lanes b and c). In *Phoenix, Washingtonia* and *Cocos*, the electrophoretic techniques show that the major seed proteins are not tissue-specific; this correlates with the histochemical similarity in the endosperm and embryo of these species with proper quiescent tissues. In the three genera, cells store abundant lipids and proteins in the form of lipid and protein bodies, the percentage of normal cell constituents (cytoplasm, nucleus and other organelles) is very small, and, at the transmission electron microscopy level, no ribosomes or endoplasmic reticulum or endomembranes are discernable in mature tissues (DeMason, 1986; 1988; DeMason and Thomson, 1981; DeMason *et al*., 1983).

In *E. edulis*, endosperm proteins resolved into 13 main bands ranging between 14 and 141 kDa (Fig. 3A, lane b) and embryo proteins resolved into 7 main bands ranging from 17 to 45 kDa (Fig. 3A, lane c). Endosperm and embryo had in common bands of approximately 17, 22, 25, 28, 35, and 40 kDa. Bands between 50 and 141 kDa, were detected clearly in the endosperm but not in the embryo. Endosperm profile included both 67 kDa - 7S globulin and 35 kDa - 11S globulin that are present in *Phoenix, Washingtonia* and *Cocos* (Chandra Sekhar and DeMason, 1988a, b; 1989a; DeMason and Chandra Sekhar, 1990).

Using the periodate-Schiff reaction (Fig. 3B), five major glycosylated bands were detected in endosperm of approximately 16, 25, 35, 50 and 55 kDa, and two bands in embryo, of approximately 25 and 50 KDa. The 25 KDa bands, which are present in both tissues, would correspond to cocosin, i.e. the basic component of the 11S globulin detected in coconut by García *et al*. (2005).

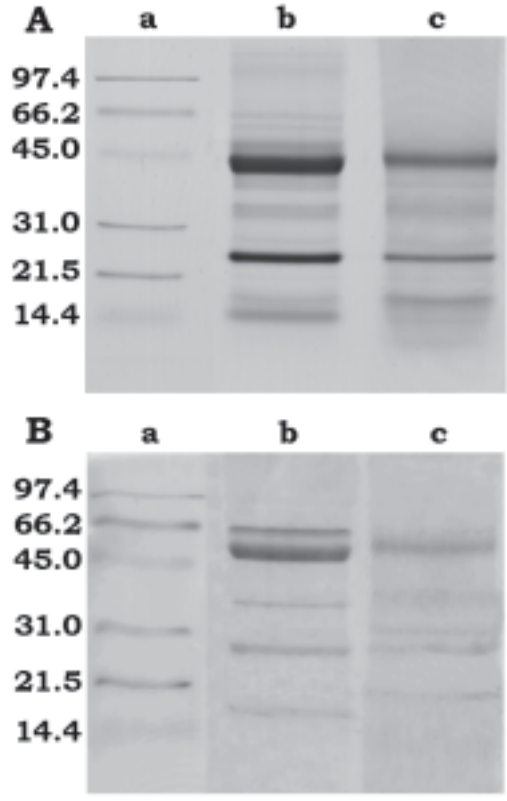

FIGURE 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of *Euterpe edulis* endosperm and embryo. For both gels: lane a = molecular weight standards; lane b= endosperm soluble proteins; lane c = embryo soluble proteins. **A**, gel was stained with 0.1% (w/v) Coomassie Blue-R-250; **B**, gel was stained with periodate-Schiff reaction (five major glycosylated endosperm bands, of approximately 16, 25, 35, 50 and 55 kDa and two major glycosylated embryo bands, of approximately 25 and 50 KDa were observed).

The 55 kDa glycosylated protein found in *E. edulis* endosperm, is present, according to DeMason (personal communication) not only in the endosperm of the three previously studied species (*Phoenix, Washingtonia* and *Cocos*) but also in all endosperm of other species she has analyzed briefly in her lab, i.e. species of the genera *Calamus, Chamaedora, Caryota, Erythea* and *Syagrus.* Establishing if the 55 kDa protein constitutes a taxonomic marker for the Arecaceae awaits further investigation in other palm species.

Acknowledgments

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