Histochemical analysis of seed reserve mobilization in *Passiflora edulis* Sims fo. *flavicarpa* O. Deg. (yellow passion fruit) during germination

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Abstract

In the present work, we analyzed the histochemical aspects of *Passiflora edulis* seeds reserve mobilization during the first ten days of germination. Our results showed that mainly lipids present in the endosperm are used as a reserve source, and their levels reduce at the same time the radicle protrudes, between the fourth and sixth day of sowing. Furthermore, protein bodies are present in the cotyledons, which are degraded as germination occurs and are almost depleted by the time of radicle protrusion. Starch grains also appear in the late germination period, and it is not clear if there is any reserve wall polysaccharide consumption in the endosperm.

Keywords: seed germination, lipid bodies, protein globules, carbohydrates.

Análise histoquímica da mobilização de reservas em sementes de *Passiflora edulis* Sims fo. *flavicarpa* O. Deg (maracujá amarelo) durante a germinação

Resumo

No presente trabalho analisamos os aspectos histoquímicos da mobilização de reservas das sementes de *Passiflora edulis*, durante os primeiros dez dias de germinação. Nossos resultados mostraram que principalmente lipídios presentes no endosperma são utilizados como reserva, com o seu nível começando a diminuir ao mesmo tempo em que ocorre a protrusão da radícula, entre o quarto e sexto dia do início da embebição. Corpos proteicos também estão presentes nos cotilédones, e são degradados à medida que ocorre a germinação e são consumidos quase totalmente quando da protrusão da radícula. Grãos de amido também aparecem no período tardio de germinação, e não está claro se há ou não consumo de polissacarídeos de reserva de parede no endosperma.

Palavras-chave: germinação de sementes, corpos lipídicos, glóbulos proteicos, carboidratos.

1. Introduction

Yellow passionfruit (*Passiflora edulis* Sims f. *flavicarpa* Deg) belongs to the Passifloraceae and is found all over Brazil, as well as in many other countries in Latin America, such as Argentina, Peru, Ecuador, Colombia, Venezuela and Paraguay (Cervi, 1997). Its economical value, and therefore, importance, resides in the gustative and pharmacodinamic qualities of its juice, fruit, skin and seeds. Also important are the by-products of its industrialization, such as the skin, pulp and seeds, used in animal feed (Manica, 1981). In traditional medicine, the plant is used as a digestive stimulant and as a treatment for gastric cancer (Morton, 1987), and its leaves have diuretic properties when prepared as a tea or in fermentation. The roots, leaves and seeds are anti-helminthic, and the leaves are also useful against bronchopulmonary irritation, insomnia and as a tranquilizer (Cervi, 1997). The juice has a much appreciated typical flavor, rich in vitamin A, calcium and phosphorus (Ferreira, 2000).

The main reserves in seeds are carbohydrates, lipids and protein, varying in quantity amidst different species. The study of the chemical composition in a seed is also of interest in seed technology because both the vigor and storage of seeds are influenced by its own reserves (Carvalho and Nakagawa, 2000). Moreover, interest in seed reserves is based not only on their nutritional value, but also on their usefulness in making industrial products (Buckeridge et al., 2004a).

Morton (1987) showed that in *P. edulis* seeds, there is 11.1% of protein and 23% of oil similar to that of soybean and sunflower, predominantly consisting of linoleic acid (68.70%), oleic acid (17.5%), palmitic acid (11%) and estearic acid (2.80%) (Pontes, 1989). Corner (1977) characterized the seed as oily and Raju (1955) also showed that seed reserve of *Passiflora calcarata* consists of starch, oil and proteins.

The the main objective of this work is the histochemical analysis of reserve mobilization patterns during the first ten days of germination in *Passiflora edulis* seeds, giving special attention to lipid mobilization as it is the main reserve in this species, as well as the other storage compounds, such as proteins and carbohydrates.

2. Materials and Methods

2.1. Seed acquisition and processing

Seeds of *P. edulis* f. flavicarpa cv. IAC 273/277 were purchased from the Instituto Agronômico de Campinas (IAC), São Paulo, Brazil, which is the institution responsible for seed processing. All seeds were delivered without any aril and ready for use.

2.2. Germination conditions

Fifteen seeds were put inside Petri dishes and lined on filter paper soaked with 10 mL of distilled water. The plates were maintained inside incubators at an alternating temperature of 20-30 °C, as recommended by Silva (2004) and under continuous white light provided by two 15 W fluorescent daylight lamps (GE, Hungary), installed over the shelves where the dishes were placed. Seeds with 2 mm long roots were considered as germinated. Six seeds from zero (t0), second (t2), fourth (t4), sixth (t6), eighth (t8) and tenth (t10) days from sowing were collected and analyzed as follows.

2.3. Histology/Histochemistry

Seeds were fixed under vacuum in 70% FAA (Johansen, 1940) over two days and then maintained in 70% alcohol until alcoholic dehydration and embedding in Leica historesin, following the kit procedure.

Eight micrometer sections were made using a Reichert-Jung Leica model 2040 Autocut, and the following techniques were used: Toluidine Blue (routine), Periodic Acid of Schiff (PAS, carbohydrates), Bromophenol Blue (proteins) (Pearse, 1985) and Nile Blue (lipids) (Hawes and Satiat-Jeunemaitre, 2001). After staining and mounting the slides with Entellan, the materials were photographed in a Leica DMLB photomicroscope using a Leica DFC280 camera and were analyzed.

3. Results

3.1. Germination

The seeds had a high germination ratio, occurring between the fourth (t4) and sixth (t6) day from sowing, with all seeds used for this essay showing radicle protrusion at t6, as well as cotyledon aperture and detachment of endosperm remnants beginning at t8.

3.2. Endosperm

Lipid levels decreased significantly over the first 10 days from sowing (Figure 1), more significantly between the sixth and eighth days from the external to the internal portion of the endosperm. It can be observed that the edges of the lipid bodies react positively to the Bromophenol Blue, indicating the presence of proteins in this region of the droplet (Figure 2). The cell walls reacted positively to the PAS and Toluidine Blue, showing the degradation of the wall, from a defined, rigid structure in the first days of germination (t0 to t6, Figures 3a-d and 4a-d, respectively) to a flaccid one, causing a loss in structure integrity, observed in the late periods of germination (t8 to t10, Figures 3e-f and 4e-f, respectively). No other reserve material was observed in the endosperm.

3.3. Cotyledons

With toluidine blue, the retake in the traditional and functional structure of the nucleus between t4 and t6 (Figure 4c-d) was observed along with a color change in the cytoplasm, from light blue to a deeper and homogeneous one. This was observed by the globules present in the initial period which disappeared in the later ones. These globules reacted very poorly with PAS, but a color change in the cytoplasm was also observed in the late period of germination, together with some starch granules at t8 and t10 (Figure 3e-f). The globules present in the initial period of germination reacted positively to Bromophenol Blue (t0 to t6) (Figure 2a-d), they disappeared in the late period of germination and also presented a color change in the cytoplasm. No significant result was observed with the Nile Blue technique.

Evidence of cellular division was observed in t6 and t8 due to the size change from a large cell to many divided ones, with formation of cell walls dividing the cytoplasm and the formation of new nuclei.

4. Discussion

Lipids play an important role in the *P. edulis* seed and are the main source of energy for the *P. edulis* seed germination. As stated before, the seed is characterized as oily (Corner, 1977) due to its lipid-rich endosperm, and this reduction in lipid levels is somewhat identical to other oily seeds. *Euphorbia heterophylla* L. seeds showed a reduction of about 50% in lipid levels after 2 to 3 days from sowing (Suda and Giorgini, 2000) coinciding with radicle protrusion, and in *Arabidopsis thaliana* (L.) Heynh. seeds the lipid level showed a 65% reduction after three and half days from sowing, almost two days after radicle protrusion (Mansfield and Briarty, 1996). *P. edulis* seeds started to show some lipid consumption around the 6th day at the same time as radicle protrusion, which happened between t4 and t6. Other species seeds maintained stable lipid levels during the early period of germination, only consuming them at the late stages, as observed in the *Ricinus communis* L. seeds (Muto and Beevers, 1974), where the lipid content remains at the same levels until the 3rd day from sowing, disappearing almost completely on the 7th day, with root-shoot axis length of about 15 cm, and in seeds of *Caesalpinia peltophoroides* Benth. (Corte et al., 2006, 2008) with lipid levels diminishing during the first ten days from sowing, with complete consumption until the

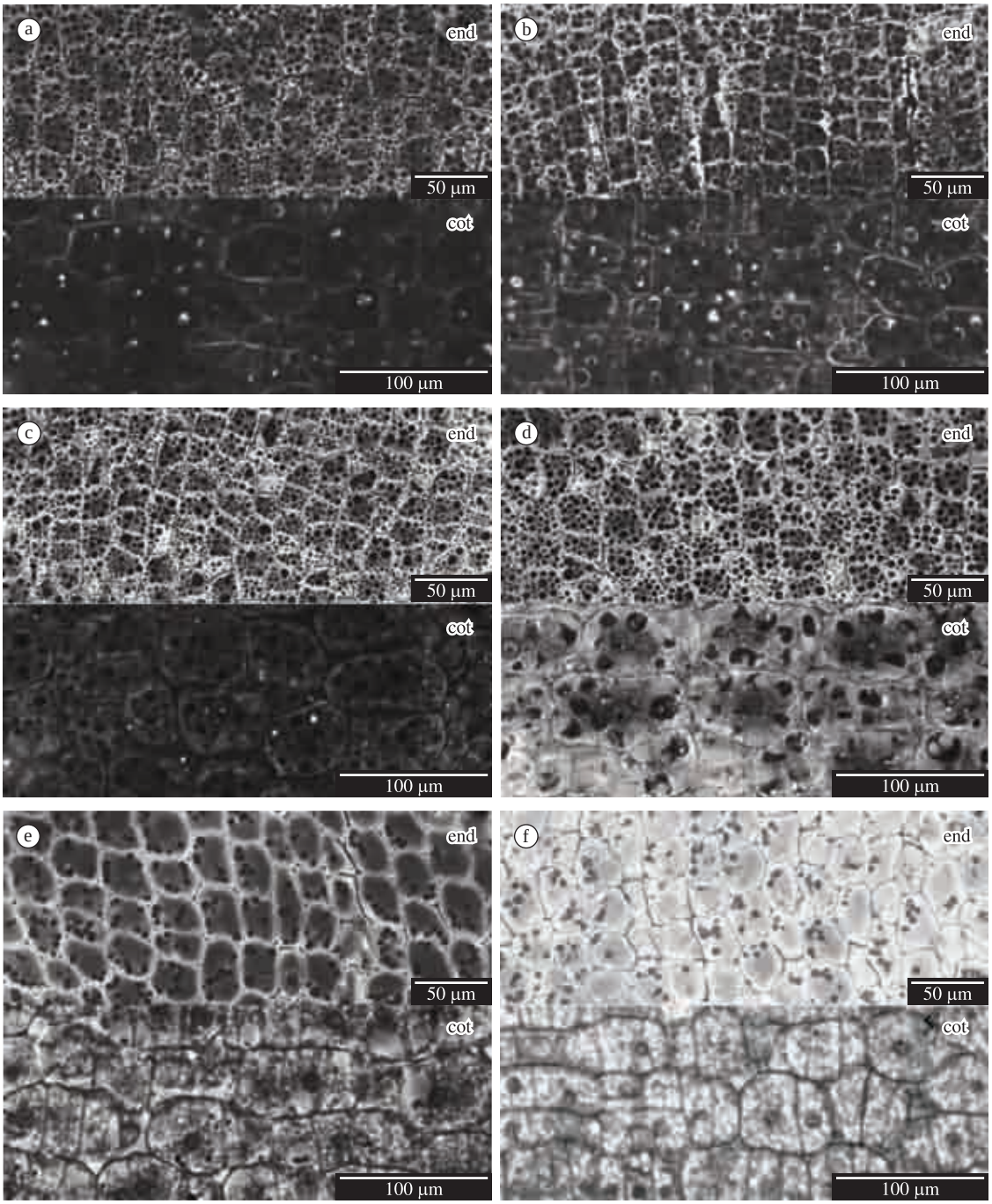

Figure 1. *P. edulis* endosperm (end) and cotyledon (cot) during seed germination stained with Nile Blue. Lipid levels start decreasing in the endosperm at t6 (d). a-t0; b-t2; c-t4; d-t6; e-t8; f-t10. Scale bar = 50 µm for the endosperm and 100 µm for the cotyledon.

20th day. Probably, the lipid present in the endosperm is used as a source material for the synthesis of carbohydrates as a positive response to PAS in the cotyledons appears just after the lipid levels start to diminish, around t6. Additionally, starch granules are present in t8 and t10, also coinciding with the decrease in lipid levels. It is known that lipid degradation in plants lead to the production of Acetil Co-A, leading to sucrose synthesis by gluconeogenesis and therefore may be stocked as starch when its levels in the cytosol are high (Buckeridge et al., 2004b; Graham, 2008; Mansfield and Briarty, 1996; Silva et al., 1998). The same is suggested by Horner and Arnott (1966) in *Yucca*

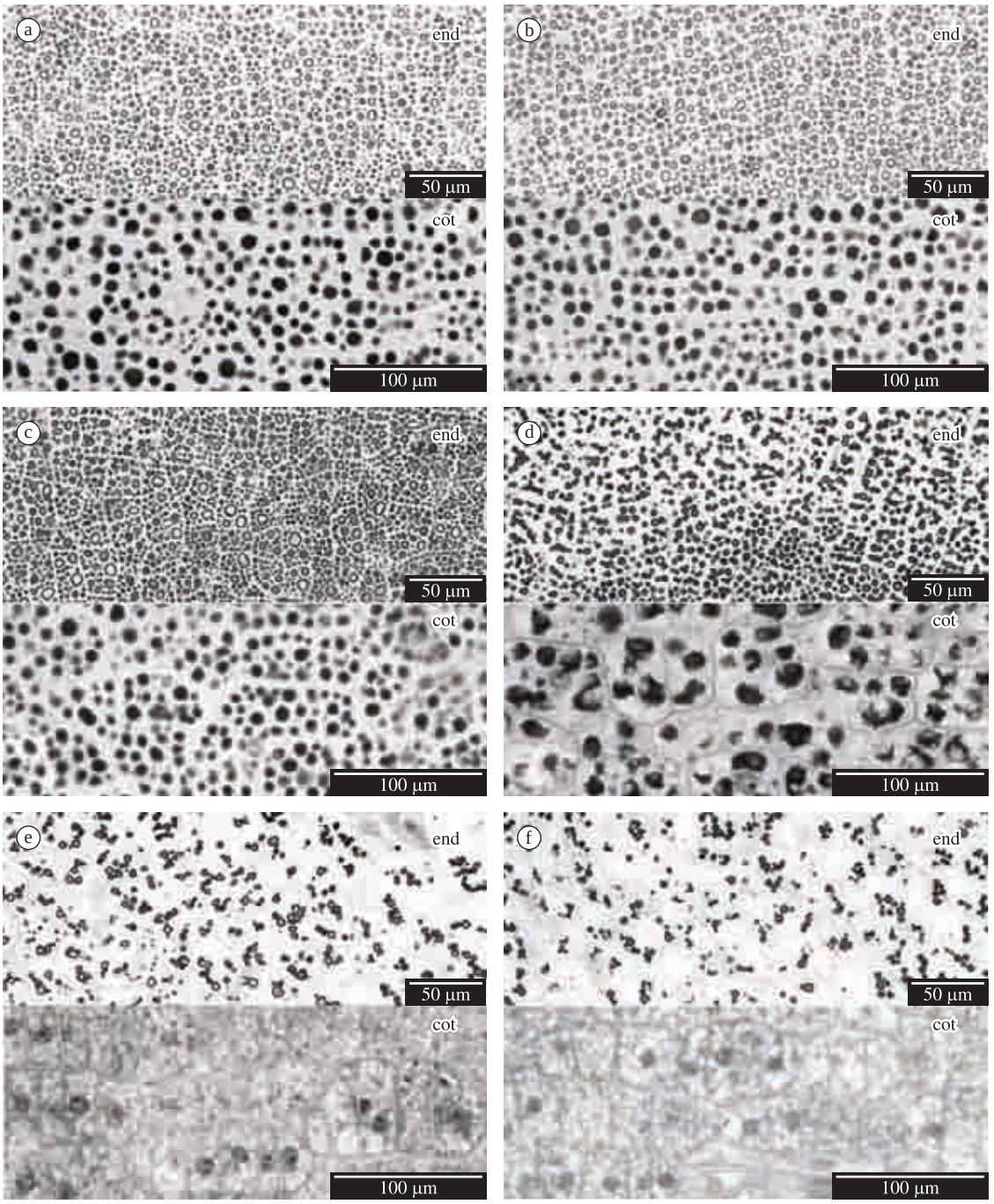

Figure 2. *P. edulis* endosperm (end) and cotyledon (cot) during seed germination stained with Bromophenol blue. Evidence of oleosins around lipid bodies in the endosperm is observed, and protein granules present at the cytoplasm of the cotyledons (a-d) are completely hydrolyzed during germination. a-t0; b-t2; c-t4; d-t6; e-t8; f-t10. Scale bar = 50 µm for the endosperm and 100 µm for the cotyledon.

schidigera Roezl. where seeds showed an increment in starch levels while lipid reserves decreased, justifying the rise in starch levels as a consequence of triglycerides degradation in fat acids and glycerol, and then converted to carbohydrates. The hypothesis to support this fact states that the primary reserve degradation occurs more rapidly than needed for the seedling initial development, and therefore to maintain osmotic balance, the excess would be converted into a "secondary" reserve, starch. Lipids can also serve as a source for the formation of membrane

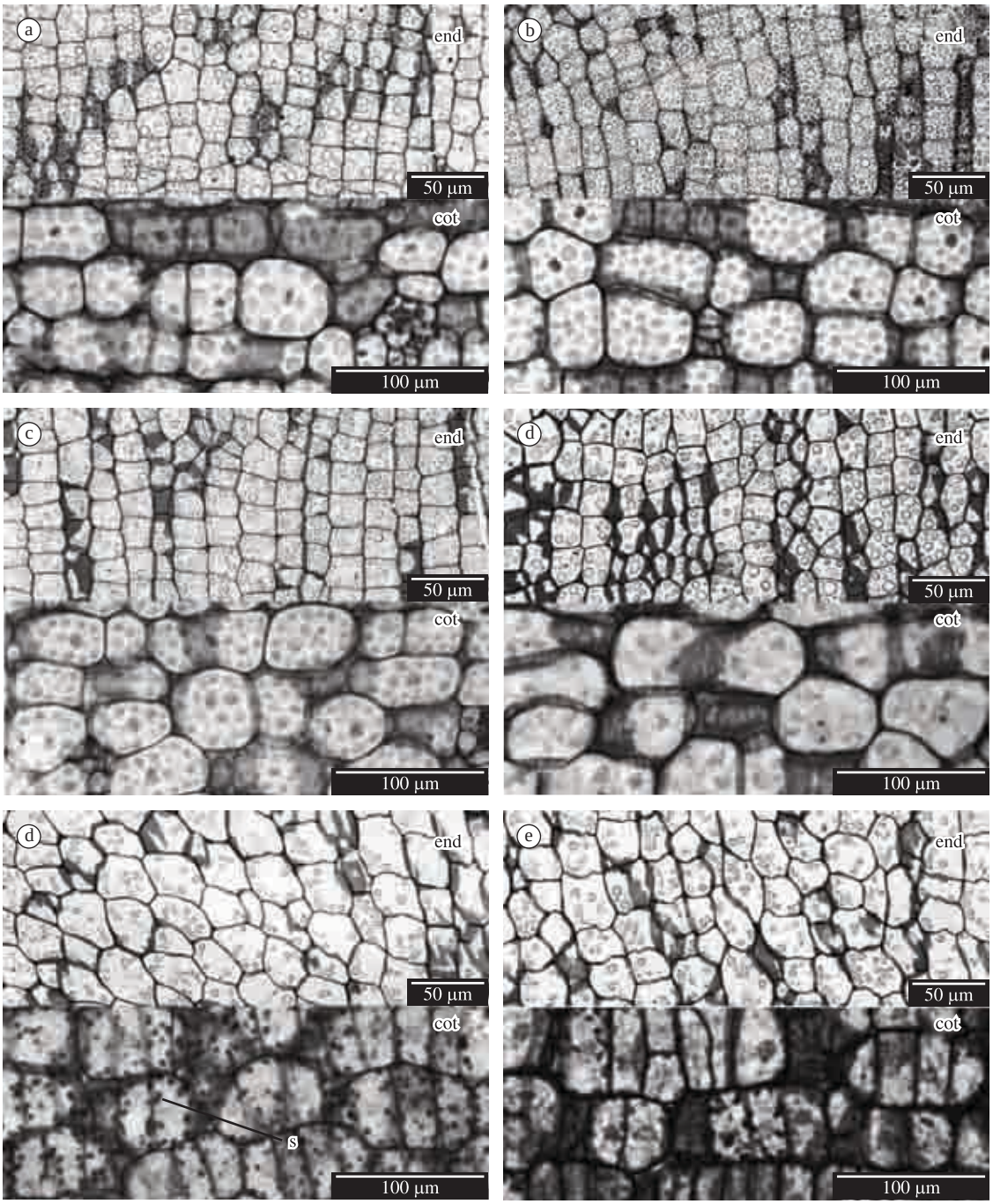

Figure 3. *P. edulis* endosperm (end) and cotyledon (cot) during seed germination stained with PAS. Loosening of the cell walls is evident in the endosperm at t8 and t10 (e-f) after sowing, with a significant change in cytoplasm staining intensity in the cotyledons, with starch grains (s) present at t8 and t10 (e-f). a-t0; b-t2; c-t4; d-t6; e-t8; f-t10. Scale bar = 50 µm for the endosperm and 100 µm for the cotyledon.

systems, such as those of the glyoxysomes, which require non polar lipids and phospholipids for their enlargement during seed germination (Chapman and Trelease, 1991).

The positive bromophenol blue staining around the lipid bodies can indicate the presence of oleosins, 15 to 26 kDa proteins adhered to the membrane of the bodies (also referred to as lipid globules, spherossomes) which store triglycerides, and are associated to lipid mobilization and maintenance of the lipid body structure during the seed maturation desiccation period (Buckeridge et al., 2004b; Somerville et al., 2000).

It is hard to say that there is actually cell wall polysaccharides mobilization in *P. edulis*. While some characteristics show that there may be, like the flaccidity

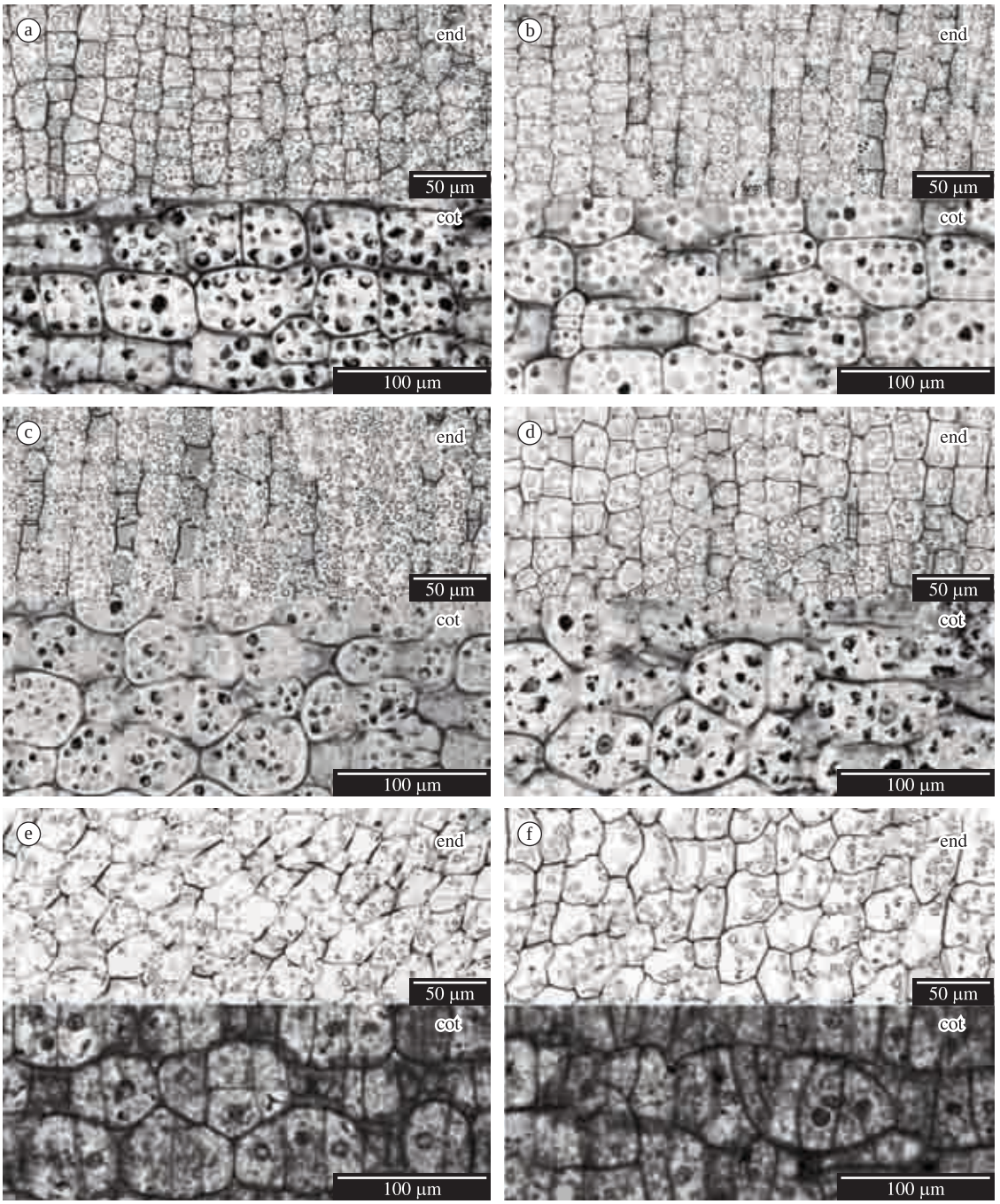

Figure 4. *P. edulis* endosperm (end) and cotyledon (cot) during seed germination stained with toluidine blue. Cell Wall loosening is also evident in the endosperm in the late germination period (e-f), and shows the retake in the nucleus traditional shape and intensity in cytoplasm staining in the cotyledons (d-f). a-t0; b-t2; c-t4; d-t6; e-t8; f-t10. Scale bar = 50 µm for the endosperm and 100 µm for the cotyledon.

observed in the cell walls of the endosperm at t8 and t10, there is no polysaccharide reserve mobilization evidence whatsoever, since there is no significant alteration in the staining intensity by PAS or toluidine blue. Horner and Arnott (1966) showed a reduction in the staining intensity by PAS during the germination period in *Y. schidigera*, also showing an organized dissolution of the perisperm cell wall close to the embryo. The loss of the endosperm cell wall integrity can also indicate the beginning of endosperm senescence, separating from the cotyledons 15 to 20 days from sowing (Pereira and Andrade, 1994).

The irregular shape and low volume of the nucleus in the initial days of germination (Figure 4c-d), as also observed by Mansfield and Briarty (1996), is an indicative of the

seed´s low metabolism, and as imbibitions occur, cellular metabolism is retaken (Castro et al., 2004), and much of the early stages of germination are focused on reorganization and development of preexisting organelles and formation of new ones (Mansfield and Briarty, 1996). Metabolism retake can be proven, as well as germination itself and the nucleus reshaping, by the cotyledon's cells deep staining by toluidine blue, which stains acid compounds, and thus can indicate the presence of mRNAs. The synthesis of new mRNAs occurs as germination proceeds, coding essential proteins for normal cellular metabolism and maintenance of normal seedling development (Bewley, 1997).

The positive staining of the cytoplasmic granules by bromophenol blue suggests a protein nature, probably acid as some regions of the granule are also stained by toluidine blue. The disappearance of the granules followed by strong cytoplasm staining suggests that there is degradation of these granules in small portions. The same was observed in seeds of *Prosopis juliflora*, where the globular protein bodies were hydrolyzed and more soluble (Gallão et al., 2007). Electrophoretic analysis of protein extractions (unpublished data) showed that as germination takes place, there is a reduction in high molecular weight bands whereas low molecular weight bands become more evident. A similar event was observed by Corte et al. (2008), during protein mobilization in *C. peltophoroides* seeds. However, the authors reported that there was an increase in the size of the protein body preceding its content degradation, which remains confined to a small space, whilst the same was not observed in our results. Nevertheless, in t8 and t10 low cytoplasm vacuolization was observed due to reserve use, as observed by Mansfield and Briarty (1996). We are inclined to believe that the low vacuolization observed in our results indicate that protein mobilization is low in the first ten days of germination, occurring after the referred period.

In short, we conclude that *P. edulis* seeds consume mainly lipids as their reserve material for germination. Its product may be accumulated as starch during the germination process and hydrolysis of protein bodies is also observed in the cotyledons during the first six days of germination.

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