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# Ultrastructural and biochemical changes induced by salt stress in *Jatropha curcas* seeds during germination and seedling development

*Nara L. M. Alencar*[A,B], *Cibelle G. Gadelha*[A], *Maria I. Gallão*[C], *Mary A. H. Dolder*[D], *José T. Prisco*[A,E] *and Enéas Gomes-Filho*[A,E,F]

[A]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza 60 440 970, Brazil.
[B]Instituto Federal de Educação Ciência e Tecnologia do Ceará, Campus Crateús, Crateús 6 370 000, Brazil.
[C]Departamento de Biologia, Universidade Federal do Ceará, Fortaleza 60 440 554, Brazil.
[D]Departamento de Biologia Estrutural e Funcional, Instituto de Biologia, Universidade Estadual de Campinas, Campinas 13 083 862, Brazil.
[E]Instituto Nacional de Ciência e Tecnologia em Salinidade (INCTSal)/CNPq, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza 60 440 970 PO Box 6039, Brazil.
[F]Corresponding author. Email: egomesf@ufc.br

**Abstract.** *Jatropha curcas* L. is a multipurpose species of the Euphorbiaceae family that is widespread in arid and semiarid regions. This study investigated the ultrastructural and biochemical changes induced by salt stress during *J. curcas* seed germination and seedling development. Salt stress negatively affected seed germination and increased $Na^+$ and $Cl^-$ contents in endosperms and embryo-axis. Lipids represented the most abundant reserves (64% of the quiescent seed dry mass), and their levels were strongly decreased at 8 days after imbibition (DAI) under salinity stress. Proteins were the second most important reserve (21.3%), and their levels were also reduced under salt stress conditions. Starch showed a transient increase at 5 DAI under control conditions, which was correlated with intense lipid mobilisation during this period. Non-reducing sugars and free amino acids were increased in control seeds compared with quiescent seeds, whereas under the salt-stress conditions, minimal changes were observed. In addition, cytochemical and ultrastructural analyses confirmed greater alterations in the cellular reserves of seeds that had been germinated under NaCl stress conditions. Salt stress promoted delays in protein and lipid mobilisation and induced ultrastructural changes in salt-stressed endosperm cells, consistent with delayed protein and oil body degradation.

**Additional keywords:** lipid, protein, salinity stress, starch, oilseed, ultrastructure.

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## Introduction

Germination is a key process that allows a seed embryo to grow and evolve into a photosynthetic organism. This process begins with water absorption by the quiescent seed, and ends with the onset of embryo axis elongation, which can be visualised through radicle emergence (Sánchez-Linares *et al*. 2012; Bewley *et al*. 2013). Following germination, seed reserves are mobilised: reserve mobilisation being one of the most thoroughly studied processes related to seedling development (Sánchez-Linares *et al*. 2012; Bewley *et al*. 2013). Lipids, proteins and starch are the main reserves that are mobilised from storage tissues, and these are used as energy sources and building blocks for seedling growth after germination (Alencar *et al*. 2012; Bewley *et al*. 2013).

Salinity is one of the most detrimental abiotic stresses that affects crops due to its deleterious effects on plant growth and development (Munns and Tester 2008). Crop productivity can be affected by alterations in salinity in soil, which leads to changes in metabolism as well as physiological and anatomical functions (Munns and Tester 2008). Germination is generally considered to be the most salt-sensitive developmental stage, particularly among crops that are exposed to hostile environments (Díaz-López *et al*. 2012). Salt stress generally inhibits or delays seed germination via water deficits and ion toxicity (Ashraf *et al*. 2003). The main physiological consequence of salt stress is diminished seed imbibition due to the low solute potential of saline growth media. Moreover, the accumulation of $Na^+$ and $Cl^-$ ions can be toxic to several biochemical and physiological processes and can reduce reserve mobilisation, hinder seedling emergence and increase mortality rates (Ashraf *et al*. 2003; Marques *et al*. 2013).

*Jatropha curcas* L. is a multipurpose species of the Euphorbiaceae family that is widespread in arid and semiarid areas of tropical and subtropical regions (Heller 1996; Silva

*et al*. 2013). This species is gaining much attention as a promising energy crop because it is a non-food crop and has several features that render it suitable for biodiesel production, including rapid growth, facile propagation, a short gestation period, low seed cost (Heller 1996), high oil content (~40–60%), and adaptability to various agro-climatic conditions such as adverse soil conditions, drought and marginal lands (Gao *et al*. 2008; Yang *et al*. 2009). Moreover, *J. curcas* seed oil can be used as an energy source, and other parts of the plant can be used as fertiliser and animal feed, as *J. curcas* seed cakes and kernels are rich in high quality proteins (Gao *et al*. 2008). This plant is also considered to be a rich source of phytochemicals, indicating its therapeutic and industrial potential (Gao *et al*. 2008).

Recent studies have described ultrastructural, cytochemical and biochemical analyses of *J. curcas* seeds under normal conditions (Yang *et al*. 2009; Reale *et al*. 2012). Most previous studies have focussed on analysing the cytology, reserve mobilisation and proteomics of this plant during and after seed germination. Some studies have reported the effects of salinity alterations on anatomical changes in the leaves and roots of *J. curcas* (Melo *et al*. 2011), osmolyte production, oxidative protection and photosynthesis (Silva *et al*. 2012). Some ecophysiological aspects of the tolerance of *J. curcas* to NaCl have also been explored (Díaz-López *et al*. 2012). Although interest in the responses of *J. curcas* to salt stress is growing, studies examining the effects of NaCl on germination and seed reserves are scarce.

We hypothesised that salt stress impairs seed germination as consequence of the increased $Na^+$ and $Cl^-$ accumulation in the embryo axes and endosperms of salt-stressed seeds, resulting in the delayed mobilisation of reserves and consequent delayed seedling development. Moreover, to explore additional negative effects on seedling development, we also examined whether NaCl stress damages lipids and protein bodies in the cell ultrastructure. Therefore, we focussed mostly on seed protein and lipid reserve mobilisation and ultrastructural changes in cells under salt stress, correlating these effects with protein and lipid degradation, which could be deleterious to initial plant development.

## Materials and methods

### Plant material and seed germination

*Jatropha curcas* L. seeds were provided by the Lagoa Seca farm (Campina Grande-PB, which is located in north-eastern Brazil) and were maintained at 4°C until use. The seeds were first disinfected with 2% Orthocid 500 fungicide (Arysta LyfeScience, Jundiaí, SP, Brazil) for 10 min and then rinsed with distilled water. Preliminary experiments were performed to test different NaCl concentrations (50, 100 and 150 mM) on *J. curcas* germination as described below. The 100 mM NaCl concentration was selected for all subsequent experiments because the 50 mM NaCl concentration was very similar to the control conditions, whereas the percentage of seed germination at the 150 mM concentration was very low, which was not appropriate for the experiment. The germination synchrony was estimated using GerminaQuant 1.0 software (Recife, PE, Brazil; Pompelli and Marques 2014). The *J. curcas* seeds were germinated using three germination towels (germination paper, JProlab, São José dos Pinhais, PR, Brazil) under one of the following two conditions: they were moistened with 50 mL of distilled water (control) or 100 mM NaCl solution for 8 days. The seeds were distributed on top of the papers, which were placed in rolls in polyethylene pots that were covered with transparent plastic. The seeds used in the germination tests were maintained in a growth chamber at a constant temperature (30°C) with a constant photoperiod (12/12 h) according to work by Andréo-Souza *et al*. (2010).

### Germination parameters and inorganic ion determination

Seed germination was scored daily for 8 days, and the following parameters were evaluated: germination speed index (GSI) according to work by Maguire (1962), percentage of seed germination (%G) and average germination time (AGT) following work by Laboriau (1983). The seeds were collected daily and divided into embryo axes and endosperms, and the dry mass of these organs was determined by freeze drying. For the determination of the ion contents in the embryo axes and endosperms, 100 mg of dry mass was extracted in 10 mL of distilled water for 1 h in a water bath at 45°C. The extracts were then centrifuged at 3000*g* for 15 min, and the supernatant was filtered in through filter paper and stored at −20°C until use. The $Na^+$ content was estimated by flame photometry, and the $Cl^-$ content was determined using the $Hg(SCN)_2$-$Fe(NO_3)_3$ method with NaCl as a standard as described by Gaines *et al*. (1984).

### Seed reserves and hydrolysis products

The seeds were collected at 0 (quiescent seed), 1, 2, 3, 4, 5, 6, 7 and 8 days after imbibition (DAI) under the control and saline conditions. Five seeds or seedlings were selected according to their uniformities. Subsequently, their seed coats were removed, and the endosperms were separated. The endosperm tissues were then freeze dried for dry mass measurements and stored at −20°C until use. These materials were used for the estimations of total lipids, total proteins, soluble sugars (reduced and non-reduced), free amino acids and starch.

For the total lipid determination, 200 mg of endosperm dry mass was ground and homogenised with 5 mL of hexane on an orbital shaker (45 rpm) for 30 min at 25°C in test tubes that had been previously weighed. Then, the homogenate was centrifuged at 10 000*g* for 10 min at 25°C, and the supernatant was transferred to another tube, which had also been previously weighed. Thereafter, the remaining pellet was re-extracted with 5 mL of hexane and submitted to all of the aforementioned extraction procedures. At the end of the procedure, the two supernatants were combined, and the organic phase was evaporated using liquid nitrogen. After the extraction step, the total lipid content was determined by gravimetry, and the remaining pellet was used for the other reserve compound determinations.

Total protein was estimated using the colorimetric method that was proposed by Baethgen and Alley (1989). To accomplish this, 50 mg of defatted endosperm powder was digested using a standard Kjeldahl procedure, and total nitrogen levels were measured. The protein content was calculated by multiplying the Kjeldahl nitrogen content by 6.25.

For the soluble sugar and free amino acid determinations, 50 mg of defatted endosperm powder was homogenised in 5 mL of 80% ethanol (v/v), and the homogenate was warmed at 75°C for 30 min. During this procedure, the tubes were vortexed at constant intervals of 10 min, after which they were centrifuged at 3000*g* for 10 min. Next, the supernatant was collected, the pellet was re-extracted twice for 30 min each, and the supernatants from both extractions were combined. The amount of total soluble sugars was then determined according to work by DuBois *et al*. (1956) using glucose as a standard. Free amino acids (FAAs) were determined using the Yemm and Cocking (1955) method with glycine as a standard. Reducing sugars were estimated according to Miller's (1959) method, whereas non-reducing sugars were estimated based on the difference between the total soluble and reducing sugars. Starch was extracted from the ethanol-insoluble pellet with 30% (v/v) perchloric acid, and its levels were determined using glucose as a standard according to Hodge and Hofreiter (1962).

### Light microscopy analyses

For the cytochemical analyses, 10 *J. curcas* seeds were sown in triplicate for each treatment (distilled water for the control and 100 mM NaCl for the salt treatment). Then, five endosperms were collected from the seeds at 0, 4 and 8 DAI. Each endosperm was divided into four equal parts and fixed with a solution of 40 g $L^{-1}$ paraformaldehyde in 0.1 mol $L^{-1}$ phosphate buffer (pH 7.2) with 10 g $L^{-1}$ glutaraldehyde for 48 h at 25°C according to work by Karnovsky (1965). The material was then dehydrated in a graded ethanol series and embedded using a Historesin Embedding Kit (Jung, Heidelberg, Germany). The tissue blocks were sectioned into 5 µm slices with a Leica RM 2065 microtome (Heidelberg, Germany). Subsequently, the endosperm sections were incubated with the protein stain Xylidine Ponceau (XP), as described by Vidal (1970). Lipid detection was performed by staining freehand sections with Sudan IV to detect lipid bodies. Cross-sections were analysed and images were obtained using a light microscope (Olympus Optical BX41, São Paulo, SP, Brazil) coupled with a digital camera (UC30), using an appropriate software for image processing.

### Ultrastructural analyses

For the ultrastructural analyses by transmission electron microscopy (TEM), the seeds were germinated in distilled water (control) or 100 mM NaCl (salt treatment) using the same conditions as previously mentioned. The seeds were collected at 0, 4 and 8 DAI for both the control and saline conditions, separated into endosperms and embryos, and transversely sectioned. The endosperm sections were fixed in a solution containing 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde with 5 mM $CaCl_2$ and 0.1 M sucrose prepared in 0.05 M sodium cacodylate buffer (pH 6.8) for 48 h at 25°C. After rinsing three times for 2 h each in 0.05 M sodium cacodylate buffer (pH 6.8) containing 0.1 M sucrose, the material was postfixed in 1% (w/v) osmium tetroxide in the same buffer for 12 h. Next, the samples were rinsed again in the same buffer without osmium and maintained in this solution until use. Dehydration was carried out in increasing concentrations of ethanol (50–100%) for 15 min each, followed by ethanol-acetone and pure acetone for 5 min each. Finally, Epon embedding was performed. Semithin sections (1–2 µm thickness) were cut with an LKB ultramicrotome that was equipped with a glass knife. The sections were then stained with toluidine blue and observed using a light microscope. Ultrathin sections (~90 nm) were cut with a diamond knife and stained in 2% uranyl acetate according to Watson (1958) in alcohol in the dark for 15 min. Subsequently, the material was washed with distilled water and then exposed to 0.2% lead citrate (10 min) in close proximity to NaOH pellets. The sections were observed using a transmission electron microscope (Zeiss, Göschwitz, Jena, Germany, Leo 906) at 80 kV.

### Experimental design and statistics

The experimental design was completely randomised in a $[(2 \times 8) + 1]$ factorial arrangement that was composed of the following two treatments: media with two different concentrations of NaCl (0 and 100 mM NaCl) and eight periods of seed imbibition (1, 2, 3, 4, 5, 6, 7 and 8 DAI) with an additional treatment at 0 DAI for the quiescent seeds. The treatments included five replicates of 20 seeds each, totalling 100 seeds for each evaluated period and treatment. For the dry mass and ion determinations, all of the seeds were used, whereas for the seed reserve mobilisation analyses, five uniform seeds or seedlings were selected from each treatment. A two-way analysis of variance (ANOVA) was performed to evaluate the differences among the factors using Snedecor's *F* test. Significant differences ($P \leq 0.05$ and $P \leq 0.01$) between means were determined for each variable by the Scott-Knott test.

## Results

### Germination parameters and seedling dry mass

The analysed germination parameters were significantly affected by salinity treatment ($P \leq 0.01$). In the preliminary experiment, the percentage of seed germination and germination speed index (GSI) were strongly reduced by impose 150 mM NaCl-stress, with 90% decrease in relation to non-stressed treatment; however, the germination of seeds was not altered by 50 mM NaCl stress (Table 1). Hence, the 100 mM NaCl-stress was selected because the germination was reduced by ~40.0% – a condition less severe and thus appropriate to the biochemical and microscopy analyses (Table 1). In the definitive experiment, by using 100 mM NaCl stress, the percentage of seed germination (%G) was also

**Table 1. Percentage of seed germination (%G), germination speed index (GSI), average germination time (AGT) and germination synchrony (Z) of *Jatropha curcas* seeds exposed to NaCl-stress during 8 days**

Means that are followed by different letters are significantly different at $P \leq 0.01$ according to Scott-Knot's test for treatments. Note: nd, not detected

| | Germination parameters | | | |
|---|---|---|---|---|
| NaCl (mM) | %G | GSI | AGT (days) | Z |
| 0 | 93.0 ± 2.2A | 12.5 ± 0.3A | 8.04A | 0.86 ± 0.06A |
| 50 | 88.0 ± 2.2A | 12.5 ± 0.3A | 8.12A | 0.68 ± 0.06A |
| 100 | 59.0 ± 4.0B | 6.9 ± 0.8B | 8.70A | 0.33 ± 0.02B |
| 150 | 11.0 ± 2.2C | 1.1 ± 0.3C | 8.70A | nd |

impaired by 39.0% (Table 2); whereas the GSI values were decreased by 48.0% relative to control. In contrast, the average germination time did not show a large change under the salt-stress conditions (9 DAI) compared with the control (8 DAI) (Table 2). Moreover, the synchronism value (Z) also revealed that the salt treatment promoted a slower and more unsynchronised germination (Table 2).

Additionally, the embryo axis dry mass significantly increased ($P \leq 0.05$) from 3 DAI, reaching a maximum at 8 DAI in the control, which corresponded to a 157.0% increase relative to the salt treatment (Fig. 1*a*). In comparison, the endosperm dry mass showed minimal changes until 2 DAI, and the most significant reduction was observed in the control at 8 DAI (22.7% relative to the salt-stress conditions). However, this parameter essentially remained unchanged under the salt-stress conditions (Fig. 1*b*).

### Ion contents

The $Na^+$ content significantly increased ($P \leq 0.05$) in both the embryo axis (Fig. 2*a*) and endosperm (Fig. 2*b*) under the salinity treatment. In the embryo axis, the $Na^+$ content was increased under the salt treatment compared with the control treatment for all of the evaluated periods, except for the first day of analysis. Under salt stress, the $Na^+$ content was 2-fold greater than the control at 4 DAI and 3-fold greater than the control at 7 and 8 DAI. In the endosperm, the $Na^+$ content also significantly increased by up to 6-fold following NaCl treatment compared with the $Na^+$ content in quiescent seeds at 8 DAI. However, in the control, slight alterations in $Na^+$ content were detected, and the most pronounced increase was observed at 8 DAI (Fig. 2*b*).

The $Cl^-$ contents in the embryo axis fluctuated during all of the evaluated periods under both the control and salinity treatments (Fig. 3*a*). In addition, significant elevations in $Cl^-$ ($P \leq 0.05$) were observed at 8 DAI in the salt-stress conditions compared with the control. In the endosperm, $Cl^-$ contents increased during the evaluated period, but there were no significant differences between the treatments until 6 DAI (Fig. 3*b*). At 7 and 8 DAI, the $Cl^-$ contents were 87.0 and 39.0% higher, respectively, in the salt treatment compared with the control (Fig. 3*b*).

### Seed reserves and hydrolysis products

The endosperm lipids represented the most abundant reserve and corresponded to 64.0% of the dry mass of the quiescent seed endosperm (Fig. 4*a*). Proteins were the second most important reserve in the *J. curcas* seeds, amounting to 21.3% of the endosperm dry mass (Fig. 4*b*). Starch was less abundant, corresponding to only 5.5% of the quiescent seed endosperm (Fig. 4*c*).

Regarding lipid content, significant differences between the treatments were not observed until 4 DAI ($P > 0.05$) (Fig. 4*a*). However, after 5 DAI (i.e. during the seedling development stage), significant differences were detected between the treatments, with higher lipid contents detected following the salt-stress treatment. At the last evaluation period (8 DAI), the lipid contents were 69.0% lower in the control compared with the quiescent seed, whereas the lipid contents of the salt-treated seeds were reduced by only 25.5% in relation to the quiescent seed (Fig. 4*a*).

The protein content did not significantly change ($P > 0.05$) until 4 DAI (Fig. 4*b*), corresponding to the radicle protrusion period. Subsequently, this reserve was strongly reduced in the control during seedling development, whereas salt stress did not significantly alter the protein content during the entire evaluation period (Fig. 4*b*). The protein content was also observed to decrease by 43.2 and 39.5% at 8 DAI in the non-stressed and salt-stress conditions, respectively, compared with the quiescent seed (Fig. 4*b*).

**Table 2. Percentage of seed germination (%G), germination speed index (GSI), average germination time (AGT) and germination synchrony (Z) of *Jatropha curcas* seeds exposed to NaCl treatment during 8 days**

Means that are followed by different letters are significantly different at $P \leq 0.01$ according to the Scott-Knot test for treatments. Note: nd, not detected

| | Germination parameters | | | |
|---|---|---|---|---|
| NaCl (mM) | %G | GSI | AGT (days) | Z |
| 0 | 95.0 ± 2.2A | 13.2 ± 0.3A | 8.0A | 0.80 ± 0.022A |
| 100 | 58.0 ± 3.4B | 6.9 ± 0.7B | 8.6A | 0.34 ± 0.026B |

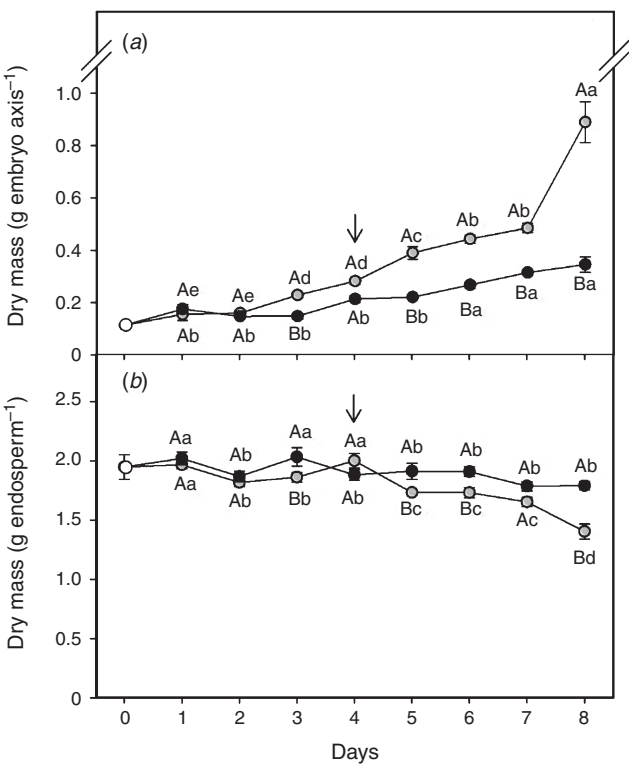


**Fig. 1.** Seed dry mass of the embryo axis (*a*) and endosperm (*b*) of *Jatropha curcas* seeds that were grown under control conditions (grey circles) or in the presence of 100 mM NaCl (black circles). Each value represents the mean of five replicates ($n = 5$) ± s.e. Means that are followed by different uppercase letters (for treatments) or by different lowercase letters (for days) are significantly different at $P \leq 0.05$ according to a Scott-Knot test. Quiescent seed values are used as a reference (white circles). An arrow indicates radicle protrusion.

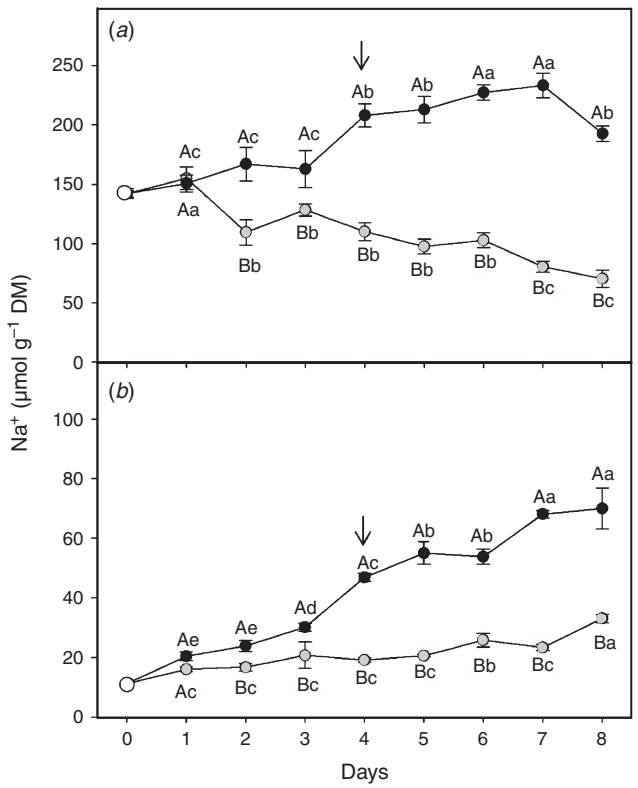


**Fig. 2.** $Na^+$ contents in the embryo axis (*a*) and endosperm (*b*) of *Jatropha curcas* seeds grown under control conditions (grey circles) or in the presence of 100 mM NaCl (black circles). Means that are followed by different upper case letters (for treatments) or by different lower case letters (for days) are significantly different at $P \leq 0.05$ according to a Scott-Knot test. Quiescent seed values are used as a reference (white circles). An arrow indicates radicle protrusion. Abbreviation: DM, dry mass.

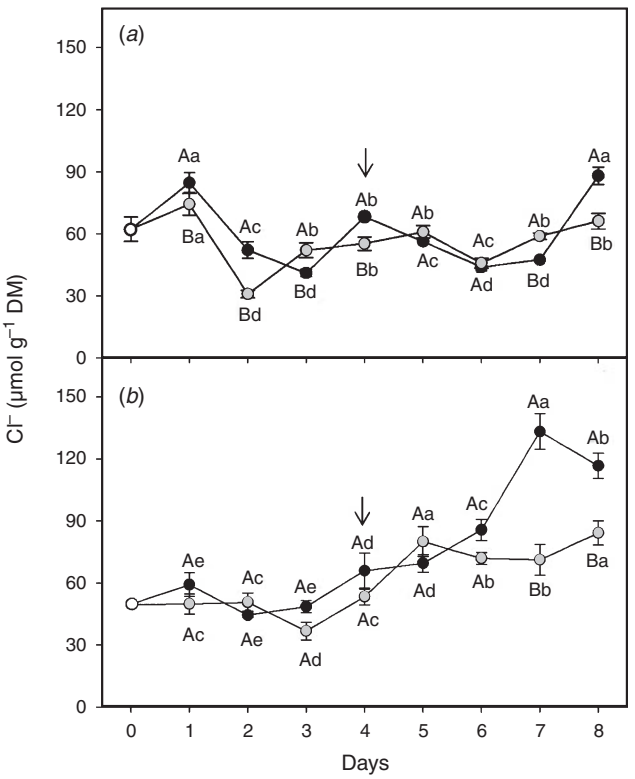


**Fig. 3.** $Cl^-$ contents in the embryo axis (*a*) and endosperm (*b*) of *Jatropha curcas* seeds grown under control conditions (grey circles) or in the presence of 100 mM NaCl (black circles). Means that are followed by different upper case letters (for treatments) or by different lower case letters (for days) are significantly different at $P \leq 0.05$ according to a Scott-Knot test. Quiescent seed values are used as a reference (white circles). An arrow indicates radicle protrusion. Abbreviation: DM, dry mass.

Under control conditions, starch transiently accumulated until 5 DAI, which signified the end of germination. Thereafter, from 5 to 7 DAI, it steadily decreased until the end of the evaluated period (Fig. 4*c*). In salt conditions, there was also a small, but significant change for starch contents during germination for salt stressed seeds (Fig. 4*c*). At 8 DAI, although the starch content, in non-stressed seedlings, was reduced by 51.0% in relation to quiescent seed, it did not significantly vary as compared with stressed seedlings (Fig. 4*c*).

Soluble sugars remained unchanged until 4 DAI, which corresponded with the radicle protrusion period, and no significant differences were detected between the control and salt treatments (Fig. 5*a*). After 4 DAI, the soluble sugar contents increased until the final evaluation period, with the highest values occurring in the control conditions compared with the salt-stress conditions (Fig. 5*a*). Non-reducing sugars were more abundant than reducing sugars under the non-stressed conditions, but significant increases ($P \leq 0.05$) in both sugars were verified in the control treatment (Fig. 5*b*, *c*). These compounds were significantly elevated beginning at 5 DAI, and at 8 DAI, the non-reducing sugars reached maximum values that were approximately 2-fold greater than those observed following the salt treatment. However, under the salt-stress conditions, the reducing sugars did not substantially change during the evaluation period (Fig. 5*b*).

The free amino acid content changed significantly ($P \leq 0.05$) in the control and salt treatments throughout the germination period (Fig. 5*d*), showing significant elevations under both treatments. However, the free amino acid levels were significantly higher ($P \leq 0.05$) under the control conditions compared with the salt-stress conditions during the entire evaluation period. Moreover, at 8 DAI, the free amino acid levels were two times greater under the control treatment compared with the salt treatment (Fig. 5*d*).

### *Cytochemical and ultrastructural analyses*

Cytochemical analyses revealed that quiescent *J. curcas* seeds have a prominent endosperm containing an abundance of protein bodies that are distributed throughout the cytoplasm; this was confirmed by intense positive XP staining (Fig. 6*a*).

The protein bodies were modified during germination, which was more pronounced at 8 DAI in control conditions; as evidenced by reduced size and their concentration in some regions of the cytoplasm (Fig. 6*d*). Moreover, at this time, several empty cells were observed in the endosperms, indicating the

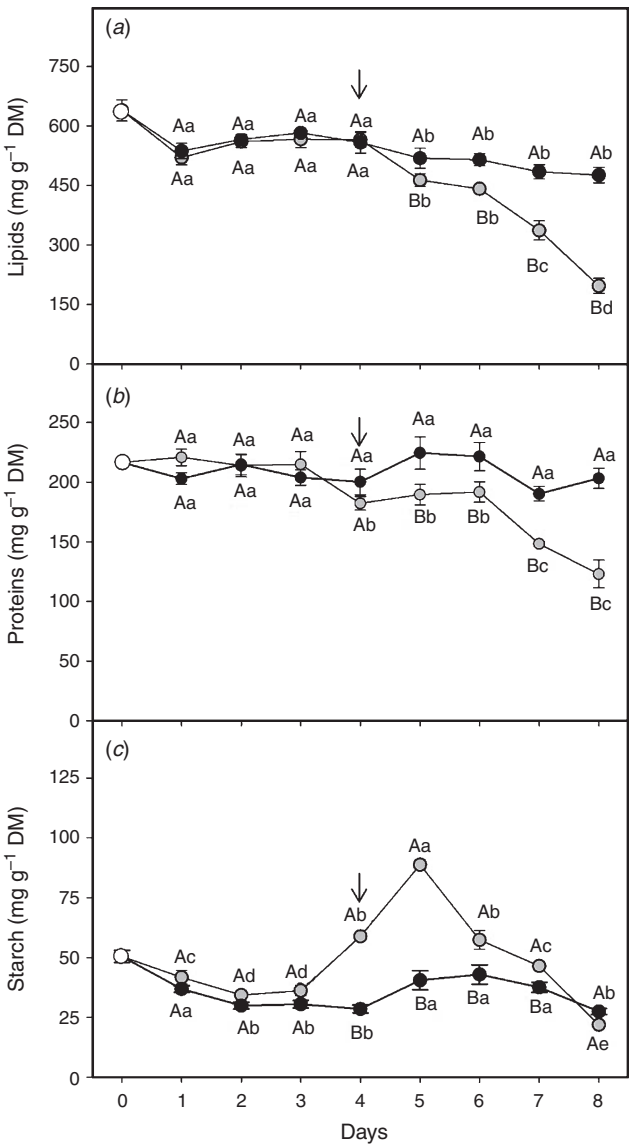


**Fig. 4.** Lipid (*a*), protein (*b*) and starch (*c*) contents in *Jatropha curcas* endosperms grown under control conditions (grey circles) or in the presence of 100 mM NaCl (black circles). Means that are followed by different upper case letters (for treatments) or by different lower case letters (for days) are significantly different at $P \leq 0.05$ according to a Scott-Knot test. Quiescent seed values are used as a reference (white circles). An arrow indicates radicle protrusion. Abbreviation: DM, dry mass.

greatest reduction and mobilisation of protein bodies (Fig. 6*d*). Under the salt-stress conditions, a delay in protein mobilisation was observed at 8 DAI, resulting in a great quantity of protein bodies in some endospermic cells (Fig. 6*g*).

*J. curcas* endosperm cells were also strongly stained with Sudan IV, revealing a large amount of lipid bodies irregularly distributed in the cytoplasm of the quiescent seeds (Fig. 6*b*). The greatest reduction in cytoplasmic lipid bodies was mainly observed at 8 DAI in the control, and in some cells, the lipid bodies disappeared completely (Fig. 6*e*). In contrast, at the same period, the salt-stressed cells were filled with lipid bodies, indicating a harmful effect of salt stress in the mobilisation of this reserve (Fig. 6*h*).

Ultrastructural TEM analyses revealed the presence of some protein bodies of different sizes in the endosperm cells of quiescent *J. curcas* seeds, suggesting that these structures were abundant in quiescent seeds (Fig. 6*c*). Although the lipid bodies were smaller than protein bodies, they were the most abundant reserve in the endosperm quiescent seeds, occupying the endosperm cells almost completely (Fig. 6*c*). In this section, it was also visualised the cell wall (Fig. 6*c*).

Protein and lipid body degradation was evident at 8 DAI under the control conditions in the seed endosperm sections (Fig. 6*f*); probably, nearly all of these structures had been degraded. Moreover, reserve material was also absent from the cytoplasm and it were observed vacuolated protein bodies (Fig. 6*f*). In contrast, salt treatment promoted the rupture of some of the cells and caused cytoplasmic disorganisation and cell shape alterations (Fig. 6*i*). Also, protein and lipid bodies were not visualised in this section; however, residual protein material was observed in the cytoplasm in addition to starch grains (Fig. 6*i*).

## Discussion

### *Germination parameters and seedling dry mass*

The negative effects of salt stress were reflected in the delayed germination period, which was confirmed by significant reductions in %G and GSI (Tables 1, 2). These results were corroborated by the finding that the embryo axis dry mass was significantly reduced following the NaCl treatment compared with the control. This reduction mainly occurred beginning at 4 DAI and could have delayed normal seedling development (Fig. 1*a*). However, although the endosperm dry mass was not greatly affected by the salt-stress treatment, the changes were significant following radicle protrusion, suggesting that reserve depletion was inhibited due to the effects of toxic ions. In contrast, under the control conditions, reserve mobilisation occurred normally (Fig. 1*b*).

The effects of salinity on *J. curcas* seed germination rates have been reported in other studies (Andréo-Souza *et al*. 2010), which have also confirmed that salt stress promotes delayed germination and that seedling growth and development are reduced when seeds are treated with a 6 dS m$^{-1}$ electrical conductivity NaCl solution. Negative effects on seed germination and seedling development under salt-stress conditions have been investigated in other oilseed plants, such as sunflowers (Liu *et al*. 2010) and cashew nuts (Marques *et al*. 2013). In such studies, delayed germination was reported, although the complete inhibition of germination did not occur (Marques *et al*. 2013). This finding may be a consequence of the reduced water potential of the substrate due to the increased salt levels in the medium, which contributed to the reduced water absorption.

### *Ion contents*

The increased $Na^+$ and $Cl^-$ contents in the embryo axes and endosperms of the salt-stressed *J. curcas* seeds and seedlings were the main consequences of the salt-stress treatment (Figs 2, 3). However, $Na^+$ accumulation was higher than $Cl^-$

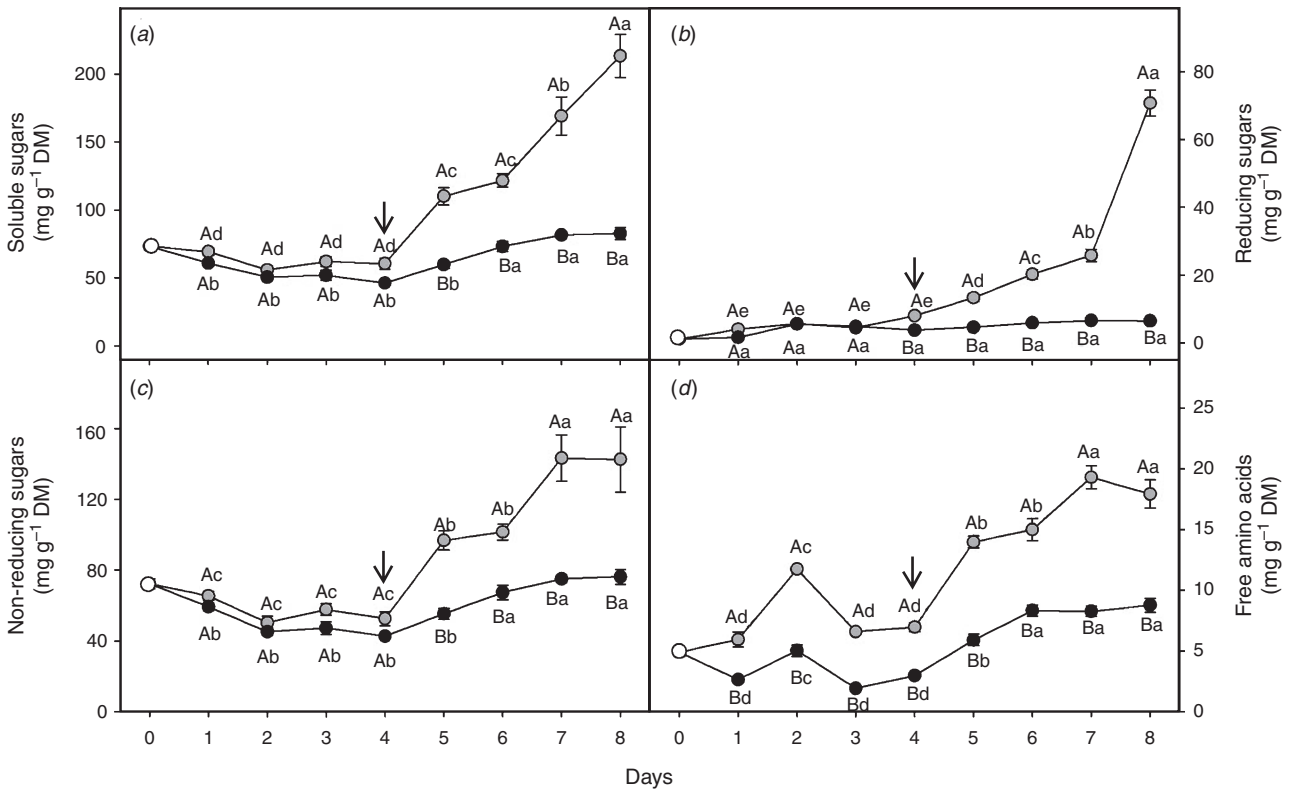


**Fig. 5.** The contents of soluble (*a*), reducing (*b*) and non-reducing (*c*) sugars and free amino acids (*d*) in *Jatropha curcas* endosperms grown under control conditions (grey circles) or in the presence of 100 mM NaCl (black circles). Means that are followed by different upper case letters (for treatments) or by different lower case letters (for days) are significantly different at $P \leq 0.05$ according to a Scott-Knot test. Additional details are provided in the legend of Fig. 1. Quiescent seed values are used as a reference (white circles). An arrow indicates radicle protrusion. Abbreviation: DM, dry mass.

accumulation in both of the analysed tissues. Thus, the high $Na^+$ levels were the main factor underlying the negative effects on seedling development and endosperm depletion (Fig. 1). Although $Cl^-$ contents were not significantly altered in embryo axis, its increment in endosperm after 6 DAI could have contributed to the inhibition of seedling development. Otherwise, $Na^+$ ion increased significantly over time starting four day after imbibition, indicating its probable deleterious effects in both germination and seedling development. Similarly, Marques *et al*. (2013) reported that $Na^+$ and $Cl^-$ contents progressively increased in the cotyledons and embryo axes of cashew nut seedlings as NaCl levels increased in the medium. Additionally, Díaz-López *et al*. (2012) subjected *J. curcas* plants for 40 days to different NaCl concentrations, and they described that the salinity negative influence was correlated with $Cl^-$ and/or $Na^+$ toxicity and a nutritional imbalance caused by increase $Na^+ : Cl^-$ ratio and the osmotic effect was discharged. These authors also reported that $Cl^-$ concentrations were higher than $Na^+$ concentrations in the leaves and roots. In addition, other studies have found similar results. For example, Ashraf *et al*. (2003) reported the negative effects of $Na^+$ and $Cl^-$ on sunflower seedlings, in which the $Na^+$ and $Cl^-$ contents in the hypocotyls increased over time under all of the tested salt treatments, whereas in the cotyledons, the salt treatments had no significant effect on $Na^+$ or $K^+$ levels. Liu *et al*. (2010) also studied sunflower seed germination and seedling development, verifying that 'the $Na^+$ and $Cl^-$ contents increased along with salinity levels in the leaves and roots and the $K^+$ contents increased along with salinity levels in the leaves and stems.

### *Seed reserves and hydrolysis products*

Lipids have been reported to be the main reserve compound of oilseeds such as *Helianthus annuus*, *Brassica napus* and *Ricinus communis*, which belong to the Asteraceae, Brassicaceae and Euphorbiaceae families, respectively (Bewley *et al*. 2013), and *Cereus jamacaru* (Alencar *et al*. 2012), which belongs to the Cactaceae family. *J. curcas* belongs to the Euphorbiaceae family, in which lipid storage occurs in endosperm tissues (Heller 1996). In this study, lipids were the main reserves that were detected in the endosperm (accounting for ~60% of its dry mass); this result was also supported by their high abundance as detected by cytochemical and ultrastructural analyses (Fig. 6*b*, *c*). Our results corroborate with Reale *et al*. (2012) and Yang *et al*. (2009), who also described, through ultrastructural analyses, the abundance of lipids in *J. curcas* endosperm cells under non-stress conditions.

Some recent studies have reported the effects of salt stress on the source-sink regulation of cotyledonary reserve mobilisation during cashew (*Anacardium occidentale*) germination and seedling establishment (Marques *et al*. 2013) and during

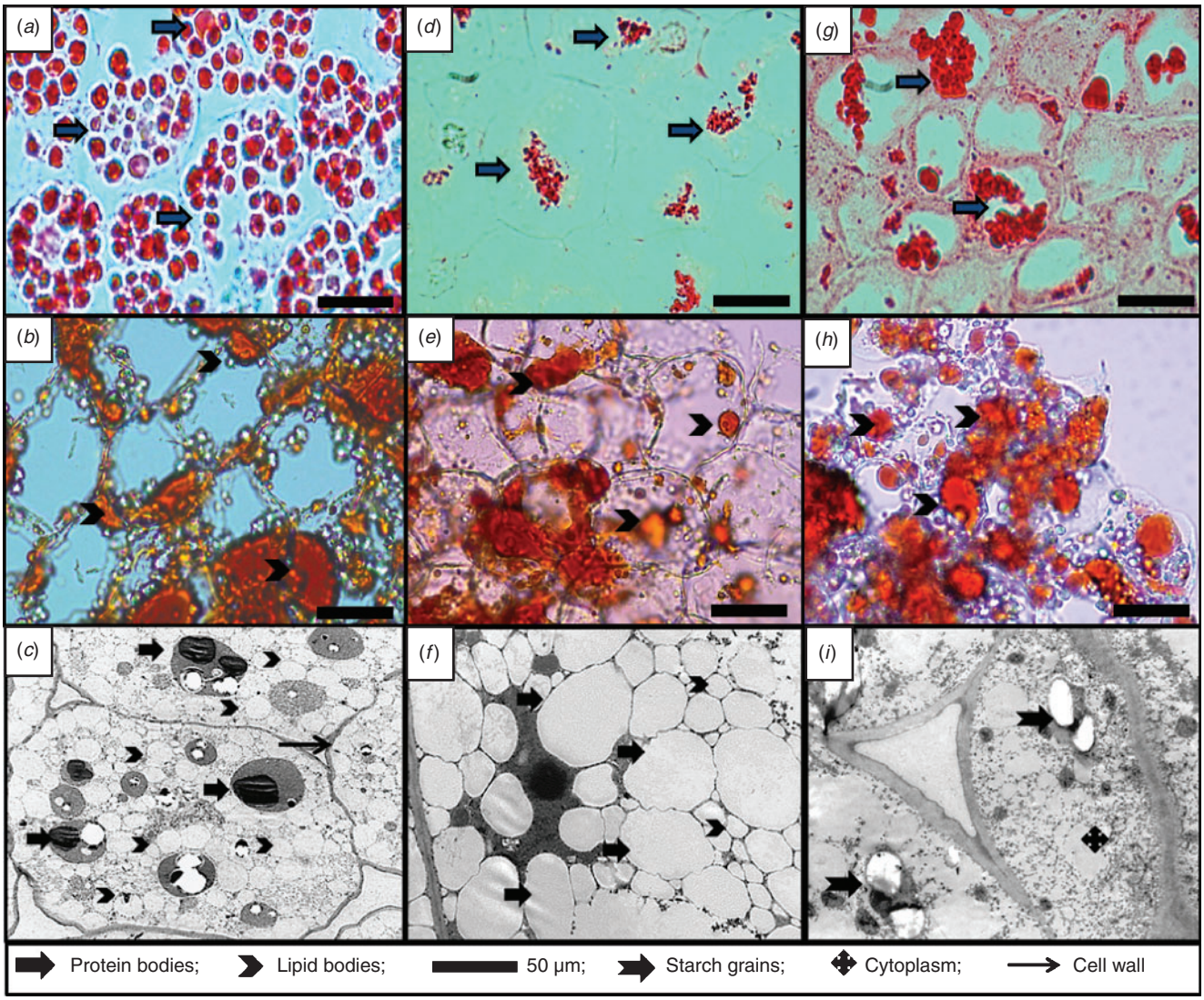


**Fig. 6.** Micrographs of endosperms of *Jatropha curcas* seeds: quiescent (*a*–*c*): at 8 DAI in control (*d*–*f*) and salt stress (*g*–*i*) in salt stress. Sections stained with Xylidine Ponceau (XP, *a*, *d*, *g*) revealing protein bodies and Sudan IV (*b*, *e*, *h*) revealing lipid bodies, ultra-micrographs with magnifications ×4646 (*c*, *f*) and ×10 000 (*i*).

sunflower seed germination (Liu *et al*. 2010). We note that the current literature contains little to no information regarding seed reserve mobilisation in *J. curcas* or its response to salt-stress conditions; therefore, our findings will contribute greatly to the current knowledge.

This study also showed that total lipid reserve mobilisation was negatively affected by salt stress, which was mainly verified at the seedling stage. Lipids were strongly mobilised in the control after radicle protrusion; in fact, ~69.0% of all lipids had already been mobilised at the beginning of the seedling stage (8 DAI) (Fig. 4*a*), indicating the importance of these reserves as carbon and energy sources for *J. curcas* seed germination. Under salt-stress conditions, the greatest reductions in lipid mobilisation (beginning at 4 DAI) could be attributed to the deleterious effects of $Na^+$ and $Cl^-$ accumulation in the endosperm cells and $Na^+$ accumulation in the embryo axis. $Na^+$ levels were shown to increase beginning at the germination phase and peak during seedling development, resulting in growth reduction.

Lipid mobilisation in the *J. curcas* endosperm under non-stressed conditions has been reported by Yang *et al*. (2009). They described a 33.7% decrease in oil concentrations at 96 h of imbibition, and at 144 h almost all of the oil was consumed. In addition, Marques *et al*. (2013) described faster lipid mobilisation rates in cashew nut seeds and seedlings under control conditions, while under salt treatment, the rates were strongly reduced. The same authors correlated the reduced rates of lipid mobilisation with decreased lipase activities under salt-stress conditions. In contrast, Voigt *et al*. (2009) observed that lipid mobilisation in cashew was very prominent under both control and salt treatments, particularly in seeds at 10 days of germination. In addition, lipid breakdown rates

were shown to decrease in response to salinity stress in other oilseed species such as sunflower (Ashraf *et al*. 2003).

It should be noted that the NaCl treatment also negatively affected protein mobilisation following radicle protrusion at 4 DAI (Fig. 4*b*), which was more pronounced during seedling development at 8 DAI. This could result in a decrease in the amount of free amino acids available for plant tissue development, which would consequently affect seedling formation. In support of this hypothesis, Marques *et al*. (2013) reported the NaCl-induced inhibition of protein mobilisation in cashew nut cotyledons in addition to the accumulation of amino acids in cotyledons at early developmental stages. Protein mobilisation was also observed to be increased in the control compared with the salt treatment, which also induced significant accumulation of free amino acids in cashew nut cotyledons (Voigt *et al*. 2009).

A transient rise in starch content was observed following radicle protrusion at 4 DAI, and thereafter, the starch was rapidly consumed, indicating that this reserve was available during seed germination and initial seedling development (Fig. 4*c*). However, although the starch content slightly varied under salinity, a more delayed consumption compared with the control was registered, indicating another salt harmful effect on the initial seedling development. Additionally, starch grains were observed at 8 DAI by polarisation and electron microscopy under the control and salt conditions, suggesting that photosynthetic activity was already occurring at that time.

The starch accumulation in salt absence (from 4 to 5 DAI) (Fig. 4*c*) was correlated with the beginning of lipid mobilisation (Fig. 4*a*), which, in turn, should be converted in soluble sugars; however, in this period, it was not detected an intense conversion of lipids to sugars, resulting in transient starch accumulation (Fig. 4). Nevertheless, this process occurred after 5 DAI, when the decrease of the lipids and starch matched with a stronger soluble sugar increment (Fig. 5*a*–*c*). This observation has been previously reported for other oilseeds, such as dwarf cashew seedlings, during seedling establishment under non-stress conditions (Voigt *et al*. 2009; Marques *et al*. 2013). In addition, Voigt *et al*. (2009) reported that transient starch synthesis could be the result of intense lipid breakdown during the radicle protrusion of cashew nut seeds, indicating carbohydrate synthesis from lipids by gluconeogenesis.

The most abundant soluble sugars that were detected in the endosperms of the *J. curcas* seeds were non-reducing sugars, suggesting their relevance in seedling development in this species (Fig. 5*c*). It can be assumed that soluble sugars increase under control conditions because the mobilisation of reserve sugars in the endosperm has not yet been completed, as this most likely occurs during seedling development. Thus, the reduced sugar accumulation under salt-stress conditions may negatively affect seedling development, considering that this reserve is one of the first to be used during this stage in addition to the following periods. The increase in soluble sugars following radicle protrusion at 4 DAI strongly correlates with the reduced lipid concentrations observed during the same period (Fig. 4*a*), suggesting that this last reserve can be converted into sugars. Some studies have also revealed that non-reducing sugars, such as sucrose, are generally the first to be used in the initial hours of imbibition, maintaining the growing embryo until germination occurs (Baranova *et al*. 2006). This was very evident in dwarf cashew nuts (Marques *et al*. 2013). The same study reported that NaCl stress inhibited non-reducing sugar consumption in the cotyledons of germinated nuts and established seeds (Marques *et al*. 2013). Moreover, the reduced accumulation of soluble sugars, mainly non-reducing sugars, under salt-stress conditions may have a negative effect on seedling development, considering that this reserve is the first to be used during germination and seedling development (Sánchez-Linares *et al*. 2012).

### *Cytochemical and ultrastructural analyses*

Decreases in cytoplasmic lipid bodies were greatest under the control conditions at 8 DAI (Fig. 6*e*, *f*), suggesting that mobilisation rates are faster in the absence of salt. However, in the salt-stressed seed sections at 8 DAI, the cells were almost completely filled with lipid bodies, indicating delayed mobilisation of this reserve due to salt stress. These findings (Fig. 6*h*) appear to confirm the biochemical analyses (Fig. 4*a*). In agreement with this study, mobilisation of storage lipids in the cotyledons of alfalfa seedlings, which was also analysed by electron microscopy, revealed that NaCl, $Na_2SO_4$ and mannitol treatments suppressed lipid mobilisation in this part of the seedling (Baranova *et al*. 2006).

Changes were observed in the protein bodies based on cytochemical and ultrastructural analyses (Fig. 6), which confirmed the biochemical analyses (Fig. 4*b*). An ultrastructural analysis of protein mobilisation was also performed in *Mucuna pruriens* (Muccifora *et al*. 2010), demonstrating that the mobilisation of this reserve in the embryo and cotyledons occurred before the end of germination The opposite was found in the present study of the *J. curcas* endosperm, in which increased protein body mobilisation was more evidenced, at 8 DAI in control treatment (Fig. 6*d*, *f*). In contrast, mobilisation in the NaCl-treated endosperm cells was greatly hindered due to protein body degradation at 8 DAI (Fig. 6*g*, *i*). This result could be related to the deleterious effects of salt on protein mobilisation that were observed at 8 DAI as reported above and as shown by the biochemical (Fig. 4*b*), cytochemical (Fig. 6*g*) and ultrastructural analyses (Fig. 6*i*). Salt stress was also shown to reduce the efficiency of lipid and protein mobilisation in *A. occidentale*, and this reduction was particularly extreme in emerged and established seedlings that were grown at the highest salt level (Marques *et al*. 2013).

## Conclusions

In conclusion, this study confirmed the great relevance of lipids as reserves for *J. curcas* seeds, revealing that these molecules are more rapidly mobilised under control conditions and might play a role in transient starch accumulation. We also examined the accumulation of $Na^+$ and $Cl^-$ in NaCl stressed seeds, which primarily occurs during the initial stages of seedling development, suggesting that these ions might be closely associated with delayed seed reserve mobilisation. In addition, cytochemical and ultrastructural analyses confirmed delayed mobilisation of protein and oil bodies under these conditions, suggesting an additional negative effect of salt stress on seedling development. The effects of salt stress were more severe during

seedling development than during seed germination. This study is the first to characterise salt-induced biochemical and ultrastructural changes in *J. curcas* seeds during germination and initial seedling development. Therefore, the results obtained herein enhance our understanding of the effects of salt stress on the initial development of *J. curcas*, and provide useful information for future studies concerning the agricultural traits of this species in arid and semiarid regions.

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