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Impact of soaking process on the microstructure of cowpea seeds in relation to solid losses and water absorption

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Abstract

A cytohistological investigation was performed to better understand the structural alterations of cowpea seeds in relation with soaking. Thin sections obtained from seeds soaked at 30°C, 60°C and 95°C were treated to specifically visualize starch, proteins, cellulose and pectin. Micropyle behavior as well as water uptake and dry matter loss were also monitored. A Soaking at 30°C induced slight alteration of parenchymatous cells of cotyledons, whereas drastic alterations were observed at 60°C and intense alterations at 95°C. All these structural modifications of cells could explain losses of nutrients and antinutritional factors at the highest soaking temperature (95°C). The size of the apertures in the micropyle sections varied depending on both soaking temperature and time. At 30°C and 60°C, the micropyle aperture enabled notable water uptake by seeds. At 95°C, water was entirely taken up through the testa. These results show that models describing nutrients losses and water uptake should account for these structure changes during soaking of legumes.

Keywords

Microscopy; legumes; cell wall; micropyle; cooking; β -elimination

43 1. Introduction

44 Legume seeds such as cowpea (*Vigna unguiculata*) are widely cropped and are of vital
45 importance to the livelihood of several million people in West Africa (Achigan-Dako et al., 2011).
46 Legume seeds are important in human nutrition (Graham & Vance, 2003) and unlike animal-based
47 products, benefit the environment (Tscharntke et al., 2012). Cowpea seeds are rich in proteins (20-
48 25 %), starch (45-55%) and micronutrients including vitamins and minerals (Sreerama, Sashikala,
49 Pratapa, & Singh, 2012). Cowpeas are used in many different traditional African dishes (Dovlo,
50 Williams, Zoaka, & others, 1976) whose preparation often requires a preliminary soaking step.
51 Soaking usually involves immersing the seeds in water at room temperature overnight. After soaking,
52 the seeds are usually cooked in boiling water for 40 to 60 min (Madodé et al., 2013). During the
53 soaking-cooking process, due to both seed heating and hydration, the seeds partially or totally
54 gelatinize and proteins may undergo thermal denaturation. All these combined phenomena strongly
55 affect seed structure and confer a softer texture..

56 In terms of structure, cowpea seeds are composed of a testa with a hilum and a micropyle, two
57 cotyledons and an embryonic axis (epicotyl + hypocotyl + radicle) (Stanley & Aguilera, 1985). The
58 cotyledon is the main nutritional component of legume seeds and previous studies have shown that
59 cotyledons are rich in starch and proteins (Swanson, Hughes, & Rasmussen, 1985). Heat treatment
60 between 60°C and 75°C during soaking causes the starch to gelatinize thereby enhancing its
61 digestibility (Coffigniez et al., 2018; Stanley & Aguilera, 1985). It also enhances protein digestibility
62 by increasing protein solubility (Alonso, Aguirre, & Marzo, 2000; Hoover, Hughes, Chung, & Liu,
63 2010). The structural integrity of cotyledons is also affected by heating. For example, Hung,
64 McWatters, Phillips, & Chinnan, (1990) showed that soaking at 50°C, 70°C and 90°C dissociated the
65 cotyledon cells. This is mainly due to pectin depolymerization and β -elimination (Njoroge et al.,
66 2016). Heating has also been shown to induce nutritional and anti-nutritional losses through diffusion
67 and thermal degradation (Ibrahim, Habiba, Shatta, & Embaby, 2002; Coffigniez et al., 2018). A

68 previous study by our team showed a low diffusion of alpha-galactosides during soaking at 30°C,
69 which was enhanced at higher soaking temperatures (60°C and 95°C) (Coffigniez et al., 2018).
70 However, the cytohistological mechanisms involved in this heat-induced destructuring remain poorly
71 understood.

72 Heat treatment also affects the water uptake capacity of the seeds. The testa can act as a barrier
73 to water uptake and affect the extent of interlocking with the cotyledons (Sefa-Dedeh & Stanley,
74 1979). In cowpea and lupin, most water absorption occurs via the micropyle and the testa of the
75 seeds (Sefa-Dedeh & Stanley, 1979; Robles-Díaz, Flores, & Yáñez-Espinosa, 2016). The size of the
76 micropyle determines the quantity of water absorbed by the seed (Sefa-Dedeh & Stanley, 1979). The
77 degree of starch gelatinization also determines the water uptake capacity of the seed (Briffaz,
78 Bohuon, Méot, Dornier, & Mestres, 2014). However, the impact of heat treatments on the behaviour
79 of the micropyle and consequently, on its role in regulating water uptake remains to be established.

80 The aim of the present study was thus to better understand the impact of seed structural
81 modifications at different levels (including cotyledons and micropyle) on water uptake, dry matter
82 losses and on the biochemical behavior of some macro constituents (starch, proteins, cell walls)
83 during soaking of cowpea seeds using microscopic and cytohistological techniques.

84 **2. Materials and methods**

85 ***2.1 Material***

86 The variety of cowpea used for this study was the *Wankoun* brownish one provided by IITA in
87 Benin. Cowpea seeds were sown in November 2014 and harvested in February 2015. The seeds were
88 kept in a vacuum pack and stored at 4°C in the dark until use.

89 **2.2 Soaking experiments**

90 Thirty grams of cowpea seeds were soaked in 120 mL deionized water using the method
91 described by Coffigniez et al., (2018) with a resulting seed-to-water ratio of 1:4 (w/w). The
92 temperatures and time ranges investigated were 30°C/0.5-38 h; 40°C/0.25-8 h; 60°C/0.16-8 h;
93 70°C/0.16-6 h; 80°C/0.16-6 h and 95°C/0.25-3 h.

94 In an additional experiment, prior to soaking at 30°C for 3 h or 60°C for 1 h, the hilum and
95 micropyle were both obstructed with superglue (cyanoacrylate) to assess their role in water uptake.

96 **2.3 Microscopy studies**

97 **2.3.1 Seed overall morphology**

98 To analyze the overall structure of the seeds, symmetric longitudinal half seed sections were cut
99 using shape razor blades and stained with lugol (to reveal the starch). Observations were performed
100 with a Nikon AZ100 stereomicroscope (Champigny sur Marne, France).

101 **2.3.2 Cytology**

102 Raw (control) and soaked seeds (see section 2.2 for soaking procedure) were immersed in a cold
103 fixative solution consisting of 4% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.2 M
104 phosphate buffer (pH=7.2). To facilitate the penetration of the fixative products, samples were placed
105 in a vacuum for 20 min. After 48 h of fixation at 4°C, the specimens were rinsed in distilled water
106 and stored in 70% ethanol at 4°C until required. They were then cut into 5×5 mm fragments from
107 different regions of the seed: cotyledons, testa with hilum and micropyle. All the samples were
108 dehydrated in increasing concentrations of ethanol (80-100% (v/v)) and embedded in methacrylate
109 resin (Kit Technovit 7100, Heraeus-Kulzer GmbH, Wehrheim, Germany) according to the
110 manufacturer's instructions. Sections (3 µm thick) were serially cut using a retraction microtome
111 (Supercut 2065, Reichert-Young, Wien, Austria) and deposited on microscope slides. For routine

112 observations, sections were stained with toluidine blue, a metachromatic dye (Clark, 1981). Sections
113 were also cytochemically stained to specifically visualize major cell components using the following
114 cytochemical reagents: periodic acid-Schiff's reagent (PAS) for starch and cellulose (pink); naphthol
115 blue-black (NBB) protein (blue) (El Maâtaoui & Pichot, 1999), Ruthenium Red (RR) (Sigma,
116 Germany) for pectin (red) and Fluorescent Brightener (FB) (Sigma, St Louis) for cellulose (Clark,
117 1981). In this latter case, the observations were made using UV illumination (365 nm) (Clark,
118 1981). All the observations were performed using a Leica DMR (Leica, Wetzlar, Germany)
119 photomicroscope equipped for bright field, dark field, phase contrast and UV illuminations. Images
120 were captured using a Leica DFC 300 FX digital camera and processed using LAS (Leica, Wetzlar,
121 Germany) and image J software (<https://imagej.net/>). For each treatment, approximately 60 sections
122 were analysed, and the best images were selected to illustrate our investigation.

123 *2.3.3 Micropyle aperture measurements*

124 Using the same experimental configuration as explained above, the aperture of the micropyle was
125 measured on 50 microscopic cross sections obtained from seeds soaked in different time and
126 temperature conditions (see section 2.2) and is expressed as an average length (μm).

127 *2.4 Dry matter losses*

128 For each soaking experiment, around 20 mL of soaking water was collected, weighed and dried
129 at 100°C for 48 h. The dry matter loss $DML(t)$ (kg / 100 kg db) in soaking water was calculated
130 against time t (h) as follows:

$$131 \quad DML(t) = \frac{m_{d,sw}(t)}{m_{d,s}(0)} * 100 \quad (1)$$

132 where $m_{d,sw}(t)$ and $m_{d,s}(0)$ are the mass (kg) of the 20 mL of soaking water after drying and the
133 mass of dried seeds at initial time respectively (kg).

134 **2.5 Water uptake kinetics**

135 Water content $WC(t)$ (kg of water/kg db) of soaked seeds was measured as a function of soaking
136 time t (h) using the method described by (Coffigniez et al., 2018) and the following equation:

137
$$WC(t) = \frac{m_{w,s}(t) - m_{d,s}(t)}{m_{d,s}(t)} \quad (2)$$

138 where $m_{w,s}(t)$ and $m_{d,s}(t)$ are the mass of the wet seeds and dried seeds versus time t (h)
139 respectively (kg).

140 **3. Results and discussion**

141 Observations of symmetric longitudinal seed sections showed that the gross structure of cowpea
142 seed is consistent with the organisation of exalbuminous *Fabaceae* (Fig. 1). The structure comprises
143 an embryo with massive and reserve-rich cotyledons (C) as well as an embryonic axis (E) embedded
144 in the testa (T), giving them a kidney-like appearance. The hilum (H) and micropyle (M) are located
145 in the concave part of seeds.

146 **3.1 Modifications of cotyledon cells**

147 The cytohistological effects of soaking process were mainly investigated in the parenchymatous
148 tissue of the cotyledons, which is the main component of the seed.

149 **3.1.1 Modification of cell content: starch and protein**

150 The cotyledons of the control (raw seeds) were composed of an epidermal layer embedding
151 massive, compact parenchymatous tissue containing many vascular bundles (not shown). PAS and
152 NBB staining (Fig. 2a) revealed numerous compact, heterogeneously sized starch granules (stained
153 pink) and a protein-rich (stained blue) cytoplasm. Proteins were dispersed in the cytoplasm
154 (amorphous), or organised as protein bodies. The parenchymatous tissue contained sizable
155 intercellular spaces, sometimes filled with protein.

156 Figure 2 (b, c and d) shows the impact of soaking treatments on cowpea cotyledon cells.
157 Compared to raw seeds (Fig. 2a), sections of cotyledon obtained from seeds soaked at 30°C for 6 h
158 showed a decrease in the intensity of the staining of the starch granules, proteins and cell walls of the
159 parenchymatous cells (Fig. 2b). Similar alterations were observed in parenchymatous cells soaked at
160 60°C for 2 h. However, PAS and NBB positive material accumulated in the intercellular space
161 (Fig. 2c). Sections of cotyledons from seeds being soaked at 95°C for 1 h showed marked alterations
162 in the structure of the parenchymatous cells (Fig. 2d). Indeed, we observed a decrease in PAS
163 staining of starch and an increase in the size of starch granules, which doubled in size (Fig. 2d)
164 compared to raw seeds (Fig. 2a). On the basis of Differential Scanning Calorimetry measurements,
165 Coffigniez et al., (2018) showed that a cooking at 95°C for 1 h resulted in full gelatinization of
166 starch. The proteins were homogeneous in the cytoplasm, but the blue color was strengthened by
167 NBB (Fig. 2d). Dense material filled the intercellular spaces (Fig. 2d).

168 *3.1.2 Modifications of cell walls*

169 Given that the major constituents of plants cell walls are cellulose and pectin, we decided to use
170 PAS, FB and RR staining reagents. Figures 2, 3 and 4 are representative micrographs illustrating the
171 effects of soaking on the behaviour of these components. In the control, PAS resulted in intense
172 staining of the components of the cell walls, which appeared to be compact and homogeneously
173 organized (Fig. 2a). This was confirmed by the results of FB (Fig. 3a) and RR (Fig. 4a) treatments
174 for cellulose and pectin respectively. After staining, the cell walls displayed a cohesive cellulose
175 structure and a middle lamella in the form of a pectin layer. After soaking at 30°C for 6 h, the cell
176 walls showed a reduced intensity using PAS reagent (Fig. 2b), and slight disruption causing the
177 migration of cytoplasmic materials which accumulated in enlarged intercellular spaces. FB staining
178 (Fig. 3b) showed loosening of the cellulose, considerably thickening the cell walls. Pectin specific
179 staining with RR (Fig. 4b) showed a reduced intensity in the pectic middle lamella, particularly at the
180 boundaries of the intercellular spaces. Soaking at 60°C induced similar but greater alterations of the

181 cell walls (Figs. 2c, 3c and 4c) with the accumulation of cytoplasmic material in intercellular spaces
182 and separation of the cells. Sections of cotyledons soaked at 95°C displayed parenchymatous cells
183 with profoundly destructured cell walls (Figs. 2d, 3d and 4d). This was confirmed by the breaks in
184 the cell walls at several places and the onset of cell dissociation where the middle lamella had
185 disintegrated. After this treatment, there was intense accumulation of cytoplasmic material in the
186 intercellular spaces.

187 The cell wall degradation and accumulation in intercellular space after soaking at 60°C and 95°C
188 (figs. 2 to 4) showed that the cell walls were permeable and that cellular content had diffused into the
189 intercellular space. Other authors also reported similar cell disruption after heat treatment of
190 legumes. Mwangwela, Waniska, & Minnaar, (2006) showed the accumulation of pectins in the
191 intercellular space after hydrothermal treatment. Waldron et al., (2003) and Varriano-Marston &
192 Jackson, (1981) reported the loss of plasmalemma and disorganization of cytoplasm after treatment
193 at high temperature. This communication with the intercellular space probably began in the
194 plasmodesmata and could be due to β -elimination and pectin depolymerization (Bernal-Lugo, Parra,
195 Portilla, Peña-Valdivia, & Moreno, 1997; Waldron, Parker, & Smith, 2003). Buren, (1979), Liu,
196 Phillips, & McWatters (1993) and Njoroge et al. (2016) showed that β -elimination was limited below
197 60°C but total after boiling at 100°C for 30 min.

198 A very slight disruption of the cotyledon cell walls appeared after soaking at 30°C. The limited
199 disruption could be linked to lack of β -elimination, but also to possible cell wall strengthening, partly
200 due to phytate hydrolysis. In cowpea, phytate concentrations range from 0.3–1.5 mg/100 mg, mainly
201 in the protein bodies of the cotyledon parenchyma. Phytates are associated with bivalent minerals
202 such as calcium, magnesium, iron and zinc (Kruger, Minnis-Ndimba, Mtshali, & Minnaar, 2015;
203 Gonçalves et al., 2016; Sandberg, 2002). Some authors have reported loss of phytates during soaking
204 of legumes at 30°C due to hydrolysis from endogenous phytase (Alonso et al., 2000; Chang,
205 Schwimmer, & Burr, 1977; Shi, Arunasalam, Yeung, Kakuda, & Mittal, 2004). This phytate

206 degradation triggers the release of bivalent minerals from the phytate-mineral complex and their
207 migration towards the cell wall of the middle lamella (Phillips et al., 2003), where the pectins are
208 usually soluble and linked to monovalent minerals. However, in this case, the released divalent
209 minerals, especially calcium, replace the monovalent minerals and the pectin becomes insoluble
210 (Kruger, Minnis-Ndimba, Mtshali, & Minnaar, 2015). The insoluble pectin forms a stronger cell
211 wall, thereby preventing cell separation (Phillips et al., 2003).

212 When legumes are soaked at between 50°C and 60°C, there is competition between pectin
213 strengthening and pectin degradation in the cell walls. However, in our cytohistological study and in
214 this temperature range, pectin degradation predominated (Figs. 2b and 4b).

215 *3.1.3 Impact of cell wall degradation on mass transfer properties*

216 The observed structural changes in the cell wall during soaking can strongly affect the mass
217 transport of several components, including nutrients and antinutrients, from the seed to the soaking
218 medium. As shown in figure 6a, soaking temperature had a strong effect on the extent of solid losses.
219 Indeed, over a soaking time of 3 h, mean dry matter losses were about 10 times higher at 95°C than
220 at 30°C. The same trend was reported by Kon, 1979 in beans with a dry matter loss of 2%, 12% and
221 12% after soaking for 5 h at 40°C, 1 h 30 at 60°C and 0.8 h at 90°C, respectively. These losses,
222 which often include nutrients and antinutrients, are thus facilitated by high soaking temperatures that
223 may increase cell wall degradation.

224 For instance, concerning antinutrients, a previous study by our team on the same cowpea
225 variety (*Wankoun*) showed that 2.2%, 58% and 48% of alpha-galactosides had diffused in water after
226 soaking at 30°C for 6 h, at 60°C for 2 h, and at 95°C for 1 h, respectively (Coffigniez et al., 2018).
227 This diffusion of alpha-galactosides from cowpea seed was modelled using Fick's law and apparent
228 diffusivities were identified, revealing a strong effect of temperature. For example, the apparent
229 diffusivity of stachyose was found to be 150 times lower at 30°C than at 60°C and 1.5 lower at 60°C

230 than at 95°C. The modification in the structure of the cell wall together with pectin degradation at
231 higher temperature may explain the resulting higher apparent diffusivities obtained at 60°C and 95°C
232 compared to 30°C. This model could be upgraded by integrating this structural factor.

233 Similarly, other components can leach out of the legume seed to an extent which varies with
234 the soaking conditions. Carbohydrates represent the main fraction which is lost during soaking. For
235 instance, Sayar et al. (2011) showed in the case of chickpea that boiling for 1 h or soaking at 20°C
236 for 15 h induced carbohydrate losses of 75% (db) and of 88% (db) respectively. For instance,
237 soaking cowpea seeds at room temperature for 8 h resulted in a loss of tannins, phytic acid and
238 trypsin inhibitors of less than 1%, whereas boiling for 45 min resulted in a loss of 41%, 6.8 % and
239 87 % of the same species, respectively (Ibrahim, Habiba, Shatta, & Embaby, 2002). In beans, Kon,
240 1979 found that increasing soaking temperature (40°C/5 h, 60°C/1.5 h, 90°C/50 min) increased the
241 diffused fraction of thiamin by 1%, 32% and 40%, respectively). All these observations show that the
242 soaking temperature can be optimized to preserve the nutritional value of seeds for human
243 consumption.

244 By comparing the results of the present microscopic study and those of a quantitative study
245 on alpha-galactosides by Coffigniez et al. (2018) using the same seed cultivar, we can confirm that
246 there is a strong link between destructuring of the cell (disruption of the cell wall and degradation of
247 pectin) that is enhanced by high soaking temperatures and molecular diffusion through the
248 intercellular space. However, other seed structures could also affect the material losses from the seed
249 during soaking such as the micropyle whose opening can facilitate diffusion, essentially at low
250 temperatures.

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3.2 Water uptake by the seeds

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3.2.1 Water uptake from the micropyle

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The behaviour of micropyle was monitored using cross sections stained toluidine blue. Cross sections from the control (Fig. 5a) showed a closed aperture embedded in three different structures: a spongy tissue (around 500 μm), a palisade layer (around 150 μm) and a group of tracheids (around 400 μm). After soaking at 30°C for 3 h and 6 h and at 60°C for 1 h and 2 h, the micropyle was open. However, the opening was twice as big after the shortest soaking time at 30°C and 60°C (Table 1). After soaking at 95°C, the micropyle remained almost closed (Table 1) at both soaking times.

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Figure 6b shows cowpea seed water uptake kinetics at different soaking temperatures ranging from 30°C to 95°C. The early water uptake rate (calculated as the linear slope from 0 to 0.5 h, data not shown) increased with soaking temperature, except at 95°C, when the lower initial water uptake rate could be due to the closure of the micropyle (Figs. 5f and 5g). These results are consistent with those obtained by Coffigniez et al., (2018) using the same cowpea material and in the same experimental soaking conditions. These authors identified apparent water diffusivities (D , $\text{m}^2.\text{s}^{-1}$) in cowpea seed through model adjustment and showed that D was two times lower at 95°C than at 60°C. In the present study, seed water content was also measured in the same experimental conditions but with the seed micropyle and hilum artificially closed using superglue (data not shown). In this configuration, the resulting water content was 39% and 33% lower after soaking at 30°C for 3 h and at 60°C for 1 h, respectively than in seeds with a free micropyle and hilum. These observations suggest that at low temperature, these structures are involved in the regulation of water uptake by the seed. Moreover, at low soaking temperatures (i.e. at 30°C and 40°C), water absorption behavior was not purely Fickian, with a short delay at the early stage of water uptake kinetics. Indeed, at 40°C for example, the initial ($t < 0.25$ h) water uptake rate was found to be lower

275 (0.88 kg/kg db/h) than that from 15 to 30 min (1.52 kg/kg db/h). These results suggest that active
276 transport might occur through the micropyle and hilum. The contribution of these structures to the
277 active control of seed water absorption at low soaking temperatures was confirmed with the aperture
278 of the micropyle at 30°C (figs. 5b and 5c).

279 Other authors also highlighted the contribution of the micropyle to the water absorption process
280 in some legume seeds. Using a dye in lentil and lupin seeds soaked at room temperature, Robles-
281 Díaz et al., (2016) showed that water uptake preferentially occurred through the micropyle. Gama-
282 Arachchige, Baskin, Geneve, & Baskin, (2013) blocked the hilum and micropyle of *Cercis*
283 *canadensis* seeds, which partly blocked water uptake. Lush & Evans, (1980) reported that the
284 behaviour of the micropyle of cowpea seeds depended to a great extent on experimental conditions.
285 Indeed, they showed that the micropyle is waterproof (closed) when the seed is dry (water content of
286 10-12 %). To absorb water, a change in cell conformation and a water content threshold (24%) are
287 required for micropyle opening.

288 **3.2.2 Water uptake through testa layer**

289 Despite the blockage of micropyle and hilum structures (section 3.2.1), 60% of water uptake was
290 maintained during soaking at 30°C and 60°C. This water uptake probably occurred through the testa
291 layer. In this case, the structure of the cowpea seed testa may play an important role in water uptake
292 (Stanley & Aguilera, 1985). The water diffusivity through the testa increased with decreasing
293 thickness and increasing temperature (Kaptso et al., 2008; Yildirim, Öner, & Bayram, 2011). At
294 95°C, water was only taken up through the testa (micropyle closed), which explained the lower water
295 uptake rate before 1 h compared with that at lower temperatures. However, the marked breakdown of
296 cell walls observed at 95°C (Figs. 2d to 4d) could facilitate water uptake and partly explain the
297 higher water uptake after 1 h compared with that at lower temperatures.

298 **4. Conclusion**

299 Our study has advanced our knowledge of changes in major cell components and structure
300 (including micropyle) of cowpea seeds during soaking and their impact on the diffusion of material
301 and uptake of water. The changes in the cells were monitored by means of cytohistological
302 investigation. During soaking at 30°C, only slight structural modifications were observed, with
303 minimal impact on mass transfer out of the cells and water mainly taken up through the micropyle.
304 At higher soaking temperatures (i.e. 60°C and 95°C), cell wall disruption occurred due to β -
305 elimination and depolymerization of pectin. The drastic effect of high soaking temperatures on the
306 cell walls was responsible for the loss of material in the soaking water that facilitated water uptake
307 by the seeds. At high soaking temperatures, water uptake mainly occurred through the testa layer. All
308 these temperature-dependent structural changes should be integrated in existing models to better
309 describe the diffusion of alpha-galactosides and the uptake of water by cowpea seeds during soaking.

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311 The authors declare no conflicts of interest. FC, MEM and LA set up the study and performed the
312 experiments. FC, MEM AB, CM and PB revised the manuscript. All the authors read and approved
313 the final manuscript.

314

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Fig 1: Micrograph showing the gross morphology of cowpea seeds. T, testa; H, hilum; M, micropyle; C, cotyledon E, embryonic axis. Lugol staining (dark blue) reveals accumulation of starch in the cotyledon. Embryonic axis appears to be starch free. Scale bars = 1630 μm .

Fig 2: Effects of soaking on the parenchymatous cells of cowpea cotyledon. Sections were stained with PAS-NBB. (a) Control (raw seeds). (b) After soaking at 30°C for 6 h. (c) After soaking at 60°C for 2 h. (d) After soaking at 95°C for 1 h. Note the decrease of stain intensity of starch and proteins in (b) and (c) compared to (a), and the presence of amorphous materials in intercellular spaces in (c) and (d) (arrows), and the intense blue colour of the cytoplasm in (d). CW: cell wall; IC: intercellular space; S: starch; Cy: cytoplasm; PB: protein body; N: nucleus. Scale bars = 28 μm .

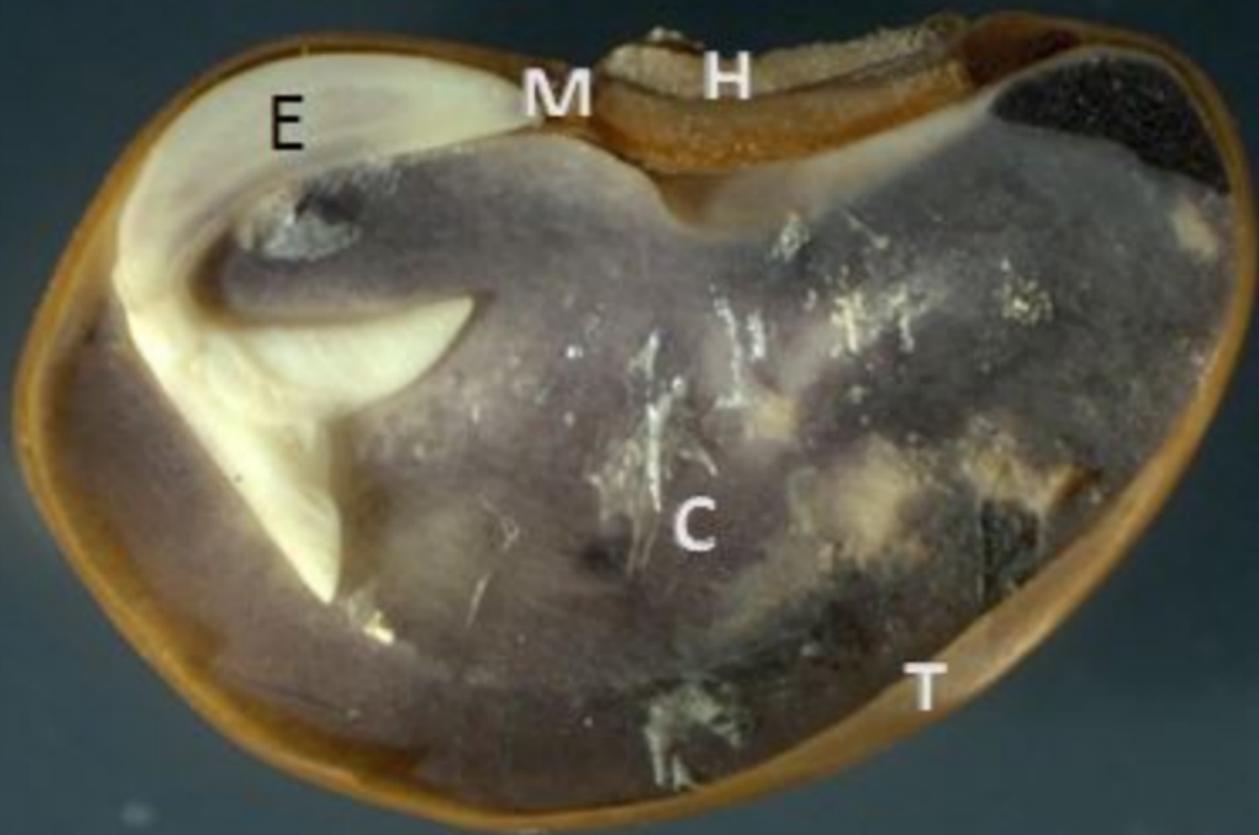
Fig 3: Effects of soaking on cellulose in the cell walls of cowpea cotyledons. Sections were stained with FB. (a) Control (raw seeds). (b) After soaking at 30°C for 6 h. (c) After soaking at 60°C for 2 h. (d) After soaking at 95°C for 1 h. Note the disruption of the cell wall in (c) and (d) (arrows), and cell dissociation in (d) (star). Scale bars = 18 μm .

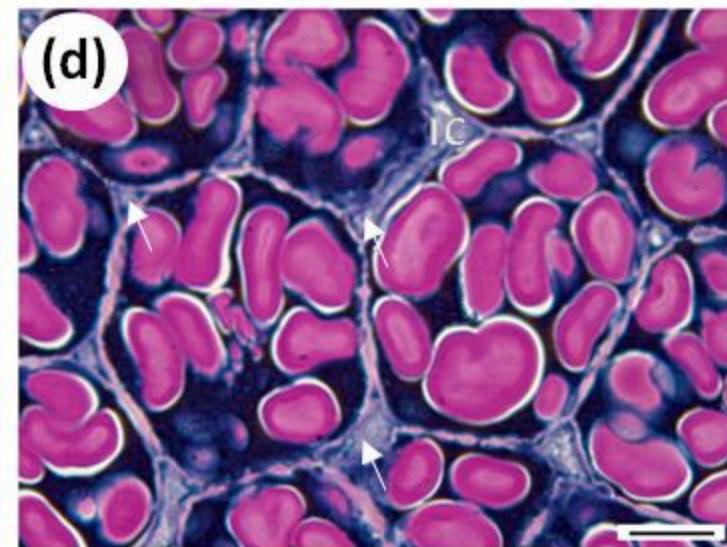
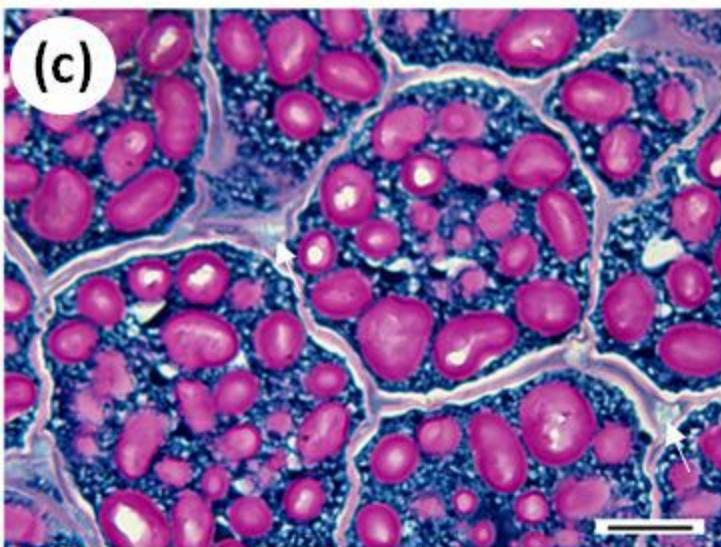
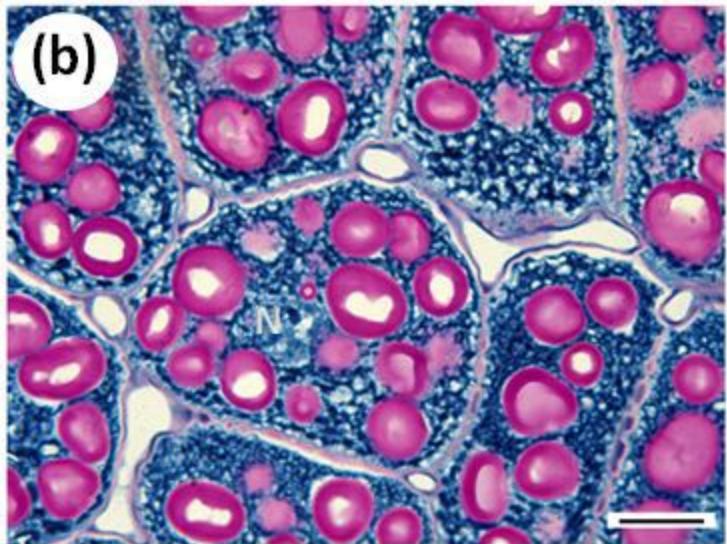
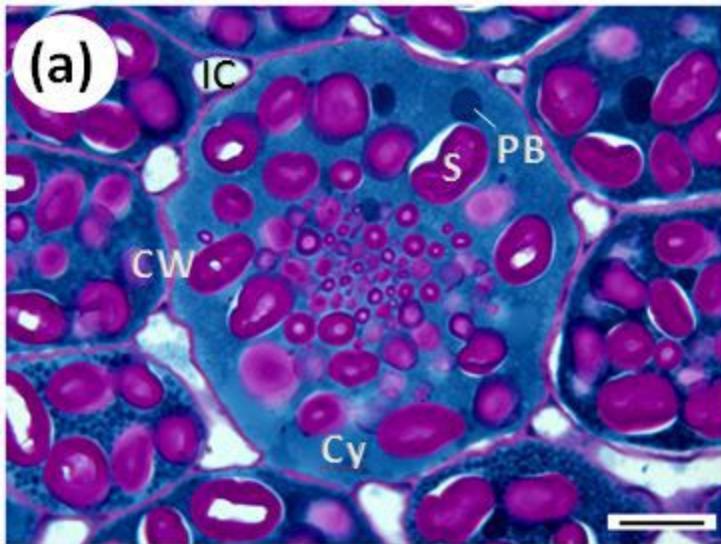
Fig 4: Effects of soaking on pectin in the cell walls of cowpea cotyledons. Sections were stained with RR. (a) Control (raw seeds). (b) After soaking at 30°C for 6 h. (c) After soaking at 60°C for 2 h. (d) After soaking at 95°C for 1 h. Note the presence of pectin in the intercellular spaces in (b), (c) and (d) (arrows), and the fusion of starch granules in (d). S, starch. Scale bars = 15 μm .

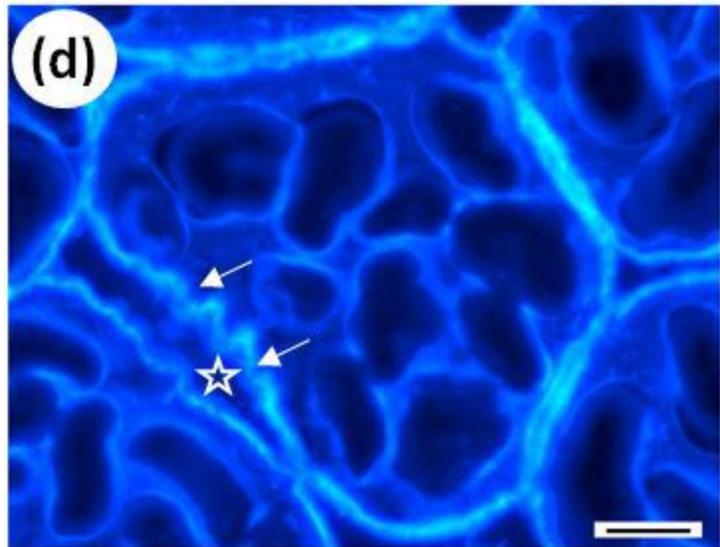
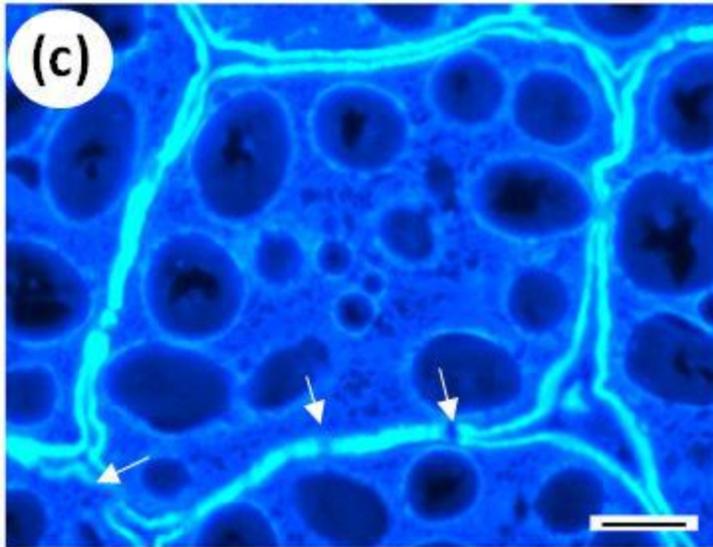
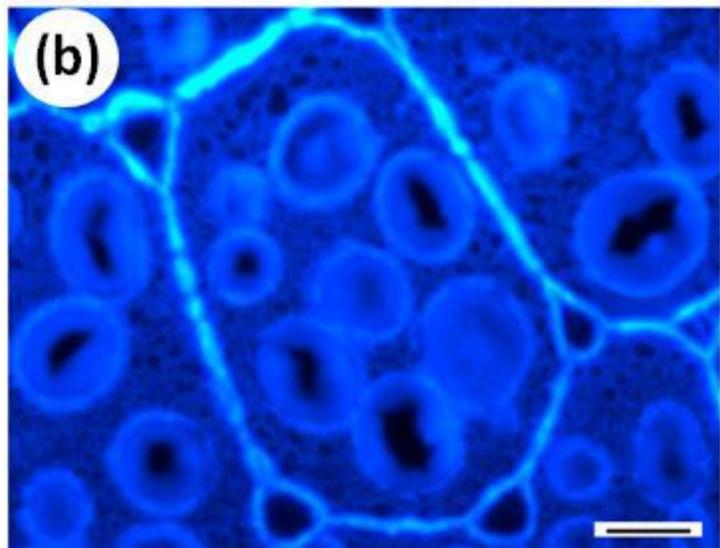
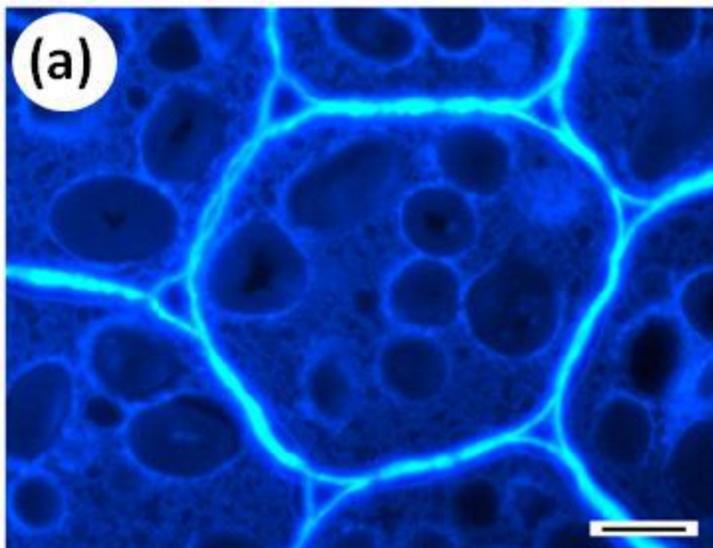
Fig 5: Effects of soaking on cowpea micropyle. Sections were stained with toluidine blue. (a) Control (raw seeds). (b) After soaking at 30°C for 3 h. (c) After soaking at 30°C for 6 h. (d) After soaking at 60°C for 1 h. (e) After soaking at 60°C for 2 h. (f) After soaking at 95°C for 0.5 h. (g) After soaking at 95°C for 1 h. Note the presence of the aperture in (b), (c) and (d). Ap: aperture; Pa: palisade; TI: tracheid island. Scale bars = 18 μm .

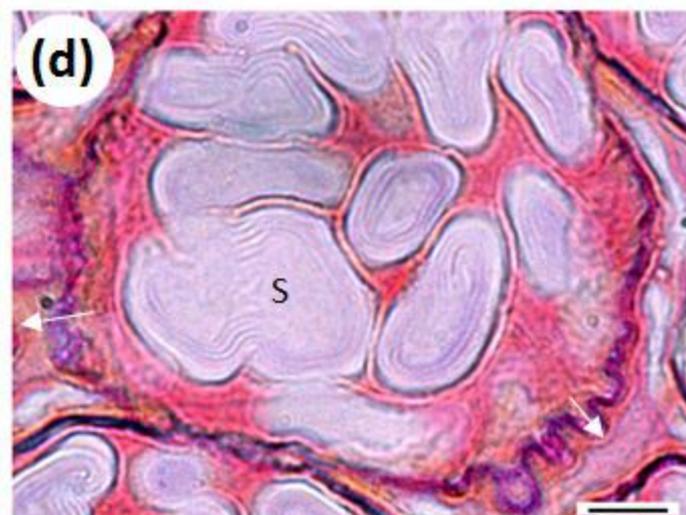
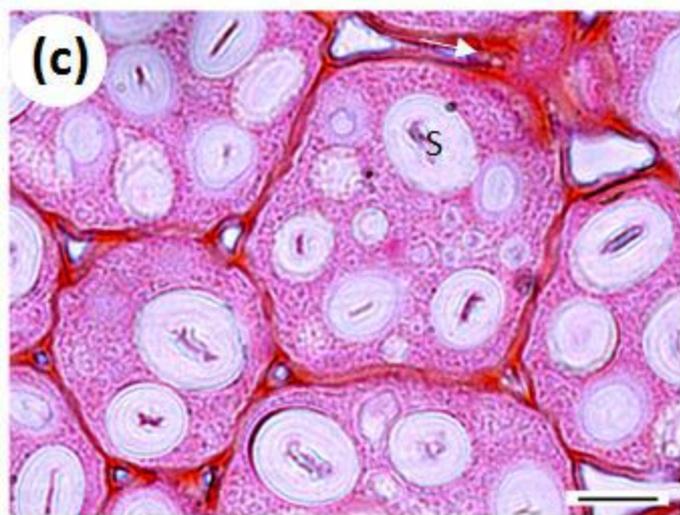
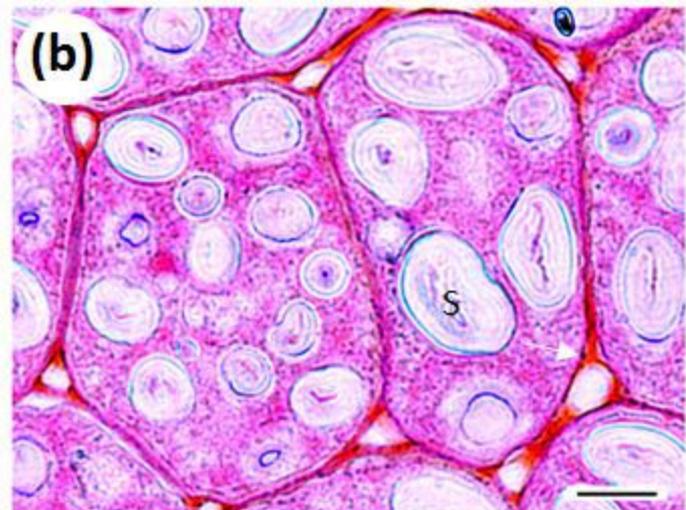
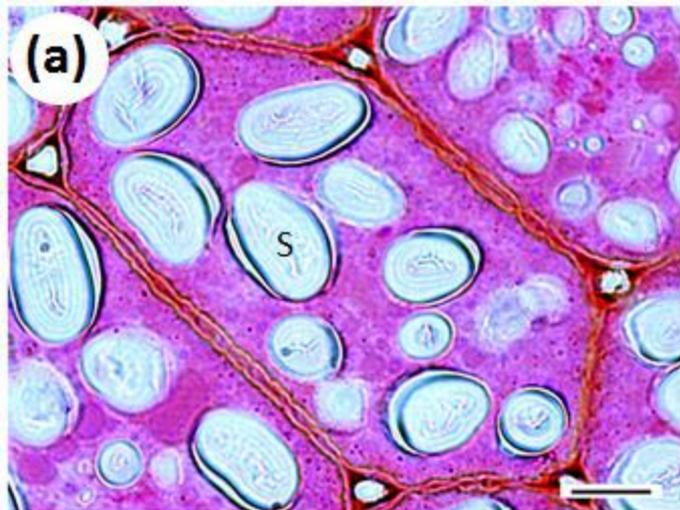
Fig 6: (a) Dry-matter loss kinetics (kg/100 kg db) of cowpea seeds during soaking at 30°C (black), 60°C (blue) and 95°C (brown). (b) Water uptake kinetics (kg of water/kg db) of cowpea seeds during soaking at 30°C (black), 40°C (red), 60°C (blue), 70°C (green), 80°C (orange) and 95°C (brown). Error bars represent the standard deviation (n=4)

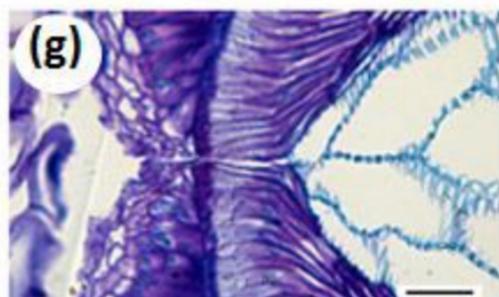
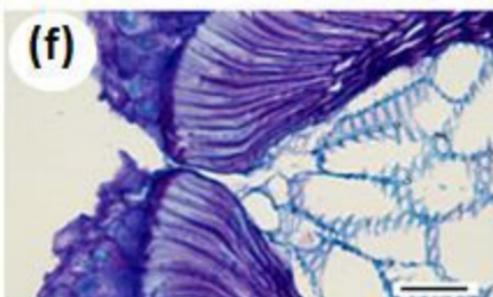
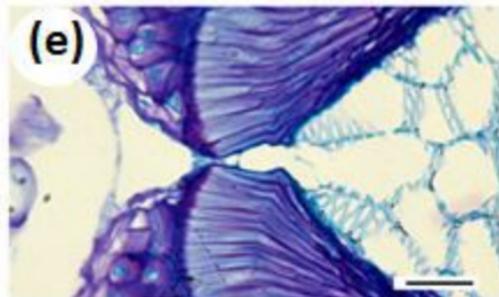
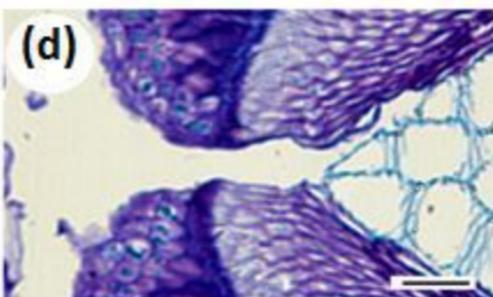
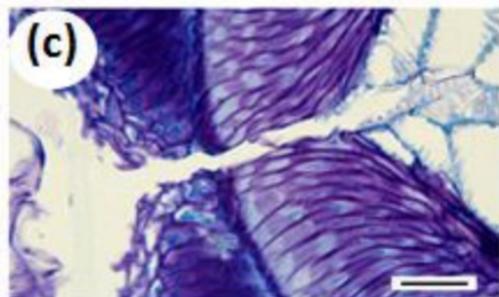
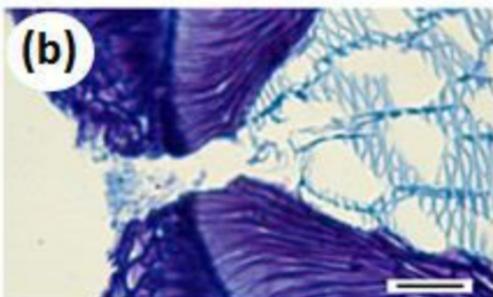
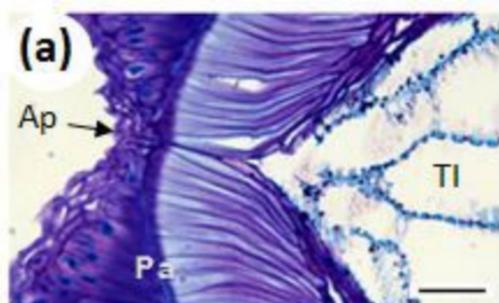
Table 1. Changes in the micropyle aperture after soaking at 30°C, 60°C and 95°C. The mean aperture was measured on the whole micropyle presented in Figure 5. (mean values \pm standard deviations determined with n=6 measurements)

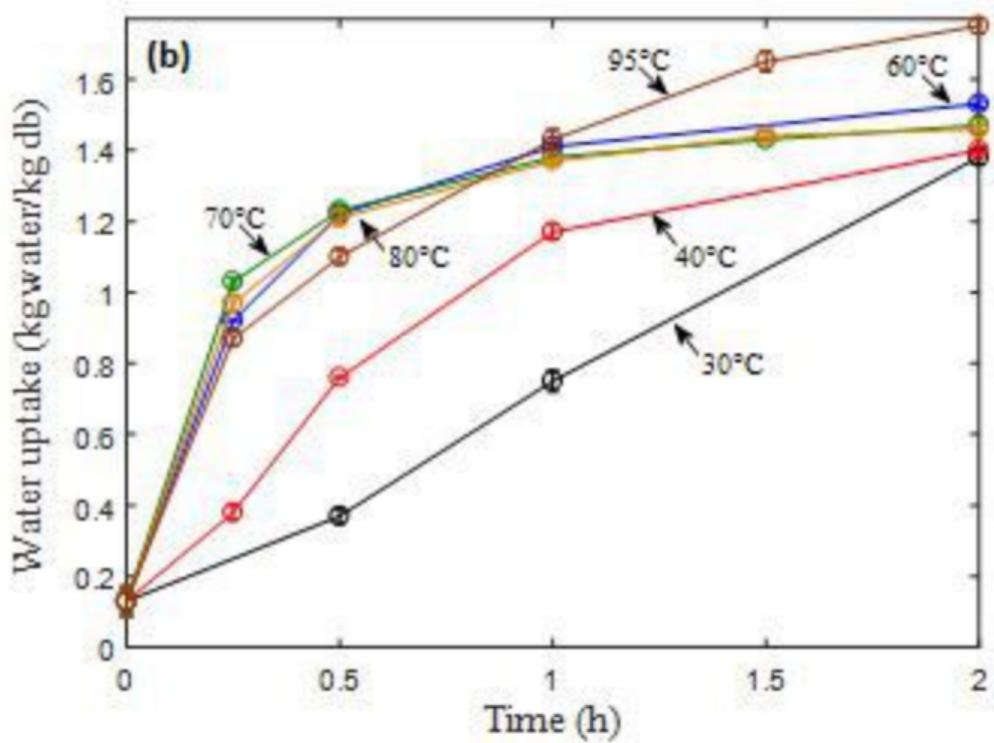
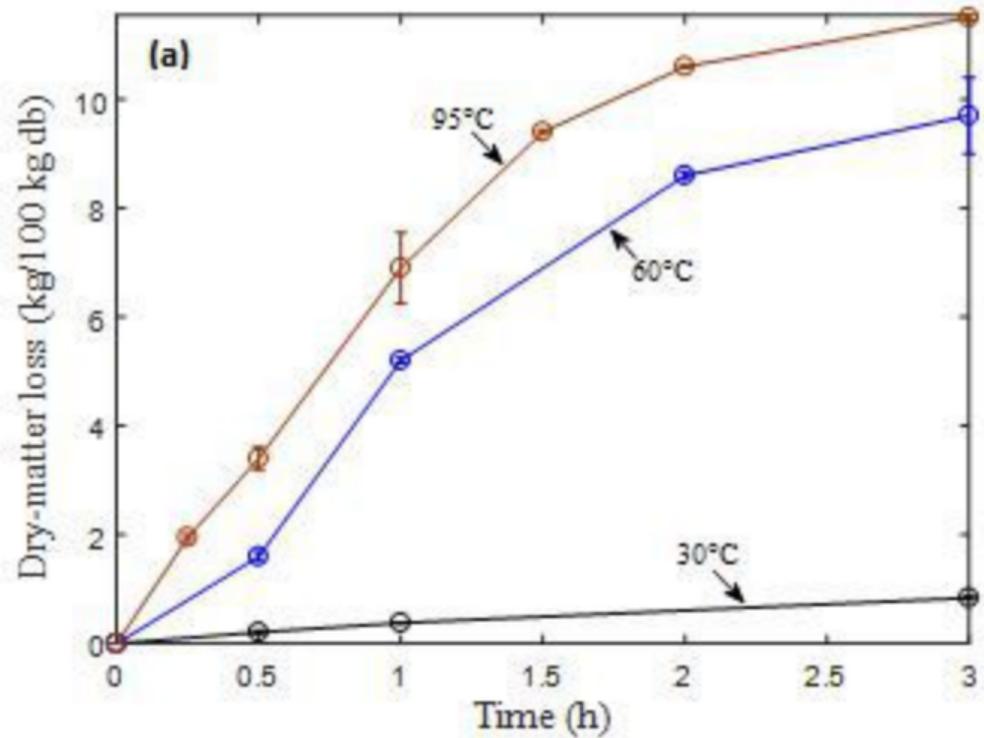












Temperature (°C)	Time (h)	Micropyle thickness (μm)
0	0	0 ± 0 ^a
30	3	12.2 ± 1.3 ^b
	6	5.8 ± 0.8 ^c
60	1	11.7 ± 2.0 ^b
	2	5.4 ± 2.6 ^c
95	0.5	1.8 ± 0.7 ^d
	1	1.3 ± 0.2 ^d

Values with different letters (a–b–c–d) are significantly different ($p < 0.05$).