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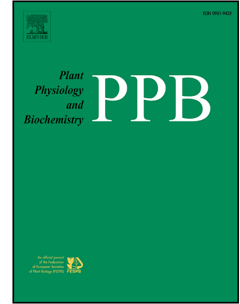

Impact of protective agents and drying methods on desiccation tolerance of *Salix nigra* L. seeds

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19 **Abstract**

20 Willow seeds are classified as orthodox, but they show some recalcitrant
21 characteristics, as they lose viability in a few weeks at room temperature. The aim of
22 this work was to improve the desiccation tolerance of willow seeds (*Salix nigra* L.), as a
23 model of sensitive materials to dehydration, through imbibition in solutions and later
24 vacuum (VD) or freeze-drying (FD). Imbibition was conducted with 45% w/v trehalose
25 or polyethylene glycol 400 –PEG– or water prior to dehydration treatments. Water- and
26 especially trehalose-imbibed seeds subjected to VD showed better germination
27 capability with respect to the freeze-dried ones. Water crystallization was mainly
28 responsible for the great loss of capability germination observed in water- or trehalose-
29 imbibed seeds subjected to FD. PEG behavior was better when seeds were FD instead
30 of VD. DSC thermograms of seeds allowed to identify two thermal transitions
31 corresponding to lipids melting and to proteins denaturation. This last transition reveals
32 information about proteins state/functionality. Dehydration of control and PEG- or
33 water-imbibed seeds affected proteins functionality leading to lower germinability. In
34 the case of trehalose-imbibed seeds subjected to VD, proteins maintained their native
35 state along dehydration, and the seeds showed a great germination capacity for all the
36 water content range. Germinated seeds showed higher luminosity (L*), greenness (a*)
37 and yellowness (b*) values than not-germinated seeds independently of the employed
38 agent. Present work reveals that the presence of adequate protective agents as well the
39 dehydration method were the main critical factors involved in willow seed desiccation
40 tolerance.

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41 **Highlights**

42 • Willow seeds were subjected to imbibition and subsequent vacuum or freeze-
43 drying

44 • Trehalose imbibition followed by vacuum drying provided >75% seed
45 germination

46 • Protein changes could be determined by DSC

47 • Dehydration affected proteins functionality leading to lower germinability

48 • The a* coordinate correlated with germinability at the first stages of germination

49

50 **Key words:** orthodox seeds; willow; dehydration, desiccation, seed tolerance, seed
51 storage; imbibition; trehalose; seed protein denaturation; color changes.

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52 **1. Introduction**

53 The conservation of labile biomolecules/structures in biological, pharmaceutical and
54 food sciences is generally performed in frozen or dehydrated systems (Santagapita and
55 Buera, 2008). Vegetal germplasm is usually conserved as seeds, which are naturally
56 dehydrated systems. The so-called orthodox seeds (the most common) tolerate
57 dehydration up to low water contents (wc), and can remain viable for several years.
58 Seeds of 24 species of *Salix* containing between 6 to 10% water content have been
59 maintained without loss in viability for 3 years in hermetic storage at –19 °C (Zasada
60 and Densmore, 1977) or 5 years when stored over a desiccant at –8 °C (Sato, 1955).
61 Although willow seeds (*Salix spp.*) are orthodox (Hong et al., 1996), they exhibit some
62 recalcitrant characteristics: the longevity does not follow the dehydration tolerance as is
63 shown for orthodox seeds. At room temperature *Salix spp*. seeds lose their viability in a
64 time frame from two days (Campbell, 1980) up to a few weeks, like *Salix alba* and *Salix*
65 *matsudana* (Maroder et al., 2000) and *Salix nigra* (Roqueiro et al., 2010), depending on
66 the species.

67 Collected seeds are usually first dehydrated at 20 °C for 3h from wc 41% to 9-12%
68 (wet basis), leaving the seeds with water activity (a_w) values between 0.8 and 0.7. Then,
69 seeds are kept at -70-80 °C in order to avoid viability loses, but this increases storage
70 time-dependent costs. This fact also limited the possibilities to find commercial seeds.

71 In a previous work Maroder and co-workers (2000) studied the effects of
72 dehydration, storage temperature and humidification on germination of *Salix alba* and
73 *Salix. matsudana* seeds. They observed that germination of the high vigor lot (100% of
74 initial normal germination) was not affected by dehydration up to 6.7% of moisture
75 content but germination decreased with further dehydration to 4.3%. The lowest vigor
76 lot (75% of initial normal germination) was more susceptible to dehydration and

77 germination decreased following dehydration to 6.7% moisture content. Seeds showed
78 improved performance at lower storage temperature when stored between -70 and 25 °C
79 and can be dehydrated to a moisture content in equilibrium with 15% relative humidity,
80 suggesting that they are orthodox in storage behavior although they are short-lived.

81 One of the strategies to improve germination stability upon drying is imbibing the
82 labile structures with osmo-protectant solutes. These solutes promote specific
83 interactions (especially hydrogen bonding) with biological structures, stabilizing them
84 during drying. Several biomolecules, structures and organisms (protein, membranes,
85 cells, seeds, and microorganisms) have been stabilized through immobilization in glassy
86 sugar matrices obtained by freeze- or spray-drying (Hoekstra et al., 2001; Tunnacliffe
87 and Lapinski, 2003; Buera et al., 2005; Santagapita and Buera, 2008). Trehalose, a non-
88 reducing disaccharide, has a protective effect on biomolecules which is not only
89 explained by the capacity of the sugar to form glassy structures, but also by the
90 intermolecular interaction between the biomolecules and sugar through hydrogen bonds
91 (Santagapita and Buera, 2008). Polyethylene glycol (PEG) is usually used as an
92 adequate cryo-protective excipient of enzymes (Carpenter et al., 1993), but it is not
93 efficient as a dehydro-protectant.

94 In recent years emphasis has been placed on the application of techniques of
95 computer vision systems and image analysis for assessing the color changes and other
96 properties related to the quality (Briones and Aguilera, 2005; Agudelo-Laverde et al.,
97 2011 and 2013). This approach represents an interesting alternative for heterogeneous
98 systems, because it is provide a large quantity of information in a fast and non-
99 destructive method (Jayas et al., 2000).

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100 The aim of this work was to improve the desiccation tolerance of willow seeds
101 (*Salix nigra* L.), chosen as a model of sensitive materials to dehydration, through
102 imbibition in solutions and later vacuum (VD) or freeze-drying (FD).

103

104 2. Results and discussion

105 2.1. *Salix nigra* seeds upon dehydration

106 A rapid decrease of germination capacity during dehydration was observed for non-
107 imbibed (control) vacuum dried seeds **(Figure 1)**. The water content (wc) decreased
108 during the dehydration procedure from the initial point (11% dry basis, pointed in
109 **Figure 1** with an arrow). It is important to take into account that at the moment of the
110 present work, the seed control-pool retained 77% of the germination capacity. Then,
111 these seeds could be classified as a low vigor lot, and could be more susceptible to
112 dehydration and to a decrease of germination after dehydration. Similar results were
113 obtained by Maroder and co-workers (2000) for *Salix alba* and *Salix matsudana* seeds.

114

115 2.2. Imbibition

116 The imbibition of seeds with dimethylsulfoxide (DMSO) or glycerol, as well as with
117 other cryo- and dehydro-protectants (such as sucrose and maltodextrin) and their
118 combinations previous to germination did not provide any improvement to germination
119 index.

120 Trehalose and water imbibed seeds showed higher significant germination values
121 (28 ± 5 and 16 ± 4%, respectively) than the control seed pool. This behavior was
122 already reported upon imbibition of *Salix spp*. seeds (Maroder et al., 2000) and is
123 related to the so called priming effect (Chojnowski et al., 1997), which involved
124 extensive repair processes which take place during imbibition prior to germination.

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125 These repair processes continues until the water content which allows division and cell
126 elongation (beginning of the seedling) is reached, improving the physiological quality
127 of the seeds (Chojnowski et al., 1997). These processes involve DNA repair
128 mechanisms (nucleotide and base excision repair, homologous recombination,
129 chromatin remodeling, small-RNAs mediated repair), replacement of damaged
130 ribosomal RNA (rRNA) and response of antioxidant mechanisms (ROS scavengers,
131 enzyme synthesis) (Ventura et al., 2012). Besides, Nakaune and co-workers (2012)
132 determined in tomato exposed to short-term priming that osmo- and hydro-priming
133 improves seed germination probably by affecting plant hormones concentrations of
134 abscicic acid (ABA) and especially gibellerin (GA). Lin et al. (2013) observed that
135 nitric oxide (NO) and ethylene cooperate to increase germination rate of *Arabidopsis*
136 seed exposed to salinity stress by reducing the accumulation of H_2O_2, one of the main
137 reactive oxygen species (ROS). This finding could be related to the protection observed
138 by priming in the seeds analyzed in present work.

139 PEG-400 imbibed seeds showed lower significant germination values (0.72 ± 0.03)
140 than the control seed pool. Such toxicity was sometimes related to the presence of
141 metallic or organic ions, but even though these ions could be removed by
142 chromatographic techniques, plants roots are not completely impermeable to PEG,
143 which has been reported to exert toxicity towards the seeds (Plaut and Federman, 1985).
144 Its uptake and translocation through the plant could cause damage by blocking water
145 pathway (inducing desiccation) (Lawlor, 1970), phosphorus transport inhibition across
146 the root to the xylem (Emmert, 1974), or to the low O_2 solubility in PEG solutions
147 (Mexal et al., 1975).

148

149 **2.3. Seed drying after imbibition**

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150 **2.3.1. Vacuum drying (VD)**

151 After imbibition, vacuum and freeze-drying were assessed as drying techniques. VD
152 has the advantage to be easy to perform, uses worldwide-available and relatively low-
153 cost equipments, and requires short processing times compared to freeze-drying.

154 Imbibed and non-imbibed (control) seeds were subjected to different times of
155 vacuum drying, which leads to different water contents of the seeds. The germination
156 capacity was determined after drying, and the results are showed in **Figure 2** (a and b)
157 for a wide range of water contents. Water imbibed seeds showed an even greater loss of
158 germination capacity along dehydration than the control seeds, showing only good
159 germination capacity values at high wc values, demonstrating their susceptibility to
160 dehydration, even higher than that shown in Figure 1. Trehalose imbibed seeds
161 maintained a very high level of germination capacity (> 75%) among all the dehydration
162 range (going from imbibed, with 161.5% of wc, to very dried, with 5% of wc). The
163 germination capacity of PEG imbibed seeds remains almost unchanged during
164 dehydration, being very low in all the range.

165 Trehalose imbibition and vacuum drying combination showed to be the best of the
166 analyzed strategies to dehydrate seeds with great conservation of the germination
167 capacity.

168 Proteins state (native or denatured) was considered as an overall marker of the
169 physiological state of the seeds. Denaturation process may occur during both imbibition
170 and/or drying, as well as during DSC scan. It is important to remark that the
171 endothermal event corresponding to protein denaturation is related to the amount of
172 native protein present in the system, which has the opportunity to occur during DSC
173 scanning. The proteins which have been previously denatured by any stress do not show
174 the typical endothermal. Thus, the denaturation enthalpy value obtained in the DSC

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175 scans correlates inversely to the amount of denatured proteins during imbibition and
176 drying (Michnik, 2003; Santagapita et al., 2007). The thermograms of the seeds showed
177 two main endothermic events: the first event between -40 and 10 °C was assigned as
178 melting of lipids; the second event, starting at temperatures > 60 °C, was assigned as a
179 protein denaturation, as shown in **Figure 3a**. *Salix* seeds contain over 20 % of lipids and
180 35 % of proteins (Maroder, 2008). Lipid crystallization was also observed during DSC
181 scans from 25 to -100 °C (data not shown). The rescan shows an unchanged melting
182 event and the disappearance of the second event, which are consistent with lipid melting
183 and protein denaturation events, respectively. These assignments were also supported
184 by comparison with thermograms of other seeds (Crane et al., 2003 and Matiacevich et
185 al., 2006 for melting of lipids in *Cuphea* and quinoa seeds, respectively, and Leprince
186 and Walters-Vertucci, 1995, and Sanchez del Angel et al., 2003 for protein denaturation
187 in bean and corn seeds, respectively). **Figure 3b** shows a detail of protein denaturation
188 event: two fractions were clearly distinguished, the first one with peak temperatures
189 between 60 and 80 °C, and the second one with peak temperatures between 100 and 115
190 °C. Along dehydration, these two fractions "move" to higher temperatures of
191 denaturation as a consequence of the reduction on mobility, and hence, more energy is
192 needed to achieve the denaturation (Hägerdal and Martens, 1976). **Figure 4** shows the
193 changes on both protein denaturation fractions for control (a), water (b), trehalose (c)
194 and PEG (d) imbibed seeds. Changes on protein denaturation temperature were
195 observed along dehydration for all the analyzed systems (**Figure 4**). All imbibed seeds
196 showed higher enthalpy of denaturation (ΔH) on the second fraction with respect to the
197 first fraction (**Figure 4b-d**). After dehydration, the enthalpy of the second fraction was
198 strongly reduced in all imbibed seeds. A similar trend was observed for the enthalpy of
199 the first fraction for control, and water- and PEG-imbibed seeds, with the exception of

200 trehalose-imbibed seeds, which showed even an increase of these values (**Figure 4c**),
201 indicating that not only the kinetics, but also the thermodynamic aspects of protein
202 denaturation were affected (Remmele and Gombotz, 2000; Santagapita et al., 2007) by
203 the interactions with trehalose. Enthalpy denaturation values of control and all imbibed
204 seeds along vacuum drying are shown in **Figure 5** as a function of germination degree.
205 In the case of trehalose-imbibed seeds, the maintenance of high enthalpy values along
206 dehydration, which implies that a great amount of proteins maintained their native state
207 along this process, correspond to the conservation of a great germination capacity along
208 dehydration. Instead, control seeds, and water and PEG-400 imbibed seeds subjected to
209 vacuum drying showed a decrease in the denaturation enthalpy values along
210 dehydration, which implies that dehydration strongly affects protein's functionality,
211 which correspond to the lower germination capacity observed (**Figure 5**).

212 Besides protein denaturation, other mechanisms could be involved in the loss of
213 germination capacity, such as membranes damage. Then, lipids changes were further
214 analyzed by DSC. In *Salix spp* seeds the chloroplasts with chlorophyll and thylakoid
215 membranes are conserved in mature seeds (Maroder et al., 2003), which could lead to
216 the production of free radicals (FRs) by auto-oxidation affecting phospholipids,
217 glycoproteins and the relation between PUFAs and SFAs (Roqueiro et al., 2010 and
218 2012). Some authors have reported that the auto-oxidation process generates FRs and
219 ROS which produce damage to molecules, membranes and organelles (Priestley and
220 Leopold, 1983; Priestley, 1986; Wilson and McDonald, 1986; Ponquett et al., 1992;
221 Maroder et al., 2003; Roqueiro et al., 2010). In the dehydrated state, molecular defenses
222 against FRs and ROS would be insufficient and/or scarcely efficient, and enzymatic
223 defenses inactive (Nandi et al., 1997; Bailly, 2004). It is to be noted that the oxidative
224 reactions take place at low water contents and they have less mobility restrictions than

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225 other deteriorative reactions, since dehydrated biological materials have surface lipids
226 and oxygen has access to them (Nelson and Labuza, 1992; Sun et al., 2002). Table 1
227 shows the enthalpy values (ΔH) of lipid melting of seeds without imbibition, subjected
228 to different vacuum drying times (and, as a consequence, samples had different wc). An
229 initial reduction on lipids enthalpy values was observed during the early stages of
230 vacuum drying (from 11.0 to 10.1% of wc, on dry basis), while the continuous
231 removing of water (from 10.1 to 2.1%) did not produced any further changes on
232 enthalpy values. Also, melting temperature changes were observed in samples dried
233 from 11.0 to 10.1%, varying both onset and peak temperatures from -24 to -15.9 °C and
234 -7.6 and -5.3 °C, respectively. These changes could indicate modifications in the ratio
235 between PUFAs and SFAs, which are in agreement to those observed by Roqueiro and
236 co-workers (2010 and 2012) attributed to the damage produced by FRs and ROS.
237 Recent studies in desiccation tolerance of maize embryos during development and
238 germination showed the importance of the antioxidant enzymes (superoxide dismutase,
239 catalase, ascorbate peroxidase, glutathione reductase and dehydroascorbate reductase) to
240 scavenge ROS species and control malonyldialdehyde (MDA) content (Huang and
241 Song, 2013). MDA is a product of lipid peroxidation which could damage membranes,
242 being responsible of the principal cause of deterioration in orthodox seeds (Smith and
243 Berjak, 1995).

244

245 **2.3.2. Freeze-drying**

246 The germination capacity of imbibed seeds after freeze-drying was also evaluated.
247 As it can be seen in **Figure 6** (black bars), FD after imbibition is not a good option to
248 preserve germination capacity when trehalose or water were used as imbibition agents.
249 However, PEG imbibed seeds maintained around 50% of the germination capacity

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250 previous to dehydration. Also, considering the results obtained using vacuum drying,
251 PEG behaviour was better when seeds were FD instead of VD, as it can be seen in
252 **Figure 6**.

253 The effect of freezing, as a previous step to freeze-drying, was studied in the
254 imbibed seeds, as shown in **Figure 6**. After freezing, a great loss of germination
255 capability was observed in trehalose and water imbibed seeds; instead, PEG seeds
256 maintained their ability to germinate. After FD (final wc of 5% db), a 30% decrease of
257 the germination capability was observed in the PEG imbibed seeds in comparison to the
258 frozen ones. This result implies that even though the freezing step was critical for all the
259 samples (especially for trehalose and water imbibed seeds), in the case of PEG imbibed
260 seeds, FD also affected the germination capacity of the seeds.

261 The loss of germination capability during freezing or later FD was possibly related
262 to freezable water, which can extensively damage the cells during freezing and thawing
263 (Wesley-Smith et al., 2004). As revealed by DSC thermograms of the imbibed seeds,
264 water melting was observed in all the imbibed seeds, and followed the increasing order:
265 PEG< trehalose< water, as shown in **Figure 7**. The lower water content and water
266 crystallization degree observed in PEG imbibed seeds in comparison with those of the
267 imbibed seeds with trehalose or water promoted a lower reduction of the germination
268 capability of imbibed seeds. Interfaces of crystals produced by water (especially) and oil
269 crystallization could cause membrane damage, affecting germination capacity as
270 observed after freezing and freeze-drying. Water crystallization was not observed in
271 control seeds. The lipids melting showed in **Figure 3a** is not observed in **Figure 7**
272 because its enthalpy is very small in comparison with water melting event.

273 Summarizing, water, and especially trehalose-imbibed seeds subjected to vacuum
274 drying showed better germination capability in comparison with the freeze-dried ones,

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275 revealing that both the amount of crystallized water and the presence of adequate
276 protective agents were the main critical factors involved in *Salix nigra* seed
277 conservation.

278

279 **2.4. Color changes of imbibed and dried seeds**

280 Macroscopic color changes on seeds were observed during germination analysis.
281 **Figure 8** shows the color parameters values of seeds imbibed in water, trehalose, or
282 PEG and after VD and FD processes at 48 h of germination. Germinated seeds showed
283 higher luminosity (L*) values than not-germinated seeds (**Figure 8a**) independently of
284 the employed protective agent.

285 The germinated seeds showed an intense green color, reflected in negatives a*
286 values (related to greenness degree, as shown in **Figure 8b**) than those of the not-
287 germinated seeds. The seeds that were not able to germinate developed brown
288 coloration along germination represented by slightly negative or even positive a* values
289 (redness). Besides, not-germinated seeds presented an important loss of yellowness (b*
290 values) in comparison with germinated seeds (**Figure 8c**).

291 It is important to note that the global color, and particularly the a* coordinate
292 allowed to distinguish the germinability at the first stages of germination, independently
293 of the drying process or protective agents employed. The not-germinated seeds were
294 darker, had a low greenness degree, with also a low value of the b* coordinate.

295

296 **3. Conclusions**

297 – Present work reveals that the presence of adequate protective agents as well as
298 the dehydration method were the main critical factors involved in willow seed
299 desiccation tolerance.

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300 – Trehalose imbibition of seeds followed by vacuum drying comprise a promising
301 method to improve willow seed desiccation tolerance.

302 – Protein denaturation determined by DSC reveals changes on germination
303 capacity for vacuum dried seeds.

304 – Freeze-drying after imbibition severely affected seed germination capacity due
305 to the ice presence during the freezing step.

306 – Color measurement (particularly the a* coordinate) was detected as a suitable
307 index to predict germinability at the first 48 h of germination.

308

309 4. Materials and methods

310 *4.1.* Seed recollection and storage

311 *Salix nigra* L. seeds were collected in Castelar (Buenos Aires, Argentina) at the
312 INTA-Castelar experimental field during October/ December 2010. Collected seeds are
313 usually first dehydrated at 20 °C for 3 h from wc 41% to 9-12% (wet basis), leaving the
314 seeds with water activity (a_w) values between 0.8 and 0.7. Seeds were stored at -70 °C
315 in individual micro-centrifuge tubes and were protected from light by using a black
316 plastic bag. Control seeds are the ones which are not imbibed and are not subjected to
317 any additional treatment (freezing, freeze-drying or vacuum drying).

318

319 4.2. Preservation treatments

320 4.2.1. Imbibition

321 Imbibition was conducted with 45% w/v trehalose, polyethylene glycol 400 –PEG–
322 or water in 5 mL glass vials at 4 °C for 16 h prior to dehydration treatments.

323 After that, the imbibed seeds were taken from the respectively solution, then were
324 placed on filter paper and cleaned with distilled water to remove excess solution.

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325

326 4.2.2. Freezing and thawing (f/t)

327 Imbibed seeds were placed in 5 mL glass vials and frozen at -20 °C (conventional
328 freezer) for 24 h. Thawing was performed at 5 °C until seeds were completely unfrozen.
329 Thawing was performed at 5°C since freezing rate was quite slow and the thawing rate
330 should not differ too much from the freezing rate in order to provide a slow re-warming
331 avoiding cells damage (Wood et al., 2003).

332

333 4.2.3. Dehydration

334 Seeds dehydration was performed by two different methods: vacuum drying (VD)
335 and freeze drying (FD).

336 VD was performed in an oven operating at a chamber pressure of 11.300 Pa at 25
337 °C, containing dried silica gel. Samples were dried from 10 min to 5 h. Seeds with and
338 without imbibition have always been treated separately so that the soaked seeds do not
339 rehydrate the other ones.

340 FD of seeds (frozen 24 h at -20 °C and exposed to liquid nitrogen) was performed
341 for 24 h in an ALPHA 1-4 LD2 freeze drier (Martin Christ Gefriertrocknungsanlagen
342 GmbH, Osterode am Harz, Germany) operating at a condenser plate temperature of -55
343 °C and a minimum chamber pressure of 4 Pa. The main drying was performed without
344 shelf temperature control. Secondary drying was performed at 25 °C.

345 After dehydration, the seeds were maintained in vacuum desiccators at 4°C until
346 their corresponding treatment/s or property determinations.

347

348 4.3. Determination of the seeds characteristics

349 4.3.1. Germination rate

350 Briefly, four replicates of 25 seeds each were set to germinate over wet paper in 6
351 cm Petri dishes at 25 ± 1 °C for 72 h (16/8 day/night light cycle) (Maroder, 2008).
352 Germination was evaluated according to ISTA Rules (ISTA, 2005): it was considered
353 that seeds had germinated when it was possible to see the development of hypocotyls,
354 cotyledons and root. At the moment of the present work, the seed control-pool retained
355 77% of the germination capacity, and had 11 %(dry basis) and 0.745 of water content
356 and activity, respectively. For the time frame of the experiment, untreated control seed
357 pool were kept in the freezer at -20°C. Using freezer or ultra-freezer (-20 or -70°C,
358 respectively) provided similar and adequate conservation of the control seed pool.
359

360 4.3.2. Thermal transitions by differential scanning calorimetry (DSC)

361 Protein denaturation and lipid melting were determined by DSC using a Mettler
362 Toledo 822 DSC (Mettler Toledo AG, Switzerland) and STAR^e Thermal Analysis
363 System version 3.1 software (Mettler Toledo AG). The instrument was calibrated using
364 standard compounds (cyclopentane and indium) of defined melting point and heat of
365 melting. All measurements were made in duplicate with 5-10 mg sample mass, using
366 hermetically sealed 40 μL aluminum pans (Mettler). The material was cooled at the
367 higher rate available for the equipment (around -40/-50 °C/min) from 25 to -100 °C and
368 then was heated from -100 °C to 140 °C at 10 ºC/min; an empty pan was used as a
369 reference. The confidence interval estimated for temperature values and for enthalpy
370 values were 2 °C and 10 mJ, respectively.
371

372 4.3.3. Water content and water activity

373 The water content (wc) of the seeds was determined gravimetrically by difference in
374 weight before and after drying in a vacuum oven for 1 h at 130 ± 2 ºC (ISTA, 1999).

375 These drying conditions were adequate to determine water content in the studied
376 systems with a confidence interval of 6% to 95% certainty. The water content was
377 expressed in dry basis (amount of water related to the dried matter, d.b.).

378 Water activity (a_w) was determined by means of a drew-point Aqualab instrument
379 (Decagon Devices, Inc, Pullman, WA, USA). A special sampler holder was used to
380 reduce the number of seeds to be placed, and the corresponding calibration curve was
381 performed with salts of known a_w (Greenspan, 1977).

382

383 4.3.4. Color measurement by image analysis

384 Seeds color changes were determined by image analysis. The computer system
385 vision consisted of three elements: a lighting system, a digital camera and a personal
386 computer. The lighting system included a D65 lamp (this illuminant corresponds to
387 solar irradiation with a color temperature of 6500 K (Agudelo-Laverde et al., 2011 and
388 2013)) inside a gray chamber (corresponding to N7 in the Munsell color space). The
389 warm up time of the lamps was 15 min. The angle between the camera axis and the
390 sample plane was 45º and the angle between the light source and the sample plane was
391 90º, in order to capture the diffuse reflection responsible for color (Yam and Papadakis,
392 2004). A high-resolution (10.1 megapixel) digital camera model EOS 40D (Canon Inc.,
393 Tokyo, Japan) was used, with an EF-S 60 mm f2.8 macro lens (Canon Inc.). The digital
394 camera was operated in manual mode, with the lens aperture at f = 6.3 and speed 1/8 s
395 (no zoom, no flash) to achieve high uniformity and repeatability. The calibration of the
396 chromatic parameters used for image capture is described in Briones and Aguilera
397 (2005). Images have a resolution of 3,888 x 2,592 pixels and were stored in JPEG
398 format using Canon's Remote Capture program (EOS Utility, Canon Inc.). The images
399 were taken using white background. The color functions selected to follow the seed

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400 color changes were the CIELAB coordinates (L*, a*, b*). For this study, fifty seeds
401 were located in Petri dishes in strict order. By the germination analysis, two groups of
402 samples were separated: germinated and non-germinated. The color change of each
403 single seed was measured along the germination time. Average and standard deviation
404 values are informed.

405

406 4.3.5. Statistical analysis

407 One way analysis of variance (ANOVA) was applied on the results of the chromatic
408 coordinates and on germination index, using the program Prism v5 (GraphPad Software,
409 Inc., San Diego, CA, USA).

410

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416

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Legends for Figures

Figure 1. Seed germination as a function of water content (wc). Different wc were reached by leaving the samples for different times under vacuum drying. The gray arrow shows the starting point for control seeds; the dotted line is only indicative.

Figure 2. Seed germination related to different water content obtained after different times of vacuum drying. Seeds were imbibed with trehalose, water or PEG. Not imbibed seeds (control) were also included for comparative purposes. Lines are only indicative.

Figure 3. a) DSC thermograms of control seeds showing lipid melting and protein denaturation events. The dotted line correspond to the rescan of the same sample. b) Detail of the protein denaturation showing two events and the corresponding changes in both temperature and enthalpy after vacuum drying (indicated by arrows). The samples correspond to water-imbibed seeds (wc of 150 % db) and control seeds (wc of 11% db). Gray dotted lines were included to show the employed criteria for transitions assignment.

Figure 4. DSC thermograms showing the changes on protein denaturation fractions of control (a), water (b), trehalose (c), and PEG (d) imbibed seeds. Imbibed and dried samples correspond to water content between 66 and 185 % d.b., and between 48 and 5 % d.b., respectively. Gray dotted lines were included to show the employed criteria for transitions assignment.

Figure 5. Denaturation enthalpy of proteins (considering the sum of both fractions) of vacuum dried seeds without imbibition (control) and imbibed in water, trehalose and PEG-400 as a function of germination. Enthalpy values were normalized by water content values.

Figure 6. Seed germination capacity of imbibed seeds after freezing, freeze-drying (FD) and vacuum drying (VD). FD and VD seeds with wc around 5 % were selected for comparative purposes. *: no germination was observed due to the damage occurred during freezing.

Figure 7. DSC thermograms of imbibed and control seeds showing water melting. Corrected enthalpy values (normalized by water content, expressed in % wet basis, wb) were included.

Figure 8. Color coordinates L* (a), a* (b) and b* (c) of water, trehalose, and PEG imbibed seeds after imbibition, FD and VD seeds at 48 h of germination. The mean and 95% confidence intervals are reported for germinated (circles) and not-germinated (empty squares) seed groups. The shaded zone correspond to germinated seeds.

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Table 1. Enthalpy values, and onset and peak temperatures related to lipid melting of control and vacuum dried-control seeds at different wc values. Enthalpy values were normalized by water content values.

wc (% db)	ΔH (J/g_{db})	T_{onset} (°C)	T_{peak} (°C)
12	-12 ± 1 [a]	-24 ± 1 [a]	-7.6 ± 0.2 [a]
11.2	-6.3 ± 0.7 [b]	-15.9 ± 0.7 [b]	-5.1 ± 0.2 [b]
9.1	-6.3 ± 0.7 [b]	-17.9 ± 0.8 [b]	-5.4 ± 0.2 [b]
7.7	-7.3 ± 0.8 [b]	-17.6 ± 0.8 [b]	-5.2 ± 0.2 [b]
4.5	-6.8 ± 0.6 [b]	-19.1 ± 0.9 [b]	-5.4 ± 0.2 [b]
2.1	-6.7 ± 0.7 [b]	-18.6 ± 0.9 [b]	-5.2 ± 0.2 [b]

Significant differences due to the drying/water content are indicated with different letters ($P < 0.05$) for each parameter.

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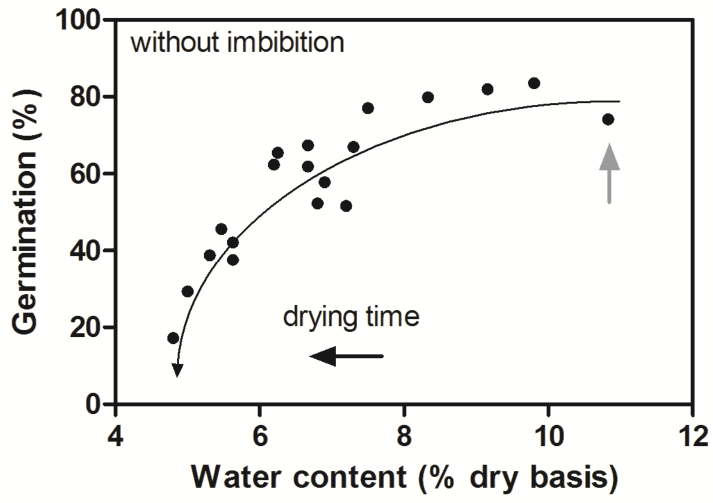

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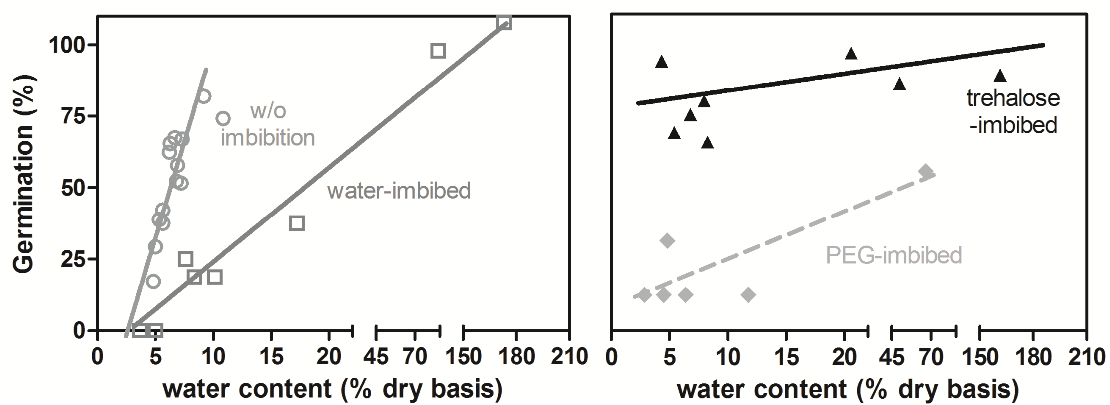

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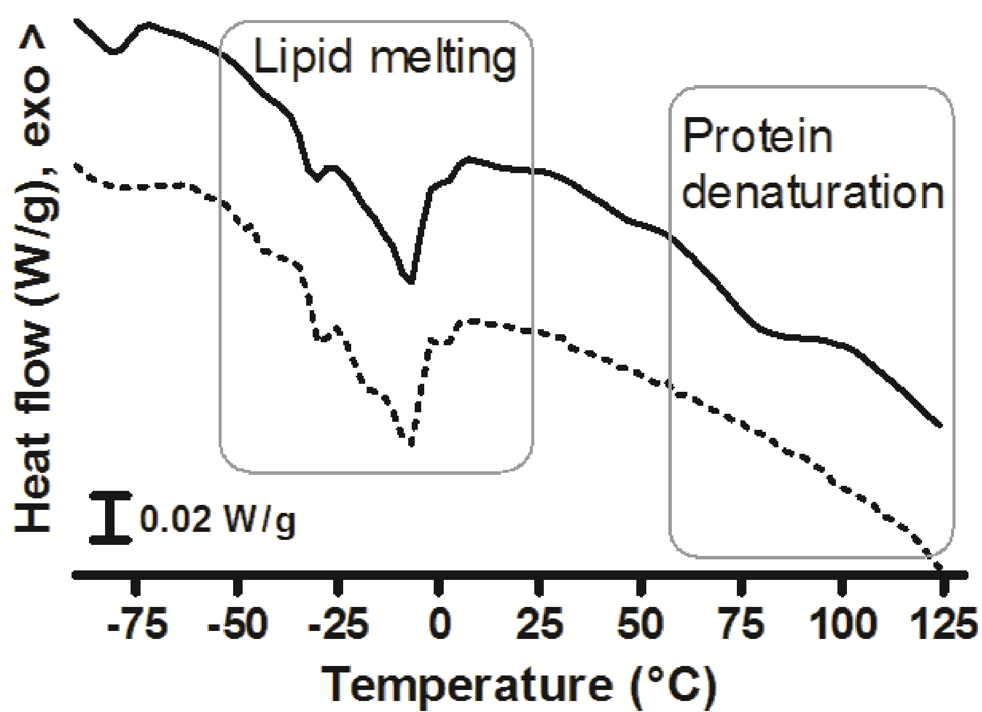
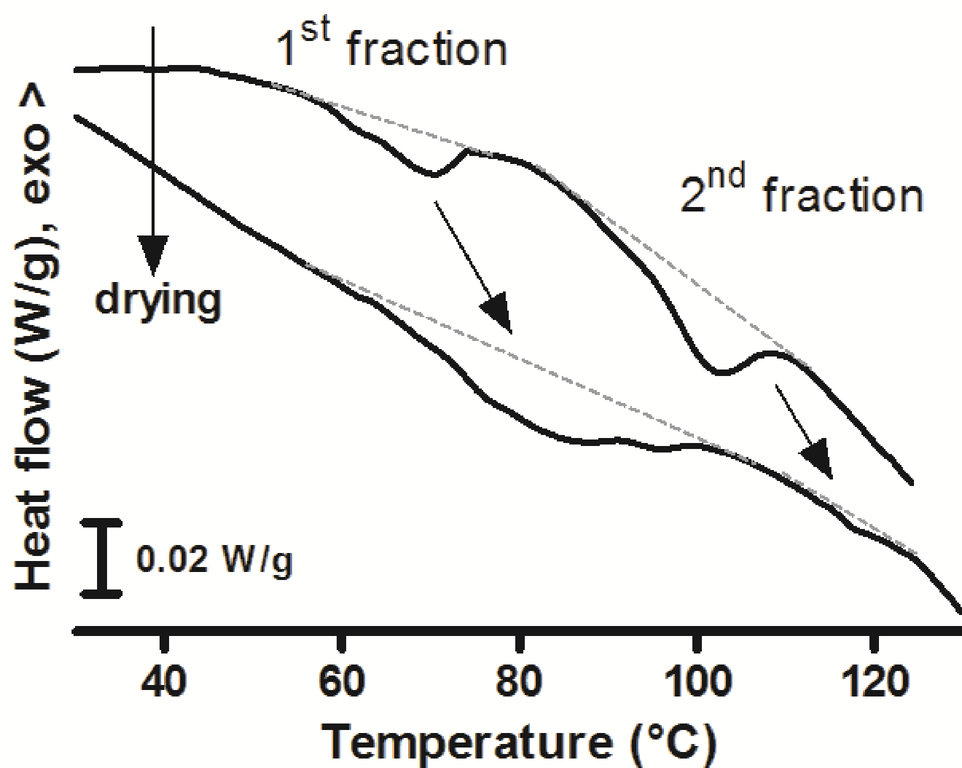

Lipid melting
Protein denaturation
Heat flow (W/g), exo >
0.02 W/g
-75 -50 -25 0 25 50 75 100 125
Temperature (°C)


1st fraction
2nd fraction
drying
Heat flow (W/g), exo >
0.02 W/g
40 60 80 100 120
Temperature (°C)

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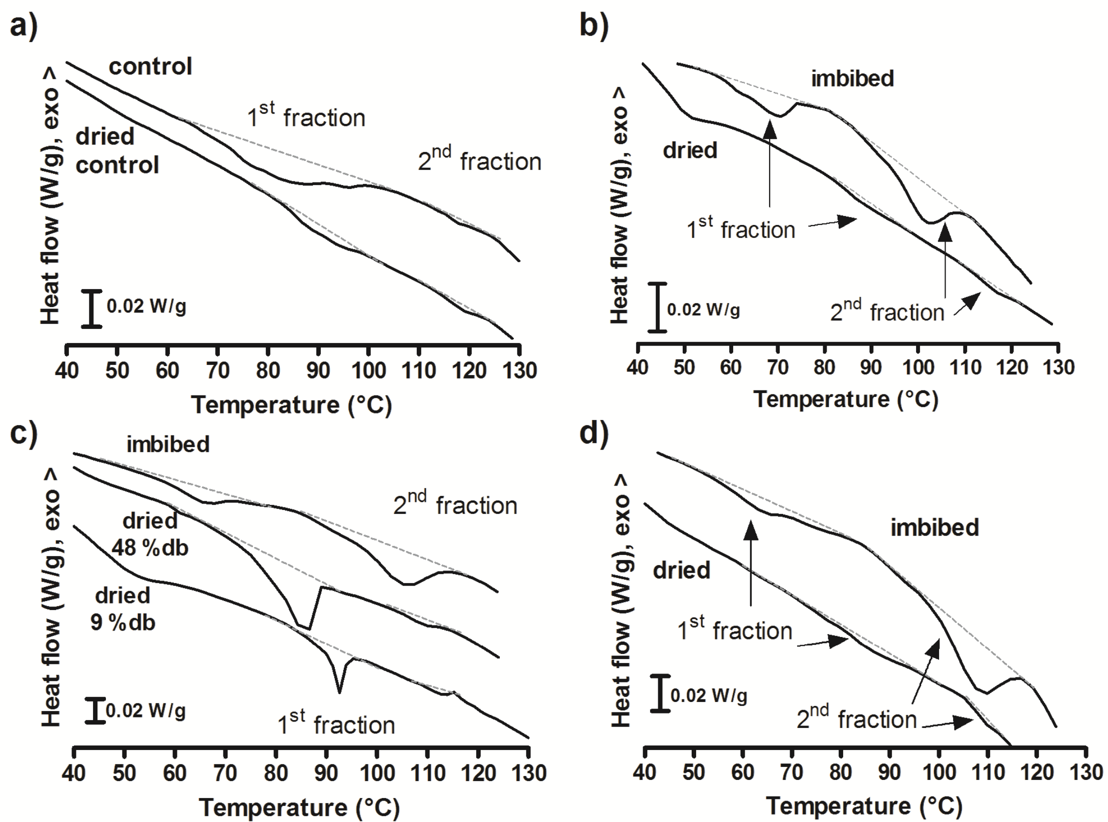

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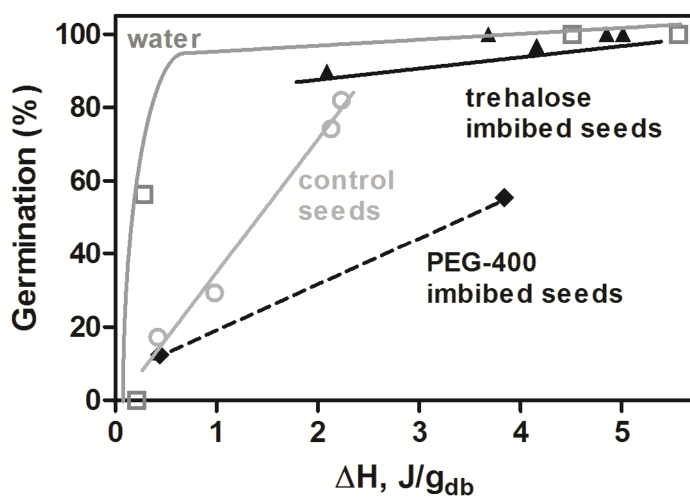

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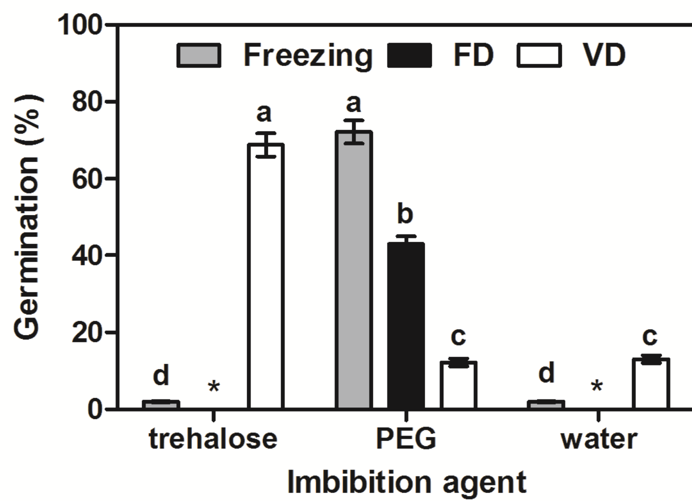

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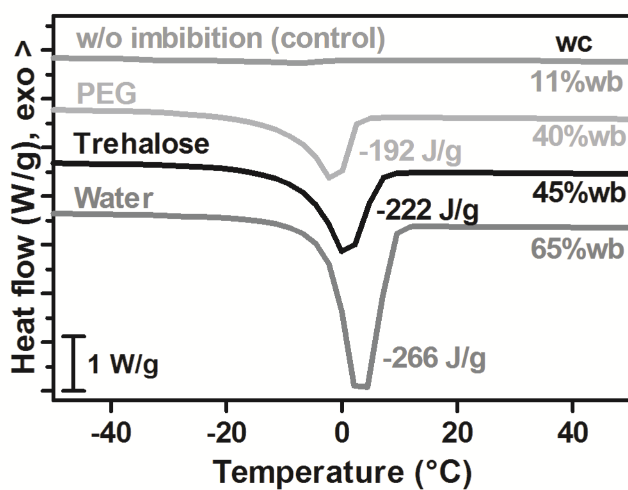

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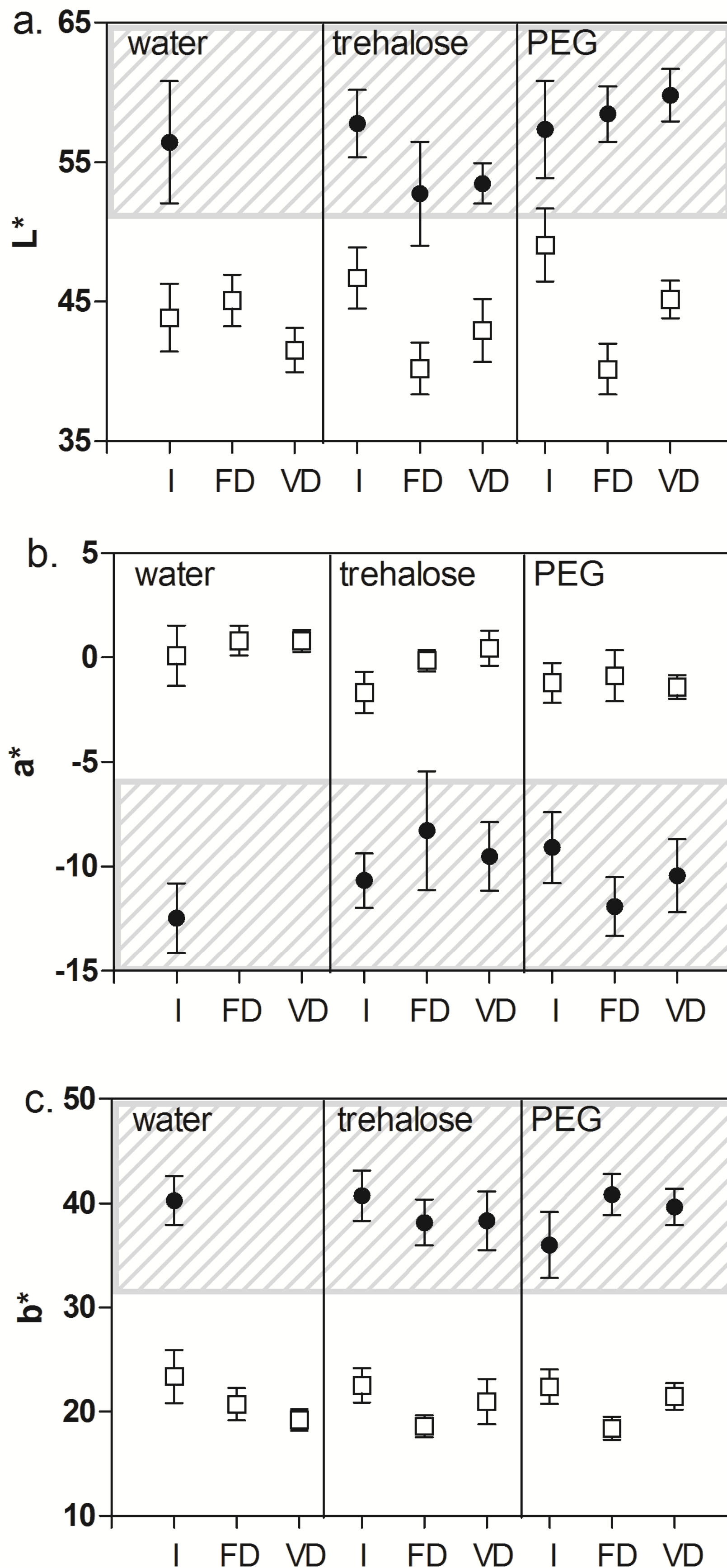

a.
L*
65
55
45
35
water
trehalose
PEG
I
FD
VD
I
FD
VD
I
FD
VD
b.
a*
5
0
-5
-10
-15
water
trehalose
PEG
I
FD
VD
I
FD
VD
I
FD
VD
c.
b*
50
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water
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Highlights

- Willow seeds were subjected to imbibition and subsequent vacuum or freeze-drying
- Trehalose imbibition followed by vacuum drying provided >75% seed germination
- Protein changes could be determined by DSC
- Dehydration affected proteins functionality leading to lower germinability
- The a* coordinate correlated with germinability at the first stages of germination

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Contribution

Impact of protective agents and drying methods on desiccation tolerance of *Salix nigra* L. seeds

Santagapita PR (1,2), Ott Schneider H (1), Agudelo-Laverde M (1,2), Buera MP (1,2,*)

Contributions of each author:

Santagapita PR: Patricio conducted all DSC determinations and data analysis. He was one of the supervisors of Helena Ott Shneider during her mobility period, and strongly contributed planning the experiments, supervising data analysis, and with manuscript writing.

Ott Schneider H: Helena conducted a huge part of the experiments. She prepared the solutions for seed imbibition and performed several analysis (germination, water content and water activity determinations), as well as freezing and dehydration treatments. Her work was a part of a mobility for her formation as Food Engineer (Agrosup Dijon, France).

Agudelo-Laverde M: Marcela conducted image acquisitions for color measurement analysis. She processed and analyzed the images. She also participated during manuscript writing and edition.

Buera MP: Pilar was one of the supervisors of Helena during her mobility period. She strongly contributed planning the experiments, discussing results, and with manuscript writing and edition.

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