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1 **Etching of the seed cuticle by cold plasma shortens imbibitional leakage in *Linum***
2 ***usitatissimum* L.**

3

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21

22

23 **Abstract**

24 Pre-sowing treatment of seeds with cold plasma, a reactive mixture of charged particles,
25 is an emerging, environmentally friendly method, shown to improve water absorption,
26 germination and seedling growth in many crop species. We studied how plasma affects
27 different structural layers in flax seed coats, and focused on the effects on some of the
28 earliest germination events, directly related to imbibition: solute leakage and mucilage
29 extrusion. We characterized the ultrastructure of flax seed coats using scanning electron
30 microscopy (SEM) and observed mucilage extrusion *in situ*, using environmental SEM.
31 Time-courses of water uptake and solute leakage during germination were recorded,
32 respectively, by weighing the seeds and semi-quantitative metabolite profiling of the
33 exudates using GC-MS, LC-MS and LC-UV. Plasma treatment perforated the cuticle of
34 flax seeds but did not visibly etch the underlying mucilage secretory cell (MSC) walls.
35 Plasma treated seeds took up more water than untreated seeds during imbibition.
36 Mucilage expansion of untreated seeds detached the distal MSC walls from the radial
37 walls and extrusion occurred, relatively slowly, via sparse cracks in the distal cell wall
38 layer. In plasma treated seeds, mucilage extrusion occurred fast, via rupture of distal cell
39 walls of almost every single MSC, and the junctions between distal and radial MSC walls
40 remained intact. Initially, imbibitional solute leakage was higher in plasma treated seeds
41 but the leakage had quasi stopped after one hour of imbibition. In untreated seeds, the
42 leakage continued amply more than two hours after the start of imbibition. Fragments of
43 the lignan macromolecule, stored in parenchymatous cells under the MCS, were released
44 more abundantly in the spermosphere of plasma treated than untreated flax seeds. It is
45 concluded that plasma treatment etches the cuticle of flax seeds and weakens the
46 underlying MSC cell walls, and that such treatment leads to significantly faster recovery
47 of membranes during imbibition, reducing leakage time in germinating seeds.

48 **Keywords**

49

50 *Linum usitatissimum* L., Seed, Plasma, Germination, Mucilage extrusion, Imbibitional

51 leakage

52

53

54

55 **1. Introduction**

56 **Low** temperature plasma is being studied as an environmentally friendly, cost efficient and
57 effective approach to improve seed performance (Powell, 2010; Randeniya and de Groot,
58 2015; Zahoranová et al., 2016). A plasma is a fluid-like mixture of free electrons, ions,
59 radicals and neutral particles, obtained by the excitation of gaseous molecules and atoms.
60 Surfaces that are exposed to this very reactive environment can undergo a variety of changes,
61 depending on the nature of the substrate, the energy, and the source gas composition (Boenig,
62 1982). **Various** studies have demonstrated that treatment of seeds with low temperature
63 plasma generated from air, N₂, O₂, water vapor, or inert gases (He or Ar), can reduce
64 pathogen or chemical contamination, improve germination efficiency, and stimulate plant
65 growth (**Randeniya and de Groot (2015)**). The mechanisms by which plasma treatment
66 influences germination and plant growth are not fully understood, and probably a
67 combination of effects is involved. For example, in seeds suffering from high mortality due to
68 fungal infections, the removal of microbial layers by plasma treatment may be the
69 determining factor leading to improved germination (Dhayal et al., 2006). However, for seeds
70 that are generally not much affected by infections or pathogen attacks, the main cause of the
71 plasma effect on germination must be different. The growth promoting action of cold plasma
72 treatment may be a response to a mild oxidative stress, resulting from reactive oxygen species
73 (ROS) and/or reactive nitrogen species (RNS) that are generated at the seed surface by air or
74 water vapor plasma (Holubová et al., 2020). The moisture content of the seeds has been
75 accounted for the creation of ROS when inert gases are used for producing plasma. Mild
76 doses of oxidative stress can trigger signal cascades that interact with phytohormone activity
77 and stimulate growth, a process which is referred to as “hormesis” (Małkowski et al., 2020).
78 On the other hand, for all plasma treated seeds with enhanced germination rate, the plasma
79 treatment had increased the hydrophilicity of the surface, which was demonstrated by the

80 decrease of the contact angle of a water droplet on the seed, and the imbibition had been
81 accelerated as compared to untreated seeds (Bormashenko et al., 2012; Ling et al., 2014). It is
82 generally agreed that altered wettability of the surface and accelerated imbibition are the
83 major reasons for the modification of germination efficiency in plasma treated seeds. Indeed,
84 seeds that do not imbibe water readily are referred to as “hard” and cause physical dormancy
85 in certain families (Baskin et al., 2000). A decrease of the contact angle by plasma treatment
86 may be caused by the removal of organic contaminants, oxidation of the surface, changes in
87 functional groups, etching and micro-roughening, or a combination of those. Scanning
88 electron microscopy images revealed increased roughness and the appearance of holes or
89 cracks at the surface of plasma treated seeds (Kitazaki et al., 2014; Stolárik et al., 2015; Tong
90 et al., 2014; Wang et al., 2017). Wang et al. (2017) suggested that cellulose in the seed coats
91 is broken down, as was observed in plasma treated wood surfaces (Jamali and Evans, 2011).
92 It has been proposed that the cracks or reduced thickness increase the permeability of the
93 seed coat (Randeniya and de Groot, 2015). However, the mechanical basis of the seed coat
94 roughening, increased wettability and permeability, needs further investigations.

95 The seed coat is a complex layered structure built of differentially specialized cell layers and
96 derived from the inner and outer integuments of the ovule. Today, it is not totally understood,
97 neither what structural components are involved in controlling water movement into the seed,
98 nor which cell layers of the seed coat are affected by plasma treatment. In the flax seed coat,
99 the mechanical layers are believed to be formed by the inner integument, which consists of an
100 innermost layer of thick-walled pigment cells, and an outer layer of longitudinally oriented
101 fibrous elements (Boesewinkel, 1980). The outer integument consists of an innermost layer of
102 sclerified cells, a layer of parenchymatous cells, and, outermost, the epidermis covered with a
103 rugose, plicate cuticle, poor in cutin (Boesewinkel, 1980). The epidermis cells of flax seeds
104 are differentiated into mucilage secretory cells (MSC), containing dehydrated pectinaceous

105 mucilage. When a flaxseed imbibes water, the mucilage expands in the MSCs, causing a
106 rupture of the cell walls and extrusion of the mucilage (Miart et al., 2019). Variability in
107 mucilage extrusion efficiency among different flax recombinant inbred lines (RILs) has been
108 related both to the mucilage swelling and hydration properties, and to the mechanical
109 properties of the distal epidermal walls (Miart et al., 2019). In *Arabidopsis* it has been shown
110 that, upon water uptake, seeds actively degrade the outer cell wall through the expression of
111 cell wall modifying enzymes, whose activity is essential for mucilage extrusion (Kunieda et
112 al., 2013; Saez-Aguayo et al., 2013). Therefore, it can be expected that the ablation of the
113 distal cell walls of flax seed integuments by plasma treatment, and the resulting changes in
114 wettability can affect the efficiency of mucilage extrusion.

115 The spermosphere is defined as a zone of elevated microbial activity around a germinating
116 seed and this microbial activity is driven largely by the carbon compounds released into the
117 soil during imbibition of the seed (Verona, 1958). Besides mucilage extrusion, which is
118 specific for mucilaginous seeds, imbibition of dry seeds leads, more generally, to an
119 immediate leakage of low molecular weight metabolites into the environment. This leakage is
120 referred to as “seed exudation”. An interesting feature of flax seeds is that, in the outer
121 integument just under the MSCs, parenchymatous cells accumulate extremely high amounts
122 of the lignan secoisolariciresinol diglucoside (SDG) (Fang et al., 2016). In contrast to free
123 lignans found in other tissues or plants, SDG of flax seed integuments accumulates under the
124 form of a polyester, called the lignan macromolecule, in which the conserved repeated
125 structure is an ester between SDG and 3-hydroxy-3-methylglutaric acid (HMGA)(Struijs et
126 al., 2009; Thiombiano et al., 2020). The localization in the outer integument suggests a role
127 for this macromolecule in the interaction with the environment. Ester bonds are less stable
128 than ether linkages, found for example in lignin, suggesting that this polyester structure could

129 facilitate the release of high amounts of lignans in the spermosphere. However, no data on the
130 exudation of flax seed SDG have been reported today.

131 Microbial communities established in the spermosphere are crucial for the implementation of
132 the future plant rhizosphere, which, in turn, can significantly influence plant growth and yield
133 through the activity of plant-growth promoting rhizobacteria (PGPR) (Backer et al., 2018;
134 Schiltz et al., 2015). Today, PGPR and plant-microbe interactions are intensively studied in
135 the context of their potential applications for the development of a sustainable green
136 agriculture (Backer et al., 2018). Because plasma treatment is known to affect the hydration
137 characteristics of seeds, we hypothesized that plasma treatment can affect exudation and
138 mucilage extrusion, two processes directly linked to seed hydration. When seeds germinate in
139 the soil, such changes in carbon deposition may alter the establishment of the microbial
140 community in the spermosphere, with potentially long-lasting impact on plant development.
141 The other way around, studying the effect of plasma treatment on imbibitional events like
142 mucilage extrusion and exudation may help to improve our understanding of how plasma
143 affects the involved seed coat layers.

144 We studied here the relation between ultrastructural changes, mucilage extrusion, and
145 exudation of cold argon plasma treated flax seeds. We quantified the wettability and water
146 uptake of differently treated seeds and studied the relation between imbibition characteristics
147 and surface ultrastructure of the seeds. We used *in situ* environmental scanning electron
148 microscopy (ESEM) to study the microstructural changes caused by increasing humidity
149 levels in the microscope chamber, finally leading to mucilage extrusion. We further analyzed
150 the chemical composition of the exudates and the effect of plasma treatment on the exudation
151 dynamics through the germination process.

152

153 **2. Materials and methods**

154 2.1. Chemicals

155 Standards for the identification of *p*-coumaric acid, ferulic acid, as well as the GC-MS
156 standards and retention time index alkanes, were purchased from Sigma Aldrich (St. Louis,
157 MO, USA). The herbacetin diglucoside standard was purchased from Chromadex (Molsheim,
158 France). The secoisolariciresinol diglucoside (SDG) standards had been extracted earlier
159 from hydrolyzed flax seed integuments and authenticated by 1D and 2D NMR (Socrier et al.,
160 2019).

161

162 2.2. Plant material

163 Flax seeds (*Linum usitatissimum* L. cv. Baladin) produced in 2010 were kindly provided by
164 the seed company Laboulet (Airaines, France).

165

166 2.3. Plasma treatment

167 Plasma treatment was performed using a radio frequency plasma cleaner device (plasma prep
168 III, SPI supplies, USA). A homemade stainless-steel mesh basket was attached to the tip of
169 the specimen stage to introduce the seeds into the plasma cleaner's chamber. The gas used for
170 plasma treatment was argon, at a pressure of 0.25 Torr (mild vacuum). The frequency and
171 power of the plasma cleaner device were set at 13.56 MHz and 10 W respectively. Plasma
172 treatment duration was 1 min, 5 min, 10 min, 15 min, or 20 min. Control samples were
173 untreated (control) or mild vacuum treated (0.25 Torr) for 20 min in the plasma cleaner
174 device. Batches of ten seeds were treated at a time.

175

176 2.4. Scanning electron microscopy

177 An environmental scanning electron microscope (ESEM, FEI Quanta 200 FEG, USA)
178 equipped with an energy-dispersive X-ray microanalyser (EDX, X-Max 80, Oxford

179 Instruments Co., UK) was used. High-resolution images were obtained under high vacuum
180 after sputter coating of the seeds with 10 nm of gold using an automatic sputter coater (BAL-
181 TEC SCD 050, Balzers, Germany). For *in situ* analysis of mucilage extrusion, humidity was
182 controlled by manipulating the chamber pressure and the sample temperature with a Peltier-
183 chip controlled cooling stage, and images were captured using a gaseous secondary electron
184 detector (GSED).

185

186 2.5. Contact angle and surface wettability measurements

187 The static contact angle of water droplets (0.5 μL) on untreated and 5 min plasma treated
188 seeds was measured after baseline adjustment and curve fitting of the droplet profiles
189 captured by a contact angle measurement device (DSA30, Krüss, Germany).

190 The distribution of a 0.5 μL droplet of potassium permanganate (KMnO_4 , 0.1 M) on seed
191 surfaces of three untreated and three independently plasma treated (5 min) seeds was
192 determined using SEM imaging coupled with EDX. A potassium permanganate solution was
193 used because of its similar surface tension to that of distilled water (71 mN m^{-1} compared to
194 72 mN m^{-1}) and because it can be traced on the surface of seeds by EDX. Droplets were
195 placed on the seed surfaces within the microscope chamber using a micropipette. The SEM
196 chamber then was pumped down and maintained at “low Vacuum” at the pressure of 1 Torr.
197 Back scattered electron (BSE) images at lower magnifications and EDX maps were acquired
198 from the surface of the seeds.

199

200 2.6. Water absorption

201 Individual seeds from differently treated batches of seeds (control, vacuum, 5, 10, 15, and 20
202 min plasma) were weighed and subsequently placed in 12-well microtiter plates (a single
203 seed per well). A 1mL sterile milli-Q water was added to each well and after 0.25, 0.5, 1, 2,

204 4, or 8 hours of imbibition, five seeds of each treatment were taken from the wells and
205 immediately re-weighed. Additional seeds from control, and vacuum and 5 min plasma
206 treated batches of seeds were selected for longer imbibition time. Five seeds from each of
207 these batches were re-weighed after 24 hours, and five other after 48 hours of imbibition. The
208 amount of water uptake during imbibition is expressed as the weight increase in mg.

209

210 2.7. Germination

211 For each treatment (control, vacuum, 5, 10, 15, and 20 min plasma), six batches of 10 seeds
212 were treated, and subsequently placed on moistened filter paper in plastic Petri dishes
213 (diameter 9 cm, 3 mL sterile milli-Q water, 1 dish per batch, 10 seeds per dish). The Petri
214 dishes were covered and placed first for 48h in the dark, and then transferred to a phytotron
215 under controlled conditions (23°C, 80% humidity, light-dark cycle 16h:8h 3350 to 3500 lux).
216 The number of germinating seeds then was counted for each treatment batch after one day
217 (24 HAI (hours after imbibition)) and three days (72 HAI). **Only seeds with split seed coat for**
218 **which radicle elongation was confirmed at 72 HAI, were maintained in the counts for 24**
219 **HAI.**

220

221 2.8. Exudate preparation

222 For each treatment (control, vacuum, and 5, 10, 15, or 20 min plasma), 30 seeds, originating
223 from 3 different treatment batches, were divided over three glass Petri dishes (diameter 15
224 cm, 1 dish per batch, 10 seeds per dish). A five mL of sterile milli-Q water was added to each
225 Petri dish, (“seed medium”), and the Petri dishes were covered. This time-point corresponds
226 to the start of imbibition. At 0.5 hour after the start of imbibition (HAI), the seed medium
227 from the three dishes per treatment were transferred to three 15 mL tubes. The Petri dishes
228 were rinsed with an additional 5 mL of sterile milli-Q water and the rinse water was pooled

229 with the corresponding seed medium. The seeds were also rinsed with water at the same time
230 and transferred into a new Petri dish with five mL of sterile milli-Q water, to pursue the
231 germination and exudation process. The process of sampling and transfer was repeated at 1,
232 2, 4, 8, 24, and 48 HAI. For each treatment and sampling time, three biological replicates
233 were thus performed. In order to eliminate the debris, the tubes were centrifuged (10 min,
234 1400 g) and the supernatants were transferred to another tube. The samples were then dried
235 by lyophilization and resuspended in 1 mL of methanol/water (1/1, v /v) to which ribitol
236 (0.02 mg mL⁻¹) was added as an internal standard for the GC-MS analysis.

237

238 2.9. Liquid chromatography–mass spectrometry (LC–MS)

239 The resuspended exudates were analyzed by reversed phase HPLC (Dionex Ultimate module
240 3000 LC system, Thermo Scientific Dionex, Sunnyvale, CA, USA), equipped with a Sunfire
241 C18 column (150 9 3 mm, 3.5 µm; Waters, Milford, MA, USA) coupled to a guard column
242 (20 9 3.0 mm; Waters), and connected to electrospray ionization-high resolution mass
243 spectrometry (LTQ Orbitrap XL hybrid FTMS, Thermo Fisher Scientific, Waltham, MA,
244 USA), operated in the negative ionization mode. The buffers, HPLC gradient, and conditions,
245 as well as the parameters and settings of the mass spectrometer, were as described previously
246 for the analysis of lignan macromolecule partial saponification products (Thiombiano et al.,
247 2020). The high resolution FTMS mass spectrometer is preceded by a linear ion trap, in
248 which data-dependent MS_n fragmentation was performed. Identification of the
249 macromolecule fragments was based on concordance of retention time, exact m/z and MS_n
250 fragmentation, with previously elucidated (partial) saponification products of the lignan
251 macromolecule, analyzed on the same instruments, in the same laboratory, under the same
252 conditions (Thiombiano et al., 2020).

253

254 2.10. Gas chromatography–mass spectrometry (GC-MS)

255 For analysis of primary metabolites by GC-MS, 250 μ L of the resuspended exudates was
256 dried. Derivatization for GC-MS analysis was performed as described in Dauwe et al. (2012)
257 and subsequent GC-MS analysis was performed as described in Nguyen et al. (2017) . For
258 each compound, the signal of a representative fragment ion was quantified. The “total GC-
259 MS signal” is the sum of the quantified signals of the 30 identified compounds, at each time-
260 point. All compounds were tentatively identified based on mass spectral and retention index
261 libraries of the Golm Metabolome Database (GMD) (Kopka et al., 2005). The identity of
262 glucose, galactose, xylose, arabinose, fucose, mannose, gluconic acid, glucaric acid, tartaric
263 acid, threonic acid, and mannitol were confirmed by GC-MS analysis, under the same
264 conditions, of an authentic standard.

265

266 2.11. High-performance liquid chromatography-ultraviolet (HPLC-UV)

267 For saponification and the analysis of released lignan macromolecule monomers (SDG,
268 HDG, ferulic acid and coumaric acid) by HPLC-UV, another 250 μ L aliquot of the
269 resuspended exudates was used. In order to release the phenylpropanoids that are contained
270 through ester linkages in exudated higher molecular weight fragments of the flax seed lignan
271 macromolecule, these aliquots were first subjected to full saponification: 5 μ L NaOH 5M was
272 added (final concentration 0.1M) and the tubes were agitated (2000 rpm) for 3 h at 60 °C on a
273 thermomixer (Eppendorf, Hamburg, Germany). The saponification was stopped by adding 5
274 μ L HCl 5M (final pH 3). Of the hydrolysates, 50 μ L was analyzed with an LC-2010cHT
275 HPLC system (Shimadzu, Champs sur Marne, France) equipped with a reversed phase
276 Sunfire C18 column (150mm x 3mm, 3.5 μ m; Waters, Milford, MA). The separation was
277 performed with aqueous 0.1% acetic acid and acetonitrile/water (99/1, v/v, acidified with
278 0.1% acetic acid) as solvents A and B. At a flow rate of 300 μ L/min and a column

279 temperature of 40 °C, the following gradient was applied: 0 min 5% B, 40 min 45% B, 45
280 min 100% B. Detection was performed at 280 nm. Peak integration was performed with the
281 LabSolutions HPLC software (Shimadzu, Champs sur Marne, France). Phenylpropanoids
282 detected by HPLC were identified based on spiking with authenticated standards.

283

284 2.12. Statistics

285 All statistical computation was performed in R version 3.6.3 (R Development Core Team,
286 2013). We used a significance level of 0.05, divided by the number of different time-points at
287 which the variable was tested (t-tests or ANOVA) (Bonferroni correction). The line plots
288 were created using the ggplot function of the ggplot2 R package (Wickham, 2016).

289

290 3. Results

291 3.1. Seed coat and surface ultrastructure

292 **High resolution scanning electron microscopy (SEM) images of the surface and transversally**
293 **cut seed coats of untreated, vacuum treated and plasma treated (1, 5, 10, 15, or 20 min) seeds,**
294 **were analyzed to evaluate the ultrastructural impact of the treatments. (Figure 1).** In images
295 of transversally cut seeds, we could distinguish **the rugose cuticle**, the different cell layers of
296 the outer and inner integument, as well as the layered structure of the mucilage within the
297 MSCs. There was no significant difference in the ultrastructure of the surface of untreated
298 seeds, vacuum treated seeds or seeds that were plasma treated for a duration of 1 min. Plasma
299 treatment of the seeds for 5 min and longer resulted in erosion and micro-roughening of the
300 surfaces. **This** erosion consisted essentially of perforations in the cuticle, without visible
301 effects on the outer periclinal cell walls of the MSCs. Superficial cracks in the outer
302 integument were not uncommon in untreated seeds but these cracks were more numerous and
303 larger in seeds exposed to longer plasma treatments (15 and 20 min). In seeds that were

304 plasma treated for 20 min, cracks that reached until the sclerified cell layer of the outer
305 integument were observed all over the seed surface. Considering the above-described
306 ultrastructural observations, we assigned the minimum duration of plasma treatment to 5 min
307 for further analyses.

308

309 3.2. Wettability

310 On untreated seeds, the contact angle θ measured between the surface of a water droplet and
311 the seed surface was above 90° (Figure 2). For seeds exposed to a 5 min plasma treatment, θ
312 was below 50° , indicating a much more hydrophilic surface (Law, 2014). The increased
313 wettability of plasma-treated seed surfaces, was confirmed by the differential spreading of
314 potassium permanganate (KMnO_4) droplets, visualized by SEM (Figure 2). In untreated
315 seeds, the droplets had smaller contact areas distinguished from the non-wetted surface by
316 their compositional contrast and crystals of KMnO_4 . In 5 min plasma treated seeds, the
317 contact area of the droplet on the surface was much larger showing a lower contrast and the
318 absence of crystalline structures. Accordingly, EDX analysis confirmed that elements of
319 KMnO_4 droplets in plasma treated flaxseeds were more diffused on the surface, showing
320 lower intensity in the acquired surface maps compared to those in untreated seeds. These
321 observations confirm that plasma treatment significantly increased the hydrophilicity and
322 wettability of the flax seed surface.

323

324 3.3. Water uptake and germination

325 The process of germination, *sensu stricto*, starts with the uptake of water by the dry seed and
326 is complete when the radicle protrudes (Weitbrecht et al., 2011). The water uptake of
327 germinating seeds occurs in three phases (Nelson, 2004). Phase I is characterized by a rapid
328 initial water uptake and is referred to as the imbibition. The second phase (phase II) is a

329 “plateau phase” during which no more water is taken up. The emergence of the radicle due to
330 cell expansion marks the transition of the second phase to the third phase. Phase III is, *sensu*
331 *stricto*, a post-germination phase, characterized by the restart of water uptake and during
332 which the seedling is growing. Because differences in wettability of the seed surface are
333 expected to affect water uptake, and thus germination, we monitored the water uptake during
334 the germination process of control and plasma treated flax seeds (Figure 3, **Supplemental**
335 **Tables 1-3**). No difference in water uptake was observed between the control and the vacuum
336 treated seeds (**Supplemental Table 1**) or between the seeds treated by different plasma
337 durations (**5, 10, 15 or 20 min**) (**Supplemental Table 2**). **Therefore, for further statistical**
338 **analyses of water uptake, we considered untreated and vacuum treated seeds together as the**
339 **control group, and seeds subjected to 5 to 20 min of plasma treatment were considered**
340 **together as the plasma treated group (Figure 3, Supplemental Table 3).** For both untreated and
341 plasma treated seeds studied here, the duration of the imbibition phase was invariably one
342 hour. The amount of water that was taken up during imbibition was, however, 33 % higher in
343 plasma treated seeds (5, 10, 15 or 20 min) (~20 mg water per seed) than in control or vacuum
344 treated seeds (~15 mg per seed), and the water content of plasma treated seeds remained
345 higher than untreated seeds until 8 HAI. At 24 HAI, the seed coat had split open for 50 to 90
346 % of the seeds (no difference observed between the treatments), indicating the start of phase
347 III between 8 HAI and 24 HAI. During this period, water uptake and weight gain resumed for
348 untreated seeds, but not for plasma treated seeds, so that the weight of untreated seeds
349 equaled the weight of plasma treated seeds at 24 HAI. Between 24 HAI and 48 HAI, all seeds
350 gained weight, showing that water uptake had also restarted for the plasma treated seeds. In
351 general, during the post-germination phase (monitored at 24 HAI and 48 HAI), no difference
352 in weight was apparent any more between control and plasma treated seeds, and no effect of

353 plasma treatment on the germination percentage could be observed at 24 HAI or 72 HAI
354 (Supplemental figure S1).

355

356 3.5. Exudates

357 The influx of water during seed imbibition leads to leakage of cellular and vacuolar
358 constituents into the environment. This leakage is referred to as seed exudation. Using GC-
359 MS, we followed the accumulation pattern of 30 primary metabolites in the exudates of flax
360 seeds during the first 48 hours after the start of imbibition, and the effect of plasma treatment
361 on these exudates (Supplemental Figure S2). Independently of the treatment, glucose,
362 fructose, sugar alcohols and organic acids were mainly detected during the imbibition phase,
363 whereas other sugars (xylose, fucose) and amino acids were only detected in the post-
364 germination phase. The measured intensities of all these compounds were never significantly
365 different between exudates of untreated seeds and exudates of vacuum treated seeds, neither
366 was a significant difference in metabolite intensities found between exudates of seeds that
367 had been subjected to 5, 10, 15 or 20 min plasma treatment (Supplemental Tables 1 and 2).
368 Therefore, for further statistical analyses of the exudates, we considered untreated and
369 vacuum treated seeds together as the control group, and seeds subjected to 5 to 20 min of
370 plasma treatment were considered together as the plasma treated group (Figure 3,
371 Supplemental Table 3). The strongest exudation was observed immediately at the start of
372 imbibition, before 0.5 HAI. Based on the total intensity of all 30 quantified metabolites in the
373 exudates accumulated from the start of imbibition till 0.5 HAI, the imbibitional leakage was
374 about 50% higher in plasma treated seeds than in untreated seeds. However, during the next
375 half an hour (0.5 HAI – 1 HAI), the levels of leakage were the same in untreated and plasma
376 treated seeds, slightly lower than the leakage from untreated seeds during the first half an
377 hour of imbibition (Figure 3, GC-MS total signal). Imbibitional leakage dropped thus faster

378 in plasma treated than in untreated seeds between 0.5 HAI and 1 HAI, and this trend
379 continued in the next hours. In the exudates of plasma treated seeds that were collected from
380 the periods between 1 HAI and 2 HAI and between 2 HAI and 4 HAI, the amounts of
381 metabolites that were released during imbibitional leakage were significantly lower than in
382 the corresponding exudates of untreated seeds. Only in the period between 4 HAI and 8 HAI,
383 imbibitional leakage of untreated seeds had dropped to the levels plasma treated seeds. The
384 graphs in Figure 3 should be interpreted with caution, taking into account that accumulated
385 metabolite levels during exudation periods of different durations are represented. For
386 example, a status quo in exudation per time unit in the periods 1 HAI to 2 HAI (one hour of
387 exudation) and 2 HAI to 4 HAI (two hours of exudation) would be represented as a value at 4
388 HAI that is twice as high as the value at 2 HAI. The faster drop in imbibitional leakage of
389 plasma treated seeds as compared to untreated seeds was observed for all constituents of the
390 imbibitional exudates and was most obvious for the most abundant ones: glucaric, tartaric and
391 citric acid. Roughly, the leakage had quasi stopped after one hour of imbibition for plasma
392 treated seeds, whereas for untreated seeds a relatively strong exudation was still observed
393 between two and four hours after the start of imbibition. In contrast, no significant effect of
394 plasma treatment could be detected on the late, post-germination exudation of primary
395 metabolites (Figure 3; Supplemental Figure S2; Supplemental Table 3).

396 To investigate whether the lignan macromolecule also contributes to the exudates, we
397 analyzed the exudates of untreated flaxseeds, obtained at 0.5, 2, 8, 24 and 48 HAI, by HPLC-
398 ESI-Hybrid Ion Trap-Orbitrap Mass Spectrometry (LC-MS). We specifically targeted
399 phenolic compounds that have been previously found in partial saponification products of the
400 lignan macromolecule, and for which the exact m/z , retention time, and MS_n fragmentation
401 spectra have been elucidated (Thiombiano et al., 2020). Eight distinct lignan macromolecule
402 monomers (secoisolariciresinol diglucoside (SDG), herbacetin diglucoside (HDG),

403 lariciresinol diglucoside (LDG), ferulic acid glucoside (FeAG), coumaric acid glucoside
404 (CouAG), a guaiacyl unit 8–O–4-coupled to ferulic acid ($G(t8-O-4)FeA$ and $G(e8-O-$
405 $4)FeA$), and a hexosylated guaiacyl unit 8–O–4-coupled to ferulic acid ($Ghex(8-O-4)FeA$)),
406 as well as lignan macromolecule fragments containing two monomers (SDG ester-linked to
407 3-hydroxy-3-methylglutaric acid (HMGA) (SDG-HMGA) and FeAG or CouAG esterified to
408 succinic acid) or three monomers (HMGA-SDG-HMGA) were detected here in the flax seed
409 exudates (Supplemental Figure S3). The strongest signal was found in the post-germination
410 phase (24 HAI and 48 HAI) for the lignan backbone fragments SDG-HMGA and HMGA-
411 SDG-HMGA and for $G(t8-O-4)FeA$ and $G(e8-O-4)FeA$, which have been shown recently
412 to be esterified to a glucose moiety of SDG in the lignan macromolecule (Thiombiano et al.,
413 2020). Although less intense, also FeAG-succinic acid and CouAG-succinic acid
414 accumulated specifically in exudates of the post-germination phase. These dimers have been
415 shown to be esterified to bigger macromolecules, but the substrates to which these
416 compounds are esterified in the lignan macromolecule have not been elucidated today
417 (Thiombiano et al., 2020). In order to evaluate the overall exudation level of the lignan
418 macromolecule during the first 48 hours after the start of imbibition, and the effect of plasma
419 treatment here upon, part of the exudates on which GC-MS primary metabolite profiling was
420 performed (see above), was subjected to a total saponification. We then relatively quantified,
421 in the fully saponified exudates, four of the major macromolecule monomers by HPLC-UV:
422 SDG, HDG, ferulic acid (FeA) and coumaric acid (CouA). Accumulation of these
423 macromolecule monomers in the saponified exudates showed that exudation of the
424 macromolecule started between two and four hours after the start of imbibition, and was the
425 strongest in the post germination phase (24 HAI and 48 HAI). During this period of
426 macromolecule exudation, no significant difference was observed between exudates of seeds
427 that had been subjected to different times of plasma treatment (Supplemental Table 2).

428 Between exudates of untreated seeds and exudates of vacuum treated seeds, no significant
429 difference in macromolecule monomer abundance was found either (Supplemental Table 1).
430 For subsequent t-tests, we considered untreated and vacuum treated seeds together as the
431 control group, and seeds subjected to 5 to 20 min of plasma treatment as the plasma treated
432 group. The accumulation of lignan macromolecule derived monomers in the saponified
433 exudates was significantly higher for plasma treated seeds than for control seeds, at all time-
434 points from 4 HAI on (Figure 3; Supplemental Table 3).

435

436 3.4. Mucilage extrusion

437 The effects of hydration on the surface ultrastructure of untreated and 20 min plasma treated
438 seeds was studied by two approaches. In the first approach, seeds were pre-wetted by placing
439 a 2 μ L droplet of distilled water on their surface, and were immediately placed in the SEM
440 chamber and observed under “low vacuum” mode (1 torr). On the surface of untreated seeds,
441 extrusion of mucilage occurred in sparse patches. In contrast, in 20 min plasma treated seeds,
442 the mucilage blanketed the entire wetted surface by the time SEM images were acquired. The
443 extruded transparent mucilage was desiccated under the low vacuum of the SEM chamber
444 and rendered an opaque white color visible on the surface of the seeds (Figure 4A). SEM
445 images also showed how swelling of the mucilage in untreated seeds had lifted the layer of
446 MSC distal cell walls, leading to ruptures in this cell wall layer, often following the junctions
447 between the cells. In the second approach, to visualize better the early phases of mucilage
448 expansion and extrusion in untreated and 20 min plasma treated seeds, changes in surface
449 ultrastructure of non-pre-wetted seeds were studied *in situ* by changing the relative humidity
450 (RH) of the ESEM chamber from 75 to 100 percent (Figure 4 B-C). In untreated seeds, the
451 increase of relative humidity initially made the cell boundaries of the MSCs less clearly
452 defined in some areas. In transversally cut seeds, we could observe how swelling of the

453 mucilage ruptured the radial MSC cell walls. Such a separation took place just underneath the
454 distal cell walls, hence creating a “patchworked” blanket of distal cell walls that was lifted
455 up. In other areas, however, individual distal cell walls started bulging when the relative
456 humidity was increased, and the connection with radial cell walls seemed preserved. Further
457 increase of the relative humidity led to two different types of ruptures. On the one hand,
458 mucilage of individual or a few neighboring MSCs was released through ruptures of the
459 junctions between distal and radial cell walls. On the other hand, larger cracks, spanning
460 many cells and often following the cell boundaries, appeared in the uplifted layer of distal
461 cell walls. Ruptures crossing the surface of single distal cell walls were rare in untreated
462 seeds. Large areas of the seed surface remained furthermore intact. In plasma treated seeds,
463 however, our *in situ* observation revealed a different mechanism of mucilage extrusion. In
464 these seeds, increases in relative humidity resulted in the formation of domed distal cell walls
465 of individual MSC cells all over the seed surface. The bulging of individual cells was more
466 extreme than what was observed in untreated seeds, and eventually led to ruptures of the
467 distal cell walls and extrusion of balloon-like mucilage bulbs from quasi every single MSC
468 cell. In contrast to untreated seeds, a reticulum of cell boundaries, corresponding to the
469 connections of distal cell walls to radial cell walls, remained strongly pronounced all over the
470 seed surface. Our observations made on transversally cut plasma treated seeds, confirmed that
471 the connections between radial and distal cell walls were preserved during the expansion and
472 extrusion of mucilage. Overall, mucilage expansion in untreated and plasma treated seeds led
473 to a different type of rupture of MSC cell walls. In untreated seeds, the ruptures were formed
474 in radial cell walls and between distal cell walls of neighboring cells. This resulted sometimes
475 in long, extended cracks, but they were relatively sparsely distributed over the seed surface.
476 In plasma treated seeds, mucilage was extruded by ruptures within the distal cell wall of
477 almost every single MSC of the seed.

478

479 **4. Discussion**

480 4.1. First things first

481 Argon plasma treatment of seeds can improve the germination rate and subsequent seedling
482 vigor, and many studies, performed on a wide variety of species, have shown these effects
483 (Randeniya and de Groot, 2015; Starič et al., 2020). The flaxseeds that were the subject of
484 our study showed a fairly good germination efficiency and our aim was not to improve it by
485 plasma treatment. Instead, we exploited here the structure of the outer integument of
486 flaxseeds, and in particular it's mucilage extrusion mechanism, to improve our understanding
487 of the effect of plasma on cuticle, cell walls, and membranes. With vague proclamations like
488 "eroded seed coat", "reduction in seed coat thickness", "ruptured seed coat", or "cracks in the
489 cell outer layer" (*sic*), a lot of uncertainty and confusion regarding the reach of plasma effects
490 on seed coats prevails indeed in the literature today (Tong et al., 2014; Wang et al., 2017;
491 Yamauchi et al., 2012; Živković et al., 2004). This aspect seems nevertheless essential to
492 understand the basis of potential subsequent physiological effects.

493

494 4.2. Etching of the cuticle

495 Plasma treatment of flax seeds resulted in a faster water uptake during the first 30 min of
496 imbibition and a significantly higher water content of the seeds until the end of phase II of
497 germination. SEM images presented substantial evidence that in flax seeds with increased
498 water uptake due to plasma treatment, the cuticle was partially etched away, creating micro-
499 perforations all over the seed surface. Although longer plasma treatments (15-20 min)
500 induced extensive cracking of the outer integument, this did not further affect the water
501 uptake. Therefore, the etching of the cuticle, early in the treatment, was probably the key
502 factor leading to increased water uptake. Micro-holes at the seed coat surface are also visible

503 in previously published SEM images of plasma treated *Chenopodium album*, *Andrographis*
504 *paniculata*, *Sophora flavescens* and *Pisum sativum* seeds, and can be, based on our results,
505 interpreted as perforations of the cuticle (Sera et al., 2008; Stolárik et al., 2015; Tong et al.,
506 2014; Yamauchi et al., 2012). The role of outermost seed cuticles in governing water entry is
507 often overlooked, probably because they are very thin and do not stain readily for lipids
508 (Shao et al., 2007). However, similar results have been reported recently in soybean, where
509 the seed coat permeability to water was correlated with the presence of small cuticular cracks,
510 visible by SEM (Ma et al., 2004). These cuticular cracks were not present in soybean
511 cultivars that do not readily imbibe water, a feature referred to as “hardseededness”, but were
512 present in “soft” seeds, which are more permeable to water. Hardseededness can be beneficial
513 for long-term seed survival, but is undesirable for the food processing industry, as it leads to
514 physical dormancy (Baskin, 2003). When whole seeds are processed, such as for the
515 production of soya milk, seeds should ideally take up water quickly and synchronously.
516 Etching of the cuticle by mild plasma treatment, as was observed here for flax seeds, could
517 therefore open possibilities to combine the benefits of hardseededness and fast imbibition.

518

519 4.3. Altered mechanical properties of the distal MSC cell walls

520 The mechanism of mucilage extrusion of flax seeds has been previously studied in detail, and
521 the mucilage extrusion by untreated flaxseeds here observed in the ESEM chamber under
522 increasing relative humidity conditions, was largely in accordance with the previously
523 described processes (Boesewinkel, 1980; Miart et al., 2019). The mucilage extrusion was
524 faster in plasma treated seeds and this acceleration was at least partially caused by the faster
525 water uptake, associated with the perforations in the cuticle. It can be expected that the
526 pressure on MSC cell walls of the fast imbibing plasma-treated seeds was higher than for
527 untreated seeds. On SEM images of transversally cut seeds no etching of the distal MSC cell

528 walls, directly beneath the cuticle, was visible. Nevertheless, *in situ* environmental SEM
529 imaging revealed a different behavior of these distal cell walls under the pressure of the
530 swelling mucilage. In untreated seeds, the distal cell walls that were detached from the radial
531 cell walls, although not thicker than those of plasma treated seeds, looked more rigid. In
532 plasma treated seeds, the individual distal cell walls were more heavily stretched by the
533 expanding mucilage, making them look like eggs in a box, before breaking open. The
534 observation that only in plasma treated seeds the distal MSC cell walls broke, and that in
535 untreated seeds tears occurred almost exclusively at the cell junctions, reveals a modification
536 of the mechanical characteristics of the MSC distal cell walls in plasma treated seeds. It
537 seems that plasma treatment has increased the flexibility and elasticity but reduced the
538 strength of these outer cell walls. It is well known that ROS, and particularly hydroxyl
539 radicals, can degrade lignocellulosic material, and they are produced by wood decay fungi to
540 initiate wood degradation and make the polymers more accessible to hydrolytic enzymes
541 (Gessler et al., 2007; Hammel et al., 2002). A similar action of ROS, which can be generated
542 by plasma treatment from the water vapor of the seed, could thus have affected the
543 mechanical properties of the distal MSC cell walls of plasma treated seeds without visible
544 etching. Compliments of our findings with chemical analyses and micro-indentation
545 experiments could provide further details about the nature of the compositional and
546 mechanical changes. In a selection of three flax RILs that were characterized by different
547 speed of mucilage extrusion, micro-indentation experiments performed on distal and radial
548 MSC walls showed that faster mucilage extrusion was associated with higher values of both
549 relative hardness and relative elasticity of these walls (Miart et al., 2019). Our observations in
550 plasma treated flax seeds indicated that variation in the mechanical properties of the MSC
551 walls may alter the mechanism of mucilage extrusion. It would be interesting to further

552 investigate if flax RILs characterized by relatively elastic MSC walls and fast mucilage
553 extrusion show an extrusion mechanism similar to plasma treated seeds.

554

555 4.4. Accelerated membrane recovery

556 The rehydration of seeds during phase I of germination, is accompanied with a leakage of
557 cellular metabolites. It is generally thought that this leakage, if it is too strong or lasts too
558 long, can delay seed germination, reduce seedling vigor, or even lead to failed germination,
559 whereas reducing the leakage can promote growth (Bradow and Bauer, 2010; Hoekstra et al.,
560 1999; Yu et al., 2015). Especially in thermophilic crops, such as maize and rice, low
561 temperatures during imbibition increase the severity of cellular leakage, which is then
562 referred to as “imbibitional chilling injury” (Bochicchio et al., 2008; Taylor et al., 1992).
563 Despite the economical relevance, no consensus exists on the exact mechanisms underlying
564 imbibitional exudation and subsequent exudation ending. Imbibitional leakage is often
565 interpreted as the result of malfunctioning, dehydrated membranes in dry seeds, so that the
566 leakage will stop as soon as the membranes are rehydrated (Cortes and Spaeth, 1994; Powell
567 and Matthews, 1981; Simon and Harun, 1972). A more sophisticated variation of this
568 hypothesis attributes solute leakage to temporarily malfunctioning membranes during the
569 transition from gel phase to hydrated liquid-crystalline state, or from hexagonal II phase to
570 lamellar phase, when the seeds take up water (Bewley, 1997; Bochicchio et al., 2008; Crowe
571 and Crowe, 1992; Scarafoni et al., 2013; Yu et al., 2015). Totally different is the oldest, but
572 still commonly proclaimed hypothesis, according to which leakage is simply due to
573 membrane damage, physically imparted by rapid water inrush (Kigel et al., 2015; Larson,
574 1968; Schiltz et al., 2015). Here, we studied the relation between water uptake, leakage and
575 recovery mechanisms, through a comparative time-course analysis of water uptake and
576 metabolite exudation in untreated and plasma-treated flax seeds. During the short period of

577 faster water uptake by plasma treated seeds, i.e. the first 30 min of imbibition, we observed
578 an increase in total exudation of primary metabolites by these seeds. Our observations thus
579 suggest that the initial imbibitional leakage was at least partially due to membrane damage
580 imparted by water inrush, and that therefore faster water uptake resulted in more membrane
581 damage. After this initial period, however, when the water uptake rate slowed down both for
582 untreated and plasma treated seeds but the water content remained significantly higher in
583 plasma treated seeds, we observed a much faster decrease in exudation in plasma treated
584 seeds, indicating a facilitated recovery of the membranes. A possible positive effect of water
585 content on the recovery suggests that, besides physical damage by water inrush, also
586 dehydration or phase transition of the membranes played a role in the leakage. It is
587 conceivable that, once the pressure of fast water inrush is over, the speed of rehydration or
588 reorganization of the membranes to a functional state is positively correlated with water
589 content. Many efforts to alleviate the susceptibility to imbibitional chilling injury focused on
590 reducing water uptake rates, for example by coating of the seeds (Priestley and Leopold,
591 1986; Tully et al., 1981). Recently, de Groot et al. reported that argon plasma treated cotton
592 seeds were more cold tolerant (less imbibitional chilling) than untreated seeds, while showing
593 significantly more cold water uptake (de Groot et al., 2018). The authors classified this
594 observation as contradictory and needing further detailed investigation. Our results show that
595 speed of water uptake and seed water content during germination phase II, are two distinct
596 factors that may have opposite effects on imbibitional leakage. Possibly, the micro-
597 perforations of the cuticle induced by plasma treatment allow an increased water inflow that
598 is sufficiently controlled to avoid harsh membrane damage, so that the positive effect of fast
599 membrane recovery prevails. Timing is a prominent feature for the events during
600 germination. Membrane reorganization has been proposed to be the most important event
601 during imbibition, given that it precedes other events and is a prerequisite for most cellular

602 processes (Yu et al., 2015). The beneficial effects of plasma treatment on germination and
603 seedling vigour, reported in many species (maize, wheat, radish, tomato, buckwheat
604 (Randeniya and de Groot, 2015)), could be related to an accelerated recovery from
605 imbibitional leakage, as in all studied species an increased water uptake was observed. The
606 mechanism underlying membrane recovery during imbibition has been the subject of recent
607 studies in maize. The mechanism seems to be not merely physical or chemical, but to rely on
608 transcriptional activation of a large number of genes and to involve intracellular vesicular
609 trafficking and exocytosis (He et al., 2019; He et al., 2017). It would be of interest to further
610 investigate the effect of plasma treatment, or of mere perforations in the seed's outer cuticle,
611 on the expression of genes involved in plasma membrane recovery.

612

613 4.5. Exudation of lignan macromolecule fragments

614 Germination ends with the extension and protrusion of the radicle through the seed coat,
615 around 24 HAI, and this event is accompanied by another wave of primary metabolite
616 exudation. These “end-of-germination” seed exudates result from the degradation of storage
617 polymers, as energy source for the non-photosynthesizing developing seedling (Nelson,
618 2004). The many amino acids detected in these exudates result from the breakdown of
619 storage proteins, and xylose and fucose are probably derived from the degradation of pectin.
620 Plasma etching did not affect the efficiency of the germination in our study, neither the
621 weight increase in the post-germination phase, nor the exudation of primary metabolites at
622 the protrusion of the radicle. However, we observed here for the first time that also parts of
623 the lignan macromolecule, which is stored in the parenchymatous cell layer just underneath
624 the MSCs, are exudated, and plasma treatment significantly increased the release of
625 macromolecular fragments in the exudates. The appearance of lignan macromolecule
626 monomers as well as higher molecular weight fragments containing two or three lignan

627 macromolecule monomers, suggests that the lignan macromolecules are hydrolysed during
628 germination, releasing phenolics in the spermosphere. The release of lignan macromolecule
629 fragments seems to be independent of the primary metabolite exudation process and of the
630 radicle appearance, since the first traces were visible in exudates collected between 2 HAI
631 and 4 HAI, during the “plateau phase”. Potentially, hydrolytic enzymes start degrading the
632 lignan macromolecule in that period and breakdown products diffuse from the
633 parenchymatous cell layer towards the spermosphere. The mucilage has been extruded well
634 before the beginning of phase II, and therefore, it could be that the disruptions of the
635 individual MSC distal cell walls all over the surfaces of plasma treated seeds facilitate this
636 diffusion as compared to untreated seeds, where large parts of the rigid layer of distal MSC
637 cell walls remain intact. The microbial community of the spermosphere is largely driven by
638 seed exudates and lignans and other phenylpropanoids are known to be involved in different
639 types of plant-microbe interactions (defense or symbiosis) (Akiyama et al., 2007; Carpinella
640 et al., 2005; Ralph et al., 2006; Szoboszlay et al., 2016; Weston and Mathesius, 2013). Here
641 we showed for the first time that phenylpropanoids from the flaxseed lignan macromolecule
642 contribute to the establishment of the flaxseed spermosphere and therefore can have far
643 reaching consequences for later plant development.

644

645 **5. Conclusion**

646 Plasma treated flax seeds with etched cuticle showed an increased wettability and water
647 uptake. Therefore we conclude that the cuticle of the MSC layer of flax seeds is a key factor
648 in the interaction of the seed coat with water. We did not identify any significant etching
649 effects below the cuticle but the distal epidermal cell walls of plasma treated seeds showed a
650 higher elasticity, leading to an altered mucilage extrusion mechanism. It appears therefore
651 that the mechanism of mucilage extrusion of flax seeds is variable and depends on the cuticle

652 and epidermal cell wall properties of the cultivar. The time lapse of imbibitional solute
653 leakage was shortened in plasma treated seeds. We deduced that the membrane recovery
654 after imbibitional damage can be accelerated by higher water content of the seeds. Lignan
655 macromolecule accumulates in the outer integument of flax seeds. This macromolecule, to
656 which no specific function could be attributed today, was fragmented and exudated early after
657 imbibition, which signifies its contribution to the establishment of the spermosphere around
658 the germinating seeds.

659

660 **Author Contributions**

661 A.J. and E.G. conceived, designed and supervised the experiments; R.R. and M.R. and B.T.
662 performed the experimental work. F.M. was involved in administration and validation of this
663 research project. R.D. performed all metabolite profiling data treatment, statistical analyses
664 and interpretation of the results. A.J. and E.G. were responsible for the research activity
665 planning and execution. R.D. and A.J. wrote the paper and all authors have read and agreed
666 to the final version.

667

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680
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868

869 **Figure captions**

870 **Figure 1: Effect of plasma treatment on the ultrastructure of the flax seed coat.** Surface
871 ultrastructure of untreated flaxseed, 20 min low vacuum treated flaxseed, and flaxseeds
872 exposed to 1 min, 5 min, 10 min, 15 min or 20 min of plasma treatment (a-g). Crack formed
873 in the outer integument of a 20 min plasma treated seeds (h, l). High resolution images of the
874 outer seed coat layers of transversally cut seeds: untreated (i, j) and plasma treated for 20 min
875 (k, l). c, cuticula; cw, cell wall; e, endosperm; f, fibrous elements; ii, inner integument; msc;
876 mucilage secretory cell; m, mucilage; oi, outer integument; pa, parenchymatous cell; pi,
877 pigmented cell; sc, sclerified cell.

878

879 **Figure 2: Comparison of surface wettability of untreated (left column) and 5 min**
880 **plasma treated (right column) flax seed.** Measurement of contact angle of water droplets on
881 the surface of the seeds showing a significant increase on plasma treated seeds (A-B);
882 Locating the emplacement of the KMnO₄ droplets deposited on the seed surfaces (brighter in
883 color) using backscattered SEM images (C-D) and elemental X-ray mapping of the same
884 survey area as the SEM images (E-F), showing increased spreading of droplets over the
885 plasma treated seeds compared to untreated seeds (manganese in red and carbon in green).

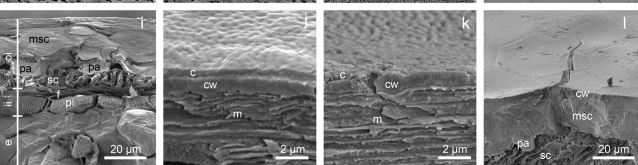
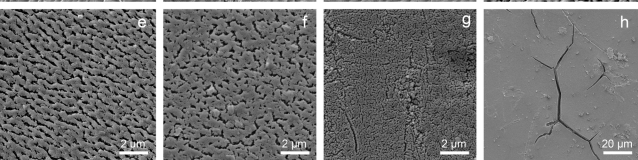
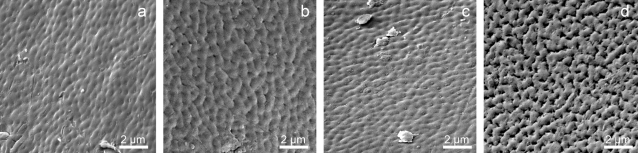
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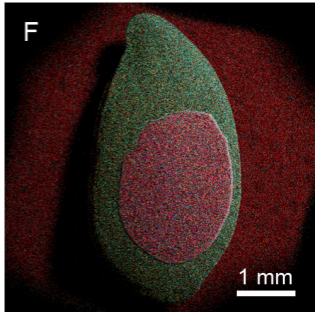
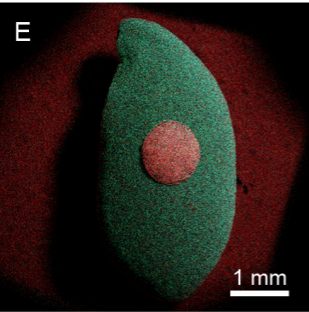
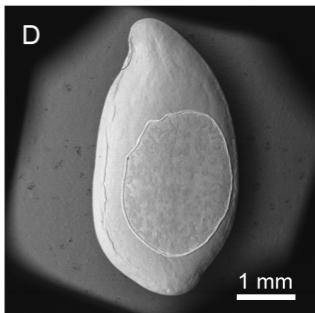
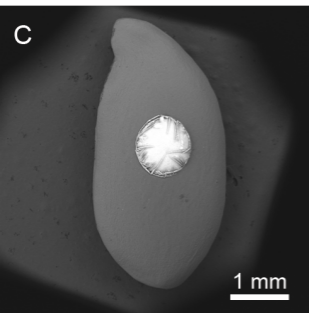
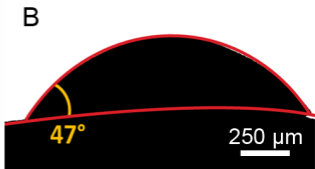
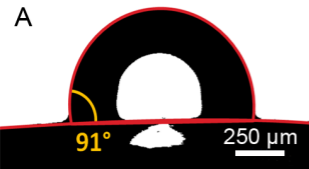
887 **Figure 3: Water uptake and compound exudation during seed germination. Red: Control**
888 **group, comprising untreated and vacuum treated seeds. Blue: Plasma treated group,**
889 **comprising seeds subjected to plasma treatments of 5, 10, 15 or 20 min.** Water uptake is
890 expressed as the weight gain in mg, with respect to the dry, pre-imbibition state of the
891 individual seeds. The “GC-MS total signal” corresponds to the sum of the **peak areas** for the
892 30 GC-MS identified metabolites. Glucaric, tartaric and citric acid were the three most
893 abundant primary metabolites, relatively quantified by GC-MS in non-saponified exudates.

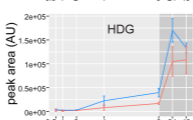
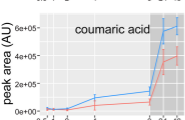
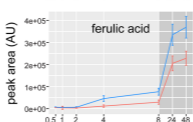
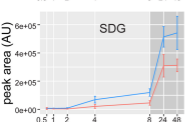
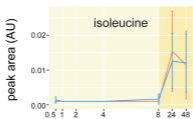
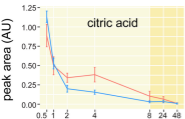
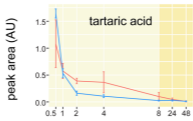
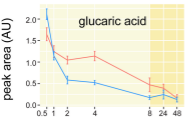
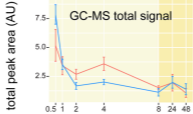
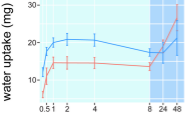
894 SDG, FeA, CouA, and HDG are lignan macromolecule monomers relatively quantified by
895 HPLC-UV in fully saponified exudates. GC-MS and HPLC quantifications of exudated
896 compounds at 0.5, 1, 2, 4, 8, 24 and 48 HAI correspond to analyses of exudates accumulated
897 during the first 30 min after the start of imbibition and during the subsequent intervals
898 between 0.5 HAI and 1 HAI, between 1 HAI and 2 HAI, between 2 HAI and 4 HAI, between
899 4 HAI and 8 HAI, between 8 HAI and 24 HI, and between 24 HAI and 48 HAI, respectively.
900 The time scale in the post-germination stage (darker shaded area after 8 HAI) is not
901 proportional. **Number of replicates (n):** Water uptake (control, all time-points): n=10 (five
902 untreated seeds and 5 vacuum treated seeds); Water uptake (plasma treated, time-points 0.5 –
903 8 HAI): n=20 (5 min plasma (n=5), 10 min plasma (n=5), 15 min plasma (n=5), 20 min
904 plasma (n=5); Water uptake (plasma treated, time-points 24 – 48 HAI): n=5 (5 min plasma);
905 Exudated compounds (control, all time-points): n=6 (three untreated seeds and three vacuum
906 treated seeds); Exudated compounds (plasma treated, all time-points): n=12 (5 min plasma
907 (n=3), 10 min plasma (n=3), 15 min plasma (n=3), or 20 min plasma (n=3)). Error bars
908 indicate sem. Mean values, sem and p-values of statistical tests comparing water uptake and
909 exudated compound intensities, within control samples, within plasma treated samples and
910 between control and plasma treated samples, at different time-points, are given in
911 Supplemental Tables 1, 2, and 3. **AU: arbitrary units.**

912 **Figure 4. Mucilage extrusion.** A. Mucilage extrusion after deposition of a water droplet on
913 the surface of untreated (a-c) and 20 min plasma treated (d-e) seeds. a, d: macroscopic view,
914 after dessication in the SEM chamber; b, c, e: low vacuum SEM images. B. Mucilage
915 extrusion by untreated seeds, induced by increasing the relative humidity (RH) in the ESEM
916 chamber. a-d, time lapse over 5 min, of a given seed surface area under increasing RH from
917 75 % (dry seed, a) until 90 % (d) in 5 min e-g, details of extruding mucilage at 90-95 % RH.
918 Distal cell walls are detached from radial cell walls and lifted by expanding mucilage (e).

919 Large cracks form in the patchworked blanket of distal cell walls, spanning many cells and
920 mainly following cell boundaries (f). Mucilage of individual or a few neighbouring MSCs is
921 released through ruptures of the junctions between distal and radial cell walls (g).
922 Transversally cut seed under 95 % RH, with swelling mucilage and distal MSC cell walls
923 detached from radial cell walls (h). C. Mucilage extrusion by 20 min plasma treated seeds,
924 induced by increasing the relative humidity in the ESEM chamber. a-d, time lapse over 4 min
925 of a given seed surface area under increasing RH from 75 % to 90 %, starting from the
926 beginning of mucilage extrusion by a few cells (a) till the total coverage of the area by
927 extruded mucilage bulbs (d). Details of extruding mucilage: individual distal MSC cell walls
928 break and release balloon-like mucilage bulbs (e-f). Transversally cut seed under 90 % RH,
929 with swelling mucilage, bulging individual cells, and junctions between distal and radial
930 MSC cell walls intact (g-h). *cw, MSC distal cell wall; e, endosperm; m, mucilage; pi,*
931 *pigmented cell.*
932







HAI

— control — plasma

