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Multiscale localization of procyanidins in ripe and overripe perry pears by light and transmission electron microscopy

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22 **Highlights**

23 Perry pear procyanidins are concentrated in exocarp and mesocarp parenchyma cells

24 Procyanidins were only detected in vacuoles in perry pear flesh

25 Procyanidins become attached to tonoplast at overripe stage.

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45 **Abstract**

46 Histochemical staining with 4-dimethylaminocinnamaldehyde (DMACA), light microscopy
47 and transmission electron microscopy (TEM) were applied to characterize procyanidin
48 localization at ripe and overripe stage in perry pear flesh (cv 'De Cloche'). Pear flesh contained
49 stone cell clusters surrounded by very large parenchyma cells. DMACA staining showed
50 procyanidins mainly located in parenchyma cells from fruit mesocarp. Under light microscopy
51 and TEM, procyanidins appeared in the vacuole of parenchyma cells as uniformly stained
52 granules, probably tannosomes. They were differently dispersed in ripe and overripe perry
53 pears, as the granules remained free inside the vacuole in ripe pears and mostly attached to the
54 tonoplast in overripe pears.

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56 **Keywords:** *Pyrus communis* L., DMACA, condensed tannins, ripening, histology

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69 1. Introduction

70 Condensed tannins or proanthocyanidins are oligomers and polymers of flavan-3-ol
71 monomers such as (-)-epicatechin, (+)-catechin, (+)-gallocatechin and (-)-epigallocatechin.
72 They are present in many fruits, e.g. apple [1-4], grape [5-7], apricot [8], peach [9], and pear
73 [10-11]. Perry pears, among pear cultivars, are used only to make perry. Perry pear flesh
74 polyphenol profile is simple and composed of phenolic acids, i.e. mainly hydroxycinnamic
75 acids, and flavan-3-ols. Flavan-3-ols class is composed of monomers detected as (+)-catechin
76 and (-)-epicatechin, and mainly highly polymerized procyanidins with a homogeneous structure
77 based on (-)-epicatechin unit [10-12]. Procyanidins are able to bind to cell walls, especially
78 pectins, and salivary proteins contributing to the astringency sensation of beverages such as
79 wine, cider and perry [4, 12-14]. The pear procyanidin contents ranged between 0.051 g/kg
80 Fresh Weight (FW) ('Conference' cultivar) and 8.6 g/kg FW ('Plant De Blanc' cultivar) in the
81 flesh and between 0.76 g/kg FW ('Conference' cultivar) and 31.8 g/kg FW ('Arbi Chiheb'
82 cultivar) in the peel [11-12].

83 Histochemical methods allow revealing procyanidin localization and distribution within
84 the cell. Dimethylaminocinnamaldehyde (DMACA) and vanillin are commonly applied to
85 detect proanthocyanidins in fresh tissues [15-20]. Under acidic conditions, DMACA is more
86 sensitive than vanillin and recommended in plants with low concentrations of procyanidins
87 [20]. The DMACA reagent stains procyanidins with a blue color by binding to meta-oriented
88 dihydroxy or trihydroxy substituted benzene rings [21]. This reagent reacts specifically with
89 the A ring of procyanidins [19, 21] and reveals specific blue coloration as described in cherries
90 [22], grapes [23], dates [19] and some legumes [15, 20].

91 Transmission electron microscopy (TEM) can complement histochemical methods to
92 better understand procyanidin localization. Polyphenols located within the cell vacuoles are
93 enclosed both by tonoplast and cytoplasmic lipid membranes, which are surrounding by the
94 plant cell wall. These polyphenols are present in free solution inside the cell vacuoles, but may
95 also be linked to the protein matrix forming vacuolar inclusions [23, 24]. Procyanidins have
96 been detected using transmission electron microscopy as entirely osmiophilic spheres without
97 apparent internal structure and they have been described indifferently as tannin vesicles,
98 accretions or vacuolar aggregates [25-27]. Recently, the localization, in chlorophyllous tissues
99 of tracheophyte species, of procyanidins was investigated [16, 28, 29], suggesting that
100 procyanidins are polymerized in a new chloroplast-derived organelle, named the tannosome, as

101 observed in grape and in dates [19, 29]. The tannosomes are formed by pearling of the
102 thylakoids into 30 nm spheres, which are then encapsulated in a tannosome shuttle formed by
103 budding from the chloroplast and bound by a membrane resulting from the fusion of both
104 chloroplast envelopes.

105 Procyanidins localized in the plant cell vacuoles are separated from the cell walls, this
106 implies that association occurs after tissue destruction induced by some external factors such
107 as mastication, food processing [30] and environmental stress (pathogen attack) [31]. These
108 interactions are primordial in determining the transfer of procyanidins from fruits to juices [32].
109 Overripening clearly decreases the extractability of procyanidins from perry pear to pear juice
110 [11, 12] although chemical composition and chemical structure of procyanidins are not
111 modified [11]. A possible explanation, which is tested here, is that overripening might modify
112 procyanidin localization inside the cell vacuole favoring cell wall-procyanidin interactions. The
113 procyanidins localization and its evolution during fruit ripening is not well known,
114 histochemical methods and transmission electron microscopy were used in this study to
115 describe them in pear flesh at a ripe and at an overripe stage.

116 **2. Materials and methods**

117 **2.1. Solvents and reagents**

118 Methanol and hydrochloric acid were provided by Merck (Darmstadt, Germany).
119 Ethanol was provided by Fisher Scientific (Strasbourg, France). 4-
120 Dimethylaminocinnamaldehyde (DMACA) was from Aldrich (Steinheim, Germany). Uranyl
121 acetate, sodium cacodylate, propylene oxide, glutaraldehyde, osmium tetroxide, toluidine blue
122 and Araldite Resin were from Delta Microscopies (Mauressac, France).

123 **2.2. Plant Materials**

124 Ripe and overripe ‘De Cloche’ perry pears (*Pyrus communis* L.) were harvested in the
125 orchard of Mr Aubry (Clécy, France). “Ripe” fruits correspond to pears at harvest, i.e.
126 November 28, 2014 and “Overripe” fruits (described as “soft under the fingers”) correspond
127 to pears stored during 10 days at 10 °C and then 3 days more at room temperature.

128 **2.3. Direct observation after DMACA staining**

129 Thick flesh pear sections from ripe and overripe fruits (circa 0.5 mm) were incubated
130 with DMACA (3 g/L) in a mixture of DMACA methanol / 6 mol/L HCl (1/1: mL/mL) for 20
131 min at 4 °C. Rinsing was carried out by dipping the thick flesh section several times in deionized
132 water to remove excess reagent. Observations of procyanidin-containing cells stained blue were
133 made by light microscopy [19, 20]. Photos of whole stained pear sections were taken by a
134 Kodack EasyShare Z812 IS camera (Camera lens: VARIOGON 36 mm -432 mm AF 12X IS
135 Optical Zoom).

136 **2.4. Fixation**

137 Fixation method used was described by Hammouda et al., (2014) with some
138 modifications. Fresh pear flesh (with or without skin) were used to cut small cubes of tissue (1
139 mm³). Small fresh flesh cubes were fixed with 25 mL glutaraldehyde in 0.1 mol/L sodium
140 cacodylate buffer (pH 7.2) for 1.5 h at room temperature. Blocks were rinsed 3 times (30 min)
141 with sodium cacodylate 0.1 mol/L and then postfixed at room temperature for 1 hour with 20
142 g/L osmium tetroxide (Os O₄) in deionized water. After postfixation, blocks were dehydrated
143 through increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%) followed by
144 dehydration by propylene oxide. Blocks were then progressively infiltrated with araldite resin
145 (GY 502). Polymerization of infiltrated samples was done for 48 h at 60 °C.

146 **2.5. Histology**

147 For light microscopy, semithin sections (1 µm) of fixed flesh pear cube were obtained
148 using an ultramicrotome (Leica microsystems, Leica, Germany) and were then stained with a
149 solution of toluidine blue 10 g/L in Na₂CO₃ 25 g/L. The observations were carried out using a
150 light microscope Olympus BX60 (Olympus Corporation, Tokyo, Japan) and images were taken
151 using a color camera Jenoptik Progress (Jenoptik, Jena, Germany).

152 **2.6. Transmission electron microscopy**

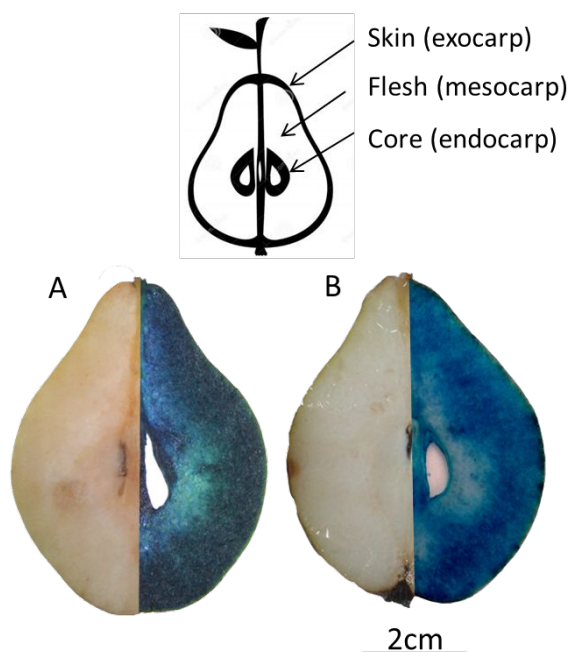
153 Ultrathin sections (60-80 nm) of fixed flesh pear cubes were contrasted with uranyl
154 acetate 20 g/L in a solution of methanol 50% during 40 minutes and then lead citrate at 0.72%
155 during 5 min. Samples were examined using a **Philips CM10 transmission electron microscope**
156 **TEM (FEI Company, Eindhoven, The Netherlands).**

157 **3. Results and discussions**

158 **3.1. Procyanidins localization by light microscopy coupled to DMACA staining**

159 The DMACA staining of procyanidins allowed to localize these phenolic compounds in
160 the edible tissues of the perry pear fruits (Figure 1). The staining was not homogeneously
161 distributed in the whole pear tissues and two distinct areas were observable at both maturity
162 stages. Transverse (data not shown) and longitudinal (Figure 1) sections revealed that the blue
163 coloration due to procyanidin staining by DMACA seemed to fill almost completely the
164 external part of the mesocarp with an uniform distribution. These observations suggested the
165 presence of highly polymerized procyanidins in perry pear cell vacuoles for both maturity
166 stages, i.e. in ripe (Figure 1A) and overripe (Figure 1B) fruits, in agreement with the high
167 concentrations detected in the fruits [10, 11]. Especially, in De Cloche cultivar, the fruit (edible
168 part of the fruit: skin plus flesh) procyanidin content ranged between 6.7 g/kg Fresh Weight
169 (FW) (ripe stage) and 5.8 g/kg FW (overripe stage) with a degree of polymerization close to 20
170 [11].

171 Moreover, transverse (data not shown) and longitudinal (Figure 1) sections revealed that
172 the core of the ripe fruit (endocarp) had a very low intensity blue coloration whereas no
173 coloration, i.e. clear areas, was observed for overripe fruit. ‘De Cloche’ perry pear core was
174 rich in stone cells as reported in other pear cultivars [33]. Stone cells are a type of sclerenchyma
175 cell formed by the secondary deposition of lignin and cellulose in the primary cell walls of
176 parenchyma cells [34] and are responsible for pear gritty texture. The very low blue coloration
177 in ripe “De Cloche” perry pear core might be explained either by the low procyanidin contents
178 or by the fact that the stone cells are strongly lignified and limit the DMACA penetration.
179 Particularly for overripe fruit, the thickness of the stone cell walls may have increased during
180 overripening due to lignification, as lignin content increase [35], which resulted in an increased
181 limitation of DMACA penetration compared to ripe fruits. To validate this statement, it would
182 therefore be necessary to know the density, the location and the volume of the stone cells around
183 the core.



184

185 **Figure 1:** Specific localization of procyanidins in perry pear flesh with DMACA. Longitudinal
 186 section of pear flesh before (on the left) and after DMACA staining (on the right) at ripe (A)
 187 and at overripe stages (B).

188 Light microscopy observations after specific DMACA staining of flavonols (Figure 2)
 189 revealed that perry pear mesocarp contained high procyanidin contents which were located in
 190 large parenchyma cells. No coloration could be observed in pear exocarp cells close to the
 191 **cuticle**. However, previous data have shown that procyanidins are more concentrated in the
 192 skin than in the flesh [10] and that using cryo-laser scanning confocal fluorescence microscopy
 193 the most fluorescent zone of apple and grape fruit is the sub-cuticular cell layers of the exocarp
 194 [36]. **The discrepancies observed between light microscopy observation and previous**
 195 **quantification results could be due to:**

196 **-a limitation of DMACA diffusion in the exocarp tissue because of stone cells**
 197 **aggregates,**

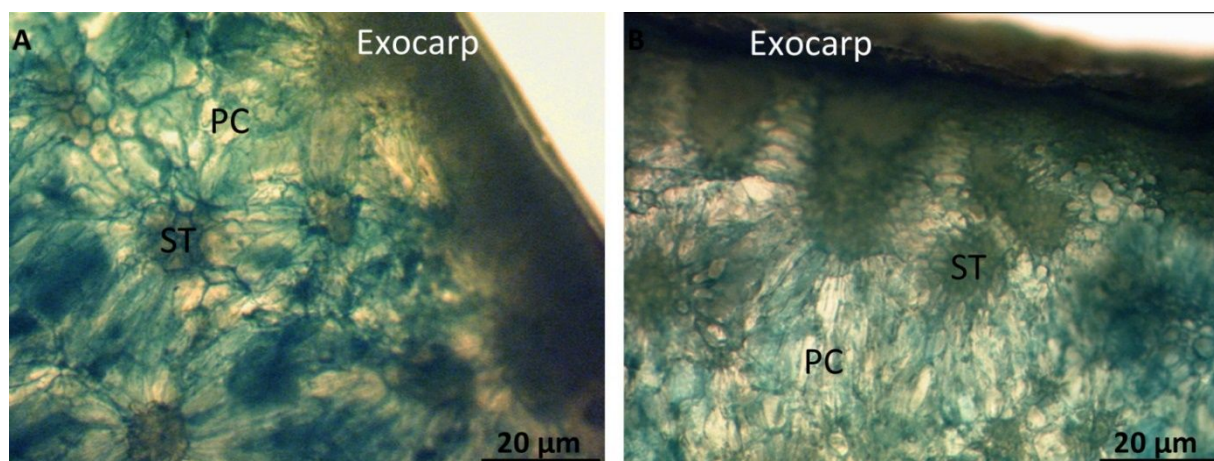
198 **-the intrinsic coloration of the exocarp and the epidermis, which prevents good**
 199 **observation of the blue coloration due to DMACA staining,**

200 **-the darkness of the layer to see whether staining had occurred because staining was to**
 201 **intense due to high procyanidin concentrations in the exocarp especially in the sub-cuticular**
 202 **cell layers,**

203 -the definitions of the skin versus exocarp, as the “skin” as used in [10] contains the
204 exocarp plus some mesocarp cell layers.

205 DMACA specific staining allowed the visualization and localization of cell vacuolar
206 flavan-3-ols in tissues and cell types. The blue coloration seems to fill almost completely the
207 intracellular area suggesting the presence of flavanols in the cell vacuoles. Most but not all the
208 cells were stained. Although DMACA staining does not distinguish between flavan-3-ol
209 monomers (catechins) and polymers (procyanidins) [15-17, 19], and the proanthocyanidin-
210 DMACA molar absorption coefficients are affected by both DMACA reagent environment and
211 proanthocyanidins structural variations [37], it is likely that those flavan-3-ols mainly
212 correspond to highly polymerized procyanidins as procyanidin concentrations are an order of
213 magnitude higher than monomeric flavan-3-ols, i.e. (-)-epicatechin, in ‘De Cloche’ pears [11].

214 Nor general observation (Figure 1) nor light micrograph (Figure 2) allowed doing a
215 visual difference in procyanidin distribution between ripe and overripe pears using DMACA
216 staining. The difference observed in color intensity was probably due to the difference in
217 thicknesses between the sections and not to the procyanidins modifications with overripening
218 (sections Figure 1A and 2A were thicker than 1B and 2B due to pear texture differences).
219 Moreover, it has been shown, using thiolytic HPLC-DAD quantification that the phenolic
220 contents and compositions of pear fruits do not change during overripening [11].



221
222 **Figure 2** : Light micrographs of ripe (A) and overripe (B) perry pear sections stained with
223 DMACA to allow procyanidins localization. ST: stone cells, PC: parenchyma cells

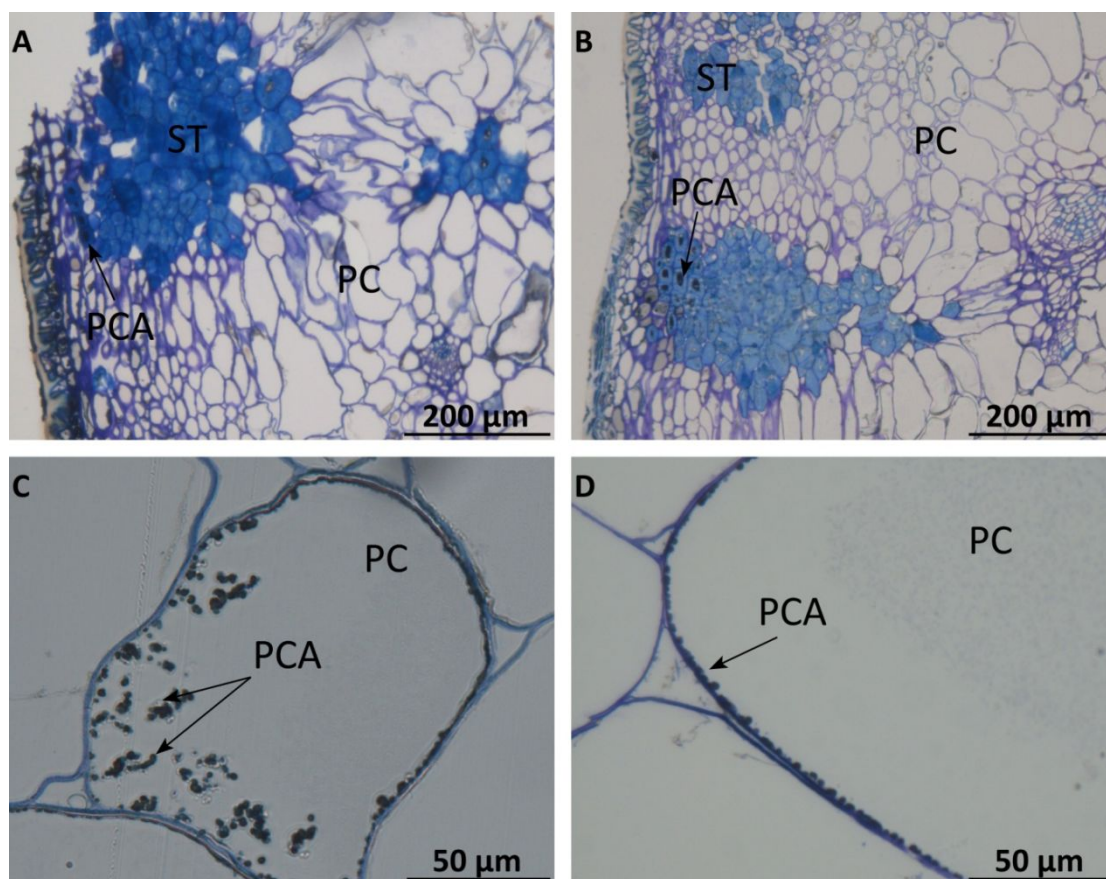
224 3.2. Intracellular localization of procyanidins

225 In order to localize procyanidin at the intracellular level, firstly sections were contrasted
226 using toluidine blue to identify cells which contain polyphenols (Figure 3) and which will be
227 then selected for observation by transmission electron microscopy. Transmission electron
228 microscopy was used, afterwards, to localize procyanidin at the subcellular scale.

229 Toluidine blue staining observed in Figure 3A and 3B seemed to reveal differences in
230 distribution depending on cell type with the presence of polyphenols in stone cells, presumably
231 procyanidins as they represent > 90% of polyphenols in “De Cloche” pears [11]. Moreover, due
232 to the thickening of secondary cells, procyanidins seemed to be embedded within the lignified
233 stone cells.

234 After treatment of samples by osmium tetroxide, dark precipitates were visible mainly
235 in the cell vacuoles of very large parenchyma cells (Figure 3C). Procyanidins of perry pear cells
236 were present as extremely dense spherical granular units, the form of which was constant in the
237 vacuole cell. The sizes of the spherical granules were however variable, ranging from 0.5 μm
238 to 1 μm . In ripe fruit vacuole, procyanidin granules appeared as cluster or to be dispersed in the
239 vacuole as uniformly stained deposits or for some particles close to the tonoplast. The granules
240 did not occupy the entire vacuole (Figure 3C). It could be speculated that these dense spherical
241 granules were tannosomes as observed in grape and in dates [19, 29]. Procyanidin dispersion
242 varied depending on maturity stage (Figures 3C, 3D) with procyanidins only located close to
243 the tonoplast at the overripe stage. Moreover, contrary to DMACA staining which filled all the
244 cells, in both Figure 3C and 3D procyanidins appeared to be present in isolated cells and were
245 present as granules rather than appearing throughout the cell.

246



247 **Figure 3:** Light micrographs of semi thin sections contrasted with toluidine blue from ripe (A,
 248 C) and overripe pear flesh (B, D) use to locate procyanidins before transmission electron
 249 microscopy. ST: stone cells, PC: parenchyma cells, PCA: procyanidins.

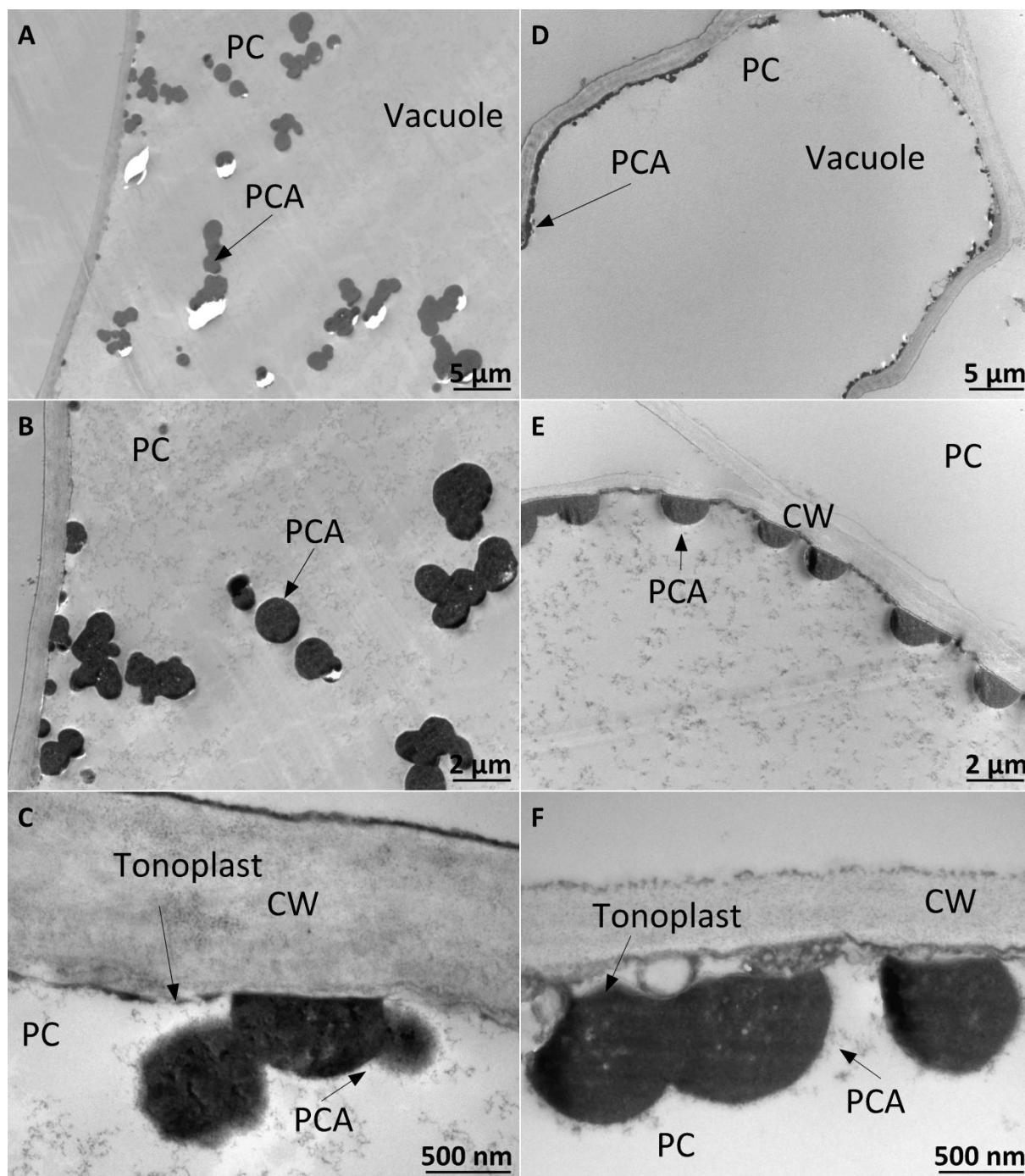
250 The DMACA and toluidine blue stain results seemed contradictory, i.e. the stone cells
 251 stained darkly with the toluidine blue whereas they did not stain with DMACA, and all cells
 252 were uniformly stain with the DMACA whereas only some localized cells stained with toluidine
 253 blue. The discrepancies between these two staining methods may be due to the lack of diffusion
 254 of the DMACA stain through the lignified cell walls of the stone cells, and their relative
 255 volumes and contents. The discrepancy also may be due to sample preparation. For DMACA
 256 staining, thick flesh sections from ripe and overripe pears were used. Cellular integrity may
 257 have been compromised during the sectioning process, inducing a diffusion of polyphenols in
 258 all cells of the tissue. Moreover, the discrepancy between the results of DMACA and toluidine
 259 may be explained also by their different procyanidin affinities, DMACA staining being highly
 260 specific to procyanidins.

261 The distribution of procyanidins accretions in the vacuoles of parenchyma cells seemed
 262 to be change by overripening. At the ripe stage, the vacuolar procyanidin accretions mostly

263 remained dispersed and free inside the vacuole, alone or as cluster, although some were close
264 or associated to the tonoplast (Figure 4A, B, C). At the overripe stage, in contrast, the vacuolar
265 procyanidins were no longer dispersed within the cell vacuole but seemed to migrate close to
266 the tonoplast, the membrane that separates the cytoplasm from the vacuole lumen [38].
267 Procyanidins formed a **continuous** granular layer extremely dense to electrons (Figure 4D, E,
268 F) that entirely covered the tonoplast (Figure 4D). **They seemed losing their spherical shape**
269 **close to the tonoplast.** The tonoplast was still there, the procyanidins were close to the cell wall
270 but still vacuolar. **The very close physical proximity between the tonoplast and procyanidins**
271 **could be explained by the high affinity of these molecules for proteins, in particular the**
272 **tonoplast proteins [39], or to fusion between the tonoplast and the tannosome membranes.**

273 **No procyanidins were detected encrusted or within the the cell walls, in contrast to**
274 **results obtained by Amrani and Mercierz (1994) [39] in grape cells using chemical analysis and**
275 **light microscopy and transmission electron microscopy. Three types of condensed tannins were**
276 **differentially localized in grape berry: free tannins in the vacuole sap, tannins bound to the**
277 **proteins at the internal face of the tonoplast and tannins bound to the cell wall polysaccharides**
278 **by osidic bonds. If the procyanidins were encrusted or within the cell walls a similar appearance**
279 **to the coating of the tonoplast may be expected. Hence, the procyanidins quantified and**
280 **characterized in pear cell walls [11, 35, 40] after cell wall preparation are an artefact of the**
281 **isolation procedure related to the adsorption of intracellular procyanidins to the cell walls.**

282 Nevertheless, as shown in our previous studies, interactions between cell walls and
283 procyanidins were promoted by pear fruit overripening, so that in overripe pear juice
284 procyanidin concentrations were lower than in ripe pear juice. Whereas, fruit procyanidin
285 concentrations were the same [11]. **Using chemical analysis of the pomace obtained after juice**
286 **extraction [11] as well as binding isotherms and isothermal titration calorimetry with purified**
287 **cell walls and procyanidins [40] it was shown that highly polymerized procyanidins are**
288 **absorbed to cell walls. The higher procyanidin retention at the overripe stage was explained by**
289 **cell wall modifications where the degradation of pectic side chains with ripening facilitates**
290 **procyanidin access to cell walls [35, 40].** In overripe pears, the particular behavior of
291 procyanidins which were physically close to the plant cell walls, might promote, when cells are
292 disrupted during fruit senescence or during perry processing, their adsorption to cell walls.
293 More investigations should be done in particular about the choice of maturity stage for pressing.
294 In order to better understand both procyanidins structure and localization in the fruit during
295 growing and maturation, different stage of fruit ripening, could be observed and analyzed.



296 **Figure 4 :** Transmission electron micrographs from ripe (A, B, and C) and overripe pear flesh
 297 (D , E, and F) observed at different magnification. PC: parenchyma cells, PCA: procyanidins,
 298 CW: cell walls.

299 The combination of different methods used in this work to characterize perry pear
 300 procyanidins showed that maturity stage affected only their localization within the vacuole of
 301 parenchyma cells mostly located in fruit mesocarp. These results are consistent with our
 302 quantitative characterization in which no difference of procyanidin compositions was detected
 303 between ripe and overripe stage [11]. However, the specifically organization of perry pear
 304 procyanidins in overripe pears, clustered at the internal face of the tonoplast, might promote

305 their interaction with cell walls when the tonoplast is ruptured at later ripening stages or during
306 perry processing lowering their concentrations in the pear juice.

307

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312

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