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Highlights

- Perry pear procyanidins are concentrated in exocarp and mesocarp parenchyma cells
- Procyanidins were only detected in vacuoles in perry pear flesh
- Procyanidins become attached to tonoplast at overripe stage.

Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Solvents and reagents

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

2.2. Plant Materials

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

2.3. Direct observation after DMACA staining

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

2.4. Fixation

Fixation method used was described by Hammouda et al., (2014) with some modifications. Fresh pear flesh (with or without skin) were used to cut small cubes of tissue (1 mm^3). Small fresh flesh cubes were fixed with 25 mL glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2) for 1.5 h at room temperature. Blocks were rinsed 3 times (30 min) with sodium cacodylate 0.1 mol/L and then postfixed at room temperature for 1 hour with 20 g/L osmium tetroxide (Os O_4) in deionized water. After postfixation, blocks were dehydrated through increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%) followed by dehydration by propylene oxide. Blocks were then progressively infiltrated with araldite resin (GY 502). Polymerization of infiltrated samples was done for 48 h at 60 °C.

2.5. Histology

For light microscopy, semithin sections (1 µm) of fixed flesh pear cube were obtained using an ultramicrotome (Leica microsystems, Leica, Germany) and were then stained with a solution of toluidine blue 10 g/L in Na_2CO_3 25 g/L. The observations were carried out using a light microscope Olympus BX60 (Olympus Corporation, Tokyo, Japan) and images were taken using a color camera Jenoptik Progress (Jenoptik, Jena, Germany).

2.6. Transmission electron microscopy

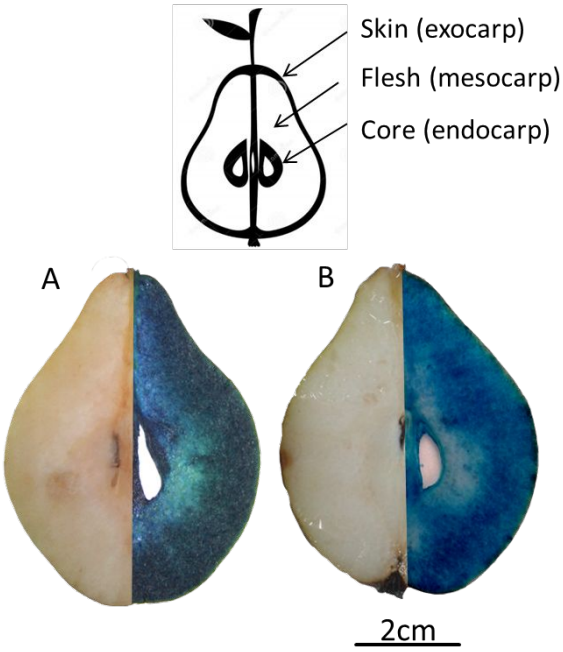
Ultrathin sections (60-80 nm) of fixed flesh pear cubes were contrasted with uranyl acetate 20 g/L in a solution of methanol 50% during 40 minutes and then lead citrate at 0.72% during 5 min. Samples were examined using a Philips CM10 transmission electron microscope TEM (FEI Company, Eindhoven, The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

3. Results and discussions

3.1. Procyanidins localization by light microscopy coupled to DMACA staining

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

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



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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 cuticule. 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,

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

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

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

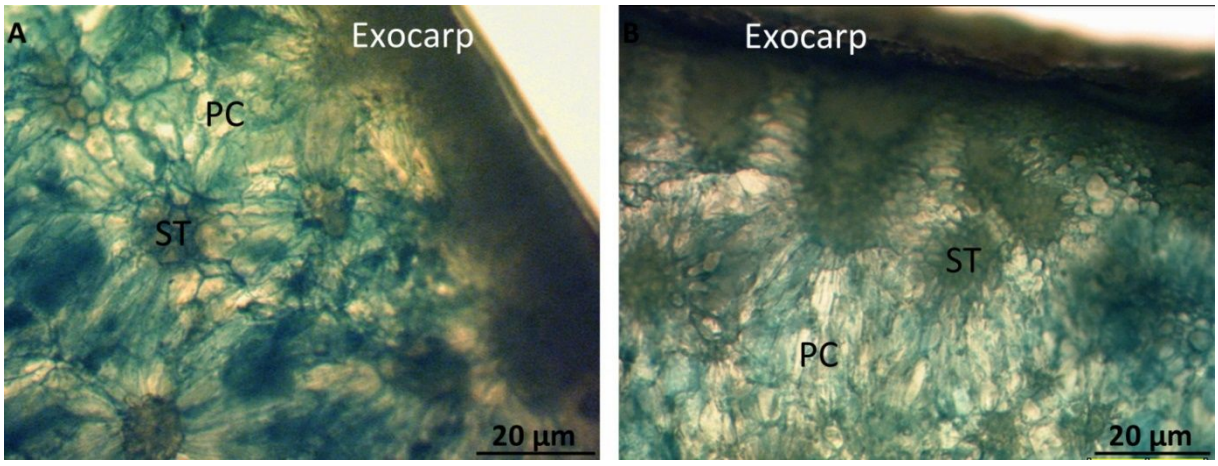

Figure 2 : Light micrographs of ripe (A) and overripe (B) perry pear sections stained with DMACA to allow procyanidins localization. ST: stone cells, PC: parenchyma cells

3.2. Intracellular localization of procyanidins

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

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

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

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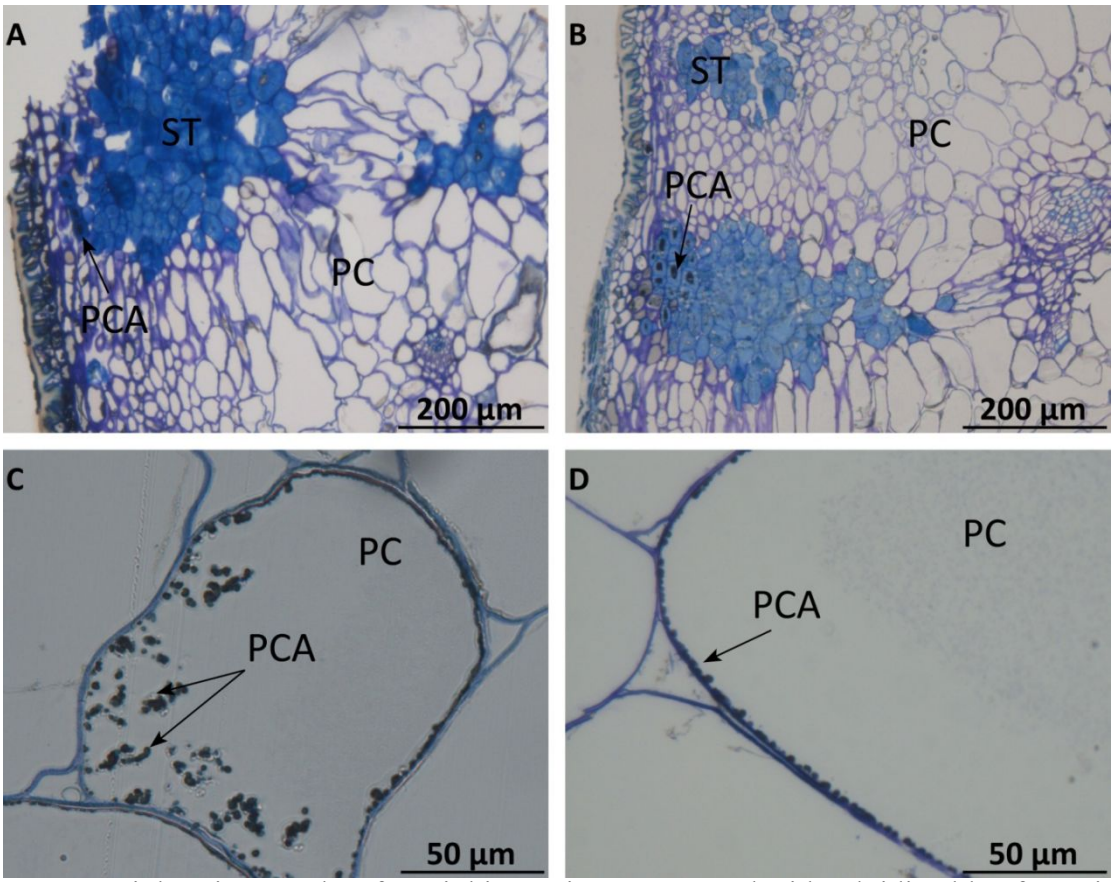

247 **Figure 3:** Light micrographs of semi thin sections contrasted with toluidine blue from ripe (A,
248 C) and overripe pear flesh (B, D) use to locate procyanidins before transmission electron
249 microscopy. ST: stone cells, PC: parenchyma cells, PCA: procyanidins.

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

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

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

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

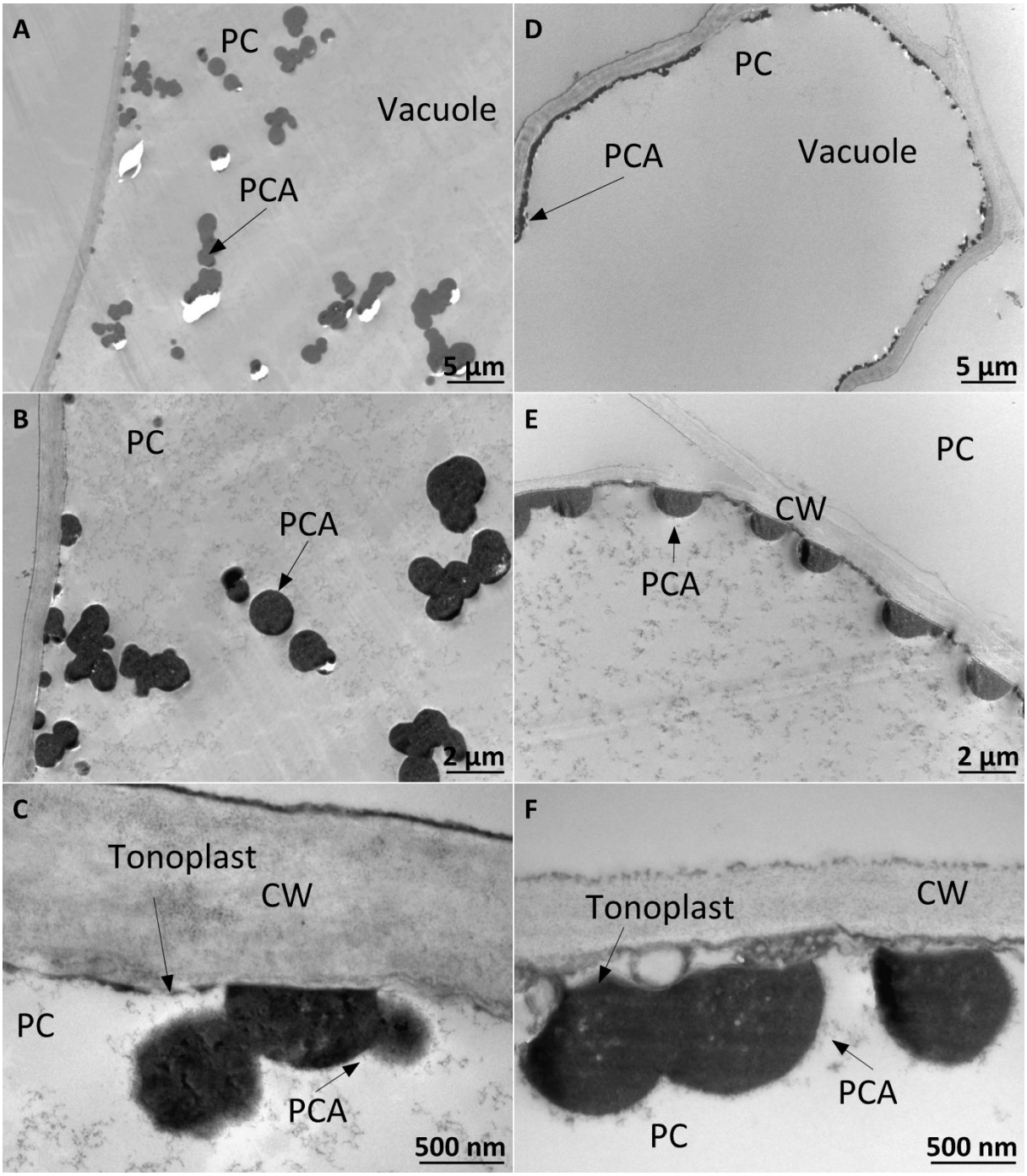

Figure 4 : Transmission electron micrographs from ripe (A, B, and C) and overripe pear flesh (D , E, and F) observed at different magnification. PC: parenchyma cells, PCA: procyanidins, CW: cell walls.

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

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

4. Acknowledgments

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