In vivo dissection of extracellular vesicles production in plants

In the last decade, the number of studies isolating and describing extracellular vesicles (EVs) has dramatically grown once their participation in intercellular communication has been confirmed. As transporters of bioactive molecules, EVs can take part in different physiological mechanisms and have been proposed and used as potential therapeutic tools.

After the discovery of extracellular vesicles (EVs) in animal cells, increasing evidences support the occurrence of MVB- and exosome-like vesicles in plants. Although the existence of plant exosomes is still controversial, in the late 60's two works already described small vesicles formed through unconventional secretory ways. More recently, numerous investigations on cell growth and differentiation, as well as on plant responses against distinct stresses, have supported the existence of plant EVs.

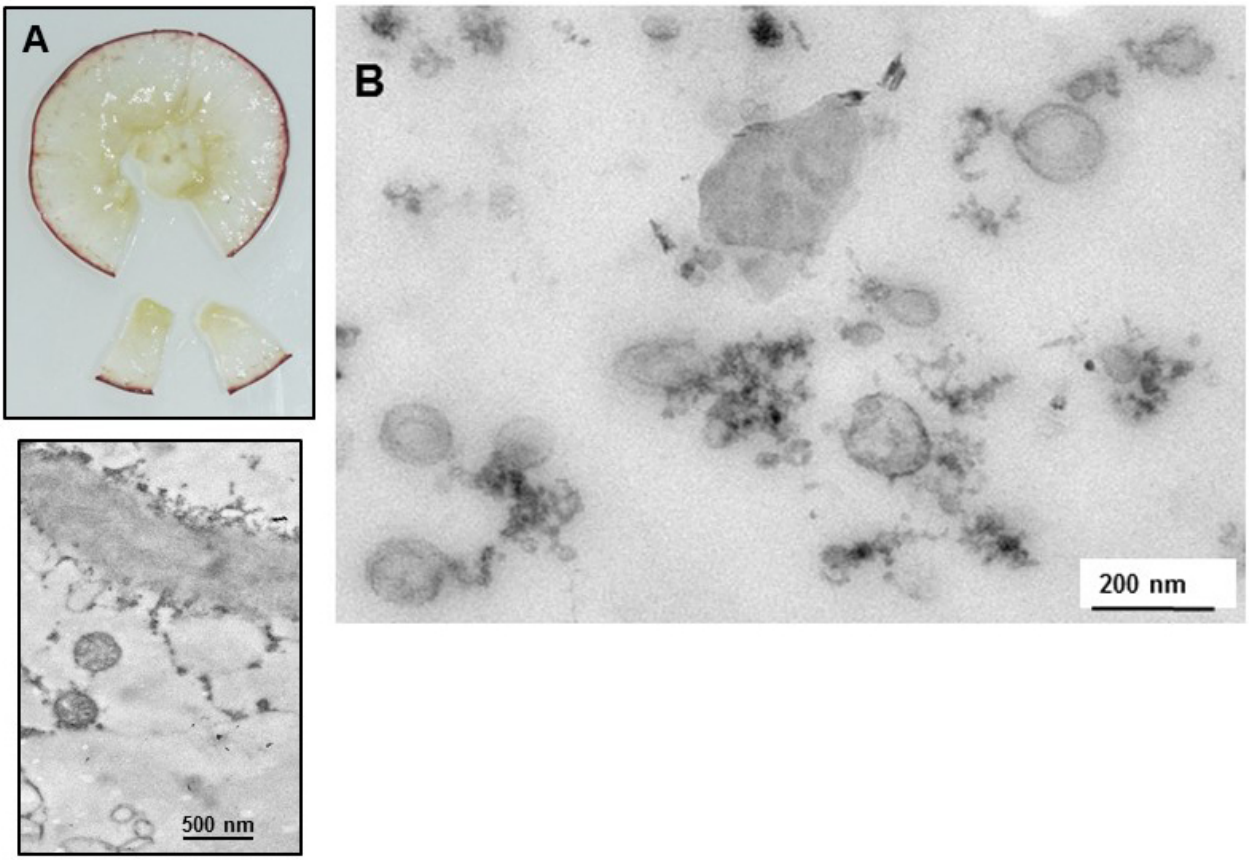

Fig. 1. A) Grape slices fixed and TEM from mesocarp; B) Extracellular vesicles isolated by ultracentrifugation.

The aims of this study were: i) to isolate and characterise EVs obtained from Bobal grape berries by using Transmission Electron Microscopy (TEM) and proteomic analyses, ii) to carry out a preliminary dissection of EVs secretion by analysing by TEM their presence in the pericarp of the grape berry.

Grape EVs isolation. Bobal grape berries were washed and homogenized in the presence of protease inhibitors. Solid residuals were removed and the collected must was concentrated using centrifugal filter devices. Concentrated juice was filtered and nanovesicles pelleted at 120000g for 60 min (4ºC). Finally, pellets were washed and re-suspended in PBS buffer.

Grape berry fixation. Eight injections of 25% glutaraldehyde were done in the equatorial plane of the fruit. In each injection point, glutaraldehyde was first injected in the endocarp, and successively in the mesocarp and exocarp. After 2 h, the equatorial disk (2 mm) was dissected and cut into small pieces that were immersed in fresh fixative for 3 h under vacuum. Finally, the samples were processed and observed under TEM.

Sample preparation and TEM observation. Both, isolated EVs and berry slices were fixed with 2.5% glutaraldehyde, filtered in resin, and polymerized at 60 ºC for 48 h. Ultrathin slides (60 nm) were stained with 2% uranyl acetate and viewed by TEM in a Jeol microscope.

Proteomic analysis and Database searches. Proteomics were performed by LC-MS/MS analysis. Database search on NCBInr databases were performed using the Protein Pilot® software v4.5.

Results
Dissection of *in vivo* EVs secretion in grape berries has demonstrated that EVs are released in all the tissues analysed. We showed the existence of both, MVBs and microvesicles driven mechanisms for EVs secretion. These data represent the first *in vivo* characterisation of typical EVs in plants (Fig. 1A).

Regarding the isolation of EVs, our results confirmed that classical ultracentrifugation, combined with previous concentration steps, was an efficient method to isolate EVs from grape berries. EVs characterized by TEM in the final pellets ranged from 30 to 200 nm (Fig. 1B).

Proteomic analyses of grape EVs showed the presence of typical EVs proteins, including membrane and vesicle-associated ones. LC-MS/MS analysis showed 246 peptides, corresponding to 121 proteins, in EVs from Bobal berries. Out of the 55 identified proteins in grape EVs, only two of them have a signal peptide.

Our data support that fruits release EVs by both MVB and microvesicle formation, and their membranes can protect their content from lysis. Plant EVs provide a convenient system for delivering therapeutic agents, so they can be delivered from the plants to our cells. Although there

are very few reports regarding this topic, current clinical trials are addressing the use of fruit-derived EVs (grape, grapefruit, watermelon, lemon, orange, etc.) as a promising treatment to inhibit proliferation in different tumor cell lines.

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