Application of an innovative technique for histological and histochemical study of different vegetal tissues

B. Dore[1], A. Ponso[2], R. Vallania[3]

[1] Department of Animal and Human Biology, University of Turin
[2] Department of Arboriculture and Pomology, University of Turin
[3] Plant Virology Institute CNR, Grugliasco Unit, Grugliasco (TO)

KEY WORDS: cryostabilization, embedding, polar resin (GMA), agar, fruit trees.

Abstract

A new original "cryostabilization" technique (Dore, 1992; Dore et al, 2004)) was applied to different animal tissues and organs. In this study, we apply this technique also to vegetal tissues (ripening and ripe fleshy fruits, buds and embryos). The proposed method preserves good tissue morphology and intracellular substances, and avoids loss of epicuticular waxes; moreover it maintains in situ some enzymatic activities. Being some samples very difficult to handle the original protocol had to be modified. The correct orientation in embedding of samples is an important requirement which is not easy to achieve when dealing with small fragments of tissues. To obtain slices oriented correctly, Vitis vinifera L. somatic and zygotic embryos, very little in size, and Corylus avellana L. leaves, are pre-included in agar 1%. This pre-inclusion in agar either facilitates the sample manipulation either allows to achieve a satisfactory precision in slicing. In a study on apricot buds (Prunus armeniaca L.), vacuum was used to facilitate reagent penetration, in spite of tissue hardness and impermeability. Observations were performed on slices cut with glass blades and stained with different histological and histochemical dyes. In Vitis vinifera the method allowed to distinguish normal and anomalous embryo development in different stages. In Corylus avellana different thickness of leave lacunose and palisade tissues could be put in relation with sun or shadow conditions of growth. In apricot buds the morphology of meristematic tissues is well preserved and starch reserves could be visualized, up the starting of hydrolysis at the inception of the new vegetative season.

Introduction

A new "cryostabilization" technique (Dore, 1992; Dore and Carnemolla, 1993; Dore *et al.*, 2004) was set up a few years ago and was applied to different animal tissues and organs. This method was also used on plant tissues (ripening and ripe fleshy fruits of apples, grapes, apricots, kiwis, cherries, tomatoes, buds and embryos) (Jona *et al.*, 1998) because it is advantageous in comparison with traditional histological techniques. It allows a soft dehydration of tissues even if they are very rich in water (e.g. fruit pulp which contains about 86% water), it protects cell structure and prevents leakage of cell and organule substances. For example it was possible to spot polyphenol in the cell (Dore *et al.*, 2005): their antioxidative properties are very important in the human diet. In grape, polyphenols are visible as soluble substances into the cell vacuoles of the first layer of the berry; on the opposite into unripe apples they are abundant and diffused into the vacuoles of the cells of six-seven layers of pericarp. Later, in ripe apples, the polyphenol reaction takes place only in the vacuoles of the first and second layer of pericarp; in kiwis the reaction is stronger in cell walls, while inside the cell the reaction is more light than in other fruits. The proposed method preserves good tissue morphology and intracellular substances such as starch, lipids and polyphenolic substances, and avoids loss of epicuticular waxes. Samples obtained by cryostabilization also maintain *in situ* some enzymatic activities, as acid and alkaline phosphatase and polygalatturonase, an enzyme involved in the ripening process of fruits which increases during the infection by *Botrytis cinerea* (Ruffa *et al.*, 2003).
Aim of this study is to modify the cryostabilization method for vegetable tissues and organs that are difficult to handle and present scarce penetration of reagents.

Materials and methods

The "cryostabilization" technique is based on the use of ethylene glycol at -20°C as cryoprotector. At the same time there is in tissues water is substituted with a mixture water/ ethylene glycol of increasing concentration (50%, 75%, 90%, 95%). Once the highest concentration is reached glycol methacrylate (GMA) can be added thus embedding the tissues: polymerization is induced at low temperature

(6-10°C). Some of our samples are very difficult to handle and the original protocol has to be modified to solve this problem. In the final embedding process the correct orientation of small samples is an important factor, usually not easy to obtain. To obtain well oriented sections, *Vitis vinifera* L. somatic and zygotic embryos, very little in size, and *Corylus avellana* L. leaves, are pre-included in agar 1%. This pre-inclusion in agar allows either manipulating the sample through the different steps of the process and better precision in slicing.

Somatic embryogenesis is a technique used in breeding programs to obtain plants from a single cell. The studied samples were obtained from ovary and anther cultures of *Vitis vinifera* cv Grignolino. We considered the fundamental developmental stages of the embryo: globular, heart, torpedo and fully formed embryo.

In *Corylus avellana*, we studied different thickness of lacunose and palisade tissue of leaves either exposed to the sun either shaded. We performed our studies on the cultivar Tonda gentile delle Langhe, largely cultivated in Piemonte (North-West of Italy). Leaves were collected approximately at the same height from both sunny and shaded south oriented part of the canopy. Leaf lamina thickness, palisade tissue percentage out of total thickness and cell layers of palisade tissue were compared between the 2 theses. Moreover the stomata density (No/mm^2) was measured.

In a study on apricot buds (*Prunus armeniaca* L.), to obtain better reagent penetration vacuum was used in order to overcome tissue hardiness and impermeability. In apricot buds, longitudinally cut, each step of ethylen glycol/water substitution was of 24 hours and was realized under controlled vacuum gradually obtained by a rotary pump (-0.95 bar). Ten twigs with vegetative buds of two varieties (San Castrese and Laycot) were weekly collected from November to February then grown under controlled conditions, partially dipped in water, in order to determine the date of dormancy breaking. Bud development was observed until full bloom. The bud sections were stained with the same dyes as leaves and with Lugol solution at pH7, in order to put in evidence starch granules in the tissues.

As the method preserves either tissues and organs morphology and their histochemical characters, some observations were performed on 2.5-3 μm thick slices obtained with glass blades by a rotary microtome, floated on water and then coloured with different histological and histochemical dyes. Toluidine blue exhibiting a characteristic metachromatic reaction (from blue to violet purple) was used to reveal the morphology. Different histochemical dyes were used to stain the epicuticular waxes and lipids (Nile red by fluorescent microscope at λ 460-480 nm), total polysaccharides i.e.cellulose, hemicellulose, pectin (PAS), nuclei (DAPI fluorescence at λ 360-380 nm), chloroplasts (autofluorescence at λ360-380 nm), starch granules (Lugol solution).

Results

Pre-inclusion in agar allows the correct section orientation also of difficult material such as embryos in early stage of development and leaf lamina. Good and easily reproducible results are obtained for meristematic apex of buds (fig. 1) and leaves. More difficult has been to obtain sections from the coriaceous bud perules.

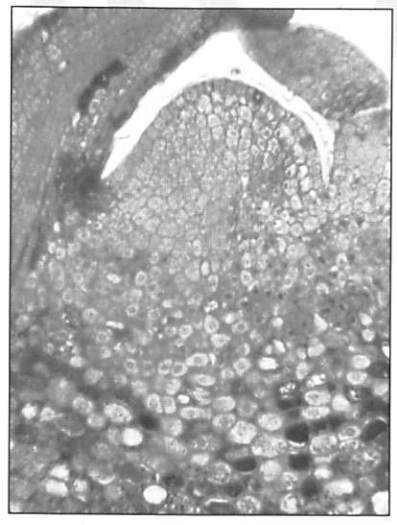


Fig. 1 - *Apricot bud morphology Toluidine blue*

In *Vitis vinifera* embryos it was possible to point out normal and anomalous development in different stages (Ponso *et al.*, 2007a). Toluidine blue allows to put in evidence protodermic cells probably containing polyphenolic blue brilliant substances dyed, blue-violet cell walls, blue-green lignified vessels, colour-less starch and accumulation of lipidic substances, and deep violet nuclei (fig. 2). Somatic

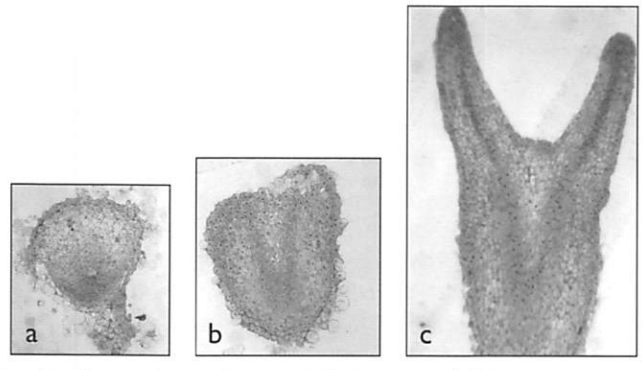
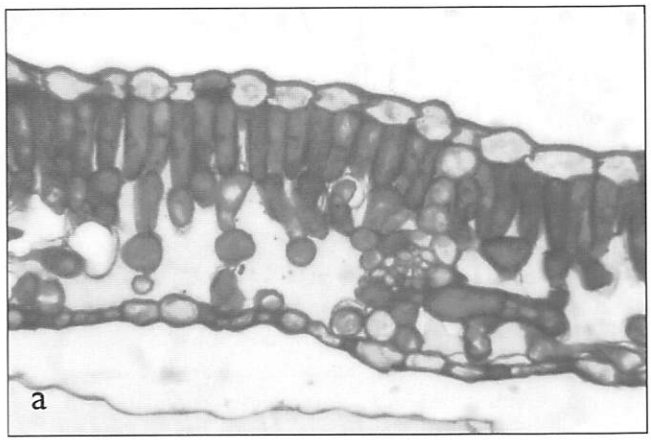


Fig. 2 - Grapevine embryos: globular stage (a), heart stage (b), torpedo stage (c). Toluidine blue.

embryos often show anomalous morphologies that do not allow them to develop in vital plants. Most of anomalies began to be detectable between heart stage and torpedo stage, during differentiation of the apical meristem. The aberrations were detected in apical meristem, in cotyledons and in hypocotyl. These different kinds of anomalies are probably due to the culture on media containing high concentrations of growth regulators, used to induce embryogenic callus and development of somatic embryos.

In fruit trees instead the productivity is correlated to light penetration through the foliage: the higher quantity of light is intercepted from leaves the higher is the frequency of flower induction in axillary buds, as assessed by many authors in different fruit trees. Training system and plant disposition in orchard both influence the quantity of light intercepted by leaves, nowadays some studies are performed on leaf photosynthetic efficiency. Since hazelnut species are less known for this aspect, morphology of leaf lamina was analyzed with Toluidine blue, while total polysaccharides were dyed with the PAS reaction; Nile red dyes epicuticolar waxes, DAPI nuclei, while lignin and chlorophyll presence exhibits yellow-green colour due to autofluorescence. In sunny leaves the palisade tissue is constituted on the average by 2.5 cell layers, while in shaded leaves the palisade tissue is constituted on the average by 1 cell layer (fig. 3). Stomata density was the



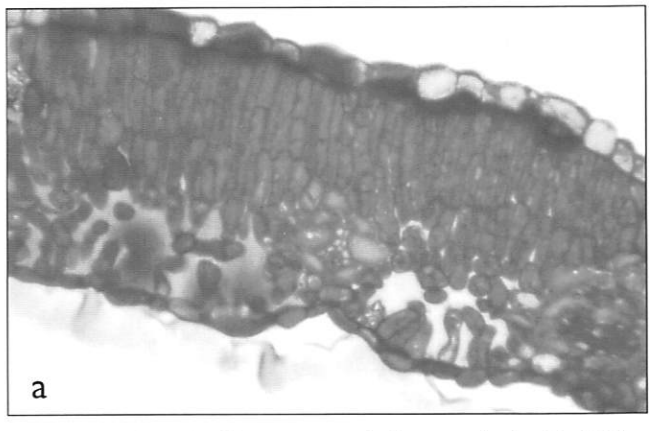


Fig. 3 - Hazelnut leaf lamina morphology in shaded leaf (a) and sunny leaf (b). Toluidine Blue.

double in sunny leaves in comparison with shaded leaves. The different leaf morphology found in 2 theses here examined helps to determine plantation density, training systems and pruning, in order to optimize sun exposition and the subsequent productivity of plants (Ponso *et al.*, 2007b).

From the observations of apricot buds it results that both cultivars were in total dormancy at the end of November. The end of dormancy occurred between the end of December for San Castrese and the beginning of January for Laycot. The presence of starch was observed as granules stained in red-violet by PAS or of dark coloration after Lugol treatment. The maximum starch presence was observed in both cultivars when bud are dormant. Starch hydrolysis started about in middle December, when the buds began to flower. When the plants have reached the end of dormancy, buds have hydrolyzed almost all starch (fig. 4). Starch was accumulated during chilling weather as

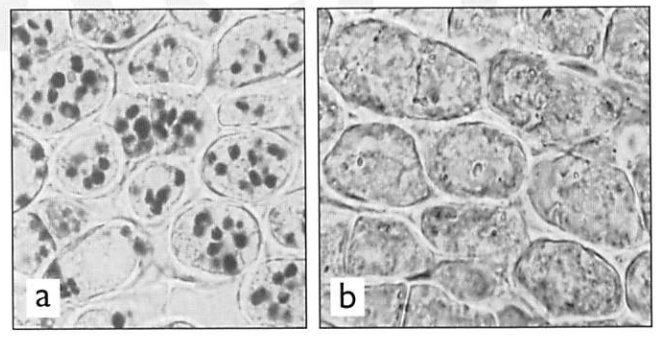


Fig. 4 - Starch in apricot dormant bud (a) and in bud at renewal of vegetative season (b). Lugol.

reserve and it was hydrolyzed and converted to sugar when the temperature started to rise. Moreover it is used by the cells during the expansion, when resumption of growth begin. In both cultivars nuclei (DAPI fluorescence) were more numerous and brightly luminous in dormancy-breaking buds (fig. 5). Lipids (Nile red) were observed as a thin layer on cells of protoderm of bud apex and perules, and also inside the cells as lipidic drops.

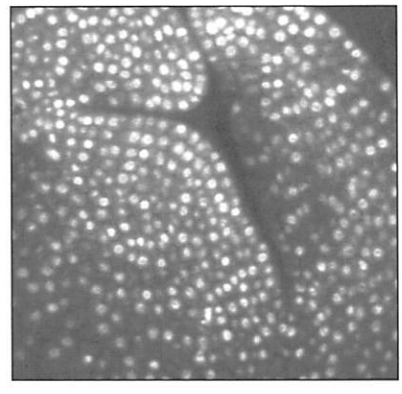


Fig. 5 - Nuclei in apricot dormancy-breaking bud. DAPI.

Conclusions

The "cryostabilization" technique is rapid, easy to perform, nor it requires expensive and dedicated instruments. Moreover the operators and environments are not exposed to VOC (formaldehyde and solvent vapours). Using both ethylene glycol solution as cryostabilizer and means of soft dehydration embedding in polar hydrophilic resin at low temperatures, good preservation of different vegetal tissues and useful and reproducible results have been achieved. Moreover this technique allows the subsequent application of histochemical protocols also if delayed. In some experiments problems in the manipulation and resin infiltration of sample were faced (e.g. coriaceous

bud perules and hard leaves grown in sunny condition). We hope to solve the observed problems extending the experimentation.

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