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Histological method to study winter physiological dormancy in leaf buds of 'San Castrese' and 'Laycot' apricot, *Prunus armeniaca* L.

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Abstract

A new method of cryostabilization and embedding in a polar resin, performed at –20°C under controlled vacuum, was used to microscopically observe morphological and histochemical changes in leaf buds of apricot collected during the rest period. In order to determine the date of endodormancy breaking, ten twigs of two varieties (San Castrese and Laycot) were collected weekly from November to February and held under controlled conditions. At the same time, ten leaf buds from each variety were collected. The stained sections obtained revealed good preservation of tissue and cell structures, which allowed the study of physiologic changes correlated to breaking of dormancy. Moreover the observation of lipids and waxes that in traditional inclusion methods were lost was allowed. The histological-cryostabilization method revealed changes of cell components, such as different amounts of starch and lipid, size of cells, differences in colour and brightness of nuclei from buds which were completely dormant to metabolically active.

Introduction

Dormancy is a plant strategy to survive environmental conditions unfavourable for growth, such as harsh winters when low temperatures would kill an actively growing plant. In the boreal hemisphere this phenomenon is normally induced in autumn, when day length and temperature decrease, and it is characterised by the inability to grow (Cronje *et al.*, 2003).
The aim of this study was to observe morphological and histochemical changes in leaf buds during resting period and link them with physiological mechanism involved in break dormancy. We correlate an empirical agronomic study of chilling requirement of apricot cultivars (Valentini *et al.*, 2004) with a histological study. The histochemical method used for the study was already successfully used for animal tissues (Dore, 1992; Dore and Carnemolla, 1993). To adapt the method to plant material and to allow a good resin infiltration in bud tissues, two important modifications were introduced: a) pre-infiltration of a dehydration series before the embedding process and b) conduction of the whole dehydration series and embedding process under controlled vacuum.

Material and Methods

Two apricot *cultivars*, 'Laycot' originally from Ontario (Canada) and 'San Castrese' from Vesuvio area (Southern Italy), were used for the study. Experiments were carried out in 2000-2001, in an apricot orchard located in Costigliole di Saluzzo, Piedmont region, Northwest Italy (long 45° 4' N; lat 7° 40' E) at 500 m a.s.l. Climatic data were recorded by meteorological stations installed in the orchard and hourly temperature data were recorded from the end of summer until full-bloom. Five plants for each variety were chosen and marked for the trial. Ten twigs of each variety, 40-50 cm long, were collected weekly from November to February. Twigs were held at 18-22°C with twig bases immersed in water, in order to determine the date of endodormancy breaking and flowering (Valentini *et al.*, 2004). Every three days, bud development was observed until full bloom. In stone fruits, flower and leaf buds develop at different rates; flowering occurs before leaf sprouting, and endodormancy is considered broken when 70% of the twigs and 50% of the flower buds on each twig begin to flower (Stage F, according to Baggiolini, 1952).
Ten leaf buds of each cultivar were weekly sampled at the same time of twigs and used for the histological, histochemical and morphometric study. The buds were longitudinally cut and pre-cryostabilized at +4°C using Ethylene Glycol (EG) / water 50% solution for 30 min. Afterwards buds were placed in water/EG solution increasing EG concentration (50%, 75%, 90%, 95%). Each step was for 24 hours and was performed at –20°C under controlled vacuum gradually obtained by a rotary pump (-0,95 bar). Then the buds were subjected to a 1:1 EG and hydrophilic polar resin GMA (Glycol Methacrylate,

Technovit 7100-Kulzer) monomer solution for 24 hours at + 4°C. The buds were then embedded using the activated resin (Benzoyl Peroxyde and hardener agent) for 6 hours at + 4°C.
Sections, 2.5 m thick, were obtained with a rotary microtome using a Ralph type glass blade, floated on a water droplet, air dried and examined using the following staining procedures:

- Toluidine Blue O is used to observe general morphology of cell walls, nuclei, and middle lamellae (Clark, 1981). Slices were immersed in 0.05% of dye in 0.1 M, pH 4 sodium acetate - acetic acid buffer solution;
- Schiff's reagent was prepared from pararosaniline according to Tomasi's method (Pearse, 1968). PAS is used to stain neutral polysaccharides. Sections were placed in 0.5% aqueous periodic acid for 20 min, washed in running water and immersed into Schiff's reagent, at room temperature for 30 min;
- Lugol solution is a histochemical test used to stain starch granules in plant tissue: slices were immersed in aqueous 1% iodine and 4% KI, at room temperature and pH 7 (Lotti, 1985);
- Nile Red is used to stain waxes and also the minute lipid droplets retained in cells avoiding chemical fixation, exhaustive dehydration and hydrophobic resin infiltration necessary in the usual paraffin or apolar resin embedding: slices were immersed for 15 min at room temperature in 1% aqueous Nile Red, then transferred to 0.1% acetone, then washed in distilled water (Fowler and Grespan, 1985; Brown *et al.*, 1992), and the chromophore observed by fluorescent microscopy with excitation at 340-380 nm (Greespan *et al.*, 1985);
- DAPI (4'-6–diamino-2-phenyl indole) is used to observe nuclei: slices are stained for 20 min in 1% of DAPI in pH 7.4 phosphate buffer, then washed and observed with excitation at 340-380 nm fluorescent light (Kapuscinski, 1995). This fluorescent stain is A-T selective for DNA and is believed to bind to the minor groove of double-stranded DNA.

For each sampling date, sections of five leaf buds of each variety were observed and measured under light microscope. On the basis of preliminary observations, buds were classified in three zones: A - meristematic zone; B – transition (determination) zone; C - elongation (differentiation) zone (Longo, 1986). The apical meristematic zone of cellular division and elongation zone cells of the buds were easily identified in the slides. Between these two zones the transition aspect of cells in the developmental process can be observed.
Cell sizes were measured to the nearest 0.1 m using an ocular micrometer. Cell starch content was estimated by visual estimations from independent observers using a starch rating scale estimation from 0 to 5. Morphometric data were analysed with ANOVA and mean values compared by Tukey's test using STATISTICA Software (StatSoft).

Results

Both *cultivars* were completely dormant at the end of November. The percentage of flower buds that bloomed increased progressively during the following weeks. The end of endodormancy occurred at the last week of December for 'San Castrese' (27 December) and at the beginning of January (3 January) for 'Laycot'.
The presence of starch was observed as granules stained red-violet by PAS (or blue by Toluidine blue O) or dark coloration after Lugol treatment. The maximum starch abundance was observed in both cultivars when buds were still dormant (29 November) in cells of differentiation zone. Starch hydrolysis began 13 December, the date on which buds began to flower. When the plants reached the end of endodormancy, essentially all starch was hydrolysed in the leaf buds.
In all the subdivision zones – meristematic, determination, diferentiation, cell diameters were greater in 'Laycot' variety compared to 'San Castrese'.
In both varieties, cells of differentiation zone showed greater dimensions than those of meristematic and determination zones. Considering each single zone, dimensions increased from complete dormancy (29 November) to the end of dormancy. However, a greater size difference was observed in cells of meristematic zone (75.5% for 'San Castrese' and 35.3% for 'Laycot') while the least size difference was in cells of differentiation zone (57.2% for 'San Castrese' and 22.4% for 'Laycot').
Buds of 'San Castrese' showed significant differences in cell dimensions of meristematic zone only between completely dormant buds (November) and the subsequent sampling dates. By contrast, cells of determination and differentiation zones exhibited more evident size increases when buds were breaking dormancy. 'Laycot' showed a progressive increase in cell size from buds sampled in November to those sampled in January. Observations of the last two sampling dates did not show significant differences in cell diameters once endodormancy was broken.
Nuclei were observed using DAPI stain and epifluorescence. In both *cultivars*, nuclei were less bright in dormant buds, whereas in dormancy-breaking buds they were more numerous and brightly luminous. In addition, dormant buds showed differences between cells of protoderm (first cellular layer) which had nuclei coloured bright blue, whereas cells of inner layers showed nuclei stained opaque blue. The difference was also evident in cells that compound protoderm of perule. No differences in nuclear colour or brightness were found in cells of buds that were breaking dormancy.
The presence and amount of lipids were observed using Nile Red staining. Lipids were observed as a thin layer in cells of protoderm of bud apex and perule, and also inside the cells as lipid droplets. In contrast to the starch evaluation, no differences in location and amount of lipids were found in buds sampled on different dates.
Buds are specialized structures to permit survival of "dormant" tissue in extreme conditions. Thus they present specialization to insulate and protect the biological material

from adverse environmental conditions. The thick cuticle wax strata represents a defence to water loss or absorption and also to the penetration or action of pathogens. Due to this type of structure it was previously very difficult to penetrate bud tissues with resin to obtain microscopic sections. In spite of this difficulty, this new method allowed good preservation of the bud tissue and to apply histochemical reactions without any apparent artefacts. One valuable characteristic of this method was to allow characterization of the lipids. By contrast, with the traditional inclusion method, lipids are lost as a consequence of paraffin solvents and the high temperature used.

Many cellular activities begin at the onset of bud dormancy until the resumption of growth capacity: reserve carbohydrate mobilisation, water content increase, energy transport, supply and utilisation of metabolites in the growing organelles (Zanol and Bartolini, 2003). Actually, changes in starch hydrolysis, cell diameter increases, and different nuclei stainability were evidenced in this study and they can be correlated with breaking of dormancy.

In both *cultivars*, dormant leaf bud cells were filled by starch granules, whereas cells were almost completely starch devoid once dormancy was broken. Starch accumulated during chilling which functioned as a metabolic energy reserve and when resumption of growth began, it was hydrolysed to sugar at warm temperatures and used in expansion of cells. Another important change was the increase of cell dimensions that is evident in the three considered zones from buds completely dormant (29 November) to those that had ended the endodormancy period. The increase of cell dimension is probably correlated with the whole cellular content and/or the increase of thickness of cell walls.

Probably the status of water is involved both in cell enlargement as well as in starch hydrolysis. In fact, bound water is reported to be rapidly converted to free status when resumption of growth begins (Crabbe, 1994).

Owens and Molder (1973) have studied dormancy in terms of the mitotic activity of cells of buds, defining buds as "dormant" when mitotic activity in the cells of bud is zero, a condition occurring from December through February in their climatic conditions. When growth is resumed, metabolic machinery of the buds is unleashed, DNA, RNA and enzyme synthesis begin and energy metabolism shifts from the pentose pathway to the tricarboxylic acid pathway (Wang *et al.*, 1991). The staining differences revealed in this trial could only suggest a different metabolic status of nuclei, but we were not able to quantify the mitotic activity of cells.

The fluorescent Nile Red dye, was applied successfully on slices cryostabilized in EG solution and thus not subjected to solvent extraction, and buds showed the different locations of lipids during breaking of dormancy, and a new migration of these lipids from cells to outside with the function of bud protection. The lipids on the surface were waxes that protected the buds from the external environment and pathogens, while the intracellular lipids were more likely employed in the synthesis of membrane lipids necessary during cellular expansion. Increase of free water in buds is one of the first signs of bud break and changes in membrane lipid composition may be associated with the mobilisation of water into cells and changes in cellular organization (Crabbe, 1994).

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