USE OF DMACA TO VISUALISE FLAVAN-3-OLS IN GRAPE BERRY SKINS (*VITIS VINIFERA* L., CV CABERNET FRANC)

UTILISATION DU DMACA POUR LA MISE EN ÉVIDENCE DES FLAVANES-3-OLS DANS LA PELLICULE DE BAIES DE RAISIN (*VITIS VINIFERA* L., CV CABERNET FRANC)

M. CHEVALIER[1], Émilie PERROCHON[2], Marie TELLIER[1] and G. BARBEAU[2]

1 : INRA, Unité d'amélioration des espèces fruitières et ornementales, 49071 Beaucouzé cedex, France
2 : INRA, Unité expérimentale Vigne et Vin, 49071 Beaucouzé cedex, France

Résumé : Les composés phénoliques revêtent une grande importance en œnologie. Ils jouent un rôle majeur dans l'élaboration des vins rouges et durant leur conservation, et contribuent fortement à leurs propriétés organoleptiques. Parmi eux, les flavonoïdes constituent le groupe le plus important présent dans la pellicule de la baie. Leur étude histologique est classiquement basée sur l'utilisation du bleu de Toluidine en tant que colorant non spécifique des différents types de polyphénols. L'utilisation du DMACA (p-dimethylaminocinnamaldehyde), spécifique des tanins (flavan-3-ols), a permis de préciser sans risque d'erreur, la localisation de ces composés et de mettre en évidence les tanins, collés au tonoplaste, lesquels ne sont pas révélés par le Bleu de Toluidine.

Abstract : Phenolic compounds are of great importance in Enology. They play a major role in the elaboration of red wines and during wine preservation, and contribute greatly to their organoleptic properties. Among them, flavonoïds constitute the major group present in berry skins. Their histological study is based classically on the use of Toluidine blue O as a staining agent, which is not specific of any type of phenolics. The use of DMACA (p-dimethylaminocinnamaldehyde), which is specific of tannins (flavan-3-ols), permitted to precise the localisation of these compounds without any risk of error ; it allowed also for the visualisation of tannins, stuck to the tonoplast, which had not been identified with Toluidine blue O.

Key words : grapevine, phenolics, staining procedure, p-dimethylaminocinnamaldehyde, Toluidine blue O

Mots clés : vigne, composés phénoliques, coloration, p-dimethylaminocinnamaldehyde, bleu de Toluidine

INTRODUCTION

Phenolic compounds are of great importance in Enology. They play a major role in the elaboration of red wines and during wine preservation, and contribute greatly to their organoleptic properties. Phenolics are found in grape berries skins and seeds. The flavonoïds - anthocyanins (red pigments), flavan-3-ols (tannins), flavonols (yellow pigments) and flavanonols - are the most important phenolic compounds found in the skin. Among them, the flavan-3-ols are known to be particularly abundant (AMRANI-JOUTEI, 1993). Within this group, cathechin, epicathechin and gallocathechin are present in the vacuoles in their polymerised forms (AMRATI-JOUTEI, 1994; BROSSAUD, 1999). Flavonoïds are reported to have a beneficial effect on human health .

Classical histological studies are realised using a freezing microtom ; in these conditions, numerous staining methods are possible. Glutaraldehyde, as a fixative agent, hampers the staining reactions. The toluidine blue O, used for routine examination in numerous laboratories, has not a good specificity ; moreover, considering the poor contrast between blue (polyphenolic compounds) and green (cytoplasm) coloured tissues, this staining method appears unsuitable for a good differentiation of the phenolic compounds (GUTTMAN, 1995). The histochimical method using the p-dimethylaminocinnamaldehyde (DMACA) was developed by FEUCHT *et al.* (1992), after glutaraldehyde fixation and embedding in metacrylate resin. This method has a good specificity to flavan-3-ols.

*Correspondance : cvlr@angers.inra.fr

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In this note, the use of DMACA was adapted to grape berry skins for specific localisation of flavan-3-ols, through light microscope observations. The DMACA reagent was compared to toluidine blue O and used to describe the evolution of phenolics during the ripening of the berries.

MATERIALS AND METHODS

I - PLANT MATERIAL

The experimental plot (CYR I) was located in Saint Cyr-en-Bourg near Saumur (France), and planted with Cabernet Franc, cl 210, grafted onto 3309C rootstock. Vines were twelve years-old in 2002. Berries at the same stage of development were collected with their pedicels, from one month before veraison up to the end of veraison. Homogeneous young green hard berries were obtained from flowers that had been previously marked, according to their date of opening, later on, soft homogeneous ripening berries were selected according to their density. The shaded face was identified in order to have comparable samples. A square portion of berry skin (0.25 cm^2) was taken off from the equator of the sun-exposed face.

II - FIXATION AND EMBEDDING

Samples were transfered immediately into a fixation solution containing 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2, for 2 hours, under vacuum. Thereafter, samples were dehydrated in a grade of ethanol series and embedded in a resin Technovit 7100 according to KROES et al. (1998). Specimens were stored at 37°C.

III - STAINING SOLUTION

The following staining solutions were used :

Toluidine blue O: 0.5 % in distilled water.

DMACA: 0.5 M sulphuric acid in 1-butanol. After cooling off, 0.1 % p-dimethylcinnamaldehyde (Acros organics, New Jersey, USA) was added and dissolved (GUTMANN and FEUCHT, 1991).

IV - STAINING PROCEDURE

1.5 µm sections were cut with a Reichert Jung microtom and mounted on clean glass slides. For toluidine blue O staining, sections were placed during 90 seconds within the solution then rinsed with distilled water and mounted, after dehydration, in a synthetic resin.

For DMACA staining, sections were placed on a hot-plate and covered with 2 ml of staining solution, during 15 minutes (until evaporation) and rinsed repeatedly with absolute ethanol. Coloured sections were mounted in a synthetic resin.

V- OBSERVATIONS

Samples were examined under BH2/RFCA Olympus microscope. With the toluidine blue O, polyphenolic compounds have a characteristic blue-green colour whereas cell-walls are blue purple and cytoplasm is green. With DMACA, flavan-3-ols are coloured in blue and cell-walls and cytoplasm are not stained.

RESULTS AND DISCUSSION

Histological observations of Cabernet franc berry skins from CYR I coloured with toluidine blue O show two types of cells: some without any precipitate or granulation into the vacuoles, and others with dense granulation, which corresponds to phenolics. Four types could be distinguished:

- uniform aspect, the vacuole is completely coloured (figure 1c)
- granular aspect, with an homogeneous distribution (figure 1a)
- small spherical vacuolar inclusions, stuck to the tonoplast (not shown here)
- large spherical inclusions, free into the vacuole (not shown here)

Samples coloured with DMACA revealed the same pattern of polyphenolic compounds (figures1 b, d): there was no visual difference between the two colorations. So the localisation of the flavan-3-ols (DMACA) coincided with that of phenolics (Toluidine blue). The DMACA coloration was intense independently of the stage of maturation. These results suggest that flavan-3-ols are present in grape berry skin at veraison stage. What is coloured with toluidine blue may be a combination of different phenolics, including flavan-3-ols, but the exact specificity of toluidine blue is unknown. The advantage of DMACA is that cell-walls or cytoplasm are not coloured. It is easier to distinguish the different types of cells. Surface of cells was not affected by heating for the coloration of DMACA.

In addition, the DMACA coloration allowed to underline a new type of flavan-3-ols which is stuck to the tonoplast (figure 1f). This compound could not be clearly identified with toluidine blue because of a possible confusion with cytoplasm (figure 1e). These particular phenolic compounds were underlined by AMRANI-JOUTEI *et al.* (1994) and DIAKOU and CARDE (2001) through Transmission Electronic Microscopy.

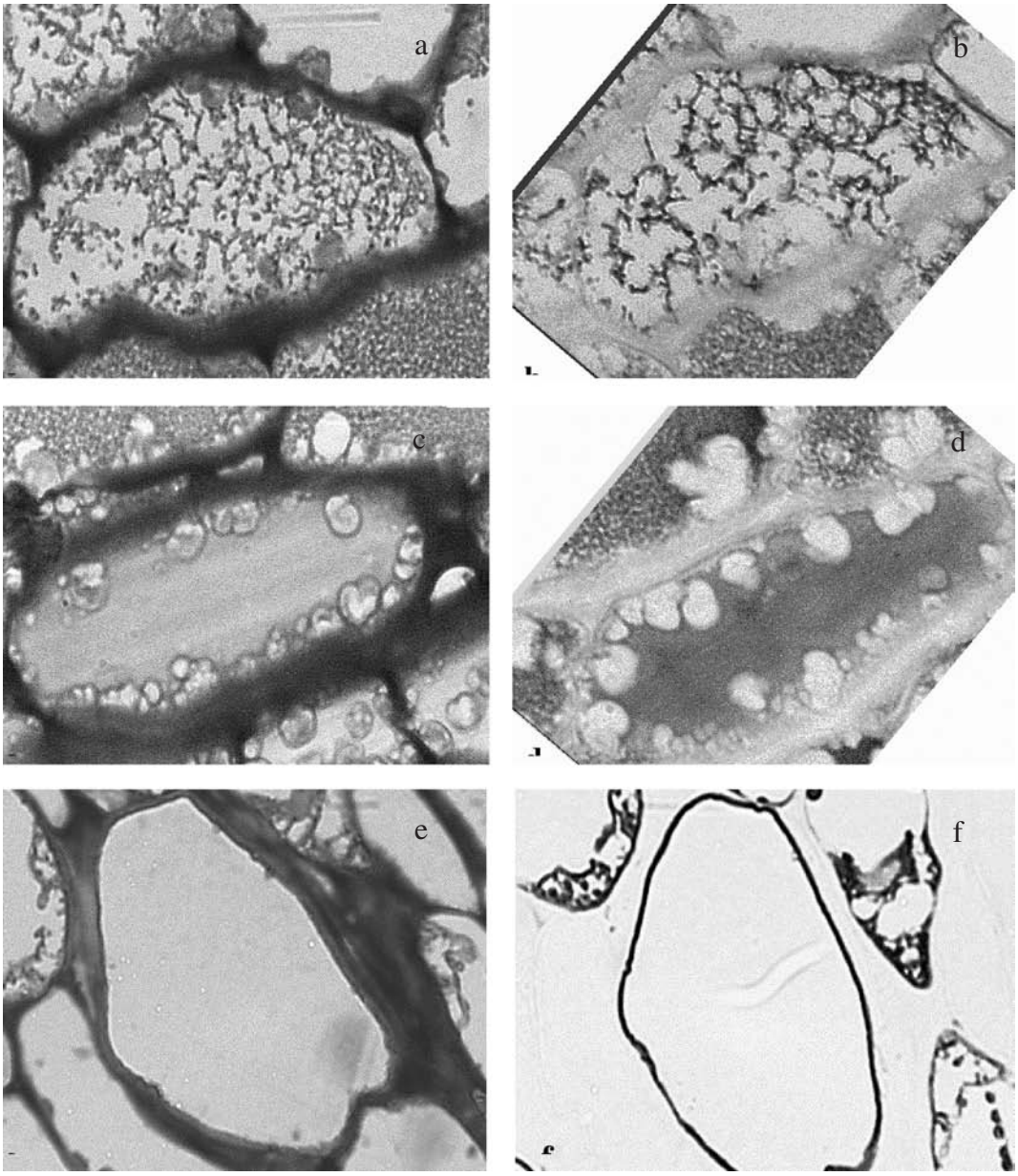

Fig. 1 - Comparison of the toluidine blue O and DMACA coloration (x 2300)
a. granular aspect, coloured with toluidine blue O
b. granular aspect, coloured with DMACA
c. uniform aspect, coloured with toluidine blue O
d. uniform aspect, coloured with DMACA
e. stuck to the tonoplast aspect, coloured with toluidine blue O
f. stuck to the tonoplast aspect, coloured with DMACA

Comparaison de la coloration au Bleu de Toluidine et au DMCA (X 2300)
a. aspect granuleux, coloration au Bleu de Toluidine
b. aspect granuleux, coloration au DMACA
c. aspect uniforme, coloration au Bleu de Toluidine
d. aspect uniforme, coloration au DMACA
e. polyphénols collés au tonoplaste, coloration au Bleu de Toluidine
f. polyphénols collés au tonoplaste, coloration au DMACA

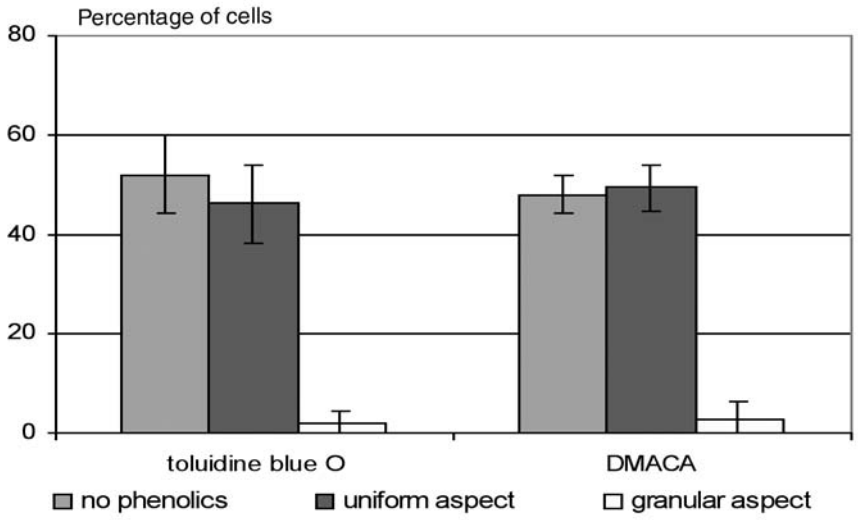

Fig. 2 - Comparison of Toluidine blue O and DMACA staining for the numbering of cells with or without phenolics (CYR I, 23/07/2002, one month before veraison)

Comparaison de la coloration au Bleu de Toluidine et au DMACA pour le dénombrement des cellules avec ou sans polyphénols (CYR I, 23/07/2002, un mois avant véraison)

The number of cells from each class of samples coloured with toluidine blue O and DMACA was counted and the results were compared (figure 2). There was no significant difference between the two results.

DMACA has many advantages: visualisation of tannins and counting of cells are facilitated because no mistakes are possible.

BIBLIOGRAPHY

AMRANI JOUTEY K., 1993. Localisation des anthocyanes et des tanins dans le raisin. Étude de leur extractabilité. Thèse de doctorat, Université de Bordeaux II, 125 p.

AMRANI-JOUTEY K., GLORIES Y. et MERCIER M., 1994. Localisation des tanins dans la pellicule de baie de raisin. *Vitis*, **33**, 133-138.

BROSSAUD F., 1999. Composition en flavonoïdes des baies et des vins de *Vitis vinifera* variété Cabernet franc. *Thèse*, docteur de l'ENSAR, INRA Angers et Montpellier, 9-43.

DIAKOU P. et CARDE J.P., 2001. *In situ* fixation of grape berries. *Protoplasma*, **218**, 225-235.

FEUCHT W., TREUTTER D. et CHRIST E., 1992: The precise localisation of catechins and proanthocyanidins in protective layers around fungal infections. *J. Plant Diseases Protection*, **99**, n°4, 404-413.

GUTMANN, M.; FEUCHT, W.; 1991: A new method for selective localisation of flavan-3-ols in plant tissues involving glycolmethacrylate embedding and microwave irradiation. *Histochemistry*, **96**, 83-86.

GUTMANN, M.; 1995: Improved staining procedures for photographic documentation of phenolic deposits in semithin sections of plant tissue. *J. Microscopy*, vol. 179, Pt 3, 277-281.

KROES G.M.L., BAAYEN R.P. and LANGEN W., 1998. Histology for root of flax seedlings (*Linum usitatissimum*) infected by *Fusarium ascyoparum* f.s.p. *lini*. *Eur. J. Plant Pathol.*, **104**, 725-736

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