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Staining methods for the investigation of *Plasmopara viticola* and its infection structures in semi-thin sections

A. Kortekamp

Institute for Phytomedicine (360), University of Hohenheim, Stuttgart, Germany

Summary

The investigation of *Plasmopara viticola*, that spends most of its life cycle inside of leaves, requires specific staining techniques. It is desirable to visualize both, host cells and parasitic structures, given their silimar chemical constitutions, especially in reference to the cell wall. On the basis of appropriate staining procedures it was possible to demonstrate parasitic structures during the infection process. Several dyes and optical brighteners were successfully used to identify hyphae, vesicles, haustoria, gametangia and oospores. The chemical determination of the stained structures is discussed.

Key words: downy mildew, fluorescence, grapevine, histochemistry, *Vitis*.

Introduction

Some plant-microbe interactions, such as the infection of grapevine with the downy mildew fungus, cause severe symptoms leading to the death of the infected tissue or the entire plant, and will therefore cause a reduction in crop yield and quality. In such a case, an investigation of the infection process, including the typical infection structures of the respective pathogen, may help to understand the progression of infection and to find the Achilles' heel of the pathogen. This requires specific staining techniques in order to visualize the pathogen in the infected cells or tissues. Unfortunately, it is difficult to clearly demonstrate parasitic structures of this obligate biotrophic pathogen in or at the plant cell given their similar chemical constitution. Therefore, several dyes and optical brighteners were used alone or in combinations to ascertain whether well-established or new staining techniques may help to identify infection structures of *Plasmopara viticola* and their chemical composition in semi-thin sections.

Material and Methods

For semi-thin sections (1.0 µm) ethanol-fixed leaf fragments of *P. viticola*-infected, field-grown grapevines (*Vitis vinifera* cv. Riesling) were embedded in methacrylate. Slices were prepared as previously described (Kortekamp *et al.* 1997) and stained for 5 min with (a) 0.01 % Acridine orange (C.I. 46005) in distilled water, (b) saturated solution of Blankophor RKH (C.I. 508150) in methanol, (c) 0.01 % Calcofluor White M2R (Fluorescent Brightener 28, C.I. 40622) in 0.075 M phosphat buffer pH 8.0, (d) 0.1 % Carboxyfluorescein, (e) 0.1 % Chlorazole Black E in distilled water, (f) 0.1 % Eosin B (C.I. 45400) in distilled water (since it gives deeper reds and better contrast than Eosin Y), (g) 0.5 % Primulin (C.I. 49000) in distilled water, (h) 0.05 % Toluidine blue O (C.I. 52040) in tap water (pH 7.0) or (i) 0.01 % Stains-all which was first dissolved in a small amount of ethanol and then diluted in distilled water at pH 7.0. Excess dye was drained off and slices were washed for one min with double distilled water. All chemicals were purchased from Sigma (Taufkirchen, Germany) except for Blankophor RKH, which was a kind gift of Brauns-Heitmann GmbH (Warburg, Germany). The samples were examined by light and epifluorescence microscopy with a Zeiss Axioskop II microscope equipped with filter sets 01 (excitation at 365 nm, emission at 397 nm) and 05 (excitation at 395-440 nm, emission at 470 nm). The images were photographed with a MC80 DX camera (Zeiss) equipped with Kodak film EPY 64 T.

Results

Toluidin blue is the dye of choice when an overview in semi-thin sections is desired. The typical fungal infection structures, such as infection hypha, vesicles, appressoria and haustoria were easily seen (Figure, a, b). The fungal cell walls showed metachromasia, in contrast to the cytoplasm which stained blue. The plant cell walls also stained red but at even lower rates. After application of the dye Stains-all, it was also possible to distinguish plant from fungal cell walls. While the former showed a bluish-purple colour, the latter appeared red to pink (Figure, c). This is also the case for the haustoria and the intercellular hyphae. Furthermore, the haustoria, which can be divided into the haustorial head and the haustorial neck, reacted quite differently to this dye. The head was stained like the fungal cell wall and the chloroplasts of the plant, but the haustorial neck behaved like the plant cell wall. After double staining of semi-thin sections with Acridine orange and Calcofluor, hyphae appeared white to pink, whereas the haustoria showed an orange colour under UV-light (Figure, d). Both structures, hyphae and haustoria, were stained in the same manner after treatment with Carboxyfluorescein and Calcofluor, but they showed a brighter fluorescence compared to the plant cell wall (Figure, e-f). Interestingly, besides the staining of cell walls of the intercellular hyphae and walls of outgrowing sporangio-

Correspondence to: Dr. A. Kortekamp, Institute for Phytomedicine (360), University of Hohenheim, D-70593 Stuttgart. Germany. Fax: +49-711-459-2408. E-mail: kortekam@uni-hohenheim.de

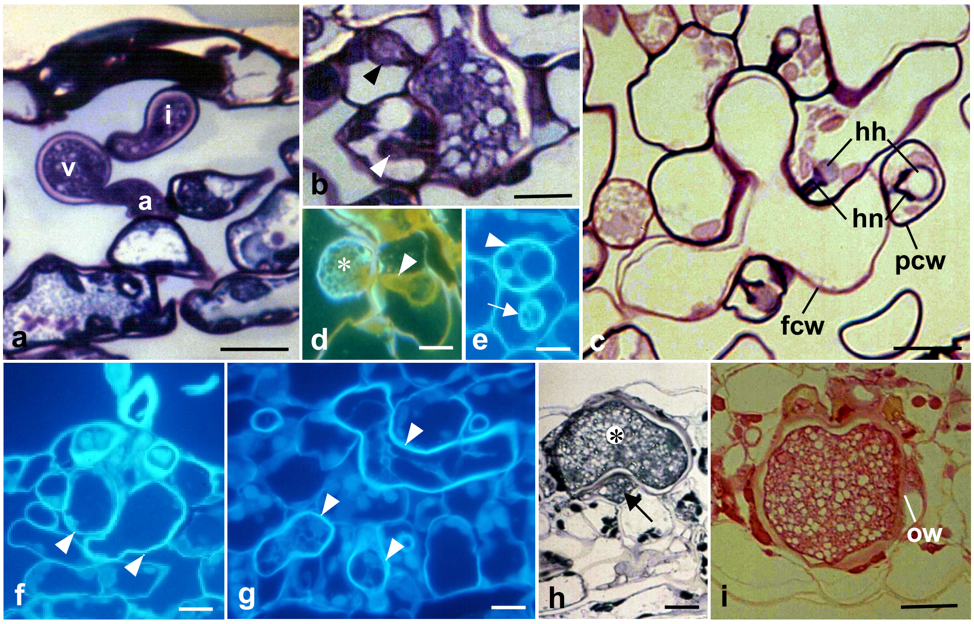

Figure: Histochemically stained semi-thin transverse sections through infected grapevine leaves, cv. Riesling, with lesions of *P. viticola* showing infected cells and fungal infection structures; a) and b) Toluidine blue O staining led to metachromasia, whereby the cytoplasm appeares blue in contrast to the cell walls that show a reddish colour (i = infection hypha, v = vesicle, a = appressorium); c) intercellular hypha that developed several haustoria in plant cells; haustorial head and neck as well as the plant and fungal cell wall are distinguishable from each other after staining with Stains-all (hh = haustorium head, hn = haustorium neck, fcw = fungal cell wall, pcw = plant cell wall; d) double staining with Acridine orange and Calcofluor White; the sectioned hypha mainly absorbed the optical brightener and thus shows a blue-white colour, whereas the haustorium shows only an affinity to acridine orange; e and f) double staining with Carboxyfluorescein and Calcofluor White leading to an accentuation of the fungal cell walls (arrow heads) and the haustorial head (arrow); also cell walls of sporangiophores and guard cells are emphasised; g) double staining with Primulin and Blankophor RKH; note enhanced fluorescence of fungal cell walls (arrow heads); h) gametangia produced during the sexual reproduction are stained with Chlorazole Black E; both, oogonium (asterisk) and antheridium (arrow) are indicated; i) mature oospore stained with Eosin B; ow = oogonial wall. Bars = 10 µm.

phores, the guard cells showed a bright fluorescence, indicating a more similar chemical composition. Enhanced fluorescence of fungal cell walls could also be achieved with the aid of Primulin in combination with Blankophor (Figure, g). Some leaves infected with *P. viticola* that were harvested in late summer, hosted the reproductive stage of the life cycle. Both, Eosin B and Chlorazole Black E, stained the oogonia or antheridia (Figure, h) as well as the wall and mainly the cytoplasm of subsequent built oospores (Figure, i).

Discussion

For semi-thin sections it is helpful to have a rapid staining method, especially, for checking how well the sectioning is progressing. In many cases Toluidine blue O has become the dye of choice. Most organelles and the cytoplasm are stained orthochromatically, whereas acidic polymers, particularly cell wall components, are stained metachromatically, leading to a shift of the transmission spectrum peak from blue to red. This results in the demonstration of sulfated polysaccharides, proteoglycans or polyphosphates but not of cellulose, callose or starch (O´Brian *et al.* 1964). In *Plasmopara*-infected grapevine leaves, the cytoplasm was stained intense blue, while host and parasite cell walls stained bluish-red. This result indicated the same binding capacity and consequently a similar chemical constitution, even though metachromasia appeared clearer in the fungal than in the plant cell wall. In addition to Toluidine blue O, leaf sections were separately stained with Stains-all in an aqueous solution. According to literature, Stains-all is able to stain lipopolysaccharides, mucopolysaccharides and other acidic polymers (Edstrom 1969, Janda and Work 1971). Since this dye also shows metachromasia by binding at proteins based on their conformation status (Sharma *et al.* 1989), it may not be possible to characterize all stained structures at the level of chemical composition. Nevertheless, in contrast to Toluidine blue, both the plant and fungal cell wall, as well as the haustorial neck and its corresponding head, were clearly distinguishable, indicating a different underlying chemistry. The nature of the haustoria is of particular interest since these structures represent the true host-pathogen interface through which the exchange of metabolites occurs. As indicated from truncated haustorial necks in Figure c, the dark colour of the neck is a result of the dye binding to the wall. Interestingly, its colour is the same as that of the plant cell wall. From the same binding behaviour, it might be concluded that their chemical composition is identical or at least similar and differs from that of the hyphal cell wall. Although the name of the dye Stains-all implies that it stains every component of the cell, it failed to stain or stained infrequently the fungal cell cytoplasm. Similar observations have been made with transmission electron microscopy. Langcake and Lovell (1980) reported that vacuolation of the fungal hyphae increases with age such that older portions of the fungus may be totally devoid of cytoplasm.

Beside Toluidine blue and Stains-all, Acridine orange also shows metachromasia after illumination with UV-light when binding to polyanionic biopolymers such as glycosaminoglycans. Although it has been used primarily in nucleic acid studies, a weak solution in water has been used as a vital dye for plant and fungal cells, where it appears to accumulate into vacuoles and fungal spores (Wilson *et al.* 1978). Hayat (1993) reported that a specific staining of glycosaminoglycans can be achieved when low concentrations of acridine orange are used, since this dye has a higher affinity for complex carbohydrates than for nucleic acids. If used in combination with Calcofluor White, it binds better to the haustorial structures than to the hyphae, whereas the latter showed a greater attraction to Calcofluor White. In this case, not only the wall but also parts of the cytoplasm showed a bright fluorescence under UV-light. Interestingly, if Calcofluor was used alone, there was no difference between plant and fungal cell walls regarding their fluorescence; but if slices were treated with Carboxyfluorescein before staining with Calcofluor White, walls of all fungal elements appeared in a broader fluorescence compared to the plant cell walls. Carboxyfluorescein is a carboxylated analogue of Fluorescein and was used as Carboxyfluorescein diacetate as a vital dye in cell suspension cultures and in suspensions of *P. viticola*-sporangia in order to determine their viability after fungicide application (Sergeeva *et al.* 2002). Unfortunately, there is no information in literature regarding the specificity of binding patterns and nothing is known about the detection of fungal infection structures in plants using the carboxylated dye. Although walls of guard cells showed a bright fluorescence, mainly fungal cell walls were stained in semithin sections, which was also achieved using Primulin. Primulin, or Direct Yellow 59, was used such as Fluorescein as a vital stain and has become quite widely used for this purpose. Nevertheless, references to this dye in biological literature are rare, especially regarding the detection of plant or fungal structures. It was used to investigate pollen wall development and to distinguish between intact and broken starch grains (for ref. see O´Brian and McCully 1981) or in combination with Calcofluor to stain lignified cell walls in spruce needles without staining the cytoplasm (Boxler 1998). Unfortunately, in grapevine this double-staining method failed to bring out fungal cells or cell walls. After double staining with Primulin and Blankophor RKH as a substitute for Calcofluor White, fungal cell walls were visible. Blankophor is currently used for studying cell wall architecture in yeasts and in medical mycology (Rüchel *et al.* 2001 and literature therein). Calcofluor White and Blankophor RKH bind non-covalently by intercalation to β-glycosidically linked polysaccharides and they can therefore be used to detect glucans in cell walls. Interestingly, fungal cell walls could not be distiguished if other optical brighteners of the same type (*e.g.* Blankophor DML) were used. Although Calcofluor White and Blankophor are used as markers for glucans, they also bind to chitin (Maeda and Ishida 1967), which is also part of the cell wall of Pythiaceae or Peronosporaceae like *P. viticola* (Werner *et al.* 2002) but not of the septa that consist mainly of glucan(s), as indicated after enzyme digestion (Kortekamp 2005). Both dyes, Calcofluor and Blankophor, give no satisfactory information about cell wall chemistry, but have no toxic effect on conidial germination or formation of appressoria and vesicles and, thus, can be used to study the development of downy mildews (Cohen *et al.* 1987).

In conclusion, several dyes and optical brighteners can be used to visualize different infections structures of the grape downy mildew pathogen and surely of the other members of the oomycetes. Since the chemical determination of the stained structure depends on the specificity of the dye, these staining procedures should be amended by other analytical methods. This may lead to a better insight into the respective infection structures and thus, in combination with a molecular analysis, to the development of appropriate and specific fungicide-based disease management strategies.

References

Boxler, C.; 1998: Einflüsse von abiotischen Faktoren auf die Ultrastruktur von Chloroplasten und die Leitbündelanatomie von Fichtennadeln. Diss. Univ. Karlsruhe.

Cohen, Y.; Peer, S.; Balass, O.; Coffey, M. D.; 1987: A fluorescent technique for studying growth of *Peronospora tabacina* on leaf surfaces. Phytopathology **77**, 201-204.

Edstrom, R. D.; 1969: A Colorimetric method for the determination of mucopolysaccharides and other acidic polymers. Anal. Biochem. **29**, 421-432.

Hayat, M. A.; 1993: Stains and cytochemical methods. Plenum Press, New York.

Janda, J.; Work, E.; 1971: A colorimetric estimation of lipopolysaccharides. FEBS Letters **16**, 343- 345.

Kortekamp, A.; 2005: Growth, occurrence and development of septa in *Plasmopara viticola* and other members of the Peronosporaceae using light- and epifluorescence-microscopy. Mycol. Res. **109** (in press).

Kortekamp, A.; Wind, R.; Zyprian, E.; 1997: The role of callose deposits during infection of two downy mildew-tolerant and two-susceptible *Vitis* cultivars. Vitis **36**, 103-104.

Langcake, P.; Lovell, P. A.; 1980: Light and electron microscopical studies of the infection of *Vitis* spp. by *Plasmopara viticola*, the downy mildew pathogen. Vitis **19**, 321-337.

Maeda, H.; Ishida, N.; 1967: Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. J. Biochem. **62**, 276-278.

O´Brian, T. P.; McCully, M. E.; 1981: The study of plant structure. Principles and selected methods. Termarcarphi, Melbourne.

O´Brian, T. P.; Feder, N.; McCully, M. E.; 1964: Polychromatic staining of plant cell walls by Toluidine blue O. Protoplasma **59**, 367-373.

Rüchel, R.; Behe, M.; Torp, B.; Laatsch, H.; 2001: Usefulness of optical brighteners in medical mycology. Rev. Iberoameric. Micol. **18**, 147-149.

Sergeeva, V., Nair, N.; Legendre, L., Darley, E.; Sponner-Hart, R.; 2002: The use of fluorochromes to determine the effect of chlorine dioxide on survival of *Plasmopara viticola* on grapevine. Aust. Plant Pathol. **31**, 295.297.

Sharma, Y., Rao, C. M., Rao, S. C.; Krishna, A. G.; Somasundaram, T.; Balasubramanian, D.; 1989: Binding site conformation dictates the color of the dye Stains all. J. Biol. Chem. **264**, 20923-20927.

Werner, S., Steiner, U.; Becher, R.; Kortekamp, A.; Zyprian, E.; Deising, H. B.; 2002: Chitin synthesis during *in planta* growth and asexual propagation of the cellulosic Oomycete and obligate biotrophic grapevine pathogen *Plasmopara viticola*. FEMS Microbiol. Lett. **208**, 169-173.

Wilson, C. L.; Jumper, G. A.; Mason, D. L.; 1978: Acridine orange as a lysosomal marker in fungal spores. Phytopathology **68**, 1564-1567.

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