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

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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 similar 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 red 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 Acidine 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-
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.
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


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