A SIMPLE DUAL STAIN FOR DETAILED INVESTIGATIONS OF PLANT-FUNGAL PATHOGEN INTERACTIONS

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Received: October 24, 2012; Accepted: January 28, 2013

Summary

Dramatic increase in confocal microscopy observation output has been gained by optimization of a simple trypan blue and aniline blue dual-stain and its application to two model pathosystems: *Pseudoperonospora cubensis*-cucumber and *Phytophthora infestans*-tomato. Comparison of two dual-stain methods for confocal microscopy studies of *P. cubensis*-challenged cucumber leaves indicated the 'mild' approach most successful. This methodology provides simultaneous detection of different pathogen structures layered with the plant defense reactions. Moreover, ImageJ-assisted quantification of plant defense responses renders this method useful for addressing the host plant resistance reactions, as well as investigating the given isolate's pathogenicity. Application of this method for the *P. infestans*-challenged tomato leaf samples resulted in detection of several fungal infection structures, along with plant defense responses. The dual-stain also enabled detection of a peculiar aniline blue-sensitive material in the pathogen cell walls at the area of its hyphae emerging through the leaf stomata. Results presented herein indicate this method is applicable for detailed (possibly quantitative) investigations of multiple plant-fungal pathosystems.

keywords: histochemical dual-stain, cucumber downy mildew, tomato late blight, pathogen infection structures, host plant resistance, resistance reaction quantification

INTRODUCTION

In the course of domestication, many plant species have undergone selection directed for utility traits, namely, yield quantity and quality, rather than pathogen or abiotic stress resistance (Foolad 2007, Wang et al. 2007, Huang et al. 2009, Mueller et al. 2009, Grandillo et al. 2011). This utilitarian selection has rendered
crops susceptible to many diseases of economic importance. As a result, phytopathologists and plant breeders worldwide commit themselves to elucidating mechanisms of plant resistance, and their introduction them into commercial cultivars (Ganeshan et al. 2009, Vleeshouwers et al. 2011, Nowicki et al. 2012a). Recently, multiple areas of investigation have yielded tremendous progress in plant-pathogens interactions. Next-generation (genome/ transcriptome) sequencing, resistance/effector gene characterization (Haas et al. 2009, Huang et al. 2009, Mueller et al. 2009, Raffaele et al. 2010, Tian et al. 2011, Woycicki et al. 2011), micro-examination of the interactions between pathogenic fungi and host plants (Hood & Shew 1996, Hardham & Shan 2009, Savory et al. 2010), and systems biology studies (Vleeshouwers et al. 2011, Woycicki et al. 2011, Nowicki et al. 2012b, Raffaele et al. 2010, Shibata et al. 2010) have all proven highly effective tools in identifying the molecular and cellular basis of pathogen resistance and susceptibility. In particular, microscopic studies provide an exciting body of evidence regarding the pathogen biology or host plant reaction upon the perceived attack (Hood & Shew 1996, Hardham & Shan 2009, An et al. 2010, Savory et al. 2010, Chen & Halterman 2011). These studies, though invaluable as a pathogen diagnostic tool or an indicator of the resistance level exhibited by the host plants, hitherto mostly failed to investigate both the pathogenic fungi and their host plants’ responses within one assay. Certain staining techniques allow plant and fungal tissues to be differentiated, thus advancing experimental research in plant pathology, (Hood & Shew 1996, An et al. 2010, Chen & Halterman 2011). More specifically, staining can aid examination of fungal infection and colonization processes, for instance, by differentiating hyphae (primary/secondary) in life cycles of different plant pathogens. Specific stains can also enable identification of fungal presence in asymptomatic plant tissue (Hood & Shew 1996, Shibata et al. 2010). Clearly, the effectiveness of a particular staining technique can vary greatly depending on the examined pathosystem.

Callose depositions formed by plant cells upon a perceived attack of fungi in some plant-fungus interactions are routinely identified by aniline blue fluorescence stain (Currier 1957, Hood & Shew 1996, An et al. 2010, Chen & Halterman 2011). Binding of the fluorochrome portion of the dye to various glucans and other plant polysaccharides has been demonstrated (Currier 1957, Hood & Shew 1996), suggesting a strong affinity of the aniline blue pigment to β-1,3-D-glucans (callose), permitting investigations of host plant responses to pathogen infection. Fluorescence derived from this dye has also been used to observe glucan-containing fungal cell walls (Currier 1957, Hood & Shew 1996, Latgé 2007). Several studies have successfully employed such method for examining the infection structures of fungal pathogens, such as Aspergillus fumigatus and the yeast Saccharomyces cerevisiae (Latgé 2007), or Plasmopara viticola, the
causal agent of grapevine downy mildew (Diez-Navajas et al. 2007).

The pathosystem Phytophthora infestans (Mont.) de Bary – potato/tomato has proven instrumental for investigations of pathogen development, as in characterization of spore germination, appressorial morphogenesis, or hyphal morphogenesis. It enabled detailed interplay analyses of ‘effectoromics’ and host plant colonization as a function of virulence and phytopathogenicity. Finally, it served as basis for elucidation of mechanisms of plant defenses, such as cell wall reinforcements, reactive oxygen species production, hypersensitive response (all above aspects reviewed in Hardham & Shan 2009, Vleeshouwers et al. 2011, Nowicki et al. 2012a). The importance of potato and tomato plant models in pathogenic interactions has significantly increased due to the completion of their genomic sequences and extensive arrays of mutants (Mueller et al. 2009, The Potato Genome Sequencing Consortium 2011). From over 200 pathogens adversely affecting potato and tomato production, the oomycete P. infestans causing late blight remains the prominent reason of losses (Nowicki et al. 2012b). By employing the hemibiotrophic mode of infection, the germinated pathogen spore first develops large biotrophic intracellular primary hyphae. Next, the pathogen switches to a necrotrophic mode, in which the secondary hyphae extensively colonize surrounding tissue. Finally, by asexual sporulation, has the potential to incite massive epidemics (Nowicki et al. 2012b).

Another example is the U.S. 2004 re-emergence of downy mildew, which impacts cucumber production worldwide. Here, the appearance of more aggressive isolates of the causal organism, Pseudoperonospora cubensis (Berk & Curt.) Rostovzev, and sudden fall of respective resistance previously introduced to cucumber (Savory et al. 2010, Lebeda & Cohen 2011) has attracted renewed scientific interest. This obligate biotroph oomycete is capable of infecting approximately 20 different genera with potential yield losses of up to 80%, threatening the long-term viability of cucurbit crop production (Savory et al. 2010). Similar to P. infestans in potato or tomato, P. cubensis develops several morphologically distinct infection structures. Germ tubes, appressoria, infection or penetration pegs, haustoria, mesophyll hyphae, and sporangiophores have all been observed, and pathogen morphology serves as one of the ways to characterize this pathosystem (Savory et al. 2010, Lebeda & Cohen 2011). These characteristics of the progressing infection make both pathosystems appropriate for detailed microscopic investigations (An et al. 2010, Savory et al. 2010, Shibata et al. 2010, Chen & Halterman 2011).

In this study, we simplify and broaden the applicability of the recently reported aniline blue and trypan blue fluorescence dual-staining technique (Bhaduria et al. 2010) for examining the infection structures of the hemibiotrophic oomycete P. infestans in tomato, and the biotrophic oomycete P. cubensis in cucumber. Combination of both staining agents
within one assay yielded a clear contrast between fungal infection structures and the host plant response structures. Laser confocal microscopy provided the necessary spectral resolution and contrast between host plant tissue, including plant defense reactions like callose depositions or hypersensitive response, and pathogen infection structures like germ tubes or haustoria, respectively. Using this methodology, numerous fungal and host structures formed during pathogen attack were observed. Moreover, quantitative measurements of host plants’ responses were obtained and are reported herein. Taken together, with the ease of sample perpetration and use, this technique holds great promise for broad use and advancement of the research on pathogen/ host relationship.

METHODS AND MATERIALS

Plant material
Two cucumber (Cucumis sativus L.) lines (DM48 and DM1, exhibiting resistance and susceptibility towards P. cubensis, respectively) and one tomato variety (Solanum lycopersicum L. cv. Rumba, susceptible to P. infestans) were tested in this study. Cucumber and tomato plants were grown in environmentally-controlled growth chamber at temperature 22 °C/18 °C day/night, and 12 h photoperiod. Fungal material and inoculation
Isolates of P. cubensis were collected from downy mildew-diseased cucumbers grown in the Research Institute of Horticulture (RIH, Skierniewice, Poland), and routinely maintained by consecutive inoculations on susceptible cucumber plants grown under controlled conditions. Pure cultures of P. infestans were derived from late blight affected tomatoes (RIH) and maintained on the artificial, standard RyeA medium. The isolate used in this study was of the complex 0,3,4,7,10,11 (Black) race, mating type A1, and mtDNA haplotype Ia. Inoculum concentration was calculated before each treatment by means of Nikon Eclipse E200 microscope using a standard hemacytometer.

Seven-week old tomato plants cv. Rumba were sprayed with a sporangial suspension of P. infestans (10^4×cm^-3). Inoculated plants were kept in a chamber at 100% RH immediately after inoculation. Asymptomatic leave tissue has been sampled with a 5 mm diameter puncher at 4 dpi (at least five plants per genotype per collection round; first true leave sampled), and cleared in 100% ethanol. Cucumber plants grown for four to five weeks (two true-leaf stage) were spray-inoculated with a suspension of P. cubensis sporangia (5×10^3×cm^-3). Challenged plants were kept in the dark in the growth chamber at 20 °C and 100% RH for 48 h. After this time, plants were grown at 23 °C and 12 h photoperiod. Cucumber leaves were sampled at 2 dpi from asymptomatic plants and 6 dpi, with 18 specimens per genotype and per sampling round and samples cleared in 100% ethanol. Trypan-aniline blue dual-stains
Cleared samples of both, cucumber and tomato plants, were re-hydrated by soaking in decreasing ethanol solutions (100, 75, and 50% ethanol). 'Harsh' trypan stain followed the
method published elsewhere (Shibata et al. 2010). Briefly, collected and cleared leaf samples were boiled for 2 min in lactophenol trypan blue stain (10 ml of H₂O, 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 10 mg of trypan blue, CarlRoth Poland). After cooling the stain-boiled leaves for 1 h at room temperature, the trypan blue stain was replaced for chloral hydrate at 1 g×cm⁻³, then stained overnight at room temperature with 0.05% aniline blue (GUR UK) in 150 mM KH₂PO₄, pH 9 (aq), and de-stained in 150 mM KH₂PO₄, pH 9 (aq). Stained samples were mounted with water for observations.

The 'mild' dual-stained samples were soaked in 0.05% trypan blue (CarlRoth Poland, aq) overnight at room temperature, then soaked in 0.05% aniline blue in 150 mM KH₂PO₄, pH 9 (aq) for 3–4 h. The leaves then were de-stained in 150 mM KH₂PO₄, pH 9, two to three times for 15 min, and mounted with water for observations.

Microscopy

Confocal laser scanning microscopy was performed with the Nikon C1 microscope using argon and diode lasers. Image acquisition of dual trypan blue - aniline blue stained samples was performed in a sequential mode to avoid spectral cross-talk. For the trypan blue channel, the 488 nm excitation line of an argon ion laser was used in a single track mode. Fluorescence was collected through a filter block with a 513-530 nm BP emission filter. Z series images were collected at 0.7-1 μm intervals through the specimens. All images were processed with the EZ C1 Free-Viewer (v.3.90; http://nikon-ez-c1-freeviewer.software.informer.com/); the digital quantifications employed ImageJ (http://rsbweb.nih.gov/ij/).

RESULTS AND DISCUSSION

'Harsh' and 'mild' dual-stain comparison

At least two methods are available for the trypan blue staining of fungal plant pathogens. 'Harsh' stain is based on boiling the samples in lactophenol trypan blue, with subsequent chloral hydrate bleach/ de-stain (Shibata et al. 2010), while 'mild' stain bases on the overnight trypan blue (aq) stain (Bhadauria et al. 2010). Thus, addressing the applicability of the dual-stain (trypan blue for pathogen structures detection, and aniline blue for visualization of plant defense reactions) required comparison of both extremely different, 'harsh' and 'mild' treatments in a dual-stain manner. Asymptomatic leaf samples collected from P. cubensis-challenged resistant and susceptible cucumber lines were subject to parallel dual-stain, using both 'harsh' and 'mild' trypan blue protocols. Careful inspection of the 'mild' dual-stained samples succeeded in detection of both, developing aerial hyphae, germinating sporangia (visible
only in DIC, suggesting lack of trypan blue-detectable chitin in *P. cubensis* sporangial walls; Fig. 1A-B), leaf surface hyphae, appressoria, and haustoria (Fig. 1A-H). Comparatively, 'harsh' dual-stain rendered the cucumber samples extremely fragile, leading to their fragmentation during the de-staining process. This was evidenced by the microscopic observations of leaf mesophyll cells floating freely upon sample mounting (Fig. 1F). ‘Harsh’ trypan blue stain has been applied with success towards the studies of tobacco - *P. infestans* system (Shibata et al. 2010), enabling visualization of the pathogen hyphae penetrating the leaf tissue; however, this treatment caused the normally sturdy cucumber leaf samples to virtually fall apart. Thus, the 'mild' dual stain was employed for all further investigations.

**Dual stain enables identification of *P. cubensis* infection structures in cucumber**

Dual stain proved particularly beneficial for pathogenesis studies in the *P. cubensis* – cucumber system (as mentioned above). The oomycete sporulates on the underside of the host plant's leaves, resulting in the production of sporangia that are dispersed by wind. Upon contact with a susceptible host, sporangia germinate in free water on the leaf surface, releasing motile zoospores that encyst and form germ tubes. These in turn develop appressoria that form penetration hyphae invading the leaf tissue. Hyphae grow through the mesophyll and establish haustoria, specialized structures for nutrient transfer and signaling between host and pathogen (Savory et al. 2010, Lebeda & Cohen 2011). Application of the 'mild' dual stain provided a wealth of visual data on the various stages of the downy mildew disease in cucumber, as well as on the pre- and post-invasion host plant defenses. Germinated sporangia on the cucumber leaf, aerial pathogen hyphae, leaf germ tubes with formed appressoria and penetration hyphae, as well as haustoria formation were all observed (Fig. 1A-H). Observation of fully-formed aerial hyphae on the leaf surface confirms the mild conditions of the stain procedure, allowing for preservation of this relatively fragile structure. It is noteworthy, that in contrast to the previously suggested penetration route (Savory et al. 2010, Lebeda & Cohen 2011), the double-stain procedure suggests the direct zoospore germination prevailing over the stomatal attack (Fig. 1D; Supplementary material 1). Indeed, significant portion of the detected appressoria were not formed on, or in the immediate vicinity of the cucumber leaf stomata.
Fig. 1. Confocal dual-stain investigations of *Pseudoperonospora cubensis* pathogenesis on downy mildew-susceptible cucumber DM1 (A-F; at 2 dpi, inoculum load: 5×10^3 sporangia×cm^-3) and downy mildew-resistant cucumber DM48 (G-H; at 2 and 4 dpi, inoculum load: 5×10^3 sporangia×cm^-3). (A) Germinating spore, observed in the trypan blue-specific channel; (B) DIC observations of A; (C) Germinated spore developing aerial hyphae on cucumber leaf surface; merge of the trypan blue and DIC channels; (D) After their release from sporangium [S], zoospores [Z] germinate and produce germ tubes, forming appressoria [A]. As first line of pathogen attack, cucumber plants deposit papillae, containing callose [C]. Dual stain with merged trypan blue and aniline blue channels; (E) Upon the fall of primary host plant defenses, *P. cubensis* develops haustorium [H]; (F) ‘Harsh’ dual stain causes cucumber tissue fragmentation, rendering this method inapplicable for this pathosystem, as observed in merged trypan blue and aniline blue channels; (G) At 2 dpi, DM-resistant cucumber DM48 exhibits several singular collapsed cells, while surrounding cells quarantine from their side with callose depositions, preventing from possible secondary infections. Shown are merged views in trypan blue, aniline blue, and DIC; (H) At 6 dpi, the collapsed area enlarges; the same applies for the callose ‘quarantine zone’. Shown are merged views in trypan blue, aniline blue, and DIC.
Fig. 2. *Phytophthora infestans* infection cycle on susceptible tomato cv. Rumba at 4 dpi (inoculum load: sporangial suspension of $10^4 \times \text{cm}^{-3}$) as visualized by dual stain and confocal laser microscopy. Red channel: Pathogen-specific trypan blue stain and plant autofluorescence; Green channel: callose-specific aniline blue stain; black and white: DIC. (A) Germinating spore [S] develops infection tube and attempts leaf tissue penetration by formation of appressorium [A]. Host plant’s primary lines of defense is rapid formation of callose-rich papilla [P] directly underneath the attempted penetration area; (B) Same as A, but visible in DIC; (C) Upon successful infection, the pathogen grows through the leaf mesophyll, as denoted by the numerous hyphae (marked 1-4); (D) At the end of the infection cycle, the hyphae emerge through the abaxial leaf side stomata, followed by sporangial development; (E) Same as D, visualized as merge of DIC and Green channel; (F) Rapid field infestation is achieved through successful colonization; descendant sporangia will drive the epidemics through the season. Notice presence of the aniline blue-sensitive material in the hyphae emerging through stomata (D-F).
**Cucumber defenses against the *P. cubensis* infection**

Sample leaves from susceptible and resistant cucumber plants challenged with *P. cubensis* exhibited extremely different responses to the perceived pathogen attack. At 2 dpi, the susceptible plants developed massive callose papillae directly underneath the pathogen appressoria (Fig. 1D; Supplementary material 1). Several studies reported accumulation of phenolics, callose, and lignin in the infected sites, but also along the surface of the pathogen mycelia; this is regarded a primary plant defense line, limiting the growth of *P. cubensis* germ tubes (reviewed in Lebeda & Cohen 2011). Comparatively, failure of pathogen attack in the downy mildew-resistant cucumber leaf tissue was probably due to the observed hypersensitive response (Fig. 1G,H). At 2 dpi, several singular dead cells were observed in the challenged downy mildew-resistant cucumber leaf samples, with cells surrounding them extensively accumulating callose (4182 ± 190 µm²; n=3; Fig. 1G). Observations of the same cucumber line at 6 dpi suggest increase of such lesion zone: Area, on which the plant cells underwent hypersensitive reaction and the cells surrounding the lesion zone accumulated callose, increased dramatically compared with 2 dpi (Fig. 1H). For the first time the dual-stain employed in such investigation helped link visually, this pathogen’s protruding germ tubes with the localized cell wall enhancements, as callose papilla.

The 'mild' dual-stain applied in these investigations enabled a quantitative approach in these resistance studies. ImageJ-assisted quantification of the papillae area indicates average papillae size of 129.4 ± 36.7 µm² (n=6) at 2 dpi in the downy mildew-susceptible cucumber line. In-depth studies of papillae formation and quantification (including alternative labeling of plant cell’s microtubule network) may help address the pathogenesis dynamics in relation to cucumber lines exhibiting different levels of resistance against *P. cubensis*. This hypothesis is based on the observed gene-for-gene interaction in exuding the cucumber resistance against *P. cubensis* (Call et al. 2012), despite only fledgling discoveries of both, pathogen avirulence agents and cucumber’s molecular responses to downy mildew (Savory et al. 2012a,b, Adhikari et al. 2012).

**Dual-stain studies of the tomato-*P. infestans* pathosystem**

While dual-stain, image-assisted analyses may help elucidate the resistance mechanisms of cucumber against the downy mildew causal agent, they have already provided crucial data in the investigations of pathosystem potato - *P. infestans* (Freytag et al. 1994, Chen & Halterman 2011). In contrast to this well-studied system, however, the tomato late blight resistance mechanisms are far less defined. This area of research gained momentum after recent resurgence of the pathogen and dramatic yield losses (Vleeshouwers et al. 2011, Nowicki et al. 2012a,b). ‘Mild’ dual stain studies
of this pathosystem resulted in the visualization of numerous pathogen infection structures, along with the plant defense responses (Fig. 2). These results demonstrate an innovative and highly effective tool for simultaneously investigating the host plant resistance mechanisms as well as the pathogen’s assault weapons within a single assay. The applied dual-stain enabled observation of germinating *P. infestans* spores on a susceptible tomato leaf at 4 dpi, with creation of appressoria and simultaneous activation of primary line of host plant-induced defense, here depicted by callose deposition (Fig. 2A,B). Callose deposition was negatively correlated with resistance levels in a recent potato-*P. infestans* study (Chen & Halterman 2011). Thus, similar to the cucumber - *P. cubensis* system, broader application of dual-stain towards challenged tomato genotypes exhibiting various late blight resistance levels and ImageJ-assisted picture analyses may drive the quantitative studies of expression of this trait.

Employment of the 'mild' dual-stain proved helpful for studies of subsequent stages of tomato leaf invasion by *P. infestans*: Pathogen hyphae were clearly observed penetrating the tomato leaf mesophyll (Fig. 2C). Intensive sporulation observed normally at the end of the *P. infestans*’ infection cycle (Nowicki et al. 2012a) was also recorded here. Numerous sporangia have been observed protruding from the stomatal opening of late blight-susceptible tomato leaf already at 4 dpi (Fig. 2D-F), clearly visualizing the noted potential for asexual sporangia to drive the late blight epidemics during the growing season (Nowicki et al. 2012a). Interestingly, clear deposition of aniline blue-detectable material at the oomycete hyphae in the area of their emergence through the stomata was observed (Fig. 2D-F; Supplementary material 2). Despite previous studies on *P. infestans*-infected plants having failed to detect this particular feature (Freytag et al. 1994, Hardham & Shan 2009, Shibata et al. 2010, Chen & Halterman 2011), the pathogen genome contains at least six putative callose synthase genes (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html; gene search: “callose synthase”). This allows for speculation on the aniline blue-sensitive material probably serving towards strengthening the pathogen cell wall against stomata-derived tension, while at the same time underlines the research potential of the dual-stain method.

In summary, significant simplification of the dual trypan-aniline blue stain was achieved in this study with regards to sample fixation, re-hydration and washes, as compared with the previous report of Arabidopsis pathogens (Bhadauria et al. 2010). Comparison of the dual-stain methods for confocal laser microscopy studies of *P. cubensis*-challenged cucumber leaves indicated the 'mild' approach most successful. This methodology provides simultaneous detection of different fungal structures layered with the plant defense reactions. Moreover, ImageJ-assisted quantification of plant
defense responses renders this method useful for addressing the host plant resistance, as well as investigating the given isolate’s pathogenicity. Broadening of the application of this method for the *P. infestans*-challenged tomato leaf samples resulted in detection of several fungal infection structures, along with plant defense responses. The dual-stain also suggested direct germination and appressorium formation on cucumber leaves by *P. cubensis* over widely accepted stomatal attack, as well as enabled detection of a peculiar aniline blue-sensitive material in *P. infestans*’ cell walls at the area of its hyphae emerging through tomato leaf stomata. Results presented herein, together with the report published on the Arabidopsis trypan blue - aniline blue dual-stain (Bhadauria et al. 2010), indicate this method is applicable for detailed (possibly quantitative) investigations of a broad array of plant-fungal pathosystems.

Z-stack *P. cuben-sis*-cucumber pathosystem, turned into a movie, visualizes the initial infection stages. Z-dimension resolution: 1 µm.

Z-stack stomatal *P. infestans* hyphae emerging from abaxial leaf side indicating presence of aniline blue-sensitive material on the pathogen cell wall. Z-dimension resolution: 1 µm.

Acknowledgements
Downy mildew-related and tomato late-blight related research carried out at the Research Institute of Horticulture (Skierniewice, Poland) is funded by the Polish Ministry of Agriculture and Rural Development HOR hn-801-7/12 grants # 89/2012 and 95/2012, respectively. Dr. Dorothy M. Tappenden Ph.D (Michigan State University) is gratefully recognized for skillful editorial help with and critical reading of the manuscript. Prof. dr. hab. Czesław Sulusarski (RIH, Skierniewice) is acknowledged for critical reading of the manuscript.

Competing Interests Statement
All and each of the authors declare no competing interests.

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SZYBKA I PROSTA METODA PODWÓJNEGO BARWIENIA
HISTOCHEMICZNEGO W CELU SZCZEGÓŁOWYCH BADAŃ
INTERAKCJI ROŚLIN Z PATOGENAMI GRZYBOWYMI

Streszczenie