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Refining a method for ascospore viability testing in overwintering chasmothecia of *Erysiphe necator* --Manuscript Draft--

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Abstract:	The vital stain fluorescein diacetate (FDA) has long been used to assess the viability of <i>Erysiphe necator</i> ascospores in chasmothecia. To determine the viability of animal, plant and other cells, more complex staining procedures, for example simultaneous double-staining procedures based on the use of FDA and propidium iodide (PI) have also been widely used. We refined this method, and replaced PI with ethidium bromide (EtBr), to be used in testing the viability of <i>E. necator</i> ascospores during overwintering in chasmothecia.	
Response to Reviewers:	Our replies and reactions to the Reviewers' comments are included in a file uploaded as 'REPLY to Reviewers Eneator ascospore viability.pdf'	

SHORT COMMUNICATION

Refining a method for ascospore viability testing in overwintering chasmothecia of

Erysiphe necator

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Abstract

The vital stain fluorescein diacetate (FDA) has long been used to assess the viability of *Erysiphe necator* ascospores in chasmothecia. To determine the viability of animal, plant and other cells, more complex staining procedures, for example simultaneous double-staining procedures based on the use of FDA and propidium iodide (PI), have also been widely used. This paper reports a refined version of this method, where PI was replaced with ethidium bromide (EtBr), to be used in testing the viability of *E. necator* ascospores during overwintering in chasmothecia.

Keywords: grapevine powdery mildew; fluorescein diacetate; ethidium bromide; propidium iodide; epifluorescence microscopy

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7 The question whether ascospores remain viable during, and after, overwintering of *Erysiphe*
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9 *necator* chasmothecia (formerly known as cleistothecia) has long been an important issue
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11 because of previous controversies about the role of chasmothecia in the life cycle of the
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13 grapevine powdery mildew fungus (Pearson & Gadoury 1987; Cortesi et al. 1997; Rossi et al.
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15 2010). The vital stain fluorescein diacetate (FDA) was used in several works to detect living
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17 *E. necator* ascospores (e.g., Gadoury & Pearson 1991; Cortesi et al. 1995; Evans et al. 1997;
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19 Miazzi et al. 2003; Hajjeh et al. 2008) based on a method originally reported by Widholm
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21 (1972) to test the viability of plant cells. FDA was also used to verify the viability of *E.*
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23 *necator* hyphae on grapevine berries (Ficke et al. 2002) and leaves (Moyer et al. 2010), thus
24
25 this stain has been largely applied in the study of the grapevine powdery mildew fungus.
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31 To determine the viability of fungal, animal, plant and other cells, more complex
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33 staining procedures have also been widely used. For example, FDA was successfully
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35 combined with propidium iodide (PI) to develop a simultaneous double-staining procedure
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37 for animal cells (Jones and Senft 1985). A similar method based on staining with FDA and PI
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39 was developed to distinguish viable and dead bacteria, and the method was successfully
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41 tested with spores of different fungal species, as well (Chen and Séguin-Swartz 2002). We
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43 refined this method, and replaced PI with ethidium bromide (EtBr), to be used in testing the
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45 viability of *E. necator* ascospores during overwintering in chasmothecia. EtBr and PI
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47 performed equally well in our tests; EtBr was preferred because its availability is more
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49 widespread in laboratories compared to PI. Here we present a detailed report of our procedure
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51 adapted to powdery mildew research to supplement earlier descriptions which did not provide
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53 all the fine points, and limits, of the methodologies used, and were based on the use of FDA
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1 only. Our method consisted of crushing chasmothecia on microscope slides directly in a
2 droplet of a solution containing FDA and PI, both at a working concentration of 20 µg/ml.
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4 This solution was always freshly prepared by diluting 20x a 1 mg/ml FDA stock solution in
5 acetone, using phosphate-buffered saline (PBS) or distilled water, and adding EtBr to reach
6 the final concentration of 20 µg EtBr/ml in the solution. Slides were incubated in dark at
7 room temperature for 5 min and then checked immediately under a Nikon Eclipse 80i
8 epifluorescence microscope. The EtBr signal was detected using the filter cube with 540/25
9 nm excitation, 565 nm dichroic mirror, and 605/55 nm barrier filter. FDA fluorescence was
10 detected with another filter cube with 465-495 nm excitation, 505 nm dichroic mirror, and
11 515-555 nm barrier filter. Photographs were taken with a Spot 7.4 Slider camera and edited
12 and merged with the Adobe Photoshop software.
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27 FDA is lipophilic, membrane-permeable, non-fluorescent, and it is hydrolyzed to
28 polar fluorescent molecules in the cytoplasm of living cells. These molecules remain in the
29 cytoplasm of living ascospores with intact cell membranes and exhibit intense green
30 fluorescence when excited with blue light (465-495 nm); lights with other wavelengths can
31 also be used to make these molecules fluoresce. In contrast, EtBr can only penetrate dead, or
32 dying, cells with damaged plasma membranes and intercalates into double-stranded nucleic
33 acids, thus mainly emits fluorescence from the nucleus, in addition to some background
34 fluorescence which was also helpful to visualize cells and asci in our samples. We used green
35 light, i.e. the Nikon 540/25 nm excitation filter, to detect an intense orange EtBr signal from
36 dead or dying *E. necator* ascospores; lights with other wavelengths can also be used to excite
37 EtBr.
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53 Although after the dual staining with FDA and EtBr it is possible to observe the
54 fluorescence signals from both dyes simultaneously, as recommended by Jones and Senft
55 (1985), in case the microscope is equipped with a proper dual band fluorescence filter set, we
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1 found more useful to observe first the FDA signal alone, using the blue light excitation, and
2 then the EtBr signal only, following excitation with green light. This two-step observation
3 procedure was more straightforward because a part of the living ascospores were damaged
4 to 10 min after crushing the chasmothecia on microscope slides, probably partly due to their
5 exposure to heat and also to the mixed FDA and EtBr solution containing acetone.
6
7 Consequently, in a part of the ascospores exhibiting bright green fluorescence at the
8 beginning of the microscopic observations (Figs. 1A-C), and no EtBr signal at all, a few
9 minutes later the nucleus started to fluoresce in orange when EtBr was excited (Figs 1D-E),
10 indicating that the cell membranes became damaged as a result of the experimental
11 procedure. These ascospores were always counted as living ones when the number of viable
12 and dead ascospores was determined. Those ascospores which emitted the EtBr signal only,
13 right from the beginning of the epifluorescent examination of a sample (Figs. 1E-F), were
14 considered as dead in the examined sample. Merged pictures (Figs. 1G-I) were also produced
15 to make the distinction between viable and dead ascospores more clear. In some cases living
16 ascospores remained viable, showing green fluorescence only, for a long time, more than 10-
17 15 min after the start of the microscopic observation (Fig. 1F).

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19 The refined double staining method provided more information compared to the FDA
20 staining alone because in this way it was possible to determine the number of dead
21 ascospores, as well, which were not visible when only the FDA signal was observed (Figs.
22 1E-F). The FDA staining alone did not result in much background fluorescence, in contrast to
23 the EtBr (or PI) staining. The orange background EtBr fluorescence, in addition to the intense
24 orange fluorescence of nuclei in the dead, or dying, ascospores (Figs. 1D-F), contributed to a
25 better visualization of the samples. This is a clear advantage of the method when pictures are
26 examined with a software for image analysis.

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Another advantage of the double staining procedure was that technical problems with FDA staining did not remain overlooked. In some cases, FDA was simply not metabolized by a part of the presumably viable cells, and, thus, these did not show green fluorescence, due to some technical problems. These cells did not exhibit the EtBr signal either, indicating a procedural matter; in these cases we always repeated the double staining with another subset of the respective sample and usually got reliable results. Such technical issues, i.e. false negative results, cannot be detected if FDA is the only stain used, and cell viability is simply assessed by switching from brightfield to fluorescence microscopy to determine the ratio between the viable vs. the total number of spores. Based on these considerations, the simultaneous FDA and EtBr staining procedure proved to be the best choice for ascospore viability assessments in overwintering chasmothecia of *E. necator* and this method can be recommended for other plant pathological studies, as well.

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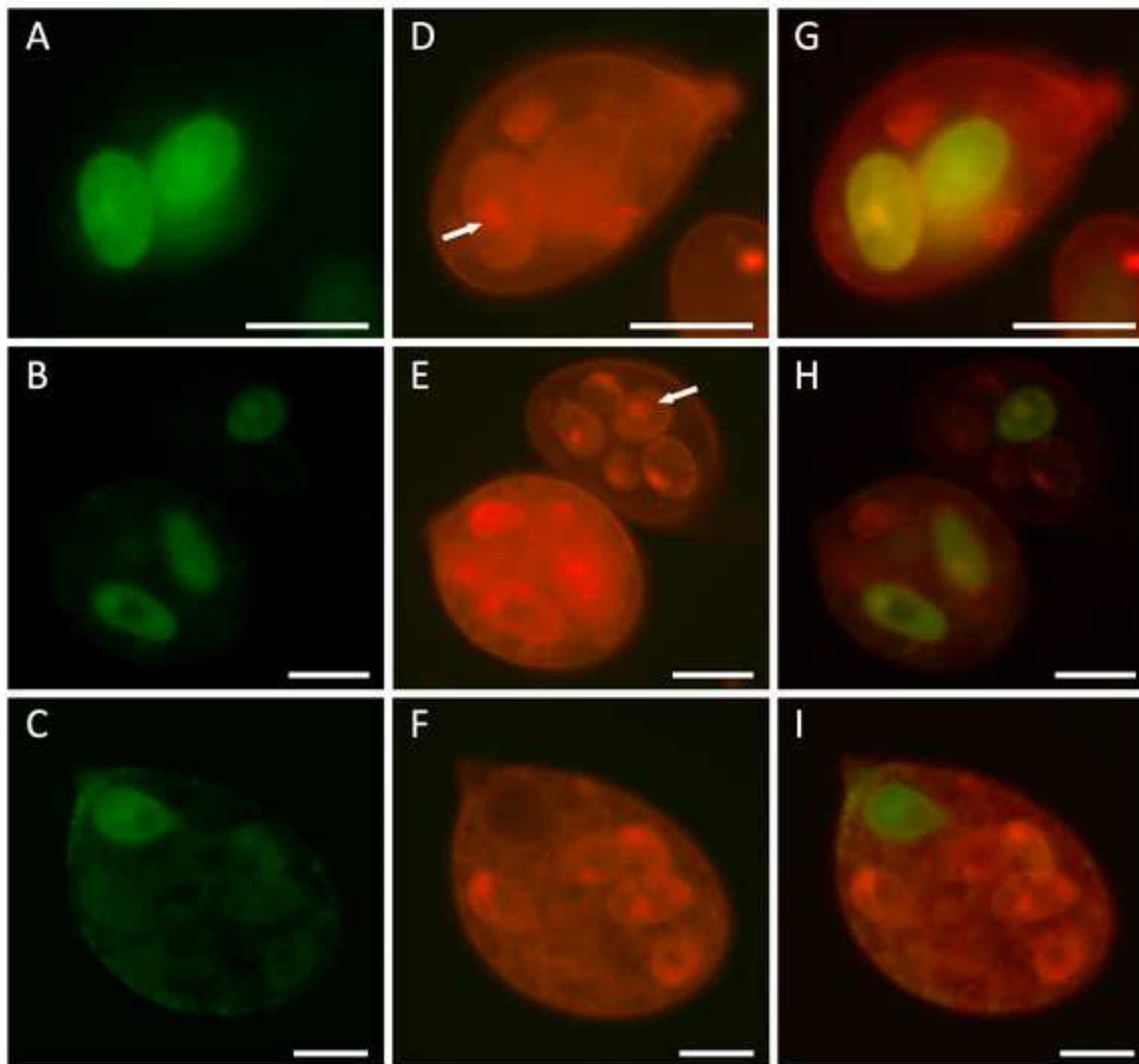
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Figure legends

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5 **Fig. 1** *Erysiphe necator* asci and ascospores stained with a double staining method based on
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7 fluorescein diacetate (FDA) and ethidium bromide (EtBr). **A.** Intense green fluorescence with
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9 FDA indicating that the two ascospores in an ascus are living. **B.** Two asci with ascospores:
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11 three ascospores exhibit green fluorescence with FDA. **C.** One ascus with ascospores: a
12
13 single ascospore exhibits green fluorescence with FDA. **D.** 7-8 minutes after the start of the
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15 examination the nucleus in one ascospore (arrow) started to fluoresce when EtBr was excited
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17 probably because the cell membrane was damaged during the study. **E.** A few minutes after
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19 the start of the study the EtBr signal appeared clearly in a nucleus of an ascospore which was
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21 alive based on the green fluorescence (arrow); other ascospores, not seen when FDA was
22
23 excited, also exhibited the EtBr signal indicating that these were dead at the start of the
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25 microscopic examination. **F.** Five ascospores with nuclei stained with EtBr; the ascospore
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27 exhibiting green fluorescence when FDA was excited remained unstained with EtBr during
28
29 the duration of the whole study (more than 10 min). **G., H. and I.** Merged pictures of the
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31 previous micrographs in the respective rows. Bars = 30 μ m.
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