1 Staining and Microscopy of Mycorrhizal Fungal Colonization in Preserved Ericoid Plant Roots

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8 Abstract

9 BACKGROUND: Visualization of ericoid mycorrhizal colonization using traditional methods relies on

10 either fresh or KOH stored samples. Increasing interest in studying ericoid mycorrhization has

11 highlighted a need for methods which can be used for preserved samples and are simple to implement

12 with commonly available equipment.

13 OBJECTIVE: The aim of this study was to improve on traditional techniques for staining ericoid

14 mycorrhizal fungi and microscopically visualizing ericoid mycorrhizal roots which have been preserved.

15 METHODS: Ericoid mycorrhizal roots were placed in KOH or frozen at -20 °C for long-term storage.

16 Traditional Trypan Blue staining methods were modified to reduce damage to fine mycorrhizal hyphae

17 and cortical cells. A high light-intensity dark-field microscopy technique was applied to clearly visualize

18 stained mycorrhizae. The novel application was compared to other commonly used practices.

19 RESULTS: Trypan Blue staining without KOH storage or clearing allowed for successful staining of ericoid

20 mycorrhizal roots stored at -20 °C. The application of high light-intensity dark-field microscopy provided

21 high contrast visualization of mycorrhizal structures.

22 CONCLUSIONS: The modified Trypan Blue staining method was effective on frozen root samples, with

23 dark-field microscopy being particularly effective at visualizing dark colored roots. Advantages to this

24 method are low cost and relatively fast application time. Therefore, this method is a realistic option for

25 large scale analyses with many samples which require long-term preservation.

26 Keywords: ericoid, mycorrhizae, dark-field microscopy, Trypan Blue staining, mycorrhizal colonization

27 ## 1. Introduction

28 The under-story vegetation in boreal forests comprises predominantly ericoid plants whose berries
29 provide an important source of nutrition for many organisms. Ericoid plants rely upon mycorrhizal fungi
30 for accessing organic nutrients in harsh, nutrient-poor environments [2]. Ericoid mycorrhizal plants are
31 important to both wild and cultivated berry production in a wide variety of ecosystems. This has led to
32 an increasing interest in studying ericoid mycorrhizal symbiosis for its ecological significance as well as
33 agricultural potential. Ericoid highbush blueberry plants (Vaccinium corymbosum L.) inoculated with
34 mycorrhizal fungi have been shown to gain improved growth and protection against plant pathogens
35 with reduced fertilization requirements [4] [9] [11]. Recent data from fungal genome studies indicate
36 that ericoid fungal species have a significant capacity for decomposition of soil organic matter as their
37 genomes harbor a wide variety of carbohydrate degrading genes [3]. Despite this capability, ecosystems
38 dominated by ericoid and ectomycorrhizal fungi store the most terrestrial C on Earth [1]. As their role in
39 terrestrial C sequestration has not yet been studied intensively, investigations focusing on ericoid plants
40 and their mycorrhizal symbionts will be of great interest in the future. There is also a need to investigate
41 the functions of ericoid mycorrhizae in commercial blueberry fields [10].

42

43 Ericoid plant roots are fine structured and ericoid mycorrhizae usually do not form a hyphal mantle,
44 therefore the presence of ericoid mycorrhizal colonization can only be verified using microscopy aided
45 by staining of fungal hyphae. There are several methods available for staining ericoid mycorrhizal roots
46 for microscopy, many of which rely on the original paper by Phillips and Hayman [8]. For instance, Koske
47 & Gemma [5], Nestby et al. [7], Sadowsky et al. [10] and Scagel [12] used modified Phillips & Hayman [8]
48 methods, including clearing of roots in KOH followed by acidification and then staining. As reported by
49 Koske & Gemma [6], the 10% KOH treatment "is harsh and may result in the loss of the cortex from

50 much of the root system". This is particularly evident in roots which must be preserved long-term and
51 require KOH storage or freezing at -20 °C, with freezing often being necessary for molecular and
52 chemical analyses. Thus, there is a clear need to develop and test methods for staining fragile and thin
53 ericoid plant roots for microscopy that have been stored frozen. Also, as many research projects dealing
54 with ericoid plants are not designed for studying ericoid mycorrhizal colonization but rather other
55 ecological or commercial aspects of ericoid plants, it would be advantageous to be able to re-analyze
56 frozen root samples from such experiments. This capability would provide better understanding of
57 ericoid mycorrhizal ecology and importance for berry cultivation.
58
59 The aim of the study was to develop a simple, fast and gentle method for staining and visualizing
60 preserved ericoid plant roots for microscopic detection of mycorrhizal intracellular colonization.
61
62 2. Materials and methods
63 The ericoid species Vaccinium myrtillus, Vaccinium vitis-idaea, and Calluna vulgaris were grown
64 experimentally for 18 months in homogenized, natural forest humus under simulated boreal forest
65 conditions [13]. The plants were allowed to naturally form ericoid mycorrhizal symbioses within their
66 roots. Root samples were hand washed with cold tap water to remove excess soil.
67
68 2.1 Staining
69 Root samples were stored at -20 °C and in 10% KOH solution to test the effects of long-term storage (6
70 months) on ericoid mycorrhizal roots. Root samples were prepared for microscopic visualization of
71 mycorrhizal colonization using a Trypan Blue staining technique modified from Phillips and Hayman [8].
72 A second set of samples were stored in 10% KOH for 1 day or 3 days at room temperature and incubated
73 in Trypan Blue at 90 °C for 30 minutes to test if reduced time in both steps prevented damage to fine

74 root cortical cells. Frozen samples were taken directly from -20 °C and incubated in a 1:1 solution of
75 lactic acid and glycerol containing 0.05% Trypan Blue at 90 °C for 30 minutes. Frozen roots were not
76 treated with KOH for clearing and therefore did not require neutralization in HCl. As a comparison with
77 the long-term preservation methods being tested, fresh C. vulgaris roots were placed in Trypan Blue for
78 10 minutes at room temperature.
79
80 Immediately after staining with Trypan Blue all root samples were placed in a de-staining solution of 1:1
81 lactic acid and glycerol at room temperature for 15 minutes to remove excess dye. Individual root
82 segments were then arranged on glass microscopy slides. Several root segments from each sample were
83 fitted in parallel under the same 20x50 mm cover slip and embedded with clean de-staining solution,
84 which was then sealed along the sides using transparent nail polish.
85
86 2.2 Microscopy
87 A Zeiss Axioskop 2 Plus microscope (Zeiss, Oberkochen, Germany) was used in transmitted light mode
88 with an achromatic-aplanatic universal condenser (0.9 H D Ph DIC) in Dark Field mode and front lens in
89 place. Each prepared slide was observed through an A-Plan 40x/0.65 (Ph2 Var2) objective with
90 maximum illumination and all diaphragms fully opened. The height of the condenser was adjusted to
91 near upper maximum position to produce optimal illumination of the samples. This was achieved by
92 oblique light rays reflecting within the embedding solution surrounding the root fibers, providing
93 increased contrast of roots and stained mycorrhizal fungal cells. Careful adjustment of focus allowed for
94 visualization of colonizing fungal features in several planes. Use of this technique on older microscopes
95 such as an Axioskop 1 (Zeiss, Oberkochen, Germany) is not ideal as their light sources are not sufficient,
96 a 100 watt light source is recommended for clear visualization.
97

98 Caution: light intensity should only be increased to maximum after the slide is in place for observation,
99 as the embedding solution reduces the light to a safe viewing level.
100
101 2.3 Estimation of Colonization Percentage
102 By using this method to clearly visualize ericoid mycorrhizal roots it is possible to quantify colonization
103 using the magnified intersections method [6]. This method is performed by recording the mycorrhizal
104 colonization status of each root fiber as it is intersected by the eyepiece crosshair along a vertical
105 transect at 400X magnification. Generally, 50 to 200 vertical transects are done along the length of the
106 slide. This method allows for a simple analysis of colonization at each intersection of the eyepiece
107 crosshair.
108
109 3. Results
110 Storage of ericoid roots in 10% KOH for both 1 and 3 days was highly damaging to cortical cells of all
111 tested species, with nearly all roots becoming completely disrupted (Figure 1A). Directly stained fresh C.
112 vulgaris roots (Figure 1B) allowed for high quality dark-field microscopic visualization of fungal
113 structures, but the freeze-dye method gave even better results , even after long storage of roots at -20
114 °C (Figure 1C). The visualization of mycorrhizal colonization with the freeze-dye method produced the
115 best results also with mycorrhizal roots of V. myrtillus and V. vitis-idaea (Figures 2A & B). The high light-
116 intensity dark field microscopy (Figure 3A) provided superior visualization of root cells when compared
117 with traditional bright field microscopy (Figure 3B).
118
119 4. Discussion
120 The combined staining and microscopy methods described here provide good visual clarity and
121 detection of ericoid mycorrhizal colonization in roots frozen for later analyses with the additional

122 benefits of simplicity, rapid application and low cost. With practice, a slide preparation of stained ericoid
123 mycorrhizal roots takes under one hour to complete and time of microscopy is reduced as the high light-
124 intensity dark-field method makes it easier to observe mycorrhizal structures in several focus planes.
125 Firstly, staining of mycorrhizal hyphae was successful after freezing of ericoid mycorrhizal roots at -20 °C,
126 while preserving fine hyphae on the surface of roots and internal hyphal structures. Traditional methods
127 describe clearing of roots in KOH followed by HCl neutralization and staining, which in our case was
128 shown to be damaging to fine ericoid plant roots and any hyphae on or within them. As the microscopy
129 utilized in this method provided enhanced visual clarity and illumination, it was possible to observe
130 mycorrhization even in darkly colored root cortical cells. Therefore, a clearing step using KOH was
131 entirely unnecessary and fragile hyphal structures were preserved.

132 Compared to more advanced and potentially cost prohibitive visualization techniques such as
133 Differential Interference Contrast (DIC) or electron microscopy, high-quality microscopy of mycorrhizae
134 using dark-field microscopy is advantageous. This method requires minimal training and is achievable
135 using the majority of modern light microscopes without expensive additional equipment. The ability to
136 clearly observe mycorrhizal structures reduces ambiguity when identifying mycorrhizal colonization and
137 provides more accurate estimation of colonization frequency.

138 The primary advantage of this method is the short application time, with quick staining and simple
139 microscopy providing the capability to analyze large numbers of samples which can also be preserved by
140 freezing at -20 °C, allowing for further molecular and chemical analyses. Reducing time of analyses in
141 commercial berry production or field scale ecological studies is critical to producing results. The
142 capability of this method may become increasingly relevant with growing interest in utilizing ericoid
143 mycorrhizae for commercial purposes, as well as current research which indicates the potential
144 importance of ericoid mycorrhizae in global carbon sequestration. The presented method produces
145 successful staining and microscopy of frozen ericoid mycorrhizal root samples. Thus, freezer-stored

146 ericoid root samples from old, completed experiments could be re-analyzed for quantification of ericoid

147 mycorrhizal colonization.

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151 Conflict of Interest

152 The authors have no conflict of interest to report.

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187 Figure Captions

188 Figure 1: A) C. vulgaris roots after KOH treatment and Trypan Blue staining resulting in total loss of root
189 cortical cells. B) Fresh C. vulgaris roots after staining in Trypan Blue for 10 minutes at room temperature
190 and displaying intracellular hyphal coiling (HC). C) Mycorrhizal C. vulgaris roots after freezing at -20 °C
191 and staining with Trypan Blue and displaying intracellular hyphal coiling (HC). Bar = 50 µm.

192 Figure 2: A) Mycorrhizal V. myrtillus roots after freezing at -20 °C and staining with Trypan Blue and
193 displaying intracellular hyphal coiling (HC). B) Mycorrhizal V. vitis-idaea roots after freezing at -20 °C and
194 staining with Trypan Blue and displaying intracellular hyphal coiling (HC). Bar = 50 µm.

195 Figure 3: A) C. vulgaris mycorrhizal roots visualized by high light-intensity dark field microscopy. B)
196 Comparative visualization of the same C. vulgaris mycorrhizal roots using bright field microscopy. Bar =
197 50 µm.

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207 Figures

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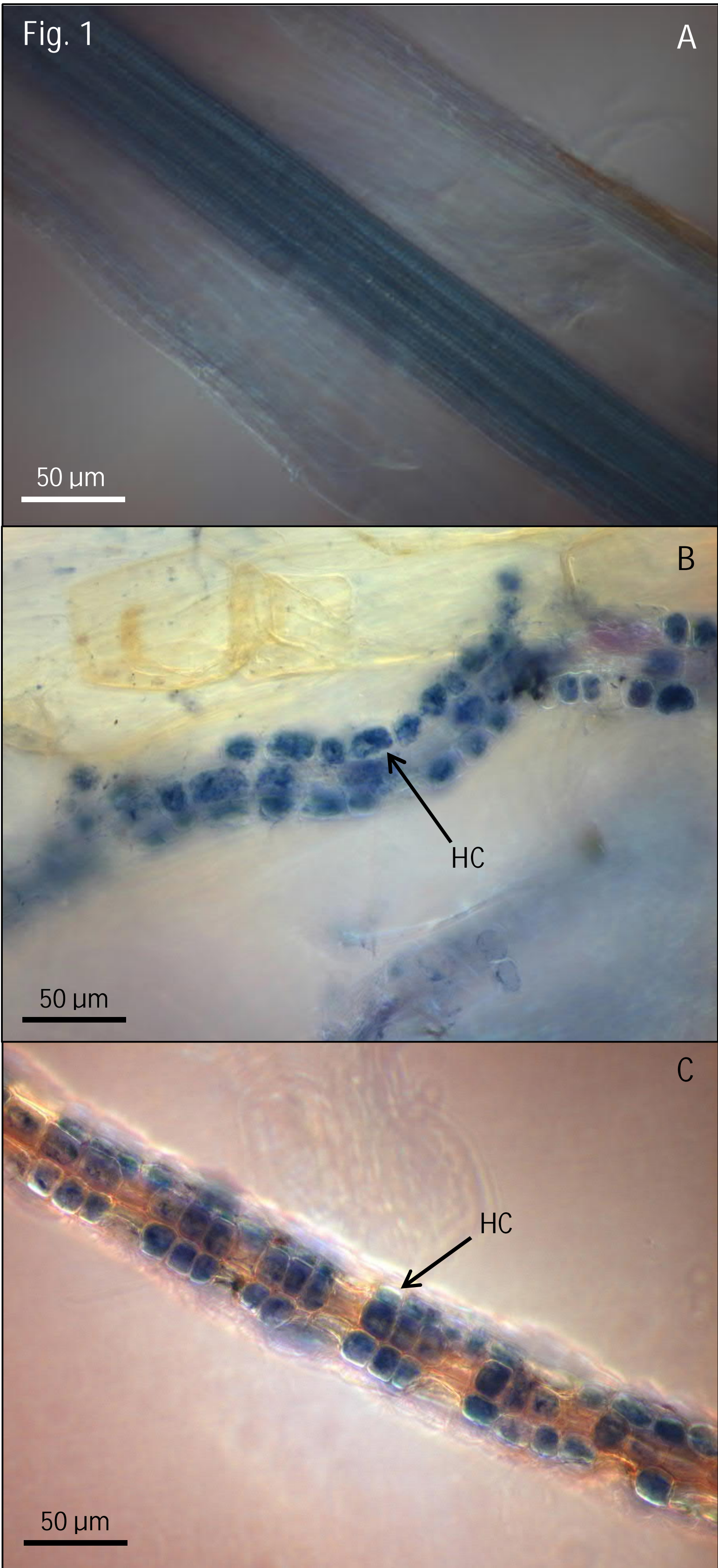

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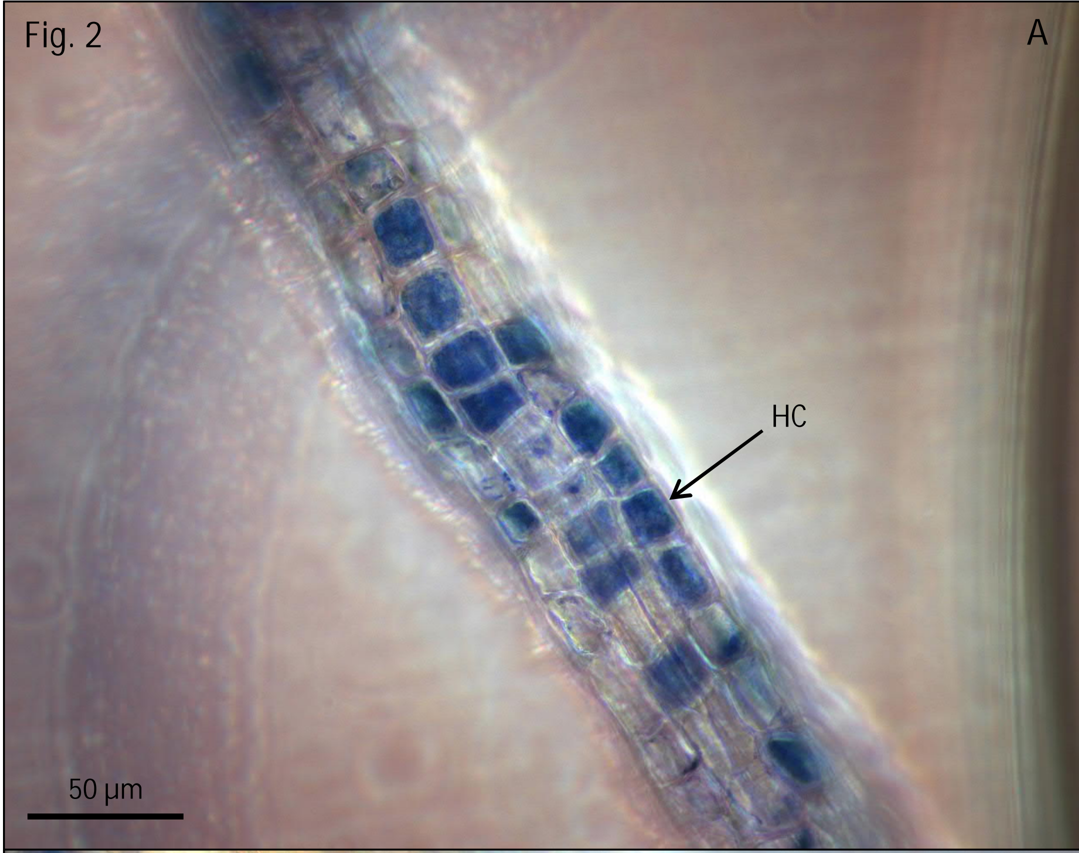

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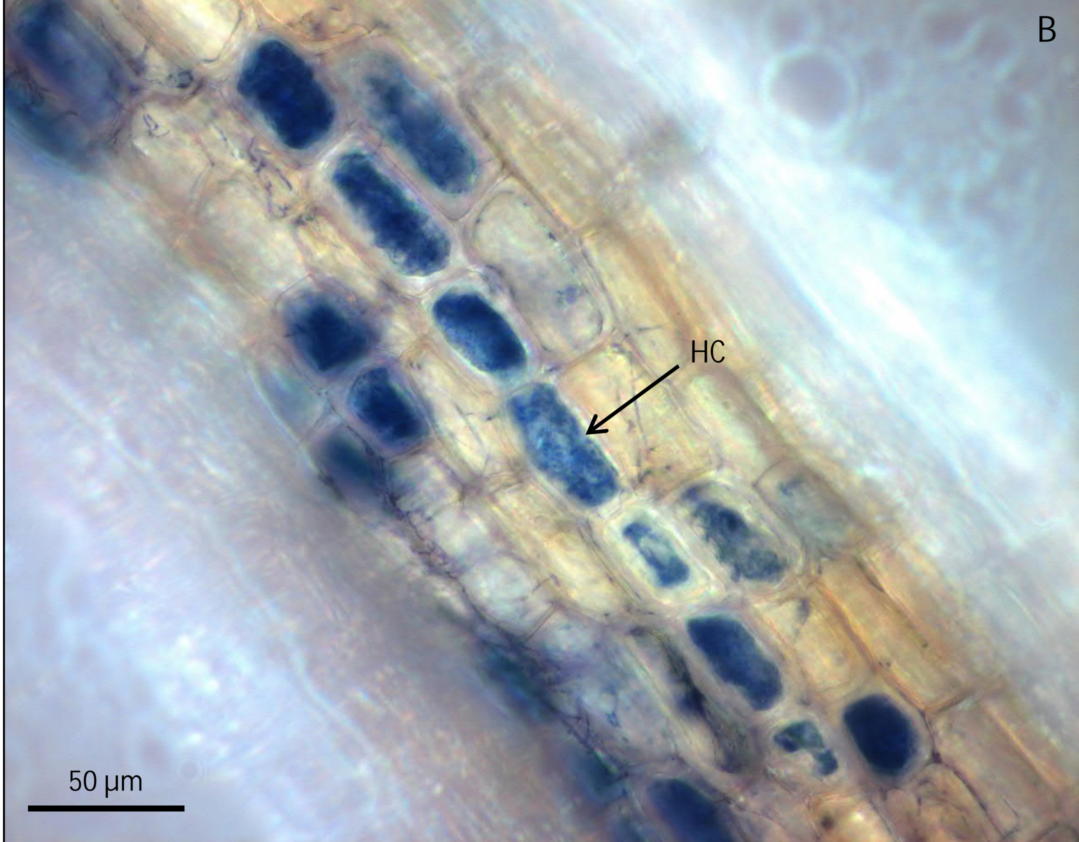

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