Visualization of Growth and Morphology of Fungal Hyphae *in planta* Using WGA-AF488 and Propidium Iodide Co-staining

Amey Redkar[1,*], Elaine Jaeger[2] and Gunther Doehlemann[2]

[1]Department of Genetics, University of Cordoba, 14071, Cordoba, Spain; [2]Botanical Institute and Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, BioCenter, Cologne, Germany

*For correspondence: ge2rerea@uco.es

[Abstract] Fungal pathogens colonizing plants show a varying degree of symptoms. Microscopy techniques have been used to study the infection and proliferation of fungal hyphae inside the host. One of the best optimized and commonly used method is the co-staining with Wheat Germ Agglutinin-Alexa Fluor 488 conjugate (WGA-AF488) and propidium iodide (PI), which stains fungal hyphae and the plant cell wall in contrasting shades. This technique is widely used to characterize the various behaviors of fungal hyphae, *e.g.,* in fungal knockout mutants being attenuated during differential stages of host colonization. We describe the protocol for sample preparation of WGA-AF488– PI staining of infected plant tissue. Here, we have used an infected sample with the basidiomycetous smut fungus *Ustilago maydis* that infects its host plant maize (*Zea mays* L.) and *Ustilago hordei* that infects barley (*Hordeum vulgare* L.). This protocol helps to understand growth, biomass and morphology of fungus *in planta* by confocal laser scanning microscopy (Doehlemann *et al*., 2011; Redkar *et al.*, 2015).

Keywords: Microscopy, Fungus, Propodium iodide, Confocal, Maize

Materials and Reagents

1. 2.0 ml microcentrifuge tubes
2. 100% ethanol
3. Double distilled water
4. Potassium hydroxide 10% (KOH)
5. Tween 20 (Sigma-Aldrich, catalog number: P1379)
6. Wheat Germ Agglutinin (WGA) Alexa Fluor 488 (Thermo Fisher Scientific, catalog number: W11261)
7. Propidium iodide (PI) (Sigma-Aldrich, catalog number: P4170)
8. Potassium phosphate monobasic (KH_2PO_4)
9. Sodium phosphate dibasic (Na_2HPO_4)
10. Potassium chloride (KCl)
11. Sodium chloride (NaCl)
12. WGA-AF488-Stock Solution (see Recipes)
13. Phosphate buffer saline (PBS, pH 7.4, self-made) (see Recipes)

14. Propidium iodide-Stock Solution (see Recipes)
15. Staining Solution (see Recipes)

Equipment

1. Vacuum Infiltrator
2. Confocal microscope (*e.g.*, Leica Microsystems, model: Leica TCS SP8)

Software

1. Leica Image Analysis Software in the SP8 confocal microscopy or with the freely available ImageJ

Procedure

A. Infection and harvesting of the samples
   1. The *U. maydis* infections are carried out as reported previously (Redkar and Doehlemann, 2016) and *U. hordei* as described by Hof *et al.*, 2014.
   2. Harvest samples at the desired time-points (2 days post inoculation (dpi) leaf samples for *U. maydis* infection and the 8 dpi leaf samples for *U. hordei* infection in this protocol).
      Note: It is recommended to collect the samples in 2.0 ml microcentrifuge tubes. In case of larger leaf samples, the infected area is cut into small sample sizes to fit in a microcentrifuge tube. Usually, 2 leaves (2 cm size around the infection area) is sufficient to carry out staining as it fits ideally into the microcentrifuge tubes (Figure 1).

B. Fixation of samples and co-staining with WGA-AF488/PI
   1. Place the harvested tissues (maize or barley leaves) in 100% ethanol for 2-3 days to undergo bleaching and complete removal of chlorophyll. This is the fixation step which can also be carried longer until the complete chlorophyll is removed (Figure 1).

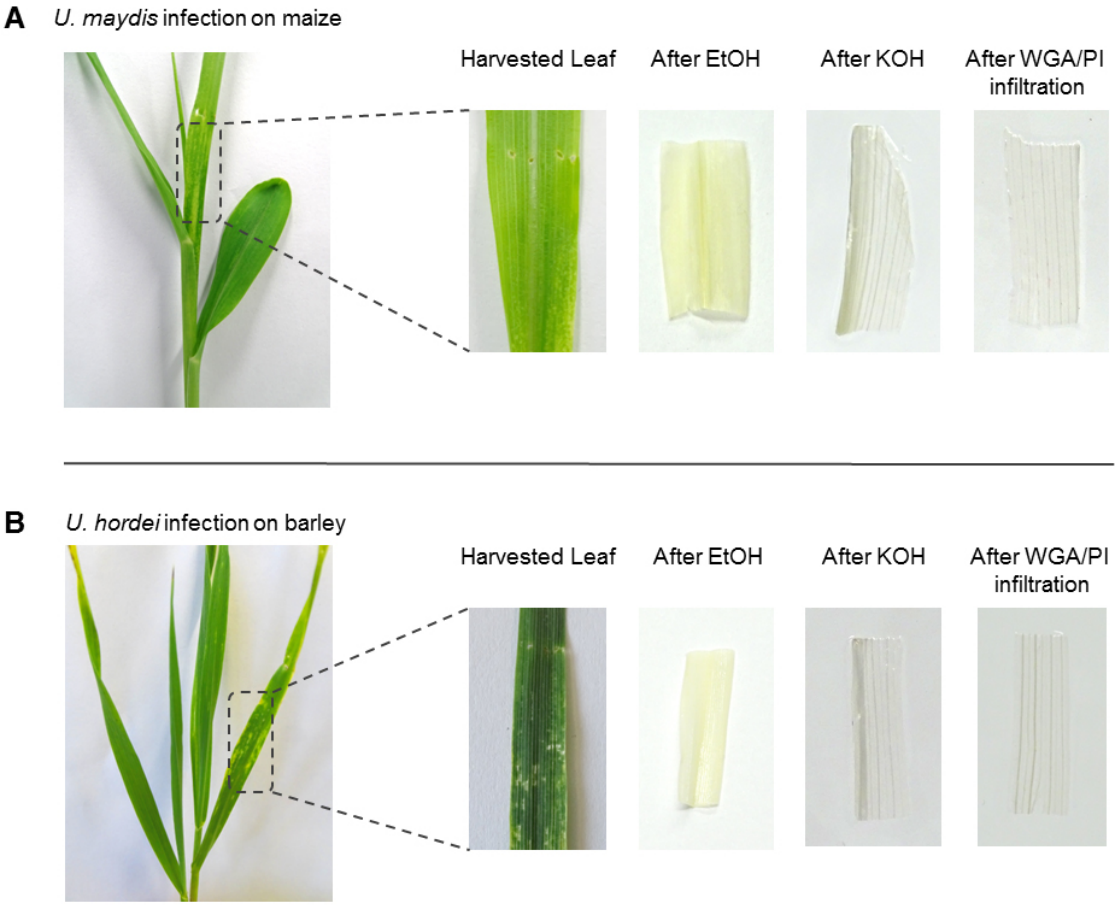

Figure 1. Representative images of infected leaves after different treatments in WGA-AF488-PI co-staining procedure. Images show pictures of (A) maize and (B) barley leaves during harvesting, after fixation in ethanol, after KOH treatment and after staining with WGA/PI in PBS.

2. After bleaching, replace the ethanol with 10% KOH; and incubate samples for 4 h at 85 °C (barley leaf samples are incubated only for 2 h). This step makes the tissue more fragile and transparent.

   Notes:

   a. *Depending on the cell wall stiffness, incubation time of this step will need to be adapted for different plant tissues. Very soft plant tissues might require only few minutes of the KOH treatment.*

   b. *Be sure to properly seal the lids of microcentrifuge tubes with parafilm or plastic sealers as a constant incubation at a higher temperature may pop off the lids.*

3. Wash the samples at least 4-5 times in PBS (pH 7.4) for neutralization. More frequent washing with PBS is necessary to lower the pH near to neutral for the efficient WGA staining.

4. Add the staining solution (WGA-AF488 and PI in PBS) to the samples in microcentrifuge tubes, make sure that the samples are completely immersed in the staining solution. The WGA-AF88

stains the fungal hyphae (green) and the propidium iodide stains the plant cell wall (red) as shown in Figures 2 and 3.

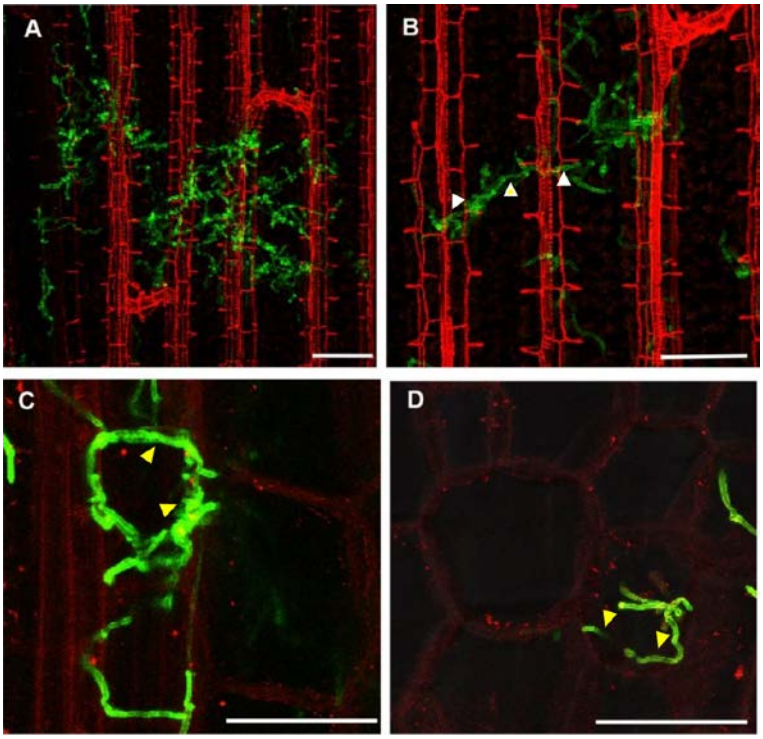

Figure 2. Representative images of WGA-AF488-PI co-stained confocal maximum projections of maize leaves infected with *U. maydis*. Samples were harvested at 2 dpi time-point. Fungal hyphae were stained with WGA-AF488 (green), plant cell walls were stained with propidium iodide (red). A. An overview picture. B-D. Close up of the biotrophic fungal hyphae showing colonization and hyphal morphology with either linear growth as shown with the white arrow-heads (B) or restricted growth in single cells as shown in (C) and (D) with yellow arrow-heads. Scale bars = 50 µm.

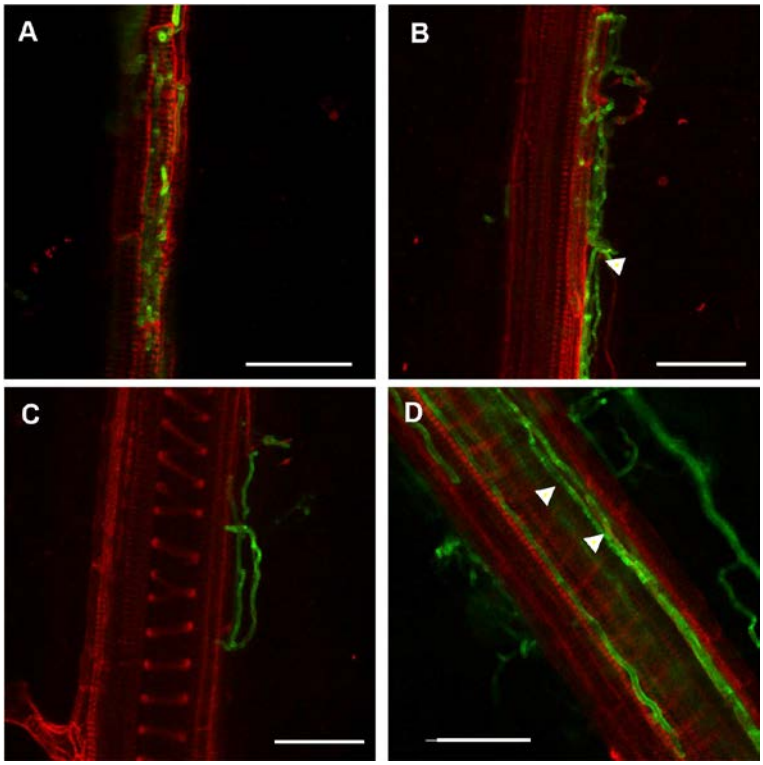

Figure 3. Representative images of WGA-AF488-PI co-stained confocal maximum projections of barley leaves infected with *U. hordei.* Samples were harvested at 8 dpi time-point. Fungal hyphae were stained with WGA-AF488 (green), plant cell walls were stained with propidium iodide (red). A and B. Overview pictures of the biotrophic fungal hyphae showing colonization and hyphal growth along the vascular tissue; C and D. Close up of hyphae. White arrow-heads indicate linear hyphal growth from cell to cell. Scale bars = 50 µm.

5. Then vacuum infiltrate the samples at 250 millibar 3-4 times for 5 min each along with a regular interval of 5 min with atmospheric pressure.
6. After the infiltration, wash the samples twice in PBS (pH 7.4), then transfer to new PBS (pH 7.4), and store at 4 °C in the dark (up to several days) until analysis by confocal microscopy.

C. Microscopy of the WGA-PI stained leaves

All confocal analysis may be performed on a TCS SP8 confocal laser scanning microscope (Leica, Germany), or any other suitable microscope. The laser channels used for confocal fluorescence analysis of excitation and detection wavelengths are summarized in Table 1.

Table 1. Lasers used and the excitation and detection wavelength

Detection	Excitation Wavelength	Detection Wavelength	Laser type
WGA AF488	488 nm	500-540 nm	Argon
Propidium iodide	561 nm	580-630 nm	561 DPSS

Data analysis

Data processing can be done by using the Leica Image Analysis Software in the SP8 confocal microscopy or with the freely available ImageJ.

Recipes

1. WGA-AF488-Stock Solution
   1 mg/ml in H_2O
   Store at 4 °C
2. PBS (pH 7.4)
   1.5 mM KH_2PO_4
   8 mM Na_2HPO_4
   2.7 mM KCl
   137 mM NaCl
   Dissolved in H_2O
3. Propidium iodide-Stock Solution
   10 mg/ml in PBS (pH 7.4)
   Store in the dark
4. Staining Solution
   20 µg/ml Propidium iodide
   10 µg/ml WGA-AF488
   0.1% Tween 20
   All added to the PBS buffer (pH 7.4)

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