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Technical note:

Fungal staining tools to study the interaction between the beneficial endophyte *Piriformospora indica* with *Arabidopsis thaliana* roots

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Abstract

Piriformospora indica, a primitive endophytic fungus of Sebacinales, colonizes the roots of all plant species investigated so far and promotes plant growth. It also increases seed production, uptake of nutrients and resistance to biotic and abiotic stress. Several studies have demonstrated the degree of root colonization is crucial for the benefits of both symbionts. Furthermore, since the fungus grows inter- and intra-celullarly, efficient tools are required for the detection of fungal hyphae and spores in and around the roots. Here we describe different staining methods to study the histology of *P. indica-Arabidopsis thaliana* interaction.

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Introduction

Piriformospora indica is an endophytic fungus of Sebacinales that colonizes the roots of many plant species including forestry, agricultural, horticultural and medicinal species (Barazani et al. 2005; Glen et al. 2002; Kaldorf et al. 2005; Oelmüller et al. 2005; Peškan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Selosse et al. 2002a, b; Shahollari et al. 2005, 2007; Sherameti et al. 2005, 2008a, b; Urban et al. 2003; Varma et al. 1999, 2001; Waller et al. 2005; Weiss et al. 2004), as well as the model plant *Arabidopsis thaliana* (cf. Peśkan-Berghöfer et al. 2004; Oelmüller et al. 2005; Shahollari et al. 2005; Sherameti et al. 2008a; Camehl et al. 2011). For all plant species tested so far, *P. indica* showed to have the potential to promote growth and biomass production indicating that the symbiosis is beneficial for the hosts and has the potential for agricultural and biotechnological applications. We use Arabidopsis as host to understand the molecular basis of the beneficial interaction between the symbionts.

P. indica hyphae can be detected on the surface of Arabidopsis root, in the outer cell layers, and within the root cells, using optical microscopy (Peśkan-Berghöfer et al. 2004). Several studies have demonstrated that the degree of root colonization is critical for establishing benefits for the plants. For the wild-type, root colonization and association of fungal hyphae results in the promotion of growth and seed yield, as well as tolerance against biotic and abiotic stress (Varma et al. 1999; Verma et al. 1998; Oelmüller et al. 2009; Oelmüller et al. 2004; Rai et al. 2004; Sherameti et al. 2008a, b; Varma et al. 2001; Waller et al. 2005), whereas overcolonization of hyphal growth can result in the loss of the benefits, and may even lead to a shift from a mutualistic to a antagonistic interaction (Johnson and Oelmüller 2009; Camehl et al. 2010a, b). Furthermore, it has been proposed that the beneficial interaction of *P. indica* with barley roots requires cell death programs (Deshmukh et al. 2006; Schäfer et al. 2007). Fungal colonization increases with root tissue maturation. The root tip meristem of barley showed no colonization, and the elongation zone showed mainly intercellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were present in dead rhizodermal and cortical cells that became completely filled with chlamydospores. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing endogenous programmed cell death. Further studies with the barley BAX inhibitor-1, a gene capable of inhibiting plant cell death, demonstrated that *P. indica* requires host cell death for proliferation in differentiated barley roots (Deshmukh et al. 2006; Schäfer et al. 2007). Recently, this study was extended to Arabidopsis (Jacobs et al. 2011). By employing transmission electron microscopy and epifluorescence microscopy along with reporter and mutant plants, the authors observed a biotrophic colonization of Arabidopsis roots by a fungus. This biotrophic stage is followed by a cell death-associated colonization phase. The genetic and molecular analyses also demonstrated the efficiency of the root innate immune system to halt microbial colonization and indicated that mutualistic colonization success is intimately dependent on efficient immune suppression strategies (Jacobs et al. 2011).

All these observations clearly demonstrate that powerful staining techniques are required to visualize hyphal growth within the roots.

Until now, the growth and morphology of *P. indica* hyphae and spores was studied by light microscopy, epifluorescense, scanning-electron (Sun et al. 2010) or confocal microscopy (Stein et al. 2008; Waller et al. 2005). However,

some of the light microscopical techniques were time-consuming and harsh (Verma et al. 1998). Recently, GFP-transformed *P. indica* strains have been reported (Zuccaro et al. 2009), which might be a powerful tool in future studies.

We use *Arabidopsis thaliana* as a host for *P. indica* and employed various simple methods for the visualization of the spores and hyphae within and around the root. This includes live cell microscopy, different staining methods and epifluorescense microscopy. The objectives of this study were to develop fast and simple methods to describe the colonization under different growth and cultivation conditions, and to obtain staining protocols that allow study of the fungal growth to some extend in and around the Arabidopsis roots.

Results and Discussion

The interaction of *P.* indica with A. thaliana roots was studied on different media including PNM (Plant Nutrient Medium) and MS (Murashige and Skoog-Salt) by light and fluorescence microscopy without chemical treatment (Figures 1-7). With all methods, the mycelium is nicely visible in *A. thaliana* roots by optical microscope. Using different

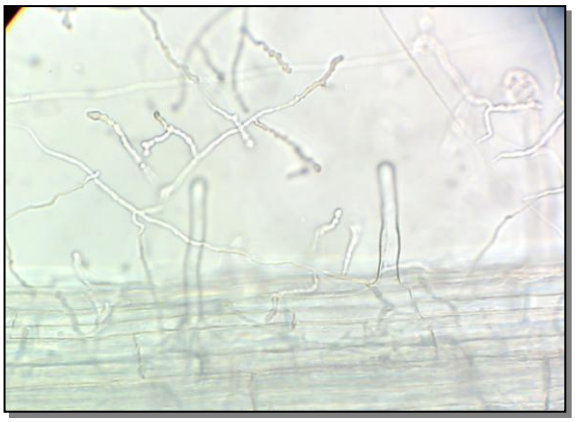

Figure 1: *Arabidopsis thaliana* roots, and hyphae of *P. indica* under the light microscope, magnification 400x. Life microscopy. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

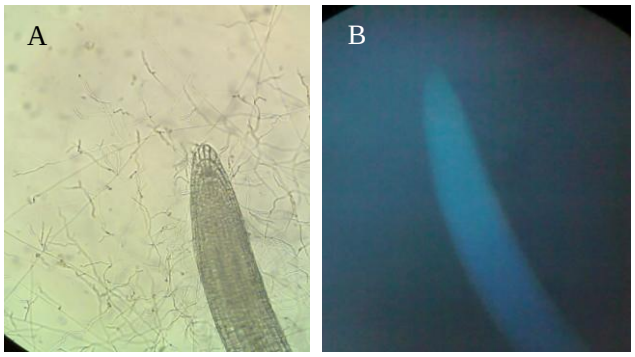

Figure 2: *Arabidopsis thaliana* roots, and hyphae of *P. indica* under the light microscope (A) and fluorescent channel at 450-490 nm (B), magnification 100 times. Life microscopy. 15 days after interaction of *P. indica* with *A. thaliana* on PNM .medium.

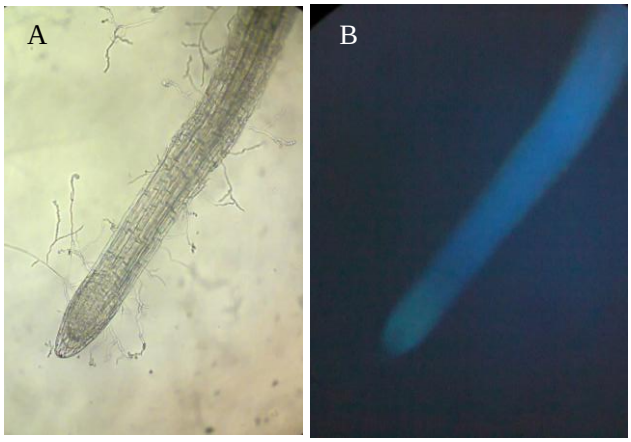

Figure 3: *Arabidopsis thaliana* roots, and hyphae of *P. indica* under the light microscope (A) and fluorescent channel 450-490 nm (B), magnification 100x. Life microscopy. 15 days after co-cultivation of *P. indica* with *A. thaliana* on MS-S medium.

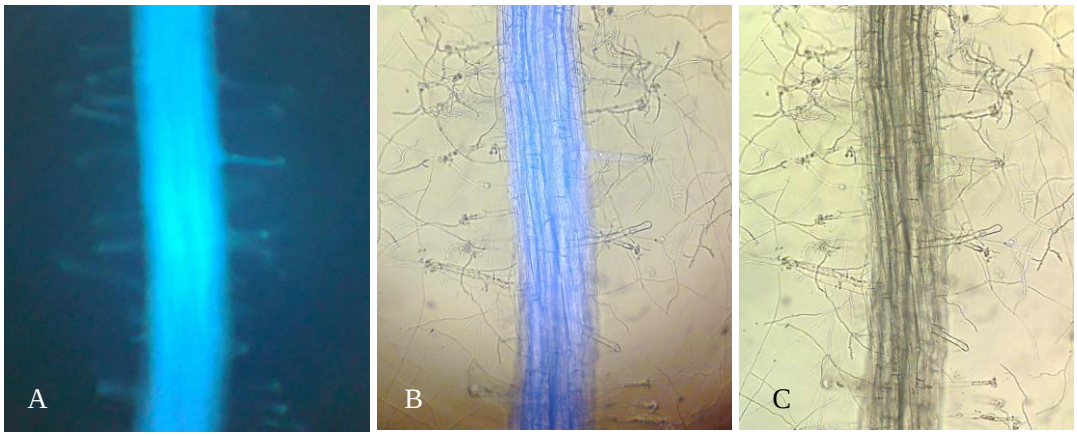

Figure 4: *Arabidopsis thaliana* roots, hyphae and chlamydospores of *P. indica* under the fluorescent channel of 450-490 nm (A), fluorescent and visible light (B) and light microscopy (C), magnification 100x. Life microscopy. 7 days after co-cultivation of *P. indica* with *A. thaliana* on MS-S medium.

magnifications (4x, 10x, 20x and 40x) and different wavelengths (visible and fluorescent channels) better resolution and discrimination of fungal and plant tissues could be achieved. Fluorescence microscopy is more sensitive compared to light microscopy. Additionally, different layers of the cell tissue can be scanned using fluorescence microscopy.

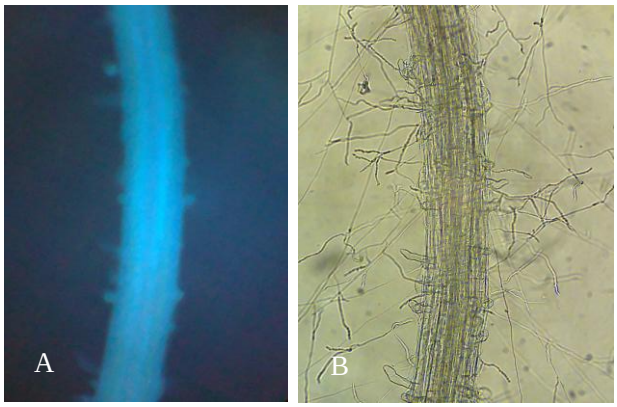

Figure 5: *Arabidopsis thaliana* root, and hyphae of *P. indica* under the fluorescent channel at 450-490 nm (A) and under the light microscope (B), magnification 100x. Live microscopy. 15 days after co-cultivation of *P. indica* with *A. thaliana* on MS-S medium.

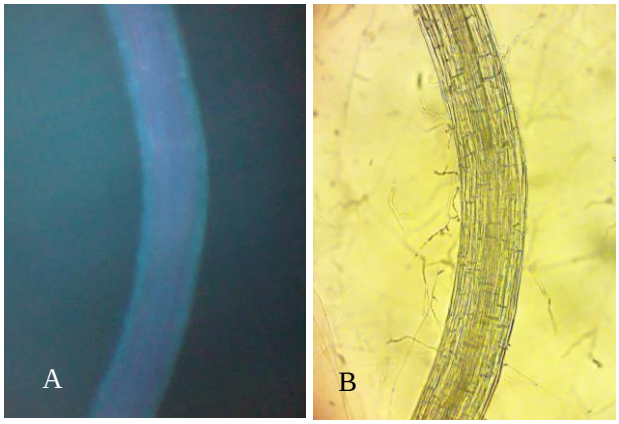

Figure 6: *Arabidopsis thaliana* root, and hyphae of *P. indica* under the fluorescent channel at 450-490 nm (A) and under light microscopy (B), magnification 100x. Live microscopy. 7 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

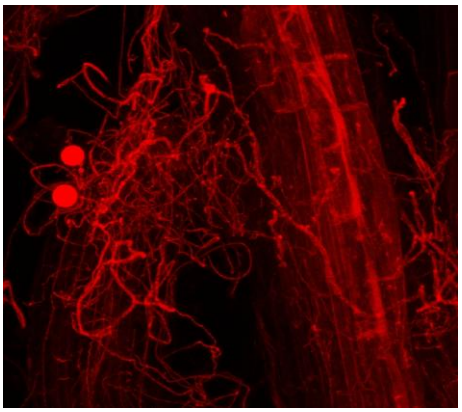

Figure 7: *Arabidopsis thaliana* root, and hyphae of *P. indica* under the fluorescence channel at 488 nm. Life microscopy. magnification 400x. 4 weeks after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

Fluorescence microscopy

Optimal results were obtained with fuchsin acid and fluorescence microscopy using different wavelengths (Figures 8-10). The method is fast and allows visualization of *P. indica* mycelia and chlamydospores in the root environment. For detection and counting of chlamydospores, we found that staining with erythrosine and safranin using the fluorescence channel at 470 and 450-490 nm resulted in contrast between spore and roots (Figure 11A and B). With this method the mycelia are not visible and the spores can be counted without light interference. Staining with fuchsin acid/cotton blue gave comparable results. With this method, all stained parts of the roots and mycelia can be seen with visible light, but at 470 nm, only chlamydospores are visible (Figure 12).

Chemical staining

The optimal time for staining with Trypan blue (0.0001 mg/ml) is 1 minute, since longer incubations also stains root tissue. This method gave best results when only the mycelium should be visualized. It shows the interaction with root environment and allows the analysis of the mycelium networks in and around the roots. Furthermore, the strong contrast between the colored mycelia and the background is suitable for the analysis of root colonization with appropriate software (Figures 13 and 14).

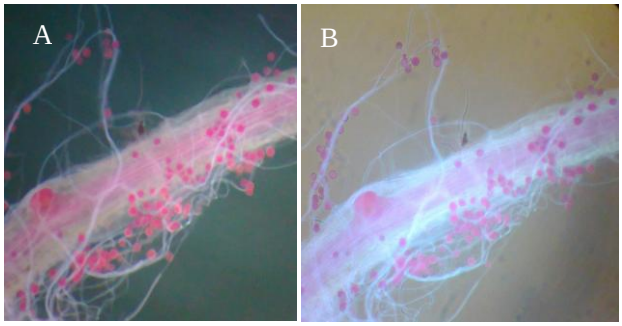

Figure 8: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 450-490 nm (A) and at 365 nm (B); magnification 100x. Fuchsin acid staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

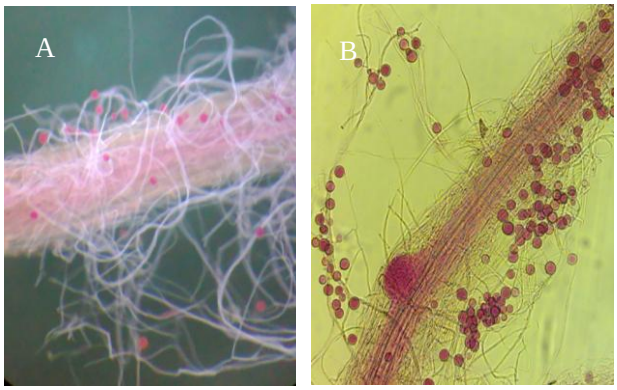

Figure 9: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 450-490 nm (A, magnification 200x) and under light microscopy (B, magnification 100x). Fuchsin acid staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

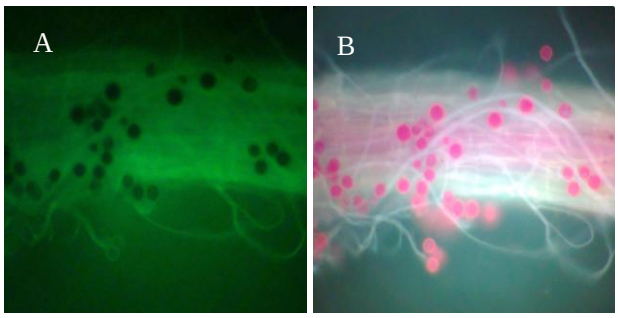

Figure 10: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 470 nm (A) and 450-490 nm (B), magnification 200x. Fuchsin acid staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

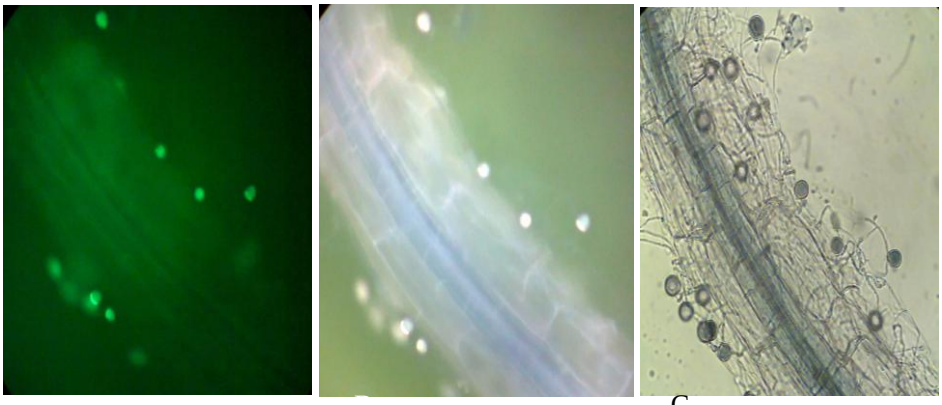

Figure 11: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 470 nm (A), 450-490 nm (B) and under the light microscope (C), magnification 200x. Erythrosine/safranin method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

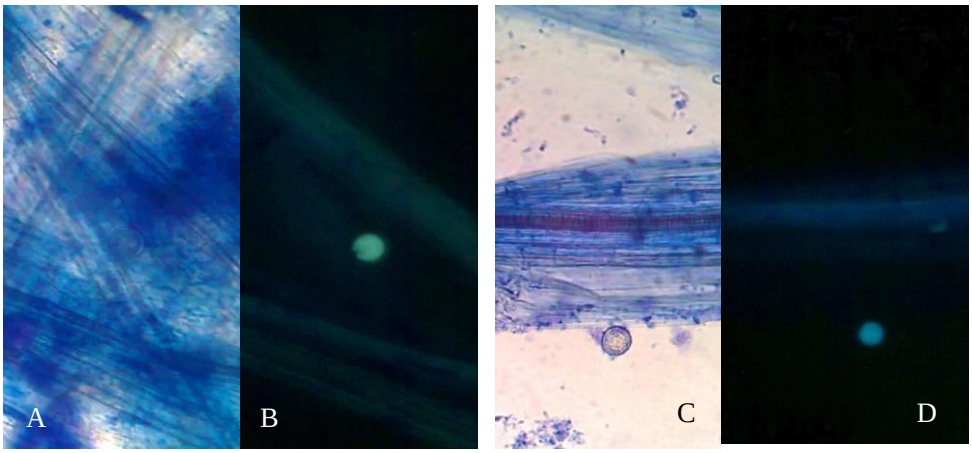

Figure 12: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope (A and C) and fluorescent channel at 470 nm (B and D), magnification 400x. Fuchsin acid / cotton blue method 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

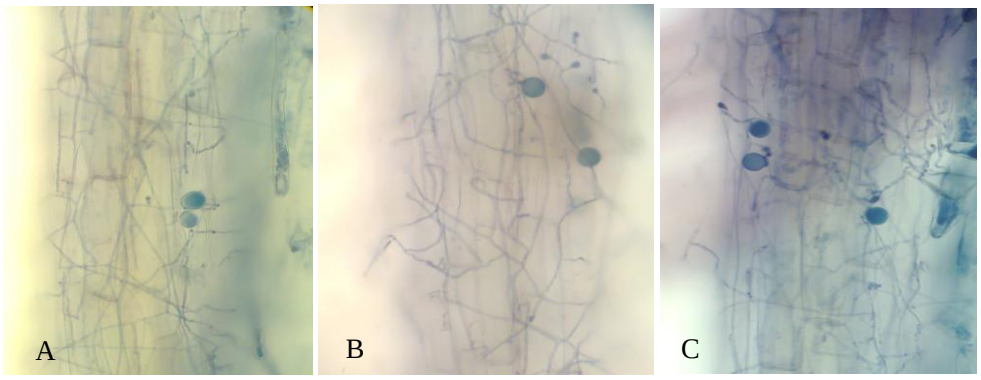

Figure 13: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* light microscopy, magnification 200x. Trypan blue (0.0001 mg/ml) method (A, B, 1 min staining; C, 5 min staining). 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

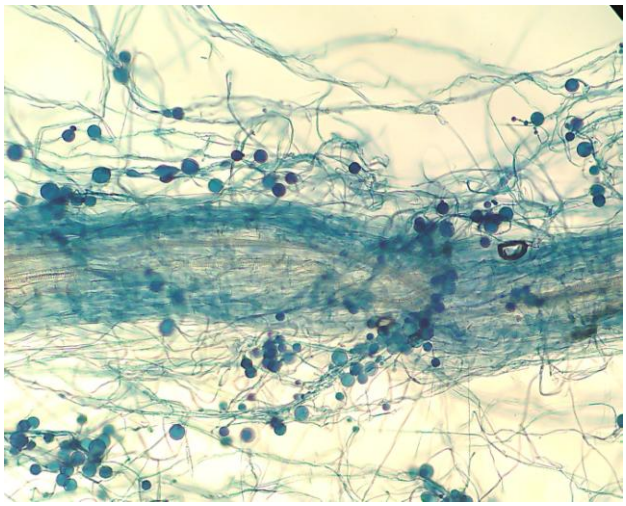

Figure 14: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 200x. Trypan blue (0.01 mg/ml) method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

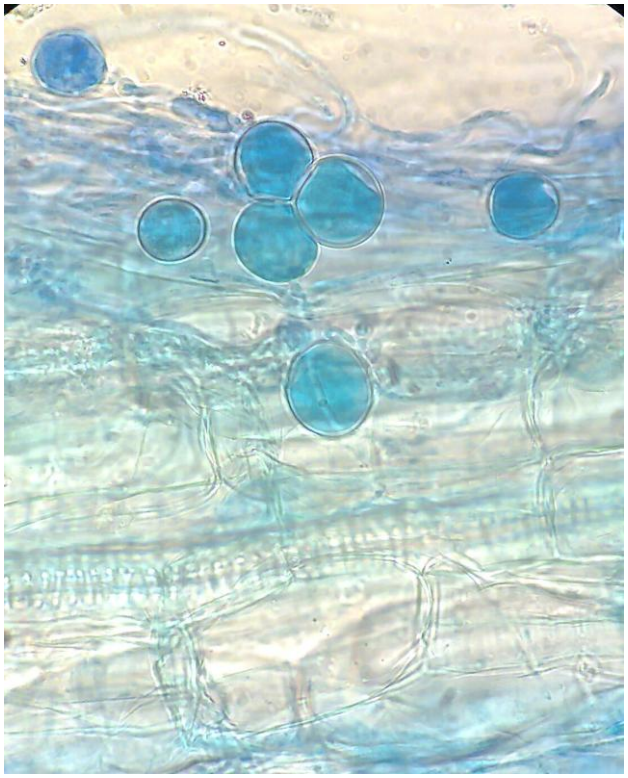

Figure 15: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under light microscope, magnification 1000x. Erythrosine/aniline blue method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

By staining spores in the root structure we obtained optimal results with the erythrosine and aniline blue stain, since it allows visualization of both root tissue and cells (Figure 15). Other methods used were staining with janus green/methyl green/natural red, methyl green/aniline blue, naphtol blue black, fuchsin acid/trypan blue, cotton blue (Figures 14-23).

In conclusion, different staining methods allow the identification of the fungal mycelium and spores in the root environment, and partly the quantification of root colonization. We used combinations of these staining methods to identify mutants which are impaired in the *P. indica*/Arabidopsis interaction and to identify plant mutants which fail to host the fungus. Besides basic research to understand the molecular mechanisms underlying root colonization and propagation of the mycelium, the comparative analysis of the staining methods may also help to analyze colonization of the roots from agriculturally important plant species.

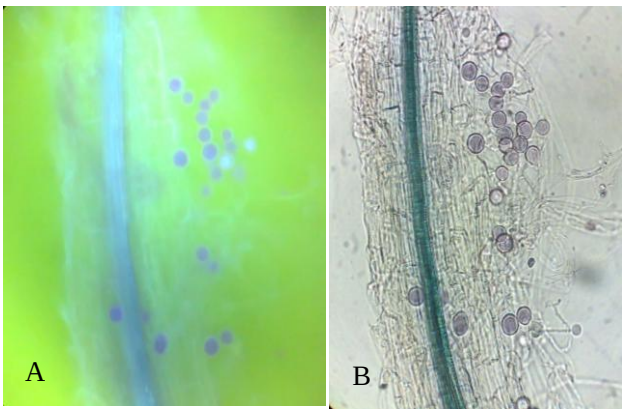

Figure 16: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the fluorescent channel at 450-490 nm (A) and under the light microscope (B), magnification 200x. Janus green/methyl green/natural red staining method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

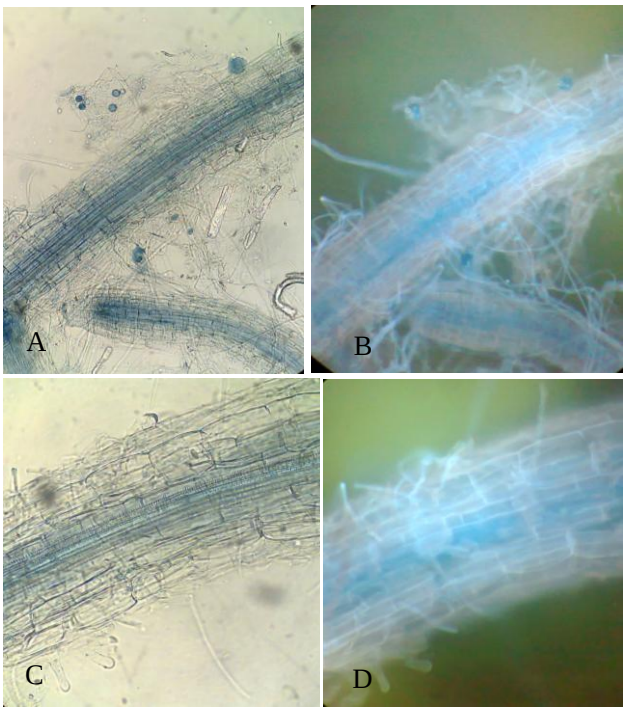

Figure 17: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope (A and C) and under the fluorescent channel at 450-490 nm (B and D), magnification 200x (A and B), 400x (C and D). Methyl green/aniline blue staining method. 15 days after interaction of *P. indica* with *A. thaliana* on PNM medium.

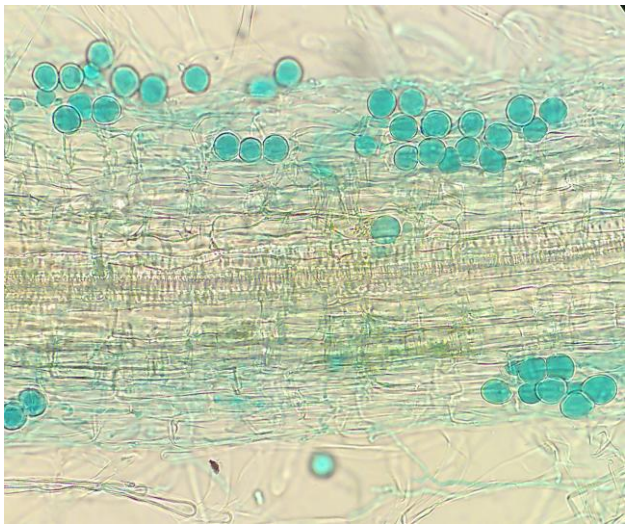

Figure 18: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 400x. Naphtol blue black method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

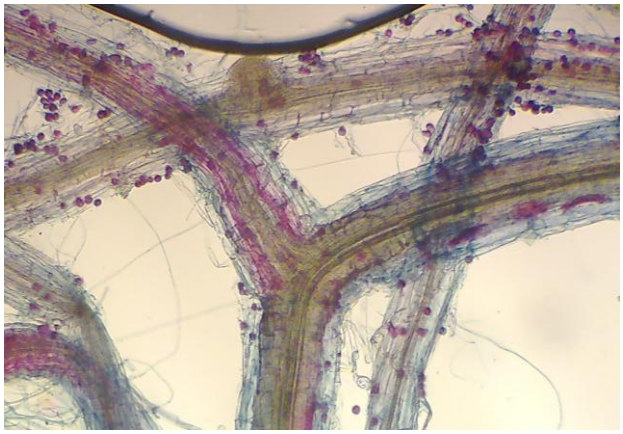

Figure 19: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 100x. Fuchsin acid/trypan blue method. 15 days after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

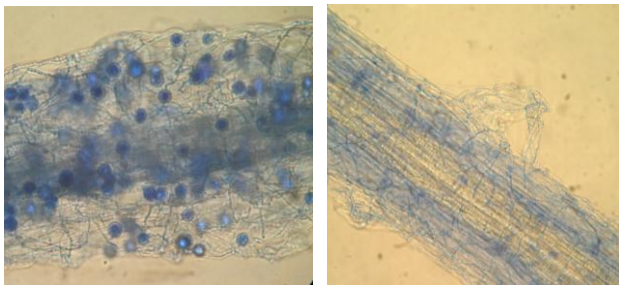

Figure 20: *Arabidopsis thaliana* root, hyphae, and chlamydospores of *P. indica* under the light microscope, magnification 400x. Cotton blue staining method. 2 weeks after co-cultivation of *P. indica* with *A. thaliana* on PNM medium.

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The Methods

Growth conditions for *Piriformospora indica* and Arabidopsis

Piriformospora indica was cultured as described previously (Verma et al. 1998; Peśkan-Berghöfer et al. 2004) in Petri dishes on a modified Kaefer's medium (KM). The plates were kept at room condition for 2 weeks. For co-cultivation experiments with Arabidopsis, *P. indica* spore solutions (1×10^6 spore/ml) were prepared from the fungus grown on KM plates. Root inoculation by *P. indica* was preformed by adding 5 µl of spore solution (1×10^6 spore/ml) to each seed on different media including MS (Murashige and Skoog) and PNM.

Life microscopy

Step 1 Plant 10 seeds of *A. thaliana* and 5 µl of *P. indica* spores ($1x10^6$ s/ml) on a PNM or MS plate
Step 2 Keep at 4°C for 2 days before transfer to light (24 hour light and 23°C) for 10 days
Step 3 Observe the roots by light or fluorescent microscopy directly in the plates.

Staining with fuchsin acid

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into fuchsin acid solution for 5 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with erythrosine/safranin

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into erythrosine solution for 1 min.
Step 3 Stain with safranin solution for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with fuchsin acid and cotton blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into fuchsin acid solution for 2 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Stain with cotton blue solution for 1 min.
Step 5 Washing the samples in distilled water for 1 min.
Step 6 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 7 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with janus green/methyl green/natural red

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into janus green and methyl green mixture solution (1:1) for 2 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Stain with natural red solution for 1 min.
Step 5 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 6 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with erythrosine and aniline blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample into erythrosine solution for 2 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Stain with aniline blue solution for 1 min.
Step 5 Washing the samples in distilled water for 1 min.
Step 6 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 7 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with trypan blue (0.0001mg/ml)

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample into trypan blue solution for 1 or 5 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths

Staining with trypan blue (0.01 mg/ml)

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample into trypan blue solution for 1 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths

Staining with naphtol blue black

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Incubate the sample in naphtol blue black solution for 1 min.
Step 3 Wash the samples in distilled water for 1 min.
Step 4 Add 50 µl GL solution on glass slide and cover with glass cover.
Step 5 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with fuchsin acid and trypan blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample in fuchsin acid solution for 10 min.
Step 3 Washing the samples in distilled water for 1 min.
Step 4 Stain with trypan blue (0.0001gm/ml) solution for 3 min.
Step 5 Washing the samples in distilled water for 1 min.
Step 6 Adding 50 µl GL solution on glass slide and cover with glass cover.
Step 7 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with methyl green and aniline blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample in methyl green (0.05%) solution for 10 seconds.
Step 3 Stain with aniline blue solution for 1 min.
Step 4 Washing the samples in distilled water for 1 min.
Step 5 Adding 50 µl GL solution on glass slide and cover with glass cover.
Step 6 Observe the samples by light and fluorescent microscopy using different wavelengths.

Staining with cotton blue

Step 1 Take samples from Arabidopsis root and wash in distilled water.
Step 2 Put the sample in cotton blue solution for 30 seconds.
Step 3 Put on glass slide and cover with glass cover.
Step 4 Observe the samples by light and fluorescent microscopy using different wavelengths.

Media and Solutions

MS Medium

MS-Salt (=Murashige and Skoog-Salt)	4.4 g
Gel-Rite	3 g
MES	0.5 g
Saccharose	13.7 g
H_2O	up to 1 litter

pH 5.7 – 5.8

PNM Medium

KNO_3	5 mM
$MgSO_4$ 1 M	2 mM
$Ca(NO_3)_2$ 1 M	2 mM
$FeSO_4$	0.01 mM
H_3BO_3	70 mM
$MnCl_2$	14 mM
$CuSO_4$	0.5 mM
$ZnSO_4$	1 mM
Na_2MoO_4	0.2 mM
$CoCl_2$	0.01 mM
Gel-Rite	3 g
KH_2PO_4 1 M	2.5 ml
H_2O	up to 1 liter

pH 5.6

Kaefer's medium

$NaNO_3$	7.0 mM
KCl	7.0 mM
$MgSO_4$	2.1 mM
KH_2PO_4	9.2 mM
EDTA	0.02 mM
$ZnSO_4$	0.77 mM
H_3BO_4	0.18 mM
$MnSO_4$	0.02 mM
$CoCl_2$	0.007 mM
$CuSO_4$	0.0065 mM
$FeSO_4$	0.02 mM
ammonium molybdate	0.001 mM
thiamine	0.003 mM
gylcine	0.005 mM
nicotinic acid	0.002 mM
pyridoxine	0.0004 mM
glucose	110 mM
peptone	2 g/l
yeast extract	1 g/l
casein hydrolysate	1 g/l
agar	10 g/l
H_2O	up to 1 liter

pH 6.5

Glycerol lactic acid solution (GL)	
glycerol	10 ml
lactic acid	10 ml
distilled water	10 ml

Trypan blue (0.0001mg/ml) solution	
GL solution	1 ml
trypan blue	0.0001 g

Trypan blue 0.01mg/ml solution	
GL solution	1 ml
trypan blue	0.01 g

Fuchsin acid solution	
GL solution	1 ml
fuchsin acid	0.001 g

Erythrosine solution	
GL solution	1 ml
erythrosine	0.01 g

Aniline blue solution	
GL solution	1 ml
aniline blue	0.005 g

Naphtol blue black solution	
GL solution	1 ml
naphtol blue black	0.0001 g

Safranin solution	
LG solution	1 ml
safranin	0.0001 g

Janus green solution	
GL solution	1 ml
janus green	0.0001 g

Methyl green solution	
GL solution	1 ml
methyl green	0.0001 g

Natural red solution	
GL solution	1 ml
natural red	0.0001 g

Cotton blue solution (Lactophenol cotton blue solution)	
glycerol	50 g
phenol	25 g
L(+)-lactic acid	25 g
cotton blue	50 mg