Method paper:

Protocols for Arabidopsis thaliana and Piriformospora indica co-cultivation – A model system to study plant beneficial traits

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The endophytic root colonising basidiomycete fungus, Piriformospora indica isolated from the Indian Thar desert, belongs to Sebacinales. The fungus interacts with the roots of many plant species including Arabidopsis thaliana and promotes growth, development and seed production and also confers resistance to various biotic and abiotic stress. The fungus can be cultivated axenically on synthetic or complex media without a host. The endophytic interaction of P. indica with the model plant A. thaliana helps to understand the molecular and physiological basis of beneficial interactions between two symbionts. To study this interaction, both partners should be grown together in harmony. Here we describe detailed and optimized protocols for in vitro and in vivo co-cultivation of P. indica and A. thaliana, which can also be used for other plant species.

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Introduction

The endophytic beneficial interaction of P. indica with a large variety of horticulturally and agriculturally important plants belonging to both monocots and dicots including Arabidopsis thaliana ultimately leads to growth promotion, increased biomass production, enhanced seed yield and resistance/tolerance against biotic and abiotic stress (Lee et al. 2011; Camehl et al. 2010, 2011; Sun et al. 2010; Vadassery et al. 2008; Shahollari et al. 2007; Sherameti et al. 2005; Waller et al. 2005; Pham et al. 2004; Varma et al. 1999, 2001). As the fungus can colonize the roots of many plant species, the interaction between the symbiotic partners should be based on general recognition and signalling processes. The complex cellular interaction between root and fungus necessitates continuous recognition and signal exchange between both partners. Once inside the roots, the fungus gets access to nutrients such as sugars, nitrogen, phosphorous, sulphur etc. which allow it to proliferate. Moreover, the fungus significantly enhances root growth thus providing more niches for its growth in roots. The plants benefit from this relationship through increased root and shoot growth, increased nutrient uptake, early flowering, enhanced seed production and tolerance from drought, salinity, root and foliar pathogens. This endophytic beneficial interaction results in reprogramming of the plant transcriptome, proteome and metabolome, in changes of the phytohormone levels and its signalling, nutrient uptake and metabolism, and finally resistance to abiotic and biotic stress. We investigate this beneficial interaction using the model plant Arabidopsis to identify the genes and signalling processes targeted by the fungus in the plant. We have standardized growth and co-cultivation conditions for the two symbionts, which are described here in details. The co-cultivation medium is designed such that both organisms can grow in harmony.

Acknowledgments

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References


Arabidopsis/P. indica co-cultivation protocols, Johnson JM et al.

Protocols for *Arabidopsis thaliana* and *Piriformospora indica* co-cultivation – A model system to study plant beneficial traits

A. *Piriformospora indica*

1. Growth conditions of *P. indica*

*P. indica* (Figure 1) is cultured on Kaefer medium (KM); (Table 1).

- Pour the medium into sterile plates (20 ml per plate) and allow to solidify.
- Make fungal plugs of 5 mm diameter (Figure 1) with a sterile Pasteur pipette or cork borer.
- Transfer fungal plugs to the center of fresh KM plates.
- Incubate at 22-24°C in the darkness for 4 weeks in a temperature-controlled growth chamber.
- Sub-culture the fungus to fresh KM plates every 4 weeks.

![Figure 1: Four-week old *P. indica* on KM medium where fungal plugs are made.](image)

2. Maintaining “root colonization efficiency” of *P. indica*

The fungus loses its “root colonization efficiency” after repeated subculturing on synthetic medium. To maintain this efficiency the fungus must be periodically inoculated to the roots of host plants (*in vitro* or *in soil*) and re-isolated from the internally colonized roots. Co-cultivation of *P. indica* with Arabidopsis is done as described in section C1.

- Cut the colonised roots into small pieces of 1-2 mm after 20 days of co-cultivation (ensure the internal colonization of the fungus inside the roots by microscopy).
- Surface sterilize the roots with sterility (for 100 ml solution: 4 ml laurylsarcosin 20%; 32 ml Klorix; 64 ml sterile H2O) or 0.1% HgCl2 for 1 minute, and then wash 4 times with sterilised H2O (or transfer the root pieces into a series of sterilised H2O).
- Treat the roots with 70% ethanol for 30 seconds and further wash them 3 times with sterilised H2O or transfer the root pieces into a series of sterilised H2O.
- Place the root pieces on KM plates and incubate at 22-24°C in a temperature-controlled growth chamber) in the dark for 8-10 days to see the growth of the fungus. The fungus is then immediately sub-cultured by taking the hyphal tip to fresh KM plates.
B. Arabidopsis thaliana

1. Growth Conditions of Arabidopsis thaliana

- Surface-sterilize *A. thaliana* wild type seeds (ecotype Columbia-0) with sterilium for 8 minutes followed by a series of washing of seeds in sterilized H2O for 7-8 times.
- Place the seeds on Petri dishes with MS medium (pH 5.6 to 5.8) containing 0.3% gelrite (Murashige and Skoog 1962). Normally 12-15 seeds are placed per Petri dish.
- Seal the plates with Parafilm strips.
- Incubate the plates at 4°C for 48 h for cold treatment to ensure uniform germination.
- After cold treatment, incubate the plates for 7 to 10 days (see 1Note) at 22°C under continuous illumination (100 μmol m⁻² s⁻¹).

For co-cultivation experiments 12-15 seeds per Petri dish are grown. Only those seedlings which are equally grown are used for growth promotion assays.

1Note:
This depends on plant growth. We suggest to use 6-8 leaf-stage.

C. Co-cultivation of *A. thaliana* and *P. indica*

1. in vitro co-cultivation

For *in vitro* co-cultivation of the two symbionts, a modified PNM medium (Table 2) is used. PNM medium is optimized in such a way that allows growth of both organisms within the time of experiment. Nine to twelve-day old (4°C for 48 h followed by 7 to 10 days in light) Arabidopsis seedlings and 4 week old *P. indica* plugs are used.

**Method 1:** *A. thaliana* and *P. indica* are transferred to PNM medium at the same time point (Figure 2A).

- Place a sterilised 80-85 mm nylon disks (Sefar Nitex 03-70/33; pore size 65-70 μm; mesh count 81 cm⁻¹; Sefar GmbH, Switzerland; see 2Note) on top of a modified PNM medium in Petri dishes.
- Transfer one or two 9-12-day-old (4°C for 48 h followed by 7-10 days in light) seedlings on the nylon membrane on the top of PNM plates. If 2 seedlings are transferred, they are kept 3 cm apart.
- Make fungal plugs or discs of 5 mm diameter from 4 week old fungal plates with a sterile Pasteur pipette or cork borer.
- Place the plugs or discs without medium about 1 to 1.5 cm away (Figure 3) from the roots (or keep the fungal plug between the roots of two plants; Figure 4). We use one or two seedlings per Petri dish.
- Use agar plugs from an un-inoculated KM plate as the control.
- Seal the plates with Parafilm strips.
- Incubate the plates at 22°C under continuous illumination from the side (80 μmol m⁻² s⁻¹) for 6-14 days (Figure 5A, B).

2Note:
Cut the nylon membrane, soak in ddH₂O, heat in the microwave for about 3 minutes, spread without folding in aluminium sheet and sterilize.
Figure 2: Scheme describing co-cultivation steps between *A. thaliana* seedlings and *P. indica*.

Figure 3: Growth promotion of *A. thaliana* seedlings on PNM media after 10 days of co-cultivation. **Left:** mock treatment; **Right:** *P. indica* treated seedling.
Figure 4: Growth promotion of *A. thaliana* and Chinese cabbage on PNM media after 10 days of co-cultivation. 
*Left:* control (1 or 2 KM plugs without fungus as mock treatment); *Right:* + *P. indica* treatment (1 or 2 fungal plugs).

Figure 5: Continuous illumination (80 μmol m⁻²·s⁻¹) from the side (*A, B*) and from the top (*C*). 
*A.* Shelves for the illumination. The upper and lower plates do not contain seedlings; *B.* Closer view of the plates; *C.* Illumination from the top.
**Method 2:** *A. thaliana* and *P. indica* are transferred to PNM medium at different time points (Figure 2B).

- Place the sterilised 80-85 mm nylon disks (as described above) on top of a modified PNM medium in Petri dishes.
- Make fungal plugs or discs of 5 mm diameter from 4 week old fungal KM plates (Figure 1) with a sterile Pasteur pipette or cork borer.
- Place the plugs or discs without medium on the center of the PNM plate.
- Seal the plates with Parafilm strips.
- Incubate the plates for 7 days in a temperature-controlled Biotrons at 22°C under 12 h/12 h light/dark cycle and a light intensity of 80 μmol m⁻² s⁻¹ from the top. The mycelia of the fungus grow about 4 cm in diameter by this time.
- Transfer 12-day-old (4°C for 48 h followed by 10 days in light) seedlings on each plate. Four (Figure 6A) to eight (Figure 6B) seedlings per Petri dish are used.
- Seal the plates with Parafilm strips.
- Incubate the plates at 22°C under continuous illumination from the top (80 μmol m⁻² s⁻¹) for 6-14 days (Figure 5C).

![Figure 6: Growth promotion of *A. thaliana* seedlings (Col-0) on PNM media under continuous illumination (from the top, 80 μmol m⁻² s⁻¹) after 10 days of co-cultivation. A. four seedlings per plate; B. eight seedlings per plate.](image)
The plates are also incubated at 22°C under low light (30 µmol m⁻²s⁻¹), normal light (80 µmol m⁻²s⁻¹), high light (250 µmol m⁻²s⁻¹), photosystem I or photosystem II light (Fey et al. 2005), short day (8 h light/16 h dark), long day (16 h light/8 h dark, Figure 7) and day neutral (12 h light/12 h dark) conditions with illumination from the top. In all these conditions A. thaliana and tobacco seedlings showed growth promotion after co-cultivation with P. indica.

Fresh weights are determined for shoots and roots at 6, 10 and 14 day after co-cultivation. For each treatment 24 to 32 seedlings per line are used and at least 5-7 independent experiments are performed. The results of the experiments are reproducible throughout the year.

Growth promotion after in vitro co-cultivation with P. indica is well established for different plants such as A. thaliana (Figure 3, 4, 6, 7A), Chinese cabbage (Figure 4), Nicotiana tabaccum (Figure 7B, 8B), Rapeseed and Timothy grass. Growth promotion is also established using split Petri dishes (Figure 8) which clearly demonstrates that volatiles including gases do not play a role in growth promotion in this system.

Figure 7: Growth promotion of A. thaliana (A) and tobacco (B) seedlings on PNM media under continuous light and long day conditions (16 h light/8 h dark) after 10 days of co-cultivation. Illumination is from the top (80 µmol m⁻²s⁻¹).
Figure 8: Split plate experiment with mock treatment and *P. indica*. A. *A. thaliana* after 10 days of co-cultivation; B. tobacco seedlings after 10 days of co-cultivation.

2. Co-cultivation of *A. thaliana* and *P. indica* in soil and vermiculite

Garden soil, garden soil-vermiculite mix (9:1) and vermiculite are used for the *in vivo* co-cultivation of *A. thaliana* and *P. indica*.

**Method 1: In soil or soil-vermiculite mix (9:1)**

- Seedlings are co-cultivated for two weeks with/without *P. indica* on PNM medium on Petri dishes as described above (see C1).
- Before transferring to soil the roots of the seedlings co-cultivated with the fungus are examined under the microscope (see below C3) to ensure that hyphae and spores are developed within the roots.
- Soil, or soil-vermiculite mix (9:1) is sterilised at 121°C for 30 min after adding water to its field capacity level.
- Fill the sterilised garden soil or garden soil-vermiculite mix in plastic pots with small holes for drainage.
- Transfer colonised seedlings and uncolonised control seedlings to pots with soil or soil-vermiculite mix (9:1).
- Keep pots with colonised and control seedlings in separate trays.
- Transfer them to a temperature-controlled growth chamber at 22°C under short day conditions (8 h light/16 h dark) until flowering followed by long day conditions (16 h light/8 h dark) after keeping tripod and araccon tubes (Figure 9A). Light intensity is 80 μmol m⁻²s⁻¹.
We grow *P. indica*-colonized and control pots under the same growth conditions. Irrigate the plants weekly with the same amount of water to the field capacity of the soil.

The sizes of the plants are continuously monitored 2, 4, 6 and 8 weeks by examining the number and area of leaves, flowering, number of pods/plant and number of seeds/pods. Seeds are harvested and quantified as gram seed per plant. The fitness of the seedlings is determined by measuring various chlorophyll fluorescence parameters viz. $F_v/F_m$ (maximum quantum yield of photosystem II), $(F_m'-F_t)/F_m'$ (quantum yield of photosystem II), $q_P$ (proportion of open photosystem II) and NPQ (non-photochemical quenching) using a Fluorocam (Oelmüller and Briggs 1990; Maxwell and Johnson 2000).

**Figure 9**: Growth promotion of *A. thaliana*, *Nicotiana tabaccum* and Chinese cabbage in soil after co-cultivation. **A.** *A. thaliana* after 4 and 7 weeks of co-cultivation; **B.** *Nicotiana tabaccum* after 5 weeks of co-cultivation. **C.** Chinese cabbage after 4 weeks of co-cultivation.

In our laboratory this method is successfully established for *in vivo* co-cultivation of *P. indica* with *Arabidopsis thaliana* (Figure 9A), *Nicotiana tabaccum* (Figure 9B) and Chinese cabbage (Figure 9C).
**Method 2: In vermiculite**

- Sterilise vermiculite (we use vermiculite approximately 2 mm diameter) in polypropylene bag.
- Fill the sterilised vermiculite to plastic pots (65 x 65 x 65 mm).
- Before transferring to vermiculite, the roots of the seedlings co-cultivated with the fungus are examined under the microscope (see below C3) to ensure that hyphae and spores are developed within the roots.
- Transfer colonised seedlings and uncolonised control seedlings to pots with vermiculite.
- Add 70 ml of sterile PNM broth to each pot.
- Keep pots with colonised and control seedlings in separate trays.
- Transfer them to a temperature-controlled growth chamber at 22°C under short day conditions (8 h light/16 h dark). Light intensity is 80 µmol m⁻²s⁻¹. We grow *P. indica*-colonized and control pots under the same growth conditions.
- Add 70 ml of sterile PNM broth per pot with colonised seedlings and uncolonised control seedlings at 10 days interval.

Growth promotion in *A. thaliana* is also established for the long-run co-cultivation experiments in vermiculite (Figure 10).

**Figure 10**: Growth promotion of *A. thaliana* in vermiculite after 6 weeks of co-cultivation.
3. Colonization of fungus: staining of fungal hyphae and spores

To monitor root colonization (Figures 11, 12), 10 small roots samples from seedlings co-cultivated with *P. indica* are used.

- Wash the colonized and uncolonized roots thoroughly in running deionized water.
- Cut roots into 1 cm long pieces and treat them with 10% KOH.
- Incubate overnight at room temperature in sterile bench.
- Wash root pieces 5 times with sterilized H$_2$O.
- Incubate roots with 1% HCl for 3 min.
- Mount roots in 0.05% trypan blue in lactophenol.
- Examine roots under the microscope.

![Figure 11](image1.png)

**Figure 11**: Root colonization and sporulation of *P. indica* in Arabidopsis. A. Mycelia and hyphae in primary and secondary roots; B. Sporulation in root especially in elongation, meristematic and root tip region; C. Young developing chlamydospores inside the root cells; D. Mature piriform shaped chlamydospores in root.

![Figure 12](image2.png)

**Figure 12**: Fluorescent image of root colonization: Extra-, inter- and intra-cellular sporulation (See Peškan-Berghöfer et al. 2004).
Table 1: Kaefer Medium (KM; Hill and Käfer 2001) composition for *P. indica*.

<table>
<thead>
<tr>
<th>Components for 1 liter</th>
<th>Amounts</th>
</tr>
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<tbody>
<tr>
<td>D-glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>peptone/trypton</td>
<td>2.0 g</td>
</tr>
<tr>
<td>yeast extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>casein hydrolysate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>macronutrient mix (20x)*</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>micronutrient mix (100x)**</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Fe-EDTA (100x)**</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>agar</td>
<td>10.0 g</td>
</tr>
</tbody>
</table>

Adjust pH to 6.5 with 10N KOH
Autoclave at 121°C for 20 min
Add 1 ml filter sterilised vitamin mix (100x)** before pouring the media (temperature 45 to 50°C) to Petri dishes.

1* macronutrient mix (20x)
For 1 liter: 12 g NaNO_3; 10.4 g KCl; 10.4 g MgSO_4·7H_2O; 30.4 g KH_2PO_4. All components are dissolved in sterile H_2O and then stored at 4°C.

2*micronutrient mix (100x)
For 1 liter: 2.2 g ZnSO_4·7H_2O; 1.1 g H_3BO_3; 0.5 g MnSO_4·4H_2O; 0.16 g CoCl_2·5H_2O; 0.16 g CuSO_4·5H_2O; 0.11 g (NH_4)_6Mo_7O_24·4H_2O (ammonium molybdate tetrahydrate). All components are dissolved in sterile H_2O and then stored at 4°C.

3*Fe-EDTA (100x)
For 50 ml: 0.77 g Na_2EDTA; 0.556 g FeSO_4·7H_2O. All components are dissolved in sterile H_2O, heat to boil and stir for 30 min while cooling and then store at 4°C.

4*vitamin mix (100x)
For 100 ml: 10 g thiamin; 0.04 g glycine; 0.01 g nicotinic acid; 0.01 g pyridoxine. All components are dissolved in sterile H_2O, then filter-sterilized and stored as aliquots of 1 ml at -20°C.

Table 2: Modified PNM medium composition used for co-cultivation experiments (*A. thaliana* and *P. indica*).

<table>
<thead>
<tr>
<th>Components</th>
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<tbody>
<tr>
<td>5 mM KNO_3</td>
</tr>
<tr>
<td>2 mM MgSO_4·7H_2O</td>
</tr>
<tr>
<td>2 mM Ca(NO_3)_2</td>
</tr>
<tr>
<td>2.5 ml Fe-EDTA*/1 liter</td>
</tr>
<tr>
<td>10.0 ml micronutrient-mix*/1 liter</td>
</tr>
<tr>
<td>10.0 g agar (Serva)/1 liter</td>
</tr>
</tbody>
</table>

Sterilize at 121°C for 20 min
Adjust pH (under sterile conditions) to 5.6 by adding 2.5 ml filter-sterilized 1 M KH_2PO_4.

1* Fe-EDTA
Add 2.5 g FeSO_4·7H_2O in 400 ml sterile H_2O
Add 3.36 g Na_2EDTA
Heat to boil in the microwave
Stir for about 30 minutes while cooling
Bring to the final volume of 450 ml.

2* Micronutrient-mix
70 mM H_3BO_3; 14 mM MnCl_2·4H_2O; 0.5 mM CuSO_4·5H_2O; 1 mM ZnSO_4·7H_2O; 0.2 mM Na_2MoO_4·2H_2O; 10 mM NaCl; 0.01 mM CoCl_2·6H_2O.

Pour approximately 20 ml of PNM medium to each plate.