

Growth Assay and Detection of TRP and Indole Derivatives in *Piriformospora indica* Culture Supernatant by LC-MS/MS

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[Abstract] The mutualistic root endophyte *Piriformospora indica* colonizes a wide range of plants and the colonization of root cells by this fungus is very often associated with beneficial effects to its host, such as growth promotion and increased biotic and abiotic stress tolerance. These traits may be based on general mechanisms and signaling pathways common to many different plant species. One such mechanism could be the recruitment of phytohormone pathways by *P. indica*. It is known, that many mutualistic microorganisms are able to synthesize and secrete phytohormones during the interaction with their host plants. This protocol has been successfully utilized to analyze tryptophan (TRP)-dependent biosynthesis of indole-3-acetic acid (IAA) and its indole derivatives by *P. indica* as well as their influence on the growth of this fungus (Hilbert *et al.*, 2012).

Materials and Reagents

1. Indole derivative:
 - a. TRP (Sigma-Aldrich, catalog number: T0254-500g)
 - b. IAD (indole-3-acetaldehyde) (Sigma-Aldrich, catalog number: I1000-100mg)
 - c. IAA (Sigma-Aldrich, catalog number: I5148-2g)
2. Neubauer improved counting chamber (Marienfeld-Superior)
3. Sterile scalpel
4. Sterile drigalski spatula
5. Miracloth filter 15 cm x 15 cm (Merck KGaA, catalog number: 475855)
6. Ruler
7. 0.3 ml polypropylene snap ring microvials
8. 0.9% NaCl
9. 0.002% (v/v) Tween water 20
10. 90% methanol (HPLC grade)
11. Acetonitrile (HPLC grade)
12. Acetic acid (HPLC grade)

13. Chlamydo spores solution
14. Microelements ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, H_3BO_3 , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, KI , $\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)
15. Glucose
16. Peptone
17. Yeast extract
18. Casamine acids
19. Agar
20. Complete medium (CM medium) (see Recipes)
21. Buffer A (see Recipes)
22. Buffer B (see Recipes)

Equipment

1. 1.5 ml Eppendorf tubes
2. 50 ml Falcon tube
3. Petri dishes (12 mm diameter)
4. Incubator (e.g. B. Braun Biotech International, Certomat BS-1)
5. Biofuge Primo R (Heraeus, Germany) (Rotor, catalog number: 7590)
6. Phenomenex Luna 250 x 4.6 mm C_{18} RP-HPLC column (Phenomenex)
7. Phenomenex Luna 10 x 4.6 mm C_{18} RP-HPLC guard column (Phenomenex)
8. ICS3000 HPLC system (Dionex)
9. QTrap 3200 triple quadrupole mass spectrometer (Applied Biosystems SCIEX)
10. Polypropylene snap ring microvial

Procedure

A. Growth Assay

1. Collect spores from 3 to 4 weeks old *Piriformospora indica* plate cultures (Figure 1).
 - a. Pour approximately 5 ml sterile 0.002% Tween water 20 on 3-4 weeks old *P. indica* plate under sterile condition at room temperature (RT).
 - b. Scratch plate with sterile Drigalski spatula and/or scalpel and mix.
 - c. Pour spore solution through miracloth filter and collect flow through in 50 ml Falcon tube.
 - d. Centrifuge for 7 min at 3,500 rpm discard supernatant.
 - e. Wash pellet with 5-10 ml 0.002% Tween water 20.
 - f. Centrifuge for 7 min at 3,500 rpm, discard supernatant.
 - g. Wash pellet with 5-10 ml 0.002% Tween water 20.

- h. Centrifuge for 7 min at 3,500 rpm, discard supernatant.
- i. Resuspend spore pellet in 10 ml 0.002% Tween water 20, count spores with counting chamber (e.g. Neubauer improved) and dilute to requested spore concentration (e.g. 500,000 spores/ml).



Figure 1. Four-week-old *P. Indica* agar plate

- a. Pour approximately 5 ml sterile 0.002% Tween water 20 on 3-4 weeks old *P. indica* plate under sterile condition at room temperature (RT).
 - b. Scratch plate with sterile Drigalski spatula and/or scalpel and mix.
 - c. Pour spore solution through miracloth filter and collect flow through in 50 ml Falcon tube.
 - d. Centrifuge for 7 min at 3,500 rpm discard supernatant.
 - e. Wash pellet with 5-10 ml 0.002% Tween water 20.
 - f. Centrifuge for 7 min at 3,500 rpm, discard supernatant.
 - g. Wash pellet with 5-10 ml 0.002% Tween water 20.
 - h. Centrifuge for 7 min at 3,500 rpm, discard supernatant.
 - i. Resuspend spore pellet in 10 ml 0.002% Tween water 20, count spores with counting chamber (e.g. Neubauer improved) and dilute to requested spore concentration (e.g. 500,000 spores/ml).
2. Inoculate 50 ml CM medium (Pham *et al.*, 2004) supplemented with appropriate indole derivative (e.g. 2.5 mM TRP; 250 μ M IAD or 1, 10, 100 μ M IAA) with 400 μ l chlamydo spores solution (500,000 spores/ml) and cultivate for 7 days at 28 $^{\circ}$ C in the dark (alternatively wrap flasks with aluminium foil). Use mock inoculated flask as a negative control.

3. Separate supernatant from mycelium using miracloth filter (check the mass of each filter before).
 4. Wash mycelium with 0.9% NaCl and let the whole miracloth filter with fungal biomass dry overnight in oven (85 °C).
 5. Measure the dry fungal biomass (= mass of miracloth filter with dried fungal biomass – mass of empty miracloth filter).
 6. Place 5 mm agar plugs from the 3 to 4 weeks old *Piriformospora indica* plate culture in the middle of a CM agar plate supplemented with the appropriate indole derivative (2.5 mM TRP, 250 µM IAD or 1, 10, 100 µM IAA). Use CM agar plate as control.
 7. Use ruler to measure colony diameter after 14 days of cultivation at 28 °C in the dark.
- B. Detection of tryptophan and indole derivatives in culture supernatant by LC-MS/MS
1. Use a 15 µl aliquot of *P. indica* culture supernatant obtained from section I point 2 of the procedure.
 2. Add 1 ml of 90% methanol.
 3. Vortex briefly and dilute an aliquot 1:10 in 90% methanol into a 0.3 ml polypropylene snap ring microvial.
 4. Analyze 10 µl of the 1:10 dilution by LC-MS/MS.
 5. IAA and ILA are separated on an ICS3000 HPLC system equipped with a Phenomenex Luna 250 x 4.6 mm C₁₈ RP-HPLC column with the following gradient

a.	0 to 5 min	hold at 80% buffer A, 20% buffer B
b.	5 to 26 min	hold at 54% buffer A, 46% buffer B
c.	26 to 27 min	ramp to 10% buffer A, 90% buffer B
d.	27 to 32 min	hold at 10% buffer A, 90% buffer B
e.	32 to 34 min	ramp to 80% buffer A, 20% buffer B
f.	34 to 45 min	equilibrate with 80% buffer A, 20% buffer B
 6. Subject the HPLC eluate to coupled electrospray ionization in the negative ionization mode and to subsequent tandem MS analysis on the QTrap 3200 mass spectrometer with the following settings:

a.	dwel time	75 ms
b.	declustering potential (DP)	-22 V (IAA), -30 V (ILA)
c.	entrance potential (EP)	-7 V (IAA), -55 V (ILA)
d.	collision cell entrance potential (CEP)	-14 V (IAA), -15 V (ILA)
e.	collision energy (CE)	-15 V (IAA), -18 V (ILA)
f.	collision cell exit potential (CXP)	0 V (IAA), -4 V (ILA)
 7. Quantitate IAA using the m/z transitions 174/130 and 174/128.
 8. Quantitate ILA using the m/z transitions 204/128 and 204/158.

9. Employ commercially available authentic substances as references.

Recipes

1. Complete medium (CM medium; Pham *et al.*, 2004)
 - a. CM medium (1 L)
 - 50 ml 20x salt solution
 - 20 g glucose
 - 2 g peptone
 - 1 g yeast extract
 - 1 g casamine acids
 - 1 ml microelements
 - 15 g agar
 - b. 20x salt solution
 - 120 g NaNO_3
 - 10.4 g KCl
 - 10.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 - 30.4 g KH_2PO_4
 - c. Microelements
 - 6 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
 - 1.5 g H_3BO_3
 - 2.65 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
 - 750 mg KI
 - 2.4 mg $\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$
 - 130 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
2. Buffer A
 - 0.75% acetic acid, pH 2.55 (adjust with acetic acid, if necessary)
3. Buffer B
 - Acetonitrile/0.75% acetic acid, pH 2.55 (adjust with acetic acid, if necessary)

Acknowledgments

This protocol is adapted from Hilbert *et al.* (2012).

References

1. Hilbert, M., Voll, L. M., Ding, Y., Hofmann, J., Sharma, M. and Zuccaro, A. (2012). Hilbert, M., Voll, L. M., Ding, Y., Hofmann, J., Sharma, M. and Zuccaro, A. (2012). [Indole derivative production by the root endophyte *Piriformospora indica* is not required for growth promotion but for biotrophic colonization of barley roots.](#) *New Phytol* 196(2): 520-534.
2. Pham, G. H., Singh, A., Malla, R., Kumari, R., Prasad, R., Sachdev, M., Luis, P., Kaldorf, M., Peskan, T., Herrmann, S. (2004). Interaction of *P. indica* with other microorganisms and plants. In: Varma A, Abbott L, Werner D, Hampp R, eds. *Plant Surface Microbiol* Heidelberg, Germany: Springer, 237-265.