Trichoderma harzianum Root Colonization in *Arabidopsis*

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[Abstract] *Trichoderma* is a soil-borne fungal genus that includes species with a significant impact on agriculture and industrial processes. In this article we show a detailed protocol of *Trichoderma harzianum* (*T. harzianum*) root invasion procedure described by Alonso-Ramírez *et al.* (2014). Some *Trichoderma* strains exert beneficial effects in plants through root colonization. They promote growth and development, modify root architecture, facilitate efficient nutrient use, or stimulate defenses against pathogens, although little is known about how this interaction takes place. For this purpose, *Trichoderma-Arabidopsis* hydroponic cultures were grown inside Phytatray II boxes, using mycelia obtained from spores of *T. harzianum* and *Arabidopsis thaliana* (*A. thaliana*) seedlings. In this way changes in root architecture, such as callose deposition, promoted by the fungus can be analyzed.

Materials and Reagents

1. *Arabidopsis thaliana* Col-0 ecotype seeds from *Arabidopsis* Information Service Collection (www.arabidopsis.info)
2. *Trichoderma harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) [referred to as T34 along the paper is the strain used in this work. *T. harzianum* T34 is grown on Potato Dextrose Agar (PDA) and spores are maintained at -80 °C in a 30% glycerol solution]
3. Murashige & Skoog medium (MS), including B5 vitamins (Duchefa Biochemia, catalog number: M0255.0050)
4. Sucrose (Applichem Panreac, catalog number: 141621.1211)
5. 85% potassium hydroxide pellets (KOH) (Applichem Panreac, catalog number: 141515.1210)
6. 0.15% agarose (Conda Pronadisa, catalog number: 8016)
7. 0.39% potato dextrose agar (PDA) (Sigma-Aldrich, catalog number: P2182)
8. 0.24% potato dextrose broth (PDB) (Sigma-Aldrich, catalog number: P6685)
9. Potato dextrose agar (PDA) (Conda, catalog number: 1022.00)
10. Potato dextrose broth (PDB) (Difco, catalog number: 254920)

11. Glass wool washed QP (Panreac, catalog number: 211376.1208)
12. Resma filter paper (420 x 500 mm) (Auxilab S.L, catalog number: 80250452)
13. Liquid nitrogen (Air Liquide)
14. Ethanol (Panreac, catalog number: 161086)
15. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
16. Sodium hypochlorite
17. Sterilization solution (see Recipes)
18. MS Medium (see Recipes)
19. Washing solution (see Recipes)

Equipment

1. Sterile distilled water purification system (EMD Millipore, model: ELIX35)
2. Brand cotton roving (Sigma-Aldrich, catalog number: BR28205)
3. Sterile stainless steel screen (Alunet, catalog number: 174562)
4. Surgical Micropore tape (3M, catalog number: 1530-0)
5. 1.5 ml microtubes (Deltalab, catalog number: 200400P)
6. Cold chamber
7. Phytatray II boxes [114 mm, 86 mm, 102 mm (W x D x H)] (Sigma-Aldrich, catalog number: P5929)
8. Laminar flow cabinet (Telstar, model: AV-100)
9. Plant Growth Chamber AGP-1400-HR (Radiber SA)
10. Shaker Certomat® R (B. Braun, model: 986302/4)
11. Petri dishes (90 x 14 mm) (Deltalab, catalog number: 200209)
12. Surgeon carbon steel (surgical blade sterile) (Jai Surgicals, catalog number: 0835147)
13. 1.5 ml microtubes with glass wool (homemade)
14. 15 ml Tubes (Deltalab, catalog number: 401402)
15. Thoma cell counting chamber (BRAND, catalog number: 7180 05)
16. Coverslip EUROTUBO (22 x 22 mm) (Deltalab, catalog number: D102222)
17. Optical microscope (Leica Microsystems AG, model: DC300F; catalog number: 10447115)
18. Erlenmeyer flask (Thermo Fischer Scientific, catalog number: 11972233)
19. Kühner shaker (Thermo Fisher Scientific, model: ISF-1-W)
20. Vacuum/pressure pump PALL (Life Sciences, model: DOA-P730-BN)
21. Safety glass (vacuum flask, 10 ml pipette and gums) (homemade)
22. X5 Graduated pipette (type1, class B, ISO 835) (Thermo Fisher Scientific, catalog number: 11912178)
23. Medical grade silicone tubing (1x 1.5, internal diameter x external diameter) (Deltalab, catalog number: 3500115)
24. Rubber plugs (VWR International, catalog number: 217-9463)

25. Forceps (stainless, L= 105 mm) (Thermo Fisher Scientific, catalog number: 10458242)
26. Vacuum flask pirex (1,000 ml) (Thermo Fisher Scientific, catalog number: 12693182)
27. Magnetic filter funnel (VWR International, catalog number: 516-7590)
28. Scissors stainless 170 mm (Thermo Fisher Scientific, catalog number: 12693182)
29. Lyophilizer Virtis Advantage (SP Scientific)

Procedure

1. *Arabidopsis thaliana* seeds have to be washed and sterilized superficially before sowing. For this purpose, approximately 200 seeds are placed in a 1.5 ml microtube, and 1 ml of washing solution is added. Shake seeds for 30 min at room temperature in a shaker (Kühner shaker). Remove washing solution. Add 1 ml of sterilization solution. Shake again for 10 min at room temperature. Remove sterilization solution and wash seeds by tube inverting four times with sterile distilled water. Finally, seeds are placed in 1.5 ml microtubes and kept in stratification at 4 °C in the cold chamber for 3 days in order to break seed dormancy and synchronize the germination.
2. Place *Arabidopsis* seeds inside Phytatray II boxes with 50 ml of liquid MS media on a sterile gauze sheet over a sterile stainless steel screen (Figures 1 and 2) in a laminar flow cabinet, using an aqueous agarose solution (0.15%) to sow seeds individually.

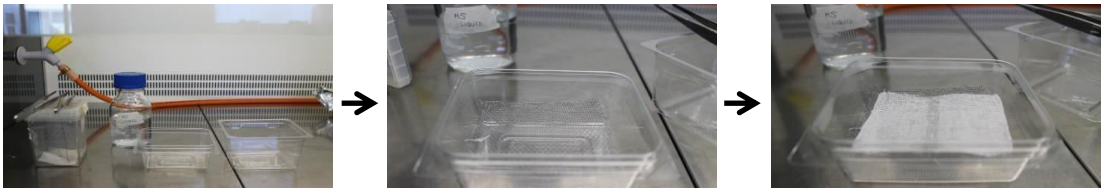

Figure 1. Set up of a sterile gauze sheet over a sterile stainless steel screen in Phytatray II boxes

Figure 2. Pictures showing the hydroponic cultures in the Phytatray II boxes

3. For the propagation of the *T. harzianum* strain, incubate a fungal plug of 5mm of diameter, grown and sporulated on a petri dish with PDA, at least 7 days at room temperature, to achieve full coverage of the plate surface with spores.
4. To harvest the spores, add 5 ml of sterile distilled water to the plate. Filter this suspension in 1.5 ml microtubes with glass wool, placed on 15 ml tubes, in order to remove traces of mycelium (Figure 3 and 4). Spores are maintained at 4 °C no more than 1 week. Finally, determine spore concentration in a Thoma cell counting chamber by pipetting 100 µl of a 1:100 dilution, using the following formula:

$$\frac{Spores}{ml} = \bar{X} \;\; 25 \;\; 10^4 \;\; dilution\ factor$$

$\overline{X}$: It is the mean of the spores counted in four different quadrants.

Finally, keep spores at 4 °C until use.

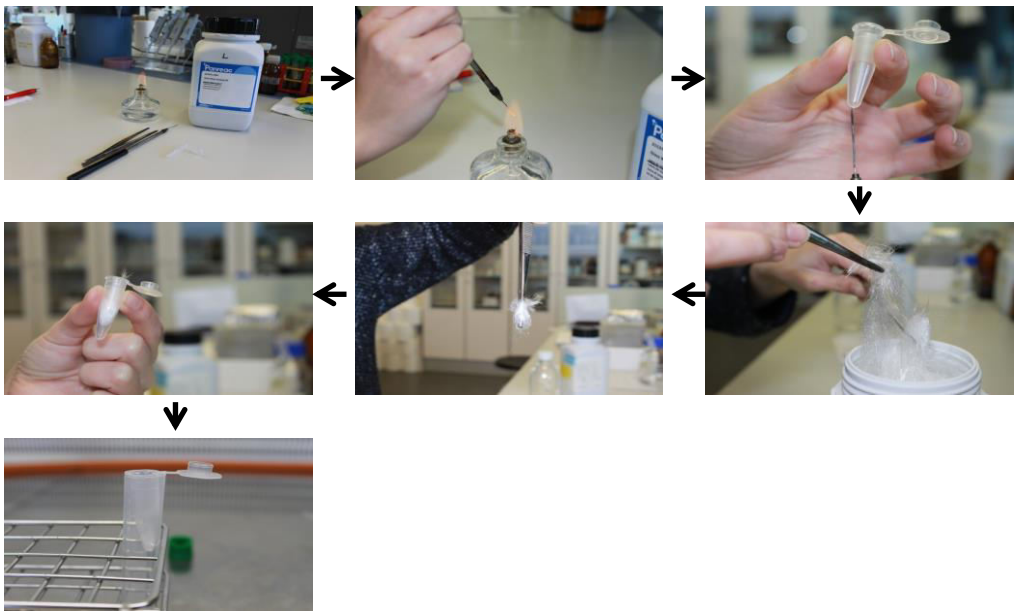

Figure 3. Set up for the glass wool in a microtube placed on a 15 ml tube

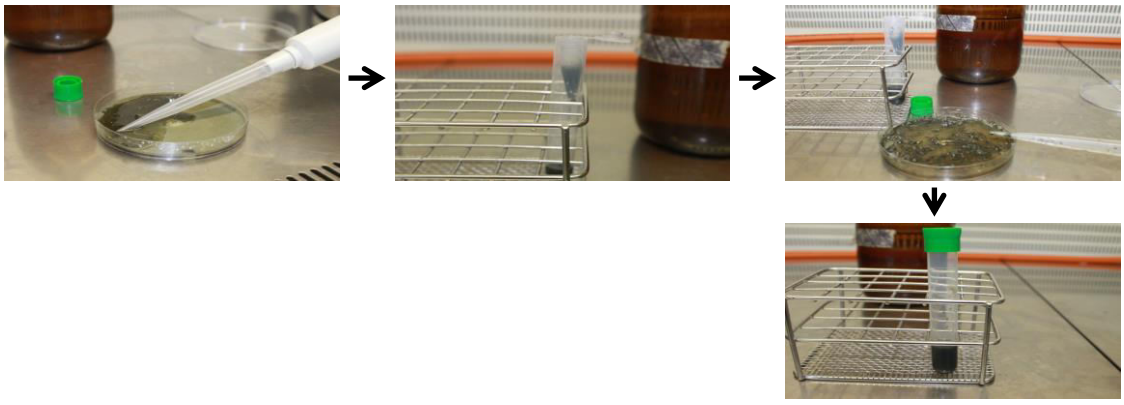

Figure 4. Harvest of *T. harzianum* spores

5. Use spores of *T. harzianum* (10^7 spores) to inoculate 250-ml flasks containing 100 ml of PDB. Maintain cultures at 25 °C and 200 rpm for 48 h. Harvest mycelia (approximately 250 mg) by filtration (filtration system: vacuum/pressure pump PALL, safety glass, rubber plugs, vacuum flask pirex and magnetic filter funnel). Wash the mycelia through the magnetic funnel with 100 ml of sterile water (Figure 5). Use the washed mycelia to inoculate the Phytatray boxes containing *Arabidopsis* plants grown for 21 days. Inoculate mycelia by lifting the stainless steel screen where the *Arabidopsis* seedlings are, with the help of sterile forceps (Figure 6). Place mycelia on MS media and shake (shaker Certomat® R) in order to promote dispersion.

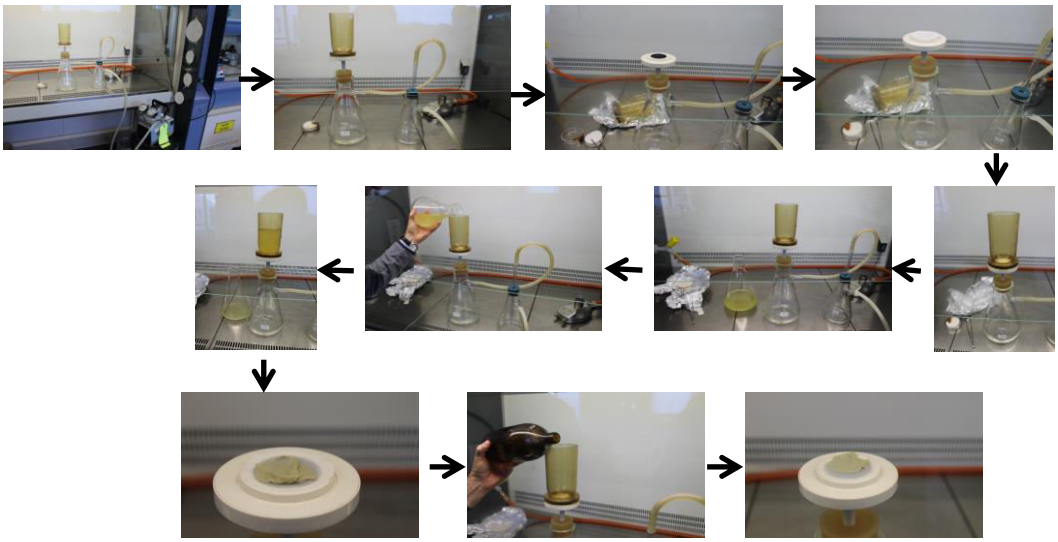

Figure 5. Set up of the filtration system to harvest the spores

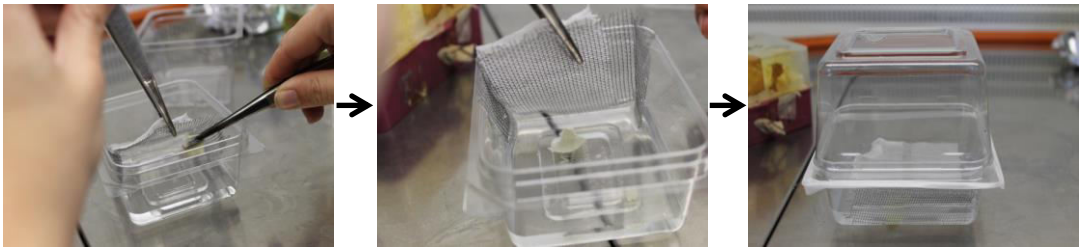

Figure 6. Procedure to inoculate the mycelia into the Phytatray boxes

6. Finally, keep hydroponic cultures for 20 days at 80 rpm and 22 °C in a plant growth chamber with 40% humidity under long daylight conditions (16 h light/8 h dark) (light intensity of 80 to 100 µE/µm^2/s). Afterwards, roots are recovered using sterile scissors on a laminar flow cabinet. Wash the roots with sterile water to remove mycelia traces. Dry the roots on filter paper and finally freeze them in liquid nitrogen. Lyophilize using a Lyophilizer Virtis Advantage until the water from the roots is entirely removed. Collected samples are ready for nucleic acid extraction or qPCR procedures. Success

of root invasion is analyzed by qPCR. The corresponding protocols are described in Alonso-Ramírez *et al.* (2014).

Recipes

1. Sterilization solution
   2.5% sodium hypochlorite
   0.005% Triton X-100
   dH_2O
2. MS medium
   9 g of Murashige & Skoog medium, including B5 vitamins, for 1 L
   1% sucrose
   Adjust pH to 5.7 with KOH
   Qs. dH_2O 1 L
   Sterilized for 20 min at 120 °C/1 atm using an autoclave
3. Washing solution
   70% ethanol
   1% Triton X-100
   dH_2O

Acknowledgments

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References

1. Alonso-Ramirez, A., Poveda, J., Martin, I., Hermosa, R., Monte, E. and Nicolas, C. (2014). Salicylic acid prevents *Trichoderma harzianum* from entering the vascular system of roots. *Mol Plant Pathol* 15(8): 823-831.