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In vitro co-culture system using a fiber-supported liquid approach

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We use this protocol and it's working

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Abstract

In vitro co-culture techniques that allow the growth of plants and pathogens under controlled environmental conditions are being used to re-create host plant infection. These approaches reduce infection times, promote reproducibility, and enable a rapid evaluation of plant-pathogen interactions. As a result, these systems have become essential in breeding programs aimed at developing plant resistance to diseases. In this study, we developed and validated an *in vitro* co-culture system to investigate the Armillaria root rot (ARR) affecting *Prunus* spp. This disease, caused by fungi *Armillaria* spp. and *Desarmillaria caespitosa*, poses a severe threat to the stone and nut fruit industry due to the susceptibility of most commercial rootstocks to infection and the lack of effective management options for its control. The system consists of a fiber-supported liquid approach in sterile plastic vessels that allows a fast and reproducible fungal infection under controlled environmental conditions. The floor of the vessels was covered with a polyester-fiber matte and a germination paper that served as an interface between the mycelia and the plant roots. The vessels were subjected to inoculation with *Armillaria mellea* and *D. caespitosa*, and three *Prunus* genotypes ('Guardian[®]', 'MP-29', and *Prunus cerasifera* '14-4') were co-cultured with both fungi. Disease progression and plant and fungal biomass were monitored during co-culture. The presented *in vitro* co-culture approach facilitates the concurrent growth of *Armillaria/Desarmillaria* spp. and *Prunus* spp., excluding most of the limitations associated with greenhouses and field experiments. This system provides consistent and reproducible conditions for investigating a prominent plant disease affecting *Prunus* spp.

Materials

Materials

- 20 × 150 mm culture tubes (Stellar Scientific; Baltimore, MD, USA, Cat. N°: SKU:GS-1522)
- Autoclavable polypropylene culture tube closures (General Laboratory Supply; Pasadena, TX, USA, Cat. N°: T3054-4)
- MagentaTM GA-7 vessels (Merck, Darmstadt, Germany, Cat. N°: V8505-25EA)
- Petri plates (VWR International, Radnor, PA, USA, Cat. N°: 391-0579)
- Parafilm 'M' laboratory film (Sigma Aldrich; Darmstadt, Germany; Cat. N°: P7668)
- 15 mL Pyrex[®] Ten Broeck tissue grinder with a pour spout (Corning, Tewksbury, MA, USA; Cat. N°: 7727-15)
- Ultra-clear porous cellophane sheet (0.1 mm thick) (Research Products International, Mount Prospect, IL, USA; Cat. N°: 1080)
- Lazy-L spreader (Merck, Darmstadt, Germany, Cat. N°: Z376779)
- 1.5 mL Eppendorf[®] tubes (Thermo Fisher Scientific Inc, Waltham, WA, USA; Cat. N°: 0030120175)
- Rectangular vessels (110 × 297 mm; Southern Sun BioSystems, Hodges, SC, USA)
- Polyester fiber matte (BioStrateTM Felt; Cropking Inc., Lodi, OH, USA)
- Germination paper (Anchor Paper Co., St. Paul, MN, USA)
- Polyvinyl chloride (PVC) sealing film (Phytotech Laboratories, Shawnee Mission, KS, USA; Cat. N°: A003)

Equipment

- Laminar flow hood
- Autoclave
- Articulated rocker arm
- Fungal growth incubator
- LED light NutriLED, Hubbell Lighting, Greenville, SC, USA)

Establishment of Plant Cultures

1 *Establishment of plant cultures from dormant shoots*

- 1.1 Collect dormant shoots, cut them (3 cm in length), and cleanse them by submerging in 70% ethanol for 1 minute, followed by rinsing with sterile deionized water. Then, immerse the shoots in a 10% bleach solution for 10 minutes, and rinse them twice with deionized water (Figure 1).

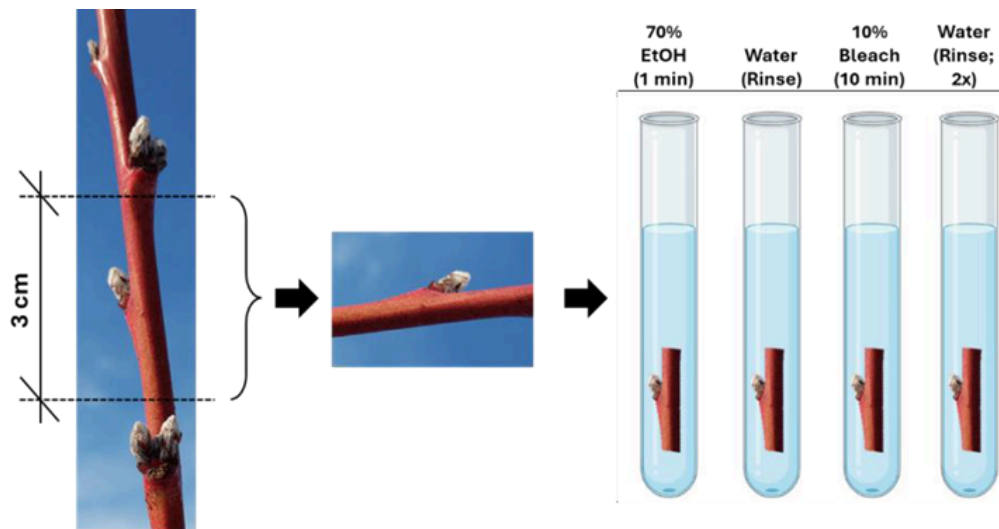


Figure 1. Cleaning process of dormant shoots

- 1.2 Peel the vegetative shoot buds and transfer them into culture tubes containing 20 mL of Murashige and Skoog agar media. Place the shoot vertically ensuring that the bud is 1 cm above the agar media.

2 *Establishment of plant cultures from seeds*

- 2.1 Clean fruit exocarps with 20% bleach for 10 minutes, followed by a 10-minute immersion in 70% ethanol.
- 2.2 Extract seeds within a laminar flow hood and transfer them aseptically into culture tubes containing Woody Plant Medium. Allow them to undergo stratification for ten weeks in darkness at 4 °C.

- 2.3 Upon germination (Figure 2), micropropagate shoot tips in culture vessels(Magenta GA-7) using an agar-based medium



Figure 2. Germinated seeds in tubes.

3 *Maintenance of stock plants*

- 3.1 Sustain stock plants in Magenta GA-7 vessels by transferring shoot tips every five weeks onto a fresh medium.
- 3.2 Maintain vessels under a photosynthetic photon flux density of 20 $\mu\text{mol/s/m}^2$, with a 16-hour photoperiod at 24°C.

Optional: When the presence of hyper multiplication, an occasional resting cycle with 16 μM indole-3-acetic acid (resting media) is recommended.

3.3

Fungi Preservation

- 4 Propagate fungal cultures in Petri plates by placing two plugs (0.5 × 0.5 cm) from the youngest part of the colony (Figure 3).



Figure 3. One-week-old *Armillaria mellea* cultures

- 5 Seal plates with parafilm and maintain in the dark at 20 °C.
- 6 Refresh every 14 days by transferring mycelial plugs to fresh MEA plates to ensure active fungal growth.

Inoculum preparation

- 7 Extract three ten-millimeter-diameter plugs from the edge of two-week-old colonies
- 8 Remove most of the agar plug and homogenize mycelium with 5 mL of sterile water using a sterilized 15 mL tissue grinder (Figure 4).

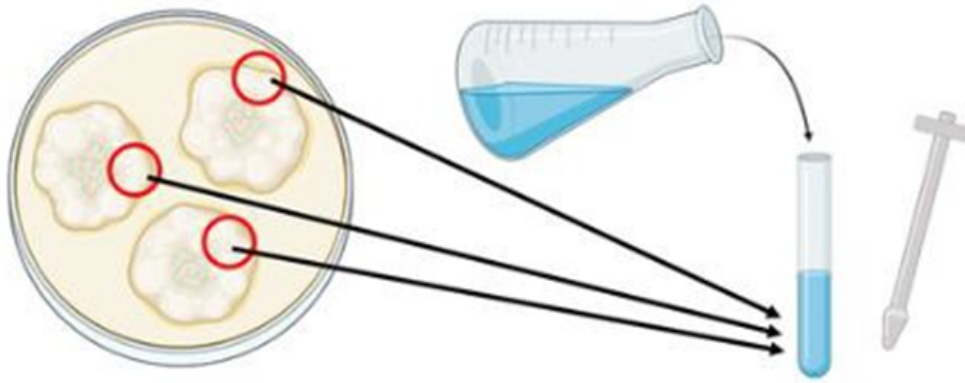


Figure 4. Fungi homogenization in sterile water

- 9 Place a sterile ultra-clear porous cellophane sheet on top of a Petri dish containing malt extract agar media and pour 600 μ L of homogenate
- 10 Spread homogenate uniformly over the entire plate using a cell spreader and incubate the plate in the dark for 14 days.
- 11 Prepare the mycelium suspension for inoculum by taking a 2 $\times$ 2 cm plug from the previously prepared plate and homogenate with 7 mL of sterile water using a tissue grinder, and aliquoted in 1.5 mL Eppendorf tubes (Figure 5).

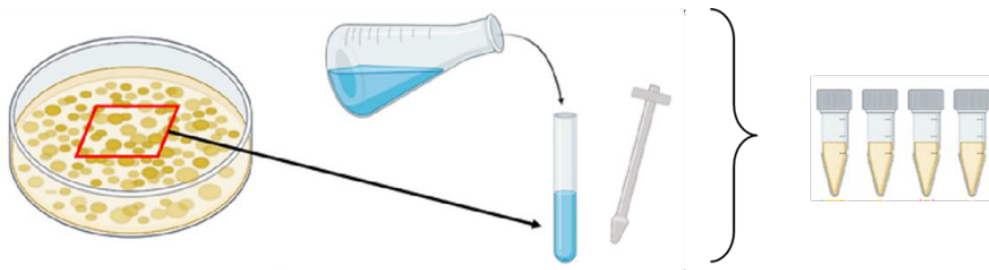


Figure 5. Preparation of the inoculum suspension for infection.

Co-culture (plant-fungi) establishment

- 12 Autoclave (121 $^{\circ}$ C for 20 min) rectangular plastic vessels (110 $\times$ 297 mm; Southern Sun BioSystems) and after cooling down to room temperature, set the fiber-supported paper on the floor of each vessel (Figure 6).



Figure 6. a) Fiber-supported paper.



Figure 6. b) Southern Sun BioSystems with fiber-supported paper inside.

- 13 Add 175 mL of plant growth regulator-free liquid 'New *Prunus* Medium' to each vessel.
- 14 Transfer fifteen *in vitro* plants from an agar-based medium to each vessel removing the agar and sealing vessels with polyvinyl chloride film (Figure 7).



Figure 7. *In vitro* plants growing in the Southern Sun BioSystems vessels.

- 15 Place vessels on a rocker's arm with an articulated shelf that provides one swing every 15 min.
- 16 Use another set of rectangular Southern Sun BioSystems vessels containing 175 mL of 'New *Prunus* Medium', fiber mat, and germination paper and add 1 mL of the mycelium suspension for inoculum.
- 17 Seal the vessels with PVC film and place them on an automatic rocker arm at 5 rpm under $\mu\text{M}/\text{m}^2/\text{s}$ LED light 2 red 1 blue and 16 h/day photoperiod at 24 °C.
- 18 After ten and seventeen days of inoculation with *A. mellea* and *D. caespitosa*, respectively, transfer the *in vitro* rooted plants from the liquid media to the inoculated vessels.
- 19 Add 60 mL of 'New *Prunus* Medium' without any plant growth regulator just before plant transferring.
- 20 Seal the vessels with PVC film and place them on an automatic rocker arm at 5 rpm under $\mu\text{M}/\text{m}^2/\text{s}$ LED light 2 red 1 blue and 16 h/day photoperiod at 24 °C (Figure 8).



Figure 8. Southern Sun BioSystems vessels on rocking platform with an articulated shelf (5rpm) exposed to $20 \mu\text{mol/s/m}^2$, 16h/day at 24°C .

- 21 Collect tissues when needed.

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