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Spore based infection assay on Pinus sylvestris seedlings with Diplodia sapinea

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We use this protocol and it's working

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Abstract

This protocol describes a spore based method for assessing *Diplodia sapinea* pathogenicity in Scots pine seedlings (*Pinus sylvestris*), including inoculation, symptom evaluation, and reisolation.

Materials

Material:
Petri dishes
D. sapinea (e.g. ex-type strain CBS 138184)
Light shelf with cold white daylight (e.g. Osram Lumilux 18W/865), intensity from 5000 to 6500 lux
Cleanbench
Centrifuge
Sterile scalpel
Ethanol (70%)
Sodium hypochlorite (3% NaOCl)
Sterile water

Plant Material:
Scots pine (*P. sylvestris*) container seedlings
Potting soil
Plastic pots

Minimal medium (VMM):
as described in Vogel, 1964
20 ml Vogel's solution
20 g sucrose
15 g agar
add 1 l water autoclave

Trace element solution:
50 g citric acid
50 g zinc sulfate (J.T. Baker)
10 g ammonium iron(II) sulfate
2,5 g copper sulfate
0,5 g manganese sulfate
0,5 g boric acid
0,5 g sodium molybdate
add 1 l water
Chloroform (1 ml) is added as a preservative, store at room temperature.

Vogel's solution:
125 g sodium citrate
250 g potassium dihydrogen phosphate
100 g ammonium nitrate
10 g magnesium sulfate
5 g calcium chloride
5 ml trace element solution
2,5 ml biotin solution

add 1 l water
Chloroform (2 ml) is added as a preservative, store at room temperature.

Biotin solution:
0,1 g biotin
add 1 l water
Store at -20°C

0,01 % (v/v) Tween:
0,1 ml Tween
add 1 l water
aliquod and autoclave

Plant Material and Greenhouse Conditions

1 Use 2 year old container seedlings of Scots pine (*Pinus sylvestris*).

2 Ensure seedlings have no visible symptoms and have undergone two annual cycles.

3 Apply the last fungicide treatment 8 weeks before the first inoculation.

4 Replant seedlings in plastic pots filled with potting soil

5 Maintain plants at 20–25°C, 16 h of light per day, and high humidity (>90% RH) for 4 d after inoculation, then switch to moderate humidity (60% RH).

Fungal Inoculum Preparation

6 Grow *Diplodia sapinea* on VMM (Vogels Minimal Medium) for 21 d at approximately 27°C under constant light (5000–6500 lx) to induce sporulation.

7 Harvest the spores from the plate by adding approximately 2 ml of 0.01% (v/v) Tween to the plate and rinse the surface of the colony several times by pipetting. Repeat the procedure for a higher yield.

8 Dilute the spore suspension to $2x10^6$ spores/ml for inoculation.

9 Assess spore viability by spreading 600 µl of the spore suspension onto three VMM plates. Incubate for 7 h at 27°C and record the germination status of 200 spores per plate. Aim for an average germination rate of 88%.

10 Confirm the absence of hyphal fragments microscopically.

Inoculation

11 Wounded or not wounded plants can be inoculated. Wounding is likely to influence symptom development.

12 Wounding: Use a sterile scalpel to make a 5 mm long cut down to the cambium on dormant seedlings and on the last year's growth segments of actively growing seedlings. Be careful that the cut does not become too deep.

13 Inoculation with Spores:

13.1 Pipetting: Apply 2 µl of spore suspension (approx. 4000 spores) directly onto the wound or unwounded stem.

13.2 Spraying: Spray approximately 360 µl (approx. 720,000 spores) from a distance of 10 cm onto the wound or unwounded stem.

13.3 Control Treatments: Use sterile 0.01% Tween 20 for mock inoculation.

Symptom Assessment

14 Evaluate the symptoms 4- and 6-weeks post-inoculation based on the classification into previously determined symptom classes, for example:
1. No symptoms
2. Necrotic needles, no symptoms on stem
3. Upper third of shoot necrotic
4. Upper two thirds necrotic
5. Seedling dead

Sampling for reisolation

15 After 6 weeks, cut off and discard the shoot tip.

16 Remove an approx. 1.5 cm long piece of the stem above the wound (if applicable), remove needles, and use for reisolation.

Surface Sterilization

17 Sterilize stem pieces:

17.1 Immerse in 70% ethanol for 30 s.

17.2 Immerse in 3% NaOCl for 60 s.

17.3 Rinse twice in sterile water for 30 s each.

17.4 Dry on sterile filter paper.

Isolation and Identification

18 Cut four approx. 2 mm long pieces from the center of the stem samples and place on MYP agar. Incubate for 27 d at room temperature and ambient daylight.

19 Sort isolates into morphological groups from day 4 of cultivation.

20 Extract fungal DNA of a representational number of isolates for molecular identification.

21 Use species-specific primers to identify D. sapinea (for example as described in Adamson et al. 2021).

Protocol references

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