Inoculation and growth with soil borne pathogenic fungi

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1. Introduction

Soil borne fungal pathogens are causal agents of legume diseases of increasing economic importance such as root rots, seedling damping-off and vascular wilts. In comparison to plant responses to foliar pathogens relatively little is known about responses to root infecting pathogens, primarily due to the difficulty in observing the early stages of the interaction and attaining synchronous infection for gene expression studies.

Medicago truncatula provides an excellent system to study plant interactions with soil borne fungal pathogens because of the range of agriculturally relevant pathogens for which *M. truncatula* is a host. Other model plants such as Arabidopsis are host to few root-infecting fungal pathogens. In addition, the use of *M. truncatula* and root-infecting fungi allows investigation of the interaction between defence and symbiosis pathways in the root which is not possible in Arabidopsis. The ability to study resistance to agriculturally relevant pathogens in *M. truncatula* is likely to lead to more successful strategies to introduce resistance to these pathogens, particularly to legume crops. The majority of root-infecting pathogens studied using *M. truncatula* are isolated from alfalfa/lucerne (*M. sativa*) presumable due to the close relationship with *M. truncatula* and its cultivation around the world. Often soil borne fungal pathogens work in disease complexes resulting in plants being infected by multiple pathogens at once. In order to study legume defences against these pathogens, inoculation systems have been developed to enable efficient infection *M. truncatula* by individual fungi.

Previous inoculation systems have relied on pre-infestation of soil for several weeks or months prior to planting seedlings however, we have found these methods are associated with variability in inoculum pressure throughout the soil and uncertainty in the time of first contact between fungus and plant. These variables have been addressed in the following inoculation protocols.

2. *Fusarium oxysporum*

Fusarium oxysporum is the causal agent of Fusarium wilt. Compared to other soil borne fungal pathogens, the wilt pathogens have a more specialized host range (classified as *forma speciales*) and are adapted to grow in the vascular system of their host. The Fusarium vascular wilt is a disease of many agricultural and horticultural crops, including banana, tomato, cotton, chickpea, lupin, among others.

Here we describe the procedure we commonly use to assess the response of a large number of *M. truncatula* seedlings to *F. oxysporum* f. sp. *medicaginis*. Temperature (28±2°C) and root trimming seem to be the major determinants of successful infections in this pathosystem. Often *Medicago sativa* cv. Sceptre is used as a positive control.

2.1 Plant material

The tests are carried out in a growth chamber at 28±2°C under 14:10 light: dark cycles. Seeds are germinated as described in “Vernalization, crossings and testing for pollen viability”. Germinated seedlings are planted in 100% vermiculite in 7x8 cells trays (4x4x7 cm each cell) or 5x6 cells trays (5x5x6 cm each cell) as individuals or triplets depending on the length of the experiment. Watering and fertilization are done according to “Growing *M. truncatula*: choice of substrates and growth conditions”.

2.2 Fungal material

Re-isolation - Suspension stocks should be regularly maintained by re-isolating the fungus from plants of highly susceptible accessions to avoid loss of pathogenicity. Seedlings 7-10 days after infection are washed free of vermiculite, submerged into a 2.5% bleach-5% ethanol solution for 2 min, dried with sterile tissue, cut into 1-1.5cm sections and plated on water agar plates (2%) containing 0.1mg/ml ampicillin; plates are incubated at 25°C for 3-4 days.

Single conidia cultures - Cultures from single conidia are produced to maintain purity and pathogenicity and to permit consistent production of conidia. Single-conidial cultures are produced by streaking a water-agar (2%) Petri dish with a conidial suspension (dishes are stored at 25°C for 12-24hs); distinct colonies are selected and transferred into PDB flasks.

Storage - Conidial suspensions at a concentration of 10^9 conidia/ml in 25% glycerol are stored in aliquots at -80°C.

Taxonomy - We recommend using genomic sequence data to complement taxonomic identification of isolates; please refer to Geiser et al (2004) for specific information on Fusarium species.

Inoculum - We recommend preparing the inoculum on the same day the seedlings are being infected. All materials, other than the fungal suspension, should be sterilized or thoroughly cleaned before use.

Materials

- *F. oxysporum* conidial suspension (refer to 'Storage' above)
- Potato Dextrose Broth (PDB - see 'Appendix ' for recipe)
- Erlenmeyer flask (500ml)
- Controlled temperature rotary shaker
- Funnel and milk filter discs (~200 mm diameter)
- Plastic centrifuge bottle (500ml)
- Sterile distilled water
- Haemocytometer
- Hand tally counter or equivalent

Procedure

a. Autoclave 150ml PDB in a 500ml-Erlenmeyer flask for 20min at 120°C.
b. Inoculate PDB with a conidial suspension to a final concentration of 10^6 conidia/ml. Addition of 150µl ampicillin (0.1g/ml) is optional.
c. Incubate suspension for 3 to 4 days on a rotary shaker at 150-170 rpm and 28±1°C. During this period of time mainly microconidia are produced in suspension.
d. Filter the culture through three autoclaved milk filter discs into a 500ml centrifuge bottle. This will separate both micro and macro-conidia from the mycelia.
e. Precipitate conidia by centrifugation, at 10^5 x g for 20-30 min at 10±4°C. The low temperature facilitates better handling of the spore pellet when discarding the supernatant.

f. Wash twice the conidia by resuspending the spores in sterile distilled water and centrifuging. Resuspend conidia in 2-3 ml water.
g. Using this procedure it is usual to obtain about 4-5ml conidial suspension at 1-2x10^9 conidia/ml. Calculate conidia concentration using a haemocytometer. Prepare 10^6 conidia/ml suspensions in autoclaved distilled water for inoculation. Keep inoculum chilled at 4°C until use on the same day.

2.3 Inoculation

Materials

- *F. oxysporum* conidial suspension (10^6 conidia/ml)
- *M. truncatula* seedlings (1-7 weeks old)
- Growing trays (8x7 cells of 3.5x3.5x7 cm each)
- Vermiculite (medium grade particle size)
- Sterile distilled water
- Forceps, scissors, spatula

Procedure

a. Uproot the seedling from its growing medium.
b. Trim off root tips.
c. Immerse root into the conidial suspension for 2 min.
d. Transplant the infected seedling into an individual cell containing fresh vermiculite watered with distilled water.
e. Apply 10ml suspension per cell.
f. Incubate infected seedlings at 28±2°C.

2.4 Disease assessment

First symptoms (leaf yellowing and wilting) start appearing at about 14 days post inoculation. Plants of susceptible accessions are usually dead 4-5 weeks after inoculation. The disease progress is assessed throughout the experiment by observing the leaves (1=dead, 2=yellow, 3=chlorotic, 4=green) and the proportion of wilted leaves from total. Four to five weeks after infection, the seedlings are removed from the vermiculite and the fresh and dry weights of roots and shoots are measured.

3. *Rhizoctonia solani*

Rhizoctonia solani causes crown rot, root rot and seedling damping-off in a wide range of field crops. The species *R. solani* (teleomorph *Thanatephorus cucumeris*) is a large, diverse and complex group of fungi. Isolates of this species are often classified into hyphal anastomosis groups (AG); these have somewhat specific host range and distribution. Little information is available on molecular aspects of the species taxonomy. It is advisable to include in the inoculation experiments the natural host/s of the specific AG as positive controls.

The preparation of *Rhizoctonia* inoculum follows the guidelines published by Büttner et al (2004) which describe the usage of blended mycelia as the source of the inoculum to achieve homogenous inoculation. For a valuable source of addition methods for inoculation with *Rhizoctonia* refer to Carling and Summer (1992).

3.1 Plant material

The response of *M. truncatula* to Rhizoctonia can be observed at the seedling stage, when seeds have just germinated in the form of damping off and/or root rot or when plants are young in the form of root and/or hypocotyl rot. Here we describe a method to infect germinated seeds that regularly gives homogenous infection.

Germinate seeds as described in "Vernalization, crossings and testing for pollen viability". To achieve seeds with homogenous radical sizes, vernalize as suggested for 3 to 4 days. Radicals should be about 1cm long.

3.2 Fungal material

Isolation - No special technique are required to isolate species of *Rhizoctonia* from host tissues. The usual methods of surface-disinfestation (0.5-1% NaOCl for 1-2min) will suffice. Disinfected tissue is incubated on water agar and hyphal tips transferred to PDA.

Storage of fungal culture - The *Rhizoctonia* isolates are kept in infected millet seeds (*Panicum miliaceum* L.) at -80°C for long-term storage. The seeds are sterilized by autoclaving for 20 min at 121°C on two successive days previous to infection. To regenerate the culture, 2 colonized millet seeds are plated on PDA for 4-6 days at 25°C.

Inoculum - All materials, except fungal culture, are to be sterilized or thoroughly clean with bleach or ethanol before use.

Materials

- *Rhizoctonia*-infected PDA discs (4-6 days old colony, see 'Storage' above)
- Potato Dextrose Broth (commercially available PDB can be used)
- Erlenmeyer flask (250ml)
- Rotary shaker
- Sieve
- Oven (50-60°C)
- Analytical balance and milligram scales
- Sterile distilled water
- Homogenizer or equivalent

Procedure

a. Autoclave 100ml PDB in a 250ml Erlenmeyer flask.
b. Inoculate PDB with 5 infected agar discs. Addition of 100µl Ampicillin (0.1g/ml) is optional.
c. Incubate the culture 11 to 14 days on a rotary shaker at 80-100 rpm at the optimum growth temperature of the particular anastomosis group to which the isolate belongs (e.g. AG8 and AG11 at 22-25°C).
d. Separate mycelium from the nutrient solution using a sieve and wash it repeatedly with sterile distilled water.
e. Drain excess water by applying gentle pressure to the mycelium.
f. Take a small sample, weigh fresh and dry weights and calculate the ration of fresh/dry weight. Weigh the remaining fresh fungal biomass and calculate total dry fungal biomass equivalent in the stock suspension.

g. Homogenized mycelium in 10-20ml sterile distilled water to a smooth homogenous suspension (stock suspension). Stock suspensions can be stored in the fridge for about a week without measurable loss of viability.

3.3 Inoculation

Dilute stock solutions on the same day seedling infection will take place. Undiluted stock can be kept at 4°C for a week without lost of viability or pathogenicity.

Materials

- *R. solani* mycelium suspension (diluted to the equivalent of 2.2mg dry weight ml^{-1})
- *M. truncatula* germinated seeds (with radicals of 1-1.5cm long)
- Growing trays (5x6 cells – 5x5x6 cm each)
- Vermiculite (medium and small grade particles)
- Sterile distilled water
- Forceps, scissors, spatula

Procedure

a. Fill up 1/3 of the growing trays cells with medium grade vermiculite particles. Water till saturation with sterile distilled water.
b. Dilute mycelium stock suspension to 2.2mg dry weight ml^{-1} (Büttner et al., 2004) and inoculate vermiculite with 5ml suspension per cell.
c. Top infected vermiculite with additional water-saturated vermiculite.
d. Incubate, at the temperature for optimal fungus growth, for 24h to allow fungal development.
e. Plant 4 seedlings per cell and fill up cells with small grade vermiculite particles.

3.4 Assessment of plant response

Emergence, survival and number of leaves per pot are assessed throughout the experiment period. Three to four weeks after infection, the seedlings are cleaned of the vermiculite, disease incidence is measured (proportion of seedlings showing rot symptoms per cell), and the fresh and dry weights of roots and shoots are measured.

4. *Pleiochaeta setosa*

Pleiochaeta setosa is the most wide spread pathogen of lupins in Australia. It can infect foliar tissue, stems and pods (brown spot) or roots (Pleiochaeta root rot) of lupins and has been recorded in all countries where lupins are cultivated (Sweetingham *et al.*1998). It can be transmitted by seed, carried on infected stubble and trash and survives, as spores, in soil. The spores are thick walled, pigmented and capable of long term survival in soil. Soil borne spores are the key component of both foliar and root infection. Foliar infection arises from rain splash of spores from the soil surface onto lower leaves. Root infection generally commences soon after germination and brown-black root lesions develop within 3-4 weeks. When severe, root lesions can sever the taproot causing seedling death.

The ability of *P. setosa* to cause root rot in *M. truncatula* was assessed at the seedling stage. Here we describe a method to challenge germinated seeds through the use of *P. setosa* infested soil with which homogenous infection is observed in susceptible hosts.

4.1 Plant material

Germinate seeds as described in "Vernalization, crossings and testing for pollen viability". To achieve seeds with homogenous radical sizes, vernalize as suggested for 3 to 4 days. Radicals should be about 1cm long.

4.1 Fungal material

Isolation –*Pleiochaeta setosa* can be isolated from infected root tissue. Wash roots in three changes of sterile distilled water, blot dry with sterile filter paper and plate on water agar containing 25ppm Aureomycin HCl. Distinctive hyphal growth can be subcultured on water agar or potato extract agar.

Storage of fungal culture - The *P. setosa* isolates are cultured on potato extract agar (Sweetingham, 1991) and infected plugs of agar are maintained in ampoules by lyophilisation. To regenerate the culture, lyophilised agar plugs are plated onto potato extract agar at 15°C for 7-14 days.

Inoculum – Inoculum is produced by:

Materials

- *P. setosa* culture on V8-agar plates
- Distilled water
- Surfactant (eg. Wettasoil ®)
- microscope slide
- binocular microscope

Procedure

a. Prepare V8 juice agar (Appendix)
b. Using a wire loop, streak spores from potato extract agar (see Storage section) to V8 juice agar, approximately 4 streaks / plate.
c. Incubate V8 plates under 12hr day/night light at 15°C for 14 days. Conidia are dark pigmented and with good growth, plate surface will appear black after 14 days.
d. Conidia are washed from agar plates using distilled water containing 0.01% surfactant (Wettasoil®).
e. Determine spore concentration by 10 replicate counts of spore number in a 2µL drop at 50x magnification on binocular microscope and calculate the required volume to achieve 800spores/g soil.
f. Spore suspension should be prepared and used on the same day.

4.3 Inoculation

The technique used is a modification of the technique described by Sweetingham (1991). Spore suspension is mixed with soil to achieve a concentration of 800spores/g soil prior to sowing seedlings. The experiment is carried out in a glasshouse or other controlled environment cabinet at 15ºC.

Materials

- *P. setosa* spore suspension
- *M. truncatula* germinated seeds (with radicals of 1-1.5cm long)
- Seed of susceptible lupin control (eg. Cv. Merrit), not pre-germinated
- A mixture of 80% coarse sand and 20% fine yellow sand (2% clay).
- Mixing equipment (eg. cement mixer)
- Cylindrical pots (white to reduce heat absorption)
- Measuring cylinder, scales
- Fine white sand

Procedure

a. Weigh the amount of soil required, thoroughly pre-mix. If the soil is extremely dry, add 1% water and mix well before adding the spores.
b. Whilst mixing, slowly add spore suspension to soil to achieve a rate of 800 spores/g soil. Continue to mix soil to ensure even distribution of spores. Mix for 2 minutes. Do not over-mix, as the spores are easily damaged and lose their viability if mixed for too long.
c. Weigh inoculated soil into pots to ensure standard quantity of soil per pot.
d. Place in the 15°C glasshouse. To ensure completely even wetting of all pots, water from below for 24 hours by placing in sealed trays 5cm deep and filling with de-ionised water. Remove from watering tray and allow to drain.
e. Using standard seeding depth (1.5cm) sow all seeds. Place a single lupin seed in the centre of each pot as a susceptible control.
f. Maintaining soil temperature is important, in glasshouse situations where exposure to sunlight occurs, use white pots and place 0.5cm fine white sand over pot to reduce heat absorption.
g. No further watering should be required for the period of the experiment.

4.4 Assessment of plant response

Twenty one days after sowing, roots of lupin control plant and *Medicago* plants are washed free of sand and rated for disease incidence and severity. The top 6cm of the tap root is rated, with the maximum lesion in each 1cm segment scored on a 0-5 scale: 0 =no lesion, 1 = shallow cortical lesion, 2 =lesion penetrating the cortex to the stele, 3 =cortex completely rotted girdling the stele, 4 = cortex completely rotted and stele partially rotted, 5 =cortex and stele completely rotted to sever the tap root (Sweetingham, 1989).

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6. Appendix

Potato Dextrose Broth (PDB) 1L

Peeled and sliced potatoes	200 g
Dextrose	20 g
Distilled Water	1000 ml

Cook potatoes for 1 hour in a steamer or 40 minutes in an autoclave in 500 ml of water. (Less precipitation will result in the finished product if they are autoclaved).
Strain and decant the potato juice, bring the volume to 1000 ml. Add and dissolve the sugar. Dispense to appropriate containers and autoclave.

V8 Agar

Calcium carbonate ($CaCO_3$)	2.7g
Agar	10.0g
V8 juice*	100ml
Distilled water	400ml

* Campbells V8 100% vegetable juice

Requires autoclaving for 40min at 121°C three times over three consecutive days. After the third autoclaving, pour in Petri dishes. If the plates are not for immediate use, store in the dark at 4°C.

Potato extract agar

Peeled and sliced potatoes	200 g
Agar	20 g
Distilled Water	1000 ml

Extract potatoes for 1 hour in a steamer or 40 minutes in an autoclave in 500 ml of water. Strain and decant the potato juice, bring the volume to 1000 ml, add and mix agar.