Medicago truncatula resistance to Oomycetes

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Two main oomycetes are considered: *Aphanomyces euteiches* which causes root rot disease on pea, alfalfa, lentils and *Phytophthora medicaginis* (formerly *P. megasperma* f. sp. *medicaginis)* which causes Phytophthora root rot on alfalfa (Hansen and Maxwell, 1991), annual medic species (DeHann *et al*., 1996) and chickpea (*Cicer arietinum*), which is a more susceptible host (Irwin *et al* 1995).

1. Resistance to *A. euteiches* (Anne Moussart, Bernard Tivoli)

This test published by Moussart et al. (2006) is a modified version of the test developed for the evaluation of pea resistance to *A. euteiches* by Moussart *et al.* (2001).

Plant material

Seeds were allowed to germinate on moist filter paper for 48 to 72 h at 25°C in the dark and were then transferred to 500 ml plastic pots containing unsterilized vermiculite (VERMEX, M). Five seeds of a single accession were sown per pot, with each pot constituting a replicate. There were four replicates per accession. Pots were arranged in a completely randomized design, in a controlled environment chamber under constant conditions (thermoperiod: 25/23°C and 16 h photoperiod). Two *Pisum sativum* genotypes — Baccara and PI180693 (USDA Plant Introduction Station, Pullman, USA), susceptible and resistant to *A. euteiches* (Wicker *et al*., 2003), respectively — were used as controls.

Pathogen inoculation and incubation

Seven days after sowing, seedlings were inoculated with a suspension of zoospores from a strain of *A. euteiches*. Zoospores were produced as previously described (Moussart *et al*., 2001), with the concentration of the suspension adjusted to 2000 zoospores/ml. Seven-day-old seedlings were inoculated by applying 25 ml of inoculum suspension per pot (10^4 zoospores per plant). To favor disease development, vermiculite was saturated with water after inoculation.

Disease assessment

Plants were removed 14 days after inoculation, and disease severity was scored on a 0 to 5 scale: 1= no symptom; 1= traces of discoloration on the roots (<25%); 2= discoloration of 25 to 50% of the roots; 3= discoloration of 50 to 75% of the roots; 4= discoloration of more than 75% of the roots; 5= the plant is dead. The screening test was repeated twice.

Data analysis

We carried out an analysis of variance (ANOVA) for the screening results, and compared means, using a Newman-Keuls test (P=0.05), in the General Linear Model procedure of SAS (SAS Institute, Cary, NC, USA).

2. Resistance to *P. medicaginis* (Deborah Samac, Nola D'Souza)

Storage of *Phytophthora* cultures

Phytophthora isolates can be reliably stored under sterile MilliQ or distilled water in McCartney bottles in the dark at 15 – 20°C. Growth of isolates on corn meal agar can be cut into approx. 1cm cubes and then added to the water. It is preferable to store a freshly isolated culture and grow fresh cultures from the original rather than continued sub-culturing, which may result in loss of virulence.

Isolation of *Phytophthora* from diseased tissue

Various methods of isolation are discussed in Erwin and Ribiero (1996). Isolation from diseased roots is facilitated by surface sterilization in 70% ethanol for 30secs-1min then washing in three changes of sterile distilled water. Roots are then placed onto *Phytophthora* selective agar (containing various anti-fungal and anti-bacterial solutions – see Erwin and Ribiero (1996)) and incubated at 20 – 24°C. Within 48 hrs *Phytophthora* hyphae can be seen growing into the selective agar and can be sub-cultured onto corn meal agar for storage.

Plant material

Seeds were mechanically scarified between two pieces of medium sandpaper then sown directly in a vermiculite/sand/perlite mixture (1:2:1, v/v) in bedding plant tray inserts with 1 1/2" x 2 3/8" inch cells. Ten seeds of a single accession were sown per cell, with each cell constituting a replicate. There were three replicates per accession and the experiment was done two times. Cells were arranged in a completely randomized design, in a controlled environment chamber under constant conditions (12-h photoperiod (300 µmol m^{-2} s^{-1}) with a 21°C-day/19°C-night regimen and 50% humidity). Two *M. sativa* genotypes, Saranac and Agate, susceptible and resistant to *P. medicaginis* (Irwin *et al*., 1979), respectively were used as controls.

M. truncatula seeds can be scarified with a mortar and pestle with a small amount of clean white sand then vernalised in the fridge covered with plastic wrap and aluminium foil for approx. 3 days on wet filter paper in a Petri dish. Seedlings will germinate at room temperature over night before planting. Various seedling trays with various experimental designs can be used to plant *M. truncatula.* A single seedling per cell grows well for up to 5 weeks in a 56 well seedling tray of cell size 4cm x 4cm x 7cm, in a temperature cooled glasshouse.

Sand mixtures (1:4 v/v of yellow sand to coarse white river sand) are used for *Phytophthora* inoculation to allow free movement of motile zoospores.

Pathogen preparation for inoculation

Phytophthora medicaginis cultures (such as strain M2019) are grown on V8 Agar for 10-14 days at room temperature for all inoculation methods.

Infested millet seed

Soak approx 100 ml of pearl millet seed in distilled water overnight in a 250mL conical flask. Drain the millet seeds then add approx 40ml of distilled water and plug the flask with cotton wool and cover with aluminium foil. Autoclave for 20 mins at 121psi on two consecutive days, 24 hours inbetween. Inoculate the millet seed with 5 discs of *Phytophthora* hyphae from a V8 plate, obtained using the large end of a 1mL plastic pipette tip. Inoculate at 20 –

24°C for 10 - 14 days with periodic manual shaking/rotation every 2nd day to ensure even inoculation throughout the medium.

Mycelial inoculum
The inoculum was prepared by homogenizing mycelium and spores from six 100-mm plates of the pathogen in a liter of water with a blendor.

Production of zoospores
Various zoospore production methods are available - see Erwin and Ribiero (1996), Pratt et al. (1975). The method of choice may require some testing though soil extract can be reliable for zoospore production. Prepare by shaking approx. 200mL of potting mix in a Schott bottle with I Lt of distilled water, then let stand on the bench overnight. Filter the liquid through two layers of paper towel then autoclave at 121 psi for 20mins.

Using the large end of a 1mL plastic pipette tip, remove a number of V8 hyphal discs from an infested plate (@20) and put into a sterile petri dish. Flood with enough soil extract to just cover the discs (approx 20mL). Alternatively remove the agar around the growing edge of hyphae and flood the whole plate with soil extract. Incubate overnight at 20-24 °C. Zoospore release can be instigated by cold shock at 5 °C for a minimum of 1 hour followed by room temperature until they are released (approx 1 hour). Alternatively there are reports that incubation overnight at 16 °C results in automatic zoospore release ready immediately. Note that non-sterile soil extract will induce a larger amount of zoospores but be aware of the possibility of other contaminants - mock inoculate controls with the plain soil extract to determine if there is an adverse affect.

Before estimating the concentration of zoospores using a haemocytometer, a 1mL aliquot of motile zoospores can be induced to encyst by vortexing for 1 minute. It is recommended that inoculation is done promptly after zoospores have released to maximize the number of motile spores exposed to the seedlings. Zoospore motility declines between 1-3 hours after release.

<u>Inoculation methods</u>
Soil inoculation
Inoculate a sand mixture with 1% w/w of infested millet seed. Once in the trays water to capacity 2 days in a row with drainage then plant germinated seedlings directly into the mixture. Water the trays to capacity 24 hours after planting, allowing the water to drain. Then water with drainage as required (approx. every 2nd day), to maintain soil moisture. Drainage water could contain *Phytophthora* spores and so should be collected and sterilized accordingly before discarding to prevent spread and contamination.

Hyphal inoculation
Ten milliliters of mycelial inoculum were dispensed onto the growing medium in each cell with 10 one-week-old seedlings. The flats were flooded for two days then watered as needed there after.

Zoospore inoculation
Trays containing seedlings at the 1st trefoil stage (approx. 7-10 days) are flooded so the water is just above the soil level. Seedlings are inoculated with 1000 zoospores per seedling using a 1mL pipette. The trays are left flooded for 3 hours before removing the trays from the water and allowing free drainage. Soil moisture should then be maintained by watering with drainage as required (approx. every 2nd day).

Disease assessment

Qualitative assessment

Plants were removed 14 days after inoculation, and disease severity was scored on a 1 to 5 scale: 1= no necrosis of roots and hypocotyls; 2=slight necrosis (25% or less) of roots and hypocotyls; 3=necrosis of roots and lower hypocotyls greater than 25-50%, slight chlorosis of cotyledons; 4=extensive necrosis of roots, hypocotyls and cotyledons (greater than 50%); 5=dead plant. A rating of 1 or 2 was considered resistant; a rating of 3, 4, or 5 was considered susceptible.

Quantitative assessment

Quantitative disease assessment involves shoot and/or root evaluation. Shoot assessment involves counting the number of leaves that are dead or dying (yellowing) in proportion to the number of healthy leaves per plant. Root assessment involves washing the sand mixture from the roots blotting dry and weighing the root fresh weight.

Data analysis

The percentage of resistant plants for each accession was determined. Accessions with more than 75% resistant plants were considered to be resistant.

ANOVA of mean root weight or proportion of shoot health can be compared between inoculated and non-inoculated seedlings to determine if there is a significant affect of the pathogen on a particular accession. Direct comparison between accessions can also be assessed.

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