Inoculation and growth with foliar pathogenic fungi

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Table of contents:

1 Necrotrophic foliar fungi
1.1 Isolation of fungi from diseased tissue
1.2 Isolate storage and monospore cultures

2 *Colletotrichum trifolii*
2.1 Growth of *C. trifolii* and preparation of seedlings
2.2 Inoculation and disease scoring
2.3 Staining and visualization of *C. trifolii*
2.4 References

3 *Mycosphaerella pinodes*
3.1 Seedling test
3.1.1 Plant material
3.1.2 Inoculation and incubation conditions
3.1.3 Disease assessment
3.2 Detached leaves
3.2.1 Plant material

3.2.2 Inoculation and incubation conditions
3.2.3 Disease assessment
3.3 References

4 *Phoma medicaginis*
4.1 Spray and spot inoculations
4.2 Disease assessment
4.3 Detached leaf assays
4.4 References

5 *Ascochyta rabiei* and *Ascochyta lentis*
5.1 Plant material and fungal isolates
5.2 Inoculation and incubation conditions
5.3 Disease assessment
5.4 References

6 *Botrytis cinerea* and *Botrytis fabae*
6.1 Plant material and fungal isolates
6.2 Whole-plant test: inoculation and disease assessment
6.3 Detached-leaf test: inoculation and disease assessment
6.4 References

7 Biotrophic fungi

7 *Erysiphe pisi*
7.1 Growth of *M. truncatula* plants
7.2 Growth and maintenance of *E. pisi*
7.3 Inoculation of *M. truncatula* plants
7.4 Visual scoring of disease development
7.5 Detached leaf assay for *E. pisi*
7.6 Staining and visualization of fungal growth
7.7 References

8 *Uromyces pisi*
8.1 Maintenance of inoculums
8.2 Screening techniques
8.2.1 Field screening
8.2.2 Screening under controlled conditions
8.2.3 *In vitro* assays
8.3 Histological observations
8.4 References

1 Necrotrophic foliar fungi

1.1 Isolation of fungi from diseased tissue

Diseased tissue is surface sterilised in a 5% ethanol and 1% hypochlorite solution, followed by three washes in sterile water. For small pieces of tissue this is best carried out in a 5ml syringe, which may be tapped and gently pressured to release trapped air bubbles. Pieces of tissue are then plated out on tapwater agar. Where bacteria are a persistent problem, filter sterilised ampicillin, neomycin, and streptomycin may be added to the agar to give 50 mg L^{-1}, 50 mg L^{-1}, and 30 mg L^{-1} respectively. Plates are incubated at 22°C and the leading edge of colonies subcultured onto appropriate growth media at 24 hr, or as hyphae become visible. Refer to the Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/) for media and incubation conditions for different fungal species.

1.2 Isolate storage and monospore cultures

Necrotrophic fungi can normally be stored in glycerol at -80°C. As pathogenic fungi can loose their virulence when subcultured *in vitro*, single use glycerols stocks remove the need for re-isolation of fungi from their host, or they can be re-isolated periodically from infected plant tissue. Conidia are harvested by rinsing plates with sterile water. Conidia produced within pycnidia may require incubation in water for up to 20 minutes before release. Condia are filtered through sterile glass wool and counted in a haemocytometer. Should homogenous cultures be required, conidia are diluted to give 5-50 conidia per plate. After overnight incubation, single colonies may subcultured onto new plates with the aid of a magnifying glass or dissecting microscope. Glycerol stocks are prepared by combining spore suspension with 40% glycerol in a 1:1 ratio by volume. Glycerol stocks should be flash frozen in liquid nitrogen before storage at -80°C. For delicate fungi, e.g. *Aphanomyces*, storage on agar slopes is necessary.

2 *Colletotrichum trifolii*

In this assay, resistant Saranac AR and susceptible Saranac alfalfa seedlings may be used as controls for *C. trifolli* virulence, although the highly susceptible *M. truncatula* genotypes F83005.5 or DZA45.5 (Torregrosa et al., 2004) could be used as well.

2.1 Growth of *C. trifolii* and preparation of seedlings

C. trifolii can be grown on the bench top on Potato dextrose agar (PDA, Difco) plates. For the cotyledon assay, plant germinated seeds at a density of 4/cell in 1 1/2" x 2 3/8" cell bedding trays filled with moistened vermiculite/sand/perlite (1:2:1, v/v) mixture. Cover bedding trays to conserve humidity until cotyledons emerge. Inoculate when seedlings are 7-days old. For whole plant assays, place seeds in larger containers filled with soil mix and incubate in the growth chamber for 3-6 weeks.

2.2 Inoculation and disease scoring

a. Prepare inoculum from 7-10 day old cultures by flooding plates with 1-2 ml sterile water, collect spores with a pipette, and adjust the spore concentration to10^6 cells/ml in 0.01%

Tween20. A sterile loop assists in dislodging the orange-colored spores. Several plates of *C. trifolii* may be required, since sporulation does not consistently occur between 7 and 10 days.

b. Spray seedlings with the inoculum until runoff, cover trays and return plants to the growth chamber. Spray control plants with 0.01% Tween20. To maintain high humidity during the first 2-3 days post-inoculation, plants should be covered tightly with a clear plastic dome, while older seedlings may be wrapped in a Saran-wrap tent. Alternatively, plants may be placed in a mist chamber (Mould et al. 1991a and b).

c. At 10 dpi, 40-60% of alfalfa Saranac AR and 0-5% of Saranac will remain standing (http://www.naaic.org/stdtests/anthracn.html). Cotyledons of resistant *M. truncatula* accessions (e.g. Jemalong) will display large dark water-soaked lesions (2-3 mm) and/or chlorosis. A large percentage of susceptible *M. truncatula* will be dead after 10 dpi, while intermediate genotypes will display extreme cotyledon chlorosis and/or shrivelling with variable trifoliate leaf necrosis. Older susceptible plants will exhibit collapsed stems with acervuli developing on stems and leaves.

d. To perform a detached leaf assay (Torregrosa et al., 2004), place trifoliate leaves on layers of wet Whatman 3MM paper in a Petri dish. Inoculate each leaflet with 6 µl spore suspension, wrap plates with parafilm, and place in the growth chamber. Add sterile water to plates as necessary to prevent leaf wilting. Lesions will be visible in the inoculated area 3 dpi, followed by a halo of chlorosis 5 dpi in susceptible genotypes.

2.3 Staining and visualization of *C. trifolii*

a. Fix and decolorize cotyledons and leaves overnight in acetic acid: ethanol (1:3 v/v). Rinse with water.

b. Immerse tissue in 40-50°C LPTB stain (10 ml 60% DL-lactic acid solution, 10 ml glycerol, 10 ml trypan blue solution (10mg/ml water), and 10 g solid phenol crystals) then place under vacuum three times for 5-10 min to expel air and to facilitate stain entry into the tissue. Next, place cotyledons under LPTB stain in a boiling water bath for 5 min before distaining overnight in a minimum of changes of saturated chloral hydrate solution (10 g/25 ml ddH2O).

c. Mount tissue on microscope slides in 50% glycerol.

2.4 References

Mould, M.J.R., Boland, G.J. and Robb, J. (1991) Ultra structure of the *Colletotrichum trifolii-Medicago sativa* pathosystem I, Pre-penetration events. Physiol. Mol. Plant Path. 38, 179-194.

Mould, M.J.R., Boland, G.J. and Robb, J. (1991) Ultra structure of the *Colletotrichum trifolii-Medicago sativa* pathosystem II, Post-penetration events. Physiol. Mol. Plant Path. 38, 195-210.

Torregrosa, C., Closet, S., Fournier, J., Hogue, T., Gamas, P., Prosperi, J.M., Esquerre-Tugaye, M.T., Dumas, B. and Jacquet, C. (2004) Cytological, genetic, and molecular

analysis to characterize compatible and incompatible interactions between *Medicago truncatula* and *Colletotrichum trifolii*. Mol. Plant-Microbe Interact. 17, 909-920.

3 *Mycosphaerella pinodes*

This methodology has been published by Moussart et al. (2006), and uses two tests to screen for resistance to *M. pinodes*. The seedling test is generally used to screen a large number of accessions. The test on detached leaves can be used to characterise disease progression.

3.1 Seedling test

3.1.1 Plant material

This test is an adaptation of the pathogenicity test for pea developed by Onfroy *et al.* (1999). 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 an unsterilized soil / sand / peat mixture (1: 1: 1 v/v). Four seeds were sown per pot, with four pots used per accession, each constituting a replicate. A fifth pot was included as an uninoculated control. Pots were arranged in trays in a completely randomized design and were placed in a controlled environment chamber under constant conditions (thermoperiod: 20/18°C and 16 h photoperiod). Two lines of *Pisum sativum*, JI296 (John Innes Institute, UK) and DP (R. Cousin's collection, INRA, France), susceptible and resistant to *M. pinodes,* respectively, were used as controls (Onfroy *et al.*, 1999).

3.1.2 Inoculation and incubation conditions

Plants were inoculated with a suspension of pycnidiospores when they reached the three- to four-leaf stage. The isolate used (*Mp* 91.31.12) was very aggressive on pea and representative of the French collection. The pycnidiospore suspension was prepared as previously described (Onfroy *et al*. 1999) and its concentration was adjusted to 10^6 spores per ml. One drop of Tween 20 was added as a wetting agent and the spore suspension was applied (0.5 ml per plant) with a hand-held garden sprayer. After inoculation, each tray was covered with a clear plastic cover to maintain 100% relative humidity and plants were regularly sprayed with distilled water.

3.1.3 Disease assessment

Disease severity was assessed on the first two trifoliate leaves, 18 days after inoculation, using a 0 to 5 scale established by Tivoli *et al*. (1996) for the *P. sativum*/*M. pinodes* pathosystem and available for the *Medicago spp.*/*M. pinodes* pathosystem: 0 = no lesion; 1 = a few scattered flecks; 2 = numerous flecks; 3 = 10-15% of the leaf area necrotic and appearance of coalesced necrosis; 4 = 50% of the leaf area dehydrated or covered by lesions ; 5 = 75-100% of the leaf area dehydrated or necrotic.

3.2 Detached leaves

This test is an adaptation of the test on detached leaves described by Heath and Wood (1969), and adapted by Onfroy (UNIP-INRA, France, pers. comm.) for the *M. pinodes*/*Pisum sativum* pathosystem.

3.2.1 Plant material

Seedlings were grown using the conditions described for the seedling test, with the same controls. When the seedlings reached the four-leaf stage, the first and the second trifoliate leaves were excised and maintained alive on a layer of water in a small clear plastic box divided into compartments.

3.2.2 Inoculation and incubation conditions

A drop of pycnidiospore suspension (10 µl, 2 x 10^5 spores ml^{-1}) was carefully applied to the center of each leaf. Four replicates of two trifoliate leaves were tested for each accession. Inoculated leaves were incubated in a controlled environment chamber (thermoperiod: 20/18°C and 14 h photoperiod), in a completely randomized design. Disease progression was observed daily.

3.2.3 Disease assessment

The number of days after inoculation required to observe 1) the appearance of flecks, 2) the coalescence of the flecks at the drop site and 3) the spread of necrosis beyond the drop site, were assessed. Three reactions are observed : 1) absence of lesions, possibly due to failed spore penetration, 2) fungal development restricted to the drop site, 3) slowing of lesion progression, followed by a halting of lesion progression.

3.3 References

A. Moussart, C. Onfroy, A. Lesné, M. Esquibet, E. Grenier, B. Tivoli 2006. Host status and reaction of M*edicago truncatula* accessions to infection by three major pathogens of pea (P*isum sativum*) and alfalfa (M*edicago sativa*). *Euphytica* (Accepted for publication).

Onfroy C, Tivoli B, Corbiere R and Bouznad Z (1999) Cultural, molecular and pathogenic variability of *Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella* isolates from dried *pea (Pisum sativum*) in France. *Plant Pathol*. **48**: 218-229.

4 *Phoma medicaginis*

P. medicaginis grows well on wheat meal agar and ½ PDA (Difco). Four and six weeks old plates produce the highest numbers of viable spores. Two methods of inoculating *P. medicaginis* are commonly used; spray and spot inoculations (Ellwood et al., 2006). Spray inoculations allow large numbers of plants to be assessed, and spot inoculations allow more precise delivery of spores where factors such as absence of leaf hairs or vertically orientated leaves affect the volume of inoculum adhering. Plants may be inoculated at 3-4 weeks of age, or at the 4th trefoil stage.

4.1 Spray and spot inoculations

Spray inoculations are performed by harvesting conidia by incubating plates with distilled water for 20 min. The suspension is filtered through glass-wool, the conidia counted in a haemocytometer, then adjusted to 10^6 spores/ml. Tween 20 is added to 0.05% as a surfactant.

Plants are sprayed with an artists airbrush (Paasche Airbrush Co., Harwood Heights, IL, USA) to runoff, ideally using a rotating platform to ensure an even distribution over plant surfaces. Inoculated plants are placed in a sealed container or humidity chamber for 24-48 hr to facilitate conidia germination.

Spot inoculations are performed using 5-10 µl droplets of spore suspension, prepared as described above and delivered through a fine pipette tip. Ideally, leaves of the same age should be inoculated, i.e the first, second and third trefoils. One leaf of each trefoil is left uninoculated as a control.

4.2 Disease scoring

A minimum of three plants per replicate pot and three replicates per accession are inoculated in a randomised design. Plants are macroscopically evaluated for disease phenotype at 7 days post inoculation (dpi), and rescored at 10 dpi to confirm more resistant disease reactions. Disease scores should be verified in independent experiments. Whole plant spays were evaluated according to the 1-5 scale devised by Salter and Leath (1992), with an additional class, 0, for complete absence of symptoms.

Spot inoculated plants were rated on a related scale as follows: 1 = small brown or black flecks at the inoculation site (typically 3mm in diameter); 2 = large lesions spreading beyond the inoculation site, chlorosis limited to a 1-2 mm margin around the lesion; 3 = necrotic lesion and chlorosis covering up to 1/3 of the leaf area; 4 = necrotic lesion and chlorosis covering up to 4/5 of the leaf area; 5 = necrotic lesion, entire leaf chlorotic or dead.

4.3 Detached leaf assays

For inoculation of leaves *in vitro*, detached leaves are maintained on 1% agar with up to 150mg/l benzimidazole as senescence inhibitor. The cut petiole should be inserted into the agar to reduce wilting. Such leaves may be inoculated in a variety of ways, for example using a plug of infected agar media. To maintain humidity, Petri dishes are wrapped with clingfilm or similar. Detached leaves remain viable for 1-2 weeks.

4.4 References

Ellwood, SR, Kamphuis, LG, Oliver RP. 2006. Identification of sources of resistance to *Phoma medicaginis* isolates in *Medicago truncatula* SARDI core collection accessions, and multigene differentiation of isolates. Phytopathology 96:1330-1336

Salter, R. M., and Leath, K. L. 1992. Spring back stem and leafspot resistance. in: Standard tests to charaterise alfafa cultivars, C. C. Fox, R. Berberet, F. A. Gray, C. R. Grau, D. L. Jessen and M. A. Petersen, eds., North American Alfafa Improvement Conference, Beltsville, MD.

5 *Ascochyta rabiei* and *Ascochyta lentis*

Typical wild-type cultures should be used for the preparation of inoculum, ideally recently isolated from diseased chickpea plants and maintained on a low nutrient medium. Isolates that

been subcultured repeatedly on rich media should be avoided as they are likely to be avirulent.

5.1 Plant material and fungal isolates

M. truncatula accessions are tested at 3 weeks or when the third trefoil is present. Susceptible chickpea (*Cicer arietinum*) or lentil (*Lens culinaris)* accessions are included as controls.

The isolates are grown on ½ PDA plates at 20^{o}C with a 12 hr day/light cycle. The optimal age for harvesting spores is when the plates are between 3 and 6 weeks old. Conidia are exuded in a sticky mass with a dark-orange colour.

5.2 Inoculation and incubation conditions

The spore suspension is prepared adding 10 ml water to a plate. This is incubated for approx. 30 min., then conidia release aided by gently rubbing the culture surface. The suspension is filtered, briefly vortexed, and a sample taken to estimate the concentration of spores. The suspension is adjusted to $1x10^6$ spores per ml, and Tween 20 and glucose added to 0.02% and 50mM respectively. The suspension is kept on ice.

Inoculations were carried out by using the air-brush system until runoff (approximately 2 ml inoculum per plant). As controls, non-infected *Medicago* plants and other legume species (host to the pathogens) are included. Plants are placed under a plastic cover to create a dew-chamber effect, then placed on greenhouse benches for 48 hours.

5.3 Disease assessment

Disease symptoms appear at 8 to 10 days past infection (dpi) on leaves and petioles and are classified from resistant (no symptoms), moderately resistant (spots on infected leafs, but still green), moderately susceptible (infected leaves, initial wilting), and highly susceptible (dead leaves). On susceptible *Medicago* accessions the fungus forms circular spots, and in the case of petiole/stem infection a severe attack may evolve into complete girdling, thereby disrupting the epidermal and cortical tissue and leaves drop prematurely. Secondary infection cycles may be obtained under appropriate conditions (dispersal of spores by 'rain splash').

5.4 Reference

Kaiser, W.J. (1991) Host range studies with Ascochyta blight pathogens of chickpea. *International Chickpea Newsletter* **25**: 25-26

6. *Botrytis cinerea* and *Botrytis fabae.*

Botrytis cinerea is an important pathogen of *Medicago sativa*, causing blossom blight in this crop (Gossen et al. 1997). Both *B. cinerea* and *B. fabae* can infect *Medicago truncatula* under controlled conditions in the laboratory. There are two different types of tests: (i) whole-plant tests, using *M. truncatula* seedlings; (ii) detached-leaf tests, which tests the reaction of detached leaves to infection. Conditions for the growth of plant material and fungal isolates are the same in both cases.

6.1 Plant material and fungal isolates

M. truncatula seeds are sown in small pots and grown in growth chambers at 20-25 ºC. Plants are inoculated at the three- to fourth- leaf stage. Monosporic isolates of *Botrytis* spp. are used for the assays. These are cultured on Petri dishes with PDA, and incubated under conditions that stimulate sporulation; 19ºC under a cycle of 12 hours of darkness and 12 hours of visible light and near U.V. radiation for two weeks (Harrison, 1988).

6.2 Whole-plant test

6.2.1 Inoculation

A spore suspension is prepared by pouring 5-10 ml of a glucose solution in sterile water (1,2g/ml) onto each dish where the pathogen is growing, and dislodging the spores by scraping the surface of the medium with a loop. This suspension is filtered through two layers of sterile cheesecloth and the spore concentration adjusted to 1,000,000 spores/ml. 0.03% v : v Tween-20 is added to the suspension.

Plants are sprayed with this suspension, 2.5 ml per plant overall and incubated 24h in darkness with a relative humidity over 90%, by placing plants in trays covered with plastic whose inner sides are sprinkled with water to maintain relative humidity.

6.2.2 Disease assessment

First symptoms may appear 48 hours after inoculation. Disease severity is measured in each individual plant as the percentage of foliar surface covered with chocolate spot lesions. Final evaluation usually takes place 10-12 days after inoculation.

6.3 Detached-leaf test

6.3.1 Inoculation

Petri dishes are filled with sterile water. Then, the third leaf of *Medicago* plants is placed gently on the surface of the water, so that it will float on it. A spore suspension is prepared in the same way as for the whole-plant test. A 10 µl drop of this suspension is placed on the upper surface of each leaflet in the Petri dish, and covered with the lid. These can be incubated under the same conditions as for the plants growth.

6.3.2 Disease assessment

Lesions can be seen 48-72 hours after inoculation. The diameter of lesions is measured on a daily basis, and area under the disease progress curve (AUDPC) may be calculated.

6.4 References

Gossen, B.D., Lan Z., Harrison L.M., Holley J. , and Smith S.R. 1997. Survey of blossom blight of alfalfa on the Canadian Prairies in 1996. *Can. Plant Dis. Surv.* **77:** 88_89.

Harrison, J.G. (1988). The biology of Botrytis spp. on *Vicia* beans and chocolate spot disease - a review. *Plant Pathol.* **37**: 168-201.

7 Biotrophic fungi

Few protocols exist for long term storage of one of the most important genera of biotrophs, powdery mildews, as the conidia are not amenable to freezing and therefore they are cultured by regular transfer to fresh susceptible plants. Infected plants can have extended life-spans if they are grown at reduced temperature (4 - 10^{o}C). Where several isolates are maintained in close proximity, they may grown on detached leaves in sealed Petri dishes with 1% agar and up to 150 mg/l benzimidazole as a senescence inhibitor as described above. Biotrotrophs may also be purified by single colony isolation, and pathotypes determined by selection on different combinations of host resistance genotypes.

Several inoculation methods have been developed for biotrophs. These include direct inoculation by use of a small camel hair artists brush or a finely drawn sterilized glass rod to dislodge conidia from infected leaves at a set height above the target leaves. To ensure an even distribution of conidia, this is performed in a draft free environment such as settling towers made from PVC piping about 100 cm in height. Biotrophs may also be delivered by spraying with an artists airbrush as described for nectrotrophs. Inoculated plants should be placed in a sealed container or humidity chamber for 24-48 hr to facilitate conidia germination.

7.1 Inoculation of *M. truncatula* with powdery mildew *Erysiphe pisi*

In this procedure, *M. truncatula* seedlings are grown in the growth chamber until five to ten trifoliate leaves develop. *E. pisi* conidiospores are shaken onto the extended leaves and allowed to settle for 20 minutes. Inoculated plants are then returned to a dedicated growth chamber, while control uninoculated plants are transferred to a separate "clean" growth chamber. Disease development is scored at four and seven days post-inoculation (dpi).

7.2 Growth of *M. truncatula* plants

Plant seedlings in water-soaked Jiffy-7 peat pellets placed 4/pot in 6'' square in plastic containers filled with Sunshine SB300 (Sungrow USA) or other potting mix. Seedlings are inoculated after 3-4 weeks growth a growth chamber (16 hr photoperiod, 22°C light/16°C dark).

7.3 Growth and maintenance of *E. pisi*

E. pisi is an obligate biotroph and must be continuously maintained on a susceptible host (Falloon, 2001). Although some *M. truncatula* genotypes may be used to host the fungus, we have found that susceptible *Pisum sativum* can produce and sustain a heavy inoculum without leaf drop for a longer period of time (about one month).

a. Plant 3 pea seeds/pot every 3-4 weeks in a clean growth chamber. Fertilize once a week with a soluble fertilizer to maintain robust growth. Transfer pots to a dedicated growth chamber containing an infected plant when peas are 2-3 weeks old. Usually peas will become lightly infected by passive inoculation. Heavily infected plants will not live as long.

b. To develop large quantities of inoculum, gently tap spores from infected plants onto 3 pots of new peas. Spores will be mature 10 days post inoculation. Heavily infected plants from 3 pots will inoculate 64 *M. truncatula* seedlings (in 16 pots) at a density of 20-100 spores/mm^2 leaf area.

c. To maintain *E. pisi* culture in isolation, peas can be grown in a growth chamber in Magenta tissue culture containers fitted end-to-end with adapters (total height of 8 inches). To do this, autoclave Magenta containers filled with 1 inch of wet Metro-mix 200 twice for at least 30 minutes. Using a laminar flow hood, plant a single surface-sterilized pea seed into each container. When the pea plants are 2-3 inches high, transfer spores from a single E. pisi colony to the plants with a sterile cotton swab or small paintbrush. Cultures should be transferred to new plants every 3-4 weeks.

d. To obtain a single-spore culture, detach an infected pea leaf containing a single colony and inoculate onto a fresh pea plant in a clean growth chamber. As soon as colonies appear, detach a new leaf containing a single colony. Using a sterile cotton swab or paintbrush, collect spores from this single colony and inoculate a fresh pea plant in a separate clean growth chamber.

7.4 Inoculation of *M. truncatula* plants

Plants are surrounded by a tall cardboard box, which is open at the top and bottom. Spores from infected pea plants are tapped into the top of the box and allowed to settle onto the leaves below.

a. Construct a cardboard settling tower from a large box opened on both ends or tape several boxes together. The total height should be at least 150 cm and the width/depth should accommodate all pots to be inoculated in a single experiment.

b. Arrange pots containing *M. truncatula* on the floor. Set hemocytometers on top of the pots in several locations. Place the settling tower around the pots and then gently tap spores from infected plants over the settling tower. Allow 20 minutes for spores to settle. Remove the settling tower and place cover slips over hemocytometers. Transfer inoculated plants to a clean growth chamber. Obtain spore densities from hemocytometers using a light microscope.

7.5 Visual scoring of disease development

Macroscopic colonies will become visible 3-4 days post-inoculation (dpi). To differentiate between highly susceptible and partially resistant genotypes, compare several of the most highly infected leaves. At 7-10 dpi, some leaves of susceptible *M. truncatula* plants will be completely covered with white velvety fungal colonies while leaves from partially resistant plants will have a mottled appearance. Leaves that emerged after inoculation will not be infected.

7.6 Detached leaf assay for *E. pisi*

a. Detached leaves of susceptible and partially resistant genotypes will support growth of *E. pisi*. Place excised trifoliate leaves on cotton saturated with 5% filter sterilised sucrose solution in Petri plates.

b. Incubate plates in the growth chamber using settings described in 2.1.12.5.1. Leaves can be scored under the microscope at 2-3 dpi. Macroscopic colonies will be visible after 5-7 days, although leaves may be chlorotic.

7.7 Staining and visualization of fungal growth

a. Prepare LPTB by mixing 10 ml 60% DL-lactic acid solution, 10 ml glycerol, 10 ml trypan blue solution (10 mg/ml water), and 10 g solid phenol crystals.

b. Fix and decolorize leaves for several hours or overnight in acetic acid:ethanol (1:3). Wash once with water.

c. Incubate leaves in LPTB stain for 30 minutes and mount on microscope slides. Fungal structures will appear blue.

d. Alternatively, leaves may be stored in lactic acid:water:glycerol (1:1:1) after fixation and stained later.

7.8 References

Falloon, R. E., and Viljanen-Rollinson, S. 2001. Powdery mildew. Compendium of pea diseases. 2nd Ed. APS Press. St. Paul, USA.

Warkentin, T.D., Rashid, K.Y., and Zimmer, R.C. (1995) Effectiveness of a detached leaf assay for determination of the reaction of pea plants to powdery mildew. *Can. J. Plant Pathol*. **17**: 87-89

8 Rust

Alfalfa rust caused by *U. striatus* is an important disease in many areas, being particularly damaging in alfalfa grown for seed (Koepper, 1942). *U. striatus* can also infect *M. truncatula* (Rubiales and Moral, 2004).

8.1 Maintainance of inoculum

Uredospores can be stored long term at -80°C, or air dried in sealed bags or capsules.

8.2 Screening techniques

8.2.1 Field screening

To screen for resistance under field conditions, infection should be uniform and severe enough to avoid escapes. To ensure uniform disease distribution artificial inoculation is recommended, by spraying with an aqueous suspension of rust spores or dusting a mixture of spores in an inert carrier such as pure talc powder or *Lycopodium* spores.

Disease severity may be assessed periodically by visual estimation of the leaf area covered with rust pustules (disease severity, DS). Resistance of accessions can be compared by the last measure of the disease severity (the final disease severity), by the area under the disease progress curve (AUDPC) or by the epidemic growth rate (*r*) (Sillero et al., 2006). In addition to the amount of infection, the infection type (IT) should be recorded using the 0–4 scale of Stakman et al. (1962), where 0 = no symptoms, **;** = necrotic flecks, 1 = minute pustules barely sporulating, 2 = necrotic halo surrounding small pustules, 3=chlorotic halo surrounding pustules, 4=well formed pustules with no associated chlorosis or necrosis. Values 0–2 are considered indicative of resistance and 3–4 of susceptibility.

8.2.2 Screening under controlled conditions

Testing under controlled conditions can be carried out in either a growth chamber or a greenhouse, allowing screening in seedling and adult plant stages. Rust inoculation can be done by dusting the plants with urediospores diluted in an inert carrier, but for quantitative measurements the use of a spore settling tower is recommended in order to ensure more uniform spore deposition. Plants are then incubated for 24 h in an incubation chamber at 20 ^{o}C in complete darkness at 100% relative humidity, and subsequently maintained in a growth chamber at 20 ^{o}C with a 14-h photoperiod.

Several macroscopic components of resistance can be measured under controlled conditions. The Latent Period (LP), the period of time between inoculation and sporulation of 50% of the pustules, is determined by counting daily the number of pustules visible in a marked area on the leaves, using a pocket lens, until the number of pustules no longer increases. Infection Frequency (IF), the number of pustules per unit area, can be calculated in the same area in which LP was estimated. Infection Type (IT) describes the external appearance of the pustules, as described above. Colony Size (CS) can be measured microscopically with the help of a micrometer from 4 to 10 days after inoculation, using epifluorescence or contrast phase microscope (Sillero and Rubiales, 2002), or estimated macroscopically on the basis of the pustule diameter.

8.2.3 In vitro assays

Several methods for testing rust resistance using detached leaves have been proposed. Herath et al. (2001) maintained excised leaves in Oasis water-retaining medium, with 5 ppm gibberellic acid, in an enclosed box in a temperature-controlled glasshouse. Detached leaves can also be maintained on tissue paper soaked with distilled water to which benzimidazole (1%) was added (Sillero et al., 2006) or floating on sterile tap water in Petri dishes (Singh and Sokhi, 1980). Excised leaflets are carefully laid, adaxial surface up, on the maintaining medium, then inoculated, incubated and maintained as described above.

8.3 Histological observations

Leaves are collected 2 days after inoculation (d.a.i.) to study phases of fungus growth prior to stoma penetration (Rubiales and Moral, 2004). The leaf samples are laid, adaxial surface up, on filter paper dipped in fixative (1:1, absolute ethanol/glacial acetic acid, v/v). When the leaf segments have been bleached by several changes of the fixative, they are transferred to filter paper moistened with tap water for at least 2 h, to soften the tissues. Next they are transferred to lactoglycerol (1:1:1, lactic acid/glycerol/water, v/v/v) for at least 2 h. To stain the samples,

a drop of Trypan blue in lactoglycerol (0.1%, w/v) is placed on a cover glass, the sample carefully laid with the adaxial surface toward the cover glass and then mounted in lactoglycerol on a microscope slide. A minimum of 200 urediospores per leaf sample are observed under 200 X's magnification and grouped into the following categories: germinated urediospores (a spore is considered germinated when a germ tube at least as long as the diameter of the spore is produced); germ tubes growing over stomata but not forming appressoria; and germ tubes forming appressoria. Of the germ tubes which form appressoria, a distinction is made between appressorium formed over a stoma or away from stomata (misplaced).

To study the phases of the fungus growth after stoma penetration leaves are collected 2 d.a.i. and stained with Trypan blue (Sillero and Rubiales, 2002). The leaves are fixed in acetic acid:ethanol (1:3 v/v) for 30 min; stained by boiling in 0.05% Trypan blue in lactophenol:ethanol (1:2 v/v) for 10 min and cleared in a nearly saturated aqueous solution of chloral hydrate (5:2 w/v) to remove Trypan blue from the chloroplast. Early stages of the infection are studied microscopically, using a phase contrast microscope at 400x magnification. A minimum of 20 random colonies are assessed per leaf. The numbers of hyphal tips and haustoria are recorded for each colony, along with the presence or absence of necrosis of the host cells associated with an infection structure. Necrosis can be identified by uptake of Trypan blue by plant cells.

8.4 References

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