



# RAPRA Protocols for Susceptibility Testing

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## *Protocol 7: Assessment of the Sporulation Potential of *Phytophthora ramorum* on Detached Foliage*

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### **Inoculum production:**

Zoospores are used to inoculate the detached leaves. The zoospore solution is made using the procedure outlined in Protocol 6.

### **Point inoculations on detached broad-leaves for assessing sporulation potential:**

- Non-wounded broad-leaved host plants are inoculated in these tests. For each species three sampling periods are set up; 4, 7 and 10 days.

### **Day 1:**

- Branches bearing fully expanded leaves of the broad-leaved trees are cut, brought into the laboratory immediately and the cut ends placed in water.
- On day 1 of the experiment leaves are rinsed with sterile water and placed on sterile paper towels to air dry.
- Approximate leaf shape, length and width measurements of each leaf are recorded.
- Leaves are placed inside a moist chamber; constructed from a plastic box lined with wet paper towel. A wire rack is placed over the paper towel onto which the leaves are placed. A single moist chamber houses the leaves of a single species; one for the control and another for each of the isolates being used.
- Each leaf is inoculated by placing a single 100  $\mu$ L drop of its designated isolate in the middle of the leaf close to the midrib.
- A black mark is made with an indelible marker pen on the leaf near the inoculation point to identify where inoculation has taken place.
- The walls of the plastic chamber are sprayed with sterile water.
- The chamber is tightly covered with plastic cling film that has also been sprayed with sterile water.
- Leaves are incubated at 20°C under continuous daylight; 60 W daylight bulbs are suspended 30 cm above the chambers and cool white light is supplied from fluorescent tubes.

### **Day 2 (24 hrs after inoculation):**

- The inoculum drop is removed from each leaf with a tissue to absorb the liquid.
- The leaves are re-incubated in the moist chambers for a further 24 hrs.

### **Day 3:**

- Leaves are atomised with sterile water to ensure the surface of the leaf remains moist at all times.
- The three sets of leaves for each sampling period are now treated differently.



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## Protocol 7: Assessment of the Sporulation Potential of *Phytophthora ramorum* on Detached Foliage - continued

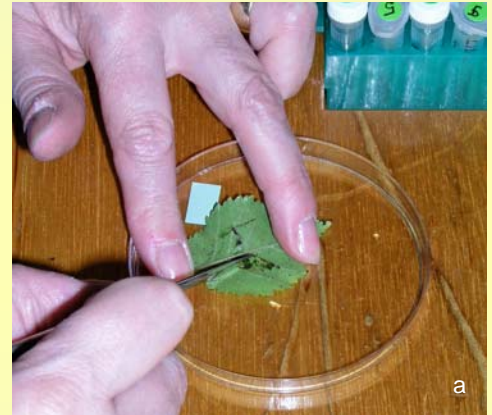


### Day 4:

- A single 100  $\mu\text{L}$  drop of sterile water is placed on the original inoculation point of the 4 day leaves and incubated further. The leaves should be checked throughout the day to ensure the water droplet is still present, if the droplet disappears the drop is replenished.

### Day 5 (96 hrs after inoculation):

- The leaves (with water droplet *in situ*) are examined using a compound microscope with the 10x objective for the presence or absence of sporangia.
- The water droplet is then pipetted off the leaf and transferred to a 1 mL microtube.
- The length and width of the lesion are measured in millimeters and the data recorded.
- The leaf surface below the water drop (including all the necrotic parts developed from inoculation) is then gently scraped with a rounded scalpel blade to free all sporangia from the leaf surface (a). A 200  $\mu\text{L}$  drop of sterile water is placed on the inoculum spot, to suspend the scalpel scrapings, the drop is pipetted up and added to the microtube.
- 5  $\mu\text{L}$  of cotton blue (5%) in lactophenol is added to the tube.
- The inoculated area of the leaf is cut out of the leaf and surface sterilised for 30 secs in 70% ethanol and rinsed in sterile water. Two or three pieces of tissue are then plated onto Synthetic Mucor Agar (SMA) to back isolate the pathogen, confirming infection of leaf tissue (or not in the case of the control).



### Days 7-10:

- The 7 and 10 day samples are atomised daily with sterile water until 24 hrs before scraping the leaf surface (i.e. at 6 and 9 days after inoculation respectively).
- For the 7 day sample the 100  $\mu\text{L}$  drops of sterile water are placed on the original inoculation points 6 days after inoculation and leaves incubated for a further 24 hrs.
- In the 10 d sample the 100  $\mu\text{L}$  drops of sterile water are placed on the original inoculation points 9 days after inoculation and incubated for a further 24 hrs.

### Preparing the sporangial suspensions for counting:

- The microtubes are kept in cold storage until they are to be prepared for counting.
- The microtubes are centrifuged at 4500 rpm for 3 minutes and all the liquid pipetted off.
- The remaining plant material is then resuspended in 20  $\mu\text{L}$  of sterile water and the contents agitated using a vortex machine for 30 seconds.
- Two 10  $\mu\text{L}$  drops are dispensed onto a glass slide and covered with a glass cover slip.
- Using a compound microscope with the 10x objective the number of sporangia in each drop are counted.

### References: