



# Protocols for Susceptibility Testing



## **Protocol 9: Determination of Mating Type of *Phytophthora ramorum* Isolates by Mycelial Mixing**

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This is a novel method for assessing the mating type of *Phytophthora ramorum* that has, up to now, proved to be largely unresponsive to common mating techniques applied to this genus.

*P. ramorum* produces large quantities of chlamydospores in culture. This method is designed to encourage gametangial formation by exploiting the early, pre-chlamydospore stage of *P. ramorum*.

### **Preparing thin carrot agar (CA):**

Petri dishes of thin carrot agar should be prepared as follow:

- liquidise 200g washed carrots in c.500mls tap water;
- filter through fine cheesecloth or muslin to remove the carrot bits;
- make the filtrate up to 1000ml with more tap water;
- add 15g Oxoid Agar Technical No.3;
- autoclave for 15 mins at 121°C/ 15psi and allow to stand and solidify overnight;
- autoclave again for 10 mins, and pour thinly (10mls/plate) into Petri dishes.

### **Setting up Subcultures:**

- Set up subcultures of all isolates to be tested, including known A1 and A2 tester isolates on the thin CA and incubate for 3 days at 20°C in the dark.
- After 3 days the subcultures should have produced mycelial growth but no chlamydospores. Make new subcultures from these plates on to fresh thin CA plates, including multiple plates of the known A1 and A2 testers. Incubate for 3 days at 20°C in the dark.

### **Determination of Mating Type:**

- For each unknown isolate, take six pieces (3mm diam.) from the growing edge of the second subcultures and place three pieces per thin CA plate to form an equidistant triangle.
- Place three A1 pieces next to the unknown pieces on one plate and three A2 pieces against the unknown pieces on the second plate, taking care to avoid cross contamination
- Using a fine sterilised needle, gently mix each pair of pieces on the plates, so as to have three discrete mixed areas each of ca. 0.5-1cm in diameter on each plate.
- Incubate the mixes for 3-7 days at 20°C in the dark.
- Search for gametangia by scanning the area under the mixed pieces through the underside of the plates using a compound microscope. The formation of gametangia by an isolate of previously unknown mating type with a tester isolate indicates that the unknown is of the opposite mating type to the tester.

**NB.** The *P. ramorum* gametangia formed in this way will be rare to infrequent and difficult to find. If none are seen initially it may help if the pieces of mixed mycelium are removed from the plates and the area underneath them re-examined.

### **Reference:**

Brasier CM & Kirk SA, (2004). Production of gametangia by *Phytophthora ramorum* *in vitro*. *Mycological Research* **108**: 823-827.