



# Protocols for Susceptibility Testing

## Protocol 12: Extraction of Oospores from Leaf Tissue



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### Method:

The oospore extraction protocol was modified from a protocol described by Van der Gaag & Frinking (1996). The leaf area of sample (1 – 5 leaflets) was determined using an interactive digitizer (Minimop, Kontron, Oberkochen, Germany). Leaflets were comminuted in 5 ml crushed ice and 5 ml tap water (4°C) using an Ultra Turrax mixer (T25 basic, IKA Labortechnik, Germany,) at 24000 rpm for 90 seconds. The resulting suspension was cooled to 4°C and comminuted a second time at 24000 rpm for 90 seconds. Cellulase (C8001, Duchefa, Haarlem, The Netherlands) and Macerase (M8002, Duchefa, Haarlem, The Netherlands) were added to a final concentration of 0.5 mg ml<sup>-1</sup> each. Suspensions were incubated on an orbital shaker (SM25, Edmund Bühler, Tübingen, Germany) at room temperature for 2 h at 100 rpm followed by sonication (Branson 2510, Branson ultrasonics corporation, Danbury, USA) for 2 x 5 minutes. Following sonication, samples were again incubated on the orbital shaker at room temperature for 2 days at 100 rpm. The resulting suspensions were washed on a set of sieves (75 µm and 20 µm), using tap water, to remove the enzymes. The residue on the 20 µm sieve was transferred to a 50 ml centrifuge tube and spun down for 3 minutes at 5000 g. The volume was reduced to 5 ml by removing supernatant. The oospore concentration in the remaining suspension was determined using a Fuchs-Rosenthal haemocytometer. Suspensions were air dried and stored in Petri dishes at 20°C for later use.

For samples with larger quantities of leaf tissue (10–35 g dry weight) a similar procedure was followed as described with some modifications. Leaf samples were weighed and stems were removed. The remaining leaflets were washed in water to remove sand and comminuted in a blender (Waring commercial, model 38BL40) in 50 ml crushed ice and 100 ml tap water (4°C) for 60 seconds at low speed followed by 30 seconds at high speed. The resulting suspensions were transferred to 500 ml Duran bottles and processed as described above. Suspensions were however washed on a 250 µm, 125 µm, 75 µm and 20 µm sieve set. Residue on the 20 µm sieve was transferred to a 500 ml Duran bottle and left to settle overnight before reducing the volume to 100 ml.

Oospore viability is determined using tetrazolium bromide (MTT, Sigma M-2128) according to Jiang & Erwin (1990).

### References:

van der Gaag DJ and Frinking HD (1996). Extraction from Plant Tissue and Germination of Oospores of *Peronospora viciae* f.sp. *psii*. *Phytopathology* **144**: 57-62

Jiang J and Erwin DC (1990). Morphology, plasmolysis, and tetrazolium bromide stain as criteria for determining viability of *Phytophthora infestans*. *Mycologia* **82**: 107-113