Species:	*Plasmopara viticola*
Product Class:	QoI and CAA fungicides, and also suited for other fungicide classes
Methods described:	microtiter plate test
Date of protocol	2003-12
Proven for	Azoxystrobin. mandipropamid
Should be suitable for	other QoI and CAA fungicides. Also other fungicide classes. Protocol adjustments may be needed due to the individual compound characteristics.
comments	• validated routine method for labs equipped with microtiter plate technique • proven methodology for the active ingredients listed above. Others not mentioned have to be evaluated carefully and methods may have to be adjusted accordingly (e.g. solvents, concentrations) to ensure valid results

Method:

1) Plant Material

Plasmopara viticola is an obligate biotrophic pathogen. Therefore it is necessary to propagate the pathogen on fresh plant material on a weekly basis.

Plant details

grape variety	Gutedel seedlings
age	5-6 weeks
leaves used	third and fourth (counted from top)
special treatment	to prevent *Uncinula necator* infection the plants are sulfurated twice a week

2) Fungicide

Azoxystrobin and mandipropamid, stock solution: 10’000 ppm (ai solved in Dimethyl sulfoxide → DMSO)

3) Pathogen

3.1 Inoculation Method

P. viticola is passaged on single grape leaves. To prevent the leaves from drying up they are placed into a petridish containing a filterpaper soaked with water.
To wash off the sporangia from an infected leaf, it’s put into a beaker and approximately 5mL of sterile deionised water are added. Then vortex. The spore density is checked under the microscope. For passaging only it is not necessary to adjust a certain concentration. To spray the spore suspension onto the lower leafside an atomiser has been used. The leaf surface should be covered evenly with the droplets.

3.2 Incubation

To reach 100% of humidity the petridishes are incubated in a plastic box also containing a water soaked filter paper. The conditions in the climate chamber are as follows:

Temperature	19°C
Light	12h day /12h night
Relative Humidity	70%

3.3 Storage

In order to store P.*viticola* for a longer time it is possible to deepfreeze at –20°C. Take a fresh sporulating leaf transfer it into a fresh petridish and let it dry in the cleanbench to reduce the residual waterdroplets. Then close the petridish with parafilm and put it into the deepfreezer. There the samples are viable for up to one year.

3.4 Sample Processing

To obtain a representative sample, lesions, from thirty to fourty leaves are cut out with a scissor and placed in a humid box containing a filter paper soaked with water.
Then the pieces are wetted with sterilized water using an atomiser. The box is closed and incubated for 24h up to three days, depending on how soon the isolate sporulates. As soon as fresh sporulation is visibile on the surface the sporangias are washed off in sterile water and fresh grape leaves are inoculated. The plates are incubated at the same conditions as mentioned above until sporulation is visible. The sample is stored at –20°C until it is used for testing.

4) Sensitivity Assay

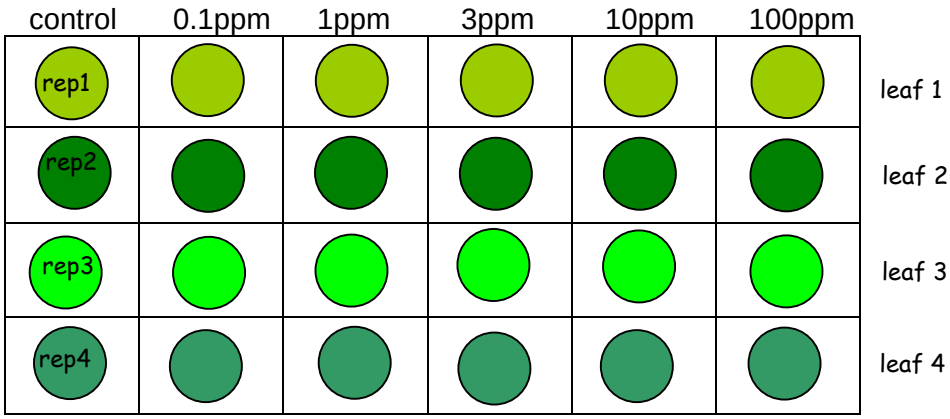
Each sample is tested twice in two autonomous sensitivity assays as described below.

4.1 Pathogen Preparation

Samples to be tested are taken out of the deepfreezer. The process of making up the inoculum is the same as with fresh leaves. Passage the sample once before testing since not all sporangias that were frozen are able to cause an infection. So it is impossible to determine the spore density. Depending on the quality of the inoculum it is possible that the incubation time must be extended by a couple of days until sporulation is visible.

4.2 Leaf Disc Assay

- The sensitivity assay is accomplished in 24-well plates
- Fill 1mL of 0.5% water agar into each well
- Punch out leaf discs (15mm diameter) from either the third or fourth leaf
- Put the discs upside down onto the agar attending to use discs from four different leaves for the single repetitions



4.3 Fungicide Application

- The application is done one day prior to inoculation
- Make up the fungicide dilutions for AMS starting from a 10'000 ppm stock solution (ai solved in Dimethyl sulfoxide → DMSO)
- The control also contains of 1% DMSO
- The application is carried out in an application machine (10 µl per well) or with a paintbrush by hand.
- Dry the leaf discs the next day in a flow cabinet

4.4 Inoculation

- Prepare the inoculum just one at a time
- Adjust the spore density to 50'000 spores/mL
- While spraying the suspension onto the leaf discs take care to spray the plate from opposite directions to achieve an even cover
- Also include a sensitive reference strain into each test

4.5 Incubation

- Close the 24-well plates with a lid

- Put them directly into the climate chamber. There is no need to use an additional plastic box to obtain necessary humidity of 100%
- Incubate for six days

4.6 Assessment

- The assessment is done visually by determining the percentage of infected leaf area
- The data are entered into a database
- The EC50 value is calculated by using AGSTAT (Syngenta internal)

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