Species:	*Botrytis cinerea*
Product Class(es):	Anilinopyridine and phenylpyrrole fungicides
Method type described:	microtiter plate test
Date of protocol:	2005-01
Proven for	Cyprodinil, Fludioxonil
Should be suitable for	other anilinopyrimidine, phenylpyrrole and other classes of Fungicides. Protocol adjustments may be needed due to the individual compound characteristics.
Version	1
comments	• validated routine method for labs equipped with microtiter plate technique • proven methodology for the active ingredient listed above. Others not mentioned have to be evaluated carefully to ensure valid results

Method:

1. Sample collection

Grape berries, with visible and sporulating *B.cinerea* lesions, are collected from different plants and locations in the field. One sample should consist of 5 sub-samples, each sub-samples constituted of at least 2 or 3 infected berries. Each sub-sample is placed in a closed plastic beaker containing a dry paper tissue. The 5 platic beakers meaning one sample are placed into a sending card box with the well filled out sampling sheet. The card boxes are immediately sent to the lab.
When the plastic beakers arrive in the lab, the berries are controlled. If the paper tissue is wet, it is changed to a dry one. The beakers are incubated for 2 or 3 days at room temperature in the lab, until the sporulation is visible.

2. Sample propagation

2.1. Medium

The modified recipe for 1L GEA medium is as follows:

- 160 g frozen peas
- 5 g Sucrose
- 20 g Bacto Agar

The frozen peas are mixed homogeneously with 250 mL H_2O bidest. Sucrose and Agar were then added. After the volume is adjusted to 1000 mL with H_2O bidest, the pH is adjusted with 1M HCL to 6.0. Then the medium is sterilized for 30 minutes at 121° C in the autoclave.

2.2. Inoculum preparation

Sporulating mycelium of each sub-sample is transferred onto the medium with a sterile piner (2 petridishes per sub-sample, 5 inoculation-points per petridish). The petridishes are incubated at 20°C, 16h white light and 60% RH for about 7 days. The dry and sporulating mycelium in then transefered on a new GEA- plate (only 1 infection-point per petridish) and incubated again for 7 days at 20°C, 16h white light and 60% RH. This procedure is repeated as often as necessary until the isolates are free of contaminations. The isolates are then tested with the fungicides.

3. In vitro “photometric Method”

3.1.Medium

The recipe for 1 L GG medium is as follows:

- 1.5 g KH_2PO_4
- 0.75 g $MgSO_4 * 7\ H_2O$
- 4 g Gelatine from porcine skin
- 4 g Glucose

dissolved in 1000 mL H_2O bidest

After 20 minutes sterilization @ 121°C in the autoclave, the medium is cooled down to room temperature.

Note: The medium on which the sensitivity of *Botrytis cinerea* to aniline-pyrimidines is evaluated must be methionine free.

3.2. Inoculum preparation

The spores are collected from the sporulating GEA-plates by scrabbing them carefully with a sterile coton-swab in GG-medium. The mycelia- spore suspension is filtered through a sterile 5ml pipette tip layered with glass wool, to remove the mycelium fragments. The spore density is adjusted to approximatively 1mio sp/ml, using a table photospectrometer. (the OD must be between 0.281 and 0.315 at

600nm). The spore suspensions can be stored at 4°C for at least 24 hours before testing.

4. Plate preparation

The sensitivity test is carried out in 96-well flat- bottomed plates.

Fungicides: Cyprodinil (CDL), as, diluted to a stock solution of 500ppm in DMSO
Fludioxonil (FDL), 10'000ppm in DMSO

Final concentrations range of both fungicides:

0 – 5 – 2 – 0.8 – 0.32 – 0.13 – 0.05 – 0.02 – 0.008 – 0.0033 – 0.0013 – 0.0005 ppm

At first, the fungicides are diluted in GG-Medium in the range of : 50 – 20 – 8 – 3.2 – 1.3 – 0.5 – 0.2 – 0.08 – 0.03 – 0.013 – 0.005 – 0 ppm in a 96 deep-well plate, and 15µ of each concentration is distributed in 96 flat-bottomed well plates (4 replicates per concentration). Each spore supension is diluted 1:9 (2ml spore suspension + 16ml GG-medium), and 135µl of it is given in each well to dissolve the fungicide 1:10 and obtain the final desired concentration.

4.1. Photometric measurment and incubation

The photometric measurment is done twice, using a photospectrometer at OD 492nm, the first time immediately after application (0h), the second time after 3 days (72h). Between those two measurments, the plates are incubated in a growth chamber at 20°C in the dark

4.2. Reference strains

For each test, the Cyprodinil sensitive strain #79 (ex CH 9.83) and the Cyprodinil resistant strain #199 (ex CH 52.00) are tested in order to be used as reference

4.3. Data analysis

The test is assessed by substracting the photometric value 0h from the value 72h for estimating the growth value in each well. The results are converted in EC-50 value using the AGSTAT- program.

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