Species:	*Rhynchosporium secalis*
Product Class(es):	QoI, SBI and AP fungicides, and also suited for other fungicide classes
Method type described:	microtiter plate test
Date of protocol:	2006-01
Proven for	Azoxystrobin, Cyprodinil, Propiconazole, Prothioconazole
Should be suitable for	other QoI-SBI-Fungicides and active ingredients from other classes. Protocol adjustments may be needed due to the individual compound characteristics.
Version	1
comments	• proven methodology for the active ingredients listed above. Others not mentioned have to be evaluated carefully to ensure valid results • validated routine method for labs equipped with microtiter plate technique

Method:

In vitro assay on AE media in 24 well plates.

Fungicides and Concentrations

Azoxystrobin (AZ)	10'000 ppm stock in Aceton 100/10/1/0.1/0.01/0 ppm
Cyprodinil (CDL)	1'000 ppm stock in Aceton 10/1/0.1/0.01/0 ppm
Propiconazole (PPZ)	EC250, 5'000 ppm in water 500/100/10/2.5/0.5/0 ppm
Prothioconazole (PTC)	EC250, 5'000 ppm in water 500/100/10/2.5/0.5/0 ppm

Procedure

- Prepare AE media (see recipe below). In order to exclude bias of alternative oxidase activity in the in vitro assays, 200µM SHAM (Salicylhydroxamic acid) is added to the AE medium for selected isolates.
- Make up fungicide stock solutions and dilutions (10x higher than the final concentration in the well): stock solutions as described above, all dilutions in sterile water. For control treatment either set up a 10% Aceton solution (AZ, CDL) or only use water (PPZ, PTC).
- Pipet from each dilution 100uL into the 24 well plate.

Plate setup:

Az	rep 1	control	0.01	0.1	1	10	100	PPZ	rep 1	control	0.5	2.5	10	100	500
	rep 2	control	0.01	0.1	1	10	100		rep 2	control	0.5	2.5	10	100	500
CDL	rep 1	control	0.01	0.1	1	10	empty	PTC	rep 1	control	0.5	2.5	10	100	500
	rep 2	control	0.01	0.1	1	10	empty		rep 2	control	0.5	2.5	10	100	500

- Add 900uL of AE media (cooled down to ca. 50°C).
- Shake the 24 well plates by hand by rotating them approximately three times.
- Let the agar get dry.
- Prepare spore suspension: take one up to two petri dishes from each *R. secalis* isolate (grown on PDA, age 10 to 14 days, incubated at 20-22°C in darkness) and add 5-10mL of sterile water. Scrub over the plate with a inoculating loop and poor off the suspension into a sterile falcon tube. Count spores per mL with a Neubauer counting chamber and adjust the density to 100'000 spores/mL or if lower note the spore densitiy achieved (when stored at 4°C the spore suspension is stable for up to one month, probably even longer but no test done yet to prove it!).
- Pipet 50uL of the spore suspensions into each well.
- Incubate the plates in the climate chamber closed in a dark box at 20-22°C for 8 days until assessment.

Assessment

- The assessment is done visually by determining the percentage of growth in one well but also the quality of growth (for example the density) is included into the rating.
- The EC50 value is calculated in AGSTAT 1.59.

AE- Medium

Receipt for 1L Medium:

- 10 g Yeast extract
- 0.5 g $MgSO_4 * 7\ H_2O$
- 6 g $NaNO_3$
- 0.5 g KCl
- 1.5 g KH_2PO_4

- 20 g Bacto Agar
- 20 mL Glycerol

Weigh the salts and dissolve them in part of the water. Measure out glycerol in a cylinder and rinse the cylinder with water. Weigh Yeast extract and agar and add it to the salts and glycerol mixture. Fill with water to 1000mL. Autoclave Medium 20 Min at 121°C.

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