Species:	*Oculimacula* spp. (*Tapesia* spp., *Pseudocercosporella* spp.) Eyespot
Product Class(es):	SBI fungicides
Method type described:	microtiter plate test
Date of protocol:	2006-05
Proven for	Prothioconazole
Should be suitable for	other SBI-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. Sampling:
   Wheat plants with clear visible eyespot symptoms are sampled randomnly within the field preferably at plant growth stage EC75. Stem base segments of about 10-20 cm length should be dried for about 24 h at room temperature before sending to the laboratory.

2. Isolation:
   Wheat stem base segments are washed under tap water for 10 min, then disinfected with NaOCl (2 %; 4 min), rinsed 2 times with sterile distilled water (5 min each) and dried on sterile filter paper. Small fragments of 5 mm^2 are cut at the edge of the necrosis and transferred on Petri dishes containing malt agar supplemented with an antibiotic substance (e.g. enrofloxacin, 25 µg/ml) and

tolclofos-methyl (0.2 µg/ml, to eliminate other upcoming, contaminating fungi). Petri dishes are then incubated at 19°C in darkness for 5 to 7 days.

3. Purification:
   When a typical mycelial colony of *Oculimacula yallundae* (grey mycelium with regular margin) or *O. acuformis* (white mycelium with irregular margin) starts to grow on Malt-agar medium, a small fragment of agar with mycelium is transferred on potato dextrose agar (PDA). Petri dishes are incubated at 19°C in darkness for 1 week and then under near-UV for 2 additional weeks to stimulate conidia production (radial growth >2 mm/d: *O*. *yallundae*; radial growth <2 mm/d: *O*. *acuformis*). A small fragment of agar with mycelium and conidia is then transferred to an Eppendorf tube containing 1.0 ml sterile distilled water. After homogenization, 100 µl of each conidial suspension is poured onto water agar medium supplemented with an antibiotic substance (for example enrofloxacin, 25 µg/ml). After 48 hours of incubation at 19°C in darkness, a single spore *Oculimacula* spp. colony is taken under the stereo-microscope with a sterile needle and transferred on potato dextrose agar (PDA). Petri dishes are incubated at 20°C in darkness for 7 to 10 days.

4. Sensitivity test:
   Potato dextrose broth (PDB) supplemented with antibiotics is autoclaved for 30 minutes at 120°C and allowed to settle at room temperature. Sterile 10 ml polystyrene tubes are filled with 5 ml of sterile PDB and inoculated with a mycelial plug of 5 mm in diameter taken with a cork borer from the margins of a 8-18 day-old *Oculimacula* spp. culture. Tubes are then incubated for 7 days at 19°C under continuous shaking (135 r.p.m.). After this incubation period, mycelium of each *Oculimacula* spp. strain is mixed for 20 seconds with an high speed lab mixer (15.000 rpm). An aliquot of each ground mycelium (0.4 ml) is then added to 4.6 ml of sterile “modified” RPMI 1640 liquid medium (addition of glucose 8 g/l) supplemented with an antibiotic substance (for example enrofloxacine, 25 µg/ml) and the pH adjusted to 7.0 with 1.0 N HCl.
   An appropriate amount of technical compound previously dissolved in dimethyl sulfoxide (DMSO) is added to “modified” RPMI 1640 liquid medium to reach the final concentration.

   Example (final test concentrations in 200 µl):

   - Prothioconazole: 0, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 mg a.i./l

   The final concentration of DMSO into fungicide-amended as well as into fungicide-unamended (control) liquid medium is equivalent to 0.1 % (v:v).
   After homogenization, each well of a 96-well microtitre plate is poured with 100 µl of “modified” RPMI 1640 liquid medium containing the fungicide (except the control). Then, each well is seeded with 100 µl of the diluted mycelium suspension prepared in “modified” RPMI 1640 liquid medium. For each *Oculimacula* spp. strain 2 replicates are tested (2 rows per strain). The microtitre plates are then incubated in the dark at 19°C for 5 days under continuous shaking (135 r.p.m.).
   Measuring the optical density of the wells containing the mycelium suspensions and fungicide concentrations assesses the extent of mycelial growth. The measurements are made at 0 and 5 days using a microtiter plate reader

(wavelength of 405 nm or 620 nm) and available computer software should be used to capture and process the data. Then, the EC_{50} of each *Oculimacula* spp. strain to the compound is determined.
Reference strains of *Oculimacula* spp. should be included in each sensitivity monitoring test series (O*culimacula yallundae* and *O. acuformis* strains).

Further comment:
Several media are suitable for the isolation (for example Klewitz agar[1]) of *Oculimacula* spp. or for the sensitivity test of SBI fungicides (for example Potato Dextrose Broth, PDB).
The above mentioned `modified´ (glucose amended) RPMI 1640 medium, available e.g. at Sigma-Aldrich, is specifically suitable for prothioconazole.

[1] Preparation of Klewitz agar:

Klewitz-agar:

Hansteen-Cranner solution	250 ml
trace element solution	0.5 ml
sterile water	750 ml
agar-agar	15 g
antibiotic substance	25 mg (if enrofloxacin is used)

Hansteen-Cranner solution:

solution a:	0.5 ml
solution b:	0.5 ml
solution c:	0.5 ml
solution d:	5.0 ml
trace element solution:	5.0 ml
sterile water:	3.5 l

solution a:	$Ca(NO_3)_2$	8.2 g
	$CaCl_2 \times 2H_2O$	4.0 g
	NaCl	1.5 g
	sterile water	1.0 l
solution b:	KH_2PO_4	4.5 g
	sterile water	1.0 l
solution c:	$MgSO_4 \times 7H_2O$	6.2 g
	sterile water	1.0 l
solution d:	$FeCl_3 \times 6H_2O$	5.0 g
	sterile water	100 ml
trace element solution:	$Al_2(SO_4)_3$	55.56 mg
	KJ	27.78 mg
	KBr	27.78 mg

TiO_2	55.56 mg
$SnCl_2 \times 2H_2O$	27.78 mg
LiCl	27.78 mg
$MnCl_2 \times 4H_2O$	388.89 mg
H_3BO_3	611.11 mg
$ZnSO_4$	55.56 mg
$CuSO_4 \times 5H_2O$	55.56 mg
$NiSO_4 \times 6H_2O$	55.56 mg
$Co(NO_3)_2 \times 6H_20$	55.56 mg
As_2O_3	5.56 mg
sterile water	1000 ml

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