Species:	*Mycosphaerella graminicola, Septoria tritici*
Product Class:	QoI, SBI fungicides, and also suited for other fungicide classes
Method described:	microtiter plate test
Date of protocol:	2006-05
a.i. s proven	Azoxystrobin, Pyraclostrobin Epoxiconazole, Cyproconazole, Prothioconazole
Should be suitable for	other QoI- and SBI-Fungicides and Fungicides from other classes. 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 ingredients listed above. Others not mentioned have to be tested to ensure valid results.

Method:

1. Sample colletion

Wheat leaves with visible symptoms preferably with pycnidia are wrapped in some dry paper towel and packed into a paper envelope and are shipped to the lab.
One sample should consist 10-30 leaves and the leaves should be collected randomly from the plot.
The leafs are dried, packed into new paper towel if necessary and stored at 4°C till the isolation. The information from the sampling information sheet is written in the sample tracking database.

1.1. Media:
1.1.1 V8 Juice Agar:

Receipt for 1L Medium:

- 200mL V8 Vegetable Juice
- 3g $CaCO_3$
- 20g Bacto Agar

Measure the V8 with a cylinder ad $CaCO_3$ and Agar and adjust with H_2O bidest. to 1000 mL. Adjust the pH before autoclaving to 6.3. Sterilise it for 30 min at 121°C.

1.1.2 V8 Juice Agar & Streptomycin& Rifampicin:

Receipt for 1L Medium:

- 200mL V8 Vegetable Juice
- 3g $CaCO_3$
- 20g Bacto Agar
- 100mg Streptomycin
- 100mg Rifampicine

Measure the V8 with a cylinder ad $CaCO_3$ and Agar and adjust with H_2O bidest. to 1000 mL. Adjust the pH before autoclaving to 6.3. Sterilise the media for 30 min at 121°C.
Cool down to 50°C and add the Streptomycin and Rifampicine. =>dissolve the 100mg Streptomycin in 1mL EtOH 100% and the 100mg Rifampicin in 5mL EtOH 100% and pipette both into the V8 Juice agar. Before preparing the plate mix it well on a stirrer plate.

1.2. Preparation of isolates and Inoculum:

For the isolation cut leaf with symptoms into 2 cm long pieces and surface sterilise for 2 minutes in 2% Javel water. Rinse samples with sterile H_20 bidest in order to remove the javel.
Leaf pieces (upper side of the leaves upwards) were placed in petri dishes with wet filter paper (use ca. 1 ml H_20 for a 6cm dish) and incubate dishes at 20°C under black Light (NUV) 24-48 hours.

Under the binocular single or several cirri are picked with a fine needle and transferred to a V8 agar plate with antibiotics. The plates are incubated for 4 to 7 days at 20°C under cool white light.
On a clean place pick some septoria conidia (S. tritici produce on V8 agar almost only conidia and no mycelium) with an inoculation loop and plate it on a new V8 plate without antibiotics and incubated it under cool white light for another 7 days at 20°C.
To use the obtained strains in biotest they are transferred (plate on) to V8 plates without antibiotics and incubated for 7 days under cool white light at 20°C.

2. In vitro „96-well Test“

2.1. Medium

2.1.1 PDA- Medium

Receipt for 1L Medium:

- 39g Difco Potato Dextrose Agar Cat#213400

Mix the PDA Powder with H_2O bidest. and adjust to 1000 mL. Sterilise the media for 20 min at 121°C.

2.2. Fungicides & concentrations:

2.2.1. Fungicides

Azoxystrobin	100 / 10 / 1 / 0.1 / 0.01 / 0.001 / 0	mg ai / L
Pyraclostrobin	10 / 1 / 0.1 / 0.01 / 0.001 / 0.0001 / 0	mg ai / L
Epoxyconazole	3 / 1.1 / 0.37 / 0.12 / 0.04 / 0.013	mg ai / L
Cyproconazole	10 / 3 / 1.1 / 0.37 / 0.12 / 0.04	mg ai / L
Prothioconazole	30 / 10 / 3 / 1.1 / 0.37 / 0.12	mg ai / L

All fungicides are used as technical material dissolved in DMSO. In order to exclude bias of alternative oxidase activity in the in vitro assays, 200µM SHAM (Salicylhydroxamic acid) is added to the PDA medium for selected isolates.

2.2.2. Preparation of the 96 well plates:

Prepare PDA, for each Isolate and Fungicide 3mL are needed. After autoclaving the medium cool down to 50°C.
Prepare the fungicide dilution series at 10 times higher than the end concentration. Pipette 20µL of the corresponding fungicide solution in each well and add 180µl medium.

2.2.3. Inoculation of the plates:

For each isolate 1 petridish is needed (Ø 6cm). With a sterile loop a piece only one eyelet of the fungus is taken and well mixed in approximate 10 mL sterile H_2O bidest. The spore suspension should have a density of 100000 Sp/ mL. Each well will be inoculated with 20µL spore suspension.
The plates are incubated for 6 days at 20°C in the dark.

2.2.4. Evaluation:

Percent growth is accessed visually and EC50 estimated using AGSTAT computer package (Syngenta internal).

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