Species:	*Pyrenophora tritici-repentis, Drechslera tritici-repentis*
Product Class(es):	QoI, SBI fungicides, and also suited for other fungicide classes
Method type described:	microtiter plate test
Date of protocol:	2006-01
Proven for	Azoxystrobin, Cyprodinil, Propiconazole
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:

1. Sample colletion

Leaf samples with symptoms or stems with pseudothecia wrapped in a paper towel are send to the lab.

1.1. Media:

1.1.1. Malt Agar & Chloramphenicol:

Receipt for 1L Medium:

- 20g Malz Extract
- 20g Bacto Agar
- 100mg Chloramphenicol

Weight Malz Extract and Agar and adjust with H_2O bidest. to 1000 mL. Autoclaved 20 min at 121°C. Cool down to 50°C and add 100mg Chloramphenicol =>dissolve 100mg Chloramphenicol in 1mL EtOH 100%.

1.1.2. PCA:

Receipt for 1L Medium:

- 20g carottes
- 20g potatos
- 20g Bacto Agar

Peal potato and carottes, and blend, add 20g Agar and adjust with H_2O bidest to 1000mL. Autoclave Medium 20 min at 121°C.

1.1.3. Wheat leaf Agar:

Receipt for 1L Medium:

- 70g fresh wheat leaves
- 20g Bacto Agar

Homogenize wheat leaves add 20g Agar and adjust with H_2O bidest to 1000mL. Autoclave Medium 20 min at 121°C.

1.2. Preparation of isolates and Inokulum:

Cut leaf or stem samples with symptoms into 2 cm long pieces and surface sterilise for 2 min in 2% Javel water. Rinse samples with H_20 bidest in order to remove the javel water.
Leaf pieces (upper side of the leaves upwards) or stems were placed in petri dishes with very wet filter paper (use 3 ml H_20 for a 8cm dish) and incubate dishes at 20°C under black light (NUV) 2-4 days.
On the leaf pieces conidiophores emerge and eventually spores are produced, predominantly at the edges of the leaves. On the stems the pseudothecia ripe and become “softer”.
Under the binocular single spores are picked with a fine needle and transferred to a malt agar plate (& Chloramphenicol). The pseudothecia from the stems are picked with a pincer, opened and also transferred to malt agar. The plates are incubated for 4 to 5 days at 20°C under black light (NUV).
A small part of the growing mycelium is transferred to new PCA plate and incubated under black light for another 8 to 10 days at 20°C.
To use the obtained strains in biotest they are transferred (mycelium disks) to wheat agar plates and incubated for 14 days under black light (NUV) at 20°C.

2. In vitro „24well Test“

2.1. Medium

2.1.1. 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.

2.2. Fungicides & concentrations:

2.2.1. Fungicides

Azoxystrobin	10 / 1 / 0.1 / 0.01 / 0.001 / 0	mg ai / L
Cyprodinil	10 / 1 / 0.1 / 0.01 / 0.001 / 0	mg ai / L
Propiconazole	100 / 10 / 1 / 0.1 / 0.01 / 0	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 AE medium for selected isolates.

2.3. Procedure:

2.3.1. Preparation of the 24 well plates:

Prepare AE Medium, for each Isolate and Fungicide 12mL are needed. After autoclaving the medium cool down to 50°C.
Prepare the fungicide dilution series at 10x. Mix 900µl medium with 100µl Fungicide in each well.

2.3.2. Inoculation of the plates:

For each isolate 1 petri dish is needed (Ø 8cm). Inoculum =>only conidiopores are scrapped with a sterile glass (microscope object holder) and suspended in 5ml water. 100µl suspension is sprayed in each well.
The plates are incubated for 5 days at 20°C at dark.

2.3.3. Evaluation:
Percent growth is accessed visually and EC50 estimated using AGSTAT computer package (Syngenta internal, also other statistical programmes are suitable for EC50 estimation).

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