Pyrenophora teres f. *maculata*, the cause of Pyrenophora leaf spot of barley in South Africa

G. F. CAMPBELL[1], P. W. CROUS[1]* AND J. A. LUCAS[2]

[1] *Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa*
[2] *IACR Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, BS18 9AF, U.K.*

Net blotch caused by *Pyrenophora teres* is a serious disease of barley in many cereal production areas world-wide, including the Western Cape province of South Africa. The pathogen occurs as two forms, *P. teres* f. *teres*, which produces net-blotch symptoms, and *P. teres* f. *maculata* which produces leaf spots. *Pyrenophora japonica* and *P. hordei*, which have also been reported in South Africa, also produce spots on susceptible barley cultivars. Using RAPD markers, spot-forming isolates from the South African population were found to be relatively uniform. Single ascospores were obtained from pseudothecia after *in vitro* mating had occurred between a verified *P. teres* net-blotch isolate from Denmark and a representative Pyrenophora leaf spot isolate from South Africa. Using amplified fragment length polymorphism (AFLP) and RAPD markers, recombination was demonstrated in the progeny which had DNA banding patterns different from the two parental isolates. Pathogenicity trials also confirmed that recombination had taken place during mating. Inoculations were conducted on differential cultivars susceptible to the net-blotch or leaf spot forms. The two parents induced typical net-blotch or leaf spot symptoms whereas the progeny mostly induced a jagged spot symptom on each cultivar. Fungicide sensitivity tests using the ergosterol biosynthesis inhibitors triademinol, bromuconazole and triticonazole showed that, due to recombination, some progeny could have increased resistance to these fungicides. Due to mating and subsequent recombination between a net blotch isolate of *P. teres* and a representative leaf spot isolate, it was concluded that the latter was *P. teres* f. *maculata*. These results contrast with the earlier belief that Pyrenophora leaf spot isolates in the Western Cape are *P. japonica* and *P. hordei*.

Correct identification of the causal agent of a disease on agricultural crops as well as knowledge of genetic variability of the pathogen (McDonald & Martinez, 1990) are important for breeding programmes directed at producing resistant cultivars, and for developing strategies to exploit resistance (Leung, Nelson & Leach, 1993).

Pyrenophora teres Drechsler (anamorph *Drechslera teres* [Sacc.] Shoemaker) the cause of net blotch of barley (*Hordeum vulgare* L. emend. Bowden) is an economically important pathogen in South Africa and throughout most other barley growing regions of the world (Shipton, Kahn & Boyd, 1973; Steffenson, Webster & Jackson, 1991; Louw, Crous & Holz, 1996). Two types of leaf symptoms are associated with net blotch, a net-like symptom which produces elongated, light brown lesions with dark brown reticulations, and a leaf spot symptom which is dark brown with a distinct halo (Smedegård-Petersen, 1971). *P. japonica* S. Ito & Kurib. [anamorph *Drechslera tuberosa* (G. F. Atk.) Shoemaker] was originally identified as causing the leaf spot symptoms, whereas *P. teres* has been associated with net-blotch lesions (Ito & Kuribayashi, 1931; Shoemaker, 1962). McDonald (1967), however, reported after successfully mating net-blotch and leaf spot isolates, that the latter were mutant forms of *P. teres*. Smedegård-Petersen (1971) repeated these matings with Danish isolates, and concluded that the two types were forms of the same biological species, for which he proposed the names *P. teres* f. *teres* for the net-blotch causing isolates, and *P. teres* f. *maculata* Smed.-Pet. for the leaf spot form. Each form is capable of causing economic yield losses (Jordan, 1981; Jordan, Best & Allen, 1985; Martin, 1985; Deadman & Cooke, 1987; Delserone & Cole, 1987; Steffensen *et al.*, 1991). Scott (1991) identified the cause of leaf spot of barley in South Africa as *P. japonica*. This was supported by Louw, Crous & Holz (1994), who also identified the teleomorph from barley stubble and from crosses between leaf spot isolates in culture as *P. japonica*. More recently *P. hordei* Wallwork, Lichon & Sivan., which also causes a leaf spot of barley, has been isolated in South Africa (Scott, 1994; Den Breeÿen, Crous & Holz, 1996). Using restriction fragment banding patterns of A + T-rich total DNA, Crous *et al.* (1995) showed that verified *P. japonica* and *P. teres*. f. *maculata* isolates were almost identical, and that *P. japonica* should be treated as a synonym of *P. teres*. Furthermore, Crous *et al.* (1995) reported successful mating between a Danish leaf spot and an Australian net-blotch isolate. When single ascospore isolates were inoculated onto differentially susceptible barley cultivars, net-blotch, leaf spot and intermediate symptoms were produced, suggesting that recombination had occurred. Pathogenicity trials conducted on putative spot × blotch hybrids in other studies by Smedegård-Petersen (1976, 1977)

* Corresponding author.

yielded similar results. Molecular genetic evidence for hybridity, however, was not obtained.

Due to conflicting reports concerning the identity of the *Pyrenophora* isolates producing leaf spots on barley in South Africa, there is still uncertainty about the species involved (Scott, 1991, 1994, 1995; Crous *et al.*, 1995; Louw *et al.*, 1995; Den Breeÿen *et al.*, 1996). The net-blotch and leaf spot forms of *P. teres* both cause disease on a number of susceptible cultivars, but are controlled by different chemical control programmes (Scott, 1995), thereby making identification of the correct species important. The aim of the present study was, therefore, to mate a verified *P. teres* net-blotch isolate with a Pyrenophora leaf spot isolate representative of a collection of leaf spot isolates from South Africa, and to subsequently analyse the progeny for recombination using amplified fragment length polymorphisms (AFLPs) (Zabeau & Vos, 1993) and random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) as molecular genetic markers. Furthermore, symptom expression of the progeny on two differential barley cultivars was assessed, and the sensitivity of the parental isolates and their progeny to the major triazole fungicides used against this pathogen was also determined.

MATERIALS AND METHODS

Isolate maintenance and mating studies

The isolates used were: Pt90-8a (Danish net-blotch); KH334 (Australian net-blotch); Nap5 (South African net-blotch) and MP4 (South African leaf spot). Isolate MP4 was chosen as a representative of the South African Pyrenophora leaf spot population which was characterised using A+T-rich DNA banding patterns (Crous *et al.*, 1995) and RAPDs (unpublished results). Isolates were maintained on potato dextrose agar (PDA) slopes at 4 °C. Matings of the four isolates in all possible combinations and as single isolates were made on barley agar (BA) consisting of sterile barley straws lain on water agar in Petri dishes (Louw *et al.*, 1995), which were kept moist in larger containers and incubated at 10° under nuv light for 1 yr. As only a few pseudothecia formed asci with ascospores after this period, plates were subsequently transferred to 4° for a further 6 mo. Protopseudothecia were periodically examined microscopically for the production of asci with ascospores. To induce ascospore release from pseudothecia, fruiting bodies were removed from barley stalks and soaked in water for 2 h. Pseudothecia were subsequently attached to the inner surface of a Petri dish lid with the aid of petroleum jelly. After 24–48 h at 25° in the dark, ascospores were ejected from the pseudothecia on to PDA plates. Single germinated ascospores were subsequently transferred to clean PDA plates, and stored on slants. Ascospore progeny were numbered GC1–GC23, and are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch, South Africa.

DNA isolation

Mycelial plugs from 7-day-old cultures were transferred to flasks containing 100 ml of a yeast extract and glucose medium (YEG) [8 g l^{-1} yeast extract and 5 g l^{-1} glucose]. Flasks were incubated on a rotary shaker at 150 rpm at 25° for 3 d. Due to excessive polysaccharide production by this fungus in liquid culture harvesting of mycelia with a Buchner funnel was difficult. Mycelia were, therefore, harvested by centrifugation at 5000 rpm for 5 min at 4°. Once harvested, mycelium was stored at −80° until required, when it was crushed in liquid nitrogen with a mortar and pestle, and transferred to an Eppendorf tube containing 500 µl extraction buffer of 50 mM Tris (pH 7·2), 50 mM NaCl, 50 mM EDTA, and 3% (w/v) SDS. Then 350 µl phenol was added followed by 150 µl chloroform/isoamylalcohol (24:1, v/v). The suspension was mixed, incubated while being shaken at room temperature for 15 min, and subsequently centrifuged at 13 000 rpm for 60 min. The aqueous phase was transferred to a clean Eppendorf tube after which 25 µl RNase (10 mg ml^{-1}) (Boehringer Mannheim Chemicals, South Africa) was added and incubated for 30 min at 37°. An equal volume of chloroform was added followed by centrifugation at 13 000 rpm for 10 min. The aqueous phase was transferred to another tube and subjected to two more chloroform extraction procedures. DNA was precipitated with 0·54 vol. isopropanol and incubated at −20° for 2 h, and pelleted by centrifugation at 13 000 rpm for 5 min. The DNA was subsequently washed twice in 70% ethanol, dried, resuspended in 100 µl TE buffer [50 mM Tris (pH 8·0), 50 mM EDTA] and stored at −20° for future use. In DNA samples with excessive polysaccharides, the volume was increased to 400 µl with TE buffer. An equal volume of Phenol/chloroform/isoamylalcohol [25:24:1 (v/v)] was added followed by centrifugation at 13 000 rpm for 10 min. The aqueous phase was extracted three more times with an equal volume of chloroform/isoamylalcohol. The DNA was subsequently precipitated by the addition of 7·5 M NH_4OAc to a final concentration of 2 M and 2 vol. 100% cold ethanol. Samples were washed with 70% cold ethanol, dried and resuspended in 100 µl TE buffer.

RAPD analysis

Amplification reactions were conducted in a final volume of 25 µl of reaction mixture. The reaction mixture contained 2·5 µl of 10X *Taq* DNA polymerase buffer [100 mM Tris HCl (pH 8·3), 15 mM $MgCl_2$, 500 mM KCl] (Boehringer Mannheim, South Africa); 200 µm of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1 U of *Taq* DNA polymerase. The final $MgCl_2$ concentration was adjusted to 4 mM. Reaction mixtures were overlaid with 50 µl mineral oil to prevent evaporation during thermocycling. Primers (Operon Technologies Inc., Alameda, U.S.A.) that gave discriminatory banding profiles for the two parental isolates and which could, therefore, reveal genetic recombination in the ascospore progeny were used for RAPD analysis; the primers as well as their respective sequences were:

OPE 7: 5′ AGATGCAGCC 3′,
OPE 15: 5′ ACGCACAACC 3′,
OPM 10: 5′ TCTGGCGCAC 3′,
OPM 20: 5′ AGGTCTTGGG 3′.

Amplifications were made in a Biometra TRIO-Thermoblock TB1 (Gottingen, Germany). Reactions underwent an initial denaturation process at 96° for 120 s, followed by 30 cycles

of 92° for 30 s, 38° for 30 s and 72° for 60 s. After the last cycle a final extension step was done at 72° for 120 s. Amplification products were separated through 1·5% (w/v) agarose gels in TAE buffer (Sambrook *et al.*, 1989).

AFLP analysis

Generation and selection of fragments. The AFLP analysis was done following the procedure of Zabeau & Vos (1993). For preparation of primary template DNA, approximately 50 ng genomic DNA was digested at 37° for 1 h using 20 U of *Pst*I, 4 U of *Mse*I and 5 µl reaction buffer (One Phor All buffer, Pharmacia Biotech) in a final vol. of 50 µl. Following digestion the adapter molecules were ligated to the cleaved DNA fragments. This was done by adding 10 µl of a mixture containing 5 pmol of *Pst*I adapter; 50 pmol of *Mse*I adapter; 1·2 µl of a 10 mM ATP solution; One Phor All buffer and 1 U of T4-ligase (Pharmacia Biotech). This 60 µl restriction fragment ligation mixture was subsequently incubated at 37° for 2 h. The sequence of the *Pst*I adapter was:

5′-biotin-CTCGTAGACTGCGTACATGCA-3′,
3′-CATCTGACGCATGT-5′.

The sequence of the *Mse*I adapter was:

5′-GACGATGAGTCCTGAG-3′,
3′-TACTCAGGACTCAT-5′.

Dynabeads M-280 streptavidine (Dynal, Oslo, Norway) were used to select biotinylated DNA fragments. Before use, the beads were washed with 1 vol. TE buffer and resuspended in 1 vol. TE buffer. To each DNA sample (60 µl) 10 µl of resuspended beads was added. Samples were subsequently incubated at room temperature for 30 min. The beads were then collected using a magnet (Dynal MPC). The supernatant was removed and the beads were washed three times with 200 µl TE buffer. After the final washing step the beads were resuspended in 50 µl TE buffer.

PCR amplification. Selective amplification was done with four AFLP primers specific for *Pst*I adapters. Each primer contained two selective nucleotides at the 3′ end. A total of four primer combinations was used as the sequence of the *Mse*I primer was kept constant. The sequences of the *Pst*I primers was:

*Pst*I.1 5′GACTGCGTACATGCAG**AC** 3′,
*Pst*I.2 5′GACTGCGTACATGCAG**AA** 3′,
*Pst*I.3 5′GACTGCGTACATGCAG**CC** 3′,
*Pst*I.4 5′GACTGCGTACATGCAG**CA** 3′.

The sequence of the *Mse*I primer was:

5′GATGAGTCCTGAGTAAACA 3′.

One primer was labelled according to the manufacturer's recommendations using 1 µCi ^{33}P-ATP (Amersham) and 0·2 U of T4-kinase (Pharmacia Biotech). Each amplification was performed with 30 ng of labelled and 30 ng of unlabelled selective primer in the reaction mixture.

Each PCR reaction contained the following: 2 µl PCR buffer [100 mM Tris-HCl (pH 8·0), 15 mM $MgCl_2$, 500 mM KCl], 1 µl of template DNA, 1 µl labelled *Mse*I primer (30 ng), 1 µl *Pst*I primer (30 ng), 3 µl of a 1·25 mM solution, 0·2 µl *Taq* polymerase (Gibco BRL) and 13 µl ddH_2O. Amplifications were done in a Stratagene Robocycler Gradient 40. Reactions underwent an initial denaturation process at 94° for 60 s. This was followed by 10 cycles of 94° for 40 s, 62° for 60 s and 72° 60 s. A further 25 cycles was conducted in which the annealing temperature was lowered to 56° for 60 s. After the last cycle a final extension step was done at 72° for 20 min.

Reaction products were denatured at 95° for 3 min, snap-cooled on ice and run through 6% (w/v) denaturing polyacrylamide sequencing gels at 35 W for 3 h in 1X TBE buffer (Sambrook *et al.*, 1989) using a Model S2 sequencing gel system (Life Technologies, Inc., Gaithersburg, MD). The gels were dried with a Model 583 Bio-Rad gel dryer and exposed to Kodak Biomax MR1 X-ray films for 12 h at room temperature.

Data analysis

For RAPD and AFLP analyses, DNA fingerprints were evaluated by visual inspection of photographs of the gels, and autoradiographs respectively. All monomorphic and polymorphic fragments which were observed as dark intense bands were used for analysis. Data was scored in the form of the presence or absence of each fragment within each individual isolate and then pooled over all fragments and primer(s) (combinations) in order to assess the degree of (co)segregated fragments. Recombination in progeny was identified by the cosegregation of bands that distinguished the parents or by the absence of a band present in both parental isolates. The proportion of cosegregated bands in the progeny to the total number of bands was also assessed.

Pathogenicity trials

Mycelial plugs from 7-day-old cultures were transferred to flasks containing 100 ml YEG medium which were incubated at 25° on a rotary shaker at 150 rpm for 3 d. Mycelia were harvested by centrifugation at 5000 rpm for 5 min and subsequently resuspended in 25 ml ddH_2O. This was followed by homogenisation of the mycelium with a Virtis homogenizer (Virtis Company Inc., U.S.A.).

The two differentially susceptible barley cvs Stirling and B87/14 (susceptible to net-blotch and leaf spot, respectively) were used in the pathogenicity trials. Plants were incubated at the two leaf stage in a glasshouse (20–15°, day/night temperature) using the technique described by Louw *et al.* (1994). Plants were initially sprayed with a solution of 0·01% (v/v) Tween 20 to reduce leaf surface tension, and subsequently sprayed to runoff with the mycelial suspensions. Plastic bags were then placed over the inoculated plants for 48 h to create moisture chambers. Control plants were sprayed with distilled water without inoculum. Plants were examined for symptom expression 10 d after inoculation.

Fungicide sensitivity

Both parental isolates and all the progeny were tested for their sensitivity to the major triazole fungicides used to control net-

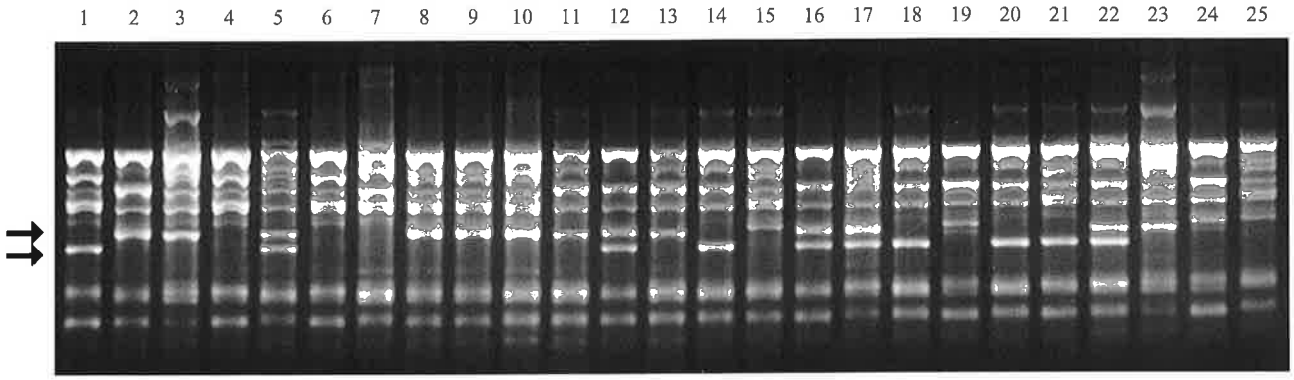

Fig. 1. Segregation pattern of the RAPD marker bands for primer OPM 10. Lanes 1 and 2 are respectively the net-blotch and leaf spot parents while lanes 3–25 show the banding patterns of the progeny. The arrows indicate the bands discriminating the net-blotch and leaf spot parents.

Table 1. Genetic recombination observed in progeny of *Pyrenophora teres* as revealed by RAPD analysis. Recombination is shown as the (co)segregation of parental bands in the progeny using four different primers

	Primers[d]										
	OPE 7		OPE 15		OPM 10		OPM 20		Total (co)segregating bands	Total bands scored[b]	(Co)segregation (%)
Isolate	N[a]	S[a]	N	S	N	S	N	S			
Net	1[c]		0		1		1		—	19	—
Spot	1[c]		1		1		1		—	20	—
GC1	1	1	0	0	0	1	0	0	3	21	14·3
GC2	1	1	0	0	0	0	0	0	2	20	10·0
GC3	1	0	0	0	1	1	1	0	4	22	18·2
GC4	1	1	0	0	0	0	0	0	2	19	10·5
GC5	1	0	0	1	0	0	1	0	3	20	15·0
GC6	1	1	0	1	0	1	0	0	4	21	19·0
GC7	0	1	0	1	0	1	0	1	4	19	21·1
GC8	0	1	0	1	0	1	1	1	5	21	23·8
GC9	0	0	0	1	0	1	0	0	2	17	11·8
GC10	0	0	0	0	1	1	0	0	2	18	11·1
GC11	0	1	0	0	0	1	0	0	2	19	10·5
GC12	0	0	0	1	0	0	0	0	2	19	10·5
GC13	0	0	0	0	0	1	1	1	3	19	15·8
GC14	0	1	0	0	1	1	0	1	4	20	20·0
GC15	0	0	0	0	1	1	0	1	3	20	15·0
GC16	0	1	0	0	0	1	0	0	2	20	10·0
GC17	0	0	0	1	0	0	0	1	3	19	15·8
GC18	1	0	0	1	1	0	0	0	3	20	15·0
GC19	1	0	0	0	1	0	0	0	2	19	10·5
GC20	0	1	0	0	1	1	1	1	4	19	21·2
GC21	1	0	0	0	0	1	1	1	5	20	25·0
GC22	1	1	0	1	0	0	0	1	4	21	19·0
GC23	1	0	0	1	0	0	0	0	3	20	15·0

[a] Net-blotch (N) and leaf spot (S) parental isolates.
[b] Total number of bands scored for all four primers.
[c] Number of bands unique to parental isolates.
[d] Dominant and (co)segregating bands.

blotch. The technique described by Robbertse, Holz & Crous (1996) was followed. Mycelial plugs (3 mm diam.) from 7-day-old cultures were placed in the centre of PDA plates amended with either triademinol, bromuconazole or triticonazole at various concentrations; these were 0, 1·0, 10·0, 30·0 and 60·0 μg ml^{-1} for triademinol and 0, 0·1, 0·3, 1·0, 3·0 and 10·0 μg ml^{-1} for the other two fungicides. All isolates were tested in triplicate at each concentration for each fungicide. Stock solutions were prepared for each fungicide by dissolving the fungicide in 70% ethanol. For each fungicide, control plates were amended to contain the same amount of solvent as plates containing the highest concentration of fungicide. Plates were incubated inverted at 25° for 5 d. Colony diameters were subsequently derived by averaging two perpendicular measurements and subtracting the diameter of the agar plug. The % inhibition in each isolate at each specific concentration was expressed as the proportion of radial growth on the fungicide-amended plates compared to growth on the control plates. After visual inspection a best curve was fitted to this data. The SAS/STAT software version 6.04

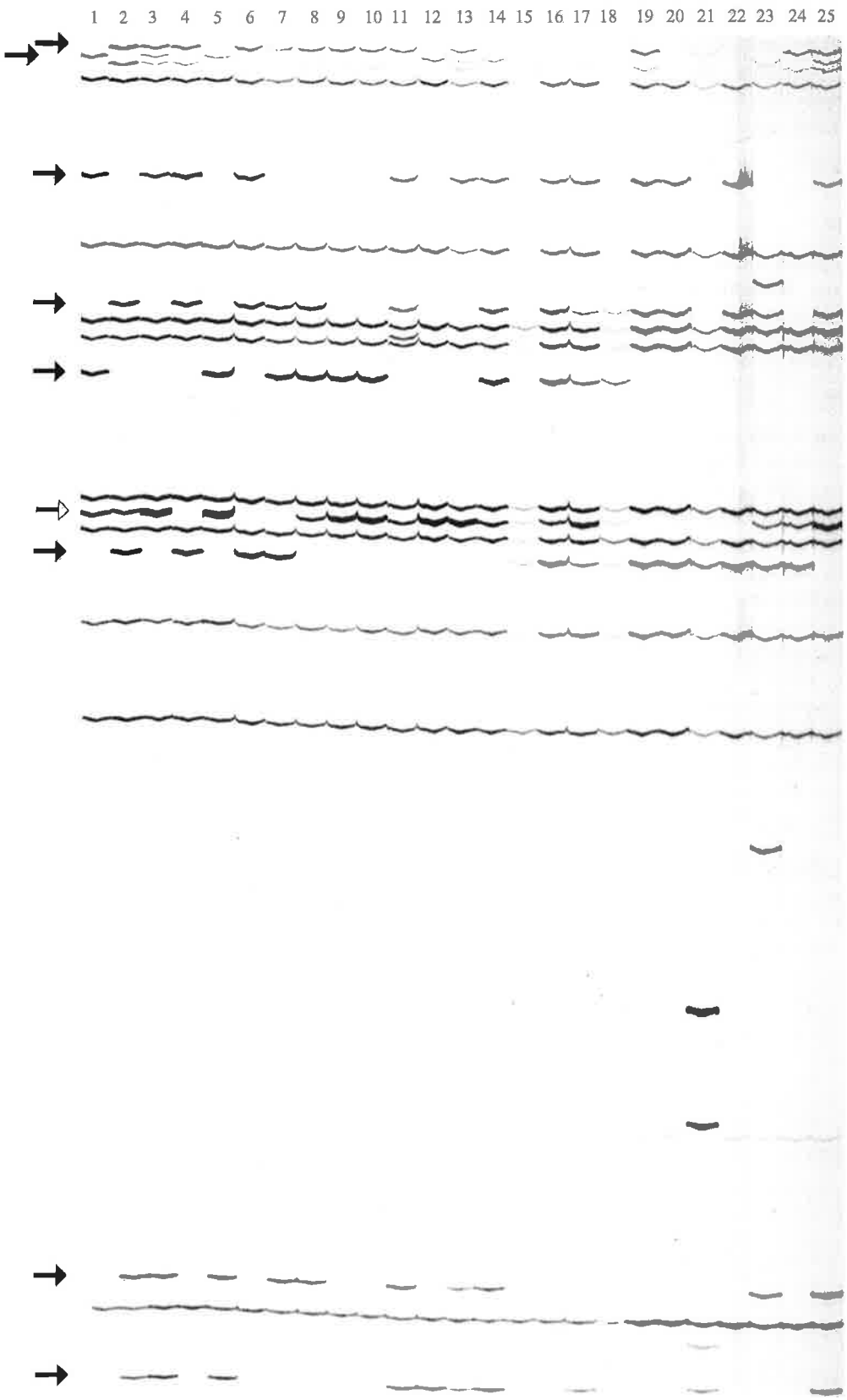

Fig. 2. Segregation pattern of the AFLP marker bands for the primer combination *MseI*/*PstI*.4. Lanes 1 and 2 are respectively the net-blotch and leaf spot parents while lanes 3–25 indicate the banding patterns of the progeny. Solid arrows indicate polymorphic AFLP bands which cosegregate as dominant markers (presence or absence). The open arrow indicates recombination in the progeny due to the loss of a fragment present in both parents.

package was used to calculate the IC_{50} values for each isolate. Nonlinear regression was carried out to calculate the IC_{50} for each isolate by regressing radial growth (as a proportion of the control) against log-transformed fungicide concentrations and using the fitted regression line to estimate IC_{50} values.

RESULTS

Mating studies

Although protopseudothecia were formed in all combinations between isolates, fertile pseudothecia containing mature ascospores were produced only by the MP4 × Pt90-8a mating. From these, 23 single ascospore cultures were obtained.

RAPD analysis

The results of the RAPD analysis of the parental isolates and their progeny are shown in Fig. 1 and Table 1. Of the primers screened OPE 7, OPE 15, OPM 10 and OPM 20 revealed DNA polymorphisms (unique bands) between the parents. All the primers with the exception of OPE 15 yielded unique marker bands in each parent. Recombination in the progeny was observed as the (co)segregation of unique parental RAPD marker bands. In the case of primer OPE 15 the two parental isolates were distinguished by the presence of a single band in the leaf spot parent which was absent in the net-blotch parent. Of the 23 progeny, 18 contained (co)segregating markers from both parents. The number of visible bands that were scored for analysis ranged from 3 to 8 for the different combinations while the number of polymorphic bands varied from 1 to 2. When all four primers were scored together 7 out of a total of 20 bands revealed differences between the parental isolates.

AFLP analysis

AFLPs were used to conclusively show that recombination had taken place during mating, and therefore, that the putative hybrids produced were actually true hybrids. Recombination was confirmed by the cosegregation of parental AFLP marker bands in the progeny. Four primer combinations were used in which AFLP markers were produced for all pairs of combinations (Fig. 2).

Tabulated electrophoretic data is depicted in Table 2. The number of visible bands that were scored for analysis ranged from 16 to 28 for the different combinations while the range of polymorphic bands was between 6 and 11. When all four primer combinations were taken into account, 36 out of a total of 81 bands revealed differences between the parents. All primer combinations revealed genetic markers which could be

Table 2. Genetic recombination observed in progeny of *Pyrenophora teres* as revealed by AFLP analysis. Recombination is shown as the cosegregation of parental bands in the progeny using four different primer combinations

	Primer combinations[f]											
	*Pst*I 1·1		*Pst*I 1·2		*Pst*I 1·3		*Pst*I 1·4					
Isolate	N[a]	S[a]	N	S	N	S	N	S	Absence of bands[b]	Total cosegregating bands[c]	Total bands scored[d]	Cosegregation (%)
Net	5[e]		2		5		3		—	—	60	—
Spot	6[e]		6		3		6		—	—	67	—
GC1	4	4	1	2	4	1	2	4	—	22	68	32·4
GC2	4	2	2	6	3	2	1	4	1	24	67	35·8
GC3	2	5	1	1	2	1	2	2	—	16	61	26·2
GC4	4	2	2	6	2	0	1	4	1	19	68	27·9
GC5	3	4	1	2	1	1	1	5	1	18	63	28·6
GC6	5	3	2	4	4	0	2	4	—	20	70	28·6
GC7	1	4	0	5	2	2	1	2	—	12	62	19·4
GC8	1	3	0	4	2	2	1	2	—	11	61	18·0
GC9	3	3	0	5	4	1	2	5	—	18	73	24·7
GC10	2	1	1	2	3	2	1	2	—	14	59	23·7
GC11	4	3	1	3	4	1	2	4	—	22	68	32·4
GC12	4	1	2	1	3	1	2	3	—	17	64	26·6
GC13	2	3	0	0	4	3	1	3	—	16	63	25·4
GC14	1	2	0	3	1	1	2	2	—	9	55	16·4
GC15	3	5	1	4	5	3	3	3	—	27	72	37·5
GC16	1	3	1	1	6	2	1	2	—	17	59	28·8
GC17	2	2	1	4	2	2	1	4	1	18	63	28·6
GC18	2	3	1	1	1	1	1	2	1	12	55	21·8
GC19	2	3	1	1	1	1	1	4	1	14	60	23·3
GC20	1	4	0	1	2	1	1	2	1	11	59	18·6
GC21	4	4	1	1	4	2	1	3	—	20	68	29·4
GC22	4	5	1	1	1	1	0	3	—	13	63	20·6
GC23	4	1	2	3	5	1	2	5	—	23	67	34·3

[a] Net-blotch (N) and leaf spot (S) parental isolates.
[b] Recombination revealed using primer combination *Pst*I 1·4 was shown by the absence of a band present in both parental isolates.
[c] Primer combinations in which one of the parental isolates yielded no segregating bands were not included in the total number of cosegregated bands.
[d] Total number of bands scored for all four primer combinations.
[e] Number of bands unique to parental isolates.
[f] Dominant and (co)segregating bands.

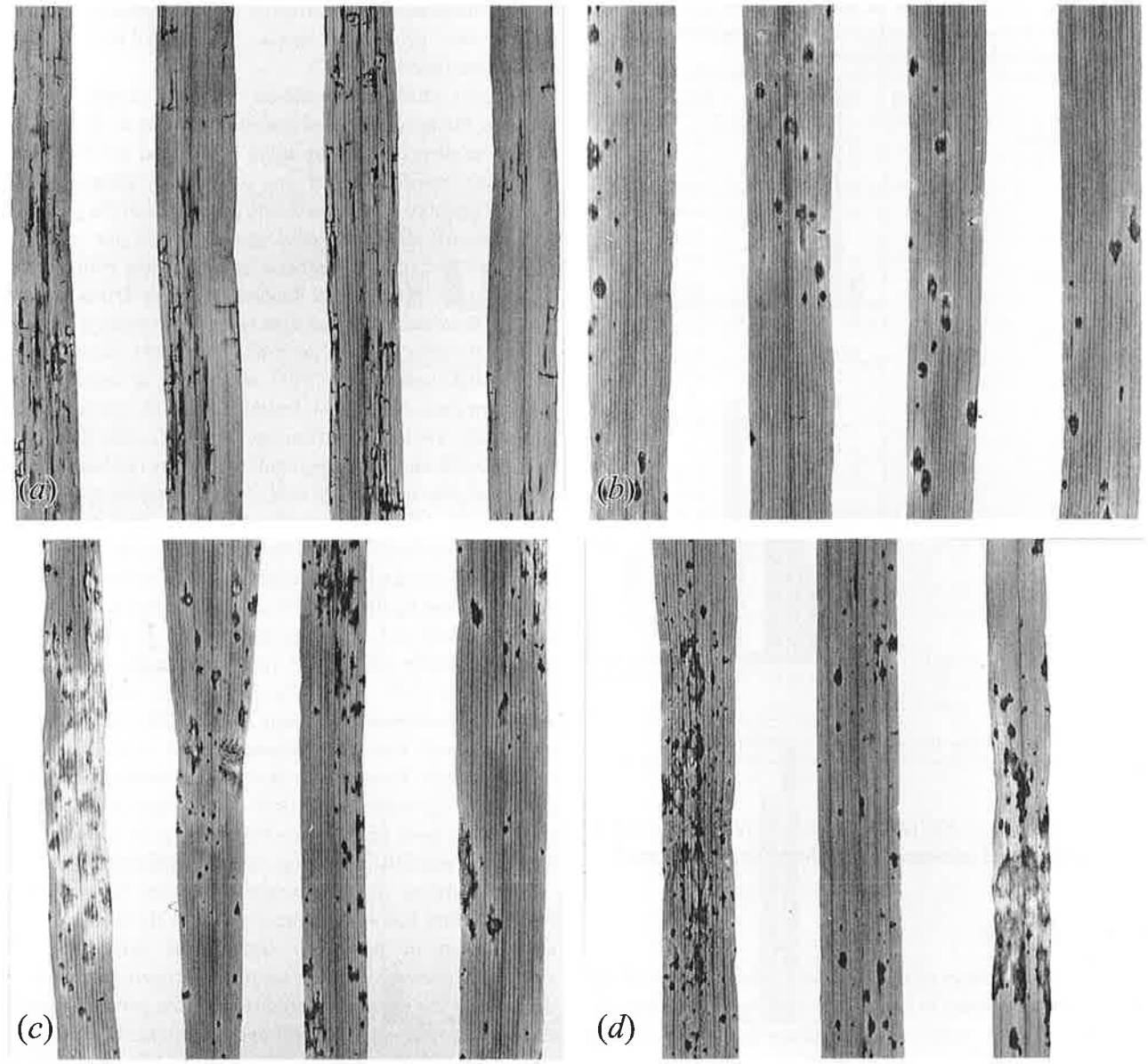

Fig. 3. Symptom expression on cvs Stirling and B87/14. (*a*) Pt90-8a (net-blotch parent) on Stirling, (*b*) MP4 (leaf spot parent) on B87/14, (*c*) isolate GC19 on Stirling and (*d*) GC19 on B87/14.

observed to have cosegregated in the progeny. Of the 23 progeny tested, three of the primer combinations showed recombination in more than 20 while the combination containing *Pst*I.2 showed 16. When the primer combinations were pooled together the range of cosegregated bands amongst the 23 progeny isolates was between 8 and 26 (mean 15·75). Primer combinations which yielded segregating bands from only one parent and not the other were not included as cosegregating bands which could reveal recombination. For the primer combination containing *Pst*I.4 recombination was observed in some isolates as the absence of a band which was present in both parental isolates.

Symptom expression

The verified parental net-blotch and leaf spot isolates produced typical symptoms on the two differentially susceptible cultivars (Figs 3*a*, *b*). Most of the progeny produced symptoms that were intermediate between typical net-blotch and leaf spot symptoms (Figs 3*c*, *d*). In comparison to the well-defined spots produced on cv. B87/14 by MP4, the intermediate symptoms caused by progeny on either cultivar displayed jagged, elongated, brown spots. Around the edges of the intermediate symptoms were areas of chlorotic tissue.

Fungicide sensitivity

The results of the fungicide sensitivity trials are shown in Table 3 and Figs 4*a–c*. For the three fungicides tested several ascospore isolates showed enhanced *in vitro* resistance compared to their net- and spot-type parents. Increased resistance to triademinol was displayed by ascospore isolates GC19 and GC21 while isolates GC18 and GC19 showed resistance to triticonazole. Four isolates showed strong resistance to bromuconazole compared to the parental isolates,

Table 3. IC_{50} values (μg ml^{-1}) of the net-blotch and leaf spot parents and their progeny determined after fungicide testing with the triazoles triademinol, bromuconazole and triticonazole

	Triademinol	Bromuconazole	Triticonazole
Net-blotch parent	0·01	0·01	4·91
Spot parent	5·97	0·14	0·72
GC1	0·01	0·12	0·08
GC2	0·85	0·19	0·64
GC3	0·81	*	0·18
GC4	5·49	0·14	0·47
GC5	10·56	0·20	1·64
GC6	6·61	0·20	1·02
GC7	0·85	*	0·33
GC8	0·11	0·01	0·50
GC9	0·12	*	0·07
GC10	3·36	0·12	*
GC11	*	0·01	0·12
GC12	4·01	0·16	*
GC13	5·42	0·24	0·16
GC14	3·67	*	0·46
GC15	0·77	0·06	0·22
GC16	0·70	5·23	0·53
GC17	*	0·21	0·26
GC18	5·31	2·49	18·78
GC19	39·47	2·51	22·86
GC20	*	1·99	*
GC21	37·65	*	0·25
GC22	5·19	0·08	0·21
GC23	0·97	0·09	0·16

* IC_{50} values fell below the range of concentrations used.

namely GC16 and GC18-GC20. Ascospore isolate GC19 showed increased resistance to all three fungicides tested.

DISCUSSION

The taxonomic status of the *Pyrenophora* leaf spot genotype associated with barley in South Africa has been uncertain. On the basis of the morphological descriptions by Sivanesan (1987), Scott (1991) reported that the *Pyrenophora* sp. causing leaf spot in South Africa was *P. japonica*. Louw *et al.* (1994) concluded that the name of the newly collected teleomorph state for the leaf spot *Pyrenophora* in South Africa was *P. japonica*. The present study supports the claims of Louw *et al.* (1995) that leaf spot isolates identified as *P. japonica* (Scott, 1991) are in fact *P. teres* f. *maculata*. Various other studies have been conducted on *P. teres* involving morphological criteria and mating tests. Smedegård-Petersen (1971) could not distinguish the two pathotypes of *P. teres* using morphological characteristics, and therefore proposed them as two forms, namely *P. teres* f. *teres* (net-blotch) and *P. teres* f. *maculata* (leaf spot). Furthermore, Smedegård-Petersen (1976, 1977) reported that net-blotch and spot-spot isolates could be mated in culture, thereby confirming their being the same species. In addition, putative hybrid progeny subjected to pathogenicity trials showed that, as a result of recombination occurring during mating, intermediate symptom types between net-blotch and leaf spot were obtained. In the present study a verified *P. teres* net-blotch isolate from Denmark mated with a South African *Pyrenophora* leaf spot isolate thereby confirming the identity of the local isolate as *P. teres* f. *maculata*. Pathogenicity trials with progeny from the mating revealed intermediate symptoms similar to those obtained by Smedegård-Petersen (1977).

Evidence that recombination occurred during mating between the net-blotch and leaf spot isolates in the present study was demonstrated by using RAPD and AFLP markers to identify novel DNA banding patterns, by comparing the parental genotypes with the novel genotypes of the progeny. The demonstration of novel genotypes in the progeny suggested that the pseudothecia formed during mating were the product of a true cross. Recombination of RAPD markers during the sexual cycle has also been demonstrated in other phytopathogenic fungi (Dyer *et al.*, 1993, 1994; Daniels *et al.*, 1995; Nicholson *et al.*, 1995) as well as in agriculturally important food crops (Echt, Erdahl & McCoy, 1992; Heun & Helentjaris, 1993). The advantage of AFLPs over RAPDs in being able to identify cosegregating markers has been shown in several crop species (Hill *et al.*, 1996; Maughan *et al.*, 1996; Sharma *et al.*, 1996; Maheswaran *et al.*, 1997; Paul *et al.*, 1997).

It is of note that only one mating was successful. The viable progeny obtained were the result of mating between a Danish net-blotch and South African leaf spot isolate. Both the net-blotch and leaf spot as well as the teleomorph of the leaf spot form are present in South Africa (Louw *et al.*, 1994, 1995, 1996; Crous *et al.*, 1995). The fact that no viable progeny were produced from the mating between the South African net-blotch and leaf spot isolates as well as for the other matings might therefore be due to sub-optimal laboratory conditions. Alternatively, isolates used in some of the matings might have been of the same mating-type, as *P. teres* has a two-allele heterothallic mating system (McDonald, 1963).

The presence of intermediate symptom types due to recombination has several implications with regards to the identification of net-blotch under field conditions. The symptoms caused by all the ascospore progeny were clearly different to the symptoms produced by the parental isolates. Although other *Pyrenophora* spp. such as *P. hordei*, *D. wirreganensis* Wallwork, Lichon & Sivan. and *P. graminea* S. Ito & Kurib. have also been reported from South Africa, recombination between the net-blotch and leaf spot forms of *P. teres* could explain the great variation of symptoms frequently observed under field conditions (Scott, 1995; Den Breeÿen *et al.*, 1996). As this study was mainly concerned with confirming hybridity the actual extent of disease which these novel genotypes might cause has still to be assessed. It has been reported that the spot symptoms caused by *P. teres* f. *maculata* vary widely with regards to their form and colour and may lead to confusion with atypical lesions induced by other barley pathogens (Toubia-Rahme *et al.*, 1995*a*). It is conceivable that symptoms caused by ascospores produced as a result of a cross between a net-blotch and leaf spot isolate may also lead to confusion in the identification of this fungus with other *Pyrenophora* spp. pathogenic to barley.

The present study also demonstrated that recombination between net-blotch and leaf spot isolates may have affected fungicide sensitivity. Tests of sensitivity to the three triazole fungicides detected altered dose responses among the progeny. The IC_{50} values for several isolates could not be obtained as they fell below the lowest concentrations used. In

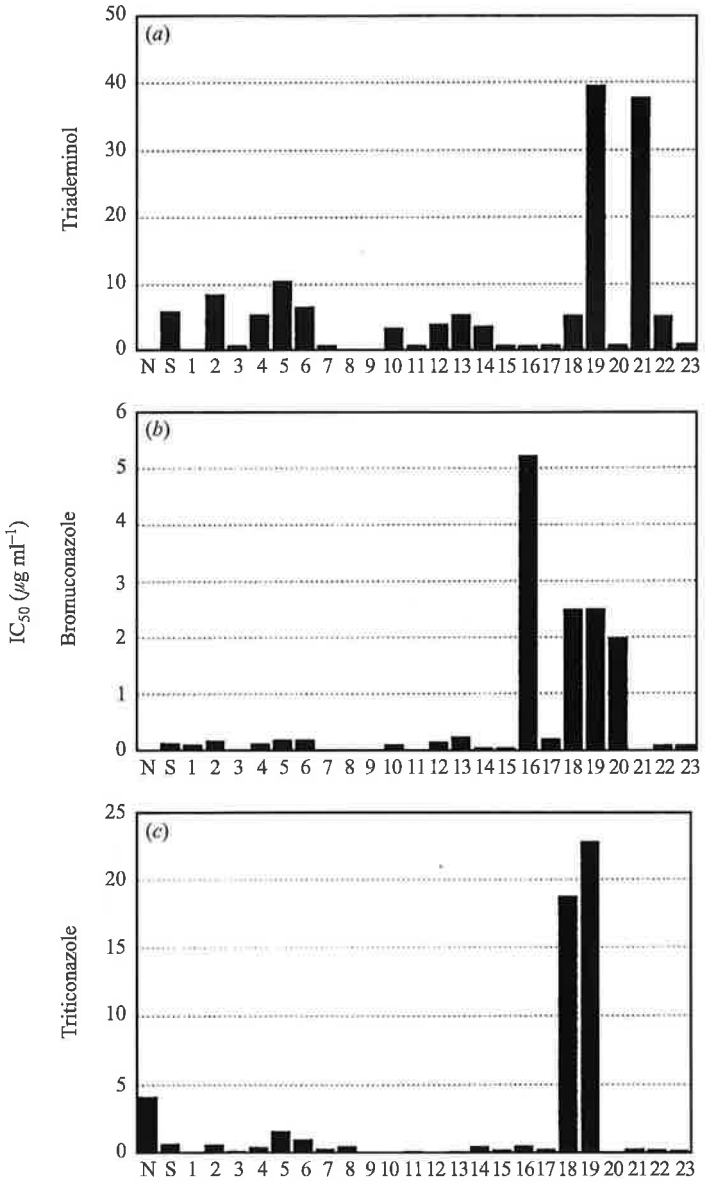

Fig. 4. IC_{50} values for net-blotch and leaf spot parents and the progeny for fungicides.

the present study both the net-blotch and leaf spot parents were sensitive to triademinol, with resistant isolates growing at 10 μg ml^{-1} (Sheridan, Grbavac & Sheridan, 1985; Peever & Milgroom, 1992). Three of the progeny, however, showed increased resistance, of which two were highly resistant. No published data are available on the activity of bromuconazole and triticonazole against *P. teres*. For both fungicides, however, progeny with increased resistance by comparison to parental isolates were again obtained. These results suggest that should sexually compatible net-blotch and leaf spot isolates be present in a mating population in the field, progeny with resistance to fungicides might arise, despite the sensitivity of parental isolates. Isolate GC19 showed increased resistance to each of the three fungicides. This suggests that recombination between isolates of *P. teres* can potentially lead to multiple resistance towards different triazoles.

Steffenson & Webster (1992) reported a large number of pathotypes present in populations of *P. teres* in California where the teleomorph is commonly produced under field conditions. The present study demonstrated that sexual recombination in *P. teres* can result in substantial changes in both disease expression in the host and to fungicide sensitivity in the pathogen. This is important with regard to the epidemiology and control of net blotch in that it provides the pathogen with the genetic flexibility to respond to selection pressures such as fungicide applications (Peever & Milgroom, 1992). In the Western Cape province there has been a shift in the *P. teres* population from the net-blotch to the leaf spot form (Louw *et al.*, 1996). Whether this shift was due to the introduction of new cultivars with increased susceptibility to leaf spot or the occurrence of mating between net-blotch and leaf spot isolates, followed by some other mode of selection for leaf spot isolates has not been established. Although the teleomorph of the leaf spot form of *P. teres* has been collected from stubble in South Africa (Louw *et al.*, 1994) it is still not clear what the implications of a net × spot mating would be to

field populations which differ regarding their cultivar (Tekauz, 1990; Arabi *et al.*, 1992; Afanasenko *et al.*, 1995) and fungicide responses (Toubia-Rahme *et al.*, 1995 *b*).

The authors gratefully acknowledge Drs J. C. Reeves (National Institute of Agricultural Botany, Cambridge, U.K.) and T. N. Kahn (Western Australian Department of Agriculture, South Perth, Australia) for placing several isolates at our disposal for study and the British Council for financial support through the U.K./S.A. Science and Technology Fund. Furthermore, we thank Dr K. J. Edwards (IACR Long Ashton Research Station, U.K.) in whose laboratory the AFLP analysis was conducted, and for his invaluable advice.

REFERENCES

Afanasenko, O. S., Hartleb, H., Guseva, N. N., Minarikova, V. & Janosheva, M. (1995). A set of differentials to characterise populations of *Pyrenophora teres* Drechs. for international use. *Journal of Phytopathology* **143**, 501–507.

Arabi, M. I., Barrault, G., Sarrafi, A. & Albertini, L. (1992). Variation in the resistance of barley cultivars and in the pathogenicity of *Drechslera teres* f. sp. *maculata* and *D. teres* f. sp. *teres* isolates from France. *Plant Pathology* **41**, 180–186.

Crous, P. W., Janse, B. J. H., Tunbridge, J. & Holz, G. (1995). DNA homology between *Pyrenophora japonica* and *P. teres*. *Mycological Research* **99**, 1098–1102.

Daniels, A., Papaikonomou, M., Dyer, P. S. & Lucas, J. A. (1995). Infection of wheat seedlings by ascospores of *Tapesia yallundae*: Morphology of the infection process and evidence for recombination. *Phytopathology* **85**, 918–927.

Deadman, M. L. & Cooke, B. M. (1987). Effects of net blotch on growth and yield of spring barley. *Annals of Applied Biology* **110**, 33–42.

Delserone, L. M. & Cole, H. (Jr). (1987). Effects of planting date on development of net blotch epidemics in winter barley in Pennsylvania. *Plant Disease* **71**, 438–441.

Den Breeÿen, A., Crous, P. W. & Holz, G. (1996). *Pyrenophora hordei* and *Drechslera wirreganensis* occurring on graminicolous crops in South Africa. *South African Journal of Science* **92**, XIV (Abstr.).

Dyer, P. S., Nicholson, P., Rezanoor, H. N., Lucas, J. A. & Peberdy, J. F. (1993). Two-allele heterothallism in *Tapesia yallundae*, the teleomorph of the cereal eyespot pathogen *Pseudocercosporella herpotrichoides*. *Physiological and Molecular Plant Pathology* **43**, 403–414.

Dyer, P. S., Papaikonomou, M., Lucas, J. A. & Peberdy, J. F. (1994). Isolation of R-type progeny of *Tapesia yallundae* from apothecia on wheat stubble in England. *Plant Pathology* **43**, 1039–1044.

Echt, C. S., Erdahl, L. A. & McCoy, T. J. (1992). Genetic segregation of randomly amplified polymorphic DNA in diploid cultivated alfalfa. *Genome* **35**, 84–87.

Heun, M. & Helentjaris, T. (1993). Inheritance of RAPDs in F_1 hybrids of corn. *Theoretical and Applied Genetics* **85**, 961–968.

Hill, M., Witsenboer, H., Zabeau, M., Vos, P., Kesseli, R. & Michelmore, R. (1996). PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theoretical and Applied Genetics* **93**, 1202–1210.

Ito, S. & Kuribayashi, K. (1931). The ascigerous forms of some graminicolous species of *Helminthosporium* in Japan. *Journal of the Faculty of Agriculture, Hokkaido University* **29**, 85–125.

Jordan, V. W. L. (1981). Aetiology of barley net blotch caused by *Pyrenophora teres* and some effects on yield. *Plant Pathology* **30**, 77–87.

Jordan, V. W. L., Best, G. R. & Allen, E. C. (1985). Effects of *Pyrenophora teres* on dry matter production and yield components of winter barley. *Plant Pathology* **34**, 200–206.

Leung, H., Nelson, R. J. & Leach, J. E. (1993). Population structure of plant pathogenic fungi and bacteria. *Advances in Plant Pathology* **10**, 157–205.

Louw, J. P. J., Crous, P. W. & Holz, G. (1994). *Pyrenophora japonica* occurring on barley in South Africa. *Plant Pathology* **43**, 420–423.

Louw, J. P. J., Crous, P. W. & Holz, G. (1996). Relative importance of the barley net blotch pathogens *Pyrenophora teres* f. *teres* (net-type) and *P. teres* f. *maculata* (spot-type) in South Africa. *African Plant Protection* **2**, 1–7.

Louw, J. P. J., Victor, D., Crous, P. W., Holz, G. & Janse, B. J. H. (1995). Characterization of *Pyrenophora* isolates associated with spot and net-type lesions on barley in South Africa. *Journal of Phytopathology* **143**, 129–134.

Matheswaran, M., Subudhi, P. K., Nandi, S., Xu, J. C., Parco, A., Yang, D. C. & Huang, N. (1997). Polymorphism, distribution, and segregation of AFLP markers in a double haploid rice population. *Theoretical and Applied Genetics* **94**, 39–45.

Martin, R. A. (1985). Disease progression and yield loss in barley associated with net blotch, as influenced by fungicide seed treatment. *Canadian Journal of Plant Pathology* **7**, 83–90.

Maughn, P. J., Maroof, M. A. S., Bass, G. R. & Huestis, G. M. (1996). Amplified fragment length polymorphism (AFLP) in soybean – species-diversity, inheritance and near-isogenic line analysis. *Theoretical and Applied Genetics* **93**, 392–401.

McDonald, W. C. (1963). Heterothallism in *Pyrenophora teres*. *Phytopathology* **53**, 771–773.

McDonald, W. C. (1967). Variability and the inheritance of morphological mutants in *Pyrenophora teres*. *Phytopathology* **57**, 747–755.

McDonald, B. A. & Martinez, J. P. (1990). DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. *Phytopathology* **80**, 1368–1373.

Nicholson, P., Dyer, P. S., Rezanoor, H. N., Lucas, J. A., Walkowiak-Cagara, I. & Hollins, T. W. (1995). Mating relationships between different pathotypes of *Tapesia yallundae*, the teleomorph of the eyespot pathogen *Pseudocercosporella herpotrichoides*. *Mycological Research* **99**, 457–465.

Paul, S., Wachira, F. N., Powell, W. & Waugh, R. (1997). Diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. *Theoretical and Applied Genetics* **94**, 255–263.

Peever, T. L. & Milgroom, M. G. (1992). Inheritance of triademinol resistance in *Pyrenophora teres*. *Phytopathology* **82**, 821–828.

Robbertse, B., Holz, G. & Crous, P. W. (1996). Sensitivity of South African *Ramulispora herpotrichoides* isolates to carbendazim and ergosterol biosynthesis inhibitors. *Plant Pathology* **45**, 270–275.

Sambrook, J., Fritsch, E. F. & Maniatis, T. A. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbour Laboratory Press: Cold Spring Harbor, NY.

Scott, D. B. (1991). Identity of *Pyrenophora* isolates causing net-type and spot-type lesions on barley. *Mycopathologia* **116**, 29–35.

Scott, D. B. (1994). *Drechslera* spp. and their teleomorphs on small-grain cereals in South Africa (Abstracts of the Fifth International Mycological Congress), p. 194. Vancouver, BC, Canada.

Scott, D. B. (1995). Helminthosporia that cause leaf spots on small-grain cereals in South Africa. In *Helminthosporia metabolites, Biology, Plant Diseases*. (ed. J. Chelkowski), pp. 107–137. Institute of Plant Genetics, Polish Academy of Sciences.

Sharma, S. K., Knox, M. P. & Hills, T. H. N. (1996). AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. *Theoretical and Applied Genetics* **93**, 751–758.

Sheridan, J. E., Grbavac, N. & Sheridan, M. H. (1985). Triademinol insensitivity in *Pyrenophora teres*. *Transactions of the British Mycological Society* **85**, 338–341.

Shipton, W. A., Kahn, T. N. & Boyd, W. J. R. (1973). Net blotch of barley. *Reviews in Plant Pathology* **52**, 269–290.

Shoemaker, R. A. (1962). *Drechslera* Ito. *Canadian Journal of Botany* **40**, 808–836.

Sivanesan, A. (1987). Graminicolous species of *Bipolaris, Curvularia, Drechslera, Exserohilum* and their teleomorphs. *Mycological Papers* **158**, 1–261.

Smedegård-Petersen, V. (1971). *Pyrenophora teres* f. *maculata* f. nov. and *Pyrenophora teres* f. *teres* on barley in Denmark. *Yearbook of the Royal Veterinary and Agricultural University (Copenhagen)*, pp. 124–144.

Smedegård-Petersen, V. (1976). Pathogenesis and genetics of net-spot blotch and leaf stripe of barley caused by *Pyrenophora teres* and *Pyrenophora graminea*. *DSR Verlag, Royal Veterinary and Agricultural University, (Copenhagen)*, p. 1–176.

Smedegård-Petersen, V. (1977). Inheritance of genetic factors for symptoms

and pathogenicity in hybrids of *Pyrenophora teres* and *Pyrenophora graminea*. *Phytopathologische Zeitschrift* **89**, 193–202.

Steffensen, B. J. & Webster, R. K. (1992). Pathotype diversity of *Pyrenophora teres* f. *teres* on barley. *Phytopathology* **82**, 170–177.

Steffenson, B. J., Webster, R. K. & Jackson, L. F. (1991). Reduction in yield loss using incomplete resistance to *Pyrenophora teres* f. *teres* in barley. *Plant Disease* **75**, 96–100.

Tekauz, A. (1990). Characterization and distribution of pathogenic variation in *Pyrenophora teres* f. *teres* and *Pyrenophora teres* f. *maculata* from Western Canada. *Canadian Journal of Plant Pathology* **12**, 141–148.

Toubia-Rahme, H., Ali-Haimoud, D. E., Barrault, G. & Albertini, L. (1995*a*). Inhibition of *Drechslera teres* sclerotioid formation in barley straw by application of glyphosate or paraquat. *Plant Disease* **79**, 595–598.

Toubia-Rahme, H., Ali-Haimoud, D. E., Barrault, G. & Albertini, L. (1995*b*). Effect of four fungicides on barley net blotch caused by *Drechslera teres*. *Journal of Phytopathology* **143**, 335–339.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531–6535.

Zabeau, M. & Vos, P. (1993). Selective restriction fragment amplification: A general method for DNA fingerprinting. *European Patent Application* No.: 92402629.7; Publication number EP 0534858 A1.

(*Accepted 29 April 1998*)

BMS Symposium on Tropical Mycology

The BMS Symposium on Tropical Mycology to celebrate the Millenium will be held in Liverpool April 25th through 29th 2000 with forays and public lectures extending into the weekend. There will be lecturers from all corners of the world and the topics covered will be conservation, physiology, genetics, mutualisms, ecology and systematics of selected groups. Tropical mycodiversity will be addressed therefore in all its facets. Prof. Jack Rogers, University of Washington, Pullman will deliver the Benefactor's lecture. Poster sessions and workshops will also be an integral part of the meeting. The Council of the British Mycological Society has generously offered five bursaries to the sum of £500 each for deserving delegates wishing to attend. Further information about the meeting and the bursaries can be obtained from Prof. Roy Watling e-mail r.watling@rbge.org.uk.