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Genetic stability of net × spot hybrid progeny of the barley pathogen *Pyrenophora teres*

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Abstract. Hybrid progeny produced from a mating between a net- and a spot-type isolate of the barley net blotch pathogen *Pyrenophora teres* were screened to assess their viability and genetic stability. Progeny (F_1) inoculated onto seedlings of the barley cultivars Stirling (highly susceptible to net-, and slightly susceptible to spot-type isolates), B87/14 and Clipper (only susceptible to spot-type isolates) produced jagged-type symptoms on all cultivars. When cultures ($F_{1\text{-}1}$) isolated from leaves inoculated with F_1 cultures were inoculated onto these cultivars, they produced similar symptoms. $F_{1\text{-}2}$ cultures isolated from $F_{1\text{-}1}$ infections also produced similar symptoms. RAPDs produced on all isolates of $F_{1\text{-}1}$ and $F_{1\text{-}2}$ progeny revealed identical profiles to those obtained for F_1 isolates. Molecular and infection data indicated that jagged-type hybrid progeny of this net × spot mating retained their virulence and fertility over time, and were genetically stable after two cycles of inoculation, mitosis and re-isolation. Hybridisation between net- and spot-type isolates of *P. teres* may, therefore, play a significant role in net blotch epidemiology in barley-growing areas.

Additional keywords: Drechslera, hybrids, mating studies.

Introduction

Net blotch, caused by the fungus *Pyrenophora teres* [anamorph *Drechslera teres*] is a destructive foliar disease of barley (*Hordeum vulgare*) in South Africa and throughout most other barley growing regions of the world (Campbell *et al*. 1999). Two types of leaf symptoms are associated with net blotch, a net-like symptom that produces elongated, light-brown lesions with dark-brown reticulations, and a spot symptom that is dark brown with a distinct halo (Smedegård-Petersen 1971). Both types are capable of causing economic yield losses (Steffenson *et al*. 1991). *P. japonica* [anamorph *Drechslera tuberose*] was originally described as the pathogen causing spot-type symptoms, whereas *P. teres* was associated with net-type lesions (Shoemaker 1962). Smedegård-Petersen (1971) mated net blotch and leaf spot isolates and concluded that they were two formae of the same biological species for which he proposed the names *P. teres* f. *teres* (net-type symptoms) and *P. teres* f. *maculata* (spot-type symptoms).

Contrary to earlier belief that Pyrenophora leaf spot isolates in South Africa were *P. japonica* (Louw *et al*. 1994), Campbell *et al*. (1999) showed that these Pyrenophora leaf spot isolates were actually *P. teres* f. *maculata*. This was shown by demonstrating that recombination, confirmed using molecular markers, had taken place following mating between a net- and spot-type isolate of *P. teres.* Although mating between net- and spot-type isolates of *P. teres* has been reported in various studies (Smedegård-Petersen 1971; Louw *et al*. 1995; Campbell *et al*. 1999), it is difficult to initiate mating under laboratory conditions (Peever and Milgroom 1992; Campbell *et al*. 1999). Not only have net × spot hybrids been shown to cause an intermediate symptom on susceptible cultivars (Smedegård-Petersen 1977; Crous *et al*. 1995; Campbell *et al*. 1999), but a proportion of hybrid progeny were found to be more resistant to commercially used fungicides following mating between two sensitive parents (Campbell *et al*. 1999; Campbell and Crous 2002). The role and genetic stability of these hybrid progeny in net blotch epidemics, however, remain unclear.

Various DNA-based techniques can be used to assess genetic stability of hybrids. RAPD markers have been used to demonstrate the formation of hybrids during the sexual cycle in various phytopathogenic fungi (Dyer *et al*. 1994; Daniels *et al*. 1995; Nicholson *et al*. 1995; Campbell *et al*.

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1999; Schoch *et al*. 2000). Although hybrid progeny have been successfully produced between net- and spot-type isolates in the past (Smedegård-Petersen 1983; Campbell *et al.* 1999), hybrids have only recently been detected in nature (Campbell *et al.* 2002). The aim of the present paper was, therefore, to determine if net × spot hybrid progeny of *P. teres* would retain their pathogenicity, fertility, symptomatology and genetic stability if passed through their host over time. This was done by screening isolates for pathogenicity and symptom expression via several successive inoculation cycles, and obtaining genetic fingerprints from re-isolated strains using RAPDs.

Methods

Symptom expression

Hybrid progeny (F_1) produced from a mating between a net- and spot-type isolate of *P. teres* (Campbell *et al*. 1999) were used to monitor genetic stability over a series of pathogenicity trials. Isolates were maintained on potato-dextrose agar (PDA) at 4°C. For cultural growth, inoculated PDA culture plates were incubated at 25°C for 7 days. In the present study, the following 14 hybrid isolates were used from Campbell *et al.* (1999) for the pathogenicity trials and RAPD stability analyses: GC2, 3, 4, 6, 7, 9, 10, 12, 16–20 and 22. This study consisted of the following phases: phase 1 — inoculation of hybrid progeny (F_1) onto barley seedlings; phase 2 — isolation of the fungal pathogen from diseased leaves to obtain $F_{1\text{-}1}$; phase 3 — inoculation of $F_{1\text{-}1}$ onto barley seedlings and finally, phase 4 — re-isolation of the fungal pathogen from diseased leaves to obtain $F_{1\text{-}2}$.

The three barley cultivars Stirling (highly susceptible to net-, and slightly susceptible to spot-type isolates), B87/14 and Clipper (only susceptible to spot-type isolates) were used in the pathogenicity trials. Production and processing of mycelia for inoculation onto seedlings was done according to the method set out by Campbell *et al*. (1999). Plants were incubated at the two-leaf stage in a glasshouse (15–20°C, night/day temperature) using the technique as explained by Louw *et al*. (1994). A solution of 0.01% (v/v) Tween 20 was initially sprayed onto the plants to reduce leaf surface tension, after which the mycelial suspensions were sprayed to runoff. Moisture chambers were created by placing plastic bags over the inoculated plants for 48 h. Plants were examined for symptom expression 5–10 days after inoculation.

Diseased barley leaves with net-blotch symptoms were removed from the plants 10 days after inoculation. Leaves were surface sterilised by immersion in 70% ethanol for 30 s followed by transfer to 2% NaOCl for 60 s and finally 70% ethanol for 30 s. Sterilised leaves were subsequently left to dry in a laminar air-flow cabinet. Dry leaves were placed onto glass slides in moisture chambers and incubated at 10°C under near-UV light to induce sporulation. After approximately 3–4 days single conidia were transferred to PDA culture plates.

RAPD analysis

Isolation of DNA, RAPD analysis and gel electrophoresis was performed according to standard procedures (Sambrook *et al*. 1989) essentially as described by Campbell *et al*. (1999) with the following modification: The reaction mixture for RAPD analysis contained 2.5 µL of 10 × NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20]; 200 µM of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1.0 unit of BIOTAQ DNA polymerase [Bioline Ltd, London, UK and Whitehead Scientific, South Africa]. RAPD analysis was done with two different primers that delineated a dominant RAPD band from each respective parental type. The sequences of the primers were the following: OPE7 — 5 ′ AGATGCAGCC 3 ′ and OPM10 — 5 ′ TCTGGCGCAC 3 ′. Amplifications were done in a Perkin-Elmer GeneAmp PCR system 2400 cycler. Reactions underwent an initial denaturation process at 96°C for 120 s, followed by 45 cycles of 92°C for 30 s, 38°C for 30 s and 72°C for 60 s. After the last cycle, a final extension step was conducted at 72°C for 120 s. Amplification products were separated through 1.5% (w/v) agarose gels in TAE buffer (Sambrook *et al*. 1989).

DNA fingerprints were observed in a SYNGENE Darkroom S/N: SYDR/1318 linked to a desktop computer. Fingerprints were captured using the SYNGENE programme GeneSnap. A molecular weight marker (λ DNA digested with *Hin*dIII) was used as a reference for comparing samples from different gels. Using the F_1 fingerprints (OPE7 and OPM10) as references, genetic stability at the molecular level was assessed in isolates from $F_{1\text{-}1}$ and $F_{1\text{-}2}$.

Results

Symptom expression

Symptoms were obtained on all three cultivars for all $F_{1\text{-}1}$ and $F_{1\text{-}2}$ isolates. Intermediate symptom expression characterised by jagged, elongated brown lesions were produced to varying degrees depending on the cultivar used. These results indicate that the *P. teres* net × spot hybrids studied remained virulent and stable, being able to induce disease on all three cultivars following a series of inoculation and re-isolation steps. Sporulation on symptomatic leaf tissue placed in moist chambers was profuse, with no visible differences between progeny and parental isolates.

RAPD analysis

Using OPE7, two marker bands (Campbell *et al*. 1999) were produced that distinguished the net- and spot- type parental isolates. The lower band, designated as marker band 2, which is characteristic of the spot-type parent (Campbell et al. 1999), was present in six of the hybrid progeny in F_1, and was also present in the respective isolates in $F_{1\text{-}1}$ and $F_{1\text{-}2}$. Marker band 1, characteristic of the net-type parent, was present in eight of the hybrid progeny in F_1, as well as in the respective isolates in $F_{1\text{-}1}$ and $F_{1\text{-}2}$.

Using primer OPM10, two marker bands were produced that distinguished the net- and spot-type parental isolates. Isolates in which both these bands were present in the hybrid progeny in F_1, were also present in the respective isolates in both $F_{1\text{-}1}$ and $F_{1\text{-}2}$ (Fig. 1). The results obtained using these two primers clearly indicated that the hybrid progeny were genetically stable as the net- and spot-type marker bands were retained through two series of inoculation and re-isolation cycles.

Discussion

Spot-type lesions of barley were originally ascribed to *P. japonica*, whereas *P. teres* was associated with net-type, and *P. graminea* with leaf stripe symptoms (Ito & Kuribayashi 1931; Shoemaker 1962, Smedegård-Petersen 1983). McDonald (1967) regarded *P. japonica* as a mutant strain of *P. teres*, whereas Kenneth (1962) considered it to be a sibling species. By comparing the total DNA profiles of verified

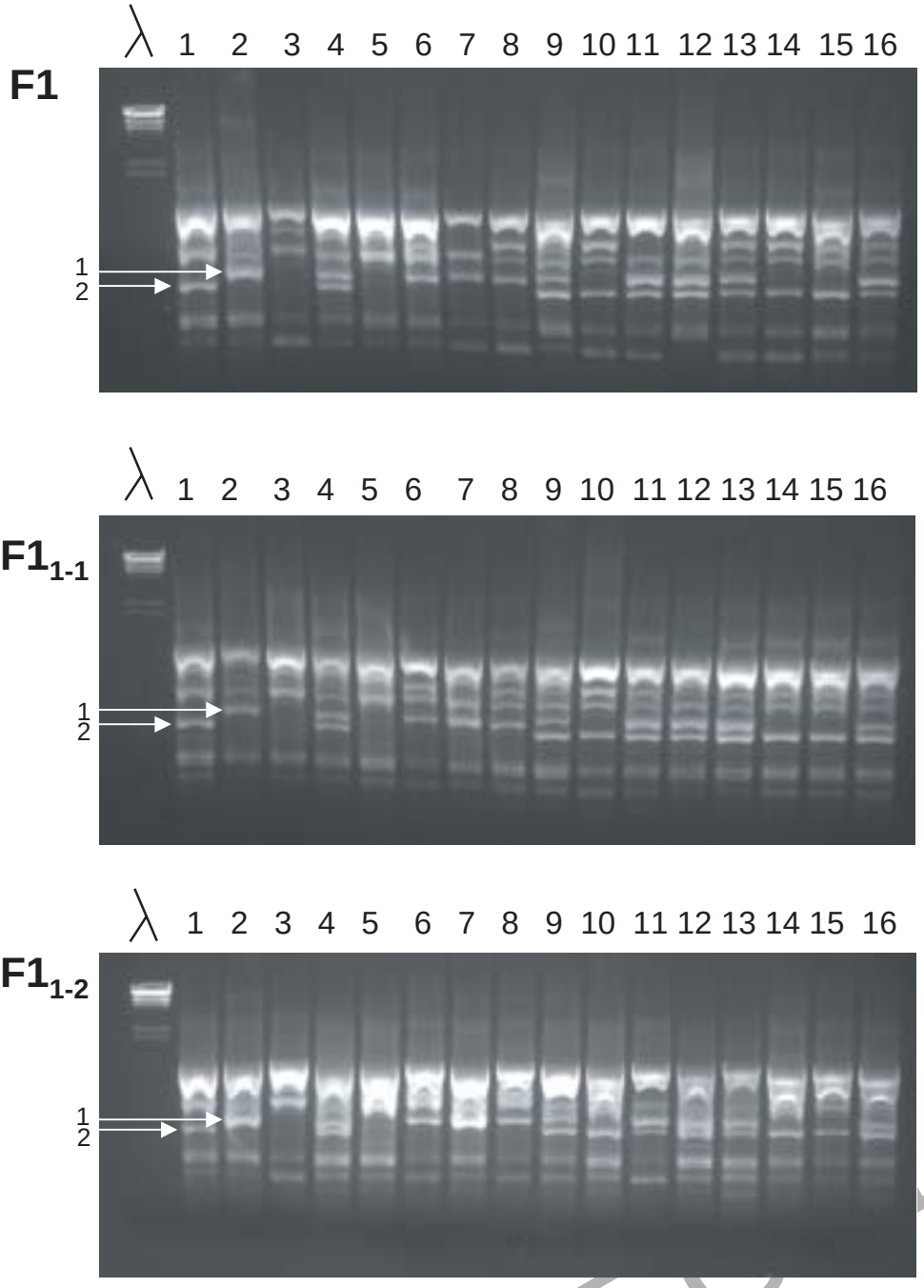

Fig. 1. RAPD fingerprints for F_1, $F_{1\text{-}1}$ and $F_{1\text{-}2}$ using primer OPM10. The first lane is λ DNA digested with *Hin*dIII. Lanes 1 and 2 are respectively the net- and spot-type parental isolates. Lanes 3–16 are the hybrid isolates. Marker bands 1 and 2 are indicated with arrows.

isolates of *P. japonica* and the spot form of *P. teres*, Crous *et al*. (1995) concluded that they were synonymous. Using RAPDs, Bulat and Mironenko (1990) demonstrated that *P. teres* and *P. graminea* were intraspecific forms of the same species. By comparing DNA sequence data of the 5.8S gene and the internal transcribed spacer (ITS-1 & ITS-2), as well as the histone H3 gene (Campbell 2001), good resolution could be obtained between different species of *Pyrenophora*, except for the barley complex consisting of *P. teres* (net- and spot-type), *P. japonica* and *P. maculata*, which appeared synonymous. Using various techniques, it has previously been demonstrated that net- and spot-type isolates of *P. teres*, as well as *P. graminea*, can freely hybridise in the laboratory (Smedegård-Petersen 1983; Campbell *et al*. 1999). When

inoculated on different cultivars, these progeny produced symptoms ranging from net-type, spot-type, intermediate, stripes or flecks (Smedegård-Petersen 1983). These findings led Smedegård-Petersen (1983) to conclude that if these hybrids occurred in nature, they should be treated as races or forms of the same biological species, *P. teres*. By employing RAPDs and by screening symptom expression of differential cultivars, Campbell *et al.* (2002) were recently able to demonstrate that such hybrids do occur in barley fields in South Africa. The aim of the present study, therefore, was to determine if hybrid progeny (F_1) of a *P. teres* net × spot cross would remain genetically stable, virulent and fertile, following a series of glasshouse experiments involving inoculation and re-isolation cycles. Results of this study have shown that these hybrids do remain fertile and virulent after having been preserved for a period of more than two years. Furthermore, they also remained stable regarding their RAPD profiles and symptom expression on differential cultivars after several inoculation and re-isolation cycles.

Our earlier work has shown that net- × spot-type hybrids have different fungicide sensitivities and cultivar preferences (Campbell *et al*. 1999). The fact that these hybrids can occur in nature (Campbell *et al*. 2002) and remain viable and stable over time, has serious implications with regards to the epidemiology of net blotch disease. The question of hybridisation is important, as it can be responsible for introducing new genotypes into natural populations. Evidence obtained through glasshouse pathogenicity trials suggests that hybrid forms between the net- and spot-type of *P. teres* may be able to survive from one season to the next on straw (Louw *et al*. 1994) remaining in fields after harvest. Crops in subsequent seasons could then be infected by means of rain splash (Jordan 1981) or by wind-borne conidia (Shipton *et al*. 1973). Straw remaining in fields after harvest is regarded as the primary inoculum source of *P. teres* (Piening 1968; Jordan 1981). Overwintering structures such as pseudothecia may also play a role in the epidemiology of net × spot hybrid isolates.

Three barley cultivars highly susceptible to either net- or spot-type isolates were used in pathogenicity trials in the present study. The hybrid isolates, however, infected all cultivars in both sets of pathogenicity trials, indicating that due to recombination the normal constraints on host resistance had been overcome. Furthermore, it has been reported by Tekauz (1990) that two malting cultivars in Canada showed equal susceptibility to both net- and spot-type isolates. This was attributed to various factors, one being the possibility for sexual recombination between net- and spot-type isolates (Tekauz 1990). Data from the present study support this hypothesis, clearly showing that hybrids possess the ability to infect cultivars resistant against either the net- or spot-type of *P. teres*.

The existence of this new jagged-type hybrid suggests that both the net- and spot-type formae have recently evolved from a common ancestor, which is also shared by *P. graminea* (Smedegård-Petersen 1983). In view of the different host specificity of the jagged-type *P. teres* hybrid, this may be an important mechanism of *Pyrenophora* to enable rapid adaptation to new genotypes. Early reports have described net-type net blotch as one of the most common barley diseases in South Africa (Smith and Rattay 1930; Putterill 1954). A recent survey conducted on 26 localities in the south-western Cape Province of South Africa found that net-type net blotch was restricted to three localities, and that spot-type symptoms were present at all but one locality (83% of all isolates sampled) (Louw *et al*. 1996). Analysis of the population structure of *P. teres* occurring in fields in the south-western Cape found evidence of sexual recombination within the spot- as well as the net-type populations (Campbell *et al*. 2002). The cultivar Clipper, which is presently the dominant barley cultivar planted, is only susceptible to spot-type isolates. As the present study has shown, however, net-type isolates can hybridise with spot-type isolates to produce jagged-type progeny, which also infect Clipper. These findings suggest, therefore, that new breeding programmes will have to consider breeding for resistance to the net-, spot- and jagged-type of *P. teres*, as well as *P. graminea*.

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