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Short communication

Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape

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Abstract

An existing PCR-based method for diagnosis of the winter oilseed rape (*Brassica napus* ssp *oleifera*) fungal pathogen *Pyrenopeziza brassicae* (cause of light leaf spot) was improved by the development of a pair of primers (PbN1 and PbN2) for use in nested-PCR reactions. The nested-PCR technique improved the detection of *P. brassicae* DNA *in vitro* by three orders of magnitude over that achieved using the first-round PCR primers (Pb1 and Pb2). In controlled environment experiments, the nested-PCR assay detected *P. brassicae* within infected *B. napus* leaves before visible light leaf spot symptoms developed and earlier than was possible by incubating infected leaves in polyethylene bags to promote sporulation of *P. brassicae*. A three-primer PCR technique using the primers PbM-1-3, PbM-2 and Mt3 was developed to distinguish between the two mating types (*MAT-1* and *MAT-2)* of *P. brassicae*. This technique was able to determine the mating types present within DNA extracted from infected plant tissue, including tissue infected with both mating types together.

Light leaf spot, caused by *Pyrenopeziza brassicae* Sutton and Rawlinson, is one of the most damaging diseases of winter oilseed rape (*Brassica napus* ssp *oleifera*) in the UK (Fitt et al., 1997). No complete resistance to light leaf spot exists currently and control of the disease depends upon the application of fungicides shortly after initial infection has occurred in the autumn (Jeffery et al., 1994; Rawlinson et al., 1984). However, the symptoms of light leaf spot often are not visible until January or February (Figueroa et al., 1995; Rawlinson et al., 1978) and, even when visible, can be confused with lesions caused by other diseases or with frost damage (Fitt et al., 1998). A method for diagnosis of pre-symptomatic light leaf spot infections would be invaluable to guide strategies for control of the disease to ensure that only those crops which are shown to be infected receive fungicide sprays, thereby reducing the numbers of fungicide treatments which are currently applied (Gilles et al., 2000a).

Diagnosis of light leaf spot is achieved by incubating plant samples in polyethylene bags for 4–5 days at 10–15 °C (Fitt et al., 1998) to promote the production of the diagnostic white spore masses (acervular conidiomata). However, the latent period (between infection and sporulation) of *P. brassicae* is approximately 160 ° days (Gilles et al., 2000b). Incubation of samples which have only recently been infected would not result in the production of spore masses using this method. Ascospores of *P. brassicae* are more infective than conidia and may be responsible for the initiation of light leaf spot epidemics in the autumn (Gilles et al., 2001a). Both mating types (*MAT-1* and *MAT-2*) of the fungus must interact to initiate sexual reproduction (Ashby, 1997; Gilles et al., 2001b). Analysis of the frequency with which both mating types are found on the same leaf would be useful in further studies of the role of the sexual stage of *P. brassicae* and it may be possible to relate this information to the development of the

sexual stage within crops and subsequent dispersal of ascospores.

Problems were experienced with a previous attempt to design a PCR-based method for the differentiation of *P. brassicae* mating types because a sequence immediately downstream from the *P. brassicae* mating-type loci, which was used as a target for the assay, was not amenable to amplification from every isolate of mating-type *MAT-2* tested (Foster et al., 1999). This paper reports work to demonstrate that nested PCR can improve the sensitivity of the existing PCR-based diagnostic assay for *P. brassicae* and presents an improved PCR-based method for the differentiation of *P. brassicae* mating types.

The isolates of *P. brassicae* used were described in Foster et al. (1999). Isolates of other fungi used to confirm the specificity of the *P. brassicae* diagnostic primers also were as described in Foster et al. (1999) but included an additional five isolates of *Leptosphaeria maculans*, two isolates of *Alternaria brassicae*, one isolate of *A. brassicicola*, three isolates of *Sclerotinia sclerotiorum*, two isolates of *Peronospora parasitica* and one isolate of *Plasmodiophora brassicae* (details of these isolates may be requested from the authors). Genomic DNA was extracted from fungal cultures according to the published protocol (Foster et al., 1999).

The *P. brassicae* diagnostic primers Pb1 (5′-CAA CAT TGC CTG GTA TTG AGA AAC-3′) and Pb2 (5′-ATC TGA TAC GCC TAC ACC GTC C-3′) (Foster et al., 1999) were used in PCR reactions with ten-fold serial dilutions (range from 100 ng per reaction to 1 fg per reaction) of *P. brassicae* DNA (diluted in 100 ng μl^{-1} *B. napus* genomic DNA) to determine the detection limits of the assay. PCR reactions using the *P. brassicae*-specific primers Pb1 and Pb2 were done according to Foster et al. (1999). Amplification of *P. brassicae* DNA using these primers produced a 753 bp amplicon. Using this assay, it was possible to detect as little as 10 pg of *P. brassicae* DNA (Figure 1). To improve the sensitivity of the assay, nested-PCR primers PbN1 (5′-TGT AGA TGG AAC CCT ACC CGT ATT G-3′) and PbN2 (5′-GTG ACC ACA ACG AAC CTT GTA TCA G-3′) were designed from the sequence of the PCR product amplified using primers Pb1 and Pb2 (Foster et al., 1999). PCR reactions using these primers were done using the same reaction mix as for the first-round primers but with a cycling programme consisting of an initial denaturation period of 10 min at 95 °C, followed by 20 cycles of 95 °C for 1 min, 72 °C for 1 min followed by an additional primer extension period of 72 °C for 10 min. Amplification using these primers with 1 μl of the original Pb1/Pb2 PCR reaction as template resulted in the amplification of the expected 408 bp amplicon from all isolates of *P. brassicae* tested. (Including an additional 37 isolates not included in Foster et al. (1999), details of which may be requested from the authors.) No products were amplified from DNA from other fungi used to test the specificity of these primers (data not shown). Using the nested-PCR protocol, it was possible to detect as little as 10 fg of *P. brassicae* DNA (Figure 1), which represents a thousand-fold increase in sensitivity over the original PCR assay.

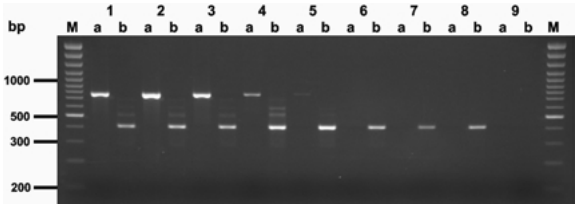

Figure 1. PCR amplification of genomic DNA from *P. brassicae* isolate PC3 serially diluted in *B. napus* DNA; 1, 100 ng DNA; 2, 10 ng DNA; 3, 1 ng DNA; 4, 100 pg DNA; 5, 10 pg DNA; 6, 1 pg DNA; 7, 100 fg DNA; 8, 10 fg DNA; 9, 1 fg DNA; M, 100 bp DNA ladder (MBI Fermentas). Lanes labelled 'a' are the result of amplifying genomic DNA using the primers Pb1 and Pb2. Lanes labelled 'b' are the result of re-amplification of 1 μl samples of the same PCR reactions using the nested primers PbN1 and PbN2.

To test the ability of the PCR-based assays to detect pre-symptomatic infection of leaves by *P. brassicae*, an infection time-course was set up. Oilseed rape (cv. Bristol) plants were grown in compost (75% peat, 12% loam, 10% grit, 3% vermiculite; Petersfield Products, Cosby, UK) in 8 cm diameter pots in a controlled-environment room (16 °C, 12 h light/dark, 190 $\mu E\, m^{-2}\, s^{-1}$ light intensity). At the 4–5 leaf stage (GS 1.4–1.5; Sylvester-Bradley and Makepeace, 1985), plants were inoculated by spraying with a conidial suspension (1×10^5 spores ml^{-1}) of a *P. brassicae* field isolate using an aerosol sprayer (Chrom Atomiser, Camlab, Cambridge, UK). The inoculum had been prepared by shaking in water leaves of *B. napus* (cv. Bristol) with sporulating *P. brassicae*, which had been taken from an affected crop at IACR-Rothamsted in May 1999. Inoculated leaves were sprayed until water droplets formed at the leaf margins. Leaves that were fully expanded at the time of inoculation were tagged with tie-on labels.

At time points 0, 3, 6, 10, 13 and 16 days post inoculation (dpi), six plants were removed from random positions within the controlled-environment cabinet. The two youngest tagged leaves from each plant were removed and visually assessed for sporulation characteristic of *P. brassicae* infection; symptoms were recorded as the percentage of the leaf area with sporulation. DNA was extracted from the leaves from three of the plants (designated set A leaves) using the same protocol as for fungal mycelium (Foster et al., 1999). Extracted DNA was resuspended in 100 µl of TE and 1 µl of a fifty-fold dilution was used as template for the *P. brassicae* diagnostic PCR assays. The leaves from the remaining three plants (designated set B leaves) were placed in polyethylene bags which had been sprayed inside with water and were incubated for 5 days at 10 °C in darkness. Following incubation, the leaves were again assessed for sporulation of *P. brassicae* and

Table 1. Visual (% sporulation) and PCR (presence or absence of *P. brassicae*-specific marker) assessments of *B. napus* leaves inoculated with a *P. brassicae* spore suspension (Plants were assessed before (set A) and after (set B) incubation in polyethylene bags)

Sample/leaf no.		Set A			Set B			
		% Sporulation[a]	PCR[b]	N-PCR[c]	% Sporulation[a,d]	% Sporulation[a,e]	PCR[b]	N-PCR[c]
0 dpi[f]	1	0	−	−	0	0	−	−
	2	0	−	−	0	0	−	−
	3	0	−	−	0	0	−	−
	4	0	−	−	0	0	−	−
	5	0	−	−	0	0	−	−
	6	0	−	−	0	0	−	−
3 dpi	1	0	−	−	0	0	−	+
	2	0	−	−	0	0	−	−
	3	0	−	−	0	0	−	−
	4	0	−	−	0	0	−	+
	5	0	−	−	0	0	−	−
	6	0	−	−	0	0	−	−
6 dpi	1	0	−	−	0	0	−	+
	2	0	−	−	0	0	+	+
	3	0	−	+	0	0	−	+
	4	0	−	−	0	0	−	+
	5	0	−	+	0	0	−	+
	6	0	−	+	0	0	−	+
10 dpi	1	0	−	+	0	<1	−	+
	2	0	−	−	0	<1	−	+
	3	0	−	−	0	<1	−	+
	4	0	−	+	0	<1	−	+
	5	0	−	+	0	25	+	+
	6	0	−	−	0	0	−	+
13 dpi	1	1	+	+	5	65	+	+
	2	<1	+	+	<1	10	−	+
	3	0	−	+	70	90	+	+
	4	35	+	+	1	3	−	+
	5	40	+	+	3	35	+	+
	6	20	+	+	7	30	+	+
16 dpi	1	25	+	+	25	55	+	+
	2	20	+	+	20	30	+	+
	3	1	+	+	35	45	+	+
	4	55	+	+	70	80	+	+
	5	5	−	+	10	20	−	+
	6	3	−	+	2	5	−	+

[a]Percentage of leaf area with visible sporulation. [b]PCR using primers Pb1 and Pb2. + and − indicate presence or absence of PCR product. [c]Nested PCR using primers PbN1 and PbN2. + and − indicate presence or absence of PCR product. [d]% sporulation prior to incubation for 5 days at 10 °C. [e]% sporulation after incubation for 5 days at 10 °C. [f]Days post inoculation.

then used for DNA extractions and PCR analysis as described above.

No sporulation was evident on set A leaves harvested prior to and including 10 dpi (Table 1). The PCR tests with primers Pb1 and Pb2 on set A leaves did not detect *P. brassicae* until 13 dpi when sporulation was also evident. Using the nested PCR, however, it was possible to detect *P. brassicae* as early as 6 dpi when 50% of the leaves tested were shown to be infected despite an absence of sporulation (Table 1).

Prior to incubation, set B leaves showed no visible signs of sporulation up to and including 10 dpi (Table 1). Following incubation, sporulation was evident on the leaves which had been harvested from 10 dpi onwards. Using the diagnostic primers Pb1 and Pb2, it was possible to detect *P. brassicae* in one asymptomatic leaf which had been harvested at 6 dpi but which did not show any signs of sporulation following incubation. Using the nested-PCR technique, it was possible to detect pre-symptomatic infection from leaves harvested as early as 3 dpi, which had been incubated but still did not show sporulation following incubation.

Two *P. brassicae* mating-type specific forward primers PbM-1-3 (5′-GAT CAA GAG ACG CAA GAC CAA G-3′) and PbM-2 (5′-CCC GAA ATC ATT GAG CAT TAC AAG-3′) were designed from the sequences of the *MAT-1-3* and *MAT-2* mating-type genes (Singh and Ashby, 1998) and were thus specific to *MAT-1* and *MAT-2* isolates, respectively. A common reverse primer Mt3 (5′-CCA AAT CAG GCC CCA AAA TAT G-3′) was designed from the flanking region that was fully homologous between the two mating types (Singh and Ashby, 1998). PCR reactions and cycling parameters were the same as those used for reactions with the primers Pb1 and Pb2. When used together in a PCR reaction, the three primers amplified a 687 bp product from *MAT-1* isolates and an 858 bp product from *MAT-2* isolates (Figure 2); the respective products were amplified from DNA from 10 *P. brassicae* isolates of each mating type (data not shown). The *P. brassicae* mating-type specific primers did not amplify any products when DNA from other fungi was used as a template (data not shown). No PCR products were amplified from healthy *B. napus* DNA using the three-primer approach (Figure 2). However, the diagnostic bands were amplified from healthy *B. napus* DNA spiked with 10 ng of *P. brassicae* DNA of each mating type alone and in combination (Figure 2), showing that failure to amplify from the healthy plant material was not due to inhibition of the PCR reaction by substances present in the plant DNA sample and also that PCR detection of mixed mating-type infections may be possible. DNA extracted from a *B. napus* leaf, taken from an oilseed rape crop, which exhibited light leaf spot symptoms resulted in the production of both the 858 and 687 bp diagnostic PCR products (Figure 2).

The nested-PCR assay for *P. brassicae* provides a substantial improvement in sensitivity over the existing assay (Foster et al., 1999). The improved technique is sufficiently sensitive to enable detection of the fungus in pre-symptomatic oilseed rape plants earlier than is possible using conventional methods. This technique may enable pre-symptomatic diagnosis of light leaf spot infection in the autumn, when fungicide spraying decisions are made. The mating-type specific primers developed in this work, PbM-1-3, PbM-2 and Mt3, also can be used to reliably differentiate between the two mating types of *P. brassicae*. This rapid test will enable large-scale surveys of mating-type distribution and will facilitate identification of mating types in genetic studies. It also may be possible for mating-type determination to be done directly on infected leaf material. This procedure will circumvent the problems associated with isolation of *P. brassicae* and the fact that not all isolates reliably reproduce sexually in culture. A survey of this type will add to data obtained previously (Ilott et al., 1984; Majer, 1997) and will provide more information to allow the importance of the

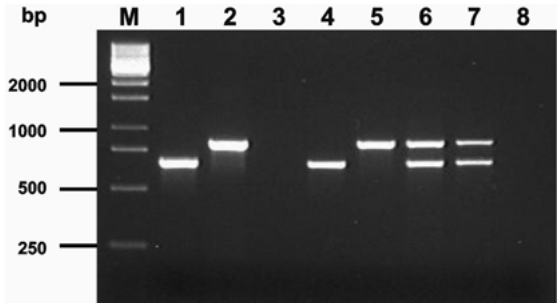

Figure 2. PCR amplification using the *P. brassicae* mating-type specific primers Mt3, PbM-1-3 and PbM-2. M, 1 kb DNA ladder (MBI Fermentas); Lane 1, 100 ng *P. brassicae MAT-1* isolate JH26 genomic DNA; lane 2, 100 ng *P. brassicae MAT-2* isolate NH10 genomic DNA; lane 3, 100 ng uninfected *B. napus* DNA; lane 4, 90 ng uninfected *B. napus* DNA spiked with 10 ng JH26 DNA; lane 5, 90 ng uninfected *B. napus* DNA spiked with 10 ng NH10 DNA; lane 6, 80 ng uninfected *B. napus* DNA spiked with 10 ng each of JH26 and NH10 DNA; lane 7, 100 ng DNA from a field sample of *B. napus* with light leaf spot symptoms; lane 8, negative (water) control.

sexual stage of the fungus in light leaf spot epidemics to be ascertained.

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