PROJECT REPORT No. 244

APPLICATION OF A NEW DIAGNOSTIC TECHNIQUE FOR THE DETECTION OF SEED-BORNE *PYRENOPHORA* SPECIES (LEAF STRIPE AND NET BLOTCH) ON BARLEY

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APPLICATION OF A NEW DIAGNOSTIC TECHNIQUE FOR THE DETECTION OF SEED-BORNE *PYRENOPHORA* SPECIES (LEAF STRIPE AND NET BLOTCH) ON BARLEY

by

Dr J E THOMAS, Dr J A BATES AND Dr D M KENYON

NIAB, Huntingdon Road, Cambridge CB3 OLE

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ABSTRACT

The aim of the project was to develop a diagnostic method for seed-borne *Pyrenophora* species on barley, based on DNA primers and the polymerase chain reaction (PCR) which was capable of delivering a rapid throughput routine test. The specific objectives were to optimise the PCR system and check the specificity of some of the primer sequences used, quantify the test for seed-borne net blotch and define a suitable extraction procedure of the fungus from seed, and finally to identify any practical problems which might arise in handling large numbers of tests.

Primer sequences derived from previous MAFF funded research were used. Optimal PCR conditions were established for the ITS (internally transcribed spacer region) primers used to detect net blotch, but it was not possible to use these in the same reaction as the specific primers for leaf stripe and separate PCR runs were needed. The ITS primers were checked against a range of other pathogens and saprophytic organisms commonly found on barley seed, and no significant cross-reactivity was detected. An extraction procedure developed for leaf stripe on barley seed also proved sufficiently reproducible and sensitive for extraction of net blotch. The PCR test for net blotch was quantified by establishing a relationship between % infection on agar plates, and the output of the PCR assay. The relationship proved accurate over a range of low to moderate infection levels when used to predict the infection % of over 80 samples of barley seed previously submitted to NIAB for testing.

The PCR test produced no false positive reactions for either leaf stripe or net blotch when no infection was expected from the agar plate test. Internal controls designed to protect against the possibility of false negatives due to DNA extraction failure were included in all tests. The extraction and testing sequence could be completed within one working day. In common with other advanced PCR systems, the equipment used at NIAB can process batches of samples sequentially throughout the day. The flow of barley samples submitted to NIAB for germination and health testing is likely to reflect the overall processing frequency of winter and spring barley seed lots. Large peaks occur in late July and throughout August, with probably in the region of 1000 lots, either farm-processed or certified seed, requiring testing each week during this period. Two or three laboratories operating high-throughput PCR systems would be able to meeting testing requirements for the majority of seed lots giving a test result within a maximum of 48 hours.

The development of a rapid diagnostic technique for these diseases offers growers the opportunity to make decisions on barley seed treatments on the basis of need without incurring impracticable delays to the processing of seed.

SUMMARY

Background

The majority of barley seed in the UK is treated with fungicides to control seed-borne diseases, the most important of which are leaf stripe (*Pyrenophora graminea*), net blotch (*Pyrenophora teres*), and loose smut (*Ustilago nuda*). Leaf stripe is a potentially serious seed-borne disease which usually results in failure of the plant to produce ears. Infection levels may increase rapidly in successive generations of untreated seed, but since the pathogen is only carried in seed, chemical treatments are a very efficient means of control. Net blotch is also transmitted by crop debris and this is generally thought to be a more important source of the disease than seed-borne infection. However, high levels of the latter could cause earlier epidemic development which may be more difficult to control with foliar applied fungicides, particularly on susceptible varieties, or introduce the disease into areas where husbandry practices had previously maintained a low level. Loose smut causes yield loss through replacement of the seed with a spore mass, and, like leaf stripe, can increase rapidly in successive generations of seed. Previous surveys of seed-borne diseases on barley in the UK have shown that the incidence and severity of leaf stripe and loose smut in seed stocks were very low, though the incidence and severity of net blotch were more variable. Results from seed health tests carried out by the Official Seed Testing Station at NIAB indicate that this situation still exists (Table 1). However, seed treatment is applied to over 95% of seed stocks for crop production, regardless of health status. Though some seed treatment products will be applied for purposes other than the control of seed-borne disease, such as reduction of foliar disease and prevention of BYDV, others have no additional effects. In the absence of seed-borne disease, growers may thus be using active ingredient which is not needed.

Table 1. Incidence and severity of leaf stripe *(lst*) net blotch *(nb) and loose smut (ls)* in seed samples 1995-1999

Year	% with *lst*	% lst> 2%	% with *nb*	% nb > 5%	% with ls	% ls>0.2%
1995	4	2	45	5	15.1	8
1996	2	0	71	20	7.9	5
1997	1	0	76	13	11.8	2
1998	2	0.5	77	17	1	0
1999	0	0	56	19	1.2	1

The risks of seed-borne diseases are such that in the generations of seed production, a prophylactic approach is advisable to safeguard the health of seed stocks. However, in seed for ware production a treatment approach based on need is more acceptable. Provided that health tests are carried out, and that not more than one

generation of untreated seed is used, it should be possible for growers to achieve savings on seed treatment costs. Given the current disease levels and considering farm-processed seed only, savings of the order of £2 million per year could be made, worth about £6/ha. However, a major barrier to achieving these savings lies in the testing techniques which are currently used to determine seed-borne disease levels. Leaf stripe and net blotch are evaluated in an agar plate test which takes seven days, and occasionally longer, to complete, requires a high level of mycological skill and is thus relatively costly. With the current laboratory capacity in the UK, it would be impossible to test large numbers of samples, particularly of winter barley, and base a treatment decision on need for these diseases within the time scale necessary for operation of mobile processing units and preparation of seed for drilling.

DNA primers and PCR testing technology

New techniques in seed health diagnostics offer the potential for rapid, high throughput tests with high sensitivity and specificity. The techniques are often based on the polymerase chain reaction (PCR), which is used to increase the concentration of short pieces of DNA which are specific to the target pathogens. In order to derive the specific DNA pieces, sequences known as primers are needed. The primers themselves are highly specific, and adhere to corresponding sequences only in the target DNA. After increasing, or amplifying, the target DNA, a system to analyse the product is required. Several approaches are possible. In the current work, a fluorescent dye was used. The dye binds to the DNA product and as it does so, the fluoresence increases and is measured by an optical fluorimeter. This system can also be used to quantify the level of infection present, provided that a robust relationship between the amount of DNA, as measured by fluoresence, and % of seeds infected, can be derived. A seed health test using this technology would have the ability to deliver results within a time scale which could provide a realistic opportunity for growers to avoid routine prophylactic treatment, and target agrochemicals where they are needed.

DNA primer sequences for leaf stripe and net blotch

A MAFF funded project at NIAB has carried out fundamental research to identify primer sequences which were specific to leaf stripe and net blotch. Though sequences specific for leaf stripe were identified, it has not yet been possible to develop sequences which only identified net blotch. However, other primer sequences (referred to as "general" primers were designed which identified both species. This provided the potential for designing a two stage process. Firstly, the primers capable of identifying both species could be used to screen samples. Positive reactions would be highly likely to contain only net blotch, and could be quantified to determine infection level. Negative reactions would not have either disease, and seed need not be treated for them. The positive reactions

would be screened again with the specific leaf stripe primers to check for presence of this disease. Positives from this could be treated either according to the level of infection, or routinely. Given the very low incidence of leaf stripe, routine treatment should be regarded as best practice to maintain a downward pressure on the background level of this disease. This project was aimed at developing such a test system, and involved optimising test conditions for the general primers, checking their specificity, quantifying the reaction for net blotch, and ensuring that the test system was capable of the routine handling of samples at their likely frequency of submission after harvest.

Optimisation of test conditions

A polymerase chain reaction consists of a number of stages to separate double stranded target DNA, join the primers to the strands, extend the region between the primers, separate it again etc. The stages proceed for varying lengths of time at different temperatures, and conditions may be quite specific for different primer sequences. "Multiplex" systems, where reactions with primers for multiple targets can be carried out together, provide the most efficient way to identify the components of a disease complex. However, optimum conditions for the different primer sets used in this work could not be achieved in the same reaction due to different temperature requirements for some steps of the PCR process. Though this has necessitated a two stage process, the PCR system is still very rapid.

Specificity of primer sequences

The specificity of the leaf stripe primers has been previously established. However, it was necessary to check the general primers against a range of barley infecting Pyrenophora species, and a number of other organisms, either pathogens or saprophytes, which might possibly be found on barley seed. DNA from two *Pyrenophora* species, *P. lolii* and *P. bromi*, was not amplified with the primers, but since these are largely found on grasses, this failure has no practical significance. Isolates of net blotch and leaf stripe from a wide geographical range all reacted positively with the primers. DNA from two non-target organims, a species of Alternaria, and spores of bunt (*Tilletia caries*), both of which could be found adhering to barley seed, was amplified weakly with the primers. However, infection levels of these interfering species would have to be extremely high (at least 1000-fold higher than *P. graminea* or *P. teres*) in order to give a false positive test result, and the likelihood of this occurring from a seed sample is very remote.

Extraction and quantification procedure for net blotch from seed samples

A rapid and reliable extraction procedure from seed samples was developed for leaf stripe within the MAFF funded project. Leaf stripe was detected in all the samples where it was expected (over 80 samples). To maximise the efficiency of the test, the same extraction procedure would need to be equally reliable for net blotch. Furthermore, since the test for net blotch needs to be quantitative, the procedure should be sufficiently reproducible to minimise extraction error in the derivation of the relationship between DNA and % infection. Initial tests with a single sample with relatively high net blotch infection using various modifications of the procedure (extended stomaching and the use of glass beads added to the stomacher bag) did not significantly improve the extraction of net blotch DNA and the leaf stripe extraction procedure was thus used for all subsequent work with net blotch. To quantify the test for net blotch, a number of samples were prepared by mixing appropriate quantities of a highly infected lot with a low infection level lot. Twelve target infection levels between 0 and 10% were constructed. Genomic DNA was prepared from a pure culture of net blotch, and different concentrations were used to derive a calibration between the degree of fluoresence measured in the PCR and DNA concentration. The PCR test was then carried out on 10 replicate samples of each target infection level, and a further 10 replicates were assessed in an agar plate test. There was a very high and significant correlation between the mean PCR results, and the mean plate test result ($r = 0.93$, $p = 0.01$, see Figure 1). A linear regression equation, $y = 5.17x$, where y is picograms of DNA and x is the % infection on plates, was used to describe the relation, assuming that zero infection would not generate any PCR product. This equation was used to predict the % infection level in a total of 88 samples of barley submitted to NIAB for testing in 1998 and 1999. Samples were re-tested on plates at the same time as the PCR test was carried out.

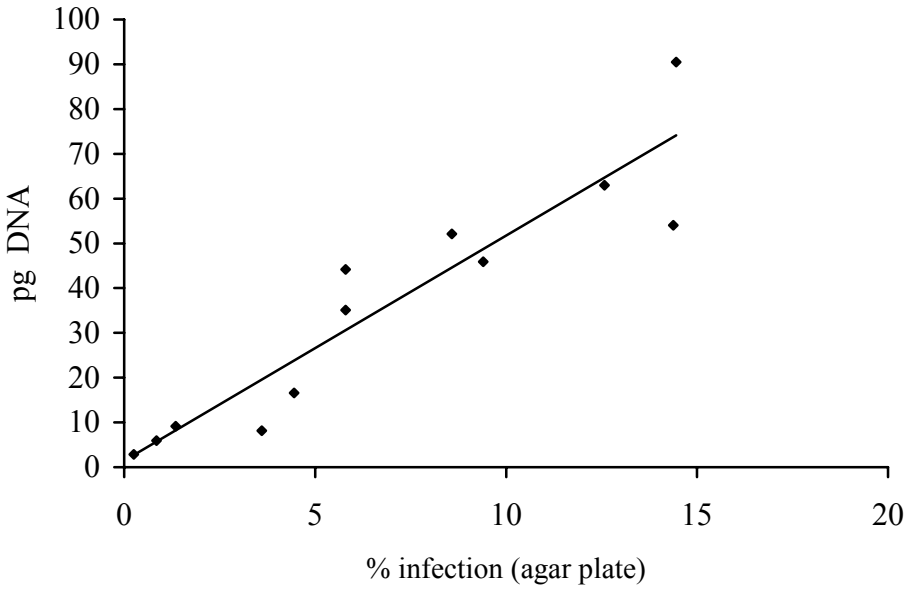


Figure 1 Relationship between picogram DNA in PCR test and % infection on plates for net blotch

The comparisons between plate test result, and predicted % infection from the PCR are shown in full in Table 2. There was a high and significant correlation (r= 0.78, p=0.001) between the plate test and the predicted result. However, the PCR gave a large overestimate of infection levels on those samples where the plate test itself gave a relatively high result. This is probably due to higher individual seed loading with fungus in those samples where infection incidence was high, but in practical terms, the overestimate has little effect, since the treatment decision would always have been the same. In three samples, the PCR predicted a lower level of infection than the plate test to the extent that the treatment decision would have changed, taking 5% infection as a treatment threshold. Even so, the underestimate of infection only occurred on samples which had a relatively low infection, and effects on future disease development through non-treatment would probably be minimal. The causes of variation between PCR prediction and plate test result, apart from the seed loading effect, may be due to variation in the efficiency of extraction from the seed. However, sample to sample variation within the same lot does occur for plate test results and this is probably responsible for most of the smaller differences between the PCR predicted % and plate test %. A comparison of PCR and plate test results for 5 repeat sub samples from 4 different lots is shown in Table 3. Variability was generally greater using % infection predicted by the PCR test, but this variability was more apparent at the higher infection level lots, where seed loading with fungus might be expected to influence results. At lower infection ranges, variation between sub samples was similar for each type of test.

Table 2 Observed % infection (plate test) for net blotch, and PCR predicted infection %

Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
0	0.6	10	3.7	34	66.2	2	17.4
9.5	6.8	1.5	1.7	28	30	9	13.9
0	4.6	9	8.9	4	21	6.3	29.7
4.5	5.8	12.5	9.3	10.5	33.1	8	14.8
23.5	22.6	20.5	14.1	46.5	74.4	4	13.6
7	12.4	4	10.8	9	17	14	21.7
3.5	3.5	0.5	1.5	0.5	6.1	3	17.2
24.5	22.1	4.5	9.6	73.5	404.3	1	16
1	5.8	6.5	6.1	78.5	170.2	10	10.8
2	5.4	19.5	31.4	2.5	7.2	6.5	28.2
36.5	75.5	7.5	10.1	3	9	4.5	15.5
5	11.5	1	13.6	69	90.8	1	2.7
3.5	3.8	6.5	6.8	42.5	165.9	3.5	3.6
7.5	10.2	3	3.2	24.5	23.2		
32.5	38.1	4.5	9.2	15	12.7		
2	3.9	3.5	10.2	61.5	75.5		
7	3.4	1	1.8	0	2.6		
32	34.6	53	120.4	2.5	11.7		
16.5	5.1	27	41.4	18.5	12.9		
16.5	19.8	76.5	87.7	34	32.4		
2	3.1	54.5	70.5	10	11.6		
11.5	20.9	89	227.3	13	9.7		
2	2.8	50.5	51.6	0	4.6		
6.5	8.5	54.5	45.9	10	11.9		
12	8.8	41.5	54	29	58.2		

Table 3 Variation in repeat sub-samples tested with PCR and on agar plates

	Lot 1	Lot 2	Lot 3	Lot 4
Plate test %				
1	27.0	73.5	3.0	9.5
2	27.5	82.5	3.0	14.5
3	36.0	90.0	4.0	15.0
4	25.5	85.0	4.0	15.0
5	25.0	87.5	2.0	7.5
Mean	28.2	83.7	3.2	12.3
SE	2.00	2.84	0.37	1.56
PCR predicted %				
1	39.7	462.1	5.0	20.2
2	36.8	209.4	6.3	10.5
3	55.9	238.4	9.6	17.4
4	22.8	298.2	9.1	18.9
5	41.4	469.8	9.0	7.8
Mean	39.3	335.5	7.81	14.96
SE	5.27	55.1	0.92	2.46

False positive and false negative screening

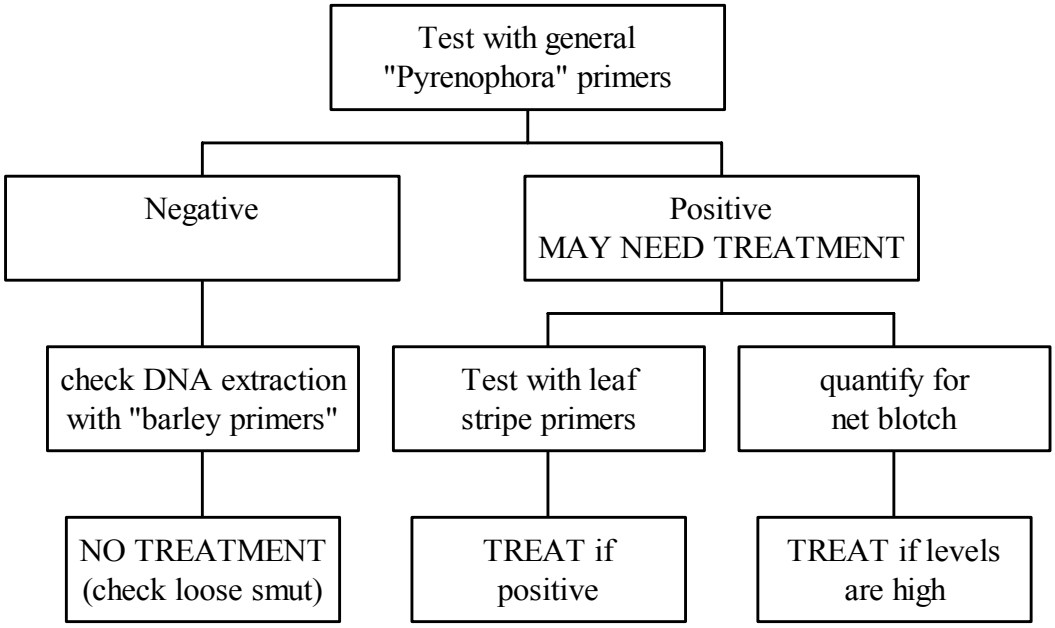
False positives could occur if unknown organisms were present which did have DNA sequences to match the primers, or if non-viable target fungus was present. To determine the risk of false positives occurring for leaf stripe, 100 samples with a nil result in the plate test were screened with the PCR test, and no positives were obtained. False positive screening for net blotch could not be carried out rigorously, since so few samples have nil infection. However, there were no unexpectedly high predicted results from samples where only low infection had been recorded on agar plates. No false negatives for net blotch occurred in 110 out of 110 samples where infection was expected. False negative tests for leaf stripe were carried out during MAFF funded project work, and one negative PCR result occurred in 60 tests where infection was expected. This was due to a DNA extraction failure, and an internal control step was introduced to detect this. The control consists of primer

sequences which can detect any barley DNA, which is always extracted with the sample. They are used if the general leaf stripe/net blotch primers fail to detect any corresponding DNA before recommending that no treatment would be necessary.

Sample flow and test logistics

Sample flow into NIAB laboratories can be assumed to give a relatively accurate picture of the frequency of seed handling and movement on farms, and projecting this frequency for an estimated total of about 15,000 seed lots (perhaps 10,000 of which are farm-processed) suggests that up to 1000 samples will need to be handled each week during the latter part of July and most of August. A single operator can extract and prepare about 60 samples per day. These can be tested on the day of preparation, or stored overnight. Each PCR "run" lasts about 30 minutes, and is operated according to the flow diagram shown below. About 10 "runs" per day could be achieved, each containing a maximum of 12 tests samples. At full capacity, a single laboratory operating this type of equipment, or its equivalent, could handle about half of the testing requirement of the UK. The techniques can be learnt quite quickly in comparison to the lengthy acquisition of mycological skills necessary for conventional testing. At present, the limiting step in sample throughput is DNA extraction rather than the PCR run, but further automation in this procedure is probably achievable in the near future.

Flow chart illustrating steps in leaf stripe/net blotch PCR tests



Conclusions

This work has achieved the objectives of optimising and quantifying a PCR test for barley net blotch, and combining this with an existing test for leaf stripe. The method has been used in routine tests at NIAB for harvest 2000, and has improved turn around time from seven days to a maximum of two. This provides growes with an increased opportunity to target seed treatment for these diseases where they are needed, and reduce inputs when they are not required. Since batch testing is possible, and the PCR method overall is less labour intensive, cost savings can be passed on to customers in a lower test price. This is particularly important for smaller farm-saved seed lots where the cost of testing using conventional methods can outweigh the cost of seed treatment. The PCR test has enabled lower pricing for leaf stripe and net blotch, but further work is needed to extend this methodolgy to loose smut. Though current methods for detecting loose smut offer relatively rapid results, the test is labour intensive and quite difficult to carry out. A PCR method with the potential of batch-testing would offer the potential for further cost reductions in barley seed health testing.

TECHNICAL REPORT

The work of the project has been prepared for publication as follows:

J. E. Thomas, E. J. A. Taylor, J. A. Bates and D. M. Kenyon. (2000) The application of a real-time PCR method to detect *Pyrenophora* species in barley seed, and implications for seed treatment strategies. *Proceedings of the EPPO Conference on Diagnostic Techniques for Plant Pests , Wageningen, in press*

J. A. Bates, E. J. A. Taylor, D. M. Kenyon and J. E. Thomas. The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed *Submitted to Molecular Plant Pathology On Line, 2000*

D. M. Kenyon, J. E. Thomas, J. A. Bates, E. J. A. Taylor. Quantitative and qualitative detection of *Pyrenophora* species on barley seed using PCR in advisory seed health testing. *Accepted for BCPC Seed Treatment Conference Proceedings, 2001.*

Parts of the following paper have been used in the preparation of these submissions

The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed and its use in advisory seed health tes

Keywords: *Pyrenophora teres*, *Pyrenophora graminea*, real-time detection, PCR quantification, seed health test

ABSTRACT

A real-time quantitative PCR technique has been used to develop a rapid and sensitive seed health test for *Pyrenophora* spp. on barley seed. Using the fluorescent reporter dye SYBR Green I for real-time detection of PCR amplification in a LightCycler™ instrument, pathogen DNA extracted from infected seed can be quantified to the picogram level. The amount of *Pyrenophora teres* DNA extracted from seed samples of an artificial infection level gradient, constructed by mixing infected and uninfected seed, correlated with good agreement (r = 0.93) to percentage infection levels of the same samples measured by agar plate testing. The proposed test was performed in three parts: (1) quantification of *Pyrenophora* spp. infection using *Pyrenophora* specific PCR primers; (2) test of any negative samples from (1) with barley-specific PCR primers to check the DNA extraction process; (3) test of positive samples from (1) for the presence of *P. graminea* using *P. graminea* specific PCR primers. All PCR reactions were performed in the LightCycler™ instrument allowing each PCR

and analysis to be completed within about 30 minutes. A single operator could extract up to 60 samples in a day, and over 100 samples could be processed in the Light Cycler ™ each day.

INTRODUCTION

There are two species of Pyrenophora which affect barley in the UK: *Pyrenophora graminea*, the causal agent of leaf stripe and *Pyrenophora teres*, which causes net blotch. *P. graminea* is strictly seed-borne and seed treatment with systemic fungicides is the only means of controlling infection. *P. teres*, however, can be either seed or debris-borne and while seed treatment can help to reduce early infection by net blotch, foliar fungicides can also be very effective if plants later become infected from infested plant residues.

Management of seed-borne Pyrenophora can be achieved by advisory seed testing. There is a voluntary rather than statutory threshold for treatment set at 2% seed infection for *P. graminea*. Although this standard may also applied to net blotch, higher levels of infection may be tolerated since there is a relatively large source of field inoculum of *P. teres* in most growing areas. Results of barley seed health tests carried out at NIAB over the last 6 years indicate that levels of leaf stripe are declining to very low incidence, with only an occasional sample failing the threshold level. However the incidence and severity of net blotch on seed is much more variable from year to year, largely depending on weather factors and the popularity of susceptible varieties.

Since barley is predominantly autumn sown in the UK, there is only a very short time period available for testing around 15,000 seed lots which need to be processed in 8-10 weeks. The current seed health test method uses agar plates (Rennie and Tomlin, 1984), takes seven days to complete and requires a high level of technical skill to identify morphological growth characteristics. As a result, most seed is treated prophylactically without any knowledge of its health status. A rapid and reliable seed health test that can deliver equivalent results to the agar plate test is therefore needed if seed treatments are to be targeted where they are required. The PCR technique has the ability to do this: not only can pathogens be specifically detected in a complex DNA background but the amount of DNA and hence level of infection, can be quantified using a real-time detection technique.

Specific PCR primers for detection of *P. graminea* have previously been developed from a RAPD product (Taylor et al., 2000). The use of these RAPD sequences was, however, unsuccessful for obtaining primers specific for *P. teres*. Sequence data obtained previously from internal transcribed spacer region (ITS) of ribosomal RNA genes of *Pyrenophora* spp. (Stevens et al., 1998) was used as an additional source of information for primer design. This region has been used by several groups for the specific detection of a number of closely related fungal plant pathogens (Henson and French, 1993; Ristaino et al., 1998; Willits and

Sherwood, 1999), as the ITS sequence region tends to vary as much within species as between them. The rRNA genes are highly repetitive and the ITS and intergenic spacer regions evolve relatively quickly (White et al., 1990) such that variations may be found within closely related species, making them attractive targets for PCR based-assays. Previous research on the ITS and 5.8s rRNA region in barley infecting Pyrenophora spp. (Stevens et al., 1998) had indicated however that the amount of intraspecific variation was extremely small, with only one base pair difference in the ITS1 region separating *P. graminea* from the other *Pyrenophora* species. We therefore designed a second set of primers specific for barley infecting *Pyrenophora* species.

We present here a method for the detection of Pyrenophora infection in barley seed together with the specific identification of *P. graminea.* In addition we demonstrate that *P. teres* infection can be quantified using a real-time PCR technique and that the amount of pathogen DNA extracted from seed correlates well with the level of infection measured using the conventional plate test. The sensitivity is such that seed lots with less than 1% infection with either species can be readily identified as positive. These PCR methods have been developed into a rapid seed health test that can be completed within a day, with increased sample throughput compared to the traditional agar plate test.

MATERIALS AND METHODS

Origin and maintenance of fungal isolates

The *Pyrenophora* spp. and other fungal isolates used in this work were obtained from geographically diverse areas and host species (Table 1). Fungal isolates derived from single conidia were identified, cultivated and maintained as described by Stevens *et al*. (1998).

Barley seed samples

Barley seed samples which had tested positive for *P. teres* and /or *P.graminea* infection by agar plate testing (Rennie and Tomlin, 1984) were obtained from the Official Seed Testing Station (OSTS), Cambridge. A further 100 samples which had tested negative for *P graminea* were also identified. An artificial infection level gradient for *P teres* was constructed by mixing different proportions of infected and non-infected seed using the thousand grain weights of each lot to determine the amounts to be used. Twenty-five replicate samples of 200 seeds were prepared for 12 different target infection levels ranging from 0% to 10% by blending appropriate proportions of 32.5 % and 0% infected seed lots of the variety Regina using a Riffle divider. Ten replicates were assessed using an agar plate test and ten with the quantitative PCR assay.

Fungal DNA extraction

Genomic DNA was extracted from fungal cultures as described by Taylor et al. (2000) adapted from a method by Raeder and Broder (1985).

DNA extraction from barley seed infected with Pyrenophora spp.

Seed extracts were prepared essentially as described by Taylor et al. (2000), with some modifications. Two hundred seeds were soaked in 20 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 5 min in an extraction bag (12 x 14 cm, Bioreba, supplied by Bond Bio-tech Ltd, UK) placed inside a second bag (11 x 23 cm, Fisher Scientific, UK). The seed soak was blended for 1 min in a stomacher (Lab-Blender 400, A.J. Seward, Suffolk, UK). 15 ml of the seed soak liquor was transferred to a 15 ml tube, placed on ice and centrifuged at 9000 g, 4^{o}C for 10 min. The supernatant was removed and the pellet freeze-dried. DNA was extracted from the pellet using the Nucleospin Plant kit (Machery-Nagel GmbH & Co, Duren, Germany) as described previously (Taylor et al., 2000).

Development of the PCR assay

Primers specific for *P.graminea* (PG2F and PG2R) were designed from sequences of RAPD products and described previously (Taylor et al., 2000). General primers for barley-infecting *Pyrenophora* spp. (ITSFF and ITSR) were designed from *Pyrenophora* ITS1 sequences with ITSFF corresponding to bases 15-37 and ITSR bases 98-121 (reverse and compliment) of *the P. teres* sequences (Figure 3 in Stevens et al., 1998). The latter primers were used in the quantification of *P. teres.* Both sets of primers were tested against DNA from other pathogens and saprophytes commonly found on barley. Primers for barley DNA (Hv5s-1 and 5s-2) were based on sequences of the 5S rRNA genes (Genbank accession number U07391, bases 67-86 and 464-485 respectively) and were used to check the DNA extraction of suspect negative samples. Primer sequences are listed in Table 2.

PCR reactions consisted of 10 µl volumes containing 1 µM of each primer, 0.5 µl SYBR Green I (Catalogue number 1765, Biogene Ltd, UK, final dilution, 1 in 20,000), 5 µl of PCR Master Mix (3mM $MgCl_2$, Biogene Ltd, UK) and 2.5 µl DNA template from the extraction protocol given above, or standard DNA of known concentration. For quantification, a serial dilution of *P. teres* genomic DNA was prepared. DNA concentration was determined using a fluorescent assay in a DyNA Quant™ 200 fluorimeter (Hoefer Pharmacia Biotech Inc., USA) using the Hoescht 33258 dye. *P. teres* standards were then prepared by dilution to 1 ng/µl, 100 pg/µl, 10 pg/µl and 1 pg/µl. Duplicate PCR reactions for both standards and unknown samples were carried out for all quantification assays.

The PCR reaction conditions for primers ITSFF and ITSR were: initial denaturation at 95°C for 30 s, followed by 35 cycles of 95°C for 0 s, 65°C for 0 s and 72°C for 6 s. For primers PG2F and PG2R conditions were: initial denaturation at 95°C for 30 s, followed by 35 cycles of 95°C for 0 s, 68°C for 0 s and 74°C for 5 s. For primers Hv5s-1 and 5s-2 the conditions were: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 0 s, 55°C for 5 s and 72°C for 17 s. A melting programme (72°C-98°C at a rate of 0.2°C/s) was included at the end of each amplification. All reactions were performed in a LightCycler™ instrument (Idaho Technology Inc., USA). PCR product accumulation was measured at the end of each amplification cycle by the fluorescence of SYBR Green I dye binding to double stranded DNA. The identity of each product was confirmed by melting curve analysis at the end of the reaction. For quantification, a standard curve was generated by plotting the log of the DNA concentration of the known standards against the cycle number at a defined point in the log-linear increase in the fluorescence of the PCR product (crossing point). From this, unknown sample template concentrations could be derived.

Accuracy and reproducibility of the assay

To test the variability of the DNA extraction technique, ten individual seed samples with *P. teres* infection were prepared as described above and the seed soaks mixed together prior to centrifugation and freeze-drying. The combined percentage infection level mean was 24.4 %. 15 ml of the mixture was aliquoted into each of 10 tubes, centrifuged and the pellets freeze-dried. DNA was extracted from the pellets as described above and PCR carried out on 2.5 µl aliquots, as above.

To investigate sample variability of naturally infected samples, five repeat sub samples of four different seed lots covering a wide range of percentage infection levels with *P. teres*, were extracted and tested by PCR as above. The original infection levels of the submitted samples were 3 %, 15 %, 27 % and 73.5 %. A further five sub samples were re-tested on agar for compariosn with the PCR test.

RESULTS

PCR screening of *Pyrenophora* spp. DNA with PCR primers

PCR primers PG2F and PG2R have previously been screened against several *Pyrenophora* spp. and other pathogens and saprophytes commonly found on barley (Taylor et al., 2000) and found to be specific for *P. graminea* for all samples tested. ITSFF and ITSR primers were designed from ITS1 sequence data obtained from a number of clones of *Pyrenophora* spp. (Stevens et al., 1998) and were tested in a similar manner with the results presented in Table 1. Primer sequences are given in Table 2. All PCR reactions were performed in the LightCycler™ instrument and the products recovered from the capillaries by centrifugation for gel analysis.

Table 1 Details of the *Pyrenophora* spp. and other isolates used in this work.

Isolate identity	Host	Country of	Date of	Amplification
DPg 95-5	n/a	Denmark	1995	+
SsPg 97-BS1	n/a	n/a	1997	+
SsPg 97-BS6-1	Barley	Yugoslavia	1997	+
SsPg 97-76a	Barley	UK	1997	+
SsPg 97-78c	Barley	UK	1997	+
SsPg 97-81g	Barley	Italy	1997	+
Pg 97-83b	Barley	UK	1997	+
Pt 93-9	n/a	UK	1993	+
Pt 95-17	n/a	UK	1995	+
Pt 95-32	n/a	Sweden	1995	+
Pt 96-1	Barley	UK	1996	+
RPt 96-2	n/a	Russia	1996	+
RPt 96-6	n/a	Russia	1996	+
RPt 97-78g	n/a	Russia	1997	+
SsPm 857	Barley	Canada	1996	+
SsPm 1881a	Barley	Canada	1996	+
P. avenae 94-1	Oats	UK	1994	+/-
P. avenae 94-3	Oats	UK	1994	+/-
P. hordei 94-1	Barley	Australia	1994	+
P. hordei 94-12	Barley	Australia	1994	+
P. bromi 94-1	Brome	Denmark	1994	-
P. lolii 95-1	Ryegrass	UK	1995	-
Alternaria tenuis 97-1	n/a	UK	1997	-
Alternaria spp. 97-1	n/a	UK	1997	+/-
Cochliobolus sativus 97-10	Wheat	UK	1997	-
Epicoccum spp. 97-1	n/a	UK	1997	-
Fusarium culmorum 98-11	Wheat	UK	1998	-
F.moniliforme 98-1	n/a	n/a	1998	-
F. oxysporum	Linseed	UK	1997	-
F. poae 95-1	Wheat	UK	1995	-
F. spp. 97-1	Bean	UK	1997	-
Microdochium nivale	Wheat	UK	1997/1998	-
Septoria nodorum 98-2	Wheat	UK	1998	-
Tilletia caries	Wheat	UK	1999	+/-

Abbreviations: DPg, ssPg or Pg = *P. graminea*; RPt or Pt = *P. teres*; ssPm = *P. teres* f. sp. *maculata*; n/a = information not available.

Table 2 Sequences of PCR primers used in the seed health test. Hv5s-1 and 5s-2 amplify barley DNA. ITSFF and ITSR amplify barley-infecting *Pyrenophora* spp. PG2F and PG2R are specific for *P. graminea*.

Primer pair	Sequence	Product length
Hv5s-1 5s-2	5'-TAGGAAGTCCTCGTGTTGCA-3' 5'-CATTAGTGCTGGTATGATCGCA-3'	419 bp
ITSFF ITSR	5'-GCAGATTGGGTAGTCCCCGCTTT-3' 5'-GAGCCCGCCAAGGAAACAAGTAGT-3'	94 bp
PG2F PG2R	5'-CTTCTTAGCTGGGGCTACCGTC-3' 5'-ACCGACTCGGGAAAAGAGCA-3'	435 bp

Of the *Pyrenophora* spp. tested, only *Pyrenophora lolii* and *Pyrenophora bromi* failed to amplify with primers ITSFF and ITSR. As these two species are found predominantly on grasses and are unimportant as pathogens in barley the negative result is of little importance to the seed test. *Pyrenophora. avenea*, the cause of seedling blight of oats, did produce a positive PCR product but was very poorly amplified even though equivalent amounts of DNA were included in the PCR reactions. This may be due to mis-match between the primer ITSFF as some of the *P. avenea* ITS sequences previously analysed were found to be highly variable in this region (Stevens et al., 1998).

Of the other pathogens and saprophytes tested, only *Tilletia caries* and an unidentified *Alternaria* species. (but not *A. tenuis*) gave any amplification products, as shown in the melting profiles in Figure 3b. When the products were analysed by agarose gel electrophoresis, however, several of the other species appeared to have faint bands of the correct size for products of ITSFF and ITSR that were not detected on the LightCycler™. As was the case for amplification *of P. avenae*, products with species other than *Pyrenophora* appear at very late amplification cycles even though the DNA concentrations in the PCR reactions were the same as for *P. graminea* and *P. teres*. This suggests that infection levels of these cross-reacting species would have to be extremely high (at least

1000-fold higher than *P. graminea* or *P. teres*) in order to give a false positive test result. In conclusion, PCR in the LightCycler™ using primers ITSFF and ITSR essentially detects *P. graminea*, *P. teres* and *P. hordei* (a recently identified barley pathogen isolated from Australia, Wallwork et al., 1992) in extracts from barley seed.

Quantification of *Pyrenophora teres* in constructed percentage infection gradients

Quantification of *Pyrenophora* spp. was carried out using a real-time PCR technique performed in a LightCycler™ instrument. *P. teres* genomic DNA standards of known concentration were run in parallel reactions to generate a standard curve from which the DNA concentration of unknown samples could be derived. Figure 4 shows the quantification of *P. teres* genomic DNA using primers ITSFF and ITSR and the corresponding standard curve. As expected for 10-fold differences in concentration, initiation of amplification occurs at approximately 3-cycle intervals.

In order to correlate percentage infection levels with the amount of pathogen DNA present on the seed, an artificial percentage infection gradient was set up by blending various amounts of *P. teres* infected and uninfected seed. Ten replicates at each of 12 different infection levels were tested both by traditional agar plate test and quantitative PCR. The results are presented in Table 3. Mean infection % and the mean concentration of DNA in ten samples at each infection level were highly correlated ($r=0.93$, $P=0.01$). Although variation between samples within any one infection level appears to be quite high using the PCR technique, it was possible using 10 replicates to obtain a mean value that measured a gradient of infection of between 0 % and 14 %. It is noteworthy that the traditional agar plate test is also subject to quite large variation, as shown in Table 3, suggesting that this is a feature of seed sampling itself and not the technique used to measure infection. These data were used to derive a linear conversion described by $y=5.17x$, where y = picogram DNA, and x = % infection level.

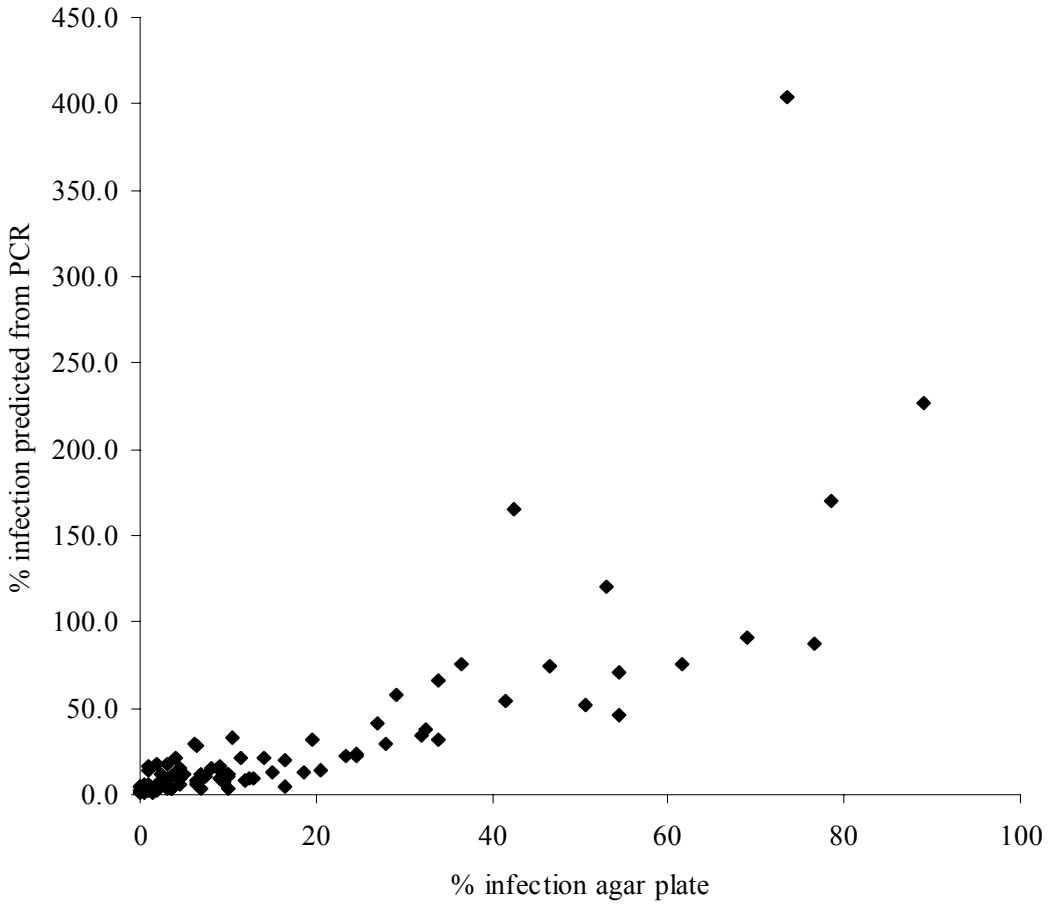
Table 3 Construction of an artificial infection level gradient for *P. teres*: results by conventional agar plate test and quantitative PCR. The range represents the lowest and highest results of ten replicate samples for each method.

Target	Agar plate test (% Infection)		Quantitative PCR (pg DNA)	
% Infection	Range	Mean	Range	Mean
0	0 - 1.0	0.3	1.3 - 6.1	2.8
0.5	0 - 1.5	0.9	1.4 - 11.2	5.9
1	0.5 - 2.5	1.4	3.0 - 22.7	9.1
2	1.5 - 6.0	3.6	3.8 - 16.1	8.1
3	3.0 - 6.5	4.5	6.3 - 31.0	16.6
4	3.0 - 9.0	5.8	14.8 - 83.2	44.1
5	3.5 - 10.5	5.8	14.9 - 76.0	35.1
6	4.0 - 11.0	8.6	25.7 - 103.8	52.1
7	5.0 - 13.5	9.4	24.4 - 77.2	45.9
8	10.0 - 16.7	12.6	22.2 - 93.2	62.9
9	12.0 - 18.0	14.4	23.0 - 135.0	54.0
10	8.5 - 20.5	14.5	51.9 - 164.3	90.5

Quantification of *Pyrenophora* spp. in seed test samples

Seed samples received by OSTS for advisory tests were also tested by quantitative PCR using primers ITSFF and ITSR. A total of 88 samples with a wide range of infection with *P. teres* was tested and the PCR results used to predict % infection level. are presented in Figure 1. A good correlation (r=0.78, P=0.001) was obtained between infection level on plates and that predicted from the PCR test although variability increased above about 40 % infection on plates. In these cases, the PCR test predicted well over 100% infection.

Figure 1 Relationship between plate test % and PCR predicted % infection with *P. teres*



Accuracy and reproducibility of the assay

The reproducibility of the DNA isolation technique was investigated by extracting DNA from ten aliquots of a pooled seed soak obtained from *P. teres* infected seeds, with a mean infection level of 24.4 %. The mean PCR result was 115.8 +/- 22.0 pg DNA with individual samples varying, on average, around 14 % from the mean. All PCRs were run in duplicate and the variation of individual PCR reactions from the mean was, on average, 15 % and was independent of the starting DNA concentration.

To investigate sample variability of naturally infected samples, five replicates of four different seed lots covering a wide range of percentage infection levels with *P. teres*, were tested (Table 4). These results illustrate that there is considerable variation in the amount of DNA extracted from each 200 seed lot, but also in the % infection levels of repeat sub samples on agar plates. A similar result was observed for the individual seed lots prepared

for the artificial percentage infection level gradient, both with the agar plate test and quantitative PCR (Table 3). This indicates that the largest source of variation results from sub-sample to sub-sample variation, and not from poor reproducibility of the PCR test.

Table 4 Variability between repeat sub samples in % infection from agar plates and PCR predicted % infection from four seed lots

	Lot 1	Lot 2	Lot 3	Lot 4
Plate test %				
1	27.0	73.5	3.0	9.5
2	27.5	82.5	3.0	14.5
3	36.0	90.0	4.0	15.0
4	25.5	85.0	4.0	15.0
5	25.0	87.5	2.0	7.5
Mean	28.2	83.7	3.2	12.3
SE	2.00	2.84	0.37	1.56
PCR predicted %				
1	39.7	462.1	5.0	20.2
2	36.8	209.4	6.3	10.5
3	55.9	238.4	9.6	17.4
4	22.8	298.2	9.1	18.9
5	41.4	469.8	9.0	7.8
Mean	39.3	335.5	7.81	14.96
SE	5.27	55.1	0.92	2.46

False positive tests

None of the 100 samples tested which had nil infection with *P. graminea* on agar plates showed any product amplification in the LightCycler™. It was not possible to test an equivalent number of samples with nil infection with *P. teres*, but there was no evidence of any very low infection samples having high predicted levels from the PCR test.

DISCUSSION

A seed health test needs to satisfy a number of requirements. Primarily, it must specifically detect the pathogen of interest at a level sensitive enough to detect tolerated thresholds. For this, it needs to be repeatable within and between samples, within the limits of sampling error. It should also be reasonably simple and robust such that the technology can be readily transferred between laboratories and from a commercial angle, rapid and economic. Conventional diagnostic methods for seed health testing include agar plate tests, seedling bioassay and microscopic observation. These methods, however, are time consuming, labour-intensive and subjective. The technology of nucleic acid test methods is expanding rapidly and these have the advantage in that they are highly specific, sensitive and rapid with the potential for automation, leading to high throughput (Reeves, 1995). The PCR test for *Pyrenophora* spp. described in this paper fulfils the requirements set out above, reducing the current test length from seven days to a maxiumum of two. The PCR reactions described in this work were performed in a LightCycler™ instrument which allows real-time detection of amplification products by the fluorescence of SYBR Green I dye binding to double stranded DNA (Wittwer et al., 1997). Real-time PCR detection instruments such as the LightCycler™ are able to quantify nucleic acids in unknown samples by direct comparison to standards amplified in parallel reactions (Morrison et al., 1998). Quantification of fungal pathogens of plants has previously been achieved using conventional PCR techniques (Hu et al., 1993; Mahuku et al., 1995; Nicholson et al., 1996; Nicholson et al., 1997) but these are time consuming and do not give an accurate measurement of a reaction in the log-linear phase of amplification, which is the most informative point of the PCR reaction. One of the main advantages of the PCR detection methods described in this paper is that there are no lengthy post PCR analysis steps, as the PCR and detection occur in the same tube. This closed-tube system also reduces the risk of cross- contamination.

In addition to simple quantification assays, real-time detection provides the facility to measure the melting profile of the PCR products (Ririe et al., 1997). At the end of the cycling reactions, the products are melted by slowly increasing the temperature at the same time as continually monitoring the fluorescence. The temperature at which the product melts is characterised by its sequence composition and length. Melting curve analysis at the end of a run provides a check that specific amplification has occurred and distinguishes products from non-specific primer-dimers and can highlight any contamination.

Real-time quantitative PCR is used widely in the clinical field for detection of viral and bacterial infections (Abc et al., 1999; Hardegger et al., 2000; Machida et al., 2000; Reischl et al., 2000) and there are increasing reports of applications in plant pathology (Bohm et al., 1999; Weller et al., 2000) and its use for detecting genetic modification of crops and foodstuffs (Vaitilingom et al., 1999).

As the measurement of SYBR Green I fluorescence is a non-specific (product independent) detection method, the Pyrenophora test is carried out in two (or three) stages. The first stage comprises the quantification reactions using primers ITSFF and ITSR, which will amplify any barley-infecting *Pyrenophora* spp., extracted from the seed. In the UK, this effectively measures the level of *P. teres* infection since *P. graminea* is rare. Any positive samples from the first stage above a threshold level set from the correlation of percentage infection levels and amount of DNA determined from constructed infection level gradients are tested with the *P. graminea* specific primers PG2F and PG2R. Any negative samples from the first stage (those which show no characteristic Pyrenophora melting curve below the threshold level) are subjected to a third test using the barley primers Hv5s-1 and 5s-2. This latter test indicates if there has been a failure in the DNA extraction procedure as some barley DNA is carried over in the extraction and can be measured independently. It is extremely rare that this part of the test needs to be performed as samples well below the "negative" threshold limit were found to give small amounts of products with a characteristic *Pyrenophora* melting curve compared to controls, thus confirming a successful DNA extraction and illustrating the sensitivity of the technique.

As indicated above, the method is extremely sensitive, being able to readily measure a fraction of a picogram of pathogen DNA from the seed extract. From the results presented in Table 3 this represents a value of less than 0.5 % infection, well within the accepted threshold limit. Even samples recorded as zero by agar plate testing were found to contain small amounts of pathogen DNA, although it cannot be certain if this came from viable pathogen or not.

For such a sensitive test, the PCR primers chosen need to be highly specific. Any number of other pathogens and/or saprophytes may be present on the seed surface and these will be extracted along with those of interest. Of the other fungal species which could potentially be found on or in barley seed, only two of those tested (an *Alternaria* spp. and *T. caries*) gave any cross-reactivity that could be measured by the LightCycler™. Amplification of these was extremely poor and indicated that very high levels of infection would have to be present to interfere with the *Pyrenophora* test.

Quantification of *Pyrenophora* DNA extracted from both naturally infected seed samples and samples blended to give target infection levels gave a good correlation with the agar plate test results over a wide range of infection levels despite large variations between some samples. Investigation into the reproducibility and potential sources of variability in the PCR test concluded that the sub-sample itself is responsible for the largest source of error. This is borne out by the fact that the agar plate test results also showed a range of infection levels in repeated sub-samples. The establishment of confidence limits for this seed health test was not part of

the work reported here, and further tests to establish these are desirable. Other sources of variation include differences in seed loading with fungus, and the possible presence of non-viable pathogen. Both of the factors would cause variation in the PCR test, but not in a plate test. The presence of non-viable pathogen material should not be a significant problem in fresh seed, though variation in seed loading was probably the cause of the large overestimate of infection in those seed lots where infection % on agar plates was high.

The PCR test described here cannot be designed to give a precise measure of *Pyrenophora* spp. infection % over a wide range of levels in different seed lots due to the factors discussed above. However, prediction % infection values were accurate in low and moderate infection ranges which are more typical for this disease. Of greater importance is the fact that the PCR test did not under-predict any infection levels which might be regarded as high risk in a field situation if the seed was sown untreated. *P. graminea* can be specifically detected if present at very low levels of infection, and it is recommended that any positive samples for this disease are treated in order to maintain a downward pressure on the pathogen population. This features, together with the rapidity of the test, could encourage the move away from unnecessary and costly prophylactic use of seed treatments and towards increased use of seed health testing.

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Note: The use of the sequences described for a diagnostic test for *P. graminea* is covered by a patent application (British Patent Application No. 9923022.9) owned by NIAB.

REFERENCES

Abc, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaks, S., Yoshiba, M. and Kohara, N. (1999) Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J. Clin. Microbiol. 37, 2899-2903.

Bohm, J., Hahn, A., Schubert, R., Bahnweg, G., Adler, N., Nechwatal, J., Oehlmann, R. and Oswald, W. (1999) Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus Glomus mosseae and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. J. Phytopath. 147, 409-416.

Hardegger, D., Nadal, D., Bossart, W., Altwegg, M. and Dutly, F. (2000) Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. J. Microbiol. Methods 41, 45-51.

Henson J.M. and French R. (1993) The polymerase chain reaction and plant disease diagnosis. Ann. Rev. Phytopath. 31, 81-109.

Hu, X., Nazar, R.N. and Robb, J. (1993) Quantification of *Verticillium* biomass in wilt disease development. Phys. Mol. Plant Path. 42, 23-36.

Machida, U., Kami, M., Fukui, T., Kazuyama, Y., Kinoshita, M., Tanaka, Y., Ogawa, S., Honda, H. and Chiba, S. (2000) Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. J. Clin. Microbiol. 38, 2536-2542.

Mahuku, G.S., Goodwin, P.H. and Hall, R. (1995) A competitive polymerase chain reaction to quantify DNA of *Leptosphaeria maculans* during blackleg development in oilseed rape. Mol. Plant-Microbe Int. 8, 761-767.

Morrison, T.B., Weis, J.J. and Wittwer, C.T. (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification Biotechniques 24, 954–962.

Nicholson P., Lees A.K., Maurin N., Parry D.W., Rezanoor H.N. (1996) Development of a PCR assay to identify and quantify Microdochium nivale var. nivale and *Microdochium nivale* var. *majus* in wheat. Phys. Mol. Plant Path. 48: 257-71.

Nicholson, P., Rezanoor, H.N., Simpson, D.R. and Joyce, D. (1997) Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* using a PCR assay. Plant Path. 46, 842-856.

Raeder, U. and Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. Letts. App. Microbiol. 1, 17-20.

Reeves, J.C. (1995) Nucleic acid techniques in testing seedborne diseases. In New Diagnostics in Crop Sciences (Skerritt, J.H. and Appels, R., eds.). CAB International, Oxon, UK, pp. 127-151.

Reischl, U., Linde, H.-J., Metz, M., Leppmeier, B. and Lehn, N. (2000) Rapid identification of methicillin-resistant *Staphylococcus aureus* and simultaneous species confirmation using real-time fluorescence PCR. J. Clin. Microbiol. 38, 2429-2433.

Rennie, W.J., and Tomlin, M.M. (1984) Barley Leaf Stripe, working sheet No. 6 (2nd edition) In ISTA Handbook on Seed Health Testing. International Seed Testing Association, Zurich, Switzerland.

Ririe K.M., Rasmussen R.P., Wittwer C.T.(1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. 245,154-160.

Ristaino, J.B., Madritch, M., Trout, C.L. and Parra, G., (1998) PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. App. Env. Microbiol. 64, 948-954.

Stevens, E.A., Blakemore, E.J.A. and Reeves, J.C. (1998) Relationships amongst barley and oat infecting isolates of *Pyrenophora* spp. based on sequences of the internal transcribed spacer regions of ribosomal DNA. Mol. Plant Path. On-Line http://www.bspp.org.uk/ mppol/1998/1111stevens

Taylor, E.J.A., Stevens, E.A., Bates, J.A., Morreale, G., Lee, D., Kenyon, D.M. and Thomas, J.E. (2000) Rapid cycle PCR detection of *Pyrenophora graminea* from barley seed. Plant Path. in press.

Vaitilingom, M., Pijnenburg, H., Gendre, F. and Brignon, P. (1999) Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. J. Agric. Food Chem. 47, 5261-5266.

Wallwork, H., Lichon, A. and Sivanesan, A. (1992) *Pyrenophora hordei*- a new ascomycete with Dreschlera anamorph affecting barley in Australia. Mycol. Res. 96, 1068-1070.

Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N. and Stead, D.E. (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. App. Env. Microbiol. 66, 2853-2858.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: a guide to methods and applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds.). USA: Academic Press Inc., pp. 315-322.

Willits D.A. and Sherwood J.E. (1999) Polymerase chain reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barley varieties. Phytopath. 89, 212-217

Wittwer, C.T., Herrmann, M.G., Moss, A.A. and Rasmussen, R.P. (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22, 130-138.