PROJECT REPORT No. 340

CEREAL SEED HEALTH AND SEED TREATMENT STRATEGIES: EXPLOITING NEW SEED TESTING TECHNOLOGY TO OPTIMISE SEED HEALTH DECISIONS FOR WHEAT

Technical Paper No. 1

Development of rapid seed health tests for *Microdochium nivale* seedling blight

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INTRODUCTION

In the UK, seed-borne *Microdochium nivale* causes a pre-emergence blight, with high levels of seed infection responsible for seedling losses where wheat crops are sown untreated (Paveley *et al*., 1996). Levels of *M. nivale* vary considerably from year to year, and high infections are associated with rainfall during anthesis (Kenyon *et al*., 2001 and Cockerell *et al*., 2002).

Winter wheat is the largest cereal crop in the UK, with approximately 2 million ha sown. For winter wheat the time between harvest and sowing is short and cereal seed producers and growers operate under severe time constraints during the autumn period. More than 1000 seed lots are processed per week during September and October (Paveley *et al*., 1996). The current seed health testing method for *M. nivale* is slow and labour intensive. The test involves plating seed on agar. The agar plates then require from five to seven days incubation, sometimes longer, before the result is available. Experienced personnel are necessary to examine the fungal colonies that grow on the agar and throughput is restricted to approximately 15 samples per analyst per day. The time required for testing and the restrictive throughput limits the usefulness of this test during the autumn period and most seed is sown treated with fungicide without any knowledge of its health status. To allow better targeting of seed treatment new seed health tests are required. They must: be quick; allow for a higher throughput; be at least as sensitive as current methodology; and they must be cost effective.

In recent years, a number of molecular techniques have been used for the detection of plant pathogenic fungi, but reports on their use in the detection of seed-borne fungi in seed samples are limited (Smith *et al*., 1996; Ophel-Keller *et al*., 1999; Bates *et al.,* 2001; Taylor *et al.,* 2001).

This paper describes the quantification of *M. nivale* using competitive and real-time PCR assays and discusses their potential as a routine test.

MATERIALS AND METHODS

Seed Samples

Wheat seed samples that had tested positive for *M. nivale* infection in agar plate testing were obtained from the OSTS for England and Wales (NIAB) and OSTS, Scotland (SASA). Seed samples were sampled and prepared in accordance with the International Rules for Seed Testing, Chapter 2 Sampling (Anon. 2003).

Agar plate tests

Method 1

Two hundred seeds were surface sterilised by immersing them for 10 minutes in a solution of sodium hypochlorite (1% available chlorine) followed by draining. Using aseptic technique, 2 x 100 seed replicates were plated onto PDA (Potato dextrose agar) + streptomycin (100ppm), with 5 seeds per 9 cm diameter plate. Plates were incubated for 5 days at 22°C under a 12 hour NUV, 12 hour dark cycling regime and then examined for *M. nivale* colonies.

Method 2

As method 1, but plates were incubated in the dark at 20°C for 7 days and then examined for *M. nivale* colonies.

DNA Extraction

Fungal DNA Extraction

Method 1

Genomic DNA was extracted from single-spore-derived fungal cultures as described by Taylor *et al*. (2001) using the method of Raeder & Broda (1985) with the modification of incubating the fungal cultures for three days in potato dextrose broth. The DNA samples were further purified with commercial resin and silica membrane columns (Wizard DNA silica-resin clean-up system, Promega, Southhampton, UK) according to the manufacturer's instructions. Genomic fungal DNA was quantified using a DynaQuant 200 florometer (Hoefer).

Method 2

Cultures of *M. nivale* and other fungi were obtained from the SASA culture collection, and aseptically plated and grown on potato dextrose agar (PDA) plates, with 100 ppm streptomycin, covered with a circle of porous gel drying film. The plates were incubated for 5-7 days at 20°C in the dark. Fungal

mycelium from colonies harvested from 7 plates was ground three times under liquid nitrogen then DNA was extracted using a modified version of the Genescan Europes' (Germany) 'GMOScreen Advanced Screening System Basic' protocol (Standard method). This modification involved the addition of a RNase digestion, followed by a further phenol/chloroform extraction. The resultant stock solution of purified fungal DNA was quantified spectrophotometrically using a GeneQuant RNA/DNA calculator.

DNA from pure fungal colonies of *M. nivale* (SASA Reference Sample) was used to produce a range of standards, which were used within the ABI Prism Real-time PCR assay, to quantify the amount of fungal pathogen present in seed samples. The stock solution of *M. nivale* DNA was diltuted using 1x Tris-EDTA buffer (Sigma-Aldrich), to produce standards of concentrations varying from 15000ng/ml to 0.4ng/ml. The amount of DNA encompassed by these standards covered the range of infection levels likely to be observed within samples submitted to SASA & NIAB for testing.

Method 3

Fungal isolates were obtained from a selection of UK seed samples examined by SASA. The isolates were grown into pure culture on PDA containing 100ppm streptomycin. A small amount of mycelia was removed from the plates using a pipette tip, placed in 0.5ml mM Tris-HCL (pH 8.0), boiled for ten minutes and centrifuged prior to use in a PCR assay.

<u>DNA Extraction from seed</u>

Method 1

Two hundred seeds were sealed in a plastic extraction bag (12 x 14 cm, Bioreba, supplied by Bond Bio-tech Ltd, UK) and crushed using a hammer or a roller mill (Marga Mulino, Italy), with rollers at setting number 2, to give a roughly broken appearance to the sample. The DNA was extracted from the resultant material, using the CTAB (cetyl trimethyl ammonium bromide) extraction method described by Edwards *et al*., (2001). The concentration of the purified whole seed DNA extract was measured using a GeneQuant RNA/DNA calculator, then diluted to a working concentration of 50µg/ml in 1x Tris-EDTA buffer (Sigma-Aldrich).

Method 2

Two hundred seeds were either heat-sealed in a plastic extraction bag before being crushed using a hammer until all the seeds were broken or milled for 10 seconds using a lab blender to produce a coarse powder. The DNA extraction method used by Edwards *et al*., (2001) was followed.

PCR Quantification

Competitive PCR

Primers

Two primers MNIV-A and FUN28-B previously designed and generated at SASA, using sequences from the 28S rRNA gene, (GenBank Accession number AF024596) were used in all competitive assays (McNeil *et al*., 2004).

Mimic construction

A composite primer-pair, each 5' end containing the fungal primer-binding site and the 3' end containing the binding site for bacteriophage M13, was used to amplify an M13 DNA template (Bayou Biolabs). The location of the binding sites for amplification of M13 was chosen with regard to the size of the resulting Mimic as a primary consideration. The PCR product was diluted 1 in 100 before being used as a template for PCR amplification using the fungal-specific primer pair. This PCR product was purified from an agarose gel usig a Sephaglas Band Prepkit and this constituted the stock solution of PCR Mimic.

Quantification

The concentration of each Mimic preparation was determined spectrophotometrically and the Mimic was then diluted to a working concentration of 2 μg/ml. This was further diluted 1 in 54 to achieve the designated Mimic dilution M_0 (100 amol/μl). Serial ten-fold dilutions were made from M_0 to give dilutions M_1 to M_8. The diluent used was a bacteriophage λ DNA solution (10 μg/ml), to reduce loss of Mimic DNA through adsorption to the plastic storage vessels, particularly at high Mimic dilutions. After titration against target extracts the Mimic dilution that gave equal band intensity to that of the target was chosen for use within the competitive PCR.

PCR conditions

Amplification conditions for 25 μl reactions were as follows: 8 pmol of each primer (MNIV-A & FUN28-B); 200 μM nucleotides; 2 mM $MgCl_2$; 10× reaction buffer without $MgCl_2$ (Promega) and 1 unit of *Taq* DNA polymerase. The amplifications were performed in an MJ Research PTC-200 thermal cycler. The following conditions were used for amplification: an initial denaturation step at 95°C for 5 minutes; followed by 35 cycles of 94°C for 30 seconds; 52°C for 30 seconds; 72°C for 60 seconds; and a final elongation step at 72°C for 10 minutes.

Post-PCR analysis was carried out by agarose gel eletrophoresis, the PCR products were stained with ethidium bromide and visualized using ultra-violet trans-illumination. Images were captured using a

digital camera and the intensity of each band (target and mimic) was calculated using image analysis software (Kodak 1-D). The ratio of target and Mimic bands was calculated for each reaction and this was used to estimate infection levels when compared to standards subjected to Mimic PCR.

Real-time PCR using the ABI Prism 7700 Sequence Detection System

The real-time PCR assay incorporated TaqMan® chemistry, using a sequence-specific probe hybridising between the forward and reverse primers, as described by Böhm *et al.,* (1999). The samples were amplified and analysed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington).

For each seed sample tested a specific cycle threshold (Ct) value was calculated. The Ct value is defined as the cycle number at which a statistically significant increase in the ΔRn (the difference between the reacted sample and unreacted sample) is first detected. This is also the cycle at which the detection of target crosses an arbitrary threshold. The threshold is defined as the average standard deviation of ΔRn for early cycles. It is placed where the detection of an increase in signal, associated with an exponential growth of PCR product, has occurred. The Ct values thus obtained are then used to calculate the initial amount of DNA present.

A method of absolute quantification was used, comparing the amount of DNA in unknown samples to that of known DNA Standards. The slope and intercept of the regression of the Ct values for standards, on their known DNA quantities (after taking logarithm base 10) were used to calculate the amount of DNA in each seed sample.

Primer and Probe Design

For the TaqMan® system, primers were designed to amplify a short segment or amplicon, 65 base pairs long, of the target nucleotide sequence on the 28S Ribosomal subunit. A fluorescent labelled probe was also designed to anneal to a sequence located between the primer pair, and this amplifies in conjunction with the primers. These were designed using Primer Express® Software (Applied Biosystems, Warrington). A BLAST (Basic Logical Alignment Search Tool) search of the GenBank nucleic acid database and sequence alignment with closely related species was undertaken on the sequence chosen for primer and probe design, to ensure there would be no amplification of other closely related species. No significant matches were revealed within the database. Primer and probe sequences were, forward primer Mn-F (5’ GTG TAA TAC CGC CGC TGG G 3’), reverse primer Mn-R (5’ TTG ATG ACC ATT ATG CCA GCA 3’) and probe Mn-Pr (5’ FAM- CTG AGG ACC GCG CTT CGG CTA G -TAMRA 3’). The TaqMan® probe was labelled at its 5’ end with the fluorescent reporter dye FAM, whereas its 3’ end was modified with the universal quencher dye TAMRA.

DNA extracts from fungal isolates were used to check primer/probe specificity. These isolates were obtained from the SASA fungal reference collection and from UK seed lots tested at NIAB. Those fungal isolates of pathogens likely to infect wheat seeds or plants were chosen, along with selected saprophytic fungi, and a range of other common agricultural fungal pathogens.

PCR conditions

PCR reactions used 1µl of the whole seed extract as a template for the reaction, and all samples were assayed in duplicate. A total reaction volume of 25 µl was used in this assay. Reactions were performed in MicroAmp® optical 96-well plates (closed by MicroAmp®optical caps(Applied Biosystems, Warrington)). Each 25µl reaction contained, 1µl template (various amounts of DNA), 8µl of HPLC grade water (Sigma), 12.5µl of 2x TaqMan® Universal master Mix (no AmpErase UNG) (which contains dNTPs, passive reference ROX, optimised bufffer components and AmpliTaq Gold® polymerase(Applied Biosystems, Warrington)), 7.5pmol of each primer and 2.5pmol of probe. The samples were amplified for 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds; these cycles were preceded by a hotstart incubation of 50°C for 5 minutes and a 95°C incubation for 10 minutes. Two wells of negative controls, containing no template, are included with each sample assay.

PCR using the LightCycler™ and SYBR Green I

Primers Mn-F and Mn-R were used in all PCR assays using the LightCycler™ and SYBR Green I.

PCR conditions

A total reaction volume of 10 µl was used for the PCR consisting of: 0.4µl 4mM $MgCl_2$; 5.0µl master-mix (Biogene Ltd, UK); 0.5µl of SYBR Green 1 (Biogene Ltd, UK); 1.0µl of each primer Mn-F and Mn-R; 2.0µl of DNA extracted from seed; and 0.1µl of H_2O. Amplification was carried out in a LightCycler™ (Idaho Technology, USA) with an initial denaturing step at 94°C for 30 seconds followed by 35 cycles of 94°C for 0 seconds, 55°C for 0 seconds, 74°C for 17 seconds and 86ºC for 0 seconds. The temperature transition rate was set at 20°C/second. Acquisitions of fluorescence signal were carried out at the end of every extension step for 50 ms. A melting curve was obtained immediately after amplification to distinguish specific products from non-specific products and primer-dimers. This was done by holding the temperature at 60°C for 5 seconds and gradually increasing the temperature to 98°C at a rate of 0.2°C/second, with the fluorescent signal acquisition mode set at step. The fluorescent data was converted into melting peaks as described by Ririe *et al*. (1997) and Woo *et al*. (1998). A graph was plotted of the negative derivative of fluorescence with respect to temperature (–dF/dT).

Experiment 1: Quantification and Calibration experiments for Competitive PCR

In each of two experiments samples were taken from seed lots with a range of *M. nivale* infection levels. Experiment 1a used 10 seed lots (Appendix) and DNA was extracted from two to five samples from each lot using DNA extraction method (1). The PCR ratio was measured twice for each DNA extract. Experiment 1b used 46 seed lots (Appendix) and the PCR ratio was measured once from each extract.

Experiment 2: Quantification using the ABI Prism 7700 Sequence Detection System

A set of 33 seed samples previously tested for *M. nivale* at NIAB or SASA (using agar plate method (1) or (2)) with infection levels ranging from 0 to 82.5 % were subjected to quantitative PCR using the ABI Prism 7700 Sequence Detection System (Appendix). Each sample was assayed in two separate runs (each run containing 33 samples) and within the assay each PCR reaction was duplicated.

Ct values were visually scanned for drop-out values, where no amplification had occurred. These values were eliminated from further analysis.

Experiment 3: Quantification of *M. nivale* in seed lots using the LightCycler™ and SYBR Green I

A series of 25 samples, with a range of *M. nivale* infection levels from 0 to 21% assessed using the agar plate method (1), were tested using quantitative PCR (LightCycler™). Eight replicate sub-samples of 200 seeds were produced of which four were tested using the agar method and four by the quantitative PCR assay with the DNA seed extraction method (2). A second series of samples with infection ranging from 21 to 53% assessed using either agar plate method (1) or (2) were also tested. Four replicate sub-samples of 200 seeds were produced and tested using the quantitative PCR assay with the DNA seed extraction method (2). Mean results for both series are presented in Appendix1.

Experiment 4: Quantification of *M. nivale* in single seeds using the LightCycler™ and SYBR Green I

Using the DNA extraction from seed method (2), DNA was extracted from individual seeds from four lots of wheat variety Charger. These had been assessed using agar plate method (1) and found to contain 2, 5.5 13.5 and 50.5% *M. nivale* infection. *M. nivale* DNA was quantified from twenty-six seeds from each sample.

Experiment 5: Calibration experiments for LightCycler™ and SYBR Green I

A subset of 91samples, with *M. nivale* infection levels ranging from 0 to 70% (agar plate method (1)) submitted to NIAB during autumn 2002, were tested using DNA extraction from seed method 2 and quantitative real time PCR (Light Cycler™). A second set of 33 samples described in Appendix were used to test the calibration set of 91 samples.

RESULTS

Check of primer and probe specificity

The primers MNIV-A and FUN28-B detected all *M. nivale* isolates tested, and no other fungi (Table 1). The Taqman® primers Mn-F and Mn-R, amplified all *M. nivale* isolates in both real-time PCR systems and no other fungi tested in either the ABI prism or LightCycler™ system (Table 1).

Experiment 1: Quantification and Calibration of Competitive PCR

Evaluation of the two data sets showed a linear relationship between PCR ratio and percentage *M. nivale* infected seeds after both were transformed logarithmically. Figures 1 and 2 show plots of the natural log PCR ratio versus the natural log percentage of infected seed.

In both experiments there was a certain lack of fit that was not wholly explained by variation within lots. This is clearly seen in experiment 1 (Figure 1) where results for a single lot are often consistently above or below the regression line. The lack of fit was quantified for experiments 1 and results are summarised in Table 2. The lack-of-fit error variance was larger than the within-lot variance.

The estimates of the intercept, slope and residual mean square error are given for the regression of the natural logarithm of the PCR ratio (mean for each lot) on the natural logarithm of the percentage of *M. nivale* infected seeds for each experiment and are given in Table 3. The results were similar across the two experiments with the same intercept and similar slopes.

Upper and lower prediction limits were calculated using the formulae detailed in Draper & Smith (1981). The upper limit is the PCR ratio, which should be exceeded if we want to be 95% (or 99%) confident that the true level of infection in a seed lot exceeds a 10% threshold level. The lower prediction limit is the PCR ratio, which should be exceeded if we want to be 95% (or 99%) confident that the true level of infection in a seed lot is less than the 10% threshold level. Table 4 gives the upper and lower limits for the confidence at 95% and 99% probability for an infection threshold of 10% in the two experiments.

Table 1 Fungi isolated from cereal seed lots used in testing the specificity of primer pairs MNIV-A and FUN28-B, and Mn-F and Mn-R for PCR.

Organism	**Primers** **MNIV-A/FUN28-B** Number of UK isolates tested	Amplification[1]	**Mn-F/Mn-R** Number of UK isolates tested	Amplification[1]
Microdochium nivale	85[5]	+	21[3, 4]	+
Alternaria spp.	25[5]	–	1[4]	–
Alternaria tenuis			1[3]	–
Botrytis spp.	7[5]	–	1[3]	–
Bipolaris sorokinia			2[3,4]	–
Drechslera avenae			1[4]	–
Drechslera graminea			1[4]	–
Drechslera spp.	2[5]	–		
Drechslera teres			2[3,4]	–
Drechslera tricepta			1[4]	–
Epicoccum spp.	5[5]	–	1[3]	–
Fusarium avenaceum	24[5]	–	1[4]	–
Fusarium culmorum	9[5]	–	2[3,4]	–
Fusarium graminearum			1[4]	–
Fusarium moiliforme			1[3]	–
Fusarium oxysporum			1[3]	–
Fusarium poae	36[5]	–	1[4]	–
Fusarium sambucinum			1[4]	–
Fusarium sp.	1[5]	–		
Microdochium sp.			1[4]	–
Penicillium sp.	1[5]	–	2[4]	–
Septoria nodorum	5[5]	–	2[3,4]	–
Tilletia tritici			1[2]	–

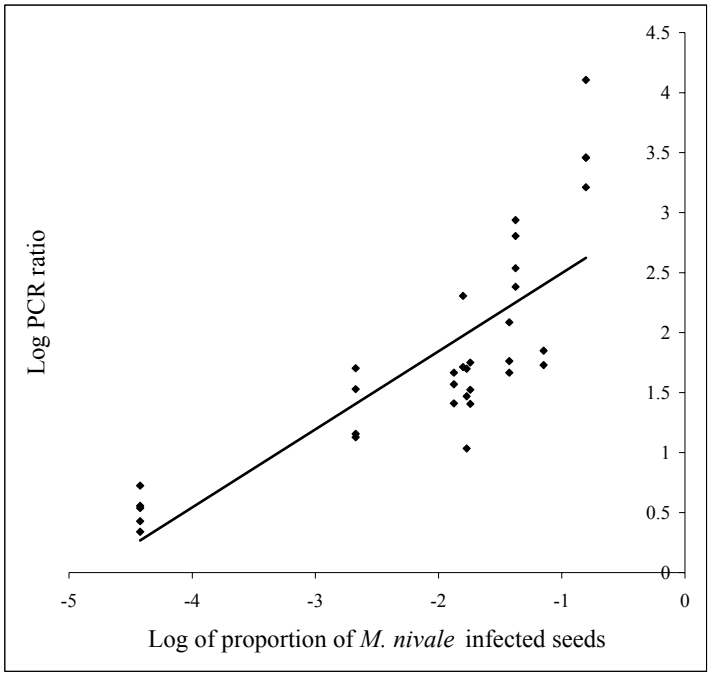
[1]Presence of amplification product; + present, – no PCR product.

[2] DNA extracted from chlamydospores (McNeil *et al., 2004*)

[3] Fungal DNA Extraction Method 1

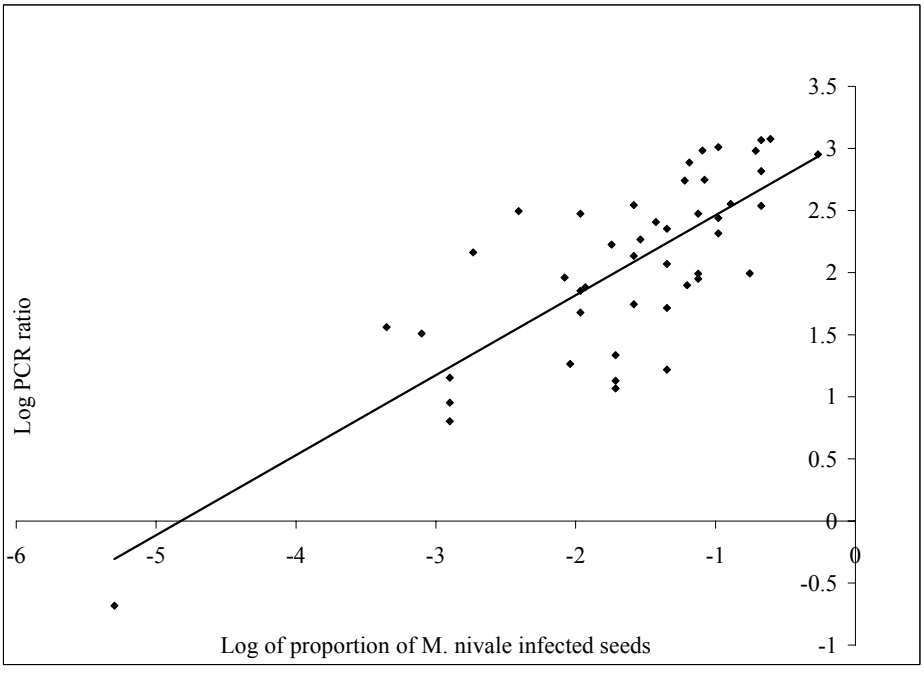
[4] Fungal DNA Extraction Method 2

[5] Fungal DNA Extraction Method 3



2-5 sub-samples per seed lot

Figure 1 Competitive PCR: Experiment 1, the relationship between Log PCR ratio and Log proportion of *M. nivale* infected seed.



1 sub-sample per seed lot

Figure 2 Competitive PCR: Experiment 2, the relationship between Log PCR ratio and Log proportion of *M. nivale* infected seed.

Table 2 Competitive PCR: Lack-of-fit statistics for Experiment 1.

Experiment	Within-lot variance	Lack-of-fit error variance	F-test p-value
1a	0.064	0.936	<0.001

Table 3 Estimates of regression parameters

Experiment	Intercept	Slope	RMS error
1a	3.0	0.63	0.27
1b	3.0	0.59	0.22

Table 4 Table of prediction limits for PCR ratio (equivalent infection percentage in brackets) within individual experiments

			Experiment	
M.nivale seed infection threshold	Confidence	Threshold	1a	1b
10%	95%	Lower	1.7 (1.9%)	2.4 (2.5%)
		Upper	14 (51%)	12 (40%)
	99%	Lower	1.0 (0.8%)	1.7 (1.4%)
		Upper	24 (100%)	17 (73%)

Experiment 2: Quantification using the ABI Prism 7700 Sequence Detection System

There was a good relationship between the agar plate test and the amount of DNA (µg) measured using the PCR assay, Figure 3. Correlation co-efficients for Run 1 and Run 2 were 0.87 and 0.74 respectively.

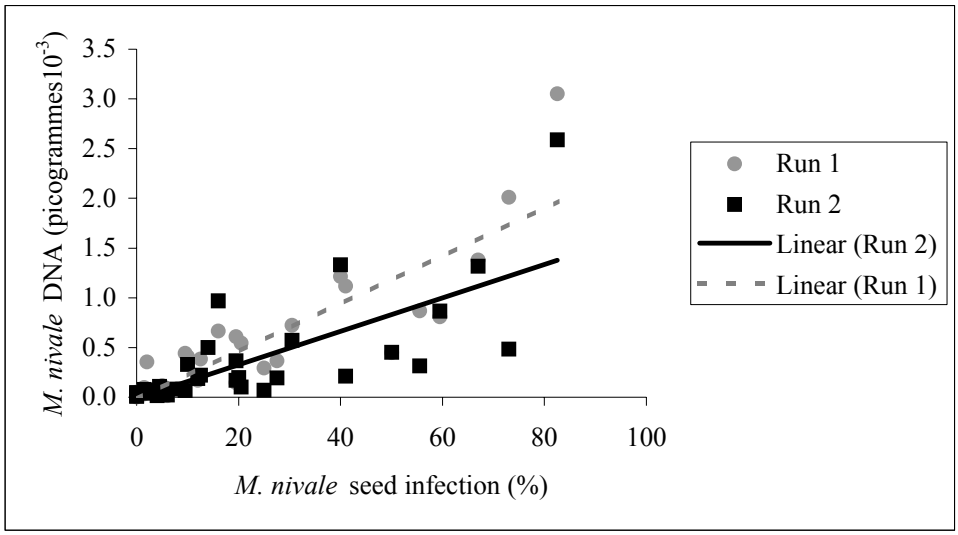


Figure 3 Real-time PCR using the ABI Prism 7700: The concentration of *M. nivale* DNA (µg) compared to *M. nivale* infection levels in an agar plate test for samples with infection levels between 0-82.5%.

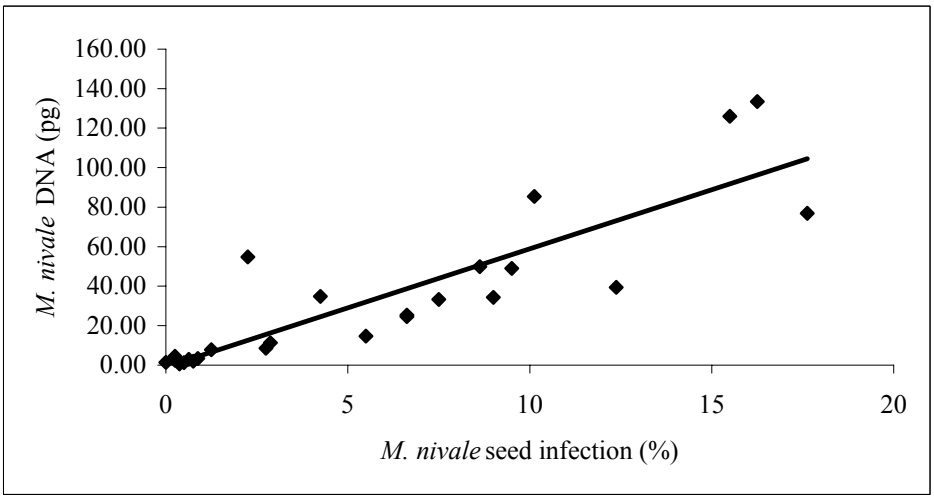


Figure 4 Real-time PCR using the LightCycler™: The concentration of *M. nivale* DNA (pg) compared to *M. nivale* infection levels in an agar plate test for samples with low infection levels (0 – 21%).

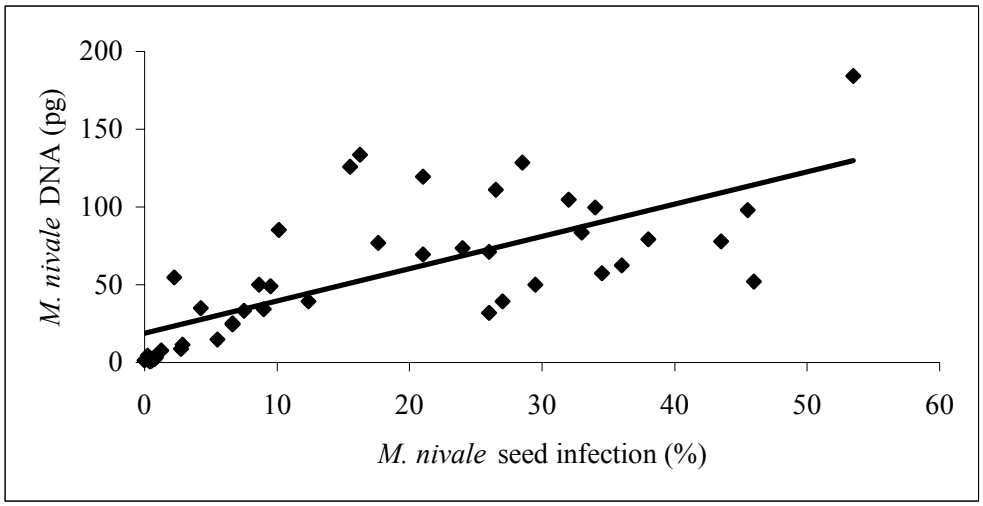


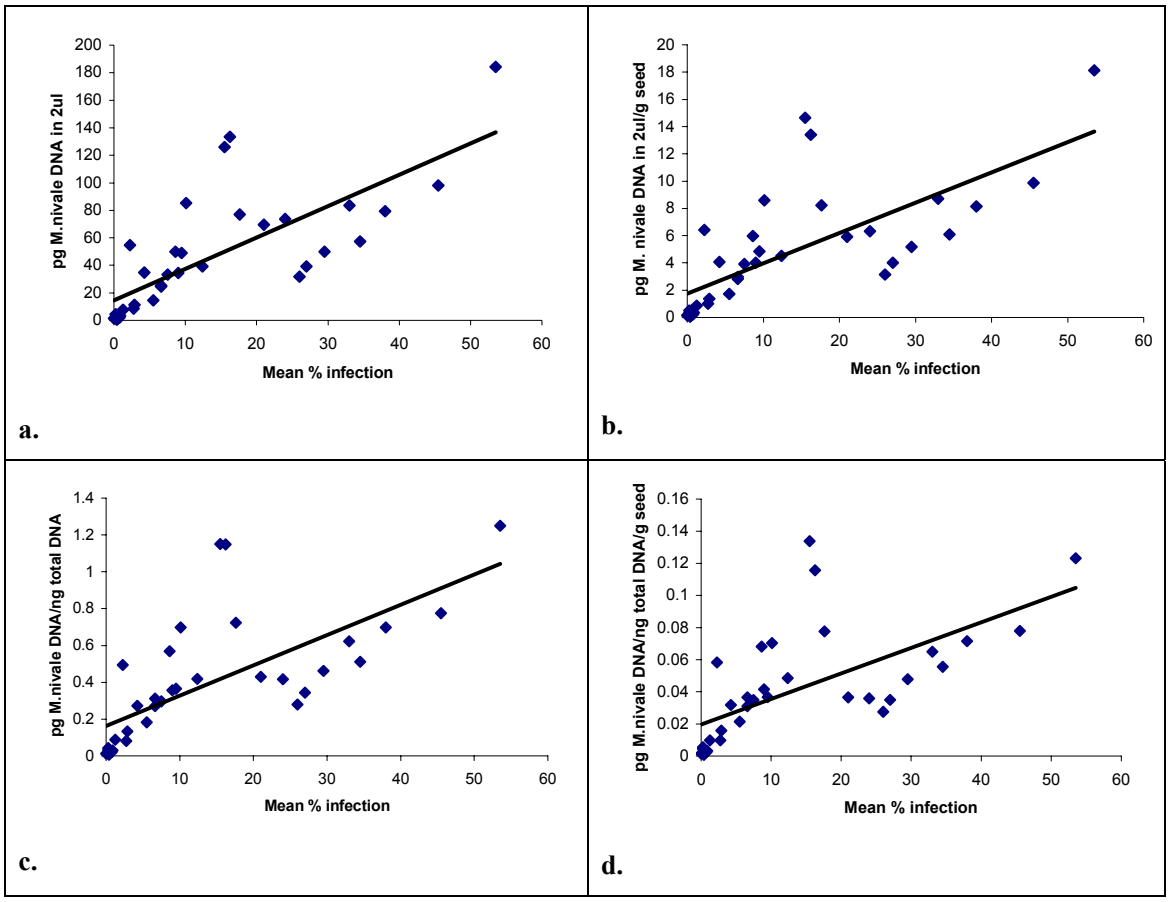
Figure 5 Real-time PCR using the LightCycler™: The concentration of *M. nivale* DNA (pg) compared to *M. nivale* infection in an agar plate test in a range of infected samples (0-53.5%).

Experiment 3: Quantification of *M. nivale* in seed lots using the LightCycler™ and SYBR Green I

Quantification of *M. nivale* DNA extracted from the samples with infection levels below 21% gave a linear relationship with a correlation coefficient of (0.87) (Figure 4). When samples with infection greater than 21% were included the relationship was less defined with a correspondingly lower correlation coefficient (0.72) (Figure 5).

Effect of seed weight and total DNA on the relationship between seed infection (measured by agar plate test method (1)) and pg DNA measured in PCR reaction using the LightCycler™ and SYBR Green I

A selection of test results from Experiment 3 (Appendix) were adjusted to take account of seed weight, (pg *M. nivale* DNA in 2µl of extract/g seed), the total amount of DNA extracted from the seed (*M.nivale* DNA/total DNA), and both seed weight and total DNA Figure 6. None of these corrections improved the relationship with a correlation coefficient before any correction of 0.75 and after corrections 0.72, 0.70 and 0.65 respectively.



All points are means of 4 replicates.

Figure 6 a: Amount of *M. nivale* DNA per 2µl aliquot, plotted against agar plate test results; b: data corrected for seed weight; c: data corrected for total DNA extracted; d: data corrected for seed weight and total DNA extracted.

Experiment 4: Quantification of *M.nivale* DNA in single seeds using the LightCycler™ and SYBR Green I

Whilst very low levels of *M. nivale* DNA were detected in the 2 and 5.5% samples there was considerable variation between individual seeds in the 13.5 and 50.5% seed lots. In the 13.5% infected seed lot three of the infected seeds detected had levels of *M. nivale* DNA greater than that seen with any seeds in the 50.5% sample. Levels of DNA measured in the seed lot with 13.5% infection are given in Table 6.

Table 6 DNA concentrations recorded from 26 individual seeds from a sample of wheat with 13.5% *M. nivale* infection.

Seed	pg DNA in 2µl	Seed	pg DNA in 2µl	Seed	pg DNA in 2µl
1	16.7	10	10.5	19	26.8
2	11.3	11	6.9	20	27.4
3	11.8	12	10.5	21	37.4
4	7.9	13	9.7	22	1124.0
5	9.5	14	19.3	23	1150.0
6	162.4	15	54.1	24	18.6
7	7.5	16	4156.0	25	21.0
8	10.4	17	6.7	26	26.5
9	8.2	18	18.9		

Experiment 5: Calibration: LightCycler™ and SYBR Green I

A correlation of r = 0.78 was found when the agar plate test (method 1) and real time PCR using the LightCycler™ were compared. Transforming both the Agar Plate % to Log_{10}(Agar Plate % +1) and PCR DNA to Log_{10}(PCR DNA +1) the regression gave an improved fit in terms of R^2 (0.6982). The observed variation, even in the transformed variables, is large and leads to the 95% confidence interval of prediction also being large (Figure 7).

A regression relationship between the agar plate method (1) and the real-time PCR using the LightCycler™ was established using the results from the 91 samples. Application of the regression to a smaller 'test' set was necessary to assess the robustness of the relationship derived from the 91 sample calibration set. The test set of 33 samples with a range of level of *M. nivale* DNA levels again showed good correlation (0.91) with agar plate test, Figure 8. In the test set, agar plate infection levels of 20.6% or greater gave rise to DNA values above 100 pg. When restricted to DNA values above 150 pg, the corresponding plate determinations were at least 28.2%. When the predictions are plotted against the 'observed' data for Agar Plate %, there is a better agreement in levels of infection between 0 and 20% than for infection levels exceeding 20% (Figure 8).

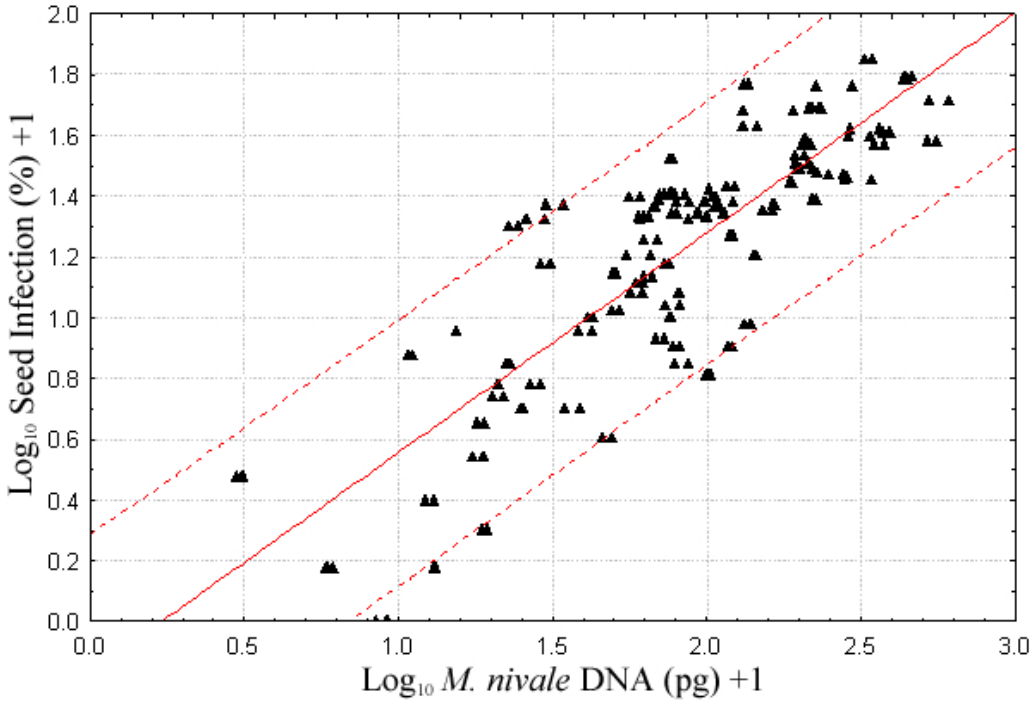


Figure 7 The log transformation of *M. nivale* DNA (pg) compared to the percentage of seeds showing infection in an agar plate test with 95% confidence interval prediction.

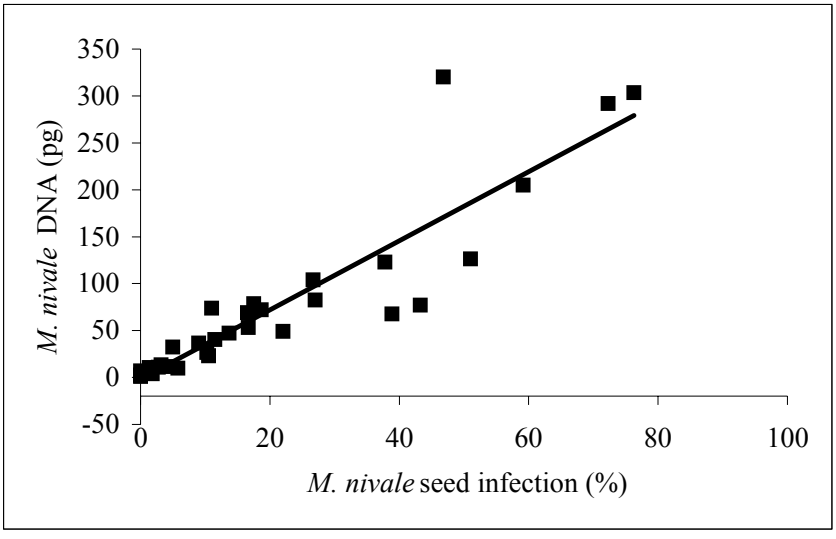


Figure 8 The concentration of *M. nivale* DNA (pg) compared to the percentage of seeds showing infection in an agar plate test.

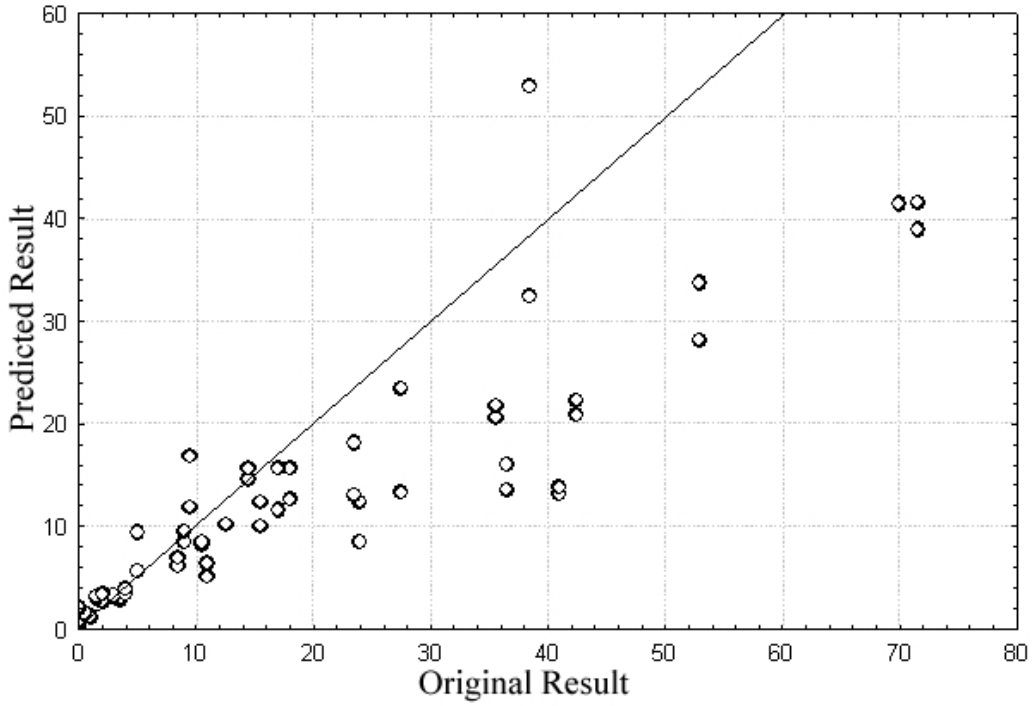


Figure 9 The percentage seeds infected predicted from *M. nivale* DNA (pg) using the standard curve compared to the percentage of seeds showing infection in an agar plate test (added line is 1:1 relationship).

DISCUSSION

In this study we outline the approach we have taken to construct competitive PCR assays and real-time PCR assays which could be used for quantification of *M. nivale* seed infection. Competitive PCR uses an exogenous DNA fragment which can be amplified by the same primers used for pathogen (target) detection, but which can be differentiated from the pathogen-specific band on the basis of size. As both target and competitor compete for the use of the primer pair, comparing the amount of target and competitor products indicates the initial molar ratio between the target and competitor. For the competitive PCR method described, 'Mimic PCR' was developed. This uses an artificially constructed competitor fragment and comprises an unrelated fragment flanked by the pathogen-specific primer sites (Seibert & Kellogg, 1996). Pathogen quantification was achieved by comparison of sample pathogen/competitor ratios to those of DNA standards. Real-time PCR uses fluorescent-based detection of PCR products during the assay (Wittwer *et al.,* 1997) and enables quantification of nucleic acids in unknown samples by direct comparison to standards amplified in parallel reactions (Morrison *et al*., 1998).

In addition to quantification assays, real-time detection using SYBR Green I allows measurement of 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 determined by its sequence composition and length. As SYBR Green I is a non-specific DNA binding dye, melting curve analysis at the end of a run provides a check that specific amplification has occurred. This distinguishes products from non-specific primer-dimers and can highlight any contamination. Two real-time PCR techniques are described, one using a LightCycler™ instrument (Roche Diagnostics, Lewes), 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), and the other using an ABI Prism 7700 Sequence Detection System (Applied Biosystem, Warrington) and TaqMan® chemistry using a pathogen specific probe.

M. nivale is found within the seed tissue; a number of extraction methods were evaluated (Mulholland *et al.,* 1999 and Anon., 2001) before a method of whole seed DNA extraction (Edwards *et al.,* 2001) was adopted where both seed DNA and *M. nivale* DNA are extracted from a 200 seed sample. This extraction method was used in all of the experiments described in this paper with small modifications made in different laboratories.

Competitive PCR uses an end-point measure of fungal contamination whereas real-time PCR examines the kinetics of PCR product formation. Both methods use a method of "Absolute Quantification" to measure the DNA levels in unknown samples. This involves creating a standard curve. The standard curve consists of a dilution series of purified pathogen DNA. In the competitive assay infection level is determined by comparing the Mimic PCR sample ratios to the ratios of DNA standards. In real time assays the standards are run within the assay alongside samples of unknown infection levels. By comparing the results of unknown samples against the curve produced from the standards, a DNA value for the unknown samples is obtained.

Nicholson *et al* (1996) published a variety-specific PCR (polymerase chain reaction) method to differentiate *M. nivale* var. *majus* and var. *nivale*. Both *M. nivale* var. *majus* and var. *nivale* are strongly pathogenic toward wheat, Simpson *et al.,* (2000), and in the current seed health method there is no requirement to routinely test for, and quantify, the presence of each variety of *M. nivale*. Therefore primer combinations that are specific for *M. nivale* but not specific for the varieties of *M. nivale* were developed and used in both competitive and real-time assays. No amplification was observed for any of the other commonly occurring seed-borne diseases and / or saprophytic fungi tested.

Real-time PCR (using both the ABI Prism 7700 and the LightCycler™) and competitive PCR assays are able to quantify the amount *M. nivale* DNA present in a sample of seeds. Although we expect the amount of DNA to be proportional to the level of seed infection, the relationship between these assays show a high degree of variability particularly in samples with seed infection levels above 20%, as measured by the agar plate test. Where tested the variability was greater than that expected for within lot variability. Potential causes of variation include the experimental conditions related to the test (PCR conditions, method of analysis, analyst, environment etc). In addition, differences in the total amount of DNA extracted may vary due to seed weight or variety and ; within infected lots the the amount of *M.nivale* fungus within each infected seed may differ.

When corrected for seed weight and total DNA there was no significant difference in the relationship between seed infection as measured by the agar plate test and levels of *M.nivale* DNA as determined in PCR assays. Single seed analysis showed that the amount of *M. nivale* DNA per seed varied as much as 25-fold between individual seeds within an infected seed lot This may be an inherent weakness in establishing a firm relationship between the PCR assay and the agar plate test and it will not be possible to resolve this through improvements to experimental design. Nevertheless, the PCR assay could provide a measure of the variability to allow the production of upper and lower limits of measured DNA. These limits could then be used to identify lots that are likely to have a higher or lower percentage of infected seeds than the advisory threshold of 10% (Cockerell *et al.,* 2004) above which seed treatment is required.

Although competitive PCR produced a reasonable level of quantification it was clear that the within run predicted confidence limits based on each of the experiments described would be of limited practical use where a 10% *M. nivale* threshold is used. A predicted result of 10% in the competitive PCR provides confidence that the agar plate test result will lie between approximately 2% and 40% *M. nivale* infection in 95% of samples tested. Although it may be possible to tighten the prediction intervals by improvements in experimental design, the competitive PCR has now been superceded by the real-time PCR. Real-time PCR has a number of significant advantages in terms of potential throughput and time saving. It is a one step process with automatic analysis compared to the multistep competitive PCR.

As with competitive PCR the two real-time PCR systems produced a good level of quantification. Using results from 91 samples with a range of *M.nivale* seed infection levels seed (0 to 76% in agar plate tests), a regression relationship was established between PCR results from the LightCycler using the SYBR Green I system and the agar plate test results. This allowed the prediction of the percentage *M. nivale* in samples with unknown infection using the PCR result. This prediction was tested using samples with known infections and when plotted against agar plate results, the agreement

was better for infection levels of between 0 and 20% (by agar plate method) than for infection levels exceeding 20%.

Neither real-time PCR system was able to reduce the within or between sample variation. Although the differences in *M. nivale* inoculum levels from seed to seed may account for much of this variation, run to run variation has been observed in the laboratory over a number of experiments using real-time PCR (an example of which is given in Figure 3). This must be further investigated. It may be possible to account for this variation in future prediction calculators.

Real-time PCR is an extremely sensitive methodology; it is able to detect a fraction of a picogram of *M. nivale* DNA from a seed extract. It has been demonstrated that seed infection levels of 0.5%, the detection threshold of the agar plate method, can be detected using PCR. However, wide variation in the amount of *M. nivale* DNA quantified at high levels of seed infection may mean that the real-time PCR test cannot be used as a direct replacement to the agar plate test. It can however, provide a reasonable level of quantification at low levels of *M. nivale* seed infection. *M. nivale* DNA can be converted to a result which indicates whether the level of *M. nivale* in a seed lot is above or below the seed treatment threshold of 10%. The major advantage of this real-time PCR test is speed, reducing the current test length from approximately 7 days to 48 hours. The test offers a greater opportunity for farmers and seed merchants in the UK to complete seed health testing within a time scale that allows a treatment decision based on knowledge of disease risks.

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Experiment 1

Experiment 1a			Experiment 1b	
% *M. nivale* seed infection	Mean Ratio (2-5 sub-samples)		% *M. nivale* seed infection	Ratio
1.2	2.063463		0.5	0.505527
1.2	1.712083		3.5	4.764368
1.2	1.404159		4.5	4.524739
1.2	1.74284		5.5	3.170601
1.2	1.535351		5.5	2.2333
6.9	3.176908		5.5	2.59215
6.9	4.614533		6.5	8.687091
6.9	3.09149		9	12.1229
6.9	5.49144		12.5	7.100017
15.33	5.288395		13	3.542357
15.33	4.805056		14	6.381094
15.33	4.094223		14	5.352184
16.5	5.548523		14	11.87694
16.5	10.02736		14.5	6.569225
17	2.814004		17.5	9.26097
17	4.346437		18	2.906325
17	5.469804		18	3.093721
17.5	4.082026		18	3.801842
17.5	5.753265		20.5	8.445318
17.5	4.590663		20.5	5.723075
24.03	5.826819		20.5	12.73094
24.03	8.056532		21.5	9.64757
24.03	5.287115		24	11.10052
25.3	12.63811		26	7.931952
25.3	18.8715		26	3.380371
25.3	10.82464		26	5.557029
25.3	16.5243		26	10.51033
31.75	5.634812		29.5	15.50644
31.75	6.358616		30	6.670639
44.75	60.74315		30.5	17.95076
44.75	31.78143		32.5	7.027995
44.75	24.80104		32.5	7.318295
44.75	31.74977		32.5	11.86749
			33.5	19.76491
			34	15.60707
			37.5	10.13429
			37.5	11.47512
			37.5	20.29957
			41	12.83583
			47	7.350322
			49	19.70238
			51	21.46331
			51	12.64973
			51	16.72478
			54.5	21.65315
			76.5	19.13958

Experiment 2

% *M. nivale* seed infection	Run 1 µg DNA	Run 2 µg DNA
0	0.0529	0.046585
0	0.04573	0.0434
2	0.35382	0.06825
4	0.038255	0.016085
6	0.014585	0.021795
8	0.084	0.08305
10	0.40737	0.330055
12	0.168075	0.18866
14		0.500845
16	0.665055	0.969635
20	0.153525	0.197635
30.5	0.724815	0.571825
40	1.213615	1.33221
50	0.4486	0.45032
59.5	0.8085	0.867225
73	2.00894	0.485315
82.5	3.05279	2.589485
0	0.00346	0.00808
0	0.01633	0.01083
1.5	0.095735	0.078605
4.5	0.081	0.108755
1.5	0.033445	0.039145
5	0.113925	0.08071
9.5	0.44313	0.06628
19.5	0.60932	0.366465
12.5	0.385095	0.22167
25	0.294295	0.071145
19.5	0.14532	0.168045
20.5	0.544775	0.103595
27.5	0.365375	0.1927
41	1.115825	0.210945
55.5	0.86785	0.31521
67	1.37929	1.31704

Experiment 3

Series of 25 samples			Second series of 19 samples	
Mean % M. nivale seed infection	Mean DNA (pg)		*% M. nivale seed infection*	Mean DNA (pg)
0	1.40		21.0	69.58
0.75	1.95		21.0	119.53
0.63	2.83		24.0	73.64
4.25	34.85		26.0	83.42
7.50	33.20		26.0	31.92
9.50	48.90		26.5	111.22
16.25	133.53		27.0	39.37
12.38	39.28		28.5	128.48
15.50	125.98		29.5	49.97
2.75	8.57		32.0	104.73
2.25	54.75		33.0	83.42
17.63	76.83		34.0	99.55
0.25	4.43		34.5	57.42
0.88	3.33		36.0	62.38
1.25	7.71		38.0	79.28
5.50	14.61		43.5	77.72
2.88	11.33		45.5	97.93
0.38	0.53		46.0	52.1
0.50	1.16		53.5	184.28
6.63	24.60			
8.63	49.86			
0.25	2.42			
6.63	25.28			
9.00	34.38			
10.13	85.40			

Experiment 5

% *M. nivale* seed infection	Mean DNA (pg)
0	6.976
0	1.076
1.33	10.701
3.17	13.458
1.83	3.800
5.00	32.515
9.00	36.710
13.67	47.043
11.00	73.960
18.67	72.178
16.67	53.385
17.50	78.565
26.67	103.935
38.83	67.663
46.83	320.300
59.17	205.100
0	2.475
0	2.436
1.25	5.162
2.75	10.900
4.50	11.430
5.75	10.072
10.50	23.055
10.25	26.790
11.50	40.425
16.50	69.155
22.00	49.085
27.00	82.480
37.80	122.900
43.25	77.113
51.00	126.300
72.25	291.850
76.25	303.45