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Quantification of *Pyrenophora graminea* in barley seed using real-time PCR

Annemarie Fejer Justesen · Henrik Jørskov Hansen · Hans O. Pinnschmidt

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Abstract A real-time PCR assay was designed to quantify seed-borne infection of *Pyrenophora graminea* in barley (*Hordeum vulgare*). Conventional tests such as the freezing blotter method cannot distinguish *P. graminea* from the closely related *P. teres*. The seed infection threshold for *P. graminea* is lower than the one for *P. teres* and is therefore applied for both species although *P. graminea* may be absent. This results in unnecessary rejections of seed lots. PCR primers and a TaqMan probe were designed to target a *P. graminea*-specific DNA sequence. The potential of the real-time PCR assay for quantifying seed-borne infection of *P. graminea* was investigated by examining seed lots harvested from *P. graminea*-infected fields. The major part (84%) of the variation in the amount of *P. graminea* DNA measured by real-time PCR could be attributed to variation between seed lots while only about 8% was due to variation within seed lots. DNA quantities of *P. graminea* were positively correlated with seed infection incidence detected by the freezing blotter method as well as with the infection incidence of plants examined in the greenhouse. Both correlations were highly significant ($P<0.001$) but the DNA quantities accounted only for 59% ($R^2=0.59$) and 56% ($R^2=0.56$), respectively, of the variation in the results obtained by the two conventional methods. Seed lots of varieties resistant to *P. graminea* contained considerable amounts of *P. graminea* DNA but showed no or only few leaf symptoms in the greenhouse test suggesting that the recommended seed infection thresholds could be raised for resistant varieties.

Keywords *Drechslera graminea* · Organic seed production · Quantitative PCR · Seed-borne · Seed infection threshold · TaqMan

A. F. Justesen (✉) · H. O. Pinnschmidt
Faculty of Agricultural Sciences,
Department of Integrated Pest Management,
University of Aarhus,
4200 Slagelse, Denmark
e-mail: AnnemarieFejer.Justesen@agrsci.dk

H. J. Hansen
Danish Plant Directorate,
Skovbrynet 20,
2800, Lyngby, Denmark

Introduction

Two *Pyrenophora* species occur in barley (*Hordeum vulgare*) seed in Denmark: *Pyrenophora teres* (imperfect state: *Drechslera teres*) causing net blotch and *P. graminea* (imperfect state: *D. graminea*) causing leaf stripe. *Pyrenophora teres* is transmitted via seed and plant debris while *P. graminea* is strictly seed-borne and persists as mycelium in the hull, pericarp and seed coat (Babadoost 1997). The mycelium infects the developing seedling and spreads systemically as the plant grows. Conidia are produced on the surface of infected leaves and are spread to nearby spikelets where they germinate and infect developing seeds (Skoropad and Arny 1956). In conventional

farming, seed transmission of the two diseases can be controlled efficiently by the use of fungicides. However, an expansion of organic farming has led to an increased demand for seed produced organically, i.e. without the use of fungicides. In some years, 50% or more of the organically produced seed lots have to be rejected in Denmark because the infection levels of *Pyrenophora* species exceed the recommended threshold levels which are 5% infected seeds for *P. graminea* and 15% for *P. teres* (Nielsen 2001).

The conventional method for testing barley seed for *Pyrenophora* species is the freezing blotter method where the surface of seeds is microscopically examined for conidia after 7 days of incubation (Jørgensen 1982a). The conventional method does not allow distinction of the two species. Consequently, the lowest seed infection threshold, which applies for *P. graminea*, must be applied for both species. Only an additional greenhouse test for leaf symptoms can determine the infection level of *P. graminea* (Jørgensen 1980). This test however needs at least 1 month for completion and in some barley varieties it can be difficult to distinguish leaf symptoms caused by the two species (Smedegård-Petersen 1983). As a result, most seed lots are tested without differentiating between *P. teres* and *P. graminea* via the greenhouse test. Since *P. teres* occurs much more frequently than *P. graminea*, some seed lots are unnecessarily rejected because the 5% *P. graminea*—threshold is applied although this pathogen might actually be absent.

In contrast to conventional seed health tests, DNA-based methods have the advantages of species-specificity, sensitivity, speed and potential for automation. The internal transcribed spacer (ITS) regions in the ribosomal DNA have been used successfully for the development of species-specific PCR primers for many phytopathogenic fungi (Ghignone and Migheli 2005). Sequencing of the ITS regions showed very low levels of variation between *P. teres* and *P. graminea* (Stevens et al. 1998). Based on the ITS sequence data, a real-time PCR assay for quantification of *Pyrenophora* spp. (Bates et al. 2001) and a single nucleotide polymorphism (SNP) assay for detection of *P. teres* have been developed (Bates and Taylor 2001). Due to the low levels of variation in the ITS region, *P. graminea*-specific PCR primers have previously been derived from a RAPD product and used for detection in seed samples, however quantification was not investigated (Taylor et al. 2001). Real-time PCR has provided a useful tool that can easily be automated and used for quantification of fungal DNA (Schena et al. 2004) but only few detailed studies investigating the usefulness for quantification of seed-borne fungi have been published (Chilvers et al. 2007; McNeil et al. 2004; Bates and Taylor 2001; Bates et al. 2001).

In order to improve the general seed health status and to circumvent unnecessary rejection of seed lots, especially within organic farming where seed with low disease levels can be in short supply, it is crucial to have specific and reliable test methods. The objective of this study was to investigate the potential of real-time PCR for quantifying *P. graminea* infection levels in barley seed lots as an alternative to the time-consuming blotter and greenhouse tests. For this purpose, a TaqMan assay for *P. graminea* was developed and relationships between the amounts of *P. graminea* DNA and the infection incidence of seeds and plants were investigated.

Materials and methods

Fungal isolates and DNA extraction

Several *Pyrenophora* spp. isolates of diverse geographical origin as well as additional pathogenic and non-pathogenic fungal species associated with barley seeds were used in this study (Table 1). Single-spore isolates were grown on PDA at 22°C under 12 h darkness and 12 h light. Fungal mycelium was harvested by scraping the mycelium from the agar surface with a scalpel. Mycelium was ground with a pestle in liquid N_2 in a mortar, and DNA was extracted using the method described by Möller et al. (1992).

Barley seed samples and DNA extraction

Fifty seed lots of the highly leaf stripe-susceptible spring barley variety Agneta were obtained from a field experiment conducted in 2002. The seed lots differed in their levels of *P. graminea* infection as revealed by results of blotter and greenhouse seed testing methods described below. Additionally, seed lots of 14 spring barley varieties representing various levels of resistance to *P. graminea* were obtained from a field resistance-screening test conducted in 2003, 2004 and 2005. The resistance levels of the varieties were judged based on their average leaf stripe rating in a series of

Table 1 Names and origins of fungal species and isolates used in this study

Fungus	Isolate	Country of origin	Source	Amplification in TaqMan assay
Pyrenophora graminea	Pg-Pallas	Denmark	1	+
	Pg-Triumph	Denmark	1	+
	Pg-Alexis	Denmark	1	+
	Pg-CI6944	Denmark	1	+
	PD-1	Denmark	1	+
	dB1	Denmark	1	+
	dB2	Denmark	1	+
	dB4	Denmark	1	+
	dD3	Denmark	1	+
	dC6	Denmark	1	+
	JJ3	Denmark	1	+
	JJ6	Denmark	1	+
	Kp30	Denmark	1	+
	Pg6	UK	2	+
	Pg11	UK	2	+
	Pg15	UK	2	+
	Pg2376	Norway	1	+
	wrs1239	Canada	1	+
Pyrenophora teres	Pt-Celtic	Denmark	1	−
	Pt-Pastorale	Denmark	1	−
	Pt-Maresi	Denmark	1	−
	Pm2	Denmark	2	−
	Pm4	Denmark	2	−
	NZ1	New Zealand	1	−
	NZ2	New Zealand	1	−
	Pt4	UK	2	−
	Pt15	UK	2	−
	Pt16	UK	2	−
	PtSVE	Sweden	1	−
	Pm1892	Canada	2	−
	Pm1893	Canada	2	−
	LewS″	USA	1	−
	M-Ital	Italy	1	−
Pyrenophora avenae	Pa3828	Norway	2	−
Pyrenophora bromi	P.bromi-kh	n.a.	1	−
Cochliobolus sativus	Es-afj	Denmark	1	−
Aspergillus spp.	ik87	Denmark	1	−
	ik12	Denmark	1	−
Alternaria spp.	1-afj	Denmark	1	−
Acremonium spp.	ik68	Denmark	1	−
Cladosporium cladosporioides	km1	Denmark	1	−
Epicoccum spp.	ik59	Denmark	1	−
Rhynchosporium secalis	Rs-lp	Denmark	1	−
Stagonospora nodorum	200602	Denmark	1	−
Fusarium culmorum	328-lb	Sweden	1	−
F. avenaceum	I-jl	n.a.	1	−
F. poae	85	Denmark	1	−
F. graminearum	362-lb	n.a.	1	−
Microdochium nivale	148–1	Denmark	1	−
Penicillium spp.	140	Denmark	1	−

n.a. information not available; *1* University of Aarhus, Faculty of Agricultural Sciences, Research Centre Flakkebjerg, Slagelse, Denmark; *2* NIAB, Cambridge, UK; *minus sign* amplicon absent; *plus sign* amplicon present

such tests conducted from 2002 to 2003 (Anonymous 2005). Two sub-samples of 200 seeds were tested per seed lot. The seeds were ground to a fine powder in a Retsch mixer mill (Retsch GmbH, Germany) and DNA was extracted from 150 mg of the powder using a NucleoSpin® Food kit (Machery-Nagel, Germany).

Design of primers and probes

Primers and a minor groove binding (MGB) probe were designed from a *P. graminea* specific sequence of unknown function (GenBank accession number EU304259) using the Primer Express programme version 2.0 (Applied Biosystems, Foster City, CA, USA). The specific sequence has previously been isolated from a genomic library of *P. graminea* and showed no cross-hybridisations to other *Pyrenophora* spp. when used as a probe in dot-blot hybridisations (Husted 1993). The primers for *P. graminea* are the forward primer pg20 (5′-GCC GCG TCT CTT GTT TGT G-3) and the reverse primer pg21 (5′-AGC CCG GAT ACG ATT TGT GA-3′). The probe Pg (5′-ACA ATC TAG CCA AGG TT-3′) was labelled at its 5′ end with 6-FAM and its 3′end was modified with a non-fluorescent quencher. The specificity of the primers and probe was tested on 1/100 and 1/1000 dilutions of DNA from a range of fungal isolates (Table 1). The PCR products were analysed in an agarose gel as well as in TaqMan PCR. An internal amplification control was based on the sequence of the alcohol dehydrogenase 1 (Adh1) gene of barley (Petersen and Seeberg 1998). The forward and reverse primers for this assay were Adh1F (5′-CTA CTT CTG GGA GGC CAA GGT-3′) and Adh2R (5′-TCG ACG GGC GAA CAT AAT TAC-3′), respectively. The MGB probe Adh (5′-ACA CTT GCT TCC GGC C-3′) was labelled at the 5′ end with VIC. This internal amplification control was used to identify samples for which DNA-extraction or PCR failed, and to derive a normalized measurement of infection, which was calculated as ng of fungal DNA per ng of barley DNA.

Real-time PCR

TaqMan reactions were performed in 25 µl using 12.5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems). Primer optimization was done according to the Applied Biosystems protocol and 300 nM of forward primer pg20, 900 nM of reverse primer pg21 and 200 nM of the FAM-labelled MGB probe Pg were found to be optimal. For the internal amplification control, 300 nM of the forward primer Adh1F, 900 nM of the reverse primer Adh2R and 250 nM of the VIC-labelled MGB-probe Adh were used. Both reactions were performed on 2.5 µl of a 1/10 dilution of DNA extractions from seeds. PCR was run in a 96 well format in an ABI prism 7000 with the following cycling conditions: one cycle of 2 min at 50°C, and 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each PCR was run in duplicate. A *P. graminea* standard curve was obtained by PCR amplification of 10-fold serial dilutions of purified *P. graminea* DNA (4 ng to 400 fg) in T0.1E (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA). The barley standard curve was prepared from DNA extracted from seeds free of *P. graminea* infection and was diluted to 20, 4, 0.8, 0.16 and 0.032 ng μl^{-1}. Amplification efficiency of *P. graminea* DNA in the presence of barley seed DNA was investigated on serial dilutions of fungal DNA (2 ng to 2 pg) in a DNA extraction from seeds free of *P. graminea* infection.

Concentrations of the *P. graminea* and barley DNA used for the standard curves were determined by a fluorometer (DyNA Quant 200, Hoefer). The concentrations were confirmed by visually comparing the band intensities of the DNA to a λ-DNA standard on an ethidium bromide-stained agarose gel. For quantification in unknown samples, standard curves were generated by plotting $\log_{10}$ of the DNA quantity of known standards against the cycle number (C_t) at which the fluorescent signal from the amplified PCR products surpassed the detection threshold. The threshold line is the level of detection at which the fluorescent signal is above background and it is set in the exponential phase of the PCR. Two reactions were run for each dilution and the standard curves were included in each PCR run. Two independent PCR runs were performed for all samples.

Freezing blotter method and greenhouse test

The freezing blotter method was performed essentially as described by Jørgensen (1982a): 200 seeds per seed lot were incubated on moist filter paper without surface-sterilization for 24 h at 20°C followed by 12 h at −20°C and finally 5 days at 20°C. After incubation the seeds were examined microscopically for the presence of *Pyrenophora* conidia. The percentage of

plants infected with *P. graminea* was assessed in a greenhouse test where 200 seeds from each seed lot were sown and plants with leaf stripe symptoms were counted after 4–5 weeks (Jørgensen 1980).

Data analysis

Relationships between DNA quantities (nanogram *P. graminea* DNA) and infection incidence (percent infected seeds in the blotter test and percent infected plants in the greenhouse, respectively) were examined. Furthermore, infection ratios (= percent infected plants in the greenhouse/nanogram *P. graminea* − DNA in the corresponding seed sample) were computed and examined for individual varieties. Prior to statistical analyses, logarithmic transformation of the data was compared with root and arcsine transformations with various fractional power terms in order to find a transformation assuring linearity between dependent and independent variables and minimizing heteroscedasticity and skewness of the data, dependence of the residuals from the estimations and other systematic patterns of the residuals. For data representing DNA quantity and seed infection incidence, the third root transformation fulfilled these criteria best. The second root transformation appeared best for plant infection incidence and the log-transformation (ln[x+1]) for infection ratios. The transformed data were subjected to further statistical analyses. DNA data were subjected to general linear mixed model analyses (GLM) using seed lot and sub-sample nested within the seed lot as random effects. Data on seed- and plant infection incidence per seed lot were regressed on the mean amount of *P. graminea* DNA per seed lot by linear regression analysis (LR). The expected random variation of the 3rd root-transformed seed infection incidence (σ_s^2) was computed as:

$$\sigma_s^2 = 1/9y_s^{(-4/3)}((y_s(100 - y_s))/n_s)$$

where y_s=percent infected seeds and n_s=number of examined seeds. The expected random variation of the second root-transformed plant infection incidence (σ_p^2) was computed as:

$$\sigma_p^2 = 1/4y_p^{(-1)}\left(\left(y_p\left(100 - y_p\right)\right)/n_p\right)$$

where y_p=percent infected plants and n_p=number of examined plants. Note that σ_s^2 and σ_p^2, respectively, are not computed based on observed data but represent the random variation to be expected if n_s seeds respectively n_p plants were sampled at a seed infection incidence of y_s respectively at a plant infection incidence of y_p. Note that the different denominators and power terms in these equations account for the different transformations used on the original data. Infection ratios of varieties were compared using Tukey's honestly significant difference test (HSD).

Results

Quantification of *P. graminea* with real-time PCR

The *P. graminea*-specific pair of primers used in the TaqMan assay amplified a 59-bp PCR product from *P. graminea* isolates tested in this study. No PCR amplification was observed when non-target fungal DNA was used as a template for PCR (Table 1). Quantifications of *P. graminea* DNA showed a linear relationship between $\log_{10}$ values of the amount of genomic DNA and cycle threshold values with R^2 values ranging from 0.994 to 0.999 (data not shown). The PCR assay for *P. graminea* allowed quantification between 10 ng and 1 pg fungal DNA. However, the variation among PCR runs increased at high Ct values. Quantification of fungal DNA in the presence of barley seed DNA was investigated by preparing serial dilutions of fungal DNA in a DNA extraction from seeds free of *P. graminea* infection and showed a linear relationship from 2 pg to 2 ng of fungal DNA (R^2=0.988). From the slope of the linear relationship an efficiency, $E=10^{(-1/\text{slope})} - 1$, of 90% was calculated indicating that quantification in barley seed extracts is reliable and with an efficiency close to the theoretical efficiency (100%) within this dilution range of target DNA (Fig. 1).

Variation of *P. graminea* DNA in naturally infected seeds

Results of GLM analyses revealed that 92.2% of the variation of *P. graminea* DNA measured by PCR in naturally infected seed lots of variety Agneta can be accounted for: 84% by the seed lot and 8.2% by the sub-sample within the seed lot (Table 2, upper part). Of the variation of normalized DNA, only 82.7% can be accounted for: 76.3% by the seed lot and 6.4% by

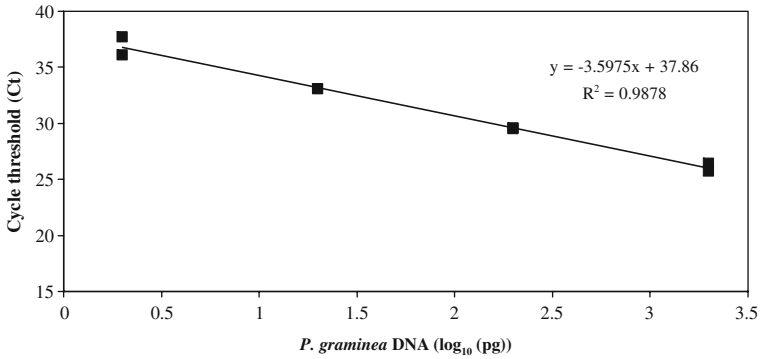

Fig. 1 The relationship between *P. graminea* DNA (isolate Pg Pallas) and cycle threshold value measured by TaqMan real-time PCR. *Pyrenophora graminea* DNA was diluted in DNA extracted from barley seeds with no infection in order to demonstrate the quantification of *P. graminea* DNA in barley seed DNA extracts. Two replicates were run for each of four dilutions

the sub-sample within the seed lot (Table 2, lower part). In the subsequent statistical analyses, we focused therefore on the non-normalized data.

Relationships between *P. graminea* DNA and infection incidence in naturally infected seeds

The infection incidence of the seed lots of variety Agneta ranged from 3% to 41% infected seeds when tested with the freezing blotter method and from 0% to 11% infected plants when tested with the greenhouse method. The amount of *P. graminea* DNA of the Agneta seed lots was positively correlated with their infection incidence, whether determined as percent infected seeds by the freezing blotter method or as percent infected plants by the greenhouse test. Both correlations were highly significant ($P<0.001$) although the corresponding R^2 values determined via LR were relatively low (0.59 and 0.56, respectively; Fig. 2a and b, respectively). According to the LR models shown in Fig. 2a and b, 5% infected seeds, which represent the currently recommended seed infection threshold, correspond to 8.6 pg *P. graminea* DNA. At this level, the 95% confidence intervals for individual predictions indicate between approximately 1% and 14% infected seeds for the blotter method and up to about 3% infected plants for the greenhouse method. Note also the positive intercept of the LR model for the blotter method and the negative intercept of the LR model for the greenhouse test. The error mean squares of the LR models presented in Fig. 2a and b are 0.12 and 0.27 for the seed infection incidence of the blotter method and the plant infection incidence of the greenhouse method, respectively. The corresponding mean random variance of observation amounts to about 1/3 of these (0.04 respectively 0.12, see caption of Fig. 2). The projections in Fig. 3a and b indicate that the random variance of observation of seed- and plant infection incidence could be drastically reduced if the number of seeds or plants examined respectively by the blotter and greenhouse methods was increased.

Variation of infection ratios of varieties

Analyses of seed samples of different varieties from different years indicated that the infection ratios, i.e. the plant infection incidence per ng *P. graminea* DNA, varied considerably across and within varieties (Table 3). But although the infection ratios are very variable even within individual varieties, as indicated by large differences between minimum and maximum values for individual varieties, they are clearly highest for variety Jersey, followed by Power, Agneta and Alliot indicating that infections are more efficiently produced per amount of fungal DNA on these varieties, compared to other varieties. In this respect, Jersey is significantly different from the varieties Barke, Cicero and Hydrogen that, beside Sebastian, exhibited no or very low infection levels in the greenhouse in relation to the amount of *P. graminea* DNA detected in their seed lots.

Discussion

Conventional seed health tests are time-consuming and require trained personnel for correct morpholog-

Table 2 Random effects of the seed lot and the sub-sample within the seed lot on the variation of *P. graminea*-DNA in seed lots of variety Agneta

Dependent variable	Source of variation		Type III sum of squares	Degrees of freedom	Mean square (ms)	Percent variance explained	*F* value	Probability value
P. graminea DNA (ng, 3rd root)	Intercept	Hypothesis	20.600	1	20.600		743.144	<0.001
		Error	1.359	49	0.028[a]			
	Sample	Hypothesis	1.362	49	0.028	84.0	10.471	<0.001
		Error	0.133	50	0.003[b]			
	Subsample (sample)	Hypothesis	0.133	50	0.003	8.2	2.066	0.001
		Error	0.127	99	0.001[c]			
P. graminea DNA/ *H. vulgare* DNA (ng/ng, 3rd root)	Intercept	Hypothesis	5.370	1	5.370		584.860	<0.001
		Error	0.450	49	0.009[d]			
	Sample	Hypothesis	0.450	49	0.009	76.3	12.252	<0.001
		Error	0.037	50	0.001[e]			
	Subsample (sample)	Hypothesis	0.037	50	0.001	6.4	0.731	0.889
		Error	0.102	100	0.001[f]			

Results of general linear model analyses.

Upper part of the table non-normalized data, *lower part of the table* data normalized with estimates of barley DNA

[a] 0.997 ms (sample) + 0.003 ms (error)

[b] Ms (sample(sample)) +.000045 ms (error)

[c] Ms (error)

[d] Ms (sample)

[e] Ms (sample (sample))

[f] Ms (error)

ical identification of fungal structures such as spores. The freezing blotter method is a frequently used method for estimating the seed infection level of various seed-borne diseases and has routinely been used for assessing the infection incidence of *Pyrenophora* spp. in barley seed lots. However, the method cannot distinguish between conidia of *P. graminea* and *P. teres*. The only conventional alternative is an even more resource-demanding greenhouse test where leaf symptoms are identified. In the present study, we designed a TaqMan real-time PCR method for detecting and quantifying *P. graminea* DNA in seed samples. DNA was extracted from whole seeds, which were ground to a fine powder to ensure that all *P. graminea* DNA in the seed can be detected no matter where in the seed it is located. We used a commercial kit developed for DNA extraction from food samples such as cereal products in order to reduce PCR inhibitors of seed origin. This method gave high quality DNA with low variation between repeated PCR reactions on the same DNA-extract. Normalization with the amount of barley DNA in order to compensate for differences in extraction efficiencies did not considerably improve the correlation between the infection incidence level and the DNA content in naturally infected samples. In subsequent analyses, normalized data was therefore not used. Nevertheless it is crucial to include the plant assay as an internal amplification control in order to avoid false negatives.

Specificity assays of primers and probe confirmed that the assay is specific for *P. graminea*. We used a TaqMan probe to avoid fluorescent signals from non-target PCR amplicons. Furthermore, the use of TaqMan probes offers the opportunity for future developments of a multiplex test for seed-borne diseases. Previously, a sequence-characterized amplified region (SCAR) has been used to design primers for *P. graminea* detection (Taylor et al. 2001). These were used in an assay mainly developed to test for the presence or absence of the pathogen rather than to quantify it, because *P. graminea* has been steadily declining in the UK and so detection seemed sufficient and quantification not necessary. The aim of our study was to investigate possibilities for quantitative and fast determination of *P. graminea* in seed lots via real-time PCR. It was inspired by the

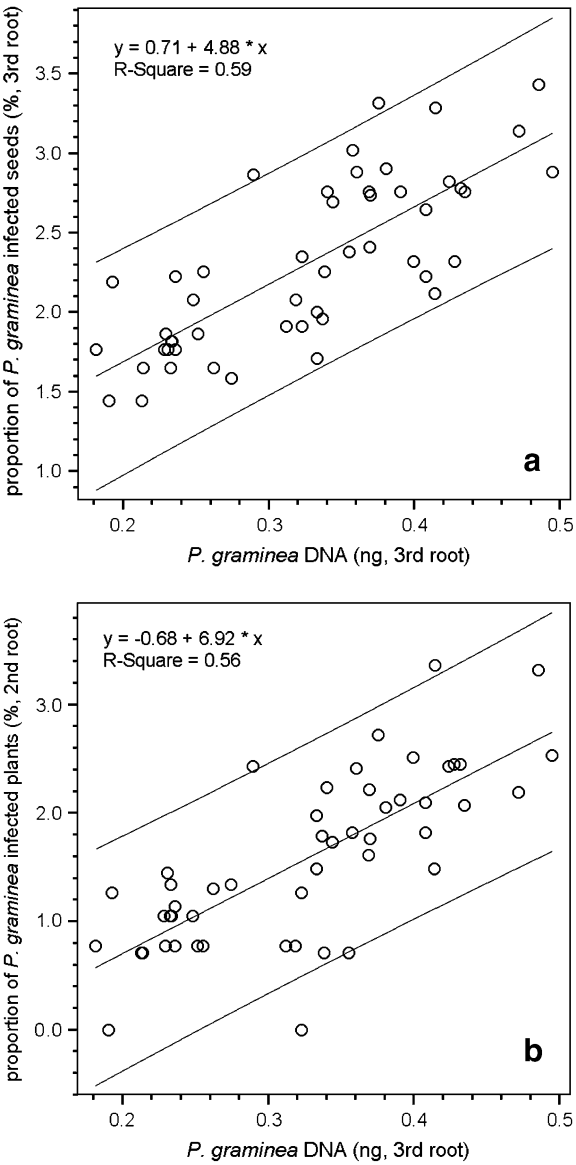

Fig. 2 Relationship between the amount of *P. graminea* DNA measured by real-time PCR and the infection level determined by the freezing blotter method (**a**; R^2=0.59, P<0.001, error mean square=0.12, mean random variance of observation=0.04) or the greenhouse test (**b**; R^2=0.56, P<0.001, error mean square=0.27, mean random variance of observation=0.12) on variety Agneta. Regression lines and 95% confidence intervals for individual predictions are shown. Each data point represents the mean of two PCR runs. Data is transformed to the fractional power (root) that resulted in minimum heteroscedasticity of the data and random pattern of the regression residuals

assumption that seed infection by *P. graminea*, although more frequent in organically than in conventionally produced seed, may often occur at low levels that do not necessitate rejection of the affected seed lots even though results of the more time-consuming conventional seed testing methods would suggest rejection. To ensure optimal amplification efficiency for accurate quantification we designed a TaqMan assay optimized for the ABI system.

The developed real-time TaqMan PCR assay was able to precisely quantify *P. graminea* DNA in barley seed DNA extractions in the range from 2 pg to 2 ng. However, it can most likely expand beyond this range as shown from the standard curves where the dynamic

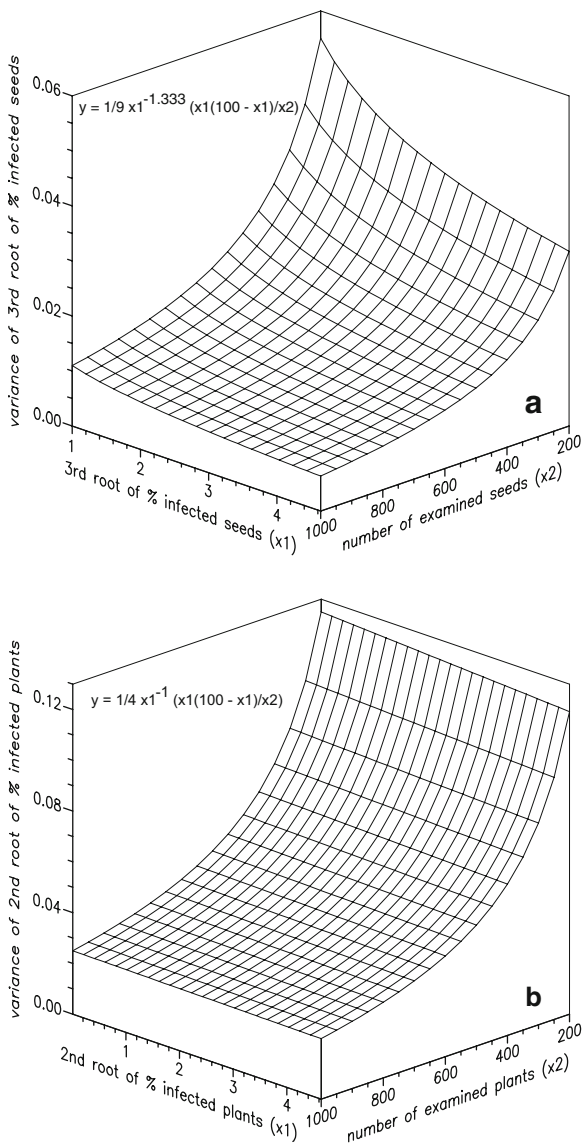

Fig. 3 Random variance of infection levels in seeds (**a**) and plants (**b**) measured by the blotter and greenhouse methods, respectively, as related to the infection level (x_1) and the number of examined seeds or plants (x_2)

Table 3 Infection ratios (= percent infected plants in the greenhouse ng^{-1} *P. graminea*-DNA in 200 seeds) of seed lots of spring barley varieties in 3 years (2003, 2004, 2005)

Variety	Minimum	Median	Mean	Maximum	Grouping (Tukey's HSD)
Agneta	0.0	72.0	81.0	300.6	–
Alliot	36.5	54.4	80.2	149.9	ab
Astoria	0.0	18.0	13.0	21.1	ab
Barke	0.0	0.0	0.0	0.0	a
Braemar	27.2	39.5	42.5	60.8	ab
Brazil	7.5	24.9	24.7	41.8	ab
Cicero	0.0	0.0	1.4	4.2	a
Class	0.0	35.0	32.1	61.4	ab
Hydrogen	0.0	0.0	5.1	15.2	a
Jersey	97.3	184.0	600.0	1518.9	b
Otira	28.5	33.1	58.7	114.5	ab
Power	0.0	7.8	94.8	276.5	ab
Scarlett	0.0	10.3	12.2	26.3	ab
Sebastian	0.0	5.7	5.8	11.7	ab
Simba	0.0	91.1	62.9	97.8	ab

Varieties with a different letter in the last column have significantly different means (P=0.05) according to Tukey's Honestly Significant Difference test (HSD) when data are log-transformed (ln[x+1]). Because the data are skewed, minimum, median, mean and maximum values of the infection ratios are shown, rather than means and standard deviations. Variety Agneta represents a separate data set (from 2002) and was therefore not included in mean comparisons.

range was from 1 pg to 10 ng. The lowest infection level observed in the naturally infected seed lots of this study was 3% as determined by the freezing blotter method. Seed lots with this infection level contained between 6.6 and 10 pg *P. graminea* DNA per reaction when tested with real-time PCR (Ct values from 30.9 to 32.8) indicating that it may be possible to detect down to 0.5%, which is the detection limit of the blotter test, although this will depend very much on the infection level of the individual seeds. The highest infection level of the tested samples was 41% infected seeds, which is unusually high. This corresponded to 0.11 ng *P. graminea* DNA per reaction. It is thus not likely that naturally infected samples will exceed the upper limit of the dynamic range of the PCR assay.

The amount of *P. graminea* DNA in naturally infected seed lots, as measured by real-time PCR, was significantly correlated with the infection incidence determined with the blotter and greenhouse methods. However, only 59% (R^2=0.59) and 56% (R^2=0.56) of the variation in seed- respectively plant infection incidence could be explained by the PCR results. Data of Bates et al. (2001) indicated a closer relationship between the results of a quantitative PCR for *Pyrenophora* spp. and a conventional agar plate test of seed samples mainly containing *P. teres* but large variation between samples of comparable infection levels was also seen. Moreover, their analysis was based on untransformed data. Chilvers et al. (2007) found no significant linear relationship between disease incidence of *Botrytis* spp. in onion seed and the amount of *Botrytis* DNA measured by real-time PCR, showing that it is not always possible to define a linear relationship between the disease incidence and the amount of pathogen DNA in a seed sample. It should also be noted that correlations between the results of the blotter method and greenhouse test in the present study were comparable to correlations between the results of the PCR test and the blotter method as well as the greenhouse test (data not shown here).

Variation in the results of the blotter and greenhouse tests that could not be accounted for by regressions on the PCR results may be partly due to 'background' infection with *P. teres* which the blotter method cannot distinguish from *P. graminea* whereas real-time PCR only measures *P. graminea*. Although the seed samples were from a field experiment inoculated with *P. graminea*, natural infection with *P. teres*, which is a widely distributed barley pathogen in Denmark in most years, was most likely present in

some samples and may have caused overestimation by the blotter method. This is also suggested by the positive intercept of the LR model for the relationship between *P. graminea* DNA and infection incidence of the blotter method. In contrast, the negative intercept of the LR model for the relationship between results of the PCR method and the greenhouse test suggests a detection limit of 0.95 pg *P. graminea* DNA (Ct= 37.9) in seed samples. Furthermore, the results of the blotter and greenhouse methods are subject to random variation that amounts to approximately one third of the variation that remains unexplained after regressing them on the PCR data. Projecting the random variation over a range of infection incidences and sample sizes (Fig. 3) indicated that if the number of seeds or plants tested in the blotter- and greenhouse methods were increased, the random variation would drastically decrease. This might also decrease the amount of variation that could not be explained by LR. Another reason for variation may be that DNA is amplified by PCR regardless of the viability of the fungal tissue and this can lead to an overestimation of the infection level in some seed lots. The degradation levels of DNA from dead fungal cells are not known and may strongly depend on the storage of the seed samples prior to PCR testing. To circumvent this, a biological amplification step could be introduced in the procedure (Schaad and Frederick 2002). But this will require more time for analysis and quantification and correlation with infection incidence will be difficult. Findings of Cockerell et al. (2004) moreover suggest that the distribution of seed-borne fungi within a seed lot may be heterogeneous and that the infection level of individual seeds may vary considerably causing variation in the amount of fungal DNA between seeds of the same lot. To circumvent this type of variation, PCR analyses of single grains or small seed batches would be optimal but this is currently not an economically viable option. However, the GLM results of the present study indicate that by far the largest source of DNA variation was the seed lot while the variation within seed lots was relatively small.

A threshold of 5% *P. graminea* infected seeds is currently recommended in Denmark for all barley varieties regardless of their degree of leaf stripe resistance. This threshold is based on estimates suggesting that 5% seed infection incidence corresponds to 1–3% infected plants in the field, depending on the particular conditions (Nielsen 2001). According to the LR models obtained for the susceptible variety Agneta, this 5% threshold of the blotter method corresponds to 8.6 pg *P. graminea* DNA and the upper 95% confidence limit for individual predictions is about 14% infected seeds and 3% infected plants as shown by the upper 95% confidence limit for the greenhouse method. The corresponding infection incidence in the field will probably be even lower as the greenhouse test tends to overestimate the field infection level (Jørgensen 1980). However, varieties vary in their infection ratios, i.e. in the infection incidence expressed in the greenhouse per amount of *P. graminea* DNA measured by the PCR. Particularly varieties Barke, Cicero, Hydrogen and Sebastian, that expressed good to moderate resistance against *P. graminea* in field experiments (Anonymous 2005; Pinnschmidt and Nielsen 2006) displayed low to very low infection ratios while Agneta, Alliot, Jersey and Power, that are known to be leaf stripe-susceptible in the field (Anonymous 2005; Pinnschmidt and Nielsen 2006), expressed high infection ratios. For resistant varieties, a higher DNA-level may thus be tolerated than for susceptible varieties at a given infection incidence threshold. The results thus suggest that it may be preferable to employ flexible DNA-thresholds that account for the leaf stripe-resistance level of individual varieties rather than to use rigid thresholds that are applied across all varieties. Results of the conventional freezing blotter method have also indicated great variation among varieties differing in leaf stripe resistance (Jørgensen 1982b). Especially for varieties with high levels of resistance, the predictive value of the freezing blotter method was very low (Jørgensen 1982b). This likewise suggests the use of seed infection thresholds that consider the degree of varietal resistance. Quantitative real-time PCR makes it possible to introduce such variety-dependent DNA-thresholds provided that the resistance levels of the individual varieties are known beforehand. If this is not the case, the low DNA-threshold for the most susceptible varieties might be applied to minimize risk.

Combining real-time PCR with a fast DNA-extraction method offers the opportunity for rapid and automated detection and quantification of *P. graminea* DNA in seed samples. This method reduces the required test time from 1 month in the greenhouse to 1 day in the laboratory. The real-time method is currently used only to identify seed lots containing *P. graminea*, rather than

to quantify fungal DNA and judge it with respect to a seed infection threshold. Nonetheless the use of the PCR method has shown that the percentage of rejected seed lots can be reduced by 31–49% in some years (Nielsen 2006). The major advantages of the PCR are its precision, specificity and speed. Dedicating more development work to alleviate its above mentioned shortcomings and to establish flexible DNA-thresholds for varieties with different resistance levels can make it a competitive method in the near future.

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