DEVELOPMENT OF MOLECULAR DIAGNOSTIC ASSAYS FOR SEED-BORNE PATHOGENS OF WHEAT

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Summary: Seed producers work under severe time constraints during the harvest period. Decisions on seed treatment during seed production is restricted by the lack of timely results from seed health assays. Currently, advisory testing is carried out using an agar plate test for *Microdochium nivale* (up to 7 days) and a seed washing/filtration method for *Tilletia tritici* (24 hours). Although the *T. tritici* test can be completed within one day, the throughput for this test is low. We describe the development of PCR assays for detection of these pathogens. The PCR Mimic technique (a form of competitive PCR), and real-time PCR were tested for quantification of fungal infection.

INTRODUCTION

Molecular biology methods for agricultural diagnostics have become an area of increasing interest recently. A significant flaw in the majority of the assays published to date is that they stop short of being of practical application in a routine plant-health context. In this study we have tried to address some of these points in the pursuance of practical PCR assays for plant health. We selected two fungal pathogens of winter wheat which have different modes of growth *in planta*.

M. nivale causes a variety of symptoms in wheat, notably seedling blight of which this pathogen is the most common cause in the UK (Paveley *et al*. 1996). Seedling blight causes the greatest proportion of seedling loss in the UK (Paveley *et al*. 1996). PCR assays have already been published for *M. nivale*. Nicholson *et al.* (1996) presented a variety-specific PCR method to differentiate *M. nivale* var. *majus* and var. *nivale*. However, as the existing agar plate identification method does not differentiate between the varieties this was considered unsuitable as a direct replacement for the current assay.

T. tritici is the causative agent of common (or stinking) bunt. Infected wheat ears can contain 'bunt balls' which replace the seed. These bunt balls break open during harvesting, and contaminate the surface of harvested seed and machinery. Uncontrolled spread of *T. tritici* can be rapid, with increases in infection from no visual crop symptoms to complete crop loss in three seasons. A laboratory assay of seed lots for bunt spores is essential to allow treatment (or rejection) of infected seed prior to planting (Paveley *et al*. 1996).

Current techniques for laboratory-based quantification of these pathogens have serious restrictions. Seed-borne infection with *M. nivale* is measured by outgrowth of mycelium from

surface-sterilised grain placed on potato dextrose agar (after incubation for up to ten days). Infection by spores of *T. tritici* is measured by microscopic examination of nitrocellulose membranes, through which seed washes have been filtered, and results are expressed as the number of spores per seed.

Previously, we have described methods for the quantitative detection of *M. nivale* and *T. tritici* in seed lots using competitive PCR assays to quantify infection levels in seed lots (Mulholland & McEwan 2000). Competitive PCR uses an exogenous DNA fragment which can be amplified by the same primers used for pathogen detection, but which can be differentiated from the pathogen-specific band on the basis of size. The ratio of the product from the pathogen compared to the product from the competitor indicates the initial molar ratio between the pathogen and competitor, as both products compete for the use of the primer pair. For the competitive PCR method, 'Mimic PCR' was chosen, which uses an artificially constructed competitor fragment, which comprises an unrelated fragment flanked by the pathogen-specific primer sites (Seibert & Kellogg 1996). Real-time PCR uses fluorescent-based detection of PCR products during the assay. The value, in cycle numbers, for an amplification to reach a threshold level (C_T) can be used to quantify the amount of starting DNA by comparison to DNA standards. A comparison of these two methods is described.

MATERIAL AND METHODS

DNA Extractions

A crude method was used to isolate PCR template from agar plate-grown cultures. A small amount of mycelia were removed from the plate using a pipette tip, placed in 0.5 ml 10 mM Tris·HCl (pH 8.0), boiled for 10 minutes and centrifuged prior to use in a PCR assay. *T. tritici* DNA was purified using the method of Gang and Weber (1995). Other DNA extraction methods from wheat seed were as previously described (Mulholland & McEwan 2000)

Competitive PCR Quantification

The concentration of each Mimic preparation was determined by spectrophotometry. The Mimic was then diluted to a concentration of 2 µg/ml, this being designated Mimic dilution M_0. Serial ten-fold dilutions were made from M_0 to give dilutions M_1 to M_8. PCR reactions containing Mimic and fungal PCR products were amplified and analysed as previously described (Mulholland & McEwan 2000).

Real-time PCR

Real-time PCR was carried out using SYBR Green Master Mix (Applied used were the same as those used in the competitive PCR assays. *M. nivale* assays were performed were placed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Warrington) and amplified using the factory-set conditions. Data analysis was performed using the supplied software (SDS 1.7). This method does not use an added competitor, but measures amount of target DNA based on the length of time, in cycles of amplification, required to exceed a fluorescence threshold. The use of the intercalating dye SYBR Green I in the reaction is the source of the fluorescence; when bound to DNA the fluorescence is greatly increased compared to unbound dye.

RESULTS

Microdochium nivale Assays

A series of wheat samples were selected for the comparison of mimic and real-time PCR methods. These samples had been extensively characterised by traditional detection methods, and between two and five sub-samples were available from each sample.

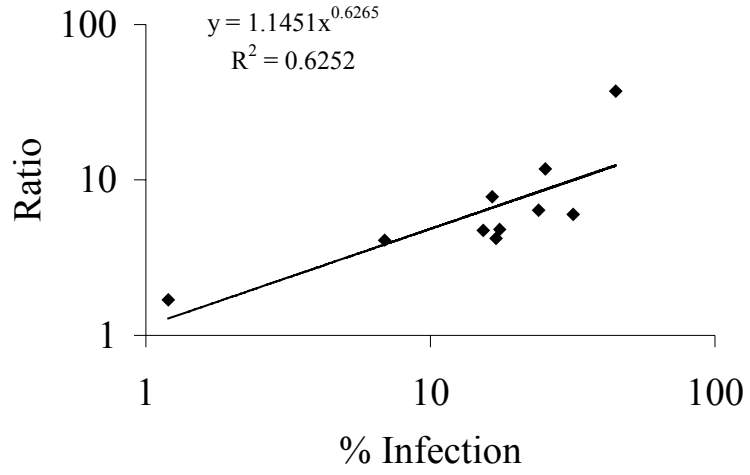

Figure 1 Use of competitive PCR for the quantification of *M. nivale* infection in wheat. The correlation coefficient (R^2) is a measure of the relationship between the results of the agar plate assay and the competitive PCR assay.

A mimic PCR quantification was performed on the samples, the results for each sub-sample was averaged to obtain a mean value for each sample and these results were compared with those obtained from the agar plate assays. A graphical representation of these data is shown in Fig. 1.

The same sets of wheat samples were also tested using a real-time PCR assay. When plotted against the infection levels determined by the agar plate method (Fig. 2) the results give a regression coefficient of $R^2 = 0.8887$. This is considerably higher than $R^2 = 0.6252$ for the competitive PCR assay compared to the agar plate assay, and is a reflection of the higher level of agreement between the real-time PCR and the agar plate assay. Comparison of various statistics shows that the variability of data from real-time assays is not significantly different from the variability in the agar plate test.

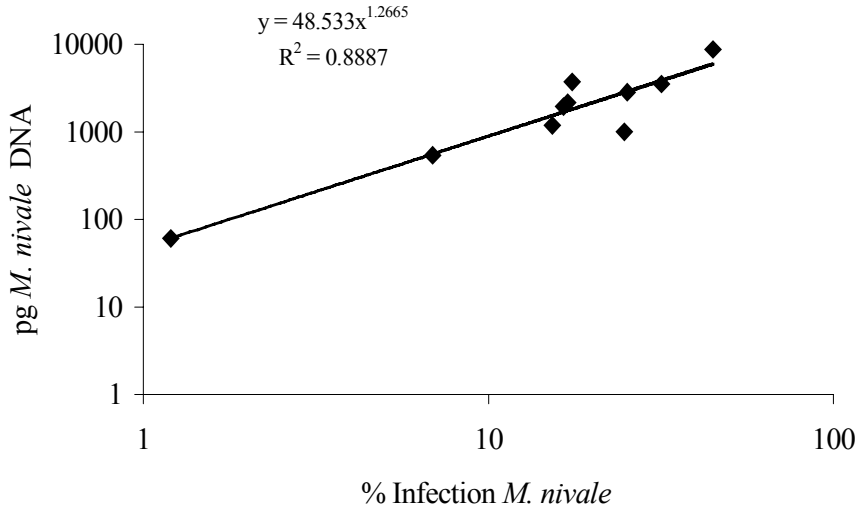

Figure 2 Comparison of real-time PCR with infection rate of *M. nivale*. Samples used were identical to those used for comparison of competitive PCR (See Fig. 1).

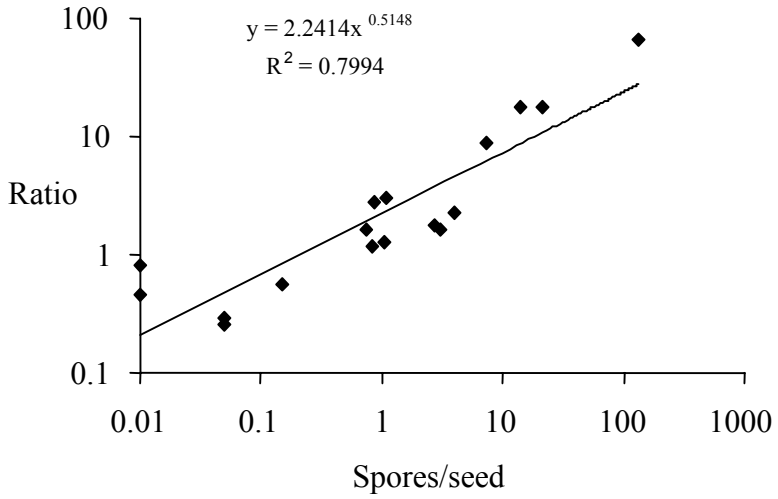

Figure 3 Correlation between seed infection levels and target-to-Mimic ratios. This graph shows the relationship between the results of PCR Mimic quantification experiments compared to infection levels (calculated by traditional methods) of seventeen *T. tritici*-infected wheat lots.

Tilletia tritici Assay

A series of 17 samples (ranging from 0 to 132 spores/seed) were selected for comparison of the quantification methods. Up to 5 replicates were taken from each sample. Quantitative PCR was performed by both competitive PCR (Fig. 3) and real-time PCR (Fig. 4). The correlation coefficients were quite similar when one compares the two methods. However, measurements

of variability for the real-time data were significantly greater than the variability encountered in the spore counts from microscopic examination.

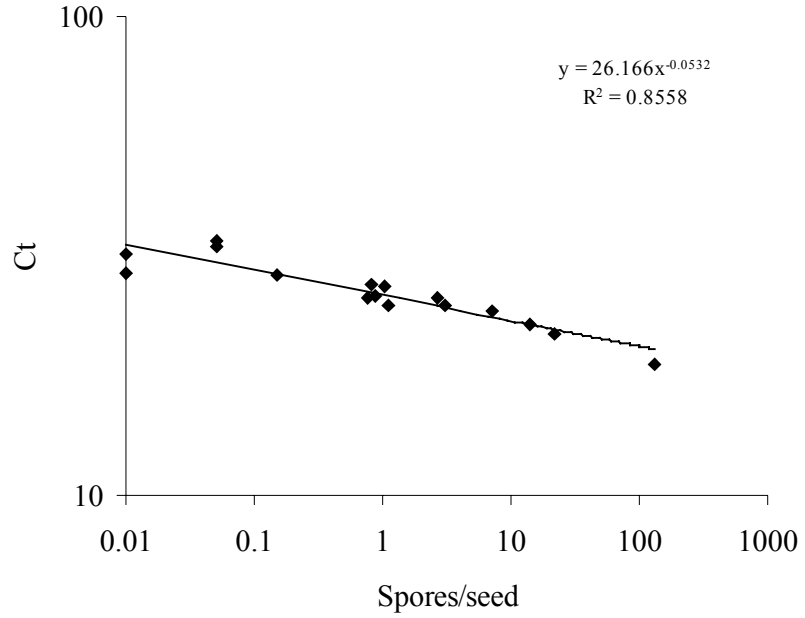

Figure 4 Comparison of real-time PCR with microscopic analysis of spores of *T. tritici*. Samples used were identical to the ones used for comparison of competitive PCR (See Fig. 3).

DISCUSSION

In this paper we have outlined some of the approaches we have taken to measure the relative accuracy of quantitative PCR assays which would be amenable to the larger-scale requirements needed for seed health testing. The correlation of both methods with the currently used techniques holds promise for the use of molecular techniques for seed health testing. However, potential sources of variability need to be isolated and eliminated to improve the precision of the methods

One potential problem that we have examined is the question of variability of the real-time assay compared to the current assay methods. For *M. nivale*, the variability is approximately comparable for both methods. However, the variation of the real-time assay for *T. tritici* is significantly greater than the variability for the microscope examination technique. We shall, in collaboration with Adrian Roberts (BioSS, Edinburgh), attempt to locate and control the largest components of this variation. This should enable changes to be made in the real-time methodology to achieve acceptable variation, not significantly different from the current method.

In conclusion, we have presented the comparison of two quantitative PCR assays for each of two fungal pathogens of wheat. These assays, particularly the real-time versions, seem to hold the promise of becoming usable methods in the replacement of the traditional detection methods for these organisms.

ACKNOWLEDGEMENTS

We would like to thank the Home-Grown Cereals Authority and the Scottish Executive Rural Affairs Department for funding.

REFERENCES

Gang, D R; Weber, D J (1995) Preparation of genomic DNA for RAPD analysis from thick-walled dormant teliospores of *Tilletia* species. BioTechniques 19, 92-97

Mulholland V; McEwan, M (2000) PCR-based diagnostics of *Microdochium nivale* and *Tilletia tritici* infecting winter wheat seeds. EPPO Bulletin 30, 543-547

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. Physiological and Molecular Plant Pathology 48, 257-271

Paveley, N D; Rennie, W J; Reeves, J C; Wray, M W; Slawson, D D; Clark, W S; Cockerell, V; Mitchell, A G (1996) Cereal seed health strategies in the UK. Home-Grown Cereals Authority, London

Siebert, P D; Kellogg, D E (1995) PCR MIMICs: competitive DNA fragments for use in quantitative PCR. In: PCR 2: A practical approach, MJ McPherson, BD Hames (Eds). IRL Press, Oxford. pp 135-148