Short Communication

Rapid Detection and Quantification of *Rhynchosporium secalis* in Barley Using a Polymerase Chain Reaction

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Abstract: PCR primers for diagnosis of *Rhynchosporium secalis* in seed samples of barley were developed. For the quantification of the pathogen in seed samples a real-time PCR with SYBR Green approach was used. Amounts from 1.8 to 419.1 pg of *R. secalis* DNA per 100 ng of total DNA were detected in 18 samples of barley seeds contaminated by *R. secalis* in field conditions. The correctness of this quantitative analysis was checked using an artificial infection of seeds with 1, 2, 5 and 20% level of infection by *R. secalis*. The level of contamination of artificially infected samples decreased with a lowering amount of added seed powder contaminated by the pathogen, the correlation coefficient for this analysis was 0.98. While the primer pair used in these analyses shows cross-reactions with other pathogens (*P. teres*, *Drechslera tritici-repentis*, *F. culmorum* and *F. poe*), it is recommended to check the products of RT-PCR by agarose-gel electrophoresis, in which these pathogens are easily distinguishable from *R. secalis* by different lengths of the amplified fragments.

Keywords: barley scald; real-time PCR; pathogen diagnostics

The scald, a foliar disease caused by *Rhynchosporium secalis* (Oudem.) J. J. Davis, is a major disease of barley (*Hordeum vulgare* L.) that occurs in all main barley growing regions of the world where it causes considerable yield losses (Turkington *et al.* 2002; Yahyaoui 2002). The scald infection also decreases grain quality, measured in terms of seed weight and percentage of plump grains (Brown *et al.* 1996). The seed-borne inoculum of *R. secalis* is important, especially in the dispersal of novel races as well as in the long-range dispersal of the pathogen (Salamati *et al.* 2000).

In recent years DNA technology has become one of the major tools for the identification and detection of fungal plant pathogens (Reeves 1995; Parry & Nicholson 1996; Doohan *et al.* 1998). In the majority of these cases, PCR was only used for specific detection of the pathogen in plant material, but not for its quantification. To date there have been several reports of the use of real-time quantitative PCR for quantification of different pathogens (Bates *et al.* 2001; McNeil *et al.* 2004; Strausbaugh *et al.* 2005).

This paper was aimed at the development of specific primers for detection and preliminary study of quantification of *R. secalis* in seed samples of barley.

The isolates RH1 of *R. secalis*, PT4 of *Pyrenophora teres* f. *teres*, PG3 of *Pyrenophora graminea* and S1-5 of *Stagonospora nodorum* were collected

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in Slovakia. The isolates of *Septoria tritici* (KS-RL-03-1 and KS-Hv-01) used in this study were provided by Prof. W. Bockus (Kansas State University, USA), isolates of *Septoria nodorum* (S3 and S4) and *Drechslera tritici-repentis* (D23, D31 and D42) were provided by Dr. M. Jalli (MTT Agrifood Research, Finland), 2 isolates of *R. secalis* (4004 and 32-2) were provided by Dr. S. Salamati (Kvithamar Research Center, Stjørdal, Norway), isolate of *Pyrenophora teres* f. *maculata* (Merlot 02 S2) was provided by Dr. V. Minaříková (Agricultural Research Institute Kroměříž, Ltd., Czech Republic), isolate of *P. teres* f. *teres* (3d 32/98) and isolate of *P. teres* f. *maculata* (10/97) were provided by Prof. K.J. Williams (Cooperative Research Center for Molecular Plant Breeding, South Australian Research and Development Institute, Urbane, Australia) and isolates of *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium poe* and *Fusarium avenaceum* were provided by Dr. S. Šliková (Research Institute of Plant Production, Slovakia). All pathogens were maintained on potato dextrose agar at 20°C. Total fungal genomic DNA was extracted from 8 weeks old single-spore-derived pathogen cultures grown on agar plates according to the method of Dellaporta *et al.* (1983).

The 1, 2, 5 and 20% level of infection of an artificially infected seed powder was reached by the addition of *R. secalis*-infected seed powder from seeds of barley cultivar SK-13991 which were artificially inoculated by pathogen cultures of *R. secalis* using an agar block 0.5 × 0.5 cm in size overgrown with the pathogen. Additional barley seed samples used in this study were collected from two barley-growing regions of Slovakia (Table 1). Samples of barley seeds were finely ground with an ultracentrifugal mill (ZM 100, Retschn GmbH & Co. KG., Haan, Germany) and 1 g of the seed powder was used for the analysis. Total DNA from barley seeds was isolated using the Adgen DNA Extraction System (Adgen Ltd.).

PCR primers RS17-F (5'-TTGAGTCCGGCTGGTCTACT-3') and RS17-R (5'-CCTACCTGATCCGAGGTCAA-3') were designed to amplify an amplicon, 67 bp long, of the target nucleotide sequence from ITS regions of rDNA of *R. secalis* (accession number AY140668). The nucleotide sequence of the pathogen was derived from the GenBank nucleic acid database for sequences (www.ncbi.nlm.nih.gov). These primers were designed using online programme Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, Rozen & Skaletsky 2000) and the specificity of primers was tested by BLAST (www.ncbi.nlm.nih.gov/BLAST). The PCR analysis of *R. secalis* was done as described previously (Gubiš *et al.* 2004). Electrophoretic detection of PCR products was performed in 3% agarose gels stained with ethidium bromide.

Real-time PCR quantification of the pathogen was carried on an ABI PRISM® 7000 SDS instrument (Applied Biosystems, USA) using SYBR® Green I fluorescence dye (SYBR® Green I Master Mix, Applied Biosystems). Reactions for the RT-PCR using SYBR Green detection consisted of 12.5 µl of SYBR® Green I Master Mix, different concentrations of 500nM forward and reverse primers, 2.5 µl of DNA template and water to the final volume of 25 µl. All PCR samples and nontemplate controls (NTC) were prepared in identical duplicate using 0.2 ml MicroAmp Optical reaction tubes and MicroAmp Optical tube caps (Applied Biosystems, USA). The amplification conditions of the reaction were according to the manufacturer's instructions (Applied Biosystems). The stock of *R. secalis* DNA solution was serially diluted using 1 × TE buffer to obtain standards 2.5, 25, 250 and 2500 pg/µl (final amounts in PCR reaction). The quantity of DNA encompassed by these standards covered the range of contamination levels likely to be observed within samples submitted for testing.

In this study the potentiality of a real-time PCR system to detect and quantify *R. secalis* in seed material was determined. Lee *et al.* (2001, 2002) and Turkington *et al.* (2005) performed quantitative detection of *R. secalis* in seeds, however competitive PCR was used to analyse the quantity of the pathogen DNA. Fountaine *et al.* (2003) were concerned with quantification of *R. secalis* in leaf samples using primers derived from the gene for cytochrome *b* from *R. secalis* and a FAM labelled TagMan probe. They compared a visual assessment of pathogen attack on plants with the results of RT-PCR and determined a correlation between them. In our analyses SYBR Green approach was used.

PCR primers for qualitative detection and preliminary study of quantitative detection of *R. secalis* in seed samples of barley were developed. Primer pairs RS17-F and RS17-R were proved to be specific with a PCR product of 67 bp amplified for all *R. secalis* isolates tested on the conventional PCR machine (Figure 1). Other genera and species of

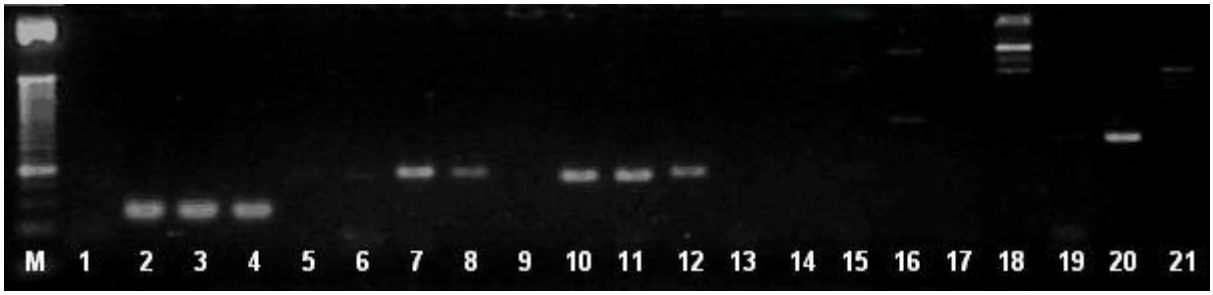

Figure 1. Agarose gel showing amplification products with primer pair RS17F and RS17R; DNA templates: M – 25 bp DNA ladder; 1 – H_2O (negative control); 2 – *R. secalis* RH1; 3 – *R. secalis* 4004; 4 – *R. secalis* 32-2; 5, 6 – *P. teres* f. *maculata*; 7, 8 – *P. teres* f. *teres*; 9 – *P. graminea*; 10,11, 12 – *Drechslera tritici repentis*; 13, 14 – *Septoria tritici*; 15, 16, 17 – *Stagonospora nodorum*; 18 – *F. culmorum*; 19 – *F. graminearum*; 20 – *F. poe*; 21 – *F. avenaceum*

fungal pathogens (*R. secalis*, *P. teres* f. *teres*, *P. teres* f. *maculata*, *P. graminea*, *Drechslera tritici-repentis*, *S. nodorum*, *S. tritici*, *Fusarium culmorum*, *F. graminearum*, *F. poe* and *F. avenaceum*) were screened for amplification with these primers. Positive reactions were observed for *P. teres* f. *teres*, *Drechslera tritici-repentis*, *F. culmorum* and *F. poe*, but amplified fragments were longer and bands were in different positions easily distinguishable from *R. secalis* in agarose gel (Figure 1).

Table 1 represents the preliminary study of quantification of *R. secalis* in seed samples of barley. Eighteen samples of barley contaminated by *R. secalis* from field conditions were analysed to verify the primers. All tested samples displayed a signal in the RT-PCR analysis. The ABI PRISM 7000 SDS allowed the quantification of *R. secalis* levels producing a specific fluorescence signal in samples from field conditions, which ranged between 1.8 and 419.1 pg of *R. secalis* DNA per 100 ng of total DNA (Table 1). The coefficient of determination for these analyses was 0.96. The trueness of *R. secalis* amplicons was verified with the help of melting curve analyses (*Tm* = 77°C). The correctness of this quantitative analysis was checked using 4 samples of artificially infected seeds with 1, 2, 5 and 20% level of infection by *R. secalis*. The level of the contamination of artificially infected samples increased with the raising level of infection (Table 1) and the correlation coefficient for this analysis was 0.98. While the primer pair used in these analyses shows cross-reactions with other pathogens (*P. teres*, *Drechslera tritici-repentis*, *F. culmorum* and *F. poe*), it is recommended to check the products of RT-PCR by agarose gel electrophoresis.

Table 1. Amounts of *R. secalis* DNA in pg per 100 ng of the total DNA in naturally-infected seeds of barley from 2 localities and artificially infected seeds of barley cultivar SK-13991

Locality	Cultivar	*R. secalis* level (pg/100ng)
Malý Šariš	Garant	19.424
	Akcent	8.224
	Ebson	8.608
	Progres	27.808
	Expres	5.104
	Annabell	3.76
	Cyril	15.696
	Jubilant	419.088
	Pasadena	792
	Bolina	6.24
	Lenka	6.64
Spišká Belá	K29192 Diamant	12.768
	Lenka	15.6
	Viktor	18.56
	Norbert	5.2
	CI 5791	3.376
	CI 4922	1.776
	Terno	2.48
	level of infection (%)	*R. secalis* level (pg/100ng)
a.i. of cv. SK-13991*	1	3750.4
	2	5625.6
	5	6195.2
	20	8606.4

*artificially infected seeds of barley cultivar SK-13991

This work was aimed at the development of specific primers for detection of *R. secalis* in seed samples of barley and preliminary study of pathogen quantification using RT-PCR with SYBR Green approach, which is an economical RT-PCR format. In our next study, Taq Man approach will be used. Taq Man is recommended for analyses of such type because SYBR Green dyes do not discriminate between the different dsDNA molecules and bind to any double-stranded DNA. For this reason these results are only preliminary, of limited practical use.

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References:

Bates J.A., Taylor E.J.A., Kenyon D.M., Thomas J.E. (2001): The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. Molecular Plant Pathology, **2**: 49–57.

Brown A.H.D., Garvin D.F., Burdon J.J., Abbott D.C, Read B.J. (1996): The effect of combining scald resistance genes on disease levels, yield and quality traits in barley. Theoretical and Applied Genetics, **93**: 361–366.

Dellaporta S.L., Wood J., Hicks J.B. (1983): A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter, **1**: 19–21.

Doohan F.M., Parry D.W., Jenkinson P., Nicholson P. (1998): The use of species-specific PCR-based assays to analyse Fusarium ear blight of wheat. Plant Pathology, **47**: 197–205.

Fountaine J., Cook A., Napier B., Shaw M., Ward E., Fraaije B. (2003): Following the epidemics of *Rhynchosporium secalis* in susceptible and resistant barley cultivars using quantitative real-time PCR assays. In: Proc. 8th Int. Congr. Plant Pathology, February 2–7, 2003, Christchurch, New Zealand.

Gubiš J., Hudcovicová M., Klčová L., Červená V., Bojnanská K., Kraic J. (2004): Detection of leaf blotches – causal agents in barley leaves and grains. Czech Journal of Genetics and Plant Breeding, **40**: 111–117.

Lee H.K., Tewari J.P., Turkington T.K. (2001): A PCR-based assay to detect *Rhynchosporium secalis* in barley seed. Plant Disease, **85**: 220–225.

Lee H.K., Tewari J.P., Turkington T.K. (2002): Quantification of seed-borne infection by *Rhynchosporium secalis* in barley using competitive PCR. Plant Pathology, **51**: 217–224.

McNeil M., Roberts A. M. I., Cockerell V., Mulholland V. (2004): Real-time PCR assay for quantification of *Tilletia caries* contamination of UK wheat seed. Plant Pathology, **53**: 741–750.

Parry D.W., Nicholson P. (1996): Development of a PCR assay to detect *Fusarium poe* in wheat. Plant Pathology, **45**: 383–391.

Reeves J.C. (1995): Nucleic acid techniques in testing for seed-borne diseases. In: Skerritt J.H., Apples R. (eds.): New Diagnostics in Crop Science. CAB International, Wallingford, 127–151.

Rozen S., Skaletsky H. J. (2000): Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S., Misener S. (eds): Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, 365–386.

Salamati S., Zhan J., Burdon J.J., McDonald B.A. (2000): The genetic structure of field populations *Rhynchosporium secalis* from three continents suggests moderate gene flow and regular recombination. Phythopatology, **90**: 901–908.

Strausbaugh C.A., Overturt K., Koehn A.C. (2005): Pathogenicity and real-time PCR detection of *Fusarium* spp. in wheat and barley roots. Canadian Journal of Plant Pathology, **27**: 430–438.

Turkington T.K., Xi K., Tewari J.P., Tekauz K., Clayton G.W., Kutcher H.R., Harker K.N., Bailey K. (2002): Management of barley leaf diseases in Western Canada. In: Proc. 2nd Int. Workshop on Barley Leaf Blight Diseases, ICARDA, April 7–11, 2002, Aleppo, 67–87.

Turkington T.K., Xi K., Tewari J.P., Lee H.K., Clayton G.W., Harker K.N. (2005): Cultivar rotation as a strategy to reduce leaf diseases under barley monoculture. Canadian Journal of Plant Pathology, **27**: 283–290.

Yahyaoui A.H. (2002): Occurrence of barely leaf blight disease in Central West Asia ad North Africa. In: Proc. 2nd Int. Workshop on Barley Leaf Blight Diseases, ICARDA, April 7–11, 2002, Aleppo, 13–18.

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