Quantification of *Tilletia caries* and *Tilletia controversa* Mycelium in Wheat Apical Meristem by Real-time PCR

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Abstract

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In the Czech Republic, three closely related species of the genus *Tilletia* belong to pathogens that cause significant losses of wheat crops by replacing grains with a mass of teliospores. A quantitative real-time qPCR assay using SYBR Green I has been developed to quantify the amount of *T. caries* and *T. controversa* mycelium in apical meristems of different wheat varieties. The real-time PCR reaction was validated by evaluating selected extraction methods, examining the specificity of designed target-specific IGS primers and verifying the optimised amplification reaction on naturally infected wheat plants. The PCR detection limit for the specific identification of fungal DNA was 0.22 ng of mycelium, and the negative correlation between the target DNA quantity and cycle threshold (Ct) was high with a coefficient of determination of R^2 = 0.992. The developed method was used to quantify pathogens mycelium in five wheat varieties in the range from 0.34 ng to 15 µg per one growing tip.

Keywords: *Tilletia* spp.; real-time PCR; apical meristem; absolute quantification

In the Czech Republic, wheat crops (*Triticum aestivum* L.) can be affected by three closely related species of the genus *Tilletia*. *Tilletia caries* (DC.) Tul. & C. Tul. (1847) [syn. *T. tritici* (Bjerk.) G. Winter (1874)] and *Tilletia foetida* (Wallr.) Liro (1920) [syn. *T. laevis* J.G. Kühn (1873)], the causal agents of common bunt (stinking smut), are mainly seed-borne pathogens replacing wheat grains in ears by smut balls consisting of teliospores on both spring and winter wheat; in contrast, *Tilletia controversa* J.G. Kühn (1874), the causal agent of dwarf bunt, is a soil-borne fungus causing excessive tillering and stunting in growth (especially of winter wheat plants) in addition to smut balls. The crushing of smut balls from all of these fungal species is followed by a characteristic foul, fishy smell of trimethylamine. Interspecific hybridisation among *Tilletia* spp. resulting in intermediate forms of teliospore morphology, teliospore germination and pathogenicity (Flor 1932; Holton & Kendrick 1956; Silbernagel 1964) can contribute to increasing genetic variability in pathogen populations as well as the ability of the fungus to overcome current disease control steps. Effective control measures against the bunts consist of seed treatment and the growth of tolerant wheat varieties. When suitable control methods are not used, bunts can cause significant yield losses by decreasing the quality of grains.

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In recent years, however, the importance of bunts has increased again, because the economic situation has put pressure on farmers to reduce the costs of chemical seed treatment and to sow seed self-supplied by farmers. Serious problems connected with outbreaks of bunts have occurred in organic farming, where the use of chemical seed dressing is prohibited. The tolerance (resistance) of new wheat varieties may be evaluated only on the basis of visual symptoms of the disease in the head of the plant in field or greenhouse trials. Therefore, it is important to find an accurate and rapid method for the identification of a sensitive or tolerant (resistant) reaction of wheat varieties. It is known that mycelia of *Tilletia* spp. can be present in plant tissue during both compatible and incompatible interactions between the host and pathogen (Gaudet *et al.* 2007), but they do not penetrate into a growing point in plants with resistance genes to *Tilletia* spp. (Hansen 1958; Swinburne 1963). The presence of fungal hyphae in wheat plants can be detected by staining (Hansen 1958) or using molecular biological and serological methods (Josefsen & Christiansen 2002; Eibel *et al*. 2005; Yuan *et al*. 2009).

To diagnose plant pathogenic fungi, the application of a polymerase chain reaction (PCR) technique using internal transcribed spacer (ITS) regions separating 18S-5.8S-28S subunits of the eukaryotic pathogen ribosomal DNA has been used most frequently. Between each copy of the rDNA cistron within the repeating unit, non-transcribed spacers are present as sequences entirely free of selective constraint. For this reason, non-transcribed spacers (NTSs), otherwise known as intergenic spacers (IGSs), are sensitive markers of evolutionary change that track drift more rapidly than ITSs (Collins & Cunningham 2000; Sterud *et al*. 2002). IGS regions provide a powerful means of distinguishing phylogenetically closely related species, as presented by Sugita *et al*. (2002) in their work using IGS regions for species identification of *Trichosporon* and genotype identification of *T. asahii*. In addition to the commonly used species-specific competitive PCR technique, real-time (quantitative) qPCR technology has opened increasing opportunities to detect and study phytopathogenic and antagonistic fungi. This method combines the sensitivity of conventional PCR with the generation of a specific fluorescent signal, providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets. In diagnostics of plant pathogens as well as in other areas, real-time qPCR has played a significant role in recent years. This technology, with its attributes of sensitivity, repeatability and specificity, has been frequently used in human, veterinary and phytopathological practice (Cullen *et al*. 2002; Lees *et al*. 2002; Berg *et al*. 2006; Belbahri *et al*. 2007; Luo *et al*. 2007; Campanile *et al*. 2008). In plant pathology, amplicon sequence-non-specific (SYBR Green I) and sequence-specific (TaqMan, molecular beacons, and scorpion-PCR) methods have been utilised the most heavily (Schena *et al*. 2004). The real-time PCR technique has been applied for measuring the expression of genes of interest, validating microarray experiments and monitoring biomarkers. Reverse-transcription quantitative PCR (RT-qPCR) has become a key tool in gene expression analysis to measure the level of mRNA and miRNA as well as for RNA species quantification (VanGuilder *et al*. 2008).

The objective of the present study was to develop an efficient method for the estimation of *Tilletia caries* and *Tilletia controversa* mycelium in a growing tip during the first stages of wheat plant growth in view of evaluating sensitive/tolerant (resistant) reactions of different wheat varieties. We tested this assay on five wheat varieties naturally infected in a field trial.

MATERIALS AND METHODS

Biological material. The main sources of fungi and plant materials used in this study were naturally infected wheat plants cultivated on *T. controversa* infested fields in Vsetín and wheat plants growing from artificially *T. caries* inoculated seed in the Agricultural Research Institute in Kroměříž, Czech Republic. Samples of five varieties of whole plants (Sulamit, Drifter, Trend, Bill, and Ebi) growing on fields in both localities were collected at the beginning of tillering (BBCH 21). Ten growing tips from each variety were excised using an optical scalpel under the stereomicroscope and used directly for genomic DNA isolation. Teliospores were collected from infected fully developed heads of plants from field trials by crushing the smut balls, and they were sieved using a 20-μm sieve. The teliospore suspension was pelleted by centrifugation (1200 × g for 3 min), surface sterilised by resuspending in 0.5% sodium chlorite and immediately centrifuged (1200 × g for 1 min). It was then washed twice in

sterile distilled water, centrifuged (1200 × g for 5 min) and finally resuspended in sterile distilled water. The suspension of teliospores was spread onto 2% water agar with 30 mg penicillin and 200 mg streptomycin per litre of agar and incubated at 15–18°C for 5–7 days (*T. caries*) or at 5°C for 3–5 weeks (*T. controversa*). After teliospore germination and sporidia formation, small pieces of agar were transferred into a Petri dish with potato dextrose agar. Pure cultures of mycelia were harvested, divided into 500-mg aliquots and stored at –80°C for subsequent analyses.

Extraction of DNA. Genomic DNA from *T. caries*, *T. controversa* and the apical meristem was extracted from the mycelium and the apical tip (Figure 1) in the stage of beginning of wheat tillering (BBCH 21) using three different extraction methods to ensure a high level of repeatability in subsequent isolations. This is necessary for the accurate quantification of mycelium in plant tissue. The biological material in a 2-ml tube was repeatedly frozen in liquid nitrogen and de-frozen in a water bath at 50°C. After addition of an extraction buffer based on a selected method and three stainless steel beads (3 mm), the samples were pulverised by shaking (10 min 30×/s) using a mixer mill (RETSCH MM 400). The first method of extraction was based on a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, USA) used according to the manufacturer's instructions. The second method consisted of using proteinase K. After homogenisation in the lysis buffer (50mM Tris, 100mM NaCl, 5mM EDTA, 1 % sodium dodecyl sulphate), the lysate was incubated in a heating block at 80°C for 10 minutes. It was then cooled to 40°C, incubated with 10 µl of 10 mg/ml proteinase K for 2 h and then incubated again at 80°C for 10 minutes. An equivalent volume of chloroform:isoamyl alcohol (24:1) was added, and the tubes were gently mixed by inversion. This was followed by incubation at 0°C for 30 min and centrifugation at 4°C for 10 min at 10 000 × g. The supernatant was recovered into a new tube, precipitated in a half-volume of isopropanol and incubated at –60°C for 1 hour. DNA was collected by centrifugation for 10 min at 4°C at 10 000 × g and subsequently washed with 70% ethanol. The DNA pellet was dried at room temperature and dissolved in TE buffer (10mM Tris, pH 7.5 with HCl, 1mM EDTA). The third method was a CTAB extraction procedure. The homogenised material in CTAB buffer (1% CTAB, 50mM Tris-HCl (pH 8.0),

Figure 1. An excised apical meristem from a wheat plant at the beginning of tillering used for DNA extraction

0.7mM NaCl, 10mM EDTA and 20mM 2-mercaptoethanol) was incubated at 60°C for 1 h, and then an equivalent volume of chloroform:isoamyl alcohol (24:1) was added and vortexed for 10 minutes. The aqueous phase was separated from cell debris by centrifugation for 10 min at 10 000 × g and carefully transferred to a new sterile tube. DNA was

precipitated in an equivalent volume of isopropanol overnight at –20°C. After centrifugation (10 min at 10 000 × g), the supernatant was discarded. The pellet was washed with 70% ethanol, centrifuged again, dried and resuspended in TE buffer. The quantity and purity of isolated DNA were measured using a spectrophotometer (Helios γ, ThermoSpectronic, Watertoun, USA). DNA purity was estimated from the A_{260}/A_{280} ratio, and DNA concentration was calculated by measuring the absorbance at 260 nm. For assaying primer specificity, DNA was isolated using a CTAB extraction method as described below from a healthy plant of wheat as a negative control and from other fungal species (*Alternaria alternata*, *Botrytis cinerea*, *Fusarium* spp., *Penicillium* spp., *Phoma* spp.) grown on potato dextrose agar or malt extract agar.

Primer design. Ten different primer pairs were designed using Beacon Designer 7 software to hybridise to a complementary template of 100–300 bp DNA sequence located in the IGS region of *Tilletia* spp. The primers were first analysed and evaluated for their specificity *in silico* by blast analysis using NCBI Blast features to exclude possible complementarity with sequences of other organisms in GenBank. Thereafter, they were assayed using conventional PCR and real-time qPCR.

PCR assay testing primer specificity. For developing qPCR, several steps were necessary. Initially, designed primer pairs were screened for their specificity to target DNA using conventional PCR. A gradient thermal cycler MJ Research PTC 200 (MJ Research, Waltham, USA) was used for optimising annealing temperature and magnesium chloride concentration. PCR reactions were carried out under thermal cycling parameters as follows: initial denaturation at 95°C for 5 min; then, 35 cycles of denaturation at 94°C for 1 min, primer annealing at 52–64°C for 30 s and primer extension at 72°C for 1 min; finally, final primer extension at 72°C for 4 minutes. The reaction was conducted in a 25-µl reaction mixture consisting of 2.5 µl 1× buffer for *Taq* polymerase (Fermentas, GmbH, St. Leon-Rot, Germany), 1.5–3.0 µl $MgCl_2$ (1.5–3.0mM), 0.25 µl dNTP (0.4µM of each nucleotide), 0.4 µl primer mix (0.4µM of each primer), 0.5 µl *Taq* polymerase (2.5 U) (Fermentas), 1.0 µl template DNA and ddH_2O up to 25 µl. PCR amplicons were electrophoresed on 1.0% agarose gel stained with ethidium bromide and visualised by UV light (260 nm).

Real-time PCR quantification. The selected forward and reverse target-specific primers from PCR were evaluated for optimising qPCR. A series of seven 10-fold serial dilutions in the range from 2 µg to 2 pg of a fungal DNA standard corresponding to 5 mg–5 ng of mycelium was prepared. To evaluate the correlation between the mycelium amount in the plant and quantified DNA, dilution series were prepared as a mixture of fungal DNA in the dilution range as above and a constant amount of pure wheat DNA extracted from 10 mg of wheat tissue. Finally, the assays were validated using naturally infested samples of wheat plants and then used for the quantification of mycelium in the selected wheat varieties. The reaction for SYBR Green was conducted in a 25-µl reaction mixture consisting of 12.5 µl Maxima^{TM} SYBR Green qPCR Master Mix, 0.3 µl primer mix (0.3µM each primers), 10.2 µl RNase-free water and 2.0 µl template DNA. The real-time qPCR amplifications were carried out on a Biorad OPTICON II under the following conditions: single steps of 50°C for 2 min and 95°C for 10 min; then, 45 cycles of 95°C for 15 s, 63°C for 30 s and 72°C for 30 seconds. All PCR reactions were performed in triplicate. At the end of each PCR run, melting curve analysis was performed for evaluating amplification specificity by heating to 95°C, cooling to 30°C, slowly heating to 95°C and reading every 1°C. The cycle threshold (Ct) values for each PCR reaction were automatically calculated and analysed by Opticon II software.

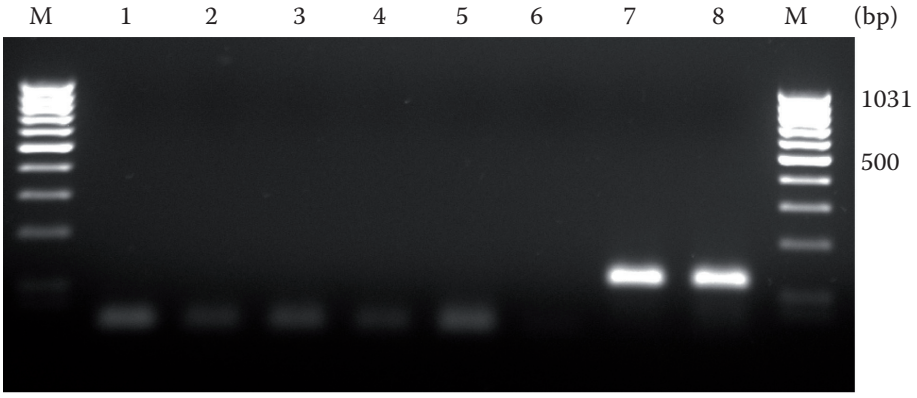
RESULTS

Extraction of DNA

The three procedures for DNA extraction from the fungal mycelium and the apical meristem were evaluated in this study. The best results with respect to DNA concentration and purity were obtained using the CTAB extraction method. This method gave the highest and most stable yield of DNA in good purity (A_{260}/A_{280} 1.8 ± 0.1) in repeated isolations of different amounts of plant tissue and fungal mycelium.

Conventional PCR with designed specific primers

For target organisms *T. caries* and *T. controversa*, ten PCR primer sets were designed based on IGS region sequences. The primer pair TillIGS2A_F 5'-TAGCGACCCGACCCGACCAG-3', TillIGS2A_R



Tilletia caries mycelium and teliospores are shown in lanes 7 and 8, respectively: lane 1: healthy wheat plant as a negative control; lane 2: *Fusarium* spp.; lane 3: *Alternaria alternata*; lane 4: *Botrytis cinerea*; lane 5: *Penicillium* spp.; lane 6: water as a negative control; lane M: MassRuler^{TM} DNA Ladder Low Range (Fermentas)

Figure 2. Agarose gel electrophoresis of the amplification products of target and non-target DNA using primers TillIGS2A_F and TillIGS2A_R

5'-CCCTCACGTTCCACGACGGG-3' provided only a single specific fragment of 122 bp just for both *Tilletia* species following upon optimisation of the PCR reaction at an annealing temperature at 63°C and using 3.0mM $MgCl_2$. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other species (Figure 2). The other nine sets of primers were excluded after optimisation and specificity testing, because they provided cross reactivity with non-target DNAs.

Real-time qPCR detection and melting curve analyses

To quantify both *Tilletia* species mycelium in growing tips, real-time qPCR using the intercalating SYBR Green dye and the primer set TillIGS2A_F and TillIGS2A_R was performed. This method was optimised for specific detection and amount estimation of the pathogen mycelium in the growing tip in the early stages of wheat plant development. The method of absolute quantification was used for comparing the amount of DNA of unknown samples with DNA standards. For generating of standard curves, seven 10-fold serial dilutions of fungal DNA and fungal-plant mixture DNA were amplified (Figure 3). DNA standard dilution sets were included in every run. The correlation between the Ct-value and the target DNA concentration was linear with a high coefficient of determination ($R^2 > 0.992$, $y = -3.573x + 24.08$). The efficiency of the PCR amplification was 1.905. Small, statistically insignificant differences between the replicated samples of both species and runs were noted without observation of any changes in threshold cycle values between the serial dilutions used. The detection limit for both *Tilletia* species was estimated at 100 fg of DNA, which corresponds to 0.22 ng mycelium (data not shown). Melting curve analysis was used to identify real-time qPCR products and to demonstrate the specificity of the amplification process. The maximum melting temperature was evaluated as 80°C, the point at which the amplicon

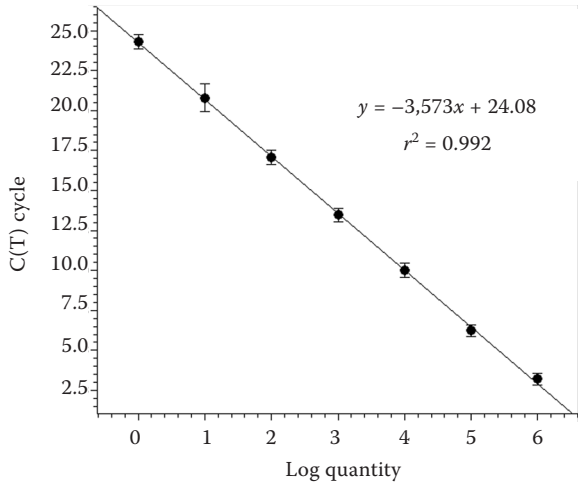


Figure 3. Standard curves used for the quantification of target *Tilletia caries* DNA in growing tips using real-time PCR Syber Green. Standard curves were obtained with amplification of a 10-fold dilution series of target DNA in the range from 2 µg to 2 pg of DNA

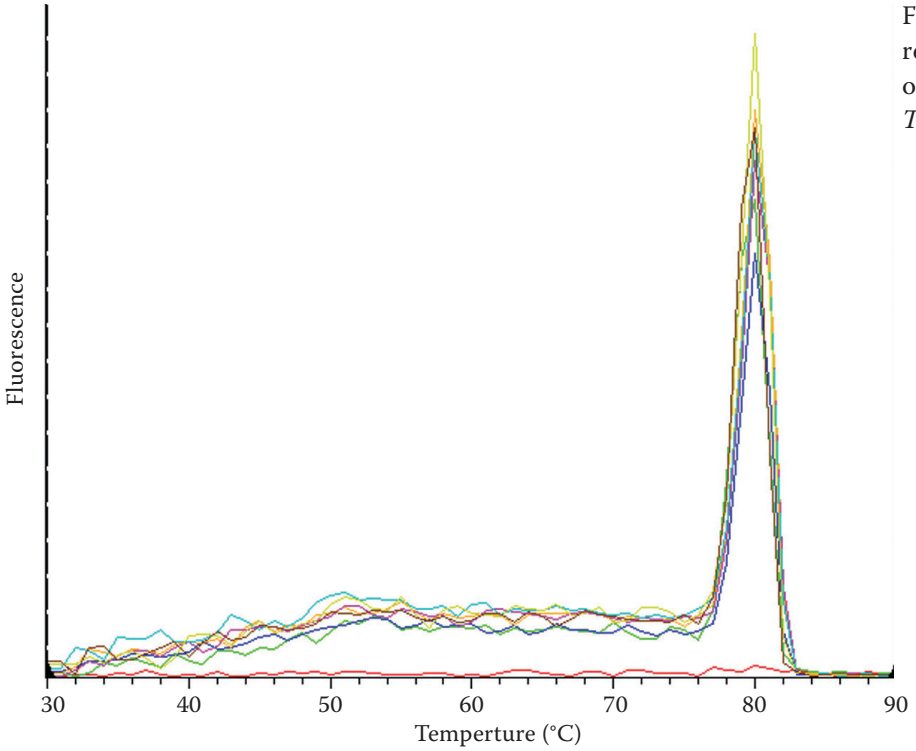
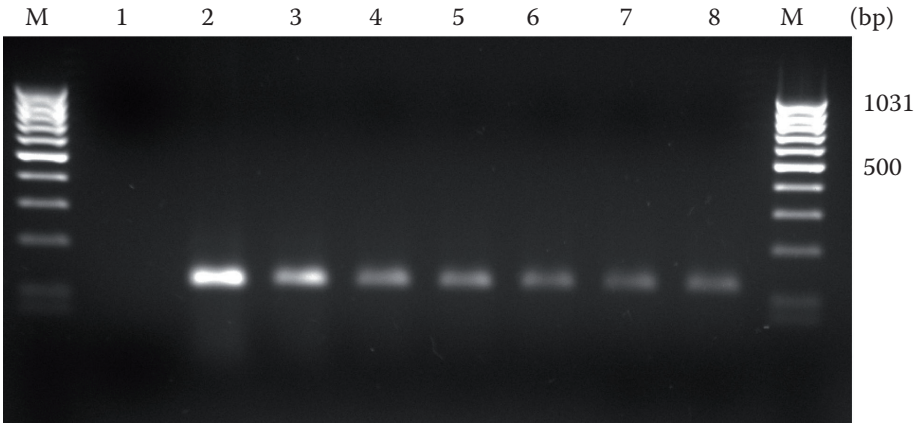


Figure 4. Melting curves (fluorescence versus temperature) of specific amplicons for both *Tilletia* species

melting profiles showed the presence of a single dissociation peak (Figure 4). Additional agarose gel electrophoresis was performed for verification of the melting curve analysis (Figure 5). The concentration of input template DNA (used as a weight standard) had no influence on melting temperature. Based on the generated standard curve, we found that one growing tip of the tested wheat varieties contained mycelium in the range from 0.34 ng to 15 µg. The optimised real-time qPCR assay for both pathogens was highly reproducible within sample replicates and among different samples as well. The obtained data shows that the amount of mycelium in growing tips varies both between wheat varieties and among samples of one variety (Figure 6).

DISCUSSION

In diagnostic methods based on polymorphisms of nucleic acid, the first basic step requires the suitable selection of an efficient and reliable extraction



Lane 1: water as a negative control (blank); lanes 2–8: 10^0 to 10^{-6} dilution of the DNA fungal standard; lane M: MassRuler^{TM} DNA Ladder Low Range (Fermentas)

Figure 5. Agarose gel electrophoresis of the real-time PCR products of serial 10-fold dilutions of a DNA fungal standard (*T. caries*) using primers TillIGS2A_F, TillIGS2A_R

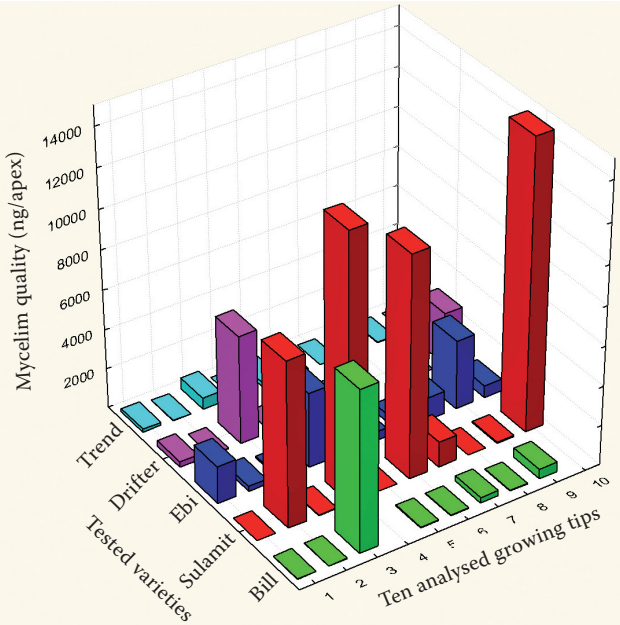


Figure 6. Graph of mycelium amount of *Tilletia caries* in ten growing tips in stage of the beginning of tillering (BBCH 21) from the five tested wheat varieties

procedure for target organism DNA to ensure the stable yield and purity of DNA subsequently used in amplification techniques. As Hauglanda *et al.* (1999) and Karakousis *et al.* (2006) presented, this study first searched for an optimal method for DNA extraction from both the mycelium and the very small amount of plant tissue in order to enable repeatability of the analysis. The procedure using the CTAB lysis buffer proved to be the method with the best results for isolating DNA from the apical meristem of wheat plants infected by *T. caries* and *T. controversa* and then using this DNA in a real-time qPCR technique. The designed primers were situated into a repetitive array of rDNA cistron to obtain a specific marker for the detection of both *Tilletia* species. The IGS sequences are conserved parts of a eukaryotic genome frequently used in the diagnosis of target organisms due to their stability in the process of species microevolution. Mogou *et al.* (2008) used intergenic spacer regions to study the microevolution of pathogens causing oak powdery mildew. Woodhall *et al.* (2007) characterised isolates of *Rhizoctonia solani* using primers amplifying the IGS region sequences as well. Using ribosomal DNA IGS regions in a pre-symptom PCR assay is suitable for the identification *Fusarium oxysporum* f.sp. *lycopersici* in infected tomato leaves (Balogun *et al.* 2008). Following its invention in 1983, PCR technology has been adapted for numerous molecular biology applications (VanGuilder *et al.* 2008). In plant pathology, this rapid and sensitive technique has been among the best available molecular tools for the detection and identification of various pathogens. However, comparative quantification of a pathogen in tissues upon detection and identification has remained a difficult task for many years. Based on pathogen quantification, it is possible to estimate the spread of inoculum, pathogen population dynamics, disease development and possible economic losses. Different molecular biology methods have been used previously to detect agents causing bunts. Josefsen and Christiansen (2002) used a technique based on a nested PCR amplification of *T. tritici* DNA in infected plant inflorescence tissue at the stage of internode elongation. Liu *et al.* (2009) developed a diagnostic molecular marker generated from an amplified fragment length polymorphism (AFLP) and sequence characterised amplified region (SCAR) marker for the rapid identification of *T. controversa*. In spite of the sensitivity, specificity and availability of these methods, quantification of the amount of pathogen is not possible using these methods. This study is the first example of the estimation of *T. caries* and *T. controversa* mycelium in a growing tip using absolute real-time PCR. A similar study on another pathogen was presented by Cullen *et al.* (2002). Real-time PCR employed for the detection of *Colletotrichum coccodes* on potato tubers and in soil was sensitive enough to detect three spores per g of soil (the equivalent of 0.06 microsclerotia per g of soil), and it detected target DNA down to fg levels. When it is impossible to identify a pathogen according to morphological characteristics or when

the amount of a pathogen in plant tissue is very small, using real-time PCR is quite advantageous. De Weerdt *et al.* (2006) presented a method for quantifying *Fusarium foetens* DNA in the range of 1 ng to 100 fg. Leisova *et al.* (2006) developed a real-time method for quantifying and monitoring the dynamics of the two forms of *Pyrenophora teres* during the barley growing season. Pre-symptomatic and accurate diagnosis is desirable for both the disease prediction and the timely application of fungicides. An example of this involves using real-time PCR for the detection of *Mycosphaerella graminicola* (Guo *et al.* 2006). McNeil *et al.* (2004) presented a model predicting that samples with 44 pg of DNA will be below one spore per seed with 95% probability. Although we tested this method, we have not succeeded in reaching the detection limit described; this is likely due to our use of a different biological material and different reaction amplification conditions. This detection limit could be insufficient for the quantification of fungal mycelium in a growing tip; therefore, this study aimed to increase the reaction sensitivity.

Additionally, further investigations should evaluate the capacity of the method developed for the detection and quantification of mycelium of causal agents of common and dwarf bunt to accurately detect the time of hyphal colonisation of an apical meristem during different wheat growth stages (especially early stages). Such future studies would be related to observations on the colonisation of wheat plants by a common bunt fungus (*T. tritici*) as well as to understanding the dynamics of compatible and incompatible interactions using fluorescent and confocal microscopy as presented by Gaudet *et al.* (2007).

Identification and quantification of bunts based on real-time PCR can be applied in the screening process for bunt resistance in wheat as well as in certification and breeding processes at early stages of plant development. It could facilitate the selection of potential resistance donors for breeding.

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