e-ISSN:2320-0189
p-ISSN:2347-2308

Research & Reviews: Journal of Botanical Sciences

Identification and Quantification of *Tilletia caries* and *T. controversa* in Seed Samples and Discrimination of the Two Species

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Research Article

Received date: 29/02/2016

Accepted date: 17/04/2016

Published date: 19/04/2016

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Keywords: Common bunt, Wheat dwarf bunt, *Tilletia caries, Tilletia controversa*, PCR, PCR-RFLP, Real-time PCR.

ABSTRACT

The stinking or common bunt, caused by *Tilletia caries*, and the wheat dwarf bunt, caused by *T. controversa* Kühn, are major seed born diseases in cereals and grassland species (Graminaceae). Contamination levels are routinely controlled by certification bodies or official control laboratories. We report several PCR assays which were developed for the identification and quantification of *Tilletia* spp. in contaminated wheat and spelt samples. A 140 bp segment of the ITS region is amplified with qualitative PCR for screening purposes or with real-time PCR for quantification. The method was validated successfully both in-house and in a proficiency test in comparison with other established methods. In addition a PCR-RFLP was developed for species discrimination. Using *HinfI* as restriction endonuclease the PRB2 sequence of *T. caries* is digested into three fragments whereas there are only two in *T. controversa*. In routine analyses and monitoring of seed lots or feed materials this PCR approach is a suitable alternative for the time-consuming and laborious traditional microscopic methods.

INTRODUCTION

Among the most important seedborn diseases in cereals and grassland species (Graminaceae) the stinking or common bunt, caused by *Tilletia caries*, and the wheat dwarf bunt, caused by *T. controversa*, played a considerable role in Central Europe in the last few years. Since 2011 there were several seasons in which the weather conditions led to strong appearances in seeds, cereals, and feed materials. Especially in organic farming this infestation is of high meaning as chemical plant protection agents are not allowed for application. Laboratory tests are essential activities to ensure that both spores do not spread through seeds and mycotoxins like trimethylamine do not endanger the health of consumers and animals. There are established methods in official control, e.g. a microscopic filtration method for the quantification of seeds, but they are laborious and time-consuming. In addition the species differentiation is sometimes disputable [1].

Several PCR methods for the characterization of the two Tilletia species were published, but they proofed to be not universal enough or not specific enough for our applications [2-4]. In previous studies the real-time PCR with Tc-F/Tc-R primers presented is applicable for wheat samples, but there is no calibration for spelt which exhibits a totally different correlation [2]. In addition we herewith report some nonspecific amplification of other fungal DNA. In contrast to this the TILf/TILr primers were highly specific in conventional PCR, but the size of the amplificate is too large for a good real-time PCR efficiency [3].

The aim of this study was to develop and to validate an efficient real-time PCR method for the quantification of T. *caries* and *T. controversa* in wheat (*Triticum aestivum*) and in spelt (*Triticum spelta*) in order to have a reliable alternative for the microscopic analyses of seeds and some feed materials. For this purpose the methods were in-house validated with naturally infected samples and they were subjected to a proficiency test. In addition we present a new specific RFLP-PCR which discriminates between the two *Tilletia* species.

e-ISSN:2320-0189
p-ISSN:2347-2308

MATERIALS AND METHODS

Sampling

From 2011-2015 the seeds were sampled by official seed samplers according to ISTA Rules (International Seed Testing Association) in the framework of seed certification.

DNA Extraction

Four initial weights of 5 g were used (this correlates with 4 × 50 spikelets) for spelt; for wheat, triticale or barley four replicates of 10 g were used (this correlates with 4 × 250 seeds).

The sample materials were filled into 50 ml tubes, suspended with 15 ml CTAB extraction buffer and incubated in a thermomixer at 60°C for 1 h. 1 ml of this extract was subsequently incubated with 20 µl Proteinase K (25 mg/ml) and 5 µl RNase (10 mg/ml) at 60°C for 1h. The extract was purified with chloroform, isolated with the Wizard Clean Up System (Promega), cleaned up with 80% isopropanol, and dissolved in elution buffer (pH 9).

PCR and Real-Time PCR Conditions

PCR primers Til122-F and Till262-R were designed to qualitatively amplify a 140 bp PCR product of the Internal Transcribed Spacer (ITS) region of Tilletia spp. **(Table 1).** Amplification control was carried out with a universal plant PCR previously described which generates an amplificate of the trnL intron of the chloroplast DNA [5]. Tilletia- and plant PCR reactions were performed in a final volume of 25 µl containing 12.5 µl HotStar Taq Polymerase Mastermix (Qiagen), 2 µl of each forward/reverse primer, 6.5 µl sterile water, and 2 µl extracted DNA. Amplification was performed with a PeqStar 2x Thermal Cycler (Peqlab) using the following PCR conditions: 95°C-15 min, (94°C-30 s, 60°C-40 s, 72°C-1 min) × 35 cycles, 72°C-10 min, 12°C~.

Table 1. Sequences of primers used in this study.

Primer	Sequence 5´- 3´	Reference
Til122-F	ACC CAT TGT CTT CGG ACT TG	This work
Till262-R	GGT GCG TTC AAA GAT TCG AT	
Til175-P	FAM-CTT GGT TCT CCC ATC GAT GAA GA-TAMRA	
Tc-F	TTG GGA TTG GCG TAT TTG C	McNeil, et al. [2]
Tc-R	ATG CCA CAT TTC TCC TAC TAT TAT CCA	
RPB2-740F	GAT GGA CGC GGT TTG TAA TG	Carris, et al. [6]
RPB2-1365R	TCG AAG AGC YAA CAC TGA GAC G	
Plant1 (c)	CGA AAT CGG TAG ACG CTA CG	Taberlet, et al. [5]
Plant2 (d)	GGG GAT AGA GGG ACT TGA AC	

Positive samples were quantified with real-time PCR using TaqMan chemistry. Assays were performed in a final volume of 20 µl containing LightCycler 480 Master (Roche, 10 µl), 20 pmol primer Til122-F (0.5 µl), 20 pmol primer Till262-R (0.5 µl), 10 pmol TaqMan probe (Til175-P, 0.3 µl), sterile water (3.7 µl), and DNA (5 µl). Amplification was performed in a LightCycler 480 (Roche) according to the following program: 95°C-10 min, (94°C-5 s, 60°C-15 s) × 55 cycles, 30°C-30 s~.

For calibration purposes the DNA of a strongly infected wheat sample was extracted and diluted in 7 steps each with a ratio of 1:5 **(Table 2).** Each dilution step correlated with a quantity of spores. Wheat and spelt samples were separated because of their different morphology. Two calibrator samples **(Table 3)** with different contamination levels were used to verify the calibration system.

Table 2. Correlation of DNA dilution and number of spores.

No	DNA dilution	Number of spores per kernel (wheat)	Number of spores per spikelet (spelt)
1	Undiluted	5,000	50,000
2	1:5	1,000	10,000
3	1:25	200	2,000
4	1:125	400	400
5	1:625	80	80
6	1:3,125	1.6	16
7	1:15,625	0.32	3.2
8	1:78,125	-	0.64

e-ISSN:2320-0189
p-ISSN:2347-2308

Table 3. Method performance data of the two methods. Ct, threshold cycle; SD, standard deviation; VR%, coefficient of variation of reproducibility.

		Real-time PCR				Microscopic method	
Calibrator sample	**Ct value**	**spores**	**mean**	**SD**	**VR%**	**spores**	**mean**
ValP2	29.07	88.4	88	7	8		91
	28.99	94.1				97.0	
	29.31	74.8					
	29.07	88.8				95.0	
	29.15	83.8					
	29.08	87.8				90.1	
	29.06	89.1					
	28.93	98.3				83.2	
ValP3	31.90	11.7	12	2	20		15
	31.72	13.3				18.4	
	32.03	10.6					
	32.64	6.9				13.2	
	31.65	13.9					
	31.65	13.9				12.1	
	31.64	14.0					
	31.72	13.3				15.8	

Each analysis was performed with an alternate standard concentration and a calibrator sample for positive control as well as a blank and a negative control. The quantity of spores was calculated with the help of the calibration system. The results of the replicates were used to determine mean values, standard deviation, and coefficient of variation.

In seeds the results were presented in spores/kernel and spores/spikelet respectively. In feed materials the ratio of spores were declared in %; 0.1% spores correspond to 10,000 spores/kernel.

PCR-RFLP for Species Discrimination

The two species were discriminated by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The PCR reactions were carried out in 25 µl containing 5 pmol of each primer RPB2-740F/RPB2-1365R [6], 2 µl extracted DNA, 12.5 µ HotStar Taq Polymerase Mastermix (Qiagen), 6.5 µl sterile water. Amplification was performed with a Touchdown (Td) PCR in a PeqStar 2x Thermal Cycler (Peqlab) using the following PCR conditions: 95°C for 15 min, (94°C-30 s, 56°C-50°C (Td-0.5°C/cycle)-40 s, 72°C-1 min) × 12 cycles, (94°C-30 s, 50°C-40 s, 72°C-1 min) × 28 cycles, 72°C-10 min, 12°C~.

RFLP fragments were produced in a 20 µl reaction mixture containing 2 µl HinfI restriction endonuclease (New England Biolabs), 2 µl buffer (New England Biolabs), 6 µl sterile water, and 10 µl RPB2 PCR product which was incubated at 37 °C for 3 h.

PCR products and RFLP fragments were visualized using ethidium bromide staining and 2.5% agarose gel electrophoresis.

Proficiency Test

In 2015 a proficiency test on the detection and quantification of Tilletia spp. was performed with seven European labs which were free to use analyses method(s) of their choice including the microscopic method (7 times), the PCR method (twice), and a different house method (once). Five naturally infected samples of wheat and spelt plus a negative control were analyzed and evaluated according to DIN38402-A45 with the statistical software ProLab (Quo Data GmbH). In short: This application automatically calculates weighted mean value, standard deviation, reproducibility, limits of tolerances, outliers, and Zu-scores for standardizing. Zu-scores > ±2 were judged as outliers. Participating labs were anonymized with code numbers. There were two objectives to perform the proficiency test: Firstly to validate the real-time PCR, secondly to validate the so-called “microscopic method” (data not yet published) which is based on the ISTA method [1].

RESULTS

Two different PCR approaches were implemented to analyze *Tilletia caries* and T. *controversa* in wheat and spelt samples. The amplification of a segment of the ITS region was suitable for identification and quantification of *Tilletia* spp. whereas a PCR-RFLP method which produces fragments of the RPB2 gene was adequate to discriminate the two species.

PCR-RFLP for the Discrimination of *T. caries* and *T. controversa*

The differentiation between *T. caries* and *T. controversa* was obtained with PCR-RFLP. Using RPB2-740F and RPB2-1365R [6] as primers **(Table 1)** a 630 bp amplificate of the RPB2 gene was generated from the DNA of the two species **(Figure 1).** *T. barclayana* and *T. sumatii* DNA was not amplified.

In silico sequence analyses of several database entries (EU257596.1, EU257597.1, EU257598.1) showed that the restriction endonuclease *HinfI* was suitable to take advantage of the sequence differences between the two species: There are two *HinfI* restriction sites in the PCR product of *T. caries* whereas there is only one in T. *controversa*. In fact we could show that three fragments of 170 bp, 210 bp, and 250 bp were formed in case of T. *caries* whereas *T. controversa* only showed two fragments of 210 bp and 420 bp **(Figure 2).**

e-ISSN:2320-0189
p-ISSN:2347-2308

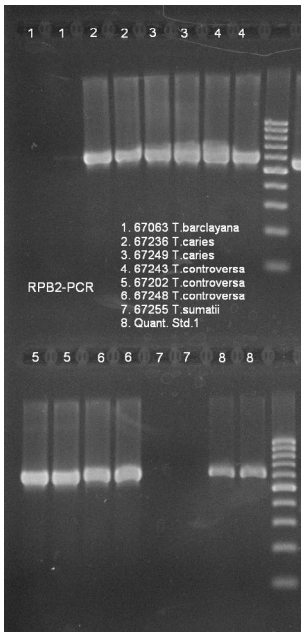

Figure 1. Electrophoresis of RPB2 PCR products of different Tilletia species. Molecular weight marker: 100 bp to 1,000 bp in intervals of 100 bp.

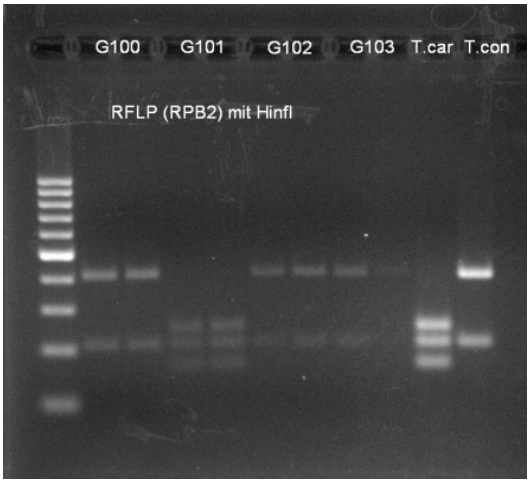

Figure 2. PCR-RFLP of *T. caries* (T. *car*), *T. controversa* (T. *con*), seed samples G100-G103.

To avoid false negative results and to ensure specificity of the PCR reactions several fungi which appear in cereals and grassland species were tested as well. Apart from *T. caries* and *T. controversa* there were some other species which showed amplification products after PCR with RPB2-740F/RPB2-1365R. But these signals were either very weak (*F. langsethiae*) or they were non-specific, i.e. of abnormal size **(Table 4).** In routine analyses such findings would be judged as negative. Other *Tilletia* species were not tested, because they are not common in central European agriculture.

Table 4. Specificity of three qualitative Tilletia PCR reactions. Amplification product -, absent; +, present; (+), weak; (*), non-specific; n.d., not determined.

No.	Fungal species	source	Til122-F/ Till262-R amplificate	RPB2-740F/ RPB2-1365R amplificate	Tc-F/ Tc-R amplificate
1	*Fusarium graminearum*	JKI[1]	-	-	-
2	*Fusarium culmorum*	JKI	-	-	-
3	*Fusarium langsethiae*	JKI	-	(+)	-
4	*Fusarium oxysporum*	JKI	-	-	-
5	*Fusarium proliferatum*	JKI	-	(*)	-
6	*Fusarium subglutinans*	JKI	-	-	+
7	*Fusarium tricinctum*	JKI	-	-	-
8	*Fusarium verticillioides*	JKI	-	-	-
9	*Fusarium sporotrichioides*	CBS[2] 115701	-	-	-
10	*Fusarium poae*	CBS 186.96	-	(*)	-
11	*Fusarium avenaceum*	CBS 408.86	-	(*)	(*)
12	*Fusarium equiseti*	CBS 119663	-	-	(*)
13	*Alternaria dauci*	DSM[3] 62017	-	-	+
14	*Alternaria linicola*	LTZ[4]	-	-	n.d.
15	*Alternaria radicina*	DSM 62029	-	-	-
16	*Colletotrichum lini*	CBS 172.51	-	-	(*)
17	*Colletotrichum graminicola*	DSM 63127	-	(*)	(*)
18	*Microdochium nivale*	DSM 62281	-	-	+
19	*Botrytis cinerea*	CBS 131.28	-	-	n.d.
20	*Phoma exigua*	DSM 63389	-	-	(*)
21	*Drechslera graminea*	DSM 63589	-	(*)	(*)
22	*Tilletia caries*	DSM 4526	+	+	+
23	*Tilletia controversa*	CBS 121952	+	+	+
24	*Tilletia sumatii*	JKI	+	-	n.d.
25	*Tilletia barclayana*	JKI	+	-	n.d.

[1]Julius Kühn Institut, Messeweg 11/12, 38104 Braunschweig, Germany. [2]Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands. [3]Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstr. 7B, 38124 Braunschweig, Germany. [4]Center for Agricultural Technology Augustenberg, Nesslerstr. 25, 76227 Karlsruhe, Germany.

The limits of detection (LOD) were determined by serial dilutions of the DNA extract to be detected by gel electrophoresis **(Figure 3).** For validation low infected samples which were also analyzed with the microscopic method were tested as well. Thus the LOD of the RPB2-740F/RPB2-1365R PCR was fixed at 40 spores/seed or spikelet.

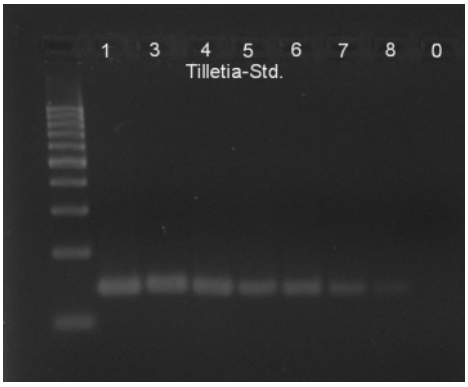

Figure 3. Electrophoresis of a dilution series of the Til122-F/Till262-R PCR product. 1, 50,000 spores; 2, 10,000 spores; 3, 2,000 spores; 4, 400 spores; 5, 80 spores; 6, 16 spores; 7, 3.2 spores; 8, 0.64 spores; 0, sterile water.

Quantification of Tilletia spores

A real-time PCR assay was developed for the quantification of Tilletia spores (see material and methods). The method was in-house validated with seed samples which were also tested with the microscopic method. In 2012 about 80 samples of winter wheat, spring wheat, spelt, and triticale were compared for method development [7]. In the following years about 10% of the routine samples were simultaneously analyzed with both methods. The review of 2015 shows a good correlation of the results of the two assays **(Figure 4).** In this period 10 samples of spelt and 13 samples of wheat were analyzed.

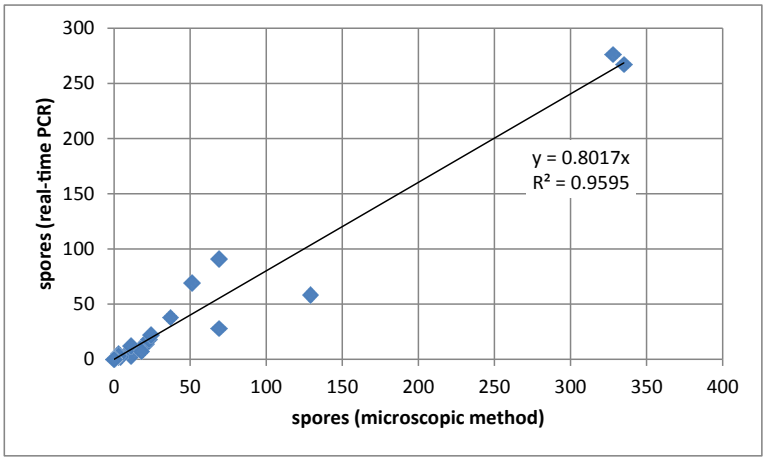

Figure 4. In-house validation with 23 routine samples in 2015.

Table 3 shows the reproducibility of the results of two spelt samples with different contamination levels. The standard deviations and the variation of reproducibility of eight replicates were satisfying. These two samples were also used as calibrator samples.

Amplification with Til122-F/Till262-R was specific with all four tested Tilletia species whereas other fungal DNA was not amplified **(Table 4).** The limit of quantitation (LOQ) of the Til122-F/Till262-R PCR was found to be 1 spore/kernel or spikelet.

Highly contaminated feed matrices (>10,000 spores/kernel) were also analyzed successfully, but there are too little data available yet for publication.

Finally we want to report some nonspecific amplification when using the Tc-F/Tc-R primers **(Table 4).**

Proficiency Test

In 2015 the Center for Agricultural Technology Augustenberg organized a proficiency test in order to validate the real-time PCR method of this publication. Naturally infected seed samples of wheat and spelt were analyzed with the microscopic method, with certain house methods and/or with this real-time PCR. The samples represented contamination levels relevant within the most interesting range for the official seed testing which is 20 spores/seed.

The outcome of the proficiency test is summarized in **Table 5** and **Figure 5**. Seven labs performed their analyses with microscopy. Three of them additionally performed real-time PCR. Among them one lab applied the real-time PCR according to McNeil et al which is only employable for wheat samples [2]. As a consequence they were not able to quantify Tilletia in spelt.

e-ISSN:2320-0189
p-ISSN:2347-2308

Furthermore there was one lab which wanted to check its microscopic house method. There even were some labs (#2 and #3) which reported the results of different lab assistants (data not shown). In this case the average results were taken for evaluation.

Table 5. Key data of the Tilletia proficiency test 2015 generated by ProLab software. Participating labs are presented as plain numbers supplemented with P, real-time-PCR; H, house method; non-supplemented numbers represent microscopic results. The results are presented in spores/kernel and spores/spikelet. E, outlier which strongly deviates from the common mean of all labs.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Lab code	**spelt**	**spelt**	**spelt**	**spelt**	**wheat**
1	8.6	7.5	14.4	4.9	16
1P	–	–	–	–	10
2	7.7	6.9	10.4	6.2	19.2
2P	12	14	24	10	
3	7.2	4	10.1	2.4	17.6
3H	7.2	4.1	10.3	1.6	22.8
4	18	13	39	16	20
5	22	14	21	8	26
7	41 (E)	21	11	37 (E)	26
8	14	10	28	5	28
8P	8	13	24	9	23
weighted mean	11.8	10.6	18.4	7.0	21.0
S_R [1]	6.8	5.9	8.4	6.6	6.5
V_R [2]	0.58	0.56	0.45	0.94	0.31
lower limit	2.3	2.1	5.5	1.0	9.5
upper limit	29.8	26.3	40.2	24.8	36.6
n=	10	10	10	10	10

[1]standard deviation of reproducibility. [2]coefficient of variation of reproducibility.

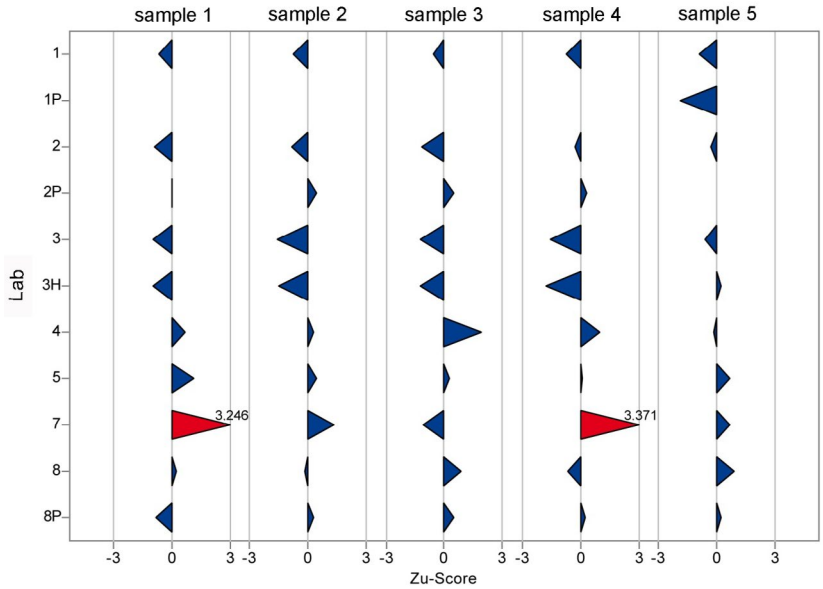

Figure 5. Zu-scores of the Tilletia proficiency test generated by ProLab software.

Considering the huge measurement uncertainty microbiological subjects normally have this proficiency test provided pleasant findings. The distribution of the results showed that all different methods produced comparable results. Especially the real-time PCR fits very well to the established microscopic method. Only one microscopic lab produced two outliers of the category E, i.e. the result related to the common mean of all labs was beyond the limits of tolerance.

In addition there was a wheat sample which served as negative control. This was identified correctly. Only one lab detected traces <0.2 spores/seed.

DISCUSSION

Amongst other questions Tilletia analyses in seeds and feeding stuffs are performed on behalf of certification bodies or official control laboratories. These boards of control are bound to use standardized and reliable methods such as established microscopic methods, e. g. the ISTA working sheet method for identification and quantification [1] which consists of a washing procedure, membrane filtration, and the enumeration of spores by microscopy. For many years this has been a well-proven practice, but there are efforts to simplify parts of the procedure (unpublished data). For that matter the presented proficiency test also served several laboratories which used an advanced version of the microscopic method.

We think that even this advanced method is too laborious. Our validation data (in-house validation and proficiency test) show that real-time PCR is a good alternative with many advantages. First of all there is no washing procedure and no filtration. The isolation of the DNA is performed directly out of the sample. In addition more samples can be analyzed simultaneously and the efficiency of the lab is increased. The proficiency test revealed that other labs were able to introduce this real-time PCR rapidly and successfully.

The variance of the results of the real-time approach is very similar to the microscopic method and its magnitude is accepted in biological systems. There is no possibility to improve accuracy any further because this is not a drawback of the method but a problem of the non-uniform dispersion of the analyte in the matrix. The occurrence of bunt balls intensifies this problem and it is sometimes a challenge to get suitable samples which have to be representative for tonnages of seed lots.

Our study shows that the Til122-F/Till262-R real-time PCR is more specific than the Tc-F/Tc-R PCR approach. In addition it is applicable not only for wheat samples but also for spelt.

Our daily lab experience in Tilletia analyses shows that the following strategy is suitable for routine laboratories: At the beginning the qualitative ITS PCR can be used as a screening method. Thus negative samples can be reported very quickly without any further efforts. In contrast contaminated samples are subsequently verified and quantified by real-time PCR. If species discrimination is necessary the RPB2 PCR can be added. Unfortunately the LOD of this PCR-RFLP method is very high. Samples containing <40 spores/seed should be pretreated with a concentrating washing procedure.

The methods presented here are suitable for upgrading if it will be necessary to analyze other Tilletia species or other sample matrices. Especially feed materials should be validated in a greater extend because this is very important for animal health and consumer protection.

ACKNOWLEDGEMENT

Thanks to Sabine Grimm (Center for Agricultural Technology Augustenberg) for statistical evaluation of the proficiency test.

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