Development of Immunochemical and PCR Methods for Qualitative Detection of *Tilletia* Species in Organic Seeds

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Abstract: A rapid and sensitive detection of *Tilletia* species in contaminated cereal grains could enhance the quality of organic seed production and would also improve quarantine regulations. The final aim is to develop methods based both on molecular and immunological techniques for determination and differentiation of fungal species causing smut diseases mainly on seeds produced under conditions of organic farming. First results demonstrate that it is possible to verify an infection with *Tilletia caries* in freshly harvested grains in less than 5 h by Western blotting and in less than 3 h by PCR. The specific primer-pair developed for *T. caries* does not show any significant reactions with *T. controversa* or other seed-borne fungus pathogens like fusarium. In Western Blots using a polyclonal antiserum against *T. caries* spores, a specific reaction in form of a single band was observed when total spore-protein extracts were analyzed in the gel. In addition, there was no cross reaction to the protein extract prepared from *T. controversa* spores. Further methods for the specific detection of *T. controversa* and *T. indica* by PCR, ELISA or Western blotting are in development.

Keywords: seed-borne fungi; *Tilletia* species; Western Blot; PCR; polyclonal antibodies

Wheat is one of the most important staple crops of the world. The production of wheat is often influenced by diseases like rust, bunts and smuts which results in poor grain quality and reduced crop yield. Among these pathogens the seed-borne *Tilletia* species are one of the most dangerous pathogens in Europe and the world. Particularly *T. indica* (which does not occur in Europe until now) causes a major concern for quarantine regulations, thus emphasizing the need for adequate methods of detection this dangerous fungus.

In this research project, two methods were developed, to detect and differentiate between the most important and most harmful seed-borne pathogens in wheat: stinking smut of wheat (*T. caries*), dwarf bunt of wheat (*T. controversa*) and the quarantine organism Karnal Bunt (*T. indica*).

For the detection of unique sequences in the genome and in the proteome of the pathogens, specific primer pairs in the PCR and appropriate polyclonal antibodies in Western Blot methods are used. In each case the HSP60 gene, which codes for a chaperon protein and occurs ubiquitously in all of this seed-borne fungi, contain this unique sequence. In addition, this gene offers a sufficient variability to be able to distinguish between individual *Tilletia* species.

MATERIAL AND METHODS

Collection of Tilletia-infected seeds. Infected wheat grains were obtained from: T. Raabe, Bavarian State Research Center for Agriculture, Freising (*T. controversa*); Prof. Spiess, Federal Biological Research Centre, Darmstadt (*T. caries*).

Spores were collected by sieving the spikes in sieves with reducing diameters (large scale) or by pooling the spores manually (small scale).

Isolation of DNA. DNA of spores was isolated by the Qiagen Plant Isolation Kit with an additional

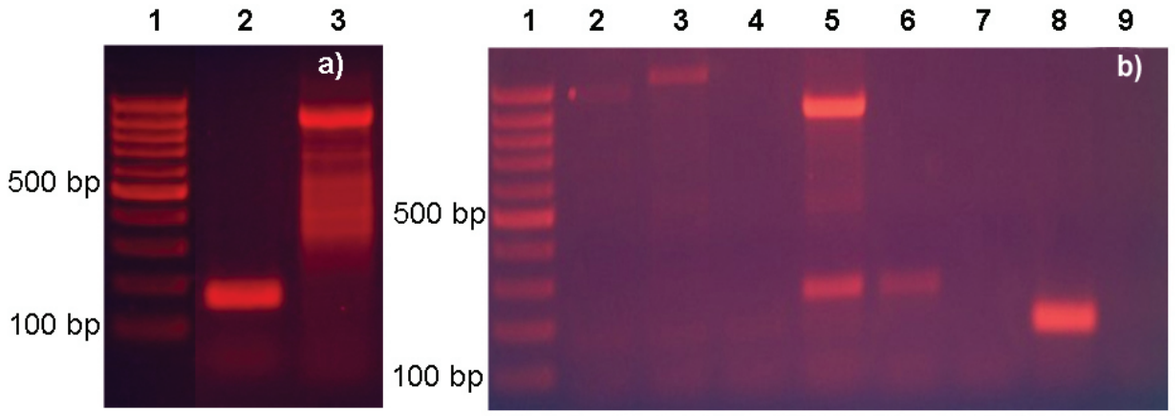

Figure 1. Detection of PCR fragments on 1.5% agarose gels

a) lanes 1–3: 100 bp marker (1); 155 bp PCR product from *T. caries* yielded by specific *T. caries* primers (2); PCR products from *T. controversa* yielded with specific *T. caries* primers (3)
b) lane 1: 100 bp marker, lanes 2–9 show the different fungal PCR products by the specific *T. caries* primer pair: *Fusarium poae* (2); *Fusarium graminearum* (3); *Fusarium culmorum* (4); *Microdochium nivale* (5); *Aspergillus fumingatus* (6); *Penicillium gladicola* (7); *Alternaria alternata* (8); *Cladosporium* ER 21 (9). None of these pathogens show the characteristic 155 bp band

crunching step using glass beads at the beginning due to their thick wall (Gang & Weber 1995). Alternatively isolation was done by microwave treatment of moistened spores for 5 min. and TE buffer (10mM Tris, 1mM EDTA, pH 7.6) was used for DNA extraction. After centrifugation the supernatant was used for further analyses (Ferreira & Glass 1996).

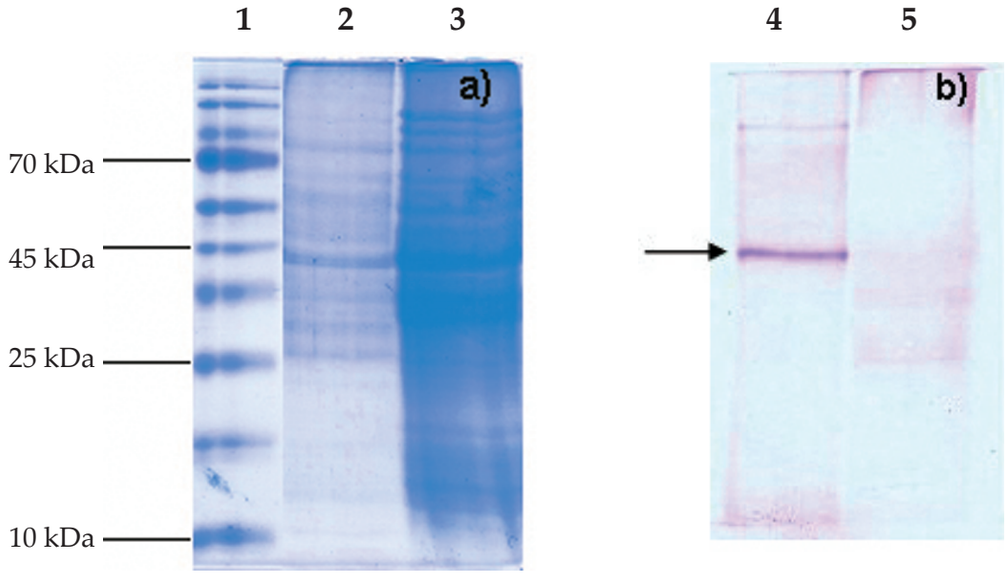

Figure 2. Detection of a specific antigen by Western Blot (marked by an arrow) after SDS-PAGE (12%)

a) SDS-PAGE; lanes 1–3: prestained molecular weight marker (1) and the total protein extract from *T. caries* (2) and *T. controversa* (3)
b) corresponding Western Blot; lanes 4 and 5: the polyclonal antibody against *T. caries* spores detects only its appropriate antigen (4) and does not show any cross reaction with *T. controversa* (5)

PCR conditions. Annealing temperature: 49°C; Elongation temperature: 72°C; 30 cycles.

Protein extraction. Spores and glass beads (1:1) were vortexed in extraction buffer (6M Urea, 2M Thiourea, 4% CHAPS, 65mM DTT, pH 8.0) and sonicated 3 times at 50°C for 1 min. Sonication was interrupted by freezing. After centrifugation the supernatant was used for further analyses (van Etten & Freer 1978; Sulc *et al.* 2005).

Generation of anti-spore antibodies. Antibodies were generated using standard protocols by Dr. F. Rabenstein, Federal Centre for Breeding Research on Cultivated Plants, Aschersleben.

Western Blot conditions. A standard protocol for running a Western Blot was used by blotting for 1 h with 50 mA per gel. The first antibody was incubated for 1 h in a PBS 0.1% Tween buffer solution (1:5000 dilution). Final detection was carried out by alkaline phosphatase (AP) conjugated α-Rabbit antibody (1:2000 dilution) in a PBS 0.1% TWEEN buffer solution.

RESULTS AND DISCUSSION

PCR Method. At first we determined the *Tilletia caries* HSP60 sequence, which was the only one not published in the ncbi sequence database (www.ncbi.nih.gov). This was performed by using a primer pair for *Tilletia controversa* sequence at a low annealing temperature. With the assumption of a high degree of homology between the two species, a PCR was performed and the PCR product was sequenced. The sequence obtained was compared with the *T. controversa* sequence from the database and appropriate sequence ranges were identified, which can be utilized for distinction. By this way, a new primer pair specific for *T. caries* was designed for the differentiation in a PCR. Using these primer pairs on the specific genomic sequence should yield a 155 bp PCR fragment. The distinction takes place via the band recorded in agarose gel electrophoresis (Figure 1a). A false primer-template-combination does not result in the 155 bp product or has non-specific PCR products. The primer pair for *T. caries* was successfully tested and can be applied for further investigations. In addition, the 155 bp PCR fragment is not obtained when other common seed-borne fungi are tested (Figure 1b).

Specific primer pairs for *Tilletia controversa* and *Tilletia indica* will be tested soon.

Western Blot method. Polyclonal antibodies were produced using *Tilletia caries* and *Tilletia controversa* spore suspension as immunogen. The purified antibodies could be used for Western Blot analyses in a 1:5000 dilution. Detection was carried out with an alkaline phosphatase conjugated second antibody (α-Rabbit IgG AP-conjugate) in a 1:2000 dilution (Figure 2).

This method offers a specific detection for *T. caries* and demonstrates the opportunity for monoclonal antibodies in future commercial applications by using the detected band as antigen.

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