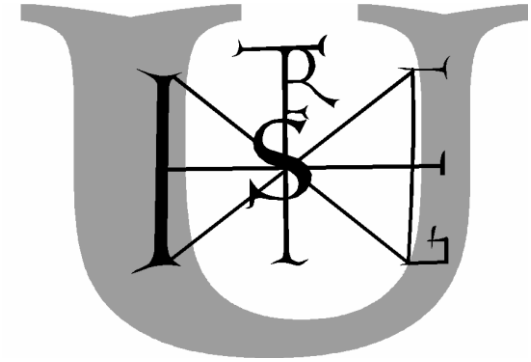

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THE DIAGNOSTICS AND SURVEY OF *TILLETIA* SPECIES ON WINTER WHEAT IN HUNGARY

PhD thesis

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1. BACKGROUND AND OBJECTIVES

An economically important crop, winter wheat (*Triticum aestivum*) is a staple food in 40% of the world as it is rich in nutrition. Only rice and corn have the same significance. With 245–250 million hectares wheat is one of the most valuable crops and has one of the biggest acreages in the world (BORSOS et al. 1995). This crop is widespread because of the broad climatic tolerance and good adaptability of the wheat species and varieties. In Hungary the acreage of wheat has been about 1.1 million hectares since the beginning of the 2000s, and the yield is 3–5 t/ha (ÁGOSTON 2009).

Winter wheat is our most important bread-crop, as well as an important source of living, so its economic and ecological protection is a major task. Wheat production is influenced by various diseases, such as rusts and smuts, affecting the quality and quantity of the crop. Seedling infecting smuts are by far the most dangerous wheat diseases (KELLERER et al. 2006).

Grain smuts are historically widely known species (SZEPESSY 1977). Mitterpacher differentiated common bunt and loose smut in 1777. Linhardt described 31 different smuts in 'Fungi Hungarica' (HORTVÁTH 1995). Smuts were one of the most damaging diseases until the introduction of intensive crop production (TÓTH 2006). Nowadays common bunt has a significance in places where seed treatment is either abandoned or not professionally applied.

In Hungary two wheat infecting smuts are established: *Tilletia caries* (DC.) Tul. and *T. leavis* J. G. Kühn. *T. contraversa* J. Kühn, the economically important wheat smut has not yet been detected in Hungary according to literature data. We have to take into consideration the regulated pest, *T. indica* Mitra, which has not been detected in Europe.

It is known that smuts nowadays have been responsible for less economic losses than fifty years ago due to innovations implemented in agriculture, particularly in cultural methods and plant protection. Recently, however, a slight decline could be observed compared to the 1970s and 1980s, because wheat production, like agriculture as a whole, fell out of authorised control (for example the size of the cultivated fields reduced, large estates fell apart, seed treatments diminished, etc.). Consequently, from the middle of the 1990s an increasing ratio of common bunt infections could be observed.

Several countries refuse the import of wheat if it is infected with *Tilletia* species. Local phytosanitary regulations are also in effect regarding *T. indica* and *T. contraversa* as part of a strategy to first hinder the disease from establishing in the country or to eradicate it and, more recently, to prohibit its spread. Because of the possible export restrictions, Hungary has to make annual surveys to prove that the country is free from *T. contraversa*. Surveys are carried out by

the Plant Health and Molecular Biology Laboratory (PHMBL), Directorate of Plant Protection, Soil Conservation and Agri-environment, National Food Chain Safety Office.

Considerable overlaps exist in the characteristics of the teliospores of *T. caries* and *T. contraversa* making identification difficult (MURRAY et al. 1998). Criteria presently used to distinguish between the two species include teliospore wall morphology (light and fluorescent microscopy) and the optimum temperatures for teliospore germination should be used simultaneously to achieve a reliable result. These techniques are time-consuming, labour-intensive and require a practiced, professional eye. That was the reason why we decided to develop or adopt one or more DNA or protein based molecular methods for making the diagnostic work faster and more reliable in the PHMBL.

Although some aerobiological studies have shown the presence of *Tilletia* teliospores in the atmosphere of ecologically diverse locations (TSE et al. 1980; SAVINO and CARETTA 1992; CROTZER and LEVETIN 1996), their aerobiology in grain warehouses has not been studied. Due to the economic importance of *Tilletia* species it is necessary to monitor the spread of the teliospores in the air of the warehouses, because of the hazard of cross-contamination and the introduction of quarantine regulations, furthermore to help the development of safe grain-handling technologies.

Our aims were:

1. To survey *Tilletia* species in Hungary in wheat grain after harvest between 2007 and 2012. To characterise *T. contraversa* morphologically and to find out the possible reasons for its recurrence in 2011.

2. To survey the aerobiological characteristics of grain warehouses with portable Hirst type volumetric impaction sampler: to analyse the air of warehouses in connection with plant and human health issues focusing on the teliospore content and dispersal.

3. To test and adopt appropriate DNA based molecular methods to differentiate *T. caries* and *T. contraversa*.

4. To develop an accurate method for distinguishing *T. caries* and *T. contraversa* with protein-based molecular techniques, to work out a reliable, fast and reproducible electrophoretic method for laboratory use.

2. MATERIALS AND METHODS

2.1. Collecting samples

Winter wheat samples were collected with by the plant-health inspectors in the framework of the annual survey for *Tilletia* species. During the sampling procedure at least 2×50 grams of wheat grain per sample were collected (from combine harvester), altogether 133, 94, 105, 103, 90 and 98 samples in the years 2007-2012, resp. (One sample consists of wheat harvested on a particular field.) The required numbers of samples were five per county. The samples were stored in a cool, dry place.

The air samples were taken with a portable Hirst type volumetric impaction sampler (Hirst type sampler) in two central grain warehouses in Budapest (Bp) and Jászapáti (Ja), and in 14 farm warehouses belonging to 10 companies. We also collected wheat samples in each sampling area, and dust samples were taken, too, from the dust deposited on the horizontal surfaces of the warehouses.

Two isolates of *T. contraversa* (TILLCO A and TILLCO B) are from Poland, and one is from Hungary (TILLCO C). Heads of *T. contraversa* infected wheat were provided by Grazyna Szkuta (Central Laboratory, Plant Protection and Seed Inspection Service (PPSIS), Torun, Poland) and Ágnes Révay (Botanical Department, Hungarian National History Museum, Budapest, Hungary). Wheat samples infected with *T. caries* were collected from different parts of Hungary from 2008 to 2009 by the plant health inspectors and András Varga mycologist. Five Hungarian isolates of *T. caries* were involved in the study (TILLCAR 1-5.)

2.2. Microscopic examination

To detect the *Tilletia* species in wheat samples we performed the washing test, and to determine the exact spore morphology x1000 magnification was used in a light microscope with an oil immersion lens (USDA 1974, OEPP/EPPO 1982, 2007). Teliospores were identified according to Vánky (2012).

The fluorescence test was performed according to the modified version of the method by STOCKWELL and TRIONE (1986). Autofluorescence was observed with an epifluorescence microscope (Leica DM LB2, Leica Microsystems GmbH, Wetzlar, Germany) with a 50 W mercury lamp and equipped with the Leica filter I3 (450-490 nm excitation and 515 nm barrier filter).

Samples collected with Hirst type sampler: Direct microscopic examination was performed at x800 in an Olympus BX51 light microscope and x1000 in a Leica DM LB2 light microscope with an oil immersion lens. Teliospores were identified according to Vánky (2012).

2.3. DNA based molecular methods for distinguishing *Tilletia contraversa* and *T. caries*

DNA extraction from teliospores

DNA extraction from *Tilletia* teliospores was done using the DNEasy Plant Mini Kit according to user manual with slight modifications.

Tilletia genus specific PCR method

Polymerase chain reaction (PCR) was performed in a BioRad iCycler Thermal Cycler according to KOCHANOVA et al. (2004) applying the TILf, TILr primer pairs specific for the genus *Tilletia*. The following PCR programme was used: 95 ^{o}C for 5 min, 50 cycles each at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and an extension step at 72°C for 4 min. Amplified PCR products were subjected to electrophoresis on an agarose gel (2%), visualized under UV light and documented. Size of the PCR product was 361 bp.

Tilletia contraversa specific PCR method

PCR was performed in a BioRad iCycler Thermal Cycler according to YUAN et al. (2009) applying the CQUTCK2, CQUTCK3 primer pairs specific for *T. contraversa*. The following PCR programme was used: 94 ^{o}C for 3 min, 40 cycles each at 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and an extension step at 72°C for 7 min. Amplified PCR products were subjected to electrophoresis on an agarose gel (1.5%), visualized under UV light and documented. Size of the PCR product was 747 bp.

We tried to reproduce the results of YUAN et al. (2009) nine times, but without success. After that we performed the PCR method applying the P23-BA0166, CQUTCK1 primer pairs, which were used to develop the former primer pair (CQUTCK2, CQUTCK3) according to the publication. The PCR programme included 94°C for 4 min, five cycles each at 94°C for 30 s, 53 °C for 45 s, 72°C for 1 min, after that ten cycles each at 94°C for 30 s, 36 °C for 45 s, 72°C for 1 min and an extension step at 72°C for 10 min. Amplified PCR products were subjected to electrophoresis on an agarose gel (1.5%), visualized under UV light and documented. Size of the PCR product was 1322 bp.

Identification of *Tilletia contraversa* with a SCAR marker based method

PCR was performed in a BioRad iCycler Thermal Cycler according to GAO et al. (2010) applying the TCKSF3, TCKSR3 primer pairs. The following PCR programme was used: 94 ^{o}C for 5 min, 30 cycles each at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and an extension step at

72°C for 10 min. Amplified PCR products were subjected to electrophoresis on an agarose gel (1.5%), visualized under UV light and documented. Size of the PCR product was 419 bp.

We gained faint bands with this programme, so we optimised the reaction as a method development process applying touch down PCR. The PCR programme included 95°C for 15 min, five cycles each at 94°C for 30 s, 45 °C for 30 s, 72°C for 30 s, after that 94°C 30 s, then 30 cycles each at 94°C for 30 s, 55 °C for 30 s, 72°C for 30 s and an extension step at 72°C for 10 min.

2.4. Protein based molecular methods for distinguishing *Tilletia contraversa* and *T. caries*

Extraction of polypeptides from teliospores

The inner protein content of *Tilletia* teliospores was extracted by an own-developed PBS (phosphate buffer salin)-based method. The protein concentration of the samples was determined by the Bradford Method (ROSENBERG 2004). The protein extracts were stored in aliquots at -70°C until use.

One dimensional gel-electrophoresis (1-D SDS-PAGE)

SDS electrophoresis was performed according to LAEMMLI (1970). Protein extract and 4x SDS sample buffer were mixed in a ratio of 1 to 3, and heated in boiling water for 3 min. 15-30 µl samples (approximately 1 µg / µl protein concentration) were loaded into the wells. 1-D SDS-PAGE was performed in an E5889 – Dual mini Sigma-Aldrich (Sigma-Aldrich Co. LLC) vertical gel electrophoresis unit using single concentration gels. A 5% stacking gel (pH 6.8) and a 10% Tris-Glycine resolving gel (pH 8.8) were applied. Electrophoresis was performed at 55 volts until the bromophenol blue tracking dye entered the resolving gel. The power was decreased to 45 volts until the tracking dye reached the bottom of the resolving gel (~ 3-4 hours). The gel was fixed in 12% trichloroacetic acid for 15 min, then stained with the blue silver method (CANDIANO et al. 2004). The gel was photographed and analysed with Quantity One 1-D Analysis software (Bio Rad).

3. RESULTS

3.1. The annual *Tilletia* surveys between 2007 and 2013

We have surveyed the economically important *Tilletia* species in Hungary since 2007, the results were documented and mapped, and the possible relations between the distribution of the *Tilletia* species and the abiotic factors were described.

133 (2007), 94 (2008), 108 (2009), 103 (2010), 90 (2011) and 98 (2012) samples were examined. According to our survey results *T. caries* has been continuously present in Hungary since 2008. *T. laevis* was detected together with *T. caries* as mixed infection in 2009, 2011 and 2013, and in one case only *T. laevis* was detected in a sample in 2009. *T. contraversa* was found twice in 2011 and in 2013 (Pölöske, Ludányhalászi) as mixed infection together with *T. caries*. As a result of the *T. contraversa* infection in 2011 a widened survey was carried out in Zala County in 2012. None of the samples collected near Pölöske was infected with *T. contraversa* or any of the *Tilletia* species.

To identify *T. contraversa* and *T. caries* the samples were examined both with the washing test and the epifluorescence microscopy method. In some light microscopical studies exact criteria were found for differentiating the teliospores of these wheat bunt fungi, but considerable overlaps still exist in their characteristics making identification difficult, especially in mixed infections. In addition, these techniques are time-consuming for routine examination, need a professional eye and not always give reliable results.

The sori of *T. contraversa* were dark brown, had a fishy odour (from trimethyl amine) and the spore mass was semi-agglutinated. The teliospores were golden to dark brown and measured 19.29±1.3 μm in diameter. The spore walls were reticulated and had 4–5 meshes per spore diameter, the meshes were 3.08±1.04 μm wide. The reticulations were 1.49±0.69 μm deep, and the teliospores had a gelatinous sheath measured 2.44±0.18 μm. The following morphological features of *T. contraversa* could be detected by using the epifluorescence microscopy method: the teliospores were orange, the cytoplasms fluoresced yellowish-greenish. The reticulated wall layer resembled a football in surface view and formed a drawn Sun in median view.

Samples infected with *T. caries* and/or *T. laevis* showed the features published in the literature. The sori of *T. caries* filled the ovaries with dark brown pulverulent spore masses (and had a fishy odour). The teliospores were globose, yellowish to light brown and measured 17.41±1.2 μm in diameter. The spore walls were reticulated and had 4–5 meshes per spore diameter, the meshes were 3.55±0.45 μm wide. The reticulations were 0.99±0.14 μm deep. The following morphological features of *T. caries* could be detected by using the epifluorescence

microscopy method: the cytoplasms of the teliospores contained bright, discrete, yellow to yellow-green fluorescing globules; the sheaths were non-fluorescent. When mounted in immersion oil the teliospores often appeared deformed.

A few samples suspected to be infected with *T. laevis* were examined with the washing test method as described above. In this case, the spore mass was brown and powdery. The teliospores were globose or ovoid, smooth and measured 16.8±0.4 μm in diameter. The colour of the spores was yellowish-brown, and contained guttulates.

3.2. The analysis of the air samples taken by Hirst type volumetric impaction sampler

Wheat cargos and air were sampled and examined to detect *Tilletia* species during the research period. The air samples were taken with a Hirst type sampler in two central grain warehouses in Budapest (Bp) and Jászapáti (Ja), and in 14 farm warehouses belonging to 10 companies. We also collected wheat samples in each sampling area, and dust samples were taken, too, from the dust deposited on the horizontal surfaces of the warehouses. The quarantine organisms, *T. contraversa* and *T. indica*, were not detected in any of the samples. *T. caries* dominated in the samples, while *T. laevis* were present only in a low concentration in the air samples collected in Bp, but this fungus was absent in Ja and in the farm warehouses.

All samples collected in Bp contained *Tilletia* teliospores. In Ja, lower teliospore concentration was measured than in Bp. In the farm warehouses, *Tilletia* species were detected in 88.9 % of the seed samples, 25.8 % of dust samples and 56.3 % of the air samples. The measured concentrations in the farm warehouses were low in all but one case: high seed and airborne *Tilletia*-levels were measured in Hódmezővásárhely (1,985 teliospores/50 g, 220 teliospores/m^3). When teliospores were detectable in the air, the seed samples were always found to be infected, but not vice versa. In 38.8 % of the cases, no teliospores were found in the air samples collected above *Tilletia*-infected wheat heaps.

Airborne teliospore levels of the two outermost sampling sites of each wheat-storing rooms were compared, but no significant difference was found ($P = 0.16$). Similarly, the volume of stored wheat did not significantly affect the airborne *Tilletia* spp. concentrations ($P = 0.64$, $U = 3.00$). (The amount of wheat was expressed with the height of the wheat heaps.) Teliospore levels in seed samples correlated with those in the air samples which were collected from above the wheat heaps ($n = 12$, $rs = 0.581$, $P < 0.05$).

Teliospores were found in high concentration in the dust samples collected in the central warehouses (Bp and Ja: avg. 53.3 and 11.1 teliospores/mg, respectively), while teliospore levels were lower in farm warehouses (avg. 1.4 teliospores/mg). Teliospore content of the dust showed medium correlation with the airborne teliospore level ($rs = 0.551$, $P < 0.05$), but no correlation

was found between dust and seed teliospore concentration. At the outdoor reference sites, in the yard and on the roof, teliospores were still present, being only 16.5 and 14.1% of the indoor level, respectively. Surprisingly, the indoor reference sites (rooms in the same warehouse containing the non-*Tilletia* host sunflower seed and maize) had high *Tilletia*-level—reaching a concentration of 86.7 and 91.5%, respectively, of the teliospore concentrations of the wheat-storing rooms.

3.3. DNA based molecular methods for distinguishing *Tilletia contraversa* and *T. caries*

DNA extracted from the *T. caries* and *T. contraversa* isolates was first tested with the method according to KOCHANOVA et al. (2004). With the use of the primer pairs TILf and TILr the expected band is a *Tilletia* genus-specific 361 bp band. Our experiment was successful, we detected the expected DNA band in the gel.

In our further studies we tested the method described by YUAN et al. (2009), which was based on a *T. contraversa* species specific 747 bp DNA product. We tried to reproduce this result but we did not succeed.

After that we tested the method of GAO et al. (2010), which is a new ISSR method, based on SCAR markers. With the application of SCAR primers (TCKSF3/TCKSR3) and some modifications in the PCR programme we gained the published 419 bp *T. contraversa* species specific band.

3.4. Protein based molecular methods for distinguishing *Tilletia contraversa* and *T. caries*

Since our aim was to extract proteins only from the inner part of the teliospores, hence they were washed with distilled water to remove the redundant material from both the teliospore suspension and the teliospore walls. The disruption of teliospore walls to release the proteins from inside was successful.

The protein extraction yielded different amounts of protein from both of the isolates of *T. caries* and *T. contraversa*. Maintaining the same parameters in the extraction procedure the protein concentration was lower from *T. contraversa* than from *T. caries* samples. There were no significant differences between the yielded protein among the isolates from the same species or between the two species. Nevertheless, differences in the staining intensity of polypeptides could be detected between species and even between the isolates of the same species. The overall intensity of the bands of *T. caries* was stronger than those of *T. contraversa* isolates. Among the three *T. contraversa* isolates the one with two extra protein bands (TILLCO B) exhibited the strongest staining intensity, whereas the protein extract of the Hungarian *T. contraversa* (TILLCO C) showed the lowest intensity.

Replicate teliospore extracts from each wheat sample yielded reproducible protein patterns. The SDS electrophoresis showed distinct protein bands for both species. Using homogenous gels, proteins in the teliospore extracts were detected in the range of 14-110 kDa. The five *T. caries* isolates had pattern stability, the isolates which originated from different geographical locations had the same pattern. The three *T. contraversa* protein extracts showed a consistent species-related difference in a 106 kDa polypeptide that appeared in each extract of *T. contraversa*, but was not present in the protein extracts of *T. caries*. One of the Polish *T. contraversa* isolates (TILLCO B) showed two additional bands at 73 and 85 kDa which could not be detected either in the other Polish (TILLCO A), or in the Hungarian *T. contraversa* isolates (TILLCO C). These two bands were not present in any of the *T. caries* protein extracts.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. The survey of *Tilletia* species in Hungary

According to the annual surveys Hungary was recognised to be free from *T. contraversa* between 2007 and 2010. Most of the samples were infected with *T. caries*, and less with *T. laevis*. To summarise 12.77% of the samples were infected with *Tilletia* species in 2008, 15.24% in 2009, 23.30% in 2010 (HALÁSZ 2012), 20% in 2011 and 19.28% in 2012. In 2011 we detected *T. contraversa* in one sample from Pölöske, Zala County. This result drew attention, because it could cause serious economic consequences. It is not recommended to draw conclusions too fast from only one positive result, it is suggested to do more investigation. During our extended survey in Zala County in 2012, we did not detect *T. contraversa* and any other *Tilletia* species in the fifteen samples. It was also interesting that we identified *T. contraversa* for the second time in 2013 in a sample from Ludányhalászi, Nógrád County. According to these results we will have to count on the further sporadic recurrence of *T. contraversa* in the future.

These results can be explained by two different aspects. The first possible reason for the recurrence of *T. contraversa* is based on regressions in agricultural practices (for example the size of cultivated fields reduced, large estates fell apart, chemical treatments diminished, etc.). Consequently, from the middle of 1990s higher number of common bunt infections could be observed. On the other hand changes could be explained with the different environmental requirements of the examined species and the persistent changes in the climatic conditions. It is widely known that the occurrence of fungal diseases is influenced by seed treatment, host plant species and climatic conditions. It depends on the fungal species which factor counts the most. Nevertheless in the progression of an epidemic the first factor is generally the climate which we can not affect.

The following risk factors have to be taken into account for the infection of *T. contraversa* according to PETERSON et al. (2009): snow cover (more than five days of snow cover means a snowy period), if it is not snowing then minimum 42 days of continuous precipitation helps the development of *T. contraversa* infection, while the soil temperature remains between –2 and +10°C during tiller development.

If we take into account the climatic conditions of Hungary, it is not common to have 42 days or more of precipitation. As a general rule, wheat germination begins usually in October, and after that period the plant is the most susceptible for the infection of *T. contraversa*. In accordance with the data of weather stations, snow cover is most likely from January to March in Hungary, while in October-December it is less frequent (5 times in 10 years). Data of 15

Hungarian weather stations were considered between 2000 and 2013 (source: OMSZ). From 2007 there were not sufficient long rainy periods or snow cover which could contribute to the occurrence or spread of *T. contraversa*, but the heavy rainy intervals could give the appropriate soil moisture conditions for the infections.

In summary, for a successful infection at least four of the following factors seem to be necessary (PETERSON et al. 2009): continuous snow cover for a given number of days, wheat has to be at the stage of germination or at the beginning of tillering, low temperature and high-moisture conditions in the soil and shallow sowing. The infection of *T. contraversa* is in most cases soil-borne, so that explains why shallow sowing is a serious risk factor: teliospores of *T. contraversa* can germinate and produce basidiospores solely on or near the soil surface, which is the first step for the infection.

It is has to be added that the coexistence of the environmental factors needed for the infection of *T. contraversa* has very little chance. This could explain why we found a single *T. contraversa* infection in 2011. After that the winter of 2011/2012 had the required long snow cover, so it ensured a possible recurrence of *T. contraversa*. Still we could not detect any *T. contraversa* in 2012 in any of the samples. However, *T. contraversa* teliospores had been identified in 2013 from a different geographic location: Ludányhalászi, Nógrád County. Further infections could be expected based on these findings.

As far as the susceptibility of the host wheat species is concerned, according to the information by Ottó Veisz and László Szunics none of the commercially cultivated wheat varieties are resistant to any of the *Tilletia* species in Hungary. Wheat varieties have not been bred for this trait in our country, as a consequence the existence of resistant or semi-susceptible varieties are just coincidence.

Our results were rated according to the kind of seed treatment applied, and the host wheat species infected. Unfortunately data fixing was not always successful. The success of seed treatment could not be evaluated because we have the necessary information only in 50% of the positive samples. The infected wheat varieties were also diverse, and a given wheat variety could have been once infected and for the second time not infected.

In the appearance of the wheat infecting common and dwarf bunts, climate, agricultural practice (e.g. depth of sowing) and host wheat variety do not play a significant role, as it is known if seed treatment is professionally applied the infection could be prevented successfully. In our opinion, as for the infection of *Tilletia* species, the first risk factor is seed treatment, the second is the climate and the third is the wheat variety.

4.2. The usability and reliability of the identification methods of *Tilletia contraversa* and *T. caries*

To identify *T. contraversa* and *T. caries* the samples were examined both with the washing test and the epifluorescence microscopy method. The drawback of the classical microscopy methods is that considerable overlaps still exist in the characteristics of the two species, so that makes identification time-consuming. Furthermore it is not always reliable. As a consequence our aim was to develop or adopt one or more suitable molecular methods for laboratory use and to make our work faster and more efficient.

During our DNA based molecular work we successfully adopted the method developed by KOCHANOVA et al. (2004). With the use of the primer pairs TILf and TILr the expected band is a *Tilletia* genus-specific 361 bp band. This newly developed primer pair is more specific than primers ITS1 and ITS4 of PIMENTEL et al. (1998 a), which produce two fragments: 550 bp and 750 bp. The former is specific for *Tilletia* species, but the latter amplifies wheat DNA. It can cause problems in practical diagnosis.

In our further studies we tested the method described by YUAN et al. (2009), based on a *T. contraversa* species specific 747 bp DNA product. This was the first publication for the differentiation of *T. caries* and *T. contraversa* by species specific primers. We tried to reproduce this result but we did not succeed in it. The reason of this failure could be that our isolates were from diverse geographical locations, thus the primer binding sequences of our isolates and theirs differ in a few base pairs.

After that we tested the method of GAO et al. (2010), which is a new ISSR method, based on SCAR markers. With the application of SCAR primers (TCKSF3/TCKSR3) and some modifications in the PCR programme we gained the published 419 db *T. contraversa* species specific band.

For the differentiation of *Tilletia* species on the basis of protein composition we successfully developed a new 1-D SDS gel-electrophoresis method. Our results differed from the results of WEBER and SCHAUZ (1985) who compared the phenol soluble proteins extracted from *T. contraversa* and *T. caries* teliospores, and found a 65 kDa polypeptide present only in *T. contraversa* extracts. Nevertheless, our findings did not meet the results of KAWCHUCK et al. (1988), who used two dimensional (2-D) electrophoresis to compare also the phenol-soluble polypeptides in the extracts from the two *Tilletia* species. They detected considerable amount of polypeptides, but did not resolve polypeptides specific for any of these fungi. Finally BANOWETZ et al. (1994) found a 116 kDa polypeptide in the extracts of *T. contraversa*, which

was not present in the extracts of *T. caries*. They applied 2x Laemmli-puffer for the extraction, while we analysed the total PBS-soluble protein extracts during our experiments, and we could not detect any of the mentioned polypeptides in our isolates. In our case the electrophoretic pattern showed a consistent species-related difference in a 106 kDa polypeptide that appeared in each extract of *T. contraversa*, but was not present in the protein extracts of *T. caries*. One of the Polish *T. contraversa* isolates (TILLCO B) showed two additional bands at 73 and 85 kDa which could not be detected in any other isolates. These two different bands could be resulted from diverse geographical locations and/or host winter wheat cultivars, too. (Unfortunately we have not got any data about the host wheat variety of TILLCO A.)

We proved that PBS-based protein extraction is more efficient than 2x Laemmli puffer based extraction. The 2x Laemmli extraction media resulted in a blurred, less distinct protein pattern, and the background of the lanes was too high. In contrast, the PBS extraction media gave a clear protein pattern, assuring easy comparison of bands and lanes by densitometry.

It is obvious that nowadays the DNA based molecular methods are more popular than the protein based ones. As a consequence, more literature and research projects can be found connected to DNA based methods. While evaluating these techniques we adopted the ISSR method of GAO et al. (2010) to our laboratory conditions. There have not been any available protein based identification methods, so we developed a new one based on 1-D SDS-PAGE.

According to our results the application of DNA and protein based methods is also suggested, the selection mostly depends on the facilities of the laboratory. Today DNA based techniques are more favourable, but protein based methods could not be forgotten. DNA based molecular methods amplify only a few base pairs, while huge numbers of polypeptides (gen-products) could be compared with the protein based ones. The sole limiting factor is the amount of the sample, in case of a relatively low amount of teliospores DNA based methods are more suitable.

4.3. The spread of *Tilletia* teliospores in warehouses

Our results suggest that teliospores spread readily, possibly by continuous warehouse activities. Teliospore dispersal takes place rapidly between rooms and floors via opentop walls and spouts, respectively. There is a noticeable risk of airborne *Tilletia* contamination of the healthy grain in the warehouses as well. It is also supposed that teliospores could be distributed from deposits originating from previous crops as teliospores were found also in the reference rooms containing the seed of non-*Tilletia* host plants (sunflower, corn). Apparently, the source of the airborne teliospores could be the settled dust. There was a medium correlation between the teliospore content of dust and air as well as the teliospore content of seed and air, but no

correlation was found between the teliospore content of seed and dust. Airborne levels of *Tilletia* teliospores could reach high concentrations especially in central warehouses, which could increase the risk of respiratory diseases. So it is suggested to wear appropriate labour safety devices (i. e. masks) during the work in warehouses.

Results shown in our study hopefully will help improve the diagnostic methods applied in laboratories for differentiating *Tilletia* species on the one hand, and will draw attention to the possible human and plant health risks of smut teliospores in the stored grain on the other.

4.4. New scientific results

1. We proved the dominant occurrence of *T. caries* in the framework of the countywide annual survey (2007-2013), and detected twice the known economically important pest, *T. contraversa*. We found that the climatic conditions highly affect the success rate of infection, but the effective seed treatment should prevent smut infection. Wheat variety has the least significance, since breeding for smut resistance has been absent in Hungary.

2. We also proved the practical usability of the SCAR primer (TCKSF3/TCKSR3) based method of GAO et al. (2009), which amplified a 419 base pair long *T. contraversa* specific DNA fragment. With the modification of the published protocol the method could be used for the confirmation of a result gained by classical microscopy method for differentiating *T. caries* and *T. contraversa*. In addition we verified the method by KOCHANOVA et al. (2004) who applied the primer pairs TILf and TILr with which a *Tilletia* genus-specific 361 bp band was amplified.

3. We developed an efficient protein extraction method from the inner part of the teliospores with rupturing the spore wall.

4. Uniquely we developed a protein-based 1-D SDS gel-electrophoresis method for the differentiation of *T. caries* and *T. contraversa.* The electrophoretic pattern showed reproducibly a consistent species-related difference in a 106 kDa polypeptide that appeared in each extract of *T. contraversa*, but was not present in the protein extracts of *T. caries*. We also proved the applicability of PBS buffer instead of the 2x Laemmli buffer for protein extraction.

5. For the first time in Hungary we examined the characteristics of the inner air of central and farm warehouses with a portable Hirst type volumetric impaction sampler. *Tilletia caries* was dominant in the samples. There was a medium correlation between the teliospore content of dust and air as well as the teliospore content of seed and air, but no correlation was found between the teliospore content of seed and dust. Moreover, it is also supposed that the grain could be contaminated by the previously stored infected crop. Apparently, the source of the airborne teliospores could be the settled dust.

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6. PUBLICATIONS RELATED TO THE THESIS

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