Laboratory Diagnostics of Common Bunt and Dwarf Bunt

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Abstract: Fungal pathogens belonging to the genus *Tilletia* infect young seedlings of cereals (wheat, barley, rye, triticale), transform their grains into sori filled with teliospores, degrade harvest quality index and cause as much as 95% loss of yield. Until full maturity there are any concrete visible symptoms on the plant, except light yellow spots and more shoots production that can have more occurrence reasons. There are three possibilities how to detect *Tilletia* spp. in plant tissue – using vital dye for mycelium staining, polymerase chain reaction and dot blot hybridization.

Keywords: detection; dot blot hybridization; DNA extraction; PCR; plant tissue staining; *Tilletia*

In the Czech Republic there can occur three species of genus *Tilletia*, *T. caries* (DC.) Tul., *Tilletia foetida* (Wallr.) Liro and *Tilletia controversa* Kühn. These fungal pathogens infect young seedlings and in the full maturity transform grains into sori filled with *Tilletia* teliospores. *Tilletia* produces strongly smelly trimethylamin, that causes unavailability of contaminated crop for food and keeping products. Bunt presence degrades harvest quality index and faces total disposal of crop, it means loss of all costs expended on crop creation, fertilization, treatment and harvest or crop disposal.

Arrays of specific oligonucleotides which can screen a single sample for different pathogens simultaneously has been developed to detect and identify species of *Pythium* and *Phytophthora* (Levesque *et al.* 1998), *Sporisorium reilianum*, which causes head smut maize, can be detected and identified using a dot blot hybridization procedure on genomic DNA (Xu *et al.* 1998). The dot blot procedure is useful for screening many samples for a particular organism. Prior amplification of a portion of the genome greatly enhances the sensitivity of a dot blot test (Mitchell *et al.* 1994).

In our case we describe how to detect most frequent and dangerous *Tilletia* species in juvenile growth stage, in phase without any visible symptoms.

MATERIAL AND METHODS

Biological material. Biological material included young wheat seedlings infected by common bunt and dwarf bunt, wheat not infected by bunt cultivated separately and *T. controversa* teliospores. Spores germinated on water agar at 8°C for four weeks, then transferred on solid T-19 medium (Wilcoxson & Saari 1996) and cultivated at 16°C until mycelium growing.

DNA extraction. Biological material was homogenized in a commercial blender with cetyltrimethylamonium bromide (CTAB) buffer and incubated in water bath in 60°C. Then homogenate was vortexed with chloroform-isoamylalcohol (24:1) and centrifuged. Upper phase was taken away, isopropanol was added and DNA was precipitated in liquid nitrogen. After this step it was centrifuged, isopropanol was decanted and pellet washed in 80% ethanol – 10mM LiCl – 1mM Tris. This solution was centrifuged, decanted again and pellet dried under vacuum. Then DNA pellet was resuspended in ddH_2O.

Preparation of probe for dot blot hybridization. Mycelium DNA was extracted. Nucleotide sequence was amplified using primers T1f (5′-TCG GTT CCT ACA ACC TTT TC-3′) and T1r (5′-ATG GGA GAA CCA AGA GAT CC-3′), thermocycler program (heating at 94°C for 4 min, thirty five cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 s and extension at 72°C for 44 s, and a final extension at 72°C for 4 min) and PCR solution (2.5 µl of buffer for Taq polymerase, 3.0 µl $MgCl_2$, 0.25 µl dNTP (0.4mM of each nucleotide), 0.4 µl primer mix (11 pmol), 0.15 µl Taq polymerase (Fermentas), 1.0 µl of extracted DNA, 17.7 µl ddH_2O. Amplified PCR product of expected length 108 bp was examinated by electrophoresis in agarose gel containing ethidium bromide and visualized using UV transilluminator. Obtained nucleotide called TC1 belonging to ITS region was labeled using Biotin ULS Labeling Kit (Fermentas) by concentration 4 ng of DNA per 1 µl of probe.

Hybridization reaction. DNA extracted from infected plant, one shoot, was denatured by boiling for five minutes, transferred onto positively charged nylon membrane and fixed in an oven at 120°C for 30 min. Then membrane was placed in a tube containing hybridization buffer (5× SSC, 0.1% N-Laurilsarcosina, 0.02% SDS, 1% agente blogueant) and prehybridizated at 53°C for one hour. After that membrane was placed in a tube containing hybridization buffer included probe (1 ml of hybridization buffer (5× SSC, 0.1% N-Laurilsarcosina, 0.02% SDS, 1% agente blogueant) obtained 12 µl of denaturated probe (by boiling in water bath) and incubated overnight. Membrane was washed in 2× SSC, 0.1% SDS at 53°C for five min, then twice in 0.1× SSC, 0.1% SDS at 53°C for five min. This buffer was always preheated at 53°C before use. Streptavidin-AP Conjugate (Boehringer Mannheim) was diluted in detection buffer (0.1M Tris, 0.1M NaCl, pH 9.5) in dilution 1:2000 and incubated at room temperature for one hour. Antibody solution was discarded and membrane was washed in washing buffer (0.1M maleic acid, 0.15M NaCl, 0.3% Tween 20) twice for 15 min. Then membrane was placed in detection buffer at room temperature for 3 min. Staining solution (7.5 µl NBT, 3.7 µl BCIP (Duchefa) per 1 ml detection buffer) was prepared and membrane was incubated in dark until exposure of purple coloration. For finishing of staining TE stop buffer (10mM Tris, 1mM EDTA) was used.

Polymerase chain reaction (PCR). The second shoot was used for PCR. Primers TILf (5′-CAC AAG ACT ACG GAG GGG TG-3′) and TILr (5′-CTC CAA GCA ACC TTC TCT TTC-3′) were designed on the basis of known *Tilletia* ribosomal DNA sequence from NCBI database (Zhang *et al*. 2001). PCR solution contained 2.5 µl of buffer for Dynazyme II, 0.25 µl dNTP (0.4mM of each nucleotide), 0.4 µl primer mix (11 pmol), 0.5 µl Dynazyme II (1.5 U, Finnzymes), 1 µl of extracted DNA, 20.35 µl ddH_2O. Amplifications were performed in a MJ Research PTC-200 thermocycler. Samples were firstly heated at 95°C for 5 min, followed by thirty five cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 4 min. Amplified PCR products were examinated by electrophoresis in agarose gel containing ethidium bromide and visualized using UV transilluminator.

Plant tissue staining. Results were confirmed by method of plant tissue staining using of light microscope. Plant tissue – the third shoot – was cleared by boiling in 30% KOH for 5 min and stained in 1% trypan blue for 10 min then visualized by light miscroscope.

RESULTS

Dot blot hybridization is specific to *Tilletia* species – *T. caries*, *T. foetida* and *T. controversa*. Using of probe TC1 reaction was negative with negative controls – plant DNA not infected by genus *Tilletia* but naturally infected by other fungal pathogens.

DNA of infected plants was extracted and amplified and PCR product of expected length 361 bp was obtained. PCR gave negative results using of negative controls.

Plant tissue staining and using of light microscope confirms presence of *Tilletia* mycelium in infected plants.

DISCUSSION

Among diagnostic methods for genus *Tilletia* is using of teliospore morphology and light microscope. Study of teliospores is possible in full plant maturity only. Until this growth phase there are light yellow spots and more shoots production on the plant that can have more occurrence reasons.

Easy method how to detect *Tilletia* in plant is staining of plant tissue. By this way it is possible to detect hyphae in plant early growing stage, in young plant seedlings. Swinburne (1963) and Hansen (1959) used solution contained vital dye cotton blue and detected mycelium in juvenile plant.

Molecular biologic diagnosis is practically usable for the fast and easy detection of plant pathogens specific DNA sequence and in the world it is used routinely (Henson & French 1993), very often primers are placed to Internal Transcribed Spacer – ITS region and for target DNA are specific (Josefsen & Christiansen 2002). A dot blot assay can analyze many samples for a single organisms, as the PCR products from the samples are blotted on the membrane and hybridized to a single probe specific for the particular organism (Utkhede & Cao 2005).

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