Utilization of Immunochemical Methods for the Detection of *Fusarium culmorum* in Winter Wheat

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**Abstract**


Two monoclonal antibodies and one polyclonal antibody were prepared and used for the detection of *Fusarium culmorum* in winter wheat. In addition, *Fusarium oxysporum*, *F. graminearum*, *F. solani*, *F. avenaceum*, *F. tricinctum*, and *F. moniliforme* were used in the tests. The antibodies were genus specific. Monoclonal antibodies did not cross-react with other fungal pathogens of winter wheat (*Gaumamnomyces graminis, Pseudocercosporella herpotrichoides, Rhizoctonia solani*). The pathogen was detected *in vitro*, in hydrated and artificially inoculated kernels and in seedlings, by PTA-ELISA, immunoprinting, dot blot and immunofluorescent microscopy. Dot blot and immunofluorescent microscopy were the most sensitive methods for recognising FCu *in vitro* and *in vivo*. Specificity and sensitivity of monoclonal and polyclonal antibodies were similar.

**Key words:** *Fusarium culmorum*; winter wheat; polyclonal and monoclonal antibodies; PTA-ELISA; immunoprinting; dot blot; immunofluorescent microscopy

*Fusarium culmorum* and other *Fusarium* species (*F. graminearum*, *F. avenaceum*, *F. solani*, *F. poae*, *F. oxysporum*, *F. nivale*) are well known pathogens of winter wheat. Infection of winter wheat with *Fusarium* species causes lower germination, death of seedlings, head blight, yellow stems and leaves, and root rot (KOVÁČIKOVÁ et al. 1992).

Recent studies have shown that reliable, fast and specific diagnoses of these pathogens cannot be based on the detection of mycotoxins because the production of some of the toxins can be modified by abiotic factors. Further, the content of toxins is not always correlated with virulence of this pathogen and/or resistance of the variety (MESTERHÁZY & BARTÓK 1992; MANKA et al. 1989). For this reason we studied the use of immunochemical methods for the detection of these pathogens in host plants. For healthy plants and a good quality of products it is particularly important to detect pathogens already in the kernels.

This paper continues our recent research, i.e., the preparation and testing of polyclonal antibodies for the detection of *F. culmorum* (KRÁTKA et al. 1997).

The aims of this study were:

- to test the specificity of poly- and monoclonal antibodies for detection of *F. culmorum* and other *Fusarium* pathogens of winter wheat,
- to determine the optimal growth stage of the host plant (after artificial inoculation of kernels) to detect the pathogen with immunochemical methods,
- to present suitable immunochemical methods for the detection of the pathogen in host plants.

**MATERIAL AND METHODS**

**Biological Material**

The virulent isolate of *Fusarium culmorum* (FCu) no. 67 was used (KRÁTKÁ et al. 1997). Analyses were also carried out with *Fusarium oxysporum*, *F. solani*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. moniliforme* and other important pathogens of winter wheat *Pseudocercosporella herpotrichoides, Gaumamnomyces graminis* and *Rhizoctonia solani*.

The fungal isolates were chosen according to tests of pathogenicity performed at the Research Institute of Crop Production (HÁKOVA, SYCHROVÁ – pers. comm.).

The winter wheat cultivar Arina with high resistance to FCu was used (BARTOS – pers. comm.). Analyses were performed with kernels, seedlings and young plants (at
the growth stage of two leaves). Fifty kernels were sterilized by immersing them in antiseptic solution SAVO (Biochemie) containing 47.2 g/l NaClO, then in 96% ethyl alcohol, and finally sterile H2O. Afterwards the kernels were placed in Petri dishes with Czapek Dox agar medium (48 h, 20°C, thermostat). Only healthy hydrated seeds (without visible presence of bacteria and fungi) were selected and inoculated with a suspension of conidia and mycelium of FCCu (cca 10^6 CFU/ml). Incubum was injected by syringe into the kernels. In the first experiment inoculated kernels were placed on a Petri dish with Czapek Dox agar medium (20°C, thermostat). Hydrated kernels and seedlings were analyzed after 24 h and 48 h. In the second experiment inoculated kernels were placed in a test tube with Czapek Dox agar medium (light thermostat, 20°C). The young plants (with two leaves) were analyzed after 6 days.

Antigen Preparation

FCCu was cultured on Czapek Dox liquid medium for 25 days. All other fungal pathogens were also cultured on that medium.

Antigens (purified protein solutions) were prepared from mycelial mats according to KRÁTKÁ et al. (1997).

Preparation of Antibodies

Polyclonal antibodies were prepared and tested according to KRÁTKÁ et al. (1997).

Monoclonal antibodies: A. Female Balb/c mice, 6–20 weeks old, were given five interperitoneal injections of 100–250 µg of proteins (FCu antigen) at 2-week intervals. Antigen was emulsified 1:1 with Freund's adjuvant. The sixth injection, containing 300 µg of proteins without adjuvant, was applied after 2 weeks. Three days after application of the sixth injection the mice spleenocytes were fused with Sp 2/0 myeloma cells using 50% polyethylene glycol 1300–1600 (Sigma).

B. Female Balb/c mice, 6–20 weeks old, were given six interperitoneal injections of an extract from seeds naturally infected with FCCu (150 seeds were extracted in 10 ml of PBS). Each immunization included 100 µl per dose mixed with Freund's adjuvant (1:1). Three days after the last injection the mice spleenocytes were fused with Sp 2/0 myeloma cells using 50% polyethylene glycol 1300–1600 (Sigma).

Hybridoma were tested using indirect ELISA on Terasaki microtitre plates. Clones were obtained from hybridoma colonies that grew on soft agar. Each positive cell line was cloned three times. The stable clones were cultured in a spinner flask to 0.5 l for large-scale production of monoclonal antibodies (MAbs).

The antibody-containing cell culture supernatants were concentrated by polyethylene glycol approximately 20 times. Antibodies (class IgG) were purified by caprylic acid (VIKLICKÝ 1987). Final concentration of IgG (FCu antibody) was 1 mg/ml. The antibodies were then frozen and stored at −18°C.

Immunochemical Methods

Preparation of plant material: The same plant samples were used in three methods:

1. Hydrated kernels, coleoptiles and roots of seedlings, leaves and stems of young plants, and mycelial mats were printed.

2. Printed samples (5 kernels or 5 coleoptile/mycelial mats were placed in 1ml of PBS at 4°C, the extracts were used for dot blot or ELISA. Extracts prepared in PBS were used for ELISA and immunoblotting without further dilutions.

3. Printed samples (5 kernels or 5 coleoptile/mycelial mats were placed in 1ml of PBS containing IgG and used for immunofluorescent microscopy.

PTA-ELISA was conducted according to KYNĚROVÁ et al. (1998) on polystyrene microtitre plates (GAMA). Absorbance was measured with an automatic reader (Dynatech) at 405 nm.

Immunoblotting (tissue immunoprinting and dot blot): Plant samples and mycelial mats or 2 µl PBS extracts were applied on nitrocellulose membranes (Millipore Corporation, 0.45 µm of porosity). The membranes were immersed in PBS pH 7.2 containing 5% skimmed milk for 1 h at room temperature, then incubated with antibodies diluted in PBS pH 7.2 with 5% skimmed milk for 1 h at room temperature. After three rinses with PBS pH 7.2 and one rinse with TBS (0.01M Tris, 0.015M NaCl, pH 8.1) the membranes were incubated with sheep anti-rabbit IgG coupled with alkaline phosphatase (Boehringer Mannheim) diluted to 250 µU/ml in TBS (polyclonal) or incubated with sheep anti-mouse IgG coupled with alkaline phosphatase diluted to 250 µU/ml (monoclonal) in PBS 0.5 h. After three rinses with TBS and one rinse with TBS-MgCl₂ (0.1M Tris, 0.15M NaCl, 0.005M MgCl₂ × 6H₂O, pH 9.1), the membranes were immersed in the BCIP/NBT solution (Sigma). The membranes were dried and evaluated. All incubation steps were performed during orbital shaking (Minishaker IKA, 300 rpm). The colour reactions were stopped by three rinses in distilled water.

Immunofluorescent microscopy: Plant samples and mycelial mats were immersed in PBS pH 7.2 containing polyclonal or monoclonal antibodies. After three rinses with PBS the plant samples/mycelial mats immersed in polyclonal IgG were incubated with SwAR/FITC (Institute of Sera and Vaccine, Prague, CZ), while material immersed in monoclonal IgG was incubated with anti-mouse Ig-fluorescein from sheep (Boehringer Mannheim). The incubations for 3 h at 37°C were performed during orbital shaking. Reactions were stopped by three rinses with PBS, rinsed material was placed in distilled water. Microscopic preparations were examined with a fluorescence microscope (Nikon).
RESULTS

Preparation and Test of Monoclonal Antibodies

Twenty positive hybridomas prepared with method A, and eight positive hybridomas prepared with method B were identified by indirect ELISA on Teraski plates. The two best monoclonal antibodies were selected for tests: MAb33/8 (method A) and MAb30/2 (method B).

Using PTA-ELISA and dot blot we found that both mono- and polyclonal antibodies were genus specific (Table 1, Fig. 1a, b). Fluorescent microscopy was sensitive enough to recognise conidia of *FCu* in a mixture of conidia of *Fusarium* species (Fig. 2a, b, c). We determined the optimal concentrations of IgGs for each method. In ELISA we used a concentration of 1 µg/ml of polyclonal IgG and 10 µg/ml of both monoclonal IgGs, for immunoblotting 1 µg/ml of polyclonal IgG and 4 µg/ml of monoclonal IgGs, and for fluorescent microscopy 4 µg/ml of all IgGs. This means that the sensitivity of polyclonal IgG was higher than that of monoclonal IgGs, especially in ELISA. The specificity was similar for all IgGs.

![Image](image-url)


Fig. 1. Dot blots of *Fusarium* sp. extracts on nitrocellulose: a – polyclonal IgG, b – MAb 30/2

![Image](image-url)

Fig. 2. Immunofluorescent microscopy – conidial mixture of various *Fusarium* species (*F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. oxysporum*, *F. solani*, *F. tricinctum*, *F. moniliforme*): a – polyclonal IgG, b – MAb30/2, c – light microscopy

Table 1. Cross-reactions of antibodies to *Fusarium culmorum* with other fungi

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Antibodies</th>
<th>PAb</th>
<th>±s</th>
<th>MAb30/2</th>
<th>±s</th>
<th>MAb33/8</th>
<th>±s</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. avenaceum</em></td>
<td>PAb</td>
<td>0.72</td>
<td>0.08</td>
<td>0.55</td>
<td>0.02</td>
<td>0.61</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>±s</td>
<td>0.03</td>
<td></td>
<td>0.25</td>
<td>0.01</td>
<td>0.31</td>
<td>0.01</td>
</tr>
<tr>
<td><em>F. graminearum</em></td>
<td>PAb</td>
<td>0.61</td>
<td>0.07</td>
<td>0.52</td>
<td>0.02</td>
<td>0.60</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>±s</td>
<td>0.03</td>
<td></td>
<td>0.12</td>
<td>0.01</td>
<td>0.21</td>
<td>0.01</td>
</tr>
<tr>
<td><em>F. moniliforme</em></td>
<td>PAb</td>
<td>0.65</td>
<td>0.03</td>
<td>0.48</td>
<td>0.02</td>
<td>0.53</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>±s</td>
<td>0.01</td>
<td></td>
<td>0.31</td>
<td>0.02</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>PAb</td>
<td>0.55</td>
<td>0.05</td>
<td>0.39</td>
<td>0.04</td>
<td>0.45</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>±s</td>
<td>0.04</td>
<td></td>
<td>0.51</td>
<td>0.04</td>
<td>0.59</td>
<td>0.04</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>PAb</td>
<td>0.80</td>
<td>0.05</td>
<td>0.69</td>
<td>0.03</td>
<td>0.60</td>
<td>0.02</td>
</tr>
<tr>
<td><em>F. sambucinum</em></td>
<td>PAb</td>
<td>0.60</td>
<td>0.04</td>
<td>0.51</td>
<td>0.04</td>
<td>0.59</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Gaeumannomyces graminis</em></td>
<td>PAb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudocercospora herpotrichoides</em></td>
<td>PAb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Rhizoctonia solani</em></td>
<td>PAb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Each value represents the mean of nine replications, ± standard errors of the mean were determined; Concentration of proteins in each antigen = 1 µg/ml; PAb: polyclonal antibodies, MAb: monoclonal antibodies.
The antibodies did not cross-react with other pathogenic fungi of winter wheat, i.e., *Gaetumannomyces graminis*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia solani* (Table 1).

**Detection of FCu in Wheat**

The pathogen was detectable in hydrated kernels and seedlings. We used both mono- and polyclonal antibodies for the detection by ELISA, immunoprinting and dot blot. The pathogen was successfully detected in kernels one day after inoculation, and two days after inoculation in coleoptiles using immunoblotting (Fig. 3a, b) and ELISA (Fig. 4). Absorbances (ELISA) of infected samples had to be higher than 0.05 (Fig. 4). The threshold for positive pathogen detection represented the mean of nine replications (three microtitre plates with three wells for each sample), ± standard errors of the means were determined. Especially the dot blot procedure was very reliable when using extracts from plant samples. Direct printing of kernels, roots and coleoptile (from seedlings) was difficult and in many cases not reproducible.

![Fig. 3. Dot blots of plant extracts (1 day after infection): a - polyclonal IgG, b - MAb 33/8](image)

1-3: different FCu antigens, 4-5: control kernel, 6-7: control coleoptile, 8-9: infected kernel, 10-11: infected coleoptile

![Fig. 4. Detection of *Fusarium culmorum* in artificially infected seedlings by ELISA. Each value represented the nine replications, the bars represent ± standard errors of the means](image)

PAb - polyclonal antibodies; MAb - monoclonal antibodies

![Fig. 5. Infected wheat coleoptile tissue visualised using polyclonal IgG under immunofluorescent microscopy (a) and light microscopy (b)](image)

Infected tissue was visualised using polyclonal antibodies and examined under an immunofluorescence microscope (Fig. 5).

It was difficult to detect the pathogen in young plants. The plants had visible symptoms, that is yellow leaves and stems, mycelial growth on stems, inhibition of growth, but the differences in absorbances (ELISA) between infected and control plants were not reproducible in all cases, and dot blots were negative. For these reasons no detailed results on this stage of the plants are described.

**DISCUSSION**

From the results it appears that detection of *Fusarium culmorum* in artificially hydrated kernels and seedlings of winter wheat with the immunomethods is most reliable. We suppose that these methods will also be reliable to detect FCu and other *Fusarium* sp. in naturally infected kernels of winter wheat. The use of hydrated kernels, and particularly seedlings, facilitates the detection of latent infection. At present we are studying this aspect. Detection of FCu in plants with visible infection was unreliable. We experienced the same difficulty while detecting *Phytophthora* sp. in host plants such as strawberry, tomato and potato (*Kynérova et al. 1998; Pešková-Kynérova & Kutíková 1999*). Similar conclusions were reached by other authors (DEWEY 1996 – pers. com.). According to WERRES (1988) and GABLER & URBAN (1995) this problem could be due to inhibition of the ELISA reaction by oxidized polyphenols which were produced in diseased plants.

The selection of suitable methods was facilitated by the use of similar plant material and determination of antibody specificity and sensitivity. We used the same plant samples in all methods. Polyclonal and monoclonal antibodies are genus-specific, they are thus not suitable to differentiate between *Fusarium* species. Beside many other advantages of ELISA (e.g., processing of numerous samples, rapidity, sensitivity, and low cost), its results in a quantitative form are particularly valuable. But the determination of the positive/negative threshold in the detection of fungal pathogens in hosts is difficult (CONVERSE & MARTIN 1990). For this reason the statistical evalu-
tion of data is very important. Based on the measurements we defined a very low positive threshold value for detection of the pathogen. We determined this level on the basis of the mean of nine replications, after calculating the standard errors of the means. Because we used the same plant material (also the same dilutions) in all tests by different methods, we were able to compare the results and confirm our conclusions. This problem was discussed recently by other authors (KYNĚROVÁ et al. 1998).

Dot blot is a fast and sensitive method which, according to our experience, could be used to detect the presence of Fusarium sp. in tissue most reliably. Immunofluorescence is a very sensitive method. The interpretation of immunofluorescence is frequently difficult due to other sources of fluorescence, such as cross-reactions of antibodies with plant metabolites, and autofluorescence of the plant material. The importance of interference has to be individually determined for each system and requires repeated observations of suitable controls, unstained samples or healthy tissue (MILLER & MARTIN 1988; GINGER 1990; WERRES & STEFFENS 1994).

Using this method we were able to localize the antigen in plant tissue. According to our experiences and results it is reasonable to use at least two immunomethods for detection of FCu in winter wheat after artificial infection.

We studied the differences between mono- and polyclonal antibodies, their specificity and sensitivity. Monoclonal antibodies for detection of FCu were genus-specific. Similar conclusions on the detection of fungi were obtained by DEWEY (1996). The prepared antibodies have approximately similar sensitivity. Compared to polyclonal antibodies, the main advantage of monoclonal antibodies is that they can be produced in unlimited quantities and preserved indefinitely (DEWEY 1996).

References


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Souhrn


Byly připraveny a otestovány monoklonální protititky (MAb30/2 a MAb33/8) pro detekci Fusarium culmorum (FCu) v pšenici ozimé. Testování bylo provedeno pomocí nepřímé ELISA metody a bylo zjištěno, že specifickost připravených MAb je rodová. Pro testování byly použity druhy rodu Fusarium (oxyphorum, solani, graminearum, avenaceum, tricinctum, moniliforme). Roz-
dily byly kvantitativní. MAbs nevykazovaly křížové reakce s dalšími houbovými patogeny pšenice (Gaeumannomyces graminis, Pseudocercosporella herpotrichoides, Rhizoctonia solani). Detekce FCu byla provedena v uměle infikovaných obilkách. Pro identifikaci patogena byly využity PTA-ELISA, imunoprint, dot blot a imunofluorescenční mikroskopie. Při všech použitých metodách byly použity poly- a monoklonálních protilátky, jejich reakce (specifickost a citlivost) byly obdobné.

Klíčová slova: Fusarium culmorum; pšenice ozimá; polyklonální a monoklonální protilátky; PTA-ELISA; imunoprint; dot blot; imunofluorescence

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