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

JIŘINA KRÁTKÁ, BLANKA PEKÁROVÁ, ANDREA ZEMANOVÁ and MIROSLAVA KUTÍKOVÁ

Research Institute of Crop Production – Division of Plant Medicine, Prague-Ruzyně, Czech Republic

Abstract

KRÁTKÁ J., PEKÁROVÁ B., ZEMANOVÁ A., KUTÍKOVÁ M. (2000): **Utilization of immunochemical methods for the detection of** *Fusarium culmorum* **in winter wheat**. Pl. Protect. Sci., **36**: 1–6.

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 (*Gaeumannomyces 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* (KRÁTKÁ *et al.* 1997).

The aims of this study were:
– to determine the optimal preparation of monoclonal antibodies,
– 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, Gaeumannomyces graminis* and *Rhizoctonia solani*.

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

The winter wheat cultivar Arina with high resistance to *FCu* was used (BARTOŠ – 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 H_2O. 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 *FCu* (cca 10^6 CFU/ml). Inoculum 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

FCu 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 emulfisied 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 splenocytes 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 *Fcu* (150 seeds were extracted in 10 ml of PBS). Each immunization included 100 µl extract per dose mixed with Freund's adjuvant (1 : 1). Three days after the last injection the mice splenocytes were fused with Sp 2/0 myeloma cells using 50% polyethylene glycol 1300–1600 (Sigma).

Hybridoma were tested using indirect ELISA on Teraski 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 after 24 h. 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-rabitt IgG coupled with alkaline phosphatase (Boehringer Mannheim) diluted to 250 mU/ml in TBS (polyclonal) or incubated with sheep anti-mouse IgG coupled with alkaline phosphatase diluted to 250 mU/ml (monoclonal) in TBS 0.5 h. After three rinses with TBS and one rinse with TBS-$MgCl_2$ (0.1M Tris, 0.15M NaCl, 0.005M $MgCl_2 \times 6H_2O$, 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.

a

b
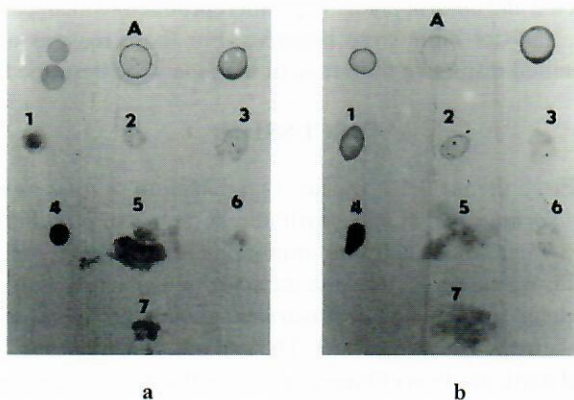
1: *F. culmorum*, 2: *F. graminearum*, 3: *F. avenaceum*, 4: *F. solani*, 5: *F. oxysporum*, 6: *F. tricinctum*, 7: *F. moniliforme*, A = antigens

Fig. 1. Dot blots of *Fusarium* sp. extracts on nitrocellulose: a – polyclonal IgG, b – MAb 30/2
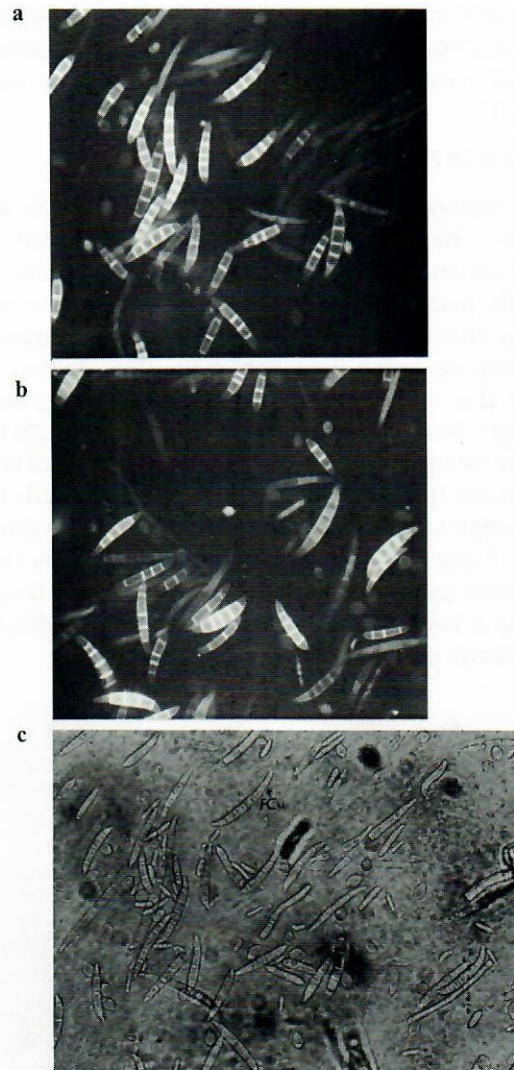
Fig. 2. Immunofluorescent microscopy – conidial mixture of various *Fusarium* species (*F. culmorum, F. graminearum, F. avenaceum, F. oxysporum, F. solani, F. tricinctum, F. moniliforme*): a – polyclonal IgG, b – Mab30/2, c – light microscopy

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

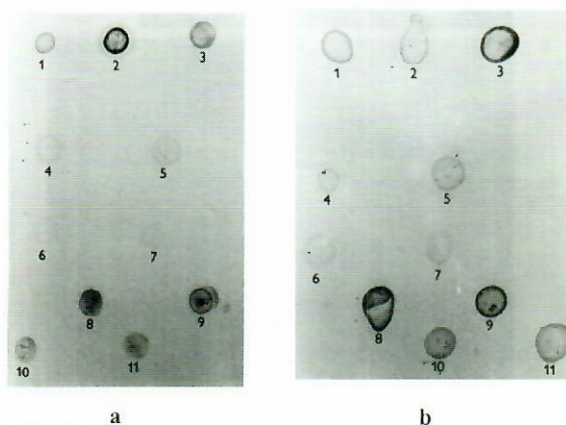| Antigens	Antibodies					
	PAb	$\pm s_x$	MAb30/2	$\pm s_x$	MAb33/8	$\pm s_x$
F. avenaceum	0.72	0.08	0.55	0.02	0.61	0.01
F. equesiti	0.40	0.03	0.25	0.01	0.31	0.01
F. moniliforme	0.61	0.07	0.52	0.02	0.60	0.02
F. nivale	0.20	0.03	0.12	0.01	0.21	0.01
F. oxysporum	0.65	0.03	0.48	0.02	0.53	0.02
F. poae	0.30	0.01	0.31	0.02	0.30	0.02
F. sambucinum	0.80	0.05	0.69	0.03	0.60	0.02
F. solani	0.55	0.05	0.39	0.04	0.45	0.04
F. tricinctum	0.60	0.04	0.51	0.04	0.59	0.04
Gaeumannomyces graminis	–		–		–	
Pseudocercosporella herpotrichoides	–		–		–	
Rhizoctonia solani	–		–		–	

Each value represents the mean of nine replications, ± standard errors of the mean were determined; Concentration of proteins in each antigen = 1µg / ml; PAb: polyclonal antibodies, MAb: monoclonal antibodies

The antibodies did not cross-react with other pathogenic fungi of winter wheat, i.e., *Gaeumannomyces 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 immunoblotings (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.

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

Fig. 3. Dot blots of plant extracts (1 day after infection): a – polyclonal IgG, b – MAb33/8
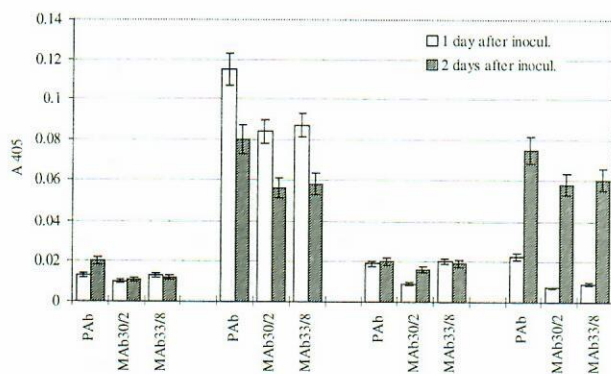
PAb – polyclonal antibodies; MAb – monoclonal antibodies

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
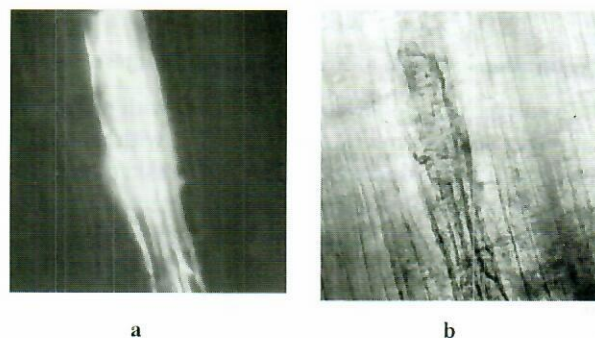

Fig. 5. Infected wheat coleoptile tissue visualised using polyclonal IgG under immunofluorescent microscopy (a) and light microscopy (b)

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 (KYNĚROVÁ *et al.* 1998; PEKÁROVÁ-KYNĚROVÁ & 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 evalua-

tion of data is very important. Based on the measurements we defined a very low positive treshold 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; GINGERY 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. Mono- and polyclonal 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

CONVERSE R., MARTIN R. (1990): Enzyme-linked immunoassay (ELISA) – viruses. In: HAMPTON R., BALL E., BOER S. (Eds.): Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. Amer. Phytopath. Soc., St. Paul, Minnesota, APS Press: 179–197.

DEWEY F. M. (1996): Production and use of monoclonal antibodies for the detection of fungi. In: BCPC Symp. – Proc. No. 65: Diagnostics in crop production: 85–91.

GABLER J., URBAN M. (1995): Evaluation of resistance differences between tomato cultivars to *Phytophthora nicotianae* by indirect ELISA. J. Pl. Dis. Protect., **102**: 275–283.

GINGERY R. (1990): Immunofluorescence – viruses. In: HAMPTON R., BALL E., BOER S. (Eds.): Serological Methods for detection and Identification of Viral and Bacterial Plants Pathogens. Amer. Phytopath. Soc., St. Paul, Minnesota, APS Press: 287–295.

KOVÁČIKOVÁ E., ONDŘEJOVÁ J., VLASÁKOVÁ A. (1992): Vztah hostitel patogen v ontogenezi pšenice ozimé po inokulaci houbou *Fusarium culmorum*. Ochr. Rostl., **27**: 27–30.

KRÁTKÁ J., KYNĚROVÁ B., ZEMANOVÁ A., SÝKOROVÁ S. (1997): The diagnosis of *Fusarium culmorum* by polyclonal antibodies – preparation and character of antigens and antibodies. Ochr. Rostl., **33**: 89–102.

KYNĚROVÁ B., KRÁTKÁ J., ZEMANOVÁ A., PODANÁ M. (1998): The diagnosis of *Phytophthora nicotianae* var. *nicotianae* and *Phytophthora infestans* by polyclonal antibodies – detection of the pathogen in plants. Plant Protect. Sci., **34**: 15–19.

MANKA M., CHELKOWSKI J., BRAYFORD D., VISCONTI A., KWASNA H., PERKOWSKI J. (1989): *Fusarium graminearum* Schwabe (telemorph *Gibberella zeae* Schw. Petch) – Cultural characterists, pathogenicity towards cereal seedlings and ability to produce mycotoxins. J. Pathol., **124**: 143–148.

MESTERHÁZY J., BARTÓK T. (1992): Resistance and pathogenicity influencing toxin (DON) contamination of wheat varieties following *Fusarium* infection. Hodow. Rósl. Aklimat. i Nasien., **37**, part. ii: 9–15.

MILLER S.A., MARTIN R. R. (1988): Molecular diagnosis of plant disease. Ann. Rev. Phytopath., **26**: 409–432.

PEKÁROVÁ-KYNĚROVÁ B., KUTÍKOVÁ M. (1999): Preparation and use of monoclonal atibodies to detect *Phytophthora nicotianae* var. *nicotianae*. Plant Protect. Sci., **35**: 41–46.

VIKLICKÝ V. (1987): Monoklonální protilátky pro diagnostické účely. ÚMG SAV, Praha.

WERRES S. (1988): Enzyme-linked immunosorbent assays (ELISA) as methods for detection of *Phytophthora fragariae* HICKMAN in strawberry roots. Nachr.-Bl. dt. Pfl.-Schutzdienst., **40**: 146–150.

WERRES S., STEFFENS C. (1994): Immunological techniques used with fungal pathogens: aspect of antigens, antibodies and assays for diagnosis. Ann. Appl. Biol., **125**: 615–643.

Souhrn

KRÁTKÁ, J., PEKÁROVÁ B., ZEMANOVÁ A., KUTÍKOVÁ M. (2000): **Využití imunochemických metod pro detekci** *Fusarium culmorum* **v pšenici ozimé**. Pl. Protect. Sci., **36**: 1–6.

Byly připraveny a otestovány monoklonální protilátky (MAb30/2 a MAb33/8) pro detekci *Fusarium culmorum* (*FCu*) v pšenici ozimé. Testování bylo provedeno pomocí nepřímé ELISA metody a bylo zjištěno, že specifičnost připravených MAbs je rodová. Pro testování byly použity druhy rodu *Fusarium* (*oxysporum, solani, graminearum, avenaceum, tricinctum, moniliforme*). Roz-

díly 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 (specifičnost a citlivost) byly obdobné.

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

Corresponding author:

RNDr. JIŘINA KRÁTKÁ, DrSc., Výzkumný ústav rostlinné výroby, odbor rostlinolékařství, Praha 6-Ruzyně, Česká Republika, tel. + 420 2 33 02 23 73, fax: + 420 2 33 31 06 36, e-mail: kratka@hb.vurv.cz