Utilization of Immunochemical Methods to detect *Phytophthora fragariae* in Strawberry Plants

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Abstract

PEKÁROVÁ B., KRÁTKÁ J., SLOVÁČEK J. (2001): **Utilization of immunochemical methods to detect *Phytophthora fragariae* in strawberry plants.** Plant Protect. Sci., **37**: 57–65.

Polyclonal antibodies anti-PfP IgG and anti-PfM IgG and monoclonal antibody MAb29 were prepared to detect a quarantine pathogen of strawberry, *Phytophthora fragariae*. Laboratory rabbits and mice were immunized using purified and unpurified protein extracts from the mycelial mass of the pathogen. Except for *Pythium ultimum*, the antibodies did not cross-react with other pathogenic fungi, such as *Botrytis cinerea, Colletotrichum acutatum, Fusarium* sp., *Verticillium albo-atrum*. PTA-ELISA was used to test the antibodies. *P. fragariae* was detected in artificially infected strawberries (cultivars Elsanta, Kama and Vanda) by means of PTA-ELISA, immunoprinting and dot blot. Detection of the pathogen was optimal in undamaged roots or roots with necrotic tips only. At a later stage of infection, when whole roots were necrotic, the crown was more suitable for successful detection. To detect *P. fragariae* at the early stages of infection it is recommended to use at least two of the three mentioned immunotechniques.

Keywords: *Phytophthora fragariae*; polyclonal and monoclonal antibodies; PTA-ELISA; immunoprinting; dot blot; strawberry

Phytophthora fragariae Hickman is a pathogen of the roots of strawberry, but if the cultivar is very susceptible, the pathogen might spread into the crown and bottoms of petioles. The pathogen was isolated, described and named by HICKMAN (1940). The disease named "red core rot" (ALCOCK 1929) is recorded in many countries (SMITH *et al.* 1997). It was recorded in the Czech Republic in 1961 for the first time and became widespread in 1977 (PETERKA *et al.* 1991). In recent years there has been no record of the pathogen in the Czech Republic.

Phytophthora fragariae is spreading in plants as oospores and chlamydospores. Its diagnosis is usually based on the observation of oospores in roots by light microscopy or on a biological test recommended by EPPO (DUNCAN *et al.* 1986). This test takes up to 5 weeks and the results might not be clear. In dubious cases, isolation of the fungus and use of a selective medium (MONTGOMERIE & KENNEDY 1983) are essential. Therefore, the use of antibodies and of some immunochemical methods is very important for the standardisation of European diagnostic methods. Until now, only polyclonal antibodies with high specificity have been used (AMOUZOU-ALLADAY *et al.* 1988; MOHAN 1988, 1989). Recently, two methods for the detection of *Phytophthora fragariae* were recommended by EPPO Reporting Service 98/070: PCR (BONANTS *et al.* 1997) and ELISA (OLSSON & HEIBERG 1997). With the exception of commercial kits, simple indirect ELISA-based assays, often referred to as plate-trapped antigen assays (PTA-ELISA) (DEWEY 1996), are widely used by practical plant protection services.

In this paper we present results on the detection of *Phytophthora fragariae* in strawberry plants by means of polyclonal and monoclonal antibodies using PTA-ELISA and immunoblotting. Our aims were (1) to prepare poly- and monoclonal antibodies suitable for the detection of *P. fragariae,* (2) to test their specificity and efficiency for diagnosis of *P. fragariae* in strawberry, and (3) to determine which of the techniques is the most suitable and sensitive for the detection of this pathogen in several strawberry cultivars.

The research was based on our previous work on the preparation and utilization of poly- and monoclonal anti-

The research was supported by a Grant No. 522/99/0444 of the Grant Agency of the Czech Republic.

bodies to detect *Phytophthora* (KRÁTKÁ *et al.* 1996, KYNĚROVÁ *et al.* 1998; PEKÁROVÁ-KYNĚROVÁ & KUTÍKOVÁ 1999).

MATERIAL AND METHODS

Pathogen Material. Three isolates of *Phytophthora fragariae* (*PFr*), No. 8138, 8140 and 8147 that had originally been isolated from *Fragaria* sp., were obtained from the Collection of Microorganisms, Brno (Czech Republic). Isolates of other fungi were obtained from the Collection of Phytopathogenic Microorganisms of the Research Institute of Crop Production, Prague-Ruzyně. Isolates of *PFr* and *P. cactorum* (*PCa*), isolated from *Malus* sp., were maintained in Petri dishes on potato dextrose agar, while Czapek-Dox agar was used for *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium* sp. isolated from *Fragaria* sp., *Gliocladium* sp. from soil substrate, *Verticillium albo-atrum* from *Cucumis* sp.

Plant Material. All experiments were done only with artificially inoculated plants. No naturally infected strawberry plants were found in the Czech Republic.

The strawberry cultivars Elsanta, Kama and Vanda were used. Meristem tissues were obtained from the Research and Breeding Institute of Pomology, Holovousy, Ltd., CR. Plants were grown in a controlled room (12L:12D, 20°C) and inoculated by immersing roots in a mycelial suspension of *PFr*, isolate No. 8140 (4×10^6 propagules/ml, counted in a Bürker chamber), 50 ml of inoculum/plant, for 24–48 h at 15–18°C, or of PCa (10^4 propagules/ml). Roots to be used as controls were immersed in distilled water. All plants were subsequently placed separately in a sterile growing substrate at 20–24°C.

Preparation of Plant Extracts. In total, 80 plants of cv. Elsanta were inoculated. Plants were sampled 30 d after inoculation and were put into six classes based on visual symptoms of their roots: 0 – no symptoms or a few necrotic tips; 1 – necrotic tips, withering secondary roots; 2 – approx. 1/3 of the primary roots with rattail appearance; 3 – approx. 1/2 of the primary roots with rattail appearance; 4 – approx. 2/3 of the primary roots with rattail appearance; 5 – rotted roots.

Crowns, roots and root tips (ca 1.5 cm long) were taken from one to five plants. One gram of each plant part was frozen by liquid nitrogen and homogenized with 5 ml of PBS containing 2% polyvinyl pyrrolidone, pH 7.2. Homogenates were extracted for 20 h at 4°C and centrifuged (5000 g, 10 min at 4°C).

Preparation of Antigens. Purified soluble protein fractions were prepared according to KRÁTKÁ *et al.* (1996). Unpurified soluble protein mycelial extracts of lyophilized mycelium, prepared according to KRÁTKÁ *et al.* (1996), were resuspended in PBS pH 7.2 (1 mg/5 ml), centrifuged at 5000 g for 10 min.

Production of Polyclonal Antibodies. Antigens isolated from a mycelial mass of *PFr* (isolate No. 8140) were used for immunization. Laboratory rabbits (*Chinchilla grandis*) were immunized by intramuscular injections with 1 ml of a purified antigen containing 2000 μg of protein per dose (anti-PfP) or with 1 ml of an unpurified antigen containing 100 μg of lyophilized mycelium per dose (anti-PfM). The first injection included Freund's complete adjuvant (day 1), subsequent injections included Freund's incomplete adjuvant (days 14, 21, 28, and 35). Blood was sampled three times at weekly intervals. Antiserum titers were evaluated by agar double diffusion according to OUCHTERLONY (1948) and by PTA-ELISA. IgGs were isolated by precipitation with ammonium sulfate and subsequent ion-exchange chromatography on a DEAE-matrix (HARLOW & LANE 1988).

Production of Monoclonal Antibodies. Female Balb/C mice, 6 to 20 weeks old, were given five intraperitoneal injections of 150–250 μg of purified soluble proteins at 2-week intervals. The antigen was emulsified for the first injection with Freund's complete adjuvant, and with Freund's incomplete adjuvant for subsequent injections. Three days after the sixth injection containing 300 μg of proteins without adjuvant, the mice splenocytes were fused with Sp 2/0 myeloma cells using 50% polyethylene glycol 1300–1600 (Sigma). Growing hybridomas were tested for antibody production 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 spinner flasks to 0.5 liter for large-scale production of MAbs. Antibodies (class IgG) were purified from supernatants of hybridoma cultures growing *in vitro* using caprylic acid (VIKLICKÝ 1987).

PTA-ELISA. PTA-ELISA using polyclonal antibodies, anti-PfP IgG and anti-PfM IgG, was used according to KRÁTKÁ *et al.* (1996). PTA-ELISA with monoclonal antibodies was conducted according to PEKÁROVÁ-KYNĚROVÁ and KUTÍKOVÁ (1999).

Immunoprinting and Dot Blot. Immunoprinting was according to PEKÁROVÁ-KYNĚROVÁ and KUTÍKOVÁ (1999) and the dot blot procedure according to KRÁTKÁ *et al.* (2000).

Prints of crowns and roots or plant extracts on a nitrocellulose membrane were digitized using a scanner and the average intensity (on a grey scale where 0 = 'black' and 255 = 'white') of individual prints was assessed using SigmaScan Pro 5.0 software.

Comparison of Immunotechniques. Immunoprinting and PTA-ELISA were used to detect *PFr* in cv. Elsanta at an early stage of the disease. Infected and control plants were sampled 5 and 10 d after inoculation. Roots and crowns were first printed on nitrocellulose, then extracted and used in ELISA. Anti-PfP IgG was used in both techniques.

PTA-ELISA and dot blot were compared using three strawberry cultivars (Elsanta, Kama and Vanda) 14 d after inoculation. Of each cultivar, three infected plants amd one control plant were used. The infected plants were either without symptoms or had a few necrotic tips (class 0–1).

Statistical Analyses. Differences between absorbances of polyclonal and monoclonal antibodies at antigen concentrations 0–5 μg/ml were studied by ANCOVA with 'antibody' (anti-PfP IgG, anti-PfM IgG, MAb29) as a factor and 'concentration' as a covariate. Absorbance values were log transformed to meet assumptions required by ANCOVA. Individual differences were studied using post-hoc contrasts (ZAR 1999).

Differences between absorbance values of the control and the infected plants observed for different IgGs were expressed as a ratio of means (= mean control/mean infected) and as a *t*-statistic (difference of means divided by pooled standard errors) in order to take account of sample variances.

ELISA and immunoprinting results of infected and control plants were analyzed separately for 5 and 10 d after inoculation using 2-way ANOVA with 'treatment' (control, infection) and 'plant part' (crown, root, root tip) as factors. Subsequently, contrasts were used to compare differences between the control and the infected material. Absorbance values were log transformed to reduce heterogeneity of variances. Grey intensity values were arcsine transformed prior to ANOVA because they are bounded.

RESULTS

Description of Antibodies and *in vitro* Detection of *PFr*

Both antiserum titers were 1:16 by double diffusion in agarose. The titer of anti-PfP evaluated by ELISA was 1:70 000 using an antigen consisting of 10 μg of *PFr* protein extract per ml. The optimal concentrations of polyclonal IgG for ELISA and immunoprinting were determined as 1 μg/ml, 1 μg/ml of MAb29 for ELISA and 4 μg/ml for immunoprinting. Fig. 1 shows a hyperbolic response curve obtained with purified *PFr* antigen coated with 1–10 μg protein/ml and anti-PfP IgG at a concentration of 1 μg/ml in ELISA. The best fit was obtained by a logarithmic model: $y = 0.386 + 0.679 \times \log(x)$. It is apparent from the model that the highest rate of increase of absorbance is between 0–5 μg/ml. If there is a difference between the sensitivity of the different antibodies, it should be most obvious within this interval. ANCOVA revealed that MAb29 had significantly lower absorbance than anti-PfP IgG and anti-PfM IgG ($P = 0.03$). This difference was also manifest in linear models describing the relationship between absorbance and concentration (0–5 μg/ml) of the antibodies (Fig. 2). Models for anti-PfP IgG and anti-PfM IgG did not differ either in slope or in intercept. The sensitivity of polyclonal IgGs was thus higher than that of the monoclonal IgG.

Specificity of the Antibodies

All tested *Phytophthora* species reacted with the antibodies. Unfortunately, MAb29 had higher cross-reactivity with *P. cactorum*, *P. cinnamomi*, and *P. cryptogea* antigens than polyclonal IgGs. There were no cross-reactions with the other fungi, except with *Pythium ultimum*, which cross-reacted markedly with polyclonal antibodies (the reaction with this species was 15% of the reaction with *P. fragariae*) (Table 1).

Detection of *PFr* by ELISA

In 63% ($N = 16$) of plants without any symptoms (class 0), oospores of *PFr* were found (Table 2, Fig. 3). Roots of such plants gave positive reactions in ELISA (i.e. values higher than mean +3.5 × SD of the control plants). The highest percentage of ELISA positive reactions in roots came from plants of class 1 and in crowns of class 3 and 4. The Anti-PfP IgG was used.

The ability to detect infection in a crown and in a root using all IgGs were compared in ELISA readings. Ten plants of class 1, and five healthy plants (i.e. control) were

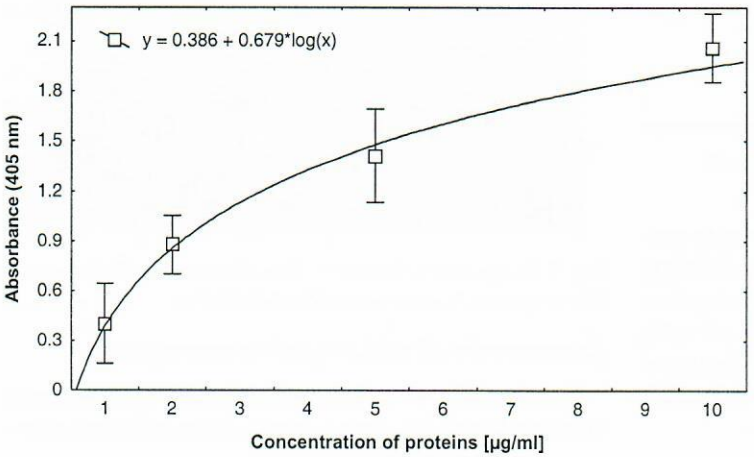


Fig. 1. ELISA response obtained with various concentrations of *PFr* antigen proteins. Wells were coated with 1–10 μg/ml. Anti-PfP IgG at concentration 1 μg/ml was used. The substrate reaction was determined after 60 min. Error bars represent SE

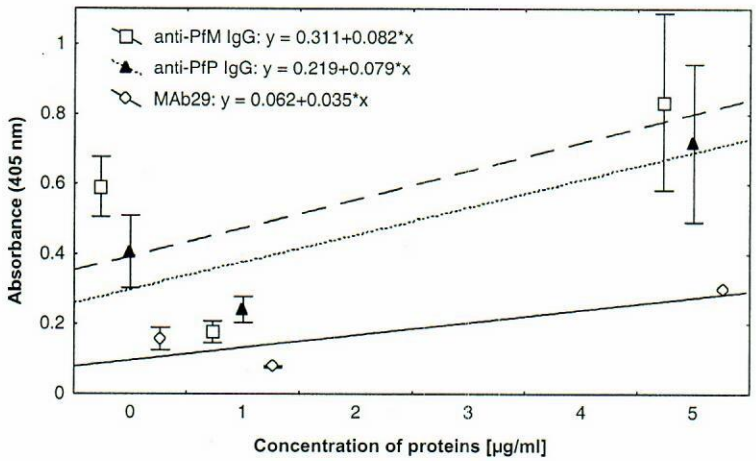


Fig. 2. Comparison of ELISA responses of anti-PfP IgG, anti-PfM IgG and MAb29 obtained with purified *PFr* antigen concentration 0–5 µg/ml. All antibodies at concentration 1 µg/ml were used. The substrate reaction was determined after 60 min. Error bars represent SE

Table 1. Relative level (%) of cross-reactivity of antibodies against *PFr* antigen with antigens of various fungi evaluated by PTA-ELISA. Wells were coated with 1 µg protein/ml. Anti-PfP IgG, anti-PfM IgG and MAb29 were diluted to concentration of 1 µg/ml. Relative level (%) of cross-reactivity was calculated as $100 \times /a$, where a is the absorbance produced by *PFr* antigen and b is the absorbance produced by other antigens (after MOHAN 1989)

Antigen	Antibody		
	anti-PfP IgG	anti-PfM IgG	MAb29
Phytophthora cactorum	30	32	45
P. cinnamomi	45	40	55
P. cryptogea	20	18	25
*P. fragariae**	100	100	100
*P. fragariae***	96	98	98
*P. fragariae****	98	100	101
P. nicotianae	15	20	10
P. parasitica	4	7	3
Pythium oligandrum	5	6	1
P. ultimum	16	15	2
Gliocladium sp.	0	0	
Verticillium albo-atrum	0	0	0
Colletotrichum acutatum	0		
Botrytis cinerea	0		

*:isolate No. 8140, ** isolate No. 8147, *** isolate No. 8138

used for this comparison. Absorbance values of both control and infected material were higher for anti-PfP IgG (0.31 on average) than for anti-PfM IgG (0.16 on average) and MAb (0.019 on average). However, in all IgGs the difference between the control and infected plants was of similar magnitude (ratio (control/infection) =1.6 ...2.1, t = 1.53...1.64).

Table 2. Relative number (%) of infected plants of cv. Elsanta with positive reaction in PTA-ELISA (absorbance values higher than 0.08)

Class	Plant part			
	N	Root Tip	Root	Crown
0	16	75	75	31
1	25	92	92	68
2	20	70	90	80
3	14	43	71	100
4	3	0	100	100
5	2	0	0	50

Plants were classified into six classes based on visual symptoms on the roots: 0 – no symptoms or a few necrotic tips; 1 – necrotic tips, withering secondary roots; 2 – approx. 1/3 of the primary roots with rattail appearance; 3 – approx. 1/2 of the primary roots with rattail appearance; 4 – approx. 2/3 of the primary roots with rattail appearance; 5 – rotted roots. *N* = number of plants in each class. Anti-PfP IgG at a concentration 1µg/ml

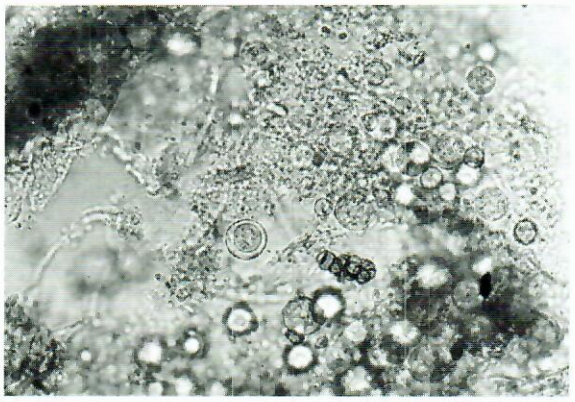


Fig. 3. Oospores of *PFr* in macerated tissue of infected strawberry root, cv. Elsanta (magnification 500×)

Comparison of PTA-ELISA and Immunoprinting

Five days after inoculation the intensity of prints ranged between 17 and 240. Prints of roots from infected materi-

al were significantly darker (ANOVA, $P = 0.007$) than those of the control (Fig. 4A). Although the prints of crowns from infected plants were also darker than those from the control, the difference was only marginally insignificant ($P = 0.06$). Absorbance values varied between 0.02 and 0.12. The values of all plant parts were larger for the infected material than for the control (Fig. 5A). The differences in root and root tip between infected and control material were highly significant (ANOVA, $P < 0.001$), but in the crown the difference was only marginally insignificant (ANOVA, $P = 0.07$).

Ten days after inoculation the intensity of prints ranged between 90 and 240 (Fig. 6). Prints of crown and root of the control material were significantly paler than those of the infected material (ANOVA, $P = 0.02$ for crown, $P = 0.03$ for root, Fig. 4B). Absorbance values varied between 0.02

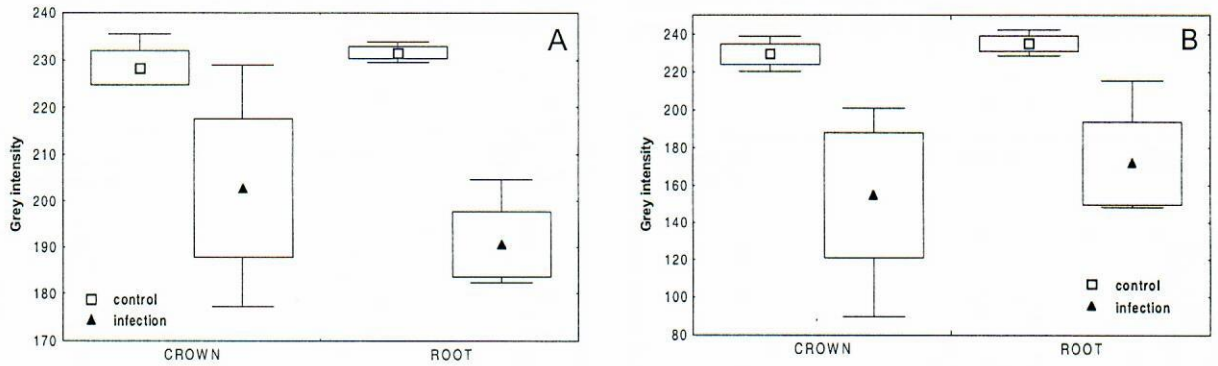


Fig. 4. Immunoprinting 5 (A) and 10 (B) days after inoculation. Comparison of print intensities (0 – black, 255 – white) (mean ± SE [box], min-max [whisker]). Plants of cv. Elsanta were inoculated with *PFr*. Anti-PfP IgG at concentration 1 µg/ml was used

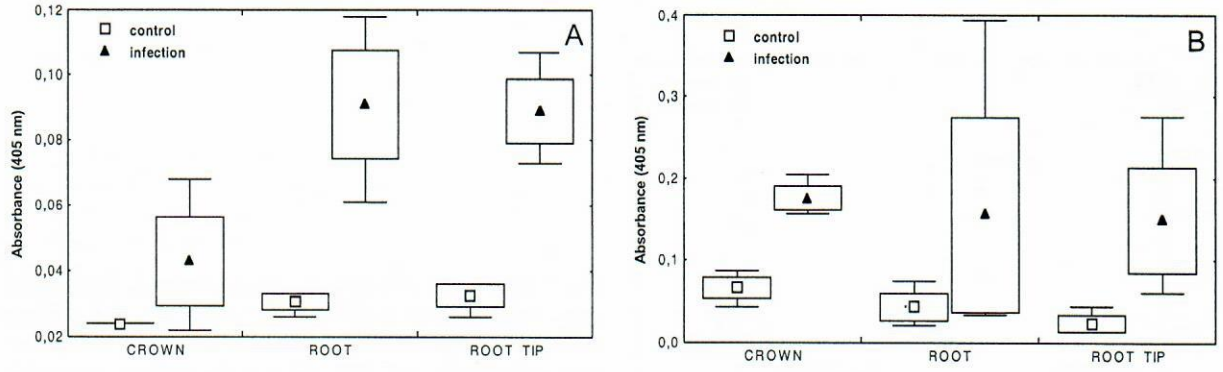


Fig. 5. PTA-ELISA 5 (A) and 10 (B) days after inoculation. Comparison of absorbances (mean ± SE [box], min-max [whisker]). Plants of cv. Elsanta were inoculated with *PFr*. Anti-PfP IgG at concentration 1 µg/ml was used

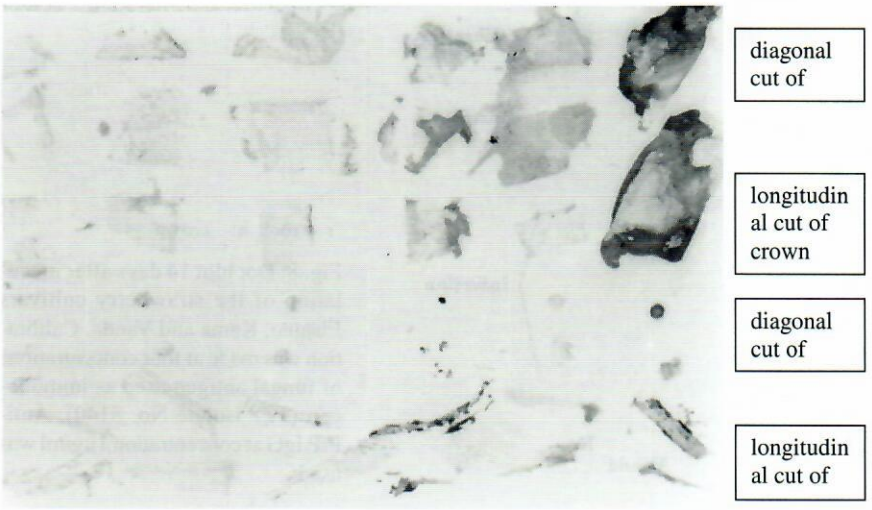


Fig. 6. Immunoprinting – strawberry plants, cv. Elsanta, 10 days after inoculation with *PFr*. Inf 1, Inf 2: class 0 (no symptoms or a few necrotic tips), Inf 3: class 1 (necrotic tips, withering secondary roots). Prints are evaluated in Fig. 4B

and 0.4. The values of crown, root and root tip of the infected material were larger than those of the control (Fig. 5B), but only the root tips differed significantly (ANOVA, $P = 0.01$). Values for the crown and the root were not significantly different (ANOVA, $P > 0.13$).

Comparison of PTA-ELISA and Dot Blot

In all cultivars, the absorbance values obtained from infected roots were significantly higher than those from the control material (ANOVA, $P < 0.001$ for all cultivars, Fig. 7A–C). The absorbance of extracts from control roots was always lower than 0.1. The differences between absorbances of infected crowns and control crowns were statistically significant only in cv. Kama (ANOVA, $P = 0.019$, Fig. 7B). Similar results were obtained using the dot blot: extracts taken from infected roots of all cultivars made markedly darker dots than those of the control plants, but extracts from crowns produced dots of a light intensity which could not be reliably distinguished from the control dots (Fig. 8).

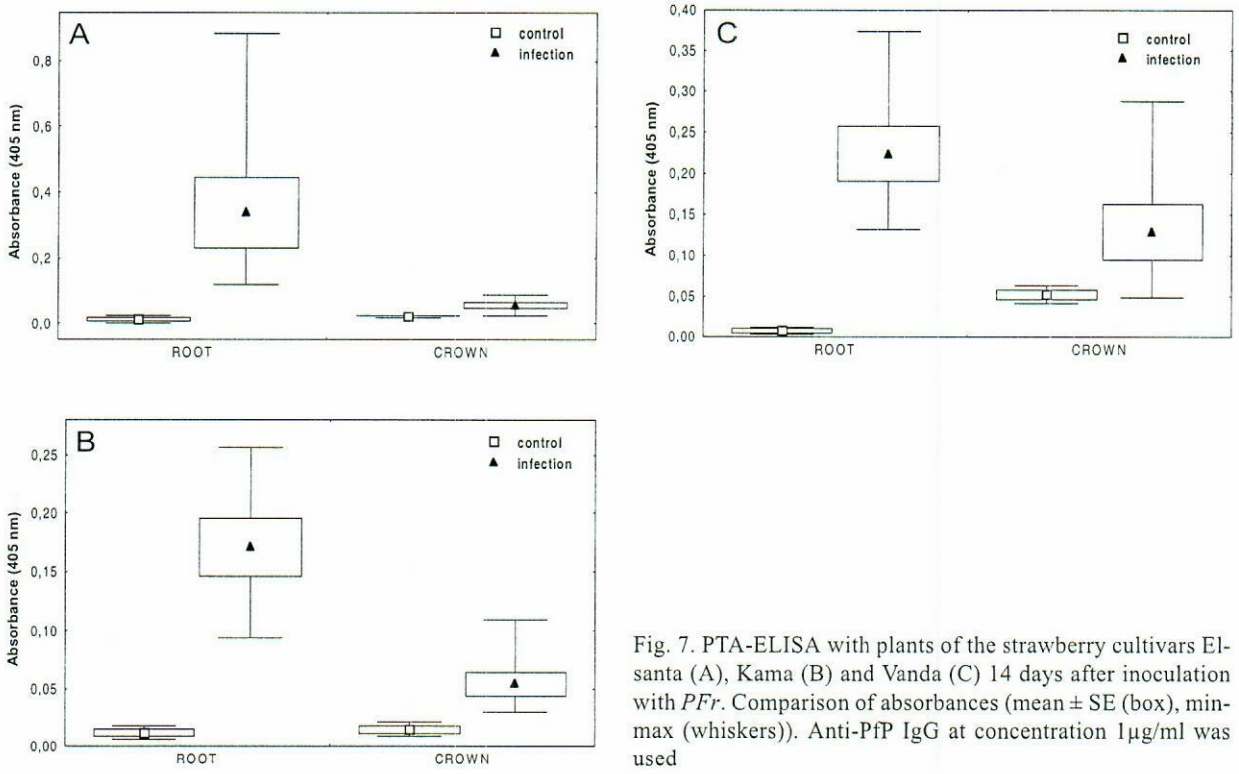
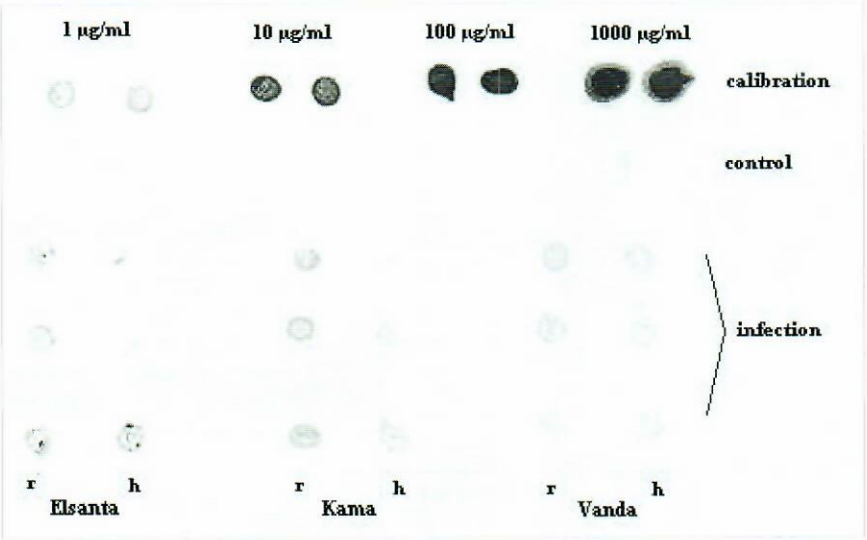


Fig. 7. PTA-ELISA with plants of the strawberry cultivars Elsanta (A), Kama (B) and Vanda (C) 14 days after inoculation with *PFr*. Comparison of absorbances (mean ± SE (box), min-max (whiskers)). Anti-PfP IgG at concentration 1µg/ml was used



r – root, h – crown

Fig. 8. Dot blot 14 days after inoculation of the strawberry cultivars Elsanta, Kama and Vanda. Calibration was made at four concentrations of fungal antigen used as immunogen (*PFr* isolate No. 8140). Anti-PfP IgG at concentration 1µg/ml was used

Detection of *PCa* in Plants

Extracts from roots of infected plants produced higher values of absorbance than extracts from roots of non-inoculated plants with all anti-*PFr* IgG (Table 3). Antibodies cross-reacted with *PCa in vivo*.

Table 3. PTA-ELISA values (mean ± SE) obtained with root extracts of strawberry cv. Elsanta inoculated with *P. cactorum*. Each extract was prepared from roots of three plants 2 weeks after inoculation, when plants appeared healthy. Mean values were calculated from two experiments. Concentrations of antibodies were 1 µg/ml

Strawberry	Antibody		
	anti-PfP IgG	anti-PfM IgG	MAb29
Inoculated	0.460 ± 0.07	0.350 ± 0.05	0.097 ± 0.02
Healthy	0.136 ± 0.02	0.100 ± 0.04	0.021 ± 0.01

DISCUSSION

In phytopathological mycology, immunochemical methods are suitable if the presence of a pathogen in a host can not be unambiguously determined either micro- or macroscopically. We tested these methods on samples of *PFr*, a quarantine pathogen of strawberry, which according to the EPPO list (SMITH *et al.* 1997) occurs in the Czech Republic.

In the beginning it was important to choose plant parts in which the pathogen would be reliably detected. In our previous experiments we found tissues which were visually damaged by the pathogen unsuitable for the detection (KYNĚROVÁ *et al.* 1998; PEKÁROVÁ-KYNĚROVÁ & KUTÍKOVÁ 1999; KRÁTKÁ *et al.* 2000). Later experiments showed us, however, that at an early stage of infection the pathogen can be reliably detected in still undamaged roots or in roots with brown tips only. If the roots were badly damaged (i.e. brown to red-brown tissue, "rattails") it would be more appropriate to identify the pathogen in the crown.

Later, when evaluating ELISA results, we had to find a threshold value for positive reactions. According to CONVERSE and MARTIN (1990) there is no absolute threshold which would separate positive ELISA values for a pathogen from background noise. The absorbance values are obviously affected by the isolation procedure of an antigen from an infected plant, dilution of the antigen, biochemical interactions in the host-pathogen system, inhibition of ELISA metabolites of a host or a pathogen etc. The absorbance value considered as the threshold for positive reactions was obtained by adding the mean value for healthy controls to 3.5 times the value of the standard deviation of the healthy controls (AMOUZOU-ALLADAYE *et al.* 1988).

In apparently undamaged roots of artificially inoculated plants we sometimes found oospores. The presence of oospores in strawberry tissues is considered as confirmation of *PFr* infection (BAIN & DEMAREE 1945). The EPPO instructions agree with this (SMITH *et al.* 1997). However, in our experiments we were often unable to find oospores in infected roots. Microscopical investigations showed us that the distribution of oospores is often clumped, i.e. the oospores were found in one or in a few roots or just a part of it. Finding the oospores was thus rather accidental. In strawberry roots, the oospores occur particularly when there are either no visible symptoms apparent or just brown tips (class 1 or 2 according to our classification).

Another aim of this study was to evaluate the utilisation of immunomethods as diagnostic tools. PTA-ELISA, immunoprinting and dot blot turned out to be suitable for quick detection of the pathogen in a tissue. Although all techniques gave very similar results, after taking into account other aspects such as time, labour and costs, the immunoprinting seems to be the best choice. Unlike in ELISA or dot blot, immunoprinting does not require physical grinding of the tissue, thus saving time and labour. Further, from the prints on the nitrocellulose it is possible to read the distribution of the pathogen in the tissue. For a precise diagnosis of a pathogen, however, it is advisable to use at least two of these techniques.

When comparing the specificity of the prepared antibodies we observed that both poly- and monoclonal antibodies were genus specific. Similar results were obtained also by other authors while investigating antibodies of some fungal pathogens (e.g. DEWEY 1998; COOK *et al.* 1996). Our MAb were only slightly less sensitive than PAb. Since the antibodies are genus specific we were able to detect *PCa* in apparently healthy roots as well. The reactions were similar to that of *PFr*. PCa causes large losses in strawberry plantings. Our experiences showed *PCa* as the most frequent pathogen in strawberry plantings in the Czech Republic. To identify this pathogen in a host plant in the early stages of infection, it is sufficient that the antibodies are genus specific.

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Received for publication August 14, 2000
Accepted for publication March 28, 2001

Souhrn

PEKÁROVÁ B., KRÁTKÁ J., SLOVÁČEK J. (2001): **Využití imunochemických metod k detekci *Phytophthora fragariae* v rostlinách jahodníku**. Plant Protect. Sci., **37**: 57–65.

K detekci karanténního patogenu *Phytophthora fragariae* byly připraveny a otestovány polyklonální protilátky anti-PfP IgG a anti-PfM IgG a monoklonální protilátka MAb29. K imunizaci laboratorních králíků a myší byl použit purifikovaný nebo nepurifikovaný proteinový extrakt z myceliální hmoty patogenu. Všechny připravené protilátky jsou rodově specifické. Křížové reakce nebyly zjištěny s patogenními houbami rodu *Fusarium*, *Verticillium albo-atrum*, *Colletotrichum acutatum*, *Botrytis cinerea*, *Trichoderma* sp. a *Gliocladium* sp. Všechny připravené protilátky mají křížové reakce s houbou *Pythium* sp. Pro testování protilátek byla použita PTA-ELISA. V jahodníku (odrůda Elsanta, Kama, Vanda) byla *Phytophthora fragariae* detekována pomocí PTA-ELISA, imunoprintu a dot blot. Detekce patogenu je optimální v nepoškozených kořenech, příp. v kořenech

s nekrotizovanými špičkami. Při větší nekrotizaci kořene je lépe stanovit přítomnost houby v kořenovém krčku. Při sledování infekce jahodníku v počátečních fázích doporučujeme použít alespoň dvě z jmenovaných imunotechnik. Všechna sledování byla prováděna na uměle infikovaném rostlinném materiálu.

Klíčová slova: *Phytophthora fragariae*; polyklonální a monoklonální protilátky; PTA-ELISA; imunoprint; dot blot; jahodník

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