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

BLANKA PEKÁROVÁ, JIŘÍNA KRÁTKÁ and JAKUB SLOVÁČEK

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

Abstract


Polyclonal antibodies anti-PIP IgG and anti-PM 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 (PETEKENA 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 immunoochemical methods is very important for the standardisation of European diagnostic methods. Until now, only polyclonal antibodies with high specificity have been used (AMOUZOU-ALLAY 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 polyclonal 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-

**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., *Glicladium* 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 × 10⁶ propagules/ml, counted in a Burker chamber), 50 ml of inoculum/planet, for 24–48 h at 15–18°C, or of PCa (10⁷ 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 root tail appearance; 3 – approx. 1/2 of the primary roots with root tail appearance; 4 – approx. 2/3 of the primary roots with root tail 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-PIP) or with 1 ml of an unpurified antigen containing 100 μg of lyophilized mycelium per dose (anti-PIM). 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 Sp2/0 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 MAb. Antibodies (class IgG) were purified from supernatants of hybridoma cultures growing in vitro using caprylic acid (VIKLICKY 1987).

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

**Immunoprinting and Dot Blot.** Immunoprinting was according to PEKÁROVÁ-KYNĚROVÁ and KUTÍKOVA (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-PIP 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 and 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-PIP 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-PIP 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-PIP 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-PIP 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-PIP 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), ooospores 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-PIP 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
Table 2. Relative number (%) of infected plants of cv. Elsanta with positive reaction in PTA-ELISA (absorbance values higher than 0.08)

<table>
<thead>
<tr>
<th>Class</th>
<th>N</th>
<th>Root Tip</th>
<th>Root</th>
<th>Crown</th>
</tr>
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<tr>
<td>0</td>
<td>16</td>
<td>75</td>
<td>75</td>
<td>31</td>
</tr>
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<td>25</td>
<td>92</td>
<td>92</td>
<td>68</td>
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<td>3</td>
<td>14</td>
<td>43</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

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 raitall appearance; 3 – approx. 1/2 of the primary roots with raitall appearance; 4 – approx. 2/3 of the primary roots with raitall appearance; 5 – rotted roots. N = number of plants in each class. Anti-PiP IgG at a concentration 1 μg/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-PiP IgG at concentration 1µg/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-PiP IgG at concentration 1µg/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

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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-PIP IgG at concentration 1μg/ml was used.

**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-PIP 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

<table>
<thead>
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<th>Strawberry</th>
<th>Antibody</th>
<th></th>
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<tbody>
<tr>
<td>Inoculated</td>
<td>anti-PfP IgG</td>
<td>0.460 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>anti-PfM IgG</td>
<td>0.350 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MAAb29</td>
<td>0.097 ± 0.02</td>
</tr>
<tr>
<td>Healthy</td>
<td>anti-PfP IgG</td>
<td>0.136 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>anti-PfM IgG</td>
<td>0.100 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>MAAb29</td>
<td>0.021 ± 0.01</td>
</tr>
</tbody>
</table>

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 (KYNEROVÁ et al. 1998; PÉKÁROVA-KYNEROVÁ & KUTÍKOVA 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, immunoinprinting 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 immunoinprinting seems to be the best choice. Unlike ELISA or dot blot, immunoinprinting 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.

References


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Souhrn


K detekci karantenního patogenu Phytophthora fragariae byly připraveny a otestovány polyclonální protištíky anti-PP IgG a anti-PFM IgG a monoklonální protištíky MAb29. K imunizaci laboratorních králíčků byl použit pyřitý nebo nepurifikovaný proteínový extrakt z mycelialní hmoty patogenu. Všechny připravené protištíky jsou rovněž specifické. Krčkové reakce nebyly zjištěny s patogennimi houby rodu Fusarium, Vorticillium albo-atrum, Colletotrichum acutatum, Botrytis cinerea, Trichoderma sp. a Gliocladium sp. Všechny připravené protištíky mají krčkové reakce s houbou Pythium sp. Pro testování protištítek byla použita PTA-ELISA. V jahodníku (odrůda Elsanta, Karna, Vanda) byla Phytophthora fragariae detekována pomocí PTA-ELISA, imunoprintu a dot blot. Detekce patogenu je optimální v nepoškozených kofenech, příp. v kofenech, ...

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

*Corresponding author:*

Ing. BLANKA PEKÁROVÁ, Výzkumný ústav rostlinné výroby, Praha 6-Ruzyně, Česká republika, tel.: +420 2 33 02 24 19, fax: +420 2 33 31 06 38, e-mail: pekarova@hb.vurv.cz