Utilisation of Immunochemical Methods for Detection of *Colletotrichum* spp. in Strawberry

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Abstract

KRÁTKÁ J., PEKÁROVÁ-KYNĚROVÁ B., KUDLÍKOVÁ I., SLOVÁČEK J., ZEMÁNKOVÁ M. (2002): **Utilisation of immunochemical methods for detection of** *Colletotrichum* spp. **in strawberry**. Plant Protect. Sci., **38**: 55–63.

Four polyclonal and two monoclonal antibodies were prepared and tested to detect a quarantine pathogen of strawberry – *Colletotrichum acutatum*. Only one of them, polyclonal IgG K91, was sensitive enough to recognize the pathogen. This antibody was genus-specific and did not cross-react with several other fungal pathogens of strawberry (*Phytophthora fragariae, P. cactorum, Verticillium albo-atrum, Botrytis cinerea, Pythium ultimum).* Four techniques, PTA-ELISA, dot blot, immunoprint and immunofluorescent microscopy were used to test the specifity and sensitivity of antibodies. After artificial infection of strawberry (cvs Elsanta, Vanda, and Kama), *Colletotrichum acutatum* was detected by PTA-ELISA, dot blot and immunoprint in roots, crowns, petioles and fruits in the latent stage of the disease. For reliable detection in the latent stage it is recommended to use at least two of the mentioned techniques.

Keywords: *Colletotrichum acutatum*; strawberry; pathogenicity; polyclonal antibodies; monoclonal antibodies; PTA-ELISA; immunoprint; dot blot; immunofluorescent microscopy

Colletotrichum is one of the most important genera of plant pathogenic fungi. Many hosts can be infected by a single species of this genus. Conversely, a single host may be infected by several species of *Colletotrichum.* This genus, together with the genera *Phytophthora* and *Fusarium,* belongs to those plant pathogenic fungi most studied in the last years (BAILEY & JEGER 1992; PRUSKY *et al*. 2000; SUMMEREL *et al*. 2001).

Colletotrichum acutatum Simmonds is infectious on many cultivated plants, but on strawberry it causes damage of economical importance. Although it is most detrimental in warmer areas, it has spread to cooler regions where strawberry is grown. According to EPPO it occurs in Central Europe, namely Germany and Austria, but the pathogen has the potential of spreading to all neighbouring countries where strawberry is cultivated. Although absent in the Czech Republic, it is included in the List of Quarantine Pests to prevent its introduction.

To detect the pathogen in a host, EPPO recently recommended a biological method (testing of petioles) and the use of antibodies. For its detection in the latent stage the use of rapid and sensitive methods is very important. Except for the preparation of specific and sensitive antibodies, the utilisation of molecular methods has been intensively studied (BARKER *et al*. 1994; COOK *et al*. 1995; FREEMAN & RODRIGEZ 1995; SMITH *et al*. 1997; CORREL *et al*. 2000; FREEMAN 2000; CANNON *et al*. 2000).

In this paper we present results on preparation and testing of poly- and monoclonal antibodies and on the detection of *Colletotrichum acutatum* in strawberry plants using immunochemical techniques. The antibodies were prepared and tested at the Research Institute of Crop Production (RICP), Prague-Ruzyně, Czech Republic.

MATERIAL AND METHODS

Plant material. All experiments were done with artificially inoculated plants because no naturally infected strawberry plants have so far been found in the Czech Republic.

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Three strawberry cultivars, Elsanta, Kama and Vanda, were used. Meristem tissues were obtained from the Fruit Research and Breeding Institute, Holovousy Ltd., Czech Republic, and grown in a greenhouse.

Strawberry plants (with four to five leaves) were inoculated by immersing roots in a mycelial and conidial suspension of the pathogen (10^4 propagules/ml, 50 ml/plant) for 24 h at 20–22°C in a light chamber. Six isolates of *Colletotrichum acutatum* (CA), as well as isolates of other *Colletotrichum* species were used (Table 1). Control plants were immersed in distilled water. All plants were subsequently placed separately in sterile water at 24°C. Immunotests were carried out after 4–7 d. The infection of plants (visual symptoms) was evaluated 8–12 d after inoculation by determining the presence or absence of lesions on petioles and leaves, wilting or drying of leaves and flowers.

Fruits were inoculated by injecting the inoculum (5 µl) under the calyx or on the surface of ripe fruits. Inoculated fruits were placed separately in a sterile thermostat (22ºC). Immunotests were carried out 1–4 d after inoculation.

Pathogen material. Six isolates of *Colletotrichum acutatum*, one isolate each of *C. coccodes*, *C. dematium*, *C. fuscum*, *C. gleosporoides*, *C. lindemuthianum*, *C. musae*, *Botrytis cinerea*, *Phytophthora fragariae*, *P. cactorum*, *Pythium ultimum* and *Verticillium albo-atrum* were cultivated on Czapek Dox medium at 22°C in Petri dishes. For re-isolation from infected plant material we used also Czapek Dox medium. Isolates were obtained from the Collection of the University of Salamanca, Spain; Collection of Microorganisms, Brno, CR; Collection of the Charles University, Prague, CR; and the Collection of Fungi, RICP Prague, CR (Table 1). Pathogenicity of CA isolates and other *Colletotrichum* species was evaluated by the frequency of symptoms on cv. Elsanta (Table 2).

Preparation of antigens. Purified soluble protein extracts were prepared from lyophilised mycelial mass (conidia and mycelium) according to KRÁTKÁ *et al*. (1996); 1mg of mycelial mass/3 ml PBS was used. Antigens for preparation and testing of antibodies were prepared from all isolates of *Colletotrichum* species, those of *Botrytis cinerea, Phytophthora* spp., *Pythium ultimum, Verticillium albo-atrum*, and from strawberry fruits attacked by *Botrytis cinerea*.

Preparation of plant extracts. Extracts from petioles and roots were prepared 7 d after inoculation. The surface of the plant parts was sterilised by immersing them in aseptic solution SAVO (Biochemie) containing 47.2 g per l NaClO, then in 96% alcohol, and finally in sterile distilled water (SDW). Petioles, crowns and roots were sampled from three plants. Petioles were cut into three parts (each approx. 3 cm long). One gram of each of the plant parts was frozen in liquid nitrogen and homoge-

Table 1. List of tested species and isolates of the genus *Colletotrichum*

Species	Collection No.	Working No.	Symbol	Country of origin	Material
Colletotrichum acutatum	324995	10C	*Ca*	USA	*Fragaria* root
Colletotrichum acutatum	345026	12A	Ca	Spain	*Fragaria* root
Colletotrichum acutatum	*345026	12C	Ca	Spain	*Fragaria* root
Colletotrichum acutatum	345577	30B	Ca	New Zealand	*Fragaria* root
Colletotrichum acutatum	348494	54A	Ca	France	*Fragaria* root
Colletotrichum acutatum	348501	60B	Ca	France	*Fragaria* root
Colletotrichum acutatum	*348501	60C	Ca	France	*Fragaria* root
Colletotrichum acutatum	351587	67B	Ca	USA	Fragaria root
Colletotrichum coccodes	14		Cc	Czech Republic	
Colletotrichum dematium	11		Cd	Czech Republic	*Prunus* fruit
Colletotrichum fuscum	12		Cf	Czech Republic	*Digitalis* sp.
Colletotrichum gloeosporioides	9		Cg	Czech Republic	seeds of *Lupinus*
Colletotrichum lindemuthianum	16		Cl	Germany	*Pisum sativum*
Colletotrichum musae	13		Cm	Czech Republic	*Musa* leaf
Botrytis cinerea	153/3		Bc	Czech Republic	*Fragaria* fruit
Phytophthora fragariae	CH33		Pf	Sweden	*Fragaria*
Phytophthora cactorum	S10		Pc	Czech Republic	*Fragaria*
Pythium ultimum	F290		Pu	Czech Republic	*Cucumis*
Verticillium albo-atrum	F526		Vaa	Czech Republic	*Acer* root

*after previous re-isolation from strawberry plants

nised with 3 ml of PBS containing 2% polyvinylpyrolidine (pH 7.2). Homogenates were extracted for 24 h at 4ºC and centrifuged (5000 g, 10 min at 4ºC). The extracts were used for PTA-ELISA and dot blot.

Tissues from fruits were sampled 1–4 d after inoculation. The surface of fruits was sterilised by 15% SAVO and in SDW. Fruits were cut up into two or three parts (basal, medium and tip). One gram of each of the tissues was homogenised in 5 ml of PBS containing 2% of polyvinyl pyrolidine (pH 7.2). Homogenates were extracted for 24 h at 4ºC and centrifuged (5000 g, 10 min at 4ºC). The extracts were used for PTA-ELISA. A diagonal cut through the tested part of fruits was directly printed on a nitro-cellulose membrane.

Preparation of polyclonal antibodies. Antigens isolated from the mycelial mass of a CA isolate were used for immunisation. Laboratory rabbits (*Chinchilla grandis*) were immunised by intramuscular injection with 1ml of antigen (antigens contained 1401–1662 µg of proteins per dose, respectively). The first injection included Freund's complete adjuvant (day 1), subsequent injections included Freund's incomplete adjuvant (days 14, 21, 28, 35). Blood was sampled three times at weekly intervals. Antisera titres were evaluated by PTA-ELISA. IgGs were isolated by precipitation with ammonium sulphate and subsequent ion-exchange chromatography on DEAE-matrix (HARLOW & LANE 1988).

Preparation of monoclonal antibodies. Female Balb/C mice 15–20 weeks old were given five intraperitoneal injections of 250 µg purified soluble proteins at two-week intervals. The antigen A 200 was emulsified with Freund's complete adjuvant, and with Freund's incomplete adjuvant for subsequent injections. Three days after the sixth injection containing 300 µg of protein without adjuvant, the mice splenocytes were fused with Sp2/0 myeloma cells. Monoclonal antibodies were prepared and tested according to PEKÁROVÁ *et al*. (2001). Antibodies (class IgG) were purified from supernatants of hybridoma cultures growing *in vitro* using caprylic acid (VIKLICKÝ 1987).

PTA-ELISA. PTA-ELISA was used according to KRÁTKÁ *et al*. 1996 (polyclonal antibodies), and according to PEKÁROVÁ-KYNĚROVÁ & KUTÍKOVÁ 1999 (monoclonal antibodies).

Immunoblot (tissue immunoprint and dot blot). The tests were done according to PEKÁROVÁ-KYNĚROVÁ & KUTÍKOVÁ (1999) and KRÁTKÁ *et al*. (2000).

Immunofluorescent microscopy. The tests were performed according to KRÁTKÁ *et al*. (2000).

RESULTS

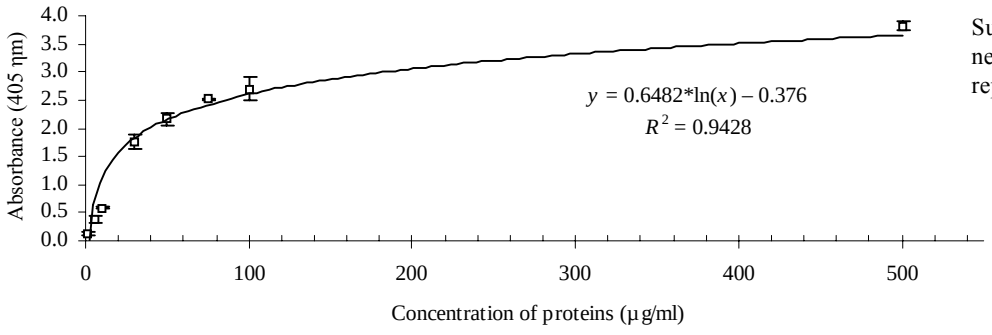
Description of antibodies, specificity and sensitivity. In preliminary experiments we found that antigen A 200 (mixture of isolates 12A and 12C) was the best immunogen. Using this antigen, we prepared four polyclonal and two monoclonal antisera. All prepared antisera were genus-specific, but only one polyclonal antiserum, IgG K91, showed high sensitivity and was suitable for subsequent tests.

The titre of this antibody, as evaluated by PTA-ELISA, was 1:50 000 using an antigen containing 10 µg of CA protein extract per ml.

Fig. 1. shows a hyperbolic response curve obtained with purified CA antigen at a concentration of 1–500 µg protein/ml and antibody IgG K91 at a concentration of 1µg per ml in ELISA. The best fit was obtained by a logarithmic model: $y = 0.648 \times \ln(x) - 0.376$, from which it is apparent that the highest rate of absorbance is between 0–50 µg/ml.

Using PTA-ELISA and dot blot we found that IgG K91 was genus-specific and did not show cross-reactions with other fungi pathogenic on strawberry (Figs. 2 and 3).

Fluorescent microscopy was sensitive enough to recognise conidia of CA (Fig. 9). The concentration of antigen in PTA-ELISA and dot blot was 10 µg/ml. The optimal concentration of IgG K91 for PTA-ELISA and dot blot was 1 µg/ml, for the immunofluorescent microscopy it was 4 µg/ml.



Substrate reaction determined after 45 min, error bars represent SD

Fig. 1. ELISA response obtained with various concentrations of *C. acutatum* antigen proteins

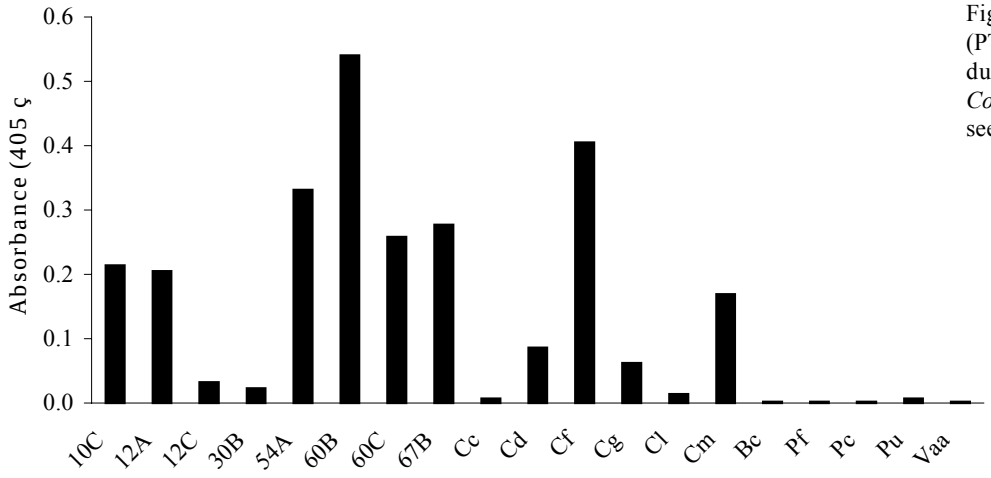
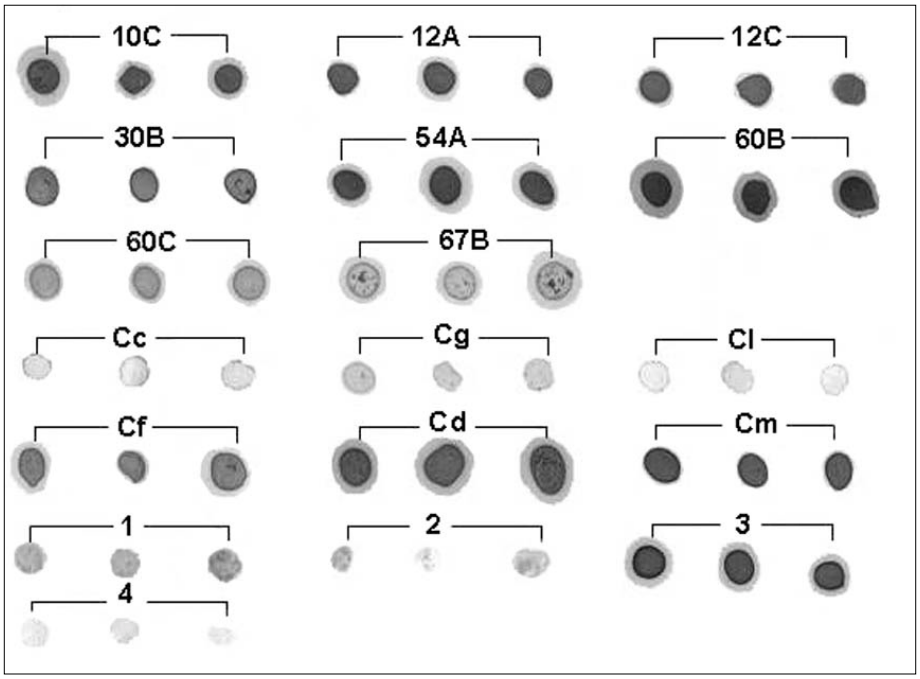


Fig. 2. Specifity of IgG K91 (PTA-ELISA) against individual isolates of the genus *Colletotrichum* (for symbols see Table 1)



1 – *Pythium ultimum;* 2 – *Botrytis cinerea*; 3 – *Phytophthora fragariae*; 4 – *Verticillium albo-atrum*

Fig. 3. Specifity of IgG K91 (dot-blot) against individual isolates of the genus *Colletotrichum* (for symbols see Table 1)

Detection of CA in strawberry plants and fruits. PTA-ELISA tests gave positive reactions with roots and crowns of all cultivars 7 d after inoculation with all isolates of CA (Table 1); at this time there were no visible symptoms. In petioles the infection was detected only in cv. Elsanta (Fig. 4). Similar results were obtained using dot blot (Fig. 5). Extracts of plant samples were used in both techniques.

Infection was manifested by the appearance of lesions on petioles, wilting of leaves or flowers and their gradual drying (spreading from dried spots) (Table 2).

Using PTA-ELISA we detected CA (all isolates) in the basal part of fruits from 1 to 4 d after inoculation. The positive reaction was confirmed by immunoprint of a cut at one-third of the fruit (Figs. 6 and 7). Symptoms were not visible at this time. They appeared quickly within a day in inoculated fruits.

In fruits, watery and often brown spots turned up first at the site of inoculation but within the next day the spots enlarged. As the first symptoms appeared (about 8 d after inoculation) no immunotests were carried out.

Inoculation of plants and fruits of cv. Elsanta with other *Colletotrichum* species showed symptoms very similar to one another, except for *C. dematium* and *C. lindemuthianum* which gave no symptoms.

The presence or absence of the pathogen was confirmed by its re-isolation from plant parts that appeared to be infected. The fact that other species of the genus *Colle-*

Table 2. Evaluation of pathogenicity of *Colletotrichum* spp. in cv. Elsanta

Isolate	Frequency and type of symptoms										Count of positive symptoms
	stipules		petioles – lesions		the oldest leaves		leaves		flowers		
	brown	rot	on base	along petioles	wilt	dry	wilt	dry	wilt	dry	
10C	**	*	**	*	**	*	**	*			12
12A	**		*	**			**	*			8
12C[1]	**	**	**	**	**		**	*			13
30A	**	*	**	**	**	**	**	**	**		17
54A	*			*	**	*	**	*	*		9
60B	*		**	*	*	*	**	*	**		11
60C[1]	**		**	**	**	*	**	**			13
67B	**	**	**	**	**	*	**	*	*	**	17
Cf	**	*	*		*	*	**				8
Cm	*	*	*		*	*	**				7
Cg	**	*	**	*	**	*	*	*	*		12
Control	*						*				2

[1] After previous re-isolation from a strawberry plant
Assessed 10 d after inoculation, 10 plants were used for every isolate
*symptoms on up to 50% of plants; **symptoms on more than 50% of plants

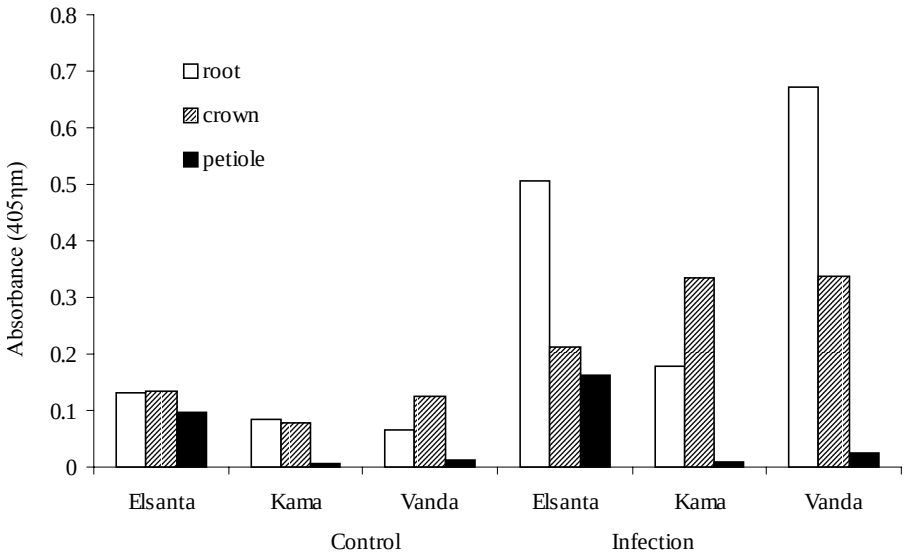
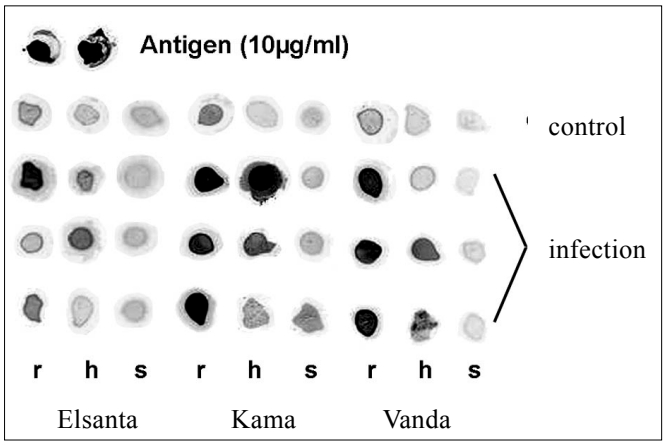


Fig. 4. Detection of *Colletotrichum acutatum* isolate 12A by PTA-ELISA in strawberry plants (cvs Elsanta, Kama and Vanda) 7 d after artificial infection



r – root, h – crown, s – petiole

Fig. 5. Detection of *Colletotrichum acutatum* isolate 30A by dot-blot in strawberry plants (cvs Elsanta, Kama and Vanda) 7 d after artificial infection

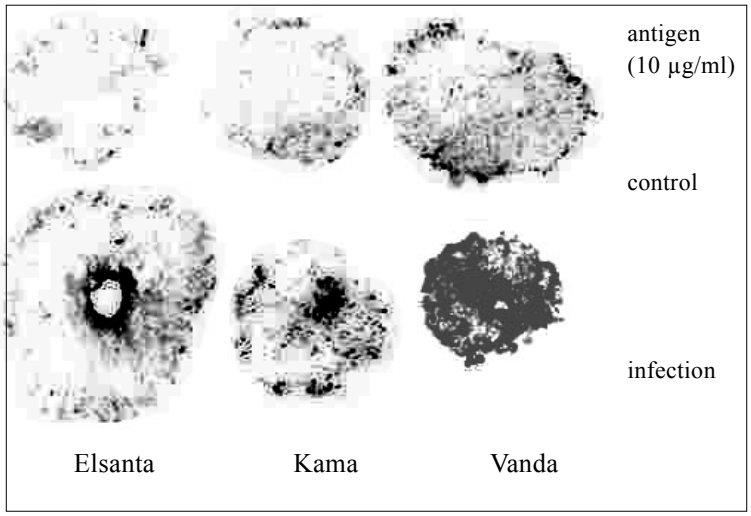


Fig. 6. Detection of *Colletotrichum acutatum* isolate 54A by immunoprinting in strawberry fruits (cvs Elsanta, Kama and Vanda) 4 d after artificial infection

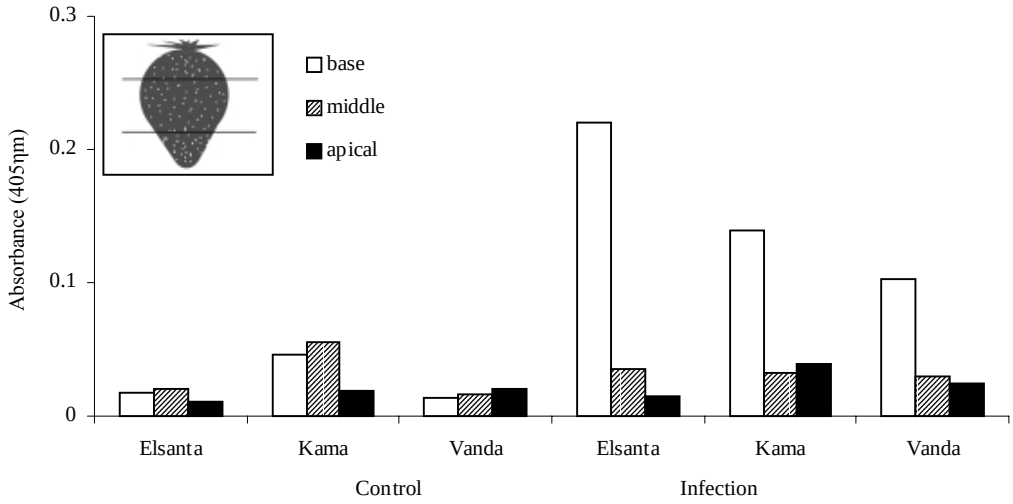
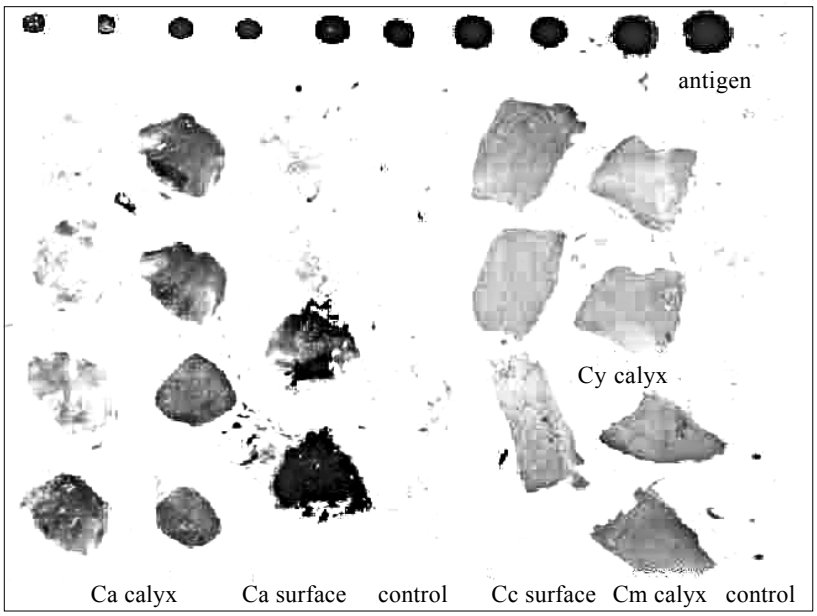


Fig. 7. Detection of *Colletotrichum acutatum* (isolate 12C) by PTA-ELISA in strawberry fruits (cvs Elsanta, Kama and Vanda) 4 d after artificial infection



Ca (Cc, Cm, Cg) calyx – infection under calyx
Ca (Cc) surface – infection on the surface of fruits
Ca – isolate 54A
Antigen at concentration 2, 20, 200, 700 and 1400 µg/ml, respectively

Fig. 8. Detection of *Colletotrichum* spp. by immunoprinting in strawberry fruits 4 d after artificial infection

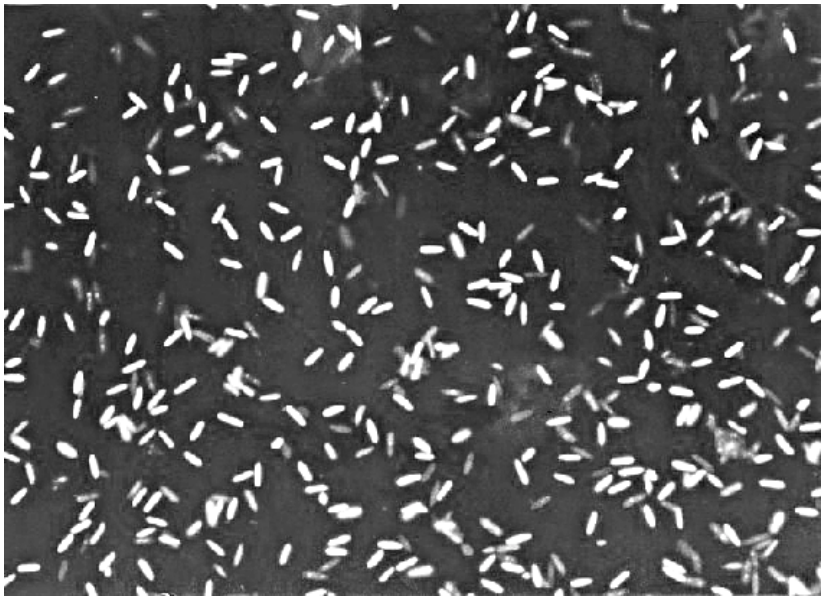


Fig. 9. Conidia of *Colletotrichum acutatum* (immunofluorescent microscopy, magnification 500×)

totrichum also infect strawberry was used to gauge the genus-specificity and sensitivity of the antibodies. The presence of *Colletotrichum* species was detected by means of immunoprint in inoculated plants and fruits when no symptoms were visible (Fig. 8.).

In all tests we used IgG K91 at a concentration 1 µg/ml and 250 mU/ml of sheep anti-rabbit IgG coupled with alkaline phosphatase. In dot blot we applied 2 µl of extracts on a nitro-cellulose membrane.

Based on the results obtained at different concentrations using ELISA we conclude that a positive reaction of antibody with antigen occurs when $A_{405} > 0.12–0.15$.

DISCUSSION

Its ability to cause a latent infection makes the genus Colletotrichum among the most important after-harvest pathogens. Identification of these pathogens is based on the morphology of the fungi growing on a natural substrate and on its appearance in culture. In some cases such information is augmented by data on host specificity. As a result of great variability in morphology, wide spectrum of hosts, and marked effect of abiotic factors (including geographical distribution) there have been many misidentifications. Therefore, taxonomists began to employ molecular methods in order to standardise the protocol used for reliable identification. There exists no suitable taxonomical system for this genus today (SUTTON 1992; FREEMAN 2000; CANNON *et al*. 2000).

Antibodies cannot be used for taxonomic purposes at present, as the majority of them are only genus-specific. But they can be used in plant protection to quickly detect latent infection in plants. For this purpose the identification of the fungal genus is often sufficient. Thanks to utilisation of modern methods and intensified taxonomical studies in recent years it is possible to expect clarification, which may allow a positive diagnosis of the causal organism (FREEMAN & RODRIGUEZ 1995; FREEMAN 2000).

Surprisingly, we observed a rapid manifestation of symptoms after artificial inoculation of strawberry with various *Colletotrichum* species. This supports partly the fact that *Colletotrichum* species have a wide range of hosts, but since screening for pathogenity was not the aim of our study we did not analyse this aspect further. Instead, our results served to verify specificity and sensitivity of the prepared antibodies. Visual symptoms confirmed the presence of the fungi in plant tissue, which was analysed using immunotesting. Positive results were obtained with healthy-looking plants.

Colletotrichum acutatum has not yet been found in strawberry in the Czech Republic. We prepared the antibodies and performed tests on isolates coming from laboratory collections. Because CA is a quarantine pathogen, all our results are based on artificial inoculations in laboratory.

BARKER *et al*. (1994) and COOK *et al*. (1995) dealt with the preparation of antibodies for the detection of *C. acutatum*. They found that an antibody prepared from a protein antigen extracted from spores may not detect the presence of the pathogen's mycelium. Therefore, the authors tested antibodies when antigen from sporulating or unsporulating cultures was used. The authors give much detailed information, showing that the preparation of suitable antibodies is not simple. They prepared genus-specific monoclonal antibodies, which were able to detect both mycelium and spores.

We prepared antigens from cultures without previous selection of or restriction to sporulation. For the preparation of antigens we used a natural mixture of mycelia and spores grown on a culture medium. This fact may influence the individual quality, sensitivity and specificity of antibodies. We stemmed from a suggestion that selection and manipulation of the initial material may affect this variable pathogen even more, and thus lower the potential to detect it in a host.

Even though the initial material for the preparation of antigens and antibodies was quite rich, we obtained only one polyclonal antibody (K 91) that was able to detect the pathogen in tissues in a latent stage and did not cross-react with other fungal pathogens of strawberry.

Based on the results we summarise that a latent infection of strawberry by *C. acutatum* can be detected using antibody K 91. After artificial infection of roots we observed gradual presence of the pathogen in roots and crown, and in cv. Elsanta even in petioles although visual symptoms were not apparent. The utilization of immunotests is possible for the protection and certification of material for cultivation. Suitable techniques are PTE-ELISA, dot blot and immunoprinting.

Using artificially inoculated fruits we verified the possibility to detect the pathogen in tissues of the host. We assume that for ordinary practical use (detection of the latent stage of the pathogen) this mode is not suitable. Since visual symptoms of the disease appear rapidly after inoculation, immunodiagnosis of the pathogen in ripening fruits would not help to assure the health condition of strawberry. To verify the presence of the pathogen in tissues by means of antibodies it is advised to use at least two tests.

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Souhrn

KRÁTKÁ J., PEKÁROVÁ-KYNĚROVÁ B., KUDLÍKOVÁ I., SLOVÁČEK J., ZEMÁNKOVÁ M. (2002): **Použití imunochemických metod ke stanovení** *Colletotrichum* spp. **v jahodníku**. Plant Protect. Sci., **39**: 55–63.

Byly připraveny čtyři polyklonální a dvě monoklonální protilátky pro detekci *Colletotrichum acutatum* – karanténního patogena jahodníku. Pouze jedna polyklonální protilátka, IgG K91, byla dostatečně vhodná k identifikaci patogena v hostiteli. Tato protilátka je rodově specifická a nevykazuje křížové reakce s dalšími houbovými patogeny jahodníku (*Phytophthora fragariae*, *P. cactorum*, *Verticillium albo-atrum*, *Botrytis cinerea*, *Pythium ultimum*). Pro testování specifity a citlivosti protilátek byly použity čtyři techniky: PTA-ELISA, dot blot, imunoprint a imunofluorescence. Po umělé infekci bylo *Colletotrichum acutatum* detekováno v jahodníku (odrůdy Elsanta, Vanda a Kama) v latentním stadiu onemocnění v kořenech, kořenových krčcích, řapících a plodech pomocí PTA-ELISA, dot blotu a imunoprintu. Pro bezpečnou detekci patogena v latentním stadiu je doporučeno použít alespoň dvě imunotechniky.

Klíčová slova: *Colletotrichum acutatum*; jahodník; patogenicita; polyklonální a monoklonální protilátky; PTA-ELISA; imunoprint; dot blot; imunofluorescence

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