Serological and Biochemical Distinguishing of *Pseudomonas syringae* Pathovars on Peas*

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Abstract

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Polyclonal antisera to detect and determine two related pathovars, *Pseudomonas syringae* pv. *pisi* and *Pseudomonas syringae* pv. s*yringae,* were prepared. Untreated bacterial cells, or fixed by formaldehyde or glutaraldehyde were used as antigens. After cross-absorption with heterologous antigens, antisera revealed a high level of specificity in slide agglutination, Ouchterlony gel double-diffusion and in DAS-ELISA and PTA-ELISA. Each of 14 *P. s. pisi* prepared polyclonal antisera could detect and determine all strains of *P. s. pisi,* regardless of race. PTA-ELISA was the most appropriate serological test to distinguish *Pseudomonas syringae* pathovars on peas. Most of the *P. s. pisi* strains from foreign collections were in serological tests confirmed as *P. s. pisi*, while most of the Czech strains suspected as *P. s. pisi* were determined as *P. s. syringae* strains. The principal biochemical reaction, i.e., use of DL-homoserine as a carbon source to grow *P. s. pisi* but not *P. s. syringae*, was proved not to be sufficiently reliable to distinguish both pathovars.

Key words: bacterial blight of peas; *Pseudomonas syringae* pv. *pisi; Pseudomonas syringae* pv. *syringae;* slide agglutination; Ouchterlony gel double-diffusion; ELISA

Two very closely related plant pathogenic bacteria are associated with peas. *Pseudomonas syringae* pv. *pisi* causes significant economic damage, particularly during cold and wet springs and summers (TAYLOR 1972; STEAD & PEMBERTON 1987; SCHMIT 1991; HOLLAWAY & BRETAG 1995). *P. syringae* pv. *syringae* is a less virulent pathogen which often spreads after hail damage or under excessively wet conditions (WIMALAJEEWA & NANCARROW 1984). The dissemination of *P. s. pisi* is restricted by quarantine and seed certification (SMITH *et al.* 1997). Bacterial blight on peas is sometimes confused with similar symptoms caused by *Pseudomonas viridiflava,* but this pathogen is usually associated with frost injury. It is possible to distinguish *P. s. pisi* from *P. s. syringae* and *P. viridiflava* by the results of a DL-homoserine test (HIDEBRAND 1973). Laboratory tests reported in the literature to distinguish both *P. syringae* pathovars include an inoculation of young bean pods and immature lemons (WIMALAJEEWA & NANCARROW 1984), stem inoculation of specific pea cultivars (MALIK *et al.* 1987) and a set of typing phages (TAYLOR & DYE 1972). LYONS *et al.* (1995) developed a rapid method that permitted simultaneous detection of bacterial, viral and fungal pathogens on peas. The method is based on the use of an enrichment medium that permitted detection of *P. s. pisi* isolates from seed-soak fluid in low concentration. The pathogen was detected by an ELISA test with polyclonal antiserum prepared against a lipopolysaccharide (LPS) extract of *P. s.* pv. *pisi* (LYONS & TAYLOR 1990). The serological methods to distinguish the mentioned pathovars in this paper are likewise based on polyclonal antibodies.

MATERIAL AND METHODS

Bacterial Cultures and Isolates: Foreign strains of *P. s. pisi* were obtained from France (Dr J. Schmit, Centre INRA d'Angers, Beaucouze cedex), Germany (Prof. K. Naumann, Federal Centre for Breeding Research on Cultivated Plants, Aschersleben), United Kingdom (Dr J. D. Taylor, Horticulture Research International, Wellesbourne), Denmark (unknown) and Hungary (Prof. Z. Klement, Plant Protection Institute, Budapest). They were compared with Czech isolates from pea fields (Table 1). The strains from England were all known races (seven). All bacterial strains and isolates were cultured on King's B medium (KING *et al.* 1954), 48–72 h at 23°C.

Use of DL-homoserine: Use of DL-homoserine as the sole source of carbon in medium C was according to DYE (1968). Filter-sterilized DL-homoserine was incorporated (1 g/l) into the basal medium adjusted to pH 7.2 (STAI-

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NER *et al.* 1966). A comparison was performed on the same medium without DL-homoserine. Thirty µl of bacterial suspension was placed on the surface of the medium; growth of the organisms was observed after 3, 7 and 14 days incubation at 23–25°C.

Preparation of Antisera: Strain *P. s. syringae* 4073 and seven *P. s. pisi* isolates (Table 2) were chosen for preparation of polyclonal antisera. The bacterial cultures were maintained in heavy suspensions in sterile saline (8.5 g NaCl, 1000 ml water, pH 7.2) and then centrifuged for 10 min at 10 000 g. The formaldehyde-fixed and glutaraldehyde-fixed antigens were prepared by treating a suspension containing 10^9 CFU/ml, with formaldehyde at a final concentration of 1%, or with glutaraldehyde at a final concentration of 0.1%. These suspensions were incubated overnight at 23°C and centrifuged for 10 min at 10 000 g. The cells were resuspended in sterile saline and maintained at 4°C until required. Antigens were adjusted to 5×10^6 CFU/ml and used to inject into each of two rabbits per strain at weekly intervals. For the first and second injections 1 ml of each antigen preparation was mixed 1 : 1 with Al-Span-Oil adjuvant and injected intramuscularly. Three subcutaneous injections of 0.5, 1.0 and 1.5 ml of bacterial suspension on days 15, 22 and 29 were then made. On the 10^{th} day after the last injection, the rabbits were bled at the marginal ear vein. The blood was clotted for 1 h at 37°C, refrigerated overnight and fractioned by centrifugation at 3000 g for 20 min. Antisera were titred in a slide agglutination by mixing equal amounts of serial dilutions (ranging from 1 : 2 to 1 : 1024) of each antiserum and a bacterial suspension (10^9 CFU per ml). All antisera were tested against homologous and heterologous antigens of *Agrobacterium, Erwinia, Clavibacter* and *Xanthomonas* genera, and against those of the related species *Pseudomonas viridiflava* and *P. fluorescens* and of related pathovars which for *P. s. pisi* it was *P. s. syringae* and *P. s. phaseolicola,* while for *P. s. syringae* it was *P. s. pisi* and *P. s. phaseolicola* (Table 2). The better of each two antisera was then used for serological distinguishing tests.

Slide Agglutination (SA): SA tests were conducted by mixing equal amounts (80 µl) of diluted antiserum and a bacterial suspension on a slide. In most studies, suspensions of living cells (10^9 CFU/ml) in saline were used to react with antiserum. The mixtures were incubated for 20–60 min in a humid chamber and observed with a stereoscope Meopta for an agglutination reaction.

Ouchterlony Gel Double-diffusion (ODD): Tests were done by the OUCHTERLONY (1958) method using two different media. The first medium (M1) contained 400 ml H_2O; 60 g electrophoretic agar; 4.3 g NaCl; 0.1 g NaN_3; 0.1 g trypan blue; pH 7.0–7.2. The second medium (M2) contained 500 ml H_2O; 3.5 g Difco agar; 0.12 g NaN_3; pH 6.8–7.0. Central wells were filled with diluted antisera, peripheral wells with concentrated suspensions (10^9 CFU/ml) of bacterial cultures (living cells). Twenty µl of antigen or antiserum was placed in each well. The plates were incubated for three days at room temperature in a humid chamber to intensify band development. Bands were observed by eye and under indirect light. Antisera with high value of heterologous titer in SA and ODD were treated by cross-absorption (AZAD & SCHAAD 1988). Sometimes it was necessary to repeat this procedure up to three times.

Enzyme-linked Immunosorbent Assay (ELISA): The DAS-ELISA technique of CLARK and ADAMS (1977) and PTA-ELISA technique of HARLOW and LANE (1988) were used to evaluate the serological relationship of different isolates. ELISA tests were optimized with different IgG, IgG-AP, anti-rabbit IgG-AP and antigen concentrations. Antigens were diluted to concentrations of 10^7–10^3 CFU/ml. The first antibody – IgGs purified from the rabbit polyclonal antisera were used in concentrations 0.25–0.05 mg/ml. Conjugate IgG-AP (HARLOW & LANE 1988) for DAS-ELISA was used in dilution 1 : 100–1 : 2000, and for PTA-ELISA anti-rabbit IgG-AP (Boehringer Mannheim Biochemica, antiserum from sheep) was used at the recommended concentration 200 mU/ml. The readings of the hydrolyzed substrate (*p*-nitrophenyl phosphate at 1mg/ml) were made at 405 nm at 60 min. Optical density values greater than twice that of the negative control (*Erwinia amylovora*, 10^6 CFU/ml) were considered positive.

RESULTS AND DISCUSSION

All foreign strains representing all seven races and the Czech isolates of *P. s. pisi* listed in Table 1 were tested for utilization of DL-homoserine as the sole source carbon. Most of the foreign reference strains of *P. s. pisi* utilized DL-homoserine, which corresponds with the literature (HILDEBRAND 1973). Negative reactions were observed with strains 265 and 267 from Denmark, and 948, 974 and 981 from France. The Czech isolates HX2 and HX5 gave a negative DL-homoserine reaction, while the others (H1, H5, H6, HX1and HX4) were positive. The culture of Czech collection P. *s. syringae* CCM 2868 utilized DL-homoserine, but *P. s. syringae* CCM 4073 did not.

This biochemical test for utilization of DL-homoserine, when used for classification and identification, does not take advantage of the strict diversity of the two pathovars *P. s. pisi* and *P. s. syringae.*

The polyclonal antisera titrations for determination of *P. s. pisi* in SA are summarized in Table 2. They ranged between 1 : 128 and 1 : 2048. Titre differences between antigens were due to variation in immunological response by the animals used (MAZAREI *et al.* 1992). All seven polyclonal antisera against *P. s. pisi* reacted with isolates from all seven races independently on form of antigen, immunisation schedule and race of used antigen.

The Ouchterlony gel double-diffusion and ELISA tests were done only with the two selected antisera PS9 and

Table 1. Origin of bacterial cultures of *Pseudomonas syringae* pathovars used in this investigation

Source	Bacterial strain	Pathovar
France	30, 815, 937, 948, 974, 981	*Psp*
Germany	PP01, 296, 298, 299, 300, 436	*Psp*
England	299(1), 202(2), 870(3), 895(4), 974(5), 1704(6), 2491(7)	*Psp*
Denmark	265, 267, 282, 286	*Psp*
Hungary	1434	*Psp*
Czech Republic	H1, H5, H6, HX1, HX2, HX4, HX5	*Psp?*
Czech Republic	4073, 2868	*Pss*

Number in bracket means a race of *P. syringae* pv. *pisi*

Psp = *P. syringae* pv. *pisi*; *Pss* = *P. syringae* pv. *syringae*

PP11. These antisera had the highest level of specificity in the SA method. There was no cross-reaction with heterologous antigens from other species. Slight reactions in SA were observed only with the collection strains of *P. fluorescens* and *P. viridiflava* (PS9 1 : 8 for both antigens, and PP11 1 : 4 for *P. viridiflava* and 1 : 16 for *P. fluorescens*). One-step cross-absorption of these two antisera PS9 and PP11 was completely susccessful. The other antisera reacted slightly with *P. fluorescens* after three absorptions (1 : 2). In a similar study on *P. s. tomato*, JONES *et al.* (1982) was also not completely successful in eliminating cross-reactions after four absorptions. This is evidence for a common heat-stable antigen present in crude lipopolysaccharide. These antigen reactions are similar to those observed with other species of *Pseudomonas* (JONES *et al.* 1982).

The antisera titrations in ODD were the following: for PS9 1 : 64 on M1 and 1 : 128 on M2 medium, for PP11 1 : 256 on both M1 and M2. The spur formation of precipitin bands mainly for heterologous antigens was easier to observe on M2 medium. The reactions with heterologous antigens in ODD were on the same level as in the SA method. There were no differences between results for all of the seven races of *P. s. pisi* and for the various forms of antigens (whole untreated cells, formaldehyde or glutaraldehyde fixed cells).

Antiserum PP11 reacted positively with Czech isolate H6 and with all foreign strains, except strain 267. Two or three precipitin bands were formed close to the antigen wells. Antiserum PS9 reacted positively with foreign strain 267, with Czech isolates H1, H5, HX1, HX2, HX4 and HX5, and with the collection strains *P. s. syringae* CCM 2868 and CCM 4073.

Table 2. List of polyclonal antisera prepared against *Pseudomonas syringae* pv. *pisi* strains and their reaction with the seven races of *P. syringae* pv. *pisi*

No. of antiserum	No. of antigen	Race of antigen	Form of antigen	Antiserum titer	Method	Race of *Pseudomonas syringae* pv. *pisi* 1	2	3	4	5	6	7
PP1	937	4	untreated	256	agglutination	++	++	++	+	+	+	++
					immunodiffusion	+	+	+	+	+	+	++
PP2	30	2	untreated	1024	agglutination	++	+	+	+	+	+	+
					immunodiffusion	+	+	+	+	++	+	+
PP5	H6	–	treated with 0.5% formaldehyde	128	agglutination	+	+	+	+	+	+	+
					immunodiffusion	+	+	+	+	+	+	+
PP8	299	5	treated with 0.5% formaldehyde	1024	agglutination	+	+	+	++	++	+	+
					immunodiffusion	+	+	++	+	++	+	++
PP10	PP01	–	treated with 0.1% glutaraldehyde	512	agglutination	++	++	+	++	++	+	+
					immunodiffusion	++	+	+	++	+	+	++
PP11	1434	–	treated with 1.0% formaldehyde	2048	agglutination	+	+	+	+	++	+	+
					immunodiffusion	++	+	+	+	+++	++	+
PP15	2491	7	treated with 0.1% glutaraldehyde	128	agglutination	+	+	+	+	++	+	++
					immunodiffusion	++	+	+	+	++	++	+++

– no reaction; +, ++, +++ intensity of agglutination or immunodiffusion reaction

Of the many ELISA variants assayed for sensitivity and false antigen-positive reactions, the indirect PTA-ELISA fulfilled the requirements. Detection limits in PTA-ELISA of *P. s. pisi* were 0.1 mg/ml IgG, 200 mU/ml anti-rabbit IgG-AP and 10^4 CFU/ml of homologous antigen. The best dilutions for identification of *P. s. pisi* isolates by DAS-ELISA were 0.1 mg/ml IgG, 1 : 500 IgG-AP and antigen at the concentration 10^5 CFU/ml. The detection limits achieved for identification of *P. s. syringae* antigens in DAS-ELISA and PTA-ELISA were similar. These results compare favourably with those obtained by UNDERBERG and SANDER (1991) for *Corynebacterium sepedonicum* (5×10^5 CFU/ml).

The *P. s. pisi* and *P. s. syringae* IgG prepared from polyclonal antisera after cross-absorption were highly specific. This was shown by the absence of cross-reactions with the other *P. syringae* pathovars and with the type-strain *Pf.* A strong positive signal for heterologous antigens can be explained by close epitopes relationship (MAZAREI *et al.* 1992). High cross-reactions can also occur because the IgG as a second antibody is absorbed to the plate (E_{405} 0.6–0.7) despite BSA blocking (MAZAREI *et al.* 1992).

All three serological techniques demonstrate antigenic homogeneity among the strains of *P. s. pisi.* There were no considerable serological differences among antigens of the seven races of *P. s. pisi.* Therefore, it is sufficient to prepare one antiserum against all of the *P. s. pisi* isolates (Table 3). After cross-absorption, our antisera could differentiate *P. s. pisi* from *P. s. syringae* isolates.

The Czech isolate H6 and the foreign strains were with all serological tests determined as *P. s. pisi.* Only the foreign strain 267, initially received as *P. s. pisi*, was serologically determined in agglutination test, immunodiffusion test and ELISA tests as *P. s. syringae.* Likewise, the Czech isolates H1, H5, HX1, HX2, HX4 and HX5, and collection strains CCM 2868 and CCM 4073 were in all serological tests determined as *P. s. syringae.*

TAYLOR (1970) and TAYLOR and DYE (1972) described a test that could differentiate *P. s. pisi* from *P. s. syringae* only when heat-killed bacteria as antigens were used. Antisera were also raised also against sonicated, glutaraldehyde-fixed cells and glutaraldehyde-fixed flagellar antigens (MAZAREI & KERR 1990; MAZAREI *et al.* 1992). We are able to differentiate both *P. s.* pathovars also with polyclonal antisera prepared against whole untreated cells.

LYONS *et al.* (1995) developed a technique for simultaneous detection of important seed-borne pathogens including bacterial blight (*P. s. pisi*), leaf and pod spot (*Mycosphaerella pinodes)* and pea seed-borne mosaic potyvirus (PSbMV) by using the same seed sample. The authors also inform about antisera prepared against lipopolysaccharide antigens from the cell wall.

Our work presents evidence that the serological specificity between *P. s. pisi* and *P. s. syringae* pathovars lies in the surface antigens. Our polyclonal antisera *P. s. pisi* and *P. s. syringae* prepared against untreated, formaldehyde-fixed and glutaraldehyde-fixed cells have a comparable specificity in all tested serological methods (Table 2). Thus, our antisera can at present be used against *P. s. pisi* and *P. s. syringae* for routine analysis of pea seed samples in diagnostic laboratories.

Table 3. Responses of bacterial strains of *Pseudomonas syringae* pv. *pisi* and pv. *syringae* to various diagnostic tests

Bacterial strain	Fluorescence	DL-homo-serine	Double-diffusion	ELISA DAS	ELISA PTA	Pathovar identification
Antiserum (IgG) PP 11						
30, 937, 1434	+	+	+	+	+	*Psp*
282, 815, PP01, 296, 298, 299, 300, 436, 299(1), 202(2), 870(3), 895(4), 974(5), 1704(6), 2491(7)	–	+	+	+	+	*Psp*
265, 948, 974	+	–	+	+	+	*Psp*
981	–	–	+	+	+	*Psp*
H6	+	+	–	+	+	*Psp*
286	–	+	–	–	+	*Psp*
Antiserum (IgG) PS 9						
2868	+	+	+	+	+	*Pss*
4073	+	-	+	+	+	*Pss*
HX1, HX4	+	+	-	+	+	*Pss*
H1, H5	+	+	-	-	+	*Pss*
HX2, 267	+	-	-	-	+	*Pss*

The number in bracket means race of *Pseudomonas syringae* pv. *pisi*
– negative reaction, + positive reaction; *Psp* = *P. syringae* pv. *pisi; Pss* = *P. syringae* pv. *syringae*

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Souhrn

PÁNKOVÁ I., KOKOŠKOVÁ B. (1999): **Sérologické a biochemické rozlišení patovarů** *Pseudomonas syringae* **na hrachu**. Pl. Protect. Sci., **35**: 79–84.

Vypracovali jsme rychlou diagnózu a rozlišení dvou blízkých, ekonomicky významných bakteriálních patogenů hrachu, bakterie *Pseudomonas syringae* pv. *pisi*, která náleží ke karanténním organismům, a bakterie *Pseudomonas syringae* pv. *syringae*. Pracovali jsme s bakteriálními kmeny z národních sbírek Francie, SRN, Dánska, Maďarska, Velké Británie a České republiky, se kterými byly porovnány bakteriální izoláty získané z polních porostů hrachu v České republice. Stěžejní biochemická reakce, tj. využívání *(P. s. pisi)* a nevyužívání *(P. s. syringae)* DL-homoserinu jako zdroje uhlíku pro růst bakterií, se pro rozlišení obou patovarů neprokázala dostatečně spolehlivou. Z hlediska úspory času a interpretace výsledků byly výhodnější sérologické metody. Pro identifikaci *P. s. pisi* bylo připraveno celkem sedm polyklonálních antisér (PP1, PP2, PP5, PP8, PP10, PP11, PP15) a pro

identifikaci *P. s. syringae* jedno polyklonální antisérum (PS9). Použité bakteriální antigeny byly buď neošetřené, nebo byly fixovány formaldehydem nebo glutaraldehydem. Titr antisér s homologním a heterologním antigenem byl stanoven sklíčkovou aglutinací (SA) a Ouchterlonyho gelovou imunodifuzí (OI). Výraznější křížové reakce proti *Pseudomonas fluorescens* a *Pseudomonas viridiflava* byly odstraněny vysycením antisér. V DAS i PTA-ELISA testu byly použity IgG z antisér s nejvyššími specifičností PS9 a PP11. Optimální ELISA kit pro oba patovary *P. syringae* obsahoval IgG o koncentraci 0,1 mg/ml, IgG-AP 1 : 500 nebo 200 mU/ml anti-rabbit IgG-AP a bakteriální suspenzi homologního antigenu o koncentraci 10^5–10^4 CFU/ml. Všechny anglické izoláty zahrnující zástupce všech sedmi ras *P. s. pisi* reagovaly spolehlivě ve všech typech sérologických testů se všemi antiséry PP bez ohledu na způsob přípravy antigenu, imunizační cyklus a příslušnost antigenu k určité rase. Připravené kity budou využity při rutinním testování vzorků hrachu v diagnostických laboratořích.

Klíčová slova: bakteriální spála na hrachu; *Pseudomonas syringae* pv. *pisi*; *Pseudomonas syringae* pv. *syringae*; sklíčková aglutinace; Ouchterlonyho gelová imunodifuze; ELISA

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