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## Serological Properties of *Pseudomonas syringae* pv *phaseolicola* Isolates Collected From Eskişehir

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**Abstract:** Thirty six bean seed samples collected from bean fields in Eskişehir-Türkiye were examined for detection and isolation of *Pseudomonas syringae* pv. *phaseolicola*. A polyclonal antiserum specific to P. s. pv. *phaseolicola* NCPPB 52 was raised in a rabbit and both biochemical and serological test were used for identification. Heat-stable O antigens of 92 isolates identified as P. S. pv. *phaseolicola* together with authentic isolates of the bacterium were compared by Ouchterlony immunodiffusion test and all the isolates had a common antigenic structure.

**Key Words:** *Pseudomonas syringae* pv. *phaseolicola*, halo blight, polyclonal antiserum, Ouchterlony immunodiffusion test.

### Eskişehir'den Toplanan *Pseudomonas syringae* pv. *phaseolicola* İzolatlarının Serolojik Özellikleri

**Özet:** Eskişehir'deki fasulye ekim alanlarında toplanan 36 fasulye tohumunda *Pseudomonas syringae* pv. *phaseolicola* bakterisi aranmış ve izole edilmeye çalışılmıştır. P. s. pv. *phaseolicola* NCPPB 52 izolatı için spesifik poliklonal antiserum tavşanda üretilmiş ve identifikasyon için hem biyokimyasal hemde serolojik testler kullanılmıştır. *Pseudomonas syringae* pv. *phaseolicola* olarak teşhis edilen 92 izolat ile bu bakterinin standart suşlarının ısıya dayanıklı O antijenleri Ouchterlony immunodiffüzyon testi ile karşılaştırılmış ve bütün izolatların ortak antijenik yapıya sahip oldukları görülmüştür.

**Anahtar Sözcükler:** *Pseudomonas syringae* pv. *phaseolicola*, hale yanıklığı, poliklonal antiserum, Ouchterlony immunodiffusion testi

### Introduction

Beans particularly dwarf beans (*Phaseolus vulgaris*) is grown in nearly every part of Turkey and the consumption of beans was 2.7 kg per person in 1993 and it has been aimed to reach 3.5 kg in 1999 (1).

*Pseudomonas syringae* pv. *phaseolicola* (Burkholder) Young, Dye & Wilkie the causal agent of halo blight, is a serious seedborne pathogen of beans worldwide (2) and can reduce yield and quality (3). *Pseudomonas syringae* pv. *phaseolicola* races 1 and 2 differentiated by their pathogenicity on *Phaseolus vulgaris* cultivars have been reported from USA and UK (4, 5, and 6). A third race of the bacterium was reported by studies carried out in CIAT (Centro Internacional de Agricultura Tropical) (7).

Contaminated seed is a source of primary inoculum (8) and the best method of control is therefore by the use of disease-free seed. Detection of this pathogen in seed is essential for effective control of the disease. Several methods have been described for testing the presence of *Pseudomonas syringae* pv. *phaseolicola* (9,10,11).

KARACA (12) first reported that halo blight was seen in Black sea region and Bursa province of Türkiye. The disease was also reported in Niğde, Nevşehir, Afyon and Eskişehir provinces with the losses being up to 50% (13).

This investigation aimed to detect and isolate *P. s. pv. phaseolicola* in samples collected from Eskişehir. The isolates were identified by conventional tests and by using a specific antiserum. The antigenic structure of the isolates were compared with authentic strains of three races.

## Materials and methods

**Microorganisms:** The bacteria used for antiserum production and for comparison are listed in Table 1.

**Antiserum production:** *P. s. pv. phaseolicola* NCPPB 52 was grown in nutrient broth at 27 °C for 2 days and washed two times in 0.01 M PBS (pH=7.2) by centrifugation. Cell suspension was adjusted to 0.7 at  $A_{550}$  (~10<sup>9</sup> cells/ml). A New Zealand white rabbit of approximately 3 kg weight was injected with an emulsion prepared from equal volumes of antigen and Freund's adjuvant. The immunisation procedure was as follows:

Day	Route	Volume	Antigen
1	Subcutaneous	1ml in nape of neck	109 cells/ml in FCA
8	Subcutaneous	1ml in nape of neck	109 cells/ml in FCA
15	Intramuscular and	0.5ml in leg	109 cells/ml in FCA
	subcutaneous	0.5 ml in nape of neck	109 cells/ml in FCA
22	Intramuscular and	0.5 ml in leg	109 cells/ml in FCA
	subcutaneous	0.5 ml in nape of neck	109 cells/ml in FCA
29	Test bleeding		
36	Intramuscular and	0.5 ml in leg	109 cells/ml in FCA
	subcutaneous	0.5 ml in nape of nek	109 cells/ml in FCA

FCA: Freund's Complete Adjuvant.

FIA: Freund's Incomplete Adjuvant.

The titre of the antiserum was determined in 96 wells microtiter plates as indicated by GÜVEN (14).

**Isolation of bacteria:** Seed material was collected from 36 different fields in Eskişehir. The

seeds were first surface sterilized by immersion in 0.5% sodiumhypochloride for 5 min, rinsed in distilled water and dried overnight at 30 °C. Seeds were ground to a flour in a mill and collected in new polyethene bags. The parts of the mill were sterilized by alcohol and then assembled for each use. The bean flour was added to sterile water (500 g/l) and shaken vigorously at room temperature for 4 hours. 0.1 ml of samples from the undiluted supernatant fluid and from each of two serial 1:10 dilutions in nutrient broth were streaked onto King's medium B agar (15). The plates were air-dried and incubated at 27 °C for 3 days. After examining under UV light at 365 nm, the single colonies giving blue-green fluorescence were purified for further studies and several colonies were chosen from each sample.

Bacterium	Strain no.	Origin	Table 1.	Microorganisms used in the assay.
<i>P. s. pv. phaseolicola</i> race 1	NCPB 52 <sup>a</sup>	Canada		
<i>P. s. pv. phaseolicola</i> race 1	T 1516 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 1	T 1484 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 1	T 1355 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 1	T 1360 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 2	T 1599 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 2	T 1817 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 2	T 1374 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 2	(Q 1495 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 3	T 1301 <sup>a</sup>	Tanzania		
<i>P. s. pv. phaseolicola</i> race 3	T 1381 <sup>a</sup>			
<i>P. s. pv. phaseolicola</i> race 3	T 1302 <sup>a</sup>	Rwanda		
<i>P. s. pv. phaseolicola</i> race 1	T PPB 4101 <sup>b</sup>	Turkey		
<i>P. s. pv. syringae</i>	T PPB 4250 <sup>b</sup>	Turkey		
<i>P. s. pv. t mato</i>	T PPB 4212 <sup>b</sup>	Turkey		
<i>P. marginalis</i>	T PPB ,4650 <sup>b</sup>	Turkey		
<i>P. cichori</i>	T PPB 4601 <sup>b</sup>	Turkey		
<i>P. fluorescens</i>	T PPB 4660 <sup>b</sup>	Turkey		
<i>Xanthomonas campestris</i> pv. phaseoli	TPPB 5001 <sup>b</sup>	Turkey		
<i>Escherichia coli</i>	B <sup>a</sup>			

<sup>a</sup> Dr. John Turner University of East Anglia, Norwich UK

<sup>b</sup> Dr. Kemal Benlioğlu, adnan Menderes University, Aydın-Türkiye

**Identification:** Conventional biochemical tests including Gram's stain, motility, utilisation of mannitol, sorbitol and inositol together with LOPAT tests were carried out following the procedure given by SANDS et al. (16) and FAHY and HAYWARD (17). Serological identification of *P. s. pv. phaseolicola* was done by microagglutination test in microtiter plates as it was in determining the titre of the antiserum.

**Pod inoculation tests:** Freshly picked young bean pods were inoculated by stabbing with a needle dipped in a bacterial suspension. Three or four inoculation points per pod were used and the pods were stored in a humid container at room temperature for 3-4 days (10).

**Immunodiffusion test:** Heat-stable O antigens of bacterium was prepared as indicated by LUCAS and GROGAN (18). Circular patterns with 6 wells 6 mm in diameter and 7 mm apart, surrounding a central well were cut in immunodiffusion test agar (0.8% Difco purified agar containing 0.05% sodium azide) plate. Fifty µl of *P. s. pv. phaseolicola* NCPPB 52 antiserum was put into the central well. Two opposite wells were used for homologous antigen suspension. The remaining four wells were filled with the test isolates. The plates were placed in humid bags and first incubated at room temperature overnight and then at 4 °C for 7 days.

### Results and Discussion

A total of 120 fluorescence cultures were isolated from 36 beans samples tested. By using biochemical tests 92 of them were identified as *P. s. pv. phaseolicola*, 26 were *P. s. pv. syringae* and 2 were *P. viridiflava* (Table 2).

The titre of the antiserum was determined as 1/1024. All the isolates identified as *P. s. pv. phaseolicola* and the authentic isolates of the bacterium listed in Table 1 agglutinated with *P. s. pv. phaseolicola* NCPPB 52 antiserum. Agglutination test confirmed the identification of 92 *P. s. pv. phaseolicola* isolates and no cross-reaction was observed indicating the specificity of the antiserum (Table 2).

Table 2. Differentiating biochemical and serological properties of *Pseudomonas* spp. isolated from bean samples

No.	Gram	Moti	Fluores	Levan	Oxida	Pectol.	Argin.	Tobac.	Patho-	Utilis.	Utilis.	Utilis.	Aggluti	Identi	
isolates	reaction	lity.	cence	produc	se	test	activity	Dihydr.	HR	genicity	Mannit.	Sorbit.	Inosit.	-nation	fication
			on KB	tion.											
92	-	+	+	+	-	-	-	+	+	-	-	-	+	A	
26	-	+	+	+	-	-	-	+	+	+	+	+	-	B	
2	-	+	+	-	-	+	-	+	+	+	+	+	-	C	

**A:** *Pseudomonas syringae* pv. *phaseolicola*

**B:** *Pseudomonas syringae* pv. *syringae*

**C:** *Pseudomonas viridiflava*

Pod inoculation tests showed the pathogenicity of *P. s. pv. phaseolicola* isolates by giving watersoaked lesions on stabbing points. Whereas, *P. s. pv. syringae* and *P. viridiflava* lesions appeared as brown-necrotic zones on pods.

Immunodiffusion tests with heat-treated soluble antigenic preparations showed that all the *P. s. pv. phaseolicola* isolates including members of three races had a single well-defined precipitin band when they interacted with *P. s. pv. phaseolicola* NCPPB 52 anti serum (Figure 1). Therefore, no antigenic difference could be detected between the three races and the Turkish isolates of *P. s. pv. phaseolicola* by the Ouchterlony immunodiffusion test.

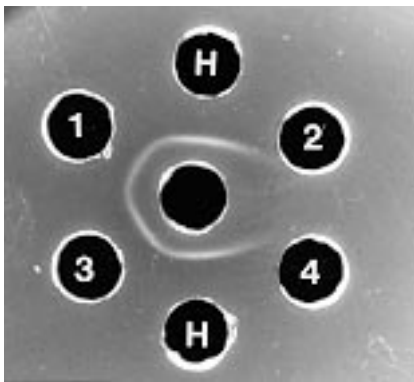


Figure 1. Heat-stable O antigen precipitin bands formed between *P. s. pv. Phaseolicola* NCPPB 52 antiserum and homologous antigen (H). *P. s. pv. phaseolicola* strains (1 and 3) isolated in our study. No precipitin band formed with *P. s. pv. syringae* (2) and *P. viridiflava* (4).

Serological tests for plant pathogenic bacteria have been known since 1918 (19) and there are several reasons for using these tests. First, they are suited for routine application because of their rapidity and easy application to large amounts of seed samples (20). Second, some of the serological tests such as immunofluorescence microscopy, are very sensitive (21); others are very specific such as Ouchterlony double diffusion (19).

Several investigators reported that prolonged heating or autoclaving of cells has been necessary to destroy non-specific antigens of *P. s. pv. phaseolicola* (18,10, 22). The work reported here shows that specific antibodies against *P. s. pv. phaseolicola* can be raised and used in serological tests. The antibody discriminated between pathovars of *P. syringae* and recognized all strains of *pv. phaseolicola* tested, which comprised the three known races and were wide-spread geographic origin.

Wyatt (23) raised an antiserum specific to surface polysaccharides of *P. s. pv. phaseolicola* and reported that all the isolates of the bacterium tested reacted similarly in the ELISA assay regardless of race or origin. We also could not detect any discriminatory property between races of *P. s. pv. phaseolicola* by using heat-stable O antigens of the bacterium. These results showed

that *P. s. pv. phaseolicola* is serologically uniform in contrast to other plant pathogenic bacteria e.g. *Erwinia carotovora* (24).

In conclusion, the antiserum raised in our study could be used in alternative serological techniques e.g. immunomagnetic separation (25). Further studies including PCR and RAPD molecular markers (26) can be employed to detect genetic variation between races of *P. s. pv. phaseolicola*.

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