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Short communication. Detection by multiplex PCR and characterization of nontoxigenic strains of *Pseudomonas syringae* pv. phaseolicola from different places in Spain

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Abstract

The efficient control of halo blight, caused by *Pseudomonas syringae* pv. phaseolicola, is primarily based on the use of pathogen-free seed. Detection of the pathogen in seeds is currently carried out with high-sensitive methods based on the detection by PCR of genes involved in the biosynthesis of phaseolotoxin, which was believed to be produced by all strains of the pathogen with epidemiological importance. However, field epidemics of halo blight in the county of Castilla y León, Spain, are often associated to nontoxigenic isolates of *P. syringae* pv. phaseolicola, which cannot be detected using current molecular and serological methods. The results presented in this work show the existence of nontoxigenic isolates of *P. syringae* pv. phaseolicola in areas other than Castilla y León, indicating the need to establish a reliable methodology for seed certification. A simple two-step methodology is presented with the aim to identify both types of isolates that is based on a multiplex enrichment PCR of seed soakates and on pathogenicity assays.

Additional key words: BIO-PCR, halo blight, phaseolotoxin, *Pseudomonas syringae* pv. syringae, races.

Resumen

Nota corta. Detección mediante PCR multiplex y caracterización de cepas no toxigénicas de *Pseudomonas syringae* pv. phaseolicola de distintas zonas de España

El control eficiente de la grasa de la judía causada por *Pseudomonas syringae* pv. phaseolicola se basa principalmente en la utilización de semilla libre del patógeno. La detección del patógeno en semilla se efectúa mediante métodos altamente sensibles basados en la detección por PCR de los genes responsables de la biosíntesis de la faseolotoxina, la cual, hasta ahora, se consideraba que era sintetizada por todas las cepas del patógeno con importancia epidemiológica. Sin embargo, en la Comunidad de Castilla y León, España, las epidemias de grasa de la judía en campo se asocian frecuentemente con cepas no toxigénicas de *P. syringae* pv. phaseolicola, que no pueden ser detectadas con los métodos moleculares y serológicos actuales. Los resultados presentados en este trabajo demuestran la existencia de aislados no toxigénicos de *P. syringae* pv. phaseolicola en zonas distintas de Castilla y León, lo que implica la necesidad de establecer una metodología fiable para la certificación de semillas de judía. Con este propósito, se presenta un sencillo protocolo en dos fases que permite la identificación de los dos tipos de aislados, y que se basa en una PCR multiplex con enriquecimiento a partir de extractos de semilla y en ensayos de patogenicidad.

Palabras clave adicionales: BIO-PCR, faseolotoxina, grasa de la judía, *Pseudomonas syringae* pv. syringae, razas.

Dry bean (*Phaseolus vulgaris* L.) is the most important legume crop in Spain. In 2004 around 137,000 tones were produced in an extension of 103,000 ha (MAPA, 2004), with the majority of this production concentrated in the county of Castilla y León, where the crop is cultivated extensively. However, in the rest of the temperate areas of the country, the production is restricted to small plots, and local landraces are cultivated, where the seeds are usually saved by the growers from previous seasons. In both systems, this crop may be very sensitive to bacterial diseases, if climatic conditions are favourable, due to (i) the use of seeds from previous crops, which may act as primary

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inoculum, (ii) the lack of crop rotations and (iii) the inefficiency of the chemical control (C. Asensio, personal communication).

The most relevant bacterioses of bean are those caused by the Gram negative bacteria *Pseudomonas syringae* pv. phaseolicola, *P. syringae* pv. syringae and *Xanthomonas campestris* pv. phaseoli, which result in important economical losses worldwide (Smith *et al.*, 1988; Saettler, 1991). *P. syringae* pv. phaseolicola, which causes halo blight of bean, is a seed-borne pathogen present in all countries were beans are cultivated, and is responsible for serious crop losses, especially in temperate areas of the world, such as Spain. Contaminated seeds, either internally or externally, constitute the primary source of infection (Schwartz, 1989) and a very low level of inoculum can result in severe epidemics under favourable environmental conditions (Trigalet and Bidaud, 1978; Webster *et al.*, 1983). Control of the disease is difficult and its efficient integrated control requires the use of seed certified free of the pathogen. Methods used for detection of this pathogen, however, have to be highly sensitive because a single contaminated seed among 2,000 or 5,000 can initiate an epidemic (Trigalet and Bidaud, 1978; Webster *et al.*, 1983).

Several highly sensitive PCR-based methods have been designed for the detection of *P. syringae* pv. phaseolicola, and all of them rely on the detection of DNA sequences involved in the biosynthesis of phaseolotoxin (Tourte and Manceau, 1991; De la Fuente-Martínez *et al.*, 1992; Prosen *et al.*, 1993; Schaad *et al.*, 1995; Audy *et al.*, 1996). It is generally accepted that the genes for the biosynthesis of phaseolotoxin are present in all the strains of *P. syringae* pv. phaseolicola found causing field epidemics (Rudolph, 1995; Schaad *et al.*, 1995). Additionally, this DNA appears to be highly specific because it has not been found in other bacterial bean pathogens, although it was also described in strains of *P. syringae* pv. actinidiae (Tamura *et al.*, 1989, 2002), which infects kiwi (*Actinidia deliciosa* Liang et Ferguson), and in a single strain of *P. syringae* pv. syringae isolated from vetch (*Vicia sativa* L.) (Tourte and Manceau, 1995). However, it was recently showed that more than 66% of the *P. syringae* pv. phaseolicola isolates causing field epidemics in Castilla y León, Spain, were nontoxigenic and did not contain DNA specific for the biosynthesis of phaseolotoxin (Rico *et al.*, 2003; Oguiza *et al.*, 2004). The strains of *P. syringae* pv. phaseolicola that lacked the phaseolotoxin gene ORF6 were designated as *tox*–, whereas those containing this gene were designated as *tox*+ (Rico *et al.*, 2003). Consequently, this designation will be used from now on because it is helpful to distinguish those strains that can (*tox*+) or cannot (*tox*–) be detected by current PCR methods using primers directed to the phaseolotoxin cluster. Incidentally, all the *P. syringae* pv. phaseolicola *tox*– strains analyzed so far also lacked other genes of the phaseolotoxin biosynthesis cluster and failed to produce phaseolotoxin (Rico *et al.*, 2003; Oguiza *et al.*, 2004). Additionally, the *tox*– isolates also failed to react with one of the few commercial antibodies for the serological detection of *P. syringae* pv. phaseolicola (Rico *et al.*, 2003), all of which are polyclonal. In consequence, the efficient certification of bean seed in Spain using current highly sensitive methods, such as PCR and serological methods, will depend on the possible distribution of *tox*– populations. The aim of this work was to examine the presence of *tox*- populations of *P. syringae* pv. phaseolicola in other bean producing areas of Spain.

In this work, the presence of *Pseudomonas syringae* in 22 bean seed samples obtained from experimental fields in Spain was examined. Seventeen samples were of cv. Alavesa from Álava, two samples were of cv. Blanca from Huesca, two samples of an unknown cultivar were from Galicia and one sample of cv. Jules from an unknown place was obtained from a Spanish Germplasm Bank (Plant Genetic Resource Centre, CRF-INIA, Alcalá de Henares). Seeds (15 to 100 g) were soaked overnight at 4ºC in sterile distilled water (1:1.5 w/v) and the water extract was recovered and centrifuged at 1,5550 × g for 5 min. The pellet was washed twice in 5 ml of distilled water and concentrated 20 times from the original extraction volume. Aliquots of 100 µl from this concentrated soakate were plated on Milk-Tween (MT) medium (Goszczynska and Serfontein, 1998), incubated overnight at 18ºC and used for enrichment-PCR analysis (Schaad *et al.*, 1995). The bacterial growth from a single plate was washed with 3 ml of sterile distilled water, and 5 µl of the resulting suspension were used as template in a multiplex PCR reaction in a total volume of 25 µl which contained 20 pmol each primer, 0.15 mM each dNTP, 1X PCR reaction buffer, 1.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Biotaq; Bioline Ltd., London). Control strains and primers used are described in Tables 1 and 2. The primers used in the multiplex PCR were primer pair P5.1-P3.1 (Schaad *et al.*, 1995; Zhang and Patil, 1997), specific of open reading frame (ORF) 6 from locus *phtE* of the phaseolotoxin biosynthesis cluster, and primer pair

Table 1. Bacterial strains used in this study

Strain	Characteristics	Source or reference
***P. syringae* pv. glycinea**		
49a90	Race 4, coronatine producer, 1990, Germany	Ullrich *et al.,* 1993
4180	Race 4, coronatine producer, 1975, New Zealand	Mitchell, 1978
***P. syringae* pv. phaseolicola**		
1149B	Race 7, 1985, Ethiopia	Taylor *et al.,* 1996
CYL304	*tox–*	Rico *et al.,* 2003
CYL325	Race 7; *tox–*	Rico *et al.,* 2003
***P. syringae* pv. syringae**		
B728a	Wild type; Rif^r; isolated from *Phaseolus vulgaris,* USA	G.W. Sundin, Michigan St. Univ., Michigan, USA
***P. syringae* pv. tabaci**		
CFBP1621	Wild type	C. Manceau, INRA, Angers, France
P. marginalis		
NCPPB247	Wild type	A. Vivian, Univ. West England, Bristol, UK
P. tolaasii		
NCPPB2192	Wild type	A. Vivian, Univ. West England, Bristol, UK

P3004L and P3004R, which amplify a 0.24 kb fragment (GenBank acc no. AJ568001) that has been observed only in nontoxigenic strains (Oguiza *et al.*, 2004). Additionally, 100 µl aliquots of a 1/10 dilution of the concentrated seed soakate were cultured on MT plates for 24-48 h at 28ºC, and putative colonies belonging to *P. syringae* pvs. phaseolicola and syringae were identified by their fluorescence and the presence or absence of haloes due to the hydrolysis of casein (Goszczynska and Serfontein, 1998). The pathogenicity of the resulting isolates was tested on bean pods of cv. Canadian Wonder, which is susceptible to all known races of *P. syringae* pv. phaseolicola (Taylor *et al.*, 1996), and cv. Perona as described previously (Harper *et al.*, 1987). Only fluorescent colonies producing water-soaking or sunken-brown lesions on bean pods, characteristic of *P. syringae* pvs. phaseolicola and syringae, respectively, were retained. Reference strains were used for comparison (Table 1).

No colonies were observed in 17 out of the 22 seed lots examined, or the few encountered were not fluorescent. In all these cases no amplification bands were produced by multiplexed PCR, and these seed lots were not further assayed. The remaining five seed lots

Table 2. Primers used in this study

Name	Sequence	Amplified band size (kb)	Source
P5.1 P3.1	5' AGCTTCTCCTCAAAACACCTGC 3' 5' TGTTCGCCAGAGGCAGTCATG 3'	0.5	Schaad *et al.* (1995)
PHA19 PHA95	5' CGTCTGTAACCAGTTGATCC 3' 5' GAATCCTTGAATGCGAAGGC 3'	0.48	Marques *et al.* (2000)
P3004L P3004R	5' CTGTCTGGCAGCCACTACAAAG 3' 5' GGCTGCAAATTGTGGGATTT 3'	0.24	This work

produced fluorescent colonies on MT or King's Medium B (King *et al*., 1954) plates, and three of them produced amplification bands upon multiplexed PCR. One of the lots, originating from Galicia, produced the specific 0.5 kb fragment after amplification with primers P5.1 and P3.1, whereas other two lots, both from cv. Alavesa, amplified the 0.24 kb fragment specific of *P. syringae* pv. phaseolicola *tox*– isolates (Table 3). The identification of the fluorescent colonies obtained from the diluted soakates from these five seed lots was performed essentially as described (Lelliott and Stead, 1987; Braun-Kiewnick and Sands, 2001) following the LOPAT scheme (Lelliott and Stead, 1987), with slight modifications (Cazorla, 1998; Rico *et al.*, 2003). Degradation of casein was examined on MT plates (Goszczynska and Serfontein, 1998). The utilisation of D-mannitol, inositol, D-sorbitol, erythritol, L+tartrate, D+tartrate, L-lactate, arabinose, adonitol, glutarate and glucuronate as the sole carbon source was examined on Ayer's minimal medium (Lelliott and Stead, 1987) containing the carbohydrates to a final concentration of 0.1% (w/v).

All the fluorescent colonies obtained (20 colonies in total) were levan positive, oxidase and arginine dihydrolase negative, and elicited a hypersensitive reaction on tobacco (LOPAT group Ia); they were therefore identified as *Pseudomonas syringae* (Lelliott and Stead, 1987). Seven colonies from two seed lots from Álava showed a biochemical profile typical of *P. syringae* pv. syringae while the 13 colonies from one lot from Galicia and two lots from Álava, produced water soaked lesions on bean pods and showed the biochemical profile typical of *P. syringae* pv. phaseolicola (Lelliott and Stead, 1987; Braun-Kiewnick and Sands, 2001) (Table 3). These data agreed with the results of the enrichment-PCR amplifications of the three seed lots soakates with the primer pairs specific for *tox*+ or *tox*– strains of *P. syringae* pv. phaseolicola (see Table 3).

From the above 13 *P. syringae* pv. phaseolicola isolates, four isolates from the seed lot from Galicia did not utilise mannitol, produced the expected amplification fragments with primer pairs P5.1-P3.1 and PHA19-PHA95, and did not produce the 0.24 kb band with primers P3004L and P3004R (Fig. 1 and Table 3); therefore, they were classified as typical *tox*+ isolates. Assimilation of mannitol is usually negative for strains of *P. syringae* pv. phaseolicola that contain the DNA for the biosynthesis of phaseolotoxin (Marques *et al.*, 2000), whereas the strains that do not contain this DNA can utilise mannitol as the sole carbon source (Rico *et al.*, 2003). Additionally, primers PHA19 and PHA95 (Table 2) direct the amplification of a 0.48 kb internal fragment from the amidinotransferase gene *amtA*, which is also essential for phaseolotoxin biosynthesis (Marques *et al.*, 2000; Hernández-Guzmán and Álvarez-Morales, 2001). The remaining nine isolates,

Table 3. Identification of toxigenic and nontoxigenic *P. s.* pv. phaseolicola strains and *P. s.* pv. syringae strains in five seed lots

Cultivar, place of isolation and lot number	D-Mannitol utilisation[1]	Enrichment PCR[2]		Isolates[3]	avr genes content[4]		
		P5.1-P3.1	P3004L-P3004R		*avrPphB*	*avrPphE*	*avrPphF*
Unknown, Galicia, L15	–	+	–	Pph *tox*+	–	+	–
Alavesa, Álava, N8	+	–	+	Pph *tox*–	–	+	+
Alavesa, Álava, N18	+	–	+	Pph *tox*–	–	+	+
Alavesa, Álava, N2	+	–	–	Psy	NA	NA	NA
Alavesa, Álava, N10	+	–	–	Psy	NA	NA	NA

[1] D-Mannitol was added as the sole carbon source to Ayer's minimal medium (Lelliott and Stead, 1987) to a final concentration of 0.1% (w/v). Those isolates that did not show significant growth after 1 week of incubation at 28ºC were considered negatives. [2] Detection of *tox*+ and *tox*– isolates in bean seed soakates with primers P5.1-P3.1 and P3004L-R, specific for each type of isolates, respectively. [3] Type of pathogens found on the isolation plates seeded with bean soakates. Pph: *Pseudomonas syringae* pv. phaseolicola; Psy: *Pseudomonas syringae* pv. syringae. [4] The avr genes content was examined in individual colonies by PCR amplification with primers specific of *avrPphB* (Jenner *et al.,* 1991), *avrPphE* (Stevens *et al.,* 1998) and *avrPphF* (Tsiamis *et al.,* 2000). +: amplification of the expected fragment. –: no amplification. Colonies positive for *avrPphE* produced an 1.2 kb amplicon, as expected for all *P. syringae* pv. phaseolicola races except for race 8, which produce an 1.32 kb amplicon. NA: not applicable.

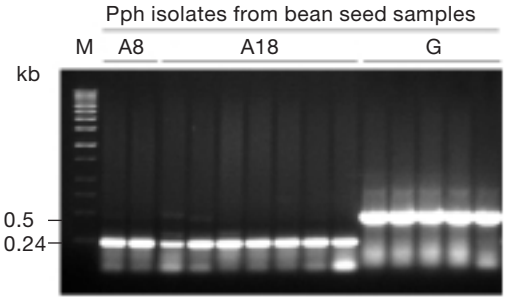

Figure 1. Multiplexed PCR using the primer pair P5.1 and P3.1 in combination with P3004L and P3004R to identify *P. syringae* pv. phaseolicola *tox*+ and *tox*– isolates. PCR products were separated by electrophoresis in a 1% agarose gel. M: 1 kb DNA ladder (Promega). A8: two colonies from seed lot A8, cv. Alavesa from Álava; A18: 7 colonies from seed lot A18, cv. Alavesa from Álava; G: 5 colonies from a seed lot of an unknown cultivar from Galicia. Sizes are indicated in kb. The 0.5 kb and 0.24 kb fragments correspond to the amplicons obtained with primer pairs P5.1-P3.1 and P3004L-P3004R respectively.

from two seed lots from Álava, could use mannitol as the sole carbon source, did not amplify the expected fragments with primers P5.1-P3.1 and PHA19-PHA95, and produced a band of 0.24 kb with primers P3004L and P3004R (Fig. 1 and Table 3); therefore, they were classified as typical *tox*- isolates.

The race assignation of the different isolates was also investigated by PCR analysis (Rico *et al.*, 2003) using specific primers for avirulence genes *avrPphB*, *avrPphE* (Stevens *et al.*, 1998) and *avrPphF* (Tsiamis *et al.*, 2000), which were used as published. The four *tox*+ strains identified in this study were tentatively assigned to race 2 and 6, since they neither amplified genes *avrPphF* nor *avrPphB*. On the other hand, the nine *tox*– strains contained *avrPphF*, and could be assigned to any of the races 1, 5, 7 or 9 (Table 3). These data agree with the race structure reported for *P. syringae* pv. phaseolicola in Castilla y León, in that all *tox*– isolates contain *avrPphF* whereas most of the *tox*+ isolates lack this gene and belong to race 6 (Rico *et al.*, 2003).

The identification of toxigenic and nontoxigenic populations of *P. syringae* pv. phaseolicola was greatly simplified by the analysis of seed soakates with the two-step strategy implemented in this work. The first step consisted on the analysis of soakates by a multiplex PCR with primers specific for *tox*+ and *tox*– *P. syringae* pv. phaseolicola strains. The second step entailed the plating of seed soakates onto semiselective medium; from the resulting growth, only those fluorescent colonies that produced characteristic lesions on bean pods were selected and further characterized. Only a few of the seed lots examined yielded fluorescent bacteria, which were identified as *P. syringae*, which might be due to the low size of most samples (around 50 g of seeds) and/or their good sanitary condition. In all cases, the seed soakates that produced specific amplicons for *tox*+ or *tox*– *P. syringae* pv. phaseolicola populations also yielded characteristic *tox*+ or *tox*– isolates, respectively, suggesting the applicability of primers P3004L/R for the detection of nontoxigenic isolates. Toxigenic and nontoxigenic isolates were not found coexisting in any of the seed lots examined, although this can be due to the small sample size, because their coexistence in disease bean plants has been repeatedly observed (C. Asensio, personal communication).

Altogether, the results obtained in this work clearly indicate that *tox*– populations of *P. syringae* pv. phaseolicola can be found in different bean producing areas of Spain, besides Castilla y León. This implies that bean seed lots from Spain cannot be certified as free of this pathogen using routine highly sensitive methods, such as the PCR detection of DNA sequences for the biosynthesis of phaseolotoxin or serological methods with commercial antibodies. Infected bean seeds are the main primary source of *P. syringae* pv. phaseolicola in field epidemics; additionally, the use of seed certified free of the pathogen is the main method for the control of halo blight (Smith *et al.*, 1988; Saettler, 1991). Therefore, the primers validated in this study can be used for the development of appropriate detection methods that can identify all the populations of the pathogen that are currently found in the country.

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