European Journal of Plant Pathology **103:** 809–814, 1997.

RAPD PCR-based differentiation of *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*

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Accepted 12 September 1997

Key words: RAPD PCR, *Xanthomonas campestris* pv. *phaseoli*, common bacterial blight, fuscous blight

Abstract

A RAPD PCR-based method was used to differentiate between isolates of *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. Using random primer OP-G11, a single, high intensity band of 820 bp was amplified from DNAs of all *X. c.* pv. *phaseoli* var. *fuscans* isolates, while multiple amplification products of varying sizes were generated from *X. c.* pv. *phaseoli* DNAs. Whereas RAPD PCR differentiation gave an unambiguous result in under 4 h, standard differentiation by recording the production of a brown pigment by *X. c.* pv. *phaseoli* var. *fuscans* isolates took up to 7 days and showed variation both between isolates and between media. The unequivocal nature of the RAPD PCR method was demonstrated when isolate 408, originally classified as *X. c.* pv. *phaseoli* var. *fuscans*, failed to produce the 820 bp band typical of *X. c.* pv. *phaseoli* var. *fuscans* isolates, and after also failing to produce a brown pigment, was re-classified as *X. c.* pv. *phaseoli*.

Introduction

Common bacterial blight and fuscous blight, caused by *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* respectively, are major world-wide seed-borne diseases of bean (*Phaseolus vulgaris*), causing yield reductions from 10 to 40% in susceptible cultivars (Vidaver, 1993; Wallen and Jackson, 1975). Although commonly regarded as a single pathogen, a variety of techniques, including isoenzyme profiling (El-Sharkawy and Huisingh, 1971), plasmid profiling (Lazo and Gabriel, 1987), restriction fragment length polymorphism (Lazo et al., 1987), DNA-DNA hybridisation (Hildebrand et al., 1990) and amplified DNA polymorphisms (Xue and Goodwin, 1993), have all revealed *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* to be two distinct classes. Caution should thus be taken when considering the relationship between these isolates and, indeed, Goodwin and Sopher (1994) have proposed that they retain distinct taxonomic status.

Pathogenic variation both between and within *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates signifies a potential problem in breeding for resistance to bacterial blight (Ekpo and Saettler, 1976; Maraite, 1989; Schuster and Coyne, 1981). Detailed analysis of such variation is thus a fundamental prerequisite to designing an informed resistance breeding program. Numerous reports have demonstrated that *X. c.* pv. *phaseoli* var. *fuscans* isolates are more pathogenic than *X. c.* pv. *phaseoli* isolates, generally causing greater stem collapse (Allen, 1983; Basu and Wallen, 1967; Leakey, 1973; Ekpo and Saettler, 1976; Rudolph, 1990; Opio et al., 1996). Moreover, a clear differential resistance to these organisms has been recorded (Burkholder and Bullard, 1946). An unambiguous, reliable method is therefore required to distinguish between *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates. Currently, *X. c.* pv.

Table 1. Bacterial isolates

Isolates	Location	Source
X. c. pv. *phaseoli* var. *fuscans*		
266	Argentina	V. Verdier
239 (ATCC 19315)	Canada	C. Hale
180	Colombia	V. Verdier
1065, 1068	Ethiopia	A.F. Opio
CFBP1816	Greece	C. Bragard
2721, 2722, 3403, 3430, 3485	New Zealand	C. Hale
1005, 1007, 1010, 1033	Uganda	A.F. Opio
072	USA	V. Verdier
X. c. pv. *phaseoli*		
123	Argentina	V. Verdier
095, 306	Colombia	V. Verdier
418	Dominican Rep.	V. Verdier
1069b	Ethiopia	A.F. Opio
408	Guatemala	V. Verdier
9076	Hungary	C. Hale
NCAIMB01523	Hungary	P. Ott
2725	New Zealand	C. Hale
364	Nicaragua	V. Verdier
1038b, 1048	Uganda	A.F. Opio

phaseoli var. *fuscans* is distinguished from *X. c.* pv. *phaseoli* by its production of a brown pigment on a variety of media, including King's B medium (KBM) (King et al., 1954), modified nutrient broth yeast agar (MNBY) (Vidaver, 1967), yeast dextrose chalk agar (YDCA) or mineral salt casein medium (MSC) (Goodwin and Sopher, 1994). However, both the time from inoculation and extent of such production can vary substantially (Goodwin and Sopher, 1994; and this study). Other biochemical characteristics are not useful in distinguishing between *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates, since these are similar for all pathovars of *X. campestris* (Dye, 1962). This report describes a fast and reliable RAPD PCR-based method for the differentiation of these two groups.

All *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates used in this study are listed in Table 1. Isolates were routinely grown on YDCA at 27 °C and stored at 4 °C for up to 2 weeks. For longer term storage, 0.5 ml of a 16 h LB culture was mixed with 0.5 ml sterile freezing medium (per litre: 12.6 g K_2HPO_4, 0.9 g $C_6H_5Na_3O_7.2H_2O$, 0.18 g $MgSO_4.7H_2O$, 1.8 g $(NH_4)_2SO_4$, 3.6 g KH_2PO_4, 88.0 g glycerol), frozen in liquid nitrogen, and kept at −80 °C in 0.1 ml aliquots.

Pigment production

X. c. pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates were tested for production of brown pigment following growth on three media, YDCA, KBM and MNBY. After inoculation from colonies grown on YDCA at 27 °C for 72 h, each isolate was grown at 27 °C in triplicate on each medium and examined daily from inoculation. As expected, after fifteen days no pigment was observed on any of the plates inoculated with *X. c.* pv. *phaseoli* isolates (results not shown). However, one isolate that had previously been classified as *X. c.* pv. *phaseoli* var. *fuscans*, isolate 408, failed to produce detectable levels of the pigment within the time-frame of this experiment and was accordingly reclassified as *X. c.* pv. *phaseoli* (Table 1). All other *X. c.* pv. *phaseoli* var. *fuscans* isolates produced a brown coloration on triplicate plates of each medium, although the time for this to be detected varied between isolates and between media. All *X. c.* pv. *phaseoli* var. *fuscans* isolates produced a strong brown coloration on KBM medium after 1-2 days, while a less intense coloration was observed on both YDCA and MNBY after 5-6 days and 5-7 days respectively. To clearly observe pigment production on YDCA and MNBY it was necessary to compare plates with an *X. c.* pv. *phaseoli* isolate as a negative control to avoid subjective and often ambiguous readings (Figure 1). African *X. c.* pv. *phaseoli* var. *fuscans* isolates consistently produced pigment one day earlier than other *X. c.* pv. *phaseoli* var. *fuscans* isolates on both KBM and MNBY media (data not shown). This was unlikely to be due to differences in growth rate as, although all isolates grew fastest on MNBY medium, there was little difference in growth rate between isolates on a given medium. Thus such differences are likely to be due to variation in either the level or time of pigment production.

We concluded that the pigment test for distinguishing between *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans*, whilst time-consuming and laborious, also varies according to the medium used. In addition, variation in the length of time for different isolates to produce the brown pigment, together with differences in pigment intensities, could potentially lead to the erroneous classification of *X. c.* pv. *phaseoli* var. *fuscans* isolates as *X. c.* pv. *phaseoli*.

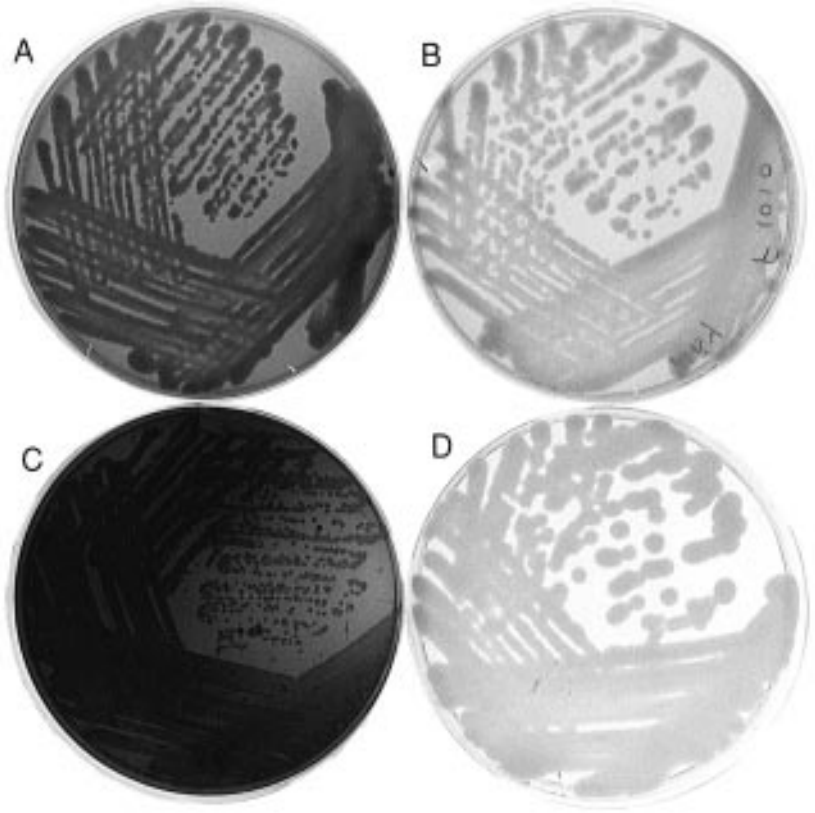

Figure 1. Brown pigment production by *X. c.* pv. *phaseoli* var. *fuscans* isolate 1010 on three media: A, MNBY; B, YDCA; C, KBM. Plate D shows *X. c.* pv. *phaseoli* isolate 408 on MNBY medium (negative control).

Differentiation between X. c. *pv.* phaseoli *and* X. c. *pv.* phaseoli *var.* fuscans *using RAPD PCR*

Total genomic DNAs (gDNAs) from all *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates were extracted using a modification of the Phytopure Plant DNA extraction kit (Scotlab Bioscience), after growth on YDCA at 27 °C for 72 h. An extra chloroform extraction step was included prior to the chloroform and silica suspension extraction step. In addition, the DNA was resuspended in 50 μl of HPLC-pure water (Sigma) and kept at 4 °C for 16 h, followed by heating to 55 °C for 5 min, centrifuged at 13 000 rpm for 2 min, and 45 μl of supernatant transferred to a fresh tube. Genomic DNA from each isolate was used in RAPD PCR analysis with the 10-mer Operon primers OP-G01, OP-G05, OP-G07, OP-G11, OP-G16, OP-G17 and OP-H17 (Operon Technologies, Alemada, Calif., U.S.A.). The sequence of the OP-G11 primer, for which RAPD PCR is reported in this study, is 5′-TGCCCGTCGT-3′. RAPD PCR was routinely performed in a 25 μl reaction mixture containing 50 ng of gDNA, 1 x reaction buffer (Gibco), 1 unit of *Taq* DNA polymerase (Gibco), 0.6 μM primer, 100 μM each of dCTP, dGTP, dATP and dTTP, and 0.75 mM $MgCl_2$. PCR reactions were performed in a Perkin Elmer 9600 thermocycler under the following conditions: denaturation at 95 °C for 5 min. followed by 31 cycles of 95 °C for 1 min, 37 °C for 2 min, and 72 °C for 1 min, with a final extension of 72 °C for 5 min. Amplified DNA fragments were electrophoresed through a 1.4% agarose gel according to Sambrook et al. (1989).

Whereas primers OP-G01 and OP-H17 amplified few DNA bands from *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* gDNA, all other primers, with the exception of OP-G11, reproducibly generated multiple amplification products (results not shown). In the case of OP-G11, a variable number of DNA bands were amplified from the gDNAs of *X. c.* pv. *phaseoli* isolates. Conversely, DNA amplified from all *X. c.* pv. *phaseoli* var. *fuscans* isolates generated a single high intensity band of 820 bp (Figure 2A). However, no such band

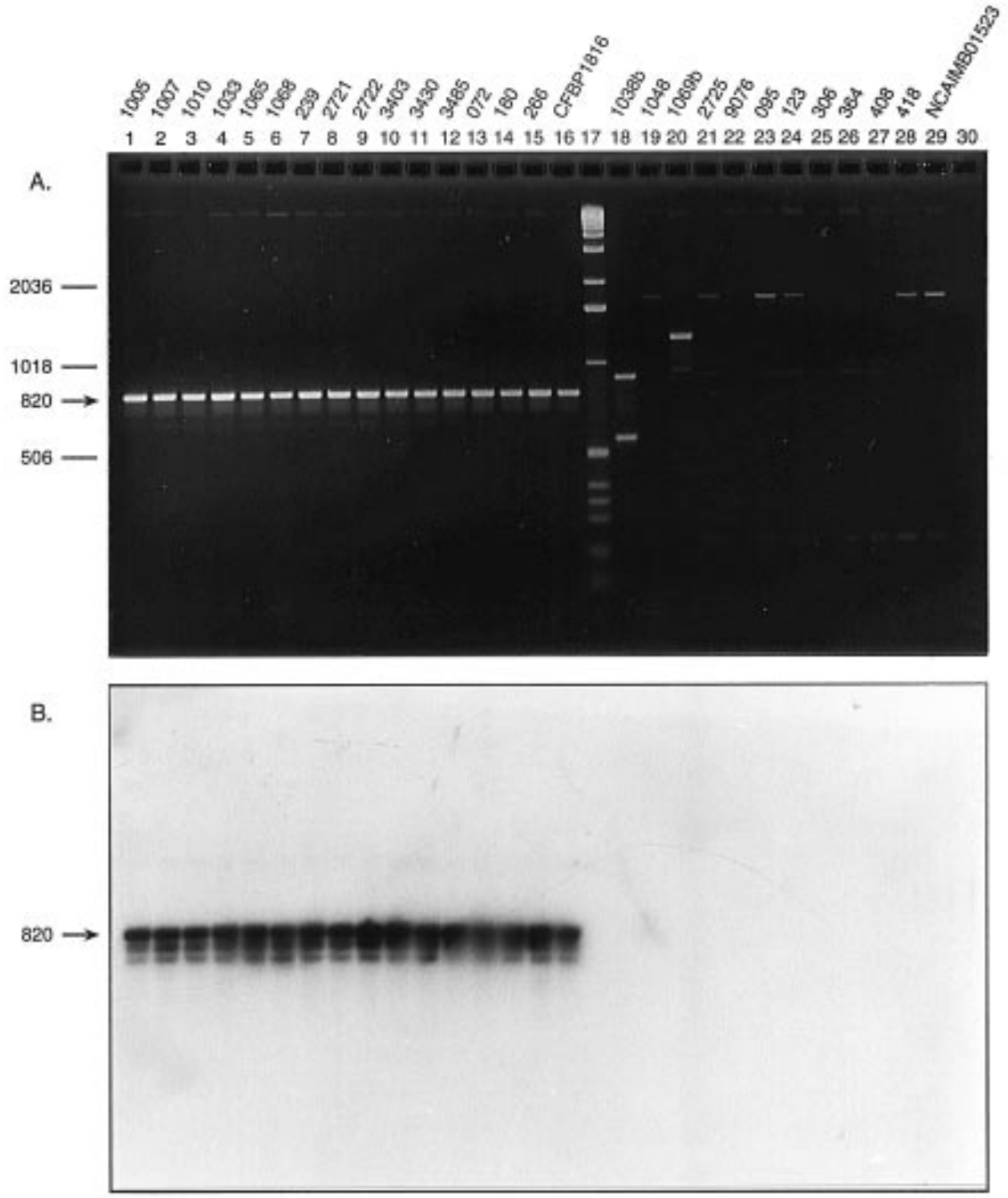

Figure 2. A) Agarose gel electrophoresis of RAPD PCR-amplified DNA using primer OP-G11. Lanes 1-16, *X. c.* pv. *phaseoli* var. *fuscans* isolates; Lanes 18-29, *X. c.* pv. *phaseoli* isolates; Lane 30, no DNA (negative control); Lane 17, 1 kb ladder (Gibco-BRL) (sizes are given in base pairs to left of panel). Arrow indicates 820 bp *X. c.* pv. *phaseoli* var. *fuscans*-specific amplification product. Isolate numbers are shown at the top of the panel. B) Southern blot of gel A probed with ^{32}P dCTP-radiolabelled 820 bp amplification product from *X. c.* pv. *phaseoli* var. *fuscans* isolate 1005. Arrow indicates 820 bp *X. c.* pv. *phaseoli* var. *fuscans*-specific amplification products.

was generated from the gDNA of isolate 408, which had previously been classified as *X. c.* pv. *phaseoli* var. *fuscans*. After re-testing for brown pigment production this isolate was re-classified as *X. c.* pv. *phaseoli* (see above).

To test whether the 820 bp DNA band was uniquely amplified from gDNAs of *X. c.* pv. *phaseoli* var. *fuscans* isolates, it was excised from the agarose gel and used as a probe in Southern analysis of DNAs amplified from all *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* isolates by the OP-G11 primer. Southern analysis was performed as described in Sambrook et al. (1989) using high stringency conditions and the probe was labelled with ^{32}P dCTP using a Random Primed Labelling Kit (Pharmacia). Hybridisation was observed only to the 820 bp band generated from *X. c.* pv. *phaseoli* var. *fuscans* gDNAs and not to bands amplified from *X. c.* pv. *phaseoli* gDNAs, suggesting that this product was, indeed, uniquely amplified from the gDNAs of *X. c.* pv. *phaseoli* var. *fuscans* isolates (Figure 2B). There are two possible explanations for this: either the region to which the OP-G11 primer anneals in *X. c.* pv. *phaseoli* var. *fuscans* gDNA is absent from *X. c.* pv. *phaseoli* gDNA, or mutation at an analogous region in *X. c.* pv. *phaseoli* gDNA results in insufficient identity for annealing of the OP-G11 sequence. These hypotheses were tested by Southern analysis of the total gDNA from each class of isolate.

Southern analysis reveals that the 820 bp region amplified from X. c. *pv.* phaseoli *var.* fuscans *gDNA is absent from* X. c. *pv.* phaseoli *gDNA*

To investigate whether sequences within the 820 bp product amplified from *X. c.* pv. *phaseoli* var. *fuscans* isolates were also present in *X. c.* pv. *phaseoli* isolates, undigested gDNAs (1 μg) from four *X. c.* pv. *phaseoli* var. *fuscans* isolates (1005, 1068, 2721 and 266) and four *X. c.* pv. *phaseoli* isolates (1038, 1067, 2725 and 306) were each digested with the restriction enzyme *Eco*RI, and Southern hybridised to the 820 bp PCR amplification product. Hybridisation, using both high and low stringency washing conditions, was observed only to the gDNAs of the *X. c.* pv. *phaseoli* var. *fuscans* isolates, suggesting that this region of DNA is indeed specific to *X. c.* pv. *phaseoli* var. *fuscans* isolates (data not shown). Hybridisation occurred only to the gDNA, suggesting that homology was to chromosomal and not to plasmid sequences, although further work would be needed to confirm this. In addition, we have hybridised this fragment to gDNA from a variety of other xanthomonad species and found no homology (unpublished results).

The standard procedure for distinguishing between isolates of *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans* involves growth on any one of at least three media, followed by visual inspection for brown pigment production after 1 to 7 days of incubation. This method is laborious and time consuming, and variation in the time taken for pigment to be generated could lead to the mis-classification of isolates. We suggest, therefore, that a PCR-based test would not only be quicker but would also prove more reliable in the future classification of such isolates. Using a RAPD PCR approach we have demonstrated that a clear, reproducible, amplification product is specifically generated from the gDNAs of *X. c.* pv. *phaseoli* var. *fuscans* isolates, so far tested, from various geographical locations in as little as 4 h, allowing their accurate and rapid distinction from *X. c.* pv. *phaseoli* isolates.

Recently, Audy et al. (1994) designed a single set of primers (X4c and X4e) for the specific PCR amplification of DNA from *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans*, which have been used as part of a system for the concurrent detection of both *X. c.* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola* (Audy et al., 1996). They found that a 730 bp product was amplified from *X. c.* pv. *phaseoli* DNAs, and either this product, one of 550 bp, or both, were amplified from *X. c.* pv. *phaseoli* var. *fuscans* isolates (Audy et al., 1994). In addition, the strength of amplification varied between isolates. Thus, not only do they fail to accurately distinguish between *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans*, but it remains unclear how well their primers will detect all *X. c.* pv. *phaseoli* var. *fuscans* isolates. We are, therefore, currently designing *X. c.* pv. *phaseoli* var. *fuscans*-specific primers from the 820 bp product generated in this study to be used in tandem with the primers designed by Audy et al. (1994), in the development of an unequivocal detection system for both varieties of *X. c.* pv. *phaseoli*.

Acknowledgements

We thank Chris Hale (Horticulture and Food Research Institute of New Zealand, New Zealand), Peter Ott (Plant Protection Institute, Hungarian Academy of Sciences, Hungary) and Valerie Verdier (ORSTROM, France and CIAT, Columbia) for sending isolates of *X. c.* pv. *phaseoli* and *X. c.* pv. *phaseoli* var. *fuscans*. Fina Opio was supported by the Kawanda Research

Station, Uganda, and Robert Taylor by the Horticulture and Food Research Institute of New Zealand. We acknowledge the support of the Scottish Office Agriculture and Fisheries Department.

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