

DETECTION OF *PSEUDOMONAS SYRINGAE* PV. *PAPULANS* IN APPLE BUDWOOD

J.L. VANNESTE and J. YU

HortResearch, Ruakura Research Centre, Private Bag 3123, Hamilton,
New Zealand

Corresponding author: JVanneste@HortResearch.co.nz

ABSTRACT

Pseudomonas syringae pv. *papulans* (Psp) causes blister spot on apples, an economically important disease of the cultivar Mutsu in eastern North America. Neither the pathogen nor the disease has been recorded in New Zealand or Australia. Since Psp can be transmitted via budwood, a protocol to specifically detect Psp in apple buds has been developed. It is based on the amplification by polymerase chain reaction (PCR) of part of the *hrpL* gene. Using this protocol, presence of Psp could be routinely detected in apple buds spiked with 100 cells of the pathogen. This protocol was used to analyse budwoods from Hawke's Bay and Waikato and apple fruit from Waikato, Hawke's Bay and Central Otago. All samples were negative, which is consistent with the pathogen never having been recorded in this country.

Keywords: blister spot, cv. Mutsu, PCR, *hrpL* gene.

INTRODUCTION

Pseudomonas syringae pv. *papulans* (Psp) is the causal agent of blister spot, a bacterial disease of apple that affects more severely and more commonly apples of the cultivar Mutsu, also known as cv. Crispin (Dhanvantari 1977). Symptoms on fruit are small, usually round raised lesions of 1-5 mm around the lenticels, with a brown centre and a dark purple border. This disease was for a long time limited to eastern North America, New York and Michigan states in the USA and the province of Ontario in Canada, where cv. Mutsu is grown commercially (Dhanvantari 1977). It has since been found in Italy (Bazzi & Calzolari 1983), British Columbia, Canada, (Sholberg & Bedford 1997) and more recently in France (Kerkoud et al. 2000) and Germany (Moltmann 2000). In France Psp was found on symptomless fruit and in Germany it was found on cv. Delbarestivale.

This disease has never been recorded in New Zealand or Australia. Psp overwinters in buds (Burr & Katz 1984) and could be transmitted via budwood. This project aimed to develop a detection method that would allow the rapid and accurate detection of Psp in apple buds. The protocol developed in this study was used to analyse budwood and apple fruit from Hawke's Bay, the Waikato and Central Otago in New Zealand.

MATERIALS AND METHODS

Bacterial strains

The 15 strains of Psp used in this study were obtained from the International Collection of Micro-organisms from Plants (ICMP), Landcare Research, Auckland, New Zealand. They were kept in a biological containment facility (PC2) at HortResearch, Ruakura Research Centre, Hamilton, New Zealand. The strains ICMP3881, ICMP4040, ICMP4041 and ICMP4043 were isolated from the USA; the strains ICMP4044, ICMP4045, ICMP4046, ICMP4047, ICMP4048, ICMP4049, ICMP4051, ICMP4052, ICMP4053, ICMP4054 and ICMP4055 were isolated from Canada. The other strains used in this study were the strain of *Pseudomonas syringae*

pv. *tomato* ICMP809, the strain of *Pseudomonas fluorescens* A506 (Lindow 1982), the strain of *Erwinia amylovora* Ea1540 from ICMP, and a strain of *Pseudomonas* species from our laboratory. These strains were grown at 28°C on Luria Broth Base (Invitrogen Life Technologies Ltd) supplemented with agar when needed.

Sample preparation

Presence of Psp was detected by amplification of a DNA fragment by polymerase chain reaction (PCR). When the PCR was carried out with total bacterial DNA, the DNA was isolated using the Wizard® genomic DNA kit (Promega).

When apple buds were being analysed, they were cut from the twig with a sterile scalpel and macerated using a Kinematica® Ag Polytron PT3000 in 500 µl of a buffer containing 140 mM NaCl, 50 mM KCl, 0.05% Tween-20 and 2% polyvinyl polypyrrolidone. The macerate was allowed to settle for 10 min, and all of the supernatant was spread onto a single Luria agar plate and incubated at 28°C for 48 h. The bacterial growth was then harvested and resuspended in 500 µl of distilled water. This bacterial suspension was kept at -20°C before being diluted 100 times and used for the PCR amplification. One PCR amplification was usually carried out for each bud.

To determine the limit of detection of this method, apple buds were artificially inoculated with Psp. An aliquot (100 µl) from an overnight culture of Psp ICMP4041 diluted in 10 mM MgSO₄ was added to the bud immediately before maceration with the Polytron. The number of bacteria added to each bud was determined by plating on Luria agar plates, using 1/10 serial dilutions in 10 mM MgSO₄ of the bacterial suspensions. The bacterial count was determined after 48 h incubation at 28°C.

When fruit were being analysed, they were washed in the smallest possible volume of 10 mM MgSO₄ (ca 10-15 ml) by rubbing the fruit gently for about 10 min in a plastic bag containing the MgSO₄. When symptoms on fruits (spots) were analysed, they were cut away with a sterile scalpel and washed in 500 µl of 10 mM MgSO₄. Those fruit or symptom washings were plated on Luria agar plates and incubated at 28°C for 48 h. Bacterial growth from those plates was harvested and processed as described above.

PCR protocol

PCR amplifications were performed in a Mastercycler Personal (Eppendorf), using the primers PapHrp1 and PapHrp2 designed by Kerkoud et al. (2002). These primers allow the amplification of a 242 base pairs fragment of the *hrpL* gene, which encodes an alternative sigma factor required for the expression of the *hrp* gene cluster in *Pseudomonas syringae* (Huang et al. 1995). After an initial denaturation step at 95°C for 2 min, 30 cycles of 92°C for 1 min, 64°C for 30 sec and 72°C for 1 min, were carried out followed by a final extension at 72°C for 1 min. Amplified DNA fragments were separated by 2% agarose gel electrophoresis in TAE buffer; they were visualised under UV light after staining with ethidium bromide (Maniatis et al. 1982).

Origin of field samples

Field samples consisted of apple buds and fruit. Ninety-four buds of apple cv. Jazz™ from Hawke's Bay were collected and analysed. Twenty-five buds from a block of cv. Royal Gala at the Ruakura Research Station near Hamilton were also analysed. The fruit analysed consisted of 25 fruit from cv. Royal Gala from the Waikato Research Centre at Rukuhia; 20 Central Otago fruit in total from four wild apple trees growing by the side of the road and from organic blocks of cv. Braeburn, cv. Royal Gala and cv. Pacific Rose™; and 20 fruit in total from 10 different cultivars from the apple collection held by HortResearch in Hawke's Bay. When fruit had spots and the spots were of similar morphology at least one spot from each fruit was analysed separately. If there were spots of different morphology at least one spot of each morphology type was analysed separately.

RESULTS

Specificity and limit of detection of Psp

When total DNA of any of the 15 strains of Psp was used as a template for the PCR, a DNA fragment of the expected size (242 base pairs) was detected. No DNA fragment was recovered after amplification using the total DNA of either *P. syringae* pv. *tomato*, *P. fluorescens*, *Pseudomonas* sp. or *E. amylovora*. When using apple buds artificially inoculated with a culture of ICMP4041, a fragment of 242 bp was detected when the buds were inoculated with ca 100 bacteria or more (Fig. 1).

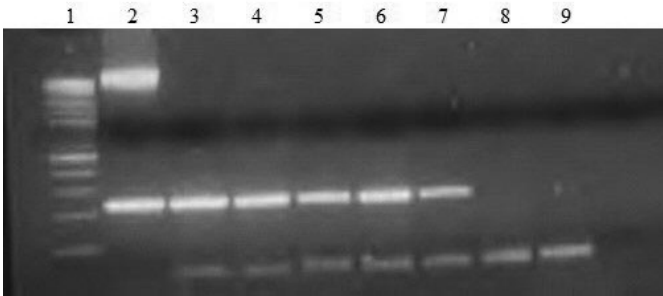


FIGURE 1: Gel electrophoresis of the *hrpL* DNA fragment amplified by polymerase chain reaction using the primers PapHrp1 and PapHrp2. Lane 1: DNA size markers (1 kb ladder from Invitrogen), Lane 2: total DNA of *Pseudomonas syringae* pv. *papulans* ICMP4041 used as a positive control, Lanes 3 to 8: apple buds inoculated with decreasing concentration of *P. syringae* pv. *papulans* ICMP4041, from 10^6 bacteria per bud (Lane 3) to 10^1 bacteria per bud (Lane 8), and Lane 9: the bud was inoculated with water and used as a negative control. The smaller fragments visible in Lanes 3 to 9 are primer-dimers.

Detection of Psp in field samples

A total of 219 samples were analysed by PCR, including 119 samples from apple buds, 65 samples from fruit and 35 samples from fruit spots. None of these samples yielded a DNA fragment of the expected size.

DISCUSSION

Blister spot, caused by Psp, is an economically important disease of cv. Mutsu (Dhanvantari 1977). Psp has never been recorded in New Zealand or Australia, but it could be introduced accidentally via budwood, since it colonises dormant buds (Burr & Katz 1984). To allow the potential screening of apple budwood for presence of Psp, a protocol for detection of this pathogen has been developed. It allowed the detection of about 100 cells of Psp in a bud.

The protocol presented in this paper was initially designed for the detection from apple buds of *Erwinia amylovora*, the fire blight pathogen (Vanneste & Yu 2005). Using a similar protocol for the detection of those two pathogens might allow their detection by duplex PCR, as it has been proposed earlier (Taylor et al. 2003). For the detection of Psp only, the sensitivity of this protocol could be improved by reducing or eliminating the enrichment step (the 48 h incubation on Luria agar plates after maceration). Kerkoud et al. (2002) report that an enrichment step on King's medium B reduced the level of sensitivity of detection of Psp by PCR in leaf extract, probably because Psp is overrun by other epiphytic bacteria.

The primers used in this protocol were chosen for their specificity (Kerkoud et al. 2002). The limited number of non Psp strains tested in this study confirms this specificity.

Using this protocol, no field samples from New Zealand were found harbouring Psp. Burr & Hurwitz (1981) reported that Psp grows epiphytically preferentially on cv. Mutsu. Since Mutsu apples are not grown commercially in New Zealand, the presence of blister spot would be of minor economic importance in this country. In the field samples analysed so far, no buds or fruit were of the cultivar cv. Mutsu. However, as cv. Mutsu apple trees have recently been located in New Zealand, they are being checked for presence of Psp using the method described in this paper.

ACKNOWLEDGEMENTS

This project was supported by Horticulture Australia Ltd (project APO1030). We thank Wendy Fluhler for technical support.

REFERENCES

- Bazzi C, Calzolari A 1983. Bacterial blister spot of 'Mutsu' apples in Italy. *Phytopathologia Mediterranea* 22: 19-21.
- Burr TJ, Katz B 1984. Overwintering and distribution pattern of *Pseudomonas syringae* pv. *papulans* and pv. *syringae* in apple buds. *Plant Disease* 68: 383-385.
- Burr TJ, Hurwitz B 1981. Seasonal susceptibility of Mutsu apples to *Pseudomonas syringae* pv. *papulans*. *Plant Disease* 65: 334-336.
- Dhanvantari BN 1977. A taxonomic study of *Pseudomonas papulans* Rose 1917. *Journal of Agricultural Research* 20:557-561.
- Huang H-C, Lin R-H, Chang C-J, Collmer A, Deng W-L 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin_{ps} secretion that are arranged collinearly with *Yersinia ysc* homologs. *Molecular Plant-Microbe Interactions* 8:733-746.
- Kerkoud M, Manceau C, Gardan L, Samson R, Paulin JP 2000. Epiphytic occurrence of *Pseudomonas syringae* pv. *papulans* (Rose) in France, where blister spot has never been seen. *European Journal of Plant Pathology* 106: 481-485.
- Kerkoud M, Manceau C, Paulin JP 2002. Rapid diagnosis of *Pseudomonas syringae* pv. *papulans*, the causal agent of blister spot of apple, by polymerase chain reaction using specifically designed *hrpL* gene primers. *Phytopathology* 92: 1077-1083.
- Lindow SE 1982. Integrated control of frost injury and fire blight of pear with antagonistic epiphytic bacteria. *Phytopathology* 72: 946.
- Maniatis T, Fritsch EF, Sambrook J 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.
- Moltmann E 2000. Die blasenfleckenkrankheit an 'Delbarestivale' verursacht durch *Pseudomonas syringae* pv. *papulans*. *Obstbau* 8:457-458.
- Sholberg PL, Bedford KE 1997. Characterization of blister spot [*Pseudomonas syringae* pv. *papulans*] in British Columbia and its potential for spread to new apple cultivars. *Canadian Journal of Plant Pathology* 19: 347-351.
- Taylor RK, Hale CN, Horner MB, White AG 2003. Improved detection of *Pseudomonas syringae* pv. *papulans* and *Erwinia amylovora* in apple budwood by the polymerase chain reaction assay. 8th International Congress of Plant Pathology 2:95.
- Vanneste JL, Yu J 2005. Distribution of *Erwinia amylovora* the fire blight pathogen in artificially inoculated apple plants. 15th Biennial Australasian Plant Pathology Society Conference Handbook. p. 58.