

## Detection of tumorigenic rhizobia in asymptomatic peach plants by PCR

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**Summary.** At present the only method for the detection of *Rhizobium radiobacter* and *R. rhizogenes* (ex *Agrobacterium tumefaciens*) in plants is by isolating the bacteria on selective medium and testing them *in vivo* for pathogenicity. This procedure is time-consuming and not appropriate for detecting low concentrations of these agrobacteria. In this study a protocol was developed for detecting tumorigenic agrobacteria in the stem and root tissues of artificially inoculated peach plants. DNA was extracted from the plant tissues by a rapid procedure and then a 246 bp sequence of the *vir* region of the pTi was amplified by PCR. The target sequence was found in all stem and root samples of asymptomatic peach plants, and was evidenced in all the samples analyzed showing the effectiveness and reliability of the method.

**Key words:** *Rhizobium radiobacter*, *Rhizobium rhizogenes*, detection, systemic activity, peach.

### Introduction

The phytopathogenic bacteria *Rhizobium radiobacter* and *R. rhizogenes* (ex *Agrobacterium tumefaciens*) cause crown gall, a serious disease on many fruit trees and ornamental plants throughout the world. Crown gall causes serious losses in the nursery because infected plants are unsaleable. *A. tumefaciens* can live as an endophytic bacterium, moving inside the vascular system of host plants, and this behaviour may explain its spread in plant propagation material (Tarbah and Goodman, 1987; Marti *et al.*, 1999; Zoina *et al.*, 2001). Crown gall is

all the more worrying disease for peach growers since most stone-fruit rootstocks commercially available are susceptible to *A. tumefaciens* infection (Zoina and Raio, 1999). The most effective method to control crown gall on peach is by treating the plants with bacterial suspensions containing the antagonist *Agrobacterium radiobacter* strain K84 (Kerr and Tate, 1984). This is however only a preventive method, which is ineffective against plants with latent infections. At present the only method to detect tumorigenic agrobacteria in plants is by isolating the bacteria on a selective medium and testing them *in vivo* for pathogenicity. This procedure is always time-consuming and does not detect latent infections, given its low sensitivity (requiring 10<sup>2</sup> cfu ml<sup>-1</sup> of plant tissue). Only the tumorigenic forms of agrobacterium harbour a plasmid (pTi) that causes the tumors in

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plants. Some sequences of pTi are highly conserved and can be used as a target for the specific detection of this pathogen by PCR.

A rapid and sensitive procedure for detecting *A. tumefaciens* in asymptomatic peach plants by PCR is described in this study.

## Materials and methods

Peach seedlings cv. Montclair were inoculated with suspensions containing the tumorigenic *A. tumefaciens* strain B49c/83 marked for rifampicin resistance. Plants were inoculated by dipping their roots for five minutes in the bacterial suspensions containing the tumorigenic strain at three different concentrations  $10^3$ ,  $10^5$ ,  $10^7$  cfu ml<sup>-1</sup>. Plants were then transplanted to pots containing sterile soil and kept in the greenhouse for about a year. Each treatment included three replications of twenty seedlings.

Both the stem and root tissues of peach seedlings inoculated with the three different suspensions were sampled for agrobacterium occurrence. For each analysis three seedlings from each treatment were processed separately. Stem and root samples were washed under running tap water, disinfected with a 0.5% NaClO solution for 5 minutes, washed three times in sterile distilled water and then dried under the hood. Samples of one gram each were placed in 50 ml Falcon tubes containing 9 ml saline solution and ground in a homogenizer for 3 min.

The procedure described by Llop *et al.*, (1999) was used to extract DNA from the plant tissues. A thermal shock step was added to this protocol in order to improve yield and the quality of DNA extracts.

A specific sequence for *A. tumefaciens* was amplified using the primers FGPvirB<sub>11+21</sub> and FGPvirG15' designed on the intercistronic region between vir B and vir G of the virulence region of the tumor-inducing plasmid pTi harboured by the tumorigenic agrobacterium (Nesme *et al.*, 1995).

PCR reactions were performed in a total volume of 50  $\mu$ l containing: PCR buffer  $1\times$  (10 mM), MgCl<sub>2</sub> (1.5 mM), dNTPs (200  $\mu$ M each), primer (0.1  $\mu$ M each), Taq (2.5 U) (Gibco, BRL, USA), extracted DNA (5  $\mu$ l). After 5' denaturation at 94°C, the profile of amplification cycle was: 94°C $\times$ 30", 57°C $\times$ 45", 71°C $\times$ 1' and a final extension step at

71°C $\times$ 3'. All amplifications were performed using a Peltier Thermal Cycler PTC-200 (MJ research, Waltham, MA, USA).

The sensitivity of the new procedure was determined by analysing samples prepared by mixing suspensions of B49c/83 Rif<sup>r</sup> at different concentrations with plant tissues. DNA was extracted and amplified according to the protocols described above.

*Agrobacterium tumefaciens* was also detected with the dilution plating method, using the 2E selective medium (Brisbane and Kerr, 1983) amended with rifampicin 100 ppm.

## Results and discussion

Only the peach seedlings inoculated with the suspension containing  $1\times 10^7$  cfu ml<sup>-1</sup> of strain B49c/83 Rif<sup>r</sup> developed tumors. However, the presence of endophytic tumorigenic agrobacteria was also assessed on asymptomatic peach plants inoculated with  $1\times 10^3$  and  $1\times 10^5$  cfu ml<sup>-1</sup> ml suspensions of the marked strain.

The PCR-based protocol developed in this study showed high sensitivity since it detected the target sequence down to a concentration of 5 cfu g<sup>-1</sup> of peach tissue.

Tumorigenic agrobacteria were always detected in the stems of peach plants inoculated with B49c/83 Rif<sup>r</sup> at all three concentrations and in the roots of plants inoculated with the highest concentration of the bacterium (Fig. 1).

The target sequence was detected from the roots of plants inoculated with  $1\times 10^5$  and  $1\times 10^3$  cfu ml<sup>-1</sup> of the tumorigenic strain only when the plant extracts were diluted 1:100. The positive amplification of the diluted extracts indicated that the extraction procedure did not eliminate chemical compounds that may act as Taq polymerase inhibitors.

The inoculated marked strain was reisolated from the roots of peach plants inoculated with  $1\times 10^7$  and from the roots and the lowest part of the stems of plants inoculated with  $1\times 10^5$  cfu ml<sup>-1</sup> of B49c/83. The dilution plating counts of the bacterium are shown in Table 1.

A comparison between the molecular procedure and the classical method showed that the new protocol detected the target sequence from the roots and stems of both galled and asymptomatic peach plants, while the isolation of the marked strain on

selective media was not always effective (Table 2).

The molecular procedure was fast and reliable, being completed within 24 hours, and was also highly sensitive and effective in detecting the tar-

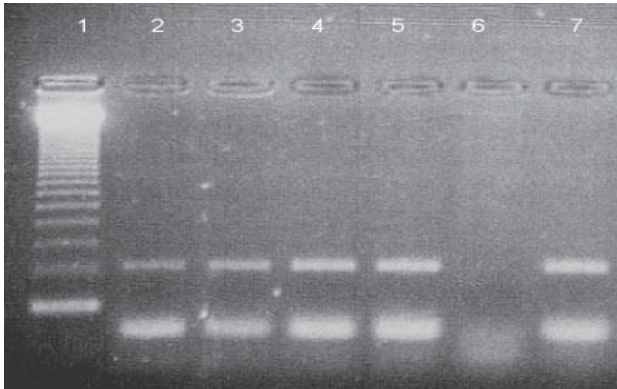


Fig. 1. Amplification of the 246 bp region in the stem tissue of peach seedlings inoculated with  $1 \times 10^3$  cfu ml<sup>-1</sup> (2);  $1 \times 10^5$  cfu ml<sup>-1</sup> (3);  $1 \times 10^7$  cfu ml<sup>-1</sup> (4). Bacterial suspensions and root tissues of plants inoculated with  $1 \times 10^7$  cfu ml<sup>-1</sup> (5). Negative control (6). Positive control (7). Lane 1, 123 bp ladder.

get sequence of the pTi of *A. tumefaciens*. The classical method, on the other hand, takes at least 20 days and does not always detect low concentrations of *A. tumefaciens*.

DNA extraction from plant tissues is a simple procedure and does not require phenol or other toxic reagents (Llop *et al.*, 1999); however some chemical compounds (such as polyphenols) are extracted together with the DNA and these compounds may act as Taq polymerase inhibitors. This problem can be overcome by diluting the extracts before amplification.

Detecting *A. tumefaciens* by PCR may also be helpful to study the systemic activity of this pathogen in plants. Preliminary results showed that *A. tumefaciens* cells were detected in the stems up to 20 cm above the crown of peach plants, indicating that the pathogen moved inside the vascular system of host plants other than those plants (chrysanthemum, grape, rose or weeping fig) in which its systemic activity is already well known. The protocol devised in this study may furnish the basis for a scheme of sanitary certification of plant

Table 1. Concentration of B49c/83 Rif<sup>r</sup> strain (cfu g<sup>-1</sup>) in peach seedlings detected with dilution plating.

Plant material	cfu g <sup>-1</sup>		
	Treatment 1 <sup>a</sup>	Treatment 2	Treatment 3
Roots	$2.8 \times 10^4$	$3.5 \times 10^2$	0
Stem (5–10 cm)	0	$1.2 \times 10^2$	0
Stem (15–20 cm)	0	0	0

<sup>a</sup> Treatment 1, 2 and 3: plants inoculated with  $10^7$ ,  $10^5$  and  $10^3$  cfu g<sup>-1</sup> of B49c/83 strain respectively.

Table 2. Comparison between the positive (+) or negative (-) results obtained by using the PCR-based protocol and the dilution plating method (DPM) in the detection of *A. tumefaciens* in peach seedlings.

	Treatment 1 <sup>a</sup>		Treatment 2		Treatment 3	
	PCR	DPM	PCR	DPM	PCR	DPM
Roots	+	+	+ <sup>b</sup>	+	+ <sup>b</sup>	-
Stem 5–10 cm	+	-	+ <sup>b</sup>	+	+ <sup>b</sup>	-
Stem 15–20 cm	+	-	+ <sup>b</sup>	-	+ <sup>b</sup>	-

<sup>a</sup> Treatment 1, 2 and 3: plants inoculated with  $10^7$ ,  $10^5$  and  $10^3$  cfu g<sup>-1</sup> of B49c/83 strain respectively.

<sup>b</sup> DNA extracts diluted 1:100.

propagation material and could be adopted for routine analysis in the laboratories charged with such certification of plant propagation material.

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