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Evidence for the occurrence of endophytic prokaryotic contaminants in micropropogated plantlets of *Prunus Cerasus* cv. 'Montmorency'

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Introduction

A significantly damaging problem encountered by the plant tissue culture industry is the presence of covert microbial contaminations [1]. In this respect, bacterial contamination is responsible for considerable losses at each step of the micropropagation process, and also for the final consumers of the products (e.g. the greenhouse and nursery industries). Moreover, the increasing applications of micropropagated material as sources of reputedly pathogen-free status is placed in jeopardy since phytopathogenic bacteria can be found among these contaminating micro-organisms [2].

This report presents evidence that aseptically 'micropropagated' *Prunus cerasus* plantlets contained populations of endophytic bacteria which persist in latent form.

Materials and methods

Plant material

Prunus cerasus L. 'Montmorency' (mericlone 8665) was 'aseptically' multiplied by clonal propagation on medium 706 as previously described [3].

Isolation of bacteria

In vitro-grown plantlets of *P. cerasus* were examined for the presence of endogenous bacteria by grinding the tissues in water or in CPW (Cell and Protoplast Washing) solution containing 13% mannitol [4]. After different incubation times (at 25 °C and in the dark) aliquots of the homogenate were either directly plated onto 868 medium (20 g l^{-1} glucose, 10 g l^{-1} peptone, 10 g l^{-1} yeast extract and 15 g l^{-1} Difco agar), or incubated in CPW solutions containing lower concentrations of mannitol before plating them onto 868 medium.

PCR detection of Pseudomonas *sp.*

DNA extracts were obtained from *P. cerasus* plantlets or axenic cultures of bacteria using the method described by Sambrook et al. [5]. Primers (PS1 and PS2) designed to amplify a region of the gene coding for the receptor siderophore in fluorescent *Pseudomonas* sp. were used according to the protocol described by de Vos [6]. The following thermal cycling scheme was used for 30 reaction cycles (Biometra cycler): template denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min and DNA synthesis at 72 °C for 2 min. Amplification products were analysed by electrophoresis in a 1% agarose gel, in Tris–acetate–EDTA buffer [5]. Bands were visualised by ethidium bromide staining.

Results and discussion

Description and identification of endophytic bacteria

Bacterial contaminations of protoplast suspensions prepared from *P. cerasus* (mericlone 8665) led us to investigate whether these contaminants originated from the material itself rather than from casual contaminations introduced during tissue culture procedures.

Plating the homogenate of *P. cerasus* (mericlone 8665) tissues onto 868 medium after grinding and incubating (up to 5 days) it in water did not reveal any bacterial contaminant. On the other hand, plating the homogenate onto 868 medium after grinding the tissues in solutions in which osmotic pressure decreased with incubation time (either the media used in the protocol of protoplasts isolation and incubation [4] or

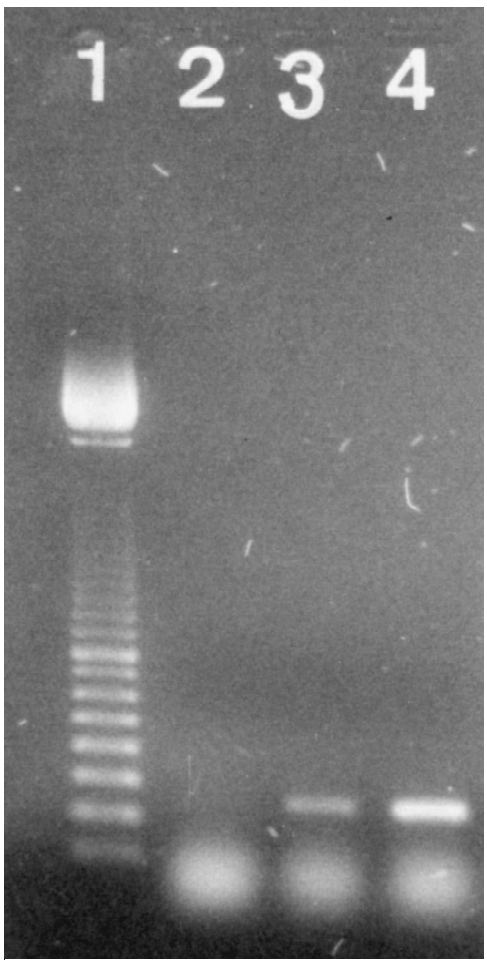

Figure 1. Analysis of PCR products amplified with PS1 and PS2 primers. Lane 1, DNA size markers. Lane 2, control without DNA. Lane 3, 270 pb amplified product from *Pseudomonas aeruginosa* (PAO1).

CPW solutions containing mannitol diluted by one-third every 3 days of incubation) allowed the recovery of bacteria (after 2 days of incubation in protoplast media or after 7 days of incubation in the successive CPW solutions). Different types of morphologically distinct colonies were observed and identified with biochemical tests as *Pseudomonas syringae* and *Agrobacterium rhizogenes* species.

Moreover, electron microscopical examinations of leaf tissues indicated that wall-less prokaryotic cells were present within the cytoplasm of mesophyll cells of the micropropagated plantlets as well as in the centrifugation pellet of the homogenate of the leaf tissues.

PCR detection of Pseudomonas*-like sequence in micropropagated plantlets*

The PCR protocol applied on total nucleic acids extracted from micropropagated plantlets and from the *Pseudomonas aeruginosa* strain (PAO1) used to design the PS1 and PS2 primers allowed the amplification of a 270 pb fragment, thus suggesting the presence of bacteria belonging to the genus *Pseudomonas* in the plant material (Figure 1). Moreover, a similar amplified product was observed from the centrifugation pellet of the leaf homogenate and from the *Pseudomonas syringae* isolate recovered from plant tissues (Kamoun, unpublished results).

Overall observations led us to conclude that *Prunus* shoot culture may contain associated bacteria which appear to be truly endophytic and which persist in latent form during the micropropagation. Similar observations of endophytic bacteria in shoot cultures have already been reported in *Dioscorea* species [7] and potato [2]. In the case of potato, evidence of wall-less prokaryotic (L-form) *Erwinia carotovora* var. *atroseptica* was reported both *in vitro* and *in vivo*. These closely associated bacteria are not directly detected by streaking explants onto classical nutrient media. Our results suggest that a preliminary incubation in solutions with decreasing osmotic pressure is a prerequisite for their recovery and growth on classical nutrient media. That preliminary step could be necessary to regenerate the cell wall of these hypothetical L-form of bacterial contaminants.

Due to its rapid execution and sensitivity threshold, the PCR technique described here can be useful for the detection of fluorescent *Pseudomonas* sp. contaminants. Compared to microbiological tests, its main limitation is its specificity. Primers targeting more conserved regions of bacterial genomes would be necessary to develop broader spectrum PCR tests to detect bacteria belonging to different species or even families.

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