IDENTIFICATION AND ELIMINATION OF BACTERIAL CONTAMINANTS FROM *Pelargonium* TISSUE CULTURES

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A B S T R A C T

In our laboratory, bacterial contamination was detected in cultures of *Pelargonium* x *hederaefolium* 'Bonete' that had been subcultured continuously for several years. Two contaminating bacteria were isolated and identified by partial sequence analysis of 16S rRNA gene as *Paenibacillus glycanilyticus* and *Lactobacillus paracasei.* Antibiotic sensitivity testing was performed on the bacterial isolates and four antibiotics were identified as being potentially useful in eliminating these contaminating bacteria: carbenicillin, cefotaxime, neomycin and streptomycin. Neomycin and streptomycin were found to be phytotoxic to *Pelargonium* shoots.

Shoots were grown on multiplication medium containing 0.5 mg l^{-1} m-topolin supplemented with either 500 mg^{-1} carbenicillin or 250 mg l^{-1} cefotaxime and transferred to fresh medium every three weeks. After growing in the presence of carbenicillin for three weeks, bacterial growth was inhibited, but not completely eliminated. Intensive formation of high-quality shoots also took place. After nine weeks of growth on medium supplemented with carbenicillin, 40% of the shoots were free of both contaminating bacteria. After three weeks of growth on medium supplemented with cefotaxime, 37% of the shoots were free of both contaminating bacteria. However, shoots grown in the presence of cefotaxime tended to be shorter and have yellow leaves more so than shoots grown in the presence of carbenicillin. Prolonged exposure to cefotaxime adversely affected shoot formation and eventually resulted in plant death.

Key words: antibiotics, bacterial identification, *in vitro*, multiplication, *Pelargonium*

Abbreviations: MS – Murashige and Skoog's medium; m-topolin – N^6-(meta-hydroxybenzyl) adenine

INTRODUCTION

Plant tissue cultures may be contaminated by a wide variety of bacteria, many of which are species specific. Bacterial contamination can reduce growth rate, retard rooting, and even cause plant death (Leifert and Waites, 1992). Tissue cultures can become contaminated at any stage of the tissue culturing process (Leifert, 2000). The most difficult bacteria to control are endogenous bacteria which do not cause any visible symptoms in the contaminated culture.

Plant tissue cultures can become contaminated with bacteria either because of poor aseptic technique while handling the cultures or because of animal vectors (Leifert et al., 1994). During micropropagation, bacterial contamination can remain undetected because the salt concentration, sucrose concentration, pH and temperature are not optimal for bacterial growth (Cooke et al., 1992). When the culture conditions are changed, bacterial contaminants, which were present in small numbers can actively multiply and damage the plant cultures (Leifert, 2000).

In our laboratory, bacterial contamination was detected in cultures of *Pelargonium* × *hederaefolium* 'Bonete' that had been subcultured continuously for several years. At first, only sporadic single shoots were visibly affected (eliminated from cultures). However, over time, whole plants started to showed signs of bacterial contamination, including slower growth and reduced shoot quality.

The aim of this study was to identify the bacteria contaminating *Pelargonium* shoots and to find out which antibiotics were most effective in eliminating them.

MATERIAL AND METHODS

Isolation and identification of bacteria

The contaminating bacteria were isolated by placing material from visibly contaminated *P.* × *hederaefolium* cultures directly on King's B medium (King et al., 1954). After incubation at 25°C for 24 h, two colony types were observed. It was difficult to obtain pure cultures of these bacteria because they grew slowly after being subcultured onto fresh medium.

The bacterial isolates were identified by sequence analysis of the products of PCR amplification of the 800 bp 5' end fragment of the 16S rRNA gene. The primers used were fD1 and 800r (Weisburg et al., 1991; Drancourt et al., 1997). The sequences obtained were compared with the sequences stored in the GenBank and EMBL databases using the BLAST N program.

Sensitivity of bacteria to antibiotics

Antibiotic sensitivity testing was performed on the bacterial isolates using a panel of twenty different antibiotics in commercially available test kits. A bacterial lawn was prepared by spreading a suspension of the bacteria (10^5 cfu·ml^{-1}) on King's B medium. Antibiotic test discs were then aseptically placed on

the medium. The plates were incubated for 24 hours at 25°C in the dark. The diameters of the inhibition zones around the disks were measured and recorded.

Antibiotics treatment of plant material

Four antibiotics were selected on the basis of their effectiveness in antibiotic sensitivity testing: carbenicillin, cefotaxime, neomycin and streptomycin.

To test their effectiveness in eliminating bacterial contamination, the antibiotics were added to multiplication medium (Wojtania and Gabryszewska, 2001) containing 0.5 mg l^{-1} m-topolin, in the following dosages:

Antibiotic	**Dosage**
Carbenicillin	250 and 500 mg l^{-1}
Cefotaxime	250 mg l^{-1}
Neomycin	100 and 200 mg l^{-1}
Streptomycin	100 and 200 mg l^{-1}

Antibiotic stock solutions were made freshly every day, filter sterilized, and added to the medium after autoclaving.

Shoot tips (0.5 cm) from contaminated *P.* × *hederaefolium* cultures were then placed on the medium and grown in individual 50-ml Erlenmeyer flasks for two or three weeks. The number of bacteria-free shoots was recorded.

Shoots with no detectable signs of bacterial contamination were individually transferred onto fresh medium without antibiotics and subcultured every three weeks to medium supplemented with 0.5 mg l^{-1} m-topolin. At every third subculture, the shoots were checked for bacterial contamination on King's medium. Growth rate and plant appearance were monitored to determine whether the antibiotics had any phytotoxic effects on multiplication, root formation and subsequent growth in the greenhouse.

Shoots with visible signs of bacteria contamination were transferred to antibiotic-containing media.

All shoot cultures were kept in a growth chamber at 19-20°C, with a sixteen-hour photoperiod under cool-white fluorescent lamps at 39 µmol $m^{-2} \cdot s^{-1}$ (Philips TLD 36W/95).

RESULTS AND DISCUSSION

The bacterial contaminants were identified by partial 16S RNA sequence analysis as *Paenibacillus glycanilyticus* and *Lactobacillus paracasei*. Although both bacteria are known to be associated with plants, their presence in tissue cultures does not necessarily mean that they were present in the original plant culture material, but may be due to handling in the laboratory (Leifert and Waites, 1992). Both bacteria are gram-positive. *P. glycanilyticus* has been recently described as being able to degrade the heteropolysaccharide produced by the cyanobacterium *Nostoc commune* (Kajiyama et al., 2002). *Lactobacilli* are commonly encountered as contaminants in tissue cultures of many different plant species (Leifert et al., 1989). Members of the *Lactobacillus casei* group occur naturally in dairy products, silage, and the human intestinal tract and mouth. The genetic

relationships among members of *L. casei* group have been described by Chen et al. (2000).

Antibiotic sensitivity testing revealed that the antibiotics most effective against the contaminating bacteria were carbenicillin, cefotaxime, neomycin and streptomycin (Fig. 1). Of these, carbenicillin and cefotaxime were chosen for detailed study, because neomycin and streptomycin strongly inhibited shoot growth. Furthermore, streptomycin was only slightly effective at reducing bacterial growth in tissue cultures even at the higher dosage.

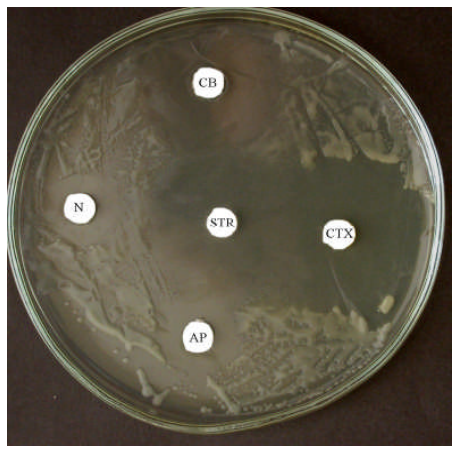

Figure 1. Inhibition of the growth of bacteria isolated from *Pelargonium* shoots in the presence of different antibiotics: AP – Ampicillin, CB – Carbenicillin, CTX – Cefotaxime, N – Neomycin, STR – Streptomycin

Of the four antibiotics tested, the one which was least phytotoxic to *P.* x *hederaefolium* cultures was carbenicillin. When shoot tips were grown for three weeks on multiplication medium supplemented with carbenicillin, bacterial growth was inhibited, but not completely eliminated. Intensive formation of high-quality shoots also took place (Fig. 2). Shoot number in cultures grown on 500 mg l^{-1} carbenicillin was actually higher that in healthy cultures grown on medium supplemented with m-topolin alone (Wojtania and Gabryszewska, 2001) (Fig. 3). Carbenicillin has been reported to enhance multiplication in *Anthirrhinum majus,* enhance callus formation in *Malus*, but inhibit callus formation in *Clematis, Delphinium, Hosta, Iris* and *Photinia* (Holford and Newbury, 1992; Yepes and Aldwinckle, 1994; Leifert et al., 1992). In our study, subculturing three times onto medium supplemented with carbenicillin eliminated both of the contaminating bacteria in 40% of the *P.* x *hederaefolium* shoots. Shoot number and quality were also reduced.

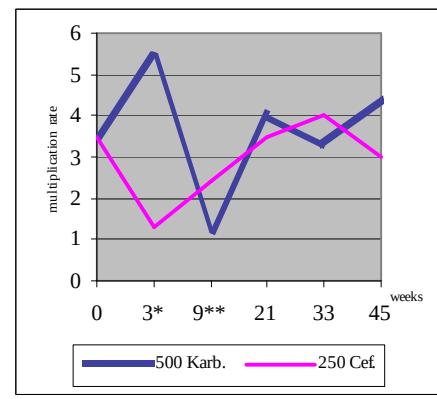

Figure 3. Multiplication rate of *Pelargonium* shoots before (week 0), during and after carbenicillin or cefotaxime treatment (*end of cefotaxime treatment, **end of carbenicillin treatment)

After three weeks on medium supplemented with 250 mg l^{-1} cefotaxime, 37% of the shoots were

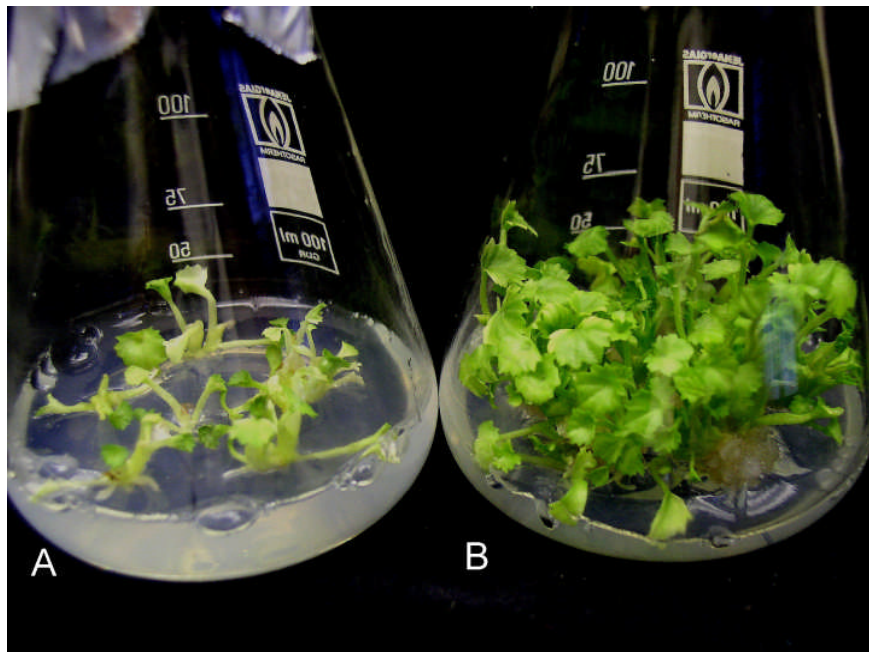

Figure 2. The *P.* × *hederaefolium* shoots after 3 weeks of the growth on the medium containing cefotaxime (A) and carbenicillin (B)

Table 1. Effect of carbenicillin and cefotaxim e on the elimination of bacteria isolated from *P.* × *hederaefolium* shoots

Antibiotic	The number of shoots free of both contaminating bacteria [%]		
	after 3 weeks*	after 6 (3+3) weeks	after 9 (3+3+3) weeks**
Carbenicillin	0	0	40
Cefotaxime	37	-	-

*end of cefotaxime treat ment because of phytotoxicity
**end of carbenicillin treatment

free of both contaminating bacteria (Tab. 1). However, shoots grown in the presence of cefotaxime tended to be shorter and have yellow leaves more often than shoots grown in the presence of carbenicillin (Fig. 2). Prolonged exposure to cefotaxime adversely affected shoot formation and ultimately resulted in plant death.

Carbenicillin and cefotaxime, either alone or in combination, are the antibiotics most commonly used to eliminate bacterial contamination in plant tissue culture. They are relatively non-toxic to plant cells (Okkels and Pedersen, 1988; Leifert et al., 1992; Barrett and Cassells, 1994). In our study, as well as in

other studies, cefotaxime was more active than carbenicillin, probably because cefotaxime is more resistant to bacterial β-lactamases (Estopa et al., 2001; Chevreau et al., 1997). Cefotaxime has been recommended for eliminating *Xanthomonas* contamination in *Pelargonium* cultures (Barrett and Cassells, 1994). Cefotaxime has also been recommended for use in *Agrobacterium*-mediated transformation in *Pelargonium* (Krishna Raj et al., 1997).

Shoots with no detectable signs of bacterial contamination were individually transferred onto fresh medium without antibiotics and subcultured every three weeks to medium supplemented with 0.5 mg l^{-1} m-topolin. At every third subculture, the shoots were checked for bacterial contamination on King's medium. All of the shoots which had been treated with antibiotics were still free of bacterial contamination after one year of subculturing. After several transfers, most of the shoots turned green, although chlorophyll-free shoots were sporadically observed (Fig. 4).

Figure 4. The bleached *Pelargonium* shoots 5 month after cefotaxime treatment

The multiplication rate of shoots treated with antibiotics was similar to that of healthy plants (Fig. 3). However, shoots treated with antibiotics were fragile and had long petioles which were often damaged during subculturing. The shoots were able to produce roots, but most of the plants did not survive in the greenhouse. Sectorial chlorophyll deficiency in leaf blades was also noted in weaning plants (Fig. 5).

Figure 5. The sectorial chlorophyll deficiency in the leaf blades observed among weaning plants

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IDENTYFIKACJA I ZWALCZANIE INFEKCJI BAKTERYJNYCH W KULTURACH *Pelargonium*

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STRESZCZENIE

Badania miały na celu izolację i identyfikację bakterii zasiedlających pędy pelargonii w warunkach *in vitro* i określenie właściwych antybiotyków do ich zwalczania.

Bakterie wyizolowano z podstaw pędów *P.* × *hederaefolium* rosnących w warunkach *in vitro*. Na podstawie analizy molekularnej bakterie zidentyfikowano jako *Paenibacillus glycanilyticus* i *Lactobacillus paracasei.* Dla wyizolowanych bakterii wykonano antybiogramy z użyciem 20 antybiotyków. Do badań wybrano cefotaksym, karbenicylinę, neomycynę i streptomycynę, które wykazywały największe ograniczenie wzrostu bakterii. Do pożywki MS zawierającej topolinę dodawano antybiotyki. Stwierdzono niekorzystny wpływ neomycyny i streptomycyny na jakość pędów pelargonii (pędy zdeformowane, bezchlorofilowe). Najmniej fitotoksyczna była karbenicylina. Po 3 tygodniach wzrostu eksplantatów na pożywce zawierającej 500 mg l^{-1} tego antybiotyku obserwowano ograniczenie wzrostu bakterii i korzystny wpływ na mnożenie i jakość pędów. 40% czystych kultur uzyskano jednak dopiero po 9 tygodniach trwania kultury (3 pasaże). Po zastosowaniu cefotaksymu w stężeniu 250 mg l^{-1} uzyskano 37% pędów wolnych od bakterii już po 3 tygodniach wzrostu pędów. Równocześnie obserwowano jednak zmiany w wybarwieniu liści.

Obserwowano następczy wpływ stosowanych antybiotyków na intensywność wzrostu i morfologię pędów pelargonii (kruchość, przebarwienia, deformacje) oraz zdolność do aklimatyzacji w warunkach szklarniowych, utrzymujący się co najmniej przez 12 miesięcy od momentu ich stosowania.

Słowa kluczowe: antybiotyki, identyfikacja bakterii, *in vitro*, namnażanie, *Pelargonium*