



## Detection and identification of bacterial contaminants of strawberry runner explants

Piyarak Tanprasert & Barbara M. Reed

USDA/ARS National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, OR 97333-2521, USA

### Introduction

Bacterial contamination is a continuing problem for research and commercial plant tissue culture labs [1,2]. Bacterial contamination is often difficult to detect [3,4]. Plants which appear healthy can contain bacteria [3,5], and some plant exudates may look similar to bacterial growth [6,7]. Contaminated plants may lack symptoms, have reduced multiplication rates, reduced rooting rates, or may die [8,9].

Bacteria in plant tissues can be detected by several types of indexing media: 523 medium [4,10]; Murashige and Skoog [11] medium alone or with additions such as yeast extract, peptone, glucose [8,12]; or coconut water [13]. Liquid MS medium alone detects most contaminants from mint explants with a few additional contaminants detected later [10]. Additional experiments with contaminated mint explants found that medium with peptone and yeast extract produced more obvious and faster bacterial growth without causing toxicity to the plants (P. Tanprasert, unpublished).

Many types of bacteria have been detected in plant cultures: *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Corynebacterium*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, *Xanthomonas* and yeasts such as *Torulopsis* [5,8,12,14]. Bacterial contaminants found in plants cultured for 4 weeks or less are often motile Gram-negative bacteria, while contaminants from plants cultured for at least 12 months are more likely to be Gram-positive [8]. Kneifel and Leonhardt [15] found that some plants contain mixtures of Gram-negative rods, Gram-positive rods, and cocci. Mint cultures are contaminated mostly with Gram-negative bacteria, such as *Agrobacterium* and *Xanthomonas*, that are usually associated with plants and soils [14]. Different types of bacteria are found on various plant species and at specific stages of plant cultures.

The goals of this study were to develop good detection methods for bacterial contaminants of strawberry explants, isolate and identify bacteria from explants, characterize the antibiotic response of the isolated bacteria and successfully treat contaminated plants.

### Procedure

#### *Plant material*

*In vitro* cultures were initiated from runners taken from over 100 cultivars of pot-grown strawberries in a screenhouse at the USDA–ARS National Clonal Germplasm Repository, Corvallis, OR. Runners were disinfested by immersion in 10% household bleach (5.25% sodium hypochlorite; Clorox, Oakland, CA) solution containing 1% Tween-20 (Sigma Chemical Co., St. Louis, MO) for 10 min, rinsed twice in sterile deionized water, and grown in individual 16×100 mm tubes containing half-strength liquid Murashige and Skoog (MS) medium [11], pH 6.9, at 25 °C for 10 days with 16 h of light (25  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

#### *Detection of contaminants*

Runners (2–12 from each genotype) from 70 genotypes of *Fragaria* spp. were collected from June to August 1994 and screened for contaminants by partially submerging them in liquid medium (above). Runners (2–12 from each genotype) from 72 additional genotypes were collected from June to August 1995, and screened as above but in medium with 265 mg l<sup>-1</sup> peptone and 88 mg l<sup>-1</sup> yeast extract.

#### *Isolation and purification of bacteria*

Contaminants were transferred with a sterile cotton swab to 0.8% nutrient broth (Difco; Sigma Chemical, St. Louis, MO) with 1% glucose and 0.1% yeast extract at pH 6.9 and incubated at 25 °C. Bacteria were

purified by repetitive streaking on nutrient agar (NA) plates.

#### *Diagnostic tests and identification of bacteria*

Gram stain, KOH, motility, oxidase, starch hydrolysis, and gelatinase tests were performed according to the methods described by Klement et al. [16]. The ability of bacterial strains to oxidize 95 substrates was tested on Biolog Gram-negative and Gram-positive Microplates (Biolog, Inc., Hayward, CA). Identification was made by comparison with standard cultures, Biolog computer analysis and Bergey's Manual [17].

#### *Minimal bactericidal concentrations (MBCs)*

MBCs were tested for sensitivity to single antibiotics (Timentin, gentamicin, streptomycin sulfate). All two-way combinations of Timentin (125 and 250  $\mu\text{g ml}^{-1}$ ) and gentamicin (6.25 and 12.5  $\mu\text{g ml}^{-1}$ ), Timentin (125 and 250  $\mu\text{g ml}^{-1}$ ) and streptomycin sulfate (250 and 500  $\mu\text{g ml}^{-1}$ ), and streptomycin sulfate (250 and 500  $\mu\text{g ml}^{-1}$ ) and gentamicin (6.25 and 12.5  $\mu\text{g ml}^{-1}$ ) were tested. All possible three-way combinations of Timentin (125 and 250  $\mu\text{g ml}^{-1}$ ), streptomycin sulfate (250 and 500  $\mu\text{g ml}^{-1}$ ), and gentamicin (6.25, 12.5 and 25  $\mu\text{g ml}^{-1}$ ) were tested.

#### *Treatment of contaminated plants*

Contaminated plants were treated (10 days) with combinations of three antibiotics (Timentin, streptomycin sulfate, and gentamicin) at (mg  $\text{ml}^{-1}$ ) 500 (T) + 250 (S) + 25 (G), 1000 (T) + 250 (S) + 25 (G), and 1000 (T) + 500 (S) + 25 (G).

## **Results and discussion**

#### *Detection of contaminants*

Contaminants were detected in 45 of 70 genotypes initiated in 1994 in basal medium (Table 1). Only in four genotypes were all explants contaminated. Similar results were seen in 1995 in enriched medium with 53 of 72 genotypes contaminated. Only in six genotypes were all explants contaminated. The majority of contaminants were bacteria and yeast. The 10% increase in contaminants detected in 1995 might be due to the use of peptone and yeast extract in the medium; however, this was only a screening process and these results need to be verified in a controlled experiment. This

technique did not distinguish between endophytic and epiphytic contaminants.

Several bacterial and yeast contaminants cannot be detected on proliferation or elongation medium, but are apparent and often lethal on rooting medium [12]. Peptone and yeast extract added to the proliferation and elongation medium allowed detection and elimination of all bacterial contaminants within two subcultures [12].

#### *Characterization and identification of bacteria*

Bacterial contaminants isolated from 22 strawberry genotypes included approximately 16 bacterial strains as determined by the Biolog database, standard cultures, and biochemical tests. One to four bacterial strains were found per genotype. More than half of the genotypes tested contained more than one bacterial strain. Most of the contaminants were Gram-negative, rod-shaped, motile, non-spore forming bacteria, and most were *P. fluorescens* types. In this study, the most common bacteria found were *P. fluorescens* type F (13 isolates). Two *Xanthomonas* spp., *Enterobacter cloacae* A, and several *Pseudomonas* spp. were identified, but five other Gram-negative bacteria, two Gram-positive bacteria, and two yeasts were not identified in this study. They are likely to be soil-, water- or plant-related micro-organisms because runners of strawberry plants are closely associated with the soil and could easily be contaminated by soil-borne bacteria.

#### *Minimal bactericidal concentrations (MBCs) and plant treatment*

Single antibiotics were ineffective for most bacterial isolates. Combinations of Timentin, streptomycin, and gentamicin showed promising results against all the bacteria. All bacterial strains were killed even at the lowest concentrations: (mg  $\text{ml}^{-1}$ ) 125 (T) + 250 (S) + 6.25 (G). Ten-day treatments of two strawberry genotypes infected with two different bacterial isolates produced 23–100% bacteria-free plants. The three antibiotics combined produced different results depending on the plant and bacterial genotypes. These antibiotics were not phytotoxic.

## **Conclusions**

Microbial contaminants were successfully detected in strawberry runners partially submerged in half-

strength liquid MS medium. Contaminants were detected in 75% of genotypes tested in 1994 and 73% of genotypes tested in 1995. Half of the contaminants were bacteria and yeasts, 25% filamentous fungi and 25% mixed. Most genotypes had some contaminated explants and in a few genotypes all explants were contaminated. This detection method made it possible to quickly identify contaminated explants.

Most of the bacteria isolated from strawberry runner explants were Gram-negative, rod-shaped, motile, non-spore forming *Pseudomonas* species. *P. fluorescens* types A, F, and G were the most common contaminants found. *P. corrugata*, *P. tolaasii*, and *P. paucimobilis*, one *X. campestris*, two *Xanthomonas* spp., *Enterobacter* spp. and *Enterobacter cloacae* were also identified. Five Gram-negative and two Gram-positive contaminants could not be identified by the Biolog test. Biochemical tests confirmed Biolog test results and were useful for bacterial characterization.

Bacterial isolates were evaluated for antibiotic sensitivity. Combinations of antibiotics were most effective for killing these bacterial isolates. Initial treatment of contaminated plants produced promising results and little phytotoxicity.

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