

CHARACTERIZATION AND IDENTIFICATION OF BACTERIA ISOLATED FROM MICROPROPAGATED MINT PLANTS

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SUMMARY

Bacterial isolates from contaminated mint shoot cultures were characterized and identified as a preliminary step in determining an elimination treatment. The 22 bacteria were characterized using biochemical and morphological tests and subjected to sensitivity tests with four antibiotics. The isolates were compared with known organisms and assigned to genera according to similarities in characteristics. Seven isolates were analyzed by fatty acid analysis carried out by a commercial laboratory. Six were classified as *Agrobacterium radiobacter*; eight as *Xanthomonas*; one each as *Pseudomonas fluorescens*, *Micrococcus* spp., *Corynebacterium* spp., and *Curtobacterium* spp.; four could not be assigned to genera. Inhibition of growth of the bacteria by most antibiotics was best at pH 7.5. Minimal inhibitory concentration and minimal bactericidal concentrations of gentamicin, rifampicin, streptomycin sulfate, and Timentin varied with genotype.

Key words: *Agrobacterium*; antibiotic; bacterial contamination; *Mentha*; *Pseudomonas*; *Xanthomonas*.

INTRODUCTION

Plant tissue culture contamination by microorganisms, especially bacteria, continues to obstruct successful experimentation and the exchange of plant material that is free of detectable microbes. Leifert et al. (1991b) consider it the most important reason for losses in micropropagation operations. Kneifel and Leonhardt (1992) recognize contamination by endophytic bacteria as "one of the most serious problems in tissue culture." Increasing attention has been directed to this problem by workers seeking to ascertain sources of contamination and develop procedures for eliminating them by avoidance, rigorous manipulation of environmental and nutritional factors, or treatment with antibiotics (DeFossard and DeFossard, 1988; Debergh and Zimmerman, 1991). Cassells (1991) pointed out that plants may develop endophytic microbial colonization through wounds and natural openings and via the root system. Both pathogenic and saprophytic bacteria have been isolated from plants used for micropropagation (Bastiaens, 1983). The most commonly isolated bacteria belong to the genera *Xanthomonas*, *Corynebacterium*, *Erwinia*, *Bacillus*, *Pseudomonas*, *Micrococcus*, *Agrobacterium*, *Arthrobacter*, and *Enterobacter* (Bastiaens, 1983).

Bastiaens (1983) emphasized the need for sound screening of donor plants and for effective decontamination followed by meticulously practiced asepsis in the handling of tissue cultures in the laboratory. Persistent endophytic microorganisms may be transferred unknowingly at each subculture and may become apparent weeks or months after the material was initiated in vitro (Leifert et al., 1989). Contaminants may be latent and fail to produce overt disease symptoms (Bastiaens, 1983). The role of endophytic organisms in the metabolism of the host plant is unknown. In the case of

plant pathogens, evidence of virulence may be limited by aspects of disease resistance in the host such as release or stimulation of naturally present inhibitors and the accumulation of agglutinins or phytoalexins (Billing, 1982; Bastiaens, 1983). None of these is sufficient to eliminate either saprophytic or latent pathogenic bacteria that may be present in any plant part used for micropropagation.

One of the recommended solutions to contamination problems is to discard all infected cultures and begin with clean material. Ensuring that endophytic contamination is absent from stock plants requires that the method used for detection be dependable and rapid (Debergh and Vanderschaeghe, 1988; Reed et al., in press). Many bacteria grow slowly or not at all in the media used in plant culture, thus escaping detection until considerable time and materials have been expended (Viss et al., 1991). A popular solution is to use antibacterial substances (e.g., antibiotics), but this approach has met with varying degrees of success (Cornu and Michel, 1987; Falkiner, 1990; Leifert et al., 1991a). In some cases, antibiotic treatments appear bacteriostatic, resulting in reduced rather than eliminated contamination (Phillips et al., 1981; Bastiaens et al., 1983; Young et al., 1984; Kneifel and Leonhardt, 1992). In other cases, the phytotoxicity of the antibiotics has precluded their use at a level high enough to destroy all contaminants (Falkiner, 1990; Leifert et al., 1991a, 1992). Selecting drug-resistant strains of bacteria from long-term exposure to antibiotics is also of concern (Cornu and Michel, 1988; Kneifel and Leonhardt, 1992). The lack of descriptive information and antibiotic susceptibilities of a large number of plant-associated bacteria further complicates the use of antibiotics (Leifert et al., 1989). Better characterization and identification of plant-associated bacteria should lead to more successful antibacterial therapies (Cornu and Michel, 1987; Cassells, 1991; Leifert et al., 1991b). Falkiner (1988) reviewed the desirable features of antimicrobial substances for use in plant tissue culture and

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outlined the testing necessary to establish antibiotic concentrations and effective combinations for treatment of infected tissues.

Our experience with the subcultivation and long-term cold storage of shoot-tip explants, especially from numerous (>400) specimens of mint, led to the observation that endophytic bacteria frequently persisted in the absence of obvious damage to the plants. Repeated subcultivation of tips from these cryptically infected plants showed characteristic haloes of bacterial growth in the plant growth medium, despite vigorous disinfection and aseptic handling of the explant tissues during and after their collection from pot-grown screenhouse plants. Often the contamination became apparent only after several subcultures.

This report describes a) the characterization and identification of bacteria isolated from persistently contaminated mint cultures using diagnostic biochemical tests, as well as tests of nutritional and environmental factors and b) the susceptibility of these bacteria to four antibiotics.

MATERIALS AND METHODS

Plant material used. The 35 infected mint accessions used were: *Mentha spicata* L. (10 accessions), (3 rugose form, *M. cordifolia* Opiz ex Fresen); *M. suaveolens* Ehrh. (4); *M. spicata* var. *Crispa* (Benth.) Danhart (3); *M. canadensis* L. (2); *M. longifolia* (L.) Hudson (1); *M. suaveolens variegata* Ehrh. (1); *Mentha* × *piperita*, ssp. *citrate* (Ehrh.) Briq. (4); *M.* × *smithiana* R. H. Graham (1); *M.* × *villosa* Hudson (1); and the following hybrids: *M. suaveolens* Ehrh. × *M. longifolia* (L.) Hudson (3), *M. citrate* Ehrh. × *M. aquatica* L. (2), *M. aquatica* L. × *M. spicata* L. (1), *M. canadensis* L. × *M. spicata* L. (1), and *M. spicata* L. × *M. suaveolens* Ehrh. (1). The plants were grown in pots in a screenhouse at the USDA-ARS National Clonal Germplasm Repository, Corvallis, OR. Explants 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 Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), with 0.5 mg · liter⁻¹ N⁶-benzyladenine, 3 g · liter⁻¹ agar (Bitec, Difco, Detroit, MI) and 1.25 g · liter⁻¹ Gelrite (Schweizerhall, South Plainfield, NJ), pH 5.7, at 26–30° C with 16 h of light (25 μmol · m⁻² · s⁻¹).

Detection and isolation of bacteria. Bacterial growth appeared as a cloudy zone in the agar medium around the shoot base or as turbidity in liquid plant medium containing infected material (Reed et al., in press). Contaminants were transferred with a cotton swab to 0.8% nutrient broth (Difco; Sigma) with glucose 1% and yeast extract 0.5% at pH 6.9 and incubated at 25° C until visibly turbid. Bacteria were purified by repetitive streaking and subsequently maintained on nutrient agar (NA). Single colonies were transferred to NA slants for short-term storage and to plates of the same agar and grown to provide inocula for the various tests.

Diagnostic tests for bacterial characterization. The following tests were performed according to the methods described by Klement et al. (1990): Gram stain, KOH, motility, oxidase, oxidative/fermentive (O/F), starch hydrolysis, gelatinase, urease, phosphatase, arginine dihydrolase, and potato slice digestion. Also employed were D-series selective media prepared as described by Kado and Heskett (1970) for the detection of agrobacteria, xanthomonads, and pseudomonads. Standard methods were used to evaluate bacterial response to temperature and pH. Preparations from broth cultures were negatively stained with sodium phosphotungstate and examined for flagella by transmission electron microscopy.

Fatty acid methyl ester analysis. Selected bacterial strains were sent to Microcheck, Inc. (Northfield, VT) to be identified by gas chromatographic fatty acid methyl ester analysis. Strains with a similarity index (SI) of ≥0.5 were identified to species, those with SI <0.5 and ≥0.1 to genus, and those with an SI <0.1 to a group (Boehm et al., 1993).

Standard bacterial cultures. Cultures of the following bacterial strains were provided by Ms. Marilyn Canfield, Department of Botany and Plant Pathology, Oregon State University: *Xanthomonas campestris*, *X. incanae*, and *X. vesicatoria*; and by Dr. Joyce Loper, USDA-ARS, Horticultural Crops Laboratory, Corvallis, OR: *Agrobacterium tumefaciens* (Strain

B49C), *Enterobacter cloacae* (EcCT-501), *Erwinia herbicola* (952), *Pseudomonas fluorescens* (PF-5), and *P. putida* (A12). These strains were used as standards to make comparisons and aid in the characterization and presumptive identification of the bacterial strains isolated in this study.

Antibiotic susceptibility. Streptomycin sulfate, gentamicin, rifampicin (Sigma), and Timentin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA) were prepared as wt/vol or vol/vol solutions in deionized water, filter sterilized, and added in appropriate concentrations to nutrient agar (pH 6.8–6.9). Drops (50 μl) of bacterial suspensions were inoculated onto the plates that were incubated at 25° C for 4–7 d and observed for total inhibition of growth.

Minimal inhibitory concentrations. Minimal inhibitory concentrations (MIC) of the antibiotics were estimated by a tube dilution method for both standard bacterial cultures and bacterial isolates from infected mints. Liquid plant medium 0.5× at pH 6.9 provided a suitable substrate for the test in which a series of tubes was set up containing doubling serial dilutions of antibiotic in a final volume of 2 ml per tube. Each tube was inoculated with 50 μl of 72 h broth culture of bacteria and incubated at 25° C for 4–7 d. Cell growth was assayed by measuring turbidity; a clear tube implied total inhibition of growth. Very slight turbidity was often difficult to detect, and led to imprecise determinations of MIC. To overcome this, we sampled one concentration below and three or four at and above the last clear tube, for plating onto sectors of the minimal bactericidal concentration (MBC) detector plates. Also included was a control sample that contained bacteria but no antibiotic, as well as one that had been incubated with diluent alone. MIC is recognized to be a preliminary test, indicating the lowest concentration of an antibiotic that will prevent bacterial growth and multiplication; it does not detect whether viable organisms remain.

A second test was used to determine the minimal bactericidal concentration (MBC). MBC, usually reported in μg/ml, was set up by inoculating 50 μl droplets from the MIC tubes (as described above) onto marked sectors of a nutrient agar plate (lacking antibiotic). Plates were incubated for 4–7 d before examining for colony formation. The MIC tube with the lowest concentration of antibiotic that resulted in complete absence of growth on a test plate was considered to be the minimal bactericidal concentration.

Bacteria in this study are designated by the local identification numbers of the mint accessions from which they were isolated.

RESULTS AND DISCUSSION

Characterization tests. Bacteria were grouped into tentative generic categories by comparisons of diagnostic tests and characteristics of known strains (Tables 1–4). Colony characteristics were based on growth on NA. Pigmentation developed on NA but was not present on the plant growth medium.

Xanthomonas group. Seven yellow pigmented forms were grouped as *Xanthomonas* species based on colony appearance, diagnostic tests, and genus descriptions found in Bergey's Manual (Krieg and Holt, 1984). The three known strains of *Xanthomonas* produced mucoid/butyrus, yellow colonies; exhibited starch hydrolysis and motility typical of xanthomonads; and gave typical responses to oxidase, gelatinase, and oxidation/fermentation (O\F) tests (Table 1). The known *X. campestris* strain digested potato slices, a characteristic associated with pathovars of the *X. campestris* group, but none of the mint isolates did. Isolates from mint cultures generally matched the test results of one or more of the known strains and three were identified by fatty acid analysis as related to *X. campestris celebensis* GC subgroup B (M26, SI 0.5; M59, SI 0.48; M363b, SI 0.36).

Isolates M128 and M214 deviated from the known strains especially by their fermentive activity; however, the xanthomonads are a diverse group, shown by the results for the known *X. vesicatoria* strain (Table 1). For example, *X. ampelina* grows poorly on NA and lacks characteristic xanthomonadins in its pigment compared with *X. campestris* pathovars. The *X. ampelina* group also differs from *X. campestris* in urease activity and failure to hydrolyze starch or gela-

TABLE 1

CULTURAL CHARACTERISTICS OF GRAM-NEGATIVE BACTERIA FROM *MENTHA* IN VITRO CULTURES TENTATIVELY IDENTIFIED AS *XANTHOMONAS* SPECIES AND KNOWN STRAINS OF *X. CAMPESTRIS*, *X. INCANAE*, AND *X. VESICATORIA* GROWN ON NUTRIENT AGAR

Characteristic/Organism	26*	45	58	59*	77	128	214	363b*	<i>X. campestris</i>	<i>X. incanae</i>	<i>X. vesicatoria</i>
Yellow pigment	+	+	+	+	+	+	+	+	+	+	+
Mucoid/butyrous	-	-	+	-	+	+	-	-	+	+	+
Oxidase	-	-	+	-	-	-	-	-	-	-	+
O/F (glucose)	-/-	-/-	+/-	-/-	-/-	-/+	+/+	-/-	-/-	-/-	+/-
Motility	-	-	-	-	-	+	+	-	+	+	+
Starch hydrolysis	+	-	-	-	-	-	+	+	+	+	+
Gelatinase	+	-	-	-	-	-	+	+	+	+	-
Flagella	+	+	+	+	-	+	-	+	nd	nd	nd

* Identified as related to *X. campestris celebensis* GC subgroup B by fatty acid analysis (M26, SI 0.5; M59, SI 0.48; M363b, SI 0.36). nd = not done; +/- = present/absent.

tin (Krieg and Holt, 1984). Some of the mint isolates grouped with the xanthomonads possessed the latter two characteristics, but were pigmented and grew readily on the NA. Xanthomonad selective medium D-5 (Kado and Heskett, 1970) failed to favor the growth of our xanthomonad isolates and instead supported growth of several other groups.

Agrobacterium group. Six isolates with beige pigmentation associated with highly mucoid, moist colonies were categorized as *Agrobacterium* species (Table 2). They were able to grow on *Agrobacterium* selective medium D-1 (Kado and Heskett, 1970). All six were oxidase positive, O/F negative and most were starch hydrolysis negative and urease positive typical of *Agrobacterium*. Four were identified further by fatty acid analysis as *A. radiobacter* (M47, SI 0.9; M76, SI 0.9; M 234B, SI 0.9; M591, SI 0.6) and two others with similar characteristics were included among the agrobacteria, based on biochemical and morphological data.

Other Gram-negative isolates. The remaining four Gram-negative isolates varied in their characteristics (Table 3). The known *P. fluorescens* strain was oxidase positive, starch hydrolysis negative, arginine dihydrolase positive and showed fluorescence on King's B medium (Klement et al., 1990) under ultraviolet light. Isolate 363a closely matched the known *P. fluorescens* strain in biochemical reactions and showed strong fluorescence (Table 3). Medium D-4 (Kado and Heskett, 1970), selective for pseudomonads, not only favored their growth but also supported the growth of several other genera.

Isolates M221 and M234a may belong to the genus *Flavobacte-*

rium (Table 3). Both isolates produced small, round, convex, moist, butyrous, yellow colonies with entire edges, conforming to the colony characteristics described in Bergey's Manual (Krieg and Holt, 1984). *Flavobacterium* strains are reported to resist many antimicrobials; this was not the case with mint isolate M221 but M234a showed resistance to streptomycin and Timentin at all pH levels tested but not to gentamicin. These isolates also resemble *Alcaligenes paradoxus*. Fatty acid analysis was inconclusive for M234a.

Gram-positive isolates. Gram-positive bacteria were assigned to several groups, based on diagnostic tests and comparison with characteristics of genera containing nonsporeformers, especially those associated with or isolated from plants (Table 4). Isolate M363c was similar in cell morphology and colony characteristics to *Micrococcus* species, however, results of the oxidase, O/F, arginine dihydrolase, and urease tests differed from those expected for many *Micrococcus* species.

Bergey's Manual (Krieg and Holt, 1984) describes several groups of Gram-positive, irregular, nonsporeforming rods, which include organisms isolated from plants and soil. Among these are *Corynebacterium*, *Aureobacterium*, *Curtobacterium*, and *Arthrobacter*. Reliable differentiation of the several *Corynebacterium* groups rests on the composition of cell wall material, including the type of peptidoglycan and the major peptidoglycan diamino acid. Based chiefly on morphological similarities, isolate M119 was placed with the coryneform group.

M557b was identified by fatty acid analysis as most closely re-

TABLE 2

CULTURAL CHARACTERISTICS OF GRAM-NEGATIVE BACTERIA FROM *MENTHA* IN VITRO CULTURES TENTATIVELY IDENTIFIED AS *AGROBACTERIUM* SPECIES AND A KNOWN STRAIN OF *A. TUMEFACIENS*

Characteristic/organism	47*	76*	228	234b*	470	591*	<i>A. tumefaciens</i>
Yellow pigment	-	-	-	-	+	-	-
Mucoid/moist	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
O/F (glucose)	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Motility	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	+	-	-
Urease	+	+	+	+	-	-	-
Growth @ pH 4.4	-	-	+	+	-	-	-

* Identified by fatty acid analysis as *A. radiobacter* (M47, SI 0.9; M76, SI 0.9; M 234B, SI 0.9; M591, SI 0.6). +/- = present/absent.

TABLE 3

CHARACTERISTICS OF GRAM-NEGATIVE BACTERIA FROM *MENTHA* IN VITRO CULTURES TENTATIVELY IDENTIFIED AS *PSEUDOMONAS* OR OTHER SPECIES AND KNOWN STRAINS OR DESCRIPTIONS OF RELATED SPECIES

Colony form Characteristic/Organism	Convex, Round, Entire				Flat, Spreading			Raised, Spreading, Entire	
	57	221	234a	<i>Flavobacterium</i> ^b	363a ^a	<i>P. fluorescens</i> ^a	<i>P. putida</i> ^a	<i>E. herbicola</i>	<i>Alcaligenes</i> ^b
Moist/butyrus	+	+	+	+	+	+	+	+	+
Yellow pigment	+	+	+	+	-	-	-	+	-/+ ^c
Oxidase	-	-	-	+	+	+	+	-	+
O/F (glucose)	-/-	+/-	-/-	v/-	-/-	-/-	-/-	+/+	+/-
Motility	-	-	-	-	+	+	+	+	+
Gelatinase	-	-	-	v	+	+	-	-	-
Growth at 5° C	-	-	-	-	+	+	+	+	na
Arginine dihydrolase	-	-	-	-	+	+	+	-	-

^a Showed fluorescence on King's B medium under uv light.

^b Description from Bergey's Manual (Krieg and Holt, 1984).

^c *Alcaligenes paradoxus*, moist, slimy, yellow colonies. v = species may vary; na = information not available; +/- = present/absent.

lated to *Curtobacterium flaccumfaciens betae* (SI 0.4). Members of the genus *Curtobacterium*, containing saprophytes and plant pathogens, exhibit morphology very similar to organisms of *Corynebacterium* species. The group *C. flaccumfaciens* is regarded as phytopathogenic. The main characteristic for differentiating *Curtobacterium* from *Aureobacterium*, *Cellulomonas*, and *Microbacterium* is the structure of the peptidoglycan (Krieg and Holt, 1984). We did not analyze for peptidoglycan structure.

Leifert et al. (1989) isolated 190 strains of bacteria from micro-propagated plant cultures of which 30% were identified as belonging to the genus *Pseudomonas*. They found that about 70% of contaminants isolated after plants had been propagated for more than 12 months were Gram-positive bacteria belonging to the genera *Staphylococcus*, *Micrococcus*, and *Lactobacillus*, commonly found among human flora. These isolates were thought to have been introduced by the workers, while others including 57 *Pseudomonas* spp. were believed to have been introduced from infected stock plants or inadequate sterilization of media or equipment (Leifert et al., 1989). Bacteria isolated from mint cultures, including the Gram-

positive isolates, were from groups generally recognized as soil or plant associated organisms.

Effect of pH on antibiotic susceptibility. Gram-negative bacteria were tested for antibiotic susceptibility on NA plates at three pH levels (Table 5). Antibiotic activity usually is affected by the pH of the carrier medium (Falkiner, 1988). Gentamicin (50 µg/ml) inhibited growth of xanthomonad isolates and known strains at pH 7.5 but five isolates grew at pH 6.5 and seven at pH 5.5. Susceptibility of the *Agrobacterium* group to gentamicin varied from total at pH 7.5 and variable at pH 6.5, to completely resistant at pH 5.5. The other Gram-negative isolates and known strains were variable in their responses to gentamicin at lower pH levels but all were inhibited at pH 7.5 (Table 5).

Rifampicin (25 µg/ml) inhibited the growth of all known strains and isolates at all pH levels except for *A. tumefaciens* and *P. putida*, which were totally resistant (data not shown). For most organisms, the bactericidal level of rifampicin exceeded the phytotoxic level for mint plants, limiting its use (Reed et al., in press).

Streptomycin results were variable; one isolate (M591, *A. radio-*

TABLE 4

CHARACTERISTICS OF GRAM-POSITIVE BACTERIA ISOLATED FROM *MENTHA* IN VITRO CULTURES AND DESCRIPTIONS OF OTHER GRAM-POSITIVE BACTERIA

Feature	Organism or Group					
	M363c	<i>Micrococcus</i> ^a	M119		M557b ^b	<i>Corynebacteria</i> ^a
Colony form	round small	round small	round small	round small	round, raised small	round, entire small
Pigment	cream	various	gray-white	pink	yellowish	various
Cell morphology	coccus	coccus	rod	rod	rod	rod
Motility	-	-	-	-	-	-
Oxidase	-	+	-	-	-	na
O/F	+/+	+/v	+/+	+/-	+/+	+/-
Starch	-	-, v	-	-	-	-, usually
Gelatinase	-	-, v	-	-, v	-	-, usually
Urease	+	-, v	-	+	-	v

^a Description from Bergey's Manual (Krieg and Holt, 1984).

^b Identified as related to *Curtobacterium flaccumfaciens batiae* by fatty acid analysis (SI 0.4).

^c Only *Micrococcus varians* and *M. nishinomiyensis* are positive for urease. v = species may vary; na = information not available; +/- = present/absent.

TABLE 5
GROWTH OF GRAM-NEGATIVE BACTERIA AS AFFECTED BY pH AND GENTAMICIN AT 50 $\mu\text{g}/\text{ml}$
OR STREPTOMYCIN OR TIMENTIN AT 500 $\mu\text{g}/\text{ml}$ IN NUTRIENT AGAR

pH	Gentamicin		Streptomycin		Timentin	
	5.5	7.5	5.5	7.5	5.5	7.5
<i>Xanthomonas</i> group						
<i>X. campestris</i>	+	+	-	-	-	-
<i>X. incanae</i>	+	-	-	-	-	-
<i>X. vesicatoria</i>	-	-	-	-	-	-
M26 ^a	+	+	-	+	-	-
M45	+	-	-	+	+	+
M57	-	-	-	+	+	+
M58	-	-	-	-	-	-
M59 ^a	nd	+	nd	nd	nd	nd
M128	+	-	-	+	+	+
M214	+	+	-	+	-	-
M363b ^a	+	+	-	-	-	-
<i>Agrobacterium</i> group						
<i>A. tumefaciens</i>	+	-	-	+	+	-
M47 ^b	+	+	-	-	-	-
M76 ^b	+	-	-	+	-	-
M234b ^b	+	+	-	-	-	-
M470	+	+	-	+	-	-
M591 ^b	+	-	-	+	-	+
Other Gram-negative rods						
<i>Enterobacter cloacae</i>	+	-	-	-	-	-
<i>Erwinia herbicola</i>	-	-	-	-	-	-
<i>P. fluorescens</i> ^c	+	+	-	-	-	+
<i>P. putida</i> ^c	+	-	-	-	-	+
M363a ^c	+	+	-	-	-	+
M234a	+	-	-	+	+	+

^a Identified as *X. campestris celebensis* GC subgroup B by fatty acid analysis (M26, SI 0.5; M59, SI 0.48; M363b, SI 0.36).

^b Identified as *A. radiobacter* by fatty acid analysis (M47, SI 0.9; M76, SI 0.9; M234b, SI 0.9; M591, SI 0.6).

^c Fluorescent on King's B medium, under ultraviolet light. nd = not done; +/- = bacterial growth/no growth.

bacter) was totally resistant at any pH while the known *A. tumefaciens* strain was susceptible, but only at pH 7.5. The other isolates were either inhibited at all pH levels or only at pH 6.5 and 7.5. Streptomycin (500 $\mu\text{g}/\text{ml}$) inhibited growth of seven xanthomonads at pH 6.5 and 7.5 but only five at pH 5.5. Four other isolates were resistant to streptomycin at all pH levels (Table 5).

Timentin, composed of the broad-spectrum antibiotic ticarcillin and a β -lactamase inhibitor, clavulanic acid, at 500 $\mu\text{g}/\text{ml}$ inhibited growth in 15 of 23 organisms at three pH levels and four other isolates at least at one pH level (Table 5). Four others were resistant at all three pH levels while most agrobacteria and xanthomonads were inhibited at all three. Two isolates, xanthomonads M45 and M128, were inhibited by Timentin at pH 7.5 but not at lower pH levels; and in two other isolates, corynebacter M119 and agrobacter M470, inhibition was present at lower but not higher pH levels.

The pH of the medium is important for the optimum activity of most antibiotics and, therefore, for the success of antibiotic treatment (Falkiner, 1988). Standard tissue culture media range in pH from 4.8 to 5.9 and are optimized for plant growth (Wolfe et al., 1986). Inhibition of bacteria from cultured mint shoots by gentamicin, and in some cases streptomycin, was ineffectual at pH 5.5, a pH usual for plant growth media, and most effective at pH 6.5 and 7.5 where plant growth could be suboptimal. Results from the tests with Timentin at 500 $\mu\text{g}/\text{ml}$ showed that its inhibitory activity is less affected by differing pH levels than that of gentamicin or streptomycin.

In preliminary tests with additional bacterial contaminants (P. Buckley and B. Reed, unpublished data), Timentin was strongly inhibitory to 26 of 56 bacteria at three pH levels, at concentrations of 250 and 500 $\mu\text{g}/\text{ml}$, and to 11 more at one or more pH levels. However, others, notably pseudomonads, were resistant at all three pH levels and at both concentrations used. Timentin at less than 1000 $\mu\text{g}/\text{ml}$ was not phytotoxic to micropropagated mint shoots. The consistent pattern of resistance exhibited by *Pseudomonas* spp. when exposed to Timentin suggests that the drug might be useful in media selective for this genus.

Results from tests with these four antibiotics illustrate the need to evaluate not only the concentrations but also the effect of pH on the activity of any antibiotics considered for treatment. Falkiner (1988) is among the few workers emphasizing the importance of pH among the other factors in treatment design.

Minimal bactericidal concentrations (MBCs). Gentamicin was bactericidal at 40 $\mu\text{g}/\text{ml}$ or less to all of the organisms in Tables 1-4 with the exception of M76 (*A. radiobacter*) (Table 6). Effective treatment of plant tissues may require gentamicin levels of up to 80 $\mu\text{g}/\text{ml}$; however, the risk of phytotoxicity increases at concentrations greater than 50 $\mu\text{g}/\text{ml}$ (Reed et al., in press).

The MBCs for rifampicin varied from ≤ 4 (M363c) to ≥ 100 $\mu\text{g}/\text{ml}$ (M47, *A. radiobacter*, *A. tumefaciens*), with most of the known strains requiring more than 60 $\mu\text{g}/\text{ml}$ for killing. Of the Gram-positive bacteria, isolate M557b (*Curtobacterium*) had an

TABLE 6

MINIMAL BACTERICIDAL CONCENTRATIONS IN $\mu\text{g/ml}$
DETERMINED AT pH 6.9 FOR FOUR ANTIBIOTICS AGAINST
KNOWN BACTERIA AND ISOLATES FROM INFECTED
MINT CULTURES

Organism/Antibiotic	Minimal Bactericidal Concentration ($\mu\text{g/ml}$)			
	Gentamicin	Rifampicin	Streptomycin	
<i>Xanthomonas</i> group				
<i>X. campestris</i>	12.5	>60	1000	500
<i>X. vesicatoria</i>	<6	>60	1000	500
M26	10	25	1000	>1000
M45	10	15	>2000	500
363b	40	>60	<125	500
<i>Agrobacterium</i> group				
<i>A. tumefaciens</i>	40	>100	>2000	500
M47	10	>100	>1000	500
M76	>80	>60	>2000	500
591	20	15	500	250
Other Gram-negative				
<i>Pseudomonas fluorescens</i>	40	30	<500	>1000
<i>P. putida</i>	20	>60	<500	>1000
M363a	<5	>60	<125	>1000
<i>Enterobacter cloacae</i>	<5	>60	<125	500
Gram-positive group				
M119	10	30	<125	1000
M363c	<5	<4	<125	<67.5
M557b	10	60	<125	500

MBC of 60 $\mu\text{g/ml}$, while M363c, a Gram-positive coccus, showed the least resistance (Table 6). Rifampicin was used in treatment but at no level higher than 30 $\mu\text{g/ml}$ because of phytotoxicity (Reed et al., in press).

Streptomycin at 1000 $\mu\text{g/ml}$ or less was bactericidal to most of the known strains and the isolates, indicating that this level, which was near or below the phytotoxicity level for many of the mint cultures, could be useful in treatment. Exceptions included the known bacterial strain of *A. tumefaciens*, and isolates M45 (*Xanthomonas*), and M76 (*A. radiobacter*), which survived exposure to streptomycin at >2000 $\mu\text{g/ml}$. Of interest is the observation that M45 and M76 were among organisms isolated from infected mints that remained positive for bacteria after antibiotic treatments and storage (Reed et al., in press). For both gentamicin and streptomycin, MBCs were lower for the Gram-positive than for many of the Gram-negative isolates. These results further emphasize the need to obtain pertinent information about contaminants before attempting treatment.

Leifert et al. (1991a) attempted to eliminate several Gram-positive and Gram-negative bacteria from micropropagated shoots using antibiotics. Their studies indicate that certain combinations of antibiotics can eliminate some contaminants, but phytotoxicity and high costs dictate that identification and antibiotic sensitivity testing must precede treatment. They cautioned against the use of antibacterial compounds for prophylaxis. Commercial micropropagators have been advised to discard contaminated cultures and start from clean donor plants (Leifert et al., 1989; Cassells, 1991). This is less practicable in cases where plant material is limited. It is encouraging to note that from an armamentarium of more than 7000 antibi-

otics, routinely successful treatment of plant materials may be forthcoming (Quesnel and Russell, 1983).

It was not surprising to find that the kinds of bacteria isolated from contaminated micropropagated mint shoots were similar to those described by workers with other plants (Bastiaens, 1983; Debergh and Vanderschaeghe, 1988). Nor was it surprising to discover a preponderance of Gram-negative bacteria that varied in their response to different antibiotics. Of the procedures that were used to characterize and identify the microorganisms, the fatty acid profile analysis is the most definitive, but the lack of definite identification for 4 of the 11 cultures analyzed implies that a more extensive inventory of plant-associated bacteria is needed for comparison. It is also possible that these unidentified bacteria have never been described or characterized. The standard laboratory tests that were most helpful in categorizing the bacterial isolates were the Gram stain, colony morphology and appearance, and the oxidase, O/F, starch hydrolysis, motility, and gelatinase tests. The selective medium D-1 (agrobacteria) provided fairly satisfactory selection, but the media D-4 (pseudomonads) and D-5 (xanthomonads) gave less clearcut results.

CONCLUSION

Contaminants of micropropagated mint shoots were mostly Gram-negative bacteria often found associated with plants and soil. The 22 bacteria were assigned to major groups based on colony characteristics and biochemical tests. Six were classified as *Agrobacterium*; eight as *Xanthomonas*; one each as *P. fluorescens*, *Micrococcus* spp., *Corynebacterium* spp., and a *Curtobacterium* spp.; four could not be assigned to genera. Microorganisms can be accurately identified by fatty acid profiles, patterns of carbon compound utilization, or nucleic acid studies. Those procedures are costly and classical methods may suffice, depending on the investigator's budget and objectives. More known strains need to be added to the data base for fatty acid and carbon source utilization to provide a greater likelihood of identification.

The best bacterial inhibition by antibiotics occurred at pH levels of 6.5 and 7.5. Effective antibiotic type and concentration varied with bacterial type. Gentamicin (50 $\mu\text{g/ml}$) and streptomycin (500 $\mu\text{g/ml}$) at pH 7.5 were effective treatments for most of the isolates. Rifampicin (25 $\mu\text{g/ml}$) was effective for some xanthomonads and Gram-positive isolates but not for agrobacteria or pseudomonads. Timentin (500 $\mu\text{g/ml}$) was very effective against most xanthomonads and agrobacteria but not pseudomonads.

Ascertaining the Gram reaction, purity of the isolate, colony growth, and a few defining biochemical characteristics along with screening for responses to selected antibiotics should prove helpful in predicting whether treatment to eliminate a contaminant will succeed.

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REFERENCES

- Bastiaens, L. Endogenous bacteria in plants and their implications in tissue culture—a review. *Med. Fac. Landbouww. Rijksuniv. Gent* 48:1–11; 1983.
- Bastiaens, L.; Maene, L.; Harbaoui, Y., et al. The influence of antibacterial products on plant tissue culture. *Med. Fac. Landbouww. Rijksuniv. Gent* 48:13–24; 1983.
- Billing, E. Entry and establishment of pathogenic bacteria in plant tissues. In: M. E. Rhodes-Roberts; F. A. Skinner, eds. *Bacteria and plants*. Vol. 10. The Society for Applied Bacteriology Symposium; New York: Academic Press; 1982:51–70.
- Boehm, M. J.; Madden, L. V.; Hoitink, H. A. J. Effect of organic matter decomposition level on bacterial species diversity and composition in relationship to *Pythium* damping-off severity. *Appl. Environ. Microbiol.* 59:4171–4179; 1993.
- Cassells, A. C. Problems in tissue culture: culture contamination. In: Debergh, P. C.; Zimmerman, R. H., eds. *Micropropagation technology and application*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1991:31–44.
- Cornu, D.; Michel, M. F. Bacterial contamination in shoot cultures of *Prunus avium* L. Choice and phytotoxicity of antibiotics. *Acta Hort.* 212:83–86; 1987.
- Debergh, P. C.; Vanderschaeghe, A. M. Some symptoms indicating the presence of bacterial contaminants in plant tissue culture. *Acta Hort.* 255:77–81; 1988.
- Debergh, P. C.; Zimmerman, R. H., eds. *Micropropagation technology and application*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1991.
- DeFossard, R. A.; DeFossard, H. Coping with microbial contaminants and other matters in a small commercial micropropagation laboratory. *Acta Hort.* 225:167–176; 1988.
- Falkner, F. R. Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. *Acta Hort.* 225:53–56; 1988.
- Falkner, F. R. The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. *Int. Assoc. Plant Tissue Cult. Newsl.* 60:14–21; 1990.
- Kado, C. I.; Heskett, M. G. Selective media isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. *Phytopathology* 60:969–976; 1970.
- Klement, Z.; Rudolph, K.; Sands, D. C., eds. *Methods in phyto bacteriology*. Budapest, Hungary: Akademiai Kiado; 1990.
- Kneifel, W.; Leonhardt, W. Testing of different antibiotics against gram positive and gram negative bacteria isolated from plant tissue cultures. *Plant Cell Tissue Organ Cult.* 29:139–144; 1992.
- Krieg, N. R.; Holt, J. G., eds. *Bergey's Manual of Systematic Bacteriology*. Vol. 1. Baltimore: Williams & Wilkens; 1984.
- Leifert, C.; Camotta, H.; Waites, W. M. Effect of combinations of antibiotics on micropropagated *Clematis*, *Delphinium*, *Hosta*, *Iris*, and *Photinia*. *Plant Cell Tissue Organ Cult.* 29:153–160; 1992.
- Leifert, C.; Camotta, H.; Wright, S. M., et al. Elimination of *Lactobacillus plantarum*, *Corynebacterium* spp., *Staphylococcus saprophyticus* and *Pseudomonas paucimobilis* from micropropagated *Hemerocallis*, *Choisya* and *Delphinium* cultures using antibiotics. *J. Appl. Bacteriol.* 71:307–330; 1991a.
- Leifert, C.; Ritchie, J. Y.; Waites, W. Contaminants of plant tissue and cell cultures. *World J. Microbiol. Biotechnol.* 7:452–469; 1991b.
- Leifert, C.; Waites, W. M.; Nicholas, J. R. Bacterial contaminants of micropropagated plant tissue cultures. *J. Appl. Bacteriol.* 67:353–361; 1989.
- Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497; 1962.
- Phillips, R.; Arnott, S. M.; Kaplan, S. E. Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of *Helianthus tuberosus*. *Plant Sci. Lett.* 21:235–240; 1981.
- Quesnel, L. B.; Russell, A. D. Introduction. In: Russell, A. D.; Quesnel, L. B., eds. *Antibiotics: assessment of antimicrobial activity and resistance*. New York: Academic Press; 1983:1–17.
- Reed, B. M.; Buckley, P. M.; DeWilde, T. N. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro Cell. Dev. Biol. Plant.* in press.
- Viss, P. R.; Brooks, E. M.; Driver, J. A. A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro Cell. Dev. Biol.* 27P:42; 1991.
- Wolfe, D.; Chin, C. K.; Eck, P. Relationship of the pH of medium to growth of “Bluecrop” highbush blueberry in vitro. *HortScience* 21:296–298; 1986.
- Young, P. M.; Hutchins, A. S.; Canfield, M. L. Use of antibiotics to control bacteria in shoot cultures of woody plants. *Plant Sci. Lett.* 34:203–209; 1984.