

## DETECTION AND ERADICATION OF ENDOPHYTIC BACTERIA FROM MICROPROPAGATED MINT PLANTS

BARBARA M. REED, PATRICIA M. BUCKLEY, and TRACI N. DeWILDE

USDA-ARS, National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, Oregon 97333-2521.

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### SUMMARY

Liquid medium and an enriched agar were used to detect endophytic bacteria in micropropagated mints (*Mentha* spp.) within 2 to 6 d of inoculation. Bacteria isolated from the cultures were screened on several antibiotic concentrations to determine bactericidal doses. Antibiotics were also tested for phytotoxic effects. Shoot tips from infected plants were treated by immersion in liquid MS medium containing antibiotics either singly or in combination. Streptomycin applied at 1000 µg/ml for a period of 10 d was effective and less phytotoxic in a larger number of cases than gentamicin (50 µg/ml), neomycin (500 µg/ml), or rifampicin (30 µg/ml). Mint cultures that tested negative for bacteria after antibiotic treatment were multiplied, retested, and cold-stored for 1 yr or longer. Upon regrowth after storage, 25 of 30 treated cultures (83%) tested negative for bacteria. Of the 25, 8 were successfully treated with streptomycin, 1 with gentamicin, 2 with neomycin, 1 with rifampicin, and 1 with streptomycin and gentamicin; 12 required more than one treatment. An early detection system, initial trial treatment with streptomycin for infected plants, and monitoring of treated cultures successfully reduced the spread of bacterial contamination. Antibiotic treatment in liquid MS medium at pH 6.9 resulted in enhanced bactericidal activity over that seen at pH 5.5.

**Key words:** antibiotics; endophytic bacteria; *Mentha*; micropropagation; contaminant detection.

### INTRODUCTION

Contamination of in vitro cultures by microorganisms, especially bacteria, is a continuing problem for commercial and research plant micropropagators (Debergh and Vanderschaeghe, 1988; Duhem et al., 1988; Cornu and Michel, 1987; Cassells, 1991). Endophytic infections are particularly troublesome because they may not be apparent when the plant material is first initiated into culture but emerge weeks later and persist through subsequent transfers (Thorpe and Harry, 1990). The occurrence of both pathogenic and nonpathogenic endophytic microbial contaminants has been reported for several plant genera (Bastiaens, 1983; Trick and Lingen, 1985; Leifert et al., 1989; Cassells, 1991; Kneifel and Leonhardt, 1992). Nonpathogenic, opportunistic bacteria may fail to produce symptoms other than reduced plant vigor (Bastiaens et al., 1983; Leifert et al., 1994). Not only may plant health and vigor suffer from the proliferation of such microorganisms but economic losses, disappointing performance of progeny, and failure to meet phytosanitation standards pose additional risks to the success and reputation of a micropropagation laboratory (Cassells, 1991). Debergh and Vanderschaeghe (1988) reported that many research and commercial laboratories are burdened with the problem of cryptic microbial contaminants that may escape detection in solidified plant media. Leifert et al. (1991a) ranked microbial contamination as one of the most important reasons for losses of micropropagated material. As a result, avoidance and elimination of contaminating microorganisms are two strongly emphasized goals of plant micropropagation laboratories.

Antibiotics successfully control bacterial contamination in plant

cultures (Phillips et al., 1981; Leifert et al., 1991a; Kneifel and Leonhardt, 1992). Several reports, however, have indicated that antibiotics may be more effective as bacteriostatic rather than as bactericidal agents (Young et al., 1984; Fisse et al., 1987; Mathias et al., 1987; Leifert et al., 1992). Phytotoxicity and the development of antibiotic resistant bacterial populations have also restricted the use of antibiotics (Dodds and Roberts, 1981; Phillips et al., 1981; Pollock et al., 1983; Leifert et al., 1992). These disadvantages are somewhat offset by the use of combinations of antibiotics at relatively lower concentrations (Leifert et al., 1992). Streptomycin, carbenicillin, rifampicin, gentamicin, cephalothin, and polymyxin alone or in various combinations with one another are frequently used to eradicate bacterial contaminants from in vitro plant cultures (Falkner, 1988; Kneifel and Leonhardt, 1992).

At the USDA-ARS National Clonal Germplasm Repository (NCGR) in Corvallis, the mint (*Mentha* spp.) collection consists of approximately 400 accessions kept as potted plants in a screenhouse. As these plants were initiated into in vitro culture, we noted that approximately one-third of the accessions were contaminated with bacteria. Repeated collection of materials from stock plants failed to avoid these bacterial contaminants. The goals of this study were to develop quick and reliable detection methods for bacterial contaminants and to seek suitable antibiotic treatments for their eradication from persistently contaminated mint plants.

### MATERIALS AND METHODS

**Plant material.** Thirty-five infected mint accessions were sampled: *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); *M. × piperita* ssp. *citrata* (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. citrata* 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). Bacterial strains were designated by the local identification numbers of the mint accessions from which they were isolated.

**Standard initiation method.** Shoot tips (8–12 mm) were obtained from pot-grown plants in a screenhouse collection of >400 *Mentha* accessions at NCGR. 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 then grown in individual 16 × 100 mm tubes containing MS medium (Murashige and Skoog, 1962) with 0.5 mg · liter<sup>-1</sup> N<sup>6</sup>-benzyladenine, 3 g · liter<sup>-1</sup> agar (Bitec, Agar Difco Laboratories, Detroit, MI), and 1.25 g · liter<sup>-1</sup> Gelrite (Schweizerhall, Inc., South Plainfield, NJ). The pH of the medium was 5.7 before the addition of gelling agents. Plants were incubated at 26–30° C with 16 h illumination (25 μmol · m<sup>-2</sup> · s<sup>-1</sup>).

#### Screening of Explants for Bacterial Contamination

1. **Liquid MS medium.** To quickly detect contamination, freshly collected disinfested shoot tips were immersed in one-half strength liquid MS medium at pH 6.9. Medium pH 6.9 was chosen to promote bacterial growth. Turbidity caused by bacterial growth could be detected after 2 to 6 d in stationary culture. Preliminary tests using liquid bacteriological media produced phytotoxic effects for the explants.

2. **523 medium.** An alternative method for rapid detection of bacterial contaminants relied on a plating agar (523 medium) recommended by Viss et al. (1991). The enriched 523 medium (casein hydrolysate, sucrose, yeast extract) contained 0.8% agar instead of the 1.2% commonly used to solidify bacteriological media, making it easy to inoculate with soft-stemmed plant material. Visible bacterial colonies could be detected as early as 17 h after inoculation, but plates were held for 7 or more d to ensure detection of slow-growing bacteria. The 523 medium plates were also used to monitor cultures after treatment or storage.

3. **Comparison of liquid medium initiation with 523 medium.** Five shoots each from four mint accessions were collected and tested for contamination by 523 medium inoculation and liquid MS medium three times at weekly intervals.

**Screening of antibiotics for phytotoxicity and bactericidal activity.** Antibiotic preparation: Streptomycin sulfate, gentamicin, neomycin sulfate, and rifampicin (Sigma) were prepared as wt/vol or vol/vol solutions in sterile deionized water and filter-sterilized using 0.22 μM membrane filters. Shoot tips known to harbor endophytic bacterial contaminants were placed individually into tubes containing 1:1 serial dilutions of an antibiotic in stationary liquid MS medium. For example, streptomycin was first tested at 500, 250, 125, and 62.5 μg/ml. The remaining antibiotics were screened in a similar fashion. As a result, maximum concentrations useful for treatment were determined.

**Effective antibiotic concentration and pH effects.** A second screening test established the effect of antibiotic concentration and pH on bacteria isolated from mint cultures. Bacteria from young broth cultures were inoculated onto antibiotic-containing nutrient agar plates at pH 5.5, 6.5, and 7.5. The plates were observed for 7 d for total inhibition of growth.

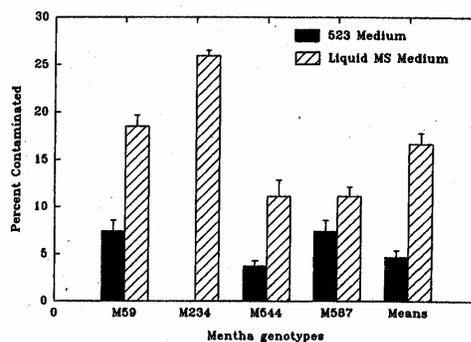


FIG. 1. Percentage of mint shoot tips found to be contaminated with bacteria using two detection methods. Five explants from each of four different mint accessions were stabbed onto 523 medium and then immersed in liquid MS medium to determine which method better detected bacteria. The percentage of contaminated plants detected by each method 10 d after inoculation is indicated for each genotype. Each bar is the mean of three replicates (15 explants) ± standard deviation.

**Antibiotic treatment of shoots.** Mint shoot tips from liquid MS medium that remained clear for 6 d and plants with no bacterial growth on 523 medium were judged to be free of bacterial contaminants and were transferred to tubes of agar-solidified MS medium for further multiplication. Shoot tips from liquid medium exhibiting turbidity or from 523 medium with bacterial growth were discarded and new tips were collected, surface-disinfested, and treated. For treatment, five shoot tips were immersed in individual tubes of MS liquid medium (pH 6.9) that had been amended with a known concentration of a given antibiotic or combination of antibiotics. A sixth tube of liquid MS medium, with a shoot tip but without antibiotic, served as the control. Ten days later the shoot tips were removed from the tubes, drained on sterile paper towels, trimmed to expose fresh tissue, and cultured on tubes of solid MS medium without antibiotics. Cultures were further monitored by streaking onto 523 medium at each transfer.

#### RESULTS AND DISCUSSION

**Comparison of liquid MS and 523 medium detection methods.** Liquid MS medium was more effective than the 523 medium in detecting bacterial contamination in freshly collected and surface disinfested mint shoots (Fig. 1). The liquid method provided contact between the medium and all surfaces of the shoots, allowing for rapid multiplication of bacterial contaminants. The 523 medium was better for detecting contamination from explants already in culture. Bacteria present at the surface of the explants are killed by disinfestation procedures and movement of endophytic bacteria out through the cut stem would require some time and thus would not appear on the 523 medium. Bacteria isolated from infected mint cultures grew well in the unsupplemented liquid MS medium and did not require medium with additional nutrients as employed by Debergh and Vanderschaege (1988) or Leifert et al. (1991a). This finding is probably due to the types of bacteria involved because further contaminants did not appear in the clean cultures

after this screening process. Later checks using the 523 medium (a supplemented medium) showed no additional contaminants. Detection of contaminants in standard plant culture medium is difficult and it is unreliable as a screening method (P. Buckley and B. Reed, unpublished data).

These detection methods meet the criteria of being easy to set up, favorable to the growth of possible contaminants, and interpreted without difficulty. In addition, they had no deleterious effects on the plant material. Bacterial contamination was not readily detected when the mint shoot tips were initiated directly onto solid MS medium (standard method) and both 523 medium inoculation and liquid MS medium improved detection of contaminants.

Bacterial contamination was detected in 47% of the mint genotypes assessed with the liquid MS medium culture method. Examination of 22 of the contaminants revealed 83% Gram-negative and 17% Gram-positive bacteria. Cultures from most of the mint plants revealed only single organisms, while mixed cultures containing two or three bacterial species were obtained from four plants. No relationship was noted between the type of infecting organism and the host genotype.

**Preliminary antibiotic testing.** Because the bacterial strains were predominantly Gram-negative, streptomycin was the first antibiotic selected for testing, followed by neomycin, gentamicin, and rifampicin. These antibiotics are often used in the treatment of plant tissue cultures. Streptomycin at 1000  $\mu\text{g}/\text{ml}$ , gentamicin at 50  $\mu\text{g}/\text{ml}$ , neomycin at 500  $\mu\text{g}/\text{ml}$ , and rifampicin at 30  $\mu\text{g}/\text{ml}$  and combinations of streptomycin at 1000  $\mu\text{g}/\text{ml}$  with gentamicin at 10 or 20  $\mu\text{g}/\text{ml}$  or with rifampicin at 30  $\mu\text{g}/\text{ml}$  were the highest antibiotic levels that permitted survival and multiplication of the treated shoot tips and still inhibited bacterial growth (Fig. 2). At a streptomycin concentration of 1000  $\mu\text{g}/\text{ml}$ , most shoots survived and bacterial growth was not detectable. Interestingly, streptomycin at concentrations  $\leq 500$   $\mu\text{g}/\text{ml}$  failed to inhibit the growth of bacteria in many of the contaminated shoots. Similarly, our results suggest that neomycin, gentamicin, and rifampicin are severely toxic to mint shoots when used at concentrations higher than those outlined. Phytotoxicity could be ranked as: neomycin > rifampicin > gentamicin > streptomycin. These results differ from those of Leifert et al. (1992) who found that streptomycin was more phytotoxic to the growth of shoots of *Clematis*, *Delphinium*, *Hosta*, *Iris*, and *Photinia* than were polymyxin, rifampicin, or carbenicillin. Neomycin was later used successfully to eliminate bacteria from two of the mint cultures (Table 1); but, because of its phytotoxicity, it was not a preferred treatment. Differential responses to antibiotic toxicity were observed among the mint genotypes evaluated in this study. When phytotoxicity occurred, it ranged from mild to severe stunting, yellowing, curling or bleaching of leaves, and for some genotypes, death (Fig. 2).

Pollock et al. (1983) reported that *B*-lactams (e.g., penicillin, carbenicillin) were the least phytotoxic among more than 20 antibiotics tested on protoplast-derived tobacco cells, and suggested the combination of rifampicin and trimethoprim for broad-spectrum use. The only *B*-lactam screened in this study was carbenicillin, a *B*-lactamase labile compound that has been shown to have activity against *Pseudomonas aeruginosa* (Quesnel and Russell, 1983) and might be expected to show activity against other Gram-negative organisms. Leifert et al. (1992) found that carbenicillin at 400  $\mu\text{g}/\text{ml}$  was toxic to several micropropagated ornamentals. Most of the bacteria isolated from our mint plants failed to be inhibited by

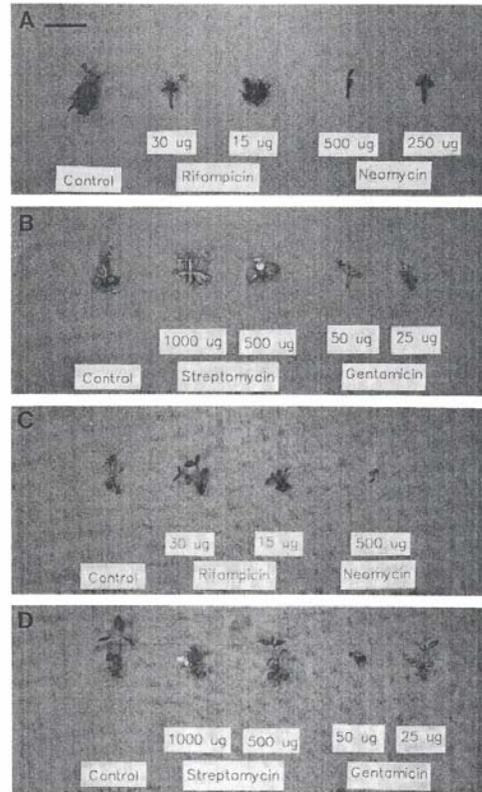


Fig. 2. Phytotoxic effects of neomycin, rifampicin, gentamicin, and streptomycin on mint shoot cultures (*M. suaveolens* and *M. canadensis*) treated for 6 d in liquid MS medium amended with various concentrations of the antibiotics. A, *M. suaveolens*: Slight inhibition of growth by rifampicin and severe phytotoxicity with neomycin. B, *M. suaveolens*: Nearly normal growth with streptomycin but inhibition and phytotoxicity with gentamicin. C, *M. canadensis*: Normal growth with rifampicin and severe phytotoxicity with neomycin. D, *M. canadensis*: Inhibition and phytotoxicity with higher concentrations of streptomycin and gentamicin and normal growth at lower concentrations. Bar = 1.25 cm.

carbenicillin at 500  $\mu\text{g}/\text{ml}$  and because of this it was not evaluated on infected plant material (data not shown). Pollock et al. (1983) did not recommend aminoglycosides because of phytotoxic effects observed at bactericidal levels.

Aminoglycosides (streptomycin, neomycin, gentamicin) undergo complex interactions with cells such as degradation of polyribosomes and disrupting or perturbing the cytoplasmic membrane leading to loss of permeability control and cell death. Their toxicity to plant cells may reflect the similarities in permeation and protein synthetic processes of bacterial and plant cells (Pollock et al., 1983). Dodds and Roberts (1981) noted that concentrations of

gentamicin in plant tissue culture media should not exceed 10  $\mu\text{g}/\text{ml}$  because of phytotoxicity to Jerusalem artichoke explants and to pith parenchyma of lettuce. We found that several mint explants could tolerate gentamicin at concentrations ranging from 30–50  $\mu\text{g}/\text{ml}$  when used as an immersion treatment for 6 d in liquid MS medium (pH 6.9) (Fig. 2). Published antibiotic activities (Sigma) showed that the antibiotics used in this study remain stable in solution at 37° C for 3–5 d. Thus, growth room conditions with temperatures of 25–30° C should not seriously reduce their activity during the treatment immersion period.

The usual pH of the MS initiation and growth media for mint propagation is 5.7. Tests of the effects of pH on the antibacterial activities of the antibiotics showed enhanced drug effects at both pH 6.5 and 7.5 compared to pH 5.5 (Buckley et al., in press). This finding supported the use of pH 6.9 MS medium in our initial detection and treatment procedures. Treatment of infected plant cultures on solid MS medium with eight different antibiotics including streptomycin, gentamicin, and rifampicin proved unsuccessful for Bastiaens et al. (1983), indicating that the use of a liquid medium at a higher pH may be the key to effective treatment.

Phillips et al. (1981) reported that rifampicin at 50  $\mu\text{g}/\text{ml}$  was fully effective against bacterial contaminants in short-term cultured explants of Jerusalem artichoke. For *Mentha* cultures, our results showed that rifampicin was markedly bactericidal to isolated microorganisms and effectively eliminated bacterial contaminants at 30  $\mu\text{g}/\text{ml}$  in some mints, but was very phytotoxic to some genotypes (Buckley et al., in press). Rifampicin binds to RNA polymerase in bacterial cells, disrupting mRNA transcription, suggesting that a similar mechanism may affect plant cells (Phillips et al., 1981).

**Treatment of contaminated plants.** Antibiotic treatments were successful for most mint cultures. Treatment with streptomycin (1000  $\mu\text{g}/\text{ml}$ ) proved successful in the largest number of cases. When a treatment appeared to be effective, cultures were multiplied without further exposure to the antibiotics and stored at 4° C. After 1 yr or more, 30 antibiotic-treated mint cultures were taken from storage, grown, and reevaluated for bacterial contamination (Table 1). In all, 83% of the mint cultures originally treated with antibiotics tested negative for bacterial contamination 1 yr after being placed into cold storage. Eight treated cultures remained free of bacterial contaminants after a single application of streptomycin (1000  $\mu\text{g}/\text{ml}$ ). Other single, effective treatments included gentamicin (1), neomycin (1), rifampicin (1), and streptomycin and gentamicin combined (2). Six others required two treatments and six were treated three or more times. Five remained contaminated after four treatments.

#### CONCLUSION

Leifert et al. (1991a, 1994) pointed out that human skin contaminants such as yeasts, fungi, *Bacillus* spp., coryneforms, and micrococci may enter plant tissue cultures due to inefficient aseptic handling during routine subculture and can result in greater plant losses than contaminants from explants at initiation. The present study detected no human-associated contaminants but found instead a predominance of Gram-negative bacteria from genera more likely to be plant or soil associated (Buckley et al., in press). All contaminants were found at or soon after initiation of explants, were present in repeated initiation attempts, and were extremely resistant to surface disinfection, suggesting an internal plant source.

TABLE 1  
EVALUATION OF ANTIBIOTIC-TREATED MINT SHOOTS  
REGROWN AFTER ONE YEAR OF COLD STORAGE

Antibiotic <sup>a</sup>	Number of		Mint Accession No. <sup>c</sup>
	Treatments <sup>b</sup>	Genotypes	
Streptomycin	1	8	18, 77, 119, 196, 201, 234, 426, 591
	2	1	21
	3	2	557, 564
	4	1	203
Gentamicin	1	1	544
	2	3	9, 31, 33
Strep + gentamicin	1	1	587
	2	2	8, 266
	3	1	59
	4	1	55
Neomycin	1	2	122, 176
Rifampicin	1	1	58
Strep + rifampicin	4	1	22

<sup>a</sup> Antibiotic levels: Streptomycin, 1000  $\mu\text{g}/\text{ml}$ ; gentamicin, 50  $\mu\text{g}/\text{ml}$ ; neomycin, 500  $\mu\text{g}/\text{ml}$ ; rifampicin, 30  $\mu\text{g}/\text{ml}$ ; streptomycin + gentamicin, 1000 + 20  $\mu\text{g}/\text{ml}$ ; streptomycin + rifampicin, 1000 + 15  $\mu\text{g}/\text{ml}$ .

<sup>b</sup> Original or final effective treatment following unsuccessful attempts with other antibiotics.

<sup>c</sup> Mint accessions resistant to treatment: 26, 45, 47, 140, 366.

Most internal bacterial contamination can be reliably detected through the use of liquid MS medium and subsequent monitoring with 523 medium. Standard treatment for contaminated shoot tips included 10 d immersion of mint shoot tips in antibiotic-amended liquid MS medium (pH 6.9), followed by transfer to tubes of solid MS medium. The use of liquid MS medium at a higher pH resulted in enhanced bactericidal activity without apparent damage to the plants. Within 2 wk, the plants had usually grown sufficiently to be tested for the presence of the bacterial contaminants on 523 medium and multiplied further if clean. If the 523 medium remained negative for bacterial growth, the treated mint cultures were screened again with 523 medium at the next transfer. If contamination was found, the plantlets were retreated and reevaluated accordingly. Treatment of contaminated cultures with streptomycin proved successful in the largest number of cases. These results demonstrate the feasibility of early detection of contaminants and of using antibiotics to eradicate bacteria from contaminated cultured mint plants.

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