In Vitro Cell. Dev. Biol.—Plant 33:227–230, July–August–September 1997

1071-2690/97 $05.00 + 0.00

DETERMINATION OF MINIMAL BACTERICIDAL AND EFFECTIVE ANTIBIOTIC TREATMENT CONCENTRATIONS FOR BACTERIAL CONTAMINANTS FROM MICROPROPAGATED STRAWBERRIES[1]

PIYARAK TANPRASERT AND BARBARA M. REED[2]

Department of Horticulture, Oregon State University, Corvallis, Oregon 97331 (P. T.) and USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, Oregon 97333-2521 (B. M. R.).

(Received 13 November 1996; accepted 6 March 1997; editor G. C. Phillips)

SUMMARY

Minimal bactericidal concentrations (MBCs) were determined for 16 bacterial strains isolated from strawberry runners. Bacteria were treated with single antibiotics: Timentin, streptomycin sulfate, gentamicin, and dihydrostreptomycin; and with combinations of two or three antibiotics: Timentin, streptomycin sulfate, and gentamicin. Combinations of the three antibiotics (12) were effective with all bacteria tested and were then used to treat contaminated plantlets. *Fragaria* × *ananassa* Duch. cv. Jucunda inoculated with *Xanthomonas campestris* pv. *vesicatoria* or *Pseudomonas corrugata* were grown for 1 wk, then treated with combinations of Timentin, streptomycin, and gentamicin. Antibiotic treatments were 100% effective in eliminating *P. corrugata* from 'Jucunda,' but only 23% of the plants inoculated with *X. campestris* pv. *vesicatoria* were freed of the bacteria. Phytotoxicity was observed only at high antibiotic concentrations. Detection of bacteria from treated plants was most effective after one subculture, as antibiotics continued to inhibit bacterial growth on detection medium immediately after treatment.

Key words: antibiotic; bacterial contamination; *Fragaria*; phytotoxicity; *Pseudomonas*; *Xanthomonas*.

INTRODUCTION

Bacteria resistant to surface sterilization or endophytic bacteria lodged within plants are continuing problems in plant tissue culture and often can be eliminated only with antibiotics (Mathias et al., 1987). Antibiotic treatments vary greatly depending on the plant and the bacterial contaminant. Antibiotics are either incorporated into culture media or used as brief treatments for specific surface contaminants (Leifert et al., 1991). Short-term antibiotic treatments are best to prevent the development of antibiotic resistance in bacterial contaminants (Kneifel and Leonhardt, 1992; Leifert et al., 1991). It is also important to determine that antibiotic treatments are bactericidal rather than bacteriostatic to avoid reoccurrence of bacteria (Mathias, 1987; Leifert et al., 1991).

Combinations of antibiotics may be more effective than single antibiotics in killing contaminants and reducing the risk of antibiotic resistance developing in the microbial population (Falkiner, 1988; Leifert et al., 1991; Kneifel and Leonhardt, 1992). If antibiotic combinations are synergistic, the effective concentration of each antibiotic can be reduced; and the reduced concentration of each antibiotic produces fewer phytotoxic side effects (Falkiner, 1988). Barrett and Cassells (1994) found that antibiotics lost some effectiveness when incorporated in tissue culture media.

Successful antibiotic treatment of infected plants requires the determination of the minimal bactericidal concentration (MBC) and the antibiotic phytotoxicity to plant materials before treatment begins (Barrett and Cassells, 1994; Buckley et al., 1995; Falkiner, 1990). Antibiotics that are effective on isolated organisms may not be effective in contaminated plant cultures due to phytotoxicity or poor penetration into plant tissues (Bastiaens et al., 1983; Viss et al., 1991; Reed et al., 1995). Bacteria may reappear months later because of transient bacteriostatic activity of antibiotics (Bastiaens et al., 1983; Cassells, 1991). Mathias et al. (1987) suggest using antibiotic treatments in liquid medium, because greater surface contact increases the uptake of antibiotics into internal tissues.

The goals of this study were to determine minimal bactericidal concentrations of antibiotics for the control of bacterial contaminants detected from strawberry runner explants, effective treatments for contaminated plants, and an effective medium to detect bacterial contaminants after antibiotic treatments.

MATERIALS AND METHODS

Bacterial isolates used. Bacteria used were isolated from strawberry runner explants: *Pseudomonas corrugata*, *P. fluorescens* types A and F (1 and 2), *P. tolaasii*, *P. paucimobilis*, *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas* spp., *Enterobacter cloacae* A, two Gram-positive and one Gram-negative unidentified species, and standard cultures (Tanprasert, 1996).

Minimal bactericidal concentrations (MBCs). MBCs were tested for single antibiotics: Timentin (T a combination of ticarcillin and clavulanic acid), gentamicin (G), streptomycin sulfate (S), and dihydrostreptomycin (DS) (SmithKline Beecham, Philadelphia, PA; Sigma Chemical Company, St. Louis, MO); combinations of two antibiotics (T + G), (T + S), (G + S); and combinations of three antibiotics (T + S + G). We estimated MBCs of the antibiotics by inoculating 50 ml of 48-h-old cultures of bacterial isolates to a series of antibiotic tube dilutions in one-half strength MS medium, at pH

[1]Parts of this manuscript are included in a summary of a poster presented at the Second International Symposium on Bacterial and Bacteria-Like Contaminants of Plant Tissue Cultures, Cork, Ireland, September 1996.

[2]To whom correspondence should be addressed.

TABLE 1

MINIMAL BACTERICIDAL CONCENTRATIONS OF FOUR ANTIBIOTICS DETERMINED AFTER FIVE DAYS' GROWTH IN HALF-STRENGTH MS MEDIUM AT pH 6.9 FOR BACTERIA ISOLATED FROM STRAWBERRY RUNNER EXPLANTS

Bacterial strain	No. of isolates	Minimal bactericidal concentration (μg/ml)			
		Timentin	Gentamicin	Dihydrostreptomycin	Streptomycin sulfate
Pseudomonas corrugata	1	>500	<3	<125	<625
	1	>500	>3	<125	<62.5
P. fluorescens type A	1	>1,000	<3	<62.5	<62.5
P. fluorescens type F (1)	1	>62.5	<3	>250	<62.5
	1	>500	>3	>1,000	<62.5
	1	>500	>3	>1,000	<62.5
	1	>500	<3	>1,000	>1,000
	1	>500	<3	<62.5	<62.5
P. fluorescens type F (2)	1	>1,000	<3	<62.5	<62.5
	1	>1,000	<3	>62.5	<62.5
P. tolaasii	1	>1,000	<3	<62.5	<62.5
P. paucimobilis	1	>125	<3	>1,000	>1,000
Xanthomonas spp.	1	>250	>6.25	>1,000	>1,000
	1	<62.5	>6.25	>1,000	>1,000
X. campestris pv. *vesicatoria*	1	>500	>6.25	<62.5	<62.5
Enterobacter cloacae A	1	>1,000	>12.5	<62.5	>500
Unknown species	1	>125	<3	<62.5	<62.5
	2	>500	>50	<62.5	<500
	1	>1,000	>50	>125	<62.5

(1) = Fluorescent on King's B medium.
(2) = Nonfluorescent on King's B medium.

6.9. The tube dilutions contained a geometric dilution series of filter-sterilized antibiotic. Two ml of the diluted antibiotic was added to each tube. After 5 d incubation at 25° C, each dilution was transferred to 1.2% nutrient agar and 0.4% tryptic soy agar plates with a sterile applicator stick and incubated at 25° C for 4–7 d to determine bacterial growth. The lowest concentration of antibiotic that showed no bacterial growth was considered to be the minimal bactericidal concentration. Antibiotic concentrations were chosen from earlier published reports (Falkiner, 1988; Buckley et al., 1995).

Antibiotic concentrations. Single antibiotics: Timentin (T), streptomycin sulfate (S), and dihydrostreptomycin (DS) (μg/ml): 62.5, 125, 250, 500, and 1000; and gentamicin (G): 3, 6.25, 12.5, 25, and 50. Combinations of two antibiotics: All combinations of (T) (125 and 250 μg/ml) and (G) (6.25 and 12.5 μg/ml), (T) (125 and 250 μg/ml) and (S) (250 and 500 μg/ml), and (S) (250 and 500 μg/ml) and (G) (6.25 and 12.5 μg/ml) were tested. Combinations of three antibiotics: All possible combinations of (T) (125 and 250 μg/ml), (S) (250 and 500 μg/ml), and (G) (6.25, 12.5, and 25 μg/ml).

Phytotoxicity. Fragaria × *ananassa* Duch. cvs. Florida Belle, Sierra, Headliner, Fortune, and Jucunda; *F. virginiana* subsp. *glauca* (S. Watson) Staudt; and *F. virginiana* subsp. *virginiana* Duch. were treated with single antibiotics (μg/ml): 1000 (T), 1000 (S), 1000 (D), and 50 (G); combinations of two antibiotics: 250 (T) + 12.5 (G), 250 μg/ml (T) + 500 (S), and 500 (S) + 12.5 (G); and combinations of three antibiotics: 250 (T) + 500 (S) + 25 (G).

Antibiotic treatment of plants. Bases of 'Jucunda' and *F. virginiana* subsp. *glauca* plantlets were dipped into bacterial cultures of *P. corrugata* or *X. campestris* pv. *vesicatoria*, planted on agar medium, and grown for 7 d to establish bacterial colonies within the plants. Contaminated plants were totally submerged in ½ strength liquid MS medium without and with combinations of three filter-sterilized antibiotics (Timentin, streptomycin sulfate, and gentamicin) at (μg/ml) 500 (T) + 250 (S) + 25 (G), 1000 (T) + 250 (S) + 25 (G), and 1000 (T) + 500 (S) + 25 (G). After 10 d of treatment, bases of plants were touched to 523 Medium (Viss et al., 1991) then transferred to multiplication medium containing 265 mg peptone per liter and 88 mg yeast extract per liter (Boxus and Terzi, 1987). Plantlets were subcultured and bacterial contaminants recorded every 3 wk for 12 wk. For statistical analysis, antibiotic treatments of 'Jucunda' and *F. virginiana* subsp. *glauca* plants were arranged in a completely randomized design, and the data were subjected to analysis of variance (ANOVA) with StatGraphics 7.0 (Statistical Graphics Corp., Rockville, MD).

Results and Discussion

MBC of single antibiotic treatments. Timentin, a combination of ticarcillin and clavulanic acid, was most effective against xanthomonads and some unknown strains (Table 1). Pseudomonads, enterobacteria, and other unknown species required higher Timentin concentrations (1000 μg/ml) for bactericidal results. Gentamicin was bactericidal at relatively low concentrations for pseudomonads (3 μg/ml), xanthomonads (6.25 μg/ml), and enterobacteria (12.5 μg/ml), but concentrations greater than 50 μg/ml were required to kill three of the four unidentified bacteria. Effective dihydrostreptomycin concentrations required to kill pseudomonads and xanthomonads varied from less than 62.5 μg/ml to more than 1000 μg/ml. Dihydrostreptomycin was bactericidal at approximately 62.5 μg/ml for enterobacteria and the unidentified isolates. Streptomycin sulfate was bactericidal at 62.5 μg/ml for most pseudomonads, except one strain of *P. fluorescens* type F and *P. paucimobilis*. Bactericidal concentrations were varied for xanthomonads and the unidentified isolates, whereas *Enterobacteria cloacae* A was killed at (S) concentrations greater than 125 μg/ml. Dihydrostreptomycin results were similar to those for streptomycin sulfate so it was not tested further.

Effective combinations of two or three antibiotics. Some combinations of (T + G), (G + S), and (T + S) were effective in killing the bacteria tested (Tables 2 and 3). The combination of (G + S) at 6.25 + 250 μg/ml killed all xanthomonads, all pseudomonads except *P. paucimobilis*, *Enterobacter cloacae* A, and one Gram-negative unidentified strain. *P. paucimobilis* were killed only at (G + S) concentrations as high as 12.5 + 500 μg/ml. Similar results were found with (T + S) combined (Table 2). Combinations of (T + G) produced unsatisfactory results. Many pseudomonads, some xanthomonads, and one unidentified Gram-negative strain were not killed even at the highest concentrations tested (Table 3).

TABLE 2

GROWTH OF ISOLATED BACTERIA AFTER SEVEN-DAY EXPOSURE IN HALF-STRENGTH MS MEDIUM AT pH 6.9 IN COMBINATIONS OF STREPTOMYCIN SULFATE AND GENTAMICIN OR STREPTOMYCIN SULFATE AND TIMENTIN

Bacterial strain	Growth of bacteria on nutrient agar plates[a] following exposure to:				
		Streptomycin sulfate + gentamicin (μg/ml)			
	Untreated	500 + 12.5	500 + 6.25	250 + 12.5	250 + 6.25
Pseudomonas paucimobilis[b]	+	−	+	+	+
Unidentified Gram-positive isolates (2)	+	+	+	+	+
		Streptomycin sulfate + Timentin (μg/ml)			
	Untreated	250 + 500	250 + 250	125 + 500	125 + 250
P. paucimobilis[b]	+	−	−	+	+
Xanthomonas spp.	+	−	−	+	+

[a]All concentrations were bactericidal to *Enterobacter cloacae* A, *P. corrugata*, *P. fluorescens* type A, F(1), F(2), G, *P. tolaasii*, one *Xanthomonas* spp., *X. campestris* pv. *vesicatoria*, and one or more unidentified isolated.

[b]+ Growth or − no growth of bacteria when spotted onto nutrient agar plates.

TABLE 3

GROWTH OF ISOLATED BACTERIA AFTER SEVEN-DAY EXPOSURE IN HALF-STRENGTH MS MEDIUM AT pH 6.9 IN COMBINATIONS OF TIMENTIN AND GENTAMICIN

Bacterial strain[a]	Growth of bacteria on nutrient agar plates[b] following Timentin + gentamicin (μg/ml) treatments				
	Untreated	250 + 12.5	125 + 12.5	250 + 6.25	125 + 6.25
Pseudomonas fluorescens type F(2)	+	+	+	+	+
P. fluorescens type G	+	−	+	+	+
P. tolaasii	+	+	+	+	+
P. paucimobilis	+	−	−	−	+
Xanthomonas spp.	+	−	−	−	+
	+	−	−	+	+
Enterobacter cloacae A	+	−	−	−	+
Unidentified Gram-positive isolate	+	−	−	+	+
Unidentified Gram-positive isolate	+	+	+	+	+
Unidentified Gram-negative isolate	+	−	−	−	+

[a]All concentrations were bactericidal to *P. corrugata*, *P. fluorescens* type A, one *P. fluorescens* type F(1), one type F(2), one *Xanthomonas* spp., *X. campestris* pv. *vesicatoria*, and one unidentified isolate.

[b]+ growth or − no growth of bacteria when spotted onto nutrient agar plates following 7 d of antibiotic treatment.

Cultures of all tested bacterial strains were killed when treated with any of the 12 combinations of the three antibiotics (T + S + G) (data not shown). The effectiveness of these combinations of three antibiotics for all bacterial strains make them good candidates for treating plant materials contaminated with unidentified bacteria.

Phytotoxicity tests. Buckley et al. (1995) found that antibiotics cause stunting, yellowing, curling, bleaching of leaves, or death of mint plants, depending upon the antibiotic used and its concentration. None of seven *F. chiloensis* genotypes treated with single and two- and three-antibiotic combinations showed toxicity to the antibiotics tested (data not shown). 'Jucunda' exhibited some leaf bleaching at antibiotic concentrations higher than those tested for phytotoxicity but recovered when removed from treatment solutions (data not shown).

Antibiotic treatment of plants. Ten percent of 'Jucunda' plants contaminated with *X. campestris* pv. *vesicatoria* were bacteria-free after treatment with (μg/ml): 500 (T) + 250 (S) + 25 (G), and 23.33% were decontaminated when treated with 1000 (T) + 500 (S) + 25 (G) or 1000 (T) + 250 (S) + 25 (G). The results of the latter two treatments (23.33%) were significantly better ($P = 0.02$) than results for treatment with (μg/ml) 500 (T) + 250 (S) + 25 (G) which produced 10% bacteria-free plants. 'Jucunda' plants inoculated with *P. corrugata* were 100% bacteria-free after all treatments. The effectiveness of the treatment varied greatly with the two bacteria tested. These differences may have been caused by the location of the bacteria in the plant, chemical properties such as mucus production, or other unknown physical or chemical factors. Both bacterial strains were lethal to *F. virginiana* subsp. *glauca*, so it was not treated with antibiotics. The mixture of three antibiotics would be most useful in cases where it is not possible to determine the type of contaminants present.

Minimal bactericidal concentrations for plant treatment. MBCs are used as reference concentrations for determining plant treatments, and treatment concentrations are usually two to four times greater than MBCs (Leifert et al., 1991). Plants in this study were treated with combinations of antibiotics, because those were the only treatments effective on all individual isolates. 'Jucunda' inoculated with *X. campestris* pv. *vesicatoria* required eight times the (T), two times the (S), and four times the (G) over the lowest MBC of the three antibiotics in combination to produce 23% bacteria-free plants. But

'Jucunda' inoculated with *P. corrugata* was 100% bacteria free when treated with a combination of the three antibiotics at concentrations four times (T), one times (S), and four times (G) over the lowest MBC.

Detection of bacteria from treated plants. When 523 Medium was used to detect bacteria immediately after antibiotic treatments, only 3% of 'Jucunda' plants appeared contaminated with *X. campestris* pv. *vesicatoria*. However, at the next transfer, treated plants were moved onto 523 Medium plus peptone and yeast extract and contamination was detected in 90% of the cultures treated with the antibiotic combination (μg/ml): 500 (T) + 250 (S) + 25 (G), and 77% of the cultures treated with the antibiotic combinations (μg/ml): 1000 (T) + 250 (S) + 25 (G) and 1000 (T) + 500 (S) + 25 (G). Bacterial detection on 523 Medium was probably affected by antibiotics from treatment solutions, resulting in poor bacterial growth. We suggest that 523 Medium be used as an indexing medium at each subculture following treatment, but not at the initial transfer since it is not effective due to antibiotic carryover.

Conclusions

Determination of minimal bactericidal concentrations (MBCs) was useful in formulating effective antibiotic treatments. Combinations of antibiotics were more effective than any single antibiotic for killing isolated bacteria. No phytotoxicity appeared when plants were treated with either a single or a combination of antibiotics. Treatments with combinations of Timentin, streptomycin sulfate and gentamicin were effective for the total elimination of *Pseudomonas corrugata* and partial elimination of *Xanthomonas campestris* pv. *vesicatoria* from inoculated strawberry plants. Treated plants should be indexed at subsequent subcultures for several months to determine the effectiveness of the treatments. Treatment regimes for contaminated strawberry cultures will be developed with the MBC information derived in this study.

Acknowledgments

The authors thank Dr. Patricia M. Buckley, Department of Botany and Plant Pathology, Oregon State University, for her advice and suggestions. This manuscript is part of a thesis submitted by P. Tanprasert in partial fulfillment of the requirements for the M.S. degree at Oregon State University.

References

Barrett, C.; Cassells, A. C. An evaluation of antibiotics for the elimination of *Xanthomonas campestris* pv. *pelargonii* (Brown) from *Pelargonium* × *domesticum* cv. 'Grand Slam' Plant Cell Tissue Organ Cult. 36:169–175; 1994.

Bastiaens, L. Endogenous bacteria in plants and their implications in tissue culture—a review. Med. Fac. Landbouww. Rijksuniv. Gent. 48:1–11; 1983.

Boxus, P.; Terzi, J. M. Big losses due to bacterial contamination can be avoided in mass propagation scheme. Acta Hort. 212:91–93; 1987.

Buckley, P. M.; DeWilde, T. N.; Reed, B. M. Characterization and identification of bacteria isolated from micropropagated mint plants. In Vitro Cell. Dev. Biol. 31P:58–64; 1995.

Cassells, A. C. Problems in tissue culture: culture contamination. In: Debergh, P. C.: Zimmerman, R. H., ed. Micropropagation technology and application. Dordrecht. Netherlands: Kluwer Academic Publishers; 1991:31–44.

Falkiner, F. R. Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. Acta Hort. 225:53–56; 1988.

Falkiner, F. R. The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. Newsletter, International Association for Plant Tissue Culture 60:13–23; 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.

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; 1991.

Leifert, C.; Waites, W. M. Bacterial growth in plant tissue culture media. J. Appl. Bacteriol. 72:460–466; 1992.

Leifert, C.; Waites, W. M. Dealing with microbial contaminants in plant tissue and cell culture: hazard analysis and critical control points. In: Lumsden, P. J.; Nicholar, J. R.; Davies, W. J., ed. Physiology, growth and development of plants in culture. Dordrecht, Netherlands: Kluwer Academic Publishers; 1994 :363–378.

Mathias, P. J.; Alderson, P. G.; Leakey, R. R. B. Bacterial contamination in tropical hardwood cultures. Acta Hort. 212:43–48; 1987.

Reed, B. M.; Tanprasert, P. Detection and control of bacterial contaminants of plants tissue cultures. A review of recent literature. Plant Tissue Culture Biotech. 1:137–142; 1995.

Tanprasert, P. Detection, identification, and antibiotic treatment of bacterial contaminants from micropropagated strawberries. M.S. Thesis, Oregon State University. 1996.

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.