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Identification and Control of Bacterial Contamination in Callus Cultures of *Ulmus americana*[1]

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Abstract

Bacillus macerans Schardinger appeared on culture media and forceps used in serial transfers of *Ulmus americana* callus tissue after several contamination-free transfers and may have originated as an endophyte in the leaf tissue used as an explant. Bacteria remained viable on forceps stored for several weeks in 95% ethanol whether or not the excess was burned off in a flame from an alcohol lamp. Bacteria were eliminated from forceps treated similarily with 85% ethanol. The bacterium on artificially contaminated forceps remained viable after immersion up to 4 hr in either 95%, 85%, 80%, or 70% ethanol with or without flaming. Artificial contamination was eliminated, either by autoclaving for 20 min at 121C (185.8F) or exposure to a bunsen burner flame for 6–8 sec. *Bacillus macerans* remained viable in both naturally and artificially contaminated ethanol at dilutions of 95%, 85%, 80%, and 70%. Thus, forceps may be contaminated by latent bacteria in callus or contaminated ethanol. Ethanol may become contaminated by storage of nonsterile forceps and airborne spores introduced during routine, septic procedures.

Index words: *Bacillus macerans*, in vitro propagation, sterilization techniques, Dutch elm disease, micropropagation.

Significance to the Nursery Industry

Micropropagation has become important as an applied technique for mass propagation of particular plant species and as a research tool for the development and improvement of nursery crops. However, bacterial contamination of cultures can be a serious problem. Commonly used sterilization procedures for tools, culture vessels, media and plant tissue can eliminate some contaminating organisms but are ineffective in destroying others. For example, *Bacillus spp.*, including *B. macerans* Schardinger, form heat- and alcohol-resistant spores. Also, some bacteria remain latent in explant tissue and do not become visible until after extended culture periods. Contaminants may be transmitted to other cultures during routine subculturing by using contaminated forceps. Sterilization procedures such as storage in ethanol or flaming excess ethanol with an alcohol lamp were ineffective in eliminating *Bacillus spp.* Autoclaving and bunsen burner flaming were most effective in completely eliminating contaminants of *B. macerans*. In addition, alcohol contaminated by air-borne bacterial spores and used in routine sterilization procedures may serve as a source of contamination of instruments and plant tissue.

Introduction

Bacterial contamination of plant tissue cultures can originate from ineffective surface sterilization of explant tissue, transfer instruments and inadequate sterilization of media and culture vessels (8). Improved aseptic techniques can reduce contamination (3). Although the addition of antibiotics to the culture media may eliminate some strains of bacteria, they may also allow growth of resistant forms (4).

Prescribed sterilization procedures for instruments used in subculturing include storage in 95% or 70% ethanol followed by alcohol lamp flaming (1, 6, 12, 13). Most bacterial contaminants occur externally on plant tissue and can be eliminated by surface sterilization (5) whereas others occur as endophytes of the tissues used in callus initiation. *Bacillus spp.* have been identified as contaminants of plant tissue (8, 10, 11). They may be difficult or impossible to eliminate since they remain latent and appear only after numerous subcultures. Delayed appearance of these bacteria may be due to a lack of optimal nutrients in culture media (5). Therefore, the following study was conducted to determine the identification, source and control of the bacterial contamination encountered in American elm callus tissue after several subcultures.

Materials and Methods

Four bacterial samples from elm callus cultures and forceps used in transfers were submitted for identification to Five Star Laboratories, Branford, CT.

The sterility of the filtered air from a laminar flow hood was tested by placing 3–4 open petri plates containing half-strength Murashige and Skoog (1/2 MS) medium (9) in the work area for 15 min. These were then incubated at 25 to 28C (77 to 82.4F) for 3–4 days in the dark. This test was repeated several times during the course of this study.

Bacterial contamination of forceps. In 1992, forceps stored in 95% and 85% ethanol between callus transfers were tested for sterility by touching their tips to media in petri plates before and after flaming using an alcohol lamp. They were then returned to storage in ethanol.

In 1994, we compared the sterilizing effects of 95% and 85% ethanol on forceps contaminated naturally during callus transfer and of 95%, 85%, 80% and 70% ethanol on forceps contaminated artificially. Both naturally and artificially contaminated forceps were stored in air at 22C (71.6F).

Prior to treatment, naturally contaminated forceps tips were touched to 1/2 MS or potato dextrose agar (PDA) to confirm contamination. They were then either dipped into 95% or 85% ethanol and flamed using an alcohol lamp or

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immersed in one of the two ethanol concentrations for either 15 min or 4 hr.

The sterilizing effects of 95%, 85%, 80% and 70% ethanol prepared from fresh stock 95% ethanol were tested on artifically contaminated forceps. Forceps tips were dipped into a prepared suspension of *B. macerans* and air dried at 22C (71.6F). The bacterium was grown on potato dextrose broth (PDB) on a wrist action shaker at 22 to 25C (71.6 to 77.0F) for 3 days in the dark. This suspension was diluted with sterile, distilled water to a concentration of approximately 300 million cells/ml (McFarland nephelometer barium sulfate standard #1). Contamination was confirmed by touching forceps tips to the medium before treatment. Treatments consisted of storing forceps in ethanol for 30 sec and touching tips to the medium surface either before or after flaming in alcohol.

The effect of autoclaving and bunsen burner flaming on bacterial contamination was determined. Forceps tips were dipped into a bacterial suspension prepared as described previously and air dried. After 24 hr, forceps tips were touched to the medium to confirm contaminaion. Contaminated forceps were then either wrapped in aluminum foil and autoclaved for 20 min at 121C (185.8F) or held above the blue cone of the bunsen burner for 6–8 sec and cooled in sterile, distilled water for 3–4 sec.

Each test consisted of touching forceps to four places on each of four or five plates. Forceps not used in callus transfers were treated similarily. Three tests were conducted 4 to 8 days apart. Plates were incubated at 25 to 28C (77.0 to 82.4F) for 48 hr in the dark.

Bacterial contamination of ethanol. The sterility of 95% ethanol used to store forceps between callus transfers, a 95% ethanol stock solution and 85%, 80% and 70% dilutions with sterile, distilled water prepared under septic conditions were tested. Sterile, 13 mm filter paper discs were dipped into ethanol concentrations and placed on PDA. Plates were incubated at 25 to 28C (77.0 to 82.4F) for 48 hr in the dark and examined for bacterial growth. Sterile discs soaked with sterile distilled water served as controls. There were eight replicated plates per treatment with four discs per plate.

We also determined survival of *B. macerans* in artificially contaminated ethanol. Dilutions were made from a sealed bottle of 100% ethanol with sterile, distilled water to 95%, 85%, 80% and 70% under aseptic conditions. Each ethanol dilution was amended with 0.1 ml of the bacterial cell suspension, prepared as described previously, in 25 ml of ethanol under aseptic conditions. Amended, sterile distilled water and the inoculum were also tested for bacterial viability. Unamended ethanol served as a control. Test suspensions of contaminated ethanol were incubated at 25 to 28C (77.0 to 82.4F) for 9 and 21 days in the dark and bacterial contamination was determined using filter paper discs as described previously. Tests consisted of five petri plates per treatment with three to five filter paper discs per plate.

Results and Discussion

Bacterial samples were identified tentatively as *B. macerans*. Contamination did not develop on media in petri plates exposed to air under the laminar flow hood.

Bacterial contamination of forceps. Results of studies on forceps are given in Table 1. In 1992, bacteria grew from 43.5% of touch points from forceps contaminated during callus transfer and stored in 95% ethanol (Table 1). When excess ethanol was flamed in an alcohol lamp, 38.2% were contaminated. Forceps treated similarly with 85% ethanol were free of bacterial contamination. This response may be due to increased wetting action of 85% ethanol providing closer contact of ethanol with bacterial cells.

In 1994, naturally occurring *B. macerans* contamination was not reduced either by a 95% or 85% ethanol soak up to 4 hr or by flaming (Table 1). Differences in the 1992 and 1994 results are difficult to explain with certainty. The concentration of bacterial cells on forceps used in 1994 may

Table 1. Detection and control of *Bacillus macerans* on forceps used for transfer of elm callus explants.[z]

Naturally contaminated forceps[y]	% Contamination	Artificially contaminated forceps[x]	% Contamination
1992		**1994**	
Ethanol storage:		Nontreated	83.3
95%	43.5		
95%, flamed[w]	38.2	Ethanol immersion, 30 sec in:	
85%	0.0	95%	100.0
85%, flamed	0.0	85%	100.0
		80%	100.0
1994		70%	100.0
		95%, flamed	89.0
Ethanol immersion, 15 min or 4 hr in:		85%, flamed	66.7
95%	100.0	80%, flamed	66.7
85%	100.0	70%, flamed	66.7
Ethanol dip:		Bunsen burner flamed: 6–8 sec	0.0
95%, flamed	100.0		
85%, flamed	100.0	Autoclaved: 20 min, 121C	0.0

[z]Forceps tips were touched to 1/2 MS in four places on each of five dishes and incubated 48 hr at 25 to 28C (77.0 to 82.4F) to determine presence of bacteria. Contamination percentage equals percent touch points with bacterial growth.

[y]Bacteria found on forceps and media following transfer of elm callus tissue.

[x]Forceps tips dipped into standardized cell suspension of *B. macerans* and air dried at 22C (71.6F).

[w]Excess ethanol burned off in an alcohol lamp flame.

Table 2. Occurrence and survival of *Bacillus macerans* in naturally and artificially contaminated ethanol.[z]

Treatment	Contaminated discs (%)		
	Naturally contaminated[y]	Artificially contaminated[x]	
		Incubation period	
		9 day	21 day
Ethanol (%)			
95	37.5	86.7	93.3
85	0.0	31.3	60.0
80	50.0	70.6	100.0
70	100.0	100.0	100.0
95 (spent)	100.0	—	—
Inoculated sterile distilled water	—	100.0	100.0
Sterile distilled water	—	0.0	0.0
Ethanol control	—	0.0	0.0

[z]Filter paper discs were dipped in test solutions of ethanol, placed on 1/2 MS and incubated for 48 hr at 25 to28C (77.0 to 82.4F) in dark. Four discs were placed on each of five plates per treatment.

[y]Stock solutions of 95% ethanol used to septically prepare dilutions and to sterilize forceps during, and to store forceps between, transfers (spent 95% ethanol).

[x]Previously unopened bottle of 100% ethanol diluted under aseptic conditions. Dilutions were contaminated with 0.1 ml of standard bacterial suspension per 25 ml ethanol, incubated at 25 to 28C (77.0 to 82.4F) and tested for bacteria using filter paper discs.

have been higher. In addition, the storage periods in 1992 were considerably longer than in 1994 providing sufficiently long contact to kill all of the cells. Since these tests are qualitative, a reduction rather than complete elimination of viable cells would not be detected.

When forceps tips were contaminated artificially, bacteria grew from 83.3% of touch points (Table 1). Following a 30 sec dip in 95%, 85%, 80% and 70% ethanol, 100% of touch points showed bacterial growth. Burning of excess alcohol in an alcohol lamp flame reduced contamination to 66.7%–89.0%. Thus, neither dipping nor flaming eliminated *B. macerans*. Temperatures produced by burning alcohol appeared too low to kill heat-resistantant spores of *Bacillus* (2). These results agree with those of Kunnemen (7) that resistance of bacteria to ethanol and short-term flaming may result in spreading bacterial contamination.

Contamination was completely eliminated from forceps flamed in a bunsen burner or autoclaved (Table 1). Autoclaving provides effective initial sterilization of transfer tools. Bunsen burner flaming is practical during transfers so long as tips are cooled adequately in sterile, distilled water.

Bacterial contamination of ethanol. Bacterial contamination occurred naturally in all dilutions of ethanol prepared septically from the 95% stock solution except 85%. Again, this might be explained by the increased wetting action of the 85% ethanol. Percentages of contaminated discs in 95%, 80%, 70% and spent 95% ethanol were 37.5%, 50.0%, 100.0% and 100.0%, respectively (Table 2). Contamination trends in artificially contaminated ethanol were similar but percentages were higher, probably due to higher cell concentrations of the artificial inoculum. Percentages of contaminated discs increased with increased length of incubation period.

Since ethanol is generally considered to be antiseptic, little consideration is given to the dangers of aerial contamination during routine use or dilution. Data herein suggest that air-borne *B. macerans* spores may contaminate stock solutions of ethanol during septic preparation and use. Also, nonsterilized measuring vessels used in making dilutions could be a source of contamination.

This study demonstrated that ethanol used as a sterilant in tissue culture procedures may become contaminated by instruments used in serial transfers and by exposure to bacterial spores during septic preparation. Once contaminated, this ethanol can serve as a source of inoculum for other transfer tools and cultures. Ethanol soaking and subsequent flaming of instruments are not reliable sterilization procedures for the complete elimination of *Bacillus macerans*. However, a bunsen burner or autoclave are effective in eliminating these sources of contamination.

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