

Growth and disinfestation of 6 different bacteria in embryogenic suspension cultures of cotton

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Summary. Growth of 6 different common laboratory bacteria (*Escherichia coli*, *Flavobacterium balustrum*, *Xanthomonas maltophilia*, *Enterobacter cloacae*, *Pseudomonas fluorescens*, and *Agrobacterium tumefaciens*) in a bacterial medium, fresh plant medium, and "spent" plant media was initially measured. In all cases, bacteria grew best in the bacterial medium followed by the fresh plant medium. The spent plant medium did not support growth of the bacteria and apparently was actively toxic to bacterial cells. Proliferating, embryogenic suspension cultures of cotton (*Gossypium hirsutum*) were then inoculated with these 6 different bacteria. Two to three d following bacterial inoculation, embryogenic tissues were placed in various concentrations of bleach for various amounts of time, rinsed with sterile water, and placed on a bacterial culture medium. Clumps of embryogenic tissue which showed no visible bacterial growth after 3 d of culture were then transferred to an agar-solidified plant tissue culture medium to determine viability of bleach-disinfested tissues. Viable, single pieces of the disinfested embryogenic tissue were then used to reinitiate embryogenic suspension cultures. Treatment of contaminated tissue with a 1% bleach solution for 1-5 min resulted in the highest recovery of viable, disinfested tissues using 5 of the 6 bacteria. It was not possible to remove *F. balustrum* from clumps of embryogenic tissue without also killing the plant tissue.

Abbreviations. 2,4-D = 2,4-dichlorophenoxyacetic acid

Introduction

The development of embryogenic suspension cultures can require input of much time and labor. Due to a high tissue-to-medium contact, suspension cultures grow very rapidly and must be subcultured more often than

callus cultures. Due to the necessity of frequent transfer of embryogenic suspension cultures and nature of subculture, these cultures are more likely to become contaminated with bacteria. In addition, bacteria often do not proliferate rapidly in plant tissue culture media and can go unnoticed before a visible "cloudiness" due to contamination can be detected. One method of disinfesting contaminated cultures is to identify the bacteria and then use selective antibiotics to kill the bacteria (Cornu and Michel 1987; Poulsen 1988). This method requires rapid identification of the bacteria and additional screening of plant tissue for resistance to the selective antibiotics.

To reduce the time loss required for identification of the bacteria and avoid the use of antibiotics, we have developed a simple procedure for disinfestation of embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.). Initially, growth of 6 different bacteria in plant and bacterial media was determined. Embryogenic suspension cultures were then intentionally contaminated with 6 different common bacteria. Embryogenic cultures were surface-disinfested using a dilute bleach solution, followed by rinsing and plating on nutrient agar. Clumps of embryogenic tissue, which showed no visible bacterial growth after 3 d of culture on nutrient agar, were used to re-establish embryogenic suspension cultures. A single piece of disinfested, embryogenic tissue could be used to reinitiate the embryogenic suspension cultures.

Materials and Methods

Embryogenic Suspension Cultures: Embryogenic suspension cultures of cotton (*Gossypium hirsutum* L. cv. Coker 310) were initiated and maintained as previously described (Finer 1988). Briefly, embryogenic cultures were initiated from hypocotyl tissue in a medium containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), 2% sucrose, and 0.5 mg/l picloram (pH 5.7). For maintenance,

the tissue was then transferred to and maintained in the cotton embryo proliferation medium (CEPM medium) which contained 5 mg/l 2,4-D in place of picloram. For subculture, 0.5 ml packed cell volume of proliferating embryogenic tissue was transferred to 35 ml of fresh CEPM medium in a 125 ml deLong flask. Subcultures were performed weekly.

Growth of Bacterial Cells: Growth rates of 6 different bacteria were determined. Overnight cultures of *Escherichia coli* (MV1190), *Flavobacterium balustrum*, *Xanthomonas maltophilia* (EN76), *Enterobacter cloacae* (EN313), *Pseudomonas fluorescens* (A478), and 2 d cultures of *Agrobacterium tumefaciens* (A281) were prepared in 10 ml of Difco Bacto nutrient broth contained in 125 ml deLong flasks. These overnight cultures were grown at 27°C with continuous agitation at 150 rpm.

Bacterial cells were inoculated at low density (10^2 - 10^3 bacteria/ml) into 30 ml of either nutrient broth, CEPM medium, or spent CEPM medium. Spent CEPM medium was prepared by filtration of the CEPM medium through a 0.45 μ m filter after the weekly subculture. Four flasks of each bacterial culture were prepared using the 3 different test media. Bacteria were grown at 27°C with continuous agitation at 150 rpm. After 1, 2, 4, and 7 d of culture, small aliquots (100 μ l) of medium were removed from each flask to determine bacterial cell density by dilution plating on nutrient agar.

Inoculation and Disinfestation of Embryogenic Suspension Cultures: Embryogenic suspension cultures of cotton, taken 4 d following subculture, were inoculated at high density (10^4 - 10^5 bacteria/ml) with the 6 bacteria grown in nutrient broth as described above. Three d following inoculation, embryogenic cotton cells were harvested for disinfestation. Clumps of embryogenic tissue were washed 3 times with sterile CEPM medium and placed in 1, 5, or 15% (v/v) commercial bleach (5.25% sodium hypochlorite) in CEPM medium. Aliquots of embryogenic suspension cultures were removed from the bleach solutions after 1, 5, 10, and 15 min. Tissues from these aliquots were washed 3 times with CEPM medium, and plated in 4x4 grids on nutrient agar plates. Three plates were evaluated per treatment.

Evaluation of Disinfestation Protocols and Re-establishment of Cultures: Clumps of tissue that showed no evidence of bacterial growth after 3-4 d were placed on Hi2iP medium (Finer *et al.* 1987) to evaluate survival following the disinfestation procedure. Embryogenic tissue that was white following 4-6 weeks culture on the Hi2iP medium was rated as dead. Tissues that showed any pigmentation (green-brown) were rated as alive following disinfestation.

For re-establishment of embryogenic suspension cultures, clumps of tissue which were plated on either the nutrient agar or the Hi2iP medium were placed back into the CEPM liquid medium.

Results and Discussion

Growth of Bacterial Cells:

Growth of the 6 different bacteria were similar within media (Fig. 1). Data for the 7 d time point is not presented because bacterial populations were stationary by day 4. Growth of each bacterial strain was most rapid in nutrient broth followed by the cotton maintenance medium (Fig. 1). In most cases, bacteria in the cotton maintenance medium could be detected by noting cloudiness. *F. balustrum* however, failed to proliferate in the cotton medium and could not be visually detected but persisted at 10^3 bacteria/ml for the

length of the experiment. Thus, some bacteria may be able to survive in plant cell cultures at low densities and never be detected (no noticeable cloudiness). These latent bacteria could affect growth of plant cultures without actually overriding them. The bacteria could grow rapidly at a later time due to changes in medium composition which could be beneficial to bacterial growth. These changes could result from release of substances (i.e. amino acids, proteins) by actively-growing plant cells into media or from simple subculture to fresh medium.

Bacteria did not survive in the spent cotton medium for longer than 2 d with all but one of the bacteria surviving less than 1 d. Apparently, the cotton cells had either depleted an essential factor from the medium or produced a factor which was toxic to the introduced bacteria. Bacterial contamination in plant cultures usually results from the introduction of low numbers of bacteria. It is possible that some potential contamination problems may never be encountered if bacteria that contaminate older cultures simply do not survive. This was clearly the case with all of the bacteria that were intentionally introduced at low density into the spent CEPM medium.

Disinfestation of Embryogenic Plant Tissue:

Following disinfestation and plating of clumps on nutrient agar, embryogenic tissues which were not contaminated with bacteria were easily identified (Fig. 2). Those clumps which showed no ring of bacterial growth were removed from the nutrient agar plates for reinitiation of plant cultures. Although usually only 2-3 d were necessary for the growth of bacteria and subsequent identification of disinfested clumps, embryogenic cotton tissue could remain on the nutrient agar plates for 2 weeks with no harmful effects.

The effects of various disinfestation procedures on percent disinfestation and percent viability of disinfested tissue is shown in Table I. As expected, all treatments with the higher concentrations of bleach and long exposures of contaminated tissue to lower concentrations of bleach resulted in high percentages of disinfestation and low viability of plant tissue. In most cases, it was possible to disinfest cultures using a short exposure of contaminated embryogenic cotton tissue to low concentrations of bleach. However, it was not possible to disinfest cultures of *Flavobacterium balustrum* without killing the plant tissue regardless of the treatment. This is the same bacterium which persisted but did not proliferate in the fresh CEPM.

Following the disinfestation treatment and viability screen, single clumps of viable tissue were placed in 35 ml of CEPM medium to re-establish embryogenic suspension cultures. The ability of embryogenic tissue of cotton to survive and proliferate at low inoculum density has been described previously (Finer 1988).

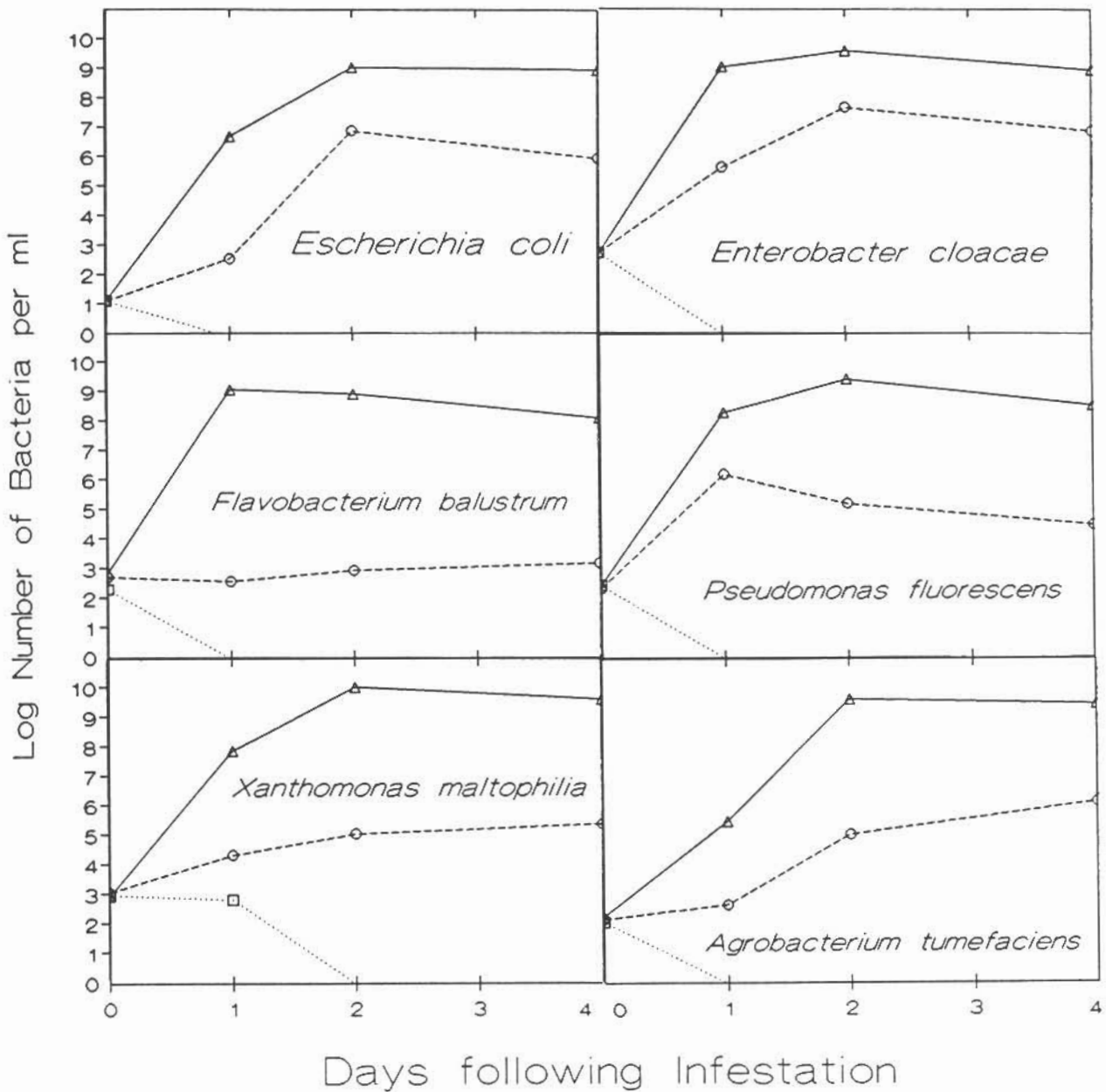


Figure 1. Growth of 6 different bacteria in nutrient broth (—Δ—), CEPM medium (---○---), and spent CEPM medium (···□···).

The procedure described in this manuscript for disinfestation of embryogenic cotton cultures may have limitations relative to contamination of different embryogenic suspension cultures and other tissue types as well as infestation by other bacteria. In this study, 6 different bacteria were used and it was not possible to recover viable embryogenic tissue following contamination with 1 of those 6 bacteria. Therefore, in some cases, it may not be possible to rid embryogenic cultures of contaminating bacteria using this protocol. All of the bacteria were capable of growth on nutrient agar, which was used as a visible score for disinfestation. Although most common bacteria proliferate rapidly on nutrient agar, some do not.

The plant tissue used for this disinfestation study was early-staged, small clumps of embryogenic tissue. Early-staged embryogenic suspension culture tissue typically does not contain vascular tissue or areas where the bacteria could be sequestered from the bleach treatment. More organized tissue, such as micropropagated shoots, could contain such regions and may not be suitable for dilute bleach disinfestation.

Low density survival and growth of embryogenic tissue was necessary for both scoring for disinfestation on nutrient agar plates and re-establishment of suspension cultures. In addition to cotton (Finer 1988), low density subculture has been used for maintenance of early-staged embryogenic suspension cultures of soybean

(Percent embryogenic tissue disinfested)/
Percent viability of disinfested tissue

[Bleach] (%)	Time (min)	BACTERIA					
		<i>Escherichia coli</i>	<i>Flavobacterium balustrum</i>	<i>Xanthomonas maltophilia</i>	<i>Agrobacterium tumefaciens</i>	<i>Enterobacter cloacae</i>	<i>Pseudomonas fluorescens</i>
1	1	(27 ± 36)/41 ± 37	(0 ± 0)/-	(39 ± 30)/61 ± 24	(90 ± 13)/90 ± 18	(100 ± 0)/75 ± 5	(0)
	5	(100 ± 0)/50 ± 12.5	(85 ± 20)/0	(71 ± 45)/20 ± 18	(100 ± 0)/10 ± 10	(100 ± 0)/67 ± 16	(15 ± 11)/58 ± 8
	10	(100 ± 0)/27 ± 16	(100 ± 0)/0	(100 ± 0)/22 ± 7	(90 ± 10)/80 ± 9	(100 ± 0)/0	(100 ± 0)/19 ± 27
	15	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/13 ± 22	(100 ± 0)/73 ± 8	(100 ± 0)/0
5	1	(100 ± 0)/50 ± 12.5	(33 ± 52)/0	(63 ± 35)/42 ± 12	(100 ± 0)/15 ± 21	(100 ± 0)/67 ± 16	(92 ± 6)/56 ± 9
	5	(100 ± 0)/62.5 ± 17	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(54 ± 41)/3 ± 3
	10	(100 ± 0)/2 ± 3	(100 ± 0)/0	(100 ± 0)/0	(85 ± 13)/66 ± 19	(100 ± 0)/0	(100 ± 0)/2 ± 3
	15	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0
15	1	(100 ± 0)/0	(0 ± 0)/-	(100 ± 0)/19 ± 17	(100 ± 0)/0	(100 ± 0)/85 ± 6	(96 ± 6)/10 ± 13
	5	(100 ± 0)/9 ± 14	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0
	10	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0
	15	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0	(100 ± 0)/0

Table I. Percent disinfestation and percent viability (\pm standard deviation) of disinfested cotton tissue following disinfestation with different bleach solutions for various amounts of time. Cultures were contaminated with the 6 different bacteria.

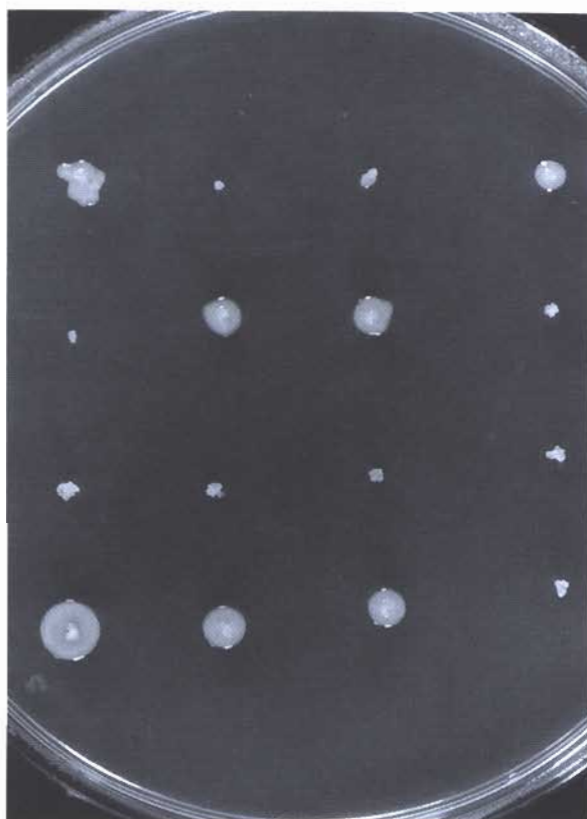


Figure 2. Nutrient agar plate showing rings of bacterial growth around cotton tissues which were not disinfested.

(Finer and Nagasawa 1988), white pine (Finer *et al.* 1989), Chinese yam (Nagasawa and Finer 1989), and maize (Finer unpubl.). Considering the similarities (early-staged nature and low inoculum subculture) between these embryogenic suspension culture systems, the procedure developed using intentionally contaminated embryogenic suspension cultures of cotton may have applications in disinfestation of contaminated embryogenic suspension cultures of other species. This procedure has been successfully used for disinfestation of embryogenic suspension cultures of cotton and Chinese yam contaminated with unidentified bacteria (Nagasawa and Finer unpubl.).

As a general recommendation for disinfestation of embryogenic liquid cultures, tissue should be washed 3 times with fresh medium, exposed to 1% bleach for 1-5 min, washed an additional 3 times with medium, and plated on nutrient agar plates for screening. After 2-3 d, clumps of viable embryogenic tissue which show no evidence of bacterial growth may then be used to reinitiate cultures.

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