Cationic Surfactants: Potential Surface Disinfectants to Manage *Agrobacterium tumefaciens* Biovar 1 Contamination of Grafting Tools

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Abstract

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Nursery production of walnut seedlings is a 2-year process, during which crown gall, caused by *Agrobacterium tumefaciens*, often develops at grafting wounds. In this study, the spread of crown gall via contaminated tools and the efficacy of several disinfectants against *A. tumefaciens* were demonstrated. The cationic surfactants benzalkonium chloride (BC), cetyltrimethylammonium bromide (CTAB), and Physan 20 eliminated 100% of the *A. tumefaciens* population in water suspensions treated at 7, 5, and 2 ppm, respectively. Sodium hypochlorite eliminated 100% of the *A. tumefaciens* population at 0.5 ppm. Sodium hypochlorite efficacy, however, was reduced by 64% in the presence of

total solids (0.7 g/ml) which are commonly found in field situations. At similar concentrations of total solids, the efficacy of cationic surfactants decreased, on average, by only 13%. The minimum effective treatment needed to eliminate *A. tumefaciens* on infested scalpels was a 5-s exposure to BC or CTAB at 5,000 ppm (0.5%). Infested scalpels treated with BC or CTAB at less than 5,000 ppm caused gall formation in $14 \pm 7\%$ of cuts made on *Datura stramonium* stems. This was significantly less than the tumor incidence (100%) in cuts made with inoculated blades not treated BC or CTAB.

Since the 1970s, the walnut hybrid *Juglans regia* L. $\times$ *J. hindsii* Jeps. ex R.E. Sm. ‘Paradox’ has been the dominant rootstock used in California walnut production (34). Paradox induces precocity and vigorous scion growth while providing variable resistance to several species of *Phytophthora* and tolerance to low populations of *Pratylenchus vulnus* (11,31,32). Unfortunately, Paradox is highly susceptible to *Agrobacterium tumefaciens* Smith and Townsend, the causal agent of crown gall (31,32).

In nursery production of walnut trees, English walnut (*J. regia*) scions are grafted onto 1-year-old Paradox seedlings, which are sold to walnut growers 10 to 12 months later (34). This 2-year process involves cutting the seedlings several times in the field. First, the English walnut scion is grafted onto a Paradox rootstock and the union is wrapped. Second, several “bleeding” cuts are often made below the graft union to reduce xylem turgor pressure, which can separate the scion from the rootstock before the graft has healed. All these plant wounds can serve as sites for *A. tumefaciens* infection via windblown dust, tools contaminated by soil, or cutting through stems systemically infected with populations of *A. tumefaciens*, as demonstrated for crops like chrysanthemum (17,27). Crown gall symptoms have frequently been observed at wound sites incurred during the grafting process in nurseries in California (L. Yakabe, *personal observation*).

The importance of grafting tool sanitation has been demonstrated for numerous crops in which plant pathogens are readily transferred from plant to plant via grafting tools (2). Several studies have documented *A. tumefaciens*-contaminated tools contributed to gall formation at the cut wounds in rose and chrysanthemum production (17,27,32).

The sanitation of tools between sequential cuts is an important first step in crown gall management in numerous crop production systems, including the walnut-propagation industry.

Sodium hypochlorite, a standard sanitizing agent in many industries, is an effective disinfectant of water and solid surfaces. However, it is corrosive and is rapidly inactivated by dissolved or suspended solids such as organic matter, which is common in field situations (15,16,44). Surfactants offer potentially effective alternatives to sodium hypochlorite for the control of microorganisms in environments with high levels of organic matter. Surfactants are commonly used in household products such as dishwashing detergents and shampoos, as agricultural wetting agents, and as disinfectants in the food industry and medical fields (20,26,35,36). Surfactants reduce surface tension at the interface between hydrophilic and hydrophobic molecules. The amphipathic nature of surfactants also alters the structure and function of cellular membranes, including modifying enzyme activity and affecting cell lysis (8,9,25). Numerous studies have reported on the ability of surfactants to disinfect solid surfaces (3,12,13,20,23,30,36,41,42). Surfactants also have been used to manage oomycete plant pathogens in water used for hydroponic cultivation of lettuce and pepper (37).

There are four main classes of surfactants used as disinfectants: nonionic, amphoteric, anionic, and cationic (8,9). In general, cationic surfactants exhibit the greatest degree of antimicrobial activity, while nonionic surfactants show the least activity. In fact, Rajagopal et al. (33) reported Gram-negative bacteria were 10,000 times more sensitive to cationic detergents than anionic detergents. However, in that study, the examination of cationic surfactant antimicrobial activity was restricted to members of the family Enterobacteriaceae. *A. tumefaciens*, a member of the family Rhizobiaceae, is hypothesized to respond similarly. Reported in this study are investigations into the potential role of grafting tools in the spread of crown gall, and the utility of sodium hypochlorite and several surfactants as grafting tool disinfectants to manage crown gall in the production of grafted walnut seedlings.

Materials and Methods

Bacterial characterization and inoculum preparation. *A. tumefaciens* isolate 186, originally isolated from a stem section of a walnut seedling in Stanislaus County, CA, was used to generate

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplemental table and one supplemental figure are available online.

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dose response curves to the compounds examined in this study. Identification of the isolate was accomplished through 16S rDNA sequencing using universal primers fd6 and rp5 (43). A positive Benedict's test confirmed identification of the isolate as *A. tumefaciens* biovar 1 (29). To facilitate recovery of the isolate from complex environments, a rifampicin-resistant mutant of *A. tumefaciens* 186, 186r, was generated by growing isolate 186 on trypticase soy broth agar (TSBA) amended with rifampicin sulfate at 100 ppm (TSBA + rif) for 3 days at 28°C. Rifampicin-resistant colonies were picked and streaked onto TSBA + rif and incubated at 28°C for 48 h. Single colonies were picked and confirmed to be rifampicin-resistant derivatives of *A. tumefaciens* 186 using 16S rDNA sequence alignment comparisons with the wild-type parent isolate. *A. tumefaciens* 186r was used in experiments examining transmission via plant wounding, tool disinfection, and effect of total solids on antimicrobial efficacy of the compounds tested.

Antibiotic-resistance profiles were assessed by swabbing 186r and the wild-type isolate 186 onto the surface of TSBA. Immediately after streaking, antibiotic disks (Sensi-disk; Becton, Dickinson and Co., Sparks, MD) treated with chloramphenicol (30 µg), ampicillin (10 µg), nalidixic acid (30 µg), minocycline (30 µg), trimethoprim (5 µg), bacitracin (10 µg), tobramycin (10 µg), kanamycin (30 µg), cefoperazone (75 µg), or rifampicin (5 µg) were placed on the agar surface. Inoculated TSBA plates were incubated at 28°C for 48 h, after which the diameter of the zone of growth inhibition of the bacterium by each antibiotic was measured. Each isolate was tested in triplicate in each of two trials in a completely randomized design. Data for the zones of inhibition were subject to an analysis of variance (ANOVA) using PROC GLM in SAS (version 9.1; SAS Institute, Cary, NC).

Growth rates of isolates 186 and 186r were also examined in 25-ml aliquots of AB liquid medium (6) shaken at 200 rpm and incubated at 28°C. Growth-curve cultures were adjusted to an initial optical density at 600 nm (OD₆₀₀) of 0.025 using bacterial cells grown in AB liquid medium for 48 h. Growth was evaluated spectrophotometrically (OD₆₀₀) every 2.5 h for the first 10 h followed by measurements every 4 h for the next 44 h. Cultures were arranged in a completely randomized design in the incubator. Two growth-curve trials were conducted with each isolate replicated in triplicate.

The ability of 186 and 186r to cause galls was tested on *Datura stramonium*, a susceptible host of *A. tumefaciens*. Fourteen-day-old seedlings were wounded on the stem with a straight-edge razor. The wounds were immediately inoculated with 1 µl of a 48-h culture of either 186 or 186r, and subsequently wrapped with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL). Plants were incubated in a completely randomized design in a greenhouse for 2 weeks, after which gall formation was recorded. Each isolate was inoculated onto three plants in each of two trials.

A. tumefaciens inoculum was prepared by adding a 3-µl loop of a 48-h culture of 186 grown on TSBA or 186r grown on TSBA + rif to 3 ml of trypticase soy broth. The cultures were incubated at 28°C on a rotary shaker (200 rpm) for 48 h. Each bacterial suspension was centrifuged at 5,468 × *g* for 10 min. The resulting pellet was washed twice using sterile distilled water and resuspended in sterile distilled water. The inoculum suspension was then adjusted spectrophotometrically to approximately 10⁸ CFU/ml (OD₆₀₀ = 0.1).

Cutting tool transmission assay. To assess whether *A. tumefaciens* can be harbored on and transmitted by cutting or grafting tools, *D. stramonium*, a crown gall indicator plant, and Paradox hybrid walnut plants were cut with scalpel blades (number 11 stainless-steel surgical blades; Miltek Inc., York, PA) dipped in either a suspension of *A. tumefaciens* 186r or sterile water. After a single dip in the inoculum or water, the scalpel was used to inoculate 10 *D. stramonium* plants in succession. Each cutting wound was wrapped immediately with Parafilm and the plants were incubated for approximately 30 days in a greenhouse, after which incidence of cuts with galls was recorded. Plants were treated and arranged in a greenhouse in a completely randomized design. In a separate experiment, Paradox walnuts seedlings were cut acro-

petally along the length of the stem six consecutive times after the grafting knife (Victorinox, Ibach-Schwyz, Switzerland) was dipped once in a suspension of *A. tumefaciens* 186r or water. Wounds were wrapped immediately with Parafilm. Plants were treated and arranged in a greenhouse in a completely randomized design. Gall development was recorded after 60 days. To allow symptom development from latent infections and examine the effects of plant dormancy on gall incidence, the walnut plants were incubated at 6°C in the dark for 120 days to induce dormancy after the initial 60-day observation time. Gall development was recorded 60 days after plants emerged from dormancy.

For each host plant experiment, the treatments were repeated six times in each of two trials. Trial was not a significant factor affecting the frequency of gall development on *D. stramonium* or Paradox within each treatment, as determined by χ^2 analysis (at $P \leq 0.05$) (38). Therefore, data from the two trials were pooled for each host. The total frequencies of gall development using inoculated versus noninoculated blades was subject to χ^2 analysis ($P \leq 0.05$). To represent the data in terms of the percentage of plants that developed galls, data for the six replicates were randomly pooled into groups of three replicates. Results for the percentage of plants that developed galls were subjected to an ANOVA using PROC GLM in SAS. When necessary, data were transformed using a log transformation or PROC RANK in SAS to meet the assumptions of ANOVA. Means separation was accomplished using Tukey's *w* test calculated in SAS (38).

Surfactants. Cationic surfactants tested were benzalkonium chloride (BC; Sigma-Aldrich, St. Louis), cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich), and Physan 20 (Maril Products Incorporated, Tustin, CA), the latter registered for tool disinfection in the United States. The non-ionic surfactants tested were Triton-X (Lab Chem Inc., Pittsburgh, PA), Tween 20 (Promega Corp., Madison, WI), and Nonidet P40 (Fluka BioChemica, Buchs, Switzerland). The anionic surfactants tested were sodium lauryl sarcosine (EMD, Darmstadt, Germany), sodium cholate hydrate (Sigma-Aldrich), sodium dodecyl sulfate (EMD), and taurocholic acid (ICN Biomedicals Inc., Aurora, OH).

Surfactant antimicrobial screening. *A. tumefaciens* 186 (20 µl, OD₆₀₀ = 0.1) was exposed to 180 µl of each surfactant at final concentrations of 10², 10³, and 10⁴ ppm in a covered, 96-well plate. The nontreated control treatment consisted of a 20-µl aliquot of isolate 186 placed in 180 µl of sterile, deionized water. The surfactant-bacterial suspensions were incubated for 30 min at room temperature (approximately 25°C) and then serially diluted on TSBA. Dilution plates were placed in an incubator in a completely randomized design for 36 h at 28°C before enumerating bacterial colonies. Three replicates were included for each surfactant at each concentration. Data were transformed to meet the assumptions of ANOVA by ranking using PROC RANK in SAS, and then subjected to ANOVA using PROC GLM. Given that CTAB and BC were the most effective at reducing bacterial populations (Table 1), these surfactants were selected for further testing.

Evaluation of BC, CTAB, Physan 20, and sodium hypochlorite. Assays used to generate dose response curves for BC, CTAB, Physan 20 (Physan), and sodium hypochlorite on *A. tumefaciens* 186 were conducted in covered 96-well plates as described above at concentrations of 0.5 to 100 ppm. The negative control treatment consisted of isolate 186 incubated in water. Each disinfectant concentration was replicated four times in each of two trials in a randomized complete block design. Trial was not a significant factor at any of the concentrations tested as determined by ANOVA ($P \leq 0.05$) using PROC GLM and, thus, data from both trials were pooled. The lethal dose required to eliminate 100% of the bacterial population (LD₁₀₀) was determined for each product based on the minimum concentration of disinfectant in which all replicate plates of the treatment yielded no recoverable bacteria.

Exposure time assays. In 1.5-ml microcentrifuge tubes, 50-µl aliquots of *A. tumefaciens* 186 were added to 450 µl of BC or CTAB at 70, 100, 500, or 5,000 ppm. The resulting suspensions were incubated for 1, 5, 10, 20, or 30 s to determine the effect of

exposure time on survival of the bacterium. To determine surviving populations of *A. tumefaciens* 186, 500 µl of each solution was serially diluted and plated on TBSA. After incubation in a completely randomized design for 36 h at 28°C, colonies were enumerated. Each surfactant concentration at each exposure time was replicated four times in each of two trials. Data were transformed to meet the assumptions of ANOVA using PROC RANK in SAS and then subjected to ANOVA using PROC GLM. The main effect of trial and interaction terms of trial with surfactants, surfactant concentration, and duration of exposure were not significant in the ANOVA ($P > 0.05$), thus data from both trials were pooled. The pooled ANOVA determined the interaction between surfactant concentration and duration of exposure was significant ($P = 0.004$); hence, means separation using Tukey's w test was calculated for all surfactant concentration and exposure time–treatment combinations.

Greenhouse cutting tool assays. To determine the efficacy of BC and CTAB for disinfecting grafting tools, noninoculated or *A. tumefaciens* 186r-inoculated scalpels were dipped in BC or CTAB at 70, 100, 500, or 5,000 ppm or sterile water, or not dipped, before sequentially cutting *D. stramonium* stems. *D. stramonium* was chosen for these assays because this indicator plant is more susceptible to *A. tumefaciens* infection than walnut and is known to produce symptoms reliably a few weeks after infection compared with walnut, which can require 4 weeks to several years for symptom development. Only the cutting edge of the blade was inoculated with *A. tumefaciens* 186r. The bacterial suspension was placed on a glass slide into which the cutting edge of the blade was dipped. The blade was then agitated in 5 ml of the appropriate disinfectant solution for 5 s, followed by a quick flick of the blade to remove excess solution. *D. stramonium* plants then were each cut once with the treated blade. To quantify the number of bacteria remaining on the blade after the cut, the blade was removed from the scalpel and submerged in 5 ml of sterile water for 10 min, after which the water was diluted serially and plated on TSBA + rif medium. The number of bacteria in the used disinfectant solution also were enumerated by dilution plating onto TSBA + rif medium. All dilution plates were incubated for 48 h at 28°C, after which colonies were enumerated. *D. stramonium* plants were arranged in a completely randomized design in a greenhouse. Gall development was evaluated after 30 days. Each treatment was replicated six times in each of two trials. Differences in the numbers of CFU detected on blades and in the disinfectant solutions were analyzed by ANOVA using PROC GLM in SAS. The fre-

quency of gall development recorded for each of the disinfectant treatments was compared with that of the sterile water control treatment using a χ^2 analysis with a Bonferroni adjusted significance of $P \leq 0.00625$ (28).

Effect of total solids on disinfectant efficacy. To generate a disinfectant solution with a high concentration of total solids, 1,500 cm³ of peat moss was steeped in 3 liters of deionized water for 7 days. After the suspension was filtered through four layers of cheesecloth to remove large particles, calcium carbonate was added to adjust the suspension to pH 7.0. Total solids of this peat-amended suspension were determined by the University of California Agriculture and Natural Resources Analytical Lab (Davis, CA). The peat-amended suspension then was mixed with deionized water to produce suspensions with total solids at 0.7, 1.5, 2.0, and 3.0 g/ml. These suspensions were used to create BC, CTAB, Physan, and sodium hypochlorite solutions containing lethal doses that killed 80% of the bacterial population (LD_{80}). LD_{80} concentrations were first estimated from a linear regression of the linear phase of the dose response curves described above, and adjusted based on preliminary experiments (*data not shown*). Linear regressions were performed using PROC REG in SAS. Tested in the same 96-well plate assay as described above, 180 µl of each of the peat-amended BC, CTAB, Physan, and sodium hypochlorite solutions were added to 20-µl suspensions of *A. tumefaciens* 186r ($OD_{600} = 0.1$), and incubated at room temperature (approximately 23°C) for 30 min. The peat-amended surfactant suspensions were made no more than 1 min prior to the addition of the bacterial suspension. Bacterial survival was determined as described above. The negative control treatment consisted of *A. tumefaciens* 186r incubated in peat-amended water for 30 min. Each treatment was replicated three times in each of two trials. The differences in efficacy between disinfectant solutions prepared in water versus peat-amended water were evaluated by ANOVA using PROC GLM.

Results

Comparison of *A. tumefaciens* isolates 186 and 186r. Except for growth in the presence of rifampicin, the rifampicin-resistant strain of *A. tumefaciens*, 186r, was indistinguishable from the wild-type parent, *A. tumefaciens* 186 (Supplementary Table S1). The growth rate of 186r was also within the 95% confidence interval of the growth rate of 186 (Supplementary Figure S1). Both isolates also caused gall formation on *D. stramonium* (*data not shown*).

Fig. 1. Incidence of crown gall from sequential cuts made on *Datura stramonium* (solid bars) and 'Paradox' walnut seedlings (hatched bars) using a cutting tool inoculated with *Agrobacterium tumefaciens*. Ten sequential cuts were made on *D. stramonium* versus six on walnut seedlings. Different letters within each plant type indicate statistically significant differences as determined by Tukey's w procedure for means separation. Plants cut with noninoculated blades (control treatment) did not develop galls. Each treatment was replicated six times in two trials. Data from both trials were pooled.

Cutting tool transmission. Wounding plants with cutting tools infested with *A. tumefaciens* 186r caused crown gall development on *D. stramonium* and Paradox walnut plants. Each inoculated blade caused the formation of at least one gall on the set of cut

Table 1. Reduction of *Agrobacterium tumefaciens* populations after 30 min of exposure to selected surfactants

Class, surfactant	Surfactant concentration (ppm)	Reduction (%) ^y
Nonionic		
Triton X	10,000	100 ab
	1,000	100 ab
	100	97 bcd
Nonidet P40	10,000	93 bcde
	1,000	92 cdef
	100	89 efg
Tween 20	10,000	96 bcd
	1,000	92 cdef
	100	88 efg
Anionic		
Sodium dodecyl sulfate	10,000	100 a
	1,000	100 a
	100	99 bc
Taurocholic acid	10,000	91 cdefg
	1,000	85 fg
	100	88 efg
Sodium cholate hydrate	10,000	86 fg
	1,000	85 g
	100	90 defg
Sodium laural sarcosine	10,000	100 a
	1,000	100 a
	100	81 efg
Cationic		
Benzalkonium chloride	10,000	100 a
	1,000	100 a
	100	100 a
CTAB ^z	10,000	100 a
	1,000	100 a
	100	100 a

^y Percent reduction in CFUs compared with the control treatment without surfactant ($n = 3$). Different letters indicate statistically significant differences as determined by Tukey's w test for means separation of ranked data.

^z Cetyltrimethylammonium bromide.

plants. The first cut in every sequence of cuts developed a gall. The probability of gall development declined with each sequential cut (Fig. 1). The probability of gall formation on *D. stramonium* declined to 58% by the third cut and 0% by the ninth cut. On walnut, the probability of gall formation declined to 51% by the second cut and 28% by the last (sixth) cut. For both walnut and *D. stramonium*, plants cut with a noninoculated blade did not develop galls. Based on the ANOVA, the frequency of gall development produced by using inoculated blades was significantly greater than the frequency of gall development using noninoculated blades (0%) ($P < 0.0001$).

Surfactant evaluation. Overall, *A. tumefaciens* isolate 186 was significantly more sensitive to cationic surfactants than to anionic or non-ionic surfactants (Table 1). BC and CTAB, the cationic surfactants evaluated, reduced populations of *A. tumefaciens* by 100% regardless of whether surfactant was used at 100, 1,000, or 10,000 ppm. In contrast, four of the seven anionic and nonionic surfactants evaluated did not reduce populations of *A. tumefaciens* by 100% even at concentrations of 10,000 ppm. Efficacy of the other three anionic and nonionic surfactants (Triton X, sodium dodecyl sulfate, and sodium laurel sarcosine) decreased with decreasing concentration of surfactant. These results led to selection of the cationic surfactants for further testing. LD₁₀₀ values for *A. tumefaciens* were 7.0, 5.0, 2.0, and 0.5 ppm when exposed to BC, CTAB, Physan, and sodium hypochlorite for 30 min, respectively (Fig. 2).

Effect of cationic surfactant exposure time. The interaction of cationic surfactant treatment (BC, CTAB, and water control treatment) with duration of exposure was significant ($P < 0.0001$) in the ANOVA for results combined over repeated experiments. Exposure of *A. tumefaciens* suspensions for <30 min to BC and CTAB required significantly greater doses to eliminate 100% of the 186r population than exposure to BC and CTAB for >30 min (Table 2). At 70 ppm, BC and CTAB eliminated 99% of the population after 5 s but up to 30 s of exposure was required to eliminate 100% of the bacterial population. Exposure of 1 s to BC or CTAB at 100 or 500 ppm eliminated 100% of the population (Table 2).

Greenhouse cutting tool assay. Trial was not a significant factor in the ANOVA ($P = 0.852$); therefore, data from both trials were pooled. The populations of *A. tumefaciens* collected in the water

Fig. 2. Dose response curve of *Agrobacterium tumefaciens* isolate 186 to benzalkonium chloride (BC), cetyltrimethylammonium (CTAB), Physan 20 (Physan), and sodium hypochlorite (hypochlorite) after a 30-min exposure to a given concentration (ppm) of each product. Each data point represents the mean and standard deviation of four replicates of each treatment averaged over each of two experiments.

used to rinse cutting tools after the blades had been inoculated with the pathogen were significantly greater than populations detected when disinfectant solutions were used on the inoculated blades ($P < 0.0001$). Similarly, there were significantly more galls on plants cut with inoculated blades rinsed in water than on plants cut with inoculated blades rinsed in disinfectant solutions prior to cutting the plants ($P < 0.00625$). *A. tumefaciens* 186r was not detected on cutting blades or in the disinfectant solutions when the blades were treated with BC or CTAB for 5 s at 70, 100, or 500 ppm. However, galls developed on 8, 8, and 25% of the *D. stramonium* stems cut with blades treated with these respective concentrations of BC, and 17, 17, and 8% of the stems cut with blades treated with these respective concentrations of CTAB (Table 3). *A. tumefaciens* 186r was not detected on cutting blades or in disinfectant solutions when the blades were treated with BC or CTAB for 5 s at 5,000 ppm and galls did not form on *D. stramonium* stems cut with the blades treated at this concentration with either disinfectant (Table 3). In contrast, when water was applied to the inoculated blades, 186r populations averaged 9.6×10^2 CFU/ml in the wash-water solutions and tumors developed in all of the stem cuts.

Effect of total solids on surfactant efficacy against *A. tumefaciens*. Trial was not a significant factor ($P = 0.623$) in the ANOVA; therefore, data from both trials were pooled. Means separation was determined by Tukey's w test on the pooled data. Suspended solids reduced the efficacy of sodium hypochlorite more significantly than the efficacy of BC, CTAB, or Physan (Table 4). Compared with distilled water with no solids, the efficacy of sodium hypochlorite at 0.02 ppm at killing *A. tumefaciens* was 64% less effective in suspensions containing total solids at 0.7 g/ml. In suspensions containing total solids at 3 g/ml, the efficacy of sodium hypochlorite at 0.02 ppm was reduced 91%. In suspensions containing total solids at 0.7 or 3.0 g/ml, the LD₈₀ concentrations of BC, CTAB, and Physan (2.5, 2.0, and 0.8 ppm, respectively) were reduced by 21, 28, and 31%, respectively.

Discussion

The walnut hybrid Paradox is the dominant rootstock used in California walnut production (34). The benefits of this rootstock are often outweighed by its susceptibility to *A. tumefaciens*. Under nursery conditions, we have observed large numbers of grafted Paradox seedlings with galls at the graft union or bleeding wounds, implicating the involvement of improperly sanitized grafting and cutting tools. This study demonstrated it is possible to spread *A. tumefaciens* on contaminated grafting tools onto *D. stramonium* as well as Paradox walnut seedlings, resulting in crown gall formation. Interestingly, each sequential cut from an inoculated cutting tool resulted in a reduced probability of crown gall developing, most likely due to some of the inoculum being rubbed into the wounds of the previous cuts.

Many nursery operations use disinfectants to clean tools between grafting cuts in order to minimize the spread of phytopathogens. Though highly corrosive, sodium hypochlorite is a common disinfectant used to clean solid surfaces to minimize the spread of pathogens. This disinfectant was effective against *A. tumefaciens* at an LD₁₀₀ of 0.5 ppm in this study. Unfortunately, the oxidizing

potential of sodium hypochlorite is significantly reduced in solutions with suspended and dissolved solids (total solids) (4,14–16,44). In this study, sodium hypochlorite efficacy against *A. tumefaciens* decreased by nearly two-thirds in a suspension with total solids at 0.7 g/ml compared with water without solids.

In this study, a class of cationic surfactants known as quaternary ammonium compounds (QACs), which includes BC, CTAB, and Physan, effectively reduced *A. tumefaciens* populations compared with selected anionic and non-ionic surfactants evaluated. Previous studies also reported greater biocidal effects of cationic surfactants compared with anionic and non-ionic surfactants (26,36). At low concentrations, QACs act on membrane permeability and cause cytolytic leakage. At high concentrations, QACs target carboxylic groups and coagulate bacterial cytoplasm (36). BC, CTAB, and Physan, exhibited LD₁₀₀ concentrations of 7, 5, and 2 ppm,

Table 3. Proportion of *Datura stramonium* stems that developed galls when *Agrobacterium tumefaciens*-inoculated grafting blades were treated with benzalkonium chloride (BC), cetyltrimethylammonium bromide (CTAB), sterile water (SW), or not treated (negative control) 5 s prior to wounding the plants with the grafting blade

Wash solution	Concentration (ppm)	Stems with galls (%) ^y
BC	70	8 ± 0.6 ^z
	100	8 ± 1.4 ^z
	500	25 ± 2.4 ^z
	5,000	0 ^z
CTAB	70	17 ± 1.4 ^z
	100	17 ± 2.8 ^z
	500	8 ± 1.4 ^z
	5,000	0 ^z
SW	Not applicable	100
Negative control	Not applicable	0

^y Mean ± standard deviation of the incidence (%) of stems on which a crown gall developed.

^z Significantly different incidence of galls from that of plants treated with sterile water, as determined by χ^2 analysis (Bonferroni adjusted significance value of $P \leq 0.00625$). Each treatment was replicated six times in two trials. Data from both trials were pooled.

Table 4. Percent reduction in *Agrobacterium tumefaciens* populations after 30 min exposure to the lethal dose that killed 80% of the bacterial population in concentrations of sodium hypochlorite, benzalkonium chloride (BC), cetyltrimethylammonium bromide (CTAB), and Physan 20 (0.02, 2.50, 2.00, and 0.80 ppm, respectively) prepared in water with various concentrations of total solids^z

Total solids (g/ml)	Sodium hypochlorite	BC	CTAB	Physan 20
0	93 a	81 a	79 a	83 a
0.7	29 b	67 b	63 b	74 ab
1.5	11 c	65 b	59 bc	67 b
2.0	4 c	63 b	54 c	54 c
3.0	2 c	60 b	51 c	52 c

^z Different letters within a column indicate statistically significant differences as determined by Tukey's w procedure for means separation. Each treatment was replicated three times in two trials. Data from both trials were pooled.

Table 2. Reduction in *Agrobacterium tumefaciens* populations exposed to benzalkonium chloride (BC) or cetyltrimethylammonium bromide (CTAB) solution

Surfactant	Concentration (ppm)	Reduction (%) per exposure time ^z				
		1 s	5 s	10 s	20 s	30 s
Benzalkonium chloride	70	99 b	99 b	99 b	99 b	100 a
	100	100 a	100 a	100 a	100 a	100 a
	500	100 a	100 a	100 a	100 a	100 a
CTAB	70	99 b	99 b	100 a	100 a	100 a
	100	100 a	100 a	100 a	100 a	100 a
	500	100 a	100 a	100 a	100 a	100 a

^z Percent reduction in CFU compared with the control treatment without surfactant. Each treatment was replicated four times in two trials. Data from both trials were pooled. Different letters indicate statistically significant differences as determined by Tukey's w procedure for means separation on ranked data.

respectively, when *A. tumefaciens* was exposed to these materials for 30 min.

The efficacy of BC and CTAB against *A. tumefaciens* was reduced by only 14 to 16% in the presence of total solids at 0.7 g/ml, whereas the efficacy of sodium hypochlorite was reduced by 64%. In the presence of total solids at 3 g/ml, efficacy was reduced by 21, 28, and 31% for BC, CTAB, and Physan, respectively, compared with 98% reduction in efficacy of sodium hypochlorite. The elevated tolerance of QACs to dissolved organic matter, a specific component of total solids, has been reported in studies examining the antimicrobial activities of QACs against foodborne bacteria (4,14,19,22). Given the tolerance of QACs to total solids, QACs are a robust alternative to strong oxidizing agents for surface-sanitizing applications under field situations. Additionally, a study conducted by Luppens et al. (24) reported BC was more efficient than sodium hypochlorite at reducing bacterial populations in biofilms. This quality increases the potential utility of QACs over sodium hypochlorite for many field applications where disinfecting solutions often accumulate high levels of total solids.

Other important attributes to consider when choosing a disinfectant are potential metal-corroding properties and phytotoxic effects. In this study, preliminary observations were noted on the corrosive and phytotoxic characteristics of sodium hypochlorite, Physan, BC, and CTAB. *D. stramonium* stems cut with blades dipped into solutions of sodium hypochlorite (6 ppm) or Physan (4 ppm) produced abnormal corky tissue around the wounds. In contrast, cuts made with BC- and CTAB-treated blades (3,000 ppm) produced wounds indistinguishable from those on stems cut with blades dipped in water (*data not shown*). We also observed that stainless-steel blades soaked in sodium hypochlorite (6 ppm) quickly rusted, whereas blades soaked in BC and CTAB (3,000 ppm) did not show any rusting after 48 h (*data not shown*). The hazards of working with chlorinated compounds also include corrosion of water pipes, yellowing or death of seedlings and mature plants, and production of noxious fumes that can endanger workers (2,10,44). These hazards do not exist when using the cationic surfactants evaluated at the concentrations tested in this study.

Exposure time to a given disinfectant can have a dramatic impact on effectiveness of the disinfectant (25,36). In this study, increased concentrations of BC and CTAB were required to eliminate *A. tumefaciens* populations if the duration of exposure of the bacterium to the surfactant was <30 min. The LD₁₀₀ for BC was 7 ppm when a suspension of *A. tumefaciens* was exposed for 30 min. The LD₁₀₀ increased to 70 ppm for BC when *A. tumefaciens* was exposed for 30 s. CTAB at 70 ppm required up to 10 s for elimination of 100% of *A. tumefaciens* 186 in an aqueous suspension. Surfactant concentrations greater than 70 ppm were needed to eliminate *A. tumefaciens* on the surface of cutting blades. Bacteria can be more difficult to eliminate from solid surfaces than in liquid suspensions, because the bacterial cells can be sheltered from sanitizers in protected microscopic environments or in established biofilms on solid surfaces (5,7). In practice, a 5-s dip in BC or CTAB at 100 or 500 ppm lowered *A. tumefaciens* populations below detection limits. A 5-s dip in BC or CTAB at 5,000 ppm (0.5% solution) was sufficient to prevent gall development on inoculated plants. BC or CTAB at 5,000 ppm also eliminated *A. tumefaciens* populations in the surfactant solution, which could act as an inoculum reservoir if organic matter concentration increased in the solution or if adequate concentrations of a bactericide were not maintained in the suspension.

Bacteria can develop resistance to many disinfectant products and QACs are no exception (1,18,35,39,40). BC use in the food industry has resulted in development of resistant forms of *Pseudomonas* spp., *Staphylococcus aureus*, and *Listeria monocytogenes* (1,14,21,23,39,40). Isolates of *Pseudomonas aeruginosa* also have been reported to be tolerant of CTAB (23). For these reasons, prudent measures should be taken to ensure appropriate concentrations of QACs are used in disinfectant solutions in walnut nursery propagation programs. QAC concentrations should be greater than the minimum LD₁₀₀ to account for decreases in efficacy from

unintentional dilution or elevated total solids. Disinfectant solutions should be refreshed as often as feasible to avoid dilution. Finally, disinfectants with different modes of action should be alternated to reduce selection of mutants resistant to any individual disinfectant.

This study indicates *A. tumefaciens* can be transferred between walnut plants by contaminated grafting tools in a greenhouse setting. QACs, notably BC and CTAB, dramatically reduced bacterial contamination on cutting blade surfaces which lowered gall formation in test plants and was less phytotoxic than the commonly used microbiocide, sodium hypochlorite. Both QAC compounds are useful additions to sterilization methods used during grafting operations where high levels of total solids are common. Field studies are being conducted to verify these results in a commercial walnut nursery setting.

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