Prevention of Crown Gall on Prunus Roots by Bacterial Antagonists

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ABSTRACT

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Biological control of crown gall on *Prunus* species was accomplished both in the greenhouse and field with strain 84 of *Agrobacterium radiobacter*. In the field, preplanting inoculation with strain 84 reduced the incidence of galling to 11% in seedlings that subsequently were inoculated with a mixture of six strains of *A. tumefaciens*, and completely protected seedlings against naturally-occurring crown gall bacteria in the soil. In contrast, more than 75% of the seedlings that had not received strain 84 before or after inoculation with *A. tumefaciens* developed galls. In the field, when the proportion of colony-forming units of strain 84 to pathogenic strains was 1:10, galling was reduced to 5% compared to 31% at 1:100. Nevertheless, 6% of the seedlings may have been infected by two of the six pathogenic strains that were insensitive to a bacteriocin produced in vitro by strain 84. When these two bacteriocin-insensitive strains were mixed individually with strain 84 and inoculated to tomato seedlings, 89% or more of the seedlings were galled, even with population ratios of 1:100 of pathogen to strain 84. Conversely, none of the seedlings became infected when inoculated simultaneously with 1:1 mixtures of strain 84 and each of the four bacteriocin-sensitive pathogens. A combined inoculum of all six pathogens mixed 1:1 or 1:10 with strain 84 resulted in >80% galled tomato seedlings contrasted to 5-6% galling of mazzard cherry seedlings inoculated in the field. Cells of strain 84 treated with chloroform did not prevent infection of tomato seedlings by strain Q51, whereas a similar number of viable strain 84 cells completely prevented galling. In the field, strain 84 was significantly more effective in reducing galling on inoculated cherry seedlings than were the bactericides commonly used in the nursery trade. Aboveground grafts of flowering cherry (*Prunus pendula* cv. 'Double Subhirtella') on mazzard cherry *(Prunus avium)* rootstocks also were protected from galling when scion wood was sprayed with strain 84 just prior to grafting. A mutant of strain 84 resistant to rifampicin and streptomycin readily colonized the roots of mazzard cherries following inoculation of the seed, roots, or crown. Three Oregon isolates of *A. radiobacter* of 32 tested prevented crown gall on mazzard cherry seedlings inoculated with *A. tumefaciens*, but two other isolates of this species significantly increased the number and size of galls on the seedlings.

In 1972, New and Kerr (8) published the first report of biological control of crown gall by using strain 84 of *Agrobacterium radiobacter*. Peach seeds stratified for 3 months were dipped in a suspension of strain 84 containing 10^8 cells per milliliter and potted in soil inoculated with a pathogenic *Agrobacterium* species. Galling was reduced from 79% to 31% on seedlings grown from the inoculated seed, and nonpathogens (presumably strain 84) became predominant on the roots. Of seven avirulent strains tested, only strain 84 was effective in reducing crown gall in greenhouse tests. New and Kerr (8) predicted that dipping plants in a cell suspension of strain 84 before planting could provide effective control of crown gall in the field.

The present study was designed to determine: (i) the extent of colonization of the host by strain 84 and its survival on host roots, (ii) the efficacy of strain 84 in preventing crown gall in the field, and (iii) the effectiveness of nonpathogenic *Agrobacterium* isolates from Oregon in preventing crown gall. A preliminary report has been published (7).

MATERIALS AND METHODS

Bacterial and plant culture.—Strain 84 of *Agrobacterium radiobacter* and strains 27 and 29 of *A. tumefaciens* were kindly supplied by A. Kerr, of the Waite Agricultural Institute, Australia. Strain B234 was obtained from J. De Vay, University of California, Davis; B6 from R. Baker, Colorado State University, Fort Collins; EU-8 from M. N. Schroth, University of California, Berkeley; and Q51 was isolated from a naturally galled cherry tree in Oregon. All pathogenic strains were ketolactose-negative (2) except EU-8 and B6, which were positive. All strains were pathogenic to *Prunus* spp. except strain 84.

Inoculum was prepared from late log-phase cultures of each of the bacterial strains grown on a rotary shaker in yeast-dextrose-peptone (YDP) broth formulated: 4 g yeast extract, 20 g dextrose, 4 g peptone, and 5 g $(NH_4)_2SO_4$ in 1 liter of distilled water adjusted to pH 7.0.

Cultures were diluted with distilled water to the desired cell concentration [3 to 7 × 10^8 colony-forming units (CFU) per ml]. Alternatively, the bacteria were cultured on a mannitol-glutamate agar medium (3), then washed from the agar surface and diluted to the desired concentration (2 to 7 × 10^8 CFU/ml).

Unless specified otherwise, the *Prunus* seedlings were grown and handled in the same manner as commercially propagated stock. Oregon nurserymen usually plant seed in October, the seed germinate in February or March, and the seedlings that develop are dug in December. Harvested seedlings are stored in a warehouse or "heeled-in" outside. Prior to transplanting, dormant seedlings are graded and the stem, tap, and lateral roots are pruned.

In the field, seedling crowns were inoculated with strain 84 or with the mixture of pathogenic strains after removing the soil and cutting a longitudinal slit 2-3 cm long and 1-1.5 mm deep on the crown with a linoleum knife. Harvested dormant seedlings were wounded similarly. Seedlings and seeds were inoculated by dipping or spraying them to run-off with the bacterial suspensions. Two sets of controls were used: (i) inoculated but unwounded, and (ii) wounded but noninoculated seedlings. All seedlings were harvested in November or December and scored for galling.

EXPERIMENTAL

Crown gall prevention by the nonpathogenic Agrobacterium radiobacter isolate 84 applied to Prunus roots before or after introduction of the pathogen, Agrobacterium tumefaciens.—To prevent infection, wounds on a susceptible host must be protected from *A. tumefaciens* residing on the roots or in the soil in which root-pruned seedlings are planted. Consequently, the pathogen may precede or follow the application of an antagonistic organism to the roots.

Root-pruned seedlings of mazzard cherry were inoculated with isolate 84 immediately before or after inoculation with the mixture of pathogenic strains. Forty seedlings for each inoculation sequence were potted in field soil and grown in the greenhouse. Galling was rated and recorded 2 months later.

Strain 84 significantly reduced the percentage of galled seedlings whether it was applied to the seedling roots before or after they were inoculated with the pathogenic mixture, but inoculation with strain 84 was more effective when it followed the pathogen (Table 1). Root surfaces were inoculated to run-off, and the first bacteria inoculated to the roots subsequently may have been displaced by the second challenge inoculation. However, replacement apparently was reduced when the seedlings were drained for 15 minutes after inoculation with strain 84 and before challenge inoculation with the pathogens.

Effectiveness of Agrobacterium radiobacter isolate 84 against various concentrations of Agrobacterium tumefaciens inoculum suspension.—To be of economic value, strain 84 must prevent crown gall in the field where various concentrations of naturally-occurring pathogens may be present. Dormant mazzard cherry seedlings were root-pruned, wounded on the crown, dipped in a washed

TABLE 2. Effectiveness of *Agrobacterium radiobacter* strain 84 in reducing galling of mazzard cherries inoculated with various concentrations of *A. tumefaciens* and then grown in the field

Number of colony-forming units per ml of inoculum[a]		Seedlings galled[b] (%)
Strain 84	Pathogen mixture	
0	3×10^8	91 z
3×10^6	3×10^8	31 y
0	3×10^7	58 z
3×10^6	3×10^7	5 x
0	3×10^6	17 y
3×10^6	3×10^6	5 x
0	3×10^5	26 z
3×10^6	3×10^5	6 x
0	0	22 z
3×10^6	0	0 x

[a]Wounded crowns of root-pruned seedlings were sprayed to run-off with strain 84, drained for 15 minutes, and sprayed to run-off with the mixture of pathogens. The number of colony-forming units (CFU) per milliliter of the pathogen mixture represents the total CFU present in the mixture.

[b]Data based on the mean of five randomly planted replications of each treatment with 20 seedlings per replication. Means followed by the same letter(s) are not significantly different ($P = 0.05$), using the chi-square test.

TABLE 1. Incidence of crown gall on mazzard cherry seedlings as influenced by the sequency of inoculation with *Agrobacterium tumefaciens* and *A. radiobacter* strain 84

Sequence of wounding and inoculations[a]				
I	II	III	IV	Seedlings galled[b] (%)
Wound	Pathogens			90 z
Strain 84	Pathogens	Wound		62 y
Wound	Strain 84	Pathogens		28 x
Wound	Pathogens	Strain 84		12 w
Wound				10 w, z
Pathogens	Strain 84	Wound		8 z
Strain 84	Drained 15 minutes[c]	Pathogens	Wound	3

[a]Seedling crowns were sprayed to run-off with 1.45 × 10^9 colony-forming units (CFU)/ml of the pathogen mixture and 6.1 × 10^8 CFU/ml of isolate 84. Roman numerals I through IV indicate the sequence of events.

[b]Mean of four replications each consisting of 10 seedlings per pot. Means followed by the same letter(s) are not significantly different ($P = 0.05$), using Duncan's multiple range test (F-test = 49.2 at $P = 0.01$).

[c]Seedlings inoculated with strain 84 were drained for 15 minutes before challenge-inoculation with the pathogen mixture.

cell suspension of isolate 84, drained for 15 minutes, and sprayed to run-off with various concentrations of the pathogen mixture. Bacterial suspensions were prepared the night before inoculation and stored at 4 C. Viability assays made immediately before inoculations showed that the colony-forming units (CFU) per milliliter for strain 84 had dropped from 2×10^8 to 3×10^6, whereas the pathogen mixture had remained stable at about 3×10^8. Wounded seedlings inoculated with isolate 84 only, and wounded but noninoculated seedlings were used as controls. Each treatment consisted of five groups of 20 seedlings randomly planted in the field. The percentage of galled seedlings was determined at harvest in October.

At all pathogen concentrations tested, strain 84 significantly reduced galling (Table 2). Wounded seedlings inoculated only with strain 84 were completely protected, whereas wounded but noninoculated seedlings became galled (Table 2). The noninoculated galled seedlings probably were infected by naturally-occurring pathogenic strains in the field soil. The complete absence of galls on seedlings inoculated only with isolate 84 suggests that these naturally-occurring pathogens were sensitive to the bacteriocin produced by isolate 84.

Bacteriocin production by Agrobacterium radiobacter strain 84.—Some galls occurred on mazzard seedlings inoculated with strain 84 and the pathogen mixture, even when populations on strain 84 were in excess of those of the pathogens (Table 2). These galls may have been caused by pathogenic strains in the mixture that were insensitive to a bacteriocin reportedly produced by strain 84 (4). Bacteriocin production by strain 84 was tested in vitro against each isolate of the pathogenic mixture by Stonier's method (11) in a medium containing mannitol, glutamate, and biotin (3). After 2 or more days of incubation, strain 84 was killed with chloroform and overlaid with a suspension of the test strains to determine if growth inhibitors were present.

Zones of growth inhibition were evident 3 days after overlaying with the four ketolactose-negative pathogens, but not with the ketolactose-positive strains (Table 3). Thus, the low frequency of galling in plants inoculated with strain 84 and challenged with the pathogenic strains may have resulted from infection by the ketolactose-positive strains of *A. tumefaciens*.

Effectiveness of Agrobacterium radiobacter strain 84 against each pathogenic strain of Agrobacterium tumefaciens on tomato seedlings.—Each of the six pathogenic strains was mixed individually with strain 84 (1:1 or 1:10, v/v) and inoculated to Bonny Best tomato seedlings. Seedlings 3-4 weeks old were wounded once with a multineedle instrument in the first internode, and 0.01 ml of the mixed inocula was placed on the wound. Control seedlings were wounded and inoculated with the same individual bacterial suspensions diluted 1:1 or 1:10 (v/v) with sterile distilled water instead of the suspension of strain 84. Each test was replicated three times with six seedlings per replication. Seedlings were grown in a commercial medium (Jiffy Mix-Plus, Jiffy Products of America, West Chicago, IL 60185) contained in 7.2-mm diameter styrofoam cups, at 27 C day and 21 C night (12-hour photoperiod) and 150 hectolux. The number of galled seedlings per test was determined after five weeks.

Strain 84 mixed with each of the four ketolactose-negative pathogens completely prevented infection, whereas the two ketolactose-positive strains essentially were unaffected (Table 3). All the seedlings inoculated with the individual pathogens alone were infected, whether diluted 1:1 or 1:10 with sterile distilled water. When the six pathogens were combined, as in the field experiments, and then mixed with strain 84, over 80% of the inoculated tomato seedlings developed galls (Table 3). The inability of strain 84 to inhibit the combination of pathogens on tomato seedlings is very different from the field data (Table 2), but similar to the results of strain 84 against strain EU-8 and B6 on tomato seedlings.

The galls induced by the pathogen when mixed with strain 84 at a 1:10 ratio usually were smaller than those induced by the 1:1 inoculum, suggesting that higher ratios of strain 84 to the ketolactose-positive pathogens might prevent infection. Accordingly, strain 84 (1.9×10^9 CFU/ml) was mixed with strain EU-8 (1.4×10^9 CFU/ml) or B6 (2×10^9 CFU/ml) at population ratios of 50:1 and 100:1 and inoculated to tomato seedlings as previously described. In addition, a 500:1 mixture of strain 84 to strain B6 was used. Over 89% of the inoculated seedlings were galled at 50:1 and 100:1 ratios of 84 to EU-8 or B6, and 61% were galled at a ratio of 84 to B6 of 500:1.

Effectiveness of viable- vs. killed cells of Agrobacterium radiobacter strain 84.—An important

TABLE 3. Effectiveness of *Agrobacterium radiobacter* strain 84 in preventing galling of tomato seedlings inoculated with single or combined strains of *A. tumefaciens*

Bacterial strains	Colony-forming units per milliliter of inoculum ($\times 10^8$) (no.)	Percent galling with ratio of pathogen to strain 84 of: 1:1	1:10	Sensitivity to bacteriocin 84
Q51	1.4	0	0	+
K27	0.89	0	0	+
K29	3.9	0	0	+
B234	2.9	0	0	+
EU-8	0.89	100	89	−
B6	2.0	100	100	−
Combined pathogenic strains	2.0	89	83	N.T.[b]

[a]Mean of three replications of six tomato seedlings each per test. Control seedlings inoculated with 1:1 or 1:10 dilutions of each pathogenic strain in water, or a combination of all six, were all galled. The concentration of strain 84 was 2×10^8 CFU/ml.

[b]The combined pathogens were not tested.

consideration in the usefulness of strain 84 as a commercial biological control, as well as in the possible mechanism of action, was whether dead bacteria would prevent infection. Use of dead cells would simplify product storage, shipment, and care and handling in the field as compared to living cultures. To test this, cultures of strain 84 grown on mannitol-glutamate agar were treated with chloroform for two hours. The bacteria were resuspended in 0.1 M phosphate buffer (pH 7.0) to a Klett reading of 171 (about 2×10^8 CFU/ml). Aliquots of the suspension of treated bacteria were diluted and plated. Most cells were killed; 2×10^4 CFU/ml developed on the plates compared to 1- to 5×10^8 CFU/ml prior to chloroform treatment. No differences in bacterial morphology before and after treatment were detected by light microscopy.

Suspensions of chloroform-treated cells of strain 84 were mixed 1:1 and 10:1 with suspensions of the pathogenic strain Q51 (1.4×10^8 CFU/ml) and inoculated to tomato seedlings as described under the previous section. Controls consisted of 1:1 and 10:1 ratios of nontreated strain 84 (2×10^8 CFU/ml) and strain Q51 (2.1×10^8 CFU/ml). All the seedlings inoculated with a mixture of Q51 and treated 84 were galled. None of the seedlings inoculated with mixtures of viable 84 and Q51 was galled.

Comparison of Agrobacterium radiobacter strain 84 and bactericidal chemical treatments for preventing crown gall.—The effectiveness of strain 84 in preventing crown gall was compared to control with agricultural bactericides commonly used in commercial nurseries. Root-pruned mazzard cherry seedlings were spray-inoculated to run-off with a suspension of *A. tumefaciens,* allowed to dry for 15 minutes, and wounded. Roots of some of the inoculated seedlings (50 per treatment) were sprayed to run-off with strain 84, and the remaining roots were dipped for 10 minutes in a bactericide solution just prior to planting. The following bactericides were used: Agristrep (21.2% streptomycin sulfate, Merck & Co., Rahway, New Jersey); Captan 50 WP, 50% N-(trichloromethyl)thio-4-cyclohexene-1,2-dicarboximide plus 5.25% sodium hypochlorite; (EPA Registration Number 476-581, Stauffer Chem., Mountain View, California); and Kocide 101 (83% cupric hydroxide, Kennecott Chemical, Houston, Texas; plus 0.47 liters (1 pint) of Volck Supreme Oil, Ortho Chemical Division, San Francisco, California). Control seedlings either were untreated but inoculated or noninoculated and untreated.

Strain 84 reduced galling more effectively than the bactericides (Table 4). Galling was more severe in all the bactericide-treated plants than in the controls. Enhanced galling on inoculated seedlings has been observed in other tests for chemical control (L. W. Moore, *unpublished*) and I suspect that natural antagonists may have been eliminated or that tissue susceptibility was increased.

Protection of aerial graft unions by strain 84.—Crown gall in the tongue grafts of weeping flowering cherry *(Prunus pendula* 'Double Subhirtella' on *Prunus avium*) has been a recurring problem in an Oregon nursery despite extensive efforts at sanitation. The efficacy of strain 84 for preventing infection of graft unions was tested. Inocula of strain 84 ($8 \times$ CFU/ml) were sprayed to run-off over the cut surfaces of the scion and stock just prior to grafting in March. Trials included: (i) inoculation of scion and stock with strain 84 followed by inoculation of the scion with the pathogen mixture (6.4×10^6 CFU/ml of the mixture); and (ii) equal volumes of the pathogen mixture and isolate 84 were mixed together and sprayed over both cut surfaces. Controls consisted of scion and stock cuts inoculated with strain 84 alone, the pathogen mixture alone, or water. All grafts were wrapped with tape. Thirty grafted trees were used for each treatment. The trees were examined in November for amount of galling.

Ninety three percent of the grafts inoculated with the pathogen mixture alone were galled, whereas only 3% of the grafts developed gall when inoculated with strain 84 before the pathogen mixture or when mixed 1:1 with the pathogen mixture. Control grafts inoculated only with strain 84 or water were free of galls.

Colonization of mazzard cherry roots by a drug-resistant mutant of strain 84.—The survival of strain 84 on field-planted seed and subsequent colonization of seedling roots was followed by means of a strain 84 variant that was resistant to streptomycin and rifampicin. This strain was obtained by repeated culturing in liquid media containing increasing concentrations of the antibiotics. The resistant strain grew on a selective medium for ketolactose-negative agrobacteria (8) containing 2.5 mg/ml each of rifampicin and streptomycin. Antibiotic production and activity against the pathogens by the resistant strain was equivalent to

TABLE 4. The effectiveness of strain 84 of *Agrobacterium radiobacter* and various bactericides in preventing crown gall on mazzard cherry seedlings inoculated with *A. tumefaciens* in the field

Treatment[a]	Concentration (μg/ml)	Seedlings galled[b] (%)
Noninoculated		11 x
Inoculated		88 y, z
Strain 84		10 x
Captan 50W + Sodium hypochlorite	4700, 210	100 z
Agristrep	1200	100 z
Kocide + Supreme Oil	3000, 1200	76 y
	6000, 1200	75 y
Supreme Oil	1200	82 y

[a]Root-pruned seedlings (50 per treatment) were inoculated with *A. tumefaciens,* drained, wounded, and sprayed with isolate 84 or dipped for 10 minutes in the indicated bactericide solutions before planting.

[b]Means followed by the same letter (s) are not significantly different ($P = 0.05$), using the chi-square test.

that of the parent (strain 84) when tested by a modification of Stonier's method (11). Seeds were dipped in a suspension of 3×10^7 CFU/ml of the resistant strain, drained, and planted in March. Eight months later, intact crowns and root systems were cut from two seedlings and assayed individually by macerating the tissue in a Waring Blendor in 100 ml of water for 5 minutes, diluting the suspension serially, and spreading three 0.1 aliquants directly from the suspension and from selected dilutions on the selective medium containing rifampicin and streptomycin.

Populations of 8.5×10^5 to 1×10^6 CFU of the drug-resistant strain per gram of fresh tissue were recovered from roots of seedlings grown from inoculated seed. No *Agrobacterium* spp. were isolated from roots of seedlings grown from uninoculated seed. The drug-resistant strain successfully overwintered on the inoculated seed and subsequently colonized the emerging root system.

To determine if the drug-resistant strain could colonize uninoculated parts of the root system of growing plants, 3×10^8 CFU were sprayed over the exposed crown of mazzard cherry seedlings in July. The soil was replaced around the crown, and the inoculated and uninoculated control seedlings were harvested 2 hours or 1 month later. In addition to assaying the total root system for the drug-resistant strain, the tap root tip (30-33 cm from the crown), lateral root tips, and cylinders of the epidermal surface 2.3 to 3 cm long (peeled from the tap root at the crown and 13 to 14 cm below the crown) were assayed separately.

The drug-resistant strain was detected only on peelings from the crown and tips of lateral roots near the crown of seedlings harvested 2 hours after inoculation (Table 5). The inability to detect the mutant on other parts of the root below the crown, as well as the total root, may have resulted from: (i) much of the inoculum sprayed over the crown was absorbed to surrounding soil and (ii) the bacteria were diluted below the level of detection. One month after inoculation, the drug-resistant strain had significantly increased in numbers and had spread to other parts of the root system. No *Agrobacterium* was isolated from roots of uninoculated seedlings.

TABLE 5. Colonization of mazzard cherry roots by a drug-resistant mutant of strain 84 of *Agrobacterium radiobacter*

Tissue sampled	Colony-forming units ($\times 10^5$) of the resistant strain following inoculation[a]	
	2 Hours	1 Month
Root tips:		
Laterals	1.2	3300
Tap	ND[b]	ND
Epidermal shavings:		
Crown	0.22	18
Midway	ND	8.8
Total root	ND	1.4

[a]Soil around the seedling crowns was removed, about 3×10^8 cells of a mutant strain of strain 84 resistant to streptomycin and rifampicin were sprayed over the crown, and the soil was replaced. Root systems of two inoculated seedlings were removed and assayed at 2 hours and 1 month following inoculation. No colonies of *Agrobacterium* were recovered from noninoculated roots handled in the same manner.

[b]Scored as nondetectable when fewer than 10 colonies developed on less than three replicated agar plates at the lowest dilution (the roots were macerated in 100 ml of water and 0.1 ml of the suspension was plated directly.).

Ability of Agrobacterium radiobacter isolates to prevent crown gall.—New and Kerr (9) reported that two of eight *A. tumefaciens* isolates they tested were not controlled by strain 84. Later tests showed that pathogens controlled by strain 84 were sensitive to a bacteriocin produced in vitro by strain 84 (4). Four of the six pathogenic isolates I used in the inoculum were sensitive to the bacteriocin produced by strain 84, but strains EU-8 and B6 were not sensitive. Consequently, 32 other nonpathogenic *A. radiobacter* isolates from Oregon were tested against the pathogen mixture. Exposed crowns of mazzard cherry seedlings growing in the field were wounded and inoculated with (i) the pathogen mixture (3×10^8 CFU/ml); (ii) *A. radiobacter* mixture (5×10^8 CFU/ml), followed by the pathogen mixture; or (iii) *A. radiobacter* mixed 1:1 with the pathogen mixture.

Three of the 32 isolates of *A. radiobacter* completely prevented crown gall regardless of the inoculation method, and two other isolates caused a significant increase in galling (85-90% as compared to 45% by the pathogen mixture alone). The effect of the remaining 26 isolates of *A. radiobacter* ranged between protection and enhanced infection.

DISCUSSION

Crown gall can be controlled effectively in the field by strain 84 of *A. radiobacter*. A few seedlings inoculated with strain 84 developed galls when challenged by a mixture of pathogenic strains, but seedlings were completely protected against natural pathogenic strains in the field. Complete protection probably occurred because of low *A. tumefaciens* populations in the field (only 22% of the uninoculated seedlings became galled) and because the predominant strain of *Agrobacterium* in Oregon is ketolactose-negative (1). In Australia, the ketolactose-negative strains were more sensitive to strain 84 than the ketolactose-positive strains (4).

Mazzard cherry seedlings are root-pruned and planted in early spring and need extended protection from infection while wound healing occurs. Strain 84 is particularly effective in providing this long-term protection, as shown by the colonization of *Prunus* roots by the drug-resistant mutant. Movement of strain 84 from the crown to the roots of seedlings inoculated during the growing season also suggests a useful way to treat susceptible ornamental plants (e.g. roses), already established at a site. Supplemental irrigations may be needed to aid downward-movement of the bacteria from the point of inoculation.

The protective action of strain 84 against a mixed population of bacteriocin-sensitive and -insensitive pathogenic strains was much greater belowground (less than 10% galling of cherry seedlings) than aboveground (more than 80% galling of tomato seedlings). Other than the different hosts used in these tests, the major difference was the inoculation site. Apparently, strain 84 functions better in the rhizosphere-rhizoplane region against

pathogens insensitive to bacteriocin 84. Schroth and Moller (10) also obtained a reduction of crown gall on almond, peach, plum, and apricot inoculated belowground with strain 84 and a mixture of three ketoglycoside-positive pathogenic strains, one of which was insensitive to bacteriocin 84. These results emphasize the need for caution in extrapolating from assays of bacterial antagonists against pathogens on tomato seedlings in the greenhouse to their potential effectiveness in preventing crown gall under field conditions. For example, Kerr and Htay (4) stated that 7 of 14 overseas strains tested were resistant to bacteriocin 84 and not subject to biological control on tomato seedlings. It should be noted, however, that the correlation between the ability of strain 84 to prevent infection of tomato seedlings and the sensitivity of the six pathogens to bacteriocin 84 that I tested was similar to that reported by Kerr and Htay (4).

The mechanism by which strain 84 prevents infection has been attributed to a bacteriocin (4), rather than to exclusion of the pathogen from the infection or attachment site (5). The data presented in this paper support the first proposition. Only viable cells of strain 84 are effective, whereas Lippincott and Lippincott (5, 6) could reduce infection with dead cells of both virulent and avirulent *Agrobacterium*. They were able to exclude the pathogen from the host cell attachment site with most *Agrobacterium* species tested. Only about 10% of the Oregon *A. radiobacter* strains tested prevented infection, indicating more specific inhibition. Lippincott and Lippincott's strains never completely inhibited infection (5), even at virulent:avirulent ratios exceeding 1:600. Furthermore, the order of inoculation was critical. Inhibiting cells had to precede the pathogen, otherwise, they were ineffective (5, 6). In my experiments, galling was greatly reduced by strain 84 whether introduced to the wound before or after inoculation with the pathogen. Lippincott and Lippincott (5) also reported that "...only a 15 minute difference in time of addition of inhibitor relative to virulent cells can result in no inhibition." The rationale for exclusion of virulent cells from the infection site by avirulent cells seems well documented as a means of reducing tumor numbers on bean leaves, but the data presented in this paper do not fit that scheme (5). Our data agree more closely with the concept of an inhibitory compound(s) produced by a living microorganism.

The larger number and size of galls, over the control, that I observed when mixed virulent and avirulent *Agrobacterium* strains were used was similar to Lippincott and Lippincott's report (6) of tumor growth complementation. However, tumor complementation was described as an increase in tumor diameter in the absence of a significant change in tumor number, except when low concentrations ($\leqslant 3 \times 10^8$ cells/ml) of the pathogen were present.

This work verifies and amplifies the phenomenon of biological control of crown gall reported by New and Kerr (9), and demonstrates that isolate 84 can be used effectively in the field to prevent infection by *A. tumefaciens*.

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