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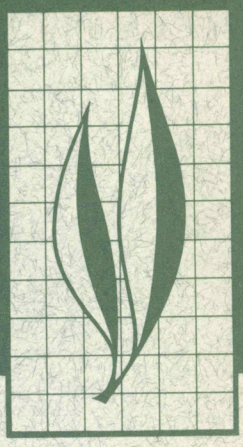

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Biology and Control of *Agrobacterium tumefaciens*

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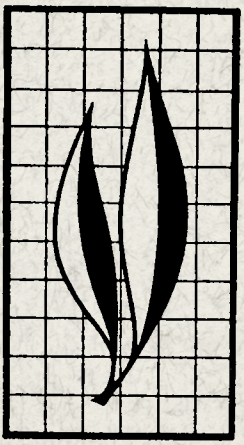
D. C. Hildebrand, and N. Ross

UNIVERSITY OF CALIFORNIA DIVISION OF AGRICULTURAL SCIENCES



This paper presents recent information on the biology of *Agrobacterium tumefaciens,* and outlines techniques for isolating and studying the bacterium. Data are presented indicating that *A. tumefaciens* is a soil inhabitant. The organism was isolated in 18 of 28 California soils, including soils not known to support host plants, and appears to be a rhizosphere organism. *In vitro* experiments showed that the bacterial cells aggregated around roots of various plants.

A. tumefaciens and *A. radiobacter* were studied for serological and physiological differences. Although no physiological differences were detected, most *A. tumefaciens* strains differed serologically from *A. radiobacter* in possessing an antigen which formed a precipitin line near the antigen well in gel-diffusion tests. The last part of the paper discusses how the disease is contracted and presents techniques for reducing loss from crown gall.

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INTRODUCTION

THE BACTERIUM *Agrobacterium tumefaciens* has an ubiquitous host range among dicotylendonous plants and causes disease in many parts of the world. Disease incidence has steadily increased in many California nurseries and orchards along with their increase in production. In 1963 crown gall was judged one of the three most important diseases on 14 major crops in California, causing an annual loss of $7 million per year (University of California Plant Pathology Statewide Conference on Plant Disease Losses Committee, 1965).

Although *A. tumefaciens* has been intensively studied for over 60 years, little data exist concerning its biology in soil. It is not known how long the pathogen survives, what factors favor its multiplication, whether it is a rhizosphere organism, and what the best methods are to eradicate it from soil. In response to the request of California growers for assistance in controlling the disease, an investigation was initiated to study the biology of the causal organism and to develop economically feasible control methods.

Some of the information resulting from these studies has already been published (Ross *et al.* 1970). Although there still are no specific answers to all of the questions mentioned, this report contains the latest information on the biology of the organism and describes our techniques for studying the organism and reducing loss from crown gall disease. Table 1 lists the organisms used in the study.

ISOLATING THE ORGANISM

Efficient methods for isolation and rapid identification of the *A. tumefaciens* are essential for studying its ecology in soil and the lack of such methods is largely responsible for the scanty attention given to this aspect of the disease cycle. Therefore, we developed two methods for selectively isolating agrobacteria from soil. The first method consists of placing soil directly on fresh carrot slices and incubating them for 10 to 15 days (Ark and Schroth, 1958). The development of galls indicates that *A. tumefaciens* is present in the soil. However, this method does not provide quantitative data about the population in the soil and has the further disadvantage that carrot slices are often rotted by other bacteria.

The second isolation procedure con-

* Submitted for publication April 30, 1970.

TABLE 1
ORIGINS OF ISOLATES USED IN THE STUDY

Genus and isolate number	Isolated from	Source
Agrobacterium radiobacter		
Rad-1	Soil	M. N. Schroth
Rad-2	Soil	M. N. Schroth
Rad-6466	Soil	American Type Culture Collection
Rad-6467	Soil	American Type Culture Collection
Red-B	Soil	M. N. Schroth
Red-C	Soil	M. N. Schroth
Red-D	Soil	M. N. Schroth
TR-1	Soil	M. P. Starr, ICPPB
TR-5	Soil	M. P. Starr, ICPPB
TR-6	Soil	M. P. Starr, ICPPB
TR-102	Soil	M. P. Starr, ICPPB
TR-105	Soil	M. P. Starr, ICPPB
A. rhizogenes		
Rhi-1	Rose	P. A. Ark
Rhi-2	Rose	P. A. Ark
A. tumefaciens		
Ach-1	Achillea	P. A. Ark
Al-1	Almond	M. N. Schroth
Al-2	Almond	Z. Volcani
Ap-1*	Apple	M. N. Schroth
Apr-1	Apricot	M. N. Schroth
Apr-A	Apricot	M. N. Schroth
Apr-B	Apricot	M. N. Schroth
B-38	Bryophyllum	P. A. Ark
B-48	Bryophyllum	P. A. Ark
B-55	Bryophyllum	P. A. Ark
Ced-1*	Incense cedar	M. N. Schroth
CG-1	Pear	P. A. Ark
Dah-1	Dahlia	Z. Volcani
Dod-1	Dodonaea	M. N. Schroth
Eu-2	Euonymus	P. A. Ark
Pe-1	Peach	M. N. Schroth
Pl-1	Plum	M. N. Schroth
Pop-1*	Poppy	M. N. Schroth
R-12	Hollyhock	Unknown
Rub-1	Blackberry	M. N. Schroth
S Bak-1	Soil	M. N. Schroth
Sba-1	Sugar beet	J. P. Thompson
TT-2	.....	M. P. Starr, ICPP
Rhizobium japonicum		
RJ-1	Soybean	Shirley Nash Smith
R. meliloti		
Rm-1	Sweet clover	Shirley Nash Smith

* Although these strains were isolated from tumors, they were not virulent and could therefore also be considered to be *A. radiobacter*.

sisted of plating soil on a selective medium (Schroth *et al.*, 1965) composed of a combination of antibiotics and nutrients that exclude over 99 per cent of the microorganisms which develop on standard media. *A. tumefaciens* and *A. radiobacter* colonies are easily distinguished from other bacterial colonies by their characteristic appearance. The medium has been useful in assaying soils for populations of *A. tumefaciens* and *A. radiobacter*, and its plating efficiency is indicated by the fact that average recovery of cells of different isolates introduced into soil was 38 per cent. However, certain strains of *A. tumefaciens* do not grow on it. Thirty-nine strains of *A. tumefaciens* were compared for growth on this medium and on potato-dextrose-peptone agar (PDP). Two-tenths ml of a 10^4 cell per ml of suspension was pipetted onto the surfaces of

TABLE 2

NUMBER OF COLONIES OF *A. TUMEFACIENS* FORMED ON VARIOUS MEDIA WITHOUT SOIL SOLUTION*

Isolate number	Medium			
	Nutrient agar	Patel's	PDP	Schroth's
	Number of colonies			
Apr A	12,300	10,000	....	8
Apr B	1,800	1,560	480	5
CG 1	6,520	5,520	880	1
Cg Alm	7,080	2,520	1,160	41
Dod 1	44	38	20	4
Eu 7	1,216	842	670	146
S. Bak 1	22	26	22	1
SBA 1	5,760	8,040	3,720	6
T 2	11,240	10,440	7,560	4

* Each figure is the mean of 5 replications and represents number of colonies formed per ml of bacterial suspension on different media. Suspensions were standardized, using a Klett reading of 20.

the media and distributed with an L-shaped glass rod. Only 17 of the 39 strains grew on the selective medium, and, surprisingly, only 28 grew on PDP. Kerr (1969) reports that some strains occuring in Australia do not grow on the selective medium. This is probably because of the Australian isolates' inability to use nitrate; we have found that these strains will grow if ammonium salts are added to the medium as the nitrogen source.

Although the selective medium is effective for isolation purposes, it is not a good medium for culturing the organism. The ability of nine strains to produce single colonies in Patel's medium (Patel, 1926), PDP, nutrient agar, and the selective medium without soil solution, was evaluated by plating a dilution series on these media. The greatest number of colonies developed on nutrient agar, and the least on the selective medium (table 2). The plating recovery of cells introduced into soil was 38 per cent, but the average number of colonies formed on the selective medium without soil solution averaged only 0.61 per cent, as compared to that on nutrient agar.

It is not surprising that the selective medium operates effectively as an isolation medium when a soil suspension is used, as the medium was developed by plating soil solutions on it, recording the results, and then adjusting the balance of nutrients and antibiotics. The selective medium probably lacks compounds required for bacterial growth but which are present in the soil solution. Thus the medium is essentially a basal medium until soil solution is added.

While developing the selective me-

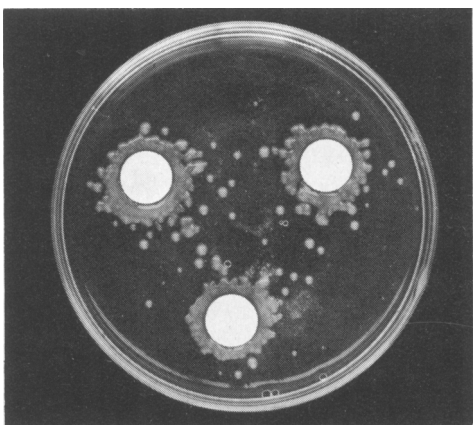

Fig. 1. Growth of *Agrobacterium tumefaciens,* isolate R-12, on potato-dextrose-peptone agar, demonstrating that culture filtrates provide required substance(s) not present in the medium. Surface of the plate was seeded with bacteria at a concentration of 10^4 cells per ml. The discs shown in this photograph contain cultural filtrate from a nutrient broth culture of R-12 grown for 2 days.

dium it was noted that some strains did not grow unless a heavy suspension or a mass transfer of cells was applied to the medium. This also occurred with other media such as PDP and nutrient agar. The problem was solved by first incorporating sterile culture filtrate into these media. Striking results were obtained by soaking filter paper discs in bacterial filtrate and placing them on a PDP dish previously seeded with the filtrate requiring strain at a cell count of 10^4 per ml (fig. 1). Growth was considerably greater about the discs, and in some cases (as with strain R-12) growth occurred only around the discs. This suggests that data collected from plate counts should be evaluated precisely, as poor nutrition or lack of growth requirements may result in highly inaccurate counts. Colonies often may develop only if there is an aggregate of cells present, and not from a single cell.

IDENTIFICATION OF *A. TUMEFACIENS*

The Bernaerts-De Ley test (Bernaerts and De Ley, 1963) for 3-ketoglycoside formation is a simple, rapid test for general identification of the *A. tumefaciens-radiobacter* group. The test is performed by spotting suspected *Agrobacterium* (4 to 6 spots per plate) on the Bernaerts-De Ley medium (0.1 per cent yeast extract, 1 per cent lactose, and 2 per cent agar). The plates are incubated at 28°C. for 1 to 2 days, then flooded with Benedict's reducing sugar reagent at room temperature. The formation of a yellow ring around the colony after 15 minutes to 1 hour is indicative of both *A. tumefaciens* and *A. radiobacter*. However, we have several strains which do not form these substances. Similar non - 3 - ketoglycoside - forming strains have been reported from Australia (Kerr, 1969).

At present there is no rapid method for distinguishing between *A. tumefaciens* and *A. radiobacter,* although serology shows promise for doing this. The best present method is to inoculate a host plant. This is most easily accomplished by inoculating either carrot slices, young tomato, or sunflower plants; tumors will appear approximately 7 to 10 days after inoculation if the bacterium is *A. tumefaciens*. Care must be taken not to confuse wound callus tissue with gall tissue.

SURVIVAL OF *A. TUMEFACIENS* IN SOIL

The ability of *A. tumefaciens* to survive in soil for long periods is controversial. Reports that the organism is not a good soil inhabitant (Hildebrand, 1941) may result from lack of adequate techniques for isolating the bacteria. The ability of the organism to survive is an important facet of the disease cycle because it effects the type of control procedure to apply (such as crop rotation, soil fallow, fumigation, or movement of a nursery to an area known not to have supported host plants).

Isolation of *A. tumefaciens* and *A. radiobacter* on the selective medium discussed above indicated that both organisms are widespread in California soils. *A. tumefaciens* was detected in 18 of 28 soils tested. Only one of 28 California soils tested failed to yield *A. radiobacter* when plated directly on the selective medium—but the organism was recovered even from this soil when the selective medium was used as an enrichment medium. For this purpose, the selective medium was prepared without agar, 1 gram of soil was added to 100 ml of the broth, and the mixture was incu-

TABLE 3

POPULATION OF *A. TUMEFACIENS* AND *A. RADIOBACTER* IN SOIL AS DETERMINED BY PLATING ON THE CROWN GALL SELECTIVE MEDIUM

Area and soil type*	Crop	Population per gram of soil†	
		A. tumefaciens	*A. radiobacter*
Bakersfield—sandy loam	Cotton field	20	9,120
sand	Weeds	5	600
Butte County—sandy loam			
Before fumigation	Nursery	272	3,400
After fumigation	Nursery	77	966
Modesto—sandy loam	Almond	150	3,300
Pittsburg—sandy loam	Almond	45	5,050
Salinas—clay loam	Bean	5	2,500

* The Bakersfield soils were from a cotton field and an area near a river; the Pittsburg soil was from a walnut orchard; the Salinas soil was from a grass pasture; and the other soils were from nurseries where stone fruits had been planted.

† Figures represent the mean of 4 replications, with the exception of the soils from Butte County where 12 soil samples were taken at random from a field before and after fumigation with methyl bromide-chloropicrin. In the assay for *A. tumefaciens*, only 2 of 12 samples contained the organism after fumigation. Pathogenicity of the isolates was tested by inoculation of sunflower and tomato plants.

bated for 48 hours after which two-tenths of a ml was plated on the standard selective medium. Table 3 shows that the ratio of *A. tumefaciens* to *A. radiobacter* in seven of the eighteen soils harboring both species varied from 1:13 to 1:500.

The lowest ratio occurred in nursery soils where stone fruits had been planted. The grass pasture (Salinas soil, table 3) had, supposedly, never been cultivated and had not supported host plants other than dicotyledonous weeds. We have also isolated *A. tumefaciens* from three other fields not known to have grown host plants other than weeds. *A. tumefaciens* was also occasionally isolated from soils of cotton and tomato fields and from fields left fallow for over 5 years, using the carrot technique. Greatest success occurred when pieces of root material with clinging soil were placed on carrot slices.

Additional studies on populations of *A. tumefaciens* in soils have not been conducted because of the inability to readily distinguish between virulent and avirulent strains. However, certain strains of *A. tumefaciens* can easily be distinguished from other strains on the selective medium because of a particular colony appearance, such as a deep-red color. These strains could be used to study population dynamics in soil.

The above findings indicate that in many areas of California *A. tumefaciens* is either a soil inhabitant or is able to persist for years after introduction. Survival most likely is affected by multiplication in the rhizospheres of plants and occasional increase by infection of susceptible weeds and commercial crops. There is no assurance, therefore, that planting of nursery trees in soil not known to have supported economic host plants will result in the production of disease-free trees. The ability of *A. tumefaciens* to survive in the soil is not unexpected because *A. radiobacter*, which is the same as *A. tumefaciens* except for virulence (De Ley, 1968), has long been considered a normal rhizosphere bacterium by microbiologists. Dickey (1961) reported that *A. tumefaciens* survived for long periods in sterilized and unsterilized soil contained in tumblers without the presence of a host, and in view of this he concluded that the organism must be considered a successful soil invader.

ATTRACTION OF AGROBACTERIA TO ROOTS

A. tumefaciens commonly attacks the crown and roots of plants. This and the observations that members of *Rhizobium,* a genus related to *Agrobacterium,* often aggregate around roots (Fahraeus, 1957; Thorton, 1936) prompted an investigation to ascertain if *A. tumefaciens* also might be attracted to roots. An abstract on this study has been published (Schroth and Ting, 1966).

Seeds of pea, mung bean, tomato, tobacco, barley, and cucumber were sterilized in a 0.5 per cent aqueous solution of sodium hypochlorite for 3 minutes and then germinated on moist filter paper in petri dishes. Excised root and intact seedlings were placed in drops of water containing bacteria, placed on microscope slides, and covered with a cover slip. Movement of bacteria was observed by phase microscopy at 400x.

Bacteria of most strains were observed to cling to root hairs and zones of elongation of roots on all plants examined (fig. 2,A, B). Strands of bacteria were often observed between two root hairs, as if there was an attraction between them (fig. 2A). Only 13 of 46 strains of *A. tumefaciens* failed to accumulate around root hairs. Generally, the accumulation of bacteria around the hairs was slow, but bacteria often could be seen darting to a specific site on a root hair, oscillating for one to several seconds, then departing in another direction. This specific site would be visited by a number of bacteria. Broken root hairs also appeared to be major sites for bacteria accumulation. There was a distinct zone of repulsion around the root-cap zone of all plants.

It is tempting to speculate that bacterial accumulation in roots and wounds may have an important role in the initiation of disease. However, it is also possible that the phenomenon may not operate in the rhizosphere because of the effect of other microflora and charged soil particles. Although this phenomenon has been observed hundreds of times, attraction of agrobacteria to root hairs does not always occur; often we make several tests before attraction occurs. Attempts to determine the source of variability have failed.

SEROLOGY OF AGROBACTERIUM

Many techniques have been unsuccessfully used in attempts to easily distinguish *A. tumefaciens* from *A. radiobacter.* The principle characteristic distinguishing *A. tumefaciens* from *A. radiobacter* is its ability to cause tumors. However, Hochster and Cole (1967) investigated the serological characteristics of four virulent and four avirulent strains of *A. tumefaciens* and concluded that serology probably could be used to identify strains of the organism. An investigation was therefore made of the serological properties of *A. radiobacter* and *A. tumefaciens,* as this would greatly assist a study of the population dynamics in soil since it is necessary to distinguish between virulent and avirulent strains. *A. rhizogenes* and *Rhizobium* species were included in the investigation for comparative purposes.

The 12 virulent strains of *A. tumefaciens* used in the study were from plum (Pl-1), almond (Al-2), peach (Pe-1), dahlia (Dah-1), pear (CG-1), apricot (Apr-1), and bryophyllum (B-38, B-48, and B-55). The four avirulent isolates tested were from apple (Ap-1), poppy (Pop-1), cedar (Ced-1), and strain Ach-1 (which became avirulent in culture) from *Achillea L.* These strains, since they are avirulent technically, probably should be called *A. radiobacter.* Because strain Ach-1 initi-

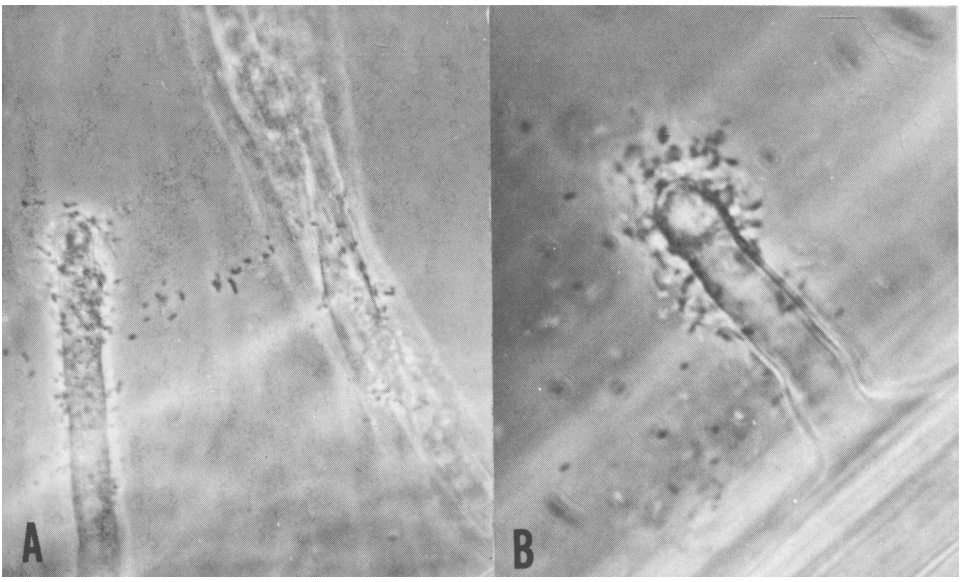

Fig. 2,*A* and *B*. Accumulation of *A. tumefaciens* cells around the root hairs of a tomato plant. *A* also shows a line of bacteria as commonly observed between two hairs.

ally was examined for purity only by streaking several times on a medium, it is possible that the culture was a mixture of *A. tumefaciens* and *A. radiobacter*.

Other organisms tested were ten strains of *A. radiobacter* (table 1), *Rhizobium japonicum,* and *R. meliloti,* and two isolates of *A. rhizogenes.*

Antisera for *A. tumefaciens* strains (CG-1, B-38, B-55, Al-2, and Eu-2) were prepared from rabbits injected with acetone-treated bacterial cells and whole cells, using techniques described by Kabat and Mayer (1961). Serological relations were determined by means of the Ouchterlony (1958) double-gel diffusion test. Titers of the antisera after concentration with $(NH_4)_2SO_4$ were between 3,000 and 5,000.

Results from challenging the antigens from *A. tumefaciens* and *A. radiobacter* strains against antisera of *A. tumefaciens* suggested that *A. tumefaciens* possessed a distinctive antigen. Four to five lines with one dominant line *a* being formed near the antiserum well and another *b* near the antigen well, consistently occurred when challenging *A. tumefaciens* antigen against antisera of *A. tumefaciens.* Some of the lines were broad and could be divided into additional lines, using different techniques. The two exceptions to this serological pattern were with strain B-38 (rose) and Dah–1 (dahlia), as a *b* line was not formed next to the antigen well when when challenged against antiserum from other strains. However, antigen of B-38 produced line *b* when reacted against its homologous antiserum. Antiserum was not prepared for Dah-1; accordingly, the antigen of CG-1 did not produce line *b* when challenged against antiserum of B-38.

The principle difference in the serological pattern between *A. radiobacter* strains, or avirulent strains, and virulent *A. tumefaciens* strains, was that the antigen from none of the avirulent strains produced line *b* when challenged against *A. tumefaciens* antisera. The one exception to this observation was strain Ap-1 (apple) which was isolated from a tumor but which did not produce galls on test plants.

Antigen from *R. meliloti* reacted with antisera of *A. tumefaciens* and produced one precipitin line next to the antiserum well and the other in the middle between the wells. A positive agglutination reaction was previously reported by Graham (1963). *R. japanicum* did not produce a reaction.

Gel-diffusion tests indicated that *A. rhizogenes* was not serologically related to *A. tumefaciens,* since precipitin lines were not formed. Attempts were made to separate the various antigens with Millipore filtration, and with differential extraction using physiological saline. The degree of separation of the antigens was tested by the double gel-diffusion technique previously used. Cells were grown in nutrient broth plus 1.0 per cent glucose, centrifuged, and washed three times with saline. The antigen extracted with each washing was precipitated by acetone and then tested. The separation of antigen was affected by pressure filtration through a 0.22–μ Millipore filter.

Both the saline and Millipore filtration technique gave some separation of antigens. Although the first washing of cells with saline contained portions of all the antigens, the second and third washings contained mostly antigen *b*. This suggests that perhaps this antigen is more closely associated with the cell wall than antigens easily removed. With Millipore filtration, antigens *a* and the middle ones passed through the filter, whereas antigen *b* was mostly retained.

Because of two exceptions to the findings that precipitin line *b* is specific to *A. tumefaciens,* results are inconclusive as to whether or not the virulent *A. tumefaciens* strain can consistently be serologically separated from avirulent strains and more work should be done to ascertain if the exceptions are authentic. Possibly, strain Ap-1 might produce a tumor if inoculated during certain environmental conditions. The fact that antigen of B-38 produced a precipitin line next to the antigen well when challenged against its homologous antiserum—but not against antisera of other *A. tumefaciens* strains—suggests that the correlation between virulence and the formation of the precipitin line next to the antigen well may still hold, but that there may be some variation in the nature of the *b* antigen in virulent strains.

These findings are similar to those of Schnathorst *et al.* (1964) who reported that virulent isolates differed in antigenic structure. They also found that the line next to the antigen well was associated with virulent isolates (J. E. De Vay, personal communication).

PHYSIOLOGICAL TESTS

Physiological tests have not appeared satisfactory for differentiating *A. tumefaciens* from *A. radiobacter.* However, several tests have been cited as useful for identification purposes with the agrobacteria.

The physiology of eight California isolates of *A. tumefaciens* and seven of *A. radiobacter* were compared, using a small number of tests to determine whether there were any gross physiological differences between the two species and to determine variation within the species. The physiological tests used in the study included nitrate reduction (Society of American Bacteriologists, (1957), oxidase test (Stanier *et al.,* 1966), 3-ketoglycoside formation (Bernaerts and De Ley, 1963), and starch hydrolysis (Society of American Bacteriologists, 1957). The production of acid from 17 carbohydrates was tested, using the basal medium described by Hugh and Leifson (1953).

Table 4 shows that virulent and avirulent isolates could not be distinguished on the basis of these tests. The tests also demonstrate the hazard of at-

TABLE 4

ACID PRODUCTION ON VARIOUS CARBON SOURCES AND MISCELLANEOUS REACTIONS BY ISOLATES OF *AGROBACTERIUM TUMEFACIENS* AND *A. RADIOBACTER**

Strains	Arabinose	Arbutin	Galactose	Glucose	Glycerol	Inulin	Lactose	Levulose	Maltose	Mannitol	Mannose	Raffinose	Salicin	Sorbitol	Sucrose	Xylose	Nitrate reduction	3-Keto-glycoside
A. tumefaciens																		
Ach-1	++	++	++	++	++	+	+	++	+++	++	+	+	++	−	++	++	−	+
A1-1	++	++	++	++	++	+	±	++	+++	++	++	+	++	+	++	++	+	+
B-38	++	++	++	++	++	+	+	++	+++	++	+	+	++	+	++	++	+	+
B-48	++	++	++	++	+	+	−	−	+++	++	+	+	++	+	++	++	−	+
CG-1	++	++	++	++	++	+	+++	+	+++	++	+	+	++	+	++	++	+	+
Dah-1	++	++	++	++	++	+	+++	++	+++	++	+	+	++	−	++	++	+	+
P1-1	++	++	++	++	++	+	+	++	+++	++	+	+	++	+	++	++	−	+
R-12	++	++	++	++	++	−	+++	+	+	++	++	+	++	+	+	+	++	−
A. radiobacter																		
Rad-6466	++	++	++	++	++	+	+++	++	+++	++	+	+	++	+	++	++	+	+
Rad-6467	++	+	++	++	++	+	+	++	+++	++	++	+	++	+	++	++	−	+
Red-B	++	++	++	++	++	+	+	++	+++	++	++	+	++	+	++	++	−	+
Red-C	++	++	++	++	++	+	++	++	+++	++	+	+	++	+	++	++	+	+
Red-D	++	+	++	++	++	+++	+	++	+++	++	+	+++	+	+	+	+	+	+
TT-1	++	++	++	++	++	+	+	++	+++	++	+	+	++	−	++	++	−	+
TT-2	+++	++	++	++	++	+	+	++	+++	++	+	+	++	+	++	++	−	+

*The reactions are recorded as ± trace, + weak, and +++ strong.

taching significant value to any particular test, as there was considerable variation among the isolates. The ability to reduce nitrate varied among strains of a species, and slight differences were noted between species. Most strains produced a positive reaction with all carbohydrates tested except glycogen. They all produced a positive oxidase test and a negative starch hydrolysis test. Isolate R-12 was the only strain which did not produce 3-ketoglycoside.

As pathogenicity has been the only basis for placing *A. tumefaciens* and *A. radiobacter* in separate species, these data and those of De Ley (1968) and Graham and Parker (1964) suggest there are no taxonomic data available to merit such separation. Even though there may be some serological differences, they alone cannot be used as a basis for speciation. It is unfortunate that the splitting of *A. tumefaciens* and *A. radiobacter,* as with many bacterial groups, has not been based on comprehensive studies.

DISEASE OCCURRENCE

Disease inception in stone fruit and other trees can occur at any time during the life of the tree as a result of a wound caused by growth, frost cracks, or mechanical injuries. The greatest incidence of disease, however, occurs in the nursery principally during (1) the germination process (as in peaches and almonds) when there is abrasion of radical or epicotyl against the rough seed coat, (2) the harvesting of liners and the subsequent planting in nursery soils, (3) final harvesting of trees for the market when a blade or other device is used to uproot the tree from the soil, and (4) the storing of trees in sawdust-sand healing-in beds. Infection is most serious when it occurs during the harvest period. Although inspectors and nurserymen cooperate to prevent shipping of infected trees, they can eliminate only those having visible symptoms because of their inability to discern diseased trees having incipient infections or which have been contaminated during harvesting. It is not uncommon for a nurseryman to discard 5 to 80 per cent of his trees because of the disease and then to sell the remainder of the so-called healthy trees. It can be safely assumed that if 5 to 80 per cent of the trees were infected and discarded, the apparently healthy, gall-free trees are contaminated, and many will be infected during the harvesting period. If infection does not occur during this process, there is an excellent likelihood that it will occur when trees are placed in healing-in beds. An analysis of the materials used in healing-in beds has shown that an incidence of the organism as high as 500 cells per gram of soil is not uncommon. Fumigants have greatly helped to reduce the amount of infection occurring in healing-in beds, although not eliminating the disease entirely. Some infection will still occur, since the compost becomes contaminated with the pathogen present in incipient galls and in soil clinging to roots of trees placed in the beds. Rigorous procedures used by nurserymen to eliminate the disease, combined with periodic inspection, have greatly reduced the number of diseased plants set in orchards, but until an effective dip treatment is developed there is no practical method of insuring that trees sold will be disease-free. Infection frequently occurs when trees are injured in the orchard during planting. We have often detected the organism in field soil (also, contaminated soil from the nursery usually clings to the roots).

Trees often become infected in the field as a result of injuries caused by cultivation, although this may not adversely affect them (depending upon their age). Crown gall tumors occuring on mature trees generally cause minor

damage to the trees, because the restrictive effect of the tumor on translocation of solutes is small. When young trees are infected, however, the tumors continue to progress as the trees mature, and by maturity the tumor radiates from the center of the tree to the exterior, and in many cases restricts flow of nutrients in the tree. This causes a serious disruption of translocation, especially when the tumor becomes moribund and, as often occurs, is invaded by heartrot fungi.

Because infection can occur during various stages of the life of the tree, it is difficult to state with certainty whether infection of a tree has occurred in the nursery or in the orchard. This has been a topic of controversy among growers and nurserymen, especially when a large amount of crown gall has been detected in young orchards.

CHEMOTHERAPEUTIC CONTROL OF CROWN GALL TUMORS

An effective chemotherapeutic treatment using Bacticin® has been developed that successfully eradicates crown gall tumors from many plants including peach, cherry, almond, pear (Schroth and Hildebrand, 1968), walnut, and rose. Bacticin selectively penetrates and eradicates neoplastic tissues. The critical time to use this material to control the disease is during the first several years of tree growth—therefore examination of the crown region of a plant for tumors should include the area below the soil line, as this is the primary site of infection. Many trees in an orchard may be affected by the disease, although growers may not be aware of it until the vigor of the trees has declined. Examination of the crown should be conducted after the first year of planting, using a method which does not cause injuries; currently, the recommended soil removal procedure is the use of an hydraulic system (Ross *et al.*, 1970).

Exposed crown gall tumors are treated by a liberal application of Bacticin to the entire tumor area, with some overlap onto the surrounding healthy tissues. The tumors should be relatively free of soil. To conserve material, some growers remove most of each tumor with a hatchet, although some reports indicate that treatment is less effective when much gall tissue is removed. The cracks and folds on the rough exterior of a tumor probably help to retain the chemicals, preventing them from flowing into the soil and allowing more to be absorbed by tumor tissue. The tumor should remain exposed at least for several weeks after treatment, since coverage with soil appears to reduce efficacy. Crown gall tumors should be dry at the time of application for better penetration of the oil carrier into the tissues. Although penetration of a tumor 4 inches in diameter occurs within 24 to 48 hours, death may not occur until after 2 or 3 months, or sometimes 4 months in winter.

One application of Bacticin is sufficient to eradicate most tumors. However, tumors over 4½ inches in diameter may sometimes require two applications, especially with pears and cherries where the tumors appear to be more resistant to the toxic action of the hydrocarbons in the material. Extensive testing by growers and farm advisers has shown Bacticin to be highly effective against crown gall on almond, apricot, peach, plum, pear, and cherry. It works equally well against olive knot tumors caused by *Pseudomonas savastanoi.* In 1966, 299 crown gall tumors were treated on stone fruit and pear trees. Of these, 274 were eradicated and 25 had 50 per cent or more of the tumor

tissues killed; most of the 25 that contained live tumor tissue after treatment had been incompletely covered during application (Schroth and Hildebrand, 1968).

When tree crowns are encompassed by tumors, only 33 per cent of the crown area should be covered with Bacticin at one application because complete coverage at one time may damage the tree. However, some browning may occur on healthy tissues adjacent to the tumor. Because translocation of materials occurs through tumorous tissues, their eradication may affect the supply of materials to the adjacent tissues. Also, the tissues immediately surrounding the tumor appear to be more susceptible to the toxic action of the compound, presumably because the disease has some effect on surrounding healthy tissue (Schroth and Hildebrand, 1968). Because high temperatures increase phytotoxicity, it may be necessary to dilute Bacticin by 20 per cent or more under certain climatic conditions.

A long-term effect of using Bacticin to control tumors has been favorable. After 4 years of observation we conclude that Bacticin does not retard callus formation around the tumor site, and that there seldom is evidence of regrowth of the tumor or production of new tumors around the original site.

Crown gall tumors have been treated over the years with other chemicals such as antibiotics (Ark and Sibray, 1957; Dye *et al.*, 1950; Dye, 1952; Sorauer, 1956) and phenolic compounds (Démétriadès, 1953; Phillips, 1895; Sorauer, 1956; Stapp, 1961). The phenolics are highly toxic to both plants and animals and their use has not been cleared by the Food and Drug Administration of the U. S. Department of Health, Education, and Welfare. Of the phenolic materials, sodium dinitro-ortho-cresolate (sold commercially as Elgetol), when mixed with methanol, has been used in treatment of crown galls in California (Ark and Scott, 1951).

FUMIGATION AND SEED TREATMENT

Previous results indicate that *A. tumefaciens* persists for long periods in many soils. Therefore, it would be highly beneficial to reduce or eliminate soil infestation with may crops, such as nursery trees and field-grown roses. Accordingly, several soil fumigants and methods of application were evaluated for the control of crown gall. Because of the possibility that the crown gall bacterium may be introduced into noninfested soil from contaminated plant parts, the effectiveness of treating planting material with disinfectants was also evaluated.

Field-grown Roses

Cuttings used for starting field-grown roses present an ideal infection court for the crown gall bacterium and therefore roses grown in infested soil often have a high level of crown gall infection, with attendant financial loss.

In the first tests, chloropicrin was applied at rates of 1, 2, and 3 ml per linear foot in a single strip down the center of the bed at a depth of 3½ to 4 inches. The bed was immediately covered with black paper through which the cuttings were inserted. Effect of surface treating cuttings with sodium hypochlorite was also evaluated. Treated cuttings were immersed in a 0.5 per cent solution of sodium hypochlorite for 1 minute just before planting. After about 9 months' growth the plants were dug and the percentage of plants with gall was ascertained.

The fumigation reduced crown gall infection but did not effectively control the disease (table 5). However, tumors on plants growing in nontreated soil were much larger than those in treated soil. This may reflect a delayed buildup

TABLE 5

EFFECT OF IN-THE-FURROW FUMIGATION WITH CHLOROPICRIN AND SURFACE TREATMENT OF CUTTINGS WITH SODIUM HYPOCHLORITE ON CROWN GALL IN FIELD-GROWN ROSES

Treatment*	Per cent infected plants‡	
	Plot 1	
Chloropicrin		
1 ml per foot	60.4	
2 ml per foot	56.1	
3 ml per foot	52.5	
Control	83.2	
	Plot 2	
	Cuttings treated	*Cuttings not treated*
Chloropicrin		
3 ml per foot	59.5†	70.6
Control	97.5	93.1

* LSD 5% level = 25.4
† Each value is the mean of 4 replications (approximately 50 plants per replication).
‡ Each value is the mean of 4 replications (approximately 100 plants per replication).

in the bacterial population and later infection. Surface treatment of the cuttings had no effect on disease severity (table 5).

Because of the slight reduction in disease severity obtained with an in-the-furrow fumigation, a second trial was conducted the following year. In this test, solid blocks of plants were treated and then tarped with polyethylene. The fumigant was a mixture of ⅔ chloropicrin and ⅓ methyl bromide applied at a rate of 320 pounds per acre at a depth of 6 inches.

This solid block tarped fumigation resulted in an appreciably greater reduction in disease incidence than did the in-the-furrow application, but still did not result in the desired degree of control (table 6).

These two plots provide an excellent example of seasonal variations of the crown gall disease. The second trial was on the same land as the first, yet disease severity in the controls varied from 83 to 98 per cent of trees infected the first year to 36 per cent the second. This could have been caused by many factors: soil moisture and temperature during rooting, condition of the cuttings, or seasonal changes in the population of the organism.

Almond Seedlings

In this trial, soil fumigation and seed treatment were evaluated, using a split-plot design. The fumigant used was a mixture of chloropicrin (⅔) and methyl bromide (⅓) at rates of 396 and 792 pounds per acre, followed by tarping with polyethylene. The seed treatments were mercuric chloride at 1 ppm plus a wetting agent (3 ounces household detergent per 10 gallons of water for 15 minutes, and 1 per cent sodium hypochlorite plus wetting agent for 15 minutes). Almond seed, previously stratified, was treated on February 25 and planted on March 5 after 4 days of pre-soaking. Trees were pulled and the percentage of crown gall determined on December 17.

Treatment of seed had no influence on the incidence of disease (table 7). Soil fumigation significantly reduced crown gall, but as with the roses, it did

TABLE 6

EFFECT OF SOLID-BLOCK FUMIGATION WITH TARPING ON CROWN GALL OF FIELD-GROWN ROSES

Treatment	Number of plants observed	Number galled	Per cent gall	Per cent reduction
Fumigation-320 lb. per acre*	236	26	11	69
Control	207	75	36	..

* Data based on 5 replications. Material was ⅔ chloropicrin and ⅓ methyl bromide.

not provide a satisfactory level of control (table 8). The failure to satisfactorily control the disease did not appear to be caused by an inadequate rate of fumigant application. Doubling the rate from 396 to 792 pounds per acre did not have any effect on crown gall severity, indicating that this was not the limiting factor.

TABLE 7
EFFECT OF SEED TREATMENT ON CROWN GALL OF ALMOND SEEDLINGS

Treatment	Infected plants
	Per cent
Mercuric chloride—0.1% for 15 minutes	14.7*
Sodium hypochlorite—1% for 15 minutes	15.8
Control	17.5

* Each figure is the mean of 12 replications, at 50 to 250 trees per replication. Difference not statistically significant.

These tests show that soil fumigation can reduce the incidence of crown gall, particularly when solid application and tarping is employed. Even under these conditions, however, the disease was not effectively controlled. It is interesting to note that with two very different hosts, and in two different areas, the percentage of reduction was similar—this suggests that these data provide a good picture of the effect of soil fumigation on crown gall.

TABLE 8
EFFECT OF SOIL FUMIGATION ON CROWN GALL OF ALMOND SEEDLINGS

Treatment	Infected plants	Reduction
	Per cent	*Per cent*
Chloropicrin-methyl bromide		
396 lb per acre	11.2*†	49
792 lb per acre	12.5†	43
Control	21.8	..

* Each figure is the mean of four replications (200 to 600 trees per replication).
† These values differ significantly from the control at the 5% level.

The reasons for this lack of control are not known. The fumigant has been shown in plastic-bag tests to be highly effective against the bacterium (Munnecke and Ferguson, 1960). It also has been effective in controlling *A. rhizogenes,* a related bacterium (Munnecke *et al.,* 1963). However, *A. rhizogenes* is a nutritionaly fastidious organism requiring vitamins, and it may be a poor soil invader and competitor. Our results (table 8) suggest that in the field a low percentage of the *A. tumefaciens* cells survive the treatment, and that the bacterium increases rapidly in fumigated soil to a level which can cause appreciable disease if a susceptible host is planted. The data of Deep *et al.* (1968) and Dickey (1962) support this supposition.

SUMMARY AND CONCLUSIONS

Agrobacterium tumefaciens was isolated from various California soils, using either a selective medium or a carrot discs trap technique. The bacterium appears to survive for long periods in these soils and the population ratio of *A. tumefaciens* to *A. radiobacter* varied from 1:13 to 1:500. Many strains of *A. tumefaciens* aggregated around roots of various seedling plants when the seedlings were placed in water containing bacterial cells. There appeared to be specific sites for aggregation: the root tap zone repulsed bacteria, but root hairs served as an attractant.

Serological studies using the Ouchterlony gel-diffusion test suggested that virulent isolates possessed (in contrast to *A. radiobacter*) a distinctive antigen that formed a precipitin line next to the antigen well. This antigen appeared to be closely associated with the cell wall, and was separable from other

antigens by filtration with a 0.22-μ Millipore filter. Physiological tests proved of no value in differentiating virulent and avirulent agrobacteria.

The occurrence of disease in stone fruits and the application of cultural practices to avoid the disease and a chemotherapeutant for eradication of crown gall tumors are discussed. Fumigation of soil with chloropicrin and methyl bromide reduced disease incidence but did not economically control the disease. Treated nursery soil was subsequently found to harbor *A. tumefaciens* as a result of incomplete eradication, or contamination by wind-blown dust, tools, or irrigation water containing the organism.

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