CROWN GALL OF STONE FRUIT

I. ISOLATION OF *AGROBACTERIUM TUMEFACIENS* AND RELATED SPECIES

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Summary

Two distinct forms of tumour-inducing bacteria have been isolated. One form, readily isolated when macerated galls were plated on Patel's medium, did not grow on Schroth's medium even when antibiotics and other bacterial inhibitors were omitted; nor did it produce 3-ketolactose when grown on lactose agar. It is considered to be the primary cause of crown gall in at least one commercial nursery.

The second form was rarely isolated from galls or from soil surrounding galls. It grew on Schroth's medium and produced 3-ketolactose, and was designated *Agrobacterium tumefaciens*. Its role in the etiology of crown gall is doubtful. *A. radiobacter* inhibited gall induction by *A. tumefaciens* and this is likely to operate in the field, where the ratio of the two species is more than 100:1.

I. INTRODUCTION

The nature of crown gall was established by Smith and Townsend (1907) who showed that the disease is caused by a bacterium which they named *Bacterium tumefaciens*, later changed by Conn (1942) to *Agrobacterium tumefaciens*.

In South Australia, crown gall is widespread and serious, particularly on peach, almond, and plum. It presents considerable problems in nurseries, where disease incidence is often high but varies markedly from year to year. Although only plants without galls are sold by a nursery, the possibility of transporting the causal organism in soil attached to the roots is very real. Yet little is known about the ecology of *A. tumefaciens* or its distribution and abundance in nurseries and orchards, either in South Australia or elsewhere. The main reason for this is the difficulty of distinguishing *A. tumefaciens* from other soil-inhabiting bacteria. Recently, Schroth, Thompson, and Hildebrand (1965) described a selective medium for the *A. tumefaciens–A. radiobacter* group of bacteria, allowing quantitative assessment of these bacteria in soil. Where there is doubt about the identity of a particular isolate, this can be dispelled by a very simple test for 3-ketolactose production (Bernaerts and De Ley 1963).

It was considered that these two techniques would allow a much more detailed study of the ecology and distribution of *A. tumefaciens* than had previously been possible. This paper describes preliminary investigations largely directed towards assessing the suitability of the selective medium described by Schroth, Thompson, and Hildebrand (1965) for isolating *A. tumefaciens* from soil and from galls.

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II. Methods

(a) *Isolation of* Agrobacterium *spp. from Soil*

The soil dilution plate method was used: 0·1 ml of an appropriate soil dilution (usually 1:10 or 1:50) was pipetted on to Schroth's medium in a 9-cm Petri dish and spread evenly over the surface with a sterile L-shaped glass rod. The plates were incubated at 27°C for 3 days and then selected individual bacterial colonies were transferred to nutrient agar slopes.

(b) *Isolation of* Agrobacterium *spp. from Galls*

Galls or parts of galls showing no sign of secondary rotting were removed from naturally infected plants and thoroughly washed in tap water; 0·5–1·0 g of tissue was surface-sterilized in 0·5% sodium hypochlorite for 10 min, washed briefly, and then macerated in a pestle and mortar with 10 ml of sterile distilled water. The macerate was transferred to a sterile Potter–Elvehjem homogenizer and the pestle depressed, giving a supernatant suspension free from large particles. The supernatant was diluted (1:100) with sterile distilled water and plated on Schroth's medium, or, in the later stages of the investigation, on Patel's medium (Patel 1926).

A similar method was used for re-isolation of bacteria from artificially inoculated plants, except that a 3·0-cm length of stem, which included the site of inoculation, was removed and macerated in an electric blender before transferring to a homogenizer.

In both cases, selected colonies were transferred to nutrient agar slopes.

(c) *Identification and Pathogenicity of Isolates*

All isolates were tested for 3-ketolactose production (Bernaerts and De Ley 1963), a quick and specific test for the *A. tumefaciens–A. radiobacter* group of bacteria. To distinguish between *A. tumefaciens* and *A. radiobacter* it is necessary to determine pathogenicity. Isolates were grown on nutrient agar slopes at 27°C for 48 hr. A heavy suspension of bacterial cells was prepared and deposited on young tomato plant stems which were then wounded by an instrument similar to that described by Huisingh and Durbin (1965) but with no cotton wool. Inoculated plants were kept at a temperature of 27°C for 3 days and were then transferred to a glasshouse. Results were recorded after 10 days. Young peach seedlings were inoculated in the same way and also by wounding roots before applying a bacterial suspension. If isolates produced 3-ketolactose, pathogens were designated *A. tumefaciens* and non-pathogens *A. radiobacter*.

III. Results

(a) *Isolations from Soils and Galls*

Eighteen soil samples were collected from the sites of crown gall-infected stone fruit trees in several localities, and 36 samples from various other sites, where no crown gall-infected trees were observed. From infested sites, only one isolate out of 195 tested was pathogenic. No pathogens were isolated from other sites (Table 1).

Because of the infrequent isolation of *A. tumefaciens* from soil, isolations from galls from six different localities were attempted using the same selective medium. From 33 galls, 385 isolates, all ketolactose-positive, were tested for pathogenicity; only five were pathogenic (Table 1).

(b) *Inoculation of Tomato Plants with* A. tumefaciens *and* A. radiobacter

Since differentiation of species is based on pathogenicity, a loss of virulence following infection could explain the difficulty of isolating *A. tumefaciens* from natural galls. Alternatively *A. tumefaciens* could be swamped by *A. radiobacter* after tumour induction. These possibilities were investigated. Tomato plants were

inoculated with *A. tumefaciens* and *A. radiobacter* both separately and mixed in the ratio of 1:3. Galls developed following inoculation with *A. tumefaciens* and with the mixture. Bacteria were isolated from inoculated plants over a period of 8 weeks using three plants per treatment. On each occasion 30 isolates were selected at random from each treatment and tested for pathogenicity. Results are given in

TABLE 1

FREQUENCY OF RECOVERY OF PATHOGENIC ISOLATES FROM GALLS AND FROM SOIL PLATED ON SCHROTH'S MEDIUM

	Source		
	Galls	Soil Surrounding Galls	Other Soils
No. of samples	33	18	36
No. of isolates	385	195	88
No. of pathogenic isolates	5	1	0
No. of samples yielding pathogenic isolates	2	1	0

TABLE 2

NUMBER (OUT OF 30) OF PATHOGENIC ISOLATES FROM TOMATO STEMS INOCULATED WITH *A. TUMEFACIENS*, *A. RADIOBACTER*, OR A MIXTURE OF BOTH (IN THE RATIO 1:3)

	No. of Pathogenic Isolates at Various Weeks after Inoculation:								Total Pathogenic Isolates
	0	1	2	3	4	5	6	8	
Inoculated with:									
A. tumefaciens	29	30	30	28	29	25	28	30	229
Mixture	3	7	6	6	9	9	9	7	56
A. radiobacter	0	0	0	0	0	0	0	0	0

Table 2. Of 240 isolates tested for each treatment, plants inoculated with *A. tumefaciens* yielded 229 pathogens, those inoculated with the mixture, 56 pathogens, and those inoculated with *A. radiobacter*, none. It can be concluded that a marked change in pathogenicity is unlikely to occur following inoculation and that *A. tumefaciens* is not swamped by *A. radiobacter* after tumour induction.

Lippincott and Lippincott (1967) have reported that the presence of non-pathogenic isolates of *A. tumefaciens* restricts tumour induction by pathogens, presumably by competing for infection sites. As the ratio of pathogens to non-patho-

gens in infested soil appears to be less than 1:100, the possibility of interference was investigated. Bacterial suspensions of *A. tumefaciens* and *A. radiobacter* were mixed to give a ratio of 1:100 and then serially diluted. The pathogenicity of the mixture was compared with that of a pure culture of *A. tumefaciens* also serially diluted. Numbers of bacteria were determined by plating before inoculation. Young tomato plants were wounded in four places with a blunt needle and 0·05 ml of bacterial suspension deposited on each wound. Stem diameters at inoculation sites were measured after 5 weeks, and the results are shown in the following tabulation:

No. of *A. tumefaciens* cells per wound	$12{\cdot}5\times10^6$	$12{\cdot}5\times10^4$	$12{\cdot}5\times10^2$	12·5	0
Mean diam. (cm)* of tomato stems following inoculation with:					
A. tumefaciens	$1{\cdot}61^1$	$1{\cdot}39^2$	$1{\cdot}08^3$	$0{\cdot}79^4$	$0{\cdot}65^5$
Mixture†	—	$0{\cdot}83^4$	$0{\cdot}69^5$	$0{\cdot}64^5$	$0{\cdot}65^5$

* Means with different superscript numbers are significantly different at the 1% level.
† *A. tumefaciens* and *A. radiobacter* in the ratio 1:100.

The presence of *A. radiobacter* markedly restricted or completely inhibited tumour development, depending on the number of *A. tumefaciens* cells per wound. These results confirm those of Lippincott and Lippincott (1967) who showed that the inhibition is fairly specific; unrelated bacteria and some strains of *Agrobacterium* failed to inhibit. This might explain why Beaud, Manigault, and Stoll (1963) found no evidence of inhibition.

(c) *Pathogenicity of Isolates to Peach and Tomato*

These results cast doubt on the value of Schroth's medium for isolating *A. tumefaciens* both from soil and from galls though there is the further possibility that isolates from stone fruit may not be pathogenic to tomato. Six pathogenic and 100 non-pathogenic isolates, obtained during the present investigation, as well as 12 pathogenic isolates supplied from other laboratories within Australia, were inoculated into both tomato and peach plants. All isolates pathogenic to tomato induced crown gall development on peach stems and roots; all other isolates were non-pathogenic to both tomato and peach.

(d) *Pathogenicity of Bacteria Isolated on Schroth's and Patel's Media*

It would seem that the most likely explanation for the infrequent isolation of tumour-inducing bacteria from galls, or from soil surrounding galls, is that the medium described by Schroth, Thompson, and Hildebrand (1965) is not suitable. This was investigated by plating galls on both Schroth's and Patel's media and determining the pathogenicity of isolates. Young galls were collected on three separate occasions from peach and plum seedlings in a commercial nursery, macerated, diluted 1:1000, and plated on both media. Isolates were obtained from individual colonies on each medium and tested for 3-ketolactose production and for pathogenicity to tomato (Table 3). Of 112 isolates from Schroth's medium, none were pathogenic, although all produced 3-ketolactose. Of the 117 isolates from Patel's medium, 85 were patho-

genic. Of the non-pathogens, approximately half produced 3-ketolactose and can be designated *A. radiobacter*; the remainder have been identified as *Enterobacter* sp. (Hayward, private communication) and were readily distinguishable from the pathogens by cultural appearance. The inability of the pathogenic isolates to grow on Schroth's medium was confirmed by plating the bacteria on this medium, when no growth was observed. Nor will they grow on Schroth's basal medium which lacks antibiotics and other bacterial inhibitors. These isolates are tentatively designated *Agrobacterium* sp. and six were tested for pathogenicity to peach. All induced crown gall development.

TABLE 3

3-KETOLACTOSE PRODUCTION AND PATHOGENICITY OF BACTERIAL ISOLATES FROM GALLS PLATED ON SCHROTH'S AND PATEL'S MEDIA

No. of Isolates	Isolation Medium	3-Ketolactose	Pathogenicity
112	Schroth	+	−
17	Patel	+	−
85	Patel	−	+
15	Patel	−	−

IV. DISCUSSION

Crown galls collected from one nursery were plated on both Schroth's and Patel's media. As tumour-inducing bacteria were isolated only on Patel's medium, it seems clear that Schroth's medium is not suitable for the isolation of these bacteria. That this applies to samples from other areas is suggested by the very infrequent isolation on this medium of pathogenic bacteria from 33 galls taken from six different localities.

Nomenclature of the isolates deserves comment. Pathogenic isolates were designated *A. tumefaciens* and non-pathogens *A. radiobacter* if they could grow on Schroth's medium and produce 3-ketolactose when grown on lactose agar. This is a very arbitrary identification but is nevertheless very convenient for distinguishing these isolates from others, tentatively designated *Agrobacterium* sp., which can induce crown gall but cannot produce 3-ketolactose or grow on Schroth's medium even when antibiotics and other bacterial inhibitors are omitted. Final identification awaits detailed taxonomic study, but all three types would be included in phenon 1 of Thornley (1967) along with other agrobacteria (Hayward, private communication). It is relevant that De Ley *et al.* (1966) found that 4 of 28 strains of *A. tumefaciens* did not produce 3-ketolactose.

Both *A. tumefaciens* and the *Agrobacterium* sp. produce identical symptoms when inoculated into tomato or peach. The frequent isolation of *Agrobacterium* sp. would suggest that it is the primary cause of crown gall in at least one nursery.

The role of *A. tumefaciens* is puzzling. It is rarely isolated from galls and this cannot be explained by a loss of virulence following infection, because when plants were inoculated, more than 95% of the re-isolates obtained over a period of 8 weeks were pathogenic. Nor was *A. tumefaciens* swamped by *A. radiobacter* when a mixture of the two bacteria in the ratio 1:3 was inoculated into tomato. The ratio of the two organisms before, and for 8 weeks after, inoculation remained relatively constant. The evidence indicates that the infrequent isolation of *A. tumefaciens* from natural samples reflects its low numbers in the field.

In soil, the ratio of *A. tumefaciens* to *A. radiobacter* is less than 1:100, but when these organisms are mixed in this ratio and then inoculated into tomato stems, gall formation is either completely inhibited or markedly reduced compared with that induced by the same number of *A. tumefaciens* cells in pure culture. In other words, *A. radiobacter* inhibits gall induction by *A. tumefaciens*. As soil is the only source of inoculum for natural infection of plants, these results suggest that *A. tumefaciens* does not cause natural infection. Nevertheless *A. tumefaciens* was isolated from two galls. It is possible that there are local pockets in the soil where the ratio of *A. tumefaciens* to *A. radiobacter* is greater than 1:100. Another possible explanation is that there is a transfer of virulence from the *Agrobacterium* sp. to *A. radiobacter*, changing the latter into *A. tumefaciens*. If this were a relatively rare occurrence, it could explain the low numbers of *A. tumefaciens* in the field.

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