STUDIES ON CROWN GALL

by

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CONTENTS

	Page
SUMMARY	vi
STATEMENT	ix
ACKNOWLEDGMENTS	x
PART A: LITERATURE REVIEW	
DESCRIPTION OF CROWN GALL	1
THE CAUSAL AGENT OF CROWN GALL	2
Taxonomy	2
Nutrition and Biochemical Tests	8
Host Range and Virulence	11
Interactions Between Strains	14
(a) Tumour stimulation	14
(b) Inhibition of tumour induction	16
Genetic Transfers	18
TUMOUR INDUCTION	22
Sequence of Events in Tumour Induction	22
The Nature of TIP	25
(a) Plant growth hormones	25
(b) Bacterial nucleic acids	27
(c) Temperate bacteriophage	33
THE ECOLOGY OF AGROBACTERIUM TUMEFACIENS	37
Ecology and Crown Gall Incidence	37
Isolation of a New Tumour Inducing Organism	41

Page

ECONOMIC ASPECTS 43

Losses Due to Crown Gall 43

Disease Control 45

PART B: IDENTIFICATION AND NOMENCLATURE OF AGROBACTERIUM ISOLATES

INTRODUCTION 48

CULTURES TESTED 49

BIOCHEMICAL TESTS 49

PATHOGENICITY OF ISOLATES 50

SEROLOGY 53

Preparation of Antigens 53

Preparation of Antisera 53

Tube Agglutination Test 54

Gel Diffusion Test 55

Fluorescent Antibody Staining 55

(a) Preparation of fluorescein conjugate 55

(b) Staining - direct method 56

(c) Microscopy 56

(d) Staining by the indirect method 58

ELECTROPHORESIS 60

Growing Bacteria for Electrophoresis 61

Disruption of Bacteria 62

Starch Gel Electrophoresis 63

Polyacrylamide Disc Electrophoresis 64

Page

Evaluation of Protein Patterns 65

Replication 66

Results 67

FLAGELLATION 68

DISCUSSION 70

PART C: DEVELOPMENT OF A SELECTIVE MEDIUM FOR A. RADIOBACTER BIOTYPE 2

INTRODUCTION 75

BASIS OF DIFFERENTIAL SELECTION BETWEEN BIOTYPES 1 AND 2 75

DEVELOPMENT OF A SELECTIVE MEDIUM BASED ON ERYTHRITOL 79

PROPERTIES AND USE OF THE SELECTIVE MEDIUM 85

Isolation From Galls and Soil 85

Growth of Other Organisms 87

Reliability of Recovery 87

PART D: THE ECOLOGY OF A. RADIOBACTER BIOTYPE 2 AND EPIDEMIOLOGY OF CROWN GALL

INTRODUCTION 90

THE NURSERY 91

ABUNDANCE OF A. RADIOBACTER BIOTYPE 2 AROUND GALLED AND HEALTHY TREES 92

Methods 92

(a) Sampling 92

(b) Plating 94

(c) Pathogenicity of isolates 94

Page

Experimental 95

(a) Balhannah - almond seedlings 95

(b) Other properties 97

EFFECT OF PATHOGEN TO NON-PATHOGEN RATIO ON GALL INDUCTION 98

Standard Methods of Wounding and Inoculation 98

(a) Tomato 98

(b) Peach 98

Artificial Inoculation of Soil 99

Experimental 99

PROPORTION OF PATHOGENS IN NATURAL GALLS 102

GALL INDUCTION IN NATURALLY INFESTED SOIL 103

INHIBITORY EFFICIENCY OF OTHER NON-PATHOGENIC ISOLATES 104

BIOLOGICAL CONTROL 105

SURVIVAL OF A. RADIOBACTER BIOTYPE 2 109

Survival Under Pasture 110

Survival in the Rhizosphere 110

Survival in Fallow Soil 113

DISPERSAL OF THE CROWN GALL ORGANISM 115

DISCUSSION 117

Epidemiology 117

Survival and Dispersal of the Crown Gall Organism 121

Biological Control 122

Mechanism of Biological Control 123

Page

APPENDICES

APPENDIX 1. Culture Collection of the Waite Institute Plant Pathology Department: Isolate Numbers 128

APPENDIX 2. Composition of Media 129

APPENDIX 3. Composition of Selective Media for *Agrobacterium* and *Rhizobium* 133

APPENDIX 4. Publications 136

REFERENCES 137

SUMMARY

Thirty-six local isolates and twenty-six named Agrobacterium isolates supplied by other laboratories were compared by biochemical tests, serology, protein patterns following gel electrophoresis, and by pathogenicity. All but one of the isolates fell into two distinct biotypes, and this was supported by their serological reactions and by their protein patterns. Both biotypes contained tumour-inducing, root-proliferating and non-pathogenic forms.

Since some pathogenic and non-pathogenic isolates were indistinguishable by physiological tests, by serology or by their protein patterns, the present division of the genus into species based on pathogenicity seems of little value. A new classification is proposed (Keane, Kerr & New, 1970): the genus Agrobacterium should be reduced to one species, A. radiobacter, with pathogenicity indicated by a varietal epithet and the biotype specified.

Available evidence indicates that A. radiobacter var. tumefaciens biotype 2 is largely responsible for crown gall in at least two South Australian nurseries (Kerr, 1969; New & Kerr, 1971). A selective medium was developed to allow a quantitative estimation of numbers of biotype 2 agrobacteria in soil and in crown gall tissue. Tumour-inducing and non-pathogenic forms of biotype 2 grew on this medium, which was used to study the ecology of A. radiobacter biotype 2.

Pathogenic forms of A. radiobacter biotype 2 were present in

soil around galled stone fruit trees, where they formed a significant proportion of the total biotype 2 population, but pathogens were rarely detected around healthy plants.

Tomato and peach seedlings were inoculated with mixtures of pathogenic and non-pathogenic biotype 2 isolates, to see whether the non-pathogens would inhibit tumour induction. Five isolates prevented gall induction when present in approximately equal numbers to pathogens, but eleven other isolates were not inhibitory. In the field, gall induction probably depends on the number and proportion of pathogens at a wound site and on the inhibitory efficiency of the non-pathogens. One inhibitory isolate was used to control crown gall biologically, and gave complete protection against the disease in a glasshouse experiment.

The survival and dispersal of biotype 2 agrobacteria was also studied. A natural population of pathogenic and non-pathogenic A. radiobacter biotype 2 survived 22 weeks in fallow soil in undiminished numbers, and non-pathogens could still be isolated after 61 weeks. The data does not show any marked difference in survival ability between pathogens and non-pathogens. Non-pathogens were stimulated by the roots of a variety of grasses and weeds, and were isolated from soil kept under pasture for four years, but there is no comparable data for pathogens.

Evidence is presented that pathogenic A. radiobacter biotype 2 is spread to commercial orchards on the roots of healthy stone fruit trees supplied by a crown gall-infested nursery.

The mechanism of tumour inhibition is discussed, and a theory presented to explain tumour inhibition, tumour induction and the acquisition of virulence by Agrobacterium isolates.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text.

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PART A: LITERATURE REVIEW

DESCRIPTION OF CROWN GALL

Crown gall is a disease characterized by the development of distinctive tumorous outgrowths from affected plants. The galls commonly develop at the crowns of plants, but also frequently on the roots and sometimes on the above-ground parts of the stem and the leaves. Crown gall affects a wide range of dicotyledons (Elliott, 1951) and is a serious problem on a number of commercial plants, including stone fruit trees.

It is caused by a rod-shaped, gram-negative bacterium, Agrobacterium tumefaciens (Smith & Townsend) Conn, which can only infect the plant via wounds. Infected plant cells become independent of the growth-regulatory mechanisms of the plant, and continue to proliferate in a disorganized and completely uncontrolled manner.

E.F. Smith (1912) found that certain host plants, such as Paris daisy, produce primary galls at the initial site of infection, and secondary galls sometimes develop on other parts of the plant. These secondary galls have the same capacity for unlimited growth as primary galls, to which they are sometimes connected by a strand of tumorous tissue, called a "tumour strand". Under certain conditions, Smith (1916) found that A. tumefaciens could induce abnormal growths with some degree of differentiation, intermediate between completely disorganized tumours and normal plant tissue. These were named

teratomas, by analogy with similar neoplastic growths in animals.

His discovery of primary and secondary tumours and tumour strands, structures which are typical of animal cancers, led Smith (1912) to exclaim that crown gall and animals tumours "... have a good deal in common, so much, in fact, that I believe we have in these particular plant overgrowths a key to unlock the whole cancer situation". Today, nearly sixty years later, his far-sighted view still prevails and research workers continue to probe the mysteries of crown gall induction, with a view to a better understanding of cancerous changes in general.

THE CAUSAL AGENT OF CROWN GALL

Taxonomy

The genus Agrobacterium Conn is one of the three genera of the family Rhizobiaceae, as listed in Bergey's Manual (6th edition: Breed, Murray & Hitchens, 1948; 7th edition: Breed, Murray & Smith, 1957); the others are Rhizobium Frank and Chromobacterium Bergonzini. The characteristics of the family are rod-shaped cells without endospores, the cells being sparsely flagellate (1 polar or lateral flagellum, or 2-4 peritrichous flagella) or non-motile, and usually gram-negative. The bacteria utilize glucose oxidatively. No gas and no appreciable amount of acid are formed on glucose and other carbohydrate media.

Chromobacterium is the only pigmented group within the Rhizobiaceae. On the basis of its DNA base composition and low DNA homology with other members of the Rhizobiaceae, Heberlein, De Ley & Tijtgat (1967) recommended that Chromobacterium be removed from the family.

Conn (1942) created the genus Agrobacterium to bring together Alcaligenes radiobacter and Phytomonas tumefaciens and Phytomonas rhizogenes. Bergey's Manual (7th edition: Breed et al., 1957) now lists seven species in the genus, the three original species plus A. rubi, A. gypsophilae, A. pseudotsugae and A. stellulatum. Recent work confirms that the first four species belong in the same genus, if not in the same species, but the last three should probably be excluded (De Ley, 1968).

Agrobacterium tumefaciens (Smith & Townsend, 1907) Conn 1942 is the type species for the genus. It was originally named Bacterium tumefaciens (Smith & Townsend, 1907) and has also been known as Pseudomonas (Stevens, 1913) or Bacillus tumefaciens (Holland, 1920). In the first edition of Bergey's Manual (Bergey et al., 1923) it was placed in the genus Phytomonas Bergey et al., which was a conglomeration of all plant pathogenic bacteria.

Agrobacterium radiobacter (Beijerinck & van Delden, 1902) Conn 1942 is a soil saprophyte and has long been recognized as being

closely related to A. tumefaciens. Riker et al. (1930), in presenting their reasons for a taxonomic division based on pathogenicity, noted that Phytomonas tumefaciens would have to be included in Bacillus radiobacter (= Agrobacterium radiobacter) if any other criteria were used. Subsequent research has confirmed that A. radiobacter is essentially identical to A. tumefaciens in its physiology (Sagen, Riker & Baldwin, 1934; Hendrickson, Baldwin & Riker, 1934; Riker, Lyneis & Locke, 1941; Starr, 1946; Huisingh & Durbin, 1967). Recent serological studies (Graham, 1963, 1971), comparisons of DNA base composition (De Ley et al., 1966) and DNA homology (Heberlein et al., 1967) support that conclusion.

The hairy-root disease of apple trees, which is characterized by abnormal root proliferation from infection sites, is caused by Agrobacterium rhizogenes (Riker et al., 1930) Conn 1942. Like A. tumefaciens, it can only infect the plant via wounds (Hildebrand, 1934).

Although quite similar to A. tumefaciens and A. radiobacter, the hairy root organism may be easily distinguished by physiological tests (Riker et al., 1930; Sagen et al., 1934; Hendrickson et al., 1934; Riker et al., 1941; Huisingh & Durbin, 1967). Its DNA base composition falls within the range of these Agrobacterium species (De Ley et al., 1966), but it shows slightly greater DNA homology with Rhizobium leguminosarum than with A. tumefaciens (Heberlein et al.,

1967).

Agrobacterium rubi (Hildebrand, 1940) Starr & Weiss 1943 is the cause of cane gall. It was distinguished from A. tumefaciens by its limited host range; it only infects plants belonging to the genus Rubus (Pinckard, 1935; Hildebrand, 1940). However, the restricted host range of the pathogen is in some doubt now; some presumed A. rubi isolates have produced galls on bean (Klein & Kelin, 1953; Lippincott & Lippincott, 1969a), broad bean and sunflower (Coleman, 1950) and carrot discs (Lippincott & Lippincott, 1969a). Banfield (1930), Pinckard (1935) and Hildebrand (1940) found that the cane gall organism differed physiologically from A. tumefaciens. Moffett & Colwell (1968) included two isolates of A. rubi in their Adansonian analysis of the Rhizobiaceae and concluded that A. rubi should remain a separate species.

On the other hand, McKeen (1954) found that eight isolates from galls on cane-fruit plants ranged from those with physiological properties typical of A. rubi, to those typical of A. tumefaciens. Pathogenicity of all these isolates was not restricted to Rubus spp. He concluded that A. rubi is merely a form of A. tumefaciens with a limited host range, a conclusion reached also by De Ley et al. (1966) and Heberlein et al. (1967) as a result of their DNA analyses and DNA hybridization experiments.

Agrobacterium gypsophilae (Brown, 1934) Starr & Weiss 1943 causes galls on Gypsophila paniculata L. and closely related plants, but is not pathogenic to many plants susceptible to crown gall.

Maas Geesteranus & Barendsen (1966) have extended its known host range to include rose, carnation and a cactus. The "spongy gall" induced by A. gypsophilae is quite different from crown gall, in that the bacteria are very numerous inside spongy galls and are seen inside the plant cells and in water-soaked areas within the gall (Brown, 1934). In contrast, crown gall tissue contains low numbers of A. tumefaciens, by comparison with other bacterial diseases of plants, and there are no water-soaked areas (Smith, 1911).

It is doubtful whether A. gypsophilae should remain in the genus. De Ley et al. (1966) examined one A. gypsophilae isolate and found that the percentage of guanine plus cytosine (% GC) in the DNA lay well below the range for Agrobacterium. Moffett & Colwell (1968) included a different A. gypsophilae isolate in their numerical taxonomic study, and concluded that the species should be removed to the Enterobacteriaceae.

Agrobacterium pseudotsugae (Hansen & Smith, 1937) Savulescu 1947 causes slow-growing galls on Pseudotsuga taxifolia (Douglas fir) and P. macrocarpa (Hansen & Smith, 1937; Smith, 1940). All recent studies have led to the conclusion that A. pseudotsugae is unlike all other Agrobacterium species and should be removed from the genus (De Ley et al., 1966; Heberlein et al., 1967; Moffett & Colwell, 1968).

Agrobacterium stellulatum Stapp & Knösel 1954 is a marine

bacterium included in the genus mainly because it forms star-like clusters of cells in certain media. The formation of stars is characteristic of the other Agrobacterium species except for A. gypsophilae and A. pseudotsugae, but is also seen in other genera such as Rhizobium, Phyllobacterium, Chromobacterium, Xanthomonas and Pseudomonas (Knösel, 1962). De Ley et al. (1966) found that the DNA base composition of A. stellulatum lies outside the range for Agrobacterium, and suggested it be included with the marine pseudomonads.

If the doubtful species are removed, three pathogenic and one saprophytic species remain in the genus Agrobacterium. A. tumefaciens and A. radiobacter almost certainly should be amalgamated, and possibly A. rubi should be included with them. Most workers believe that A. rhizogenes should be retained as a separate species.

De Ley (1968) has suggested the following revision of the genus, which he includes in Rhizobium. This is not the place to discuss the advisability of combining the genera; suffice it to say that there is both caution ('t Mannetje, 1967) and support (Graham, 1964; Moffett & Colwell, 1968) for the move.

De Ley's proposed genus Rhizobium (simplified):

A. Small, motile rods with up to 6 peritrichous flagella; DNA base composition 58 to 63.5% GC.

a. Root nodule bacteria - R. leguminosarum : 59 to 63.5% GC
R. meliloti : 62 to 63.5% GC

b. Causes hairy root disease on apple and other plants -
R. rhizogenes : 61 to 63% GC
(Note: former Agrobacterium rhizogenes)

c. Frequently producing galls on angiosperms. Most strains produce 3-ketoglycosides : 59.5 to 63% GC
R. radiobacter : non-pathogenic
R. radiobacter var. tumefaciens : crown gall bacteria
(Note: pools the former Agrobacterium tumefaciens, A. radiobacter and A. rubi)

B. Small, motile rods with 1 subpolar flagellum; usually slow-growing; root nodule bacteria; 59.5 to 65.5% GC.
R. japonicum

Nutrition and Biochemical Tests

Agrobacterium tumefaciens and A. radiobacter have very simple nutrient requirements; most isolates will grow in a medium containing essential minerals, including inorganic nitrogen, either ammonium or nitrate, and a suitable source of organic carbon (Sagen et al., 1934; Riker et al., 1941; Starr, 1946). A. rhizogenes and A. rubi cannot utilize inorganic nitrogen; they require L-glutamic acid as the nitrogen source and the following growth factors: biotin for A. rhizogenes; biotin, nicotinic acid and pantothenic acid for A. rubi (Starr, 1946). The growth factor requirements of A. rhizogenes and A. rubi were not accurately known before publication of Starr's paper, but most workers supplied them in the form of yeast extract.

The different minimal nutrient requirements seemed a promising means of distinguishing some of the Agrobacterium species, but since then, naturally-occurring forms of A. tumefaciens with vitamin requirements have been isolated. Braun (1950) isolated strains from a Euonymous gall which required biotin, nicotinic acid and calcium pantothenate, and Demaree & Smith (1952) obtained isolates from blueberry galls which needed biotin and nicotinic acid for growth on a medium containing nitrate.

Lippincott & Lippincott (1969a) found that a number of isolates of A. tumefaciens required one or more of the vitamins biotin, calcium pantothenate and nicotinic acid for growth on a sucrose-mineral salts medium; some also required glutamic acid. Of these isolates, at least one (ICPB: TT133) is identical with the new group of tumour-inducing bacteria isolated by Kerr (1969) and examined in detail by us (Keane, Kerr & New, 1970; this thesis).

There is no correlation between nutritional requirements and pathogenicity of Agrobacterium isolates; A. radiobacter is non-pathogenic, yet has identical requirements to A. tumefaciens, as has at least one attenuated A. tumefaciens strain (Riker et al., 1941). However, there is some data suggesting that virulence of auxotrophic mutants and vitamin-requiring isolates is limited by lower bacterial growth rates in wounds (Manigault & Beaud, 1966; Lippincott & Lippincott, 1969a; Schmidt, Lippincott & Lippincott, 1969); in some

cases, virulence could be increased by adding vitamins or the deficient chemical to inoculated wounds.

There have been many biochemical studies of the various Agrobacterium species, including those by Riker et al. (1930); Muncie & Suit (1930), Sagen et al. (1934), Hendrickson et al. (1934), Pinckard (1935), Hildebrand (1940), McKeen (1954), Graham & Parker (1964), Huisingh & Durbin (1967) and Moffett & Colwell (1968). As mentioned earlier, A. tumefaciens could not be reliably distinguished from A. radiobacter, but was easily distinguished from A. rhizogenes. Some A. rubi isolates were quite distinct, but others seemed to grade into A. tumefaciens.

I do not propose to discuss in detail the results of the various tests, save to mention the 3-ketoglycoside test. A description and discussion of some biochemical tests and their results may be seen in Keane et al. (1970) (Appendix 4).

The production of 3-ketoglycosides from disaccharides and bionic acids is a reaction which is apparently limited to A. radiobacter and A. tumefaciens, and does not occur in any other class of bacteria. (Bernaerts & De Ley, 1963, 1967). A simple, specific test has been devised for these bacteria (Bernaerts & De Ley, 1963): Cultures are grown for one or two days on lactose-yeast extract agar, then the Petri dish is flooded with Benedict's solution. The 3-ketolactose

reduces the reagent at room temperature, precipitating a yellow ring of cuprous oxide around ketolactose-positive colonies within one hour.

Since the discovery of the 3-ketolactose test, occasional ketolactose-negative isolates of A. tumefaciens have been noted (De Ley et al., 1966; Lippincott & Lippincott, 1969a). Kerr (1969) isolated ketolactose-negative agrobacteria from galls, and concluded that they were the major tumour-inducing form in at least one South Australian stone fruit nursery. He tentatively designated these isolates Agrobacterium sp. This thesis will consider the relationship between Agrobacterium sp., A. radiobacter, A. tumefaciens, A. rhizogenes and A. rubi.

Host Range and Virulence

The species A. tumefaciens is composed of a heterogeneous group of strains which differ from one another in host range and virulence. Many workers have reported strains with limited host ranges (e.g. Stapp, 1940; Braun, 1950; Demaree & Smith, 1952) and Wormald (1945) has divided the British isolates of A. tumefaciens into four races, depending on host range.

A. tumefaciens isolates show appreciable variation in the size and vigour of the galls they induce, and bioassay methods have been developed in the hope of expressing bacterial virulence in terms of a measurable plant response. Several methods involve measuring

growth of a gall which has been induced in a standard wound by a standardized inoculum. Klein & Tenenbaum (1955) inoculated discs of carrot phloem tissue; Beaud & Manigault (1964) used Datura plants with a cylindrical wound in the stem, and Kurkdjian, Manigault & Beardsley (1969) inoculated the cut surface of decapitated pea seedlings.

Lippincott & Heberlein (1965) developed a leaf assay method, similar to that used in infectivity studies of mechanically transmissible viruses: The primary leaves of Pinto bean seedlings (Phaseolus vulgaris L.) are wounded by gently rubbing with carborundum powder, and a standard volume of inoculum is spread over them. The number of leaf tumours induced is proportional to the number of bacteria in the inoculum and to their "specific infectivity", which is the number of galls induced per bacterial cell and is of the order of 10^{-7} (Heberlein & Lippincott, 1967).

This bioassay system has been used to study the effect of certain treatments on bacterial virulence, and to investigate the inhibition and stimulation of tumour initiation by mixed inocula. It is not clear just what relationship exists between "specific infectivity" and bacterial virulence and the growth rate of galls.

Attenuated isolates seem to lie at the lower extreme in virulence; they do not induce tumours except in the presence of growth

hormones supplied by another gall on the same plant or artificially applied to the inoculation site (Locke, Riker & Duggar, 1938; Braun & Laskaris, 1942). Attenuated cultures can arise spontaneously from virulent cultures (Hendrickson et al., 1934), or may be artificially produced by successive transfers on media containing glycine (Longley et al., 1937) or certain other amino acids (Van Lanen, Baldwin & Riker, 1940). Some attenuated cultures are stable, but others gradually regain virulence, following successive sub-culturing on a medium without the attenuating chemical; the permanence of attenuation is partially dependent on the number of transfers in the amino acid medium after attenuation.

Stapp (1951) reported that two A. tumefaciens isolates completely lost their virulence towards certain host plants, following successive sub-culturing on α-alanine or leucine, but were still weakly or even fully virulent towards other hosts. Although the mechanism of attenuation is not known, this report suggests the interesting possibility that tumour induction is mediated by a number of tumour inducing principles within the bacterial cell, each with a certain host specificity and capable of being lost independently of the others (Braun, 1954).

Interaction Between Strains

(a) Tumour stimulation

When two pathogenic strains or a pathogenic and a non-pathogenic strain of Agrobacterium are applied to the same wound, the resulting tumour is often larger and faster growing than tumours induced by either strain alone. Beaud, Manigault & Stoll (1963) reported such a tumour stimulation when a pathogenic strain was mixed with a non-pathogen before inoculation, or added to the wound 12 hours later. Manigault (1970) observed greater tumour growth when 1 : 1 mixtures of certain virulent and avirulent cultures were added to decapitated pea seedlings.

Lippincott & Lippincott (1969b) mixed pairs of isolates and inoculated them onto Pinto bean leaves. They found that certain pairs, consisting of a pathogen and either a pathogen, a non-pathogen or a root-proliferating form, induced faster growing tumours than when inoculated separately. They ascribed the stimulation to the production of a "tumour growth factor" (TGF) by the host plant under the influence of one member of the pair, resulting in stimulation of the galls produced by the other member. Manigault (1970) explained his observed stimulation in a similar way. There is some evidence linking TGF with the amino acid derivatives lysopine and octopine (Lippincott & Lippincott, 1970a).

Another kind of stimulatory effect of mixed inocula has been

reported by Lippincott & Lippincott (1970b), who observed an increase in the number of tumours initiated. The phenomenon only occurred at total inoculum densities of less than 10^9 cells/ml. They suggested that tumorigenic "donor" strains produce, or induce in the host, a substance which diffuses through the leaf to conditionally tumorigenic "receptor" strains, enabling them to induce tumours. The mechanism of increased tumour initiation is not identical with the mechanism of increased tumour growth, as there is not complete correspondence between the lists of TGF-positive isolates and "donor" strains, or between TGF-negative isolates and "receptor" strains.

From these data, it is not possible to determine which phenomenon observed by Lippincott & Lippincott corresponds with the stimulation observed by Beaud et al. (1963) and Manigault (1970). With comparatively large wounds, increased gall growth could be due to more cells being transformed, or to increased growth of the same number of tumour cells, or to both.

There have been reports of induction of more rapidly growing tumours by mixtures of virulent and attenuated cultures (Beaud & Manigault, 1964; Manigault, 1970). At least some of the increased growth may be due to multiplication of tumour cells transformed by the attenuated strain, in the presence of growth hormones from the virulent strain or from plant cells transformed by the virulent strain.

(b) Inhibition of tumour induction

Using their leaf bioassay technique Lippincott & Lippincott (1969c) found that certain avirulent or root-proliferating Agrobacterium isolates reduced the number of galls induced by a virulent strain, when the isolates were mixed. The inhibition was fairly specific; no other genera could inhibit gall induction, and some Agrobacterium isolates were not inhibitory. However, heat-killed cells (60°C, 20 min.) were equally effective in inhibiting tumour induction, which suggests a passive function of the inhibitor cells, perhaps linked with the surface properties of the cells.

Lippincott & Lippincott's mathematical analyses led them to propose that virulent and avirulent cells were competing for infection sites; if a non-pathogen cell attached to a site, it blocked a pathogen cell from inducing a tumour in that wound. According to Lippincott & Lippincott there was only one infection site in each wound.

Where larger wounds are used, many infection sites would be expected. Manigault & Beaud (1967) calculated that there were between 10^5 and 10^6 infection sites in their standard cylindrical wounds in Datura stems. In similar wounds to this, Kerr (1969) reported complete inhibition of tumour development by non-pathogenic agrobacteria present in a ratio of 100 non-pathogens to 1 pathogen, and Schilperoort (1969) obtained essentially the same result. At ratios of 10 : 1 and even 1 : 1 Schilperoort reported partial inhibition of tumour induction.

Schilperoort confirmed that Escherichia coli does not inhibit gall induction (Lippincott & Lippincott, 1969c), but found that heat-killed A. radiobacter cells (100°C or 60°C, 1 hr) were also ineffective as tumour inhibitors. It seems that a different mechanism may be involved in tumour inhibition in large stem wounds, from that in small leaf wounds, although Schilperoort was prepared to attribute the inhibition to competition for infection sites. In such very different systems, it is perhaps not surprising that different mechanisms may operate.

Beaud et al. (1963) did not observe any decrease in tumour weight when avirulent mutants of A. tumefaciens were added to wounds on Datura, before or at the same time as pathogens. This may simply be because non-inhibiting isolates were used.

Manigault (1970) reported tumour inhibition by avirulent and attenuated strains of A. tumefaciens, but only if the inhibitory strains were added to the wound 24 hours before the pathogens; inoculation of both isolates at the same time did not inhibit tumour induction, but sometimes stimulated it. In Manigault's experimental conditions, complete occupation of infection sites was probably achieved; he added 2×10^8 non-pathogenic cells per wound, with a period of 24 hours in which to multiply up to the saturation level of 5×10^8 cells per wound.

This thesis will describe interactions between pathogenic and non-pathogenic agrobacteria.

Genetic Transfers

Understandably, most reports of genetic transfers by Agrobacterium have concerned transfer of virulence.

Coleman & Reid (1949) attempted to transfer virulence by the classical method for in vitro virulence transfer in pneumococci (Dawson & Sia, 1931). Although unsuccessful in this aim, they claim to have transferred the character of avirulence from A. radiobacter to A. tumefaciens, but theoretical considerations and a scrutiny of their methods suggest that this is unlikely; continued exposure to its homologous antiserum may have made the A. tumefaciens strain avirulent or selected avirulent strains already present.

They reported that production of non-capsulated cultures from normal, capsulated A. radiobacter also involved acquisition of virulence, but this claim can probably be dismissed; the bacteria re-isolated from the gall reacted with antiserum to capsulated A. tumefaciens.

Klein & Klein (1953, 1956) treated non-virulent and weakly virulent Agrobacterium isolates and Rhizobium leguminosarum with sterile extracts of crown-gall tissue, with suspensions of heat-killed cells (60°C, 45 min.), and with culture filtrates or DNA extracts of virulent bacteria. In all cases they claimed that the recipient cultures became tumorigenic or their virulence increased, and that

the host range of the transformed cultures corresponded with that of the donor bacteria. They did not isolate the transformed cells, but inoculated test plants with a mixture of untransformed and presumed transformed cells. Using their carrot bioassay (Klein & Tenenbaum, 1955) they calculated a transformation efficiency of 30% in one experiment. Apart from the possibility of contamination, which was not covered by the use of genetic markers in the donor or recipient strain, such a high transformation efficiency casts very grave doubts on the validity of their results; transformation efficiencies as high as 10% are very rare indeed, and most transformations alter less than 1% of the recipient cells (Hayes, 1968).

Kern (1965a) successfully repeated Klein & Klein's work, transferring virulence to Rhizobium leguminosarum by means of either a suspension of heat-killed cells (60°C, 45 min.) or a DNA preparation from A. tumefaciens. His experiments are subject to the same criticisms, in that he did not isolate transformed cells and did not use genetic markers, such as antibiotic resistance. Further evidence to refute his claims comes from his comparison of the "transformed" cultures with the parent strains. They were intermediate between the parent strains in morphological, physiological and biochemical properties (Kern, 1965b) and in DNA hybridization results (Kern, 1965c). By implication, approximately 50% of the A. tumefaciens genome must have

been incorporated in the transformed bacteria, which is not possible according to present knowledge of transformation; usually less than 1% of the donor genome appears in the recipient cells (Hayes, 1968). Despite stringent precautions to ensure sterility of his donor extracts, most of Kern's results would be best explained by contamination of the recipient culture with viable cells of A. tumefaciens. Kern (1969) also transferred antibiotic resistance between A. tumefaciens and R. leguminosarum. This work seems beyond dispute, but the transformation efficiencies were only $4\text{-}7\times10^{-6}$ (A. tumefaciens $\longrightarrow$ R. leguminosarum) and $13\text{-}6\times10^{-6}$ (R. leguminosarum $\longrightarrow$ A. tumefaciens).

Most Agrobacterium species form star-like clusters of cells in certain liquid media. The details of the process of star formation have been studied by Stapp (Stapp & Bortels, 1931; Stapp, 1942; Stapp & Knösel, 1954) and confirmed by Braun & Elrod (1946). Stapp (1942) and Stapp and Knösel (1954) observed a migration of nuclear material in the cells to the centre of the star, and interpreted this as a sexual process of conjugation. The observation was confirmed by Braun & Elrod (1946), who warned that crosses between different isolates were needed, to establish unequivocally the sexual role of star formation. To my knowledge, this has not been accomplished. Kerr (1971) was unsuccessful in inducing transfer of virulence under

conditions which favoured star formation of donor and recipient strains.

Kerr (1971) described a virulence transfer between isolates with quite distinct biochemical properties and with antibiotic resistance markers as an extra precaution to detect contamination. Virulence transfer took place within 7-14 days under optimal conditions and was only observed when non-pathogenic recipient cultures were swabbed onto the surface of tomato galls induced by the donor strain. The recipient strain was re-isolated by plating gall macerates onto a selective medium on which the pathogenic strain could not grow. Usually 50-75% of the recipient colonies were pathogenic.

Such a high frequency of acquisition of virulence could not be due to transformation, and virulence was not transferred when the donor and recipient were grown in conditions likely to favour conjugation. It is possible that the virulence factor is transferred first to the tumour cells, and thence to the recipient bacteria. This interesting possibility could be tested by attempting virulence transfer from bacteria-free tumours.

TUMOUR INDUCTION

Of the various aspects of the crown gall disease, the mechanism whereby a normal plant cell is converted to a tumour cell has stimulated the most interest and persistent study. The literature is far too extensive to review in detail, so I will concentrate mainly on the newer developments in the field. There are several good reviews which draw together earlier papers on crown gall induction (e.g. Braun, 1954, 1962; De Ropp, 1951; Klein & Link, 1955; Riker & Hildebrandt, 1951; White, 1951).

Sequence of Events in Tumour Induction

Several requirements must be fulfilled for tumorous transformation of plant cells to take place: The cells must first be conditioned by wounding of nearby cells, then the tumorigenic bacteria must be in contact with the conditioned cells for at least a minimum period of time.

The cells become "conditioned", or capable of undergoing a tumorous change, approximately 24 hours after wounding and remain in that state for three or four days (Braun, 1943, 1947, 1952; Braun & Mandle, 1948). Conditioning of cells appears to be induced by substances released from nearby injured cells, and is part of the normal process of wound healing.

The minimum period of contact between conditioned cells and

bacteria, for tumour induction, depends on whether the bacteria have been environmentally adjusted or not. Approximately 8 hours in a conditioned or unconditioned wound is the minimum requirement for bacterial environmental adjustment (Lipetz, 1966). It is not clear what "environmental adjustment" involves, but Manigault (1970) proposed that it is the time required for the bacteria to become attached to "infection sites" in the wound.

Environmentally adjusted bacteria require at least 8-10 hours' contact with conditioned cells to induce small, slow-growing galls (Braun & Mandle, 1948; Lipetz, 1966). Longer periods of transformation result in larger, more rapidly growing galls. Complete transformation takes approximately 48 hours.

Crown gall bacteria are present in tumours in very low numbers, compared with other bacterial diseases of plants (Smith, 1911), and seem to be confined mainly to the intercellular spaces (Riker, 1923b; Stonier, 1956). Tumours may be completely freed of bacteria by heat treatment at 46-47°C for 2-3 days (Braun, 1943) or 41°C for 7 days (Manasse & Lipetz, 1971), and the sterile tumours grow as vigorously as tumours containing bacteria. It appears that some fundamental change occurs during transformation, making the tumour cells capable of unlimited, autonomous proliferation. This change is induced by a

hypothetical "tumour inducing principle" (TIP) (Braun, 1947; Braun & Mandle, 1948), an unidentified product of the interaction between the conditioned host and crown gall bacteria.

Braun and his co-workers have established several important facts about the nature of the tumorous transformation in crown gall:

1. Transformation is a gradual process. The size and vigour of galls depends on the period of contact between the conditioned host and virulent bacteria, under favourable conditions for transformation (Braun, 1943; Braun & Mandle, 1948).

2. The tumorous state is heritable, as bacteria-free tumour tissue proliferates indefinitely (Braun, 1943, 1951a; Braun & White, 1943).

3. Recovery of tumours may be demonstrated by forcing rapid growth of tobacco teratoma tissue, by successive graftings onto vigorous plants (Braun, 1951b, 1959). This was interpreted as loss by dilution of a self-duplicating cytoplasmic entity, the heritable principle responsible for maintaining the tumorous state, which could not divide as quickly as the rapidly dividing cells.

The Nature of TIP

Braun (1947) made five suggestions about the origin and nature of the tumour inducing principle, which still seem to be valid. He said that TIP may be:

1. a metabolic product of A. tumefaciens,
2. a normal host constituent, converted to TIP by the action of the bacteria,
3. a chemical fraction of the bacterial cell (e.g. DNA),
4. a virus or other agent, which is present in association with the crown gall organism, or
5. the bacteria themselves, so changed in morphology and physiology as not to be demonstrable by the methods of isolation or microscopy employed up to the present time.

Among numerous suggestions about the nature of TIP, two groups of compounds have been very seriously considered, plant growth hormones and bacterial nucleic acids.

(a) Plant growth hormones

The discovery that tumorigenic agrobacteria produce auxin (Brown & Gardner, 1936; Dame, 1938) and that tumours contain high levels of auxin (Link & Eggers, 1941), once made it seem likely that

TIP would be identified as a growth hormone. However, it is now known that the hyperplasias induced by auxin are different from crown gall, in that they cease growth when removed from the influence of growth hormones (De Ropp, 1947a) and do not proliferate when grafted onto another plant of the same species (Braun & Laskaris, 1942).

The process of "habituation", or "accoutumance" (Gautheret, 1942), is more akin to the tumorous transformation in crown gall. It is a rare occurrence involving a sudden, usually permanent change from growth hormone-auxotrophy to prototrophy, in normal tissues cultured in vitro on media containing indole acetic acid or naphthalene acetic acid (Gautheret, 1946). Like crown gall tissue, habituated tissue may be cultured indefinitely on media without growth hormones, and Limasset & Gautheret (1950) reported that habituated tobacco tissue continued to proliferate when grafted onto a tobacco plant. On the other hand, Braun & Morel (1950) found that habituated grape tissue soon ceased its uncontrolled growth when grafted onto a healthy vine. Whether grafted habituated tissue continues to proliferate may depend on the plant species involved.

It is unlikely, however, that the tumorous transformation in crown gall is an habituation induced by bacterial growth hormones. Habituation is a sporadic occurrence and usually involves tissue culture in the presence of growth hormones for at least 5 months (Gautheret, 1946), whereas crown gall may be regularly and reproducibly induced

within 5 days by inoculation with Agrobacterium tumefaciens.

(b) Bacterial nucleic acids

It is at present suspected that the tumour inducing principle is bacterial nucleic acid, because actively replicating bacterial DNA has been found in tumour cells and this would account for the heritability of the tumorous condition. There is supporting evidence from the inhibition of tumour induction by nucleic acid inhibitors, but most attempts to induce galls by sterile bacterial nucleic acids have been unsuccessful.

There is no doubt that A. tumefaciens DNA (Schilperoort et al., 1967; Quétier, Huguet & Guillé, 1969) and RNA (Milo & Srivastava, 1969) are present in sterile crown gall tissue. Within 24 hours of inoculation, self-replicating A. tumefaciens DNA and a plant-bacterial DNA complex appear in the plant cells and bacterial RNA is synthesized (Stroun et al., 1971). There is a partial or total shut-off of transcription of host cell DNA.

However, the uptake of active bacterial DNA is not restricted to A. tumefaciens. Stroun and his co-workers have reported the same results following uptake of live E. coli or Pseudomonas fluorescens cells by cut tomato stems (Stroun, 1970; Stroun, Anker & Auderset, 1970). But only A. tumefaciens DNA must contain the necessary genetic information for tumour induction.

The non-pathogenic A. radiobacter contains very similar DNA to A. tumefaciens (Heberlein et al., 1967), and nucleic acids from A. radiobacter have given similar results to nucleic acids from A. tumefaciens in hybridizations with nucleic acids from crown galls (Schilperoort, 1969; Milo & Srivastava, 1969). This means that only a small portion of the A. tumefaciens genome must be involved in tumour induction, although probably the whole bacterial genome is taken up by a plant cell (Quétier et al., 1969).

Although galls are not formed in the absence of wounding, Stroun, Gahan & Sarid (1969) and Stroun (1970) reported that active A. tumefaciens DNA was taken up from living bacteria into the cells of unwounded tomato shoots. They did not find any differences between wounded and unwounded plants, in the uptake and subsequent replication and transcription of bacterial DNA. If the processes of tumour induction involve only a small portion of the A. tumefaciens DNA, it may be that Stroun's technique was not sensitive enough to detect the difference.

The role of wounding may also be linked with the production in the plant of a "nuclear heavy" satellite DNA (Nh DNA), rich in guanine and cytosine, which may be responsible for the greater part of a transient increase in DNA content following wounding (Kupila & Stern, 1961; Guillé, Quétier & Huguet, 1968). Quétier et al. (1969) found partial homology between A. tumefaciens DNA and Nh DNA from wounded plants, and proposed that tumour induction first involves the formation

of a complex between the bacterial DNA and plant Nh DNA. If this is so, it would explain the biochemical basis of conditioning in tumour induction.

As far as the inhibition of tumour induction by chemicals is concerned, there is no unequivocal evidence for the role of A. tumefaciens nucleic acids, mainly because all the chemicals have been more or less inhibitory to A. tumefaciens.

For example, Bopp (1960, 1961, 1964) inhibited tumour induction by adding to inoculated wounds, fluoro- and bromo- derivatives which antagonize the pyrimidine bases of nucleic acids or their nucleosides. Tumour induction was only inhibited if the chemicals were added during the four-day induction period, and the inhibition could be reversed by the addition of larger amounts of the antagonized nucleic acid component. However, these results do not provide conclusive proof of the involvement of bacterial DNA in tumour induction, because all the inhibitory chemicals also inhibited bacterial growth (Lipetz & Stonier, 1961; Beardsley & Lipetz, 1966).

Mitomycin C, an antibiotic which appears to cross-link purines in DNA and produces extensive DNA breakdown (Hayes, 1968), inhibits tumour induction when applied at 1 μg/ml to inoculated wounds (Gribnau & Veldstra, 1969). These workers claimed that there was no significant decrease in bacterial growth or virulence following incubation with

mitomycin C, but their claim can be questioned; Heberlein & Lippincott (1967) found that concentrations in excess of 0.5 μg/ml caused breakdown and release of bacterial DNA and a decrease in the number of viable cells.

Stroun et al. (1971) reported that rifamycin SV, an inhibitor of bacterial DNA-dependent RNA polymerase, inhibited tumour induction in bacteria-infiltrated plants if applied within 10 hours after wounding. They claim that the tumour inhibition is due to the prevention of RNA transcription from bacterial DNA, but it could equally be due to death of the virulent bacteria; they admit that rifamycin is toxic to the bacteria in the plant. To date, Stroun has not established the point which is implicit in this report, that A. tumefaciens DNA within plant cells will induce a gall when the plant is wounded in the absence of living bacteria.

Braun & Wood (1966) have proposed that bacterial RNA may be the agent of tumour induction. Gall induction was inhibited when a fairly high concentration of ribonuclease A was added to the wound 1-2 hours before inoculation, but desoxyribonuclease had no such effect. Although Braun & Wood found no deleterious effect of RNA-ase on the bacterial growth rate or on wound healing, Nevins, Grant & Baker (1970) have since found subtle changes in the bacteria; there was an increased rate of incorporation of macromolecular precursors from the medium and

an increased leakage of intra-cellular components, presumably proteins or nucleic acids or their degradation products. Ribonuclease-induced tumour inhibition may be due to degradation of bacterial RNA, but a loss of bacterial virulence caused by the other changes in the bacterial cell cannot be ruled out.

If bacterial DNA is the tumour inducing principle, the failure of DNA-ase to prevent gall induction suggests that there is some mechanism to protect the DNA, as it is transferred from the bacteria to the host cell.

The only certain way to demonstrate the causal role of bacterial DNA in crown gall tumour induction is to initiate tumours with bacteria-free A. tumefaciens DNA. The literature is replete with claims of sterile gall induction and with subsequent retractions of these claims.

Klein (1954) claimed the first sterile induction of crown gall with filtrates of plant sap in which A. tumefaciens had been grown. Klein & Braun (1960) have since shown that the sterility controls in this and a similar report (Klein & Knupp, 1957) were inadequate. Similar accounts by Manigault, Commandon & Slizewicz (1956) and Bender & Brucker (1958, 1959) have since been dismissed for the same reason (Manigault & Stoll, 1960).

Tumours have been induced on carrot phloem discs by a sterile

culture filtrate containing A. tumefaciens DNA (Thomas & Klein, 1959) but the so-called tumours ceased proliferation after one week (Klein & Braun, 1960). Manigault & Stoll (1958, 1960) produced short-lived proliferations on Datura stems with a sterile filtrate whose active principle was a DNA extract from A. tumefaciens or from a crown gall. The tumours looked like typical crown galls, and grew for three to four weeks before necrosing.

Kovoor (1967) reported that Scorzonera tissue was transformed in vitro by DNA extracted from A. tumefaciens. Only a small proportion of treated calluses became protoprophic for auxin, and the controls were too few to rule out habituation by the auxin in the medium.

In addition to the reports of successes which have since been dismissed or challenged for various reasons, there are many reports of unsuccessful attempts to induce crown gall by sterile extracts (Manil, Delcambe & Fourneau, 1955; Kern, 1965a; Bieber & Sarfert, 1968; Gribnau & Veldstra, 1969). Beltrá & Rodriguez de Lecea (1971) claim to have induced crown gall tumours on carrot discs and Pinto bean stems with bacterial DNA. The tumours satisfied all requirements of growth on minimal media and continued proliferation following grafting, but in view of Bieber & Sarfert's (1968) failure to induce galls with DNA extracted by essentially the same method, this report must be regarded with caution.

Leff & Beardsley (1970) have recently succeeded in inducing

crown gall tumours with DNA extracted from a temperate bacteriophage of A. tumefaciens. The report has not been independently confirmed, but may explain why it has been impossible to induce tumours in the absence of living bacterial cells; the induction of prophage in living bacteria may be the necessary first step in crown gall induction.

(c) Temperate bacteriophage

De Ropp (1947b) was the first to seriously suggest that crown gall may be due to a virus, and the discovery that many isolates of A. tumefaciens are lysogenic (Beardsley, 1955; Zimmerer, Hamilton & Pootjes, 1966) stimulated renewed speculation along these lines. Bacteriophage could not be detected in all strains of A. tumefaciens, but this may be due to the use of insensitive indicator strains (Zimmerer et al., 1966).

With one exception (Hohl, 1961), phage-like particles have never been seen in electron microscope studies of crown gall tissue (Gee, Sun & Dwyer, 1967; Lipetz, 1970; Manocha, 1970), except for a limited period during the induction phase. However, bacteriophage has been isolated from sterile gall tissue by incubating phage-sensitive agrobacteria with extracts of macerated tissue (Parsons & Beardsley, 1968; Tourneur & Morel, 1970). These bacteriophages had the same morphology as the temperate phages from A. tumefaciens, namely, a polyhedral head, 60-100 mμ in diameter, and a tail approximately 200 mμ long. They all contained DNA.

It was with the DNA from a bacteriophage from sunflower crown gall tissue, that Leff & Beardsley (1970) induced tumours on sunflower and tobacco. The intact bacteriophage did not induce tumours. This is not unusual; it is well known that free viral nucleic acids can be active in hosts highly resistant to the intact virus (Stroun et al., 1971). Almost all plant viruses contain RNA, but synthesis of a DNA phage of E. coli has been demonstrated when tobacco leaves were inoculated with phage DNA, but not with the intact phage (Sander, 1964).

Although phage-like particles are rarely seen in electron microscopy of mature galls, they have been seen in thin sections of inoculated pea seedlings, during the induction phase. Phage-like particles were observed 28 hours after inoculation in bacterial cells and lysed cell debris in the intercellular spaces of the plant, but could not be seen at 10 or 72 hours after inoculation (Kurkdjian, Beardsley & Manigault, 1968; Kurkdjian, 1968, 1970). Manocha (1971) claims to have seen phage particles between 30 and 60 hours after inoculation of pea seedlings, but his electron micrographs show particles which seem to be too variable in size to be phage (R.I.B. Francki, personal communication).

In sections of inoculated Datura stramonium wounds, Hoursiangou-Neubrun & Puiseau-Dao (1969) saw phage particles within the bacterial cells, free in the intercellular spaces and even within living plant

cells. They did not state how long after inoculation the phages could be seen.

These reports do not constitute proof of the tumour-inducing role of temperate A. tumefaciens phages, as induction of prophage may simply be associated with other changes involved in the lysis of bacteria in the wound, and not directly related to tumour induction. Only a few strains of A. tumefaciens have been examined so far, and phage-like particles have been seen in most of them, including one avirulent clone (Kurkdjian, 1970). The attenuated strain A66, which only forms tumours if supplemented with auxin, did not produce phage in wounded pea seedlings.

Kurkdjian's (1970) results do not mean that bacteriophage cannot be the tumour-inducing principle, because some avirulent strains may carry non-tumorigenic phages. It is harder to explain the absence of phage in the attenuated strain, especially in view of Braun & Laskaris' (1942) theory that attenuated strains actually transform the host cells, but that the transformed cells do not divide, due to lack of sufficient auxin.

What is really needed is an electron microscopic comparison of a pathogenic isolate which should contain phage, with a non-pathogenic isolate which, theoretically, should not contain phage. One of Kerr's (1971) non-pathogenic recipient cultures would fall into the second

category; it could be compared with the daughter pathogenic culture formed after transfer of virulence.

If the tumour-inducing principle is a bacteriophage, this would explain a number of phenomena. Exposure of A. tumefaciens to ultra-violet light increases the virulence of the surviving cells (Duggar & Riker, 1940; Heberlein & Lippincott, 1965). This may be due to UV induction of prophage (Lwoff, Siminovitch & Kjeldgaard, 1950). Treatment with low concentrations of mitomycin C also increases virulence of A. tumefaciens, possibly by induction of prophage (Heberlein & Lippincott, 1967).

When bacteria-free tumour tissue was grafted in vitro onto normal sunflower stems, tumours sometimes developed from the healthy plant tissue (De Ropp, 1947b; Camus & Gautheret, 1948; McEwen, 1952), suggesting the transfer of a transmissible principle, such as a virus, from the gall tissue (De Ropp, 1947b). However, habituation due to growth hormones produced by the gall has not been ruled out (McEwen, 1952).

Aaron-da Cunha (1969) has achieved a similar transformation by grafting X-Ray irradiated sterile crown gall tissue onto a healthy tobacco stem. Growth of the gall was prevented by the high radiation dose, but a tumour developed in nearby healthy tissue. She proposed that the X-Rays released a tumorigenic principle, probably a phage.

Tourneur & Morel (1970) have since isolated an A. tumefaciens bacteriophage from this transformed tissue, but in many attempts they have never isolated phage from habituated or normal tissue.

The transfer of virulence reported by Kerr (1971) could be well explained in terms of a tumorigenic phage. According to this theory, the phage is transferred from A. tumefaciens to healthy tomato cells, which become tumorous. The phage is then transferred from the tumour cells to non-pathogenic recipient agrobacteria, which become lysogenic and tumorigenic.

THE ECOLOGY OF AGROBACTERIUM TUMEFACIENS

Ecology and Crown Gall Incidence

Our knowledge of the ecology of the crown gall organism has been quite limited until recently, mainly because of the lack of suitable methods for isolating A. tumefaciens from soil.

Some workers have obtained a rough estimate of the presence or absence of pathogens by planting wounded, susceptible plants (Reddick & Stewart, 1924; Muncie, 1926a; Niemeyer, 1935) or discs of fleshy vegetable roots (Ark & Schroth, 1958; Pereira, Lima & Zagatto, 1968) in the soil; these form galls if A. tumefaciens is present in sufficient numbers. Apart from not being quantitative, this method has the disadvantage that the test plant or root disc may first be attacked by rot-producing organisms, and that other antagonistic

organisms may prevent A. tumefaciens from inducing galls.

Patel (1926) devised a partially selective medium which has been widely used for the quantitative isolation of agrobacteria. Many contaminants can grow on it, so it is only suited to isolating A. tumefaciens, A. radiobacter and A. rhizogenes from locations where they are present in fairly high numbers relative to the contaminants, such as in galls and hairy root infections, on root surfaces and in soil near infected plants, and in artificially inoculated soils. Hendrickson et al. (1934) used a partially selective mannitol-yeast extract agar medium containing aniline blue to isolate A. tumefaciens from galls, but no other workers have used this medium.

Schroth, Thompson & Hildebrand (1965) have devised a selective medium which enables easy, quantitative isolation of agrobacteria from soil, even when they are present in quite low numbers. Like the earlier selective media, it does not distinguish between A. tumefaciens and A. radiobacter. A. tumefaciens can only be distinguished by inoculating isolates onto crown gall-susceptible plants, such as tomato. A. rhizogenes and A. rubi do not grow on the medium.

There have been few reports of isolations of A. tumefaciens from natural soil populations (Patel, 1928; Pereira et al., 1968; Kerr, 1969; Schroth et al., 1971), and in all but one, A. tumefaciens was only found in soil around galled plants. This may be partly due to inadequate isolation techniques. Schroth et al. (1971) isolated

A. tumefaciens from several fields of cotton and tomatoes, and from four fields where crown gall hosts had never been grown, except possibly for dicotyledonous weeds.

There is good evidence that A. tumefaciens can survive long periods in the soil. Pathogenic agrobacteria have been isolated from artificially inoculated, unsterilized soils after $5\frac{1}{2}$ months (Muncie, 1926b), 9 months (Dickey, 1961), 14 months (Banfield, 1934), and 22 months (Patel, 1929). There have been no experiments to determine quantitative survival in naturally infested soils, but Hildebrand (1941) and Schroth et al. (1971) have isolated A. tumefaciens at pathogenic levels 2 and 5 years, respectively, after the removal of susceptible crops. However, the bacteria may have survived in the rhizosphere of weed plants; Hildebrand gave no details of where he sampled, but Schroth et al. obtained best results when pieces of root with adhering soil were placed on carrot root discs.

In the past, most workers have implied that A. tumefaciens is primarily a plant pathogen, and that numbers in the soil are maintained only by leakage of the bacteria from galls. The data of Schroth et al. (1971) show that A. tumefaciens may be a successful soil saprophyte, in the absence of any host plants. This is not surprising, because the almost-identical non-pathogen, A. radiobacter, is recognized as a successful soil saprophyte and rhizosphere inhabitant (Fåhraeus &

Ljunggren, 1968). However, there is quite good evidence that A. tumefaciens is released in large numbers from the surface of intact galls (Robinson & Walkden, 1923; Banfield, 1928, 1934; Dickey, 1962), and this could account for its greater ease of recovery from soil where galled plants have been grown.

A. tumefaciens can only infect a plant via a wound less than one week old (Riker, 1923a; Riker & Banfield, 1932). In the nursery, wounding may be due to cultural practices, the activities of soil animals and normal growth processes of the plant.

Siegler & Bowman (1940) proposed that crown gall bacteria often gain entry at wounds produced in the germination of peach seeds. They observed that stems of seedlings are frequently bruised by a sharp projection of the hard endocarp, and linked this with the frequent occurrence of galls at the same position in more mature seedlings. Natural root branching also involves tearing of cortical tissue, and many galls are seen at the junction of roots.

Banfield (1934) demonstrated that root-feeding arthropods cause damage which can become an infection site for crown gall bacteria. Root-knot nematodes are sometimes associated with crown gall and have been shown experimentally to increase its incidence (Nigh, 1966; Griffin, Anderson & Jorgenson, 1968; Orion & Zutra, 1971).

Digging up, trimming and replanting of nursery stock is

probably responsible for providing suitable sites for infection. Grafting was held responsible for a proportion of crown galls in apple nurseries, but Riker & Keitt (1926) found that 86% of so-called "crown galls" at graft unions on apple trees were really wound overgrowths, caused by badly-fitting grafts. The method of above-ground budding in stone fruit crops offers little opportunity for infection.

A. tumefaciens cells are actively attracted towards expressed sap (Riker, 1923a) and plant roots (Schroth & Ting, 1966) but the significance of this attraction in ensuring colonization of wounds in the field, is not known.

The dispersal of crown gall bacteria has been little studied. As well as in visible galls, it appears that A. tumefaciens may be dispersed to new areas in incipient galls (Banfield, 1934; Banfield & Mandenberg, 1935) and on the roots of healthy plants. Although there is no published data for A. tumefaciens, the same situation probably applies as for A. rhizogenes, which has been isolated from the surface of roots of seedling apples. Healthy plants may become contaminated from storage in contaminated soil or in proximity to galled plants (Banfield & Mandenberg, 1935).

Isolation of a New Tumour Inducing Organism

Kerr (1969) used the medium of Schroth et al. (1965) to isolate A. tumefaciens from crown galls and the surrounding soil, but found it

present only in low numbers, and outnumbered by A. radiobacter by at least 100 : 1 in soil and 75 : 1 in galls. When a 100 : 1 mixture of A. radiobacter and A. tumefaciens was inoculated onto tomato plants, no gall developed, so he concluded that A. tumefaciens was probably not the primary pathogen in the nursery in question.

Using Patel's (1926) partially selective medium, Kerr (1969) isolated a tumour inducing bacterium which did not grow on the medium of Schroth et al. (1965); he designated the new isolates Agrobacterium sp. This organism was present in relatively high numbers in galls, and appeared to be the primary cause of crown gall in the nursery.

It may be that Agrobacterium sp., rather than A. tumefaciens, is the cause of crown gall in other parts of the world. In California, Schroth et al. (1971) detected A. tumefaciens in 18 out of 28 soils tested. Where present, the ratio of A. tumefaciens to A. radiobacter varied from 1 : 13 to 1 : 500. These results raise doubts as to whether A. tumefaciens is the primary pathogen in California.

Kerr (1969) was only able to isolate Agrobacterium sp. from galls, where it outnumbered A. radiobacter by about 5 to 1 and was readily distinguishable from contaminants on Patel's (1926) medium. This medium is unsuitable for isolation from soil, because of the growth of many contaminants, so there is no data on the abundance of

Agrobacterium sp. in soil.

This thesis will describe the development of a highly selective medium for Agrobacterium sp. The medium has been used in an ecological study of this organism in relation to crown gall incidence.

ECONOMIC ASPECTS

Losses Due to Crown Gall

Crown gall affects many dicotyledons (Elliott, 1951), but its severity varies from one geographical location to another, and from one host to another. Although it may affect vines and cherry, plum and pome fruit trees, it does not appear to be a serious problem in these crops in England or Europe (Riker, 1928; Niemeyer, 1935; Lelliott, 1971).

Crown gall is one of the most serious diseases of nursery peach trees in many parts of the U.S.A., especially in sections of the southern and western states (Siegler & Bowman, 1940). In 1963, crown gall was judged one of the three most important diseases on fourteen major crops in California, where it causes an estimated annual loss of $7 million (Schroth et al., 1971). However, Reddick & Stewart (1924) thought that the disease did not cause much loss of productivity on apple or peach trees in the colder climate of New York State.

In South Australia the disease is very severe in stone fruit nurseries, especially on almond and peach. Apple is recorded as a host, but where the Northern Spy rootstock is used, crown gall is never observed.

Crown gall adversely affects the host plant by preferential uptake of nutrients (Link et al., 1953) and by partial or complete disruption of the sap flow. Crown gall decreases the water conductivity of stems of two year old apple trees by 30% (Melhus, Muncie & Ho, 1924) and of two year old peach trees by about 82% (Muncie, 1926b).

Mature trees can survive with quite large galls on their roots and crowns, but the effect on the yield and longevity of the trees is not known. Ross et al. (1970) ventured the opinion that "hundreds of almond trees in California die or are blown over every year, due to heartrot caused by crown gall infections occurring many years earlier". They say that widespread infection in fruit orchards may reduce their economic life by as much as 20 per cent.

Crown gall is probably most important during the first few years' growth of a tree, because galls frequently girdle young trees and kill them. Although sale of galled trees is illegal in many countries, the disease still appears in orchards, due to incipient infections, or to pathogenic bacteria introduced on nursery stock or already present in the soil.

Disease Control

Until recently there has been no reliable cure for galled trees, and disease control measures have aimed at preventing the introduction of the pathogen to new areas on contaminated material and at reducing the opportunities for wounds to become infected. In the case of root-grafted apples, Riker et al. (1934) advised grafting onto clean, dry roots. Many workers advise growers to dip the roots of all nursery stock in disinfectant, before planting out. Mercuric chloride (Riker et al., 1934), sodium hypochlorite (Ross et al., 1970) and terramycin (Guengerich, Goodman & Millikan, 1969) have been suggested.

Once present in the soil, A. tumefaciens cannot be economically eradicated by soil fumigation (Dickey, 1962; Deep, McNeilan & MacSwan, 1968; Ross et al., 1970). Schroth et al. (1971) suggested that agrobacteria surviving fumigation can multiply rapidly up to levels which cause appreciable disease in susceptible hosts.

Chemical protection against crown gall infection in the field may, in some cases, be achieved by the use of seed dips, soil drenches and spray-applied systemic fungicides. Siegler & Bowman (1940) greatly reduced the incidence of crown gall by dipping peach seeds in 2.5% mercurous chloride, before planting. On the other hand, Schroth et al. (1971) did not achieve any beneficial effect by using 1% sodium

hypochlorite or 0.1% mercuric chloride as a seed dip; the level of mercury treatment was probably too low to have any permanent anti-bacterial effect in the soil. Guengerich et al. (1969) reduced crown gall incidence on peach by applying "Morsodren", an organic mercury compound, as a soil drench accompanying seed planting. However, the use of mercury compounds is not desirable because of their human toxicity and their contribution to environmental pollution.

Two systemic fungicides, cycloheximide thiosemicarbazone and cycloheximide acetate, protected cherry trees against development of aerial galls, if applied before the trees were artificially inoculated. There was some injury to the sprayed trees, which became evident in the following season (Helton & Williams, 1968).

A number of chemicals have been proposed as treatments to cure established crown galls, but most of them are unsatisfactory because of low effectiveness, high phytotoxicity or high cost. Antibiotics have completely eradicated galls under laboratory conditions (Brown & Boyle, 1944; Hampton, 1948; Blanchard, 1951; Klemmer, Riker & Allen, 1955), but they have not been adopted for commercial use, possibly for economic reasons. For example, crown galls on pecan trees were cured by yearly applications of terramycin for four years (Cole, 1969). The application rate was 5 gallons of a 0.04% solution of 20% terramycin per tree. The treatment sometimes had the undesirable effect of causing a tree to shed its nuts.

Ark (1941) tested preparations containing sodium dinitro-o-cresol, iodine or clove oil as crown gall eradicants. The iodine preparation was not consistently effective and clove oil was too expensive, but a 1 : 4 mixture of sodium dinitro-o-cresol and methanol was quite effective when painted onto galls. This is the present recommended treatment in South Australia (Thomas, 1968), but it has not been cleared by the Food and Drug Administration for use in the U.S.A.

Schroth & Hildebrand (1968) have devised a very successful gall eradicant, marketed as "Bacticin", which consists of five hydrocarbons and hydrocarbon derivatives in a kerosene-water emulsion. It is based on the observation that crown galls are permeable to oils, but healthy tissue is relatively impermeable. Thus the active constituents, dissolved in kerosene, selectively penetrate and eradicate gall tissue. Exposed galls painted with "Bacticin" usually die within 2 to 3 months (Ross et al., 1970).

Although "Bacticin" appears to be effective, the method of application is laborious and expensive. The crowns and main roots of orchard trees have to be exposed, generally by a strong jet of water, before galls can be located and treated.

This thesis describes the development of a simple and efficient method of biological control.

PART B: IDENTIFICATION AND NOMENCLATURE OF AGROBACTERIUM ISOLATES

INTRODUCTION

Four of the seven species of Agrobacterium Conn, listed in the 7th edition of Bergey's Manual (Breed, Murray & Smith, 1957) clearly belong to this genus, whether or not they remain as separate species (De Ley et al., 1966), but the remaining three, A. gypsophilae, A. pseudotsugae and A. stellulatum, should probably be excluded (De Ley, 1968). The first four species are distinguished mainly by their pathogenicity and host range. A. tumefaciens and A. rubi both cause crown gall, but the former has a wide host range, whereas A. rubi is supposedly restricted to Rubus spp. (Banfield, 1930; Hildebrand, 1940). A. rhizogenes causes only slight swelling with marked pathological root proliferation, on apple and a number of other hosts. A. radiobacter is not pathogenic.

From galls on stone fruit trees, Kerr (1969) isolated a tumour-inducing form of Agrobacterium which did not conform with any previously described species; such isolates were designated Agrobacterium sp. This form is apparently the primary cause of crown gall in South Australia.

In a co-operative study with Mr. P.J. Keane and Dr. A. Kerr, Agrobacterium sp. was intensively studied and compared with previously described nomen-species (Keane et al., 1970, Appendix 4). For clarity

it has been found necessary to present some results obtained by Keane and Kerr, but where this is done, it will be clearly stated.

CULTURES TESTED

Table 1 lists the cultures tested, their origins, the substrates from which they were isolated, the suppliers and suppliers' numbers. Of the local isolates, all those between 1 and 43 were isolated by Dr. A. Kerr, and the rest were isolated by me on the selective medium prepared for *Agrobacterium* sp. (Part C). The isolates are numbered according to the system used in Keane *et al.* (1970); corresponding numbers in the Waite Institute culture collection are given in Appendix 1.

The cultures were stored in sterile distilled water at 10°C for routine use (De Vay & Schnathorst, 1963). Two-day old cultures on nutrient agar were used as inoculum in most tests. Long-term storage was by freeze-drying in ampoules which were stored at 5°C.

BIOCHEMICAL TESTS

Most isolates were tested for biochemical reactions by Kerr (personal communication) and were divided into two biotypes as a result of these tests (Table 2). All isolates originally designated *Agrobacterium* sp. were placed in biotype 2.

The two biotypes could be distinguished by ten biochemical

Table 1

The sources of 61 cultures of *Agrobacterium* spp. and one unnamed isolate

Isolate No.	Designation	Isolated from:	Origin	Supplier and Supplier's Number*
1 and 2		*Prunus cerasifera* gall	South Australia	
3		Peach gall	South Australia	
4		Pear gall	South Australia	
5–10		Soil	South Australia	
11			Canada (A.G. Lockhead: 426)	A.C. Parker (WU 10)
12	*A. radiobacter*		Canada (A.G. Lockhead: 590)	A.C. Parker (WU 11)
13				A.C. Parker (WU 80; ICPB: TR1)
14				A.C. Parker (WU 81; ICPB: TR4)
15		Soil	South Australia	
16–19		Peach gall	South Australia	
20 and 21		*Prunus cerasifera* gall	South Australia	
22			Wisconsin	R. Mushin (3)
23			London	R. Mushin (5)
24				A.C. Parker (WU 18)
25			Canada (W.H. Cook: B70)	A.C. Parker (WU 21)
26		Apple gall	Edinburgh (W. Blyth via D.W. Dye: A4)	A.C. Parker (WU 78)
27	*A. tumefaciens*	Poplar gall	Edinburgh A.M. Paton via D.W. Dye: A6)	A.C. Parker (WU 79)
28		Peach gall	New South Wales Forestry Commission (109A)	A.C. Parker (WU 89)
29		Peach gall	New Zealand (D.W. Dye: A1)	A.C. Parker (WU 75)
30		Peach gall	New Zealand (D.W. Dye: A3)	A.C. Parker (WU 77)
31			New Zealand	J. DeLey (NCPPB: 223)
32		Almond gall	Israel (Z. Volcani)	J. DeLey (ICPB: TT133)
33–37		Peach gall	South Australia	
38–40	*Agrobacterium* sp. (tumorigenic)	Plum gall	South Australia	
41–42		Almond gall	South Australia	
43	*A. rubi*			J. DeLey (ICPB: TR2)
44				J. DeLey (ICPB: TR3)
45				M.P. Starr (ICPB: TR7)
46				J. DeLey (ICPB: TR101)
47				M.P. Starr (ICPB: TR105)
48				J. DeLey (ICPB: TR107)
49	*A. rhizogenes*			M.P. Starr (ICPB: TR104)
50				M.P. Starr (ICPB: TR108)
51				M.P. Starr (ICPB: TR106)
52				M.P. Starr (ICPB: TR109)
53	*A. tumefaciens*		Victoria	A.C. Parker (WU 74)
54–57	*Agrobacterium* sp. (tumorigenic)	Soil	South Australia	
58–61	*Agrobacterium* sp. (non-pathogenic)	Soil	South Australia	
62	Unnamed	Soil	South Australia	

* ICPB, International Collection of Phytopathogenic Bacteria, Davis, California; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England.

Table 2

Division of isolates into biotypes
(Kerr, personal communication)

Biotype	Isolate numbers
1	1-28, 49-52
2	29-48, 54, 55, 58, 59, 61
neither biotype	62
not tested	53, 56, 57, 60

tests (Keane et al., 1970). One of the quickest and most reliable was that for production of 3-ketolactose (Bernaerts & De Ley, 1963); all biotype 1 isolates were ketolactose-positive and all biotype 2 isolates were ketolactose-negative.

PATHOGENICITY OF ISOLATES

The tumour-inducing ability of isolates was determined by inoculation onto young tomato plants (Lycopersicum esculentum Mill.). A heavy suspension of bacteria was smeared on the stem of each plant, which was then pierced by a multi-needle wounding instrument (Kerr, 1969) (Plate 1A). The instrument is sterilized by dipping in alcohol and flaming.

Most galls developed within two weeks (Plate 1B), but non-galled plants were examined again four weeks after inoculation. The pathogenicity results are given in Table 7.

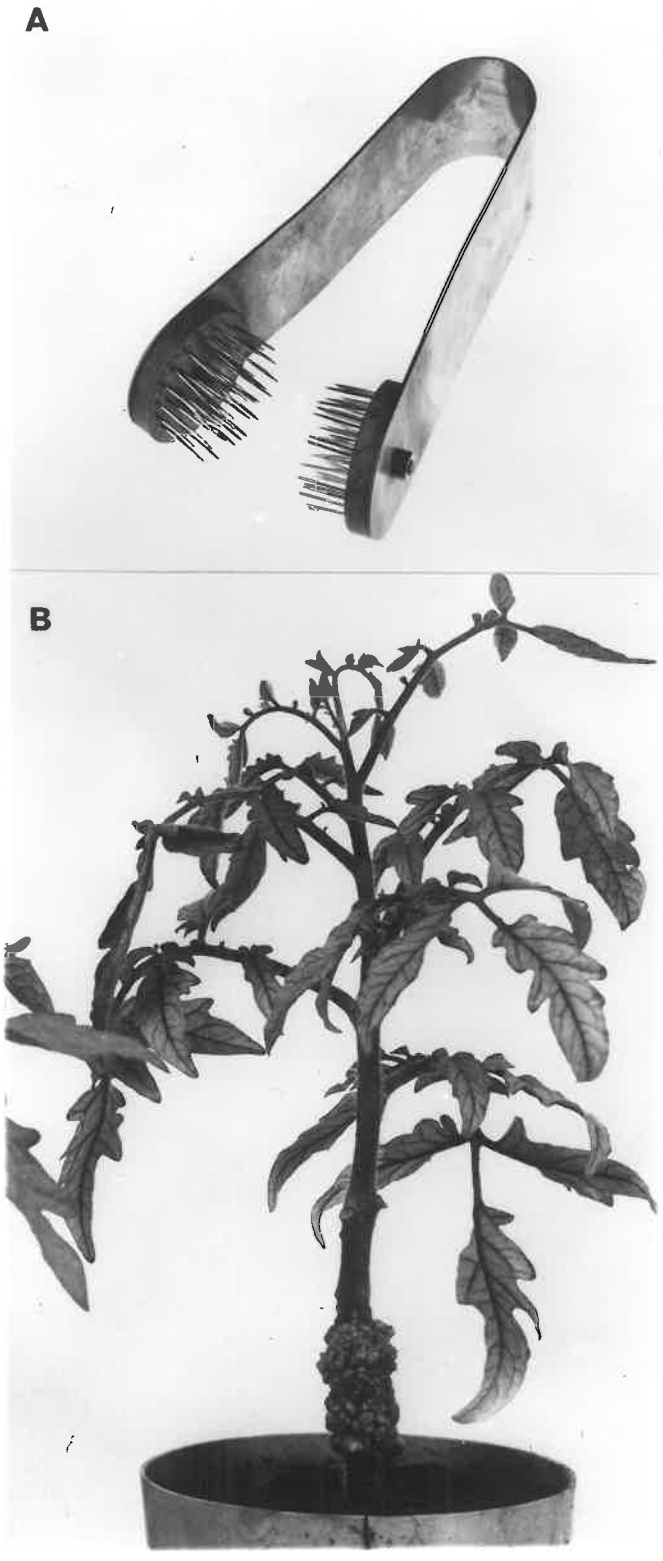
Kerr (1969) showed that tomato plants gave exactly the same galling response as peach seedlings with 18 pathogenic and 100 non-pathogenic biotype 1 isolates. As only 6 biotype 2 isolates were tested by Kerr (personal communication), a further 11 isolates, 6 pathogenic and 5 non-pathogenic to tomato, were inoculated onto peach seedlings.

A heavy bacterial suspension was smeared onto the stem, just at or below soil level, and the stem was wounded with a scalpel. Galls

Plate 1

A. Multi-needle wounding instrument for testing pathogenicity of isolates to tomato seedlings.

B. Gall on tomato produced after inoculation with a tumorigenic isolate.


A
B

appeared within one month of inoculation.

There was complete agreement between the results on peach and tomato (Table 3); another 3 isolates which are not listed here were not pathogenic to tomato or peach. Therefore tomato is satisfactory for testing tumorigenicity of these isolates.

It seemed possible that the non-tumorigenic biotype 2 isolates could, like A. rhizogenes, induce pathological root proliferation. This possibility was tested for five of these isolates by inoculation of carrot discs (Ark & Thompson, 1961), along with seven A. rhizogenes isolates and one biotype 2 isolate known to induce tumours.

Carrots from a local store were peeled and surface-sterilized by dipping into alcohol and flaming. The upper 2 cm and the terminal 1.5 cm were discarded and each root was cut into discs about 1 cm thick, which were placed upside down on sterile moistened filter paper in Petri dishes; preliminary tests with A. rhizogenes isolates showed that apical facing surfaces gave a more vigorous pathological response, a phenomenon previously only reported for tumour-inducing isolates (Klein & Tenenbaum, 1955; Wells & Baker, 1969).

A bacterial suspension (approx. 10^9 cells/ml) was spread over the discs, each isolate being inoculated onto discs from several different carrots, usually four or eight. Four discs, inoculated with only one isolate, were stored in each dish. Control discs for each carrot

Table 3

Tumorigenicity of local biotype 2 isolates on tomato and peach seedlings

Isolate No.	Tumorigenicity on	
	Tomato seedlings	Peach seedlings
34	+	+
35	+	+
40	+	+
55	+	+
56	+	+
57	+	+
58	-	-
59	-	-
60	-	-
61	-	-
62	-	-

were inoculated with sterile distilled water. Three separate tests were carried out. The results are given in Table 4.

None of the non-tumorigenic biotype 2 isolates induced any response on carrot discs. Eleven other non-tumorigenic isolates, not listed here, also failed to induce root proliferation. From this, it is assumed that all non-tumorigenic isolates are non-pathogenic; this is probably a safe assumption, since hairy root has never been recorded in Australia.

Isolate 34 induced typical galls on carrot discs (Plate 2A).

Only three of the seven supplied A. rhizogenes isolates induced root proliferation (Plate 2B), and one produced a galling response on carrot. The negative reactions could be ascribed to differences in host range of the isolates. Apparently isolate 50 has been incorrectly identified as A. rhizogenes. Hendrickson et al. (1934) and Starr (1946) have also described tumour-inducing isolates which were designated A. rhizogenes.

The carrots varied greatly in the magnitude and speed of their response to pathogenic isolates. Galls or roots appeared within 15 days of inoculation in the first two tests, but only after four weeks in the third and several unreported tests, although experimental conditions were the same in each case. In each test a certain proportion of carrots produced a weak or negative response to pathogenic isolates; this was especially noticeable in the third test, when three out of

Plate 2

A. Reaction of carrot discs to a tumorigenic isolate.

B. Reaction of carrot discs to a root-proliferating isolate.

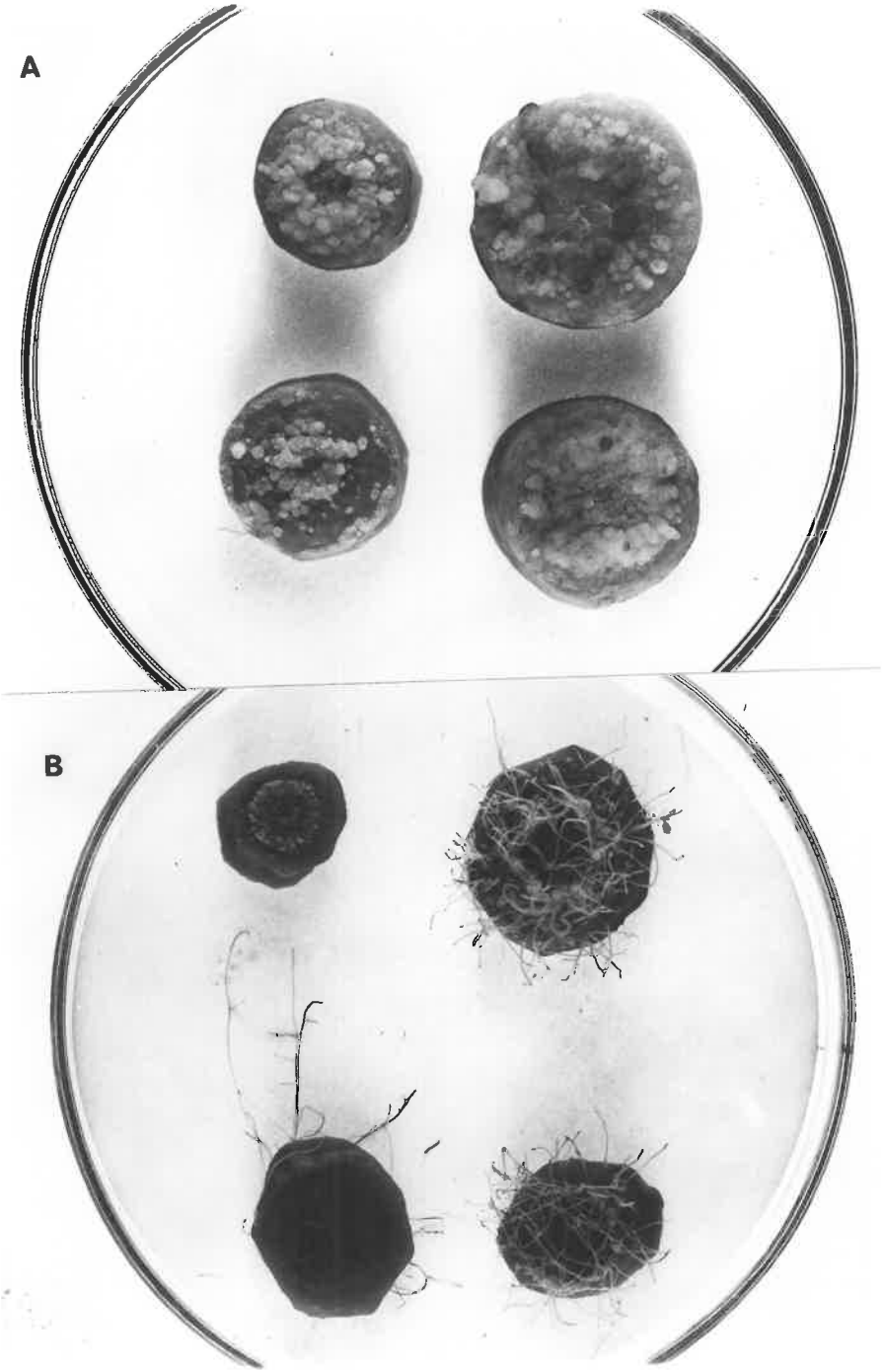

A
B

Table 4

Reaction of carrot discs to inoculation with seven isolates of *A. rhizogenes* (45-52) and six local biotype 2 isolates, one of them pathogenic (34) and five not pathogenic to tomato seedlings (58-62)

Isolate No.	Reaction on carrot discs	Proportion of discs showing pathological reaction in 3 tests		
		1	2	3
34	galling	2/4	3/3	2/7
45	root prolif.	-	-	1/7
46	root prolif.	3/4	4/4	2/7
47	root prolif.	-	-	1/8
48	-	0/4	0/4	0/8
50	galling	2/4	3/3	-
51	-	-	0/3	-
52	-	-	0/3	-
58	-	0/4	-	0/7
59	-	-	0/3	0/8
60	-	-	0/3	0/8
61	-	-	0/3	0/8
62	-	-	0/3	0/5

sixteen carrots were not reactive. This variation has been reported previously for tumorigenic isolates (De Ropp, 1950; Klein & Tenenbaum, 1955); De Ropp found that 10% of the carrots he used gave no response to inoculation with A. tumefaciens. From the results obtained here, it seems that South Australian carrots are too variable for reliable routine use of carrot discs for pathogenicity testing.

SEROLOGY

Preparation of Antigens

Isolates 1, 15 and 34 were grown in mannitol-nitrate-biotin broth (Appendix 2) for 24 hr and harvested by centrifugation. After 2 washings in normal saline (unbuffered), the cells were taken up in the original volume of normal saline to give a suspension of approximately $5x10^8$ cells per ml. These suspensions were used in the preparation of the antisera.

Antigens for the tube agglutination test were prepared by growing the isolates in mannitol-casamino acids broth (Appendix 2) for 48 hr. The cells were harvested by centrifugation, washed in sterile distilled water and diluted to a concentration of approximately 10^8 cells per ml in normal saline (pH 7.2).

Preparation of Antisera

The three isolates were injected into three separate rabbits,

each being given an injection once a week for 3 weeks. The first two injections, of 1 ml, were given subcutaneously and consisted of an emulsion of equal proportions of bacterial suspension and Freund's adjuvant. The third injection consisted of 2 ml of bacterial suspension without adjuvant, and was given intravenously.

Ten days later the rabbits were bled and antiserum obtained by allowing the blood to clot and centrifuging the serum at 4,000 g for 10 min to remove red blood cells. The antisera had low titres in the tube agglutination test, so each rabbit was given a booster injection of 2 ml intravenously and bled again after ten days. These antisera had acceptable titres. They were stored at -40°C.

Tube Agglutination Test

Successive two-fold dilutions of antisera were prepared in Pyrex serology tubes by serial dilution of 0.5 ml of each antiserum in 0.5 ml aliquots of buffered saline. An equal volume of bacterial suspension was then added to the 0.5 ml of diluted antiserum in each tube and the tubes were incubated 3 hr in a 37°C water bath. The tubes were left overnight at 4°C before agglutination end-points were determined. The three antisera were tested with all three antigens.

Results of the tube agglutination test (obtained in conjunction with P.J. Keane) are presented in Table 5. It is clear that isolate 34 (*Agrobacterium* sp.) is distinct serologically from isolates 15

Table 5

Agglutination titres of representative *Agrobacterium* isolates

Antiserum	Antigen	Titre
A. tumefaciens (isolate 15)	*A. tumefaciens*	4096
	A. radiobacter	4096
	Agrobacterium sp.	< 4
A. radiobacter (isolate 1)	*A. radiobacter*	8192
	A. tumefaciens	2048
	Agrobacterium sp.	< 4
Agrobacterium sp. (isolate 34)	*Agrobacterium* sp.	512
	A. tumefaciens	< 4
	A. radiobacter	< 4

(A. tumefaciens) and 1 (A. radiobacter) and that the latter two are closely related.

Gel Diffusion Test

Gel diffusion tests on many of the isolates were carried out by Keane and Kerr (personal communication) and for comparison with other tests, are presented in Table 7.

Fluorescent Antibody Staining

The fluorescent antibody method, pioneered by Coons, Creech & Jones (1941), has been widely used for the specific staining of microorganisms, including rhizobia in soil and nodules (Schmidt, Bankole & Bohlool, 1968; Trinick, 1969). This method was adopted for easy identification of isolates serologically related to isolate 34 (Agrobacterium sp.).

Preparation of fluorescein conjugate

Antiserum against isolate 34 was conjugated with fluorescein isothiocyanate (Sigma Co., St. Louis, Missouri) by a modification of the method of Hill and Gray (1967). The fluorescent dye (FITC) was added to the purified globulin at pH 9.5 (0.5M carbonate buffer) to give a theoretical ratio of 0.04 mg FITC/mg protein, and left overnight at $4^{o}C$. Unfortunately an error in protein determination (Biuret method; Gornall, Bardawill & David, 1949) resulted in a ratio of only 0.006 mg

FITC/mg protein.

Unconjugated dye and protein were removed by filtration through a 20-cm long Sephadex gel column (G-25 coarse) equilibrated with pH 7.3 saline. The conjugate filtered through in 15-30 minutes, whereas the unbound components took several hours. The fluorescent antiserum was dispensed into small tubes for long-term storage at -40°C. When in use, it could be stored at 4°C for several weeks without deterioration.

Staining - direct method

Bacteria were thinly smeared on a clean glass slide, heat-fixed and a drop of labelled antiserum added. After 30 min - 1 hr at 25°C in a moist chamber, the slide was rinsed in several changes of normal saline (pH 7.3) and blotted dry. The preparations were mounted in carbonate-buffered glycerol (glycerol : water 9 : 1 (v/v), pH 9.5). Up to 20 isolates could be stained on one slide.

Microscopy

The preparations were examined under a Leitz Ortholux microscope fitted with a 200-W mercury vapour lamp. The most successful filter combination, in terms of maximum fluorescence with minimum background, consisted of a BG38 (4 mm) and two BG12 (5 and 1 mm) primary filters and a K510 suppression filter.

Initially the cells were observed with an oil immersion objective (x100), but later a phase contrast system with an H.P. objective (x40) was used. This slightly decreased the brightness of stained cells, but made their location in white light much more easy.

A series of trial stainings showed that the fluorescent antiserum selectively stained the local isolates of Agrobacterium sp. and some imported biotype 2 isolates, but not biotype 1 isolates or Escherichia coli. The homologous isolates occasionally stained quite brightly, but more often only fluoresced dimly. Sometimes there was even doubt as to whether staining had occurred.

The medium on which the bacteria had been cultured, proved to be important for fluorescent staining. Cells cultured in mannitol-glutamic-biotin broth or on lactose agar, yeast mannitol agar, nutrient agar plus glucose (Appendix 2) or the erythritol-based selective medium (Table 17) usually reacted well with the fluorescent antiserum. Cells grown on nutrient agar fluoresced dimly or not at all.

This is probably due to the production of very little capsular polysaccharide on a medium with a low carbohydrate level. Keane (personal communication) concluded that most of the soluble antigens of Agrobacterium sp. were polysaccharides, and this is probably also the case with cell-surface antigens involved in the fluorescent antibody test.

The fluorescent antiserum was diluted in several stages up to 1 : 10 in normal saline (pH 7.3), and tested with smears of isolate 34, but only the 1 : 1 dilution and the undiluted antiserum gave any fluorescence. Undiluted antiserum was used in all other tests. The very low titre of the fluorescent antiserum is probably due to the suboptimal FITC : protein ratio of the conjugation mixture (Lewis et al., 1964).

Most isolates were tested by the direct fluorescent antibody method, during harvesting of cultures for electrophoresis. The results are given in Table 7.

Staining by the indirect method

Weller & Coons (1954) describe an indirect fluorescent antibody technique which gives about ten times brighter staining than the direct method. A modification of their technique was used (Francki & Carter, 1970): Heat-fixed bacterial smears were flooded with antiserum to isolate 34, incubated 15 minutes in a moist chamber and rinsed with normal saline (pH 7.3). A small drop of fluorescein-conjugated goat anti-rabbit-globulin serum (The Sylvana Co., Milburn, New Jersey) was then mixed with the film of saline on the slide, which was incubated a further 15 minutes, rinsed, blotted dry and mounted in buffered glycerol (pH 9.5).

Smears of isolates 15, 34 and 35 were stained by the indirect method and controls were prepared using serum from a non-immunised rabbit. The controls did not fluoresce, but cells of all three isolates fluoresced brightly in the treatments with isolate 34 antiserum. This can be attributed to the presence of a common antigen in all isolates (Keane et al., 1970), which the indirect method is sensitive enough to reveal.

In an attempt to prevent staining of isolate 15 with the antiserum to isolate 34, the antiserum was diluted in steps up to 1/320 in normal saline (pH 7.3). Table 6 shows that at dilutions in excess of 1/40 isolate 15 did not fluoresce, while isolates 34 and 35 fluoresced brightly. A dilution of 1/80 was adopted as standard, to give bright staining of homologous and closely related bacteria with no possibility of cross-reaction with biotype 1 agrobacteria. For routine use, the 1/80 dilution was prepared in 50% glycerol : 50% normal saline (pH 7.3), because this preparation can be stored below 0°C without freezing.

A number of isolates were tested by the indirect method, using the 1/80 antiserum dilution, and gave the same results as by the direct method. Isolates 54-62 were only tested by the indirect method. The results are presented in Table 7.

Table 6

Fluorescence of isolates 15, 34 and 35 with dilutions of isolate 34 antiserum. The indirect fluorescent antibody method was used

Dilution of antiserum	Level of fluorescence isolate 15	isolate 34	isolate 35
1/4	++	++	++
1/8	+	++	++
1/16	+	++	++
1/40	-	++	++
1/80	-	++	++
1/160	-	++	++
1/320	-	++	+

++ bright or moderately bright

\+ weak fluorescence

\- no fluorescence visible

All biotype 2 agrobacteria fluoresced, with the exception of A. tumefaciens isolates 29 and 30 and A. rhizogenes isolates 47 and 48; these isolates gave a weak reaction against isolate 34 antiserum in gel diffusion tests (Keane et al., 1970). No fluorescence was obtained with biotype 1 agrobacteria or with other bacteria, such as E. coli, Aerobacter aerogenes or Rhizobium trifolii.

Isolate 62 fluoresced and gave a moderate gel immunodiffusion reaction with isolate 34 antiserum. It was isolated from soil near a eucalypt trunk, on the selective medium for biotype 2 agrobacteria, but is biochemically distinct from this group (Kerr, personal communication).

ELECTROPHORESIS

Gel electrophoresis of proteins was pioneered by Smithies (1955) and is used to separate a mixture of proteins into bands determined by the rate of migration of proteins through a gel in an electric field. The position of a band is characteristic of the protein of which it consists, and in closely related organisms, say, within a species, it is probably reasonable to assume that proteins with the same mobility are homologous (Turner, 1969). Insofar as the protein complement of an organism is an expression of its genotype, comparison of electrophoretic patterns may be a useful taxonomic tool.

Discontinuous gel electrophoresis, which gave better resolution of proteins into thinner bands than Smithies' technique, was developed by Ornstein & Davis (Ornstein, 1964). It differs from Smithies' zone electrophoresis, in that the proteins pass through a coarse-pore gel of lower pH to concentrate them into a thin band, before being separated in the higher pH fine-pore gel. "Disc" electrophoresis of proteins has already been used for some isolates of Agrobacterium tumefaciens and A. radiobacter and A. rhizogenes (Huisingh & Durbin, 1967), as well as in the characterization of Erwinia amylovora and other bacteria associated with fireblight (Smith & Powell, 1968).

In the present study the protein patterns of 52 isolates were determined.

Growing Bacteria for Electrophoresis

Most isolates were grown in mannitol-glutamic acid-biotin broth (Appendix 2). In the case of the A. rubi isolates (43 and 44), the medium also contained 100 ug/l. each of filter-sterilized nicotinic acid and calcium pantothenate. The A. rhizogenes isolates 47 and 49-52 grew poorly on the basal medium; for these isolates, it was supplemented with 0.01% yeast extract.

The medium was inoculated with two-day old nutrient agar slopes, shaker incubated at 25°C for 2 days and the bacteria harvested by centrifugation (20,000g, 15 minutes). The bacterial paste was washed

once in sterile distilled water, spun down and stored at -40°C. To check purity of inoculum, all isolates were re-tested during harvesting for pathogenicity to tomato plants, for ketolactose production (Bernaerts & De Ley, 1963) and for fluorescence with the fluorescent isolate 34 antiserum.

The possibility that the protein patterns may vary with age of cultures was tested by disc electrophoresis of proteins from 24-hour (log phase) and 60-hour (stationary phase) cultures of representative isolates 1 (A. radiobacter), 15, 24 and 29 (A. tumefaciens) and 34 and 39 (Agrobacterium sp.). There were slight differences in protein patterns of bacteria harvested at different times. A few bands varied in intensity, were slightly displaced or even absent, but these small differences were no greater than for organisms grown and harvested on separate occasions, under nearly identical conditions.

As time of harvest was shown to have little effect on the protein pattern, it was not necessary to closely specify incubation time. A two day incubation period gave reasonable yields of bacteria in the minimum time, about 1 g wet weight of cells from 250 or 500 ml of culture medium.

Disruption of Bacteria

For starch gel electrophoresis, the three isolates were disrupted in Eaton's (1962) modification of the Hughes Press, in which frozen

bacteria are forced through a narrow orifice under pressure. They were then suspended in their own volume of Tris-citrate buffer (0.076M, pH 9.0) and ultracentrifuged (130,000g, 45 min.) to remove cell debris. The supernatant was electrophoresed the same day.

The ultrasonic disruption method of Huisingh & Durbin (1967) was used to prepare all the extracts used for polyacrylamide disc electrophoresis. The bacterial paste, diluted in 3 to 15 times its weight of protein extraction medium (Appendix 2) to give a final volume of more than 3 ml, was cooled in an icebath and treated for 5 minutes in an MSE ultrasonic disintegrator. Cell debris was spun down (25,000g, 30 min.) and the supernatant stored at -40°C until required for electrophoresis. The protein content of the supernatant was determined by the Biuret method (Gornall et al., 1949)

Starch Gel Electrophoresis

The method of Clare, Flentje & Atkinson (1968), which is a modification of Poulik's (1957) discontinuous buffer system, was used. Protein extracts of isolates 1, 15 and 34 were absorbed in pieces of filter paper (5x8 mm) and placed in slots in the gel; the protein load was doubled by placing two filter paper rectangles in some slots. Electrophoresis at a constant current of 30 mA continued for approximately 2 hr, until the citrate-borate buffer interface had migrated 7.5 cm past the sample slots towards the anode. The gels were then sliced

horizontally and stained with amido black 10B (Chroma Gesellschaft, Stuttgart, W. Germany).

About 20 protein bands could be distinguished. The patterns for isolates 1 and 15 appeared to be identical and fairly similar to that of isolate 34, if only band position were considered; isolate 34 differed only in having two fine bands in a position where isolates 1 and 15 had one, and in apparently lacking one other band. If band intensity is considered, isolate 34 is easily distinguished from the other two.

Because polyacrylamide disc electrophoresis subsequently gave better resolution of protein bands, and had been used for agrobacteria by other workers (Huisingh & Durbin, 1967), the starch gel method was not used for any other isolates.

Polyacrylamide Disc Electrophoresis

Disc electrophoresis was carried out by the method of Davis (1964) in an apparatus essentially the same as that described by Ornstein (1964). Electrophoresis could be conducted concurrently in 12 gels, each of which was 6 mm in diameter and consisted of 1 cm coarse-pore gel over 5 cm fine-pore gel.

Protein extract was layered on top of each gel, as the extract was dense and viscous enough to avoid turbulent mixing with the buffer solution. A load of 50-70 μg protein nitrogen gave best results.

This is approximately twice the load used by Huisingh & Durbin (1967), though probably only one-third greater on a basis of protein per cross-sectional area of gel. A drop of bromophenol blue was then added to the buffer (Tris-glycine, pH 8.3) in the upper (cathode) reservoir and a current of 4 mA per tube applied until the dye marker band had reached 5 mm from the end of the gel. This usually took 1 hr 20 min. The whole apparatus was kept inside a refrigerator at 2°C, to prevent undue electric heating of the gels.

Initially, the gels were stained in Amido Black and destained electrophoretically (Davis, 1964), but this method was soon replaced by the method of Chrambach et al. (1967), in which protein bands are fixed in 12.5% trichloracetic acid and stained with Coomassie Brilliant Blue. Comparisons of the patterns of the six isolates used in the age-of-cultures experiment showed no difference between the two stains. The Coomassie Blue method eliminates the need for electrophoretic destaining, but the bands fade slowly and the gels shrink irreversibly.

Evaluation of Protein Patterns

In the past, the presence or absence of bands has often been taken as the only criterion for the comparison of protein patterns (e.g. Clare et al., 1968). In a short gel with a relatively large number of bands (30 bands in 4.5 cm) this approach becomes very difficult, especially when bands are slightly displaced. Apart from controllable

factors such as slight variations in gel length, displacement of bands is sometimes due to differences in electrical conductivity, caused by differing protein compositions of different samples (Smithies, 1955).

Peterson & Latch (1969) point out that some bands must be multiple in nature, that is, composed of more than one protein. Therefore band intensity should also be considered in conjunction with band position. Huisingh & Durbin (1967) apparently have adopted another criterion in comparing patterns for Agrobacterium isolates; they only map the seven or eight "major" bands, which are consistently present in the extracts from each species, and disregard the "minor" bands which indicate variability between isolates within a species.

The somewhat subjective approach, involving both band position and intensity, has been adopted in the comparison of the protein patterns in this study. Closer examination of representative patterns confirmed that this approach resulted in the same groupings of patterns, as when band position was the only criterion used.

Replication

Each isolate was grown, harvested and electrophoresed on two separate occasions. On the few occasions that the protein patterns of the replicates did not match, a third extract was prepared. In each case, two of the three patterns were identical and the different pattern could be attributed to contamination, on the basis of the purity checks.

Results

The protein patterns could be divided into five classes (Table 7). The biotype 2 isolates form a very well-defined electrophoretic class (Group B); all the protein patterns were almost identical to those of isolates 30, 32, 39, 46 and 59, except for isolate 60, which is however more similar to group B than to any of the other patterns (Plate 3B). Group B contains tumour-inducing, root-proliferating and non-pathogenic forms.

All the ketolactose-positive A. tumefaciens and A. radiobacter isolates formed a more variable group (A). That it is a valid group is rather hard to say, but comparison of all the patterns did not reveal any smaller, more coherent groups within A, so the variability in protein patterns is probably an expression of the natural range of variation of this class of bacteria.

Contrary to the results of Huisingh & Durbin (1967), there was no division corresponding to the pathogenicity difference between the two species. Moreover, the so-called "major bands" illustrated in their paper did not appear to match any continually reappearing bands observed in the present study. The interspecific differences they observed may be due to their having tested too few isolates, namely, only five of A. radiobacter and six of A. tumefaciens. Plate 3A shows two pairs of patterns in which similarity between A. radiobacter and

Table 7

Characteristics of the 62 isolates

Isolate	Biotype*	Reaction to* antisera of isolate: 15	34	Reaction to fluorescent antibody of isolate 34**	Protein pattern group	Pathogenicity
1	1	+++	-	- 1,2	A	NP
2	1	+++	-	n.t.	n.t.	NP
3	1	++	-	n.t.	n.t.	NP
4	1	++	-	n.t.	n.t.	NP
5	1	++	-	- 1	A	NP
6	1	+	-	- 1	A	NP
7	1	++	-	- 1	A	NP
8	1	++	-	n.t.	n.t.	NP
9	1	++	-	- 1	A	NP
10	1	++	-	- 1	A	NP
11	1	++	-	- 1,2	A	NP
12	1	++	-	- 1	A	NP
13	1	++	-	- 1	A	NP
14	1	++	-	- 1	A	NP
15	1	+++	-	- 1,2	A	TI
16	1	++	-	n.t.	n.t.	TI
17	1	+++	-	- 1,2	A	TI
18	1	+++	-	n.t.	n.t.	TI
19	1	++	-	- 1,2	A	TI
20	1	++	-	- 1	A	TI
21	1	++	-	n.t.	n.t.	TI
22	1	++	-	- 1,2	A	TI
23	1	++	-	- 1	A	TI
24	1	++	-	- 1,2	A	TI
25	1	+	-	- 1	A	TI
26	1	++	-	- 1	A	TI
27	1	++	-	n.t.	n.t.	TI
28	1	++	-	- 1	A	TI
29	2	-	+	- 1,2	B	TI
30	2	-	+	- 1,2	B	TI
31	2	-	+	+ 1,2	B	TI
32	2	-	++	+ 1,2	B	TI
33	2	-	++	+ 1	B	TI
34	2	-	+++	+ 1,2	B	TI
35	2	-	++	+ 1,2	B	TI
36	2	-	++	n.t.	n.t.	TI
37	2	-	++	+ 1	B	TI

Table 7 (continued)

Isolate	Biotype*	Reaction to* antisera of isolate: 15	34	Reaction to fluorescent antibody of isolate 34**	Protein pattern group	Patho-genicity
38	2	-	+++	+ 1	B	TI
39	2	-	+++	+ 1	B	TI
40	2	-	+++	+ 1,2	B	TI
41	2	-	++	+ 1	B	TI
42	2	-	++	n.t.	n.t.	TI
43	2	-	-	- 1	C	TI
44	2	-	-	- 1	C	TI
45	2	-	+	+ 1,2	B	RP
46	2	-	++	+ 1,2	B	RP
47	2	-	+	- 1,2	B	RP
48	2	-	+	- 1,2	B	RP
49	1	+	-	- 1	D	RP
50	1	-	-	- 1,2	D	RP
51	1	+	-	- 1	D	RP
52	1	-	-	- 1	D	RP
53	n.t.	n.t.	n.t.	+ 1	B	TI
54	2	-	++	+ 2	B	TI
55	2	-	-	+ 2	B	TI
56	n.t.	n.t.	n.t.	+ 2	B	TI
57	n.t.	n.t.	n.t.	+ 2	B	TI
58	2	-	-	+ 2	B	NP
59	2	-	+	+ 2	B	NP
60	n.t.	n.t.	n.t.	+ 2	B	NP
61	2	-	++	+ 2	B	NP
62		-	++	+ 2	E	NP

+++, strong reaction; ++, moderate reaction; +, weak reaction, -, no reaction. NP, non pathogenic; TI, tumour-inducing; RP, root-proliferating (suppliers' designation); n.t., not tested.

* From Keane, Kerr (personal communication).

** Determined by, 1. the direct method, 2. the indirect method.

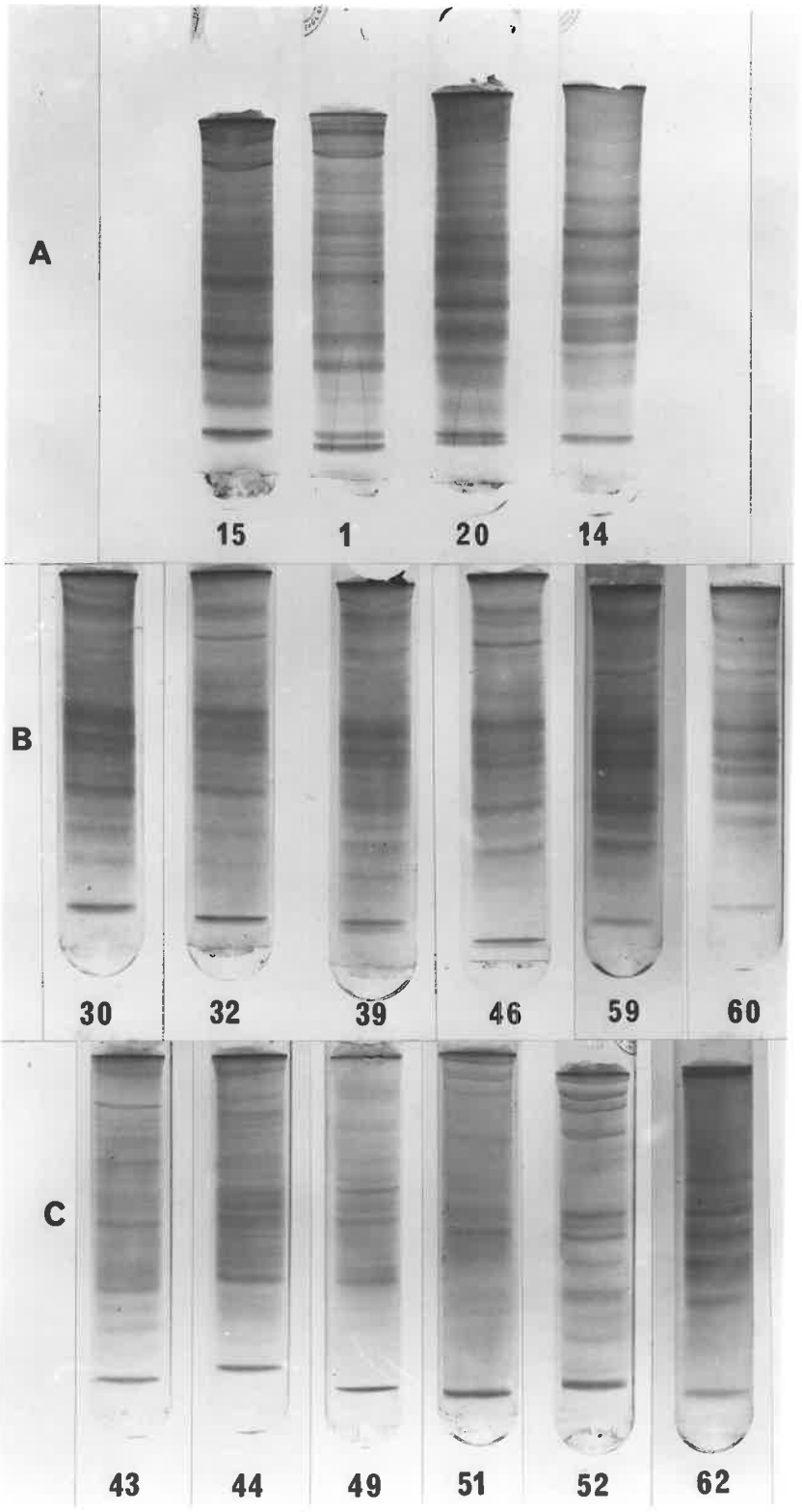
Plate 3

Protein patterns of Agrobacterium isolates following disc electrophoresis.

A. Electrophoretic group A. Isolates 15 (tumorigenic) and 1 (non-pathogenic); 20 (tumorigenic) and 14 (non-pathogenic).

B. Electrophoretic group B. Isolates 30, 32 and 39 (tumorigenic); 46 (root-proliferating); 59 and 60 (non-pathogenic).

C. Electrophoretic groups C, isolates 43 and 44; D, isolates 49, 51 and 52; E, isolate 62.


A
15
1
20
14
B
30
32
39
46
59
60
C
43
44
49
51
52
62

A. tumefaciens is greater than the similarity between the patterns in the same species.

The two A. rubi isolates (43 and 44) and the biotype 1 A. rhizogenes isolates (49 - 52) have been placed in separate electrophoretic classes, C and D respectively (Plate 3C). Within each of these classes there is considerable pattern uniformity; they are distinct from group B and it has not proved possible to include them within the range of variability of group A.

Isolate 62 had a pattern, designated "group E" (Plate 3C), which was quite unlike that of any other isolate. Although it was isolated on the selective medium for Agrobacterium sp., its reaction in biochemical tests is quite different from other isolates of Agrobacterium sp. (Kerr, personal communication), so its flagellation was examined to see whether it could be distinguished by this character.

FLAGELLATION

The genus Agrobacterium Conn has degenerate peritrichous flagellation (Conn, 1942) which may be mistaken for monotrichous flagellation in some cases (Conn & Elrod, 1947). Keane (personal communication) showed that Agrobacterium sp. (isolate 34) also has peritrichous flagella. The flagellation of a non-pathogenic form of Agrobacterium sp. (isolate 58) and of the anomalous isolate 62 is reported here. Hanging drop preparations of both isolates contained some motile

cells.

Bacteria were examined under an electron microscope following negative staining by a method similar to that used by Basit, Francki & Kerr (1970). The bacteria were grown for two days in nutrient broth (Appendix 2) without agitation. Formvar-coated electron microscope grids were inverted for one minute on the surface of the broth cultures and air-dried. At this point, some grids were gently washed by immersion in distilled water to remove the salts of the nutrient broth, which might obstruct the electron beam. The preparations were stained for about one minute by inversion on the surface of a drop of 0.5% phosphotungstic acid (pH 6.8). The liquid on the grid was reduced to a film by draining off the excess with a filter paper, and the grid allowed to air-dry.

The cells of both isolates were bacilliform, but very few flagellate cells were seen. The simplest procedure gave the best results; washing the grids by immersion in distilled water also washed off some of the bacterial cells, and gave no advantage in clarity of preparations. Some of the bacterial suspensions were mixed with 5% bovine serum albumin (Difco Laboratories, Inc., Detroit) to help stick the bacteria and flagella to the grid, but the extra manipulations resulted in fewer flagellate cells being seen.

About 20% of isolate 62 cells had flagella, which were arranged

peritrichously, one or a few per cell. Fewer isolate 58 cells had flagella, but they were also peritrichous.

Therefore, isolate 62 cannot be distinguished from the Agrobacterium isolates by flagellation.

DISCUSSION

The results of biochemical tests (Table 2) indicated that almost all the isolates studied fell into two distinct biotypes, which could be distinguished by ten critical tests. This division is paralleled in the classes formed as a result of serology and gel electrophoresis of the bacterial proteins.

Biotype 1 contains tumour-inducing, root-proliferating and non-pathogenic forms. Isolates 1-14 are non-pathogenic and appear to be typical of A. radiobacter. Isolates 15-28 induce tumour formation in tomatoes, but otherwise cannot be distinguished from isolates 1-14, and to assign them to a separate species, A. tumefaciens, on the basis of pathogenicity alone can hardly be justified.

Huisingh & Durbin (1967) reported that A. radiobacter and A. tumefaciens could be distinguished by their electrophoretic patterns. The differences they observed were probably due to testing too few isolates of each species; with fourteen isolates of each, I was unable to find any constant difference between A. radiobacter and A. tumefaciens.

Serological studies by Schroth *et al.* (1971) suggested that tumorigenic isolates possess a distinctive antigen which is usually absent in *A. radiobacter*; however, one of their fourteen strains of *A. radiobacter* possessed the antigen.

On the other hand, evidence from other serological work (Graham, 1963, 1971), from comparison of DNA base compositions (De Ley *et al.*, 1956), from DNA homology (Heberlein *et al.*, 1967) and from numerical taxonomy involving over 100 characters (Graham, 1964; Moffett & Colwell, 1968) all supports the amalgamation of *A. radiobacter* and *A. tumefaciens* in one species.

The position of the remaining isolates in biotype 1, the *A. rhizogenes* isolates 49-52, is not so clear. They differ from the other isolates in biotype 1 in requiring growth factors, in their protein patterns and in their weak or negative serological reaction. However, except in their reputed root-proliferating characteristics, they are quite different from *A. rhizogenes* as described by Riker *et al.* (1930) and biochemically are much closer to other biotype 1 isolates than to isolates 45-48, which are also designated *A. rhizogenes*. The most satisfactory solution would seem to be inclusion in *A. radiobacter* biotype 1, with a varietal epithet to specify pathogenicity. Isolate 50 induced gall formation on carrots and should be assigned to var. *tumefaciens*.

On a biochemical basis, biotype 2 coincides with the definition (Riker et al., 1930) of A. rhizogenes, but it includes tumour-inducing and non-pathogenic isolates, as well as root-proliferating forms. With the exception of isolates 43 and 44, the biotype 2 isolates form a remarkably uniform group. De Ley et al. (1966), Heberlein et al. (1967) and De Ley (1968) reported small differences in the range of variation of DNA base composition (%GC) and slightly lower DNA homology between A. rhizogenes and A. radiobacter - A. tumefaciens, than between A. rhizogenes and Rhizobium leguminosarum. They proposed the removal of A. rhizogenes from Agrobacterium to Rhizobium. The discovery of tumorigenic and non-pathogenic forms so closely related to A. rhizogenes means that all these biotype 2 agrobacteria would have to be placed in Rhizobium. It is doubtful whether the differences between the biotypes are sufficient to justify separation into two separate genera. However, if the A. rhizogenes data (De Ley et al., 1966; Heberlein et al., 1967) applies to the whole of biotype 2, there would seem to be a small, but fundamental, difference in the DNA of biotypes 1 and 2.

The position of the two A. rubi isolates (43 and 44) is doubtful. They can be distinguished from other isolates in biotype 2 by their negative serological reaction, by their distinct protein patterns, and by their growth-factor requirements. In fact, DNA homology would place A. rubi isolate 43 in the A. tumefaciens - A. radiobacter group,

rather than with ketolactose-negative A. rhizogenes in biotype 2 (Heberlein et al., 1967). Many more isolates would have to be tested to clarify the position, but it is clear that the pathogenicity of A. rubi is not restricted to Rubus spp., so there is no good reason for separating it from other tumorigenic agrobacteria (McKeen, 1954; De Ley et al., 1966). McKeen (1954) described some cane-gall organisms which gave an acid reaction in litmus milk, whereas others produced an alkaline reaction. Further studies could reveal this limited-host-range tumorigenic nomen-species to contain both biotype 1 and 2 organisms.

Isolate 62 seems to be biochemically distinct from the other isolates and has a distinct protein pattern following disc electrophoresis, but it reacted serologically with biotype 2 antiserum and was isolated on the selective medium for biotype 2 agrobacteria (Part C). It could not be distinguished from other Agrobacterium isolates by its flagellation. It is probably a member of a third biotype of Agrobacterium, although it may belong to some other group of soil-inhabiting bacteria.

The present division of the genus Agrobacterium into species according to pathogenicity is unsatisfactory for several reasons. On the one hand, isolates which would be designated A. radiobacter under the present system of classification are indistinguishable biochemically, serologically and electrophoretically from others that would be designated A. tumefaciens. On the other hand, biotype 2 contains otherwise identical

non-pathogenic, tumorigenic and root-proliferating isolates which are readily distinguishable from biotype 1 non-pathogens and pathogens by the above tests.

The report (Kerr, 1971) that A. radiobacter can be converted to A. tumefaciens through transfer of virulence is further evidence that pathogenicity is an unsatisfactory basis for speciation.

Therefore A. radiobacter, A. tumefaciens, A. rhizogenes and A. rubi should be included in one species which, according to the International Code of Nomenclature of Bacteria and Viruses (1958), should be A. radiobacter unless Agrobacterium is incorporated into Rhizobium, as proposed by Graham (1964), De Ley (1968) and Moffett & Colwell (1968).

With the exception of isolate 62, all the isolates studied could be accurately defined by giving a varietal epithet to indicate pathogenicity and by specifying biotype. The 61 isolates would be grouped as follows:

A. radiobacter var. radiobacter biotype 1 - isolates 1-14

A. radiobacter var. tumefaciens biotype 1 - isolates 15-28, 50

A. radiobacter var. rhizogenes biotype 1 - isolates 49, 51, 52

A. radiobacter var. radiobacter biotype 2 - isolates 58-61

A. radiobacter var. tumefaciens biotype 2 - isolates 29-44, 53-57

A. radiobacter var. rhizogenes biotype 2 - isolates 45-48

PART C: DEVELOPMENT OF A SELECTIVE MEDIUM FOR *A. RADIOBACTER* BIOTYPE 2

INTRODUCTION

Tumorigenic biotype 2 agrobacteria are apparently more important than biotype 1 as the cause of crown gall in at least one South Australian nursery (Kerr, 1969), but a study of their ecology was restricted by the lack of a suitable selective medium to isolate biotype 2 organisms from soil.

An excellent medium for most biotype 1 agrobacteria is available (Schroth *et al.*, 1965), though it does not distinguish tumorigenic bacteria from non-pathogens. The earliest selective medium for agrobacteria (Patel, 1926) is unsuitable because it allows growth of both biotypes, and contamination of plates by other soil organisms can make identification of *Agrobacterium* impossible.

In 1968, P.J. Keane (personal communication) began to develop a selective medium for biotype 2 agrobacteria (Appendix 3), based on differential antibiotic sensitivity of the two biotypes. Although this medium was not successful, the information he obtained on antibiotic effects has been very useful in developing a selective medium.

BASIS OF DIFFERENTIAL SELECTION BETWEEN BIOTYPES 1 AND 2

The high selective efficiency of the medium of Schroth *et al.* (1965) (Appendix 3) is due mainly to the minimal requirements for

growth of A. radiobacter biotype 1. A very large number of soil microorganisms, including biotype 2 agrobacteria, are eliminated by their inability to grow on a medium containing only essential minerals, mannitol, and sodium nitrate. The remaining microorganisms are inhibited by antibiotics and by toxic substances such as berberine, calcium propionate and sodium selenite.

A. radiobacter biotype 2 requires biotin but otherwise has the same nutrient requirements as biotype 1; the root-proliferating forms and a variant form of isolate 34 also require glutamic acid (Keane, personal communication). The most likely way to selectively inhibit growth of biotype 1 would be by antibiotics or by the use of a carbon-source which only biotype 2 could utilize.

These two possibilities were explored, using isolates 15 and 34 as test organisms; they were considered to be representative of biotype 1 and biotype 2 respectively.

Antibiotics were tested for differential inhibition of the two isolates, by the filter paper disc method. Each isolate was spread over the surface of several plates of mannitol-glutamic acid-biotin agar (Appendix 2) at a rate of 10^6 to 10^7 cells per plate, and antibiotic discs (Multodiscs 11-14B, 11-14C, 11-15F, S1; Oxoid Ltd, London) were placed on the agar after the surface had dried. After two days' incubation the bacteria had formed a continuous lawn over all the plates

except for zones of inhibition around some antibiotic discs.

Most antibiotics were at least as inhibitory to isolate 34 as to isolate 15; only with novobiocin and neomycin was there greater inhibition of isolate 15 than isolate 34, and the differences were not great (Table 8). With neomycin, the zone of inhibition was only slightly greater than the diameter of the antibiotic disc. Novobiocin was used in Keane's selective medium, but is unstable in solution and gave variable results, so it was not considered for inclusion in the proposed biotype 2 selective medium. None of the antibiotics so far tested could be considered as a possible basis of a biotype 2 selective medium.

The other possibility, of using a carbon source as the basis of selectivity, was examined. A. radiobacter biotype 2 differs from biotype 1 in being able to utilize malonate or erythritol as a source of carbon (Keane et al., 1970). D.C. Sands (personal communication) found that an A. rhizogenes isolate could utilize L-tyrosine and isobutanol, as well as erythritol and malonate, whereas three A. tumefaciens isolates could not. Since many A. rhizogenes isolates belong to biotype 2, these chemicals were also tested. The four compounds were each included as sole carbon source in basal media containing all the other requirements for growth of biotype 2 agrobacteria (mannitol-glutamic acid-biotin agar minus mannitol,

Table 8

Inhibition of isolates 15 and 34 by antibiotic discs

Antibiotic	concentration (μg per disc)	Zone of inhibition (diam., mm) Isolate 15	Isolate 34
bacitracin	5	-	-
chloramphenicol	10	± 9	± 12 r
	50	+ 22 r	+ 22
erythromycin	10	± 10	+ 20 r
	50	+ 14	+ 30
neomycin	10	+ 10	± n.m.
nitrofurantoin	200	+ 20	+ 30
novobiocin	5	+ 20	+ 18
	30	+ 34	+ 30
oleandomycin	5	-	-
	10	-	-
penicillin G	0.94*	-	-
	3.14*	-	-
polymixin B	10	± n.m.	+ 13
streptomycin	10	-	-
	25	-	-
sulfafurazole	100	+ 40	+ 50 r
	500	+ 48 r	+ 60 r
tetracycline	10	+ 26	+ 44
	50	+ 32	+ 52

- no inhibition

± variable result

r resistant colonies in the zone of inhibition

n.m. not measured

* 1.5 and 5 international units

Appendix 2) and growth of isolates 15 and 34 was compared on each medium.

The carbon sources, except for L-tyrosine which was not sufficiently soluble, were added as filter-sterilized solutions to the autoclaved molten basal medium just before pouring plates. L-tyrosine was added as the dry powder. The final concentration of the carbon sources was low (0.1%), to minimize the chance of spurious results due to possible impurities in the chemicals. Two-day old nutrient agar cultures of isolates 15 and 34 were suspended in sterile distilled water and plated on the media at dilutions sufficient to give discrete colonies. The results are summarized in Table 9.

Only erythritol showed promise as a selective carbon source. Both isolates produced minute colonies on the other carbon sources, and isolate 15 grew no better on erythritol. Despite some crowding of colonies, isolate 34 grew well on erythritol.

Thirteen other isolates were streaked from nutrient agar onto the erythritol medium. Biotype 1 isolates 5, 9, 11, 19, 20, 23 and 24 did not grow, but biotype 2 isolates 29, 32, 35, 37, 39 and 47 all grew well. This confirmed that erythritol could be used as the basis of the biotype 2 selective medium.

Biotype 1 isolates fail to grow on the erythritol medium simply because they are unable to utilize erythritol, and not because of any

Table 9

Comparison of carbon sources: Growth of isolates 15 and 34 on minimal media with each compound as the sole carbon source

Carbon source	Growth of	
	Isolate 15	Isolate 34
sodium malonate	-	-
isobutanol	-	-
erythritol	-	+
L-tyrosine	-	-

toxic effect. If a small amount of some other carbon source, such as glucose, is added to the medium, they grow normally.

DEVELOPMENT OF A SELECTIVE MEDIUM BASED ON ERYTHRITOL

The erythritol medium used in the previous section prevents growth of biotype 1, while permitting growth of biotype 2 agrobacteria, but it would not be suitable for isolating biotype 2 from soil, because it supports growth of many other soil microorganisms. The possibility of inhibiting some of these contaminants with antibiotics was examined.

In the course of testing various antibiotic combinations, the composition of the basal erythritol medium was altered in several respects. The compositions of the modified basal media are given in Table 10. Erythritol is stable to heat, so it was added to the other ingredients before autoclaving.

The antibiotics and inhibitory substances considered for inclusion in the medium were those of the biotype 1 medium of Schroth *et al.* (1965) and those of the *Rhizobium* medium of Graham (1969) (Appendix 3). It seemed likely that at least some of the antibiotics in these media would prove useful in the biotype 2 medium, because of the similarity of these groups to *A. radiobacter* biotype 2. Polymixin B was the only antibiotic tested which was not in these media; it is surface-active against gram-negative bacteria and was used in an attempt

Table 10

Composition (g per litre) of the basal media used in the development of the biotype 2 selective medium

		(g/l)	Basal Medium G	LG	LN	HN	MS
erythritol			1	1	1	5	1
N-source:	L-glutamic acid		2	0.5	-	-	0.5
	$NaNO_3$		-	-	0.5	2.5	-
Minerals:	$CaCl_2$	0.2	+	+	+	+	-
	$NaHCO_3$) $MgCO_3$)	0.075	-	-	-	-	+
calcium propionate		1.2	-	-	-	-	+
Minerals:	KH_2PO_4	0.1))					
	$MgSO_4.7H_2O$	0.2))					
	NaCl	0.2))					
	FeEDTA (0.65%)*	2 ml))	+	+	+	+	+
biotin		2 µg))					
agar		18))					
cycloheximide		0.250)					

pH 7.0

* See Appendix 2.

to inhibit growth of certain gram-negative cocci which formed spreading colonies on some basal media.

The various antibiotic solutions were added to the molten basal media at 50-55° and plates were poured immediately. The anti-fungal antibiotic, cycloheximide, was not inhibitory to isolate 34 (Keane, personal communication) and was used in all media.

Two-day old nutrient agar cultures of isolate 34 were suspended in sterile distilled water and plated out at a dilution giving approximately 50 colonies per plate; only those antibiotic combinations which allowed good colony growth were considered for the selective medium. Their efficiency in inhibiting other soil microorganisms was determined by plating out a mixed suspension of isolate 34 cells and a 1 : 10 or 1 : 100 dilution of Berri sand, which is devoid of biotype 2. A 0.1 ml aliquot of the suspension was spread over each plate, and the effectiveness of the potential selective medium was judged from the growth of contaminants and the ease of identifying colonies of isolate 34.

First, basal medium G was used with various combinations of three of the antibiotics, chloramphenicol, neomycin and penicillin, of the medium of Graham (1969). The other antibiotics in Graham's medium were omitted, PCNB because it unnecessarily duplicated the anti-fungal activity of cycloheximide, and sulfathiazole because it was not available and is probably highly inhibitory to isolate 34, like the other sulfa

antibiotic, sulfafurazole (Table 8). Cycloheximide was present at 200 ppm, which is slightly lower than the usual level (Table 10).

The results (Table 11) confirmed that 25 ppm penicillin is definitely inhibitory, when used in combination with the other antibiotics (Keane, personal communication), and showed that chloramphenicol at 5 and 10 ppm reduces growth of isolate 34. The combinations were not tested for their ability to inhibit other soil microorganisms.

Three of the antibiotics, bacitracin, tyrothricin and streptomycin, and one of the inhibitory chemicals, sodium selenite, of the medium of Schroth et al. (1965) were used in conjunction with medium G. The concentrations used are given in Table 12. The other inhibitory compounds in the Schroth medium, calcium propionate, penicillin and berberine, were omitted; Keane (personal communication) found that berberine was inhibitory to isolate 34, when used in combination with other antibiotics.

This medium slightly inhibited the growth of colonies of isolate 34 (Table 12), but was satisfactory for isolating biotype 2 agrobacteria from a gall on tomato. Colonies of isolate 34 could be easily recognized five or six days after plating out the bacteria-soil suspension, but many were over-run by large, translucent colonies of gram-negative cocci, which appeared at day 3. Colonies identified as A. radiobacter biotype 2 were tested with fluorescent antiserum against isolate 34, and 44 out of 46 gave a positive reaction.

Table 11

Growth of isolate 34 on basal medium G plus some antibiotics of Graham's (1969) medium

Antibiotics*	Growth of isolate 34
P, C(10), Neo[1]	-
C(10), Neo[1]	+ (inhibited)
Neo[2]	+
C(10)[2,3]	+ (inhibited)
C(5)[3]	+ (inhibited)
C(1)[3]	+

1,2,3 The media were prepared and assessed at different times, as indicated by the superscripts.

* P, penicillin 25 ppm; C(1), C(5), C(10), chloramphenicol 1, 5 or 10 ppm; Neo, neomycin 2.5 ppm.

Table 12

Growth of isolate 34 on two basal media plus antibiotics, and recovery from a suspension of isolate 34 plus soil

Basal medium	Antibiotics*	Growth of isolate 34	Soil suspension + isolate 34 recovery	contaminants
G	B, T, Na, S[1]	+ (slightly inhibited)	+	spreading bacteria
	nil[2]	+ (moderately inhibited)	n.t.	n.t.
MS	B, T, Na, S[3]	-	-	few contaminants
	C, Neo[2]	-	-	extensive spreading bacteria

* B, bacitracin 100 ppm; T, tyrothricin 1ppm; Na, sodium selenite 100 ppm; S, streptomycin 3 ppm; C, chloramphenicol 2.5 ppm; Neo, neomycin 2.5 ppm.

1,2,3 The media were prepared and assessed at different times, as indicated by the superscripts.

n.t. not tested.

In an attempt to inhibit the "spreading colonies" of gram-negative cocci, which were a problem on medium G plus bacitracin, tyrothricin, streptomycin and sodium selenite, a basal medium (MS, Table 10) containing calcium propionate was used with the same antibiotics and also with chloramphenicol and neomycin. The basal medium moderately inhibited isolate 34, and there was complete inhibition in the basal medium plus antibiotics (Table 12); calcium propionate, the most likely cause of inhibition in the basal medium, was not included in any other media.

Various combinations of bacitracin, tyrothricin, sodium selenite, streptomycin and polymixin B were used in medium LG, which is similar to MS, but lacks calcium propionate and carbonates. It contains a lower level of glutamic acid than G and should restrict growth of any contaminants unable to utilize erythritol, but able to utilize glutamate as a source of both carbon and nitrogen.

The results (Table 13) show that streptomycin is inhibitory in LG. In medium G only slight inhibition was noted (Table 12); possibly the higher level of glutamate in that medium partly off-set the inhibitory effect of streptomycin. The various LG-based media were given a "contamination rating", a value on a scale of 1 to 6 which expressed increasing level of contamination. Of the combinations which permitted growth of isolate 34, bacitracin, tyrothricin, and sodium

Table 13

Comparison of antibiotic combinations in the basal medium LG: Growth of isolate 34 and recovery from a suspension of isolate 34 plus soil

Antibiotics*	Isolate 34		Contamination rating**
	Growth	recovery from soil	
PB(1)	+	-	6
PB(10)	-	-	6
B,T	+	+	5
Na,S	-	-	2
B,T,Na	+	+	3
B,T,S	± (inhibited)	+	4
B,Na,S	-	-	1
T,Na,S	-	-	1
B,T,Na,S	-	-	1
B,T,Na,S,PB(1)	-	-	1

* B, bacitracin 100 ppm; T, tyrothricin 1 ppm; Na, sodium selenite 100 ppm; S, streptomycin 3 ppm; PB(1), PB(10), polymixin B 1 or 10 ppm.

** On a scale 1-6, increasing growth of contaminating colonies from the soil

selenite gave best control of contaminants, including almost complete control of the spreading contaminants.

Polymixin B (1 ppm), chloramphenicol (1 ppm), neomycin (2.5 ppm) or chloramphenicol plus neomycin were added to medium LG plus bacitracin, tyrothricin and sodium selenite, and the various combinations assessed. None of the added antibiotics significantly reduced contamination, so the simplest combination, bacitracin, tyrothricin and sodium selenite, was retained. Whenever a medium contains these antibiotics it will be coded thus: G', LG', etc., to distinguish it from the basal medium (G, LG, etc.).

Medium LG' was used to isolate and count biotype 2 agrobacteria in eight natural peach galls, collected at a nearby nursery (Balhannah), and seven soil samples from around them. The results with soil were disappointing; of 147 colonies tentatively identified as biotype 2 seven days after plating, only 77 fluoresced with fluorescent antiserum to isolate 34.

It was possible that some soil bacteria growing on LG' could not utilize nitrate as the sole nitrogen source, so medium LN' was used in an attempt to eliminate these contaminants. A mixed suspension of Berri sand and isolate 34 was plated on LN' and LG' media. Numbers of biotype 2 colonies on the two media did not differ significantly (Table 14), showing that both are equally efficient in recovering

Table 14

Comparison of colony counts of isolate 34 on media LG' and LN'. Isolate 34 was recovered from a suspension of Berri sand

Soil dilution	Colony count (average of 3 plates)	
	LN'	LG'
10^{-2}	132	124
10^{-3}	13	14

Table 15

Comparison of media LG' and LN': Numbers of biotype 2 isolated from natural soil, and number transferred from LG' to replica plates of LN'

Soil sample	Soil dilution	Average colony count LG'	Average colony count LN'	Colonies per plate/accuracy of identification* LG'	Colonies per plate/accuracy of identification* LN' replica
1	10^{-2}	15^{1}	17	15/8	7/7
2	10^{-3}	13	19^{1}	16/16	16/16

* Number of A. radiobacter biotype 2 colonies, as determined by reaction with the fluorescent antiserum to isolate 34.

1 Only one plate at this dilution.

isolate 34.

The possibility that a significant proportion of South Australian biotype 2 isolates would be unable to utilize nitrate as sole nitrogen source, like a variant form of isolate 34 (Keane, personal communication) was investigated by replica plating (Lederberg & Lederberg, 1952). Two Balhannah soil samples were plated out at 10^{-2} and 10^{-3} dilutions on both LG' and LN'. Four days later, small colonies about 0.5 - 1 mm in diameter could be seen, and replica plates of LG' and LN' were inoculated from the original LG' plates, using a sterile velvet-covered disc as the replicator.

The numbers of biotype 2 colonies recovered on the two media, from a natural population, did not differ significantly (Table 15). Of 31 colonies on LG' which were tentatively identified as biotype 2, 24 were genuine; 23 of them appeared on the replica plates of LN', but the seven wrongly identified colonies did not develop on LN'. This suggested that medium LN' would reduce contamination by other organisms likely to be confused with A. radiobacter biotype 2.

Medium LN' had one serious drawback. Biotype 2 colonies were quite small when present in large numbers on the plate, due to competition for the carbon and nitrogen sources, which were present at low concentrations. This made it more difficult to distinguish biotype 2 colonies from contaminants. Therefore the levels of erythritol and

Table 16

Comparison of media LG', LN' and HN': Numbers of biotype 2 isolated from natural soil, and accuracy of identification

Soil dilution	Average colony counts on the selective media		
	LG'	LN'	HN'
$1 : 10^2$	-*	223	314
$1 : 10^3$	31	21	37
Accuracy of identification of biotype 2**	10/11	12/16	15/16

* Colonies small, with much contamination.

** Proportion of colonies reacting with the fluorescent anti-serum to isolate 34.

Table 17

Composition of the selective medium for *A. radiobacter* biotype 2

agar	18.0 g
erythritol	5 g
$NaNO_3$	2.5 g
KH_2PO_4	0.1 g
$CaCl_2$	0.2 g
NaCl	0.2 g
$MgSO_4.7H_2O$	0.2 g
FeEDTA (0.65%)*	2 ml
biotin	2 µg
distilled water	1 litre
1 N NaOH to pH 7 (approximately)	

The basal medium is autoclaved and cooled to 50-55°, when the following antibiotics and inhibitory chemical are added to give a final concentration of:

cycloheximide	250 ppm
bacitracin	100 ppm
tyrothricin	1 ppm
sodium selenite	100 ppm

* See Appendix 2.

sodium nitrate were increased to 0.5% and 0.25% respectively (medium HN, Table 10).

Medium HN' compared favourably with media LN' and LG' in its efficiency in isolating biotype 2 from one of the Balhannah soil samples (Table 16). The biotype 2 colonies were larger than those on LN'; on a plate with about 300 colonies, they were convex and 2-2.5 mm in diameter and could be easily identified.

Six soil samples were collected from around galled stone fruit at another nursery (Barmera) and plated on medium HN'. Biotype 2 colonies were isolated from all samples. Seventy-one colonies were tested with the fluorescent antiserum to isolate 34 and all gave a positive reaction.

The final composition of the biotype 2 selective medium was that of the basal medium HN, plus the antibiotics bacitracin, tyrothricin and sodium selenite. Its detailed composition is given in Table 17.

PROPERTIES AND USES OF THE SELECTIVE MEDIUM

Isolation from Galls and Soil

A. radiobacter biotype 2 may be isolated from soil and from galls by spreading 0.1 ml of the appropriate dilution of soil or gall extract over the medium in a 9 cm Petri dish, with an L-shaped glass

rod. A 1 : 10 or 1 : 100 soil suspension is used, depending on bacterial numbers and the type of soil. The gall, or a piece of it, is macerated with approximately 10 times its own weight of sterile distilled water with a mortar and pestle or electric blender and dilutions prepared as described by Kerr (1969). This extract is diluted 1 : 100 to 1 : 10,000 for plating. Plates were usually incubated at 25°C.

Colonies of biotype 2 first appear after 3 days, and are counted at day 5 or 6, when they are translucent, greyish-white to fawn in colour, and approximately 2-3 mm in diameter. Other bacteria grow from soil dilutions, but colonies of biotype 2 may be distinguished by their roundness, entire margin and convexity. A pearly glow is very characteristic of the colonies when they are viewed through the bottom of the Petri dish by reflected light (Plate 4).

The selective medium was developed for tumorigenic A. radiobacter biotype 2; at that stage only tumorigenic and root-proliferating forms of biotype 2 were known. During the course of the development of the medium, non-pathogenic forms were isolated from the Balhannah soil, first on the LG' medium, then on LN' and then on the final selective medium (HN'). They are identical with the tumorigenic forms in all respects except pathogenicity (Section B) and were designated A. radiobacter var. radiobacter biotype 2.

Plate 4

An upturned Petri dish of the selective medium, showing 6-day old biotype 2 colonies isolated from a natural soil. The biotype 2 colonies (A) are easily distinguished from contaminants (C) by the diffuse semi-circle of light reflected from them. Light is incident from the direction of the top of the page, at an angle to the bottom of the Petri dish.


A
C

Growth of Other Organisms

Biotype 1 bacteria are unable to grow on the selective medium because they cannot utilize erythritol, the sole carbon source. The root-proliferating forms of biotype 2 are unable to grow because of a minimal requirement for glutamic acid as the nitrogen source. So far, hairy-root has never been reported in Australia.

The genus Rhizobium is closely related to Agrobacterium, so three strains of R. trifolii were streaked on the biotype 2 selective medium and also on Patel's (1926) partially selective medium for A. radiobacter biotypes 1 and 2. They failed to grow on either, indicating that there is unlikely to be a problem of rhizobial growth on the selective medium.

Certain other soil bacteria form colonies on the selective medium, but these are easily distinguished from the very distinctive biotype 2 colonies. Isolate 62 was isolated on the selective medium from soil beneath eucalypts; its colony was white and opaque and quite distinct from the translucent biotype 2 colonies.

Reliability of Recovery

To test whether the medium gives a reliable estimate of the number of viable biotype 2 cells in soil, bacterial suspensions were added to soils, and the bacteria recovered by dilution plating. The two natural soils used, Berri sand and Urrbrae clay loam, were both

devoid of biotype 2. Two isolates of A. radiobacter var. tumefaciens biotype 2 (isolates 34 and 37) were diluted in 5 five-fold steps from an initial suspension containing approximately $2x10^6$ cells/ml, determined by dilution plating on lactose agar and nutrient agar. A sample of 0.5 ml of each bacterial dilution was added to 10 g soil, allowed to stand for 15 min. and dilution plates prepared.

The logarithmic plots of bacterial numbers recovered against numbers added to the soil (Fig. 1 (A) - (D)) are linear, with slopes not significantly different from unity. It follows that the numbers recovered are proportional to the numbers added. The percentage recoveries were quite variable for each combination of soil and bacterial isolate; average values of 50 and 30% were obtained for isolates 34 and 39, respectively, in Urrbrae clay loam, but recovery was not significantly different from 100% for either isolate in Berri sand.

To determine the accuracy of identification of biotype 2 in the presence of other bacterial colonies, 20 colonies tentatively identified as biotype 2 were randomly selected from the plates corresponding to each bacterial dilution, and their identity checked using the indirect fluorescent antibody method. The accuracy of identification of biotype 2 colonies varied with the type of soil and the soil dilution used (Table 18). With Urrbrae clay loam, biotype 2 colonies could be

Figure 1

Logarithmic plots of the recovery of Agrobacterium radiobacter var. tumefaciens biotype 2 from soil, using the selective medium: A. Isolate 34, Urrbrae clay loam; B. Isolate 34, Berri sand; C. Isolate 39, Urrbrae clay loam; D. Isolate 39, Berri sand. S.E. = standard error of the slope.

FIG. 1

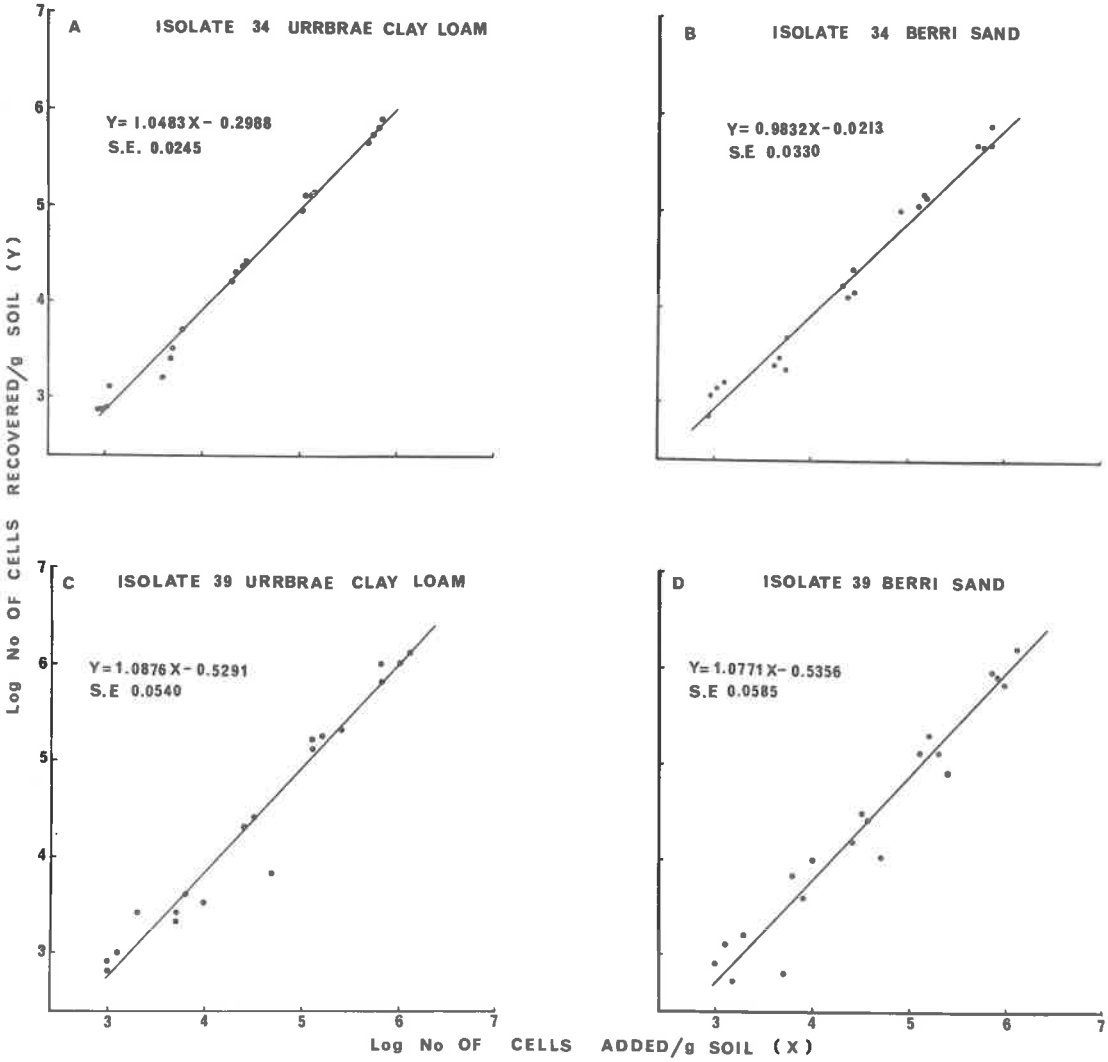

A ISOLATE 34 URRBRAE CLAY LOAM
Y= 1.0483X - 0.2988
S.E. 0.0245
B ISOLATE 34 BERRI SAND
Y= 0.9832X - 0.0213
S.E 0.0330
C ISOLATE 39 URRBRAE CLAY LOAM
Y= 1.0876X - 0.5291
S.E 0.0540
D ISOLATE 39 BERRI SAND
Y= 1.0771X - 0.5356
S.E 0.0585
Log No OF CELLS RECOVERED/g SOIL (Y)
Log No OF CELLS ADDED/g SOIL (X)
3
4
5
6
7

Table 18

Effect of soil dilution on the accuracy of identification of Agrobacterium radiobacter var. tumefaciens biotype 2 on the selective medium

Isolate	Accuracy (%) of identification of biotype 2 in soil when present at a concentration/g of				
	1.6×10^3 1:10*	8×10^3 1:10*	4×10^4 1:40*	2×10^5 1:200*	10^6 1:1000*
34, Urrbrae clay loam	70	95	96	-	-
34, Berri sand	24	46	76	-	-
39, Urrbrae clay loam	83	97	100	100	100
39, Berri sand	53	67	87	-	95

* Optimum soil dilution used.

accurately identified on plates prepared from a 1 : 10 soil dilution, particularly when numbers in the soil sufficient to give 50-100 colonies per plate. For Berri sand, a soil dilution of less than 1 : 40 was unsatisfactory, as the high numbers of other bacterial colonies made it difficult to distinguish biotype 2 colonies. Using suitable soil dilutions, it was possible to identify biotype 2 colonies with an accuracy of more than 95%.

PART D: THE ECOLOGY OF A. RADIOBACTER BIOTYPE 2 AND EPIDEMIOLOGY OF CROWN GALL

INTRODUCTION

Most previous studies of the ecology of the crown gall organism have been limited by lack of a good selective medium for quantitative isolation of agrobacteria from soil. Some workers have obtained a rough estimate of the presence or absence of pathogens by planting wounded, susceptible plants (Reddick & Stewart, 1924; Muncie, 1926a; Niemeyer, 1935) or discs of fleshy vegetable roots (Ark & Schroth, 1958; Pereira et al., 1969) in soil; these form galls if pathogens are present in sufficient numbers, but the method is not quantitative.

A partially selective medium (Patel, 1926) has enabled quantitative isolation of agrobacteria from locations where they were present in fairly high numbers relative to contaminants, such as in galls and hairy root infections, on root surfaces and in soil near infected plants, and in artificially inoculated soils. At low soil dilutions, the growth of contaminants permits only qualitative assessment of agrobacteria. A. radiobacter biotypes 1 and 2 both grow on Patel's medium and in most of these studies it is not clear which biotype was involved.

An excellent selective medium for A. radiobacter biotype 1

has been developed by Schroth et al. (1965) and was used by Schroth et al. (1971) in an ecological study of the organism in California. Where present, biotype 1 pathogens were outnumbered from 13 : 1 to 500 : 1 by non-pathogens. At low ratios of pathogen to non-pathogen, gall induction can be completely inhibited (Kerr, 1969) and this raises doubts as to whether biotype 1 is the primary pathogen in California.

A. radiobacter biotype 2 is the primary pathogen on several South Australian stone fruit-growing properties and biotype 2 pathogens are known to occur in Greece (C.G. Panagopoulos, personal communication), Israel, New Zealand, New South Wales, Victoria (Part B, this thesis), and the U.S.A. (L.W. Moore, personal communication). Following the development of a highly selective medium for biotype 2 agrobacteria (Part C), a detailed study of these bacteria in the field was initiated.

THE NURSERY

Most of the data presented here were obtained from samples from a nursery in the Adelaide hills (Balhannah), with a long history of crown gall on stone fruit.

Cultural practices at the nursery are as follows: Almond seeds and 1 year old peach seedlings are planted in rows about 75 cm apart in September, where they grow for almost two years. They are budded the February after planting, cut back in August and dug up the following June. The peach seeds are planted in germination beds in

April where they overwinter and make one year's growth before transplanting.

After a nursery crop, the land is rested till the following Autumn, when it is cropped with oats. After three more years of volunteer pasture, the land is again ploughed for nursery use.

Most samples of trees with their upper roots intact in a block of soil were collected between March and July 1970 from an almond plot planted in September 1969, which will be referred to as the "one year old plot". Several almond seedlings and some weed roots were sampled from a plot one year older, the "two year old plot", which was about half a mile away. Soil was also sampled from a pasture adjacent to the one year old plot. The top-soil at all the sampling sites was a loamy clay. Almonds were sampled in preference to peaches, as the former reputedly have a higher incidence of crown gall.

ABUNDANCE OF A. RADIOBACTER BIOTYPE 2 AROUND GALLED AND HEALTHY TREES

Methods

(a) Sampling

Two methods were used to obtain almond seedlings with upper roots and soil intact, so that the distribution of biotype 2 could be studied. The first involved digging out the roots in an approximately 30 cm cube of soil. This had the disadvantage that the soil tended to crumble away

from the roots during transportation.

The second method was more successful in preventing crumbling of the soil block and was used for the remaining plants. A 30-cm length of 30-cm diameter cast-iron pipe was placed around a seedling and hammered 15-20 cm into the ground. The taproot and other deep roots were severed by a spade, and the soil core containing the intact crown and upper roots of the plant was lifted, protected by the pipe. The soil was broken and scraped away in the laboratory to expose the plant roots for sampling (Plate 5). Closely adhering soil was shaken from the roots and designated "rhizosphere soil".

Because of the possibility of different levels of biotype 2 on roots of different ages, the roots were divided into three categories (Plate 6A): R1 were young healthy roots, up to 3 cm long and including the root tip, R2 were old, decorticated terminal fine roots and R3 were decorticated connective roots with secondary thickening, up to 1.5 mm in diameter.

Soil cores were collected from between the rows at varying distances from the stems of the seedlings in 10 cm diameter metal cylinders. The soil cores were then expelled and broken, so that only soil which had not been in contact with the surface of the cylinder was used for bacterial isolation. Soil collected with a trowel about 5 cm below the soil surface, from around the stem of a plant, was designated

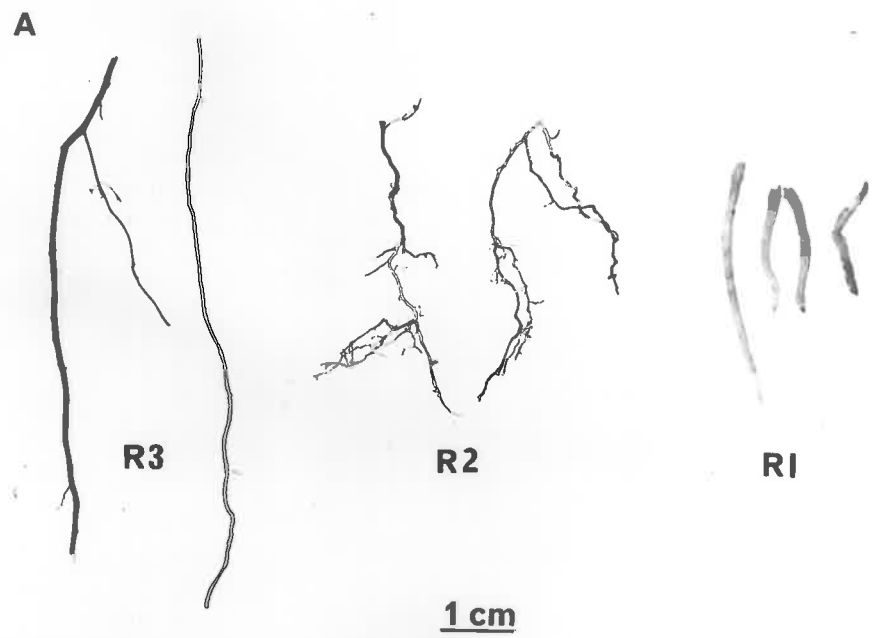
Plate 5

Intact crown and upper roots of an almond seedling, with soil, sampled in a 30-cm diameter pipe. The soil has been partially removed to expose some roots.

Plate 6

A. Categories of almond roots. R1, young healthy roots, up to 3 cm long, including the root-tip; R2, old decorticated terminal roots; R3, decorticated connective roots with secondary thickening.

B. Implements used for sampling galled and healthy almond seedlings and the surrounding soil


A
R3
R2
RI
1 cm


B

"crown soil".

All the implements used are shown in Plate 6B. They were sterilized by alcohol.

(b) Plating

Soil samples of approximately 10 g or root samples of 0.02-1 g (fresh weight) were added to 90 ml sterile distilled water and shaken 5 min. on a wrist-action shaker. Subsequent dilutions were prepared and shaken 1 minute. Numbers of A. radiobacter biotype 2 were determined by plating 0.1 ml of an appropriate dilution on the selective medium. Root suspensions were diluted 1 in 10 and 1 in 100. The initial soil suspensions and two ten-fold dilutions were plated out.

Surface-sterilized galls were macerated with 10 times their own weight of sterile distilled water with a mortar and pestle and dilutions prepared as described by Kerr (1969), except that dilutions of 10^{-2}, 10^{-3} and 10^{-4} were plated out.

Colonies were counted after 5 or 6 days' incubation at 25°C, and some were tested for pathogenicity to tomato. All bacterial counts are expressed as number per g fresh weight of roots or gall or per g soil at natural soil moisture.

(c) Pathogenicity of isolates

Biotype 2 colonies were randomly selected from plates of selective medium and tested for pathogenicity to tomato seedlings (see

Part B). Two methods of testing were compared. Isolated colonies on the selective medium were either transferred directly to tomato stems or subcultured on nutrient agar slopes before transfer. Direct transfer of 30 pathogenic and 50 non-pathogenic isolates gave four false negative results, an acceptable level of error; direct transfer was used for all routine testing because of the considerable saving in labour.

Experimental

(a) Balhannah - almond seedlings

In a first attempt to gain information about the distribution of biotype 2, almond roots were stuck onto adhesive tape (Plate 7A), which was then pressed, adhesive side down, onto plates of the selective medium.

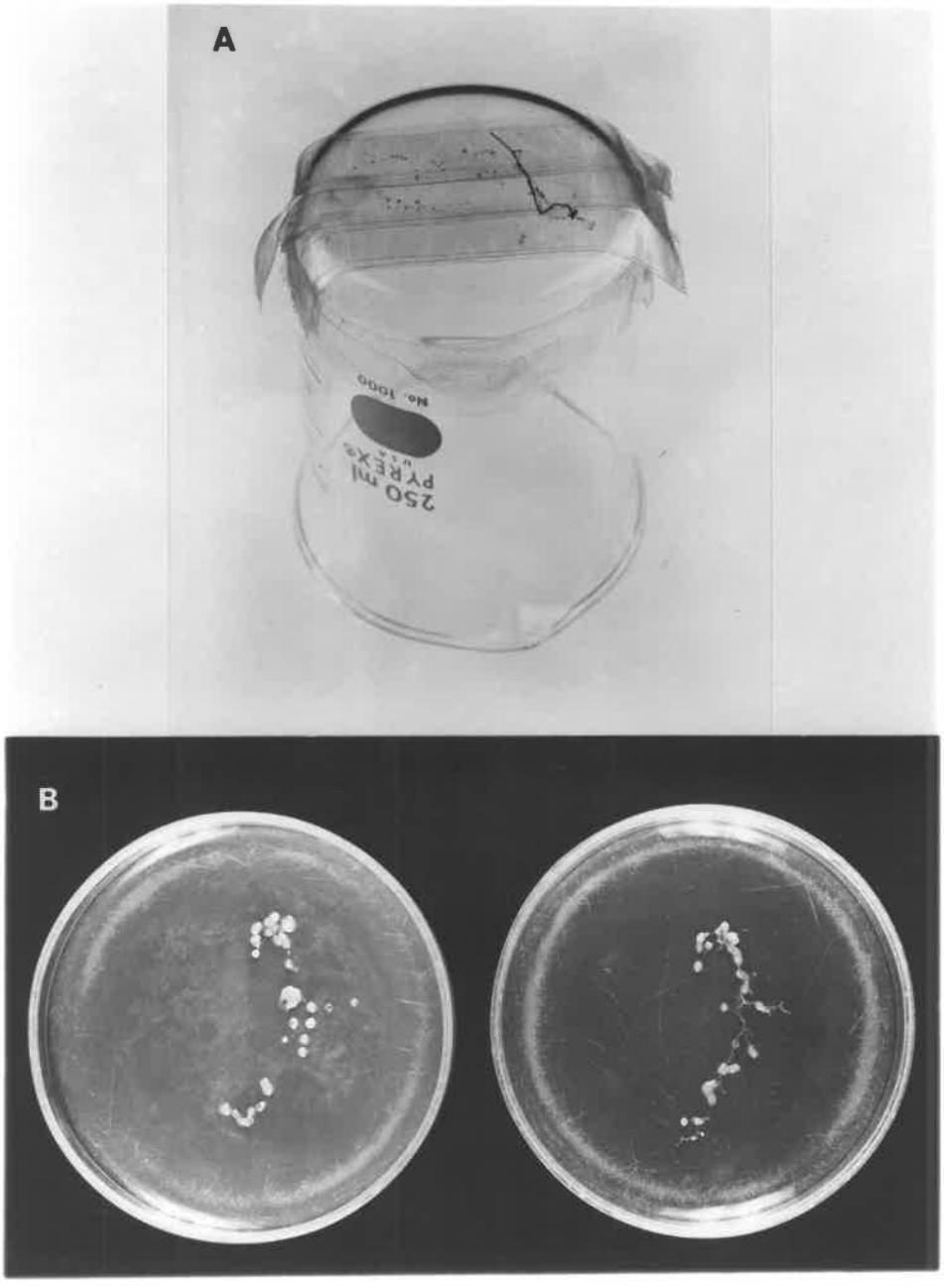
Colonies developed all along the imprints of the roots at points of contact and frequently coalesced, but there was no marked concentration of biotype 2 colonies at any particular points (Plate 7B). There was no obvious difference between the three different classes of root. Although these results suggested that stratified sampling of the roots would not be necessary, the categories R1, R2 and R3 were retained in dilution plating.

Soil and root samples from four galled and three healthy almonds from the one year old plot and two healthy almonds from the two year old

Plate 7

A. Method of making root impressions on the selective agar medium. The root, held in place on adhesive tape, is pressed gently onto the surface of the medium.

B. Biotype 2 colonies growing along a root impression (left) and along a root (right) on the selective medium.


A
250 ml
PYREX
No. 1000
B

plot were plated out. As there was no significant difference in numbers of agrobacteria between the three root categories, the results are amalgamated in Fig. 2.

The numbers of biotype 2 were quite variable. Only data from the one year old plot is included in the statistical analysis, as the results in the two year old plot were more variable, and possible differences due to soil and age of the trees could be confounded with differences between the diseased and healthy states.

There were between 10^6 and 10^7 biotype 2 cells per g root and there was no significant difference between galled and healthy trees. In all soil categories, significant differences of approximately ten-fold were found between bacterial numbers around diseased and healthy trees. There was a significant rhizosphere effect; numbers of biotype 2 agrobacteria in the rhizosphere and crown soil were approximately 10^3 times greater than in the inter-row soil. The build-up from inter-row numbers to rhizosphere numbers occurs within 8 cm of the roots or crowns of the plants, as there was no significant decline in numbers from 8 to 40 cm from the stems of the plants.

In the one year old plot, the proportion of pathogenic biotype 2 agrobacteria (Table 19) shows a good correlation with the incidence of crown gall. Pathogens were found only in close association with the galled trees; the one pathogenic isolate from inter-row soil was found only 8 cm from the stem of a galled plant sampled by the first method,

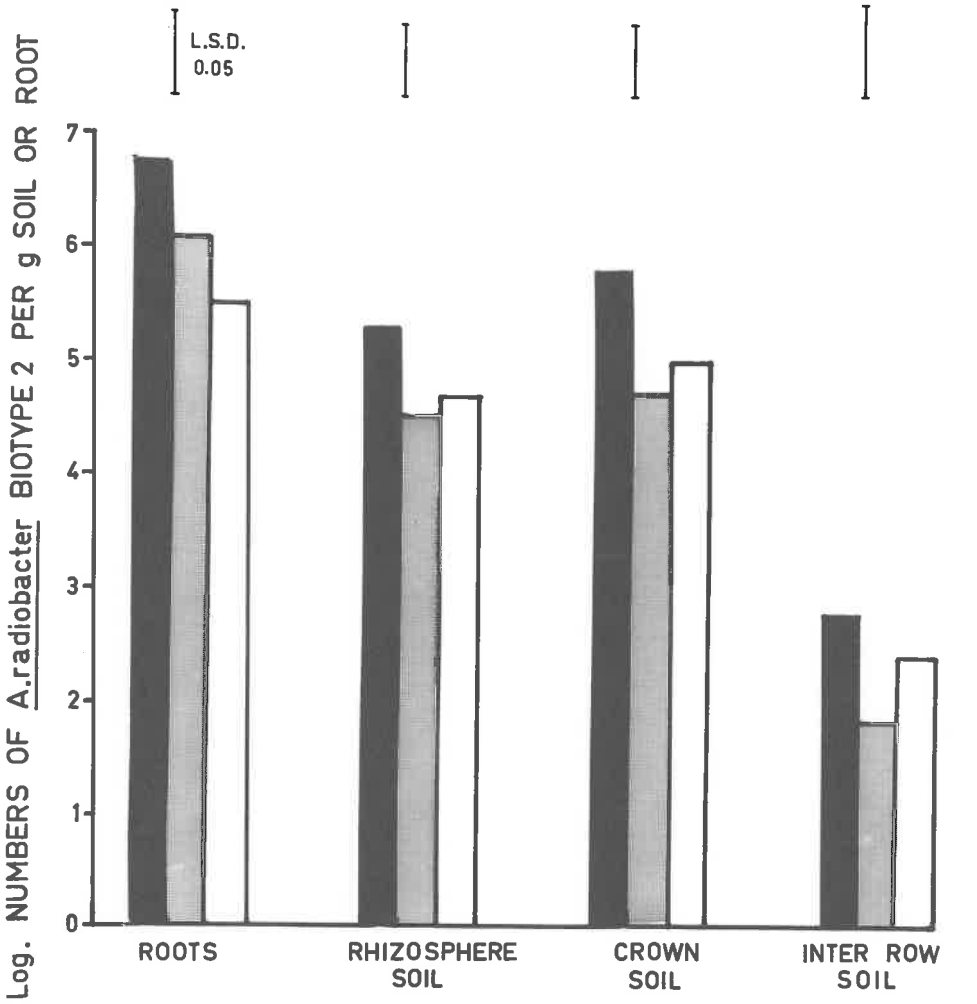


FIG. 2 NUMBERS OF A.radiobacter BIOTYPE 2 AROUND GALLED ■ AND HEALTHY ▭ ALMOND SEEDLINGS FROM THE YEAR OLD PLOT AND HEALTHY ▭ ALMOND SEEDLINGS FROM THE 2 YEAR OLD PLOT

Table 19

Ratio of pathogenic to non-pathogenic bacteria associated with galled and healthy almond seedlings

	Plant No.	Sampling method*	Roots	Rhizosphere soil	Crown soil	Inter-row soil
Galled** 1-yr-old plot	1	1	2/7	3/3	4/4	1/12
	2	1	0/8	0/3	5/5	0/3
	3	2	3/54	0/10	8/20	0/26
Healthy, 1-yr-old plot	1	1	0/18	0/20	-	0/23
	2	2	0/59	0/20	0/20	0/39
	3	2	0/20	-	0/20	0/34
Healthy, 2-yr-old plot	1	1	0/20	0/20	-	0/2
	2	2	1/60	3/20	0/20	9/79

* Intact crown and upper roots sampled by 1. the first method, 2. the second method.

** No figures were obtained for the proportion of pathogens around the fourth galled seedling.

and contamination from crown soil was a real possibility.

In the two year old plot, pathogens were isolated from around one of the trees. The relatively high proportion of pathogens in the inter-row soil may be due to transport of pathogens during harvesting operations; the sample was collected from soil disturbed by recent passage of a plough used in harvesting.

Crown soil samples from five other galled almond seedlings in the one year old nursery plot yielded pathogens in every case.

(b) Other properties

Crown soil samples were collected from around galled and healthy stone fruit trees on several other properties, a stone-fruit nursery at Barmera, the Waite Institute orchard and two orchards at Angle Vale, newly planted on land which had not previously been planted with stone fruit. Table 20 details the sources of the samples, the numbers of A. radiobacter biotype 2 and the proportions of pathogens.

Pathogens were isolated from soil around a peach tree at Waite Institute, on which no gall could be seen. This brings the number of healthy trees associated with pathogens to two out of six sampled.

High proportions of pathogens were isolated from soil around galled trees on the other properties, all of which had a high incidence of crown gall. In the case of the Angle Vale orchards, the pathogens

Table 20

Recovery of *A. radiobacter* biotype 2 from soil around galled and healthy stone fruit trees on several properties

Property and soil type	Tree	No. biotype 2 per g soil	Proportion of pathogenic biotype 2
Waite Institute orchard (clay loam)	4-yr old peach (healthy)	8×10^5	6/20
Barmera nursery (sand)	plum (galled)	6×10^4	5/5
	peach (galled)	5×10^4	3/4
	apricot (galled)	7×10^4	3/3
	apricot (galled)	5×10^4	3/3
	apricot (galled)	8×10^3	19/19
Angle Vale orchard 1 (loamy sand)	1-yr old almond (galled)	3×10^6	9/10
Angle Vale orchard 2 (loamy sand)	1-yr old almond (galled)	-	20/20

were probably introduced on planting material, as no biotype 2 agrobacteria were detected in unplanted soil on one property (orchard 2) or from uncleared land in the same district.

The question arises as to whether the high proportion of pathogens in soil around a gall is because of the proximity of the gall, or whether a tree becomes galled because of a high proportion of pathogens in the soil. The second possibility seemed to offer more hope of explaining the epidemiology of the disease and was examined in the following experiments.

EFFECT OF PATHOGEN TO NON-PATHOGEN RATIO ON GALL INDUCTION

Standard Methods of Wounding and Inoculation

(a) Tomato

Stems of four-week old tomato plants were wounded to a depth of 3 mm with a blunt cylindrical rod 2 mm in diameter: 0.004 ml of a bacterial suspension was then deposited in each wound by a micrometer syringe. The method is similar to that described by Beaud & Manigault (1964). Inoculated plants were sub-irrigated to prevent wound contamination.

(b) Peach

Peach seedlings grown in vermiculite for 8-15 weeks were washed to remove the vermiculite, potted individually in infested soil and

wounded either immediately or one week after planting. To facilitate wounding, soil was removed to expose the crown and some roots. A sterile scalpel was used to make a single cut 1.5 cm long and 1-2 cm deep in the underground part of the stem, just above the cotyledons, and two or three lateral roots were cut off as near as possible to the tap root.

Pots were watered individually, to prevent contamination between treatments, due to splashing.

Artificial Inoculation of Soil

A washed suspension of bacteria grown on nutrient agar was added to a sandy loam soil devoid of biotype 2 agrobacteria at the rate of 100 ml per 2 Kg soil, to give between 10^4 and 10^5 pathogens per g soil. The inoculated soil was thoroughly mixed by hand. The concentration of bacteria in the inoculum was determined by dilution plating on the selective medium.

Experimental

Lippincott & Lippincott (1969c) have reported that non-pathogenic isolates of biotype 1 restrict tumour induction by biotype 1 pathogens, and this was confirmed for South Australian isolates by Kerr (1969). To test whether a biotype 2 non-pathogen can inhibit tumour induction by a biotype 2 pathogen, varying numbers of isolate 58 were mixed with a

constant number of the biotype 2 pathogen, isolate 34, and inoculated into tomato seedlings. Another series of plants was inoculated with mixtures containing a biotype 1 non-pathogen, isolate 1, in place of isolate 58.

Bacteria were grown on nutrient agar and bacterial numbers in the inoculum were determined by plating on nutrient agar before mixing the isolates; approximately $4x10^5$ cells of the pathogen were added to each wound. There were five replicates of each treatment. Stem diameters at the site of inoculation were measured after five weeks (Table 21).

Isolate 58 completely inhibited gall induction in all treatments, even when present only in equal numbers to the pathogenic isolate (Plate 8). Isolate 1 did not inhibit gall induction and reduced gall diameter significantly only when present at approximately 50 times the number of pathogens. A mixture of both non-pathogenic isolates prevented gall induction by the pathogen; this effect can doubtless be attributed mainly to isolate 58.

The inhibition of galling by non-pathogenic biotype 2 isolates could be of great importance in the field, where pathogen : non-pathogen ratios were often lower than 1 : 1. The possibility of gall inhibition on stone fruit seedlings was investigated. Ten-week old peach seedlings were individually potted in a range of artificially-infested soils

Table 21

Effect of pathogen to non-pathogen ratio on gall induction in tomato seedlings. Different numbers of non-pathogenic isolates 1 and/or 58 were mixed with a constant number ($4x10^5$ cells/wound) of isolate 34 cells before inoculation

Non-pathogenic isolate	Pathogen:non-pathogen ratio	Mean stem diameter (mm) at inoculation site
-	1 : 0	11.7
1	1 : 2.7	11.2
	1 : 11	10.1
	1 : 27	10.5
	1 : 54	9.4
58	1 : 0.7	7.7
	1 : 2.5	7.3
	1 : 6.5	7.6
	1 : 13	7.9
1 and 58 (isol. 34:1:58)	1 : 11 : 2.5	6.6
Control (distilled water)	-	7.6
$L.S.D._{0.05}$	-	1.7

Plate 8

Control of crown gall in tomato seedlings by non-pathogenic isolate 58 when mixed with pathogenic isolate 34 before inoculation.

Right, plant inoculated with isolate 34 alone;

Left, plant inoculated with a 1 : 1 mixture of isolates 34 and 58.

Table 22

Effect of ratio of pathogenic to non-pathogenic bacteria in soil, on crown gall induction in peach seedlings

	Pathogen : non-pathogen ratio in soil						
	1:0	14:1	7:1	4.2:1	0.7:1	1:3	Control
No. of seedlings with crown gall (out of 5)	4	5	4	5	0	0	0

containing one level of the pathogen, isolate 34, and six different levels of the non-pathogen, isolate 58. There were five replicates of each treatment. At the time of potting, there were 3.5×10^4 cells of the pathogen per g soil and pathogen to non-pathogen ratios were 1 : 0, 14 : 1, 7 : 1, 4.2 : 1, 0.7 : 1 and 1 : 3. Peach was chosen instead of almond purely for convenience, a plentiful supply of peach seedlings being available at the time of the experiments.

One week later, the plants were wounded by the standard method, except that in one replicate the stems were pierced once with a sterile needle, rather than slit with a scalpel; the resulting galls were smaller, but there was no significant difference in the number of galls induced. The excised roots from three of the five plants in each treatment were plated out. The number of biotype 2 cells had increased approximately one-hundred-fold in the rhizosphere, but the pathogen to non-pathogen ratio had changed significantly in only one treatment; the 0.7 : 1 ratio was 1.4 : 1 at the time of planting.

Seven weeks later the seedlings were uprooted and any crown galls were cut off and weighed. There was complete gall inhibition when the pathogen to non-pathogen ratio was approximately 1 : 1 or less (Table 22). The galls which formed varied considerably in size within treatments, and there was no significant difference in gall weights between treatments.

PROPORTION OF PATHOGENS IN NATURAL GALLS

The data so far seem to suggest that galls are only induced when a wound is associated with a high proportion of pathogenic biotype 2. If this is so, natural galls should contain a high proportion of pathogens. If pathogens and non-pathogens are localized in small discrete pockets or microcolonies in the soil, a small wound in the host could be infected with 100% pathogens or non-pathogens, which would result in galls containing 100% pathogens.

Fifteen natural galls on almond seedlings were macerated and plated on the selective medium. From each plating, fourteen isolated colonies were selected at random and tested for pathogenicity (Table 23).

Total numbers of biotype 2 ranged from $2x10^5$ to $5x10^7$ per g tissue and there was no correlation between number and the proportion of pathogens. Pathogens were detected in all galls and from more than half the galls only pathogens were isolated; these galls may have been induced by microcolonies of 100% pathogens.

Five of the galls contained more than 50% non-pathogens. According to the results of the previous section, galls should not be induced at pathogen to non-pathogen ratios less than 1 : 1. Either some non-pathogenic isolates do not inhibit tumour induction as efficiently as isolate 58, or the proportion of pathogens in galls is not constant from the time of wounding. Kerr (1969) found that the

Table 23

Frequency of isolation of pathogenic bacteria from fifteen galls on almond seedlings

Number of pathogenic biotype 2 (out of 14)	14	13	12	6	5	4	1
Frequency (No. of galls)	8	1	1	1	1	1	2

ratio of pathogenic to non-pathogenic biotype 1 in tomato galls did not alter over eight weeks from inoculation, but there is no data for biotype 2 in peach galls, or for a longer period of time.

GALL INDUCTION IN NATURALLY INFESTED SOIL

The phenomenon of gall inhibition by non-pathogenic A. radiobacter biotype 2 raises the question of whether the proportions of pathogens around galls are sufficiently high to have induced the galls. To resolve this question, peach seedlings were grown and wounded in soil collected from within 15 cm of the crowns of four galled almond seedlings in the one year old plot.

Although the soil was sieved (0.6 cm) and mixed thoroughly, the pathogen : non-pathogen ratios still varied from pot to pot. Three random samples from the mixed soil, before potting, contained 3, 3 and 10 out of 15 pathogens. Total numbers of biotype 2 agrobacteria were approximately 5×10^4 cells/g in each sample.

Eight week old peach seedlings were individually potted and were wounded one week later by a sterilized needle, which pierced the crown region of the stem three times and the roots several times. Five plants were uprooted and examined for crown galls after two weeks, five after five weeks and the remaining ten after nine weeks.

Only the plants harvested nine weeks after wounding had galls

(Table 24). It is likely that galls had been developing slowly but were not apparent in some of the plants harvested earlier; the probability that the only six galled plants would occur by chance in the last ten pots sampled is very low (approximately 0.005).

INHIBITORY EFFICIENCY OF OTHER NON-PATHOGENIC ISOLATES

Crown galls were induced in plants growing in natural soil despite the presence of approximately 50% non-pathogenic biotype 2, and more than 50% non-pathogens were isolated from several natural galls. The possibility that some non-pathogenic isolates do not inhibit gall induction as efficiently as isolate 58 was tested in two experiments in which non-pathogenic biotype 2 isolates were mixed with pathogenic isolate 34 at a ratio of approximately 1 : 1 and inoculated on tomatoes.

In the first experiment, seven non-pathogenic isolates were tested. There were approximately 6×10^5 pathogenic cells per wound. The habitats from which the non-pathogens were isolated are presented with the results in Table 25. Through a miscalculation, the actual ratio of pathogen : non-pathogen was only 1 : 20 in the case of isolate 64.

Only isolate 58 inhibited gall induction. None of the other isolates had any significant effect on gall size.

In the second experiment, ten non-pathogenic isolates from two

Table 24

Proportion of peach seedlings with crown gall after growth in natural soil for 2, 5 and 9 weeks following wounding

Time after wounding (wks)	2	5	9
Proportion of galled plants	0/5	0/5	6/10

Table 25

Gall inhibition by various non-pathogenic isolates, when mixed in equal numbers with the pathogenic isolate 34 (first experiment)

Isolate No.	Source	Mean stem diameter (mm) at wound after 5 weeks
58	soil around old peach gall	7.3
59	soil around healthy apple tree	11.3
60	bare ground	11.3
61	soil, healthy peach tree	11.2
63	soil around almond gall	11.0
64*	almond gall	10.6
65	soil, healthy plum tree	10.7
Control (34 alone)	-	11.3
$L.S.D._{0.05}$		1.1

Approximate level of pathogen = 6×10^5 cells per wound

* Pathogen : non-pathogen ratio approximately 1 : 20.

Table 26

Gall inhibition by various non-pathogenic isolates, when mixed in equal numbers with the pathogenic isolate 34 (second experiment)

Isolate No.	Source	Gall inhibition
58	soil around old galled peach stump 1	+
66	"	-
67	"	+
68	"	+
69	"	+
70	soil around old galled peach stump 2	-
71	"	-
72	"	-
73	"	-
74	"	+

Approximate level of each isolate = 3×10^5 cells per wound

soil samples were tested. Both soil samples were collected around old peach galls. There were approximately $3x10^5$ cells of the pathogenic isolate per wound. As inhibition of gall induction was an all-or-nothing response, Table 26 records whether inhibition occurred or not. Five isolates, including isolate 58, completely inhibited gall induction (Table 26).

In two experiments, a total of 16 non-pathogenic biotype 2 isolates were tested and only 5 were highly inhibitory to crown gall induction.

BIOLOGICAL CONTROL

In the field, the presence of inhibitory non-pathogenic agrobacteria must decrease the chance of a plant becoming galled. If the proportion of inhibitory non-pathogens around susceptible plants could be artificially increased, this would provide a simple and effective method of biological control. With stone fruit trees, a practicable way to achieve this would be by dipping seeds or the roots of young plants in a suspension of a suitable non-pathogenic isolate before planting in pathogen-infested soil.

In a preliminary test of this possibility, the roots of fifteen-week old peach seedlings were washed and briefly dipped in a suspension of washed isolate 58 cells ($1.5x10^8$ cells/ml) before planting in soil

artificially infested with the pathogenic isolate 34 ($2x10^4$ cells/g). Controls were dipped in sterile distilled water. Half the plants were wounded by the standard method immediately after planting, and half were wounded after one week, to simulate two possible situations in the field; some plants would be wounded during transplanting, whereas others would be wounded at some later time.

Plants were uprooted at six and ten weeks. None of the eleven protected plants had any galls, but four of the five controls were galled (Table 27).

The concentration of the isolate 58 suspension required for protection was determined by dipping the roots and crowns of peach seedlings in suspensions of varying concentrations, before planting them in soil artificially infested with pathogenic biotype 2. Plants were dipped in order from the lowest to the highest concentrations of isolate 58, to minimize contamination of low-level treatments. The number of cells of isolate 58 absorbed by the roots was determined by dilution plating the complete root systems of two seedlings per treatment on the selective medium immediately after dipping (Table 29). Because of the low numbers involved, numbers absorbed could not be reliably determined for treatments of less than 10^5 cells/ml.

Half the plants were wounded by the standard method immediately after planting and half after one week. The number of biotype 2

Table 27

Control of crown gall by dipping roots and crowns of peach seedlings into a suspension of non-pathogenic isolate 58 before planting in pathogen-infested soil

	Proportion of galled plants at two intervals after planting			
	Unprotected (6 weeks)	Dipped in isolate 58 suspension		
		5 weeks	9 weeks	Total
Wounded immediately before planting	2/2	0/2	0/2	0/4
Wounded 1 week after planting	2/3	0/2	0/5	0/7
Total	4/5		0/11	

Soil contained 2×10^4 cells isol. 34 per g soil.
Suspension contained 2×10^8 cells isol. 58 per ml.

agrobacteria and the percentage of pathogens on the surface of excised roots were measured at one week. The incidence of galling was assessed ten weeks after planting.

In the first experiment the isolate 58 suspensions ranged in concentration from 10 to 10^6 cells/ml. There were four replicates. Galls were found on the roots and crowns of almost all the protected plants (Table 28a). This can be explained by the relatively high proportion of pathogens found on the roots one week after planting.

The experiment was repeated using higher concentrations of isolate 58 and six replicates of each treatment. The number of pathogens in the soil at the time of planting was 10^4 cells per g. After 10 weeks, all the unprotected plants and two out of twelve plants dipped in the lowest concentration of isolate 58 were galled; all other plants were healthy (Table 28b, Plate 9).

Using higher concentrations of isolate 58 reduced the percentage of pathogens on the root surface to a very low level and concentrations of $3x10^6$ and $3x10^7$ per ml provided complete protection.

Comparison of the pathogen levels in the soils and the concentrations of isolate 58 used in both experiments suggests that the proportion of pathogens recovered from the roots is too high at the 10^6 treatment in the first experiment. This is apparently due to the fact that, in the first experiment, the numbers of isolate 58 on the roots

Table 28

Attempted control of crown gall by dipping roots and crowns of peach seedlings into various concentrations of non-pathogenic isolate 58, before planting in pathogen-infested soil

(a) Experiment 1 - Number of galled plants (out of 4)

Concentration per ml of non-pathogen	10^6	10^5	10^4	10^3	10^2	10	0
Wounded immediately	4	4	4	4	4	4	4
Wounded one week after planting	3	4	4	4	4	4	3
% pathogens on roots at one week after planting	38	65	94	95	98	83	-

(b) Experiment 2 - Number of galled plants (out of 6)

Concentration per ml of non-pathogen	$3x10^7$	$3x10^6$	$3x10^5$	0
Wounded immediately	0	0	2	6
Wounded one week after planting	0	0	0	6
% pathogens on roots at one week after planting	< 1.7	< 1.7	5	-

Plate 9

Attempted control of crown gall by dipping roots and crowns of peach seedlings into various concentrations of non-pathogenic isolate 58, before planting in pathogen-infested soil. Pairs of seedlings were wounded immediately (W) or one week (W1) after planting. Concentrations of isolate 58 were 0 (distilled water control), $3x10^5$, $3x10^6$ and $3x10^7$ per ml. g = gall.

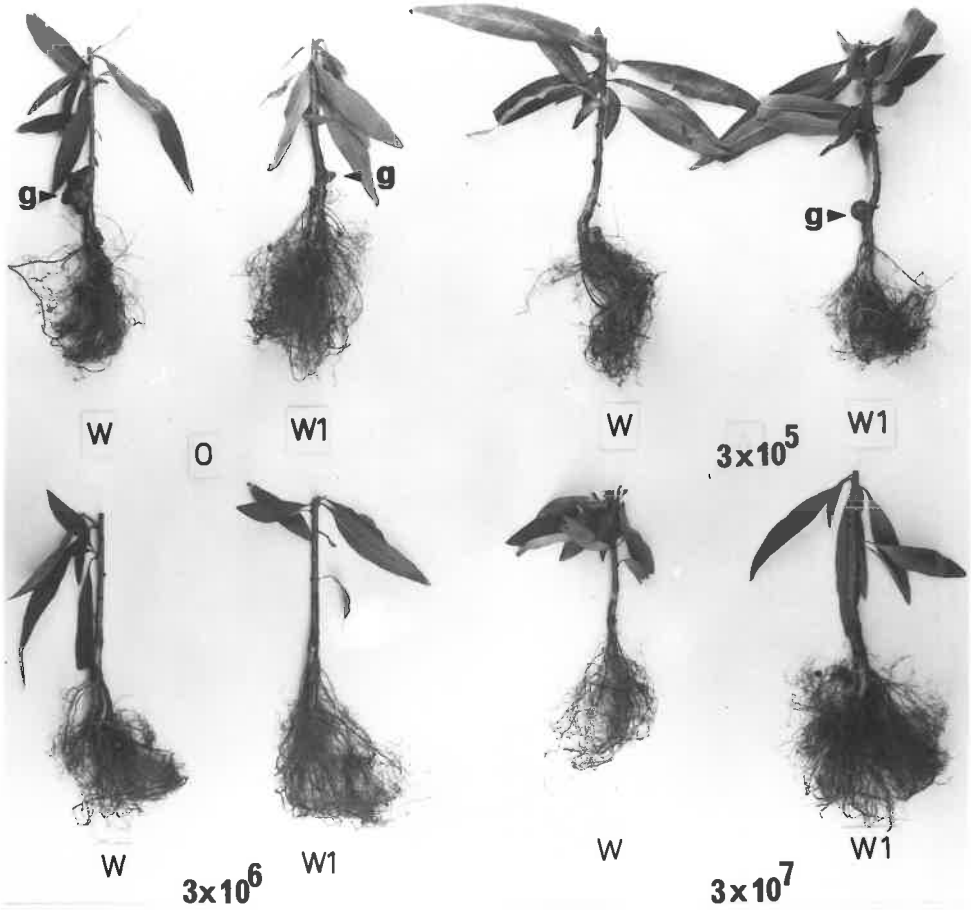

g
g
g
W
W1
W
W1
0
3x10^5
W
W1
W
W1
3x10^6
3x10^7

Table 29

Level of non-pathogenic isolate 58 on peach roots immediately after dipping in a suspension of the organism and one week later

	Level of treatment (isolate 58 cells per ml)	Number of isolate 58 cells per g root	
		Immediately after dipping	One week after planting
Experiment 1	10^5	2.5×10^4	6.5×10^5
	10^6	7.5×10^4	1.5×10^6
Experiment 2	3×10^5	3.5×10^5	4.2×10^6
	3×10^6	2×10^6	2.5×10^7
	3×10^7	3.5×10^7	9×10^7

immediately after dipping were much lower than expected (Table 29). Preliminary experiments showed that the roots of peach seedlings absorb approximately 1 ml suspension per g root weight, which is in good agreement with the results of experiment 2 (Table 29).

It appears that there was a decrease in the number of viable cells in the isolate 58 suspensions used in experiment 1, especially in the 10^6 suspension which was not used until $5\frac{1}{2}$ hr after preparation. Dilute distilled water suspensions of agrobacteria decline in viability on storage for several hours (Htay, personal communication). However, the suspensions in experiment 2 were held for 6 - $9\frac{1}{2}$ hr before use, without any appreciable decline in numbers. There is no satisfactory explanation for this, other than that the more concentrated suspensions in experiment 2 may be expected to survive better, due to carry-over of nutrients with the cells.

The numbers of isolate 58 around the roots had increased from three to twenty-six times by one week after planting, an important consideration if biological control is used in the field.

The effectiveness of this control method has not yet been tested in the field, where one of the unknown factors is the ability of isolate 58 to inhibit gall induction by a variety of tumorigenic strains. Kerr (personal communication) tested 8 pathogens and found that 6, including 2 in biotype 1, were completely controlled by isolate

58 at a pathogen : non-pathogen ratio of 1 : 1. Only two biotype 1 isolates were capable of initiating galls in the presence of isolate 58. Gall induction by all 4 local isolates and by all 4 biotype 2 isolates, including one from overseas, was completely inhibited. So prospects for control of crown gall in the field are quite good.

SURVIVAL OF A. RADIOBACTER BIOTYPE 2

Several workers have studied the survival of the crown gall organism in the absence of susceptible hosts. Patel (1928, 1929), Banfield (1934) and Dickey (1961) measured the decline in numbers of crown gall bacteria in artificially infested soil, by dilution plating on Patel's (1926) partially selective medium. Their work has the theoretical disadvantage that survival of artificially inoculated bacteria may differ from survival in naturally infested soil; however Patel used finely ground galls as the inoculum, which may be similar to the natural situation.

There is no quantitative data on the survival of pathogenic agrobacteria in naturally infested soils. Hildebrand (1941) isolated crown gall bacteria from soil 2 years after the removal of galled trees; he probably used Patel's (1926) medium. Schroth et al. (1971) used the carrot disc method (Ark & Schroth, 1958) to isolate agrobacteria at pathogenic levels from fields left fallow for over 5 years. In both

these cases, it is not certain which biotype was involved; both biotypes grow on Patel's medium and induce galls on carrot discs.

This study will report data on the survival of natural populations of A. radiobacter biotype 2.

Survival Under Pasture

Twenty soil cores were sampled from a pasture at the Balhannah nursery and numbers of biotype 2 were determined by plating on the selective medium. No crown gall-susceptible crop had been grown in the soil for four years.

The numbers of biotype 2 ranged from zero to 9×10^4 per g soil (Fig. 3). Out of 250 isolates tested, none was pathogenic.

It was thought that the variability of the biotype 2 counts may be due to differences in soil organic matter content, so the content of easily extractable organic matter in each sample was determined by the Walkley and Black wet combustion method (Jackson, 1958; p. 219). The level of organic matter ranged from 6.8 to 29.9 mg carbon per g soil, but there was no correlation between organic matter content and numbers of biotype 2 in the samples.

Survival in the Rhizosphere

The recovery of A. radiobacter biotype 2 from soil after four years under pasture raises the question of whether the organism survives

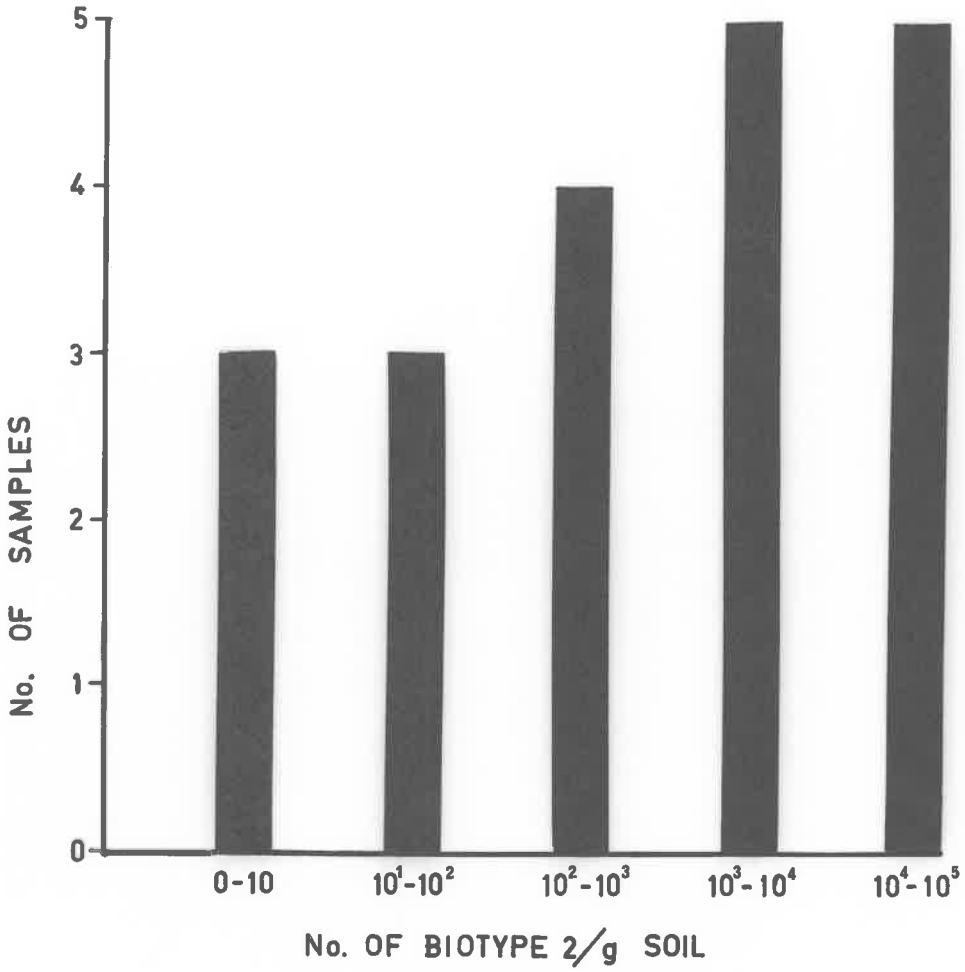


FIG. 3 FREQUENCY DISTRIBUTION OF NUMBERS OF A.radiobacter BIOTYPE 2 IN SOIL UNDER PASTURE, 4 YEARS AFTER REMOVING NURSERY TREES.

passively in the soil, or whether it becomes established in the rhizosphere of weeds, grasses and other plants.

Crown soil or root samples of five other plant species growing in the nursery, including three common weeds, were plated on the selective medium. Biotype 2 agrobacteria were isolated from around the crown or roots of four of the five species, sometimes in high numbers (Table 30). Pathogens were only found around one plant, a subterranean clover growing in the one year old almond plot. The samples from around eucalypt trees were collected from a field which had never been used for growing nursery stock, so the absence of A. radiobacter biotype 2 may reflect the original state of the nursery.

Numbers around most plants were comparable with those around almonds, which suggests that there is rhizosphere stimulation of A. radiobacter biotype 2 by these plants. This was investigated by growing six species common in the nursery 4 to 16 weeks in soil containing low numbers of biotype 2, and determining numbers of biotype 2 in the rhizospheres of the plants.

Soil was collected from fallow ground in the nursery, sieved (0.6 cm), mixed and dispensed into pots. Seeds of all species except the eucalypts were planted in the pots; small eucalypt seedlings, collected in the Waite Institute grounds, were transplanted at the same time. There were three replicate pots for each plant species and for

the unplanted controls, and these were harvested at three different times. Not all plants germinated at the same time; the ages of the plants from germination to sampling are given in Table 31.

Each plant species was also grown in three pots of Angle Vale soil, which is devoid of biotype 2 agrobacteria. At each time of harvesting, no biotype 2 could be detected in the rhizosphere of these plants, thereby proving that the organisms were not present on the seeds or roots at planting.

Numbers of biotype 2 in the rhizosphere soil were determined by plating on the selective medium. Complete root systems were shaken relatively free of soil and added to 90 ml of sterile distilled water. This suspension and three ten-fold dilutions were plated out. The weight of rhizosphere soil was estimated by evaporation of a 20 ml sample of the soil suspension obtained after removal of roots from the bottle containing the original dilution; the weight of soil in the sample was multiplied by 4.5. This was a simplification of the true relationship which seemed justified in view of the magnitude of other variations in the results.

Although very variable, the results (Table 31) show a marked rhizosphere stimulation by all plants. With the exception of one isolate from ryegrass none of 400 isolates tested were pathogenic. The one pathogen in 400 isolates may be an indication that pathogens are not

Table 30

Number of *A. radiobacter* biotype 2 around weeds and other plants

Plant	Sample from	Concentration of biotype 2 per g soil or roots	Proportion of pathogenic biotype 2
Apple 1	roots	4×10^6	0/20
Apple 2	crown soil	10^5	0/20
Eucalyptus sp. (5 trees)	crown soil	0	-
Malva sp.	roots	10^5	0/20
Trifolium subterraneum			
plant 1	roots	4×10^5	0/18
plant 2	crown soil	10^3	3/19
Cynodon dactylon	roots	7×10^5	0/20

Table 31

Rhizosphere stimulation of *A. radiobacter* biotype 2

Plant	Age (weeks)	No. bacteria ($x10^{-5}$) per g of root	No. bacteria ($x10^{-5}$) per g of rhizosphere soil	R:S*
Trifolium subterraneum	6	12	15	200
	11	9	13	40
	12	39	84	8,000
Trifolium repens	5	6	10	150
	10	22	46	130
	14	21	41	4,000
Lolium perenne	6	3	3	40
	11	2	2	6.5
	15	55	33	3,200
Cynodon dactylon	4	3	1	20
	9	3	3	9
	13	48	41	4,000
Chenopodium album	6	22	18	1,800
	6	11	6	540
Eucalyptus sp.	7	31	60	840
	12	4	11	30
	16	8	37	3,500

Soil counts (fallow pots)	at planting	$4x10^3$
	first sampling	$7x10^3$
	second sampling	$3.5x10^4$
	third sampling	$1x10^3$

* R:S number of biotype 2 cells/g rhizosphere soil : number/g in fallow control soil.

confined to the soil around galled plants, but are usually only present in very low numbers elsewhere. The variability is probably due to the heterogeneity of the natural soil populations of biotype 2. Many more samples would be needed, to provide a reliable estimate of numbers, but these results are quite adequate to illustrate the rhizosphere stimulation.

The reason why biotype 2 agrobacteria were not isolated from Eucalyptus sp. in the field is not due to failure to grow in the rhizosphere; there was marked stimulation of numbers of biotype 2 around eucalypt roots in the glass-house test. Therefore biotype 2 can never have been present around the gum trees, which suggests that the organism is introduced rather than endemic in the nursery, which was originally under Eucalyptus spp.

Survival in Fallow Soil

From time to time the crown gall organism may have to survive in fallow soil, although probably it is not long before volunteer plants grow. The survival of biotype 2 was measured in soil which was collected in April 1970 from within 15 cm of a galled almond, sieved (0.6 cm) and held in two 20-cm pots. All weeds were pulled out as they appeared.

The pots were watered immediately after each sampling and at

approximately weekly intervals between samplings. Pot 1 was watered to a constant weight corresponding to 21% moisture and Pot 2 to 24% moisture. Between waterings the soil moisture levels occasionally dropped below wilting point (8.5%) in both pots. The pots were stored in an open-sided glass-house; over the period of the experiment, the highest maximum temperature was 39.1°C and the lowest minimum 3.7°C. Numbers of biotype 2 were first determined in June 1970 and samples were then taken at fairly regular intervals until August 1971.

Soil samples were collected between 2 and 5 cm depth by means of a metal tube of 2.2 cm internal diameter, which was divided longitudinally into two halves, to facilitate removal of the soil core. The soil moisture content was also estimated using soil in the core. Each time, a single sample was taken from each pot, except in the first and last samplings, when two and four samples respectively were taken and average values calculated. The results are given in Table 32.

The numbers of biotype 2 remained more or less constant for almost 6 months, then showed a decline over December 1970 and January 1971, a nine week period of high temperatures and low soil moisture levels, during which no samples were taken. Had the pots been watered more frequently, the bacteria may have survived better. Even 61 weeks after the first sampling, 400 non-pathogenic biotype 2 cells per g could be isolated from the soil in Pot 2, which had been kept wetter than

Table 32

Survival of A. radiobacter biotype 2 in fallow soil

Time (weeks)	Pot 1		Pot 2	
	No. biotype 2 per g soil	Proportion of pathogens	No. biotype 2 per g soil	Proportion of pathogens
0	$7x10^4$	24/39	$9x10^4$	23/37
3	$9x10^4$	10/20	$5x10^4$	13/19
5	$2x10^4$	5/19	$3x10^4$	6/20
7	$12x10^4$	17/20	$7x10^4$	10/20
9	$6x10^4$	7/17	$5x10^4$	5/11
13	$4x10^4$	13/18	$6x10^4$	14/19
22	$3x10^4$	13/20	$3x10^4$	12/19
31	$2x10^1$	-	$1x10^3$	4/20
32	$3x10^2$	1/1	$1x10^3$	6/20
61	$6x10^0$	0/1	$4x10^2$	0/20

Pot 1; it has been shown that biotype 2 can multiply up to high levels in the rhizosphere from levels almost as low as this (Table 31).

The proportion of pathogens did not change significantly during the first 32 weeks, which emphasizes the essential similarity between the survival ability of pathogenic and non-pathogenic biotype 2 agrobacteria. Further work is needed to establish whether pathogens have slightly lower survival ability than non-pathogens, as suggested by the results at 61 weeks.

DISPERSAL OF THE CROWN GALL ORGANISM

Sale of galled trees is illegal here and in the United States, but many apparently healthy trees become galled after transplanting in the orchard, and this has been ascribed to pathogens carried on the roots and to incipient infections in the nursery. Banfield (1934) surface-sterilized the roots of raspberry canes bearing no visible galls and planted them in sterilized soil, but 15% of plants developed galls. This he ascribed to development of incipient tumours which were initiated before the antiseptic treatment.

Riker et al. (1934) isolated hairy root bacteria, which are physiologically similar to biotype 2 crown gall bacteria, from the surface of roots of healthy apple seedlings. It has long been assumed, but never proved, that tumorigenic agrobacteria are carried on the roots of healthy plants; plants with root galls which broke off during

harvesting would be particularly likely to be contaminated with pathogens.

In the Balhannah nursery, young stone fruit trees are tied in bundles after excess soil has been shaken or hosed from the roots. The trees are later separated; galled trees are discarded and healthy trees are trimmed and re-tied in bundles of 10 to 15.

The numbers of A. radiobacter biotype 2 on the roots of some plants being prepared for sale were determined; small roots were cut from two bundles of almond and four of peach and plated on the selective medium. Only one bundle contained galled plants, yet five of the six bundles yielded pathogenic biotype 2 (Table 33). This result is not surprising because one of the five healthy trees sampled in this nursery was associated with pathogens in the field; on average, 2 to 3 trees per bundle would be expected to have pathogens on their roots.

The trees in two bundles of peach (nos. 5 and 6, Table 33) were planted in a commercial orchard in positions where old, healthy peaches had been rooted out the previous year. It was not possible to sample the sites before planting, but soil cores were taken 1 m uphill of five randomly selected trees and crown soil and small roots growing near the crowns of the plants were sampled and plated on the selective

Table 33

Numbers of A. radiobacter biotype 2 and proportion of pathogens on roots of trees being prepared for sale

Plant	Bundle No.	No. of biotype 2 per g root	Proportion of pathogens
Almond	1	$2x10^4$	1/19
"	2	$2x10^5$	1/10
Peach	3	$2x10^3$	0/20
"	4*	$2x10^3$	5/20
"	5/	$8x10^5$	13/19
"	6/	$5x10^5$	10/20

* Bundle contained two galled plants.

/ Bundle temporarily planted in sand.

Table 34

Numbers of A. radiobacter biotype 2 and proportions of pathogens around peaches from the nursery, transplanted into an orchard

Tree No.	No. of biotype 2 per g			Proportion of pathogenic biotype 2		
	Soil 1 m from tree	Crown soil	Roots	Soil 1 m from tree	Crown soil	Roots
1	$4x10^3$	$2x10^5$	$2x10^5$	0/20	12/20	7/20
2	$2x10^3$	$5x10^4$	$2x10^6$	4/20	3/20	9/19
3	8x10	$2x10^3$	$6x10^5$	0/7	4/20	2/9
4	$1x10^3$	$3x10^4$	$5x10^6$	1/20	4/20	9/18
5	$1x10^2$	$1x10^5$	$2x10^6$	1/9	0/19	7/20
Total				6/77	23/99	35/96

medium. The results are given in Table 34.

Pathogenic biotype 2 agrobacteria were absent or few in the soil cores. The proportions of pathogens on the roots and in crown soil were significantly higher than in the soil cores. This is good evidence for the introduction to another property of crown gall bacteria on the roots of healthy trees from a pathogen-infested nursery.

DISCUSSION

Epidemiology

Numbers of A. radiobacter biotype 2 around galled and healthy almond seedlings were studied by dilution plating on the biotype 2 selective medium. Although numbers were slightly, but sometimes significantly, greater around diseased trees, the main difference was in the ratio of pathogenic to non-pathogenic agrobacteria. Pathogens were always isolated from around galled trees, where the ratio was usually high; around healthy trees it was sometimes too low to be detected.

The hypothesis was investigated that trees become galled only when there is a high ratio of pathogens to non-pathogens in the surrounding soil. Using isolate 58, the first non-pathogenic biotype 2 strain to be isolated, it was found that gall induction was completely inhibited in both tomato and peach seedlings at a pathogen to non-pathogen ratio of 1 : 1 or less.

At first it seemed that galls could only be induced where the pathogen to non-pathogen ratio exceeded 1 : 1. If this were so, gall induction in a natural soil with an overall ratio lower than this value could be ascribed to the heterogeneity of the soil and the presence of pockets of bacteria in which pathogens outnumbered non-pathogens. The discovery of natural almond galls with pathogen : non-pathogen ratios appreciably less than 1 : 1 suggested that galls may sometimes be induced when pathogens are outnumbered by non-pathogens.

Other isolates were tested and, out of a total of 16 non-pathogenic biotype 2 isolates, only 5, including isolate 58, inhibited crown gall induction when present in equal numbers to pathogens. This would explain why some natural galls contain less than 50% pathogenic biotype 2. So far, all the inhibitory non-pathogens have been isolated from two soil samples from similar habitats. Many more isolates will have to be tested, to determine the abundance and distribution of inhibitory isolates.

For a susceptible plant to become galled, initially two things are required, a wound and sufficient tumorigenic bacteria at that wound. If these requirements are satisfied, whether a gall is induced will depend on the ratio of pathogens to non-pathogens and on the inhibitory efficiency of the non-pathogens.

Even though the average pathogen : non-pathogen ratio around a

plant may not amount to a tumorigenic mixture, the bacteria may exist in pockets containing a high proportion of pathogens or non-pathogens, so there is an element of chance in whether a wound will be in contact with a pocket containing predominantly pathogens or non-pathogens. That galls are frequently induced by pockets of pure pathogens is supported by the recovery of 100% pathogens from half the almond galls macerated.

According to this theory, pathogens should occasionally be isolated from around healthy trees, which have escaped infection because wounds have not been contaminated by a high proportion of pathogens. Two cases have been found in the six healthy trees sampled; a four year old peach in the Waite Institute orchard yielded six pathogens out of twenty biotype 2 isolates from a crown soil sample, and pathogens were associated with the roots of a healthy almond tree sampled from the two year old plot.

If some non-pathogens have the effect of protecting plants against crown gall, the disease should be more severe in areas where non-pathogens are absent or only present in low numbers. The results from Barmera and Angle Vale, where high incidence of crown gall was correlated with low proportions of non-pathogens, seem to bear this out. To firmly establish this point, more extensive sampling will be needed.

Finally, to return to the initial hypothesis that stone fruit trees become galled due to the presence of a high ratio of pathogenic

to non-pathogenic agrobacteria in the surrounding soil, rather than the high proportion of pathogens being due to the presence of the gall, it must be recognized that the hypothesis is only partly true. Certainly, a high proportion of pathogens is required to induce a gall, but just how high depends on the inhibitory efficiency of non-pathogens at the wound. On the other hand, pathogens are almost certainly released from the surfaces of intact galls (Robinson & Walkden, 1923; Banfield, 1928, 1934; Dickey, 1962). To some extent, the high proportion of pathogens in soil around galls may be due to leakage from the galls.

In ten out of fifteen almond galls, more than 50% of the biotype 2 agrobacteria were pathogenic. It seems that the factors determining the proportion of pathogens in galls, namely, the proportion of non-pathogens which are inhibitory and the size of wounds relative to the size of pockets of bacteria in the soil, are such that leakage from galls increases the overall proportion of pathogenic agrobacteria in surrounding soil. As with other plant diseases, if the primary inoculum is high enough it will initiate disease which, in turn, results in the release and build-up of inoculum. In other words, there is likely to be a positive feed-back mechanism operating.

Survival and Dispersal of the Crown Gall Organism

A. radiobacter biotype 2 is evidently a very successful soil saprophyte; it can survive long periods in fallow soil under unfavourable conditions of soil moisture and temperature, and was stimulated in the rhizosphere of stone fruit trees and all the weed species tested. In the field it was recovered from under pasture, four years after any trees susceptible to crown gall had been grown in the soil.

Pathogenic biotype 2 were not recovered from the pasture and were not present in appreciable numbers in the soil used to test rhizosphere stimulation by weeds, but they survived almost as well as non-pathogens in the fallow soil. Their physiological identity with non-pathogens should enable them to react in exactly the same way in the rhizosphere. This means that once introduced to a property, crown gall organisms could not be eliminated and probably not even reduced in numbers by crop rotation.

The absence of biotype 2 agrobacteria in virgin soil under eucalypts on the property of the Balhannah nursery is good evidence for the disease having been introduced to the area, probably on planting material. There is good evidence that pathogenic as well as non-pathogenic A. radiobacter biotype 2 is transported on the roots of healthy plants from infested nurseries and becomes established in new areas where the trees are planted.

Biological Control

Biological control of crown gall by the use of a highly inhibitory non-pathogenic strain promises to be the first truly effective control measure against the disease. Although it has not yet been proved in the field, isolate 58 should provide long-term protection from the disease because it appears to be identical with biotype 2 pathogens in all respects except pathogenicity. Thus it should survive or multiply under all conditions which permit survival or multiplication of the crown gall organism. It completely inhibited gall induction by all local pathogens and all the biotype 2 pathogens tested, including one from overseas, which gives reason to believe that it will be effective against the majority, if not all, of South Australian pathogenic strains.

The ability of isolate 58 to colonize new roots as they grow is not known, but it multiplied and became well established on roots which had been dipped in suspensions of it. Peach stocks in the nursery are transplanted after a year's growth, so they could be protected by dipping during transplanting, preferably before or soon after operations which are likely to cause wounding, because Kerr (personal communication) has found that if isolate 58 is added to a wound more than 2 hr after the pathogen, there is not complete gall inhibition.

Seedling almonds are not transplanted while in the nursery, but they and peaches to be grown in germination beds could be protected by

dipping the seeds in a suspension of isolate 58 before planting. The organism should become well established in the crowns of the seedlings, because of multiplication in the rhizosphere of developing roots.

Assuming that isolate 58 becomes well established in the crowns of susceptible plants, there are only two possible ways the control could break down. The first is by acquisition of virulence by isolate 58 (Kerr, 1971). This is unlikely, as Kerr (personal communication) was unable to transfer virulence to isolate 58. In any case, virulence has only been transmitted via the gall (Kerr, 1971) and if a gall is present, the control must have been applied too late.

The other way that control could break down, would be by an increase in frequency of pathogens which are not efficiently controlled by isolate 58. If such isolates arise or already exist in South Australia, another inhibitory isolate could probably be found.

Mechanism of Biological Control

Lippincott & Lippincott (1969c) noted that certain non-pathogenic agrobacteria reduced the number of galls produced by a constant number of pathogens in a Pinto-bean leaf assay (Lippincott & Heberlein, 1965) and ascribed this to exclusion of pathogenic cells from hypothetical wound sites by non-pathogenic cells. In their system, the individual wounds are small, averaging 9 epidermal cells (Lippincott

& Lippincott, 1965), and their results suggested that there was only one site per wound.

In this study, the wounds were relatively large; in similar wounds in Datura stems, Manigault & Beaud (1967) calculated that there were between 10^5 and 10^6 infection sites. That being so, if Lippincott & Lippincott's (1969c) theory of competition for sites is correct, at a pathogen to non-pathogen ratio of 1 : 1 approximately half the sites should be occupied by pathogens and half by non-pathogens. This would result in a decrease in final tumour weight, but not complete inhibition of tumour induction. Using a similar technique to mine, Schilperoort (1969) did find a decrease in gall weight which could be due to competition for wound sites between pathogenic and non-pathogenic agrobacteria.

However, the Lippincotts' model cannot apply to the complete inhibition of gall induction by isolate 58, when present in equal numbers to pathogens. Nor can the dramatic inhibition be ascribed to inhibition or killing of the pathogenic organism; Kerr (personal communication) found that the multiplication of the pathogen in the wound was slightly reduced by the presence of isolate 58 cells, but not enough to account for the complete prevention of gall induction. In another experiment, Kerr (personal communication) found that isolate 58 cells killed by heating at 60° were not able to inhibit tumour induction. In other

words, the living bacterial cell is probably needed, in much the same way as the living pathogenic cell appears to be indispensable in tumour induction.

Bearing in mind that competition for bacterial attachment sites cannot explain the inhibition of tumour induction by isolate 58, another theory is proposed. This proposed mechanism of infection and immunity also accounts for the phenomenon of transfer of virulence (Kerr, 1971). It is presented diagrammatically in Figure 4.

Available evidence indicates that the hypothetical "tumour inducing principle" (TIP) produced by pathogenic agrobacteria (Braun, 1947) is DNA, possibly a temperate bacteriophage. It is proposed that cells of inhibitory isolates contain a similar DNA entity to the TIP found in pathogenic cells, with one important difference; it is unable to induce the tumorous state in host cells.

"Defective TIP" is released into host cells in exactly the same way as true TIP, which it inhibits in some way, perhaps by blocking an essential biochemical pathway in tumour induction. It is well known that virus-infected plant cells and lysogenic bacteria may be immune to subsequent infection by a closely related virus or phage (Kassanis, 1963; Bertani, 1953). Provided there is at least one inhibitor bacterium per conditioned host cell and the "defective TIP" can irreversibly block an essential biochemical chain, inhibition of tumour induction in 100% of conditioned cells could be achieved.

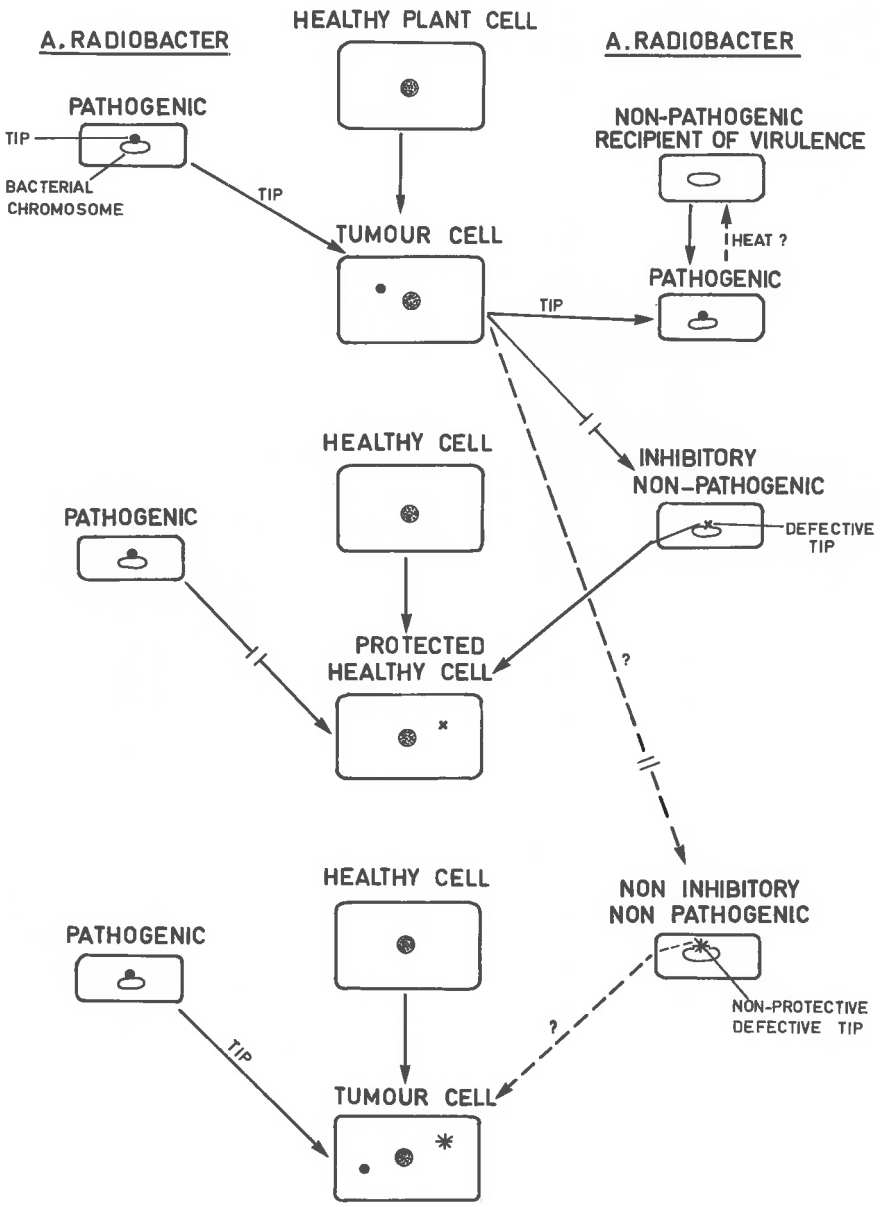


FIG. 4 PROPOSED MECHANISM OF TUMOUR INDUCTION, TUMOUR INHIBITION AND TRANSFER OF VIRULENCE.

This theory also explains the mechanism of virulence transfer. Kerr (1971) suggested that the tumour inducing principle was transferred from pathogenic bacteria to host cells and from them to non-pathogenic recipient bacteria. If recipient bacteria contain no "defective TIP" it would be possible for them to incorporate TIP released by tumour cells and become pathogenic. Pathogenic daughter cultures are serologically and biochemically identical to the untransformed recipient cultures, which means that only a small fraction of the pathogenic bacterial genome, probably a phage or other episomal DNA, is transferred.

It is significant that isolate 58 could not acquire virulence, probably because of "immunity" conferred by the presence of defective TIP. It is perhaps also significant that all recipient strains were isolated from potting soil which had undergone partial sterilization by heat, a treatment which could possibly free bacterial cells of prophage or other episomal DNA.

The other category of agrobacteria, the non-inhibitory non-pathogens isolated from the field, presumably contain a "defective TIP" which does not inhibit the action of true TIP in plant cells. The fact that these non-pathogens have been isolated from galls suggests that they also are prevented by the defective TIP from acquiring virulence.

It is proposed here that whether an Agrobacterium isolate is tumorigenic or non-pathogenic, inhibitory or non-inhibitory, depends on

whether its cells contain TIP or inhibitory or non-inhibitory defective TIP. If techniques of electron microscopy and isolation and proof of pathogenicity of a bacteriophage can be used to prove that TIP is a temperate phage, it may be possible to use similar techniques to observe a similar phage in inhibitory bacteria or to isolate it from them. If TIP is not phage, it will be more difficult to prove the existence of defective TIP. It may be possible, however, to obtain circumstantial evidence by subjecting plants inoculated with inhibitory bacteria to various treatments known to affect tumour induction by pathogenic bacteria, and noting whether inhibition of tumour induction is affected in the same way.

Crown gall has considerable economic importance as a plant disease, but by far the greatest interest in this plant tumour has been in its supposed similarity to animal cancers. In this thesis, a method of biological control of crown gall is presented and a theory proposed to explain the mechanism of control. It has been pointed out that the hypothetical tumour inducing principle and a tumour inhibiting principle may be temperate bacteriophages.

While it is not certain that all cancers are of viral origin, many are known to be induced by virus (Huxley, 1958; Dulbecco, 1967) and the discovery of a control of crown gall may have profound implications for the control of these animal tumours.

APPENDICES

APPENDIX 1. Culture Collection of the Waite Institute Plant Pathology Department: Isolate Numbers

APPENDIX 2. Composition of Media

APPENDIX 3. Composition of Selective Media for *Agrobacterium* and *Rhizobium*

APPENDIX 4. Publications

APPENDIX 1. Culture Collection of the Waite Institute Plant Pathology Department: Isolate Numbers

Isolate No. (Table 1)	Culture collection number	Isolate No. (Table 1)	Culture collection number
1	22	39	29
2	23	40	49
3	20	41	35
4	21	42	36
5	15	43	45
6	16	44	63
7	17	45	65
8	18	46	46
9	43	47	66
10	44	48	47
11	42	49	67
12	1	50	69
13	8	51	68
14	9	52	70
15	14	53	3
16	19	54	99
17	24	55	106
18	25	56	107
19	40	57	108
20	30	58	84
21	31	59	98
22	11	60	103
23	12	61	104
24	2	62	101
25	41	63	111
26	6	64	112
27	7	65	113
28	10	66	123
29	4	67	124
30	5	68	125
31	48	69	126
32	62	70	127
33	26	71	128
34	27	72	129
35	33	73	130
36	34	74	131
37	37		
38	28		

APPENDIX 2. Composition of Media

Mannitol-nitrate-biotin broth:

mannitol	1 g
$NaNO_3$	4 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
biotin	1 μg
NaOH to pH 7	
distilled water	1 litre

Mannitol-casamino acids broth:

mannitol	10 g
'Difco' casamino acids	6.7 g
KH_2PO_4	0.01 g
$CaCl_2$	0.2 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
FeEDTA solution*	2 ml
NaOH to pH 7	
distilled water	1 litre

* FeEDTA solution (0.65%):

$FeSO_4.7H_2O$	278 mg
Na_2EDTA	372 mg
H_2O	to 100 ml

APPENDIX 2 (continued)

Mannitol-glutamic acid-biotin broth:

mannitol	10 g
L-glutamic acid	2 g
KH_2PO_4	0.1 g
$CaCl_2$	0.2 g
NaCl	0.2 g
$MgSO_4$	0.2 g
FeEDTA*	2 ml
biotin	2 µg
NaOH to pH 7	
distilled water	1 litre

Nutrient broth:

'Difco' nutrient broth	8 g
distilled water	1 litre

Nutrient agar:

as for nutrient broth, plus 15 g agar

Nutrient agar plus glucose:

as for nutrient agar, plus 10 g glucose

(autoclaved separately)

APPENDIX 2 (continued)

Lactose agar:

lactose	10 g
yeast extract	1 g
agar	20 g
distilled water	1 litre

Yeast mannitol agar:

mannitol	10 g
K_2HPO_4	0.5 g
$CaCl_2$	0.2 g
NaCl	0.2 g
$MgSO_4$	0.2 g
$FeCl_3$	0.01 g
yeast extract	1.0 g
agar	15.0 g
distilled water	1 litre

APPENDIX 2 (continued)

Protein extraction medium (Staples & Stahmann, 1963):

Tris (Tris(hydroxymethyl)aminomethane)	1.21 g
sucrose	17 g
ascorbic acid	0.1 g
cysteine hydrochloride	0.1 g
1 N HCl to pH 8.0	
distilled water	to 100 ml

APPENDIX 3. Composition of Selective Media for *Agrobacterium* and *Rhizobium*

A. radiobacter biotype 1 (Schroth et al., 1965)

agar	20 g
mannitol	10 g
$NaNO_3$	4 g
$MgCl_2$	2 g
Calcium propionate	1.2 g
$Mg_3(PO_4)_2.6H_2O$	0.1 g
$NaHCO_3$	0.075 g
$MgCO_3$	0.075 g
distilled water	1 litre

Antibiotics and inhibitory chemicals:

cycloheximide	250 ppm
bacitracin	100 ppm
tyrothricin	1 ppm
sodium selenite	100 ppm
streptomycin	30 ppm
penicillin G	60 ppm
berberine	275 ppm

$\frac{N}{10}$ HCl to pH 7

APPENDIX 3 (continued)

A. radiobacter biotype 2 (P. Keane)

agar	15 g
mannitol	10 g
L-glutamic acid	2 g
K_2HPO_4	0.5 g
$CaCl_2$	0.2 g
NaCl	0.2 g
$MgSO_4$	0.2 g
FeEDTA (0.65%)*	2 ml
biotin	2 µg
distilled water	1 litre
1 N NaOH to pH 7 (approximately)	

Antibiotics and inhibitory chemicals:

cycloheximide	250 ppm
bacitracin	100 ppm
tyrothricin	1 ppm
sodium selenite	100 ppm
novobiocin (freshly suspended)	1 ppm

APPENDIX 3 (continued)

Rhizobium spp. (Graham, 1969)

agar	20 g
mannitol	5 g
lactose	5 g
yeast extract	0.5 g
K_2HPO_4	0.5 g
NaCl	0.2 g
$CaCl_2.2H_2O$	0.2 g
$MgSO_4.7H_2O$	0.1 g
$FeCl_3.6H_2O$	0.1 g
distilled water to	1 litre

Antibiotic and inhibitory chemicals:

cycloheximide	200 ppm
penicillin G	25 ppm
chloramphenicol	10 ppm
neomycin	2.5 ppm
pentachloronitrobenzene (PCNB)	100 ppm
sulfathiazole	25 ppm
1% Congo red solution	2.5 ml (optional)

pH adjusted to 7.0

APPENDIX 4. Publications

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