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BIOLOGICAL CONTROL OF CROWN GALL

by

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SUMMARY

Crown gall on stone fruit trees (peach, *Prunus persica*, cultivar Wight) was biologically controlled by a nonpathogenic strain (84) of *Agrobacterium radiobacter* var. *radiobacter* biotype 2 (New and Kerr, 1972). The efficiency of the strain was investigated under field conditions. Strain 84 was inoculated at the time of sowing seeds (seed inoculation), the time of transplanting (root inoculation), or both. Marked prevention of crown gall on plants grown in naturally and artificially infested soils was achieved by either seed or root inoculation, however, root or combined seed and root inoculation gave better control than seed inoculation alone. Application of strain 84 into the soil around the crowns of peach plants two weeks after transplanting did not give any protection against crown gall.

In tests on the practical application of inoculation it was found that inoculum made by mixing the bacteria from one culture bottle in one gallon of water (approximately 10^8 cells/ml) gave as high a control as suspensions with higher numbers of bacteria. Survival of strain 84 was better in water with the addition of some soil than in water alone, buffered water, saline, or buffered saline water. Gamma irradiated peat was tested and found to give satisfactory growth of strain 84. Peat inoculum packed in polythene bags is suggested for commercial distribution in future.

The mechanism involved in biological control of crown gall by strain 84 was investigated by examining the interactions between various pathogenic and nonpathogenic strains of *A. radiobacter* on wounded tomato seedlings and in liquid culture. Strain 84 inhibited the growth of many

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pathogens and prevented gall formation by them. It was later found that biological control of pathogenic strains correlated highly with the sensitivity of these strains to bacteriocin produced by strain 84 (Kerr and Htay, 1974).

Subsequent study examined the hypothesis that bacteriocin 84 was the mechanism behind biological control by strain 84. Bacteriocin 84 and marked inhibition of the growth of pathogens subject to biological control were detected at the same time in liquid cultures when strain 84 and a pathogen were grown together. Sterile bacteriocin extracted from cultures of strain 84 not only inhibited the growth of susceptible pathogens but also prevented gall induction by them. However, production of bacteriocin on wounded tomato stems where strain 84 operated biological control has still to be determined.

Limited attempts were made to discover other selective nonpathogenic strains which would produce a bacteriocin against two bacteriocin-84-insensitive pathogenic strains and to control these pathogens. None of 110 soil isolates tested was found to produce a bacteriocin and none of 17 isolates controlled either of the pathogens at a ratio of 1:1 pathogenic to nonpathogenic cells. However, it was found that high proportions (70:1 and 180:1) of nonpathogenic cells of 4 strains, including strain 84, markedly inhibited gall formation by these pathogens.

STATEMENT

This dissertation has not previously been submitted for a degree at this or any other university, and is the original work of the writer, except where due reference is made in the text.

KHIN HTAY

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GENERAL INTRODUCTION

Crown gall is a universally known plant disease caused by the bacterium *Agrobacterium radiobacter* var. *tumefaciens* (Keane, Kerr and New, 1970). The bacterium induces an uncontrolled tumorous growth of plant tissue which in nature is similar to cancerous growth of animal tissue (Smith, 1912, 1916). Consequently, crown gall is one of the most studied plant diseases.

Crown gall occurs on many different plants belonging to at least 142 genera, distributed over 61 widely separated families of plants (Elliot, 1951). Common susceptible plants are stone fruit trees, apple, pear, grapes, rose, hop, poplar, as well as many herbaceous species. Although many conifers and almost all dicotyledonous plant species are susceptible none of the monocotyledons has shown an unequivocal response (Wood, 1972).

Outbreaks of this disease have been reported from many countries in the world. It occurs in Asia, Africa, Europe, Australia, New Zealand and U.S.A., but its severity varies from place to place and on different hosts. Thus, the economic loss caused by crown gall is different in different countries. For instance, when California alone was having an estimated annual loss of \$7 million due to this disease (Schroth, Weinhold, McCain, Hildebrand and Ross, 1971) crown gall was considered not important in England and Europe (Lelliot, 1971; Riker, 1928; Riker, Berbee and Smalley, 1959).

In South Australia peaches, almonds and plums are seriously

2.

affected but not apricots. Especially in nurseries it is a considerable problem and its incidence is often high but varies markedly from year to year (Kerr, 1969). In Greece the incidence and severity of the disease on fruit trees in nurseries has increased in recent years. Stone fruit trees, grape vines, apple, pear, pistachio, orange, olive, cotton and rose are reported susceptible plants in Greece (Panagopoulos and Psallidas, 1973).

In different countries of Europe the disease has been known by a variety of names such as Canker, Kerbs, Wurzelkropf, Broussins and Rogna (Smith, 1911). However, as its tumorous outgrowths occur mostly on the crown portions of affected trees, internationally it has been called crown gall.

The causal bacterium was first isolated by Cavara of Italy in 1897 (Lum, 1970) from galls on grape vines. It was originally named *Bacterium tumefaciens* by Smith and Townsend (1907), later changed to *Agrobacterium tumefaciens* by Conn (1942). But like the disease itself, the causal bacterium has had many different names:

Pseudomonas tumefaciens (Stevens, 1913)

Bacillus tumefaciens (Holland, 1920)

Phytomonas tumefaciens (Bergey, Harrison, Breed, Hammer and
Huntoon, 1923)

Agrobacterium tumefaciens (Conn, 1942)

Erwinia tumefaciens (Dowson, 1957)

Rhizobium radiobacter var. *tumefaciens* (De Ley, 1968)

Agrobacterium radiobacter var. *tumefaciens* (Keane et al., 1970)

A. radiobacter (Beijerinck and van Deldon) Conn, is a soil saprophyte and has long been recognised by many investigators as closely resembling *A. tumefaciens*. Both organisms have been considered to be one species (De Ley, 1968; Graham, 1964; Heberlein, De Ley and Tijtgat, 1967). Keane *et al.* (1970) classified both organisms as one species with pathogenicity indicated by a varietal epithet. Thus, *A. radiobacter* var. *tumefaciens* is for pathogenic isolates and *A. radiobacter* var. *radiobacter* is for nonpathogens.

A. radiobacter var. *tumefaciens* is an aerobic, gram negative rod-shaped bacterium with dimensions of 0.7 - 0.8 and 2.5 - 3.0 μm (Bergey *et al.*, 1923; Breed, Murray and Smith, 1957). It may have 1-4 flagella (one polar flagellum or more than one peritrichous flagella). The optimal growth temperature is 25 - 28°C. ~~*A. radiobacter* var. *radiobacter* is smaller than *A. tumefaciens* with dimensions of 0.15 - 0.75 and 0.3 - 2.3 μm .~~

One of the characteristics of *A. radiobacter* var. *tumefaciens* is star formation on a medium rich in carbohydrate but containing little nitrogen. Stapp (1957) considered this star formation to be the preliminary sexual stage of the bacterium (Kiraly, Klement, Solymosy and Vörös, 1974).

The crown gall bacterium infects susceptible plants via wounds (Riker and Berge, 1935; Braun, 1954, 1962). Recently Kerr (1972) observed an association of galls and lenticels on the roots of young peach trees and suggested that bacteria penetrate the loose cells of lenticels. The bacterium occurs in intercellular spaces in host tissue and transforms

normal host cells into tumor cells under the influence of a hypothetical tumor-inducing principle (TIP) (Braun, 1947).

The transformation of a normal cell to a fully autonomous tumor cell is not a one-step process but occurs progressively over a 3 to 4 day period. The total process of tumor formation has been divided into two phases: (1) the inception phase, which is concerned with the transformation of normal cells to tumor cells and (2) the developmental phase, which refers to the continued self-proliferation of the tumor cells once the normal cells have been completely transformed (Braun and Laskaris, 1942; Wood, 1972).

The inception phase has been divided into two parts; conditioning and induction. Conditioning is when normal plant cells are irritated by wounded cells nearby (Braun, 1943). The conditioning period lasts for 3 - 4 days (Braun, 1943, 1947, 1952; Braun and Mandle, 1948). Before the actual induction takes place, the bacterium requires at least 8 hours to attach itself to infection sites of the conditioned cell (Lipetz, 1966; Manigault, 1970). Then, during the next 8 - 10 hours, the conditioned cell is induced to become a slow-growing tumor cell by the influence of TIP derived from the bacterium. In the case of lenticel infection, Kerr (1972) suggested that the bacteria penetrated the loose cells of lenticels and came into contact with actively dividing cells which were susceptible to crown gall induction. However, further study on lenticel development and structure has been suggested in order to understand crown gall induction on underground portions of plants (Kerr, 1972). After induction is completed, continued abnormal and autonomous growth of the tumor cells

occurs. This is referred to as the developmental phase which can be carried out without the presence of any causal bacteria.

Braun (1943) was able to demonstrate the continuous development of tumor cells after killing bacteria present in these cells by heat treatment at $46 - 47^{\circ}$ for 2 - 3 days or 41° for 7 days (Manasse and Liptez, 1971). This continuous growth of bacteria-free tumor tissue was also demonstrated by White and Braun (1941, 1942) who grew the tumor tissue *in vitro* with the complete absence of bacteria. De Ropp (1947a) grafted bacteria-free sunflower tumor tissue into normal stem fragments and found that a new secondary tumor appeared from the normal stem segment. He suggested that a transmissible agent might be present in crown gall tumor tissue. However, the nature of the tumor-inducing principle which induces normal cells to become tumor cells is still a matter of controversy although it is undoubtedly the most intriguing problem in crown gall research. This, as yet, uncharacterized agent has been suggested variously by many workers as: plant growth hormones produced by bacteria (Brown and Gardner, 1936; Link and Wilcox, 1937); a temperate bacteriophage (Beardsley, 1955; Heberlein and Lippincott, 1967; Parsons and Beardsley, 1968; Zimmer, Hamilton and Pootjes, 1966), or bacterial nucleic acid (RNA or DNA) (Klein and Braun, 1960; Braun and Wood, 1966; Schilperoort, 1969; Chadha and Srivastava, 1971; Stroun and Anker, 1971; Yajko and Hegeman, 1971).

Since the characteristics of hyperplastic tissue induced by growth hormones were different from crown gall tissue and no correlation between pathogenicity and production of auxin was observed (Locke, Riker and Dugger, 1938; Braun and Laskaris, 1942; Braun and Morel, 1950;

Galsky and Lippincott, 1969), the theory of TIP being bacterial hormone is invalid.

Most attempts to induce galls by purified bacteriophages isolated from pathogenic agrobacteria have been unsuccessful (Klein and Beardsley, 1957; Schilperoort, Sittert and Schell, 1973; Stonier, McSharry and Speitel, 1967; Zaenen, Van Larbeke, Teuchy, Van Montagu and Schell, 1974); likewise with purified phage DNA (Beiderbeck, Heberlein and Lippincott, 1973).

Although a number of studies have indicated rather convincingly that bacterial nucleic acid or DNA from *A. radiobacter* var. *tumefaciens* is the inducing agent, this theory is also still not conclusively proven because of inability to induce crown gall by nucleic acid or DNA of pathogenic bacteria (Bieber and Sarfert, 1968; Gribnau and Veldstra, 1969; Braun and Stonier, 1958). Moreover, Heberlein *et al.* (1967) observed that nonpathogens had similar DNA to that of pathogens and Schilperoort (1969) found that nucleic acid of *A. radiobacter* var. *radiobacter* gave similar results to that from *A. radiobacter* var. *tumefaciens* in hybridization experiments with nucleic acid from gall tissue.

Recently some workers have observed a correlation between induction of either of the guanidines, octopine or nopaline, in crown gall tissue, and the utilization of either of these compounds by pathogenic isolates of *Agrobacterium* in culture (Petit, Delhayé, Tempe and Morel, 1970; Lippincott, Beiderbeck and Lippincott, 1973). Petit and Tourneur (1972) also found an association between loss of pathogenicity and loss of

ability to utilize the guanidine in culture. This evidence suggested that either of these guanidines could be the agent for TIP or could be associated with TIP. However, observation of the presence of a small amount of octopine in normal plant tissue (Johnson, Guderian, Eden, Chilton, Gordon and Nester, 1974) means the above suggestion needs further confirmation.

The presence of a large circular DNA plasmid in virulent, but not avirulent cells of *Agrobacterium* has also been reported and it is suggested that the plasmid is TIP (Van Larebeke, Engler, Holster, Van den Elsacker, Zaenen, Schilperoort and Schell, 1974; Zaenen *et al.*, 1974). But Kado, Heskett and Langley (1972) and Roberts (1975) could not find any evidence of plasmid in virulent cells in their experiments.

Since none of the above agents, but only viable and metabolically active tumorigenic *A. radiobacter* var. *tumefaciens* cells are, as yet, able to induce crown gall, any of the above interpretations of the TIP has still to be proved.

The formation of tumorous outgrowths especially on the crowns of plants disturbs the uptake of nutrients (Link, Wilcox, Eggers and Klein, 1953) and decreases the water conductivity of young trees (Melhus, Munice and Ho, 1924). Mature trees are not affected as seriously as young trees (Riker *et al.*, 1959), but Ross, Schroth, Sanborn, O'Reilly and Thompson (1970) reported that hundreds of almond trees in California died or were blown over every year due to heart rot following crown gall infection occurring years earlier. They estimated that a loss of 20% of the economic life of a fruit orchard might be caused by the disease.

Although it is illegal to sell young trees with galls and these are not transplanted in South Australian orchards, this disease continues to appear every year. This could be due to incipient infections. New (1972) found that in a commercial nursery in South Australia, 10 - 40% of young trees in bundles to be sold carried this pathogen. Cameron (1972) was also able to isolate pathogenic cells of *A. radiobacter* var. *tumefaciens* from symptomless trees. The sale of infected young trees from which galls had been cut accidentally or in ignorance of their nature, could be one of the causes of the appearance of the disease in new orchards.

Control of crown gall has been attempted by many workers during the last sixty years. The earlier control measures emphasized reducing the probability of contact of the pathogen with planting materials and preventing the introduction of the pathogen to a new area. For instance, growers were advised to use cloth wrappers at graft unions of apple trees (Melhus and Maney, 1922) and to use clean root stocks (Steward, 1924; Riker and Keitt, 1926).

Eradication of the pathogen from planting materials was practised by dipping roots of all nursery stocks in disinfectants or antiseptics and applying chemicals to grafting unions. Mercuric chloride (Riker, Keitt, Hildebrand and Banfield, 1934) and Terramycin (Guenerich, Goodman and Millikan, 1969) were suggested for use for dipping roots.

Soil fumigation has also been used to control this disease (Dickey, 1962; Deep, McNeilan and MacSwan, 1968; Ross *et al.*, 1970). But Schroth *et al.* (1971) found that although fumigation could reduce the

incidence of crown gall, the disease was not effectively controlled. Moreover, they found that the low percentage of cells of *A. radiobacter* var. *tumefaciens* which survived in fumigated soil increased rapidly to a level that could cause appreciable disease in a susceptible host. This result agreed with those of Deep *et al.* (1968) and Dickey (1962).

Pathogenic bacteria fail to survive in soil of low pH. Thus, acidification of soil by treatment with sulphur has been suggested (Ark, 1941; Dickey, 1962; Shierbakoff, 1925; Siegler and Bowman, 1940). Hussin (1962) and Hussin and Deep (1965) have studied the influence of mineral nutrients on gall development. The size of galls on tomatoes and cherries decreased if the levels of N and P were increased. Galls failed to develop in boron-deficient soil. However, the manipulation of mineral nutrients was not applied in the field to control the disease.

Some workers have looked for resistant varieties of susceptible plant species. For instance, Brown (1923) developed resistant varieties from artificially inoculated Paris Daisy plants and from hybrid rose varieties. However, no permanent resistance to crown gall was observed in any variety. Smith (1924) studied 40 species of *Prunus* and found that *Prunus mume* and *P. besseyi* appeared to be resistant to crown gall. De Vay, Nyland, English and Schick (1962) developed resistant plants from irradiated root-stocks of *Prunus mahaleb* (cherry), *P. persica* (Lovell peach), *P. amygdalus* (bitter almond) and *P. cerasifera* (myrobalan plum). However, the susceptibility of these irradiated plants to crown gall was found to increase with age.

Some protection against the disease has been achieved by dipping

seeds in fungicides. The incidence of galls was reduced by dipping peach seeds in 2.5% mercurous chloride before planting (Siegler and Bowman, 1940). But Schroth *et al.* (1971) failed to achieve satisfactory control by seed treatment with 0.1% mercuric chloride and 1% sodium hypochloride. Morsodren, an organic mercury compound was also able to reduce gall incidence when it was used as a soil drench accompanying planting seeds (Guenerich *et al.*, 1969). Cycloheximide thiosenic carbazone and cycloheximide acetate, systemic fungicides, protected cherry trees against development of aerial galls if the fungicides were applied before the trees were artificially inoculated with the pathogen (Helton and Williams, 1968). However, most chemical fungicides were found injurious to the trees.

and

Antibiotics such as penicillin, terramycin, streptomycin have been suggested for use as eradicants of galls (Brown and Boyle, 1945; Braun, 1948; De Ropp, 1951; Hampton, 1948; Klemmer, Riker and Allen, 1955; Cole, 1969). Although eradication of galls by these antibiotics seemed satisfactory in experimental work their use was not practical in commercial orchards because of cost and undesirable side effects on the trees.

Chemicals such as colchicine (Brown, 1939), iodine, clove oil and Elgetol (Ark, 1941; Ark and Scott, 1951) were also proposed for use as eradicants of gall tissue by painting or smearing the gall surface. A mixture of sodium-dinitro-ortho-cresol and methanol at a ratio of 1:4, was reported an effective paint for galls but it was not cleared for use by the U.S.A. Food and Drug Administration (Ross *et al.*, 1970).

A very successful gall eradicator, which is commercially known as 'bacticin' was developed by Schroth and Hildebrand (1968). Bacticin consists of five hydrocarbons and hydrocarbon derivatives in a kerosene-water emulsion. The active contents dissolve in kerosene, and selectively penetrate the gall tissue which gradually dies within 2 to 3 months (Ross *et al.*, 1970). However, as galls normally form below ground level, application of bacticin to a gall involves the removal of soil around the base of a tree before the bacticin is painted on the gall surface. To minimize damage to trees, Ross *et al.* (1970) recommended a hydraulic system (a strong jet of water) for the removal of soil around galls. Although this treatment gave a 95% successful eradication of galls, the method of application of bacticin consumes much time and labour, which in turn, affects the economy of commercial growers.

Recently, an economical, simple control measure has been reported by New and Kerr (1972) and Kerr (1972) in South Australia. This control involves the use of a biological inoculum of a strain of *A. radiobacter* var. *radiobacter* (strain 84) which is used for seed and root treatments.

In short term experiments, Kerr (1972) and New and Kerr (1972) achieved 69% and 100% control of crown gall in artificially infested soil by inoculating seeds and roots respectively with this inoculum. They also reported that strain 84 controlled most of the pathogenic strains tested but a few were only slightly controlled by strain 84 in glasshouse experiments. However, they could not find any other nonpathogenic strains which were as effective as strain 84 in biological control of crown gall.

This thesis describes experiments on biological control of crown gall by strain 84 in naturally infested soil as an approach to field conditions. The interaction of pathogenic and nonpathogenic strains of *A. radiobacter* in liquid cultures and on wounded tomato stems were also investigated to gain an understanding of the mechanism involved in biological control of crown gall which has not previously been investigated. Such information would give more understanding of prevention as well as the formation of this disease, so that, if necessary, improvements could be made in the methods of biological control in the field.

GENERAL MATERIALS AND METHODS

1. Strains of *Agrobacterium radiobacter* used:

Most strains of *Agrobacterium radiobacter* used in this work, were supplied by Dr. A. Kerr. General information on each strain is given in Table 1. All cultures were freeze-dried and stored at 5°C. Those cultures used extensively were also stored on porcelain beads (Norris, 1963), and when required, subcultured on yeast mannitol (YM)* agar slopes.

The nomenclature of Keane, Kerr and New (1970) was used for bacterial strains:

Agrobacterium radiobacter var. *radiobacter* biotype 1 or 2 for nonpathogenic strains,

A. radiobacter var. *tumefaciens* biotype 1 or 2 for pathogenic strains.

2. Media:

The medium of Schroth, Hildebrand and Thompson (1965) was used for biotype 1 strains and the medium of New and Kerr (1971) was used for biotype 2 strains. Yeast mannitol agar, Mannitol glutamic acid (MGA) liquid and Stonier's (1960a) liquid and agar were used as general media for both biotypes.

3. Inoculation of tomato seedlings:

Stems of 4 - 6 week old tomato seedlings (*Lycopersicum esculentum*

* The formulae and preparations of media are given in Appendix 1.

Table 1. Origin and information on the strains of *Agrobacterium radiobacter* used.

Strain	Classification	Biotype	Origin and General Information
84	<i>A. radiobacter</i> var. <i>radiobacter</i>	2	Soil around peach gall, South Australia; P.B. New. Inhibitory. Produces bacteriocin 84.
27	<i>A. radiobacter</i> var. <i>tumefaciens</i>	"	Peach gall, South Australia; A. Kerr. It can transform some biotype 1 non-pathogens to acquired pathogens <i>in vivo</i> . Sensitive to bacteriocin 84, subject to biological control.
130	<i>A. radiobacter</i> var. <i>radiobacter</i>	"	Soil, South Australia; A. Kerr. Not inhibitory. Not sensitive to bacteriocin 84.
128	"	"	Soil around peach gall, South Australia; P.B. New. Not inhibitory. Sensitive to bacteriocin 84. Produces bacteriocin 128 to which strain 84 and 130 are sensitive.
108	<i>A. radiobacter</i> var. <i>tumefaciens</i>	"	Almond gall, South Australia; P.B. New. Sensitive to bacteriocin 84. Produces bacteriocin 108 to which strain 84 is sensitive. Not subject to biological control.
147	<i>A. radiobacter</i> var. <i>radiobacter</i>	1	Soil, South Australia; A. Kerr. Not sensitive to bacteriocin 84. Can be transformed to pathogen by acquiring the virulence from biotype 2 pathogen strain 27. Not inhibitory.
152	<i>A. radiobacter</i> var. <i>tumefaciens</i>	"	Acquired pathogen; parent nonpathogen is 147. Sensitive to bacteriocin 84. Subject to biological control.
24	"	"	Peach gall, South Australia; A. Kerr. Sensitive to bacteriocin 84; is used as standard indicator strain for bacteriocin 84. Subject to biological control.
6	"	"	Gall on apple; Edinburgh, Scotland; W. Blyth via D.W. Dye (A4); supplied by A.C. Parker (WU78). Not sensitive to bacteriocin 84. Not subject to biological control.
10	"	"	Peach gall, New South Wales Forestry Commission (109A), supplied by A.C. Parker (WU89). Not sensitive to bacteriocin 84. Not subject to biological control.

cv. South Australian Early Dwarf Red) were wounded by piercing with a multi-needle instrument (Kerr, 1969). A few drops of sterile distilled water were placed on the wound and a loopful of bacterial culture was, then, gently rubbed on the wound. Inoculated tomato seedlings were kept in a 25°C glasshouse for 2 - 4 weeks. Isolates which formed galls at inoculation sites were recorded as pathogens.

To inoculate with a specified number of bacterial cells, a standard wound was made by piercing the stem of tomato seedling to a depth of 3 mm with a cylindrical rod 2 mm in diameter (New and Kerr, 1972). A bacterial suspension (0.004 ml) of specified concentration was prepared by mixing 2-day-old culture with sterile distilled water and measuring the concentration of cells by optical density using an EEL colorimeter (Absorptiometer). A suspension with optical density 6 at 603 mμ gave approximately 10^8 cells/ml (Lum, 1970).

The inoculated tomato seedlings were subirrigated to avoid contamination by splashing and incubated in a 25°C glasshouse for 2 weeks to examine galls and 5 weeks to measure the diameter of galls.

4. Test for bacteriocin 84 production:

Cultures to be tested for bacteriocin production were spot-inoculated on plates of Stonier's agar medium (Stonier, 1960) and the plates incubated at 25°C for 24 hours. The colonies were then exposed to chloroform for 15 minutes by placing a lid with an attached filter paper moistened with chloroform over the plate. The lid was then replaced with one without filter paper and excess chloroform vapour was removed by slightly opening the lid of the plate for 5 - 10 minutes. Pathogenic

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strain 24 was used as the indicator strain susceptible to bacteriocin

84. A loopful of 2-day-old culture was inoculated to 10 ml of sterile Stonier's liquid medium and incubated at 25°C for 2 - 3 days. Half a ml of this liquid culture was mixed with 2.5 ml of soft buffered agar (Stonier, 1960) at 45°C and evenly spread over the test plate. Bacteriocin (84) production was indicated by a zone of inhibition round a colony.

SECTION A

THE USE OF NONPATHOGENIC STRAIN 84, *AGROBACTERIUM RADIOBACTER*,
VAR. *RADIOBACTER* BIOTYPE 2, TO CONTROL CROWN GALL

INTRODUCTION

Serious infection of crown gall on peach, almond and vines has been reported in South Australia for a long period (Kilpatrick, 1958). This disease is common in the Murray River districts, seriously affecting nursery trees, especially at Loxton and Renmark. An infection of 30 - 80% in nurseries is not uncommon. Young trees under 10 years of age are economically affected.

Apart from tumors on the crown and roots of peach and almond noticeable symptoms included general unthriftness or stunting, or sometimes debilitated growth on part of a tree, with purple pigmented leaves. Seriously affected trees had a short period of productivity and then a rapid decline which caused serious economic losses. Apricot trees were also affected but not as seriously as were peach and almond. The Gordo variety of vine was reported as the most susceptible variety of the disease. The incidence of crown gall was much greater in light sandy soils than in heavier alluvial types.

No satisfactory control was known. While the sale of infected trees was illegal, because of the difficulties of recognising incipient infection, infected young trees were still being sold. In a nursery, although 40% of the young trees showed infection, a further 10 - 40% of the trees might have unrecognisable early infection (Kilpatrick, 1958; New, 1972). Chemical sterilization of nursery soil did not give complete

control of the organism. Treatment of galls with Dinoc or antibiotics was impracticable and costly. Until 1968 commercial growers eradicated the infected trees simply by uprooting, burning, replacing infested soil with clean soil, and diseased trees with young healthy trees. These control practices were not sufficient to prevent the incidence of crown gall.

From 1969, work on eradication of galls was carried out. Eradication was by painting the gall surface with the commercial preparation 'Bacticin' (Schroth and Hildebrand, 1968). Although the treatment was effective (95% of painted galls died within 2 or 3 months) and the cost of bacticin itself was cheap, its application in the orchard was time-consuming and expensive in cost of labour since the galls had to be exposed by removing soil using a method which did not cause injuries to the trees (Ross *et al.*, 1970). Then gall tissue was chipped off to a basal 1/2 to 3/4". The gall surface was left to dry for 24 hours, then painted with bacticin. The painted gall was then left exposed for another 24 hours before the hole was refilled. Re-examination was also recommended after 4 - 6 months and retreatment given if there were any living galls present.

Dipping the roots of young trees in bacticin before transplanting, was also suggested. Dipping in organic mercurial compounds such as Ceresan and Aretan was also recommended to protect cut surfaces of young trees at transplanting. The main drawback was that both of these chemicals are extremely poisonous to man.

Although crown gall disease has been partially controlled by the above methods, their cost was very high. Attempts, therefore, were made to find a more effective and less expensive method of control.

Research on crown gall in South Australia was commenced by Kerr and his team from the Waite Agricultural Research Institute in 1967. The work started with an investigation of the ecology of the crown gall organism *Agrobacterium tumefaciens* (Smith and Townsend) Conn. The first problem encountered was difficulty in isolating the pathogen. After a number of attempts, pathogenic isolates were eventually isolated from gall tissue. However, these isolates did not conform to published descriptions of *A. tumefaciens* (Kerr, 1969). The South Australian isolates of this pathogen were then compared with isolates from England, Canada, New Zealand and Israel (Keane, Kerr and New, 1970). The local isolates were found to belong to a different biotype of *Agrobacterium*. The differentiation of biotypes was based on a number of biochemical tests, serological reactions and protein patterns following disc electrophoresis.

The division of the genus *Agrobacterium* into species according to pathogenicity was found to be unsatisfactory for several reasons. Keane, Kerr and New (1970) proposed combining *A. tumefaciens* with *A. radiobacter*, with pathogenicity indicated by a varietal epithet and the biotype specified. Both biotypes contain crown gall (tumor-inducing) bacteria, hairy root (root-proliferating) bacteria and nonpathogenic forms (Keane, Kerr and New, 1970). Thus, their proposed nomenclature for these bacteria is *A. radiobacter* var. *radiobacter* biotype 1 or 2 for non-pathogenic bacteria, *A. radiobacter* var. *tumefaciens* biotype 1 or 2 for crown gall pathogens and *A. radiobacter* var. *rhizogenes* biotype 1 or 2 for hairy root pathogens.

Most gall-forming isolates from South Australia belong to biotype 2 and they do not grow on Schroth *et al.*'s (1969) medium which has been reported as highly selective for crown gall bacteria. New and Kerr (1971) developed a selective medium for biotype 2 isolates on the basis of their knowledge that biotype 2 isolates could utilize erythritol as a source of carbon and grow on a medium containing sodium selenite (100 ppm) and the antibiotics such as cycloheximide (250 ppm), bacitracin (100 ppm) and tyrothricin (1 ppm) which inhibited the growth of other microorganisms.

The development of this selective medium for biotype 2 agrobacteria made it possible to investigate the ecology of tumor-inducing bacteria in South Australian stone-fruit nurseries. Although biotype 2 bacteria from gall tissue as well as soil were easily isolated, it was not possible to distinguish pathogens from nonpathogens on the medium.

Isolates had to be inoculated into test plants, tomato, to determine if they were pathogenic.

New and Kerr's (1972) study on the ecology of crown gall bacteria gave information not only on the population of these bacteria in soil but also on the natural relationships of pathogenic and nonpathogenic agrobacteria. The number of *A. radiobacter* biotype 2 that appeared was as high on roots of healthy plants as on those of diseased plants. However, the number of these bacteria was significantly higher in soil around the crowns of diseased plants. The most striking observations were that the ratio of pathogenic to nonpathogenic bacteria in soil round a gall ranged from 1:2 to 8:1, and only nonpathogens were found in soil around healthy

trees. This observation prompted them to investigate the influence of ratios of pathogen to nonpathogen on crown gall induction.

The first investigation of the effect of nonpathogens on the prevention of gall formation was carried out by Kerr (1969). Marked inhibition of gall formation was observed when he inoculated a mixture of pathogenic to non-pathogenic cells at a 1:100 ratio into tomato wounds. In 1972 New and Kerr isolated a biotype 2 nonpathogenic isolate from the soil round a peach gall. This nonpathogenic isolate (strain 84) was found to have the ability to prevent gall formation by many pathogenic strains of both biotypes 1 and 2.

Complete inhibition of gall induction was achieved from the inoculation of a mixture of a pathogenic strain (isolate 27) and the nonpathogenic strain 84 at a 1:1 ratio into wounds on tomato seedlings. Complete prevention of gall formation was also observed on wounded peach seedlings which were grown in soil inoculated with pathogenic cells and nonpathogenic cells at a ratio of 1:1. Dipping the roots of peach seedlings in a suspension of isolate 84 (3×10^6 or 3×10^7 cells/ml) before the seedlings were grown in artificially inoculated soil (10^4 pathogenic cells of isolate 27/g soil) also gave complete protection against the disease (New and Kerr, 1972).

Soon after these successful glasshouse experiments, Kerr (1972) demonstrated satisfactory control of crown gall by dipping peach seeds in a suspension of strain 84 (approximately 10^8 cells/ml) before the seeds were grown in soil artificially infested with pathogenic cells of isolate 27 (7.9×10^3 cells/g soil). This experiment was done in large

pots, kept in the open and run for 2 growing seasons. The first disease assessment was made after 6 months growth. Unharvested seedlings were then budded with a commercial variety and cut back to the bud. Final assessment was recorded after 2 years. Significant control was achieved on both occasions. The most marked effect was on the number of dead plants. Forty-two percent of plants grown from uninoculated seeds died compared with none in the seed inoculation treatment. As death of nursery trees is not often associated with crown gall infection in the field, Kerr suggested that it could be due to too high an infestation of pathogenic cells established in the soil.

In commercial practice, stone-fruit trees are normally grown from seeds in nursery beds. After 9 - 12 months the young plants are shifted to orchards. In some cases, seeds are sown in fields and the budded seedlings not moved until they are sold after 2 or 3 years. Thus, there are two chances to treat planting materials with inhibitory inoculum before trees are grown permanently in an orchard; (i) before the seeds are sown, and (ii) before the young trees are transplanted into the orchard. By inoculating seeds or roots or both, inhibitory non-pathogenic cells can be established in soil around the plants and prevent disease induction by pathogens which are present in nursery or orchard.

This section presents an investigation of the effect of the inhibitory nonpathogenic strain 84 applied to seeds, roots, or both, on the control of crown gall disease on young peach plants grown in naturally or artificially infested soil. The viability of strain 84 in various liquid media which could be used to prepare effective suspensions

of strain 84 for the inoculation of seeds or roots in commercial orchards, is also examined.

EXPERIMENTS AND RESULTS

1. Biological control of crown gall

The Wight variety of peach was used. Seeds were removed from stones and surface sterilized by soaking them in Thimerosal solution (Gilmore, 1950) for 5 minutes at room temperature. The seeds were then washed 10 times in sterile distilled water, and then artificially stratified by storing them in a cold room at 2°C for 3 months.

The stratified seeds were dipped in a cell suspension of strain 84 (approximately 10^8 cells/ml) harvested from a 2-day-old culture on a YM agar slope. The seeds were then grown in a sandy soil collected from Nildottie, Murray District, South Australia. The reason for using this soil was that few biotype 2 agrobacteria could be detected (< 3.3 cells/g soil; Kerr, 1969) so that the establishment of strain 84 cells around the seedlings would not be greatly influenced by the native soil agrobacteria. Non-treated seeds were also grown in the same soil in separate pots. The pots were watered carefully to avoid contamination by splashing soil from one pot to another.

After 12 months, plants from both treated and non-treated seeds were removed from the soil for transplanting. In the rhizosphere of plants which grew from treated seeds, there were 10^6 cells of nonpathogenic biotype 2 agrobacteria/g of soil (presumably strain 84). None were detected in the rhizosphere soil of the plants which grew from non-treated

seeds. Root inoculation was applied to some plants of both treated and untreated seeds before transplanting: thus, there were 4 treatments: seed inoculation, root inoculation, seed and root inoculation, and no inoculation. The plants were then transplanted into naturally, or artificially infested soil in metal drums (18 litre capacity) to give a total of 8 treatments with 26 replicates of each.

Both naturally and artificially infested soils were sandy loam, collected from an almond orchard at Angle Vale, South Australia. The naturally infested soil was dug from a circle 0.3 m radius and 0.3 m deep round each of 15 galled trees (Fig. 1). The natural infestation of this soil was measured. It contained 9.0×10^3 cells/g soil of pathogenic biotype 1 and 4.4×10^3 cells/g soil of pathogenic biotype 2 agrobacteria. Uninfested soil was collected from 12 randomly chosen positions between the rows of trees which were 6.1 m apart. No pathogenic cells of either biotype were detected in the inter-row soil which was later inoculated with a cell suspension of pathogenic strain 27 (biotype 2) to have approximately 10^4 cells/g of soil.

The experiment was run in the open over two growing seasons. In dry weather, the plants were subirrigated using a Triklon drip irrigation system (Fig. 2). This system was specially set up so that each plant received an equal volume of water with a measured accuracy of 5 to 10 per cent.

The incidence of crown gall was determined by measuring the number of galls, the dry weight of galls and the percentage of galled plants (Tables 2, 3). Disease incidence was markedly reduced in all

- Fig. 1.
- A. An infected almond tree.
 - B. Crown-gall on crown and roots of the infected tree.

Fig. 1



Fig. 2. Triklon drip irrigation system.

Fig. 2



Table 2. The effect of inoculation with strain 84 on mean number of galls and mean dry weight of gall tissue per peach tree growing in naturally and artificially infested soil.

Inoculation Treatment	Mean number of galls/plant			Mean dry weight of gall tissue/ plant (g)		
	Naturally infested soil	Artificially infested soil	Overall Treatment means	Naturally infested soil	Artificially infested soil	Overall Treatment means
None	18.08	16.00	17.04	11.64	17.09	14.37
Seed	3.04	8.25	5.64	2.50	9.50	5.87
Root	0.46	2.58	1.52	0.59	5.69	3.14
Seed and Root	0.35	2.31	1.33	0.14	2.33	1.24
L.S.D. (P=0.05)*		2.81				2.58
(P=0.001)		3.69				3.39

* In number of galls/plant, there was a significant interaction between treatments and soils. Therefore, the L.S.D.s of 2.81 and 3.69 apply to any two treatment-soil means. In mean dry weight of gall tissue/plants, there was no significant interaction between treatments and soils. Therefore the L.S.D.s of 2.58 and 3.39 apply to the overall treatment means.

Table 3. The effect of inoculation with strain 84 on the sites of galls on peach trees growing in naturally and artificially infested soil.

Inoculation Treatment	Naturally infested soil % plants* with galls on				Artificially infested soil % plants with galls on			
	Crown	Primary root	Lateral roots	Any part	Crown	Primary root	Lateral roots	Any Part
None	31	77	100	100	62	54	96	100
Seed	0	4	96	96	8	8	85	88
Root	4	4	31	35	12	12	73	77
Seed and Root	0	8	23	31	0	0	54	54

* There were 26 plants in each of the 8 treatment-soil combinations. Because nil and 100% infection were frequently recorded, valid statistical analyses were not possible.

inoculated treatments. Significantly more and larger galls were formed on trees growing in artificially infested soil compared with those in naturally infested soil. This may have been due to high infestation of pathogenic cells in artificially infested soil, but, unfortunately the number of pathogenic cells was not measured at the end of the experiment.

The effect of crown gall formation on the growth of the young trees was also assessed by measuring the diameter of the main stem, 2 cm above the junction of stem and root, and the dry weight of roots after removal of galls (Table 4). Plant growth was significantly improved in the root inoculated treatment.

The nearest approach to field conditions was in the naturally infested soil and probably the most satisfactory method of assessing disease incidence is by measuring dry weight of gall tissue/plant because this integrates percentage infection and number and size of galls formed. The data on dry weight of gall tissue/plant in naturally infested soil show that combined seed and root inoculation achieved 99 percent control ($\frac{11.64 - 0.14}{11.64} \times 100$). Root and combined seed and root inoculation gave better control than seed inoculation alone. But seed inoculation itself had a significant effect on the prevention of disease incidence. Thus, it is still important to apply this treatment in nurseries where young trees are generally seriously infected.

A distinctive effect of inoculating seeds with control inoculum was that few galls were observed at the crown of young trees in artificially infested soil and none were observed in naturally infested soil (Table 2). This showed that the number of inhibitory nonpathogenic cells at the crown was sufficient to give complete or marked prevention of gall

Table 4. The effect of inoculation with strain 84 on mean stem diameter and mean root dry weight of peach trees growing in naturally and artificially infested soil.

Inoculation Treatment	Mean dry weight of roots (g)			Mean stem diameter (mm)		
	Naturally Infested Soil	Artificially Infested Soil	Overall Treatment Mean	Naturally Infested Soil	Artificially Infested Soil	Overall Treatment Mean
None	24.68	24.55	24.62	1.37	1.27	1.32
Seed	29.95	28.91	29.43	1.45	1.35	1.40
Root	51.36	34.55	42.96	1.53	1.40	1.47
Seed and Root	42.72	31.18	37.00	1.47	1.48	1.47
L.S.D. ($P=0.05$)*	8.12					0.07
($P=0.001$)	10.74					0.09

* In dry weight of roots there was a significant interaction between treatments and soils. Therefore the L.S.D.s of 8.12 and 10.74 apply to any of two treatment-soil means. In stem diameter, there was no significant interaction between treatments and soils. Therefore the L.S.D.s of 0.07 and 0.09 apply to the overall treatment means.

formation. This led to consideration of whether there would be any effect if control inoculum was applied to soil around the crown portion of the trees which were already growing in infested soil.

2. The effect of applying strain 84 to soil around the crowns of peach plants

In New's (1972) study of gall induction in naturally infested soil, small galls at the wounded portions of 8 week old peach seedlings were observed 9 weeks after transplanting. In an experiment here, visible but small galls were observed on the roots of one-year-old peach plants 15 weeks after they had been transplanted into naturally infested soil (Fig. 3). These observations suggested that gall induction by pathogens in soil was completed during a period before 9 or 15 weeks depending on the condition and age of susceptible hosts. Thus the following experiment was conducted to see the effect of application of control inoculum to soil around the crowns of peach seedlings before visible galls were formed.

One-year-old peach seedlings were individually grown in small pots (1½ litre capacity) filled with naturally infested soil which had pathogenic bacteria approximately 2.0×10^3 cells/g of soil of biotype 1 and 4.1×10^4 cells/g of soil of biotype 2. Roots of half of the seedlings were dipped in a cell suspension of strain 84 (approximately 10^8 cells/ml) before they were transplanted.

At various times, 8 ml of strain 84 cell suspension of the same concentration (10^8 cells/ml) was applied into the soil around the crown




Fig. 3. Galls induced on the roots of a young peach
plant (15 weeks after transplanting).

Fig. 3



portions of both inoculated and uninoculated seedlings. This amount of cell suspension was considered sufficient to wet the whole root system of a seedling. The crown application was done at different times, thus there were 5 treatments for both inoculated and uninoculated plants: (1) no application, (2) application at 2 weeks, (3) at 4 weeks, (4) at 8 weeks and (5) at 2, 4 and 8 weeks. This gave a total of 10 treatments, each of which had 7 replicates. After 21 weeks, the plants were uprooted and the number and the dry weight of galls were recorded.

No significant ~~effect on the~~ reduction of gall formation at the crown or any part of the roots was observed in any treatments except where the roots had been dipped in a suspension of strain 84 (Table 5). This showed that the control inoculum had a significant effect on the prevention of gall formation if it was inoculated to the roots before transplanting the seedlings into infested soil. In contrast, application of the inoculum to the soil even 2 weeks after transplanting did not give any prevention of gall formation. This suggested that gall induction by the pathogen in the roots of susceptible plants in nature was completed within 2 weeks. Braun (1943, 1947) observed that gall induction by pathogens inciting in artificial wounds in periwinkle (*Vinca rosea* L.) plants under laboratory conditions took 4 days to reach the maximum level of gall induction. Htay and Kerr (1974) reported that in tomato, no prevention of gall formation was observed in wounds which were inoculated with strain 84, 4 hours after inoculation with a pathogen into the same wounds. Strain 84 was effective at preventing gall formation when it was inoculated within 2 hours after inoculation

Table 5. Effect of application of strain 84 cell suspension into the soil around the crowns of peach plants.

Soil Application at	Plants with galls at crown portion	Mean No. of galls/plant		Mean dry weight of gall tissue/plant (g)	
		Uninoculated Roots	Inoculated Roots	Uninoculated Roots	Inoculated Roots
2 weeks		9.71	0	0.09	0
4 weeks	2/7	19.85	0	0.14	0
8 weeks		8.00	0	0.26	0
2 + 4 + 8 weeks		13.43	0	0.37	0
No application	2/7	12.57	0	0.06	0
L.S.D. (P=0.05)		4.79		0.04	

with a pathogen. They suggested that if the results of the above experiment with tomato plants was also applicable for stone fruit trees in nature, strain 84 should be applied to transplants within 2 hours. However, inoculating seeds and roots with the control inoculum, before they are in contact with infested soil (before they are grown in nurseries or orchards) is the safest way of applying this control measure.

3. The effect of concentration of strain 84 suspension on the prevention of gall formation

For commercial use a cell suspension of strain 84 in water in the proportion of one 200 ml culture bottle (Fig. 4A) in one gallon of water has been suggested. This suspension gives approximately $10^7 - 10^8$ cells per ml. In the work reported previously this number of cells of strain 84 gave satisfactory control of the disease (Table 3) though some infected plants still occurred. This could be due to pockets on the surface of the roots where only pathogenic cells were present or where the number of pathogenic cells was higher than nonpathogenic cells. If this were the case, then perhaps by using a higher concentration of strain 84 cell suspension, the occurrence of such pockets could be eliminated. An experiment was carried out to investigate effect of different concentrations of strain 84 cell suspension on the prevention of gall formation.

Roots of one-year-old peach plants were washed with water and excess water was drained for a few seconds. The roots of different batches of plants were then dipped in different concentrations of strain

Fig. 4. Strain 84 inoculum.

A. YM-agar bottle.

B. Peat in polythene bag.

Fig. 4

A



B



84 cells suspension. These cell suspensions were made at the ratios of one culture bottle per (1) one gallon, (2) 1/2 gallon, (3) 1/4 gallon, or (4) 1/8 gallon of water. The inoculated plants were grown individually in small pots filled with naturally infested soil which was the same soil used in the preceding experiment. The roots of control plants were not dipped in any suspension. Each treatment had 6 replicates.

After 21 weeks, the plants were harvested and the number and dry weights of galls were measured. Only uninoculated plants had galls (Table 6). This showed that the concentration of cell suspension at the ratio of one culture bottle to one gallon of water was as effective as the higher concentrations prepared.

Nevertheless, in long-term inoculation experiments, some gall formation, though low, still occurred (Kerr, 1972; Htay and Kerr, 1974). This could be due to the transference of virulence from pathogens to nonpathogens in soil (Kerr, 1969, 1971), but this could not happen in the case of strain 84 since nonpathogenic cells of this strain do not acquire virulence from a pathogen (Kerr, unpublished data). Another possible explanation could be the presence of pathogenic strains which are not controlled by strain 84 (New and Kerr, 1972; Kerr and Htay, 1974). If this is the case, then further inhibitory non-pathogenic strains of these unknown pathogens need to be discovered.

The suspension of strain 84 in water has given satisfactory results in experiments where comparatively small numbers of seeds and roots were used. In a commercial nursery or orchard, however, there will

Table 6. The effect of concentration of strain 84 suspension
on the prevention of gall formation on peach plants.

Inoculation rate	Mean No. of galls/plant	Mean dry weight of gall tissue/plant (g)
One culture bottle in:		
1 gallon of water	0	0
1/2 gallon of water	0	0
1/4 gallon of water	0	0
1/8 gallon of water	0	0
No inoculation	19.80	0.44

be large numbers of seeds or roots to be treated and inoculation may take several hours or days. It is important to know what type of liquid medium gives maximum survival of strain 84 cells, and how long the cells will survive in such a liquid medium. In the following experiment the survival of strain 84 in different liquid media was examined.

4. Survival of strain 84 in different liquid media

A three-day-old culture of strain 84 on YM agar in a 200 ml flat bottle (which is now in commercial use) was used.

Survival of strain 84 in the following liquid media was tested:

- (1) sterile distilled water (SDW),
- (2) sterile saline water (0.85% NaCl),
- (3) sterile buffered water (0.2M sodium phosphate buffer at pH 7.0),
- (4) sterile buffered saline (10 ml of buffer + 90 ml of saline), and
- (5) SDW with addition of soil (10 gm of soil in 90 ml of SDW).

A few ml of thick cell suspension of a 3-day-old culture of strain 84 on YM agar was added to different liquid media bottles and the volume in each bottle was made up to 100 ml. The concentration of cells in each bottle was about 10^7 /ml, approximately equivalent to that given by one culture bottle in one gallon of water. There were 2 replicates in each liquid suspension. Bottles were shaken thoroughly and kept on the laboratory bench throughout the experiment.

To follow any change in number of cells in the different suspensions dilutions were made immediately, and at 4, 8, 24, 48, 72 and 168 hours (one week) after the stock suspensions were made. Appropriate dilutions (10^{-8} to 10^{-10})

and 10^{-7} were plated out on the selective medium for biotype 2 (New and Kerr, 1971). There were 3 replicate plates for each dilution, and the number of colonies after 5 - 6 days incubation at 25°C, was counted.

Strain 84 grew well in all liquid media (Fig. 5), but markedly better in the soil solution. In this solution an increase in the number of cells was observed at 24 hours and although the number of cells decreased after 3 days, the number of surviving cells was still high. The initial number in soil solution at the beginning of the experiment was 4×10^7 cells/ml and the number of surviving cells after one week was 10^7 cells/ml.

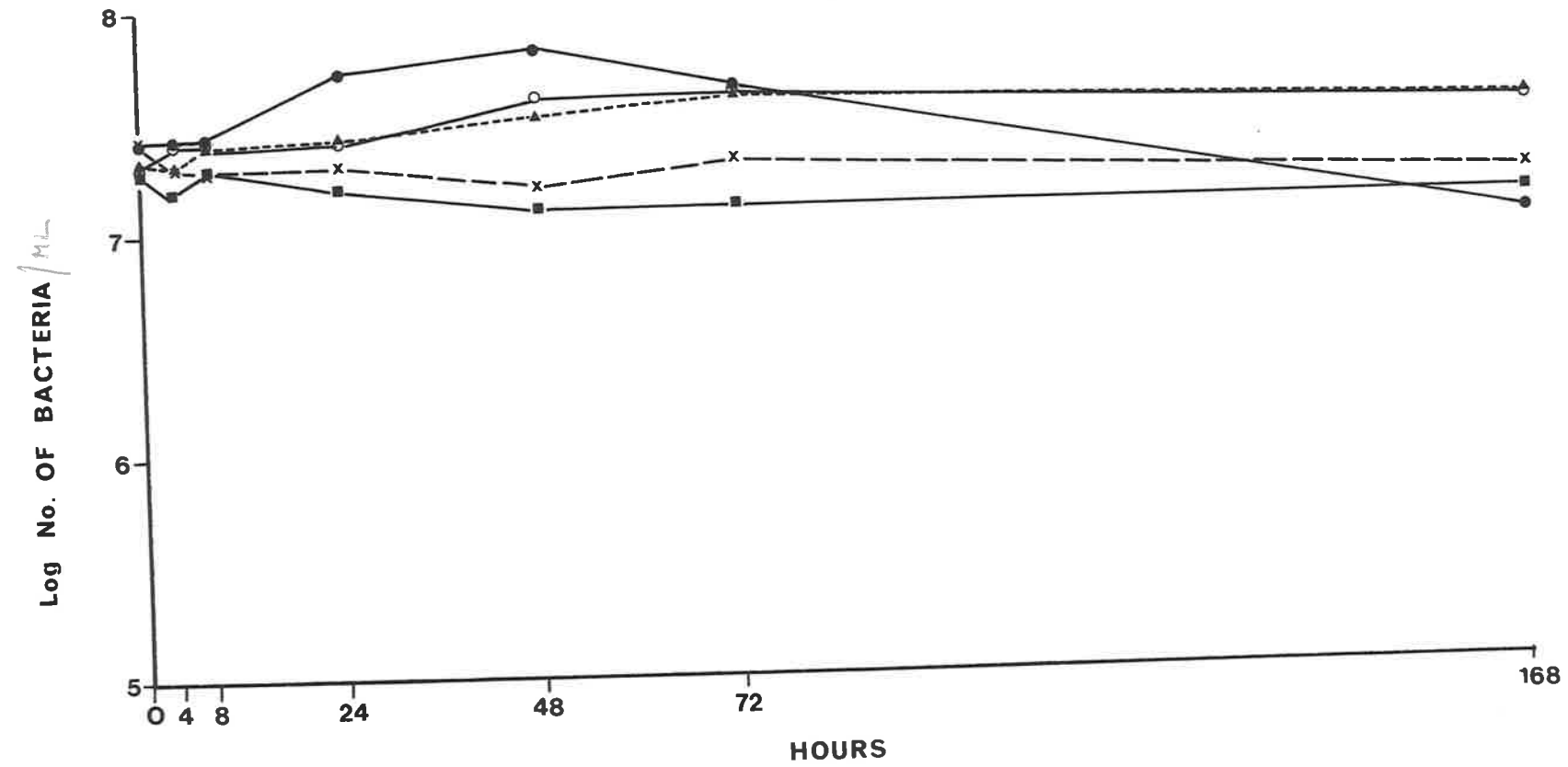
Besides being a good liquid medium for survival of strain 84, a soil suspension is an easily applicable one for commercial growers, because of its simplicity and lack of cost. For seed inoculation, a handful of soil in a gallon of water makes a satisfactory liquid medium for strain 84. Roots which already have some soil adhering to them only need to be dipped in water suspension. But, if the roots have been washed, it is better to add some soil to the inoculum suspension.

Since a grower would use a handful of soil from his orchard or nursery to prepare the control inoculum suspension it was necessary to see whether different types of soil might have different effects on the survival of strain 84. The following experiment investigated the survival of strain 84 in solutions prepared from different soils.

Fig. 5. Survival of strain 84 in different liquid media.

- — ● Water + soil.
- ▲ --- ▲ Buffered water.
- — ○ Buffered saline.
- X --- X Saline.
- — ■ Water.

Fig. 5



5. Survival of strain 84 in different types of soil solution

Soil samples from Loxton, Renmark, Angle Vale and Balhannah were collected. Those from Loxton and Renmark were red sandy soils with pHs of 7.8 and 6.4 (soil : CaCl_2 , 1 : 2 - Smiley and Cook, 1972), respectively. Angle Vale soil was also a red sandy loam with a pH of 6.2 and Balhannah soil was a black loam with a pH of 6.4.

Stock cell suspensions in SDW were prepared as described in the preceding experiment. Ten grams of each soil were added to each of two stock cell-suspension bottles. Survival of strain 84 was measured at various intervals.

The number of ^{viable} ~~surviving~~ cells increased at 24 hours in all soil suspensions (Fig. 6). Although fewer cells were recorded in Loxton soil until 48 hours, strain 84 grew better in this soil thereafter. At 168 hours (one week), the lowest number of cells (2×10^6 cells/ml) was recorded in the Balhannah soil suspension. However, strain 84 was found to grow in all tested soil suspensions without being greatly affected.

6. Survival of strain 84 in peat inoculum suspension

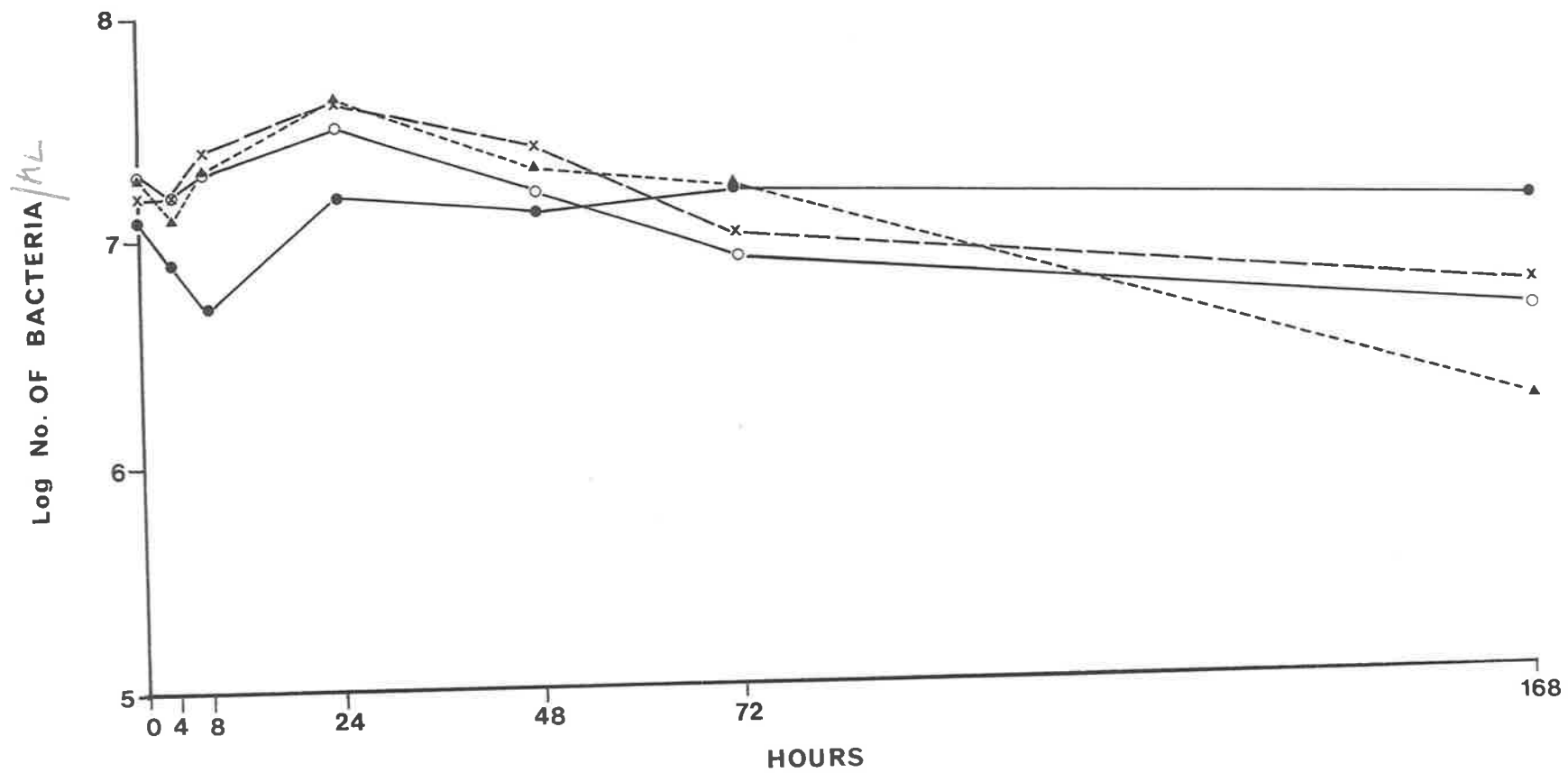
Distribution of inoculum has been widely made not only in Australia but also in Canada, Britain, South Africa, Turkey, Greece, New Zealand and the United States. For distribution overseas, it would be more economical if some cheaper and lighter container for the culture medium could be used instead of a glass bottle with agar medium.

For inoculating legumes, *Rhizobium* species have been successfully distributed as peat inoculum packed in polythene bags for commercial use

Fig. 6. Survival of strain 84 in different types
of soil solution.

● — ●	Loxton soil.
X — X	Angle Vale soil.
▲ — ▲	Balhannah soil.
○ — ○	Renmark soil.

Fig. 6



throughout the world (Roughley and Vincent, 1967; Brockwell and Dudman, 1968a, 1968b). Since *Agrobacterium* is closely related to *Rhizobium* (Graham, 1964; White, 1972) and is a successful soil colonizer (New and Kerr, 1972; Kerr, 1974; Starkey, 1929), it might be expected to grow in peat as well as does *Rhizobium*. In fact, Roughley (unpublished data) tested and found that strain 84 grew well in sterilized peat at 25°C for up to 16 weeks and was comparable to the ~~survival~~^{growth} of *Rhizobium* in peat.

A peat packet (Fig. 4B) is lighter than a culture bottle (\pm 60 grams compared with \pm 215 grams for the weight of a culture bottle). However, the ability of strain 84 to survive in peat inoculum suspension which would be used for inoculation of seeds and roots needed to be investigated.

Peat packets inoculated with strain 84 were supplied by Mr. Roughley (Horticultural Research Station, Narara, New South Wales). The peat had been sterilized by irradiation at the dose of 5M rads. Twenty ml of strain 84 broth culture containing 7.3×10^8 cells/ml had been injected into each sterilized peat packet. The injection hole was then sealed and the peat inoculum was incubated at 26°C for 6 days and then kept at 5°C. The following experiment was done 3 weeks later.

Peat inoculum suspension was prepared to have the concentration equivalent of one peat packet in one gallon of water. There were 2 replicate-peat inoculum packets and 4 stock suspension bottles of each inoculum. Two bottles of each were added with 10 grams of soil. Further steps of the experiment were carried out as described in the

preceding experiments. Although the initial number of cells in peat inoculum was about ten times less than that in culture bottles, strain 84 grew as well in peat inoculum suspension as in YM-agar-culture suspension (Fig. 7). There was no marked effect of addition of soil to peat inoculum suspension.

However, a striking phenomenon was observed. Small colonies of different shape developed on all dilution plates of peat inocula together with normal strain 84 colonies. The unknown colonies were 4 - 5 times smaller in size and about 8 times more numerous than strain 84 colonies (Fig. 8A). The unknown colonies were round in shape and had a flat surface with concentric rings and a small knob in the centre (Fig. 8B), whereas strain 84 colonies were round with a smooth, convex surface. The morphology of individual cells, under electron microscope, were found to be the same as strain 84 (Fig. 8C and D).

Not only did the unknown colonies grow on the selective medium for biotype 2 (New and Kerr, 1970), as did strain 84, but they also gave the same biochemical reactions, i.e. they did not produce 3-ketolactose but did utilize malonate. They also produced a bacteriocin which had the same reaction to pathogenic and nonpathogenic strains of *A. radiobacter* as bacteriocin 84. Further, when the unknown cells were mixed with the cells of pathogenic strain 27 at a 1:1 ratio and inoculated into wounded tomato plants, gall formation by strain 27 was completely inhibited. Although 5M rad is not sufficient to kill all bacteria originally present in the peat (Roughley, personal communication), the above evidence strongly suggested that unknown colonies were slower growing mutants of strain 84.

Fig. 7.

Survival of strain 84 in agar-culture and peat-culture suspension.

- — ● Strain 84 in agar-culture suspension
(with addition of soil).
- — ○ Strain 84 in peat-culture suspension
(without soil).
- X — X Strain 84 in peat-culture suspension
(with addition of soil).
- ▲ --- ▲ Unknown bacterial cells in peat-culture
suspension (without soil).
- — ■ Unknown bacterial cells in peat-culture
suspension (with soil).

Fig. 7

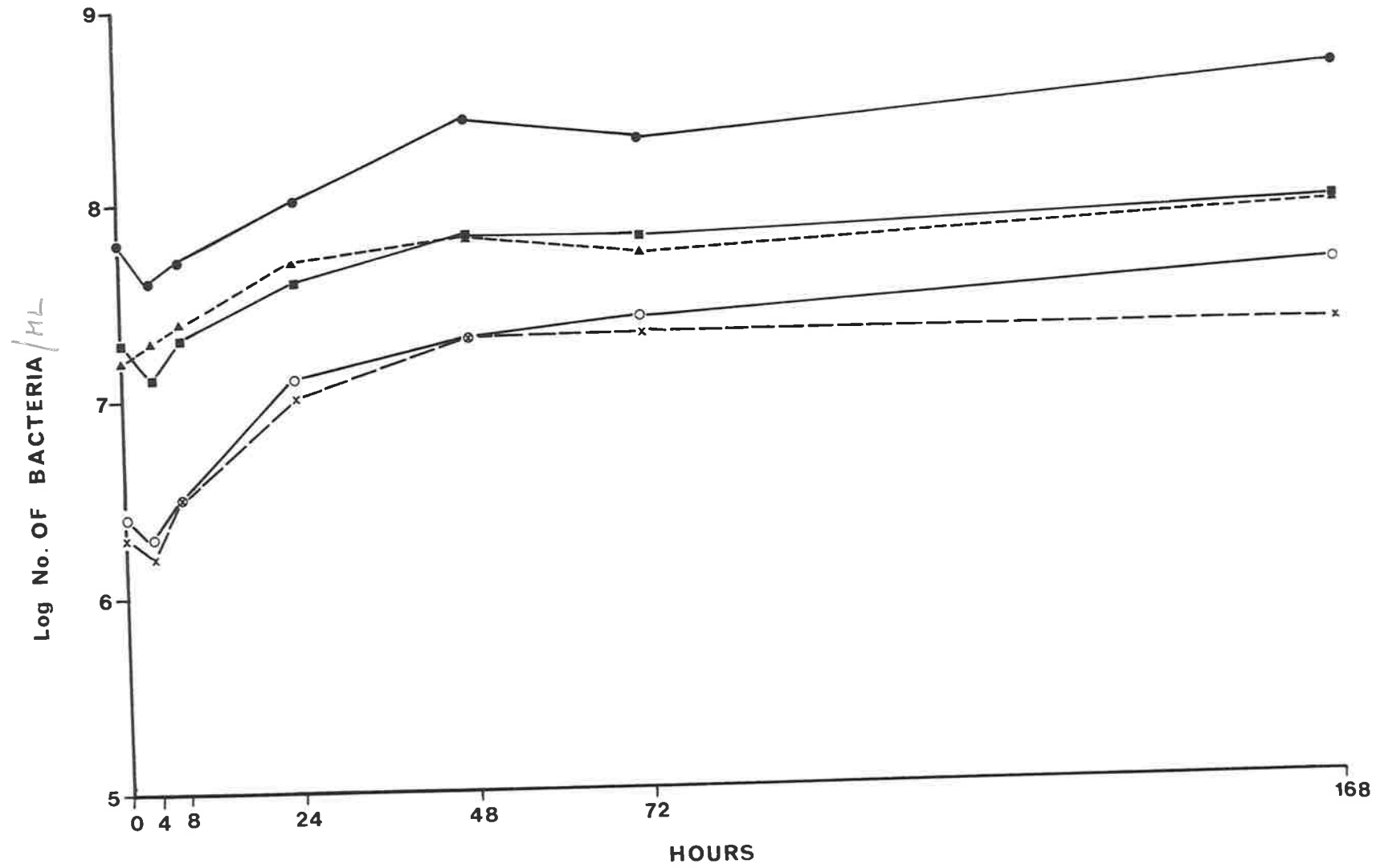
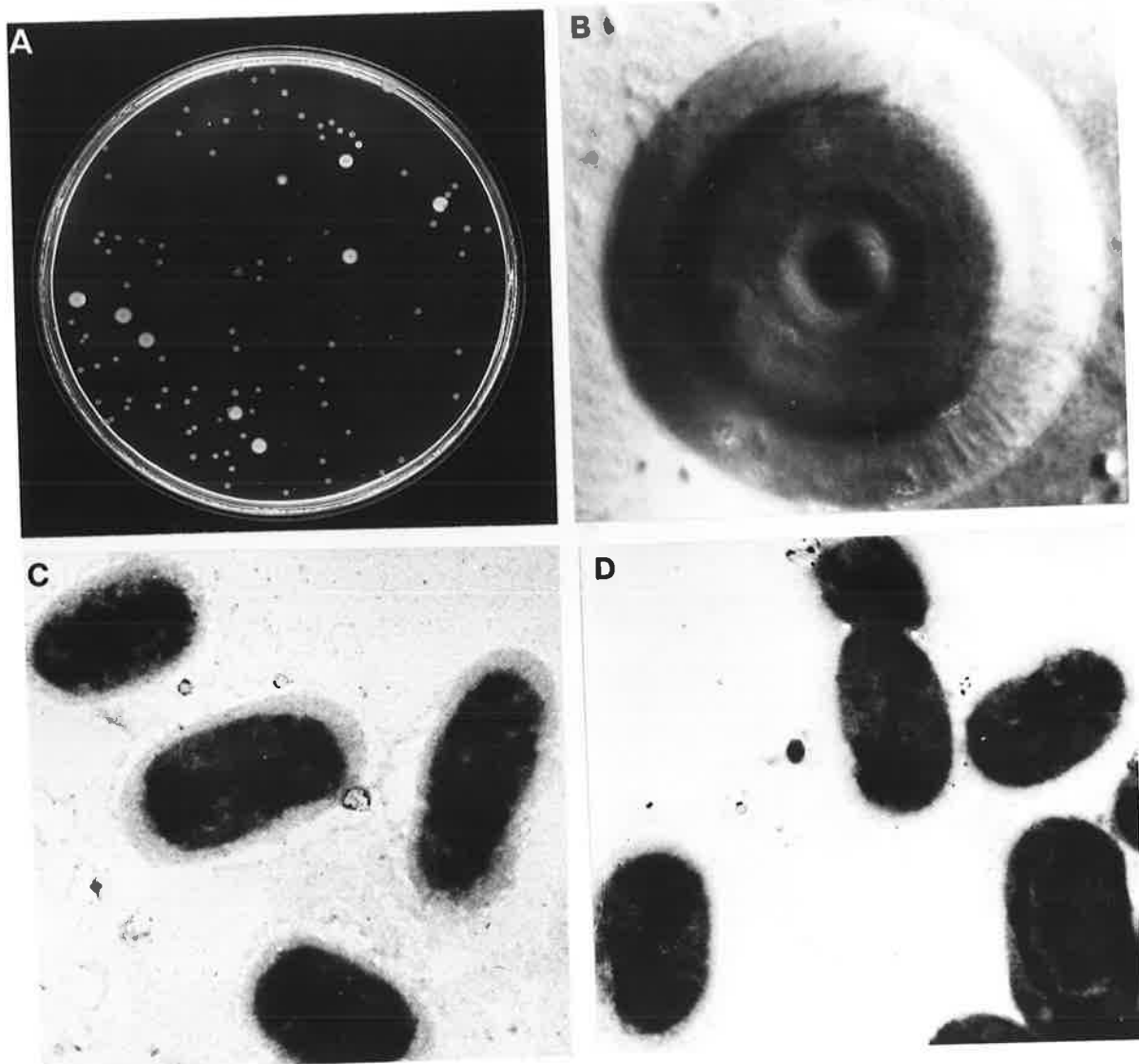


Fig. 8. Unknown bacterium from strain 84-peat inoculum.

- A. Unknown small colonies with normal strain 84 colonies on dilution plate of New and Kerr's medium.
- B. An unknown colony (Mag. x100).
- C. Individual cells of strain 84 observed under electron microscope (Mag. x26,000).
- D. Unknown bacterial cells observed under electron microscope (Mag. x 26,000).

Fig. 8



The combined number of normal sized strain 84 colonies and small colonies present in a peat inoculum packet gave as high a number as that in a YM-agar-culture bottle.

The reasons ^{for} ~~of~~ the presence of these small colonies in peat inoculum packet are not known. However, other peat inocula freshly prepared in this laboratory or in the New South Wales laboratory have not produced any small colonies. Since the bacteria from small colonies also gave biological control of susceptible pathogenic strains, their presence would not invalidate the use of peat as a medium for the production of inoculum.

DISCUSSION

In South Australia, the most serious damage from crown gall is caused to crowns and roots of peach and almond trees. To prevent this disease by ^{using strain 74 as an agent of} ~~inoculating~~ biological control, ~~inoculum (Strain 84)~~ there are two occasions when the inoculation is feasible; when sowing seeds of root stocks and when young trees are transplanted one year, or sometimes two years later.

In the reported experiment, root, and combined root and seed inoculation, gave better control than seed inoculation alone. Although root inoculation alone was not significantly different from combined seed and root inoculation, the former treatment cannot be recommended unless it is known that seed will be sown in soil free from *A. radiobacter* var. *tumefaciens*. In commercial nurseries, it will be practically impossible to determine this. So it seems clear that both seed and root inoculation

Root mince - not in A. radiobacter infected soil. 41.

should be used. Both inoculations are, in fact, now widely practised by commercial growers in South Australia. Although precise data from commercial application are not yet available, incidence of crown gall which has been serious in several nurseries for many years has been negligible following inoculation. It is estimated that inoculation with strain 84 will save South Australia's stone-fruit industry \$750,000 a year (Kerr, unpublished data).

Not only is the biological control inoculum, strain 84, highly effective in the control of crown gall, but also the method of its application is very simple. Bacteria from a culture bottle are suspended in one gallon of water with the addition of some soil to aid survival of the bacteria. Commercial growers have been advised to use non-chlorinated water and a clean plastic bucket. This suspension can be used for at least 48 hours. Since strain 84 was found to survive well in sterile peat, it is suggested that peat inoculum of strain 84 can be used for commercial distribution in future.

Although data on the survival of strain 84 in the rhizosphere soil of nursery and orchard trees, are not available yet, *A. radiobacter* has been recognized as a rhizosphere soil inhabitant (Starkey, 1929; Schroth, 1969; New, 1972; New and Kerr, 1972; Kerr, 1974). New (1972) isolated *A. radiobacter* from soil under the pasture where crown gall susceptible trees had not been grown for 4 years. But he was not able to record any pathogenic forms in this soil. This indicates the better viability of nonpathogenic forms in soil in the absence of a susceptible host.

However, the efficiency of strain 84 in biological control of

crown gall could be interfered with ^{by} ~~the~~ environmental factors in the field such as pH of soil, temperature or other organisms which may antagonize strain 84. These factors need examination in order to eliminate any possible interference of biological control of crown gall by strain 84.

Although successful biological control of crown gall by strain 84 had been achieved in the laboratory and under field conditions, the mechanism involved in this control has not been investigated. New and Kerr (1972) hypothesized that ^a ~~the~~ temperate phage ^{which might be} carried by strain 84 (if the phage theory of Leff and Beardsley (1970) for tumor induction can be confirmed) ^{could} ~~interfered~~ with the induction process of crown gall pathogens. Experiments on the mechanism behind successful control of crown gall by strain 84 are reported in the next section of this thesis.

SECTION B

BASIC INTERACTIONS BETWEEN NONPATHOGENIC
AND PATHOGENIC AGROBACTERIA

INTRODUCTION

New and Kerr (1972) isolated a nonpathogenic strain (84) of *A. radiobacter* var. *radiobacter* biotype 2 from soil around a peach gall. When they inoculated tomato seedlings with a mixture of strain 27 (pathogenic) and strain 84 (nonpathogenic) at the ratio 1:1 gall induction was completely prevented. Strain 84 was also found to prevent gall formation on peach seedlings which were dipped into a suspension of strain 84 and grown in soil artificially infested with pathogenic cells (strain 27). However, they did not elucidate the mechanism involved in this highly effective biological control by strain 84.

Several workers have tried to explain the mechanism involved in the prevention of gall formation obtained by inoculating mixtures of pathogenic and nonpathogenic agrobacteria. Lippincott and Lippincott (1969a) observed a 50 percent inhibition of the number of tumors on leaves of pinto bean when they inoculated leaves with a mixture of virulent (strain B6) and avirulent (strain 11BNV6) cells at 1:1 ratio. The inhibition was found to increase with the log. of the number of avirulent cells. They suggested that an antibiotic, unusual products of avirulent cells, or a hypersensitive reaction induced by avirulent cells, could not be involved since heat-inactivated (60°C for 20 min)

cells of both virulent and avirulent strains were found to inhibit tumor formation. The addition of avirulent cells before the virulent cells also gave the same result but not when virulent cells preceded avirulent cells. However, they did not consider that the inhibition was due to preimmunity because only 15 minutes separated the time of addition of inhibitory cells relative to virulent cells. This was probably not long enough for immunity to develop. Lippincott and Lippincott (1969a) proposed that the inhibition of tumor formation was due to the attachment of ^avirulent cells to a specific wound site, thus excluding the virulent cells from the site. In this, they assumed that specific complementary binding of virulent bacterium to a wound site exposed by the inoculation procedure was an essential event in crown gall tumor initiation.

The above proposal was supported by Schilperoort (1969) and Bogers (1972) who, under electron microscope, observed the attachment of both *A. radiobacter* var. *tumefaciens* and *A. radiobacter* var. *radiobacter* to the plant cell wall. They also found partial inhibition of tumor formation in the wound of *Kalanchoë daigremontiana* plants which were inoculated with an inoculum containing virulent and avirulent cells at 1:10 or 1:1 ratios and complete inhibition ^{at} of 1:100 ratio. They suggested that inhibition of tumor induction could be due to competition for the limited number of bacterial attachment sites on the walls of plant cells. However, they did not observe inhibition of gall formation by heat-treated cells (60°C or 100°C for 1 hour). This could be because of the use of different infectivity assays.

Marked inhibition of gall formation was observed by Kerr (1969) when he inoculated the mixture of a pathogen and a nonpathogen at 1:100 ratio into the tomato wounds. Kerr also supported the suggestion of the above workers that competition for infection sites was the mechanism involved in the inhibition of gall formation. Complete inhibition of tumor formation on *Datura* plants was achieved by Manigault (1970) when nonpathogenic cells were inoculated 24 hours before the pathogen. This result could be explained by the multiplication of nonpathogenic cells and their occupation of sites before the arrival of pathogenic cells.

If competition for infection sites by nonpathogenic cells was the mechanism behind the prevention of tumor formation, this ability of the nonpathogen would seem to be specific: Lippincott and Lippincott (1969a) observed that several other unrelated bacteria and certain strains of *Agrobacterium* failed to inhibit tumor induction; Schilperoort (1969) and Bogers (1972) could not detect inhibition by *E. coli* or heat-treated cells of *A. radiobacter*; and Beaud, Manigault and Stoll (1963) did not observe any decrease in gall formation by the addition of avirulent mutants of *A. tumefaciens* to the wounds on *Datura* plants before or at the same time as the pathogen. Moreover, certain strains of *Agrobacterium* even stimulated gall formation (Beaud *et al.*, 1963; Manigault, 1970; Lippincott and Lippincott, 1969b, 1970a).

In the case of nonpathogenic strain 84, which gave complete inhibition of gall formation at a 1:1 ratio with a pathogenic strain, the inhibition by strain 84 must be due to more than just competition.

New and Kerr (1972) could not find any other nonpathogenic strain which was as effective as strain 84 in the prevention of gall formation. They considered that since the degree of inhibition was higher than that observed by other workers at a 1:1 ratio of pathogen to nonpathogen, competition for infection sites could not account for the inhibition by strain 84. Nevertheless, they observed neither antagonism between pathogen and nonpathogen, nor a hypersensitive reaction induced by the nonpathogen. Thus, they hypothesized that if crown gall was induced by a temperate bacteriophage carried by a pathogen, as suggested by Leff and Beardsley (1970) and other workers, gall induction could be inhibited by a closely related phage carried by a nonpathogen. However, since no phage has been detected from gall tissue (Schilperoort *et al.*, 1973) and no galls have been induced by either purified bacteriophages isolated from pathogenic agrobacteria (Stonier *et al.*, 1967) or by purified phage DNA (Beiderbeek, Heberlein and Lippincott, 1973), the bacteriophage theory has still to be confirmed.

Disease inhibition by a nonpathogen or a saprophyte has also been reported in other bacterial diseases. Farabe and Lockwood (1957, 1958) isolated saprophytic bacteria from Fireblight cankers of apples and pears, and found that many of them inhibited growth of the pathogen, *Erwinia amylovora*, *in vitro*. They explained that the inhibition was due to the production of acid by the saprophytes. Seven out of nine saprophytes which inhibited the pathogen *in vitro* prevented disease development. Similar results were obtained when apple shoots were inoculated with an avirulent strain of *E. amylovora* prior to the virulent strain (Goodman,

1964, 1965, 1967).

The saprophytic bacterium, *Pseudomonas fluorescens*, isolated from dead bean plants was found to produce an antibiotic, which inhibited the growth of a number of bacterial plant pathogens *in vitro* (Teliz-Ortiz and Burkholder, 1960). The inoculation of bean plants with this saprophyte prior to their inoculation with the pathogen, *P. phaseolicola*, was found to reduce the severity of Halo Blight disease markedly.

Lovrekovich, Lovrekovich and Stahmann (1968) discovered that heat-killed cells of *Pseudomonas tabacci*, which were inoculated into tobacco leaves prior to living cells of the same organism, increased peroxidase activity of leaf tissue. This led to increased resistance to the disease. Peroxidase itself, however, had no inhibitory effect on growth of the pathogen *in vitro*. It was suggested that the peroxidase-induced Wildfire resistance might be dependent upon the presence of plant metabolites.

An agglutinating factor which was associated with the prevention of Bacterial Wilt in tobacco cuttings was found by Main (1968). The cuttings were pretreated with avirulent mutants of the bacterium *Pseudomonas solanacearum*, and the virulent cells were grown in the extract of the pretreated cuttings. The virulent cells lost motility, became agglutinated and failed to multiply. This factor was also found in the extract of non-treated resistant tobacco plants.

Crosse (1965) isolated a saprophyte from the leaf of a cherry tree and found that this bacterium was capable of reducing the incidence and/or severity of leaf-scar infection by *Pseudomonas mors-prunorum*. He considered that the main effect of the saprophyte was associated with some

living activity because the effect was not found with heat-killed cells. Since no antibiosis was observed, he suggested that it might be stimulation of the resistance of the host, or a direct inhibition of the virulence of the pathogen by the saprophyte.

These mechanisms suggested by the above workers may not account for the inhibition of gall induction by the nonpathogenic strain 84 of *A. radiobacter*, as the mechanism involved in the inhibition of disease development by a nonpathogen or saprophyte may differ in different diseases. However, it was considered worthwhile to investigate the reaction of strain 84 on various pathogenic strains of *A. radiobacter* *in vivo* and *in vitro* in order to gain information that might lead to understanding the mechanism behind the highly effective prevention of gall formation by strain 84.

This section describes a number of experiments on the basic interactions between several pathogenic and nonpathogenic strains of *A. radiobacter* both *in vivo* and *in vitro*.

EXPERIMENTS AND RESULTS

1. Interactions between strain 84 and a completely controllable or a partially controllable pathogen *in vivo*

Strain 84 is a biotype 2 nonpathogenic *Agrobacterium* which cannot be distinguished by any biochemical means from pathogens of the same biotype (Roberts and Kerr, 1974) but by a tedious pathogenicity test (Keane *et al.*, 1969). Strain 84, however, inhibits many biotype 1 and 2 pathogens (New and Kerr, 1972). Since only biotype 1 bacteria grow on

the selective medium developed by Schroth *et al.* (1969) and only biotype 2 on New and Kerr's medium (1970), it was convenient to use strain 84 and a biotype 1 pathogen to investigate their interactions.

There were two biotype 1 pathogens which were reported to be only partially controlled by strain 84 (New and Kerr, 1972). A comparison of the effect of strain 84 on a completely controllable pathogen and a partially controllable pathogen was considered to be worth investigation. Strain 24, which is a biotype 1 pathogen and controlled by strain 84, and strain 10 which is the same biotype but only partially controlled by strain 84, were used.

Bacterial cell suspensions were made by adding sterile distilled water to 2-day-old cultures on YM agar slopes. The suspensions were adjusted to optical density 6 with an EEL colorimeter at 603 m μ to give approximately 10^8 cells/ml (Lum, 1970). Five or six-week-old tomato seedlings were wounded on their stems to a depth of 3 mm by piercing with a blunt cylindrical rod (2 mm in diameter) and each wound was inoculated with 0.004 ml of cell suspension by a microsyringe. Inoculation treatments were: strains 10, 24 and 84 alone; strains 24 and 84 at a 1:1 ratio; and 10 and 84 at the same ratio. Inoculated plants were kept at 25°C in the glasshouse. Plants were harvested at various time intervals for the isolation of bacteria and five plants of each treatment were kept for 5 weeks for the observation of gall formation.

At various times (0, 1, 2, 3 and 10 days after inoculation), the stems of three tomato seedlings from each treatment were cut one centimetre above and below the site of inoculation. Each length of

inoculated stem was macerated separately in 10 ml sterile distilled water with a mortar and pestle. Further dilutions were made and 0.1 ml of each of the 10^{-3} and 10^{-4} dilutions were placed on the surface of a dried agar medium and spread with an L-shaped glass rod. Plates of both Schroth *et al.*'s medium and New and Kerr's medium were inoculated, incubated at 25°C and the colonies appearing on the plates counted after 5 - 6 days.

The data showed that the growth of strain 24 on tomato stems was markedly inhibited by strain 84 after one day whereas only slight inhibition of strain 10 at 1 and 2 days was observed (Fig. 9). Strain 84 also completely prevented gall induction ^{by} of strain 24 but strain 10 ^{was} only partially ^{inhibited} (Fig. 10). A further experiment showed that strain 84 only slightly inhibited the growth of pathogenic strain 6 which was another biotype 1 pathogen not subject to biological control (Fig. 11). In the above experiments, strain 84 itself grew very well on tomato stems (Figs. 9, 11), but its growth was affected by the pathogens.

These observations on the effect of strain 84 suggested that the inhibition of growth of pathogenic strain 24 by strain 84 might be the cause of the prevention of gall induction by strain 24 on tomato stems. This suggestion was further supported by the result of the following experiment.

2. Interactions between a pathogen and either a controlling or a noncontrolling nonpathogen

Since strain 84 was the only strain which was involved in biological control (New and Kerr, 1972), it was used as the controlling

Fig. 9.

Interactions between strain 84 and a completely or a partially controllable pathogenic strain (strain 24 or 10) on wounded tomato stems.

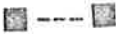


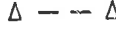

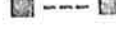
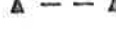
- A:  Strain 24.
 Strain 24 (+ 84).
- B:  Strain 10.
 Strain 10 (+ 84).
- C:  Strain 84.
 Strain 84 (+ 24).
 Strain 84 (+ 10).

Fig. 9

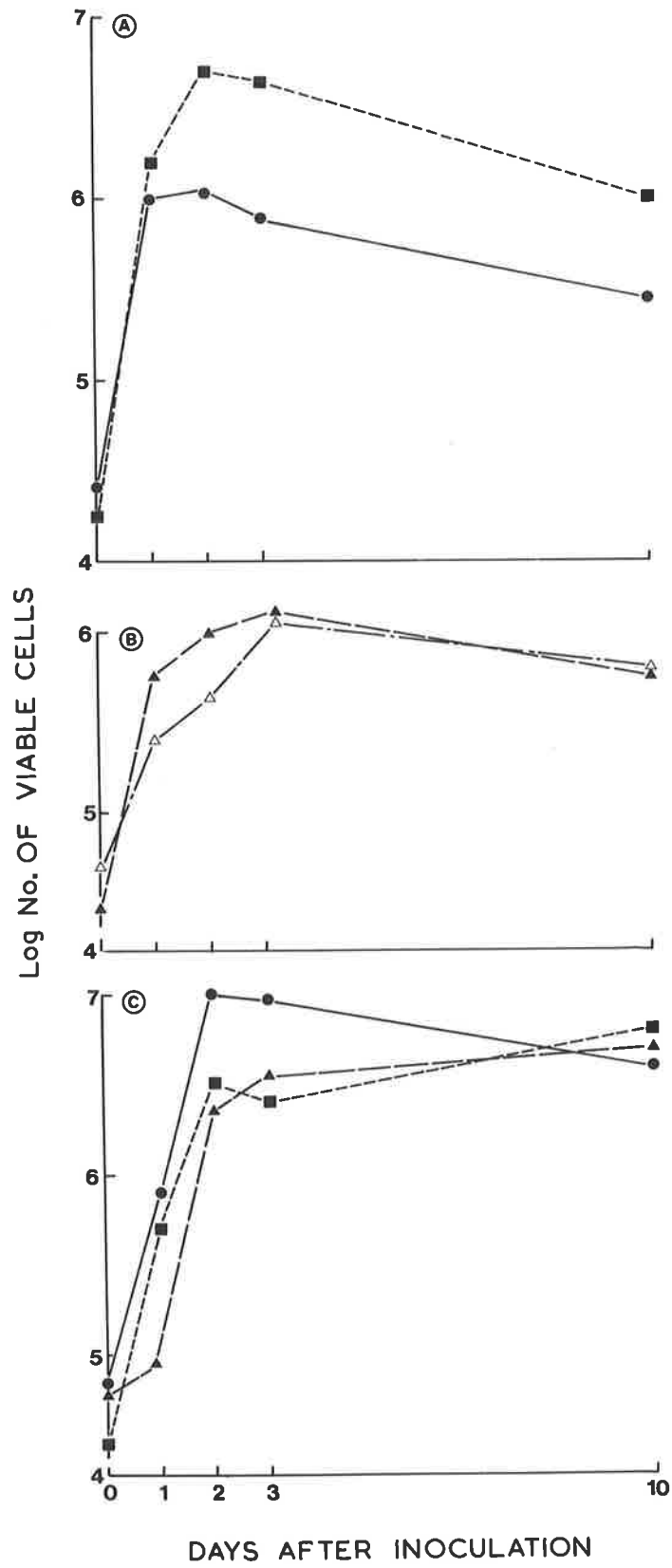
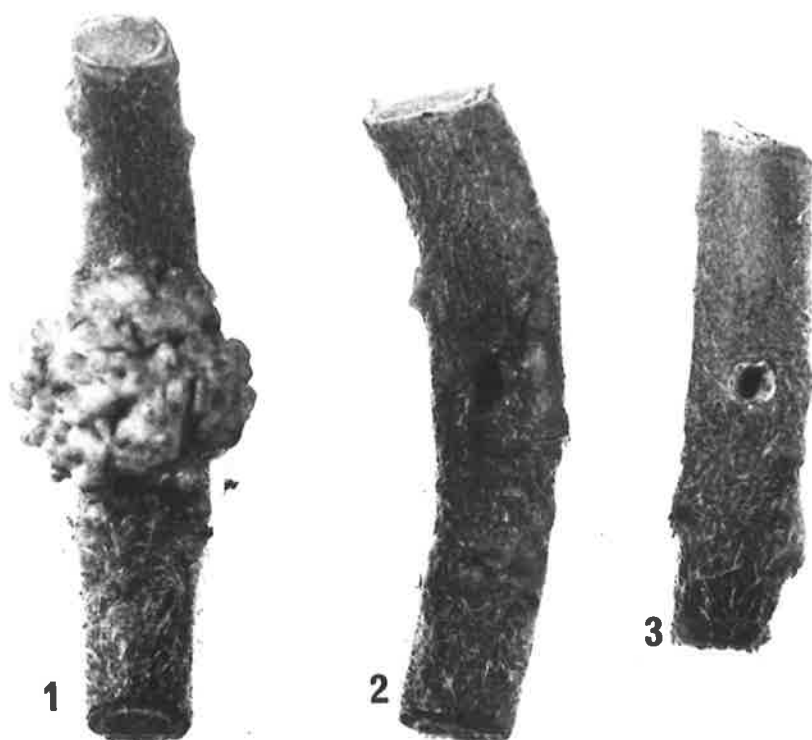


Fig. 10. Effect of strain 84 on gall formation by pathogenic strains.

- A.
 - 1. A seedling inoculated with pathogenic strain 24 alone (Mag. x3).
 - 2. A seedling inoculated with a 1:1 mixture of strains 24 and 84.
 - 3. A seedling inoculated with strain 84 alone.
- B.
 - 1. A seedling inoculated with pathogenic strain 10 alone (Mag. x2).
 - 2. A seedling inoculated with a 1:1 mixture of strains 10 and 84.
 - 3. A seedling inoculated with strain 84 alone.

Fig. 10

A



B

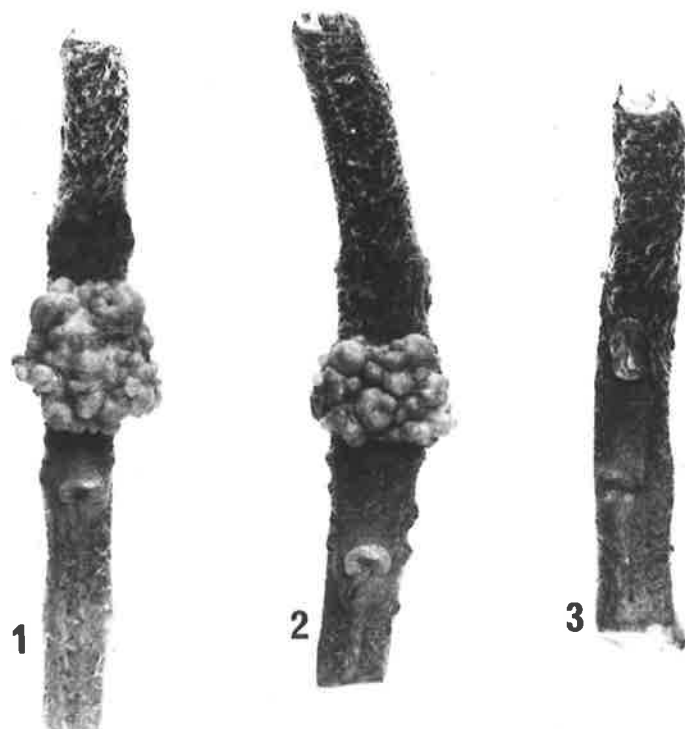
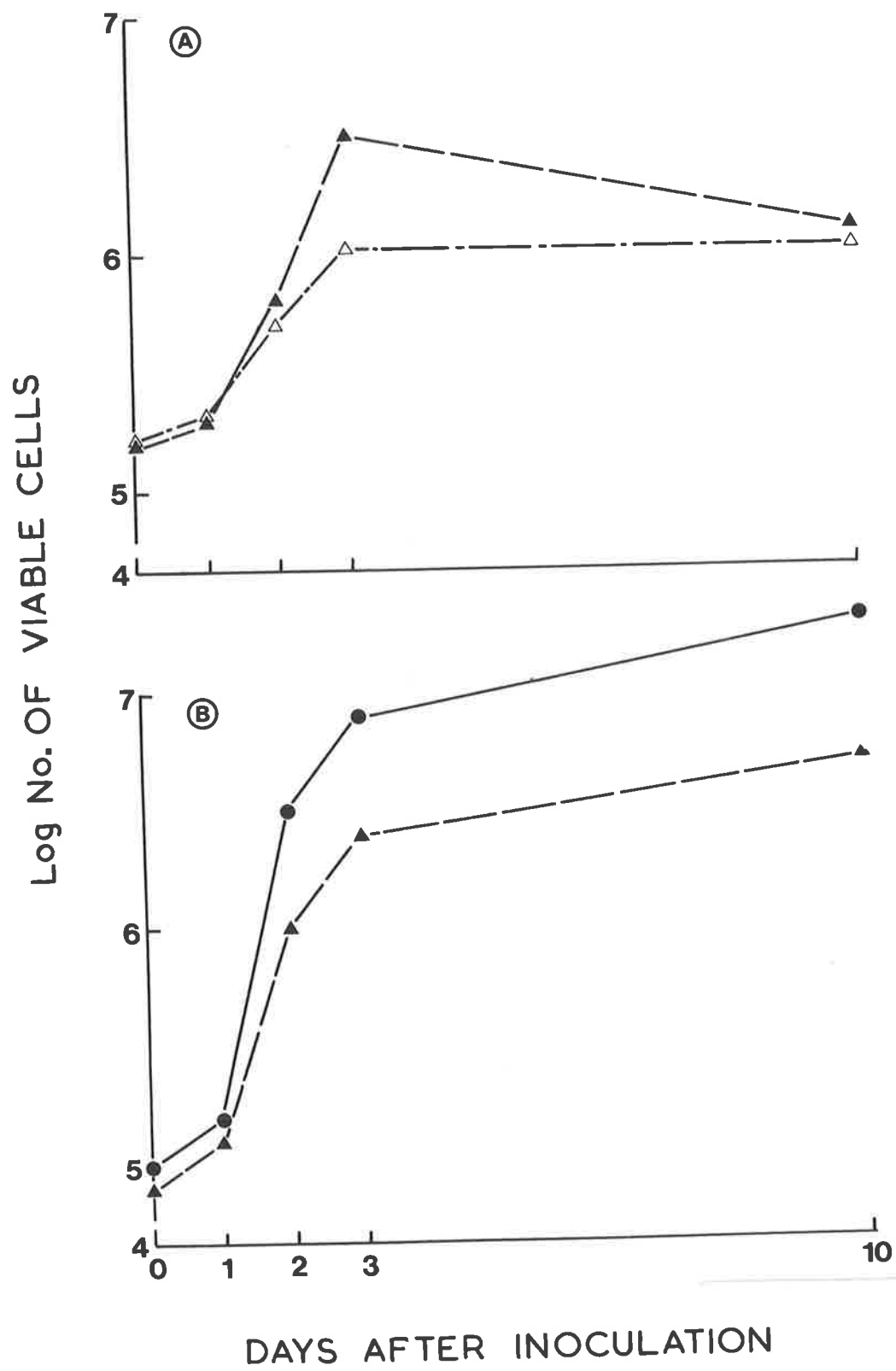


Fig. 11. Interactions between strain 84 and partially
controllable pathogenic strain 6, on tomato
stems.

A: ▲ --- ▲ Strain 6.
 Δ --- Δ Strain 6 (+ 84).

B: ● ——— ● Strain 84.
 ▲ --- ▲ Strain 84 (+ 6).

Fig. II



nonpathogen in this experiment. Strain 130 which was another biotype 2 nonpathogen which did not give biological control, was used as the noncontrolling nonpathogen. Strain 24 was again used as the pathogen to confirm the previous effect of strain 84 in its growth. The procedure was the same as that described in the former experiment.

The data (Fig. 12A) showed that strain 84 markedly inhibited the growth of pathogenic strain 24, but strain 130, however, did not, but slightly stimulated its growth after one day. The growth of both non-pathogenic strains was not markedly affected by strain 24 (Figs. 12B, C).

The above observations on the effect of a controlling and a noncontrolling nonpathogen on the pathogen supported the suggestion that strain 84 controlled the pathogen by inhibiting its growth. However, it was still to be proved whether strain 84 inhibited the growth of other pathogenic strains subject to biological control. This was investigated.

3. Effect of strains 84 and 130 on the growth of an acquired pathogen and its parent nonpathogen

Kerr (1969) reported that some nonpathogenic strains of *Agrobacterium radiobacter* could be transformed into pathogens by acquiring virulence from a pathogenic strain. Strain 147 is a biotype 1 nonpathogen and strain 152 is the same strain after it has acquired virulence from strain 27, a biotype 2 pathogen. Strains 147 and 152 cannot be distinguished by any biochemical means ^{only by} a pathogenicity test. Strain 152 forms galls on tomato seedlings and is subject to biological control by strain 84. Strain 147 does not cause gall formation.

Fig. 12. Interactions between pathogenic strain 24 and a controlling or a noncontrolling nonpathogenic strain (strain 84 or 130) on tomato stems.

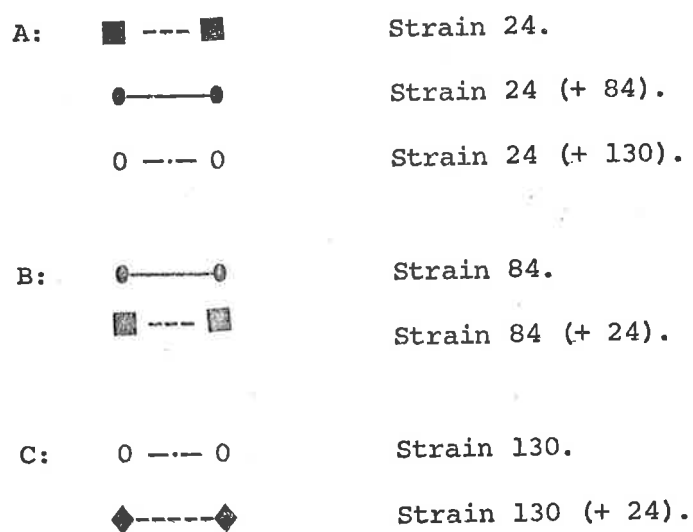
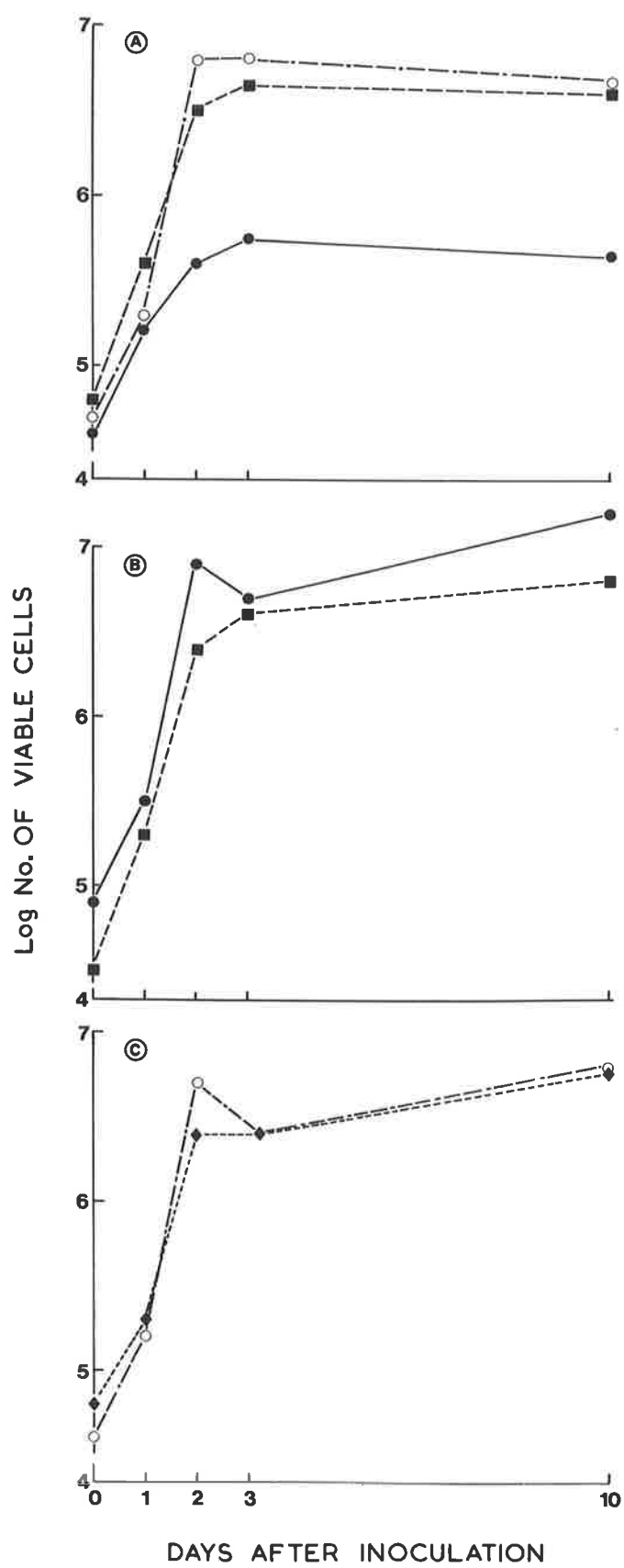


Fig. 12



Since strains 147 and 152 are genetically ^{similar} ~~the same~~ the effect of strain 84 on the growth of both strains 147 and 152 was considered to be worth investigation. In the preceding experiment the different effect of a controlling and a noncontrolling nonpathogen was observed. To confirm this observation, strain 130 which does not control the pathogen 152 was also used in this experiment.

Tomato seedlings were inoculated with strain 152, 147, 84 or 130 alone. Other seedlings were inoculated with a mixture of strain 152 with 84 or 130, or strain 147 with 84 or 130, at 1:1 ratio. The experiment was carried out as described before.

There was no significant difference in growth of the parent nonpathogen strain 147 alone, or of the pathogen, strain 152 alone, at all time intervals (Figs. 13A and B). Galls developed on the plants inoculated with strain 152 alone, but not on the plants inoculated with strain 147. This showed that both strains had the same surviving ability *in vivo* but were different in virulence.

Strain 84 inhibited the growth of pathogenic strain 152 markedly from day 2 onwards (Fig. 13A). Although strain 152 was not inhibited as much as was strain 24, no galls were formed by strain 152 on the inoculated plants even though high numbers of viable cells of strain 152 (10^7 at day 2 and 8×10^6 at days 3 and 10) were recorded on the plants. This suggested that the inhibition of the growth of pathogenic strain 152 by strain 84 was highly effective in preventing gall induction by 152, or alternatively, strain 84 seemed to inhibit not only the growth but also the virulence of the pathogen. However, the latter suggestion needed

Fig. 13. Interactions between strains 84 and 130, and an acquired pathogen (strain 152) and its parent nonpathogen (strain 147).

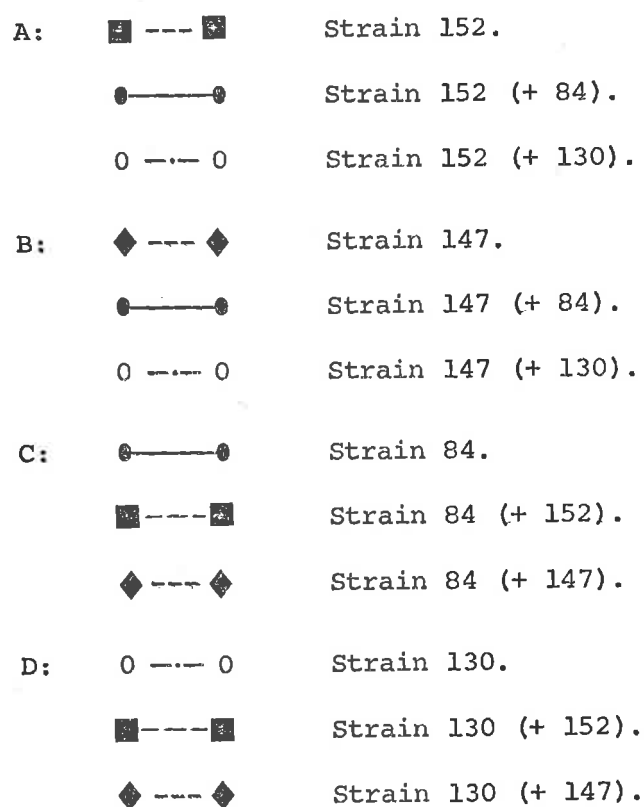
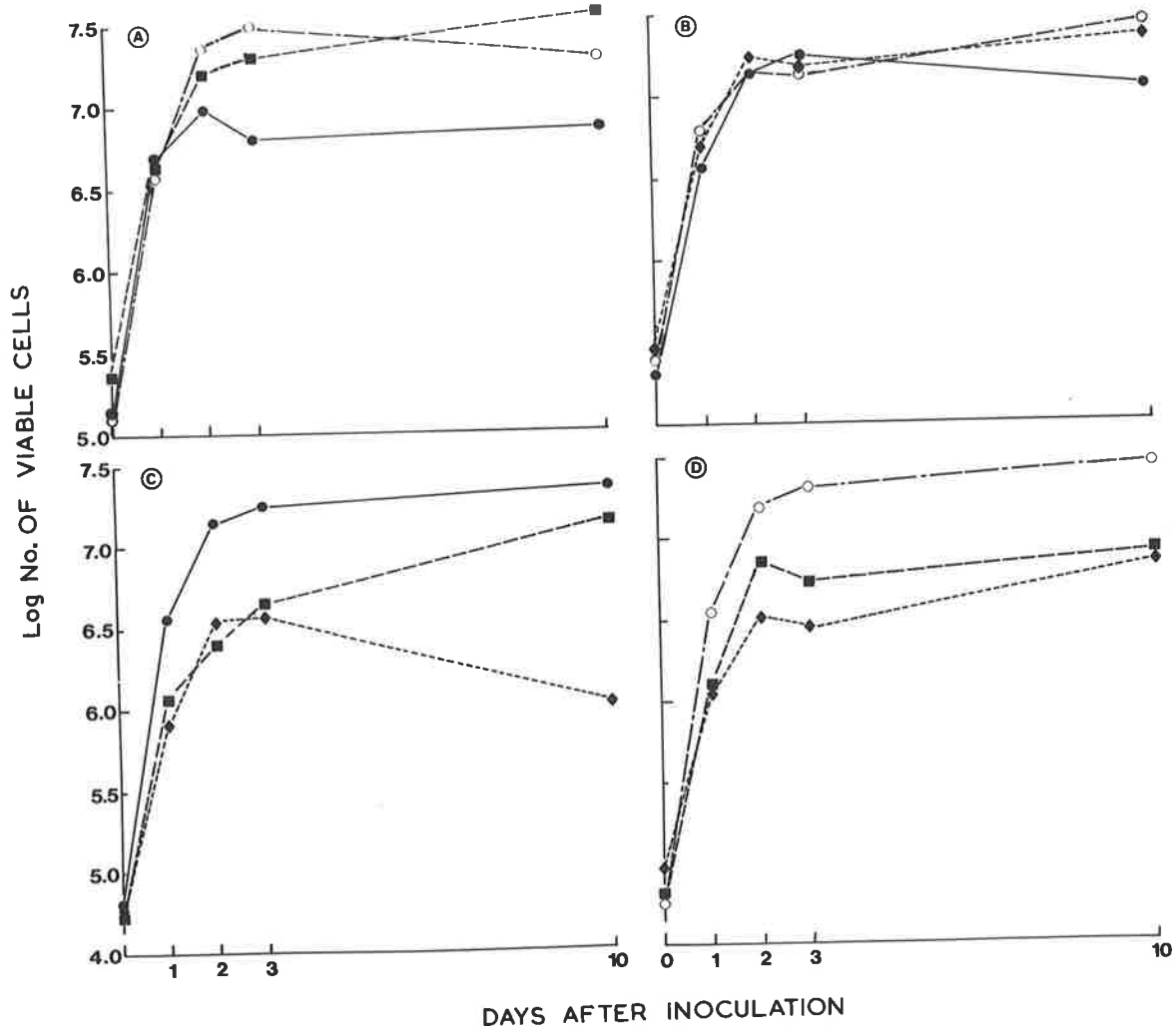


Fig. 13



further investigation.

A striking effect of strain 84 was observed: it inhibited the growth of pathogenic strain 152, but not the parent nonpathogenic strain 147 (Figs. 13A and B); strain 147 was even slightly stimulated by strain 84. Strain 84 was, in fact, expected to have a similar reaction to both strains 152 and 147 since they were genetically the same.

The data with strain 130 (Figs. 13A and B) showed that neither strain 152 nor 147 was inhibited and that gall induction by pathogenic strain 152 was not prevented. Strains 84 and 130 were found to be inhibited by both strains 147 and 152 (Figs. 13C and D).

The above experiments showed that strain 84 inhibited the growth of strains 24 and 152 markedly and also prevented gall induction by them completely, but it did not greatly inhibit pathogenic strains 6 and 10 and hardly prevented their gall induction. The other specific effect was that the nonpathogenic strain 147 was not affected by strain 84 but once strain 147 acquired virulence, its growth was significantly inhibited by strain 84.

The mechanism behind these effects of strain 84 was unknown. It could be considered as competition for infection sites and nutrients by strain 84 since strain 84 alone grew as well on tomato plants as pathogens alone did (Figs. 9C, 11B, 12B, 13C). However, if this was the explanation, plants inoculated with a mixture of strains 24 or 152 with strain 84 at a 1:1 ratio would be expected to have some galls. But strain 84 prevented gall induction by strains 24 and 152 completely. Moreover, strain 84 was also affected by the pathogenic strains since its growth was inhibited by either of the pathogens. These observations suggested that

strain 84 must have possessed some other factor which reacted differently to different strains of pathogens.


Since the inhibitory effect of strain 84 was observed on living plants, it could be suggested that the inhibitory factor was induced by the host plant in the presence of strain 84. To clarify this, the effect of strain 84 on the growth of pathogenic strain 24 in liquid culture was investigated.


4. Interaction between strain 84 and pathogenic strain 24 *in vitro*


The interaction between strains 84 and 24 was first investigated in liquid culture using Mannitol glutamic acid (MGA) medium. Bacterial suspension was prepared to have 10^8 cells/ml and 0.4 ml aliquots added to 100 ml lots of sterile MGA liquid medium (Keane *et al.*, 1969) in 250 ml flasks. There were 3 treatments: pathogenic strain 24 alone; strain 84 alone; and a mixture of strains 84 and 24 at a ratio of 1:1. Each treatment had 2 replicates. The culture flasks were shaken on a rotary shaker in a 25° constant temperature room. The growth of the bacteria was measured at various intervals (0, 1, 2, 3 and 10 days) by preparing dilutions and plating out on Schroth *et al.*'s, and New and Kerr's media.

The growth of strain 24 was not inhibited by strain 84 and was even stimulated at some time intervals (Fig. 14A). But the growth of strain 84 was markedly affected by strain 24 (Fig. 14B). Possible explanations of these results include: that the pH of the liquid medium influenced the interaction of the 2 strains; that the medium used was only favourable for a biotype 1 pathogen; or that strain 84 might not have an inhibitory

Interactions between strains 84 and 24 in MGA liquid medium.

A:  Strain 24.

 Strain 24 (+ 84).

B:  Strain 84.


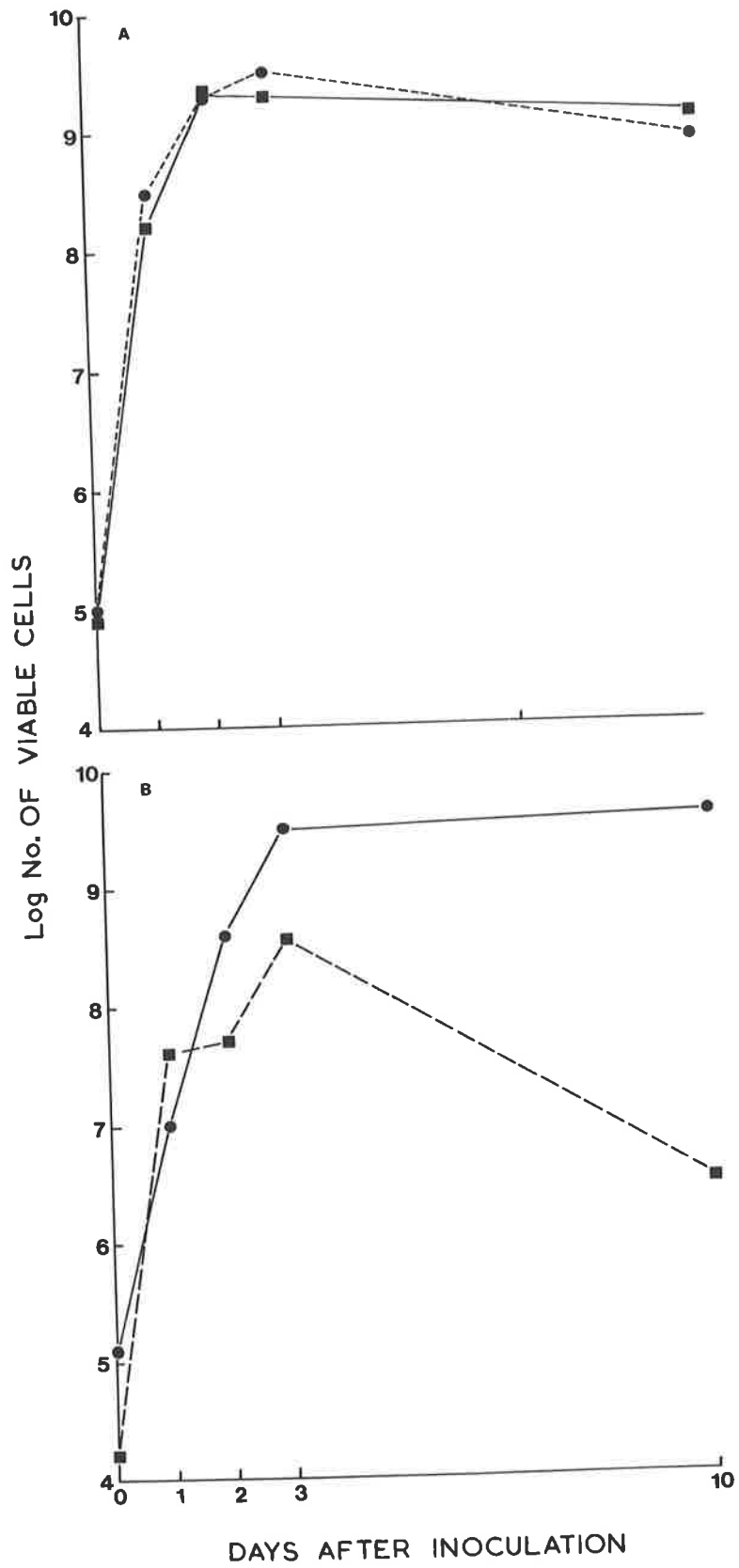
 Strain 84 (+ 24).

Fig. 14



effect on strain 24 *in vitro* at all.

The pH of the medium after autoclaving was found to be 7.6, although initially it had been adjusted to 7.0. The pH changes of the cultures were, unfortunately, not recorded. Therefore, to examine the influence of pH of the liquid medium on the interaction between strains 84 and 24, the following experiment was carried out.

5. Interaction between strains 84 and 24 in MGA liquid media at different pHs

MGA liquid medium was prepared and adjusted to 3 different pHs, 5.5, 6.1 and 7.1, using 1N HCl or 1N NaOH. There were 3 treatments at each pH and 2 replicates for each treatment, which were the same as in the former experiment. The growth of the bacteria was measured as described previously and pH of the cultures were recorded at all intervals (0, 1, 2, 3 and 10 days).

Strain 24 was inhibited by strain 84 in all media: at 1 and 3 days at pH 5.5 and 6.1; at 2 and 3 days at pH 7.1; and after 3 days in all media (Figs. 15A, 16A, 17A). Strain 84 was also affected by strain 24 in all media (Figs. 15B, 16B, 17B), but not as much as in the former experiment. The data of pH changes showed that all culture media gradually became alkaline (Table 7). The reason why inhibition of strain 24 was not observed in the former experiment could be the initial alkaline pH of the medium which was 7.6, and may have become higher.

These observations suggested that the pH of the medium influenced the interaction between pathogenic strain 24 and nonpathogenic strain 84.

Fig. 15. Interactions between strains 84 and 24 in MGA liquid medium at pH 5.5.



Fig.15

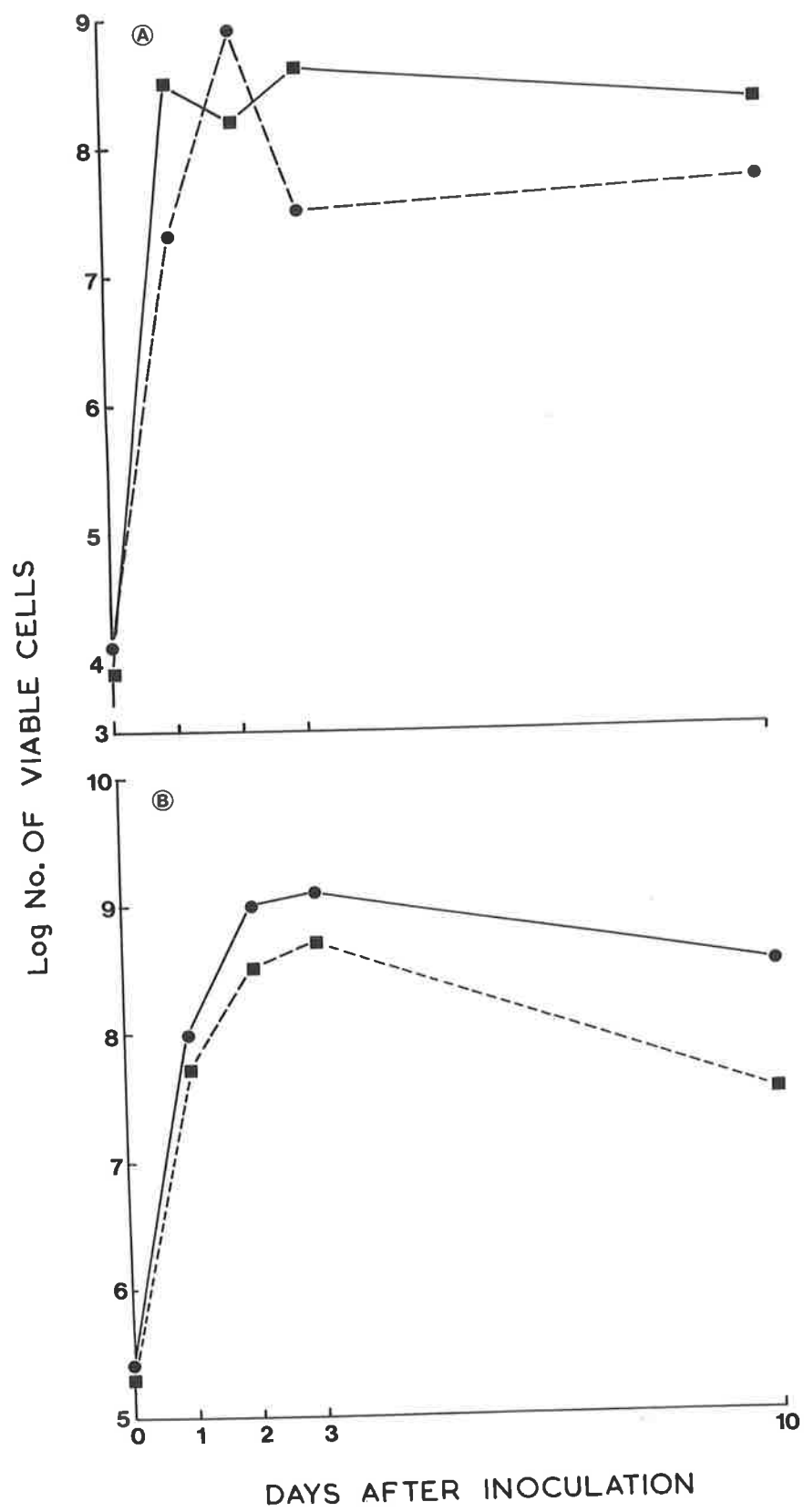


Fig. 16. Interactions between strains 84 and 24 in MGA liquid medium at pH 6.1.

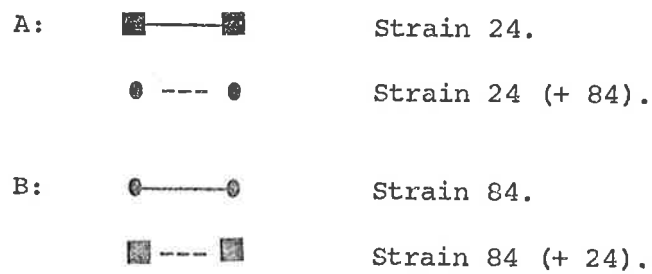


Fig. 16

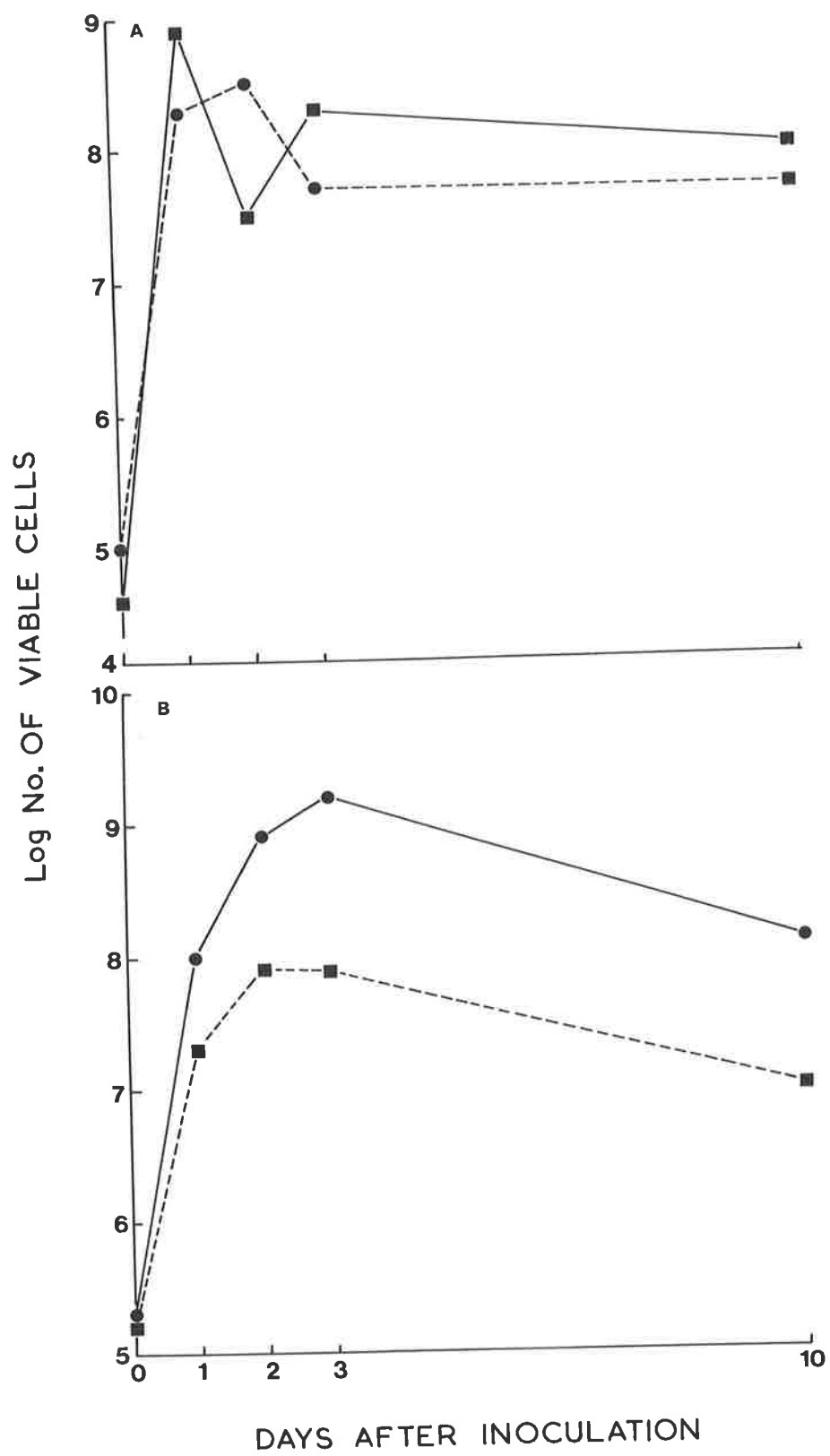


Fig. 17. Interactions between strains 84 and 24 in MGA liquid medium at pH 7.1.

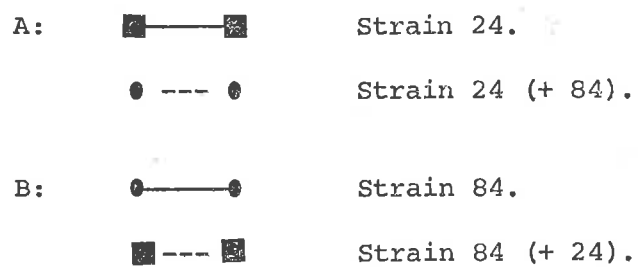


Fig.17

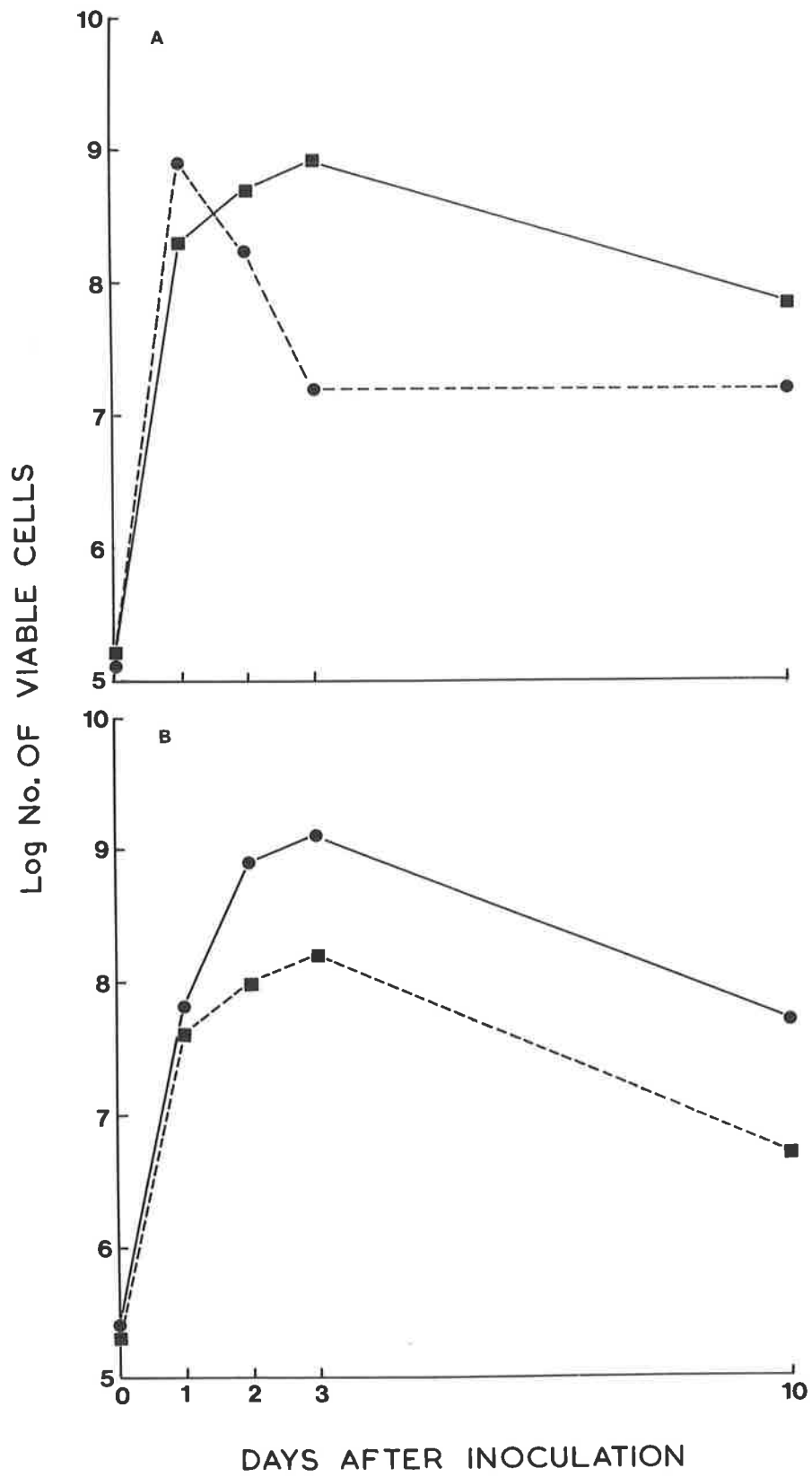


Table 7. pH changes of cultures of nonpathogenic and pathogenic strains of *A. radiobacter* in Mannitol glutamic acid liquid medium.

Strain	pH of the culture at (days)				
	0	1	2	3	10
84 (nonpathogen)	5.5*	5.8	6.6	6.7	7.6
	6.1	6.2	6.8	6.9	7.8
	7.1	7.1	7.1	7.2	7.6
24 (pathogen)	5.5	6.4	7.0	7.1	7.5
	6.1	6.7	7.1	7.4	7.8
	7.1	7.1	7.3	7.5	8.1
24 + 84	5.5	6.2	6.8	6.9	7.0
	6.1	6.7	7.0	7.0	7.1
	7.1	7.1	7.2	7.3	7.6

* Figures are the means of 2 replicates.

Therefore, another experiment was conducted to investigate the effect of buffering the medium to hold the initial pH, on the interaction between strain 84 and 24.

6. Interactions between nonpathogenic strains 84 and 130 and pathogenic strain 24 in buffered Mannitol ammonium sulphate liquid medium

The medium was prepared in the same way as in the previous experiments, but ammonium sulphate instead of glutamic acid was used as the nitrogen source in an attempt to hold a stable pH. The medium was buffered by adding ten ml of 0.2M sodium phosphate buffer at pH 7.0 (Appendix 1) to 90 ml of the medium.

In this experiment nonpathogenic strain 130 which did not inhibit pathogenic strain 24 *in vivo* was also used to observe its effect on strain 24 *in vitro*. The procedure was as described in the preceding experiments. There were 5 treatments: strain 24 alone, 84 or 130 alone; a mixture of strains 24 and 84; and a mixture of 24 and 130 at 1:1 ratio. There were 3 replicates in each treatment.

Strain 24 was found strongly inhibited by strain 84 after day 1 (Fig. 18A), but it was not markedly affected by strain 130. However, a marked decrease of the growth of strain 24 alone was also observed after day 2. Strains 84 and 130 were also found to be markedly affected by strain 24 at day 2 and 3 (Figs. 18B and C). All culture media remained at the initial pH until day 1 but thereafter became acid (Table 8).

These observations showed that an alkaline pH of the medium favoured the growth of the pathogenic strain 24 but an acidic pH did not.

Fig. 18. Interactions between pathogenic strain 24 and nonpathogenic strains 84 and 130 in buffered Mannitol ammonium sulphate liquid medium.

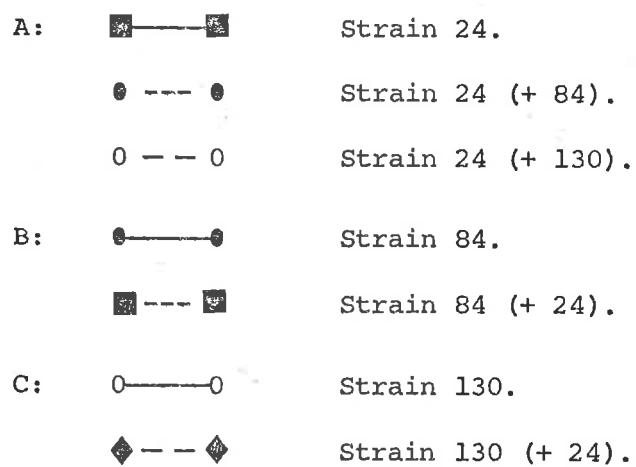


Fig. 18

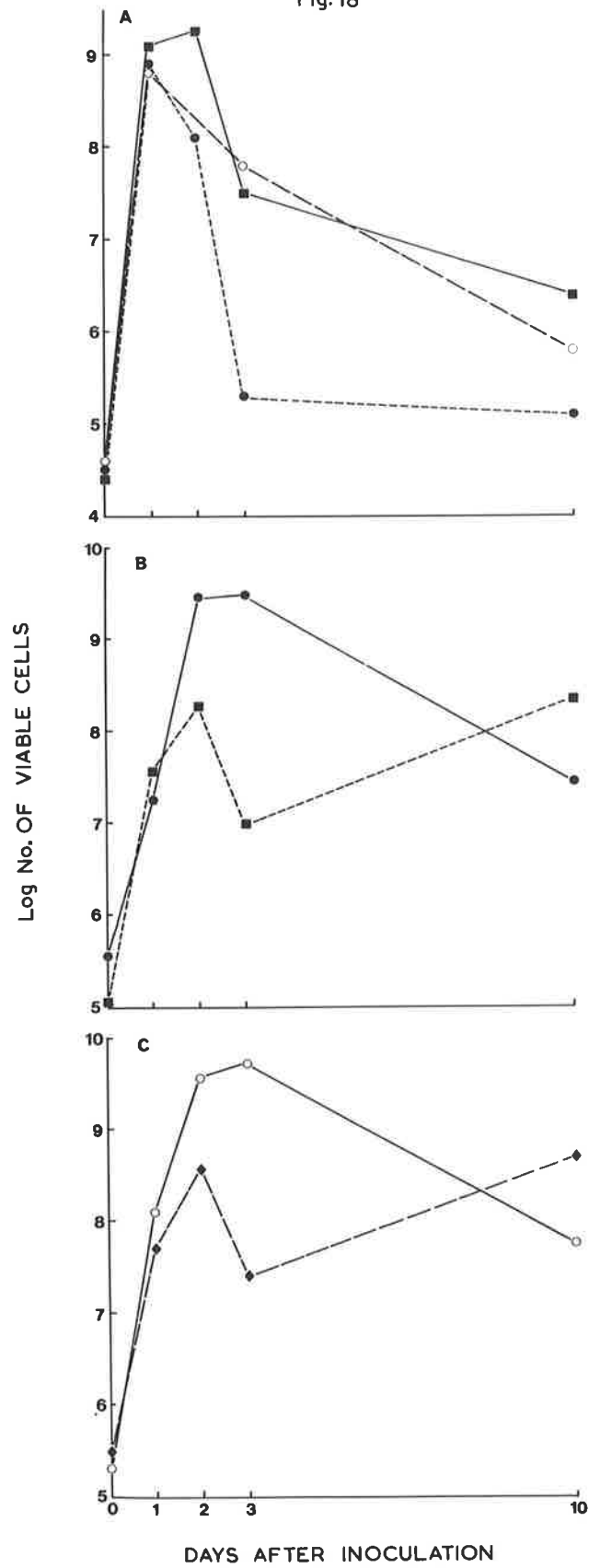


Table 8. pH changes of cultures of nonpathogenic and pathogenic strains of *A. radiobacter* in buffered Mannitol ammonium sulphate liquid medium.

Strain	pH of the culture at (days)				
	0	1	2	3	10
84 (nonpathogen)	6.9*	6.9	6.4	5.7	4.9
130 (nonpathogen)	6.9	6.9	6.3	5.8	5.0
24 (pathogen)	6.9	6.7	5.7	5.4	4.8
24 + 84	6.9	6.7	5.9	5.6	5.0
24 + 130	6.9	6.7	5.9	5.4	5.3

* Figures are the means of 3 replicates.

The acidic pH, in turn, seemed to favour the inhibitory effect of strain 84. Alternatively, the inhibitory effect of strain 84 in the buffered Mannitol ammonium sulphate medium depended on the nitrogen source, ammonium sulphate *versus* glutamic acid.

Shortly after these experiments, it was established that strain 84 produced a bacteriocin. Pathogens which were subject to biological control by strain 84, were sensitive to the bacteriocin (84) produced by strain 84, but pathogens which were not subject to biological control and nonpathogens were not sensitive to bacteriocin 84 (Kerr and Htay, 1974; Roberts and Kerr, 1974). The observations on the effect of bacteriocin showed that it not only inhibited a large number of pathogenic strains but that most of the resistant bacteria which developed in the inhibition zone of the bacteriocin were no longer pathogenic (Kerr and Htay, 1974). This suggested that to operate biological control *in vivo*, strain 84 not only inhibited the growth of pathogens but also caused a loss of pathogenicity of resistant cells of the pathogenic strains through bacteriocin production. Since the effect of strain 84 on the inhibition of the pathogenic growth had been observed, its effect on the loss of pathogenicity of resistant cells was investigated in the following experiment.

7. The effect of strain 84 on the pathogenicity of resistant cells of pathogenic strains 152 and 10 on tomato plants

Batches of fifteen tomato seedlings were inoculated with strain 152 alone; with a mixture of strains 152 and 84 at a 1:1 ratio; with strain 10 alone; and with a mixture of strains 10 and 84 at a 1:1 ratio.

At 0, 1, 2, 3 and 10 days after inoculation, the cells of the pathogenic strains from three seedlings of each treatment were isolated on Schroth's medium. After 5 - 6 days incubation, 50 colonies from each treatment at each interval were randomly chosen and inoculated singly into healthy tomato seedlings. After 2 weeks incubation in 25° glasshouse, plants with galls were recorded.

Most plants were galled except some which were inoculated with colonies of strain 152 reisolated from the plants ^{on} ~~of~~ which strain 152 had been grown with strain 84 (Table 9). All the colonies tested for pathogenicity were also tested for bacteriocin sensitivity. All strain 10 colonies and those of strain 152 which did not produce galls on tomato plants were found insensitive to bacteriocin 84. This showed that the high correlation between pathogenicity and sensitivity to bacteriocin 84 *in vitro* reported by Kerr and Htay (1974) also occurred *in vivo*.

As strain 84 produced bacteriocin 84 which was easily detected by an indicator strain (pathogenic strain 24), strain 84 could be distinguished from other biotype 2 agrobacteria by using a bacteriocin sensitivity test which was a slight modification of that described by Stonier (1960) (Kerr, personal communication). Thus, the effect of strain 84 on the biotype 2 pathogenic strain 27 could be investigated.

8. Interaction between strain 84 and biotype 2 pathogenic strain 27, *in vivo*

Batches of fifteen tomato seedlings were separately inoculated with strain 27 alone, 84 alone, and a mixture of strains 27 and 84 at a 1:1 ratio. At 0, 1, 2, 3 and 10 days after inoculation, bacterial cells from 3 seedlings of each treatment were isolated as described previously,

Table 9. The effect of strain 84 on the pathogenicity of resistant cells of pathogenic strains 152 and 10 on tomato plants.

Treatment	No. of pathogenic cells/50 resistant cells at (days)				
	0	1	2	3	10
Pathogenic strain 152 alone	50	50	50	50	50
Strain 152 (+ strain 84)	50	50	45	45	28
Pathogenic strain 10 alone	50	50	50	50	50
Strain 10 (+ strain 84)	50	50	50	50	50

and plated on New and Kerr's medium. Fifty colonies from each treatment where strains 84 and 27 were combined were randomly chosen and tested for bacteriocin. Bacteriocin production as indicated by a zone of inhibition round a colony, indicated strain 84 colonies (Fig. 19), the remainder were strain 27. The data (Fig. 20A) showed that strain 27 was markedly inhibited by strain 84 at all intervals whereas strain 84 was affected by 27 only at day 7 (Fig. 20B). All reisolated colonies of strain 27 were tested and found pathogenic as well as sensitive to bacteriocin 84. This observation was different from that in the preceding experiment with biotype 1 reisolated cells of strain 152, some of which had lost their pathogenicity and sensitivity to bacteriocin 84 after they had been exposed to strain 84 *in vivo*.

Moreover, different types of inhibition zones of bacteriocin 84 against pathogens of different biotype were also observed. The zone against biotype 1 pathogenic strains was clearer, with a sharper margin and appeared earlier than that against biotype 2 pathogens (Fig. 21). The reason why bacteriocin 84 has different effects on pathogens of different biotype has still to be explained.



However, it was clear that strain 84 had a similar effect on the pathogenicity of the resistant bacterial cells in plants as bacteriocin 84 had *in vitro*. To confirm the suggestion that bacteriocin 84 was involved in biological control by strain 84 (Kerr and Htay, 1974), the effect of sterile bacteriocin 84 on the growth of pathogenic strain 27 and on gall formation by this pathogen was investigated.

Fig. 19. Bacteriocin production. Colonies surrounded
by clear zones indicate production of bacteriocin.

Fig. 19



Fig. 20. Interactions between strain 84 and biotype 2
pathogenic strain 27 on tomato stems.

A:  Strain 27.
 Strain 27 (+ 84).


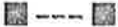
B:  Strain 84.
 Strain 84 (+ 27).

Fig. 20

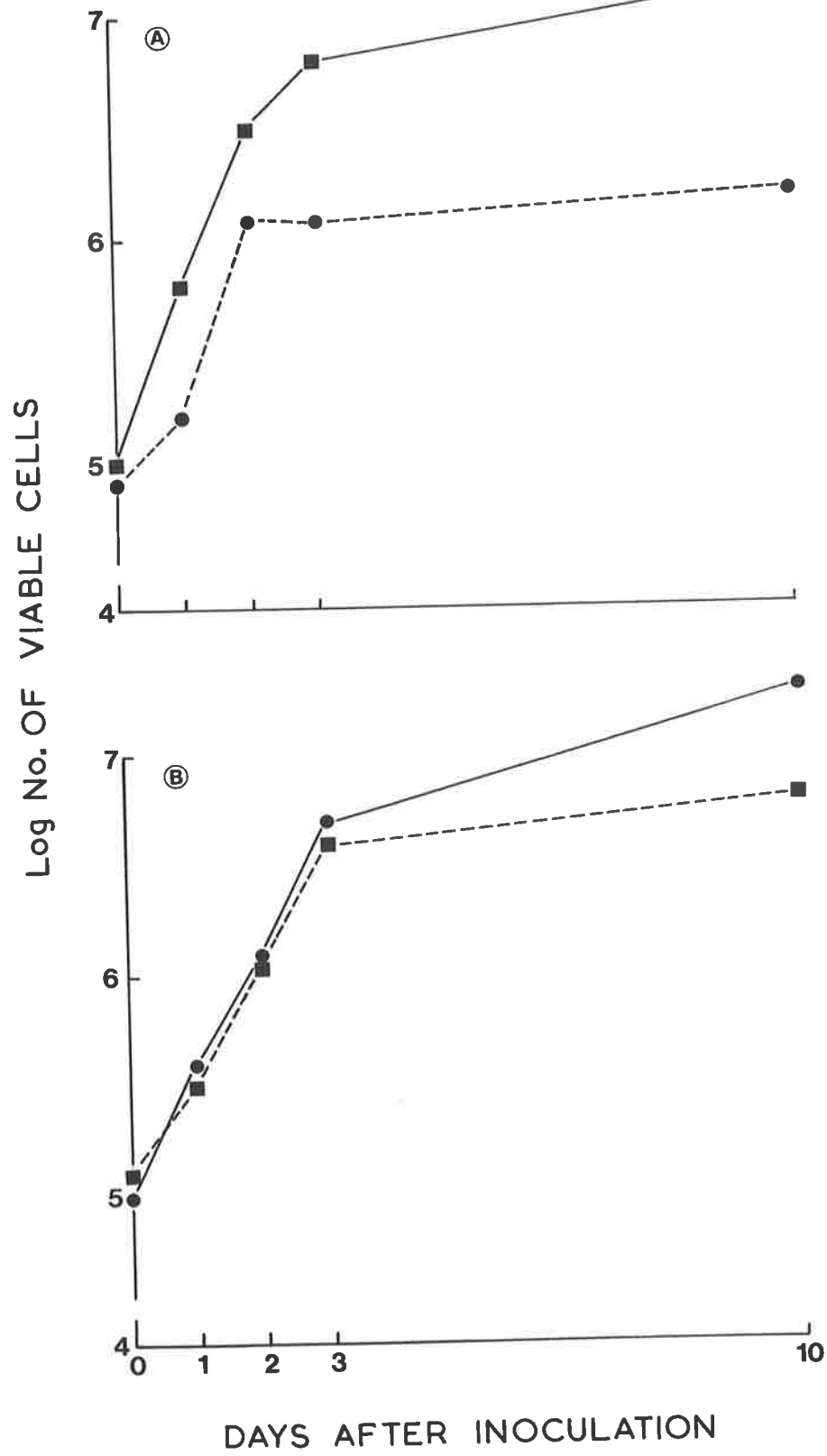
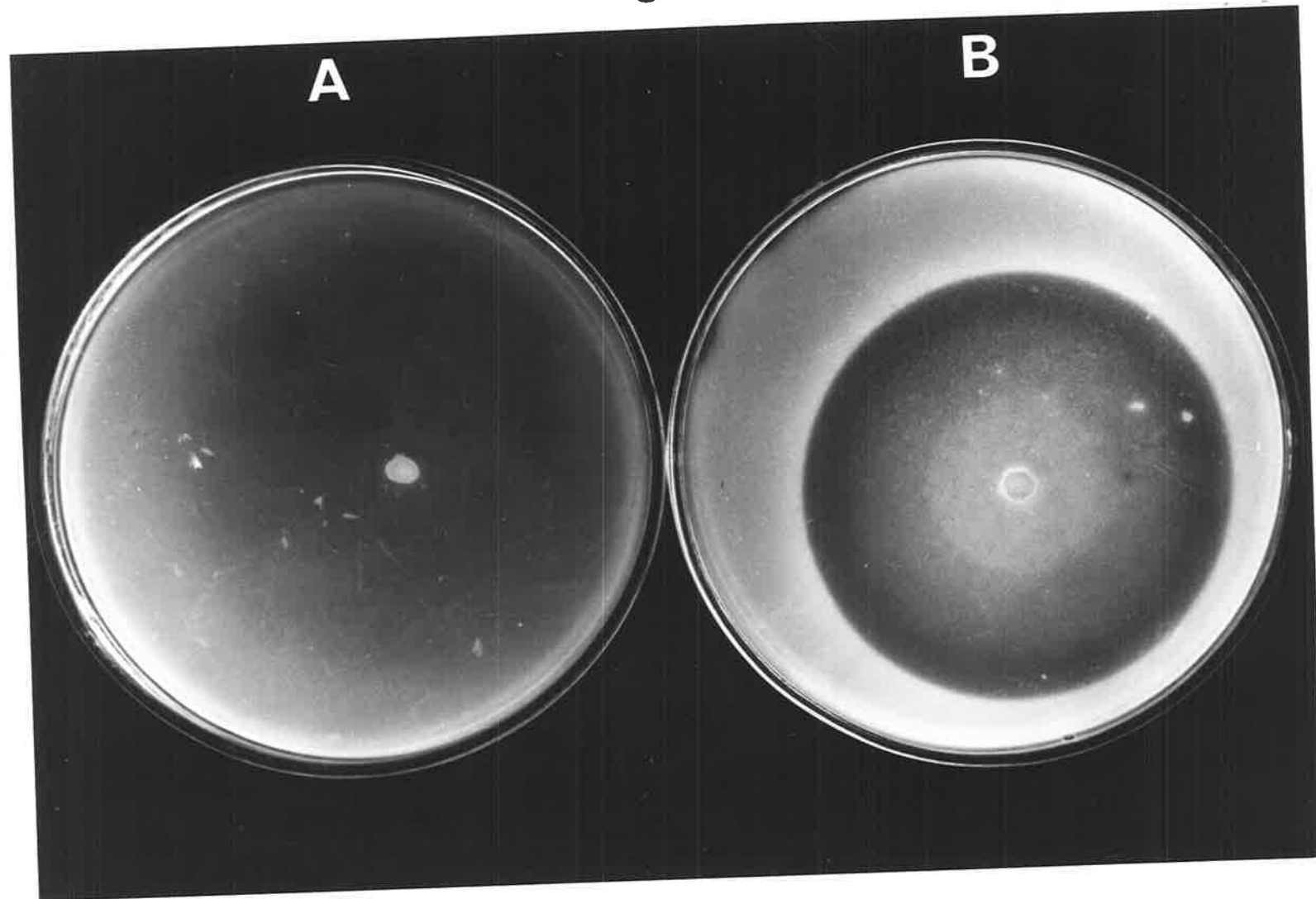


Fig. 21. Different types of inhibition zones of bacteriocin 84 against pathogens of different biotypes.

- A. Diffuse inhibition zone against a biotype 2 pathogen.
- B. Sharp-edged inhibition zone against a biotype 1 pathogen.

Fig. 21



9. The effect of sterile concentrated bacteriocin 84 on pathogenic strain 27 on wounded tomato seedlings

Bacteriocin 84 was extracted from liquid cultures of strain 84 as described by Roberts (1975). A thick cell suspension was prepared by mixing a two-day-old culture of strain 84 on a YM-agar slope with 10 ml of SDW. One ml of the thick suspension was inoculated to 200 ml of the sterilized Stonier's liquid medium in each of twenty 500 ml conical flasks. The flasks were kept on the rotary shaker in a 25° constant temperature room for 3 days. Before extracting bacteriocin 84, the pH and optical density of the liquid culture were measured. Cultures at pH 8.0, with optical density 90 gave the highest yield of bacteriocin, the bacteriocin is unstable at pHs higher than 8.5 (Roberts, 1975).

The liquid culture was centrifuged at 8,000 rpm (7719 g) for 10 min. The supernatant liquid was shaken with chloroform 3:1 v/v and centrifuged again at 6,000 rpm (4342 g) for 10 min. The supernatant liquid was removed and adjusted to pH 7.0 with 1N HCl. The bacteriocin was concentrated by rotary evaporation keeping the liquid extract at less than 45°. Normally 4 litres of liquid extract were evaporated to dryness and the residue was made up to 400 ml volume with SDW to have 10 times the concentration of the original culture extract. This was "concentrated bacteriocin", which could be stored with a few drops of chloroform at 4°C for 6 - 8 weeks, and at 0°C for a longer period with full activity.

To test its effectiveness thirty microlitres of bacteriocin were placed in a 5 mm diameter well, cut in a plate of Stonier's agar medium.

The agar surface was exposed to chloroform vapour by sticking a filter paper moistened with chloroform to the lid of the plate to kill any bacteria that might be present. After 10 - 15 minutes, the chloroform was removed by opening the lid and removing the paper. The plate was then poured with indicator strain 24 in cooled soft buffered agar (Stonier, 1960), and incubated at 25°C overnight. The concentration of bacteriocin was assessed by measuring the diameter of the inhibition zone round the well. In this case, an inhibition zone of 3.5 cm diameter was recorded.

One ml of cell suspension of strain 27 (10^8 cells/ml) was mixed with 5 ml of concentrated bacteriocin 84 and 0.004 ml of the mixture was inoculated into each of 20 tomato seedlings. Another 20 seedlings were inoculated with a mixture of cell suspension and concentrated Stonier's liquid medium as a control treatment. Stonier's liquid medium was concentrated in the same way as was the bacteriocin extract except that the pure sterile liquid medium instead of culture extract was used.

At 0, 1, 2, 3 and 10 days after inoculation, the growth of strain 27 from both treatments was measured by isolating the bacteria from three plants of each treatment and plating on New and Kerr's medium. Five plants from each treatment were kept in the glasshouse for 5 weeks to measure the diameters of the galls formed at the inoculation sites.

The growth of strain 27 was observed to be inhibited markedly by bacteriocin at 0, 1 and 2 days. But from 3 days onwards, no marked difference in the growth of strain 27 in the two treatments was recorded (Fig. 22). The reason why there was such a marked difference between the two treatments at day 0. could be explained in terms of the length

Fig. 22. The effect of sterile concentrated bacteriocin 84 on the growth of strain 27 on tomato stems.

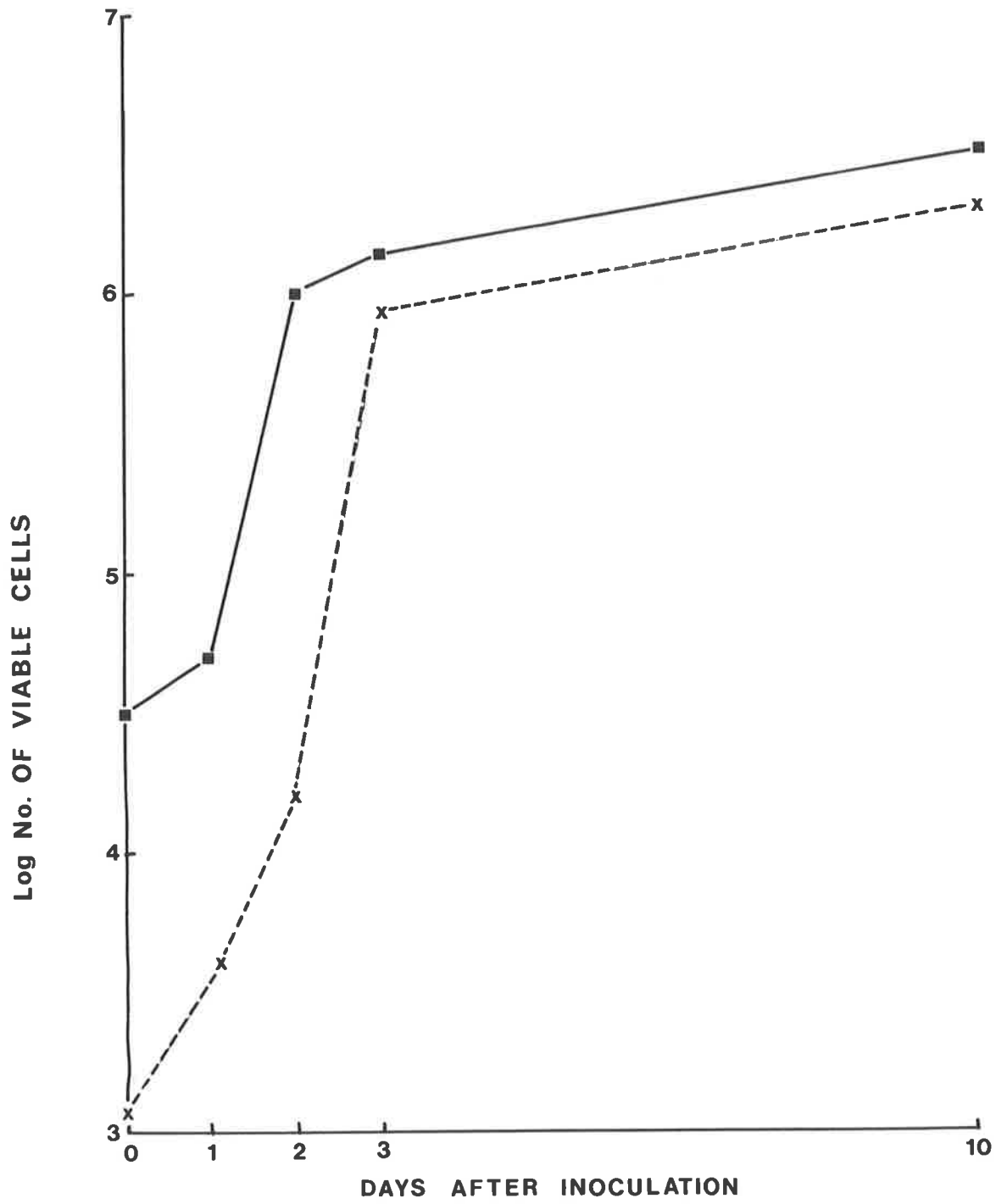


Strain 27.

X --- X

Strain 27 (+ bacteriocin 84).

Fig. 22



of time during which bacterial cells were directly exposed to concentrated bacteriocin in the test tube before they were inoculated into tomato seedlings. The mixture of cells and bacteriocin was kept for about 15 minutes while the control plants were inoculated. The reisolated colonies from both treatments were tested and found pathogenic and sensitive to bacteriocin 84.

Galls that developed on the control plants were significantly bigger than those on 3 out of 5 bacteriocin-treated plants - no galls were observed on the remaining two plants (Table 10). These data showed that bacteriocin 84 also had an inhibitory effect on the growth of pathogenic strain 27 and on gall formation by this pathogen *in vivo*.

Kerr and Htay (1974) reported that among the tested pathogenic strains which were sensitive to bacteriocin 84 and subject to biological control, there was one exceptional pathogenic strain (108) which was not subject to biological control by strain 84 although it was sensitive to bacteriocin 84. The reason was that strain 108 produced a bacteriocin to which strain 84 was sensitive. If bacteriocin 84 was the mechanism behind biological control, gall formation by strain 108 would be inhibited by bacteriocin 84 but not by living cells of strain 84 since these were sensitive to the bacteriocin produced by strain 108. Thus, the effect of sterile bacteriocin 84 and that of living cells of strain 84 on the gall formation by strain 108 were investigated in the following experiments.

10. The effect of sterile concentrated bacteriocin 84 on gall formation by strain 108

One ml of cell suspension of strain 108 (10^8 cells/ml) was mixed

Table 10. The effect of sterile concentrated bacteriocin 84 on the inhibition of gall induction by pathogenic strain 27.

Treatment	No. of galled plants	Mean diameter of galls (mm)
S27 + bacteriocin	3/5	0.9*
S27 + Stonier's medium	5/5	3.3
L.S.D. (P=0.05) 1.36		
(P=0.01) 2.41		

with 5 ml of sterile concentrated bacteriocin 84, and 0.004 ml of this mixture was inoculated into each of ten wounded tomato seedlings. For the control plants, a mixture of cell suspension of strain 108 and sterile concentrated Stonier's liquid medium was inoculated into another ten tomato seedlings. Plants were subirrigated and kept at 25°C in a glasshouse. After 5 weeks incubation, the number of galled plants were recorded and the diameters of galls were measured.

Four of 10 bacteriocin-treated plants, and all 10 control plants were found with galls. Significantly smaller galls were observed on bacteriocin-treated plants (Table 11).

11. The effect of strain 84 on gall formation by strain 108

Kerr and Htay (1974) reported a biotype 2 nonpathogenic strain (128) which was sensitive to bacteriocin 84 and also produced a bacteriocin (128) to which strain 84 was sensitive. The influence of bacteriocin 128 on the biological control of strain 84 was also tested in this experiment. Therefore, this experiment concerned not only the effect of strain 84 on gall formation by strain 108, but also the influence of the other bacteriocin-producing strain on the biological control ^{exerted by} ~~of~~ strain 84.

Tomato seedlings were separately inoculated with cell suspension of strain 108 alone, a mixture of strains 108 and 84 at 1:1 ratio, and a mixture of strains 108, 84 and 128 at 1:1:1 ratio. The number of pathogenic cells was 10^5 cells per wound in all treatments. Plants were subirrigated and kept in a 25°C glasshouse. After 5 weeks incubation, the diameter of galls on plants were measured.

Table 11. The effect of sterile concentrated bacteriocin 84 on gall induction by pathogenic strain 108.

Treatment	No. of galled plants	Mean diameter of galls (mm)
S108 + bacteriocin 84	4/10	0.5**
S108 + Stonier's medium	10/10	3.2
	L.S.D. (P=0.05)	0.03
	(P=0.01)	0.88

All plants were found with galls but plants inoculated with strains 108 and 84, or strains 108, 84 and 128 had significantly smaller galls. There was no marked difference in the sizes of galls on plants treated with either of the above combinations (Table 12).

Evidences strongly suggested that the bacteriocin produced by strain 84 was the mechanism involved in the successful biological control by strain 84. Final proof would be to demonstrate that strain 84 produced bacteriocin in plants.

12. Attempted detection of bacteriocin 84 in tomato seedlings inoculated with strain 84

Approximately 4×10^5 cells of strain 84 were inoculated into each of fifteen tomato seedlings. At 0, 1, 2, 3 and 10 days, two cm lengths at the inoculation sites of each of three seedlings were soaked in sterile distilled water with a few drops of chloroform overnight at room temperature. Each extract was freeze-dried using a Dyna-Vac centrifugal freeze-drying machine. Each residue was redissolved in 0.1 ml of sterile distilled water and inoculated into a well for a bacteriocin test. No bacteriocin was detected from any of the extracts.

High voltage paper electrophoresis (Roberts, 1975) was also used in an attempt to detect the bacteriocin. Residue suspension was loaded across the centre of a 15 cm by 57 cm strip of Whatman 3 mm paper. Bacteriocin extracted from strain 84 liquid culture was also used as a running marker. The suspensions were loaded at the origin as thin streaks at a rate of 0.1 ml/cm. The paper was then moistened with citrate buffer at pH 5.0, allowing it to creep slowly up to the load

Table 12. The effect of strain 84 on gall induction by strain 108 in the presence or absence of strain 128.

Treatment	No. of galled plants	Mean diameter of galls (mm)
S108 alone	7/7	5.14
S108 + S84	7/7	3.57*
S108 + S84 + S128	7/7	3.43*
L.S.D. (P = 0.05) 1.36		

streak. Excess buffer was then blotted off. The paper was placed in a carbon tetrachloride-cooled, high voltage paper electrophoresis apparatus (Tate, 1968) and run at 1,500 volts for one hour. The paper was then removed, and dried and assayed.

One cm squares were cut along the run, placed on agar plates of Stonier's medium, sterilized with chloroform vapour, then poured with the sensitive indicator strain in soft buffered agar. The plates were incubated for 1-2 days. No inhibition zones around the paper squares of extract from tomato stems were observed but those with bacteriocin extract from strain 84 liquid culture developed inhibition zones.

The reason why bacteriocin 84 was not detected from inoculated tomato plants is not known. It could be simply due to technical faults or because bacteriocin was produced in minute amounts, so that it was not detected by the techniques used. It could also be the fact that strain 84 did not produce bacteriocin in tomato cell sap. This was investigated.

13. Detection of bacteriocin 84 in cultures of strain 84 in tomato-stem-extract

Tomato-stem-extract was prepared using the technique of Brathwaite and Dickey (1970). One hundred g of 5 to 6-week-old tomato stems was macerated with 400 ml distilled water in an electric blender for 5 minutes. The mixture was filtered through 6 layers of cheese-cloth and the liquid well shaken with 200 ml of chloroform to remove coarse materials and chloroplasts. The mixture was let stand for an hour and

the clear supernatant aqueous liquid removed to another flask and made up to one litre in volume. The pH of the tomato extract was adjusted to 7.0 with 1N NaOH. The extract was used as a liquid medium.

One hundred ml of extract in a 250 ml flask was sterilized by autoclaving. Bacterial cell suspension was prepared to have 10^8 cells/ml and 0.4 ml of the suspension added to the flask. The culture was grown on the shaker at 25°C for 3 days. The culture was then centrifuged at 8,000 rpm for 10 minutes and the supernatant liquid was mixed with a few ml of chloroform, well shaken and centrifuged at 6,000 rpm for 10 minutes. Forty microlitres of supernatant aqueous liquid were placed in a well cut in agar plate and assayed for bacteriocin production. The mean diameter of the 5 replicate-zones of the culture extract was 1.5 cm whereas that of the strain 84 culture extract using Stonier's liquid medium was 2.0 cm.

These observations on the production of bacteriocin 84 in tomato-stem-extracts suggested that inability to detect bacteriocin 84 in tomato plants did not necessarily mean that strain 84 did not produce bacteriocin in plant cell sap. However, further investigation is still needed to demonstrate the production of bacteriocin by strain 84 *in vivo*.

Since strain 84 produced bacteriocin in Stonier's liquid medium, the interaction between strains 24 and 84 in this medium was carried out in the following experiment.

14. Interaction between strains 84 and 24 in Stonier's liquid medium

Bacterial cell suspension was prepared to have 10^8 cells/ml and 0.4 ml lots added to a 100 ml of sterile Stonier's liquid medium at pH 7.0

in 250 ml flasks. There were 3 treatments: pathogenic strain 24 alone; nonpathogenic strain 84 alone; and a mixture of strains 24 and 84 at a ratio of 1:1. Each treatment had 2 replicates. The culture flasks were kept on the rotary shaker in a 25°C constant temperature room. The growth of the bacteria was measured at 0, 1, 2, 3 and 7 days after inoculation as described previously.



The growth of strain 24 was found to be significantly inhibited by strain 84 at day 2 and 3 (Fig. 23A). Strain 84 was not affected by 24 (Fig. 23B) as it was in MGA and buffered Mannitol ammonium sulphate liquid media.

The production of bacteriocin by strain 84 was not recorded in this experiment which was only concerned with the determination of the inhibitory effect of strain 84 in Stonier's liquid medium. Since the inhibitory effect of strain 84 on the growth of pathogenic strain 24 was observed in Stonier's liquid medium, this effect was also tested on the other pathogenic strains 152 and 10 as well as nonpathogenic strain 147.

15. Interactions between strain 84 and pathogenic strains 152, 10 and nonpathogenic strain 147 in Stonier's liquid medium

The procedure was as described in the preceding experiment. There were seven treatments: strain 84, 152, 147 or 10 alone; a mixture of strains 84 and 152, 84 and 147, and 84 and 10, all at a ratio of 1:1. Each treatment had 2 replicates. The growth of each strain was measured at 0, 1, 2, 3 and 7 days and pH changes of the cultures were

Fig. 23. Interactions between strain 24 and 84 in Stonier's liquid medium.

A:  Strain 24.
  Strain 24 (+ 84).



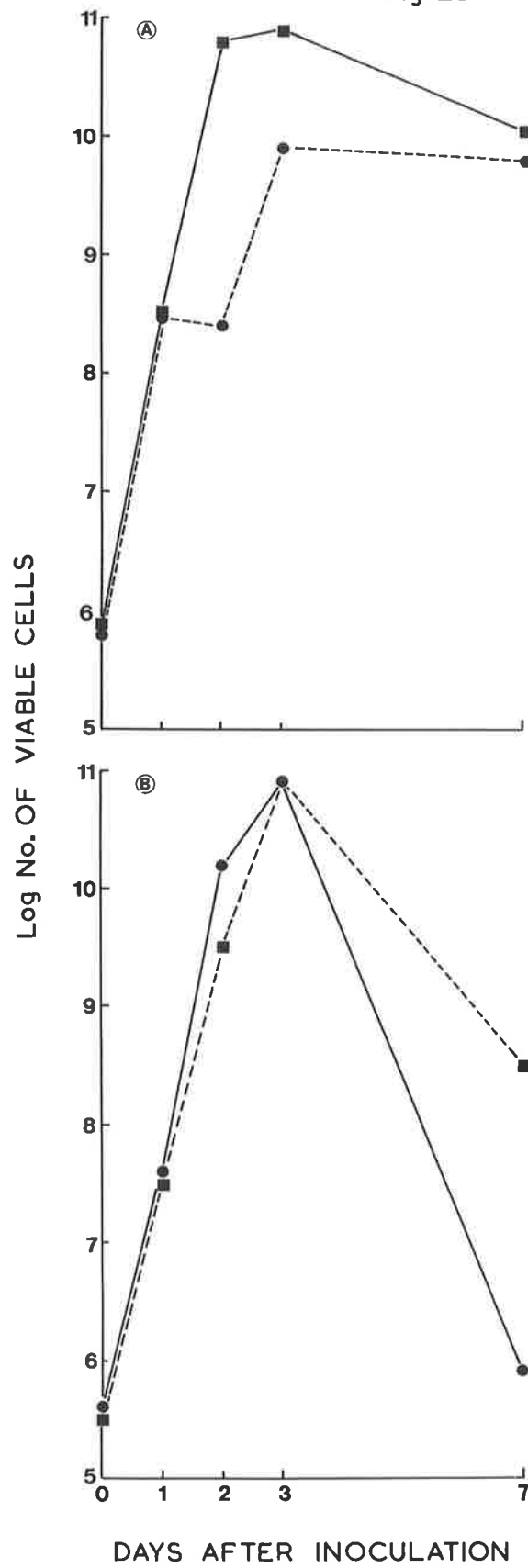
B:  Strain 84.
  Strain 84 (+ 24).

Fig. 23



also recorded.

In this experiment the effect of strain 84 on the pathogenicity of the resistant cells of the pathogens and production of bacteriocin 84 were tested at various intervals. To detect its effect on the pathogenicity of resistant cells, fifty reisolated colonies of each strain 152 or 10 from each treatment where they were grown alone or together with strain 84 in liquid medium, were randomly chosen at various intervals and singly inoculated into healthy tomato seedlings. After two weeks incubation in a 25^o glasshouse, the galls developed on the plants were recorded.

To detect bacteriocin 84 in the liquid cultures, two ml of liquid from every flask of each treatment was withdrawn at the different time intervals. Each sample was shaken with a few drops of chloroform and let stand at 5^oC overnight. The next day, forty microlitres of supernatant liquid were placed in a well cut in a plate of Stonier's agar medium and assayed for bacteriocin.

At days 3 and 7 strain 152 was markedly inhibited by strain 84 (Fig. 24A), whereas strains 147 and 10 were only slightly affected at day 2 onwards (Fig. 24B and C). Strain 84 was markedly affected by pathogenic strain 10 at days 1 and 2, and by nonpathogenic strain 147 at days 2 and 3, but not at all affected by strain 152 (Fig. 24D). However, data on the growth of strain 84 at day 7 was not recorded, since no colonies developed on the New and Kerr's medium. Possibly the wrong dilutions were plated on this medium. There was no marked difference between pH changes of cultures in all treatments. All cultures changed to an

Fig. 24. Interactions between strain 84 and pathogenic strains 152 and 10, and nonpathogenic strain 147 in Stonier's liquid medium.

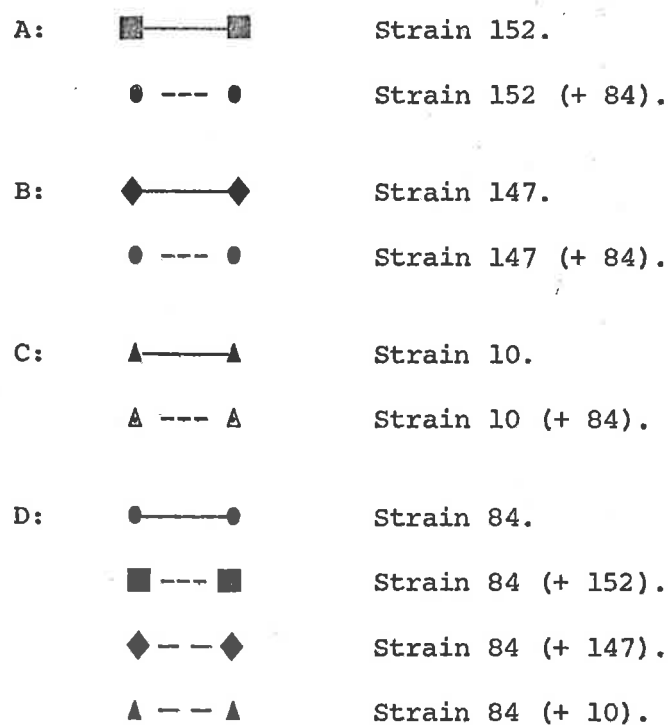
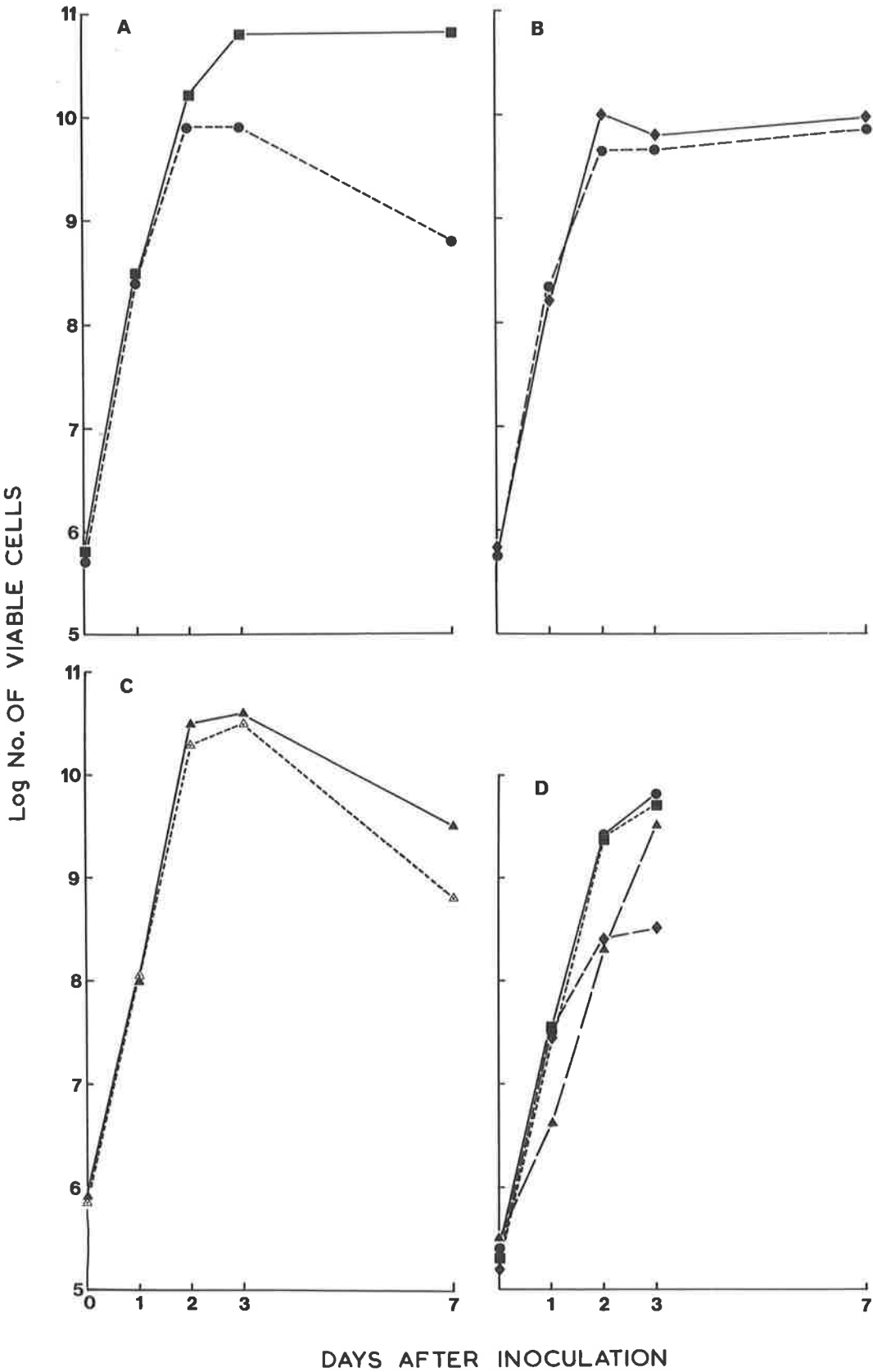


Fig. 24



alkaline pH after day 1 (Appendix 2.1). Bacteriocin 84 was only detected in the cultures where strain 84 alone or 84 combined with strain 152 was grown (Fig. 25). The plants which were inoculated with reisolated colonies of strains 152 and 10 were found with galls except some inoculated with the colonies of 152 that had been exposed to strain 84 (Table 13).

Since the inhibitory effect of strain 84 on the growth of controllable biotype 1 pathogenic strains 24 and 152 was found in liquid culture, its effect on the controllable biotype 2 pathogenic strain 27 was also investigated.

16. Interaction between strains 84 and 27 in Stonier's liquid medium

The procedure was the same as that in the preceding experiment. Strain 84 alone, strain 27 alone, and a mixture of strains 84 and 27 at a ratio of 1:1 were separately inoculated into ten liquid medium flasks. At 0, 1, 2, 3 and 7 days after inoculation, the appropriate dilutions were made and plated on New and Kerr's medium. Fifty colonies from each of the treatment where strains 84 and 27 were combined, were randomly chosen and tested for bacteriocin 84 production. The colonies which produced inhibition zones were recorded as strain 84 and the rest were strain 27. At various intervals the pH changes of the cultures were recorded. The production of bacteriocin 84, the loss of pathogenicity and the sensitivity of the reisolated colonies to bacteriocin 84 were also tested.

Strain 27 was inhibited from day 2 onwards (Fig. 26A). All the reisolated colonies showed pathogenicity and sensitivity to bacteriocin 84.

Fig. 25. Detection of bacteriocin 84 in liquid cultures
at 0, 1, 2, 3 and 7 days after inoculation.

$7_1, 7_2$: strain 147 alone.

$+7_1, +7_2$: strain 147 + 84.

$9_1, 9_2$: strain 84 alone (note the inhibition
zone of bacteriocin 84).

$2_1, 2_2$: strain 152 alone.

$+2_1, +2_2$: strains 152 + 84 (note the inhibition
zone of bacteriocin 84).

Fig. 25

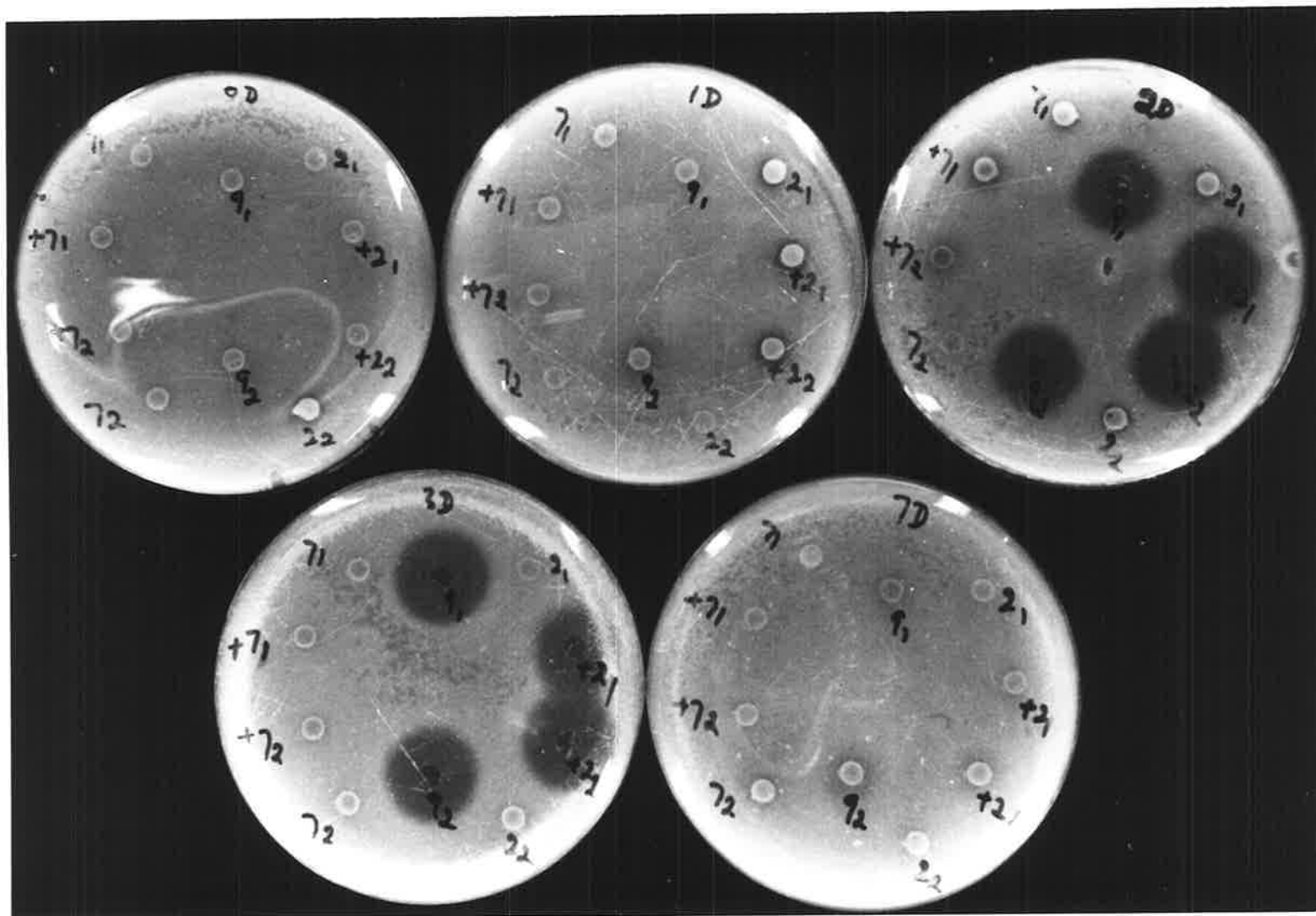
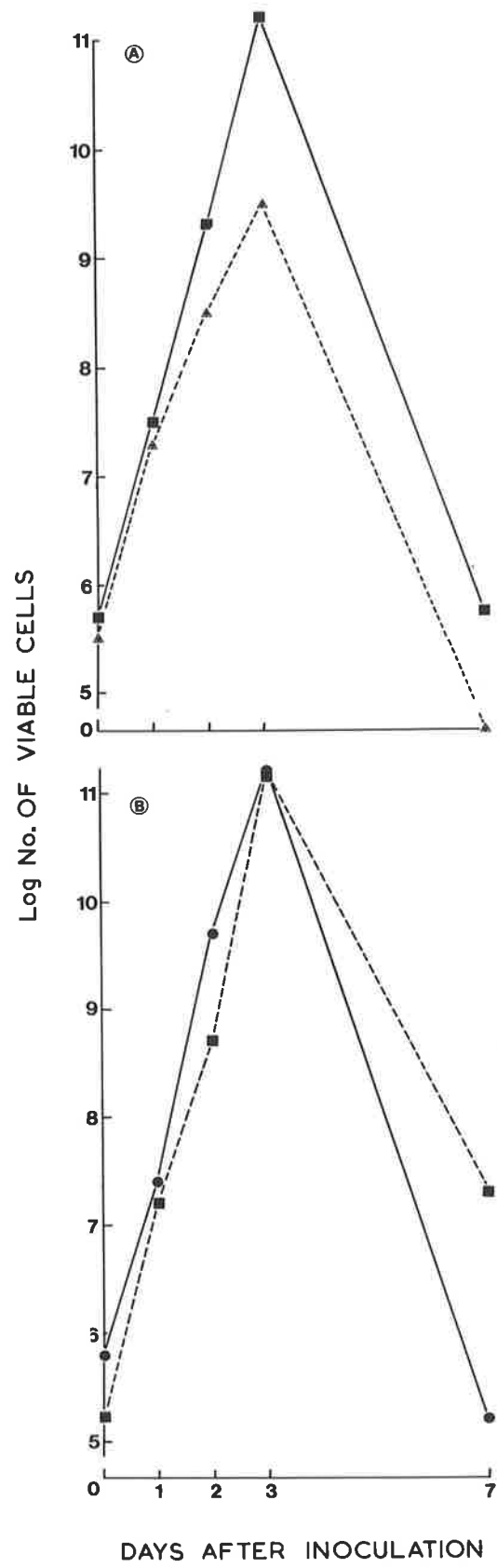


Table 13. The effect of strain 84 on the pathogenicity of resistant cells of strains 152 and 10 in liquid culture.

Treatment	No. of pathogenic cells/50 reisolated cells at (days)				
	0	1	2	3	7
Strain 152	50	50	50	50	50
Strain 152 (+ 84)	50	50	46	32	18
Strain 10	50	50	50	50	50
Strain 10 (+ 84)	50	50	50	50	50

Fig. 26



The growth of strain 84 was ~~found affected~~ ^{reduced} only at day 2 (Fig. 26B). Bacteriocin 84 was detected at days 2 and 3 (Fig. 27). The pH of all cultures changed to alkalinity (Appendix 2.2).

In the above experiments, the observations of the presence of bacteriocin 84 at days 2 and 3, the inhibition of the growth of the strains 152 and 27, and the loss of pathogenicity of resistant cells of strain 152 at day 2 onwards suggested that bacteriocin produced by strain 84 was the major cause of the effects of strain 84. This was supported by the observation of the absence of the bacteriocin 84 associated with no inhibition of growth of strain 10 or 147 in the cultures where strains 84 and 10 or 147 were growing.

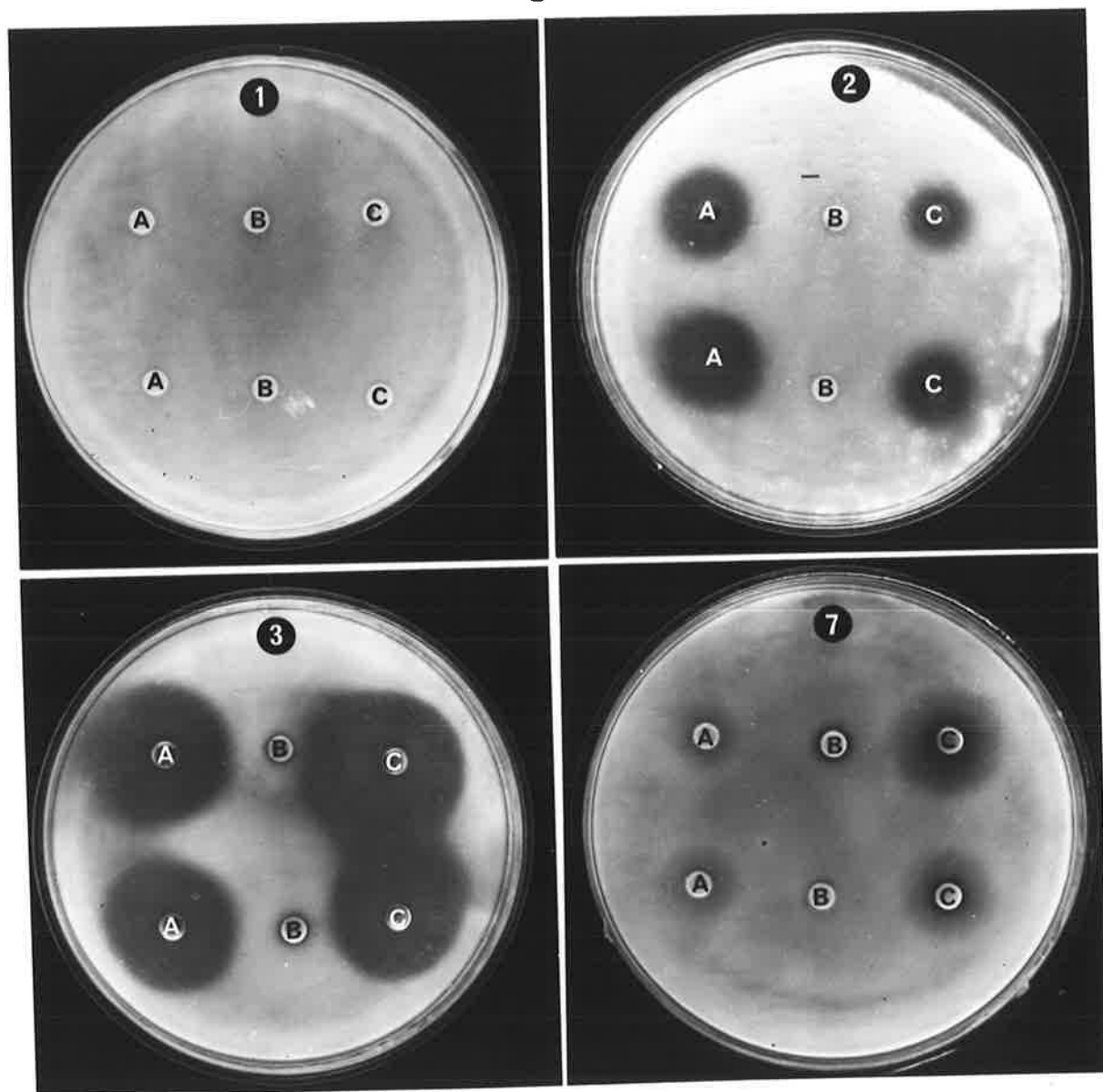
Since bacteriocin 84 was detected in the cultures where the inhibitory effects of strain 84 were recorded, it was clear that bacteriocin 84 was also involved in the interactions of strain 84 and other agrobacteria.

The effect of strain 84 on gall formation by the bacteriocin producing pathogenic strain 108 in wounded tomato seedlings was reported in the earlier part of this section. Although strain 84 did not prevent gall formation by strain 108 since it was sensitive to bacteriocin 108, it reduced the size of the galls significantly, even in the presence of the other bacteriocin-producing nonpathogenic strain 128. Not only the interactions of these three strains, 84, 108 and 128, but also the interactions of their bacteriocins would give useful information on the mechanism behind the prevention of the galls formed by strain 108. Thus,

Fig. 27. Detection of bacteriocin 84 in liquid cultures
at 1, 2, 3 and 7 days after inoculation.

- A. Strain 84 alone.
- B. Strain 27 alone.
- C. Strains 84 + 27.

Fig. 27



the following experiment was carried out to investigate the interactions of these bacteriocin-producing strains in liquid cultures.

17. Interactions between strain 84 and two biotype 2, bacteriocin-producing strains, 108 and 128

Strain 108 is a pathogen which produces bacteriocin 108 and strain 128 is a nonpathogen which produces bacteriocin 128. They both are sensitive to bacteriocin 84 and strain 84 is sensitive to both bacteriocins 108 and 128. The interaction between these three strains *in vitro* was investigated.

Stonier's liquid medium was used. The procedure was carried out as described in the former experiments. There were 7 different inoculation treatments: (1) strain 84 alone, (2) strain 108 alone, (3) strain 128 alone, (4) a mixture of strains 108 and 84 at a 1:1 ratio, (5) a mixture of strains 108 and 128 at a 1:1 ratio, (6) a mixture of strains 108, 128 and 84 at a 1:1:1 ratio, and (7) a mixture of strains 128 and 84 at a 1:1 ratio. These treatments were inoculated separately into the liquid medium. Cells were reisolated on New and Kerr's medium at 0, 1, 2, 3 and 7 days after inoculation. Strain 84 was distinguished by detecting bacteriocin 84 using indicator strain 24 and strain 108 was distinguished from 128 by a pathogenicity test. Two ml of each culture from each treatment was sampled and shaken with a few drops of chloroform and let stand at 5° overnight and then tested for bacteriocin.

All 3 strains grew very well in the cultures where they were alone. The growth of strain 108 was inhibited in all treatments in the presence of strain 84 (Fig. 28A). No marked effect on the growth of strain 108 by

Fig. 28. Interactions between strain 84 and other bacteriocin producing strains 128 (nonpathogen) and 108 (pathogen) *in vitro*.

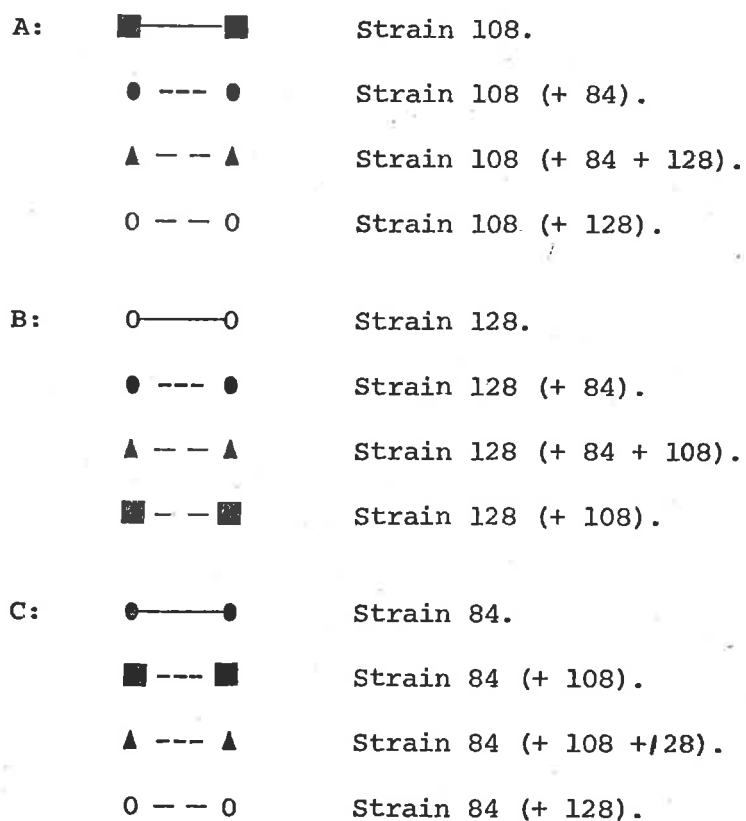
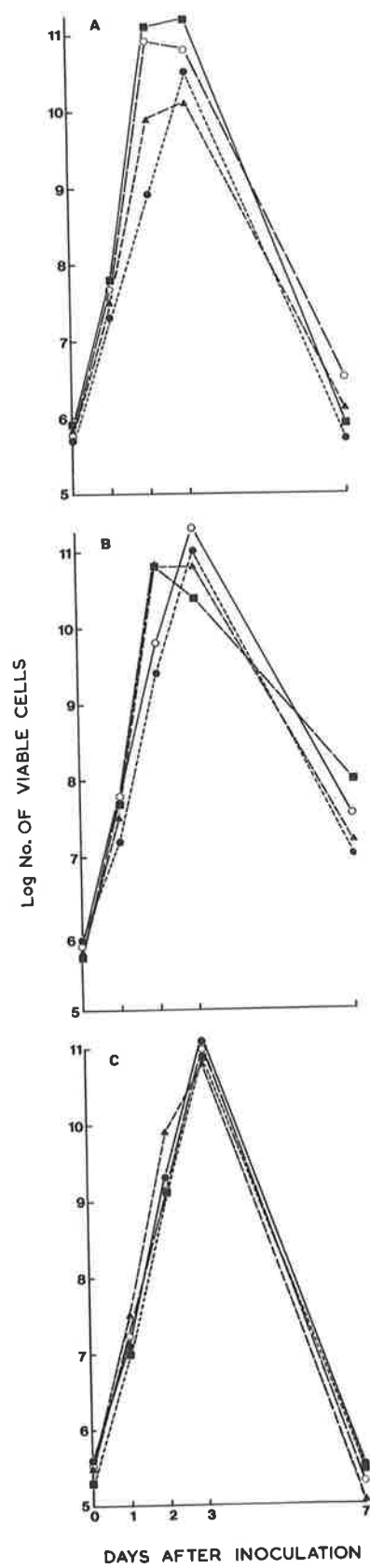


Fig. 28



strain 128 was observed. Strain 128 was only slightly affected by strain 84 and stimulated by 108 at day 2 and by the combination of strains 108 and 84 at days 2 and 7 (Fig. 28B). There was no marked effect on the growth of strain 84 in all treatments except that at day 1 and 2 it was slightly stimulated by the presence of strains 108 and 128 (Fig. 28C). The pH of cultures changed to alkalinity after day 1 but there was no significant difference of pH changes between the cultures (Appendix 2.3).

Bacteriocin 84 was detected at day 2 and 3 in all cultures where strain 84 was present, and there was no marked difference between the size of inhibition zones of bacteriocin 84 in all treatments (Fig. 29). Bacteriocins 108 and 128 were not detected in any cultures. The reason for this was not clear. It could be that strains 108 and 128 produced minute amounts of bacteriocin 108 and 128 respectively, or it could be a technical fault. However, observation of the characteristics of the inhibition zones of bacteriocins 84, 108 and 128 on Stonier's agar medium showed that the zone of bacteriocin 84 was bigger than those of bacteriocins 108 and 128 (Fig. 30). Other reasons could be: bacteriocins 108 and 128 lost their activities quickly or that strain 84 was able to compete with strains 108 and 128 for nutrient since they all were of same biotype so that the latter strains could not produce bacteriocin. However, the actual reason has still to be ~~confirmed~~ ^{discovered}.

The above evidence showed that the inhibitory effects of strain 84 were always associated with the presence of bacteriocin 84 in the liquid culture. To prove the major responsibility of bacteriocin 84 for these effects of strain 84, the effects of sterile bacteriocin 84

Fig. 29. Detection of bacteriocin 84 in liquid cultures at 2 and 3 days after inoculation. It was not detected at 0, 1 and 7 days.

- A. Strain 84.
- B. Strains 84 + 128 + 108.
- C. Strains 84 + 108.
- D. Strains 84 + 128.

Fig. 29

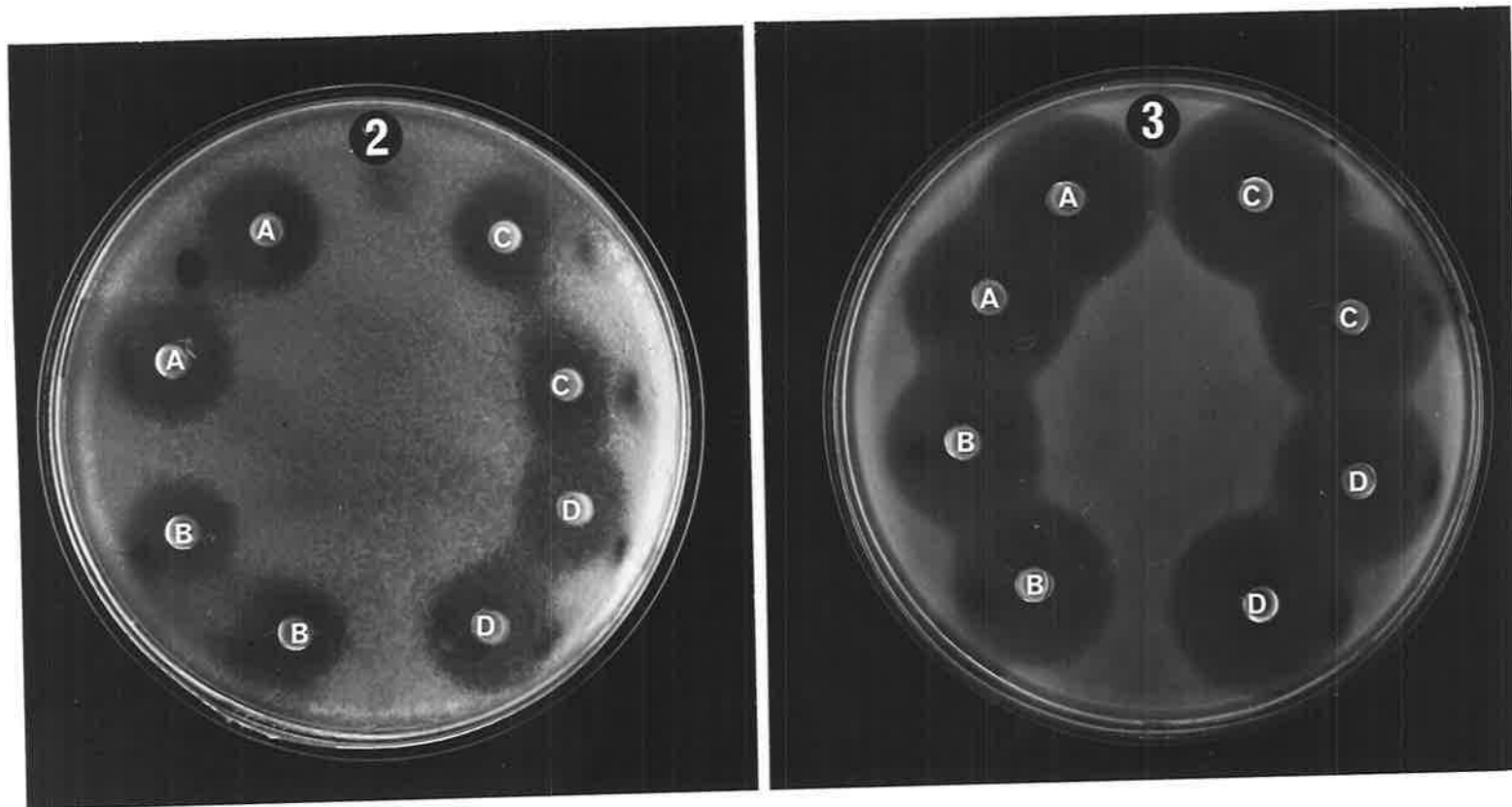
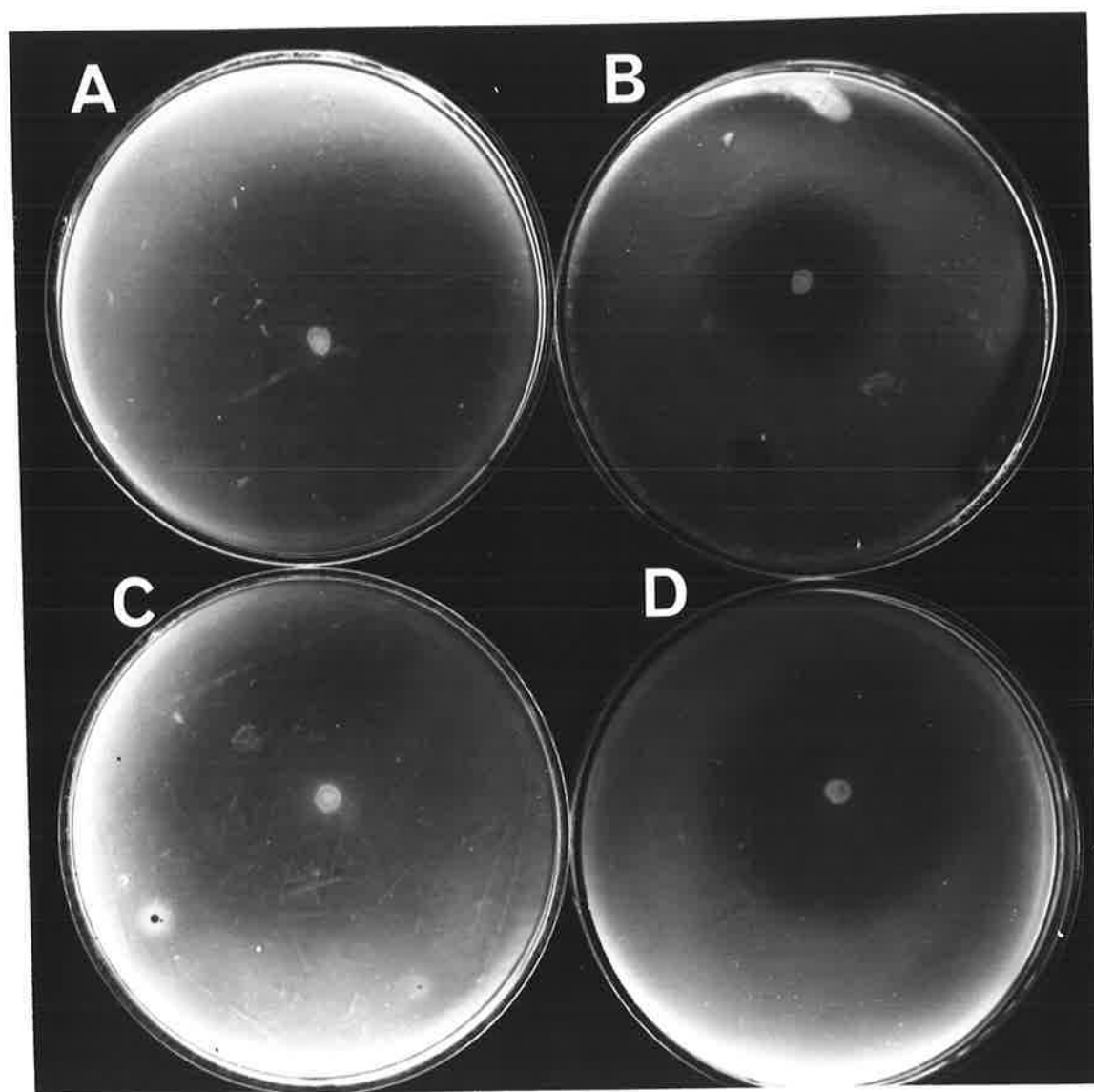


Fig. 30. Inhibition zones of different bacteriocin.

- A. Bacteriocin 84 against strain 108.
- B. Bacteriocin 108 against strain 84.
- C. Bacteriocin 84 against strain 128.
- D. Bacteriocin 128 against strain 84.

Fig. 30



on strain 152, 147, 10 and 27 were tested in liquid culture.

18. The effect of sterile concentrated bacteriocin 84 on strains 152, 147, 10 and 27 in liquid culture

Ten ml of sterile concentrated bacteriocin 84 was added to 90 ml of Stonier's liquid medium. As the bacteriocin had been stored with chloroform, excess of chloroform was disposed by removing the cottonwool plug of the culture flasks and keeping the flask open in a laminar flow for 30 minutes. For the treatment without bacteriocin, 10 ml of concentrated sterile Stonier's liquid medium was added to 90 ml of ordinary Stonier's liquid medium.

Cell suspensions (10^8 cells/ml) of strains 152, 147, 10 and 27 were prepared separately and 0.4 ml of the suspension of each strain was separately inoculated into bacteriocin-treated or nontreated flasks of medium. Cultures were incubated and measurement of growth of bacteria was carried out by plating dilutions of strains 152, 147 and 10 on Schroth's medium and strain 27 on New and Kerr's medium. The bacteriocin was resampled from the treated flasks at various intervals and its activity tested. Tests of pathogenicity and bacteriocin-sensitivity were carried out for reisolated colonies of strains 152, 10 and 27. The pH changes of the cultures were measured.

Strains 152, 147 and 10 grew very well in the control flasks but strain 27 grew very slowly. The growth of strains 147 and 10 was not affected by bacteriocin (Fig. 31B and C), whereas the growth of strains 152 and 27 was inhibited (Fig. 31A, D). The inhibition of strain 152 was more marked than that of strain 27. All reisolated colonies of

Fig. 31. The effect of sterile concentrated bacteriocin 84 on the growth of strains 152, 147, 10 and 27 in liquid culture.

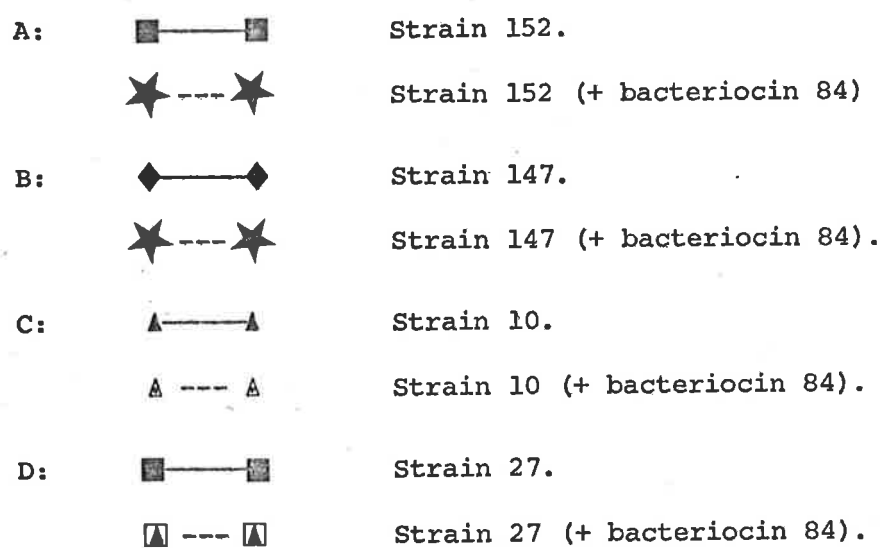
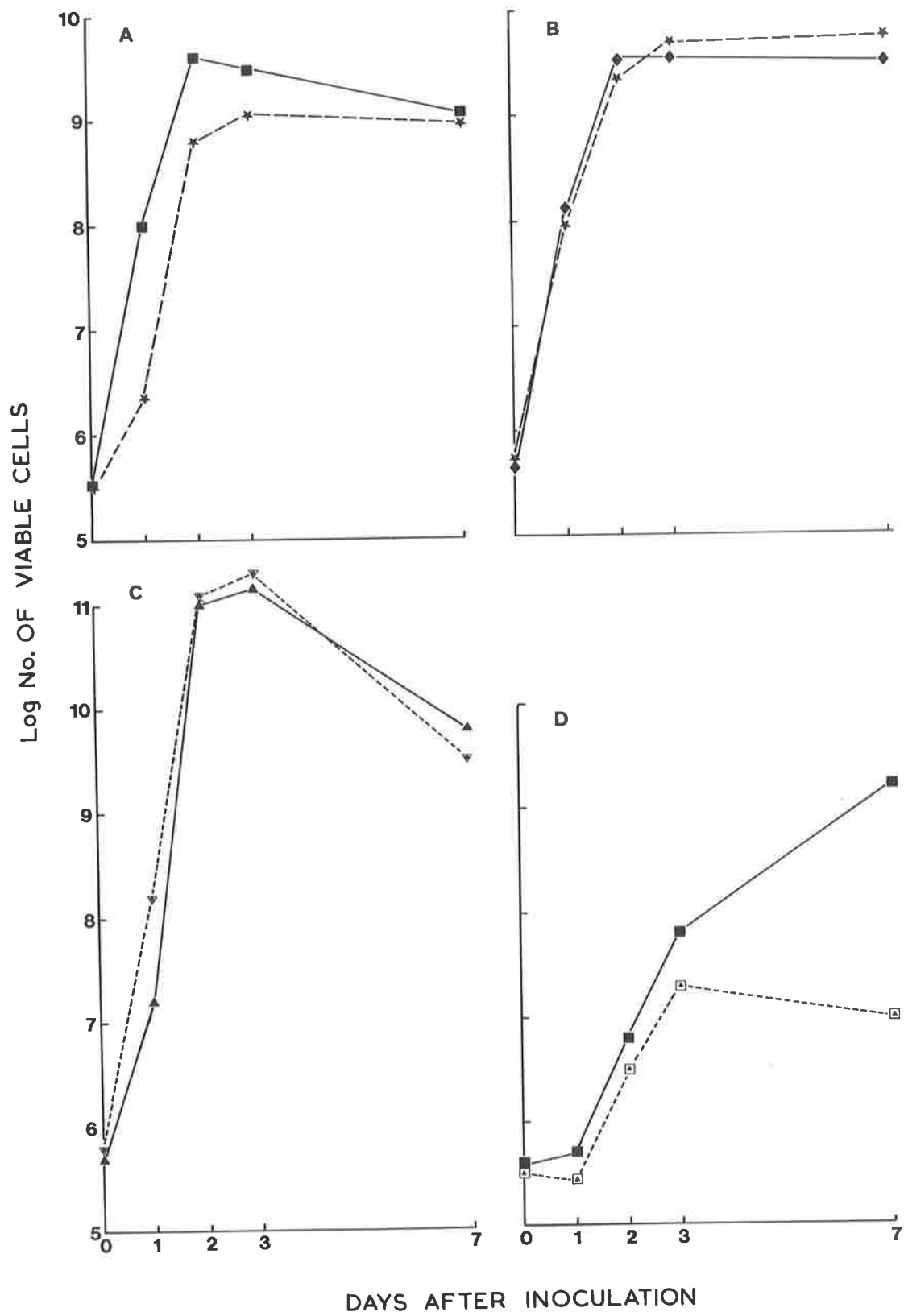


Fig. 3I



strains 10 and 27 were pathogenic, and those of strain 27 were sensitive to bacteriocin 84. A large proportion of the reisolated colonies of strain 152 which had been exposed to bacteriocin were found no longer pathogenic (Table 14), nor sensitive to bacteriocin.

The activity of bacteriocin detected in every culture flask was found to decrease gradually (Figs. 32, 33). The pH of all the cultures changed to alkalinity (Appendix 2.4).

DISCUSSION

Nonpathogenic strain 84 of *Agrobacterium radiobacter* var. *radiobacter* biotype 2 prevents gall formation of many pathogenic strains of *A. radiobacter* var. *tumefaciens* biotypes 1 and 2 (New and Kerr, 1972). In this study of the interaction between pathogenic and nonpathogenic strains of *A. radiobacter* it was found that strain 84 inhibited the growth of pathogenic strains 24, 152 and 27, and prevented gall induction by them.

The degree of disease prevention seemed to be directly related to the degree of growth inhibition of a particular strain by strain 84. Strain 84 inhibited the growth of pathogenic strains 24, 152 and 27 markedly and prevented their gall formation completely. But it did not greatly inhibit the growth of pathogenic strains 6 and 10, and hardly ~~prevented~~ ^{affected} gall formation by these strains. These results suggested that the inhibition of the growth of a pathogen by strain 84 correlated with inhibition of gall induction by that pathogen. Strain 130, however, which is a biotype 2 nonpathogen and which is not involved in biological

Table 14. The effect of sterile concentrated bacteriocin 84 on the pathogenicity of resistant cells of strains 152, 10 and 27 in liquid culture

Treatment	No. of pathogenic cells/50 resistant cells at (days)				
	0	1	2	3	7
Strain 157 (+ medium)	50	50	50	50	50
Strain 152 (+ bacteriocin)	50	11	2	1	0
Strain 10 (+ medium)	50	50	50	50	50
Strain 10 (+ bacteriocin)	50	50	50	50	50
Strain 27 (+ medium)	50	50	50	50	50
Strain 27 (+ bacteriocin)	50	50	50	50	50

Fig. 32. Activity of concentrated bacteriocin 84 in liquid culture at 0, 1, 2, 3 and 7 days after inoculation.

- A. Bacteriocin 84 in strain 152 culture.
- B. Bacteriocin 84 in sterile liquid medium.
- C. Bacteriocin 84 in strain 147 culture.

Fig. 32

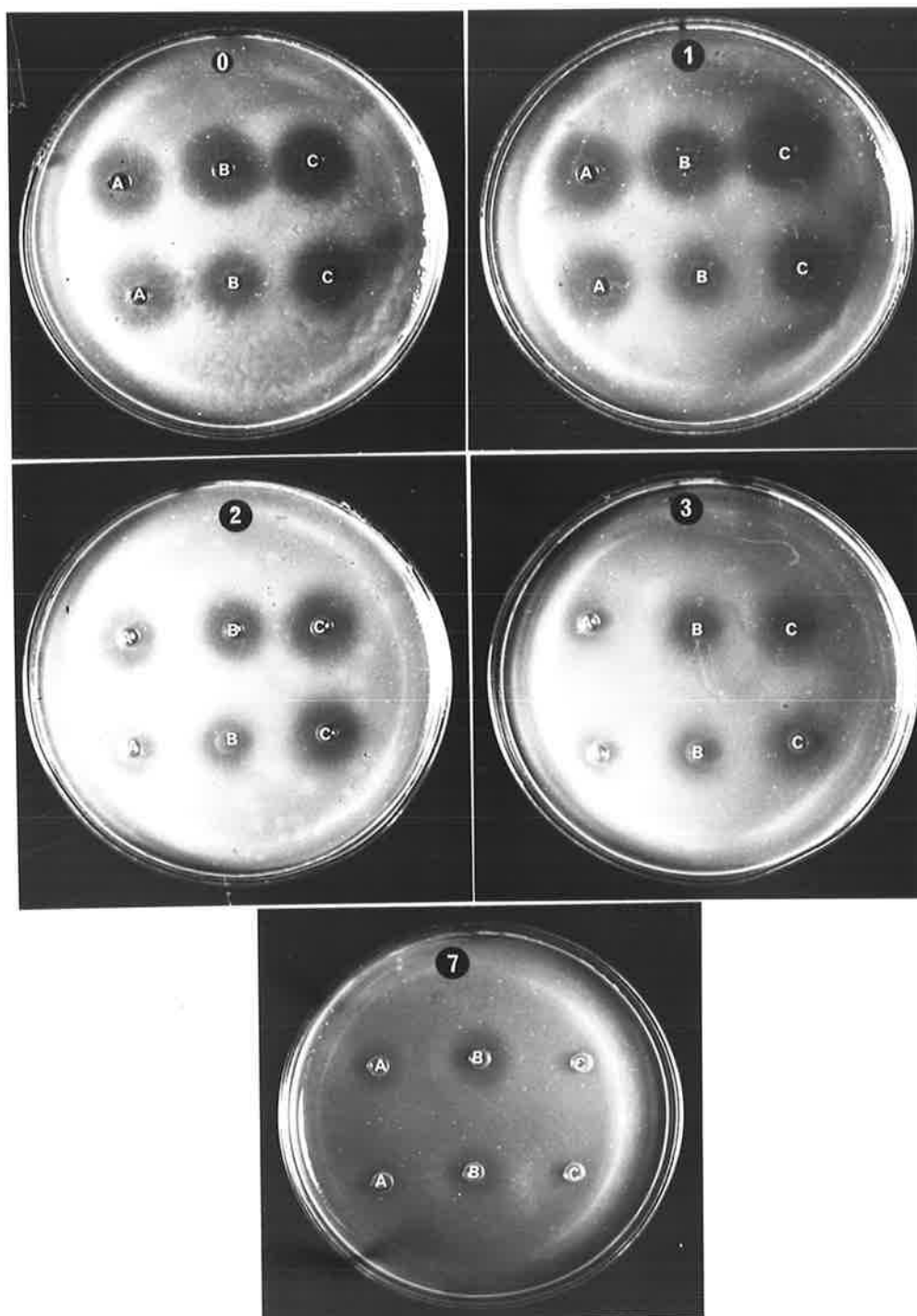
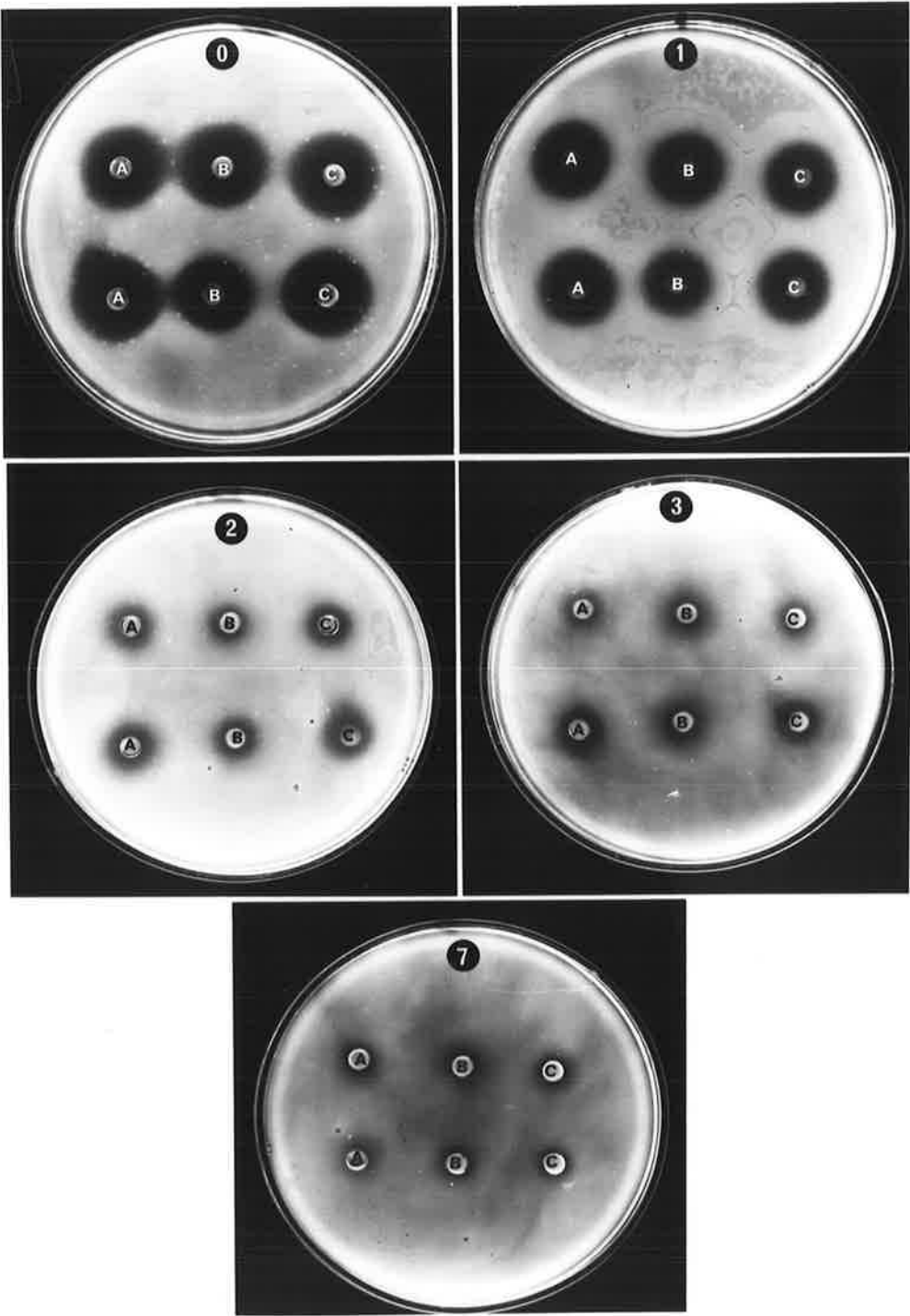


Fig. 33. Activity of concentrated bacteriocin 84 in liquid culture at 0, 1, 2, 3 and 7 days after inoculation.

- A. Bacteriocin 84 in strain 27 culture.
- B. Bacteriocin 84 in sterile liquid medium.
- C. Bacteriocin 84 in strain 10 culture.

Fig. 33



control, was found to have no effect on the growth of the pathogens tested, and not to prevent their gall induction. This result supported the above suggestion on the correlation between inhibition of growth of a pathogen and inhibition of its gall induction by strain 84.

Since this correlation was found *in vivo*, factors induced by the host in the presence of strain 84 could be suggested as the cause of the correlation. Numerous workers have found that resistance could be induced by hosts after they were inoculated with bacterial cells. Two kinds of induced resistance have been reported (Klement, 1972):

(i) Premunity: which was a form of resistance gained by host plants that were inoculated with a saprophyte or killed bacterial cells prior to being inoculated with the pathogen (Klement and Goodman, 1967; Lovrekovich and Farkas, 1965).

(ii) Hypersensitive Reaction: which was produced when incompatible or resistant hosts were inoculated with pathogens (Kennedy and Crosse, 1966; Klement, Farkas and Lovrekovich, 1964; Klement and Goodman, 1967; Lelliott, Billing and Hayward, 1966; Lozano and Sequeira, 1970; Stall and Cook, 1968). Hypersensitivity also occurred when a sensitive host came into contact with a pathogen which had lost its virulence (Klement and Goodman, 1967; Lozano and Sequeira, 1970).

However, neither type of resistance can account for the effect of strain 84 *in vivo*. Strain 84 was always inoculated at the same time as the pathogen, so the inhibition of growth of the pathogen could not be due to the premunity of the host. As for hypersensitivity, it was not

observed when strain 84 was inoculated into tomato and tobacco leaves at 25° in the glasshouse under normal light. Whether or not delayed necrosis might occur in the same experiment in the dark as suggested by Hildebrand and Riddle (1971) remains to be investigated. So, it seemed unlikely that the inhibition of a pathogen was due to resistance induced in a host by the presence of nonpathogenic strain 84 and this was supported by the observation of inhibition of growth of pathogens by strain 84 *in vitro*.

Growth of pathogenic strains 24, 152 and 27 was inhibited by strain 84 in all experiments *in vitro* except one. In this experiment mannitol glutamic acid (MGA) liquid medium at pH 7.6 was used. It was later found that MGA liquid medium at pH 7.6 favoured growth of the pathogenic strain 24, but not the inhibitory effect of strain 84. Acid or neutral MGA medium or buffered mannitol ammonium sulphate medium seemed to favour the inhibitory effect of strain 84. The growth of pathogenic strain 24 alone, was also found to be affected by a lower pH of the medium. These results *in vitro* suggested that the growth inhibition of pathogens was likely to be due to the effect of strain 84 itself rather than resistance induced by the host.

There were a number of possibilities any one of which would account for the inhibitory effect of strain 84: competition for nutrients and infection sites; production of metabolites such as acids or antibiotics; an agglutinating factor or peroxidase activity as suggested by many other workers in many other diseases.

If competition for nutrients or infection sites was the cause of the inhibitory effect of strain 84, partial gall development should occur on the plants which were inoculated with the combination of pathogenic strains 24, 152 or 27 with strain 84 at 1:1 ratio. But, in all cases, complete prevention of gall formation was observed, even though the growth of strain 84 itself was slightly affected by either of pathogens 24 or 152. These observations led to the consideration that competition could not satisfactorily be regarded as the sole mechanism of biological control by strain 84. However, whether any of the other suggested possibilities was involved in the control remained to be investigated.

Although the cause of the inhibitory effect of strain 84 was not known, the following specific characteristics of the effect were observed:

- (a) Different degrees of inhibition of pathogenic strains and different degrees of prevention of gall induction by different pathogens: growth of strains 24, 152 and 27 was markedly inhibited by strain 84 and their gall induction was completely prevented, but no marked growth inhibition and gall prevention by strain 84 was observed on pathogenic strains 6 and 10.
- (b) Different reaction to pathogens and nonpathogens: growth of pathogenic strain 152 was inhibited but not nonpathogenic strain 147 which was the same strain before it acquired virulence.
- (c) A period of time was necessary for a marked effect on the growth of pathogens. The inhibition of growth of pathogenic strains was normally observed at day 2.

When Kerr and Htay (1974) reported that strain 84 produced bacteriocin 84 on Stonier's agar medium, the inhibitory effect of strain 84 was suggested to be the production of bacteriocin 84. This suggestion was based on their finding a very high correlation between biological control of pathogenic strains by strain 84 and their sensitivity to bacteriocin 84 produced by strain 84, and sensitivity of pathogens tested (with a few exceptions) to bacteriocin 84 but insensitivity of nonpathogens (Kerr and Htay, 1974; Roberts and Kerr, 1974).

Another characteristic of bacteriocin 84 that Kerr and Htay (1974) reported was its effect on the pathogenicity of resistant cells of biotype 1 pathogens: most bacteria which developed resistance to the bacteriocin were no longer pathogenic. This suggested that if strain 84 operated biological control through bacteriocin production, it could also produce this effect in wounded tomato stems where it operated biological control. This was tested and it was found that some reisolated cells of strain 152 which had been exposed to strain 84 in tomato stems for 2 days or longer, had lost their pathogenicity and sensitivity to bacteriocin 84. This effect was not found on the pathogenicity of reisolated cells of biotype 2 pathogenic strain 27. However, bacteriocin 84 also did not cause the loss of the pathogenicity of biotype 2 resistant cells (Kerr, unpublished data). The reason why biotype 1 resistant cells lost pathogenicity but biotype 2 did not, was not clear. It could be due to the different inhibitory effect of bacteriocin 84 produced by strain 84, on pathogens of different biotypes: it killed biotype 1 pathogens and caused mutant cells of biotype 1 pathogen, strain 152, to lose pathogenicity,

but it only slowed down the growth of biotype 2 pathogen, strain 27, and did not cause the slow-growing cells to lose pathogenicity. Further evidence that showed different reactions of biotype 1 and 2 sensitive pathogens to bacteriocin 84 was the production of different types of inhibition zones against pathogens of different biotypes. However, this observation does not explain the reason why bacteriocin had such different reactions. Roberts and Kerr (1974) hypothesized that tumor-inducing bacteria must have a specific configuration on their cell surface acting as a receptor site for bacteriocin; when the configuration was changed pathogenicity was lost. If this were so, then cells of different biotype must have different forms of surface configuration, since biotype 2 cells did not lose their pathogenicity after they were treated with bacteriocin 84. However, to understand how bacteriocin acts upon sensitive cells of different biotypes, further work on the purification of bacteriocin 84 and on its precise reaction on the sensitive cells needs to be carried out.

Bacteriocin 84 was detected ~~in vitro~~ ^{any} cultures in which strain 84 was growing alone or with ~~either~~ of pathogenic strains 24, 152 or 27, but not in cultures in which strain 84 was growing with pathogenic strain 10 or nonpathogenic strain 147. This absence of bacteriocin 84 in the last 2 cultures could be associated with a growth inhibition of strain 84 by either strain 10 or 147. In wounded tomato stems bacteriocin 84 was, unfortunately, not detected although strain 84 grew very well. But bacteriocin 84 was detected ^{after growth of strain 84} in tomato stem extract liquid. This showed that tomato stem tissue did not ^{adversely} affect the production of bacteriocin 84 by strain 84. The reason why bacteriocin was not detected in the plants

inoculated with strain 84 could be due to the technique which might not be sensitive enough to detect the bacteriocin produced by strain 84 *in vivo*.

Since the effect of strain 84 on the growth of pathogens and nonpathogens *in vivo* coincided with that *in vitro*, and these effects *in vitro* were found to be associated with presence of bacteriocin 84 produced by strain 84 in liquid culture, it strongly suggested that the inhibitory effect of strain 84 *in vivo* was due to the production of bacteriocin 84. The above suggestion was also supported by the effect of sterile bacteriocin on the growth of pathogens and nonpathogens *in vivo* and *in vitro*. Sterile concentrated bacteriocin was found to produce the same effect as strain 84, i.e. inhibition of growth of pathogenic strains 152 and 27 but not that of pathogenic strain 10 and nonpathogenic strain 147, and the loss of pathogenicity of biotype 1 resistant cells. It also significantly depressed gall induction by strain 27 on tomato seedlings. The reason why sterile bacteriocin 84 did not prevent gall induction as completely as did strain 84, could be due to loss of activity of bacteriocin 84 by being diluted in plant cell sap.

However, sterile bacteriocin 84 was found to have a stronger effect on the inhibition of gall induction by strain 108 than the presence of strain 84 itself. The reason was simple. Bacteriocin 84 inhibited strain 108 since strain 108 was sensitive to bacteriocin 84, but strain 84 could not inhibit strain 108 as much as bacteriocin 84 did because of

its own sensitivity to bacteriocin 108 produced by strain 108. This evidence again suggested that bacteriocin 84 played a major role in biological control of crown gall by strain 84.

The inhibition of growth of the pathogenic strains by strain 84 on wounded tomato stems and in liquid cultures seemed to be due to a reduction in the rate of normal increase of pathogenic cells rather than a killing of the original cells since appreciable numbers of viable cells which were higher than the number of initially inoculated cells, were recorded. This suggested that bacteriocin 84 affected cell division of sensitive cells. Roberts (1975) did not observe any effect of bacteriocin 84 on the respiration rate of sensitive cells although bacteriocin was found to reduce the increase in optical density of sensitive bacterial cultures with time. Roberts (1975), therefore, suggested that bacteriocin appeared to stop cell division without grossly affecting the energy metabolism of sensitive cells.

The simultaneous occurrence of bacteriocin production by strain 84 and the inhibition of the growth of bacteriocin-84-sensitive pathogenic strains resulting in inhibition of gall formation suggests a strong causal link between the two phenomena. If this causal link is discovered, it would give greater understanding not only of how strain 84 produces the bacteriocin which plays a major role in the biological control of crown gall by strain 84 but also ^{of} ~~on~~ the nature of the tumor-inducing principle elaborated by pathogenic strains of *A. radiobacter*. However, further research on the causal link remains to be carried out.

SECTION C

ATTEMPTS TO DISCOVER A NONPATHOGENIC STRAIN TO CONTROL BACTERIOCIN-
84-INSENSITIVE PATHOGENS

INTRODUCTION

New and Kerr (1972) reported that strain 84 almost completely prevented gall formation by 6 of 8 pathogenic isolates of both biotype 1 and 2. All controlled isolates were from South Australia; the other 2, which were not subject to biological control, were noted as non-Australian isolates. These strains which were not controlled were later observed to be insensitive to bacteriocin 84 (Kerr and Htay, 1974). Kerr and Htay (1974) reported that gall induction by 34 out of 44 pathogenic strains was completely inhibited by strain 84. The controlled pathogens were all found sensitive to bacteriocin 84. With one exception, the remaining pathogens were insensitive to bacteriocin 84. The exception was strain 108, which was sensitive to bacteriocin 84 but still formed galls. The reason was that strain 108 produced a bacteriocin to which strain 84 was sensitive (Kerr and Htay, 1974). Also another biotype 2 nonpathogenic strain 128, was found to produce a bacteriocin (128) to which strain 84 was sensitive (Kerr and Htay, 1974). Thus, the ability of strain 84 in biological control would be affected by other bacteriocin-producing strains. Such strains may occur in large number in rhizosphere soil, since soil itself is a medium already occupied by an enormous variety of microorganisms and plant roots stimulate various members of the microflora

by producing plant exudates (Rovira, 1965; Wallace, 1958).

However, these observations of the presence of bacteriocin-84-insensitive pathogenic strains and the other bacteriocin-producing strains which affected strain 84, suggested a need to discover other nonpathogenic strains to inhibit these exceptional strains.

New and Kerr (1972) found that strain 84 was not typical of all biotype 2 nonpathogenic strains; it was the only isolate out of 7 tested that significantly inhibited gall induction by many pathogenic strains. Kerr (1974) discovered 4 other nonpathogenic strains which were as effective as strain 84 in prevention of gall induction by a pathogenic strain (strain 27). But nonpathogenic strains which were able to inhibit the pathogens that are not controlled by strain 84 have not yet been reported.

Since strain 84 itself was discovered from soil around a peach gall (New and Kerr, 1972) other selective inhibitory strains for pathogens which are not inhibited by strain 84 may also be found from soil. A large number of nonpathogenic cells of *Agrobacterium radiobacter* biotype 2 were reported to be present in rhizosphere soil around healthy as well as diseased almond trees (New and Kerr, 1972; Kerr, 1974).

In Californian soils, Schroth *et al.* (1971) found that *A. radiobacter* (nonpathogen) was more common than *A. tumefaciens* (pathogen). The ratio of nonpathogen to pathogen in 7 out of 18 soils examined by them was found to vary from 13:1 to 500:1. (Both species reported in their work should be regarded as biotype 1 strains of *A. radiobacter* as classified by Keane *et al.* (1970) since Schroth *et al.*'s medium is selective for biotype

1 strains.)

In the present study of the population of *A. radiobacter* in an infested almond orchard, Angle Vale, South Australia, biotype 1 agrobacteria were as common as biotype 2. In soil around galled almond trees, the number of biotype 1 cells was approximately 11×10^3 cells and biotype 2, 7×10^3 cells per gram of soil. The number of non-pathogenic cells in this infested soil was found to be approximately 2×10^3 cells of biotype 1 and 3×10^3 cells of biotype 2 per gram of soil. In the inter-row soil (the infected almond trees were 6.1 m apart) the number of nonpathogenic cells was 3×10^3 biotype 1 and 6×10^6 biotype 2 per gram of soil. However, no pathogenic cells were detected in the inter-row soil.

These reports on the abundance and frequent presence of non-pathogenic agrobacteria in soil prompted an investigation for other nonpathogens which would control the pathogens that strain 84 did not, through bacteriocin production.

EXPERIMENTS AND RESULTS

1. Attempted isolation of inhibitory nonpathogenic strains of *A. radiobacter* from soil

Soils from two different places were collected:

- (i) Soil around the crown portion of galled rose plants* in a rose

* No pathogenic cells of either of biotype 1 or 2 were isolated from the galls on the rose plants, although biotype 2 pathogenic cells were detected in the soil. The reason could be that the pathogenic cells lost pathogenicity in the gall tissue or they were of another biotype which did not grow on Schroth *et al.*'s or New and Kerr's media (Kerr, personal communication). However, further study of the ecology of crown gall on rose plants is needed.

nursery, Elizabeth, South Australia;

(ii) Soil around a galled almond tree in an infested orchard, Angle Vale, South Australia.

Soil dilutions were prepared from freshly collected soil samples and the appropriate dilutions (10^{-2} , 10^{-3} and 10^{-4}) were plated on New and Kerr's and Schroth's selective media. The plates were incubated at 25° for 6 days. Randomly chosen isolates of colonies that grew on the plates were tested for 3-ketolactose and malonate utilization to confirm whether they were biotype 1 or 2. One hundred isolates of each biotype were separately inoculated into tomato seedlings to test their pathogenicity.

Seven biotype 1 and 10 biotype 2 isolates were randomly chosen from those which did not cause galls on tomato seedlings. Each isolate was mixed with pathogenic strain 10, which was not inhibited by strain 84, at 1:1 ratio and inoculated into 5 tomato seedlings. All isolates failed to inhibit gall induction by pathogenic strain 10. All these 17 nonpathogenic isolates and 93 of the remaining soil isolates of both biotype (45 biotype 1 and 48 biotype 2) were tested and found not to produce a bacteriocin against pathogenic strain 10 or 6, strains which are insensitive to bacteriocin 84.

Failure to discover an inhibitory strain for the pathogenic strains 6 and 10 led me to consider other possible inhibitory effects of strain 84 which has been introduced into the field. There is the possibility that strain 84 might be able to compete with the bacteriocin-84-insensitive pathogenic isolates for nutrients or infection sites.

Numerous workers have reported that inhibition of gall induction by different nonpathogenic isolates was due to their competition with pathogens for infection sites (Kerr, 1969; Lippincott and Lippincott, 1969; Schilperoort, 1969). Other diseases have also been found to be protected by saprophytes or nonpathogens which successfully competed with the pathogen for nutrient or space. For instance, infection by the pathogen *Pseudomonas tolaasii* which causes brown blotch disease in mushrooms, was effectively prevented by several saprophytes isolated from soil and peat. This prevention was achieved by Nair and Fahy (1972) who inoculated a mixture of saprophyte to pathogen at 80:1 ratio, to mushroom beds. Because there was no direct effect of saprophytes on the pathogen either by lysis, or inhibition of growth, they suggested that a possible mode of action of the saprophytes was competition for nutrients. This seemed particularly likely with one of their saprophytes, *Ps. fluorescens* C₁₂, which could be only distinguished from the pathogen *Ps. tolaasii* by its inability to brown mushrooms.

This report agreed with Lincoln's (1940) suggestion, that the disease severity resulting from inoculating with a given culture was related to relative proportion of virulent to avirulent cells in the inoculum. This was supported by Averre III and Kelman (1964) who achieved marked reduction of disease severity of the wilt pathogen *Ps. solanacearum* by inoculating tobacco, tomato and egg plant seedlings with an inoculum containing avirulent to virulent cells at a 95:5 ratio.

This evidence of successful competition of soil saprophytes led to an investigation of a possible competitive effect of strain 84 on crown gall pathogens not inhibited by bacteriocin 84.

2. Effect of various proportions of cells of strain 84 on the inhibition of gall induction by pathogenic strains 6 and 10

Mixtures of strain 84 and pathogenic strain 6 were prepared at ratios of 0:1, 1:1, 5:1, 12:1 and 70:1. Tomato seedlings were separately inoculated with each of the mixtures. Pathogenic cell number was approximately 10^5 cells per wound in all treatments. Plants were sub-irrigated and kept at 25°C in the glasshouse for 5 weeks. Seven out of 10 plants which were inoculated with the combination at 70:1 ratio were found without galls. The galls on the other 3 plants inoculated with this combination (70:1) and all those inoculated with 12:1 were significantly smaller than those on other plants inoculated with other combinations (Table 15).

This experiment was repeated using pathogenic strain 10. Here the ratios were 0:1, 1:1, 30:1 and 180:1. Five of 10 plants, which were inoculated with the combination 180:1 had no galls at their inoculation sites. The five other plants of the same treatment had significantly smaller galls than the other treatments (Table 15).

The above results showed that a high proportion of nonpathogenic cells of strain 84 gave a significant inhibition of gall induction of strains 6 and 10. However, it was wondered whether strain 84 was the only nonpathogen which had this competitive effect on these pathogens, strains 6 and 10. To clarify this the following experiment was conducted.

Table 15. Effect of various ratios of nonpathogenic (strain 84) to pathogenic (strains 6 and 10) cells on the prevention of gall induction on wounded tomato seedlings.

Strains (nonpathogenic and pathogenic)	Ratio of nonpathogenic to pathogenic cells	No. of plants with galls	Mean diameter of galls (mm)
84 and 6	0 : 1	10/10	4.3
	1 : 1	10/10	3.8
	5 : 1	10/10	3.6
	12 : 1	10/10	2.7**
	70 : 1	3/10	0.2**
L.S.D. P=0.01, 0.9			
84 and 10	0 : 1	10/10	3.9
	1 : 1	10/10	3.7
	30 : 1	10/10	3.1
	180 : 1	5/10	0.9**
L.S.D. P=0.01, 1.2			

3. Effect of high ratios of nonpathogenic (strains 84, 130, 147 and 128) to pathogenic (strains 6 and 10) cells on the prevention of gall induction on tomato seedlings

The procedure was the same as in the preceding experiment. Eight tomato seedlings were separately inoculated with each of the mixtures of nonpathogenic strain 84, 130, 147, 128 or combined 84 and 128, and pathogenic strain 6 or 10 at ratios of 0:1, 70:1 and 180:1. Seedlings were sub-irrigated. After 5 weeks incubation in a 25° glasshouse, the diameter of the galls developed at the inoculation sites was measured.

The data showed that all nonpathogenic strains had significant effect on the reduction of the size of galls induced by either pathogenic strain 6 or 10 at both ratios, 70:1 and 180:1 (Table 16). However, strain 84 seemed to compete with the pathogens better than the other strains since it completely prevented gall formation by either strain 6 or 10 on some plants (Table 16). Combined cells of strain 84 and 128 also significantly inhibited the gall induction by either strain 6 or 10. The data suggested that the interaction between these two bacteriocin-producing nonpathogenic strains (84 and 128) did not markedly influence their competition with pathogenic strains.

Table 16. Effect of high ratios of nonpathogenic (strains 84, 130, 147 and 128) to pathogenic (6 and 10) cells on the prevention of gall induction on wounded tomato seedlings.

Strains (nonpathogenic and pathogenic)	Ratio of nonpathogenic to pathogenic cells	No. of plants with galls	Mean diameter of galls (mm)
Nil + 6	0 : 1	8/8	4.77
84 + 6	70 : 1	5/8	1.00**
"	180 : 1	4/8	0.79
130 + 6	70 : 1	8/8	2.22
"	180 : 1	7/8	1.57
147 + 6	70 : 1	7/8	1.47
"	180 : 1	7/8	1.16
128 + 6	70 : 1	8/8	2.57
"	180 : 1	"	2.25
(128+84) + 6	(70+70) : 1	"	1.79
L.S.D. P=0.01, 1.08			
Nil + 10	0 : 1	8/8	6.13
84 + 10	70 : 1	"	2.32**
"	180 : 1	4/8	1.13
130 + 10	70 : 1	8/8	2.35
"	180 : 1	"	1.25
147 + 10	70 : 1	"	4.63
"	180 : 1	"	2.57
128 + 10	70 : 1	"	3.72
"	180 : 1	"	2.41
(128+84) + 10	(70+70) : 1	"	2.38
L.S.D. P=0.01, 1.34			

DISCUSSION

One of the factors which would interfere with the biological control of crown gall by strain 84 is the presence of bacteriocin-84-insensitive pathogenic strains, since strain 84 operates through the production of bacteriocin. However, high proportions of cells of strain 84 gave a significant inhibition of gall formation by pathogenic strains 6 and 10, both of which are insensitive to bacteriocin 84. This effect of high ratios of nonpathogenic to pathogenic cells on the prevention of gall formation seemed to be a competitive effect of nonpathogenic cells with pathogenic cells for infection sites. In nature, pathogenic cells in rhizosphere soil come into contact with the surface of roots and penetrate wounds or the loose cells of lenticels of the crowns and roots of susceptible plants. These wounds and lenticels could be blocked by nonpathogenic cells established on the root surface. Thus, inoculation of seeds or roots with high concentrations of cell suspension of strain 84, before the seeds or roots are in contact with the infested soil should give some protection even against bacteriocin-84-insensitive pathogenic strains. Gall formation by strains 6 or 10 was also found to be significantly inhibited by high proportions of cells of other nonpathogenic strains such as 130, 147 or 128. This suggested that the competitive effect of high proportion of strain 84 was not a special effect of this strain but it is normal for nonpathogens to compete with pathogens.

The fact that nonpathogenic strain 84 is selectively effective in control of many pathogens of biotype 1 and 2 suggests that there could be selective inhibitory strains for strains 6 and 10. However, limited

attempts to discover such inhibitory strains for strains 6 and 10 in two South Australian soils tested were unsuccessful. This leads to the suggestion that perhaps such inhibitory strains are most likely to be discovered in soils of other countries since strains 6 and 10 are overseas isolates. A third biotype of *A. radiobacter* in Greek soils has been reported by Panagopoulos and Psallidas (1973). It would be worth investigating the biological control of crown gall by nonpathogenic strains of the third biotype.

APPENDIX 1

FORMULAE AND PREPARATION OF MEDIA

Yeast-Mannitol Agar (YM Agar)

K_2HPO_4	0.5 g
$MgSO_4$	0.2 g
NaCl	0.2 g
$CaCl_2$	0.2 g
$FeCl_3$	0.01 g
Yeast extract	1.0 g
Mannitol	10.0 g
Agar	15.0 g
Distilled water	1 litre

Selective Medium for *A. radiobacter* biotype 2 (New and Kerr, 1972)

Erythritol	5.0 g
$NaNO_3$	2.5 g
KH_2PO_4	0.1 g
$CaCl_2$	0.2 g
NaCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
FeEDTA (0.65%)*	2.0 ml
Biotin	2.0 µg
Agar	18.0 g
Distilled water	1 litre

1N NaOH to pH 7 (approximately)

The basal medium is autoclaved and cooled to 50-55°, then 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

APPENDIX 1 (continued):

FeEDTA (0.65%) Solution*:

FeSO ₄ ·7H ₂ O	278 mg
Na ₂ EDTA	372 mg
H ₂ O	to 100 ml

Selective medium for *A. radiobacter* biotype 1 (Schroth *et al.*, 1965)

Mannitol	10 g
NaNO ₃	4 g
MgCl ₂	2 g
Calcium propionate	1.2 g
Mg ₃ (PO ₄) ₂ ·6H ₂ O	0.1 g
NaHCO ₃	0.075 g
MgCO ₃	0.075 g
Agar	20.0 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 1 (continued)Mannitol-Glutamic Acid Medium (MGA)

Mannitol	10 g
Glutamic acid	2 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
Biotin	2 μ g
Distilled water	1 litre

1N NaOH to pH 7

Mannitol-Ammonium Sulphate Medium

The ingredients are the same as in MGA medium but 1 g of ammonium sulphate was used instead of 2 g of glutamic acid. This medium is buffered by adding 10 ml of 0.2M sodium phosphate buffer at pH 7* to 90 ml of the medium at pH 7.

0.2M Sodium Phosphate Buffer at pH 7*

Solution A.	Na_2HPO_4	-	28.4 g/litre distilled water
B.	NaH_2PO_4	-	31.2 g/litre distilled water

Solutions A and B mixed at a proportion of 61:39 respectively.

APPENDIX 1 (continued)Stonier's Medium (Stonier, 1960b)

CaSO_4	0.1 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
NaCl	0.2 g
NH_4NO_3	2.7 g
Potassium citrate	10.0 g
Glutamic acid	2.0 g
NaH_2PO_4	0.3 g
K_2HPO_4	0.9 g
$\text{Fe}(\text{NO}_3)_3$	5.0 mg
MnCl_2	0.1 mg
ZnCl_2	0.1 mg
Biotin	2 μg
Distilled water	1 litre

1N NaOH to pH 7.

For solid medium, agar (15 g) was added.

APPENDIX 2.1. pH Changes of Cultures of Nonpathogenic and Pathogenic Strains of *A. radiobacter* in Stonier's liquid medium.

Strain	pH of the culture at (days)				
	0	1	2	3	7
84 (nonpathogen)	6.7*	6.8	7.7	8.0	8.8
24 (pathogen)	6.7	6.8	7.8	8.3	8.8
24 + 84	6.7	6.7	7.3	8.2	8.8
152 (pathogen)	6.7	6.8	7.8	8.2	8.2
152 + 84	6.7	6.8	7.5	8.2	8.6
147 (nonpathogen)	6.7	6.8	7.8	8.2	8.6
147 + 84	6.7	6.8	7.7	8.0	8.3
10 (pathogen)	6.7	6.7	7.5	8.4	8.7
10 + 84	6.7	6.7	7.4	8.3	8.8
27 (pathogen)	6.8	6.8	7.6	8.0	9.0
27 + 84	6.8	6.8	7.3	8.0	8.9

* Figures represent means of 2 replicates.

APPENDIX 2.2. pH Changes of Cultures of Nonpathogenic and Pathogenic Strains of *A. radiobacter* in Stonier's Liquid Medium.

Culture	pH of cultures at (days)				
	0	1	2	3	7
84	6.7*	6.7	7.2	8.2	9.0
108	6.7	6.7	7.3	8.2	9.0
128	6.7	6.7	7.2	8.2	9.0
108 + 84	6.7	6.7	7.4	8.0	9.0
108 + 128	6.7	6.7	7.6	8.3	9.0
108 + 128 + 84	6.7	6.7	7.6	8.2	9.0
128 + 84	6.7	6.7	7.3	8.2	9.0

* Figures are means of 2 replicates.

APPENDIX 2.3. pH Changes of Cultures of Nonpathogenic and Pathogenic Strains of *A. radiobacter* in Stonier's Liquid Medium.

Culture	pH of the culture at (days)				
	0	1	2	3	7
152 + medium	6.7*	6.7	6.7	6.7	8.0
152 + bacteriocin	7.2	7.5	7.5	7.5	8.0
147 + medium	6.7	6.7	7.3	7.5	8.7
147 + bacteriocin	7.2	7.6	7.6	7.9	8.7
10 + medium	6.7	6.7	7.8	8.1	8.2
10 + bacteriocin	7.2	7.2	7.3	8.1	8.9
27 + medium	6.7	6.7	7.8	8.2	8.6
27 + bacteriocin	7.2	7.3	7.8	8.4	8.8

* Figures are the means of 2 replicates.

APPENDIX 3. PUBLICATIONS

Kerr, A., and Htay, Khin (1974).

Biological control of crown gall through bacteriocin production.

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Biological control of crown gall: Seed and root inoculation.

Journal of Applied Bacteriology 37 : 525-530.

Biological control of crown gall through bacteriocin production

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(Accepted for publication June 1973)

Biological control of crown gall through bacteriocin production

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Crown gall caused by *Agrobacterium radiobacter* var. *tumefaciens* can be controlled biologically by a closely related non-pathogenic bacterium, *A. radiobacter* var. *radiobacter*. The control mechanism operates through the production of a bacteriocin by the controlling organism. With one exception, pathogenic strains sensitive to the bacteriocin are effectively controlled. The exception is a strain which produces another bacteriocin that inhibits the controlling organism. Pathogenic strains insensitive to the bacteriocin produced by the controlling organism are not subject to biological control. When a bacteriocin-sensitive pathogenic strain is exposed to bacteriocin, resistant colonies develop but these are no longer pathogenic.

INTRODUCTION

An effective method of biological control of crown gall was recently reported from this laboratory [4, 6]. Basically the method involves the control of a pathogen, *Agrobacterium radiobacter* var. *tumefaciens* by a closely related non-pathogen, *A. radiobacter* var. *radiobacter*. Although the method is highly effective and is now widely used by commercial growers in Australia, the mechanism of control was not elucidated in the earlier papers. However, the available evidence indicated that it was not by competition for hypothetical infection sites as proposed by Lippincott & Lippincott [5] to explain their data on inhibition of tumour induction by non-pathogens.

This paper presents evidence that the mechanism of control is through production of a bacteriocin, a highly specific antibiotic substance, by the non-pathogen.

MATERIALS AND METHODS

Cultures

The nomenclature of Keane, Kerr & New [1] was used. Details of origin of cultures not isolated in this laboratory are given below.

All strains were freeze-dried and stored at 5 °C; those used extensively were also stored on porcelain beads [7].

A. radiobacter var. *tumefaciens* biotype 1: Strains 6 [ex W. Blyth, via D. W. Dye (A4) supplied by A. C. Parker (WU 78)], 10 [ex N.S.W. Forestry Commission (109A) supplied by A. C. Parker (WU 89)], 11 [ex R. Mushin (3)], 12 [ex R. Mushin (5)], 14, 19, 24, 31, 41 [ex W. H. Cook (B 70) supplied by A. C. Parker (WU 21)], 64, 74, 76, 78, 116 [ex R. E. Beardsley (B2AC)], 119 [ex R. E. Beardsley (B6Sm)], 120, 122, 136 (NCPPB 398), 139 (NCPPB 4), 140 (NCPPB 1001), 142 (NCPPB 925), 145 (NCPPB 396), 148, 152, 190 [ex G. Morel (T37)].

A. radiobacter var. *tumefaciens* biotype 2: Strains 26, 27, 29, 33–37, 48 (NCPPB 223), 49, 62 [ex J. DeLey (ICPB TT133)], 71 (ex L. J. Penrose), 105–108, 114, 118, 163, 166 (NCPPB 1651).

A. radiobacter var. *radiobacter* biotype 1: Strain 147.

A. radiobacter var. *radiobacter* biotype 2: Strains 84, 128, 130. Strain 84 has been lodged with the NCPPB, England.

Bacteriocin production

The method of Stonier [10] was used. The method consists of spot-inoculating a test strain on to a defined nutrient agar medium and incubating for 2 days; the test strain is then killed by chloroform and an indicator strain spread over the plates to form a continuous lawn. Bacteriocin production is indicated by a zone of inhibition in the bacterial lawn around the test strain.

Cycloheximide (250 parts/million) was added to the medium to inhibit growth of fungal contaminants.

Individual bacteriocins are referred to by the number of the producer strain. Thus bacteriocin 84 is the bacteriocin produced by strain 84.

Biological control

Tomato seedlings were inoculated as described by New & Kerr [6]. Plants were wounded with a blunt rod and half were inoculated with a pathogen alone and half with the same pathogen mixed with a non-pathogen in the ratio of 1 : 1. Approximately 10^6 cells of a pathogen were added to each wound.

Selective medium

The selective medium of Schroth, Thompson & Hildebrand [9] was used. Plates were poured 2 days before required and 0.1 ml of a bacterial suspension placed on the agar surface and spread with a sterile L-shaped glass rod. Plates were incubated at 27 °C and colonies counted after 5 days.

RESULTS

Specificity of biological control and of bacteriocin sensitivity

New & Kerr [6] reported that strain 84 controlled six out of eight pathogens tested. This work was extended by testing strain 84 against 44 pathogenic strains. With one exception, results were never equivocal; isolate 84 either did or did not control any particular strain. The exception was strain 142 which induced very slow-growing galls when mixed with strain 84 and fast-growing galls when inoculated by itself.

The same 45 pathogenic strains were tested for sensitivity to bacteriocin 84. Results are presented in Table 1.

There is a very high correlation between biological control of pathogenic strains by strain 84 and the sensitivity of these strains to bacteriocin 84. Even strain 142 gives an intermediate reaction in both tests. The only exception is strain 108 which induces tumours when mixed with strain 84 but is sensitive to bacteriocin 84. It is possible that strain 108 produces a bacteriocin which inhibits strain 84. This was tested and proved to be true. Seven other pathogenic strains, 19, 24, 27, 31, 122,

148 and 152, all sensitive to bacteriocin 84 and subject to biological control by strain 84, were tested for production of bacteriocins able to inhibit strain 84. No inhibition was detected.

New & Kerr [6] reported that only one non-pathogenic strain of *A. radiobacter* out of seven tested, inhibited gall induction by pathogens. Two of the non-inhibiting strains, 128 and 130, were tested for bacteriocin production, using selected strains as indicators. Results are presented in Table 2. Strain 128 produces bacteriocin 128 which inhibits strains 84 and 130 but does not inhibit any of the pathogenic indicator strains. There was no evidence that strain 130 produced a bacteriocin.

TABLE 1
Biological control of 44 pathogenic strains by strain 84, and their sensitivity to bacteriocin 84

Strain	Biotype	Biological ^a control	Bacteriocin 84 ^b sensitivity	Strain	Biotype	Biological ^a control	Bacteriocin 84 ^b sensitivity
6	1	-	-	74	1	+	+
10	1	-	-	76	1	+	+
11	1	+	+	78	1	+	+
12	1	+	+	105	2	+	+
14	1	+	+	106	2	+	+
19	1	+	+	107	2	+	+
24	1	+	+	108	2	-	+
26	2	+	+	114	2	+	+
27	2	+	+	118	2	+	+
29	2	+	+	119	1	-	-
31	1	+	+	120	1	+	+
33	2	+	+	122	1	+	+
34	2	+	+	136	1	-	-
35	2	+	+	139	1	-	-
36	2	+	+	140	1	-	-
37	2	+	+	142	1	±	sl.
41	1	-	-	145	1	-	-
48	2	+	+	148	1	+	+
49	2	+	+	152	1	+	+
62	2	+	+	163	2	+	+
64	1	+	+	166	2	+	+
71	2	+	+	190	1	+	+

^a + = subject to biological control; - = not subject to biological control; ± = intermediate reaction.

^b + = sensitive; - = resistant; sl. = slightly sensitive.

TABLE 2
Bacteriocin production by four test strains as indicated by the sensitivity of 15 indicator strains^a

Test strains	10	19	24	27	31	84	116	119	122	128	130	139	145	148	190
84	-	+	+	+	+	-	-	-	+	-	-	-	-	+	+
128	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
130	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
190	-	+	+	-	+	-	+	+	+	-	+	-	+	+	-

^a + = sensitive; - = resistant.

Strain 190 was also tested against the same indicators. Strain 190 was supplied by G. Morel as strain T37 which was used by Stonier [10] who showed that it produced a bacteriocin that affected a large number of strains of *A. radiobacter*. The results show (Table 2) that bacteriocin 190 is different from bacteriocins 84 and 128.

Although the zones of inhibition resulting from bacteriocin action were always clearly defined, some bacterial growth within the zones occurred (Plate 1), indicating that resistant bacteria quickly developed. This seemed surprising since Kerr [4] reported that biological control was evident several months after inoculating peach seeds with strain 84. If resistant bacteria quickly develop, control should be only transient. This was investigated.

Bacteria both from within and without the inhibition zones of indicator strains 11, 19, 24, 120, 122, 148 and 152 were suspended in sterile distilled water and streaked on to lactose agar plates. Individual colonies that developed were sub-cultured on yeast-mannitol agar slopes and tested for pathogenicity [2]. Ten colonies from each strain were tested, five originating from within and five from without the inhibition zone. Results are presented in Table 3. With two exceptions, bacteria isolated from inside the zone of inhibition were non-pathogenic whereas 34 of the 35 isolates from outside were pathogenic. The exceptions, re-isolates from strains 19 and 152, were tested for sensitivity to bacteriocin 84. Both were resistant. The one non-pathogenic isolate, from outside the zone of inhibition, was also tested against bacteriocin 84. It was sensitive. All other re-isolates were tested for resistance to bacteriocin 84. All pathogens were sensitive, all non-pathogens resistant.

TABLE 3
Pathogenicity of bacteria isolated from inside and outside zones of inhibition resulting from action of bacteriocin 84. Number of isolates pathogenic (out of 5)

Isolated from	Strains						
	11	19	24	31	122	148	152
Within zone of inhibition	0	1	0	0	0	0	1
Outside zone of inhibition	5	5	4	5	5	5	5

Interactions on tomato plants

It is clear that there is a very high correlation between the biological control of pathogenic strains by strain 84 and the susceptibility of these strains to bacteriocin 84. Although it is tempting to assume a causal relationship, important supporting evidence would be provided if it could be shown that growth inhibition of pathogens occurred on a host plant.

To measure numbers of a pathogenic strain of *A. radiobacter* when it is mixed with a non-pathogenic strain of the same species, it is necessary to use two different biotypes and a selective medium to inhibit the non-pathogen. Strain 84 is the isolate used in biological control and as it belongs to biotype 2, it was necessary to use biotype 1 pathogens and the selective medium of Schroth *et al.* [9].

Pathogenic strain 24, which is controlled by strain 84 and sensitive to bacteriocin 84, was mixed separately with strain 84 and with strain 130, a non-pathogen ineffective

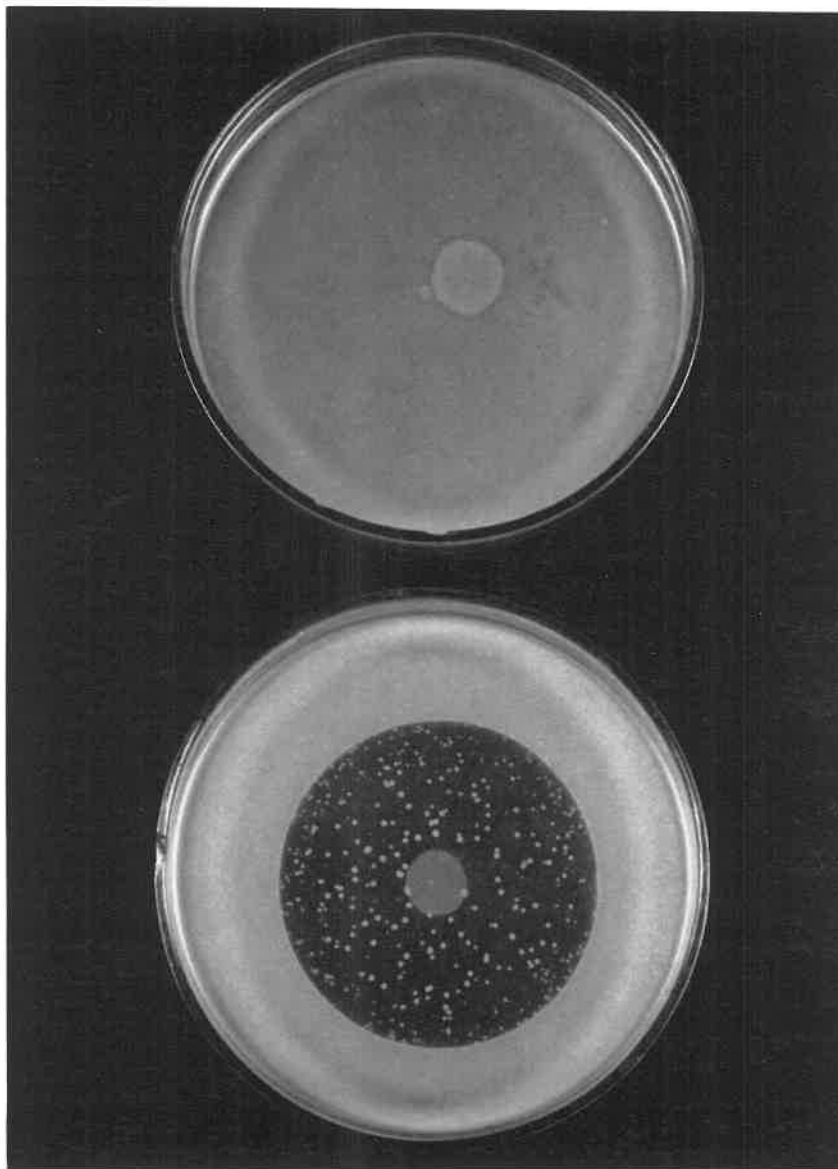


PLATE 1. Indicator strains resistant (top) and sensitive (bottom) to bacteriocin 84. Strain 84 is in the centre of both plates. Colonies resistant to bacteriocin can be seen within the zone of inhibition of the sensitive strain.

[facing page 40]

in biological control and which does not produce bacteriocin. Tomato seedlings were inoculated with strain 24 alone and also with the same strain mixed with the two non-pathogenic strains. There were 15 replicates of each treatment. At various times after inoculation, the stems of three tomato seedlings from each treatment were cut 1 cm above and 1 cm below the site of inoculation and the 2-cm lengths of stem macerated separately in a mortar and pestle with 10 ml sterile distilled water. After dilution, 0.1 ml was plated on Schroth, Thompson & Hildebrand's selective medium. Results are given in Fig. 1. It is clear that the pathogenic strain is markedly inhibited by strain 84 but not by strain 130.

In another experiment, strain 10 which is not subject to biological control and is not susceptible to bacteriocin 84 was mixed with strain 84. Tomato seedlings were inoculated with strain 10 alone and also with the mixture. The method of assessment was the same as in the previous experiment. Strain 10 was only slightly inhibited by strain 84, no more than would be expected from competition for nutrients (Fig. 2).

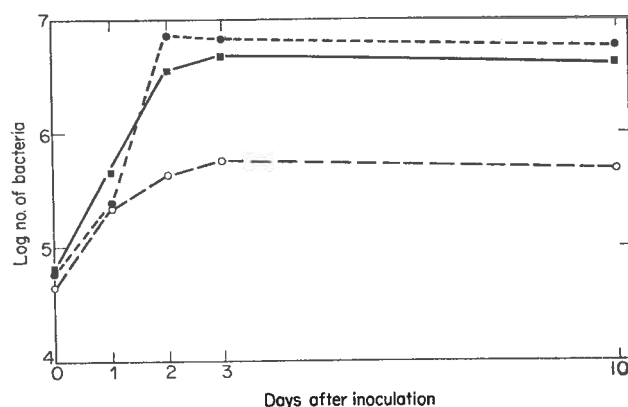


FIG. 1. Numbers of strain 24 following inoculation of tomato seedlings: (■—■) strain 24 alone; (○—○) strain 24 mixed with strain 84; (●—●) strain 24 mixed with strain 130.

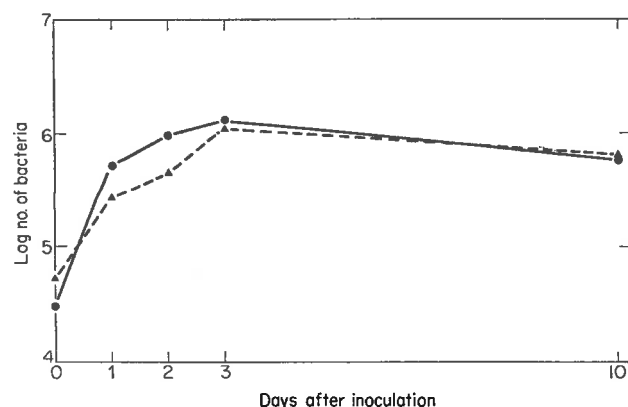


FIG. 2. Numbers of strain 10 following inoculation of tomato seedlings: (●—●) strain 10 alone; (▲—▲) strain 10 mixed with strain 84.

These results support the hypothesis that biological control of crown gall operates through production of bacteriocin 84. It could be argued, however, that the combination of strains 24 and 84 was the only treatment that did not lead to tumour induction. Perhaps this is why numbers of pathogens are restricted and not because of bacteriocin production. This was investigated.

Some non-pathogenic strains of *A. radiobacter* can acquire virulence [3]. Strain 147 is non-pathogenic and strain 152 is the same strain after it has acquired virulence from strain 27, a biotype 2 pathogen. Strains 147 and 152 cannot be distinguished serologically, by enzyme patterns or by many other tests (unpublished data of W. P. Roberts), but strain 152 is pathogenic and strain 147 is not. Tomato seedlings were inoculated with these two strains separately and also with each strain mixed with strain 84. Numbers were measured as in the previous experiments and results presented in Fig. 3.

Over the 10-day period when measurements were taken, there was no significant difference in numbers of the non-pathogen, strain 147 and the pathogen, strain 152, indicating that lack of gall development does not lead to a marked decrease in numbers of *A. radiobacter*. Strangely enough, the pathogen was more markedly affected by the presence of strain 84 than was the non-pathogen ($P < 0.01$), suggesting that they might differ in sensitivity to bacteriocin 84.

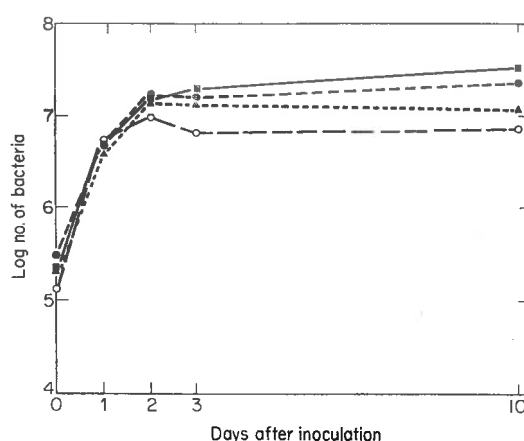


FIG. 3. Numbers of strains 147 and 152 following inoculation of tomato seedlings: (●—●) strain 147 alone; (▲---▲) strain 147 mixed with strain 84; (■—■) strain 152 alone; (○---○) strain 152 mixed with strain 84.

DISCUSSION

There can be little doubt that biological control of crown gall operates through the production of bacteriocin 84 and through the sensitivity of pathogens to it. With one exception, all pathogens sensitive to bacteriocin 84 are subject to biological control by strain 84. The exception is strain 108 which produces another bacteriocin, 108, that inhibits strain 84. Non-pathogens like strains 128 and 130, which apparently do not produce bacteriocins which inhibit pathogens, are ineffective as agents of biological control. Similar results were obtained following inoculation of tomato plants with various combinations of pathogen and non-pathogen.

Not only does bacteriocin 84 inhibit a large number of pathogens but most bacteria that develop resistance to it are no longer pathogenic. So if strain 84 is established around the roots and crown of a plant, it is likely to inhibit gall induction for a long period. It is possible that under these conditions strains like 108 will have a selective advantage and increase in numbers. It remains to be seen how long this process will take. However, as the mechanism is now known, it should not be difficult to select bacteriocin-producing non-pathogens that are insensitive to bacteriocin 108.

The problem of controlling pathogens that are insensitive to bacteriocin 84 remains. This does not seem to be an important economic problem in Australia because of 30 Australian pathogenic strains tested, 29 were sensitive; only strain 10 was resistant. However, 7 of the 14 overseas strains tested were resistant to bacteriocin 84 and were not subject to biological control. It seems likely that strains of *A. radiobacter* will be found to produce a wide array of bacteriocins. In this work, bacteriocins 84, 108, 128 and 190 are quite distinct and Stonier [10] has reported the production of several bacteriocins but provided details of only one. It should be possible to isolate bacteriocin-producing strains which inhibit the pathogens resistant to bacteriocin 84.

There appear to be two quite distinct types of pathogen; those sensitive to bacteriocin 84 and those resistant to it. The reason for this is not clear but other workers have divided crown gall pathogens into two types. For example Petit *et al* [8] divide them into octopine inducers and nopaline inducers. It would be of interest to know if the induction of the two guanidines is correlated with sensitivity to bacteriocin 84.

One of the most interesting aspects of the work is that, within the group sensitive to bacteriocin 84, there is a very high correlation between pathogenicity and sensitivity to bacteriocin 84. Resistance to bacteriocin 84 entails loss of pathogenicity and there is some evidence from the tomato inoculation experiments that a change from non-pathogen to pathogen involves a change from bacteriocin 84 resistance to sensitivity. We are aware of the likely significance of this in the mechanism of tumour induction, and this aspect of the work is being investigated.

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NOTE:

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