

'THE EPIDEMIOLOGY AND BACTERIOLOGY
OF AGROBACTERIUM TUMEFACIENS'

by

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ABSTRACT

Studies on the epidemiology of crown gall in an F12/1 cherry layer bed showed that there was a rapid and progressive increase in disease incidence over three years. Certain parts of the layer bed system e.g. the bends on one-year-old layers and, particularly, shoot scars on two-year-old layers were highly susceptible to infection. Crown gall introduced into blackberry plantations with the planting material spread from the original infection foci to all bushes within 5 years.

In an attempt to resolve the controversy of whether or not the presence of galls was detrimental to tree growth, field data were collected from over 1200 trees of up to 8 years' old but no consistent or major effects of galls on tree growth were found. There was likewise no evidence that crown gall decreased either cherry layer bed productivity or blackberry crop. Crown gall therefore remains chiefly a nursery problem of a cosmetic nature.

Isolates of the pathogen from cherry, blackberry and loganberry were compared in standard biochemical, physiological and pathogenicity tests, together with studies of their bacteriophage and bacteriocin relationships. The cherry and loganberry isolates formed a homogeneous group conforming to biotype 2 while the blackberry isolates were of both biotypes 1 and 2 and also intermediate forms.

Disease susceptibility and rate of gall development were maximal, and the latent period minimal, during the

growing season. When the data were related to cultural operations in the field it was found that while cuts on cherry were performed at times when risk of infection was low, those on blackberry, especially summer pruning of laterals, involved considerable risk of spreading infection.

Experiments on cultural and chemical methods of control of crown gall on cherry proved ineffective; there was often a higher incidence of disease on treated stocks than on the undipped controls. Crown gall infection was effectively controlled when the antagonistic A. radiobacter, strain 84, was applied simultaneously to cherry leaf scars. The prospects for such biological control of crown gall in the field in England are discussed.

PREFACE

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INTRODUCTION

Crown gall disease is widespread in temperate and subtropical regions but is rarely reported from tropical areas (Raabe, 1964). A wide range of herbaceous and woody dicotyledonous plants are susceptible, Elliot (1951) lists 142 genera, but the Papilionaceae and monocotyledons are seldom, if ever, attacked (Tamm, 1954). Fruit crops on which the disease has been recorded include stone fruits (almond, apricot, cherry, peach and plum), pome fruits (apple, pear, quince), cane fruits (blackberry, loganberry, raspberry) and also grapes and hops.

Toumey (1900) first described the disease and later demonstrated its infectious nature by inoculating parts of galls into healthy plants. The pathogen is a soil-inhabiting organism which enters the host plant through wounds, normally at or below soil level, i.e. the crown, where it stimulates the surrounding cells to enlarge and divide to form an undifferentiated mass of parenchymatous tissue which develops into a macroscopically visible gall in as little as 4-10 days or in more than a year depending on the plant host and time of infection. Young galls are small, pale, soft and smooth but enlarge, darken and become hard, irregular and often deeply fissured on ageing especially on woody plants; on Rosaceae galls have been found weighing more than 40 kg. The galls develop during the growing season but in autumn and winter rot either completely or partially; new gall tissue may subsequently develop on another part of, or immediately adjacent to, the old gall.

a. The causal organism

The causal bacterium was first isolated by Smith & Townsend (1907) from the Paris daisy (Chrysanthemum frutescens) and they named it Bacterium tumefaciens. It was subsequently renamed Pseudomonas tumefaciens (Smith & Townsend) Duggan (1909); Phytomonas tumefaciens (Sm. & Towns.) Bergey (1923) and Bacillus tumefaciens (Sm. & Towns.) Israilsky (1926). It was assigned to the genus Agrobacterium by Conn in 1942 and for many years was known as Agrobacterium tumefaciens. However, within the last decade, there have been four proposals to change the name yet again following studies on DNA homology, GC ratios, numerical taxonomy etc. The close relationship between the saprophytic A. radiobacter and the pathogen of crown gall led De Ley, Bernaerts, Rassel & Guilmot (1966) to assign both to the species A. radiobacter and to recognise the pathogen by the varietal epithet tumefaciens. The Australian workers Keane, Kerr & New (1970) also considered there should be this one species but they designated the saprophytic and pathogenic forms var. radiobacter and var. tumefaciens respectively. However, they further distinguished two biotypes, which cut across the varietal divisions, which differed in a small number of biochemical tests. Other workers consider that the differences between Rhizobium and Agrobacterium are not sufficiently great to merit separate generic status.

Thus, White (1972) on the basis of numerical taxonomy concluded, in agreement with Heberlein, De Ley & Tjittgat (1967), that the crown gall pathogen should be named Rhizobium radiobacter 'state tumorigenic' and the non-pathogenic form be distinguished from it by 'state saprophytic'.

The close taxonomic relationships between the saprophytic form, the causal organisms of crown gall and hairy root and the fast-growing rhizobia are clear. However, the plant pathologist is not primarily concerned with taxonomic problems but with the fact that differences exist which are demonstrable by routine pathogenicity tests and are manifest in the field. The recognition of the pathogen of crown gall from its related forms by simple laboratory tests, for many years an unfulfilled and seemingly unattainable aim, may now be possible through the utilisation of octopine or nopaline (Lippincott, Heiderbeck & Lippincott, 1973) although these substances have not yet been very widely tested and the correlation between their utilisation and pathogenicity, though high, is not absolute.

Lack of standardisation between tests and materials used by different workers undoubtedly accounts for some of the variation reported among isolates of the crown gall organism. However, the existence of four different physiologic races, or pathotypes, in England was reported by Wormald (1945) the two from weed hosts having a wider host range than those from apple and tomato. Two serological groups were distinguished among 15 Egyptian

isolates (El Helaly, El Dahab & El Nil, 1969); one group comprised serologically related isolates having a wide host range and the other group, of serologically unrelated types, had a very restricted host range. Lippincott & Lippincott (1967) noted differences in host range and virulence in 35 strains of A. tumefaciens and concluded those with restricted host range, or reduced virulence on many hosts, were the more nutritionally exacting strains. The existence of two biotypes, which differed in certain biochemical tests, among stone fruit isolates was reported by Keane et al. (1970) and these two types and also a third or intermediate type have been found to occur in Greece (Panagopoulos & Psallidas, 1973).

Thus, the crown gall organism is composed of isolates which differ in pathogenicity, biochemical and serological reactions. The exact taxonomic position of these isolates is not yet stabilised and it is therefore proposed, in the interest of brevity, to adhere to the well-established name of Agrobacterium tumefaciens throughout this work.

Over the past 70 years a vast literature on the disease has accumulated. Work has centred mainly on the mechanisms of gall initiation and development because of the parallels between plant crown gall and animal cancers. Studies, mainly on greenhouse grown plants inoculated under carefully controlled conditions, have been reviewed by Braun (1954, 1957), Braun & Stonier (1958), Klein & Link (1955), De Ropp (1951) and Stapp (1958). However, studies

of the disease on field crops have been very limited and data on the epidemiology of crown gall of fruit crops, thinly scattered through the literature, is often either contradictory or inconclusive.

b. Economic importance and effect on plant growth

Crown gall is not only of academic interest as a subject of tumour growth studies and host-parasite relations but is also of agricultural importance.

The disease has been reported to be serious in the U.S.A. on stone-fruits, especially on peach, and particularly in the nursery (Siegler & Bowman, 1940; Gambrell & Gilmer, 1956). Loss of nursery stock was estimated at \$1200 per acre in Oregon in the 1950's (Young, 1954; Deep, 1958). In California where the disease has been one of the more serious, on 14 major crops an annual loss of \$6-7 million since 1963, excluding nursery losses, has been reported (Ross, Schroth, Sanborn, O'Reilly & Thompson, 1970). Ross et al., (1970) also suggested that, due to infection in early years resulting in susceptibility to strong winds, the economic life of almond orchards has been reduced by 20%.

Peach is also the host most affected by the disease in Australia where rejection of up to 80% of nursery stock has occurred in some years (Hutton, 1949; Stubbs, 1971). In the peach growing areas of Europe, too, severe crown gall infection has been reported (Khristov, 1940; Brichet, 1944; Kaskaloglu, 1949; Behr, 1958). The extent of the disease in English fruit production nurseries, the subject of a recent survey (Lelliott, 1971),

shows that crown gall is not generally severe, although there have been a few cases of serious infection.

Economic losses of pome and stone fruits due to crown gall in Europe are not well documented but it is certain that the effect of the disease on these hosts is greatest in the nursery and is probably of more widespread occurrence than is reported in the literature.

Crown gall on grape vines occurs predominantly on the aerial parts of the plant and is reported to be graft transmitted (Lehoczky, 1968; Zinca, 1967). On this host the disease has been reported to reduce yields over 3 years by 13-19% and to impair quality, depress growth by 25% and kill 10% of the vines (Malenin, 1971).

The effect of the presence of galls on tree growth is a continuing subject of controversy. Early studies suggested that growth and/or trunk diameter of galled trees was less than that of healthy ones (Greene & Nelhus, 1919; Riker, 1939) and a progressive decline in vigour, leading to the death of both young and older apple trees in Kansas, was reported by Kainski (1964). On the other hand Harris & Pearce (1938) found no difference in growth and cropping of apple trees with and without galls after 18 years' growth at East Malling while Wormald (1945) observed increased vigour of galled trees.

Crown gall also occurs on cane fruits and its severity on raspberry has destroyed the sources of certified stock in Nova Scotia (Gourley & Harrison, 1961) and curtailed raspberry growing in Kansas (Kainski, 1964). It appears

however, that the vigorously growing Himalaya Giant blackberries can be severely infected with crown gall without any apparent injurious effect (McKeen, 1954).

c. Factors favouring infection

The inability to distinguish between A. tumefaciens and the ubiquitous soil saprophyte A. radiobacter, other than on the basis of pathogenicity, has made soil population studies difficult and subject to error. Nevertheless, the pathogen has been reported to survive in the soil for more than 2 years (Patel, 1926). Increasing coarseness of texture, depth and temperature are reported to be accompanied by decreasing populations of the organism, these being more pronounced in non-sterile than in sterile soils (Dickey, 1961).

The incidence of crown gall is generally thought to be higher on alkaline than on acid soils and it has been suggested that the comparative freedom from the disease in New Zealand is attributable to the generally low soil pH of 5.5 (Atkinson, 1971). Seigler & Bowman (1940) found that the incidence of crown gall on peach seedlings in a soil of pH 8.5 was six times greater than in a soil of pH 5.8. A direct relationship between populations of A. tumefaciens and soil pH was thought to exist (Dickey, 1961) but there is clearly no simple relationship since severe infection in a fen peat of pH 5.9 has been reported (Lelliott, 1971). In this case the high moisture content of the soil was probably a contributory factor since this has long been implicated in influencing the degree of crown gall infection. Hedgecock (1910)

recorded a higher incidence of apple crown gall on a heavy, wet loam compared to a light, dry loam and more recently Kainski (1964) has shown that irrigation increases disease incidence.

Studies of soil nutrient status in relation to crown gall have shown that regimes which increase plant vigour, such as high levels of nitrogenous fertilisers, increase the disease susceptibility of plants (Link, et al. 1953; Zinca, 1967). However, where nitrogen applications are sufficiently high to reduce the availability of boron, gall size may be reduced (Hussin & Deep, 1965). The effect of potassium fertilisers, if any, is to decrease susceptibility to the disease (Hussin & Deep, 1965; Zinca, 1967). In general, plants growing vigorously under optimum conditions are more susceptible to crown gall than weaker ones (Klein, 1952; Riker & Hildebrand, 1934).

The environmental factor which has been most closely studied in relation to crown gall is temperature. Temperature and humidity optima (22°C , 60% RH) for crown gall development in herbaceous hosts were found to be slightly lower than those for optimum growth of the host ($22-30^{\circ}\text{C}$, 80% RH) while gall formation is apparently inhibited at temperatures over 30°C (Riker, 1926; Riker, Lyneis & Locke, 1941; Braun, 1947). Destruction of the tumour-inducing principle at 32°C has been propounded (Braun, 1947) while Lippincott & Lippincott, (1966) concluded, in their studies with pinto beans, that it was the bacteria which were affected by the high temperature. However, tumour initiation on mazzard cherry

roots has been demonstrated at 35°C and even up to 37°C, above which the host plant suffered damage (Deep & Hussin, 1962; 1965).

At the other end of the scale low temperature injuries to plants allowing ingress of the pathogen on vines (Henner, 1964; Raikov, 1967; Zinca, 1967) and also on stone-fruits (Kaskaloglu, 1949) are quoted as the reason for high levels of infection in years following severe winters.

In recent years increased incidence of crown gall has been reported where there were high soil populations of nematodes. Simultaneous inoculations of plants with A. tumefaciens and Meloidogyne spp. have resulted in a higher level of crown gall infection than in comparable plants inoculated with the bacterium alone (Nigh, 1966; Griffin, Anderson & Jorgenson, 1968). The exact role of nematodes, whether as vectors or merely to provide wound sites for bacterial entry, has not been determined.

d. Control

Bacterial diseases are notoriously difficult to control and, since much of the literature on field aspects of crown gall that has accumulated since the beginning of the century is devoted to this aspect, it is clear that this disease is no exception. In a review of control measures Stapp (1961) concluded "we shall not analyse them in detail because no positive, practical conclusions have yet been reached". The USDA still recognise this situation and recommend the planting of disease-free stock in pathogen-free soil since no curative treatments are generally available.

Disease control measures have ranged from cultural practices to chemical methods of prevention, such as the use of preplanting treatment of seeds, cuttings or maiden trees and soil fumigation, and cure by selective destruction of galled tissues. Within the last decade an exciting breakthrough has been made with biological control (Kerr, 1972).

In the absence of any adequate chemical control, Hutton (1949) advocated using only healthy plant material, exercising great care when grafting, and crop rotation to prevent build-up of the pathogen. Gambrell & Gilmer (1956) asserted cultural methods were the only ones available for crown gall control and recommended that fields with a history of infection should be totally avoided and others cropped with susceptible nursery stock not more frequently than triennially. Even as recently as 1970 the advice in America has been to plant only healthy, certified and inspected stock as a means of combating the disease (Ross et al., 1970). The best long term solution of the problem probably lies in the breeding of resistant rootstocks. Little appears to have been done on the relative resistance of host varieties or species to crown gall. Smith (1925) found no resistance in almond or peach but P. domestica cultivars he divided into groups of low, moderate and high susceptibility; P. umbellata, P. insititia and the St. Julien plum were all in the most resistant group. Crown gall resistance has been reported to be less than 2% in Prunus spp. (Khristov, 1940) and less than 5% in pome fruits (Wormald & Grubb, 1924; Dame, 1938). Wormald & Grubb concluded

there was no direct correlation between inherent vigour and susceptibility to crown gall in the apple rootstocks they examined. Until crown gall resistant stocks are produced by the fruit breeders, the problem of disease control must be tackled in other ways.

Some success has been claimed for chemical methods especially where mercurial compounds have been used as preplanting treatments for fruit trees (Wormald & Garner, 1945; Behr, 1958) or for seed dressing (Ark, 1942; Siegler & Bowman, 1940; Guengerich, Goodman & Millikan 1969). Common household bleach (0.5% sodium hypochlorite) was reported to be an effective treatment of peach seeds prior to stratification (Ross et al., 1970). However, the level of control attained by the many inorganic chemicals tested on a wide variety of hosts has been neither very good nor very consistent. Many antibiotics have been examined and Dickey (1965) found 6, aureomycin, terramycin, tetracycline, neomycin, novobiocin and polymixin B active against A. tumefaciens in vitro against many strains of the pathogen. Several of these had already shown some activity in greenhouse tests (Blanchard, 1951; Klemmer, Riker & Allen, 1955; Deep, 1956; Ark & Sibray, 1957; Boyle & Price, 1963). The levels of control attained on various hosts has ranged from 40% to total gall inhibition but the cost of antibiotics and regulations restricting their use agriculturally effectively prevent their use on a commercial scale.

In any new plantation, crown gall infection may be introduced within or adhering to the planting material.

Even if this is prevented by a preplanting treatment or use of 'clean' stock, freedom from disease cannot be ensured if the soil is infested with the pathogen. Eradication of the soil-inhabiting pathogen has been attempted by soil fumigation (Dickey, 1962; Deep & Young, 1965; Deep, MacNeillan & McSwan, 1968; Schroth, Weinhold, McCain, Hildebrand & Ross, 1971).

The levels of control achieved varied with the season, soil moisture and temperature, physiological state of the planting material and seasonal changes in the populations of the pathogen. In several cases the more vigorously growing plants had more galls than their less vigorous neighbours in non-fumigated land. Where the pathogen is only partially controlled or is reintroduced on planting material, the pathogenic population builds up rapidly. Of the various fumigants tested methyl-bromide-chloropicrin mixtures gave the best control but the cost and efficiency of soil fumigation has not found general acceptance.

Selective destruction of galls by painting on them petroleum products has proved successful (Ark, 1941; Charles, 1961; Dye, 1951; Schroth et al., 1970) but such treatments are practicable on only a small scale and are more likely to be of use on specimen trees rather than in general commercial practice.

The most recent and also most outstanding advance in the fight against crown gall is the development of a method of biological control. Kerr (1972) reported that a strain of A. radiobacter isolated from peach soil could, through the action of a bacteriocin it produced, inhibit growth of A. tumefaciens. (Kerr & Hay, 1974). Dipping peach seedlings in a culture of the antagonistic bacterium has resulted in successful control of peach crown gall in South Australia and has stimulated much interest elsewhere (Dhanvantari, 1976, Schroth & Noller, 1976).

SECTION I

FIELD OBSERVATIONS ON THE DISTRIBUTION AND SPREAD OF CROWN GALL

1. Crown gall in cherry layers

The rootstock commonly used for cherries in England is a Prunus avium seedling, F12/1, selected many years ago at East Malling Research Station as a vigorous stock suitable for high working with sweet cherry varieties and resistant to bacterial canker. The stock is normally propagated by layering, a technique usually adopted for plants that do not root readily. The parent layers are planted in spring at an angle of 45° and are pegged horizontally on the soil in the following January or February in a shallow trench (after risk of being lifted again by frost has diminished). Before the buds swell the layers are covered with 2-3 cm fine soil and the young developing shoots are half covered with fine soil in April. To ensure etiolation at the base of the shoots, soil is applied 2 or 3 further times during the growing season to provide a suitable environment for root development, which occurs from July onwards. In November the soil is removed and the young rootstocks are cut from the parent layers. At regular intervals a shoot is left for pegging down in the following season.

The layer bed at East Malling Research Station consisted of 12 rows of layer, each 110 m long, and laid down in 1968 from ENLA F12/1 stock on a soil of pH 7.5.

Six of these rows were used for the detailed studies of crown gall between 1970-1972. The layers are normally below soil level for 8 months, from April to November. Disturbance of soil during harvesting of stocks covers over some of the younger surface layers but is not sufficient to expose older more deeply embedded layers. Once exposed, galls become desiccated and shrink in very cold weather. To fully expose layers and record the galls is a laborious task and it must be carried out as quickly as possible after harvesting before layers are covered or galls shrivel. To accomplish the task in the time available, recording was restricted to sectors of not less than 60 m. This was considered sufficient to give data representative of the layer bed as a whole and to provide enough galls for statistical treatment of results.

Each of the sectors was divided into 1 m blocks and a record was made of the number of galls, their position and diameter, and sometimes also the age of the layer on which they were situated. The precise age of galls could not be determined but they were designated young if they were pale, smooth and fairly soft and old if they were dark, convoluted and woody. Gall sites on the layer were recorded as:-

bend, on the few cm where the layer had been bent
for pegging down

fork, where layers divided

scar, from where rooted shoots had been harvested

base, below the scar on the remaining portion of the
rooted shoot

node, where there was a cluster of roots not
associated with a shoot

end, at the broken end of a layer

mid, where there was no apparent association with
shoot scar or node

root, anywhere on the root system (Fig. 1).

Where age of layers was taken into account they were
designated:-

new, first season layers from which rootstocks had
only just been harvested

2-year, from which the first crop of rootstocks had
been harvested the previous year, leaving many
shoot scars

old, any older layers.

The majority of galls, especially when young, are
spherical but as they age they may become flattened in one
plane and all eventually become quite irregular in shape.
To simplify recording, gall diameter only was measured
which was regarded as sufficient for comparison of galls of
different ages, on various sites and layer ages.

a. Sites of infection

The numbers and diameters of total, old and young
galls were recorded on 2 sectors of the layer bed (B, E1/F1
Fig. 2; 170 m) in 1972. The data are presented in Table 1.
Taken as a whole galls are fairly evenly distributed among
the specified sites at each of which c. 20% galls occur.
However, when old and young galls are considered separately
it is seen that the numbers of old galls were greatest at

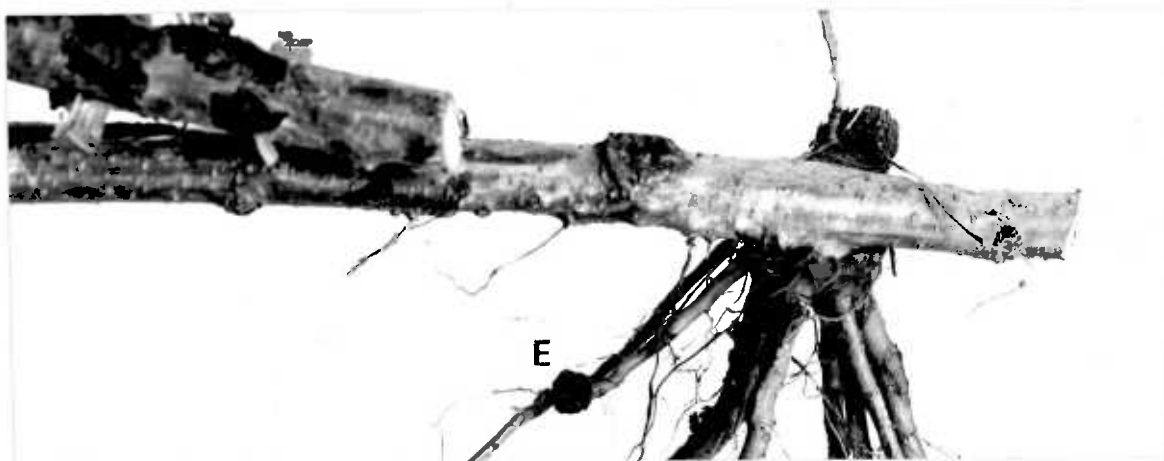
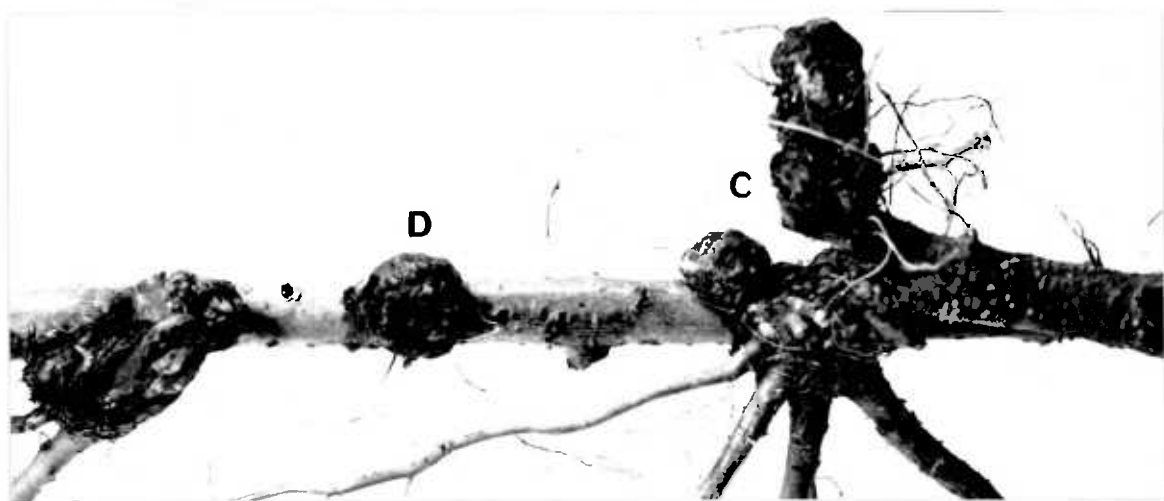
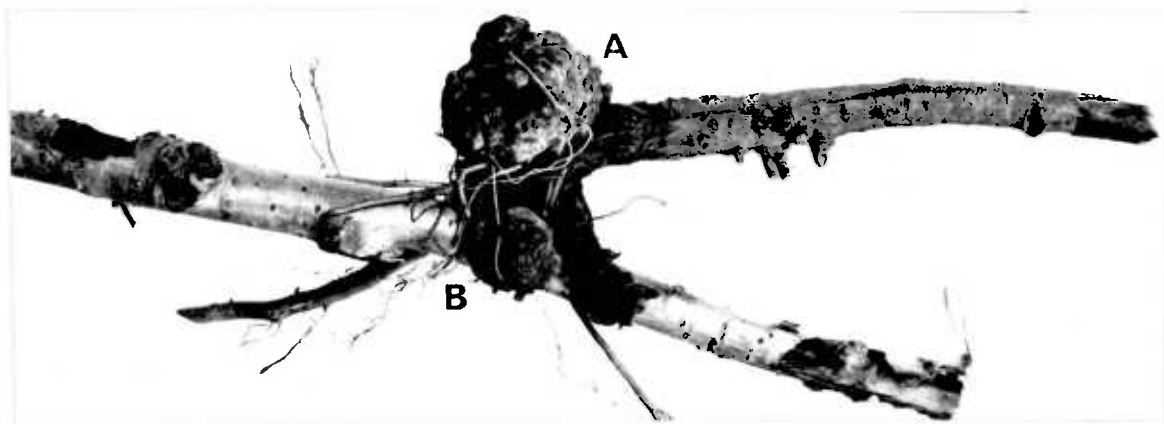


Fig. 1 Galls on F12/1 cherry rootstock layers, situated at a bend (A); a fork (B); the base (C); mid layer (D); on a root (E).

EMRS F12/1 layer bed

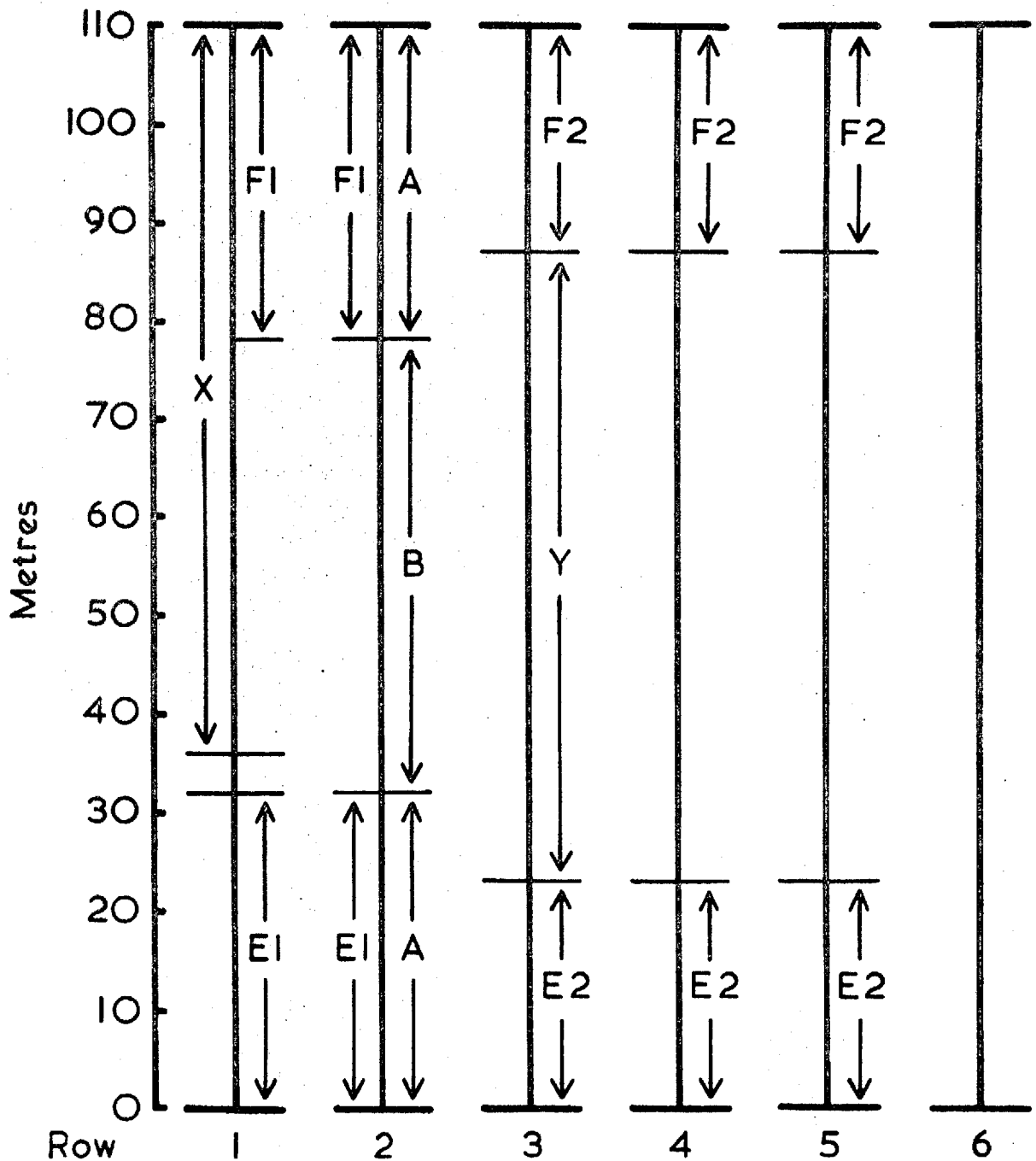


Fig. 2 Plan of F12/1 cherry rootstock layer bed.

Table 1. Number, percent and diameter of old and young galls
in relation to their site on the layer bed

Site	<u>total galls</u>		<u>old galls</u>		<u>mean diam.</u>		<u>young galls</u>		<u>mean diam.</u>	
	% per site		no.	%	(mm)		no.	%	(mm)	
Bend	19.9		75	29.3*	57.04		162	17.3	28.0	
Fork	16.7		69	26.9*	53.09		130	13.9	31.05	
Mid	20.1		31	12.1	41.79		209	22.3†	14.61	
Scar	17.2		19	7.4	40.0		187	20.0†	26.2	
Root	15.2		25	9.8	19.37		156	16.6	15.67	
Other	10.9		37	14.4	-		93	9.9		

* significantly greater than percentages at other sites ($p < 0.001$ and $p < 0.01$)

† significantly greater than percentages at bend ($p < 0.01$ and $p < 0.05$)

bends and forks; these sites together accounted for 56% of old galls. The numbers of galls at all other sites were comparatively small. The largest galls were those at bends and forks while root galls were smallest.

Young galls were more numerous than old at all sites; the numbers at forks and bends being twice, and at other sites six times, those of old galls. The distribution of young galls also differed in that mid layer and scar galls each accounted for about 20% while those at other specified sites were only slightly smaller percentages of the total. The young galls were smaller than the old at all sites but were largest at bends and forks, slightly smaller at the scars and only half the size at mid layer sites and on the roots.

b. Seasonal effects

i. Number of galls: Sector A (Fig. 2) comprising c. 70 m of layer bed was recorded annually for 3 years from 1970-1972 inclusive. The results showed a sharp increase in numbers of galls over the 3 year period, particularly in the last year (Table 2, Fig. 3). This very large increase in numbers in 1972 was also reflected on other sectors of the layer bed.

Over the 3 year period the mean diameter of old galls increased steadily from 40.9 mm to 49.8 mm but the mean diameter of young galls was 25-28 mm in each year and was in fact smallest in 1972.

A proportion of old galls will continue to expand from one year to the next, others will die or the outer tissues become sloughed off while galls which were new in

Table 2 Seasonal effect on the number of galls
per 100 m of layer bed for the years
1970-1972

	1970	1971	1972
No. of old galls	28	62	124
No. of young galls	45	67	697
Total no. of galls	73	129	821

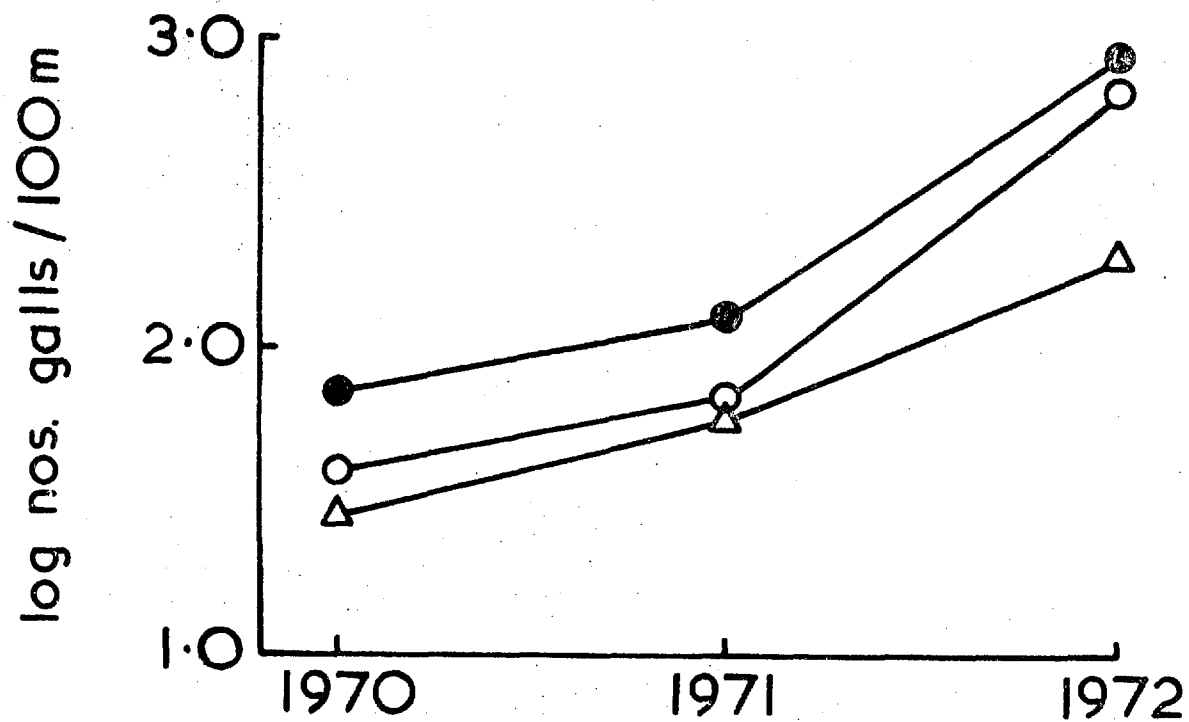


Fig. 3 Incidence of galls in a sector of layer bed from 1970-1972 inclusive
 ● total galls; ○, young galls; △, old galls.

1970 would be classified as old in 1971 and would still be enlarging. On the other hand each year's record of new galls is only of those which have recently developed and therefore the mean size would not differ greatly from year to year unless abnormal environmental conditions allowed a longer, or shorter, growth period.

ii. Distribution of galls: To determine whether the distribution of the galls over the layer bed was random or not the numbers of galls occurring on 15 cm lengths of layer bed were compared. A Poisson distribution and a negative binomial distribution were fitted to each of the selected variates using the method of maximum likelihood. The results (Table 3) clearly show that distribution is not random but in clusters fitting the data expected from a negative binomial distribution very closely (Fig. 4).

The effect of the presence of galls in a given region in one year on the presence of galls in the following year was determined from Kendall's rank correlation coefficient. The value of τ for galls on old layers was 0.038 and on all layers was 0.036 both of which are significant at $p < 0.001$. Thus there is a small correlation between numbers of galls in a given region in successive years.

Table 3 Values of observed percentage of 15 cm lengths
which are galled and as expected by fitting a
Poisson or negative binomial distribution by
the method of maximum likelihood

	<u>Expected</u> (Poisson)	<u>Observed</u>	<u>Expected</u> (neg. bin.)
Old galls on old layer	8.9	7.1	7.1
Young galls on old layer	12.6	7.1	7.1
Old galls on 2-yr layer	2.7	2.5	2.5
Young galls on 2-yr layer	28.4	17.2	17.4
Young galls on new layer	16.5	9.4	9.4
Old galls on all layers	11.4	9.3	9.3
Young galls on all layers	47.7	29.3	29.7

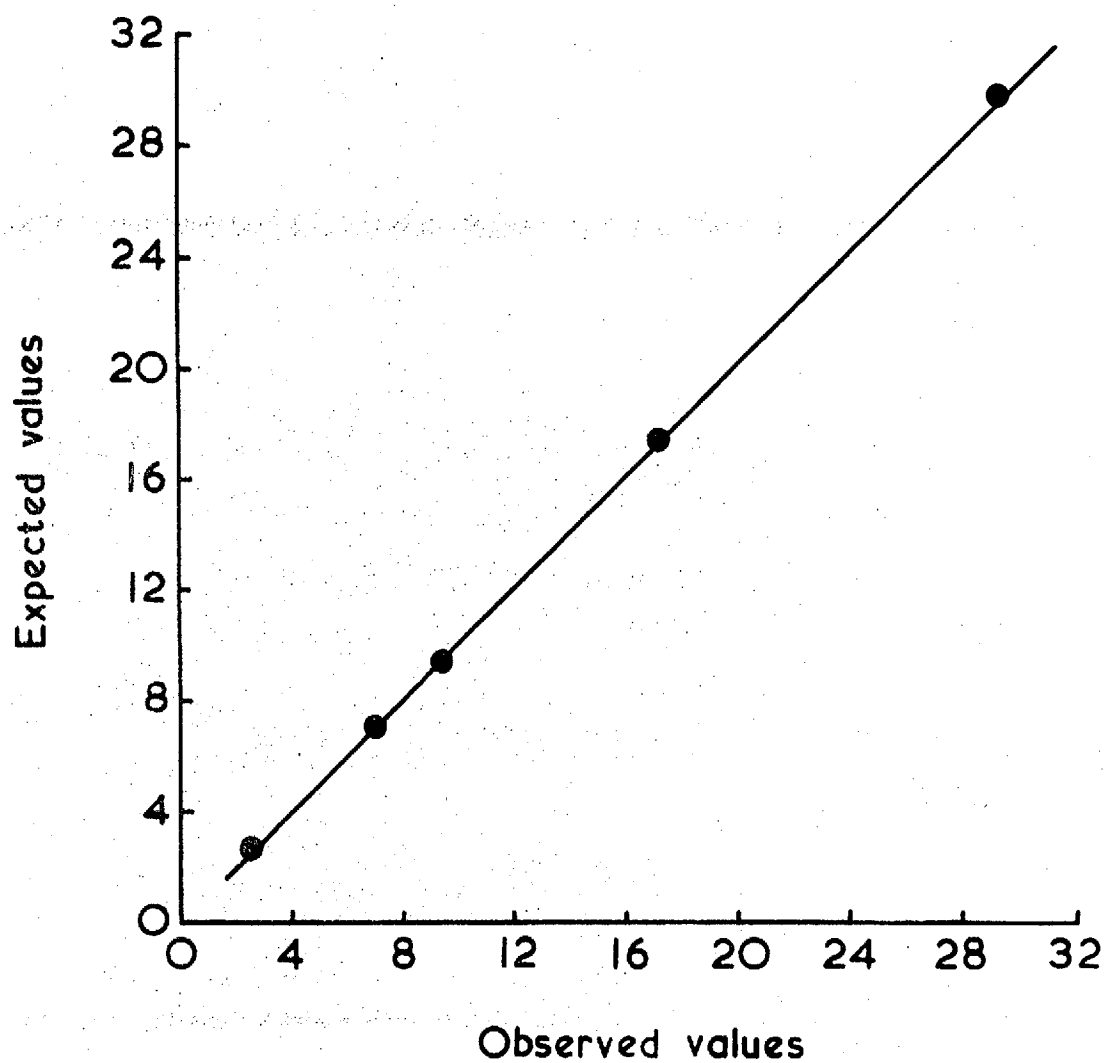


Fig. 4 Relationship between observed and expected percentages of galls on 15 cm lengths of layer bed assuming a negative binomial distribution.

c. Effect of layer age

The numbers and distribution of galls on layers of different ages in sectors X and Y (Fig. 2) is shown in Table 4.

The total number of galls was highest on 2-year-old layers, where there was an average of one every 3.75 cm compared with one every 22.14 cm on the new layers. However, the frequency of galls (no./unit length) was highest on older layers (less than 2.0 cm apart on average). This reflected the clustering of galls at the bends, the rest of the older layers having rotted away. The majority of old galls were found on the older layers and their frequency was significantly higher than ^{on} 2-year-old layers ($p < 0.001$). The majority of new galls on the other hand occurred on the 2-year-old layers although their frequency was not significantly higher than on old layers due to the shorter length of the latter (see Table 4). The number of galls on new layers was significantly less than on the 2-year-old and the older layers ($p < 0.001$ and $p < 0.01$ respectively).

The distribution of galls amongst the different infection sites was influenced by the age of the layers. Thus 47% of the young galls on the new layers were at bends or forks and a further 20% at the mid layer and shoot base positions. By contrast, the greatest number of galls on 2-year-old layers were located at shoot scars or mid layer positions; these accounted for half of the young galls with galls at bends and forks being relatively less common than on new layers.

Table 4 Relationship between age of layer and numbers of galls and their distribution

Layer age	gall age	no. of galls	no./unit length of layer*	<u>% galls at different sites</u>					
				mid	base	scar	fork	bend	root
old	all	254	3.58	16.8	6.7	5.6	28.4	29.5	12.6
2-year	all	442	2.66	24.4	12.7	23.8	21.0	5.5	12.5
new	all	82	0.45	19.7	19.7	9.1	24.2	22.7	4.5
old	old	138	1.95	13.4	6.7	1.3	30.9	40.9	6.7
2-year	old	72	0.43	17.4	4.3	18.8	34.8	11.6	13.0
old	young	116	1.64	20.6	6.6	11.0	25.7	16.9	19.1
2-year	young	370	2.23	25.6	14.2	24.6	18.7	4.5	12.4
new	young	82	0.45	19.7	19.7	9.1	24.2	22.7	4.5

* Total lengths of new, 2-year and older layers were 181.6 m, 165.7 m and 70.6 m respectively. The shorter length of the old layers was due to rotting, leaving only short lengths of the bent-over portion.

Most galls on older layers were positioned at bends and forks, with a higher proportion of young galls on roots than was the case with the younger layers.

The size of galls, young and old, increased with the age of layers but there was no marked effect of layer age on the relative size of galls at the different sites of infection (see Table 1).

2. Incidence of galls on Fl2/1 cherry rootstocks
taken from an infected layer bed

a. Numbers of galls

In spite of the considerable and increasing infection on the layer bed the incidence of galls on stocks out from it was negligible in all years. However, when stocks were examined 1 or 2 years after planting out at other sites on the Research Station a substantial proportion were found to be galled (Table 5). Only one of the planting sites, Crabtree, had a previous history of chronic crown gall infection. Nevertheless the level of infection was broadly similar at all sites, suggesting that most of it originated from bacteria introduced with the stocks as contaminants or, more probably, in view of the failure of pre-planting chemical treatments (see Section V, p159), as latent infections. The fact that on Crabtree the number of galled stocks lifted between 1970 and 1973 increased with infection in the layer bed (see p27) strongly supports this hypothesis.

Rootstocks grafted with scion material, and therefore left in the nursery for two years, had a higher percentage of infection than younger stocks. As age of tree increased so the numbers of galls per tree increased (Table 6). On 6-year-old trees, 55.5% of which were galled, 40% had 3 galls or more; many of these were root galls which probably originated as secondary infections from primary sources of inoculum.

Table 5 Incidence of galls on 'healthy' cherry stocks
planted-out after harvesting from layer beds

Planting site	year harvested (in November)	% † stocks galled after	
		1 year	2 years
Crabtree	1970	5.3	-
	1971	21.6	35.0
	1972	26.4	51.0
North Park	1973	-	48.1
	1974	18.5	-
South Park	1971	-	27.0
Scott	1971	-	46.5
Kilnfield	1973	-	40.0

† percentages based on samples of from 150 to 607
individual stocks.

Table 6 Distribution of multiple infections
on cherry rootstocks

Age of stocks (years)	% stocks with	
	1 gall	2 or more galls
1	90.3	9.7
1	67.9	32.1
2	79.7	20.3
2	65.6	34.4
6	40.0	60.0

b. Positional distribution and size of galls

A large wound is made at the base of the young rootstock when it is severed from the layer and many small wounds are made when the root system is trimmed prior to planting. Examination of many trees showed (Table 7) that on one-year-old stocks infections at the base accounted for about a quarter of the galls while half were in the crown area (Fig. 5). After a second year of tree growth galls were larger (Fig. 6) and the distribution between crown and basal galls was reversed while on older trees the majority of galls were on the root system.

Root galls were generally the smallest while those at the base and crown were larger, but of approximately comparable size (Table 8). For all positions gall size increased as age of rootstock increased.

c. Determination of time of gall appearance

A sample of young rootstocks was lifted each month during the first season of growth and the number and size of galls on them recorded (Table 9). The first galls were noted in June but few had appeared by early September; the majority (65%) up to this time were in the basal position. Higher numbers of galled stocks were present in later samples and were more evenly distributed between the base, crown and root positions. Gall diameter increased as the season progressed.

d. Effect of virus-free status on crown gall of cherry

Virus-free (EMLA) and virus-infected trees of six ornamental cherry cultivars were examined for crown gall. The results (Table 10) showed that, although virus-infected

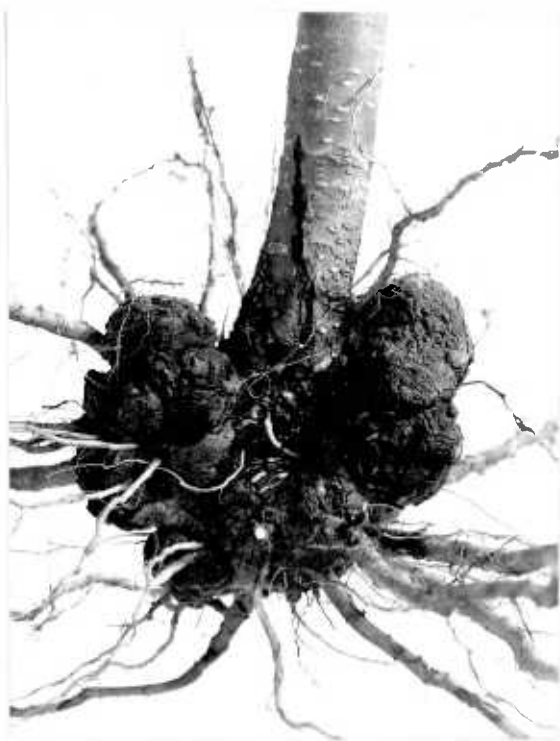


A



B

Fig. 5 One-year-old F12/1 cherry rootstocks infected with Agrobacterium tumefaciens. A, gall at the base; B, gall in the crown region.



A



B

Fig. 6 Two-year-old F12/1 cherry rootstocks infected with Agrobacterium tumefaciens. A, gall at the base; B, gall in the crown region.

Table 7 Positional distribution of galls on cherry
rootstocks of different ages

Age of rootstock (years)	No. trees	% galls per position		
		Base	Crown	root
1	536	26.3	50.4	23.3
2	169	49.1	31.9	15.4
6	40	20.6	25.0	54.4

Data combined from several plots for 1 and 2-year trees.

Table 8 Mean diameter (mm) of galls on cherry
rootstocks of different ages

Age of rootstock (years)	No. galls	Position of gall		
		Base	Crown	root
1	536	19.1	16.7	9.8
2	166	65.7	42.9	31.2
6	136	108.4	118.9	76.6

Data combined from several plots for 1 and 2-year trees.

Table 9 Number and size of galls on cherry rootstocks
at monthly intervals during their first
growth season

Sampling date	No. galled stocks (max.80)	Mean no. galls/ stock	Mean gall diam. (mm)
6th May	0	-	-
1st June	2	1.0	2.0
6th July	4	1.25	5.4
30th August	3	1.0	10.0
7th September	6	2.0	15.3
11th October	13	1.3	17.2
1st November	12	1.3	18.2
13th December	14	1.1	15.1

640 rootstocks were planted, in spring 1976 and 80, in
8 randomised blocks, were sampled each month.

Table 10 Effect of virus status of ornamental scions
on the incidence of crown gall in the
2-year-old P12/1 rootstocks on which they
were grafted

Cultivar	Virus free		Virus infected	
	no. trees	% galled	no. trees	% galled
Azaleogawa	9	44.4	64	54.7
Kanzen	49	44.9	48	41.7
Shirofugen	50	50.0	48	41.7
Shirotae	45	53.3	29	41.4
Ukon	12	41.7	73	37.0
Yoshino	38	55.3	53	49.1

trees of the cv. Amanogawa had a higher percentage galled, for most of the cultivars it was the virus-free trees which had the higher percentage of galled stocks. Analysis showed that these differences were just significant.

e. Rootstock/scion interaction in crown gall of cherry

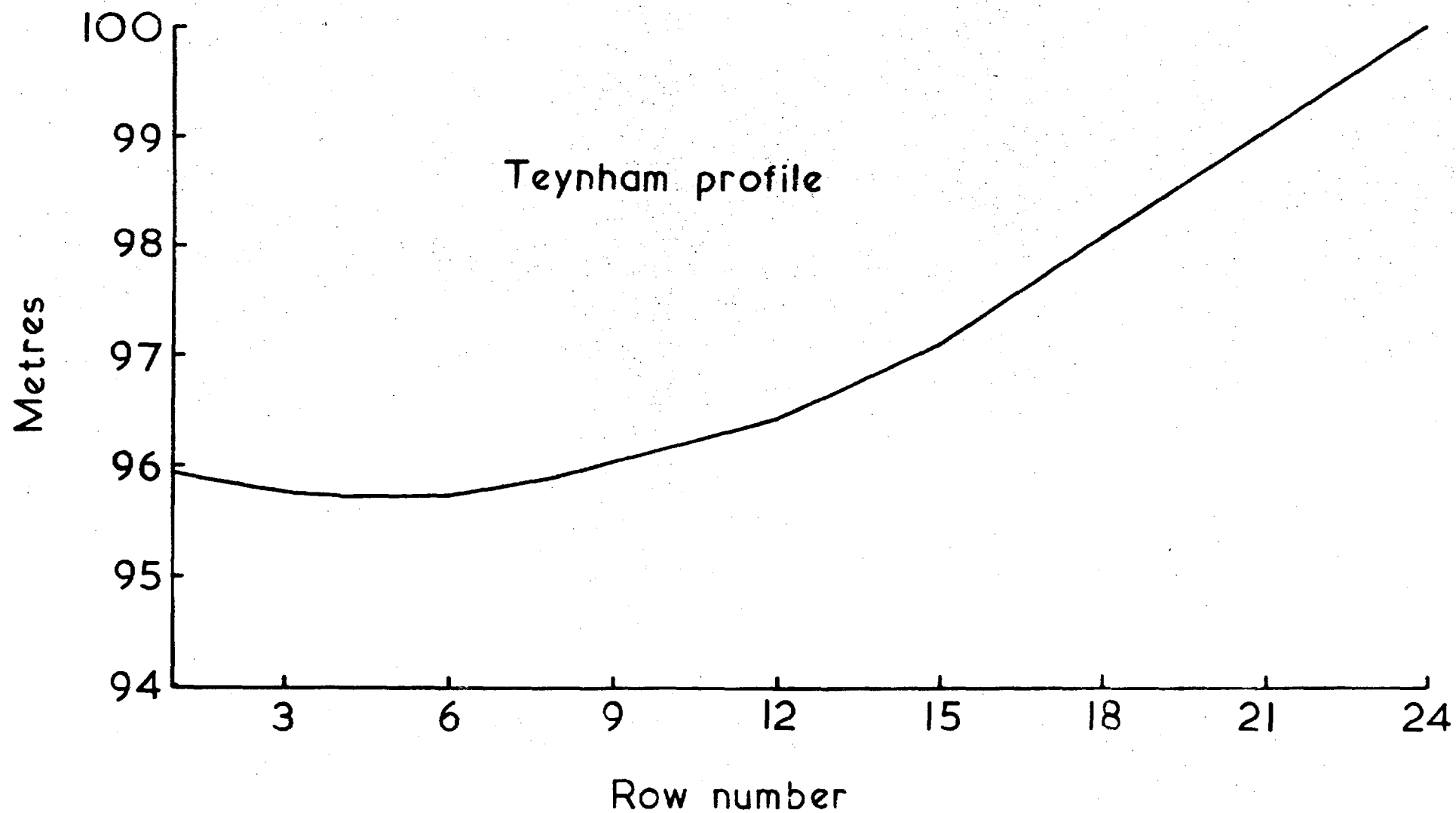
Field observations on over 2000 2-year-old trees of 13 sweet cherry, 14 ornamental cherry and 2 acid cherry cultivars on Fl2/1 rootstocks gave no clear or consistent indication of a rootstock/scion interaction in crown gall. No scion cultivars conferred immunity to the disease on the Fl2/1 rootstock. However, there was some evidence that the acid Morello cherry and the sweet cherry Napoleon (see Section II) may increase rootstock susceptibility to A. tumefaciens infection.

3. Crown gall in blackberry

a. Spread in plantations

1. Newlands Farm, Teynham: A four-acre site was planted with blackberry cv. Bedford Giant in autumn 1967. The site had been a hop garden for the previous 8 years and existing wire-work was used for the blackberries; rows were 4.27 m (14 ft) apart and bushes were at 2.13 m (7 ft) spacing within the row.

Fig. 7 Profile of the site of the blackberry plantation at Teynham.
Horizontal scale 1 mm = 445 mm.



The northern end of the site sloped downwards (1 in 20 gradient) from west to east from row 24 to row 6; the ground was level to row 4 but sloped upward to row 1 (Fig. 7). The rows were herbicide treated but there was abundant weed growth in the alleyways (nettles, thistles, dock, fat-hen and grasses).

Canes that formed during the summer trailed on the ground until tied-in during the winter (late autumn to early spring) when old canes were cut out leaving a short stub of c. 5-10 cm. There was no systematic orchard hygiene and galled canes or pieces of galls were often left lying within or between rows. A complete tidy-up and cultivation along the rows i.e. at right angles to the slope of the land, was carried out in winter 1972-3 when old canes were cut to ground level.

Gall incidence: The grower had not noticed any galls in 1968 but found patches of heavily galled crowns in 1969. On the advice of an ADAS inspector the majority of galled bushes together with c. 1 m³ of surrounding soil were removed and replaced with fresh soil and stock propagated by the grower from his original supply.

Distribution and spread within the plantation: In 1969 there were 27 infected plants in the southerly half (1.4%) and 340 (18.9%) in the northerly half of the plantation of which 2/5 were in the 6 rows towards the top (western side) of the slope. Assuming infection originated on the planting material either more severely infected plants were put into the higher numbered rows or infection was fairly evenly distributed but conditions were more conducive to disease development in these upper rows.

A detailed study of spread was made in the northern half of the plantation in a sector of 360 plants, 15 in each of the 24 rows. This sector was examined annually in May/June and the gall record checked by a further examination after leaf fall. The numbers of infected plants per row for 1969-73 are illustrated in Fig. 8. As distribution of infection was not random, the sector was divided into 4 blocks of 6 rows. The percentage of galled plants in each block is given in Table 11.

Until 1971 infection was virtually absent from rows 1-6. Infection was initially most prevalent in rows 19-24 but, after removal of infected plants in winter 1969-70, there was a high proportion of young replants in this block, which accounts for the lower level of infection in the following year compared to rows 13-18 where older plants were more predominant. Infection spread rapidly both down the slope and along the rows so that by 1973, i.e. in 5 years, the plantation was almost totally infected. The costly removal of galled plants in 1969 retarded spread in this area by only a year or two.

ii. Bifrons Gardens, Canterbury: This 1 acre walled garden, previously cropped as a market garden, was planted in winter 1969-70 with blackberry cv. Bedford Giant. The garden was on level ground, soil pH c. 7.5, and was divided into 2 halves, A and B, each of approximately 250 plants in rows 3.7 m (12 ft) apart and planting distance within the row of 2.4 m (8 ft). The garden was kept weed-free by herbicide treatment. New canes were tied-in as soon as practicable and laterals on fruiting canes were

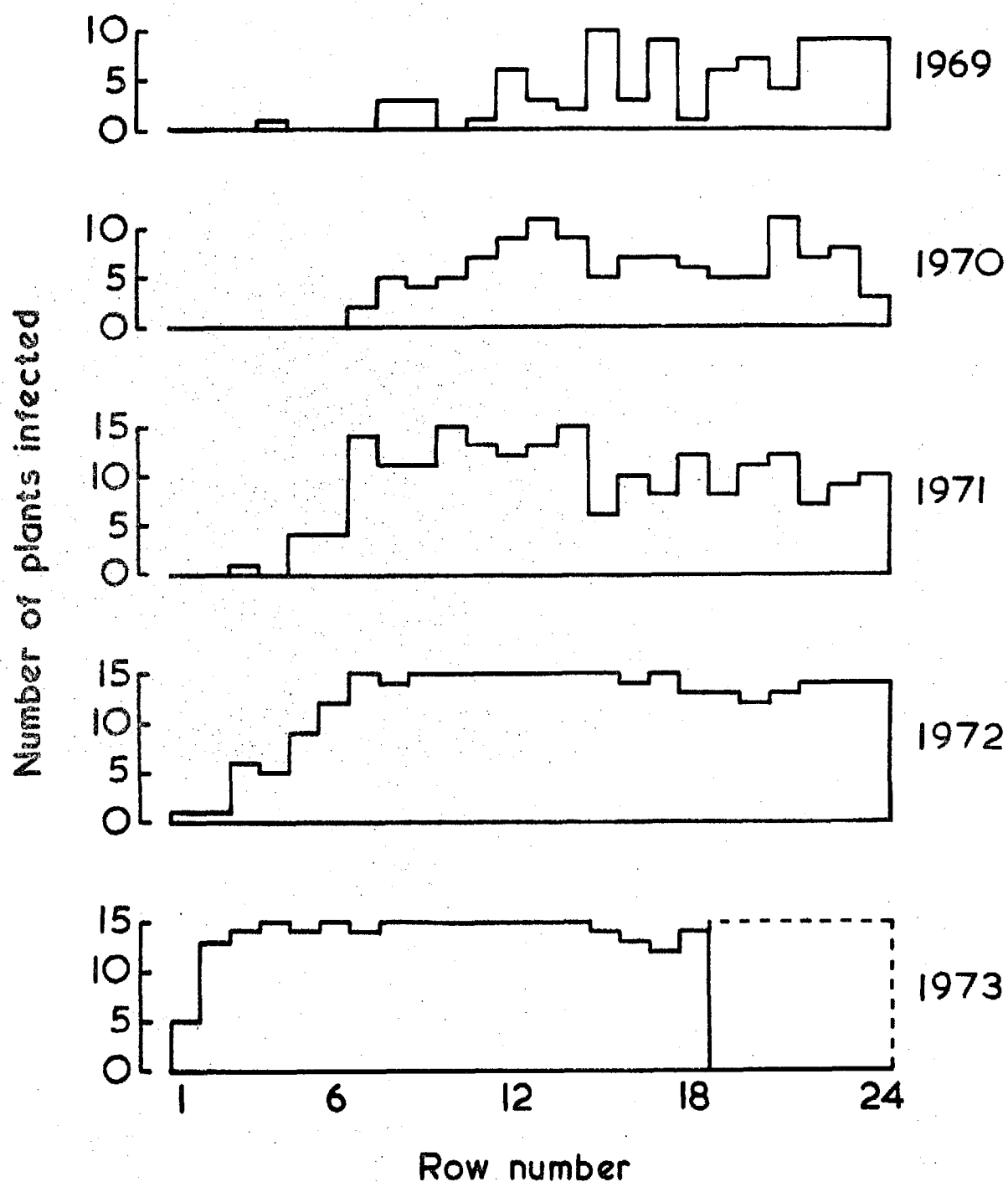


Fig. 8 Numbers of infected blackberry bushes (cv. Bedford Giant) per row, at Teynham, for the years 1969-1973 inclusive. Dotted area not recorded but assumed to be 100%.

Table 11 Percentage galled blackberry plants at
Teynham per 6 row block from 1969-1973

Block	Rows	1969	1970*	1971*	1972	1973
1	1-6	1.1	-	10.1	37.7	84.4
2	7-12	14.4	38.9	92.8	98.8	98.8
3	13-18	31.1	62.9	93.5	96.6	92.2
4	19-24	48.8	54.5	84.2	88.8	
Totals						
	Rows 1-24	23.8	35.2	66.1	80.5	91.8

* figures for these two years are percentages of mature plants infected only. Young replacement plants had about 20% and about 25% infection in 1970 and 1971 respectively.

pruned back during the summer. After harvesting, old canes were cut out, normally to ground level but with 15 cm snags in winter 71/72 only in half the garden (A).

Gall incidence: Galls were first noted by the grower on young plants in the summer of 1970. When the garden was first inspected in September 1971, many plants were galled, mainly at the crown. At least 2 plants, each having only 2 canes, were very severely affected and later died.

The number of infected bushes in Bifrons Gardens A increased from c. 50% in 1971 to nearly 100% in 1973 and in Bifrons Gardens B from 70% in 1972 to 94% in 1973. Each half of the garden was divided into 4 blocks and the percentage infection in each block over the period of study is given in Table 12.

The distribution of infected plants in this plantation was more patchy than at Teynham. At first there were islands of healthy plants in Bifrons Gardens A (Fig. 9) but these could not be related to differences in soil pH as this varied little within the plantation (Table 13).

Distribution and spread within one season: In Bifrons Gardens in winter 1971, canes were cut to leave stubs in area A but were cut to ground level in area B from where all pieces of old gall were removed. Records during 1972 showed a progressive increase in the number of galled plants in both areas (Table 14).

Table 12 Crown gall infection in blackberry in the
four blocks of Bifrons Gardens A and B

<u>Bifrons A</u>	Percentage incidence of galled plants		
	1971	1972	1973
Block 1	64.5	79.0	100
2	40.3	51.6	95.2
3	51.6	66.1	97.0
4	45.0	65.0	96.8
Totals	50.6%	66.1%	98.0%

<u>Bifrons B</u>	Percentage incidence of galled plants	
	1972	1973
Block 1	59.2	95.9
2	75.5	97.8
3	64.9	98.7
4	81.5	93.8
Totals	70.3%	94.2%

Note: not exactly same numbers in each block, 60-63 in
Bifrons A (later years replants counted as mature)
and from 45-77 in blocks in Bifrons B.

Table 14 Distribution of galls on Bedford Giant
blackberry plants at Bifrons Gardens in 1972

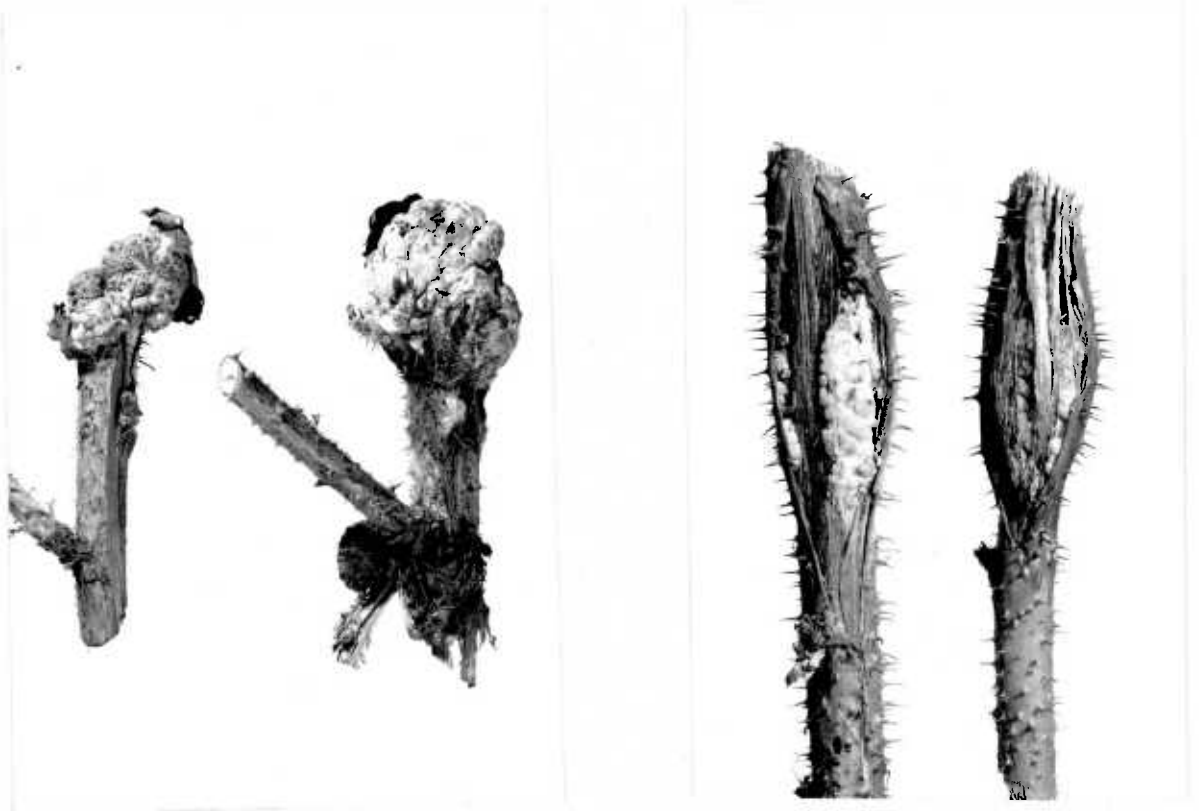
Plantation	Site of gall(s)	No. of galls recorded		
		25 May	21 June	1 Aug.
Bifrons Gardens A (246 plants)	Crown	3	4	5
	Crown & stub	14	18	2
	Crown & cane	2	5	32
	Crown, stub & cane	-	-	19
	Stub	1	1	1
	Stub & cane	14	15	18
	Cane	37	67	84
Total no. plants infected		70	110	161
% crown infections		27.1	24.5	36.0
Bifrons Gardens B (236 plants)	Crown		1	1
	Crown & cane		42	70
	Cane		84	94
Total no. plants infected			127	165
% crown infections			33.8	43.0

The percentage of plants infected at the end of the season was lower in Bifrons A than B. In Bifrons A even at the May record, only 1 plant had the infection limited to the stub and for all 3 records there were approximately 20% of the infections in the crown. There was a progressive decrease in those infected on old canes only, to those in which infection spread to the fruiting canes. However, throughout the season the number of plants with infection on fruiting canes only, was high and rapidly increased especially between May and June.

In Bifrons B about one third of the infected plants had crown galls, nearly all of them associated with galls on the fruiting canes both basally and nodally. Here also the majority of infections, both in June and later in the season, were on fruiting canes on plants having no crown galls.

b. Development of galls on blackberries

Stub galls appeared first, according to the season between 3rd and 10th May, when new lateral shoots were about 20 cm long and new canes about 40-50 cm long and growing strongly from the crown. The stubs of old canes, especially the thicker ones, were often still green and some had lateral shoot growth. Approximately 44% of the new stub galls recorded at Teynham in 1971 were at the top of the stubs, 38% emerged some distance below (Fig. 10) and the remainder at ground level.



A

B

Fig. 10 Stubs of old blackberry canes (cv. Bedford Giant) with galls A, originating at the top; B, originating below the cut surface.
Photographed in May.

New crown galls could be seen in the first two or three weeks of May but only if the soil or pieces of old gall were removed to expose them. They originated through the surfaces of old canes that had been cut at ground level. Later in the season crown galls were clearly visible, often adjacent to or on top of old galls (Fig. 11).

Galls on the fruiting canes generally appeared after stub or crown galls, sometimes as late as August. The majority were at nodes, either surrounding the base of a growing lateral or, more frequently, around the stump of a summer pruned lateral. A cane rarely had a single gall only; by mid to late summer it was not unusual to see as many as 10 nodal galls on a cane, especially at Bifrons Gardens (Fig. 12A). Basal cane galls were often seen in June on canes issuing from the crown at an acute angle where the canes tended to crack on being tied in to the wires (Fig. 12B).

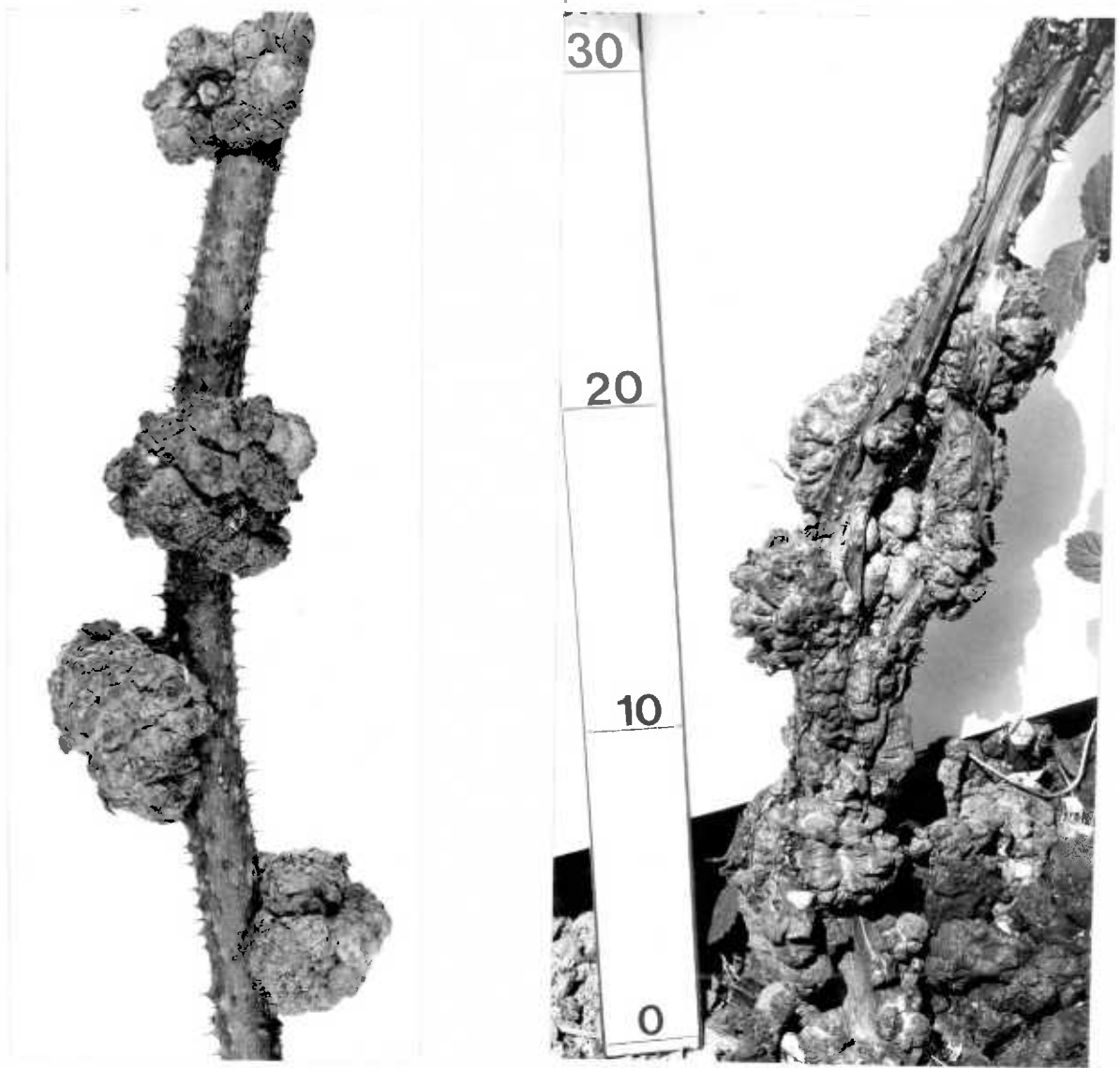
c. Rate of development

Growth of galls was initially very rapid through the months of May and June. Thereafter many galls ceased to expand and became hard, woody and fissured. This occurred particularly with stub and crown galls where the check to growth was associated with the decay of the old canes on which they were situated. Galls on fruiting canes continued to expand for a longer period but their initial growth rate was not maintained. In 1976 a late-season flush of gall expansion occurred between October and November following an exceptionally hot dry summer and a wet September.



Fig. 11 Bedford Giant blackberry with old
and young basal galls.

Photographed 8th July, 1976.



A

B

Fig. 12 Canes of blackberry (cv. Bedford Giant) with galls: A, at successive nodes (July) and B, at the base (August).

d. Seasonal variations in infection

Only 15 blackberry bushes (5.5%) of the 270 recorded at Teynham from 1969-73 inclusive remained free from infection with crown gall. Once infected the majority of bushes developed new galls in each succeeding year; only 13 bushes behaved inconsistently in this respect. These included 7 bushes that showed no visible signs of infection in one year and 3 bushes that were infected in one year only (1972). In Bifrons Gardens all except one of the bushes infected in 1971 were infected in the following year and only 4 infected in 1972 appeared free from infection in 1973.

4. Incidence of disease in loganberry

Crown gall infection was reported from Offham, in August 1972 on a level site with a soil pH 7.2. Loganberry bushes (170) of clone LY59, supplied by a local nurseryman and planted in 1967/8, were free from infection but 14 bushes (8.5%) of a Thornless clone planted in spring 1969 were galled. Some infection had been noted in 1970 when the first old canes were cut out.

DISCUSSION

These field observations on the distribution and spread of crown gall clearly show that if the disease is left unchecked its incidence will increase progressively from year to year. This is contrary to the view expressed by Lelliott (1971) that there was no evidence of a seasonal increase in crown gall in cherry layer beds.

The clustering of galls on the layer bed in a negative binomial distribution suggests that the concentration of the pathogen, and hence the probability of new infection, is greatest in the vicinity of existing galls. The non-random distribution of galls also reflects the fact that the layer bed is not a homogeneous plant system and that different parts of the system e.g. bends of new layers and the shoot scars of two-year layers, are inherently more prone to infection than others. There is no evidence that the presence of crown gall on the layers significantly reduces rootstock production but, apparently healthy stocks cut from an infected bed can carry infection, since the increase in the percentage of stocks which developed disease one or two years after planting-out was proportional to the increased incidence of disease on the layers. On stocks lifted after one year of growth galls were more often within the crown than directly on the base, whereas the proportion of basal galls was higher on stocks lifted after 2 years of growth. Assuming that many of the stocks become infected when cut from the layer bed, then either the latent period is more than a year or galls from infections originating when the stocks are cut do not always develop at the site of infection but a few centimetres distant, as is apparently the case of galls on blackberry stubs. As age of the rootstocks increased there was a corresponding increase in the percentage with multiple galls, again suggesting spread of inoculum from the primary gall to other susceptible sites nearby.

There was an indication that virus-free ornamental trees were slightly more prone to infection with crown gall than virus-infected ones. The appearance of serious crown gall infection on the East Malling layer beds, after nearly 30 years of sporadic and minor infection, followed the establishment of the first layer bed using EMLA virus-free F12/1 rootstocks. This may be coincidental, but further investigation into the effect of virus-free status on the predisposition of rootstocks to crown gall might be interesting.

There seems little doubt that the infection in the two blackberry plantations under close study, and two others in addition to the loganberry plantation, were introduced on the plant material which originated from a common, specialist nursery source. None of the planting sites had a previous history of crown gall. At Teynham the disease was apparently introduced in plants at the top of the slope and spread from these to the rest of the plantation. On the level Bifrons Gardens site, the islands of non-infected plants soon became infected and in both sites investigated the spread of infection was rapid and virtually complete in 4 years. Within each bush there was a high correlation between infection in one year and the next and often infection in one site in one year was followed by more widespread infection on other parts of the bush in the following year.

The size of galls on layers, rootstocks and blackberry crowns increased with age and on blackberry in particular galls could develop very rapidly and be very large by mid summer.

The perennial nature of cherry layer beds and blackberry plantations and the cultural practices adopted with these hosts creates an ideal situation for the spread of crown gall.

SECTION II

EFFECT OF CROWN GALL DISEASE ON GROWTH AND CROPPING

1. Effect on layer bed productivity

A newly established layer bed normally yields increasing numbers of rooted shoots during the first few years of production. However, productivity of the F12/1 cherry rootstock layer bed at East Malling declined over the period 1969-71 (Table 15). The main decrease from 1969-71 was in the number of top quality rootstocks; there was also a rise in the number of non-rooted shoots which had to be grown-on for a further year. Detailed records made on 6 of the 12 rows of layer bed showed that the incidence of crown gall was not correlated with the yield of larger shoots.

The improved yield in 1972 followed the application of benomyl drenches in May 1972 to control infection by Thielaviopsis basicola, the cause of replant disease in stone fruits (Sewell, Howard & Wilson, 1975). It is probable that any effect of A. tumefaciens was obscured by the action of T. basicola. There was therefore no conclusive evidence on the effect of crown gall on layer bed productivity.

2. Effect of crown gall on growth of cherry trees

Although there was no direct evidence of an effect of crown gall infection on the productivity of a cherry rootstock layer bed, the subsequent incidence of the

Table 15 Production of F12/1 cherry rootstocks from
a layer bed from 1969-72

Grade of harvested stocks	Year of harvest from layer bed			
	1969	1970	1971	1972
Good	4,050	3,550	2,837	3,789
Medium	2,870	2,000	2,379	3,046
Poor	2,300	2,875	2,380	3,400
Non-rooted shoots	150	200	339	393
Total shoots	9,370	8,625	7,935	10,628
% of 1969 total	100	92.06	84.7	113.5

disease on young rootstocks obtained from the infected bed (Table 5) was sufficiently high to be economically significant. Galled stocks are generally unmarketable, and in some countries their sale is prohibited. Experienced nurserymen assert that it is impossible to predict from a row of cherry trees those that will have galls when lifted and there is no conclusive evidence that subsequent performance of rootstocks is significantly impaired by the disease. Incidental observations were therefore made on various stone fruit trees in which there was A. tumefaciens infection and on which measurements of girth, plant height or shoot growth had been recorded.

a. One-year-old rootstocks

Observation of F12/1 cherry rootstocks lifted after one year of growth in 1972 suggested that stocks with galls were slightly larger than the healthy stocks. In the following year some F12/1 rootstocks were measured when lifted for gall assessment. The mean height of 322 healthy trees (98.2 cm) was significantly less ($p < 0.01$) than that of 118 galled trees (104.2 cm).

b. Two-year-old rootstocks

i. Ornamental cherries on F12/1 rootstocks More than 200 EMMA F12/1 cherry rootstocks worked with virus-free ornamental cherry cultivars were lifted in spring 1974 for planting in a field trial on virus diseases. There had been no evidence to suggest that the plants were infected but galls were found on 48% of them. The small differences in height between diseased and healthy trees (Table 16A) were not significant ($p > 0.05$).

Table 16 Effect of crown gall on growth of P12/1
rootstocks worked with ornamental cherry
cultivars

Cultivar	<u>Healthy</u>		<u>Galled</u>	
A. <u>Lifted 1974</u>	No. trees	mean ht (cm)	No. trees	mean ht (cm)
Amanogawa	7	167	4	186
Kanzan	27	190	22	187
Shirofugen	24	180	25	177
Shirotae	20	169	23	169
Ukon	7	179	4	171
Yoshino	19	188	20	180
B. <u>Lifted 1976</u>	No. trees	mean shoot length (cm)	No. trees	mean shoot length (cm)
P12/1 non-worked	29	395	19	389
Autumn cherry	6	887	2	675
Shirotae	10	597	5	497
Schmittii	10	522	4	427
<u>P. avium</u>	9	504	9	428
Umeniko	9	734	9	507
Yoshino	7	442	8	403

In spring 1976 136 F12/1 EMLA cherry rootstocks, 48 non-worked and 88 worked with ornamental cherry cultivars, were lifted and about 40% were found to have galls. Although the mean total shoot length for all cultivars was less on the galled than on the healthy trees (Table 16B) the differences were not significant ($p < 0.05$).

There were insufficient numbers of galled trees of each cultivar for statistical assessment of the effect of gall position on tree size. However, of the 19 galled non-worked F12/1 trees, those with crown and basal galls were smaller than healthy trees, whereas those with root galls were larger than the healthy ones. There was a negative correlation ($p < 0.05$) between total shoot growth and log gall 'volume' (i.e. the product of width x height x depth). There was a similar correlation for the trees of Prunus avium, ($p < 0.1$), but none for the 9 diseased trees of cv. Umeniko or the 8 cv. Yoshino.

11. Sweet cherries on F12/1 rootstocks Cherry rootstocks (F12/1 EMLA) worked with the sweet cherry cultivars Napoleon and Roundel were lifted in spring 1976 and many of them bore galls. The heights of all diseased trees and of 10 healthy trees of each cultivar were measured (Table 17). Galled trees of Napoleon were significantly shorter ($p < 0.01$) than healthy trees; the effect on Roundel was not significant.

The trees were destined for a future field experiment on bacterial canker and all healthy trees were planted out,

together with some galled trees of Napoleon. Additional galled trees of each cultivar were planted on an adjacent plot for growth studies to be made over the next 5 years. Girth measurements were taken immediately after planting on 88 trees of each cultivar, of which 40 Napoleon and 28 Roundel were galled. In both cultivars the mean girth of galled trees was less ($p < 0.05$) than that of healthy trees (Table 17).

The relationship between gall position (Fig. 13) and growth of Napoleon and Roundel trees is shown in Table 18. Infected Napoleon trees were significantly shorter than healthy trees, irrespective of the gall positions. The largest effects were associated with galls on the base, crown or root ($p < 0.001$); Roundel trees with galls did not generally differ significantly from healthy trees but those with root galls were significantly smaller ($p < 0.01$). For all gall positions the girths of diseased Napoleon trees were smaller than those of healthy ones (Table 19), especially where galls were at the stem base ($p < 0.01$). Positional effects of galls on girths of Roundel were all non-significant.

c. 6- and 8-year-old trees

i. Sweet cherry, Schrecken, on Fl2/1 rootstock A plot of 6-year-old trees of the cultivar Schrecken on Fl2/1 rootstocks, was grubbed in winter 1973-4 and the uprooted stumps of the trees were examined for galls. It was assumed that the trees were infected at, or shortly after, the time of planting. Girth measurements had been taken annually. The final mean girth of the non-galled trees,

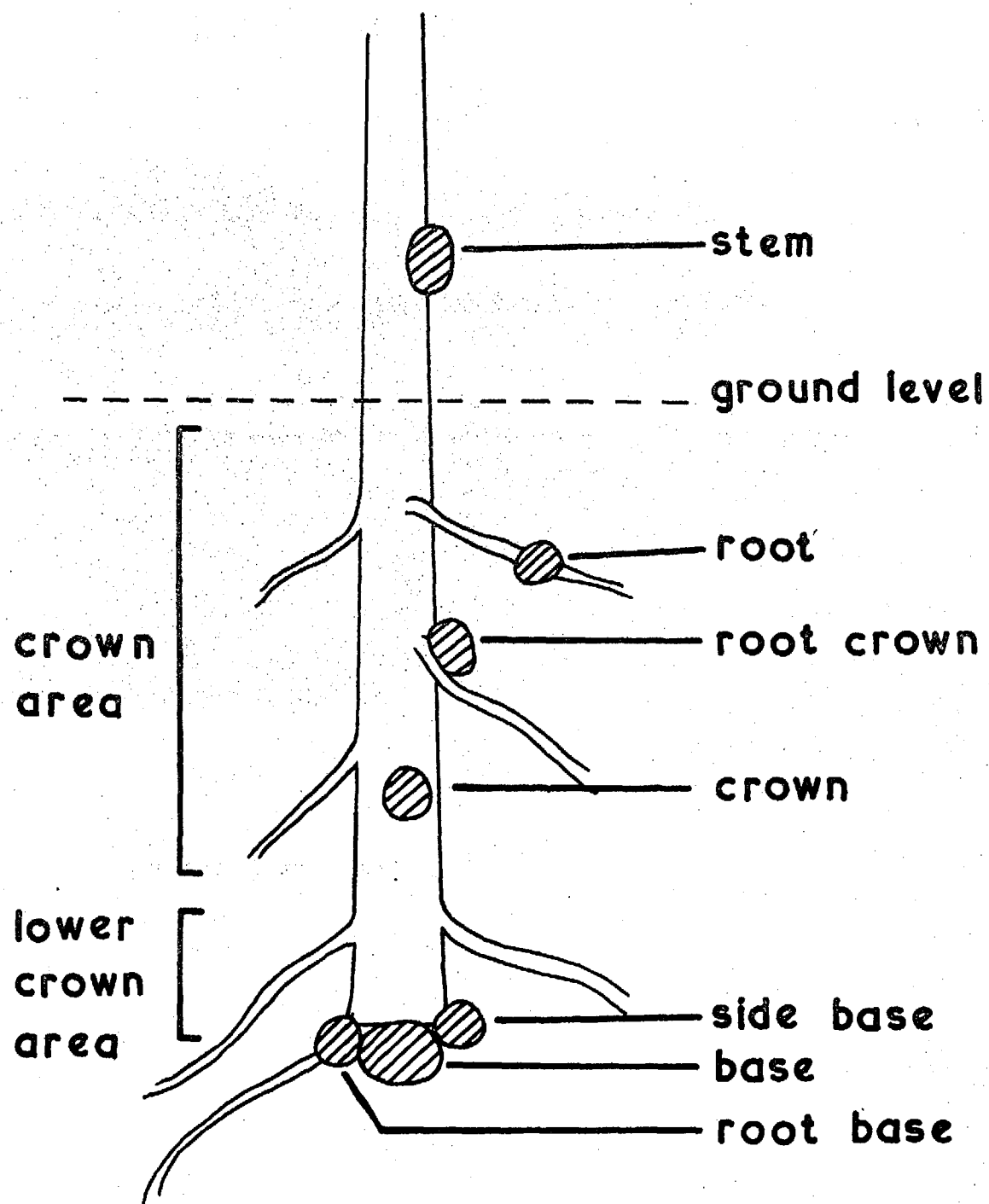


Fig. 13 Gall positions on cherry rootstock.

Table 17. The effect of crown gall on the height and
girth of 2-year-old Napoleon and Roundel
trees on P12/1 rootstocks

		no. trees	mean height (cm)	no. trees	mean girth (mm)
Napoleon	Healthy	10	161.3	48	52.2
	Galled	72	141.3**	40	47.6*
Roundel	Healthy	10	153.5	60	51.8
	Galled	40	143.2	28	48.6*

*, **, significantly smaller than healthy trees at
 $p < 0.05$, $p < 0.01$ respectively.

Table 18 Effect of gall position on height of
2-year-old Napoleon and Roundel cherry trees

Status	Napoleon		Roundel	
	No. trees	mean height (cm)	No. trees	mean height (cm)
Healthy	10	161.3	10	153.5
Galled	72	141.3**	40	143.2
Gall position:				
Base	28	141.0***	20	144.2
Crown	5	131.4***	1	142.0
Lower crown	8	147.9*	4	141.5
Side base	15	144.7**	7	142.0
Root	5	135.0***	3	127.0**
Root base	4	137.2**	2	148.0
Root crown	4	142.5*	1	157.0
Root base crown	3	142.0*	2	155.0

*, **, ***, significantly smaller than healthy trees
at $p < 0.05, < 0.01, < 0.001$ respectively.

Table 19 Effect of gall position on girth of
2-year-old cherry trees of Napoleon
and Roundel

	<u>Napoleon</u>		<u>Roundel</u>	
	no. trees	mean girth (mm)	no. trees	mean girth (mm)
Healthy	48	52.2	60	51.8
Galled	40		28	
basal	13	45.2 **	12	49.6
base and crown;				
base, crown	12	50.2 *	8	47.6
and root				
crown	7	46.9 *	4	50.5
base and root;				
crown and	8	48.4 *	4	46.0
root; root				

*, **, differences between diseased and healthy
trees differed at $p < 0.05$, $p < 0.01$
respectively.

Data only for trees planted in experimental plot
(see text).

35.8 cm, was slightly greater than that for galled trees 33.9 cm.

However, regression of girth increments during 2-year periods against final 'volumes' of basal galls showed a significant reduction in growth rate for the period 1969-71 (Table 20); the rate subsequently increased and infected trees were only slightly smaller than healthy ones when they were grubbed. However, trees with crown galls showed a significant reduction in growth rate for the second 2-year period i.e. 1969-71, and the growth increment of these trees during 1967-73 was significantly smaller than that of healthy trees. It therefore appears that the effect of crown galls on growth may be greater than that of basal galls.

No correlation was found between the final girth and the total 'volume' of all galls on the stem and roots.

ii. Sweet cherry cultivars on F12/1 rootstock with different interstocks Data were available from a trial in which different interstocks on F12/1 rootstocks were worked with either the cultivar Merton Glory (10 trees/interstock) or cv. Van (9 trees/interstock) some of which, when grubbed at 8 years old in spring, 1976, were found to be infected with A. tumefaciens. Differences in total shoot weight between galled and healthy trees of each cultivar/interstock combination were not significant (Table 21) except for one very small tree of Van on Montmorency interstock ($p < 0.01$).

Table 20 Regression coefficients for girth increments
of Schrecker against volume of galls at the
base and crown for periods between 1967 and
1973

	Basal galls	Crown galls
1967-69	- 2.97*	- 1.09
1969-71	+ 0.44	- 2.31*
1971-73	+ 1.17	- 1.41
1967-73	- 1.78	- 2.23*

*, results significant at $p < 0.05$.

Table 21 Mean weight (kg) of 8-year-old trees of
Norton Glory and Van, with and without
crown gall, on six interstocks on F12/1
rootstocks

Interstock	<u>Norton Glory</u>		<u>Van</u>	
	Healthy	Galled	Healthy	Galled
F12/1	42.6 (7) [†]	44.8 (3)	32.2 (8)	29.9 (1)
Montmorency	46.1 (9)	58.3 (1)	35.8 (8)	16.8 (1)**
<u>P. avium</u> x <u>P.</u> <u>pseudocerasus</u>	35.6 (8)	43.9 (2)	26.9 (3)	32.7 (6)
<u>P. avium</u> x <u>P.</u> <u>incisa</u>	41.8 (9)	28.7 (1)	32.2 (7)	23.5 (2)
27.27	47.1 (9)	51.1 (1)	34.6 (5)	45.5 (4)
4.158	38.9 (8)	41.3 (2)	31.8 (9)	-

[†] number of trees recorded in parenthesis.

** significantly smaller than healthy trees $p < 0.01$.

Analysis of the tree weight data failed to show any correlation with gall size for the 6 trees of Van on Prunus pseudocerasus or 4 trees on genetic dwarf 2727. Although there were too few galled trees of other cultivar/interstock combinations for analysis, there did not appear to be a relation between tree growth and gall size.

3. Effect of galls on growth and cropping of blackberry

As a measure of plant vigour and cropping potential the numbers of new canes tied-in each year on each bush were counted at ground level and at the third training wire.

During 4 years of records at Teynham no clear differences in cane production between healthy and infected bushes were found. In 1974 the mean cane number on healthy bushes, 7.6, was very slightly lower than that for infected bushes, 7.9. At Bifrons Gardens A in 1974 the mean cane number for healthy bushes, 4.2, was slightly higher than for infected, 4.0. There was no correlation between the number of years a bush had been infected, nor of the severity of infection in one year on plant vigour in the following year. Similarly, in Bifrons Gardens B there was no evident correlation between infection and plant vigour.

Growers at both the farms under observation maintained high yields in spite of infection increasing to 100%.

Precise data on cropping of individual plants could not be obtained because the sites were remote from East Malling and because the picking season extends from early July to September involving over 20 separate pickings. An attempt to obtain crop data from a plot at East Malling was precluded because the bushes became heavily infected with Bedford Giant mosaic which reduced the crop.

DISCUSSION

Reports of the effects of crown gall on tree growth have been largely based on observation, without experimental data. Hutton (1949) surmised that galled trees were more liable to succumb under stress conditions than healthy trees. In a 3-year survey Kainski (1964) attributed death of peach trees up to 20 years old to root galls, and not to winter injury as had first been reported. In an orchard of 10-year-old Montmorency sour cherry trees, all infected with crown gall, the death of 5 trees appeared to be due to their multiple galls. Death of apple trees was rarely attributable to crown gall.

Growth and cropping of plums and pears infected with crown gall was reported to be comparable with that of healthy trees (Popova, 1973a) but multiple galls on wild pear rootstocks caused severe damage and subsequent death (Popova, 1973b).

There have been some attempts to determine the effects of galls on growth experimentally. Harris & Pearce (1938) inoculated potted apple rootstocks and measurements taken after two years showed that galled trees grew slightly more than the controls. In the U.S.A. Riker (1939) inoculated

Yellow Transparent apple trees and after 6 years could detect no significant difference in height or girth between galled and non-infected trees. More recently Dahl & Johansen (1970) claimed from 6-year experiments that crown gall did not depress the growth of apple trees; since they compared galled trees with those from which galls had been removed or dipped in chemical, and had no controls, their results should be treated with caution.

The data reported here, based on observations of over 1200 naturally infected F12/1 cherry rootstocks of different ages and grafted on different scion cultivars, have not revealed any consistent or major effects of crown gall on tree growth. On one-year-old rootstocks crown gall tended to be associated with larger plants but on older stocks and grafted trees the reverse was more generally true and bears out the assertion of nurserymen that it is impossible to pick out galled trees until they are lifted and the subterranean parts exposed. It is not clear whether any of the growth differences observed were the consequence or cause of crown gall infection.

On grafted trees there were indications of an interaction between stock and scion. Thus, stocks worked with Napoleon had significantly more galls than those worked with Roundel, and on Napoleon infection was associated with significantly less growth compared with healthy trees, whereas Roundel appeared much less affected by gall infection. There were also indications that growth differences were more closely associated with the position of galls than with their size.

None of the effects observed on tree growth, however, can be considered of major importance and the main economic importance of the disease appears to be more of a cosmetic nature. Similarly there is no clear evidence that crown gall affects the growth of the blackberry cv. Bedford Giant, although infection has reportedly decreased productivity of the Thornless loganberry clone (pers. comm.); an effect on growth and cropping of other less vigorous blackberry or loganberry cultivars cannot therefore be excluded.

It should, however, be emphasised that the above conclusions are based entirely on evidence of association and that there is no direct proof that crown gall affects growth. This can only be obtained by inoculation experiments with a high degree of replication, using several rootstock/scion combinations or cultivars and taking account of different infection sites on the hosts. However, some useful data should in the meantime be obtained from the planting of Napoleon and Roundel trees whose size and gall status at planting-out from the nursery are well documented.

SECTION III

BACTERIOLOGY

This study was motivated by epidemiological considerations with the aim of determining the biotypes of the organisms causing crown gall on cherry and blackberry and to see whether any host related characters could be distinguished. Therefore, only isolates which were pathogenic to test plants were included and compared with strains from other culture collections of the pathogen.

1. Media and Methods

a. Isolation and storage of cultures

i. Method As far as possible young galls were selected. They were quickly washed in running tap water then surface sterilised by immersion in 2% chloramine T or 0.5% sodium hypochlorite for 10 min and then washed thoroughly in sterile distilled water. Alternatively galls were dipped in 96% alcohol, the surplus liquid was shaken off and the gall flamed momentarily. Whole galls, if 5 mm diameter or less, or tissues shaved from the gall surface, were ground in a mortar or in a Colworth Stomacher, 80, in a suitable quantity of sterile distilled water. After a minimum of 2 hr, ten-fold dilutions were made and plated on selective media and/or nutrient agar. After up to 8 days incubation, colonies were selected for growth in pure culture and for testing for pathogenicity on greenhouse plants.

ii. Media a) non-selective NA, nutrient agar (0.8% Difco nutrient broth with 1.5% agar) was used.

b) selective. The selective media described by Patel (1926), Clark (1969), New & Kerr (1971) were used and the medium of Schroth, Hildebrand & Thompson (1965), less some of its antibiotic constituents.

After isolation, cultures were routinely stored on slopes of NA, or NA plus 2% glycerol, at 4°C.

b. Morphological, physiological and biochemical tests

Unless otherwise stated, media were adjusted to c. pH 7.0 and cultures were incubated at 25°C.

Flagella staining was by Garrett, Panagopoulos & Crosse's (1966) modification of Gray's (1926) method.

Colony form, Gram reaction, mode of utilisation of glucose, sodium chloride tolerance, nitrate reduction, and oxidase activity from growth on NA were all examined according to the methods described by Garrett et al. (1966).

3-ketolactose production was determined by the method of Bernaerts & De Ley (1963).

Utilisation of compounds as sole carbon sources was examined on the basal medium of Ayers, Rupp & Johnson (1919) with the test compounds incorporated at a final concentration of 0.1% (sodium formate, sodium malonate, sodium tartrate, leucine and raffinose) or at 0.5% (erythritol, inositol, mannitol, and sorbitol).

Citrate utilisation was tested by Simmons' (Cowan & Steel, 1965) method.

c. Pathogenicity tests for routine diagnosis and cross-infection

Inoculum was prepared from 1-3 day-old bacterial growth on NGA slopes suspended in sterile distilled water to give a moderately turbid suspension of c. 10^8 cells/ml.

Greenhouse plants were inoculated by pricking the stem with a sterile needle or fine scalpel immediately before application of the bacterial suspension or through a drop of suspension already placed there. Rooted blackberry cuttings cv. Bedford Giant were inoculated near the base of the stem in May; tomato plants cv. Moneymaker were inoculated, when c. 20 cm high, half way up the stem; sunflower seedlings were inoculated c. 11 days after sowing 2 cm above the cotyledons.

Field blackberries were prick inoculated in June or July near the tip of succulent young canes. Stems of potted F12/1 cherry rootstocks were surface sterilised with 70% alcohol and then prick inoculated as above.

Plants to which sterile distilled water was similarly applied served as controls in all experiments. All greenhouse plants were retained for one month and field and potted plants were observed for 3 months to allow symptom development.

d. In vivo growth of isolates

Young succulent canes of blackberry (cv. Bedford Giant) were inoculated c. 15 cm behind the tip with representative isolates of cherry, blackberry or loganberry strains of A. tumefaciens. Sterile distilled water applied to other canes served as controls. At intervals up to 128 days, the canes were sampled to estimate bacterial populations.

On each occasion from 3-5 replicate canes were sampled; the bark and prickles were removed from 2 cm portions of cane, with the inoculated area central, cut in half longitudinally and the inoculated halves ground in 10 or 20 ml of sterile distilled water. After 2 hr dilutions were made and plated on NA.

When galls were present at sampling, these were carefully removed, ground and treated separately from the cane tissue for estimation of bacterial populations.

e. Bacteriophage relationships

i. Media The media of Crosse & Garrett (1963) were used.

ii. Isolation methods a) from soil. The method of Crosse & Garrett (1963) was adopted and later modified in various ways such as using rhizosphere soil; adding the enrichment culture in phosphate buffer pH 7.0 or 6.3; incubating at 18° or 25° for periods of 1, 4 or 6 days and removing the bacteria from filtrates by centrifugation at 8,000 g for 20 min or passage through 0.22 μ millipore filters. b) from sewage. To 10 ml nutrient broth was added 0.1 ml sewage sample, untreated or after millipore filtration, and 0.1 ml of a 24 hr culture of A. tumefaciens. After 24 hr incubation the cultures were centrifuged at 8,000 g for 4 min and then either 0.1 ml of the resultant supernatant was spread on indicator plates or 0.5 ml of the supernatant was added to 18 hr phage broth cultures of indicators, incubated then centrifuged and the supernatant tested for phage on indicator plates.

c) from galls. Young galls were comminuted in a little phage broth and broth cultures of A. tumefaciens were added. After 48 hr incubation the preparations were centrifuged at 8,000 g for 4 min and the supernatants were tested for phage.

d) from lysogenic strains. Detection of lysogeny was by the method of Garrett & Crosse (1963).

Phage purification, preparation of high titre stocks, determination of routine test dilution (RTD) and method of storage were all according to methods previously described (Crosse & Garrett, 1963; Garrett & Crosse, 1963).

f. Antagonistic action against A. tumefaciens by bacteria from soil

Test isolates were streaked diametrically across the dried surface of NA or NA plus 0.2% glycerol plates. After 3-4 days incubation growth was scraped off and residual bacteria killed by inverting the plates for 30 min over chloroform-impregnated filter papers. The lids were left partly open for a further 30 min to allow traces of vapour to escape. Growth from 24 hr NGA slopes of indicator strains was then streaked at right angles to the producer strain and the plates were examined for inhibition after 24 and 48 hr.

Bacteriocin production was also examined by Stonier's (1960) method.

2. Results

a. Isolation

The pathogenic isolates obtained are listed in Table 22, together with isolates from other sources. Preparations from galls from Fl2/1 cherry rootstock layer

Table 22 List of pathogenic isolates of *A. tumefaciens* from cherry, loganberry and blackberry and of isolates from other sources

<u>Host</u>	<u>Source</u>	<u>Date of isolation</u>	<u>Prefix</u>	<u>Isolates numbers</u>
Cherry	layer beds	Nov. 1971	At	62-66
	" "	Mar. 1972	At	150, 154, 159 160, 188, 191
	maiden trees	Mar. 1976	At	210,- 213
Loganberry	Offham	Aug. 1972	AL	2-7, 10-11, 15
	"	May 1973	AL	20-22
Blackberry	Teynham	June 1975	T	98-9, 110-112, 116, 123, 125-8, 132
	Bifrons Gardens	May 1976	T	200-2, 205-6, 211, 213, 217
National Collection of Plant Pathogenic Bacteria			N	4, 396, 397, 925 [†] , 1001, 1641 [†] , 1649, 1650 [†] , 1651, 1674
International Collection of Phytopathogenic Bacteria [†]			I -	TT9, TT1, TR2 B6, 0362
A. Kerr, S. Australia			K	14, 17, 20, 24, 27, 29, 30, 34, 35, 84
C.G. Panagopoulos, Greece			Ag	1, 22, 23, 43, 53

[†] strains obtained via J. De Ley, Belgium.

beds in November 1971 were plated on NA and on Clark's medium and yielded fairly pure cultures on the latter. Some of the selected colonies were pathogenic to greenhouse test plants and Clark's medium was thereafter used in isolations from cherry galls. Between May 1971 and November 1972 from over 150 selected colonies, 12 were found to be pathogenic. A further 3 isolates were obtained in March 1976.

Samples of crown galls on loganberry bushes at Offham, Kent (see p. 60) taken in August 1972 and May 1973 yielded colonies on New & Kerr's medium 11 of which, from 24 selected, were pathogenic to test plants. NA and Patel's media yielded a variety of organisms from which selection of the pathogen was difficult.

None of 120 blackberry gall samples collected between May 1971 and May 1974 inclusive and plated on a variety of non-selective and selective media yielded isolates pathogenic to sunflower or potted blackberry seedling test plants although nearly 300 colonies were selected and tested. From 1975 onwards, only NA was used as the isolation medium and resulted in the selection of c. 100 colonies, 20 of which were pathogenic to greenhouse tomato test plants. No non-pathogenic isolates were retained for characterisation.

b. Morphological, physiological and biochemical tests

The cherry and the loganberry isolates were uniform in colony morphology and in reaction to the limited number of tests employed but the blackberry isolates varied (Table 23).

Table 23 Differential characteristics of 40 isolates of Agrobacterium tumefaciens from cherry, loganberry and blackberry

Isolates	Colony on NSA	NaCl tolerance (%)	Utilisation of							
			malonate	erythritol	formate	tartrate	mannitol	inositol	leucine	raffinose
Biotype 1†		4	-	-			+	+		+
Biotype 2		1	+	+			+	+		-
12 At*isolates	Levan	1 (one 4)	+	+	+	+	+	+	+	-
10 AL*isolates	Levan (2 raised)	1	+	+	+	+	+	+	+/-	- (AL20+)
18 T*isolates:										
T110, T128	Levan	1	+	+	+	+	+	+	+	+
T123	Levan	4	+	-	+	+	+	+	+	-
T215	Levan	1	+	-	-	-	+	+	-	+
T125	Raised	4	-	-	-	-	+	+	-	+
T112, T126	Raised	1	+	-	-	-	+	-	-	+
T127	Raised	1	-	+	-	-	+	-	-	-
T42	Raised	4	-	-	-	+	+	+	+	+
T200, T201,										
T202, T205	Raised	4	-	-	-	-	+	+	-	-
T206, T211, T213	Raised	4	-	-	-	-	+/-	+	-	-
T132	Raised	4	-	-	-	-	+		+	-
T116	Raised	4	-	-	-	-	-	-	-	-

† Differential characters of biotypes 1 and 2 as reported by Keane, Kerr & New (1970) and Panagopoulos & Psallidas (1973).

* At isolates, cherry; AL isolates, loganberry; T isolates, Blackberry of which T200-206 were from crowns, remainder from canes.

Strains of biotypes 1 and 2 were used as standard controls in all tests and gave the expected results (Keane, Kerr & New, 1970; Panagopoulos & Psallidas, 1973).

i. Common characters. All isolates were Gram negative and motile by peritrichous flagella; all metabolised glucose oxidatively, possessed oxidase activity and utilised citrate and sorbitol as sole sources of carbon. None gave a positive result in the 3-ketolactose test or reduced nitrates.

ii. Characters of cherry and loganberry isolates. These had a low tolerance of sodium chloride (1-2%) and utilised malonate, erythritol, formate, tartrate, mannitol, inositol and leucine but not raffinose as sole sources of carbon. With two exceptions (AL11 and AL20) they formed convex levan-type colonies on NSA. As far as was possible to make direct comparison, they therefore conformed to the description of biotype 2 of the pathogen.

iii. Characters of blackberry isolates. Four of the eighteen isolates produced convex colonies on NSA (T110, T123, T128, and T125). Two resembled biotype 2 in most tests but did not use raffinose as a sole carbon source (T110, T128). T123 agreed with biotype 2 except for sodium chloride tolerance and its failure to grow on erythritol, while T215 agreed with biotype 1 except for sodium chloride tolerance and its ability to grow on malonate. Only one isolate, T125, was typical of biotype 1 (although in common with all the isolates described it gave a negative reaction on 3-ketolactose). Ten isolates could be broadly grouped as biotype 1 although they differed in reaction on raffinose (T42), mannitol (T116, T123) and leucine (T42, T132).

Intermediate reactions were shown by T112, T126 and T127; these had a low sodium chloride tolerance, failed to utilise inositol and utilised malonate (T126), or erythritol (T127) but not raffinose (T127).

The heterogeneous group of blackberry isolates thus comprised one biotype 1 and many intermediates, three of which seemed closer to biotype 2 and ten of which were very similar to biotype 1. The type was not related to source of the isolates.

c. Pathogenicity and cross infection

In routine pathogenicity tests isolates produced visible galls on rooted blackberry cuttings in 10-28 days, on tomato in from 10-20 days and on sunflower in 7-10 days (Fig. 14). In general galls were induced more rapidly by cherry and loganberry isolates than by those from blackberry. In the field galls appeared on blackberry canes and cherry stems, in from 3 weeks to 3 months, according to the isolate and time of inoculation (Fig. 14). In cross infection experiments all cherry and loganberry isolates induced galls in all the four test hosts except At64 and At66 from cherry, which were negative in blackberry and AL7 from loganberry which was negative in sunflower and in cherry (Table 24).

The pathogenicity of the blackberry isolates was more variable. They all induced galls on tomato and blackberry and two (T112, T125) also did so on sunflower and cherry. In this respect they behaved like the biotype 2 isolates although they were intermediate and biotype 1 respectively. Six isolates, including two which appeared biochemically closer to biotype 2 (T123, T128), failed to induce galls on either sunflower or cherry and a further 4 gave a positive



A



B



C



D

Fig. 14 Galls on test plants. A, on a blackberry seedling; B, on a blackberry cane; C, on a tomato stem and D, on a sunflower stem.

Table 24 Cross infection of English isolates from
three hosts on four test plant hosts

Plant source	Isolates	Test hosts			
		Tomato	Sun- flower	Cherry	Black- berry
Cherry	13 isolates	+	+	+	+
	At64, At66	+	+	+	-
Loganberry	9 isolates	+	+	+	+
	AL7	+	-	-	+
Blackberry	T42, T116, T123, T127-8, T132	+	-	-	+
	T112, T125	+	+	+	+
	T126	+	+	-	+
	T110, T211, T213	+	-	+	+
	T201-2, T205-6	+	+	N.T.	+
	T214, T215	+	N.T.	N.T.	+

+, gall produced; -, no gall produced; N.T., not tested.

result on only one or other but not both of these hosts. Six isolates were not tested for reaction on cherry and/or sunflower (Table 24).

Thus differences in pathogenicity to even a limited range of test hosts was not correlated with source or biotype of the isolates.

d. Growth of *A. tumefaciens* isolates in blackberry

The isolation plates from canes usually yielded pure cultures of *A. tumefaciens* but at 72 days many saprophytic bacteria were encountered in canes inoculated with blackberry isolates. No bacteria, and few fungi, were found initially in control canes but saprophytic bacteria were isolated from some later samples.

The population trends of the pathogen in vivo are shown in Figs 15A and 15B. Populations of the loganberry isolates increased rapidly in the first 18 days and thereafter maintained a fairly steady level. Both cherry and blackberry isolates increased immediately after inoculation but from 8-41 days they declined, especially the blackberry isolate. However, at the final sample the populations of cherry and blackberry isolates were similar though both were lower than the initial level.

Galls first appeared on canes 36 days after inoculation with loganberry isolates. Attempts to reisolate the pathogen at this stage were unsuccessful but it was recovered from samples taken after 72 and 128 days (Table 25). One cane each of the 3 which were sampled 18 days after cherry and blackberry isolate inoculations had galls. When recorded 4 days later, 18th June 1976, 80% of the canes inoculated with blackberry isolates were

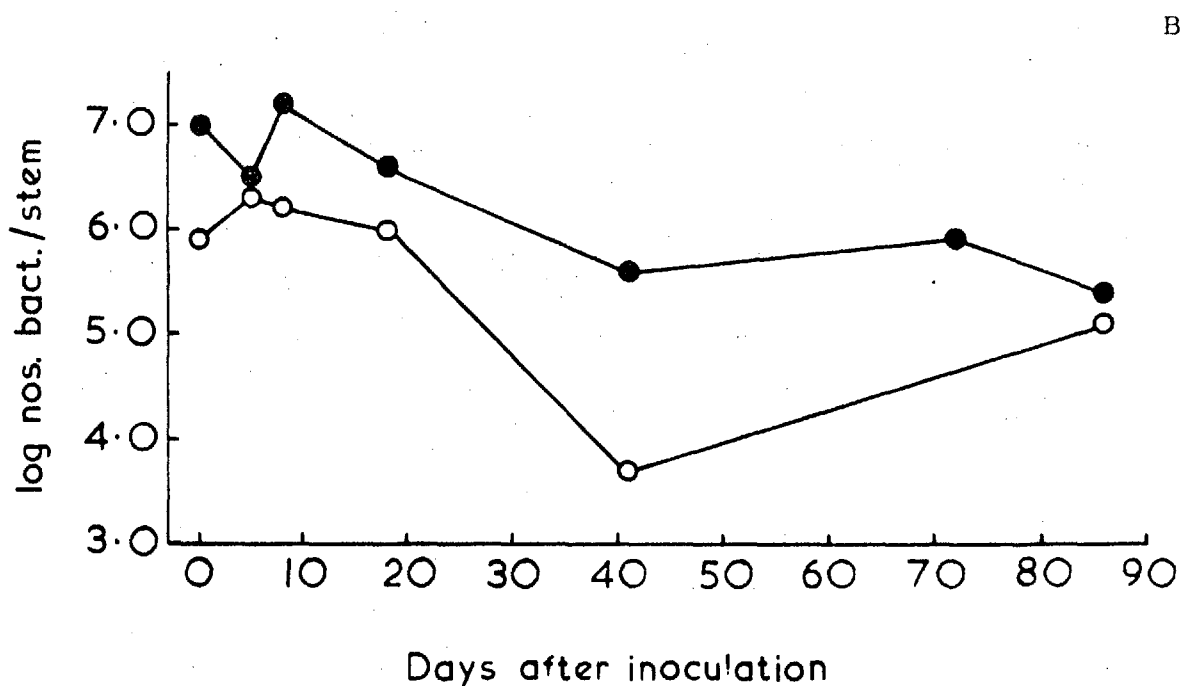
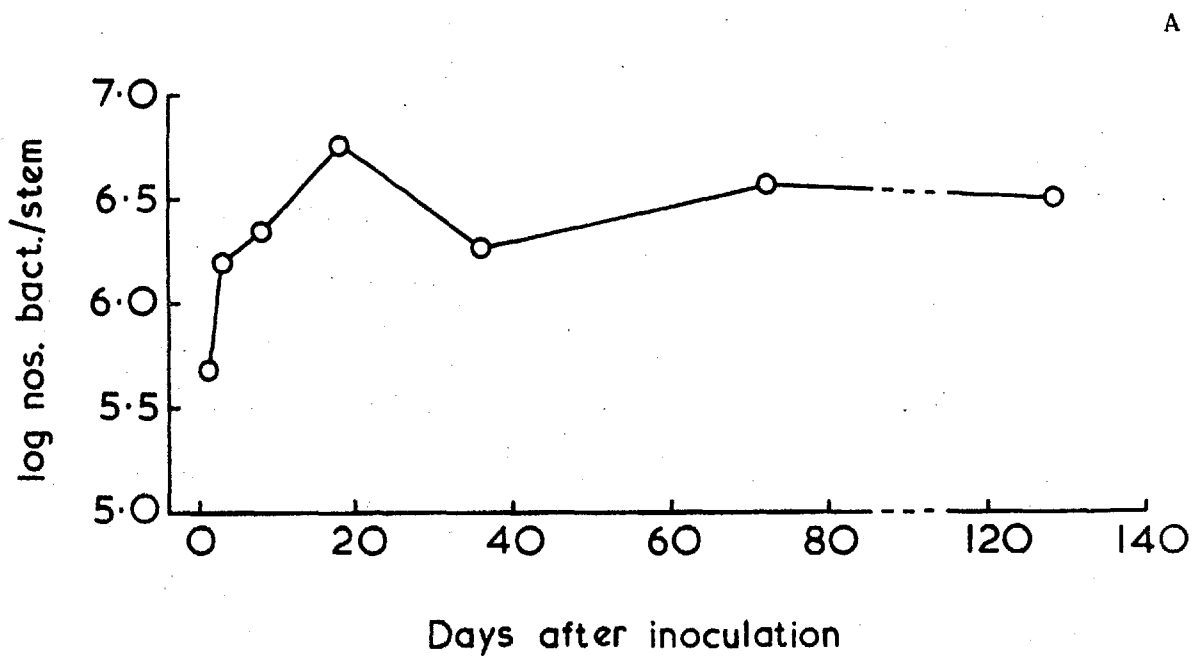


Fig. 15 Numbers of *Agrobacterium tumefaciens* in blackberry canes. A, loganberry isolates AL5 and AL21 inoculated 8th July 1975. B, cherry isolates At66 and At191 (●) and blackberry isolates T112 and T126 (○) inoculated 27th May 1976.

Table 25 Populations of *A. tumefaciens* recovered from
blackberry galls following cane inoculation
with English isolates from 3 hosts

Days after inoculation	Bacterial population/gall from canes inoculated with isolates from		
	loganberry (AL5, AL21, in 1975)	cherry (At66, At191, in 1976)	blackberry (T112, T126, in 1976)
18	-*	-	6.2×10^5
36 or 41	0	4.0×10^3	7.0×10^3
72	4.6×10^4	-	-
86	-	7.3×10^3	5.2×10^5
128	2.9×10^3	-	-

* -, no count made

galled compared with only 12% of those inoculated with cherry isolates. Pure cultures of the pathogen were isolated from most gall samples but the 72 day samples had high saprophytic populations. The population of the pathogen per gall appeared to be higher with blackberry isolates than with cherry isolates.

Thus, the pathogen was readily reisolated from the blackberry cane tissue although recovery of the homologous type was the least successful. The latent period for the homologous type was less than for the cherry isolates even though the latter reached higher population levels. Isolation from galls gave inconsistent results and proved more difficult than from cane tissue.

e. Bacteriophage relationships

1. Isolation of phages

Details of the phages isolated are given in Table 26.

One phage was obtained from cherry layer bed soil enriched with cherry isolates At150 and At159 using the routine method. Weak lysis resulted on plates of ITT9 indicator but not on the two enrichment strains. No other phages were obtained by any of the modified procedures adopted from 12 other samples from soil in which galled cherries were growing.

More than 20 phages, at least 13 with a different host range, were isolated from sewage samples using various A. tumefaciens isolates for enrichment. The two isolation methods used were equally successful.

The phage isolated from gall tissue follow^{ed} the addition of strain K14 (Australia) to a ground blackberry gall using ICPB TT9 and 0362 (Belgian isolates) as indicator strains.

Table 26 List of phages obtained which showed some activity against isolates of A. tumefaciens

Phage no.	Source	Enrichment strain	Indicator strain
P1	sewage, Aylesford	K 14	K 14
P2	" "	B6	N 397B
P4	" Malling	N 1649	K 14
P7	" "	N 1649	N 1649
P10	" Aylesford	B6	B6
T1	" "	B6	B6
T6	" Malling	N 397B	N 1649
T7	" Aylesford	N 1649	N 1649
TT9A	" "	I TT9	I TT9
TT9C	" Canterbury	I TT9	I TT9
TT9M	" Malling	I TT9	I TT9
TT9R	" Rochester	I TT9	I TT9
A14	" Aylesford	K 14	K 14
150M	" Malling	At150	At150
159M	" "	At159	At159
159Crt	soil, E. Malling	At159	I TT9
14g	gall, blackberry	K 14	I TT9
B6/N 397b Strain B6			N 397B
N 1001/N 397b Strain N 1001			N 397B

Lysogeny was detected in the standard reference strain D6 and also in NCPPB 1001 but not in isolates from cherry (9) or loganberry (11) or in 14 other isolates of which 10 were Australian.

ii. Phage sensitivity tests. Forty-seven isolates of A. tumefaciens were tested with 19 phages at RTD. Twenty-two isolates, including all the English cherry and loganberry isolates tested and all those from Greece, were insensitive to all phages. Results for the remaining 15 isolates are shown in Table 27.

Phage P10 lysed 10 of the isolates but other phages were more specific and four of them lysed only one isolate. A different pattern of phage sensitivity was shown by each isolate except for the Australian strains K14 and K35. The blackberry strains were isolated after the typing was completed and have not been tested.

f. Antagonistic action against A. tumefaciens

i. Bacteriocin activity. In initial tests 47 isolates (10 cherry, 8 loganberry, 15 from the NCPPB and ICPB, 9 Australian and 5 Greek) were examined as producer and indicator strains in reciprocal tests on NA plus 0.2% glycerol medium. Only 4 Australian isolates, including A. radiobacter var. radiobacter strain 84 of Kerr, were found to be bacteriocinogenic. The sensitivity of the other strains to the bacteriocins produced is shown in Table 28.

The composition of the growth medium for detecting bacteriocin production was reported to be critical by Kerr (pers. comm.) who recommended Stonier's (1960) medium. When this medium was subsequently used all of 10 English

Table 27 Sensitivity of 15 strains of A. tumefaciens to 19 phages

Strain	Phages (at Routine Test Dilution)																		
	P1	P2	P4	P7	P10	T1	T6	T7	TT9A	TT9C	TT9M	TT9R	159Crt	150M	159N	A14	14G	B6/N 397B	N 1001/N 397B
N 396	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
N 1674	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
N 397B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N 1649	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N 1001	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N 4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I TR2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I TT9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I TT11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0362	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K 14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K 17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K 24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K 35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

-, isolate insensitive to the phage; +, isolate sensitive to the phage giving confluent or nearly confluent lysis.

Table 28 Sensitivity of isolates of Agrobacterium to bacteriocins produced by 3 Australian isolates of A. tumefaciens and one of A. radiobacter in NA + 0.2% glycerol medium

<u>Indicator strains</u>	<u>Bacteriocins produced by strains</u>			
	K14	K17	K35	K84
N1001, N1641	+	+	+	-
N396, N1649, N1674	+	-	+	-
N4, N397B	+	-	-	-
N397A, N925, N1650, N1651	-	-	-	-
I TT9, O362	+	+	+	-
B6	-	-	+	-
I TR2	-	-	-	-
K34	+	+	+	+
K84	+	+	+	-
K20	+	+	-	+
K24, K29, K35	-	+	-	+
K14	-	-	-	+
K17, K27	-	-	-	-
10 cherry isolates	U.K.	-	-	-
8 loganberry isolates				
5 isolates	Greece	-	-	-

cherry isolates tested and 8 cane fruit isolates were found to be sensitive to bacteriocin 84 (Fig. 16). Growth of other isolates on Stonier's medium was too poor to evaluate their sensitivity to bacteriocin 84.

ii. Antagonism by soil organisms. The lack of sensitivity of English isolates of A. tumefaciens to bacteriocin 84 in initial tests prompted a search for other organisms antagonistic to the pathogen. Fifty-eight bacterial antagonists were eventually isolated from soils (Table 29) and most of those with fairly strong activity (Fig. 17) were identified as pseudomonads. One isolate, Z80, showing marked antagonism against cherry isolates of A. tumefaciens in the preliminary screen was tested against other bacteria. It strongly inhibited 10/17 A. tumefaciens and 10/13 A. rhizogenes isolates and also single strains of Pseudomonas fluorescens, P. morsprunorum, P. syringae, Erwinia amylovora and Xanthomonas albilineans. Single strains of P. marginalis, E. herbicola and Escherichia coli were not affected.

In mixed culture plates of A. tumefaciens (cherry isolate At150) with Z80 at a 1:1 ratio the soil organism reduced both number and size of colonies of the pathogen by more than 50%.

Although the activity of Z80 was non-specific its inhibitory effect on A. tumefaciens was considered worthy of further investigation and it was therefore included in some in vivo tests (see p. 152).

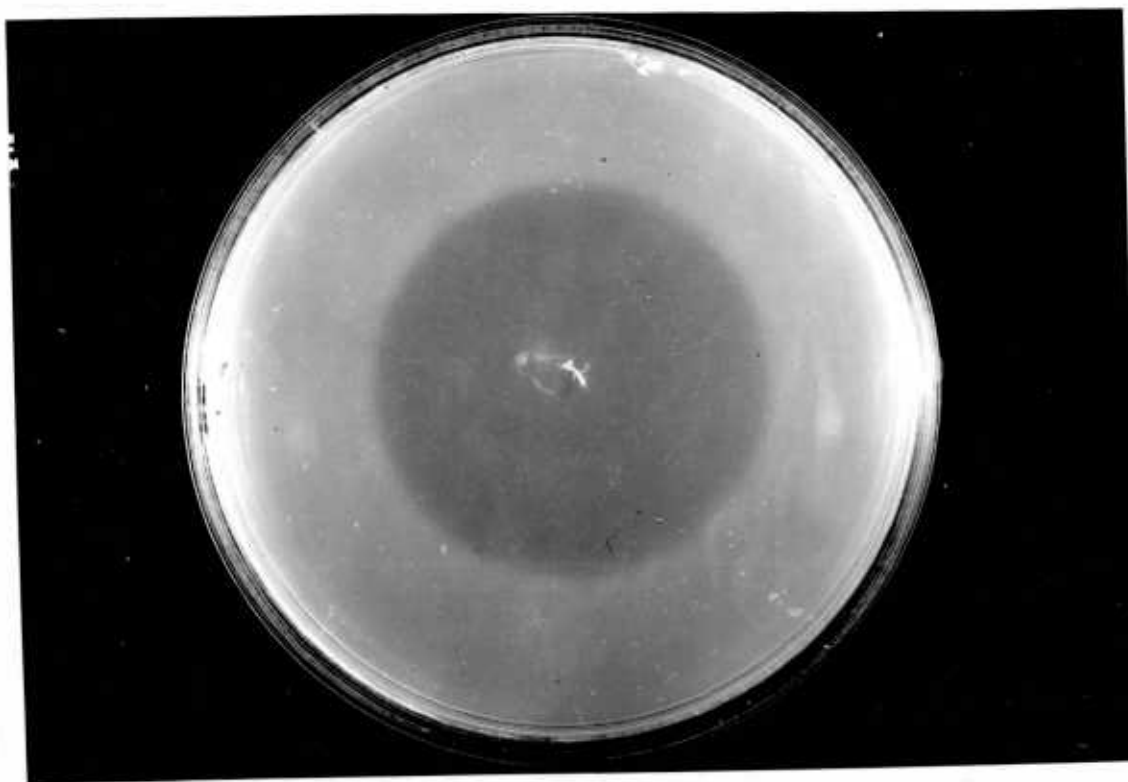


Fig. 16 Bacteriocin production by a colony of Agrobacterium radiobacter strain 84 at the centre of the plate resulting in a zone of inhibition in the growth of A. tumefaciens.

Table 29 List of organisms found to be antagonistic
to the growth of A. tumefaciens in vitro

Isolates		Date isolated	Source	Isolates identified
Culture no.	total			
Z1-7	7	Jul. 1974	pea soil	3 pseudomonads
Z14-19	6	May 1974	cherry soil (orchard)	2 pseudomonads 3 actinomycetes
Z20	1	May 1974	cherry soil (layer bed)	actinomycete
Z30-33	4	Oct. 1974	cherry soil (boxed)	3 pseudomonads
Z34-35	2	Nov. 1974	raspberry gall	2 coliforms
Z42-68	27	Jan. 1975	blackberry soil	11 pseudomonads
Z70-80	11	Mar. 1975	cherry soil (pots)	5 pseudomonads

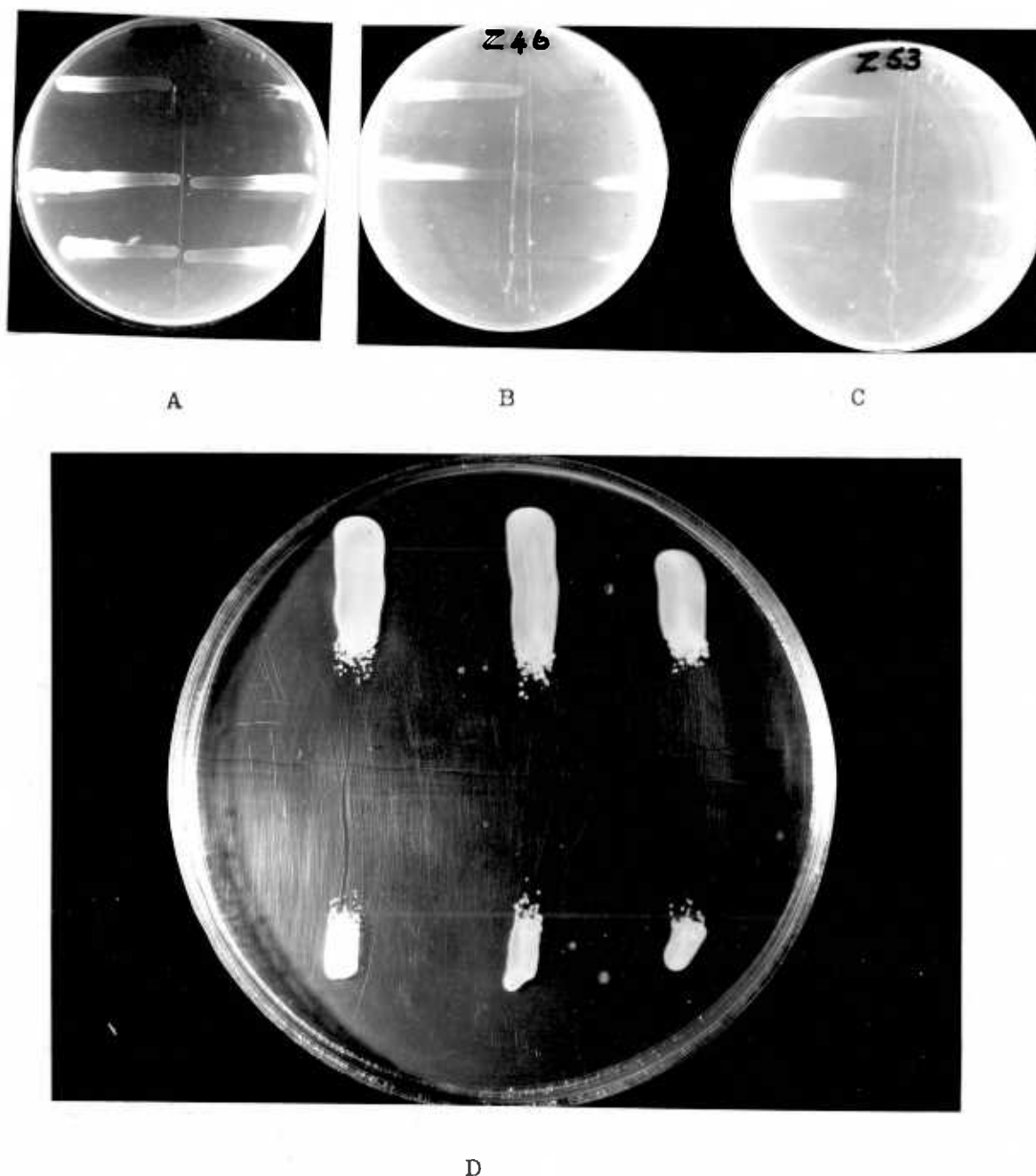


Fig. 17 Inhibition of growth of test isolates of Agrobacterium tumefaciens at right angles to diametrically streaked soil pseudomonads. Activity against 6 loganberry isolates from A, Z42; B, Z46; and C, Z53. D, activity of Z80 against 3 cherry isolates (At65, At150, At160)

DISCUSSION

A major initial difficulty in this work was the isolation of the pathogen from blackberry. The selective media used were either too selective, containing inhibitors or lacking essential nutrients, or insufficiently selective allowing overgrowth of the pathogen by saprophytes.

Nutrient agar proved the most valuable for isolation; this medium is also routinely used for preference by Panagopoulos & Psallidas (1973) and by both M. Ridé and R.A. Lelliott (pers. comm.) although some selective media may also be useful. The results of in vivo growth tests showed that the pathogen could not always be isolated readily from galls, especially very young or old ones.

Proof of pathogenicity of selected isolates was for a time determined exclusively on sunflower but the advantages of this indicator, used 11 days after sowing and taking up minimal glasshouse space, were offset by the fact that not all blackberry isolates induced galls on this host which probably contributed to the earlier failure to obtain the pathogen from blackberry.

The fact that they came from a single locality, were isolated on one selective medium, and were further selected by pathogenicity tests in a restricted range of hosts, probably contributed to the homogeneity observed amongst cherry & loganberry isolates. Examination of isolates from a wider range of localities using more varied techniques may reveal greater diversity than was apparent in this work.

The blackberry isolates were obtained from two plantations but the source of the original planting material was the same. The heterogeneity among these isolates may reflect isolation on a non selective medium, involvement of a host factor or infection with strains from the soil, although there is no evidence for the latter. Variation among these isolates was not related to type or site of gall or geographical locality.

It might be argued that the blackberry isolates should be assigned to the species A. rubi, created by Hildebrand (1940) to accommodate the organism which formed elongated galls on raspberry canes and differed from crown gall in its restricted host range and some physiological characteristics. The earlier fairly lengthy description of this species in Bergey's Manual (7th edition, 1957) has been replaced (8th edition, 1974) by only brief comment on host range and symptoms. Recent studies on the taxonomy of agrobacteria, based on DNA homology and numerical taxonomy do not provide any justification for maintaining separate specific rank for this organism (De Ley et al., 1966; White, 1972). McKeen (1954) concluded that although there are differences in host range and symptom expression between the crown gall and cane gall organisms there are also intermediate types. The blackberry isolates studied in this investigation are therefore considered to be forms of A. tumefaciens; they do not conform exactly to descriptions of biotypes 1 and 2 nor to the limited descriptions (Kersters, De Ley, Sneath & Sachin, 1973) of the so-called 'species' A. rubi. The majority, however, were relatively slow growing organisms with specific, but so far undetermined, requirements for

optimum growth and in these respects they resemble the type referred to as A. rubi.

Although the blackberry and loganberry plants studied here originated from the same commercial nursery, the infections on them did not have a common source since the bacteria isolated from the two host plants were different biotypes. It is likely that they are host adapted forms of the pathogen that are transmitted from generation to generation on the propagating material.

Contrary to experience with the aerial pathogens Pseudomonas morsprunorum and Erwinia amylovora (Crosse & Garrett, 1963; Billing, Crosse & Garrett, 1960) the isolation of phages for A. tumefaciens from soil, its natural environment, proved difficult. Sewage was the best source of phage but, as others have found (Roslycky, Allen & McCoy, 1962; Boyd, Hildebrandt & Allen, 1970) no phages lysed many strains of the pathogen and a large proportion of isolates were insensitive to all phages. Lysogeny was detected in two strains only; only 8% of over 130 strains examined by other workers have been shown to be lysogenic (Roussaux, Kurkdjian & Beardsley, 1968; Kurkdjian, Beardsley & Manigault, 1968), the temperate phages having a narrow activity spectrum (Zimmerer, Hamilton & Pootjes, 1966; Böttcher, 1972). The poor results obtained using English cherry isolates as enrichment cultures coupled with their insensitivity to the various phages isolated indicated that prospects for diagnosis of the pathogen by phage or the erection of a phage typing scheme for strain differentiation were extremely limited.

The study of phage relationships was therefore discontinued.

Production of bacteriocins by plant pathogenic bacteria had been little studied before Matthews (1964) and Panagopoulos (1966) detected activity among certain host-adapted forms of Pseudomonas morsprunorum and P. syringae respectively. The correlation between bacteriocin production by A. radiobacter strain 84 and biological control of the pathogen (Kerr & Htay, 1974) has stimulated interest in this field. On the simple nutrient medium routinely used for pseudomonads, bacteriocin production by strain 84 against other Australian isolates was readily confirmed but gave misleading negative results against English cherry isolates. However, although Stonier's medium gives clear unequivocal results with many isolates, it is not a universally good medium for the detection of bacteriocin sensitivity as a number of blackberry isolates (and peach isolates, Lopez, pers. comm.) grow very poorly on the medium although Kerr & Htay (1974) found no difficulty with either biotypes 1 or 2. The strong in vitro antagonism by the soil organism 280 is non-specific and therefore due to some unidentified toxic metabolite and not to bacteriocin. However, it is clear that within the soil environment are many organisms, particularly pseudomonads, with potential antagonistic activity but their role, if any, in the development of crown gall disease remains to be elucidated.

SECTION IV

PLANT INOCULATIONS

A. CHERRY

1. Seasonal variation in susceptibility and gall development on cherry rootstocks

F12/1 cherry rootstocks, potted in spring 1972, were inoculated with Agrobacterium tumefaciens (NCPPB 1651 and 8 East Malling cherry isolates) either (a) by pricking the stems c. 5 cm above ground level with a sterile needle and depositing inoculum on the wounded area, or (b) by wounding the exposed, washed stem a little below ground level with a file and depositing the inoculum on to the surface of the damaged tissues before the soil was replaced. Nine replicate trees, one for each isolate, were inoculated at approximately monthly intervals from April 1972 to July 1973 inclusive.

a. Rate of infection

The percentage of stocks developing galls by January 1974 from the prick inoculations is given in Fig. 18A. The rate of infection was generally high during the period of active growth, from April to August 1972. With the approach of dormancy the rate declined to a minimum in December and then increased again to a maximum in March 1973. It remained at this level until July. Results from the larger subterranean file wounds followed a similar pattern (Fig. 18B)

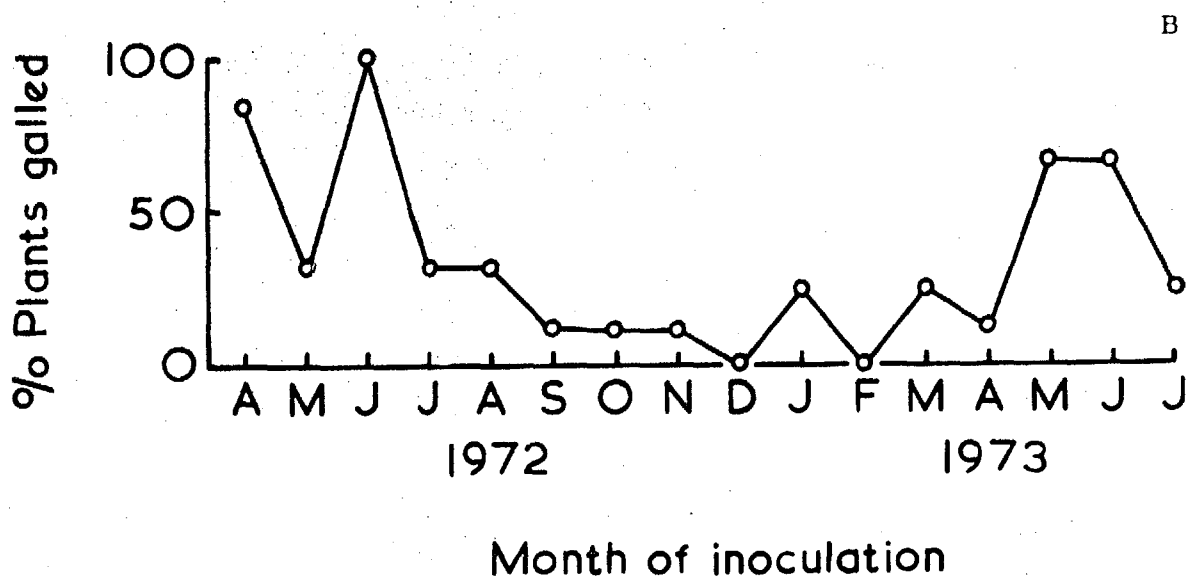
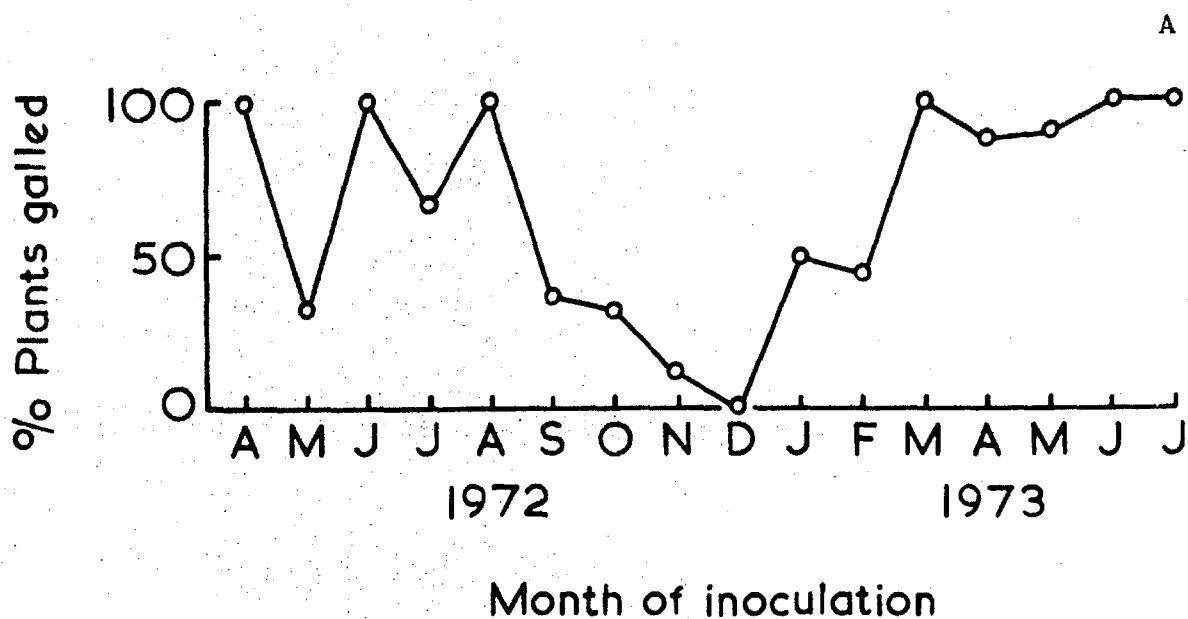


Fig. 18 Effect of time of inoculation on Fl2/1 cherry rootstocks with Agrobacterium tumefaciens on the incidence of crown gall. Inoculations were made at monthly intervals, from April 1972 to July 1973 inclusive, to A, stem pricks 5 cm above ground level and B, subterranean wounds.

but the rate of infection was generally lower than for the stem prick inoculations; from September 1972 to April 1973 inoculations it did not exceed 25% and reached 100% on only one occasion, in June 1972.

b. Latent periods

These are shown for stem prick inoculations in Fig. 19. The latent period was shortest between February and June, with a minimum of approximately 9 weeks in May. From July onwards the latent period increased to a mean maximum of approximately 10 months for the August to December inoculations.

c. Rate of gall enlargement

This was generally most rapid from May and onwards throughout the growing season (Fig. 20). With the approach of tree dormancy in autumn the rate declined and was virtually nil during the winter. Gall expansion resumed in the following May or June.

d. Gall size

When the final records were taken in January 1974, the largest galls (mean diam. 41 mm) from prick inoculations were associated with the earliest inoculations, i.e. those made from April-June 1972. Gall size was not, however, a simple function of the developmental time since it was also influenced by seasonal variations in the rate of gall enlargement. Galls from spring 1973 inoculations were similar in size (mean diam. 30 mm) to those resulting from inoculations made in late summer 1972. Galls from the latter

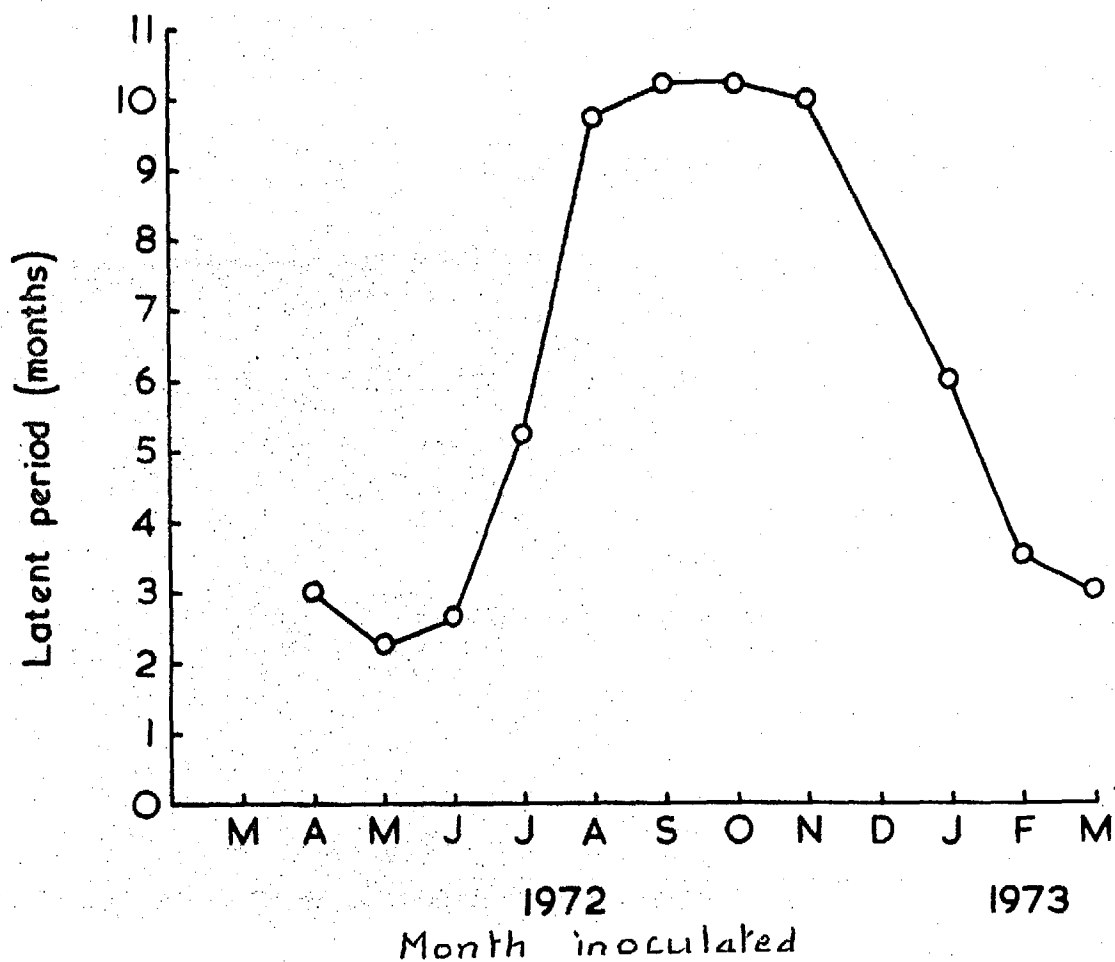


Fig. 19 Mean latent period in F12/1 cherry rootstocks prick inoculated with Agrobacterium tumefaciens at monthly intervals from April 1972 to July 1973.

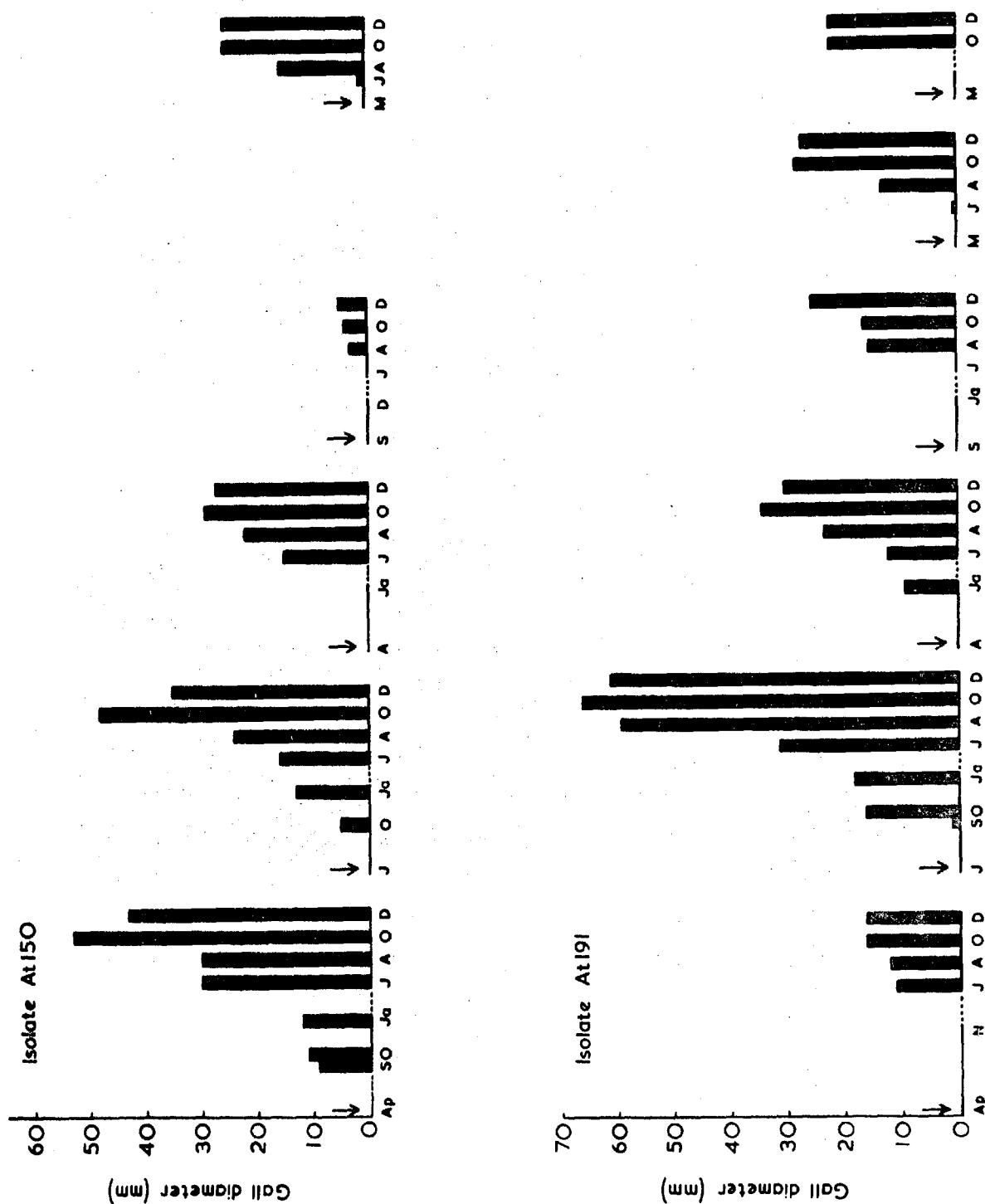


Fig. 20 Rate of gall enlargement resulting from inoculation of Fl2/1 cherry stems at monthly intervals with Agrobacterium tumefaciens. Data shown for isolates At150 and At191 for selected months only. ↓, month inoculated.

inoculations were slow to develop and those that appeared by the end of 1972 were generally small. Their development in the following year was markedly slower than that of galls from the spring inoculations. The size of galls resulting from June-August 1973 inoculations was proportional to the developmental time and was smallest (6 mm diam.) for the later inoculations.

Results for subterranean galls at file wounds were generally similar, except that gall size varied less with time of inoculation.

Typical galls from prick and file inoculations are shown in Fig. 21.

2. Susceptibility of different types of wounds

In naturally infected layer beds there is a high incidence of galls at shoot scars and at wounds occurring on bends. The susceptibility of these sites at different times during autumn and winter was investigated using potted or boxed Fl2/1 cherry rootstocks.

a. Simulated shoot scars

To simulate the scars left when shoots are harvested from the layer beds, potted rootstocks were cut back to 4-6 cm at monthly intervals from August to December 1973 inclusive and inoculated immediately after cutting. Four replicate stocks were inoculated each month with each of isolates At65, At150 and At160 of A. tumefaciens. Two stocks were treated with sterile water on each occasion to serve as controls. Leaf fall preceded the October



Fig. 21 An F12/1 cherry rootstock in January 1975 with galls resulting from inoculation of stem prick and subterranean wounds with Agrobacterium tumefaciens isolate At150 in June 1973.

inoculations. Results are shown in Table 30. Stocks were most susceptible in August and September when there was 100% infection. Susceptibility decreased markedly in October, after leaf fall, and then increased again in November and December.

All stocks inoculated in August developed galls within a month and although some leafed-out in the spring, many were dead by early June. Galls from September inoculations did not develop until late April of the following year, although some At65 inoculations resulted in galls by January. First galls from the later inoculations were not seen until late April but they continued to develop throughout the summer. The galls initially developed around the periphery of the cut surface but soon enveloped the whole area (Fig. 22A).

b. Layer bends

To simulate layer bends, 6 rootstocks were planted at one end of bushel boxes and bent through holes in the opposite end. At approximately monthly intervals from December 1973 to March 1974 they were bent further and secured horizontally by tying the ends to a cross-strut in the box. They were inoculated, immediately after the second bending, through natural cracks or file wounds with A. tumefaciens. Two stocks were treated with water on each occasion to serve as controls.

The first galls were not detected until the end of April 1974. After 20th May galls appeared at frequent intervals, particularly on stocks inoculated in March (Fig. 22B). Galls were normally located at the wounded area on the upper side of the stock, but occasionally they

Table 30 Percentage galled stocks resulting from inoculation to rootstocks cut back at monthly intervals throughout the autumn
(Recorded in August 1974)

Month inoculated (1973)	% stocks galled (out of maximum of 12)
August	100
September	100
October	42
November	58
December	87

Table 31 Percentage infection and mean gall diameter resulting from inoculation of boxed Fl2/1 cherry rootstocks at bends at intervals during the winter
(Recorded in July 1974)

Time of inoculation†	% stocks galled	mean gall diam. (mm)
20th December 1973	65	7.5
11th January 1974	25	6.3
14th February 1974	58	8.0
5th March 1974	83	9.2
13th March 1974	75	11.6

† a mixed inoculum of cherry isolates of A. tumefaciens
At65, At150, At160 at a concentration of c. 10^8 cells/ml



A



B

Fig. 22 Galls on F12/1 cherry rootstocks in August 1974 resulting from inoculation with Agrobacterium tumefaciens.

A, potted rootstock inoculated in September 1973 on the cut surface, to simulate the shoot scar
B, boxed rootstocks tied down horizontally and inoculated at the bend in March 1974.

developed on the under-side only or on both sides.

Stocks were most susceptible in March and least susceptible in January and February (Table 31). Galls, recorded in July 1974, were smallest from the December and January inoculations but were larger as time of inoculation approached resumption of active plant growth.

1. Conclusions These experiments show that cherry rootstocks are susceptible to infection at any time of year but that susceptibility is greatest when the plants are meristematically active, i.e. during the growing season, and are least susceptible to infection during dormancy. The rate of gall development was also greatest during the period of meristematic activity.

Gall size cannot be taken as a good indication of when infection has occurred in field plants. It is evident that gall size is not simply related to developmental time. The size of the gall is also influenced by seasonal variations in rate of development, by the nature of the wound through which the pathogen enters and by the location of the gall, which governs the conditions under which the gall develops, i.e. whether on the aerial or subterranean parts of the plant.

The investigation into the susceptibility of wounds simulating layer bed shoot scars and bends at different times suggests that the current times for harvesting stocks from the layer beds and for pegging down new layers are probably optimum from the point of view of minimising crown gall infection. There would therefore be no advantage in altering the timing of these operations.

3. Leaf scars as an experimental avenue of infection on cherry

The majority of galls caused by A. tumefaciens on cherry trees occur below ground level and are only seen when the trees are transplanted or grubbed. Although galls on cherry can readily be induced through wounds, the limited degree of replication possible in wound inoculation is a major restraint in infectivity titrations with the crown gall organism.

In bacterial canker of cherry caused by Pseudomonas morsprunorum leaf scars are a natural avenue of infection, and leaf scar inoculations have proved a valuable and sensitive method of disease assay in this system because of the exceptionally high level of replication possible on comparatively few experimental trees. It was therefore decided to investigate the possibility of using leaf scar infections for disease assay in crown gall. An added advantage was that galls would be readily visible and thus development could be continuously monitored.

a. Preliminary experiment

Leaf scars on fruiting spurs of F12/1 potted cherry rootstocks were inoculated on 7 occasions between June and September 1974 and the number and 'volume' of the developing galls recorded over the next 18 months. Gall volume was measured as the product of width x height x depth and this was considered accurate enough for comparative purposes. The results of the experiment are summarised in Table 32. All the June inoculations resulted in galls and there was a generally high level of infection until September when it fell to g. 10%. Galls from the June and July inoculations

Table 32: Inoculations of cherry leaf scars with *A. tumefaciens*

Date *	no. spurs inoculated	no. infected				% infected	log. mean ** gall volume
		30.9.74	23.1.75	27.5.75	18.6.75		
June 24	12	8	10	12	12	100	4.101
July 4/7	18	15	16	16	16	88.9	4.559
July 12	12	5	5	7	7	58.3	4.058
Aug. 2	6	0	0	4	4	66.7	3.718
Aug. 23	6	0	0	4	5	83.3	4.140
Sept. 30	6	-	-	1	1	10.6	3.527

* mixed inoculum of isolates At65, At150, At160

** log of product gall diameter x height x depth in June 1975

were mostly visible in September, but those from the later inoculations did not develop until April of the following year (Fig. 23).

Galls increased progressively in size throughout the growing season but were virtually static from the onset of leaf fall and during the dormant season (Fig. 24 A & B). Occasionally, however, galls made little or no growth during the summer (gall C on tree 14) or had a relatively static period in mid-summer (gall D on tree 46). When the final record was taken, the three largest galls were on spurs inoculated on 4th July 1974, and many (94%) of the spurs inoculated at this time had strong shoots growing out through the centre of the galls. Galls from August and September inoculations were generally smaller; none developed shoot outgrowths.

b. Effect of inoculum dose on leaf scar infection

A logarithmic series of inocula in the range 10^1 to 10^9 cells/ml was applied to leaf scars on potted 2-year-old F12/1 cherry rootstocks on 30th May 1975; field trees of Roundel were similarly inoculated on 4th June. The final record of gall development was made on 21st October.

i. Rate of infection No galls developed on either the F12/1 rootstocks or Roundel trees with inoculum doses of 10^4 cells/ml or less. The percentages of galled spurs resulting from higher doses of inoculum followed typical sigmoid dosage/response relationships (Table 33). When the log inoculum dose was plotted against the probit a straight line resulted. The data for Roundel are given in Fig. 25 from which the EC 50 can be estimated at c. log 6.5.



A



B



C

Fig. 23 Galls arising from inoculation of leaf scars of F12/1 cherry rootstock with Agrobacterium tumefaciens.

A and B, inoculated on 12th July 1974 with isolate At65, photographed 9 weeks later.

C, inoculated with isolate At150 on 28th June 1973 photographed 31st January 1975, in greenhouse.

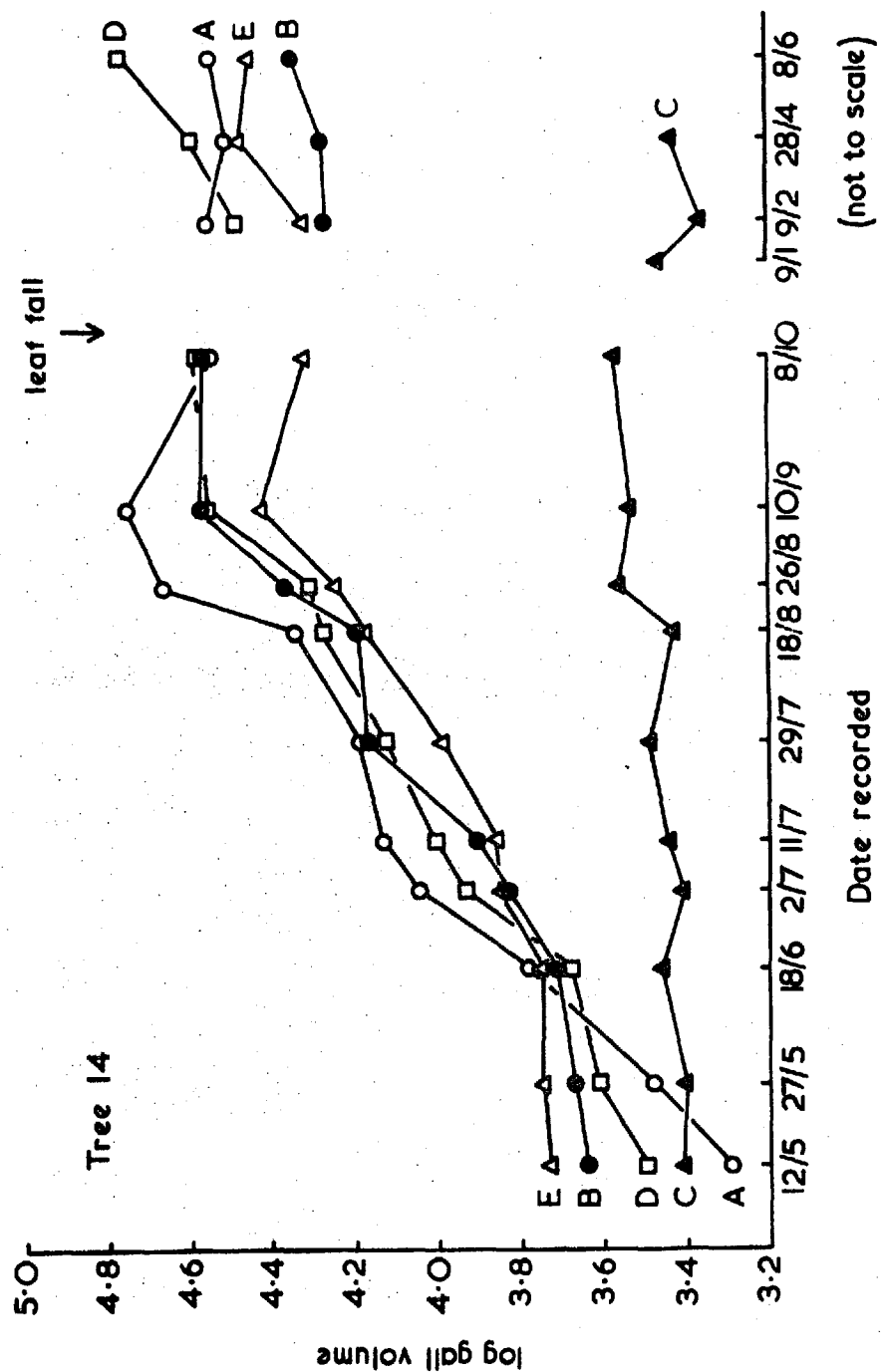


Fig 24a Growth of 5 galls resulting from leaf scar inoculation with Agrobacterium tumefaciens on each of two F12/1 potted cherry rootstocks. Tree 14 inoculated 4th July 1974.

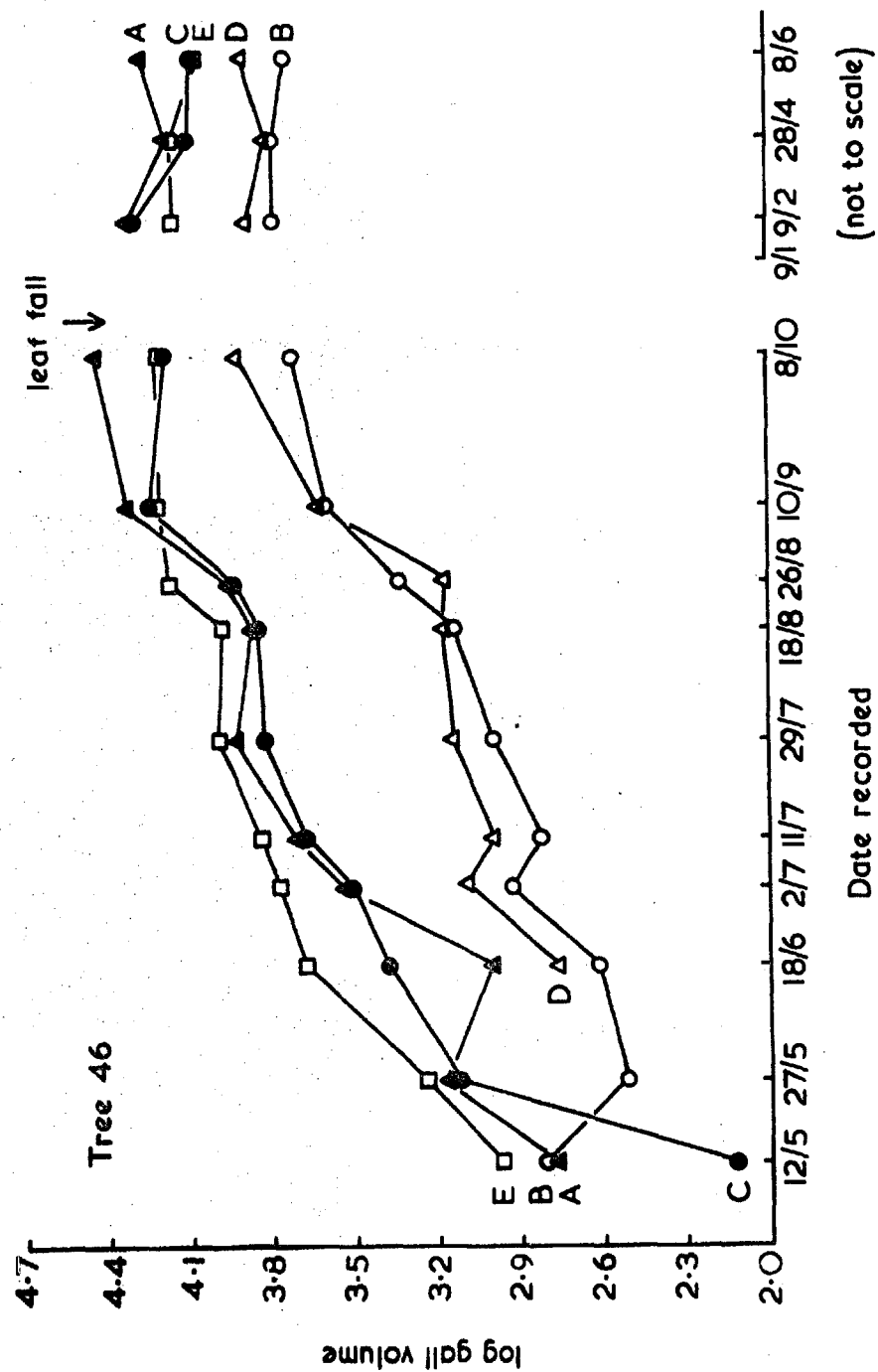


Fig. 24b Growth of 5 galls resulting from leaf scar inoculation with Agrobacterium tumefaciens on each of two F12/1 potted cherry rootstocks. Tree 46 inoculated 23rd August 1974.

Table 33: Percentage infection of cherry spurs from inoculation with a log series of *A. tumefaciens*

Bacteria† cells/ml	% spurs infected on	
	(a) F12/1	(b) Roundel
10^1 - 10^4	0	0
10^5	18.57	7.5
10^6	33.33	24.5
10^7	44.00	57.1
10^8	41.67	86.8
10^9	N.T.	82.1

† a mixed inoculum of *A. tumefaciens* isolates At150
At159 and At160

(a) each inoculum dose applied to 6-10 spurs on each
of 3 branches on different trees

(b) each inoculum dose applied to 6-11 spurs per branch
on each of 5 replicate trees.

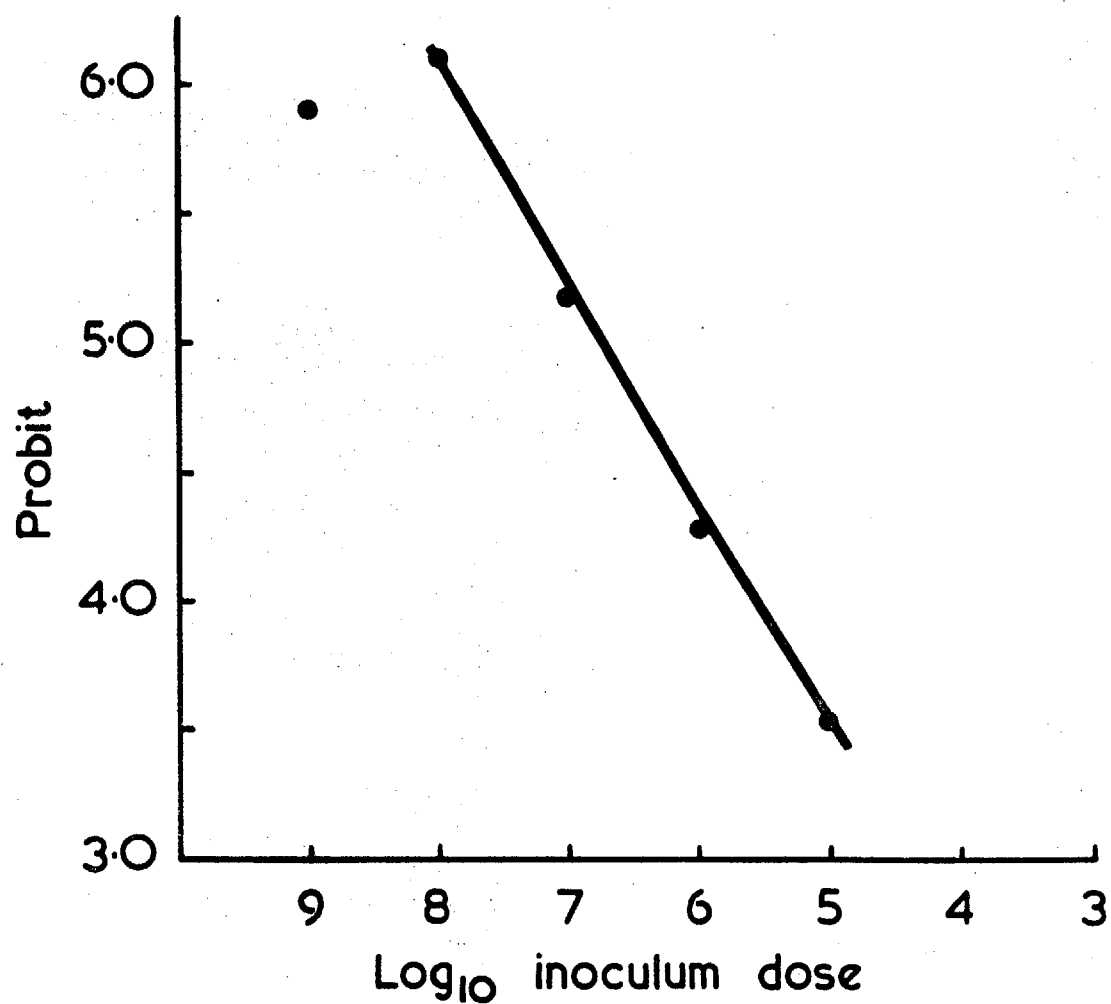


Fig. 25 Probit of cherry leaf scar infections in relation to log inoculum dose of Agrobacterium tumefaciens.

ii Latent period On Fl2/1 the first galls were noted 7 weeks after inoculation, on 17th July. By 21st August, of the spurs which were to develop galls from inoculum doses of 10^7 and 10^8 cells/ml, 70% were already positive but only 33% and 20% of those which were to develop following inoculation with 10^6 and 10^5 cells/ml respectively.

On Roundel the first record was taken on 12th August when over 90% of the positives were evident on spurs inoculated with the two highest inoculum concentrations and by 21st August about 60% of those inoculated with 10^5 cells/ml were present.

Conclusions The minimum threshold concentration of inoculum for infection was between 10^4 - 10^5 cells/ml for both the Fl2/1 rootstocks and Roundel. The minimum dose for infection is dependent upon the volume of inoculum that penetrates the leaf scars and, although this is not known precisely, it is probably very small. Infection, therefore, might have originated from comparatively few bacterial cells, suggesting that this method is quite sensitive. The relation between inoculum dose and latent period may reflect the time necessary for a threshold level of bacterial population to be reached within the host tissues.

The dosage/response relationship between inoculum concentration and rate of infection parallels the result one obtains with P. morsprunorum in cherry. It also confirms that this method could prove very sensitive for disease assay, particularly at inoculum concentrations

near the LD 50 when maximum changes in infectivity, as for example resulting from the inclusion in the inoculum of an antagonistic organism, are likely to be obtained.

4. Susceptibility of new clonal cherry rootstocks to crown gall

The breeding programme at East Malling includes the selection of new rootstocks for both sweet and ornamental cherry to replace the vigorous clone F12/1. Some of the more promising selections have been tested for susceptibility to crown gall infection. They included Colt (Fb2/58/21), a semi-dwarfing rootstock for sweet cherries that is now being extensively propagated for distribution to the stone-fruit industry.

From 7-10 replicate plants from each new clonal selection, and 4 standard F12/1, were inoculated through prick wounds at ground level on 24th May 1974 and the final results assessed on 12th December (Table 34).

All the inoculations were successful and the clones differed only in gall size and rate of gall development. On these criteria all the Fb2/58 series of clones were more susceptible than F12/1. Among the clonal selections the smallest galls occurred on nos. 22 (Cornflower) and 21 (Colt) and the latent period was longer on these clones. Thus, only about half the replicate plants in these clones showed galls by mid July, when most of the plants in the other clones were already bearing galls.

The susceptibility of Colt may not be a major disadvantage since it is propagated from cuttings and it should be easier to protect from crown gall infection than

Table 34 Susceptibility of Fb2/58 clonal
rootstocks to crown gall infection

Clone Fb/2/58	No. stocks infected †	Mean ht (cm)	Mean gall diam. (mm)
50	9/9	73.1	54.7
17	10/10	89.9	48.9
38	10/10	137.1	36.9
15	10/10	107.9	35.8
22	7/10	117.8	24.6
21 (Colt)	10/10	121.5	13.4
F12/1	4/4	130.5	12.7

† The inoculum used was a mixture of A. tumefaciens isolates At65, At150 and At160 at a concentration of c. 10^8 cells/ml.

F12/1 and other clones that must be propagated commercially by layering.

B. BLACKBERRY

5. Variation in susceptibility

a. During winter

Field infection of blackberries commonly occurs on stubs of old fruited canes that are cut out during the winter at, or just above, ground level. The time of cutting varies between November and April according to weather conditions and availability of labour.

To determine susceptibility to infection during this period canes of Bedford Giant blackberry were cut out in November, January and March (1975-76) and inoculated immediately or 8-14 days after cutting. Final disease records were taken in early September (Table 35).

Infection occurred readily at all times of inoculation but was most frequent on canes inoculated immediately after cutting in November and March. In January, where the interval between the two series of inoculations was only 8 days, the rates of infection were equal. The mean number of galls per cane was higher from the November than from the January and March inoculations (1.7 per cane compared with 1.3 and 1.2 respectively).

On all canes the inoculum was deposited carefully on the cut surface but no other precaution was taken to prevent the inoculum running down. Only 23% of the galls were

Table 35 Development of galls on blackberry canes (cv. Bedford Giant) inoculated with *A. tumefaciens*⁺ on three occasions, immediately after cutting and a few days later

Date* cut	Time of inoculation (days after cutting)	No. canes cut	No. died	% died	No. surviving canes infected at			% infected
					top	below top	near side shoot	
Nov.	0	21	6	28.6	2	7	4	86.7
	12	22	15	68.2	0	3	1	57.1
Jan.	0	17	6	35.3	2	1	4	63.6
	8	15	4	26.7	2	3	2	63.6
March	0	25	6	24.0	4	4	5	68.4
	14	28	7	38.9	1	2	0	27.3
Totals		128	44	34.4	11	20	16	
					23.4	42.5	34.0	
Mean gall diam.					36.9	23.9	26.9	

⁺ Mixed inoculum of T112 and T126 at a concentration of $\underline{c. 10^9}$ bacteria/ml

* Canes cut approximately 20-30 cm above ground level

located on the cut surface; the majority appeared a few centimetres below, often around the base of a side shoot emerging below the cut (Fig. 26). Galls on or near the cut surface were generally larger than those in other positions.

Approximately 34% of all the inoculated canes had died back by the end of May 1976 thus preventing gall development. The dead canes were significantly thinner than the survivors with a mean diameter of 13.7 mm compared with 16.01 mm ($p < 0.001$).

The results of this experiment indicate that, in a mild winter, canes are liable to infection whenever they are cut out and remain so for several days. Where a stub is left, galls develop near the cut surface or just below and it would be feasible therefore to remove them as soon as they appear, and thus minimise the risk of initiation of secondary galls or the build up of inoculum. It would be quite impracticable however to remove galls from canes cut to ground level.

b. During summer

At both blackberry plantations nodal galls on the fruiting canes were common although the cultural practices at the two sites differed: at Teynham new canes were left to trail on the ground during summer while at Bifrons Gardens these were tied-in as soon as they were long enough but there was extensive summer pruning of laterals.



A



B

Fig. 26 Galls on stubs of old blackberry canes
(cv. Bedford Giant) on 27th May resulting
from inoculation with Agrobacterium tumefaciens
(T112 and T126)

A, galls originating below tip and round base
of side shoot

B, gall a little below the tip.

The susceptibility of leaf scars and laterals to crown gall infection was therefore investigated on canes of Bedford Giant tied down horizontally about 60 cm above ground level, in autumn 1975, to facilitate inoculation. From 35-60 leaf scars, or wounds resulting from removal of laterals, were inoculated on from 2-4 canes on 8 occasions from October 1975 to August 1976 inclusive with a mixture of blackberry isolates, T112 and T126, at a concentration of $\approx 10^8$ cells/ml. Final results were recorded on 19th October 1976; the percentage of infections increased from the October inoculations to a maximum from those made in March while inoculation through the summer months resulted in a constant high level of infection (Fig. 27). The apparent failure of the August inoculations might reflect the longer latent period required at this season.

Galls resulting from October to March inoculations (Fig. 28) first became visible on 17th May. At the final record the galls from April and May inoculations were the largest while those from October and November inoculations, which had had the longest developmental time, and from June inoculations, for which the developmental time was shortest, were the smallest.

The high level of susceptibility to infection at all times of inoculation, particularly during the growing season, indicate that while summer pruning of laterals may be desirable culturally it involves considerable risk of spreading any infection which may be present in a plantation. The practice of allowing new canes to trail on the ground during the summer, where breakage of leaves or other wounds may allow ingress of the pathogen, should be avoided.

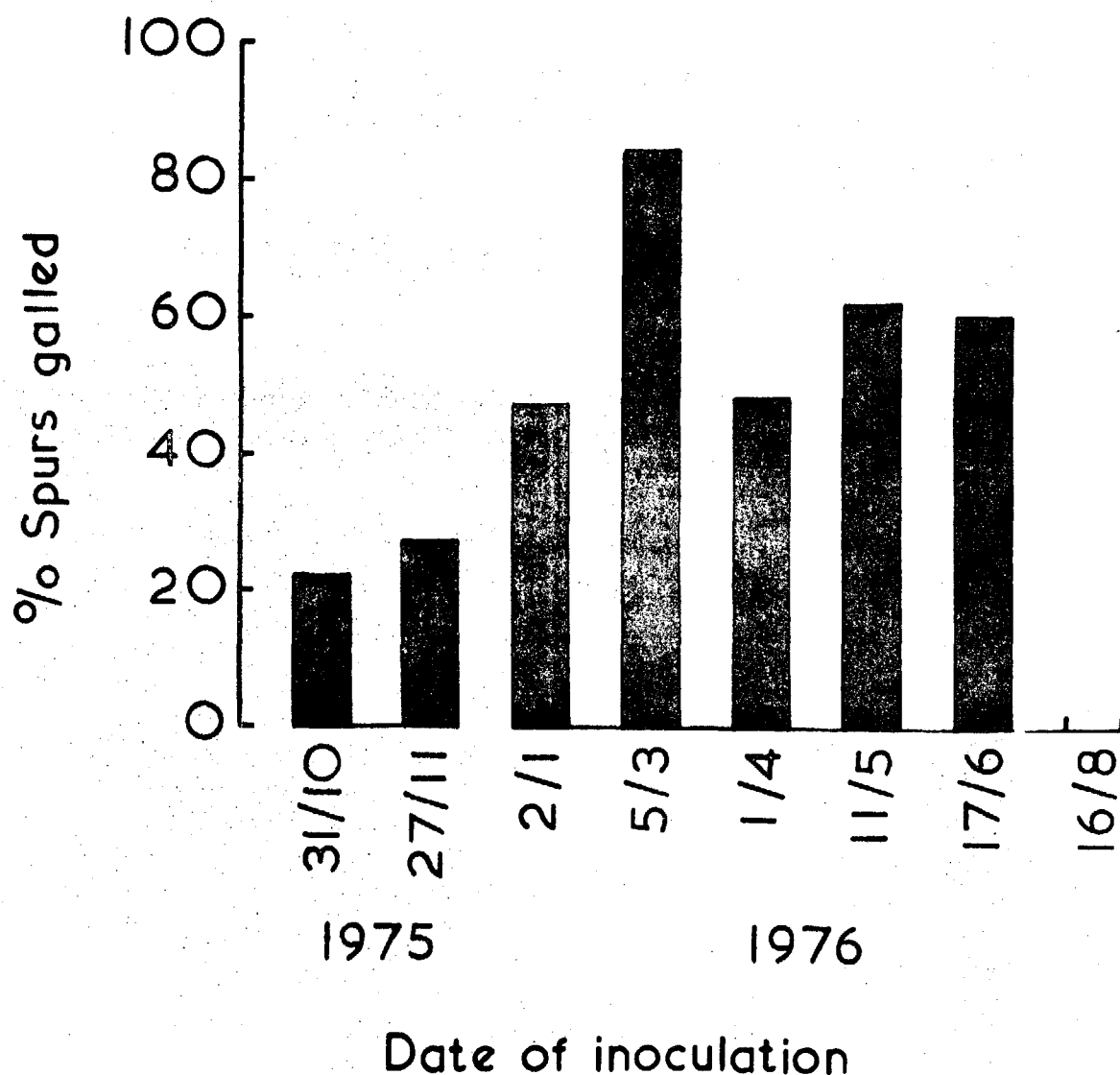


Fig. 27 Incidence of infection of blackberry (cv. Bedford Giant) leaf scars or nodes to infection resulting from inoculation with Agrobacterium tumefaciens (isolates T112 and T126) at intervals from October 1975 to August 1976. Data of record taken 19th October 1976.



A

B

Fig. 28 Blackberry canes (cv. Bedford Giant) with galls resulting from inoculation with Agrobacterium tumefaciens, isolates T112 and T126, to nodes (A) and leaf scars (B).

DISCUSSION

The results of inoculation experiments on cherry confirm general opinion that host susceptibility and rate of gall expansion are greatest and the latent period shortest during the period of meristematic activity of the host i.e. the growing season. The same general principle applies in blackberry.

The experiments using simulated shoot scar and layer bend material indicated that the cultural practices involved in layer bed production of cherry rootstocks are performed at times when the risk of infection is low. The data in Section I showed that 'healthy' stocks cut from an infected layer bed can develop galls in the first year of growth. The experimental results support this finding since stocks infected shortly before or during harvesting, i.e. at a time when the latent period is 10 months, would not appear infected when planted in spring but galls might develop from June onwards and be seen when the young trees were lifted in the following autumn.

All the new clonal cherry rootstocks were susceptible to crown gall when experimentally inoculated in the stem but their reaction to natural infection in the field has still to be assessed. Although Colt and Cornflower were susceptible the latent periods were longer than for the other clones suggesting that these two clones may not be seriously infected in the field under low natural levels of inoculum. The increasing use of rootstocks such as Colt, propagated from cuttings, should help to reduce considerably

the incidence of crown gall in the nursery provided they are planted into fumigated land. The use of the cherry leaf scar as an experimental avenue of infection provides an interesting extension of a well-established method of disease assay on this host and might be used in determinations of the efficacy of other antagonistic organisms or chemicals in disease control or for comparative virulence tests of isolates of the pathogen.

In blackberry the winter pruning of old canes and summer pruning of laterals will increase the risk of spreading any crown gall infection which may be present in the plantation. These operations are necessary for maintenance of crop yields and keeping the bushes manageable. The experimental results suggest that pruning after November and leaving 15 cm stubs would reduce risk of extensive infection and facilitate the early removal of young galls so restricting disease spread; provided the tools used were disinfected between each bush. When canes are cut to ground level crown gall removal is impractical and disease spread inevitable.

The isolates used in field experiments on blackberry were subsequently found to be biotype 1 and therefore not truly representative of the isolates from this host. This does not, however, invalidate the experimental results.

A phenomenon noted occasionally on cherry and more frequently on blackberry was that galls appeared initially at a site several centimetres distant from the inoculation

wound (Figs. 22B and 26A). The possibility of the inoculum running down the stem externally cannot be excluded but if the pathogen penetrates the vascular system then application of sprays to control the disease would need to be applied to the freshly cut surface to have any chance of success; application after wound healing would be ineffective.

SECTION V

CONTROL

Attempts over many years to reduce losses from crown gall have met with little success. There is evidence that nursery stock taken from infected layers is more likely to develop crown gall than stocks from healthy layers. Theoretically, therefore, it would be best to institute control measures in the layer bed. However, because a bed remains in production for many years and not all parts of it are exposed during normal cultural operations, chemical and other direct control measures are difficult to apply. Apart from one investigation of cultural control on the layer bed, experimental control measures were therefore applied to stocks after their removal from the layer bed and before planting. Chemical and, later, biological methods of control were investigated.

1. Cultural control of crown gall on layer beds

Since the presence of a gall in one year significantly increased the probability of infection in that area in the following year (see Section I), the effect of removing galls on the subsequent incidence of disease was tested.

Galls were recorded in 1971 on one 130 m area at each end of the layer bed. The galls were left in half of each area (Fig. 2 E1, F1) but were removed from the other half (E2, F2). The numbers and positions of galls were recorded in 1972. The results are given in Table 36.

Table 36 The effect of gall removal on gall incidence
in the following year on an F12/1 cherry
rootstock layer bed

		<u>Numbers of galls</u>					
		<u>position on layer</u>					
<u>Galls left</u>							
<u>in situ</u>		total	mid	fork	bend	scar	root
	<u>gall</u> <u>age</u>						
1971	old	64	13	22	19	3	5
	young	75	13	24	8	13	13
	total	139	26	46	27	16	18
1972	old	153	24	46	45	12	15
	young	651	174	113	98	133	75
	total	804	198	159	143	145	90
<u>Galls removed</u>							
1971	old	70	18	20	23	5	4
	young	92	25	26	18	5	15
	total	162	43	46	41	10	19
1972	old	80	9	21	39	3	6
	young	628	112	89	143	136	115
	Total	708	121	110	182	139	121

a. Numbers of galls

The number of galls increased from 139 to 304 where they were left in situ and from 162 to 708 where they were removed. Old galls on the untreated area in 1972 approximately equalled total numbers recorded there in 1971. The presence of 80 old galls on the treated area in 1972 indicated that removal from the area in 1971 had been incomplete; most of these galls occurred on partly submerged bends and, to a lesser extent, on forks. There were over 600 young galls on each area in 1972. The total number of galls on the two areas differed significantly ($p < 0.1$) but the difference was entirely attributable to differences in numbers of old galls.

b. Sites of infection

In 1971 there were similar numbers of old and young galls on both areas but old galls were more prevalent on bends and young galls on roots. The greatest increases in young galls in 1972 were at scar and mid layer sites when galls had been left in situ and at scars, mid layer and root sites where galls had been removed. This increase in root galls might have been partly due to infection through wounds made when the layers were being exposed for gall removal in 1971. The removal of mid and fork galls in 1971 may have reduced the numbers of galls on these sites in 1972 on this sector.

However, removal of galls did not reduce the number of infections at the most vulnerable site, the shoot scar. The results suggest that even if complete removal of galls was practicable, the labour costs would probably be

prohibitive, and any advantage gained from the reduction in the inoculum supply would be more than offset by the damage caused in fully exposing the layers.

2. Chemical control by preplanting dips

Attempts to control crown gall by chemical treatment of material before planting are probably more numerous than any other control measures. Some of the chemicals that have been reported to give promising results on other hosts (Schroth, et al., 1971; Wormald & Garner, 1945) were tested as preplanting dips for the control of crown gall on cherry rootstocks. For each experiment EMLA F12/1 cherry rootstocks were harvested from infected layer beds. Treatments, which always included water-dipped controls, were fully randomised at planting out; the stocks were lifted after one season's growth for disease assessment.

a. Experiment 1 Trial of sodium hypochlorite and chloramine T for control of crown gall of cherry

One thousand selected rootstocks were divided into bundles of 25 after trimming their roots. Ten bundles were dipped for 30 seconds in either 0.25% sodium hypochlorite or 0.7% chloramine T; two sets of 10 bundles served as controls. The rootstocks were planted on 25th March 1970. The results (Table 37) showed that sodium hypochlorite was highly phytotoxic at the concentration used, although there was an apparent reduction in the incidence of galls on the surviving stocks. No demonstrable effect was observed with chloramine T.

Table 37 Trial of sodium hypochlorite (0.25%) and
chloramine T (0.7%) for control of crown
gall of cherry

	sodium hypochlorite	chloramine T	control	
			1	2
% stocks dead	24.6 20%	3.2	2.0	4.8 3.4%
% surviving stocks galled	2.66	5.78	4.90	9.66
			7.25	

250 stocks were dipped for each treatment

b. Experiment 2 The effects of three chemicals at different concentrations and times of application

The following were used for root-dipping treatments:-

Bacticin at 1%; 0.2%; 0.04%

Chloramine T at 3.75%; 0.75%; 0.15%

or Sodium hypochlorite at 0.25%; 0.05%; 0.01%

i.e. for each chemical there was a five-fold concentration series.

Rootstocks were harvested in November 1970 and put into bundles of 10; three bundles were used for each treatment, and were dipped for 1 minute. The procedures before which treatments were applied were:-

- 1) heeling-in (6th November, 1970)
- 2) early planting (16th December, 1970)
- 3) late planting (23rd February, 1971)
- 4) heeling-in, and before early planting
- 5) heeling-in, and before late planting

Thus, there were 45 combinations of chemical, concentration, and time of application; seventy control stocks were planted early and late. The roots of all stocks planted early were pruned on 16th December but weather conditions delayed planting until 14th January 1971. The roots of stocks planted on 23rd February were pruned the day before.

Phytotoxicity results are summarised in Table 38. Highest mortality was generally associated with chloramine T, high concentrations and with late planting. At the highest concentration chloramine T killed 45% of the stocks that were planted on 23rd February and many more showed retarded growth in the spring.

Table 38 Phytotoxicity of chemicals tested for the
control of crown gall on cherry rootstocks
expressed as percentage stocks killed

Chemicals (means for all concentrations and times)

nacticin 3.5	sodium hypochlorite 2.5
chloramine T 9.6	untreated controls 0.7

Concentrations (means for all chemicals and times)

High 10.3	medium 3.3	low 2.2
-----------	------------	---------

Times of application (means for all chemicals and concentrations)

Heeling-in 2.6	early planting 1.5
Heeling-in and early planting 5.3	
Late planting 7.1	
Heeling-in and late planting 9.5	

The incidence of galls on the surviving stocks in the various treatments is shown in Table 39. None of the treatments reduced the number of galls except possibly sodium hypochlorite at the medium concentration. There were indications of enhanced gall development at the medium and low concentrations of the other materials. The effect of time of application is shown in Table 40. Late planting appeared to increase galls on treated stocks and there was a comparable increase on the controls.

c. Experiment 3 Efficiency of chloros and agrosan in crown gall control

Six hundred rootstocks harvested in November 1971 were taken from store on 16th March 1972 and dipped for 1 min in water, 0.25% 'chloros' or 0.01% a.i. Agrosan (an organo-mercury compound) soil slurry. Roots of half the stocks for each treatment, c. 100, were trimmed prior to dipping and half were trimmed on the following day. All were held in store until planted on 24th March as 5 replicates of 20 stocks/treatment.

There were more dead stocks in all the chemical treatments compared with the controls but the only substantial mortality ($>30\%$) followed the use of agrosan after root pruning (Table 41).

None of the treatments reduced the incidence of galls and there were slight indications that some of them may have increased it. This was in line with the results of the previous experiments although none of the differences in this experiment were significant.

Table 39 Effect of three chemicals, each at three concentrations, on the incidence of crown gall on F12/1 cherry rootstocks
(means for all times of application)

Treatment	<u>% viable stocks galled</u>		
	concentration of chemical		
	high	medium	low
Dacticin	5.3	12.7	9.6
Chloramine T	4.7	12.2	13.2
Sodium hypochlorite	11.6	3.4	8.3
Untreated controls	5.4		

Table 40 Effect of time of application of three chemicals on the incidence of crown gall on F12/1 cherry rootstocks

Time of application before:	<u>% stocks galled</u>	
	Treated	Controls
Heeling-in	10.6	
Heeling-in and early planting	9.6	
Heeling-in and late planting	10.1	
Early planting	3.8	1.4
Late planting	12.1	8.9

Table 41 Experiment 3

Phytotoxicity of sodium hypochlorite and
agrosan and their effects on the incidence
of crown gall in cherry rootstocks

Time of treatment	Treatment	No. stocks	% died	% of viable galled
After root pruning	Control	101	3.0	20.4
	sodium hypochlorite	98	6.1	27.2
	agrosan	94	30.8(a)	21.5
Before root pruning	Control	98	4.1	25.0
	sodium hypochlorite	98	8.2	25.6
	agrosan	98	9.2(b)	31.5

(a) was significantly higher than (b) at the $p < 0.01$ level.

3. Biological control

A new and more promising approach to the control of crown gall originated recently in Australia where Kerr (1972) pioneered the use of the antagonistic soil bacterium A. radiobacter, strain 84, for biological control of the disease on peach. In laboratory tests, English cherry isolates of A. tumefaciens were found to be sensitive to the bacteriocin produced by strain 84. The in vivo activity of strain 84 was examined in wound inoculations of glasshouse tomatoes and in leaf scar inoculations of cherry before proceeding to control experiments in the field.

a. Tomato inoculations

i. Antagonism of strain 84 in tomato inoculations

Fifteen isolates of A. tumefaciens (ten from cherry, two from blackberry and three from loganberry) were prick-inoculated into duplicate tomato plants at 10^7 cells/ml. All induced galls within 10 days. In parallel inoculations with the same isolates in mixed inocula with strain 84, at 10^8 cells/ml, no galls appeared within 6 weeks.

ii. The effect of delaying inoculation with strain 84.

Strain 84 (10^8 cells/ml) was inoculated to duplicate tomato plants simultaneously with A. tumefaciens cherry isolate At160 (10^7 cells/ml) and then at intervals of from 3.5 hr to 4 days afterwards through, or immediately adjacent to, the wounds made for the inoculation of the pathogen. Results were recorded after 5 weeks. No galls developed when strain 84 was inoculated simultaneously with or up to 24 hr after A. tumefaciens. When the antagonist was



Fig. 29 Biological control of gall formation in tomato stems: 28 days after prick inoculation with Agrobacterium tumefaciens cherry isolate At160 (10^7 cells/ml). From left to right: inoculated with the pathogen alone; with A. radiobacter strain 84 added after 24 hr, 2 days and 4 days.

inoculated after 2 or 3 days gall development was delayed and gall size reduced (Fig. 29). Strain 84 had no effect on gall production when added after 4 days. The results are contrary to those of Htay and Kerr (1974) who found strain 84 to be ineffective as an inhibitor if its inoculation was delayed by as little as 4 hr.

b. Cherry inoculations

i. Antagonism of strain 84 in leaf scar inoculations

An antagonistic soil organism, Z80 (see Section III) and a strain of A. tumefaciens (NCPPB 1641) reported to be non-pathogenic in Oatara and sunflower were also tested in this experiment. The latter was included because Lippincott & Lippincott (1969) had reported the inhibition of virulent strains of A. tumefaciens by avirulent strains, which they suggested was due to competition for infection sites. In leaf scar infections of cherry most of the inoculum applied is drawn into the vessels of the leaf traces and there is a possibility of blockage with concentrated bacterial suspensions. To eliminate the possibility that gall development was influenced by physical factors like this, mixed inoculations were also made with heat-killed cells of the added bacteria.

The inoculum contained approximately equal numbers of two cherry isolates of A. tumefaciens, At66 and At160, at a total concentration of $\approx 2.5 \times 10^7$ cells/ml. In the mixed inocula the other bacteria, alive or dead, were present at final concentrations of $\approx 2.5 \times 10^8$ cells/ml. Leaf scars of potted 2-year-old Fl2/1 cherry rootstocks were inoculated on 11th June 1975; each of the 7 treatments, plus a water control, was applied to 40-50 spurs

distributed over 6 replicate branches. Final results were recorded on 21st October, while observation of the trees one year later showed that there was no change in infection levels but galls had increased in size (Fig. 30).

None of the heat-killed bacteria had any demonstrable effect on the pathogen (Fig. 31) which, when inoculated alone, induced galls in c. 75% of the spurs. The rate of infection was reduced by about a third in the presence of cells of 280 and of NCPPB 1641, but the difference was not significant. Gall formation was, however, completely inhibited in the presence of strain 84.

ii. The effect of delaying inoculation with strain 84
Strain 84 (10^8 cells/ml) was inoculated simultaneously with, or 5 hr, 29 hr, or 72 hr after A. tumefaciens cherry isolate At160 (10^7 cells/ml) to leaf scars on one-year-old potted F12/1 cherry rootstocks. Two methods were used for the delayed inoculations with strain 84; the suspensions were applied directly to the existing scar tissues or, alternatively, to freshly exposed tissues resulting from shaving-off the top 1 mm of the scar. Each treatment, and a water control, was replicated 10 times, each tree receiving inoculum on 4-9 leaf scars on each of 3 branches. The inoculations were begun on 24th June 1976 and final records were taken on 22nd September.

All treatments with strain 84 significantly reduced the incidence of galls ($p < 0.001$) except when the antagonist was applied to unshaven leaf scars 72 hr after the pathogen (Fig. 32). It was most active when applied simultaneously with the pathogen and its activity decreased



Fig. 30 Cherry rootstock F12/1 16 months after leaf scar inoculation with Agrobacterium tumefaciens alone (centre branch) or with A. radiobacter added simultaneously as live inoculum (right branch) or as dead cells (left branch).

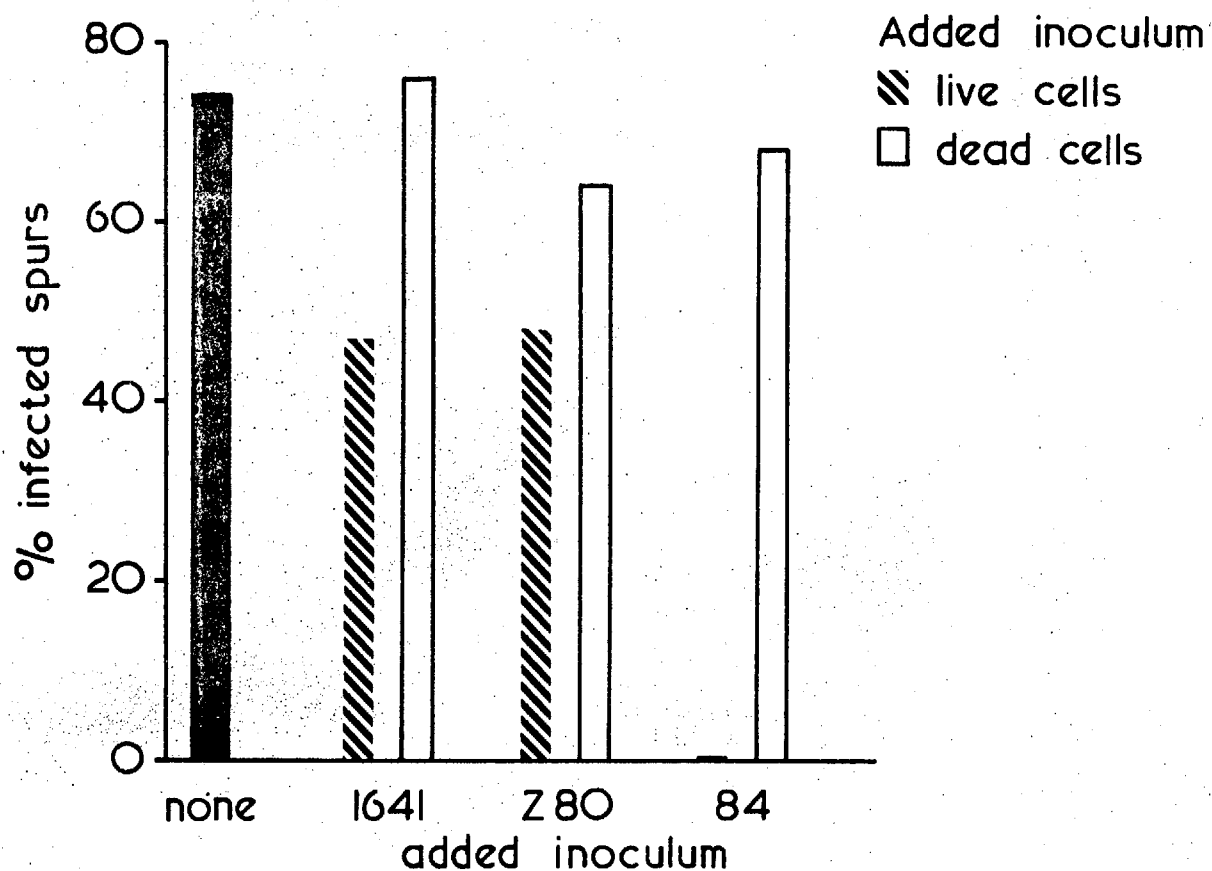


Fig. 31 Percentage cherry leaf scar infection resulting from inoculation with Agrobacterium tumefaciens alone or with the addition of live or dead cells of A. tumefaciens NCPPB 1641, soil organism Z80 and A. radiobacter strain 84.

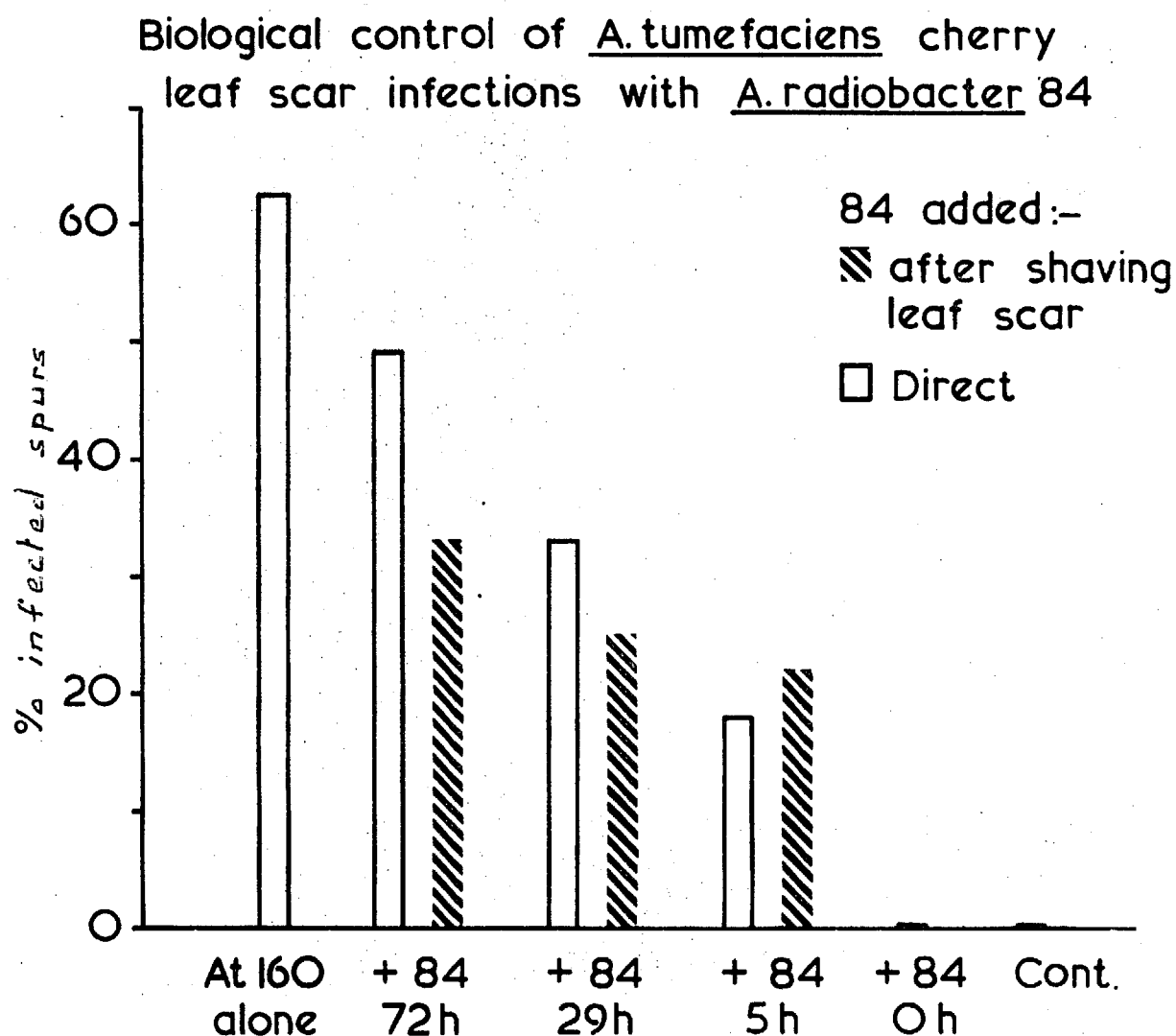


Fig. 32 Biological control of cherry leaf scar infection with Agrobacterium tumefaciens (At160) by the addition of A. radiobacter strain 84 simultaneously and at intervals up to 72 hr later.

Table 42 The effect of delaying inoculation with
A. radiobacter strain 84 on cherry leaf
scar infection by A. tumefaciens (At160)

Strain 84 (10 ⁸ cells/ml) added after:	leaf scars	% leaf scars infected †	χ^2	sig. level
5 hr		18.18	26.07	xxx
29 hr	unshaven	32.81	11.35	xxx
72 hr		49.15	2.46	
5 hr		22.03	20.16	xxx
29 hr	shaven	25.42	16.87	xxx
72 hr		32.78	11.09	xxx
not added		65.47		

†, no infection observed in water controls;

xxx, significant at $p < 0.001$

progressively as the time of addition after the pathogen increased (Table 42). No major or consistent effects were observed from shaving leaf scars before application of strain 84.

DISCUSSION

Control of crown gall is likely to be costly in both labour and materials and doubts have been expressed as to whether it is really necessary, especially in the absence of any conclusive evidence that it adversely affects growth and cropping. However, for nurserymen, who are prohibited from selling galled plants, or cannot sell them for cosmetic reasons, effective control measures are urgently required.

The standard advice of extension and advisory services in Britain, U.S.A. and Australasia is to plant disease-free stock in pathogen-free soil and to avoid wounding. Chemical treatments have not proved consistently effective, except perhaps the spot treatment of galls with bacticin (Schroth & Hildebrand, 1968) which is completely impracticable on a large scale. The unaccountable failure of pre-planting chemical treatments that have proved successful on other occasions has been variously attributed to different soil and environmental conditions, physiological condition of the plant and to other factors.

None of the chemical treatments investigated during the present studies for the control of galls on cherry rootstocks showed any prospect of commercial value. Phytotoxicity was a major hazard and there were indications also that some of the treatments actually increased the

incidence of galls compared with untreated controls. The reasons for the failure of the chemical treatments are not known but may be due to the inaccessibility of bacteria in latent infections present on stocks when they are lifted from the layer beds.

The biological control of crown gall on stone fruits is the most effective method so far tested and has the advantages of being both relatively cheap and highly specific. The Australian A. radiobacter strain 84 prevented gall formation by cherry and cane fruit isolates of the pathogen in tomato inoculations and by cherry isolates in cherry leaf scar inoculations, even when applied several hours after the pathogen. This apparent conflict with the results of Kerr & Htay (1974) may be attributable to differences in host plants, bacterial strains or experimental conditions.

The slight reduction in cherry leaf scar infections with the avirulent strain NCPPB 1641 and Z80 was not due to blockage of the leaf trace vessels but there may have been some competition for infection sites, although there was no direct evidence for this. The lack of activity of Z80 may be attributed to its inability to produce, or rapid breakdown of, its antibiotic substance in vivo.

Biological control of crown gall on a commercial scale has so far been limited to the pre-planting application of strain 84 to cuttings and seedlings raised in sterile soil (Kerr & Htay, 1974; Dhanvantari, 1976).

The results of a trial in progress at East Malling on the pre-planting treatment of hardwood cuttings of the rootstock Colt are awaited with optimism. However, where the disease is already present as in latent infections in established layer beds or plantations of perennial crops such as blackberry, curative rather than preventive action is required and the prospects for biological control are less promising. Nevertheless, experiments have been initiated to determine the efficacy of strain 84 as a pre-planting dip on rootstocks from an infected layer bed and as a spray on the fresh layer bed shoot scars immediately after harvesting and on blackberry cane stubs after winter pruning. The results of the delayed applications of strain 84 suggest that it might be feasible, in commercial practice, to delay treatment at least until the end of the day when these operations are carried out.

The heterogeneity of the 'species' Agrobacterium tumefaciens, and the knowledge that vine isolates are insensitive to the activity of bacteriocin 84 (Panagopoulos, pers. comm.) indicate that biological control is not a panacea for crown gall unless other antagonistic organisms with a different spectrum can be isolated.

SUMMARY AND CONCLUSIONS

1. The pathogen is introduced into 'clean' land through infected nursery stock. This is probably the most common method of crown gall dissemination and where greatest vigilance is needed.

The origin of infection on the layer bed studied, established on fumigated land, is unknown but disease was almost certainly introduced into a later established layer bed with 'healthy' material from the infected bed. The cane fruit plantations studied were all supplied with plants from the same specialist nursery. The initial distribution pattern of crown gall at Bifrons Gardens was attributable to infection in random bundles of the nursery stock. Freedom from crown gall depends on planting pathogen-free stock.

2. Spread of the disease from the initial foci of infection was rapid and extensive.

Establishment of the cherry layer bed on fumigated land, where the population of natural antagonists would have been depleted or eliminated, would allow the

A. tumefaciens population to increase rapidly. In the culture of cherry rootstocks and blackberry crops the many wound sites allowing ingress of the pathogen provide better conditions for disease spread than, for example, in orchards. The lack of adequate control measures resulted in the rapid spread of infection on the layers and in the blackberry plantations, and led to the grubbing of the layers after only 4 years' productivity (compared with a normal 20 years) and to 100% infection of blackberry bushes within 5 years. The Importance of planting healthy

material in clean land is again emphasised.

3. There can be no guarantee that rootstocks harvested from an infected layer bed are free from infection.

Prohibiting the sale of galled nursery stock will reduced the dissemination of crown gall but will not prevent it. Apparently healthy rootstocks used to establish a new cherry layer bed on fumigated land soon became galled; preplanting chemical dips failed to control the disease; the rate of infection on the layer bed and on the 'healthy' rootstocks taken from it when lifted after one year's growth increased in parallel over several years. There is no doubt that the rootstocks were latently infected when harvested from the layers although galls did not show until the following summer.

To prevent dissemination of the disease from the nursery, ideally, no plant material from land where crown gall is known to be present should be sold. Such a drastic measure would not be accepted nor could it be justified.

4. No clear evidence was found that crown gall adversely affected growth of cherry and blackberry or the cropping of the latter.

Nevertheless certain circumstances may combine, e.g. basal infection on Fl2/1 rootstock in the nursery before grafting with EMLA Napoleon, to bear out the assertion of some growers that growth is affected by the disease. The determination of the precise conditions leading to significant adverse effects on growth cannot be regarded as of such high priority as disease control in the nursery.

The effect of virus-free status on susceptibility to the disease might, however, be worthy of investigation.

5. The causal organism of crown gall, A. tumefaciens, exists in different biotypes and pathotypes. Successful isolation of the pathogen depends on choice of gall, isolation media and range of indicator test plant hosts. These studies confirmed that bacteriophages showed no potential for diagnosis or phage typing of the different strains of the pathogen. However, the in vitro sensitivity of many isolates to the bacteriocin of K84 has already made important changes in the approach to field control.

6. Although there is as yet no evidence that crown gall has any major adverse effect on growth and cropping, in certain special situations it is of considerable economic importance, e.g. in cherry layer beds where infection can render stocks unsaleable and in the ornamental trade. In these situations control measures to prevent widespread and indiscriminate dissemination of a pathogen with a wide host range are essential.

The only way of ensuring freedom from crown gall has been to plant pathogen-free stock in pathogen-free soil. The raising of cherry rootstocks from hardwood cuttings and biological control of the pathogen with a preplanting treatment of strain K84 of A. radiobacter have greatly increased the possibility of producing cherry trees free from the disease. The use of K84 is not, however, a panacea; not all strains of the pathogen are sensitive to its bacteriocin nor has it any demonstrable curative action.

The search for other organisms antagonistic to A. tumefaciens could prove rewarding and extend the range of biological control.

Some of the contradictory results reported for soil fumigation may have been due to the reintroduction of the pathogen as latent infections in the planting material. Fumigation may therefore have greater potential in disease control than is realised and carefully controlled experiments where any risk of introducing the pathogen on planting material is eliminated should clarify this.

The only curative treatment, of painting galls with e.g. bacticin, though effective on specimen trees or certain ornamentals, is not practical on a large scale. Spread of the disease might be retarded by careful timing of pruning operations to coincide with periods of low susceptibility, disinfection of pruning tools and high standards of nursery and orchard hygiene. Future investigations into control by chemical means could include modification of the time of application and improvement of penetration. The leaf scar inoculation technique might prove useful in the preliminary screening of any new products showing some potential in crown gall control.

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