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Biological Control of Crown Gall

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ABSTRACT : Crown gall of stonefruit and nut trees is one of the very few plant diseases subject to efficient biological control. The disease is caused by the soil-inhabiting bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* and the original control organism was a non-pathogenic isolate of *A. rhizogenes* strain K84. Control is achieved by dipping planting material in a cell suspension of strain K84 immediately before planting. Strain K84 produces a plasmid-encoded antibiotic called agrocin 84 which specifically inhibits pathogenic strains containing a nopaline Ti plasmid. Because the agrocin 84-encoding plasmid (pAgK84) is conjugative, it can be transmitted from the control strain to pathogenic strains which, as a result, become immune to agrocin 84 and cannot be controlled. To prevent this happening, the transfer genes on pAgK84 were located and then largely eliminated by recombinant DNA technology. The resulting construct, strain K1026, is transfer deficient but controls crown gall just as effectively as does strain K84. Field data from Spain confirm that pAgK84 can transfer to pathogenic recipients from strain K84 but not from strain K1026. The latter has been registered in Australia as a pesticide and is the first genetically engineered organism in the world to be released for commercial use. It is recommended as a replacement for strain K84 to prevent a breakdown in the effectiveness of biological control of crown gall. Several reports indicate that both strains K84 and K1026 sometimes control crown gall pathogens that are resistant to agrocin 84. A possible reason for this is that both strains produce a second antibiotic called agrocin 434 which inhibits growth of nearly all isolates of *A. rhizogenes*, both pathogens and non-pathogens. Crown gall of grapevine is caused by another species, *Agrobacterium vitis*. It is resistant to agrocin 84 and cannot be controlled by strains K84 or K1026. It is different from other crown gall pathogens in several characteristics, including the fact that, although a rhizosphere coloniser, it also lives systemically in the vascular tissue of grapevine. Pathogen free propagating material can be obtained from tissue culture or, less surely, by heat therapy of dormant cuttings. A number of laboratories are searching for a biocontrol strain that will prevent, or at least delay, reinfection. A non-pathogenic *A. vitis* strain F/25 from South Africa looks very promising in this regard.

Crown Gall of Stonefruit

Crown gall of stonefruit and nut trees is caused by the soil-inhabiting bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (28). Pathogenicity is dependent on the presence

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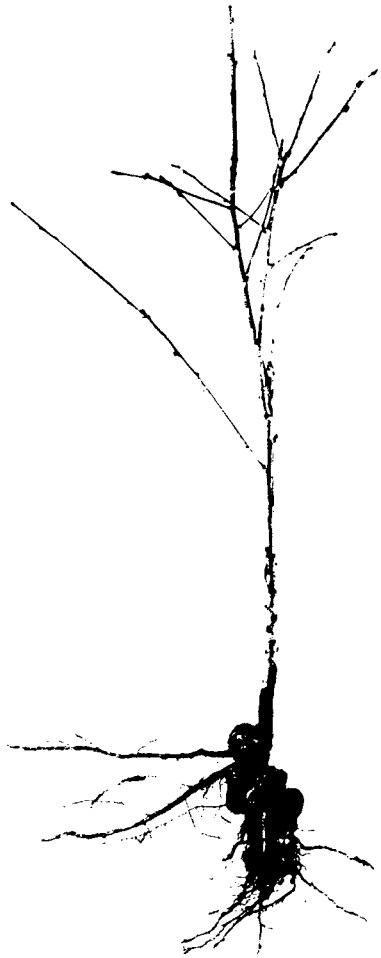
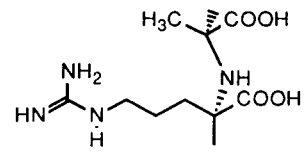
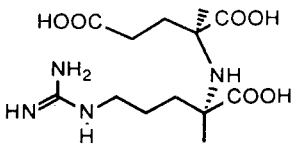


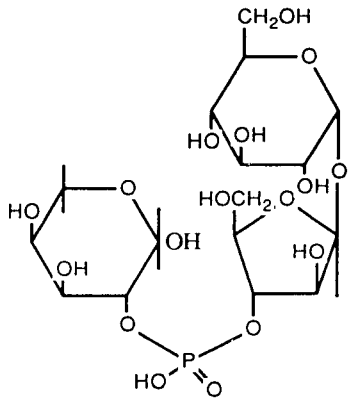
Fig. 1. Crown gall on a young peach seedling.



OCTOPINE



NOPALINE



AGROCINOPINE A

Fig. 2. Chemical structures of some opines (Courtesy of M. E. Tate).

of a large tumour-inducing plasmid (pTi) (52, 54) and during the infection process, a small piece of this plasmid, the T-DNA, is transferred to a plant cell (8) which, as a result, grows and divides rapidly to form a large tumour or gall (Fig. 1). Several kinds of pTi have been described and are usually distinguished by their "opine" characteristics. Opines are unusual compounds present in crown gall tissue but found nowhere else in the plant kingdom (50, 51). Examples of opines are octopine, nopaline (Fig. 2) and agropine and their synthesis is coded for by T-DNA. If octopine is synthesized in a gall, the inducing bacteria can also catabolise octopine but not nopaline and vice versa. Most non-pathogens and other soil bacteria cannot utilise opines. Thus, opines provide a reserved source of nutrient for pathogenic agrobacteria Only bacteria containing a nopaline Ti plasmid are subject to efficient biological control. Fortunately, these are the bacteria that cause most economic damage, particularly to stonefruit trees, nut trees and a few horticultural plants such as rose, *Prunus* and *Euonymus*.

Biological control

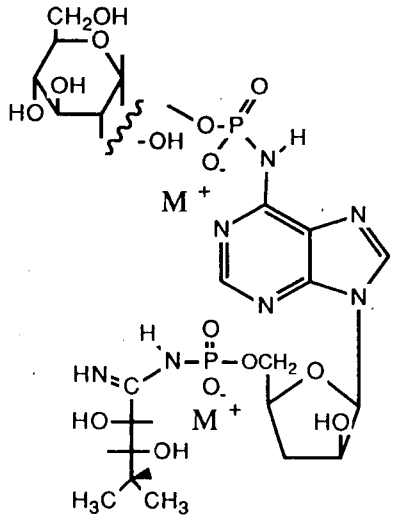
Strain K84. Biological control of crown gall is achieved by dipping planting material in a cell suspension of a non-pathogenic strain of *A. rhizogenes* (This strain is often referred to as *A. radiobacter* but recent taxonomic studies clearly indicate that it belongs to *A. rhizogenes* even though it is non-pathogenic) strain K84 (21, 26). It was isolated near Adelaide, South Australia. Nearly 100 percent control is achieved on stonefruit trees (26) (Table 1) and rose bushes (17, 36). Strain K84 produces an unusual antibiotic or bacteriocin called agrocin 84 (29) (Fig. 3) which inhibits the growth of pathogenic agrobacteria containing a nopaline Ti plasmid (16). Most other pathogens and soil-inhabiting bacteria are unaffected. The remarkable specificity is an uptake phenomenon (39, 40, 49); susceptible strains possess an agrocin 84 permease gene whereas resistant strains do not. In fact, the "normal" function of the permease gene is for the uptake of an opine called agrocinopine A (14), (Fig. 2) but it also transports into the cell agrocin 84, which inhibits growth by preventing DNA replication (11, 31). The permease gene is located on the nopaline Ti plasmid (16, 19, 20) and this explains the specificity, which is highly desirable because only pathogenic target organisms are affected. Biological control is now practised in most countries where susceptible crops are grown.

Strain K1026. A threatened breakdown of biocontrol of crown gall was first reported

Table 1. Biological control of crown gall in naturally infested soil

Treatment	Mean dry weight of gall tissue per plant (g)	Control (%)
Water	11.64	–
Seed inoculation with strain K84	2.50	78.5
Root inoculation with strain K84	0.59	94.9
Seed and root inoc-ulation with strain K84	0.14	98.8

Source : (26)



AGROCIN 84

Fig. 3. Chemical structure of the antibiotic agrocin 84 produced by strains K84 and K1026 (Courtesy of M. E. Tate).

from Greece (43). Panagopoulos and his colleagues noticed that when strain K84 was mixed with pathogenic strains before inoculating peach trees, efficient control was not achieved. From the galls formed, strains were isolated which were pathogenic but also produced agrocin 84. These strains were not subject to biological control. The phenomenon can be explained by plasmid transfer (13). The synthesis of agrocin 84 is encoded by a 48 kb plasmid called pAgK84 (17, 18), which can be transmitted from cell to cell by conjugation (15, 18). Following plasmid transfer, the transconjugants produce agrocin 84 and are immune to it (44); pathogenicity is unaffected (Fig. 4).

To prevent a breakdown in the efficacy of biocontrol, pAgK84 was made transfer deficient (Tra-). First, a physical (47) and then a functional (18) map of the plasmid were constructed (Fig. 5). Then Tn5 Tra- mutants were selected and shown to be efficient biocontrol agents (46). To construct a Tra- deletion mutant, the BamH1 fragment B1 covering the transfer region was cloned and, by various manipulations, EcoR1 fragments D1 and H were removed; the deleted BamH1 fragment B1 was then substituted for the intact region in strain K84 (23). The new strain is called K1026; it is identical with strain K84 except that most of the transfer region of pAgK84 is lacking. It shows normal agrocin 84 production (Fig. 6) but is incapable of transferring pAgK84 at a detectable frequency. Strain K1026 is as efficient as K84 in controlling crown gall (22, 24) (Table 2).

Strains K84 and K1026 possess another plasmid (pAtK84b) (Fig. 9) which plays an important role in biological control. It carries genes for plasmid incompatibility (inc) of the same type as nopaline Ti plasmids (9). Two plasmids of the same incompatibility group cannot exist together in the same cell. Plasmid pAtK84b therefore protects both strains K84 and

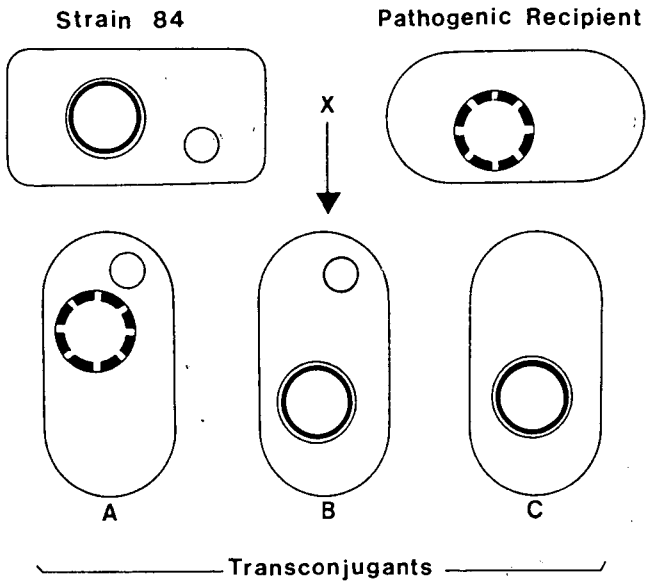


Fig. 4. Diagrammatic representation of a cross between strain K84 and a pathogenic strain of *Agrobacterium tumefaciens*. Chromosomal DNA and the large cryptic plasmid are not shown. Strain K84 contains two plasmids, the smaller one coding for agrocin 84 synthesis and for immunity to agrocin 84 . The pathogen has one plasmid (pTi) coding for tumour induction and agrocin 84 sensitivity as well as for many other characters including opine synthesis and catabolism. The cross results in three common plasmid transconjugants. Transconjugant A combines pathogenicity with immunity to agrocin 84. Such strains are no longer subject to biological control by strain K84. Source: (27).

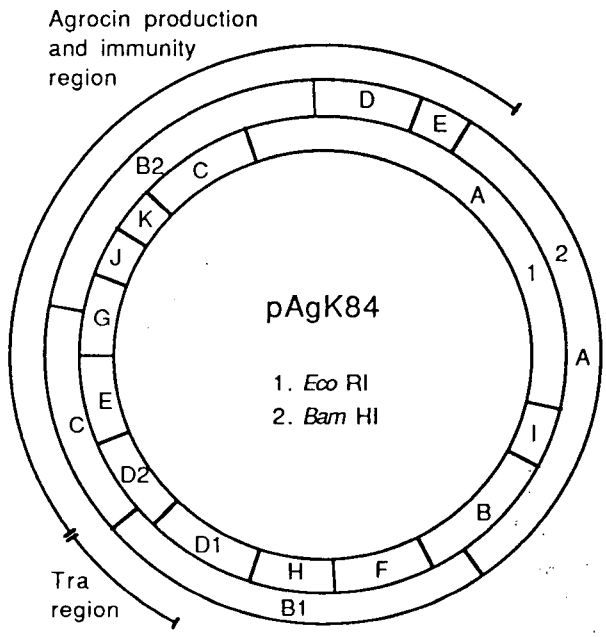


Fig. 5. *Bam*HI and *Eco*RI restriction maps of the agrocin plasmid pAgK84 showing the transfer, agrocin synthesis and agrocin immunity regions. Source: (18).

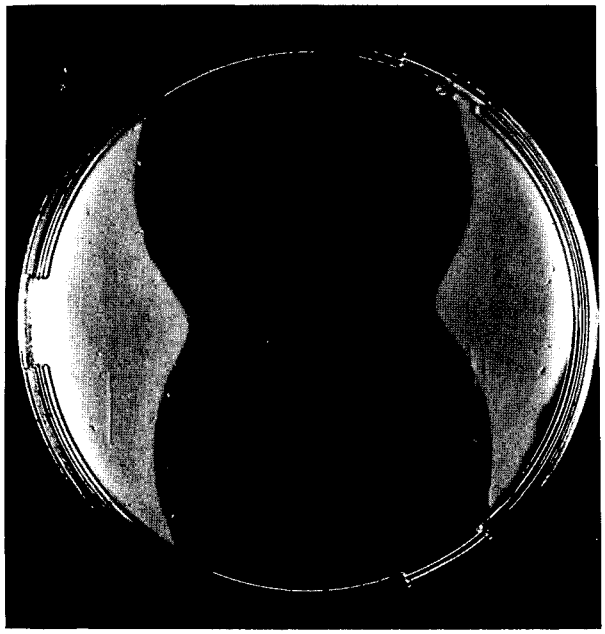


Fig. 6. Bioassay for the production of agrocin 84 by strains K84 and K1026. Agrocin production is indicated by the zones of inhibition in the growth of a sensitive strain. Source: (23)

Table 2. Biological control of crown gall by strain K84 and its genetically engineered derivative strain K1026

Treatment	% plants galled	No. of galls per plant
Water	100	46.33
K84	20	0.20
K1026	27	0.67

Source : (22)

K1026 from acquiring a Ti plasmid which would make them pathogenic. Strain K1026 has been registered in Australia for commercial use as a pesticide. It was the first and so far, is the only commercial release anywhere in the world of a living genetically engineered organism (27). Strain K1026 is an ideal organism for commercial release because of its obvious safety to humans, animals, plants and the environment. Some of these safety features are listed below:

(1) K84, the progenitor of K1026, has been registered as a pesticide and used commercially in many countries for more than 15 years; there have been no reports of harm.
(2) K1026 is identical to K84 except that it lacks functional transfer genes on the agrocin 84 plasmid, thereby preventing plasmid transfer.
(3) No foreign DNA is present in K1026.
(4) K1026 contains no Ti-plasmid encoded genes involved in crown gall induction (9).
(5) K1026 cannot grow at 37℃, human body temperature.

(6) Agrocin 84 is specific for agrocinopine-catabolising agrobacteria, most of which are crown gall pathogens; other organisms are unaffected. So, just as no ecological damage has resulted from production of agrocin 84 by K84, none will result from production of agrocin 84 by K1026.

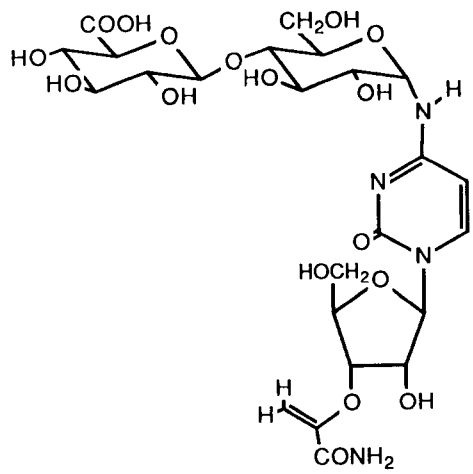
In the laboratory, it is easy to establish that strain K84 is transfer positive and strain K1026 transfer negative. However, it is much more difficult to confirm this under field conditions. Supporting data have been published from Spain (53). Lopez and her colleagues have shown that transfer of pAgK84 is uncommon in soil but can be detected when strain K84 is used as the biocontrol agent; no transfer was detected from strain K1026. Their results "…suggest the use of K1026 as a safer organism than K84 for biological control of crown gall". Strain K1026 is marketed under the name of NoGall by Biocare Technology Pty Ltd, Woy Woy, NSW, Australia. To reduce the probability of a breakdown in the effectiveness of biological control of crown gall, we recommend that wherever possible, strain K1026 is used instead of strain K84.

Agrocin 434. There have been several reports of strains K84 and K1026 controlling patho-

Table 3. Biocontrol of an agrocin 84 sensitive and resistant strain of *Agrobacterium tumefaciens*

Agrocin 84 sensitivity of pathogen	Treatment	% plants with galls	Mean fresh wt. of gall tissue per plant
Sensitive	Water	91.9	30.21
	K84	17.4	0.11
Resistant	Water	94.4	52.82
	K84	46.9	1.76

Source : (35)



AGROCIN 434

Fig. 7. Chemical structure of the antibiotic agrocin 434 (Courtesy of M. E. Tate).

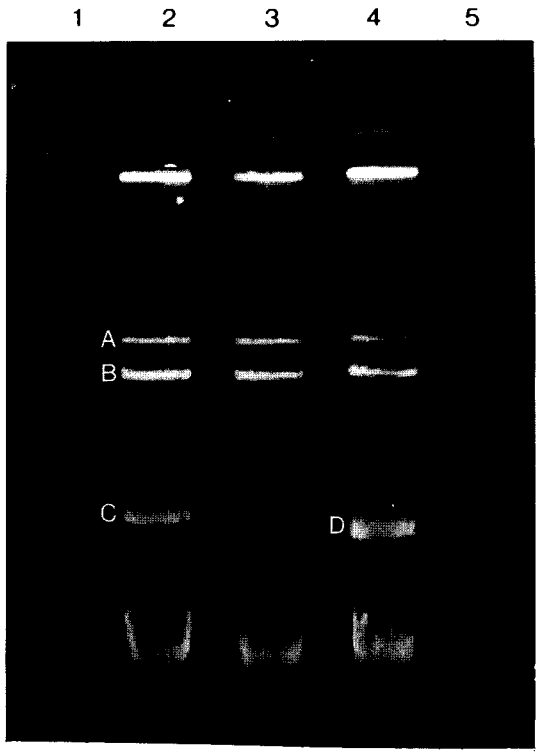


Fig. 8. Plasmids contained within strains K84, K434 and K1026. Left: strain K84; centre: strain K434; right: strain K1026. Band A contains plasmid pAgK434 (previously known as the cryptic plasmid); band B, pAtK84 b which codes for immunity to pTi; band C, pAgK84; band D, pAg1026.

genic strains that are resistant to agrocin 84 e.g. (35, 38, Table 3). Also, a non-agrocinogenic mutant of strain K84 significantly reduced crown gall incidence (10).

A possible reason for this is that both strains produce a second antibiotic called agrocin 434 (Fig. 7) which inhibits growth of nearly all isolates of *A. rhizogenes*, both pathogens and non-pathogens (12). In fact, the only resistant strains of *A. rhizogenes* are those which produce agrocin 434. Synthesis of agrocin 434 is encoded by genes situated on a large (300~400 kb) plasmid (pAgK434) also present in both strains (Fig. 8). The importance of this new antibiotic in the biological control of crown gall is being investigated. Competition for infection sites (34) may also be important in the control of strains resistant to agrocin 84 (see last section).

Crown Gall of Grapevine

Crown gall of grapevine is caused by another species, *Agrobacterium vitis* (42), which is resistant to agrocin 84 and agrocin 434 and is therefore not subject to efficient biological control by strains K84 and K1026 (30). *A. vitis* differs from *A. tumefaciens* and *A. rhizogenes* in several characteristics, including the fact that it lives systemically in its host (32). The

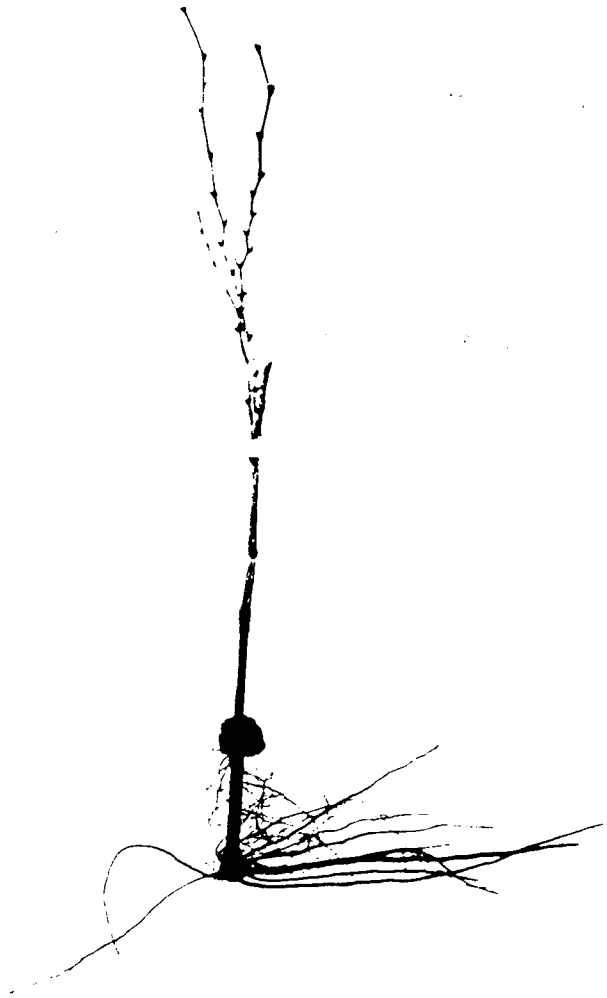


Fig. 9. Crown gall on grapevine cutting.

disease takes two distinct forms. The more destructive form is cane gall which occurs in countries with low winter temperatures. Canes and trunks are damaged by frost and this allows the systemic bacteria to transform wounded cells. In the following spring, masses of galls appear along the length of the canes and even on the trunks. In warmer climates such as those of California and Australia, galls are largely confined to the bases of cuttings used for propagation (Fig. 9) or to major graft wounds. It is particularly noticeable where a new cultivar has been top-grafted on to established vines in a vineyard. *A. vitis* does not survive for long periods in soil but can be detected in plant debris in soil for at least two years (1). The main source of infection is planting material, not soil (3). This means that the strategy to control crown gall of grapevines is quite different from that for crown gall of stonefruit.

The first essential is to ensure that planting material is healthy. This can be achieved

in two ways. First, and more reliable is to propagate from shoot tips using tissue culture (4). Shoot tip propagated vines are now commercially available and, so far, have remained free of the pathogen when planted in soil not previously used for grapevine cultivation (Burr, personal communication). It is important that both stock and scion are propagated in this way. The second method of obtaining healthy propagating material is to use heat therapy of dormant cuttings --50℃ for 30 minutes is generally recommended (5) but complete eradication of *A. vitis* from infected cuttings is not always achieved. Heat treatment is probably a satisfactory method of disease control for normal propagation of grapevines in areas where severe frosts do not occur. Crown gall in the nursery is dramatically reduced (Table 4) and establishment of plants is usually improved. Once a vineyard is established in such areas, later infections are probably of little importance unless top-grafting is practised. Heat treatment is not considered satisfactory for the establishment of disease-free nuclear stocks for widespread distribution and propagation. Nor is it satisfactory for normal propagation in areas subject to severe frosts where zero infection is desirable.

Although healthy propagating material can be obtained by shoot tip propagation and by heat therapy, the likelihood of reinfection has not yet been established. *A. vitis* is not a soil-inhabiting organism but new vineyards are frequently established on the sites of old vineyards and the chance of reinfection from old vine debris must be quite high, especially as *A. vitis* possesses pectinase genes (37) which enable it to cause root rotting (2). Re-infestation through rotting roots seems a likely possibility. As *A. vitis* is not sensitive to agrocin

Table 4. Effect of hot water treatment (50℃ for 30 minutes) on crown gall formation in the field

Scion/rootstock combination	Treatment	Number of vines	% plant with galls
Chardonnay/	No heat	111	5
Ramsey	Heat	125	0
Chardonnary/	No heat	99	48
K51-40	Heat	77	3
Zante Currant/	No heat	136	1
Ramsey	Heat	66	0
Zante Currant/	No heat	80	58
K51-40	Heat	35	0

Source : (42a)

Table 5. Strains used in studies on biocontrol of crown gall on grapevines

Strain	Species	Pathogen	Genetic modification	Inhibitory to K1345	Source
K1345	*A. vitis*	yes	Tn501	no	This study
K1069	*A. vitis*	no	〃	no	Kerr
F26	*A. vitis*	no	〃	yes	Liang
F2/5	*A. vitis*	no	〃	yes	Staphorst
K315	*Pseudomonas*	no	〃	yes	Kerr
HLB2	*A. tumefaciens*	no	〃	yes	Chen

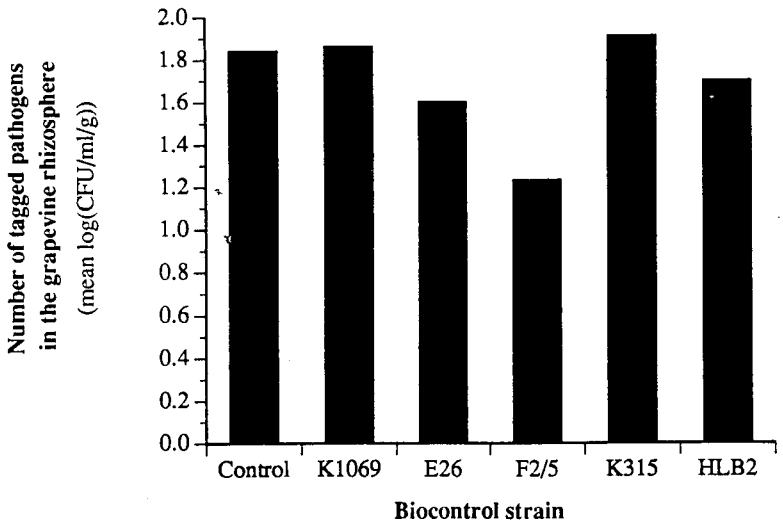


Fig. 10. Colonisation of the grapevine rhizosphere by a pathogenic strain of *Agrobacterium vitis* following treatment with various potential biocontrol strains.

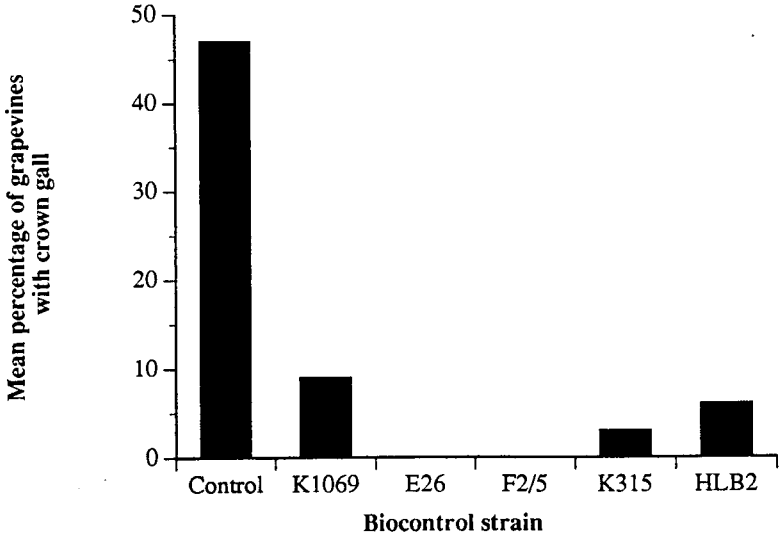


Fig. 11. Incidence of crown gall on grapevine cuttings following treatment with various potential biocontrol strains.

84, strains K84 and K1026 cannot be used to protect grapevines. Many persons are searching for a suitable organism and there have been several reports of potential biocontrol strains (6, 7, 33, 48, 55, 56).

In our laboratory, we have tested several bacterial strains isolated in different laboratories (Table 5), as possible biocontrol agents. They were applied individually to heat-treated cuttings before roots were formed and after. A genetically marked pathogen, strain K1345 was then applied either to the cuttings before planting or to soil after the cuttings were planted.

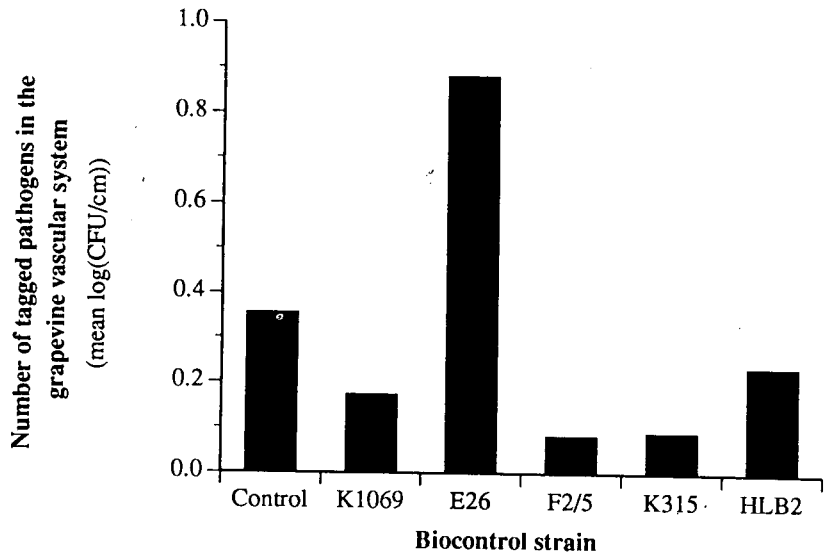


Fig. 12. Colonisation of the vascular tissue of heat-treated cuttings by a pathogenic strain of *Agrobacterium vitis* following treatment with various potential biocontrol strains.

Only one strain, *A. vitis* F2/5, significantly reduced colonisation of the rhizosphere by the pathogen (Fig. 10). However all strains significantly reduced the incidence of crown gall on the cuttings (Fig. 11), the most effective being *A. vitis* strains F2/5 from South Africa and E26 from China where no galls were detected. These two strains both inhibited *A. vitis* in culture but a non-inhibitory strain (K1069) also significantly reduced the incidence of crown gall. So it is not known how important antibiotic production is to achieve biological control. No strain completely prevented recolonisation of the vascular tissue by the pathogen (Fig. 12) but a significant reduction was achieved by most strains tested; greatest reduction resulted from inoculation with A. vitis strain F2/5 and an antibiotic producing strain of *Pseudomonas fluorescens* K315. Considering that the pathogen was applied as a cell suspension of about $10^9\,ml^{-1}$, which is much higher than one would expect to find in the field, the results are very promising, particularly for areas which do not experience severe frosts. **The most promising biocontrol strain appears to be *A. vitis* F2/5, a South African isolate** (48). It controls crown gall induction and reduces colonisation of both vascular tissue and the rhizosphere. Similar results have been reported from America (6) and South Africa (48).

Speculations

It is 22 years since New and Kerr (41) reported the successful biological control of crown gall by strain K84. Since then, there have been many reports of biological control of other plant diseases but very few, if any, have led to successful commercial products. Instead of strain K84 being a fore-runner of many biocontrol agents, it seems to stand alone. Why is this? Let us consider how the biocontrol of crown gall of stonefruit works. We have

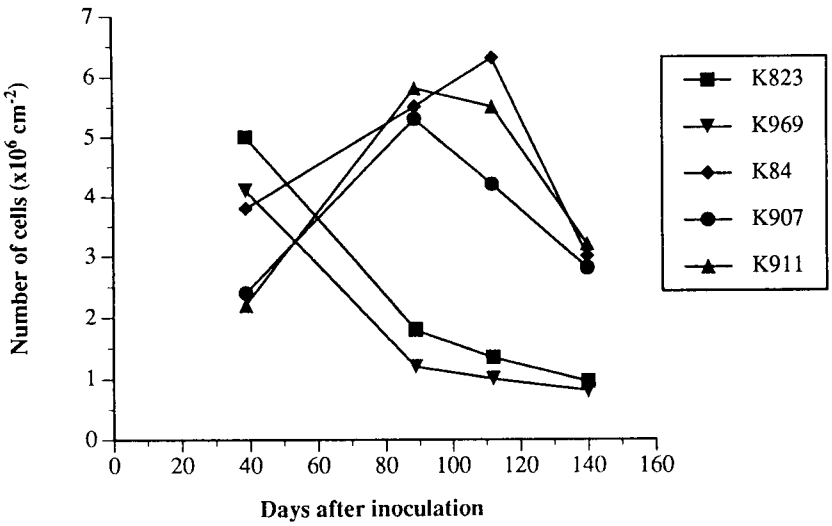


Fig. 13. Colonisation of almond roots by *A. tumefaciens* (strains K823 & K969) and *A. rhizogenes* (strains K84, K907 & K911). Almond seedlings were dipped in bacterial suspensions of 10^7 cells/ml. Source: (46).

come to the conclusion that the vast majority of galls develop from lenticels. Lenticels are not randomly distributed on the tap roots of stonefruit trees. Most are located near the point where a lateral root emerges (25). If peach or almond seeds are sown in soil heavily infested with *A. tumefaciens* and the emerging seedlings examined at frequent intervals, galls do not appear until lenticels are present. In fact, they can be seen developing from lenticels (25). There is some circumstantial evidence that *A. rhizogenes* can colonise lenticels more efficiently than can *A. tumefaciens*. Fig. 13 shows the cell numbers of three strains of *A. rhizogenes* and two strains of *A. tumefaciens*. All were inoculated at the same concentration. Thirty nine days after inoculation (before lenticels would have appeared), *A. tumefaciens* was more abundant than *A. rhizogenes*. After 89 days (after lenticels would have appeared), the reverse applied. Not strong evidence admittedly, but suggestive. We propose that following biocontrol treatment, strain K84 or K1026 colonises the rhizosphere and also the lenticels as they develop. This in itself would probably confer some biological protection by restricting lenticel colonisation by pathogens and by competing for infection sites (34). However, the biocontrol strains also produce agrocin 84 which not only inhibits growth of pathogens but terminates DNA replication (11, 31). Transfer of T-DNA from *A. tumefaciens* to a host cell requires DNA replication and this would be prevented by agrocin 84. So not only is colonisation of infection sites restricted in two ways (prior colonisation and growth prevention) but also the actual infection process is inhibited. This is why we think the biocontrol of crown gall of stonefruit has been so successful and may indeed be unique. We are not optimistic that biocontrol of other plant diseases by applying a single organism will be widely successful. A much more promising approach is through the genetic engineering of plants following an increased understanding of pathogenicity and resistance.

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