

# Biological Control of Crown Gall Through Production of Agrocin 84

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It is not often that an entirely new method of disease control is developed. Since 1973, commercial stone fruit and rose growers in Australia have protected their crops from crown gall by dipping their planting material in a suspension of bacterial cells, achieving nearly complete control of the disease. It is the first commercial use of a specific microorganism to control a plant pathogen in soil and it is the first commercial use of a bacterium to control any plant disease.

## Development of the Method

Crown gall is caused by *Agrobacterium radiobacter* var. *tumefaciens*, a bacterium that lives in soil. The bacterium enters plants through wounds and induces unregulated cell division leading to massive gall formation (Fig. 1).

In Australia, almond, peach, and rose are the crops most severely affected by crown gall. During a study of agrobacteria in soil of a stone fruit nursery, in addition to pathogenic forms, agrobacteria were found that did not induce crown gall when inoculated into plants (10). By all tests used, these nonpathogens could not be distinguished from the pathogens. The ratio of pathogens to nonpathogens was high around diseased plants but low around healthy plants. This prompted a study of the interactions between these two kinds of agrobacteria. Strain 84 was the nonpathogenic isolate used. Tomato seedlings were used in preliminary experiments. When the ratio of pathogen to nonpathogen was one or less than one, no galls developed after inoculation. The experiment was repeated with peach seedlings, and results were the same.

At this stage of the investigation we realized that control of crown gall in the

field might be possible. The objective was to ensure that the ratio of pathogen to nonpathogen at the root surface never exceeded one. This might be achieved by establishing high numbers of the nonpathogen around the roots of susceptible plants by means of inoculation. In the propagation of almond and peach, the only two practical opportunities for inoculation are when sowing the seed of the rootstock or when transplanting young trees.

Soil naturally infested with pathogenic agrobacteria was collected from an orchard where almond trees were being removed because of a high incidence of crown gall. The soil was transferred to large pots (23 L) kept in the open. An experiment was designed to test the effect of nonpathogenic agrobacteria on the incidence of crown gall of peach. Three inoculation treatments with strain 84 were applied: 1) seed inoculation only, 2) root inoculation only, and 3) both seed and root inoculation. Noninoculated plants were used as controls. Seed was sown in soil free of pathogenic agrobacteria, and 1 yr later the plants were transferred to the naturally infested soil in the large pots. Results were assessed 9 mo later (Table 1). Approximately 95% control of crown gall was achieved by root inoculation and 99% by combined seed and root inoculation. Seed inoculation alone, applied nearly 2 yr before results were assessed, achieved 78% control (5).

Field experiments in several countries, including Canada, France, Greece, Hungary, Italy, New Zealand, South Africa, and the United States, have confirmed the effectiveness of strain 84 in controlling crown gall. Where naturally infested soil was used, the level of control was 100% or nearly so (1,8,9).

## Commercial Application

The Waite Agricultural Research Institute has supplied commercial growers with agar cultures of strain 84 since 1973. Commercial firms market the organism

in New South Wales, New Zealand, California, and Oregon. Strain 84 is also used commercially in South Africa. The cultures from New South Wales and New Zealand are supplied in peat, using the techniques developed for *Rhizobium* cultures. The other suppliers provide agar cultures that are easier to prepare but more difficult to distribute. Very little critical work has been done on effective dosage rates; the rate generally recommended is between  $10^7$  and  $10^8$  bacterial cells per milliliter.

Because the control method involves a living organism, some precautions are necessary to ensure effective treatment. For example, the bacteria should not be suspended in chlorinated water, exposed to very high or very low temperatures, or exposed to direct sunlight. The treatment is simple, and sophisticated equipment is not required. Seeds, cuttings, or roots of young plants are dipped in the bacterial suspension and sown or planted immediately. In several nurseries that have had a serious crown gall problem for 10 yr or longer, disease incidence was negligible after treatment. The treatment is also inexpensive, only a few cents per tree for combined seed and root inoculation. Cheap, simple, and effective—what more could be desired? Perhaps an extension of the method to other crops, because the method cannot be applied to all crops subject to crown gall, including grapevine and some floricultural crops, such as chrysanthemum and dahlia.

## Mechanism of Control

Strain 84 produces an unusual kind of antibiotic that selectively inhibits most pathogenic agrobacteria (6). The antibiotic has been called agrocin 84 and belongs to a new group of highly specific antibiotics known as nucleotide bacteriocins. It is a fraudulent adenine nucleotide with two substitutions and several unique features (Fig. 2) (12). Details of its chemical structure and mode of action are being investigated.

Evidence for the involvement of agro-

(Opposite page) Grower treating peach seed with strain 84. The bacteria are suspended in water, and the seeds are dipped in the suspension and planted immediately.

# Problems brought to you by Mother Nature.

*Barnyardgrass*

*Yellow  
Nutsedge*

*Giant Foxtail*

*Fall Panicum*

*Quackgrass*

*Crabgrass*

*Johnsongrass*

*Wild Oats*

*Bermudagrass*

*Broadleaf  
Signalgrass*

*Field Bindweed*



cin 84 in the biological control of crown gall is based on correlations and on genetic data.

**Correlations.** Only those nonpathogenic agrobacteria that produce agrocin 84 control crown gall; nonproducers are ineffective (6). Only pathogenic agrobacteria sensitive to agrocin 84 in a laboratory test can be controlled; resistant strains such as those isolated from grapevine are not subject to control (7). This explains why crown gall on grapevine cannot be controlled. The correlations are perfect except for strain 108, which is

sensitive to agrocin 84 but cannot be controlled; it produces another antibiotic, agrocin 108, which inhibits strain 84 (6).

**Genetic data.** The production of agrocin 84 is coded for by a plasmid (J. Schell, *personal communication*), an extrachromosomal piece of circular DNA. This plasmid can be considered a second, very small chromosome. Many plasmids are conjugative, meaning they promote conjugation with a related bacterium that lacks the plasmid. After conjugation, the plasmid is transferred to the other (recipient) bacterium. As far as is known, the agrocin 84 plasmid is not conjugative. However, strain 84 has another plasmid that codes for the catabolism of nopaline (14), an unusual amino acid derivative found only in crown gall tissue. This plasmid is conjugative. It not only promotes conjugation but also mobilizes the agrocin 84 plasmid, which can then be transferred to a nonpathogenic recipient bacterium (2). The recipient can now produce agrocin 84 and has also acquired the ability to control crown gall. Again, control is dependent on agrocin 84 production.

Other genetic evidence supports the hypothesis. It was recently established that the genes controlling pathogenicity in *Agrobacterium* are located on a large plasmid, the Ti plasmid (15,16). In strains sensitive to agrocin 84, the genes controlling sensitivity are located on the same plasmid (4). In other words, there is a very close genetic linkage between pathogenicity and sensitivity to agrocin 84. This is probably why the method of control is so effective. When a pathogen is exposed to agrocin 84, resistant colonies arise (Fig. 3), but most are nonpathogenic (6). Either the Ti plasmid has been lost or a deletion mutation has occurred that results not only in resistance to agrocin 84 but also in nonpathogenicity. Infrequently, a pathogenic, agrocin 84-resistant strain can be isolated after exposure of a sensitive strain to agrocin 84. It is no longer subject to biological control by strain 84. The evidence seems overwhelming that biological control of crown gall operates through production of agrocin 84.

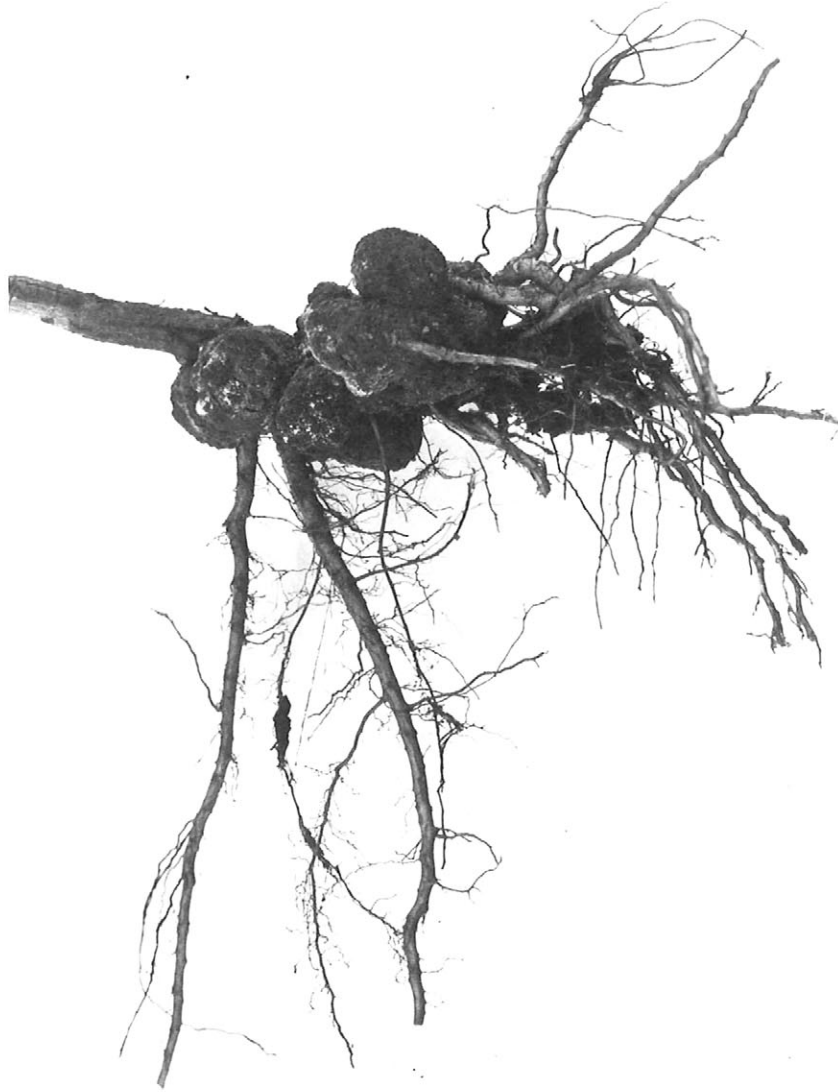


Fig. 1. Crown gall on a young peach tree.

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

| Treatment                                | Mean dry weight of gall tissue per plant <sup>a</sup> (g) | Control <sup>b</sup> (%) |
|--|---|--------------------------|
| None                                     | 11.64   | ...                      |
| Seed inoculation with strain 84          | 2.50  | 78.5                     |
| Root inoculation with strain 84          | 0.59  | 94.9                     |
| Seed and root inoculation with strain 84 | 0.14  | 98.8                     |

<sup>a</sup>Least significant difference of mean dry weights 0.97 ( $P = 0.05$ ).

<sup>b</sup>Percentage control is the difference between the weight of gall tissue on inoculated and noninoculated plants expressed as a percentage of the weight of gall tissue on noninoculated plants.

### Possible Breakdown in Control

With an entirely new method of disease control, it is always possible that something will go wrong. For example, the pathogenic bacteria might mutate and no longer be subject to control. In Australia, there is no evidence that this has happened in the field, but in Greece, three strains isolated from peach could not be controlled because they were resistant to agrocin 84 (7). It is possible that such strains will become more common when inoculation with strain 84 is widely practiced.

An even more dangerous situation has been described from Greece (11). In field experiments in which peach seedlings were inoculated with a 1:1 mixture of strain 84 and a pathogenic strain, many

galls developed. Pathogenic, agrocin 84-producing strains were isolated from these galls. All agrocin 84-producing strains so far examined are resistant to agrocin 84 and therefore cannot be controlled by strain 84. These characteristics were found in 16.5% of all isolates.

The inference from the data is that the genes controlling agrocin 84 production (and resistance) can be readily transferred from strain 84 to a pathogenic recipient. This was confirmed by carrying out crosses between strain 84 and pathogenic agrobacteria under controlled laboratory conditions (3). Such a cross is represented diagrammatically in Fig. 4. Three plasmids are involved: two in strain 84 and one in the pathogen. Strains that acquire a plasmid after conjugation are called plasmid transconjugants. The six possible types of transconjugant are represented in Fig. 4, and all were isolated. It would appear that after conjugation, the two plasmids from strain 84 transfer independently, while the Ti plasmid in the recipient may or may not be retained. Transconjugants B and C represent the hazard; they are pathogenic, produce agrocin 84, and are resistant to it.

Why is there no evidence for breakdown in the effectiveness of biological control in Australia where the method has been practiced for 6 yr? Although plasmid transfer from strain 84 to pathogenic recipients can be readily achieved in the laboratory, it can be achieved only in the presence of nopaline (3). When nopaline is absent, conjugation is completely repressed; when present, nopaline derepresses the genes controlling conjugation and plasmid transfer. In other words, nopaline acts as a bacterial aphrodisiac. Because nopaline occurs naturally only in crown gall tissue, plasmid transfer will occur only under natural conditions in the presence of crown gall tissue. In Australia, biological control has been nearly 100% effective. There is little or no gall tissue present and therefore little or no plasmid transfer. In Greece, artificial inoculation with patho-

gens must have resulted in high numbers of virulent cells on and around the roots. Presumably, strain 84 did not adequately control them; galls developed, nopaline was produced, and plasmid transfer occurred.

Although transfer of the agrocin 84 plasmid has not yet been detected in commercial nurseries or orchards where strain 84 has been applied as a routine control measure, there can be little doubt that pathogenic, agrocin-producing strains will eventually appear. It is unrealistic to expect 100% control in every field every year. Can anything else be done to minimize the risk of a breakdown in the effectiveness of biological control of crown gall? Yes, a

mutant of strain 84 with a defective plasmid transfer or mobilization system could be used. Such mutants are known in other bacteria and if found in strain 84 either would prevent cell conjugation and plasmid transfer or would prevent mobilization of the agrocin 84 plasmid by the conjugative plasmid. Either mutation would seem to be an ideal solution to the problem, and a determined search for such mutants seems justified. A breakdown in the effectiveness of the biological control of crown gall not only would result in an annual loss of millions of dollars (13) but also would undermine confidence in the introduction of biological methods for the control of plant diseases in general.

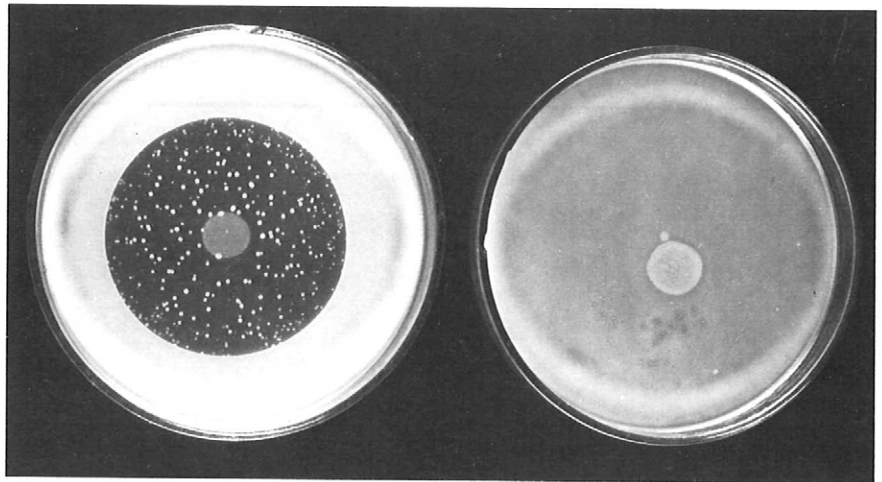


Fig. 3. The effect of agrocin 84 on (left) a pathogenic strain of *Agrobacterium tumefaciens* and (right) a nonpathogen. The resistant colonies within the zone of inhibition are predominantly nonpathogens.

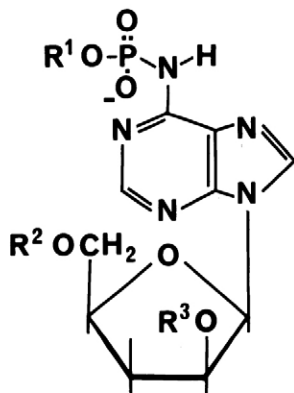


Fig. 2. Molecular structure of agrocin 84.

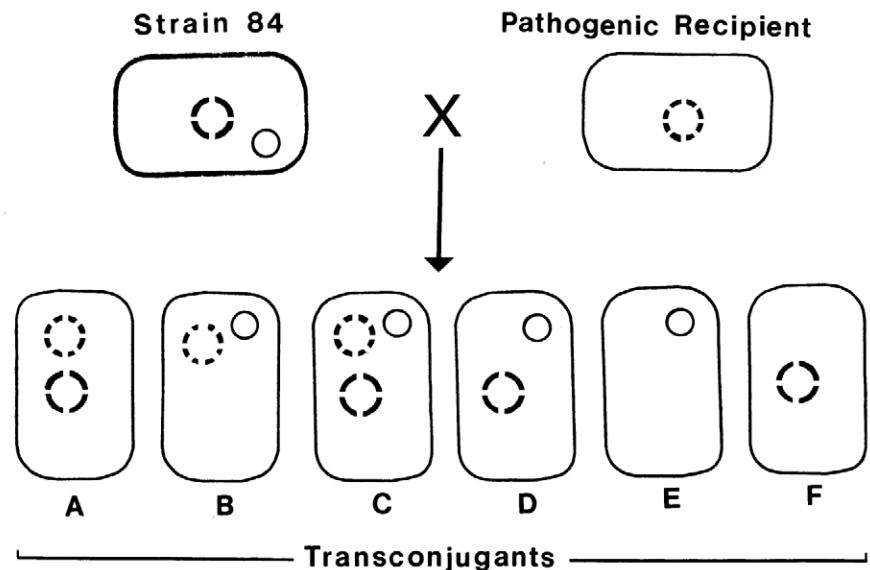


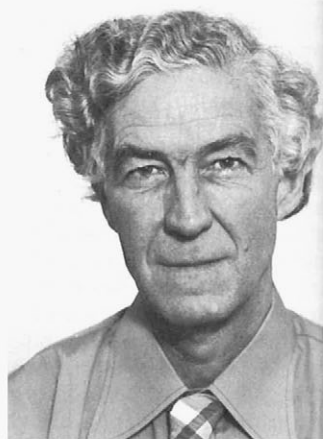
Fig. 4. Diagrammatic representation of a cross between strain 84 and a pathogenic recipient of *Agrobacterium tumefaciens*. Chromosomes are not shown. Strain 84 contains two plasmids, one (solid line) coding for agrocin 84 production and resistance to agrocin 84 and the other (broken line) coding for nopaline catabolism and for conjugation. The pathogen has one plasmid (dotted line) that codes for pathogenicity and for agrocin 84 sensitivity as well as for other characters not discussed in the text. The cross results in six plasmid transconjugants. Transconjugants B and C combine pathogenicity with resistance to agrocin 84.

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Dr. Kerr was born in Edinburgh, Scotland, and graduated from the University of Edinburgh in 1947. He spent the next 4 yr at the North of Scotland College of Agriculture, Aberdeen, and then accepted a position as lecturer in plant pathology at the Waite Agricultural Research Institute, University of Adelaide, where he studied the behavior of soilborne pathogens on and around plant surfaces. During 1963-1966, he served at the Tea Research Institute of Ceylon. On returning to Adelaide, he began his studies on crown gall, and in 1978 he was elected a Fellow of the Australian Academy of Science.