# A full factorial analysis of nine factors influencing in vitro antagonistic screens for potential biocontrol agents

**G.A. Dickie and C.R. Bell**

**Abstract**: The effect of nine factors on the outcome of classic in vitro screens testing the antagonistic action of endophytic bacterial isolates from grape vines against virulent *Agrobacterium vitis* has been examined. These factors were (*i*) the strain of *A. vitis*, (*ii*) the strain of endophyte, (*iii*) the growth medium of the pathogen, (*iv*) the growth medium of the endophyte, (*v*) the temperature of growth of the pathogen, (*vi*) the temperature of growth of the endophyte, (*vii*) the pH of growth of the pathogen, (*viii*) the pH of growth of the endophyte, and (*ix*) the medium of the assay plate. Analyses of variance of the full factorial design incorporating main effects and two- and three-way interactions accounted for 66% of the variance. All nine factors had a significant effect on the diameter of inhibition zones ($p < 0.001$). An examination of the three-way interactions revealed that generalizations were difficult to draw; each target agrobacterium had a specific response to a given antagonistic isolate. It was possible to determine that the growth history of bacterial strains, before they were administered to an assay plate to test for antagonism (especially the composition of the growth medium and the temperature of growth), had a profound effect on the outcome of the test. Generally the more chemically defined media produced less inhibition whereas the lower growth temperature of 15°C produced more inhibition. These findings could be relevant to in situ inhibitory activity. The method used to conduct the inhibitory screen (order of strain application and the medium of the assay plate) had a profound influence on the results. These influences add to the caution necessary in the use of in vitro antagonistic screens for finding successful biocontrol agents.

*Key words*: in vitro antagonism, endophytic bacteria, grape crown gall disease, biocontrol, screening strategy.

**Résumé** : L'effet de neuf facteurs sur les résultats de sélection classique in vitro, dans des essais sur l'action antagoniste d'isolats bactériens endophytes obtenus de vignes contre l'espèce virulentes *Agrobacterium vitis*, a été étudié. Ces facteurs sont (*i*) la souche d'*A. vitis*, (*ii*) la souche endophyte, (*iii*) le milieu de croissance de l'agent pathogène, (*iv*) le milieu de croissance de la souche endophyte, (*v*) la température de croissance de l'agent pathogène, (*vi*) la température de croissance de la souche endophyte, (*vii*) le pH de croissance de l'agent pathogène, (*viii*) le pH de croissance de la souche endophyte et (*ix*) les milieux de culture en plaques. Les sources de variation du dispositif factoriel complet, soit les effets principaux, les interactions doubles et triples, ont expliqué 66% de la variance totale, lors des analyses. Chacun des neuf facteurs a eu un effet significatif sur le diamètre des zones d'inhibition ($p < 0{,}001$). L'examen des interactions triples a révélé qu'il était difficile de formuler des généralisations, vu que chaque agrobactérie-cible a fourni une réponse spécifique pour chaque isolat antagoniste particulier. Il a été impossible de déterminer si le profil de croissance des souches bactériennes auraît un effet marqué sur le résultat des essais, avant de les déposer sur les plaques de culture pour tester l'antagonisme (en particulier la composition des milieux et les températures de croissance). Généralement, les milieux les plus chimiquement définis ont produit moins d'inhibition, alors qu'une faible température de croissance de 15°C a produit plus d'inhibition. Ces résultats pourraient être applicables à de l'activité inhibitrice in situ. La méthode utilisée pour mener la sélection inhibitrice, soit l'ordre d'application des souches et les milieux composant les plaques, a eu une influence marquée sur les résultats. Ces traits d'influence ajouent à la circonspection nécessaire lors de la sélection d'antagonistes in vitro pour détecter des agents efficaces de biocontrôle.

*Mots clés* : antagonisme in vitro, bactéries endophytes, gale de la couronne de la vigne, biocontrôle, stratégie de sélection.
[Traduit par la Rédaction]

## Introduction

The dilemma of how much faith to place in in vitro antagonistic screening to determine the relationship between microorganisms in biocontrol research is longstanding. Every recent review on the subject includes a cautionary note on the use of such screens and their inconsistent record as predictors of

Received September 28, 1994. Revision received January 5, 1995. Accepted January 9, 1995.

**G.A. Dickie and C.R. Bell.**[1] Department of Biology, Acadia University, Wolfville, NS B0P 1X0, Canada.

[1] Author to whom all correspondence should be addressed.

Table 1. Bacterial strains used in this study.

| Strain | Species | Source of isolate | Location of isolate | Supplied by |
|---|---|---|---|---|
| JC573 | *P. corrugata* | Grape sap | Canada | C.R. Bell, Canada |
| JC577 | *R. aquatilis* | Grape sap | Canada | C.R. Bell, Canada |
| JC583 | *P. corrugata* | Grape sap | Canada | C.R. Bell, Canada |
| AA25 | *A. vitis* | Grape gall | Afghanistan | G.L. Ercolani, Italy |
| Ag63 | *A. vitis* | Grape gall | Greece | C.G Panagopoulas, Greece |
| CG64 | *A. vitis* | Grape gall | U.S.A. | T.J. Burr, U.S.A. |
| JP308 | *A. vitis* | Grape gall | Canada | C.R. Bell, Canada |
| K306 | *A. vitis* | Grape gall | Australia | A. Kerr, Australia |
| NW161 | *A. vitis* | Grape gall | Germany | E. Bien, Germany |

in planta performance (Campbell 1994; Becker and Schwinn 1993; Cook 1993; Deacon 1991; Merriman and Russell 1990). Yet a sampling of the current literature reveals that in vitro screening is still very much in evidence, either as secondary experiments to determine the mode of action (Kraus and Loper 1992; Lemanceau et al. 1993; Maurhofer et al. 1992), or as primary screens (Crawford et al. 1993; Elabyad et al. 1993; Huang et al. 1992).

The most likely mechanisms causing zones of inhibition on an in vitro assay would be antibiotic, siderophore, or in some cases HCN production (O'Sullivan and O'Gara 1992). Because of the considerable successes of *Agrobacterium radiobacter* K84 and agrocin 84 in the biocontrol of crown gall disease (Moore and Warren 1979; Ryder and Jones 1990), most workers in this particular field have focused on controlling crown gall with similar bacteriocins (Chen and Xiang 1986; Hendson et al. 1983; Staphorst et al. 1985; Thomson 1986; Webster et al. 1986).

Surprisingly, despite the literature on *A. radiobacter* K84, little is known about the metabolic factors that control the expression of agrocins. In a study of genetic factors influencing agrocin 84 production, Ryder et al. (1987) observed that production was unaffected by nopaline, agrocinopine A, acetosyringone, and low or high levels of ferric iron. Formica (1990) surveyed a range of sugars and sugar alcohols and found D-fructose to be the best for agrocin 84 production. He also examined the effect of opines and various phytohormones as supplements. Although these two studies are useful they do not address questions on how to optimize a screen for agrocin activity as has been done for antibiotics of pharmaceutical importance. Fundamental abiotic factors such as medium, pH, temperature, and agitation have not been studied as they have been so exhaustively in the industrial context (Elander 1987; Hewitt and Vincent 1989).

In research on crown gall biocontrol, it is invariably the protocol of Stonier (1960) as exploited so successfully by Kerr (Kerr and Htay 1974; New and Kerr 1972) that has become the traditional method for performing agrocin sensitivity assays (Moore et al. 1988). This paper examines several factors that could influence the outcome of screenings against virulent *Agrobacterium vitis*, including the type of growth medium, the pH of the medium, the temperature of growth, and the type of assay plate.

## Materials and methods

### Source of bacterial cultures

The antagonistic endophytic bacteria were obtained from xylem sap from an assortment of grapevine cultivars. The sap was collected using the vacuum extraction method of Bazzi et al. (1987). Full details of these extractions are given elsewhere (Bell et al. 1995*a*) but essentially all vines were taken from vineyards in the Annapolis Valley, Nova Scotia, Canada. Approximately 1 mL of sap was aseptically removed from each vine and the bacteria within were grown on plates of 1/10 strength tryptic soya agar (1/10 TSA) at 30°C. Colonies were restreaked to purity on 1/10 TSA before being stored at −70°C in 15% (w/v) glycerol. A total of 851 isolates were collected in this manner. After the three in vitro antibiosis screens (details given below), three endophytic isolates, JC573, JC577, and JC583, were chosen on the basis of strong and consistent antagonistic activity. These isolates were identified by Biolog™ GN plates (Biolog Inc, Hayward, Calif.) as *Pseudomonas corrugata*, *Rahnella aquatilis*, and *Pseudomonas corrugata*, respectively.

A total of six tumorigenic *A. vitis* strains (Table 1) were used in screening the collection of endophytic bacteria for antagonism.

### In vitro antibiosis screens

Three separate screens were conducted to identify inhibitory activity (data not shown here; full details in Bell et al. 1995*b*). In screen 1, the endophytic isolates were grown in Luria broth (LB) at 20°C for 48 h. The two *A. vitis* indicator strains, Ag63 and CG64 (Table 1), were grown in mannitol glutamate broth (MG) (Bell 1990) at 30°C for 48 h. Five-microlitre aliquots of each endophyte were spotted onto LB and MG plates in triplicate. The plates were incubated at 20°C for 24–36 h. Each plate was then sprayed with the 48-h *A. vitis* indicator culture and incubated for another 36 h at 20°C. Endophytic isolates showing a zone of inhibition greater or equal to 2 mm from the edge of the endophytic colony were selected.

In screen 2, all the procedures remained the same except that additional *A. vitis* indicator strains were included. These were strains K306, AA25, NW161, and JP308 (Table 1).

In screen 3 the order of application of endophyte and indicator strains was reversed. Thus, MG and LB plates were first sprayed with the seven *A. vitis* indicator strains. After 30 min of absorption at 20°C, 5-μL aliquots of the endophytic

bacterial cultures were applied in triplicate. The plates were incubated at room temperature for 48 h before being scored for inhibition zones.

**The full factorial analysis on inhibition**

The effects of the following nine factors on the in vitro screens were examined. (*i*) Pathogen (×3): strains AA25, JP308, and CG64 were used. (*ii*) Antagonist (×3): strains JC573, JC577, and JC583 were used. (*iii*) Growth medium for the pathogen (×3): tryptic soya broth (TSB), glucose nitrogen broth (GN broth, after Herbert and Bell 1977), and MG were tested. (*iv*) Growth medium for the antagonist (×3): TSB, GN, and MG were tested. (*v*) Temperature of growth for the pathogen (×2): 15 and 30°C were tested. (*vi*) Temperature of growth for the antagonist (×2): 15 and 30°C were tested. (*vii*) pH of growth for the pathogen (×2): pH values of 6.0 and 8.5 were tested. (*viii*) pH of growth for the antagonist (×2): pH values of 6.0 and 8.5 were tested. (*ix*) Plate for visualizing inhibition zones (×2): plates of MG and LB were compared.

All tests were performed in triplicate, yielding a data deck of 7776 measurements for each combination of factors. The average of the triplicates was calculated and the resulting data were then analyzed with program EXAMINE of SPSS/PC+. The Levene test together with spread and level plots indicated that a reciprocal transformation of zone diameters was the most satisfactory treatment for optimizing normality and equality of variance. An analysis of variance (ANOVA) was then performed with program ANOVA for main effects, and two- and three-way interactions. Multiple comparison tests were performed on the three-way interaction groups to determine statistically significant differences between groups using program ONEWAY with the least significant difference (LSD) statistic. Interaction diagrams were drawn with the back-transformed data (from reciprocal to linear) and the means expressed in Figs. 1–5 with asymmetric 95% confidence intervals of the mean.

To perform this large experiment, the strains of *A. vitis* and the antagonists were recovered as needed from cold storage at −70°C by streaking onto MG plates and incubation at 25°C for 48 h. One litre of each of the three broths (TSB, MG, and GN) was prepared and half of each was adjusted to pH 6.0 while the other half was adjusted to pH 8.5 with HCl and NaOH, respectively. Aliquots (60 mL) were then dispensed into 125-mL Erlenmeyer flasks and 10-mL aliquots were dispensed into test tubes. Strains of *A. vitis* were inoculated into flasks of each broth type and pH to give two sets of six flasks each. One set was incubated at 30°C, the other at 15°C, both with agitation at 200 rpm. The antagonists were similarly inoculated into the test tubes and incubated at the two temperatures on a rotating carousel. All cultures were grown for 48 h.

Fine-mist spray bottles were sterilized with two washes of 70% ethanol followed by two rinses with sterile water. The *A. vitis* culture (adjusted to an optical density at 600 nm ($OD_{600\ nm}$) of 0.3) was poured into these bottles and sprayed onto four MG and four LB plates. The plates were allowed to dry for 1–2 h. Antagonist strains were adjusted to the above turbidity and 5-μL spots were arranged on the MG and LB plates. Three rows of three spots were prepared for each plate; row 1 was antagonist grown on TSB, row 2 antagonist grown on MG, and row 3 antagonist on GN. Plates were incubated at 25°C for 48 h before being scored for zones of inhibition.

**Effect of the order of application on inhibition**

In screen 3, in which the order of application of agrobacterium and antagonist was reversed, many different results were observed compared with those of screens 1 and 2. The basis for these differences was further explored in an experiment using only *A. vitis* CG64 as the indicator strain with the three antagonists, JC573, JC577, and JC583. Two different broths were used for growing the bacteria (LB and GN) and two media for measuring the resulting inhibition zones (MG and LB plates). All bacterial cells were grown in the appropriate broth at 25°C for 48 h and adjusted to an $OD_{600\ nm}$ of 0.30 (approximately $10^9$ cells/mL). Aliquots were then either spotted or sprayed on plates as described above for screens 2 and 3. Inhibition zones (five replicates for each condition) were measured after 48 h growth at 25°C. A four-way analysis of variance was performed on the data with the ANOVA program of SPSS/PC+.

## Results

**Major factors influencing in vitro inhibition assays**

The results from the ANOVA (Table 2) revealed that a model comprising up to three-way interactions accounted for 66% of the variance. All nine main factors were significant ($p < 0.01$) plus 20 two-way interactions and 24 three-way interactions. Subsequent computation involving four-way and higher interactions did not greatly enhance the efficacy of the model, was extremely expensive in terms of computational time, and was also very difficult to interpret biologically. The data in Figs. 1–5 have been chosen to demonstrate the relationships of the most biologically meaningful three-way interactions influencing this in vitro screen.

Figure 1, which corresponds to three-way interaction 1 of Table 2 (agrobacterial strain × growth medium of the agrobacteria × growth temperature of the agrobacteria), was the most influential interaction. A detailed examination of this figure highlights the complicated nature of the interactions that were typical of this experiment. In particular, the unique effects of each agrobacterial strain on the process of antagonism became evident: JP308 showed great variability over the three media, and AA25 was very constant under all conditions tested, whereas CG64 showed an intermediate range of variability. JP308 was inhibited most strongly (with the largest inhibition zones) on MG medium and least strongly on GN medium. Although this trend remained for JP308 at both growth temperatures of 15 and 30°C, strain CG64 showed marked differences between the two temperatures. Thus, growth on TSB by CG64 at 15°C produced the smallest inhibition zones: on the other hand, at 30°C, TSB produced the largest zones. It becomes evident that it is impossible to draw generalized trends from this diagram. The nature of the agrobacterial strain, plus its recent growth history (medium of growth and temperature) have a profound influence on its behaviour in an in vitro screen such as this.

The peculiarities of the effects of each agrobacterial strain on the process were further compounded through interactions with the antagonist isolates. Figure 2 (three-way interaction 2 in Table 2) shows that the most efficient antagonist depended

**Table 2.** ANOVA table of major significant effects ($p \leq 0.01$).

| Description | Sum of squares | df | $F$ | Significance of $F$ |
|---|---|---|---|---|
| Main effects | 0.221 | 13 | 90.0 | <0.001 |
| 1. Media for antagonists (GROW_B) | 0.081 | 2 | 213.7 | <0.001 |
| 2. Growth temp. for antagonists (TEMP_B) | 0.022 | 1 | 113.7 | <0.001 |
| 3. Agrobacterial strain (AGRO) | 0.039 | 2 | 101.8 | <0.001 |
| 4. Media for agrobacteria (GROW_A) | 0.034 | 2 | 88.9 | <0.001 |
| 5. Antagonist strain (BCA) | 0.034 | 2 | 88.7 | <0.001 |
| 6. Growth pH for agrobacteria (PH_A) | 0.006 | 1 | 29.3 | <0.001 |
| 7. Assay plate (PLATE) | 0.004 | 1 | 21.3 | <0.001 |
| 8. Growth pH for antagonists (PH_B) | 0.002 | 1 | 10.0 | 0.002 |
| 9. Growth temp. for agrobacteria (TEMP_A) | 0.002 | 1 | 9.1 | 0.003 |
| Two-way interactions | 0.363 | 74 | 25.9 | <0.001 |
| 1. AGRO × PLATE | 0.122 | 2 | 322.4 | <0.001 |
| 2. GROW_A × PLATE | 0.062 | 2 | 164.1 | <0.001 |
| 3. AGRO × GROW_A | 0.039 | 4 | 51.9 | <0.001 |
| 4. PLATE × TEMP_A | 0.01 | 1 | 51.6 | <0.001 |
| 5. AGRO × BCA | 0.007 | 4 | 37.1 | <0.001 |
| 6. BCA × GROW_B | 0.026 | 4 | 35.0 | <0.001 |
| 7. PLATE × PH_B | 0.006 | 1 | 29.2 | <0.001 |
| 8. GROW_B × PLATE | 0.008 | 2 | 19.8 | <0.001 |
| 9. PLATE × PH_A | 0.004 | 1 | 18.5 | <0.001 |
| 10. BCA × GROW_A | 0.013 | 4 | 16.6 | <0.001 |
| 11. AGRO × TEMP_B | 0.006 | 2 | 15.5 | <0.001 |
| 12. GROW_A × PH_A | 0.005 | 2 | 12.6 | <0.001 |
| 13. BCA × PLATE | 0.005 | 2 | 12.2 | <0.001 |
| 14. GROW_B × PH_B | 0.004 | 2 | 11.5 | <0.001 |
| 15. BCA × PH_A | 0.003 | 2 | 9.2 | <0.001 |
| 16. AGRO × PH_B | 0.003 | 2 | 9.2 | <0.001 |
| 17. BCA × TEMP_A | 0.003 | 2 | 8.5 | <0.001 |
| 18. AGRO × GROW_B | 0.004 | 4 | 4.7 | 0.001 |
| 19. GROW_B × TEMP_B | 0.002 | 2 | 6.6 | 0.001 |
| 20. BCA × PH_B | 0.002 | 2 | 4.9 | 0.008 |
| Three-way interactions | 0.237 | 242 | 5.2 | <0.001 |
| 1. AGRO × GROW_A × TEMP_A | 0.035 | 4 | 45.7 | <0.001 |
| 2. AGRO × BCA × TEMP_A | 0.026 | 4 | 34.9 | <0.001 |
| 3. AGRO × BCA × PLATE | 0.018 | 4 | 23.3 | <0.001 |
| 4. BCA × GROW_A × PLATE | 0.017 | 4 | 22.9 | <0.001 |
| 5. GROW_A × PLATE × TEMP_A | 0.008 | 2 | 20.6 | <0.001 |
| 6. BCA × PH_A × TEMP_B | 0.005 | 2 | 14.0 | <0.001 |
| 7. AGRO × BCA × GROW_B | 0.019 | 8 | 12.2 | <0.001 |
| 8. AGRO × BCA × PH_A | 0.008 | 4 | 11.2 | <0.001 |
| 9. BCA × PLATE × PH_A | 0.004 | 2 | 10.5 | <0.001 |
| 10. BCA × PLATE × TEMP_A | 0.004 | 2 | 10.3 | <0.001 |
| 11. BCA × PLATE × PH_B | 0.004 | 2 | 9.4 | <0.001 |
| 12. BCA × GROW_A × TEMP_A | 0.006 | 4 | 8.3 | <0.001 |
| 13. AGRO × BCA × GROW_A | 0.012 | 8 | 8.1 | <0.001 |
| 14. AGRO × GROW_A × PLATE | 0.005 | 4 | 7.1 | <0.001 |
| 15. AGRO × GROW_B × PLATE | 0.005 | 4 | 6.0 | <0.001 |
| 16. AGRO × BCA × TEMP_B | 0.004 | 4 | 5.9 | <0.001 |
| 17. AGRO × GROW_A × PH_A | 0.004 | 4 | 5.4 | <0.001 |
| 18. BCA × GROW_A × PH_A | 0.003 | 4 | 4.4 | 0.001 |
| 19. AGRO × PLATE × TEMP_A | 0.003 | 2 | 7.5 | 0.001 |
| 20. GROW_A × PH_A × TEMP_A | 0.002 | 2 | 6.5 | 0.002 |
| 21. PH_A × PH_B × TEMP_A | 0.002 | 1 | 8.9 | 0.003 |
| 22. AGRO × PH_B × TEMP_B | 0.002 | 2 | 5.3 | 0.005 |
| 23. AGRO × PLATE × TEMP_B | 0.002 | 2 | 5.1 | 0.006 |
| 24. BCA × PH_A × TEMP_B | 0.002 | 2 | 5.0 | 0.007 |
| Explained | 0.821 | 329 | 13.2 | <0.001 |
| Residual | 0.428 | 2262 | | |
| Total | 1.249 | 2591 | | |

**Fig. 1.** Interaction diagram of agrobacterial strain (AGRO) × growth temperature for agrobacteria (TEMP_A) × medium for agrobacteria (GROW_A). Each symbol represents the mean of 144 determinations; error bars represent the 95% confidence intervals of the mean. Symbols bearing similar letters are not significantly different (LSD, $p \leq 0.05$).

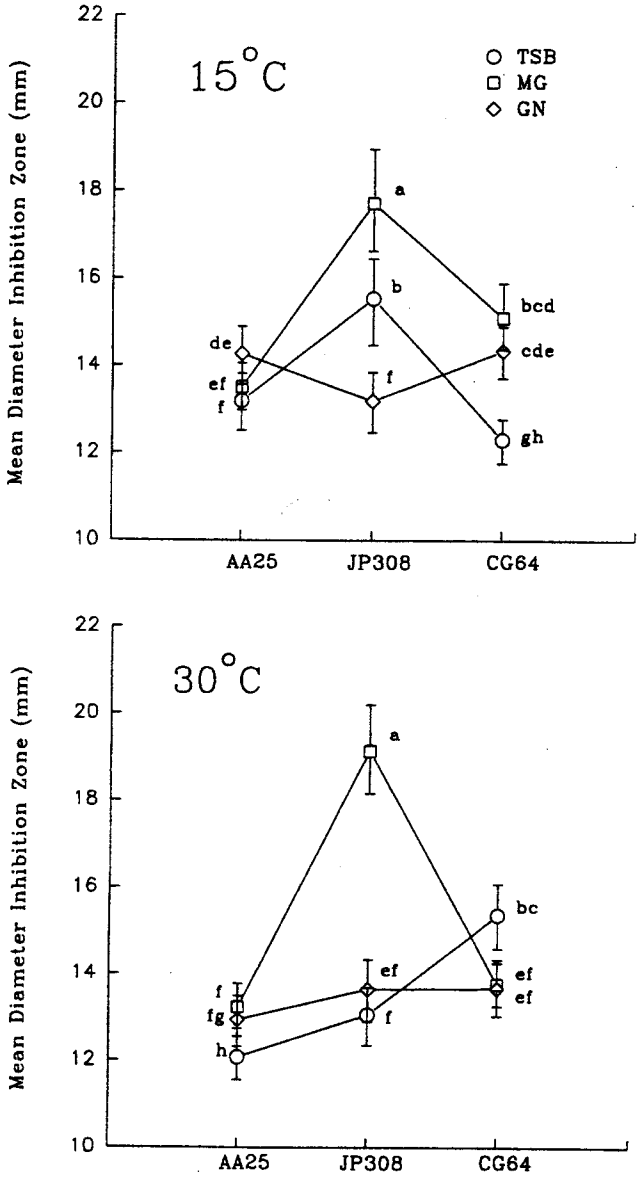
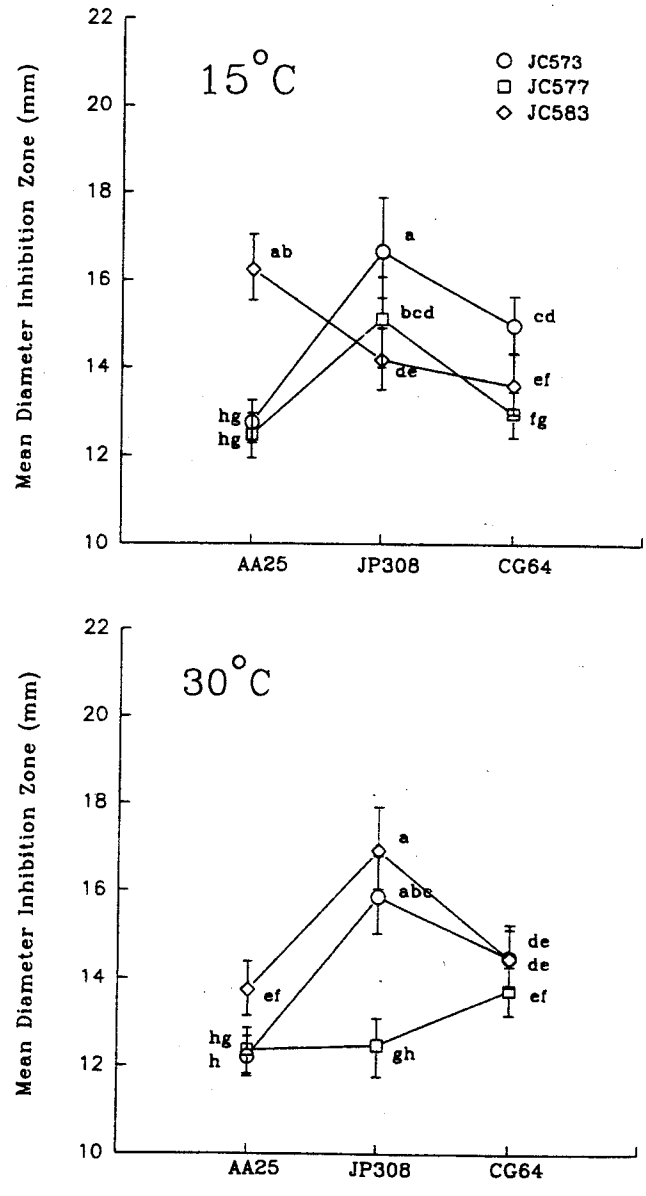



**Fig. 2.** Interaction diagram of agrobacterial strain (AGRO) × antagonistic strain (BCA) × growth temperature for agrobacteria (TEMP_A). Other conventions as in Fig. 1.




on which agrobacterial strain was the target plus the temperature at which the agrobacteria were grown. At 30°C, the pattern may appear obvious, with relative efficiencies in the order JC583 > JC573 > JC577. Unfortunately this relationship only held for one of the agrobacteria (AA25) at 15°C; with JP308 and CG64, isolate JC573 exhibited significantly stronger inhibition than the other two antagonists.

The growth history of the antagonists (i.e., medium and growth temperature) had an effect on inhibition but the sum of squares and $F$ statistic of Table 2 indicated that this was less significant than the growth history of the agrobacteria. In fact, the graphs of the interactions of agrobacteria × antagonist × growth temperature of the antagonists (Fig. 3; interaction 7 in Table 2) demonstrated that the major effect was the reduced inhibition of all three isolates when grown on GN medium. Most of the other responses were comparable.

In Fig. 4, where the interactions of antagonist growth temperature × antagonist × pH of the agrobacterial medium (interaction 6 in Table 2) are plotted, a trend arose, with the stronger inhibition being produced when the antagonists were grown at 15°C rather than 30°C. This figure also clearly demonstrates the minor influence of pH on the process of inhibition. Of the 12 means plotted, it is only the single value

F_ 3. Interaction diagram of agrobacterial strain (AGRO) × antagonistic strain (BCA) × medium for antagonists (GROW_B). Each symbol represents the mean of 96 determinations. Other conventions as in Fig. 1.

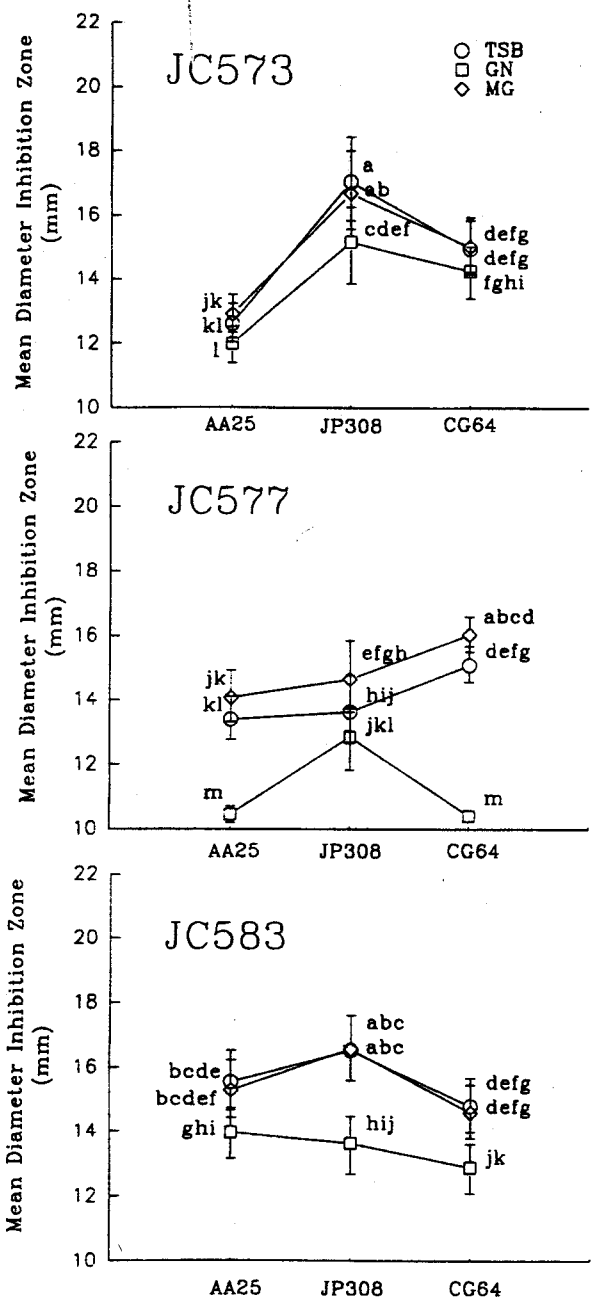
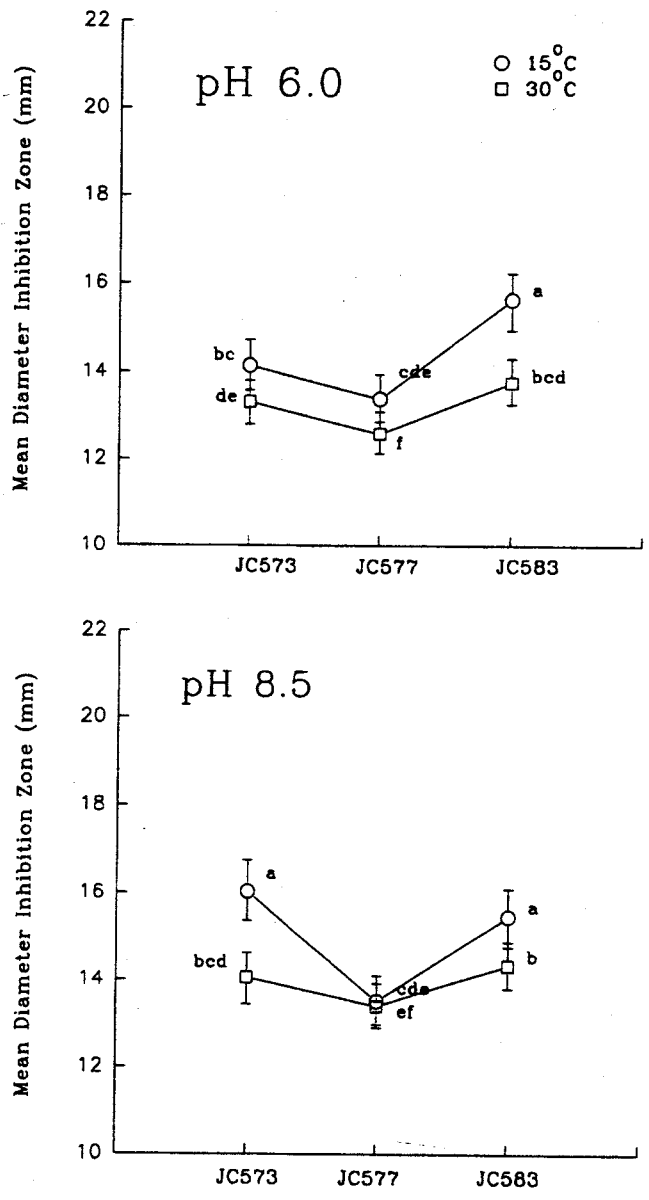


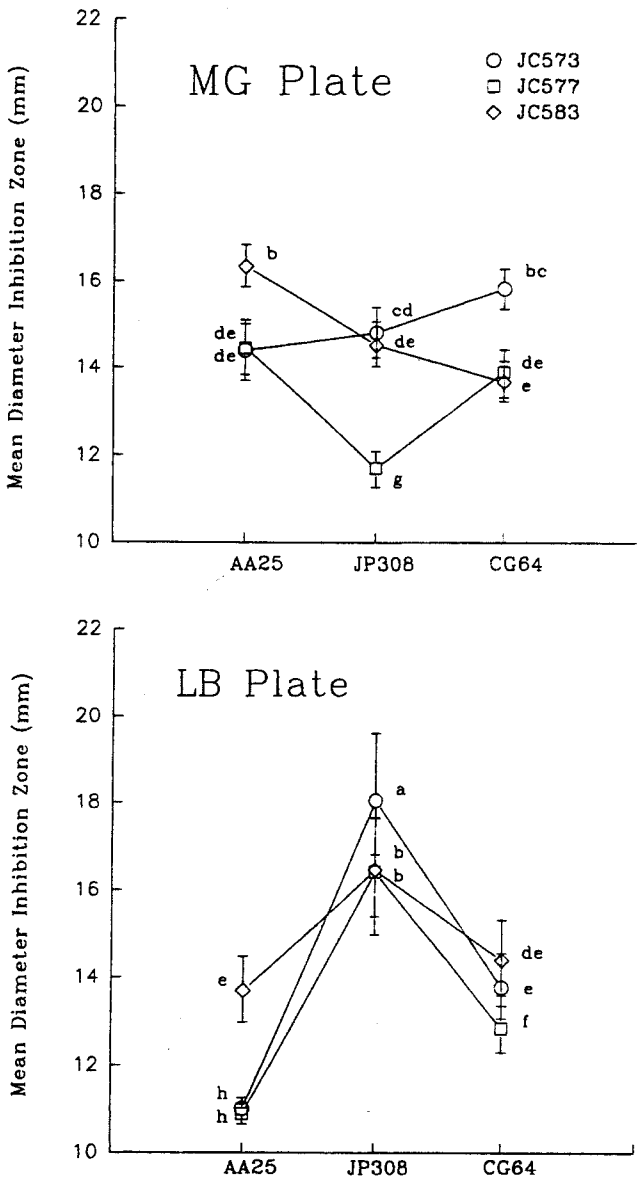

Fig. 4. Interaction diagram of antagonistic strain (BCA) × growth pH for agrobacteria (PH_A) × growth temperature for antagonist (TEMP_B). Each symbol represents the mean of 216 determinations. Other conventions as in Fig. 1.




for JC573 when grown at 15°C and reacted with agrobacteria grown at pH 8.5 that is significantly different.

Finally, Fig. 5 (together with Fig. 6 below) demonstrates how the mechanics of performing the screen affected the outcome. The influence of the assay plate (interaction 3 in Table 2) on the final results was significant. There was an overall trend involving isolate JC577, which proved to be the weakest inhibitor against all three agrobacteria on both MG and LB plates. However, the conclusions for the strongest inhibitor were not so clear cut. For instance, JP308 showed great susceptibility to inhibition by all three antagonists but only on the LB plates, with JC573 being the strongest. Overall, the response of JP308 was to produce much larger zones of inhibition (on average 3.31 mm larger) on LB plates than on MG plates. When AA25 was the target strain, isolate JC583 proved to be the strongest inhibitor on both plates. All zones were consistently smaller on LB agar with AA25. When CG64 was the target, JC573 was the strongest inhibitor on MG plates, with zones generally being smaller on LB agar.

Fig. 5. Interaction diagram of agrobacterial strain (AGRO) × antagonistic strain (BCA) × assay plate (PLATE). Other conventions as in Fig. 1.




### Effect of the order of application

The protocol for the factorial experiment applied the agrobacteria to the plate before applying spots of antagonist culture. Many investigators adopt a protocol after that of Stonier (1960) in which the antagonists are applied first. An investigation of the effect of this order of application revealed that it did make a significant difference (Fig. 6). The histograms demonstrate that the trend of obtaining greater inhibition zones with order 2 (applying the antagonists first) was broken only with JC577 and only when tested on LB agar.

## Discussion

Despite the fact that the ANOVA revealed that all nine factors and numerous two- and three-way interactions had a significant effect on the size of inhibition zones, the screen did prove effective in demonstrating differences in the antagonism of the potential biocontrol agents (Figs. 2 and 3) and also differences in the susceptibility of the agrobacterial strains to these antagonists (Fig. 1). Of course, most researchers embarking on a project to identify biocontrol agents would not take the time to do a factorial experiment this exhaustive. It is worth pointing out that 798 combinations of factors out of a total of 2592 (30.8%) did not produce any appreciable inhibition zones (mean diameter approximately 10 mm). Conversely, some combinations produced zones larger than 25 mm, which often cleared the entire Petri plate. In a smaller experimental design, this percentage of noninhibition could easily lead the researcher to false conclusions about the in vitro capability of isolates.

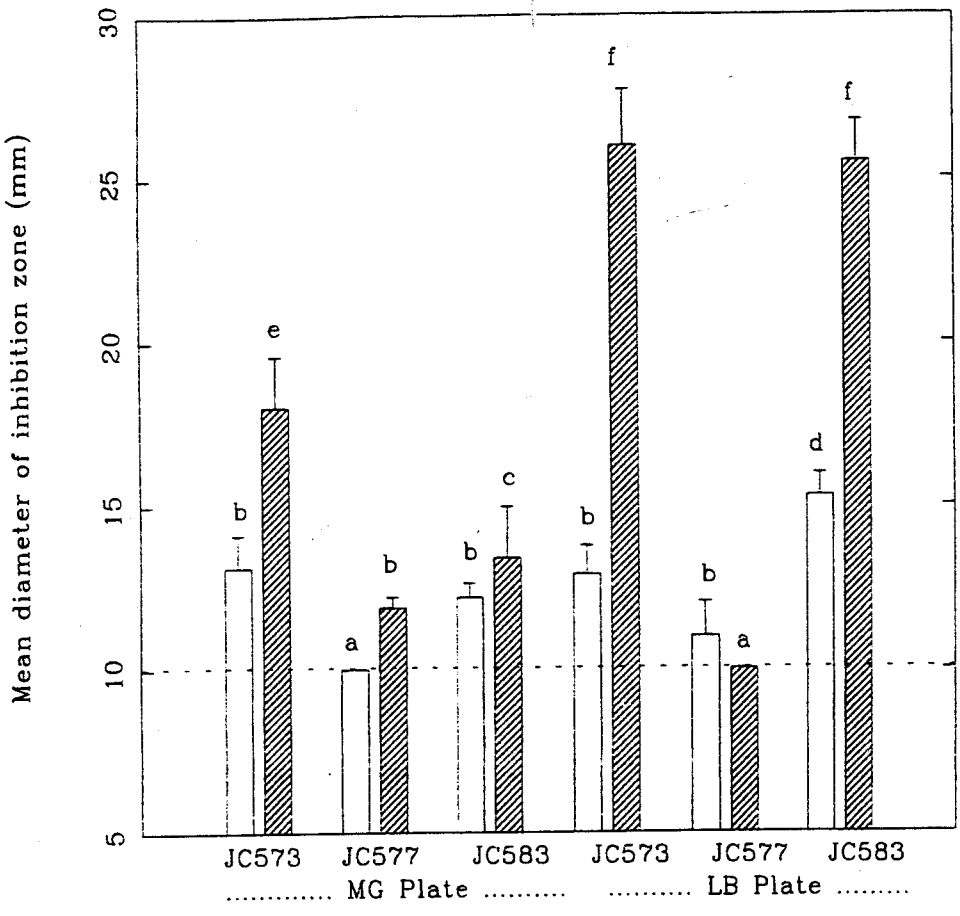
The effect of the medium on the results of in vitro antagonism trials has been well documented. Iron content is obviously fundamental to the production of siderophores (Swinburne 1985), but there is also a body of literature attesting to the effects of the medium on production of antibiotics and bacteriocins. Thus, Whipps (1987) observed stronger inhibition by antagonistic fungi on potato dextrose agar (PDA) than on either soil extract agar or tap water agar. Working with rhizobacteria, Hebbar et al. (1992) found that in vitro inhibition of *Fusarium moniliforme* varied depending on whether PDA or King's agar B was used. These observations are consistent with the findings reported here on the effect of MG or LB agar as assay medium.

The interesting aspect of this data set, however, is that it shows the significant effect of growth history *before* the bacteria are applied to the assay plate. Thus, the composition and temperature of the medium used to produce inocula for the assay plates had a major effect on the antagonism; the pH of the medium had a significant though smaller effect. Studies in which antagonistic cells are killed with chloroform, or in which sterile supernatant is used in bioassays, confirm that growth conditions can influence antibiotic production. Vidaver et al. (1971) observed that the age of the culture, the colony type, and the temperature affected bacteriocin production by phytopathogenic *Pseudomonas* spp. Formica (1990), working with *A. radiobacter* K84, found that agrocin 84 production was affected by the sugar substrate and the pH of the medium.

Of course the optimal manipulation of growth conditions for antibiotic production has become axiomatic in industry (Wang et al. 1979). The three media used in this experiment were chosen to reflect a continuum of complexity and richness with TSB > MG > GN. The two latter media are known to be carbon limited at the formulations used and it was interesting to note that it was often the chemically defined medium, GN, that produced the least inhibition (Figs. 1 and 3). Such data imply that physiological adaptations occurring during the initial growth of inocula, such as those cited above, must carry through and affect performance on the assay plate. It also raises the issue that the conditions of the in situ growth of these bacteria in the soil or on the plant, and the form of nutrient limitation in force, must affect their inhibitory potential.

The ecological consequences of the temperature of growth are similarly compelling. Figures 1 and 2 revealed that inhibition was very different when the target agrobacteria were grown at 15°C compared with 30°C. Figure 4 demonstrated that inhibition was generally stronger when the antagonists were grown at 15°C. Other researchers have noted similar

Fig. 6. Effect of the order of application on inhibition zones. Open histograms represent trials in which agrobacteria were applied first. Hatched histograms represent trials in which antagonists were applied first. Histograms topped with different letters are significantly different (LSD, $p \leq 0.05$).




effects of lower temperature. Tronsmo and Dennis (1978) found that the production of nonvolatile inhibitors by different species of *Trichoderma* was greater at the lowest temperature tested (5°C). Vidaver et al. (1971) observed that the bacteriocinogenic activity of pseudomonads was better at 20 and 24°C than at 28 and 32°C. Perhaps, when antagonistic screens are used as predictors of in situ performance, lower temperatures would be advisable.

It becomes apparent that antagonistic activity in vitro is influenced by a variety of factors. This study has indicated that generalizations concerning the interaction of a given target and a potential antagonist are difficult to draw and need to be assessed on a case by case basis. This interaction is influenced by a great number of factors. Many of these are perhaps expected from an a priori knowledge of bacterial metabolism but some, such as the extended effects of inoculum growth conditions or even the order of antagonist and target strain application (Fig. 6), are surprising. The fact that the initial growth conditions of an inoculum can still exert an effect on antagonistic behaviour following 48 h of growth under totally different conditions is intriguing and adds another layer to the thorny question of the reliability of in vitro screens. It is perhaps not too surprising, in the light of these vagaries with in vitro antagonistic assays, that opinions on the usefulness of such screens are contentious and often contradictory (Fravel 1991; Lindow 1988; Weller and Thomashow 1990). It certainly prompts added caution in their use and highlights the need for meticulous standardization, especially in conditions close to those operating in the court of infection, when their use is unavoidable.

## Acknowledgements

This work was funded under the Technology Development Program of the Canada/Nova Scotia Agri-Food Development Agreement, 1988–1993.

## References

Bazzi, C., Piazza, C., and Burr, T.J. 1987. Detection of *Agrobacterium tumefaciens* in grapevine cuttings. EPPO Bull. **17**: 105–112.

Becker, J.O., and Schwinn, F.J. 1993. Control of soil-borne pathogens with living bacteria and fungi: status and outlook. Pestic. Sci. **37**: 355–363.

Bell, C.R. 1990. Growth of *Agrobacterium tumefaciens* under octopine limitation in chemostats. Appl. Environ. Microbiol. **56**: 1775–1781.

Bell, C.R., Dickie, G.A., Harvey, W.L.G., and Chan, J.W.Y.F. 1995*a*. Endophytic bacteria in grapevine. Can. J. Microbiol. **41**: 46–53.

Bell, C.R., Dickie, G.A., Harvey, W.L.G., and Chan, J.W.Y.F. 1995*b*. Submitted for publication.

Campbell, R. 1994. Biological control of soil-borne diseases: some present problems and different approaches. Crop Prot. **13**: 4–13.

Chen, X.Y., and Xiang, W.N. 1986. A strain of *Agrobacterium radiobacter* inhibits growth and gall formation by biotype III strains of *A. tumefaciens* from grapevine. Acta Microbiol. Sin. **26**: 193–199.

Cook, R.J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Annu. Rev. Phytopathol. **31**: 53–80.

Crawford, D.L., Lynch, J.M., Whipps, J.M., and Ousley, M.A. 1993. Isolation and characterization of actinomycete antagonists of a fungal root pathogen. Appl. Environ. Microbiol. **59**: 3899–3905.

Deacon, J.W. 1991. Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Sci. Technol. **1**: 5–20.

Elabyad, M.S., Elsayed, M.A., Elshanshoury, A.R., and Elsabbagh, S.M. 1993. Towards the biological control of fungal and bacterial diseases of tomato using antagonistic *Streptomyces* spp. Plant Soil, **149**: 185–195.

Elander, R.P. 1987 Microbial screening, selection and strain improvement. *In* Basic biotechnology. *Edited by* J. Bu'Lock and B. Kristiansen. Academic Press, London. pp. 217–251.

Formica, J.V. 1990. Conditions for the production of agrocin 84 by *Agrobacterium radiobacter* K84. J. Ind. Microbiol. **5**: 33–44.

Fravel, D.R. 1991. Role of antibiosis in the biocontrol of plant diseases. Annu. Rev. Phytopathol. **26**: 75–91.

Hebbar, K.P., Davey, A.G., and Dart, P.J. 1992. Rhizobacteria of maize antagonistic to *Fusarium moniliforme*, a soil-borne fungal pathogen: isolation and identification. Soil Biol. Biochem. **24**: 979–987.

Hendson, M., Askjaer, L., Thomson, J.A., and van Montagu, M. 1983. Broad-host-range agrocin of *Agrobacterium tumefaciens*. Appl. Environ. Microbiol. **45**: 1526–1532.

Herbert, R.A., and Bell, C.R. 1977. Growth characterisitics of an obligately psychrophilic *Vibrio* sp. Arch. Microbiol. **113**: 215–220.

Hewitt, W., and Vincent, S. 1989. Theory and application of microbiological assay. Academic Press, San Diego, Calif.

Huang, H.C., Kokko, E.G., Yanke, L.J., and Phillippe, R.C. 1992. Bacterial suppression of basal pod rot and end rot of dry peas caused by *Sclerotinia sclerotiorum*. Can. J. Microbiol. **39**: 227–233.

Kerr, A., and Htay, K. 1974. Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. **4**: 37–44.

Kraus, J., and Loper, J.E. 1992. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. Phytopathology, **82**: 264–271.

Lemanceau, P., Bakker, P.A.H.M., DeKogel, W.J., Alabouvette, C., and Schippers, B. 1993. Antagonistic effect of nonpathogenic *Fusarium oxysporum* Fo47 and pseudobactin 358 upon pathogenic *Fusarium oxysporum* f.sp. *dianthi*. Appl. Environ. Microbiol. **59**: 74–82.

Lindow, S.E. 1988. Lack of correlation of in vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. Phytopathology. **78**: 444–450.

Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D., and Defago, G. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology. **82**: 190–195.

Merriman, P., and Russell, K. 1990. Screening strategies for biological control. *In* Biological control of soil-borne plant pathogens. *Edited by* D. Hornby. C.A.B. International, Wallingford. Oxon. p. 490.

Moore, L.W., and Warren, G. 1979. *Agrobacterium radiobacter* strain K84 and biological control of crown gall. Annu. Rev. Phytopathol. **17**: 163–179.

Moore, L.W., Kado, C.I., and Bouzar, H. 1988. *Agrobacterium*. *In* Laboratory guide for identification of plant pathogenic bacteria. 2nd ed. *Edited by* N.W. Schaad. APS Press, St. Paul, Minn. pp. 16–36.

New, P.B., and Kerr, A. 1972. Biological control of crown gall: field measurements and glasshouse experiments. J. Appl. Bacteriol. **35**: 279–287.

O'Sullivan, D.J., and O'Gara, F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56**: 662–676.

Ryder, M.H., and Jones, D.A. 1990. Biological control of crown gall. *In* Biological control of soil-borne plant pathogens. *Edited by* D. Hornby. C.A.B. International, Wallingford, Oxon. pp. 45–63.

Ryder, M.H., Slota, J.E., Scarim, A., and Farrand, S.K. 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. J. Bacteriol. **169**: 4184–4189.

Staphorst, J.L., van Zyl, F.G.H., and Groenewold, Z.E. 1985. Agrocin-producing pathogenic and nonpathogenic biotype 3 strains of *Agrobacterium tumefaciens* active against biotype 3 pathogens. Curr. Microbiol. **12**: 45–52.

Stonier, T. 1960. *Agrobacterium tumefaciens* II. Production of an antibiotic substance. J. Bacteriol. **79**: 889–898.

Swinburne, T.R. (*Editor*). 1985. Iron, siderophores and plant diseases. Plenum Press, New York.

Thomson, J.A. 1986. The potential for biological control of crown gall disease on grapevines. Trends Biotechnol. **4**: 219–224.

Tronsmo, A., and Dennis, C. 1978. Effect of temperature on antagonistic properties of *Trichoderma* species. Trans. Br. Mycol. Soc. **71**: 469–474.

Vidaver, A.K., Mathys, M.L., Thomas, M.E., and Schuster, M.L. 1971. Bacteriocins of the phytopathogens *Pseudomonas syringae*, *P. glycinea*, and *P. phaseolicola*. Can. J. Microbiol. **18**: 705–713.

Wang, D.I.C., Cooney, C.L., Demain, A.L., Dunnill, P., Humphrey, A.E., and Lilly, M.D. 1979. Fermentation and enzyme technology. John Wiley & Sons, New York.

Webster, J., Dos Santos, M., and Thomson, J.A. 1986. Agrocin-producing *Agrobacterium tumefaciens* strain

active against grapevine isolates. Appl. Environ. Microbiol. **52**: 217–219.
Weller, D.M., and Thomashow, L.S. 1990 Antibiotics: evidence for their production and sites where they are produced. *In* New directions in biological control: alternatives for suppressing agricultural pests and diseases. *Edited by* R.R. Baker and P.E. Dunn. Alan R. Liss Inc., New York. pp. 703–711.
Whipps, J.M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. New Phytol. **107**: 127–142.