FIELD TRIAL STUDY MAY - AUGUST 1993

Biological control of *Botrytis cinerea* on strawberries using *Bacillus pumilus* and *Pseudomonas fluorescens*

By

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Sponsored by Horticultural Development Council

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INTRODUCTION

1.0 INTRODUCTION

Botrytis cinerea (Pers.) Fr. is the causal agent of grey mould in strawberries, an important disease of green and ripe strawberry fruits worldwide (Bristow *et al.*, 1986). The fungus is a common necrotroph and is responsible for considerable losses of soft fruits, vegetables and cut flowers (Bossi & Dewey, 1992). The fungus overwinters in plant debris as sclerotia or as mycelium. The dead tissues of over 200 known host species have been recognised as potential inoculum sources. Conidia are produced under favourable conditions and may infect leaves, flowers, fruit and crowns of strawberry plants (Sutton, 1991). Generally infection can appear on any part of the fruit but development is more common around the calyx (McClellan & Hewitt, 1973).

Epidemics of grey mould are caused mainly by conidia produced locally within the crop. The major inoculum source is dead leaves, with minor sources of inoculum including mummified fruits, weeds and straw used for mulching (Braun & Sutton, 1987). Leaves are very susceptible to infection at the bud stage or during expansion. The fungus remains quiescent in the epidermis until the leaves have senesced (Braun & Sutton, 1988). The conidia infect the flowers, with the disease becoming quiescent until the fruit ripens (Bristow *et al.*, 1986). The conidia germinate on the anthers and the hyphae penetrate down the filaments and into the receptacle. Infection also occurs through open wounds and late in the season under favourable conditions, directly through uninjured skin of the fruit (Janisiewicz, 1991). Infections on the fruit, especially near the ground, result from infected organic debris (Jarvis, 1962) and infected flower parts shed after bloom adhering to the fruit surface (Sutton & Peng, 1990). Frost-damaged flowers are particulary vulnerable to infection. Direct penetration of the fruits from conidia on the surface is rare. Epidemics of grey mould occur in cool, wet, and humid weather (Williamson & McNicol, 1986).

Long wet periods during flowering also increase the incidence of disease.

Botrytis cinerea can cause severe losses in the field and during storage. Damage can be minimised by implementing effective control strategies aimed at pre- and post-harvest infections. Improved knowledge about host-pathogen relationships, the process of infection and the role of different environmental conditions has led to the development of a more rational approach to the application of chemical and cultural disease control strategies. Grey mould fruit rot of strawberries is managed conventionally through an integrated programme of fungicidal treatments and cultural practices (Evans *et al.*, 1988).

The control of grey mould on stored strawberry fruits is dependent upon the protection of the inflorescence, which is the main site of fungal entry (Bristow *et al.*, 1986). The main form of pre-harvest control is the use of fungicides. Those currently recommended to control *Botrytis* infections on strawberries in the United Kingdom include benomyl, captan, carbendazim, chlorothalonil, dichlofluanid, iprodione, thiram and vinclozolin. Maximum protection against grey mould development has been achieved by careful attention to the timing of spray applications to prevent infection of the flowers and the establishment of latent infections of the fruit (Bristow *et al.*, 1986). Spray programmes are timed to protect the newly-opened flowers. A reduction in disease control can result from effective chemicals becoming ineffective, due to resistance by pathogens (Delp, 1980) and fungicides being banned because of recently recognised hazards to humans and the environment.

Biological control of plant diseases involves the use of microorganisms to suppress or destroy disease-causing organisms. This includes either the introduction, or increase in numbers, of one or more antagonistic microorganisms as a result of changing the environmental conditions. The ideal candidate will be able to colonize the host plant rapidly and effectively, without any deleterious effects, and be resistant to extreme conditions and changes in light, humidity and

temperature (Lukezic *et al.*, 1990). The frequency and rate of application of the biocontrol agent depends upon the host to be protected and pathogen to be controlled. The capacity of the antagonist for survival and growth after it has been applied will affect the number of applications made. Their durability and effectiveness depends upon the type of propagule applied. Vegetative bacterial cells and germinating fungal spores are most effective at the time of application, but bacterial spores and chlamydospores can be more effective in the later stages of infection due to their persistence. Therefore the duration of biocontrol can be extended by using a mixture of propagules (Janisiewicz, 1991).

Pathogens which cause quiescent infections should be controlled in the field. Direct application of a biological control agent should therefore be applied as a pre-harvest application. The majority of cases of pre-harvest treatments involve spraying suspensions of the biocontrol agent onto the flowers of the crop to try and reduce the inoculum potential at fruit-set (Jeger & Jeffries, 1988). The activity of both antagonists and pathogens on the phylloplane is influenced by microclimatological conditions and the chemical environment. The morphology of the surface of leaves can also have a significant effect. Surface water affects the colonisation of the leaves, increasing the growth of bacteria and filamentous fungi. The populations of microorganisms on the phylloplane fluctuate over a period of time. Bacteria are relatively more abundant on leaves early in the growing season. Yeasts increase more slowly, so relatively high numbers do not occur until the middle of the season and finally the aerial parts are colonised by pathogenic and saprophytic filamentous fungi.

The majority of antagonistic interactions which have been successfully screened for bacteria and fungal pathogens on foliar surfaces, have involved bacteria from two genera, *Pseudomonas* and *Bacillus* (Blakeman, 1988), and fungi from a range of genera. The ultimate test for the candidates is their performance in the field, where most potential biocontrol agents fail. Many isolates are

disregarded because of their lower levels of disease control compared to the chemical fungicides. Although biological control may not measure up to chemical control for many diseases, it will still be the best alternative if effective fungicides are not an option. Isolates in initial trials could show sufficient control for further research to continue and improve performance. Particular attention to inoculum production methods and formulations can increase efficiency. Spray additives can be important in improving colonisation. The use of 'sticker' compounds can increase adhesion to the plant surface. The survival of bacteria applied in aerosol sprays can be increased by including inositol, di- and tri-saccharides (Cullen & Andrews, 1984a). Finally, the addition of U.V. protectants may increase the survival of antagonists on the plant surface.

Many attempts have been made to control grey mould on a number of different crops, with varying results. The fungi *Gliocladium roseum*, a *Penicillium* sp. and *Trichoderma viride* suppressed the number of conidiophores of *B. cinerea* by 97-100% in greenhouse studies and 58, 64 and 48% respectively in field plots (Sutton & Peng, 1993a). Other fungi which have reduced grey mould disease include *Cladosporium herbarum* and *Aureobasidium pullulans* on strawberry flowers in both greenhouse and field trials (Bhatt & Vaugh, 1962). Research for antagonistic bacteria to control grey mould compared with fungi has been less intense. One bacterial and one yeast isolate have been found which significantly suppressed grey mould on strawberry flowers (Peng & Sutton, 1990). Although biocontrol agents are generally less effective and have smaller markets than conventional fungicides, the economic barriers may be weakening against them because of changing attitudes towards chemical pesticides. Biological control on the phylloplane has potential as a non-chemical alternative.

Attempts have been made to control foliar diseases using sprays with fungicides in combination with compatible biocontrol agents, mainly filamentous fungi. Since the antagonists are sprayed along with pesticides, the biocontrol preparations must be suitable for spray equipment.

The concept of integrated control is both feasible and agriculturally acceptable. Research suggests that future control strategies of plant pathogens will not rely on a single component. Much evidence suggests that a high proportion of disease problems will be controlled by the use of biological agents as components of integrated disease management systems and high priority should be given to developing the extensive use of biological control agents.

The purpose of this field trial was to assess whether the biocontrol agents chosen from the first field trial (1992) had the potential to replace conventional fungicidal treatments as the control strategy used to reduce *B. cinerea* infections on strawberries. The efficacy and persistence in the environment of the two antagonists, *B. pumilus* and *P. fluorescens* was also studied.

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Microbiological growth media

The constituents of the liquid and solid media used in this project are given in Appendix A. Both the liquid and solid media were prepared in 2l Erlenmeyer flasks and the pH adjusted using 0.2M hydrochloric acid (HCl) or 0.2M sodium hydroxide (NaOH). The agar media were poured into medicine bottles (3 x 400ml) and either 50ml or 100ml of the broth transferred to 250ml Erlenmeyer flasks for sterilisation at 121°C for 15min. The agar was allowed to cool to 50°C and poured into 9cm Sterilin plastic petri plates.

2.2 Preparation of *B. pumilus* and *P. fluorescens* broth cultures

2.2.1 Two-day broth cultures

Erlenmeyer flasks (1 x 250ml) containing 50ml NB (pH 7.5) were inoculated with three plugs (5mm) removed from NA streak plate cultures of either *B. pumilus* or *P. fluorescens*. The flasks were incubated on an orbital shaker (160rpm) at 25°C for 24 - 48h. Erlenmeyer flasks (3 x 250ml) containing either 50ml or 100ml NB (pH 7.5) were inoculated with 0.5ml samples of either the *B. pumilus* or *P. fluorescens* cultures and incubated on a rotary shaker (160rpm) at the required temperature and time.

2.2.2 Resuspended cultures

Resuspended cultures of either *B. pumilus* or *P. fluorescens* were prepared by centrifuging 25ml of the cultures provided in section 2.4.2.1 on a J2-21 Beckman centrifuge at 15,000xg^{v} for 10min at 4°C. The filtrate was discarded and the bacterial cells washed twice by resuspending the cells in 50ml phosphate buffer (pH 7.5), centrifuging at 15,000xg^{v} and discarding the filtrate. The

cells were resuspended in 50ml NB (pH 7.5).

2.2.3 Preparation of the sticking agent

The bacteria prepared from the 2d cultures were resuspended in Erlenmeyer flasks (3 x 2l) containing 700ml NB with 5% (w/v) Speswhite clay and 1% (w/v) sticker (section 2.2.2). The sticker contained Hyphis 5, Nid 100S (surfactant) and water. Due to the hydrophobic nature of Hyphis 5, an emulsion was prepared in a glass beaker (100ml), before applying it to the cultures. 70g of Hyvis 5 was added to 3.5g of Nid 100S. This was mixed using a tissue culture homogeniser at 14,000rpm. To this mixture, 33ml of sterile dH_20 was gradually added and the solution mixed until an emulsion was formed.

2.2.4 Miles and Misra drop count method

A dilution series (10^{-1} to 10^{-7}) of the bacterial or yeast suspension was prepared in phosphate buffer (pH 7.5). Three 20μl aliquots of the six dilutions were pipetted onto NA plates and allowed to dry in a laminar flow for 15min. The plates were incubated at 25°C for 24h (16h light/8h dark). The number of colony forming units (cfu) was recorded from the dilution showing distinct and countable colonies.

2.3 Field Trial 1993

2.3.1 Field trial site

The effectiveness of different formulations of *B. pumilus* and *P. fluorescens* against natural infections of *B. cinerea* in a commercial field was studied in a field trial. The trial used strawberry c.v. Elsanta and was set up at Ratling Court Farm, near Aylesham, Kent (O.S. sheet 179, coordinates $^{6}24,^{1}54$). Rows of strawberries had been planted 0.5m wide, spaced 1m apart and

covered with a black plastic mulch. The plants were in their first year of June fruiting and spaced 15cm apart in double lines along each bed. The plants were trickle-irrigated during dry weather. Straw was applied to the rows on 3 May 1993. The herbicides, Flexidor applied at 0.117l $acre^{-1}$, Simazine w.p. applied at 0.454kg $acre^{-1}$ and Kerb 50W (0.270kg $acre^{-1}$) were applied to the site on 2 February 1993. No other agrochemicals were applied to the field trial site.

2.3.2 Experimental design

A randomised block design was used with three replicates of 30 treatments, each replicate containing six strawberry plants (Appendix B). The plants in each treatment were arranged in a two by three design. Neighbouring treatments were separated by a minimum of two guard plants.

2.3.3 Experimental treatments

Cultures of *B. pumilus* and *P. fluorescens* were each prepared in Erlenmeyer flasks (3 x 2l) containing 1000ml of NB and incubated on an orbital shaker (160rpm) at 25°C for 48h. *B. pumilus* and *P. fluorescens* were then prepared as follows (Table 2.3.3):

(a) as the neat 2d suspensions with no modifications,

(b) washed once in phosphate buffer (pH 7.5) and resuspended in 750ml NB (pH 7.5),

(c) washed once in phosphate buffer (pH 7.5) and resuspended in 750ml NB + 5% (w/v) Speswhite clay (pH 7.5),

(d) washed once in phosphate buffer (pH 7.5) and resuspended in 750ml NB + 5% (w/v) Speswhite + 1% (w/v) sticker (sticker preparation is described in section 2.2.3),

(e) washed once in phosphate buffer (pH 7.5) and resuspended in H_20 + 5% (w/v) Speswhite + 1% (w/v) sticker (pH 7.5),

(f) neat mixed cultures of *B. pumilus* and *P. fluorescens* grown for 2d and applied with no further modifications,

(g) mixed cultures of *B. pumilus* and *P. fluorescens*, washed once in phosphate buffer (pH 7.5) and resuspended in formulation (d),

(h) resuspensions of *B. pumilus* or *P. fluorescens* in formulation (d) applied during fruiting, integrated with dichlofluanid applied during flowering,

Resuspended cultures were prepared by centrifuging 200ml of the 2d culture on a J2-21 centrifuge at 15,000xg^{v} for 10min. The filtrate was discarded and the cells washed once in 200ml phosphate buffer (pH 7.5) and resuspended in 1l Erlenmeyer flasks containing 500ml of the formulation (section 2.2.2). The resuspended cultures contained 10^{9} - 10^{10}cfu ml^{-1}. Other treatments applied to the field trial included:

(i) control treatments consisting of the media of preparations (a), (c), (d) and (e) without the bacteria,

(j) a no treatment control where no application was made to the treatment plot,

(k) dichlofluanid applied during flowering with no application during fruiting,

(l) a combination of dichlofluanid applied during flowering and iprodione applied at fruiting.

2.3.4 Application of Sprays

The formulations containing *B. pumilus* and *P. fluorescens* was applied to all above-ground portions of the strawberry plants on 5, 11, 17, 24, 31 May, and 7, 14 and 21 June. Dichlofluanid treatments were sprayed on the 5, 17, 31 May. The treatments applied in an integrated programme with dichlofluanid (i.e. iprodione, and *B. pumilus* or *P. fluorescens* applied in formulation (a)), were applied on 7, 14 and 21 June. All inoculations were made 2h before sunset with a Hozelock

Table 2.3.3 Treatments applied to the field trial in 1993. Cultures were prepared in 2l Erlenmeyer flasks containing 1000ml of the medium and incubated on an orbital shaker at 25°C for 48h.

	BACTERIA		MEDIUM		BLEND		ADDITIVES		FUNGICIDE	
CODE	**Ind.**	**Mix.**	**NB**	**H_20**	**Res.**	**2d**	**U.V.**	**Stic.**	**Dich.**	**Ipro.**
a	+	-	+	-	-	+	-	-	-	-
b	+	-	+	-	+	-	-	-	-	-
c	+	-	+	-	+	-	+	-	-	-
d	+	-	+	-	+	-	+	+	-	-
e	+	-	-	+	+	-	+	+	-	-
f	-	+	+	-	-	+	-	-	-	-
g	-	+	+	-	+	-	+	+	-	-
h	+	-	+	-	+	-	+	+	+	-
i *1*	-	-	+	-	+	-	-	-	-	-
2	-	-	+	-	+	-	+	-	-	-
3	-	-	+	-	+	-	+	+	-	-
4	-	-	-	+	+	-	+	+	-	-
j	-	-	-	-	-	-	-	-	-	-
k	+	-	-	+	-	-	-	-	+	-
l	-	-	-	+	-	-	-	-	+	+

BACTERIA: **Isolate(s) used in the treatment**

Ind. Either *P. fluorescens* or *B. pumilus* used,

Mix. A mixture of *P. fluorescens* and *B. pumilus* used

MEDIUM: **Culture medium used for the treatment**

NB Nutrient Broth

dH_20 Distilled water

BLEND: **Preparation of the bacteria**

Res. Bacteria centrifuged, washed and resuspended in fresh NB

2d Two-day culture of the isolates

ADDITIVES: **Amendment(s) to the treatment**

U.V. The U.V. protectant, Speswhite clay (5% w/v) applied

Stic. The sticker, Hyphis 5+Nid 100S (1% w/v) applied

FUNGICIDE: **Fungicides applied to the plots**

Dich. Dichlofluanid fungicide applied during flowering only

Ipro. Iprodione fungicide applied during fruiting only

Polyspray 2, at the rate of 150ml per plot. A chamber (2m x 0.5m x 0.5m high), constructed with a wooden frame covered with clear plastic, was placed over the plots during inoculations to prevent any spray drift to neighbouring plots. During the trial no other agrochemicals were applied to the site.

2.3.5 Quantification of infection of flowers

Infection of petals and stamens by *B. cinerea* was quantified by the method of Peng and Sutton (1991). 15 flowers were randomly collected on 13 and 27 May from each plot treated. The stamens and petals were removed from the flowers with sterile tweezers and surface sterilised in 70% (v/v) ethanol for 5s and 0.6% (v/v) NaOCl for 60s followed by 3 rinses in sterile dH_20. In total 100 stamens and 50 petals from the flower samples were plated onto PCA, incubated at 20°C for 7d (16h light/8h dark) and examined with a dissecting microscope for sporulation of *B. cinerea*. The number of infected stamens and petals with conidiophores was recorded.

2.3.6 Infection of fruits

The fruits were harvested on 22 June and 1 July with the help of pickers. All ripe fruits were picked from each plot with their stalks intact and stored in 2kg punnets. Any fruits with grey mould infection were recorded and discarded. The harvested fruits were stored at 4°C for 5d, the approximate time period for transport and shelf life in commercial practice. The total number of fruits collected were counted and the number of fruits showing symptoms of grey mould. Once the data were collected, any fruits with missing stalks or showing any disease symptoms were discarded. From the remaining fruits, 45 were randomly selected, placed in 3 x 2kg punnets on a single layer of kitchen towel and incubated at 20°C for 5d (constant light). Strawberries were arranged so there was no contact between neighbouring fruits, minimising the risk of cross-

contamination. After the incubation period, the fruits were examined with a dissecting microscope and assessed for grey mould infection.

2.3.7 Meteorological data

The information was recorded by the meteorological station, number 03797, based at RAF Manston, Kent (O.S. sheet 179, coordinates $^{6}34,^{1}66$). The station is 44m above mean sea level (MSL). Daily temperatures (°C) were recorded every hour on a mercury dry bulb thermometer. The amount of rainfall (mm) was recorded every 6h on a rain gauge 30.5cm above the ground and with a rim diameter of 5". The humidity (%) was recorded every hour on a hygrometer 45.1m above MSL. The readings were taken between the 5 May - 1 July, 1993.

2.4 Population studies of *B.pumilus* and *P. fluorescens*

2.4.1 Selection of antibiotic-resistant mutants (ARM) of B. pumilus and P. fluorescens

An initial study to determine whether *B. pumilus* or *P. fluorescens* were naturally resistant to a variety of antibiotics was conducted using Multodiscs (Oxoid) impregnated with 1.5μg penicillin G, tetracycline, 10μg streptomycin, 10μg chloramphenicol, 10μg erythromycin, 2μg ampicillin and 10μg sulphafurazole. Three NA plates were spread with a 0.5ml suspension of each bacterial isolate. The plates were dried in a sterile laminar flow hood for 30min before the antibiotic discs were applied to the surface with sterile forceps. The plates were incubated for 24h at 30°C and examined for any natural resistance to the antibiotics. This was shown by the absence of an inhibition zone around the antibiotic discs.

It was decided to select for streptomycin and novobiocin resistance in *B. pumilus* and for streptomycin and chloramphenicol resistance in *P. fluorescens*. Erlenmeyer flasks (3 x 250ml) containing 50ml NB were inoculated with each isolate and incubated at 25°C for 24h. The *B.*

pumilus isolate was resuspended in 50ml NB amended with 50μg ml^{-1} streptomycin (Sigma), 25μg ml^{-1} novobiocin (Sigma) and 100μg ml^{-1} cycloheximide (Sigma) and *P. fluorescens* was resuspended in 50ml NB amended with 50μg ml^{-1} streptomycin, 50μg ml^{-1} chloramphenicol (Sigma) and 100μg ml^{-1} cycloheximide (section 2.2.2). The antibiotics were added to the cooled NB at 50°C after sterilisation at 121°C for 15min. The inoculated flasks were incubated for 24h at 25°C on an orbital shaker (160rpm). The procedure was repeated, increasing the antibiotic concentrations in the NB by 25μg ml^{-1} novobiocin, 50μg ml^{-1} streptomycin and 50μg ml^{-1} chloramphenicol, until a spontaneous mutant of *B. pumilus* (58MNS) was obtained which could grow on NA amended with 75μg ml^{-1} novobiocin, 200μg ml^{-1} streptomycin and a mutant of *P. fluorescens* (168MCS) was obtained which could grow on NA amended with 200μg ml^{-1} streptomycin and 200μg ml^{-1} chloramphenicol.

2.4.2 Stability of antibiotic-resistant mutants

The effect on the stability of the ARM after growth for several successive generations in NB was studied. Erlenmeyer flasks (3 x 250ml) containing 49ml sterile NB were inoculated with a 1ml of either 168MCS or 58MNS and incubated on an orbital shaker (160rpm) at 25°C. After 24h, 1ml suspension of the culture was removed to inoculate 49ml sterile NB and the flasks were incubated under the same conditions as previously described. This procedure was repeated a total of 15 times. The number of viable cells of ARM was determined after every fifth inoculation using the Miles and Misra drop count method (section 2.2.4). The dilutions were plated onto NA amended with and without the antibiotics (100μg ml^{-1} streptomycin, cycloheximide + 50μg ml^{-1} novobiocin for 58MNS and 100μg ml^{-1} streptromycin, chloramphenicol and cycloheximide for 168MCS). Comparing the numbers of viable cells on the amended and non-amended NA indicated the stability of the ARM.

2.4.3 Studies on survival of bacteria mutants

Cultures of the *B. pumilus* and *P. fluorescens* ARM were prepared. The isolates were each prepared in Erlenmeyer flasks (2l) containing 1000ml and 1250ml of NB. The flasks were incubated on an orbital shaker (160rpm) at 25°C for 48h. *B. pumilus* and *P. fluorescens* were then prepared in formulations (a), (c), (d), (e) and (g) as described in section 2.3.3. The formulations containing the *B. pumilus* and *P. fluorescens* ARM were applied to all above-ground portions of the strawberry plants on 11, 24 May and 21 June. The plants sprayed with the ARM of *B. pumilus* and *P. fluorescens* were assessed for their persistence on the flowers. Altogether, 15 flowers were collected from each plot 0, 12, 48 and 120h after the application on 24 May. The flowers were added to 250ml Erlenmeyer flasks containing 100ml of sterile phosphate buffer (pH 6.5) and 10g of glass beads (5g of 3mm beads and 5g of 5mm beads). The flasks were placed on an orbital shaker and shaken (230rpm) for 90min. The number of viable cells were calculated using the Miles and Misra drop count method (section 2.2.4). The dilutions were plated onto the selective NA for either *B. pumilus* (58MNS) or *P. fluorescens* (168MNS), depending upon the treatment. The serial dilutions of the *B. pumilus* ARM were also placed in a water bath at 80°C for 20min to kill the vegetative cells. The dilutions were plated onto the selective medium for 58MNS and the number of spores calculated. The plates were incubated at 25°C for 2d.

Viable counts of the mutants were also determined on fruits sprayed on 21 June. Four fruits were harvested from each plot at 0, 12, 24 and 96h after the spray application. The fruits were cut into quarters with a sterile knife and placed in 250ml Erlenmeyer flasks containing 100 ml sterile phosphate buffer (pH 6.5) and 10g of glass beads (5g of 3mm beads and 5g of 5mm beads). The samples were treated the same way as the flower samples and the number of viable cells calculated using the Miles and Misra drop count method (section 2.2.4).

2.5 Statistical treatment of data (Gomez & Gomez, 1984)

2.5.1 Mean values

All the treatment means represented in the figures are means of the untransformed data (x). Where appropriate the treatment means are shown with bars representing the ± standard error of the data. Any of the experiments involving percentage data (%x) lying between 0 to 30% and 70 to 100% were transformed prior to statistical analysis. Two types of transformation were used depending upon the range of the percentage values. For experiments containing percentage data lying within the range of 0 to 30% or 70 to 100%, but not both, the *square-root transformation* was used:

(a) data values < 10, especially with zeros, $(\%x + 0.5)^{1/2}$ was used,

(b) data values > 10, with no zeros, $\%x^{1/2}$ was used.

Any experiments with percentage data lying between both 0 - 30% and 70 - 100% data were transformed using the *angular transformation*:

$$(180 / \pi) \times \arcsin(\sqrt{(\%x / 100)})$$

2.5.2 Analysis of variance (ANOVA)

In order to assess whether there was a significant difference occurring between the treatments in an experiment, an ANOVA was performed on the data using the computer package 'Genstat 5, release 2.1 (Vax/VMS5), copyright 1990, Lawes Agricultural Trust, Rothamsted Experimental Station (Appendix C)'. The F value was computed and compared with the tabular F value at the 1% level of significance. A probability of $P < 0.01$, indicated there was a significant difference between the treatment means. The treatment means were then compared using *Duncan's multiple range test* (DMRT).

RESULTS AND DISCUSSION

3.0 RESULTS AND DISCUSSION

3.1 Inhibition of infection by *B. cinerea*

The effect of the bacterial antagonists *Bacillus pumilus* (IRSF58) and *Pseudomonas fluorescens* (IRSF168) in reducing *B. cinerea* infection on flowers and harvested fruits (section 2.3) was studied. The inhibition on the flowers was greatest on stamens (Figure 3.1.1) and petals (Figure 3.1.2) assessed from the first flower sample on 13 May (Figures 3.1.1a & 3.1.2a). This reduction in grey mould was not repeated on flowers collected from sample two on 26 May (Figures 3.1.1b & 3.1.2b).

Bacillus pumilus failed to reduce infections due to *B. cinerea* on stamens and petals removed at both sampling times. The only application which reduced the number of diseased stamens was F3, containing the sticker and UV protectant (9 infected stamens). However, this was not significantly different ($P > 0.01$) from the control (19 infected stamens). Flowers treated with *B. pumilus* suspended in the F4 and F6 formulations had the highest number of diseased stamens (24 and 22 infected stamens respectively) and petals (27 and 25 infected petals respectively) recorded in the first sample. Mixed cultures containing both *B. pumilus* and *P. fluorescens* did not reduce disease on the stamens and petals of flowers from sample two. However, in the first flower sample, the mixed isolates resuspended in NB with the sticker and UV protectant (F3) reduced grey mould disease on the petals (15 infected petals) and stamens (8 infected stamens) to levels significantly different ($P < 0.01$) from the no treatment and F3 controls. The dichlofluanid treatment did not inhibit *B. cinerea* on the stamens in either of the samples. Conversely, the dichlofluanid treatment reduced the number of infected petals on flowers collected from the first sample (10 infected petals), but not the second (35 infected petals). All of the formulation controls and no treatment applications resulted in similar numbers of infected stamens and petals. Flowers

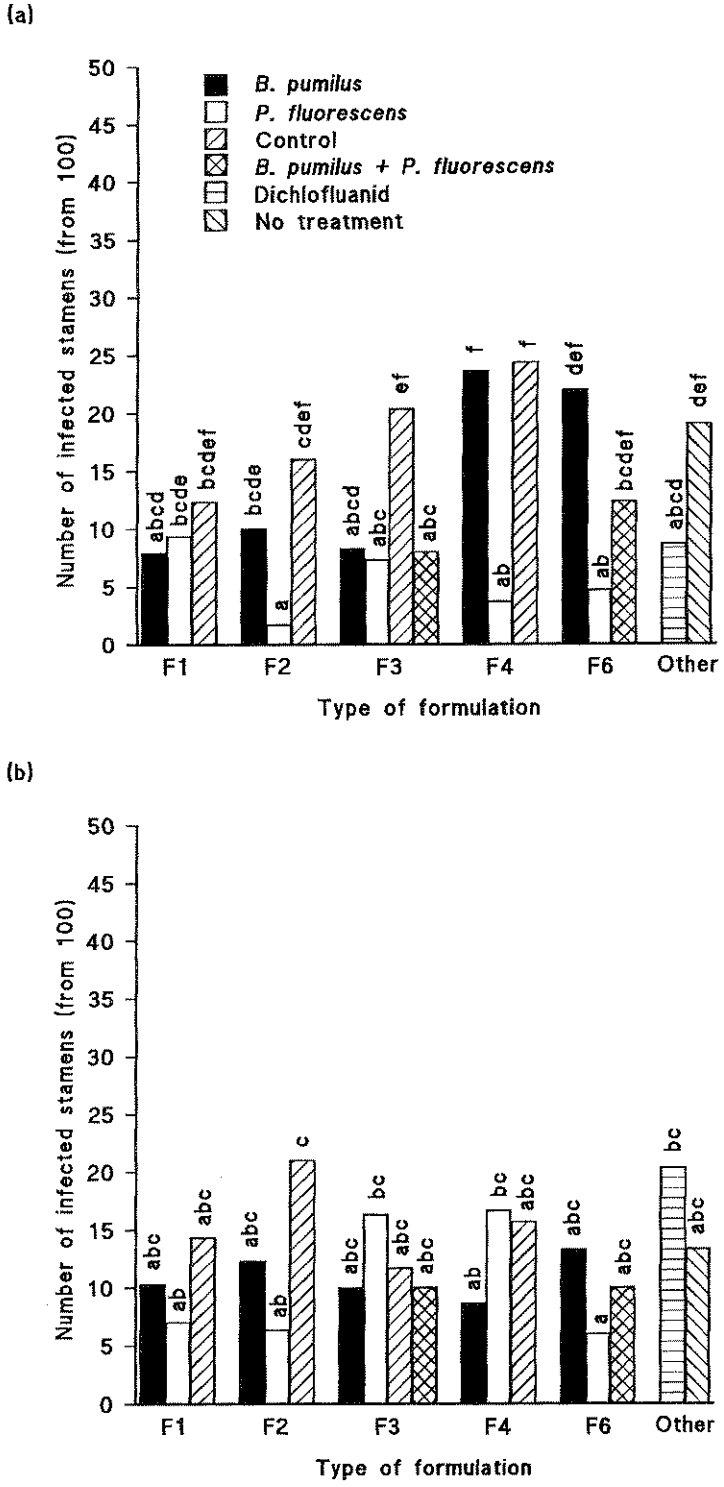

Figure 3.1.1 Inhibition of *B. cinerea* on 100 stamens removed from flowers sprayed with the different antagonist treatments and sampled on (a) 13 May and (b) 27 May. The stamens were incubated at 20°C for 5d on PCA. The different treatments consisted of *B. pumilus* or *P. fluorescens* resuspended in: NB (**F1**), NB + 5% (w/v) Speswhite (**F2**), NB + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F3**), H_20 + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F4**) and 2d NB cultures (**F6**). The control treatments contained the formulation constituents without the bacteria. Treatments annotated with a common letter are not significantly different (at P=0.01) according to DMRT.

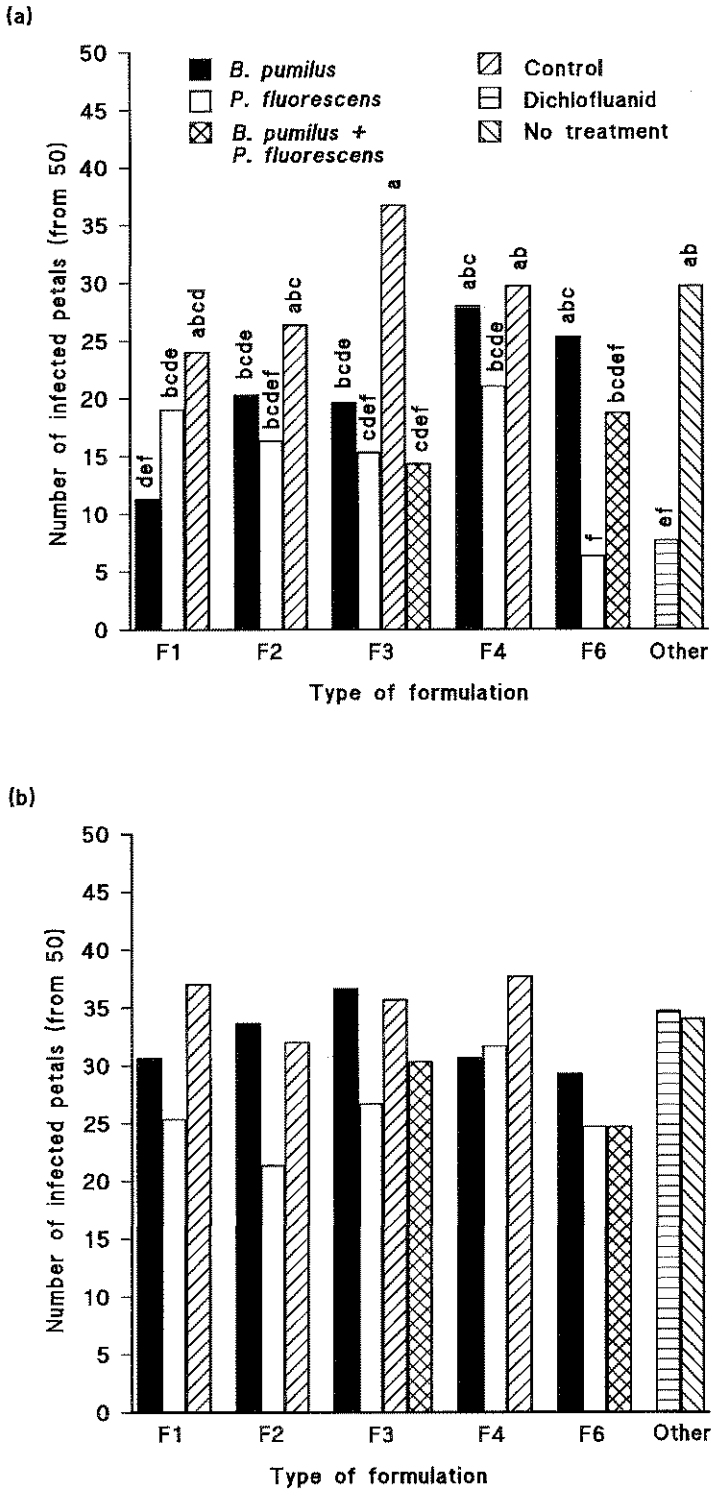

Figure 3.1.2 Inhibition of *B. cinerea* on 50 petals removed from flowers sprayed with different antagonist treatments and sampled on (a) 13 May and (b) 27 May. The petals were incubated at 20°C for 5d on PCA. The different treatments consisted of *B. pumilus* or *P. fluorescens* resuspended in: NB (**F1**), NB + 5% (w/v) Speswhite (**F2**), NB + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F3**), H_20 + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F4**) and 2d NB cultures (**F6**). The control treatments contained the formulation constituents without the bacteria. Treatments annotated with a common letter in Figure (a) are not significantly different (at P=0.01) according to DMRT. Treatments were not significantly different (P>0.01) in Figure (b).

sprayed with NB plus the sticker and UV protectant (F3 control) developed the largest number of diseased petals in sample one (36 infected petals) with similar numbers also recorded in sample two (43 infected petals). The control treatment comprising of water plus the sticker and UV protectant (F4) sprayed on flowers from the first sample resulted in the highest number of diseased stamens (23 infected stamens).

Pseudomonas fluorescens was the most effective antagonist, consistently reducing grey mould in all five formulations on the stamens from the first flower sample. Greatest inhibition (2 infected stamens) was achieved on the flowers treated with the isolate resuspended in NB and UV protectant (F2), with a similar level of control in the later sample (6 infected stamens). The *P. fluorescens* isolate resuspended in either water or NB containing the sticking agent plus UV protectant (F3 and F4) or as a 2d suspension (F6) also successfully inhibited *B. cinerea* on stamens from sample one (7 infected stamens). Treatment with the same formulations resulted in similar low levels of disease on stamens from the second sample, but did not show a significant difference ($P > 0.01$) from the no treatment and formulation controls. The *P. fluorescens* isolate inhibited *B. cinerea* infections on the petals as a 2d suspension assessed from the first sample (5 infected petals). Low numbers of infected petals from sample one flowers were also recorded for the other formulations but no significant differences were noted from control treatments. The *P. fluorescens* isolate applied in all the formulations also failed to reduce grey mould on petals from sample two.

An assessment of the number of harvested fruits exhibiting grey mould symptoms represented the ability of the different treatments to reduce *B. cinerea* infection during flowering and further infection during fruiting. Fruits incubated under commercial conditions at 4°C, showed no significant difference in the efficacy of the 21 treatments to reduce *B. cinerea* infections (Figure 3.1.3). A larger number of infected fruits was recorded after harvest one (> 15% infection) compared with harvest two (> 10% infection). The most successful treatment reducing grey mould

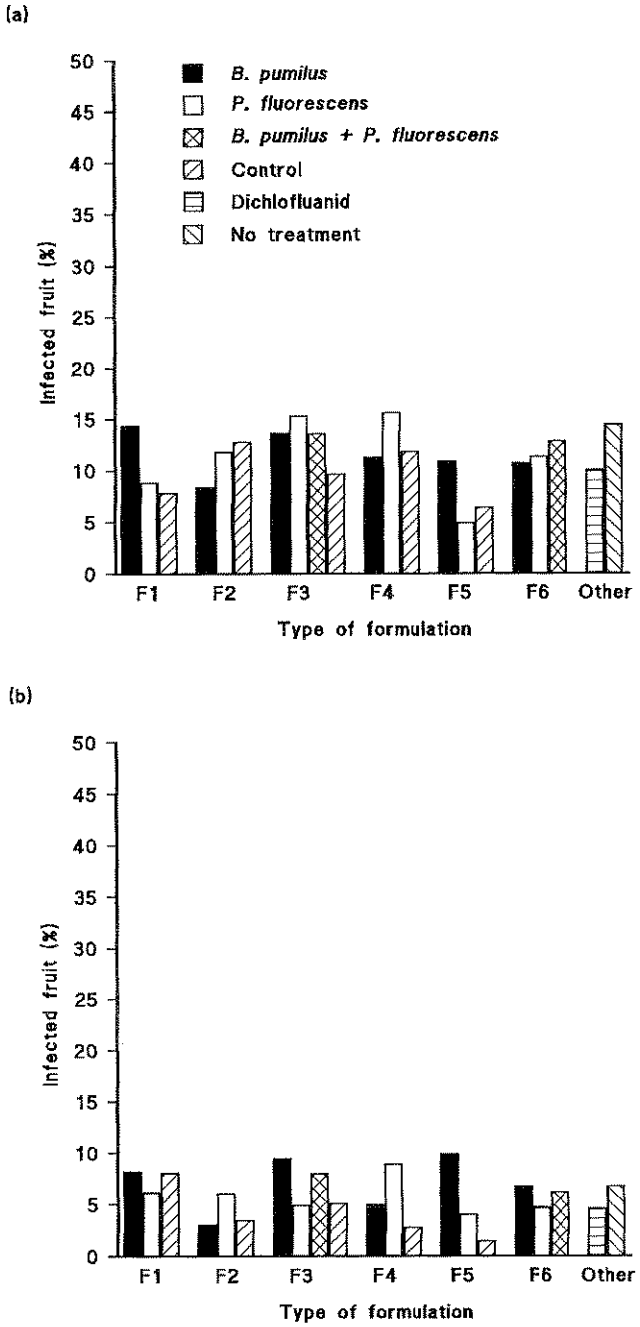

Figure 3.1.3 Inhibition of *B. cinerea* on fruits harvested on (a) 22 June and (b) 1 July picked from plants sprayed with different antagonist treatments. The fruits were incubated at 4°C for 5d in 2kg punnets. The different treatments consisted of *B. pumilus* or *P. fluorescens* resuspended in: NB (**F1**), NB + 5% (w/v) Speswhite (**F2**), NB + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F3**), H_20 + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F4**), as formulation F3 applied at fruiting following dichlofluanid applications during flowering (**F5**) and 2d NB cultures (**F6**). The control treatments contained the formulation constituents without the bacteria, except F5 where dichlofluanid was applied during flowering and iprodione at fruiting. Results from all the treatments were not significantly different according to ANOVA ($P > 0.01$).

on fruits from harvest one was *P. fluorescens* applied as a short-term treatment during fruiting (F5) following dichlofluanid treatments applied during flowering (5% infected fruit). This treatment also produced similar results in the second harvest (8% infected fruit). The highest number of infected fruit (15% infected fruit) was harvested from plants treated with the *P. fluorescens* isolate resuspended in either NB or water with Speswhite and Hyphis 5 (formulations F3 and F4 respectively) and the no treatment control. The lowest number of infected fruit collected from harvest two (1% infected fruit) were treated with the conventional spray programme of dichlofluanid during flowering and iprodione during fruiting (F5 control). The highest number of infected fruit collected from harvest two (10% infected fruit) were recorded from plants treated with *P. fluorescens* applied in the F4 formulation and *B. pumilus* applied in the F3 and F5 formulations. Treatments with the mixed cultures of *P. fluorescens* and *B. pumilus* applied in the F3 and F6 formulations, consistently resulted in high numbers of infected fruit compared with the other treatments (13% and 7% infected fruit in harvests one and two respectively), but these values were not significantly different ($P > 0.01$) from the controls.

Incubation at 20°C caused faster disease development in the fruits from both harvests relative to fruits incubated at 4°C. Larger numbers of diseased fruit were observed in harvest two compared to harvest one (Figure 3.1.4). The *B. pumilus* isolate applied in each of the six formulations did not reduce *B. cinerea* infections compared with the control treatments on fruit sampled from both harvests. The largest reduction in grey mould achieved by the *B. pumilus* isolate was recorded in harvest one (55% infected fruits) when it was applied to the flowers and fruit in the F4 formulation. This level of disease reduction was significantly different from the no treatment control (75% infected fruit) but was not significantly different from the F4 control (70% infected fruit) and dichlofluanid treatments (60% infected fruit). Fruit assessed from the second harvest, treated with *B. pumilus* applied in the F3 and F5 formulations contained the largest number

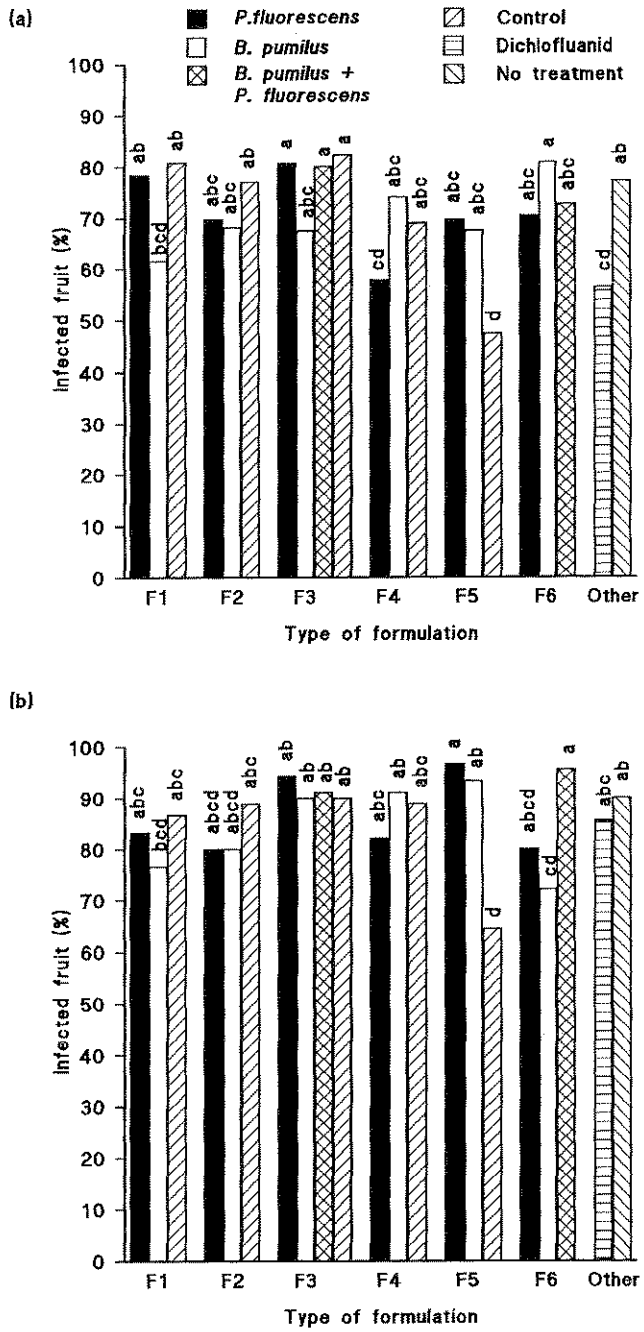

Figure 3.1.4 Inhibition of *B. cinerea* on fruits harvested on (a) 22 June and (b) 1 July picked from plants sprayed with different antagonist treatments. The fruits were incubated at 20°C for 5d in 2kg punnets. The different treatments consisted of *B. pumilus* or *P. fluorescens* resuspended in: NB (**F1**), NB + 5% (w/v) Speswhite (**F2**), NB + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F3**), H_20 + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F4**), as formulation F3 applied at fruiting following dichlofluanid applications during flowering (**F5**) or 2d NB cultures (**F6**). The control treatments contained the formulation constituents without the bacteria, except F5 where dichlofluanid was applied during flowering and iprodione at fruiting. Treatments annotated with a common letter are not significantly different (at P=0.01) according to DMRT.

of disease of fruit recorded during the field trial (95% infected fruit).

Similarly at 20°C incubation, the *P. fluorescens* isolate applied in any of the six formulations failed to reduce the number of diseased fruit from both harvests. Overall, *P. fluorescens* inhibited *B. cinerea* infection better than the *B. pumilus*, but this did not show significance at P=0.01. The largest reduction in disease development resulting from treatments with *P. fluorescens* was on fruit treated with the isolate resuspended in NB containing no additives. This reduction was similar in fruit collected from harvest one (60% infected fruit) and harvest two (75% infected fruit), but neither showed significance (at P>0.01) from the no treatment and formulation controls. The *P. fluorescens* isolate also reduced the number of diseased fruit in harvest two (70% infected fruit) when applied as a suspension grown in NB for 2 d (F6). This reduction was significantly different from the no treatment control, but not significantly different from the F6 control. However, the same treatment did not have the same effect on fruit collected from harvest one and caused one of the highest recorded percentages of diseased fruit (80% infected fruit). Mixed cultures of *P. fluorescens* and *B. pumilus* in NB applied as a 2 d suspension (F6) or resuspended with Speswhite and Hyphis 5 (F3) also failed to inhibit *B. cinerea* on fruits from both harvests. Disease levels were highest in plants treated with the combined isolate culture resuspended in the F4 formulation with 80% and 90% infected fruit from harvests one and two respectively.

Inhibition of *B. cinerea* infections on fruit using the conventional fungicide, dichlofluanid, was inconsistent. The dichlofluanid treatment applied during flowering reduced the number of diseased fruit from harvest one (60% infected fruit), but failed to exert any control on fruit collected from harvest two (85% infected fruit). The greatest inhibition of infection by *B. cinerea* in both harvests was accomplished when dichlofluanid was applied during flowering followed by iprodione at fruiting (F5 control). The percentage of diseased fruit in harvest one (50%) and

harvest two (60%) were significantly different ($P<0.01$) from the no treatment control and other formulation controls. Similar high numbers of diseased fruit in both harvests were recorded in the no treatment control and formulation controls, with between 70% - 80% fruit infected at harvest one and 85% - 90% at harvest two.

The weather can have a pronounced effect on the behaviour of both the antagonist and pathogen during the field trial. The weather during flowering in May was very wet with a total of 16d rain (Figure 3.1.5). Rain was recorded between every treatment application. The amount of rain was lowest (0.9mm) between application one on 5 June and application two on 11 June. In between the following two applications, on 11 May and 16 May, a total of 7.4mm rain fell on 3 consecutive days. The highest total recorded rainfall (27.2mm) fell on 5 consecutive days between applications 3 (17 May) and 4 (24 May), with 15.8mm recorded on 19 May and 10.4mm on 20 May. Likewise, rain was recorded on 5 successive days between applications 4 and 5 (31 May), but at a much lower volume (12.6mm). Rain was not recorded either between the treatment applications (11 May and 24 May) or the sampling times of flowers taken for assessing infection by *B. cinerea* (13 and 26 May).

The average humidity during May varied between 68% (23 May) and 95% (25 May). Generally, humidity values were very high with three periods during flowering where hourly humidity readings exceeded 94%. The first occurred between 9 and 15 May with a total of 23h, the second between 19 and 21 May with 18h and the last between 25 and 29 May with 38h. Temperature also varied dramatically during May but never exceeded 16°C. Following the first application, temperature consistently averaged 9°C with a sudden increase to 14°C the day before the second application (10 May). After the second application, temperature fluctuated daily between 11°C and 9°C. The highest temperature during May, 16°C, was recorded on 17 May, the day of the third application. Further daily decreases of 1°C were recorded over the following

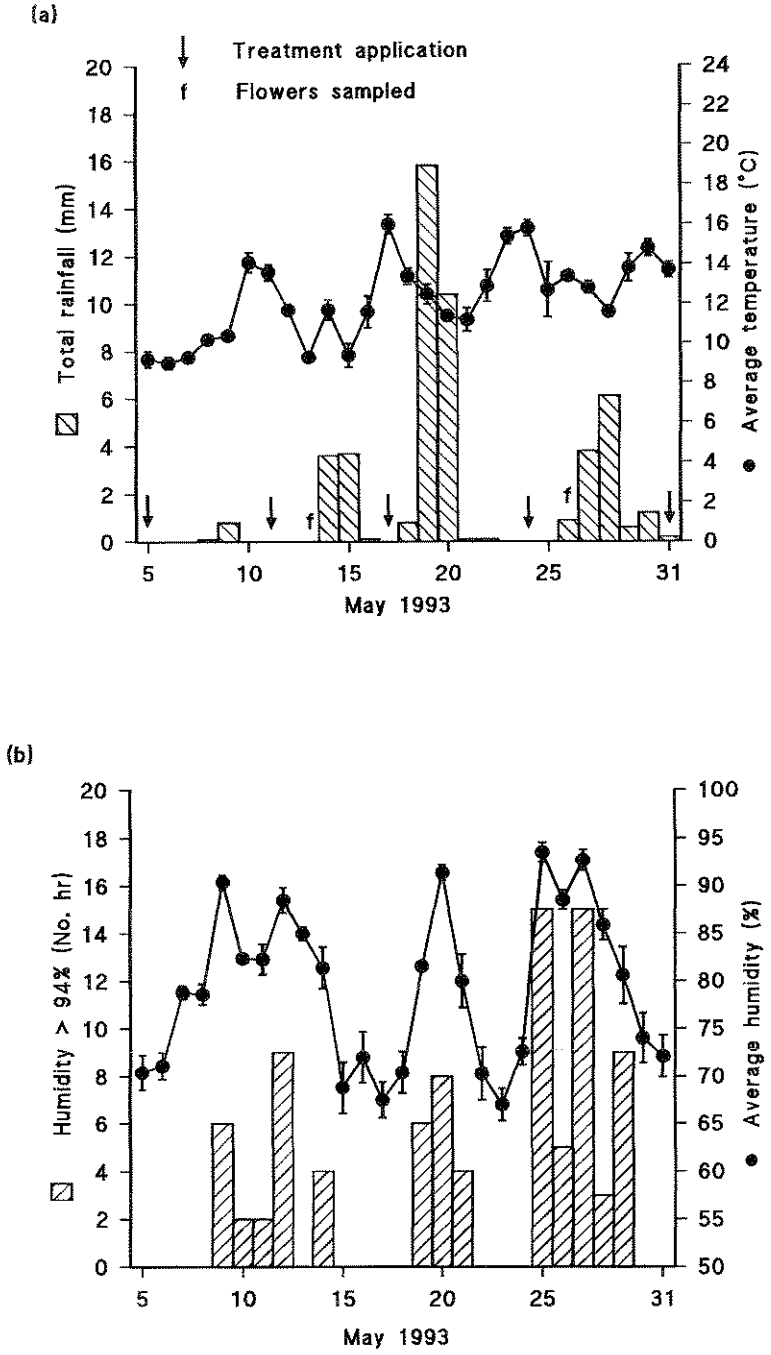

Figure 3.1.5 Average daily (a) temperature (°C) and rainfall (mm), (b) humidity (%) and number of hours humidity exceeded 94% (h). The data was recorded at the weather station, number 03797, at RAF Manston, Kent during May 1993.

4d, reaching a minimum temperature of 11°C on 21 May. Following the fourth application temperatures dropped from 16°C (24 May) to 12°C (28 May) and then increased to 15°C (30 May).

The weather was also varied during fruiting in June (Figure 3.1.6). There was only one day of rain between the fifth (31 May) and sixth (7 June) treatment sprays on 2 June (10.2mm). Conversely, rain was recorded every day between the sixth and seventh applications (20.1mm), with 5.4mm recorded immediately after the treatment application on 14 June. The highest daily rain was recorded on 16 June (16.2mm). Rain was also recorded on 18 June (2.2mm), the day of the first fruit sample. There were 2d of rain recorded on 23 June (4.4mm) and 24 June (4.6mm) between the last treatment application (21 June) and final fruit sample (30 June). Negligible rain (0.1mm) was recorded on the day of the second fruit sample.

Average humidities between 64% and 95% fluctuated wildly during 1 June to 18 June. The average humidity readings then stabilised to around 74% up until the final fruit sample on 30 June. The hourly humidity readings from 11 June to 19 June consistently exceeded 94%. During this period humidity readings exceeding 94% for over 10h were recorded on 11 June (11h), 12 June (11h), 14 June (18h) and 16 June (10h). Temperatures during June were consistently higher than those recorded during May. The air temperature steadily increased by 1°C each day from 3 June (13°C) to 9 June (20°C). The temperature then dropped to 15°C on 12 June, where it stabilised between 13°C and 15°C until a sudden increase to 19°C on 26 June. A further drop to 15°C on 28 June, followed by a steady increase to 18°C on 30 June was recorded. The highest average daily temperature was measured on 9 June (20°C) and the lowest on 3 June (13°C).

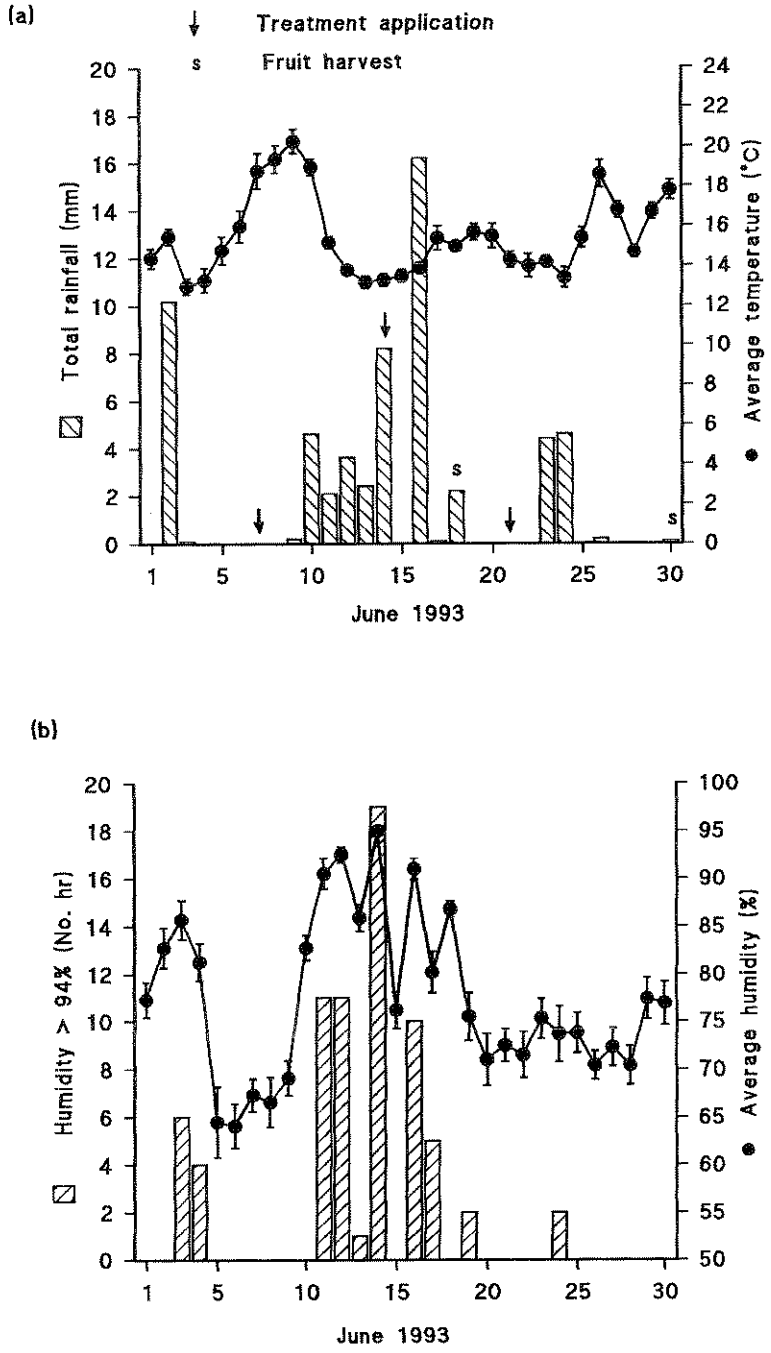

Figure 3.1.6 Average daily (a) temperature (°C) and rainfall (mm), (b) humidity (%) and number of hours humidity exceeded 94% (h). The data was recorded at the weather station, number 03797, at RAF Manston, Kent during June 1993.

3.2 Survival studies on the *B. pumilus* and *P. fluorescens* isolates

Studies of the survival of *B. pumilus* and *P. fluorescens* on the flowers and fruit were performed using antibiotic-resistant mutants (section 2.4). The persistence of the antibiotic-resistant bacteria after successive cultures in NB varied between the two isolates (Table 3.2.1). *Bacillus pumilus* was very stable after successive transfers in NB with 100% of the bacterial cells exhibiting resistance to novobiocin + streptomycin. The resistance to the antibiotics was maintained through all 15 NB transfers. However, *P. fluorescens* was not as stable on the NA amended with streptomycin and chloramphenicol. Results showed that only 41% of the bacteria were able to grow on the antibiotic-amended agar compared with 100% on normal NA after 5 transfers in NB, and this level was reduced to less than 1% after 10 transfers. No antibiotic-resistant mutants were detected after 15 transfers.

Survival of *B. pumilus* on flowers was similar for the four formulations investigated (Figure 3.2.1a). Similar numbers of bacteria resuspended in the NB formulations F1, F2 and F3 were applied to the flowers ($5x10^7$cfu ml^{-1}). A much lower concentration (10^5cfu ml^{-1}) was observed on the flowers treated with *B. pumilus* resuspended in water (F4). In all four formulations, the numbers of viable bacteria declined over 96h. The F2 formulation resulted in the lowest death rate recorded with a 10 fold decrease in cell numbers over the 4d. Similar numbers were obtained at the end of 4d for the F1 and F3 formulations (10^5cfu ml^{-1}), with survival on the F1 treated flowers slightly lower than the F3 formulation. The lowest survival was recorded for *B. pumilus* resuspended in water over 12h (10^4cfu ml^{-1}), 24h (10^3cfu ml^{-1}) and 96h (10^2cfu ml^{-1}).

The number of spores produced by *B. pumilus* on the flowers was also assessed (Figure 3.2.1c). No spores were isolated from flowers sampled immediately after the treatment application. After 12h similar numbers of spores were detected on flowers treated with all four formulations (100 spores ml^{-1}). However, after a further 12h no spores were observed on any of the flowers

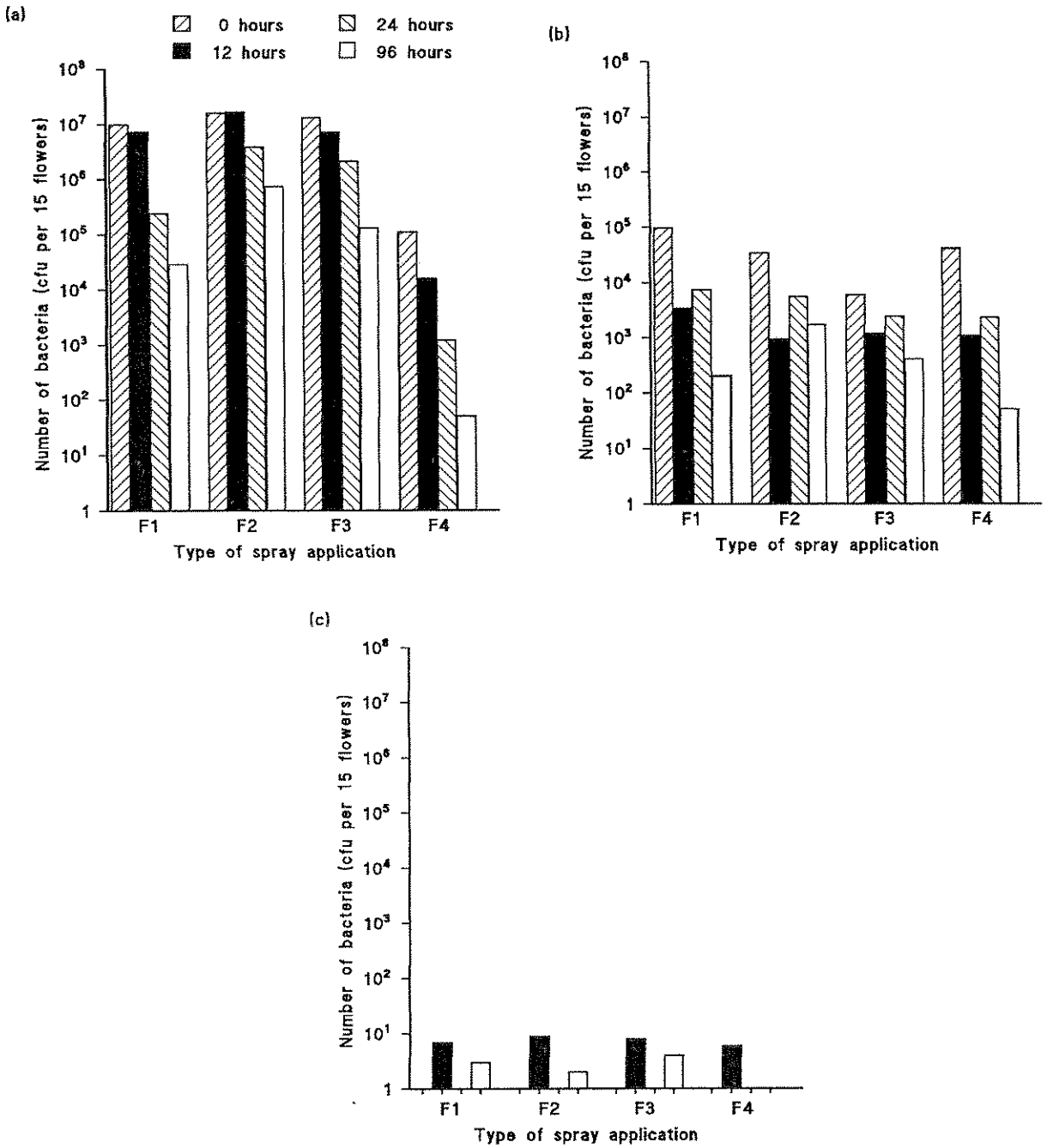

Figure 3.2.1 Survival of antibiotic-resistant mutants: (a) *B. pumilus* vegetative cells, (b) *P. fluorescens* vegetative cells and (c) *B. pumilus* spores, on 15 flowers sampled on 24 May. The different treatments consisted of *B. pumilus* or *P. fluorescens* resuspended in: NB (**F1**), NB + 5% (w/v) Speswhite (**F2**), NB + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F3**) and H_20 + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F4**). 15 flowers were sampled at 0, 12, 24 and 96h, washed and numbers of bacteria assessed using the Miles and Misra technique.

Table 3.2.1 Growth of the antibiotic-resistant mutants of *B. pumilus* and *Pseudomonas fluorescens* on antibiotic-amended and unamended media. The antibiotic-resistant mutants were transferred through 1, 5, 10 and 15 passages in non-selective NB and antibiotic stability was determined. The NB cultures were incubated at 25°C (160rpm) for 24h.

Number of sub-cultures in NB	*Bacillus pumilus*			*Pseudomonas fluorescens*		
	A (10^8 cfu ml^{-1})	**B** (10^8 cfu ml^{-1})	**C** (%)	**A** (10^8 cfu ml^{-1})	**B** (10^8 cfu ml^{-1})	**C** (%)
G = 1	16	17	**106**	15	4.7	**31**
G = 5	13	13	**100**	13	5.3	**41**
G = 10	16	17	**106**	7	0.2	**>1**
G = 15	80	72	**90**	9.1	0	**0**

where: **G** - Number of transfers into NB,
A - Number of 10^8 cfu ml^{-1} on non-amended NA,
B - Number of 10^8 cfu ml^{-1} on antibiotic-amended agar,
C - Percentage of viable cells exhibiting the antibiotic-resistant marker

sampled. Finally, over the next 3d, the number of spores then increased on flowers treated with the F1, F2 and F3 formulations. The largest increase occurred on flowers treated with *B. pumilus* resuspended in F3 (700 spores ml^{-1}) and the lowest in F2 (100 spores ml^{-1}). No spores were detected on the flowers treated with the F4 formulation.

Survival of *P. fluorescens* in all four formulations on the flowers was similar, but different from the behaviour exhibited by *B. pumilus* (Figure 3.2.1b). The initial number of viable cells isolated from the treated flowers (10^5cfu ml^{-1}) was lower than cells isolated from flowers treated with *B. pumilus* (10^7cfu ml^{-1}). For *P. fluorescens* applied in all the formulations, the trend was for an initial large decrease over the first 12h, followed by a small increase over the next 12h and a further reduction over the remaining 3d. *Pseudomonas fluorescens* resuspended in NB with Speswhite (F2) showed the smallest decrease in viable cells during the study (10^3cfu ml^{-1}). The largest decrease was exhibited by flowers treated with *P. fluorescens* resuspended in water with Speswhite and Hyphis 5 (10^2cfu ml^{-1}).

Survival of *B. pumilus* on the fruits was similar for all four formulations investigated (Figure 3.2.2a). Analogous numbers of bacteria resuspended in the F1 and F2 formulations were applied to the fruits ($5x10^4$cfu ml^{-1}). The two applications containing the sticker and UV protectant (F3 and F4) contained fewer vegetative cells ($5x10^3$cfu ml^{-1}). Survival over 96h was similar for the formulations F2, F3 and F4. The bacterial numbers gradually increased over 24h after their initial application. The largest increase was observed in the treatments containing the UV protectant and sticker (F3 and F4). The cells increased from $5x10^3$cfu ml^{-1} to $1x10^5$cfu ml^{-1}, with the largest increase occurring during the daylight. *Bacillus pumilus* in F1 was the only treatment where cell numbers decreased overnight and then increased during the following day. Over the remaining 3d the number of bacterial cells declined in all the formulations to approximately $5x10^3$cfu ml^{-1}.

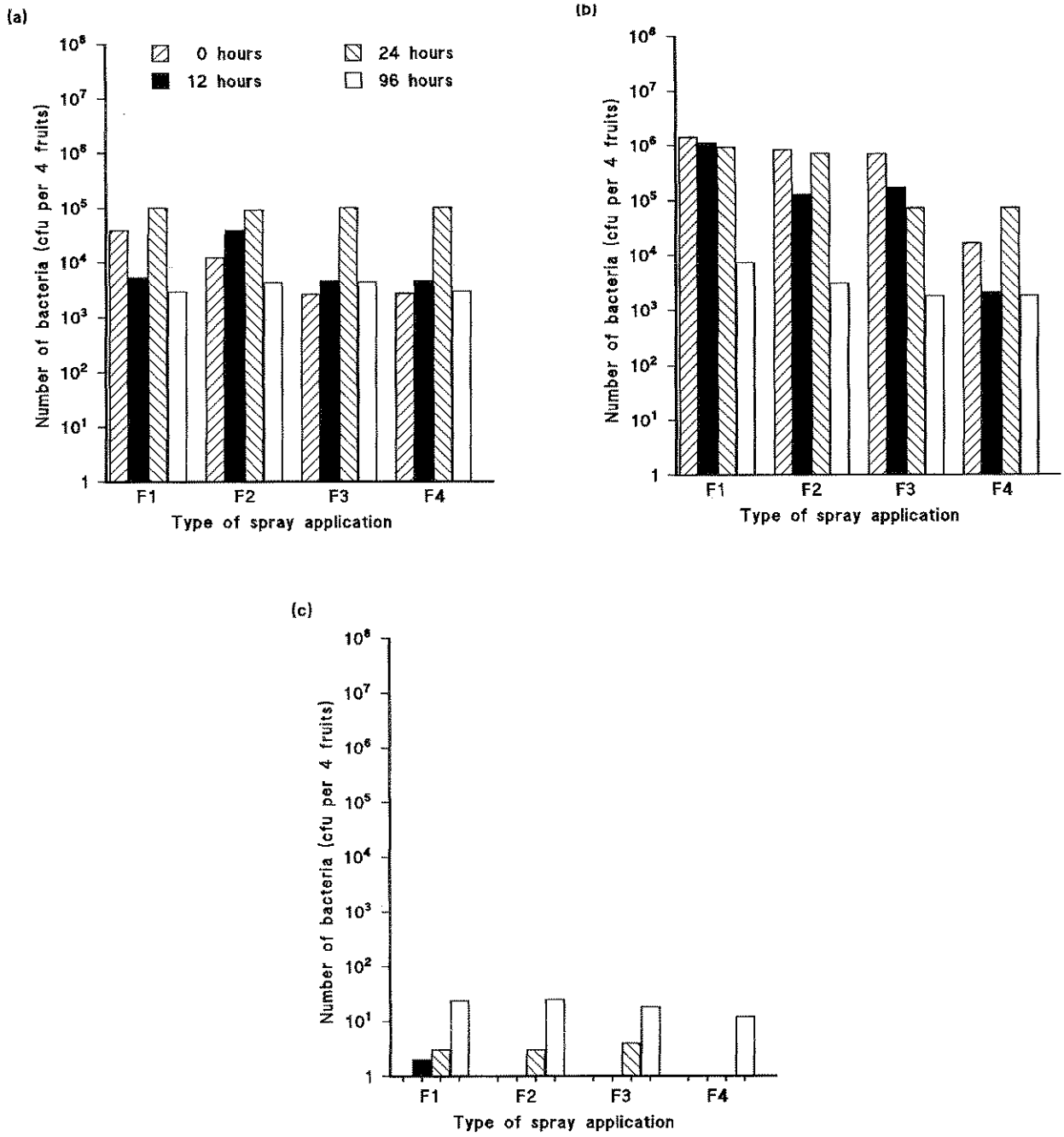

Figure 3.2.2 Survival of antibiotic-resistant mutants: (a) *B. pumilus* vegetative cells, (b) *P. fluorescens* vegetative cells and (c) *B. pumilus* spores, on 4 fruits sampled on 22 June. The different treatments consisted of *B. pumilus* and *P. fluorescens* resuspended in: NB (**F1**), NB + 5% (w/v) Speswhite (**F2**), NB + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F3**) and H_20 + 5% (w/v) Speswhite + 1% (w/v) Hyphis 5 (**F4**). 15 flowers were sampled at 0, 12, 24 and 96h, washed and numbers of bacteria assessed using the Miles and Misra technique.

The number of spores produced by *B. pumilus* on the fruit was also assessed (Figure 3.2.2c). No spores were isolated from the fruits sampled immediately after the treatment application. After 12h, spores were only detected on fruits treated with NB containing no additives (20 spores ml^{-1}). Similar numbers of spores were removed after 24h from fruits treated with the F1, F2 and F3 formulations (40 spores ml^{-1}). No spores were detected on the F4 formulation. During the following 3d, the numbers of spores on the fruits treated with the four formulations gradually increased to approximately 200 spores ml^{-1}, with the lowest number recorded on fruits treated with F4 (100 spores ml^{-1}).

Survival of *P. fluorescens* on fruits was variable (Figure 3.2.2b). The concentration of *P. fluorescens* on the fruit surface immediately after its application was higher (10^6cfu ml^{-1}) than fruit treated with *B. pumilus* (10^4cfu ml^{-1}). As with *B. pumilus*, the number of viable cells of *P. fluorescens* was much lower on fruits treated with F4 compared to the other three formulations. The number of viable bacteria on fruit treated with F1 remained constant over 24h (10^6cfu ml^{-1}), declining over the following 3 d (10^4cfu ml^{-1}). The number of viable bacteria on fruit treated with the F3 formulation gradually decreased over the 4d to 10^3cfu ml^{-1}. The survival of *P. fluorescens* in the F2 and F4 formulations followed similar patterns. A slight decrease was observed overnight followed by an increase in bacteria numbers during the day, with a further decrease over the remaining 3d (10^3cfu ml^{-1}).

3.1 Discussion

3.1.1 Inhibition of infection by B. cinerea

The successful use of a biocontrol agent in the field to reduce infections by *B. cinerea* on strawberry fruits has been limited to the fungal antagonists, *Trichoderma* spp. (Tronsmo & Dennis, 1977), *Gliocladium roseum* and *Penicillium* spp. (Peng and Sutton, 1991). The establishment of

the antagonist on the phylloplane and activity against the pathogen are major considerations when determining the reasons for the success or failure of a biocontrol agent. Blakeman (1973) identified the microclimate and chemical environment as important factors influencing the activity of the antagonist and pathogen on the phylloplane. The survival of *B. pumilus* and *P. fluorescens* on the flowers and fruits is discussed later in section 3.2. However, the survival of the antagonists does not necessarily correspond with their antagonistic activity against *B. cinerea*, which will ultimately determine their success.

In contrast with the field trial completed in 1992, both the antagonists failed to conclusively inhibit *B. cinerea* infections. This emphasises the observation by Tronsmo and Ystaas (1980) that final decisions on the efficacy of a biological control agent should not be based on the results of one field trial. In other studies, both *Pseudomonas* and *Bacillus* spp. have given excellent results controlling a range of fungal pathogens in field tests (Cook and Baker, 1983). *Bacillus pumilus* reduced infection of *Puccinia recondita* on cereal leaves (Morgan, 1963) and the closely related species *B. subtilis* reduced infection by *Uromyces phaseoli* (bean rust) by over 75% (Baker *et al.*, 1985).

The loss in performance of both isolates, but notably *B. pumilus*, can largely be attributed to the weather conditions experienced during the trial. Results examining the inhibition of *B. cinerea* during flowering showed that the *B. pumilus* strain was unable to reduce infections on either the petals or stamens. Even the incorporation of a sticker and UV protectant into the formulation was unable to improve the control. This loss of control compared to the promising results obtained from the 1992 field trial was probably a result of the lower air temperatures recorded during the trial, especially during flowering. Gordon (1989) demonstrated that the minimum temperature for *B. pumilus* growth was between 5 - 15°C. The average daily temperature for May rarely exceeded 13°C and would have seriously affected the performance of

this antagonist. Boudreau & Andrews (1987), suggested that a loss in disease control on the phylloplane may be caused by a combination of the removal, degradation or deactivation of antibiotic inhibitors from the plant surface.

In contrast, *P. fluorescens* inhibited *B. cinerea* infections on stamens in four of the five formulations on flowers from sample one. Studies on the activity and growth of *P. fluorescens* showed this isolate was extremely tolerant to low temperatures. The only treatment which failed to reduce grey mould on the stamens was the F1 formulation containing no additives. This suggested that the UV protectant and sticking agent were important in increasing the activity of the isolate. The importance of the additives was confirmed when the *P. fluorescens* was applied with the UV protectant and sticking agent (F3) as this was the only treatment to reduce *B. cinerea* infections on the petals in sample one. Knudsen & Spurr (1985) reported that short dry spells were responsible for high death rates in pseudomonad populations. The UV protectant and sticker additives may have been important in sustaining the activity of the *P. fluorescens* isolate during such extreme conditions. The increased control of plant pathogens by antagonists applied with sticker compounds had been reported by Spurr (1981) and Cullen & Andrews (1984b). Unfortunately, the antagonistic ability of the *P. fluorescens* isolate was variable and the inhibition of *B. cinerea* observed on the flowers sampled first was not repeated during the second sampling.

The absence of any significant reduction of *B. cinerea* on the flowers by the *B. pumilus* and *P. fluorescens* isolates accounts for the large amount of disease recorded on the harvested fruit. Infection studies of *B. cinerea* on strawberry flowers by Bristow *et al.* (1986) identified stamens as one important pathway for the development of post-harvest grey mould. Another was senescent petals, infected early in newly opened flowers, which were an important inoculum source for subsequent infection during fruiting (Powelson, 1960). Therefore, inhibition of *B. cinerea* had to be accomplished during the flowering period or high levels of grey mould disease would occur on

post-harvest fruit. Therefore, the large amount of disease recorded on fruit from both harvests was probably due to the inadequate control exerted during flowering. This limited control can be attributed to two factors. Firstly, the application of the biocontrol agents at weekly intervals probably limited the protection available for the rapidly developing flowers and fruits. Sutton (1990) encountered the same problem trying to reduce infection by *B. cinerea* in strawberry field trials. Secondly, poor application of the treatments to the target site caused by the dense foliage. This was minimised by using spray guns instead of commercial equipment. This suggests that a significant improvement in the application methods is required, as well as the treatments. The effect of the weather conditions on the pathogen also needs to be examined when interpreting the field observations. Studies by Bulger *et al.* (1987) on the pattern between meteorological parameters and grey mould development on strawberries showed the optimum temperature for flower infection was 20°C with 100% infection occurring in 24h wetness. The presence of free water increases the amount of flower and fruit infection. These were precisely the conditions observed during flowering in May. In this field trial none of the treated flowers developed pre-harvest grey mould (flower blight). Jarvis (1962) reported that flower blight usually occurred in conditions when the flowering period was wet. The absence of flower blight in this field trial, despite the ideal conditions, was probably caused by an initial lack of inoculum resulting from the trial consisting of first year plants. The weather recorded during June was also favourable for secondary infection and mycelial growth on the developing fruits. These conditions increased the number of infected fruit caused by cross-contamination from diseased neighbouring fruit and infection by conidia produced from secondary inoculum sources. The reduction of grey mould on fruit treated with a combination of dichlofluanid at flowering and iprodione at fruiting compared with the dichlofluanid treatment only applied during flowering, corroborates the evidence of additional infection occurring during fruiting.

The failure of the biocontrol agents to reduce grey mould in the field was a combination of poor antagonist activity and optimal conditions for pathogen infection caused by the low temperatures and wet conditions. Rewal *et al.* (1991) reported dichlofluanid was an effective fungicide treatment against *B. cinerea* infections with no resistance problems. Therefore, the subsequent failure of the dichlofluanid treatment during flowering in this field trial emphasises the importance the climate had on increasing grey mould disease. This highlights the problem of variable weather conditions encountered by biocontrol agents applied to the phylloplane, experienced in a temperate climate. This stresses the importance of not only selecting biocontrol agents that are ecologically competent on the host but also adapted to the highly variable, temperate climate. The *B. pumilus* and *P. fluorescens* isolates fulfilled the first criterion but not the second.

3.3.2 Survival studies on the B. pumilus and P. fluorescens isolates

Quantitative assessment of microbial populations reintroduced to the phylloplane by incorporating antibiotic-resistant markers into the antagonists has been used before. The selection of the specific antibiotic-markers for studying microbial populations, particulary in a combined spray programme tracing two populations, was critical. Ideally, the antibiotic-resistant marker must: (i) be stable and not revert back to the wild type and (ii) not alter the ability of the isolate to survive in its environment. The antibiotic-resistant markers selected for *B. pumilus* and *P. fluorescens* were selected as a result of the resistance shown by the isolates to Multodiscs impregnated with different antibiotics. The survival of the antibiotic-resistant mutants showed that *B. pumilus* was very stable, whereas *P. fluorescens* was comparatively unstable. The *P. fluorescens* antibiotic-resistant mutant was still used in the field trials due to the shortage of time to incorporate a different marker into the isolate. Also during this study the interaction between *B. pumilus* and *P. fluorescens* in a combined spray application could not be studied, because the

P. fluorescens mutant was naturally resistant to the novobiocin-marker incorporated into the *B. pumilus* mutant. This created problems when trying to distinguish between the two populations on the relevant antibiotic-selective agar.

The number of cfu determined for each isolate *in vitro* may not be truly representative of concentrations residing on the flowers or fruits. The antibiotic-resistance marker may cause secondary aberrations such as changing the growth rates (Compeau *et al.*, 1988) and reducing the ability of the isolate to persist in the environment (Turco *et al.*, 1986). A loss in stability of the antibiotic-resistant mutants with time in the field may result in fewer vegetative cells being detected on the selective agar. This was more likely to have occurred with the *P. fluorescens* antibiotic resistant mutants which showed a lower stability than the *B. pumilus* mutant. Further losses may also be caused by the isolation technique failing to remove all of the viable bacteria from the flower and fruit surface. This was most likely to occur with treatments containing the sticker, Hyphis 5, which bound the bacteria to the plant surface. Inefficient isolation of the bacteria would be further increased in samples where dry periods caused evaporation of water from the formulation, thereby increasing the concentration and overall effectiveness of the sticker.

The establishment and continued survival of the antagonist on the phylloplane are of primary importance when determining the efficacy of an isolate. The large numbers of *P. fluorescens* and *B. pumilus* detected on the flowers and fruits immediately after their application indicated the success of the spraying technique. Conditions were also manipulated to favour the survival of the antagonist during application and was mirrored by the survival rate of both antagonists on the phylloplane. The vegetative cells were harvested during the stationary phase of culture growth relative to the log-phase, thereby increasing the survival of the bacteria in aerosols (Goodlow & Leonard, 1961). The use of a spray gun as the delivery system was also more effective than any conventional systems, increasing the antagonist inoculum levels detected on the target sites. A

commercial sprayer would have applied lower levels of the antagonist to the flowers and fruit, as a result of the high amount of foliage reducing spray penetration to the target sites. The survival of the *B. pumilus* and *P. fluorescens* isolates was high on the flowers. The low number of epiphytic microorganisms resident on the newly-opened flowers would have placed less competitive pressure on the antagonists resulting in survival in high numbers. The higher levels of *B. pumilus* detected initially on the flowers compared with *P. fluorescens* may be caused by a loss in the instability of the *P. fluorescens* antibiotic-resistant mutants. However, the more likely explanation for this difference was a variation in the number of bacteria caused by a combination of the formulation preparation, death rate during spraying and consistency in their application to the target sites.

The *B. pumilus* and *P. fluorescens* isolates survived for longer periods on the fruit surface than on the flowers. This difference can be attributed to the nutrient-rich, moist environment created on the fruit surface compared with that on flowers. Regular plant leakages, deposits of pollen, organic debris can supply abundant amounts of nutrients for any epiphytic microorganisms (Janisiewicz, 1988b). In addition, the presence of surface characters, such as waxes and hairs, increases water retention and creates a moist atmosphere, essential for maintaining any antagonistic population. Usually these high nutrient conditions favour the multiplication of yeasts at the expense of the bacteria. This is due to the limitation in the availability of amino acids (Blakeman & Brodie, 1977). However, the survival of *B. pumilus* and *P. fluorescens* observed on the fruit surface can be attributed to the additional amino acids in the formulation reducing competition. The importance of the additional nutrients supplied in the NB was further emphasised by the lack of spore germination of *B.pumilus* resuspended in water.

The climatic conditions also influenced microbial interactions and survival of the antagonist on the flower and fruit surfaces. Resistance to desiccation, cycles of wetting and drying, extremes

of temperature and ultraviolet radiation are significant factors determining the survival of an antagonist (Cullen & Andrews, 1984b). Growth of microorganisms on the phylloplane is usually limited to periods of dew which create free water or high humidity. During flowering the high relative humidities and low temperatures favoured the survival of *B. pumilus* and *P. fluorescens* on the flowers. Most microorganisms require high humidities (>95%) to survive, while in direct sunlight the average RH on the leaf surface is between 40 and 65%. The low temperatures also accounted for the absence of growth in *B. pumilus* which multiplies in much higher temperatures. However the low temperatures did not explain the lack of growth observed in *P. fluorescens*, which was less susceptible to these temperatures.

Like the commercial plants, the plants grown in this trial were trickle-irrigated during dry spells, maintaining humid conditions on the phylloplane and reducing the death of the antagonists on the flowers and fruits. This may explain the retention of high numbers of *P. fluorescens* and *B. pumilus* detected on the fruit surface over the first 24h, despite low humidity readings and high temperatures. Gowdu & Balasubramanian (1988) reported that dry spells where no irrigation was used caused a significant reduction in the number of microorganisms on the phylloplane. The relatively high daily temperatures following the application would also account for the increase in *B. pumilus* on the fruit surface, which grows better in higher temperatures. The rain detected on 23 and 24 June would also have removed some of the inoculum and this may account for the lower numbers of both antagonists detected on the fruit sampled 4d after the initial application.

Successful application and establishment of the biocontrol agent can be achieved by incorporating nutrients, sticking agents and UV protectants into the formulation (Spurr, 1981). Rapid decreases in antagonist populations may be caused by the lack of adhesion (Cullen & Andrews, 1984b). However, the survival of *P. fluorescens* and *B. pumilus* on the flowers and fruit surfaces were mostly unaffected by the incorporation of such additives. Leben *et al.* (1965)

suggested the application of bacteria resuspended in nutrients as opposed to water was also important in increasing survival. This may account for the lower survival on flowers of *B. pumilus* resuspended in the water plus sticker and UV protectant treatment, although this was not repeated after the fruit application. The difference observed between the survival on the flower and fruit by *B. pumilus* resuspended in H_20 may be due to the high nutrient levels on the fruit surface sustaining inoculum levels similar to the NB formulations. Ultimately, the purpose of the additives was to improve establishment and hence antagonism (Cullen *et al.*, 1984). This proved unsuccessful with the additives used in this field trial.

In summary, both the *B. pumilus* and *P. fluorescens* isolates failed to conclusively reduce grey mould disease on fruits. *Bacillus pumilus* did not reduce *B. cinerea* infections on the flowers or fruits in any of the treatment formulations. *Pseudomonas fluorescens* reduced grey mould on the flowers when applied with the UV protectant and sticker but this control was not repeated on the harvested fruits. The conventional fungicides also showed variable control. Grey mould was only reduced when dichlofluanid was applied at flowering followed by iprodione during fruiting. The UV protectant and sticker slightly improved the survival of the antagonists on the fruit and flower surface. Isolates resuspended in the nutrient-rich broth also increased numbers on the target sites. The overall survival of *B. pumilus* and *P. fluorescens* on the flowers and fruits over 4d was sufficiently high to cause a reduction in *B. cinerea* infections on the flowers and post-harvest fruit. The lack of control exhibited by the two isolates was probably caused by a reduction in activity due to the low air temperatures, high humidities and constant rain, rather than lack of survival. The weather conditions recorded during the field trial were unfavourable for antagonist growth and activity but favourable for growth and infection by *B. cinerea*.

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APPENDICES

APPENDIX A

Microbiological growth media

Malt Extract Broth (MEB)

Malt extract (Oxoid)	17.00g
Mycological peptone (Oxoid)	3.00g
dH_2O	1000ml

Nutrient Agar (NA)

Nutrient agar No.1 (Oxoid)	28.00g
dH_2O	1000.00ml

Nutrient Broth (NB)

Nutrient broth No.2 (Oxoid)	25.00g
dH_2O	1000ml

The pH of the medium was adjusted to 6.5 before sterilisation

58MNS Selective Agar

Nutrient agar No.1 (Oxoid)	28.00g
Streptomycin (Sigma)	0.10g
Novobiocin (Sigma)	0.05g
Cycloheximide (Sigma)	0.10g
dH_2O	1000ml

168MNS Selective Agar

Nutrient agar No.1 (Oxoid)	28.00g
Streptomycin (Sigma)	0.10g
Chloramphenicol (Sigma)	0.10g
Cycloheximide (Sigma)	0.10g
dH_2O	1000ml

Paraquat-Cloramphenicol Agar (PCA, Sutton 1988)

Technical agar No.3 (Oxoid)	5.00g
dH_2O	1000ml

After cooling to 65°C the following chemicals were added:

Active paraquat (ICI)	0.02g
Cloramphenicol (Sigma)	0.20g

Phosphate Buffer Medium (PBM)

NaH_2PO_4 (Sigma)	31.20g
$Na_2HPO_4.7H_20$ (Sigma)	53.65g
dH_2O	1000ml

Add 367.5 ml of NaH_2PO_4 to 132.5 ml of Na_2HPO_4 to produce a buffer with a pH of 6.4

APPENDIX B

Field Trial design 1993

	BLOCK 1	BLOCK 2	BLOCK 3
N.B.+ F58 Resusp.	A14	B09	C10
N.B.+ F58 Susp.	A18	B26	C14
N.B.+ F168 Resusp.	A11	B19	C06
N.B.+ F168 Susp.	A25	B12	C17
N.B.+ U.V.+ F58 Resusp.	A30	B24	C22
N.B.+ U.V.+ F58 Susp.	A17	B04	C23
N.B.+ U.V.+ F168 Resusp.	A03	B10	C09
N.B.+ U.V.+ F168 Susp.	A28	B15	C01
N.B.+ MUT.F58 Resusp.	A01	B22	C16
N.B.+ MUT.F58 Susp.	A10	B20	C11
N.B.+ MUT.F168 Resusp.	A21	B01	C03
N.B.+ MUT.F168 Susp.	A27	B08	C30
N.B.+ U.V.+ MUT.F58 Resusp.	A04	B16	C02
N.B.+ U.V.+ MUT.F58 Susp.	A29	B11	C26
N.B.+ U.V.+ MUT.F168 Resusp.	A02	B28	C19
N.B.+ U.V.+ MUT.F168 Susp.	A07	B05	C05
N.B.+ U.V.+ STICKER + MUT.F58 Resusp.	A09	B13	C07
N.B.+ U.V.+ STICKER + MUT.F168 Resusp.	A05	B06	C13
N.B.+ F58/F168 Susp.	A08	B03	C04
N.B.+ U.V.+ F58/F168 Resusp.	A06	B25	C20
ELV.+ N.B.+ F58 Susp.	A16	B30	C27
ELV.+ N.B.+ F168 Susp.	A13	B17	C12
N.B.+ U.V.+ STICKER + F58 Resusp.	A23	B23	C18
N.B.+ U.V.+ STICKER + F168 Resusp.	A12	B27	C24
N.B. ONLY	A19	B18	C21
N.B. + U.V.	A15	B29	C28
N.B. + U.V. + STICKER	A24	B14	C15
ELVARON + ROVRAL	A20	B22	C08
ELVARON ONLY	A07	B21	C25
NO TREATMENT	A26	B02	C29

APPENDIX C

Programme for calculating ANOVA using 'Genstat 5, release 2.1'

```
UNITS [60]
TEXT [N=1] T
FACTOR [LEVELS=5] BLOCK
FACTOR [LEVELS=2; LABELS=!T('BC2','BC3') ISOLATE
FACTOR [LEVELS=6; LABELS=!T('5','10','15','20','25','30') TEMPERATURE
BLOCK BLOCK
TREAT ISOLATE*TEMPERATURE
OPEN 'EXPERIMENT1'; CHANNEL=2
FOR x=%GROWTH
READ [CHANNEL=2] T
READ [CHANNEL=2; serial=yes; print=data] x
GENE ISOLATE, TEMEPERATURE, BLOCK
PRINT ISOLATE, TEMEPERATURE, BLOCK, x
ANOVA [PRINT=aov, m, %cv; fprob=yes] x

"ALL ANALYSIS FROM HERE ON IS TRANSFORMED"

CALCULATE x=x*0.5
PRINT ISOLATE, TEMEPERATURE, BLOCK, x
ENDFOR
STOP
```