Project title:	Tomatoes: development of biocontrol as a component of an integrated, sustainable strategy for the control of grey mould (*Botrytis cinerea)*
Project number:	PC 174
Project leader:	Dr T M O'Neill, ADAS Arthur Rickwood
Report:	Annual report, September 2002
Previous reports:	Annual report, March 2001
Key workers:	Dr K Green, ADAS Arthur Rickwood Dr T Pettitt, HRI Wellesbourne Prof J Whipps, HRI Wellesbourne
Location of project:	ADAS Arthur Rickwood, Mepal, Ely, Cambs. HRI Wellesbourne, Wellesbourne, Warwicks. HRI Efford, Lymington, Hants.
Project co-ordinator:	Dr P Challinor, Humber VHB
Date project commenced:	1 April 2000
Date completion due:	31 March 2004
Key words:	Tomato, grey mould, *Botrytis cinerea*, biological control, antagonists, bioassay

The results and conclusions in this report are based on a series of experiments conducted over a two-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

I declare that this work was done under my supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr T M O'Neill
Principal Research Scientist
ADAS Arthur Rickwood

Signature ..... Date .....

Dr K Green
Research Scientist
ADAS Arthur Rickwood

Signature ..... Date .....

Dr T Pettitt
Plant Pathologist
HRI Wellesbourne

Signature ..... Date .....

Professor J Whipps
Plant Pathologist
HRI Wellesbourne

Signature ..... Date .....

Report authorised by:

Dr M Heath
Team Manager
ADAS Arthur Rickwood

Signature ..... Date .....

Professor P Mills
Head of Department
HRI Wellesbourne

Signature ..... Date .....

CONTENTS

PRACTICAL SECTION FOR GROWERS

Commercial benefits of the project

None at present. The commercial objective is to reduce the need for applying chemical fungicides to tomato crops by identifying biocontrol products, and seeking micro-organisms from amongst those naturally resident on tomato plants, with activity against *Botrytis cinerea*, the cause of grey mould. Industrial partners to commercialise efficacious micro-organisms will be sought.

Background and objectives

With increasing concern from retailers and consumer groups at the continued use of pesticides on food crops, several leading tomato growers have set in place a long-term objective of zero pesticide use. This has not presented a major problem for continued effective pest control; indeed UK tomato growers currently lead the world in achieving successful IPM strategies with nil or minimal use of insecticides. However, with disease control, fungicides remain a key component of effective disease management strategies, and grey mould is a principal target of these fungicide treatments.

Grey mould, caused by the fungus *Botrytis cinerea*, has been an economically important disease of the tomato crop for many years. Previously the pathogen caused extensive quality losses due to 'ghost spotting' on the fruit. However, in recent years, ghost-spot has not been a significant problem (due primarily to improved environmental conditions in the glasshouse) but stem *Botrytis* has become more problematic. Instead of the disease affecting fruit quality, it now causes a significant yield reduction due to plant losses from girdling stem lesions. Most growers now incur considerable labour costs in an effort to minimise losses due to the disease.

Fungicides e.g. benomyl and iprodione, have been used intensively in the past to improve control, though in recent years their efficacy has been compromised by the development of resistant and/or less sensitive strains of *B. cinerea* in the pathogen population. In the short-term, novel fungicides (e.g. pyrimethanil (Scala) and azoxystrobin (Amistar)), appear to be alleviating the *Botrytis* problem. However, in the longer-term, the aim is to minimise and, hopefully, eliminate pesticide use and therefore alternative strategies must be sought.

One alternative strategy is biological control. There are already a few antagonists with claimed activity against *B. cinerea* either available commercially or in the process of commercial development (e.g. Aspire, Mycostop and Trichodex) though unfortunately none are registered for use in the UK. It is also recognised in the literature that micro-organisms antagonistic to Botrytis occur naturally on the leaf and stem surfaces of various hosts (Church, 1992; Cohen *et al*., 1996; Cook *et al*., 1996; Elad *et al*., 1994; Hausbeck & Pennypacker, 1991; Schmidt *et al*., 1996, Sutton & Peng, 1993; Walter *et al*., 1996; Whipps & McQuilken, 1993). It is possible that the widespread use of broad-spectrum fungicides on the tomato crop and disinfectants during end of season clean-up procedures may have reduced or eliminated many of the naturally occurring antagonists thereby exacerbating disease severity. The full potential of naturally-occurring antagonists in UK tomatoes has not been investigated

in-depth previously, though studies on tomatoes at INRA, France (Nicot *et al*., 1996) and in Israel (O'Neill *et al*., 1996) suggests it is an avenue of research worthy of further investigation.

The overall objective is to develop a sustainable strategy that integrates effectively with IPM practices. We need to generate a broader understanding of the interactions between *B. cinerea* and naturally occurring antagonists on the leaf/stem/truss surfaces and, where possible, harness those most effective for commercial exploitation. Such ecologically based strategies have been successful in developing several commercially available bio-control agents for other host-pathogen systems (Whipps, 1997).

The aims of the project are:

(i) to identify existing biological control agents with efficacy against *B. cinerea;*
(ii) to determine their efficacy in relation to currently approved fungicides;
(iii) to investigate potential novel sources of biological control agents on nurseries where *Botrytis* stem rot does not appear to be problematic in tomatoes;
(iv) to evaluate candidate organisms for their efficacy against *B. cinerea* compared with existing biocontrol agents and fungicides.

Work completed in previous years (2000)

- Eighteen bio-control products and isolates (BCAs) comprising 8 fungal and 10 bacterial, were collected from commercial companies and research organisations to form a project reference collection.

- Attendance at the XIIth International Botrytis Symposium, Reims, France allowed contact to be made with other researchers working on Botrytis and biological control of diseases and to provide an update on current research world-wide in these fields.

- A range of five isolates of *Botrytis cinerea* were isolated from commercial tomato crops and maintained in the laboratory for use in the BCAs screens.

- All the BCAs in the reference collection were successfully screened *in vitro* against a range of *B. cinerea* isolates. This was carried out by observing the growth of *B. cinerea* in the presence of individual BCAs in Petri dishes in the laboratory. Some of the BCAs exhibited large zones of inhibition of the growth of *B. cinerea.* The BCAs screened were ranked on their performance.

- Leaf and stem bioassays were devised and used in the laboratory to see which would allow the most effective screening of the BCAs on tomato plant tissue. It was decided that the stem bioassay, using conidial inocula, allowed the most realistic and reproducible conditions similar to infection development and pressure under commercial growing conditions.

Work plan for 2001/2002

To complete dual culture and tomato stem piece screening of biocontrol products and of micro-organisms isolated from tomato plants and thereby draw up a short-list of materials for whole plant studies. To devise a reliable procedure for generating aggressive botrytis stem lesions on tomato plants.

Summary of results and conclusions

- Twenty-one biocontrol products or isolates with reported antagonism against *B. cinerea* have now been obtained for use in subsequent experiments. Agreements for use of these products have been made with the suppliers, where required.

- At present, three of these products are sold in the UK. They are marketed as biological growth promoters rather than as bio-fungicides because of difficulties in registration of bio-fungicides.

- A total of 106 morphologically distinct micro-organisms were collected from UK tomato crops, representing different sites, sampling times and plant tissues. Leaves generally yielded a wider range of species than stems or fruit trusses. The isolates comprised fungi, bacteria and yeasts. Isolates were cleaned and cultures put into long-term storage for future use in this project.

- A preliminary identification of isolates is as follows:

Acremonium type	11
Aspergillus	1
Bacteria/yeasts	21
Botryosporium longibrachiatum	1
Cladosporium	3
Trichoderma	1
Sclerotinia	1
Unknown	18
Poor growth	24

- A stem piece bioassay was developed which, although not perfect gives reasonably consistent development of botrytis stem rot. Replicate tomato stem pieces (3 cm long) are inserted in moist, autoclaved vermiculite, the upper end is wounded by gently crushing, and the damaged stem ends inoculated with the candidate biocontrol agent (100 µl) and then with 10^4 primed *B. cinerea* conidia (20 µl of 5 x 10^5 spores/ml). Pots of inoculated stem pieces are incubated at 15^0 C, 80% RH and low light intensity (16 h day/ 8 h night) for around 7 days and then assessed for extent of stem rotting and degree of botrytis sporulation.

- The above method was used to assess the biocontrol potential of 21 products or isolates with known antagonistic activity against *B. cinerea*. Five were demonstrated to have the ability to reduce botrytis rot in tomato stem pieces. These were:
  Gliomix (fungal product)
  Clonostachys roseum (fungal isolate)

Stimagro (Streptomycete product)
QRD 131 (bacterial product)
Yield Plus (yeast product)

- Some products (e.g. MBI 600, Trichodex) which are registered and used for control of botrytis in tomato crops in other countries were not effective in the stem piece bioassay. This may be due to differences in application rates, or the relatively severe challenge of our assay, designed to select products/isolates most likely to be of practical benefit to UK tomato growers.

- A method was devised for generating aggressive botrytis lesions on tomato stems. It involves inoculating fresh de-leafing wounds with primed conidia of *B. cinerea* and using tomato plants at least 5 months old. This method will be used as a basis for screening candidate biocontrol products and isolates for activity in reducing botrytis stem rot in 2002.

Action points for growers

None at present. A number of commercial products and isolates have been obtained and screened. However, further screening work is required to fully assess the efficacy and suitability of these biocontrol agents for controlling Botrytis in tomato crops.

Anticipated practical and financial benefits

Losses due to *Botrytis* have been estimated to be in the region of 5-10% per annum in recent years. With the farm gate value of the UK tomato industry at around £130M, losses as low as 1-2% are highly significant. Individual companies have reported losses due to botrytis ranging from £50,000 to £350,000 in a single season. Therefore, any measures, which can be taken to reduce botrytis, are likely to have a significant financial benefit both in terms of reducing plant losses and in minimising additional labour costs to the business.

This work should generate a better understanding of the microbial diversity in the tomato crop and, hopefully, generate an increased awareness of the potential for microbial antagonism against *B. cinerea*. In addition, this work could potentially lead to new bio-control products effective against *B. cinerea*, although, further studies to support registration would be necessary before any novel bio-control products could be developed commercially. The potential UK market for any bio-control product is relatively small, assuming any bio-control products generated are specific to *B. cinerea* in tomato. The EU market on tomatoes however is significantly larger. Also, any effective antagonists identified could have a much broader appeal on other crops where the market size is much greater in comparison e.g. vines.

If effective control of this disease with zero or minimal use of fungicides could be achieved there would be a considerable impact on the industry. Apart from the immediate benefit of favourable consumer reaction to reduced fungicide input, pest control using IPM strategies may be improved and labour costs from managing the disease significantly reduced. There would also be a perceived environmental benefit.

SCIENCE SECTION

Introduction

The work in the first year of the project focused on the collection of known microbial antagonists towards *Botrytis cinerea*, either commercialised bio-control products or antagonistic isolates (BCAs) from around the world. From this reference collection, a series of laboratory screens were carried out to examine the efficacy of the BCAs to affect the growth of *B. cinerea in vitro* during agar plate challenge inoculation tests. In addition to this work, laboratory bioassays for measuring the efficacy of these BCAs was developed using 'live' tomato plant tissue.

The main objectives for the second year of the project were:

1. To complete the project reference collection of commercial products and isolates with known activity against *B. cinerea* (June 2001).
2. To collect potential antagonists from four commercial tomato crops in the UK (November 2001).
3. To complete dual culture tests of potential antagonists against *B. cinerea* (March 2002).
4. To undertake presumptive identification of potential antagonists and store isolates in liquid nitrogen (fungi) or glycerol (bacteria) (March 2002).
5. To complete development of a bioassay to screen micro-organisms (June 2001).
6. To screen reference collection products and isolates in the bioassay (September 2001).
7. To screen tomato crop isolates in the bioassay (June 2002).
8. To develop a protocol for screening products/isolates in comparison with fungicides on tomato plants (September 2001).
9. To screen products and isolates in comparison with fungicides on tomato plants (June 2002).
10. To produce summary interim reports for the project consortium members every quarter and a draft annual report (March 2002).

Milestones (revised schedule as agreed July 2001)

Objective	Achievement Indicators	Target Date
1.1	Reference collection of commercial or reported antagonists secured	August 2000
1.2	Preparation of short summary report	September 2000
1.3	Further isolates sourced and secured	June 2001
2.0	Isolates with potential antagonism to *B. cinerea* collected	October 2001
3.1	All sourced isolates/products screened in dual culture plates	June 2001
3.2	All isolates (from tomato crops) screened in dual culture plates & short-listed	December 2001
4.0	Short-listed isolates identified (presumptive) and lodged in collection	April 2001
5.0	Laboratory bioassay to screen candidate micro-organisms developed	June 2001
6.1	Screen isolates/products in laboratory bioassay	September 2001
6.2	Screen micro-organisms (isolated from tomato crop) in laboratory bioassay	May 2002
7.0	Identify most promising isolates in bioassay (confirmatory)	September 2002
8.1	*In vivo* screening of products in comparison with fungicides completed	September 2001
8.2	*In vivo* screening of isolates (from tomato crops) in comparison with fungicides completed	June 2002
8.3	Small scale glasshouse trials	December 2002
9.0	Assessment of environmental factors on the efficacy of antagonists	September 2003
10.0	Evaluation of antagonists for commercial production	September 2003
11.1	Performance of most effective antagonists determined under commercial conditions in long-season tomato crop	December 2003
11.2	Performance of most effective antagonists on a commercial nursery site	December 2003
12.1	Preparation of Annual Report, year 1	March 2001
12.2	Preparation of Annual Report, year 2	March 2002
12.3	Preparation of Annual Report, year 3	March 2003
12.4	Preparation of Final Report, summarising 4 years work	March 2004
13.0	Reporting developments in biocontrol techniques from the XIIth International Botrytis Symposium as summary report in tandem with Objective 1.2	September 2000

1. Sourcing biocontrol products and isolates with antagonism to *Botrytis cinerea* (milestones 1.3 and 3.1)

Study Director: Dr K R Green
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Site Manager: Ms A Shepherd
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Status of work: Completed

Period covered: May - October 2001

Introduction

The objective of this study was to secure a reference collection of commercial and reported antagonists of *Botrytis cinerea,* for use in subsequent experiments on their efficacy for control of grey mould on tomatoes, in comparison with currently approved fungicides.

Approach

Existing knowledge and personal contacts were used to secure reference isolates and products. In addition, useful information was obtained from a comprehensive web page describing commercial biocontrol products (http://www.barc.usda.gov).

Results

ADAS and HRI Stockbridge House obtained 18 products prior to March 2001. Two yeast products have subsequently been obtained by ADAS. In addition, a liquid formulation of *Bacillus subtilis* (QRD 131) has now been obtained as well as the powder formulation previously obtained (QRD 713). These products are commercially available in the USA as Serenade.

At a progress meeting in July 2001, it was decided that there was no need to seek further products. New products may be considered for testing if it is felt that they are substantially different from current isolates/products or offer particular promise.

In order to obtain certain products, experimentation agreements were developed and signed in consultation with an ADAS legal adviser and collaborators at HRI Stockbridge House (prior to March 2001). Satisfactory agreements are now in place, or close to being finalised, for all products where requested (see Appendix 1).

To fulfil requests from BCA suppliers, progress reports indicating the performance of individual BCAs against *B. cinerea* isolates in agar challenge plate tests and how they ranked in relation to other BCAs (coded), were sent in March 2001 to Mr T. Ricard (BINAB), Dr R. Finlay, Fargro (Gliomix and Stimagro), Dr P. Fiddaman, MicroBio (MBI 600) and Dr L. Grant, EcoScience Corp. (BioSave products).

Isolate sub-cultures and product samples are being maintained at ADAS Arthur

Rickwood and will also eventually be stored at HRI Wellesbourne. Details of the 21 products/isolates obtained are given in Table 1.1.

Industry implications

Yeasts

Reports from the XIIth International Botrytis Symposium (2000) indicate that yeasts show potential as BCAs against Botrytis as they are less fastidious for nutrients, do not produce toxins, can be grown easily on media, are usually unaffected by fungicides and are tolerant of a wide temperature range. For example, Anchor Bio-Technologies in South Africa report promising results using YieldPlus (*Cryptococcus albidus*) to protect flower bulbs against *Botrytis* spp. While, the yeast product obtained from Israel (code: A10) has been used in field tests for pre- and post-harvest diseases of strawberry and grapes.

Registration of biological control agents

At present, only three of the 21 products/isolates obtained are registered for use in the UK. These are registered as biological growth promoters rather than as bio-fungicides because of difficulties in registration. EU directive EC/91/414 (regulating pesticides and, through its Annex 1, biological control agents) is going to be superseded by, or combined with Directive EC/36/201 entitled 'Marketing of phytochemicals'. In it there will be a new Annex for microbial biocontrol agents in detail.

The new directive will need information on:

- Materials for production and formulation
- Standardisation of marketable products
- Details of the identity of organisms (to pathovar level or below)
- Cost benefit analysis (efficacy data needed)
- Impact analysis – health, consumers and the environment (the latter is relatively new compared with 91/414).

The best information on current activities on directive EC 91/414 is in the PSD web site: http://www.pesticides.gov.uk/ec_process/ec_home.htm

Currently, the 'grey' area of plant growth promoters and strengtheners still remains. These materials do not require registration under EC/91/414 if they do not make claims for biological control activity.

Future prospects

The products/isolates obtained have been used in agar challenge tests (see Annual Project Report, March 2001) and tomato stem bioassays. The most effective ones will now be tested by *in vivo* tests at HRI Efford.

Table 1.1. Obtained isolates and products with reported antagonism to *B. cinerea*

Code	Product name	Micro-organism	Source/company	Experimentation agreement	Cfu/g product
H1	-	*Clonostachys roseum*	University of Guelph	No	-
H2	Bio-Save 10LP	*Pseudomonas syringae* 10LP	EcoScience Corp.	No, info. requested on product performance	-
H3	Bio-Save ESC 11	*Pseudomonas syringae* ESC11			-
H4	Biomex SA	*Trichoderma* spp.	Omex Agriculture	No	-
H5	Trichodex	*Trichoderma harzianum*	Mahkteshim Chemical Works Ltd, Israel	No	min. 10^{10} cfu/g product
H6 H7	- -	* *	* *	Agreement to be finalised	- -
H8	-	*	*	Yes	-
H9	QRD 713 (powder)	*Bacillus subtilis*	Commercially available in USA as Serenade.	No. Info. requested on product performance	min. 10^{9} cfu/g product
H10	QRD 131 (liquid)	*Bacillus subtilis*			min. 10^{9} cfu/ml product?
A1	Stimagro	*Streptomyces griseovirides*	Fargo, UK	Info. requested on product performance	min 10^{8} cfu/g product
A2	Gliomix	*Gliocladium catenulatum*			min. 10^{7} cfu/g product
A3	MBI 600	*Bacillus subtilis*	MicroBio Ltd, UK	Yes	min. 10^{11} cfu/g product
A4	*	*Trichoderma harzianum*	*	Email agreement, not signed.	10^{8} cfu/g product
A5 A6	- -	*Bacillus pumilis* 13374 *Pseudomonas fluorescens* 13373	NCIMB, Aberdeen (Swadling & Jeffries, 1996, 1998)	No	- -
A7 A8	- -	*Bacillus subtilis* 39 *Bacillus subtilis* 83	Dr S. Rossall Univ. Nottingham		- -
A9	*	*Trichoderma polysporum*, *T. harzianum*	*	Yes	min. 10^{5} cfu/g product
A10	Yeast ex Minrav AgroGreen	Not provided	Minrav AgroGreen, Israel	Info. requested on product performance	min 3 x 10^{10} cfu/g product
A11	YieldPlus	*Cryptococcus albidus*	Anchor BioTechnologies, S. Africa	Info. requested on product performance	1.5 g/l gives 10^{8} cfu/ml

*****Information witheld due to confidentiality agreements

2. Collection of micro-organisms from commercial tomato crops to evaluate for antagonistic activity against *Botrytis cinerea* (milestones 2.0 and 3.2)

Study Director: Dr K R Green
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Site Manager: Ms A Shepherd
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Status of work: Completed

Period covered: January - November 2001

Introduction

Products and isolates reported to be antagonistic against *B. cinerea* have been collected as part of this project. However, it is also recognised in the literature that antagonists to *Botrytis* occur naturally on the leaf and stem surfaces of various hosts. The aim of this work was to isolate, select and store micro-organisms from tomato plant samples (leaves, stems and trusses) collected from four commercial tomato crops at approximately two-monthly intervals during the growing season (3 sampling times). Selected isolates will subsequently be evaluated against *B. cinerea* using agar plate tests, a stem bioassay and *in vivo* studies using small tomato plants.

Materials and methods

Four commercial tomato crops (two in the North of England, two in the South) were selected for collection of potential micro-organisms. One crop of the two selected in each region received routine pesticide applications, while the other was a crop was organically grown. HRI Stockbridge House was responsible for the northern sites at the first sampling time and thereafter ADAS Arthur Rickwood was responsible for sampling at all sites.

At approximately two monthly intervals commencing from January 2001, the sites were visited on three occasions and samples were collected. The samples comprised younger leaves, older leaves (from near the base), spent fruit trusses and slivers from stem pruning wounds, taken randomly from within the crop. At the first site visit the plants were immature and only leaf and stem samples were available. However, as the crop matured there was opportunity to sample from pruning wounds and trusses. Twenty samples in total were collected at each site visit (e.g. five samples each of leaves, slivers etc). Sampling took place no less than 7 days after the last pesticide application.

Tomato plant samples collected were individually placed in paper bags or wrapped in newspaper and then plastic to minimise ‘sweating ‘ of the leaf material and then returned as soon as possible to the laboratory for isolation of micro-organisms from their surfaces.

At each site, crop records were obtained to include information on:

- variety
- the cropping system
- applied fungicides, insecticides and wetting agents and
- date of these applications.

Each of the 20 leaf, stem, pruning wound or fruit truss samples were placed in a conical flask containing 100 ml of 5 % dilute phosphate buffered saline (see Appendix 2.1) using one flask per sample. Samples were left intact or roughly chopped if they did not readily fit inside the flask. The flasks were shaken for 30 min. A serial dilution using this 100 ml suspension was performed with sterile distilled water, up to a 10^{-2} dilution with 100 µl aliquots of the suspension being spread onto the agar following vortexing. Dilutions were plated onto 2 different media to encourage the isolation of fungi, yeasts and bacteria with 3 replicate plates per dilution. The media were nutrient dextrose agar (bacteria and yeasts) and potato dextrose agar with antibiotics (total fungal). The recipes for these two media are given in Appendix 2.1.

All morphologically distinct colonies were sub-cultured and assigned numbers. Records of all isolates were made as follows: isolate code, site code, sample type (leaf, stem etc), sampling date and micro-organism type. Once pure cultures had been obtained, isolates were maintained until after the third sampling time. Duplicate isolates obtained from different sites/sampling times were discarded in addition to isolates of *B. cinerea* and fast-growing saprophytes such as *Mucor* spp. Sub-cultures of the remaining isolates were sent to Prof J Whipps (HRI Wellesbourne) for preliminary selection of 50 promising isolates for subsequent bioassays.

Isolates obtained from Site 3 at the first sampling time were tested for their antagonistic potential against five isolates of *B. cinerea* from tomato stems, in agar plate tests at HRI Stockbridge House. The protocol used for these agar plate tests and details of *B. cinerea* isolates are given in the first annual report (March 2001).

Results and discussion

Crop and sampling details are shown in Table 2.1. Each nursery grew a different cultivar and used a different cropping system. By the end of the growing season, botrytis levels were severe at Sites 1 and 2 (south), while only a trace of the disease was observed at Sites 3 and 4 (north).

Table 2.1. Site, crop and sampling details for collection of potential antagonists from commercial tomato nurseries.

Crop details	Site 1	Site 2	Site 3	Site 4
Region	South	South	North	North
System	Organic	Conventional	Organic	Conventional
Cultivar	Benefit	Elegance	Carousel	Espero
Planting date	24.01.01	24.01.01	01.02.01	1st week Jan, 2001
Planting system	Open soil	Rockwool slabs, ground polythene covered	Soil-grown with straw on central pathways	Perlite
Sampling dates	14.03.01 22.05.01 14.08.01	14.03.01 22.05.01 14.08.01	January 2001 22.05.01 15.08.01	January 2001 23.05.01 16.08.01
Chemical applications	None	Savona 07.03.01 20 g/l Thiovit 06.04.01 2 g/l Savona 23.04.01 20 g/l Elvaron 11.05.01 1 g/l Rovral 31.05.01 1 g/l Thiovit 11.06.01 2 g/l Scala 13.07.01 1 ml/l Thiovit 03.08.01 2 g/l Scala 09.08.01 1 ml/l	None	Scala 26.03.01 Torq 24.04.01 Thiovit 05.06.01 Thiovit 18.07.01 Dynamec 20.07.01
Botrytis severity	Severe at 3rd sampling	Severe at 3rd sampling	Trace at 3rd sampling	Trace at 3rd sampling

At the first sampling time, samples were collected from leaves (young and old) and stem pieces at Sites 1 and 2, and leaves only (young, medium and old) at Sites 3 and 4. At the later sampling times, samples were collected from leaves (young and old), stem pruning wounds and spent fruit trusses at each site.

At each sampling time, fungi, bacteria and yeasts grew on both media types, despite the use of nutrient dextrose agar intended for selective isolation of bacteria and yeasts, and total fungal agar. The high levels of antibiotic amendments in the total fungal agar inhibited fungal as well as bacterial growth, such that colonies were much smaller and slower to develop than on nutrient dextrose agar. The latter was found to be more useful for selecting morphologically distinct colonies.

At the first sampling time (southern sites), samples from Site 1 (organic) gave a higher number of morphologically distinct isolates compared with Site 2 (Table 2). In addition, there were more colonies per plate, indicating higher densities of micro-organisms on the foliage due possibly to cropping in soil rather than rockwool over polythene. At both sites, there were higher numbers of colony forming units (cfu) and more species isolated from leaves than stems; the number of isolates from young and old leaves was similar. The majority of isolates from leaves were fungal while bacteria and yeasts predominated on stems. No isolates of *B. cinerea* were obtained. *Penicillium* spp. were abundant.

Table 2.2. Number of morphologically distinct isolates obtained from two commercial tomato nurseries in March 2001

Site	Fungi	Bacteria	Yeasts
1	24	12	1
2	12	8	2

At the second sampling time (southern and northern sites), there was less of a difference in the number of micro-organisms isolated from organic and conventional sites than for the first sampling time (data not shown). The range of organisms isolated from younger leaves was narrower than for older leaves. Otherwise, the trends observed at the first sampling time were repeated.

At the third sampling time (southern and northern sites), a higher number of morphologically distinct colonies were isolated than on previous occasions (Table 2.3). There was greater similarity in the number of fungi and bacteria/yeasts isolated than previously. In addition, similar numbers of species were obtained from the different plant tissues. *B. cinerea* was isolated from all sites.

Table 2.3. Number of morphologically distinct isolates obtained from four commercial tomato nurseries in August 2001

Site	Young leaf		Old leaf		Stem wound		Fruit truss	
	B/yeast[a]	Fungi	B/yeast	Fungi	B/yeast	Fungi	B/yeast	Fungi
1	10	9	11	15	4	5	8	12
2	8	15	6	17	6	5	6	17
3	11	9	8	9	8	10	8	7
4	9	11	10	14	8	8	4	10

[a]Bacteria (B) and yeast were not examined microscopically to distinguish them until later sub-culturing

In agar plate tests, 26 micro-organisms from Site 3 (first sampling time) were tested against each of four isolates of *B. cinerea*, while a sub-set of 17 were tested against a further one isolate of *B. cinerea*. The collection of micro-organisms finally sent to HRI Wellesbourne included seven isolates from Site 3 (one bacteria, one yeast and five fungi). Results from the agar plate tests showed that the yeast isolate (XHAPP 01/17, ex medium-age leaf) gave noticeable zones of inhibition and also inhibited radial growth of 3 out of 4 isolates of *B. cinerea*. In addition, one of the fungal isolates (XHAPP 01/11, ex old leaf) showed inhibition against 3 out of 5 isolates.

A total of 106 isolates were sent to HRI Wellesbourne including fungi, bacteria, yeasts and Streptomycetes, representing isolations from different sites, sampling times and plant tissues. The majority of isolates sent were fungi because these were more frequently isolated from plant tissue. In addition, there were difficulties in maintaining and sub-culturing several of the bacterial and yeast species that were originally isolated. Details of the isolates selected for use in subsequent bioassays are given in Table 2.4.

Conclusions

The techniques used enabled isolation of a wide range of micro-organisms from tomato foliage at each sampling time. A higher number of fungal species were isolated than bacteria and yeasts. Leaves generally yielded a wider range of species than stem wounds or fruit trusses. An organic nursery (Site 1) provided a greater diversity of species than a conventional nursery (Site 2) at the first sampling time but this difference was not observed at subsequent sampling times.

After discarding isolates due to duplication, contamination or poor ability to grow, 106 morphologically distinct micro-organisms were sent to HRI Wellesbourne, for selection of 50 isolates to use in subsequent bioassays. Two of the isolates sent to HRI (one fungus and one yeast) showed good levels of antagonism against *B. cinerea* in agar plate challenge tests and it is recommended that these should be included in subsequent bioassays if preliminary identification suggests that they are appropriate.

Table 2.4. Isolates selected for bioassay tests

Number	Isolation code	Preliminary identification	Isolated from: Tissue	Cultivar	Site
1.	XHAPP 01/3	1.1 c/1	Young leaf	cv. Benefit	Site 1
2.	XHAPP 01/5	1.5 a/1	Young leaf	cv. Benefit	Site 1
3.	XHAPP 01/7	2.4 c/1	Young leaf	cv. Elegance	Site 2
4.	XHAPP 01/8	2.20 c/1	Stem piece	cv. Elegance	Site 2
5.	XHAPP 01/9	B 0.1 **1**	Old leaf	cv. Carousel	Site 3
6.	XHAPP 01/14	RRM 2/4	Medium aged leaf	cv. Espero	Site 4
7.	XHAPP 01/15	B 0.1/5	Old leaf	cv. Carousel	Site 3
8.	XHAPP 01/17	RRM 1/5	Medium aged leaf	cv. Espero	Site 4
9.	XHAPP 01/19	1.7 c/2	Young leaf	cv. Benefit	Site 1
10.	XHAPP 01/22	1.18 c/2	Fruit truss	cv. Benefit	Site 1
11.	XHAPP 01/25	2.11 c/2	Stem wound	cv. Elegance	Site 2
12.	XHAPP 01/27	2.17 b/2	Fruit truss	cv. Elegance	Site 2
13.	XHAPP 01/28	3.6 c/2	Young leaf	cv. Carousel	Site 3
14.	XHAPP 01/29	3.9 a/2	Young leaf	cv. Carousel	Site 3
15.	XHAPP 01/31	3.15 a/2	Stem wound	cv. Carousel	Site 3
16.	XHAPP 01/36	4.6 a/2	Young leaf	cv. Espero	Site 4
17.	XHAPP 01/37	4.7 a/2	Young leaf	cv. Espero	Site 4
18.	XHAPP 01/40	1.1 a/3	Old leaf	cv. Benefit	Site 1
19.	XHAPP 01/41	1.1 a/3	Old leaf	cv. Benefit	Site 1
20.	XHAPP 01/42	1.1 b/3	Old leaf	cv. Benefit	Site 1
21.	XHAPP 01/45	1.2 b/3	Old leaf	cv. Benefit	Site 1
22.	XHAPP 01/46	1.2 c/3	Old leaf	cv. Benefit	Site 1
23.	XHAPP 01/47	1.2 c/3	Old leaf	cv. Benefit	Site 1
24.	XHAPP 01/48	1.3 b/3	Old leaf	cv. Benefit	Site 1
25.	XHAPP 01/49	1.5 b/3	Old leaf	cv. Benefit	Site 1
26.	XHAPP 01/52	1.6 b/3	Young leaf	cv. Benefit	Site 1
27.	XHAPP 01/53	1.9 a/3	Young leaf	cv. Benefit	Site 1
28.	XHAPP 01/56	1.11 b/3	Stem wound	cv. Benefit	Site 1
29.	XHAPP 01/57	1.11 c/3	Stem wound	cv. Benefit	Site 1
30.	XHAPP 01/60	1.18 b/3	Fruit truss	cv. Benefit	Site 1
31.	XHAPP 01/62	2.2 c/3	Old leaf	cv. Elegance	Site 2
32.	XHAPP 01/63	2.2 c/3	Old leaf	cv. Elegance	Site 2
33.	XHAPP 01/64	2.3 c/3	Old leaf	cv. Elegance	Site 2
34.	XHAPP 01/65	2.4 b/A3	Old leaf	cv. Elegance	Site 2
35.	XHAPP 01/66	2.4 b/B3	Old leaf	cv. Elegance	Site 2
36.	XHAPP 01/71	2.14 a/3	Stem wound	cv. Elegance	Site 2
37.	XHAPP 01/72	2.14 a/3	Stem wound	cv. Elegance	Site 2
38.	XHAPP 01/75	2.19 a/3	Fruit truss	cv. Elegance	Site 2
39.	XHAPP 01/77	2.20 c/3	Fruit truss	cv. Elegance	Site 2
40.	XHAPP 01/81	3.4 c/3	Young leaf	cv. Carousel	Site 3
41.	XHAPP 01/82	3.6 b/3	Old leaf	cv. Carousel	Site 3
42.	XHAPP 01/83	3.9 a/3	Old leaf	cv. Carousel	Site 3
43.	XHAPP 01/84	3.11 b/3	Stem wound	cv. Carousel	Site 3
44.	XHAPP 01/85	3.12 b/3	Stem wound	cv. Carousel	Site 3
45.	XHAPP 01/87	3.17 c/3	Fruit truss	cv. Carousel	Site 3
46.	XHAPP 01/90	4.1 b/3	Young leaf	cv. Espero	Site 4
47.	XHAPP 01/93	4.4 a/3	Young leaf	cv. Espero	Site 4
48.	XHAPP 01/94	4.4 c/3	Young leaf	cv. Espero	Site 4
49.	XHAPP 01/96	4.7 c/3	Old leaf	cv. Espero	Site 4
50.	XHAPP 01/97	4.8 b/3	Old leaf	cv. Espero	Site 4
51.	XHAPP 01/98	4.8 c/3	Old leaf	cv. Espero	Site 4
52.	XHAPP 01/104	4.16 a/3	Fruit truss	cv. Espero	Site 4
53.	XHAPP 01/106	1.7 c/3	Young leaf	cv. Benefit	Site 1

Appendix 2.1

Phosphate Buffered Saline – Stock Solution

For 1 litre Stock

80g NaCl
2g KH_2PO_4
14.4g $Na_2HPO_4.2H_2O$
2g KCl

Adjust to pH 7.2

Dilute 1:10 with sterile de-ionised water for normal use.
In this protocol use a 5% solution of this diluted stock for washing plant parts.

MATERIALS AND METHODS FOR PREPARING SELECTIVE MEDIA

Nutrient Dextrose Agar (NDA)

1L H_2O
28g Nutrient Agar (Oxoid)
10g D-Glucose

Autoclave at 121ºC for 15mins

Incubate Nutrient Dextrose Agar for 5-7 days at 25°C.

Selective Media for Total Fungi

Basal media = ¼ Potato Dextrose Agar

For 300ml media –
3g PDA
3.6g Technical Agar
3ml Streptomycin sulphate Stock
3ml Tetracycline hydrochlorate stock
0.6ml Triton X-100

Stock solutions:
Streptomycin sulphate = 0.2g/20ml SDW – Use 1ml/100ml basal media.
Tetracycline hydrochlorate = 0.02g/20ml SDW – Use 1ml.100ml basal media.
Incubate total fungal plates (¼ PDA) at 20°C for 7 days

3. The development of a bioassay to measure the potential of microbial antagonists (milestone 5.0)

Study Director: Dr K R Green
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Site Manager: Ms A Shepherd
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Status of work: Completed

Period covered: April - September 2001

Introduction

Experiments were conducted during Year 1 to develop an appropriate bioassay to quantify the potential of microbial antagonists (BCAs) against *B. cinerea*. Recommendations and conclusions from these preliminary studies were as follows:

- Use stem pieces rather than leaves or small whole plants for initial bioassay work.
- The addition of nutrients (glucose and potassium dihydrogen phosphate) to a spore suspension of *B. cinerea* increases the incidence of stem infection.
- A spore suspension of *B. cinerea* amended with nutrients should be prepared 3 h prior to use, so that spores are primed but remain ungerminated at the time of inoculation.
- Incubate the stems at a low VPD after inoculation.
- Determine whether stem wounding increases susceptibility to infection; this may enable the time between BCA application and inoculation with *B. cinerea* to be extended and reduce the requirement for nutrient amendments to the spore suspension.

Two experiments undertaken to finalise the stem bioassay method are described.

Materials and methods

Experiment 1: To determine the effect of stem-piece damage on the period of time taken to develop wound resistance to infection by *B. cinerea* from conidial inocula.
Tomato stem pieces to be inoculated with *B. cinerea* were prepared either by cutting with a sharp knife, or by cutting followed by stem-end bruising. For each type of stem piece preparation, the pieces were inoculated with *B. cinerea* either immediately, after 24 h or after 3 days. For each treatment there were four replicates of five stems in a randomised block design.

Stem pieces of 3 cm in length were cut from side shoots of glasshouse-grown tomato plants (cv. Espero). The top 10 cm of stem were discarded. The stem pieces were rinsed in sterile distilled water and wrapped in moist tissue paper until required, ensuring that the upper stem- end could be later identified. For half of the stem pieces, the upper end was damaged by crushing gently using a pair of pliers. Plant

pots (9-cm-diameter) were filled with autoclaved vermiculite, moistened with distilled water. Five stem pieces were vertically inserted into each pot, representing a plot.

A spore suspension (5 x 10^5 spores/ml) of *B. cinerea* was prepared 3 h before it was required for each inoculation. A sporulating culture of *B. cinerea* isolate BC02, originally collected from stem lesions on tomato, was used. The spore suspension was amended with 0.1 M glucose and 0.07 M potassium dihydrogen phosphate. Each stem end was inoculated with a 20 ul droplet of spore suspension (to give 10^4 spores per stem), either immediately, 24 h, or 3 days after stem piece preparation.

Pots for each replicate were placed on a shallow plastic tray containing water to a depth of 1 cm. The trays were each enclosed in a large polythene bag before transferring to a controlled environment (CE) cabinet for incubation in the dark at 15^{o}C and 80 % RH. The plants were misted twice daily, unless there was excess condensation in the bags, when bags were left unfastened for a few hours. Stems awaiting inoculation were kept under the same conditions as the inoculated stems.

The severity of stem infection was assessed 8 days after inoculation (first two inoculations) and 11 days after inoculation (third inoculation) by measuring the length of stem lesions (mm). A sporulation index for each stem was also recorded:

0 = No sporulation
1 = Sporulation on stem end only
2 = Sporulation on up to 25 % of stem length
3 = Sporulation on 25-50 % stem length
4 = Sporulation on 50-75 % stem length
5 = Sporulation on >75 % stem length

Experiment 2: Evaluation of the stem-piece bioassay to determine the antagonistic potential of four BCAs

For each of the following treatments, there were four replicates of ten stem-pieces in a randomised block design. In addition, ten wounded stem-pieces were monitored as an uninoculated untreated control. Stem pieces were prepared as described for Experiment 1 except that side shoots of tomato cv. Solairo were used. Plant pots (9 cm diameter) were filled with autoclaved vermiculite and ten stem pieces were inserted into each pot with the top ends orientated upwards. The top ends of the stem pieces were wounded by gently crushing with pliers prior to application of BCAs or inoculation with *B. cinerea*.

BCA	BCA code	Formulation of BCA used	Cfu applied per stem (in 100 ul)
Sterile distilled water (control)	-	-	0
MBI 600	A3	0.1 g in 100 ml SDW	10^7
Stimagro	A1	1 g in 10 ml SDW	10^6
-	A4	1 g in 10 ml SDW	10^6
-	H8	1 x 10^7 spores/ml	10^6

A spore suspension of *B. cinerea* was prepared 3 h before required for inoculation, as

described for Experiment 1. For each treatment, a 100 ul droplet of the BCA was placed on each stem end (4 pots of 10 stems). Once the BCA had been absorbed by the stem tissue, the stem end was inoculated with 20 ul of *B. cinerea* spore suspension (10^4 spores per stem).

Pots for each replicate were placed within individual perforated polythene bags (left open), on a plastic tray containing water to a depth of approximately 1 cm. Each tray was completely enclosed in a large polythene bag and transferred to the CE cabinet for incubation at 15^{o}C, 80 % RH (lowered to 60 % RH after 3 days) and a low light intensity (16 h day/ 8 h night). The stems were misted once daily to maintain high relative humidity within the bags, but the bags were left open periodically to prevent accumulation of excess condensation. One pot containing ten stems for the uninoculated check was incubated within an individual polythene bag in the CE cabinet. The experiment was assessed 8 days after inoculation as described in Experiment 1.

Results and discussion

Experiment 1

Stem lesion development occurred for all treatments, although for each inoculation time, mean lesion length and lesion incidence was greater for the wounded stems compared with unwounded stems (Table 3.1). For non-wounded stems mean lesion length was similar (<4 mm) irrespective of inoculation time, while for wounded stems, mean lesion length was considerably higher on stems inoculated immediately, rather than 1 or 3 days later. The incidence of sporulation and mean sporulation index was markedly higher for wounded stems inoculated immediately compared with all of the other treatments.

The results indicate that wounding did increase stem susceptibility to infection by *B. cinerea* and that lesion development still occurred when stem pieces were prepared 3 days prior to inoculation. Stem piece wounding could therefore allow BCAs to be applied up to 3 days prior to inoculation with *B. cinerea* in subsequent bioassays, enabling BCA colonisation to occur on stems before pathogen introduction. Nevertheless, it was considered that the levels of lesion severity recorded when inoculation was delayed were insufficient to allow effective quantification of BCA performance and that bioassays should involve immediate inoculation of wounded stem pieces with nutrient-amended spore suspension of *B. cinerea*.

The relatively low level of sporulation recorded in the experiment was attributed to lack of lighting during incubation in the CE cabinet. It was decided that low levels of lighting would subsequently be used to encourage sporulation without reducing % RH.

Table 3.1. Lesion development and sporulation on tomato stems after wounding treatments and inoculation with *B. cinerea* spore suspension at three time intervals (Experiment 1)

Stem-end wounding	Period before inoculation (h)	Lesion incidence (out of 5)	Mean stem lesion length[a] (mm)	Mean incidence of sporulating stems (out of 5)	Mean sporulation index[a]
No	0	3.3	3.6 (1.32)	0.8	0.2 (0.12)
Yes	0	5.0	24.0 (2.18)	4.0	1.9 (0.25)
No	24	4.5	3.6 (0.75)	0.0	0.0 (0.00)
Yes	24	5.0	9.0 (1.21)	0.3	0.1 (0.10)
No	72	2.3	2.6 (0.81)	0.0	0.0 (0.00)
Yes	72	5.0	6.9 (0.69)	0.0	0.0 (0.00)

[a]Standard errors in parentheses

Experiment 2

Consistent lesion development and sporulation on the control stems (Table 2) confirmed that the inoculation and incubation technique used were appropriate for infection and lesion development to occur. There was also a significant treatment effect with lesion development being reduced by treatment with Stimagro and A4. In addition, *Botrytis* sporulation was reduced by treatment with MBI 600, Stimagro and A4.

Table 3.2. Lesion development and sporulation on tomato stems after inoculation with *B. cinerea* spore suspension and four microbial antagonists (BCAs) (Experiment 2)

BCA	BCA code	Lesion incidence (out of 10)	Mean stem lesion length (mm)	Mean no. sporulating stems (out of 10)	Mean sporulation index
Control (SDW)		10.0	28.4	9.3	4.5
MBI 600	A3	10.0	23.6	5.8	2.3
Stimagro	A1	10.0	12.5	0.3	0.1
-	A4	10.0	20.9	5.3	2.1
-	H8	10.0	28.4	9.5	4.2
SED (12 df)			2.691		0.694
Significance			P<0.001		P<0.001

Conclusions

It was concluded that the method described in Experiment 2 could be used as the basis for subsequent bioassays, given that it enabled consistent stem-piece infection with *B. cinerea* and the ability to differentiate between BCAs according to their antagonistic potential versus *B. cinerea.*

4. Use of a tomato stem bioassay to evaluate micro-organisms for antagonistic activity against *Botrytis cinerea* (milestone 6.1)

Study Director: Dr K R Green
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Site Manager: Ms A Shepherd
ADAS Arthur Rickwood, Mepal, Ely, Cambs. CB6 2BA

Status of work: Completed

Period covered: January - December 2001

Introduction

Twenty-one potential biological control agents (BCAs), including commercially available products and isolates with reported activity against *B. cinerea,* have been collected for use in this project (see section 1). Using a technique developed and evaluated as part of this project, a series of stem bioassays were undertaken to evaluate the potential of the BCAs for control of *B. cinerea* on tomatoes.

Materials and methods

Experimental design
Each bioassay included seven BCA treatments and an untreated control. For each treatment, there were four replicates of ten stem pieces in a randomised block design. In addition, for each bioassay, ten stem-pieces were monitored as an uninoculated untreated check. Two of the BCA treatments, Gliomix (code: A2) and MBI 600 (code: A3), were included in all of the bioassays as 'standards' to help determine whether results obtained from different bioassays were consistent. The two products represented contrasting levels of BCA efficacy with Gliomix giving a significant reduction in lesion development in Bioassay 1 and a preliminary trial, while MBI 600 did not reduce lesion development in comparison with the untreated control.

Treatments (Table 1)
Since BCAs were applied to stem pieces almost immediately prior to inoculation with *B. cinerea,* there was little opportunity for BCA colonisation of stem-pieces. For this reason, BCAs were applied at higher concentrations than *B. cinerea*, in order to simulate prior colonisation of stem tissue. Following advice from J. Whipps (pers. comm) and previous research (Dik *et al*., 1999), the following concentrations were used:

B. cinerea	10^4 spores per stem
Fungal BCAs	10^6 spores per stem
Yeast and actinomycete BCAs	10^7 cfu per stem
Bacterial BCAs	10^8 cfu per stem

Table 4.1. BCA treatments used in four tomato stem bioassays versus *B. cinerea*

Treatment	Code	Min. product concentration (cfu/g product)	Formulation in SDW	Min. cfu applied
Bioassay 1				
Sterile distilled water	SDW	-	-	-
Trichodex	H5	10^{10}	0.1g in 1 l	10^{6}
QRD 713	H9	10^{9}	10g in 100ml	10^{8}
Stimagro	A1	10^{8}	10g in 100ml	10^{7}
Gliomix	A2	10^{7}	10g in100ml	10^{6}
MBI 600	A3	10^{11}	0.1g in 100ml	10^{8}
-	A4	10^{8}	1g in 100ml	10^{6}
Yeast ex Israel	A10	3×10^{10}	0.1g in 300ml	10^{7}
Bioassay 2				
Sterile distilled water	SDW	-	-	-
Clonostachys roseum	H1	10^{6}	-	10^{6}
-	H8	10^{6}	-	10^{6}
QRD 131	H10	10^{9}	10ml in 90ml	10^{8}
Gliomix (standard)	A2	10^{7}	10g in100ml	10^{6}
MBI 600 (standard)	A3	10^{11}	0.1g in 100ml	10^{8}
-	A9[a]	10^{5}	10g in 100ml	?
YieldPlus	A11	10^{8}	0.15g in 1 litre	10^{7}
Bioassay 3				
Sterile distilled water	SDW	-	-	-
Bio-Save 10LP	H2	10^{8}	-	10^{8}
Bio-Save ESC 11	H3	10^{8}	-	10^{8}
Biomex SA	H4	10^{6}	-	10^{6}
-	H6	10^{8}	-	10^{8}
-	H7	10^{8}	-	10^{8}
Gliomix (standard)	A2	10^{7}	10g in 100ml	10^{6}
MBI 600 (standard)	A3	10^{11}	0.1g in 100ml	10^{8}
Bioassay 4				
Sterile distilled water	SDW	-	-	-
Gliomix (standard)	A2	10^{7}	10g in 100ml	10^{6}
MBI 600 (standard)	A3	10^{11}	0.1g in 100ml	10^{8}
Bacillus pumulis	A5	10^{8}	-	10^{8}
Pseudomonas fluoresecens	A6	10^{8}	-	10^{8}
Bacillus subtilis 39	A7	10^{8}	-	10^{8}
Bacillus subtilis 83	A8	10^{8}	-	10^{8}
YieldPlus[b]	A11	10^{8}	1.5g in 1 litre	10^{8}

[a]Because of the low concentration of the product coded A9, the required formulation for application of 1ml could not be prepared, so stem pieces were treated by dipping in a product suspension.

[b]YieldPlus was included in Bioassay 4 in addition to Bioassay 2, using a higher rate closer to the manufacturer's recommended rate.

Preparation of plant material

Stem sections (3 cm length) were cut from side-shoots of tomato cv. Espero, avoiding the top 10 cm of the shoots. 320 stem pieces were required for each bioassay plus ten stem pieces for the uninoculated control. The stem pieces were rinsed in distilled water and wrapped in moist paper towel until required, ensuring that the upper end of the stem pieces could be subsequently identified. Plant pots (9-cm diameter) were filled with autoclaved vermiculite (121^{o}C, 20 min) and wetted with distilled water (70 ml per pot). Ten stem pieces were vertically inserted into each pot with the top ends orientated upwards. Stem pieces were wounded prior to application of BCAs and inoculation with *B. cinerea* by gently crushing the end to be inoculated using a pair of pliers.

Inoculum preparation

A spore suspension (5 x 10^5 spores/ml) of *B. cinerea* was prepared 3 h before it was required for inoculation. Isolate BC02, originally collected from stem lesions on tomato, was used for all bioassays. The spore suspension was amended with 0.1 M glucose and 0.07 M potassium dihydrogen phosphate.

Application of BCAs and *Botrytis* inoculum

For each BCA treatment in a bioassay, 1 ml was applied to each stem end (4 pots of 10 stems). Once the BCA had been absorbed by the stem tissue, each stem end was inoculated with 20 ul of the spore suspension of *B. cinerea* to give 10^4 spores per stem.

Incubation

Pots were placed within individual polythene bags (left open), in plastic trays containing water to a depth of approximately 1 cm. The individual bags were used to minimise cross contamination between treatments. Each tray was completely enclosed in an autoclave bag and transferred to a controlled environment (CE) cabinet for incubation at 15oC and 80% RH, with a 16 h day/8 h night light regime (low light intensity). The stems were misted regularly (e.g. twice daily) to maintain high relative humidity, but the autoclave bags were left open periodically to prevent build-up of excess condensation. One pot containing ten wounded stem pieces for the uninoculated check was incubated in an individual polythene bag in the CE cabinet.

Assessments

The severity of stem infection was assessed 6-8 days after inoculation by measuring the lesion length on each stem. A sporulation index for each stem was recorded as shown below. Data were subjected to analysis of variance.

0=no sporulation
1=sporulation on stem end only
2=sporulation on up to 25% of stem length
3=sporulation on 25-50% of stem length
4=sporulation on 50-75% of stem length
5=sporulation >75% of stem length

Results and discussion

Bioassays 2 and 4 each had to be repeated 2-3 times due to inconsistent results. The types of problems encountered were as follows:

1. The virulence of *B. cinerea* isolate BC02 was apparently reduced, resulting in negligible lesion development even on the untreated inoculated control stems. This probably occurred due to repeated sub-culturing of the isolate. To overcome this problem, the isolate was used to infect healthy stems and was then re-isolated from infected tissues. In addition, single spore isolates were set up to ensure that there was no contamination of cultures.

2. Conditions in the CE cabinet during the incubation period were critical. If the stem pieces were not misted sufficiently in the first 1-2 days following inoculation, lesion development for individual treatments was inconsistent. However, if excess condensation accumulated within the autoclave bags, rapid lesion development possibly accelerated by bacterial rotting occurred on all stems.

3. The use of stem-pieces from the tops of side shoots in one bioassay resulted in negligible lesion development irrespective of treatment, supporting previous reports that this tissue is more resistant to infection by *B. cinerea.*

The results from successful runs of the bioassays are described below:

Bioassay 1 (Table 4.2)
Gliomix in particular, and also Stimagro, reduced lesion development and also sporulation compared with the untreated control. For Gliomix, a white deposit was visible on treated stems which microscopic examination showed to be mycelial development and sporulation of the BCA. There was no lesion development on the uninoculated untreated stem pieces apart from slight blackening due attributed to pliers wounding. This result was consistent for all bioassays.

Bioassay 2 (Table 4.3)
Lesion development was reduced by *Clonostachys roseum* and Gliomix. There was also trend (non-significant) for reduced lesion development with QRD 131. The sporulation index was significantly reduced with *C. roseum* and QRD 131.

Bioassay 3 (Table 4.4)
Lesion development and sporulation was reduced only be Gliomix in this bioassay. None of the other BCA treatments differed from the untreated inoculated control.

Bioassay 4 (Tables 4.5 and 4.6)
Lesion development and sporulation was slight compared with previous successful bioassays. Results should be treated with caution since the Gliomix standard did not reduce lesion development in comparison with the untreated inoculated control. Longitudinal splitting of stem-pieces indicated that lesion development had occurred to a greater extent within stems than on the stem surface. YieldPlus, used at a higher rate than previously in Bioassay 2, was the only BCA to apparently reduce the length

of lesions both on the stem surface and within the stem, compared with the control. Ideally this bioassay needs repeating when stem material is again readily available.

Table 4.2. Lesion development and sporulation on tomato stems after inoculation with *B. cinerea* spore suspension and seven microbial antagonists (BCAs): Bioassay 1

Treatment	Code	Lesion incidence (out of 10)	Mean stem lesion length (mm)	Mean no. sporulating stems (out of 10)	Mean sporulation index
Sterile distilled water	SDW	10	26.4	8.0	2.5
Trichodex	H5	9	24.1	7.5	2.2
QRD 713	H9	9	25.6	8.3	2.4
Stimagro	A1	8	17.2	7.0	1.6
Gliomix (standard)	A2	4	7.0	0.8	0.1
MBI 600 (standard)	A3	9	24.5	8.3	2.3
-	A4	9	23.1	7.8	2.2
Yeast ex Israel	A10	8	21.8	7.5	2.1
SED		1.3	2.88	1.20	0.33
Df		21	21	21	21
Significance		0.010	<0.001	<0.001	<0.001

Table 4.3. Lesion development and sporulation on tomato stems after inoculation with *B. cinerea* spore suspension and seven microbial antagonists (BCAs): Bioassay 2

Treatment	Code	Lesion incidence (out of 10)	Mean stem lesion length (mm)	Mean no. sporulating stems (out of 10)	Mean sporulation index
Sterile distilled water	SDW	10	29.5	9.5	3.8
Clonostachys roseum	H1	10	13.7	4.0	0.5
-	H8	10	27.6	10.0	3.7
QRD 131	H10	10	24.8	6.25	1.9
Gliomix (standard)	A2	10	20.5	8.5	2.2
MBI 600 (standard)	A3	10	27.0	8.8	3.4
-	A9	10	29.5	10.0	3.9
YieldPlus	A11	10	26.6	9.0	3.2
SED		0	2.35	1.30	0.59
Df		21	21	21	21
Significance		0	<0.001	0.002	<0.001

Table 4.4. Lesion development and sporulation on tomato stems after inoculation with *B. cinerea* spore suspension and seven microbial antagonists (BCAs): Bioassay 3

Treatment	Code	Lesion incidence (out of 10)	Mean stem lesion length (mm)	Mean no. sporulating stems (out of 10)	Mean sporulation index
Sterile distilled water	SDW	9	26.0	8.8	2.6
Bio-Save 10LP	H2	8	24.3	8.3	2.5
Bio-Save ESC 11	H3	8	24.1	8.3	2.4
Biomex SA	H4	9	26.0	8.8	2.7
-	H6	9	25.3	8.5	2.4
-	H7	9	26.2	9.0	2.6
Gliomix (standard)	A2	4	12.0	3.5	1.1
MBI 600 (standard)	A3	9	25.0	8.9	2.4
SED		0.575	1.74	0.68	0.22
Df		21	21	21	21
Significance		<0.001	<0.001	<0.001	<0.001

Table 4.5. Lesion development and sporulation on the outside of tomato stems after inoculation with *B. cinerea* spore suspension and seven microbial antagonists (BCAs): Bioassay 4

Treatment	Code	Lesion incidence (out of 10)	Mean stem lesion length (mm)	Mean no. sporulating stems (out of 10)	Mean sporulation index
Sterile distilled water	SDW	3	5.8	1.0	0.3
Gliomix (standard)	A2	1	3.0	0.0	0.0
MBI 600 (standard)	A3	1	2.3	0.0	0.0
Bacillus pumulis	A5	3	8.2	1.5	1.3
Pseudomonas fluorescens	A6	2	3.6	0.3	0.1
Bacillus subtilis 39	A7	4	8.9	1.758	0.6
Bacillus subtilis 83	A8	1	3.1	0.3	0.1
Yield Plus	A11	1	1.6	0.0	0.0
SED		0.882	2.19	0.48	0.51
Df		21	21	21	21
Significance		0.029	0.019	0.004	0.220

Table 4.6. Lesion development and sporulation on the inside of tomato stems after inoculation with *B. cinerea* spore suspension and seven microbial antagonists (BCAs): Bioassay 5

Treatment	Code	Lesion incidence (out of 10)	Mean stem lesion length (mm)	Mean no. sporulating stems (out of 10)	Mean sporulation index
Sterile distilled water	SDW	4	9.3	1.5	0.4
Gliomix (standard)	A2	4	9.5	0.8	0.1
MBI 600 (standard)	A3	4	9.7	1.0	0.1
Bacillus pumulis	A5	5	11.8	2.5	0.5
Pseudomonas fluorescens	A6	3	8.3	1.0	0.2
Bacillus subtilis 39	A7	4	9.9	2.3	0.6
Bacillus subtilis 83	A8	4	8.8	1.0	0.2
Yield Plus	A11	3	4.9	0.5	0.1
SED		0.890	2.05	0.56	0.18
Df		21	21	21	21
Significance		0.523	0.136	0.018	0.041

Conclusions

- The bioassay technique used was effective in enabling the antagonistic potential of 21 BCAs against *B. cinerea* on tomato stem tissue to be quantified, once problems of isolate pathogenicity, incubation conditions and variation in host plant resistance had been reduced.

- Promising candidates for subsequent work are:
  - Gliomix (fungal product)
  - *Clonostachys roseum* (fungal isolate)
  - Stimagro (Streptomycete product)
  - QRD 131 (bacterial product)
  - YieldPlus (yeast product)

- It was interesting to note that MBI 600 and Trichodex, which are registered and used for control of *B. cinerea* in tomato crops in Japan and Israel respectively, were not effective in these bioassays, perhaps due to application rate differences. When used commercially, the products are applied frequently and at high rates, and often in sequence with fungicide products.

5. Development of a method to screen micro-organisms for activity in reducing botrytis stem rot of tomato plants (milestones 8.1 and 8.2)

Study Director: Dr T Pettitt
HRI Wellesbourne, Wellesbourne, Warwick CV35 9EF

Site Manager: Mr M Wainwright
HRI Efford, Lymington, Hants SO41 4LZ

Status of work: Continuing

Period covered: April 2001 - March 2002

Introduction

Biocontrol products and isolates with reported antagonistic activity against *B. cinerea*, and candidate biocontrol agents collected from UK commercial tomato crops, are being tested against *B. cinerea* by dual culture plate tests and for activity against botrytis stem rot by a tomato stem piece bioassay. Isolates performing well in these tests will then be tested for their activity in reducing botrytis stem rot on whole tomato plants. As a precursor to these tests, it is necessary to devise methods for reliably producing botrytis stem rot in whole tomato plants using a standardised inoculum of *B. cinerea*.

Materials and methods

Experiment 1
Twelve week old tomato plants, cv. Espero, grown in rockwool slabs in M block at HRI Efford, were inoculated on the stem on 2 May 2001 with conidia of *B. cinerea* (isolate BC02) which had been collected dry and then primed by suspension in 0.1M glucose and 0.07M potassium dihydrogen-phosphate for 3 h. Inoculations were applied to de-leafing wounds on the stems prepared by the following methods:

Treatments
1. No petiole stub, no extra damage
2. No petiole stub, with a healed de-leafing wound
3. No petiole stub, stem damaged by scalpel cuts across the de-leafing wound
4. Petiole stub, (*c.* 1 cm), no extra damage
5. Petiole stub, (*c.* 1 cm), stub crushed with pliers
6. Petiole stub, (*c.* 1 cm), stem damaged by scalpel cuts across the de-leafing wound

Plants were grown under a conventional regime with supplementary nightly periods of high humidity induced by using a misting system between 0100 and 0500 hrs.

Experiment 2
Rockwool grown crops of cv. Espero in M12 (5 months old) and B block (10 months old) at HRI Efford were inoculated on 19 November 2001 with conidia of *B. cinerea* (isolate BC02) which had been collected dry and then prepared as described above. Freshly prepared de-leafing wound sites on tomato stems at the canopy base, were

inoculated with 20 µl of a $5x10^5$/ml spore suspension (10^4 spores/inoculation site). Glasshouses were maintained at 21^0 C and ambient RH until disease assessments were carried out, 21 days after inoculation.

Treatments

1. No petiole stub, no extra damage
2. No petiole stub, stem damaged by scalpel cuts across the de-leafing wound
3. Petiole stub, (*c*. 1 cm), no extra damage
4. Petiole stub, stem damaged by scalpel cuts across the de-leafing wound

There were a total of 25 and 50 replicates of each treatment in Experiments 1 and 2 respectively (in some cases, more than one treatment was applied to a single stem in the uninoculated wound treatments).

Results and discussion

Experiment 1

No stem lesions had developed after 16 weeks, when the last plants were removed from the house. Widespread infection was detected in re-isolations from inoculation sites and in all treatments, with more than 50% infection detected in each treatment. Possible reasons for this lack of stem rot development are: i) physiological age and condition of the plants after inoculation, which possibly was not conducive to stem rot symptom expression (plants were still young and stem tissues still green, this possibly parallels disease in other hosts where infections can remain latent in younger tissues, only showing symptoms as tissues age; ii) loss of pathogen virulence is unlikely as the same isolate was used successfully in other parts of the project and infections were successfully established; iii) the environmental conditions in the greenhouse were inappropriate for widespread stem rot symptom development. Precise conditions for stem rot symptom development have not been defined. This first trial was run in spring and early summer, a time when stem rot symptoms are less frequently seen. In addition, the high humidity treatment could possibly have had an adverse impact on disease development by allowing unfavourably long periods of surface wetness to occur (the effects of surface wetness on botrytis disease development are variable, whilst short periods of a few hours can stimulate germination and infection, longer periods can be inhibitory.

Experiment 2

A high incidence of girdling botrytis stem lesions had developed in all treatments 3 weeks after inoculation (Table 5.1). De-leafing wound sites in the crops which were not inoculated with *B. cinerea* did not show any evidence of botrytis stem rot.

Table 5.1. Effect on stem inoculation methods and wound treatment on development of botrytis stem lesions in tomato - 2001

Plant age	% inoculation sites developing stem lesions after 3 weeks			
	Petiole stub present		No petiole stub	
	Wounded	No extra wound	Wounded	No extra wound
Younger plants (c. 5 months old)	60	40	50	37
Mature plants (c. 10 months old)	100	68	90	62

Conclusions

- All inoculation methods resulted in a relatively high incidence of botrytis stem rot. De-leafing stubs that were not inoculated with *B. cinerea* remained free of botrytis stem rot.
- Extra wounding at inoculation sites increased the incidence of stem lesions.
- The presence of a petiole stub marginally increased the incidence of botrytis.
- There was noticeably greater botrytis development on the 10-month-old plants than on the 5-month-old plants. This difference may be due to different growing conditions (the crops were in different glasshouse compartments) and not just plant age.
- These results indicate we now have suitable inoculation techniques for evaluation of candidate biocontrol agents.

6. Summary of recent publications on biocontrol of botrytis (milestone 13.0)

Lui W, Sutton JC, Huang R & Owen-Going N (2001). Effectiveness of *Clonostachys rosea* against *Botrytis cinerea* in stems of hydroponic greenhouse tomatoes. *Canadian Journal of Plant Pathology* 23, 201.

Conidia of the fungus *Clonostachys rosea* (10^6 /ml) were applied in water to de-leafing wounds on tomato stems, at 0-48 h after de-leafing. Inoculation with *B. cinerea* was by natural dispersals from within the glasshouse. No botrytis stem lesions had developed after 11 weeks, even at sites not inoculated with the BCA. However, isolation from stem tissue around de-leafing sites revealed latent infection by botrytis. The % area of stem tissue affected by sporing botrytis was reduced by *C. rosea* from 89% (untreated) to 45-13% (10^3 -10^6 conidia/ml of *C. rosea*). Treatment with *C. rosea* at 0-48 h after deleafing reduced sporulation on stem pieces (latent botrytis) by 72-84% compared with controls i.e. inoculation with the BCA at 48 h after deleafing was no less effective than immediate inoculation of the wound site. It was concluded that *C. rosea* established endophytically in stems and persisted for at least 11 weeks.

Chiou AL & Wu WA (2001). Isolation, identification and evaluation of bacterial antagonists against *Botrytis elliptica* on lily. *Journal of Phytopathology* 149, 319-324.

Of 700 micro-organisms isolated from lily plants and screened by dual culture, 62 were inhibitory to *B. elliptica* on low-nutrient agars (e.g. dilute PDA). Ten isolates with the greatest inhibitory effect were then screened by bioassay on detached lily leaves, and in greenhouse trials. Bacterial isolates identified as *Burkholderia gladioli* and *Bacillus amyloliquefaciens* by the BIOLOG system were as effective as flusilazole in field trials, providing *c*. 63-76% reduction in disease severity. Results in the detached leaf bioassay correlated with those in the field trial confirming the value of such bioassays in saving time and resources. *B. amyloliquefaciens* has previously been shown to control post-harvest botrytis rot on tomatoes. *B. gladioli* causes a brown rot of tulip bulbs and leaf scorch of iris, limiting its application as a BCA

Guetsky R, Shtienberg D, Elad Y & Dinoor A (2001). Combining biocontrol agents to reduce the variability of biological control. *Phytopathology* 91, 621-627.

Control of *B. cinerea* on strawberry leaves by the BCAs *Pichia guilermondii* (a yeast) and *Bacillus mycoides* (a bacterium) applied separately was highly variable ranging from 38% to 98% disease reduction. Control was greatest when conditions were sub-optimal for botrytis. When applied as a mixture, control was more consistent (80-99%) under all conditions.

Kohl J, Gerlagh M & Grit G (2000). Biocontrol of *Botrytis cinerea* by *Ulocladium atrum* in different production systems of cyclamen. *Plant Disease* 84, 569-573.

The fungus *Ulocladium atrum* spray applied to cyclamen (at $1x10^6$ conidia/ml), gave control of botrytis, equal to that of fungicides, when applied at 4 week intervals. *U. atrum* competitively excludes *B. cinerea* from colonising necrotic leaves. Biocontrol worked under a range of cyclamen growing systems, including both sub-irrigation and overhead watering. Application just twice, early in crop production, was less effective than application every 4 weeks throughout crop production.

Fruit L & Nicot P (1999). biological control of *Botrytis cinerea* on tomato stem wounds with *Ulocladium atrum*. *IOBC Bulletin* 22, 81-84.

B. cinerea (10^4 conidia/ml) was applied to fresh petiole stubs (5-10 mm long) on 3 month old plants and *U. atrum* (10^6 conidia/ml) applied 30 mins later. Botrytis was first observed on inoculated stubs after 12 days. 33% of wound sites inoculated with *B. cinerea* had developed sporing botrytis after 55 days in a glasshouse crop. In a polytunnel crop, 60% of botrytis-inoculated petiole stubs developed botrytis after 55 days compared with 40-45% of stubs treated with *U. atrum* or fungicide. In the glasshouse crop, stem lesions developed only in the control (botrytis only) inoculated plants, with lesions at 25% of sites after 60 days. In the polytunnel crop, stem lesions developed at 40% of botrytis-only inoculation sites after 60 days, and none on the fungicide or *U. atrum* treated plants. Similar results were obtained over 2 years (1997 and 1998). *U. atrum* thus shows good potential for use as a BCA to protect tomato de-leafing wounds.

Kohl J, Lombaers-van der Plas CH, Holhoek WML, Kessel GJT & Goossen-van der Geijn HM (1999). Competitive ability of the antagonists *Ulocladium atrum* and *Gliocladium roseum* at temperatures favourable for Botrytis spp. development.

The optimum temperature for conidial germination and myclelial growth of both these BCAs is high, $27\text{-}30^0$ C. At low temperatures (5^0 C) there was 50% germination of *U. atrum* and *G. roseum* after 18 and 96 h respectively. Conidia of *U. atrum* germinated rapidly over a broad temperature range, even on water agar (no nutrients). *G. roseum* germinated only in the presence of exogenous nutrients and not on water agar (possibly due to the small size of its conidia).

On dead onion leaves, *U. atrum* suppressed sporing of *B. cinerea* by more than 85% at all temperatures ($6\text{-}24^0$ C).

On dead cyclamen leaves, *G. roseum* showed no antagonistic activity below 21^0 C though it was more effective than *U. atrum* at $21\text{-}24^0$ C. [Others have found more cold-tolerant strains of *G. roseum*, effective on *B. cinerea* between $10\text{-}25^0$ C].

On dead hydrangea leaves, *U. atrum* reduced *B. cinerea* sporulation even at 1^0 C.

On onion leaves, *G. roseum* was more effective than *U. atrum* under continuously

moist conditions, but very sensitive to interruptions of leaf wetness. In contrast, *U. atrum* was resistant to interruptions of leaf wetness.

The estimated maximum temperature for *U. atrum* growth was 35.7^0 C; for *G. roseum* it was 36^0 C.

Sclerotial production by *B. cinerea* on dead cyclamen leaves increased with temperature, from zero at 9^0 C, to a maximum of 1.9/leaf at $15\text{-}21^0$ C, and slight development at 12 and 24^0 C.

It was concluded that in warm greenhouse crops (>18^0 C) with long wetness periods, *G. roseum* is likely to give better control than *U. atrum* of *B. cinerea* sporulation. But for cool glasshouse crops (e.g. cyclamen), or outside, or where there is interrupted wetness, *U. atrum* will perform better.

Elad Y (2000). *Trichoderma harzianum* T39 preparation for biocontrol of plant diseases - control of *Botrytis cinerea, Sclerotinia sclerotiorum* and *Cladosporium fulvum. Biocontrol Science & Technology* 10, 499-507.

Trichodex (*T. harzianum* T39) was applied to tomato crops at two rates (0.2 and 0.4 g ai/l) with and without the addition of oil. Disease control was similar at the 2 rates. Addition of oil did not improve control. Botrytis stem lesions were reduced from 1.4 to 0.8 per stem by the BCA, dead plants from 25% to 12%. Tolylfluanid (Elvaron Multi) at 1.5 g/l gave similar levels of control.

Five sprays were applied at c. 7 day intervals, at 1,000 l/ha and 5 bar pressure. Biocontrol of *B. cinerea* by *T. harzianum* T39 arises from i) reduction of conidial germination and germ tube growth, ii) competition for nutrients, iii) reduced activity of botrytis pectolytic enzymes (due to production of a protease enzyme by *Trichoderma*), iv) induced resistance (dead cells of T39 give some control).

Peng G, Sutton JC & Li DW (1996). Sites of infection in tomato stems by *Botrytis cinerea*. *Canadian Journal of Plant Pathology* 18, 97 (abstract).

Tomato stem de-leafing wounds were only highly susceptible to infection from *B. cinerea* conidia for 3 days after leaf removal. These results imply BCAs need to be applied to fresh de-leafing wounds, if they act by preventing site colonisation.

Fravel D (1999). Commercial biocontrol products for use against crop diseases. www.barc.usda.gov/psi/bpdl/bpdlprod/bioprod.html (13 pages).

Summary of micro-organisms reported to control *B. cinerea* in recent publications

BCA	Crop and tissue	Reference
Fungi		
Ulocladium atrum	Tomato stem wounds	Fruit & Nicot, 1999
	Cyclamen leaves	Kohl *et al*., 2000
Gliocladium roseum[a]	Cyclamen leaves	Kohl *et al*., 1998
Gliocladium catenulatum	Tomato stems	Dik *et al*., 1999
Chaetomium globosum	Tomato stems	Dik *et al*., 1999
Trichoderma harzianum	Tomato	Elad, 2000
Penicillium spp.		
Clonostachys rosea	Tomato stems	Liu *et al*., 2001
Yeasts		
Rhodothorula glutinis	Tomato	Elad *et al*., 1994
Cryptococcus albidus	Tomato	Elad *et al*., 1994
Cryptococcus laurenti	Apple fruit	Roberts, 1990
*Pichia guilermondii**	Strawberry leaves	Guetsky *et al*., 2001
Aureobasidium pullulans	Tomato leaves	Dik & Elad, 1999
Bacteria		
Bacillus mycoides	Strawberry leaves	Guetsky *et al*., 2001
Bacillus subtilis	Lettuce leaves	Fiddaman *et al*., 2000
Brevibacillus sp.	Tomato leaves	Seddon, 2000
Pseudomonas spp.	Tomato stems	Dik *et al*., 1999
Bacillus amyloliquefaciens	Lily leaves (BE)	Chiou & Wu, 2001
Burkholderia gladioli	Lily leaves (BE)	Chiou & Wu, 2001

[a] *Gliocladium roseum* has been re-classified as *Clonostachys rosea*
[b] *Gliocladium virens* has been re-classified as *Trichoderma virens*
* Isolated from tomato leaves

7. Overall conclusions

1. Twenty-one biocontrol products and isolates (BCAs), comprising 8 fungi and 11 bacteria and 2 yeasts were obtained from commercial companies and research organisations and placed in the reference collection. Where necessary, contractual agreements to allow use of these products and isolates within this project were negotiated.

2. A total of 106 morphologically distinct micro-organisms were collected from commercial tomato crops, cleaned and placed in long-term storage. Preliminary identification of isolates was made.

3. A stem piece bioassay for use in the laboratory to allow time, space and resource-efficient screening of candidate BCAs was developed. The stem bioassay uses primed conidial inocula, stem end wounding and incubation at 15^0 C and 80% RH for 7 days.

4. The stem piece bioassay was used to assess biocontrol potential of 21 products and isolates. Five of them showed promise and will be evaluated further.

5. A method was devised for generating aggressive botrytis lesions on tomato stems. This method will be used as a basis for screening candidate BCAs on whole tomato plants in 2002.

8. Technology transfer

Article

O'Neill TM (2001). Biocontrol options for tomato stem botrytis. *HDC News* **74**, 18-19.

Summary progress reports to consortium members

1. Report 1, April 2001
2. Report 2, August 2001
3. Report 3, December 2001

Scientific progress meetings

Project meeting, ADAS Arthur Rickwood, 27 July 2001.
Project review meeting, ADAS Arthur Rickwood, 6 March 2002

9. Acknowledgements

We would like to thank Dr Nigel Dungey, Mr Paul Challinor, Mr Dave Palmer, Mr Phil Pearson, Mr Gerry Hayman and Mr John Overvoorde for their support and advice in helping to steer the project. In addition, we would like to thank all of the companies who have given product samples or antagonistic isolates as well as technical information for use in this project. We also thank Mr Rodney Edmonson, Biometrics Department, HRI Wellesbourne and Mr Doug Wilson, ADAS statistician for their support in experimental design and statistical analysis.

10. References

Andrews JH, Berbee FM & Nordheim EV (1983). Microbial antagonism to the imperfect stage of the apple scab pathogen, *Venturia inaequalis*. *Phytopathology* **73**, 228-234.

Church VJ (1992). Epidemiology in relation to control of grey mould (*Botrytis cinerea*) on sunflower. *Proceedings Brighton Crop Protection Conference 1992*, 669-674.

Cohen A. Elad Y, Abir H, Balum B & Barqzani CA (1996). Control of grapevine grey mould with Trichodex (*Trichoderma harzianum* T39). *Proceedings Kith International Botrytis Symposium, Wageningen, June 1996*, 74.

Cook DWM, Long PG, Cheah L, Ganesha S & Neilson HF (1996). Attachment of micro-organisms for bio-control of *Botrytis cinerea* in-vivo and in post-harvest kiwi fruit. *Proceedings XIth International Botrytis Symposium, Wageningen, June 1996*, 68.

Decognet V, Trottin-Caudol Y, Foumier C, Leyre JM & Nicot P (1999). Protection of stem wounds against *Botrytis cinerea* in heated tomato greenhouses with a strain of *Fusarium* sp. *IOBC Bulletin* **22**, 53-56.

Decognet V,& Nicot P (1999). Effect of fungicides on a *Fusarium* sp., biological control agent of *Botrytis cinerea* stem infections in the perspective of an integrated management of fungal diseases in greenhouse tomatoes. *IOBC Bulletin* **22**, 49-52.

Dik AJ & Elad Y (1999). Comparison of antagonism of *botrytis cinerea* in greenhouse grown cucumbers and tomato under different climate conditions. *European Journal of Plant Pathology* **105**, 123-130.

Dik AJ, Koning G & Kohl J (1999). Evaluation of microbial antagonists for biological control of *Botrytis cinerea stem infection on cucumber and tomato. European Journal of Plant Pathology* **105**, 115-122.

Dik, A.J., Koning, F. & Köhl, J. 1999. Evaluation of microbial antagonists for biological control of *Botrytis cinerea* stem infection in cucumber and tomato. *European Journal of Plant Pathology* **105**: 115-122.

Eden MA, Hill RA & Stewart A (1995). Biological control of *Botrytis* stem infection of greenhouse tomatoes. *Plant Pathology* **45**, 276-284.

Elad Y, Kohn J & Fokkema NJ (1994). Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic yeasts. *Phytopathology* **84**, 1193-1200.

Fruit L & Nicot P (1999). Biological control of *Botrytis cinerea* on tomato stem wounds with *Ulocladium atrium*. *IOBC Bulletin* **22**, 81-84.

Hausbeck MK & Moorman GW (1996). Managing *Botrytis* in greenhouse-grown flower crops. *Plant Disease* **80**, 1212-1219.

Hausbeck MK & Pennypacker SP (1991). Influence of grower activity and disease incidence on concentrations of airborne conidia of *Botrytis cinerea* among geranium stock plants. *Plant Disease* **75**, 798-803.

Jackson AJ, Walters DR & Marshall G (1994). Evaluation of *Penicillium chrysogenum* and its antifungal extracts as potential biological control agents against *Botrytis fabae* on faba beans. *Mycological Research* **98**, 1117-1126.

Jackson AJ, Walters DR & Marshall G (1997). Antagonistic interactions between the foliar pathogen *Botrytis fabae* (Chocolate spot) with *Penicillium brevicompactum* and *Cladosporium cladosporioides. Biological Control* **8**, 97-106.

Köhl J, Gerlach M, De Haas BH & Krijger MC (1998). Biological control of *Botrytis cinerea* in Cyclamen with *Ulocladium* and *Gliocladium roseum* under commercial growing conditions. *Phytopathology* **88**, 568-575.

Köhl J, van der Plas CH, Molhoek ML & Fokkema NJ (1995). Effect of interrupted leaf wetness periods on suppression of sporulation of *Botrytis allii* and *B. cinerea* by antagonists on dead onion leaves. *European Journal of Plant Pathology* **101**, 627-637.

Machowicz-Stefaniak Z & Kuropatwa E (1996). Studies on the possibilities of limiting *Botrytis cinerea* pers. on grapes by antagonistic fungi and bacteria. *Proceedings XIth International Botrytis Symposium, Wageningen, June 1996*, 58.

McPherson GM & O'Neill TM (1997). Control of stem *Botrytis* in protected tomato. *Final report for project PC 98a, Horticultural Development Council* 55pp.

Melgarejo P, Carrillo R & Sagasta EM (1985). Mycoflora of peach twigs and flowers and its possible significance in biological control of *Monilinia laxa. Transaction of the British Mycological Society* **85**, 313-317.

Moline H, Hubbard JE, Karns JS, Buyer JS & Cohen JD (1999). Selective isolation of bacterial antagonists of *Botrytis cinerea. European Journal of Plant Pathology* **105**, 95-101.

Nicot PC, Morrison N & Decognet V (1996). Protection of pruning wounds on greenhouse tomatoes by saprophytic micro-organisms against infection by *Botrytis cinerea*. *Proceedings XIth International Botrytis Symposium, Wageningen, June 1996*, 70.

O'Neill TM (1994). Resurgence of tomato stem botrytis. *Grower 21 July 1994*, 54-55.

O'Neill TM, Niv A, Elad Y & Shtienberg D (1996).. Biological control of *Botrytis cinerea* on tomato stem wounds with *Trichoderma harzianum. European Journal of Plant Pathology* **102**, 635-643.

O'Neill TM, Shtienberg D & Elad Y (1997). Effect of some host and micro-climate factors on infection of tomato stems by *Botrytis cinerea. Plant Disease* **81**, 36-40.

Purkayastha RP & Bhattacharyya B (1982). Antagonism of micro-organisms from jute phyllosphere towards *Colletotrichum corchori. Transactions of the British Mycological Society* **78**, 509-513.

Schmidt L, Ferreira JHS & Holz G (1996). Further evaluation of bio-control of *Botrytis cinerea* by *Trichosporon pullulans* on table grapes. *Proceedings XIth International Botrytis Symposium, Wageningen, June 1996*, 86.

Sutton JC & Peng G (1993). Bio-control of *Botrytis cinerea* in strawberry leaves. *Phytopathology* **83**, 615-621.

Sutton JC, De-Wei Li, Gang Peng, Hai Yu & Pinggao Shang (1997). *Gliocladium roseum:* a versatile adversary of *Botrytis cinerea* in crops. *Plant Disease* **81**, 316-328.

Swadling IR & Jeffries P (1996). Isolation of microbial antagonists for bio-control of grey mould disease of strawberries. *Bio-control Science and Technology* **6**, 125-136.

Swadling IR & Jeffries P (1998). Antagonistic properties of two bacterial bio-control agents of grey mould disease. *Bio-control Science and Technology* **8**, 439-448.

Swadling, I.R. & Jeffries, P. 1996. Isolation of microbial antagonists for bio-control

of grey mould disease of strawberries. *Bio-control Science and Technology* **6**, 125-136.

Swadling, I.R. & Jeffries, P. 1998. Antagonistic properties of two bacterial bio-control agents of grey mould disease. *Bio-control Science and Technology* **8**, 439-448.

Walter M, Boyd-Wilson KSH, Elmer PAG & Kohl J (1996). Selection of antagonistic saprophytes for suppression of *Botrytis cinerea* sporulation of kiwi fruit tissues. *Proceedings XIth International Botrytis Symposium, Wageningen, June 1996*, 89.

Whipps JM & McQuilken MP (1993). Aspects of bio-control of fungal plant pathogens. *Exploitation of Micro-organisms*, 45-79.

Whipps JM (1997). Developments in the biological control of soil-borne plant pathogens. *Advances in Botanical Research* **26**, 1-134.