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
Report:	Final report, March 2004
Previous reports:	March 2001, March 2002 and April 2003 annual reports
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Project co-ordinator:	Dr P Challinor
Date project commenced:	1 April 2000
Date completion due:	31 March 2004
Key words:	Tomato, grey mould, *Botrytis cinerea*, biological control, antagonists, bioassay, *Trichoderma harzianum, Clonostachys rosea,* Gliomix, Stimagro

The results and conclusions in this report are based on a series of experiments conducted over one year. 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.

Use of pesticides

Only officially approved pesticides may be used in the UK. Approvals are normally granted only in relation to individual products and for specified uses. It is an offence to use non-approved products or to use approved products in a manner that does not comply with the statutory conditions of use except where the crop or situation is the subject of an off-label extension of use.

Before using all pesticides and herbicides check the approval status and conditions of use.

Read the label before use: use pesticides safely.

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

Dr K Green
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CONTENTS

GROWER SUMMARY

PC 174: Tomatoes: development of biocontrol as a component of an integrated, sustainable strategy for the control of grey mould (*Botrytis cinerea)*

Headline

Several biocontrol products and micro-organisms, including an isolate of *Trichoderma harzianum* obtained from a commercial tomato crop during this project, gave significant reductions of tomato stem botrytis in laboratory tests and glasshouse trials. In a full-season crop trial, *Clonostachys rosea* and Gliomix (*Gliocladium catenulatum*) applied every 14 days from May to October reduced the number of botrytis stem lesions by ~50%. These treatments were less effective than a six-spray fungicide programme of alternating sprays of dichlofluanid, pyrimethanil and iprodione. Old fruit trusses were confirmed as an important infection route leading to stem botrytis. Potential methods for large-scale production of *T. harzianum* were devised.

Background and expected deliverables

With increasing concern from retailers and consumer groups at the continued use of pesticides on food crops, the UK Tomato Growers Association have set in place a long-term objective of zero pesticide use by 2008. Currently fungicides remain a key component of effective disease management strategies, and grey mould, caused by the fungus *Botrytis cinerea*, is a principal target of these fungicide treatments. Stem botrytis is the most problematic phase of the disease.

The potential of biocontrol as a component of an integrated sustainable strategy for control of botrytis in tomato crops has been demonstrated elsewhere, for example in Israel and France. The potential under UK cropping conditions has not been thoroughly investigated.

The **expected deliverables** from this project are:

- Identification of existing biological control agents with efficacy against *B. cinerea;*
- Determination of their efficacy in relation to currently approved fungicides;
- Novel biological control agents isolated from UK tomato crops;
- Evaluation of candidate organisms for efficacy against *B. cinerea*, compared with fungicides.

Summary of the project and main conclusions

Sourcing existing biocontrol products and isolates

Twenty one bio-control products and isolates (BCAs) with reported antagonism against *B. cinerea* were collected from commercial companies and research organisations to form a project reference collection. Most originate from overseas. Agreements for experimental use of these products were negotiated. Some of these products (e.g. Gliomix, Stimagro) are currently sold in the UK for use as biological growth promoters, though not as bio-fungicides.

Obtaining micro-organisms from UK tomato crops

A total of 106 morphologically distinct micro-organisms were collected from UK tomato crops (including from organic 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 filamentous fungi, bacteria and yeasts. Isolates were cleaned and cultures put into long-term storage for future use. Fifty isolates were selected for further study to determine their potential use in controlling *B. cinerea*.

Testing for biocontrol activity by agar plate challenge tests

All the candidate BCAs in the reference collection and 49 of the isolates obtained from UK tomato crops 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. Often, botrytis grew over the candidate BCA. Twelve of the BCAs however (6 from the reference collection and 6 from UK tomato crops) exhibited strong competition and grew over botrytis (Fig. 1).

Figure 1. Overgrowth of *Botrytis* by *Trichoderma* in an agar plate challenge test

Identification of new candidate biocontrol micro-organisms

The most promising isolates were identified as *Trichoderma harzianum* (IMI 390736) (01/14), *Scopulariopsis candida* (01/56) and *Geotrichum candidum* (01/62). These fungi are all common and have been found in a range of environments, including on plant surfaces. *Geotrichum candidum* is sometimes found on fallen or split tomato fruit, causing sour rot. *S. candida* has been found associated with lesions on humans, so further work on this fungus was discontinued. The *T. harzianum* (01/14), isolated from a tomato crop in northern England, is the most promising isolate for potential commercial use.

Devising a tomato tissue bioassay

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 piece bioassay, using conidial inocula, allowed the most realistic and reproducible conditions similar to infection development under commercial growing conditions.

The stem piece bioassay gives reasonably consistent development of botrytis stem rot. Replicate tomato stem pieces (3 cm long) are inserted in moist, autoclaved vermiculite, and damaged stem ends are 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.

Figure 2. Tomato stem piece bioassay, used for large-scale screening of micro-organisms for biocontrol activity

Testing for biocontrol activity using the stem piece bioassay

From the 21 micro-organisms in the reference collection, five were demonstrated to have the ability to reduce botrytis rot in tomato stem pieces:

Gliomix (fungal product)
Clonostachys rosea (fungal isolate)
Stimagro (Streptomycete product)
QRD 131 (bacterial product)
Yield Plus (yeast product)

From the 49 isolates from UK tomato crops, two (01/56 and 01/62) significantly reduced stem rotting. Isolate 01/14 (*T. harzianum*) grew over *B. cinerea* in dual culture challenge tests and was also selected for use in further experiments.

Although no treatment gave a high level of control using the stem piece test procedure, where a high inoculum of botrytis was deliberately used, the degree of control in a glasshouse crop, with living plants and natural inoculum dispersal, may be greater.

Effect of temperature and humidity on biocontrol activity

The effect of temperature and humidity on biocontrol efficacy was examined for seven leading BCAs using the stem piece bioassay. Stimagro showed activity at all temperatures, reducing stem lesion length by at least half compared with the untreated control (Table 1). *C. rosea* and Gliomix showed moderate activity at 15-20 ^{0}C and none at 25 ^{0}C. Yield Plus showed slight activity at all temperatures while QRD 131 appeared active at 15 and 25^0 C; the result at 20^0 C appears anomalous.

The results with *C. rosea* were confirmed in a further experiment in 2003. Gliomix showed better activity at 20^0 C than at 15 or 25^0 C, whilst *T. harzianum* (01/14) was more effective at 20-25^0 C than at 15^0 C.

Table 1. Effect of temperature on the effectiveness of eight micro-organisms in controlling tomato stem rot (lesion length) caused by *B. cinerea*

Biocontrol product or micro-organism	% control of stem rotting at:		
	15°C	20°C	25°C
2002			
C. rosea	22.5	21.2	0.0
QRD 131	25.6	0.0	46.3
Stimagro	62.1	85.4	51.7
Gliomix	39.6	26.1	0.0
Yield Plus	19.4	6.6	18.3
Geotrichum sp. (01/62)	19.4	0.0	0.0
Scopulariopsis sp. (01/56))	2.4	0.0	0.0
2003			
C. rosea	87.3	70.3	14.7
Gliomix	0.0	35.6	27.7
T. harzianum (0/14)	0.0	30.7	57.6

Comparing humidities at a constant 20^0 C, Stimagro showed greater efficacy at high

(85%) than at lower (70%) humidities. The other BCAs gave only slight control in this experiment and no treatment effects were discernible.

Effect of BCA rate and combined BCA treatments

For three of the leading BCAs, bioassay tests were conducted in which the ratio of the number of BCA to botrytis spores was set at 10:1, 1:1 and 1:10 in order to provide more information on the robustness of each treatment. *C. rosea* appeared more effective when tested at the two higher ratios; Gliomix gave significant reductions at all three ratios; *T. harzianum* (isolate 01/14) only reduced stem lesion length when tested at the highest ratio. These results suggest that the Gliomix treatment is the more robust with regard to rate, and there may be scope for reducing the application rate of Gliomix. Application of high inoculum concentrations of *C. rosea* and *T. harzianum* may be required for reliable performance of these materials.

In a set of further stem piece bioassays, *C. rosea*, Gliomix and *T. harzianum* (01/14) were tested individually and in all 2-way combinations. The rate of use of each BCA was the same whether applied alone or in mixture. In two of the tests, BCA treatment failed to reduce lesion length or the incidence or intensity of botrytis sporulation. In the third test, *C. rosea* applied with Gliomix reduced lesion length by 51%, Gliomix alone by 43% and *C. rosea* alone by 29%, suggesting a possible beneficial effect from joint application of these two BCAs. *C. rosea* and Gliomix appeared more effective than *T. harzianum* (01/14) in reducing sporulation by botrytis.

Potential for commercial production of *Trichoderma harzianum*

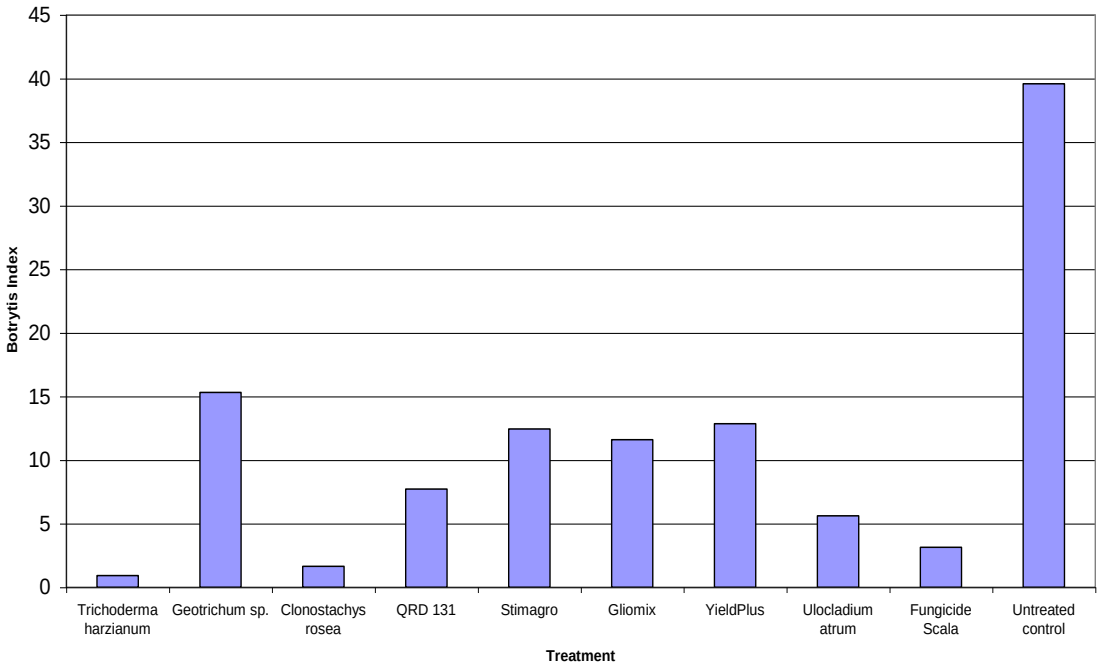
Following the screening tests and formal identification of lead strains, the *T. harzianum* isolate (01/14) was considered the novel isolate with the best potential for commercial production. A range of solid substrates and liquid fermentation media were examined for their suitability for future large-scale production of this fungus. It was found that it can be successfully grown on solid substrates using a range of common nutrient sources (rice, barley, maize meal + Perlite, peat + wheat bran, wheat, bran + vermiculite, bran + sand, oats) and in liquid culture (molasses – yeast liquid medium, potato dextrose broth). Production of both mycelial biomass and conidia was successful in both production systems.

Performance in crop trials

A 6-week trial was conducted on a mature crop of cv. Espero, in late autumn 2002. A single spray of each BCA, or a fungicide treatment (Scala), was applied to fresh de-leafing wounds prior to inoculation with botrytis. Although no stem lesions developed in the 6-week period between inoculation and crop termination, *B. cinerea* was readily recovered from stem pieces and internal stem browning was often visible. The extent of internal stem browning was used to assess biocontrol activity (Fig. 3). Four BCA products (Gliomix, QRD 131, Stimagro and YieldPlus) and five micro-organisms (*Brevibacillus brevis, Clonostachys rosea, Geotrichum candidum, Trichoderma harzianum* (01/14) and *Ulocladium atrum*) gave statistically significant reductions of botrytis stem rot development. Preventative sprays of *C. rosea* and *T. harzianum* (01/14) were equivalent in efficacy to a preventative spray of Scala.

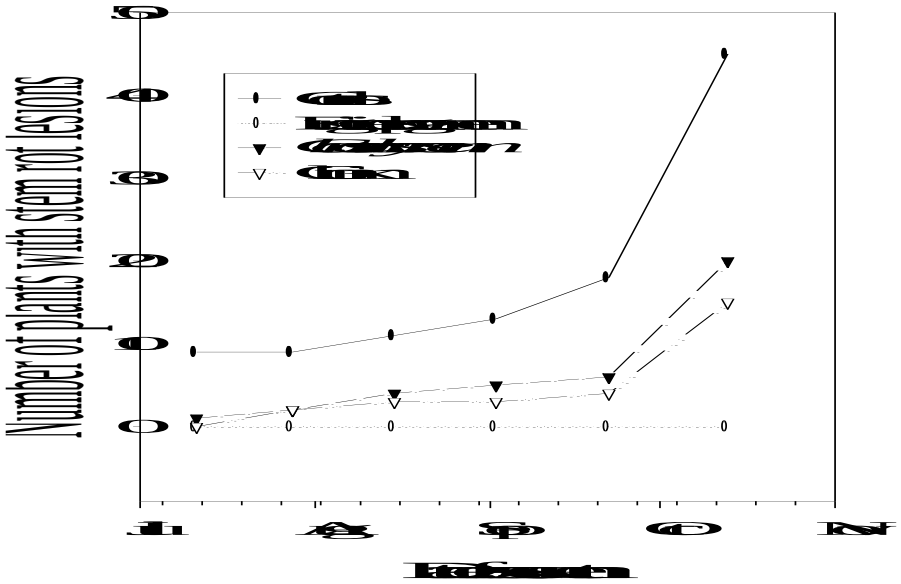
Figure 3. Effect of eight BCA treatments and a fungicide in controlling internal stem

browning of tomato associated with botrytis (glasshouse trial, autumn 2002; 6-weeks after inoculation)



A long-season trial was conducted on a crop of cv. Espero in 2003. *C. rosea* and Gliomix applied every 14 days from early May to end of October (11 sprays in total) were compared with a 6-spray alternating fungicide programme (alternating sprays of dichlofluanid, pyrimethanil and iprodione) and an untreated control. No fruit trusses were removed, fresh plates of sporulating *B. cinera* were placed in the crop weekly, and glasshouse heating was reduced from early September, all to encourage development of botrytis. A low incidence of stem botrytis was present by early September but this increased rapidly to cause widespread plant death by the end of October. The two biocontrol treatments and the fungicide spray programme all gave significant reductions in stem rot and plant death; on 13 October, the two biocontrol treatments were less effective (56-67% control) than the fungicide programme (100% control) in reducing the number of botrytis stem lesions (Fig 4). At the end of the trial, plant death because of botrytis stem rot had reached 91 % in the untreated and only 8% where the 6-spray fungicide programme was used. *C. rosea* reduced the incidence of plant death to 67 % and Gliomix to 48 %. The rapid increase in botrytis stem lesions during October followed a deliberate reduction in heat and ventilation 8 weeks earlier; this resulted in the mean daily RH increasing from around 80 % frequently to over 90 %. The majority of stem lesions arose from spent fruit trusses, confirming the importance of these dying tissues as an infection route.

Figure 4. Effects of *Clonostachys rosea* and Gliomix on the total number of botrytis stem lesions per 48 plants in comparison with a commercial fungicide spray programme



Financial benefits

None at present. 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 £130 million, 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.

Action points for growers

None at present. A number of commercial products and isolates have been demonstrated to be capable of providing effective control of stem botrytis. It is suggested that the TGA and HDC, on behalf of UK tomato growers seek to encourage companies who market biocontrol agents or other interested parties, to register with PSD products shown to be effective in this work, so that growers can use them on UK crops. For example, the efficacy data reported in this project could be made available to a company seeking product registration; the *T. harzianum* isolate (01/14) obtained during this project could be offered to a biocontrol company for further development work with a view to registration.

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 ability of these BCAs was developed using 'live' tomato plant tissue.

In the second year of the project, we completed the project reference collection of commercial products and isolates with known activity against *B. cinerea*, undertook dual culture tests of potential antagonists against *B. cinerea* and presumptive identification of potential antagonists. The bioassay to screen micro-organisms was finalised and isolates were screened. Inoculation methods on tomato plants were compared and a suitable method was devised. Summary interim reports for the project consortium members were produced, in addition to the annual report (March 2002).

In the third year of the project, we completed dual culture tests, identified promising biocontrol isolates obtained from UK tomato crops, completed laboratory bioassays of all micro-organisms and conducted a 6-week glasshouse trial that evaluated the six most promising BCAs against a fungicide treatment (Scala). Summary interim reports and an annual report (April 2003) were produced.

The key work areas in this final year were:

1. To complete assessment of the effect of temperature on antagonist efficacy (milestone 9.0)
2. Evaluate the potential of antagonists for commercial production (milestone 10.0)
3. Evaluate the performance of antagonists in a long-season trial (milestone 11.0)
4. Investigate the effect of timing of BCA application in relation to botrytis, application rate, and the potential of BCA mixtures, on control efficacy in bioassay tests (replacement for milestone 11.2)
5. Prepare a final report (milestone 12.4)

Milestones (revised schedule as agreed October 2003)

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	April 2002
4.0	Short-listed isolates identified (presumptive) and lodged in collection	April 2002
5.0	Laboratory bioassay to screen candidate micro-organisms developed	June 2001
6.1	Screen isolates/products in laboratory bioassay	April 2002
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
8.0	*In vivo* screening of products and selected isolates from tomato crops in comparison with fungicides completed	March 2003
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	Evalute the effect of BCA application rate, timing of application and use of BCA mixtures on efficacy of botrytis control using bioassay tests	March 2004
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. Confirmatory identification of leading candidate BCAs (Objective 7)

Study Director: Professor J Whipps
HRI Wellesbourne, Wellesbourne, Warwicks

Status of work: Completed

Period covered: April – December 2003

Introduction

Following the finding that the two lead isolates, XHAPP 01/56 (IMI 389400) *Scopulariopsis candida* (Guég.) Vuill and XHAPP 01/62 (IMI 389401) *Geotrichum candidum* Link were unsuitable for further commercial development on potential environmental and health risk grounds, focus changed to isolate XHAPP 01/14, tentatively identified as a *Trichoderma* species. *Trichoderma* species have a considerable history of safe use as biological disease control agents and plant growth promoters. However, for any further development a full identification is required.

Materials and methods

XHAPP 01/14 was plated onto potato dextrose agar (PDA) and examined microscopically and was tentatively identified as *Trichoderma harzianum* Rifai. However, as *Trichoderma* species are currently undergoing taxonomic revision it was sent to CABI Biosciences identification services for confirmation.

Results and discussion

The following is based on the report received from CABI and additional information from CBS database in The Netherlands.

XHAPP 01/14 (IMI 390736). *Trichoderma harzianum* Rifai. This isolate had smooth, more or less globose, pigmented conidia less than 3 μm diameter. *Trichoderma harzianum* is an example of the anamorphic (imperfect) state of the ascomycete fungus *Hypocrea*. It has a very common occurrence worldwide, being isolated from a wide range of substrates, especially soils; it has been shown to cause lesions on maize and some isolates can cause extreme damage to the edible mushroom, *Lentinus edodes*; well known antagonist of many fungi.

In view of the background history of safe use as a biological disease control agent , *T. harzianum* (IMI 390736) would seem a good strain to follow up. The only proviso would be that some assessment of pathogenicity on common plant species and other fungi such as mushrooms would probably be needed in the future if *Botrytis* control on tomato was reproducibly successful.

2. Effect of temperature on biocontrol efficacy (Objective 9.0)

Study Director: Dr Kim Green
ADAS Arthur Rickwood

Status of work: Completed

Period covered: April 2002 – December 2003

Introduction

Studies were carried out in 2002 to determine the effect of temperature on the efficacy of selected biocontrol agents (BCAs) against tomato botrytis. These studies were continued in 2003, in order to include *Trichoderma harzianum* (ADAS 01/014) that has appeared promising as a BCA.

Methods

BCA treatments were as follows:

Treatment	Code	Min. product concentration (cfu/g product)	Formulation
1. Sterile distilled water	SDW	-	-
2. *Clonostachys rosea*	H1	-	1 x 10^6 / ml
3. Gliomix	A2	10^7	10g in 100ml
4. *T. harzianum*	01/14	-	1 x 10^6 / ml

The following temperature treatments were tested: 15, 20 and 25^{o}C.

The experiment was planned as a counter balanced split-plot incomplete block design, with four replicates of each temperature treatment. Each week, pair-wise comparisons of temperature treatments were made, using two controlled environment cabinets running simultaneously. In each cabinet, there was one plot of each BCA treatment (1 pot with 10 stems) with the arrangement of BCA treatments randomised. Runs in weeks 4, 5 and 6 were planned to enable the effects of time and cabinet, if any, to be identified.

Week	**Cabinet 1**	**Cabinet 2**
1	15	20
2	20	25
3	25	15
4	20	15
5	25	20
6	15	25

In reality, week 1 temperature regimes were repeated three times in an attempt to get reasonable infection levels on the inoculated control treatment. Once good infection of the control was achieved, week 2 temperature regimes were set up. Again problems with the botrytis isolate prompted us to repeat week 2 temperature regimes. Time constraints halted any further progress with these experiments.

Preparation of plant material

From side shoots of tomato cv. Espero, 3 cm stem sections were cut, avoiding the top 10 cm of the plant. The stem pieces were rinsed in distilled water and wrapped in moist paper until required, ensuring that the upper end of the stem pieces could be identified.

Preparation of pots

Each 9 cm pot was filled with autoclaved vermiculite (121ºC, 20 min) and wetted with distilled water (70 ml per pot). Stem pieces were wounded before application of BCAs and inoculation with *B. cinerea*, by gently crushing the end to be inoculated with a pair of pliers.

Preparation of inoculum

A 7-14 day old sporulating culture of *Botrytis cinerea* (BC02), which was originally isolated from stem lesions on tomato, was used. A spore suspension (5×10^5 spores / ml) of *B. cinerea* was prepared in a 0.1 M glucose and 0.07 M potassium dihydrogen phosphate solution, 3 h prior to inoculation. After experiencing problems with the botrytis isolate, it was necessary to increase the spore concentration to 5×10^6 spores /ml and prepare it 5-6 h in advance of inoculation. Spore suspensions of *Clonostachys rosea* and XHAPP 01/14 were prepared from 7-14 day old cultures, 15 minutes prior to use.

Application of BCAs and *Botrytis* inoculum

The stem pieces for each treatment were dipped into their respective BCA formulation, ensuring that the stem pieces were completely covered with the BCA. The formulation was agitated as necessary before dipping to ensure the BCA was evenly distributed. The stem pieces were then inserted vertically into pots containing sterile vermiculite, with the top ends orientated upwards. Once the stem tissue had absorbed the excess BCA, the stem pieces were inoculated with 20 ul of the spore suspension of *B. cinerea*, to give 10^4 spores per stem. When the spore concentration of 5×10^5 spores / ml was increased to 5×10^6 spores / ml the actual spore application rose from 10^4 to 10^5 spores per stem.

Incubation

Two CE cabinets were run simultaneously each week. For each cabinet, four pots of stem pieces (one of each BCA treatment) were placed within individual perforated polythene bags (left open) in 1 cm depth of water, in a large white plastic tray. The tray was completely enclosed in a polythene bag, ensuring that the bag did not come into contact with the stem pieces. The trays were transferred into a controlled environment cabinet for incubation at one of the test temperatures at 16 h light / 8 h dark, 50% relative humidity regime for 7 days. The bags were checked daily to ensure that excess condensation did not develop (by leaving bags open) or that stems did not dry out (by misting with distilled water from a spray bottle).

An uninoculated check was set up for each temperature treatment, using one pot of ten stems placed in a small tray of water, which in turn was contained within an individual bag, within the controlled environment cabinet.

Results and discussion

Data for single runs of the experiment are presented in Table 2.1. Despite difficulties in replicating the experiment over time, some interesting trends were observed. *C. rosea* seemed to be more effective in reducing stem lesion length at 15-20°C, than at 25°C. This observation reflected results obtained in 2002 (Annual Report, April 2003). In contrast, *T. harzianum* appeared more effective at higher temperatures, while Gliomix showed best activity at 20°C. There is potential to use mixtures of BCAs with different temperature optima.

Table 2.1. Lesion development, sporulation and internal rotting of tomato stem pieces after BCA treatment, inoculation with *Botrytis cinerea* and incubation for 7 days at three temperatures.

	Incubation temperature					
Treatment	15°C		20°C		25°C	
Lesion length (mm)						
SDW	10.2	-	10.1	-	23.8	-
C. rosea	1.3	(87.3)	3.0	(70.3)	20.3	(14.7)
Gliomix	13.5	(-32.4)	6.5	(35.6)	17.2	(27.7)
T. harzianum	14.9	(-46.1)	7.0	(30.7)	10.1	(57.6)
Sporulation index (0-5)						
SDW	2.0	-	2.5	-	0.0	-
C. rosea	0.0	-	0.2	-	0.0	-
Gliomix	1.9	-	0.9	-	0.7	-
T. harzianum	2.3	-	1.1	-	0.0	-
Internal rotting (mm)						
SDW	12.0	-	7.6	-	-	-
C. rosea	7.2	(40.0)	4.8	(36.8)	-	-
Gliomix	9.4	(21.7)	5.0	(34.2)	-	-
T. harzianum	9.9	(17.5)	14.6	(-92.1)	-	-

Figures in brackets are % control compared with the water treatment; - not tested

Diary

Date	**Activity**
19 June 2003	Run 1 : 20ºC and 15ºC experiments set up
26 June 2003	Run 1 experiment assessed
15 July 2003	Run 1 first repeat set up
23 July 2003	Run 1 first repeat assessed
28 July 2003	Run 1 second repeat set up
04 August 2003	Run 1 second repeat assessed
	Run 2 : 20ºC and 25ºC experiments set up
11 August 2003	Run 2 experiment assessed
22 August 2003	Run 2 repeat set up
27 August 2003	Run 2 repeat assessed

3. Investigation of some factors influencing antagonistic activity against *Botrytis cinerea* (replacement for Objective 11.2)

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: April – December 2003

Introduction

Studies were carried out to investigate the following factors that could influence the antagonistic activity of selected BCAs against *B. cinerea* on tomato stems:

- Timing of BCA application in relation to infection by *B. cinerea*
- BCA application rates
- Effects of combined BCA applications

3.1 Application timing in relation to infection events

Objectives

To determine the effect of application timing in relation to inoculation with *Botrytis cinerea* on the efficacy of selected biological control agents (BCAs) against *B. cinerea* on tomato stems.

Methods

The effects of inoculation timing on the efficacy of the following BCAs were tested:

Treatment	Code	Min. product concentration (cfu/g product)	Formulation
1. Sterile distilled water	SDW	-	-
2. *Clonostachys rosea*	H1	-	1 x 10^6 / ml
3. Gliomix	A2	10^7	10g in 100ml
4. *Trichoderma harzianum*	01/14	-	1 x 10^6 / ml

Three timings for BCA inoculation of stems were tested:

Timing Treatment	0 h	24 h	48 h
1. Preventative	BCA	Wound / botrytis	-
2. Co-inoculation	-	Wound / botrytis / BCA	-
3. Curative	-	Wound / botrytis	BCA

The experiment was a split-plot design, with timing as main plots and BCAs as split plots. There were four replicate blocks. A single BCA treatment was applied to ten stem pieces in one pot.

An analysis of variance was performed on data where possible. Where data did not fulfil the normality and homogeneity assumptions for parametric ANOVA, the results were examined by Friedman's non-parametric test.

Preparation of plant material

From side shoots of tomato cv. Carousel, 3-cm stem sections were cut, avoiding the top 10-cm of the plant. The stem pieces were rinsed in distilled water and wrapped in moist paper until required, ensuring that the upper end of the stem pieces could be identified.

Preparation of pots

Each 9-cm pot was filled with autoclaved vermiculite (121^{o}C, 20 min) and wetted with distilled water (70 ml per pot). The stem pieces were inserted vertically into pots containing sterile vermiculite, with the top ends orientated upwards. Irrespective of BCA inoculation timing, stem pieces were wounded immediately before inoculation with *B. cinerea*, by gently crushing the end to be inoculated with a pair of pliers. Stems, which had already been inoculated with BCA treatments, were dipped in ethanol and flamed between pots to prevent BCA cross-contamination.

Preparation of inoculum

A sporulating culture of *Botrytis cinerea* (BC02), originally isolated from stem lesions on tomato, was used. A spore suspension (5 x 10^6 spores / ml) of *B. cinerea* was prepared in a 0.1M glucose and 0.07M potassium dihydrogen phosphate solution, 6 h prior to inoculation. Spore suspensions of *Clonostachys rosea* and *Trichoderma harzianum* (ADAS 01/014) were prepared 15 minutes prior to use.

Application of BCAs and Botrytis inoculum

For each BCA treatment, at each inoculation time, 1 ml of the formulation was applied to each of 10 stems in each of four pots. At the 2nd BCA application time, each stem piece was wounded and inoculated with 20 ul of the *B. cinerea* spore suspension to give 10^5 spores per stem.

Incubation

Four pots of stem pieces (one of each BCA treatment at one inoculation time) were placed within individual perforated polythene bags (left open) in 1 cm depth of water, in a large white plastic tray. The tray was completely enclosed in a polythene bag, ensuring that the bag did not come into contact with the stem pieces. The trays were transferred into a controlled environment cabinet for incubation at 20^{o}C at 16 h light / 8 h dark, 50% relative humidity regime for 14 days. The bags were checked daily to ensure that excess condensation did not develop (by leaving bags open) or that stems did not dry out (by misting with distilled water from a spray bottle).

An uninoculated check was set up for each temperature treatment, using one pot of ten stems placed in a small tray of water, which in turn was contained within an individual bag, within the controlled environment cabinet.

Results and discussion

Irrespective of BCA application time, development of botrytis on tomato stems was minimal even in the inoculated control treatment, with lesions rarely extending beyond the plier wounds on the upper end of the stem pieces (Table 3.1.1). Botrytis sporulation did not develop on stems treated with Gliomix or *C. rosea.*

Because of poor botrytis development on stem pieces even in the inoculated control treatment, incubation conditions in subsequent experiments were modified. Small-scale studies (data not presented) indicated that more consistent disease development occurred when inoculated stem pieces were incubated in natural light conditions. For subsequent trials, inoculated stem pieces were incubated at ambient temperature (20ºC) in ambient lighting (on laboratory bench adjacent to a window).

Table 3.1.1. The effect of BCA timing and BCA treatment on lesion development (mm) of tomato stem pieces after incubation for 14 days at 20ºC

	Treatment			
BCA Timing	Control (SDW)	*C. rosea*	Gliomix	*T. harzianum*
24 h before inoculation with *B. cinerea*	7.57	6.70	5.85	5.28
Co-inoculation with *B. cinerea*	5.60	4.52	4.90	4.05
24 h after inoculation with *B. cinerea*	5.40	4.85	6.35	6.15
Significance	0.746 (skewed)			
Df	27			
SED	1.718			

*****maximum 30 mm stem length

Table 3.1.2. The effect of BCA timing and BCA treatment on *B. cinerea* sporulation (0-5 index) of tomato stem pieces after incubation for 14 days at 20ºC

	Treatment			
BCA Timing	Control (SDW)	*C. rosea*	Gliomix	*T. harzianum*
24 h before inoculation with *B. cinerea*	0.78	0.0	0.0	0.08
Co-inoculation with *B. cinerea*	0.00	0.0	0.0	0.00
24 h after inoculation with *B. cinerea*	0.03	0.0	0.0	0.03
Significance	0.001 (skewed)			
Df	27			
SED	0.144			

*****Sporulation index of 0-5

Diary

Date	Activity
15 September 2003	BCAs applied
16 September 2003	Stem wounding, Botrytis inoculation and BCAs applied
17 September 2003	BCAs applied
30 September 2003	Inoculation timing experiment assessed

3.2 Effect of BCA application rate

Objective

To determine the effect of BCA application rate on the efficacy of selected biological control agents (BCAs) against *B. cinerea* on tomato stems when the BCA and *B. cinerea* are co-inoculated onto freshly wounded stems. This information will help to show how robust are different BCA treatments.

Methods

The effect of three leading BCAs (one product and two isolates) and three rates of application were tested as follows:

Treatment	BCA spores per stem piece	BCA : botrytis ratio	Formulation in SDW (cfu/ml)	Volume applied per stem (ul)
1. SDW (inoculated control)	0	-	-	100
2. *Clonostachys rosea*	10^4	1 : 10	1 x 10^7	1
3. *Clonostachys rosea*	10^5	1 : 1	1 x 10^7	10
4. *Clonostachys rosea*	10^6	10 : 1	1 x 10^7	100
5. Gliomix	10^4	1 : 10	1 x 10^6 (1 g in 10 ml)	10
6. Gliomix	10^5	1 : 1	1 x 10^6 (1 g in 10 ml)	100
7. Gliomix	10^6	10 : 1	1 x 10^6 (1 g in 10 ml)	1000
8. *Trichoderma harzianum**	10^4	1 : 10	1 x 10^7	1
9. *Trichoderma harzianum**	10^5	1 : 1	1 x 10^7	10
10. *Trichoderma harzianum**	10^6	10 : 1	1 x 10^7	100
11. SDW (uninoculated control)	0	-	-	100
12. SDW (inoculated control)	0	-	-	100

*Isolate ADAS 01/14 from tomato

The experiment was a randomised block design with three replicate blocks of 12 treatments. The treatments included two inoculated control treatments and an uninoculated control. A single BCA treatment was applied to ten stem pieces in one pot.

Preparation of plant material

From side shoots of tomato cv. Carousel, 3-cm stem sections were cut, avoiding the top 10-cm of the plant. 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 identified.

Preparation of pots

Thirty-six 9-cm pots were filled with autoclaved vermiculite (121^{o}C, 20 min) and wetted with distilled water (70 ml per pot). Stem pieces were wounded before application of BCAs and inoculation with *B. cinerea*, by gently crushing the end to be inoculated with a pair of pliers. After wounding, the stem pieces were inserted vertically into pots containing sterile vermiculite, with the top ends orientated upwards.

Preparation of inoculum

A 5 month-old sporulating culture of *Botrytis cinerea* (BC02), which was originally isolated from stem lesions on tomato, was used. A spore suspension (5×10^6 spores / ml) of *B. cinerea* was prepared in a 0.1 M glucose and 0.07 M potassium dihydrogen phosphate solution, 6.5 h prior to inoculation. Spore suspensions from isolates of *Clonostachys rosea* and *Trichoderma harzianum,* and the product Gliomix, were prepared 15 minutes prior to use.

Application of BCAs and *Botrytis* inoculum

For each treatment, the BCA formulation was applied to each of 10 stem pieces in each of three pots, using the volumes described previously. Once the stem tissue had absorbed the excess BCA, the stem pieces were inoculated with 20 ul of the *B. cinerea* spore suspension, to give 10^5 spores per stem. Stem pieces for Treatment 11 remained uninoculated.

Incubation

Twelve pots of stem pieces (one of each BCA treatment) for each replicate were placed within individual perforated polythene bags (left open) in 1 cm depth of water, in a large white plastic tray. The tray was completely enclosed in a polythene bag, ensuring that the bag did not come into contact with the stem pieces. The trays were transferred to a laboratory bench adjacent to a window for incubation at 20^{o}C, in ambient light for 12 days. The bags were checked daily to ensure that excess condensation did not develop (by leaving bags open) or that stems did not dry out (by misting with distilled water from a spray bottle).

Assessments

The severity of stem infection was assessed 12 days after inoculation by measuring the lesion length on each stem. A sporulation index for each stem was also recorded as follows:

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 on >75 % of stem length

Lesion length data were subjected to analysis of variance (ANOVA) and Duncan's test. Friedman's non-parametric test was used where the data were not suitable for analysis by ANOVA.

Results and discussion

The incidence of stems that developed lesions was high for all inoculated treatments (>90 %). There was a significant effect of treatment on stem lesion length (Table 3.2.1). *C. rosea* was most effective when applied at 10^5 spores per stem piece, with a slight reduction in efficacy when applied at a higher rate. Gliomix significantly reduced stem lesion length in comparison with the inoculated control, irrespective of the application rates used in this experiment. This suggests that there may be potential for reducing application rates without loss of efficacy, if the treatment were scaled-up for glasshouse use. In contrast, *T. harzianum* was only effective in reducing stem lesion length when applied at the highest rate (10^6 spores per stem piece).

Table 3.2.1. Effects of BCA product and rate of application on lesion development and sporulation of tomato stem pieces after incubation for 12 days at 20°C

Treatment	BCA spores per stem piece	Mean stem lesion length (mm)	**	Mean incidence of stems with lesions*** (out of 10)	Mean sporulation index***
1. SDW inoculated control*	0	11.8	c	10.0	1.8
2. *C. rosea*	10^4	9.9	c	9.7	0.2
3. *C. rosea*	10^5	6.4	c	9.7	0.0
4. *C. rosea*	10^6	6.8	bc	10.0	0.0
5. Gliomix	10^4	6.3	b	10.0	0.2
6. Gliomix	10^5	5.5	b	9.3	0.0
7. Gliomix	10^6	6.3	b	9.7	0.0
8. *T. harzianum*	10^4	8.5	bc	10.0	1.5
9. *T. harzianum*	10^5	7.4	bc	10.0	1.6
10. *T. harzianum*	10^6	5.7	b	10.0	0.7
11. SDW (uninoculated control)	0	0.0	a	0.0	0.0
Significance		0.002			
Df		23		10	10
SED		2.233			
p-value				0.004	0.003

*Treatments 1 and 12 (inoculated controls) combined
**Duncan's suffix; treatments sharing a common letter are not significantly different at P<0.05
***Analysed using Friedman's non-parametric analysis

The higher rates of *C. rosea* and Gliomix (10^5 and 10^6 spores per stem) eliminated botrytis sporulation on the tomato stem pieces, compared with a sporulation index of 1.8 in the inoculated control.

Diary

Date	Activity
02 October 2003	Experiment set up
14 October 2003	Stem pieces assessed

3.3 Combined BCA applications

Objectives

To determine the effect of selected BCAs used either singly or in combination against *B. cinerea* on tomato stems when the BCA(s) and *B. cinerea* are co-inoculated onto freshly wounded stems.

Methods

The effects of the BCA products, used either singly or in combination, on botrytis development were evaluated in three experiments, detailed below:

Experiment 1:

	BCA treatment	BCA spores per stem piece	BCA : botrytis ratio	Formulation in SDW (cfu/ml)	Volume to be applied per stem (ul)
1	SDW (inoculated control)	0	-	-	20
2	*Clonostachys rosea*	10^6	1:1	5×10^6	20
3	Gliomix	10^5	1:10	1×10^6 (1g in 10 ml)	100
4.	Gliomix + *C. rosea* (co-inoculation)	10^5 10^6	1:10 1:1	5×10^6 1×10^6 (1g in 10 ml)	20 100
5	SDW (uninoculated control)	0	-	-	20

Experiment 2:

	BCA treatment	BCA spores per stem piece	BCA: botrytis ratio	Formulation in SDW (cfu/ml)	Volume to be Applied per stem (ul)
1	SDW (inoc control)	0	-	-	20
2	*Clonostachys rosea*	10^6	1:1	5×10^6	20
3	Gliomix	10^5	1:10	1×10^6 (1g in 10 ml)	100
4	*Trichoderma harzianum*	10^6	1:1	5×10^6	20
5	*C. rosea* + Gliomix (co-inoculation)	10^6 10^5	1:1 1:10	5×10^6 1×10^6 (1g in 10 ml)	20 100
6	*C. rosea* + *T. harzianum* (co-inoculation)	10^6 10^6	1:1 1:1	5×10^6 5×10^6	20 20
7	7. Gliomix + *T. harzianum* (co-inoculation)	10^5 10^6	1:10 1:1	1×10^6 (1g in 10 ml) 5×10^6	100 20

Experiment 3:

	BCA treatment	BCA spores per stem piece	BCA: botrytis ratio	Formulation in SDW (cfu/ml)	Volume to be Applied per stem (ul)
1	SDW (inoculated control)	0	-	-	20
2	*Trichoderma harzianum*	10^6	1:1	5×10^6	20
3	*T. harzianum* + *Clonostachys rosea* (co-inoculation)	10^6 10^6	1:1 1:1	5×10^6 5×10^6	20 20
4	*T. harzianum* + Gliomix	10^6 10^5	1:1 1:10	5×10^6 1×10^6 (1g in 10 ml)	20 100

Each experiment was a randomised block design with four replicate blocks of five treatments. A single BCA treatment was applied to ten stem pieces in one pot. Where an uninoculated control was not included as part of the main experiment (Experiments 2 and 3), two pots of uninoculated, untreated stems (10 per pot), were set-up and maintained adjacent to the main experiment.

An analysis of variance was performed on data where possible. Where data did not fulfil the normality and homogenicity assumptions for parametric ANOVA, the results were examined by Friedman's test.

Preparation of plant material

From side shoots of tomato cv. Carousel, 3-cm stem sections were cut, avoiding the top 10-cm of the plant. The stem pieces were rinsed in distilled water and wrapped in moist paper until required, ensuring that the upper end of the stem pieces could be identified.

Preparation of pots

Each 9-cm pot was filled with autoclaved vermiculite (121^{o}C, 20 min) and wetted with distilled water (70 ml per pot). Stem pieces were wounded before application of BCAs and inoculation with *B. cinerea*, by gently crushing the end to be inoculated with a pair of pliers. The stem pieces were then inserted vertically into pots containing sterile vermiculite, with the top ends orientated upwards.

Preparation of inoculum

Sporulating cultures of *Botrytis cinerea* (BC02), originally isolated from stem lesions on tomato, was used. A spore suspension (5×10^6 spores / ml) of *B. cinerea* was prepared in a 0.1 M glucose and 0.07 M potassium dihydrogen phosphate solution, 6.5 h prior to inoculation. Spore suspensions from isolates of *Clonostachys rosea* and *Trichoderma harzianum* (ADAS 01/014) and the product Gliomix were prepared for each experiment as described in the tables above.

Application of BCAs and *Botrytis* inoculum

For each treatment, the BCA formulations were applied to each of 10 stem pieces in each of three pots, using the volumes described above. Once the stem tissue had absorbed the excess BCA, the stem pieces were inoculated with 20 ul of the *B. cinerea* spore suspension, to give 10^5 spores per stem. Stem pieces for Treatment 5 in Experiment 1 remained uninoculated.

Incubation

For each replicate, one pot of stem pieces for each BCA treatment was placed within

individual perforated polythene bags (left open) in 1 cm depth of water, in a large white plastic tray. The tray was completely enclosed in a polythene bag, ensuring that the bag did not come into contact with the stem pieces. The trays were transferred to a laboratory bench adjacent to a window for incubation at 20°C in ambient light for 7 days. The bags were checked daily to ensure that excess condensation did not develop (by leaving bags open) or that stems did not dry out (by misting with distilled water from a spray bottle).

Disease assessments

The severity of stem infection was assessed 7 days after inoculation with *B. cinerea* by measuring the length of lesion on each stem. In addition the sporulation on each stem was recorded as follows:

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 50-75 % of stem length
5 = sporulation on >75 % of stem length

Each stem was then cut longitudinally, and the length of internal rotting within the stem was measured from the top of the stem downwards.

Results and discussion

Experiment 1

All BCA treatments gave significant reduction in stem lesion length and internal rotting compared with the inoculated control treatment (Table 3.2.1). There was no botrytis sporulation on stems treated with BCAs. Marginally better control was obtained using Gliomix either singly or combined with *C. rosea*, compared with *C. rosea* on its own.

Table 3.3.1. The effect of BCA products used singly or in combination on botrytis development on tomato stem pieces after incubation for 7 days at 20°C (Experiment 1)

Treatment	BCA spores per stem piece	Mean stem lesion length (mm)	Mean incidence of lesions (out of 10)*	Mean sporulation index (0-5)*	Stem internal rotting (mm)
1. SDW (inoculated control)	0	9.2	10.0	2.1	13.2
2. *Clonostachys rosea*	10^6	6.5	10.0	0.0	11.7
3. Gliomix	10^5	5.2	10.0	0.0	8.3
4. Gliomix + *C. rosea* (co-inoculated)	10^5 10^6	4.5	9.8	0.0	9.0
5. SDW (uninoculated control)	0	1.1	2.3	0.0	1.5
Significance (12df)		<0.001			<0.001
SED		1.09			1.08
p-value (4 df)			0.006	0.003	

*Analysed using Friedman's non-parametric test

Experiment 2

There was no significant effect of treatment on either lesion development (Table 3.2.2) or internal stem rotting (data not presented). As in Experiment 1, there was no botrytis sporulation when *C. rosea* and Gliomix were applied either singly or in combination. Low levels of botrytis sporulation still occurred when *T. harzianum* treatments were applied.

Table 3.3.2. The effect of BCA products used singly or in combination on botrytis development on tomato stem pieces after incubation for 7-8 days at 20°C (Experiment 2)

Treatment	BCA spores per stem piece	Mean stem lesion length (mm) (max. 30mm)	Mean incidence of lesions (out of 10)*	Mean sporulation index (0-5)*
1. SDW (inoculated control)	0	8.6	9.8	2.0
2. *Clonostachys rosea*	10^6	9.9	10.0	0.0
3. Gliomix	10^5	9.8	10.0	0.0
4. *T. harzianum*	10^6	6.9	10.0	1.1
5. *C. rosea* +	10^6	9.8	10.0	0.0
Gliomix (co-inoculation)	10^5			
6. *C. rosea* +	10^6	12.6	10.0	0.1
T. harzianum (co-inoculation)	10^6			
7. Gliomix +	10^5	10.5	10.0	0.1
T. harzianum (co-inoculation)	10^6			
Significance (18 df)		0.553		
SED		2.68		
Significance (6 df)			0.424	0.001

*Analysed using Friedman's non-parametric test

Experiment 3

As in Experiment 2, there were no significant treatment effects on stem lesion development (Table 3) or internal rotting (data not presented). *T. harzianum* in combination with either Gliomix or *C. rosea* resulted in nil botrytis sporulation.

Table 3.3.3. The effect of BCA product used singly or in combination on lesion development and sporulation of tomato stem pieces after incubation for 7 days at 20°C (Experiment 3)

Treatment	BCA spores per stem piece	Mean stem lesion length (mm) (max. 30mm)	Mean incidence of stem lesions (out of 10)	Mean sporulation index (0-5)
1. SDW (inoculated control)	0	8.8	10	2.0
2. *T. harzianum*	10^6	7.4	10	0.8
3. *T. harzianum* + *Clonostachys rosea* (co-inoc)	10^6 10^6	8.5	10	0.0
4. *T. harzianum* + Gliomix	10^6 10^5	8.7	10	0.0
Significance (9 df)		0.698	N/A	
SED		1.273		
Significance (3 df)			N/A	0.008

Diary

Experiment 1:

Date	**Activity**
17 October 2003	Combination experiment 1 set up
24 October 2003	Stem pieces assessed

Experiment 2:

Date	**Activity**
28 October 2003	Combination experiment 2 set up
4-5 November 2003	Stem pieces assessed

Experiment 3:

Date	**Activity**
11 November 2003	Combination experiment 3 set up
18 November 2003	Stem pieces assessed

4. Evaluation of antagonists for commercial production (Objective 10)

Study Director: Professor J Whipps
HRI Wellesbourne, Wellesbourne, Warwicks

Status of work: Completed

Period covered: April – December 2003

Introduction

Following the screening exercises, formal identification of the lead strains and assessment of their potential for future use, only one new strain, XHAPP 01/14 (IMI 390736) *Trichoderma harzianum* Rifai remained. Consequently, production systems were focussed on the ability to produce inoculum of this fungus only.

In general there are two basic procedures for large-scale inoculum production of fungal biological disease control agents – solid substrate fermentation using some kind of grain or nutrient soaked support material, and liquid fermentation in a nutrient broth (McQuilken and Budge, 1995; McQuilken *et al*., 1997). The complete fermentation product can be used as inoculum in some cases, but generally, spores are recovered and subsequently formulated for application in the glasshouse or field.

Consequently, the aim of this work was to examine a range of solid substrates in solid fermentation and two liquid media for suitability as systems for future large scale production of inoculum of *T. harzianum*.

Materials and methods

1) Production of solid-substrate inocula

Eight solid-substrate media all used previously from production of fungal BCAs were tested for producing inocula of *T. harzianum*.

1. Barley (1 l barley grain + 450 ml tap water)
2. Bran-vermiculite (1.3 l wheat bran + 500 ml graded horticultural vermiculite + 600 ml tap water)
3. Bran-sand (1.6 l wheat bran + 150 ml silver sand + 300 ml tap water)
4. Maizemeal-perlite (2 l micronised flaked maizemeal + 10 l horticultural grade perlite + 400 ml tap water)
5. Oats (1 l oat grain + 450 ml tap water)
6. Peat-bran (1 l Irish sphagnum moss peat + 1 l wheat bran + 900 ml tap water)
7. Rice (600 ml American long grain rice + 400 ml tap water)
8. Wheat (1 l wheat grain + 450 ml tap water)

Mixtures of each medium in mushroom spawn bags (22.5 x 56 cm with a microporous strip 15 cm wide; Van Leer UK Ltd., Poole, Dorset) were autoclaved twice on two consecutive days at 121 ^{o}C for 15 min. Three replicate bags of each solid-substrate were each inoculated with *c.* 100 ml of spore suspension containing *c.* 2 x 10^6 spores ml^{-1} in sterile distilled water (SDW). The spore suspension was prepared by flooding 14 d old Potato Dextrose Agar (PDA) Petri dish cultures of *T. harzianum* with SDW and gently scraping the colony surfaces with a spatula. Bags were incubated at 20 ^{o}C with a 12 h daylength for 14 d during which time they were agitated periodically.

Following incubation, three replicate 1 g samples of inocula contained spores and mycelium were removed from each bag and serial dilutions made in SDW and plated on to PDA containing Triton X-100 (2ml l^{-1}) and chlortetracycline (2 ml l^{-1}). The plate cultures were incubated at 20 ^{o}C with a 12 h daylength for 4 d and colonies of *T. harzianum* counted and colony forming units (cfus) g^{-1} fresh inoculum calculated.

2) Production of liquid culture inocula

The growth of *T. harzianum* in terms of biomass, cfus and spore production in two liquid media was examined.

1. Molasses-yeast liquid medium (MYM) (30 g cane molasses + 5 g autolysed yeast l^{-1} distilled water)
2. Potato Dextrose Broth (PDB)

100 ml of each medium was dispensed into 500 ml conical flasks and autoclaved at 121 ^{o}C for 15 min. Spore suspensions of *Trichoderma* sp. for inoculating the liquid media were prepared as described previously. Three replicate flasks of each medium were inoculated with 3 ml of spore suspension containing *c.* 2 x 10^6 spores ml^{-1} and incubated at 20 ^{o}C with a 12 h daylength. The flasks were incubated either statically or shaking for 7 and 14 d after which time the fungal biomass was homogenised for 2 min and an aliquot retained to quantify conidial production. The biomass was then subjected to vacuum filtration on to Whatman no. 4 filter paper. A 0.1 g sample of fresh biomass from each flask was removed from the filter paper and serial dilutions made in SDW and plated on to PDA containing Triton X-100 (2 ml l^{-1}) and chlortetracycline (2 ml l^{-1}). The plate cultures were incubated at 20 ^{o}C with a 12 h daylength for 4 d and colonies of *Trichoderma* sp. counted and cfus g^{-1} fresh inoculum calculated. The remaining biomass was air dried for 14 d and then weighed.

Results and discussion

Trichoderma harzianum grew on all the solid-substrates tested (Table 1). Growth, in terms of cfu g^{-1} substrate, was similar on most of the substrates with only rice giving a slightly lower cfu count.

Table 4.1. Growth of *Trichoderma harzianum* on different solid-substrates in spawn bags at 20 ^{o}C after 14 d. Values are the mean of three replicates.

Solid-substrate	**Colony forming units g^{-1}**		
	Bag 1	**Bag 2**	**Bag 3**
Barley	7.15 x 10^8	1.26 x 10^9	1.97 x 10^9
Bran + Vermiculite	2.33 x 10^9	2.53 x 10^9	4.47 x 10^9
Bran + Sand	2.50 x 10^9	1.60 x 10^9	2.08 x 10^9
Maizemeal + Perlite	2.07 x 10^9	2.27 x 10^9	1.80 x 10^9
Oats	2.10 x 10^9	2.40 x 10^9	2.80 x 10^9
Peat + Bran	3.00 x 10^9	2.63 x 10^9	1.50 x 10^9
Rice	4.40 x 10^8	6.57 x 10^8	3.03 x 10^8
Wheat	1.60 x 10^9	1.30 x 10^9	1.12 x 10^9

Trichoderma harzianum grew in the two liquid media (MYM and PDB) tested, in both static and shaking culture (Table 4.2). Growth of the isolate was different in the two media and in the static and shaking cultures. The isolate grew better in MYM than in PDB in terms of the biomass, cfu and spores produced. In addition, growth in both media in terms of biomass produced was improved when the cultures were shaken.

Table 4.2. Growth of *Trichoderma harzianum* in static and shaking liquid culture (MYM and PDB). Values are the mean of three replicates. ND = not determined.

	Dry Biomass (g)	**CFU g^{-1} wet biomass**	**Spore ml^{-1} medium**
STATIC			
7 d harvest			
MYM	0.27	6.33 x 10^8	1.58 x 10^8
PDB	0.04	7.27 x 10^6	5.77 x 10^6
14 d harvest			
MYM	0.35	3.40 x 10^8	2.50 x 10^8
PDB	0.14	3.90 x 10^7	7.17 x 10^6
SHAKING			
7d harvest			
MYM	1.09	4.40 x 10^8	3.17 x 10^7
PDB	0.28	2.81 x 10^7	2.39 x 10^7
14 d harvest			
MYM	0.95	4.57 x 10^8	ND
PDB	0.46	1.17 x 10^6	ND

Conclusions

- *Trichoderma harzianum* can be successfully grown in both solid substrate and liquid fermentation using a range of common nutrient sources.
- As production of both mycelial biomass and conidia was successful in both culture systems it should be possible to work in collaboration with variety of commercial companies to produce inocula of this BCA in the future if desired.

References

McQuilken, M.P. & Whipps, J.M. 1995. Production, survival and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum*. European Journal of Plant Pathology, **101**, 101-110.

McQuilken, M.P., Budge, S.P. & Whipps, J.M. 1997. Production, survival and evaluation of liquid culture-produced inocula *of Coniothyrium minitans* against *Sclerotinia sclerotiorum*. Biocontrol Science and Technology, **7**, 23-36.

5. Evaluation of potential BCAs in a long-season glasshouse trial (Objective 11.1)

Study Director: Dr Tim Pettitt
HRI Wellesbourne, Wellesbourne, Warwicks

Site manager: Mike Wainwright, HRI Efford

Status of work: Completed

Period covered: April – December 2003

Introduction

One BCA product and one isolate were selected for further evaluation. They were tested for their activity in reducing botrytis stem rot on whole tomato plants in a glasshouse trial at HRI Efford.

Materials and methods

A glasshouse trial was conducted from April to October 2003 on tomato plants cv. Espero. The performance of *C. rosea* and Gliomix was compared against an untreated control and a commercial programme of six fungicide sprays. The two BCAs used were selected from the isolates and products found to be most promising in the stem bioassay and by the glasshouse trial in 2002. The *T. harzanium* isolate (01/14) was not included because, at the time the trial commenced, this fungus had not been fully identified and its potential for use on tomato was unknown. Although Stimagro performed well in bioassay tests, this product was not tested further because of the relatively high treatment cost if it were to be used as a spray on tomatoes.

The trial comprised 1 compartment of M Block at HRI Efford, with 192 plants (see Appendix 1 for trial plan). There were three replicate blocks of four treatments arranged in a randomised block design. Each plot consisted of two adjacent slabs carrying 4 propagation cubes, each with 2 single-headed plants (16 plant heads per plot).

Slab contact was on 25 March. The plants were grown according to commercial practice (following the advice of G. Hayman), with the exception that spent fruit trusses were not removed. This was to create a high disease pressure by encouraging potentially aggressive botrytis infections to develop in these senescent tissues.

Inoculations of BCA treatments were conducted using a Hozelock 'Polyspray 3' pressure sprayer, directed at the stem from the base up to and including the lowest layer of leaves. Spraying was carried out to the point of whilst avoiding much runoff. This was relatively straightforward to judge visually, especially with the highly visible Gliomix preparation. BCAs were freshly prepared for inoculation in liquid shake culture immediately prior to each inoculation (application). The two BCA treatments were applied around every 14 days from one week prior to the first de-leafing (9 May), through to 30 September (12 BCA applications in total). The precise timing of BCA sprays was determined by the timing of de-leafing operations (BCAs were always applied after de-leafing operations) and, where possible, avoided hot weather

conditions. The interval of application ranged from 11 to 15 days, application dates being: 16 and 30 May, 10 and 25 June, 8 and 22 July, 5 and 19 August, 2, 16 and 30 September and 14 October.

Fungicide sprays were applied using the same Hozelock 'Polyspray 3' pressure sprayer as used for the BCA treatments. The treatments were Elvaron WG (16 May), Scala (30 May), Rovral WP (18 June), Scala (17 July), Elvaron WG (19 August) and Scala (16 September). For commercial realism, the fungicide spray timings were set according to the prevailing potential disease risks, in consultation with G. Hayman and T. O`Neill, and using disease intelligence and local climatic data for judgements.

Sporulating cultures of *B. cinerea* (isolate BC02) were placed in the glasshouse, 1 per plot on 30 May, and fresh plates were introduced weekly. Additional isolates, collected from other tomato crops at HRI Efford (isolates B109-B112), were also introduced from 26 August onwards.

By the end of August 2003 very little disease was seen in any of the treatments. It was therefore decided to attempt to increase the potential disease pressure by altering the greenhouse conditions. This comprised a reduction of the minimum pipe temperature settings from 20ºC to no pipe heat at all and changing the venting temperatures from 17º heat and 18º vent to 15º heat and 15.5º vent at night, and altering the venting for the morning from 18º heat and 19º vent to 18º heat and 22º vent until 10:30 a.m. when it was switched back to 18º heat and 19º vent. The changed venting was to maximise the possibility of condensation occurring on the stems. In addition, the paths in the greenhouse compartment were wetted down with a hose-pipe at the end of each day. These changes were carried out towards the end of week 36 (see Appendix 2 for records of daily mean temperatures and humidities within compartment M13 where the trial was located).

The incidence and severity of botrytis stem lesions were assessed on 11 July, 28 July, 15 August, 2 September, 22 September and 13 October. Each stem was examined and lesions were classed according to their origin (fruit truss die-back, de-leafing wound, stem crack, other) and the extent of stem rot (1 = slight; 2 = moderate; 3 = severe). Using this information, a stem rot severity index was calculated using the formula:

$$\frac{\text{(no. slight lesions x 1) + (no. moderate lesions x 2) + (no. severe lesions x 3)}}{\text{total no. plants in treatment}}$$

At the end of the trial (27 October) the numbers of dead heads were counted in each treatment.

Results were examined for statistical significance by Fisher's exact test. Additionally, not withstanding the problems of pairwise comparisons, certain pairwise comparisons were also carried out as a guide to aid interpretation of results.

For the Fisher test, the total number of potential stem infection sites per treatment was assumed to be 240 (i.e. 5 sites/plant) on 28 July and 480 (i.e. 10 sites/plant) on 22 September and at later assessments.

Results and discussion

Botrytis stem rotting was first observed on 27 June and developed slowly over the next

12 weeks. The fungus was first observed on leaf debris during the same week as stem lesions were first noticed. The progress of disease was slow until October when there was a large increase in lesion numbers (Figure 5.1) and the severity of disease (Figure 5.2) in all treatments except the fungicide programme.

There were significant differences between treatments in the total number of botrytis stem lesions, and also in the numbers of dead plants at the end of the trial (Table 5.1). A pairwise comparison of stem lesion data for 22 September, indicates that the two biocontrol treatments and the fungicide are significantly different from the untreated. Also, that there was no significant difference between the *C. rosea* and Gliomix in their efficacy. By the end of the trial (27 October), over 91% of untreated plants had died from botrytis stem rot, whilst only 8% were dead in the fungicide treated plots. The two BCA treatments reduced disease both in terms of incidence and severity. Although these treatments showed 67% (*Clonostachys rosea*) and 48% (Gliomix) mortality by the end of October, they had successfully and significantly reduced the rate of disease development (Figures 5.1 and 5.2), indicating promise as potential components for an integrated control strategy potentially to reduce the number of fungicide spray treatments.

Table 5.1. Effect of two biocontrol agents and a fungicide programme on incidence of botrytis stem lesions and plant death – 2003.

Treatment	Total number of stem lesions per 48 plants					Total number of dead plants (of 48)
	11 July	28 July	2 Sep	22 Sep	27 Oct	27 Oct
Untreated	9	9	13	18	45	44
Fungicide programme	0	0	0	0	0	4
C. rosea	1	2	5	6	20	32
Gliomix	0	2	3	4	15	23
Fisher statistic	-	11.9	-	24.6	62.4	80.1
Probability	-	0.003	-	<0.001	<0.001	<0.001

Table 5.2. Pairwise comparison of the effect of selected treatments on the total number of stem lesions – 22 September 2003.

Comparison	Fisher statistic	Probability
Untreated vs Fungicide	21.96	0.000
Untreated vs C. rosea	6.17	0.021
Untreated vs Gliomix	9.37	0.004
Untreated vs C. rosea	6.11	0.031
Fungicide vs Gliomix	3.72	0.124
C. rosea vs Gliomix	0.419	0.753

Observations on the sources of stem lesions in all treatments showed that whilst small numbers originated from splitting wounds and leaf scars, the majority of lesions arose at points of fruit truss die-back (Table 5.3).

Reduction of the minimum pipe temperature to zero and changing the vent settings in

early September was associated with an increase in mean 24 hour RH from around 80 % (HD 3.8) to over 90 % (HD 1.7). The rapid increase in appearance of botrytis stem lesions occurred 8 weeks later. This observation supports the use of humidity control to reduce the risk of stem botrytis in tomato.

Gliomix resulted in a very noticeable white deposit on fruit. Further work is needed on formulation and/or targeted application, if development of this product for registration as a biocontrol agent for use on tomato crops is to be pursued.

Table 5.3 Origins of botrytis stem rot infections in the 2003 Efford trial in greenhouse compartment M13

Assessment date	Origin of infection		
	Leaf scar	Spent truss	Splits in stem
11 July	2	7	1
28 July	2	11	1
15 August	4	13	1
2 September	4	16	1
22 September	4	23	1
13 October	7	72	1

Conclusions

- Under high botrytis disease pressure, the two BCA treatments tested (*Clonostachys rosea* and the Gliomix formulation) gave significant disease control.
- The best botrytis stem rot disease control was achieved using a programme of six fungicide sprays from May to September.
- The two BCA preparations showed promise as potential components of an integrated disease control strategy and their efficacy and compatibility with fungicides warrants further investigation.

Figure 5.1 Effects of *Clonostachys rosea* and Gliomix on the total number of botrytis stem lesions per 48 plants in comparison with a commercial fungicide spray programme

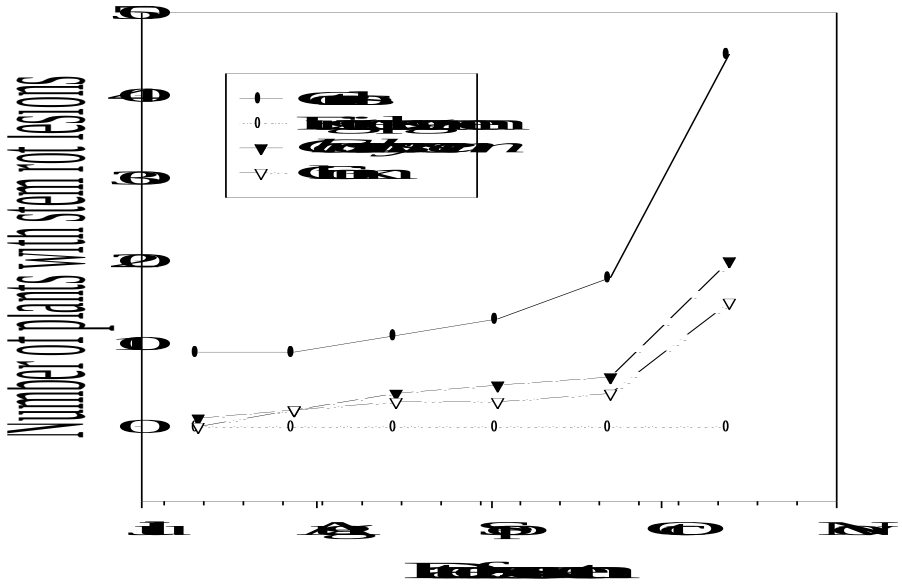
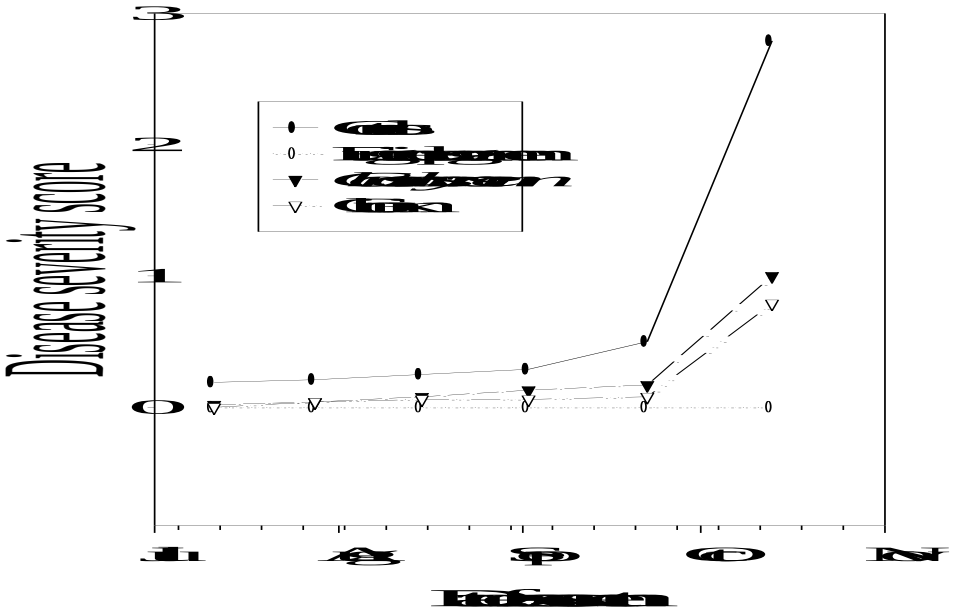


Figure 5.2 Effects of *Clonostachys rosea* and Gliomix on the severity of botrytis stem rot (as measured by a symptom severity score, 0-3) in comparison with a commercial fungicide spray programme.



5. Overall conclusions

Potential biocontrol agents

1. Six out of 49 microorganisms isolated from UK tomato crops were shown by agar plate challenge tests to have useful activity against *B. cinerea*. All isolates have been placed in long-term storage at HRI Wellesbourne.

2. A tomato stem piece bioassay was used to assess biocontrol products and microorganisms. Five showed useful levels of activity (*Clonostachys rosea*, Gliomix, QRD 131, Stimagro and Yield Plus).

3. The isolates identified as showing useful activity against *B. cinerea* by *in vitro* and *in vivo* tests were not identical. For example, *T. harzianum* (01/14), which subsequently performed well in a crop trial, showed good activity *in vitro* and not *in vivo* (stem piece bioassay).

Potential for commercial production

4. A range of solid and liquid fermentation media, using common nutrient sources (e.g. cereal grains), is suitable for future large-scale production of *T. harzianum* (isolate 01/14).

Effect of temperature and humidity on activity

5. CE cabinet bioassay studies on tomato stem pieces showed that Stimagro gave control at all temperatures tested being most effective at 20°C. *C. rosea* showed activity at 15-20°C and little at 25°C. Gliomix activity was most consistent at 20°C. YieldPlus showed slight activity at all temperatures. *T. harzianum* (01/14) was more effective at 20-25°C than at 15°C.

6. At 20°C, Stimagro exhibited greater efficacy in reducing stem rot at 85% RH than at 70% RH.

Effect of BCA rate and combined treatments

7. Gliomix was as effective in controlling botrytis stem rot in bioassays was when used at a low rate as at a high rate, suggesting this may be a more robust treatment than the two other BCAs tested (*C. rosea* and *T. harzianum* 01/14).

8. A combined treatment of *C. rosea* with Gliomix reduced botrytis lesion development in a stem piece bioassay by 51%, compared with 43% when Gliomix alone was used and 29% with *C. rosea* alone.

Performance in crop trials

9. In a short-term trial on a mature crop of cv. Espero in 2002, although no stem lesions had developed 6 weeks after inoculation of deleafing sites, the fungus was readily recovered from internal stem tissue at the inoculation sites. Assuming such infections eventually develop into stem lesions, this suggests there may be a latent period of at least 6 weeks between infection of wound sites by *B. cinerea* conidia and the occurrence of visible stems lesions.

10. Assessment of biocontrol efficacy in this 2002 trial, judged by extent of internal

stem browning beyond the inoculation site, indicated that four biocontrol products and five microorganisms reduced disease. Particularly effective were *T. harzianum* (isolate 01/14) and *C. rosea*, equal in effectiveness to the fungicide Scala when used as a preventative spray.

11. In a full-season trial on cv. Espero in 2003, *C. rosea* and Gliomix applied at 14-day intervals (12 sprays in total) to the lower stem, gave significant reductions (~50%) in stem botrytis. Control was less effective than that of a 6-spray fungicide programme of alternating sprays of dichlofluanid, pyrimethanil and iprodione up to early October.

6. Technology transfer

Articles

- O`Neill TM (2001). Biocontrol options for tomato stem botrytis. *HDC News* **74**, 18-19.
- Green K, O`Neill TM & Pettitt T (2003). Biocontrols for tomato botrytis. *HDC News* **92**, 24-25.
- O'Neill TM, Green K, Pettitt T & Whipps J (2004). Biocontrol of tomato stem botrytis – a step closer. *HDC News* (in press).
- O'Neill TM, Green K, Pettitt T & Whipps J (2004). Screening of micro-organisms for biological control of tomato stem rot caused by *Botrytis cinerea. Proceedings XIII International Botrytis Symposium,* Antalya, Turkey, 26-30 October 2004 (abstract submitted).

Summary progress reports to consortium members
Report 1, April 2001
Report 2, August 2001
Report 3, December 2001
Report 4, August 2002
Report 5, January 2003
Report 6, July 2003

Scientific progress meetings
Project start up meeting, HRI Wellesbourne, 14 March 2000
Bioassay progress meeting, ADAS Arthur Rickwood, 28 November 2000
Project review meeting, ADAS Arthur Rickwood, 27 July 2001
Project progress meeting, HRI Efford, 4 December 2002 (TMO, MW)
Project progress meeting, HRI Efford, 24 March 2003 (TMO, MW)
Project review meeting, ADAS Arthur Rickwood, 11 April 2003

Presentations

1. Southern Tomato Growers Seminar, HRI Stockbridge House, 13 June 2000 (MMcP)
2. Report to TGA Technical Committee, FEC, Warwicks, September 2002 (TMO)
3. Progress with biocontrol of tomato stem botrytis, Tomato Conference, Coventry, 2 October 2003 (TMO)

Project review meetings
HRI Wellesbourne, 6 March 2001

ADAS Arthur Rickwood, 6 March 2002
ADAS Arthur Rickwood, 11 April 2003
ADAS Arthur Rickwood, 25 February 2004

7. Acknowledgements

We thank Martin McPherson of HRI Stockbridge House who was instrumental in getting this project started and who, with his colleagues Andy Jackson, Cheryl Brewster and Catharine Lambourne, worked on this project in year 1, until closure of the site by HRI. We also thank John Overvoorde, John Drew, Paul Challinor, Philip Pearson and Gerry Hayman for their support and advice in helping to steer the project. In addition, we thank all of the companies and researchers who have given product samples or antagonistic isolates as well as technical information for use in this project. We also thank Rodney Edmonson, Biometrics Department, HRI Wellesbourne and Doug Wilson, ADAS statistician for their support in experimental design and statistical analyses.

Appendix 1. Trial plan for the Efford experiment carried out in greenhouse compartment M13 in 2003.

Row 1	Plot 3	Plot 6	Plot 9	Plot 12
	4	**2**	**3**	**1**
Row 2	Plot 2	Plot 5	Plot 8	Plot 11
	3	**1**	**4**	**2**
Row 3	Plot 1	Plot 4	Plot 7	Plot 10
	1	**3**	**2**	**4**

Appendix 2

Week 14

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	24.6	18	21.5	66	70	67.9	0	0	0	80.9	54.7	12.8	3.1
2	20.1	17.7	19	83	75	79.3	2.9	3.8	3.3	85.8	66	5.2	2.5
3	23.3	18	20.9	73	76	74.4	5.8	3.6	4.8	84.6	61.5	9.5	2.6
4	23.6	18.1	21.1	73	80	76.2	6	3	4.6	85.6	62.5	9.3	2.5
5	25	18.3	22	63	80	70.7	8.9	3.2	6.3	85.8	47.8	13.9	2.5
6	24.7	18.2	21.8	69	78	73.1	7.5	3.4	5.6	86.2	52.2	12.7	2.4
7	21.7	17.7	19.9	84	79	81.8	3.1	3.2	3.1	87	74.3	5	2.2
Week 15													
1	25.2	17.4	21.7	78	71	74.9	5.5	4.3	5	84	70.2	8.6	2.5
2	25	17.4	21.6	74	70	72.2	6.3	4.4	5.5	84.4	60.4	11.2	2.6
3	22.1	17.4	20	83	73	78.6	3.4	4	3.7	87.1	66.3	5.4	2.3
4	22.4	17.9	20.4	81	73	77.5	4	4.1	4	86.7	69	6.9	2.4
5	22.4	17.9	20.4	85	72	79.3	3	4.2	3.5	88.4	69.7	4.7	2.4
6	23.5	18.1	21.2	82	80	81.1	3.9	3.2	3.6	88.3	69.3	6.2	2.4
7	23.4	18.3	21.2	83	84	83.4	3.6	2.6	3.2	88.3	76.3	5.1	2.3
Week 16													
1	22.7	18.2	20.8	84	82	83.1	3.3	2.8	3.1	86.8	78.7	4.6	2.3
2	24.6	18.4	22	79	80	79.4	4.9	3.1	4.1	89.4	71.5	8.2	2.1
3	25.2	18.6	22.4	68	80	73.1	7.8	3.2	5.9	88.1	53.7	12.7	2.3
4	25.7	18.6	22.7	73	80	75.9	6.7	3.2	5.2	89.3	55	10.6	2.2
5	25.2	18.2	22.3	73	75	73.8	6.5	3.9	5.4	89.1	51.2	10.6	2.3
6	20.5	18.1	19.5	85	79	82.5	2.7	3.2	2.9	88.7	65	5.8	2.2
7	22.8	18.2	20.9	83	83	83	3.5	2.7	3.2	89.3	74.7	6.3	1.9

Week 17

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	23.3	18.1	21.2	84	76	80.7	3.4	3.7	3.5	89.3	77.3	5.2	2
2	24.4	18.1	21.8	80	78	79.2	4.6	3.4	4.1	89.8	71.2	6.7	2.1
3	24.6	18.4	22.1	85	82	83.8	3.4	2.9	3.2	88.8	73.9	4.6	2.5
4	23.3	19.2	21.6	86	85	85.6	2.8	2.5	2.7	92.3	78.7	3.9	1.5
5	20.5	18.2	19.6	88	83	86	2.1	2.6	2.3	91.7	80.7	3	1.6
6	23.4	18.2	21.3	87	83	85.4	2.6	2.7	2.6	91.4	80.7	3.5	1.7
7	22.2	18.2	20.6	87	83	85.4	2.5	2.7	2.6	90.2	80.9	3.3	2
Week 18													
1	20.2	18.1	19.4	87	81	84.6	2.3	2.9	2.5	89.8	78.9	3.7	1.7
2	22	17.4	20.2	85	85	85	2.9	2.3	2.7	89.1	79.3	3.7	2.2
3	22.9	17.1	20.6	84	82	83.2	3.4	2.6	3.1	91	75.1	5.3	1.8
4	22.6	17.4	20.6	83	84	83.4	3.4	2.4	3	89.1	77.6	4.9	2.2
5	21.2	16.9	19.5	85	81	83.5	2.7	2.7	2.7	90.5	81.3	3.6	1.9
6	21.9	17.2	20.1	86	83	84.8	2.8	2.5	2.7	88.8	80.3	3.9	2.2
7	23.8	17.7	21.5	77	82	78.9	5	2.7	4.1	89.8	64.5	8.1	2.1
Week 19													
1	23	17.1	20.8	81	78	79.9	4	3.2	3.7	89.1	68.7	6.2	2.1
2	22.6	17	20.5	72	81	75.4	5.9	2.7	4.7	89.1	59.1	9.9	2.1
3	23.2	17	20.9	76	83	78.6	5.2	2.5	4.2	86.6	67.2	8.2	2.4
4	22	17.1	20.2	78	82	79.5	4.3	2.6	3.7	88	65.2	7.6	2.1
5	22.6	16.2	20.2	74	82	77	5.4	2.5	4.3	86.8	67.9	7.5	2.3
6	20.2	16	18.7	84	84	84	2.8	2.2	2.6	86.8	79.2	4	2.1
7	20.6	16	18.9	84	83	83.6	3	2.3	2.7	88	78.4	4.3	2

Week 20

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	20.6	15.9	18.9	84	82	83.3	3	2.5	2.8	87.3	77	4.2	2.2
2	21.5	15.9	19.5	74	81	76.5	5.1	2.6	4.2	86.8	58.8	8.8	2.3
3	22.1	16	19.9	68	81	72.6	6.5	2.6	5.1	86.5	50.8	10.5	2.4
4	21.7	16.6	19.9	75	84	78.2	4.9	2.3	4	86.7	66.1	7.1	2.3
5	19.1	17	18.4	87	88	87.4	2.1	1.7	2	89.2	84.3	2.4	1.7
6	19.9	15.8	18.5	87	85	86.3	2.3	2	2.2	89.2	84.2	3.1	1.5
7	18.9	16.1	17.9	87	86	86.7	2.1	1.9	2	88.5	84.1	2.4	1.8

Week 21

1	20.7	15.9	19	81	82	81.3	3.5	2.4	3.1	87.3	70.5	6.2	1.8
2	20.6	15	18.7	79	83	80.4	3.8	2.2	3.3	85.8	66.9	6.5	2.1
3	20	15.6	18.5	85	89	86.4	2.7	1.5	2.3	88	80	4.1	1.6
4	18.9	15.1	17.6	87	89	87.7	2.2	1.4	1.9	91	83.2	3.2	1.2
5	19.1	15.1	17.7	87	87	87	2.3	1.7	2.1	91.7	83.5	3	1.1
6	21	15.1	19	79	85	81	4.1	2	3.4	90.8	65.2	7.9	1.2
7	21.1	15.2	19.1	71	82	74.7	5.7	2.3	4.6	87	58.1	8	1.6

Week 22

1	21.7	16.2	19.9	74	87	78.4	5.2	1.8	4.1	88.3	65.5	7.7	1.6
2	19.7	16.3	18.6	85	91	87	2.8	1.3	2.3	91.9	70.3	6.6	1.1
3	22.9	15.9	20.6	75	87	79	5.7	1.9	4.4	93.1	62.5	9.3	0.9
4	24.5	16.9	22	71	85	75.7	7.1	2.3	5.5	90.2	60.6	12.2	1.3
5	24.1	17.1	21.8	68	82	72.6	7.5	2.7	5.9	90.1	58.5	12.1	1.4
6	22.8	16.2	20.6	75	86	78.7	5.3	1.9	4.2	87.8	66	7	1.8
7	21.6	16.4	19.9	84	87	85	3.2	1.8	2.7	92.8	76.4	5.7	1.1

Week 23

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	21.4	16.5	19.8	79	87	81.6	4.2	1.8	3.4	93.2	68.6	6.9	1
2	20.1	15.9	18.7	87	90	88	2.3	1.3	2	91.7	77.8	3.9	1.3
3	20.7	15.3	18.9	83	86	84	3.4	1.9	2.9	93.3	71.7	6.1	0.9
4	21.2	16.1	19.5	80	88	82.6	3.9	1.7	3.2	89.3	73.3	5.8	1.4
5	19.8	17.2	19	90	93	91	1.7	1.1	1.5	92.7	87.3	2.2	1.2
6	20.9	16.9	19.6	86	90	87.3	2.7	1.4	2.3	95	77.5	4.9	0.7
7	21.2	15.4	19.3	79	86	81.3	2.7	1.9	3.4	93.5	67.2	7	0.9
Week 24													
1	21.6	16.8	20	77	84	79.3	4.5	2.3	3.8	90.2	67.1	7.3	1.3
2	21.1	16.6	19.7	81	86	82.6	3.6	2	3.1	92.5	71	5.5	1.2
3	21.2	16.5	19.7	79	88	81.9	4	1.7	3.3	88.9	69.9	6.4	1.5
4	22.2	15.6	20.1	88	100	91.8	2.5	0	2.5	100	66.7	7.6	0
5	22.4	16.9	20.6	72	78	73.9	6.2	3.1	5.2	99.9	55.5	10.2	0
6	24.2	17.8	22.2	71	78	73.2	6.7	3.4	5.7	85.3	61.9	10.8	2.3
7	23.6	16.9	21.5	67	83	72.1	7.4	2.4	5.8	85.8	55.6	10.7	2
Week 25													
1	24.7	16.8	22.2	64	79	68.7	8.6	3.1	6.9	86.5	48.8	13	1.9
2	23.2	16.1	21	71	76	72.6	6.5	3.4	5.5	87.9	55.8	11.5	1.8
3	20.4	17.2	19.4	84	90	85.9	2.9	1.5	2.5	89.6	73.3	3.8	1.6
4	21.7	16.4	20	83	86	83.9	3.5	2	3	93.3	75.8	5.5	1
5	23.6	16	21.2	60	80	66.2	9.1	2.8	7.1	88.3	46.1	13.3	1.6
6	24.2	17.6	22.1	67	82	71.7	7.8	2.8	6.2	86	57	11.6	2.1
7	21.5	17.6	20.3	84	82	83.4	3.2	2.8	3.1	93.1	75.6	5.1	1.2

Week 26

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	24.2	15.8	21.6	61	81	67.3	8.8	2.6	6.9	93	52.8	10.7	1.1
2	22.9	16	20.7	72	85	76.1	6	2.1	4.8	86.4	63.5	8.1	1.9
3	23.2	18	21.6	73	84	76.5	6.1	2.6	5	89.2	59	11.2	1.6
4	22.9	18.8	21.6	79	86	81.2	4.7	2.2	3.9	92.8	67.3	9.5	1
5	21.8	15.4	19.8	81	82	81.3	3.7	2.4	3.3	89.7	67.8	6	1.7
6	22.3	15.3	20.1	76	82	77.9	5.1	2.4	4.2	90.3	66.1	7.9	1.6
7	24.5	19.1	22.8	72	83	75.5	6.7	2.8	5.5	86.1	63.1	11	1.9

Week 27

1	21.7	16.6	20.1	84	87	85	3.3	1.9	2.9	94.1	74.1	6	1
2	22.8	16.4	20.7	80	81	80.3	4.2	2.6	3.7	93.6	66.2	6.3	1
3	21.5	16.3	19.8	84	88	85.3	3.2	1.7	2.7	95.3	74.3	6.1	0.8
4	21.7	15.8	19.9	76	85	78.9	4.8	2.1	3.9	82.4	58.9	9.2	2.3
5	21.9	16.8	20.2	77	81	78.3	4.7	2.7	4.1	94.3	63.8	8.6	0.8
6	21.2	17	19.8	81	85	82.3	3.7	2.2	3.2	94.1	68.8	6	0.9
7	22.9	15.9	20.6	76	86	79.3	5.2	1.9	8	93.9	64.2	8.2	0.9

Week 28

1	22.2	16.3	20.3	79	89	82.3	4.3	1.6	3.4	92	71	6.5	1.1
2	22.2	17	20.5	85	92	87.3	3.1	1.2	2.5	94.5	79.3	4.9	0.8
3	25.8	17.8	23.2	74	89	78.9	6.3	1.6	4.8	88.7	70.1	7.5	1.8
4	24.3	18.2	22.3	79	90	82.5	5	1.6	3.9	93.3	70.9	7.3	1.1
5	24.2	16.4	21.6	69	81	73	7.4	2.8	5.9	91.8	57.3	11.4	1.3
6	24.5	16.6	21.9	75	88	79.3	6	1.8	4.6	89.4	66.8	8.7	1.4
7	26.4	18.4	23.7	71	82	74.7	7.8	2.9	6.2	92.3	59.6	11.9	1.1

Week 29

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	28.1	20.4	25.5	67	74	69.3	9.8	4.7	8.1	90.8	58.1	15.2	1.5
2	26.8	18.8	24.1	69	82	73.4	8.4	3	6.6	87.7	59.5	13	2.2
3	22.4	18.8	21.2	85	92	87.4	3.1	1.3	2.5	91.3	75	6.3	1.4
4	21.3	17.5	20	86	87	86.3	2.7	1.9	2.4	95.1	78.2	5.1	0.8
5	23.3	18.1	21.5	81	86	82.7	4.2	2.2	3.5	94.3	72.6	6.2	1
6	23.4	17.9	21.5	80	87	82.4	4.3	2.1	3.6	92.2	71.4	6.7	1.3
7	23.2	17.6	21.3	80	84	81.4	4.4	2.4	3.7	94.4	73.7	6.3	0.9

Week 30

1	22.6	17.5	20.9	83	87	84.4	3.5	1.9	3	94.8	75.9	5.3	1
2	23.1	17.8	21.3	82	85	83	4.1	2.3	3.5	95.3	73.8	6.2	0.9
3	21.9	17.6	20.4	85	90	86.7	2.9	1.5	2.4	94.2	78.4	5.1	1
4	22.6	17.6	20.9	80	85	81.7	4.2	2.2	3.5	94.3	75.2	5.8	0.9
5	21.4	17	19.9	85	84	84.6	3	2.3	2.8	95	68.8	5.5	0.8
6	21.4	16.7	19.7	90	93	91.1	1.9	1	1.6	95.1	85.9	2.7	0.8
7	22.3	16.6	20.3	78	87	81.2	4.7	1.9	3.7	94.8	67.3	7.5	0.7

Week 31

1	22.5	17.9	20.9	81	91	84.6	3.9	1.3	3	94.5	77.1	5.3	0.8
2	20.9	17.8	19.8	89	92	90.1	2	1.2	1.7	95.3	85	2.9	0.7
3	23.3	17.2	21.1	78	85	80.5	4.8	2.2	3.9	95	63.7	9.6	0.8
4	22.3	17.4	20.5	80	85	84.8	4.1	2.2	3.4	94.3	70.2	7.3	0.9
5	20.4	16.5	19	91	90	90.6	1.7	1.4	1.6	96.2	86.4	2.2	0.6
6	24.2	16.2	21.2	76	86	79.7	5.8	1.9	4.4	93.2	67.1	8.8	1
7	25.8	17.3	22.6	75	84	78.3	6.8	2.4	5.2	92.8	63.7	11.9	1

Week 32

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	28.3	22.3	26.1	73	80	75.6	8.3	3.9	6.7	92.2	64.3	13.8	1.1
2	29.3	22.5	26.7	66	75	69.4	10.4	5.1	8.4	83.1	53.9	17	3.2
3	25.4	19.9	23.3	80	89	83.4	5	1.9	3.8	92	70.1	7.6	1.4
4	26.7	21.6	24.8	76	83	78.7	6.4	3.3	5.2	92.2	68.1	10.1	1.3
5	26.4	19.1	23.6	78	89	82.2	5.9	1.8	4.3	90.8	72.1	8.6	1.5
6	28.7	20.9	25.7	72	84	76.6	8.9	3	6.6	92.8	56.7	14.9	1.1
7	28.6	22.8	26.4	73	82	76.5	8.1	3.7	6.4	90.2	63.3	12.8	1.6

Week 33

1	27.9	21.6	25.4	77	87	80.9	6.7	2.6	5.1	87.1	69.3	10.8	2.5
2	26.5	20.3	24.1	81	83	81.8	5.2	3.1	4.4	92	74.5	8.5	1.4
3	23.8	18.9	21.9	79	75	77.4	4.8	4	4.5	91.6	72.6	7.3	1.4
4	24.6	16.8	21.5	74	86	78.8	6.5	2.1	4.8	90.9	71	7.4	1.6
5	23.7	18	21.4	75	79	76.6	5.6	3.3	4.7	No Data	No Data	No Data	No Data
6	22.2	17.9	20.5	82	79	80.8	3.8	3.2	3.6				
7	24.2	18.4	21.9	79	90	83.4	5	1.6	3.6				

Week 34

1	21.9	16.2	19.6	99	100	99.4	0.2	0	0.2	No Data	No Data	No Data	No Data
2	22.1	16	19.6	99	100	99.4	0.2	0	0.2				
3	22.1	17.2	20.1	99	100	99.4	0.2	0	0.2				
4	22.1	19.1	20.9	99	100	99.4	0.2	0	0.2				
5	24.2	18.4	21.8	99	100	99.4	0.2	0	0.2				
6	24.3	20.1	22.5	99	100	99.4	0.2	0	0.2				
7	24.4	17.7	21.6	99	100	99.4	0.2	0.1	0.2				

Week 35

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	22.4	17.1	20.2	99	100	99.4	0.2	0.1	0.2	No Data	No Data	No Data	No Data
2	23.2	17.4	20.7	99	100	99.4	0.2	0	0.2				
3	24.4	18	21.7	99	100	99.4	0.2	0	0.2				
4	21.2	16	19	89	89	89	2.2	1.6	1.9				
5	19	15.5	17.5	87	88	87.4	2.2	1.6	1.9				
6	22.1	15.7	19.3	66	85	74.3	7	2	4.8				
7	22.1	16.2	19.5	66	80	72.1	7	2.7	5.1				

Week 36

1	21.3	15.6	18.8	73	87	79.2	5.3	1.8	3.8	No Data	No Data	No Data	No Data
2	21.6	17	19.6	76	81	78.2	4.9	2.7	3.9				
3	23.7	17.5	20.9	83	92	87	4	1.2	2.7				
4	26.3	18.4	22.7	82	93	86.9	4.9	1.2	3.2				
5	24.2	19.4	22	91	95	92.8	2.2	0.9	1.6				
6	24.5	17.4	21.3	90	95	92.3	2.4	0.7	1.6				
7	24.2	18.2	21.5	92	96	93.8	2	0.6	1.4				

Week 37

1	24.8	18.6	21.9	93	96	94.4	1.7	0.6	1.2	No Data	No Data	No Data	No Data
2	24	19	21.7	94	98	95.8	1.4	0.4	0.9				
3	25	19.8	22.6	96	99	97.4	1	0.2	0.6				
4	23.7	19.1	21.5	96	97	96.5	1	0.5	0.8				
5	25	18.4	21.9	97	100	98.4	0.7	0.1	0.4				
6	25.5	18	22	99	100	99.5	0.3	0	0.3				
7	26.1	17.4	22	99	100	99.5	0.3	0	0.3				

Week 38

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	25.5	17.6	21.7	99	100	99.5	0.2	0	0.2				
2	25.4	17.5	21.6	99	100	99.5	0.3	0	0.3				
3	25.4	18.9	22.3	99	100	99.5	0.2	0	0.2				
4	23.7	18.9	21.4	99	100	99.5	0.2	0.1	0.2	100	97.8	0.5	0
5	24.1	17.8	21	99	100	99.5	0.3	0	0.3	100	93.9	1.5	0
6	25.6	19.4	22.6	99	100	99.5	0.2	0	0.2	100	96.3	0.9	0
7	23.7	19.9	21.8	99	100	99.5	0.2	0	0.2	100	97.4	0.6	0

Week 39

1	19.9	20.1	20	100	99	99.5	0	0.3	0.3	100	94.7	1.2	0.1
2	20.6	13.5	17	99	100	99.5	0.1	0	0.1	100	97.5	0.7	0
3	23.6	14.7	19.1	99	100	99.5	0.2	0	0.2	100	96.3	0.5	0
4	23.8	15.7	19.7	99	100	99.5	0.2	0	0.2	100	95.8	1.1	0
5	23.4	17.9	20.6	99	100	99.5	0.2	0	0.2	100	96.9	0.7	0
6	23.3	16.5	19.8	99	100	99.5	0.2	0	0.2	100	96.3	0.9	0
7	23.7	15.1	19.3	99	100	99.5	0.2	0	0.2	100	95.8	0.8	0

Week 40

1	23.5	17.3	20.3	99	100	99.5	0.2	0	0.2	100	96.8	0.7	0
2	23.6	18.4	20.9	99	100	99.5	0.3	0	0.3	100	96.8	0.7	0
3	19.7	17.1	18.3	100	100	100	0.1	0	0.1	100	98.2	0.3	0
4	21.2	18.6	19.8	99	100	99.5	0.1	0	0.1	100	97.2	0.7	0
5	23.7	15.8	19.5	98	100	99.1	0.5	0	0.5	100	90.3	2.4	0
6	22	13	17.2	99	100	99.5	0.3	0	0.3	100	95.4	1.1	0
7	22.7	15.7	19	99	100	99.5	0.2	0	0.2	100	96.4	0.6	0

Week 41

	Air Temperature			RH			Humidity Deficit			Max & Mins			
Day	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**Day**	**Night**	**24hrs**	**RH Max**	**RH Min**	**HD Max**	**HD Min**
1	23	15.8	19.2	98	100	99.1	0.3	0	0.3	100	94.2	1.3	0
2	20.9	15.2	17.8	99	100	99.5	0.1	0	0.1	100	98.2	0.4	0
3	22.8	17	19.7	99	100	99.5	0.3	0	0.3	100	95.9	0.9	0
4	22.7	18.1	20.2	99	100	99.5	0.2	0	0.2	100	94.5	1.1	0
5	23.3	17.5	20.1	98	100	99.1	0.4	0	0.4	100	89.6	2.6	0
6	24.2	16	19.7	99	100	99.5	0.2	0	0.2	100	94.5	1	0
7	23	18	20.3	99	100	99.5	0.2	0	0.2	100	95.8	1	0

Week 42

1	22.5	17.7	19.9	99	100	99.6	0.3	0	0.3	100	95.1	1.3	0
2	22.2	15	18.2	99	100	99.6	0.2	0	0.2	100	95.7	1	0
3	22.8			99			0.2			100	92.5	1.2	0
4										100	95.7	0.6	0
5													
6		12.6			100			0		100	96.8	0.7	0
7	20.8	13.8	16.8	99	100	99.6	0.1	0	0.1	100	97	0.4	0