ROOT MAT IN TOMATO

ANNUAL REPORT PC 241

Protected hydroponic tomato: investigating the potential for various novel non-chemical techniques for the suppression or control of root-mat disease.

February 2007

Project Title:	Protected hydroponic tomato: investigating the potential for various novel non-chemical techniques for the suppression or control of root-mat disease.
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The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiments were carried out and the results obtained have been reported with detail and 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 application.

AUTHENTICATION

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

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Grower Summary

PC 241

Protected hydroponic tomato: investigating the potential for various novel non-chemical techniques for the suppression or control of root-mat disease.

Headlines

- An experimental system for hydroponic tomato production using slow sand and slow rockwool filters was established at STC and a tomato crop grown for over 20 weeks but following inoculation, severe root mat symptoms did not develop in any of the experimental plots so the effect of the filters on establishment and symptom expression remains unknown.

- However, the genetic material that causes root mat symptoms was recovered at much lower levels from plants inoculated via the filters than from plants inoculated directly indicating that the filters may have some effect.

Background and expected deliverables

Root mat was first reported in the UK in the 1970s on soil and straw bale grown cucumbers. It is caused by a small circular DNA element called an Ri-plasmid (Ri = root inducing) which is harboured by rhizosphere-associated bacteria, primarily from the genus *Agrobacterium*. The disease causes massive over-production or proliferation of roots on affected plants (plate 1) which ultimately results in increased vegetative growth of the foliage. This can cause many problems in terms of crop management and poor crop quality. By the end of the 1970s the disease had disappeared in soil and straw bale crops. Root mat re-appeared in hydroponic cucumbers in 1993. Outbreaks continue, though both incidence and severity in cucumbers has reduced in recent years, largely due to a change to the number of crops produced each year, which makes management of infected crops much easier.

The disease has now occurred in tomato crops where it has persisted at a severe level in hydroponic crops on some nurseries in the UK. There is a natural concern that it could spread to infect further nurseries in the future. As there is a current lack of proven effective control measures, root-mat poses a significant potential risk to economic production of tomatoes throughout the UK. It has been estimated that losses due to root-mat in tomato are currently in the region of £0.75M/annum for one company (caused by an increase in secondary disease and crop management costs) though the potential for greater loss is considerable if the disease spread more widely to other tomato nurseries.

Plate1. Tomato with severe root mat symptoms

Chemical ‘disinfection’ strategies have so far failed to control the disease. A recently completed Defra project (HH2308SPC) indicated that increasing the microbial diversity within the rockwool growth substrate led to a suppression of root-mat symptoms in hydroponic cucumber crops. Observations on commercial nurseries where a natural decline of root-mat symptoms occurred over a number of seasons in biologically diverse, organic, soil-grown cucumber and tomato crops support this hypothesis. This led us to consider the possibility that increasing the population of naturally-occurring microbial antagonists might suppress or prevent the development of root-mat. This study has therefore looked at a number of alternative, non-chemical strategies to try to minimise or eliminate the risk of root mat in hydroponic tomatoes. It is hoped that results of this investigation, can be applied to commercial glasshouse production and provide a successful and cost effective control method.

Summary of the project and main conclusions to date

Four primary objectives were set:

a) To evaluate the potential of different filtration techniques based on the principle of slow sand filtration (SSF), but incorporating organic substrates including soil, to mimic the disease suppressive effects observed commercially in organic tomatoes.
b) To investigate the impact of formulated (non-regulated) microbial preparations on root-mat through increased microbial diversity.
c) To investigate the potential of grafting onto alternative rootstocks e.g. Aubergine as a means of suppressing or preventing root-mat in tomatoes.
d) To determine whether the principle of cross-protection, as it applies to other pathogens is effective against root-mat of tomatoes.

Work on objectives a), b) and d) has been initiated in the first year of this project (2006), further work on these objectives and objective c) will be carried out in subsequent years.

<u>Objective a</u> : Investigating the possible effects of slow sand filtration techniques on root-mat in tomatoes

An experimental standard cherry tomato crop cv Claree was grown using a re-circulating hydroponic system under near-commercial conditions at STC Ltd. A series of 6 filters were designed and constructed using the principle of slow sand or slow rockwool filtration, but with additional organic amendments to improve the diversity of the biologically active layer which forms in such filters over the priming period. Each filter provided nutrient solution for two rows of plants (44 plants in total); additional plots were included as inoculated and uninoculated controls and received unfiltered feed solution on a run-to-waste system. The details of the filters (treatments) are shown below:

1. Uninoculated control
2. Inoculated control
3. Inoculated – conventional slow sand filter (SSF)
4. Inoculated – slow rockwool filter (SRF)
5. Inoculated – SSF + organic soil ‘sandwich’
6. Inoculated – SRF + organic soil ‘sandwich’
7. Inoculated – SSF + soil/straw ‘sandwich’
8. Inoculated – SSF with soil/straw throughout filter.

The trial was inoculated using a strain of *Agrobacterium* collected from severely affected roots exhibiting root-mat.

The crop established well, however, despite two attempts to inoculate the crop, satisfactory symptom expression did not occur in the inoculated control plants. A few very early root-mat like symptoms were observed in the inoculated control plots, however the symptoms did not develop further. This in itself is intriguing as subsequent molecular testing has demonstrated the continued presence of the Ri-plasmid in the inoculated root tissues yet, for some unexplained reason, root-

mat symptoms failed to be expressed. Interestingly, molecular analysis at CSL of root samples from each treatment indicated a considerable reduction in *Agrobacterium* where the slow sand and rockwool filters had been used. Whilst it is particularly disappointing that root mat symptoms did not develop as expected in this trial the molecular analyses do appear to suggest that filtration and/or increased microbial diversity may be beneficial in reducing the risk of root mat in tomato. There is also the possibility of strain differentiation and the use of weakly virulent or avirulent cultures may potentially protect plants from more aggressive strains. These aspects will require further investigation in 2007.

Objectives b & d : Evaluating formulated microbial products and the principle of cross-protection for root-mat control in tomato

Investigation into the suppression of root-mat using existing microbiological products was undertaken in quarantine glasshouse cubicles at CSL. A range of proprietary microbiological products (listed below) were applied to tomato seedlings cv Claree one week after germination and thereafter, at weekly intervals. Two plants per treatment were included with 250ml of each product being applied to each propagation cube. Four weeks post-germination the seedlings were inoculated with *Agrobacterium* containing the root-inducing plasmid.

Details of Bio-control products under investigation

Treatment	Manufacturer	Active ingredient or organism	Rate of application (per 500ml)
1. Uninoculated (negative) control	-	-	-
2. Inoculated untreated (positive) control	-	-	-
3. Biomex SA	Omex Agriculture	*Trichoderma* spp.	0.5ml
4. Companion	Growth Products Ltd	*Bacillus* spp.	0.5ml
5. Garlic Barrier Plus	Garlic Farms	Garlic	50µl
6. Gliomix	Fargro Ltd	*Gliocladium* sp.	1g
7. Seasol	Seasol International	Bull kelp concentrate	1.7ml
8. GLD	Omex	Garlic extract and salicylic acid derivative	50µl
9. Stimagro	Fargro Ltd	*Streptomyces* sp.	0.25g
10. Seasol + Biomex-SA	Seasol International Omex Agriculture	Seaweed concentrate *Trichoderma* spp.	1.7ml + 0.5ml
11. Seasol + Companion	Seasol International Growth Products Ltd	Seaweed Concentrate *Bacillus* spp.	1.7ml + 0.5ml

Typical root-mat symptoms did not develop in any of the plants including the positive control 15 weeks post-inoculation. Interestingly, rhizogenic *Agrobacterium* was found to be present in the roots of all plants with the exception of those treated with Gliomix and the negative non-inoculated control plants following molecular analysis eight weeks post-inoculation.

It has been reported (Dr P Morley, pers. com.) that root mat symptoms in tomato differ in severity in two commercial tomato nurseries in the south of England. Tests have shown that all the *Agrobacterium* isolated at nursery A contain a different Ri-plasmid than at nursery B. This suggests a correlation (in this instance) between Ri plasmid type and symptom severity. It was postulated that inoculation with the plasmid type producing slightly weaker symptoms might be providing some cross-protection effect and prevent the more virulent strain causing more severe symptoms.

In a small scale experiment at CSL young tomato plants cv Claree were inoculated with various strain combinations of *Agrobacterium* to investigate this hypothesis. The inoculation regimes investigated were:

1. Plant inoculated with buffer only (negative control)
2. Plants inoculated with Agrobacterium type A (less aggressive)
3. Plants inoculated with Agrobacterium type B (more aggressive)
4. Plants inoculated with a mix (equal concentrations) of A and B
5. Plants inoculated with A then B one week later
6. Plants inoculated with B then A one week later

Again no definitive symptoms appeared in any of the inoculated plants 15 weeks post-inoculation.

The lack of symptom expression in each of the experiments undertaken during 2006 may be due to a number of factors. These include lack or loss of pathogenicity in the *Agrobacterium* isolate used, the choice of cultivar used[1] or other as yet unknown factors relating to growing conditions, pH of water and feed regimes.

Further work on each of the objectives will be undertaken in 2007.

Financial benefits

Root mat is a serious root disease of tomato and cucumber that interferes with the normal root production of the host allowing it to proliferate uncontrolled. This affects plant physiology, crop management, and susceptibility to other pathogens, yield and overall fruit quality. In addition to the direct impact of root mat the indirect effect of secondary pathogens, especially *Pythium* and *Botrytis*, can also be very important not only because of the direct commercial loss but also because of the need for fungicide intervention. Increased use of pesticides conflicts with the overall pesticide minimisation 'goals' of the Tomato Growers Association. It has been estimated that losses due to root mat in tomato are currently in the region of £0.75M/annum though the potential for greater loss is considerable if the disease spread more widely to other tomato nurseries.

[1] This cultivar was found to be susceptible in commercial crops in the South of England

Assuming the disease spread to affect most tomato nurseries and caused estimated losses of 10-15% the cost to the industry could be between £3-6M/annum. There is therefore a significant financial incentive to identify and implement effective control measures for this pathogen before it becomes more widespread throughout the UK.

Action points for growers

Continue to monitor crops for symptoms of root mat and alert the Project Leader to any unusual symptoms or new developments.

Project Co-ordinator Comments

"Root Mat continues to be a significant problem in hydroponic crops in the south of England. In 2006 approximately 50% (around 10ha) of individual crops were affected by the end of the season. Growers have tried various methods to deal with the symptoms including increasing the volume of rockwool available to the affected roots and removing the plastic from rockwool slabs. Growers have also (naturally) tried to deal with the prevention aspects. Water is chlorinated, pathways regularly cleaned and a strict turn round policy and procedure been tuned to address the issue of root mat. Nevertheless, only in organic crops has a significant reduction in symptoms year on year been seen.

In conclusion, it is not just the presence of root mat, in terms of extra management time which is cause for continued concern but the economic loss which can be directly attributable to the extensive and seemingly uncontrollable spread of infection. Increased levels of Pythium infection, Botrytis and a reduction in fruit quality have been part of the suite of secondary infections which have lead to an estimated 5-7% reduction in yield after infection manifests".

Dr Phil Morley, January 2007

"Outbreaks of root-mat continue, although incidence and severity in cucumbers has reduced in recent years, partly because of action taken to reduce the problem in propagation and also because of the change to have up to three crops of shorter duration that prevents the problem building up in any one crop.

It is important to remember when dealing with this problem that the Agrobacterium can be easily dealt with by steam sterilisation of the growing media but the plasmid that causes the problem is not destroyed by such steam sterilisation. The plasmid left behind in the growing media can then be picked up by Agrobacterium that can re-invade the growing media the following season. Extensive sterilisation and disinfection across nurseries has never successfully eradicated the disease and therefore will allow the problem to be carried over from one season to the next. Infested growing media should not be re-used – even if steam sterilised."

Derek Hargreaves, January 2007

SCIENCE SECTION

Introduction

Root-mat was first reported in cucumbers in the UK in the 1970s. The disease persisted for several seasons then disappeared from commercial crops even though there had been no specific intervention to control the problem. The symptoms re-surfaced in hydroponically-grown cucumber crops in 1993. The disease has persisted in several nurseries since then. Of considerable concern is that in the last eight years it has also appeared in large-scale hydroponic commercial tomato crops. Root-mat is characterised by an over-proliferation of roots. The development of the extended root system affects the plant physiology, increasing vegetative growth in the aerial parts of the plant, making crop management (where some plants are affected; others not) very difficult. Fruit quality can also be affected and the susceptibility of the crop to other pathogens such as *Pythium* and *Botrytis* has been seen to increase.

Plate 2. Tomato roots severely affected by root-mat

The symptoms are caused by a small circular DNA element called an Ri- plasmid. On infection, a piece of this plasmid (T-DNA) is transferred from the bacteria to the root cell where it is incorporated into the root cell nucleus. Genes encoded on the T-DNA induce root proliferation (Plate 2) via transformed cells. And also make the roots produce an opine (cucumopine) which provides a nutrient source for the *Agrobacterium*. Growers of crops like roses, chrysanthemum or raspberries are perhaps more familiar with the Ti-plasmid (**T**umor-**i**nducing) associated with *Agrobacterium tumefaciens*, the causal agent of crown gall disease.

Previous studies sponsored by the HDC (PC 149) and Defra (HH2308SPC) have focused mainly on investigating and controlling the problem in cucumbers. Evidence collected during these studies suggests that increasing the microbial diversity in the rockwool slabs (and glasshouse environment) may be important in reducing root-mat symptoms perhaps through direct competition or antagonism. This concept has been developed further following commercial observations that root-mat symptoms reduced season by season in an organic soil-grown crop reduced year on year without specific intervention leading to the possibility that naturally occurring antagonists were, in some way, out-competing the root-mat pathogen.

Although root-mat is still present in commercial cucumber crops, in many cases, the problem has been alleviated by changing cropping practices particularly by increasing the number of crops per season. This decreases the time during which individual plants may be affected by the disease, hence alleviating symptom expression and therefore making management of the crop easier.

The investigation reported here focused on root-mat in long-season tomatoes where adjustments to cropping frequency are not as feasible, a single, long-term crop is grown each season. The aim of the project is to find a practical and economic solution to root-mat for the UK tomato industry, through the use of one or more non-chemical intervention strategies.

This study has four primary objectives:

a) To evaluate the potential of different water filtration techniques based on the principle of slow sand filtration, but incorporating organic substrates including soil into the filters, to mimic the disease suppressive effects observed commercially in organic tomatoes.
b) To investigate the impact of formulated (non-regulated) microbial preparations (thereby through increased microbial diversity) on root-mat incidence.
c) To investigate the potential of grafting tomato plants onto alternative rootstocks e.g. Aubergine as a means of suppressing or preventing root-mat in tomatoes.
d) To determine whether the principal of cross-protection, as it applies to other pathogens is effective against root-mat of tomatoes.

Work on objectives a), b) and d) has been initiated in the first year of this project (2006), further work on these objectives and objective c) will be carried out in subsequent years. Separate elements of the work will be carried out at Stockbridge Technology Centre (STC), the Central Science Laboratory (CSL) and, in later parts of the study, on commercial tomato nurseries in southern England.

Methods & Materials

<u>Objective a</u> : Investigating the possible effects of slow sand filtration techniques on root-mat in tomatoes

Work to investigate the possible effects of slow sand filtration techniques on root-mat was carried out at STC during the 2006 season. A crop of cherry tomatoes cv Claree was grown using a re-circulating hydroponic system using methods which 'mirrored' near-commercial practice. A series of six filters were designed and constructed, each one containing different components chosen to potentially enhance the diversity of the biologically active layer – or 'schmutzdecke'. It was hoped that organisms or metabolites in this layer would either out-compete or eliminate *Agrobacterium* strains harbouring the Ri-plasmid; these filters formed the 'treatments' in this study. Each filter was used to produce filtered feed solution to irrigate two rows of tomato plants (22 plants/row (plot)) on a re-circulating system as shown in Figure 2. Inoculated and uninoculated control treatments which were not linked to filters were incorporated into the trial but for practical purposes were irrigated using a run-to-waste system (RTW) via a double Dosatron unit.

<u>Treatments</u>:

1) Uninoculated control – RTW
2) Inoculated control - RTW
3) Inoculated – conventional slow sand filter (SSF)
4) Inoculated – slow rockwool filter (SRF)
5) Inoculated – SSF + organic soil sandwich
6) Inoculated – SRF + organic soil sandwich
7) Inoculated – SSF + soil/straw sandwich
8) Inoculated – SSF with soil/straw throughout filter.

A schematic of a basic slow sand filter is shown in Figure 1 overleaf, followed by a photograph of the filters during construction in March 2006. The slow sand filters were filled using two grades of gravel at the base, to aid drainage, followed by two layers of sand of different particle size, separated by a layer of fleece (Appendix 5). Soil was collected from an organic tomato crop in the south of England where root-mat had been severe but had subsequently abated. The hypothesis was that the suppressive micro-flora introduced via the organic soil would add to the diversity of the biologically active layer in the filter. This soil layer was therefore positioned 2-3cm below the top of the sand layer in treatments 5 & 6. It was not possible to source organic barley straw, therefore conventionally produced barley straw was used instead. The straw was cut into 3-4cm pieces prior to incorporation. The soil and straw used in treatment 8 was incorporated evenly throughout the layer of the finest sand. The rockwool filters had a layer of the coarsest gravel in the base and were filled with rockwool granulate product supplied by Grodan.

Figure 1: Schematic diagram of a basic slow sand filter design

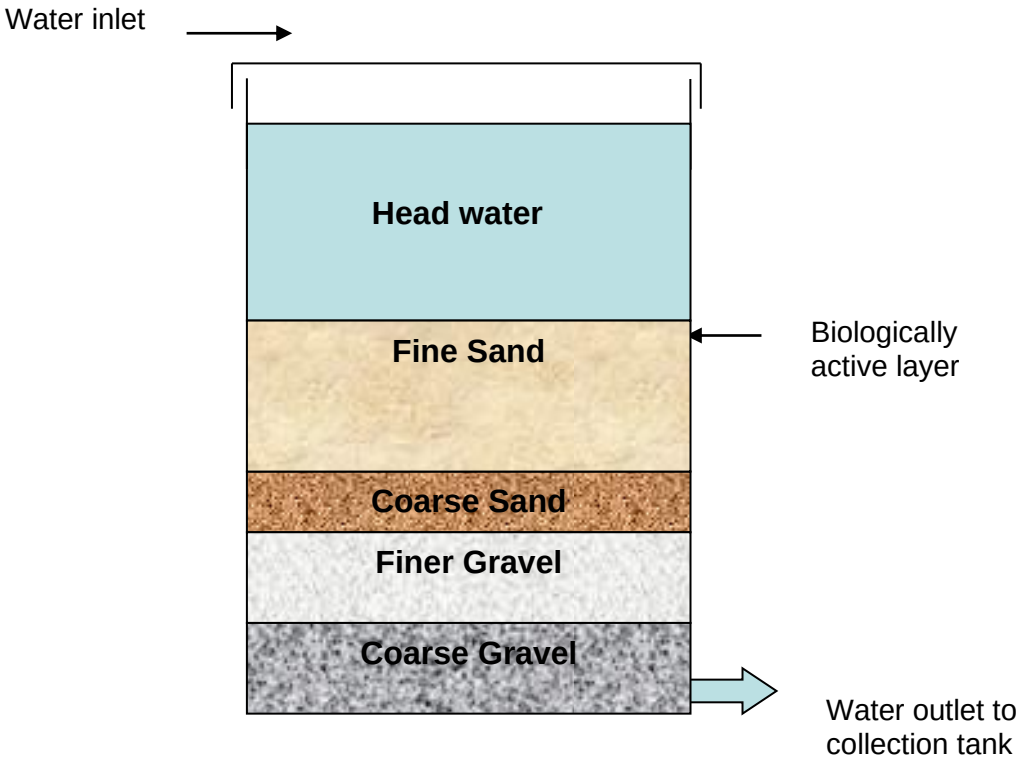


Plate 3. Filter arrangement during construction in semi-commercial glasshouse trial at STC

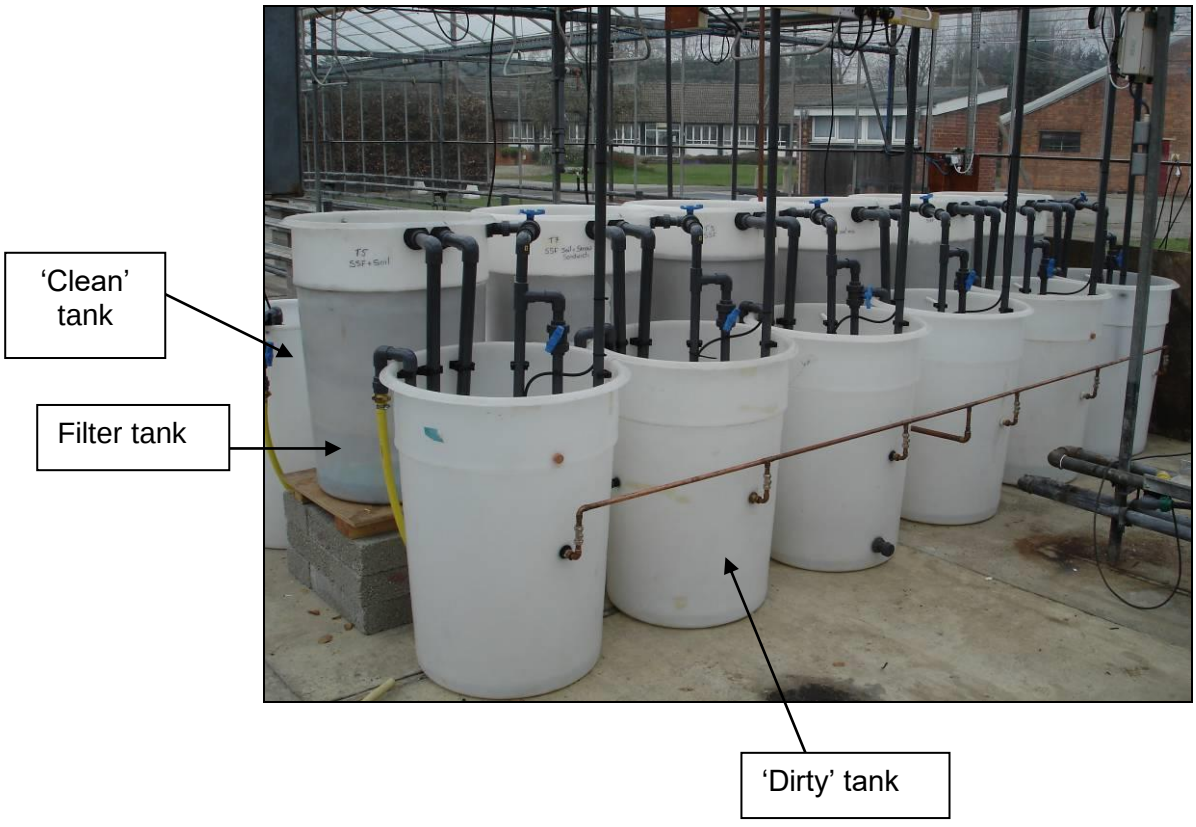
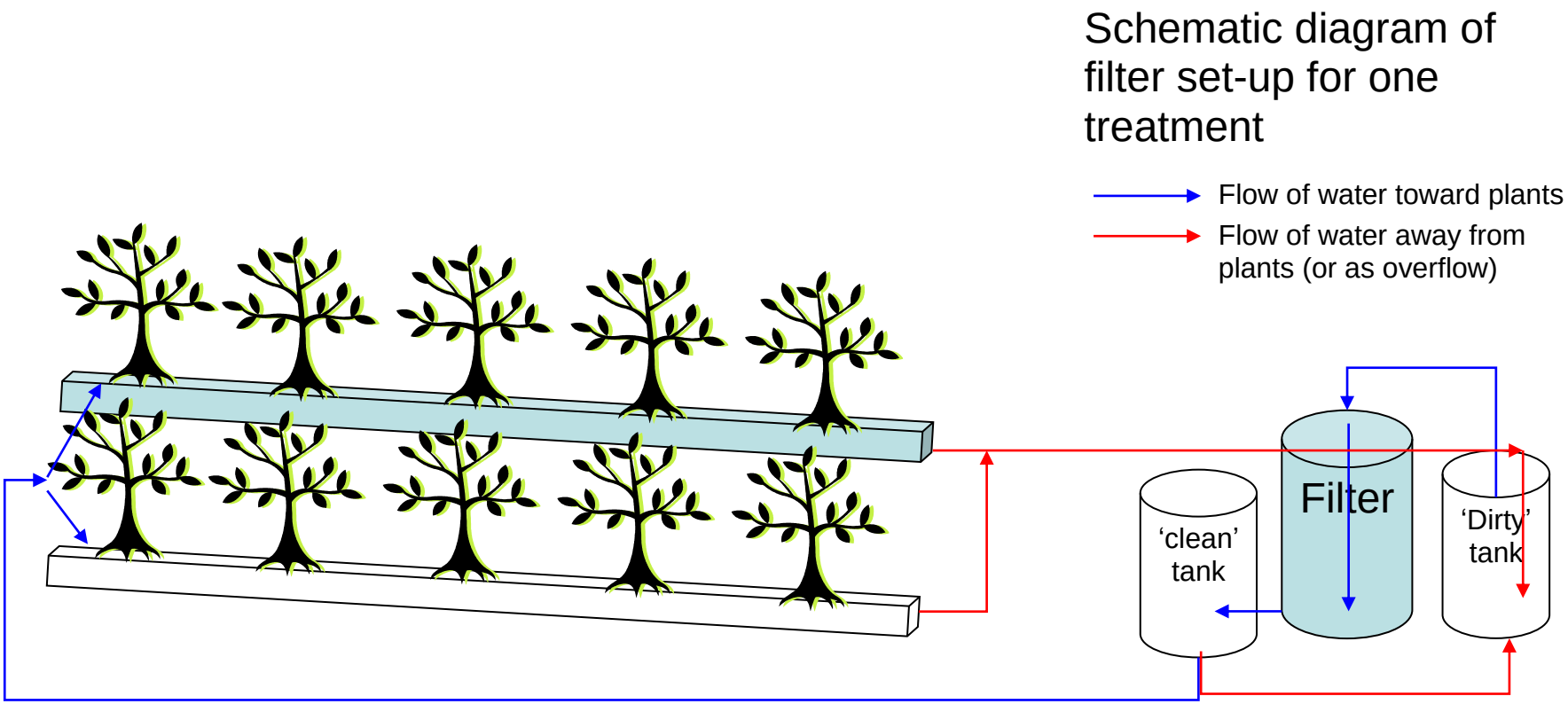


Figure 2. Investigation into control of root mat in tomatoes
E301 2006



The plumbing required for each of the six filters and associated tanks and pipe-work proved to be very complex and involved the incorporation of systems to allow for topping-up with mains water, adding concentrated feed solution to maintain a balanced conductivity between the different treatments, dealing with over flows and re-circulation during priming in addition to the basic needs of collecting water coming back from the irrigated plants, filtering it, collecting it and then pumping it back out to the crop. Once all the construction work was completed on the filters each pre-filtration tank was filled with reservoir water which was circulated through each filter for approximately five weeks prior to the introduction of the crop. This allowed the filters to be 'primed' via the development of the biologically active layer within each filter.

Crop Diary

Mar/Apr	Filters designed and constructed
19.4.06	Filters filled with filter media.
24.4.06	Tomato cv Claree sown
24.4.07	Filter filled with priming water and set to re-circulate.
22.5.06	Water samples collected from 'clean' tanks, checked for presence of *Pythium* or *Phytophthora* sp.
1.6.06	Crop planted in glasshouse
9.6.06	Crop inoculated with *Agrobacterium*.
28.7.06	2nd inoculation with *Agrobacterium*.
14.8.06	First visible signs of root-mat in inoculated control.
9.11.06	Root samples collected for PCR analysis.
15.11.06	Plants removed, root blocks and slabs left *in situ*.
21.11.06	Final root assessment carried out

Inoculation methodology

Ri-plasmid harbouring *Agrobacterium* cultures originally isolated from a severely infected crop in southern England was prepared at CSL (Isolate CSL 5083). The crop was inoculated in two different ways in order to gather as much information from the study as possible. Firstly, 1 litre of inoculum (1×10^6 cfu/ml) was poured into the head water of each filter, secondly all plants in one row of each treatment (see trial plan in Appendix 1) were directly inoculated with 5ml of the same concentration of *Agrobacterium* applied with a syringe in the area of the dripper. The inoculated control plants (T2) were all directly inoculated. This direct inoculation within the row was included as a control to confirm virulence of the introduced pathogen and demonstrate whether or not the re-circulating solution coming from the filters would have any effect on direct infection i.e. would the increased microbial activity within the filter, either directly or indirectly via metabolites, impact on *Agrobacterium* establishment or symptom expression, or even produce some sort of immune response which would enable the plants to resist infection e.g. the triggering of systemic acquired

resistance. The inoculation via the filters was used to a) ensure the inoculation procedure via the filters was robust (it had not been undertaken in this way previously) and b) to ensure that any potential absence of root mat in the crop was (or was not) due to effective removal of the inoculum, either through physical or microbiological action, rather than simply loss of virulence of the pathogen (*Agrobacterium*/plasmid).

Growing methodology

The glasshouse was maintained at a day & night temperature of 19ºC with venting set at 21 ºC. Irrigation timing and frequency were carried out automatically via a Vocom system and was adjusted to fit the demands of the crop throughout the season. A concentrated feed solution was mixed automatically from separate A & B tanks using a standard tomato feed regime (as advised by Derek Hargreaves). This was automatically 'dosed' into the 'clean' tanks post filtration. However because of the constantly fluctuating water levels in the tanks combined with the fact that solution returning to the 'dirty' tanks as run-off from the plots contained varying amounts of nutrition (depending on the weather conditions) it proved difficult to maintain a standard fertigation concentration. The electrical conductivity (EC) of the solution was monitored on a regular basis (every 2-3 days), the 'dirty' tanks were topped-up with fresh mains water and the EC was adjusted in both the clean and dirty tanks until a value of 3 – 4ms could be achieved. The uninoculated and inoculated control plots which were not connected to filters were each fed via separate A & B tanks attached to independent Dosatron units.

Strict hygiene precautions were maintained in the glasshouse with restricted access, foot dips at both access points and alcohol sprays used on all monitoring equipment. Gloves were worn for any crop or filter work whilst work required on the crop e.g. twisting, side-shooting, harvesting was carried out on the untreated control plots prior to moving into the remainder of the crop.

Crop monitoring

Prior to the introduction of the crop the water in both the clean and dirty tanks was sampled to investigate total bacterial counts. Water samples were also taken post-filtration to check for the presence of *Pythium* or *Phytophthora* spp.

Regular monitoring of the flow rates from each filter was carried out throughout the duration of the trial[2]. During the initial filter priming period (before the crop was planted) some of the filters were observed to be running at a much slower rate than others (the two rockwool filters in particular were running at a very fast rate). It was decided that the filters would be 'limited' so that they all ran at as close to the slowest recorded rate as possible to try and maintain consistency between

[2] Flow rate was measured in ml/min (Y) but has been converted to L/m^2/hr (z) using the following formula:
$\frac{Y \times 60}{1000} \times 3$ (multiplication by 3 necessary as the surface area of the filters = approximately ⅓ metre).

the filters. In general flow rates for slow sand filters are considered to be suitable for the task at between 100 – 150L/m^2/hr.

The crop was regularly monitored for the development of root-mat symptoms throughout the duration of the trial. Root samples were collected on the 9th November (22 weeks post-inoculation) and tested for the presence of rhizogenic *Agrobacterium*. Four separate samples were collected from the 1st, 5th, 10th and 15th plant in each row. Samples were covered with sterile phosphate buffer and vortexed. 0.1mls of each suspension was added to 10 mls of an *Agrobacterium* selective Medium 1A broth. These broth cultures were incubated for 72 hours. At this time 0.1mls of the broth culture was removed and boiled for 5 minutes. These lysates were then used as templates for the *rol* real-time PCR which tests for pathogenic Ri-plasmid DNA.

A final assessment for root-mat was carried out following the removal of the crop on the 21st November. The root-mat symptoms were scored using the following 0-3 severity scale:

Root-mat assessment (0-3 severity scale)

0 – No symptomatic root development

1 – A few root-mat-like roots visible around the dripper area.

2 – Moderate amount of root mat roots over more of the block surface

3 – Large amount of root-mat roots present – block swollen.

Objective b : Investigation into the control or suppression of root-mat in tomatoes using existing microbial products

This work was carried out at CSL during the spring and early summer of 2006. A total of ten treatments were chosen (including positive and negative control plants) and the details of these are shown below (Table 1). Tomato seedlings cv Claree were treated with the products at the rates stated one week after germination and thereafter at weekly intervals. Plants were inoculated with 5ml of a 10^7 cfu/ml rhizogenic *Agrobacterium radiobacter* (CSL 5083) suspension 4 weeks post germination.

Table 1: Details of Bio-control products under investigation

Treatment	Manufacturer	Active ingredient or organism	Rate of application (per 500ml)
1. Uninoculated (negative) control	-	-	-
2. Inoculated untreated (positive) control	-	-	-
3. Biomex SA	Omex Agriculture	*Trichoderma* spp.	0.5ml
4. Companion	Growth Products Ltd	*Bacillus* spp.	0.5ml
5. Garlic Barrier Plus	Garlic Farms	Garlic	50µl
6. Gliomix	Fargro Ltd	*Gliocladium* sp.	1g
7. Seasol	Seasol International	Bull kelp concentrate	1.7ml
8. Stimagro	Fargro Ltd	*Streptomyces* sp.	0.25g
9. Seasol + Biomex-SA	Seasol International Omex Agriculture	Seaweed concentrate *Trichoderma* spp.	1.7ml + 0.5ml
10. Seasol + Companion	Seasol International Growth Products Ltd	Seaweed Concentrate *Bacillus* spp.	1.7ml + 0.5ml

Samples were taken from the roots of plants at 4 and 8 weeks post inoculation to check for the presence of the Ri-plasmid. The experiment was repeated later in the year but substituting GLD (a garlic extract with salicylic acid derivative product) for Stimagro and Garshield for Garlic Barrier Plus.

Objective c : (Year 2 onwards)

Objective d : Investigation into the control or suppression of root-mat in tomatoes using cross-protection

This investigation, also carried out at CSL, was prompted by a series of observations in badly infected crops at two nurseries, situated very close to each other, in southern England. It was noticed that symptoms at one of the nurseries (Nursery A) were generally less severe than at Nursery B. Molecular tests showed that all the *Agrobacterium* isolated at nursery A contained a different Ri-plasmid to the common cucumopine Ri-plasmid present in the rhizogenic *Agrobacterium* strains isolated at nursery B and more generally in other root-mat affected crops. This suggests a correlation, in this instance, between Ri-plasmid type and symptom expression. It was postulated that inoculation of plants with the less virulent *Agrobacterium*/Ri-plasmid from Nursery A might provide some cross-protection against the more highly symptomatic *Agrobacterium*/Ri-plasmid combination from Nursery B.

As in the previous experiments cherry tomato cv Claree was used in the study. Six inoculation regimes were used (8 plants/regime) as follows:

1. Plants inoculated with buffer (negative control)
2. Plants inoculated with a rhizogenic *Agrobacterium* from nursery A
3. Plants inoculation with a rhizogenic *Agrobacterium* from nursery B

4. Plants inoculated with a mix (at equal concentrations) of rhizogenic *Agrobacterium* from A & B
5. Plants inoculated with *Agrobacterium* from nursery A, then one week later with *Agrobacterium* from nursery B.
6. Plants inoculated with *Agrobacterium* from nursery B, then one week later with *Agrobacterium* from nursery A.

Plants were inoculated by injecting 10ml of a 10^8 cfu/ml suspension around the base of four week old plants, and subsequently monitored (over a period of 15 weeks) for characteristic symptoms of root-mat.

Results

<u>Objective a</u> : Investigating the possible effects of slow sand filtration techniques on root-mat
in tomato

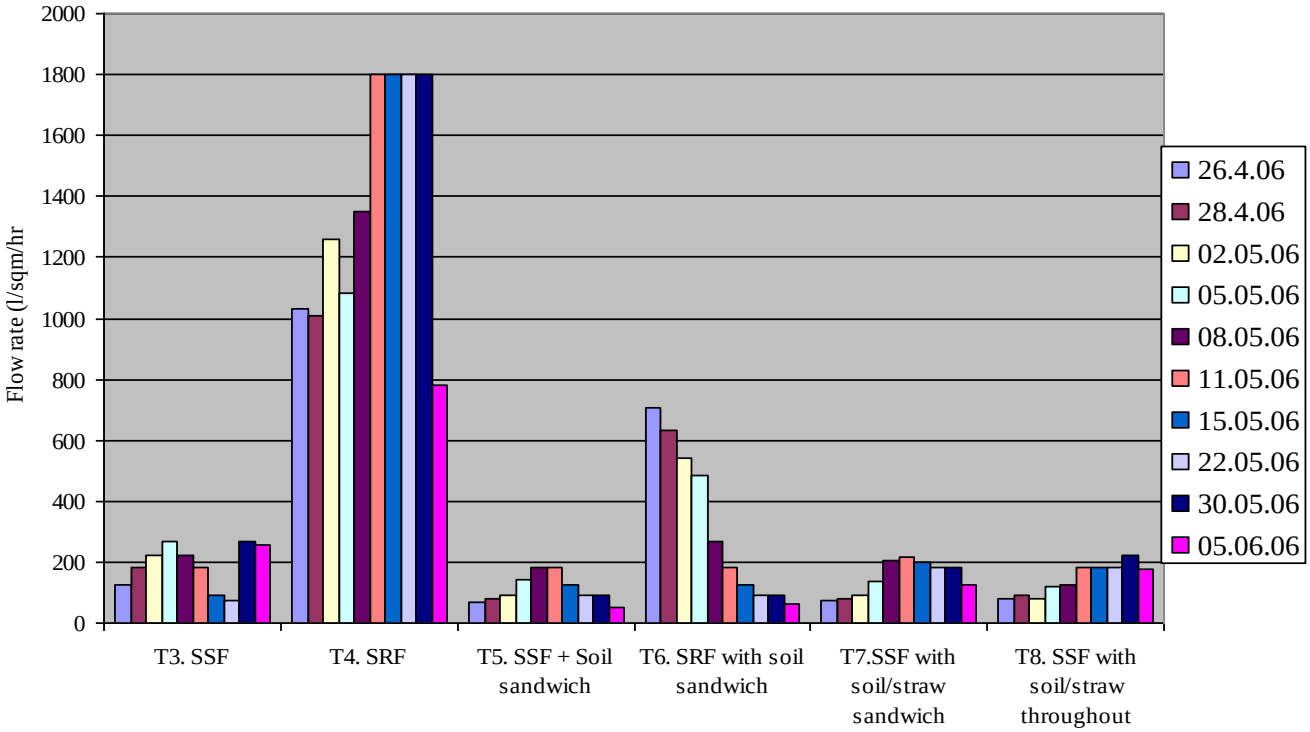
The trial established well and the filters ran as expected (Plate 4).

Plate 4. A general shot of the crop *in situ*.

The flow rates of each filter were monitored during the priming period (see chart 1).
The flow rates of the two rockwool filters was very much faster than those seen in the sand filters. The inclusion of the soil in the rockwool filter (T6) had a surprisingly large impact on the flow rate, reducing the output by just over 30% at the start of the priming period.

During the priming period the flow rate of the SRF (T4) fluctuated, though averaged out at approximately 1371L/m^2/hr. However, the flow rate of the SRF with the soil sandwich (T6) decreased rapidly over the same period dropping from a starting flow rate of almost 710L/m^2/hr to a final rate of only 63.9L/m^2/hr.

Figure 1. Filter flow rates during priming period



The flow rates in all the filters filled with sand were markedly slower than the two rockwool filters throughout the priming period, and although the SSF without any soil or soil/straw components did flow more quickly than those with the organic additions, the difference in flow rate was far less striking than had been observed with the rockwool filters. As seen with the SRF (T4), the flow rates of all the SSF fluctuated during the 5 week priming, although the flow rate of the majority of the filters did increase overall. This was deemed to be due to settlement and the gradual reduction of air pockets within the filter media. Throughout the priming period we observed that the SSF with the soil sandwich (T5) was the slowest running filter overall with the final flow rate being slightly reduced from the earlier measurements.

Such large differentials in flow rates would potentially impact on the comparison of filter efficacy and it was therefore agreed to limit the flow in the faster filters and attempt to maintain a similar flow rate in all the filters. This involved slowing them down via the outlet flow tap to the rate of the slowest filter (T5). This was undertaken to coincide with the start of the trial when the plants were placed in the channels. Overall flow rates of between 250 -300 ml/min (50L/m^2/hr) were maintained for each filter over the trial period. The flow rate of the filter for T5 continued to decrease over time dropping to 24.3L/ m^2/hr by the end of the trial. In the later stages of the trial it was not possible to limit the flow of the other 5 filters sufficiently to match T5, however they were maintained at the slowest rate that could be managed. Full details of the flow rates for all the filters during the planted period of the trial are shown in Appendix 2.

Samples of water were taken from the 'dirty' and 'clean' tanks on the 24th May, just prior to the introduction of the crop to investigate if there were differences between the total viable bacterial counts of the water pre and post filtration (Table 2).

Table 2. Results of the pre- and post-filtration bacterial testing carried out on 24th May 2006

Sample point	Filter (Treatment No.)					
	3	4	5	6	7	8
Dirty Tanks	1.0×10^5	5.0×10^4	2.0×10^5	7.5×10^5	1.1×10^6	4.0×10^5
Clean Tanks	1.0×10^5	0.0	1.0×10^5	1.1×10^6	7.0×10^5	4.0×10^5

The tabulated results show that total viable bacterial counts in the tanks were not entirely similar at this stage in the trial, although interestingly the slow rockwool filter (T4) had the lowest microbial count. This was not totally unexpected as the rockwool was likely to be semi-sterile on delivery.

Water samples were also collected from the 'clean' tanks on the 22nd May. These were tested for the presence of *Pythium* and *Phytophthora* spp. using a membrane filtration method. No evidence of either organism was detected at this time.

The crop was inoculated with the rhizogenic *Agrobacterium* suspension for the first time on the 9th June when the plants had been *in situ* for 1 week. Symptom expression with root-mat normally occurs 6-8 weeks post inoculation. However, by the middle of July no early root-mat symptoms were visible either in the planted crop at STC or in either of the experiments being carried out at CSL . The *Agrobacterium* strain used in the experiment (CSL 5083), although isolated from a heavily infected tomato crop, was not isolated from cv. Claree, the crop used in this trial. Whilst cv. Claree is known to be highly susceptible to root-mat it is thought that one possible explanation for the lack of apparent virulence is an incompatibility between cv. Claree and *Agrobacterium* CSL 5083. Thus, two further Ri-plasmid harbouring strains (CSL 6399 and 6400) were isolated from a root-mat infected crop of cv. Claree and used for a further inoculation on the 28th July. During a routine crop inspection on the 14th August possible early root-mat-like symptoms were observed in 4-5 plants in the inoculated control plots (See Plates 4 & 5 below).

Plates 4 & 5. Early root mat symptoms in inoculated control plants

The plants were carefully monitored, but the early symptoms failed to develop further and subsequently disappeared from the top of the blocks. No evidence of swelling of blocks or slabs was observed during the trial period.

By late September many plants in the trial were failing to thrive and began to experience nutrient deficiency symptoms (Plates 6 & 7). A number of plants were collapsing and a close inspection of the roots showed them to be discoloured. A *Fusarium* sp. was isolated from the root tissues although no evidence of vascular staining was found in any of the affected plants and it was concluded that this was a secondary opportunist and not primarily responsible for the symptoms observed.

Plate 6. Nutrient deficiency symptoms in crop **Plate 7. Plant in foreground with nutrient**

(T5) **deficiency symptoms (T6)**

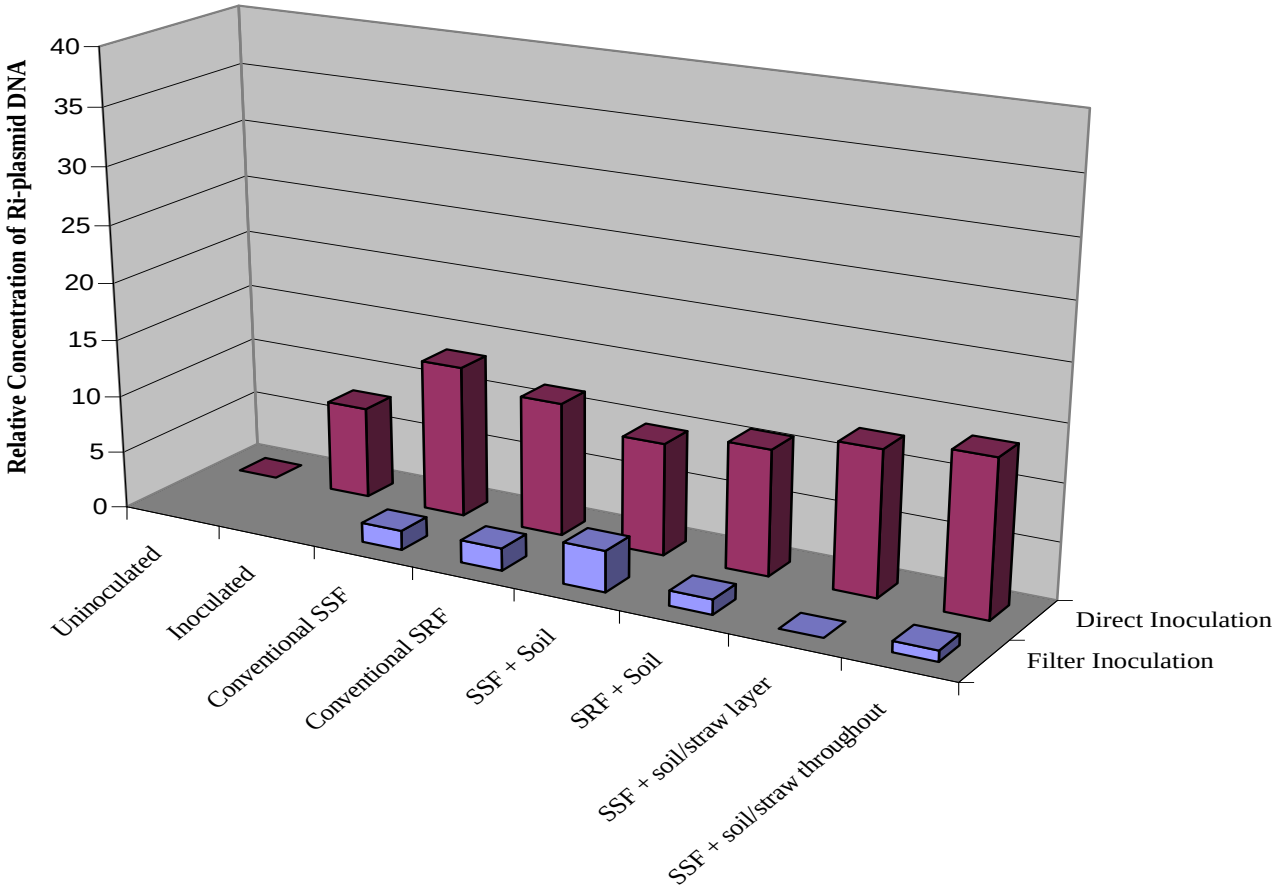
Plants in the uninoculated (T1) and inoculated (T2) control plots which were being fed by Dosatron units were noticeably greener, fuller and healthier at this point. Difficulties in maintaining a steady and correct feed regime had been experienced due to the fluctuating nature of the tank volumes. Analysis of the feed solutions from T2 (Dosatron fed), T3 (SSF) and T4 (SRF) did indicate lower levels of Ammonia N in the plots fed via the filters than in the Dosatron fed plots and this potentially accounted for the nutrient deficiency symptoms in the crop (full analyses in Appendix 3). However, it was also felt that some of the organic components contained in the filters e.g. the Barley straw in Treatments 7 & 8 may have 'locked-up' the available nitrogen and added to the nutritional problems observed. By the end of the extended trial period in mid-November 2006, no further root-mat symptoms were apparent and a decision was taken to terminate the experiment.

Root samples collected from plants in each plot were tested for the presence of the Ri-plasmid using TaqMan PCR. The results are shown graphically overleaf with a full table of un-meaned results in Appendix 4. Four root samples were collected from each row (plot) in the trial, Figure 2 shows the mean of the four values recorded/plot.

The two rows of plants which made up each treatment had been inoculated in different ways (see methods & materials section). A suspension of *Agrobacterium* containing the Ri-plasmid was applied into the top of each of the filters and the filtrate from these was used to irrigate both rows of plants in each treatment. Also, and as insurance in case the plasmid containing *Agrobacterium*

was completely eliminated by the filters, a direct inoculation to each plant in one row/treatment was also carried out. Although no symptom expression was observed during the trial, the PCR analysis of the root samples shows some very interesting results. Firstly, the uninoculated controls remained completely free from rhizogenic *Agrobacterium* showing that the organism did not spread within the glasshouse. Secondly, all the plots which had received the direct inoculation, including the inoculated control, showed the presence of the plasmid, whilst the rows of plants which had only received inoculation via the filter showed a very much lower incidence. In the majority of these latter samples, only 1 of the 4 root samples had given a weak positive result, and in the case of the SSF with the soil/straw layer no DNA was detected suggesting that the filter was 100% effective.

Figure 2. Results of PCR testing on root samples from the trial



A low C_T value indicates more target DNA present than a higher number as determined by the C_T values from the enriched *rol* real-time PCR.

NOTE:

- The C_T value or threshold cycle is an indication of which PCR cycle, in a 40 cycle PCR, detectable fluorescence is generated.
- PCR's where no fluorescence is detected within 40 cycles are considered to be negative results. In this chart the actual C_T values are expressed as (40- the recorded value) with negative results equalling zero.

Following removal of the plants on the 16th November a careful examination of the top surface of the blocks was made. A few slight root-mat like symptoms were visible on a limited number of blocks and therefore a final assessment of the root blocks was carried out on the 21st November (Table 3).

Although only very slight root-mat-like symptoms were observed, in the majority of cases these occurred in the directly inoculated rows rather than those rows which had been inoculated via the filter alone. These results cross reference with the PCR analysis on the root samples.

Table 3. Results of the final assessment for root-mat symptoms on 21st November 2006

Treatment	Plot	Mean RM severity (0-3 scale)
1. Uninoculated control	9 (-)	0.0
	10 (-)	0.0
2. Inoculated control	3 (D)	0.0
	4 (D)	0.0
3. SSF	7 (F)	0.0
	8 (D)	0.14
4. SRF	13 (D)	0.14
	14 (F)	0.04
5. SSF with soil 'sandwich'	1 (F)	0.0
	2 (D)	0.32
6. SRF with soil 'sandwich'	15 (D)	0.10
	16 (F)	0.10
7. SSF with soil/straw 'sandwich'	5 (F)	0.0
	6 (D)	0.10
8. SSF with soil/straw incorporated	11 (D)	0.32
	12 (F)	0.0

F = Filter inoculated D = Direct inoculation

<u>Objective b</u> : Investigation into the control or suppression of root-mat in tomatoes using existing microbial products

<u>Experiment 1.</u> The experiment ran for 15 weeks post-inoculation (with rhizogenic *Agrobacterium* CSL 5083). No root mat symptoms appeared in any of the plants during this time. Samples were taken at 4 weeks and 9 weeks post-inoculation and tested for the presence of rhizogenic *Agrobacterium* by an enriched real-time PCR assay (Table 4).

Table 4. Results of PCR assay on roots treated with microbiological products (Experiment 1).

Treatment	Rhizogenic *Agrobacterium* found to be present	
	At 4 weeks post-inoculation	At 9 weeks post-inoculation
Negative control	Negative (X)	X
Positive control	Positive (✓)	✓
Biomex SA	✓	✓
Companion	X	✓
Garlic Barrier Plus	X	✓
Gliomix	X	X
Seasol	✓	✓
Stimagro	✓	✓
Seasol + Biomex SA	X	✓
Seasol + Companion	X	✓

Experiment 2 This experiment was conducted over a 16 week trial period post-inoculation. No typical root mat symptoms were observed in any of the plants. Samples were taken at 4, 7 and 13 weeks post inoculation and tested for the presence of rhizogenic *Agrobacterium* by an enriched real-time PCR assay (Table 5).

Table 5. Results of PCR assay on roots treated with microbiological products (Experiment 2).

Treatment	Rhizogenic *Agrobacterium* found to be present		
	At 4 weeks post-inoculation	At 7 weeks post-inoculation	At 13 weeks post-inoculation
Negative control	X	X	X
Positive control	✓	✓	X
Biomex SA	X	✓	X
Companion	X	✓	X
Garshield	✓	X	X
Gliomix	✓	✓	✓
Seasol	X	✓	✓
GLD	X	✓	✓
Seasol + Biomex SA	✓	✓	X
Seasol + Companion	X	✓	✓

The results from the sampling do suggest a build up of *Agrobacterium* populations from between 4-7 weeks post-inoculation. However, the samples taken at 13 weeks indicate a subsequent decrease. As this decrease was also observed in the positive control plants it would appear that this may be a natural phenomenon in the disease cycle rather than an effect from the continued application of the microbiological control products.

Objective c : (Year 2 onwards)

Objective d : Investigation into the control or suppression of root-mat in tomato using cross-protection

Following inoculation no definitive symptoms of root-mat appeared in any of the inoculated plants after 15. The experiment was allowed to run for a further 4 weeks, again without symptoms appearing, and then terminated. No samples were taken during the course of the experiment.

Discussion

Although each of the experiments carried out in year 1 of this 3 year project were successfully established and carried out with care, consistent development of root-mat symptoms were not observed in the positive controls used for each study. However molecular testing on root samples collected from the glasshouse trial at STC showed that the rhizogenic *Agrobacterium* was present in the positive control plants, and also in the plants which had been directly inoculated. Yet much lower levels of the rhizogenic plasmid DNA were found to be present in the plants which had only been subjected to inoculum via the filters, suggesting perhaps that this provided a viable means of suppressing or preventing root-mat dissemination via irrigation systems.

In the experiments carried out at CSL to investigate possible control via applications of readily available microbiological products the plasmid was detected (by real-time PCR) in the roots of all the treated plants with the exception of those treated with Gliomix (Fargro Ltd) in the 1st experiment. However, the importance of this finding is not clear due to the lack of symptom expression in the positive control plants in both experiments.

The cross-protection experiment was similarly hampered by the lack of root-mat symptom expression and conclusions cannot be drawn from the results.

The reasons for the lack of symptom expression in the experiments at both CSL and STC are unclear. The isolate of rhizogenic *Agrobacterium* used in the majority of the experiments, when inoculated on the first occasion was CSL 5083. This had been isolated from a tomato crop with very severe root-mat symptoms at nursery B in southern England in 2003. Unfortunately, information on the tomato cultivar from which it was isolated was not available. All of the work to date in this study has used the cherry tomato cultivar Claree as this had been badly affected by root-mat at nurseries during 2005 (Dr P Morley, *pers com*). However, there are areas of uncertainty regarding the effect of interactions between Ri-plasmid type and cultivar choice on pathogenicity. Although the STC glasshouse trial was inoculated a second time with a rhizogenic *Agrobacterium* (suspension bulked up following isolations from highly symptomatic roots from tomato cv Claree), symptom expression still did not occur. If we could identify the precise reason for this failure to establish/express symptoms then root-mat control was potentially within our grasp.

One hypothesis was that if the plasmid type/*Agrobacterium* strain and cultivar were not ‘matched’, the inoculation with the first isolate may have ‘switched-on’ the immune system in the inoculated plants; the isolate being insufficiently virulent to cause the development of classic root-mat symptoms. The second inoculation with the cultivar-specific rhizogenic *Agrobacterium* may then have proved ineffective due to the possible ‘cross-protection’ provided by the first inoculation. Although a similar phenomenon had previously been observed when *Agrobacterium* strains isolated from tomato were inoculated onto cucumber plants (and *vice versa*) this was the first time that a similar phenomenon had been observed

with strains isolated from tomato and re-inoculated onto tomato. However, further to these observations, the result of the repeated microbiological products experiment carried out at CSL showed that when plants were inoculated with *A. radiobacter* strains CSL 6399 and 6400, root-mat symptoms still failed to develop in the positive control plants after 22 weeks post-inoculation. Hence unknown factors are responsible for the lack of symptom expression and investigation of these factors must form the basis of subsequent studies within this project as they potentially offer a route to control of the problem.

In year 2 of this project, as well as repeating the glasshouse experiment at STC, it may prove advantageous, if possible, to create a small-scale SSF/SRF system at one of the badly affected nurseries in the south of England. If this system was used to irrigate a small number of tomato plants e.g. one row in a glasshouse with a history of severe root mat it would provide a good challenge for the system and potentially demonstrate what level of disease control SSF or SRF could be achieved.

Investigation of the possible benefits of grafting tomato onto a potentially less root-mat prone rootstock such as Aubergine will also commence in 2007 primarily on commercial tomato nurseries.

Conclusions

- A technically complex experimental system for hydroponic tomato crop production was established at STC to investigate the possible effects of slow sand and slow rockwool filters containing additional organic components on the development of root mat symptoms.

- In this study root-mat symptoms did not develop as expected following the inoculation with rhizogenic *Agrobacterium radiobacter*. A second inoculation was therefore carried out. A few early possible root-mat symptoms were observed in the inoculated control plants in mid-August. However, further symptom expression did not occur and symptoms that had been initially disappeared from the surface of the rockwool blocks. During the final assessment of the blocks, at trial termination, a few, very weak root mat-like symptoms remained though it was unclear due to the high level of algal build-up by this stage.

- Root samples from the crop which were analysed using real-time PCR, showed that the plasmid was present in the inoculated plots. Higher quantities of the plasmid were present in the plots which had been directly inoculated, whilst those plants which had only received inoculum via the filter showed a significant reduction in the amount of plasmid DNA, and in one case (T7) no plasmid DNA was detected at all.

- Experiments were carried out at CSL to investigate the possible benefits of applying readily available microbiological products to root mat inoculated plants. Once again symptom expression was not achieved, though molecular tests appeared to show that plants treated with GLD (a garlic extract/salicylic acid product from Omex) did not contain the plasmid. A similar result was obtained following the application of Gliomix (Fargro Ltd) in Experiment 1 though in this case the Ri-plasmid was detected in the Gliomix treatment in Experiment 2.

- CSL also carried out an investigation into the possible cross-protection properties of applying a weakly pathogenic rhizogenic *Agrobacterium* to tomato plants prior to inoculating them with a more virulent strain. Unfortunately, no symptoms developed in any of the plants and it was not possible to draw conclusions regarding the efficacy of this control strategy.

- The lack of symptom development in each of the experiments carried out to date in this project is of concern but perhaps suggests that commercial control of root-mat is a real possibility providing that the reason for the lack of symptom expression in the inoculated crops can be determined. Further investigation into the possible causes which might include; lack of pathogenicity in the bacterial isolate – plasmid type – tomato cultivar combination, loss of virulence of the plasmid over time, pH of water, differences in cultivation methods etc. all need to be examined during the approaching season.

Technology Transfer

- Root mat update: Seminar to Wight Salads managers, Arreton Valley, Isle of Wight, 3rd October 2006 (Tim O'Neill)
- A number of interested grower groups which have visited STC during 2006 were shown the tomato glasshouse trial.
- Derek Hargreaves visited the crop on several occasions during 2006 to provide technical input to the R&D programme.
- Dr P Morley and Wight Salads grower Paul Howlett visited the trial and discussed the crop management aspects on 22nd November 2006.

Acknowledgements

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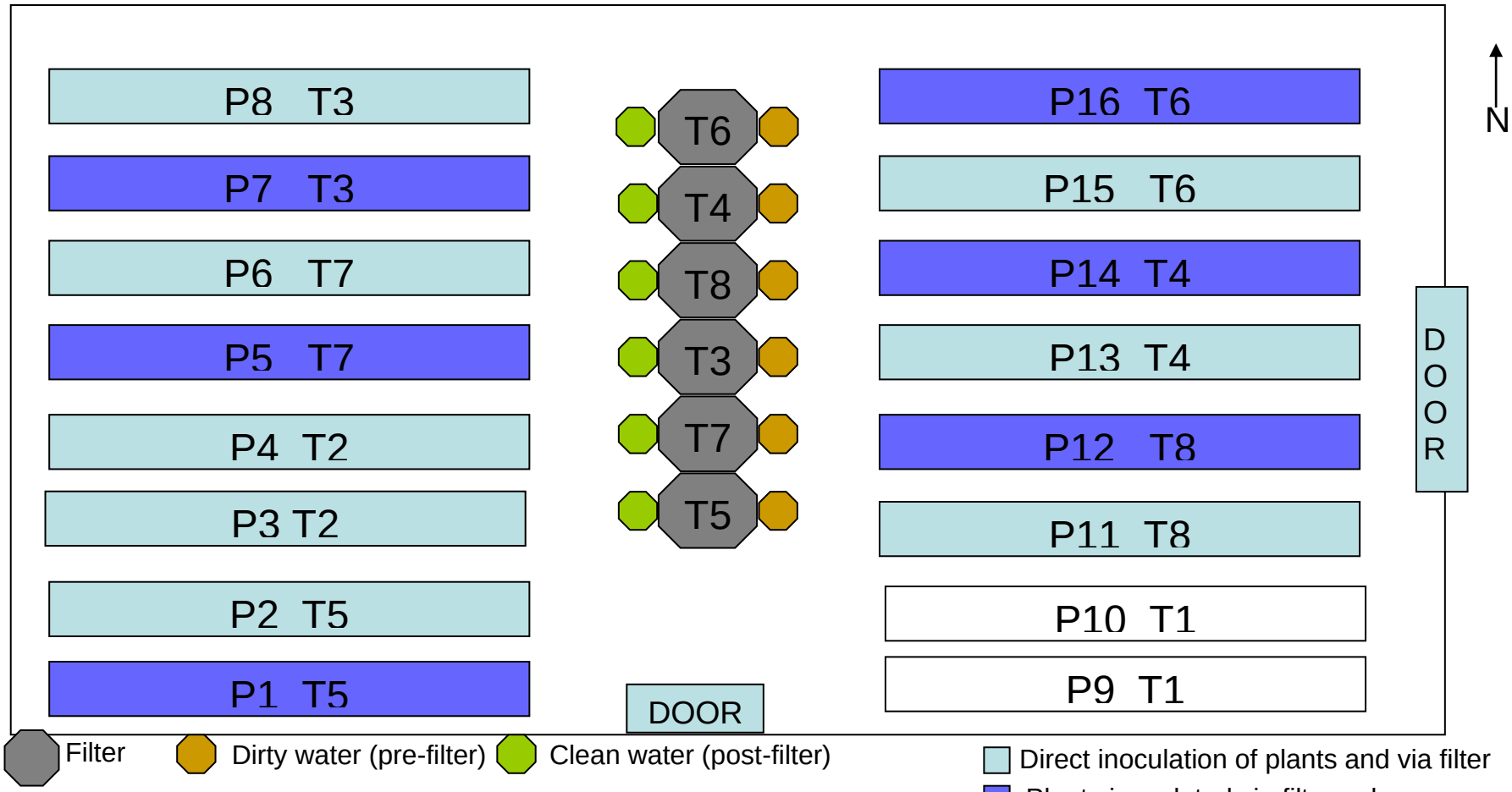
Appendix 1

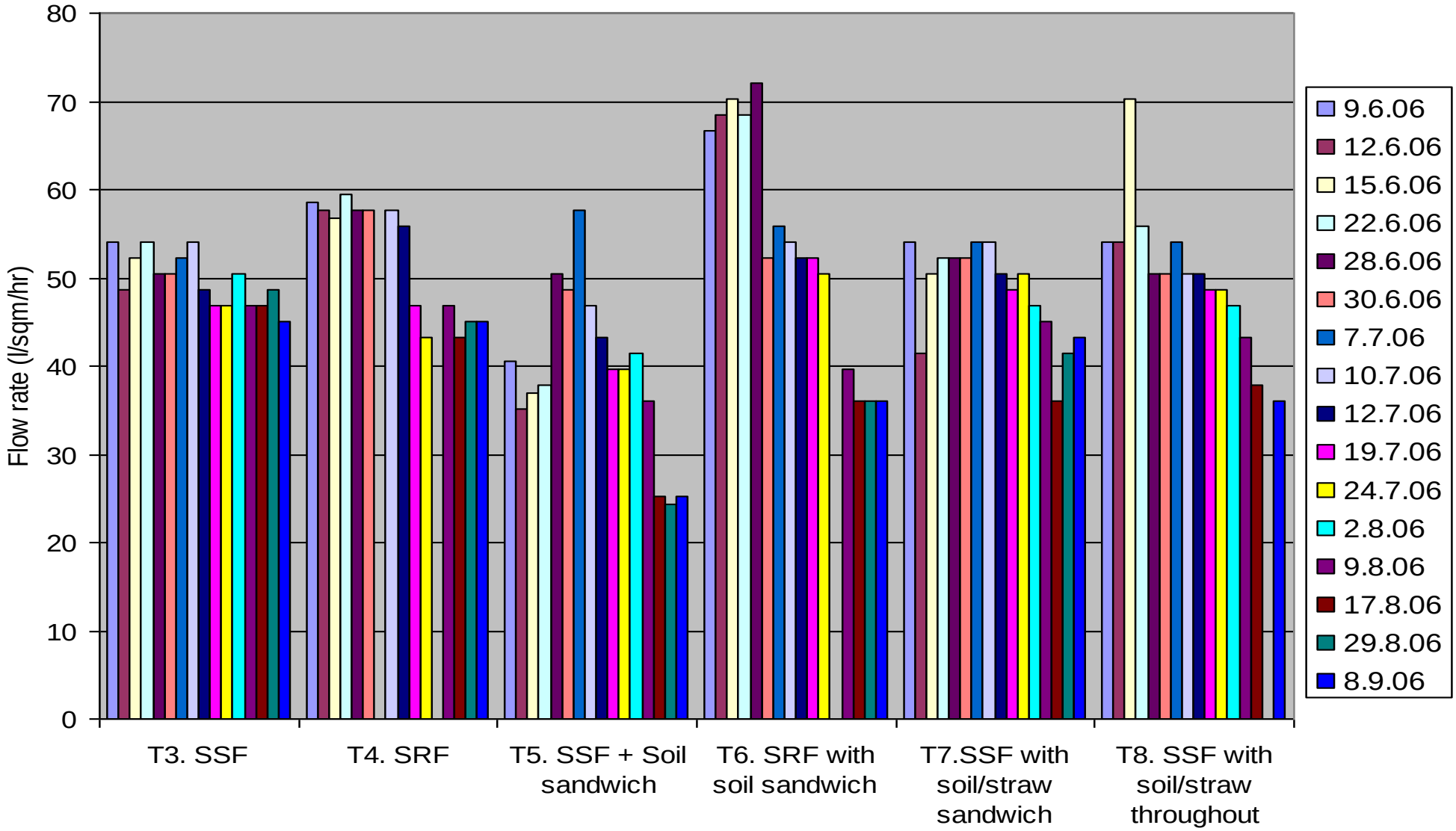
Investigation into control of Root mat in Tomatoes
E301 2006 Trial Plan



Treatments **cv. Claree**

T1 – Uninoculated control – RTW
T2 – Inoculated control – RTW
T3 – Inoculated + conventional SSF
T4 – Inoculated + slow rockwool filtration (SRF)
T5 – Inoculated + SSF with organic soil
T6 – Inoculated + SRF with organic soil sandwich
T7 – Inoculated + SSF with soil/straw sandwich
T8 – Inoculated + SSF with straw/soil mix throughout filter.

Appendix 2 Chart showing flow rates of all filters during trial period (post-planting)



NB – gaps on particular dates occurred due to the level of water in the 'clean' tank being too high to allow flow-rate measurement.

Appendix 3 – Nutrient solution analyses for Tomato Root Mat Trial at STC in 2006

Test	Sample taken on 4.08.06			Samples taken on 29.08.06		
	T2 (Dosatron)	T3 (SSF)	T4 (SRF)	T2 (Dosatron)	T3 (SSF)	T4 (SRF)
Calcium mg/l	881	601	456	400	643	636
Magnesium mg/l	450	305	221	158	330	383
Manganese mg/l	0.60	0.03	0.03	0.19	0.01	0.01
Boron mg/l	1.87	1.08	0.82	0.72	0.79	1.07
Copper mg/l	1.03	0.51	0.35	0.24	0.15	0.39
Molybdenum mg/l	0.25	0.18	0.14	0.17	0.19	0.19
Iron mg/l	3.40	0.78	0.37	1.48	0.20	0.17
Zinc mg/l	1.65	1.20	0.85	0.69	0.41	1.17
Sulphur mg/l	608.0	514.0	379.0	212.0	6.0	612.0
Phosphorus mg/l	72.0	1.00	1.0	20.00	<1.0	<1.0
Potassium mg/l	2506.0	1478.0	1106.0	998.0	1119.0	1558.0
pH	6.2	7.8	7.8	6.7	7.7	7.3
Nitrate N mg/l	1452.0	870.0	581.0	522.0	546.0	631.0
E.C. mmhos/cm	14.60	9.94	7.15	6.06	8.81	10.30
Ammonia N mg/l	8.10	0.45	0.03	2.40	0.60	0.60
Sodium mg/l	93.00	147.0	117.0	34.0	198.0	239.0
Chloride mg/l	113.0	125.0	125	45	125	174
Bicarbonate mg/l	92.0	189.0	226	99	300	334

Samples analysed by Lancrop Laboratories, Pocklington, Yorks.

Appendix 4. Results of the Analysis of root samples from tomato slow sand filter trial (STC) for presence of rhizogenic *Agrobacterium* sp.

Sample (Plot No. : Plant No.)	Treatment	Inoculation method	*rol* PCR & C_t value
1:1	T5 – SSF with organic soil	Inoculum added to top of filter	-ve
1:5			**+ve (39.71)**
1:10			**+ve (25.92)**
1:15			-ve
2:1		Inoculum applied directly to root blocks	**+ve (31.31)**
2:5			**+ve (29.16)**
2:10			**+ve (31.04)**
2:15			**+ve (29.34)**
3:1	T2 – Inoculated control (Run to waste)	All directly inoculated to root blocks	**+ve (31.55)**
3:5			**+ve (32.44)**
3:10			**+ve (31.71)**
3:15			**+ve (34.53)**
4:1			**+ve (30.05)**
4:5			**+ve (30.08)**
4:10			**+ve (30.07)**
4:15			**+ve (35.61)**
5:1	T7 – SSF with soil/straw sandwich	Inoculum added to top of filter	-ve
5:5			-ve
5:10			-ve
5:15			-ve
6:1		Inoculum applied directly to root blocks	**+ve (27.35)**
6:5			**+ve (29.58)**
6:10			**+ve (26.29)**
6:15			**+ve (26.07)**
7:1	T3 – Conventional SSF	Inoculum applied directly to root blocks	**+ve (33.26)**
7:5			-ve
7:10			-ve
7:15			-ve
8:1		Inoculum added to top of filter	**+ve (27.77)**
8:5			**+ve (27.14)**
8:10			**+ve (25.68)**
8:15			**+ve (26.46)**

(table continued on next page)

Sample (Plot No. : Plant No.)	Treatment	Inoculation method	*rol* PCR & C_t value
9:1	T1 – Uninoculated	-	-ve
9:5			-ve
9:10			-ve
9:15			-ve
10:1		-	-ve
10:5			-ve
10:10			-ve
10:15			-ve
11:1	T8 – SSF with straw/soil mixed throughout top sand layer	Inoculum applied directly to root blocks	**+ve (27.78)**
11:5			**+ve (28.55)**
11:10			**+ve (25.38)**
11:15			**+ve (23.57)**
12:1		Inoculum added to top of filter	**+ve (36.19)**
12:5			-ve
12:10			-ve
12:15			-ve
13:1	T4 – Slow rockwool filtration	Inoculum applied directly to root blocks	**+ve (28.07)**
13:5			**+ve (27.54)**
13:10			**+ve (29.16)**
13:15			**+ve (28.64)**
14:1		Inoculum added to top of filter	-ve
14:5			-ve
14:10			-ve
14:15			**+ve (32.21)**
15:1	T6 – SRF with organic soil sandwich	Inoculum applied directly to root blocks	**+ve (28.92)**
15:5			**+ve (26.92)**
15:10			**+ve (34.61)**
15:15			**+ve (25.71)**
16:1		Inoculum added to top of filter	**+ve (34.75)**
16:5			-ve
16:10			-ve
16:15			-ve

Real-time analysis facilitates quantification of the amount of sample DNA present in the reaction by ascertaining when (i.e. during which PCR cycle) fluorescence in a given reaction tube exceeds that of a threshold (Threshold Cycle (C_T)), with lower values indicating higher concentrations of target DNA than higher C_T values.

Appendix 5 – Details of slow sand filter construction

Sand and gravel particle sizes:	Coarse gravel -	16 -20 mm diameter
	Finer gravel -	4-6mm diameter
	Coarse sharp sand –	1-1.4mm
	Fine silver sand –	0.2 – 1mm

T1 Uninoc control RTW

T2 Inoc control RTW

T3 Inoc conventional SSF
4 green bags of coarse gravel, covered with an empty green bag
2 bags of finer gravel spread evenly across the top
Layer of fleece
The contents of 1 bag coarse sharp sand
Layer of fleece
The contents of 7 bags fine silver sand

T4 Inoc Slow rockwool filter (SRF)
4 green bags of coarse gravel, covered with an empty green bag
Rockwool granulate up to lip on tank – compressed

T5 Inoc SSF with organic soil sandwich
As T3 but with 5 Litres of sieved soil (AVN) sandwiched between fleece 2 cm below lip.
The contents of 6 bags of fine sand, then soil, then 7th bag of sand on top.

T6 Inoc SRF with organic soil sandwich
As T4 but with 5 Litres of sieved soil (AVN) sandwiched between fleece 2 cm below lip.

T7 Inoc SSF with soil/straw sandwich
A T5, but with 2.5 litres soil, and 2.5 litres finely cut (3cm pieces) of barley straw.

T8 Inoc SSF with straw soil mix throughout
4 green bags of coarse gravel, covered with an empty green bag
2 bags of finer gravel spread evenly across the top
Layer of fleece
The contents of 1 bag coarse sharp sand
Layer of fleece
The contents of 7 bags fine silver sand with soil/straw mix incorporated throughout.