Project title:	Tomato: epidemiology and control of *Verticillium* wilt in hydroponic and soil-grown crops
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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.

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.

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CONTENTS

GROWER SUMMARY

Headline

- Techniques for specific and sensitive detection of *V. albo-atrum* are being used to examine potential sources of the fungus on a nursery. So far, after limited testing, the fungus has not been detected in air, soil, water, insects on sticky traps, or on drip pegs.

- Several disinfectants readily killed *V. albo-atrum* spores but eradication of mycelium was more difficult.

- The potential of disease spread on hands was demonstrated; removal of *V. albo-atrum* spores from hands is possible with thorough rubbing with Med Gel.

- Some host physiology and environmental factors were found to influence development of Verticillium wilt symptoms in tomato plants.

Background and expected deliverables

HDC project PC 186 (O'Neill & Fletcher, 2002) forewarned UK growers of a new form of *Verticillium* wilt that is a potential serious risk to the economic production of tomatoes. The problem affected at least 10 nurseries in 2001 and was confirmed on a similar number in 2002, affecting crops in England, Wales and Scotland. This work, in conjunction with Defra project HH3222SPC, is intended as pre-emptive research to devise a solution before the problem becomes widespread and common.

Key points from the 2002 review were:

- The causal fungus is slow-growing and not easy to isolate from plants.
- Many varieties and rootstocks have been affected.
- The disease is a 'slow wilt' in tomatoes and may be confused with wilting caused by root rotting fungi (e.g. *Pythium*).
- Yield losses were estimated by growers at 10-15%.
- The disease is difficult to eradicate from a nursery; it tends to occur in the same glasshouses each year.
- Source of the disease and how it spreads is unknown.
- Bavistin DF (carbendazim) root drench gives some control if applied early.
- Peppers and cucumbers are also susceptible; two lettuce varieties tested were unaffected.
- No alternative sources of resistance to *Verticillium*, either varieties or rootstocks, are currently available.

This project aims to increase our understanding of the cause and spread of *Verticillium* wilt in tomato crops and of crop cultural and management practices that influence disease expression, and to devise and test practical control measures.

Summary of the project and main conclusions

Literature review

The fungus *Verticillium albo-atrum*, its life cycle, the infection process, host resistance, disease epidemiology and diagnosis are reviewed.

Sources and spread of *V. albo-atrum* on a nursery

A PCR method for the specific and sensitive detection of *V. albo-atrum* that was developed in a parallel Defra-funded project (HH3222SPC) was used to test various potential sources of the fungus. Although the fungus was not detected on tomato seed, in air, soil, water, insects on sticky traps, or drip pegs, all collected from an affected crop, further testing is required to determine with greater certainty whether or not these are potential infection sources. The detection limit of the current PCR test is around 1,000 spores; a method to improve the sensitivity is being developed.

Inoculum level and plant age

An inoculum level of just 100 spores applied as a drench to tomato seedlings (2-6 true leaves) resulted in development of wilt symptoms after around 5 weeks. Not all inoculated plants had developed symptoms by 10 weeks after inoculation. The severity of symptoms increased with spore concentration from 100 to 1,000,000 spores/plant.

Detection of *V. albo-atrum* within stems

Ten weeks after inoculation of plants by drenching spores into the root zone, *V. albo-atrum* was confirmed by a specific PCR test in vascular tissue at the stem base and 50 cm above the stem base. Using this method, the fungus was detected in plants inoculated with 10^6 spores/ml and not with the lower inoculum levels even though these treatments showed wilt symptoms. Further work comparing the sensitivity of different methods for detection of *V. albo-atrum* is required.

Infection at leaf scars

Following inoculation with *V. albo-atrum* spores of fresh leaf scars on tomato seedlings, created by removal of leaves with a scalpel, the fungus was detected by PCR in surface sterilised stem tissue 1 cm above and 1 cm below the inoculation point. This result indicates stem colonisation to this extent. The experiment is being repeated.

Plant growth and symptoms

Experiments on tomatoes grown in peat bags indicated that the severity of Verticillium wilt symptoms is affected by fruit load and occurrence of side shoots. Wilt severity was reduced by reducing the fruit load and was increased if sideshoots were allowed to develop.

Environmental factors

Following drench inoculation with a standard inoculum of *V. albo-atrum* spores, plants grown in compost that was kept moist developed Verticillium wilt symptoms whereas plants grown dry did not. Possibly this was due to death of conidia in the plants watered infrequently and allowed to dry out; an experiment on the effect of air-drying on spore viability is in progress.

Eight weeks after inoculation, pot-grown plants simultaneously inoculated with *Phytophthora cryptogea* and *V. albo-atrum* were significantly reduced in height, by 14-20

cm, compared with plants inoculated with either fungus alone. The incidence of plants infected by *Verticillium* following inoculation (70%), and the degree of stem colonisation by this fungus at the plant base, were not increased by co-inoculation with *P. cryptogea*.

Chemical disinfectants

Six disinfectants (Harvest Wash, Jet 5, sodium hypochlorite, Trigene, Unifect G and Virkon S) were all fully effective against conidia of *V. albo-atrum* when used at their standard rate and with a contact time of 5 minutes or greater. Citric acid had no effect even after 8 hours.

When tested against *V. albo-atrum* mycelium grown on filter paper discs, only Panacide M and Unifect G eliminated the fungus following a 5 minutes contact time. Sodium hypochlorite was effective after 30 mins, Trigene after 1 hr and Jet 5 and Virkon S after 8 hours. Citric acid and Harvest Wash were ineffective.

Contamination and disinfection of hands

Spores of *V. albo-atrum* were found to be suprisingly persistent on hands and were relatively difficult to remove by washing. Once contaminated by touching a sporulating stem lesion, a thumb was still transmitting *V. albo-atrum* after 160 sequential contacts with an agar plate. Even after washing with warm water and soap for 30 seconds, transmission of *V. albo-atrum* occurred for over 50 sequential contacts. Bare hands were effectively cleaned of *V. albo-atrum* by rubbing with Med Gel for 1 minute; washing in warm water and soap for 1 minute was not effective. Latex gloves also transmitted *V. albo-atrum* but were easier to clean. Both Med Gel (1 minute) and warm water and soap (1 minute) were effective.

Detection of *V. albo-atrum* in commercial crops

Damp incubation and microscopic examination of leaf petioles slices can be used to detect *Verticillium* in tomatoes without destroying the plant. No *Verticillium* was detected by plating out petiole sap onto agar.

Monitoring of *Verticillium* wilt development in a commercial crop

A rockwool crop in Kent on a nursery with a history of Verticillium wilt was monitored for the disease at intervals throughout 2004. Petioles of lower leaves were tested for Verticillium by incubation of tissue slices. Wilting was first seen in the crops, at a very low level, at the end of January, and Verticillium was confirmed. There was little visual affect of the disease for most of the season, although the plants thinned in the head in midsummer. Yields overall were relatively poor and much of the loss was attributed to the use of grafted plants which were too vigorous. At the end of the season, there were many plants dead and almost all of them had a severe *Botrytis* stem lesion, which could have accounted for the death. Dead stem bases of the variety Chloe had sporing *Verticillium* and large populations of springtails. The incidence of *Verticillium* at crop termination in October could not be related to the occurrence or severity of vascular staining. There was a higher level of *Verticillium* (88.6%) in grafted plants of cv. Encore than in ungrafted (65.1%) plants of cv. Chloe.

Financial benefits

The UK area of protected tomatoes in 2003 was estimated at 179 ha (4 ha unheated), with a total farm-gate value of around £80.1 million (Defra – Basic Horticultural Statistics for the United Kingdom), Assuming that 5% of the cropped areas suffers a 10% loss due to Verticillium wilt, this equates to £400,425 per annum. Development of an effective strategy to control Verticillium wilt will thus have a significant financial benefit.

Action points for growers

1. Be aware that after touching a sporulating *Verticillium* stem lesion, hands will be contaminated with spores of the fungus and they could spread the disease (e.g. via fresh de-leafing or side-shooting scars) to healthy plants.
2. Hands or latex gloves contaminated with *V. albo-atrum* spores can be disinfected by rubbing in Med Gel for 1 minute.
3. The disinfectants Harvest Wash, Jet 5, sodium hypochlorite, Trigene, Unifect G and Virkon S are all fully effective against conidia of *V. albo-atrum* when used at their standard rate and with a contact time of 5 minutes or greater.
4. Only Panacide M and Unifect G eliminated *V. albo-atrum* mycelium grown on filter paper discs following a 5 minutes contact time. Sodium hypochlorite was effective after 30 mins, Trigene after 1 hr, and Jet 5 and Virkon S after 8 hours. Consider using Panacide M or Unifect G to disinfect areas contaminated with *V. albo-atrum* and organic matter.

Science Section

1. Introduction and literature review

During the past several years, *Verticillium* wilts have become an increasing problem in the tomato industry. In 2001, yield losses of tomato crops due to *Verticillium* wilts were estimated by growers to be around 10-15%, equivalent to a loss of around £75,000 per annum. Tomato varieties that previously showed resistance to the disease (*Ve*-resistant tomatoes) have now become susceptible to the *Verticillium* wilts. The disease has affected production of hydroponic (rockwool and NFT) and soil-grown tomato crops. It has been recently confirmed that some UK isolates of *Verticillium albo–atrum* are capable of overcoming the *Ve*-resistance gene (O'Neill, 2002).

Molecular characterisation of new isolates of *V. albo-atrum*, from *Ve*-resistant tomatoes, indicates that they do not represent a single new clone or a closely related group of isolates. They were not separated from two isolates obtained from hops over twenty years ago so there has not been a single introduction of a new strain. It is more likely that there has been multiple introductions of, or selection, for aggressiveness to *Ve*-resistant varieties (O'Neill & Barbara, 2002b).

The commercial objective of this work is to find an effective strategy for managing this new disease by an increased understanding of the causal fungus, disease epidemiology, symptom expression, and methods of control.

1.1 The pathogen

Taxonomy:

The fungus *Verticillium albo-atrum* can be classified as follows:

- Kingdom: Fungi
- Phylum: Ascomycota
- Subphylum: Pezizomycotina
- Class: Sordariomycetes
- Subclass: Sordamycetes incertae sedis
- Order: Phyllachorales
- Suborder: Mitosporic Phyllachorales
- Genus: Verticillium
- Species: *V. albo-atrum* Reinke and Berthold (1879)

This soil-born pathogenic fungus spreads through the plant in the xylem and is associated with wilt diseases in a wide range of hosts including hop, lucerne (alfalfa), tomato, potato and ornamental plant species. *V. albo-atrum* appears to be limited to the regions that do not exceed an average air temperature of 21-24^{0}C for much of the growing season. This fungus can attack and multiply in many common weeds. It persists in soils for long periods. It remains alive by means of dark resting mycelium, which it forms in diseased plant parts.

The genus Verticillium was established in 1816 by Nees von Essenbeck based on

characteristic verticillate conidiophore, which is shown on Figure 1.1.1 below. At present there are five species that are considered vascular pathogens, including *Verticillium albo-atrum*, *Verticillium dahliae*, *V. nigrescens*, *V. nubilum* and *V. tricorpus*, although only the first two cause significant disease problems.

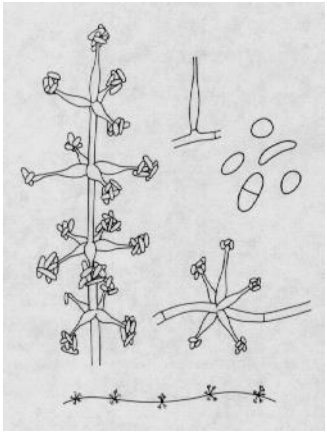


Figure 1.1.1 Verticillium is characterized by whorls of phialides produced along the length of undifferentiated filaments on conidiophores.
(From http://www.botany.utoronto.ca/ResearchLabs/MallochLab/Malloch/Moulds/Verticillium.html)

Reinke and Berthold described *V. albo-atrum*, isolated from diseased potato plants. In addition to characteristic verticillate conidiophores, they described resting structures, which they called 'dauermycelium' or resting mycelium. In 1913, Klebahn described *V. dahliae*, which he considered distinct from *V. albo-atrum*, based primarily on the formation of true sclerotia rather than the resting mycelium described for *V. albo-atrum* (Mace *et al.*, 1981).

Morphology of *V. albo-atrum*

The fungus consists of hyphae, conidiophores, conidia, and later dark, thick-walled resting structures. Hyphae and conidia of *Verticillium albo-atrum* are mostly haploid (Tolmsoff, 1973). Most cells are monokaryotic but hyphal tips may be multinucleate. Conidia (phialospores) are formed in clusters in a mucilaginous slime on elongated conidiogenous cells called phialides. These phialides are borne in whorls on branched aerial hyphae (Hawksworth & Talboys, 1970). *V. albo-atrum*, while possessing verticillate conidiophores, has complicated branches and many whorls. Reinke and Berthold illustrated a darkened base of the conidiophore in their description of *V. albo-atrum*. Isaac (1949) and Smith (1965) described larger conidiophores with dark pigmented bases of *V. albo-atrum*, when compared with the smaller, completely hyaline conidiophores of the *V. dahliae* (Pegg & Brady, 2002).

Morphology of an isolate of *V. albo-atrum* AR01/036 from Ve-resistant tomato was compared with an old UK of the fungus isolate (HRI 1974) growing in different agar media (PLYA, PDA, V8, MEA- malt extract agar). Length of conidia and diameter of the colonies were measured. Preliminary analysis showed no variation among these isolates in their morphology and sizes (Krishnamurthy, pers. comm.).

Life cycle of the pathogen

Several phases can be distinguished in the disease cycle of *V. albo-atrum* (Fig.1.1.2): survival and spread of the fungus, infection of the root, colonisation of the vascular tissue, and symptom development.

V. albo-atrum can overwinter as a dark resting mycelium within perennial hosts, in propagative organs, or in plant debris. In *V. albo-atrum*, hyphal sections differentiate into thick-walled melanized cells, the 'dauermycelium' of Reinke and Berthold. Catechol stimulates the production of dark mycelium in the fungi (Pegg & Brady, 2002).

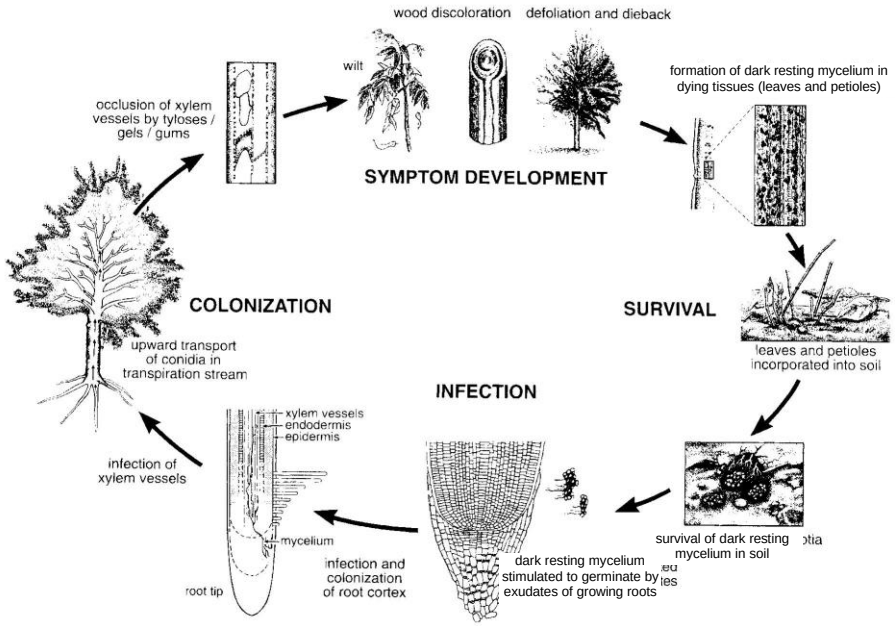


Fig.1.1.2 The disease cycle of *Verticillium albo-atrum* in tree hosts (courtesy of J.A. Hiemstra and A.J. Termorshuizen; drawing by P.J.Kostense)

Infection process

The infection process in plants caused by *V. albo-atrum* starts when the fungus invades the root tissues through wounds, which may be caused by nematodes, or via the apical meristem, and grows intercellularly until it enters the xylem vessels. There, the mycelium proliferates and produces conidiospores, which are released and carried upwards in the transpiration stream (Pegg & Brady, 2002). Eventually, spores are trapped in bordered pits or at vessel end walls; later, germ tubes are produced which penetrate into adjacent vessel elements and begin a new infection cycle. In this manner the pathogen progresses up the plant. The xylem parenchyma cells surrounding each site respond to the immediate presence of the pathogen by deploying an array of defence responses both anatomical (structural changes) and biochemical (Heinz *et al*., 1998). Vascular occlusion by the fungus, caused by cell wall degradation and by the plant's response to restrict fungal spread, results in water stress and reduced transpiration. Water stress leads to reduced photosynthesis and reduction in leaf size and fruit yield. Cell wall degrading enzymes and toxins may also be involved (Durrands & Cooper, 1988).

1.2 *Ve*-resistant tomatoes

In tomato varieties, the *Ve* gene is implicated in race-specific resistance to infection by *Verticillium* species causing crop disease. A single gene *Ve* from the Peruvian wild species *Lycopersicon pimpinellifolium* was incorporated into commercial cultivars of *Lycopersicon esculentum* by Blood in 1925 and confers resistance to race 1 of *V. dahliae* and to strains (host pathotypes from tomato and hop) of *V. albo-atrum* (Pegg & Brady, 2002). Grogan suggested that race 2 of *V. dahliae* was already in existence with race 1 and increased under the selection pressure from greater planting of *Ve*-resistant lines of tomatoes. Race 2 isolates showed a continuum of virulence suggestive of genetic variation at loci other than the *Ve* gene (Pegg & Brady, 2002).

Nature of *Ve*-resistance

Characterisation of the *Ve* locus involves positional cloning and isolation of two closely linked inverted genes. Expression of individual *Ve* genes in susceptible potato plants conferred resistance to an aggressive race 1 isolate of *V. albo-atrum*. The deduced primary structure of *Ve*1 and *Ve*2 included a hydrophobic N-terminal signal peptide, leucine-rich repeats containing 28 or 35 potential glycosylation sites, a hydrophobic membrane-spanning domain, and C-terminal domain. A leucine zipper–like sequence occurs in the hydrophobic N-terminal signal peptide of *Ve*1 and a Pro-Glu-Ser-Thr (PEST)-like sequence resides in the C terminal domain of *Ve*2. These structures suggest that the *Ve* genes encode a class of cell-surface glycoproteins with receptor-mediated endocytosis like signals and leucine zipper or PEST sequences (Kawchuk, *et al*., 2001)

1.3 Epidemiology: symptoms

In field-grown tomato, an early symptom of infection seen typically is the epinasty (downward curling) of the lower leaves. This is followed by adventitious root production from the stem. Under good conditions of moisture and nutrition, yellow blotches on the lower leaves may be the first symptoms, then brown veins appear and finally chocolate brown dead spots. The leaves may wilt and drop off. The disease symptoms progress up the stem, and the plant becomes stunted. Only the top leaves stay green. Fruits remain small, develop yellow shoulders and may sunburn because of loss of leaves (Sherf, 1980)

In glasshouse tomato crops, Verticillium wilt shows as yellowing and wilting of older, lower leaves first. During the early stages of attack, wilting may be severe in the day with apparent recovery at night. Reduced growth of the plant (stunting) and very thin stems in the plant head also occur. Initially the root system appears to be healthy but, as the disease progresses, some secondary rots may occur. If the stem of an affected plant is cut across, a pronounced brown discoloration is seen in the vascular tissue. This may be traced from soil level to a metre or more above the ground (Fletcher, 1984).

Spores of the fungus move rapidly up the xylem or sap-conducting channels. Its activity there results in interference with the normal upward movement of water and nutrients. The pathogen produces a toxin that contributes to the wilting and spotting of the leaves. A low molecular weight toxin produced by *V. albo-atrum* from tomato was active in tomato seedling assay (Pegg & Brady, 2002). Ether-soluble neutral and basic fractions of culture filtrates caused both wilting and growth inhibition, whereas an acidic fraction was solely growth-inhibitory (Mace, 1981).

Dixon and Pegg (1969) found conidia of the fungus in the extremities of tomato stems and petioles 24 hours after inoculation of susceptible cultivars. Heinz *et al.* (1998) reported on cyclical colonisation of *V. albo-atrum* in susceptible tomato Craigella cultivars following conidial inoculation at the four–leaf stage. The pathogen was systemic, reaching a peak at 2-4 days followed by a period of fungal elimination) and a second peak 12-15 days post-inoculation (see Dixon & Pegg, 1969).

1.4 Diagnosis

Visual diagnosis

Tentative diagnosis of the disease involves making a vertical slice of the main stem around 0.5 m above the rockwool cube or soil line and observing a brown colour in the conducting tissues. This discoloration can be traced upwards as well as downwards into the roots. It is not definitive for *Verticillium* as fusarium wilt (*F. oxysporum* f. sp. *lycopersici*) causes similar browning.

Classical methods

- Isolation by plating surface-sterilised stem tissue of plants on to agar.
- Damp incubation. This means putting surface sterilised pieces of stem tissue on damp filter paper and incubating for a week or more, followed by microscopic identification of the developing fungus.

Molecular

Kening *et al*., (1995) found PCR primers that amplified a 700-base pair region of the mitochondrial rRNA gene of *Verticillium*. These primers bound uniformly with conserved sites in ten species of *Verticillium*. Although priming sites were conserved, the region amplified provided probes specific for *V. albo-atrum*, *V. dahliae* and *V. tricorpus* (Kening *et al*., 1995).

2. Sources of *V. albo-atrum* on a nursery

Introduction

Plants can be infected in glasshouses by different routes. Possible sources of infection and ways by which *V. albo-atrum* can enter into greenhouses are:

- Greenhouse soil
- Windblown dust
- Dirt carried by feet, machinery and implements
- Insects
- Airborne spores from crop debris
- Seeds
- Contaminated irrigation water

The objective of this part of the project is to examine these potential sources of *V. albo-atrum* as possible sources of the disease in greenhouse tomato crops.

2.1 Seed

Tomato seed contamination has been reported but is very rare (Fletcher, 1984). This is believed to be of *V. dahliae* (Kadow, 1934; Van Koot & Brons, 1949). *V. albo-atrum* on lucerne has been transmitted by seed (Huang *et al.*,1985) and was probably the main means of spread to new areas in North America.

In autumn 2002, tomato fruit was collected by John Fletcher from plants showing symptoms of Verticillium wilt and the seed extracted as follows. The fruit were cut into halves and the contents were squeezed into a plastic container. The resultant seed slurry was kept at approximately 20°C for several days with the occasion stir. After 8 days, when the mucilage was broken down, the seeds were washed out of the liquid using clean water and sieves and then spread out thinly across a large plastic tray to dry. Once, dry the seeds were collected into a sterile Petri dish which was sealed with parafilm. They were stored in a dark drawer until tests were carried out.

In March 2004, the seeds were tested for *V. albo-atrum* by planting out onto agar. 20 plates of quarter strength PDS+S were set up, each with 5 seeds per plate. Seeds were not surface sterilised; tweezers were flamed between plates. All plates were incubated at 20°C for 7 and 14 days.

95-98% germination was recorded at 7 and 14 days respectively. No growth of Verticillium was identified from any of the seeds. Only one colony of a *Penicillium* sp. and 2 bacterial colonies were identified from the 100 seed tested.

2.2 Water

A method for detection of *V. albo-atrum* in water samples by PCR is being developed in the linked Defra project. Irrigation and run-off water samples were collected from a tomato nursery and will be tested by PCR in the near future.

2.3 Insects

Sticky tapes were collected in October 2003 from a tomato nursery in Kent with a history of Verticillium wilt over several years. The literature says that insects can carry the pathogen (Harper & Huang, 1984). Testing is in progress.

2.4 Soil

V. albo-atrum survives as resting bodies in the soil and can also multiply to a limited extend in some soils in the absence of host plants. The fungus is most likely to be moved into new areas by human transport of soil, plants accompanied by soil (bulbs and tubers) or other planting material, and on contaminated implements. Soil samples have been collected from tomato nurseries for testing.

2.5 Weeds

Many plants growing as weeds in an apparently disease-free environment can harbour *V. albo-atrum* in their vascular system without showing detectable parasitic symptoms, disease only manifesting itself with the introduction of a susceptible crop (Pegg & Brady, 2002). Weeds, which will be collected in commercial nurseries in the future, will be tested by DNA extraction from them and PCR tests.

2.6 Crop debris

Verticillium wilt is essentially a soil-borne or debris-borne disease. The fungus survives for longer periods on soil particles or plant remnants, as a resting bodies. PCR identification has been done on fresh tomato stems and *V. albo-atrum* was identified. Testing of debris is in progress.

3. Spread of *V. albo-atrum* in air

Introduction

Conidia of *V. albo-atrum* are produced on diseased lucerne tissue from resting mycelium under cool moist conditions. These conidia can become airborne and have been trapped over lucerne fields (Howard, 1985). Conidia dispersed by air currents could land on cut stems resulting in infection. Wilt in lucerne was induced by disseminating conidia in the plant canopy (Christen & Peaden, 1982). Rapid secondary spread of Verticillium wilt in lucerne was reported to occur following dissemination of spores produced on infected stems, and by contact of these and transported fragments of diseased tissues with wounded surfaces of recently cut lucerne plants (Isaac, 1957). A similar scenario may occur in tomato. Sporulation of *V. albo-atrum* is occasionally seen in commercial crops on stem base lesions of plants infected by the fungus (O'Neill, unpublished). Season-long spore trapping on a tomato nursery with a history of Verticillium wilt was therefore undertaken.

3.1 Spore trapping

A Burkard spore trapping machine (see Figure 3.1.1) has been installed in tomato nursery in Kent in November 2003, where it is collecting air samples aspirating air at 10 L/min from a point in the crop 1.5 m above ground level. The spores from the air are trapped on vaseline-coated glass slide. For detection of air-borne spores, a test extraction was carried out using spores, which were added to vaseline-coated glass slides at the University of Nottingham. At the moment, the current molecular method can detect 1,000 spores per glass slide. The aim is to look at the relationship between spore numbers and Verticillium symptoms throughout the season once the sensitivity of *V. albo-atrum* detection has been improved. This method will be used for testing glass slides from a commercial nursery in Kent.

Figure 3.1.1 Spore Trap
(see description below)

The 7-day recording volumetric spore trap is a compact unit with built-in vacuum pump, designed to sample airborne particles such as fungus spores and pollen, continuously for periods of up to seven days without attention. Particles are impacted on adhesive coated transparent plastic tape supported on a clockwork-driven drum.

4. Pathogenicity of *V. albo-atrum*

4.1 Inoculum level and plant age

Introduction

Commercial tomato plants of cv. Espero, which contains the Verticillium resistance *Ve* gene, were used in a pathogenicity test of an isolate of *Verticillium albo-atrum*, AR01/036 obtained from a *Ve* resistant crop. The aim of this experiment was to observe symptoms development of plants infected at different growth stages of young plants following inoculation with different concentration of spores.

Methods

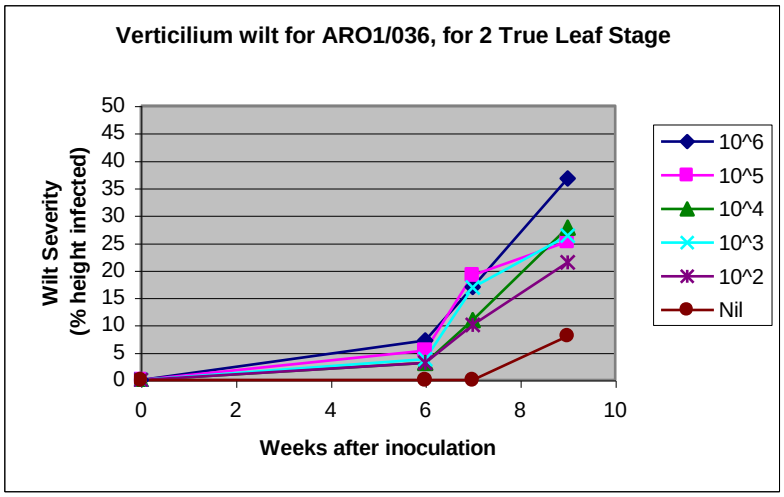
Tests were done at growth stages 2, 4, and 6-true leaf with spore suspensions in concentrations of 10^6, 10^5, 10^4, 10^3 and 10^2 conidia and with sterile distilled water (SDW) as a control. Plants grown in peat-based compost in 5-inch pots were infected by putting 1 ml of spore suspension directly into the root area of the plant. There were 10 plants per treatment. Pots were placed on the benches in a glasshouse, 1 metre between treatments in order to minimise the risk of spread by water-splash between treatments. The experiment was done in February – March using supplementary high-pressure sodium lamps to create a 16h daylength. The glasshouse was heated to maintain 20°C and vented at 22°C.

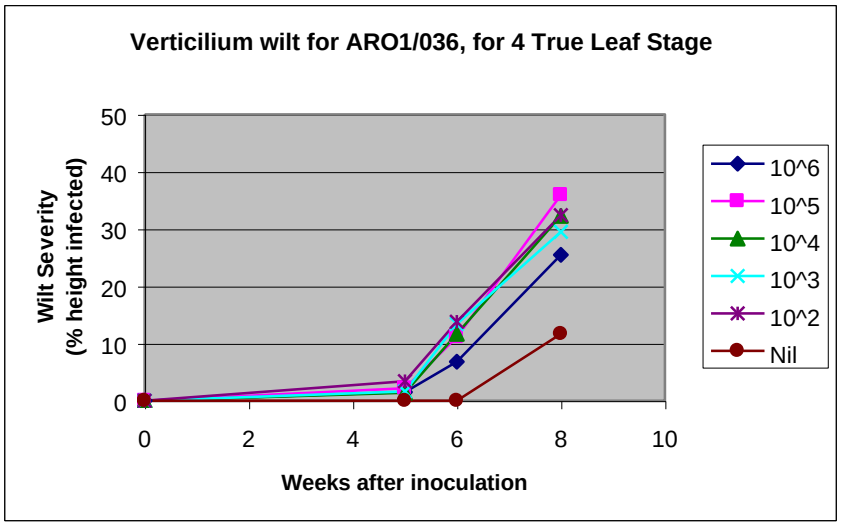
Wilt severity was estimated as follows: the height of the plant and the maximum height of the yellow-brown leaf discoloration were measured, and then the percentage was calculated for each plant compared with uninoculated control plants. The average wilt severity for each treatment was calculated.

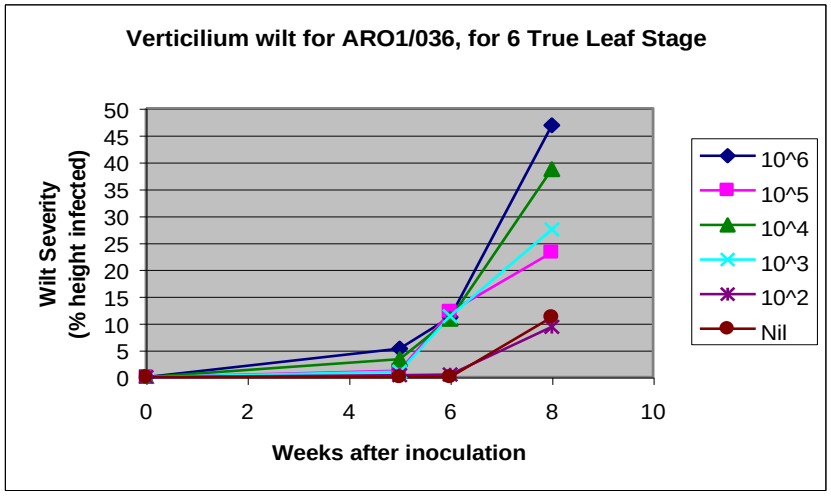
Also, stems were collected from the areas 1-2 cm above and 50 cm above the soil for each treatment. These were then stored at - 80^0C in a freezer for DNA extraction and PCR.

Results and discussion

Plants inoculated with 10^6 conidia/plant first showed Verticillium wilt symptoms 28 days after inoculation, as yellowing of cotyledons and wilting and yellowing of lower leaves with recovery at night. Symptoms progressed and after 5-6 weeks browning of lower leaves, dropping off the plants was observed. A lot of treated plants remained stunted compared to control plants.







Looking at the results of the pathogenicity test with *V. albo-atrum* isolate AR0/036, it can

be seen that with as little as 100 spores/ml, the fungus can cause Verticillium wilt in Ve-resistant tomatoes Espero F1. There was no marked difference in the susceptibility of plants at the three growth stages; plants inoculated on 2-true leaves stages started showed symptoms around a week later than plants inoculated on 4 true leaf stage.

A low severity of wilt symptoms occurred in the uninoculated control. It is not known whether this was leaf yellowing was wrongly classified as Verticillium wilt; or whether it was true Verticillium wilt following spread of the fungus to the uninoculated plants by an unknown method.

4.2 Distribution of *V. albo-atrum* within stems

After symptoms were described and the wilt severity measured, plants were harvested for identification of the pathogen in stem tissue.

Methods

Stems were collected just above soil level and 50-cm above soil level at 8 weeks after inoculation. DNA extraction was done using QIAGENE mini kit, using 100 mg of fresh tissue. The tissue selected for testing was a vascular tissue. PCR amplification was done using unique primers (supplied by Invitrogen), which can amplify *V. albo-atrum*. Primers 2/3 were used for identification of the fungus *V. albo-atrum*:

5'- ATG GAC CGA ACA GCT AGG TA-3'
5'- TCT CAG ATA TAT GCT GCT GC-3'

The expected product size is 300 bp.

Results and discussion

As can be seen in the figure, *V. albo-atrum* can be detected in tomato stems by PCR. In stem, which were inoculated with spore concentration 10^6 spores/plant, *V. albo-atrum* was detected in lower and upper (50 cm above soil level) stems. In stems inoculated with fewer spores (10^5 to 10^2 spores/plant) it was not possible to identify *V. albo-atrum* by this method.

The failure to detect *V. albo-atrum* in the stems of plants inoculated with 10^5-10^2 spores/plant may be due to i) insufficient sensitivity of the PCR test, ii) discontinuous distribution of *V. albo-atrum* in the stem and selecting 100 mg of tissue that was not colonized.

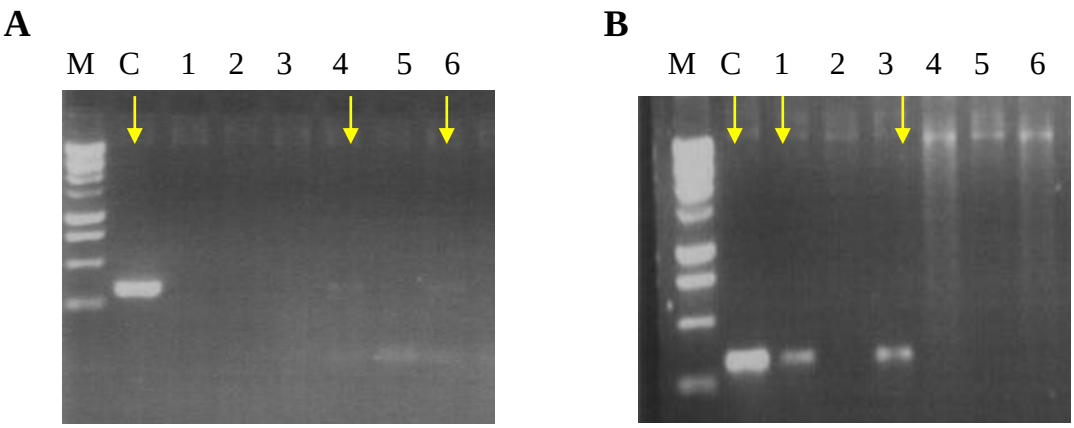


Figure 4.2.1. Agarose gel electrophoresis of PCR products of DNA extracted from tomato plants infected with 10^6 spores/ ml of *V. albo-atrum* spores. Arrows indicate samples that showed positive detection of *V. albo-atrum* from the stem tissues.
A) Upper stem tissues. Lane M, DNA Mass ladder; C, *V. albo-atrum* positive control; 1-3 uninfected tomato plants (controls); 4-6 infected tomato plants
B) Lower stem tissues. Lane M, Mass ladder; C, *V. albo-atrum* positive control; 1-3 infected tomato plants; 4-6 uninfected tomato plants (controls).

4.3 Stem infection

Introduction

The objective of this work was to investigate the potential of stem infection on Ve-resistant tomatoes. For this, a wound was created on the stem by cutting off the leaf with a scalpel blade.

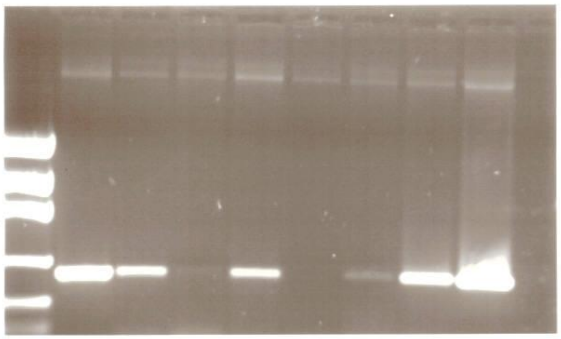
Methods

Tomato plants Espero F1 were infected at 3-4 true leaf stage through wounds at the 1/ 2nd true leaf position on each plant with 10 µl of spore suspension in concentration 10^3 and 10^2 spores/ml, so this means that per inoculation site on the plant were put approximately10 spores and 1 spore, and sterile distilled water as a control. Leaves were cut off using a scalpel.

Plants were inoculated at fresh, 3-days old and 14-days old wounds. Plants were grown after inoculation for 7 weeks and stem tissues were collected afterwards for identification of *V. albo-atrum* in stems. First, the stems were surface sterilised by putting them in 5% bleach solution and then in 70% ethanol, then rinsed well in water. DNA extraction was done using QIAGENE plant kit from pieces of stem 1-cm below and 1-cm above the wound inoculation point. 100 mg of fresh tissue in total was used selecting primarily vascular tissue; PCR amplification was done with these samples.

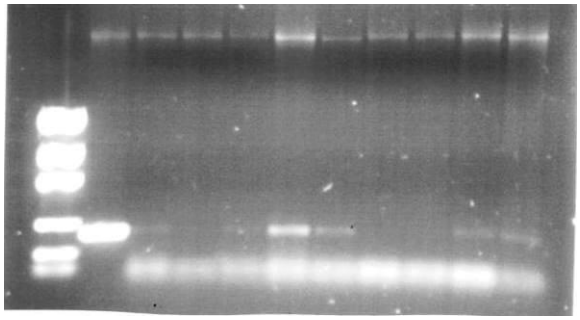
Results and discussion

As can be seen in Figure 4.3.1, *V. albo-atrum* was detected in 4 plants out of 6 when it was infected via fresh wounds at a concentration of 10^3 spores/ml (i.e. 10 spores per plant). And also in 3 stems out of 5 plants inoculated through fresh wound with a concentration of just 1 spore/plant (see Figure 4.3.2). Because of the low spore concentrations use, these experiments are being repeated to confirm the results.



1 DNA Marker
2 *V. albo-atrum* from mycelium
3 tomato stem no. 1 (10 spores per plant)
4 tomato stem no. 8 (10 spores per plant)
5 tomato stem no. 5 (10 spores per plant)
6 tomato stem no. 2 (10 spores per plant)
7 tomato stem no. 7 (10 spores per plant)
8 tomato stem no. 4 (10 spores per plant)
9 *V. albo-atrum* from mycelium
10 Negative control

Figure 4.3.1. PCR amplification of DNA samples from plants infected through a fresh wound with 10 spores/plant.



1 DNA Marker
2 *V. albo-atrum* from mycelium
3 tomato stem no. 3 (10 spores per plant)
4 tomato stem no.6 (10 spores per plant)
5 tomato stem no.9 (10 spores per plant)
6 tomato stem no.10 (10 spores per plant)
7 tomato stem no.1 (1 spore per plant)
8 tomato stem no.3 (1 spore per plant)
9 tomato stem no.4 (1 spore per plant)
10 tomato stem no.6 (1 spore per plant)
11 tomato stem no.7 (1 spore per plant)

Figure 4.3.2. PCR amplification of DNA samples from plants infected through a fresh wound with 10 spores / wound and 1 spore/wound.

These results show that, after inoculation of a fresh stem wound, *V. albo-atrum* develops, or moves, into the stem, up to 1 cm above and below the inoculation point. Futher work is required to determine if the fungus develops systemically within the stem following leaf-scar inoculation. Previous studies with alfalfa failed to show systemic infection following inoculation of leaves with *V. albo-atrum* (Jimenez-Diaz & Millar,1986) although field observations on disease spread in alfalfa indicated infection via stems.

5. Effect of host physiology on symptom expression

5.1 Flowering, fruit load and side shoot production

Introduction

Observations in chrysanthemum indicate that plants most commonly develop symptoms of Verticillium wilt at the flowering stage. Potatoes often develop symptoms of Verticillium wilt only late in the season, when tubers are bulking up. Grower experience in Holland indicates that reducing the fruit load on tomato plants infected with Verticillium wilt can reduce leaf-wilting symptoms and plants apparently recover from the disease. An experiment was devised to determine the effects of flowering, fruit load and side-shoot production of the development of Verticillium wilt in tomato.

Materials and methods

Plants of cv. Espero were grown in peat grow-bags raised on upturned plastic crates, in an unheated polythene tunnel. There were 3 plants/bag, planted on 26/5/04. Plants were inoculated with 50 ml of a 10^6/ml spore suspensions of isolates AR01/36 and AR01/129 on 8 July, poured around the stem base when plants had four trusses with fruit on them. Inoculation was done 4 weeks after treatments had commenced. The experiment was arranged in a randomised block design with four replication. Each plot consisted of one bag of three plants. Treatment 1b, 2b and 3b were all grown according to standard commercial practice. At termination of the experiment on 6 October, transverse sections of stem were removed every 20 cm on all plants, surface sterilised and examined microscopically for the presence of *Verticillium* after humid incubation for 7 days. Results were examined by analysis of variance and Fishers exact test as appropriate.

Treatments

There were three pairs of contrasting treatments:

1a. All flower trusses removed, before flowers opened; normal removal of sideshoots.
1b Normal flowering and fruit set; normal removal of sideshoots.
2a Fruit were removed from each truss at pea size to leave just 3 fruit; normal removal of sideshoots.
2b Normal flowering and fruiting; normal removal of sideshoots.
3a Normal flowering and fruit set, all side-shoots removed, before they were 1-cm long.
3b All side-shoots allowed to develop; normal flowering and fruiting

Results and discussion

The reduction in fruit load for treatment 2b was achieved (Table 5.1). Plants in treatment 1a produced yellow, curled leaves throughout the experiment after removal of flower trusses, and were stunted.

Wilting was first observed on 24/8/04 around 5 weeks after inoculation. At this time and 3 weeks later, there was less wilting where flower trusses were removed, where sideshoots were removed, and where the fruit load was reduced (Table 5.1). These

results indicate that reduction of fruit load or leaf area on a single stem reduces the likelihood of Verticillium wilt symptoms showing, possibly because of a reduced demand for moisture. Observation in crops suggest that plants with more than one head tend to show Verticillium symptoms more commonly than plants with a single head.

Laboratory examination of stem pieces confirmed the presence of *Verticillium* in all treatments except 1a, where all flowers were removed. The fungus developed most frequently from the stem base tissue (Table 5.3). There was a lower incidence of infection in plants where the fruit load was reduced (3/12 plants), than where normal fruiting was allowed (7/12 plants). Although the numbers of plants involved in this experiment were relatively small, and therefore the results need to be treated with caution, they indicate that removal of flowers and reduction of fruit load reduces the incidence of *V. albo-atrum* infection in stems.

Table 5.1 Effect of treatments on fruit yield of first four trusses and final plant height (5 October)

Treatment	Mean fruit weight (kg) per plot (3 plants)	Mean plant height (m)
1a. Flowers removed	0	1.43
1b. Flowers on	6.5	1.94
2a. Fruit load reduced	3.4	2.21
2b. Normal fruiting	6.2	1.70
3a. Side shoots removed	6.8	1.74
3b. Side shoots left on	5.3	1.04
Significance	<0.001	<0.001
SED (15 df)	0.03	0.127

Table 5.2 Effect of flowering, fruit load and side shoot production on Verticillium wilt in tomato.

Treatment	Mean number plants (of 3) with wilt		Mean wilt severity (0-3 score)
	26 Aug	16 Sep	16 Sep
1a. Flowers removed	0.8	3.0	1.5
1b. Flowers on	2.5	3.0	1.7
2a. Fruit load reduced	1.3	2.3	1.0
2b. Normal fruiting	2.0	3.0	1.5
3a. Side shoots removed	1.3	2.9	1.3
3b. Side shoots left on	2.3	3.0	1.8
Significance	-	-	0.29
SED (15 df)			0.356

Table 5.3 Effect of flowering, fruit load and sideshoot production on incidence of plants with *V. albo-atrum* in the stems and the degree of stem colonisation.

Treatment	Total number plants infected (of 12) at stem height			Mean % circumference with sporulating Vaa at:		
	0	20	100 cm	0	20	100 cm
1a. Flowers removed	0	0	0	0	0	0
1b. Flowers on	2	2	0	17	13	0
2a. Fruit load reduced	3	2	1	19	17	8
2b. Normal fruiting	7	2	1	54	13	8
3a. Side shoots removed	7	3	1	54	21	13
3b. Side shoots left on	9	2	0	45	17	0
Significance	-	-	-	0.009	0.608	0.765
SED (15 df)				14.9	11.8	10.9

Paired treatments were examined for significant differences in the incidence of stem infection by Fisher's exact test. Probability values are shown below. No differences were statistically different at $P<0.05$.

Comparison	Probability of a significant difference in the number of plants infected at different heights in the stem:		
	0	20	100 cm
1. Flowers on vs off	0.47	0.47	1.00
2. Fruit load full vs half	0.21	0.64	1.00
3. Side shoot on vs off	1.00	1.00	1.00

Additionally, each treatment was compared with the sum of treatments 2, 4 and 5, as these three treatments were identical control treatments. Effect of flower removal was

statistically significant at $P<0.05$.

Comparison	Probability of a significant difference in the number of plants infected at different heights in the stem.		
	0	20	100 cm
1. Flowers off vs off	0.003	0.088	1.00
2. Fruit load reduced vs control	0.310	0.705	1.00
3. Side shoot left on vs control	0.179	0.705	1.00

6. Effect of some environmental factors on symptom expression

6.1 Daylength

Introduction

The objective of this study was to find out how daylength influences symptom development in Ve-resistant tomatoes, and if symptoms differ in short and long light conditions.

Methods

Spore suspensions were prepared from 14-day old culture of *V. albo-atrum* AR01/036 was grown on PDA and a sporulating culture washed with 1 ml of sterile distilled water. The concentration was adjusted to 10^6 and 10^4 spores/ml and 10 plants were used as a control inoculated with water. Tomato plants cv. Espero F1 were grown to the 2-true leaves stage and infected then by pipetting, using Gilson Pipette, 1 ml of the fungal spore suspension into the root area. Plants were grown in the growth room with a set 16-hour day length and at a constant temperature of 20°C. Five weeks after inoculation the experiment were stopped and the growth room was switched to an 8 hour day length. Twenty plants at the 2-true leaf growth stage were inoculated with the same 10^6 and 10^4 spores/ml concentration and 10 control plants with SDW.

Results and discussion

Long daylength (16 hr)
First symptoms were seen 2 weeks after inoculation on plants infected with a concentration of 10^6 spores/ml. This was yellowing and falling off of cotyledons, and 3 plants were showing wilting of lower true-leaves, with recovery at night. Three weeks after inoculation, plants infected with a concentration of 10^4 spores/ml started to show the same symptoms. Four weeks after inoculation, 12 out of 20 infected plants were stunted compared with the control plants. Also 8 out of 20 infected plants started to show browning of the lower leaves. The progression of symptoms every 5–7 days after they were first seen is shown Figures 6.1.1-6.1.2.

Short daylength (8 hr)
Due to space constraints it was not possible to run the two daylength treatments concurrently in different growth rooms or sequentially in the same growth room. It is therefore planned to repeat this work in 2005.

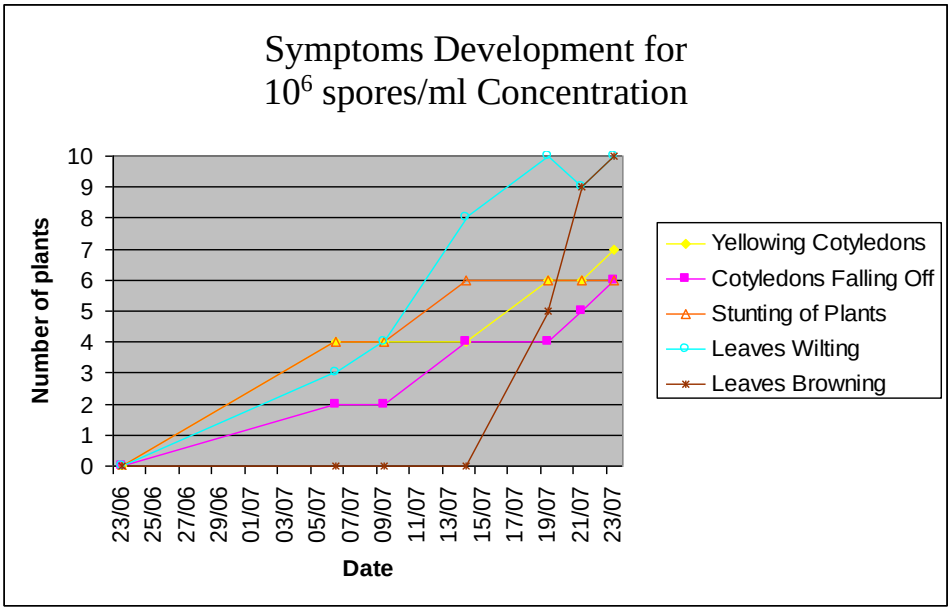


Figure 6.1.1. Symptom development in plants grown in 16-hour day length inoculated with concentration of *V. albo-atrum* at 10^6spores/ml

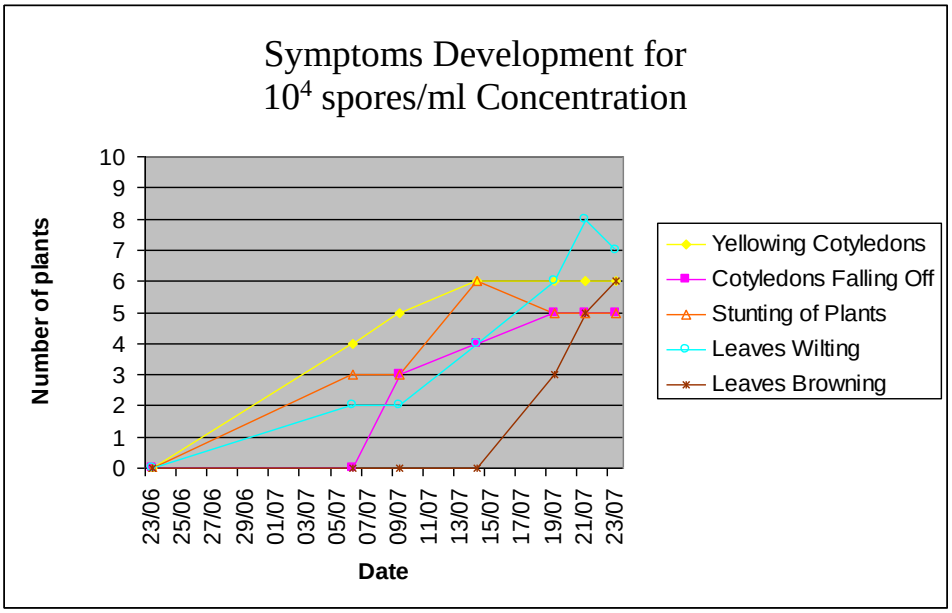


Figure 6.1.2. Graph showing symptoms development in plants grown in 16-hour day length inoculated with concentration of *V. albo-atrum* 10^4 spores/ml

6.2 Effect of water stress on susceptibility to *V. albo-atrum*

Objective

To determine if lack of water increases the susceptibility of young tomato plants to *V. albo-atrum*.

Materials and methods

Plants of cv. Espero were grown in peat-based compost (Levington M3) in 13-cm plant pots in an unheated polythene tunnel. Each plant was stood on an upturned plant pot saucer, so that excess water could drain away and the plant pot was not in contact with the soil cover. Application of 4 watering regimes commenced on 25 June. Plants were inoculated with 50 ml of a 10^6 conidia/ml spore suspension of *V. albo-atrum* isolate AR01/036 and AR01/129 on 28 July, when plants were around 30 cm tall, by pouring it around the stem base. All plants in treatments 1, 2 and 4 had been watered on the day of inoculation and T3 was watered the previous day. The compost of T1, T2 and T4 was moist at the time of inoculation. The experiment was arranged in a randomised block design with 10-fold replication; plots consisted of one plant. The experiment was terminated on 1 September, 5 weeks after inoculation. Results were examined by analysis of variance and Fisher's exact test as appropriate.

Treatments

1. Standard watering; once daily (increased to 2 x daily on 4 August)
2. Reduced watering: once every 2 days (increased to daily on 4 August)
3. Very reduced watering: once every 4 days (increased to every 2 days on 4 August)
4. Standard watering; uninoculated control

Results and discussion

Reduced watering resulted in significantly reduced plant growth ($P<0.001$), as expected (Table 6.2.1).

No wilting or other symptoms of Verticillium wilt were observed during crop growth. No vascular browning was found within the stems at the final assessment.

V. albo-atrum was recovered from plants stems of treatment 1 (6 out of 10 plans), and not at all from treatments 2-4. Where *V. albo-atrum* was confirmed, it was generally found in several stem sections per plant, at heights up to 50 cm above the stem base.

One possible explanation for the observed for the observed effect is that the *V. albo-atrum* spores did not survive drying out for prolonged periods. An experiment is in progress to determine the effect of air drying on viability of *V. albo-atrum* conidia.

Table 6.2.1 Effect of water stress on infection of stems by *Verticillium albo-atrum*.

Treatment	Number plants Infected (of 10)	Mean plant height (cm)
Standard watering (x 1 daily)	6	89
Reduced watering (x 1/2 days)	0	76
Very reduced watering (x 1/4 days)	0	54
Standard watering – uninoculated	0	87
Significance	-	<0.001
SED (27 df)		3.04

6.3 Effect of *Phytophthora* root rot on susceptibility to *V. albo-atrum*

Introduction

Observations in commercial crops suggest that plants infected with both *V. albo-atrum* and a virus disease (pepino mosaic and/or tomato mosaic) develop severe wilting symptom and often declined rapidly and die, whereas plants infected with either disease alone are less badly affected and may recover. Because of the difficulty in containing spread of PepMV and ToMV, an experiment was devised to investigate interaction of *V. albo-atrum* with another fungal pathogen, *Phytophthora cryptogea*, cause of root rot.

Materials and methods

Plants of cv. Espero were grown in peat-based compost (Levington M3) in 4 litre pots in an unheated polythene tunnel. Each pot was stood in a saucer. Plants were potted on 24 June and inoculated with *V. albo-atrum* (50 ml of 10^6/ml spore suspension) and /or *P. cryptogea* (15 ml of a macerated mass of mycelium incorporated into the compost surface) on 10 August when they were around 100 cm tall. The experiment was arranged in a randomised block design with 10-fold replication; plots consisted of one plant. Plants were assessed on 6 October, 8 weeks after inoculation. Results were examined by Fisher's exact test.

Treatments

1. Uninoculated control
2. *V. albo-atrum*
3. *P. cryptogea*
4. *V. albo-atrum + P. cryptogea.*

Results and discussion

No symptoms of Verticillium wilt, or other foliar symptoms developed in this experiment. However, simultaneous inoculation with the both pathogens (T4) resulted in a significant reduction ($P<0.01$) in plant growth (Table 6.3.1). When the experiment was terminated, vascular browning suggestive of Verticillium wilt was found extending to 20 cm, or higher, up the stem in one plant from each of treatments 2 and 4, the two treatments inoculated with. *V. albo-atrum.* The fungus was subsequently confirmed at a greater frequency in plants from these treatments than in treatments 1 and 3 (Table 6.3.2). There was a significant difference between treatments in the incidence of infected plants, determined at soil level ($P<0.02$), and in stem colonisation by the fungus at 0 and 20 cm up the stem.

Table 6.3.1 Effect of co-inoculation with *V. albo-atrum* and *P. cryptogea* on plant height

Treatment	Mean stem height
Uninoculated control	1.74
V. albo-atrum	1.62
P. cryptogea	1.68
V. albo-atrum + *P. cryptogea*	1.48
Significance	0.006
SED (27 df)	0.069

Table 6.3.2 Occurrence of *Verticillium* in stems of plants inoculated with *V. albo-atrum*, *P. cryptogea*, both fungi, and neither fungus.

Treatment	Total number plants infected (of 10) at stem height			Mean % stem circumference with sporulating Verticillium at:		
	0	20	100 cm	0	20	100 cm
1. Uninoculated control	2	1	0	18	5	0
2. Vaa root drench	7	5	2	65	40	10
3. Phytophthora	2	1	0	15	5	0
4. Vaa + Phytophthora	7	4	2	70	30	15
Significance	-	-	-	0.013	0.034	0.239
SED (27 df)				20.2	13.7	8.7

	Probability of a significant difference between treatments in incidence of infection at different stem heights		
	0	20	100 cm
P value:	0.02	0.13	0.29

7. Efficacy of chemical disinfectants

Introduction

The objective of the study was to find out which disinfectants work against *V. albo-atrum* spores and mycelium. This is needed for preventing or delaying entry of *V. albo-atrum* into glasshouses and for elimination of any contamination present.

Methods

A culture of *V. albo-atrum* AR01/036 was grown on PDA plates for 14 days. Spore suspensions (conidia) were collected from these plates by washing with 1 ml of sterile distilled water and adjusting the concentration to 10^6conidia/ml. To the spore suspension was added the recommended rate of disinfectant, and later 1:2, 1:4 and 1:8 dilutions of recommended doses of disinfectants. After mixing with the test solutions for specific time periods (total time) 5 minutes, 30 minutes, 1 hour, 4 hours, and 8 hours, spores were spin down in a centrifuge (13,000 g for 60 seconds) then re-suspended in water, and 5 µl of solution were placed in the middle of freshly prepared PDA in big Sterilin plates with square wells. Germination of spores after 7 days incubation in 20^0C was visible as growth of the fungus (Figure 7.1).There were 3 replicate plates per disinfectant.

Also, for testing the efficacy of disinfectants on mycelium, small pieces of sterile filter paper (approximately 0.5 cm^2) were placed on the surface of an actively sporulating culture of *V. albo-atrum* growing on PDA. These were left for 7 days to allow some fungal mycelium to penetrate the paper. No chlamydospores of melanised hyphae were observed in the fungal growth on filter papers. Then, these filter papers were immersed in the test solutions of disinfectant for specific time periods: 5 minutes, 30 minutes, 1 hour, 4 hours, and 8 hours. Treated pieces of paper were drained, rinsed 3 times in SDW to remove disinfectant, drained again and plated onto PDA with antibiotics. Results were scored 7 days later. As a control, contaminated but untreated pieces of filter paper were placed on PDA plates with antibiotics.

Table 7.1 Details of disinfectants tested

Disinfectant	**Active ingredient**	**Label recommended dose used in tests**
Citric acid	Citric acid	0.2%
Harvest Wash	2% chlorine dioxide	1:100
Jet 5	5% peroxyacetic acid, 10 % acetic acid, 25% hydrogen peroxide	1:125
Panacide M	30 % dichlorophen Na salt	1:60
Sodium hypochlorite	hypochlorite 8% available chlorine	2.5% (2,000 ppm hypochlorite)
Trigene	halogenated tertiary amine	1:50
Unifect G	Glutaraldehyde <15%, Ammonium compounds	4 %
Virkon S	potassium peroxymonosulfate surfactant, organic acids	2%

Results and discussion

Effect on spores

The most effective disinfectants against spores of *V. albo-atrum* were Virkon S, Unifect G, sodium hypochlorite and Trigene, which showed no growth from spores 7 days after treatment at all doses and immersion times used (Fig. 7.1). The recommended doses of Jet 5 and Harvest Wash worked well against spores but greater dilutions of these disinfectants, were less effective (Fig 7.2). The results with Panacide M are surprising and should be treated with caution. Full results are tabulated in Appendix 1.

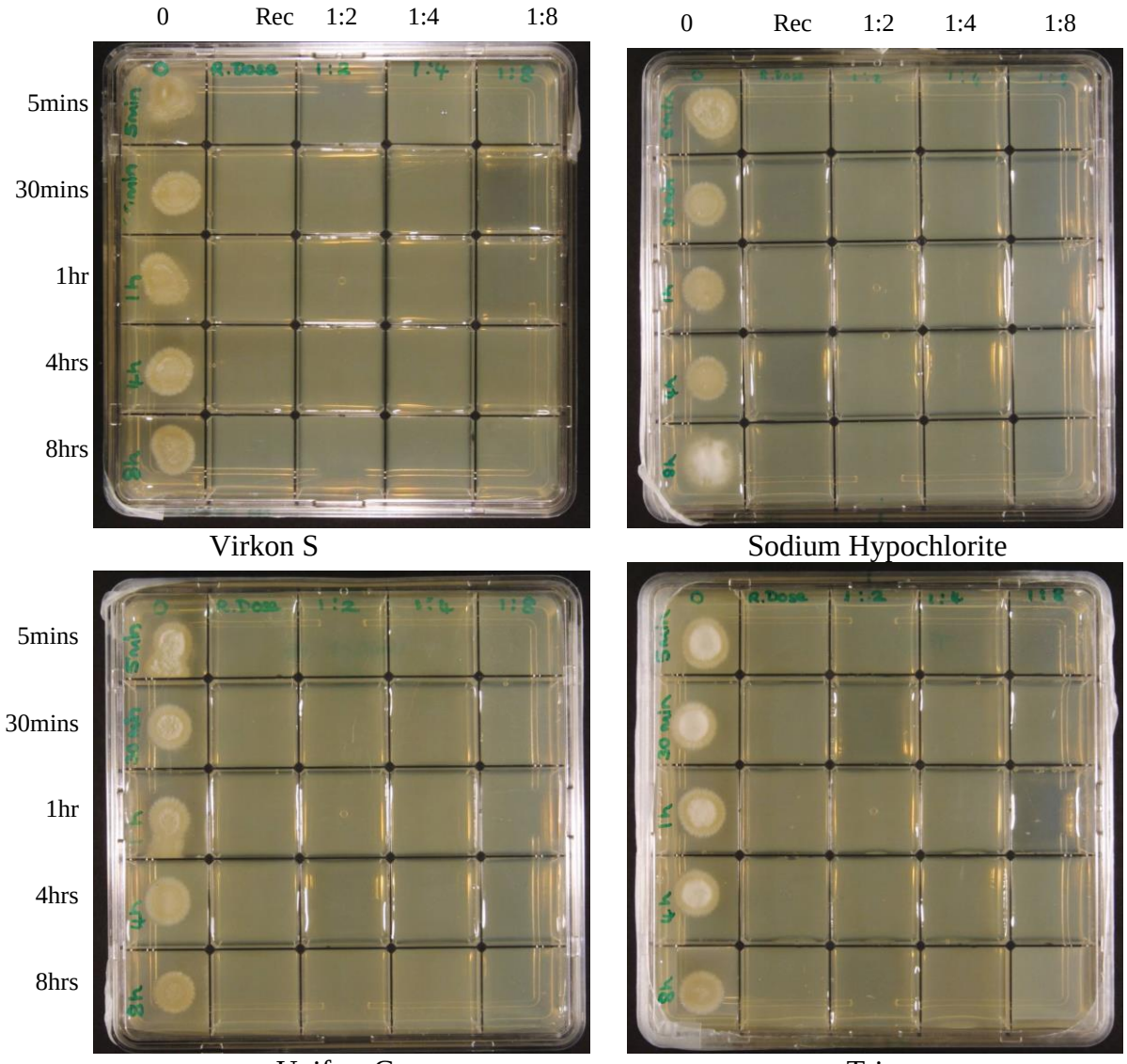


Figure 7.1. Petri dishes of spores after treatment with Unifect G, Trigene, Virkon S and sodium hypochlorite. Growth of *V. albo-atrum* is visible only in the untreated controls (left hand column of each plate).

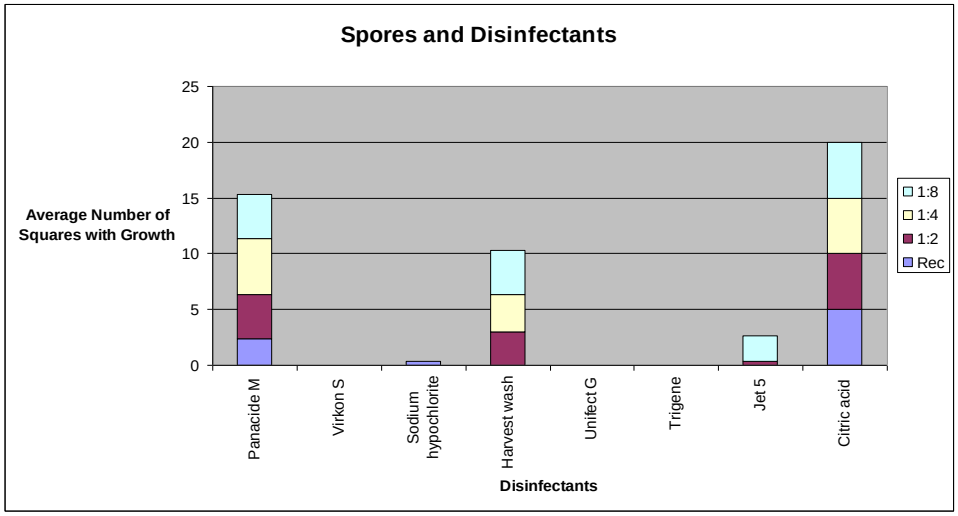


Figure 7.2. Efficacy of disinfectants against spores of *V. albo-atrum* (total average growth over all immersion times per dose).

Effect on mycelium

Results with fungal mycelium on filter paper were different (Figure 7.3). Trigene worked best in all concentrations, except at 1:8 dilution after 5 minutes contact time. At the recommended dose and ½ rate, Unifect G was worked well. Panacide M worked well at the recommended dose and ½ rate after 30 minutes treatment. Sodium hypochlorite worked at the recommended dose only. Harvest Wash and Citric acid at their recommended rates were ineffective against mycelium in filter paper discs with a contact time of 8h or less; Jet 5 was effective after 8h but not after 4h. Full results are tabulated in Appendix 2.

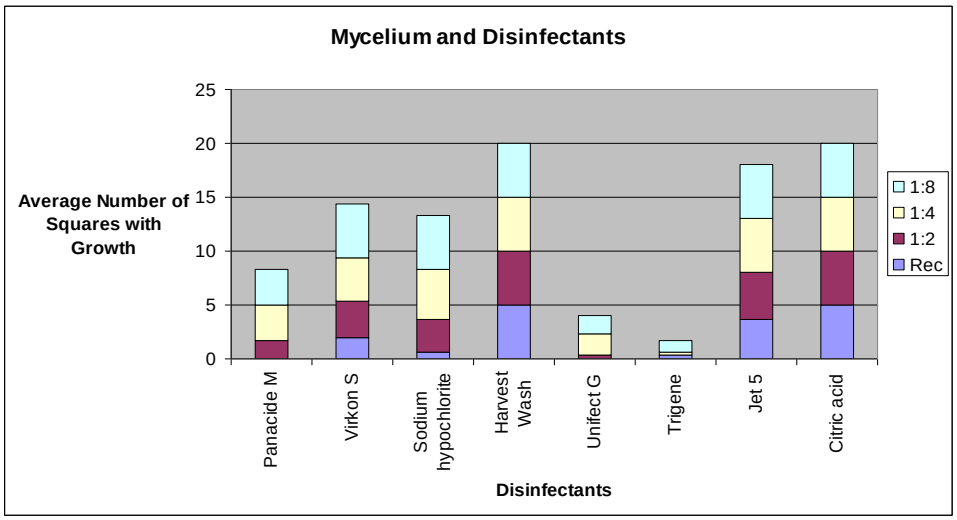


Figure 7.3. Efficacy of disinfectants on mycelium of *V. albo-atrum* (total average dose over all immersion times per dose).

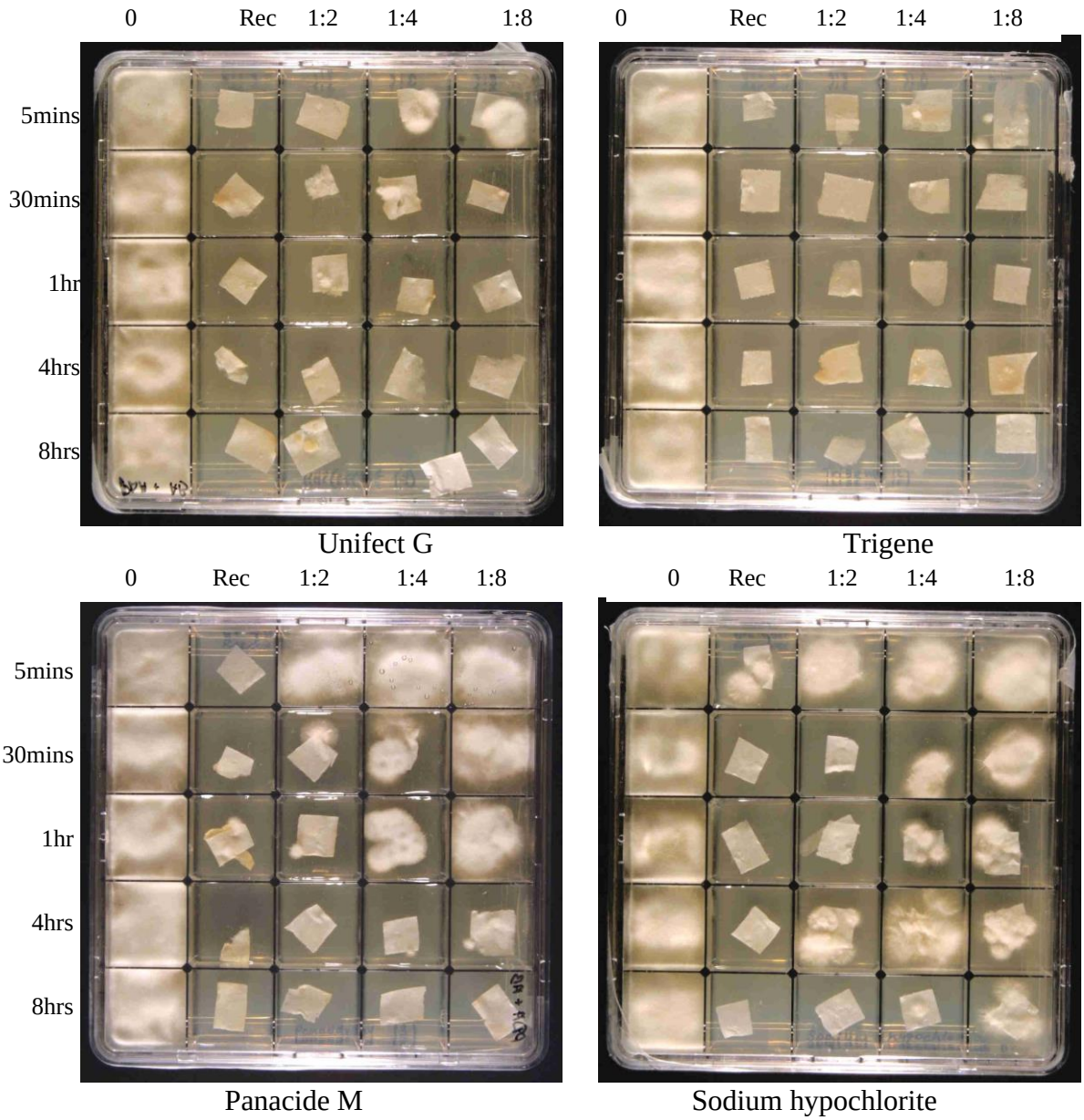


Figure 7.4. Petri dishes with treated filter papers with mycelium on them, with the 4 best working disinfectants.

As shown in Figure 7.4, after treatment of mycelium with Unifect G and Trigene, there was no growth of fungi at the recommended dose and ½ rate. Panacide M worked well after 5 minutes at the recommended dose but only after 4 hours with treatment at half rate. Sodium hypochlorite worked at the recommended dose after 30 minutes treatment. Others tested disinfectants were not effective against mycelium.

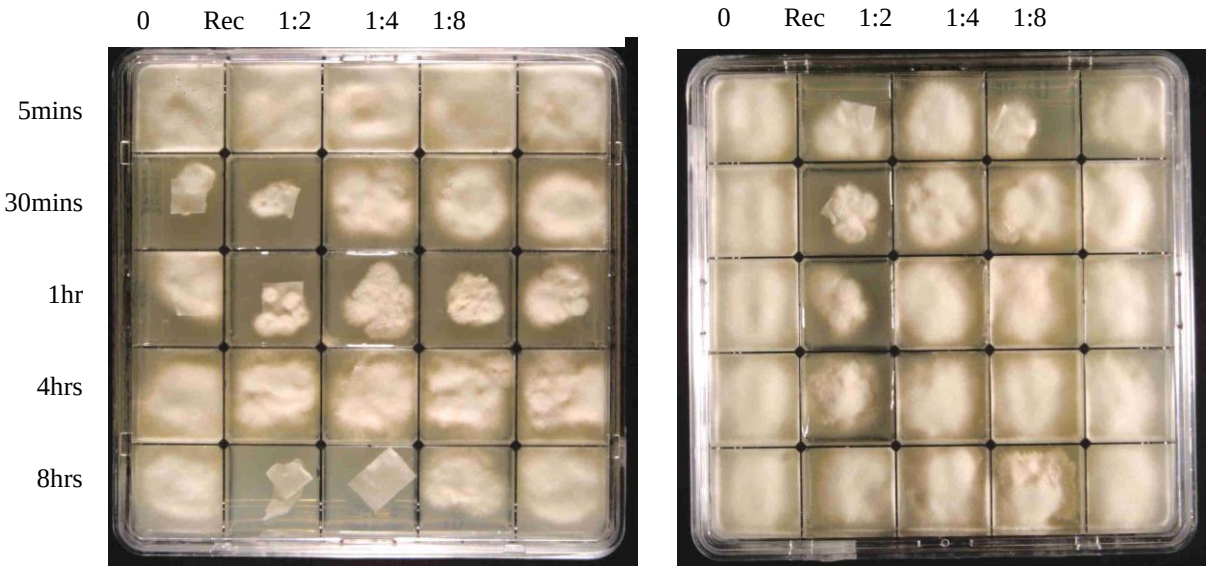


Figure 7.5. Petri dishes with mycelium of *V. albo-atrum* treated with Jet 5, Harvest Wash.

As can be seen on Figure 7.5, Jet 5 and Harvest Wash, did not work at any concentration even at the doses and immersion times used.

Effect of peat and clay contamination

In commercial glasshouses, surfaces, which must be treated with disinfectants could be contaminated with organic matters like peat or soil. Therefore, a severe test of disinfectant was made by adding 100 mg each of peat and clay (kaolin) to the disinfectants (1ml) using the 4 disinfectants that previously had shown good results.

As can be seen in Figure 7.6, after treatment of spores with disinfectants in a solution containing added peat and clay, the best results at the recommended doses were obtained with Unifect G and sodium hypochlorite.

Later, filter papers with mycelium of *V. albo-atrum* growing on them, were treated with the same disinfectants with added peat and clay. The best working disinfectants were Trigene and Unifect G, which worked at the recommended dose and at ½ rates better than other products.

The full results of these experiments are tabulated in Appendix 3. It is planned to repeat this experiment testing peat and soil contamination of disinfectants separately, and allowing a standing time (e.g. 1 hours) for the contamination to react with the disinfectant (if at all), before addition of *V. albo-atrum* spores. Nevertheless, the preliminary results from this initial experiment suggest that peat and soil do reduce the efficacy of disinfectants (e.g. sodium hypochlorite activity against mycelium).

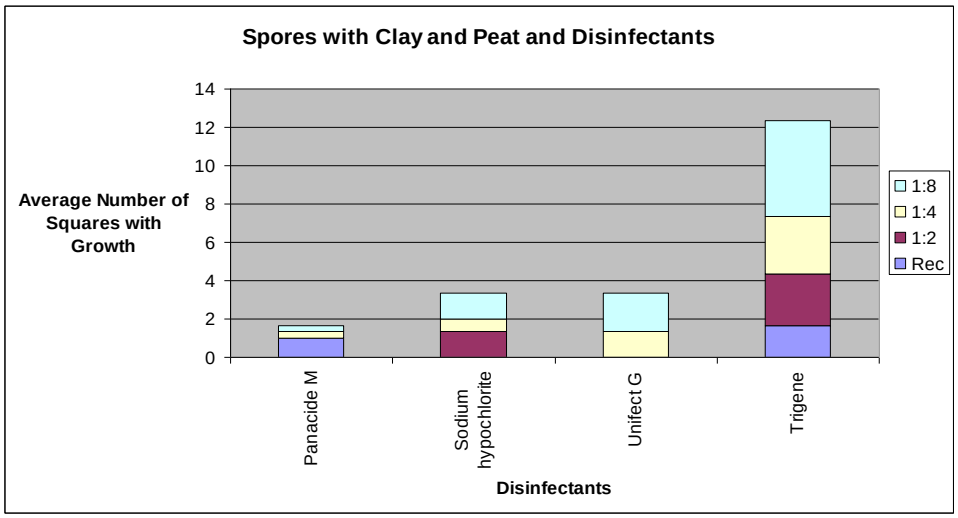


Figure 7.6. Efficacy of disinfectants with added clay and peat on spores of *V. albo-atrum* (total average growth over all immersion times per dose)

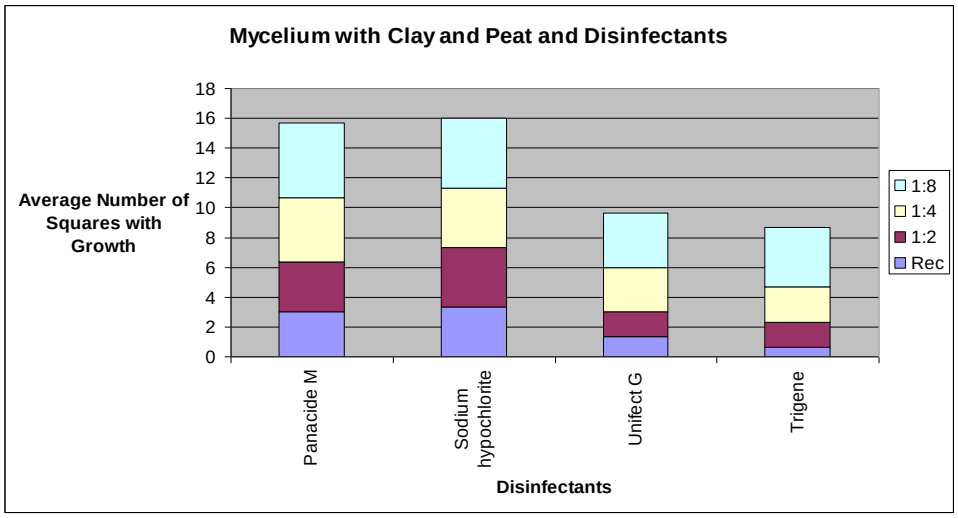


Figure 7.7. Efficacy of disinfectants with added clay and peat on mycelium of *V. albo-atrum* (total average does over all immersion times per dose).

Effect of immersion times

Apart from Citric acid and Panacide M, all of the disinfectants when tested at their standard rates were fully effective against conidia of *V. albo-atrum* after a contact time of just 5 minutes. Panacide M was only fully effective after 4 hours. When tested against mycelium in filter paper, Panacide M and Unifect G were fully effective with a contact time of 5 minutes or greater. Sodium hyphochlorite was fully effective after 30 minutes, Trigene after 1 hour and Virkon S and Jet 5 after 8 hours. Citric acid and Harvest Wash had no effect even after 8 hours. Further test with exposure periods of less than 5 minutes are planned for 2005.

8. Contamination and disinfection of hands

Introduction

V. albo-atrum has been observed on stem lesions of severely affected plants. As *V. albo-atrum* is a vascular pathogen that has been isolated from tomato stem tissue more than 1m above soil level, it is possible that spores of the fungus may also be present in plant sap on fresh stem wounds created by de-leafing and side-shooting. Handling of sporulating lesions, or stem wounds, may thus contaminate hands. If wound sites on healthy plants are subsequently touched, this could lead to disease spread. Experiments in another project have shown that incubation of wounded leaf veins and fresh de-leafing scars can lead to stem infection. A series of laboratory experiments were therefore undertaken to examine (1) the persistence of *V. albo-atrum* on hands and (2) methods of cleaning hands to reduce this risk.

Materials and methods

Contamination of hands

An isolate of *V. albo-atrum* from tomato (AR01/36 AR04/76) were grown on PDA + streptomycin for 10-14 days. A thumb was placed on a sporulating colony, or sporulating stem lesion and then onto a fresh agar plate, making 10 impressions around the circumference of the plate. This was continued on new plates until 160 impression had been made. Plates were incubated and examined for colonies of *Verticillium* after 6 days.

Washing and disinfection of hands

After touching a sporulating culture or stem lesions, various methods of decontaminating hands were evaluated. Hands were washed in soap and water for 30-60 seconds and then rinsed under a tap; or rubbed with Med Gel (an alcohol-based hand cleaner), and then 10 thumb impression made on an agar plate. Each test was repeated three times. Both bare and gloved hands were tested. Gloves were disposable latex types (Manufacturer: Sempermed Industrial).

Results

Persistence on hands

After touching sporulating *V. albo-atrum*, the first 160 out of 200 sequential contacts on agar all led to development of *V. albo-atrum* colonies (Table 8.1). Washing in soap and water reduced this number to 54; growth of colonies at each impression was less than on unwashed hands.

Washing and disinfection of hands

Washing in warm soap and water for 30 s, or rubbing in Med Gel for 30 s, or washing following by rubbing with Med Gel, were largely ineffective (Table 8.2). Increasing the duration of washing or rubbing to 1 minute significantly increased efficacy. Gloves were easier to clean than hands (Table 8.3).

Table 8.1 Persistence of *V. albo-atrum* on hands.

Treatment	Number of contacts of 200 on agar developing Vaa	
	Total	Continuous
1. Stem lesion touched	160	160
2. Stem lesion touched, then Hands washed in soap and water (30s)	54	54

Table 8.2 Washing and disinfection of hands – 30 seconds treatments – after touching a culture colony and a sporulating stem lesion.

Treatment	Number of Vaa colonies per plate (of 10)					
	Culture plate			Stem lesion		
Rep	1	2	3	1	2	3
1. Negative (clean thumb)	0	0	0	0	0	0
2. Positive control (contaminated thumb)	10	10	10	10	10	10
3. Warm soap + water (30s)	10	10	10	10	10	10
4. Med Gel (30s)	9	9	1	10	10	10
5. Warm soap + water (30s), then Med Gel (30s)	10	10	10	10	10	10

Table 8.3 Washing and disinfection of gloved and ungloved hand – 60 second treatments – after touching a sporulating culture plate colony.

Treatment	Number of Vaa colonies per plate (of 10)					
	Gloved hand			Ungloved hand		
Rep	1	2	3	1	2	3
1. Negative (clean thumb)	0	0	0	0	0	0
2. Positive control (contaminated thumb)	10	10	10	10	10	10
3. Warm soap + water (60s)	0	0	0	10	10	10
4. Med Gel (60s)	0	0	0	0	0	0
5. Warm soap + water (60s), then Med Gel (60s)	0	0	0	0	0	0

9. Detection of *V. albo-atrum* in a crop by testing leaf petioles

Introduction

The symptoms of Verticillium wilt in tomato are not always obvious. Sometimes the only symptom is a transient wilting on sunny days, or thin growth in the plant head, both of which could be caused by pathogens or factors other than *V. albo-atrum*. A pale brown vascular staining extending 50 cm or more above the stem base is often found in plants severely affected by Verticillium wilt. However, plants with a mild infection may show no vascular browning, and pathogens other than *V. albo-atrum* can cause vascular browning. Hence stem base vascular browning cannot be used as a definitive indicator for *V. albo-atrum*. Currently, the usual way to test plants for Verticillium is by a destructive test on the basal portion of the stem.

The vascular tissue in the stem is plated out onto agar (e.g. PDA + S) or incubated in a damp-chamber and examined for verticillate growth characteristic of *V. albo-atrum*. Vascular tissue (100 mg samples) can also be tested for *V. albo-atrum* by PCR.

Leaf petioles were collected to determine if they could be used to monitor for early detection of *V. albo-atrum* in a crop without destroying plants.

Materials and methods

Samples of lower leaves were collected at random, 1 leaf/plant, from visually healthy plants in glasshouse blocks with a history of Verticillium wilt in Kent (nursery A) and West Sussex (nursery B). Petiole transverse sections from the node (ADAS) and 10 sequential slices from the node (JTF) were placed in damp chambers and examined for verticillate growth after 7-14 days. Additionally, on samples of petioles collected from wilting plants on 20 July, juice was squeezed from 10 petioles and plated onto PDA. Also at the Kent nursery, 48 stems of apparently healthy plants of Chloe and Encore were examined for vascular browning at 0.5 m above the slab, and tested for Verticillium by damp incubation of transverse sections, at crop removal on 29 October.

Results and discussion

<u>Testing for Verticillium</u>
Damp incubation of petiole slices proved an effective method of testing plants for infection by Verticillium without destroying the plant. When 10 slices were taken from individual petioles and tested individually, infection was found to be discontinuous with often just one or up to four slices infected.

A small number of petiole slices that produced growth of Verticillium were also tested by PCR. *V. albo-atrum* was not confirmed. Further comparison of the sensitivity of testing methods will be undertaken in 2005.

These results indicate *V. albo-atrum* may occur within plants early in the season (January – March) and sometimes when no Verticillium wilt symptoms are present. It would be useful to tag plants shown to be infected at this time and observe if and when obvious Verticillium wilt foliar symptoms develop.

Development of wilt symptoms (Kent nursery)
February 16. 24 plants showing very slight wilting symptoms, this was first seen end of January; 23 cv. Chloe, all ungrafted, and 1 grafted (Beaufort) cv. Encore. One plant sampled and Vaa confirmed.
March 25. Leaf petioles (lowest leaf) taken from Chloe in D block and Encore in B block. 10 slices from each petiole incubated on damp paper. Positive results from 2 Chloe (1 out of 10 slices and 11 out of 11) and 3 Encore (all one out of 10)
April 14. 31 lower leaf petioles taken from Chloe and 10 slices incubated from each. All negative.
June 22. No additional wilters since the original 24. 80 lower leaf petioles taken from B block cv Encore and 10 slices of each incubated. Four positive (three 1 in 10, and one 2 in 10).
July 20. A number of Chloe plants (about 20) showing very marked wilting symptoms. 10 samples and 10 slices of the lowest petiole incubated. All negative. Juice squeezed from the 10 petioles and plated onto PDA. No Verticillium recovered.
October 1 52 lowest petioles taken from the cv. Encore in B block and incubated on damp paper 10 slices per petiole. 11 positive (10, 1 in 10 and 4 in 10)

Conclusions

Testing for *Verticillium*

- Damp incubation of petiole slices proved an effective method of testing plants for infection by *Verticillium* without destroying the plant.
- When 10 pieces were sliced from individual petioles and tested individually, infection was found to be discontinuous.

Crop monitoring in Kent

- Verticillium was first seen in the crops at a very low level early in the season
- There was little visual affect of the disease for most of the season, although the plant stem thickness in the head thinned in midsummer
- Yields overall this year were not good and much of the loss has been attributed to the use of grafted plants which were too vigorous.
- At the end of the season there were many plants dead and almost all of them had a severe *Botrytis* stem lesion which could have accounted for the death.
- At the end of the season, dead stem bases of the variety Chloe had sporing *Verticillium* and large populations of springtails
- At the end of the crops the incidence of *Verticillium* could not be related to the vascular staining
- The was a higher level of *Verticillium* (88.6%) in the grafted than in the ungrafted (65.1%)

Table 9.1 Detection of Verticillium in leaf petioles and stem sections from commercial crops – 2004

Sample date	Variety (and rootstock)	Number leaf petioles tested	Positive for Verticillium	
Kent (nursery A)			Number	%

16 February	Chloe (ungrafted)	1*	0	-
25 March	Chloe	31	2	6.5
	Encore on Beaufort	31	3	9.7
14 April	Chloe	31	0	0
22 June	Encore on Beaufort	80	4	5.0
20 July	Chloe	10*	0	-
1 October	Chloe	52	11	21.2
29 October (stems)	Chloe	63	-	65.1
	Encore on Beaufort	35	-	88.6
West Sussex (nursery B)				
10 March	Encore on Beaufort	30	1	6.7
	Flavorino on Beaufort	15	0	0
	Santa on Maxifort	15	1	3.3

* Plants with wilting symptoms

Table 9.2. Occurrence of vascular staining and Verticillium in the stems of apparently healthy plants – Kent, 29 October 2004

Variety	Stem vascular staining at 0.5 m height			
	None	Slight	Moderate	Severe
Chloe (ungrafted)				
Staining	32	18	10	3
Verticillium present	22	12	7	2
Encore on Beaufort				
Staining	14	19	2	0
Verticillium present	12	18	1	0

10. Overall conclusions

1. Verticillium wilt continues to be a problem in UK crops and affects varieties with the Ve-resistance gene. In 2004, the disease was confirmed in crops in Kent, West Sussex, Worcs and Yorkshire.

2. A PCR-method for specific detection of *V. albo-atrum* developed in a parallel Defra-funded project was successfully used to confirm identity of *V. albo-atrum* fungal cultures and to detect *V. albo-atrum* in stem vascular tissue.

3. A method was devised for early detection of Verticillium in crops without destroying the plants. Transverse sections of petioles of lower leaves are damp-incubated and then examined microscopically.

4. The spores of *V. albo-atrum* adhere to hands. Handling plants after touching a sporulating stem lesion *Verticillium* is a potential means of disease spread.

5. Hands can be disinfected of *V. albo-atrum* by rubbing with Med Gel for 1 minute. Latex gloves can be disinfected more easily, possibly indicating that spores adhere to gloves less readily than to hands.

6. Spores of *V. albo-atrum* are more readily killed by chemical disinfectants than mycelium in filter paper. Hypochlorite activity against *Verticillium* mycelium was reduced by the presence of peat and clay.

7. The severity of Verticillium wilt symptoms increased with the inoculum level of *V. albo-atrum* applied. An inoculum of just 100 spores applied as a root drench was able to cause Verticillium wilt, symptoms appearing after 5 weeks.

8. Infection of tomato leading to development of Verticillium wilt symptoms can occur through the roots and also through fresh de-leafing scars on the stem.

9. Preliminary experiments show that crop factors that influence occurrence of Verticillium wilt symptoms are flowering and fruit load.

10. Moisture level around roots was found to influence occurrence of Verticillium infection, with reduced infection in a dry growing medium.

11. The incidence of infected plants in a crop can be greater than the incidence of plants with symptoms of Verticillium wilt.

11. Technology transfer

1. Project initiation meeting, ADAS Arthur Rickwood, 10 July 2003.
2. Project review meeting, Nottingham University, 15 January 2004.
3. Project review meeting, ADAS Arthur Rickwood, 24 June 2004.
4. New results on tomato Verticillium wilt. Presentation at HDC/TGA/HRIA Tomato Conference, Coventry, 30 September 2004 (TMO).
5. Update to several UK growers and consultants during consultancy work (TMO).

12. Acknowledgements

We are grateful to John Overvoorde, Delfland Nurseries Ltd, for helpful comments and supply of plants for some of the experiments described here. Also to Certis UK and Sands Agricultural Services Ltd for provision of samples of Jet 5 and chlorine dioxide, respectively. Vicky Worthington thanks Vinodh Krishnamurthy for training in PCR methods and helpful discussion.

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Appendix 1. Effect of concentration and exposure time on efficacy of chemical disinfectants against *V. albo-atrum* conidia.

Product exposure	Number of replicates, of 3, developing growth of *V. albo-atrum* Rate F	1/2	1/4	1/8	Rate F	1/2	1/4	1/8
Citric acid (0.2%)					**Sodium hypochlorite** (2,000ppm)			
5 min	3	3	3	3	0	0	0	0
30 min	3	3	3	3	0	0	0	0
1 hr	3	3	3	3	0	0	0	0
4 hr	3	3	3	3	0	0	0	0
8 hr	3	3	3	3	0	0	0	0
Harvest Wash (1:100)					**Trigene** (1:50)			
5 min	0	3	3	3	0	0	0	0
30 min	0	3	3	3	0	0	0	0
1 hr	0	3	3	3	0	0	0	0
4 hr	0	0	1	3	0	0	0	0
8 hr	0	0	0	0	0	0	0	0
Jet 5 (1:125)					**Unifect** G (4%)			
5 min	0	1	0	3	0	0	0	0
30 min	0	0	0	2	0	0	0	0
1 hr	0	0	0	1	0	0	0	0
4 hr	0	0	0	0	0	0	0	0
8 hr	0	0	0	1	0	0	0	0
Panacide M (1:60)					**Virkon S** (2%)			
5 min	3	3	3	3	0	0	0	0
30 min	1	3	3	3	0	0	0	0
1 hr	3	3	3	3	0	0	0	0
4 hr	0	3	3	3	0	0	0	0
8 hr	0	0	3	0	0	0	0	0

F = full rate used, shown after each product name. *V. albo-atrum* grew on all untreated inoculated controls.

Appendix 2. Effect of concentration and exposure time on efficacy of chemical disinfectants against *V. albo-atrum* mycelium

Product exposure	Number of replicates, of 3, developing growth of *V. albo-atrum* Rate F	1/2	1/4	1/8		Rate F	1/2	1/4	1/8
Citric acid (0.2%)					**Sodium hypochlorite** (2,000ppm)				
5 min	3	3	3	3		2	3	3	3
30 min	3	3	3	3		0	1	3	3
1 hr	3	3	3	3		0	1	3	3
4 hr	3	3	3	3		0	3	3	3
8 hr	3	3	3	3		0	1	2	3
Harvest Wash (1:100)					**Trigene** (1:50)				
5 min	3	3	3	3		0	0	1	3
30 min	3	3	3	3		1	0	0	0
1 hr	3	3	3	3		0	0	0	0
4 hr	3	3	3	3		0	0	0	0
8 hr	3	3	3	3		0	0	0	0
Jet 5 (1:125)					**Unifect** G (4%)				
5 min	3	3	3	3		0	0	3	3
30 min	3	3	3	3		0	1	3	0
1 hr	2	3	3	3		0	0	0	2
4 hr	3	3	3	3		0	0	0	0
8 hr	0	1	3	3		0	0	0	0
Panacide M (1:60)					**Virkon S** (2%)				
5 min	0	3	3	3		2	3	3	3
30 min	0	1	3	3		3	3	3	3
1 hr	0	1	3	3		0	2	3	3
4 hr	0	0	1	1		1	2	3	3
8 hr	0	0	0	0		0	0	0	3

Appendix 3. Effect of organic matter and clay on efficacy of four disinfectants against *V. albo-atrum* conidia and mycelium

Product, rate and exposure time	Number of replicates (of 3) developing growth of *V. albo-atrum*			
	No organic matter or clay		Peat and clay contamination	
	Conidia	Mycelium	Conidia	Mycelium
Panacide M (1:60)				
5 min	3	0	2	3
30 min	1	0	0	3
1 hr	3	0	1	0
4 hr	0	0	00	3
8 hr	0	0	0	0
Sodium hypochlorite (2,000 ppm)				
5 min	0	0	0	3
30 min	0	0	0	3
1 hr	0	0	0	2
4 hr	0	0	0	2
8 hr	1	0	0	0
Trigene (1:50)				
5 min	0	0	1	0
30 min	0	1	2	1
1 hr	0	0	0	0
4 hr	0	0	1	0
8 hr	0	0	1	0
Unifect G (4%)				
5 min	0	0	0	3
30 min	0	0	0	1
1 hr	0	0	0	0
4 hr	0	0	0	0
8 hr	0	0	0	0