Project title:	Chrysanthemums: to investigate new symptoms of *Verticillium* wilt and determine pathogen variation
Project number:	PC 195
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Key words:	Chrysanthemum, wilt, *Verticillium dahliae*

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

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Grower Summary

Headline

- An HDC Information sheet 02/02 '*Verticillium* wilt of chrysanthemums' was prepared in August 2002 providing details of typical and atypical symptoms of *Verticillium* wilt disease.
- Just under half of the growers that responded to the HDC survey (11 out of 25) reported problems with atypical *Verticillium* wilt in chrysanthemum crops.
- All of the isolates of *Verticillium* collected from diseased plant material, irrespective of symptom expression, were identified as *V. dahliae.*
- The 'atypical' isolates formed a tight cluster based on molecular analysis whilst the typical isolates were more diverse.
- The development of symptoms during flowering makes detection in propagating stock plants unlikely unless appropriate disease indexing is completed.

Background

Verticillium wilt of chrysanthemums became a serious problem on a number of large nurseries in 2001 due to unusual symptom expression with concerns in the industry about development of a new strain of the fungus. Traditionally, *Verticillium* wilt in chrysanthemums has been caused primarily by *Verticillium dahliae,* although *V. albo-atrum* has been associated with the disease. There have been recent reports of sub-species variation within both *V. albo-atrum* in UK and Dutch tomatoes (O'Neill, 2002) and *V. dahliae* in Dutch chrysanthemums (*pers. comm.* Termorshuizen, Wageningen Univ.). Therefore, there was a need to establish the extent of the problem in chrysanthemums and to determine the species and sub-species variation. The project involved collaboration between UK and Dutch workers comparing isolates of the fungus from both countries.

Objectives

1. To increase grower awareness about *Verticillium* wilt.
2. To estimate the prevalence of *Verticillium* wilt in chrysanthemums.
3. To determine the species of *Verticillium* involved.
4. To ascertain if there is any sub-species variation.
5. To collect further isolates of *V. dahliae* from diseased plant material exhibiting typical symptoms of wilt and compare with existing atypical isolates.
6. To determine if there are two discrete populations of isolates associated with symptom type.
7. To review findings and report to chrysanthemum growers.

Summary of the project and main conclusions

1. To increase grower awareness about *Verticillium* wilt.
    - An HDC Information sheet 02/02 '*Verticillium* wilt of Chrysanthemums' was produced in August 2002 and sent to 250 registered growers.
    - This illustrated and described the symptoms of both typical and atypical wilt, the latter only causing symptoms towards the end of the crop affecting pedicel extension, flower shape and colour.
    - Informal progress reports were presented to the UK Chrysanthemum Growers Association by its Chairman, Dave Abbott.

2. To estimate the prevalence of *Verticillium* wilt in chrysanthemums.
    - A questionnaire was designed and sent to growers with the information sheet; twenty-five forms were returned.
    - Fifteen growers reported a problem with *Verticillium* wilt of which three reported typical symptoms only, six reported symptoms of atypical wilt alone and a further five had symptoms of both atypical and typical wilt. One grower was unsure if the infection in his garden mums was due to *Verticillium*.

3. To determine the species of *Verticillium* involved.
    - All of the isolates of *Verticillium* collected from diseased plant material, irrespective of symptom expression, were identified as *V. dahliae* using both morphological features and molecular methods.
    - No *Verticillium albo-atrum* was detected.
    - Therefore based on this study, atypical symptoms of chrysanthemum *Verticillium* wilt are not the result of the introduction of a new species.
    - For fresh isolates, identification using morphological features was as reliable as molecular methods.

4. To ascertain if there is any sub-species variation.
    - There was no evidence of a distinct difference between *V. dahliae* isolates obtained from chrysanthemums grown in the UK or the Netherlands.
    - No evidence of a difference between isolates collected in this study and older isolates held in culture at CSL was found.
    - There was no evidence of host specificity to chrysanthemum found based on molecular characterisation.
    - There was no correlation between the country of origin of cuttings and molecular characteristics of isolates.
    - The initial study indicated that the one isolate collected described as giving typical symptoms was different from the much larger number of isolates from plants with atypical symptoms. A project extension was granted to collect further isolates to see if initial molecular distinction between typical and atypical symptoms could be further substantiated.

5. Collect further isolates of *V. dahliae* from diseased plant material exhibiting typical symptoms of wilt and compare with existing atypical isolates.
    - A further fifteen isolates of *V. dahliae* were obtained.
    - Nine of these isolates came from the Netherlands but the symptom type was unknown and could not be determined.

- Six isolates came from the UK, three were described as giving typical symptoms, two as atypical and one from a two week old plant so symptoms could not be determined.

6. Determine if there are two discrete populations of isolates.
    - The new isolates were tested using molecular methods and compared with the isolates of known symptom type analysed in the initial study.
    - The 'atypical' isolates formed a tight cluster based on molecular analysis whilst the typical were more diverse.
    - Based on molecular analysis some of the Dutch isolates of unknown symptom type were very similar to the UK atypical isolates but some were more diverse.
    - The Dutch population structure is similar to that of UK isolates based on the relatedness of old and new isolates using molecular characters. Unfortunately, it cannot be used to predict symptom expression of isolates.
    - These results are consistent with a diverse population giving typical symptoms and a more constricted population giving atypical symptoms.

7. To review findings and report to chrysanthemum growers.
    - Interim project reports were submitted to HDC following two progress meetings.
    - Dave Abbott informed the UK Chrysanthemum Growers Association of progress.
    - A final report was submitted to the HDC in May 2004.

Financial benefits

The project alerts UK chrysanthemum growers to a new risk from *Verticillium* wilt that previously may have gone unrecognised due to the late onset of symptoms. It is caused by a closely related group of isolates of *Verticillium dahliae* that usually only express symptoms prior to harvest causing flower deformation. This development of symptoms during flowering makes detection in propagating stock plants unlikely. It is also unlikely to be observed in cuttings and difficult to observe in flower production crops. It alerts propagators and growers for the need to ensure disease freedom by testing for *V. dahliae* using proven laboratory techniques and not relying on symptom expression alone. This project should help workers within the industry to be familiar with the range of symptoms caused by *Verticillium* wilt.

Action points for growers

- Ensure chrysanthemum cuttings are purchased only from sources with a proven track record of disease-indexing including fungal pathogens.
- Cuttings suppliers must regularly monitor the health of stock plants to ensure they remain disease free.
- Disease introduction may occur due to new planting stock from a contaminated source or carryover of infection at the nursery.
- Encourage good growing conditions to reduce water stress on plants.
- Take care to minimise root damage as *Verticillium* colonises damaged roots.
- Look out for symptoms of wilt and have suspicious plant material tested to confirm the presence of *Verticillium dahliae* as other diseases and disorders may cause similar symptoms. See HDC Information sheet 02/02 '*Verticillium* Wilt of chrysanthemums' for details of typical and atypical wilt symptoms.
- Once symptoms are confirmed remove affected plants including the stem base and root ball and destroy all infected material.
- The only means of eliminating carryover involve removal of all plant debris followed by thorough soil sterilisation.
- Carry out good hygiene measures to help prevent dispersal within the crop and carry over to the next crop.

Science Section

Introduction

Verticillium wilt is a serious fungal disease of chrysanthemums. Traditionally, leaves of affected plants are yellow and limp although this is initially confined to one or more of the lower leaves. Eventually, more leaves become affected and older ones turn brown and die. Unfortunately, symptom expression is not very distinctive and can be quite variable preventing early recognition of the problem. However, since 2001 atypical symptoms of *Verticillium* wilt have been observed on commercial premises. The crop appears healthy until pedicels elongate when several on one side of the inflorescence fail to develop at the same rate as the others. Leaves on this side at the top half of the plant may start to break down, petals on the affected flowers remain quilled and colour is poor. This results in affected stems being unmarketable leading to significant losses. Due to this unusual expression of symptoms causes such as TSWV, INSW, stress etc., have been assumed rather than *Verticillium*. A similar problem on chrysanthemums has been reported in the Netherlands (*pers. comm.* A. Termorshuizen, Wageningen University). The Dutch have speculated that a new strain of *V. dahliae* may be the cause.

Expression of *Verticillium* wilt symptoms has been related to environmental factors, such as photoperiod length causing physiological stress, cultural factors, such as drought/water-logging, temperature or plant variety. During rapid vegetative growth by the plant, the fungus cannot keep pace with the plant growth and produces only minor or mild symptoms. When the growth of the plant is slowed by the development of flower buds, then more severe symptoms may develop. Busch & Schooley (1970) were the first to report in the scientific literature that symptom expression in *Verticillium* wilt of chrysanthemum was correlated with flowering. In these experiments, when the photoperiod encouraged vegetative growth no symptoms were observed. However, in the 1960's growers observed typical symptoms of *Verticillium* wilt on vegetative stock plants under conditions of active growth when infection levels were high (Abbott, *pers. comm.*). Pegg & Jonglaekha (1981) carried out similar experiments on chrysanthemums but found that reduced photoperiods had minimal effect on disease severity but affected the amount of fungal mycelium in stems, with a peak at flowering, and leaves with a peak one week after flowering. This work demonstrates that the expression of symptoms is not fully understood and is probably the result of the interaction of several factors, such as variety and environmental parameters, with the fungus.

V. dahliae produces both asexual spores, called conidia, and thick-walled survival structures, called microsclerotia, which can survive for up to 10-15 years in soil in association with plant material. The fungus infects the root system directly or through wounds caused naturally by root growth through soil or the action of other organisms. It spreads in the xylem vessels preventing water uptake and causing plants to wilt. Sources of inoculum include contaminated growing media, infected cuttings (which rarely display symptoms), fungal spores (conidia) that are sometimes produced on the surfaces of infected plants and may be dispersed in air-currents or water-splashed over short distances, and to a lesser extent root contact, insects, and movement in re-circulation water.

V. dahliae is known to be a variable species in many ways. Previous research using molecular methods, such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and sequence analysis, has demonstrated the presence of sub-specific groupings within *V. dahliae* (Morton *et al.*, 1995, Barbara *et al.*, 1998). In the UK most isolates belong to groups A and B which are not host associated but may differ in pathogenicity. Host associated groups include M, isolates affecting *Mentha* x *piperita* (peppermint), and D, amphihaploid strains (all others are haploid), affecting cruciferous hosts (Okoli *et al*., 1993, 1994). Different groupings are found in other countries e.g. Japan where four were described (Carder & Barbara, 1994) but more are now known to occur. Currently it is not known if any (or all) chrysanthemum isolates are host-adapted or whether sub-species variation occurs among them.

Since 2001, *Verticillium* has had significant effects on productivity leading to up to 10% loss and downgrading of crops. In the longer term, misidentification of the problem will lead to increased losses due to failure to eradicate the fungus from stock plants and/or the growing media resulting in pathogen inoculum build-up and carry through on cuttings.

The initial project was designed to investigate the simplest explanation of the problem; the development of a new strain of *Verticillium*. Molecular work during this project indicated there may be a difference between the atypical and typical isolates but due to insufficient data from the latter no firm conclusions could be drawn. Therefore, a six month extension was granted by the HDC (August to December 2003) to collect and characterise additional isolates.

Materials and Methods

1. To increase grower awareness about *Verticillium* wilt.

Text and photographs of atypical and typical symptoms of *Verticillium* wilt were supplied to the HDC in order to produce an 'Information sheet' (Appendix 1).

2. To estimate the prevalence of *Verticillium* wilt in chrysanthemums.

A questionnaire was drafted by Ruth Finlay in consultation with Dave Abbott and Charles Lane and about 250 were sent out by the HDC in October 2002 (Appendix 2). Returned questionnaires were assigned a unique letter to maintain anonymity.

3. To determine the species of *Verticillium* involved.

Grower sites for sampling were selected from the returned questionnaires and packaging for samples and information on sampling methods were supplied. Samples were assigned a unique reference number on receipt. A thorough visual examination was carried out, then representative pieces of tissue were excised aseptically and either incubated in a humid chamber or surface sterilised and small rings (3-5 mm) cut from the stem and plated out onto potato dextrose agar amended with antibiotics (streptomycin and penicillin). Cultures and humid chambers were incubated in the laboratory (ca. 22°C, 12 h light/dark) and assessed after about 7 days for the presence of typical fungal structures (verticils [spore bearing structures] and microsclerotia [minute resting structures composed of fungal hyphae]). If either structure was observed, material was transferred aseptically to fresh agar to obtain the fungus in pure culture. If no fungal structures were seen, material was incubated for a further 14-21 days.

Isolates were initially identified using well described morphological features and then confirmed by molecular techniques using standard Polymerase Chain Reaction (PCR) with the primer pair 19/22 which are known to be species specific for *V. dahliae* (Carder *et al*., 1994). For DNA extraction mycelium of all isolates was grown on sterile cellophane discs placed on standard prune lactose yeast agar (Talboys, 1960), a standard medium for differentiating *Verticillium* species which stimulates production of resting structures. Harvested mycelium was lypohilised and DNA extracted from the dried material using a commercially available column purification procedure (Qiagen DNeasy).

4. To ascertain if there is any sub-species variation.
5. Collect further isolates of *V. dahliae* from diseased plant material exhibiting typical symptoms of wilt and compare with existing atypical isolates.
6. Determine if there are two discrete populations of isolates.

Twenty-seven isolates of chrysanthemum were entered for analysis by AFLP at HRI. Eleven (with four as pairs of cultures) of these could be described as 'new UK' [collected since 2001], 2 as 'old UK' [collected prior to 2001], 5 as 'new NL', and 9 (1 as a pair) as 'old NL'. During the AFLP testing process, five cultures were eliminated either for technical reasons (*e.g.* no or impure DNA) or because they were clearly not *V. dahliae*; a total of 27 cultures representing 22 isolates appeared in the final results.

Molecular confirmation of the cultures as *V. dahliae* was made using standard PCR with the primer pair 19/22 which are known to be species specific for *V. dahliae.* For comparing isolates, a standard AFLP procedure with only minor modifications made during application to *V. dahliae* was used. Briefly, for each isolate extracted DNA was digested with two restriction endonucleases that left 'sticky ends' to the fragments. Standard linker/primers were then ligated to the ends of the fragments and a subset of the fragments amplified using PCR and primers with single base 'selective' extensions. These primers have the same sequence as the linkers previously ligated on to the fragments but are slightly longer at the 3-prime end. It is these extra bases that provide the specificity of the amplification. For this project four primer combinations, selected as effective at revealing polymorphisms in *V. dahliae* on the basis of work in earlier projects, were used. These all had single base extensions. The primers were labelled with fluorescent dyes and the mixtures of amplicons resulting from the PCR reactions analysed using a standard Applied Biosystems sequencer. AFLP banding patterns for each isolate were compared using a standard unweighted pair group method and Jacquard's coefficient.

The second group of isolates to be obtained (materials and methods 5 & 6) were directly compared to the 10 isolates from the first group for which symptom types were available. For comparing isolates, a standard AFLP procedure with only minor modifications made during application to *V. dahliae* was used. For this project four primer combinations, selected as effective at revealing polymorphisms in *Verticillium dahliae* on the basis of work in earlier projects, were used. AFLP banding patterns for each isolate were compared using a standard unweighted pair group method and Jacquard's coefficient.

7. To review findings and report to chrysanthemum growers.

A project meeting of all project partners was held (May 2003) leading to the recommendation to seek a project extension by a further 6 months to permit additional molecular work. A further project meeting was held in January 2004 to discuss project findings, to assist in preparation of the final report and to ensure good technology transfer to the industry.

Results and Discussion

1. To increase grower awareness about *Verticillium* wilt

An HDC 'Information sheet' 02/02 *Verticillium* wilt of chrysanthemums was produced in August 2002 and sent to all relevant HDC registered growers. (Appendix 1)

2. To estimate the prevalence of *Verticillium* wilt in chrysanthemums.

Twenty-five completed questionnaires were returned, the premises were coded using letters from A to Y and the responses to a range of questions in summarised in Appendix 3. Fourteen growers reported *Verticillium* wilt problems and provided detailed information, whilst a further eleven reported no problems and one grower was unsure if *Verticillium* had been a problem.

The year round pot grower who reported problems due to *Verticillium* wilt obtained new stock of the extremely susceptible varieties (Princess Anne). The grower producing both spray and pots AYR reported *Verticillium* wilt only in the cut flower programme. Of the two garden mum producers one reported no infection whilst the other thought there might have been a problem but was not sure. Of the cut flower growers, covering year round, spot, and natural season crops six reported no problems due to *Verticillium* whilst 13 had experienced the disease in their crops.

In response to question 6 concerning details of cultural practices (Appendix 3), the onset of infection with *Verticillium* could not be shown to result from changes in soil cultivation, soil sterilisation or crop hygiene. Also, there was no obvious difference in any of these areas between those growers reporting *Verticillium* and those free from the disease. Only two growers changed any part of their soil cultivation methods after the onset of infection. Seven growers changed either their method or more often their frequency of soil sterilisation following infection and six growers, only three of whom had changed their soil sterilisation, upgraded the standard of hygiene following infection. Most commonly these growers introduced rouging the crop and destruction of plants showing visible symptoms of infection.

In response to question 7 concerning measures to counteract an outbreak, four growers considered the steps they had taken to control the disease were largely unsuccessful although two gave considered answers as to where the problems probably lay and what needed to be done to improve control and aid eradication (detailed in Appendix 3).

3. To determine the species of *Verticillium* involved.

Sources of isolates

Initially, samples and cultures were obtained from a number of grower sources in the UK and NL (Table 1). Several of the isolates went to HRI as pairs of cultures selected from isolates originally from individual sources; the partners in these pairs of cultures were morphologically distinct and were treated as individual samples for the molecular analysis. In summary, a total of 9 UK isolates were obtained from chrysanthemums which had displayed typical or atypical symptoms of *Verticillium* infection. Material was collected as a result of returned HDC questionnaires, directly from PHSI and growers. Additional isolates were obtained from the Netherlands chrysanthemum industry (via Aad Termorshuizen) and from NL and UK culture collections giving a total of 22 isolates from the first and second parts of the study.

Morphological identification

The majority of these isolates produced microsclerotia in culture confirming their identity as *V. dahliae*. This demonstrated that infection in chrysanthemum is normally caused by *V. dahliae* and ruled out the possibility that atypical symptoms are associated with another species (e.g. *V. albo-atrum*). The few isolates that did not produce microsclerotia did not produce any resting structures and could not be morphologically identified. These were old isolates and loss of resting structure is common in *Verticillium* isolates kept in culture. That some of the isolates collected in this project did not produce resting structures was therefore not surprising.

Identity of isolates by PCR

When tested by PCR and primers 19/22, three of the first group of cultures gave no band and were presumed to not be *V. dahliae*. Four cultures gave weak bands and 'smearing'; these cultures were presumed mixed cultures (e.g. *V. dahliae* contaminated during culturing) with only a low proportion of *V. dahliae* present (Table 1).

The second group of isolates collected for the extension to the project were similarly tested by PCR with the same primers and all confirmed as being *V. dahliae*.

Table 1. Numbers of cultures of isolates, designation used for molecular testing and species identity by PCR (primers 19/22)

Code	*Original expression*	*Year/Country*	*Host*	*PCR(19/22)*	*Species identity*	*Symptom*
1	CC29	1979/UK	Xmum	+ve	*V. dahliae*	not known
6	2013962	2001/UK	Xmum	+ve	*V. dahliae*	atypical
7	2014153	2001/UK	Xmum	+ve	*V. dahliae*	atypical
8	2016262	2002/UK	Xmum	+ve	*V. dahliae*	atypical
9a	2020021	2002/UK	Xmum	no band		
9b				+ve	*V. dahliae*	atypical
10	2028353	2002/UK	Xmum	+ve	*V. dahliae*	atypical
11a	2028353	2002/UK	Xmum	weak/smear	*V. d.* + mixed	
11b				+ve	*V. dahliae*	atypical
12	2025567/2	2002/UK	Xmum	+ve	*V. dahliae*	atypical
13	2027474	2002/UK	Xmum	+ve	*V. dahliae*	atypical
14	2027510	2002/UK	Xmum	+ve	*V. dahliae*	typical
15a	2028409	2002/UK	Xmum	+ve	*V. dahliae*	atypical
15b				+ve	*V. dahliae*	atypical
16a	2029384	2002/UK	Xmum	weak/smear	*V. d.* + mixed	
16b				+ve	*V. dahliae*	atypical
17	-	1989/NL	Xmum	no band		
18	-	1990/NL	Xmum	+ve	*V. dahliae*	NK
19	-	1991/NL	Xmum	+ve	*V. dahliae*	NK
20	-	1991/NL	Xmum	+ve	*V. dahliae*	NK
21	-	1991/NL	Xmum	+ve	*V. dahliae*	NK
22	-	1991/NL	Xmum	+ve	*V. dahliae*	NK
24w	-	1991/NL	Xmum	weak/smear	*V. d.* + mixed	
24b				weak/smear	*V. d.* + mixed	
25	-	1993/NL	Xmum	+ve	*V. dahliae*	NK
26	DCH12064	?/UK	Xmum	no band		
	DUY	2002/NL	Xmum	+ve	*V. dahliae*	NK
	MA1	2002/NL	Xmum	+ve	*V. dahliae*	NK
	V. dahliae	2002/NL	Xmum	+ve	*V. dahliae*	NK
	VS	2002/NL	Xmum	+ve	*V. dahliae*	NK
	VU	2002/NL	Xmum	+ve	*V. dahliae*	NK
	J1		Other	+ve	*V. dahliae*	
	J5		Other	+ve	*V. dahliae*	
	J6		Other	+ve	*V. dahliae*	
	J8		Other	+ve	*V. dahliae*	
	J9		Other	+ve	*V. dahliae*	

NB. Isolates J1,5,6,8,9 were not received as part of this project and are only included to illustrate what a 'host specific' grouping, possibly based on a single introduction to a crop/region, might be expected to look like in this type of analysis.

4. To ascertain if there is any sub-species variation.

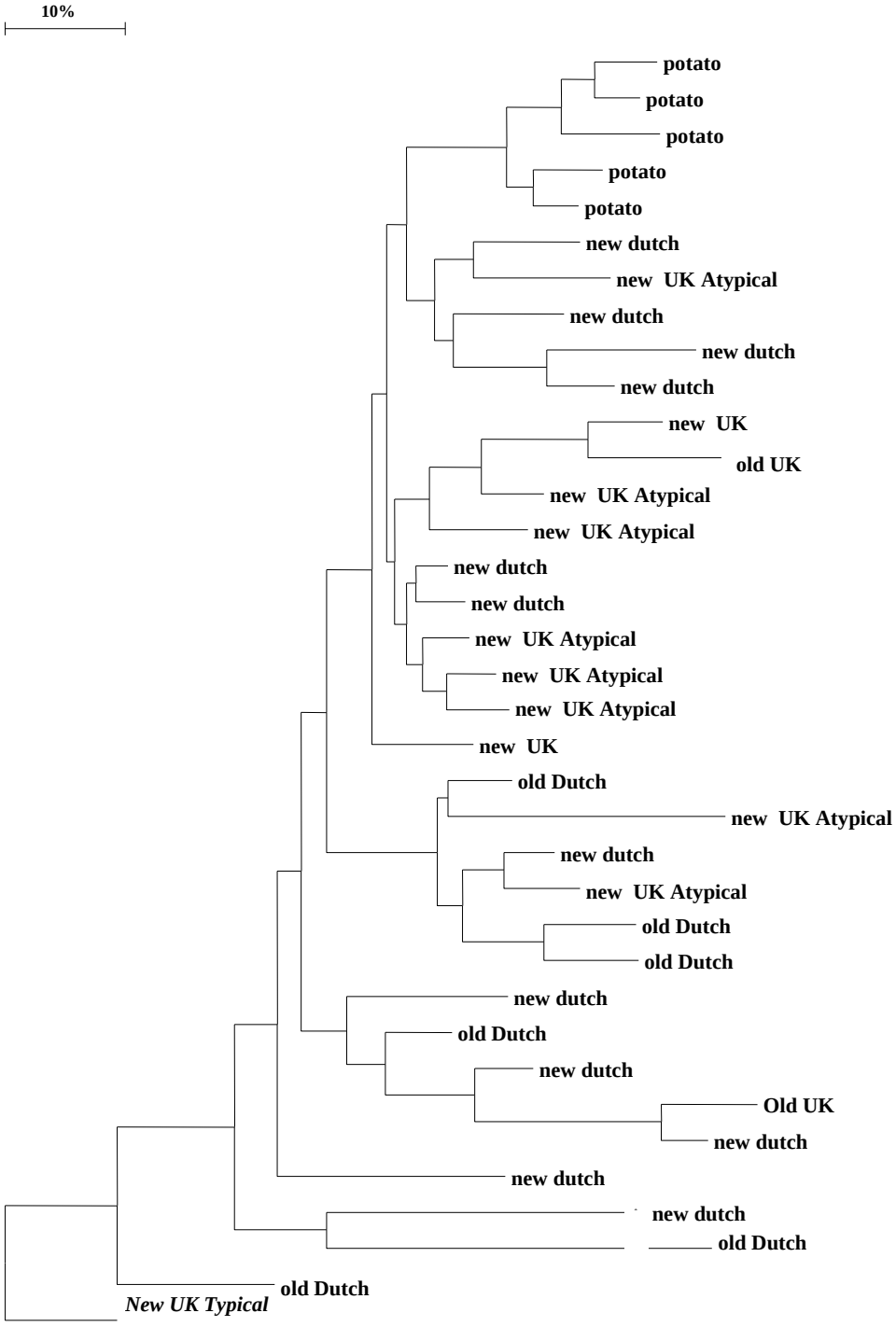
AFLP patterns grouped the cultures identified by PCR as *V. dahliae* together with other *V. dahliae* isolates collected as part of an EU project, further confirming their species identity (results not shown). This general comparison of chrysanthemum isolates with non-chrysanthemum isolates (which included isolates from potato, hop, strawberry, lime, cherry and other north European hosts) did not reveal a distinct group (or limited groupings) associated with chrysanthemum. Therefore, no evidence of host specificity was found. Many of the chrysanthemum isolates did appear more similar to each other than expected from the overall variation in the isolates. However, non-chrysanthemum isolates were also present in this broad grouping. Other groupings of 'related' isolates (e.g. some Spanish soil isolates) were seen during this exercise, suggesting that lack of grouping in the chrysanthemum isolates was not a technical artefact. This pattern confirms what has been found in similar projects running concurrently and suggests that the majority of *V. dahliae* isolates are polyphagous (*i.e.* not specialised for particular hosts).

When chrysanthemum cultures from the UK and NL were compared there was no distinct association or clusters of isolates related to country of origin or age (Fig. 1). A distinct host-associated cluster of isolates from another host (potato) collected as part of a separate project is shown to illustrate what might have been expected (Fig 1). This lack of grouping amongst chrysanthemum isolates in relation to origin was perhaps surprising at first. However, almost all chrysanthemum cuttings planted in the UK and NL are obtained from stock plants grown in a third country, usually in the tropics. These stock plants have in turn been derived from relatively small quantities of base material produced by a very limited number of propagators. The methods and standards of disease-indexing practices may vary resulting in differing degrees of disease risk. No direct correlation between the country of origin from which the stock material was used to produce cuttings for flower production and molecular characteristics of isolates was found.

The type of symptom expression was only recorded for isolates collected as a direct result of the project. The comparison of those inducing atypical with those inducing typical symptoms is potentially of the greatest interest to this project. Nine new UK 'atypical' isolates clustered together and appeared different from the one new UK 'typical' isolate available. With there being only a single 'typical' isolate, it is obviously difficult to infer a great deal from this finding. However, it did highlight the need to compare molecularly a greater number of isolates inducing known symptoms. Isolates of *Verticillium* spp. readily lose pathogenicity

in culture and it was considered impractical to determine the symptom expression of old isolates (*i.e.* those most likely to induce 'typical' symptoms). Therefore, further fresh isolates of known symptom type were sought and eventually six were collected (Table 2).

Fig 1. Cladogram representing the relationship of the chrysanthemum isolates from the UK and the Netherlands (old and new) and five Dutch isolates from potato.



5. Collect further isolates of *V. dahliae* from diseased plant material exhibiting typical symptoms of wilt and compare with existing atypical isolates.

Following the initial molecular analysis, a further 15 isolates were obtained (Table 2). Nine of these were from the Netherlands; however no details of the symptom type induced by these isolates were available or could be obtained. Of the six UK chrysanthemum isolates three were described as inducing typical symptoms, two atypical symptoms and one was from a two week old plant so symptoms could not be determined. Four of the six isolates came from the same nursery and cannot be guaranteed to be 'independent' of each other.

Table 2. *Verticillium dahliae* isolates obtained for molecular analysis in second phase of project.

Code	*Identifier*	*Nursery*	*Country*	*Variety*	*Symptoms*	*Isolated*
1	4784	O	UK	Delianne	not known	2003
2	4786	O	UK	Euro	atypical	2003
3	7584/1	O	UK	NK	typical	2003
4	7584/2	O	UK	NK	typical	2003
5	4937	V	UK	Golden	atypical	2003
6	13713	X	UK	Delianne	typical	2003
7	1	-	NL	Dracula	NK	2002
8	34	-	NL	NK	NK	2002
9	62	-	NL	Euro	NK	2003
10	86	-	NL	Orange Reagan	NK	2003
11	114	-	NL	Super Yellow	NK	2003
12	120	-	NL	Improved Rivalry	NK	2003
13	121	-	NL	Bradford	NK	2003
14	122	-	NL	Yellow Delianne	NK	2003
15	232	-	NL	Cream Reagan	NK	2003

6. Determine if there are two discrete populations of isolates.

AFLP analysis was carried out as previously described (except only the three most informative primers were used) and included not only the 15 fresh isolates but also all those from the first phase for which symptom type was available. In the resulting cladogram (Fig. 2a) it can be seen that the atypical isolates cluster together but that the typical isolates are more diverse and do not form a tight distinct cluster. The nine Dutch isolates collected during the second phase of the project gave a similar distribution, some clustering with the known atypical isolates and others being more diverse (Fig 2b).

As can be seen in Figure 2a one typical isolate clustered effectively within the atypical isolates and a second was only slightly removed. Two of the typical isolates (one from the first phase of the project and one from the second) were clearly distinct from the atypical.

Taken with the initial finding that chrysanthemum isolates did cluster specifically within a larger population of *V. dahliae* isolates, the best explanation of these results is that isolates inducing typical symptoms are part of the 'aboriginal' or earliest known population and are not host adapted. The isolates inducing atypical symptoms then appear to be selected from this population, appearing as a relatively tight cluster within the cladograms in Fig 2. At first sight, the isolates within this tight cluster appear to be too variable to represent a single clone (which should appear identical) but there may have been some diversification after selection. Without biological testing it cannot be said whether these atypical isolates are host-specific, in the sense of being able to only infect chrysanthemums, or a population selected by virtue of being able to induce different symptoms in this host. Whilst selection for a different symptom type may appear unlikely, the late expression of symptoms in the atypical disease could lead to less efficient removal of infected plants both at the plant propagation stage where these isolates are effectively symptomless and in flower crops where late and unusual development of symptoms may allow the problem to go unrecognised and untreated.

If after further investigation the build-up of 'symptomless' isolates in propagation material proves to be the main source of infection then a programme of testing the stocks and testing/ sterilisation of soils at sites used for propagation will be necessary to eliminate infection with these isolates.

Fig 2a. Cladogram (unrooted tree) representing the relationships of the isolates of known symptom type. All isolates are from nurseries in the UK (T = typical symptoms; A = atypical; * indicates isolates from the first phase of the project).

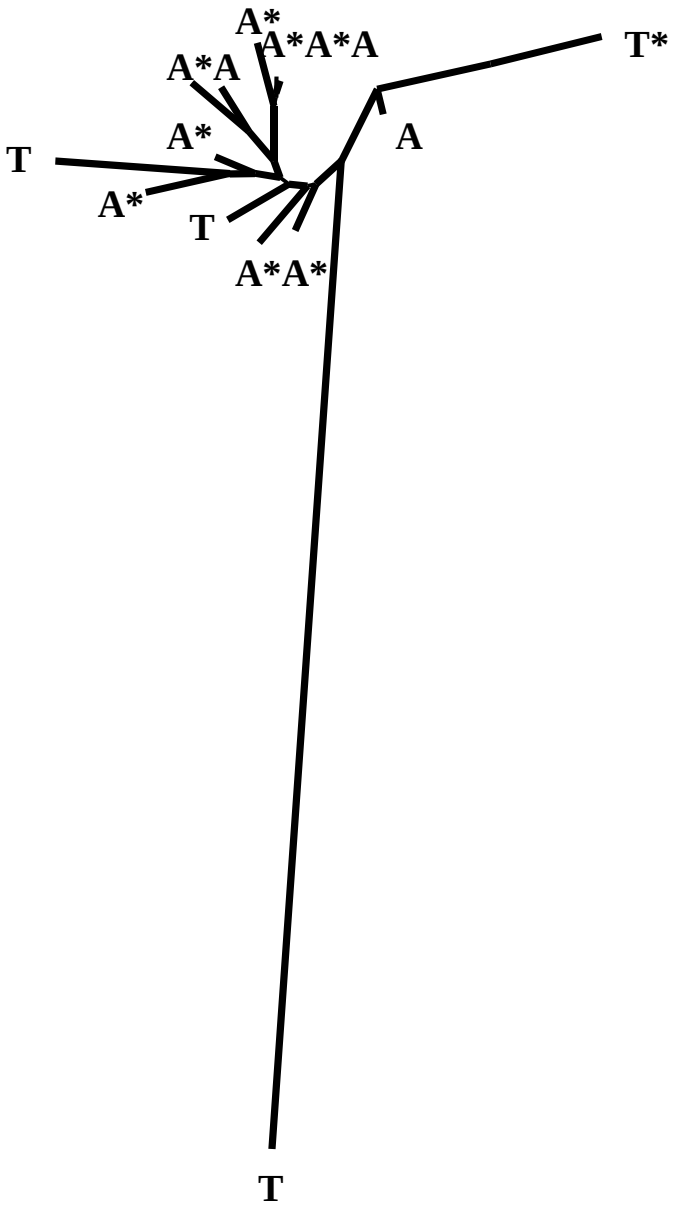
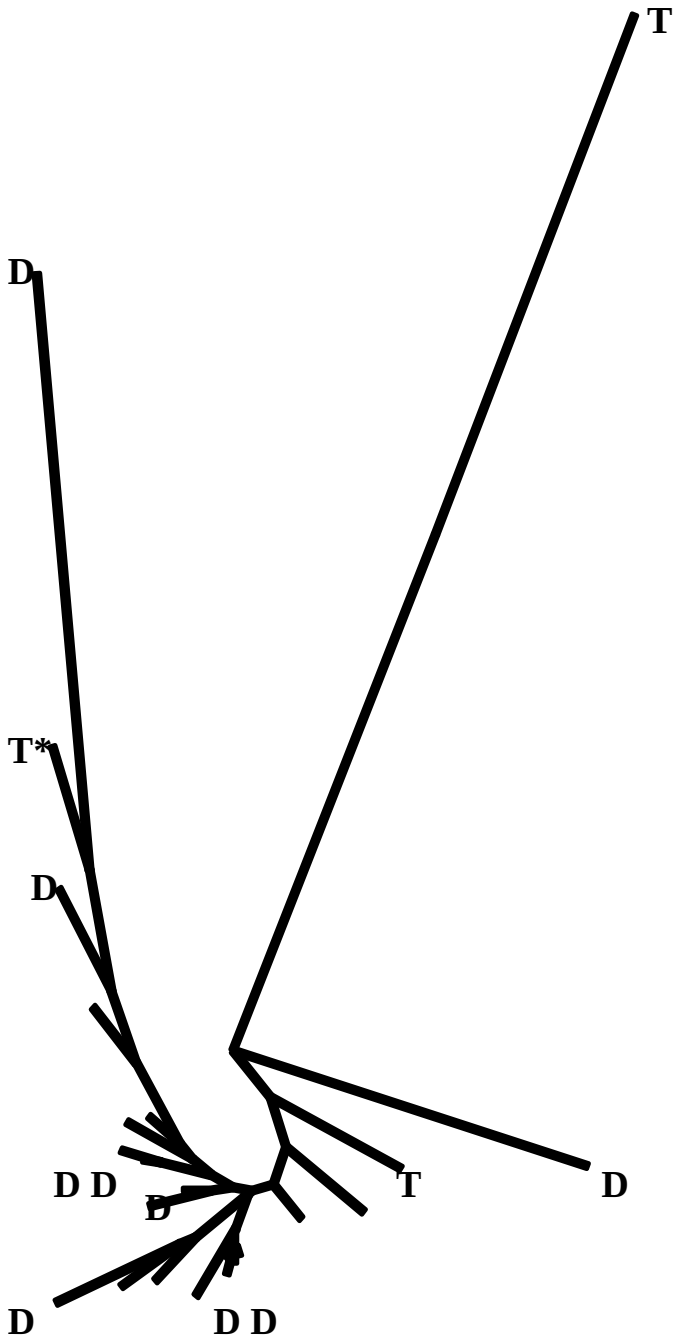


Fig 2b. Cladogram (unrooted tree) representing relationships of isolates of known symptom type. As figure 2a but with the addition of the nine recent Dutch isolates. For clarity only the typical isolates (T) and the Dutch isolates (D) are labelled. All other branches represent UK atypical isolates.



7. To review findings and report to chrysanthemum growers.

Two project meetings were held (May 2003 and January 2004) to review progress and to assist in technology transfer to the UK chrysanthemum growers by informal reports by David Abbott.

Conclusions

The main conclusion of the molecular work undertaken during this project is that most isolates of *Verticillium dahliae* from chrysanthemum are unspecialised members of the larger population of isolates of this fungus from a wide range of hosts but that those associated with the atypical symptoms form a discrete, closely related population. This latter tightly clustered population has almost certainly arisen by selection from within the broader overall population. There is no evidence that these atypical isolates infect chrysanthemums more easily then do other isolates (although this has not been tested directly). It is very likely that the selection that made these isolates more common was due to roguing by plant breeders, propagators and growers; these isolates are effectively symptomless prior to the onset of flowering and would not be removed during normal propagation procedures as stock plants are not allowed to flower. The late onset and unusual symptoms in flower production may have resulted in diseased material not be recognised as *Verticillium* wilt thus increasing the likelihood of carryover of atypical isolates as compared to typical isolates.

Effective disease-indexing including screening for fungal pathogens is essential and must be practiced by all plant breeders and propagators. It is particularly important that screening for fungal pathogens includes techniques and sufficient incubation time for *Verticillium* to develop and to be identified to species accurately. This should include both incubation of infected material in 'damp chambers' as well as isolation onto agar medium. Cuttings producers and growers have a part to play in regular monitoring of their crops, early eradication of diseased plants, testing for pathogens and effective soil sterilisation.

As with all infections with *V. dahliae*, prevention of carryover in individual nurseries is dependent on effective removal and destruction of plants, soil sterilisation and cleaning of glasshouse structures. It is notable in this respect that two of the growers reporting difficulties with control at their sites mention poor distribution of soil sterilant and inappropriate soil conditions as being factors in lack of control.

Recommendations for further work

- To investigate the disease with respect to stock plants and cuttings to ascertain symptom development and methods for monitoring for the pathogen and improve disease-indexing.

- To develop improved methods for monitoring for the pathogen in soil and plant material in order to predict risk prior to planting. Application of molecular methods may permit more rapid and potentially quantitative assessment of *Verticillium* and ascertain the effectiveness of control strategies. This could be of value not only to the chrysanthemum industry but could be applied to other crops susceptible to *Verticillium* wilt.

Technology transfer

August 2002 - HDC 'Information sheet' 02/02 *Verticillium* wilt of chrysanthemums'

March 2003 – United Kingdom Phytodiagnosticians Discussion Group. Oral communication to UK plant disease diagnostic meeting.

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Appendix 1

HDC Information Sheet 02/02 '*Vertillium* Wilt in Chrysanthemums'

Appendix 2

HDC grower survey on *Verticllium* Wilt in chrysanthemums (September, 2002)

Appendix 3

Total sent out: approx. 250
Respondents: 25
Returned questionnaires were assigned a letter from A to Y.

Questions 2 & 3. Cropping details and presence of *Verticillium*.

	Affected by *Verticillium* wilt	Unaffected by *Verticillium* wilt
AYR cut only	N, R, S, V, X, Y	F
AYR pot only	L	D, H
AYR cut & pot	Q	K
AYR cut & spot crop	O	A
AYR cut, spot and N.S.	M	
Spot crop only	T	E
Natural season (NS) only	I, U, W	B, G
Pot spot crop		C
Garden mums	P	J

Positive responses were placed into one of three groups:

	Typical (t)				Atypical (a)						Typical (t) & Atypical (a)							
	I	L	M	U	O	R	S	V	W	X	N		Q		T		Y	
	t	t	t	t	a	a	a	a	a	a	a	t	a	t	a	t	a	t
Historical problems on site > 2 yrs	Y	Y	Y			N	Y			Y		Y	Y		Y	Y	Y	Y
Within the last two years	Y	Y	Y	Y		N	Y	Y	Y	Y	Y	Y	Y					
Last summer (2001)		Y	Y	Y		Y	Y	Y		Y	Y	Y	Y				Y	Y
This summer (2002)		Y	Y		Y	N	Y		Y	Y	Y	Y	Y	Y			Y	
At present			Y			N	Y			Y			Y					

Comments: R – 14 plants only found in 2002; U – gave up flowers in 2002.

Question 4. Extent of *Verticillium* wilt outbreak – displaying TYPICAL symptoms only.

Question	I	L	M	U
Crops affected	NS	AYR pot	AYR, spot & N.S.	N.S.
Extent of area affected (m^2)	-	2400	15739	660 of 1,500
Start of outbreak	-	04/02	05/01	09/01
End of outbreak	-	08/02	Ongoing	11/01
Where did the outbreak first appear	Mature	Young crop	Mature	Mature
Cvs most affected	-	Princess Anne vars	Reagan AYR Creamist Nat season	Peninine pink – 4 colours Claudia + Red Claudia White Gernie Hock/Enbe wedding 5 colours
Suspected source of infection	Infected cuttings	Infected cuttings	Prev. hist. & infected cuttings	Infected cuttings

Question 5. Extent of *Verticillium* wilt outbreak – displaying ATYPICAL symptoms only

Question	O	R	S	V	W	X
Crops affected	AYR & N.S.	AYR	AYR	AYR	N.S.	AYR
Extent of area affected (m^2)	30,000	75% of 15,000	Variable of 26,500	26% of 24,600	-	90% of 27,192
Start of outbreak	02	7/01	Spring 01	6/01	Historic	11/00
End of outbreak	02	11/01	Autumn 02	9/01		Ongoing
Where did the outbreak first appear	Mature	Mature	Mature	Mature	Mature	Mature
Cvs most affected	-	Reagans (esp. orange) Fresco	Reagans (esp white)	Reagans (esp. white)	Ryland Gem	Reagans
Suspected source of infection	Infected cuttings	Infected cuttings	Infected cuttings	Infected cuttings	Historic	Infected cuttings

Question 5. Extent of *Verticillium* wilt outbreak – both TYPICAL and ATYPICAL symptoms.

Question	N	Q	T	Y
Crops affected	AYR	AYR	N.S.	AYR
Extent of area affected (m^2)	14,101	20% of 22,000	10% of 208	20,000
Start of outbreak	5/02	Historic	Historic	1998
End of outbreak	8/02	8/02	-	2001
Where did the outbreak first appear	Mature	Young & mature	Mature	Mature
Cvs most affected	Le Mans, Herby Biarritz	Calabria & Reagans	Shoesmith salmon family & Sprays	Reagans
Suspected source of infection	Previous history for typical. Infected cuttings atypical.	Previous history typical. Infected cuttings for atypical.	Historic	Historic for typical. Infected cuttings for atypical.

Question 6. Details of cultural practices in general and in response to a *Verticillium* outbreak with respect to soil cultivation, soil sterilisation and crop hygiene. (Premise code in upper case letters).

SOIL CULTIVATION

Key to soil cultivation: a tractor rotavator; b pedestrian rotavator; c spader - between every crop; d spader – occasionally/infrequently; e sub-soiler - between every crop; f sub-soiler - occasionally/infrequently; g other

Details of cultural practices of cut flower growers reporting to be free from *Verticillium*

Year	A	B	E	F	G	K
Prior to 1999	b,f	a,b	a	a	a,d	b,f
1999	b	a,b	a	a	a,d	b,f
2000	b,d	a,b	a	a	a,d	b,f
2001	b	a,b	a	a	a,d	b,f
2002	b,d	a,b	a	a	a,d	b,f

Details of cultural practices of cut flower growers reporting *Verticillium*. (atypical [a], typical [t]).

Year	I	M	U	O	R	S	V	W	X	N	Q	T	Y
	t	t	t	a	a	a	a	a	a	a,t	a,t	a,t	a,t
prior to 1999	a	a,e	a	b,c	a	b	a	b	b,d,e,g	b	e,d,b	b	-
1999	a	a,e	a	b,c	a	b	b	b	b,d,e,g	b	e,d,b	b	a
2000	a	a,e	a,f	b,c	a	b	b	b	b,d,e,g	b	e,d,b	b	a,e
2001	a	a,e	a	b,c	a	b	b	b	c,b	b	e,d,b	b	a,e
2002	a	a,e	-	b,c	a	b	b	b	b	d	e,d,b	b	a,e

Response of growers to an outbreak of *Verticillium*. (atypical [a], typical [t]).

Year	I	M	U	O	R	S	V	W	X	N	Q	T	Y
	t	t	t	a	a	a	a	a	a	a,t	a,t	a,t	a,t
prior to 1999	-	-	-	-	-	d	-	-	-	-	-	-	-
1999	-	-	-	-	-	d	-	-	-	-	-	-	-
2000	-	-	-	-	-	f	d	-	-	-	-	-	-
2001	-	-	-	-	-	f	d	-	-	-	-	-	-
2002	-	-	-	-	-	-	d	-	-	-	-	-	-

SOIL STERILISATION

Key to soil sterilisation: 1. Steam sterilisation after every crop; 2. Steam sterilisation – 2 times per year; 3. Steam sterilisation – annually; 4. Steam sterilisation every other year; 5. Methyl bromide – annually; 6. MeBr – every other year; 7. MeBr – when pest or disease dictate;8. No sterilisation treatment; 9. Basamid, 10. Revive,.

Details of cultural practices of cut flower growers reporting to be free from *Verticillium*

Year	A	B	E	F	G	K
Prior to 1999	2	9	9	3	-	4 + 7
1999	2	8	9	3	6	4 + 7
2000	3	8	9	3	-	4 + 7
2001	3	9	9	3	6	4 + 7
2002	3	8	9	3	-	4 + 7

Details of cultural practices of cut flower growers reporting *Verticillium*. (atypical [a], typical [t]).

Year	I	M	U	O	R	S	V	W	X	N	Q	T	Y
	t	t	t	a	a	a	a	a	a	a,t	a,t	a,t	a,t
Prior to 1999	9*+3‡	7	8	3	2	6	8	8* + 2	4	8	3	5	8
1999	9*	7	9	3	2	4	8	8* + 2	4	8	3	5	8
2000	9*	7	8	3	2	4	7	8* + 2	4	8	3	9	8
2001	9*	4	8	2	2	4	7	8* + 2	5	8	3	9	8
2002	9*	4	8	3	1	4 + 2	8	8* + 2	5	7	3	9	8

Response to outbreak of *Verticillium* outbreak (atypical [a], typical [t]).

Year	I	M	U	O	R	S	V	W	X	N	Q	T	Y
	t	t	T	a	a	a	a	a	a	a,t	a,t	a,t	a,t
Prior to 1999	-	-	-	-	-	-	-	-	-	-	1	10*	-
1999	-	-	-	-	-	-	-	-	-	-	1	10	-
2000	-	-	-	-	-	-	-	-	-	-	1	10	-
2001	-	-	9	-	1 + 7	-	-	-	-	-	1	10	-
2002	-	-	-	-	-	2	-	-	4 + 1	4	1	10	-

* Outdoor cultivation

‡ Under glass

CROP HYGIENE

Key to crop hygiene: I. Blocks/haulms routinely removed after every crop; II Blocks/haulms removed seasonally; III Blocks/haulms not removed; IV Rouging/destruction of infected plants; V Movement restrictions on personnel; VI Movement restrictions on equipment; VII Sterilisation of equipment; VIII Other.

Details of cultural practices of cut flower growers reporting to be free from *Verticillium*

Year	A	B	E	F	G	K
Prior to 1999	III	I + III	I	III	I	III
1999	III	I + III	I	III	I	III
2000	I	I + III	I	III	I	III
2001	I	I + III	I	III	I	III
2002	I	I + III	I	III	I	III

Details of cultivation practices of cut flower growers reporting *Verticillium* (atypical [a], typical [t]).

Year	I	M	U	O	R	S	V	W	X	N	Q	T	Y
	t	t	t	a	a	a	A	a	a	a,t	a,t	a,t	a,t
Prior to 1999	IV	III	I	III	III	II + IV	III	II	II	III + IV	III	III	III
1999	IV	III	I	III	III	II + IV	III	II	III	III + IV	III	II	III
2000	IV	III	I	III	III	II + IV	II	II	III	III + IV	III	II	II + IV
2001	IV	III	I	III	III	II, IV + VI	I	II		III + IV	III	II	II + IV
2002	IV	III	I	III	III	II, IV + VI	I	II		III + IV	I	II	II + IV

Response to outbreak of *Verticillium* (atypical [a], typical [t]).

Year	I	M	U	O	R	S	V	W	X	N	Q	T	Y
	t	t	t	a	a	a	a	a	a	a,t	a,t	a,t	a,t
Prior to 1999	-	-	-	-	-	-	-	-	-	IV	-	IV	-
1999	-	-	-	-	-	-	-	-	-	IV	-	IV	-
2000	-	-	-	-	-	-	I	-	-	IV	-	IV	-
2001	-	-	V, VI + VII	-	IV + VI	-	I	-	I, V, VI + VII	IV	-	IV	-
2002	-	-	-	-	-	-	I	-	I, V, VI + VII	I	-	IV	-

Question 7. If you took actions to counteract the *Verticillium* outbreak were they successful?
Yes – I, M, N, O, Q, R, S, V
No – T, W, X, Y
Stopped production of chrysanthemum U

If No, can you give reasons why not.
T- Whatever we try has little effect.
W- Almost impossible to clear from outdoor land.
X- Sterilisation temperature and time was not to blame, poor distribution of the sterilant, inappropriate soil condition and moisture may be a problem, deep cultivation and poor hygiene were suspected. Reintroduction on infected material was not suspected.
Y- Inappropriate soil condition (lack of body and fibre); reintroduction of infected plant material (sometimes not enough time to remove debris).