FINAL REPORT

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HDC Project PC 253

Verticillium wilt of chrysanthemums – protecting UK growers by ensuring a safe propagation chain

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Commercial - In Confidence

Grower Summary

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Project Title	PC 253
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Signed on behalf of: **Warwick HRI**

Signature*:**……Date*:** 14 May 2007

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Deputy Director

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

- Following the establishment of an effective inoculation method, preliminary results suggest that isolates of *Verticillium dahliae* from other hosts are either less able or unable to infect chrysanthemums. This means that new infections are more likely to arise by spread from pre-existing infections of chrysanthemums.

Background and expected deliverables

Verticillium dahliae is a typical soil-borne pathogen which in nature persists in soils, either free or on plant debris, as microsclerotia (long-lived melanised resting structures). Microsclerotia are usually produced as the plant dies and the disease cycle is completed when these are returned to the soil with plant debris. Fungicides are generally ineffective on infected plants and for glasshouse crops the main controls are preventing the disease from entering houses or soil-treatments to remove existing infestations.

Verticillium dahliae has long been known as a potential problem of chrysanthemums. The 'typical' form of the disease could occur at any stage of the crop with clear foliar symptoms. An 'atypical' form of *Verticillium* wilt is known to have appeared in chrysanthemums by 2001/2 but may have been present some years earlier (T Pettitt, *pers .comm*.). This new 'atypical' disease primarily appears towards the end of the crop and is generally restricted to flower symptoms (see HDC information sheet 02/02 for illustrations and further details of the symptoms). This form of the disease became a widespread problem in both the UK and the Netherlands.

Initial infections are thought to be normally introduced into growers' glasshouses on infected cuttings (but possibly in some unknown way from other crops) but may persist in some houses due to inadequate soil pasteurisation. No work on possible carryover in glasshouse soil is being undertaken in this project which is concentrating on the epidemiology of *Verticillium dahliae* in relation to the propagation chain in order to determine the risk of introduction via this route.

At present, *Verticillium dahliae* seems to have been eliminated from material supplied by the major propagators but a greater understanding of the epidemiology of the disease is needed to ensure this situation is maintained, particularly as it is probable that other propagators will begin to supply the UK market. This project aims to provide some of the information

necessary to fully understand *Verticillium dahliae* in chrysanthemums and to provide the guidelines and testing resources necessary to protect British growers by ensuring their supply chain for cuttings is as free from of all forms of *Verticillium dahliae* as is reasonably possible at an economic cost.

An important part of any scheme designed to protect UK growers from introducing *Verticillium dahliae* is a rapid and effective screening service that may be used to monitor the scheme. Traditionally, *Verticillium* infections have been detected through various forms of plating assays. These can be very cost-effective (and will be used where appropriate in this project) but may lead to some incorrect diagnoses and, perhaps more importantly, are not suitable for testing multiple plants as single samples. An effective and sensitive laboratory technique (PCR assay) is available for *Verticillium dahliae* but has not been validated for use on chrysanthemums.

Summary of the project and main conclusions

Four objectives were to be addressed in whole or part in the first year

1. To find an effective inoculation method for the inoculation of chrysanthemums with *Verticillium dahliae*
2. Examine the propagation chain for possible sources of infection.
3. Determine the susceptibility of chrysanthemums to isolates from other hosts.
4. Determine the rate of transmission to cuttings.

Progress to date

1. Several methods of inoculation were compared and a relatively simple-to-perform procedure called ‘drench and damage’ was chosen as the most appropriate for this project. It has the disadvantage that it does not mimic the natural infection process but the more natural system was not very effective and ’drench and damage‘ is efficient whilst allowing the level of challenge‘ to the plants to be varied.
2. This objective has been deferred until year 2 of the project (subject to funding approval).
3. Only one experiment has been carried out so far. This showed a clear division between seven isolates from UK/Netherlands (originally from hop, strawberry and direct from soil) along with five chrysanthemum, of which all but one infected chrysanthemum, and nine from southern Europe, USA and the tropics, none of which infected chrysanthemums. This suggests that infections are not getting into the crop directly from other hosts in areas where cuttings for commercial sale are produced. Similarly, a generally lower pathogenicity of non-chrysanthemum isolates for this host

suggests that even in north-west Europe most infection of new stocks are due to spread from pre-existing infections in the crop. This is in agreement with results from a previous project (PC 195) which showed the now prevalent 'atypical disease' isolates mainly fell into a tight cluster suggesting a discrete 'chrysanthemum population' circulating within the crop.

Financial benefits

The financial benefits of this project to growers will be determined by the number of outbreaks of Verticillium avoided and this obviously cannot be determined directly. The success of the project will be measured in the ability of growers to buy from all sources with confidence which in turn will be determined by the uptake and application of the project's findings.

Action points for growers

- None at this stage.

Science Section

Introduction

Verticillium dahliae is a typical soil-borne pathogen which in nature persists in soils, either free or on plant debris, as microsclerotia (long-lived melanised resting structures). Microsclerotia are usually produced as the plant dies and the disease cycle is completed when these are returned to the soil with plant debris. Fungicides are generally ineffective on infected plants and for glasshouse crops the main controls are preventing the disease from entering houses or soil-treatments to remove existing infestations.

Verticillium dahliae has long been known as a potential problem of chrysanthemums. The 'typical' form of the disease could occur at any stage of the crop with clear foliar symptoms. An 'atypical' form of *Verticillium* wilt is known to have appeared in chrysanthemums by 2001/2 but may have been present some years earlier (T Pettitt, pers .comm.). This new 'atypical' disease primarily appears towards the end of the crop and is generally restricted to flower symptoms (see HDC information sheet 02/02 for illustrations and further details of the symptoms). This form of the disease became a widespread problem in both the UK and the Netherlands.

An earlier HDC project, (PC195: *Chrysanthemums: to investigate new symptoms of Verticillium wilt and determine pathogen variation*) looked into whether a distinct type of *Verticillium dahliae* was associated with the problem. This was hampered by very few isolates associated with symptoms of the "typical" type being available but did not support the idea that a distinct new type of chrysanthemum isolate was causing delayed symptoms. In a separate collaboration with the Netherlands some evidence for host specificity was found (Dutch potato isolates were non-pathogenic on chrysanthemum) but there was no evidence that repeated steaming had selected for particularly heat tolerant chrysanthemum isolates (Ispahami *et al.*, submitted for publication). The Dutch have previously found evidence that fixed steam pipe layouts may not give effective destruction of *Verticillium* microsclerotia, particularly below the level of pipes (Termorshuizen, pers comm.).

Initial infections are thought to be normally introduced into growers' glasshouses on infected cuttings (but possibly in some unknown way from other crops) but may persist in some houses due to inadequate soil pasteurisation. No work on possible carryover in glasshouse soil is being undertaken in this project which is concentrating on the epidemiology of *Verticillium dahliae* in relation to the propagation chain in order to determine the risk of introduction via this route.

At present, *Verticillium dahliae* seems to have been eliminated from material supplied by the major propagators but a greater understanding of the epidemiology of the disease is needed to ensure this situation is maintained, particularly as it is probable that other propagators will begin to supply the UK market. This project aims to provide some of the information necessary to fully understand *V. dahliae* in chrysanthemums and to provide the guidelines and testing resources necessary to protect British growers by ensuring their supply chain for cuttings is as free from of all forms of *Verticillium dahliae* as is reasonably possible at an economic cost.

An important part of any scheme designed to protect UK growers from introducing *V. dahliae* is a rapid and effective screening service that may be used to monitor the scheme. Traditionally, *Verticillium* infections have been detected through various forms of plating assays. These can be very cost-effective (and will be used where appropriate in this project) but may lead to some incorrect diagnoses and, perhaps more importantly, are not suitable for testing multiple plants as single samples. An effective and sensitive fluorescent PCR assay, using species-specific primers, is available for *V. dahliae* but has not been validated for use on chrysanthemums.

Four objectives were to be addressed in whole or part in the first year.

1. To find an effective inoculation method for the inoculation of chrysanthemums with *V. dahliae*

Several methods have been used to inoculate plants with *V. dahliae* but not all are suitable for use with all species. The major objective for the first year was to develop a method which gave efficient infection but using as few propagules as possible; this is important as the severity of symptoms and/or transmission rates in cuttings is likely to be affected by the level of initial infection.

2. Examine the propagation chain for possible sources of infection.
3. Determine the susceptibility of chrysanthemums to isolates from other hosts.

How new infections arise in chrysanthemum stock is an important aspect of control. Do they mainly arise by transmission from existing infections in other stocks or are they entering the crop for the first time from other hosts (either crops or weeds)?

4. Determine the rate of transmission to cuttings.

The degree of testing required in any possible certification scheme is dependent on the rate of transmission to cuttings from stock plants. If the rate is low then detection in material will require exhaustive testing but if wherever infection occurs transmission to cuttings is

frequent then only small numbers of plants need testing if the sampling regime is well designed.

Materials and Methods

Objective 1. To find an effective inoculation method for the inoculation of chrysanthemums with V. dahliae.

Five methods were tested with methods i) & ii) being difficult to quantify the disease challenge but relatively natural whilst methods iii) – v) are easy to quantify the challenge presented to the plants but are relatively unnatural methods because they primarily involve conidia (asexual non-melanised spores important for spread within infected plants but not normally involved in natural spread between plants).

All inoculum production was carried out at approximately 20ºC. Primarily microsclerotial inoculum was prepared by inoculating oat seed which had been soaked and sterilized in 500 g glass jars with *V. dahliae* and leaving to grow for 2-3 weeks after which time large numbers of microsclerotia had formed on the seed. For inoculating plants, this was incorporated into compost at different rates (see below) and rooted cuttings planted into the amended compost. Mixed microsclerotial and mycelium/conidial inoculum on agar plated was grown on standard agar plates (of prune lactose yeast agar). After two weeks, each 9 cm agar disk was cut into quarters and a quarter of a disc placed on some compost in a pot such that a rooted cutting could be place sitting on the agar during transplanting. Conidial suspension was prepared by growing shake cultures (in prune lactose yeast broth) for approximately two weeks. Plants were inoculated with conidial suspension by three methods (i) drenching compost around plants with 10 ml of inoculum (ii) as *(i)* but previously giving minor damage to the roots by plunging a pencil into the compost ca 2cm from the main stem in two places and pouring the inoculum into the holes (iii) washing the compost from the roots of plants, further damaging them by trimming the roots to 2-3 cm, soaking in an excess of inoculum for 5 minutes then repotting

The five methods are referred to as

i) infested oats
ii) agar culture
iii) soil drench
iv) drench and damage
v) root dipping

Plants were inoculated at approximately 20 days after potting the rooted cuttings. Inoculated plants were observed for symptoms for 6 to 8 weeks then isolations were made by surface sterilization of petioles from two leaves low on the plant (as determined to be the

most likely infected in an initial experiment where multiple leaves and stem segments were tested), rinsing in sterile distilled water and plating onto water agar plates. Petioles were considered positive if mycelium was seen to grow from the tissue (usually primarily from the cut ends) and microsclerotia were produced, either by the mycelium on the agar or in the tissue. Plates were examined at 10-14 days using a low power binocular microscope.

All experiments used cv EuroSpeedy and some also included Kingfisher. After a preliminary experiment, three *V. dahliae* isolates were used in the main experiments *viz*. 52 (old UK - from a plant with "typical" disease), 60 (new UK - from a plant with "atypical" disease) and 74 (old Dutch).

Objective 3. Determine the susceptibility of chrysanthemums to isolates from other hosts.

Plants (five or 10/isolate) were inoculated by drench and damage (iv above) using "drench and damage" with ca. 5 x 10^5 spores/ml

Results and Discussion

Progress to date

Objective 1. To find an effective inoculation method for the inoculation of chrysanthemums with *V. dahliae*

Five methods were tested with methods i) & ii) being difficult to quantify the disease challenge but relatively natural whilst methods iii) – v) are easy to quantify the challenge presented to the plants but are relatively unnatural methods because they primarily involve conidia (asexual non-melanised spores normally important for spread within infected plants but not involved in natural spread between plants).

After three experiments (see table 1 for illustrative results from the first two experiments which were the most comprehensive for comparing methods; selection of the three isolates used here was based on a preliminary experiment) it was clear that "drench and damage" was the most practical, controllable form of inoculation and will be adopted for the rest of the project. A spore concentration of 5.10^5 /ml with 10 mls per pot seemed a good compromise between efficient, reproducible infection and ease of producing sufficient conidial suspension for larger experiments

"Root dipping" was more effective than drench and damage in that lower levels of inoculum could be used to get a similar level of infection but involved much more work at the point of inoculation (washing roots free of compost, cutting them and dipping into inoculum,

repotting) and severely damaged the plants, checking their growth after repotting. There was no gain in “naturalness” as both involved inoculation with conidial suspensions.

“Drench” without damage was less efficient and required such high level of spores that producing them was much more and so despite the ease carrying out of the actual inoculation process and lack of plant damage, this method was not used.

Inoculation with infested oats was very inefficient and required the addition of a large proportion of the infested oats to the compost (30% by volume) to get even moderate levels of infection. The results were also irreproducible. Although relatively natural in involving at least partly microsclerotial inoculum, the inefficiency and poor reproducibility meant this method was not adopted.

Placing infested agar at the bottom of pots suffered similar deficiencies to the infested oats and although easy to carry out was not adopted.for these reasons.

Overall Kingfisher seemed slightly more resistant to infection than Eurospeedy but whether this difference is biologically significant is not known.

Typical verticillium symptoms of leaf yellowing and senescence, and some height reduction were seen to varying degrees in infected plants. In a preliminary assessment, all types of symptoms were proportional to the strength of challenge (i.e. higher spore numbers gave more severe symptoms, more quickly). However, at this stage the plants are being grown in small pots and the symptoms seen an acute response to infection; as such they are unlikely to be typical of chronically infected plants or plants infected by a low level challenge and grown in open beds.

	Variety		Isolates	Isolates	
Method		Spore Concentration	52 (old UK)	60 (new UK)	74 (old Dutch)
Expt 1.					
Oats mixed at 1:3	EuroSpeedy	n/a	2/2	0/2	0/2
1:10	EuroSpeedy	n/a	10/10	1/10	2/10
Spore Drench	EuroSpeedy	5.10^6 / ml (10 mls used)	nd	1/10	nd

Drench & Damage	EuroSpeedy	5.10^5 / ml (10 mls used)	10/10	0/10	10/10
Root Dip	EuroSpeedy	1.10^3 / ml	10/10	0/10	9/10
Expt. 2					
Oats mixed at 1:10	EuroSpeedy	n/a	0/10	nd	nd
	Kingfisher	n/a	0/10	nd	nd
1:30	EuroSpeedy	n/a	0/10	nd	nd
	Kingfisher	n/a	0/10	nd	nd
Agar	EuroSpeedy	n/a	10/10	nd	5/10
	Kingfisher	n/a	5/10	nd	4/10
Spore Drench	EuroSpeedy	5.10^6 / ml (10 mls used)	0/10	nd	nd
Drench & Damage	EuroSpeedy	2.10^6 / ml	10/10	nd	8/10
	Kingfisher	(10 mls used)	7/10	nd	10/10
	EuroSpeedy	2.10^5 / ml	5/10	nd	10/10
	Kingfisher	(10 mls used)	3/10	nd	10/10
	EuroSpeedy	2.10^4 / ml	4/10	nd	10/10
	Kingfisher	(10 mls used)	3/10	nd	0/10

Table 1. Results for isolation onto water agar from chrysanthemum plants of two varieties inoculated using three isolates and five different methods. Not all combinations were done in any single experiment ("nd" in table).

Objective 2. Examine the propagation chain for possible sources of infection.

This objective was deferred until the continuation of this project into its second year had been confirmed.

Objective 3. Determine the susceptibility of chrysanthemums to isolates from other hosts.

In the first year one experiment has been carried out for this objective. In addition to the three isolates used previously, a further 18 isolates (Table 2) were used to inoculate either five or 10 plants of Euro Speedy. Infection was assessed by isolation at 39 days. Eight of these new isolates infected chrysanthemum (Table 2) including all the isolates from north-west European temperate crops or soil except one. None of the isolates from southern Europe, California or the tropics infected these plants in this experiment. Taking proportion of plants infected as a measure of virulence, the chrysanthemum and soil isolates were broadly more virulent than were the isolates from other hosts (Table 2).

Identifier	Original Host	Country of Origin	Disease Type (Chrysanth isolates)	Plants infected/ tested
UK/Holland				
12079	Soil*	U.K.	-	7/10
12085	Soil*	UK	-	7/10
210	Chrysanthemum	Holland	Atypical	9/10
211	Chrysanthemum	Holland	Atypical	7/10
2341	Hop	UK	-	4/10
321	Strawberry	UK	-	1/5
332	Strawberry	UK	-	3/10
DC36	Hop	UK	-	0/5
DC59	Hop	UK	-	5/10
Rest of world				
318	Aubergine	Brazil	-	0/5
3440	Aubergine	Brazil	-	0/5
Ca63	Bell Pepper	USA (California)	-	0/10
Ca156	Bell Pepper	USA (California)	-	0/10
Ep1	Eggplant	Spain	-	0/10
Ep47	Eggplant	Spain	-	0/10
P14	Tomato	Brazil	-	0/5
IMI	Mint	Zimbabwe	-	0/5
V008	Artichoke	Spain	-	0/5
Previous isolates				
52	Chrysanthemum	UK	Typical	3/5
60	Chrysanthemum	UK	Atypical	3/5
74	Chrysanthemum	Holland	Typical	4/5
Control (water)				0/10

Table 2. Identity and results of isolations for 18 further isolates used to inoculate EuroSpeedy plants. All controls were uniformly free of infection. “Previous isolates” are those used in earlier experiments

Although this is only a preliminary result and further isolates need testing, these results suggest that stock plants/cuttings are not likely to be becoming newly infected during the production of cuttings in warmer climates with isolates occurring in local crops or weeds. As chrysanthemum isolates seem more virulent than other isolates it is also likely that infections of new varieties/stocks are due mainly to spread from already infected chrysanthemums, but whether this occurs in temperate or tropical situations is not known.

Spread primarily from already infected chrysanthemums is in agreement with an earlier finding (HDC project PC 195) that “atypical disease” isolates formed a tight cluster by amplified fragment length polymorphisms (AFLPs), suggesting within crop spread.

Objective 4. Determine the rate of transmission to cuttings.

Experimental work for this objective is mainly set to take place in years 2 & 3. Preliminary work during the first year established that symptom-free side and basal shoots are formed on plants confirmed as infected with *V. dahliae* by isolation on two occasions. Some of this material was also confirmed to be infected by isolation but this was not done in a formally designed experiment to measure the rate, merely as a preliminary test of whether transmission in symptomless cuttings is possible and to support the greater effort to be put into this objective in years 2 & 3 as originally planned.

Objective 5. Effect of date of isolation on virulence (additional task).

Taken over all the inoculation experiments (see 1), there seemed to be a trend for new isolates to be less virulent than older ones, possibly correlating with a typical/atypical disease split. This was unexpected as isolates of *V. dahliae* are normally expected to lose virulence when kept *in vitro* due to random mutations, meaning that older isolates often become non-infectious if not regularly "refreshed" by maintaining them in plants.

However, as the experiments were not designed to test this we cannot present stringent evidence of this and a separate experiment was set up to directly address this possible difference in virulence which may be an important epidemiological factor in the milder "atypical" form of the disease. In this experiment 10 plants of Eurospeedy were inoculated with 11 isolates (3 old Dutch, 3 new Dutch, 4 new UK and the sole old UK available to us) as were 8 plants each of Tomato (cv Santa). Tomato was included as a "universally susceptible" host to ensure that isolates were still generally competent at infection and hadn't just generally lost virulence.

This experiment took place in mid winter and no symptoms were seen even after eight weeks, by which time they were starting to senesce due to the small pots size used (intended only to keep the plants healthy for 6 weeks). As *V. dahliae* naturally switches to a necrotrophic stage in senescent tissue, it was felt that attempting to isolate from this experiment would not give a good reflection of the levels of initial infection and the experiment was abandoned (although a few trial isolations did suggest that many plants were infected.

This experiment will be repeated.

Objective 6. Confirmation of the presence of V. dahliae in an outbreak in a commercial house (additional task).

Plants with and without symptoms of "atypical" wilt were received from a probable outbreak in a commercial house. All four plants with symptoms and one of the four without symptoms tested gave fungal cultures forming typical microsclerotia on the tissue 14 days after plating onto water agar. This confirms that the outbreak was of *V. dahliae*.

Conclusions

- A suitable method for inoculating plants was chosen from among five tested. This gave good levels of infection reproducibly but was optimized to use a low as reasonably possible level of conidia to prevent massive infection and rapid death of plants. This method was simple and suitable for all the subsequent experiments planned. Using a low level of inoculum is presumed to mimic natural infection and therefore give infected which behave (in terms of transmission into cuttings) reasonably like natural infections.
- The general inability of isolates obtained from countries other than the UK and the Netherlands to infect chrysanthemums and that chrysanthemum isolates are seemingly more virulent for this host than other isolates suggests that new infections (i.e. primary infections of stock plants) are likely to be coming from pre-existing infections in chrysanthemums. This is agreement with an earlier finding (HDC project PC 195) that “atypical disease” isolates formed a tight cluster by AFLPs suggesting within crop spread. Whether spread is occurring during the early stages of propagation or later is not clear.

Technology transfer

A presentation was made to the Chrysanthemum Growers Association research committee but the results are not yet sufficient for further technology transfer.