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Host specificity, but not high-temperature tolerance, is associated with recent outbreaks of *Verticillium dahliae* in chrysanthemum in the Netherlands

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Abstract Two hypotheses which might explain a recent increase in the incidence of verticillium wilt of chrysanthemums in glasshouses in the Netherlands were investigated, *viz* whether selection for increased resistance to elevated temperatures has occurred due to frequent steaming of soils in the glasshouses, or whether the strains of *Verticillium dahliae* occurring in chrysanthemum glasshouses are particularly virulent towards this host. Following artificial inoculation, five isolates of *V. dahliae* from chrysanthemum were pathogenic on chrysanthemum but five isolates from potato were non-pathogenic for this host. When inoculated onto potato plants, all isolates caused early senescence with no significant difference between the two groups of isolates. In amplified fragment length polymorphism analysis, the potato isolates formed a cluster distinct from all other isolates. As a group the chrysanthemum isolates were no more diverse than the potato isolates but did not form a cluster distinct from 12 other isolates tested. This suggests that high pathogenicity to chrysanthemum has developed on several occasions but that the group of potato isolates were possibly monophyletic. Microsclerotia produced in vitro from the chrysanthemum isolates had significantly lower average lethal temperature tolerance than those from the five potato isolates suggesting that being able to resist the effects of soil sterilisation by steam is not a factor in wilt of chrysanthemums in the Netherlands.

Keywords Microsclerotia · Host specificity · Temperature tolerance

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Verticillium dahliae is a widespread soil-borne plant pathogen causing wilt in a broad range of crops (Pegg and Brady 2002). In general, haploid *V. dahliae* isolates have been viewed as not being host-specific (Strausbaugh 1993), although specific pathotypes or host specialisation have been reported for some crops e.g. cotton (Ashworth 1983), peppermint (Horner 1954), bell pepper and eggplant (Bhat and Subbarao 1999) and these may be correlated with molecular properties e.g. Japanese pepper and tomato pathotypes (Carder and Barbara 1994). However, isolates from cruciferous hosts, once seen as a very clear case of host-specialisation in '*V. dahliae*', have been shown to be genetically quite distinct from the majority of

isolates, being amphihaploid interspecific hybrids (Barbara and Clewes 2003), and may be best thought of as a distinct species (Karapapa et al. 1997).

In the Netherlands, verticillium wilt is common in glasshouses, significantly affecting rose, eggplant and chrysanthemum. During the last 5 to 10 years, anecdotal reports by growers have suggested an increase in the incidence and severity of verticillium wilt, not only in the Netherlands, but also in the UK (personal communication, Dr C. Lane, Central Science Laboratories, York, UK). To manage verticillium wilt, glasshouse soils are usually steamed. This kills microsclerotia, the survival structures of *V. dahliae*, wherever the temperature reaches 40–47.5°C for at least 30 min (Bollen 1985). There are two obvious reasons as to why verticillium wilt might reoccur in a glasshouse following steaming. Firstly, chrysanthemums root deeply into the soil and microsclerotia occurring below the treated layer of soil may escape the steaming process. Secondly, infection can be reintroduced, either on fresh chrysanthemum planting material or from surrounding soil or crops. In the Netherlands, *V. dahliae* occurs in most agricultural soils, mainly because most soils have a potato cropping history. During a potato crop, large numbers of *V. dahliae* microsclerotia are formed on the senescing potato stems and petioles (Mol et al. 1996) and it has been hypothesized that potato has been important in the history of most Dutch isolates of *V. dahliae*, including ones isolated from other crops.

It was thought that the escalating problem of verticillium wilt in glasshouse-grown chrysanthemums might be associated with either (1) selection for increased temperature resistance in the microsclerotia as a result of many cycles of steam treatment, or (2) enhanced pathogenicity of the *V. dahliae* isolates resulting from selection imposed by the continuous cultivation of a single host. This paper reports some experiments intended to differentiate these two hypotheses.

Ten *V. dahliae* isolates were collected from diseased chrysanthemum and potato plants from various parts of the Netherlands (Table 1). Potato isolates were chosen for comparison with the chrysanthemum isolates because, as noted above, it is thought that potatoes have been important hosts for most Dutch isolates of *V. dahliae* obtained from outside glasshouses. Pure cultures were prepared by incubating an infected stem for 1 day on wet filter paper and touching a conidiophore of *V. dahliae* with a sterile needle and transferring spores to potato dextrose agar (PDA Oxoid) plates. Chrysanthemum isolates were single-spored shortly before the experiments. Potato isolates were stored as spore suspensions in glycerol at −80°C using the Microbank® Bacterial and Fungal Preservation System (Pro-Lab Diagnostic Inc., Ontario, Canada) until the start of the experiments.

Temperature sensitivity of microsclerotia was determined using material produced on cellophane according to the procedure described by Francl et al. (1987). Microsclerotia and mycelium were scraped off the cellophane and washed through 106 and 20 μm nested sieves. Microsclerotia retained on the latter sieve were used. Approximately 300 microsclerotia per isolate were placed into microfuge tubes containing 0.5 ml tap water. Using water baths at 25°C, 40°C, 45°C and 50°C (all±1°C), microsclerotia samples for all isolates were simultaneously exposed to each temperature for 30 min. Temperature in each bath was monitored continuously using thermocou-

Table 1 Origin of isolates of *V. dahliae* from the Netherlands used in the pathogenicity and temperature-tolerance studies

Isolate code	Host	Location (town, province)	Isolation date
M	Chrysanthemum	Made, Brabant	April 05, 2002
S	Chrysanthemum	Honselersdijk, Zuid-Holland	April 15, 2002
D	Chrysanthemum	Naaldwijk, Zuid-Holland	April 15, 2002
U	Chrysanthemum	Maasland, Zuid-Holland	April 15, 2002
V	Chrysanthemum	the Hague, Zuid-Holland	April 15, 2002
JKG1	Potato	Wageningen, Gelderland	September 01, 2000
JKG5	Potato	America, Limburg	September 04, 2000
JKG6	Potato	Heide, Limburg	September 04, 2000
JKG8	Potato	Schore, Zeeland	September 22, 2000
JKG9	Potato	Achterberg, Utrecht	September 22, 2000

ples and a data logger. After treatment, the microsclerotia were spread on sterile filter paper in a laminar airflow cabinet and for each treatment 50 placed individually using a fine syringe needle onto modified soil extract agar plates (Harris et al. 1993) amended with 50 ppm oxytetracyclin. Plated microsclerotia were incubated at 20°C for 7 days and the number germinated scored.

Rooted cuttings (4 week-old) of *Chrysanthemum coronarium* cv. Gold-Peas were kindly provided by Fides–Straathof (Maasdijk, The Netherlands). Homogeneous potato plants (*Solanum tuberosum* cv. Bintje) were obtained by excising eyes from potato tubers with a round scalpel and growing these for 1 week in perlite on Hoagland's solution, followed by 2 weeks in potting compost. For each of the ten isolates, a plug of mycelium from an actively growing PDA-culture was transferred to Czapek Dox liquid medium (Oxoid). After incubating for 1 week at 20°C with shaking (100 rpm), the culture was filtered through cheesecloth to remove the mycelium. The resulting conidial suspensions were adjusted to 1×10^6 conidia ml^{-1} and for each isolate eight chrysanthemum and eight potato plants were inoculated by dipping washed roots into the suspensions for 2 min. Roots of 20 plants of each species were dipped into sterile water as controls. Immediately after inoculation, each plant was potted into compost in a 10 cm plastic pot and placed in a randomised layout in a controlled environment chamber at 20°C with a 16/8 h light/dark regime. Disease severity was scored weekly. For chrysanthemum plants the disease scale used was: 0, no symptoms; 1, ≤7 leaves showing verticillium symptoms; 2, between 7 leaves and 50% of the plant showing symptoms; 3, majority of the plant showing symptoms; 4, few leaves (usually at the top) without symptoms; 5, dead plant. For potato plants the senescence scale used was as follows: 0, no necrosis; 1, 1–5% of plant showing necrosis; 2, 6–25%; 3, 26–75%; 4, 76–95%; 5, 96–100%. The area-under-the-disease-progress-curve (AUDPC) over 80 days was calculated for every chrysanthemum plant and the area-under-the-senescence-progress-curve (AUSPC) over 73 days was calculated for every potato plant (Campbell and Madden 1990). The presence of an effect of host on AUDPC or AUSPC was tested by analysis of variance and pairwise multiple comparisons were carried out with the Tukey test at $P=0.05$ using SAS version 8.0 (SAS Institute, Inc., Cary, NC, USA).

Amplified fragment length polymorphism (AFLP) analysis was used to compare isolates molecularly. A commercial kit (AFLP Analysis System II for small genomes; Invitrogen, Paisley, UK) was used according to the manufacturer's instructions. The primers used were based on earlier work with *V. dahliae*; for pre-amplification, the *Eco*RI primer had no additional nucleotides while the *Mse*I primer had additional C or G bases at the 3′ end. For selective amplification the *Eco*RI primer with one of three pairs of additional bases was used with the *Mse*I pre-amplification primers (*Mse*I + C with *Eco*RI + GA, *Eco*RI + AT or *Eco*RI + GC; *Mse*I + G with *Eco*RI + CC). The *Eco*RI primers for selective amplification were labelled with either Fam or Hex and PCR products were separated on an Applied Biosystems capillary sequencer. Products were sized and analysed using standard software. For comparison, a wider population of *V. dahliae* isolates from chrysanthemums from the UK and the Netherlands were analysed simultaneously. These extra isolates were collected over an extended period as part of other studies and are not detailed here. Prior to AFLP analysis all isolates used were shown to be *V. dahliae* by polymerase chain reaction amplification of the ribosomal RNA internal transcribed spacer/5.8*S* regions, digestion of the products with restriction endonucleases and comparison of the digestion patterns with well-characterised isolates (Collins et al. 2003).

Microsclerotia from all ten isolates were killed by heat treatment at 50°C and all survived after treatment at 25°C. After treatment at 40°C and 45°C, microsclerotia originating from potato plants were significantly ($P<0.05$, *t*-test after arcsine-root transformation of proportion germinated) more heat resistant than those from chrysanthemum plants (Fig. 1). Chrysan-

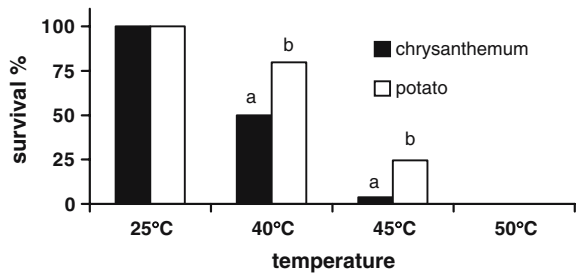

Fig. 1 Survival of *V. dahliae* microsclerotia produced in vitro from five isolates of potato (*open bars*) and chrysanthemum plants (*solid bars*) incubated for 30 min. in water at four temperatures. Within temperature treatments, different letters indicate results which differ significantly at $p<0.05$

themum plants inoculated with chrysanthemum isolates began to show verticillium wilt symptoms from the 17th day onward and eventually showed severe wilt symptoms (Fig. 2). However, chrysanthemum plants inoculated with potato isolates did not show any disease symptoms and were not distinguishable from the control plants at the end of the experiment. All potato plants showed similar AUSPCs irrespective of the origin of the isolate used, and significantly greater senescence than showed by control plants (Fig. 2). AFLP analysis of the tested isolates formed two distinct clusters with comparable levels of genetic diversity within the two (both up to approximately 25%) but with greater diversity between any two isolates across the clusters (38–53%; Table 2). When compared with 12 other chrysanthemum isolates collected over 25 years (six from the Netherlands and six from the UK), the potato isolates still formed a discrete cluster whilst the chrysanthemum isolates used in the other tests here did not (Fig. 3).

Microsclerotia formed by the potato isolates showed significantly higher tolerance of raised temperatures than those formed by the chrysanthemum isolates. As the microsclerotia used for the temperature sensitivity assay were produced in vitro on cellophane and under the same conditions for all isolates, we presume this difference is genetically controlled. At the outset of this work, one of the possible explanations for the upsurge in verticillium wilt in glasshouse-grown chrysanthemums was that steaming of soils in glasshouses over many consecutive years had selected for isolates tolerant of higher temperature. Heat adaptation of isolates of *V. dahliae* has been reported (Castejón-Muñoz and Bollen 1993). In the light of the data presented here, this has not occurred in the chrysanthemum-cultivated glasshouse soils. There is no obvious explanation as to why isolates from potato should exhibit this higher temperature tolerance as open fields, such as are used for potatoes, are generally exposed to lower temperatures than glasshouse soils. It may be that lower than usual tolerance of elevated temperatures has been selected for in the chrysanthemum isolates as a corollary of selection for host specificity (see below), possibly because of some interaction between the two traits. However, it seems more likely that a higher than average temperature-tolerance is associated with the potato isolates, probably as an inadvertent side effect of their monophyletic origin as suggested by the molecular analysis.

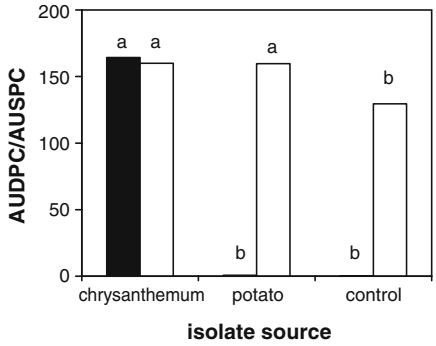

Fig. 2 Area under the disease progress curve (*AUDPC*) for chrysanthemum plants (*solid bars*) and area under the senescence progress curve (*AUSPC*) for potato plants (*open bars*) inoculated with chrysanthemum or potato isolates. Within plant species, different *letters* indicate results which differ significantly at $P<0.05$. The high AUSPC level for control potato plants reflect the natural senescence of the potato plants during the course of the experiment

The isolates of *V. dahliae* tested from potato did not cause any symptoms in chrysanthemum although they caused early senescence on potato plants showing that they had not lost their general ability to cause disease. However, the isolates tested from chrysanthemum were all highly virulent for chrysanthemum plants. Isolates of *V. dahliae* are known to be frequently unstable in culture, often losing pathogenicity and their ability to produce microsclerotia over time in an unpredictable manner. Loss of pathogenicity in culture is usually general in nature and is a natural phenomenon, but it is unlikely that one, let alone all of these five potato cultures, have specifically lost the ability to infect chrysanthemums but retained their ability to infect potatoes whilst at the same time none of the chrysanthemum isolates have lost their ability to infect this host. Moreover, these were all recent isolates at the time of these experiments and had been stored frozen at −80°C, a procedure which minimizes changes in the isolates. These results suggest that some degree of host specialisation of *V. dahliae* for chrysanthemum is associated with infection of this host in glasshouses in the Netherlands. It is too early to say whether this is the sole cause of the recent increase in verticillium wilt as we do not yet know how specialized the isolates present in the crop were prior to the increase.

Although undesirable for growers, development of host-specialisation of *V. dahliae* for chrysanthemum

Table 2 Genetic diversity (%) between five isolates of *V. dahliae* from chrysanthemum and from potato as determined by AFLP analysis

	Potato isolates					Chrysanthemum isolates				
	1	5	6	8	9	M	S	D	U	V
1	–	12.3	19.8	13.4	13.4	39.8	41.3	40.8	49.1	38.1
5		–	25.3	17.2	17.2	43.2	44.7	44.2	52.5	41.5
6			–	18.5	18.5	45.5	47.0	46.4	53.2	43.8
8				–	0.0	39.1	40.6	40.0	48.3	37.4
9					–	39.1	40.6	40.0	48.3	37.4
M						–	12.1	11.5	20.7	15.5
S							–	8.3	12.3	17.4
D								–	17.4	16.4
U									–	26.2

Bands were sized using GeneScan and scored using Genotyper (both Applied Biosystems) and genetic relationships estimated using Treecon (http://bioinformatics.psb.ugent.be) with the similarity coefficient of Nei and Li (1979)

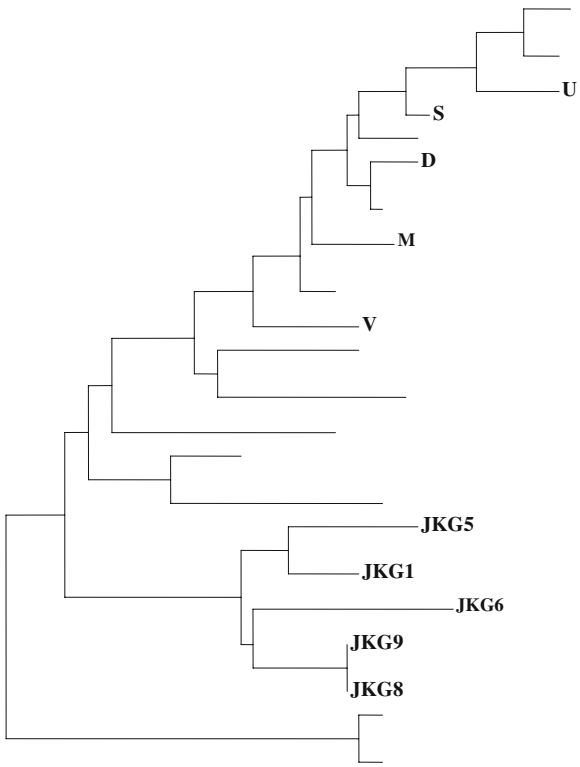

Fig. 3 Schematic representation of the genetic relationship, as determined by AFLP analysis, for five isolates of *V. dahliae* from potato (*JKG1-9*), the five isolates from chrysanthemum used for pathogenicity and temperature sensitivity testing (*D*, *M*, *S*, *U*, *V*) and 12 other isolates from chrysanthemum (six from the UK and six from the Netherlands, *unlabelled branches*). Actual relationships for labelled isolates are shown in Table 2. Cladograms were constructed from the genetic relationships shown in Table 2 using Treecon (http://bioinformatics.psb.ugent.be) and Neighbour Joining

might be circumvented by crop rotation but more information is required before rational disease management plans can be designed on this basis. For example, how quickly a non-specialised isolate (such as one from potato) would become highly pathogenic to chrysanthemum plants is not known, nor is the rate at which chrysanthemum isolates would lose their pathogenicity during serial passage through other hosts. Potentially both processes may be rapid. For example, Fordyce and Green (1963) showed that some mint isolates initially not pathogenic to tomato became so after only one to five passages through tomato, simultaneously losing pathogenicity for mint. Also, it is known that in some crop systems *V. dahliae* isolates show reciprocal high pathogenicity, e.g. cotton and olive isolates from Spain Rodríguez-Jurado et al. (1993).

The host specificity encountered in this study suggests that *V. dahliae* is surviving soil steaming in situ in glasshouses used for chrysanthemum growing, rather than being introduced from unspecialized sources to each new crop. In general, *V. dahliae,* including the isolates from chrysanthemum tested here, has a low survival temperature (Bollen 1985) which is normally easily reached by soil steaming. However, in a deep rooting crop such as chrysanthemum, some microsclerotia may be produced deep in the soil where lethal temperatures are not reached with current techniques. If this is the case, more effective treatments such as more intensive steaming or introduction of a root-impermeable layer at an appropriate depth in the soil will be needed.

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