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Genetic variability and virulence among *Verticillium albo-atrum* isolates from hop

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Abstract

Verticillium wilt, caused by *Verticillium albo-atrum* or *V. dahliae*, is an important disease of many worldwide crop species. In Europe, *V. albo-atrum* isolates infecting hop express different levels of virulence, inducing mild or lethal disease syndromes, and it is therefore an attractive model for studying the virulence of this pathogen. In this work, eleven amplified fragment length polymorphism (AFLP) primer combinations were used to analyze genetic variability among 55 *V. albo-atrum* hop isolates from four European hop growing regions, as well as isolates from other hosts and *V. dahliae* isolates. Cluster analysis divided *V. albo-atrum* and *V. dahliae* isolates into two well-separated groups. Within the *V. dahliae* cluster, isolates were separated without host specific grouping, although no host adapted isolates were included. In *V. albo-atrum*, the alfalfa isolates were distinct from isolates of other hosts, where a high association with virulence was observed in hop and tomato isolates. All lethal hop isolates were genetically different from mild hop isolates. The lethal hop isolates from England and Slovenia expressed the same virulence phenotype, although they showed a different AFLP pattern. The mild hop isolates formed two subgroups, to which isolates clustered irrespective of geographical location. These data suggest multiple origins of *V. albo-atrum* hop isolates, and the possible appearance of new virulent isolates in the future in other hop growing regions.

Introduction

Within the fungal genus *Verticillium*, *V. albo-atrum* and *V. dahliae* are well-known soil borne plant pathogens causing vascular wilts in a wide range of mainly dicotyledonous hosts (Engelhard, 1957). Most isolates of the two species are not host-adapted, although a few physiological races and pathotypes have been found. For example, *V. albo-atrum* isolates from alfalfa (*Medicago sativa*) and hops (*Humulus lupulus*) (Heale and Isaac, 1963; Sewell and Wilson, 1984), and *V. dahliae* from tomato (*Lycopersicon esculentum*), mint (*Mentha* spp.) and cotton (*Gossypium hirsutum*) (Horner, 1954; Alexander, 1962; Schnathorst and Mathré, 1966) have expressed different levels of physiological specialisation.

More detailed genetic structures of *Verticillium* isolates have been elucidated by molecular analysis using restriction fragment length polymorphism (RFLP) (Carder and Barbara, 1991; Typas et al., 1992), random amplified polymorphic DNA (RAPD) (Barasubiye et al., 1995; Koike et al., 1996;) and sequencing of the intergenic spacer (IGS) and internal transcribed spacer (ITS) of ribosomal RNA genes (Robb et al., 1993; Morton et al., 1995; Collins et al., 2003). These studies demonstrated two basic groups (Grp) in *V. albo-atrum*; Grp I, which includes a subgroup of isolates pathogenic to alfalfa (designated L) and a

subgroup of isolates from all other hosts (designated NL), and Grp II, which represents a special group of *V. albo-atrum* isolates associated with *V. tricorpus*.

The occurrence of different virulent strains or pathotypes of *V. albo-atrum* has been well documented in hops. The first record of hop infections was in England in 1924, which caused mild vascular wilt disease (Harris, 1927). Only a few years later, in 1933, more severe outbreaks appeared, known as progressive (lethal) wilt. The appearance of these two wilt syndromes was attributed to pathogen virulence, the sensitivity of hop cultivars and ecological factors (Isaac and Keyworth, 1948; Sewell and Wilson, 1984). In general, the mild wilt varies in intensity from year to year and rarely causes plant death, whereas lethal wilt is less influenced by seasonal climatic variations, and causes very severe symptoms with rapid plant withering. In England, one mild (M) and three types of lethal (PV1, PV2, and PV3) *V. albo-atrum* isolates have been identified by using virulence testing on different sets of hop cultivars (Sewell and Wilson, 1984; Clarkson and Heale, 1985b). There have also been reports of mild Verticillium hop infections in Germany, Poland, Belgium, France and, outside Europe, in New Zealand and USA (Oregon), caused either by *V. albo-atrum* or *V. dahliae* (Neve, 1991).

Lethal *V. albo-atrum* isolates were geographically limited to English hop-growing regions until 1997, when lethal outbreaks were registered in Slovenia. An epidemic of lethal wilt in the hop region of the Savinja Valley caused significant economic damage and, to date, more than 180 ha of hop gardens have been affected (Annual Report for 2005: Monitoring Survey of Hop Wilt (*Verticillium albo-atrum* and *V. dahliae*) in 2005. MAFF; Republic of Slovenia, unpublished). Virulence testing and molecular analysis of *V. albo-atrum* hop isolates collected in all Slovene hop growing regions identified two pathotypes, which were designated PG1 (mild) and PG2 (lethal) (Radišek et al., 2003).

The appearance of different strains or pathotypes requires more detailed knowledge of the population structure and virulence of *V. albo-atrum* isolates, which could contribute to resistance breeding, disease management and an understanding of the evolutionary behaviour of this organism. A comprehensive study of *V. albo-atrum* isolates from various hosts, including 35 hop isolates from England, was performed by Griffen et al. (1997). Molecular analysis, using RFLPs of ribosomal DNA, polymorphism of mtDNA and amplified polymorphic DNA (APD), confirmed the genetic distinction of alfalfa isolates from isolates of other hosts. High APD polymorphism was detected among hop isolates, but no correlation was found with the virulence, origin, hop cultivar, or date of isolation. In our previous study, amplified fragment length polymorphisms (AFLP) resolved two distinct hop pathotypes (PG1 and PG2) and seventeen AFLP markers correlated with the virulence were identified (Radišek et al., 2003).

Slovene *V. albo-atrum* isolates were thus well characterized but we did not have any evidence on the origin of PG1 and PG2 pathotypes. We therefore extended the molecular and virulence study to *V. albo-atrum* hop isolates from other European hop-growing regions, as well as to isolates from other hosts and *V. dahliae* isolates. The objectives of this study were to determine (i) whether there was a relationship between AFLP polymorphisms and variation in virulence, host specificity and the geographical origin of isolates and (ii) to compare the virulence of *V. albo-atrum* isolates from England and Slovenia that cause the lethal form of Verticillium wilt on hops.

Materials and methods

Fungal isolates

In total, 71 isolates of *V. albo-atrum* were used in this study (Table 1). Fifty-five were from infected hop (*Humulus lupulus*) plants, of which 34 isolates originated from Slovenia, 15 from England, five from Germany and one from Poland. These isolates represent most of the European hop-growing areas in which Verticillium wilt on hops has been observed (Neve, 1991). The remaining isolates were from other hosts and geographic locations, and included five isolates from alfalfa (*Medicago sativa*), six from tomato (*Lycopersicon esculentum*) and one each from potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*), petunia (*Petunia hybrida*), fat-hen (*Chenopodium album*)

Table 1. Fungal isolates analysed in this study, with results of virulence testing and AFLP analysis

Isolate designation	Host[a]	PathogenicityGroup[b]	AFLPgroup	AFLP-6-2[c]	Geographical origin	Source[d]
V. albo-atrum						
Or00	Hop	PG2	A I	+	Slovenia	IHPS
Prev	Hop	PG2	A I	+	Slovenia	IHPS
Or99	Hop	PG2	A I	+	Slovenia	IHPS
Pust	Hop	PG2	A I	+	Slovenia	IHPS
Vran	Hop	PG2	A I	+	Slovenia	IHPS
Laz	Hop	PG2	A I	+	Slovenia	IHPS
Tr98	Hop	PG2	A I	+	Slovenia	IHPS
Roz	Hop	PG2	A I	+	Slovenia	IHPS
Ciz	Hop	PG2	A I	+	Slovenia	IHPS
Ranc	Hop	PG2	A I	+	Slovenia	IHPS
Pov	Hop	PG2	A I	+	Slovenia	IHPS
Tr99	Hop	PG2	A I	+	Slovenia	IHPS
Jer	Hop	PG2	A I	+	Slovenia	IHPS
Rot	Hop	PG2	A I	+	Slovenia	IHPS
T2	Hop	PG2	A I	+	Slovenia	IHPS
T6	Hop	PG2	A I	+	Slovenia	IHPS
Vran01	Hop	PG2	A I	+	Slovenia	IHPS
Led	Hop	PG2	A I	+	Slovenia	IHPS
Pov02	Hop	PG2	A I	+	Slovenia	IHPS
Pec	Hop	PG2	A I	+	Slovenia	IHPS
Rov	Hop	PG2	A I	+	Slovenia	IHPS
Roj	Hop	PG2	A I	+	Slovenia	IHPS
Nat	Hop	PG2	A I	+	Slovenia	IHPS
Slan	Hop	PG2	A I	+	Slovenia	IHPS
Drc	Hop	PG2	A I	+	Slovenia	IHPS
Urs	Hop	PG2	A I	+	Slovenia	IHPS
Jel	Hop	PG2	A I	+	Slovenia	IHPS
Ocer	Hop	PG1	B I	−	Slovenia	IHPS
Kres99	Hop	PG1	B II	−	Slovenia	IHPS
CasA	Hop	PG1	B II	−	Slovenia	IHPS
Zup	Hop	PG1	B II	−	Slovenia	IHPS
Rec	Hop	PG1	B II	−	Slovenia	IHPS
Mo3	Hop	PG1	B II	−	Slovenia	IHPS
KresCe	Hop	PG1	B II	−	Slovenia	IHPS
11041	Hop	PV1	A II	+	Great Britain	1
11047	Hop	PV1	A II	+	Great Britain	1
11052	Hop	M	A II	−	Great Britain	1
11055	Hop	PV1	A II	+	Great Britain	1
11056	Hop	PV1	A II	+	Great Britain	1
11097	Hop	PV1	A II	+	Great Britain	1
298092	Hop	M	B I	−	Great Britain	CABI
298095	Hop	M	B I	−	Great Britain	CABI
298099	Hop	Lethal	A II	+	Great Britain	CABI
298100	Hop	Lethal	A II	+	Great Britain	CABI
298101	Hop	Lethal; supervirulent	A II	+	Great Britain	CABI
298102	Hop	Lethal; supervirulent	A II	+	Great Britain	CABI
1974	Hop	Lethal	A II	+	Great Britain	2
1985	Hop	Lethal	A II	+	Great Britain	2
1953	Hop	M	B I	−	Great Britain	2
Sol	Hop	n.t	B II	−	Poland	3
1140	Hop	n.t	B I	−	Germany	4
16/00	Hop	n.t	B I	−	Germany	4
14/93	Hop	n.t	B I	−	Germany	4
6/99	Hop	n.t	B II	−	Germany	4
15/98	Hop	n.t	B II	−	Germany	4

Table 1. Continued

Isolate designation	Host[a]	PathogenicityGroup[b]	AFLPgroup	AFLP-6-2[c]	Geographical origin	Source[d]
11	Alfalfa	n.t	C	+	Canada	5
41	Alfalfa	n.t	C	+	Canada	5
107	Alfalfa	n.t	C	+	USA	5
140	Alfalfa	n.t	C	+	USA	5
Luc	Alfalfa	Virulent	C	+	Great Britain	6
Surf	Petunia	n.t	B	–	Slovenia	IHPS
Kum	Cucumber	n.t	B	–	Slovenia	IHPS
11066	Potato	n.t	A	+	Great Britain	1
11077	Fat-hen	n.t	A	+	Great Britain	1
11081	Chrysanthemum	n.t	A	–	Great Britain	1
AR01/067	Tomato	Virulent on resistant tomato	A	+	Great Britain	2
AR0/140	Tomato	Virulent on resistant tomato	A	+	Great Britain	2
AR01/JS1	Tomato	n.t	A	+	Great Britain	2
T179	Tomato	Avirulent on resistant tomato	B	–	Great Britain	2
PD2000/4186a	Tomato	n.t	B	–	Holland	2
PD83/53a	Tomato	n.t	B	–	Holland	2
V. dahliae						
CasD	Hop	n.t	E	–	Slovenia	IHPS
MoD	Hop	n.t	D	–	Slovenia	IHPS
KresD	Hop	n.t	D	–	Slovenia	IHPS
KresDa	Hop	n.t	D	–	Slovenia	IHPS
12042	Hop	n.t	D	–	Great Britain	1
12099	Hop	n.t	D	–	Great Britain	1
2390	Hop	n.t	D	–	Germany	4
Pap	Green pepper	n.t	E	–	Slovenia	IHPS
Pap02	Green pepper	n.t	E	+	Slovenia	IHPS
JKG1	Potato	n.t	D	–	Holland	7
JKG8	Potato	n.t	D	–	Holland	7
JKG10	Potato	n.t	E	+	Holland	7
DJK	Chrysanthemum	n.t	E	–	Holland	7
MH	Chrysanthemum	n.t	E	–	Holland	7
JKG2	Southern catalpa	n.t	D	–	Holland	7
JKG7	Southern catalpa	n.t	D	–	Holland	7
JKG3	Japanese maple	n.t	D	–	Holland	7

[a]Alfalfa (*Medicago sativa*), Chrysanthemum (*Chrysanthemum* spp.), Cucumber (*Cucumis sativus*), Fat-hen (*Chenopodium album*), Green pepper (*Capsicum annuum*), Hop (*Humulus lupulus*), Japanese maple (*Acer palmatum*), Linden (*Tilia* spp.), Petunia (*Petunia hybrida*), Potato (*Solanum tuberosum*), Southern catalpa (*Catalpa bignonioides*), Tomato (*Lycopersicon esculentum*).

[b]PG1, PG2, PV1, M – *V. albo-atrum* hop pathotypes (Sewell and Wilson, 1984; Radišek et al., 2003), n.t – not tested by pathogenicity test.

[c]AFLP-6-2 marker amplified (+) or not amplified (–).

[d]IHPS-Slovenian Institute for Hop Research and Brewing, Žalec, Slovenia; CABI-CABI Bioscience, Genetic Resources Collection, Bakeham Lane, Surrey, UK; 1-Dr. G. Down, Disease Management, Horticulture Research International, West Malling, UK; 2-Dr. D. J. Barbara, Horticulture Research International, Wellesbourne, Warwick, UK; 3- Dr. E. Solarska, Institute of Soil Science and Plant Cultivation, Pulawy, Poland; 4- Dr. E. Seigner, Bayer Landesanstalt fur Bodenkultur und Pflanzenbau, Freising, Germany; 5-Dr. J. Robb, University of Guelph, Molecular Biology and Genetics, Canada; 6-Dr. R. Cooper, University of Bath, Department of Biology and Biochemistry, Bath, UK; 7-Dr. J.K. Goud, Biological Farming Systems, Wageningen, Holland; MZKI-Microbiological Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia.

and chrysanthemum (*Chrysanthemum* spp.). In addition, 17 isolates of *V. dahliae* from various hosts, including hop, were analysed in this study (Table 1).

The 34 Slovene *V. albo-atrum* hop isolates had been previously characterised as PG1 or PG2 pathotypes by virulence testing and SCAR markers (Radišek et al., 2003, 2004). The virulence characterisation of other isolates (Table 1) was specified by the supplier. All isolates used in this study were maintained in the culture collection of the Slovenian Institute for Hop Research and Brewing, Slovenia,

as monospore cultures on potato dextrose agar (PDA; Fluka, Buchs, Switzerland) at 4 °C, or stored as cultures in general fungal medium (Weising et al., 1995) in 20% glycerol at - 80 °C.

Virulence assessment

The virulence of six uncharacterised hop isolates of *V. albo-atrum* from England (11041, 11047, 11052, 11055, 11056 and 11097) (Table 1) was evaluated on four reference hop cultivars, susceptible Fuggle and Celeia, moderately tolerant Wye Challenger and tolerant Wye Target. Plants were grown in a growth chamber (Kambič, RK-13300) under a 12 h photoperiod of fluorescent light (L 58W/77; Fluora, Osram). Temperature and relative humidity were 22 °C and 65 % during the light period, and 20 °C and 70% during the dark period. Due to space limitations, isolates were tested in two separate experiments. Experiment I included isolates 11041, 11052, 11055 and 11097, and experiment II consisted of the two remaining isolates, 11047 and 11056. In both experiments, isolate Tr99 from Slovenia, previously characterised as PG2 hop pathotype, was used as the reference isolate with a high level of virulence (Radišek et al., 2003). Five plants of each hop cultivar grown as a single bine were inoculated per isolate by stem – puncture inoculation in the basal part of the bine. Control plants were similarly inoculated with sterile distilled water (SDW). Inoculum preparation and symptom assessment were carried out according to Radišek et al. (2003). Wilt was considered to be severe (lethal) when the scores of mean severity index for an isolate–cultivar combination were greater than 40. For re-isolation, the xylem tissue above the inoculation point was plated on PDA, and the species identification of isolates was checked by light microscopy. Pathotype classification was designated by the nomenclature as described by Sewell and Wilson (1984) and the pathogenicity studies of Clarkson and Heale (1985b).

DNA extraction and AFLP analysis

Isolates were cultured in general fungal medium (Weising et al., 1995) by agitation on a rotary shaker (50 rpm) at room temperature for 5 days in the dark. Mycelia were harvested by centrifugation (5 min, $4000 \times g$) and rinsed twice with SDW. Genomic DNA was extracted by the sodium dodecyl sulphate (SDS) method of Lee and Taylor (1990), with modifications as described previously (Radišek et al., 2003).

An optimised AFLP assay for analysis of *V. albo-atrum* and *V. dahliae* was used (Radišek et al., 2001). The primary template was prepared by digesting 500 ng of genomic DNA for 3 h at 37 °C, with 2.5 U each of *Eco*RI and *Msp*I restriction enzymes. Restriction fragments were ligated with *Eco*RI and *Msp*I adapters by using T4 Ligase (New England Biolabs GmbH, Frankfurt, Germany). Ligation was performed for 3 h at 37 °C and adapter-ligated fragments served as the template for primers in the polymerase chain reaction (PCR). For pre-amplification, a 10-fold diluted ligation mixture was amplified for 20 cycles at 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s, using *Eco*RI (5′GACTGCGTACCAATTC 3′) and *Msp*I (5′GATGAGTCCTGAGCGG 3′) primers having no selective nucleotides.

Selective amplification was conducted with the 11 *Eco*RI/*Msp*I primer combinations with two selective bases that were used in our previous studies (Radišek et al., 2003, 2004) (Table 3). The *Eco*RI primers were labelled with CyChrome Cy5 at the 5′ end. The PCR programme for the selective amplification was 13 cycles of 30 s at 94 °C, 30 s at 65 °C, 60 s at 72 °C, in which the annealing temperature was subsequently reduced by 0.7 °C each cycle. This was followed by 23 cycles of 30 s at 94 °C, 30 s at 56 °C, 60 s at 72 °C.

The amplification products were mixed with an equal volume of loading dye (5 mg ml^{-1} dextran blue, 1 mM EDTA, 98 % formamide), denatured by heating for 4 min at 94 °C and quickly chilled on ice. The AFLP fragments were analysed on an ALFexpress II automated sequencer (Amersham Biosciences) using 6% denaturing polyacrylamide gels (Promega, Madison, WI, USA) in $1 \times$ TBE buffer (90 mM Tris–borate, 2 mM EDTA, pH 8,9). The 50–500 bp DNA ladder (Amersham Biosciences) was used to determine the fragment sizes.

Data analysis

The AFLP fingerprints were manually scored for presence (1) or absence (0) of bands, and entered into a binary matrix. Only strong and reproducible

bands were scored, and each band was considered as a single locus. The estimate of genetic similarity among isolates was calculated according to Jaccard's coefficient, using the SIMQUAL programme in the NTSYS 2.02-pc software package (Rohlf, 1998). Dendrograms were generated via the unweighted paired group method average (UPGMA) clustering procedure. The goodness of fit of the clustering to the data was determined using COPH and MXCOMP procedures of NTSYS 2.02-pc (Rohlf, 1998). The robustness of the dendrograms was determined by bootstrap analysis with 1000 replications using the FreeTree computer programme (Pavlicek et al. 1999).

Isolation, cloning, and DNA sequencing

To obtain the sequence of the AFLP fragment of interest (previously designated AFLP-6-2) (Radišek et al., 2004), silver staining of polyacrylamid gels was conducted according to the Promega Silver Sequence protocol, with some modifications (Echt et al., 1996; Jakše et al., 2001). Selective amplification was carried out as described above by using pre-amplification reactions of three isolates (Tr99, 11041, Luc) and a corresponding primer pair *Eco*RI-GT/*Msp*I-GA, with unlabelled *Eco*RI primer. The PCR products were separated on 6 % denaturing polyacrylamide gels using a vertical electrophoresis system (Life Technologies, Paisley, UK). The AFLP-6-2 fragment of isolates was excised from the gel and re-amplified by the same primer pair that generated it. The amplification products were electrophoresed on 1.2 % agarose gel in $1 \times$ TBE buffer. Following visualisation under UV light, the fragment was excised from the gel and extracted using a QIAquick Gel Extraction kit (Qiagen). Purified DNA fragment was ligated into pGEM®-T Easy Vector in accordance with the manufacturer's instructions (Promega, Madison, USA). Plasmid DNA was extracted from an overnight culture (cultivated in 4 ml of Luria Bertani liquid medium containing 100 μg ml^{-1} of ampicillin) using a QIAprep Spin Miniprep kit (Qiagen). DNA sequencing was carried out by a commercial service (Macrogen, South Korea). The sequences of the AFLP 6-2 fragment were deposited in the GenBank under a separate accession number for each isolate: DQ008581, DQ008582, DQ008583.

Results

Virulence assessment

The virulence of the six *V. albo-atrum* hop isolates from England was determined by artificial inoculations of hop reference cultivars. Disease symptoms and isolate re-isolations revealed that each isolate could infect all hop cultivars, indicating their pathogenic potential on this host. Measurements of disease severity for each of the four hop cultivars revealed differences in the virulence (Table 2). Isolates 11041, 11055, and 11097 tested in experiment I, and isolates 11047 and 11056 from experiment II, induced severe symptoms on the susceptible cvs Fuggle and Celeia, and only mild symptoms on tolerant Wye Challenger, and Wye Target. Based on the pathotype nomenclature of Sewell and Wilson (1984), these isolates were classified as pathotype PV1. Isolate 11052 manifested a lower virulence level, inducing mild symptoms on all hop cultivars and was thus classified as M pathotype. In both experiments, reference isolate Tr99 (pathotype PG2) showed no significant differences in disease severity with isolates classified as PV1, indicating the same virulence level between Slovene PG2 and English PV1 hop isolates. A high consistency in incubation period and disease severity was observed between experiments I and II (Table 2).

AFLP and cluster analysis

AFLP analysis was conducted on 88 *V. albo-atrum* and *V. dahliae* isolates by using eleven *Eco*RI/*Msp*I primer combinations. In total, 417 DNA fragments were scored in a range of 50 to 600 bp, and the number of amplified fragments per primer pair varied from 28 to 56. All primer combinations showed polymorphisms between isolates, and the number and portion of polymorphic loci revealed in each primer combination are shown in Table 3. Fluorescent fragment detection showed consistency with our previous work (Radišek et al., 2003), in which silver staining was used. In addition, the AFLP-6-2 fragment, identified as a virulence linked marker, was amplified in *V. albo-atrum* hop isolates causing the lethal wilt in alfalfa, and virulent tomato isolates (AR01/067, AR0/140, AR01/JS1) in *V. dahliae*, potato isolate JKG10 from Holland, and in Slovene green pepper isolate

Table 2. Virulence of *Verticillium albo-atrum* hop isolates from England assessed on four hop cultivars

Isolate	Exp[a] (I/II)	Days after inoculation	Cultivar[b]					Pathotype[e]
			Wye Target	Wye Chall.	Celeia	Fuggle	Mean[c]	
11041	I	18	6.7	6.7	21.4	23.8	14.7a	PV1
		25	18.2	20.2	36.1	40.4	28.7a	
		32	28.9	28.6	63.6	65.9	46.8a	
11052	I	18	1.5	2.1	4.4	5.2	3.3b	M
		25	3.5	4.3	6.1	7.9	5.4b	
		32	3.8	5.2	6.6	9.2	6.2b	
11055	I	18	9.4	13.3	20.8	21.9	16.4a	PV1
		25	19.6	25.9	37.2	34.9	29.4a	
		32	22.8	33.8	55.9	62.4	43.7a	
11097	I	18	9	11.6	19.2	22.4	15.6a	PV1
		25	20.9	24.6	32.3	35.5	28.3a	
		32	23.9	29.8	59.1	60.8	43.4a	
Tr99[d]	I	18	8.5	6.4	19.3	17.7	12.9a	PV1
		25	20.9	23.3	31.9	35.8	27.9a	
		32	27.7	31.7	58.7	58.2	44.1a	
11047	II	19	6.9	8.9	34.1	22.7	18.2a	PV1
		26	17.6	22.3	50.3	44.1	33.6a	
		33	25.5	30.8	70.6	60.3	46.8a	
11056	II	19	5.9	9.3	26.6	20.2	15.5a	PV1
		26	14.2	21.8	44.6	46.3	31.7a	
		33	24.6	38.9	58.9	64.6	46.7a	
Tr99[q]	II	19	7.1	6.3	19.8	20.2	13.3a	PV1
		26	19.5	24.1	38.6	38.1	30.1a	
		33	24.5	34.4	61.6	64.0	46.1a	

[a]Experiment I or II.
[b]Mean severity index for 5 replicated plants for each isolate–cultivar combination.
[c]Means followed by the same letter were not significantly different at the 5% level (Duncan's multiple range test).
[d]Reference isolate of *V. albo-atrum* (pathotype PG2) (Radišek et al., 2003).
[e] Hop pathotypes determined according to Sewell and Wilson (1984).

Table 3. Number of all fragments and portion of polymorphic fragments amplified by 11 primer combinations in AFLP analysis of *V. albo-atrum* and *V. dahliae* isolates

Primer combination	Fragments (No.)	Polymorphic fragments (No.)				Polymorphic fragments (%)			
		[a]*V.a*/*V.d*	[b]*V.aNL*	[c]*V.aL*/*V.aNL*	[d]*V.d*	[a]*V.a*/*V.d*	[b]*V.aNL*	[c]*V.aL*/*V.aNL*	[d]*V.d*
E-GA/*M*-AT	50	38	4	12	4	76.0	8.0	24.0	8.0
E-GA/*M*-TA	41	26	1	12	2	63.4	2.4	29.3	4.9
E-GA/*M*-AG	47	29	3	9	7	61.7	6.4	19.2	14.9
E-GA/*M*-CG	45	21	1	15	5	46.6	2.2	33.3	11.1
E-GA/*M*-GT	52	38	3	10	3	73.1	5.8	19.2	5.8
E-GT/*M*-GA	40	29	2	9	3	72.5	5.0	22.5	7.5
E-GT/*M*-CG	56	34	2	15	1	60.1	3.6	26.8	1.8
E-AC/*M*-TC	40	31	2	9	5	77.5	5.0	22.5	12.5
E-AC/*M*-TA	34	18	5	6	6	52.9	14.7	17.6	17.6
E-AC/*M*-GT	38	30	2	11	4	78.9	5.3	28.9	10.5
E-TC/*M*-TA	28	21	1	4	2	75.0	3.6	14.3	7.1
Total	471	315	26	112	42	/	/	/	/
Mean	42,8	28,6	2,3	10,1	3,8	67.1	5.6	23.4	9.2

[a]*V.a*/*V.d*: Comparison between *V. albo-atrum* and *V. dahliae* isolates.
[b]*V.aNL*: Comparison among *V. albo-atrum* non-alfalfa (NL) isolates.
[c]*V.aL*/*V.aNL*: Comparison between *V. albo-atrum* isolates from alfalfa (L) and *V. albo-atrum* isolates from other hosts (NL).
[d]*V.d*: Comparison among *V. dahliae* isolates.

Pap02, thus showing a correlation between this marker and the virulence of *V. albo-atrum* isolates as shown in Table 1. The AFLP-6-2 fragments obtained from the three different isolates revealed the same 247 bp sequence but did not show any homologies with sequences in the GenBank.

The UPGMA cluster analysis based on Jaccard's similarity matrix generated a dendrogram dividing *Verticillium* isolates into two main clusters corresponding to *V. albo-atrum* and *V. dahliae*, with a genetic similarity value of 0.320 (Figure 1). The cophenetic correlation between the dendrogram and the similarity matrix revealed a high goodness of fit with the correlation value (*r*) 0.99. High bootstrap values (100%) indicated on the dendrogram, showed that all group-defining branches are well supported.

The cluster of *V. albo-atrum* isolates was further separated into three subgroups, designated A, B and C. Subgroup A contained 27 Slovene PG2 isolates and all English isolates from various hosts, except mild hop isolates 298092, 298095 and 1953, and avirulent tomato isolate T179. The latter isolates clustered into subgroup B, together with mild Slovene, German and Polish hop isolates, petunia and cucumber isolates, and two tomato isolates from Holland. A high genetic similarity (0.954) between *V. albo-atrum* sub-groups A and B was observed, whereas subgroup C, containing alfalfa isolates, was more distinct, with a similarity level of 0.640.

Within the *V. dahliae* cluster, isolates were divided into subgroups D and E. Subgroup D contained a homogeneous group of five hop isolates originating from Slovenia and England, which showed high similarity with potato and southern catalpa isolates from Holland. The rest of subgroup D consisted of hop isolate 2390 from Germany and Dutch Japanese maple isolate JKG3. Smaller sub-group E was more heterogeneous and consisted of two green pepper isolates and a hop isolate CasD from Slovenia, and Dutch isolates from potato and chrysanthemum.

Molecular variability among V. albo-atrum *hop isolates*

To obtain a clearer picture of genetic relationships among 55 *V. albo-atrum* hop isolates, an additional dendrogram (Figure 2) was constructed using the AFLP data set of these isolates. Cluster analysis revealed two distinct groups of isolates that well correlated with their virulence classification (bootstrap value 100%). The first group included all isolates causing the lethal form of hop wilt, and the mild isolates formed the second group, with the exception of English isolate 11052 (mild pathotype M), which clustered with the lethal isolates. The lethal group was further separated into two subgroups designated A I and A II, with a low bootstrap support of 52% (Figure 2). Subgroup A I contained 27 Slovene PG2 isolates with the same genetic pattern, and twelve isolates from England formed subgroup A II. The English isolates, of which five were classified as PV1 pathotype, four as lethal isolates and two as supervirulent lethal isolates, revealed the same AFLP profile but mild (M) isolate 11052 clustered separately in A II.

The cluster of 16 mild isolates was divided into subgroups B I and B II; subgroup B I included three English isolates classified as pathotype M, three isolates collected in Germany, and one Slovene PG1 isolate. Subgroup B II consisted of two German isolates, six Slovene PG1 isolates and an isolate from Poland. The grouping of mild isolates was not well supported by bootstrap analysis (48%).

Discussion

AFLP molecular analysis showed high genetic variability between the two *Verticillium* species, as previously reported (Collins et al., 2003; Fahleson et al., 2003; Radišek et al., 2003). In *V. albo-atrum*, the alfalfa isolates were clearly separated from isolates of other hosts, in accordance with the generally accepted division of GrpI isolates into L and NL groups. The same genetic pattern of alfalfa isolates from North America and England supports earlier reports of host adaptation and clonal population of these isolates (Correll et al., 1988; Griffen et al., 1997).

Genetic variation among *V. albo-atrum* isolates from other hosts (group NL) showed no host-specific groups, although a high association with virulence was observed in hop and tomato isolates. The group of hop isolates from Slovenia and England causing the lethal wilt, and two English isolates from tomato (AR01/067 and AR0/140) classified as virulent on resistant tomatoes grouped

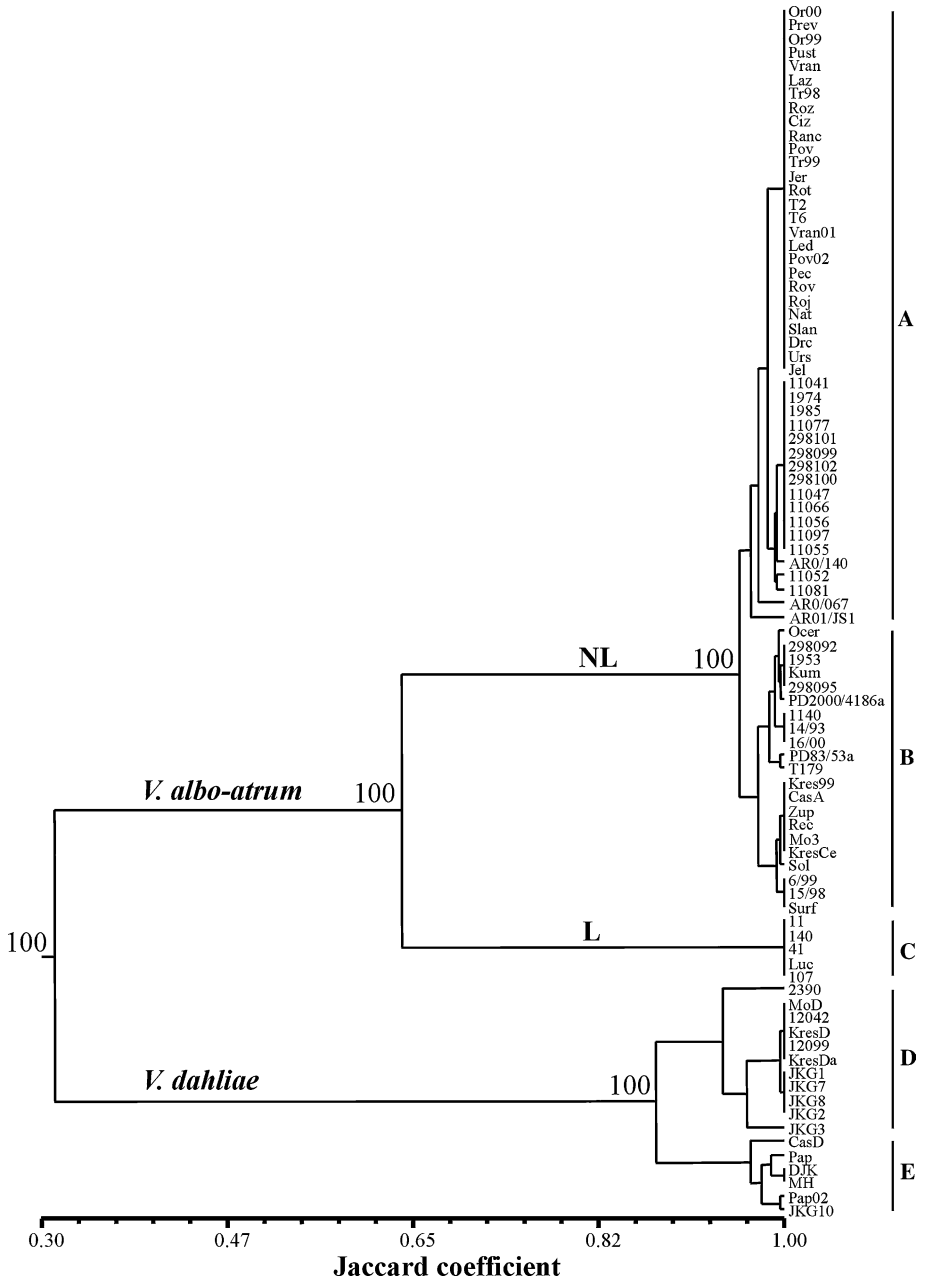

Figure 1. Dendrogram of 88 *V. albo-atrum* and *V. dahliae* isolates based on AFLP analysis, using the Jaccard coefficient of similarity and UPGMA clustering. Numbers at the nodes of major clusters denote bootstrap values (1000 replicates). L = alfalfa isolates, NL = non-alfalfa isolates. The five clusters are discussed in the text.

in cluster A (Figure 1). However, the majority of mild hop isolates from Slovenia, England, Germany and Poland, and avirulent tomato isolate T179 from England, were placed in cluster B (Figure 1). The rest of the NL isolates in this study have no virulence classification but, according to the molecular characterisation, they are related either to virulent or avirulent isolates (Figure 1, Table 1). In addition to the virulence association, molecular marker AFLP-6-2, identified in our earlier study as a fragment specific to lethal pathotype PG2 (Radišek et al., 2003), was amplified

only in more virulent *V. albo-atrum* hop and tomato isolates, in alfalfa isolates and in genetically more distinct *V. dahliae* isolates originating from potato and green pepper (Table 1). These results are in accordance with our previous study, in which five SCAR markers specific for the PG2 hop pathotype revealed associations among virulent hop, tomato and alfalfa isolates in *V. albo-atrum* (Radišek et al., 2004). However, the amplification of markers specific for virulent hop isolates in alfalfa and *V. dahliae* isolates was unexpected, since they show high genetic dissimilarity (Figure 1) and

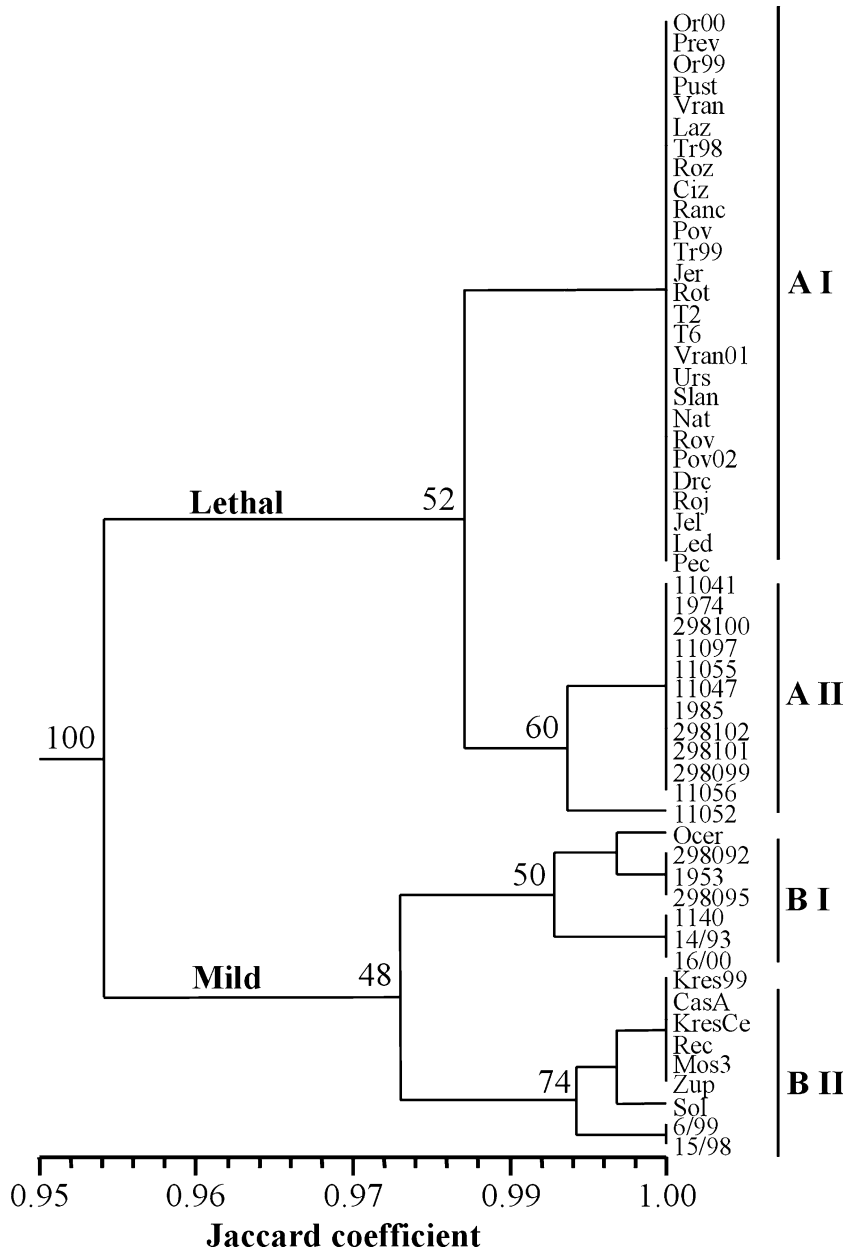

Figure 2. Dendrogram of 55 *V. albo-atrum* hop isolates based on AFLP analysis, using the Jaccard coefficient of similarity and UPGMA clustering. Numbers at the nodes of major clusters denote bootstrap values (1000 replicates). Lethal = isolates causing the lethal form of Verticillium wilt on hops, Mild = isolates causing the mild form of Verticillium wilt on hops. The four clusters are discussed in the text.

low vegetative compatibility to hop isolates (Typas and Heale, 1976; Correll et al., 1988); gene-flow between these isolates is thus hindered.

Similar associations of molecular markers with virulence patterns have been previously reported in *V. dahliae* isolates pathogenic to cotton and olive, in which RAPD analysis revealed differences between defoliating pathotype D and non-defoliating pathotype ND (Perez-Artes et al., 2000; Korolev et al., 2001). Nevertheless, such close correlations are rare and to date have been reported mostly in asexual reproducing fungi such as *Fusarium oxysporum* f.sp. *vasinfectum*, *Magnaporthe grisea* and *Puccinia recondita* (Levy et al., 1993; Assigbetse et al., 1994; Kolmer et al., 1995).

In the case of *V. dahliae*, AFLP analysis divided isolates into two clusters designated D and E (Figure 1), without host-specific grouping, although no host-adapted isolates were included. Within cluster D, it is interesting that hop isolates from England and Slovenia revealed an identical AFLP pattern, in contrast to *V. albo-atrum* isolates, and the German hop isolate 2390 was most dissimilar. Zinkernagel (1982) reported on more aggressive *V. dahliae* hop isolates from Germany, which partly explains such results. However, more isolates from Germany must be analysed to establish their true genetic relationship. The remaining cluster D isolates (two potato and two southern catalpa isolates from Holland) showed the same genetic pattern and high similarity with hop isolates from England and Slovenia. Such results might imply a common origin of these isolates. The second cluster E was more heterogeneous, showing similarities between the small group of Dutch isolates from chrysanthemum and potato and two Slovene isolates from green pepper and hop.

In general, such relationships among of *V. albo-atrum* and *V. dahliae* isolates are difficult to explain, because in most cases isolates are classified by reference to the host from which they were isolated, without information about cropping history that might suggest other host associations. However, in this study, by knowing for example that many agricultural fields in Holland have a potato growing history with *V. dahliae* infections, and that tree nurseries are often established on such fields (Goud, 2003), we can explain the same genetic pattern of *V. dahliae* isolates from potato and southern catalpa in cluster D. A similar explanation may also apply in the case of the genetic identity of isolate 11077 from fat-hen, potato isolate 11066 and a group of lethal hop isolates from England within the *V. albo-atrum* dendrogram group A (Figure 1). England is a hop-growing country, where lethal *V. albo-atrum* hop isolates have been widespread in many agricultural fields (Talboys, 1987) so it is possible that 11066 and 11077 are lethal hop isolates, isolated from infected potato and fat-hen.

Hop is a perennial plant that has a long-standing monoculture tradition in the majority of world hop-growing regions (Neve, 1991). The genetic relationships among isolates affecting hops are therefore more easily understandable than in the case of isolates obtained from plants with which crop rotation is intensively performed. Hop wilt due to *V. dahliae* is comparatively rare and it is mild in character, whereas *V. albo-atrum* shows a higher preference to hop and causes the majority of outbreaks in the mild and lethal forms (Neve, 1991). The recent occurrence of lethal *V. albo-atrum* isolates classified as pathotype PG2 in Slovenia have led to speculations about its origin.

In this study, the comparison of the disease severity on reference hop cultivars caused by five English PV1 isolates and one Slovene PG2 standard isolate, showed no significant differences (Table 2). The same virulence phenotype suggests that PG2 and PV1 isolates can be considered to be the same pathotype and that the appearance of lethal wilt in Slovenia might be through the introduction of PV1 from England. However, it is well known that virulence testing alone may not reflect the true genetic variability or evolutionary history of isolates, as in our case, in which genetic analysis separated these isolates into two clusters AI and AII (Figure 2). The identical genetic pattern of 24 Slovene PG2 isolates (AI group) obtained from different outbreaks in a radius of 7 km showed their clonal nature, which is attributable to newly evolved genotypes. These results suggest that PG2 is a new genotype of pathotype PV1, which has emerged in Slovenia. In order to avoid different pathotype nomenclature in the future, we propose the standardisation of nomenclature according to Sewell and Wilson (1984).

Cluster AII of English isolates consisted of five isolates classified as PV1 pathotype, four isolates designated as 'lethal', two 'super-virulent' isolates and isolate 11052 classified as pathotype

M. According to Talboys (1987), English isolates designated as 'lethal' correspond to pathotype PV1, while the designation 'super-virulent' corresponds to the PV2 or PV3 pathotypes. However, molecular analysis revealed no genetic variation among PV pathotypes, despite their virulence variation and the fact that isolates were collected in two different hop-growing regions, Kent and West Midlands (data not shown). The molecular classification of mild isolate 11052 into the group of lethal isolates is the only exception in the distinct clustering of mild and lethal isolates in this study. The genetic differences of this isolate suggest the possibility of virulence attenuation, although the AFLP-6-2 fragment, which has been amplified only in highly virulent isolates, was absent in this isolate.

In the case of mild *V. albo-atrum* hop isolates, two subgroups were identified (Figure 2), to which isolates clustered irrespective of their geographical location. The first subgroup, designated BI, included all mild isolates from England, three isolates collected in Germany and one Slovene isolate classified as PG1. The second subgroup, BII, consisted of the remaining Slovene PG1 isolates, an isolate from Poland and two isolates from Germany. Although such clustering of mild isolates was not supported by bootstrap analysis, we could speculate that mild isolates have at least two different origins, of which England is most likely one of them, since *V. albo-atrum* on hop was first identified there. It is also important to consider that mild isolates revealed higher genetic variability than lethal isolates, and no high host specificity was displayed, since Slovene isolates from cucumber (Kum) and petunia (Surf) showed genetic identity with some mild hop isolates from England and Germany (Figure 1). It seems that, in both *Verticillium* species, the mild form of hop wilt is also caused by isolates from other hosts that show little or no specialisation toward hop, as was previously proposed by Clarkson and Heale (1985a).

Taken together, our results suggest that *V. albo-atrum* isolates causing Verticillium wilt on hops have multiple origins, and that the appearance of new virulent isolates can be expected in the future in other hop-growing regions. A similar demonstration of multiple origins has been reported in the case of virulent *V. dahliae* isolates able to overcome the *Ve* resistance gene in tomato (Dobinson et al., 1998). Possible explanations for the evolution of new strains or pathotypes in asexual fungi have frequently been suggested as being due to genetic mechanisms such as mutations, transposition events, mitotic and parasexual recombinations. Based on numerous parasexual studies, Heale (1988) suggested that gene exchange via a parasexual cycle in *Verticillium* is probably an extremely rare event, but still could have considerable significance in the evolution of virulence and the survival of the pathogen.

On the other hand, Goodwin et al. (1994) showed that a high rate of mutations could occur under natural conditions in response to the selection pressure exerted by host resistance. The extremely wide host range of *V. albo-atrum* and *V. dahliae* shows a high capability for adaptation to changing host substrates, and not least the capacity to respond to the selection pressure brought by the introduction of host cultivars with enhanced degrees of resistance (Talboys, 1987). The emergence of highly virulent *V. albo-atrum* hop isolates in England is such example. The old hop cultivars, such as Fuggle, were substantially tolerant to the mild wilt occurring in hops before 1930. Presumably, the selection pressure of these cultivars yielded the first lethal PV1 pathotype, and later the introduction of new tolerant cultivars caused the emergence of supervirulent PV2 and PV3 (Talboys, 1987). We can draw parallels with the recent situation in Slovenia with a 50 year delay: in 1974, a mild pathotype was identified and most Slovene cultivars expressed tolerance to it (Dolinar, 1975), but after 33 years, selection pressure resulted in highly virulent isolates in the western part of the Savinja Valley. The majority of Slovene cultivars contain predominantly characteristic English hop germplasm (Šuštar-Vozlič and Javornik, 1999), so the pathogens have been subjected to similar selection pressures, which could explain the same virulence phenotype of lethal isolates from the two hop-growing regions.

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