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Population studies on *Phytophthora infestans* on potatoes and tomatoes in southern Germany

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Abstract Fifty-seven isolates of *Phytophthora infestans* from blighted potato foliage were collected in 1995 in southern Germany and analysed for mating type and sensitivity to metalaxyl. Fifty-six of them were characterised as A1 and one as A2 mating types. Resistance to metalaxyl was observed frequently: 53 isolates were resistant, three were partially sensitive, and one was sensitive. In a subsequent field study in 1999, 84 isolates collected from blighted potato and tomato foliage were analysed for mating type. Seventy-two were characterised as A1 and twelve as A2 mating types. The response of 76 isolates to metalaxyl and to propamocarb was tested. The majority (42) of the 76 isolates was classified as resistant to metalaxyl; 31 were partially sensitive and only three isolates were sensitive. The results with propamocarb were less discrete; 10 isolates were classified as resistant and three were clearly sensitive. AFLP fingerprinting was used to examine the genetic structure of the southern German *P. infestans* population collected in 1999 and indicated that the tested population can be sub-divided into a tomato group, a potato group and a mixed group containing isolates collected from both crops. The presence of Ia and IIa mitochondrial DNA haplotypes indicates that the German *P. infestans* isolates belong to the new pathogen population that has also been reported in neighbouring regions of Europe. The present study indicates that at the beginning of the season only a few genotypes were present, and the population became genetically more variable at the end of the growing season.

Keywords DNA fingerprinting · Fungicide sensitivity · Mating type · Metalaxyl · mtDNA haplotype · Propamocarb

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Introduction

Late blight, caused by the heterothallic oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato and tomato worldwide. Until the 1980s, the global population of *P. infestans* outside of central Mexico, the presumed centre of origin, was marked by a single clonal lineage of *P. infestans* (Goodwin et al. 1994), the so-called ‘old population’ or ‘US-1 clonal lineage’. This population of the

pathogen was restricted to asexual reproduction since only A1 isolates were present (Goodwin et al. 1994). Analysis of North American and European *P. infestans* isolates supported the notion that in many parts of the world the 'old population' has been displaced by another, more aggressive, 'new population' with both A1 and A2 mating types (Fry et al. 1993). In Germany the first indications of the presence of both A1 and A2 mating types in *P. infestans* pathogen populations date back to 1980 (Daggett et al. 1993). Analyses of these populations have yielded evidence suggesting an increase in complexity of virulence phenotypes and resistance to the systemic fungicide metalaxyl (Gisi and Cohen 1996). Sexual reproduction leads to the formation of sexual resting structures, termed oospores. The role of oospores in the biology of European late blight epidemics is not fully understood. Meiotic recombination of genetic traits in *P. infestans* by sexual reproduction will lead to an increase in the genetic variability of the pathogen (Fry et al. 1993; Goodwin 1997). However, this impact is dependent on the presence of viable and functional oospores. Asexually generated genetic variability is also thought to increase the ability of the pathogen to adapt to the environment (Goodwin 1997). The combined effect of clonally and sexually generated genetic variation in *P. infestans* may facilitate the directional selection of more virulent or fungicide-resistant genotypes.

The two main hosts of *P. infestans* are potato and tomato and the level of cross-infection of potato and tomato strains is of practical significance where both hosts are cultivated in close vicinity. Evidence for some host specialisation in *P. infestans* has been provided in the past, in both 'old' and 'new' populations. Legard et al. (1995) compared isolates from North America and The Netherlands, and differentiated between tomato-aggressive and non-aggressive isolates. All isolates were aggressive on potatoes, but few on tomatoes. Population surveys showed host-related differences in the nature and frequency of *P. infestans* clones present (Fry et al. 1992; Goodwin et al. 1995; Oyarzún et al. 1998). Lebreton and Andrivon (1998) found that both mating types were present among the collections from both hosts, but isolates with the A2 mating type were found on potato only in one garden crop, adjacent to tomato. Furthermore, they observed a greater diversity among tomato isolates, a marked difference in the frequencies of genotypes present, and tomato isolates generally had a lower virulence complexity than potato isolates. In a further study, Lebreton et al. (1999) found that isolates from potato and tomato showed differential pathogenicity, which contributes to the differentiation between *P. infestans* populations present on potato and tomato. Using molecular markers, Knapova and Gisi (2002) were able to differentiate 22 genotypes in Swiss and French field populations, of which seven were specific for tomato, 11 for potato, and four were isolated from both hosts. The reports of specialised strains with differential pathogenicity to potato and tomato fueled speculation about the presence of host preference and specialisation within *P. infestans*.

Metalaxyl is an acylalanine phenylamide and has proved to be a very versatile oomycete-specific fungicide in terms of biological activity and systemic properties. Metalaxyl inhibits sporulation and mycelial growth inside the host tissue, but has little effect on sporangium or zoospore germination (Schwinn and Margot 1991). Soon after introduction of the commercial fungicide Ridomil, resistance to metalaxyl developed rapidly in *P. infestans*. Since metalaxyl acts highly selectively at a single target site, a mutation might easily lead to resistance by changing the target site affinity (Schwinn and Margot 1991).

Another systemic fungicide used to control late blight is propamocarb (propyl 3-(dimethylamino) propylcarbamate hydrochloride), which disturbs the synthesis of fatty acids in oomycetes. Reports from crop consultants and potato growers over the last few years seem to be indicating an unsatisfactory efficacy of propamocarb on *P. infestans*. Hannukkala (2001) reported isolates sporulating in the presence of 1,000 ppm. These observations have fueled discussions about whether reduced sensitivity of *P. infestans* isolates to propamocarb is present in field populations of the pathogen.

Little information is available on *P. infestans* populations in Germany. Rullich et al. (2002) found, mainly for the northern part of Germany, that since 1993/1994 there was a replacement of the old population and this process is not yet finished. The mitochondrial haplotypes Ia and IIa of the new population have both mating types and there are resistant as well as sensitive strains towards metalaxyl. The portion of complex pathotypes was found to be higher in the new haplotypes Ia and IIa than in the

old haplotype Ib (Rullich et al. 2002). Bouws and Finckh (2007) reported that in the central part of Germany (Nord Hessia near the city of Kassel, see Fig. 1) the *P. infestans* populations studied from 2000 to 2002 were highly diverse with respect to pathotypes, and rep-PCR fingerprints and both mating types occurred in all three years at various proportions. Flier et al. (2007) found in a survey carried out in Europe that populations from France, Switzerland and the UK were mainly clonal populations showing restricted levels of genetic diversity, whilst those from Norway were mixed A1 and A2 mating type populations with high levels of genetic diversity, suggesting periodical sexual reproduction.

The observation made in other regions concerning the differentiation of the pathogen between potato and tomato crops raised questions about the situation in southern Germany and about the characteristics and relationships between *P. infestans* populations present on each host plant in the sampled region. The present study reports phenotypic characteristics of *P. infestans* isolates collected from potato in 1995, and phenotypic and genotypic characteristics from potato and tomato in 1999 in Germany. Isolates collected from infected potatoes and tomatoes from southern Germany in 1999 were genetically characterised for mating type, mitochondrial DNA haplotype and AFLP fingerprints. The level of genetic variation present in the pathogen population of southern Germany was compared to the genetic variation present in two reference populations of *P. infestans* which are typical for asexually (central Africa) and sexually (Valle de Toluca, México) reproducing populations. In addition, mating type, sensitivity to the fungicides propamocarb (only in 1999) and metalaxyl were tested on detached leaflets from isolates collected in 1995 and 1999. The objectives were (i) to screen the present population for its sensitivity to metalaxyl and propamocarb to adapt decision-support systems for fungicide-treatment advice to the current population, (ii) to determine the occurrence of A1 and A2 mating types within the population, (iii) to find the clonal lineages of the present population, (iv) to compare the genotypic and phenotypic characteristics of *P. infestans* isolates collected from potato and tomato, in an attempt to better understand the structure and possible interactions between populations present on these hosts, and (v) to compare the early population (sampling within 5 weeks after disease outbreak) and late population of *P. infestans*.

Fig. 1 Collection sites of isolates of *Phytophthora infestans* in 1999. The numbers within the map represent the first two digits of the postal CIP codes given in Table 1

Materials and methods

Sources and cultivation of isolates

Single-lesion isolates were collected in Germany during 1995 and 1999. During 1995, a total of 57 samples was collected successfully from naturally infected plants in commercial potato fields. The sites of sampling in 1995 were within the region Nürnberg–Ulm–Landsberg and Passau in the southern part of the Federal State of Bavaria (Fig. 1). During 1999, 84 *P. infestans*-infected leaf and stem samples were collected successfully from blighted potato (from potato fields and allotment gardens) and tomato (from allotment gardens), mainly from the same region as in 1995 (Fig. 1). In 1999 samples were also collected from organic fields, in order to have isolates from fields without fungicides used in the current growing season and in the previous growing season. The isolates were numbered according to the sampling date; the higher the isolate number the later the sampling date. Twenty-seven of the isolates were collected during

the early stages of the disease within 5 weeks after the disease outbreak in late June (isolate number < 28), the remainder during the latter stages of the epidemic from August onwards (isolate number > 27). Sixty-two of the samples came from potato crops and 22 from tomato crops from allotment gardens. Isolates were maintained on V8-agar medium and kept at 15°C in the dark for a period of ca. 3 weeks prior to analysis.

Fungicide sensitivity

All experiments were conducted with detached leaf discs from leaflets of potato plants (cv. Agria) propagated in growth chambers for 6 to 8 weeks at 20 °C with 16 h light. Leaf discs of 25 mm diam were dipped into the fungicide solution (control treatment: water) for 30 sec and six leaf discs were then transferred to a Petri dish containing benzimidazole agar (in 1995: 15 g agar l^{-1} H_2O + 1 g of benzimidazole) or water agar (in 1999: 15 g agar l^{-1} H_2O). Each leaf disc represented a single replicate. The concentration of the fungicide solution was 10, 50, 100 and 500 ppm (in 1995 additionally 200 ppm) for metalaxyl, while the concentration of propamocarb was 100, 500, 1,000 and 5,000 ppm. The Petri dishes containing the leaf discs were placed in a growth chamber at a constant temperature of 15°C and 12 h light. After 24 h the leaf discs were inoculated with zoospore suspensions of the isolates. Each zoospore suspension was obtained from a sporangial suspension of *P. infestans* at a concentration of 4×10^4 sporangia ml^{-1}. This suspension was chilled at 4°C to induce the release of zoospores. After 2 h, 50 µl of the suspension was inoculated with the help of a pipette on to the middle of each leaf disc. Six to eight days after inoculation the infection of the individual leaf discs was assessed. The inoculation experiments were replicated twice.

Growth response of each isolate to metalaxyl and propamocarb was assessed by comparing mycelial growth and sporulation on treated leaves to that on the fungicide-free leaf disc controls. Final assessments of the percent leaf disc area covered by sporulating mycelium were made when the inoculated control showed abundant sporulation throughout the leaf disc, typically at 5 to 6 days post-inoculation. Four classes of sporulation intensity were identified: 0 = no mycelia, no sporulation; 1 = mycelium with few sporangia, mycelium identifiable only under the microscope; 2 = mycelium showing intermediate number of sporangia; and 3 = mycelium showing abundant sporulation. In the latter two classes the mycelium was typically observed without the need of a microscope.

Phytophthora infestans isolates were designated as metalaxyl-resistant or sensitive according to the criteria used by Sozzi et al. (1992), which are based on the percent growth in the presence of 10 µg (10 ppm) of metalaxyl ml^{-1}, relative to the untreated control. For each isolate, dose-response data were used to compute an ED_{50} value (the concentration or dose inhibiting the radial growth of the pathogen by 50%). Isolates were classified as sensitive if $ED_{50}<$ 10 ppm, as partially resistant if $ED_{50}>10$ ppm and < 100 ppm and as resistant if the $ED_{50}>100$ ppm. Farmers commonly apply concentrations between 200 and 650 ppm metalaxyl when spraying. For propamocarb, isolates were classified as sensitive if $ED_{50}\leq100$ ppm, as partially resistant if $ED_{50}>$ 100 ppm and < 1,000, and as resistant if the $ED_{50}\geq$ 1,000 ppm.

Mating type determination

Mating type was determined by pairing isolates individually with tester strains of known mating type, provided by B. Schöber-Butin, Biologische Bundesanstalt für Land- und Forstwirtschaft in Braunschweig, Germany. The pairings were made by placing an agar block containing mycelium of the unknown isolate in a 100× 15 mm Petri dish containing V8 agar and by placing equivalent inoculum blocks of the tester isolates in the Petri dish at a distance of ca. 2 cm. To identify self-fertile isolates, self-pairings of each isolate were carried out. Cultures were incubated for 8–12 days in the dark at 15°C. The plates were scored for the production of oospores after 12 days using a microscope (100 × magnifications). If oospores were observed microscopically at the hyphal interface between the unknown isolate and the A2 mating type tester, the unknown isolate was designated A1. The unknown isolate was designated A2 if oospores were formed in pairings with the A1 mating type tester.

Genetic structure

To compare the genetic structure of the German isolates with other *P. infestans* populations, eight

isolates from central Africa (all belonging to the 'old population') and 26 isolates from the Valle de Toluca, central México, were included as samples taken from asexually and sexually reproducing populations, respectively. Isolates from Mexico were from the PRI/IPO collection, Wageningen, The Netherlands, and isolates from central Africa were provided by Greg Forbes, CIP, Lima, Perú. Genotypes were defined based on differences in the combined phenotypic and genotypic marker footprint, i.e., if isolates differed in marker data, they were considered as different genotypes.

DNA extraction To obtain pure mycelium of *P. infestans* six blocks (1 cm×1 cm) of each isolate kept in pure culture on autoclaved V8 agar for several weeks after isolation were introduced and grown for 10 to 14 days at 20°C in Erlenmeyer flasks with a sterilised nutrient solution consisting of demineralised water (800 ml), vegetable juice (200 ml) and calcium carbonate (2 g). The flasks were kept on a shaker for 10 to 14 days, until a compact mycelial tuft of 1 to 2 cm diam developed. After decanting the nutrient solution and lyophilisation, the mycelium was ground in a mortar. After harvesting, the mycelium was lyophilised and stored at −68 °C. Lyophilised mycelium (30 to 60 mg) was ground in microcentrifuge tubes with a pestle and sterile sand, and total DNA was extracted using a Puregene kit (Gentra/Biozym, Landgraaf, The Netherlands) according to the manufacturer's instructions. DNA was dissolved in 100 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and stored at −20°C.

Mitochondrial DNA haplotypes Mitochondrial DNA (mtDNA) was amplified by PCR using four sets of primers designed to amplify specific regions (P1 to P4) of the mitochondrial genomes of *P. infestans*, as described by Griffith and Shaw (1998). The PCR products were digested using restriction enzymes *Cfo*I (P1), *Msp*I (P2), and *Eco*RI (P3 & P4). Ten microlitres of the amplified product were digested with 1 unit of the restriction enzyme for 4 h. The digested products were run on 1.8% agarose gels in TBE buffer at 10 V cm^{-1}.

Fluorescent amplified fragment length polymorphisms (AFLP) Fifty-two isolates were successfully cultivated for AFLP analyses. DNA (250 ng) was digested in a 50-µl reaction volume with *Eco*RI (10 U) and *Mse*I (10 U) for 6 h at 37°C in restriction-ligation buffer (10 mM Tris/Ac [pH 7.5], 10 mM MgAc, 50 mM KA, 5 mM DTT, 50 ng μl^{-1} BSA). Digestion was confirmed on agarose gels. *MseI* and *EcoRI* adapters were ligated to restriction fragments using 0.1 µM *EcoRI* adapter, 1.0 µM *Mse* adapter, 0.2 mM ATP and 2.4 U of T4 DNA ligase (Pharmacia, Uppsala, Sweden). Ligation was performed overnight at 10°C and the ligation products were diluted × 10 with MilliQ ultra-pure water. Non-selective PCR amplification was performed using primers E00 (5′-GACTGCGTACCAATTC) and M00 (5′-GATGAGTCCTGAGTAA) to amplify all restriction fragments. Non-selective PCR amplifications were performed in a PTC200 thermal cycler (MJ Research, Watertown, MA). The amplified restriction fragment products were checked on 1.0% agarose gels. Selective PCR was performed in a 20-µl reaction volume with 5 µl of 20 × diluted amplification products, 200 µM dNTP, 5 ng of Cy5-labelled fluorescent E21 primer and 30 ng of *MseI* 16 primer. Products were loaded on Sequagel (Biozym) polyacrylamide gels and run on an ALFexpress automatic sequencer (Amersham).

Data analysis

Each isolate was classified based on its origin (potato vs. tomato, commercial fields vs. allotment gardens, pre-treated with fungicides vs. not treated, organic vs. conventional crops), mating type and mtDNA haplotype. Restriction fragments of isolates were visualised on agarose gels using ethidium bromide under UV illumination, and classified according to mtDNA haplotype. AFLP patterns were analysed using Imagemaster ID software (Amersham Pharmacia Biotech), manually correcting for faint bands and exclusion of controversial bands. A total of 111 distinct and reproducible AFLP bands was identified. Bands were treated as putative single AFLP loci and a binary matrix containing the presence or absence of these reproducible bands was constructed and used for further analysis. Bands of the same electrophoretic mobility were assumed to be identical. Statistical analyses were conducted using TFPGA (Tools for Population Genetic Analyses, version 1.3). Each AFLP band was assumed to represent the dominant

genotype at a single locus, while the absence of that same band represents the alternative homozygous recessive genotype.

Genotypic diversity analysis was used to determine the distribution of diversity among populations (Germany, central Africa, and México). Pair-wise measures of Roger's modified genetic distance and population differentiation was estimated using an exact test (Raymond and Rousset 1995) to assess the significance of the different statistics for the null hypothesis of no differentiation at the corresponding hierarchical level. Permutation and re-sampling tests (bootstrapping) were carried out. Cluster analysis of AFLP genotypes was based on allele frequencies observed for each sub-population. The similarity of the multiallelic genotypes was identified by the Jaccard coefficient. To compare the German with the African and the Mexican populations, virtual heterozygosity and percent polymorphic loci (95% criterion) were estimated for each of the three populations. A phenogram was constructed using the unweighted pair-group method of averages (UPGMA) algorithm from a Rogers´modified genetic distance matrix. Bootstrap sampling (1,000 replicates) was performed for parsimony analysis of the constructed phenogram. Differentiation among populations was estimated using an exact test (Raymond and Rousset 1995) to assess the significance of the different statistics for the null hypothesis of no differentiation at the corresponding hierarchical level.

In a further more detailed analysis of the German population a dendrogram was constructed using the UPGMA algorithm contained in the computer programme package Phylip 3.67 (Felsenstein 2004). Bootstrap analyses were used (1,000 replicates) to assess the degree of support for each group on the extended majority rule tree of the constructed phenogram.

Results

Sensitivity to metalaxyl and propamocarb

In 1995, 57 isolates were tested for sensitivity to metalaxyl. Only one isolate was classed as sensitive (> 50% inhibition with 10 ppm metalaxyl), two were partially sensitive (> 50% inhibition with 100 ppm metalaxyl) and 54 were fully resistant (< 50% inhibition with 100 ppm metalaxyl).

In 1999, 76 isolates were tested for sensitivity to metalaxyl and propamocarb. Eleven isolates had been collected from fields treated with metalaxyl and seven isolates from fields treated with propamocarb prior to collection. Only three of 76 tested isolates were highly sensitive to metalaxyl (Table 1), all of which came from fields not treated with fungicides prior to sampling. Mycelial growth of 30 of the isolates classified as non-sensitive was inhibited > 50% at a concentration of 100 ppm and they were therefore classified as partially sensitive. Fourty-one isolates were classified as resistant to metalaxyl (Table 1). Sixty of the isolates with partial and complete resistance originated from fields without metalaxyl applications prior to sampling, including all isolates collected from organic crops, which were not treated with the tested fungicide prior to sampling. Additionally, organic crops for seed tuber production were not treated with the tested fungicide the year before. Even at a concentration of 500 ppm, mycelial growth of 26 isolates (corresponding to 34% of the sample) was inhibited < 50% by metalaxyl; 14 isolates showed an inhibition of < 10% and were classified as highly resistant. Only three of these received a pre-treatment with metalaxyl. No correlation between level of metalaxyl resistance and mating type was found in the tested population. The data from all tested early (n=25) and late (n=51) samples showed no differences in percentage of resistant isolates.

For propamocarb, only three isolates had an ED_{50} of 100 ppm and were classified as highly sensitive in 1999 (Table 1). These same isolates tested very sensitive to metalaxyl. Ten isolates were classified as resistant to propamocarb, and three were highly resistant, showing an inhibition of < 50% at concentrations of 5,000 ppm. None of these ten isolates was from fields treated with propamocarb prior to collection of the infected leaves. Four originated from tomato plants and only one had the A2 mating type.

Mating types

A total of 56 A1 and a single A2 mating type were detected among the German *P. infestans* isolates collected in 1995. In 1999, 72 isolates were mating type A1 and 12 were A2. The distribution of mating types was different for isolates collected from potatoes and tomatoes. Nearly 20% of the isolates collected from potato plants were of the A2 mating

Table 1 German *Phytophthora infestans* isolates sampled in 1999, their specific origin, haplotype, mating type and fungicide sensitivity

Number[a]	Date of collection	Origin (postal CIP code)[b]	Field[c]	Host[d]	Mating type	Haplotype	Metalaxyl[e]	Propamocarb[e]	Number[a]	Date of collection	Origin (postal CIP code)[b]	Field[c]	Host[d]	Mating type	Haplotype	Metalaxyl[e]	Propamocarb[e]
1	26.06	92439	C	P	A1	Ia	R	I	43	12.08	37218	O	P	A2	IIa	R	I
2	05.07	85551	C	P	A1	Ia	S	S	44	12.08	37218	O	P	A2	IIa	–	I
3	15.07	94527	C	P	A1	Ia	R	I	45	19.08	81247	A	P	A2	–	–	I
4a	16.07	82281	O	P	A1	Ia	R	I	46c	19.08	85375	A	P	A1	Ia	R	I
5	16.07	85235	O	P	A2	Ia	I	I	47	19.08	81247	A	T	A1	Ia	R	R
6b	16.07	85236	O	P	A1	Ia	R	I	48c	19.08	80687	A	P	A1	Ia	R	I
7	16.07	93333	C	P	A1	–	R	I	49	19.08	80687	A	T	A1	Ia	I	I
8a	16.07	86551	C	P	A1	Ia	R	I	50	19.08	80687	A	T	A1	Ia	I	R
9	16.07	86551	C	P	A2	–	I	I	51	19.08	80992	A	T	A1	IIa	I	I
10b	16.07	86368	C	P	A1	Ia	R	I	52	19.08	80992	A	P	A2	Ia	I	I
11	16.07	86159	C	P	A1	–	R	I	53d	19.08	85764	A	T	A1	Ia	R	I
12a	16.07	96231	C	P	A1	Ia	I	I	54d	19.08	85764	A	T	A1	Ia	I	R
13a	18.07	89335	C	P	A1	Ia	I	I	55d	19.08	85764	A	T	A1	Ia	I	I
14	16.07	82269	O	P	A1	–	–	–	56e	19.08	85764	A	T	A1	Ia	I	I
15b	16.07	85235	O	P	A1	Ia	R	I	57	19.08	80639	A	P	A1	Ia	R	I
16	16.07	86511	O	P	A1	Ia	R	I	58f	19.08	80639	A	T	A1	Ia	I	R
17	18.07	85298	O	P	A1	IIa	R	I	59e	19.08	80639	A	T	A1	Ia	I	I
18b	18.07	85298	O	P	A1	Ia	R	I	60g	19.08	80639	A	P	A1	Ia	R	I
19	19.07	85649	C	P	A2	Ia	–	–	61	19.08	80639	A	T	A1	Ia	S	S
20a	27.07	86551	C	P	A1	Ia	R	I	62d	19.08	80639	A	T	A1	Ia	I	I
21b	27.07	86551	C	P	A1	Ia	R	I	63h	19.08	80995	A	T	A1	IIa	I	I
22b	27.07	86551	C	P	A1	Ia	R	I	64	19.08	80995	A	P	A1	Ia	I	R
23	27.07	86666	C	P	A1	Ia	I	R	65h	19.08	80995	A	T	A1	IIa	I	I
24a	27.07	86666	C	P	A1	Ia	R	I	66h	19.08	80995	A	T	A1	IIa	I	I
25	28.07	86551	C	P	A1	Ia	I	I	67	19.08	80995	A	P	A1	Ia	R	I
26	28.07	86666	C	P	A1	–	I	H	68i	18.08	85551	C	P	A1	Ia	R	I
27	28.07	86666	C	P	A1	–	I	H	69g	18.08	81247	C	P	A1	Ia	R	I
28	07.08	81247	A	P	A2	Ia	I	I	70	18.08	81247	C	P	A2	Ia	R	I
29	07.08	81247	A	T	A1	Ia	R	I	71f	19.08	80687	A	T	A1	Ia	S	S
30	07.08	81247	A	P	A1	Ia	R	I	72	25.08	85298	C	P	A1	Ia	R	I

Table 1 (continued)

Number[a]	Date of collection	Origin (postal CIP code)[b]	Field[c]	Host[d]	Mating type	Haplotype	Metalaxyl[e]	Propamocarb[e]	Number[a]	Date of collection	Origin (postal CIP code)[b]	Field[c]	Host[d]	Mating type	Haplotype	Metalaxyl[e]	Propamocarb[e]
31	09.08	85391	C	P	A1	–	–	–	73g	25.08	82256	C	P	A1	Ia	R	I
32	09.08	85411	C	P	A1	–	–	–	74i	25.08	82256	C	P	A1	Ia	R	I
33	09.08	85354	C	P	A1	Ia	I	I	75	25.08	80687	C	P	A1	Ia	R	I
34	09.08	81247	A	P	A1	Ia	–	–	76	25.08	81241	C	P	A2	Ia	I	I
35	09.08	85354	C	P	A1	Ia	R	I	77i	29.08	87767	C	P	A1	Ia	R	I
36	09.08	85298	C	P	A1	Ia	R	R	78g	29.08	87700	C	P	A1	Ia	R	I
37	09.08	85298	C	P	A1	–	–	–	79	26.08	94315	C	P	A1	–	R	I
38	09.08	85298	O	P	A1	Ia	R	I	80	05.09	87727	A	T	A1	IIa	I	I
39	09.08	86551	O	P	A1	Ia	R	R	81	08.09	85354	A	T	A1	Ia	I	I
40	12.08	85452	C	P	A1	–	R	I	82	16.09	34117	A	T	A2	Ia	I	I
41	12.08	37218	O	P	A2	Ia	R	H	83	17.09	34117	A	T	A1	Ia	I	I
42	12.08	37218	O	P	A1	IIa	R	I	84	21.09	85354	A	T	A1	Ia	I	I

[a] An identical letter after the isolate number shows a AFLP genotype, as indicated by identical AFLP bands

[b] An identical postal CIP code indicates the same origin within a small area

[c] C = conventionally managed field; O = organically managed field; A = allotment garden

[d] P = potato; T = tomato

[e] R = resistant; I = intermediate; S = sensitive; H = highly sensitive

type, while only a single A2 isolate (5%) was collected from tomato (Table 1). No differences in the ratio of A1 and A2 mating types were found in allotment gardens compared to commercial fields, or fungicide-treated fields versus untreated organic crops. Furthermore, no shifting in the ratio of A1 and A2 mating types was found during the growing season. In neither year were self-fertile isolates detected.

mtDNA haplotyping

Sixty-nine of the 84 isolates collected from German crops in 1999 were tested. All of the tested strains were characterised as either haplotype Ia or IIa based on the restriction fragments visualised after digestion of the amplified mtDNA regions (Table 2). The majority (n=60) were Ia, with fewer (n=9) IIa types. The 32 isolates originating from the Valle de Toluca were of the Ia haplotype and all eight African reference isolates showed restriction fragments typical of the 'old', asexually reproducing population with haplotype Ib (Table 2). No association between mtDNA haplotype and mating type was found in the German population of *P. infestans* (Table 1); 51 of the 58 isolates with the A1 mating type were Ia haplotype, whereas nine of the 11 isolates with the A2 mating type were of this haplotype. There was also no association between mtDNA haplotype and host.

AFLP fingerprinting

Fifty-two isolates collected in 1999 in Germany were successfully cultivated for analysis of AFLP fingerprints. The results based on the exact test for population differentiation indicated a significant population sub-structuring between *P. infestans* populations originating from Mexico, Bavaria and Africa (Fig. 2); χ^2 test statistic probabilities for pair-wise

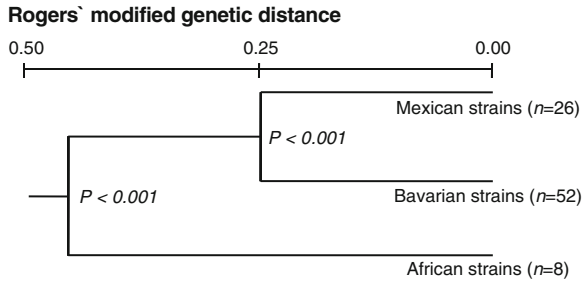

Fig. 2 Phenogram based on unweighted paired-group method with arithmetic mean (UPGMA) clustering of Rogers` modified genetic distances between the African, Mexican and German populations of *P. infestans*. Differentiation among populations was estimated using an exact test and significance levels are presented at the nodes

comparisons between the three populations led to the rejection of the null hypothesis of an absence of population differentiation (P>0.001). The percentage of clonality (as represented by 100 × (1-(number AFLPgenotypes/number isolates)) was 42%, lower than that observed in the African isolate sample (50%) but higher than that among isolates from central Mexico (19%). With this estimate of clonality, small population samples tend to underestimate clonality so it is conceivable that the clonal nature of the African *P. infestans* population will be > 50%. In any case, clonality in the Bavarian *P. infestans* population is lower than observed in the old, US1 clonal lineage population present in Africa. Virtual heterozygosity in *P. infestans* isolates varied considerably for the three populations studied. Observed virtual heterozygosity was highest in the Mexican isolates (20%), followed by German strains (16%), and was lowest in the African isolates (10%). The proportion of polymorphic loci was 56.5% and 29.4% for Mexican and African strains, respectively. German strains showed 47.5% polymorphic loci.

UPGMA grouped the German population of 29 multilocus AFLP genotypes among the 52 tested isolates based on 111 putative dominant loci into four

Table 2 Characteristics of the African, Mexican and German populations of *Phytophthora infestans*

Population	AFLP analysis		mtDNA haplotypes		
	No. of isolates	No. of genotypes	Ib	Ia	IIa
Africa	8	4	8	0	0
Mexico	32	26	0	32	0
southern Germany	69	30	0	60	9

distinct clusters (Fig. 3). Cluster 1, consisting of eight genotypes combining isolates collected from tomato as well as from potato hosts, was clearly separated from the isolates collected from potatoes. Isolates 63, 65 and 66 were collected within the same allotment garden from different host plants (Table 1) and were of the same AFLP genotype, whereas isolate 64 from the same origin belonged to cluster 2 (Fig. 3). Isolates 51 and 52 were from different host plants of the same geographical origin (Table 1), but they were of different AFLP genotype.

Clusters 2 and 3 contained isolates exclusively from potatoes. Cluster 2 consisted of seven AFLP genotypes, mainly from late-sampled isolates. Isolates 68, 74 and 77 were of the same AFLP genotype and were collected from different sites, far away from each other. The same is true for isolates 60, 69, 73 and 78. Isolates 43 and 44 were of identical geographical origin and from the same host, but they differed in AFLP-genotype. Cluster 3 consisted of four genotypes, mainly from early-sampled isolates within approximately one month after disease outbreak. The isolates within each of the AFLP genotypes were partially of different geographical origin. Cluster 4 consisted exclusively of isolates collected from tomato hosts and was completely different from the other three clusters. It consisted of 10 AFLP genotypes. Isolates 53 to 55 belonged to the same AFLP-genotype and were collected from the same geographic origin but different host plants. Isolate 62 clustered with the 53–55 genotypes, but was of different geographical origin.

Other selection criteria such as farming system (organic/conventionally managed farms) with partially different seed origins or pre-treatment with fungicides did not produce subgroups according to the dendrogram in Fig. 3. Isolates collected within the same allotment garden from different host plants showed different patterns regarding the subdivision in potato and tomato isolates: in one of the sampled allotment gardens (isolates 57–62) potato isolate 60 belonged to a completely different cluster than the four other isolates from tomato plants (isolates 58, 59, 61 and 62). In another allotment garden (isolates 63–67) we found identical AFLP genotypes in potatoes and tomatoes (isolates 63, 65, 66), as well as different AFLP genotypes (isolate 64).

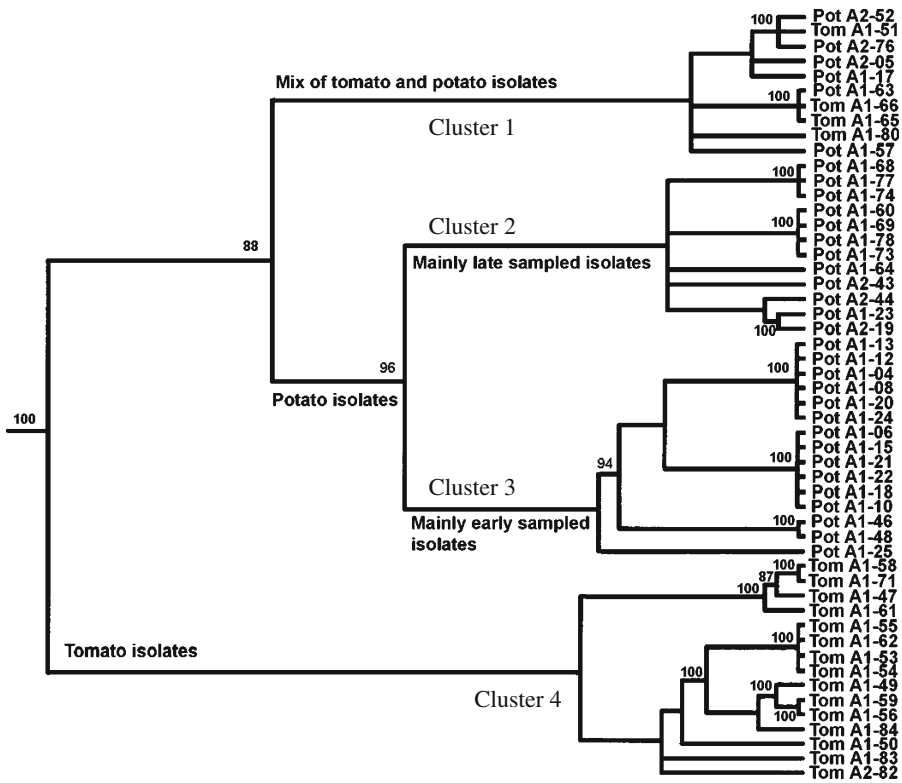

Fig. 3 Dendrogram showing the relationships within the German *Phytophthora infestans* isolates. Cluster analysis was performed by the unweighted paired-group method with arithmetic mean (UPGMA) on a distance matrix calculated with the Jaccard similarity coefficient from an AFLP phenotypic description of each isolate. The bootstrap values, noted as numbers on the main branches of the dendrogram, indicate the percentage of the interactions in which the major groups of isolates were formed. Only bootstrap > 70% are indicated

Sensitivity to metalaxyl differed partially within individual AFLP genotypes: the resistant isolate 71 belonged to the same AFLP genotype as the very sensitive isolate 58. Also the very resistant isolate 74, the partially sensitive isolate 77 and the very sensitive isolate 68 formed part of one group of clonally-identical isolates. The isolates of the group sampled at the beginning of the growing season (isolates with a sequential number < 27), all tested as highly resistant to metalaxyl.

Discussion

In the sampled southern German population of *P. infestans* there is a high degree of resistance to metalaxyl. Like Chycoski and Punja (1996) in Canada and Matuszak et al. (1994) in Mexico, no association between metalaxyl applications and metalaxyl resistance was detected. Nevertheless, most metalaxyl applications are effective against *P. infestans*. Dowley et al. (2002) reported that during an 18-year period, resistance was always lower at the beginning of the season and increased as the season progressed. Furthermore, they stated that phenylamide-resistant strains of *P. infestans* do not overwinter as effectively as sensitive strains. Gisi and Cohen (1996) found that at the end of the growing season, when host leaf area is severely limited at the end of the epidemic, the slower lesion expansion of the sensitive subpopulation together with its longer infectious period works in its favour. A further explanation for the apparent high effectiveness of metalaxyl applications in spite of the insensitivity found within the populations, might be that metalaxyl was applied together with another agent (mancozeb). Therefore, the effect of mancozeb together with a residual effect of prior applications of fungicides may counteract the higher degree of insensitivity to metalaxyl within the population. During the 1990s metalaxyl resistance was associated with the A1 mating type in many European countries (Gisi and Cohen 1996; Day et al. 2004; Lehtinen et al. 2008). In the present study, no association between mating type and metalaxyl sensitivity was found. Lehtinen et al. (2008) postulated that sexual reproduction, if present, should break any associations unless the genes for resistance and mating type are closely linked. This hypothesis was extensively tested in *P. infestans* population studies in central Mexico by Grünwald et al. (2001). No association between mating type and metalaxyl resistance was found in sexually reproducing *P. infestans* populations from both cultivated potatoes and wild *Solanum* species.

The fact that metalaxyl resistance was found also on untreated fields agrees very well with findings of other authors (Fraser et al. 1999; Day et al. 2004). Our own data suggest that a high rate of migration of resistant isolates into unsprayed fields took place, as reported previously by other authors (Fraser et al. 1999). Fraser et al. (1999) found that the frequency of metalaxyl-resistant isolates was not significantly greater in metalaxyl-sprayed than in unsprayed fields. Other authors have shown that the application of metalaxyl can select for individuals in a population with increased insensitivity to the fungicide (Daggett et al. 1993).

Metalaxyl resistance has been widely investigated, while resistance to other fungicides has attracted much less attention. Results showed that some isolates were able to sporulate on 1,000 ppb propamocarb. Therefore, the sensitivity of some strains to this fungicide was reduced. No association between prior propamocarb application and resistance was detected. This is in accordance with the monitoring results from Finland reported by Lehtinen et al. (2007), who reported that only a very low proportion of sampled isolates showed increased insensitivity to propamocarb, and that during the period from 1997–2000 there was a shift towards increased insensitivity to propamocarb. The data also agree with the monitoring results of a European population sampled by Bardsley et al. (1996). Furthermore, no indication of cross-resistance between metalaxyl and propamocarb has been found.

Temporal fluctuations in the A1:A2 mating type frequencies as found in the present study between 1995 and 1999 have also been found in some other studies carried out in Europe (Gisi and Cohen 1996; Bouws and Finckh 2007; Lehtinen et al. 2007). The reasons for the fluctuations are unexplained. The results demonstrating that the A2 mating type was more frequent on potato than on tomato crops were in contrast to findings reported from other studies (Lebreton and Andrivon 1998; Lebreton et al. 1999; Ordoñez et al. 2000), and contradicts the evidence from other regions of an association between the host and the mating type by

host-plant specificity (Ordoñez et al. 2000). It seems that there are no obvious rules governing the A1:A2 ratio within the potato and tomato populations of *P. infestans* (or at least none we can identify), and the results found by different authors are casual.

The frequency of the mating types was similar in gardens, organic and conventional crops. This contrasts with results from southern Flevoland in The Netherlands (Zwankhuizen et al. 2000), and from Scandinavia (Lehtinen et al. 2007), where A2 mating types occurred more commonly in gardens and allotments than commercial fields. Lehtinen et al. (2007) pointed out that blight epidemics are usually much more severe in gardens and organic fields than in conventional fields, as the use of fungicides is prohibited in organic fields and infrequent in gardens. More severe epidemics together with virtually continuous potato cropping in gardens increases the risk of oospore-derived infections and consequently may have led to the more common presence of both mating types than in conventional fields (Lehtinen et al. 2007). Our results agree well with those reported by Day et al. (2004). It seems that—similar to the situation comparing potatoes and tomatoes—the A1:A2 ratio of a specific subpopulation is casual rather than forced by any kind of management practice, or it depends on the interaction of local factors and local populations in a specific year.

As demonstrated for East Germany during the early 1990s by Daggett et al. (1993), the hypothesis that the 'old' population of *P. infestans* is still predominant in the southern part of Germany can also be rejected since mtDNA haplotype IB was absent in the southern German *P. infestans* population during 1999. Also other recently characterised German populations were dominated by individuals belonging to the 'new' population (Rullich et al. 2002; Bouws and Finckh 2007). A substantial level of genetic variation was observed in the sampled population of *P. infestans* using mtDNA haplotyping and AFLP fingerprinting, as also found by Bouws and Finckh (2007). The variation at the DNA level was smaller compared to that found for isolates originating from the Valle de Toluca, but was much greater compared to the variation found in isolates from a clonal, asexually reproducing population. We conclude that also in southern Germany the old population has been displaced by a new *P. infestans* population, as found for many other regions in Europe (Drenth et al. 1993; Fry et al. 1993; Lebreton and Andrivon 1998).

Overall, the *P. infestans* populations studied were highly diverse with respect to fungicide resistance and AFLP fingerprints. While few AFLP genotypes dominated the population early in the season, the population of *P. infestans* became more diverse during the season. The results of AFLP analysis showed that *P. infestans* can build up a separate population in potatoes and tomatoes, even within a small area such as an allotment garden, where potatoes and tomatoes are grown side by side. For example, isolates 57–62 originated from a single allotment garden in Munich. The potato isolate 60 belonged to a completely different cluster than the four other isolates from tomato plants (58, 59, 61 and 62). This finding is consistent with previous reports (Legard et al. 1995; Lebreton and Andrivon 1998; Oyarzún et al. 1998; Lebreton et al. 1999), and supports the view that some specificity to the host plant and host adaptation exists within *P. infestans*. However, there is also a subpopulation which is able to attack both hosts, as shown by the dendrogram in Fig. 3. Furthermore, findings in the allotment garden 'Feldmoching' showed identical AFLP genotypes in potatoes and tomatoes (isolates 63, 64 and 66), showing some exchange of population between hosts. This was in line with some other findings reported in the available literature. Knapova et al. (2002) reported that many isolates were highly aggressive to both hosts, but simultaneously some isolates preferred tomato and potato plants, respectively. Shattock (2002) found clonal lineages particularly aggressive to potato, and clonal lineages which attacked potato and tomato equally well; another clonal lineage was particularly aggressive towards the latter, and has been identified as a sexual recombinant. Lebreton and Andrivon (1998) stated that the differences in population structures may result from asexual vs. sexual reproduction, but might also depend on the cultural calendar of the two crops. It can be concluded that gene flow between *P. infestans* populations on tomato and potato took place, but the flow has been restricted by the need for specific adaptation, as argued, e.g., by Lebreton et al. (1999).

The fact that at the beginning of the growing season only low genotypic variation was detectable provides some indications that oospores were not a major source of primary infection. *Phytophthora*

infestans populations are characterised by patchiness and high rates of extinction and recolonisation from one season to the next (Fry et al. 1992). The detected changes in population structure in time and space during a season are explainable by the infection by oospores later in the growing season, or by immigration of *P. infestans* strains from other fields or regions. Montarry et al. (2008) also interpreted the lack of specific host adaptation at local and regional scales as a consequence of extensive gene flow between populations across regions, coupled with a wide distribution of specific cultivars. Bouws and Finckh (2007) found clear effects of the origin of seed potatoes with respect to pathotypes and rep-PCR fingerprints. It seems that in the sampled year 1999 the infections early in the season originate only from a few primary foci within the sampled area, as demonstrated by the low genotypic variability early in the season, confirming observations made by van der Zaag (1956) in The Netherlands that only few primary foci are responsible for the outbreak of the epidemic within a large growing area. Thus, the epidemiology of *P. infestans* populations by asexual reproduction during the epiphytotics in the growing season, followed by the winter 'bottleneck', may mean that each year, the pathogen populations depend entirely upon which few 'founder' isolates happened to survive the winter (Shaw 1994). As shown by results presented in Fig. 3, during the growing season the genetic structure of the population became more diverse. Bouws and Finckh (2007) also found changes and a reduction of the genetic diversity during the winter months and interpreted this result as a consequence of the genetic bottlenecks a predominantly asexually reproducing population has to go through each winter.

The fact that most of the isolates collected from organic crops were resistant to fungicides, and the results of the dendrogram showing some population similarities between organic and conventional crops, support the notion that there is an intensive migration of the pathogen between fields within the whole sampled region, including potatoes in allotment gardens.

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