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Inoculum sources and genotypic diversity of *Phytophthora infestans* in Southern Flevoland, the Netherlands

M.J. Zwankhuizen[1,2], F. Govers[1,3,*] and J.C. Zadoks[1,2]
[1]*Laboratory of Phytopathology, Wageningen University,* [2]*C.T. de Wit Graduate School for Production Ecology & Resource Conservation,* [3]*Graduate School for Experimental Plant Sciences, Wageningen, The Netherlands;* [*]*Author for correspondence: Laboratory of Phytopathology, Wageningen University, Binnenhaven 9, 6709 PD, Wageningen, The Netherlands (Fax: +31-317-483412; E-mail: francine.govers@medew.fyto.wau.nl)*

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Abstract

Genotypic changes in populations of *Phytophthora infestans* in Southern Flevoland (150 km^2) were analysed by characterising isolates from potato refuse piles, conventional and organic potato fields, and potatoes and tomatoes in allotment gardens for mating type (1712 isolates) and DNA fingerprint pattern using probe RG57 (1048 isolates). The overall percentages of genotypes (and of isolates) that were A2 varied from 32 (4) in 1994 to 45 (56) in 1996. Among the 1048 isolates 170 different genotypes were identified, of which 138 (81%) were 'rare' (i.e., detected in only one sampling site in the research area during 1993–1996). Many rare genotypes were encountered in organic potato fields and in allotment gardens. In 1994 and 1995, four genotypes were abundant. The highest percentages of isolates with these 'common' genotypes were encountered in refuse piles and conventional potato fields. The common genotypes were nearly absent in 1996, suggesting that the population may have passed through a bottleneck at the transition from 1995 to 1996. The Shannon index of genotypic diversity was high in allotment gardens and in organic potato fields. For the total populations the normalised Shannon index of genotypic diversity increased from 0.34 in 1994, with weather favourable to late blight, to 0.61 in 1996, with unfavourable weather. The high numbers of rare genotypes detected every year indicate that oospores may act as an infection source in commercial potato fields. However, refuse piles were identified as the most important infection sources for commercial fields in 1994 and 1995. In 1996 disease in commercial organic fields was probably initiated by a few genotypes originating from seed tubers. In allotment gardens oospores were probably the most important infection source.

Abbreviation: Rc – refuse pile, Fc – conventional potato field, Fo – organic potato field, A_p – potato plot in allotment garden, A_t – tomato plot in allotment garden.

Introduction

Since the 1840s, when *Phytophthora infestans* (Mont.) de Bary migrated from Mexico to other parts of the world (Goodwin et al., 1994b), late blight has been a serious threat to potato production world-wide (Hooker, 1981). Despite frequent fungicide applications, late blight epidemics have sometimes been uncontrollable, especially in recent years (Fry et al., 1993; Goodwin et al., 1995). Populations of 'new' genotypes of *P. infestans*, originating from central Mexico, displaced the old populations, which consisted mainly of a single asexual lineage (e.g., Spielman et al., 1991; Fry et al., 1992; 1993). Sexual reproduction

occurs in Europe as a result of the introduction in the 1970s of 'new' genotypes of both A1 and A2 mating type (Drenth et al., 1993; 1994; Sujkowski et al., 1994).

Diversity in the population may alter the epidemiology of *P. infestans* in two ways. First, if sexual reproduction occurs, oospores are formed which can survive in the soil in the absence of a host (Drenth et al., 1995). These oospores may serve as an inoculum source, additional to tuber-borne inoculum. Second, some 'new' immigrant genotypes, or recombinant progeny from oospores, may have higher pathogenic fitness than 'old' genotypes (Day and Shattock, 1997; Kato et al., 1997), resulting in increased epidemic growth rates.

The availability of isozyme markers (Tooley et al., 1985) and of molecular markers, e.g., probe RG57 (Goodwin et al., 1992a), has stimulated research on populations of *P. infestans*. Most of these studies are focused on population changes, migrations, and the possible occurrence of sexual reproduction, and concern large geographic areas, i.e., counties, states, countries, or continents (Drenth et al., 1993; Forbes et al., 1997; Goodwin et al., 1994a; 1995; Koh et al., 1994; Sujkowski et al., 1994). Our studies focused on a small area of approximately 150 km^2 in size, located in Southern Flevoland (the Netherlands) where seed and warehouse potatoes are common crops. Previously analysis of the small-scale epidemiology of late blight in this area showed that refuse piles with asexual inoculum were the most important sources for the initiation of epidemics (Zwankhuizen et al., 1998). In addition, analyses of disease gradients showed that infested fields of organically grown potatoes could act as major infection sources for conventional crops and comparison of DNA fingerprint patterns from isolates collected in disease foci suggested that oospores played a role as initial inoculum in a few cases.

In this paper, the genotypic composition of pathogen populations in Southern Flevoland are analysed, with the objective to assess the role of oospores in initiating disease at various kinds of sampling sites and in various years.

Materials and methods

Research area, surveys and site selection

During the growing seasons of 1994, 1995 and 1996, late blight development was monitored over an area of 150 km^2 in Southern Flevoland, a polder in the central part of the Netherlands. Details are described in Zwankhuizen et al. (1998). We sampled five types of sites: refuse piles with potato plants (Rc), conventional (Fc) and organic (Fo) potato fields, and potato (A_p) and tomato (A_t) plots in allotment gardens of four different compounds (Aa, Ah, An, Asp).

At the start of each growing season, refuse piles at randomly selected conventional farms were inspected. Subsequently, fields with volunteer plants, potato fields, and allotment gardens were selected at random and inspected. In each compound of allotment gardens, 40 randomly chosen potato plots were inspected per inspection round. If present, tomato plots were inspected in each individual garden in which potatoes were inspected. Sites were visited regularly throughout the growing season. Some potato sites, where disease developed rapidly, were visited more frequently than others. Occasionally, potato fields and refuse piles in potato production areas adjacent to the research area were visited, particularly when late blight appeared earlier in those areas than in the research area.

Collection of isolates

Isolates of *P. infestans* were collected in 1993 at the end of the growing season and during the growing seasons of 1994, 1995, and 1996 (Table 1). Nine infested refuse piles were encountered in 1994, seven of which were sampled. In 1995, two infested refuse piles were found and sampled and in 1996 none. However, in 1996 isolates were collected from a large refuse pile (500 m^2) in Wezep, located 28 km north-east of the research area.

In 1994, isolates were collected from 30 conventional fields. Isolates from 17 of these fields were DNA fingerprinted. Four of these conventional fields, sampled during the early outbreak in June, were amongst the first infested fields in the area. The other 13 conventional fields were sampled during a second outbreak of the disease in August and September (Zwankhuizen et al., 1998). In 1995 and 1996, when disease pressure was low, isolates from nearly all infested conventional and organic potato fields were characterised.

In each compound of allotment gardens, five to eight of the first diseased potato plots were sampled each year. In 1994 and 1996, only isolates from the compounds An and Ah were included in the fingerprint analyses. In 1996, five randomly selected tomato plots from the compounds An and Ah were sampled and characterised.

Isolates were obtained from single lesions. The sampling units were either disease foci, or entire sites.

Table 1. Numbers of isolates and genotypes of *P. infestans* collected from various types of sampling sites in Southern Flevoland, and characterised for mating type and RG57 fingerprint

Year	Sampling period	Site[1]	No. of sites sampled	Isolates collected	Characterised by fingerprinting (no. of sites)	Genotypes[2] (rare)	Percentage of A2 isolates (genotypes)
1993	10/09–21/10	Fc	3	104	40 (3)	6 (2)	4 (17)
1994	28/04–10/06	Rc	7	62	47 (7)	8 (4)	2 (13)
	03/06–23/09	Fc	30	292	140 (17)	8 (2)	1 (38)
	15/06, 19–26/08	Fo	6	70	57 (5)	9 (3)	6 (33)
	01/07–09/08	A_p	21	108	33 (9)	8 (2)	11 (13)
Total			64	532	277 (38)	22 (11)	4 (32)
1995	13/06–19/06	Rc	2	43	40 (2)	4 (2)	0 (0)
	20/06–26/07	Fc	4	73	58 (2)	11 (6)	25 (36)
	05/07–07/08	Fo	9	209	105 (6)	19 (9)	9 (27)
	27/06–07/08	A_p	24	205	180 (21)	39 (31)	31 (46)
Total			39	530	383 (31)	66 (48)	19 (39)
1996	08/08	Rc[3]	1	30	29 (1)	2 (0)	0 (0)
	20/09	Fc	2	11	9 (2)	5 (4)	9 (20)
	25/07–16/08	Fo	7	203	162 (6)	44 (37)	32 (45)
	01/08–06/09	A_p	22	221	81 (11)	26 (16)	90 (58)
	22/08–06/09	A_t	10	81	67 (10)	28 (20)	51 (36)
Total			42	546	348 (30)	97 (77)	56 (45)
Grand total			148	1712	1048 (102)	170 (138)	25 (44)

[1]Rc = refuse pile, Fc = conventional potato field, Fo = organic potato field, and A_p = potato plot in allotment garden, A_t = tomato plot in allotment garden.
[2]Numbers of genotypes (mating type+RG57 pattern) do not add up because they can occur in more than one type of site, and in more than 1 year.
[3]In 1996 the only Rc site that was sampled was located 28 km north-east of the research area in Wezep.

In the early phase of the epidemics, disease occurred in foci; 10 or 30 isolates per focus were collected (Zwankhuizen et al., 1998). Entire potato sites (refuse piles, conventional and organic fields) were sampled when disease levels were so high that disease was found all over the site (e.g., potato fields sampled later in the season), or when no disease foci could be found, as in refuse piles because of mechanical disturbance. In the infested organic fields, 30 isolates were collected randomly along two transects, unless stated otherwise. From conventional fields, a total of 10–30 isolates was obtained, generally along two transects (Zwankhuizen et al., 1998). In allotment gardens, one isolate per randomly chosen diseased plant was collected, up to 10 per plot.

Individual isolates were obtained from single lesions on stems or leaves (Davidse et al., 1989). Pure cultures were obtained by plating the mycelium on Rye A medium (Caten and Jinks, 1968), amended with antibiotics, and maintained on Rye A medium at 18 °C in the dark. For long-term storage the isolates were transferred to liquid nitrogen.

Characterisation of isolates

Isolates were analysed for mating type (Fry et al., 1991) and for nuclear DNA fingerprint using probe RG57 (Goodwin et al., 1992a). Culturing of isolates, DNA extraction, digestion with restriction enzyme *Eco*RI, and Southern blot analysis were carried out as described by Drenth et al. (1993). A multilocus phenotype (here referred to as genotype) was constructed for each isolate by combining mating type and DNA fingerprint data (Goodwin et al., 1995). Genotypes were arbitrarily numbered, with a prefix NL. A genotype was designated 'rare' when one or more isolates of this genotype were detected at only one sampling site in the research area between 1993 and 1996.

Bioassay

In allotment gardens, compost heaps are frequently made of diseased potato and tomato foliage. The material is often used as organic amendment in the

following season. Six samples from both tomato and potato waste piles were composted from September 1996 to May 1997. Shredded compost was baited with potato leaflets to see if oospores were present. The oospore bioassay was performed as described by Drenth et al. (1995).

Data analysis

The absolute and relative frequencies of genotypes per sampling site are the response variables in this study. Contingency Chi-square analyses were conducted to detect differences in genotypic composition among populations of *P. infestans* in time and space. In the case of 2 × 2 tables, Fisher's Exact Test (Sokal and Rohlf, 1981) was applied.

Genotypic diversity includes both genotype richness (i.e., the number of different genotypes in the sample) and genotype evenness (i.e., the relative frequencies of the genotypes in the sample) (Peet, 1974). The spatial component of diversity was considered by analysing diversity among categories of potato sites, and among sites of one category. A special case concerned the diversity of *P. infestans* in 1994 at different downwind distances along a disease gradient, originating from organic potato fields (Zwankhuizen et al., 1998). Diversity in time was studied within and among seasons.

The index used for diversity was the Shannon index h_0 (Bowman et al., 1971) defined as

$$h_0 = -\sum_{i=1}^{k} p_i \cdot \log_e p_i$$

where p_i is the frequency of isolates with the ith genotype and k is the number of genotypes in the sample. As a normalised Shannon statistic, we used $h_0' = h_0/\log_e N$ where h_0 is the original Shannon index and N is the total number of isolates (Goodwin et al., 1992b). This statistic, presenting the Shannon index as a fraction of the maximum diversity in the sample, has been found to be relatively stable and has provided a good measure when sample sizes varied (Sheldon, 1969). Depending on the hypotheses to be tested, either single sites or groups of sites were considered. When single sites were used, sites from which only one isolate was characterised were excluded from the analyses. Differences between the diversities of pairs of samples were calculated using the *t*-test of Hutcheson (1970). Calculations were made using BIODIV, version 5.1 (Baev and Penev, 1995).

Genotypic distance D was calculated as $1 - F$ where F is the Dice coefficient (Rohlf, 1993)

$$F = 2N_{XY}/(N_X + N_Y)$$

and N_X and N_Y refer to the number of DNA fragments in isolates X and Y, respectively, and N_{XY} is the number of fragments shared by the two isolates. In these calculations, the mating type locus was treated as a DNA locus.

Results

Late blight disease during 1993–1996

During 1993, samples were taken at the end of a moderate epidemic. During 1994, 1995 and 1996 the sampling frequencies and the numbers of isolates obtained reflect the different epidemic patterns (Table 1). Late blight development differed substantially between these years (Zwankhuizen et al., 1998). Briefly, rapid epidemic development due to favourable weather in 1994 resulted in disease throughout the research area by mid-June (early outbreak). Disease intensities decreased during a hot and dry period in July, after which late blight development was enhanced again by favourable weather, resulting in the spread of *P. infestans* over many fields (second outbreak). In 1995, disease developed about 3 weeks later than in 1994, and ceased by the end of July, due to hot and dry weather. The overall disease levels were lower than in 1994. In 1996, disease was first recorded by the end of July in organic potato fields and allotment gardens. Weather conditions were more or less favourable for the disease from the last week of July until the end of the growing season. In all 3 years, disease in allotment gardens was first observed in potato plots and developed on adjacent tomato plants within 1 month.

Overview of genotypes and mating types

Characterisation of the 1048 isolates collected on potatoes and tomatoes, 1993 through 1996, revealed 154 different RG57 DNA fingerprints. Including the mating type, 170 different multi-locus genotypes were distinguished (Table 2). In the 3 years that sampling took place throughout the growing season the number of genotypes detected varied from 22 in 1994 to 97 in 1996.

Table 2. Genotypes of 1048 isolates of *Phytophthora infestans* collected from September 1993 to September 1996 in Southern Flevoland and adjacent areas in the Netherlands

Genotype[1]	MT	RG57 fingerprint[2]	N[3]	Year	Genotype[1]	MT	RG57 fingerprint[2]	N[3]	Year
NL-1	A1	01000000011111001110101	1	94	NL-54	A2	11010001001110000110111	1	95
NL-2	A2	01011001011110101110011	3	95	NL-55	A2	11010001011011001110011	1	94
NL-3	A2	01011001011111011010011	10	95	NL-56	A1	11010010011110001110101	11	95
NL-4	A2	01011001111110101110011	1	95	NL-57	A1	11010010011111000110111	1	95
NL-5	A1	10000000001111001110101	3	95	NL-58	A2	11010010011111001110101	2	94
NL-6	A2	10000000011110000110101	3	93	NL-59	A2	11011000011110100110101	1	95
NL-7	A2	10010000001111001110101	1	95	NL-60	A2	11110100001111001110101	1	95
NL-8	A1	10010000011110000110101	13	95–96	NL-61	A1	11110100011110101111101	1	95
NL-9	A2	10010000011110001110101	17	95–96	NL-62	A1	11110100011111000110101	10	95–96
NL-10	A1	10010000011111000110101	2	95–96	NL-63	A2	11110100011111000110101	1	95
NL-11	A2	10010000011111010110101	9	95	NL-64	A1	11110100011111001110101	4	95–96
NL-12a	A1	10010010001110000110101	5	95	NL-65	A2	11110101011111000110101	2	95
NL-12b	A2	10010010001110000110101	5	95	NL-66	A2	11110101011111001110101	4	95
NL-13	A2	10010010011111000110101	2	95	NL-68	A2	11110110011111000110101	2	95–96
NL-14	A2	10010010011111001110001	2	95	NL-69	A1	11110110011111000110101	39	94–95
NL-15	A1	10010010011111010110101	2	95	NL-70	A2	11110110011111000110111	6	95
NL-16	A1	10010011011111000110101	1	95	NL-71	A1	11110110011111001110011	2	95
NL-17	A1	10011001011101010011101	10	95	NL-72	A1	11111100011111000010111	10	95
NL-18	A1	10011011011110001110101	1	95	NL-73	A1	11111100011111000110101	1	95
NL-19	A2	10110000011110000110101	5	94	NL-74	A1	11111101011111000110101	4	94
NL-20	A2	10110100001110010110101	1	95	NL-75	A1	11111101011111001110101	62	94–96
NL-21	A1	10110100001110101111101	3	95	NL-76	A1	11111110011111000110101	163	93–95
NL-22	A1	10110100001111000110101	1	95	NL-77	A2	11111110011111000110101	3	94
NL-23	A1	10110100011110100110101	1	95	NL-78	A1	11111110111111010110101	1	94
NL-24	A2	10110100011111001110101	11	95–96	NL-79	A1	11111111011111000110101	1	95
NL-25	A2	10110101011011001110101	2	95	NL-80	A1	11111111011111001110101	2	95–96
NL-26	A1	10110101011111000110101	8	95	NL-81	A2	10010000001111000110011	1	96
NL-27	A2	10110110001110000110101	1	94	NL-82	A2	10010000001111001110011	19	96
NL-28	A1	10110110001111000010101	1	95	NL-83	A1	10010000001111001110011	2	96
NL-29	A1	10110110011111000110101	14	93 + 95	NL-84	A1	10010001011110000110101	1	96
NL-30	A1	10110110011111001110101	9	93	NL-85	A1	10010010001110011110101	1	96
NL-31	A2	10110110011111001111101	1	95	NL-86	A2	10110100011110000110101	4	96
NL-32	A2	10111100011110001110101	9	95	NL-87	A2	10110100011110001110001	1	96
NL-33	A1	10111100011111000010101	1	95	NL-88	A2	10110100011110101110101	1	96
NL-34	A1	10111100011111001111101	2	95	NL-89	A1	10110101011110101110001	2	96
NL-35	A1	10111101011111000110101	1	95	NL-90	A1	10110101011111001110001	3	96
NL-36	A1	10111101011111001110101	3	94–96	NL-91	A1	10110101011111010110101	2	96
NL-37	A1	10111110011101000010101	8	95	NL-92	A1	10110110011110001110001	1	96
NL-38	A1	10111110011111000110101	1	93	NL-93	A2	10111100011110000010101	6	96
NL-39	A1	10111110011111001110101	1	94	NL-94	A1	10111101011110101110011	1	96
NL-40	A1	10111111011110001110101	1	95	NL-95	A2	10111110001110100110101	2	96
NL-41	A1	10111111011111001110101	186	93–96	NL-96	A2	10111110011110100110101	2	96
NL-42	A1	10111111011111011110101	1	94	NL-97	A2	10111110111100100110011	1	96
NL-43	A2	11000000001110001110001	1	95	NL-98	A2	10111110111100100110101	6	96
NL-44	A2	11000000011110001110101	9	95	NL-99	A2	10111110111110100110101	1	96
NL-45	A2	11000000011110011110101	11	95	NL-100	A2	11000000001111001110001	1	94
NL-46	A1	11000010011111000110101	1	95	NL-101	A1	11000000011110100110011	1	96
NL-47	A1	11001000011110011110101	1	95	NL-102	A1	11000000011110100110101	1	96
NL-48	A1	11001000011110101110101	29	94–95	NL-103	A1	11000000011110100110111	1	96
NL-50	A2	11010000011111000110101	2	95	NL-104	A1	11010000001110101110011	1	96
NL-51	A1	11010000011111000110101	3	95	NL-105	A2	11010001011111001110101	1	94
NL-52	A1	11010000011111000110111	1	95	NL-106	A2	11010010001111001110011	1	96
NL-53	A1	11010000011111001110101	1	95	NL-107	A1	11010110011111000110101	1	94

Table 2. Continued

Genotype[1]	MT	RG57 fingerprint[2]	N[3]	Year	Genotype[1]	MT	RG57 fingerprint[2]	N[3]	Year
NL-108	A1	11011000001110101110101	1	96	NL-140	A2	10011000011111001110101	1	96
NL-109	A2	11110100011110000110101	1	96	NL-141	A2	10011100011111001110101	1	96
NL-110	A1	11110100011110000110111	1	96	NL-142	A2	10110000011111000110101	2	96
NL-111	A1	11110100011110100110011	1	96	NL-143	A1	10110100011110000110101	3	96
NL-112	A1	11110100011110101110001	22	96	NL-144	A1	10110100011110010110111	1	96
NL-113	A1	11110100011110101110011	30	96	NL-145	A2	10110100011111111110101	1	96
NL-114	A1	11110100011110101110101	1	96	NL-146	A1	10110101011110000110101	1	96
NL-115	A1	11110100011111001110001	1	96	NL-147	A1	10110110011110000110101	1	96
NL-116	A1	11110100111110101110011	1	96	NL-148	A2	10110110011110000111101	4	96
NL-117	A2	11110110011110100110101	2	96	NL-149	A1	10110110011110001110111	1	96
NL-118	A2	11110110011111001110101	1	96	NL-150	A1	10110110111111000110101	1	96
NL-119	A2	11111100011110000110101	1	96	NL-151	A1	10111000011110000110101	1	96
NL-120	A2	11111100011110000110111	1	96	NL-152	A1	10111100011110000110101	1	96
NL-121	A1	11111100011110000110111	1	96	NL-153	A1	10111111011111000110101	2	94
NL-122	A1	11111100011111001110101	1	96	NL-154	A1	11010000001110001110001	2	96
NL-123	A1	11111100011111001111101	24	96	NL-155	A1	11010000001110101110101	1	96
NL-124	A1	11111100111110101110011	1	96	NL-156	A1	11010000011110001110001	1	96
NL-125	A2	11111101011111000110101	1	96	NL-157	A2	11010000011111001110101	2	96
NL-126	A1	10110100001110101110001	8	96	NL-158	A2	11010010011110001110001	1	96
NL-127	A1	10110100111110101110011	1	96	NL-159	A1	11010010011111011110101	1	96
NL-128	A2	10000000011110001110101	1	96	NL-160	A2	11011000001111000110101	6	96
NL-129	A1	10010000011110000110011	2	96	NL-162	A1	11110100011111001010101	3	96
NL-130	A2	10010000011110000110101	1	96	NL-163	A2	11110100011111001110101	1	96
NL-131	A1	10010000011110000110111	4	96	NL-164	A1	11110101011111000110101	1	96
NL-132	A2	10010000011111001110101	3	96	NL-165	A1	11111000011111000110101	7	94
NL-133	A1	10010100011110000110101	1	96	NL-166	A2	11111100001111000110101	2	96
NL-134	A2	10010100011111000110101	1	96	NL-167	A2	11111100011110101110101	1	96
NL-135	A1	10010100011111000110101	2	96	NL-168	A2	11111100011111001110101	1	96
NL-136	A2	10011000011110000110101	7	96	NL-169	A1	11111101111111001110101	1	96
NL-137	A2	10011000011110001110101	38	96	NL-170	A2	10110110011110000110101	1	96
NL-138	A1	10011000011110001110101	6	94–96	NL-171	A1	10110100011111000110101	1	96
NL-139	A2	10011000011110011110101	3	96	NL-172	A2	10110100011111000110101	1	96

[1]Genotypes are composed by combining mating type phenotype (MT) and RG57 DNA fingerprint data, and are numbered arbitrarily; numbers do not correspond to those used by Drenth et al. (1993), Goodwin and Drenth (1997), and Forbes et al. (1998). Genotype numbers do not range from 1 to 170 because some genotypes, initially identified as distinct (numbers 49, 67, 161), were assigned to existing numbers (but number 12 is split into a and b).

[2]DNA fingerprint bands revealed by the moderately repetitive probe RG57 (Goodwin et al., 1992a). Presence of a band is indicated by 1, absence by 0. Bands listed from left to right are: 1, 2, 3, 5, 6, 7, 8, 9, 9a, 10, 13, 14, 14a, 16, 17, 18, 19, 20, 21, 23, 24, 24a, 25. Band numbers correspond to those used by Goodwin et al. (1992a,b; 1994b) and Drenth et al. (1993,1994). Bands 4 and 22 could not be scored unambiguously. Bands 11 and 15 have never been detected in the Netherlands.

[3]N=number of isolates.

The percentage of isolates with the A2 mating type differed dramatically between years and sampling sites (Table 1). The overall percentage of A2 isolates in 1996 (56%) was 14 times higher than in 1994 (4%). When the percentage of A2 genotypes instead of isolates was considered, differences between mating type ratios were less extreme. For example, the ratio of A1 : A2 isolates in the allotment gardens in 1996 was 199 : 22, whereas the ratio of A1 : A2 genotypes was 47 : 34, a ratio not significantly different from 1 : 1 (Fisher's Exact Test; $P = 0.52$).

Genotypic composition of P. infestans *populations*

Over the years 1993–1996, 149 genotypes were identified among 981 isolates collected from potatoes in refuse piles, commercial fields and allotment gardens. Four A1 genotypes (NL-41, NL-69, NL-75, and NL-76) were found in many sampling sites and in at least 2 years. In 1994, the percentage of isolates with these 'common' genotypes was highest in refuse piles and conventional fields (Table 3). The percentages

Table 3. Percentages of isolates of *P. infestans* belonging to one or more of the common genotypes NL-41, NL-69, NL-75, and NL-76, found in potato sites in Southern Flevoland

Year	Refuse piles	Conventional fields	Organic fields	Allotment gardens	Total (all sites)	Sample size
1993	—	65	—	—	65	40
1994	92	94	63	52	82	277
1995	90	52	52	23	42	383
1996	100*	0	3	0	12	281
Total	93	76	30	20	47	981

*Isolates collected from a refuse pile in Wezep, located 28 km northeast from Southern Flevoland.

of isolates belonging to these genotypes differed significantly over the years ($P < 0.001$), and between commercial potato sites (Rc + Fc + Fo) and allotment gardens within years ($P < 0.001$). On average, 70% of the isolates collected from commercial fields (Fc + Fo) during 1994 and 1995 belonged to these genotypes. In 1996, however, no common genotype was found in conventional fields whereas in organic fields common genotypes occurred at a low percentage (3%). In contrast, all isolates collected in 1996 from the infested refuse pile at Wezep were either NL-41 or NL-75.

The majority of the genotypes (81%), encountered on either potato or tomato from 1993 through 1996, was 'rare' (138 out of 170 genotypes, identified among 1048 isolates). When testing distributions of isolates, total percentages of rare genotypes were significantly different over years ($P < 0.001$; with the highest percentage in 1996), and over types of potato site within years ($P < 0.05$; with the highest percentages in organic fields and allotment gardens). When testing distributions of genotypes, these differences became smaller. Highest numbers of rare genotypes were found in the organic fields in 1996 (37), and in the allotment gardens of 1995 (31) and of 1996 (16 and 20, on potato and tomato respectively) (Table 1).

The number of different genotypes encountered in conventional potato fields was usually two or three. However, in June 1995 a conventional grower found disease scattered over an area of about 2 ha of his field (field Fc2). No sources of late blight were identified in the area surrounding this field. Analysis of the 30 isolates collected revealed a mating type ratio A1 : A2 of 12 : 18, not significantly different from 1 : 1. The isolates belonged to 10 genotypes, six of which were A1. Six genotypes were rare and none was common.

Characterisation of 162 isolates, collected from six organic fields in 1996, revealed 44 different genotypes (Table 1), 37 of which were rare. Two of these fields (Fo2 and Fo5) had an extremely diverse population, with 14 and 17 different genotypes, respectively, among a total of 48 isolates collected. The first disease focus in the research area found in 1996 was in organic field Fo1. Two rare genotypes were found among 9 isolates initially collected from that focus. One week later, another four genotypes, among which one was rare and one common (NL-75), were detected among 30 isolates collected in field Fo1.

In the allotment gardens, 52% of the isolates collected from potato in 1994 belonged to one of the common genotypes (occurring in four out of nine sampled plots in total) (Table 4). In 1995 and 1996, most potato plots contained rare genotypes. In 1995, all four potato plots in compound An contained common genotypes. In one of these, three common genotypes (NL-41, NL-69, NL-76) were identified. In the other three compounds, only one out of 17 plots contained a common genotype. In 1996, none of the 11 potato plots of two compounds contained one of the common genotypes, most of the genotypes being rare.

In 1996, 28 different genotypes were detected on tomatoes in allotment gardens (Table 1), most of them rare. In compound An, only two genotypes (NL-62, NL-131) out of 28 (7.1%) were detected on both tomato and potato. Genotype NL-62 was found in one potato plot and in three tomato plots, genotype NL-131 was found in one potato plot and one tomato plot. In compound Ah, three genotypes (NL-136, NL-137, NL-143) out of 21 genotypes (14.3%) were detected on both tomato and potato plants. Genotype NL-136 was found in two potato plots and in one tomato plot, genotype NL-137 was found in four potato plots and three tomato plots, and genotype NL-143 was found in one potato plot and one tomato plot.

Genotypic diversity

Total genotypic diversity was low in 1994 and high in 1996 (Table 5). The percentage of maximum diversity (normalised Shannon index) increased from 34% in 1994 to 61% in 1996. The increase of genotypic diversity for the organic fields over the 3-year period was significant. Diversity in the allotment gardens was highest in 1995 and lowest in 1994.

Table 6 shows genotypic diversity over sites. Average diversities differed significantly between organic fields and allotment gardens, being comparatively high in A_p in 1994 and 1995 and low in A_p in 1996. In 1994, the difference in genotypic diversity between

Table 4. Number of potato plots sampled in allotment gardens in Southern Flevoland in 1994, 1995, 1996 and numbers of isolates and genotypes of *P. infestans* characterised

Year	Site[1]	Plots sampled	Isolates characterised	Genotypes identified	Rare genotypes (%)	Common genotypes (%)[2]
1994	A_pn	4	13	4	1 (25)	5 (39)
	A_ph	5	20	4	1 (25)	12 (60)
Total		9	33	8	2 (25)	17 (52)
1995	A_pn	4	36	7	3 (89)	32 (89)
	A_ph	6	50	15	13 (87)	0 (0)
	$A_p$sp	5	48	10	7 (70)	9 (19)
	A_pa	6	46	8	8 (1)	0 (0)
Total		21	180	39	31 (80)	41 (23)
1996	A_pn	5	20	14	9 (64)	0 (0)
	A_ph	6	61	12	7 (58)	0 (0)
Total		11	81	26	16 (62)	0 (0)

[1] A_p = potato plot in allotment garden; a, h, n, sp indicate four different compounds.
[2] Genotypes NL-41, NL-69, NL-75, NL-76.

commercial fields (Fc and Fo) and potato plots in allotment gardens (A_p) was significant ($P < 0.05$). Significant differences in diversity existed among compounds of allotment gardens in 1995 and 1996, but not in 1994. No pattern is discernible.

Only the year 1994 provided sufficient data for the analysis of diversity in farmers' fields over time within the growing season (Table 5). Refuse piles, although sampled early (April–June), were more diverse than conventional potato fields. Early-infested conventional potato fields (FcE; sampled in June) had remarkably low diversity, whereas conventional fields sampled later in the season, August and September (FcL), had significantly higher diversity. Diversities of organic fields (Fo; sampled in August) and refuse piles were not different.

Diversity along the gradient assessed in 1994 at distances of about 0 (VS), 1 (FcI), and 4 km (FcII) from the source (Zwankhuizen et al., 1998) was independent of distance and remained practically constant. In 1996, no significant difference was found between potato and tomato plots in both compounds.

Genotypic distance in space and time

Genotypic distance analysis was conducted both for complete samples (including all isolates) and censored samples (corrected for genotypes with more than one isolate). For most analyses, censored and uncensored samples gave similar results. The genotypic distance of the individual sampling sites ranged from 0 in many sites where only one genotype was detected to 0.27 in a conventional field (Fc2) in 1996 where four genotypes, NL-84, NL-92, NL-106, and NL-115, with substantially different DNA fingerprints, were found.

Table 7 shows how many sampling sites had low, intermediate, and high genotypic distance each year. Chi-square tests showed that the high genotypic distance class ($D \geq 0.07$) was under-represented in 1994, whereas the low genotypic distance class ($D < 0.01$) was under-represented in 1996. Apparently, isolates became less related after 1994. Correlations between the Shannon indices and the average genotypic distances of the sampling sites were calculated. For the data set 1994–1996, Spearman's rank correlation coefficient was -0.91 (-0.79 censored) ($P < 0.001$).

Epidemiological considerations

The majority of genotypes appeared to be rare (138 of the 170 genotypes, Table 1). The other 32 genotypes were detected at least twice. Four of these (NL-41, NL-69, NL-75, NL-76) were common. The remaining 28 genotypes were encountered in two to seven sampling sites (one or more years). These genotypes are subdivided into two categories, one with an epidemiological explanation for the occurrence in more than one site (16 genotypes), and the other without such an explanation (12 genotypes).

The first category consists of genotypes which were often found in contiguous potato fields or adjacent plots in allotment gardens, or in fields near an infection source containing the same genotype. For example, NL-48 was detected in two organic potato fields

Table 5. Genotypic diversities of *P. infestans* populations, as measured by the Shannon index (h_0), in potato and tomato sites in Southern Flevoland (the Netherlands) over time

	No. of sites	No. of isolates	No. of genotypes	h_0[1]
Total diversity per season[2]				
1994	38	277	22	1.89a
1995	31	383	66	3.24b
1996	30	348	97	3.43b
Diversity between seasons, organic fields (Fo)				
1994	5	57	9	1.30a
1995	6	105	21	2.28b
1996	6	162	44	2.96c
Diversity between seasons, allotment gardens (A_p)				
1994	9	33	8	1.85a
1995	21	180	39	3.26c
1996	11	81	26	2.46b
Diversity in one season, 1994 [3]				
Rc	7	47	8	1.57c
FcE	4	61	4	0.71a
Fo	5	57	9	1.30bc
FcL	13	79	8	1.10b

[1]Diversities (h_0) within the sections of the column followed by a common letter are not significantly different at $P \leq 0.05$ according to the *t*-test of Hutcheson for pairwise comparisons (see text).
[2]The normalised Shannon index h_0', expressing the Shannon index as a fraction of the maximum diversity was 0.34, 0.54, and 0.61 for 1994, 1995, and 1996, respectively.
[3]The development of the epidemic in 1994 is described in detail in Zwankhuizen et al. (1998). Rc = refuse pile; FcE = early infested potato field; Fo = organic potato field; FcL = late infested potato field.

Table 6. Genotypic diversities of *P. infestans* populations, as measured by the Shannon index (h_0), in potato and tomato sites of Southern Flevoland (the Netherlands)

Site[1]	No. of sites	No. of isolates	No. of genotypes	h_0[2]
Diversity among categories of sites, 1994				
Rc	7	47	8	1.57ab
Fc	17	140	8	1.27a
Fo	5	57	9	1.30a
A_p	9	33	8	1.85b
Diversity among categories of sites, 1995				
Fo	6	105	21	2.28a
A_p	21	180	39	3.26b
Diversity among categories of sites, 1996				
Fo	6	162	44	2.96b
A_p	11	81	26	2.46a
Diversity among compounds of allotments, 1994				
A_pn	4	13	4	1.22a
A_ph	5	20	4	1.16a
Diversity among compounds of allotments, 1995				
A_pn	4	36	7	1.44a
A_ph	6	50	15	2.36c
A_ps	5	48	10	1.90b
A_pa	6	46	8	1.80ab
Diversity among compounds of allotments, 1996				
A_pn	5	20	14	2.48bc
A_tn	5	33	16	2.55c
A_ph	6	61	12	1.71a
A_th	5	34	12	2.03ab

[1]For abbreviations see footnote of Table 1.
[2]Diversities (h_0) within the sections of the column followed by a common letter are not significantly different at $P \leq 0.05$ according to the *t*-test of Hutcheson for pairwise comparisons (see text).

(Fo3, Fo4; distance 8 km) in 1994, and was found again in 1995 in field Fo4 (at ca. 2 km from the location of field Fo3 in 1994). NL-113 was identified in two contiguous organic fields (Fo1, Fo2) in 1996. NL-136 and NL-137 were found in several potato and tomato plots in allotment garden Ah in 1996. The largest distance between those plots was 80 m.

The second category consists of genotypes often found in allotment gardens and organic fields. No reasonable epidemiological explanation other than sharing the same genotype by random recombination is applicable to these genotypes. For example, NL-56 was found on June 27, 1995, in plot 3 of allotment garden Asp, which was the first infested plot of that garden in that year. One week later, this genotype was also recovered from conventional field Fc2. This field was one of the first infested potato fields in the area, had a very diverse population (as described above), and was located approximately 14 km west from allotment garden Asp.

Infectivity of potato and tomato waste in allotment gardens

In the bioassay performed to monitor the presence of oospores in waste material, infections were recorded in four of the six tomato waste samples. None of the potato waste samples showed infected leaflets. Five isolates, collected from the tomato samples, were characterised for mating type and fingerprint pattern. The isolates had genotypes not found in the population of the research area during the 4 years that samples were collected (data not shown). A few samples of

Table 7. Annual distributions of the frequencies of sampling sites of *P. infestans* in Southern Flevoland and adjacent areas (the Netherlands), 1994–1996, with relatively low, intermediate and high genotypic distance (*D*)*

Year	Number of sites			Total	Average genotypic distance
	$D < 0.01$	$0.01 \leq D < 0.07$	$D \geq 0.07$		
1994	15	13	7	35	0.04
1995	12	10	9	31	0.05
1996	5	9	16	30	0.08
Total	32	32	32	96	

*The Chi-square test indicated a barely significant ($P = 0.047$) interaction between genotypic distance classes and years. Chi-square tests applied to pairs of years (2×3 tables) showed a significant interaction between genotypic distance classes and 1994 and 1996 ($P = 0.012$), but no interaction for the pairs of years 1994 and 1995 ($P = 0.69$) and 1995 and 1996 ($P = 0.087$).

infected tomato leaves, collected at allotment garden An in autumn 1996, were comminuted in a commercial blender. Microscopic examination of the suspensions showed that the waste material contained oospores.

Discussion

Our sampling was far from unbiased. Some potato sites were visited and sampled more frequently than others because of the predominance of the disease. In 1993, samples were collected only at the end of the growing season. In 1995 and 1996, years with limited late blight, almost every infested site was sampled, whereas in 1994 a selection had to be made. Hence, statistical conclusions must be considered with care.

Infection sources supposedly initiated by asexual inoculum (i.e., refuse piles and foci originating from infected seed tubers) were of major importance for late blight development in the commercial potato fields, compared to infection sources attributed to oospores.

Field observations indicated that refuse piles were the most important infection source for the commercial potato fields sampled in 1994 and 1995 (Zwankhuizen et al., 1998). This conclusion is supported by the genotypes found. Ninety-one per cent of the isolates collected from infested refuse piles belonged to the common genotypes NL-41, NL-69, NL-75, and NL-76, as did 70% of the isolates collected from the commercial potato fields. These common genotypes apparently overwinter well in tubers. Field observations did not indicate a significant role for infected seed tubers (Zwankhuizen et al., 1998). At times, however, infected seed tubers can be important inoculum sources (Van der Zaag, 1956; Davidse et al., 1989; Goodwin et al., 1995). Circumstantial evidence from this study indicates that infected seed tubers cannot be excluded as a source. For example, NL-48 was only detected at two organic farms in 1994 and 1995, which might indicate that this genotype survived in the seed tubers grown on these farms.

The year 1996

Disease development in 1996 showed a pattern quite different from the previous 2 years. No disease was found in refuse piles, except at Wezep, located outside the research area. The isolates from this refuse pile, containing potato waste from Flevoland and deposited by a potato processing company, belonged to common genotypes.

Between 1995 and 1996 a major change took place. The genotypes common in 1994 and 1995 nearly disappeared, though they seem to overwinter easily in tubers (as evidenced by the 1996 data from the refuse pile at Wezep). A particular sequence of events might explain the change. (i) Due to the dry weather in 1995 the absolute amount of inoculum available for tuber infection was much lower than in 1994. (ii) The weather conditions late in 1995 were not particularly conducive to tuber infection, so that few infected seed tuber were planted in 1996. (iii) The season of 1996 was so dry that early infection from seed tubers and ensuing blanket spread of early genotypes was prevented. (iv) Thus, the many rare genotypes stemming from sexual recombination were not outcompeted by the common genotypes and hence attained high visibility in 1996. (v) This visibility was enhanced by the selective sampling, because little late blight appeared in the conventional potato fields.

Evidence for sexual recombination

The evidence for the role of oospores in the development of disease in 1994 and 1995 is sparse and indirect. The ratios of A1 and A2 genotypes, and the high percentages of rare genotypes observed each year suggest that sexual reproduction probably did occur. In the absence of sexual reproduction, one would expect the number of genotypes to decrease over time but it did not. The analysis of field Fc2 in 1995 provides evidence

for the involvement of oospores as initiating inoculum. Ten genotypes, including several rare ones, were found in this field. No infection source was found outside the field. The grower told the first author that the previous potato crop in this field (1992) had been severely diseased. In 1994, the grower found severely diseased volunteer plants in this field. These observations suggest that infections in this field may have originated from oospores formed in that field before 1994.

In 1996, only one of the common genotypes (NL-75) was found in two adjacent organic fields, but at a low percentage (only five isolates). In these fields, disease could have been initiated by infected seed tubers. However, it is very unlikely that the high number of rare genotypes can be explained by massive survival of asexual genotypes within seed tubers, because (i) conditions at harvest time (August 1995) in Southern Flevoland, where the seed was grown, were unfavourable for the infection of seed tubers and (ii) no infected seed tubers were found during inspections of seed tuber lots in the spring of 1996. The high diversity cannot be explained by immigration of genotypes from other sources in Southern Flevoland or elsewhere, because the organic fields were among the earliest infested fields in the Netherlands in 1996, and other sources were practically absent.

The high level of diversity in the infested organic fields in 1996 suggests a significant role for oospores, although a contribution of soil-borne oospores to disease development in these fields seems unlikely when the cropping history of these fields is considered. In three of the six fields, potatoes were grown for the first time since the area was reclaimed. On the other three fields, potatoes were grown once in 6 or 7 years which is common practice for organic farming in the Netherlands. Furthermore, the foliage of infested organic potato crops is always flamed, which reduces the possibility of contamination of the soil with oospores formed in the crop. The high level of diversity in the organic fields might be explained as follows: (i) a few genotypes survived the 1995–1996 winter in infected seed potatoes and initiated disease early in the 1996 season; (ii) asexual descendants from these genotypes spread throughout the crop and recombination between strains with opposite mating type took place during the season; (iii) a highly diverse progeny reinfected the crop. So most of the isolates which were collected could have been progeny of matings within the potato foliage earlier in the season. The occurrence of in-the-green-crop sexual reproduction has been hypothesised previously by Drenth et al. (1995). The alternative explanation of influx of late blight inoculum into the research area seems unlikely because of the lateness and scarcity of *P. infestans* in the Netherlands during 1996. Additional experiments are needed to thoroughly test the hypothesis of in-the-green-crop sexual reproduction.

In three compounds of allotment gardens analysed in 1995, and in the two analysed in 1996, all sampled plots contained different genotypes, many of them being rare, and A1 and A2 genotypes were mostly in a 1 : 1 ratio. These results support the hypothesis that disease in allotment gardens generally originates from oospores. Growers in allotment gardens often use the potato and tomato waste to enrich the soil with organic matter. This material may contain oospores which can survive the winter period and infect host plants in the next season. Our finding that composted tomato waste material collected in allotment gardens was infectious is consistent with this hypothesis

In the allotment gardens in 1994 and in compound An in 1995, the majority of isolates belonged to common genotypes. In 1994, these genotypes were already prevalent throughout the commercial potato growing area and spores from the commercial fields might have initiated disease in allotment gardens. Influx of inoculum was also the most likely source for compound An in 1995. Disease in this compound was observed about 2 weeks later than in the other compounds. Some infections might have originated from oospores but may have been outcompeted by infections with inoculum from commercial potato fields. There are no indications that infected seed tubers played a significant role.

Genotypic diversity

In the central highlands of Mexico, the centre of origin of *P. infestans*, Goodwin et al. (1992b) found that almost every isolate from commercial crops had its own genotype. In Table 8, our study is compared with three extensive studies (≥ 1000 km^2) in the Netherlands (Drenth et al., 1993), Poland (Sujkowski et al., 1994), and North America (Goodwin et al., 1995). Genotypic diversity in the North American populations of 1992 and 1993 was relatively low ($h'_0 = 0.31$). The overall levels of diversity in Poland between 1985 and 1991, in the Netherlands in 1989, and in our present intensive 4-year (150 km^2) investigation were relatively high (0.45–0.65).

Comparison of diversities in space revealed significant differences among categories of sampling sites. Relatively high genotypic diversities were found in

Table 8. Comparison of genotypic diversities of *P. infestans* at different scales of distance between sampling sites (varying from approximately 22 to 4000 km)

Research area[1]	Sampling years	Distance (km)	Markers	Isolates	Genotypes	Rare genotypes (%)	$h_0'^2$
The Netherlands	1989	300	26[3]	153	35	21 (60)	0.45
Poland	1985–91	800	29[4]	175	81	69 (85)	0.65
North America	1992–93	4000	28[5]	130	9	4 (44)	0.31
Southern Flevoland	1993–96	22[6]	24[7]	1048	170	138 (81)	0.54

[1]From studies by Drenth et al., 1993 (the Netherlands), Sujkowski et al., 1995 (Poland) and Goodwin et al., 1995 (Northern America), and from this study (Southern Flevoland).
[2]Normalised Shannon diversity (see text).
[3]26 RG57 fingerprint loci.
[4]26 RG57 fingerprint loci, mating type and two allozyme loci (*Gpi* and *Pep*).
[5]25 RG57 fingerprint loci, mating type and two allozyme loci (*Gpi* and *Pep*).
[6]With the refuse pile at Wezep located outside the research area included, the largest distance is 50 km.
[7]23 RG57 fingerprint loci and mating type.

allotment gardens and organic fields and most genotypes at these sites were 'rare'. These findings are consistent with the hypothesis that oospores initiated disease.

Significant differences within and between seasons were found. Diversity increased over 3 years, being highest in 1996, the year with the lowest disease levels. In 1994, the level of diversity followed the epidemic pattern: at the beginning of the season a high level in the refuse piles, a lower level in the early infested fields, a higher level in the organic fields after a hot and dry period, and, towards the end of the season, a lower level in the infested conventional fields. In 1994, influx of genotypes from other sites located outside the research area cannot be excluded.

The analysis of the genotypic distance distributions over the years revealed a significant population change. The *P. infestans* population in Southern Flevoland shifted from relatively low genotypic distances in 1994 to relatively high genotypic distances in 1996. This shift is consistent with the hypothesis that the contribution of oospores to the development of disease increased over the course of the years.

Epidemiology and population structure

Sexual reproduction is probably the main mechanism generating the relatively high and increasing diversity levels in the research area. The effect of sexual recombination on the genotypic composition of populations will be most evident in dry years, such as 1996. In wet years, of which 1994 was, but, a modest example, populations may be dominated by a few genotypes spreading early and rapidly from refuse piles thus masking whatever genotypes might result from sexual reproduction. Low infection levels in dry years might appear to limit the general applicability of our findings, but instead, they allowed us to link genotypic analyses to epidemiological phenomena.

An epidemic model of population structure (Maynard Smith et al., 1993) is characterised by frequent recombination and the subsequent occurrence of a few, highly successful genotypes during the asexual phase of the epidemic. This model might be partially applicable to our populations. The structure will be confounded by overwintering asexual inoculum, putative sexual reproduction within the green crop during the growing season and, in dry years, by reduced spread of genotypes resulting in diverse subpopulations across the area. For example, in 1995, a dry year, the populations of *P. infestans* in allotment gardens differed in genetic composition from each other and from those of commercial fields whereas in 1994, a wet year, the isolates collected in the allotment gardens apparently belonged to the population of the commercial fields. Both on a global and regional level, the geographic and temporal structuring of *P. infestans* populations is a dynamic process (Andrivon, 1994).

Prediction of the direction in which the population structure will evolve in the future is nearly impossible, even in our small research area, because of the many interactions which can have an impact on the establishment and development of a *P. infestans* population. The number of fields infested by oospores will probably increase gradually. Potato crops will be continuously

confronted with an unpredictable pathogen, which may make potato growing increasingly dependent on disease control measures, such as the use of resistant cultivars and fungicides.

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