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DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands

ANDRÉ DRENTH[1,2]*, INGE C. Q. TAS[1] and FRANCINE GOVERS[1]**
[1] *Dept. of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands;* [2] *Research Institute for Plant Protection, Binnenhaven 12, 6709 PD Wageningen, The Netherlands; *Present address: CRC for Tropical Plant Pathology, The University of Queensland, Brisbane QLD 4072, Australia; **Author for correspondence*

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Abstract. The oomycetous fungus *Phytophthora infestans* (Mont.) de Bary, which causes late blight disease in potatoes, is heterothallic with two known mating types, A1 and A2. From 1845 until 1980 only A1 mating type isolates were found in Europe. In 1980, the A2 mating type appeared permitting sexual reproduction. Here we show that virulence properties and DNA fingerprint patterns of isolates collected in the Netherlands before and after the appearance of A2 mating type isolates are different. Before 1980, eight different races were found in which virulence factors 1, 2, 3, 4 and 10 were most common. After 1980, new virulence factors (i.e. 5, 6, 7, 8 and 11) showed up and the diversity for virulence increased tremendously: 73 different races were detected among 253 isolates analyzed. DNA fingerprint analyses of isolates collected before 1980 revealed that, for at least two decades, only one RG-57 fingerprint genotype was present in Europe. Among 179 isolates collected after 1980 134 distinct RG-57 fingerprint genotypes were identified. The dramatic increase in genetic diversity strongly suggests that the *P. infestans* population in the Netherlands is now propagating sexually. The change from asexual to sexual reproduction, and the resulting increased adaptability and ability to survive outside the host, may interfere drastically with the regular disease control methods.

Introduction

The oomycetous fungus *Phytophthora infestans* (Mont.) de Bary causes late blight, one of the most devastating diseases of potato worldwide. In Europe the first late blight epidemic occurred in 1845 and this resulted in the notorious Irish famine. *P. infestans* is heterothallic with two known mating types, A1 and A2. When A1 and A2 mating type thalli grow in close vicinity sexual spores (i.e. oospores) can be formed. Formation of male (antheridia) and female (oogonia) sexual organs on each thallus is induced by hormones [Ko, 1980]. Physical contact of an antheridium and an oogonium of opposite mating types leads to the production of oospores. Oospores are thick walled, hard hulled spores, which enable the fungus to survive in soil outside the living host plant. Oospores have been found in central Mexico, which is thought to be the center of origin of *P. infestans*.

There two interacting species, *P. infestans* and its solanaceous hosts, *Solanum* spp., co-evolved [Gallegly and Galindo, 1958; Smoot et al., 1958]. The isolate(s), which initially originated from this area and spread world-wide, had the A1 mating type only. From 1845 until 1980 indeed only A1 mating type isolates were found in Europe, restricting *P. infestans* for at least 135 years to asexual reproduction.

In 1984 it was reported that isolates with the A2 mating type were present in Switzerland since 1981 [Hohl and Iselin, 1984]. This finding called for mating type screening of field samples and isolates of *P. infestans* maintained in culture collections. The screenings revealed that in addition to A1 mating type isolates, A2 mating type isolates had been present in Europe since at least 1980 [Spielman et al., 1991; Fry et al., 1992, 1993; Drenth et al., 1993b]. Around the same time outbreaks of the disease in the Netherlands and other European countries began to occur more often and became more difficult to control. It was hypothesized that these phenomena were caused by the appearance of A2 mating type isolates which, in the presence of the already existing A1 mating type isolates, enabled *P. infestans* to reproduce sexually.

In order to determine whether acquisition of sexual reproduction was truly the cause of the observed phenomena we analyzed the changes in genetic diversity in *P. infestans* populations by comparing virulence properties and DNA fingerprint patterns of A1 mating type isolates collected before 1980 (called 'old' isolates) and A1 and A2 mating type isolates collected after 1980 (called 'new' isolates).

Materials and methods

Phytophthora infestans *isolates.* The majority of *P. infestans* isolates used in this study was obtained from the *P. infestans* culture collection maintained at Wageningen Agricultural University. All these isolates are stored in liquid nitrogen. Only five Dutch isolates collected before 1980 were maintained in liquid nitrogen and these were the only 'old' Dutch isolates available for DNA fingerprint analyses. 'Old' isolates collected in the United Kingdom and Germany, and maintained by R. C. Shattock, and E. Götz and B. Schöber, respectively, were also subjected to DNA fingerprint analyses.

Virulence. Virulence was determined by assaying compatibility and incompatibility in a differential set of potato lines. Detached leaflets from different potato lines carrying single resistance genes 1 to 11, with the exception of 9, and combinations 1-2, 1-3, 1-4, 2-3, 2-4, 1-2-3, 1-3-10, 1-4-10 and 1-2-3-4, were sprayed with a spore suspension of 10^4 spores per ml. After six days of incubation at 15 °C, with 16 h light per day, the leaflets were examined for the occurrence of late blight lesions. If sporula-

tion was observed, the interaction was rated compatible; if no sporulation was observed, the interaction was rated incompatible. For most isolates the virulence was determined at least twice. Virulence characteristics of 148 'old' isolates (from 1967–1971) and 38 'new' isolates (from 1981) were obtained from data published by Mooi [1967, 1968, 1971] and Davidse et al. [1981]. They used the same method and the same differential set.

DNA fingerprint analyses. For DNA fingerprint analyses, the moderately repetitive DNA probe RG-57 was used. This probe, obtained from a genomic *P. infestans* library, hybridizes to several unlinked loci [Goodwin et al., 1992b] and provides isolate-specific DNA fingerprints [Goodwin et al., 1992b,c; Drenth et al., 1993a]. DNA isolation, *Eco*RI digestion, Southern blotting and hybridization with probe RG-57 were performed as described [Drenth and Govers, 1993; Drenth et al., 1993a]. The numbers of the hybridizing fragments are according to Goodwin et al. [1992c].

Data analyses. Genetic diversity was measured using the normalized Shannon diversity index: $H_S = H/H_{MAX}$, in which H is the usual Shannon diversity index over genotypes, and H_{MAX} is ln(N), the maximum diversity of a sample of size N [Goodwin et al., 1992a]. The statistic is relatively stable when sample sizes vary [Sheldon, 1969]. Diversity indices were calculated separately for (i) presence and absence of virulence factors and (ii) presence and absence of DNA fragments hybridizing with probe RG-57.

Results

Among the 'old' isolates, diversity for virulence was limited to only five of the eleven known virulence factors and these five (i.e. 1, 2, 3, 4, 10) corresponded to the resistance genes used in the common potato cultivars grown in the Netherlands (Fig. 1). Only eight different races (defined by their ability to overcome specific resistance genes, or combinations of specific resistance genes, in the host) were identified among 148 isolates tested (Table 1). After 1980, the same five virulence factors occurred but now they rapidly appeared in many new combinations and as a consequence new races developed. When only the 'old' virulence factors 1, 2, 3, 4 and 10 were taken into consideration, 26 races with different virulence patterns were found among 253 isolates tested. New virulence factors (i.e. 5, 6, 7, 8 and 11), not strictly required to colonize the prevailing potato cultivars grown in the Netherlands, showed up (Fig. 1) and increased the total number of races with different virulence patterns from 26 to 73 among the 253 isolates tested (Table 1). Obviously, the diversity for virulence increased significantly, a conclusion supported by statistical analysis using the normalized Shannon diversity index [Sheldon, 1969; Goodwin et al., 1992c] (Fig. 3).

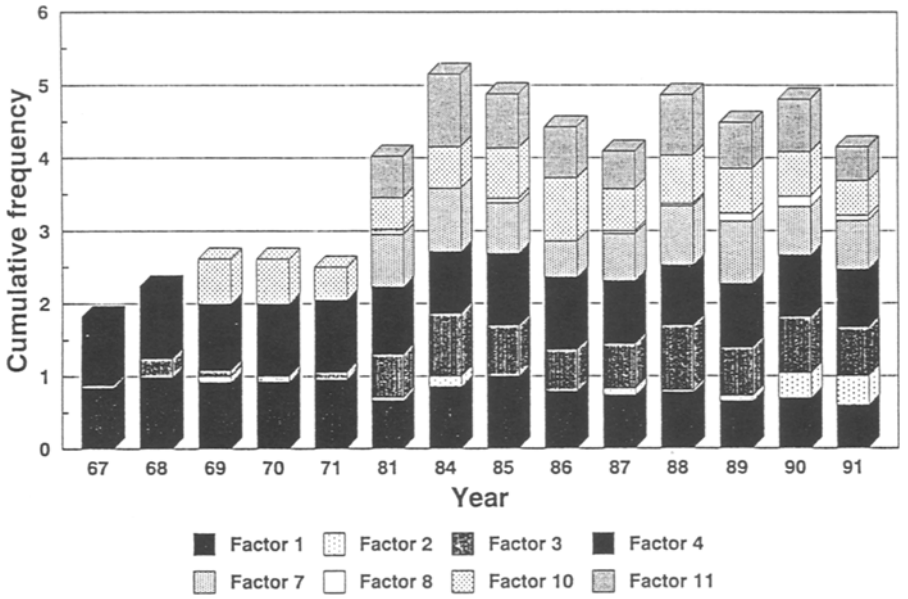


Fig. 1. Yearly cumulative frequency of virulence factors in 148 'old' (1967–1971) and 253 'new' (1981–1991) *P. infestans* isolates. For the number of isolates and races per year see Table 1. Virulence factors 5 and 6 are not included in the bars. They were not found in the 'old' isolates and only at very low frequency in the 'new' isolates (0.011 and 0.023, respectively).

For DNA fingerprint analyses the moderately repetitive DNA probe RG-57 was used. On genomic blots containing DNA isolated from 'old' *P. infestans* isolates, collected in the Netherlands between 1966 and 1978, probe RG-57 hybridizes to fifteen different DNA fragments, none of which is polymorphic (Fig. 2a,b). Sixteen other 'old' isolates collected in Europe before 1980 and available for DNA fingerprint analyses (ten from Germany and six from the United Kingdom) exhibit the same RG-57 genotype (data not shown). One of the six UK isolates (collected in 1978) is slightly different. Although this isolate displays the same RG-57 pattern as the others, it has one additional RG-57 hybridizing fragment. The DNA fingerprint data show that over at least 12 years the *P. infestans* population in western Europe consisted almost exclusively of one RG-57 genotype which is consistent with the notion that *P. infestans* reproduced asexually. From the 'new' isolates collected in the Netherlands between 1981 and 1991, 179 were analyzed by DNA fingerprinting and among those 134 different RG-57 genotypes were found (Table 1). The number of hybridizing RG-57 fragments per isolate varied between 10 and 19, and in total, 26 different RG-57 hybridizing fragments could be detected of which 23 were polymorphic (Fig. 2a,b). Apparently, genetic diversity of the *P. infestans* population increased dramatically and this is again supported by statistical analyses (Fig. 3). Every isolate collected in 1981 (5 in total), 1984 (4), 1985 (13)

Table 1. The number of *P. infestans* isolates analyzed for virulence and DNA fingerprint patterns, and the number of different races and RG-57 genotypes identified in 'old' and 'new' isolates

Year[1]	Virulence		DNA fingerprinting	
	#Isolates	Races[2]	#Isolates	RG-57 genotypes
66			1	1
67	39[3]	5		
68	63[3]	3	1	1
69	13[3]	4		
70	13[3]	4	1	1
71	20[3]	4		
74			1	1
78			1	1
Totals "old"	148	8[4]	5	1
81	46[5]	19 (12)	5	5
84	7	5 (5)	4	4
85	16	6 (4)	13	13
86	14	8 (4)	9	7
87	23	15 (2)	17	17
88	32	15 (8)	30	25
89	56	25 (14)	57	40
90	44	29 (18)	30	24
91	15	12 (9)	14	12
Totals "new"	253	73[6] (26)	179	134

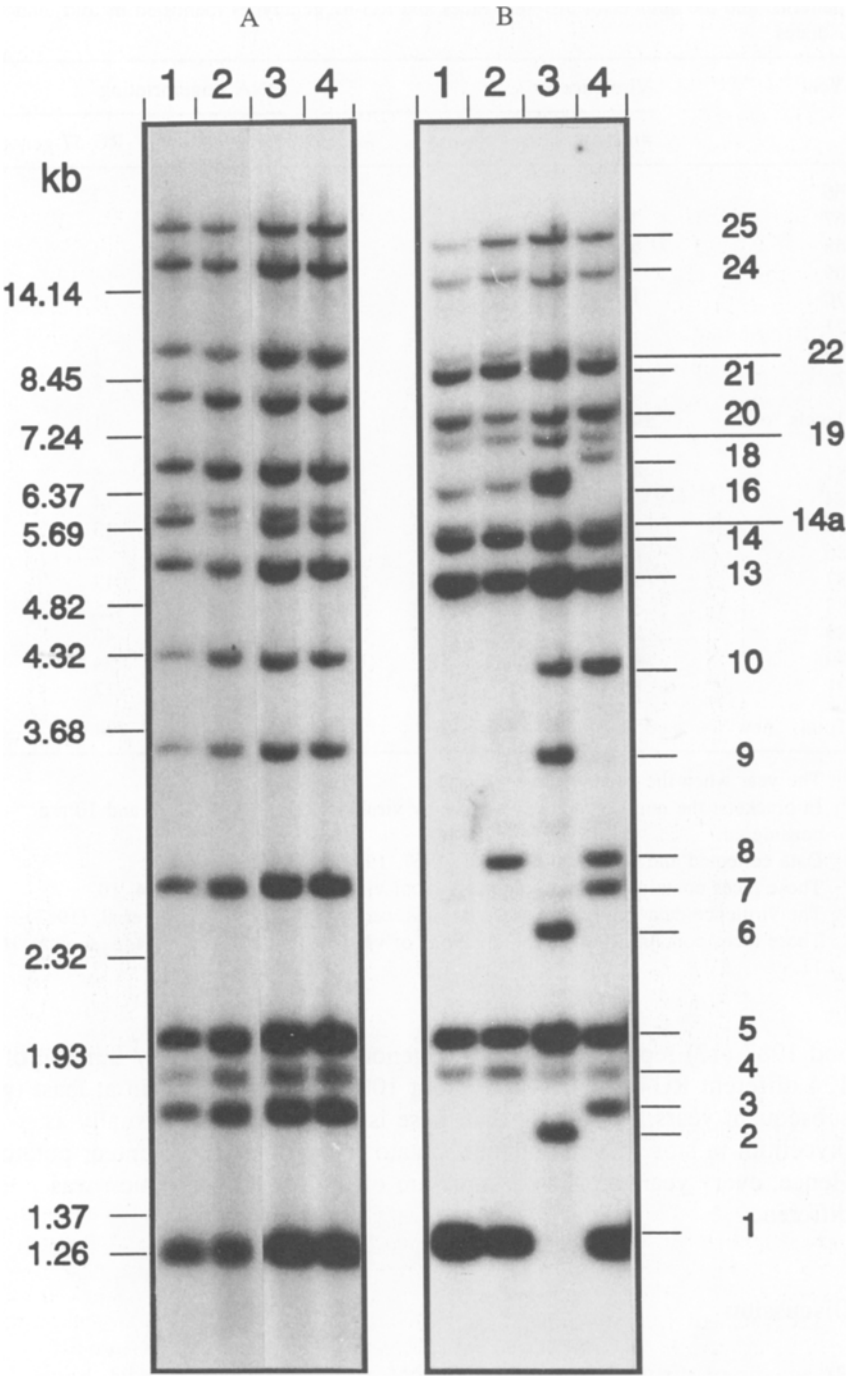
[1] The year when the isolates were collected.
[2] In brackets: the number of races when only virulence factors 1, 2, 3, 4, and 10 are considered.
[3] Data collected and published by Mooi (1967, 1968, 1971).
[4] These races contain different combinations of virulence factors 1, 2, 3, 4, 10.
[5] The virulence data from 38 of these isolates were collected by Davidse et al. (1981).
[6] These races contain different combinations of virulence factors 1, 2, 3, 4, 5, 6, 7, 8, 10, 11.

and 1987 (17) represented a unique genotype (Table 1). Only thirteen of the 134 different RG-57 genotypes, about 10%, were identified in at least two subsequent years, suggesting that these isolates survived asexually as mycelium in stored seed potatoes, potato refuse piles or volunteer potatoes. Hence, every year the genetic structure of the fungal population was different.

Discussion

By means of virulence testing and DNA fingerprint analyses we have shown that there has been a sudden change in the level of genetic diversity

[a]


A
B
1 2 3 4
1 2 3 4
kb
14.14
8.45
7.24
6.37
5.69
4.82
4.32
3.68
2.32
1.93
1.37
1.26
25
24
22
21
20
19
18
16
14a
14
13
10
9
8
7
6
5
4
3
2
1

in the Dutch *P. infestans* population around 1980. The change from little, if any, diversity in the 'old' population to a very high level of diversity in the 'new' population coincided with the appearance of new A1 and A2 mating type isolates in Europe.

The observation that the predominant RG-57 genotype found in 'old' isolates was not found in 'new' isolates proves the hypothesis by Spielman et al. [1991] that the current, 'new', population consisting of A1 and A2 mating type isolates completely replaced the 'old' A1 mating type population. There are ample indications that the 'new' population originated from Mexico [Spielman et al., 1991; Niederhauser, 1991]. Many different virulence factors and complex races are known to occur there [Rivera-Peña, 1990]. All RG-57 hybridizing fragments, which are found in the 'new' *P. infestans* population in the Netherlands, were also found in Mexican *P. infestans* isolates [Goodwin et al., 1992c; Drenth et al., 1993a].

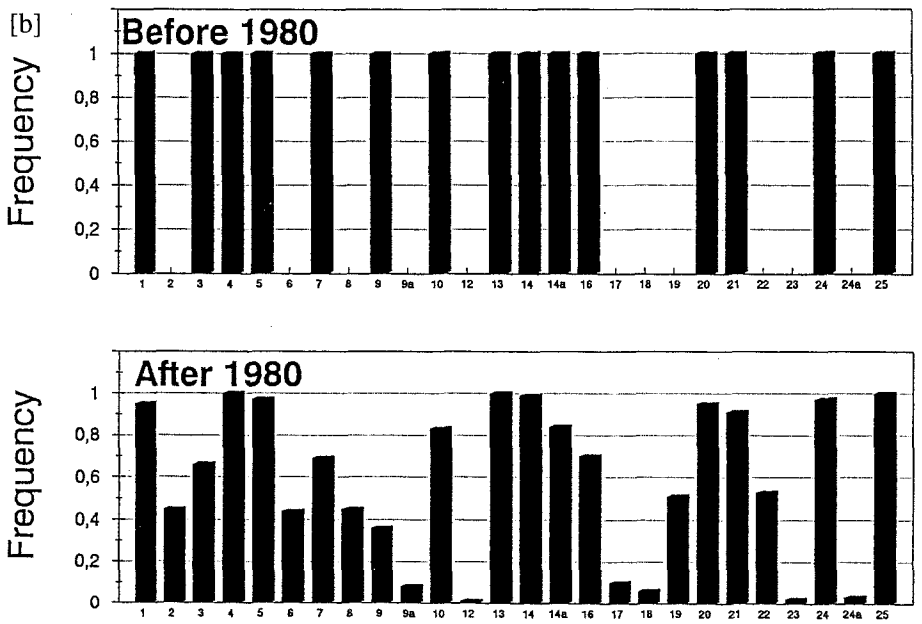


Fig. 2.[a] RG-57 DNA fingerprint patterns from 'old' and 'new' *P. infestans* isolates. The 'old' isolates shown in (A) were collected in 1966 (1), 1968 (2), 1974 (3) and 1978 (4). The 'new' isolates shown in (B) were collected in 1986 (1) and in 1988 (2, 3 and 4). Size markers in kilobases (kb) are indicated on the left. RG-57 fragment numbers, corresponding to those used by Goodwin et al. [1992b,c] and Drenth et al. [1993a], are indicated on the right. Fragments 9a, 12, 17, 23 and 24a are missing in the four RG-57 genotypes shown here but are present in other 'new' RG-57 genotypes not shown in this figure. *[b]* Relative frequency of RG-57 hybridizing fragments in 5 'old' and 179 'new' *P. infestans* isolates. The fragment numbers indicated on the x axis correspond to those indicated on the autoradiograph in [a]. The total number of different genotypes that were identified are summarized in Table 1. When 'old' UK and German isolates are included the relative frequency does not change.

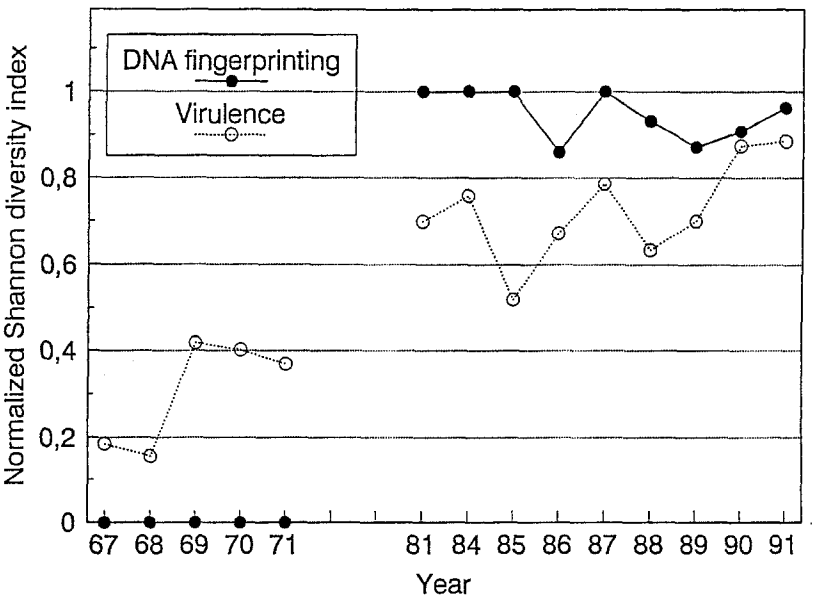


Fig. 3. Genetic diversity as calculated by the normalized Shannon diversity index for virulence and DNA fingerprint analyses by probe RG-57 for *P. infestans* populations collected between 1967 and 1991.

There are two possible explanations for the marked increase in genetic diversity: (i) the 'new' population introduced from Mexico consisted of a large number of heterogeneous isolates or (ii) only a limited number of 'new' isolates was introduced but the introduction of A1 and A2 mating type isolates allowed sexual reproduction of the pathogen. The observed genetic diversity is then caused by acquisition of sexual reproduction of the *P. infestans* population. Comparison of allozyme profiles from Mexican and European isolates make the first explanation very unlikely. In Mexico seven alleles are known for the allozyme *Gpi* and three for *Pep* [Fry et al., 1993]. In the new population in Europe only two alleles for each enzyme (90 and 100 for *Gpi* and 83 and 100 for *Pep*) have been identified [Spielman et al., 1991; Fry et al., 1991, 1992]. The *Gpi* allele 90 and the *Pep* 83 allele occur in very low frequency in the Mexican *P. infestans* population [Goodwin et al., 1992c]. Moreover, the predominant allozyme combination found after 1980 in Europe, *Gpi* 90 with *Pep* 83, has never been found in the Mexican *P. infestans* population. Hence, if an introduction of a large population of diverse genotypes would have occurred, more alleles, in combinations similar to those occurring in the Mexican *P. infestans* population, should have been detected in Europe. This is clearly not the case. Moreover, in an asexual propagating pathogen population, such as the 'old' *P. infestans* population, the genetic diversity is more likely to decrease. In the European agricultural system such a population goes through a bottle-

neck each year after the crop is harvested and only some isolates successfully overwinter asexually as mycelium in potato seed tubers. Population genetic data, based on DNA fingerprint analyses indicate that less than 10% of the genotypes detected survived asexually as mycelium between years. Over 90% of the genotypes which were found each year was different from the genotypes present in the previous years. For example, one genotype which made up half of the 153 isolates collected in 1989 [Drenth et al., 1993a] was not observed before or after 1989. Hence, every year the genetic structure of the fungal population was different and this is typical for a sexually reproducing population with non-overlapping generations [Maynard Smith, 1971]. In summary, our results strongly suggest that the acquisition and occurrence of sexual reproduction in the *P. infestans* population is the cause of the high level of genetic diversity. This suggestion is supported by the fact that we demonstrated unambiguously that oospores can survive and infect potato leaves after exposure to natural weather conditions during the winter in soil [Drenth et al., 1994].

Whether or not sexual reproduction has advantages over asexual reproduction has been a point of much debate [Stearns, 1987; Michod and Levin, 1988; Charlesworth, 1989]. In the case of *P. infestans* the advantages are obvious. Firstly, *P. infestans* is a hemibiotropic pathogen of which the asexual spores, in contrast to oospores, cannot survive for long periods outside the host plant. With the production of oospores *P. infestans* has acquired the ability to survive in soil between growing seasons, thereby increasing its possibilities to colonize new host plants and to propagate. Secondly, sexual reproduction results in a wider variety of genotypes and this, in turn, leads to an increased ability to respond to changes in the environment and to selection pressure [Maynard Smith, 1978]. This is illustrated by the speed at which the 'new' *P. infestans* population developed resistance to the fungicide metalaxyl. Soon after introduction of the 'new' population in Europe, around 1980, metalaxyl resistant isolates appeared in the field [Davidse et al., 1981]. In some countries, where the 'new' sexually propagating population did not yet appear, metalaxyl is still used successfully to control late blight. So far, metalaxyl resistant isolates have not been detected in 'old' asexually propagating *P. infestans* populations throughout the world [Fry et al., 1993]. Thirdly, the increased diversity of virulence patterns and the appearance of new virulence factors through sexual recombination, enables the fungus to avoid recognition by any specific resistance gene, or combinations of resistance genes, in the potato population.

Established disease management strategies are based on the biological characteristics of the 'old' asexual *P. infestans* population. With the asexual propagating population infections originated from mycelium retained in seed potatoes or in potato refuse piles which initiated typical focal epidemics. Hence, control measures involved disease free seed potatoes and removal of potato refuse piles. With the acquisition of sexual reproduction,

resulting in the presence of oospores in soil, late blight epidemics can start anywhere in the field as soon as potatoes have emerged. Thus, a general epidemic, originating from oospores, may appear despite sufficient removal of infected tubers containing mycelium. Moreover, in a sexually propagating population more aggressive strains can easily emerge and this will seriously interfere with the current efforts of breeders to create potato cultivars with durable resistance. At present it is difficult to envisage all practical implications for crop protection of a sexually propagating and more diverse *P. infestans* population and, therefore, it is a challenge for the future to cope with sexual reproduction of the potato late blight fungus.

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