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Characterisation of isolates of *Phytophthora infestans* from Hungary

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Abstract

A total of 36 single-lesion isolates were collected from 9 crops of potato and 13 of tomato in different regions of Hungary in the past decade, particularly in 1998. These were analysed for mating type, sensitivity to metalaxyl, allozyme genotype at glucose-6-phosphate isomerase and peptidase loci and genotype at 24 loci detected using the multilocus RFLP probe RG57. The ratios of the mating types A1 to A2 were 8 : 9 and 4 : 15 among isolates recovered from potato and tomato, respectively. Resistance to metalaxyl was found more frequently among isolates from potato and in the A1 mating type. The populations were not clearly differentiated on the basis of host origin. All isolates were homozygous (100/100) at the locus for glucose-6-phosphate isomerase. Unlike in other European countries, the most common peptidase allele was 96. Genotypes at the peptidase locus were 96/96 (50%), 96/100 (27.7%) and 100/100 (16.6%). In addition, one isolate from 1993 and another from 1998, were defined as 83/96, a genotype that had not been described elsewhere. The 18 RG57 fingerprints that were observed among 36 isolates, with one exception, seem to be unique to Europe. On the basis of combined genotypic traits, 20 multilocus genotypes were designated. These data, which reveal a remarkable variability with unique genotypes of the late blight pathogen, suggest that migration and sexual and/or asexual recombination have a role in the recent evolution of the pathogen in Hungary.

Introduction

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is one of the most devastating diseases of potato and tomato worldwide and in Hungary. In the past two decades, the disease has re-emerged in certain parts of the world as a more aggressive pathogen of these crops (Fry and Goodwin, 1997). The higher aggressiveness is connected to the global changes in populations of this oomycete, the first indication of which was the early report of the A2 mating type of *P. infestans* outside central Mexico (Hohl and Iselin, 1984). A concomitant increase in the complexity of virulence phenotypes and in the tolerance to the specific fungicide metalaxyl was observed worldwide (Fry et al., 1991; Daggett et al., 1993; Sujkowski et al., 1994; 1996; Marquinez, 1995; Schöber-Butin et al., 1995). Nevertheless, the scattered occurrence of complex races and resistance to metalaxyl had been reported long before the first detection of the A2 mating type (Malcolmson, 1969; Shattock et al., 1977; Davidse et al., 1981; Dowley and O'Sullivan, 1981). Analyses of allozyme markers (Tooley et al., 1985; Shattock et al., 1986) and DNA fingerprints (Goodwin et al., 1992a,b; Drenth et al., 1993) of isolates from a number of locations of the world supported the notion that the aforementioned changes resulted from the displacement of an 'old' world-wide clonal lineage (US-1) by a new population of *P. infestans* (Spielman et al., 1991; Fry et al., 1993; Drenth et al., 1994; Koh et al., 1994; Goodwin and Drenth, 1997). The new population consists of both A1 and A2 mating types that enable the pathogen to reproduce sexually, and is thought to have migrated at various times from central Mexico (Fry et al., 1993).

Extensive studies on diversity of *P. infestans* have been carried out all over the world including most Western European countries. In contrast, Poland (Sujkowski et al., 1994) is the only country in Central Europe where populations of the pathogen have been characterised. However, the occurrence of the A2 mating type in association with a complex virulence phenotype and insensitivity to metalaxyl (Bakonyi and Érsek, 1997; Bohár et al., 1999) and the characteristics of several other isolates collected between 1991 and 1997 (Bakonyi et al., 1998) suggested the presence of a new *P. infestans* population in Hungary.

In order to test the hypothesis that increases in the severity of late blight in potato and tomato in Hungary result from a change in genetic diversity, we characterised isolates of *P. infestans* sampled across Hungary. Specific goals were to assess: (i) the level of genetic diversity of *P. infestans*; (ii) the sensitivity to metalaxyl of the isolates; (iii) the extent of differentiation of populations from potatoes and tomatoes and (iv) the frequencies of isolates of the A2 and A1 mating type.

Materials and methods

Collection and culture of isolates

Blighted material was collected randomly from seriously diseased patches of potato and tomato fields, mostly by officers of the Hungarian Plant Protection Service. Usually, three samples were collected within each field at the points of a triangular pattern that varied in size depending on the area of the field. Samples were received as infected foliage or fruits. Isolations were attempted from a single lesion of the blight-affected plant material by transferring a fragment of infected plant tissue to pea-broth agar (Tuite, 1969) containing ampicillin (500 $\mu g\,ml^{-1}$), pimaricin (10 $\mu g\,ml^{-1}$) and rifampicin (10 $\mu g\,ml^{-1}$). Isolates recovered represented a collection from 20 distant locations in Hungary.

Isolates used as standards in phenotypic and molecular characterisation were as follows: 4/91 and 15/93 (courtesy of B. Schöber-Butin, Biologische Bundesanstalt, Braunschweig, Germany); US930287, US940501, PO880033 and JP880001 (courtesy of W.E. Fry, Cornell University, Ithaca, NY, USA); NL-91006, NL-85138 and NL-91015 (courtesy of F. Govers, Wageningen Agricultural University, Wageningen, the Netherlands); 1561 and 1568 (G.A. Forbes, International Potato Center, Quito, Ecuador). Pure cultures were maintained on pea-broth agar at 16 °C.

Mating type determination

Each sample isolate was grown together with known A1 and A2 testers in a Petri dish containing pea-broth agar (Bakonyi et al., 1998). Plates were scored for oospore formation at the hyphal interface between the developing colonies after growth for 10–14 days at 20 °C. An isolate with unknown mating type was designated A1 if it produced oospores in abundance in pairing with the A2 tester isolate. The mating type of an isolate was designated A2 if oospores were formed in pairing with the A1 tester.

Response to metalaxyl

Response to metalaxyl was determined by measuring the diameter of mycelial growth in Petri dishes containing pea-broth agar amended with metalaxyl (technical grade, Novartis Co., Budapest, Hungary) at final concentrations of 5 or 100 $\mu g\,ml^{-1}$ active ingredient. Growth on amended agar was compared to growth on unamended control plates (Daggett et al., 1993). Isolates were classified as: (i) sensitive if diameters were less than 40% of the control at both 5 and 100 $\mu g\,ml^{-1}$, (ii) intermediate when growth was greater than 40% of the control at 5 $\mu g\,ml^{-1}$, but less than 40% of the control at 100 $\mu g\,ml^{-1}$ and (iii) resistant when growth was greater than 40% of the control at both 5 and 100 $\mu g\,ml^{-1}$ (Daggett et al., 1993; Therrien et al., 1993).

Allozyme analysis and DNA fingerprinting

Proteins for allozyme analysis were extracted from mycelium grown in pea broth as described in Bakonyi et al. (1998). Cellulose acetate electrophoresis (CAE) was performed according to Goodwin et al. (1995). Agar overlays were used to detect activities of glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9) and peptidase (*Pep*, EC 3.4.3.1). Standards included in CAE were US940501 (US-1 genotype), US930287 (US-8 genotype), JP880001 (JP-1 genotype) and PO880033 (PO-6 genotype).

Total DNA for fingerprinting was extracted from lyophilised mycelium as described by Goodwin et al. (1992a) digested with *Eco*RI (Amersham Pharmacia

Biotech, Budapest, Hungary) and electrophoresed in 0.8% agarose gel. Blots on Hybond-N^+ were probed with RG57 (courtesy of W.E. Fry, Cornell University, Ithaca, NY, USA) that was labelled with digoxigenin-11-dUTP. Standard isolates used in fingerprinting were US930287, NL-91006, NL-85138, NL-91015, 1561 and 1568.

Data analysis

Nei's gene diversity analysis (Nei, 1973) was conducted on the basis of allele frequencies at the two (*Gpi* and *Pep*) allozyme loci to assess the genetic differentiation between populations from potato and tomato as well as populations of A1 and A2 mating types. (The RG57 fingerprint loci were not included in this test, because it is difficult to determine whether the genotype is heterozygous or homozygous.) The mean of gene diversities of two sampling units ($\bar{H}_S$), and the proportion of the total gene diversity (H_T) due to differentiation between sampling units (G_{ST}) were calculated according to the methods of Nei and Chesser (1983) to correct for bias in sampling between subunits. This analysis was completed with Nei's unbiased genetic distance test (Nei, 1978).

To assess genotypic diversity, mating type, allozyme genotypes and 24 RG57 fragments were combined. These multilocus genotypes differing only at one or at most two allozyme or DNA fingerprint loci were distinguished from each other by placing a decimal point and a number after the genotype name according to Forbes et al. (1998). Polymorphic bands of the RG57 fingerprints were scored as present (1) or absent (0). Genotypic diversity was calculated by the normalised Shannon diversity index (H_S) developed for comparing phenotypic diversities when sample sizes vary (Sheldon, 1969): $H_S = -\sum g_i \ln g_i / \ln N$, where g_i is the frequency of the ith multilocus genotype and N is the sample size. This index ranges from 0 (there is no diversity) to 1 (each isolate represents a unique genotype).

Cluster analyses were performed to measure the similarity among multilocus genotypes. A similarity matrix was generated for all possible pairwise comparisons among different genotypes based on the Jaccard similarity coefficient. Here, mating type data were treated as binary characters (A1 = 1; A2 = 0). Each allozyme allele detected and RG57 loci were also scored either as present (1) or absent (0). RG57 loci 4, 11, 12 and 15 were excluded because the coefficient discounts matching negative characters. A dendogram was constructed from the similarity matrix data by the unweighted pair group method analyses (UPGMA).

Results

Thirty-six isolates were recovered from 12 potato fields at 9 sites and from 16 tomato fields at 13 sites (Figure 1). In 6 potato fields only A1 and in 4 potato fields only A2 isolates were sampled. Two potato fields at sites #9 and #15 had both A1 and A2 mating types. At collection site #9 (southwest Hungary), where the most serious damage to potato was recorded in 1998, one A1 and two A2 isolates were identified from a single field. Of the 16 tomato fields sampled, 2 had only A1 and 13 had only A2 isolates; one field at site #3 had both A1 and A2 mating types.

Of 20 isolates that were screened for sensitivity to metalaxyl 10 were sensitive, 2 intermediate and 8 resistant (data not shown). Sensitivity and resistance were detected in both A1 and A2 mating types and among tomato and potato isolates. The number of sensitive, intermediate and resistant isolates was 3 : 1 : 6 and 7 : 1 : 2 for isolates from crops of potato and tomato, respectively. The 9 A1 isolates tested comprised 2 sensitive, 1 intermediate and 6 resistant isolates whereas the 11 A2 isolates comprised 8 sensitive, 1 intermediate and 2 resistant isolates.

For glucose-6-phosphate isomerase, only one allele, that is the 100/100 genotype, was detected. In contrast, three alleles, 83, 96 and 100 were identified for peptidase (Table 1). These alleles formed the genotypes 83/96 (2 isolates), 96/96 (18 isolates), 96/100 (10 isolates) and 100/100 (6 isolates). Whether our *Pep* genotype defined as 83/96 by means of CAE is a novel combination or, is the same as *Pep* 83/100 previously determined by starch gel electrophoresis in other laboratories (Tooley et al., 1993; Sujkowski et al., 1994; Lebreton and Andrivon, 1998), remains to be further tested, because the isolate PO880033 determined as *Pep* 83/100 showed a pattern identical with that of our *Pep* 83/96.

A total of 18 different RG57 fingerprints were detected, but four genotypes, that is I, II, VIII and XVII represented almost half of the isolates (44%). Fingerprint II was the only one recovered from both potato and tomato. Bands 2, 3, 5, 6, 7, 8, 9, 10, 16, 17, 18, 19, 21 and 24 were polymorphic.

In total, 20 multilocus genotypes, 8 A1 (40%) and 12 A2 (60%), were found when mating type, allozyme

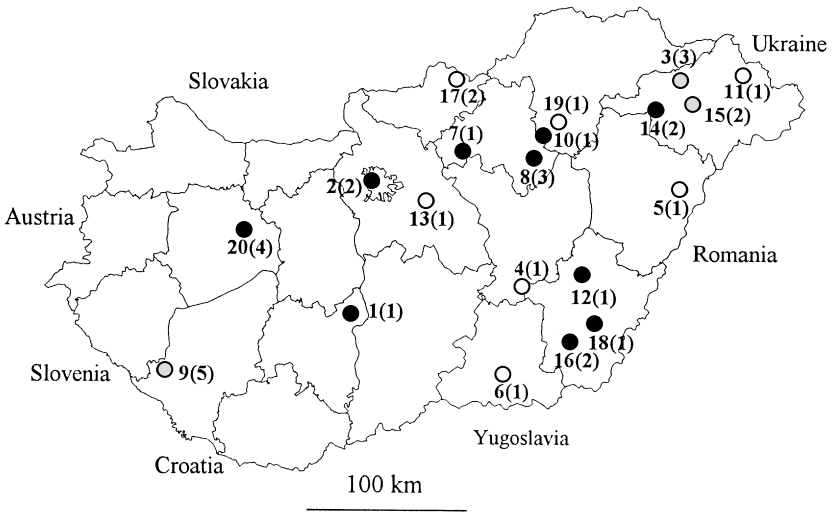

Figure 1. Locations of the sampling sites in Hungary. 1 = Bölcske, 2 = Budapest, 3 = Buj, 4 = Cserkeszőlő, 5 = Debrecen, 6 = Forráskút, 7 = Hatvan, 8 = Heves, 9 = Inke, 10 = Kerecsend, 11 = Kisvárda, 12 = Mezőmegyer, 13 = Monor, 14 = Nagycserkesz, 15 = Nyíregyháza, 16 = Orosháza, 17 = Őrhalom, 18 = Szabadkígyós, 19 = Szomolya, 20 = Zirc; Numbers in parentheses indicate the sample size collected at each site; ○ = A1 mating type; ● = A2 mating type; ● = A1 plus A2 mating types.

Table 1. Multilocus genotypes[1] of *P. infestans* in Hungary (1991–1998)

Genotype	Site[2]	Mating type	*Pep* genotype[3]	RG57 genotype[4]		Sample size[5]
HU-1	6, (13)	A1	83/96	111101001111101111011	IV	2 (1/93)
HU-2	(20)	A2	96/96	110110000111000111011	XVI	1 (1/96)
HU-3	20	A2	96/96	110100001110101111011	I	2
HU-3.1	(8), 9	A2	96/96	110100001111001111011	II	2 (1/97)
HU-3.2	9, 20	A2	96/100	110100001110101111011	I	3
HU-3.3	15	A2	96/100	110000001110101111011	XIII	1
HU-4	2, 9	A2	96/100	110100001111001111011	II	3
HU-5	(10)	A2	96/96	111101101110101111011	III	1 (1/91)
HU-6	7	A2	96/96	111101001111101101011	V	2
HU-7	12, 16, 18	A2	96/96	110110001111010101011	XVII	4
HU-8	3, 14	A2	96/96	111001101111001101001	VIII	3
HU-9	19	A1	96/96	111101001111100101011	VI	1
HU-9.1	5	A1	96/96	110101001111100101011	VII	1
HU-10	15	A1	96/96	110000001110100111011	XII	1
HU-11	1	A2	96/100	101100001111100111011	XVIII	1
HU-12	3	A1	96/100	110100001111011111111	XV	2
HU-13	8	A2	100/100	100100100110001111011	XI	1
HU-14	(17)	A1	100/100	110100011111001111011	XIV	2 (2/97)
HU-15	(4), 9	A1	100/100	100100011111000111011	IX	2 (1/97)
HU-15.1	11	A1	100/100	100100011111100111011	X	1

[1]Multilocus genotypes were identified according to Forbes et al. (1998).
[2]Number in parentheses indicates site of collection before 1998; also see Figure 1.
[3]The *Gpi* was monomorphic (100/100).
[4]RG57 fragments were identified according to Goodwin et al. (1992a). Presence and absence of fragments are indicated by 1 and 0, respectively. Bands listed from left to right are: 1, 2, 3, 5, 6, 7, 8, 9, 10, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25. Fragment 4 was excluded because it was reported to be inconsistent, whereas fragments 11, 12 and 15 were not detected in Hungary.
[5]Numbers in no parentheses indicate total size whereas those in parentheses refer to sample size/year of collection before 1998.

Table 2. Gene diversity[1] and genetic distance[2] analysis

Sampling units	$\bar{H}_S$	H_T	G_{ST}	Genetic distance[2]
Potato : tomato host comparison	0.225	0.240	0.062	0.0439
A1 : A2 comparison	0.239	0.261	0.084	0.0642

[1]The mean of gene diversities of two sampling units ($\bar{H}_S$), the total gene diversity (H_T) and the proportion of the total gene diversity due to differentiation between sampling units (G_{ST}) were calculated according to Nei and Chesser (1983) to correct for expected bias due to finite sampling.
[2]Nei's (1978) unbiased genetic distance.

and DNA fingerprint data were combined (Table 1). Nine of these 20 clonal genotypes were detected only once, whereas the rest of them occurred twice or more. Each of the genotypes HU-1, HU-3.1, HU-3.2, HU-4 and HU-15 could be found at distant locations, 100–200 km apart. With exception of HU-1, these genotypes also co-existed in a single potato field. Moreover, HU-1, HU-3.1 and HU-15 were detected not only in 1998, but also in 1993, 1997 and 1997, respectively.

There was a low level of genetic differentiation between the sampling units as indicated by the G_{ST} values calculated on the basis of allele frequencies at *Gpi* and *Pep* loci (Table 2). Six per cent and 8% of the overall gene diversity, respectively, was due to differentiation between populations from potato and tomato as well as populations of the A1 and A2 mating types. These low levels of population subdivision was supported by Nei's unbiased genetic distance analysis (Table 2).

Genotypic diversity measured by the normalised Shannon diversity index was greater for RG57 genotypes ($H_S = 0.705$) than for allozymes ($H_S = 0.324$). Total diversity that was determined for multilocus genotypes (mating type, *Gpi, Pep* and RG57 fingerprint) in the entire collection was $H_S = 0.805$. On this basis, isolates from potato and tomato were almost equally diverse with $H_S = 0.816$ and 0.767, respectively.

Similarity values measured by UPGMA cluster analysis ranged between 0.58 and 0.938 among the multilocus genotypes (Figure 2). UPGMA grouped the 20 multilocus genotypes into 5 clusters. A clear influence of the host origin was evident within cluster 4 containing genotypes exclusively from potatoes and cluster 5 consisting of genotypes from tomatoes only. Clusters 1 and 3 contained genotypes mainly from tomatoes, and

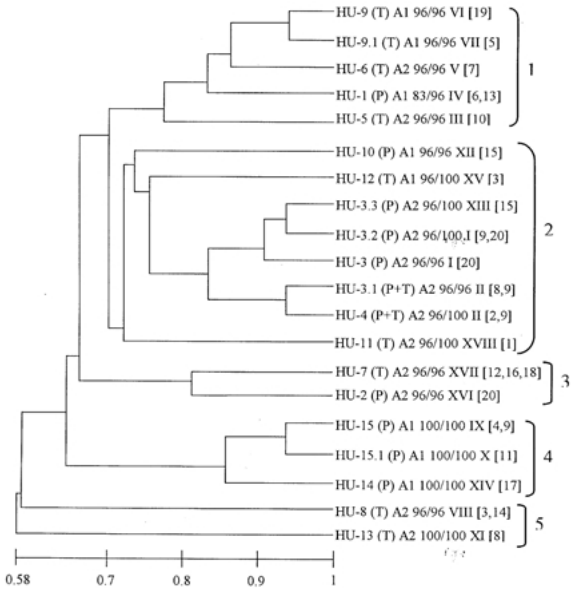

Figure 2. Dendogram showing the relationships among the multilocus genotypes of *P. infestans*. The corresponding host plants (P = potato, T = tomato), mating types, peptidase, RG57 genotypes and (sampling sites), as in Table 1 and Figure 1, are indicated here. (Glucose-6-phosphate isomerase was monomorphic (100/100) for each multilocus genotype.) UPGMA cluster analysis was performed on the basis of the Jaccard similarity coefficients. Similarity values are at the bottom.

genotypes either from potato or tomato, respectively. The largest cluster (no. 2) grouped genotypes found on both hosts with those from either host. The analysis of similarity values within and among clusters thus indicate a low level of differentiation on the basis of host origin. Although the spatial pattern of population structure showed some geographic substructuring, the extent of genetic similarity was not closely associated with regional distribution (cf. Figures 1 and 2). Clustering genotypes HU-9, HU-9.1, HU-6 and HU-5, were all collected in the northeast region but HU-1 from the same cluster (no. 1) was from the South. In contrast, closely related genotype pairs such as HU-3.1/HU-4 or HU-3.3/HU-3.2 were both found at three distant locations.

Discussion

A considerable diversity was identified among isolates of *P. infestans* collected in Hungary between 1991 and 1998. These isolates share some characteristics with isolates from other European countries, particularly

Poland and the Netherlands, from where Hungary imports large quantities of potato, including seed potato. All the isolates characterised can be ascribed to the 'new' population on the basis of presence of the A2 mating type, tolerance to metalaxyl, RG57 fingerprints and allozyme alleles including 100 for *Gpi* and 83, 96 or 100 for *Pep*. These results are consistent with a replacement of 'old' isolates by 'new' migrant ones in European populations of *P. infestans* (cf. Fry et al., 1993). Due to a lack of collections before 1991 and of population data prior to 1998, however, we do not know with certainty when the replacement started and how rapidly the changes have taken place in Hungary. The occurrence of the same clonal genotypes at two or more distant locations provide further evidence for migration of the pathogen that might have been distributed via seed tuber over long distances.

The Hungarian population of *P. infestans*, however, has some unique features. First, the proportion of the A2 mating type to the A1 is surprisingly high. In light of earlier reports on low ratios of A2 to A1 (Gisi and Cohen, 1996; Day and Shattock, 1997; Lebreton and Andrivon, 1998; Brurberg et al., 1999; Zimnoch-Guzowska, 1999) or on the seasonal/regional variations of these ratios (Schöber and Turkensteen, 1992; Sujkowski et al., 1994; Anoshenko, 1999; Sedegui et al., 2000; Zwankhuizen et al., 2000), it is a question whether the high frequency of the A2 isolates in Hungary is temporary or has been maintained consistently for several years.

Our observation that the A2 mating type is more frequent on tomato than on potato, is in accordance with data from elsewhere (Fry et al., 1991; Fraser et al., 1995). Response to metalaxyl of our isolates is in agreement with the situation in other European countries in that resistance occurs at a greater frequency in isolates from potato than in isolates from tomato and, is associated with the A1 rather than the A2 mating type (cf. Gisi and Cohen, 1996). Although isolates from potato and tomato appear different in this regard, the presence of the same multilocus genotypes on each crop and statistical data of genetic analyses indicate a low level of population subdivision, as compared to France (Lebreton and Andrivon, 1998), for instance.

Allozyme genotypes also differ remarkably from those detected in other European countries, where alleles 86, 90 and 100 for *Gpi* and alleles 83, 92 and 100 for *Pep* are the primary alleles found. We identified only the 100 allele for *Gpi* as in the UK (D.S. Shaw, pers. comm.) and alleles 83, 96 and 100 for *Pep*. The 96 allele for *Pep*, which seems to be the most frequent one in Hungary like in East Asia (Mosa et al., 1993; Koh et al., 1994), has been detected at low frequencies in a few countries including Poland from Europe (Sujkowski et al., 1994; Forbes et al., 1997; 1998).

Genotypic diversity for allozymes is considerably lower than that for RG57 genotypes ($H_S = 0.324$ versus 0.705). Most of the 18 RG57 fingerprints characterised among 36 isolates are apparently unique to Hungary; only fingerprint IX is identical with the Norwegian fingerprint N-36 (Brurberg et al., 1999) and similar to the French profile FR-06 (Lebreton and Andrivon, 1998). In addition, genotypes II, XII, XIV or XVI are similar to most recently published Dutch fingerprints NL-102, NL-105, NL-157 or NL-160 (Zwankhuizen et al., 2000).

The result of greatest interest might be that a large variety of mutilocus genotypes was found and none of them was identical to those reported from other countries. This unique diversity of genotypes would be unexpected in a population that reproduced completely asexually. Despite the lack of direct evidence (oospores) for sexual recombination, the large numbers of unique genotypes in Hungary and the co-existence of both mating types in a single field, as found at three locations, indicate the potential for sexual reproduction. The low G_{ST} value for genetic differentiation between mating types is one of the most likely indications of genetic recombination through the sexual cycle. A comparable situation has been recorded in Poland (Sujkowski et al., 1994), that is reminiscent of the situation in central Mexico, the centre of diversity and presumed centre of origin of *P. infestans*, where almost as many genotypes as isolates exist and the sexual recombination among them appears unlimited. Most recent reports on the possible sexual reproduction of *P. infestans* has been published from The Netherlands and from the USA (Gavino et al., 2000; Zwankhuizen et al., 2000). Since sexual recombination and oospore formation may affect the epidemiological dynamics of the disease and the genetic diversity of the pathogen, this aspect merits further study in Hungary on larger samples.

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