Occurrence and Distribution of Mating Types A1 and A2 of *Phytophthora infestans* (Mont.) de Bary in the Czech Republic

Jana MAZÁKOVÁ[1], Vladimír TÁBORSKÝ[1], Miloslav ZOUHAR[1], Pavel RYŠÁNEK[1], Ervín HAUSVATER[2] and Petr DOLEŽAL[2]

[1]Department of Plant Protection, Faculty of Agrobiology, Food and Natural Resources, Czech University of Agriculture in Prague, Prague-Suchdol, Czech Republic;
[2]Potato Research Institute Havlíčkův Brod, Ltd., Havlíčkův Brod, Czech Republic

Abstract

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A total of 199 *Phytophthora infestans* isolates were obtained from leaves, tubers and fruits of infected crops of potato and tomato in different regions of the Czech Republic in 2003, 2004 and 2005. They were analysed for mating type using the conventional pairing assay and PCR markers; 107 isolates were of A1 and 92 of A2 mating type. No self-fertile isolate was found. Our study is the first report of the presence and distribution of the A2 mating type of *P. infestans* in the Czech Republic. The co-existence of the two mating types may enable the pathogen to reproduce sexually, thus enhancing the diversity of its population countrywide.

Keywords: potato late blight; mating types; PCR; CAPs

Under conditions in Central Europe, potatoes are practically every year infected by late blight, caused by *Phytophthora infestans* (Mont.) de Bary. In the Czech Republic during the last 5 years, periods of intensive development of the pathogen alternated with periods of low occurrence. In the "late blight years" 2000–2002, the occurrence of late blight was very high throughout the vegetative period. In 2003, the disease was found at the beginning of the growing season, but was then stopped by dry weather that persisted to the end of the growth of potatoes. During 2004 and 2005 the fungus caused medium to strong levels of infection that were about 3 weeks later than normal.

P. infestans, causal agent of late blight, belongs to heterothallic species, therefore presence of hyphae of opposite mating types A1 and A2 are necessary for sexual reproduction and oospores forming (Drenth 1994). Although in 1958 common appearance of both mating types was reported for the first time (Gallegly & Galindo 1958), until the 1980s only mating type A1 was known all over the world. In Europe the first report of occurrence of mating type A2 comes from the Switzerland (Hohl & Iselin 1984), then from England and Wales (Gunn 1990). Subsequently the appearance of mating type A2 was confirmed in many countries of Europe, e.g. the Netherlands (Frink-

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ing *et al*. 1987), Poland (Sujkowski *et al*. 1994), France (Lebreton & Andrivon 1998), Hungary (Bakonyi & Érsek 1997), Finland (Kankila *et al*. 1995), Norway (Hermansen & Amundsen 1995) and in America and Asia (Fry *et al*. 1993, Fry & Goodwin 1995; Goodwin *et al*. 1995). The introduction of A2 mating type and new pathogen populations, composed of both mating types, within the second migration of the pathogen from Mexico (Fry & Goodwin 1995) contributed to its increased diversity and is thus also associated with an increased level of resistance against phenylamide fungicides (Goodwin *et al*. 1995). Oospores (Figures 1 and 2) overwintering in soil can start a dispersed late blight infection. They can survive unfavourable weather conditions in potato leaves and stems and start infection when conditions become suitable within the season. The most important aspect of the existence of oospores lies in the production of new recombinant races through sexual reproduction that results in genetically more diverse *P. infestans* populations (Drenth *et al*. 1995).

The objective of this study was to determine the mating types of *P. infestans* isolates collected in the Czech Republic in 2003, 2004 and 2005. For this, we used a conventional pairing method and molecular techniques based on polymerase chain reaction (PCR) and the cleaved amplified polymorphic sequence (CAPS) analysis.

MATERIAL AND METHODS

Collection of infected plants. During 2003, samples of infected haulm tissue and tubers were collected from an early potato-growing region around the localities Lysá nad Labem and Okřesaneč (Central Bohemia Region), and from a late potato-growing region and main seed-potato area around localities Želiv and Olešná (Vysočina Region). During 2004, samples of infected plants were collected from a late potato-growing region and main seed potato crop area, locality Vitice, Petrův Vrch (Vysočina Region); from an early potato-growing region, locality Czech Farm (Central Bohemia Region); from the Potato Field

Table 1. List of potato cultivars from 2005

Kolinec-Vlčkovice	Semice	Horažďovice	Valečov	Lípa	Žabčice	Keřkov
Vaneda	Impala	Baltica	Kordoba	Lady Christal	Ambra	Komtesa
	Rosara	Flavia	Ditta	Verona	Keřkovské rohlíčky	Kornelie
		KE 221/28	Dali	Valeta	Astoria	Kordoba
		Tegal	Karin	Leoni	Kornelle	Rivier
		VE 5/4	Asterix	Everest	Sázava	
		Angela	Saturna	Arnika	Karin	
		Flora	Adéla	Rosella	Velox	
		Goldika		VY 9/5	Flavia	
		Ramos		Clarissa	Berber	
		Ikar		Esprit	Kordoba	
		Adéla		Satina	Rosara	
		Kordoba		Redstar	Krasa	
		Arabela		VE 117/2	Katka	
		Sonate		Agria		
		Finka		Korela		
		Vitesse		Santé		
		Santana		Delianne		
		Valetta		KE 572/15		
		Tomensa				

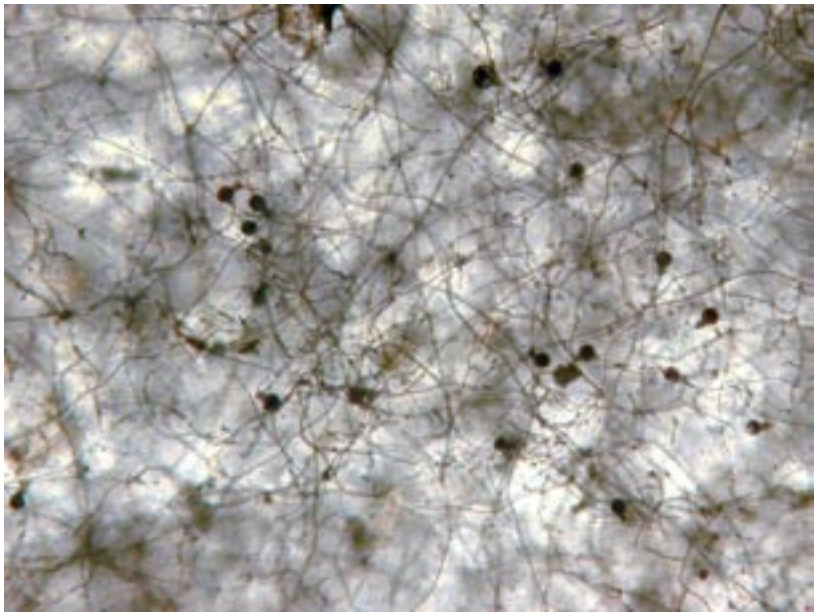


Figure 1. Heterothallic hyphae and oospore of *P. infestans* (Orig. microscopic photo Mazáková, Táborský)

Station Valečov (Vysočina Region); from private small gardens from the South and Central Bohemia Regions, and from experimental fields of the Czech University of Agriculture (CUA) in Prague-Suchdol. Also, infected parts of tomato plants were obtained from private gardens. During 2005, samples of infected potato plants were mostly collected in the late potato-growing region and main seed potato crop area. Table 1 lists the potato cultivars collected in 2005.

***Isolation and maintenance of** P. infestans*. Leaves and stems with lesions or tuber slices of infected potato and leaves, stems and fruits of infected tomato were placed into a moist chamber (an inverted Petri dish with water agar). After visible intensive sporulation, mycelium with sporangia was transferred onto a small piece of a surface-sterilised tuber of cv. Ditta. These pieces were put on B rye agar (Caten & Jinks 1968) with antibiotics (Polymyxin B sulfate, Ampicillin, rifamycin) and fungicides (TCNB, Fundazol 50 WP). Cultures were incubated in the dark at 15–18°C and transferred every 3–4 weeks.

Pairing test. The mating type of an isolate was determined by pairing of unknown isolates with standard A2 isolates on B rye agar or V8 juice agar. The isolate to be tested was placed on one side of the Petri dish, the A2 standard isolate on

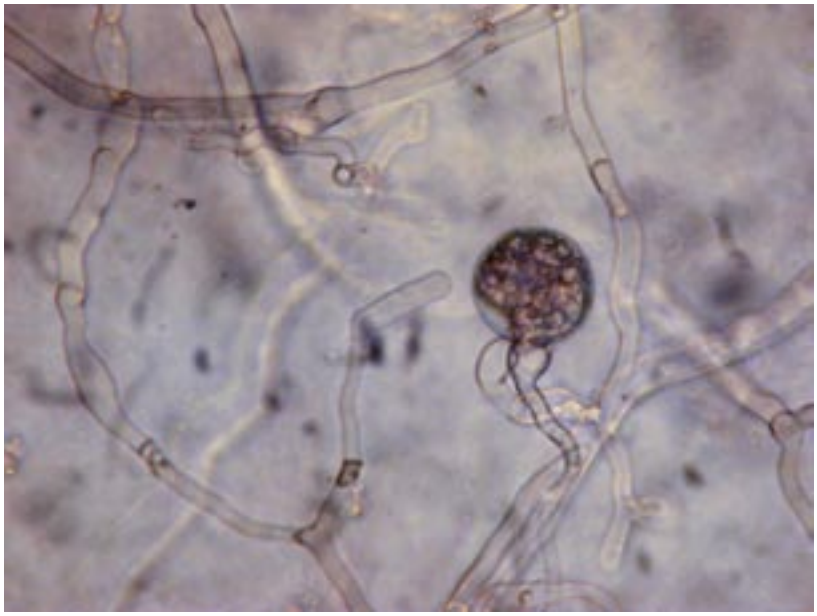


Figure 2. Oospores of *P. infestans* in Petri dish (Orig. photo Mazáková, Táborský)

the other side. The Petri dishes were incubated at 15–18°C in the dark for about 10 days or until oospores were formed. On the basis of the appearance of oospores a tested isolate was classified as mating type A1 or A2 (Tooley *et al*. 1989). If it produced oospores with the A2 standard isolate it was classified as A1 mating type; an isolate that did not create oospores was determined as A2 mating type. From the group of A1 mating type isolates, one isolate was chosen as A1 standard isolate and all isolates were paired with this isolate to confirm the mating type classification. All isolates were again tested with standard isolates of A1 and A2 that had been obtained from the Scottish Crop Research Institute, Dundee, in 2004.

Extraction of DNA from pure cultures. The pathogen mycelium was homogenised by grinding under liquid nitrogen and CTAB extraction buffer (50mM Tris-HCl pH 8.0, 0.7mM NaCl, 10mM EDTA, 1% CTAB, 20mM mercaptoethanol). After incubation for 60 min at 65°C in a water bath, an equivalent amount of chloroform-isoamylalcohol (24:1) solution was added. The tubes were vortexed and centrifuged (7000 × g) for 10 min, the aqueous phase was removed into new tubes and isopropanol was added. Tubes were mixed gently and precipitated. After the following centrifugation the DNA pellet was washed in 70% ethanol and centrifuged. The DNA pellet was dried and resuspended in deionisated water (dd H_2O). DNA was then used for PCR to amplify fragments that enable distinctions between the two mating types of the pathogen.

PCR assay. For detection of isolates of A2 mating type, the PHYB-1 and PHYB-2 primers were used for amplification of a specific DNA fragment (Kim & Lee 2002). PCR was performed and amplification conditions were as described above except that the annealing temperature was 59°C and the number of cycles was 35.

CAPs assay. W16-1 (5'-AACACGCACAAGGCATATAAATGTA-3') and W16-2 (5'-GCGTAATGTAGCGTAACAGCTCTC-3') primers (Judelson *et al*. 1995) were used for amplification of the DNA fragment of both mating types. PCR was performed in 25 µl reaction volume containing 2.5 µl (1×) buffer for DyNAzyme polymerase, 0.25 µl dNTP (0.125µM each nucleotides), 0.4 µl primer mix (0.4µM each primer), 0.5 µl DyNAzyme polymerase (0.5 U) (Finnzymes), 20.35 µl ddH_2O and 1 µl template DNA. The PCR assay was performed in thermocykler MJ Research PTC 200 and amplification conditions were as follows: an initial denaturation at 94°C for 5 min, after which 29 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 53°C) and primer extension (1 min at 72°C). PCR products were cleaved with *Hae*III (*Bsu*RI) restriction enzyme to distinguish both mating types from each other (Judelson *et al*. 1995). The restriction products were loaded on 1.5% agarose gel and visualised by means of ethidium bromide (1 mg/ml) and UV transilluminator.

RESULTS

Collection and isolation of *P. infestans*

Of the isolates collected, eventually 199 were maintained in pure culture and tested for mating type, 31 isolates from the year 2003 and 64 from 2004. Most isolates originated from infected potato leaves and stems. One isolate was from an infected tomato leaf (2004) and one from an infected fruit (2003); the 104 isolates collected in 2005 came from potato leaves and tomato leaves and fruits.

Pairings

All isolates from 2003, 2004 and 2005 were used in the biological test. In 2003, 21 isolates of *P. infestans* were classified as A1 mating type, 10 isolates as A2. Mating type A2 was found only in collections from localities Lysá nad Labem and Okřesaneč (Central Bohemia Region, early potato-growing area). In 2004, from a total of 64 isolates, 30 were determined as A1 mating type and 34 as A2. Mating type A2 was found in isolates from localities Czech Farm (Central Bohemia Region, early potato-growing area), Vitice, Valečov, Petrův Vrch and Pacov (Vysočina Region, late and seed potato-growing area), and in several private gardens from the South Bohemia Region. From the year 2005, 10 isolates from Kolinec-Vlčkovice and 6 from Horažďovice (both Pilsen Region), 7 from Semice (Central Bohemia Region), 21 isolates from Lípa, 5 from Valečov and 2 from Keřkov (all in Vysočina Region) and 6 isolates from Žabčice (South Moravia Region) were of A1 mating type; whereas 3 isolates from Semice and 14 from Horažďovice, 5 isolates from Lípa, 9 from Valečov and 2 from Keřkov, and 14 isolates from Žabčice were of A2 mating type (Table 2).

Table 2. Localities of collection, number of isolates and number of isolates of A1 and A2 mating types

Locality	Number of isolates	A1 mating type	A2 mating type
2003			
Olešná 1. collection	5	5	–
Olešná 2. collection	5	5	–
Olešná 3. collection	8	8	–
Lysá nad Labem	9	1	8
Želiv	1	1	–
Tuber u. o.	1	1	–
Okřesaneč	2	–	2
2004			
Želiv-Vitice	11	–	11
Petrův Vrch	9	–	9
Czech farm	10	6	4
Valečov	6	4	2
Blatnice	1	-	1
Pacov	3	1	2
CUA	5	5	-
Gardens	18	12	6
Tomato u. o.	1	1	–
2005			
Kolinec-Vlčkovice	10	10	0
Semice	10	7	3
Horažďovice	20	6	14
Valečov	14	5	9
Lípa	26	21	5
Žabčice	20	6	14
Keřkov	4	2	2

PCR assay

PCR assay was performed by PHYB-1 and PHYB-2 primers that amplified DNA fragment with 347 bp. This DNA fragment is specific for the A2 mating type, therefore after visualisation only isolates of A2 mating type were identified by this band, while isolates of A1 mating type cannot be visualised in gel patterns (Figure 3).

CAPs assay

557 bp fragments amplified by primers were present in isolates of both mating types. After cleaving by *Hae*III, isolates in lines with 457 bp fragments were A2 mating type. Both 457 and 557 bp fragments were observed in isolates of A1 mating type (Figure 4).

DISCUSSION

The monitoring of A1 and A2 mating type ratios is important to aid in the prediction of the extent of sexual recombination and thus the risk of long-lived oospores serving as primary inoculum sources. A study of the distribution of the A1 and A2 strains of *P. infestans* is fundamental to understanding the significance of mating type to both the generation and maintenance of genetic diversity and to disease aetiology (Cooke & Lees 2004). Migration of new

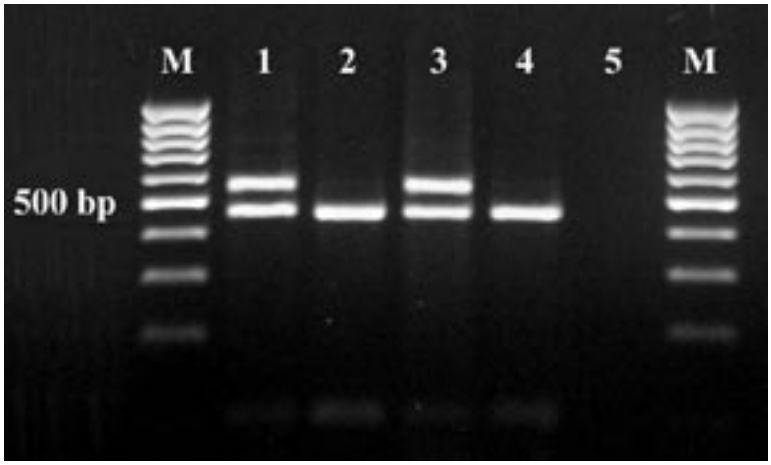
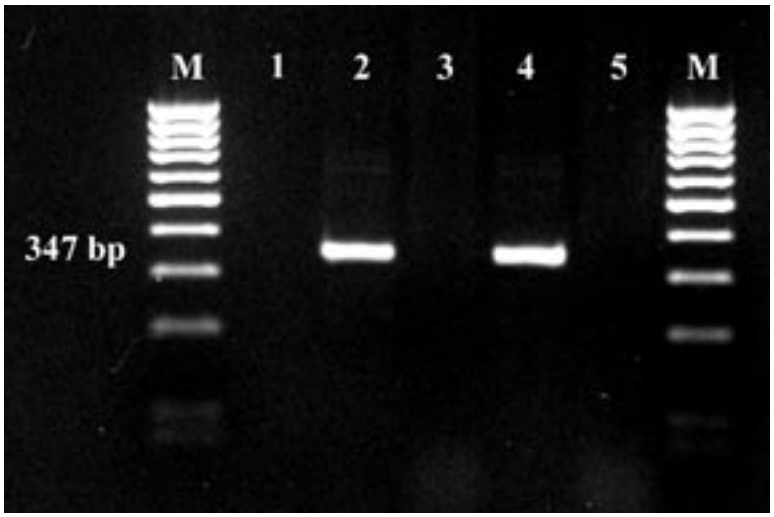


Figure 3. Products of restriction digestion by *Hae*III enzyme after amplification by W16-1 and W16-2 primers

lane M – molecular marker
lane 1 – A1 mating type standard isolate
lane 2 – A2 mating type standard isolate
lane 3 – unknown isolate of A1 mating type
lane 4 – unknown isolate of A2 mating type
lane 5 – negative control



lane M – molecular marker
lane 1 – A1 mating type standard isolate
lane 2 – A2 mating type standard isolate
lane 3 – unknown isolate of A1 mating type,
lane 4 – unknown isolate of A2 mating type
lane 5 – negative control

Figure 4. PCR products after amplification using PHYB-1 and PHYB-2 primers

genotypes of both mating types and the possibility of sexual reproduction created an increased interest in the investigation of biology and genetic variability of *P. infestans* by various markers. Mating types, together with virulence and fungicide resistance, belong to biologically significant markers. Among neutral markers, allozymes and DNA markers based on DNA polymorphism are included (DNA probe RG-57, mtDNA) (Drenth & Govers 1994; Drenth 1994).

In the Czech Republic, the pathogen *P. infestans* occurs regularly in potato-growing areas every year and targeted protection must be used to suppress and reduce the spread of the pathogen from plant to plant and from locality to locality. Yet protection measures could be complicated by the presence of oospores in soil and plant tissue. Since 2003 we are able to isolate *P. infestans* from infected potato and tomato plants and maintain the isolates in pure cultures. We can thus use pathogen mycelium to test isolates for their mating types as described above. All three methods provided identical results of mating type determination of the maintained isolates. On an electrophoreogram of CAPS products (Figure 4) it is evident that mating type A2 is present in homozygous state while mating type A1 is present in a heterozygous state, only the DNA fragment of one allele is cleaved.

In 2003 the ratio of A1 to A2 mating types was 70:30, in 2004 the ratio was 44:56 for the benefit of A2 mating type, one isolate from tomato was A1 mating type. The ratio of A1 to A2 mating types of 2005 is 55:45 for the present. According to our results it seems that till now, mating type A2 occurs mainly in stands established from seed potatoes imported from the Netherlands (localities Želiv-Vitice, Petrův Vrch). But we do not know exactly since when the A2 mating type strain was present in Czech pathogen populations, because regular monitoring of both mating types had begun in 1994 (Hausvater & Rasocha 1999) and during the period 1994–2000 no A2 mating type isolate has

been detected in the isolates collected (Hausvater & Rasocha 2000). From central European countries, A2 mating type was detected in Hungary in 1996, although it could have occurred in Hungary as early as 1991 because one isolate from infected tomato from 1991 was A2 mating type (Bakonyi *et al*. 2002). Studies from Poland also confirmed the presence of new *P. infestans* populations composed of both mating types (Sujkowski *et al*. 1994). A2 mating type was determined in Austria in the 1990s (Rauscher 2003) and in Slovakia (Forišeková, personal communication, unpublished data).

These results thus confirm the first detection of A2 mating type of *P. infestans* in the Czech Republic and show the distribution of both mating types in several regions. All three methods for testing of mating type were suitable for this purpose. *P. infestans* is a very important pathogen all over the world and it causes large damages under favourable conditions. Therefore, attention should be focused on monitoring the variability of the pathogen by various markers, especially mating types and resistance to systemic fungicides.

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Corresponding author:

Ing. Jana Mazáková, Česká zemědělská univerzita v Praze, Fakulta agrobiologie, potravinových a přírodních zdrojů, 165 21 Praha 6-Suchdol, Česká republika
tel.: + 420 224 382 601, fax: + 420 224 382 593, e-mail: mazakova@af.czu.cz