Analysis of Molecular Variability in *Plasmopara halstedii*, Causal Agent of Sunflower Downy Mildew. Use for the Characterisation of the Species and its Different Races

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Plasmopara halstedii (Farl) Berl. De Toni is the causal agent of downy mildew in *Helianthus annuus* (sunflower). The interaction between sunflower and *P. halstedii* seems to conform to the gene-for-gene hypothesis. Resistance to the disease appears to be race specific. Until 1981, only two races were known, race 1 in Europe and race 2 in North America. Vranceanu *et al.* (1981) described a race with different characteristics at the Plant Breeding Station at Fundulea in Romania. The same year, Fick and Auwater (1981) isolated a new race in North Dakota, USA, (race 3). Gulya and Urs (1985) identified race 4 in South Dakota, USA, and then Gulya *et al.*(1991) described three new races. In France, the last authors cited races 4 and 6 in addition to race 1, whereas Mouzeyar *et al.* (1994) isolated two races, A and B whose reactions with differential sunflower lines are not those published for 4 and 6. These apparently contradictory results may be due to different resistance test procedures. Roeckel-Drevet *et al.* (1997) identified two races C and D (which have not been differentiated from race 2 and 3) in France. The necessity to find new methods of pathotype characterization and to survey the evolution of downy mildew prompted us to analyse genetic variability in *P. halstedii.* The technique known as Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990) has been used successfully in various genetic studies (Wolff and Peters-van Rijn, 1993; Castagnone-Sereno *et al.* 1994) and seemed suitable for the analysis of intra- and inter-race variability in *P. halstedii* we report here. In addition, for the detection of the pathogen in seeds, a simple method of identification has been developed. RAPD analysis, based on the polymerase chain reaction (PCR)

makes possible the amplification of products which are species-specific. This study presents the definition of nucleotidic primers that can be used to detect, by PCR, the presence of the pathogen in sunflower plants.

Materials and Methods

Plasmopara halstedii isolates

French isolates of *P. halstedii* have been collected in 20 departments throughout France from 1970 to 1993. One isolate of races C and D, 5 isolates of race 1, 31 isolates of race A and 22 isolates of race B from different geographical origins were analysed.

For security reasons, no race was imported live into France. Samples of zoosporangia and zoospores were provided in 80% ethanol. Pathotype determination was made in the country of origin.

Other fungal strains

Seed pathogen isolates are maintained in the culture collection of the SNES-GEVES (Angers ; France) and were send to us as cultures on solid media (Malt 1% ; agar 1.5%).

DNA extraction and RAPD procedure

Spores of *P. halstedii* isolates were produced on the susceptible sunflower variety Peredovick and stored at -20°C or were provided in 80% ethanol. For all the other fungi, they were propagated on liquid media (malt 1%) prior to DNA extraction. The mycelium was recovered after filtration of the liquid culture on Whatman paper, and placed into a microcentrifuge tube (1.5 ml). The spores stored in ethanol 80% were recovered after centrifugation (10000 rpm, 4°C, 2 min) and elimination of the surpernatant. DNA was extracted according to Rogers and Bendich (1985). RAPD analysis (random decamer primers, amplification in a thermal cycler Perkin-Elmer Cetus 9600, electrophoresis of amplification product) was conducted according to Roeckel-Drevet *et al.*, 1997).

Cloning and sequencing of amplification products

Amplification products were gel purified before cloning. PCR^{TM}II vector (Invitrogen, Leek, The Netherlands) was used in cloning experiments which followed manufacturer's instructions (Original TA cloning Kit, Invitrogen). Plasmid amplification and preparation, using the *E. coli* INVαF' (Invitrogen) followed routine methods (Sambrook et al, 1989).

The insert DNA was sequenced by Eurogentec (Seraing, Belgium) using Sp6 and T7 primers.

Diagnostic PCR

SCAR primers were designed from the sequence of the insert of the clone which was obtained by the cloning of one RAPD band after amplification with OPJ15 primer (Operon) and DNA from *P.halstedii* race 1 as template.

The primers were synthesised by Eurogentec company (Seraing, Belgium). PCR was carried out on a thermal cycler Cetus 9600. When the amplification products were digested, the following restriction enzymes were *used: Alu I, Hha I, Hinf I, Mbo I* and *Rsa I* (Amersham). The amplification products from the diagnostic PCR experiment were visualized by electrophoresis on 1.4% agarose gels in Tris-acetic acid-EDTA (TAE) buffer stained with ethidium bromide.

Data analysis

Relationships between isolates were determined after calculation of Jacquard's coefficients (Sneath and Sokal, 1973). The dendrogram was constructed after cluster analysis of the similarity coefficients by unweighted pair-group method using arithmetic average (UPGMA) and single linkage (Sneath and Sokal, 1973).

Results

The RAPD technique only requires a few nanogrammes of DNA (Rafalski *et al.*, 1993), so it was quite appropriated in the study of obligate parasites which cannot be cultivated on artificial medium. DNA was extracted from spores which are produced by the fungus outside the plant host.

Variability of French races

Primer screening

Initial screening was carried out among decamer primers using one isolate of races 1, A and B. Among the 196 primers tested, either no amplification or a smear was observed with 67 primers (34%). When DNA fragments were amplified, polymorphism between races 1, A and B was observed with 30 (15%) primers and monomorphism with 101 (52%) primers. Since the spores used for DNA extraction were collected from Peredovik cotyledons, a control was made with sunflower genomic DNA.

Table 2 : DNA collection of *Plasmopara halstedii*

Continent	Name of strain	Code	Country	From	Race	Year Collected
Europe	CL151	F 1	France	Phillppon	1	1970
Europe	CL153	F 2	France	Walser	A	1988
Europe	CL152	F 3	France	Walser	B	1989
Europe	CL154	F 4	France	Walser	C	1995
Europe	CL155	F 5	France	Walser	D	1995
Europe	1650		Bulgaria	Gulya	3	1989
Europe	A		Spain	Melero-Vara	?	?
Europe	B		Spain	Melero-Vara	?	?
Europe	C	S1	Spain	Melero-Vara	?	?
Europe	D	S2	Spain	Melero-Vara	?	?
Europe	1555		Yugoslavia	Gulya	1	1988
Europe	H1-H	Y7	Yugoslavia	Masirevic	1	?
Europe	U 10	Y6	Yugoslavia	Masirevic	1	?
Europe	H1-NK1		Yugoslavia	Masirevic	1	?
Europe	2192		Yugoslavia	Gulya	2	1992
Europe	U 1	Y1	Yugoslavia	Masirevic	4	?
Europe	U 2	Y2	Yugoslavia	Masirevic	4	?
Europe	U 3		Yugoslavia	Masirevic	4	?
Europe	U 4		Yugoslavia	Masirevic	4	?
Europe	U 5		Yugoslavia	Masirevic	4	?
Europe	U 6	Y3	Yugoslavia	Masirevic	4	?
Europe	U 7	Y4	Yugoslavia	Masirevic	4	?
Europe	U 8		Yugoslavia	Masirevic	4	?
Europe	U 9	Y5	Yugoslavia	Masirevic	4	?
Europe	H 50		Yugoslavia	Masirevic	4	?
Europe	R 4		Yugoslavia	Masirevic	4	?
Europe	A stillité		Yugoslavia	Masirevic	?	?
Europe	2339		Hungary	Gulya	1	?
Europe	2169		Hungary	Gulya	1	?
Europe	2204		Hungary	Gulya	3	?
Europe	2036		Hungary	Gulya	3	?
Europe	2055		Hungary	Gulya	4	1991
Europe	1961		Hungary	Gulya	4	1990
Middle East	Karaj	I 1	Iran	Zad	?	1995
Middle East	Mazandaran		Iran	Zad	?	1995
Middle East	22	I 2	Iran	Zad	?	1995
Middle East	32	I 3	Iran	Zad	?	1995
Middle East	38	I 4	Iran	Zad	?	1995
Africa	M 1	M 1	Morocco	Achbani	1	1995
Africa	M 2	M 2	Morocco	Achbani	1	1995
Africa	M 3	M 3	Morocco	Achbani	1	1995
Africa	M 4	M 4	Morocco	Achbani	1	1995
Africa	2509		Morocco	Gulya	1	1994
Africa	M 5	M 5	Morocco	Achbani	2	1995
Africa	2702		S. Africa	Gulya	1	1997
Africa	2703		S. Africa	Gulya	2	1997
Africa	2678		S. Africa	Gulya	5	1996
Asia	C 1	C 1	China	LI	?	1996
Asia	C 2	C 2	China	LI	?	1996
Asia	C 3	C 3	China	LI	?	1996
Asia	C 4	C 4	China	LI	?	1996
Asia	1914		China	Gulya	3	1990
S. America	A 94	A 1	Argentina	Bazzalo	?	1994
S. America	A 96	A 2	Argentina	Bazzalo	?	1996
N. America	2262		Canada	Gulya	2	1992
N. America	1907		Canada	Gulya	4	1990
N. America	1434		Canada	Gulya	4	1985
N. America	1978		Canada	Gulya	4	1988
N. America	1409		Canada	Gulya	4	1985
N. America	2213		Canada	Gulya	4	1990
N. America	1796		Canada	Gulya	4	1990
N. America	2275		Canada	Gulya	9	1992
N. America	R 2	U 1	USA	Gulya	2	?
N. America	1227		USA	Gulya	2	1984
N. America	1458		USA	Gulya	2	1986
N. America	2022		USA	Gulya	2	1991
N. America	2636		USA	Gulya	2	1996
N. America	R 3	U 2	USA	Gulya	3	?
N. America	2156		USA	Gulya	3	1982
N. America	1231		USA	Gulya	3	1984
N. America	1317		USA	Gulya	3	1985
N. America	1974		USA	Gulya	3	1988
N. America	1623		USA	Gulya	3	1987
N. America	2065		USA	Gulya	3	1991
N. America	2172		USA	Gulya	3	1992
N. America	R 4	U 3	USA	Gulya	4	?
N. America	1521		USA	Gulya	4	1987
N. America	1411		USA	Gulya	4	1988
N. America	1984		USA	Gulya	4	1990
N. America	2533		USA	Gulya	4	1993
N. America	2622		USA	Gulya	4	1996
N. America	1574		USA	Gulya	5	1988
N. America	2299		USA	Gulya	5	1992
N. America	1297		USA	Gulya	7	1985
N. America	2305		USA	Gulya	8	1992

Analysis of intrarace variability

When several isolates of the same race were available, the analysis of intrarace variability was carried out. We used 16 primers chosen randomly among the 30 primers revealing polymorphism between races 1, A and B, allowing us to amplify at least 100 DNA fragments from each race. No variability at all was observed between isolates of the same race.

Analysis of inter-race variability (Table 1)

Having shown above that no intrarace variability was observed for race 1, A and B, we used only one isolate of these races for the following analysis. Seventy-five primers were chosen randomly among the 131 primers which gave good DNA amplification in the primer screening process. At least 397 DNA fragments were amplified from each race. Similarity coefficients are reported in Table 1 and indicate that the isolates studied are very similar to each other.

Table 1 : Relationships between French races of *Plasmopara halstedii*, Jacquard similarity between races 1, A, B, C and D.

	1	A	B	C	D
1	**1.00**				
A	**0.91**	**1.00**			
B	**0.91**	**0.93**	**1.00**		
C	**0.93**	**0.93**	**0.96**	**1.00**	
D	**0.98**	**0.89**	**0.89**	**0.92**	**1.00**

Variability of international strains of 32 isolates

Origins of 32 *P. halstedii* strains analysed by RAPD were presented in table 2 : column « code »

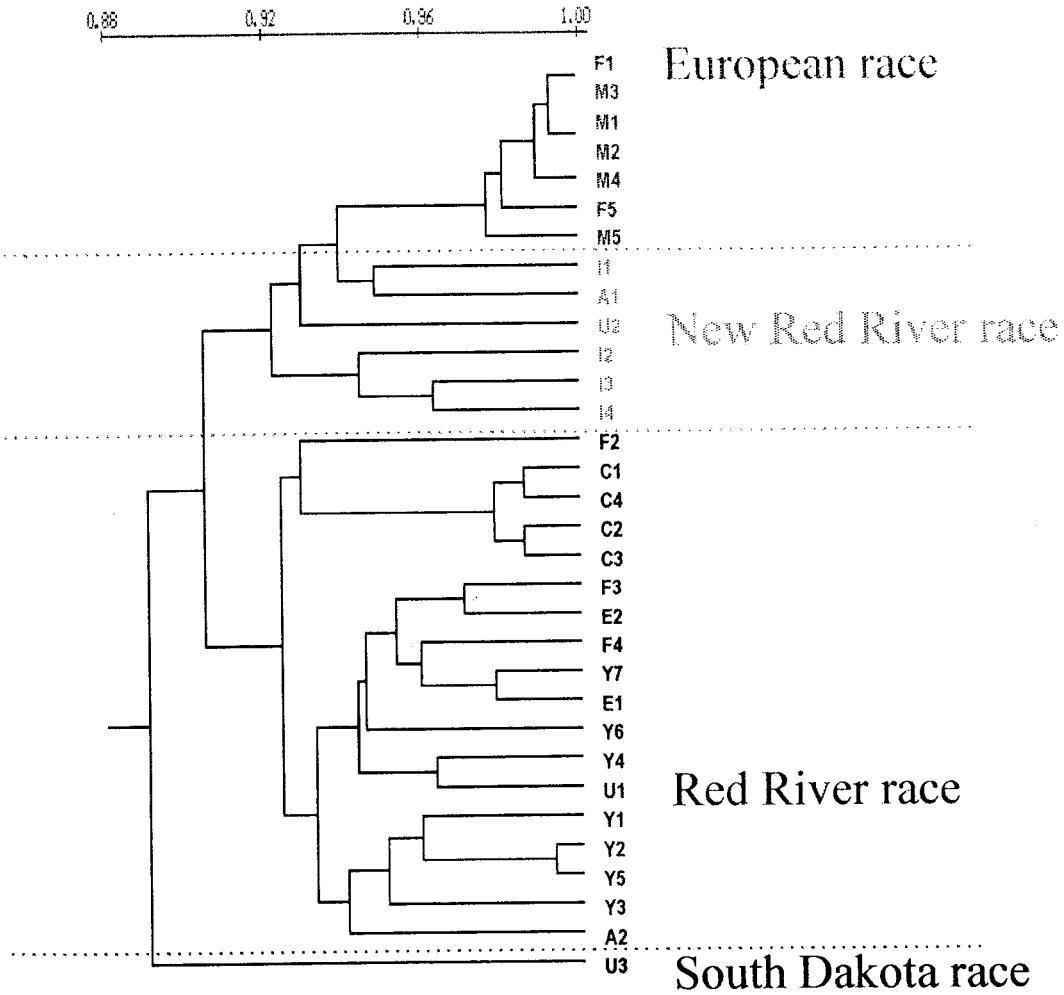
Amplification

The number of DNA fragments amplified by any one primer (on 26 primers) varied from 1 to 13. Thirteen primers showed no polymorphism. A total of 134 bands were revealed.

Classification of isolates

DNA amplifications of 32 isolates from 8 different countries show considerable similarities in RAPD profiles obtained (from 85% to 100%). These results are rather different from those of Borovkova *et* al. (1992) who found frequent polymorphism within and between downy mildew

Fig 1 : Relationships between isolates of *Plasmopara halstedii* from different countries. Dendrogram of 32 isolates (143 bands revealed by 26 primers in RAPD)



F = France, M = Morocco, I = Iran, A = Argentina, U = U.S.A., C = R.P. China, S = Spain, Y = Yugoslavia

races, on 22 isolates mainly collected in the U.S.A. !. The dendrogram is given in Fig. 1. The hierarchical classification indicates close genetical relation between the Moroccan isolates and the French isolates F1 and F5. These seven isolates would appear to have a common European origin (European race isolate F1). The other isolates appear to have greater genetic differentiation and can be grouped into two classes related to two American races (Red River race = isolate UI, New Red River race = isolate U2). The isolate U3 (South Dakota race) is separated. These results suggest that movements of seed are important in the evolution of downy mildew isolates in different countries.

Identification of downy mildew in seeds

Diagnostic PCR

Some fragments amplified with RAPD primers were sequenced to design a pair of primers, each pair being specific of one locus of the fungal genome. One of the pairs of primers was used with template DNA from different *P. halstedii* strains, and also from different species (Table 3). An amplification product of the expected size was generated with all isolates of *P. halstedii* tested. In contrast, no amplification was detected when template DNA was not from *P. halstedii* origin. In order to check the specificity of the PCR amplification which appeared to be restricted to *P. halstedii* races, Southern blotting of agarose gels with amplification products from various species was carried out using the cloned PCR fragment as probe. Hybridization signals were detected only when *P. halstedii* DNA had been used as template in diagnostic PCR, thus confirming the specificity of the PCR amplification. When digestion of the PCR amplification products was performed with restriction enzymes, no polymorphism was produced between the different *P. halstedii* isolates.

Table 3 : Specificity of SCAR.

Fungus with amplification SCAR	Fungus without amplification SCAR	
Downy mildew « French races » :		
1, A, B, C, D	*Albugo tragonopodis*	*Alternaria sp.*
	Aspergillus sp.	*Botrytis cinerea*
Strains of Downy mildew :	*Cladosporium sp.*	*Diaporthe helianthi*
Spain (3)	*Epicoccum sp.*	*Fusarium sp.*
U.S.A. (3)	*Mucor sp.*	*Penicillium sp.*
Argentina (1)	*Phoma sp.*	*Pythium sp.*
Iran (1)	*Rhizopus sp.*	*Sclerotinia sclerotiorum*
Marocco (3)	*Stemphyllium sp.*	*Trichoderma sp.*
Yugoslavia (3)	*Trichothecium sp.*	

Detection level

With the aim of controlling the presence of *P. halstedii* in seeds stocks, it was of interest to estimate the sensitivity of the diagnostic PCR. As a preliminary approach, we mixed DNA from sunflower *H. annuus* and *P. halstedii* in varying proportions, and carried out PCR reactions using specific primers. The results shows that it is possible to detect an amplification product up to the limit where 0.5 pg of *P. halstedii* had been mixed with 5 ng of *H. annuus* DNA prior to amplification. The diagnostic PCR would make it possible to show the presence of *P. halstedii* in infected plants that had not yet sporulated.

Perspectives

These results appear of sufficient interest for us to continue the development of molecular techniques :

- to develop a sytem for checking the absence of downy mildew in seed shipments ;
- to find race-specific markers. Such markers would make it possible to determine a race directly from an infected plant, without need for fungal isolation and tests on differential sunflower genotypes ;
- to study relations between races and between isolates from different countries. This study will be made from the collection of isolates now available (Table 2).

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