Differences in some DNA RAPD-loci of *Plasmopara halstedii* races affecting sunflower in Krasnodar region of Russia

Tatiana Antonova, Saida Guchetl, Maria Iwebor, Tatiana Tchelustnikova
All Russia Research Institute of Oil Crops "V.S. Pustovoit",
Filatov str., 17, Krasnodar, 350038, Russia, antonova-ts@mail.ru

ABSTRACT

Forty-three isolates of *P. halstedii* races 300, 310, 330, 700, 710, 730 affecting sunflower in Krasnodar region were studied by means of 22 RAPD-loci. Primer P38 did not produce amplification in the studied isolates. The other primers produced a total of 92 fragments with an average frequency of 4.4 fragments per primer. Eight primers (L14, Y11, P28, P46, P5, OPM08, OPM20, OPJ15) were not polymorphic among the studied isolates. Thirteen primers (OPB07, OPB17,OPC08, OPC15, OPD 11, OPD 18, OPE 03, OPD 20, OPF09 OPG02 OPG05, OPG06, and OPJ13) allowed the identification of race 300 from the other races, based on the presence of amplified DNA fractions lacking in other races, or the absence of fractions typical for races 310, 330, 700, 710, 730. The resemblance measured between races 330, 700, 710 and 730, counted by means of Jacquard coefficient, was near 1.00. The resemblance between race 300 and the others was 0.29. Accordingly, the genetic distance (D_{xy}) between race 300 and the others was 0.71. These data suggested that local races 310, 330, 700, 710 and 730 in Krasnodar region could not originate from race 300. One primer (OPG06) showed intraracial polymorphism on the presence-absence of a fragment with a length of 1125 bp in all races, except 700 and 710. Monomorphic condition (invariable presence of the 1125 bp fragment) of locus OPG06 in race 710 isolates from five remote districts of Krasnodar region pointed to its stability. The monomorphic condition of this locus (invariable absence of the 1125 bp fragment) in race 330 isolates from Kanevskoy district and polymorphic condition in one isolate from Viselkovskiy district are discussed.

Key words: downy mildew – molecular markers – *Plasmopara halstedii* – races – sunflower – RAPD markers.

INTRODUCTION

The fungus *Plasmopara halstedii* (Farl.) Berl. et de Toni is an obligate parasite of sunflower, causing downy mildew, a worldwide major disease of this crop. The pathogen exists as many physiological races, which over the last few decades has grown into a complex, with at least 36 pathotypes being identified in different countries (Gulya, 2007). Physiological races of obligate parasites are always difficult to differentiate. Different physiological races of this pathogen have been described according to their reactions on various sunflower lines. An international nomenclature based on a series of well defined host plants is starting to be used, which should make it possible to establish the presence of the same races in different continents and to define the specific races in each country (Gulya et al., 1998; Tourvieille de Labrouhe et al., 2000). This international method of *P. halstedii* races differentiation and their new nomenclature application was successfully used by many pathologists from different countries (Rozynek, Spring, 2000; Molinero-Ruiz et al., 2002; Shindrova, 2000, 2005; Shirshikar, 2005; Antonova et. al, 2006; Iwebor et. al., 2005, 2007). But sometimes this cannot guarantee the clear differentiation of some races, especially if they are from different countries. The molecular methods for genomic analysis of this fungus are especially applied at present (Roeckel-Drevet et al., 1997, 2003; Giresse at al., 2007). The relationships between all known races of *P. halstedii* from different countries have been investigated by means of 21 RAPD primers (Tourvieille, 2000; Roeckel-Drevet et al., 2003), but races of fungus from Russia were not used in that investigation. The downy mildew pathogen on sunflower in Russia until recently has been scarcely studied either on racial structure or on the molecular structure of the genome. At this time, a successful control of the disease demands a regular survey of pathogen populations and incorporation of resistance to as many races as possible in sunflower breeding programs.

The objective of our investigation was the analysis by means of RAPD-PCR markers of molecular-genetic polymorphism of *P. halstedii* races present in Krasnodar region of Northern Caucasus.

MATERIALS AND METHODS

The research included 43 field isolates of *P. halstedii* belonging to six races of the pathogen with code numbers: 300, 310, 330, 700, 710, 730 (accordingly a quantity of isolates: 2, 1, 12, 4, 13, 11). The isolates were collected from the affected sunflower plants in different areas of Krasnodar region in 2005-2007. The leaves with sporulation were kept in polyethylene bags at -80°C. The seedling inoculations were implemented by using the method described in these Proceedings (Antonova et al., 2008). The physiological races were determined according to the international nomenclature, which has been proposed by Tourvieille de Labrouhe et al. (2000). All isolates were maintained on seedlings of sunflower open-pollinated variety VNIIMK 8883, which has never been used in breeding for resistance to downy mildew. DNA was extracted from conidial sporulation of the Oomycete on cotyledons of sunflower seedlings; these were artificially infected by zoospores of every isolate separately. Spores were collected and kept at -20°C until DNA extraction, which was performed within 1 month. DNA was extracted by a modified method based on Zolan and Pukkila (1986).

For RAPD-analysis, 22 decamer primers (L14, Y11, P 28, P 53, M 08, M 20, J 15, P38, B 07, B 17, C 08, C 15, D 11, D 18, E 03, D 20, F 09, G 02, G 05, J 13, G 06) were used. The first six primers were used by us early on sunflower (Guchetl et al. 2004). The others have been used for differentiation of 5 races of *P. halstedii* collected from different districts of France (Roeckel-Drevet et al., 1997; Tourvieille et al., 2000). These primers were kindly given to us by those authors. Each 25 μL of reaction volume contained 67 mM tris-HCl, ph 8.8; 16.6 mM $(NH_4)_2SO_4$; 1.5-3.0 mM $MgCl_2$; 0.001 % Tween 20; 0.2 mM deoxynucleoside triphosphates, 10 μM primer; 10 ng template DNA and 1.0 unit *Tag* DNA polymerase (Gosniigenetic, Russia). Amplification was performed in thermocycler (AO DNA-technology, Russia). PCR was conducted at regime standard for RAPD-primers: 1 cycle at 94°C for 2 min (initial denaturation) and 30 cycles – in consecutive temperature change: 1 min. at 94°C (denaturation), 1 min. at 36°C (annealing), 2 min. at 72°C (elongation), 4 min.at 72°C (final elongation).

Electrophoresis of PCR products was carried out in agarose gel (1.5 % agarose, 1x TAE-buffer in horizontal camera during 1.5-2.0 h at I=50 mA, U= 70-90 V; 10 μL of reactionary mixture were introduced in gel together with dye-stuff bromphenol blue. GeneRuler 1 kb DNA Ladder (MBI "Fermentas") was used as marker for DNA fragments lengths. Ethydium Bromide was used for subsequent staining of DNA fragments. Data were documented by means of trans-illuminator and video system (AO DNA-technology, Russia) with computer program "Gel-Imager 2". Experiments were carried out in triplicate.

The differences between isolates were expressed by the presence or the absence of bands on gel corresponding to DNA fragments of the definite length. The resemblance measure between races was calculated by means of Jacquard coefficient (Sneath and Sokal, 1973) using the formula: $\mathbf{J_{xy} = n_{xy} / n_x + n_y}$, where $\mathbf{J_{xy}}$ is the Jacquard's coefficient; $\mathbf{n_{xy}}$, is the number of DNA fragments of patterns **x** and **y** coinciding by their electrophoretical mobility; $\mathbf{n_x}$ and $\mathbf{n_y}$ are the number of amplified DNA fragments of patterns **x** and **y.**

RESULTS AND DISCUSSION

In the first stages of DNA experiments, both the parasite and its host variety VNIIMK 8883 were amplified. The major DNA fragments of the Oomycete and sunflower reproduced always differed by the quantity of nucleotides pairs (Fig. 1). This suggested the correctness of the pathogen sporulation picking up. From 22 RAPD-primers used, one (P38) did not give any amplified DNA spectra. The others produced a total of 92 fragments with average frequency of 4.4 fragments per primer. Eight primers: L14, Y11, P28, P46, P5, OPM08, OPM20, and OPJ15 were not polymorphic in the studied isolates.

Thirteen primers: OPB07 OPB17OPC08, OPC15, OPD 11, OPD 18, OPE 03, OPD 20, OPF09 OPG02 OPG05, OPG06, and OPJ13 allowed the identification of race 300 isolates from other races, based on the presence of amplified DNA fragments lacking in other races, or the absence of fractions typical for races 310, 330, 700, 710, 730 (Fig. 2). These 13 primers produced a total of 76 fragments (from 1 to 12 polymorphic fragments per primer). From them, we identified 62 polymorphic loci. Our results showed that these 62 polymorphic loci only allowed a clear identification of race 300 from the six races studied.

The resemblance measure between race 300 and the others was accounted for by means of Jacquard coefficient (J_{xy}). This method is more suitable for accounting RAPD-data (Link et al., 1995). The resemblance measure between races 330, 700, 710 and 730 was near to 1.00. The resemblance measure between race 300 and the others was defined by a fairly small value of 0.29. Accordingly, the genetic

distance (D_{xy}) between race 300 and the others (which is calculated by the formula $D_{xy}=1- J_{xy}$) was 0.71. The resemblance measure between races 300 and 710 in France was 0.88 (Roeckel-Drevet et al., 1997).

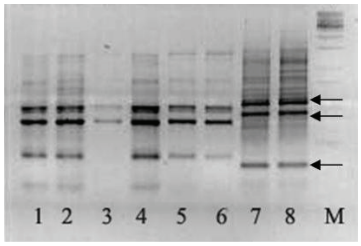

Fig. 1. Amplified DNA electrophoretical spectra of *P. halstedii* and sunflower obtained with primer L 14. Lanes: 1-6 - isolates of *P. halstedii*; 7, 8 – sunflower variety VNIIMK 8883. M – molecular mass marker (GeneRuler 1 kb DNA Ladder, MBI "Fermentas") The arrows show the reproduced DNA fractions having the length: 550 bp, 460 bp and 160 bp (respectively from top to bottom).

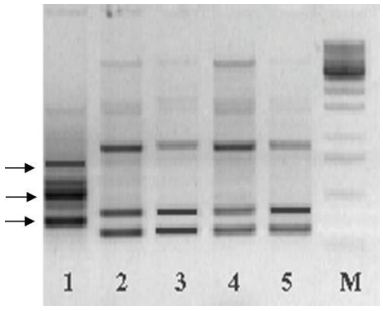

Fig. 2. Amplified DNA electrophoretical spectra of *P. halstedii* obtained with primer OPJ13. Lanes (races): 1 - 300, 2 - 330, 3 – 700, 4 -710, 5 – 730. M – molecular mass marker (GeneRuler 1 kb DNA Ladder, MBI "Fermentas" . The arrows show race 300 DNA fragments with length (from top to bottom) 710bp., 480 bp and 330 bp that distinguished it from the others.

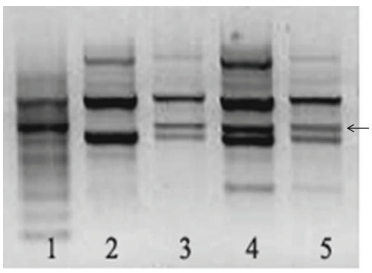

Fig. 3. Amplified DNA electrophoretical spectra of *P. halstedii* obtained with primer OPG06. Lanes (races): 1 - 300, 2 - 330, 3 – 700, 4 -710, 5 – 730. The arrow shows DNA fraction 1125 bp.

Although the race composition of *P. halstedii* population in Russia was not controlled for a long time, since first investigations date back to the beginning of the 1980s, it may be assumed that race 300 appeared in Krasnodar region later than race 100 (Antonova et al., 2000). Apparently, it was introduced into the former USSR with sunflower seeds. Its origin from another continent could explain the the distinctions of race 300 from the others on RAPD-loci studied in our experiments. Our data agreed with investigations of Tourvieille et al. (2000) grouping in separate clusters European and American isolates of this Oomycete.

Only one primer OPG06 produced intraracial polymorphism on the presence-absence of one fraction whose length is 1125 bp (shown as $OPG06_{1125}$) (Fig. 3, Table 1). Two isolates of race 300 which were

Table 1. The characteristic of DNA RAPD-locus $OPG06_{1125}$ of *P. halstedii* races, collected on sunflower from different districts of Krasnodar region, Russia, 2005

Isolate number	Race	District of isolate collection	$OPG06_{1125}$*
1	300	Fields of VNIIMK	1
2	300	Labinskiy	0
3	310	Viselkovskiy	0
4	330	Viselkovskiy	0
5	330	Viselkovskiy	0
6	330	Viselkovskiy	1
7	330	Fields of VNIIMK	1
8	330	Fields of VNIIMK	1
9	330	Fields of VNIIMK	1
10	330	Kanevskoy	0
11	330	Kanevskoy	0
12	330	Kanevskoy	0
13	330	Kanevskoy	0
14	330	Kanevskoy	0
15	330	Kanevskoy	0
16	700	Labinskiy	1
17	700	Labinskiy	1
18	700	Viselkovskiy	1
19	700	Krilovskoy	1
20	710	Fields of VNIIMK	1
21	710	Fields of VNIIMK	1
22	710	Viselkovskiy	1
23	710	Viselkovskiy	1
24	710	Viselkovskiy	1
25	710	Viselkovskiy	1
25	710	Viselkovskiy	1
27	710	Viselkovskiy	1
28	710	Viselkovskiy	1
29	710	Labinskiy	1
30	710	Labinskiy	1
31	710	Kanevskoy	1
32	710	Krilovskoy	1
33	730	Fields of VNIIMK	0
34	730	Fields of VNIIMK	0
35	730	Fields of VNIIMK	0
36	730	Fields of VNIIMK	1
37	730	Viselkovskiy	0
38	730	Viselkovskiy	0
39	730	Viselkovskiy	1
40	730	Viselkovskiy	1
41	730	Viselkovskiy	1
42	730	Viselkovskiy	1
43	730	Viselkovskiy	1

* 0- absence, 1- presence of amplified DNA fragment

collected in different districts of Krasnodar region had different genotypes for this character. The only isolate of race 310 studied showed the absence of fraction. Races 300 and 310, which were found only sporadically in the districts of Krasnodar region, gave an extremely poor sporulation and we failed to collect enough material for analysis. Twelve isolates of race 330 from three districts have shown interesting results. All the six isolates from Kanevskoy district lacked this fraction. All three isolates from fields of VNIIMK have shown its presence and, although two out of three isolates from the Viselkovskiy district did not have it, the third one did. All four isolates of race 700 have shown the presence of fraction 1125 bp in locus OPG06 and they were collected in three districts which are situated quite far from each other (Table 1). The distances between Viselkovskiy district and two others: Krilovskoy and Labinskiy are about 130 and 250 km, respectively. Therefore, despite the small quantity of studied isolates, we presume that for race 700 the presence of this fraction is uniform.

Thirteen isolates of race 710 were collected in five different districts and all of them have shown the presence of this fraction (Table 1). Eleven isolates of race 730 collected in two districts have shown the presence or absence of this fraction. Data of Table 1 show some stability of race 710 in locus OPG06 because the isolates were collected in five different districts and all of them had the fraction 1125 bp. As shown in another manuscript of these proceedings (Antonova et al., 2008), race 330 was predominant in Kanevskoy district, up to 100% in the majority of studied fields, and its isolates from there have shown the condition of locus OPG06 as the absence of the fragment. In some fields of the Viselkovskiiy district, races 710 and 730 predominated, whereas in the others there was race 330. The presence-absence of fraction varied in isolates of races 330 and 730, but it was always present on the isolates of race 710. This gives an idea about the hybridization of races in that district. Apparently, locus OPG06 is not coupled with genes of virulence. In this case, the coexistence in one population of individuals with different allelic conditions is possible.

In conclusion, our study has confirmed the considerable molecular homogeneity previously observed among the French races (Roeckel-Drevet et al., 1997; Tourvieille et al., 2000). Our data suggests that the origin of local races 330, 700, 710 and 730 in Krasnodar region could not be race 300. However, this should be further confirmed, because of the scanty quantity of race 300 isolates available for this study.

Monomorphic condition of locus OPG06 of race 710 isolates from five remote districts of Krasnodar region suggested its stability, i.e. the invariable presence of fraction 1125 bp. Monomorphic condition of locus OPG06 (the absence of fraction 1125 bp.) of race 330 isolates from one district (Kanevskoy) may be connected with this pathotype's prevailing domination there of up to 100% in the majority of studied agrocenoses. And we suppose that for "pure" race 330 the absence of fraction 1125 bp in OPG06 locus is a constant character. The polymorphic condition of this locus revealed in isolates of race 330 from Viselkovskiy district may possibly be explained as the result of hybridization between races 330 and 710 or 730 in that place.

ACKNOWLEDGEMENTS

We wish to thank Dr. D. Tourvieille de Labrouhe and Dr. J. Tourvieille for providing the RAPD- primers used in this study. This research was supported by the Russian Foundation of Fundamental Investigations (RFFI) and Administration of Krasnodar region, grant 06-04-96641.

REFERENCES

Antonova, T.S., N.M. Araslanova, A.V. Golovin, T.A. Tchelustnikova, and S.L. Saukova. 2000. To the question about the race belonging of sunflower Downy Mildew casual agent in Northern Caucasus. Sci. Techn. Bulletin of VNIIMK, 123:21-23 (In Russian).

Antonova, T.S., N.M. Araslanova, and M.V. Iwebor. 2006. New races of sunflower downy mildew causal agent. Proceedings (Vestnik) of Agricultural Academy of Russia 3:37-38 (In Russian).

Antonova, T., M. Iwebor, and N. Araslanova. 2008. Races of *Plasmopara halstedii* on sunflower in separate agrocenosises of Adigeya Republic, Krasnodar and Rostov regions in Russia (these Proceedings).

Giresse, X., D. Tourvieille de Labrouche, S. Richard-Cervera, and F. Delmotte. 2007. Twelve polymorphic expressed sequence tags-derived marker for *Plasmopara halstedii*, the causal agent of sunflower downy mildew. Mol. Ecol. Notes 7:1363-1365.

Gulya, T.J. 2007. Distribution *Plasmopara halstedii* races from sunflower around the world. In: Proceedings of the 2nd International Downy Mildews Symposium. Advances in downy mildew

research. Palacky University in Olomouc and JOLA. v.o.s., Kostelec na HanI (Czech Republic) 3:121-134.

Gulya, T., D. Tourvieille de Labrouhe, S. Masirevic, A. Penaud, K. Rashid, and F. Viranyi. 1998. Proposal for standardized nomenclature and identification of races of *Plasmopara halstedii*. p. 130-136. In: Symposium III: Sunflower Downy Mildew, Fargo, ND, 13-14 January. Int. Sunflower Assoc, Paris.

Guchetl, S.Z., T.A. Tchelustnikova, S.A. Ramazanova, and T.S. Antonova. 2004. Molecular-genetic characteristic of sunflower inbred lines on isozymes markers and DNA-profiles. Scientific-Technical Bulletin of VNIIMK, 2(131):42-46 (in Russian).

Iwebor, M.V., T.S. Antonova, and N.M. Araslanova. 2005. New races of *Plasmopara halstedii* – the causal agent of sunflower downy mildew in Northern Caucasus. Science of Kuban 2:162-164 (in Russian).

Iwebor, M.V., T.S. Antonova, and N.M. Araslanova, 2007. The identification of race composition of *Plasmopara halstedii* population in regions of Northern Caucasus. Science of Kuban 2:47-51 (in Russian).

Link, W., C. Dixkens, M. Singh, M. Schwall, and A.E. Melchinger 1995. Genetic diversity in European and Mediterranean faba bean plasma reveal by RAPD markers. Theor. Appl. Genet. 90:27-32.

Molinero-Ruiz, M.L., J. Dominguez, and J.M. Melero-Vara. 2002. Races of isolates of *Plasmopara halstedii* isolates from Spain and studies on their virulence. Plant Disease 86:736-740.

Novotelnova, N.S. 1966. Downy Mildew of sunflower. M.;L. Science. 150 p. (in Russian).

Roeckel-Drevet, P., V. Coelno, J. Tourvieille, P. Nicolas, and D. Tourvieille de Labrouhe. 1997. Lack of genetic variability in French identified races of *Plasmopara halstedii*, the cause of downy mildew in sunflower *Helianthus annuus*. Can. J. Microbiol. 43:260-263.

Roeckel-Drevet, P., J. Tourvieille, T.J. Gulya, G. Charmet, P. Nicolas, and D. Tourvieille de Labrouhe. 2003. Molecular variability of sunflower downy mildew, *Plasmopara halstedii* from different continents. Can. J. Microbiol. 49:492-502.

Rozynek, B., and O. Spring. 2000. Pathotypes of sunflower downy mildew in Southern parts of Germany. Helia 23(32):27-34.

Shindrova, P.S. 2000. Distribution and race composition of downy mildew (*Plasmopara halstedii* Farl. Berl. and de Toni) in Bulgaria. Helia 23(33): 25-32.

Shindrova, P.S. 2005. New nomenclature of downy mildew races in sunflower (*Plasmopara halstedii* (Farl.) Berl. and de Toni) in Bulgaria (race composition during 2000-2003). Helia 28(42):57-64.

Shirshikar, S.P. 2005. Present status of sunflower downy mildew disease in India. Helia 28(43):153-158.

Sneath, P.H., and R.R. Sokal. 1973. Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman (ed.). San Francisco.

Tihonov, O.I., and V.F. Zaichuk. 1981. New race of Downy Mildew. Oil Crops 6:20-21 (in Russian).

Tourvieille, J., J. Millon, P. Roeckel-Drevet, P. Nicolas, D. Tourvieille de Labrouhe, and T.J. Gulya. 2000. Molecular variability of *Plasmopara halstedii*. p. 67-72 (vol. 1). In: Proc. 15th Int. Sunflower Conf., Toulouse, France.

Tourvieille de Labrouhe, D., T.J. Gulya, Y.K. Rashid, and F. Viranyi. 2000a. New nomenclature of races of *Plasmopara halstedii* (sunflower downy mildew). p. 161-166 (vol. 2). In: Proc. 15th Int. Sunflower Conf., Toulouse, France.

Zolan, M.E., and P.J. Pukkila. 1986. Inheritance of DNA methylation in *Corpinus cinereus*. Mol. Cell Biol. 6:195-200.