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ABSTRACT

- Sunflower downy mildew caused by the obligate fungal pathogen *Plasmopara halstedii* (Farl.) Berl.et de Toni – is one of the most harmful diseases of this culture worldwide. New pathogen biotypes of the fungus (nowadays their number is up to 36) appear practically in all countries where the sunflower is cultivated. In spite of the difference in pathogenicity and geographical origin, it was found out, that the degree of the genetic similarity among races from different European countries, revealed by molecular markers is very high. We have identified 7 races of downy mildew causal agent of sunflower in the Northern Caucasus for the first time. It was proved by the means of RAPD of DNA derivatives that the race 300 stands apart from the others, its origin from the other continent was established. Other races could not be defined. In the present research we continue to study the molecular-genetic structure of the downy mildew causal agent of the sunflower in Northern Caucasus region of Russian Federation. The purpose of the research was to study intraspecific polymorphism of *P. halstedii*, revealed by PCR-derivational molecular markers.
- The objects of the research were 42 isolates of 6 races: 300, 310, 330, 700, 710 and 730 *P. halstedii*, collected from the affected sunflower plants in different areas of Krasnodar region. The artificial inoculation of the sunflower lines-differentials and varieties was realized by means of the standard methods. DNA was extracted from conidial sporulation of the fungus on the cotyledon leaves of the sunflower seedlings, using CTAB method. 36 RAPD and ISSR primers and combinations of primers were used for PCR-analysis. To define the differences among the isolates of races *P. halstedii*, PCR-analysis data were processed by Ward method. Genetic distance was calculated as a Euclidian metric distance.
- The degree of DNA polymorphism of studied isolates belonging to different races was estimated as a weak one. Only 10 RAPD and ISSR primers and combinations of primers had revealed polymorphism of DNA fragments. On the whole 58 fractions of DNA were found, 40 of them were polymorphic (from 2 to 11 fragments for the primer). The loci of DNA, which help to clearly define the races, were not found.

  Cluster analysis had shown that all studied isolates of *P. halstedii* had been divided into 2 big clusters with genetic distances 17,5 between them. All isolates of the race 710 were grouped in the first cluster and the isolates of the race 330 mostly in the second one. The race 730 had an even distribution in both clusters, between the subclusters of the races 710 and 330. That means that it could be the result of hybridization of these two races.
- The distribution of the isolates between the clusters and subclusters depended mostly on the racial belonging, then on the location of the area where the isolate was collected. On the whole, the indistinct grouping of the races in separate clusters and subclusters proves that modern races in Krasnodar region were formed recently.
- Thus, the races of *P.halstedii*, spread in Krasnodar region, are genetically close. That corresponds with data from the countries of Central and Western Europe. Our researches make up a deficiency in world knowledge of population structure of *P. halstedii* in the regions of Russian Federation.

Key words: *Plasmopara halstedii* – polymorphism - RAPD markers – SSR markers - sunflower

INTRODUCTION

Downy mildew of the sunflower caused by obligate fungus pathogen *Plasmopara halstedii* (Farl.) Berl. et de Toni (class Oomycetes) is one of the most harmful diseases of this crop worldwide. The new pathogen biotypes of the fungus appear practically in all countries cultivating sunflower. If in 2002-2003, there were 11-17 races according to different researches (Vear et al., 2003; Viranyi, 2002), at the present time there are 36 pathogens pathotypes discovered (Gulya, 2007).

Differentiation of the *P. halstedii* races, like races of others pathogens, is made according to their ability to affect the line-differentiators of the host. But besides this traditional method of the race distinction, there are quite effective molecular-genetic ways of the estimation of population's pathogen polymorphism. PCR methods often allow to differentiate physiological races of fungi-pathogens without using the line-testers, and to establish the degree of genetic similarity between races and isolates within a species, phylogenetic relationship between species of fungi (Bulat, Mironenko, 1996). However, physiological races of fungi are often similar in molecular-genetic traits due their genetically similarity. Isolates of fungi analyzed with the help of polymerase chain reaction methods with RAPD - SSR-primers showed the high level of genetic uniformity (Balmas et al., 2003; Roeckel-Drevet et al., 1997).

Among *P. halstedii* isolates, chosen from different regions of Germany and analyzed with the help minisatellites and simple-sequence repeat primers the found polymorphisms didn't correlate neither with the geographical origin nor with a physiological race isolates. At the same time, the amplification patterns generated by primers combinations allowed to differentiate all tested isolates (Intelmann, Spring, 2002). Roeckel-Drevet et al. (1997) with the help of the polymerase chain reaction method with 30 RAPD primers had differentiated 58 isolates of five pathotypes of *P. halstedii*, collected throughout France. The relationships between the races 100, 300, 700, 330, 710, 703, 730, 770 from different countries were studied by means of 21 RAPD primers. The weak polymorphism, irrespective of race and a geographical origin (Roeckel-Drevet et al., 2003) was detected. Giresse et al. (2007) had tested 12 EST the markers revealing the high level of genetic diversity among 32 *P. halstedii* isolates of the sunflower from France and Russia.

We had identified 7 races of downy mildew causal agent of sunflower in the Northern Caucasus for the first time. By means of RAPD of DNA derivatives it was shown, that the race 300 stands apart from the others, its origin from the other continent was also proved. Other races could not be defined. (Guchetl et al., 2008; Antonova et al., 2008b).

In the present research we continue to study the molecular-genetic structure of the downy mildew causal agent of the sunflower in the Northern Caucasus region of Russian Federation. The purpose of the research was to study intraspecific polymorphism of *P.halstedii*, revealed by PCR-derivational molecular markers.

MATERIALS AND METHODS

The research included 42 *P. halstedii* isolates belonging to six races of the pathogen with code numbers: 300, 310, 330, 700, 710 and 730 (accordingly a quantity of isolates: 2, 1, 12, 3, 13 and 11).The isolates were collected from the affected sunflower plants in different district of Krasnodar region in 2005-2007 (tab). The difference in number of studied isolates of different races is connected with the frequency of their detection in agrocenosises of the Northern Caucasus (Antonova et al., 2008, a). The race belonging was determined according to the international nomenclature, which has been proposed by Tourvieille de Labrouhe and co-authors (2000). The seedlings inoculations were implemented by means of the standard method of the immersion of seedlings roots in suspension of zoospores (Antonova et al., 2000).

DNA was extracted from conidial sporulation of fungus on cotyledon leaves of sunflower seedlings; the latter were artificially infected by zoospores of every isolate separately. The spores were collected and kept at -80^{o}C until DNA extraction. DNA was extracted by the modified method of Zolan and Pukkila (1986). All isolates were maintained on seedlings of open-pollinated variety VNIIMK 8883 sunflower, which was never used in breeding before for the resistance to downy mildew.

For PCR-analysis 21 primers were used:

-2 minisatellite and 8 SSR (simple-sequence repeat): M13, T3B, GATA, GTG5, TCC5, GAA6, GACA4, CA 8, CAC 5, GGAT4 and 15 their pair combinations (M13+T3B, GAA6+GACA4, TCC5+GAA6, T3B+GTG5, M13+GAA6, GAA6+GTG5, T3B+GAA6, TCC5+GTG5, GACA4+TCC5, GACA4+M13, GACA4+T3B, GACA4-GTG5, TCC5+M13, TCC5+T3B, M13+GTG5) (Intelmann, Spring, 2002);

-2 ISSR (inter-simple-sequence repeat) primers (CA) 10G and (CAC) 7T;

-SSR primers Pv39 and Pv17, revealed molecular polymorphism between physiological races of the causal agent of grape downy mildew (Delmotte et al., 2006);
- ITS1, ITS 2 (internal transcribed spacer), G1, G2, G3 (Says-Lesage et al., 2002);
- 2 RAPD (random amplified polymorphic DNA) primers PTO and OPG 06 (Operon Technologies, Inc., the USA), previously showed the polymorphism between the physiological races of the downy mildew causal agent of the sunflower (Guchetl et al., 2008; Antonova et al., 2008b).

PCR-amplifications were used with 10 ng of genomic DNA in 25 μL reactions. 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 and 1.0 unit *Tag* DNA polymerase (Gosniigenetic, Russia). Amplification was made in the thermal cycler (AO DNA-technology, Russia). PCR was conducted at regime: 1 cycle at 94°C for 2 min (initial denaturation) and 30 cycles – in consecutive temperature change: 1 min- 94°C (denaturation), 1 min – 36°-60°C (annealing depending on primers), 2 min - 72°C (elongation), 4 min - 72°C (final elongation).

Electrophoresis of PCR products was carried out in agarose gel (2 % agarose, 1x TAE-buffer in horizontal camera during 1.5-2.0 h at I=50 mA, U= 70-90 V; 10 mkL of reactionary mixture were introduced in gel together with dye-stuff bromphenol blue. GeneRuler 1 kb DNA Ladder (UAB "Fermentas") was used as marker for DNA fragments lengths. Ethydium Bromide was used for subsequent staining of DNA fragments. The 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 denrogram was constructed after cluster analysis of Ward method. Genetic distance was calculated as a Euclidian metric distance. The calculations were done with the software package of the Statistica 6.0 programs.

RESULTS AND DISCUSSION

For the detection of the polymorphism in the sunflower populations of downy mildew pathogen in Krasnodar region, a collection of 42 *P. halstedii* isolates with an application 36 primers and primer combinations was analyzed (tab). The first stage of the experiment aimed to the screening of primers for the subsequent differentiation between the five randomly selected isolates, representing the most widespread races: 300, 700, 330, 710 and 730 (fig. 1). Primers of GATA, (CA) 10G, (CAC) 7T, ITS 1 hadn't given any amplified DNA spectra. 24 primers and primer combinations were not polymorphic in the studied isolates. 10 primers and primer combinations have detected the polymorphism: PTO, OPG 06, T3B, M13, TCC5, GTG5, GAA6, GAA6+GACA4, T3B +GAA6, TCC5+T3B. It made only 28 % from all tested primers and indicates a weak intraspecific variability of *P. halstedii*, affecting sunflower in Krasnodar region. By means of these primers all the others isolate of fungus were analyzed. Thus 58 fragments of DNA were revealed. 40 of them were polymorphic (from 2 to 11 fragments per primer), characterized by presence-absence of DNA fraction of certain length. The level of the polymorphism was 0.68. Amplified fragments, depending on the primer, had the size from 80 to 2500 bp.

Table. The characteristic of *P. halstedii* isolates, analyzed with an application of PCR-markers

Race	Isolate number	District of isolate collection	Cluster, subcluster
300	1	Fields of VNIIMK	I a*
300	2	Labinskiy	I a
310	3	Fields of VNIIMK	I c
330	4	Fields of VNIIMK	II e
330	5	Fields of VNIIMK	II e
330	6	Fields of VNIIMK	II e
330	7	Fields of VNIIMK	I c
330	8	Kanevskoy	II e
330	9	Kanevskoy	II e
330	10	Kanevskoy	II e
330	11	Kanevskoy	II e
330	12	Kanevskoy	II e
330	13	Kanevskoy	II e
330	14	Viselkovskiy	I c
330	15	Viselkovskiy	II e

700	16	Viselkovskiy	I c
700	17	Krilovskoy	I c
700	18	Labinskiy	I b
710	19	Krilovskoy	I b
710	20	Krilovskoy	I b
710	21	Fields of VNIIMK	I c
710	22	Fields of VNIIMK	I c
710	23	Viselkovskiy	I c
710	24	Viselkovskiy	I c
710	25	Viselkovskiy	I c
710	26	Viselkovskiy	I c
710	27	Viselkovskiy	I c
710	28	Viselkovskiy	I b
710	29	Viselkovskiy	I b
710	30	Labinskiy	I b
710	31	Labinskiy	I b
730	32	Fields of VNIIMK	II d
730	33	Fields of VNIIMK	II e
730	34	Fields of VNIIMK	I c
730	35	Viselkovskiy	I b
730	36	Viselkovskiy	I b
730	37	Viselkovskiy	I c
730	38	Viselkovskiy	I c
730	39	Viselkovskiy	I c
730	40	Viselkovskiy	I c
730	41	Viselkovskiy	II d
730	42	Viselkovskiy	II e

* I, II -clusters, a, b, c, d, e - subclusters.

The loci of DNA that could help to clearly distinguish the races were not found. Moreover, some of the races isolates 330, 710 and 730 were similar (fig 1; fig. 2). Among these isolates the identical ones in general belonged to the same race. Only in one case the similar DNA-spectrum had shown 2 isolates, belonging to different races – 23 of race 710 and 16 of race 700. The genetic similarity of these isolates is impossible to explain by the general localization because they are collected from the different fields of Viselkovskiy district. Probably, that isolate of race 700 belonged actually to race 710 as the differentiation of races by means of lines-differentiators is not always reliable.

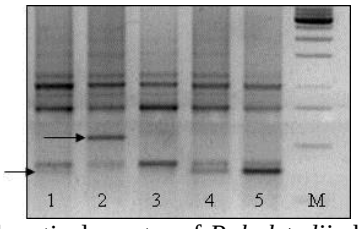

Fig. 1. Amplified DNA electrophoretical spectra of *P. halstedii* obtained with primer T3B. Lines (races): 1 - 300; 2 - 330; 3 - 700; 4- 710; 5 – 730. M – Molecular mass marker (GeneRuler 1 kb DNA Ladder, UAB “Fermentas”). The arrows show polymorphic DNA fragments with length (from top to down) 560 bp. and 360 bp.

But despite that, the fragments of the amplified DNA, unique for some isolates have been observed. Thus for example, the fragments of DNA $G06_{1135}$, $G06_{1225}$, $(T3B+GAA6)_{553}$, $T3B_{521}$ were unique accordingly to the isolates 17, 20, 28, 38. And there was no connection between the uniqueness of a genotype and its racial characteristics.

To determine the differences between the races isolates of *P. halstedii*, the results of PCR analysis were presented in the form of a conditions matrix of binary attributes in which the presence or the absence of the identical in size DNA fragments in electrophoresis spectra corresponded to 1 and 0. On the basis of conditions matrix the cluster analysis was carried out using the Ward method and the dendrogram of the genetic relationships between studied isolates was made (fig. 2).

All of the studied isolates of *P. halstedii* were divided into two clusters **I** and **II**, with a genetic distance of 17,5 between them. The cluster **I** included three subclusters, designated as **a**, **b** and **c**. Cluster **II** included 2 subclusters - **d** and **e**. Cluster **I** had grouped the isolates of races 300, 310, 700, 710, 5 isolates of races 730 and 2 isolates of races 330. Both isolates of races 300 were allocated in separate subcluster **a**, with a distance of 7,8 from united clusters that included other genotypes. This fact also supports our opinion that the race 300 is genetically far from the others because of its origin from another continent and other races have the common origin, possibly, from the former European race 1 (100) (Guchetl et al., 2008; Antonova et al., 2008b). Isolates of races 700, 710 and 730 were evenly distributed in the subclusters **b** and **c**, with a genetical distance of 5,18.

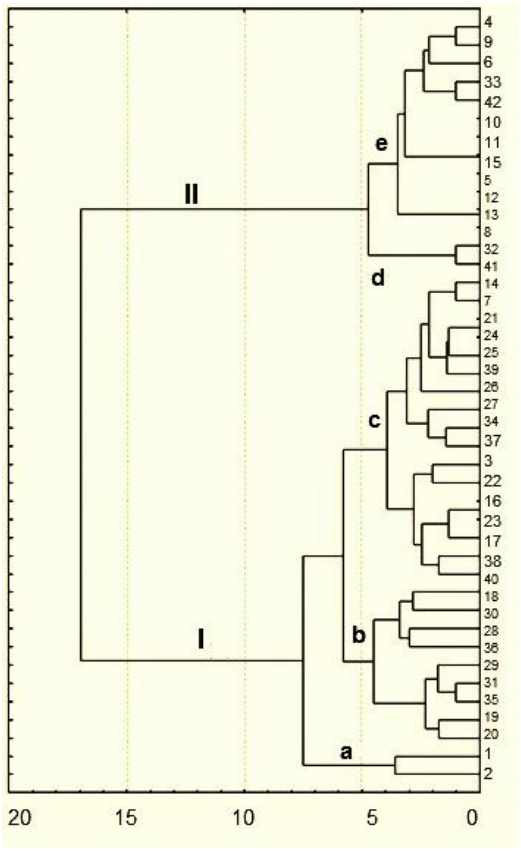

Fig. 2. Dendrogram of the genetic similarity *P.halstedii* isolates on the basis of polymorphic amplified DNA fragments

Cluster **II** has united mostly the isolates of races 330 (in subcluster **e)** and 4 isolates of races 730 which hadn't grouped in cluster **I** (fig. 2; tab**)**. Such distribution of the race 730 can indicate that it probably originates from hybridization of the races 710 and 330.

The distribution of the isolates between the clusters and subclusters depended mostly on the racial belonging, then on the location of the area where the isolate was collected.

Our researches have shown that mini- and microsatellite loci are more polymorphic in *P. halstedii* genome, than RAPD-PCR loci. In the previous works out of 22 RAPD primers used only 13 distinguished race 300 from the others, and only 1 showed the variability of the downy mildew causal agent *P. halstedii*, affecting sunflower in Krasnodar region (Guchetl et al., 2008; Antonova et al., 2008b).

The isolates of the race 300 and the majority of the isolates of race 330 are united in separate subclusters, **Ia** and **IIe** accordingly, demonstrating a big intraracial similarity. The isolates of the races 310,700, 710 are in the subclusters **b** and **c** of the cluster **I**. The greatest genetic heterogeneity refers to the isolates of the races 730, as the analysis of their molecular characteristics permitted to group them to different clusters and subclusters. Probably, all these data have a connection with a new growth of races on the territory of the Krasnodar region.

On the whole, the indistinct grouping of the races in separate clusters and subclusters proves that modern races in Krasnodar region had been formed recently.

Thus, the races of *P.halstedii*, spread in Krasnodar region, are genetically close. That corresponds with data from the countries of Central and Western Europe. Our researches make up a deficiency in world knowledge of population structure of *P. halstedii* in the regions of Russian Federation.

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