

Immunogenic breeding program. Stage I-phytopathological screening of the grape gene pool

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Abstract. At the present stage of science development it is necessary to develop and implement the grape breeding programs for immunity basing on the international level of knowledge about the genetics of immunity to pathogens that cause culture diseases. The extensive material on genes of grape resistance to *Plasmopara viticola* and *Erysiphe necator* (*Uncinula necator*), which cause mildew and oidium diseases, has been collected thanks to the MAS technology. There is an evidence of necessity to pyramidize genes of resistance to these pathogens in one progeny genome for consistent field resistance to the complex of these pathogens. However, even at present, the issue of study the transfer of specific genes of resistance from parents to the progeny, their combining ability in one genome, and gene expression during pathogenesis remains relevant. This publication discusses the formation of grape breeding program in the FSBSI Institute Magarach of the RAS on introgression of resistance genes of the species *Vitis rotundifolia* (*Muscadinia rotundifolia*), and also presents the results of phytopathological screening of populations resulting from crossing with this species.

1 Introduction

According to the entries of botanical classification and systematics, all diversity of grape forms is combined in one family *Vitaceae*, which includes 14 genera and about 900 species. Main areas of their distribution are East Asia and North America, with about 25-30 and 30-40 taxa growing there, respectively [1].

The Eurasian species *Vitis vinifera* L. has the greatest commercial distribution all over the world, occupying about 94% of the area of commercial vineyards. However, the varieties of *V. vinifera* species are generally not resistant to pathogens causing diseases. Their protection from disease is carried out using chemicals that have an impact on the environment, economy and society. Of the second part, the other species of grapes are disease resistant, but have poor quality of berries. Therefore, one of key directions of formation breeding programs is the breeding of grapes, aimed at obtaining new varieties

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that combine disease resistance with high quality of berries. Similar breeding programs based on generative hybridization have been developed since the 19th century both in the Old (Europe) and the New World to solve the problem of successful development of viticulture. The prospects of breeding for disease resistance are under discussion now, including new breeding methods such as cisgenesis and genome editing [2, 3].

For the past decade, in addition to the growing demand for innovations, the commitment to sustainable viticulture is resolved by selection of new varieties with medium resistance to main pathogens endangering grapes in the sub-humid climate. With this view the Foundation of Edmund Mach (FEM) has implemented a two-stage breeding program. Firstly, nine reference microsatellite markers (simple sequence repeats- SSRs) have been used for precise identification of varieties through international and private databases, where is possible. Secondly, 50 informative SSR markers were evaluated and applied to analyze the origin of varieties, identify relationships and confirm available ancestry information. In addition, all studied samples were analyzed for 12 loci obtained from *Vitis* spp., related to resistance to pathogens (R) according to the literature sources. This new All-vs-All approach made it possible to detect unexpected combinations of R-loci in traditionally bred material. Moreover, the loci distribution, associated with mildew (Rpv) and oidium (Run / Ren) resistance, as well as field resistance, have identified exclusive and potentially new genetic resources. At the next stage, the breeding program by means of markers using the obtained information was developed. Currently, 32% of the selected genotypes combine two Rpv loci and two Run / Ren loci, 6% combine three Rpv loci and three Run / Ren loci, and the selection of genotypes carrying up to seven R-loci is underway [4].

In 2000, a new breeding program to create grape varieties resistant to mildew and oidium and possessing the quality of berries required for high cultivars wine production, called INRA-ResDur, was launched. Various American and Asian sources of resistance have already been known for a long time. During the last decade, intensive genetic analysis of some of them has uncovered several loci of resistance. But the loss of resistance has already been observed for *Rpv3* locus (resistance to *Plasmopara viticola* carried by the resistant variety 'Bianca') and for the *Run1* locus (resistance to *Uncinula necator*, obtained from *Vitis rotundifolia*). To ensure the viability of resistance, the INRA-ResDur program used marker assisted selection (MAS) to summarize genes of resistance obtained from multiple sources. Thus, MAS allowed to trace six loci of resistance, *Rpv1*, *Rpv3* and *Rpv10* for mildew and *Run1*, *Ren3* and *Ren3.2* for oidium. This strategy followed to creation of varieties that carry not only one, but also two, or three genes of resistance to each pathogen. Four new resistant varieties: 'Artaban', 'Floreal', 'Vidoc' and 'Voltis' were registered in 2018, and a range of about 20 cultivars should be registered additionally by 2024. This project is the result of collaboration between INRA and IFV, French Institute of Vine and Wine and other European Institutes of breeding [5].

Breeding of varieties with various degree of resistance is an important strategy to obtain steady resistance in field conditions. This strategy requires a) identification of resistance sources and development of markers to control this resistance, b) characteristics of resistance mechanisms, c) marker assisted breeding for introduction of selected loci into new varieties, and, finally, d) understanding of the pathogen biology and the response to different sources of resistance. Resistance is confirmed by detailed assessment of development of a pathogen and, as a consequence, a disease in the environment of field, greenhouse or by *in vitro* analysis. Multidimensional testing of plants for resistance contributes to a better assessment of pathogen biology and interactions with different loci of resistance, and also allows make a decision to combine the resistance loci [6].

Breeding program developed in the University of Udine (Italy) is focused on pyramidizing genes involved in mildew and oidium resistance into a high-quality genetic

“*vinifera* background” for selection of new wine and table grape varieties. Parent members were selected from group of elite wine or table varieties and resistant genotypes. The progeny with pyramidizing genes was obtained from 64 initial crossing combinations. The hybrids were genotyped using molecular markers coming from four recognized mildew resistance genes (*Rpv1*, *Rpv3*, *Rpv10*, *Rpv12*) and four oidium resistance genes (*Run1*, *Ren1*, *Ren3*, *Ren4*) [7].

Grape genetic resources of the *Vitaceae* family from different centers of origin and regions of grape growing in the world are stored in one of the oldest Eurasian grape collections in the Institute Magarach. They were examined for identification of genetically determined traits of frost and pathogen resistance to fungal diseases. It was found that the trait of frost resistance and resistance of grapes to causative agents of fungal diseases in genome was formed and evolutionally anchored in individual forms of different centers of origin. Estimated sources of high frost resistance and resistance to causative agents of fungal diseases of various botanical taxa from different centers of origin were also selected to choose grape varieties resistant to frost and causative agents of fungal diseases [8].

Institute Magarach is implementing a breeding program for selection of resistant grape varieties to mildew and oidium based on the introgression into the *Vitis vinifera* genome the resistance genes from *Vitis rotundifolia* (*Muscadinia rotundifolia*), as one of the most resistant grape species, to the pathogens that cause these diseases. Studies of progeny from hybridization of *Vitis vinifera* with *Vitis rotundifolia* in the Institute Magarach began in 1974 when DRX hybrid seeds were imported from France. First populations F₂ were generated using DRX 58-5 and DRX 60-24 forms. Hybridization of DRX (F₂) hybrids and varieties of *Vitis vinifera* gave the progeny (F₃), characterized by high productivity. Hybrids combined characteristics of viability and resistance of *Vitis rotundifolia* to pathogens with qualities of *Vitis vinifera*. After 2010, the studies of the gene pool including the genome of *Vitis rotundifolia* continued. Hybrids from Moldova took part in the process of hybridization, where in 1982 hybrids F₃ (2n = 39) and then F₅ (2n = 39 and 2n = 38) were obtained from crossing of DRX-55 × (‘Aramon’ × *Vitis riparia*) and DRX-55 × XIX-30 / 33. The study also included hybrids 2000-305-163 and 2000-305-143 received from the Geilweilerhof Institute of Grape Breeding (Germany), obtained from the crossing of the French form MTP3082-1-42, bearing loci of resistance to oidium and mildew *Vitis rotundifolia* with the variety ‘Regent’. The progeny obtained from hybridization with these forms was analyzed according to the selection significance and heterosis indices. The hybrids obtained in the Institute Magarach demonstrated significant amplitude of variability in morphological characteristics, productivity and resistance to pathogens. They showed characteristic features of color, shape, structure of leaves specific for *Vitis rotundifolia*. Typical signs of *Vitis vinifera* include an elongated leaf blade, downiness on the under-side of the leaf, bunch size, relatively thin and less harsh skin, often without muscat flavor, more tender and melting pulp easy seed separable with long seed beaks [9].

However, in order to study genes introduced into genome and the action of genes, determining the resistance to pathogens in the process of pathogenesis, at first, it is necessary to select and adapt the optimal method of assessing the resistance. Field and laboratory methods of assessment *in vitro* and the disk-test method are known [10]. This publication presents the results of evaluating the resistance to mildew and oidium using the disk-test method, as suggestively the most optimal one to study the action of resistance genes in the process of pathogenesis [11].

2 Materials and methods

2.1 Plant material

In accordance with the breeding program accepted in the Institute Magarach, one of research directions is the study of introgression of genes that determine the resistance of grape plants to pathogens of *Plasmopara viticola* and *Erysiphe necator*, causing the development of mildew and oidium diseases, from the genome of *Vitis rotundifolia* into a genome with predominance of *Vitis vinifera* genes [9]. The object of the study is the recombinant lines of two populations from crossings carried out in the Institute Magarach: ♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790] (65 forms) and ♀M. No. 31-77-10 x 2000-305-143 (43 forms). To provide the greatest reliability of the received entries on the gene transfer to hybrid progeny, the maternal form taken for crossing possessed a functionally female type of flower, excluding the possibility of self-pollination and, therefore, all progeny was heterogeneous from the point of view of two specific genomes of original forms.

2.2 Phytopathological screening technique

In order to exclude the influence of side biotic and abiotic factors and to obtain reliable results, phytopathological screening was carried out using the disk-test method. For the disk-test, the leaves of recombinant lines were collected in duplicate in June – July. The fourth and fifth young leaf starting from the shoot tip was taken from each hybrid. Leaves were collected from grape plants not treated with fungicides. To destroy the surface infection, the leaves were pre-soaked in a sodium hypochlorite solution for 30 seconds, followed by washing in a large amount of running and distilled water. The disinfected leaves were placed in agar medium in Petri dishes.

Inoculation. Inoculation of leaves placed in Petri dishes with nutrient medium was carried out by applying 50 µl of an aqueous suspension of spores *Plasmopara viticola* to the under-side of the leaf. The approximate titre of *Plasmopara viticola* spores was 15000-20000 spores / ml. Inoculation of dry spores and an aqueous suspension of spores *Erysiphe necator* was carried out to the upper side of the leaf. Local isolates were studied, as the spores of *Plasmopara viticola* and *Erysiphe necator* were collected from the Southern Coast of Crimea.

Incubation. Further, the infected leaves, placed in Petri dishes, were incubated at air temperature of + 25 ° C, air humidity of 80-90%, day / night mode - 16 h / 8 h. *Vitis rotundifolia* and ‘Chardonnay’ served as the example varieties.

Assessment of resistance. Visual assessment of the recombinant line resistance was carried out 6-12 days after inoculation by the method of visual observation using the OIV descriptors 452-1 (Resistance degree of leaves to *Plasmopara viticola* in laboratory conditions (disk-test)), 455-1 (Resistance degree of leaves to *Erysiphe necator* in laboratory conditions (disk-test)) [12]:

The OIV rate scale 452-1 (Resistance of leaves to *Plasmopara viticola* in laboratory conditions):

1 point - very low: extensive pathogen damage to the leaf blade surface with clearly defined sporulation; the percentage of leaf affection by sporulation is 50.1-100%;

3 points - low: extensive pathogen damage to the leaf blade surface with clearly defined sporulation; the percentage of leaf affection is 25.1 - 50%;

5 points - average: spots with 1–2 cm diameter, fungus sporulation is from medium to strong, necrotic spots do not appear every time, 10.1 - 25.0%;

7 points - high: small single spots on leaves with and without sporulation; the percentage of leaf affection is 5.1 - 10%;

9 points - very high: small single spots without fungus sporulation are acceptable, the necrotic spots are absent; the percentage of leaf affection is 0.1 - 5%.

Rate scale 455-1 (Resistance of leaves to *Erysiphe necator* in laboratory conditions):

1 point - very low: heavy mycelium and fungus sporulation on leaves, complete or almost complete leaf fall; the percentage of leaf affection by sporulation is 50.1-100%;

3 points - low: extensive spots with fungus sporulation on leaves; the percentage of leaf affection is 25.1 - 50%;

5 points - average: well-defined spots with sporulation in diameter of 2-5 cm; the percentage of leaf affection is 10.1-25%;

7 points - high: small, up to 2 cm in diameter, spots with not clearly defined sporulation; the percentage of leaf affection is 5.1 - 10%;

9 points - very high: no mycelium on the leaf blade, slight leaf rolling; the percentage of leaf affection is 0.1 - 5%.

3 Results and discussion

As discussed above, the main goal of the research was to study the introgression of the *Vitis rotundifolia* (*Muscadinia rotundifolia*) genes, determining the resistance to fungal pathogens *Plasmopara viticola* and *Erysiphe necator*, into the genome of *Vitis vinifera* or the genome with the prevalence of *Vitis vinifera* genes during the process of pathogenesis. However, at the first stage, it is necessary to evaluate the genealogy of the studied forms and to work out the methodology for assessing the resistance and the process of pathogenesis.

3.1 Genealogy of the studied grape forms

Study of hybrid forms obtained from crossing combinations: ♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790] and ♀M.No.31-77-10 x 2000-305-143, allowed to analyze their genealogy. The hybrid form 'Magarach No. 31-77-10' was obtained by crossing the 'Nimrang' variety having functionally female type of flower with the 'Seibel 13666' variety, which is a complex interspecific hybrid obtained through the use of the species of the *Euvtis* subgenus of the *Vitis* genus: *V. riparia*, *V. berlandieri*, *V. cinerea*, *V. aestivalis*, *V. lincecumii*, *V. labrusca*, *V. rupestris* with almost 45% of *Vitis vinifera* genes in the genome. In its turn, all forms of DRX and 2000-305-143 originate from the same hybrid form based on crossing of *Muscadinia rotundifolia* with *Vitis vinifera*, followed by saturation of genomes with genes of species used in the breeding of the 'Seibel 13666' form. Knowing of genealogy from original forms to the modern ones of the entire genealogical tree, and their presence in the international ampelographic collection of the Institute Magarach, will make it possible to trace the original forms, transmitting the resistance genes to the progeny under study, which might be different from the *Muscadinia rotundifolia* species.

3.2 Resistance to *Erysiphe necator*

Infection of leaves of recombinant lines was carried out with dry spores and an aqueous suspension of *Erysiphe necator* spores. As a result of the research, it was found that after inoculation with dry spores of *Erysiphe necator*, the appearance of first spots of disease development was registered on the 4th – 6th day after inoculation, while after inoculation

with an aqueous solution of spores, the first spots of oidium were observed on the 8th -12th day. The research results showed that the degree of resistance to *E. necator* in general significantly varied between genotypes. The assessment of 65 hybrid forms of the first recombinant line (♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790]) on resistance to *E. necator* using the OIV scale allowed to divide them into the groups. The group with very high resistance (9 points, the OIV scale 455-1) included 25 hybrids. On leaves of this group the percentage of sporulation development was less than 5%.

The group with high resistance (7 points, the OIV scale) included 38 hybrids. The development of single spots of sporulation was observed on leaves of this group, the percentage of sporulation development was less than 10% (Figure 1).

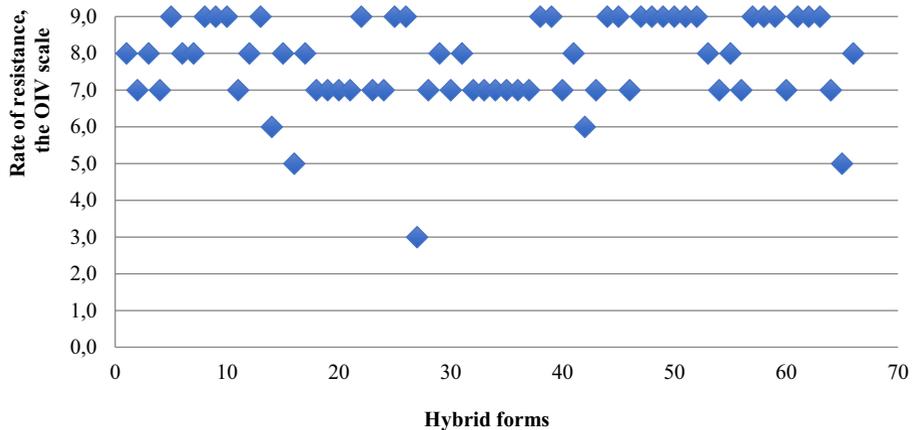


Fig. 1. Differentiation of hybrid forms by resistance to *Erysiphe necator* according to the OIV scale 455-1. Crossing combination ♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790]

In the hybrid form M.No.2-11-6-58 the resistance was medium and amounted to 5 points (Figure 2). In the hybrid form M.No.2-11-1-33 the resistance was very low; intensive sporulation was registered on the entire leaf blade surface. The resistance rate - 3 points.



Fig. 2. Development of sporulation of *Erysiphe necator* on leaves of the hybrid form M.No.2-11-6-58, resistance rate - 5 points. Crossing combination ♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790]

In the second recombinant line ♀M.No. 31-77-10 x 2000-305-143 (43 hybrid forms) (Figure 3), very high resistance to *Erysiphe necator* (9 points) was registered in 7 forms. The development of single small spots of sporulation was noted on leaves. High resistance (7 points) was observed in 31 forms. The development of single small spots of sporulation on leaves was noted.

Average resistance to *Erysiphe necator* (5 points) was observed in 4 hybrids: M.No.3-11-2-30, M.No.3-11-2-10, M.No.3-11-2-8, M.No.3-11-2-7, low resistance (3 points) - in the hybrid M.No.3-11-2-44. The percentage of leaf surface affection was up to 50%.

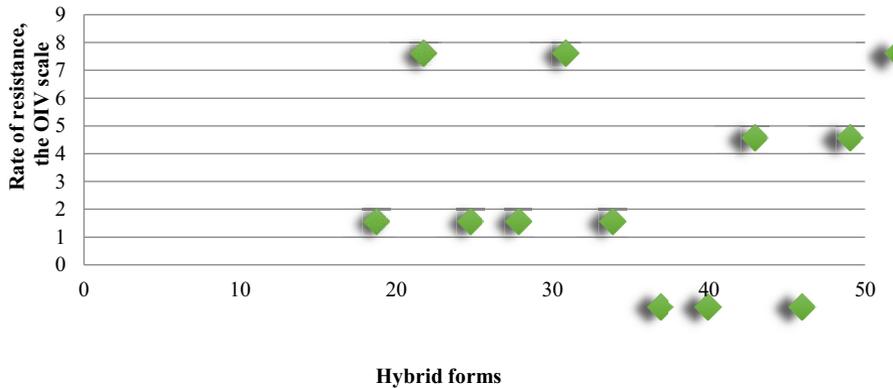


Fig. 3. Differentiation of hybrid forms by resistance to *Erysiphe necator* according to the OIV scale 455-1. Crossing combination ♀M.No.31-77-10 x 2000-305-143.

3.3 Resistance to *Plasmopara viticola*

Analysis of data on testing the resistance of hybrid forms to *Plasmopara viticola* in the first and second recombinant groups by the disk-test method allowed to receive the following results. In the first recombinant line (a combination of crossing ♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790]) very high resistance was observed in 34 out of 65 studied genotypes (Figure 4). Single small spots of sporulation were noted on the underside of the leaf.

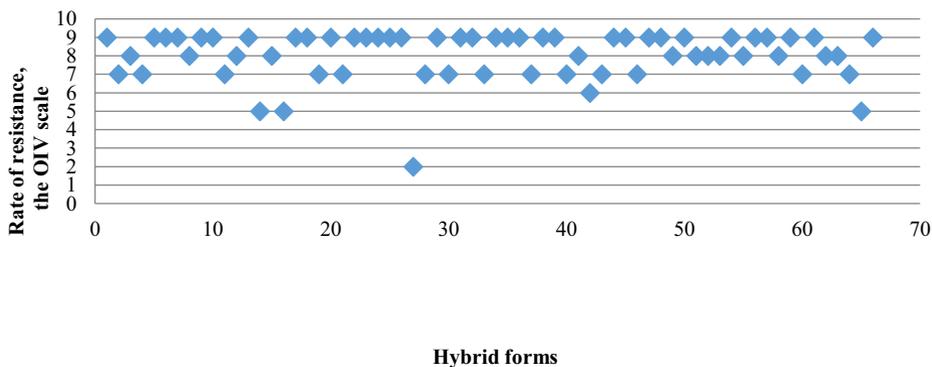


Fig. 4. Differentiation of hybrid forms by resistance to *Plasmopara viticola* according to the OIV scale 452-1. Crossing combination ♀M.No.31-77-10 x [DRX-M5-734 + DRX-M5-753 + DRX-M5-790].

Very high resistance (9 points) was indicated in 26 hybrids. Single spots of sporulation were also noted on the under-side of the leaf. Average resistance (5 points) was observed in hybrids M.No.2-11-1-19, M.No.2-11-1-21, M.No. 2-11-6-58 and M.No.2-11-1-50. Low resistance was registered for the hybrid M.No.2-11-1-33. The percentage of sporulation development consisted of about 50%.

In the second recombinant line ♀M.No.31-77-10 x 2000-305-143 (43 hybrids), very high resistance to *Plasmopara viticola* was noted in 18 genotypes. Single spots with or without sporulation were observed, necrotic spots were absent. High (7 points) and average (5 points) resistance was observed for 8 and 15 genotypes, respectively. Low resistance was registered in 2 hybrids (M.No.3-11-2-44 and M.No.3-11-2-30). The development of 2-4 small spots with sporulation was noted, necrotic spots were absent (Figure 5).

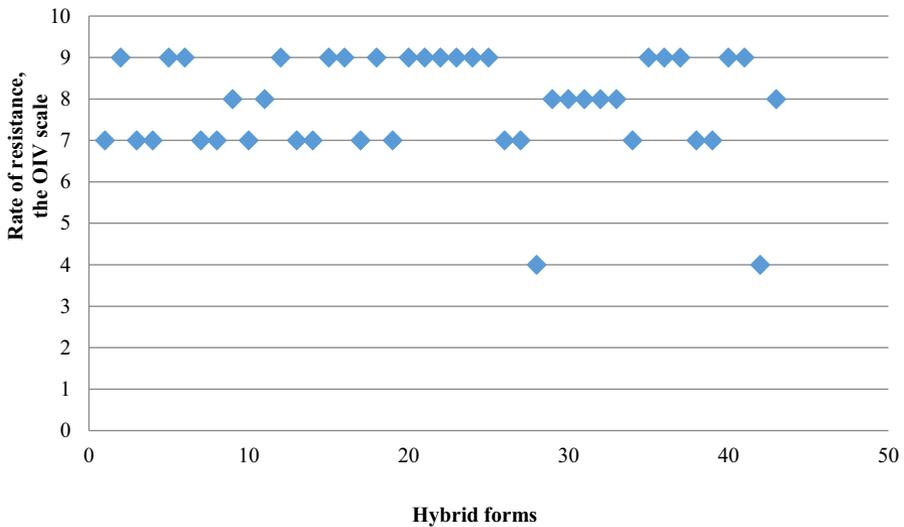


Fig. 5. Differentiation of hybrids by resistance to *Plasmopara viticola* according to the OIV scale 452-1. Crossing combination ♀M.No.31-77-10 x 2000-305-143.

4 Conclusion

The provided studies have allowed to establish that the assessment of resistance of grape genotypes to the *Plasmopara viticola* and *Erysiphe necator* pathogens, causing the development of mildew and oidium diseases, makes it possible to quickly and reliably conduct phytopathological screening of populations of hybrid grape forms using the disk-test method in order to identify genotypes, meaning the potential carriers of genes resistant to fungal diseases. It should be noted that genotypes of either, high and low resistance were liberated in the progeny in both crossing combinations. Genotypes with different combining of the resistance degree to two pathogens were identified. When comparing with the results of testing genotypes for the presence of resistance genes by DNA-marking methods, the obtained results will make it possible to identify genes or gene groups, responsible for resistance to individual or a complex of pathogens. Such data will help to answer the question of genes to be pyramided in the progeny to obtain the most complex-resistant genotype.

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