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Rapeseed Bacterial Diseases and their REP-PCR Analysis

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Box, ERIC and REP " genomic fingerprints of 12 isolated and 10 typical pathogenic for rape bacterial strains *Pseudomonas, Xanthomonas* and *Pectobacterium* genera have been analyzed. The affinity of isolated strains with representatives of *P. marginalis* pv. *marginalis, Pseudomonas fluorescens* and *Xanthomonas campestris* pv. *campestris* species has been determined.

Key words: Rape's bacterial diseases, bacteria *Pseudomonas, Xanthomonas* and *Pectobacterium* genera, REP-PCR analysis, Box, ERIC and REP " genomic fingerprints.

Despite a strategic importance of rape for the national economy the data on the genetic variability of populations of bacterial diseases of this crop in the literature is very limited[5]. Earlier we studied the phenotype properties of population of pathogens bacterial diseases of rape isolated in 2010-2012 years and it is shown that 60% of isolated strains belong to *Pseudomonas*, genus and 40% " to *Xanthomonas* genus. Particularly, we found that the strains, which are assigned to the *Xanthomonas* genus, are more related to representative of the *Xanthomonas campestris* pv. *campestris* species by the complex of phenotype features[3,15]. Instead, to perform completely identification of isolated strains, which are referred to the genus *Pseudomonas*, at the level of species was failed due to close affinity of phenotypic species *Pseudomonas fluorescens* and *Pseudomonas marginalis*[15]. Taking this into account, th5 aim of our researches were the genetic profiling of population of bacterial diseases of rape using REP-PCR analysis.

As many researches think that the most successful, for valuation of genetic structure of either individual taxons as well as the whole populations, is group of methods so called «fingerprinting genome» which include random amplified polymorphic DNA method AP/ RAPD"PCR method and the method of amplification of genetic elements, which repeat (REP-PCR), and also the method of amplification of fragments of polymorphic DNA of different length (AFLP"PCR) [2,4,7,10]. AP/RAPD-PCR or REP-PCR are the most used because it rates the genetic variability of the whole genome, effective while bacteria identification on the species, subspecies or strain level and don't need additionally conducting of restriction analysis, as while AFLP-PC[6,8,9,13]. REP-PCR is more effective in

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case of exact taxonomic researches because it gives opportunities to receive three (REP, ERIC, %) independence genetic profile simultaneously because, unlike the other methods genome fingerprinting, including AP/RAPD-PCR)[4,6,7,10,11]. That's why this method is often used for researches in the field of microorganisms systematics[7,10].

MATERIALS AND METHODS

The following collection and typical strains of phytopathogenic bacteria, which are able to affect rape: *Pseudomonas fluorescens* B-17^T (biovar), *Pseudomonas fluorescens* 8573, *Pseudomonas fluorescens* B-28 (biovar), *Pseudomonas fluorescens* B-36 (biovar), *Pseudomonas fluorescens* B-41 (biovar V), *Pseudomonas fluorescens* B-52 (biovar V), *Pseudomonas fluorescens* B-53 (biovar V), *Pseudomonas marginalis* pv. *marginalis* 9175^T, *Pseudomonas syring05* pv. *syringa5* B-1027^T, *Pectobacterium carotovorum* subs. *carotovorum* B-1075^T, *Xanthomonas campestris* pv. *campestris* B-1049^T and *Xanthomonas campestris* pv. *campestris* 820 were used in work. The isolation and purification of chromosome DNA were conductd with using a set of reagents "DNA-sorbet-». The DNA concentration was defined with spectrophotometry BioPhotometr. In our work such universal primers were: REP 1R -*5'-IIIICGICGICATCIGGC-3'*, REP 21 -*5'-ICGICTTATCIGGCCTAC-3'*; ERIC 1R -*5'-ATGTAAGCTCCTGGATTCAC-3'*, ERIC 2 -*5'-AAGTAAGTGACTGGGGTGAGCG-3*; BOX A1R-*5'-CTACGGCAAGGCGACGCTGACG-3'*. The amplification was carried out using thermal cycler Veriti 96 Well Thermal Cycler 9902, of Applied Biosystem firm (USA) under experimentally selected conditions. For *Pseudomonas* sp.strains: additional denaturation of DNA – 96^0!/6 min. and the main denaturation of DNA – 94^0!/ 1min. (the same for all kinds of REP– PCR); primers annealing – 44^0!/1min. (REP– PCR with REP primers), 52^0!/ 1min. (REP– PCR with ERIC primers) and 53^0!/1 min. (REP– PCR with BOX primers); DNA elongation – 72^0!/2min. (the same for all types of PCR reactions) and final DNA synthesis – 65^0!/ 8min. (the same for all types of REP– PCR). The amplification conditions for representatives of genera *Xanthomonas* were such: additional DNA denaturation – 95^0!/7 min. and the main DNA denaturation – 94^0!/ 1min. (the same for all kinds of REP– PCR); primers annealing – 44^0!/1min. (REP– PCR with REP ptimers), 52^0!/1min. (REP– PCR with ERIC primers) and 53^0!/1 min. (REP– PCR with BOX primers); DNA elongation – 72^0!/8min. (the same for all types of PCR reactions) and final DNA synthesis – 65^0!/15min. (the same for all types of REP– PCR). The products of reaction were distributed in 1.5% agarose gel, during 4 hours under the electric field of 1,5 V/cm. Gel Doc Universal Hood of Apllied Biosystem (USA)firm was used to visualize received genetic profiles. The affinity of received REP, ERIC and Box-profiles were evaluated with computer program DENDRO UPGMA,which based on using unweighted pair group method with averages, UPGMA).

RESULTS

Strains *Pseudomonas* sp. 40, 60, 70, 5*, 7*, 8*, 9*, 14 are closely related to the typical strain *Pseudomonas marginalis* pv. *marginalis* 9175 ^{T} (Fig. 1). It should also be noted that isolated and studied strains have also three common PCR reaction products with typical strain *Pseudomonas syring05* pv. *syringa5* B-1027^T and two commons *Pseudomonas fluorescens* 8573, *Pseudomonas fluorescens* -52, *Pseudomonas fluorescens* -53, which indicates their phylogenetic remoteness. Thus strains *Pseudomonas* sp. 6a, 7a, 5*, 7*, 8*, 9* and 14* (fig.2) formed two (similar to each other) clusters, that the most related to the typical strain *Pseudomonas marginalis* pv. *marginalis* 9175^T (fig.2). The rest of selected strains *Pseudomonas* sp. 2O, 3A, 2A and 4A found no significant affinity with any typical or collectible strain on the stage of preliminary REP, ERIC and, Box genome profiling.

The genetic remoteness of strains of *Pseudomonas* sp. 2O, 3A, 2A and 4A from the typical strain of *P. fluorescens* B-17T, probably due to significant heterogeneity of species *Pseudomonas fluorescens*, which comprises five biovar, some of which are opportunistic for plants[2].

Analysis of ERIC – profiling also revealed the affinity of seven selected *Pseudomonas* sp. 60, 70, 5*, 7*, 8*, 9*, 14* strains with typical *Pseudomonas marginalis* pv. *marginalis* 9175^T strain. As shown in fig. 3 these strains has fourteen detected products of PCR reaction which are

common with a typical representative of the *Pseudomonas marginalis* species This proves their close phylogenetic relationship. In addition, the above-mentioned *Pseudomonas* sp. strains also reveals two DNA - fragments which are common with the P. fluorescens B-28 and P. fluorescens B-36 strains.

Based on the results of cluster analysis these strains has formed a single homogeneous cluster, that most closely associated with a cluster consisting of typical strain *Pseudomonas marginalis* pv. *marginalis* 9175T (Fig. 4).

It was also determined that the isolated *Pseudomonas* sp. 2O, 3A and 4A strains are highly related to the typical *Pseudomonas fluorescens* B-52 and *Pseudomonas fluorescens* B-53 strains. In particular, these strains are related to typical representatives of fifth biovar *Pseudomonas*

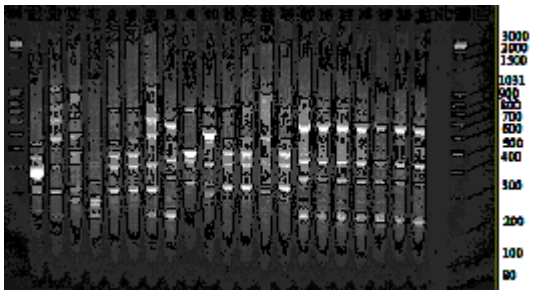

Fig.1. Electrophoretic distribution of the PCR reactions products using BOX-primers in 1,5% agarose gel.: m – markers of molecular mass (markers with molecular mass); 1.- *P. fluorescens* 8573; 2.- *P. fluorescens* B-28; 3.- *P. fluorescens* B-36; 4.- *P. fluorescens* B-41; 5.- *P. fluorescens* B-52; 6.- *P. fluorescens* B-53; 7.- *P. fluorescens* B-17^{T}; 8.- *P.marginalis* pv. *marginalis* 9175 ^{T}; 9.- *P. syringae* pv. *syringae* B-1027^{T}; 10. - *P. carotovorum* sp. *carotovorum* B-1075^{T}; 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21. – isolated strains *Pseudomonas* sp. 2O, 3A, 2a, 4a, 6a, 7a, 5*, 7*, 8*, 9*, 14*, NC – negative control.

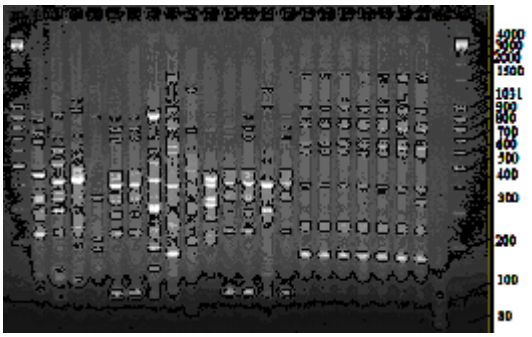

Fig. 3. Electrophoretic distribution of the PCR reaction products using ERIC-primers in 1,5% agarose gel: m " markers with molecular mass; 1.- *P. fluorescens* 8573; 2.- *P. fluorescens* B-28; 3.- *P. fluorescens* B-36; 4.- *P. fluorescens* B-41; 5.- *P. fluorescens* B-52; 6.- *P. fluorescens* B-53; 7.- *P. fluorescens* B-17^{T} ; 8.- *P. marginalis* pv. *marginalis* 9175 ^{T}; 9.- *P. syringae* pv. *syringae* B-1027^{T}; 10. - *P. carotovorum* sp. *carotovorum* B-1075^{T}; 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21. – isolated strains *Pseudomonas* sp. 2O, 3A, 2a, 4a, 6a, 7a, 5*, 7*, 8*, 9*, 14*, NC – negative control.

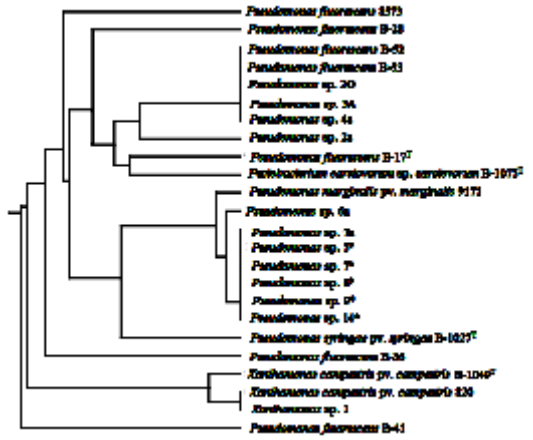

Fig. 2. Dendrogram of affinity of pathogenic bacteria of *Pseudomonas* for rape, which is based on the results of BOX-profiling.

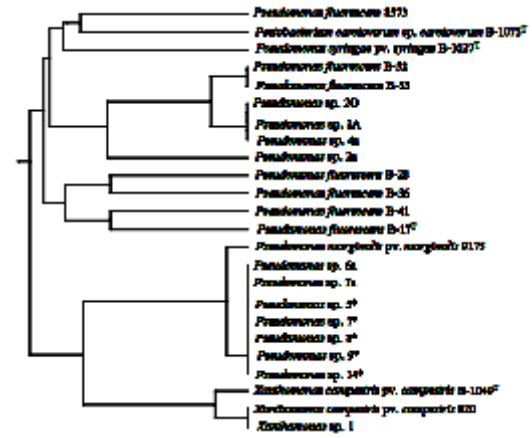

Fig. 4. Dendrogram of affinity of pathogenic bacteria of *Pseudomonas* for rape, which is based on the ERIC –profiling results.

fluorescens species by nine common products of reaction with molecular weight from 200 to 840 b.p. (Fig. 3). Instead, isolated *Pseudomonas* sp. 2a strain also reveals affinity with the typical collectible strains mentioned above, only by 3 common products of PCR reactions. In addition, this strain has one DNA-fragment which is common with the following strains: *Pseudomonas syring05* pv. *syringa5* B-1027^T, *P. fluorescens* B-17^T B0 *P. marginalis* pv. *marginalis* 9175 ^{T} (fig.3). Phylogenetic relationship of *Pseudomonas* sp. 2a strain with the typical representatives of fifth biovar of *Pseudomonas fluorescens* species is confirmed by the results of the cluster analysis. As shown in Figure 4 strain is most closely associated with the two clusters that formed in accordance with *Pseudomonas fluorescens* -52 B0 *Pseudomonas fluorescens* -53 strains, and also isolated *Pseudomonas* sp. 2, 3 B0 40 strains.

The results of REP-genome profiling of *Pseudomonas* sp. 6a, 7a, 5 *, 7 *, 8 *, 9 *, 14 * strains and a typical strain of *Pseudomonas marginalis* pv. *marginalis* 9175T also confirm the high degree of phylogenetic relationship. In particular, six DNA-fragments which are common with the typical strain of *Pseudomonas marginalis* pv. *marginalis* 9175T are identified in REP-profiles of selected strains. The *Pseudomonas* sp. 2O, 3A and 4A strains have nine DNA-fragments which are common with the typical strains of *Pseudomonas fluorescens* B-52 and *Pseudomonas fluorescens* B-53, that confirms their close phylogenetic relationship. In these strains two PCR reaction products are also revealed, which are common with the *Pseudomonas fluorescens* 8573 strain and one product – with *Pseudomonas fluorescens* B-28, *Pseudomonas fluorescens* B-41 strain. Cluster analysis confirmed the results REP-PCR profiling.

During the researches the noted genetic variability of individual strains within the genus *Pseudomonas* is consistent with the literature[9,12], and suggests the necessity of using for correctly taxonomy of representatives of the genus of the polyphasic taxonomy[2,7,10]. It also should be admitted that our isolated *Pseudomonas* sp. *20* strain by the summarized number of these DNA-fragments in BOX, ERIC and REP-profiles is the most related to the typical *Pseudomonas fluorescens* B-52 and *Pseudomonas fluorescens* B-52 strains,which belong to the fifth biovar as part of *Pseudomonas fluorescens* species. In addition, we obtained results that correlate with the previous analysis of complex phenotype features, which also indicated a significant relationship of this strain with the representatives *Pseudomonas fluorescens* species[3,15]. Thus, the results of genome fingerprinting are that about 64% of pathogenic bacteria of *Pseudomonas fluorescens* genus of rape is attributed to *Pseudomonas marginalis* pv. *marginalis* species, and other 36 % - to *Pseudomonas fluorescens*. It should also be noted that isolated, from infected rape plants, strains of *Pseudomonas* sp. 2O, 3A, 4A and 2A are heterogeneous in terms of aggressiveness[15] and by results of this research and the results of this research the most phylogenetically related with the saprophytic strains by typical representatives of fifth biovar of *Pseudomonas fluorescens* species, which isolated from rhizosphere of wheat and sundial lupin. It is known from the literature that some strains as part of this species can cause, under certain conditions, diseases of grain legumes[2,5]. Thus, this fact once again confirms the well-known pattern about the high biochemical and ecological plasticity of species representatives of *Pseudomonas fluorescens* and their ability in certain circumstances to pass from saprophytic to pathogenic mode of existence[2].

By results of ERIC-profiling the *Xanthomonas* sp. *1* strain is more related to the typical strain of *Xanthomonas campestris* pv. *campestris* 820 than with typical *Xanthomonas campestris* pv. *campestris* B-1049^T strain. In particular, in the ERIC-profiles of the aforementioned isolated strain it is found four DNA-fragments which are common with the collectible strain of *Xanthomonas campestris* pv. *campestris* 820, and three - with typical strain of *Xanthomonas campestris* pv. *campestris* B-1049T, which confirms belonging of *Xanthomonas* sp. 1 to the *Xanthomonas campestris* species and to the one-named patovar in its composition. Our obtained results of REP and ERIC - profiling are also confirmed by data of PCR reaction with Box-primers. In particular, in Box-genome profiles of isolated *Xanthomonas* sp. 1 strain it is identified five products of reaction that are common with the typical strain of *Xanthomonas campestris* pv.

campestris B-1049T. The PCR results of are consistent with the data of cluster analysis (Figure 2, Figure 4). It should also be mentioned that established genetic heterogeneity of *Xanthomonas campestris* pv. *campestris* strains,was found as a result of REP, ERIC and , Box-genome profiling, and is confirmed by the literature data for representatives of this genus and encourages research to a wider range of strains in order to develop correctly database of DNA "fingerprinting"[1,6,11,14].

The analysis of REP, ERIC and Box - genomic profiling of strains isolated by us showed that the population of bacterial diseases of rape is taxonomically heterogeneous. In particular, 37,5% of isolated strains by the complex of genotype and phenotype features is attributed to *Pseudomonas marginalis* pv. *marginalis*, 25% " *Pseudomonas fluorescens* biovar V and 37,5% to " *Xanthomonas campestris* pv. *campestris*. That is, in nature bacteria pathogenic to rape belonging to the genus Pseudomonas (62,5% of isolated strains) are more numerous than representatives of the *Xanthomonas* genus (37,5% of isolated strains). In our opinion this may be due to high environmental plasticity of exactly the *Pseudomonas* genus. Furthermore, highly specialized pathogens weren`t detected among the identified range of strains. All identified phytopathogenic bacteria are either classical polyphage, that able affect wide range of plant or opportunistic pathogens species that can, only in certain circumstances, cause a wide range of plant diseases. This fact suggests the potential dangers of incorrect introduction of rape, as a possible source of bacterial pathogens of polyphage nature and opportunistic for plant species. The careful design of correct crop rotation by farmers is also needed. It should also be noted that in the case of rape pathogens representatives of the *Pseudomonas* genus, the genotype methods are the most effective taxonomic approach including methods of "fingerprinting' of genomic DNA.

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