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DEVELOPMENT OF SPECIFIC PRIMERS FOR 16S rRNA GENE ANALYSIS IN THE DETECTION OF *Ralstonia solanacearum* SPECIES COMPLEX

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Members of *Ralstonia solanacearum* species complex (RSSC) are causal agents of vascular wilt disease in more than 450 crop species, including solanaceous plants such as potatoes, tomatoes, bell pepper, eggplant, etc. These phytopathogens cause serious yield loss mostly in solanaceous crops which are grown in tropical, subtropical, and temperate regions of the world. Yield losses comprise 80–100% in potato, up to 91% for tomato, 10–30% in tobacco, 33–90% in banana, and reduce crop productivity and yield. PCR-methods are specific, sensitive and cost-effective approaches for the detection and identification of RSSC members.

The *objective* of this study was to compare specificity of routinely used primer mix for PCR RSSC detection with the newly developed pairs of species-specific primers for ease of use diagnostics in a laboratory.

Materials and Methods. The conserved genomic regions of the 16S rRNA sequences of *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* were selected for the design of primers for this study. Newly created primer species specificity was tested in PCR using the DNA of the two targets and 12 non-target strains of bacteria.

Results. Three pairs of newly created primers Rs-28(F)/Rs-193(R), Rs-28(F)/OLI-160(R), Rs28(F)/OLI248(R) produced single specific fragments for bacterial strains of *Ralstonia solanacearum*: 166 bp, 132 bp, and 220 bp. products respectively. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other bacterial species.

Conclusion. Designed primers can be used for the development of PCR system for the qualitative and quantitative detection of RSSC members.

Key words: PCR, *Ralstonia solanacearum*, RSSC, bacterial wilt.

Ralstonia solanacearum species complex (RSSC) is a diverse group of plant pathogens (*R. solanacearum, Ralstonia pseudosolanacearum, and Ralstonia syzygii*), which belongs to the family *Burkholderiaceae* [1, 2]. Members of RSSC are gram-negative soil-borne pathogens that cause devastating losses in the world agrarian economy due to lethal wilting in plants as a result of vascular dysfunction [3–5] in susceptible hosts [6–9]. RSSC causes bacterial wilt disease in over 450 different crop plant species covering more than 54 botanical families such as *Cucurbitaceae, Brassicaceae, Solanaceae, Fabaceae, Musaceae, Zingiberaceae*, and *Asteraceae* over the world [10–13].

Bacterial wilt is one of the most spread diseases of the potato. High-level expansion of the pathogen is associated with latently infected planting material. The disease is liable for causing substantial losses to the potato industry where it exists [9, 14–16]. In European Union (EU), RSSC was included in the EPPO A2 List of pests recommended for controlling and eradicating [2]. This actualizes necessity of regular epidemiological monitoring of RSSC. For routine practice, a detection assay should be rapid, specific, and quite sensitive. In addition, the methodology for the extensive monitoring should not be labor intensive and time-consuming. According

to the EU control directive, wide range of methodological approaches are acceptable for primary screening and monitoring, including serological techniques, the enzyme-linked immunosorbent assay, and molecular techniques such as PCR [17–20]. The latter is reliable and unequivocal method, which meets the majority of aforementioned requirements and provides high levels of confidence in identification, high sensitivity and specificity.

RSSC is highly heterogenic. Initially, *R. solanacearum* was divided into five races and six biovars based on the host plants and biochemical features. Currently, RSSC is assorted into four phylotypes based on the analysis of the ITS region [9, 19] and strains' geographic origins: Phylotype I (Asia) —*Ralstonia pseudosolanacearum*; Phylotype II (America) — *R. solanacearum*; Phylotype III (Africa and India Ocean) — *Ralstonia pseudosolanacearum*; Phylotype IV (Indonesia) — *Ralstonia syzygii* [9, 19, 20]. The most widely used PCR assays for quarantine purposes or to confirm the introduction of the pathogen to areas where it was not present are usually based on the amplification of ribosomal gene sequences (i.e., 16S or 16S-23S intergenic spacer region of the ribosomal DNA [rDNA]) with the use of species-specific RS primers for detection of all RSSC strains [21].

The objective of this study was to compare specificity of routinely used primer mix for PCR RSSC detection with newly developed pairs of species-specific primers for ease of use in a diagnostic laboratory.

Materials and Methods

Bacterial strains and growth conditions. The 14 strains from the Ukrainian National Collection of Microorganisms were kindly provided by Prof. V. Patyka (Department of Phytopathogenic Bacteria, D. K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv). These reference strains, isolated from a variety of hosts and geographic locations, are representative causal agents of the bacterial diseases of solanaceous plants (are listed in Table 1.) Bacteria were grown at 28 °C on potato agar (potato 500g/L, NaCl 5g/L, agar 20g/L) for 24–48 h.

DNA preparation. Bacterial genomic DNA was extracted using the Agrosorb NK kit (LLC Agrogen Novo, Ukraine). The purity and quantity of isolated DNA were determined spectrophotometrically by absorbance measurement of A260/A280 using a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, USA) and DNA concentration is estimated by measuring the absorbance at 260 nm.

PCR analysis. A pair of primers OLI-1/Y-2 was used for molecular identification of *Ralstonia solanacearum* strains [2, 22]. The conserved genomic regions of the 16S rRNA sequences of *R. solanacearum, R. pseudosolanacearum*, and *R. syzygii* were selected for the design additional primers for this study (Table 2).

Polymerase chain reaction was performed in a final volume of 10 µl, and reaction mixture consisted of 3.4 µl double-distilled water, 3.1 µl 2×PCR SuperMix (Bio-Helix, Taiwan) 1 µl primer mix (concentration 5 µM), 2.5 µl template DNA. Sterile double-distilled water was used as a negative control to test for the presence of contamination in PCR reagents.

PCR was carried out on a T100™ Thermal Cycler (Bio-Rad Laboratories Ltd., USA). After the initial denaturation step for 96 °C at 2 min, reactions of amplification were performed using 40 cycles. Cycles consisted of a 20 s denaturation at 94 °C, 20 s of primer annealing at 66 °C and 30 s extension at 72 °C, followed by a final step at 72 °C 10 min and cooling to 4 °C.

PCR-amplified fragments were electrophoresed in the horizontal 2% (m/v) agarose gel with ethidium bromide in SB (Sodium Borate or Sodium Boric Acid) buffer at 100 V for 1 hour.

Visualization of amplified fragments was conducted by UV light (260 nm) and photographed using Bio-Rad Gel DocTM XR+ gel documentation system (Bio-Rad Laboratories Ltd., USA).

Results and Discussion

The *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ATCC 11696 (ICMP 5712), also known as K60, is *R. solanacearum* type strain, which belongs to phylotype II, sequevar 7. K60 was isolated in 1953 from a Marglobe tomato (*Lycopersicon esculentum*) in Raleigh, North Carolina, USA, and has been used extensively for bacterial wilt research [23]. According to Seal et al. (1993) [22], the use of specific primer OLI1 together with the non-specific primer Y2 in PCR with an annealing temperature of 68 °C allows specific amplification of a single 287–288 bp product from the strain K60.

***Table 1*. Bacterial strains used in the study**

Species	Strain code
Ralstonia solanacearum (Smith 1896) Yabuuchi et al. 1992	B-1109T = ATCC 11696, CFPB 2047, ICMP 5712, ICPB PS256, LMG 2299, NCPPB 325 ← Mypac B.A., 9049 ← ICMP 5712 ← NCPPB 325
Ralstonia solanacearum (Smith 1896) Yabuuchi et al. 1992	B-1110 = ICMP 7859 ← Mypac B.A., 9081 ← ICMP 7859.
Clavibacter michiganensis subsp. *michiganensis*	Ac-1996
Clavibacter michiganensis subsp. *sepedonicus*	Ac-1995
Clavibacter michiganensis subsp. *sepedonicus*	Ac-1997
Pectobacterium carotovorum	B-1075
Pectobacterium atrosepticum	B-1084
Pectobacterium atrosepticum	B-1103
Pectobacterium carotovorum subsp. *carotovorum*	B-1097
Pectobacterium carotovorum subsp. *carotovorum*	B-1079
Pseudomonas syringae pv. *syringae*	B-1022
Pseudomonas syringae pv. *syringae*	B-1028
Xanthomonas campestris pv.*campestris*	B-1070
Xanthomonas vesicatoria	B-1060

Table 2. **Primer sets used in this study**

Primer	Sequence (5′-3′)	Direction	Reference
OLI*	GGGGGTAGCTTGCTACCTGCC	Forward	Seal et al. (1993)
Y2	ACTCCTACGGGAGGCAGCAGTGGG	Reverse	Seal et al. (1993)
OLI160	CGGCCGCCTCCTATAGCATGA	Reverse	Designed in the study
OLI248	AGTCCCAGTGTGGCTGATCG	Reverse	Designed in the study
Rs28	TGGCGAACGGGTGAGTAATA	Forward	Designed in the study
Rs193	GGCCTTTACCCCACCAACTA	Reverse	Designed in the study

* All primers were synthesized by Metabion, Germany.

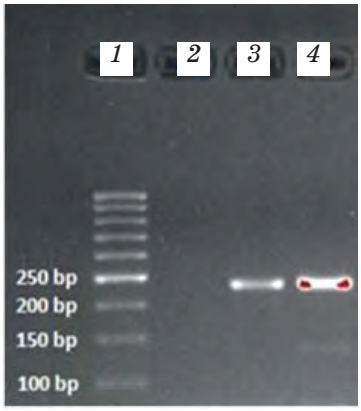

Fig. 1. **PCR result using OLI1/Y2 specific primers:**
1 — DNA size markers, Step Ladder 50 bp; *2* — negative control;
3 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 5712;
4 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 7859.

The *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 7859 was isolated by I. Herrera in 1974 from a potato (*Solanum tuberosum* L.) in Morropon, Piura, Peru, and was deposited in ICPM in 1983. Data concerning PCR identification of this isolate is absent. ICMP 5712 and ICMP 7859 both belong to the same biovar I [24].

According to Seal et al. [22], Y2 primer was designed by Young et al. (1991) [25] to amplify a partial segment (corresponding to positions 4&337 of *Escherichia coli* 16S rRNA) of most DNAs encoding 16s rRNA. And, for specific PCR amplification of specimens containing DNA of the RSSC members, the non-specific primer Y2 is used together with a specific primer OLI1 primer. In our experiments, OLI1/Y2 pair of primers produced the unexpectedly short 250-bp-long-fragment instead of expected 288-bp-long-fragment from both *R. solanacearum* strains: ICMP 5712 and ICMP 7859 (Fig. 1). Relative humidity- or/and temperature-induced transition from B-DNA to A-DNA could be one of the reasons of obtaining PCR product which is shorter than expected, since in a B-A transition, the long and narrow B-duplex is converted to the under wound and compact A-DNA structure [26–28].

Next, we tested specificity of newly constructed primers for the amplification of parts of consensus sequence for RSSC [22]. The homology of the suggested primer sequences was analyzed using the Basic Local Alignment Search Tool (BLAST) to avoid primer-template mismatches and to increase their specificity to the wide range of isolates. The primers were designed to obtain relatively short PCR products that would distinctly differ in length after the electrophoresis. Reverse primers were constructed (OLI-160 and OLI-248) on the basis of forward primer, proposed by Seal et al. (1993) [22]. Additionally, a pair of primers Rs-28 and Rs-193, which formed the amplification product within 166 bp was selected. For the testing of newly created primers specificity, we used two abovementioned strains of *R. solanacearum*, as well as 12 strains of other phytopathogenic bacteria.

Seven pairs of constructed primers were combined with the expected amplification products of 160, 248, 193, 166, 132, 220, and 260 bp correspondingly. The pairs of primers OLI/OLI-160, OLI/OLI-248 and OLI/Rs193 did not give any products. As one can see on the Fig. 2, four of seven tested pairs of primers provided only single specific fragments for bacterial strains of *Ralstonia solanacearum* of expected length. Namely, pairs of newly created primers Rs-28(F)/Rs-193(R), Rs-28(F)/OLI-160(R), Rs28(F)/OLI248(R) produced 166 bp, 132 bp, and 220 bp. products respectively. Rs28(F)/Y2(R) pair produced 260-bp-long-fragment. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other bacterial species.

Since the development of primers was carried out in the perspective of creating a PCR test system for the detection and quantitative analysis of RSSC in potato biomaterial, phytopathogens affecting both solanaceous and some non-solanaceous plants were chosen for the specificity testing (Table 1).

For *Clavibacter michiganensis* subsp. *michiganensis*, tomato is the most important host. In addition, other solanaceous plants including potato are susceptible upon natural infection and/or artificial inoculation [29].

Clavibacter sepedonicus is not widely distributed in the areas where potato evolved. Nevertheless, *C. sepedonicus* has the tendency to exist asymptomatically as latent infections in potato. Involuntary spread of the bacterium to new places of production occurs with the movement of latently infected seed tubers used for planting [30].

Pectobacterium spp. are pectolitic necrotrophic pathogens responsible for a blackleg and soft rot disease of potatoes in many areas. These quarantine and threatening pathogens cause considerable economic and yield losses, both in field production and storage [31].

Xanthomonas vesicatoria causes bacterial spot disease in tomato and pepper, as well as in various other *Solanaceae*, and can be detected on fruits of potato [32]. The remaining two phytopathogens did not affect solanaceous plant including potato: *Pseudomonas syringae* affects woody and herbaceous host plants [33] and *Xanthomonas campestris* pv. *campestris* causes black rot of crucifers [34]. Such a wide range of phytopathogenic bacteria of different host specificity allowed us to thoroughly test the specificity of newly created primers.

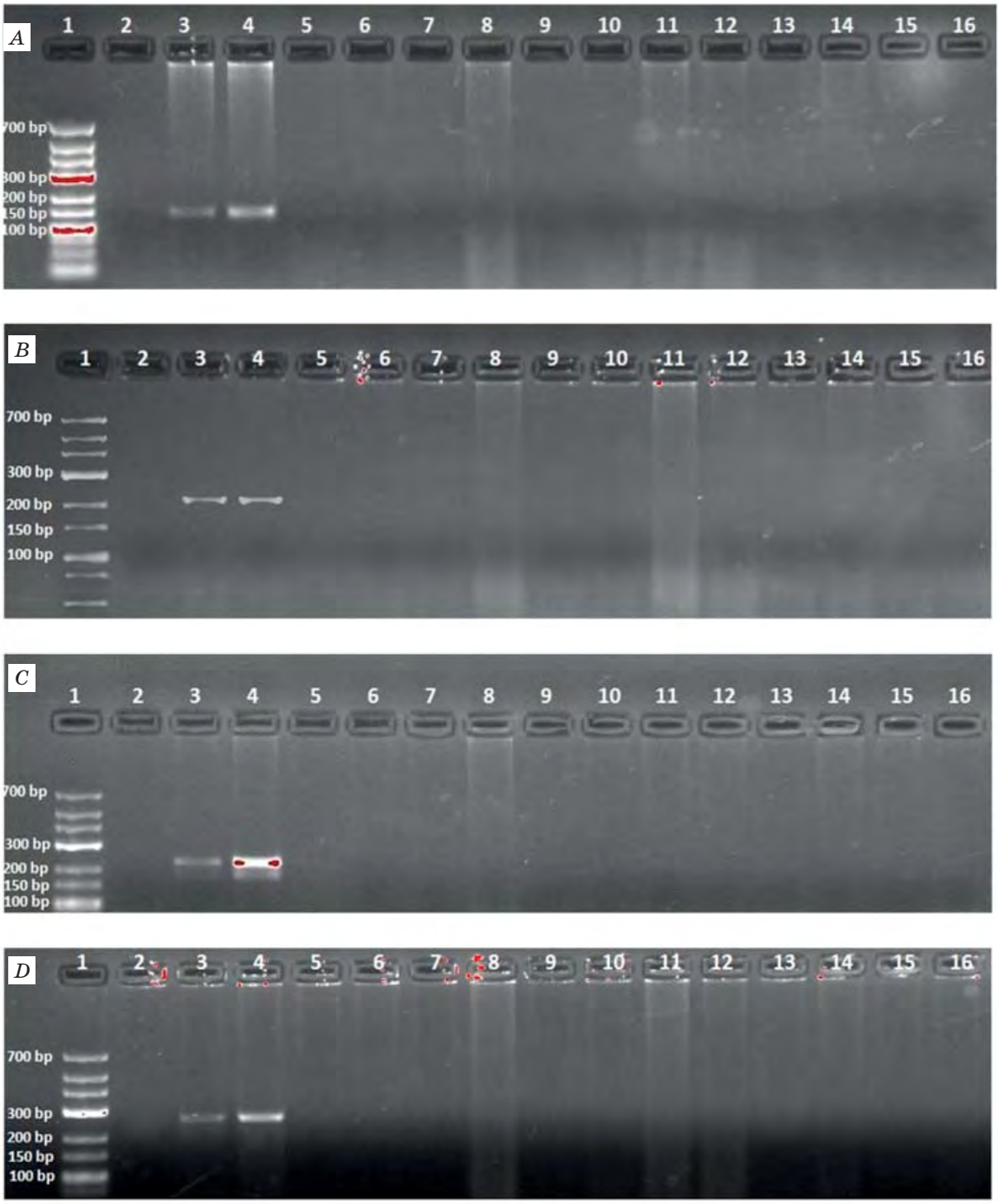

Fig. 2. **PCR results using Rs-28/Rs-193 (*A*), Rs-28/OLI-160 (*B*), Rs28/ OLI248 (*C*) and Rs28/Y2 (*D*) pairs of primers:**
1 — DNA size markers, Step Ladder 50 bp; 2 — negative control; 3 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 5712; 4 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 7859; 5 — *Clavibacter michiganensis* subsp. *michiganensis* (Ac-1996), 6 — *Clavibacter michiganensis* subsp. *sepedonicus* (Ac-1995), 7 — *Clavibacter michiganensis* subsp. *sepedonicus* (Ac-1997), 8 — *Pectobacterium carotovorum* (B-1075), 9 — *Pectobacterium atrosepticum* (B-1084), 10 — *Pectobacterium atrosepticum* (B-1103), 11 — *Pectobacterium carotovorum* subsp. *carotovorum* (1097), 12 — *Pectobacterium carotovorum* subsp. *carotovorum* (B-1079), 13 — *Pseudomonas syringae* pv. *syringae* (B-1022), 14 — *Pseudomonas syringae* pv. *syringae* (B-1028), 15 — *Xanthomonas campestris* pv.*campestris* (B-1070), 16 — *Xanthomonas vesicatoria* (B-1060).

Conclusion

Based on 16S RNA and OLI1/Y2 primer pairs, four primers specific for the RSSC were designed and tested for species specificity. Results of species specificity testing evidence that three pairs of designed primers, as well as constructed specific forward primer RS28 paired with routinely used non-specific Y2 reverse primer can be used for the development of PCR system for the qualitative and quantitative detection of RSSC members.

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Conflicts of Interest

Authors declare no conflict of interest.

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СТВОРЕННЯ СПЕЦИФІЧНИХ ПРАЙМЕРІВ ДЛЯ АНАЛІЗУ ГЕНА 16S РРНК У ВИЯВЛЕННІ ВИДОВОГО КОМПЛЕКСУ *Ralstonia solanacearum*

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Представники видового комплексу *Ralstonia solanacearum* (RSSC) є збудниками судинного в'янення більше ніж 450 видів сільськогосподарських культур, включаючи пасльонові рослини, такі як картопля, помідори, болгарський перець, баклажани тощо. Ці фітопатогени спричиняють серйозні втрати врожаю переважно пасльонових культур, які вирощують у регіонах світу з тропічним, субтропічним та помірним кліматом. Втрати врожаю коливаються від 80 до 100% для картоплі, до 91% для помідорів, 10–30% для тютюну, 33–90% для бананів, знижуючи продуктивність і врожайність зазначених культур. ПЛР є специфічним, чутливим та економічно ефективним методом для виявлення та ідентифікації представників RSSC.

Мета. Порівняння специфічності стандартно використовуваної суміші праймерів для виявлення представників RSSC методом ПЛР з парами новостворених видоспецифічних праймерів для зручності використання в діагностичній лабораторії.

Матеріали та методи. Консервативні геномні ділянки послідовностей 16S рРНК *R. solanacearum*, *R. pseudosolanacearum* і *R. syzygii* було відібрано для створення праймерів. Видоспецифічність новостворених праймерів перевіряли за допомогою ПЛР з використанням ДНК двох цільових і 12 нецільових штамів бактерій.

Результати. Три пари новостворених праймерів Rs-28(F)/Rs-193(R), Rs-28(F)/OLI-160(R), Rs28 (F)/OlI248(R) продукували специфічні фрагменти для штамів бактерій *Ralstonia solanacearum*: 166 пн, 132 пн і 220 пн. Жодних продуктів ПЛР не було отримано під час ампліфікації з негативним контролем або нецільовими матрицями ДНК з інших видів бактерій.

Висновок. Створені праймери можна використовувати для розроблення ПЛР тест-системи з метою якісного та кількісного виявлення представників RSSC.

Ключові слова: ПЛР, *Ralstonia solanacearum*, RSSC, бактерійне в'янення.