Study of the microbiome associated with stem crown galls of grapes

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Abstract. The work investigated the species composition of the bacterial microbiome associated with stem crown galls on the grape variety Kishmish radiant (Cardinal x Kishmish pink). The result of the identification of bacterial isolates using MALDI-TOF mass spectrometry, molecular genetic analysis, and the cultural method on nutrient media made it possible to identify them in stem crown galls of grapes is presented. The following microorganisms were identified: *Pseudomonas koreensis, Roaultella planticola, Enterobacter cloacae, Pseudomonas fulva, Xanthomonas bonasiae, Winslowiella toletana*. Using a biological test on carrots, the pathogenicity of two species *Xanthomonas bonasiae*, *Winslowiella toletana* was determined by the ability to form galls, while no virulence genes were detected by PCR analysis using specific primer pairs of genes.

1 Introduction

Viticulture is one of the most rapidly developing industries in the south of Russia. An important problem in ampelocenoses is contrasting climatic conditions, which leads to damage and a decrease in the general immunity of plants, and as a consequence, to the development of serious diseases, including bacterial cancer. One of the main signs of bacterial canker on the grapevine is crown galls. The presence of bacterial cancer sharply reduces the yield; after flowering, inflorescences fall off, lungs fall and the death of the plant is observed [1].

It is currently known that bacterial canker of grapes is initiated by bacterial microorganisms containing oncogenic Ti or Ri plasmids in their cells [1]. In this case, in relation to grapes, the most important are virulent strains of microorganisms containing a tumor-inducing plasmid (Ti), which ensures the transfer of a DNA carrier (T-DNA) into the plant genome, the process is accompanied by the virulence genes (Vir) of the plasmid (Ti) [2]. Genes located on T-DNA express enzymes responsible for the production of opine and plant hormones [3]. Opines are a source of nutrients not only for virulent microorganisms, but also for other types of bacteria that produce enzymes that metabolize opine [4]. Altered levels of auxins and cytokinins at the site of transformation cause uncontrolled cell division and ultimately the development of a crown gall. The main carriers of the oncogenic plasmid are bacteria of the genus *Agrobacterium*, but after the reclassification, other taxa were

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identified, for example, *Allorhizobium* with the type species *A. vitis* (Ophel and Kerr 1990) Mousavi et al. 2016 [5]. At the same time, a number of works [6] highlight the mechanisms of interaction of genetic material between bacteria and plant cells without direct transformation, but ensuring the expression of microorganism genes inside the plant cell. Such functional diversity within a microbial biotope does not exclude various types of ecological interaction, both direct and indirect, including antagonism towards oncogenic species or synergism of microorganisms during the formation of a crown gall [7]. In this regard, it is relevant to determine the composition of the microbial community associated with crown galls of grapes.

2 Materials and methods

Phytopathological and molecular biological studies were carried out in 2024 in the laboratory of phytopathology of the biotechnology center of the Kuban State Agrarian University named after I. T. Trubilin. The selection of cuttings with symptoms of bacterial cancer was carried out in February in the Temryuk district of the Krasnodar Territory, Russia. The localization coordinates of the studied ampelocenosis are 45°11'45"N, 37°01'05"E. (geographic coordinates are given in WGS-84 format). According to climatic characteristics, the surveyed territory belongs to the zone of temperate continental climate with high humidity. The object of research was the microbiome associated with stem crown galls of grapes, isolated from samples of the Kishmish radiant variety (Cardinal x Kishmish pink). Isolates of bacterial microorganisms were isolated by cultural methods using Roy and Sasser nutrient medium [8]. The plant material was thoroughly washed with running water, then prepared with sterile water, and the vine was segmented into fragments about four millimeters in size. A sample of the prepared plant material weighing about one hundred grams was transferred to a glass container and filled with sterile physiological solution until the water level above the sample reached about one centimeter. After one day of exposure at 4°C, the suspension was inoculated onto RSM medium. After 6-7 days of thermostatting, subcultivation of the grown bacterial colonies was carried out in test tubes with slanted potato dextrose agar.

Species identification of microorganisms was carried out based on analysis of the cell proteome using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS). Pure cultures of the bacterial mass were applied to a steel target plate using a plastic sticker and dried in air. Then, 1 µl of formic acid with a mass fraction of 70% was applied to the sample for protein extraction, and then 1 µl of a solution of 50% acetonitrile (Sigma-Aldrich, Poland) containing 10 mg/ml HCCA (a-cyano-4-hydroxycinnamic acid , Sigma-Aldrich, Poland) and 2.5% trifluoroacetic acid (TFA, Sigma-Aldrich, Poland). Then, for crystallization, they were kept in air at room temperature. Mass spectra of characteristic ribosomal proteins of the samples were obtained on a BactoSCREEN MALDI-TOF mass spectrometer (Litekh, Russian Federation).

Daily bacterial cultures were used to identify pathogenicity genes using PCR analysis. DNA was isolated from plant tissue and pure cultures of microorganisms by extraction using a solution of lysis buffer based on cetyltrimethylammonium bromide (CTAB) [9]. Amplification of target products was carried out with specific primer pairs for the virulence genes VirD2A (5'-ATGCCCGATCGAGCTCAAGT) and VirD2C (5'-TCGTCTGGCTGACTTTCGTCATAA), amplifying a 224 bp fragment. plasmids virD2 of the biological genus *Agrobacterium* spp. To synthesize the VirE2 gene of the genus *Agrobacterium* sp., but not *A. vitis*, containing a vitamin-type Ti-plasmid, primers VirE2PF (5'-CGTGCTGCCGTCTCTACA) and VirE2PR (5'-ACTGAACGCGATCCCACA), amplifying a fragment of 753 bp, were used. [10].

To clarify the species identity of the isolated bacteria, DNA sequencing was performed using the Sanger method using primer pairs specific to the 16S rRNA sequence of about 1.5 kb in size: 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-CCGTCAATTCMTTTRAGTTT-3'. PCR amplification conditions: 25 µl of the reaction mixture contained 2.5 µl of 10X Tersus Plus buffer, 0.5 mM forward primer, 0.5 mM reverse primer, 50X dNTP mixture 0.5 µl, 0.7 mg/ml bovine serum albumin (Merck, Germany), 50X Tersus polymerase 0.5 µl (Evrogen, Russia), 100 ng sample DNA, and sterile distilled water. Amplification was carried out in the following modes: 1 minute at 95 ºC, 32 cycles of 30 seconds at 95 ºC, 30 seconds at 60 ºC, and 90 seconds at 72 ºC, final extension 10 min at 72 ºC [11]. To amplify a genome fragment at 16S rRNA, the following mode was used: 1 minute at 95 ºC, 32 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC, and 1.5 min at 72 ºC, final elongation 10 min at 72 ºC. DNA sequencing was performed by capillary gel electrophoresis on an Applied Biosystems 3130 instrument (Applied Biosystems, USA), using the BrilliantDye™ Terminator (v3.1) Cycle Sequencing Kit (NimaGen, Holland).

De novo gene assemblies and alignment to the reference sequence were performed using the UGENE program (Unipro, Russia). Statistical data analysis was carried out in the R environment using the Bioconductor package [12].

The ability of bacterial strains to cause the formation of galls on host tissues was assessed on carrot root discs (*Daucus carota subsp. sativus*) using standard methods 3-4 weeks after inoculation with 10^8 CFU/ml bacterial suspension or distilled water as a negative control [13].

3 Results and Discussion

As a result of the research, 120 grapevine samples with signs of bacterial cancer were studied. Morphologically, the examined galls with a diameter of about 5–7 cm on a section were living, overgrown xylem cells with a degraded core. The material was taken from various parts of the vine (Figure 1). Thus, galls were located both at the base of the trunk and on the perennial woody tissue of the plant's arms. However, callus formations were not observed on annual growth vines. The condition of leaves and shoots was satisfactory, but growth inhibition was observed. No other deviations were observed; the size of internodes and the number of shoots corresponded to the varietal description.

Fig. 1. Crown galls on the stems of the grape variety "Kishmish radiant" from the middle part of the vine (left) and on the internode (right).

When analyzing pure cultures of bacterial isolates obtained by the cultural method, all morphologically and tinctorially different types of bacterial colonies were selected for the working collection. The total number of isolated bacteria associated with crown galls of

grapes on the stems of the Kishmish radiant variety was 36 isolates. The study of the morphological characteristics of the colonies made it possible to note that most of the colonies were round in shape, small in diameter (dew-colored), glossy, with a smooth edge. About 66% of the colonies were pigmented, 62% of them were white, 23% were yellow, 15% were orange or pink. The highest frequency of occurrence was in strains with opaque colonies. Convex, flat and folded colonies were noted, the center was characterized as depressed or raised. The cell morphology was consistent with rod-shaped bacterial microorganisms.

When studying the taxonomic composition of the bacterial biome of grape crown galls, identification using MALDI TOF MS was used for mass screening of microorganisms. However, due to the lack of characteristic proteomic mass spectra in the reference database of the instrument used, not all strains were identified. The taxonomic position of 36 strains of isolated bacteria was established with varying degrees of certainty (Table 1).

Table 1. Identification by MALDI-TOF of bacterial isolates isolated from galls of the Kishmish radiant grape variety.

Taxon name	Identification accuracy using MALDI-TOF
Enterobacter cloacae	Identified down to the species
Pseudomonas fulva	
Pseudomonas koreensis	
Roaultella planticola	
Bacillus atrophaeus	Identified to the exact genus
Boudabassia marimamarum	
Erwinia billingiae	
Microbacterium phyllosphaerae	
Paenibacillus peoriae	
Paenibacillus polymyxa	
Roaultella omnitinolytica	
Bacillus sp.	Estimated generic names have been established
Erwinia sp.	
Paenibacillus sp.	
Pantoea sp.	
Peptoniphilus sp.	
Pseudomonas sp.	
Rhodococcus sp.	
Shaalia sp.	

Thus, the stem galls of the studied grape plants contained at least ten biological genera of bacteria. Strains belonging to five biological genera were identified with accuracy to the species: *Enterobacter cloacae* (Jordan 1890) Hormaeche and Edwards 1960, *Pseudomonas koreensis* Kwon, et al. 2003, *Pseudomonas fulva* Iizuka and Komagata 1963, *Roaultella planticola* (Bagley et al. 1982) Drancourt et al. 2001, *Xanthomonas bonasiae* Mafakheri et al. 2022, *Winslowiella toletana*. (Rojas et al. 2004) Brady et al. 2023 Only some of them are described in the literature as phytopathogenic species (*X. bonasiae, W. toletana*) [14, 15]. Sequencing of 16S fragments of these isolates confirmed their species identity.

To determine virulent strains containing the oncogenic Ti-plasmid of the octopine type, primer pairs were used for the VirD2 virulence gene and the VirE2 plant single-stranded DNA binding gene. *Agrobacterium tumefaciens* VKPM B-2271 was used as a reference strain. Electrophoretic analysis of amplification products for virulence genes showed that most of the isolated microorganisms do not contain the pathogenic plasmid with the VirD2/VirE2 virulence genes, specific for phytopathogenic strains of *Agrobacterium* sp.

(positive control) infecting plants. When amplifying total DNA isolated from plant tissues, no amplification products were detected for the target gene fragments. However, some cultures that had morphological and cultural characteristics similar to bacteria of the genus *Rhizobium* and *Xanthomonas* had amplification products of the VirD2 gene, from 224 to 250 bp.

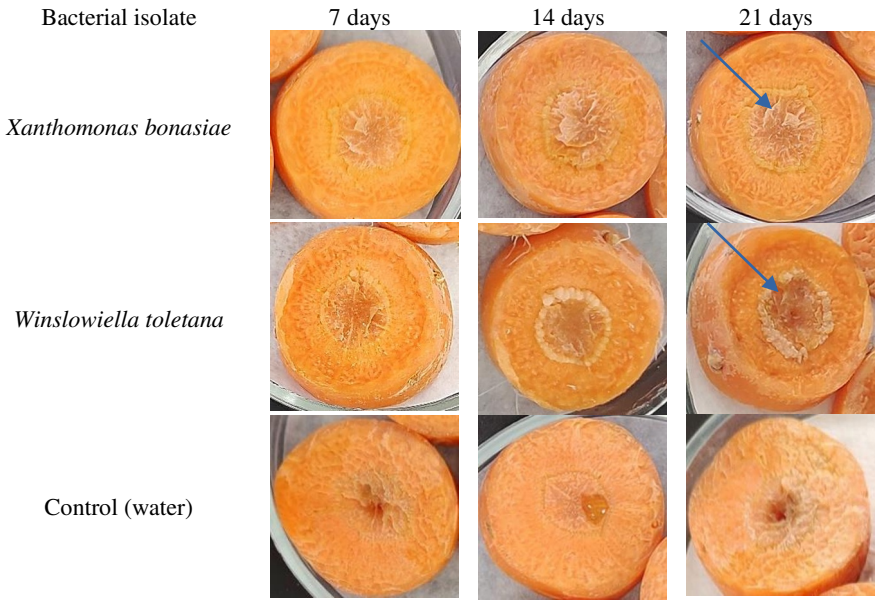

Fig. 2. Cell proliferation on carrot discs under the influence of *Xanthomonas bonasiae* and *Winslowiella toletana* cultures.

Isolates of *W. toletana* and *X. bonasiae* identified by MALDI-TOF mass spectrometry as *Erwinia sp*. and *Rhizobium sp*., contained minor products corresponding in molecular weight to the VirD2 gene (≈224 bp) and did not contain fragments of VirE2 amplification products. This may be due to the presence of either chimeric or mutant genes in these bacteria. Analysis of 16S rRNA gene amplification products in the studied objects showed genetic affinity with reference strains from the NCBI database = *W. toletana* - 96.5%, *X. bonasiae* - 93%. At the same time, the ability of bacterial isolates to cause the induction of plant cell proliferation was successfully proven on carrot disks for these two microorganisms. The formation of callus cells under humid chamber conditions occurred 7-10 days after inoculation of plant discs with the culture of the corresponding isolates (Figure 2).

Our results of the distribution of bacterial taxa in the microbiome associated with stem crown galls of grapes confirm the results of other studies. Previously, these strains were isolated from crown galls of plants of other crops. *X. bonasiae* was first isolated and described as a bacterium associated with the crown galls of *Amaranthus* sp. and *Ficus benjamina* in Iran [14]. W. toletana was first described as associated with canker wounds on sycamore (*Platanus x acerifolia*) and common linden (*Tilia x europaea*) [15]. These microorganisms were isolated and identified as separate species recently, and the reasons for the properties of these bacteria to form callus cells are not yet clear.

Strains of *X. bonasiae* and *W. toletana* were deposited in the Collection of Eubiotic and Epiphytic Microorganisms of the Kuban State Agrarian University named after I. T. Trubilin under numbers CEEM B-110 and CEEM B-126, respectively.

The results obtained indicate that the presence of a plasmid with the VirD/VirE genes is not a prerequisite for the initiation of plant cell proliferation. Moreover, the most pronounced formation of galls was observed during inoculation with *W. toletana*. Unlike *Agrobacterium vitis* (syn. *Allorhizobium vitis*), the microorganisms *W. toletana* and *X. bonasiae* existed for a long time in galls, including in growths 3 or more years old. While *A. vitis* was not detected by PCR analysis in formations for more than one year. It is also known that bacteria of the genus *Pseudomonas* are involved in the suppression of gall formation. Thus, the bacterial community associated with galls may play an important role in the development of plant diseases caused by *Agrobacterium* sp., both through stimulation of the oncogenic process and through its inhibition.

4 Conclusion

During the analysis of 120 grape plants affected by bacterial canker, 36 bacterial strains from ten genera were identified. Five species were identified with accuracy to the species: Pseudomonas koreensis, *Roaultella planticola, Enterobacter cloacae, Pseudomonas fulva. The species Xanthomonas bonasiae, Winslowiella toletana* were identified to species level by 16S rRNA sequencing. Using a biological test on carrots, the pathogenicity of two species *Xanthomonas bonasiae* and *Winslowiella toletana* was determined by the ability to form galls, while no virulence genes were detected by PCR analysis using specific primer pairs of genes.

Microbial communities within crown galls can be diverse. It is important to identify microorganisms that initiate the formation of galls and endophytic organisms that do not participate in the formation of galls. Thus, studying the microbial communities of crown galls will determine the relationships between microorganisms and their influence on the development of bacterial cancer.

References

1. S. Gelvin, Microbiol, Mol. Biol. Rev, **67**, 16-37 (2003)
2. L. Otten, P. Ruffray, E. Momol, M. Momol, T. Burr, Mol Plant Microbe Interact, **9**, 782–786 (1996)
3. T. Burr, L. Otten, Annu Rev Phytopathol, **37**, 53– 80 (1999)
4. C. Bell, L. Moore, M. Canfield, Appl Environ Microbiol, **56**, 2834 –2839 (1990)
5. A.Oren, G. Garrity, Int J Syst Evol Microbiol, **66**, 4299-4305 (2016)
6. C.E. Alvarez-Martinez, G.G. Sgro, G.G. Araujo, M.N. Paiva, B. Matsuyama, C.R. Guzzo, M.O. Andrade, C.S. Farah, Computational and Structural Biotechnology Journal, **19**, 279-302 (2021)
7. T. Hosni, C. Moretti, G. Devescovi, Z.R. Suarez-Moreno, The ISME J, **5**, **12**, 1857–1870 (2011)
8. M. Roy, M. Sasser, Phytopathology, **73**, 810 (1983)
9. S.O. Rogers, A. J. Bendich, Plant molecular biology, **5**, 69-76 (1985)
10. I. Genov, I. Atanassov, I. Tsvetkov, A. Atanassov, Vitis, **45**, 97-101 (2006)
11. J.W. Jung, Tuberculosis and respiratory diseases, **79**, 165 (2016)
12. E. Frichot, O. Francois, "LEA: an R package for Landscape and Ecological Association studies." Methods in Ecology and Evolution, http://membres-timc.imag.fr/Olivier.Francois/lea.html

13. M. Bouri, H. Gharsa, L. Vial, C. Lavire, B. Glick, A. Rhouma, Phytopathologia Mediterranea, **56**, 1 (2011)
14. H. Mafakheri, S. Taghavi, S. Zarei, P. Portier, I. Dimkić, R. Koebnik, N. Kuzmanovic, E. Osdaghi, Int J Syst Evol Microbiol 72 (2022)
15. C. Brady, S. Kaur, B. Crampton, D. Maddock, D. Arnold, S. Denman, Front Microbiol. 13 (2022)