Species of the genus Xanthomonas infecting cereals and oilseeds in the Russian Federation and its diagnostics

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Abstract. Plant pathogenic bacteria of the genus *Xanthomonas* display high levels of genetic diversity and cause remarkable damage to about 400 plant species. In 2001–2008, a new group of strains of *Xanthomonas arboricola* has been found as pathogens on novel host plants such as wheat, rye, barley, tomato, sunflower, and brassicas in Russia. Physiological tests and multilocus sequence typing (MLST) analysis confirmed their position within the *Xanthomonas arboricola* species. The obtained draft genome sequence of *Xanthomonas arboricola* strain 3004 from barley plants, also virulent to sunflower, brassicas, and chestnut, has demonstrated an evidence for the lateral gene transfer (LGT) of the virulence genes. It can be suggested that the *virE* and other genes of T4SS, obtained due to LGT, may contribute to the host range extension. Thus, T4SS genes can be used as the target for group-specific PCR analysis of this emerging pathogen of cereals and oilseeds. We propose to use *virB3*, *virB4*, and *virB9* genes to design a detection system.

1 Introduction

The genus *Xanthomonas* is composed almost entirely of phytopathogenic species capable to infect Algae, Pteridophytes (ferns), Dicotyledonous and Monocotyledonous plants. There are many economically valuable crops (over 400 species) among the host plants [6]. Representatives of the genus are able to reduce both the quality and quantity of the crop yield. Due to the host specialization, *Xanthomonas* strains are a good model to investigate the mechanisms of host plant-pathogen virulence [11]. It was proved that the host specialization of the existing pathovars within *Xanthomonas* species occurred over the past few centuries, concomitant with the intensification of agriculture [13]. *Xanthomonas* causing leaf streak (CLS) or black chaff on cereals, and vascular (e.g. black rot) or leaf spot diseases on different oilseeds and greens, are the most harmful pathogens in Russia [10]. Cereals and oilseeds are among the main economically important crops.

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Previously, we have found isolates of *X. arboricola* capable to infect cereals (fam. *Poaceae*), tomatoes (*Solanum* spp.), brassicas oilseeds (*Brassica napus*, *B. rapa*), and sunflower (*Helianthus annum*) in Russia [8]. The species *X. arboricola* is comprised of ten pathovars causing bacterial diseases of stone fruit and pomaceous fruit trees, walnut, hazelnut trees, poplar, banana, strawberry, and euphorbia [15]. Since new pathogens were able to extend the host range and retain the ability to infect initial host plants (e.g. walnut), they are further referred to as "atypical strains" or "atypical pathovars".

Strains of *X. arboricola* cause symptoms similar to *X. translucens* and *X. campestris* in the respective host plants and this feature significantly complicates their diagnostics. On the cereal leaves, the water-soaked stretched light green spots appear 10-20 days after inoculation, depending upon the average air temperature. Blackening of the upper part of spikelet scales and lateral stripes along the spikelet scales is observed on spikes. In sunflower, the pathogen causes leaf blight as well as leaf and stem spots. In oilseed rape, the pathogen causes wilting, V-shaped yellow or brown lesions and darkening of the veins. Later, the severely damaged leaves dry up or fall off.

X. arboricola is a common pathogen across the world. In Russia, 4 out of 10 pathovars were isolated: *X. arboricola* pv. *pruni* (bacterial spot of stone fruits and almond), *X. arboricola* pv. *corylina* (bacterial blight of hazelnut), *X. arboricola* pv. *populi* (bacterial canker of poplar), and *X. arboricola* pv. *juglandis* (bacterial walnut blight). However, their identity was not confirmed by molecular genetic approaches and genetic diversity was not investigated. The atypical strains capable to infect cereals, oilseeds, and other crops were isolated in Central Black Earth Region, Krasnodar and Stavropol Krai (region), Rostov, Saratov, Moscow Regions, Republics Adygea and North Ossetia-Alania [8,10].

Seeds are the most important source of primary inoculum for *Xanthomonas* pathogens, among rainwater, contaminated soil, insect pest, and equipment [17]. *X. arboricola* was the most frequently found pathogenic species on the seeds of cereals in Russia — 22 seed lots of 181 tested ones were infected by this pathogen. Rice seeds had in average 7.1% of *X. arboricola* infection, wheat, barley and rye – 29.1%. Other xanthomonads together infected 16.9% of rice seed lots and 58% seed lots for other cereals, including species that have not been reported as *Poaceae* pathogens: *X. campestris*, *X. cynarae*, *X. hortorum*, *X. pisi*, *X. gardeneri*, and *X. vesicatoria* [5].

The rapid and precise diagnostics of ethe seed infection is the main approach used to reduce crop losses caused by the bacterial pathogens. Traditional methods of detection of phytopathogenic bacteria in seeds include the plate-washing assay either on selective or semi-selective agar media, but there was no reported methods for the isolation of *X. arboricola* from cereals and oilseeds.

2 Materials and Methods

During the examination of the seed lot, we selected seeds with healthy appearance and those with visual symptoms of the disease: shrivelled, underdeveloped, grey, with withered yellowish exudate. Isolated bacteria were cultured on YDC medium at 27 °C for 48 hours. Single colonies of bacteria similar to xanthomonads were sub-cultured and stored in 15% glycerol at –70 °C. Identification of the isolated strains was done by PCR with genus-specific primers and MLST as described elsewhere [7, 18]. All the xanthomonads were tested for hypersensitive reactions (HR) on leaves of tobacco, and the positive ones were selected for pathogenicity tests. The tests of pathogenicity for HR-positive strains were conducted on plants of families *Poaceae*, *Brassicaceae*, and *Compositae*.

3 Results

3.1 Plating assay

We have developed the following protocol of *X. arboricola* isolation from seeds [4, 5]:

(1) Sterilize seeds in 0.5% sodium hypochlorite (10% commercial bleach solution) for 2 min; (2) Wash seeds 3 times in sterile tap water; (3) Transfer seeds into PBS+Tween 20 solution (750 ml of 1x PBS with 0.01% Tween 20 for 500g of seeds); (4) Chill and incubate at +4°C for 24 hours; (5) Place the seeds on the shaker for 30 minutes and 210 rpm; (6) Homogenize for 2 minutes at a rate of 8; (7) Filter the homogenate through the bacteriological filter and transferred sediment to the nutrient medium after 10-fold serial dilution. Application of succinate-quinate (SQ) selective medium (succinic acid - 10g/l, quinic acid – 5g/l, K_2HPO_4 – 1,5g/l, $(NH_4)_2SO_4$ – 1g/l, yeast extract - 0,5g/l) is a conventional method for the detection of *X. arboricola*. On the SQ medium, bacterial colonies are smooth, oleiferous, sticky, and have smooth edges. Young bacterial colonies are yellow-pigmented. As they age, bacteria start to produce a melanin-like pigment. YPGA (yeast extract - 5g/l, peptone - 5g/l, glucose – 10g/l, agar-agar – 12g/l) and YDC (glucose – 10g/l, $CaCO_3$ – 20g/l, yeast extract - 10g/l, agar-agar – 18g/l) media with antibiotics also are used for the bacterial colonies morphology analysis. Bacteria *X. arboricola* form bright yellow convex colonies on both YPGA and YDC agar. Morphologically typical colonies should be sub-cultured, purified, and stored. The suspected isolates must be tested for identity by PCR/IFA and for pathogenicity to the corresponding host plants.

The following scheme is used to assess pathogenicity to the host plants [5]:

(1) The plants are grown up to 3 or 5 leaves in 15-cm pots with compost under optimal light and temperature conditions in a greenhouse; (2) Each plant is inoculated by cutting the leaf tips with scissors dampened in a bacterial suspension (108 CFU/ml), the bacterial suspension is prepared from a 2-day bacterial culture on the YDC medium at 28°C; (3) The plants are incubated after inoculation at 24-28°C, depending on the plant species. The disease symptoms are evaluated within the period of 21 days. The plant stems or leaves can also be pricked with a needle passed through a pure colony or sprayed with a bacterial suspension. In the latter case, the plants are incubated for 48 hours in a humid chamber after spraying [2]. The main disadvantage of such detection method is a laborious and time-consuming process that needs high level of standardization of all operations. BIO-PCR in real time is used for an accurate and rapid detection of pathogenic bacteria.

3.2 PCR assay

To date, there are no recommended PCR diagnostic methods for phytopathogenic *X. arboricola* that affects cereals and oilseeds. The protocol for detection of *X. translucens* employs PCR system specific for the short DNA sequence located in 16S - 23S rRNA spacer flanking an alanine-tRNA gene. The primers amplify a 139-bp target fragment [2]. For detection of pathovars of *X. campestris*, a two-step PCR system was proposed. It includes a universal bacterial primer (reaction control) and primers specific to the *hrpF* gene (part of the type III secretion system (T3SS)) [1]. Other T3SS genes are used as targets for the detection of *X. arboricola* pathovars. Such test system is based on the amplification of the *hrpC-hrpD* region [14]. In most of cases the detection PCR methods for *Xanthomonas* pathogens were developed for well-studied strains with a known range of host plants. Our previous study has shown that the PCR target based on T3SS genes was unsuitable for atypical strains of *X. arboricola*. PCR analysis with several T3SS gene-specific conservative primers did not reveal any fragments of T3SS and T3SS Effector (T3E) genes in the strain 3004 as well as in other 32 strains of *X. arboricola* isolated in the

Russian Federation. Genome analysis of representative strain 3004 confirmed the lack of T3SS genes in the atypical X. arboricola strains [9].

In 2014, Egorova with colleagues [4] proposed a seed assay for X. oryzae and some strains of X. arboricola via BIO-PCR based on *gyrB* (DNA gyrase subunit β) gene sequences. However, the expected accuracy of such detection for atypical pathovars of *X.* arboricola is below 50%. It is related to the genome features of new strains of *X. arboricola*. The *gyrB* gene sequence, as well as another 5 housekeeping genes: *rpoD*, *dnaK*, *purA*, *prpC*, and *fabB*, were close to *X. campestris*, *X. euvesicatoria* and *X. oryzae* species in the nucleotide sequence. Diversity of MLST genes was affected by the adaptation, thus, it eliminates the possibility of using MLST genes as a targets for the detection of the atypical *X. arboricola* strains [12].

3.3 Bioinformatical analysis of *X. arboricola* genomes

Next Generation Sequencing (NGS) technologies expanded the number of available for analysis genomes of genus *Xanthomonas*, and modern bioinformatics algorithms allow for more quick and easy comparative analysis. We used methods of comparative genomics, such as multiple and pair-wise alignments, to identify potential target genes for detection of atypical pathovars of *X. arboricola*. Pair-wise alignment of the genome sequences of str. 3004, which is a type strain of the atypical strains and a type strain of the *X. arboricola* pv. *juglandis* (ATCC 49083=NCPPB 411) was performed using the Sequence-based comparison algorithm (RAST software). Multiple sequence alignment of the genome sequences of str. 3004 and complete and annotated genome sequencing of 13 other bacteria of the genus *Xanthomonas* were performed using the progressive Mauve algorithm (Mauve software). The pair-wise alignment revealed 249 unique nucleotide sequences against the reference genome. A detailed analysis of the found sequences showed that they have a high level of homology with *X. campestris*, *X. hortorum*, and *X. gardneri* species that may indicate a mosaic genome structure. This fact should be considered in the development of detection systems because the use of pseudo-unique genes can lead to false results.

During the analysis of the results of multiple genome alignment, we also noted the almost complete absence of unique sequences in the genome of *X. arboricola* str. 3004. Unique nucleotide sequences found during the multiple sequence alignment corresponded to the phage Xantho Xp10 NC 004902 and acriflavin resistance gene. However, we noted that regions demonstrating a low level of homology to the other genus members exist in the genome of str. 3004. The functional annotation of the nucleotide sequences of the found regions revealed that they belong to the type IV secretion genes (T4SS).

T4SS genes of *Xanthomonas* have a limited level of homology within both the genus and the species. In addition, the T4SS genes also have the required level of conservatism. This is explained by the fact that mutations in the key genes such as *virD4*, *virB8*, *virB9*, *virB10*, and *virB11* can completely disrupt the functions of the secretion machine [16]. We are exploring the possibility of using them for the detection of other members of the genus *Xanthomonas*. T4SS cluster of str. 3004 consists of 10 *virB* (*B1-B6*, *B8-B11*) genes and a *virD4* gene. We estimated the level of genetic variability of the proteins to assess the possibility of using the genes as targets for the detection system. During the analysis, genes with a relatively high level of variability, highly conservative genes, as well as the *virB6* gene, which is a multi-copy gene, were excluded from the target genes. We have used *virB3*, *virB4*, and *virB9* genes to design a novel detection system for the atypical strains. Now we are testing the developed detection systems for atypical strains of *X. arboricola* in seeds and in the affected plants.

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