Bacterial Diseases of Wheat Caused by *Xanthomonas* sp. in the Southern Ural: Identification Issues

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The identification of pathogenic agents isolated from infected wheat seeds and plants, collected in the Southern Ural and characterized by an atypical manifestation of bacterial infection (yellow leaf tips), has been carried out by direct microbiological and molecular methods. In addition, an indirect identification by the hypersensitive reaction (HR) approach has been performed simultaneously with the pathogenicity assessment of isolates using an indicator plant *Plectranthus australis* R. Br. Isolated samples have been microbiologically identified as *Xanthomonas* sp. According to the results of the PCR identification with species-specific primers, isolated samples represent *Xanthomonas translucens*; the reliability of the obtained results is corroborated by the hypersensitive reaction approach used also to assess the pathogenicity of isolates. Winter wheat, especially cv. Pionerskaya 23, where both *X. translucens* and *Pantoea agglomerans* were found, suffered more severe symptoms than spring wheat that was only affected by strains of *X. translucens*. To improve the reliability of the primary diagnostics of a bacterial character of seed infection, the provoking of the pathogen development by temperature or pH stress is proposed.

Keywords: wheat, bacterial diseases, *Xanthomonas translucens*, *Pantoea agglomerans*, pathogenicity, hypersensitive reaction, diagnostics.

In recent years, an increase in the crop infection with both fungal and bacterial diseases is observed in Russia as well as in other countries (Ignatov, 2014). This phenomenon is based on the changes of climatic conditions, failures in the technologies of cultivation, harvesting and storage of seeds, and the lack of timely and accurate plant pathogen diagnostics. A great variety of phytopathogenic bacterial strains and isolates makes their identification difficult for researchers.

In spite of a significant harm by bacterial diseases, the majority of farmers are unaware of the real origin of the reduced plant productivity, since the symptoms of bacterial infection are similar to those of other infectious diseases or abiotic stress factors. Never-

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theless, visual diagnosis still remains to be the primary action in the course of monitoring surveys, because it provides a quite accurate diagnostics of a bacterial infection based on revealed symptoms, especially when they are of a specific nature. According to our earlier studies, bacterial diseases of wheat in the steppe zone of the Southern Ural are caused mainly by bacteria from the genus *Xanthomonas* (Glinushkin, 2013).

Sometimes it is impossible to say that plants or soil are infected by plant pathogenic bacteria based only on the manifestation of infection on plants or on the appearance of bacterial colonies cultivated on artificial medium (Kharchenko, 2012). For example, the study of winter wheat samples, collected in the Moscow, Ryazan and Lipetsk regions, performed at the All-Russian Research Institute of Phytopathology, showed that, despite the presence of the corresponding symptoms, only 25% of analyzed samples were really infected with phytopathogenic bacteria (Ignatov et al., 2012; Egorova et al., 2014). According to the above-mentioned authors, in addition to pathogenic *Pseudomonas syringae* and *P. marginalis*, which infect many cultivated and wild plants, some nonpathogenic *Pseudomonas*, such as *P. fluorescens*, are also present in soil and on plant roots, stems and leaves and often antagonize phytopathogens. The indirect evaluation of bacterial infection by the level of deterioration of physical properties of flour or by a visual evaluation of a slimy seed fraction during germination also does not able to provide the exact and correct picture, and, therefore, are also unsuitable for correct diagnosis.

Initially, plant pathogenic bacteria were identified by microscopy or microbiological methods with the confirmation of their pathogenicity by Koch's postulates. Since the beginning of the 21st century the methods used to identify these bacteria have significantly changed, and the diagnostics of pathogenic objects and their infectious cycles became more precise and sensitive (Schaad et al., 2003). New highly-sensitive methods, such as the serologic identification with species-specific bacterial proteins (ELISA and immunofluorescence assay) and PCR, became common. The pathogenicity of isolates is now evaluated by testing their ability to evoke an HR on indicator plants. In recent years, DNA sequencing of bacteria is also used for identification purposes.

During earlier field surveys we noted that the harmfulness and level of development of bacterial diseases increased under extreme environmental conditions. Therefore, we supposed that the reliability of the primary diagnostics of bacterial infection can be improved via the artificial modeling of stress situations providing stronger infection of plants with bacteria as facultative parasites. As a rule, such stress situations are provided by the use of heavy metal salts or by salinization (Prasada, et al., 2005). However, in many small regional institutions and organizations, researchers do not have reagents required for such experiments. Therefore, the need in more simple methods exists.

The purpose of this study was the identification of pathogens causing bacterial diseases of wheat in crops and seed material in Southern Ural and the optimization of their diagnostics via the use of temperature and pH as possible stress factors enhancing disease manifestations.

Materials and Methods

Sample collection

In the course of the study, the crops of spring and winter wheat located in the steppe zone of the Southern Ural were examined. The field survey and sample collection was performed in 2011–2012. The total number of samples was 194. The level of seed infection was assessed for the following wheat cultivars:

– winter wheat: Orenburgskaya 105, Povolzhskaya 86, Pionerskaya 32, Kolos Orenburzhya;
– spring wheat: Saratovskaya 42, Albidum 188, Uchitel, Orenburgskaya 10, Orenburgskaya 21, Varyag.

The initial detection of bacterial infections was performed by several methods including the visual assessment, manifestation of infection in a moist chamber, seed germination in filter paper rolls with the further counting of infected seeds, and agro-ecological monitoring. Then the infected material was studied by common microscopic and microbiological methods used in plant pathology.

Isolation of bacteria

Seeds or fragments of plants with obvious signs of infection on leaf tips (Fig. 1) were placed into gauze pouches and soaked in 0.5% potassium permanganate for 3-5 min. Then pouches were placed into tap water for 5 min, then placed for 3 min between filter paper sheets to remove the excess of water; this procedure was repeated three times, but the duration of the second and third soaking in water was 1-2 min. Finally, surface-sterilized samples were taken out of pouches, placed on starvation agar (1% agar without any supplements), and then single bacterial colonies were obtained from the resulted bacterial mass by serial dilution method using standard semi-selective peptone-sucrose agar (PSA) and yeast-dextrose-calcium carbonate agar (YDS) media (Hayard, 1960; Vidaver, 1989).

Morphological and microbiological identification

The morphological, cultural, and pathogenic properties of obtained colonies suspected of being *Xanthomonas* were studied comparing to well-known reference *Xanthomonas translucens* pv. *undulosa* strain 926 from the State Collection of Phytopathogenic Microorganisms (All-Russian Research Institute of Phytopathology). Gram staining was done as described by Schaad et al. (2001). The morpho- and cytometry, as well as the determination of cell sizes and capsule formation of bacteria were carried out by the morphometric analysis of smears of 2–9-day-old gram-stained cultures and also by microscopic examination of bacteria, directly isolated from infected seeds and plants, using a Micros MSD 500 microscope (Austria) equipped with a digital camera, eyepiece-micrometer MOU-1-15x1500 (Russia) and a Test Morpho 4.0 software. Bacterial motility was visually determined under the microscope. The titer of bacteria was determined by serial dilution followed by inoculation on the PSA medium with the further counting of grown colonies.

Fig. 1. Manifestation of bacterial infection on wheat seedlings (yellow leaf tips)

Molecular identification

The molecular identification of the pathogens from infected plant material was carried out by a PCR approach. Bacterial DNA was isolated using a modified alkaline method (Birnboim and Doly, 1979). The species-specific primers F25 (CCTACAGGCGTCCT-CACAAGTAA) and R25 (TGATCTCAAACGCTCGCTACGTC) used in this study were specially designed at the Center of Bioengineering of the Russian Academy of Sciences (Punina et al., 2008). The PCR reaction mixture (15 μl) was composed of 0.375U Taq polymerase (Fermentas), 1x Taq polymerase buffer, 0.3 mM dNTP mix, 3.3 mM $MgCl_2$, 0.7 pM of forward primer, 0.7 pM of reverse primer, and 0.5–1 μl of template DNA. PCR was performed using a Mastercycler personal thermal cycler (Eppendorf, Germany) including negative and positive control reactions. The amplification reaction was performed as follows: primary initial activation step at 95 ºC for 2 min, followed by 35 cycles of 92 ºC for 20 sec, 67 ºC for 40 sec, and 72 ºC for 45 sec, followed by final extension of 72 ºC for 5 min. The generated amplicons were visualized on 1.5% agarose gel electrophoresis stained with ethidium bromide and illuminated with a UV transilluminator.

Indirect identification and pathogenicity evaluation by hypersensitive reaction (HR) approach

An additional indirect identification of bacteria isolated from infected seed and plant samples was carried out for a subset of 32 samples using an indicator plant *Plectranthus australis* R. Br. offered by researchers of the Center of Bioengineering of the Russian Academy of Sciences (see Results and Discussion section for more detailed information).

Leaves of *P. australis* were inoculated with bacterial cells at approximately 2×10^8 CFU/ml in a 0.85% NaCl solution. The pathogen was introduced into the leaf

apoplast by infiltration through the lower epidermis using a syringe with no needle fitted. Plant responses were observed 12 to 24 h after inoculation. The pathogenicity was assessed according to the development of necroses; the severity of infection was assessed by the size of resulting necroses and their development rate.

Evaluation of the temperature effect on the germination rate and manifestation of bacterial seed infection

Four replicates of 50 wheat seeds (cv. Albidum 188) were tested for their germination rate according to the International Seed Testing Association rules (ISTA, 1966). Germination in paper rolls was assessed after 7-day incubation at different temperatures within the range of 5–40 ºC. The standard temperature (25 ºC) was used as the control. The level of bacterial infection was evaluated at the end of the incubation period by the fraction of seeds with obvious manifestations of bacterial infection.

Evaluation of the pH effect on the germination rate and manifestation of bacterial seed infection

Four replicates of 50 wheat seeds (cv. Uchitel) were tested for their germination rate according to the International Seed Testing Association rules (ISTA, 1966). Germination in paper rolls was assessed after 7-day incubation at different pH within the range of 7–10. To provide the required pH values, paper rolls with seeds were moistened with distilled water (control) or distilled water mixed with 0.1 M KOH solution in different ratios. The pH values were determined by a pH meter. The level of bacterial infection was evaluated at the end of the incubation period by the fraction of seeds with obvious manifestations of bacterial infection.

Results and Discussion

Morphological and microbiological identification

The identification of the genus of bacteria isolated from typical infected seeds and plants was carried out using microbiological methods in accordance with the Koch's postulates. We could not reliably determine the species composition of pathogens; however, according to the modern approach to the classification of microorganisms, due to a high variability of microorganisms, including their pathogenic characteristics, the identification of the genus of a pathogen is sufficient (Vauterin et al., 1995).

Since the identification of the causal agents of a bacterial infection, manifested as light-yellow necroses at the tips of leaves and causing the maximum damage to wheat in the region, was the most difficult, the results of its diagnostics are described in details below.

The bacteria from the genus *Xanthomonas,* presumably *X. translucens* were isolated from the infected samples of spring and winter wheat. These bacteria dominated

not only in surveyed crops, but also during their isolation and cultivation on artificial media; they dominated not only in their number, but also in the growth and development rate of their colonies. The isolated bacteria were Gram-negative, aerobic, monotrichous, rod-shaped with rounded ends, and sporeless (but formed capsules). The size of observed bacteria varied with the prevalence of $0.1 \times 2.0\ \mu$m (Fig. 2). Some long structures with the size of $0.05 \times 0.5\ \mu$m and width-to-length ratio of 1:50 were observed. However, it was not clear either these structures represented chains of nonseparated cells, or were unusually long cells, so this question requires some additional studies.
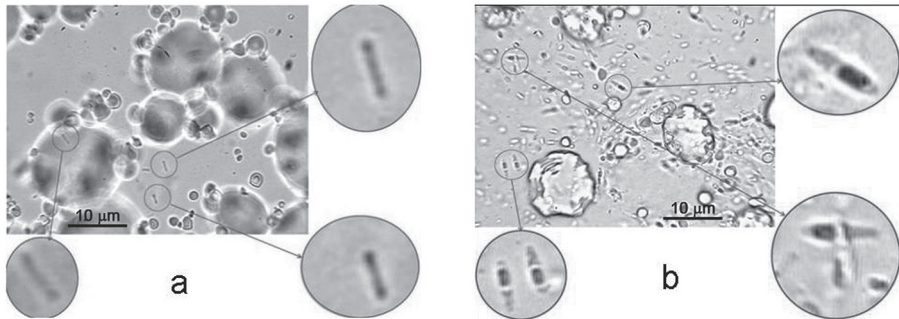

Fig. 2. *Xanthomonas translucens* bacteria in the endosperm part of spring wheat seeds: *a*, 72 h of incubation; *b*, 200 h of incubation

The grown colonies were round-shaped with the diameter up to 4–6 mm and had a smooth and sometimes slightly mucous surface. The transparency of colonies made 20–40 units, the color was whitish with a yellowish tint, and the edges were even. Based on these morphological characteristics and some biochemical data (not shown), we concluded these bacteria belonged to the *Xanthomonas* genus.

The concept of the microbiological identification method is intended to confirm the bacterial infection of sampled plants, to transfer pathogens on artificial medium, and to test their pathogenicity after their isolation from nutrition medium.

PCR identification

The microbiological identification of bacteria, isolated from plants with typical symptoms (leaf tip necroses), as *Xanthomonas* sp. and specifically *X. translucens* was confirmed by the PCR analysis (Fig. 3, Table 1). According to the PCR data, the seed material of both spring and winter wheat was infected with plant pathogenic *X. translucens*, described in a classic phytopathological literature as the causal agent of the black bacteriosis of wheat (Matveeva et al., 2006).

Table 1

Comparative identification of *Xanthomonas translucens* on winter and spring wheat by different methods and the severity of natural infection depending on the presence of *Pantoea agglomerans*

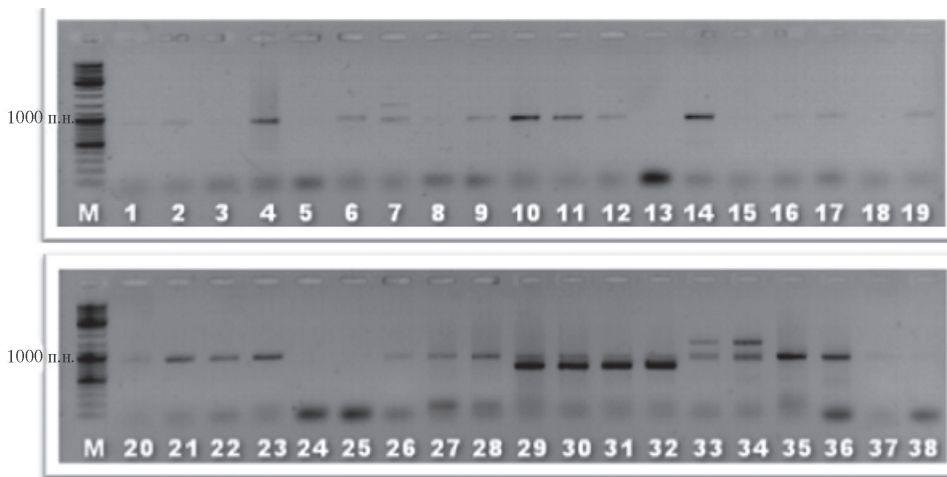| Wheat cultivar	Identification method for *Xanthomonas translucens*			Severity of infection in wheat	Presence of *Pantoea agglomerans*
	Microbiological	PCR	HR on Plectranthus		
Winter wheat					
Orenburgskaya 105	+	+	+	+++	+
Povolzhskaya	+	+−	+	+++	+
Pionerskaya	+	+	+−	++++	+
Kolos Orenburzhya	+	+	+	++	+
Spring wheat					
Uchitel	+	+	+	+++	+
Varyag	+	n/d*	n/d*	+	−
Orenburgskaya 10	+	n/d	n/d	+	−
Orenburgskaya 21	+	n/d	n/d	+	−

* not determined.

Fig. 3. Electrophoresis of PCR products obtained by the use of species-specific F25/R25 primers for identification of *Xanthomonas translucens* in wheat samples. M, molecular weight marker (Fermentas). Lanes 1–17 and 20–36: samples collected from winter wheat cvs. Orenburgskaya 105 (lanes 1–2, 5–8, 15–16, 17–20), Povolzhskaya 86 (lanes 3–4); Pionerskaya 32 (lanes 9–10, 21–22) Kolos Orenburzhya (lanes 13–14, 23–24), spring wheat cv. Uchitel (lanes 25–32, 35–36), oats cv. Skakun (lanes 11–12), barley cv. Anna (lines 33–34). Lanes 19 and 37: positive control (DNA of the reference *X. translucens* pv. *undulosa* strain 926). Lanes 18 and 38: negative control (no sample DNA)

Pathogenicity assessment and indirect identification

The pathogenicity of *X. translucens* isolates was tested by a hypersensitivity reaction (HR) approach using the indicator plant *Plectranthus australis*. According to the studies of Rogachev (1986) and Samokhvalov et al. (1986), who tested 10 potential indicator plants for their HR towards *Xanthomonas campestris, P. australis* was characterized by a clear HR towards this pathogen, and its sensitivity significantly exceeded that of other indicator plants; moreover, *P. australis* was much more sensitive to *X. campestris* comparing to some other pathogenic bacteria, such as *Pseudomonas* spp. (Samokhvalov et al., 1986). The testing of different strains of *X. campestris* showed a good correspondence between their pathogenicity towards the host plant, determined by the classical seed soaking method, and the corresponding HR of *P. australis* (Rogachev, 1986; Table 2). The sensitivity of the method was 10^4 CFU/ml. Based on these results, authors proposed *P. australis* as an indicator plant specific to *Xanthomonas* bacteria.

Next day after artificial inoculation, round brownish necroses were observed on the bottom side of inoculated leaves (Fig. 4). A wide HR variation was observed among the isolates tested; the most intensive reaction was observed in the case of inoculum isolated from winter wheat cv. Pionerskaya 32. Note that isolates collected from spring wheat demonstrated less intensive HR than those from winter wheat (Table 1). A good correspondence between the indirect indicator method of *X. translucen*s identification and its molecular identification by PCR was observed.

Effect of co-infection with Pantoea agglomerans

In addition to *X. translucens*, another bacterium, *Pantoea agglomerans*, was co-isolated from some of collected samples. This bacterium is considered to be symbiotic and nonpathogenic, and its metabolites are considered to have some immunomodulating properties for humans (Soma et al., 2006). However, in our case, all *X. translucens* isolates, which caused an intensive HR, were isolated from samples co-infected with *P. agglomerans* (Table 1). This fact provides a reason to pay more attention to this group of non-pathogenic bacteria. Probably, the harmfulness of *P. agglomerans* is manifested in the enhancement of the negative effect of *X. translucens*. The observed phenomenon requires a more thorough investigation under laboratory conditions.

Improvement of bacterial infection detection in seeds

In the course of the monitoring of surveyed wheat fields, we noted that the harmfulness and level of development of bacterial diseases, particularly those, which were manifested in the form of yellowish leaf tip necroses, increased under extreme environmental conditions due to increased growth of bacterial mass. Thus, the modeling of stress factors under laboratory conditions would be potentially useful to enhance the disease manifestation during seed assessment and, therefore, improve the primary diagnostics of bacterial diseases. In this study, pH and temperature were assessed as stress factors to find

Table 2

Comparison of the pathogenicity of *Xanthomonas campestris* isolates determined by the hypersensitive reaction of *Plectranthus australis* and by a cabbage seed soaking method*

Isolates tested	Hypersensitive reaction	Infection of cabbage seedlings, %
1	–	0
2	–	0
3	–	0
4	–	0
5	+	5
6	+	25
7	+	100
8	+	15
Control (water)	–	0

* Cyted from Rogachev, 1986.

Fig. 4. Hypersensitive reaction of *Plectranthus australis* leaves inoculated with *Xanthomonas translucens* isolates: *a*, bottom side of a leaf; *b*, top part of a leaf

the optimum values for the maximum disease manifestation. The results of the study are shown in Table 3.

According to the obtained results, the decrease in a germination temperature down to 5 ºC from the control (25 ºC) reduced the germination rate of wheat seeds by 22.4%; in the case of the temperature increase up to 40 ºC, the corresponding decrease in the germination rate was 14.5%. Temperature changes influenced the manifestation of total bacterial infection: the level of observed seed infection at 5 ºC almost doubled, reaching 35.3%, whereas increased temperature (40 ºC) provided less significant effect, increasing the level of infection by 4.3% comparing to the control (25 ºC).

An increase in the pH value of incubation solution decreased the germination rate of wheat seeds by 11% and simultaneously increased the manifestation of bacterial infec-

Table 3

The effect of temperature and pH on the germination rate of wheat seeds and level of their bacterial infection*

Temperature effect, cv. Albidum 188			pH effect, cv. Uchitel		
Temperature, ºC	Germination rate, %	Level of infection, %	pH	Germination rate, %	Level of infection, %
5	71.0 ± 2.5	35.3 ± 6.1	Distilled water	84 ± 6	52 ± 6
10	75.0 ± 3.4	31.7 ± 5.0	7.0	87 ± 3	44 ± 3
15	82.1 ± 3.3	24.5 ± 5.5	7.5	86 ± 5	46 ± 7
20	89.5 ± 4.1	21.1 ± 2.2	8.0	86 ± 4	54 ± 5
25	93.4 ± 2.5	16.7 ± 3.4	8.5	84 ± 3	59 ± 3
30	82.7 ± 1.8	20.6 ± 2.7	9.0	73 ± 4	58 ± 3
35	80.9 ± 2.7	20.0 ± 3.3	9.5	73 ± 3	57 ± 4
40	78.9 ± 3.1	20.6 ± 2.7	10.0	73 ± 3	59 ± 2

*The data shown represent average values ± standard errors.

tion by 7% as compared with the control. The similar results were obtained for the use of 0.1 M Na_2CO_3 and mix of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ (data not shown).

Thus, the decreased temperature of incubation and increased pH value are able to stimulate the manifestation of bacterial infection in wheat seeds and, therefore, increase the reliability of a primary diagnostics of a bacterial nature of infection by the paper roll method. These approaches can be recommended for the use as an alternative to the addition of heavy metal salts to the incubation medium commonly used as an artificial stress factor inducing the development of bacterial infection.

Conclusion

Microbiological identification of pathogenic agents isolated from infected wheat seeds and plants, collected in the Southern Ural region and characterized by an atypical manifestation of bacterial infection (yellow leaf tips), confirmed their belonging to the genus *Xanthomonas*. The reliability of primary diagnostics of bacterial infection of seeds by paper roll method can be improved by simple decrease in the temperature of incubation or by pH increase which provoke the manifestation of hidden infection.

The PCR analysis of isolates using species-specific primers provided their identification as *X. translucens*; the reliability of the obtained results was comparable with those obtained by the HR approach. The pathogenicity of the studied isolates varied. Plants, especially winter wheat, from which not only *X. translucens* but also *P. agglomerans* could be isolated, showed more severe disease symptoms than spring wheat, where only *X. translucens* was found.

Acknowledgements

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