

Beneficial traits of grain-residing endophytic communities in wheat with different sensitivity to *Pseudomonas syringae*

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Deep insight into compositional and functional features of endophytic bacterial communities residing in wheat grains opens the way to the use of their plant growth promoting and biocontrol abilities in agricultural biotechnology. The aim of this work was to compare grain-residing endophytes from winter wheat varieties with different sensitivity to *Pseudomonas syringae* pv. *atrofaciens* (McCulloch) and to examine their plant-beneficial traits and antagonistic effects. Grain-residing bacteria were isolated from surface-sterilized grains of three wheat varieties sown in Ukraine following a culture-dependent protocol, and were screened for their plant growth promotion (PGP) and antagonistic properties. Bacterial morphotypes were represented by gram-negative rods, endospore-forming bacilli and gram-positive cocci. Different resistance to phytopathogenic pseudomonads was associated with distinctive quantitative and functional features of grain-residing endophytic communities. High resistance to *P. syringae* was coupled with the prevalence of gram-negative rods in the endophytic community, the highest proportion of endophytic bacteria possessing three PGP activities (phosphate solubilization, nitrogen fixation and production of indolic compounds) simultaneously, and with the most potent antagonistic activity of grain-residing endospore-forming bacilli. In total, five grain-residing isolates, which were obtained from three wheat varieties (two isolates from varieties with medium and high resistance and one – from a low-resistant variety), demonstrated ability to restrain *P. syringae* pv. *atrofaciens* (McCulloch) growth. Two isolates (P6 and P10) which were obtained from the high-resistant wheat variety Podolyanka and were assigned to *Paenibacillus* and *Brevibacillus* genera according to their biochemical profiling and MS-DS identification, showed the most potent antagonistic effects as indicated by maximum inhibition zone in agar well diffusion assay. These results shed light on the association of the features of grain-residing endophytic bacteria with wheat resistance to phytopathogenic pseudomonads. Isolates from the high-resistant wheat variety can be recommended for grain dressing as plant growth promoting and biocontrol agents for *P. syringae* pv. *atrofaciens* (McCulloch).

Keywords: basal glume rot of wheat; leaf blight; agrobiotechnology; *Bacillus*; *Paenibacillus*; *Brevibacillus*; wheat grain-residing endophyte.

Introduction

Endophytes are microorganisms (including bacteria, fungi etc.) that live asymptotically inside different plant tissues. Endophytic bacteria exert beneficial effects on their host plant by various direct and indirect mechanisms. The direct mechanisms include manufacturing of phytohormones, the solubilization of phosphate, nitrogen fixation, and uptake of iron to plant tissues (Papik et al., 2020). In addition, endophytic microorganisms can exert direct antagonistic effects to phytopathogens. The endophytes can compete with the phytopathogen for nutrients, produce inhibitory chemicals etc. (Karthikeyan et al., 2021). The indirect mechanism consists of the ability of endophytes to activate plant defence responses against phytopathogens. Thus, endophytic symbionts represent superior models for insight into plant-microbe interactions (Afzal et al., 2019; Morales-Cedeño et al., 2020). Moreover, beneficial properties of endophytic microorganisms exhibit great promises for their use in agrobiotechnology for sustainable agriculture (Maheshwari, 2017; Bezpal'ko et al., 2020). At the present time, the great majority of bacterial endophytes are non-cultivable. Study of bacterial endophytic community using culture-independent approaches is primarily intended to extend current knowledge concerning diversity of plant tissue bacterial inhabitants (Eevers et al.,

2015). Biotechnological use of endophytes involves in turn the study and isolation of cultivable microorganisms. Biotechnologies based on cultivable endophytes are gaining significance for improving soil properties and enhancing crop yield, especially in principal cereals, such as wheat, rice etc. (Rana et al., 2015). Endophytic bacteria inhabit the intercellular area of different cereal tissues (Eid et al., 2021). Currently, most scientific reports have been concentrated on the isolation and biotechnological use of rhizosphere and root endophytic bacteria while data concerning endophytes inhabiting wheat grains are scarce (Makar et al., 2021). Nevertheless, it is grain endophytes that are of particular interest in terms of their use in agrobiotechnology due to their unique properties including the capacity to be transmitted vertically between generations (Compant et al., 2020; Kuźniar et al., 2020). Wheat grains seem to be one of the most important plant organs which harbour endophytic bacteria. Grain-stored bacteria are characterized by the unique ability to reside in dormant grain and adapt to unfavourable conditions, as well representing a starting point for the establishment of endophyte communities in seedlings, influence significantly whole plant endophyte formation, and eventually, seed germination, plant growth and productivity (Herrera et al., 2016; Geisen et al., 2017; Ridout et al., 2019; Kuźniar et al., 2020).

In our previous study we revealed different sensitivity of winter wheat varieties sown in Ukraine to *P. syringae* pv. *atrofaciens* (McCulloch, 1920) and its lipopolysaccharide (LPS) *in vitro* (Pastoshchuk et al., 2018). *P. syringae* is gram-negative phytopathogenic bacterium and causal agent of a variety of bacterial spot, speck, and blight diseases on a wide range of plants, including such important cereals as rice and wheat (Lamichhane et al., 2015). The contribution of *P. syringae* to crop loss is not well understood and therefore remains largely underestimated. It has been shown that grain infestation, as well as affected weeds in wheat agrophytocenoses are very important in the epidemiology of the disease (Pasichnyk, 2016; Umesha, 2020). Isolates of *P. syringae* are taxonomically divided into pathovars, based substantially on their host. There are about 50 pathovars described for *P. syringae*. The *Pseudomonas* strains causing the wheat disease “basal glume blotch” area or “basal glume rot” are classified as *P. syringae* pv. *atrofaciens* (McCulloch) (Valencia-Botin, 2012). In many countries, the occurrence of *P. syringae* pv. *atrofaciens* has only been reported once (Argentina, Australia, New Zealand, Italy, Pakistan, Iran) or has not been published (Belgium, Ethiopia and Denmark). Therefore, yield losses have never been thoroughly estimated, and no control measures for routine application have as yet been established (Kazempour et al., 2010). In an attempt to decrease the prevalent use of chemicals for preventing and treatment of phytopathogen damage to plants, the use of plant symbiotic microorganisms, including endophytic bacteria, as bio-control agents seems to be a promising approach for eco-friendly and sustainable agriculture (Muthukumar et al., 2017).

The objectives of this pilot study were to 1) isolate and compare grain-residing endophytic communities from winter wheat varieties with different sensitivity to *P. syringae* pv. *atrofaciens* (McCulloch) and its LPS; 2) examine plant-beneficial traits and antagonistic effects of grain-residing endophytic bacteria towards *P. syringae* pv. *atrofaciens* (McCulloch) *in vitro*; 3) evaluate and compare antioxidant enzyme responses in seedling of winter wheat varieties with different sensitivity to *P. syringae* pv. *atrofaciens* (McCulloch) after the grain exposure to phytopathogen.

Materials and methods

Grains of three winter wheat (*Triticum aestivum* L.) varieties with different sensitivity to *Pseudomonas syringae* pv. *atrofaciens* (McCulloch, 1920) Young, Dye & Wilkie 1978: UKM B-1013 (Table 1) were first washed under running tap water for 15 min, then were surface disinfected using ethanol 72% (30 s), followed by 2% trichloroisocyanuric acid (4.5 min) and again, ethanol 72% (30 s), with six rinses for 15 min in sterilized distilled water using shaker (220 rpm). To test the effectiveness of grain surface sterilization, control was provided by plating 1 mL of sterile water used for the final rinse onto R2A agar (Scharlau, Spain). No microbial growth was detected on the medium after 7 days of incubation at 28 °C. This result indicated successful surface sterilization for killing or inhibiting the growth of the epiphytic bacteria. Thus, microbial isolates were considered to be true endophytes. For the counting bacterial endophyte CFU, surface sterilized (as described above) wheat grains (1 g) were transferred to a sterile mortar with 9 mL of 10 mM phosphate buffer (pH 6.5) and homogenized using a sterile pestle. A volume of 1 mL was transferred to 9 mL of 10 mM phosphate buffer. A serial dilution was made, and each dilution was plated in triplicates on: potato agar for total bacteria count; McConkie agar for Gram-negative bacteria count (HiMedia, India), MYP agar (HiMedia, India) for bacilli count. Plates were incubated at 24 °C, and examined regularly for visible bacterial growth.

Table 1
Characteristics of winter wheat cultivars used in the study

Cultivar name	Winter hardiness	Drought tolerance	Fungal diseases resistance	Resistance to <i>P. syringae</i> pv. <i>atrofaciens</i> (McCulloch) (Pastoshchuk et al., 2018)
Podolanka	high	high	medium	high (seed germination is non-affected; root growth is inhibited by 20%)
Discus	high	high	high	medium (seed germination is slightly retarded; root growth is inhibited by 38%)
Favoritka	high	medium	high	low (seed germination is inhibited by 28%; root growth – by 4 times)

For isolate characterization and identification, ten surface-sterilized grains were placed on Petri dishes containing R2A culture medium (five replicates). All Petri dishes were then incubated at 28 °C for 72 h. The term of incubation was limited by an extensive endophytic fungi growth from grains of some varieties after 72 h. Representative colonies emerging on the majority of Petri dishes were then selected and grouped according to their morphological characteristics, including margin, shape, colour, viscosity, elevation and opacity in order to select different colonies.

Several isolates were obtained from each wheat cultivar after repeated subcultures. These isolates were then characterized using standard protocols based on morphology, Gram staining, spore formation. Additionally, bacterial isolates were screened for potential plant growth promoting activities, as well as for antagonistic activity towards *P. syringae* pv. *atrofaciens* *in vitro*. A strain of *P. syringae* pv. *atrofaciens* (McCulloch) Young, Dye & Wilkie, 1978: UKM B-1013 was used for the *in vitro* assay of endophytic bacteria antagonistic activity. Phytopathogenic bacteria were grown on potato agar at 28 °C for 24–48 h. Biochemical profiling and MALDI-TOF mass spectrometry were used for the identification of microorganisms.

The screening of endophytic bacteria for indolic compounds' production was performed by cultivating in liquid medium supplemented with L-tryptophan (250 µg/mL) (HiMedia, India) at 28 °C in the dark under constant agitation of 140 rpm for 48 h. The presence of indolic compounds was estimated by adding Salkowski's reagent (Sadaf et al., 2009).

The ability of the isolates to solubilize inorganic phosphate was evaluated by observing halo formation around bacterial colonies after incubation for 7 days at 28 °C in Muromtsev agar (0.2 g/L K₂SO₄, 0.2 g/L MgSO₄·7H₂O, 10 g/L glucose, 1.0 g/L asparagine, 3.3 g/L CaCl₂, 3.8 g/L Na₃PO₄, and 15 g/L agar) supplemented with 3.0 g/L of Ca₃(PO₄)₂ (pH 6.8) (Alihani et al., 2006). Screening of oligonitrotrophic isolates was performed using the inoculation of fresh colony onto nitrogen-free Ashby's mannitol agar (Woźniak et al., 2019).

The ability of endophytic bacterial isolates to inhibit the phytopathogenic strain of *P. syringae* pv. *atrofaciens* was determined using agar well diffusion assay as described by Agarwal et al. (2020), with slight modifications. The overnight culture of phytopathogenic bacteria (OD₆₀₀ ~ 0.4) and endophytic isolates (OD₆₀₀ ~ 0.4) in PBS were prepared. Sterilized Petri dishes containing R2A agar (Scharlau, Spain) were spread inoculated with 100 µL of *P. syringae* pv. *atrofaciens* culture. A well (1 cm diameter) was made in the centre, and 60 µL of each endophytic isolate was added and incubated at 28 ± 2 °C for 24 h. A plate inoculated with sterile water was used as control. Antagonistic activity was evaluated based on the production of inhibition zone around the well.

Biochemical profiling of isolates was performed using Vitek® 2Compact (bioMérieux SA, France) according to the system manufacturer recommendations. Briefly, a pure 18–24 h bacterial culture was accumulated on appropriate nutrient media (bioMérieux, France). A bacterial suspension was adjusted to a McFarland standard of 1.8–2.2 in a Vitek Saline Solution (bioMérieux SA, France) using DensiChek Plus (bioMérieux SA, France). Analysis was done using the identification BCL cards. Data were analyzed using the Vitek 2C software version 08.01 according to the manufacturer's instructions (www.biomerieux.pl).

For the identification by Maldi-Tof mass spectrometry, a pure 18–24 h bacterial culture was accumulated on appropriate nutrient media. A portion of not more than half of the microbiological loop with a diameter of 1 mm was smeared onto the spots of Vitek MS-DS disposable target slides and then the spot was covered with 1 µL of Vitek MS-CHCA solution (bioMérieux SA, France). Spots were completely air dried. After this, the slides were placed on an adapter and inserted to Vitek MS instrument. Spectra were generated using the Myla software version 3.0.0, Myla version 4.6.1 (bioMérieux, France) and the identification was automatically done with the Vitek MS. The software compares the spectrum obtained to the expected spectrum of each organism. After this, the percent probability, a quantitative value, is calculated for each sample. The range of percent probabilities for a correct identification is from 60 to 99 with values closer to 99.9 indicating a closer match. When the obtained percent probability is under 60, then it was considered as no identification. Confidence level is determined with percent probability and number of choices (Rychert et al., 2013).

For the determination of anti-oxidant systems of wheat grains in response to phytopathogen exposure, 20 surface sterilized wheat grains were placed on moisturized filter paper in Petri dishes. 5 mL of distilled water (control) or 5 mL of phytopathogenic bacteria suspension at the concentration of 10^9 CFU/mL (treated) were then applied to the Petri dishes. Grains were germinated for 7 days in humidified atmosphere at 27 °C. Seven-day-old seedlings were then used for the determination of thiobarbituric acid reactive substances (TBARS), the antioxidant enzyme assays, and proline content. All these experiments were repeated at least three times. Total TBARS (expressed as malondialdehyde (MDA) equivalents) was examined as described by Kumar & Knowles (1993). Total Superoxide Dismutase (SOD, EC 1.15.1.1) activity was examined using a modified NBT method (Beyer et al., 1987). Catalase (CAT, EC 1.11.1.6) activity was examined according to Aebi (1984). The content of proline was examined spectrophotometrically as described by Bates et al. (1973).

All experimental results are presented as means \pm standard deviation ($x \pm SD$) of at least three independent experiments. Statistical differences were calculated using ANOVA with Tukey's post-hoc test for multiple comparisons, and a two tailed T-test for single comparisons. Differences were considered significant at $P < 0.05$.

Results

A total of thirty two endophytic bacteria isolates were obtained from dry grains of three commercial winter wheat varieties with different susceptibility to *P. syringae* pv. *atrofaciens* (Fig. 1). Ten bacterial isolates were obtained from grains of Favorytka – wheat variety with medium-to-high stress-tolerance and low resistance to *P. syringae* pv. *atrofaciens* (Table 1). Eleven isolates – from wheat variety with high stress-tolerance and medium resistance to the phytopathogen (Discus). The same number of grain-residing endophytic bacteria (eleven isolates) were obtained from Podolianka – wheat variety with medium-to-high stress-tolerance and high resistance to *P. syringae* pv. *atrofaciens* (McCulloch). Bacterial morphotypes were represented by gram-negative rods, endospore-forming bacilli and gram-positive cocci. Gram-negative rods slightly predominated in grain-residing endophytes of high-resistant wheat variety. Endospore-forming bacilli were the highly predominant morphotype in grain-residing endophytes of wheat cultivar with medium resistance to the phytopathogen. In wheat cultivars with low resistance to phytopathogenic pseudomonads, the proportion of gram-positive cocci in the grain-residing endophytic community was higher than in varieties with high and medium resistance.

The abundance of endophytic bacteria in the grains of different wheat cultivars varied from 1.7×10^2 to 2.5×10^5 CFU per g of dry weight. The highest value ($2.5 \pm 1.0 \times 10^5$) was found in grains of Discus wheat cultivar with medium phytopathogen resistance, and the lowest value ($1.7 \pm 0.5 \times 10^3$) was registered in grains of Favorytka wheat cultivar with low resistance to phytopathogenic pseudomonads.

Out of 32 bacterial isolates, 20 (62.5%) exhibited different PGP activities: 10 isolates exhibited the ability to solubilize phosphates, 10 – to grow on nitrogen-free Ashby's medium (oligonitrotrophs), and 10 – to produce indolic compounds. The highest proportion of bacteria with PGP activities was revealed in endophyte harbouring grains of highly phytopathogen-resistant wheat variety Podolianka. This grain-residing endophytic community contained members with all mentioned PGP activities. Phosphate-solubilizing gram-negative rods with the ability to produce indolic compound predominated there. PGP potential of grain-residing community from the wheat variety with medium resistance to phytopathogenic pseudomonads (Discus) was represented mainly by oligonitrotrophic endospore-forming bacilli with weak ability to produce indolic compound. Endophytes of low-resistant variety contained the lowest proportion of bacteria with PGP activities. Oligonitrotrophic indole-producing endospore-forming bacilli as well as phosphate-solubilizing gram-negative rods were present (Fig. 2). Surprisingly, gram-positive cocci from grain-residing endophytic communities in all studied winter wheat varieties exerted no PGP activities. Some of the endophyte isolates exhibited two or even three different PGP activities simultaneously (Fig. 3). It is necessary to point out that the highest proportion of grain-residing bacterial isolates exhibiting all three PGP activities was registered in the wheat variety with

highest resistance to phytopathogenic pseudomonads (Fig. 3c). At the same time, oligonitrotrophs without any additional PGP activity were absent in this endophytic community. A distinctive feature of the endophyte of the wheat variety with low resistance to the phytopathogen (Fig. 3a) was increased content of bacteria producing indolic compounds.

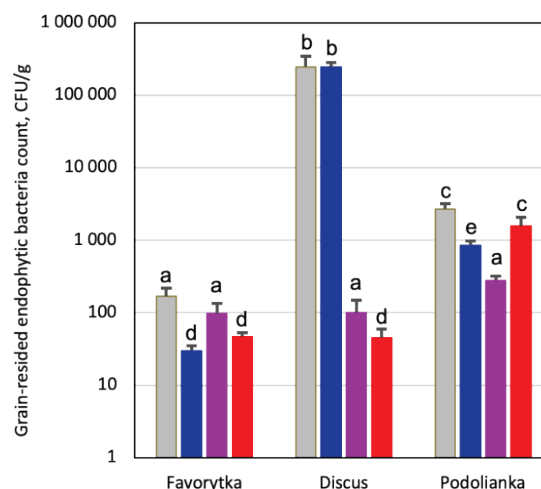


Fig. 1. Quantitative characteristics and morphotypes of grain-residing bacterial endophytic communities in wheat cultivars with different sensitivity to *Pseudomonas syringae* pv. *atrofaciens* (McCulloch): grey columns – total bacteria count; blue columns – endospore-forming bacilli; pink columns – cocci; red columns – gram-negative rods; values in bar graphs are presented as $x \pm SD$ ($n = 3$); different letters indicate significant differences between varieties (Tukey post-hoc test with Bonferroni correction, $P < 0.05$)

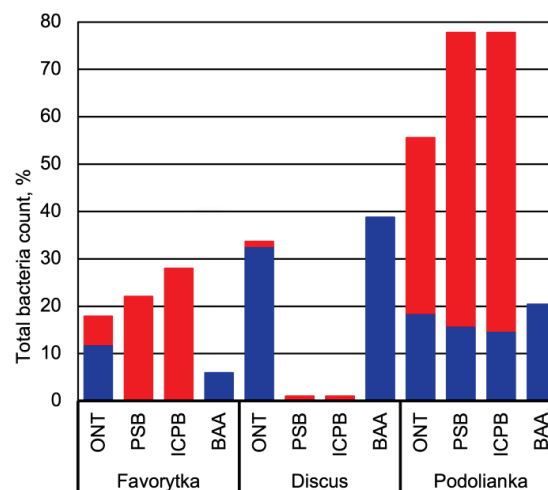


Fig. 2. Plant-beneficial characteristics of grain-residing bacterial endophytic communities in wheat cultivars with different sensitivity to *Pseudomonas syringae* pv. *atrofaciens* (McCulloch): blue – endospore-forming bacilli; red – gram-negative rods; values in bar graphs are presented as proportions in total bacteria count ($x \pm SD$, $n=5$); ONT – oligonitrotrophs, PSB – phosphate solubilizing bacteria, ICPB – indolic compound producing bacteria, BAA – bacteria with antagonistic activity against *P. syringae* pv. *atrofaciens* (McCulloch)

Antagonistic activity of the isolated grain-residing endophytic bacteria was checked against *P. syringae* pv. *atrofaciens* in vitro. Endophytic bacteria with antagonistic activity against phytopathogenic pseudomonads were present in the grain-residing communities of all studied wheat varieties. The proportion of bacteria with antagonistic activity was highest in the endophytic community of Discus (39%, 95000 CFU/mL), followed by the endophytic community of Podolianka (20%, 550 CFU/mL). The lowest proportion (6%, 10 CFU/mL) of endophytic antagonists inhabited grains of the low-resistant variety Favorytka. All endophytic bacteria

with antagonistic activity belonged to the endospore-forming bacilli morphotype (Fig. 2). Out of 32 tested isolates, 5 showed varying extents of antagonistic potential against the *P. syringae* pv. *atrofaciens* by forming characteristic clear zones inhibiting the growth of the pathogen around the well. Bacteria with antagonistic activity were then preliminarily identified by their biochemical profile and using MALDI-TOF mass-spectrometry. Two isolates with antagonistic activity (P6 and P10) were obtained from Podolianka variety. P10 showed the most pronounced antagonistic activity (inhibition zone diameter 27.0 ± 3.0 mm), and was identified as *Paenibacillus peoriae* according to its cultural and morphological properties and biochemical profiling (Fig. 4a, Table 2). P6 also demonstrated potent

antagonistic activity (inhibition zone diameter 23.0 ± 1.0 mm), and was identified as *Brevibacillus* spp. (Fig. 4b, Table 2). Two isolates (D2 and D5) were obtained from Discus variety, and were identified as *Bacillus* spp. and *Bacillus pumilus* correspondingly (Fig. 4c, d, Table 2). These isolates exerted moderate and strong antagonistic effects: inhibition zone diameters 15.0 ± 2.0 and 24.0 ± 4.0 mm correspondingly. One isolate (F1) with antagonistic activity (inhibition zone diameter 21.0 ± 3.0 mm) from Favorytka was identified as *Bacillus* spp. (Fig. 4e, Table 2). It should be noted that P6 and P10 isolates were characterized by potent phosphate-solubilizing activity and moderate oligonitrotrophy in addition to their antagonistic action.

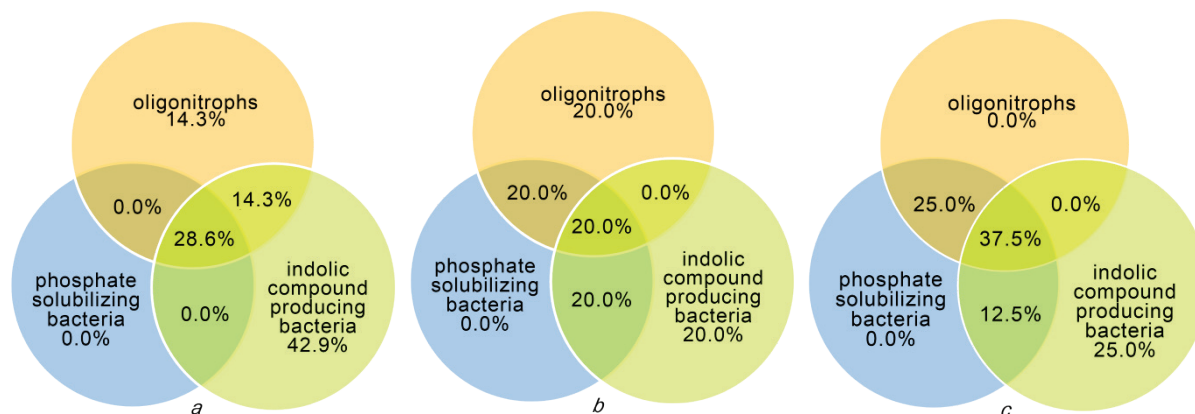


Fig. 3. Venn diagrams of grain-residing endophytic bacterial communities with single and multiple PGP activities: numerical labels represent proportions in total count of functionally active bacteria; a – grain residing endophytic community from Favorytka; b – grain-residing endophytic community from Discus; c – grain-residing endophytic community from Podolianka

Identification by biochemical profiling was additionally validated by the identification by MALDI-TOF mass spectrometry. Isolate P6 was assigned to *Paenibacillus peoriae* with confidence value 99.9%, isolate P10 – to *Brevibacillus* spp. with confidence value 99.9%, isolate D2 – to *Bacillus* spp. with confidence value 99.9%, isolate D5 – to *Bacillus pumilus* with confidence value 99.9%, and isolate F1 – to *Bacillus* spp. with confidence value 99.9%.

Differences in the composition and properties of the endophytic grain-residing community were associated with different manifestations of pathogen-induced oxidative stress and antioxidative responses in seedlings of wheat varieties with different sensitivity to *P. syringae* pv. *atrofaciens*. As one can see from the Figure 5a, TBARS content increment in the 7-day seedlings, which were germinated from grains treated with *P. syringae* pv. *atrofaciens* (as compared to their untreated counterparts), correlated with the sensitivity to the phytopathogen. Oxidative stress, as indicated by statistically significant increase of TBARS content, was registered only in seedlings of Favorytka variety, which is the least resistant to phytopathogenic bacteria according to our previous observations. It is necessary to note that TBARS content in untreated seedlings of Favorytka variety was slightly lower than that in resistant varieties Podolianka and Discus.

Antioxidative enzyme activity in seedlings germinated from grains subjected to *P. syringae* pv. *atrofaciens* varied markedly in wheat varieties with different sensitivity to these phytopathogenic bacteria. In treated seedlings of the wheat variety with lowest resistance to the phytopathogenic pseudomonads (Favorytka), SOD activity was 2.0 times higher than in untreated (Fig. 5b). At the same time, SOD activity in treated seedlings of wheat variety with moderate sensitivity to the phytopathogen (Discus) was 5.0 times lower than in untreated. SOD activity in treated seedlings of highly resistant variety did not differ significantly as compared to the control.

Similar to SOD activity, the fluctuation of CAT activity after the exposure to phytopathogen in studied varieties was different (Fig. 5c). CAT activity of treated seedlings of wheat varieties with low and moderate resistance to the phytopathogen was on average 2.2 times higher as compared to the corresponding controls. By contrast, CAT activity in the treated highly resistant variety was slightly lower as compared to the control. Changes in the equilibrium between the formation of hydrogen peroxide from superoxide dismutation and its decomposition by other enzymes (CAT) in wheat seedlings can be expressed by the ratio R =

SOD/CAT. This ratio varied significantly in wheat varieties with different sensitivity to phytopathogenic pseudomonads, which were characterized by the different compositional and functional characteristics of grain-residing endophytic communities. R value in untreated seedlings of Favorytka was 3.0 times higher in comparison with that in Podoliyanka and did not differ from that in Discus (0.22 versus 0.07 and 0.21 correspondingly). After the exposure to the phytopathogen, the R value of Favorytka remained higher than that of resistant varieties: 3.0 times as compared to Podoliyanka and 10 times as compared to Discus (0.20 versus 0.11 and 0.02 correspondingly).

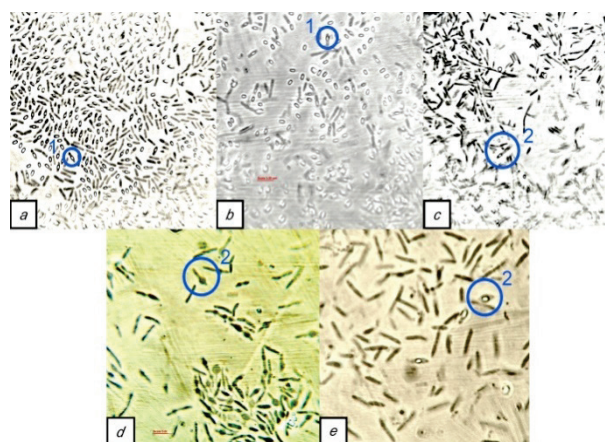


Fig. 4. Phase contrast micrographs displaying the cell morphology of wheat grain-residing bacteria with antagonistic activity against *Pseudomonas syringae* pv. *atrofaciens* (McCulloch); bar = 5 μ m; a – F1 isolate from Favorytka variety, assigned to *Bacillus* spp.; b – D2 isolate from Discus variety, assigned to *Bacillus* spp.; c – D5 isolate from Discus variety, assigned to *Bacillus pumilus*; d – P6 isolate from Podoliyanka variety, assigned to *Paenibacillus peoriae*; e – P10 isolate from Podoliyanka variety, assigned to *Brevibacillus* spp.; 1 – gram-positive rod with a sub-centrally situated ellipsoidal spore; 2 – gram-positive rod with a sub-centrally situated ellipsoidal spore that bulged the bacillary wall

Table 2
Biochemical details of designated endophytic bacterial isolates using VITEK/BCL card

Well	Test Substrate	Mnemonic	Amount mg/well	Isolate				
				F1 <i>Bacillus</i> spp.*	D2 <i>Bacillus</i> spp.*	D5 <i>Bacillus pumilus</i>	P6 <i>Paenibacillus peoriae</i>	P10 <i>Brevibacillus brevis/Brevibacillus agri</i>
1**	Beta xylosidase	BXYL	0.0324	+	+	+	+	–
3	L-lysine arylamidase	LysA	0.0228	–	–	–	–	–
4	L-aspartate arylamidase	AspA	0.0240	(+)	–	–	+	(+)
5	Leucine -arylamidase	LeuA	0.0234	+	+	+	+	–
7	Phenylalanine arylamidase	PheA	0.0264	+	+	+	+	(–)
8	L-proline arylamidase	ProA	0.0234	–	–	–	–	+
9	Beta-galactosidase	BGAL	0.0360	+	+	+	+	–
10	L-pyrrolydonyl- arylamidase	PyrA	0.0180	+	+	–	–	+
11	alpha-galactosidase	AGAL	0.0360	+	+	+	+	–
12	Alanine arylamidase	AlaA	0.0222	–	–	+	–	–
13	Tyrosine arylamidase	TyrA	0.0282	+	+	+	(–)	–
14	Beta-N-acetyl-glucosaminidase	BNAG	0.0408	–	–	–	–	–
15	Ala-Phe-Pro arylamidase	APPA	0.0384	–	–	–	+	+
18	Cyclodextrin	CDEX	0.3000	–	+	–	–	–
19	D-galactose	dGAL	0.3000	–	–	–	+	–
21	Glycogen	GLYG	0.1875	+	(+)	–	+	–
22	Myo-inositol	INO	0.3000	+	+	(–)	–	–
24	Methyl-A-D-glucopyranoside acidification	MdG	0.3000	+	+	+	+	–
25	Ellman	ELLM	0.0300	+	+	+	–	–
26	Methyl-D-xyloside	Mdx	0.300	–	–	–	–	–
27	Alpha mannosidase	AMAN	0.0360	–	–	+	–	–
29	Maltotriose	MTE	0.3000	+	+	–	+	–
30	Glycine arylamidase	GlyA	0.0120	(–)	+	+	–	–
31	D-mannitol	dMAN	0.3000	+	+	+	–	(–)
32	D-mannose	dMNE	0.3000	+	+	+	+	–
34	D-melezitose	dMLZ	0.3000	–	–	–	–	–
36	N-Acetyl-D-glucosamine	NAG	0.3000	–	–	–	–	–
37	PAI atinose	PLE	0.3000	+	+	–	+	–
39	L-rhamnose	IRHA	0.3000	–	–	–	–	–
41	Beta-glucosidase	BGLU	0.0360	+	+	+	+	–
43	Beta-mannosidase	BMAN	0.0360	–	(–)	–	–	–
44	Phosphoryl choline	PHC	0.0366	+	–	–	–	+
45	Pyruvate	PVATE	0.1500	+	+	+	–	–
46	Alpha-glucosidase	AGLU	0.0360	+	+	–	–	–
47	D-tagatose	dTAG	0.0300	–	–	+	–	–
48	D-trehalose	dTRE	0.0300	+	+	+	+	–
50	Inulin	INU	0.1200	+	+	–	+	–
53	D-glucose	dGLu	0.0300	+	+	+	+	–
54	D-ribose	RIB	0.0300	+	+	+	+	–
56	Putrescine assimilation	PSCNa	0.2010	–	–	–	–	–
58	Growth in 6.5% NaCl	NaCl 6.5%	1.9500	+	+	(+)	–	–
59	Kanamycin resistance	KAN	0.0060	–	–	–	+	+
60	Oleandomycin resistance	OLD	0.0030	–	–	–	+	–
61	Esculin hydrolysis	ESC	0.0225	+	+	+	+	–
62	Tetrazolium red	TIZ	0.0189	+	+	+	(+)	–
63	Polymixin B resistance	POLYB R	0.0009	+	+	+	+	+

Note: * – assigned to *Bacillus subtilis* / *amyoliquefaciens* / *atrophaeus*; ** – other well numbers not designated in this table are empty; *** – reactions result that are shown in parentheses “(–)” or “(+)” indicate weak reactions that are near to the threshold values.

Treatment of wheat grains with phytopathogenic pseudomonads affected proline content in seedling tissues (Fig. 5d). There was a significant difference between the wheat varieties in basal proline content, so that the pathogen-sensitive variety Favorytka showed significantly less basal free proline than resistant varieties Discus and Podolanka. After the exposure to the phytopathogen, proline content in Favorytka seedlings was 8.5 times higher than that in untreated. In resistant wheat varieties, we did not observe proline accumulation after the treatment with phytopathogenic bacteria.

Discussion

Winter wheat diseases caused by *P. syringae* lead to reduction of plant growth and cereal productivity worldwide. Infected grains are one of the key sources of wheat plant colonization with this pathogen. It necessitates a better understanding of mechanisms of wheat grain resistance to phytopathogenic pseudomonads. Endophytic bacteria are considered one of the determinants of the plant resistance to phytopathogens. A major objective of this pilot study was to compare composition and biological

features of grain residing endophytic bacterial communities in winter wheat varieties with different sensitivity to *P. syringae* pv. *atrofaciens* (McCulloch). For this purpose, we screened grain-residing endophyte collections from wheat varieties with different resistance to this phytopathogen *in vitro* (Pastoshchuk et al., 2018) for beneficial plant traits. Three wheat varieties were used in this study: Podolanka, Discus and Favorytka. According to the manufacturer data, Discus and Favorytka are characterized by high resistance to fungal diseases, Podolanka – by medium resistance. Our previous results indicate that resistance to fungal diseases does not correlate with the resistance to phytopathogenic pseudomonads (Pastoshchuk et al., 2018; Smimov et al., 2020). Our current results revealed distinctions in quantitative and functional characteristics of grain-residing bacterial endophytic communities from wheat varieties with different sensitivity to phytopathogenic pseudomonads. Three bacterial morphotypes were obtained from wheat grains of all three varieties following a culture-dependent protocol: gram-negative rods, endospore-forming bacilli, and gram-positive cocci. These results confirm the existence of rich microbial diversity in wheat grains, described by other scientific groups (Herrera et al., 2016; Kuzniar et al., 2020). Resistance to *P. syringae* pv. *atrofaciens*

was associated with a high total culturable endophytic bacteria count and with the predominance of gram-negative rods or endospore-forming bacilli, but not gram-positive cocci (Fig. 6). The latter is pretty logical, since all plant beneficial traits were inherent in gram-negative rods, endospore-forming bacilli, and were absent in gram-positive cocci. High resistance to *P. syringae* pv. *atrofaciens* (McCulloch) was also associated with the highest proportion of grain-residing endophytic bacteria possessing three PGP activities simultaneously: phosphate solubilization, nitrogen fixation and production of indolic compounds. Promotion of plant growth is a multitask challenge. Therefore, endophytic bacteria possessing multiple PGP activities seem to be more powerful allies and more effective partners of the host plant as compared with their counterparts with only single PGP activity. In addition to PGP properties, culturable wheat grain-residing bacteria exerted antagonistic effect against *P. syringae* pv. *atrofaciens*. All endophytes with antagonistic activity belonged to endospore-forming bacilli of *Bacillus*, *Paenibacillus* and *Brevibacillus* genera. Resis-

tance to *P. syringae* pv. *atrofaciens* was associated with a high proportion of antagonists in the total culturable endophytic bacteria count. Moreover, the grain-residing endophytic community of a highly resistant wheat variety contained *Brevibacillus* with most potent antagonistic activity. Our findings are consistent with literature data, according to which different *Bacillus* species including *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* etc. exert antimicrobial activity against phytopathogenic pseudomonads. Lipopeptides produced by these bacteria belong to the surfactin, iturin, and fengycin families, and are responsible for inhibition of necrotroph and hemibiotroph plant pathogens such as *P. syringae* (Nikolić et al., 2019). The efficacy of *Bacillus* isolates in experiments concerning *P. syringae* biocontrol is evidenced in several recent studies. Bacteria of *Brevibacillus* genus, were shown to produce non-ribosomal peptide with antimicrobial activity (marthiapeptide A), streptocidin D, and an unusual lysophospholipid, which are active against Gram-negative bacteria (Mougou et al., 2018).

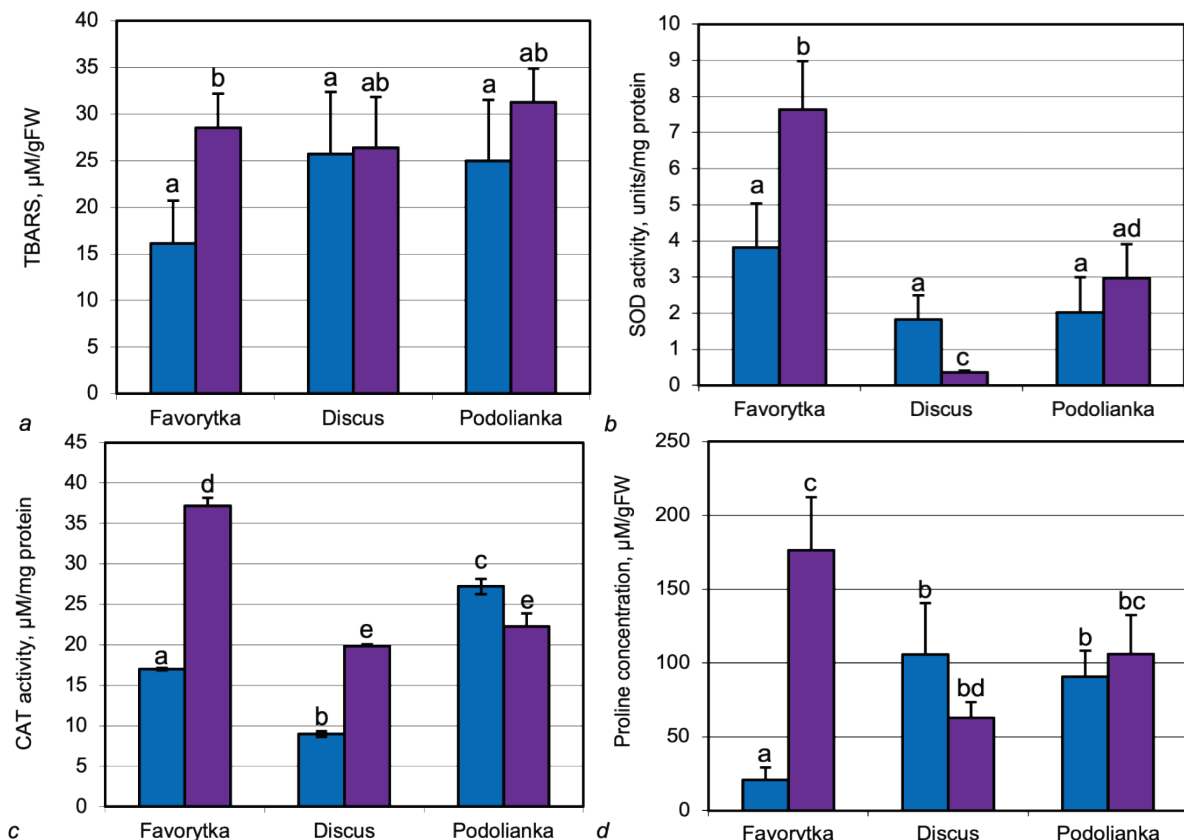


Fig. 5. Oxidative stress and antioxidative responses in wheat seedlings after grain exposure to *Pseudomonas syringae* pv. *atrofaciens* (McCulloch): *a* – thiobarbituric acid reactive substances (TBARS) content; *b* – superoxide dismutase (SOD) activity; *c* – catalase (CAT) activity; *d* – proline content; FW – fresh weight; values in bar graphs are presented as $\bar{x} \pm \text{SD}$ ($n = 5$); different letters indicate significant differences between varieties (Tukey post-hoc test with Bonferroni correction, $P < 0.05$), except for measurements in seedlings after the exposure to phytopathogen, which were compared with their untreated counterparts individually using a two tailed T-test, $P < 0.05$ versus corresponding untreated control

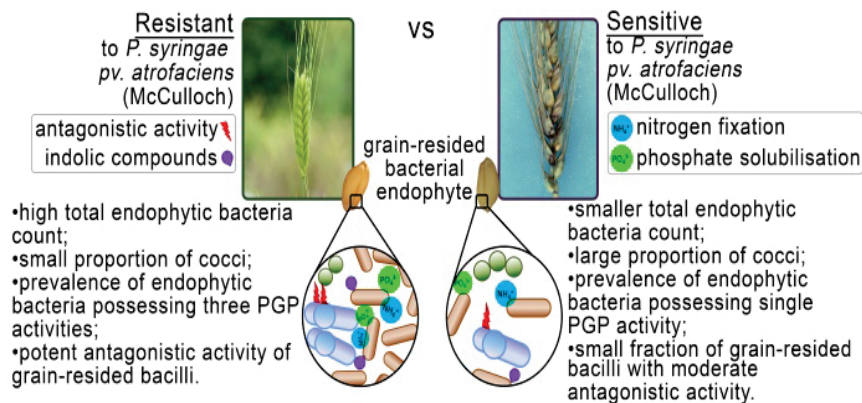


Fig. 6. Schematic representation of the features of grain-residing endophytic bacteria in winter wheat varieties with different sensitivity to *Pseudomonas syringae* pv. *atrofaciens* (McCulloch)

Dissimilarities in grain-residing bacterial endophytic communities were also associated with different manifestation of defense reactions in response to grain exposure to phytopathogen *in vitro*. Resistance to *P. syringae* pv. *atrofaciens* was associated with the absence of oxidative stress in seedlings from grains exposed to phytopathogen. The cell susceptibility to free radicals relies on the balance between the generation of hydrogen peroxide from superoxide in the dismutation reaction catalyzed by SOD and its degradation by CAT and glutathione peroxidase, rather than on the action of separate antioxidant enzymes (Imlay, 2008). In resistant winter wheat varieties both basal and post-exposure SOD/CAT ratio values were higher than in the cells of sensitive varieties, which might result in raised hydrogen peroxide generation in response to the treatment with phytopathogen. Also, no substantial free proline accumulation was observed in resistant cultivars. Low resistance to phytopathogenic pseudomonads was associated with oxidative burst in seedling tissues along with a dramatic increase in free proline content. Wang et al. (2020) reported on the ability of endophytic bacteria to maintain oxidative-antioxidative balance in plant tissue by regulating the concentrations of H₂O₂, MDA and proline, increasing the activities of antioxidative enzymes. Mishra et al. (2018) described endophytic bacteria with the capability not only to enhance defense enzymes and antioxidant activity but also augment the expression of salicylic acid- and jasmonic acid-responsive genes in plants under pathogenic stress. We would like to pay special attention to the data on the accumulation of free proline. Recently, data on the involvement of proline in plant protection against pathogens and in the regulation of cell redox potential have appeared in the literature. Proline accumulation in plant tissue can serve to neutralize reactive oxygen species (ROS) (Meena et al., 2019). On the other hand, the accumulation of proline causes an intensification of its oxidation by proline dehydrogenase with the formation of ROS (Zeier et al., 2013). Based on the literature data (Tamosiune et al., 2017), we are inclined to assume that in this case, disproportionate free proline accumulation can contribute to ROS generation in Favorytka seedlings, whereas grain-residing endophytic plant communities of resistant cultivars can participate in the regulation of proline metabolism and therefore can prevent its inadequate accumulation. However, this assumption warrants experimental verification.

Conclusion

Our results extend current knowledge of the wheat grain-residing endophytic community and its association with the resistance to *P. syringae* pv. *atrofaciens* (McCulloch). To the best of our knowledge, this is the first report concerning the fluctuation of the proline content in wheat under biotic stress and its potential association with compositional and biological features of grain-residing endophytic bacteria.

Our results give reason to believe that it is necessary to search for promising strains or consortia in the endophytic community of plant varieties resistant to phytopathogens with the aim of using them in agricultural biotechnology. P6 and P10 grain-residing endophytic bacteria can be used for wheat grain dressing as PGP and biological control agents of phytopathogenic bacteria, whose contribution to wheat grain yield losses progresses at a steady pace. For this purpose, isolate identification using 16S rRNA Gene Sequencing is warranted, as well as testing PGP and protective effects of isolates used alone and in composition in future in plant experiments.

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