NRPS genes provide antifungal activity to new *Bacillus* and *Paenibacillus* strains and change their expression in the presence of phytopathogens in a strain-specific manner

Vasilchenko N.G.[a,b], Kulikov M.P.[a]*, Prazdnova E.V.[a], Gorovtsov A.V. [a], Usatov A.V. [a], Chistyakov V.A.[a]

[a] *Southern Federal University, D.I. Ivanovsky Academy of Biology and Biotechnology, 344090, Russian Federation.*

[b] *Almetyevsk State Oil Institute, Biotechnological Research and Education Center, 423458, Russian Federation, Republic of Tatarstan.*

* Corresponding author at: Academy of Biology and Biotechnologies, Southern Federal University, 344090 Rostov-on-Don, Russia.

E-mail address: 1001011100m@gmail.com (Kulikov M.P.)

Abstract

Fusarium diseases cause serious damage to agricultural crops. In agricultural practice, various chemical fungicides, usually based on triazole, are often used to control fusariosis. This leads to the emergence of resistant forms among pathogenic fungi. An alternative to chemical fungicides treatments may be the use of biopreparations based on natural antagonists of *Fusarium*. *Bacillus* and *Paenibacillus* bacteria isolated from farmland soils exhibit a high level of antagonism against phytopathogenic fungi of the genus *Fusarium*. PCR-based analysis revealed that genes of non-ribosomal peptide synthases are present in the genomes of isolated bacterial strains, while these strains do not have any chitinase activity, so the antifungal effect seems to be provided predominantly by non-ribosomally synthesized peptides. The expression of the detected genes is increased in the presence of fungi during co-culturing, and this effect is strain-specific and pathogen-dependent. In light of these findings, the development of pathogen-specific drugs seems promising.

Keywords

Bacillus, Paenibacillus, Fusarium, NRP, non-ribosomal synthesis, gene expression

1. Introduction

The vast majority of *Fusarium* species are plant parasites capable of causing serious damage to agricultural crops, causing significant losses of commodity grain up to 40% and leading to a reduction in the overall quality of the harvested crop (Nikitin D. A. et al. 2023; Ekwomadu T. I. et al. 2021).

Various chemical fungicides are often used to control fusariosis in agricultural practice. Among all fungicides used, the most common are triazole-based preparations, as well as combinations of triazoles with other active substances - prochloraz or spiroxamine (Feksa H. R. et al. 2019; Chen Y. et al. 2020; Li C. et al. 2022; He D. et al. 2023). Such a wide use of triazoles in crop production is a serious danger. The use of substances from the same class of compounds for a long period of time quite naturally leads to the gradual emergence of resistant forms among pathogens (Yerkovich N. et al. 2020). This phenomenon is also typical for agricultural use of fungicides. The emergence of powdery mildew resistant to triazoles and a number of other fungicides was first noted back in 1987 (Weete and Wise 1987), and is currently observed in a number of other plant diseases (Resendiz Sharpe A. et al. 2018).

Members of the genera *Bacillus* and *Paenibacillus* have various mechanisms of antifungal activity, which makes them promising agents for the development of biopreparations based on them for the control of fungal phytopathogens (Soni and Keharia 2021). This group of aerobic spore-forming bacteria is known for its pronounced antagonistic activity against various organisms: gram-negative and gram-positive bacteria (Olishevska S. et al. 2019; Soni and Keharia 2021), a wide range of fungal species (Olishevska S. et al. 2019; Vasilchenko N. et al. 2022; Costa A. et al. 2022) and some insect species (Ruiu L. 2020). The ability to inhibit the growth of such a variety of living organisms by these groups of bacteria is provided by the synthesis of various

molecules and enzymes. The enzymes that determine antagonistic activity against pathogenic fungi, for example, include chitinases and beta-glucanases, which contribute to the destruction of the cell wall (Brzezinska M. S. et al. 2020; Thakur N. et al. 2022). In addition to enzyme complexes, bacteria of these genera are capable of synthesizing many low molecular weight compounds: non-ribosomal synthesis peptides (NRPs), polyketides (PCs), and volatile organic compounds (Olishevska S. et al. 2019; Li X. et al. 2020).

NRPs are of particular interest for both fundamental and applied research, as new promising antibiotics, antimycotics and biosurfactants are constantly being discovered in this group of compounds (Ranjan A. et al. 2023; Olmedo C. et al. 2022).

The aim of this work was to study the contribution of NRPS genes and their expression to the antifungal effect of the newly characterized strains. It was found that the expression of these genes increases when co-cultured with *Fusarium* strains in a strain-specific manner.

2. Methods

2.1. Strains

Cultures of fungal pathogens *Fusarium oxysporum* (strains Ras6.1 and Ras6.2.1) and *Fusarium graminearum* (strains Fus1 and Fus5), strains with strong antifungal activity *Paenibacillus polymyxa* (K1.14, O1.27, O2.11, R3.13, R4.5, R4.24, R5. 31, R6.14), *Bacillus velezensis* strains (V3.14, R4.6) and strains with weak activity (listed below in the order from 1 to 20) were isolated from soils of agricultural fields of Krasnodar region.

2.2. Primary screening of antagonistic strains

For initial selection of antagonist strains of *Fusarium*, we used the agar plate method of co-cultivation on nutrient medium (0.5 Malt extract agar : 0.5 nutrient agar), pre-inoculated with culture of either *F. oxysporum* or *F. graminearum*. A fungal culture was inoculated by plating the inoculum on the agar medium, then a pasteurized soil suspension (in a volume of 50 µl) was plated on top of the fungal culture on the same Petri dish. The double culture dishes were incubated for

3-5 days at 27-28°C. All bacteria around which a distinct zone of fungal growth suppression was formed were selected. The isolated cultures were tested against *F. graminearum* (Fus1 and Fus5) and *F. oxysporum* (Ras6.1, Ras6.2.1). Only strains capable of suppressing all 4 strains of *Fusarium* were selected.

2.3. Determination of chitinase activity of antagonistic strains

To determine the chitinase activity of the strains, the strains were sown on chitin agar according to the method of Rishad (2017). The strains were cultured for 3 days at 27 °C. The presence or absence of this activity was recorded by the presence of a zone of clarification of the nutrient medium around the colony of the microorganism.

2.4. Identification of bacterial and fungal strains

The isolated phytopathogenic fungi and ten selected most active strains of bacteria-antagonists of phytopathogenic fungi were subjected to identification based on DNA analysis in the Russian National Collection of Industrial Microorganisms (VKPM), and deposited in this collection.

Identification was based on sequencing of 16s rRNA gene sequences and further phylogenetic analysis in the case of bacterial strains, and based on sequencing and phylogenetic analysis of 18s rRNA, 5.8s rRNA and internal transcribed spacer genes *ITS1* and *ITS2* when identifying phytopathogenic fungi, the results are described in our previous paper (Gorovtsov A. V. et al. 2019).

2.5. Extraction of nucleic acids

DNA extraction from bacterial cells was performed using silica sorbtion and heat-based kit for DNA extraction "DNA Sorb C-M" (Amplisens, Russia). DNA was extracted from overnight culture grown on Malt extract-Meat peptone agar. The isolated DNA was purified using CleanMag DNA kit (Eurogen, Russia).

RNA isolation from bacterial cells was performed using a commercial extraction kit "Extract RNA" (Eurogen, Russia). The isolation was performed from bacterial culture grown on Malt

101 extract- nutrient agar in co-culture with fungi and without co-culture as a control. The isolated
102 RNA was purified using a commercial kit "CleanRNA Standard" (Eurogen, Russia).

103 The production of complementary single-stranded DNA on RNA matrix was performed using
104 the "MMLV RT kit" (Eurogen, Russia). The concentration and quality of synthesized RNA and
105 cDNA were measured using a spectrophotometer Nano Photometer P330 (Implen, USA).

106 **2.6. NRPS gene expression analysis**

107 NRPS genes were searched by PCR using a commercial kit "ScreenMix" (Eurogen) and DNA
108 as a matrix. PCR results were visualized in 1.5% agarose gel with ethidium bromide and Gel-Doc
109 gel-documentation system (Bio-Rad, USA). The primers presented in Table 1 were used to search
110 for NRPS genes.

111 Each PCR was performed according to the program:

112 1. Pre-denaturation 95°C for 5 minutes

113 2. Denaturation 95°C 30 seconds,

114 3. Primer annealing with a gradient from 54°C to 65°C 30 seconds,

115 4. Elongation at 70°C 60 seconds.

116 Steps 2 to 4 were repeated 40 times.

117 Temperatures chosen experimentally were used for primer annealing. After each reaction,
118 PCR products were incorporated into wells of 1.5% agarose gel with ethidium bromide.
119 Electrophoresis was performed at 143V, 9.4 W, 120 mA, for 15-50 minutes with periodic
120 visualization of the resulting fragments every 15 minutes in the Gel Dock system (Bio-Rad, USA).
121 For amplicon size determination, 100+bp and 50+bp length markers (Eurogen, Russia) were used.
122 As a negative PCR control, a complete reaction sample was used, in which 5 µl of nuclease-free
123 water was added instead of DNA.

The expression levels of non-ribosomal peptide synthetase genes were assessed using a real-time amplifier CFX96 (Bio-Rad, USA) and a commercial “PCR-mix SYBR Green I kit” (Syntol, Russia).

To study the effect of fungi presence on the change of NRPS gene expression patterns, fungi and bacteria were co-cultured on dense nutrient medium. RNA was then isolated from bacterial cells and single-stranded cDNA was produced. A full description of the isolation and processing conditions is presented in section 2.5.

The changes in relative gene expression in antagonist bacterial strains in the presence of different *Fusarium* species were studied separately. Experiments were performed in 3 biological replicates. The following amplification program was used in each staging:

1. DNA pre-denaturation at 95 °C for 5 minutes

2. Denaturation at 95 °C for 30 seconds

3. Primer annealing at the temperatures listed in Table 3 for 30 seconds.

4. elongation at 70 °C for 40 seconds.

Steps 2 to 4 were repeated 45 times. Melting curves between 65 and 95 °C in 0.5 °C steps every 5 seconds were analyzed to assess the specificity of amplification.

Fluorescence reading was performed using the FAM channel after the primer annealing step. The threshold of the quantification cycle was set according to the software manufacturer's algorithm without modifications.

The $2^{-\Delta\Delta CT}$ method (Rao X. et al. 2013) was used to assess the relative expression level. 16s rRNA was used as a reference gene. The obtained values of relative gene expression were presented as arithmetic mean and 95% confidence interval, which were further used for statistical calculations in STATISTICA12 or IBM SPSS Statistics 26 using non-parametric Mann-Whitney criteria or Kruskal–Wallis analysis of variance with Dunn's post-hoc test (Gasque G. et al. 2013).

3. Results

3.1. Characterization of selected strains

Based on the results of the screening for antifungal activity, 10 bacterial strains were selected: 2 strains of the genus *Bacillus* (V3.14, R4.6) and 8 strains identified as bacteria of the genus *Paenibacillus* (K1.14, O1.27, O2.11, R3.13, R4.5, R4.24, R5.31, R6.14). The strains are described in more details in our previous paper (Gorovtsov A. V. et al. 2019).

3.2. Chitinase activity detection

The test for detection and evaluation of chitinase activity in antagonistic bacterial strains was conducted to identify one of the most common mechanisms of antifungal activity (Chang et al. 2010). All 10 selected bacterial strains do not possess chitinase activity, as evidenced by the absence of a zone of hydrolysis of colloidal chitin during growth on the surface of the nutrient medium.

3.3. NRPS gene screening

The available genes of non-ribosomal peptide synthetases were analyzed. The result of the gene search in the selected antagonistic bacterial strains is presented in Table 2. Based on the data presented in Table 2, it can be concluded that in strains belonging to the genus *Bacillus* (V3.14, R4.6), in addition to the genes responsible for the synthesis of surfactin (*srfAA*) and fengicin synthetases (*fenC*), the *fusA* gene responsible for the synthesis of fusaricidin synthetase was detected. Although the presence of fusaricidin synthetase synthesis genes is usually characteristic only for bacteria of the genus *Paenibacillus*, the presence of this gene in bacteria of the genus *Bacillus* can be explained either by horizontal gene transfer or by the presence of a close analog of this gene (ortholog). The presence of the reference gene 16s subunit of the ribosome (*16s Bam*), used as a positive control of the methodology, was also expectedly detected in all strains.

In strains belonging to the genus *Paenibacillus*, only genes responsible for the synthesis of fusaricidin and polymyxin (*pmxE*) synthetases were detected among all studied targets. These

genes were detected in all 8 strains belonging to this genus of bacteria. Strains 1 - 20 showed almost no antagonistic activity against the fungus *F. graminearum* Fus1. As can be seen from the table, only 3 weak antagonistic strains 3,5,9 had NRPS genes, and only one NRPS gene was detected in each of the strains.

3.4. Analysis of NRPS gene expression in bacterial antagonistic strains and strains with weak antagonistic activity against *Fusarium*

The expression of non-ribosomal peptide synthetase genes was analyzed in bacterial strains with high antagonistic activity against *Fusarium* strains with low activity but carrying NRPS genes. In this experiment, strains with different levels of antagonistic activity were cultured both together with the *F. oxysporum* Ras6.2.1 and without it.

Although some weak-antagonistic bacteria showed the same genes as the antagonistic bacterial strains, they did not show activation of these genes in the presence of the fungus *F. oxysporum* Ras6.2.1. On the contrary, strong activation of NRPS gene expression in the presence of fungus can be seen in antagonist bacterial strains. Thus, it was found that activation of NRPS gene expression in the presence of fungi occurs only in bacterial strains with high antagonistic activity. The results are summarized in Figure 1.

Next, we tested the effect of different species of phytopathogenic fungi on changes in the expression patterns of NRPS genes in selected bacterial strains with a high level of antagonism.

Changes in gene expression patterns are presented for each strain as separate graphs (Figures 2-3). On figure 2 it can be seen that the expression of fusaricidin in cells of *B. velezensis* V3.14 was markedly higher when the bacterial strain was co-cultured with the Ras6.1 and was not statistically significantly different from the control in the presence of the Fus5, while the expression of fengycin increased in the presence of the Fus5 and decreased in the presence of the Ras6.1. It was noted that *B. velezensis* strain R4.6 showed an increase in fusaricidin gene expression and a decrease in fengycin gene expression when co-cultured with both fungal strains.

The greatest increase in fusaricidin gene expression was observed in the variant of co-cultivation with the Ras6.1. In this variant of the experiment, the expression of fusaricidin increased by 12.8 times compared to the control level.

With regard to the expression of surfactin, we can only say that its expression was at the level of control values in both bacterial strains in the presence of different species of fungi, which may indirectly indicate an auxiliary role of this non-ribosomal peptide in antifungal antagonism of bacteria of this species.

The next step of this work was to analyze the expression patterns of the detected NRPS genes in the presence and absence of *Paenibacillus polymyxa* (Figure 3).

Thus, the expression of *fusA* gene (gene responsible for the expression of fusaricidin synthetase) and *pmxE* gene (gene responsible for the expression of polymyxin synthetase) was detected in strain K1.14. The expression of these loci had statistically significant differences compared to the control. As can be seen from the graph, the expression of NRPS genes changes differently relative to the control depending on the species of *Fusarium*, which was co-cultured together with the antagonist strain. The graph shows that the expression of fusaricidin decreased in the experiment variant when the strain was co-cultured with the Ras6.1 and increased in the experiment variant when the strain was co-cultured with the Fus5. At the same time, polymyxin expression was higher than the control values only when the strain was co-cultured with *F. graminearum* Fus5.

In turn, in strain O1.27, changes in expression patterns were observed only for the gene responsible for the synthesis of subunit A of fusaricidin synthetase. At the same time, the expression level of this gene also differs in different variants of the experiment. In the case of co-cultivation of strain O1.27 with Fus5, the expression of fusaricidin decreased up to 0.3-fold compared to the control, while in the experiment variant with Ras6.1 it increased by 1.4-fold.

When analyzing the data on the expression of NRPS genes in strain O2.11, it was revealed that only fusaricidin and polymyxin synthetase are expressed in this strain. And in this case, it can be seen that the expression level varies depending on the species of fungi that was co-cultured with the antagonistic bacterial strain. In this case, expression synchronously increased for the fusaricidin gene (up to 6.5-fold in the presence of Fus5, and up to 2.8-fold in the presence of Ras6.1) and decreased for polymyxin (up to 0.4 and 0.2, respectively).

A completely opposite expression pattern was revealed in strain R3.13. This strain also expresses fusaricidin and polymyxin, while in comparison with the previous strain there is a synchronous increase in polymyxin expression (up to 1.5 and 1.6) and a decrease in fusaricidin expression (up to 0.1 and 0.9) in the presence of Fus5 and Ras6.1, respectively.

Further analysis of NRPS gene expression was done for strain R4.5. It can be observed that in this strain in case of co-cultivation with Fus5 the expression of fusaricidin synthetase was lower or at the same level as the control level, and in case of co-cultivation with Ras6.1, there was an increase in the expression of fusaricidin synthetase gene (4.4 times higher compared to the control). No statistically significant changes were detected in polymyxin gene expression in both co-cultivation variants compared to the control level.

Expression dependent on the presence of different types of fungi was also detected in strain R4.24. It can be seen that this strain reacts differently to the presence of different pathogens. Thus, in the presence of Fus5, the expression of polymyxin and fusaricidin decreased (to 0.2 and 0.1, respectively), while in the presence of Ras6.1 for the same genes increased (to 1.9 and 2.3).

In turn, *P. polymyxa* strain R5.31 was found to have increased expression of fusaricidin synthetase (up to 4.28 and 2.48) and decreased expression of polymyxin synthetase (up to 0.8 and 0.3, respectively) in both co-cultures.

A completely different expression spectrum was characteristic of *P. polymyxa* strain R6.14. Thus, in this strain, in response to the presence of Fus5 in the environment, there was an increase

in the expression of the gene responsible for the synthesis of fusaricidin synthetase, and there was a decrease in the expression of this gene in the presence of Ras6.1. At the same time, the expression of polymyxin gene did not differ from the control level in both cultivation variants. The results of measuring gene expression levels are summarized in Table 3.

4. Discussion

Bacillus and *Paenibacillus* have a wide range of antifungal activities, for example, in one study, *B. subtilis* PRBS-1 and *B. subtilis* AP-3 strains inhibited the growth of five pathogenic soybean pathogens such as *Rhizoctonia solani*, *Colletotrichum truncatum*, *Sclerotinia sclerotium*, *Macrophomina phaseolina* and *Phomopsis spp*. under in vitro conditions (Araújo et al. 2005). In a similar work, *B. amyloliquefaciens* strain provided protection of oilseed rape (*Brassica napus*) against fungal pathogens such as *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans* and *Verticillum longisporum* (Danielsson et al. 2007). In a number of other works, a high antagonistic potential of representatives of the genus *Bacillus* against phytopathogenic fungi of the genus *Fusarium*, such as *F. oxysporum* (Zhao et al. 2014), *F. verticilloides* (Martínez-Álvarez J. C. et al. 2016) and *F. graminearum* (Ntushelo K. et al. 2019) was demonstrated. A wide range of pathogens whose growth was able to inhibit bacteria of the *B. subtilis* species was shown in the work of the team of authors Kaspar F. et al. (2019). In this work, *B. subtilis* strain was found to have fungicidal activity against various toxigenic mold fungi of genera such as: *Fusarium, Alternaria, Penicillium, Penicillium*, *Botrytis* and *Rutstroemia* (Haddoudi I. et al. 2021). A high level of antagonistic activity against fungal phytopathogens of plants was also revealed in representatives of the genus *Paenibacillus*. Thus, various representatives of this genus were able to inhibit the growth of such fungi as *F. oxysporum* (Naing et al. 2015; Shi et al. 2017), *F. graminearum* (Timmusk et al. 2019), *F. moniliforme* (Zhang C. M. et al. 2010), *Aspergillus parasiticus, Aspergillus versicolor, Aspergillus ochraceus, Fusarium verticillioides, Fusarium solani, Penicillium expansum, Penicillium verruculosum* (Tupinambá et al. 2008), *Botrytis*

cinerea, Colletotricum acutatum, Magnaporthe oryzae, Phytophthora capsici, Rhizoctonia solani, and *Sclerotium cepivorum* (Xu et al. 2014).

Antifungal compounds produced by the *Bacillus* and *Paenibacillus* group of bacteria are mainly represented by polypeptides, polyketides, aminoglycosides and enzymes of the chitinolytic complex. The compounds of the first group include cyclic lipopeptides synthesized without the participation of ribosomes, thanks to the work of enzymes with modular organization, which belong to the NRPS family (non-ribosomal peptide synthetases) (Palazzini et al. 2016). This group of enzymes is capable of synthesizing peptides ranging in size from 2 to 48 amino acids. Unlike ribosomally synthesized peptides, peptides synthesized by NRPS can contain not only proteinogenic amino acids, but also methylated, hydroxylated and glycosylated residues, D-amino acids and even non-proteinogenic amino acids (Süssmuth and Mainz 2017). Non-ribosomal peptides can be synthesized as a heterogeneous mixture of isoforms differing in alpha chain length. Some studies have shown that lipopeptides are resistant to high temperatures (up to 100°C) and proteinase K (Zalila-Kolsi et al. 2016).

In our study it was shown that none of the strains has chitinase activity, which implies that their antifungal activity is due to the work of other mechanisms. As a result of our experiments, it was found that in 10 new bacterial strains of *Fusarium* antagonists' antagonistic activity is provided, apparently, mainly due to non-ribosomally synthesized metabolites, which was confirmed by the analysis based on PCR amplification of target genes involved in non-ribosomal synthesis. In turn, only 3 strains with weak antifungal activity (strains 3,5,9) were found to have NRPS genes, and their NRPS expression level was significantly lower than that of strains with antagonistic activity.

The 10 antagonist bacterial strains used in this study were identified as strains of *Paenibacillus polymyxa* (strains K1.14, O1.27, O2.11, R3.13, R4.5, R4.24, R5.31, R6.14) and strains of *Bacillus velezensis* (V3.14, R4.6). The spectrum of produced NRPs in the bacterial antagonist strains of

297 *Bacillus* and *Paenibacillus* against 2 different *Fusarium* species was analyzed. The highest number
298 of NRPS genes was found in *B. velezensis* V3.14 and R4.6 strains. When analyzing PCR products
299 with specific primers, the genes of surfactin and fengycin synthetases, as well as closely related
300 genes of fusaricidin synthetase were detected in these strains. In turn, genes responsible for
301 fusaricidin and polymyxin synthetase synthesis were detected in all *P. polymyxa* strains.

302 The expression level of the gene responsible for fusaricidin synthesis was increased in the vast
303 majority of bacterial antagonist strains isolated from soil when co-cultured with the Ras6.1. Only
304 one bacterial strain out of 10 (V3.14) showed increased expression of fengycin, and only when
305 this strain was co-cultured with the fungus Fus5. This may indicate that most often, in response to
306 the presence of fungi, the antagonist bacterial strains studied in this work produce the most
307 effective agent against a particular pathogen that is available for synthesis in a given strain.

308 For example, in the case of the fungus species *F. oxysporum*, this is overwhelmingly
309 fusaricidin, and in the case of *F. graminearum*, fengycin or also fusaricidin. Cases in which the
310 expression of both fusaricidin and fengycin was decreased in the presence of either fungi may
311 indicate that these strains are capable of synthesizing other non-ribosomal peptides or other
312 antagonistic agents.

313 When assessing the number of expressed NRPS genes in the bacterial strains presented in this
314 work, we note that we observed expression of only one NRPS gene in strains: O1.27, R4.5, R6.14
315 (the gene responsible for the synthesis of fusaricidin synthetase); 2 genes, polymyxin and
316 fusaricidin synthetase in strains: R5.31, O2.11, R3.13, R4.24, K1. 14; and only 2 strains out of 10
317 had altered expression of genes responsible for fusaricidin and fengycin synthesis: V3.14 and R4.6.
318 Interestingly, strain V3.14 showed induction of fusaricidin gene expression and suppression of
319 fengycin gene expression in response to the presence of the Ras6.1, and induction of fengycin gene
320 expression and suppression of fusaricidin gene expression in response to the presence of the fungus

Fus5. In the case of strain R4.6, when it was co-cultured with both types of fungi, there was an activation of fusaricidin gene expression and a decrease in fengycin expression.

It was revealed that the expression of NRPS genes statistically significantly changed compared to the control level in the presence of fungi. At the same time, each individual strain responded differently to the presence of a particular species of *Fusarium*, which may indirectly indicate the use of different strategies by the bacteria against different fungal species.

The relative gene expression of non-ribosomal peptide synthetase genes was increased on average 6-7 times relative to the control level. These differences between strains may be due to both differences in the NRPS gene clusters themselves and differences in the function and structure of regulatory genes (e.g. *degQ*, *abrB*, etc.) (Lilge et al. 2021; Vasilchenko et al. 2022).

Also, the differences between strains may be due to the influence of the epigenome, as different mechanisms of epigenetic regulation of gene expression have recently been discovered in this group, which may have a significant effect on gene expression (Vasilchenko et al. 2022).

It is also worth noting the induction of polymyxin expression by strain R3.13 in response to the presence of Fus5 and Ras6.1 (1.5- and 1.6-fold above control levels, respectively), which occurred with a concomitant decrease in fusaricidin expression (0.9- and 0.1-fold below control levels, respectively). Although in the literature polymyxin is mainly attributed to its role as an inhibitor of bacterial growth, in this situation the slight increase in expression of this gene may be due to the involvement of polymyxin in suppressing fungal growth. It is also possible that the NRPS responsible for polymyxin synthesis participates in the assembly of another compound active against fungi, which may indirectly explain the decrease in fusaricidin expression.

An interesting direction for further research is the study of the spectrum of metabolites and toxins released by different *Fusarium* strains, since it is unclear to which specific stimuli the antagonist bacterial strain responds by changing the expression spectrum and adapting it to a particular pathogen.

Conclusion

It can be concluded that in each of the bacterial strains of the genera *Bacillus* and *Paenibacillus* with high antagonistic activity against *Fusarium* strains 2-3 genes of NRPS were detected by PCR, whereas in bacterial strains with low activity, only 3 out of 20 studied strains had 1 gene each. The antagonistic activity of the selected bacterial strains with a high level of antagonism against phytopathogenic strains of *Fusarium* is probably to a greater extent due to the action of non-ribosomal peptide synthetases, which is proved by the absence of chitinases.

In experiments on co-cultivation of bacteria with different levels of antagonism and fungi of the genus *Fusarium*, induction of NRPS gene expression was detected only in strains with high antagonistic activity. At the same time, the level and spectrum of NRPS gene expression in bacterial strains with high antagonistic activity depends on both the fungus species and the bacterial strain when they are co-cultured.

Species- and strain-specificity of bacterial response to different antagonist fungi is a factor that should be taken into account in the development of antifungal drugs, as well as their use. In the light of the data obtained, the development of drugs adapted to specific pathogens seems promising.

Acknowledgements

The research was financially supported by the Ministry of Science and Higher Education of the Russian Federation (no. FENW-2023-0008) and by the Strategic Academic Leadership Program of the Southern Federal University ("Priority 2030", СП-12-23-04). The authors express their gratitude to LLC "Rostov Management Company" for the materials provided.

References

1. Araújo FF, Henning AA, Hungria M (2005) Phytohormones and Antibiotics Produced by Bacillus subtilis and their Effects on Seed Pathogenic Fungi and on Soybean Root Development. World J Microbiol Biotechnol 21:1639–1645. https://doi.org/10.1007/s11274-005-3621-x

2. Chang W-T, Chen M-L, Wang S-L (2010) An antifungal chitinase produced by Bacillus subtilis using chitin waste as a carbon source. World J Microbiol Biotechnol 26:945–950. https://doi.org/10.1007/s11274-009-0244-7

3. Chen Y, Yao K, Wang K, et al (2020) Bioactive-guided structural optimization of 1,2,3-triazole phenylhydrazones as potential fungicides against Fusarium graminearum. Pesticide Biochemistry and Physiology 164:26–32. https://doi.org/10.1016/j.pestbp.2019.12.004

4. Costa A, Corallo B, Amarelle V, et al (2022) Paenibacillus sp. Strain UY79, Isolated from a Root Nodule of Arachis villosa, Displays a Broad Spectrum of Antifungal Activity. Applied and Environmental Microbiology 88:e01645-21. https://doi.org/10.1128/AEM.01645-21

5. Danielsson J, Reva O, Meijer J (2007) Protection of Oilseed Rape (Brassica napus) Toward Fungal Pathogens by Strains of Plant-associated Bacillus amyloliquefaciens. Microb Ecol 54:134–140. https://doi.org/10.1007/s00248-006-9181-2

6. Ekwomadu TI, Akinola SA, Mwanza M (2021) Fusarium Mycotoxins, Their Metabolites (Free, Emerging, and Masked), Food Safety Concerns, and Health Impacts. International Journal of Environmental Research and Public Health 18:11741. https://doi.org/10.3390/ijerph182211741

7. Feksa HR, Do Couto HTZ, Garozi R, et al (2019) Pre- and postinfection application of strobilurin-triazole premixes and single fungicides for control of fusarium head blight and deoxynivalenol mycotoxin in wheat. Crop Protection 117:128–134. https://doi.org/10.1016/j.cropro.2018.12.003

8. Gasque G, Conway S, Huang J, et al (2013) Small molecule drug screening in Drosophila identifies the 5HT2A receptor as a feeding modulation target. Sci Rep 3:srep02120. https://doi.org/10.1038/srep02120

9. Gorovtsov, A. V., et al. "The influence of soil type and preceding crop on the suppression of fusarium by indigenous spore-forming bacteria." Periodico Tche Quimica 16.33 (2019): 225-240.

10. Haddoudi I, Cabrefiga J, Mora I, et al (2021) Biological control of Fusarium wilt caused by Fusarium equiseti in Vicia faba with broad spectrum antifungal plant-associated Bacillus spp. Biological Control 160:104671. https://doi.org/10.1016/j.biocontrol.2021.104671

11. He D, Shi J, Qiu J, et al (2023) Antifungal activities of a novel triazole fungicide, mefentrifluconazole, against the major maize pathogen Fusarium verticillioides. Pesticide Biochemistry and Physiology 192:105398. https://doi.org/10.1016/j.pestbp.2023.105398

12. Kaspar F, Neubauer P, Gimpel M (2019) Bioactive Secondary Metabolites from Bacillus subtilis: A Comprehensive Review. J Nat Prod 82:2038–2053. https://doi.org/10.1021/acs.jnatprod.9b00110

13. Li C, Fan S, Zhang Y, et al (2022) Toxicity, bioactivity of triazole fungicide metconazole and its effect on mycotoxin production by Fusarium verticillioides: New perspective from an enantiomeric level. Science of The Total Environment 828:154432. https://doi.org/10.1016/j.scitotenv.2022.154432

14. Li X, Wang X, Shi X, et al (2020) Antifungal Effect of Volatile Organic Compounds from Bacillus velezensis CT32 against Verticillium dahliae and Fusarium oxysporum. Processes 8:1674. https://doi.org/10.3390/pr8121674

15. Lilge L, Vahidinasab M, Adiek I, et al (2021) Expression of degQ gene and its effect on lipopeptide production as well as formation of secretory proteases in Bacillus subtilis strains. MicrobiologyOpen 10:e1241. https://doi.org/10.1002/mbo3.1241

16. Martínez-Álvarez JC, Castro-Martínez C, Sánchez-Peña P, et al (2016) Development of a powder formulation based on Bacillus cereus sensu lato strain B25 spores for biological control of Fusarium verticillioides in maize plants. World J Microbiol Biotechnol 32:75. https://doi.org/10.1007/s11274-015-2000-5

17. Naing KW, Nguyen XH, Anees M, et al (2015) Biocontrol of Fusarium wilt disease in tomato by Paenibacillus ehimensis KWN38. World J Microbiol Biotechnol 31:165–174. https://doi.org/10.1007/s11274-014-1771-4

18. Nikitin DA, Ivanova EA, Semenov MV, et al (2023) Diversity, Ecological Characteristics and Identification of Some Problematic Phytopathogenic Fusarium in Soil: A Review. Diversity 15:49. https://doi.org/10.3390/d15010049

19. Ntushelo K, Ledwaba LK, Rauwane ME, et al (2019) The Mode of Action of Bacillus Species against Fusarium graminearum, Tools for Investigation, and Future Prospects. Toxins 11:606. https://doi.org/10.3390/toxins11100606

20. Olishevska S, Nickzad A, Déziel E (2019) Bacillus and Paenibacillus secreted polyketides and peptides involved in controlling human and plant pathogens. Appl Microbiol Biotechnol 103:1189–1215. https://doi.org/10.1007/s00253-018-9541-0

21. Olmedo C, Koch A, Sarmiento B, et al (2022) Antifungal potential of biosurfactants produced by strains of Bacillus spp. (Bacillales: Bacillaceae) selected by molecular

439 screening. Revista de Biología Tropical 70:636–646.
440 https://doi.org/10.15517/rev.biol.trop.2022.49716

441 22. Palazzini JM, Dunlap CA, Bowman MJ, Chulze SN (2016) Bacillus velezensis RC 218 as
442 a biocontrol agent to reduce Fusarium head blight and deoxynivalenol accumulation:
443 Genome sequencing and secondary metabolite cluster profiles. Microbiological Research
444 192:30–36. https://doi.org/10.1016/j.micres.2016.06.002

445 23. Ranjan A, Rajput VD, Prazdnova EV, et al (2023a) Nature's Antimicrobial Arsenal: Non-
446 Ribosomal Peptides from PGPB for Plant Pathogen Biocontrol. Fermentation 9:597.
447 https://doi.org/10.3390/fermentation9070597

448 24. Ranjan A, Rajput VD, Prazdnova EV, et al (2023b) Nature's Antimicrobial Arsenal: Non-
449 Ribosomal Peptides from PGPB for Plant Pathogen Biocontrol. Fermentation 9:597.
450 https://doi.org/10.3390/fermentation9070597

451 25. Rao X, Huang X, Zhou Z, Lin X (2013) An improvement of the 2ˆ(–delta delta CT) method
452 for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma
453 Biomath 3:71–85

454 26. Resendiz Sharpe A, Lagrou K, Meis JF, et al (2018) Triazole resistance surveillance in
455 Aspergillus fumigatus. Medical Mycology 56:S83–S92.
456 https://doi.org/10.1093/mmy/myx144

457 27. Ruiu L (2020) Plant-Growth-Promoting Bacteria (PGPB) against Insects and Other
458 Agricultural Pests. Agronomy 10:861. https://doi.org/10.3390/agronomy10060861

459 28. Shi L, Du N, Shu S, et al (2017) Paenibacillus polymyxa NSY50 suppresses Fusarium wilt
460 in cucumbers by regulating the rhizospheric microbial community. Sci Rep 7:41234.
461 https://doi.org/10.1038/srep41234

29. Soni R, Keharia H (2021) Phytostimulation and biocontrol potential of Gram-positive endospore-forming Bacilli. Planta 254:49. https://doi.org/10.1007/s00425-021-03695-0

30. Süssmuth RD, Mainz A (2017) Nonribosomal Peptide Synthesis—Principles and Prospects. Angewandte Chemie International Edition 56:3770–3821. https://doi.org/10.1002/anie.201609079

31. Swiontek Brzezinska M, Kalwasińska A, Świątczak J, et al (2020) Exploring the properties of chitinolytic Bacillus isolates for the pathogens biological control. Microbial Pathogenesis 148:104462. https://doi.org/10.1016/j.micpath.2020.104462

32. Thakur N, Nath AK, Chauhan A, Gupta R (2022) Purification, characterization, and antifungal activity of Bacillus cereus strain NK91 chitinase from rhizospheric soil samples of Himachal Pradesh, India. Biotechnology and Applied Biochemistry 69:1830–1842. https://doi.org/10.1002/bab.2250

33. Timmusk S, Copolovici D, Copolovici L, et al (2019) Paenibacillus polymyxa biofilm polysaccharides antagonise Fusarium graminearum. Sci Rep 9:662. https://doi.org/10.1038/s41598-018-37718-w

34. Tupinambá G, Da Silva AJR, Alviano CS, et al (2008) Antimicrobial activity of Paenibacillus polymyxa SCE2 against some mycotoxin-producing fungi. Journal of Applied Microbiology 105:1044–1053. https://doi.org/10.1111/j.1365-2672.2008.03844.x

35. Vasilchenko N, Kulikov M, Stacenko V, et al (2022) Regulation of Nonribosomal Peptide Synthesis as a Mechanism of Antifungal Activity of Probiotics Based on the Bacteria Genera Bacillus and Paenibacillus. In: Beskopylny A, Shamtsyan M (eds) XIV International Scientific Conference “INTERAGROMASH 2021". Springer International Publishing, Cham, pp 111–121

36. Weete JD, Wise ML (1987) Effects of triazoles on fungi: V. Response by a naturally tolerant species,Mucor rouxii. Experimental Mycology 11:214–222. https://doi.org/10.1016/0147-5975(87)90007-7

37. Xu SJ, Hong SJ, Choi W, Kim BS (2014) Antifungal Activity of Paenibacillus kribbensis Strain T-9 Isolated from Soils against Several Plant Pathogenic Fungi. Plant Pathol J 30:102–108. https://doi.org/10.5423/PPJ.OA.05.2013.0052

38. Yerkovich N, Cantoro R, Palazzini JM, et al (2020) Fusarium head blight in Argentina: Pathogen aggressiveness, triazole tolerance and biocontrol-cultivar combined strategy to reduce disease and deoxynivalenol in wheat. Crop Protection 137:105300. https://doi.org/10.1016/j.cropro.2020.105300

39. Zalila-Kolsi I, Ben Mahmoud A, Ali H, et al (2016) Antagonist effects of Bacillus spp. strains against Fusarium graminearum for protection of durum wheat (Triticum turgidum L. subsp. durum). Microbiological Research 192:148–158. https://doi.org/10.1016/j.micres.2016.06.012

40. ZHANG Chu-Mei SC-J (2010) Identification of an Endophytic Paenibacillus polymyxa Strain Producing Antifungal Protein and the Inhibition to Fusarium moniliforme causing Rice Bakanae Disease. China Biotechnology 30:84–88

41. Zhao P, Quan C, Wang Y, et al (2014) Bacillus amyloliquefaciens Q-426 as a potential biocontrol agent against Fusarium oxysporum f. sp. spinaciae. Journal of Basic Microbiology 54:448–456. https://doi.org/10.1002/jobm.201200414

Table 1 Characteristics of primers used to search for NRPS genes

Target	Primer sequence	Tm °C	Amplicon length, bp. n	Source
Non-ribosomal peptide synthetases				
Surfactin synthetase, *srfAA* gene	F: TTTAATAGCGGCCATCTG R: GAAGTGTCTTCATCAGATCC	54	993-1036*	(Coutte et al. 2010)
Fengicin synthetase, *fenC* gene	F: CTGAATCTCTTGCGCCATGT R: TGATCTGCTGTGCTCCTTCA	60	214	(Zihalirwa et al. 2017)
Fusaricidin synthetase, *fusA* gene	F:GCAGAGGATGATAGTGTTGGTC R: CAGCACATCATGCGTTCC	60	110	(Raza et al. 2015)
Polymyxin synthetase, *pmxE* gene	F: GAATCGGCTCGTCTCTCCAG R: GATGTGGACATTACGCGCAC	60	775	Primer Blast
Iturin synthetase, gene *ituA*	F: TCCAGACAATGACGGATGGC R: TTGAAGGACCACGAGTTCGG	57	994	(Narendra et al. 2017)
Reference genes				
Bacillus 16s rRNA ribosome small subunit gene	F: CCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	64	195	(Sun et al. 2013)
Paenibacillus rRNA 16s ribosome small subunit gene	F: CATTCATCGTTTACGGCGT R: TGTTAATCCCGAGGCTCACT	64	210	(Raza et al. 2010)

*The amplicon size variation is due to nucleotide sequence variation between primer binding sites

Table 2 Genes of different non-ribosomal peptide synthetases detected in bacterial strains by PCR and electrophoresis methods

			Gene						
			srfAA	*fenC*	*fusA*	*pmxE*	*ituA*	*16sBam*	*16sPmx*
Bacterial strains	(symbols in the graphs below)	1	-	-	-	-	-	+	-
		2	-	-	-	-	-	+	-
		3	-	-	+	-	-	+	-
		4	-	-	-	-	-	+	-
		5	-	+	-	-	-	+	-
		6	-	-	-	-	-	+	-
		7	-	-	-	-	-	+	-
		8	-	-	-	-	-	+	-
		9	+	-	-	-	-	+	-
		10	-	-	-	-	-	+	-
		11	-	-	-	-	-	+	-
		12	-	-	-	-	-	+	-
		13	-	-	-	-	-	+	-
		14	-	-	-	-	-	+	-
		15	-	-	-	-	-	+	-
		16	-	-	-	-	-	+	-
		17	-	-	-	-	-	+	-
		18	-	-	-	-	-	+	-
		19	-	-	-	-	-	+	-
		20	-	-	-	-	-	+	-
		K1.14(VI)	-	-	+	+	-	+	+
		O1.27(I)	-	-	+	+	-	+	+
		O2.11 (VIII)	-	-	+	+	-	+	+
		R3.13 (II)	-	-	+	+	-	+	+
		V3.14 (IX)	+	+	+	-	-	+	-
		R4.5 (V)	-	-	+	+	-	+	+
		R4.6 (IV)	+	+	+	-	-	+	-
		R4.24 (X)	-	-	+	+	-	+	+
		R5.31 (III)	-	-	+	+	-	+	+
		R6.14 (VII)	-	-	+	+	-	+	+

* "+" - presence of the gene, "-" - absence of the gene

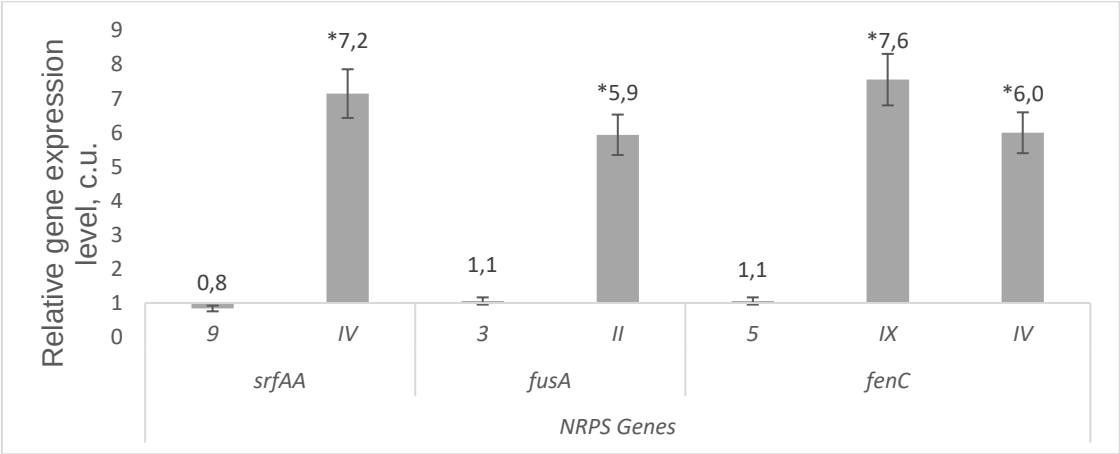
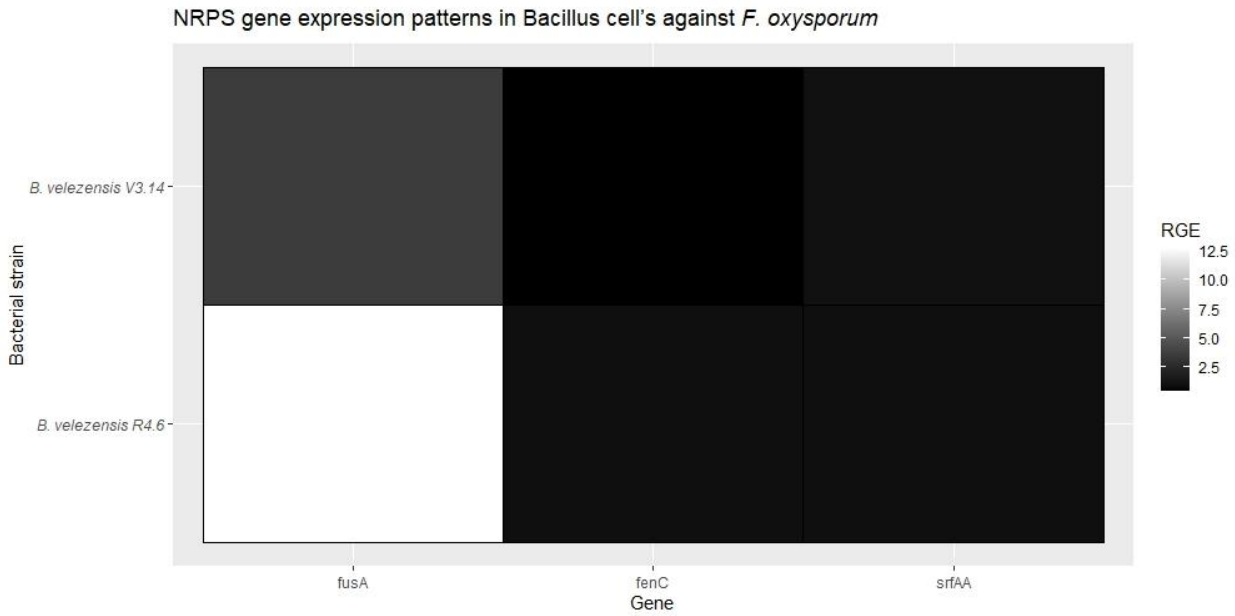
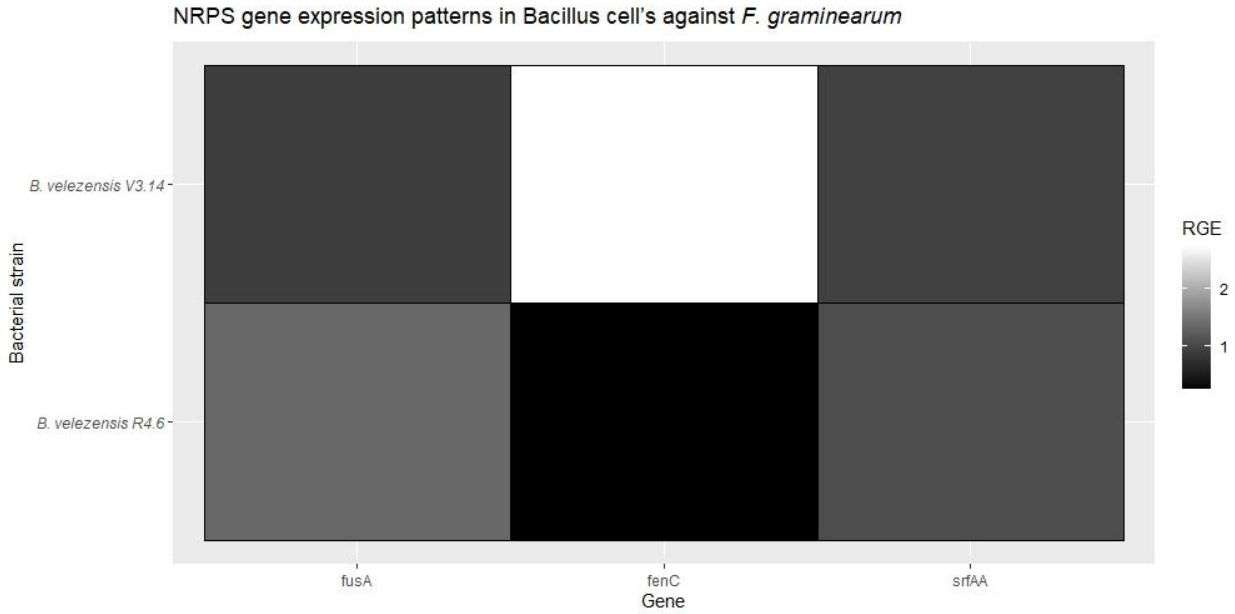


Fig. 1 Analysis of relative expression of NRPS genes in different bacterial strains in the presence of the fungus *F. oxysporum* Ras6.2.1. Numerical designations on the X-axis: Arabic numerals denote bacterial strains without expressed antagonistic properties but in which NRPS genes were detected. Roman numerals - antagonist bacterial strains. * - statistically significant differences compared to the control at $p < 0.05$



(A)

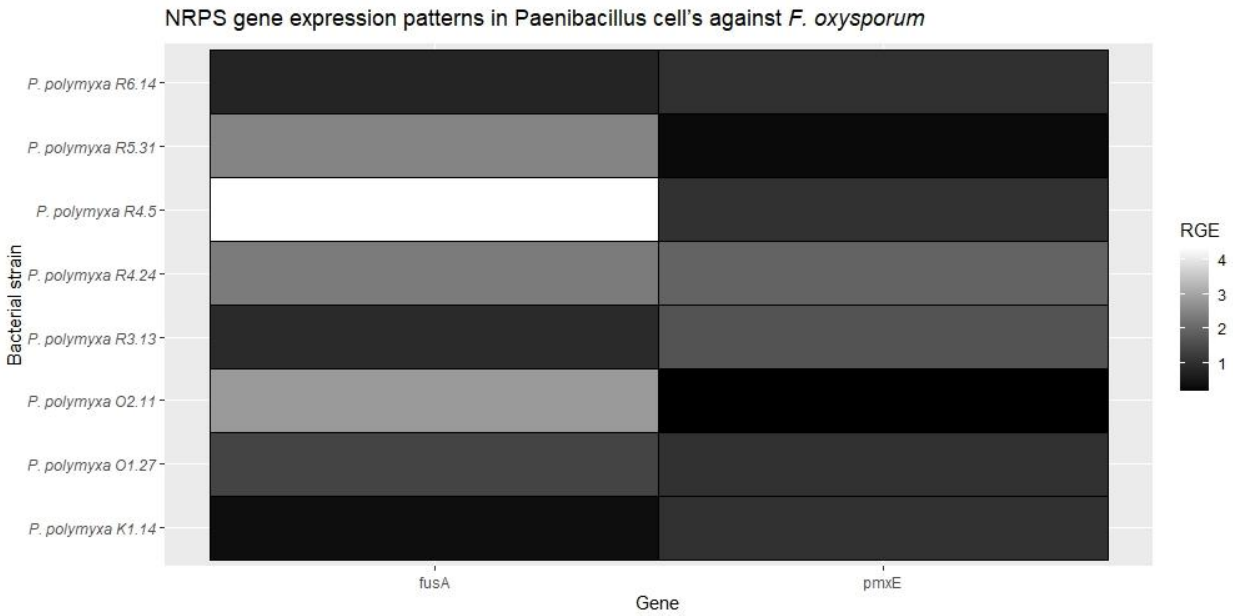


(B)

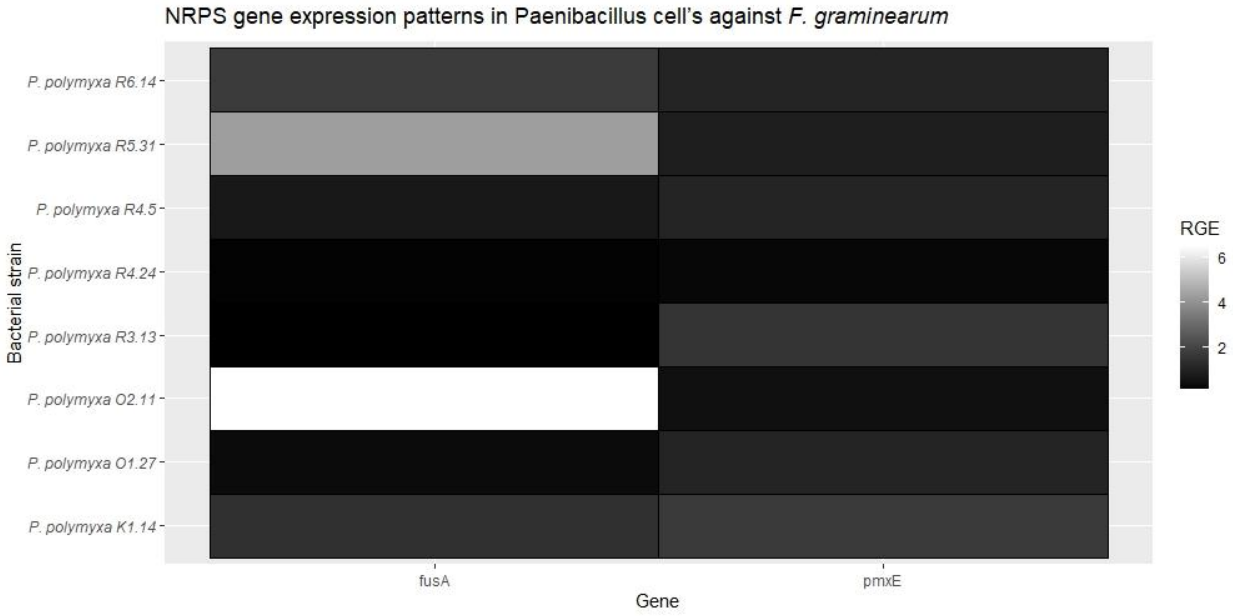
Fig. 2 Changes in NRPS gene expression patterns in strains of *B. velezensis*.

(A) in the presence of *F. oxysporum* strain Ras6.

(B) in the presence of *F. graminearum* strain Fus5



(A)



(B)

Fig. 3 Changes in NRPS gene expression patterns in strains of *P. polymyxa*

(A) in the presence of *F. oxysporum* strain Ras6

(B) in the presence of *F. graminearum* strain Fus5

Table 3 Changes in NRPS gene expression patterns in antagonist bacterial strains of *Fusarium*

Bacterial strain	**Fungal strain**							
	Fus5 (*Fusarium graminearum*)				**Ras 6.1** (*Fusarium oxysporum*)			
	Changes in relative gene expression							
	fenC	*fusA*	*pmxE*	*srfAA*	*fenC*	*fusA*	*pmxE*	*srfAA*
K1.14 (*P. polymyxa*)		▲1,4 ±0,14*	▲1,7 ±0,17			▼0,3 ±0,03	▬	
O1.27 (*P. polymyxa*)		▼0,3 ±0,01	▬			▲1,4 ±0,07	▬	
O2.11 (*P. polymyxa*)		▲6,5 ±0,65	▼0,4 ±0,04			▲2,8 ±0,28	▼0,2 ±0,02	
R3.13 (*P. polymyxa*)		▼0,1 ±0,00	▲1,5 ±0,08			▼0,9 ±0,04	▲1,6 ±0,08	
V3.14 *(B. velezensis)*	▲2,7 ±0,27	▬		▬	▼ 0,4 ±0,04	▲3,5 ±0,35		▬
R4.5 (*P. polymyxa*)		▼0,6 ±0,06	▬			▲4,4 ±0,44	▬	
R4.6 *(B. velezensis)*	▼0,3 ±0,03	▲1,4 ±0,14		▬	▬	▲12,8 ±1,28		▬
R4.24 (*P. polymyxa*)		▼0,1 ±0,01	▼0,2 ±0,02			▲ 2,3 ±0,23	▲ 1,9 ±0,19	
R5.31 (*P. polymyxa*)		▲4,3 ±0,43	▼0,8 ±0,08			▲2,5 ±0,25	▼0,3 ±0,03	
R6.14 (*P. polymyxa*)		▲1,7 ±0,08	▬			▼0,8 ±0,04	▬	

▲, up arrow - increase in expression; ▼, down arrow - decrease in expression; ▬, rectangle - expression did not differ from control level; empty cells - absence of gene; arrows show only * - statistically significant differences at $p<0.05$.