

Proceedings

Enhanced Performance of Endophytic *Bacillus subtilis* in Composition with Salicylic Acid Meliorated Simultaneous Drought and *Fusarium* Root Rot Stresses in *Triticum aestivum* L. †

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Abstract: Plants are constantly faced with both abiotic and biotic stresses, which seriously reduce their productivity. The potential of endophytic bacteria *Bacillus subtilis* (strain 10-4) alone and in the mix with salicylic acid (SA) to meliorate drought and *Fusarium* root rot (FRR) stresses in *Triticum aestivum* L. were evaluated. All microbiological, molecular, and physio-biochemical parameters were assessed using classical, and modern methods. The findings demonstrated *B. subtilis* 10-4 alone and especially in the mix with SA significantly improve wheat growth and ameliorated the damaging influence of drought, FRR, and drought+FRR. An important contribution to observed growth-stimulating and protective effects of *B. subtilis* 10-4 and SA on plants most likely makes the revealed ability of bacteria to produce auxins, siderophores, lipopeptides surfactin, and to colonize internal plant tissues. Also, bio-priming of seeds with *B. subtilis* 10-4 alone and especially in the mix with SA decreased stress-induced (drought, FRR, drought+FRR) lipid peroxidation and amino acid proline accumulation in plants, thereby indicating on protecting the plant cells against reactive oxygen species and osmotic damages. Current research provides novel insights into the potential and mechanism of *B. subtilis* 10-4 and SA in the mitigation of combined drought+FRR stresses in wheat.

Keywords: endophytic *Bacillus subtilis*; salicylic acid; *Triticum aestivum* L.; morpho-physiological traits; drought and *Fusarium* infection; induced tolerance

1. Introduction

Triticum aestivum L. (wheat) is a valuable food crop with great importance in ensuring food security around the world [1]. One of the most pressing problems of agriculture and food industries are wheat yield losses (up to 50–82%) from drought, *Fusarium*-caused diseases and combinations of these stresses [1–3]. Traditionally, chemical pesticides have played a central role in plant protection against diseases and abiotic stresses. However, the negative impact of pesticides on the environment and human health due to their high toxicity and the ability to accumulate in products in quantities exceeding sanitary standards lead to the need to reduce their use in food production and stimulates scientists around the world to show interest in the development of environmentally friendly and safe biologicals based on beneficial bacterial endophytes, such as *Bacillus subtilis* [4]. *B. subtilis* capable to improve host-plant growth and induce systemic resistance/tolerance to a wide range of pathogens and abiotic stresses [1,4]. One of the main reasons hindering the development of *Bacillus*-based biologicals is the lack of knowledge underlying interactions between host-plants and endophytic *B. subtilis* under biotic/abiotic stresses. Moreover, it is difficult to select an individual effective microbial strain with a broad spectrum of simultaneous activity against a range of pathogens and abiotic

stresses. That is why interest is co-application of *B. subtilis* with other biological methods and unraveling the mechanisms of their actions [1]. Of particular interest is the use of endophytic *B. subtilis* in combinations with natural and safe signaling molecules with a pronounced anti-stress activity, such as salicylic acid (SA)—a recognized inducer of plant systemic resistance/tolerance to diseases and abiotic stresses [4,5]. To date, a large body of information has accumulated indicating the participation of SA in the regulation of defense reactions of different plant species to phytopathogens and abiotic stresses (salinity, drought, hypo/hyperthermia, heavy metals, etc.) [5,6]. However, the information on the joint use of *B. subtilis* and SA is limited, especially in relation to combined biotic/abiotic stresses.

This study aimed to investigate the effect of endophytic bacteria *Bacillus subtilis* 10-4 and its mix together with salicylic (SA) on some physio-biochemical traits of *Triticum aestivum* L. (wheat) plants under the influence of combined biotic (*Fusarium* infection) and abiotic (drought) stresses.

2. Experiments

The experiments were carried out in hydroponically growth wheat (*Triticum aestivum* L., variety Omskaya 35) seedlings in the early stages of ontogenesis (the first 1–3 weeks). The seeds before sowing were treated with *B. subtilis* (10^5 CFU mL⁻¹) [7,8], SA (0.05 mM) [5], *B. subtilis* (10^5 CFU mL⁻¹) + SA (0.05 mM) or distilled water (Control) by immersing in the solutions for 1 h. Then treated and non-treated seeds were grown on filter paper moistened with water for 3 days ($t = 20\text{--}22$ °C, 16-h light period, 12–16 000 lux). Then, the seedlings were transplanted into the glasses with solutions of 12% PEG-6000 (drought condition) or water (normal condition). Infection of wheat with the phytopathogenic fungus *Fusarium* spp. was carried out by introducing into the base of the stems of 5 days old seedlings of a suspension of fungus conidia in water (10^6 spores mL⁻¹). The intensity of diseases development was assessed by visual symptoms (0 points—no symptoms, 1 point—damage from 1 to 25%, 2 points—from 26 to 50%, 3 points—from 51 to 75%, 4 points—more than 75%; 5 points—dead plant). In each variant, 50 plants were used in 2–3 repetitions. The tested strain 10-4 was isolated and identified in our previous work [7]. Phytopathogenic fungus *Fusarium culmorum* was obtained from the collection of BRIA UFRC RAS (Ufa, Russia).

Comparative analysis of the influence of *B. subtilis* 10-4, SA and *B. subtilis* 10-4 + SA on the length of seedlings (roots, shoots) of wheat (variety Omskaya 35) and fresh (FW) and dry (DW) biomass accumulation under normal growing conditions, when infected with phytopathogenic fungi *Fusarium* spp. and exposed to drought (12% PEG-6000) were carried out using classical physiological methods [9]. In each variant, 50 plants were used in 2–3 repetitions.

The concentration of amino acid proline (Pro) accumulation and the degree of lipid peroxidation were determined according to the methods described by Bates et al. [10] and Health and Packer [11], respectively. The ability of *B. subtilis* 10-4 to colonizing for the internal parts of seedlings was assessed using surface-sterilized plant segments and RAPD-PCR analysis [7], indole-3-acetic acid (IAA)-production using LB medium with L-tryptophan [12], phosphate solubilization using Pikovskaya medium [13], nitrogen fixation using gas chromatography [14], siderophore production using Chrome Azurol S dye [15]. Other physio-biochemical properties of strain 10-4 were tested using test-system Set №2 (MicroGen, Nizhny Novgorod, Russia). The ability of *B. subtilis* 10-4 to produce metabolites was tested on the MOLP medium, extracted with n-butanol, and analyzed by the UHPLC-MS method [16]. Shortly, cells of *B. subtilis* 10-4 were cultured in MOLP medium (130 rpm, 36 °C, 6 days), cells were pelleted by centrifugation (5000 rpm, 60 min). LP was extracted with n-butanol, the organic phase was evaporated on a rotary evaporator, the residue was lyophilized, dissolved in 10% methanol, and analyzed). *B. subtilis*-produced metabolites and extracted LP antifungal activity was tested according to [8,16].

All experiments were carried out in three biological and three analytical replicates. The data were presented as the mean of standard error (\pm SEM). Statistically significant differences between the mean values were evaluated using analysis of variance (ANOVA), followed by the Tukey test ($p < 0.05$).

3. Results and Discussion

3.1. Characteristics and Properties of the Tested Strain *Bacillus Subtilis* 10-4

3.1.1. Some Plant Growth Promoting (PGP) and Physio-Biochemical Properties

Bacterial strain 10-4 was previously isolated from the arable soils (Republic of Bashkortostan, Russia) and identified as *Bacillus subtilis* using 16S rRNA gene sequence analysis (similarity 99.8%) [7]. Further studies allowed to found that *B. subtilis* 10-4 have such plant growth-promoting (PGP) characteristics as the production of indole-3-acetic acid (IAA), siderophores, and fixing atmospheric nitrogen (Table 1). Moreover, strain 10-4 showed oxidase-negative and catalase-positive reactions, did not utilized galactose, did not dispose of inositol, and did not form indoles and hydrogen sulfide. Additionally, strain 10-4 gave a positive Voges–Proskauer reaction and a negative for PAD activity (Figure 1a). Among PGP traits, IAA plays a vital role in plant growth and development [17]. Our finding demonstrated that in presence of L-TRP, which is considered to be the main precursor of IAA formation in microorganisms [18], *B. subtilis* 10-4 produce IAA (Figure 1a). Another important PGP trait detected for strain 10-4 is siderophore production which significantly influences plant growth due to contributing to the transport of Fe³⁺ inside the plant cells [17]. The results showed *B. subtilis* 10-4 in different concentration improved 6 days old wheat seedlings growth (Figure 1b) but as the most optimal concentration in stimulating both roots and shoots length and their FW and DW accumulation was 10⁵ CFU mL⁻¹ (Figure 1c). Obviously, the detected ability of *B. subtilis* 10-4 to produce bioactive compounds (Figure 1a) makes a significant contribution to plant growth. Also, plant growth stimulation may occur due to the direct penetration of bacteria into the internal tissues and organs of plants [19].

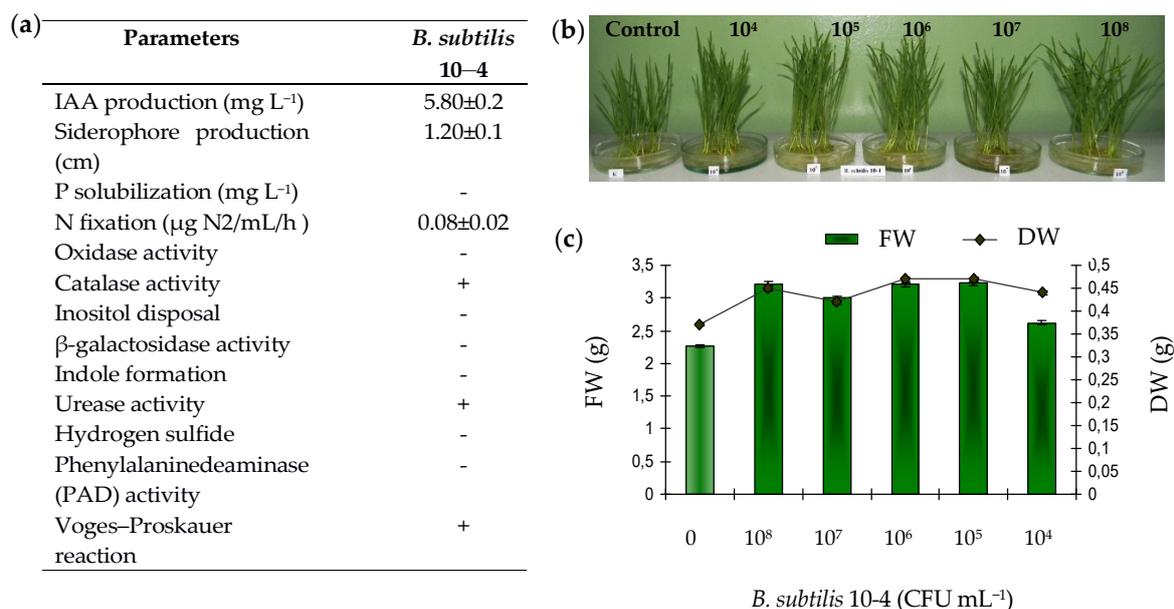


Figure 1. Some PGP traits and physio–biochemical properties of bacterial strain *B. subtilis* 10-4 (a) and effect of pre-inoculation with *B. subtilis* 10-4 in different concentration (0 (control), 10⁴, 10⁵, 10⁶, 10⁷, 10⁸ CFU mL⁻¹) on the growth of 6 days old wheat seedlings (b) and their fresh (FW) and dry (DW) biomass accumulation (c).

3.1.2. The Ability of *B. subtilis* 10-4 to Colonize Internal Wheat Tissues

Among PGP bacteria a special interest in endophytic ones due to their ability to colonize internal host-plant tissues and make more effective relationships without hampering a negative influence on plants [18,19]. Using surface-sterilized wheat seedlings (grown in sterile conditions) and RAPD-PCR analysis we observed that *B. subtilis* 10-4 effectively colonize internal wheat tissues after pre-sowing inoculation (Figure 2). Around control (non-inoculated) plant segments, bacterial growth was absent (Figure 1a), while around *B. subtilis* (10-4)-inoculated one's endophytic bacteria growth was observed (Figure 2b). RAPD-PCR analysis confirmed that bacteria grown around plants are identity to native bacterial strain 10-4 used for pre-sowing inoculation of seeds (Figure 2c).

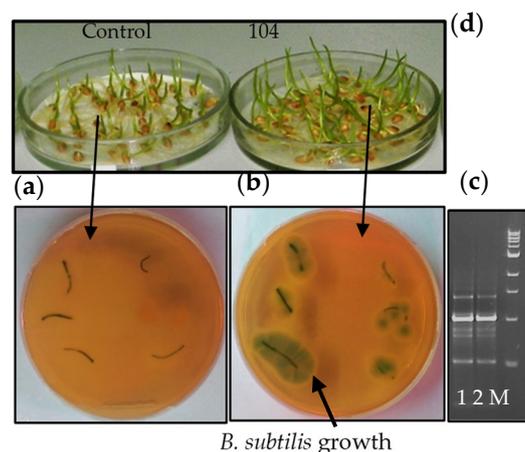


Figure 2. Testing the ability of *B. subtilis* 10-4 to colonize internal wheat seedling (3 days old) upon pre-sowing inoculation of surface-sterilized seeds and after growing in sterile conditions for 3 days. (a) the absence of bacterial growth around non-bacterial inoculated plant segments; (b) bacterial growth (*light green colonies) around the surface-sterilized leaf and roots segments of wheat seedlings pre-inoculated with *B. subtilis* 10-4; (c) electropherogram of PAAG: 1—DNA of origin *B. subtilis* 10-4; 2—DNA of bacteria isolated from the bacteria growing around wheat segments; M—DNA marker.

3.1.3. Biocontrol Activity of *B. subtilis* 10-4 Cells, Metabolites and Lipopeptides against *Fusarium* spp.

The obtained results of *in vitro* assays showed *B. subtilis* 10-4 exerts antagonistic activity against phytopathogenic fungus *F. culmorum* growth (Figure 3a). The microscopic observation showed the mycelia structure was well organized in the absence of the bacterial cells (Figure 3b), while numerous gaps of mycelia appeared in the presence of the *B. subtilis* 10-4 (Figure 3c). Most likely the observed fungal suppression (Figure 3a) related to the ability of *B. subtilis* 10-4 to produce various metabolites with strong antibiotic activities (Figure 3d). Our results showed *B. subtilis* 10-4 produce LPs surfactin C14, C15, and C15 (Figure 3e), which are characterized by strong antifungal activity (Figure 3f).

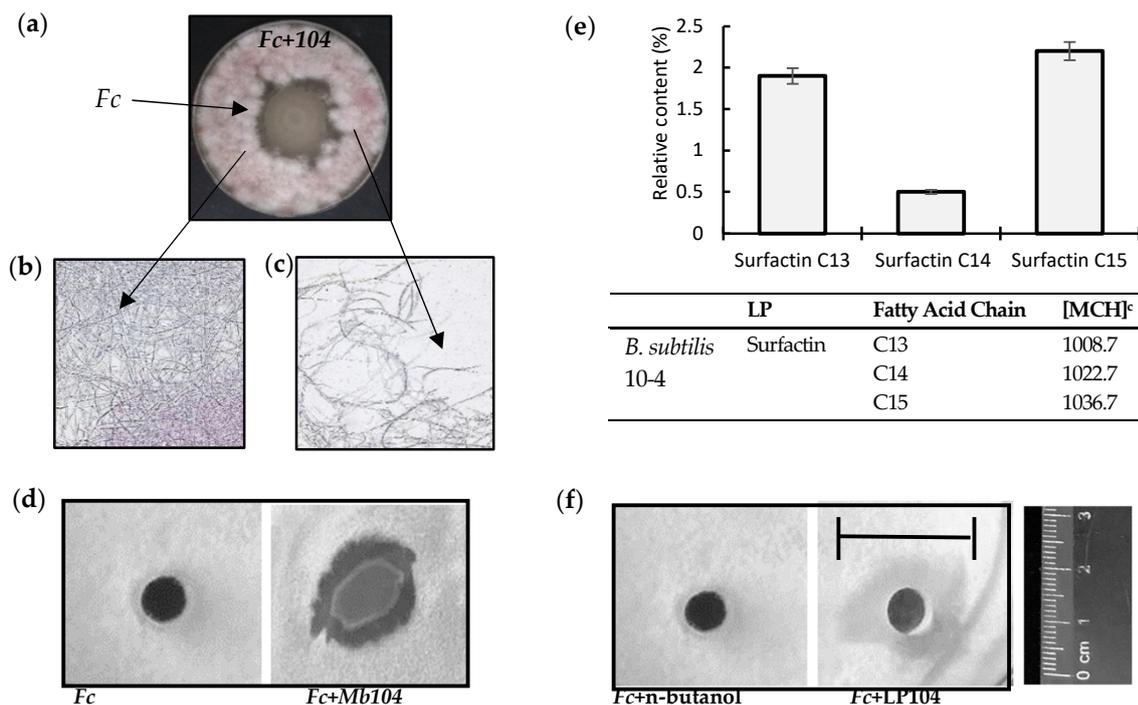
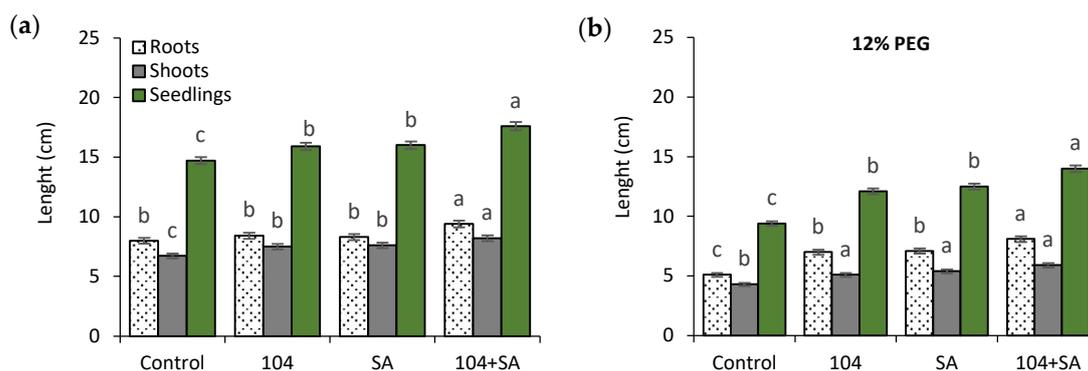


Figure 3. In vitro antagonistic activity of *B. subtilis* 10-4 (Bs104) against *Fusarium culmorum* (Fc) (a) and microscopic visualizations of the *Fc* fungal growth and morphology in the absence (b) and on the border with Bs104 (c). (d) inhibition of *Fc* by Bs104-produced metabolites (Mb104); (e) the relative content of lipopeptides (LP) produced by Bs104; (f) inhibition of *Fc* by Bs104-produced LP (LP104).

3.2. Effect of *B. subtilis* 10-4 and SA on Wheat Growth under Drought, *Fusarium* Infection and a Combination of These Stresses

In nature, plants are constantly exposed to various abiotic and biotic stresses or their combinations that can significantly inhibit the growth and productivity of major agricultural crops, including wheat [1–5]. Among biotic and abiotic stresses the impact of *Fusarium* spp.-caused diseases and drought—the most common and important combinations of stresses significantly reducing wheat yield [1–5].



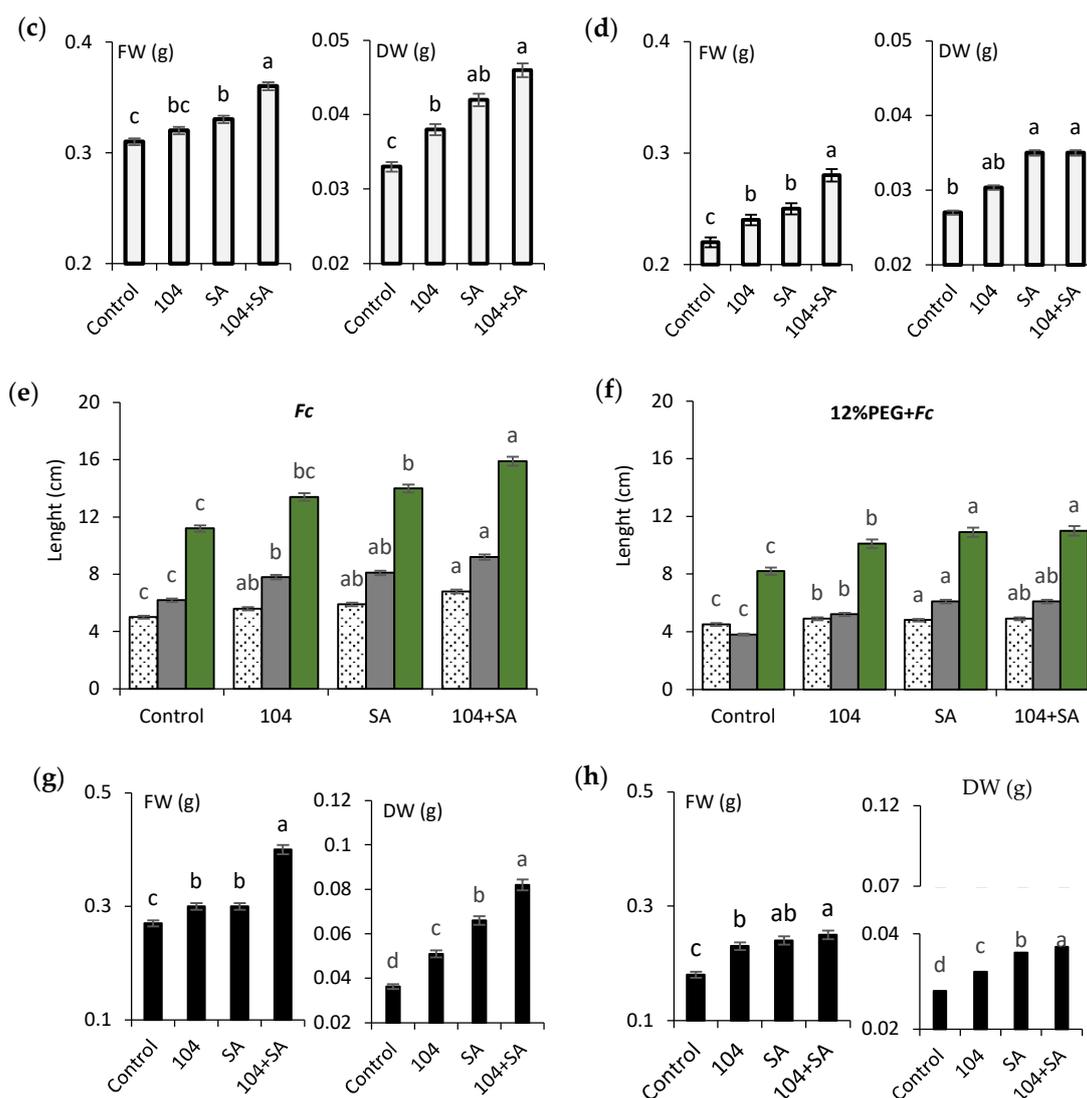


Figure 4. Effect of pre-sowing treatment with endophyte *B. subtilis* 10-4 (104), SA, and *B. subtilis* 10-4 + SA on growth of wheat seedlings (6 days old) (length of roots, shoots) and their biomass accumulation under normal (a,c), drought (12%PEG) (b,d), *Fusarium culmorum* infection (Fc) (e,g), and combined drought + *F. culmorum* infection (12%PEG+Fc) (f,h) conditions.

It was found that pre-inoculation endophyte *B. subtilis* 10-4 alone and together with SA under normal growing conditions promoted the activation of seedling growth (Figure 4a), with the best growth-stimulating after the joint application of endophyte and SA (Figure 4a). Drought, FRR, and combined drought+FRR resulted in decreased seedlings growth (Figure 4b,e,f). While pre-treatment with *B. subtilis* 10-4, SA, and *B. subtilis* 10-4 + SA softened (to varying degrees) the level of the damaging effect of these stresses on plant growth, which was reflected in higher indicators of root length and shoots of plants in comparison with untreated control. Moreover, the greatest protective effect from the damaging effect of these stresses on growth was observed when endophyte was used together with SA (Figure 4b,e,f). Similar nature of the influence of *B. subtilis* 10-4, SA and *B. subtilis* 10-4 + SA was also revealed during the assessment of plants' fresh (FW) and dry (DW) weights accumulation, both under normal conditions (Figure 4c) and in case of drought (Figure 1d), FRR (Figure 1j), and drought+FRR (Figure 2h). The findings indicate that the most effective in stimulating of growth processes under normal conditions and protecting wheat plants from the negative impact of drought, FRR, and drought+FRR is the use of *B. subtilis* 10-4 together with SA, since it is with this combination the maximum positive effect on growth indicators, including growth in the lower (roots) and aboveground (shoots) tissues and their accumulation (FW, DW) were observed (Figure 1a–d).

3.3. Changes in the Level of Lipid Peroxidation (MDA) and Proline (Pro) Content in Wheat Plants Pre-Treated with *B. subtilis* 10-4, SA, and *B. subtilis* 10-4 + SA under Combined Drought and Fusarium Infection

Abiotic/biotic stresses and their combinations result in strong oxidative and osmotic damages in cellular level due to abnormal formation of reactive oxygen species (superoxide radicals, hydrogen peroxide, etc.), and water regime disturbance [1,4,20]. As biomarkers of the development of plant's defence reactions against oxidative and osmotic stresses are the final product of lipid peroxidation—malondialdehyde (MDA) and osmoprotectant proline (Pro), which also plays role of an antioxidant and low molecular weight chaperone and involved in maintaining the native structure of enzymes reducing the degree of damage to cellular structures caused by dehydration [1,10,20]. Our results showed that seeds bio-priming with endophytic 10-4 alone and especially in the mix with SA resulted in decreased stress-induced (drought, FRR, drought+FRR) lipid peroxidation (Figure 5a) and Pro accumulation in plants (Figure 5b), thereby indicating on protecting the plant cells against reactive oxygen species and osmotic damages [20].

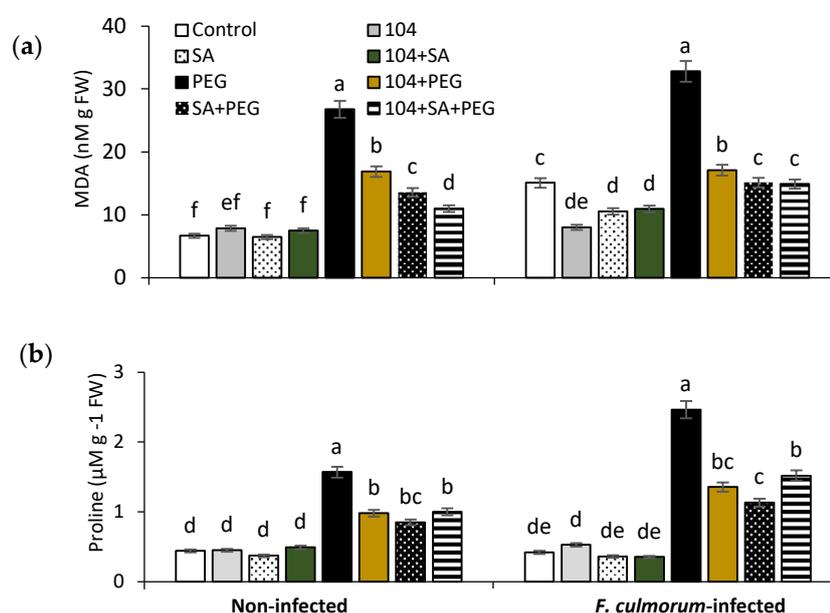


Figure 5. Changes in the content of malondialdehyde (MDA) (a) and osmoprotectant proline (Pro) (b) in healthy (Non-infected) and *Fusarium culmorum* (*F. culmorum*)-infected seedlings pre-treated before sowing with *B. subtilis* 10-4 (104), SA, and *B. subtilis* 10-4 + SA and grown under normal and combined drought stress conditions.

4. Conclusions

Thus, endophytic bacteria *B. subtilis* 10-4 alone and especially in the mix with SA significantly enhance wheat growth and softened the damaging influence of drought, FRR, and drought+FRR. An important contribution to observed growth-stimulating and protective effects of *B. subtilis* 10-4 and SA on the plants make the revealed ability of bacteria to produce bioactive compounds such as auxins, siderophores, LP surfactin (C13–C15), and colonize internal plant tissues; thereby forming close interaction with host-plant. In favor of the formation of effective interaction between *B. subtilis* 10-4 and wheat indicate the data on decreased stress-induced oxidative (MDA) and osmotic (Pro) damages of plant cells upon the influence of *B. subtilis* 10-4. Moreover, the results clearly demonstrated SA significantly increased the positive actions of *B. subtilis* 10-4 on the plant under normal and stress conditions. In total, the findings indicate *B. subtilis* 10-4 alone and especially in the mix with SA has a huge potential to be used as an eco-friendly agent to improve wheat growth and tolerance under drought, FRR, and drought+FRR stresses.

Author Contributions: O.L. conceived, designed the experiments; O.L., D.G. and A.I. performed the experiments; O.L. analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

SA	Salicylic acid
LP	Lipopeptide
FRR	<i>Fusarium</i> root rot
MDA	Malondialdehyde
Pro	Proline

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