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Improving the Biocontrol Potential of Endophytic Bacteria *Bacillus subtilis* with Salicylic Acid against *Phytophthora infestans*-Caused Postharvest Potato Tuber Late Blight and Impact on Stored Tubers Quality

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Abstract: Potato (*Solanum tuberosum* L.) tubers are a highly important food crop in many countries due to their nutritional value and health-promoting properties. Postharvest disease caused by *Phytophthora infestans* leads to the significant decay of stored potatoes. The main objective of this study was to evaluate the effects of the endophytic bacteria, *Bacillus subtilis* (strain 10–4), or its combination with salicylic acid (SA), on some resistance and quality traits of stored *Ph. infestans*-infected potato tubers. The experiments were conducted using hydroponically grown potato mini-tubers, infected prior to storage with *Ph. infestans*, and then coated with *B. subtilis*, alone and in combination with SA, which were then stored for six months. The results revealed that infection with *Ph. infestans* significantly increased tuber late blight incidence (up to 90–100%) and oxidative and osmotic damage (i.e., malondialdehyde and proline) in tubers. These phenomena were accompanied by a decrease in starch, reducing sugars (RS), and total dry matter (TDM) contents and an increase in amylase (AMY) activity. Moreover, total glycoalkaloids (GA) (α-solanine, α-chaconine) notably increased in infected tubers, exceeding (by 1.6 times) permissible safe levels (200 mg/kg FW). Treatments with *B. subtilis* or its combination with SA decreased *Ph. infestans*-activated tuber late blight incidence (by 30–40%) and reduced oxidative and osmotic damages (i.e., malondialdehyde and proline) and AMY activity in stored, infected tubers. Additionally, these treatments decreased pathogen-activated GA accumulation and increased ascorbic acid in stored tubers. Thus, the results indicated that endophytic bacteria *B. subtilis*, individually, and especially in combination with SA, have the potential to increase potato postharvest resistance to late blight and improve tuber quality in long-term storage.

Keywords: endophytic bacteria *Bacillus subtilis*; *Phytophthora infestans*; salicylic acid; potato; storage; postharvest decay; resistance; tuber quality

1. Introduction

Potato (*Solanum tuberosum* L.) is the world's fourth most produced agricultural crop after maize, wheat, and rice [1], and its tubers are used for human and animal consumption, as well as for raw materials in the production of starch [2–4]. Postharvest losses from diseases are ~50–70% annually and pose serious threats to agriculture and food security/safety worldwide [3,5].

One of the most dangerous diseases of potatoes during the cultivation, handling, transportation, and especially storage is tuber late blight, caused by the fungal-like organism, *Phytophthora infestans* (Mont.) de Bary [6–8]. As a primary inoculum, it is exposed to potato tuber seeds via oospores that overwinter in soil (thick-walled reproductive structures of *Ph. infestans*) or via zoosporangia, transported by the wind from infected plants. Most infections during a season are initiated through asexual zoosporangia, which can germinate directly or indirectly by cleaving their multinucleated cytoplasm into mononuclear zoospores [9,10]. Growth within a plant primarily involves the expansion of intercellular hyphae, which insert haustoria into host cells. As a hemibiotroph, *Ph. infestans* only attacks living tissue, extracting nutrients from the apoplast or extrahaustrial space during most of the disease cycle. *Ph. infestans* is capable of killing an entire plant, destroying the leaves and stems in 7 to 10 days, causing large-scale potato losses [10]. From aboveground parts, the zoosporangia (together with water) penetrate the soil, and the released zoospores infect tubers. However, tubers, for the most part, become infected during the harvesting period when they come into direct contact with affected tops. The oomycete penetrates into the tuber tissue through lentils, eyes, and also places where the peel has been damaged. However, symptoms of the disease are only detected 20–25 days after placing the potatoes into storage. Brown spots appear on the skin of infected tubers, and reddish-brown markings appear in their flesh. *Phytophthora*-infected tubers also may become quickly infected with other fungi and bacteria, resulting in the typical dry or wet rot of tubers, which cause the massive decay of tubers. Moreover, *Ph. infestans* has strong genetic variability, which allows it to quickly overcome the resistance of varieties and chemical fungicides [8]. Chemicals have traditionally played a central role in the control of postharvest diseases of fruits and vegetables; however, due to the toxicological risk of residual chemical fungicides in food products, their application in the postharvest period has been limited and is now completely prohibited in some countries. It is important to discover and implement safe, new alternative strategies, which can induce the natural defense mechanisms of plant organisms against postharvest diseases, without harmful effects on the environment and humans [8,11,12]. In recent years, there has been growing interest in eco-friendly and biosafe approaches to controlling the postharvest decay of potatoes, such as the application of non-pathogenic antagonistic strains of *Bacillus* sp. [11,12].

At present, a number of published studies have demonstrated the efficiency of *Bacillus* sp. as a biocontrol agent against a range of postharvest diseases of different fruits and vegetables, including potato [12–14]. Endophytic *B. subtilis*, which colonizes the same ecological niches as pathogens, is considered a promising agent for pathogen control and has a generally recognized as safe (GRAS) designation from the food industry [15–17]. Being that they are inside plant tissues, endophytes have a significant advantage over their epiphytic counterparts due to a stable pH, moisture, nutrient flow, and lack of competition from other microorganisms and adverse environmental factors in the rhizosphere [18]. Moreover, endophytes, once introduced into plant tissues, can contribute to the formation of host protection for a long time [19,20]. It was shown that the pre-planted treatment of potatoes with endophytic strain *B. subtilis* 26D resulted in a lower degree of pathogen damage during both the growing season and postharvest storage time [19]. The protective effect of *B. subtilis* can be enhanced by the co-application of other biological agents [11,12,21]. The use of endophytic *B. subtilis*, together with the signaling molecule salicylic acid (SA), results in a pronounced anti-stress activity [22]. A number of studies have revealed the effectiveness of SA application in increasing the consumer properties and resistance of different types of vegetables, fruits, and berries to diseases and stresses during storage [23–26]. Recently, the combined use of endophytic *B. subtilis* with SA was proven to be more effective than their separate use in the biocontrol of potato diseases, both during growing and storage [13,20]. These findings suggest that *B. subtilis* decreases fusarium root rot disease incidence in potato by colonizing internal tissues and protecting cells inside against pathogens development and oxidative cell damages, therewith controlling senescence processes [13]. However, the mechanisms underlying *B. subtilis* actions both

alone and in combination with SA on potato under the infection of postharvest pathogens (i.e., *Ph. infestans*) are largely unknown and require detailed investigations to fully realize their potential in agricultural/food industries.

In this study, we tested the exogenous application of *B. subtilis* on potatoes in long-term storage and measured the protective effect against tuber late blight symptoms. We used *B. subtilis* 10–4, an endophytic strain, and tested the application alone and in combination with SA. These applications were carried out on potatoes that were infected with *Ph. infestans*, and corresponding control treatments were carried out with non-infected potatoes.

2. Materials and Methods

2.1. Plant Material

The experiments were carried out on hydroponically grown potato (*Solanum tuberosum* L., cv. Bashkirsky) mini-tubers at the laboratory of Potato Breeding and Seed Production at the Bashkir Research Institute of Agriculture UFRC RAS (Ufa, Russia) using the original laboratory method described previously [13]. In vitro plant seedlings were cultivated in a hydroponic system (Minivit, KD-10, DokaGene, Zelenograd, Russia) with a continuously supplied nutrient solution (pH 5.6) (0.4–0.6%, for one week; 0.8%, for two weeks; 1.2–1.4%, for three weeks; 1.5–1.8%, from the fourth week to the end of the growing season (approximately 60 d)). The lighting mode was divided into three main periods of 120,000, 150,000, and 80,000 lux/h. Freshly harvested hydroponic potatoes (4–6 g per tuber) had an oval–round shape, smooth red skin, white flesh, and medium-depth eyes.

Cv. Bashkirsky is an early maturing table potato of Russian selection [27]. It was created by breeders of the Bashkir Research Institute of Agriculture UFRC RAS (Ufa, Russia) and the All-Russian Research Institute of Potato of A.G. Lorch (Moscow, Russia) from botanical seeds obtained by crossing the cv. Belousovsky and the hybrid 289/82-3. The plant is medium height, intermediate type, and semi-upright. It has a medium to large leaf, which is intermediate and green. The waviness of the edge is strong. Its corolla are large and red-violet. The tuber is oval–round with eyes of medium depth. The peel is smooth and red. The pulp is white. It is easy to care for and tolerates drought. It is resistant to potato wart and slightly susceptible nematode infection. The aerial parts are moderately susceptible to late blight, but the tubers are very susceptible [27].

2.2. Bacterial Strain, Pathogen, and Salicylic Acid Solution

The tested strain of endophytic bacteria *B. subtilis* 10–4 was previously isolated from the arable soils of the Republic of Bashkortostan (Russia) at the Bashkir Research Institute of Agriculture UFRC RAS (Ufa, Russia), identified using 16s rRNA analysis, described in detail and deposited in the All-Russian Collection of Industrial Microorganisms (registration number B-12988) [28]. *B. subtilis* was grown in Luria–Bertani (LB) solid medium at 37 °C. The inoculum of strain 10–4 containing 10^8 colony forming units (CFUs) per mL was prepared according to the 0.5 McFarland Turbidity Standard and monitored at 600 nm (OD_{600}) using a spectrophotometer SmartSpecTM Plus (Bio-Rad, Hercules, CA, USA). It was thereafter diluted down to 10^7 CFU mL^{-1} using solutions of SA (0.05 mM) in sterile water (to obtain the composition of *B. subtilis* 10–4 with SA) [13].

Phytopathogenic oomycete *Ph. infestans* (Mont.) de Bary (the causative agent of potato tuber late blight) isolate was obtained from the laboratory of Plant–Microbe Interactions of the Bashkir Research Institute of Agriculture UFRC RAS (Ufa, Russia). The isolate was originally isolated from potato tubers affected by tuber late blight and identified on the basis of cultural and morphological characteristics [29]. The isolate affected tuber slices of the sensitive cv. Bashkirsky in a short time, i.e., it was characterized by high pathogenicity. *Ph. infestans* were grown on potato dextrose agar (PDA) (pH ~ 6.6) at 28 °C [29]. The inoculum of *Ph. infestans* in the form of zoosporangia suspension (10^6 zoosporangia mL^{-1}) was prepared using a Goryaev chamber [29].

2.3. Experiment Design

The experiment was carried out in accordance with the method described earlier [13]. In brief, potato mini-tubers were infected (immediately before placing in storage) via their immersion into a solution of spores of *Ph. infestans* (10^8 spores mL^{-1}) for 30 min. Control group tubers were immersed into sterile water. The solutions were drained, and the tubers (after drying for 30 min at 20 °C) were immersed into the solutions of cells of *B. subtilis* 10–4 (10^8 CFU mL^{-1}) or cells of *B. subtilis* 10–4 (10^7 CFU mL^{-1}) and SA (0.05 mM) for 30 min. The concentrations of *B. subtilis* 10–4 and SA were previously selected as optimal in postharvest diseases suppression [13]. Thereafter, the solutions were drained, the were tubers dried for 30 min at 20 °C, and they were stored at 18 ± 1 °C for 2 weeks and then 3 ± 1 °C for 6 months (TVL-K 120 thermostat, Insovt, Russia). Each group had 30 tubers in 3 replications.

2.4. In Vivo and In Vitro Assay of Antagonistic Activity of B. subtilis 10–4 against Ph. infestans

Visual symptoms of tuber late blight incidence in stored potato tubers were evaluated using a five-point scale (0 points—no symptoms, 1 point—1–25% affected area, 2 points—26–50%, 3 points—51–75%, 4 points—75% and more, 5 points—100% affected). Disease development intensity on tuber slices was evaluated according to [13].

An in vitro assay of the antagonistic activity of *B. subtilis* against *Ph. infestans* was evaluated using the co-culture of bacterial strain and oomycete previously plated on Petri dishes with PDA (the antagonistic activity of *B. subtilis* 10–4 in mix with SA was evaluated in PDA medium with the addition of SA (0.05 mM)) [29]. Briefly, a spore suspension of *Ph. infestans* was applied to Petri dishes with PDA (pH 7.0), spread over all surfaces, and kept at room temperature for 2 h. Thereafter, a bacterial culture medium aliquot (150 µL) was filled in a well (5 mm diameter) made in the middle of the pathogen-plated dishes and incubated at 30 °C for the next five days. Then, the zone of pathogen growth inhibition was measured. To obtain metabolites of *B. subtilis* 10–4, the cells of strain 10–4 cultivated in medium optimal for lipopeptide production (MOLP) (130 rpm, 36 °C, six days) and cells were palleted via centrifugation at 5000× *g* for 60 min [30]. The cell-free supernatant was then used for the assessment of the antifungal activity against *Ph. infestans* in PDA plates, as mentioned above. Each experiment was performed in three replicates.

2.5. Determination the Activity of Hydrolytic Enzymes: Amylases (AMY), Proteinases (PRO), Cellulases (CEL), and Inhibitors of Exogenous Hydrolases

The activity of AMY, PRO, CEL, and inhibitors of exogenous hydrolases was determined according to [31–35]. Tuber samples were homogenized, resuspended in a single volume of buffer solution (0.05 M Tris-HCl (pH 8) for PRO; 0.2 M phosphate buffer (pH 6) for AMY); 0.05 M citrate buffer (pH 4.8) for CEL and incubated at 4 °C for 30 min. The extracts were centrifuged twice (10,000 rpm, 10 min at 4 °C) using a centrifuge (Eppendorf® Microcentrifuge 5417R, Hamburg, Germany). The supernatant was used as a source of proteolytic, amylolytic, cellulolytic, and inhibitory activity. To determine *B. subtilis* AMY inhibitors and trypsin inhibitors, supernatants were incubated with a standard enzyme solution (1:1 volume ratio). Solutions of enzymes in distilled water were used as a control. As a standard enzyme solution, commercial preparations of AMY *B. subtilis* (Sigma-Aldrich, St. Louis, MO, USA), trypsin (Sigma-Aldrich, St. Louis, MO, USA) and CEL *Trichoderma reesei* (Sigma-Aldrich, St. Louis, MO, USA) were used. A portion of the *B. subtilis* AMY preparation was dissolved in phosphate buffer (100 µg mL^{-1}), while trypsin was dissolved in Tris HCl buffer (20 µg mL^{-1}) and CEL *T. reesei* in citrate buffer (100 µg mL^{-1}). Carboxymethylcellulose and starch were used as substrates to determine the tubers' CEL and AMY activity, respectively.

Enzymatic and inhibitory activities of AMY and CEL were assessed via the hydrolysis of a substrate immobilized in a polyacrylamide gel plate [31,32]. The enzyme activity was expressed in arbitrary units obtained via digital processing of the stained areas of the gel

using an original software [33]. The activity of the inhibitor was calculated via the difference in the activity of the free enzyme and the mixture of the enzyme and the inhibitor.

Proteolytic activity was determined using the Erlanger method with our modifications [34], and the activity of trypsin inhibitors was determined via the Gofman and Vaisblai method with our modifications using a photometric analyzer of enzyme immunoassay reactions AIFR-01 UNIPLAN (Picon, Russia) [35].

For 1 conditional milliunit of inhibitor activity (mIU), an amount of inhibitor was taken that suppressed 1 conditional mU of free enzyme activity. The activity of enzymes and inhibitors was calculated in 1 mL of extract and converted in g of FW of tubers.

2.6. Determination of Lipid Peroxidation (MDA) and Proline

Lipid peroxidation was assessed according to the concentration of malondialdehyde (MDA) [36]. Fresh tuber samples (0.2 g) were homogenized with 10% trichloroacetic acid (1 mL) and centrifuged (10,000 rpm, 10 min). Thereafter, the supernatant (1 mL) was mixed with 20% trichloroacetic acid containing 0.25% thiobarbituric acid and was heated for 30 min at 95 °C, quickly cooled in an ice bath and centrifuged (10,000 rpm, 10 min). The absorbance of the supernatant was read at 532 and 600 nm using the Bio-Rad SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmoL g^{-1} FW.

To determine proline concentration [37], the samples of fresh tubers (0.5 g) were extracted with boiled water (2.5 mL). The extract (2.0 mL) was mixed with 2.0 mL of ninhydrin solution (1.25 g of ninhydrin dissolved in 30 mL of glacial acetic acid, and 20 mL of 6 M phosphoric acid) and glacial acetic acid. Then, the samples were incubated for 1 h at 100 °C and cooled in an ice bath. Proline concentration was determined at 522 nm using the Bio-Rad SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.7. Analysis of Starch, Total Dry Matter (TDM), Reducing Sugars (RS), and Ascorbic Acid (AsA)

The content of starch and TDM in potato tubers was calculated with the help of specific weight both in air and water [38,39]. The RS content was determined using Samner's reagent [39]. AsA was determined via the titration method [40] using 2.6-dichlorophenolindophenolate sodium. Briefly, the samples of fresh tubers (1 g) were homogenized with hydrochloric acid (1 mL) and brought up to 100 mL with hydrochloric acid using volumetric flask. The extract was incubated for 10 min, mixed, and filtered. The obtained extract (10 mL) was titrated with a 2.6-dichlorophenolindophenolate sodium solution until a slightly pink color appeared without fading for 15–20 s. Total AsA content was calculated as described in the methodology [40].

2.8. Quantification of Glycoalkaloids (GA)

GA (α-solanine, α-chaconine) content was quantified using high-performance liquid chromatography (HPLC) [41]. Samples of diced tubers (10 g) were homogenized in extraction solution (40 mL) (water: acetic acid: sodium hydrogen sulfide, 100:5:0.5, *v*/*v*/*w*) for 15 min in a homogenizer Ultra Turrax T-50 (Daigger Scientific, Vernon Hills, IL, USA). The homogenized solution was centrifuged (3000 rpm, 10 min), and the supernatant was collected and stored at +4 °C for up to 24 h in the dark. To clean the extract, SPE columns were used (Sep-Pak C18: silica-based octadecyl bonded phase with strong hydrophobicity, 500 mg sorbent per cartridge, particle size 55–105 µm; Waters Corp., Milford, MA, USA) that were first conditioned with acetonitrile (5 mL) and then with extraction solution (5 mL). The extracts (10 mL) were passed through the column, washed with 15% acetonitrile (4 mL) and eluted with mobile phase (4 mL); the volume was then adjusted to 5 mL with the mobile phase [41]. The sample (20 µL) or the standard solution was injected to the equipment, and the GA was decomposed using isocratic elusion with 50% acetonitrile in a HPLC system equipped with a C18 Atlantis column (5 µm, 3.9 mm × 150 mm; Waters Corp., Milford MA, USA) at a flow rate of 1.5 mL min^{-1} and 0.01 moL L^{-1} phosphate

buffer (10% 0.1 moL L^{-1}, pH 7.6). The results were obtained by taking measurements at 202 nm with a UV-detector and by comparing the regions covered by the standards of α-solanine and α-chaconine. Total GA content was calculated as the sum of α-solanine and α-chaconine [41] and expressed in mg kg^{-1} FW.

2.9. Statistical Analysis

All experiments were performed in three biological and analytical replicates. The data were presented as the mean ± standard error (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

3.1. Antagonistic Activity of Endophytic Strain of B. subtilis 10–4 against Ph. infestans

Artificial infestations of potato tubers prior to storage with *Ph. infestans* resulted in the gradual increase in tuber late blight disease incidence in tubers over time, reaching 100% by six months of storage (Figure 1). Treatment with *B. subtilis* 10–4 or a combination of *B. subtilis* 10–4 and SA notably suppressed late blight incidence in stored tubers by 30% and 40%, respectively. Additionally, the infected, stored tubers treated with a combination of *B. subtilis* 10–4 and SA looked completely healthy and fresh, while control infected tubers, along with complete defeat by late blight, were characterized by a lost turgor.

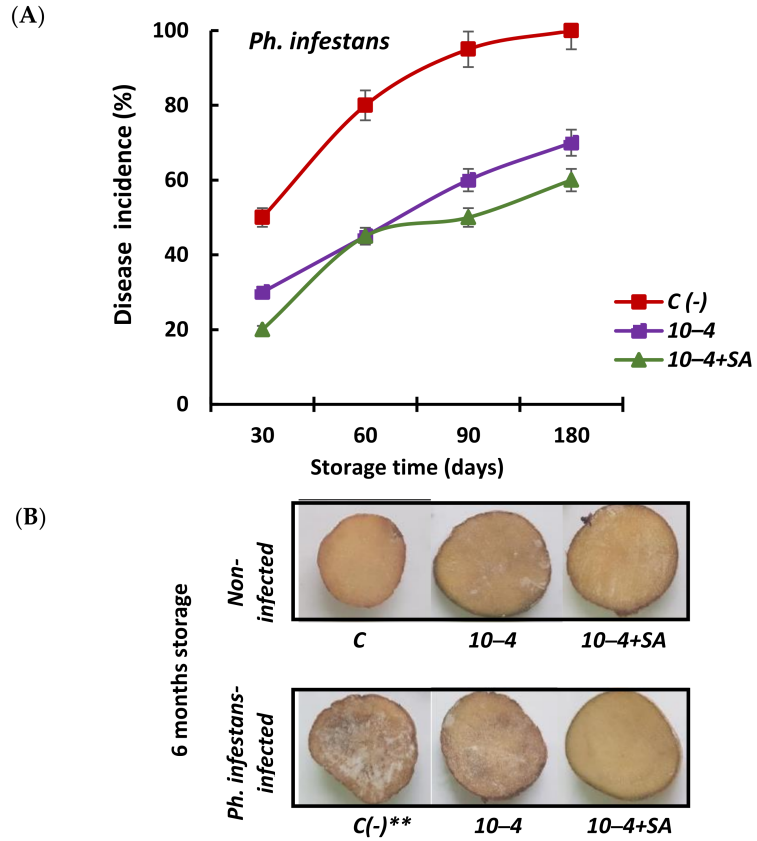

Figure 1. Influence of endophytic bacteria *B. subtilis* 10–4 (10–4) individually and in combination with salicylic acid (SA) (10–4 + SA) on *Ph. infestans*-caused tuber late blight disease incidence in potatoes during long-time storage (**A**) and visual appearance of potato tubers (cutaway view) after six months' storage (**B**). C—control tubers without treatments; C(-)—control tubers infected with *Ph. infestans*. The error bars show the average of three replicates ± standard error of the mean (SEM) (n = 30). **—the tubers lost turgor.

In vitro assays also showed antagonistic activity of *B. subtilis* 10–4 against *Ph. infestans* grown in Petri dishes with PDA (Figure 2A). The microscopic observation of *Ph. infestans* fungal mycelia clearly revealed morphological variations. The structure of *Ph. infestans* mycelia was well organized in the absence of the bacterial cells, while numerous gaps of mycelia appeared in the presence of bacterial cells. The metabolite synthesis by *B. subtilis* 10–4 in MOLP medium also showed the ability to suppress *Ph. infestans* growth in vitro in PDA plates (Figure 2B). The study also showed that mycelium growth was decreased with the increase in *B. subtilis* 10–4 concentration in PDA plates (Table 1). Additionally, the addition of SA increased the antagonistic activity of *B. subtilis* 10–4 against *Ph. infestans*.

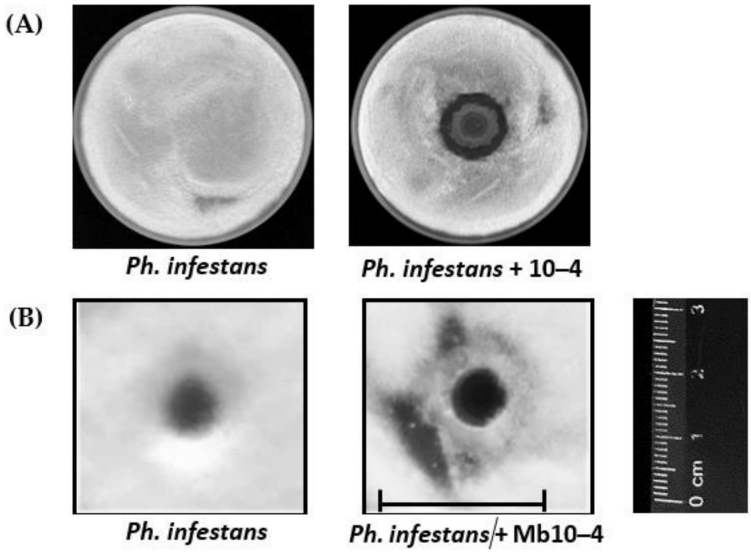

Figure 2. In vitro antagonistic activity of *B. subtilis* 10–4 (10–4) (**A**) and metabolites synthesized by *B. subtilis* 10–4 (Mb10–4) after cultivation in medium optimum for lipopeptide production (MOLP) against *Ph. infestans* in PDA plates (**B**).

Table 1. The inhibitory effect of *B. subtilis* 10–4 (10–4) or *B. subtilis* 10–4 with salicylic acid (10–4 + SA) in vitro on the growth of *Ph. infestans* in PDA plate.

10–4 (CFU mL^{-1})	0	10^3	10^4	10^5	10^6	10^7	10^8
Pathogen growth inhibition zone (mm) *	0	12.1	13.3	14.9	16.4	18.9	20.3
10–4 (CFU mL^{-1}) + SA (0.05 mM)	0	10^3	10^4	10^5	10^6	10^7	10^8
Pathogen growth inhibition zone (mm)	0	13.5	15.1	16.3	18.0	20.3	21.8

* Measured after five days of incubation at 30 °C.

3.2. Activity of Hydrolytic Enzymes Protease (PRO), Amylase (AMY), Cellulase (CEL), and Inhibitors of Hydrolases

The activity of AMY and exogenous AMY inhibitors in extracts was slightly elevated following treatment with *B. subtilis* 10–4 (Figure 3A,B). However, the level of activity of both AMY (Figure 3A) and AMY inhibitors of *B. subtilis* (Figure 3B) sharply increased upon treatment with *B. subtilis* 10–4 and SA.

In samples infected with *Ph. infestans*, the activity of AMY inhibitors decreased to zero (Figure 3B), while the activity of AMY was significantly increased (up to 400%) and was the highest when compared with other variants of the experiment (Figure 3A). The treatment of tubers with spores of *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA led to a decrease in the level of amylolytic activity in pathogen-infected potato tissues. Upon *B. subtilis* 10–4 treatment, the activity of exoamylase inhibitors was fixed at a low level (0.18 mIU/g FW under control 0 mIU/g FW) and was zero in *B. subtilis* 10–4- and SA-treated potatoes (Figure 3B).

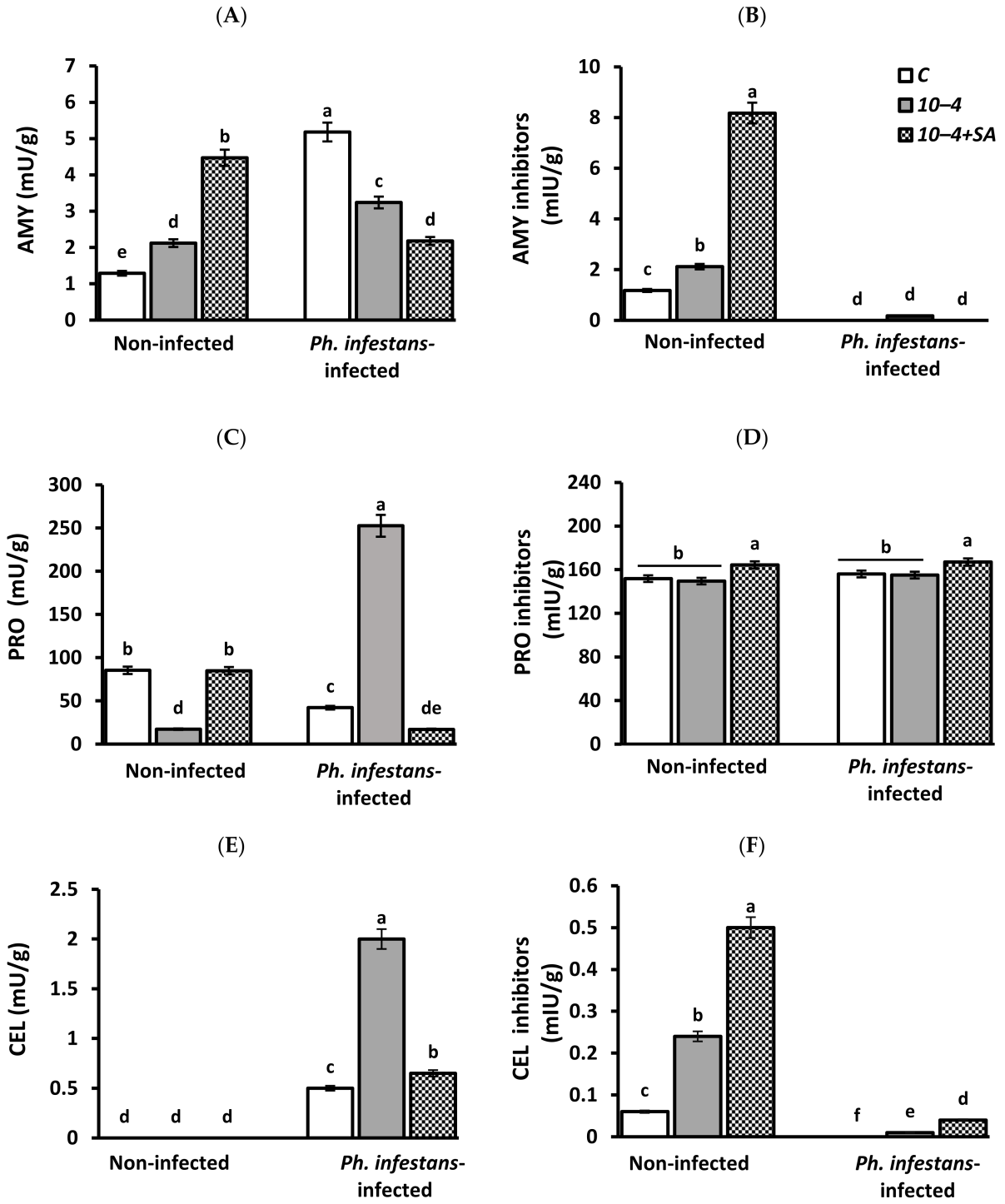

Figure 3. Influence of endophytic bacteria *B. subtilis* 10–4 individually or in combination with SA (10–4 + SA) on the activity of hydrolytic enzymes amylase (AMY) (**A**), protease (PRO) (**C**), cellulase (CEL) (**E**), and inhibitors of exogenous AMY (**B**), trypsin (**D**), and cellulase of *T. reesei* (**F**) activities in non-infected and *Ph. infestans*-infected, stored potato tubers. Time of tubers' storage—six months. C—control tubers. The bars indicate the mean values of three repetitions ± SEM. Different letters indicate a significant difference between the means at the level of $p < 0.05$.

Determination of the activity level of trypsin inhibitors in all variants of the experiment did not reveal significant differences (Figure 3D). Infection with *Ph. infestans* resulted in a very insignificant increase (by 103%) (within the margin of error) in the activity of trypsin inhibitors (Figure 3D), while the activity of exogenous AMY inhibitors was not detected

(Figure 3B). *B. subtilis* 10–4, when applied individually, did not affect the activity of PRO inhibitors in both infected and non-infected tubers (Figure 3D). However, the combined use of *B. subtilis* 10–4 with SA led to a slight increase (up to 107–110%) in the activity of PRO inhibitors in both infected and non-infected tubers (Figure 3D). The activity of AMY inhibitors was about zero in the groups of pathogen-infected tubers treated with *B. subtilis* 10–4 and SA (Figure 3B), whereas when individually treated with *B. subtilis* 10–4, the AMY inhibitors increased slightly (Figure 3B).

The CEL activity in non-infected tubers was not determined (Figure 3E). However, in all variants with *Ph. infestans* infection, the activity of CEL was determined at a high level, and in bacterial-treated tubers, it was significantly increased (by 1.3–4 times) in comparison with control tubers. The most significant effect (four-fold increase) was observed when *B. subtilis* 10–4 was used individually. It was found that pathological processes caused by *Ph. infestans* neutralize the activity of CEL inhibitors (Figure 3F). However, treatment with *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA causes a slight synthesis (possibly the activation) of these compounds. However, in non-infected tubers, the activity of CEL inhibitors significantly increased upon *B. subtilis* (by 4 times) or *B. subtilis* with SA (by 8.3 times) treatment.

3.3. Malondialdehyde (MDA) and Proline Content

Infection with *Ph. infestans* led to an increase in the MDA (up to 21%) (Figure 4A) and proline (up to 68%) (Figure 4B) content in stored potatoes. *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA helped to reduce the pathogen-caused increase in MDA content by 22% and 49%, respectively. In non-infected, stored tubers, MDA content also was reduced by 34% and 69% upon treatments with *B. subtilis* 10–4 and *B. subtilis* 10–4 with SA, respectively.

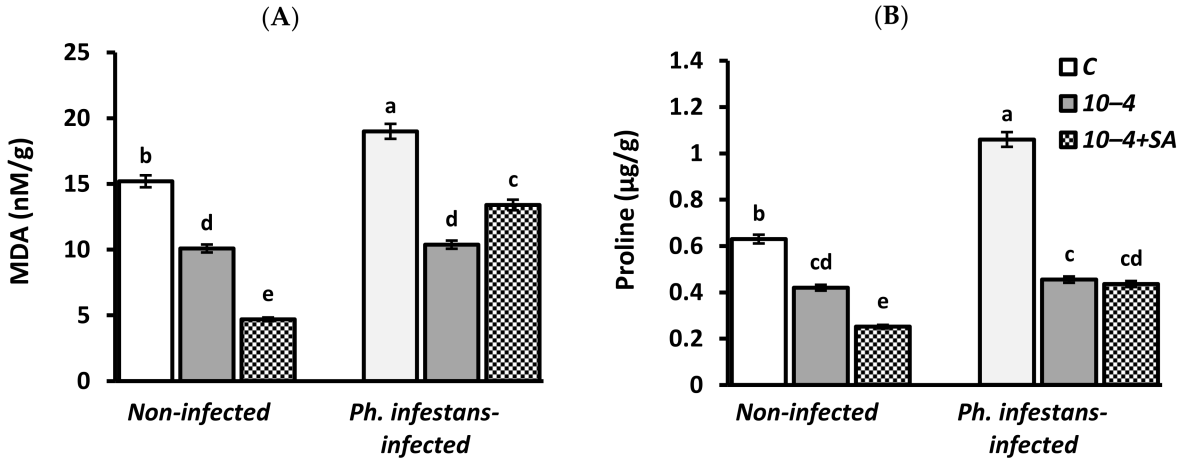

Figure 4. Changes in the contents of MDA (**A**) and proline (**B**) in non-infected and *Ph. infestans*-infected potato tubers after application of *B. subtilis* 10–4 (10–4) or combination of *B. subtilis* 10–4 and SA (10–4 + SA). C—control tubers. The bars indicate the mean values of three repetitions ± SEM. Different letters indicate a significant difference between the means at the level of $p < 0.05$.

As shown in Figure 4B, the application of *B. subtilis* 10–4 or *B. subtilis* 10–4 and SA also declined *Ph. infestans*-caused osmotic stress biomarker proline accumulation by 57% and 58%, respectively. In non-infected potatoes, upon these treatments, the content of proline was reduced by 33% (*B. subtilis* 10–4 and *B. subtilis* 10–4 with SA) in comparison with the control.

3.4. Starch, Total Dry Matter (TDM), Reducing Sugars (RS), and Ascorbic Acid (AsA) contents

Infection with *Ph. infestans* resulted in a reduction in starch content by 80.6% (from 14.4% in control to 2.8% in pathogen-infected) (Figure 5A), total dry matter (TDM) by 58% (from 20.2% in control to 8.5% in pathogen-infected) (Figure 5B), and RS by 15% (from 0.2% in control to 0.17% in pathogen-infected) (Figure 5C) over six months in stored potato tubers. Treatment with *B. subtilis* 10–4 separately and together with SA (*B. subtilis* 10–4 + SA)

prevented such *Ph. infestans*-caused reductions in starch and TDM. The content of starch and TDM in pathogen-infected potatoes upon *B. subtilis* 10–4 or *B. subtilis* 10–4 + SA treatments was counted, respectively, as 4.8% and 8.1% (starch) or 10.5% and 13.8% (TDM) (Figure 5A,B).

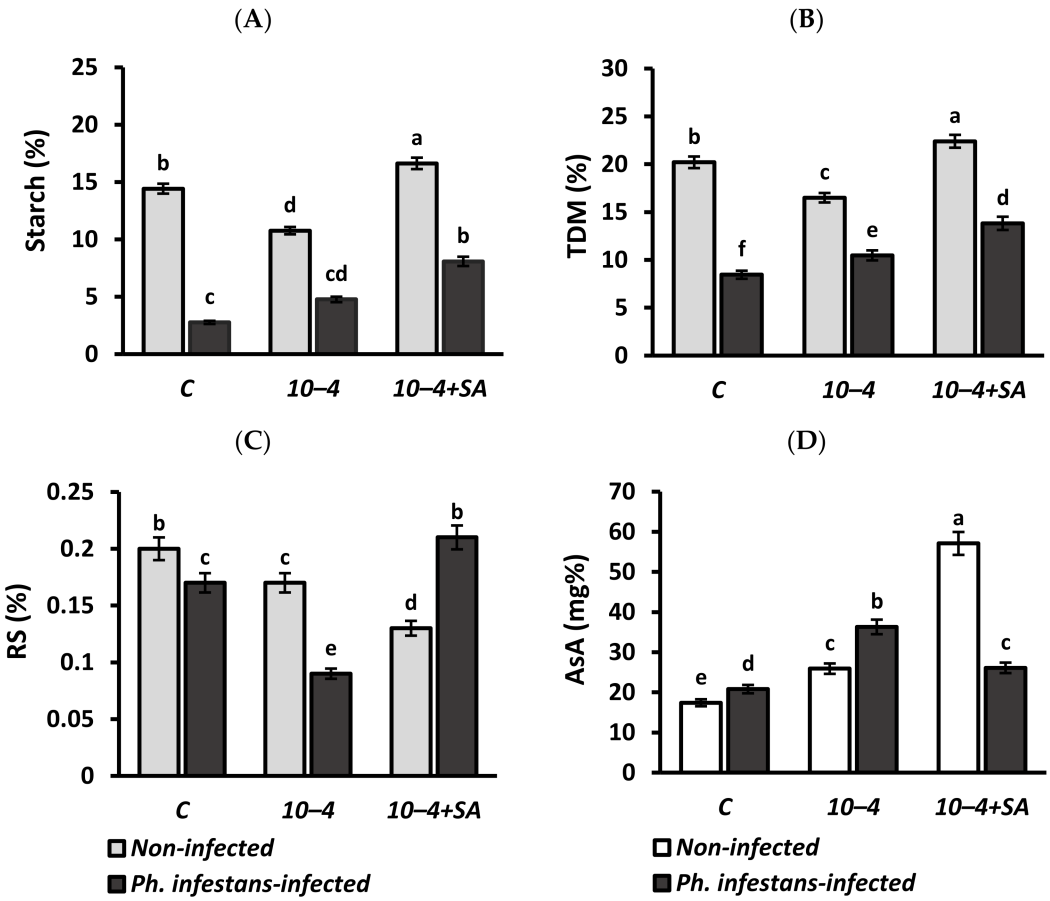

Figure 5. Influence of *B. subtilis* 10–4 (10–4) or *B. subtilis* 10–4 with SA (10–4 + SA) on the contents of starch (**A**), total dry matter (TDM) (**B**), reducing sugars (RS) (**C**), and ascorbic acid (AsA) (**D**) in non-infected and *Ph. infestans*-infected, stored potato tubers. Time of storage—six months. C—control tubers. The bars indicate the mean values of three repetitions ± SEM. Different letters indicate a significant difference between the means at the level of $p < 0.05$.

The content of RS in *B. subtilis* 10–4-treated, pathogen-infected potatoes was additionally decreased by 47.1% (from 0.17% in infected control to 0.09%), while in *B. subtilis* 10–4- and SA-treated tubers, the RS content remained at the level of the non-infected controls (Figure 5C).

Upon *Ph. infestans* infection, AsA in potato tubers stored for six months increased up to 119%, which was 20.8 mg% (under control 17.4 mg%) (Figure 5D). *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA led to a more significant AsA accumulation in these pathogen-infected, stored potatoes and reached up to 209% (36.3 mg%) and 150% (26.1 mg%), respectively. In non-infected, stored tubers, upon *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA treatments, the AsA also increased, respectively, up to 149% (25.9 mg%) and 328% (57.1 mg%) (under control 17.4 mg%) (Figure 5D).

3.5. Glycoalkaloids (GA) α-Solanine and α-Chaconine Contents

It was found that the infection of tubers with *Ph. infestans* caused an increase in the level of GA, both α-solanine (up to 360%) and α-chaconine (up to 350%), in potato tubers stored for six months (Figure 6A,B). *B. subtilis* 10–4, individually or in combination with SA, decreased the pathogen-caused α-solanine accumulation in stored tubers by 340% and 200%, respectively. The content of α-chaconine also reduced in infected tubers upon

influences of *B. subtilis* 10–4 (by 260%) or *B. subtilis* 10–4 and SA (by 240%). In contrast, in non-infected tubers, there were no significant differences from control in GA contents upon individual use of *B. subtilis* 10–4. However, upon joint use of *B. subtilis* 10–4 and SA, an increase in α-solanine (by 230%) and α-chaconine (by 180%) was revealed.

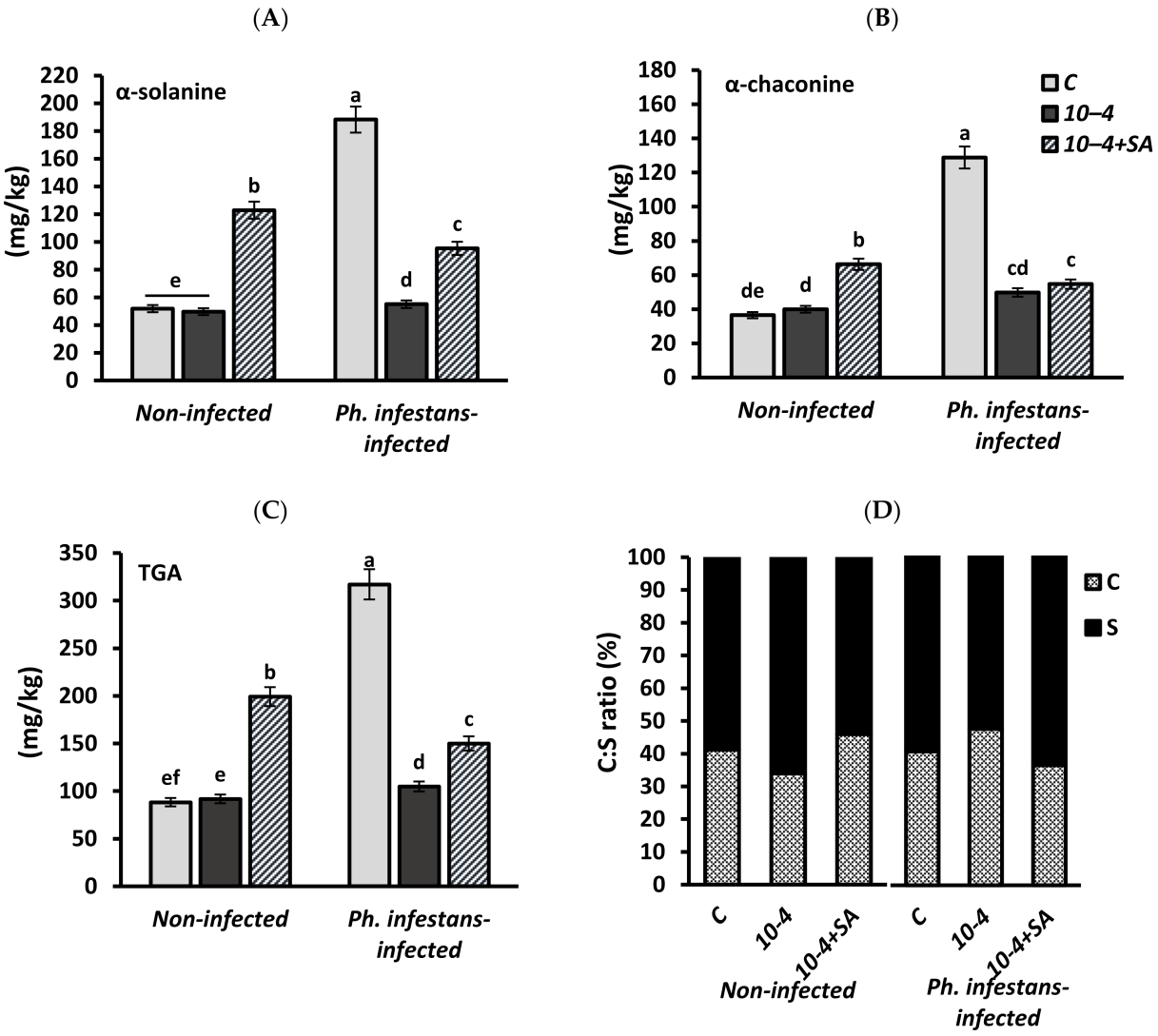

Figure 6. Influence of *B. subtilis* 10–4 (10–4) or *B. subtilis* 10–4 with SA (10–4 + SA) on the content of glycoalkaloids α-solanine (**A**), α-chaconine (**B**), total glycoalkaloids (TGA) (**C**), and the ratio of α-chaconine to α-solanine (C:S ratio) (**D**) in non-infected and *Ph. infestans*-infected potato tubers stored for six months. C—control tubers. The bars indicate the mean values of three repetitions ± SEM. Different letters indicate a significant difference between the means at the level of $p < 0.05$.

In general, in potato tubers stored for six months, the content of total GA (TGA) upon *Ph. infestans* infection increased up to 360% (317.2 mg kg^{-1} fresh tubers under control 88.4 mg kg^{-1}) and exceeded the maximum permissible level (200 mg kg^{-1}) (Figure 6C). Meanwhile, the application of *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA reduced such pathogen-caused TGA accumulation by 300% or 333%, respectively. In non-infected tubers, the TGA slightly (but not significantly) increased after the individual use of *B. subtilis* 10–4 (up to 104%) in comparison with the control, while it increased by 220% (reaching up 190 mg kg^{-1} under control 88.4 mg kg^{-1}) after the joint use of *B. subtilis* 10–4 and SA.

The proportion of α-chaconine to α-solanine (C:S ratio) ranged from 42:58 (in control) to 34:66 (10–4) to 46:54 (10–4 + SA) in fresh, non-infected tubers (Figure 6D). In contrast,

Ph. infestans-infected, stored tubers exhibited a C:S ratio of 41:59 (in control), 48:52 (10–4), and 36:64 (10–4 + SA).

4. Discussion

The results of the current study show that the application of the endophytic strain of bacteria *B. subtilis* 10–4 prior to storage reduced *Ph. infestans*-activated late blight disease in stored potato tubers and positively influenced tuber quality. This effect was pronounced when *B. subtilis* was applied alone and became even more significant when applied in combination with SA. These data are consistent with the data on the ability of *Bacillus* bacteria and SA separately to increase the resistance of different types of vegetables, fruits, and berries to diseases and stresses during storage, leading to enhancement of the nutritional quality with extension of the shelf life [12,24,25]. Recently, similar to our results, Lyosfi et al. (2021) also demonstrated improvements in the biocontrol potential of antagonistic bacteria *B. amyloliquefaciens* SF14 and *Alcaligenes faecalis* ACBC1 used in combination with SA against postharvest brown rot disease and impacts in nectarine [42]. The revealed ability of bacteria and SA to more effectively suppress disease development after their combined use may be explained by the fact that SA, as a natural and safe signal molecule, enhances and accelerates the spread of the systemic immunizing effect of *B. subtilis* 10–4 in potato tuber tissues. SA and several components of the SA pathway, including the methylated derivative of SA, are known to be among the signals contributing to systemic acquired resistance (SAR) [43,44]. The role of SA in SAR and its relationship with various SAR signals have been well reviewed [26,43]. Moreover, SA might trigger supplementary protective mechanisms responsible for preventing postharvest potato losses. Since SA can exhibit the properties of a preservative or potentially inhibits ethylene biosynthesis, it probably helps *B. subtilis* more effectively slow down senescence processes, thus prolonging the life of stored products while maintaining freshness [24,25].

The main mode of action driving the antifungal effect of antagonistic microorganisms including *Bacillus* spp. might be due to their antagonistic effect on pathogens' cell wall through the production of various metabolites with strong antifungal activity (such antibiotics, lipopeptides, biosurfactants, enzymes, hydrogen cyanide, siderophores, etc.) [45–47] and natural competition for nutrients and space suitable for colonization [48–50]. Previously, we revealed that bacterial strain *B. subtilis* 10–4 has the ability to produce siderophores [51], as well as the ability to effectively compete with pathogens and colonize the internal tissues of potatoes [13]. The current study showed in vitro antagonistic activity of *B. subtilis* 10–4 against *Ph. infestans*, and the presence of SA enhanced this activity. The revealed ability of metabolites syntheses by *B. subtilis* 10–4 in medium optimal for lipopeptide production (MOLP) to suppress *Ph. infestans* growth in vitro in PDA plates (Figure 2B) suggest their involvement in the resistance of stored potatoes to tuber late blight disease. The antifungal effects of *B. subtilis* 10–4 treatment may also promote the maintenance of firmness, because it protects potato tubers against fungal physiology, which can involve the secretion of cell-wall-degrading enzymes. Overall, further detailed investigations both of the spectrum of metabolites produced by *B. subtilis* 10–4 and changes in physiological, biochemical, and molecular defense responses in cells of stored potatoes to further our understanding of the mechanisms underlying interaction between endophytic bacteria *B. subtilis* (in absence and/or presence of SA) and stored potato tubers upon pathogen infection will be quite interesting.

When analyzing enzymatic activity against the background of infection, attention is drawn to the increase in hydrolytic activity when using *B. subtilis* 10–4. The antagonistic function of *B. subtilis* can be associated with the synthesis of hydrolases, the role of which is the destruction of structural polysaccharides of the fungal cell wall and the lysis of fungal hyphae. As a result, plants are protected from the penetration and spread of phytopathogens [12,52]. A correlation between antagonistic activity to various pathogenic fungi and the synthesis of hydrolases such as PRO, AMY, CEL, xylanases, mannanases, and lipases by bacteria has been established [12,53]. This, as a rule, occurs not due to an increase in the concentration of constitutive compounds, but due to the synthesis of new

forms of inhibitors. To date, the presence of various AMY with molecular masses of 47 kDa (*amyA*, *amyE* genes), 60 kDa (*amyS gene*), and 75 kDa (*amyM* gene) was described for *Bacillus* bacteria [54–59]. Probably, the increase in amylolytic activity in the pretreated tubers is due to bacterial enzymes as a result of the colonization of tissues by bacilli. Amylolytic activity was also found in most taxonomic groups of fungi, and these enzymes are almost always represented by constitutive proteins [60]. However, AMY is absent in oomycetes of the *Phytophthora* genus. *Phytophthora* pathogens activate the biosynthesis of potato's own AMY in damaged tissues to break down starch [61]. Our results showed an increase in the activity of AMY in stored potato tubers upon *Ph. infestans* infection (Figure 3A), indicating the intensive pathogen development in tubers' tissues. Since pathogen-produced hydrolytic enzymes are their main weapon of attack, the cell walls of plants are destroyed [53]. However, upon the application of *B. subtilis* 10–4 or *B. subtilis* 10–4 with SA, such pathogen-induced AMY activation decreased, demonstrating the protective effect of these treatments against *Ph. infestans* development in stored tubers with the best effect after the use of combined cells of *B. subtilis* 10–4 with SA. This is also confirmed by data on the visual appearance of potato tubers (Figure 1). We suggest the observed differences in the AMY activity are the result of the regulation of potato genes' expression by a combination of acting factors, particularly salicylate inducible, and not the effect of the activity of AMY inhibitors. Additionally, the low activity of AMY inhibitors is also a consequence of this regulation, or their inactivation by fungal proteases. When treating non-infected tubers, the activity level of bacillary AMY inhibitors correlated with an increase in amylolytic activity in potato tissues. Thus, it could be part of the mechanism that regulates the development of symbionts in tissues. When untreated tubers are infected, the activity of AMY increases significantly, which demonstrates the sensitivity of this potato variety to the pathogen. In tubers treated with *B. subtilis* 10–4 + SA, against the background of infection, amylolytic activity in tissues is significantly reduced. It is possible that the AMY activity shown during this treatment is the result of the action of predominantly bacterial enzymes.

It should be noted that CEL activity in non-infected tubers was not detected (Figure 3E). However, in all groups with infection, the CEL activity was at a high level. The effect was more pronounced after the individual use of *B. subtilis* 10–4. Since it is known that some strains of *Phytophthora* pathogens synthesize CEL [62], the revealed increase in CEL activity in the infected tuber tissues can be attributed to the process of pathogen development. Additionally, bacilli are capable of synthesizing this group of depolymerases [63]. However, the cell wall of oomycetes contains cellulose, so we can assume that in this case, the increase in CEL activity in tissues is due to the antagonistic action of bacteria. Potato tubers contain water-soluble compounds able to suppress the activity of exogenous cellulases, and their activity is determined by variety specificity [64]. It is likely that cv. Bashkirsky used in our study possesses a constitutive synthesis of these compounds; when treated with the bacterial preparations, their level significantly increased (Figure 3F). In pathogen-infected tubers, the activity of these compounds was absent, while it was partially retained in pre-treated tubers. The protein nature of PRO and AMY inhibitors has been confirmed by numerous studies but such data are not available for cellulase inhibitors. It is known that inhibitors of cellulolytic enzymes are compounds of oligosaccharide or phenolic natures [65,66] in particular flavonoids, which can be synthesized in potato tissues.

Hydrolase inhibitors prevent the activity of both microbial and a plant's own enzymes, thereby reducing the level of tissue degradation [60,62]. Protein inhibitors of plants make a significant contribution to the regulation of the activity of hydrolytic enzymes by suppressing the activity of intrinsic and foreign enzymes of pathogenic fungi [67]. In a number of cases, the intensity of the disease in plants (necrotic processes) depends on the level of proteolytic activity [44,68,69]. High proteolytic activity both ensures the growth and development of the pathogen due to amino acids and also neutralizes the protective proteins of potatoes—inhibitors of hydrolases and lectins. It has been shown that inhibitors of trypsin protein from potato and legume tubers can suppress exogenous proteinases of the phytopathogenic fungus *Rhizoctonia solani* Kuhn [70]. It was reported that proteolytic

activity in a cell-free preparation obtained from a spore suspension of *Ph. infestans* oomycete correlates with the development of necrosis upon injection into potato plant tissues [71]. There is also evidence of the possibility of *Ph. infestans* to express PRO of different families: serine and cysteine PRO, proteases of the *Hsl*, *Fts*, and *Clp* families [68,72]. Recent studies showed the cysteine proteases PpCys44 and PpCys45 of the *Ph. parasitica* oomycete trigger NPK1-dependent cell death in various plant species of *Nicotiana* spp. [73]. As a rule, in plants, an increase in the activity of hydrolase inhibitors occurs due to the expression of their genes [44,74]. Recently, Sorokan et al. (2021) found the decrease in the intensity of late blight symptoms on the leaves of potato plants treated with *B. thuringiensis B-5351* and SA was accompanied with *PR6* gene transcript accumulation, as well as the activity of its protein product, indicating the importance of PRO inhibitors in protecting potatoes from late blight [44]. Earlier, on sugar beet plants, we also showed that the introduction of *B. subtilis*-based biologicals promoted PRO inhibitors' synthesis and protected plants against the penetration and development of pathogenic microorganisms [69]. In the current study, we observed that PRO inhibitors were slightly activated in stored *Ph. infestans*-infected potato tubers upon treatment with the combination of *B. subtilis* 10–4 and SA, while upon their individual treatments, no significant differences from the control were observed (Figure 3C). In response to *B. subtilis* 10–4 with SA, there was also a significant increase in AMY inhibitors' activity both in non-infected and pathogen-infected tubers. This suggests the potatoes had protective reactions to the development and the involvement of these compounds in creating obstacles to the penetration and further spread of the pathogen. However, in *Ph. infestans*-infected tubers, the inhibitor activity was almost zero, which was most likely due to the fact that the potato cultivar is susceptible to late blight [27], and the use of the studied treatments may contribute to some AMY inhibitors' activation. It can serve as one of the factors in the formation of resistance against *Ph. infestans* upon their influence.

One of plant cells' responses to stresses including pathogen infection is oxidative and osmotic stress, biomarkers of which are the degree of malondialdehyde (MDA) and proline, respectively [75–77]. They can be not only the primary stress mediators, but also inducers of the corresponding defense mechanisms in plant cells. Our results showed the decrease in pathogen-induced MDA accumulation in stored tubers upon *B. subtilis* 10–4 and SA treatments, indicating their protection against oxidative damages (Figure 4A). This is consistent with data from several other studies on the capability of *Bacillus* spp. to increase stress resistance of different type of plants through the decrease in oxidative damages [12,14,28]. Many findings reported an increase in proline in stressed plants and its importance as another factor for plant survival in extreme situations [76]. Proline is a multifunctional plant stress metabolite playing the role of an antioxidant, osmolyte, and chaperone maintaining the native enzymes' structure [76,77]. The observed ability of *B. subtilis* 10–4 with SA to reduce pathogen-caused proline accumulation in stored potatoes demonstrates the protective effect of these treatments against osmotic damages. This implicates proline in *Bacillus*-mediated postharvest potato resistance against tuber late blight (*Ph. Infestans*) as a biomarker of the resistance level of tubers to diseases during storage.

As a result of stress exposure in potatoes, abnormal amounts of other compounds are synthesized and many new or unusual compounds emerge, including glycoalkaloids (GA), in levels exceeding those found in healthy tissues [78,79]. These newly synthesized compounds play an important role in creating the natural resistance of tubers to diseases. At the same time, some GA, i.e., α-chaconine and α-solanine (95% of the total GA content) has a potential toxic effect on human health (200 mg kg^{-1} tubers FW is considered as critical) [78,80]. GA formation can be influenced by many factors, including genotype, growing conditions, transportation, storage, temperature, germination, exposure to light, and phytopathogens [78,81], herbicides, and biostimulants [82]. However, the greatest GA accumulation in potatoes usually occurs during postharvest storage. The mechanisms of GA action in potatoes are not fully understood, but it is believed that they are associated with antimicrobial and antifungal activities [83,84]. It was showed that GA as a secondary plant metabolite provides resistance to microbial

diseases [78]. However, at the point in time that this study commenced, the correlation between GA in stored potato tubers infected with *Ph. infestans* and the use of endophytic bacteria *B. subtilis* or *B. subtilis* with SA in the available literature had not been established. Our results showed *B. subtilis* 10–4, both individually and together with SA, decreased pathogen-caused GA accumulation in stored potato tubers. In non-infected tubers, upon the separate use of *B. subtilis* 10–4, there was no significant difference from the control in GA. Meanwhile, upon joint use of *B. subtilis* 10–4 with SA, the content of GA increased but did not exceed critical values. On the one hand, this indicates a positive effect of the bacteria on the quality of stored products under normal storage conditions (when tubers are not infected with pathogens), and on the other hand, it indicates the possible involvement of GAs on potatoes resistance against tuber late blight upon application of *B. subtilis* 10–4 with SA. It is most likely that SA, as a signaling molecule, induces elevated GA accumulation (before stress), which plays an important role in the pre-adaptation of tubers to stresses [78,79]. Probably, this is one of the mechanisms which makes a contribution to the induction of the natural resistance of stored potatoes, which manifested in decreasing the disease development and keeping a healthy appearance of healthy and pathogen-infected potato tubers stored for six months (Figure 1B).

Changes in reducing sugars (RS), starch, total dry matter (TDM), and ascorbic acid (AsA) can serve as biochemical markers for the formation of resistance and important indicators, which largely determine the consumer and table qualities of potatoes [85–88]. Our results indicate that *B. subtilis* or *B. subtilis* with SA contribute to the preservation of starch, RS, AsA, and TDM in *Ph. infestans*-infected tubers. The treatment of tubers with bacteria significantly reduces the loss of starch by curbing the development of infection. AsA, along with the quality traits of tubers during storage, also may act as a biomarker of the physiological state due to the fact that it is a non-enzymatic antioxidant [76]. However, AsA is a very labile substance, easily and irreversibly oxidized under the influence of stresses. The data available in the literature indicate the ability of *B. subtilis* to increase resistance to pathogen attack during storage while maintaining a high level of their consumer properties, particularly thanks to increased ascorbic acid [89]. The current study also showed that *B. subtilis* 10–4 or the combination of *B. subtilis* with SA increases the AsA, both in non-infected and *Ph. infestans*-infected stored tubers. Moreover, in groups with the combined use of *B. subtilis* 10–4 and SA, a more enhanced AsA accumulation was observed. This may explain the observed fresh appearance in this potato group after long-time storage. It is known that AsA may play a role as a preservative. The role of AsA is not only limited to triggering protective reactions but makes an important contribution to slowing down the senescence process, which is reflected in maintaining a fresh appearance and extending the shelf life of potato tubers.

Thus, the obtained results indicate that exogenously applied endophytic bacteria *B. subtilis* 10–4 or the combination of *B. subtilis* 10–4 with SA contribute an important role in the formation of postharvest potato resistance to tuber late blight. This is linked with both the regulation of complex signaling plant protection systems and the synthesis of identified protective compounds. This ultimately manifests itself in a decrease in tuber late blight incidence, slowing down the senescence process and lengthening the shelf life of stored potato tubers while preserving the appearance and biological value of the product. The results of this study can be used to improve technologies for the environmentally oriented storage of potatoes and contribute to solving the problem of introducing effective biotechnologies into agriculture.

5. Conclusions

The present investigation revealed that endophytic bacteria *B. subtilis* 10–4 reduces *Ph. infestans*-caused late blight development and associated symptoms (i.e., oxidative and osmotic damages and AMY activity) in stored tubers, and this positive effect is magnified by its delivery in combination with SA. The reduction in disease symptoms was accompanied by decreasing pathogen-caused toxic GA (α-solanine, α-chaconine) accumulation, preserving starch, RS, TDM, and increased AsA. Generally, the results suggest that *B. subtilis*

10–4 with SA has potential to be used as a postharvest biocontrol agent to increase tuber late blight (*Ph. infestans*) resistance and improve the quality of stored potato tubers.

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