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Optimization of Growing Medium and Preservation Methods for Plant Beneficial Bacteria, and Formulating a Microbial Biopreparation for Raspberry Naturalization

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Abstract: The current study focuses on the optimization of bacterial growing medium composition, including the carbon and nitrogen source in different concentrations, the pH value of the medium and the temperature. Optimization was performed for four environmental bacterial isolates belonging to the genera *Arthrobacter*, *Pseudomonas* and *Rhodococcus*, which were previously obtained from wild raspberries. These bacteria proved to be potent antagonists against certain fungal and fungal-like plant pathogens. Furthermore, three preservation methods and three sample preparation techniques were evaluated. In addition, a prebiotic supplementary blend based on previous research was tested. The research included a pot experiment to estimate the influence of bacterial cultures on the growth of plant shoots and roots, on the soil enzymatic activity and the content of macronutrients, minerals and nitrogen in the soil depending on the naturalization strategy. The best carbon and nitrogen source were chosen. The addition of a supplementary blend resulted in the increased growth of two bacterial isolates. Bacterial inoculum applied to the roots and watering resulted in increased shoot mass in objects infected with plant pathogens, although in plants without the pathogen infection, bacterial inoculum resulted in the decreased mass of plants. Naturalization strategy should be matched to the pathogens present at plantations.

Keywords: biopreparation; organic farming; fungal plant pathogens; optimization; preservation; pot experiment

1. Introduction

Microorganisms have been utilized more and more often in recent years for plant growth and resistance stimulation, which includes biocontrol, as well as soil microbial activity and functionality maintenance or improvement. Bacterial biopreparations and bioproducts are now common in organic farming. Current trends in plant cultivation and pest control are shifting towards methods safe for the environment and microbial diversity [1]. The European Union promotes the use of organic methods of crop production, including pest control, but also bans compounds that are damaging to environmental biodiversity and are detrimental to soil microbial communities [2]. The European Union Biodiversity Strategy for 2030 clearly states that ecological biodiversity degradation must be stopped and one of the most important factors for achieving this goal is agricultural and horticultural production systems based on natural solutions, including beneficial microbes, as well as decreasing the application of mineral fertilizers and reducing the application of chemicals used in pest control. Furthermore, WE No. 834/2007 states that using chemical pesticides must be limited to the absolute minimum and that farmers are encouraged to use substances of natural origin [3]. Biopreparations may consist of microorganism inoculum, a carrier and supplements that support the growth of beneficial microorganisms and their survivability in different ecological niches [4,5]. Biopreparations are products composed of living microorganisms or their metabolites. Currently, there are biopreparations available to farmers, i.e., Micosat F

or Polyversum WP based, respectively, on the fungi *Trichoderma* spp. and *Pythium oligandrum*. There are other biopreparations available that inhibit the growth of soft fruit pathogens such as *Phytophthora* spp. (PREV-AM), *Colletotrichum* spp. (chitosan), and *Verticillium* spp. (Bioczos S) [6–8]. However, there is still a niche available for another agroecology product, combining plant growth and resistance stimulation, as well as being appropriate for the maintenance of soil microbial activity and diversity, and thus appropriate for use in the organic production of fruits.

Since the number of plant protection products targeting phytopathogens which are allowed to be used in organic farming is decreasing, because they are being steadily forbidden on an official basis, the demand for new methods of plant protection is rising. The most promising alternatives to these products are biopreparations based on local and highly effective bacterial isolates. The application of bacteria that are locally occurring in wild plants to plants in plantations can be called a naturalization. In our previous research, we isolated and characterized four bacterial isolates belonging to three different genera *Arthrobacter* sp., *Bacillus* sp., and *Rhodococcus* sp., and examined their ability to inhibit the growth of fungal and fungal-like plant pathogens such as *Botrytis* spp., *Colletotrichum* spp., *Phytophthora* spp. and *Verticillium* spp. Moreover, we selected four chemical compounds that have the ability to stimulate bacterial growth and are not metabolized by pathogens and might therefore be used as a supplementary prebiotic blend [5].

In this paper, we focus on the development of biopreparation formulations based on previously isolated and examined bacterial isolates. The experiments conducted concentrated on the optimization of the bacterial growth and drying of bacteria, including testing the prebiotic supplementary blend and pot experiment to examine the influence of selected bacterial isolates on plants and pathogens. The optimization of the growing media can have a high degree of influence over the speed of growth for different microorganisms. Different microorganisms utilize different chemical compounds such as a carbon or nitrogen sources. Environmental isolates may show a preference for different medium pH values or growing temperatures that are often difficult to predict. Optimization can also influence the production of enzymes or other metabolites [9–11]. Preservation methods may differ for different bacterial species. Appropriate preservation techniques ensure that survivability is maintained and that the biopreparation is stable and the shelf life of the future product is sufficiently long. It is also worth noting that an appropriate choice of carrier may shorten the drying time by increasing the surface area of the dried sample.

The aim of this research was to formulate a biopreparation for plant growth and resistance stimulation for raspberry naturalization, defined as the application of previously chosen beneficial bacterial strains, isolated from natural habitats of wild raspberries. The optimization process of the growing medium was focused on the carbon source, the nitrogen source, the pH value of the medium, the growing temperature, carrier choice and supplementary blend additive. The optimization of the drying process focused on finding a drying method and sample preparation that ensured the highest degree of survivability. We also examined the influence of pure cultures of those bacteria on raspberry plants in the pot experiment, evaluating the dry mass of shoots, the wet mass of roots, and soil dehydrogenase activity in experiments treated with different types of naturalization strategies using prepared biopreparations. The hypothesis of the presented research is that environmental isolates of bacteria belonging to *Arthrobacter, Pseudomonas*, and *Rhodococcus* genera are effective in promoting the early growth of raspberry plants and furthermore are suitable to be a part of biopreparation formulation.

2. Materials and Methods

2.1. Isolation and Identification of Bacterial Strains

Bacteria were isolated from raspberry roots and soil collected in the following Forest Districts: Łuków, Świdnik, Janów Lubelski, Kraśnik, and Siedlce. A full description of the methodology of the sampling techniques used is presented in Oszust et al. (2020) [12]. The bacteria were genetically identified based on a 16S rDNA fragment as described in

Pylak et al. (2020) [5]. The sequences obtained were submitted to the National Center for Biotechnology Information (NCBI) database: *Rhodococcus* sp. B12/18 (GenBank: MW255650), *Pseudomonas* sp. B37/18 (GenBank: MW255651), *Arthrobacter* sp. B58/18 (GenBank: MW255652), *Rhodococcus* sp. B75/18 (GenBank: MW255653). The antagonistic and metabolic abilities of the isolated strains, including important features for plant growth promotion, were evaluated and presented in previous research [5]. These strains were isolated from the rhizosphere of wild raspberry, taking into account that plant–microbe interactions in the rhizosphere determine plant health, as well as soil productivity and fertility [13].

2.2. Optimization of the Growing Medium for Bacterial Isolates

Optimization of the growing media was performed for four isolates of bacteria. The basic medium used for optimization consisted of the residues of beer filtering on diatomite (1 L), $NaHPO_4$ (7 g/L), KH_2PO_4 (3 g/L), NH_4Cl (1 g/L), and microelements solution (10 mL/L) containing 100 mg/L $MnCl_2*4H_2O$, 50 mg/L $ZnCl_2$, 50 mg/L $CuCl_2*2H_2O$, 50 mg/L $CoCl_2*6H_2O$, and 50 mg/L $Na_2MoO_4*2H_2O$. The optimization process focused on 3 sugars: glucose, lactose and sucrose were tested as additional carbon sources, including 3 different concentrations: 3%, 6% and 9%, and 4 different nitrogen sources: $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, including 3 different concentrations: 1%, 3% and 6%. The pH value was set to 7.2 using 1 M NaOH. A total of 15 mL of medium subjected to optimization was poured into 50 mL Erlenmeyer flasks; these were plugged with cellulose stoppers and sterilized at a temperature of 121 °C for 20 min. After sterilization, the medium in the flasks was inoculated with a 1.5 mL solution of bacteria in the water, for which the turbidity was set to 90% of transmittance using a turbidimeter (Biolog®, Hayward, CA, USA), which represents the optical density of 0.23–0.28 (0.23 for B37/18, B75/18 and 0.28 for B12/18, B58/18) McFarland measured with the use of a DEN-1B densitometer (Biosan®, Riga, Latwia) or absorbance value of 0.1–0.12 (0.1 for B37/18, B75/18 and 0.12 for B12/18, B58/18) measured at the wavelength of 600 nm with the use of an Infinite M200PRO spectrophotometer (Tecan®, Männedorf, Switzerland). Bacteria used for inoculum preparation were cultured for 48 h on PDA plates at a temperature of 30 °C. After choosing the best carbon and nitrogen sources and their concentrations, the culturing temperature and pH value of the medium were subjected to optimization. The following temperatures, 18 °C, 24 °C, 30 °C, and the set of pH values 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 were reviewed. For each of the aforementioned variants, carbon source, nitrogen source, the pH value of the medium and temperature of cultivation were optimized. After 72 h of cultivation in the tested conditions, 1 mL of medium with bacteria was diluted with water using the serial dilution method. From the chosen dilutions (10^{-6}, 10^{-7}, 10^{-8}) 100 μL was spread on a PDA plate using a sterile cell spreader [14]. The plates were then cultivated for 48 h at 30 °C. Afterwards, the colonies were counted and the composition of the optimized medium was evaluated.

2.3. Optimization of the Preservation Method for Bacterial Isolates

The optimization of the preservation method was performed for bacteria growing on an optimized medium. Three techniques were chosen: D—drying in a conventional dryer (Termaks® TS 9053, Bergen, Norway), VD—drying in a vacuum dryer (Memmert® Vacuum Oven VO49, Büchenbach, Germany) and LF—lyophilization (Labconco® FreeZone 2.5, Kansas City, MO, USA) of the bacterial isolates. For the conventional dryer, the temperature was set at a the level of 35 °C for the first 24 h, 42 °C for the second 24 h and 50 °C for the final 24 h and the whole drying process took 72 h. Vacuum drying was performed at 30 °C and a pressure of 50 mbar was applied for 72 h in the presence of $CaCl_2$ as a drying agent. Lyophilization was performed at a pressure of 0.01 mbar and the temperature of the condenser was set at −56 °C. There were three methods used for sample preparation for drying. The first one was to dry the whole growing medium with bacteria, the second was the centrifugation of the growing medium and only drying the bacterial residue after the

removal of the supernatant, and the third involved mixing the whole growing medium and bacteria with the carrier—diatomite. In the centrifugation variant, the bacterial suspension was centrifuged for 10 min at 2880 RCF, then the supernatant was removed and the pellet was subjected to the drying process. In the process of mixing the carrier variant with the medium containing bacteria after culturing, 10% (*w/w*) dextrose was added, as Kumar, Mallik and Sarkar (2017) showed that dextrose can increase the survivability of bacteria during the drying process.

Furthermore, the addition of trehalose up to a concentration of 0.1 M in the growing medium and the drying process were evaluated in a similar way to dextrose to increase bacterial survivability [15,16]. After culturing the medium was mixed with diatomite CELATOM FW-14 (EP Minerals, Reno, NV, USA) in a 2:1 (*w/w*) ratio. A later preparation was subjected to drying in the presented conditions. After the drying process was complete, 1 g of the preparation with bacteria was suspended in water and shaken for at least 20 min, and then diluted with water using the serial dilution method. From the chosen dilutions (10^{-6}, 10^{-7}, 10^{-8}), 100 μL was spread on the PDA plate using a sterile cell spreader [14]. The plates were then cultivated for 48 h at 30 °C. Afterwards, the colonies were counted and the composition of the optimized medium was evaluated. Each bacterium was dried in a separate container and each dryer was sterilized via heating up to 200 °C for 2 h between batches.

2.4. Prebiotic Supplementary Blend

The enhancement of bacterial growth of the 4 tested isolates by the supplementary blend consisting of D-malic acid, N-acetyl-D-glucosamine, α-keto-glutaric acid, and γ-aminobutyric acid (in an equal weight ratio, in total 10 g/L) was evaluated during 144 h of culturing in liquid culture. The medium used in the experiment was an M9 Minimal Salts medium (Merck, Darmstadt, Germany) with the 10 mL/L addition of micronutrient solution ($MnCl_2*4H_2O$ (100 mg/L), $ZnCl_2$ (150 mg/L), $CuCl_2*2H_2O$ (50 mg/L), $CoCl_2*6H_2O$ (50 mg/L), $Na_2MoO_4*2H_2O$ (50 mg/L)). Culturing took place under aerobic conditions, at pH 7.2, at 28 °C in 50 mL flasks, 15 mL of medium, 105 rpm rotation, and 50 μL of 10^6 bacterial cells per flask of inoculum. Control was provided for each isolate culture, which constituted the medium and culture conditions but without the addition of supplements. The cultures were performed in triplicate ($n = 3$). Every 24 h, 200 μL from each flask was placed in a 96-well plate and the optical density of the bacterial culture at a wavelength of 600 nm (OD600nm) was measured using a spectrophotometric microplate reader, Infinite M200PRO (Tecan®, Männedorf, Switzerland). If flocculation appeared and its severity might have had an influence on the measurement, the samples were lightly homogenized or mixed via pipetting to destroy the aggregates.

In detail, the supplements were as follows: D-malic acid (Nanga®, Złotów, Poland), N-acetyl-D-glucosamine (Glentham Life Sciences®, Corsham, UK), α-keto-glutaric acid (MyProtein®, Manchester, UK), and γ-aminobutyric acid (Ostrovit®, Zambrów, Polska). These supplements were chosen according to previous research by [5] concerning certain metabolic characteristics and features of selected bacterial isolates using GEN III plates (Biolog®, Hayward, CA, USA).

2.5. Phytotron Raspberry Pot Experiment

Seedlings of raspberry (variety *Polana*) were planted in pots, the diameter of which was 19 cm. Each pot contained 3 kg of soil collected from the top layer at the organic raspberry field, carefully sieved through a 5 mm sieve beforehand to remove stones and roots. According to District Chemical and Agricultural Station in Lublin (Lublin, Poland), the soil was classified as dense. The soil was not sterilized to ensure that the conditions were close to natural. One raspberry seedling was planted in one pot, with its roots and shoots trimmed to the same size as the others, which is a standard procedure used by farmers when establishing new raspberry plantations. After planting the surface of the soil was thoroughly covered with a blend of vermiculite and perlite (mixed 1:1) to prevent water loss from the soil due to evaporation. Soil moisture was measured using a method of

TDR (Time Domain Reflectometry) sensor FOM/mts (ETest, Poland) and kept constant at 18–20% throughout the experiment. Water was poured into the stands (diameter 23 cm) according to the TDR measurement method. The temperature in the phytotron was kept constant at 20–22 °C. The constant photoperiod of the day and night system was set at 16 h for the day and 8 h for the night. The duration of the pot experiment was two months (January and February 2020). The pot experiment was set up in triplicate (three pots per variant).

The following five variants of contamination with pathogens (pathosystems) were used: *Botrytis cinerea* G277/18 (B), *Verticillium* sp. G296/18 (V), *Colletotrichum acutatum* G172/18 (C), and *Phytophthora* sp. G408/18 (P), the variants of all of the above-mentioned tested pathogens, and controls without the addition of pathogen, but with the addition of water instead.

Pathogen inoculation was as follows: 10 mL of a freshly prepared sterile water suspension of the respective pathogens containing 10^8 conidia per ml was applied per each pot in the case of a single pathogen variant (B, V, C, P). For all of the tested pathogen variants, the suspension consisted of 2.5 mL of 10^8/mL conidia of each pathogen applied together. Pathogen inoculum was applied twice during the pot experiment. The first inoculation was performed during raspberry planting (pipetted into the soil near the roots) and the second one month after raspberry planting. In this case, the *Phytophthora* sp. and *Verticillium* sp. suspension were introduced to the soil near the roots, while *Botrytis cinerea* and *Colletotrichum acutatum* were inoculated on the plant using sterile inoculation sticks.

For every variant of pathogen contamination, four methods of naturalization strategy, including the application of beneficial bacterial strains selected from natural habitats of wild raspberries, were tested. These options correspond to situations in which biopreparations might be used in the future. The first (R) was to apply the bacterial consortium on the roots of plants during planting in pots—this method might be used when setting up a new raspberry plantation. The second was to apply the bacteria both during planting and one month after naturalization watering with the bacterial consortium (RW), which reflects newly set up plantations with both methods being used. The third one (W) corresponds to already existing plantations where it is only possible to apply microorganisms to all plants during watering. The last one was planted without any naturalization to serve as a control. Table A1 presents the experimental design of the pot experiment.

As with the pathogen inoculum, the bacterial consortium was applied in a 10 mL total volume of bacterial suspension (2.5 mL of each bacterial strain) with an abundance of 10^8/mL into the soil regardless of the naturalization variant (R, RW, W). Bacterial abundance in the inoculum was determined using the Thoma cell counting chamber and verified using serial dilution, spreading 100 μL on PCA plates and culturing for 48 h at 30 °C. After that time, colonies were counted and based on their number the number of bacteria in the inoculum was confirmed.

To obtain the appropriate number of bacteria for the pot experiment, the tested isolates were cultured on an optimized medium for 4 days. Then, the required concentration was provided through serial dilution, based on Thom's cell counting chamber results (BLAUBRAND®, Wertheim, Germany).

After the experiment, soil, root and plant samples were collected, measured and stored for future analysis. The soil samples to be used for soil analyses were stored at 4 °C. Root and shoot samples were weighed and dried, and then stored at room temperature.

2.6. Plant and Soil Analyses

The physicochemical analyses of plant and soil material from the pot experiment were performed by the District Chemical and Agricultural Station in Lublin (Lublin, Poland), according to standard procedures used in the Station described below.

Plant material (shoots) testing was performed to determine foliar feeding (N, P, K, Ca, Mg). In the soil, there was a determination of the N mineral content, the pH, P_2O_5, K_2O, Mg, the organic carbon and the soil organic matter contents.

The determination of P in plant material after mineralization in H_2SO_4 and H_2O_2 was performed using the vanadomolibdate method (KQ/PB-24) [17]. The method is based on the spectrophotometric measurement of the intensity of the yellow colour of the phosphorus–vanadium–molybdic acid complex, which is formed by orthophosphate and vanadium ions in the presence of molybdate in an acidic environment through the use of a colorimeter.

The determination of N in plant material was performed through the use of the distillation method after mineralization in H_2SO_4 and H_2O_2. Determination of nitrogen content consists of converting the amide form of nitrogen into ammonia through mineralization in concentrated sulfuric acid (VI), distilling ammonia from the alkaline medium, absorption in a known volume of a standard sulfuric acid solution, and the titration of excess acid with a standard sodium hydroxide solution (KQ/PB-70) using the Kjeldahl method with Buchi B-324 distillation apparatus and a digital burette.

The amount of Mg content in raspberry shoots was determined after mineralization in H_2SO_4 and H_2O_2 (KQJPB-26). The principle of the method consists of measuring the absorption of radiation by magnesium atoms released when the test solution is sprayed into an acetylene–air flame using a Perkin ELMER atomic absorption spectrometer.

The determination of K and Ca content in shoots was determined after mineralization in H_2SO_4 and H_2O_2 (KQ/PB-25). It involved the measurement of radiation emitted by a suitably excited sample. Excitation source—burner; flame—propane–butane air. The equipment used was a Jenway flame photometer.

The determination of nitrate and ammonium content in the soil (N-NO_3, N-NH_4) was performed using a colorimetric method which involved the use of the SKALAR SCAN ++ SYSTEM flow autoanalyzer after extraction in 1% K_2SO_4 (KQ/PB-71). Nitrate was determined using the nitrogen-reduction method with the use of cadmium. As for ammoniacal nitrogen, the modified Berthelot reaction was used.

The organic carbon content was determined using Tiurin's method with a heating plate and a Titronic R 300 digital burette being used. The soil organic matter (SOM) was evaluated through the use of a weight method using a laboratory balance, SLW 115 dryer and a Nabarthetm muffle furnace [17].

The pH in KCl was determined (PN-ISO 10390:1997) using pH-meter CP-505; the phosphorus (P_2O_5) and potassium content (K_2O) were evaluated by following the Egner–Riehm method (PN-R-04022:1996 + Az1:2002), using a Sherwood flame photometer and Genesys 6 spectrophotometer; and the magnesium content was detected through the use of the Schachtschabel method (PN-R-04020:1994 + Az1:2004), using an AAS-3 atomic absorption spectrometer.

At the end of the study, the above-ground part of the plants was cut off, and the dry weight (g), as well as height (cm) of the plants and the number of branches, were determined. Soil adhered to the roots was removed prior to weighing, shaking and washing in distilled water. In addition, the fresh weight (g) of the roots of the plants was measured. Both measurements were carried out using a laboratory balance (RADWAG, WLC 0.6/A1/C/2). All samples were stored at 4 °C before analysis for about 1 week.

For the determination of the enzymatic activity of fresh soil samples with natural moisture, samples were taken and stored at 4 °C before analysis. In the soil samples, the activity of dehydrogenase (EC 1.1.1.1) was determined using the Thalmann method [18] and modified by Alef [19] in three repetitions. Formazan equivalents released in the reaction were converted to SI units presented as cm^3 H_2. All samples were analysed on the same day.

2.7. Statistical Analysis

The colony count in the growing medium and the drying method optimization mean value were calculated based on the repetitions. For the analysis of supplementary blend influence, graphs were generated based on the mean value calculated from three replicates. For the early effect of naturalization strategies on plant growth, the mean value and

standard deviation of dehydrogenase activity in the soil were calculated and a two-way ANOVA with $p < 0.05$ was performed to check differences, and Tukey tests were also performed to reveal statistically significant differences between naturalization strategies. For the analysis of the effect of the prebiotic supplement on the growth of bacteria the mean value and standard deviation were calculated and a one-way ANOVA with $p < 0.05$, $p < 0.01$, and $p < 0.001$ was performed to check differences, and Tukey tests were also performed to reveal its statistical significance.

3. Results

3.1. Optimization of the Growing Medium

Optimizing the growing medium for bacterial growth focused on four parameters: carbon source, nitrogen source, culturing temperature and the pH value of the growing medium. As presented in Table 1, sucrose addition was optimal in supporting bacterial growth. Both glucose and lactose were also utilized by the bacteria. Peptone addition was not crucial, however, as it intensified growth when combined with a 3% concentration of lactose. In further experiments, a 3% addition of sucrose without peptone was chosen. The variant with bacteria on the control M9 medium was also considered to compare the growth rate on a classic store-bought medium to the growth achieved on a medium based on supernatant from diatomite used for beer filtration. The number of bacteria growing on the M9 medium was one order of magnitude lower for *Arthrobacter* sp. B58/18, and similar for the other tested microorganisms when compared to the 3% sucrose variant.

Table 1. The effect of different carbon sources (glucose, lactose, sucrose), their concentrations 3%, 6%, 9% and the addition of peptone on the growth of bacteria in liquid culture. The control was prepared using the M9 minimal medium containing KH_2PO_4 15 g/L, NaCl, 2.5 g/L, Na_2HPO_4, 33.9 g/L, NH_4Cl, 5 g/L. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, and *Rhodococcus* sp. B75/18, pH = 7.2, culturing temperature = 30 °C.

Carbon Source		Peptone Concentration		Colony-Forming Units (CFU/mL)			
Sugar	Concentration	Additive	Concentration	B12/18	B37/18	B58/18	B75/18
Glucose	3%	Peptone	0%	2.45×10^{11}	1.50×10^{12}	6.15×10^{10}	5.46×10^{11}
	3%		0.20%	$<10^8$	1.37×10^{12}	1.80×10^{11}	1.50×10^{12}
	6%	Peptone	0%	1.00×10^9	1.50×10^{12}	3.00×10^9	1.25×10^{10}
	6%		0.20%	6.80×10^9	4.45×10^{10}	5.00×10^8	1.66×10^{11}
	9%	Peptone	0%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
	9%		0.20%	$<10^8$	3.00×10^9	$<10^8$	$<10^8$
Lactose	3%	Peptone	0%	9.10×10^{11}	7.50×10^{11}	$<10^8$	7.96×10^{11}
	3%		0.20%	1.50×10^{12}	1.50×10^{12}	1.00×10^{10}	6.25×10^{10}
	6%	Peptone	0%	7.61×10^{11}	7.50×10^{11}	$<10^8$	$<10^8$
	6%		0.20%	4.00×10^{10}	5.20×10^{10}	$<10^8$	3.50×10^9
	9%	Peptone	0%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
	9%		0.20%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
Sucrose	3%	Peptone	0%	1.50×10^{12}	1.22×10^{12}	1.50×10^{12}	1.28×10^{12}
	3%		0.20%	1.50×10^{12}	1.50×10^{12}	1.50×10^{12}	1.50×10^{12}
	6%	Peptone	0%	1.50×10^{12}	1.50×10^{12}	8.66×10^{11}	1.50×10^{12}
	6%		0.20%	8.50×10^9	5.20×10^{10}	7.00×10^9	2.03×10^{11}
	9%	Peptone	0%	2.24×10^{11}	1.00×10^{10}	1.50×10^{10}	3.29×10^{11}
	9%		0.20%	2.15×10^{11}	6.20×10^{10}	1.30×10^{10}	1.80×10^{11}
Control using M9 medium				1.11×10^{12}	1.50×10^{12}	1.42×10^{11}	1.46×10^{12}

The results presented in Table 2 show that out of all of the tested nitrogen compounds, ammonium nitrate provided the best growth conditions for the bacteria *Pseudomonas* sp. B37/18 and *Arthrobacter* sp. B58/18. A 6% addition of this N source caused bacteria to grow to the highest concentration of 5.20×10^9. Isolate *Rhodococcus* sp. B12/18 grew in the narrowest

spectrum of nitrogen compounds and their concentrations, while *Rhodococcus* sp. B75/18 utilized all tested nitrogen compounds in the broadest spectrum of their concentrations. Ammonium nitrate (NH_4NO_3) was utilized by a broad spectrum of bacteria, but only in a higher concentration of 6%. For future experiments upon growth optimization, 6% of NH_4NO_3 was chosen as the best nitrogen source for all tested bacteria.

Table 2. The effect of different nitrogen sources ($NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl) and their concentrations of 1%, 3%, 6%, on the growth of bacteria in a liquid culture. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, and *Rhodococcus* sp. B75/18, pH = 7.2, culturing temperature = 30 °C.

Nitrogen Source		Colony-Forming Units (CFU/mL)			
Compound	Concentration	B12/18	B37/18	B58/18	B75/18
$NaNO_3$	1%	2.50×10^9	$<10^8$	6.50×10^9	4.4×10^{11}
	3%	$<10^8$	$<10^8$	$<10^8$	3.1×10^{11}
	6%	$<10^8$	$<10^8$	$<10^8$	$<10^8$
NH_4NO_3	1%	$<10^8$	$<10^8$	1.30×10^{10}	2.05×10^{10}
	3%	$<10^8$	1.10×10^{11}	2.00×10^{10}	2.7×10^{10}
	6%	5.20×10^9	2.23×10^{11}	1.20×10^{10}	2.6×10^{11}
$(NH_4)_2SO_4$	1%	3.00×10^9	1.00×10^9	$<10^8$	$<10^8$
	3%	1.00×10^9	1.50×10^{10}	$<10^8$	$<10^8$
	6%	4.00×10^9	6.00×10^9	1.40×10^{10}	3.5×10^{11}
NH_4Cl	1%	4.26×10^8	4.50×10^9	1.50×10^9	6.47×10^{11}
	3%	$<10^8$	$<10^8$	3.00×10^9	5.26×10^{11}
	6%	6.00×10^9	4.00×10^9	$<10^8$	4.67×10^{11}

As presented in Figure 1, the temperature affected the growth of bacteria. The most optimal temperature for all bacteria was a temperature of 24 °C. The results also indicate that a temperature of 30 °C provided sufficient growth of bacteria, except B58/18 (*Arthrobacter* sp.). From all of the bacteria tested, only isolate B12/18 was able to grow in acidic conditions, when the medium had a pH value of 5.5–6, and isolate B37/18 grew in a medium with a pH value of 6.5. The most optimal pH values of the optimized medium which allowed for the growth of all examined bacteria ranged between 7 and 8.
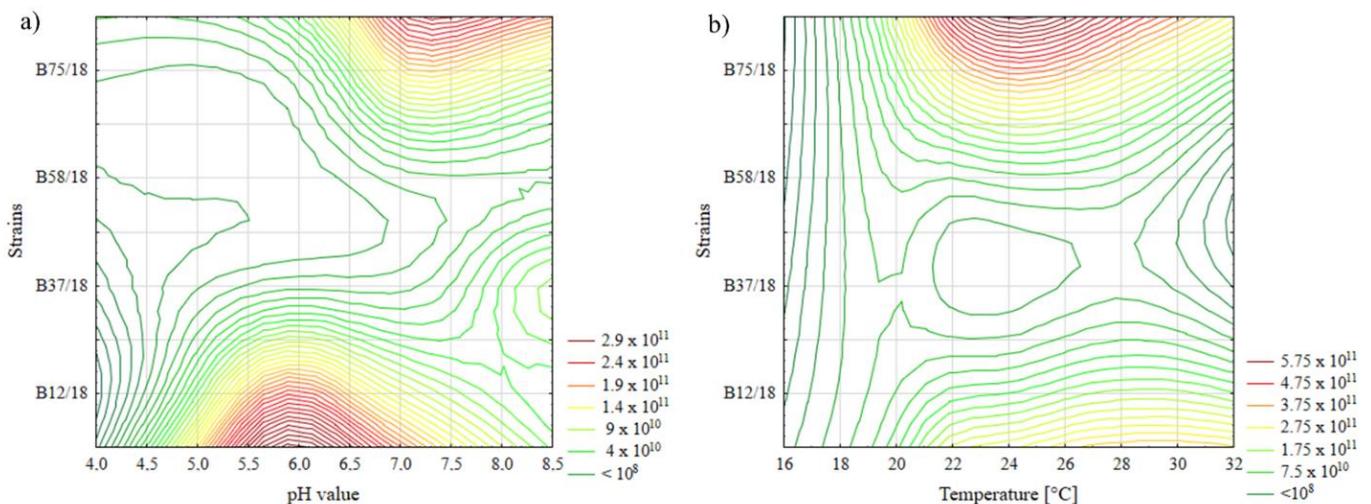

Figure 1. The effect of different medium pH values—(**a**) and temperatures—(**b**) on the growth of bacteria in a liquid culture. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, *Rhodococcus* sp. B75/18.

3.2. Influence of Bacterial Prebiotic Supplement Addition to the Growing Medium

The enhancement of growth is presented in Figure 2; this was caused by the 1% addition of the supplementary blend and was observed for two out of the four tested bacterial isolates growing in the M9 medium. An increase in optical density measured at 600 nm was observed for *Rhodococcus* sp. B12/18 and *Pseudomonas* sp. B37/18. After 100 h of cultivation, both *Arthrobacter* sp. B58/18 and *Rhodococcus* sp. B75/18 isolates began to flocculate, which resulted in the need to lightly homogenize the cultures to destroy the aggregates and ensure the accuracy of the measured optical density. All cultures were lightly homogenized to ensure a similar testing environment. Isolates B58/18 and B75/18 presented similar growth results with or without the prebiotic supplementary blend.
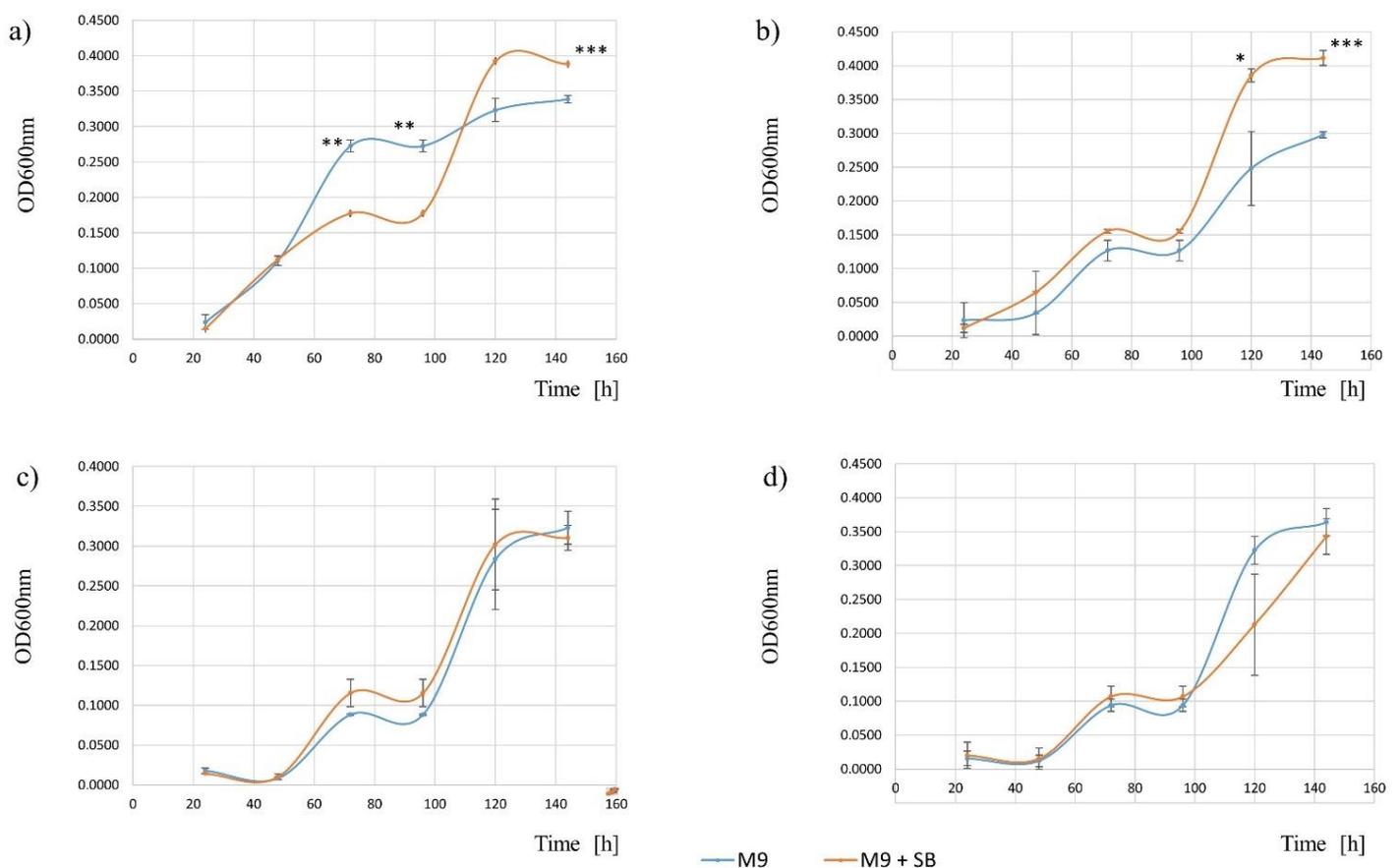

Figure 2. Effect of the 1% supplementary blend addition (D-malic acid, N-acetyl-D-glucosamine, α-keto-glutaric acid, γ-aminobutyric acid) on the growth of bacteria during 144 h of culturing in the M9 medium. Figures represent OD600nm measured during cultivation every 24 h for particular strains of bacteria accordingly: (**a**) *Rhodococcus* sp. B12/18, (**b**) *Pseudomonas* sp. B37/18, (**c**) *Arthrobacter* sp. B58/18, (**d**) *Rhodococcus* sp B75/18; n = 3, error bars represent standard sample deviation. Stars represent the level of significance (*p*-value) of the differences within the particular hour. Three stars—*p*-value < 0.001, two stars—*p*-value < 0.01, one star—*p*-value < 0.05.

3.3. Optimization of Preservation Technique

In conventional drying (D) of the bacterial pellet, for the medium with bacteria and the medium with bacteria absorbed on diatomite the number of bacteria after drying ranged from 10^6–10^8, through to 10^6–10^8 to 10^6–10^{10}, respectively. In vacuum drying (VD) of the bacterial pellet, for the medium with bacteria and the medium with bacteria absorbed on diatomite the number of bacteria after drying ranged from 10^6–10^7, through to 10^6–10^8 to 10^6–10^9, respectively. In the lyophilization (LF) of the bacterial pellet, in the medium with bacteria and in the medium with bacteria absorbed on diatomite the number of bacteria after drying ranged from 10^6 to 10^8 depending on the sample preparation strategy.

All drying techniques, including D—drying, VD—vacuum drying, and LF—lyophilization, and sample preparation had a significant influence on the survivability of the bacteria. The controls included fresh culture and bacterial pellet after centrifugation at the speed of 2800 rcf for 10 min to examine the influence of spinning on several viable bacteria in the sample. As presented in Table 3, the number of viable cells after centrifugation was reduced by up to two orders of magnitude, as in the case of *Rhodococcus* sp. B12/18 or by 5.6 times as for *Rhodococcus* sp. B75/18. Lyophilization resulted in the lowest decrease in bacterial number in the samples without the differentiation in sample type. Lyophilization of the bacterial pellet resulted in the survival of all tested isolates in a number higher than 10^6. The vacuum drying of the medium with bacteria absorbed on diatomite resulted in the survival of most strains in a number higher than 10^8 except for *Rhodococcus* sp. B12/18. This type of drying was chosen as the most effective and least costly in terms of energy compared to the other preservation methods, based on the results achieved. Even though lyophilization of the bacterial pellet resulted in improved survivability compared with vacuum drying, its energy cost and difficulty to adapt in commercial processes, when compared to vacuum drying, resulted in the resignation of this method for future research. Further preparation of biopreparation was achieved with the use of vacuum drying.

Table 3. The effect of different drying methods and sample preparations on the survivability of bacteria used in naturalization biopreparation. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, *Rhodococcus* sp. B75/18, D—drying, VD—vacuum drying, LF—lyophilization.

Drying Technique and Sample Type		Colony-Forming Units (CFU/g)			
		B12/18	B37/18	B58/18	B75/18
Control	Fresh culture	7.50×10^{10}	6.00×10^8	4.10×10^9	8.50×10^9
	Bacterial pellet	1.00×10^8	2.00×10^7	2.00×10^8	1.50×10^9
D	Bacterial pellet	$<10^6$	$<10^6$	1.00×10^6	1.00×10^8
	Medium with bacteria	$<10^6$	$<10^6$	$<10^6$	1.00×10^8
	Medium with bacteria absorbed on diatomite	4.00×10^{10}	1.00×10^6	9.90×10^8	$<10^6$
VD	Bacterial pellet	$<10^6$	$<10^6$	1.00×10^7	2.00×10^7
	Medium with bacteria	2.00×10^8	$<10^6$	$<10^6$	$<10^6$
	Medium with bacteria absorbed on diatomite	$<10^6$	5.30×10^9	7.00×10^8	4.70×10^9
LF	Bacterial pellet	7.00×10^6	1.00×10^7	2.00×10^6	2.00×10^8
	Medium with bacteria	3.00×10^8	$<10^6$	1.00×10^7	1.00×10^8
	Medium with bacteria absorbed on diatomite	7.00×10^8	$<10^6$	1.00×10^6	1.00×10^8

The effect of the addition of trehalose to the culturing of bacteria subject to conventional drying (D) is presented in Table 4. The results show an increase in the units (CFU) of *Arthrobacter* sp. B58/18 after drying by one order of magnitude from 10^8 to 10^9. The addition of trehalose to the culturing of bacteria subjected to vacuum drying (VD) resulted in an increase in CFU for *Rhodococcus* sp. B75/18 after drying by one order of magnitude, from 10^9 to 10^{10}. The addition of trehalose both for the culturing and drying of bacteria subjected to conventional drying (D) resulted in a survival rate that ranged from 10^7 to 10^8 for *Rhodococcus* sp. B12/18 and *Arthrobacter* sp. B58/18, respectively. The addition of trehalose to the culturing and drying of bacteria subjected to vacuum drying (VD) resulted in a survival rate that ranged from 10^8 to 10^9 for *Pseudomonas* sp. B37/18 and *Rhodococcus* sp. B75/18, respectively. Comparing the CFU after the conventional drying (D) of mixed batches combining *Rhodococcus* sp. B12/18 and *Arthrobacter* sp. B58/18 with the trehalose added only during culturing and for both culturing and drying, it may be noted that the results were 1.50×10^9 and 1.67×10^8, respectively. However, from the CFU after the vacuum drying (VD) of mixed batches that combined *Pseudomonas* sp. B37/18 and *Rhodococcus* sp. B75/18 with the trehalose added only

during culturing and during both culturing and drying, it may be noted that the results were 3.97×10^9 and 8.87×10^9, respectively.

Table 4. The effect of trehalose addition and the chosen drying methods on bacterial survivability in drying (D) and vacuum drying (VD) processes used in manufacturing and in the naturalization biopreparation. *Rhodococcus* sp. B12/18, *Pseudomonas* sp. B37/18, *Arthrobacter* sp. B58/18, *Rhodococcus* sp. B75/18, D—drying, VD—vacuum drying.

Bacterial Isolate	Trehalose Addition		Drying Technique	Colony-Forming Units (CFU/g)
	To the Culture	To Drying		
B12/18	0.1 M	-	D	$<10^7$
B58/18	0.1 M	-	D	1.20×10^9
B37/18	0.1 M	-	VD	2.00×10^8
B75/18	0.1 M	-	VD	1.96×10^{10}
B12/18	0.1 M	0.1 M	D	$<10^7$
B58/18	0.1 M	0.1 M	D	1.00×10^8
B37/18	0.1 M	0.1 M	VD	1.00×10^8
B75/18	0.1 M	0.1 M	VD	5.50×10^9
B12/18 + B58/18	0.1 M	-	D	1.50×10^9
B37/18 + B75/18	0.1 M	-	VD	3.97×10^9
B12/18 + B58/18	0.1 M	0.1 M	D	1.67×10^8
B37/18 + B75/18	0.1 M	0.1 M	VD	8.87×10^9

The addition of trehalose during culturing increased the survivability of *Rhodococcus* sp. B75/18 in vacuum drying and *Arthrobacter* sp. B58/18 during conventional drying.

The addition of trehalose during culturing and after culturing did not produce as much of a significant result as the addition which occurred only during culturing. It is worth mentioning that drying bacteria in mixed batches did not result in a reduction in survivability.

3.4. The Early Effect of the Bacterial Consortium on Raspberry Plants and on Soil Properties

The influence of bacterial isolates on raspberry plants was evaluated based on four parameters: dry mass of the shoots which allows for a comparison to be made with regard to plants size, the wet mass of the roots which may correlate with microorganisms influencing the plant rhizosphere, overall soil dehydrogenase activity which is correlated with the influence of the biopreparations on the soil microbial community, and the effect of naturalization strategies on soil micro- and macronutrients content.

3.4.1. The Early Effect of the Bacterial Consortium on Plant Mass

As presented in Figure 3, the early effect of the bacterial consortium used on raspberry plants depended on the mass of shoots and differed depending on the variant used, but the bio-stimulating effect of the developed biopreparation was observed in plants that were inoculated with pathogens (Figure A1). In general, the mass of shoots was higher in raspberry plants treated with biopreparation. In the variant with the *Botrytis cinerea* pathosystem, the highest mass of shoots was measured for watering with the addition of a biopreparation, which suggests that this method of biopreparation application might be the most appropriate choice against *B. cinerea* infection. For the *Verticillium* sp. inoculation variant, the highest mass was observed for the plants that were treated with biopreparation during planting (Figure 3).
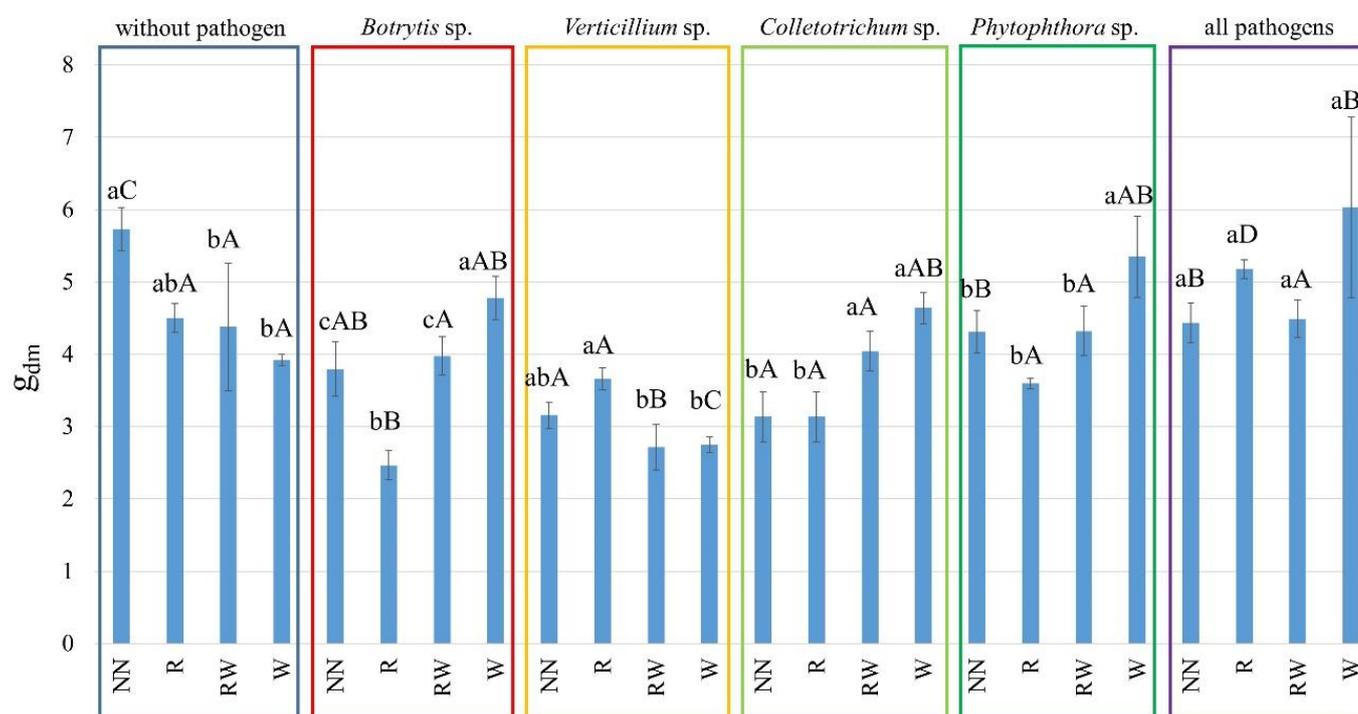
Figure 3. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depends on the pathosystem and naturalization strategy applied. The dry biomass of shoots varies, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis, Verticillium, Colletotrichum, Phytophthora*) used and also without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). The error bars indicate a standard deviation, *n* = 3, different lowercase letters above the bars indicate differences within the particular pathosystems, and different capital letters above the bars indicate differences within the particular naturalization strategies (according to ANOVA with *p* < 0.05).

Other tested naturalization strategy variants were less successful in inhibiting the negative effect of pathogens on plants. For inhibiting the influence of *Colletotrichum acutatum* on raspberries, the most effective method was watering with the addition of biopreparation, or this method combined with root naturalization during planting. Only root naturalization had no effect on the plants. Again, for plants inoculated with *Phytophthora* sp., the most effective method was watering the plants with the addition of biopreparation. Root inoculation during planting without additional treatment was not effective enough. However, the plants that were treated with all four pathogens had the highest dry mass of shoots when watered with the biopreparation. Root naturalization during planting was more effective than no biopreparation or watering naturalization combined with root naturalization.

Appendices C and D Figure A3 present the changes in the number of shoots and the average length of plants for different pathosystems.

3.4.2. The Early Effect of the Bacterial Consortium Used on Root Mass

Figure 4 shows that the roots collected from plants that were not inoculated with any pathogens were the heaviest for variants without biopreparation application. Application during planting and application during watering combined with further application with watering was less effective than watering application. Inoculation of the plants with *Botrytis cinerea* caused a decrease in the mass of the roots compared to the variants without the pathogen. The application of bacterial biopreparation resulted in an increasing mass of roots in the plants that were treated during planting as compared to the plants without any bacterial inoculum applied. In the *Verticillium* sp. variants, the application of the tested biopreparation during planting or watering caused an increase in root mass in comparison with the application during planting and after that with water. *Colletotrichum acutatum* increased the mass of

the root in objects without any naturalization treatment when compared to the control, although the difference was not statistically significant. The application of biopreparation was not effective in plant biostimulation in pathosystem with *C. acutatum*. In the plants inoculated with *Phytophthora* sp. root growth was reduced both in the variants without naturalization and when all naturalization strategies were applied. In plants that were inoculated with all 4 pathogens and had no naturalization strategy applied the growth of the roots was decreased in comparison with the objects without any naturalization strategy applied. The most effective method in inhibiting the negative influence of phytopathogens was watering with biopreparation and its application during planting along with subsequent watering with biopreparation.
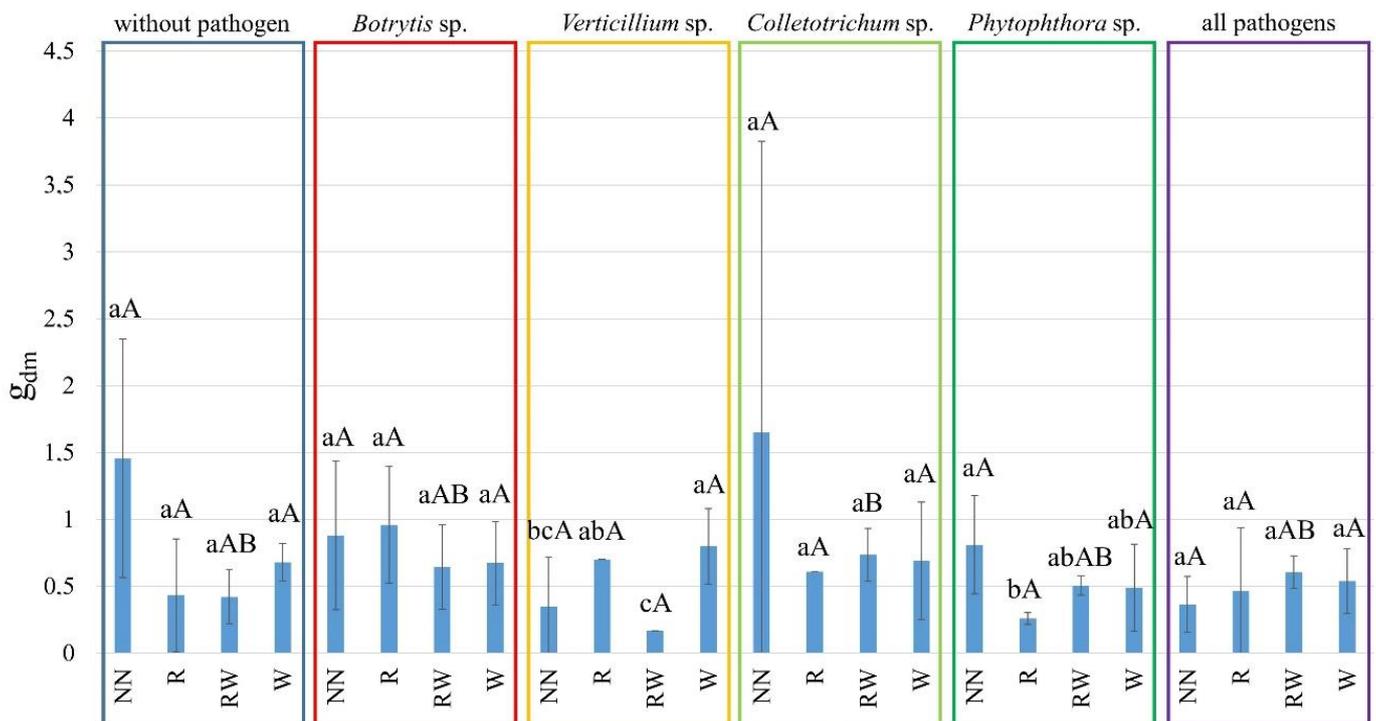

Figure 4. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depended on the pathosystem and naturalization strategy applied. The wet biomass of the roots, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). The error bars indicate a standard deviation, *n* = 3, different lowercase letters above the bars indicate differences within the particular pathosystem, and different capital letters above the bars indicate differences within the particular naturalization strategies (according to ANOVA with *p* < 0.05).

3.4.3. The Early Effect of the Bacterial Consortium on Soil Dehydrogenase Activity

The results of the naturalization strategies with regard to dehydrogenase activity are shown in Figure 5. Both the application of the biopreparation and the presence of a pathogen influenced dehydrogenase activity in the soil. For experiments conducted without the presence of pathogens, dehydrogenase activity was highest in the experiments in which both types of naturalization strategies were applied—root naturalization and watering naturalization.
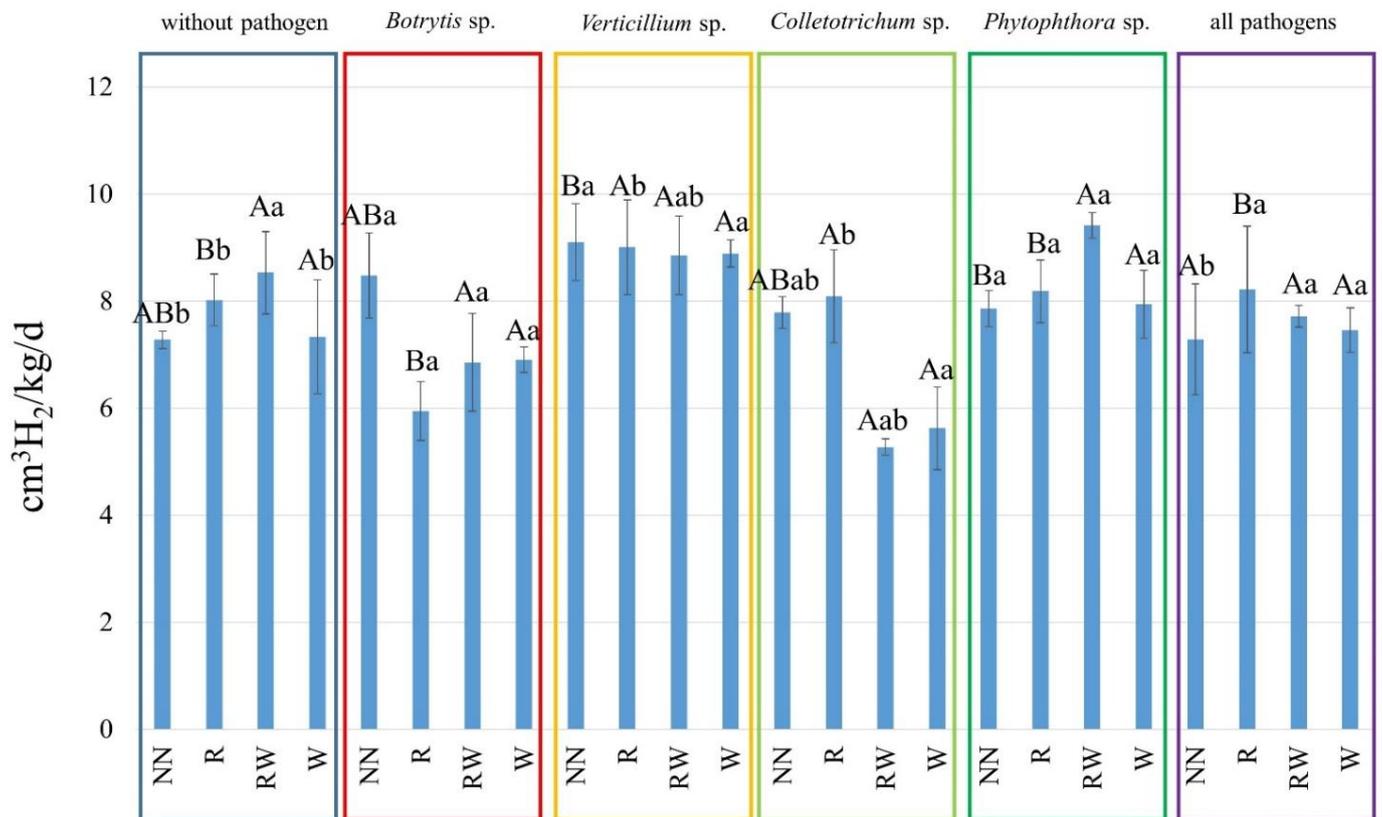
Figure 5. The effect of the bacterial consortium on dehydrogenase activity in the soil samples depending on the pathosystem and naturalization strategy applied. Dehydrogenase activity varied according to the pathosystems used, *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). Error bars indicate the standard deviation, *n* = 3, different lowercase letters above the bars indicate differences within the particular pathosystem, and different capital letters above the bars indicate differences within the particular naturalization strategies (according to ANOVA with *p* < 0.05).

The second highest activity was observed in naturalization strategies with only root inoculation, while watering with biopreparation resulted in no effect on the dehydrogenase level when compared to the strategies without the naturalization applied. For plants inoculated with *Botrytis cinerea*, dehydrogenase activity was higher without any naturalization, and a decrease in the dehydrogenase activity to lower levels than in the control was observed after the application of biopreparation. The highest levels of dehydrogenase activity, despite the overall decrease, were observed when both naturalization strategies were applied including watering naturalization and roots naturalization. In experiments carried out in the presence of *Verticillium* sp., enzymatic activity was comparable to the control regardless of any naturalization strategies, although activities in those objects were the highest among all other trials. In *Colletotrichum acutatum*, the pathosystem activity of the dehydrogenase was the highest while root naturalization was applied; however, it was only a tendency, without significant differences. Furthermore, both watering naturalization and combined naturalization resulted in reducing dehydrogenase activity in this pathosystem. In experiments involving an inoculation with the fungal-like pathogen *Phytophthora* sp., a combined naturalization strategy was most effective in increasing dehydrogenase activity. Other application methods had a similar effect and were comparable with the no-naturalization variant. In experiments that were subjected to inoculation with all four pathogens, dehydrogenase activity was the highest for experiments treated with biopreparation during planting. The second highest result was achieved by the

combined naturalization strategy, and last but not least watering with the addition of the biopreparation.

3.4.4. The Early Effect of the Bacterial Consortium on Soil Macronutrients, Minerals and Nitrogen Content

The amount of macronutrients according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp. and all pathogens (*Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp.) and without pathogens, within different biopreparation naturalization strategies (no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W)) was evaluated, and the results are presented in Table 5.

Table 5. The effect of the bacterial consortium on the macronutrient amount in the plant, root and in the soil samples depends on the pathosystem and naturalization strategy applied. The amount of macronutrients varied according to the pathosystem used, *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp. and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W).

Pathogen Contamination	Naturalization	Macronutrients in Shoots					Absorbable Forms of Minerals in the Soil			Nitrogen in Soil		
		N	P	K	Ca	Mg	P_2O_5	K_2O	Mg	N-NO$_3$	N-NH$_4$	N_{min}
				% d.m.				mg/100 g Soil			mg/kg d.m.	kg/ha
without pathogen	NN	1.44	0.29	2.47	1.22	0.44	18.3	32.5	14.3	4.41	6.26	45.90
	R	1.44	0.3	2.63	1.38	0.48	17.6	31.8	12.9	4.18	6.34	45.20
	RW	1.4	0.27	2.15	1.2	0.48	20.3	33.9	14.5	1.41	7.87	39.90
	W	1.72	0.29	2.46	1.12	0.48	20.5	34.3	15	3.33	7.10	44.80
Botrytis cinerea	NN	1.41	0.28	2.12	1.13	0.46	19.6	35.6	12.6	3.65	7.71	48.80
	R	1.52	0.29	2.65	1.28	0.48	20.2	35.3	12.7	10.7	3.35	60.40
	RW	1.48	0.3	2.29	1.21	0.44	21.1	36.1	13.9	3.11	7.40	45.20
	W	1.34	0.29	2.21	1.22	0.42	20.5	35.4	14.9	2.83	6.61	40.60
Verticilium sp.	NN	1.51	0.29	2.04	1.2	0.42	16.1	31.8	14	2.23	9.37	49.90
	R	1.66	0.3	2.31	1.16	0.4	15.5	29.8	13.5	2.75	8.02	46.30
	RW	1.65	0.29	2.35	1.15	0.32	16.1	31.7	14.6	2.45	8.94	49.00
	W	1.34	0.27	2.05	1.33	0.4	18.1	33.9	14.8	1.34	8.74	37.60
Colletotrichum acutatum	NN	1.18	0.26	2.01	1.33	0.38	17.5	31.8	12.8	2.78	7.45	44.00
	R	1.6	0.27	2.03	1.43	0.44	18	33.1	12	1.36	8.06	40.50
	RW	1.62	0.29	2.17	1.34	0.4	18	33.6	13.7	9.36	3.72	56.20
	W	1.67	0.29	2.63	1.36	0.42	18.4	33.2	13.4	12.1	1.96	60.70
Phytophthora sp.	NN	1.6	0.28	2.56	1.36	0.4	18.5	32.3	14.4	4.40	6.61	47.30
	R	1.47	0.24	2.14	1.3	0.38	19.7	36.7	15	4.33	5.36	41.70
	RW	1.16	0.24	2.04	1.17	0.32	15.5	32.9	13.9	2.84	7.85	46.00
	W	1.61	0.28	2.81	1.39	0.42	17.4	29.2	13.1	3.38	9.83	56.80
all pathogens	NN	1.54	0.27	2.23	1.24	0.39	16.7	30.4	12.9	4.32	9.15	57.90
	R	1.57	0.27	2.21	1.22	0.38	14.9	28.8	13.1	4.11	9.55	58.70
	RW	1.44	0.3	2.19	1.33	0.44	16.3	32.4	13.4	4.59	7.8	53.30
	W	1.34	0.3	2.26	1.2	0.38	18.4	34.7	13.9	2.65	9.39	51.80

The amount of nitrogen in shoots was the highest in the control experiments without any pathogens, but with the biopreparation applied via watering. For all tested pathosystems, the application on roots during planting caused an increase in the level of nitrogen in the shoots as compared to the experiments without the application of naturalization. Differences in the phosphorus level in the dry mass of the plants were negligible. The amount of potassium in the plants differed between all of the samples studied. The highest level was observed in the *Botrytis cinerea* pathosystem when roots naturalization was applied and in the *Phytophthora* sp. pathosystem when watering naturalization was applied. For *Botrytis cinerea* and *Verticillium* sp. variants, root application and combined naturalization were the most effective methods. For *Colletotrichum acutatum* and *Phytophthora* sp., application via watering was the most effective method, and in both cases levels of this element were higher than in the control.

The calcium levels were the highest in *B. cinerea* and *C. acutatum* pathosystems with roots treated during planting, and in *Verticillium* sp. and *Phytophthora* sp. treated during watering. The amount of magnesium in plants was the highest in the control experiments and *B. cinerea* variants with treated roots. Levels of this element were low in all other pathosystems except for *C. acutatum* experiments treated during planting and experiments with both naturalization strategies applied in pathosystem composed of all four pathogens.

In the control experiments, without the presence of pathogens, the highest amount of absorbable forms of minerals in soil were measured under plants that had been treated using a combined naturalization strategy of watering and the root application of biopreparation, and under plants that were watered with biopreparation.

The number of absorbable forms of minerals in the soil in experiments involving an exposure to pathogens was highest in the *B. cinerea* pathosystem, and in this case, the most effective method was watering with biopreparation. A high amount of minerals in the soil was observed in experiments involving an exposure to *Phytophthora* sp. with roots treated during planting.

The concentration of nitrogen in the soil was evaluated in terms of its three most important forms: nitrate-nitrogen (N-NO$_3$), ammoniacal nitrogen (N-NH$_4$) and mineral nitrogen (N$_{min}$). The highest levels of the nitrate form of nitrogen were found in soil samples collected in experiments involving an exposure to *Botrytis* sp. and naturalized with biopreparation during planting. High levels of this type of nitrogen were found in *Phytophthora* sp. variants treated with both root naturalization and naturalization watering and variants only treated with naturalization watering. Ammoniacal nitrogen was found in the soil at its highest concentration in samples collected from experiments involving an exposure to *Verticillium* sp., *Phytophthora* sp. and all four pathogens treated with both naturalization strategies, watering and roots naturalization, respectively. Mineral nitrogen content was the highest in soil collected from experiments involving an exposure to *Botrytis cinerea* involving the application of root treatment. *Colletotrichum acutatum* and *Phytophthora* sp. pathosystems also contained high levels of soil mineral nitrogen. Experiments involved combined naturalization strategies and watering naturalization for *Colletotrichum acutatum* and a naturalization for *Phytophthora* sp., respectively. In the pathosystem including all tested fungal and fungal-like plant pathogens, mineral nitrogen levels were high for variants with roots naturalization, and lower for variants that included watering with a biopreparation, such as the combined and watering naturalization strategies.

4. Discussion

4.1. Optimization of Growing Medium

The optimization of the growing medium is an essential part of designing any microbial biopreparation to be used in agriculture. This type of process can be applied to reduce the cost of microbial culturing and increase the number of viable cells in the finished biopreparation. This may result in decreasing the costs of the final product and the energy that must be used during the manufacturing processes [20–22]. Additionally, it is important to mention that changes in the growing medium can influence bacterial antagonistic properties against fungal plant pathogens [23]. All of the tested isolates of bacteria were able to utilize all three tested sugars as carbon sources, but sucrose was the only sugar that in the lowest examined concentration of 3% allowed all four isolates to reach numbers as high as 10^{12} in 48 h of culturing. Peptone addition did not provide any notable advantage which might suggest that the supernatant from diatomite used for beer filtering might contain a source of substances similar to those in peptone, such as an organic nitrogen source, peptides and polypeptides, vitamins and amino acids [24]. Comparing the obtained results of bacterial growth on the proposed medium with a 3% sucrose content to the commercially available M9 medium, it may be noted that growth on both media is similar with one order of magnitude higher on the tested medium as compared to the M9 medium for *Arthrobacter* sp. B58/18.

Ammonium nitrate has been previously proven to be efficient as a carbon source in bacterial growth similar, to other tested mineral nitrogen sources [25–27]. It is a cheap and efficient nitrogen source not only for bacteria but also for plants. It may be assumed that residues of ammonium nitrate in biopreparation can be utilized by bacteria and plants after the application of the biopreparation. It might be critical to evaluate the temperature and pH value of the culturing medium while optimizing the growing medium, especially for environmental isolates that have not been cultured and fully understood previously. The optimal soil pH value for most plants and plant-associated bacteria is slightly acidic or neutral. As we have proven for these environmental isolates, the optimal pH value of the growing medium is in the range between 7 and 8.

Carefully chosen medium additives have the potential to influence bacterial growth in a very effective way. In previous research we chose four chemical compounds based on their efficient utilization by bacteria [13]. D-malic acid, N-acetyl-D-glucosamine, α-keto-glutaric acid, and γ-amino-butyric acid were added to the medium in a 1% m/v total concentration. D-malic acid may be a stimulant for soil bacteria since some plants secrete this compound to the soil near their roots to possibly attract plant-growth-promoting bacteria [28]. N-acetyl-D-glucosamine takes part in cell signaling and in the construction of the cell wall [29,30]. The α-ketoglutaric acid molecule is an antioxidant; this organic acid is also a part of the Krebs cycle and its addition may stimulate bacterial growth [31,32]. The γ-amino-butyric acid molecule is an amino acid that can be used as a carbon or nitrogen source by some bacterial isolates, e.g., *Pseudomonas* sp. [33,34]. Although supplement addition was only 1% of the total medium mass, it had a positive impact on the growth of two isolates—*Rhodococcus* sp. B12/18 and *Pseudomonas* sp. B37/18. Two other isolates were not affected by the addition of supplements when compared to the control.

4.2. The Preservation of Bacteria

The preservation of bacterial cells to ensure their viability after long-term storage is not an easy task. What is more important is that the isolates used in this study were non-spore-forming Gram-negative bacteria. This makes their preservation much more difficult, and thus it is important to optimize this process correctly, ensuring a broad spectrum of methods, and at the same time it must be borne in mind that this process needs to be scalable to industrial processes. Chosen methods such as conventional drying, vacuum drying and lyophilization are typically used in the preservation of different strains of bacteria. Survivability depends on the type of bacteria and process parameters [15,35–37]. This research proved that environmental isolates of non-spore-forming bacteria can be preserved using many methods and sample preparation techniques. It has been proven that spinning bacterial cultures affected the number of viable cells in the medium. Another study indicated that the longer the storage time and the higher the RCF, the more bacteria are destroyed [38].

In this research, centrifugation resulted in a decrease in the number of viable cells, from five times up to over two orders of magnitude. Different sample preparation techniques produce many advantages and disadvantages. Preserving only the bacterial pellet results in a decrease in the drying time due to the removal of most of the water with the supernatant, but also the removal of secondary metabolites and substances that might help bacteria deal with the temperature and moisture stress [39]. Drying the whole medium with the bacteria ensures that the bacteria are surrounded by all of its metabolites and any remaining sucrose that might help bacteria to survive the drying process, but the increasing osmotic pressure caused by removing water from the drying medium might negatively affect bacteria during the drying process. The removal of large amounts of liquid takes more time by lengthening the drying process and subjecting bacteria to longer periods of high temperature [40,41]. The sample that consisted of medium absorbed on diatomite dried far more rapidly than the sample without diatomite due to its much larger surface area. The location of the absorbing medium with bacteria into diatomite results in the bacteria being surrounded by its growing medium, but because diatomite is a very porous material it is a great binder

to bacteria. However, diatomite is proven to have a negative ion load that may tightly bind bacteria without their ability to escape after biopreparation application [42]. Another advantage of using diatomite in this type of biopreparation is that it is chemically inert and can store large amounts of water, which, after the application of biopreparation, may help both plants and bacteria by improving soil water retention [43]. A classic sample drying resulted in the Maillard reaction and the process took over 72 h. Even though lyophilization was effective in the preservation of almost every sample type and bacterial isolate, due to its energy cost, sample preparation time (freezing), and the small amounts that can be lyophilized at once, it was decided that this type of drying technique will not be used in future biopreparation production. Vacuum drying was chosen as the best option for those particular isolates and for future application in biopreparation manufacturing due to its easy scalability to industrial processes. Furthermore, drying using vacuum drying resulted in the biopreparation having the form of a light and airy powder with few to no harder granules. This may help with ease of use by farmers and may also result in less of a caking tendency.

Trehalose is known to serve a role as a cryoprotectant during lyophilization and microbiological preparation drying. Its ability to both stabilize biomolecules and decrease the negative effect of drought and high temperatures makes it an ideal additive for the preservation of bacterial cells [44,45]. Research by Crove, Reid and Crowe (1996) suggests that trehalose may be superior to other sugars for preserving biomaterials [15,46]. Research has also shown that the addition of trehalose to the growing medium before culturing may stimulate bacteria to prepare for temperature and drought stress, thereby improving their survivability during preservation and enhancing the production of heat shock proteins [47]. The addition of trehalose to growing medium and preparation before drying had an impact on the survivability of some isolates such as *Rhodococcus* sp. B75/18. Since trehalose may facilitate the drying process, it was included in the final growing medium as one of the components.

4.3. Effect of Biopreparation on the Early Growth of Plants

Scientists agree that the early growth period is a crucial time for plants, which can affect their whole life. It is not only important to ensure that the soil is well prepared but also that it is rich in microorganisms that can enhance the growth of young plants such as raspberry seedlings and protect them from pathogens that are present in the soil [48–50]. Soilborne pathogens can be transported from the nursery to newly set up plantations, and from soil to leaves by soil splash that periodically occurs, e.g., during the rain [51,52]. The early effect of selected strains of bacteria on the growth of raspberry plants varied depending on the phytopathogen contamination variant and naturalization strategy. All experiments involving contamination with pathogens benefitted due to naturalization when compared with the experiments conducted without naturalization, in all pathosystems except *Phytophthora* sp. Additionally, for all pathogens, the highest shoot mass was lower than the control without any pathogens. This leads to the conclusion that the addition of biopreparation to plants infected with pathogens resulted in a higher plant mass compared to experiments without bacterial treatments. Inoculation with pathogens resulted in a decrease in plant mass as compared to the control, which shows that the plants were truly affected by the pathogens. Although the shoot mass suggested that biopreparation may be an important factor in inhibiting the effects of pathogen infection in plants, the mass of the roots decreased in most experiments involving a microbial treatment. All pathogens except *Colletotrichum acutatum*, which along with *Verticillium* sp. was inoculated on shoots, caused a decrease in root mass compared with the control. In plants without pathogen infection, naturalization caused a decrease in plant mass. This is an important observation, and may be connected to the stress of plants due to the colonization of plant tissues by microorganisms. However, the experiment was conducted for 8 weeks, which might be not enough to observe the full naturalization effect. It is known that stress-induced bacterial genes are associated with plant–bacterial interactions and plant-

associated bacteria can coordinate interactions with plants [13]. Therefore, we anticipate that after the first stress, plants will adapt to naturalization conditions and a positive effect will be possible, as was observed in pathosystems combined with naturalization strategies. Moreover, according to the literature [13], in the future there lies great potential in plant growth-promoting microorganisms as inoculants of agricultural soils.

Dehydrogenase activity is often used as an indirect indicator of overall soil microbial activity, because it occurs intracellularly in all living microbial cells and does not accumulate in the soil [53–55]. An increase in enzymatic activity might suggest that the soil microbiota present a high degree of activity and that the soil with the naturalization presents a sufficient medium for microbial life [54,56]. In experiments not involving pathogen infection, dehydrogenase activity was higher than in experiments treated with the naturalization treatment. In all pathosystems except *Botrytis cinerea*, at least one biopreparation application method caused an increase in soil enzymatic activity. Root naturalization and combined naturalization strategy (root treatment and naturalization watering) were the most effective treatment. This suggests that overall biopreparation has a positive effect on soil microbial communities by enhancing the dehydrogenase activity. It is worth noting that even though *Botrytis cinerea* and *Colletotrichum acutatum* were applied to the shoots of raspberries, they negatively affected the soil activity by the highest factor. This might be due to the response of the plant to pathogens and a defensive strategy that might involve secreting chemical compounds affecting microbes in the rhizosphere [57].

The macronutrients content in plant shoots can vary depending on many different factors, such as the stage of growth, the soil microbial community, environmental conditions, and the presence of pests [58–63]. The experiments without pathogen root naturalization resulted in the highest increase in macronutrients in plant shoots. This may be due to an increase in the solubility of compounds near the roots which leads to easier absorption. Root naturalization also proved its effectiveness in the presence of pathogens. It is worth noting that inoculation with *Botrytis cinerea* caused an increase in the absorbable forms of minerals in soil when compared with the control. Combined naturalization strategies and naturalization watering were the most effective for most of the pathosystems. Microorganisms in the soil may contribute to enhancing the solubility of some compounds that might be beneficial for plants [64]. The amount of nitrogen in the soil was not highly dependent on the tested naturalization strategy, although we found that some particular pathogens increased the amount of nitrogen in soil when compared with the control, especially *Verticillium* sp. and all pathogens combined. Some research shows that fungal and fungal-like plant pathogens such as *Phytophthora* sp. may benefit from soil nitrogen imbalance and a higher amount of nitrogen in soil may promote the growth of phytopathogens [65].

5. Conclusions

- Optimization of bacterial media composition and growing conditions leads to improved growth.
- Supplementary prebiotic additives enhance the growth of bacteria even in low concentrations, such as 1%.
- Vacuum drying growing medium absorbed on diatomite is effective in supporting bacterial survivability. The trehalose additive may be an important factor increasing survivability.
- Soil dehydrogenase activity was positively affected by the biopreparation used in control experiments, and in the presence of pathogens dehydrogenase activity varied.
- Different naturalization strategies affected the amounts of nutrients in the soil and shoots differently.
- Naturalization via watering with bacterial inoculum caused the biostimulation of plant growth through an increase in the mass of shoots in pathosystems with pathogen contamination. Therefore, naturalization strategy should be matched to the pathogens occuring at the plantations.

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Data Availability Statement: The data are presented within the manuscript, Appendices A–D, as well as the obtained nucleotide sequences are deposited in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: MW255650 (*Rhodococcus* sp. B12/18), MW255651 (*Pseudomonas* sp. B37/18), MW255652 (*Arthrobacter* sp. B58/18), MW255653 (*Rhodococcus* sp. B75/18).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

PCA	Plate count agar
PDA	Potato dextrose agar
IF-A	Biolog® GEN III Inoculating Fluid
D	Drying
VD	Vacuum drying
LF	Lyophilization
CFU	Colony-forming units
NN	No naturalization
R	Roots naturalization
RW	Roots + watering naturalization
W	Watering naturalization
NCBI	National Centre for Biotechnology Information
RCF	Relative centrifugal force
RPM	Rotations per minute
TDR	Time Domain Reflectometry

Appendix A

Table A1. Design of plant pot experiment including pathosystems, naturalization strategies and repetitions.

	Pathosystem	Naturalization Strategy	Repetitions		
1	without pathogens	no naturalization	a	b	c
2	without pathogens	bacteria roots	a	b	c
5	without pathogens	bacteria roots plus watering	a	b	c
8	without pathogens	bacteria watering	a	b	c
11	Infection *Botrytis cinerea* G277/18	no naturalization	a	b	c
12	Infection *Botrytis cinerea* G277/18	bacteria roots	a	b	c
15	Infection *Botrytis cinerea* G277/18	bacteria roots plus watering	a	b	c
18	Infection *Botrytis cinerea* G277/18	bacteria watering	a	b	c
21	Infection *Verticillium* sp. G296/18	no naturalization	a	b	c
22	Infection *Verticillium* sp. G296/18	bacteria roots	a	b	c
25	Infection *Verticillium* sp. G296/18	bacteria roots plus watering	a	b	c
28	Infection *Verticillium* sp. G296/18	bacteria watering	a	b	c

Table A1. *Cont.*

|	Pathosystem	Naturalization Strategy	Repetitions		
31	Infection *Colletotrichum* sp. G172/18	no naturalization	a	b	c
32	Infection *Colletotrichum* sp. G172/18	bacteria roots	a	b	c
35	Infection *Colletotrichum* sp. G172/18	bacteria roots plus watering	a	b	c
38	Infection *Colletotrichum* sp. G172/18	bacteria watering	a	b	c
41	Infection *Phytophthora* sp. G402/18	no naturalization	a	b	c
42	Infection *Phytophthora* sp. G402/18	bacteria roots	a	b	c
45	Infection *Phytophthora* sp. G402/18	bacteria roots plus watering	a	b	c
48	Infection *Phytophthora* sp. G402/18	bacteria watering	a	b	c
51	Infection with all 4 pathogens	no naturalization	a	b	c
52	Infection with all 4 pathogens	bacteria roots	a	b	c
55	Infection with all 4 pathogens	bacteria roots plus watering	a	b	c
58	Infection with all 4 pathogens	bacteria watering	a	b	c

Appendix B
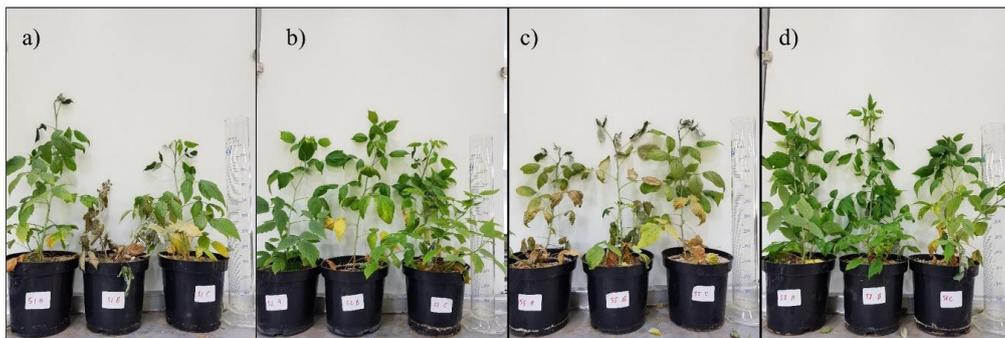
Figure A1. Effect of plant growth stimulation caused by the different naturalization strategies on plants infected with fungal and fungal-like plant pathogens *Botrytis cinerea* (G277/18), *Colletotrichum* sp. (G172/18), *Phytophthora* sp. (G408/18), and *Verticillium* sp. (G296/18): (**a**) no naturalization strategy, (**b**) naturalization of roots, (**c**) roots naturalization and naturalization watering, (**d**) naturalization watering.
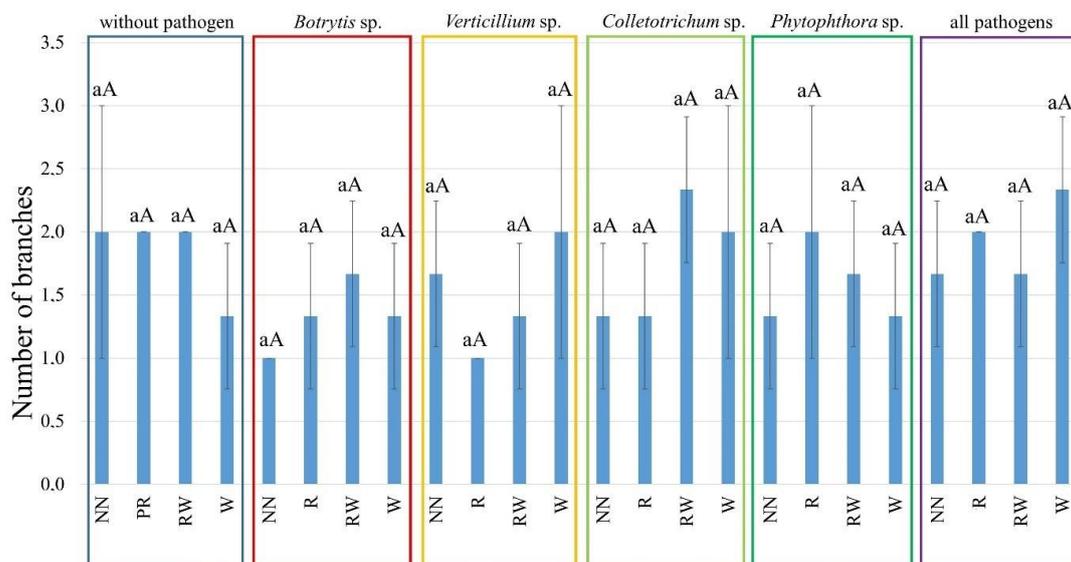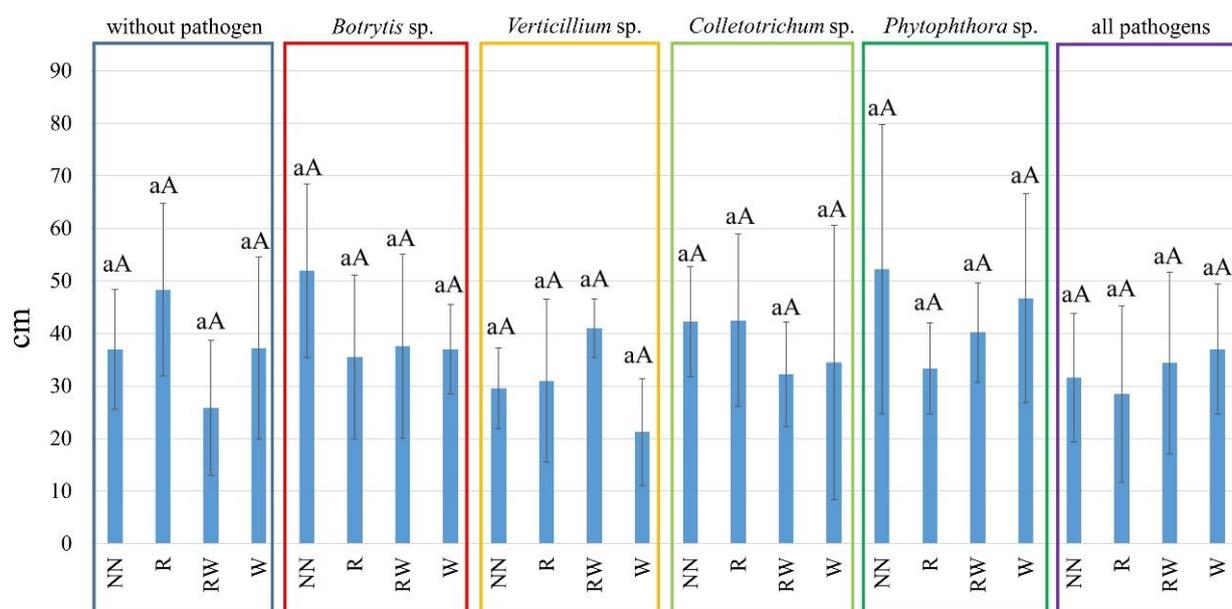
Appendix C

Figure A2. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depended on

the pathosystem and naturalization strategy applied. The average number of stems, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). Error bars indicate a standard deviation, $n = 3$, different lowercase letters above the bars indicate differences among the particular pathosystem, and different capital letters above the bars indicate differences among the particular naturalization strategies (according to ANOVA with $p < 0.05$).

Appendix D

Figure A3. The early effect of the bacterial consortium on *Polana* raspberry plant growth in the pot experiment depended on the pathosystem and naturalization strategy applied. The average length of the stems, according to pathosystems *Botrytis cinerea*, *Verticillium* sp., *Colletotrichum acutatum*, *Phytophthora* sp., and all pathogens (*Botrytis*, *Verticillium*, *Colletotrichum*, *Phytophthora*) and without pathogens, within different biopreparation application strategies: no naturalization (NN), root inoculations (R), root inoculations and watering (RW), and watering (W). Error bars indicate a standard deviation, $n = 3$, different lowercase letters above the bars indicate differences among the particular pathosystem, and different capital letters above the bars indicate differences among the particular naturalization strategies (according to ANOVA with $p < 0.05$).

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