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Microbial Preparations Combined with Humic Substances Improve the Quality of Tree Planting Material Needed for Reforestation to Increase Carbon Sequestration

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Abstract: Restoring forests in areas where they once stood is an important step towards increasing carbon sequestration. However, reforestation requires an increase in current levels of seedling production in the tree nurseries. The purpose of this work was to study the effectiveness of preparations based on bacteria and humic substances (HSs) to stimulate the growth of tree seedlings in a nursery. Two selected strains of *Pseudomonas* and humic substances were used to treat pine and poplar plants. The treatment of seedlings was carried out during their transplantation and after it, and the effects of treatment on shoot elongation, shoot and root mass were evaluated. Treatments with both bacterial strains enhanced the growth of poplar and pine shoots and roots, which was explained by their ability to synthesize auxins. *P. protegens* DA1.2 proved to be more effective than *P.* sp. 4CH. The treatment of plants with humic substances increased the nitrogen balance index and the content of chlorophyll in the leaves of poplar seedlings, which can elevate carbon storage due to the higher rate of photosynthesis. In addition, the combination of humic substances with *P. protegens* DA1.2 increased shoot biomass accumulation in newly transplanted pine plants, which indicates the possibility of using this combination in plant transplantation. The increase in length and weight of shoots and roots serves as an indicator of the improvement in the quality of planting material, which is necessary for successful reforestation to increase capture of carbon dioxide.

Keywords: decarbonization; woody plantations; seedling growth; *Pseudomonas* species; humic sustances

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1. Introduction

Deforestation is one of the largest anthropogenic sources of increased GHG emissions in the world [1]. Planting trees has the potential to increase the capacity of forests to sequester carbon [2] and reforestation helps mitigate climate [3]. However, most of the forest trees face severe problems in successful regeneration [4] and young pine seedlings experience slow growth in the first years after planting [5]. Meanwhile, reforestation requires increasing the number and quality of tree seedlings produced in nurseries. Improving cultivation of tree seedlings for reforestation has attracted the attention of researchers [6]. The nursery stage of tree planting is a very important phase for the later success of field plantations and has been shown to need critical care to ensure their long-term productivity [7]. A recent report highlights that carbon sequestration resulting from tree planting and reforestation depends on nursery management [8].

One of the ways to solve the problem of obtaining high-quality tree seedlings for woody plantations is the introduction of modern physiologically active preparations based

on bacteria and humic substances into the technology of their cultivation. Their use is considered an environmentally friendly mechanism of plant growth promotion compared to large amounts of fertilizers and is becoming more widespread in the agricultural industry [9]. It is well known that rhizospheric bacteria [10–12] as well as humate substances (HSs, products of degradation of organic matter extracted from brown coal, peat, and other sources) [13–15] have a positive effect on plant growth when introduced separately. However, there are significantly fewer reports of their effects on trees than on herbaceous plants. Nevertheless, it was shown that humates increased the growth of trees and the yield of oranges and grapefruits [16], while humic acids enhanced the growth of rubber tree planting materials [17]. Rhizosphere microbes enhanced the growth of teak seedlings (Tectona grandis) [7], and bacteria of the *Pseudomonas fluorescens* strain promoted the growth of aspen seedlings under nutrient stress [18]. Plant-growth-promoting (PGP) rhizobacteria have a beneficial effect on plants, including trees, by increasing the availability of soil nutrients to the plant and the production of metabolites such as plant hormones [19]. Reports on agricultural crops have shown that the combined effect of humates and bacteria turned out to be more effective than the use of each of them separately [20,21]. However, there are practically no works devoted to the combined use of plant growth promoting rhizobacteria (PGPR) and humic substances for tree nursery management. In this regard, the aim of this work was to study the effectiveness of preparations based on bacteria and HSs for stimulating plant growth as a means of improving the quality of tree planting material and more sustainable production of seedlings for reforestation in order to optimize carbon sequestration. The novelty of this work lies in the study of the complex effect of humates and microorganisms on the growth of tree seedlings, which, as far as we know, has not been carried out before. We hypothesized that the combined use of bacteria and humic substances may be more effective in stimulating the growth of tree seedlings in nurseries than the use of each separately.

2. Materials and Methods

2.1. Bacterial Strain and Cultural Media

We used two strains of Gram-negative bacteria from the collection of microorganisms of the Ufa Institute of Biology isolated from natural sources: *Pseudomonas protegens* DA1.2 (deposited in All-Russian Collection of Microorganisms B-3542D) described in the article by Chetverikov et al. [22] and *Pseudomonas* sp. 4CH (deposited in the collection of microorganisms UIB-57). These bacterial strains were chosen for treating tree seedlings since in previous experiments their combination with humates stimulated the growth of herbaceous plants (wheat) [20]. Bacteria were cultivated in Erlenmeyer flasks with King's B medium (2% peptone, 1% glycerol, 0.15% K_2HPO_4, 0.15% $MgSO_4$*$7H_2O$) on an Innova 40R shaker (New Brunswick, NJ, USA) (160 rpm) for 48 h at 28 °C. The number of cells in cultures was measured by applying serial dilutions to King B medium with agar-agar (15 g L^{-1}) and then counting the number of colony-forming units (CFU). The bacterial culture was diluted with sterile water to give a solution for treatment of plants. The ability to mobilize phosphates was assessed by measuring the size of transparent zones on Pikovskaya medium, and acetylene reduction assay was used as a measure of bacterial nitrogenase activity as described [23].

2.2. Taxonomic Affiliation of Bacterial Strain

For the taxonomic affiliation of the 4CH bacterial strain, the nucleotide sequence of 16S rRNA gene was determined. Total DNA from bacterial colonies was isolated using the RIBO-sorb reagent kit (Amplisens®, Central Research Institute of Epidemiology, Moscow, Russia) according to the manufacturer's recommendations. Amplification of the 16S rRNA gene fragment was performed using universal primers: 27F (5′-AGAGTTTGATCTGGCTCAG-3′) and 1492R (5′-ACGGTACCTTGTTACGACTT-3′) [24]. 16S rRNA sequencing was performed using the BigDye Terminator sequencing kit (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) using a Genetic

Analyzer 3500 xL (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). To sequester cycle-sequencing reaction components, we used the BigDye® XTerminator™ purification kit (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). The search for 16S rRNA nucleotide sequences similar to the corresponding sequences of the studied strains was performed in the GenBank sequence database using the BLAST software package (http://www.ncbi.nlm.nih.gov/blast accessed on 2 February 2023).

2.3. Extraction of Humic Substances

The source of humic substances was the brown coal from the Tyulganskoe deposit in the Orenburg region of the Russian Federation. Coal was mixed with 0.1 M KOH in a ratio of 1:10 and HSs were extracted for two hours with stirring at 1500 rpm. The precipitate was removed by centrifugation at 12,000 rpm for 10 min.

2.4. Plant Growth Conditions and Treatments

We used generally accepted technology for growing tree seedlings in nurseries, while the combined treatment of tree seedlings with bacteria and humates was used for the first time in this study. We modified the technology that was previously successfully used on wheat plants [20]. Experiments were carried out in the tree nursery of the Bashkir Agrarian University (54°80′ N, 55°84′ E, 170 m a.s.l., Ufa region of Bashkortostan, Russian Federation). For the experiment, we used cuttings of Bashkir pyramidal poplar (a hybrid of Italian pyramidal poplar and black poplar (*P. italica pyramidalis* × *P. nigra*), obtained in the late 1930s at the Bashkir forest experimental station). These tree species were chosen for the present experiments as they are often used for reforestation and urban greening in many regions. In the first ten days of April, with the beginning of intensive snowmelt, poplar branches of last year's generation were selected, from which 20 cm long cuttings were separated and laid for monthly stratification. Before treatment with preparations based on bacteria and HSs, the cuttings were placed in water for the germination of the first roots. The cuttings were planted in the ground for 2/3 of their length at an angle of 45° to the ground surface.

Before planting, poplar seedlings were soaked in 2 L of water, to which 50 mL of bacterial suspension ($(4 \pm 0.5)\ 10^9$ CFU mL^{-1}) and HSs (2 g L^{-1}) were added singly or in combination. Seedlings were watered 2 times each month with 2 L of the mixture of bacteria and HSs of the same concentration. Control plants were treated with the same amount of water without additives. Three months after planting the poplar seedlings, the length of lateral shoots was measured.

Two-year-old seedlings of *Pinus sylvestris* L. from the nursery were planted at a distance of 50 cm from each other. Before planting they were soaked in suspension of bacteria, humates, or their mixture and then watered with the same solutions as described above. In parallel, pine seedlings transplanted from the nursery 1 and 2 years prior to the present experiments (denoted below as 3- and 4-years old seedlings, respectively) were watered in the same way with bacteria and HSs (singly or in mixture) 3 times. Pine growth rate was assessed by the change in the length of the main and side shoots within 4 months. Shoots and roots of two-year-old pine seedlings were sampled after the end of seedling growth in the current year. Roots taken at the same time were washed with tap water and both shoots and roots were dried in a ventilated oven at 60 °C for 48 h to measure their dry mass.

2.5. Analysis of the Content of Pigments

The content of chlorophyll (a + b), flavonoids, and nitrogen balance index (NBI) [24] in the leaves was measured using a DUALEX SCIENTIFIC+ device (FORCE-A, Paris, France) according to the manufacturer's recommendations.

2.6. IAA (Auxin, Indoleacetic Acid) Assay in Bacterial Cultural Media

On the second day of cultivating bacteria, immunoassay of culture media was performed. IAA was partitioned from culture media of bacteria with diethyl ether as described [25]. Briefly, 1 ml of bacterial culture media was diluted with distilled water and acidified with HCl to pH 2.5 to extract IAA with diethyl ether. Then, hormones were partitioned from diethyl ether into $NaHCO_3$ solution and re-extracted with diethyl ether from the acidified aqueous phase. IAA analysis was carried out with enzyme-linked immunosorbent assay using specific antibodies against IAA as described [26]. The reliability of the method is due to specificity of antibodies to auxins and the use of an extraction method that makes it possible to efficiently extract hormones while reducing the amount of impurities by decreasing the volume of extractants at each stage of solvent partitioning. The efficiency of purification of IAA prior to immunoassay was confirmed by the study of chromatographic distribution, which showed that the peaks of immunoreactivity coincided only with the positions of the IAA standards.

2.7. Statistics

The data were processed using Statistica version 10 (Statsoft, Moscow, Russia) and are presented in the tables and figures as means ± standard errors. The statistical significance of differences between the mean values was assessed using analysis of variance followed by Duncan's test ($p < 0.05$). In the figures, mean values that are statistically different from each other are indicated by different letters. The number of replications (n) is provided in the figure legends.

3. Results

To identify the 4CH strain, the nucleotide sequence (1401 bp) of the 16S rRNA gene was determined; it was deposited in the GenBank database as OQ381088. Its comparison with other known sequences made it possible to attribute the studied microorganism to *P. chlororaphis* with a high degree of probability (99.93% similarity with the type strain of *P. chlororaphis* subsp. *aureofaciens* NBRC 3521(T) was found). Based on the data on the nucleotide sequence of the 16S rRNA gene, a phylogenetic tree was constructed to identify relationships with other species of the genus *Pseudomonas* (Figure 1).

Studied bacterial strains showed nitrogenase activity and the ability to solubilize phosphates and synthesize auxins (Table 1).

Table 1. Properties of plant-growth-promoting bacteria.

Strain	Nitrogenase Activity, nmol C_2H_4 h^{-1} mL^{-1}	Synthesis of Auxins, ng mL^{-1}	Phosphates Solubilization, mm
Pseudomonas protegens DA1.2	20.8 ± 0.3	870 ± 44	18 ± 2
Pseudomonas sp. 4CH	20.0 ± 0.2	837 ± 55	15 ± 2

Measurement of the shoot length 4 months after transplanting of 2-year-old pine seedlings showed that the rate of shoot elongation was significantly accelerated by treatment with *P. protegens* DA1.2 and its combination with HSs (Figure 2). The rates of shoot elongation in plants treated with *Pseudomonas* sp. 4CH and its combination with HSs were intermediate between control plants and plants treated with *P. protegens* DA1.2. The mean increase in shoot length of plants treated with HSs in combination with *Pseudomonas protegens* DA1.2 was significantly different from that of plants treated with HSs alone, while there was no difference between plants treated with HSs or these bacteria alone (Figure 2).

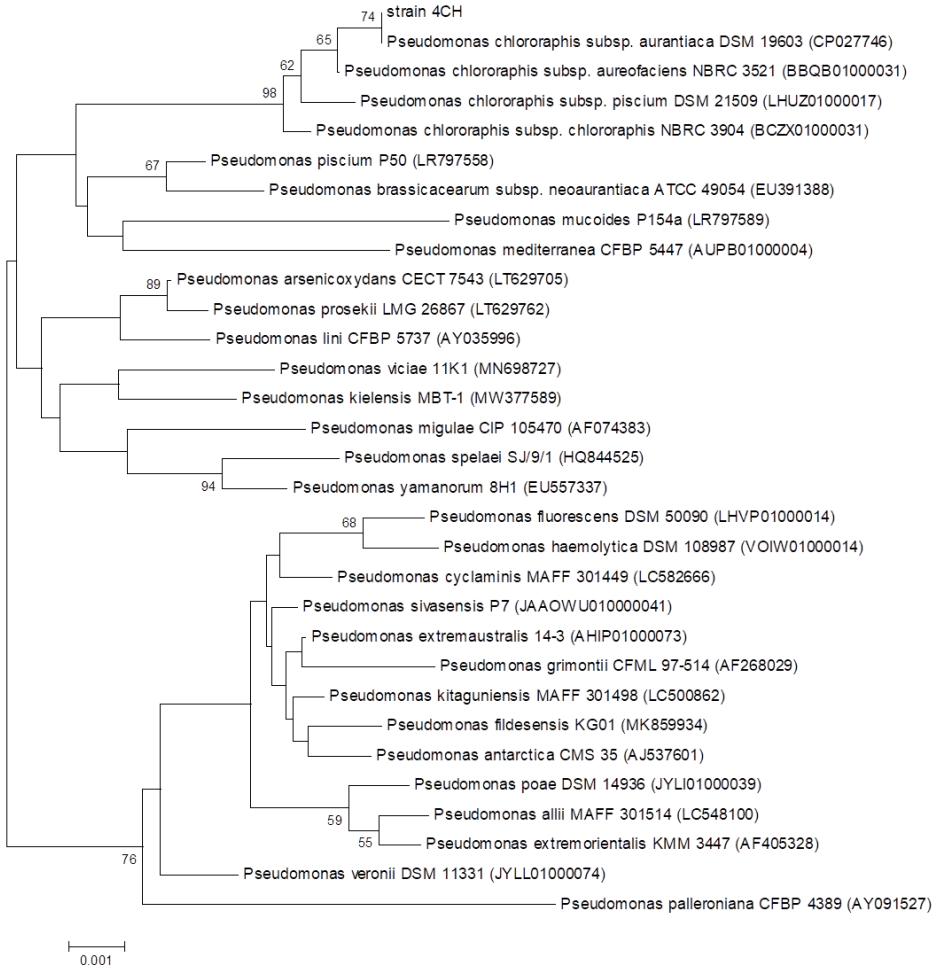

Figure 1. Phylogenetic position of the strain *Pseudomonas* sp. 4CH according to the analysis of the nucleotide sequence of the 16S rRNA gene (the evolutionary distance corresponding to 1 nucleotide change in every 1000 is shown on a scale, the numbers are the statistical significance of the branching order determined with bootstrap analysis, and the indicator values above 50% are shown).

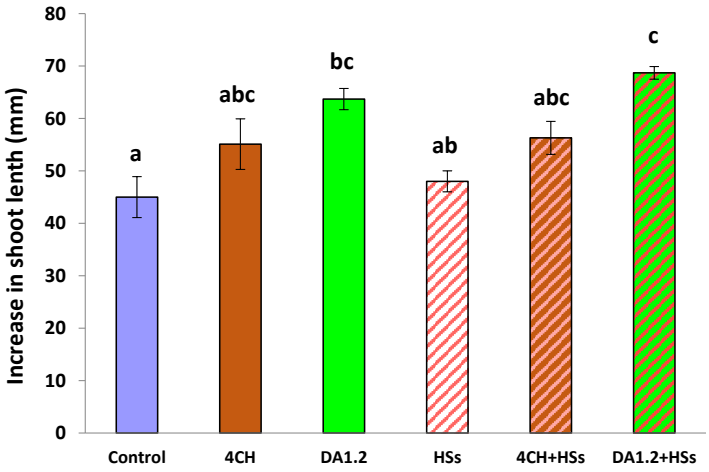

Figure 2. The increase in shoot length (averaged values for the main and lateral shoots) of 2-year-old pine seedlings 4 months after their transplantation and triple watering with the bacterial suspensions of *Pseudomonas* sp. 4CH, *Pseudomonas protegens* DA1.2, and humic substances (HSs) applied alone or in combination (4CH + HSs and DA1.2 + HSs). Means that are statistically different from each other are marked with different letters, $p \leq 0.05$, $n = 10$ (ANOVA followed by Duncan's test).

When pine seedlings were treated with both bacterial preparations, the mass of shoots or roots increased compared to the control (Figure 3). Humates did not affect the accumulation of root mass, but increased the mass of shoots when applied alone or in combination with any of the bacteria. The mass of shoots of plants treated with any bacterium in combination with HS was larger than in plants treated only with the corresponding bacteria.

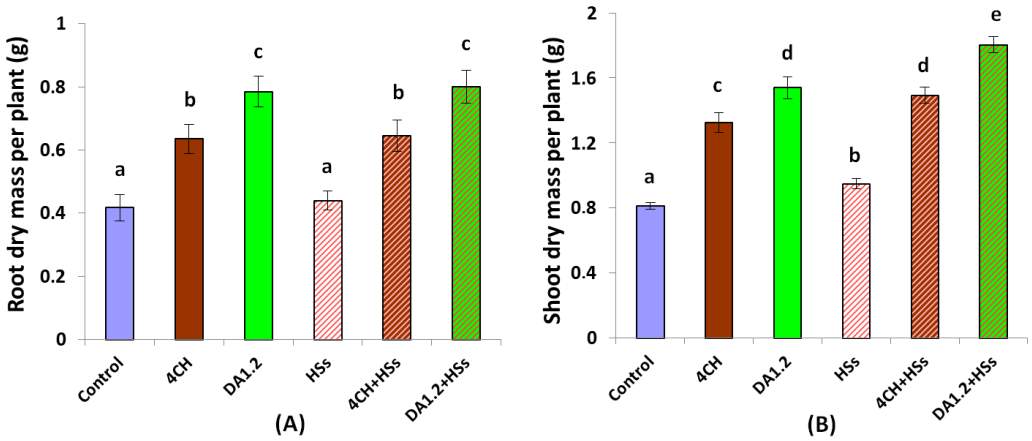

Figure 3. Dry mass of roots (**A**) and shoots (**B**) of 2-year-old pine seedlings sampled after the end of seedling growth in the current year. Mean values that are statistically different from each other are marked with different letters, $p \leq 0.05$, $n = 10$ (ANOVA followed by Duncan's test).

Bacterial treatment of pine seedlings transplanted three years before the present experiments accelerated elongation of their shoots compared with the control plants (Figure 4). The rate of shoot elongation of plants treated with HSs did not differ from that in the control or in plants treated with *Pseudomonas* sp. 4CH, while in the case of combination of *Pseudomonas* sp. 4CH and HSs the increase in shoot length was significantly greater than in plants treated with HSs alone.

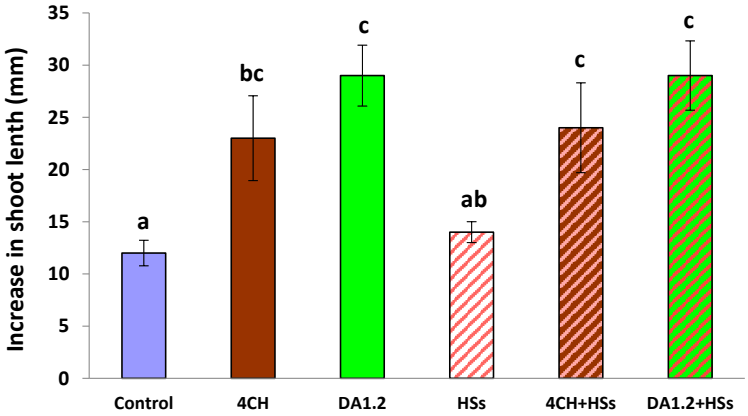

Figure 4. The increase in the shoot length (averaged values for the main and side shoots) of 3-year-old pine seedlings in 4 months after triple watering with suspensions of bacteria *Pseudomonas* sp. 4CH, *Pseudomonas protegens* DA1.2, and humic substances (HSs) used alone or in combination (4CH + HSs and DA1.2 + HSs). Mean values that are statistically different from each other are marked with different letters, $p \leq 0.05$, $n = 10$ (ANOVA followed by Duncan's test).

The treatment of 4-year-old seedlings with bacteria and HS separately and in combination accelerated the elongation of their shoots (Figure 5). However, the effect of HSs applied alone was significantly lower than that of bacterial treatments used alone or in combination with HSs.

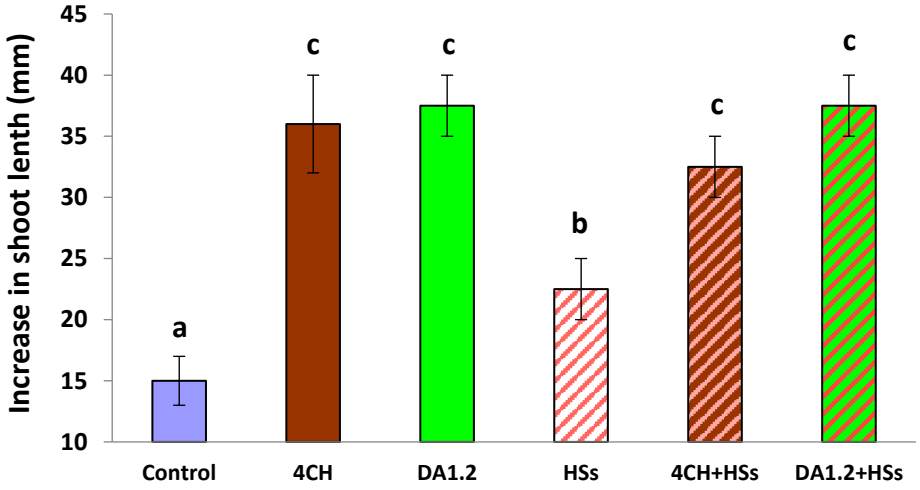

Figure 5. The increase in the shoot length (averaged values for the main and side shoots) of 4-years-old pine seedlings in 4 months after triple watering with the suspensions of bacteria *Pseudomonas* sp. 4CH, *Pseudomonas protegens* DA1.2, and humic substances (HSs) used alone or in combination (4CH + HSs and DA1.2 + HSs). Mean values that are statistically different from each other are marked with different letters, $p \leq 0.05$, $n = 10$ (ANOVA followed by Duncan's test).

Statistically significant increase in the shoot length of poplar plants compared to the control was found only when they were treated with *P. protegens* DA1.2 applied alone (Figure 6).

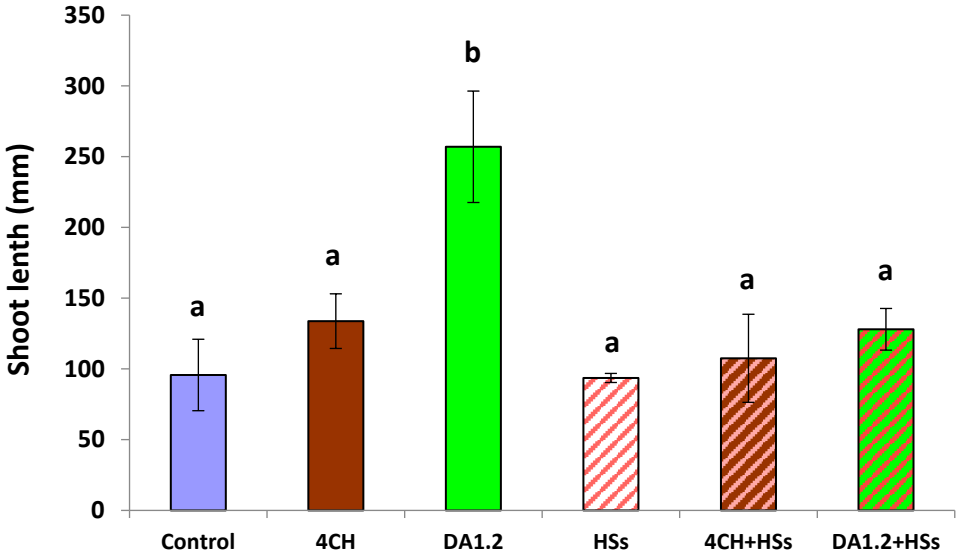

Figure 6. Length of shoots of the poplar plants after watering with bacterial suspensions *Pseudomonas* sp. 4CH, *Pseudomonas protegens* DA1.2, and humic substances (HSs) used alone or in combination (4CH + HSs and DA1.2 + HSs). Mean values that are statistically different from each other are marked with different letters, $p \leq 0.05$, $n = 6$ (ANOVA followed by Duncan's test).

None of the treatment options increased the content of flavonoids in poplar plants (Figure 7A). The content of chlorophyll and NBI increased only when plants were treated with HSs. (Figure 7B,C).

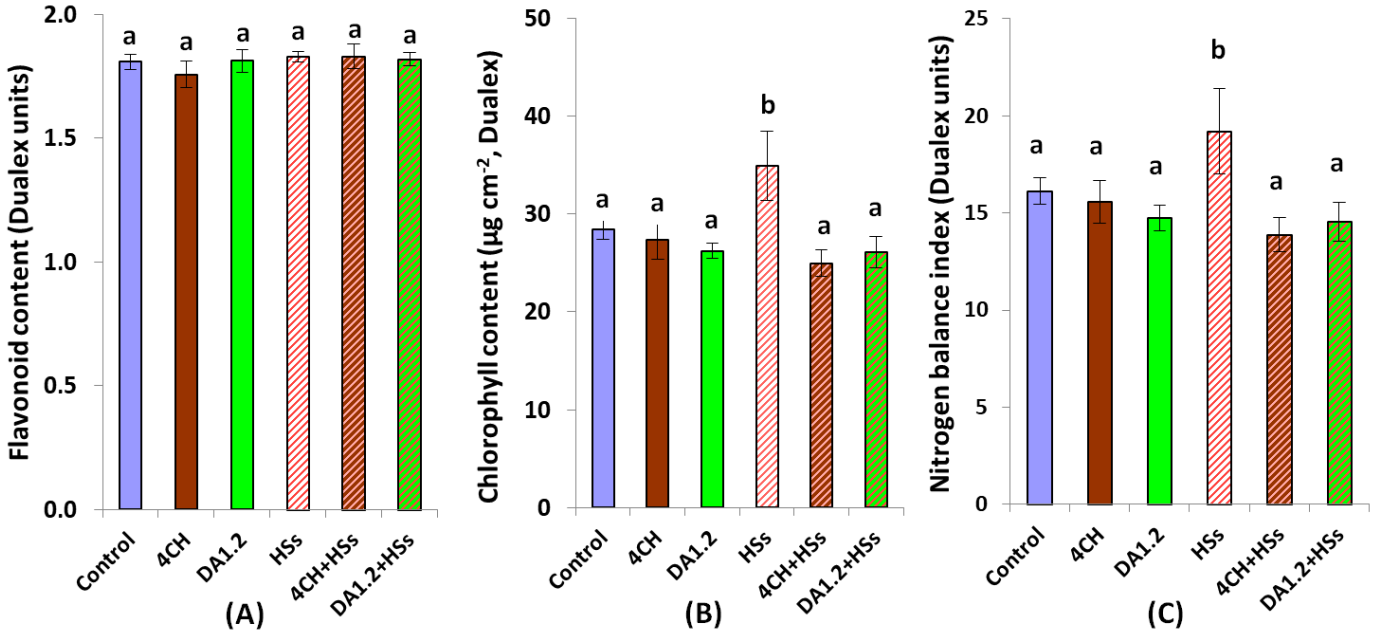

Figure 7. Content of flavonoids (**A**), chlorophyll (**B**), and nitrogen balance index (NBI) (**C**) of the poplar plants after watering with suspensions of bacteria *Pseudomonas* sp. (4CH), *Pseudomonas protegens* DA1.2, and humic substances (HSs) used alone or in combination (4CH + HSs and DA1.2 + HSs). Mean values that are statistically different from each other are marked with different letters, $p \leq 0.05$, $n = 15$ (ANOVA followed by Duncan's test).

4. Discussion

We were able to demonstrate that both bacterial treatments increased elongation of pine shoots (Figures 2 and 4). In addition, larger mass of shoots and roots was found in bacteria-treated plants (Figure 3). The use of microorganisms to increase plant growth and productivity is an important practice required for agriculture [27,28]. During the past decades, the use of PGPR for sustainable agriculture has greatly increased [29]. However, there are significantly fewer reports of their effects on trees than on herbaceous plants. Our data are consistent with a report that showed increased growth of tree seedlings under the influence of rhizospheric microbes as a sustainable way to optimize forestry [7]. Inoculation of *Pinus taeda* seedlings with *Bacillus subtilis* increased root and shoot biomass [30]. Shoot and root growth of *Swietenia macrophylla* was also stimulated by *Bacillus* spp. under nursery conditions [31].

In the present experiments, bacterial effects can be explained by the ability of the used bacterial strains to synthesize auxins, their nitrogenase activity and ability to solubilize phosphates (Table 1). Auxins are known to stimulate cell extension [32] and auxin production by these bacterial strains was detected in the present (Table 1) and previous [20] experiments. The capacity of PGPR to synthesize auxins is considered one of the most important mechanisms through which microbes regulate plant growth [10,14]. Increased root biomass found in the present experiments is consistent with the data of other researchers. Thus, inoculation with *Azospirillum brasilense* and *Pseudomonas geniculata* strains increased the root mass and length of *Linum usitatissimum* [10]. Stimulation of root growth by bacteria contributes to increased water and nutrient uptake by plants, thereby promoting their growth [10,14]. The increase in length and weight of shoots and roots found in the present experiments serves as an indicator of the improvement in the quality of planting material [33], which is necessary for successful reforestation to mitigate climate change and capture carbon dioxide [3].

The increment in shoot length was greatest in 2-year-old pine seedlings, transplanted just before the present experiments (about 45–70 mm (Figure 2) versus no more than 35 mm

in older plants (Figures 4 and 5)). Bacterial preparations were most effective on 4-year-old pine seedlings transplanted 2 years before the present experiments. In this case, treatment with both bacterial strains led to a 2.5-fold increase in the length of shoots compared with the control (Figure 5). Bacterial treatments were less effective in recently transplanted plants (Figure 2): they only resulted in an increase in the length of the shoots by about 1.5 times compared to the control in the case of *P. protegens* DA1.2, while in plants treated with *Pseudomonas* sp. 4CH the rate of shoot elongation of 2-year-old pine seedlings was close to the control. In most cases, *P. protegens* DA1.2 bacteria were more effective than *Pseudomonas* sp. 4CH. Thus, the effectiveness of the action of bacteria on shoot elongation depended on the type of microorganisms and the age of the plants.

Bacterial treatment of poplar plants was less effective than in the case of pine plants, and only the treatment with *P. protegens* DA1.2 led to a statistically significant increase in the length of poplar shoots compared with the control (Figure 6).

The effect of HSs was lower than that of bacterial treatments when each was applied separately. Humates increased the biomass of shoots, but not roots. This can be explained by the presence of cytokinin-like substances in humates [34], and it is known that these hormones stimulate the growth of shoots and inhibit root growth [35]. Our data are consistent with reports indicating an increase in the growth rate of orange and grape trees by humates [16] and an acceleration of the growth of rubber planting materials with foliar application of humic acid [17].

The additive effect of the combination of bacteria and HSs was less pronounced than in previous experiments with herbaceous plants [20]. Nevertheless, the shoots of 2-year-old pine plants treated during their transplantation were significantly heavier in the case of combination of HSs with either *P. protegens* DA1.2 or *Pseudomonas* sp. 4CH compared with corresponding bacterial treatments applied alone. Thus, the combination of HSs and these bacteria may be recommended for use during transplantation of pine plants.

HSs increased concentrations of chlorophyll and NBI (nitrogen balance index) [24] in the poplar leaves (Figure 7B,C). Humic substances stimulated the uptake of nitrate by roots and the accumulation of the anion at the leaf level of maize [36]. In our previous experiments we found an increased accumulation of total nitrogen in the shoots of wheat plants that had received organomineral fertilizers with humates [15]. The chlorophyll molecule contains nitrogen, which makes availability of this element an important factor in the development of the photosynthetic apparatus. Therefore, an increase in NBI and chlorophyll in leaves of HSs-treated poplar plants is likely to contribute to enhancing photosynthesis and improving carbon accumulation by the plants [37].

In order to increase the production of tree seedlings, nurseries are currently using large amounts of fertilizers that can lead to environmental pollution [38]. Furthermore, this technology produces individual trees which are unbalanced in size and more likely to suffer infections from phytopathogenic fungi [33]. Bacteria and humic substances may be important for plant nutrition by increasing N and P uptake by the plants without addition of excessive amounts of fertilizers.

The results of our research show that the use of bacteria and humic substances improves the quality of tree planting material for reforestation, while reforestation is a means of increasing carbon sequestration. The combination of bacteria and humic substances can work better than either of them alone.

5. Conclusions

We were first to study the combined effects of bacteria and humates on the growth of tree seedlings. Our studies have shown the ability of bacterial preparations to accelerate the growth of shoots of poplar and pine plants. *P. protegens* DA1.2 proved to be more effective than *P.* sp. 4CH, which indicates the prospects for further search for more effective strains. The treatment of plants with humic substances increased nitrogen balance index and chlorophyll content in the leaves of poplar seedlings, which is likely to increase carbon storage due to increased photosynthesis. In addition, combination of HSs with *P. protegens*

DA1.2 increased shoot biomass accumulation of recently transplanted pine plants, which suggests the possibility of using this combination during plant transplantation.

The nursery stage is believed to be a very important phase for the later success of field plantations. Nevertheless, further studies are needed to confirm the long-term positive effects of humates and bacteria on the behavior of trees in field plantations. Study of the effects of inoculating the rhizosphere of seedlings of other tree species with various bacterial strains and treating them with humates is needed to find an effective combination to achieve successful reforestation as a means of increasing the carbon storage capacity of forests under various climatic conditions. Nevertheless, the data obtained in the present study demonstrate promising prospects and the feasibility of such a study.

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Abbreviations

HSs	humate substances
GDP	gross domestic product
GHG	greenhouse gas
PGPR	plant growth promoting rhizobacteria
NBI	nitrogen balance index
IAA	Indole-3-acetic acid (auxin)

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