

Article

Populus tremula × *P. alba* Microshoot Secondary Metabolism Response after *Paenibacillus* sp. Inoculation In Vitro

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Abstract: Bacterial biostimulants are an eco-friendly alternative to chemical fertilizers. However, before their introduction into open ecosystems, broad-scope studies need to be carried out. *Paenibacillus* sp. was shown to positively affect poplar root growth. It was hypothesized that alongside these improvements, the *Paenibacillus* sp. inoculant may affect its host's secondary metabolism. *Populus tremula* × *P. alba* microshoots were inoculated in vitro. Microshoots were tested for chlorophyll, carotenoid, total flavonoid (TFC), total phenol content (TPC) and free radical scavenging capacity during primary growth after 4, 6 and 8 weeks. The results showed that the inoculation decreased shoot phenolics and free radical scavenging capacity after 6 and 8 weeks. Chlorophyll *b* amounts increased after 6 and 8 weeks. Carotenoid content decreased after 6 weeks, while chlorophyll *a* and carotenoid levels increased after 8 weeks. Correlation and principal component analyses showed that the inoculant changed the way in which the photosynthesis pigment content relates to TPC, TFC and radical scavenging activity. Overall, these data suggest that the inoculant does statistically significantly affect *Populus* tree secondary metabolism in the later stages of the initial growth period. This effect may potentially be compensatory in nature.

Keywords: poplar; bacteria; total phenols; total flavonoids; ABTS; DPPH; chlorophyll; carotenoids



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1. Introduction

In recent years research on bacterium-based biostimulants has been on the rise, due to the negative effects that chemical fertilization and pesticide use have on the environment [1–4]. However, most of it is directed towards food crops, and comparatively fewer studies have been carried out on biostimulant use on trees, even though, in the face of climate change and loss of biodiversity, reforestation often plays a large role.

Multiple studies have demonstrated that plant-growth-promoting bacteria (PGPB, or plant-growth-promoting rhizobacteria (PGPR)) can serve as effective biostimulants [5–10] and biocontrol agents [11–13], both in controlled settings and in field trials. PGPB may benefit their hosts in a variety of ways: through help with nutrient availability, phytohormone production, induced systemic resistance, antagonistic effects on plant pathogens, response to stress, etc. [1–3,11,12,14].

To determine the advantages of using microbial biostimulants, various variables can be taken into consideration: visually observable vegetative growth parameters [8], antagonism to pathogens [12], photosynthesis pigment content [10,14–16], antioxidant activity [17,18] and secondary metabolites (SMs) such as phenolics [15,19].

According to Schulz et al., “Most plants are healthy, in part due to metabolic interactions between the holobiont's host and its microbiome...” [20]. Hence, it is obvious that the modification of the plant's microbiome can have multifaceted outcomes for the plant. Furthermore, it has been demonstrated that modifying the plant's environment with bacteria may have both species-specific and genotype-specific outcomes [5,8,15,21–26]. Additionally,

these outcomes may also be shaped by the plant's growth stage [25] and environmental conditions [10,14]. Thus, it is extremely important to fully comprehend the consequences that may follow the introduction of new biostimulants to the microbiome of plants and the open ecosystems, especially since microorganisms are key elements in all biogeochemical cycles and could potentially affect the condition of the entire ecosystem [27].

It is noteworthy that while the field of bacterial biostimulants is expanding, broad-ranging studies into the assorted effects that one microorganism species can have on their plant host are still necessary. To our knowledge, there are not any studies that represent bacterial-inoculant-induced time-dependent metabolite changes during the initial growth stages of woody plants.

Poplars are valuable model organisms that represent all trees and other woody plants in a variety of studies. Poplar microshoot cultures also serve as a model experimental system for woody plant–microorganism interaction research. This is due to several factors, including their relatively easy *in vitro* propagation and clonal tissue culture initiation, fast growth, wide habitat range and the accessible information about their whole genome [27]. Moreover this genus is grown widely for profit [28].

PGPB, *Paenibacillus* sp., was recently isolated from oaks (*Quercus robur*) and tested on two distinct model poplar (*Populus* spp.) microshoot cultures, resulting in the enhancement of their root system [8]. Additionally, this bacterial species was shown to have several plant-growth-promoting properties (phytohormone indole-3-acetic acid (IAA) production; both phosphate solubilization and mineralization *in vitro*) [8], making it a potentially effective biostimulant for trees.

Thus, we hypothesize, that *Paenibacillus* sp. may have an impact on poplar's photosynthesis pigment content, antioxidative response and phenolics and hence could potentially induce systemic resistance, which would help with poplar's stress responses during the timeframe of initial growth.

The results of the experiments showed that *Paenibacillus* sp. inoculation had an effect on poplar microshoot secondary metabolism that is not suggestive of induced systemic resistance, although an antioxidant compensatory mechanism was also observed.

2. Materials and Methods

The experiment was conducted using a model hybrid poplar *Populus tremula* × *P. alba* *in vitro* clone microshoot culture, established in the laboratory of the Forest Research Institute, Poland, from the vegetative buds of 6–7-year-old cloned trees. At the time of the experiment, the culture was kept up for 4 years via bimonthly transfers (5.25 ± 0.25 mL of solid Murashige and Skoog (MS) media, supplemented with 2% sucrose and 0.4% gelrite (gelling agent), *w/v*, pH 5.6 ± 0.1) to fresh media and grown under a 16/18 h 25/20 °C cycle with cool white fluorescent light (Osram, Munich, Germany, 2×36 W, $22 \mu\text{mol m}^{-2} \text{s}^{-1}$) in 20 mm glass tubes. All the media and other components were purchased from Duchefa Biochemie (Haarlem, The Netherlands) and used per the manufacturer's instructions.

2.1. Inoculation and Growth Conditions

Paenibacillus sp. was used to inoculate poplar microshoots based on previously documented plant-growth-promoting abilities on this exact microshoot culture [8].

The tests were performed under previously described conditions, using Woody Plant media (WPM) (additionally 0.4% gelrite, 2% sucrose, *w/v*, pH 5.6 ± 0.1). In total, 30 two-month-old poplar microshoots were divided into three independent replicates per one group at one time period. For the uninoculated control groups, microshoot leaves were gently removed, and the stems were divided into 10 mm segments with 2–3 growing nodes on each stem (exempting the primary apical top). Every segment was individually placed into a $\varnothing 20$ mm glass tube with 5.25 ± 0.25 mL of WPM. The tubes were capped and placed in a growth chamber.

For groups inoculated with *Paenibacillus* sp., the inoculant was prepared prior to inoculation. A swab from a single bacterial colony was transferred to a fresh plate with

low-salt Lysogeny broth (LB) medium (pH 7.2 ± 0.1 , tryptone—10 g/L, NaCl—5 g/L, yeast extract—5 g/L) and grown at $+25 \pm 1$ °C overnight. The next day, prior to the transfer of poplar segments, tubes were inoculated with a swab using an inoculation needle [8]. Then, poplar segments were carefully placed into the stab area.

Sample measurements were taken after 4, 6 and 8 weeks (this represents three different groups, as the measurements required the destruction of the samples). Chlorophyll *a* and *b* (CHA, CHB, respectively), carotenoids (CAR), total phenol content (TPC), total flavonoid content (TFC) and antioxidant capacities using DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assays were measured.

2.2. Sample Collection and Preparation for Metabolic and Physiological Assays

After each growth period, samples were collected and immediately prepared for future metabolic and physiological assays.

For the photosynthesis pigment analysis, a total of 0.5 g of fresh aboveground biomass (stems as well as leaves, as at these growth stages, they still serve a purpose in photosynthesis) was collected from both the control group and the inoculated group after 4, 6 and 8 weeks at each time point. The total biomass was divided into three biological replicates per each time point. Immediately after collection and weighing, fresh biomass was stored at -20 °C until use.

For the other metabolic tests and DPPH and ABTS assays, 75% methanol (MeOH (Chempur, Piekary Śląskie, Poland)) was used for extraction. In total, 1.1 g of fresh aboveground biomass was collected from both the control group and the inoculated group after 4, 6 and 8 weeks at each time point. The total biomass was divided into seven biological replicates per each time point. Samples were homogenized by ceramic mortar and pestle and placed in inert plastic screw cap tubes; then 10 mL of 75% MeOH was added. Samples were subsequently placed in a thermal shaker for 24 h at $+25$ °C, $1 \times g$, in the dark. Next, samples were filtered using filter paper with a retention of 5–8 µm. Extracts were then stored at -20 °C until use.

2.3. Metabolic and Physiological Assays

2.3.1. Measurements of Photosynthesis Pigments

Relative amounts of CAR, CHA and CHB were measured via their absorbance. Frozen leaves were ground with mortar and pestle using pure acetone (30 mL) (Chempur) as extracting agent. This extract was then filtered as previously described and used for absorbance measurements with a UV-VIS spectrophotometer 80+ (PG Instruments, Lutterworth, UK) at 440, 662 and 664 nm (CAR, CHA and CHB, respectively). Three technical replicates were measured for each sample at each wavelength independently. For the calculation of pigment content, the following formulas were used [29] (mg g^{-1}):

$$\text{CHA} = \frac{(9.784 \times A_{662} - 0.99 \times A_{644}) \times V}{P \times 1000} \quad (1)$$

$$\text{CHB} = \frac{(21.426 \times A_{644} - 4.65 \times A_{662}) \times V}{P \times 1000} \quad (2)$$

$$\text{CAR} = \frac{(4.695 \times A_{440} - 0.268 \times (\text{CHA} + \text{CHB})) \times V}{P \times 1000} \quad (3)$$

where A—absorbance, V—total extract volume (mL), P—fresh aboveground biomass (g).

2.3.2. Total Phenol Content

The methanol extracts were used to determine TPC. A modified method utilizing the Folin-Ciocalteu reagent (VWR Chemicals, Radnor, PA, USA) was applied [30]. Briefly, 0.1 mL of extract was mixed with 0.1 mL of the reagent (2 N) and 2.5 mL of distilled water (dH_2O) and incubated for 6 min. Then, 0.5 mL of 20% (*w/v*) Na_2CO_3 (Molar Chemicals

KFT, Hungary) was added. The mixture was then left to incubate at 22 °C for 30 min. Absorbance was measured using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Bad Friedrichshall, Germany) at 760 nm (75% methanol as blank). Three technical replicates were measured for each sample. TPC was expressed as chlorogenic acid (TCI, Tokyo, Japan) per gram of fresh weight (mg CAE g^{-1}). The standard calibration curve equation used was $y = 5.5358x - 0.0423$ ($R^2 = 0.9975$).

2.3.3. Total Flavonoid Content

TFC was determined using a modified method [19,31]. The methanol extract (1 mL) was mixed with 0.3 mL of 5% (*w/v*) NaNO_2 (VWR Chemicals) and incubated for 5 min. Next, 0.5 mL of 2% (*w/v*) AlCl_3 (Alfa Aesar, Haverhill, MA, USA) was added and incubated for 6 min. Subsequently, 0.5 mL of NaOH (Merck, Darmstadt, Germany) (1 M) was added. The absorbance of the mixture was measured at 470 nm (75% methanol as blank) on the Synergy HT Multi-Mode Microplate Reader. Three technical replicates were measured for each sample. TFC was expressed in milligrams of catechin (Merck) per gram of fresh weight (mg CE g^{-1}). The standard calibration curve equation used for TFC was $y = 11.616x + 0.0634$ ($R^2 = 0.9983$).

2.3.4. DPPH Assay

The total free radical scavenging capacity of the extracts was determined using a modified method [19,32]. The methanol extract (0.1 mL) was mixed with 1 mL of DPPH (TCI) solution (0.1 mM; in 75% methanol) and incubated in the dark (+22 °C) for 16 min. Absorbance was measured at 515 nm on a Synergy HT Multi-Mode Microplate Reader (0.1 mL of 75% MeOH and 1 mL of DPPH solution as a blank). Three technical replicates were measured for each sample. The standard calibration curve equation used was $y = 0.2074x - 0.004$ ($R^2 = 0.9907$). The radical scavenging capacity was expressed as antioxidant Trolox (vitamin E analogue) (Merck) equivalent per gram of fresh weight (mM TE g^{-1}):

$$\text{TE} = \frac{c * V}{m} \quad (4)$$

where c —Trolox equivalent concentration (mM mL^{-1}), V —total extract volume (mL), m —fresh aboveground biomass (g).

2.3.5. ABTS Assay

The free radical scavenging capacity was also determined using the ABTS radical cation decolorization assay [19]. One day before the measurements were taken, the ABTS solution was prepared; 0.056 g of ABTS (>99% purity, Alfa Aesar) was dissolved in 50 mL of dH_2O . All of the ABTS stock solution was mixed with 200 μL of 70 nM $\text{K}_2\text{S}_2\text{O}_8$ (0.1982 g $\text{K}_2\text{S}_2\text{O}_8$ (Molar Chemicals KFT, Halásztelek, Hungary) dissolved in 10 mL dH_2O). The mixture was held in the dark at +4 °C for 16 h before it was used. Next, if necessary, the mixture was diluted with dH_2O until it reached 0.700 ± 0.2 absorbance (734 nm) (dH_2O as a blank); otherwise, 50 μL of the sample methanol extract was mixed with 2 mL of ABTS solution and placed in the dark for 10 min. Subsequently, the absorbance was measured on a Synergy HT Multi-Mode Microplate Reader (50 μL of 75% methanol and 2 mL of ABTS solution were used as a blank). Three technical replicates were measured for each sample. Trolox was used as the standard. The radical scavenging capacity was expressed as antioxidant Trolox equivalent per gram of fresh weight (mM TE g^{-1}), Formula no. 4 was used for calculations.

2.4. Statistical Analysis of the Data

Pooled data from three independent experiments were used for statistical analysis, which was performed using the open access R software (version 4.0.5; accessed on 7 January 2022) [33]. All the data points stemmed from three technical replicates and 3–7 biological replicates, depending on the metabolite group. Thirty poplar microshoots were used for

each inoculated and control group at each time point divided into three independent replicates. Data were tested for normal distribution of residuals (Shapiro-Wilk test) and homogeneity of variance (Levene's Test) with "car" package functions [34]. ANOVA was then performed using the *aov* from the "stats" function. For data that showed a significant deviation from the normal distribution of residuals, a non-parametric analog of ANOVA, Kruskal–Wallis test, was performed. Tukey Honest Significant Differences tests (HSDs) were performed for multiple pairwise comparisons between the means of groups using the *TukeyHSD* function from the "stats" package.

To build the correlation matrix, the average for each group was calculated. For log-transformed data, the Pearson correlation coefficient was calculated using the *rcorr* function from the "Hmisc" package [35]. To visualize the correlation matrix, the *corrplot* function from the "corrplot" package was used [36]. Visualization of feature distribution (principal component analysis, PCA) was carried out using the "ggplot2" package [37].

The 7 measured bioactive compounds were simplified and classified into components through PCA with *prcomp* from the "Factoextra" package [38].

3. Results

ANOVA data on the seven tested poplar secondary metabolism and physiological parameters are displayed in Figure 1. After 6 weeks, all the data were statistically significant except the amount of CHA ($p > 0.05$), and all the data after 8 weeks were statistically significant. TPC (−8.2%), TFC (−13.7%), CAR (−15.3%), ABTS (−22.7%) (this abbreviation will henceforth be used for free radical scavenging capacity measured using the ABTS assay) and DPPH (−7.7%) (this abbreviation will henceforth be used for free radical scavenging capacity measured using the DPPH assay) metrics were lower in the inoculated group, namely, the poplar microshoots inoculated with *Paenibacillus* sp., after 6-week incubation, while CHB metrics were higher by 32.9% in regard to their respective control groups.

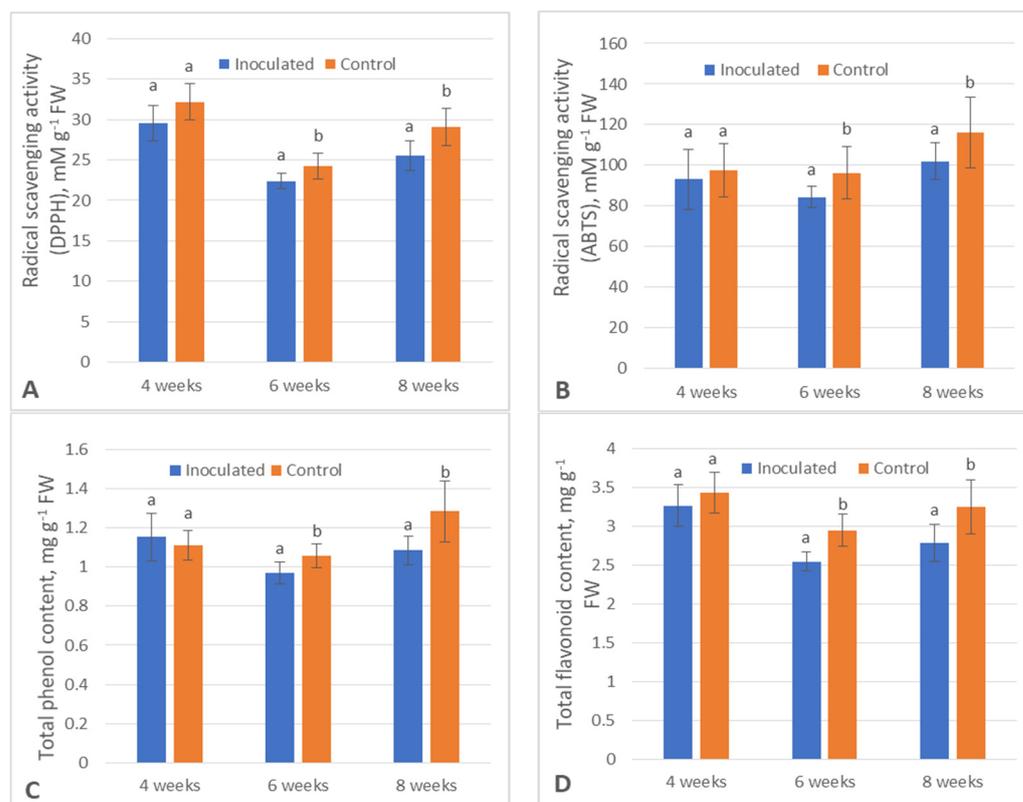


Figure 1. Cont.

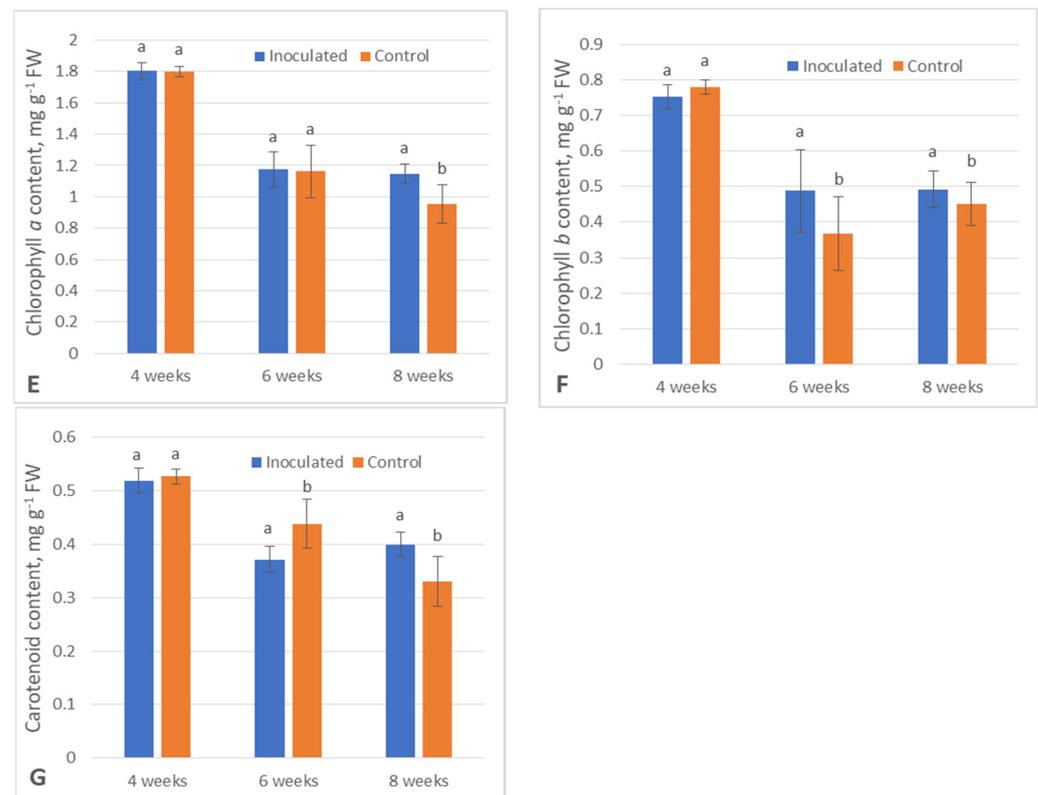


Figure 1. The concentrations of tested bioactive compounds in the control (uninoculated) and *Paenibacillus* sp. inoculated groups throughout the experiment: (A)—antioxidant scavenging (DPPH), (B)—antioxidant scavenging (ABTS), (C)—total phenolic content (TPC), (D)—total flavonoid content (TFC), (E)—chlorophyll a (CHA), (F)—chlorophyll b (CHB), (G)—carotenoids (CAR). Different letters above data from each week indicate significantly different results between control and inoculated groups, $p \leq 0.05$.

Comparatively, TPC, TFC, ABTS and DPPH were lower after 8 weeks by 15.6%, 14.4%, 21.4% and 12.4%, respectively, and all photosynthesis pigment contents were higher (CHA—+20.2%, CHB—+9.2%, CAR—+21.1%) in the inoculated plants in regard to their respective control groups.

There was a clear upward trend regarding the amounts of plant phenolics (TPC and TFC) as well as free radical scavenging capacity (ABTS and DPPH) over the duration of the experiment in both control and experimental groups; however, a significant divergence occurred between the two after the initial incubation period of 4 weeks.

On the other hand, the photosynthesis pigment content did not present a clear trend either way in either of the groups. After 6 weeks, the amounts of CAR decreased in the inoculated plants compared to the control, while CHB increased, and after 8 weeks, all the photosynthesis pigment contents significantly increased in the experimental group.

Correlation analysis allows for statistically significant relationships to be observed between different variables and shows whether that relationship is directly proportional or directly opposite, which, in this case, allowed for assumptions to be made regarding the varied mechanisms that regulate poplar's metabolism. Figure 2 shows the correlation between the amount of bioactive compounds in the control and experimental groups after the 8th week of incubation. In both the experimental and control groups, the amounts of TPC–TFC, TPC–ABTS, DPPH–CHB, CHA–CHB, CHA–CAR and CHB–CAR displayed moderate to strong positive correlations. Simultaneously, a moderately positive correlation between TFC and ABTS, and a moderately negative correlation between TPC and CAR and ABTS–CAR were observed in the control group, and moderately positive correlations in TFC–CHA, TFC–CHB, TFC–CAR, DPPH–CHA and DPPH–CAR were noted in the

experimental group. Specifically, inoculation lowered the correlation between TFC, TPC, ABTS and DPPH and the pigments.

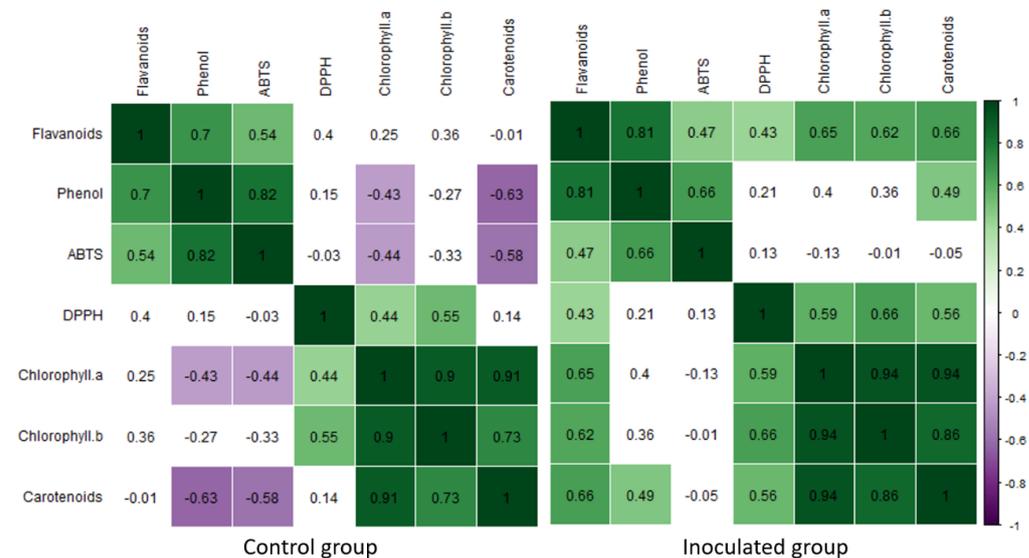


Figure 2. Correlation matrix for the data acquired after the 8th incubation week with *Paenibacillus* sp. (right) and their respective control groups (left), based on the Pearson correlation coefficient. Significant ($p \leq 0.05$) correlation coefficients are colored.

Principal component analysis is a technique used to simplify data when multiple complex data points and variables are available. This group's data, based on multivariate similarities, and similarly to correlation analysis, allows one to gauge the relationships between variables and how much they influence the grouping results and total variance. In this work, PCA analysis was performed to evaluate overall relationships between the levels of bioactive compounds after the eight experimental weeks (Figure 3). The results complimented the correlation analysis, showing some overlap between the two groups; however, there were diverging group mean values, which corresponded with the ANOVA results.

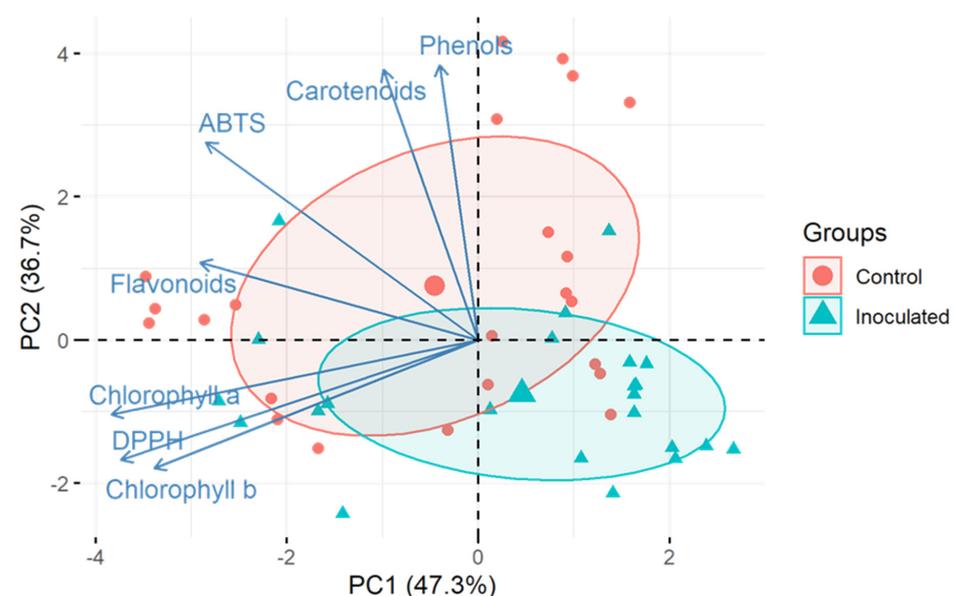


Figure 3. Concentration of tested metabolic and physiological parameters throughout the experiment via principal component analysis (PCA) displaying the main principal components (PCs), which represent 84.41% of cumulative variance. Loading vectors show the correlation between tested variables.

The seven principal components explained the total variance and three explained 92.3% of the total variance of the parameters (Table 1).

Table 1. Total variance of the cumulative data gathered throughout the experiment explained by principal components.

Importance of Components:	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Standard deviation	1.82	1.603	0.76054	0.50628	0.39011	0.31013	0.19162
Proportion of variance	0.473	0.367	0.08263	0.03662	0.02174	0.01374	0.00525
Cumulative proportion	0.473	0.84	0.92266	0.95927	0.98101	0.99475	1

Loading vector analysis showed that TPC, CAR and ABTS were all highly positively linked with principal component 2 (PC2), as was TFC, but to a lesser extent. CHA, CHB and DPPH were all negatively linked with PC1. Further relationships between the PCs and measurements can be seen in the rotated component matrix (Table 2), which suggests a possible meaning of these three principal components, as follows: PC1—pigments; PC2—ABTS and phenolics; and PC3—DPPH.

Table 2. Rotated principal component matrix and individual proportion of variance for each measured metric.

	PC1	PC2	PC3	PC4
Flavonoids	−0.37443	0.412508	−0.24301	0.143112
Phenols	−0.13049	0.563135	−0.23495	0.463554
ABTS	−0.05269	0.573278	−0.0509	−0.67733
DPPH	−0.38247	0.161549	0.869776	−0.04712
Chlorophyll <i>a</i>	−0.49104	−0.25004	−0.15893	0.064167
Chlorophyll <i>b</i>	−0.5043	−0.15491	0.022111	0.248416
Carotenoids	−0.44529	−0.26731	−0.31764	−0.48766

The results showed that the first two principal components (PCs) accounted for 84.41% of the total variation (Figure 3). PC1 (47.3%) was mainly explained by the chlorophyll and CAR content. In turn, PC2 (36.7%) was mainly explained by the ABTS activity and phenolics (although TFC to a lesser extent). PC3 could mainly be linked to DPPH data, although the total input of PC3 was only ~8.2%.

4. Discussion

Changes within a plant's metabolome illuminate how a specific plant may react to new conditions [39]. Bacterial inoculants have been shown to affect plant metabolism in previous studies [15,21,40–42]. The latest research suggests that they often act in a regulatory role [43].

Plant SMs are often linked with the plant's resistance to environmental stressors [26,44–48]. Phenols, flavonoids specifically, can act in various roles: as antioxidants [44,45], antimicrobials [45], etc. Studies pointing to the increases in SM production due to microbial inoculation posit that such a response creates induced systemic resistance (ISR) and will likely help with future responses to pathogen attacks [49,50] or abiotic stress [51]. The data of our experiment showed that the SMs and free radical scavenging activity decreased within the studied timeframe; thus, it could be assumed that the tested bacteria did not induce systemic resistance during initial growth in the tested plants.

Wallis and Galarneau conducted a meta-analysis on this subject (data from 2008 to 2017). In contrast with our own results, they confirmed that total phenolics increase when colonized by both bacteria and fungi. More importantly, their results demonstrated that the type of bacteria (beneficial or not) did not merit different reactions, but the type of fungi did (TPC increased after introduction to beneficial fungi) [52]. Other research demonstrated that

SM production decreased after fungal pathogen attack [26,53] or increased after inoculation with PGPB *Bacillus subtilis* [19], seemingly confirming these findings.

In comparison with our study, *Lolium perenne* grass inoculated with different strains of beneficial fungal endophyte *Neotyphodium lolii* expressed variations of both decreases and increases in phenolics and antioxidants that were endophyte strain dependent [54]. During our studies, both TPC and TFC were affected in a similar manner, with a significant decrease in the inoculated group after 6 and 8 weeks. Additionally, a strong positive correlation ($R > 0.7$) was observed between these effects after 8 weeks in the inoculated group as well as in the control group. This suggests that TFC is likely a large contributor to changes in TPC in our study.

In plants, flavonoids act in an antioxidative capacity, but they may also modulate plant-microorganism interactions [45]. A similar decrease in poplar phenolics, and flavonoids specifically, was noted in a study in which poplars faced a water shortage [55], showing that this type of reaction may not necessarily be unique to our studied bacteria and may potentially be indicative of stress. Consequently, the experimental design using microshoots could be the reason behind similar trends in TFC and TPC in both inoculated and control groups and provide a potential explanation as to why TFC and TPC were relatively lower in the inoculated group as a response to bacteria inoculation, indicating that they were perceived as an additional stressor.

DPPH and ABTS assays are both used to determine the total antioxidant capacity within the sample and thus are indicative of the oxidative stress level [56]. In our study, after 6 and 8 weeks of incubation, inoculated microshoot extracts exhibited a significant decrease in DPPH and ABTS metrics, suggestive of reduced free radical scavenging activity within inoculated poplars. Data on ABTS and TPC/TFC were shown to correlate irrespective of poplar inoculation status and, as phenolic compounds have been reported to act in an antioxidative capacity [44], the drop in phenolics and free radical scavenging activity could stem from the same biochemical changes.

In various studies, plant photosynthesis pigment content can be linked to the general health and vigor of the plant [10,57–59]. However, CHA, CHB and CAR have secondary functions within plants.

CAR act as secondary agents within photosynthesis; however, they also participate in antioxidative stress management and operate as precursors to phytohormones (e.g., abscisic acid, ABA) and other molecules [39,60]. Phenolics and antioxidant activity followed similar trends over the duration of our experiment, even between groups. CAR content was inconsistent throughout, with a relative decrease in the inoculated group after 6 weeks, but a complete opposite reaction after 8 weeks. Within the scope of analogue studies, the increase in CAR can be deemed to be beneficial when this increase can correlate with enhanced plant growth, as was shown in a study using an arbuscular mycorrhizal fungi-grape model [59].

The chlorophyll content within the plant is primarily linked to plant vitality [59,61]. Secondly, chlorophylls can also work as antioxidants [62]. CHB is considered an accessory to CHA [57]. In this experiment, CHB in inoculated plants was enhanced after 6 and 8 weeks, but CHA was only significantly affected after 8 weeks, with increases post-inoculation. Just as with CAR, analogous conclusions were drawn regarding the increase in chlorophyll content for grape [59], avocado [10], cotton [7], peanut [58], eucalyptus [63] and bean [14].

A correlation analysis, performed after 8 weeks of incubation, uncovered some illuminating information. It showed that the inoculation changed the way in which photosynthesis pigments related to four other tested parameters between the control and inoculated groups. Specifically, inoculation seemed to affect TPC, TFC and free radical scavenging activity in relation to photosynthesis pigment content (see Figure 2).

Post inoculation, a moderate negative correlation emerged between ABTS and CAR, as well as between TPC and CAR. This potentially revealed a compensatory mechanism, whereby the inoculation negatively impacts TPC, which correlates with lower free radical scavenging activity (ABTS), which, in turn, raises CAR levels to, at least in part, com-

compensate for the antioxidant level drop. A similar compensation was observed in several studies [64–68]. In a 2020 study, the authors revealed a compensatory mechanism between the plastid prenyllipid antioxidants tocopherol and plastoquinol and ascorbate peroxidase (APX) enzyme [64]. In a study with tomatoes, researchers observed a negative correlation between lycopene and chlorophyll (-0.6209), also suggestive of a compensatory mechanism. This relates to results from our study, whereby after inoculation, carotenoid content was negatively linked with ABTS (-0.58) and TPC (-0.63) ($p \leq 0.05$). Thus, it can be inferred that TFC, TPC, ABTS and pigment amounts correspondingly organize in relation to each other to ensure homeostasis, as noted by Sarker and Oba [68].

PCA uncovered that the inoculated group and control group diverged based on their secondary metabolite levels, and most of this was due to changes in pigments, phenolics and ABTS, while DPPH data strongly determined a small fraction of all variation ($\sim 8.2\%$).

Moreover, a long-term effect on plant growth and secondary metabolism was observed after one-time inoculation, as similar results were achieved using mutant *Paenibacillus polymyxa* on western red cedar [69] and pine [70] after a 12-month period, corn after a 3 month period [24] and *Caballeronia sordidicola* on pine and spruce after a year-long period [71]. This is potentially indicative that further changes might occur during a longer growth period, hence boosting the possibility of using *Paenibacillus* sp. as a long-acting agent. Long-term benefits would be advantageous in tree nurseries, the wood industry, or orchards, where plants are grown for years before harvest, or in cases where periodical fertilization is not feasible.

5. Conclusions

This research contains a more thorough look into *Populus* sp. metabolic and physiological changes post *Paenibacillus* sp. bacterial inoculation during the timeframe of initial growth. This is a necessary input into comprehensive studies to create biofertilizers that could be used in tree nurseries and other forestry related enterprises. These data add to the knowledge regarding the mechanisms that infer plant growth and health. It shows that bacteria may affect different aspects of secondary metabolism in different ways at the same time, i.e., different photosynthesis pigment contents as opposed to phenolic contents and antioxidant capacity. However, additional data need to be collected to further understand *Populus* tree–*Paenibacillus* interactions and pinpoint the exact compounds responsible for these changes. Overall, the data gathered here, alongside previous studies of these bacteria, provide a good example of how microbial inoculants can have complex impacts on their hosts' growth and health.

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