Plant Stress 18 (2025) 101077

Contents lists available at ScienceDirect

Plant Stress

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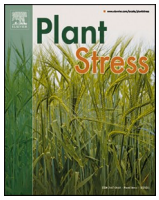

Changes in silver birch biochemical profiles and leaf-associated fungi across genetic markers induced by seed treatment with DBD plasma

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ARTICLE INFO

Keywords:
Defense
Genetic properties
Molecular biology
Plant priming
Tree stress

ABSTRACT

New approaches are needed in the forestry sector to improve tree resistance to various biotic and abiotic stress factors and mitigate their negative impacts on forest health. This study aimed to evaluate the effect of seed treatment with the dielectric barrier discharge (DBD) plasma on the changes in biochemical processes and fungal communities of silver birch (*Betula pendula* Roth.), considering tree half-sib families. The potential of DBD plasma treatment for the modulation of birch seedling properties was estimated in seven selected half-sib families by analysis of the composition of biochemical compounds (from first and second vegetation leaf samples) and fungal communities (from second vegetation leaf samples). Seed treatment with DBD plasma has potential to enhance bioactive compounds, reduce fungal pathogens, thereby improving silver birch seedling traits. The families 73 and 86 demonstrated the most distinct responses to 2-minute-DBD plasma treatment. The activity of antioxidant enzymes (CAT, APX, and GR) increased by ~50 % in the 73 family (the first vegetation), and by ~39 % in the 86 family (the second vegetation). The increase in flavonoid (by ~44 % in the first vegetation and by ~30 % in the second vegetation) and photosynthetic pigment amounts (by ~55 % in the first vegetation and by ~60 % in the second vegetation) was also determined. The family 86 exhibited a reduced relative abundance of the most abundant pathogen, *Phyllactinia betulae*. In the current study, the effect of silver birch seed treatment with DBD plasma on leaf samples was investigated at multiple levels, including biochemical, metagenomic, and genetic aspects.

1. Introduction

Forests have been increasingly impacted by environmental changes over recent decades, leading to reduced growth and disrupted reproduction (Patacca et al., 2022). Advancing technologies that enhance seed quality and tree resilience are key to sustaining healthy forest ecosystems. One of the promising fields in forestry is the application of dielectric barrier discharge (DBD) plasma, as studies have shown that DBD is an eco-friendly technology and does not cause genetic mutations in biological organisms (Attri et al., 2020; Foroughbakhch et al., 2019). DBD plasma consists of different components: an electric discharge, electromagnetic and UV radiation, and a dynamic composition of reactive chemical species, electrons, and photons (Mildaziene et al., 2022). Research indicated that seed treatment with non-thermal plasma induce both external and internal changes in seeds (Leti et al., 2022). The external changes involve modifying the seed coat to improve the hydrophilic process (e.g., water penetration during germination), while the internal changes are related to systemic modulation of cellular

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https://doi.org/10.1016/j.stress.2025.101077
Received 17 July 2025; Received in revised form 12 September 2025; Accepted 7 October 2025
Available online 8 October 2025

processes, which can be observed later through alterations in the metabolic process (Leti et al., 2022). Recent studies have demonstrated that seed treatment with DBD plasma induce shift in hormonal balance in seed tissues, with experiments on radish seeds showing rapid changes in the ratio of abscisic acid and gibberellins (Degutyte-Fomins et al., 2019). The changes induced by plasma treatment in dry and germinating seeds impact numerous processes in growing plants in a long time scale (Mildaziene et al., 2022). Beyond hormonal regulation, plasma treatments have also been linked to changes in gene expression and transcriptional activation, with several genes upregulated in agricultural species, including hemp, sage, wheat, and alfalfa (Mildaziene et al., 2022).

Pre-sowing exposure of planting material to physical stressors is a contemporary approach to enhancing plant growth by inducing various physiological and biochemical changes (Adhikari et al., 2020a; Attri et al., 2020; Mildaziene et al., 2022). However, most of the studies have been focused on agricultural plants. For example, exposure of *Echinacea purpurea* seeds to low-temperature plasma significantly enhanced the vitamin C and phenolic acid content in seedling leaves three months post-sowing. In addition, alterations in secondary metabolite levels following seed treatments with low-temperature plasma were also noted in red clover (Mildaziene et al., 2020) or common buckwheat (Ivankov et al., 2021). The influence of seed treatments on microbial dynamics, phytochemical composition, and biochemical activities in plant tissues is a multifaceted topic that has not been thoroughly examined. Specifically, studies investigating the impact of plasma-based pre-sowing treatments on perennial plants are still limited.The combined impact of global climate change amplifies the higher risk of emerging disease invasions, endangering forest health and productivity (Stenlid & Oliva, 2016). Silver birch (*Betula pendula* Roth.) is a widely distributed species with significant potential to enhance the sustainability and multifunctionality of European forests (Matisons et al., 2022). *B. pendula* is a valuable model for studying northern tree adaptation to climate change due to advances in birch genomics, extensive research on key physiological traits, and its high genetic diversity (Silfver et al., 2020; Tenkanen et al., 2019). Therefore, *B. pendula* is a convenient tree species to analyze plant resistance to biotic and abiotic stress through biologically active compounds production and its interactions with microorganisms, both of which play an essential role in plant health.

Reactive oxygen species (ROS) are steadily formed within plant cells as unintended byproducts of key metabolic pathways, especially those associated with photosynthesis and cellular respiration (Singh, 2016). Although ROS contributes to vital signaling pathways, an imbalance resulting in an excessive amount can trigger oxidative stress, thereby disturbing cellular equilibrium. Primary outcomes of oxidative stress are lipid peroxidation, which impairs the structural integrity and biological function of essential biomolecules. A marker of oxidative damage is malondialdehyde (MDA), which forms during the peroxidation process (Havaux, 2023). As a regulator of ROS levels, the antioxidant system supports critical biological processes in plants, including their growth, hormonal signaling, development, cell cycle progression, and adaptive responses to biotic and abiotic stress. Plant biochemical profiles encompass both enzymatic and non-enzymatic components that regulate physiological functions. The enzymatic antioxidant system comprises enzymes, including catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.8.1.7), superoxide dismutase (SOD; EC 1.15.1.1), and peroxidase (POX; EC 1.11.1.5). These antioxidant enzymes are essential for preserving cellular redox homeostasis by directly scavenging specific ROS and their derivatives (Arena et al., 2019; Fimognari et al., 2020; Rajput et al., 2021). Whereas the non-enzymatic components primarily consist of photosynthetic pigments and secondary metabolites (Arena et al., 2019; Česnienė et al., 2024; Fimognari et al., 2020).

Interactions between trees and their associated microbiota constitute a fundamental mechanism underlying forest ecosystem health and resilience (Rwizi et al., 2025). Trees host diverse foliar fungal communities, which may act in three different ways: beneficial, antagonistic, or have no impact on the tree (Trogisch et al., 2021). The diversity of foliar fungi in trees varies across large geographical scales (Millberg et al., 2015), and it also can differ among genotypes within the individual species (Bálint et al., 2013) or even among different leaves (Nguyen et al., 2016). Given the high diversity of foliar fungi across different tree biological traits and their important role in tree resilience, there is a strong need to assess how these fungal communities respond to treatments aimed at improving forest health.

Several studies demonstrated that synthesis of secondary metabolites and photosynthetic pigments is highly dependent on a tree's genetic properties (Marčiulynas et al., 2019; Matisons et al., 2022). Therefore, selecting half-sib families with distinct genetic properties and comparing their responses can help identify the most positively responsive families, which may be targeted for seed treatments to enhance stand stability. In widely distributed species such as silver birch, focusing exclusively on genetic adaptation within populations may enhance tree survival, growth, and reproduction, but it can also limit the ecological plasticity of genotypes (Eckert et al., 2019; Matison et al., 2022). This study aims to advance our understanding of the effects of DBD plasma by addressing existing knowledge gaps, with a particular focus on microbiota shifts and biochemical changes in young woody plants, considering their genetic characteristics.

2. Materials and methods

2.1. Genetic selection of silver birch

The seeds of seven different silver birch half-sib families were collected from a plantation established in 2012 (in the Dubrava Regional Division, 54°51′20.6″ N, 24°03′04.8″ E). The selected families were: 60, 73, 86, 112, 125, 171, 179.

2.2. Seeds treatment with dielectric barrier discharge (DBD) plasma

Collected seeds were divided into three different treatment groups: not-treated seeds (control); seed treatment with atmospheric air DBD plasma 1 minute (DBD1); and seed treatment with DBD plasma 2 minutes (DBD2). The durations of these treatments were selected based on pilot studies, which demonstrated that 1 and 2 minutes had the most pronounced effects on primary physiological traits. Number of control and treated seeds per each half-sib family: 216 × 3 (treatments) = 648; total number of sown seeds: 648 × 7 (half-sib families) = 4,536. Seeds were treated with DBD plasma using a controlled atmospheric dielectric barrier discharge device at Vytautas Magnus University, produced in Kyushu University, Japan (Sarinont et al., 2016). The homogeneous treatment area was 4 × 4.38 cm^2. The detailed methodology for seed treatment using DBD plasma parameters is described in the publication by Česnienė et al. (2024). The treatment was conducted at atmospheric pressure and air humidity (no less than 45 % and no more than 55 %). Following the treatments, the seeds were maintained for a period of four days to allow for alterations in phytohormone content (Degutyte-Fomins et al., 2019).

2.3. Sowing and cultivation of seeds

After treatment, seeds were sown in 18-cell cassettes with a peat substrate (SuliFlor SF2) pH range of 5.5 to 6.5 (Fig. 1 A). For up to 2 months, the seedlings were cultivated under controlled conditions (+25–32 °C at daytime; >+10 °C at night) (Česnienė et al., 2025). After two months, the seedlings were relocated to an outdoor area. One-year-old seedlings were transplanted into pots (1 L) containing 1000 mL of peat substrate (Fig. 1 B and C).

Fig. 1. Silver birch seedlings two months after sowing in cassettes in the greenhouse (**A**); one-year-old seedlings in cassettes (**B**); and two-year-old seedlings, transplanted into pots (**C**).

2.4. Sample Collection for Laboratory Analysis

Leaf samples from seedlings of different silver birch half-sib families were collected from both untreated (control) and DBD plasma-treated groups during August–September of the 2020 and 2021 growing seasons. For each group (control, DBD1, and DBD2), 3–5 leaves were sampled per biological replicate, and in total, three biological replicates of each group were used for analysis. The quantitative assays of biochemical compounds, stress markers (MDA levels), and the antioxidant system (enzymes and antioxidant capacity) were performed using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Bad Friedrichshall, Germany).

2.5. Amounts of pigments, soluble sugars, and phenolic compounds

The analyses were performed based on Česnienė et al., 2025 methodology. The content of photosynthesis pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) was determined at the 664 nm, 648 nm, and 471 nm wavelengths, respectively. Soluble sugars (SS) were determined based on a 0.1 % anthrone reagent, and the absorbance was measured at 620 nm wavelength. The content of phenols (TPC) was determined based on Folin–Ciocalteu reagent reaction with 725 nm wavelength. The content of flavonoids (TFC) was established based on the formation of a flavonoid-Al (III) complex with a 415 nm wavelength.

2.6. Qualitative analysis of secondary metabolites

Dried and milled silver birch leaves were extracted using 70 % methanol (v/v). The mixture was subjected to an ultrasound bath at the boiling point of the respective solvent for 30 min. and cooled. After cooling, the extract was processed by centrifugation (4000 rpm) and subsequently filtered through polyvinylidene fluoride (PVDF) syringe filters with a pore size of 0.22 µm (Frisenette, Knebel, Denmark). The identification of secondary metabolites was performed using Kazlauskaitė et al. (2024) methodology. The LC/MS system consisted of a Shimadzu Nexera X2 LC-30AD high-performance liquid chromatography (HPLC) unit coupled with an LCMS-2020 mass spectrometer (Shimadzu, Tokyo, Japan).

Chromatographic separation was carried out on a YMC-Triart C18 column (150 mm × 3.0 mm, 3 µm) at 25 °C using a linear gradient of 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B). A 10 µL sample was injected for each silver birch extract, with a flow rate of 0.5 mL/min following a specified gradient elution profile. Mass spectra were acquired over an m/z range of 50–2000, with a scan speed of 15,000 µ/s and a step size of 0.1 m/z (Kazlauskaite et al., 2024).

Spectral data were obtained using both positive and negative ionization modes. Metabolite identification was achieved through analysis of λ_{max} (nm), mode of ionization (ESI-/ESI+), fragmentation pathway [m/z], retention time, and comparison with previously reported literature data. Metabolites found in two-year-old birch leaf samples were identified using the databases (MassBank and PubChem) and literature sources (Ncube et al., 2014; Mateos et al., 2018; Tang et al., 2020).

2.7. Stress markers

The levels of stress markers were determined using lipid peroxidation analysis based on the TBA-MDA method (Murshed et al., 2013). The levels of malondialdehyde (MDA) were measured. The quantitative assays of MDA levels were measured with absorbance at 440 nm, 532 nm, and 600 nm wavelengths.

2.8. Activity of antioxidant enzymes

Sample preparation and evaluation of the activity of antioxidant enzymes were performed according to Beniušytė et al., 2023. 0.2 g of fresh silver birch leaf samples were ground with liquid nitrogen and filled with extraction buffer (5 mL), which consisted of K-phosphate buffer (pH 7.8; 150 mM), Triton X-100 (1 %), polyvinylpolypyrrolidone (0.3 g), and ascorbate (5 mM).

Total protein content (PROT) was determined based on the reaction between peptide bonds and the reagent, during which Cu^{2+} ions are reduced to Cu^{+} (Smith et al., 2020). The activity of SOD was determined based on the inhibition of nitroblue tetrazolium (NBT) photoreduction (Qu et al., 2024). The activities of CAT, APX, and POX assays were determined based on the consumption of H_2O_2 (Zhang et al., 2022). The activity of GR was determined based on NADPH oxidation in the presence of 0.5 mM NADPHas (Zhang et al., 2022).

2.9. Total antioxidant capacity (TAC)

TAC was determined using the ABTS and DPPH radical scavenging methods. The 0.5 g of silver birch leaves was homogenized with an analytical mill (Laboratory Equipment, Staufen, Germany) as described in Lučinskaitė et al., 2021. DPPH radical scavenging activity were determined based on 0.1 mM DPPH solution; ABTS radical scavenging activity were performed based on ABTS solution (consisting of ABTS and 70 nM potassium persulfate stock). The quantitative assays of TAC were measured with absorbance at 515 nm (DPPH assays) and 734 nm (ABTS assays) wavelengths.

2.10. Fungal diversity

Leaves of silver birch were randomly collected from the second vegetation seedlings. A total of 315 birch samples (7 families × 3 treatments × 15 biological replicates from different seedlings) were used for DNA extraction.

DNA was extracted and amplified using the procedures described by Menkis et al. (2022). For DNA extraction, 3 % cetyltrimethylammonium bromide (CTAB) method was used (Menkis et al., 2022). After DNA extraction, the concentration and quality of the extracted DNA were measured using a NanoDrop One spectrophotometer (Thermo Scientific, Rochester, NY, USA). DNA amplification was performed using specific fungal primers gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990), with unique identification markers. The reaction mixture consisted of template DNA (10 ng/μL) – 6.7 %, DreamTaq DNA Polymerase (5 U/μL) – 0.5 %, 10 × DreamTaq® Green buffer – 10 %, dNTPs (10 mM) – 2 %, $MgCl_2$ (25 mM) – 2 %, MilliQ water – 68.8 %, and primer gITS7 MIX (10 μM) – 10 %. The amplifications were performed using the "Applied Biosystems 2720 thermal cycler" (Applied Biosystems, Foster City, CA, USA). The PCR reaction was initiated using the following parameters: 5 min at 95 °C, followed by 35 cycles of 95 °C (30 s), 52 °C (30 s), and 72 °C (30 s), and then 72 °C for 7 min. For cleaning, a 96 % ethanol and 3 M sodium acetate (pH 5.2) (Applichem GmbH, Darmstadt, Germany) mixture (1:2) was used. The samples were mixed based on their final concentrations and subjected to high-throughput PacBio RSII sequencing at SciLifeLab in Uppsala, Sweden.

Sequence quality control and clustering were carried out using the SCATA NGS pipeline (http://scata.mykopat.slu.se). Quality filtering involved removing sequences shorter than 200 base pairs, those with an average quality score below Q20, as well as primer dimers and homopolymer-rich reads. Clustering of sequences was performed using a 98 % similarity level. Taxonomic assignments were made by comparing the most abundant genotype of each OTU against the GenBank (NCBI) database using the BLASTn algorithm (Bourret et al., 2023). Assembled representative sequences of fungal non-singleton OTUs have been submitted to GenBank under accession numbers PV948095–PV948176.

2.11. Statistical analysis

R (Version 4.2.1) with RStudio (Version 1.1.456) was used for data analysis and visualization. The packages that were used: (1) *ggplot2* (Wickham et al., 2016), (2) *multcompView* (Graves et al., 2015), (3) *corrplot* (Wei et al., 2017), (4) *dplyr* (Silge & Robinson, 2016), (5) *vegan* (Oksanen et al., 2007).

2.11.1. Biologically active compounds, antioxidant enzymes, and total antioxidant capacity

Shapiro–Wilk test was used to test data normality. For statistical significance, Kruskal–Wallis test was performed (McKight & Najab, 2010), followed by Dunn's test (Dinno, 2015) and Bonferroni correction (Sedgwick, 2012). Bar plots were created to display mean values of each treatment, with error bars representing standard errors (SE).

Pairwise Pearson correlation analyses were performed separately for each treatment group at first vegetation and second vegetation samples to examine relationships among biochemical parameters. Mean values were calculated for each treatment, family, time, and biochemical compound combination. Correlation matrices were computed using complete pairwise observations and visualized using circular correlation plots, where bubble size and color represented the strength and direction of the correlations.

To evaluate the differences in biochemical trait expression among half-sib families within each treatment group, the Wilcoxon rank-sum test with Bonferroni correction was performed for each combination of treatment and parameter. The results were visualized as heatmaps, where each tile represents a comparison between two half-sib families. Green-colored cells indicated the presence of a comparison, and significance levels were displayed as asterisks within the corresponding cells.

2.11.2. Fungal communities

To determine the overlap and uniqueness of fungal OTUs among the different treatment groups, a Venn diagram was generated using the online tool Interactive Venn (https://www.interactivenn.net/). OTU presence/absence data were compiled for each treatment—Control, DBD1, and DBD2—and used to construct a three-set diagram illustrating the number of shared and treatment-specific OTUs. Shannon's diversity index was used to assess the diversity of fungal communities (Nachar, 2008), and it was calculated for each replicate sample. The differences among treatments (Control, DBD1, and DBD2) were evaluated using the nonparametric Mann–Whitney U test (Nachar, 2008). To assess community similarity between treatments, the Sørensen qualitative similarity index (Chao et al., 2006) was calculated based on presence–absence data pooled across replicates for each treatment.

The differences in fungal OTUs composition among treatments were visualized with Non-metric Multidimensional Scaling (NMDS). Bray–Curtis dissimilarity was calculated on the species abundance matrix. Each point represents a fungal species, colored by the treatment (Control, DBD1, or DBD2) in which it was most abundant. Ellipses represent 95 % confidence intervals for each treatment group. PERMANOVA (*adonis2*) was conducted using Bray–Curtis dissimilarities with 999 permutations.

2.11.3. Correlation between fungal communities and biochemical compounds

Pearson correlation analysis was performed to evaluate the relationships between fungal functional groups and plant biochemical parameters under different treatments (C, DBD1, DBD2). For each treatment, abundances of fungal functional groups (endophytes, mycorrhizal fungi, pathogens, saprotrophs, and unknown fungi) were correlated with biochemical variables (antioxidant enzyme activities, pigments, phenolic compounds, and soluble sugars). Correlation coefficients (r), significance values (p), and sample size (n) were calculated. Correlation matrices were visualized as heatmaps, where positive and negative associations were indicated by color gradients, allowing

direct comparison of treatment-specific patterns.

3. Results

3.1. Changes in leaf biochemical composition

The results of the analysis of different biochemical compounds in seedling leaves growing from control, DBD1-, and DBD2-treated seeds, performed in seven silver birch half-sib families, are shown in Fig. 2. The results reveal that differences in amounts of all analyzed biochemical compounds are dependent on genotype, treatment duration, and year of vegetation. Significant differences between half-sib families in control, DBD1- and DBD2-treated groups were also observed (**Supplementary Figure 1**). More pronounced differences among families were noted in the first vegetation season when evaluating TPC, whereas in the second year, greater variation was observed in TFC and MDA levels.

3.1.1. Content of TPC and TFC

In the second vegetation control samples, the accumulation of total TPC was higher compared to the first vegetation birch samples (Fig. 2). Changes in TPC content induced by seed treatment were determined in five half-sib families in the first vegetation birch samples. In leaves of three families (60, 73, and 86), TPC increased (by 7-56 %), while in one family (125), TPC was reduced by 15 % in DBD1-treated group. A similar trend was observed in DBD2-treated group – TPC was increased by 14-42 % in three families (60, 73, and 125) and reduced by 26 % in one family (86). The accumulation of TPC in two families was treatment-duration-dependent: (1) in the 86 family in DBD1-treated samples TPC was increased, while in DBD2-treated samples – reduced; (2) on the other hand, in the 125 family, the effect of DBD was contrary – in DBD1-treated samples – TPC was reduced, meanwhile in DBD2-treated – increased. In the second vegetation samples, in the DBD1-treated group, only a reduction in TPC was observed – five out of seven families showed a decrease in TPC of 15-34 %. In DBD2-treated group, an increase in TPC by 13 % was observed in one family (60), but in another family (112), the accumulation of TPC was reduced by 20 %. The accumulation of TPC in the 60 family was treatment-duration-dependent – in DBD1-treated samples, TPC was reduced, while in DBD2-treated samples, it was increased, compared to control.

Changes of total flavonoid content (TFC) were determined in both DBD groups, regardless of the age of seedlings (Fig. 2). In the first vegetation seedlings, changes in TFC content induced by seed treatment with DBD1 were determined in four half-sib families – in three families (60, 73, and 86), TFC increased by 5-56 %, while in one family (125), it was reduced by 17 %. In DBD2-treated group, a more noticeable effect was observed. TFC of four half-sib families (60, 73, 171, and 179) increased by 10-57 %, while in two families (86 and 112) it decreased by 14 %. The 86 half-sib family exhibited a treatment-duration-dependent response. In the DBD1-treated group, TFC was increased, whereas in the DBD2-treated group, it was reduced compared to the control. In the second vegetation samples, the treatment effect was less noticeable than in the first vegetation samples. In DBD1-treated group, TFC was increased by 29 % in one family (112). In DBD2-treated group, TFC changes were observed in two families (60 and 86) – in both of them, TFC increased by 14-27 %.

The most abundant secondary metabolite compounds in two-year-old silver birch leaf extracts were identified as polyphenols and flavonoids. Most of the compounds were flavonoids, such as catechin, myricetin 3-O-glucoside, eriodictyol 7-O–D-glucoside, isoquercetin, and quercetin 3-O-arabinoside. Also, chlorogenic acid, coumarin acid, 3-caffeoylquinic acid, and coumaroyl quinic acid were identified using the HPLC analysis.

3.1.2. Membrane lipid peroxidation

The changes in the amount of the marker of lipid peroxidation, MDA, were observed in both years' seedlings. In the first vegetation samples, seed treatment with DBD1 changed the amount of MDA in three half-sib families (Fig. 2). Lipid peroxidation was reduced by 19-23 % in two families (112 and 125). However, in one family (171), MDA levels increased by 35 %. The same trend was observed in DBD2-treated groups. Two families (86 and 112) showed a reduced amount of MDA by 17-27 %, and in one family (179), the MDA amount increased by 25 %. The level of MDA was reduced in three families in both DBD1- and DBD2-treated groups (in different half-sib families) by 8-28 %. In was determined that lipid peroxidation was highly dependent on treatment time duration – in DBD1-treated samples, MDA was reduced in two half-sib families (112 and 125), however, in DBD2-treated group, lipid peroxidation increased in one family (86).

3.1.3. Total soluble sugars (TSS)

The analysis of TSS showed that in some half-sib families, TSS content was reduced, despite treatment duration in the first vegetation birch samples (Fig. 2). Four half-sib families showed a reduced TSS content – 25 % in DBD1-treated group and 15-82 % in DBD2-treated group. In one family (73), TSS content increased by 28 % in DBD2-treated group. A similar trend was observed in the second vegetation seedlings. In DBD1-treated group, three families (60, 73, and 125) showed a 45-83 % reduction in the accumulation of TSS. In DBD2-treated group, the amount of TSS in one family (86) increased (by 73 %), and in one family (125) it was reduced (by 53 %).

3.1.4. Photosynthetic pigments

The differences in the content of photosynthetic pigments were observed between half-sib families as well as between the effects of seed treatments in silver birch seedling leaves collected in both years (Table 1). Significant differences between half-sib families in control, DBD1- and DBD2-treated groups were also observed, evaluating photosynthesis pigments changes (**Supplementary Figure 1**). The differences between half-sib families in both chlorophyll *a* and chlorophyll *b* were more noticeable in first-year seedlings.

In two half-sib families (60 and 73), chlorophyll content was increased in DBD1-treated samples in both the first and second vegetation seasons by 28-57 % (Table 1). However, more noticeable effect was observed in DBD2-treated samples. The 60 and 73 half-sib families exhibited increased contents of chlorophylls (by 59-95 %) and carotenoids (by 3-7 %) after DBD2 treatment in the first vegetation samples. In the second vegetation samples the changes in chlorophyll accumulation were more noticeable, compared to the first vegetation. In DBD2-treated group, three families (60, 86, and 171) showed an increased accumulation of chlorophylls by 33-80 %. In addition, in three families (73, 86, and 112), the content of carotenoids in DBD2-treated group was increased by 12-24 %.

3.1.5. Antioxidant system: antioxidant enzyme activity and total antioxidant capacity (TAC)

Effects of seed treatments on the antioxidant system in seedling leaves of silver birch families were determined, and the results are provided in Fig. 3 **and Supplementary Table 1**. All of the analyzed parameters were affected, revealing distinct genotype-, treatment duration-specific, and year-dependent responses. It was found that the changes in the activity of antioxidant enzymes were more noticeable in second-vegetation seedlings compared to first-vegetation seedlings. Significant differences between half-sib families in control, DBD1- and DBD2-treated groups were also observed, evaluating the antioxidant system (**Supplementary Figure 1**). More pronounced differences in antioxidant enzymes (except APX) among families were noted in the second vegetation season samples. On the other hand, more noticeable differences among half-sib families in TAC were observed depending on the assay method. DPPH radical scavenging activity showed greater variation among families in the first-year vegetation samples, whereas ABTS radical scavenging activity exhibited more pronounced differences in the second-year vegetation samples.

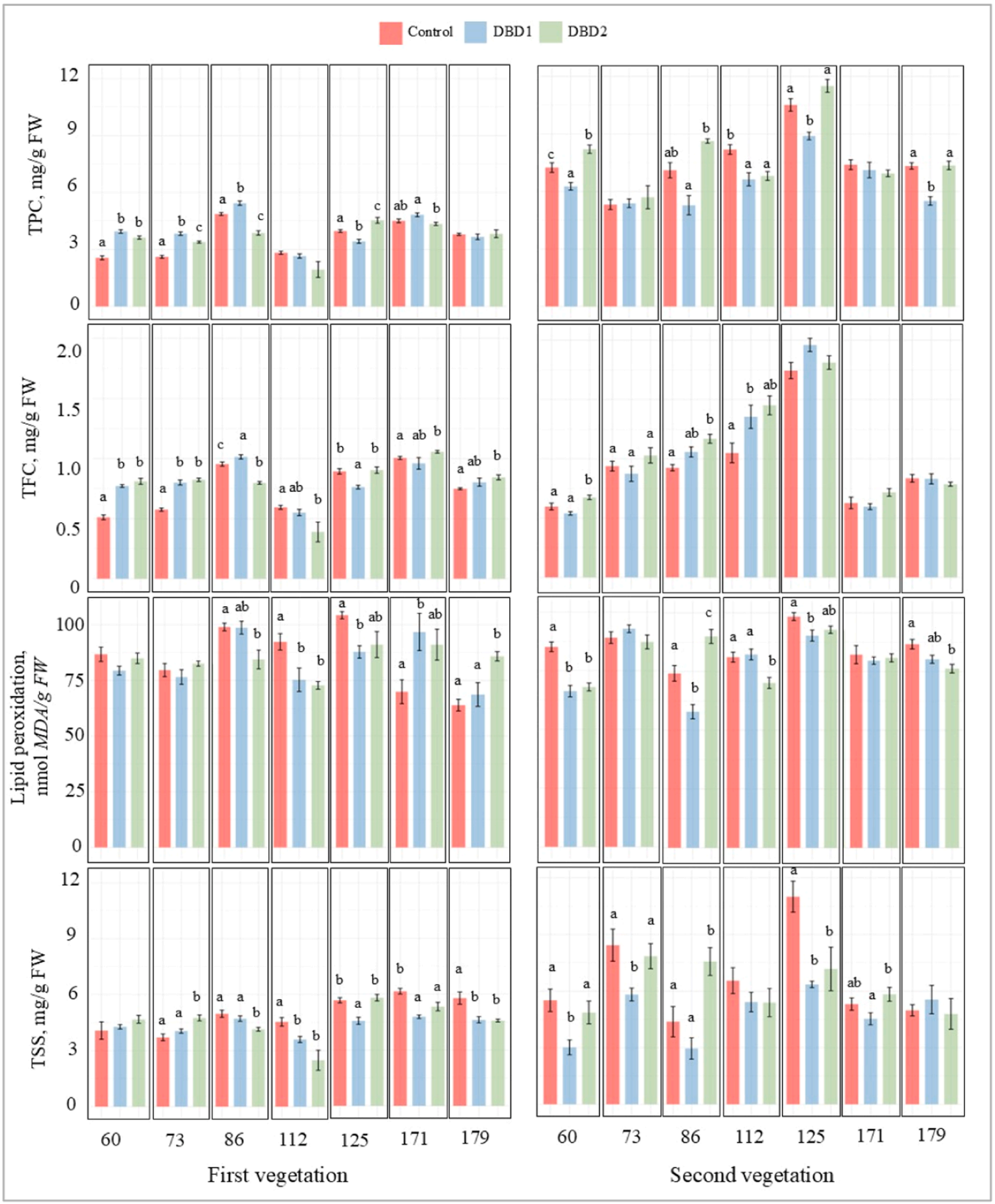

Fig. 2. The content of biochemical compounds (TPC – total phenolic content; TFC – total flavonoid content; MDA content – malondialdehyde, and TSS – total soluble sugars) in leaf samples of different silver birch half-sib families collected during the first and second vegetation season. Different letters above the columns indicate statistically significant differences between means ($p < 0.05$), as determined by the Kruskal–Wallis test followed by Dunn's test. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min.; DBD2 – seed treatment with DBD plasma 2 min.

Table 1
Photosynthetic pigment content in samples of different silver birch families during the first and second vegetation season. Different letters above the columns indicate statistically significant differences between means ($p < 0.05$), as determined by the Kruskal–Wallis test followed by Dunn's test. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min.; DBD2 – seed treatment with DBD plasma 2 min.

Half-sib Family	Treatment	Chlorophylls and carotenoids content (μg/g), ±SE					
		First vegetation samples			Second vegetation samples		
		Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Chlorophyll *a*	Chlorophyll *b*	Carotenoids
60	Control	208.0 ± 27.5^{b}	101.6 ± 10.8^{b}	14.5 ± 0.2^{c}	255.7 ± 21.9^{c}	216.6 ± 8.7^{c}	66.1 ± 3.4^{a}
	DBD1	327.1 ± 4.9^{a}	151.9 ± 2.2^{a}	15.8 ± 0.1^{a}	363.9 ± 18.9^{a}	278.4 ± 13.8^{a}	55.7 ± 1.5^{ab}
	DBD2	405.5 ± 34.7^{a}	180.1 ± 14.6^{a}	15.0 ± 0.0^{b}	460.5 ± 21.6^{b}	340.4 ± 13.3^{b}	53.9 ± 1.1^{b}
73	Control	244.6 ± 7.4^{c}	120.3 ± 3.1^{c}	15.1 ± 0.1^{a}	256.8 ± 9.6^{b}	196.2 ± 5.9^{a}	61.7 ± 2.1^{c}
	DBD1	325.1 ± 8.2^{a}	154.5 ± 3.2^{a}	15.2 ± 0.1^{a}	356.6 ± 23.5^{a}	255.7 ± 15.9^{b}	53.7 ± 1.4^{a}
	DBD2	388.3 ± 7.4^{b}	184.5 ± 3.3^{b}	16.2 ± 0.1^{b}	305.9 ± 26.8^{ab}	229.8 ± 18.39^{ab}	70.1 ± 2.0^{b}
86	Control	395.8 ± 4.3^{ab}	187.5 ± 1.8^{b}	14.0 ± 0.1^{ab}	357.6 ± 36.1^{a}	261.3 ± 20.3^{a}	32.5 ± 1.4^{a}
	DBD1	443.8 ± 6.3^{a}	207.7 ± 2.8^{a}	13.7 ± 0.1^{a}	333.9 ± 28.6^{a}	255.8 ± 18.5^{a}	36.6 ± 0.4^{a}
	DBD2	389.9 ± 17.8^{b}	186.6 ± 7.7^{b}	14.9 ± 0.3^{b}	616.7 ± 44.3^{b}	397.5 ± 24.5^{b}	40.3 ± 0.6^{b}
112	Control	225.8 ± 10.1	111.3 ± 4.1	17.4 ± 0.0^{a}	285.9 ± 26.1	196.9 ± 14.6	46.9 ± 2.2^{b}
	DBD1	241.5 ± 18.5	121.1 ± 7.4	16.6 ± 0.3^{ab}	331.3 ± 4.8	219.7 ± 3.6	48.9 ± 1.2^{ab}
	DBD2	178.4 ± 37.7	86.1 ± 18.2	15.8 ± 0.2^{b}	288.1 ± 12.9	193.8 ± 5.8	52.7 ± 1.0^{a}
125	Control	488.3 ± 9.5^{b}	236.1 ± 3.7^{b}	14.0 ± 0.1	371.4 ± 28.4	271.4 ± 13.6^{b}	48.1 ± 1.6
	DBD1	400.3 ± 7.2^{a}	196.5 ± 3.2^{a}	13.6 ± 0.2	422.9 ± 18.8	299.4 ± 10.1^{ab}	43.7 ± 0.9
	DBD2	461.7 ± 11.3^{b}	227.3 ± 4.8^{b}	14.1 ± 0.1	443.6 ± 10.9	323.0 ± 10.3^{a}	43.3 ± 1.8
171	Control	415.2 ± 14.3^{b}	199.5 ± 6.8^{b}	14.8 ± 0.2^{b}	255.9 ± 23.3^{a}	192.1 ± 11.5^{b}	48.9 ± 1.2
	DBD1	330.6 ± 6.4^{a}	158.8 ± 2.9^{a}	15.9 ± 0.2^{a}	340.6 ± 9.1^{ab}	247.0 ± 6.3^{a}	49.1 ± 1.3
	DBD2	423.8 ± 8.6^{b}	199.9 ± 3.7^{b}	14.9 ± 0.1^{b}	376.8 ± 26.5^{b}	254.9 ± 16.2^{a}	52.5 ± 2.4
179	Control	303.5 ± 4.1	140.9 ± 0.9	15.9 ± 0.1	435.6 ± 32.5	274.4 ± 16.6	48.4 ± 0.9
	DBD1	325.7 ± 43.7	152.7 ± 18.4	16.1 ± 0.6	466.3 ± 30.1	284.2 ± 14.9	47.2 ± 0.9
	DBD2	358.2 ± 16.2	162.6 ± 7.2	16.6 ± 0.2	446.4 ± 24.9	268.0 ± 11.3	49.4 ± 1.5

CAT: in the first vegetation seedlings, the changes in the activity of CAT were observed only in two silver birch families. In DBD1-treated group, the activity of CAT was increased by 50 % (73 family), in DBD2-treated group, the activity of CAT increased by 37-53 % in two families (73 and 125). Meanwhile, in the second vegetation, changes in CAT activity were more pronounced compared to the first season. The activity of CAT was increased in two families (86 and 179) by 33-88 % in DBD1-treated group, however in one family (112) the activity of CAT was reduced by 34 %. In the DBD2-treated group, the activity of CAT was increased in two families (86 and 179) by 42 % and reduced in three families (73, 112, and 171) by 20-74 %.

APX: in the first vegetation seedlings, the changes in APX activity were observed only in DBD2-treated group. The activity of APX increased by 53 % in one family (86), while in another family (73), APX activity was reduced by 111 %, compared to the control. In the second vegetation samples of the DBD1-treated group, the activity of APX was increased in two families (86 and 179) by 35-314 % and reduced in the other two families (73 and 125) by 13-46 %. In the DBD2-treated group, the activity of APX increased in two families (86 and 179) by 42-220 % and decreased in three families (60, 125, and 171) by 20-45 %.

POX: in the first vegetation seedlings, the changes in POX activity were observed only in 73 family (activity of POX increased by 58 %) in DBD1-treated group. In DBD2-treated group, the activity of POX enzyme increased in three families (73, 125, and 179) by more than 60 %. In the second vegetation samples, three families (86, 125, and 179) increased the activity of POX (by 24-73 %) in DBD1-treated group. DBD2 induced changes in the activity of POX enzyme in five half-sib families: in three families (73, 125, and 179), the activity of POX was increased by 20-74 %, and in two families (60 and 112), it was reduced by 53-97 %.

GR: in the first vegetation birch seedlings only in leaves of the 73 family GR activity was increased (by 39 %) in both DBD1- and DBD2-treated samples. In the second vegetation samples, changes in the activity of GR were determined in three half-sib families in DBD1- and DBD2-treated groups. In two families (86 and 179) the activity of GR was increased by 41-86 % in DBD1- and DBD2-treated groups, however, in 73 family, the activity of GR was reduced by 11 % (in DBD1-treated group) and in 60 family, it was reduced by 78 % (in DBD2-treated group).

SOD: in the first vegetation seedlings, in four families the activity of SOD enzyme was changed in DBD1-treated group. In three families (73, 86, and 125) SOD activity increased by 14-74 %, and in one family (60) it was reduced by 19 %. In DBD2-treated group, only a reduction (by 37-54 %) of SOD enzyme activity was observed in two families (60 and 171). In the second vegetation samples, three families (73, 125, and 179) showed increased activity of SOD (by 30-83 %) and in one family (60) it was reduced (by 147 %) in DBD1-treated group. In DBD2-treated group, a more noticeable effects were observed compared to the DBD1-treated group. In four families (73, 125, 171, and 179) activity of SOD enzyme was increased by 32-109 %, and in two families (60 and 86) it was reduced by 44-211 %.

Total antioxidant capacity (TAC) in different silver birch half-sib families was evaluated based on DPPH and ABTS assays (**Supplementary Table 1**). It was found that TAC was remarkably higher in leaves of the second vegetation seedlings, compared to the first vegetation in the control seedlings of all half-sib families (with the only exception of DPPH radical scavenging activity in 171 family). The results indicated that, in most families, DBD treatment had no significant positive effect on TAC activity or even reduced it compared to the control (**Supplementary Table 1**). In DBD1-treated group, TAC was reduced in three families (125, 171, and 179) by 23-77 % in the first vegetation samples. In DBD2-treated group, TAC was reduced in the 86 half-sib family (by 81 %). However, in the second vegetation samples, TAC was reduced (applying both DPPH and ABTS assays) only in the 86 family in DBD1-treated group (by 8-22 %).

3.1.6. *Correlation analysis*

The overall patterns of effects of seed treatments on biochemical parameters of the first- and second-year seedlings are provided in Figs. 4 and 5, irrespective of the silver birch genetic properties. Noticeable changes were observed in the treated samples across both years.

A strong positive correlation (r=0.85) was observed in the control first vegetation samples between the amounts of chlorophyll *a* and *b*, total antioxidant capacity (DPPH and ABTS), secondary metabolites (TPC and TFC), and total soluble sugars (TSS) (Fig. 4). In contrast, carotenoid (caro) content showed a negative correlation (r = –0.55) with both chlorophylls and secondary metabolites. In DBD1-treated samples, the negative correlation (r = – 0.95) between carotenoids and chlorophylls was more pronounced. Strong positive correlation (r =

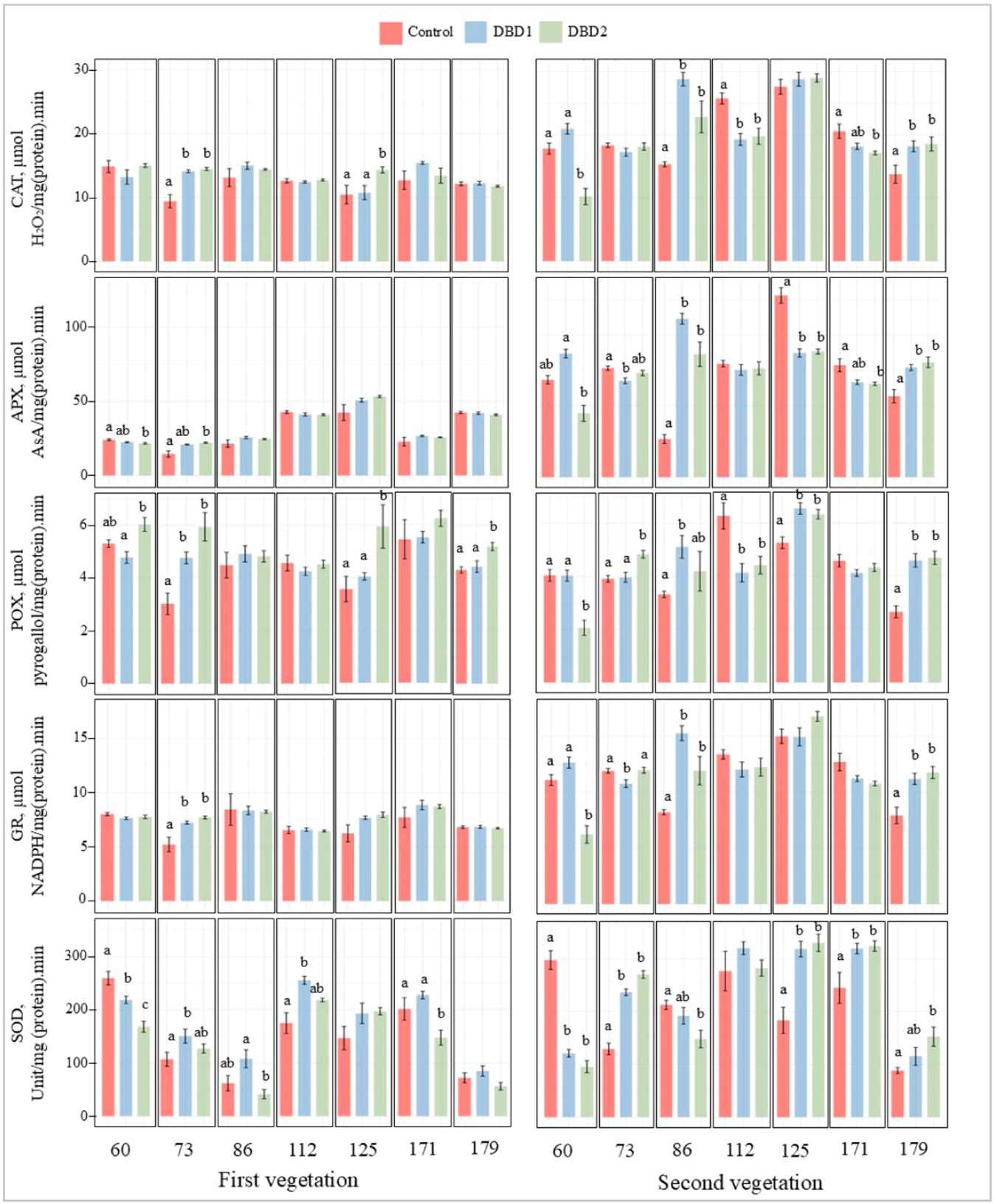

Fig. 3. . Antioxidant enzyme (CAT, APX, POX, GR, and SOD) activity in silver birch seedling leaves from different half-sib families in samples collected during the first and second vegetation season. Different letters above the columns indicate statistically significant differences between means ($p < 0.05$), as determined by the Kruskal–Wallis test followed by Dunn's test. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min.; DBD2 – seed treatment with DBD plasma 2 min.

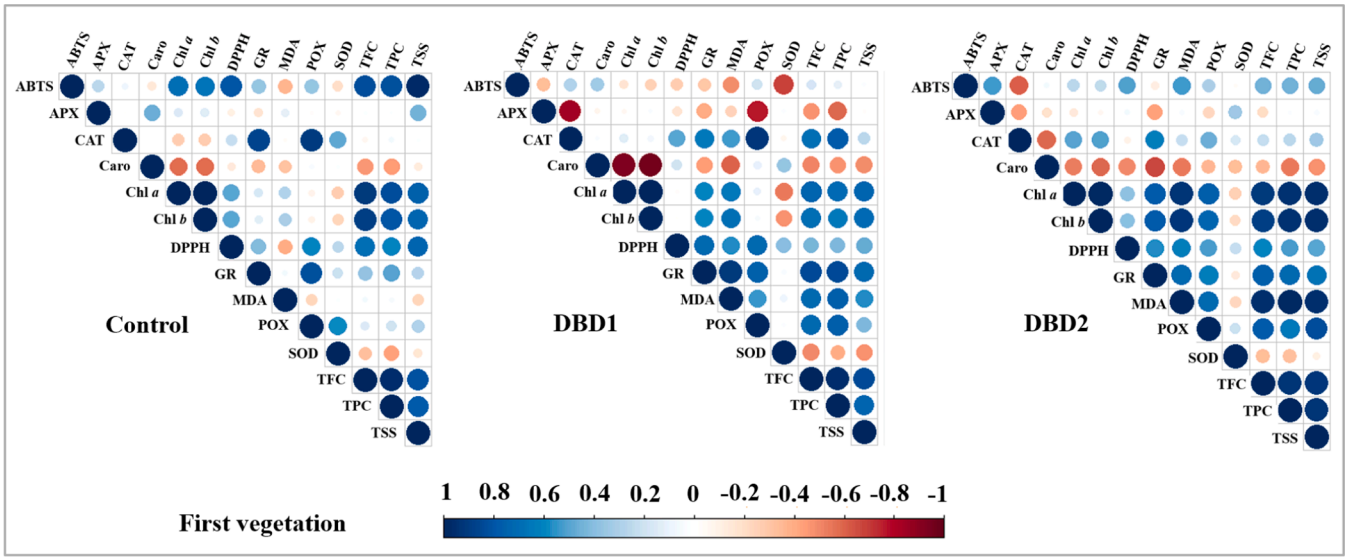

Fig. 4. Correlation of variables measured during the first growing season of silver birch seedlings, using the Pearson correlation coefficient. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min. DBD2 – seed treatment with DBD plasma 2 min. Larger bubbles indicate higher correlation values.

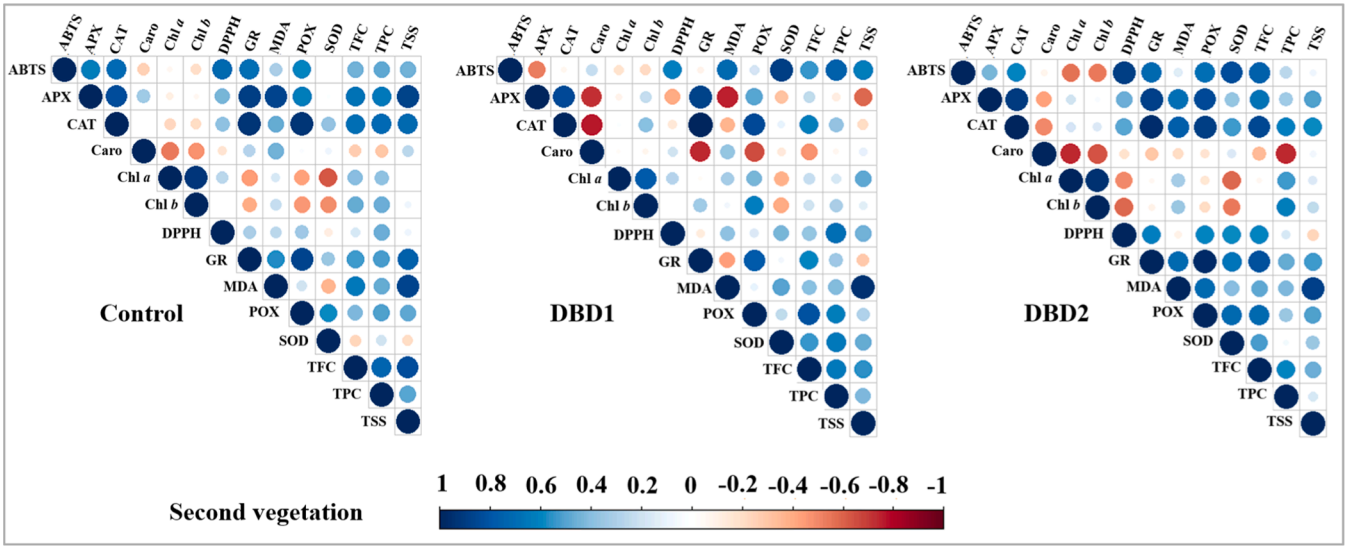

Fig. 5. Correlation of variables measured during the second growing season of silver birch seedlings, using the Pearson correlation coefficient. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min. DBD2 – seed treatment with DBD plasma 2 min. Larger bubbles indicate higher correlation values.

0.75) was determined between secondary metabolites and antioxidant enzymes (CAT, GR, and POX).

The most noticeable changes between control and treated samples were observed in the DBD2-treated group. In these samples, amounts of carotenoids negatively correlated (r = –0.50) with all other analyzed parameters. Conversely, chlorophylls showed a positive correlation (r = 0.80) with most other parameters, except SOD. Furthermore, positive correlations (r = 0.72) were found between secondary metabolites, TSS, and the GR and POX enzymes.

A positive correlation (r = 0.45) was observed in the control second vegetation samples between chlorophyll *a* and *b* and secondary metabolites (TPC and TFC) (Fig. 5). Additionally, secondary metabolites showed a positive correlation (r = 0.50) with the activity of all antioxidant enzymes, except SOD. Interestingly, SOD activity in the control samples was negatively correlated (r = – 0.40) with almost all other analyzed parameters.

In contrast, carotenoid content in control samples exhibited a negative correlation with chlorophylls and secondary metabolites. In DBD1-treated samples, a strong positive correlation (r = 0.95) was found between secondary metabolites and the activity of POX and SOD. Conversely, amounts of carotenoids showed a negative correlation (r = – 0.60) with the antioxidant enzymes APX, CAT, GR, and POX.

In DBD2-treated samples, carotenoid amounts strongly negatively correlated (r = – 0.45) with all analyzed parameters. Notably, unlike in the control samples, SOD activity in DBD2-treated seedlings positively correlated with nearly all other parameters. Also, secondary metabolites demonstrated a positive correlation (r = 0.60) with all antioxidant enzymes.

3.2. *Diversity of fungal communities associated with silver birch leaves*

The analysis of microbiota was performed to evaluate changes in fungal diversity in two-year- old silver birch seedlings following pre-sowing seed treatment with DBD plasma. When all treatments were taken together, there were 82 fungal OTUs, of which 17 OTUs were unidentified. 50 OTUs belonged to control samples, 35 OTUs to DBD1-

treated samples, and 50 OTUs to DBD2-treated samples (Fig. 6 and **Supplementary Table 2**). Metabarcoding analysis showed that the fungal diversity and relative abundance were altered in samples treated with DBD plasma. Among all fungal OTUs found in silver birch leaves, 20 OTUs were exclusively found in the control leaves, 8 OTUs in DBD1-treated leaves, and 20 OTUs in DBD2-treated leaves. In total, 19 fungal OTUs were shared among all samples (Fig. 6).

DBD treatment did not have an impact on fungal diversity and showed insignificant variations ($p > 0.05$) in Shannon's index, which was 1.61 in control samples, 1.87 in DBD1-treated samples, and 1.94 in DBD2-treated samples **(Supplementary Table 3)**. The similarity index (Sørensen's) of fungal communities was moderate (from 0.53 to 0.54) between the control and DBD1 or DBD-2 treatment.

Pathogenic fungi were the most abundant, and their relative abundance was higher (86.6 %) in control samples compared to DBD1- and DBD2-treated samples (79.9 % and 77.2 %, respectively) ($p > 0.05$). Also, the relative abundance of saprotrophs and endophytes in both (DBD1 and DBD2) treated samples had a trend to be higher than in control samples (varied from 6.9 % to 7.7 %, while in control samples, it was 4.1 %) (Fig. 7).

All identified fungi with their relative abundance (%) are presented in **Supplementary Table 2**. The ten most common fungal taxa in silver birch leaves represented 88.2–92.5 % of all high-quality fungal sequences in seedling leaves growing from seeds treated with DBD plasma (Table 2). The most abundant fungi in all leaf samples were *Phyllactinia betulae* (58.3 %), *Aureobasidium pullulans* (10.5 %), *and Cladosporium cladosporioides* (7.5 %). NMDS analysis (Stress = 0.13) revealed substantial overlap in the distribution of fungal OTUs across the treatments (**Supplementary Figure 2**). While some visual clustering was observed, the overlap among treatments suggests limited treatment-specific structuring. This was supported by a PERMANOVA analysis, which indicated no significant differences in fungal community composition among treatments ($R^2 = 0.083$, $F = 0.82$, $p = 0.59$), implying that most of the observed variation was not attributable to treatment effects. However, it was observed that both DBD1- and DBD2-treated samples had a trend to reduce the relative abundance of the most common pathogen, *Phyllactinia betulae,* in the birch leaves ($p > 0.05$). Also, the relative abundance of *Melampsoridium botulinum* and *Taphrina carpini* in DBD2-treated samples was lower compared to the control ($p > 0.05$). However, the relative abundance of *Taphrina betulina* and *Alternaria angustiovoidea* pathogens was increased (Table 2). The relative abundance of saprotrophs (*Aureobasidium pullulans* and *Cladosporium cladosporioides)* in DBD1-treated samples showed a trend to increase (increment by 15 % and 43 %, respectively).

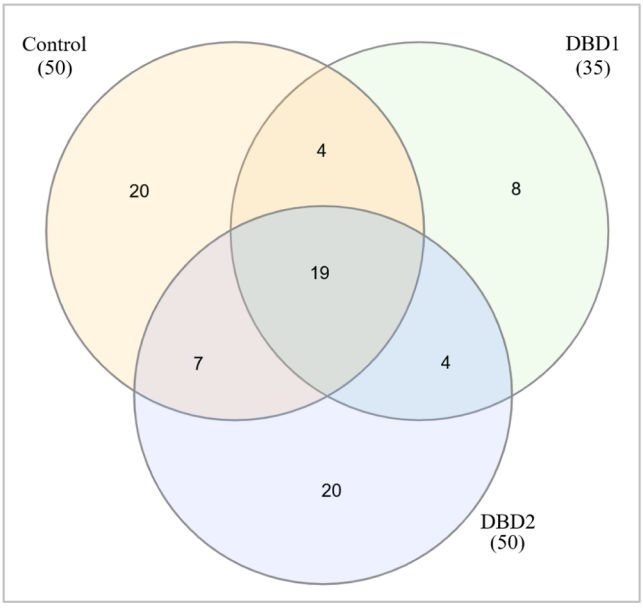

Fig. 6. Venn diagram showing the diversity and overlap of fungal OTUs in silver birch leaves. Control – untreated samples; DBD1 – seed treatment with DBD plasma for 1 min, and DBD2 – seed treatment with DBD plasma 2 min. Different tree half-sib families are combined.

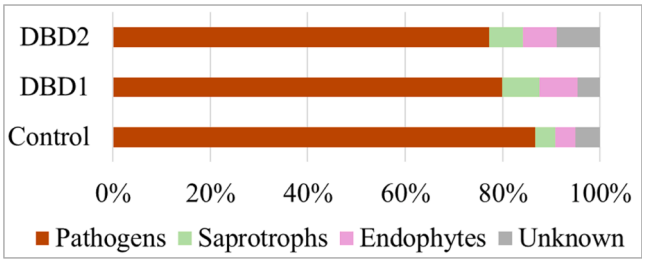

Fig. 7. Relative abundance (%) of functional groups in silver birch leaves, after seed treatment with DBD plasma. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min.; DBD2 – seed treatment with DBD plasma 2 min. Different tree half-sib families are combined.

Based on the family, the relative abundance of pathogens was reduced in DBD-treated samples of the 171 half-sib family (**Supplementary Table 4**). Additionally, the relative abundance of saprotrophs was increased by DBD treatment. Similarly, in the 60 and 112 families, the abundance of pathogens decreased, and the abundance of saprotrophs increased in both DBD1- and DBD2-treated samples. Four half-sib families (60, 86, 125, and 171) had a trend to reduce *Phyllactinia betulae* in DBD2-treated samples ($p > 0.05$).

3.3. Correlation between fungal communities and biochemical compounds

Correlation patterns between fungal functional groups and biochemical parameters from the second growing season are shown in Fig. 8. For endophytes, correlations were consistently negative in the control (DPPH r = –0.75; TPC r = –0.58), but TPC shifted to positive under DBD1 (TPC, r = 0.62). The effect was strongest in DBD2-treated samples, where endophytes showed highly significant positive associations, particularly with TPC (r = 0.86). Mycorrhizal fungi also exhibited treatment-specific shifts. In the control, they were negatively associated with antioxidants enzymes such as POX (r = –0.66) and SOD (r = –0.61). The negative correlations were determined in DBD1-treated samples (e.g., GR r = –0.58; CAT r = –0.51), but in DBD2-treated samples, a strong positive correlation with carotenoids was detected (r = 0.77). Pathogens were most strongly correlated with antioxidant enzymes in the control, where they were highly positively associated with SOD (r = 0.91) and POX (r = 0.74). These correlations weakened and shifted under DBD1 treatment, where only moderate correlations remained (SOD, r = 0.60). Under DBD2 treatment, pathogens were negatively correlated with Chl *b* (r = –0.72) but positively correlated with DPPH (r = 0.56). Saprotrophic fungi were negatively correlated with SOD (r = –0.65) and DPPH (r = –0.62) in the control samples. In the DBD1 treatment, they positively correlated with antioxidant enzymes (APX r = 0.71; GR r = 0.65). In DBD2-treated samples, the strongest response was observed, with saprotrophs highly positively correlated with Chl *b* (r = 0.81) but negatively correlated with DPPH (r = –0.75).

4. Discussion

An experimental study investigated how silver birch pre-sowing seed treatment with DBD plasma can change the contents of biochemical compounds, antioxidant activity, and fungal diversity in tree leaves. Our hypothesis that DBD plasma can increase the accumulation of biochemical compounds was based on literature studies, which showed the beneficial treatment effects on the growth and secondary metabolite profiles of *Echinacea purpurea* (Mildaziene et al., 2017), *Trifolium*

Table 2
Relative abundance of the 10 most abundant fungal OTU in silver birch leaves. C – control (untreated samples); DBD1 – seed treatment with DBD plasma for 1 min.; DBD2 – seed treatment with DBD plasma for 2 min. All analyzed half-sib families (seven) are combined.

Phylum	Fungal OTU	Trophic Group	Genbank Reference	Similarly, bp (%)	Relative Abundance, %			
					C	DBD1	DBD2	All
Ascomycota	*Phyllactinia betulae*	Pathogen	ON073890	271/272 (99)	63.9	53.5	54.1	58.3
Ascomycota	*Aureobasidium pullulans*	Saprotroph	OP704223	249/249 (100)	10.7	12.3	9.1	10.5
Ascomycota	*Cladosporium cladosporioides*	Saprotroph	OP963820	243/243 (100)	6.3	9.0	8.1	7.5
Ascomycota	*Taphrina betulina*	Pathogen	MN540705	293/293 (100)	2.4	5.7	6.7	4.6
Ascomycota	*Unidentified sp. 5636_79*	Unknown	KP892076	304/304 (100)	1.7	1.2	4.6	2.6
Basidiomycota	*Melampsoridium betulinum*	Pathogen	MH908487	313/313 (100)	1.2	0.8	0.4	0.8
Ascomycota	*Taphrina carpini*	Pathogen	OQ066577	293/293 (100)	2.7	3.1	1.3	2.3
Ascomycota	*Alternaria angustiovoidea*	Pathogen	OQ066859	253/253 (100)	1.0	1.8	1.4	1.3
Ascomycota	*Pezizomycotina sp. 5636_180*	Endophyte	KJ827240	239/239(100)	1.5	0.4	1.8	1.4
Ascomycota	*Venturia ditricha*	Endophyte	MH855707	246/246 (100)	0.9	1.4	0.9	1.0
				Total	92.5	89.1	88.2	90.3

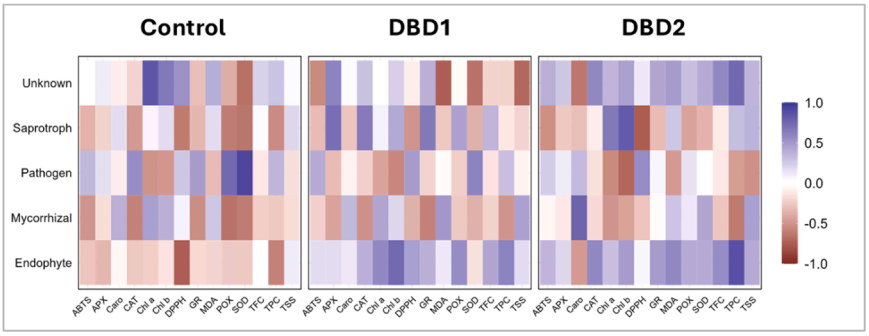

Fig. 8. Correlation between fungal functional groups and biochemical compounds measured during the second growing season of silver birch seedlings, using the Pearson correlation coefficient. Control – untreated samples; DBD1 – seed treatment with DBD plasma 1 min. DBD2 – seed treatment with DBD plasma 2 min.

pratense (Mildaziene et al., 2020), and *Cannabis sativa* (Ivankov et al., 2020). However, there is a literature gap regarding the analysis of biochemical compounds and microbiome changes in tree tissues induced by DBD. In the current study, silver birch seed treatment with DBD plasma was examined on leaf samples at multiple levels, including biochemical, metagenomic, and genetic aspects.

In previous studies, the application of DBD plasma was explored to enhance the germination of seeds in cotton (Groot et al., 2018). Increased seed germination and growth were determined in soybean (Ling et al., 2014) and red clover flora (Mildaziene et al., 2020). Seed treatment with DBD plasma in tomatoes affected plant growth regulation and modulated H_2O_2 levels, as well as the activities of peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase. Furthermore, in tomato seedlings, seed treatment with DBD plasma has been shown to effectively mitigate damage from cold, as evidenced by increased maximum photochemical efficiency of PSII and a lower chilling injury index (Li et al., 2021). It demonstrates the DBD plasma potential to enhance seedling growth and resistance to stress.

Studies on effects of tree seed treatment with DBD plasma are scarce, which may be primarily attributed to the longer lifespan of trees compared to annual agricultural plants. It has been demonstrated that the response of Norway spruce to seed irradiation with DBD plasma (effects in germination and early seedlings growth) varies across tree genetic properties (Sirgedaitė-Šėžienė et al., 2021). In addition, seed treatment with low-pressure plasma for two to seven minutes revealed that only the duration of five minutes significantly inhibited the seedling emergence of Norway spruce (Pauzaite et al., 2018). Moreover, our previous studies on Norway spruce confirmed that pre-sowing seed treatment with DBD plasma can enhance the content of biochemical compounds and antioxidant enzymes. However, the response was highly dependent on treatment duration and tree genetic properties (Česnienė et al., 2024, 2025).

In the present study, seed treatment with DBD plasma for 1 (DBD1) and 2 min. (DBD2) induced changes in secondary metabolites (TPC and TFC), and these changes were positively correlated with antioxidant enzymes, regardless of genetic differences in silver birch leaves. In earlier studies, a correlation between TPC and antioxidant activity has been observed in different genotypes of plants, such as red grape cultivars (Orak, 2007) and cornelian cherry (Hassanpour et al., 2011). Notable differences between the half-sib families were also revealed in our study. In DBD1-treated samples, the 73 family showed the most positive response, characterized by an increase in TFC and chlorophylls. Enhanced enzyme activities, specifically catalase (CAT), peroxidase (POX), glutathione reductase (GR), and superoxide dismutase (SOD), were also found, while other parameters remained essentially unchanged during the first vegetation season. Similarly, in DBD2-treated samples, the 73 half-sib family showed the most significant biochemical responses, including elevated TFC, enhanced content of photosynthetic pigments, and increased CAT, APX, POX, and GR enzyme activities. In contrast, in the second vegetation season, the most notable effects were observed in the 86 family, particularly after DBD2 treatment. This family exhibited higher TFC concentrations, as well as higher levels of photosynthetic pigments and sugars. It also showed an increased activity of CAT, APX, and GR. Antioxidant enzymes support the preservation of photosynthetic pigments by neutralizing ROS, thus protecting the photosynthetic apparatus and maintaining overall leaf function, especially under stress (Sherin et al., 2022). Conversely, changes in pigment levels can signal oxidative stress and the need for increased antioxidant activity. Our studies, along with those of other authors, confirm that biologically active compounds vary depending on the plant's genetic properties. In the current study, a more pronounced effect was evident in the DBD2-treated group, suggesting that a longer treatment duration may induce a more intense response to stress. This suggests that longer treatment durations may have a greater impact on the seed coat, as the initial reaction to the treatment occurs through the seed surface (Mildaziene et al., 2020). Studies with cotton seeds have

shown that plasma treatments, ranging from 3 to 27 minutes, affect the chemistry of the seed surface and impact water absorption, which can hinder further plant development (Wang et al., 2017).

We showed that DBD plasma as a seed treatment induced a reduction in MDA levels in certain birch families, and this decline was more evident in seedlings during their second year of growth. The decrease in MDA content, along with increased antioxidant enzyme activity, possibly indicates that the enhanced antioxidant defense system effectively mitigated oxidative stress-induced lipid peroxidation. This pattern was observed in our study across different tree genotypes, confirming that the genetic properties of the trees highly influence the response to seed treatment. For instance, in the DBD1-treated group, the 86 half-sib family exhibited increased activity of antioxidant enzymes, accompanied by a reduction in MDA levels. Similarly, in the DBD2-treated group, the 179 family showed a similar response, with enhanced antioxidant enzyme activity and decreased MDA content. This observation partially confirms Paužaitė et al. (2018) study, where seed treatment with DBD plasma modulates the production of hydrogen peroxide in Norway spruce seeds. Similarly, authors demonstrated that DBD plasma-induced hydrogen peroxide in tomato seeds acted as a signaling molecule, influencing gene expression associated with elevated downstream abscisic acid biosynthesis, thereby supporting enhanced stress response mechanisms (Li et al., 2021).

Plants produce a diverse array of secondary metabolites (SMs) with complex chemical structures, which play a crucial role in mitigating the effects of both biotic and abiotic stresses by serving as protective responses to various environmental challenges (Isah, 2019). In the current study, the identified SMs in two-year-old silver birch leaves were chlorogenic acid, catechin, coumarin acid, 3-caffeoylquinic acid, myricetin 3-O-glucoside, eriodictyol 7-O–D-glucoside, isoquercetin, and quercetin 3-O-arabinoside. They contribute to structural reinforcement of cell walls, which helps to deter pathogen or insect invasion, providing a biochemical barrier against biotic stressors. Their involvement in signaling pathways further supports the activation of stress-responsive genes, enhancing the plant's resilience (Kundu & Vadassery, 2019; Ortiz & Sansinenea, 2023). Our study revealed that the prevalence of these SMs, particularly flavonoids, influences the increase in other resistance indicators, including antioxidant enzymes and photosynthetic pigments. These SMs are closely related to functional status of photosynthetic pigments and may enhance the enzymatic antioxidant response, and maintain the structural and functional integrity of the photosynthetic system. Their accumulation is both a marker and a mechanism of enhanced stress tolerance in plants (Foyer and Shigeoka, 2011; Lingwan et al., 2023).

We observed that in most cases, the total antioxidant capacity (TAC) was not significantly affected in the DBD treatment groups. Meanwhile, the activity of antioxidant enzymes was noticeably increased in a few half-sib families. The decrease in DPPH and ABTS activity likely reflects a functional shift from non-enzymatic to enzymatic antioxidant strategies, a typical and adaptive response in plants (Gulcin, 2025). It indicates that the plant may be maintaining redox homeostasis more efficiently through enzymatic detoxification, while SM levels used in radical scavenging assays may temporarily decline due to utilization or regulatory adjustments.

Shifts in microbial communities associated with plants can influence their health, disease resistance, and resilience, as the altered microbial structure may affect nutrient uptake in plants. Our results revealed that fungal communities can correlate with secondary metabolites and antioxidant enzyme activities after seed treatment with DBD plasma. Specifically, we found that the abundance of endophytic fungi in birch leaves was positively associated with both antioxidant enzymes and phenolic compounds following plasma treatment. Similar patterns have been reported in previous studies, where endophytic fungi enhanced basal drought tolerance in *Moringa oleifera* by upregulating the APX antioxidant enzyme (Javed et al., 2022). Likewise, the interaction between leaf fungal composition and phenolic compounds has been linked to plant stress responses, such as olive leaf spot disease in olive trees (Gomes et al., 2023). In sunflower (*Helianthus annuus*), DBD plasma irradiation modified the structure of plant-associated bacterial assemblies, leading to the dominance of spore-forming *Mycobacterium* species in above-ground tissues. This shift in microbial composition was correlated with stimulated root and lateral organ growth, suggesting that DBD plasma-induced microbiome alterations contribute to enhanced plant development (Tamošiūnė et al., 2020). Our study showed that in DBD-treated samples, fungal diversity in silver birch leaves exhibited a tendency to shift. Although the overall fungal composition did not differ significantly between treatments, our study revealed a reduced relative abundance of pathogenic fungi in samples treated with DBD plasma. Notably, the most prevalent pathogen, *Phyllactinia betulae*, exhibited a consistent trend of reduced relative abundance across both DBD treatment groups. Furthermore, our findings confirm that in studies of this nature, it is essential to consider tree genetics, which is a relevant factor for fungal communities. In particular, the 86 half-sib family, which showed enhanced biochemical responses in DBD2-treated samples during the second vegetation season, also exhibited a notable reduction in *Phyllactinia betulae* relative abundance in birch leaves. Moreover, the DBD treatment and the inherent genetic variability may modulate the presence of pathogens, potentially acting synergistically to enhance overall plant health.

Studies that analyse the microorganism's diversity in the seedlings after seed treatment with plasma are rare. However, seed treatment with DBD plasma can be used as a primary disinfection from seed infection by microorganisms, and to activate protective biological processes in plants (Mukherjee et al., 2025; Sirgedaitė-Šėžienė et al., 2021). Numerous studies reported that seed treatment with plasma can reduce the occurrence of seed-borne pathogen diseases (Mildaziene et al., 2022; Adhikari, et al., 2020b). The conducted investigations (Ji et al., 2015; Mildaziene et al., 2022; Ivankov et al., 2020; Tamošiūnė et al., 2020) have substantiated that both DBD and low-pressure plasma treatment possess the capability to instigate alterations in the microbiome associated with plants. However, all these studies analyzed agricultural plants. These changes have the potential to act as mediators for secondary impacts on both plant physiology and the broader agroecosystem environment (Mildaziene et al., 2022). There are currently no other studies on changes in the microbiome of perennial plants caused by seed treatment with DBD plasma. Therefore, our research provides pilot valuable insights into fungal community shifts in second-year birch seedlings, particularly in relation to the trees' genetic characteristics. These findings lay the groundwork for further exploration of plant-microbiome interactions in young seedlings. The first year of vegetation is crucial for tree seedling establishment, therefore, the selection of genetic families that tend to respond to seed priming with plasma by coordinated enhancement of their protective molecular systems may be of importance for the development of innovative forestry technologies.

5. Conclusion

This study shows the significance of inter-species genetic variation in shaping the effectiveness of seed treatments with DBD plasma, as evidenced by the distinct responses among half-sib families. Our findings confirm the evidence on the potential of DBD plasma to enhance the accumulation of bioactive compounds and reduce the relative abundance of fungal pathogens, thereby improving physiological traits in silver birch seedlings. Genotype-specific effects underline the importance of genetic selection for optimizing DBD plasma use in forestry. In this study, the families 73 and 86 demonstrated the most distinct responses to seed treatment with DBD plasma, with notable increases in most analyzed biochemical indicators. Additionally, the family 86 had a tendency to reduce the relative abundance of the most prevalent pathogen in birch seedlings. These families are therefore suitable candidates for further investigations, such as direct pathogen inoculation and gene

expression analysis to gain deeper scientific knowledge on tree defense mechanisms and associated microbial communities.

CRediT authorship contribution statement

Ieva Česnienė: Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vytautas Česna:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Vida Mildažienė:** Writing – review & editing, Resources, Methodology. **Diana Miškelytė:** Writing – review & editing, Resources, Methodology, Investigation. **Liudas Ivanauskas:** Resources, Methodology. **Mindaugas Marksa:** Resources, Methodology, Investigation. **Audrius Menkis:** Writing – review & editing, Methodology. **Kazunori Koga:** Resources, Methodology. **Masaharu Shiratani:** Resources, Methodology. **Vaida Sirgedaitė-Šėžienė:** Supervision, Resources, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.101077.

Data availability

Data will be made available on request.

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