The effects of different combinations of simulated climate change-related stressors on juveniles of seven forest tree species grown as mono-species and mixed cultures

ALFAS PLIŪRA1,2*, GINTARĖ BAJERKEVIČIENĖ1, JUOZAS LABOKAS3, VAIDOTAS LYGIS3,4, JURGA JANKAUSKIENĖ3, VYTAUTAS SUCHOCKAS1,2 AND RITA VERBYLAITĖ1

1 Institute of Forestry, Lithuanian Research Centre for Agriculture and Forestry, Liepy str. 1, LT-53310 Giriūnys, Kaunas reg., Lithuania
2 Vytautas Magnus University Agriculture Academy, Studentų str. 13, LT-53361 Akademija – Kaunas, Lithuania
3 Institute of Botany of Nature Research Centre, Akademijos str. 2, LT-08412 Vilnius, Lithuania
4 Kaunas Forestry and Environmental Engineering University of Applied Sciences, Liepy str. 1, LT-53310 Giriūnys, Kaunas reg., Lithuania

* Corresponding author: alfas.pliura@lammc.lt; phone: +37068267811

The aim of the study was to assess changes in performance and competition for light of juveniles of seven forest tree species, Pinus sylvestris, Picea abies, Betula pendula, Alnus glutinosa, Populus tremula, Quercus robur and Fraxinus excelsior, grown in mono-species and mixed cultures with isolated potted roots under the impact of different combinations of climate change-related stressors, simulated in a phytotron under the elevated CO₂ concentration during one growing season, as follows: i) heat + elevated humidity (HW); ii) heat + frost + drought (HFD); iii) heat + elevated humidity + increased UV-B radiation doses + elevated ozone concentration (HWUO); and iv) heat + frost + drought + increased UV-B radiation doses + elevated ozone concentration (HFDUO). For the mixed cultures, three typical species’ mixtures were used: i) P. sylvestris, B. pendula and P. abies, ii) P. abies, B. pendula and Q. robur and iii) F. excelsior, A. glutinosa and P. tremula. For the control, the same material was grown outside the phytotron in ambient conditions.

Analysis of variance (ANOVA) revealed that the effects of the complex treatments, species and species by treatment interactions were highly significant in most of the biomass, growth, physiological and biochemical traits studied. Pattern of species culture had highly significant effect on physiological and biochemical traits (except for H₂O₂ concentration); meanwhile it was of low significance for biomass and growth traits. Pattern of species culture by treatment interaction was highly significant in all traits, suggesting that the effects of the applied complex treatments vary depending on the pattern of species culture.

Under the hot wet conditions the highest stem volume index, tree biomass, and growth were observed in deciduous P. tremula, A. glutinosa and B. pendula with more clearly pronounced differences in performance between different patterns of species cultures than in ambient conditions showing that the enhanced growth conditions facilitate revealing the potential and specific requirements of the fast-growers. P. abies in all treatments had lower stem volume index and tree biomass than in ambient conditions with no significant differences between the patterns of species culture, indicating that it suffered irrespectively of light availability in different cultures. The differences between performances of most tree species in mono- and mixed cultures in HFD treatment were rather small and nonsignificant. A complex HWUO treatment caused further reduction in tree biomass in all species and culture patterns except for mono-species cultures of A. glutinosa and B. pendula. The most complex HFDUO treatment had the strongest negative effect on biomass of almost all tree species compared to that observed in HW treatment, except for Q. robur and P. sylvestris which biomass and height increments remained higher than those in ambient conditions. This was due to relatively high drought tolerance and compensatory effects of the increased CO₂ concentration and temperature. Physiological and biochemical responses of species in different patterns of species cultures across treatments were very variable although often did not reflect in the effects on growth and biomass traits. The observed changes in performance of different tree species in different patterns of species cultures under various complex treatments allowed inferring that climate change may condition certain changes in competitiveness of some tree species resulting in atypical ecological successions of species and forest ecosystems.

Keywords: trees, complex treatments, controlled environment, competition, biomass, growth, physiology, biochemistry, phytotron
Introduction

It has been observed that in the course of climate change, an increase of air temperature and alterations in rainfall patterns result in more frequent periods of drought and temperature extremes (e.g., Schlyter et al. 2006, Bhardwaja and Sawant 2013, Shah 2015). It is expected that the increase of temperature in temperate and boreal regions will have positive effects on forest growth (e.g., Reyer et al. 2014). However, the temperature extremes may negatively affect plant growth and development (Wahid et al. 2007). High temperatures and drought stress alter physiological and biochemical responses of plants, affect plant morphology, leaf and wood anatomy, and susceptibility to diseases (Wahid et al. 2007, Chakraborty et al. 2011, Song et al. 2014, Topić et al. 2013, Stojnić et al. 2016). Late spring frosts are also predicted to become more frequent and occur during prolonged period of time (e.g., Schlyter et al. 2006). The probability of occurrence of late spring frosts increases in forest clear-cut sites where temperature fluctuations are more pronounced. This may result in an increase of spring frost damages to tree seedlings and even mature trees, thus reducing foliage area and photosynthetic capacity, weakening of trees and making them more susceptible to other stressors (e.g., Kramer et al. 2000).

Studies showed that elevated CO₂ concentrations increase net photosynthesis and concentration of carbohydrates in plant cells, thus enhancing growth and biomass accumulation (Pettersson and McDonald 1992, Morison and Lawlor 1999, Long et al. 2004). Other studies report a negative impact of exposure to elevated CO₂ concentrations on photosynthetic rate in some plant species (e.g., Sage et al. 1989; Bunce 1992).


Climate change-related factors such as temperature extremes, drought, elevated UV radiation, ozone and CO₂ concentrations, etc., may act interactively, in additive or synergistic manner (e.g., Kull et al. 1996, Pliūra et al. 2008, Yamaguchi et al. 2011), thus complicating investigations on their impacts. Elevated CO₂ concentrations and prolonged drought are well known to affect sensitivity of forest tree species to ozone (e.g., Kull et al. 1996, Karnowsky et al. 1998, EPA 2006b). Meanwhile, other studies show that increased concentrations of CO₂ can reduce or even neutralise the negative impact of ozone on photosynthesis and growth of some tree species (e.g., Dickson et al. 1998, Matsumura et al. 2005). Moreover, Watanabe et al. (2010) showed that for certain tree species simultaneous exposure to elevated concentrations of ozone and CO₂ can even induce growth stimulation. Water stress can also counteract the negative effect of ozone (Watanabe et al. 2005).

There are widespread concerns that trees, as long-lived organisms, may not be able to cope with such rapid and complex changes of climate and environment. Increasing frequency and amplitude of abiotic and biotic disturbances may compromise the sustainability of forest ecosystems, forest regeneration and persistence of natural ecological successions in forest ecosystems. A high pressure by climate change-related stressors may alter plant physiology and growth and intensify natural selection, which may result in reduction of genetic diversity and, subsequently, decline in adaptability potential of tree populations (Koskela and Lefèvre 2013). Multiple studies have reported large variations in plasticity and responses of forest tree species to changing environmental conditions or impact by various single or few climate change-related stressors (e.g., Skre 1991, Weiser 1995, Kleinschmit et al. 1996, Baluckas 2002, Pliūra and Eriksson 2002, Pliūra and Kundrotas 2002, Baluckas and Pliūra 2003, Lauteri et al. 2004, Eriksson et al. 2005, Pliūra et al. 2008, 2016, Petrokas and Pliūra 2014, etc.). Tree species differ from each other by a complex of biological and ecological traits, such as succession stage in an ecosystem, character of pollination and seed dispersal, social status in an ecosystem, distribution patterns in a natural range, size and structure of populations, particularities of post-glacial migration, etc. (Hamrick and Godt 1989). All these may have different impact on species plasticity and response to different climatic conditions and impact of climate change-related stressors. Broadleaves differ from conifers in the lifespan of leaves and their phylogenetic histories that underlie differences in other phenotypic features such as leaf structure, crown architecture and wood composition which translate into different adaptation strategies (Wyka et al. 2012). It was found that shade tolerance is negatively related to drought and waterlogging tolerances in temperate northern hemisphere trees and shrubs supporting the existence of functional trade-offs in adjusting to multiple environmental limitations (Abrams 1994, Niinemets and Vallarde 2006). Following the climate change scenarios, the temperate deciduous forests are predicted to shift northwards, where they are likely to replace mixed forests (Bachelet et al. 2001). Lithuania is located in the transition zone between temperate and hemiboreal forest vegetation zones (between continental and boreal biogeographical regions of Europe, European Environment Agency 2003),
thus, shifting of species’ climate envelope northwards may have a serious impact on species composition and stability of local forest ecosystems as well as significantly change the landscape.

Despite of extensive research efforts on forest tree tolerance to the primary abiotic stresses there is still a lack of knowledge on tolerance to simultaneous stresses despite the ubiquitous coexistence of multiple stresses in nature (Niinemets and Valladares 2006). Therefore, it is hard to predict, how different tree species will respond to rapidly changing environment and co-acting of multiple extreme climate-change-related stressors, and how this will change the performance and competitiveness of different tree species. Moreover, it is well known that inter- and intra-specific competition between plants for external resources is a critical process for plant growth in natural and managed ecosystems (e.g., Gayler et al. 2006). Based on model simulations, for some tree species a large influence of initial environmental conditions and stand density on the competition between the species was postulated (Gayler et al. 2006). As tree species of different biological and ecological features have different strategies and limits to cope with stressful environment, we hypothesize that impact of simultaneously acting stressors on tree physiology, biochemistry and growth may be influenced by inter- and intra-specific competition between trees for external resources. Therefore, the aim of the present study was to evaluate changes in performance and competition for light of juveniles of seven forest tree species, *Pinus sylvestris*, *Picea abies*, *Betula pendula*, *Alnus glutinosa*, *Populus tremula*, *Quercus robur* and *Fraxinus excelsior* grown as mono-species and mixed cultures with isolated potted juveniles of seven forest tree species, each species was represented by the progenies from three populations native to three respective ecoclimatic regions of Lithuania and one (*Fraxinus excelsior*) from the North East Poland (Table 1). Prior to the experiment, the seedlings were grown in an open-air nursery. In spring 2016, 2- and 3-year-old saplings of each tree species were transplanted into the squared plastic pots (15 × 15 × 20 cm) filled with 3.5 l of peat soil (Klasmann KTS-1, pH 5.5–6.0, N – 140 mg l⁻¹, P₂O₅ – 160 mg l⁻¹, K₂O – 18 mg l⁻¹, Mg – 85 mg l⁻¹, Klasmann-Deilmann, Lithuania). In spring 2017, pots with saplings were transferred into a phytotron (Institute of Forestry of Lithuanian Research Centre for Agriculture and Forestry, Central Lithuania, 55°52’ N 24°07’ E) and placed on irrigation tables with 30 pots m⁻².

**Treatments**

The experiment was carried out in four chambers of the phytotron. The area of each chamber covered 60 m²; and the interior volume was 240 m³. To study the effect of interaction in competition for light without competition between roots among different tree species, two types of culture blocks were established in each chamber: i) pure (mono-species) and ii) mixed (multi-species) cultures. Mono-species blocks of each of seven tree species were placed on two irrigation tables, and each block contained three subblocks with 27 potted saplings of each population arranged in three rows (nine plants per row). Three patterns of species mixtures typical for natural stands were established in every chamber on two additional irrigation tables: i) *P. sylvestris*, *B. pendula* and *P. abies*, ii) *P. abies*, *B. pendula*, *A. glutinosa*, *P. tremula*, *Q. robur* and *F. excelsior*.

**Materials and methods**

**Tree species studied**

Performances of juveniles of seven major forest tree species, Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* (L.) Karst.), pendunculate oak (*Quercus robur* L.), European ash (*Fraxinus excelsior* L.), black alder (*Alnus glutinosa* (L.) Gaertn.), silver birch (*Betula pendula* Roth.) and common aspen (*Populus tremula* L.) were studied in the present work. Each species was represented by the progenies from three populations native to three respective ecoclimatic regions of Lithuania and one (*Fraxinus excelsior*) from the North East Poland (Table 1). Prior to the experiment, the seedlings were grown in an open-air nursery. In spring 2016, 2- and 3-year-old saplings of each tree species were transplanted into the squared plastic pots (15 × 15 × 20 cm) filled with 3.5 l of peat soil (Klasmann KTS-1, pH 5.5–6.0, N – 140 mg l⁻¹, P₂O₅ – 160 mg l⁻¹, K₂O – 18 mg l⁻¹, Mg – 85 mg l⁻¹, Klasmann-Deilmann, Lithuania). In spring 2017, pots with saplings were transferred into a phytotron (Institute of Forestry of Lithuanian Research Centre for Agriculture and Forestry, Central Lithuania, 55°52’ N 24°07’ E) and placed on irrigation tables with 30 pots m⁻².

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ies, B. pendula and Q. robur, iii) F. excelsior, A. glutinosa and P. tremula. Each row in mixed cultures consisted of saplings of different tree species potted in plastic pots, thus with isolated roots; each species was represented by saplings from 3 populations in two rows each, 9 plants per row. In total, 5,670 saplings were tested in the experiment. For the evaluation of performance of each B. pendula and P. abies in mixed cultures, the first (i) and the second (ii) patterns of mixture were pooled together. This allowed making a simpler scheme of mixture patterns, i.e., one species in mono-culture and one species in mixed culture.

In a phytotron, four different complex treatments with different combinations of simulated climate change-related stressors, heat, drought, frost, elevated humidity, UV-B radiation and ozone concentration were applied:

i) **Combined heat and elevated humidity treatment (HW).** Air temperature in the phytotron during growth season was set to 7°C higher than ambient temperature, so it ranged between 25°C and 35°C during day and between 15 and 20°C at night. Air humidity was kept between 65 and 85% using automated fog sprinkle system. The plants were regularly watered by temporary (0.5 hour a day) flooding pots on irrigation tables to fully saturate soil and keep soil moisture at 80–95% of the full moisture capacity (FMC) throughout the experiment. Natural UV-B irradiance dose in phytotron at midday in clear days ranged between 0.7–1.2 kJ m$^{-2}$ h$^{-1}$. The HW treatment lasted for two months (July and August, 2017).

ii) **Combined heat, frost and drought treatment (HFD).** In May 2017, when all investigated tree species reached bud flushing phenology of about 3 pts, the saplings were temporarily transferred to a freezing chamber where temperature was gradually reduced from ambient to -5°C, fixed for 0.5 hour and then raised back to ambient. Each reduction and rise of temperature lasted for one hour. Then plants were transferred back to the phytotron chamber. Air temperature during growing season ranged between 25–35°C during day and between 15–20°C during night. In July, artificial drought conditions were created by reducing watering of trees for four weeks to keep pot soil moisture between 20 and 30% of FMC. To estimate soil water content and evapotranspiration, 15 potted saplings of each tree species (five saplings per population) in each treatment were weighted every second day. When water content for certain species fell down to 10% of FMC, a brief irrigation by pot bottom flooding was applied. Air humidity in the climatic chamber was set to range between 45 and 55%. After this drought treatment plants were watered to reach full water holding capacity of the soil.

iii) **Combined heat, elevated humidity, elevated UV-B radiation and ozone concentration treatment (HWUO).** All conditions were set the same as in HW treatment, except that in addition saplings were simultaneously treated with high UV-B dose (10 kJ m$^{-2}$ h$^{-1}$) and high ozone concentration (300 mg m$^{-3}$) (=150 ppb) for 5 hours a day at midday during a four-week period in July 2017.

iv) **Combined heat, frost, drought, elevated UV-B radiation and ozone concentration treatment (HFDUO).** All conditions were set the same as in HFD treatment, except that in addition saplings were simultaneously treated with high UV-B dose (10 kJ m$^{-2}$ h$^{-1}$) and high ozone concentration (300 mg m$^{-3}$) for 5 hours a day at midday during a four-week period in July 2017.

In the phytotron, concentration of CO$_2$ was set to 700 ppm, i.e., two times higher than that in ambient conditions (outside the phytotron). Light intensity (PAR 400–700 nm) at the tops of sapling crowns ranged between 1220–1370 μmol m$^{-2}$ s$^{-1}$ in clear days, while outside the phytotron it ranged between 1670–2090 μmol m$^{-2}$ s$^{-1}$. Saplings were fertilized periodically based on monitored indications of electric conductivity of soil to keep it at about 2.0 mS cm$^{-1}$. Fertilization was done by dissolving fertilizers in the water used for irrigating plants by flooding from below: N − 190 mg l$^{-1}$, P$_2$O$_5$ − 44 mg l$^{-1}$, K$_2$O − 202 mg l$^{-1}$, Mg − 85 mg l$^{-1}$ and microelements.

For the control, the same set of species, populations and their mixtures as in each of the four treatments were grown in ambient conditions outside the phytotron. In 2017, when the experiment was carried out, there were no natural droughts or heat waves at the experiment site. Thus, the control treatment in general corresponded to ambient climatic conditions almost unaffected by climate change-related stressors. Outside air temperature in June and July ranged between 18–28°C during day and between 13–18°C during night, and day and night air humidity ranged between 40–60% and 70–90%, respectively. The control saplings were watered when necessary throughout the experiment to maintain soil moisture in pots between 70–90% of FMC. Ambient concentration of ozone ranged between 50–60 mg m$^{-3}$ (=25–30 ppb). Ambient UV-B irradiance dose at midday in clear days ranged between 1.6 and 7.0 kJ m$^{-2}$ h$^{-1}$. The total duration of the control treatment was four months (from May to August 2017).

Ozone was generated using the SR-16 Ozone generator (Ozone Solutions, Inc., USA) and supplied to each phytotron chamber by tubes. Ozone concentrations were monitored using Portable Ozone Monitor OS-4 (Ozone Solutions, Inc., USA) connected to a computer. Lamps TL 40W/12 RS UV-B Medical (Philips Lighting), mounted 2.5 m above the potted trees, served as UV-B light source. The intensity and doses of UV-B light were measured using radiometer HD2302.0 LightMeter with LP 471 UVB probe (Delta OHM). The CO$_2$ was supplied to the phytotron chambers from liquid CO$_2$ pressurized tanks by a network of tubes. CO$_2$ concentrations were monitored using CO$_2$ transmitter CO112-AOS (KIMO Instruments, France) connected to a computer.

In each phytotron chamber, climatic conditions, irrigation and fertilization were monitored and controlled automatically using Eco-Innovation Climate System and Climagro 3.0 software (Incoa Sistemas S.L., Spain).
Assessment of sapling growth and physiological parameters

The height and stem diameter at a root collar were measured twice for each sapling: before and by the end of the growing season. Height and diameter increments were determined for each sapling as differences of the two consecutive measurements. Stem volume index of each sapling was estimated as product of tree height and squared tree diameter (SVI = H × D² / 4). Stem and root biomass were estimated as weight of absolutely dry stems and roots of randomly chosen 21 saplings of every tree species in each mono-species and mixed cultures block in every treatment. For this, saplings were removed from pots at the end of growing season, roots were washed with water and saplings dried in an ambient room temperature for 2 weeks and then moved to a drying oven and dried to constant weight for 48 hours at 80 °C and weighted immediately after removing from oven.

Sapling health condition was scored immediately after freezing tests and in the autumn on a categorical scale ranging from 5 (excellent condition, no visible damages) to 1 (very bad condition, dying plant). Survival was assessed in the autumn as a ratio between the number of survived trees and the total number of living trees before the treatments. Timing of leaf phenology was estimated using a five-point categorical scale, where 1 corresponded to very late flushing (buds are dormant), 2 – late flushing (buds are swollen), 3 – flushing of moderate earliness (buds are bursting, tips of leaves/needles are appearing), 4 – early flushing (leaves/needles are unfolding), and 5 – very early flushing (leaves/needles are fully unfolded, growth of shoots has started).

Leaf Area Index (LAI) was measured for each row with potted 9 trees using device SunScan SS1 (Delta-T Devices, England). A photometric stick of 1 m length was placed across irrigation table along each row of trees at pots’ top edge 10 cm north from tree stems as well as across well-lit empty neighbouring irrigation table in the same chamber at midday (from 12:00 A.M. to 2:00 P.M.).

To estimate sapling evapotranspiration, 15 potted saplings of each tree species (five per population) in each treatment were weighted every second day before and after watering during two weeks in the middle of drought treatment period (mid July 2017). The evapotranspiration was estimated as a percentage of weight loss of potted saplings compared to the weight of potted saplings with fully saturated soil right after irrigation.

Rates of photosynthesis as net CO₂ assimilation (A, μmol m⁻² s⁻¹), transpiration (E, mmol m⁻² s⁻¹), stomatal conductance (gₑ, mol m⁻² s⁻¹) and intercellular CO₂ concentration (Cᵢ, μmol mol⁻¹) were recorded using a LCpro-SD portable photosynthesis system (ADC BioScientific Ltd, UK). Light conditions inside a leaf chamber were set using the LCpro-SD light unit to emit 1000 μmol m⁻² s⁻¹ of photosynthetically active radiation (PAR). An air supply unit provided airflow to the leaf chamber at a constant rate of 200 μmol s⁻¹. Air humidity was set at 20 mBar of partial water pressure; temperature and CO₂ concentration were at ambient levels. Readings were taken in 0.5–1 minute after the steady-state conditions have been reached. In each treatment, photosynthetic gas exchange was assessed for nine randomly selected saplings of each of the seven species from each population (Table 1). Gas exchange of deciduous trees was measured on a fully expanded and exposed to sunlight fifth leaf from the top of the leader shoot of each sapling. For conifers, measurements were done on the first from the top lateral annual shoot with all needles. The measurements were carried out at the end of the experiment (beginning of August 2017) between 10:00 A.M. and 2:30 P.M.

Further, to estimate the response of photosynthesis processes to different stressors, the amounts of produced malondialdehyde (MDA), hydrogen peroxide (H₂O₂), and photosynthetic pigments (chlorophylls a and b and carotenoids) were determined. For the analysis of concentrations of MDA and H₂O₂, fresh leaf material (0.5 g) was homogenized using 5% (w/v) trichloracetic acid (TCA). Lipid peroxidation was estimated as the amount of MDA produced by 2-thiobarbituric acid (TBA) (Kramer et al. 1991). The homogenates were centrifuged at 12,130 g for 17 min, and 2.0 ml of supernatant was added to 3.0 ml of 20% (w/v) TCA containing 0.50% (w/v) TBA. The homogenates were incubated at 95°C for 30 min and subsequently cooled on ice. After cooling, the homogenates were centrifuged at 12,130 g for 17 min, and MDA content was determined by subtracting the absorbance value recorded at 600 nm from the absorbance value recorded at 532 nm and using an absorbance coefficient of 155 mM⁻¹ cm⁻¹. Concentration of H₂O₂ in leaves was determined according to Velikova et al. (2000). A 0.5 ml of supernatant was added to 0.5 ml of potassium phosphate buffer (10 mM, pH 7.0) mixed with 1.0 ml of potassium iodide (1 M). The reaction solution was incubated at 25°C for 30 min in the dark. The absorbance of the supernatant was measured at 390 nm wavelength. The concentration of H₂O₂ was calculated using a standard curve based on known concentrations.

The photosynthetic pigments were extracted from 0.05 g of fresh leaf/needle material in 3 ml of N, N’-dimethylformamide (DMF). The light absorption was measured at 480, 664, 647 nm wavelengths. Chlorophyll a/b ratios and concentrations of chlorophylls a and b and carotenoids were calculated according to Wellburn (1994).

All spectrophotometrical measurements were made using spectrophotometer SPECORD 210 Plus (Germany). All data were expressed on leaf fresh weight basis.

Statistical analysis

The variance analysis of data was done using MIXED procedure in SAS (SAS® Analytics Pro 9.4, 2017) which is based on Mixed model equations (MME) and restricted maximum likelihood (REML) method. The significance of fixed effects was tested using an F-test. The following lin-
ear model was used for joint analyses of all factors together:

\[ Y_{ijklm} = \mu + T_j + S_k + P_{ik} + S_{ik} + T_j + P_{ik} + B_{im} + B_{ik} + T_j + P_{ik} + e_{ijklm}, \]

where \( Y_{ijklm} \) is an observation on the \( i \)th tree from the \( j \)th population in the \( k \)th tree species in the \( m \)th block of different species mixture pattern in the \( p \)th treatment, \( \mu \) is the overall mean, \( T \) is the effect of the \( j \)th treatment, \( B \) is the effect due to the \( i \)th block, \( e \) is an observation on the \( l \)th tree from the \( k \)th population, \( S \) is the effect due to the \( k \)th tree species, \( P \) is the effect of the \( p \)th population in the \( k \)th species, \( B \) is the effect of interaction between the \( j \)th treatment and \( i \)th block, \( B \) is the effect of interaction between the \( j \)th treatment and \( p \)th block, \( B \) is the effect of interaction between the \( j \)th block and \( p \)th treatment, \( B \) is the effect of interaction between the \( j \)th block and \( k \)th species, \( B \) is the effect of interaction between the \( j \)th block and \( p \)th population in the \( k \)th species, \( e \) is the random residuals. All effects in the models were assumed as fixed.

Assumptions of normal distribution of residuals and homogeneity of variance for each trait were tested by using the Shapiro and Wilk-tests (Sabin and Stafford 1990) for residuals. All effects in the models were assumed as fixed.

Pearson’s correlation between growth, physiological and biochemical traits in juvenile progeny of three populations of each of seven forest tree species in mono- and mixed cultures following four complex treatments and in ambient conditions (control): F-criteria and levels of significance (\( F \)) of the factors’ effects. For trait and treatment descriptions see Materials and methods.

### Results

The results of multifactor analysis of variance showed that treatment effect on saplings’ biomass, growth, physiological and biochemical traits was highly significant (\( P < 0.001 \)) except for survival rate (Table 2).

Species mixture pattern had highly significant effect on physiological and biochemical traits (except for \( \text{H}_2\text{O}_2 \)), meanwhile it was of low significance for biomass and growth traits (except nonsignificant effects on diameter increment, leaf area index and tree condition) (Table 2). Species mixture pattern-by-treatment interaction was highly significant in growth, physiological and biochemical traits (Table 2). This interaction is indicated by differing slopes of response and rank changes of monocultures and mixed cultures across the treatments (Figures 1, 2). Species mixture pattern-by-treatment interaction was nonsignificant or of low significance for biomass traits, tree condition and survival (Table 2).

Species effect and species-by-treatment interaction were highly significant in most of the traits studied, indicating species-specific reactions to the applied complex treatments (Table 2). This interaction is indicated by differing slopes of reaction and species rank changes across treatments (Figures 1, 2, 3). Mixture pattern-by-species interaction was highly significant in most of the traits (except for stem biomass, stem/root biomass ratio and tree condition), indicating that the effect of mixture pattern on these traits depended on the species involved (Table 2).

The highest variation in species performances in most growth and biomass traits was observed in HW treatment (Figure 1). Coefficients of variation of species means in tree height increment, stem diameter increment, stem volume index and stem biomass were 102.9, 53.2, 97.7 and 56.7% respectively (data not shown). In comparison to the control in ambient conditions, the most substantial

### Table 2

Results from joint ANOVA of different biomass, growth, condition, physiological and biochemical traits in juvenile progeny of three populations of each of seven forest tree species in mono- and mixed cultures following four complex treatments and in ambient conditions (control): F-criteria and levels of significance (\( F \)) of the factors’ effects. For trait and treatment descriptions see Materials and methods.

<table>
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<tr>
<th>Factors</th>
<th>Treatment</th>
<th>Mixture pattern</th>
<th>Species</th>
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<tr>
<td>Stem volume index</td>
<td>46.0 ***</td>
<td>3.7 *</td>
<td>1280.1 ***</td>
<td>28.6 ***</td>
<td>11.5 ***</td>
<td>2.0 *</td>
<td>0.6</td>
</tr>
<tr>
<td>Tree biomass</td>
<td>20.5 ***</td>
<td>5.2 *</td>
<td>265.2 ***</td>
<td>16.8 ***</td>
<td>7.0 ***</td>
<td>1.6 *</td>
<td>2.7</td>
</tr>
<tr>
<td>Stem biomass</td>
<td>11.3 ***</td>
<td>3.9 *</td>
<td>408.9 ***</td>
<td>17.8 ***</td>
<td>7.8 ***</td>
<td>1.6 *</td>
<td>3.0</td>
</tr>
<tr>
<td>Root biomass</td>
<td>29.5 ***</td>
<td>5.2 *</td>
<td>140.0 ***</td>
<td>13.9 ***</td>
<td>5.2 ***</td>
<td>2.1 *</td>
<td>2.5</td>
</tr>
<tr>
<td>Stem/root biomass ratio</td>
<td>25.9 ***</td>
<td>4.4 *</td>
<td>267.3 ***</td>
<td>4.9 ***</td>
<td>3.7 ***</td>
<td>1.3 *</td>
<td>1.4 n.s.</td>
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<tr>
<td>Height increment</td>
<td>374.7 ***</td>
<td>7.1 *</td>
<td>1221.1 ***</td>
<td>15.3 ***</td>
<td>47.5 ***</td>
<td>3.1 ***</td>
<td>4.4 ***</td>
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<td>Diameter increment</td>
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<td>202.6 ***</td>
<td>28.8 ***</td>
<td>9.2 ***</td>
<td>1.9 ***</td>
<td>15.1 ***</td>
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<tr>
<td>Height/Diameter ratio</td>
<td>228.3 ***</td>
<td>9.6 **</td>
<td>2912.5 ***</td>
<td>5.5 ***</td>
<td>27.3 ***</td>
<td>1.6 ***</td>
<td>8.8 ***</td>
</tr>
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<td>Leaf area index</td>
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<td>0.1 n.s.</td>
<td>68.7 ***</td>
<td>9.6 ***</td>
<td>2.6 ***</td>
<td>0.9 n.s.</td>
<td>6.2 ***</td>
</tr>
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<td>Tree condition</td>
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<td>0.1 n.s.</td>
<td>432.3 ***</td>
<td>4.0 ***</td>
<td>44.9 ***</td>
<td>2.4 ***</td>
<td>0.6 n.s.</td>
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<td>Survival</td>
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<td>7.9 *</td>
<td>92.9 **</td>
<td>7.4 ***</td>
<td>2.5 ***</td>
<td>1.4 *</td>
<td>3.0 *</td>
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<td>Transpiration rate</td>
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<td>25.5 ***</td>
<td>324.3 ***</td>
<td>2.6 ***</td>
<td>17.6 ***</td>
<td>1.5 ***</td>
<td>19.7 ***</td>
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<td>Smolal conductivity</td>
<td>5.9 ***</td>
<td>68.9 *</td>
<td>214.0 ***</td>
<td>5.9 ***</td>
<td>17.6 ***</td>
<td>4.7 ***</td>
<td>34.8 ***</td>
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<td>Photosynthetic rate</td>
<td>16.2 ***</td>
<td>33.1 ***</td>
<td>105.8 ***</td>
<td>1.6 ***</td>
<td>8.3 ***</td>
<td>1.8 ***</td>
<td>20.7 ***</td>
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<tr>
<td>Chlorophyll a</td>
<td>221.8 ***</td>
<td>6.9 **</td>
<td>533.4 ***</td>
<td>10.9 ***</td>
<td>38.7 ***</td>
<td>5.5 ***</td>
<td>59.1 ***</td>
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<td>Chlorophyll b</td>
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<td>27.0 ***</td>
<td>553.3 ***</td>
<td>11.5 ***</td>
<td>38.4 ***</td>
<td>5.5 ***</td>
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<td>Carotenoids</td>
<td>242.5 ***</td>
<td>706.8 ***</td>
<td>901.0 ***</td>
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<td>37.0 ***</td>
<td>5.5 ***</td>
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<td>74.8 ***</td>
<td>85.2 ***</td>
<td>478.0 ***</td>
<td>10.5 ***</td>
<td>51.2 ***</td>
<td>5.7 ***</td>
<td>11.0 ***</td>
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<td>H2O2</td>
<td>686.1 ***</td>
<td>0.3 n.s.</td>
<td>393.2 ***</td>
<td>9.5 ***</td>
<td>100.1 ***</td>
<td>6.0 ***</td>
<td>16.5 ***</td>
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* Levels of significance: * – \( P \leq 0.05 \), ** – \( 0.001 < P \leq 0.01 \), *** – \( P \leq 0.001 \); n.s. – not significant at \( P < 0.05 \).
Figure 1. Values of juvenile growth and biomass traits of seven tree species in mono- and mixed cultures under four complex treatments in phytotron with elevated CO₂ concentration each (HW – heat + elevated humidity; HFD – heat + frost + drought; HWUO – heat + elevated humidity + increased UV-B radiation doses + elevated ozone concentration; HFDUO – heat + frost + drought + increased UV-B radiation doses + elevated ozone concentration) and in control (ambient conditions outside phytotron). Error bars indicate standard errors. For details on traits and treatments see Materials and methods.
increase in stem volume index, tree biomass, tree diameter and height growth was observed following HW treatment in the fast-growing deciduous species, *P. tremula*, *A. glutinosa*, *B. pendula*, and *Q. robur*. In comparison to ambient conditions, differences in stem volume index and some other growth traits between different patterns of species mixture were more pronounced in these fast-growing deciduous trees following HW treatment (Figures 1a, b, c, d). *P. abies* in HW and in all other treatments had lower stem volume index and tree biomass than in ambient conditions with no significant differences between patterns of mixture.

Variation in species performances in most growth and biomass traits in HFD treatment was lower than in HW and in all other treatments (Figure 1). Coefficients of variation of species means in height increment, stem diameter increment, stem volume index and stem biomass were 82.7, 34.3, 84.6 and 39.2% respectively (data not shown). In comparison to HW treatment, biomass in HFD treatment was reduced in fast-growing deciduous *P. tremula*, *A. glutinosa* and *B. pendula* (Figure 1b). Broadleaves, *Q. robur* and *F. excelsior*, and conifer *P. sylvestris* demonstrated better biomass growth in HFD treatment than in HW treatment. In HFD treatment, differences in tree biomass between mono- and mixed cultures were not pronounced, except for biomass of *B. pendula* and *P. tremula*. No significant differences between the patterns of species mixtures in volume indices of tree species were found in HFD treatment (Figure 1a). Meanwhile, tree diameter increment of *A. glutinosa*, *F. excelsior*, *P. tremula* and *P. abies* was significantly higher in mono- than in mixed cultures, while diameter increment of *B. pendula* was higher in mixed cultures (Figure 1d).

![Figure 2](image-url)

**Figure 2.** Values of physiological parameters of juveniles of seven tree species in mono- and mixed cultures under four complex treatments in phytotron with elevated CO2 concentration each (HW – heat + elevated humidity; HFD – heat + frost + drought; HWUO – heat + elevated humidity + increased UV-B radiation doses + elevated ozone concentration; HFDUO – heat + frost + drought + increased UV-B radiation doses + elevated ozone concentration) and in control (ambient conditions outside phytotron). Error bars indicate standard errors. For details on parameters and treatments see Materials and methods.
In comparison to HFD treatment, complex treatment with elevated UV-B radiation doses and ozone concentration in HWUO has caused further reduction of tree biomass in all tree species and patterns of mixture except for the monocultures in HWUO has caused further reduction of tree biomass in all tree species and patterns of mixture except for the monocultures in HWUO.

The most stressful HFDUO treatment caused substantial damages to tree leaves that seemingly had strongest negative effect on biomass growth of almost all tree species compared to that observed following HW treatment, except for both conifers, *P. abies* and *P. sylvestris*. Biomass of *Q. robur* and *P. sylvestris* and height increment of all tree species in HFDUO treatment still was higher than that in ambient conditions which most likely was due to the positive compensatory effect of the increased CO₂ concentration and temperature (Figure 1b). In HFDUO treatment, significant differences in tree biomass between the patterns of mixture were observed only for *P. tremula* and *B. pendula*.

In comparison to ambient conditions, the ratio of tree height to diameter was higher in all treatments and in all species except for conifers, *P. abies* and *P. sylvestris* (Figure 1e). The most substantial differences in the height to diameter ratio between the patterns of mixture were established in *B. pendula* in HW and HWUO treatments and in *P. tremula* in HFDUO treatment.

The magnitude of tree stem to root biomass ratio varied a lot depending on treatment and tree species with increased ratio in *P. tremula*, *Q. robur*, *F. excelsior* and *P. sylvestris* in mixed cultures in HFDUO treatment. In monocultures, an increased tree stem to root biomass ratio...
was observed in *P. sylvestris* in ambient conditions and in HWUO treatment, in *A. glutinosa* and *B. pendula* in HFD treatment and in *Q. robur* in HW treatment (Figure 1f). No significant differences between performances in monocultures vs. mixed cultures of hardwoods, *Q. robur* and *F. excelsior*, and conifers, *P. abies* and *P. sylvestris*, were observed (except for tree biomass of *Q. robur* in HWUO treatment and stem diameter increment in *F. excelsior* in HFD and HWUO treatments).

Compared to the control (ambient conditions), transpiration rate was higher in all four treatments and especially in HW and HWUO treatments for all investigated species except for *P. sylvestris* and *P. abies* (Figure 2b). Differences in transpiration rate between different patterns of species mixture were rather large in most of the species, although they varied a lot depending on treatment and tree species. As regards stomatal conductance and photosynthetic rates, very different reactions of different tree species and patterns of mixture, both negative and positive (compared to control), were observed (Figure 2c,d). The conifers showed the least variation among the patterns of mixture across the treatments (Figure 2c,d). The highest stomatal conductance was observed in monocultures of *B. pendula* and *A. glutinosa* in HW treatment and *Q. robur* in ambient conditions. The highest photosynthetic rate was found in monocultures of *B. pendula* and *F. excelsior* in ambient conditions and HFD treatment. Meanwhile, in the HW and HFDUO the highest photosynthetic rate was in *B. pendula* and *A. glutinosa* mixed cultures (Figure 2d). The photosynthetic rate in *P. sylvestris* in ambient conditions and in all treatments was much higher in monocultures than in mixed cultures, while in *P. abies* these differences were rather small, except HFDUO treatment.

Compared to the control, the treatments HW, HFD and HFDUO induced an increase in the concentrations of chlorophyll *a* and *b* in almost all tree species (except *P. tremula* and *P. sylvestris* in HW treatment), while in HWUO treatment, these concentrations were slightly higher or of a similar magnitude as those in ambient conditions (Figure 3a,b). In ambient conditions, the concentrations of chlorophyll *a* and *b* in *A. glutinosa*, *B. pendula* and *F. excelsior* were higher in mixed cultures than those in monocultures, meanwhile in most of the treatments chlorophyll *a* and *b* concentrations were higher in monocultures (Figure 3a,b). *Q. robur* in mixed cultures had higher chlorophyll *a* and *b* concentrations in all treatments, meanwhile *P. abies* – always the lowest. The most variable species-specific and pattern of mixture-specific performances across treatments were found in concentrations of MDA (Figure 3d). In all deciduous tree species, the concentrations of *H₂O₂* were much higher in ambient conditions, than those in all four treatments, while in both conifers, *P. sylvestris* and *P. abies*, the concentrations of *H₂O₂* were very low in control and in all treatments. Differences in *H₂O₂* concentrations between mono- and mixed cultures were highly pronounced for many tree species (except for *P. sylvestris*) in ambient conditions as well as in HW and HFD treatments, while in HWUO and HFDUO treatments, the differences became much smaller and often nonsignificant (except for *P. abies* in HFDUO treatment, Figure 3e).

As high B-type correlations between population means of biomass and growth traits in mono- and mixed cultures in individual treatments with stressors and in ambient conditions showed, the performance of populations in both patterns of species mixture was rather similar (Table 3). Regarding physiological traits, B-type correlations were moderate in ambient conditions and in HFD and HFDUO treatments, and high to moderate in HW and HWUO treatments (Table 3). Regarding biochemical traits, B-type correlations were high in ambient conditions but moderate in all treatments.

**Discussion**

Significant between-species effects and species-by-treatment interaction found in the present study for almost all growth, physiological and biochemical traits suggest that tree responses to complex treatments are species-specific. Highly significant mixture pattern-by-treatment interaction in growth, physiological and biochemical

<table>
<thead>
<tr>
<th>Table 3. B-type correlations between population means of the same trait in mono- and mixed cultures in separate complex treatments and in ambient conditions. For details on traits and treatments see Material and methods</th>
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<tr>
<td>Trait</td>
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<tr>
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<tr>
<td>Stem volume index</td>
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<tr>
<td>Tree biomass</td>
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<tr>
<td>Stem biomass</td>
</tr>
<tr>
<td>Root biomass</td>
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<tr>
<td>Stems/root biomass ratio</td>
</tr>
<tr>
<td>Height increment</td>
</tr>
<tr>
<td>Diameter increment</td>
</tr>
<tr>
<td>Height/Diameter ratio</td>
</tr>
<tr>
<td>Leaf area index</td>
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<td>Tree condition</td>
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<td>MDA</td>
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<td>HoO₂</td>
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</table>
traits indicates that the effects of mixture pattern on these traits depend on applied complex treatments, i.e. performance of mono-species and mixed cultures varies across treatments. Thus, competitiveness of different tree species in mixed cultures across treatments might be different and diverge from those in monocultures. As saplings in the present experiment were grown in individual pots with no root interaction and no competition for nutrients, the interaction between saplings of different tree species occurred predominantly via the competition for better light conditions which changed along with the developing of tree canopy and sheltering of neighbouring trees. This resulted in different performance of each tree species in mono- vs. mixed cultures. Some allelopathic interaction between species also might be present. It is known that leaves exhibit structural and functional plasticity in response to the crown light conditions, however the strength of this kind of response was found to be species-specific (Sack et al. 2006). These responses reflect an adaptive mechanism that lead to changes of whole plant gas exchange and resource investment strategy (Givnish 1988).

As indicated by the highest variation in species performances in most growth and biomass traits (see Figure 1), the HW treatment can be defined as adaptive environment of highest selectivity. Meanwhile variation in species performances in HWDUO was rather similar to one in HW treatment and much lower in HFD and HFDUO treatments. Similarly, largest differences between performances in mono-species and mixed cultures were observed in HW and HWUO treatments, indicating that hot wet environments facilitate revealing differences in performance.

Limited water availability during growth period in treatment HFD and especially in combination with elevated UV-B radiation doses and ozone concentrations in treatment HF DUO has caused premature partial leaf shedding in the fast-growing deciduous P. tremula, A. glutinosa and B. pendula and this contributed to the reduction of height and diameter increments. Similarly, Oksanen and Saleem (1999) found that ozone exposure results in various carry-over effects and prolonged reduction in biomass in Betula pendula. Compared to HW treatment, lower height increment of P. tremula, A. glutinosa and B. pendula in treatments with summer drought indicates that these fast-growing tree species are the most susceptible to drought. These species are shade intolerant, therefore the findings on their low drought tolerance do not correspond to those of Niinemets and Vallares (2006) who concluded that shade tolerance is negatively related to drought tolerance. In our study Q. robur and P. sylvestris benefited from drier conditions. These findings correspond to those of Correia et al. (2018) who studied performance of 33 tree species across temperature and humidity gradients and found that B. pendula showed the greatest growth reduction along with increasing dryness index, while the growth of Q. robur decreased very little. Other studies also show that Q. robur demonstrates higher drought tolerance and might gain some competitive advantages under the projected drought in some areas in the course of climatic changes (Scharnweber et al. 2011). In our experiment, lower height increments of P. tremula, A. glutinosa and B. pendula in HFD treatment may mean that in mixed cultures these species are prone to lose in performance and competing ability in comparison to Q. robur and P. sylvestris which perform better in these drier conditions. In the present experiment P. tremula and B. pendula show better growth in mixed than in monocultures, which most likely is due to better light conditions when they are surrounded by smaller trees of slower growing species. As pioneer species, P. tremula, A. glutinosa and B. pendula, still are much faster growing than Q. robur and P. sylvestris they cannot be largely negatively affected by the latter slower growing ones. Thus, although performance of P. tremula, A. glutinosa and B. pendula may somewhat deteriorate under the most stressful drier climatic conditions (as in HF DUO treatment) or on drier sites, their competing ability against broadleaves or conifers will most likely not decline in mixed forest regeneration. Nevertheless, some studies show that if the formerly dominant trees become less competitive, even small changes in climate may lead to relatively strong shifts in species composition (e.g., Lindner et al. 1997).

According to susceptibility to spring frosts the investigated species could be ranked as follows (in descending order): F. excelsior, Q. robur, P. abies, P. sylvestris, P. tremula, A. glutinosa and B. pendula. With the increasing occurrence of spring frosts in the course of ongoing climate change, the growth of the broadleaved tree species, F. excelsior and Q. robur, may be negatively affected and their competing ability may decline.

Different tree species demonstrated specific combined strategies to cope with the complex stressors: A. glutinosa and B. pendula closed stomata to reduce stomatal conductance and transpiration rate, P. tremula increased transpiration rate, and all these three species partially shed leaves, while P. sylvestris and P. abies increased their transpiration rate in drought treatments but not exhibited defoliation. The fact that P. abies in all treatments had lower stem volume index and tree biomass than in ambient conditions and there were no significant differences between the patterns of mixtures, means that this tree species suffers from every combination of stressors irrespectively of light availability level in different patterns of mixtures. Comparing the growth and biomass traits of P. abies was compared with those of the other species in ambient conditions, it could be observed that P. abies outperformed hardwoods and even P. sylvestris. This is particularly valid for tree biomass of P. abies in mixed hardwoods (Figure 1b). A better growth of P. abies in mixed culture with Q. robur correspond to the findings of Kozovitz et al. (2005) who reported that spruce was a stronger competitor than beech, as displayed by its higher aboveground biomass increments in mixed culture compared with monoculture.
An absence of significant differences between performance of noble hardwoods, *Q. robur* and *F. excelsior* (except for tree biomass of *Q. robur* in HWUO treatment and stem diameter increment in *F. excelsior* in HFD and HWUO treatments), and conifers, *P. abies* and *P. sylvestris*, in mono- vs. mixed cultures may be attributed to too short time of the experiment for these comparatively slow-growing tree species to be suppressed by the fast-growing deciduous ones. In general, although the physiological and biochemical responses of species in different mixture patterns across the treatments were much more variable, they often were not reflected in the traits of growth and biomass of trees.

Significant population-by-treatment interaction found in the present study for most of the studied growth, physiological and biochemical traits indicates different response and plasticity of the investigated tree populations to the applied treatments that may also point to the presence of specific site-related or climate-related adaptations. This is in agreement with the results of the study carried out by Jensen and Hansen (2010) on performance of *Q. robur* populations in a greenhouse irrigation experiment, which showed a significant population-by-watering interaction effect for stem diameter and tree height. Meanwhile, our study was not able to find any significant interactions between populations and patterns of species mixtures.

We would like to emphasize that this study is based on observations of juvenile tree behaviour in different tree communities under severe competition for light and, although this is a very important phase of forest regeneration and further development, we cannot directly deduce the responses and behaviours of mature trees. This study presents important information on juvenile performance of different tree species in juvenile communities under various possible climate and environment change scenarios and may be useful for the forestry, currently challenging with adaptation to climate change, although further studies are required to reliably predict a long-term performance of forest trees and the related changes of forest ecosystems.

Conclusions

The present study showed general and species-specific reactions as revealed by the highly significant effects of the applied multifactor treatments, between species and species by treatment interaction in most of the biomass, growth, physiological and biochemical traits of juvenile progeny of seven forest tree species studied.

The largest differences among growth performances of different tree species as well as between mono- and multispecies cultures that were observed in HW treatment indicate that such hot-wet environment is favourable for revealing differences in performances and can be defined as adaptive environment of highest selectivity.

The pattern of species mixture had highly significant effect on the most of physiological and biochemical traits, meanwhile it was of low significance for biomass and growth traits. The pattern of mixture by treatment interaction was highly significant in all traits meaning that the effect of the applied complex treatments depended to some extent on the pattern of mixture. Although, the most significant differences between performances in mono- vs. mixed cultures were observed in the fast-growing pioneer tree species, those differences were small in noble hardwoods and conifers.

Under hot-wet conditions, the highest stem volume index, tree biomass, and diameter and height increments were observed in deciduous *P. tremula*, *A. glutinosa* and *B. pendula* with more clearly pronounced differences between the patterns of mixtures than in ambient conditions showing that enhanced growth conditions facilitate revealing the potential and specific requirements of the fast growers. *P. abies* in HW and in all other treatments had lower values of stem volume index and tree biomass than those in ambient conditions with no significant differences between the patterns of mixture, thus it suffered from every combination of stressors irrespectively of light availability in cultures. This indicates a presumable decline of this species performance and competitiveness irrespectively of climate change pattern.

Differences in performances of all tree species in mono- and mixed cultures in HFD treatment were rather small and nonsignificant, except for tree biomass and tree diameter increment of *F. excelsior*. In comparison to HFD treatment, complex treatment HWUO which included an elevated UV-B radiation dose and ozone concentration has caused further reduction of tree biomass in all tree species and patterns of mixture (except for the ones in monocultures of *A. glutinosa* and *B. pendula*). The most complex HFDUO treatment had the strongest negative effect on biomass of almost all tree species compared to that observed in HW treatment (except for *Q. robur* and *P. sylvestris*). Biomass of *Q. robur* and *P. sylvestris* and height increment in HFDUO treatment still remained higher than that in ambient conditions which was most likely due to relatively high drought tolerance of these species and positive compensatory effects of the increased CO₂ concentration and temperature.

The species-specific changes of stem to root biomass ratio across the treatments and mixture patterns of species indicate that balancing of biomass allocation between stem and roots is one of the strategies of species to reach better acclimation/adaptation to the changed environmental conditions.

In general, physiological and biochemical responses of species in different mixture patterns across the treatments were much more variable, although often did not reflect in the traits of growth and biomass of trees.

The observed different physiological and biochemical reactions to different combinations of stress factors, heat, drought and elevated UV-B radiation and ozone concentration, and declining performance of individual tree species and their populations at the most susceptible (juvenile) stage of stand development may lead to losses in their intra-specific competitiveness when they establish...
multiples species communities. This may compromise forest regeneration and change species composition, thus causing atypical successions of forest ecosystems.

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