Warming and elevated ozone differently modify needle anatomy of Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*)

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Abstract

Acclimation of conifer needle anatomy to climate change is poorly understood. We studied needle anatomy, shoot gas exchange, current year shoot length and stem diameter growth in spruce (*Picea abies*) and pine (*Pinus sylvestris*) seedlings exposed to elevated ozone (1.35-1.5 x ambient concentration) and elevated temperature (0.9-1.3 °C + ambient temperature) alone and in combination for two exposure seasons in two separate experiments in an open-field in Central Finland. Pines grew also at two soil nitrogen levels. In spruce, warming increased mesophyll intercellular space and reduced gas exchange and shoot growth, and made needles narrower and epidermis and hypodermis thinner. In pine, warming made needles bigger, increased shoot and stem growth, stomatal row number, proportions of vascular cylinder, phloem and xylem and reduced proportion of mesophyll. These responses indicate that pine benefited and spruce suffered from moderate warming. Ozone caused a thickening of epi- and hypodermis and a lower stomatal conductance in both species, reduced stomatal density in spruce, and increased proportions of phloem, xylem and sclerenchyma, and reduced growth in pine. Ozone-responses suggest increased oxidative stress defense. Stomatal responses were affected by interactions of elevated temperature and ozone in both species. Nitrogen availability modified ozone and temperature responses particularly in the vascular tissues in pine.

Key words: O₃, elevated temperature, global change, conifer, microscopy

Introduction

Ongoing climate change is affecting boreal forests. In the boreal zone, air temperatures are projected to increase by 1-2 °C from 1986-2005 level to 2016-2035 (Kirtman et al. 2013) mostly due to continuously increasing atmospheric carbon dioxide (CO₂) concentrations (IPCC 2013). The concentrations of tropospheric ozone, a greenhouse gas and air pollutant with substantial phytotoxic properties, increased from concentrations of 10 ppb in pre-industrialized times until the beginning of 2000's, after which ozone concentrations have stabilized to the level 30-40 ppb in the Northern Hemisphere (Cooper et al. 2014). Increased photosynthesis, growth or biomass production reported in field experiments where seedlings or young trees have been exposed to moderate temperature elevation (1 – 6 °C) in closed chambers or under IR-heaters indicate that species like Scots pine, *Pinus sylvestris*, (Peltola et al. 2002), silver birch, *Betula pendula*, and European aspen, *Populus tremula*, benefit of warming (Mäenpää et al. 2011), while Norway spruce, *Picea abies*, has shown photosynthesis and growth suppressing responses (Kivimäenpää et al. 2013). On the other hand, the prevailing ozone concentrations can cause several harmful effects on forest trees: they may e.g. decrease of photosynthesis, stomatal conductance and growth (Wittig et al. 2009), induce microscopic and macroscopic injuries, and predispose trees to biotic and abiotic stresses (Huttunen and Manninen 2013). Since nitrogen (N) is one of the growth-limiting factors for Scots pine in the north (Hyvönen et al. 2007) N fertilization can be hypothesized to modify growth responses to warming and ozone.

Change in leaf anatomy is an important mean of plants in adaptation (slow evolutionary process) and acclimation (shorter-term adjustment) to new environmental conditions. Leaf structure affects e.g. carbon fixation (photosynthesis), water relations and stress tolerance, and thus, growth and survival. For example, a species adapted to dry conditions can have thicker epidermis, sunken stomata and less intercellular space in the mesophyll to avoid drought (Esau 1977) or a species adapted to sunny habitats can have thicker mesophyll tissue and higher stomatal density to achieve larger photosynthetic yields (Larcher 1995). Acclimation to elevated ozone concentrations include

e.g. increase in stomatal density (Paoletti and Grulke 2005) or thickening of epidermis in the upper leaf side (Hartikainen et al. 2009) to protect leaves of deciduous trees from oxidative ozone stress. Anatomical acclimation to warming includes e.g. thinning and enlargement of the leaves of deciduous trees which has been regarded as an efficient adaptation to maintain photosynthesis in favorable growing conditions (Hartikainen et al. 2009).

While the responses of conifer needle cell organelles to ozone (e.g. Holopainen et al. 1996) and warming (e.g. Kivimäenpää et al. 2014) have been studied, the effects of moderately elevated temperature and ozone on needle anatomy of conifers are poorly understood. This is partly because earlier studies have generally lasted less than one growing season and most anatomical characteristics are unaffected in needles developed before the beginning of the exposures. For example, Virjamo et al. (2014) did not find anatomical changes in one-year-old Norway spruce seedlings exposed to elevated temperature (target +2 °C) under IR (infrared)-heaters in a short-term study where buds had not formed under warming. To our knowledge, the only long-term study about the effects of warming on needle anatomy of Scots pine is by Luomala et al. (2005). They reported increased intercellular space, reduced thicknesses of needle and vascular cylinder, and reduced stomatal density in the needles of young Scots pine trees exposed to three-year-lasting warming (ambient +2.8-6.2 °C) in closed-top chambers. The long-term studies where buds were formed and burst under ozone exposure did not show ozone-induced changes in the dimensions of needle cross-section, the size of vascular cylinder, or the size and number of resin ducts of Scots pine (Utriainen and Holopainen 2001a) or Norway spruce needles (Kivimäenpää et al. 2003). Excess N fertilization (250 – 1000 kg N ha⁻¹), on the other hand, made needles of mature Scots pine trees larger and mesophyll thicker (Jokela et al. 1995). Larger needle cross-sections were also reported in three-year-old Scots pines grown at N fertilization level ‘150 % of optimal’ (Utriainen and Holopainen 2001a). How ozone, warming and N availability in interaction would affect needle anatomy is still largely not known. Using Scots pine growth as an example, warming (Peltola et al. 2002) and N fertilization (Utriainen and Holopainen 2001a) in general are expected to increase

growth, but ozone to decrease it (Huttunen and Manninen 2013) in the northern latitudes. Therefore, it could be assumed that e.g. ozone and warming counterbalance the effects of each other or warming and N fertilization enhance the effects of each other. Such interactions could in an early phase be seen as alterations in needle anatomy and further reflected to needle function.

The aim of this study was to determine 1) how elevated temperature and elevated ozone alone and in combination affect anatomy of Norway spruce needles and 2) how elevated temperature and elevated ozone and N availability alone and in combination affect the anatomy of Scots pine needles. Mature needles were collected from two separate open-field experiments during the second exposure season of the experiments. To support the anatomy results, basal stem diameter, current year's main shoot and needle lengths at the end of the growing season, and gas exchange measurements performed closest to the day of microscopy sampling, are reported.

Material and methods

Multifactorial open-field experiments were carried out at the University of Eastern Finland, Kuopio campus open-air exposure field (62° 53' 42'' N, 27° 37' 30'' E, 80 m a.s.l). Norway spruce seedlings were exposed to elevated ozone and elevated temperature during two growing seasons 2009 and 2010, with elevated temperature also throughout the winter 2009-2010. Scots pine seedlings were exposed to elevated ozone, elevated temperature and two soil N availability levels during the growing seasons 2011-2013. In both experiments, needle anatomy was studied during the second year (in 2010 for spruce and 2012 for pine). For the spruce study, cell organelle structure of the needles (Kivimäenpää et al. 2014), growth and VOC (volatile organic compound) emissions from the first year of exposure (Kivimäenpää et al. 2013), and phenology, needle metabolomics, gas exchange and freezing tolerance from the first growing season and the following spring (Riikonen et al. 2012) have been published earlier. For the pine study, VOC emissions, needle cross-section area and resin canal characteristics from the second year of exposure have been published by Kivimäenpää et al. (2016).

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117 **Plant material and experimental set-up**

118 Details of plant material and experimental set-up for spruce experiments have been published by
119 Kivimäenpää et al. (2013) and for pine experiment by Kivimäenpää et al. (2016). Briefly, Norway
120 spruce seedlings were three-year old when the exposures started on 9 June 2009. They were planted
121 to 2:1 mixture of mull:sand bed fertilized with 4 kg m⁻³ of Peatcare Slow Release 1 (9 % N, 3.5 %
122 P, 5 % K, 4.8 % Mg; Yara, Finland) to give a total amount of 42.6 kg N ha⁻¹a⁻¹. Scots pine seedlings
123 were one-year-old when the experiment started on 31 May 2011. The seedlings had received basic
124 fertilization (dose 77 kg N ha⁻¹ a⁻¹) and were planted to 5-litre pots with 2:1 mixture of sand:peat
125 (Kekkilä White F6, containing 16 % N, 4 % P, 17 % K). Half of the Scots pine seedlings were
126 fertilized with Peatcare Slow Release 1 (9 % N, 3.5 % P, 5 % K, 4.8 % Mg; Yara, Finland) to give a
127 target dose 120 kg ha⁻¹a⁻¹ (denoted +N seedlings) and the other half did not receive additional
128 fertilization (denoted -N seedlings). Seedlings in both experiments were distributed to eight circular
129 (diameter 10 m) exposure plots. In the middle of each plot there were two rectangular subplots
130 where the seedlings were planted (spruce) or pots placed (pine). At the beginning of the experiment,
131 there were 24 experimental spruce seedlings and 22 additional side seedlings (to equalize
132 microclimatic conditions and competition) or 18 experimental pine seedlings and 12 side seedlings
133 in each subplot. Water content of the top soil was measured with a soil moisture sensor (Theta
134 probe, type ML2, Delta-T Devices, Cambridge, UK) and the seedlings were kept well-watered by
135 watering them with lake water approximately three times per week during the growing seasons if
136 there was no rain.

137 At the end of the second growing season 2010, N concentration of the current year spruce
138 needles (determined by Kjeldahl method) was on average 2.52 % of DW, and significantly
139 increased by elevated temperature (2.38 % in ambient temperature, 2.69 % in elevated temperature,
140 $P = 0.027$ for the temperature main effect). Lammas growth was recorded in 40 % of the spruce
141 seedling in 2010, and N concentration of the lammas growth was on average 1.8 %. In the current

year pine needles, N concentration was on average 1.3 % of DW in both N levels, and was not affected by ozone and temperature, at the end of the second growing season 2012. Pine seedlings in +N had lammas growth in 2011, but not in 2012.

Ozone was generated from pure oxygen and released through vertical perforated tubes around the circular plots between 8-22 every day during the growing seasons, except during rain, very high or low wind velocities or if the ambient concentration was below 10 ppb. Ozone concentrations were monitored at 1.5 m height from the center of the plots. Four of the plots received elevated ozone concentrations (target 1.5 x ambient ozone concentration) and the other four ambient ozone concentrations. Monthly means for the daytime averages of ozone concentrations and ozone exposure in terms of AOT40 (Accumulated Ozone exposure over a Threshold of 40 ppb, a sum of average hourly ozone concentrations exceeding 40 ppb, calculated from daylight hours 8:00-22:00 for the growing season with ozone exposure; CLRTAP 2004) are shown in Table 1 for the spruce experiment and in Table 2 for the pine experiment. For spruce, the average increase in ozone concentration was 1.4 x ambient in both years. For pine, the increases were 1.5 x ambient in 2011 and 1.35 x ambient in 2012.

Warming exposures were conducted in the subplots with one elongated, rectangular IR-heater installed above one of the subplots per plot, and kept 70 cm above the canopy. A wooden bar of similar shape and color was installed above the control subplot. Monthly daytime averages of air temperatures and cumulative temperature sum (degree-days above a threshold of 5 °C) for spruce experiment are shown in Table 1 and for pine experiment in Table 2. As a daily average for the growing season, IR-heaters increased air temperatures by 1.3 °C in 2009 and 1.1 °C in 2010 in the spruce experiment, and 0.9 °C in 2011 and 1.0 °C in 2012 in the pine experiment. Elevation in air temperature by 1 °C by IR-heaters was expected to increase needle temperature by an additional 1 °C (cf. Kivimäenpää et al. 2013).

Insecticides or pesticides were not used. Aphids were occasionally observed on the current year shoots of a few seedlings during the growing seasons 2010 and 2012. At the end of the

growing season 2012, some of the Scots pine seedlings showed symptoms of fungal disease *Dothistroma pini*. We estimated that biotic stress did not affect gas exchange and needle anatomy of the sampled shoots and needles, since the seedlings sampled were free of visible symptoms of biotic stress. Ozone or warming did not cause any visible symptoms on needles.

The following abbreviations are used for elevated temperature and ozone treatments: C = ambient ozone, ambient temperature; T = ambient ozone, elevated temperature, O = elevated ozone, ambient temperature, OT = elevated ozone, elevated temperature.

Microscopy

Samples for needle anatomical studies were collected from two seedlings per subplot (32 seedlings for spruce, 64 for pine) on 3 August 2010 (spruce) and 1 October 2012 (pine). Five green, current year needles that were fully matured (cf. Sutinen et al. 2006) at the time of the sampling, were carefully detached from the needle base by tweezers from different lateral shoots of the second (spruce) or first (pine) whorl from top of the crown. For spruce, needles were specifically collected from the upper side of the shoot, so that abaxial side of the sampled needles was facing the sky and IR-heaters and adaxial side was facing the stem. The needles were put into cold (+ 4 °C) pre-fixative containing 2.5 % glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.05 M phosphate buffer, pH 7.2 (spruce) or 2.5 % glutaraldehyde in 0.075 M cacodylate buffer, pH 7.2 (pine). A 1-1.5 mm long segment from the middle of each sampled needle was cut with a razor blade in cold pre-fixative, rinsed with the buffer (3 x 10 min, + 4 °C) post-fixed in 1 % buffered (same as in prefixative) osmiumtetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) for 5 hours (+ 4 °C), rinsed with the buffer (3 x 10 min, + 4 °C), dehydrated in increasing ethanol series (50 %, 70 %, 94 %, 100 %, 3 x 10 min each, + 4 °C), treated with propylene oxide (Sigma-Aldrich, Steinheim, Germany) for 3 x 10 min at room temperature (ca. + 20 °C), mixture of propylene oxide:Ladd's epon (1:1) for 24 h (+ 20 °C), embedded in Ladd's epon (Burlington, Vermont, USA) in flat embedding molds made of silicon (Electron Microscopy Sciences, Hatfield, PA, USA), kept

at room temperature for 24 hours and polymerized at + 60 °C for 72 hours. Semi-thin (1.5 µm) sections for light microscopy (LM) were prepared of two needles per seedling as described by Kivimäenpää et al. (2010). Needle cross-sections were photographed with a digital camera (Leica CD Camera, Heerbrugg, Switzerland) at x 10 objective magnifications under a light microscope (Leica DM2500, Wetzlar, Germany).

Additional five needles from the same shoots from both species were sampled for scanning electron microscopy (SEM) studies at the same time with LM sampling. Samples were air-dried, ca. 1 cm segments from the middle of the needle were cut, placed (convex side up for pine) on double-sided copper tape on aluminum stubs, sputtered with ca. 50 nm layer of gold (Automatic Sputter Coater B7341, Agar Scientific Ltd., Stansted, UK) and studied by SEM (Philips XL30 ESEM-TMP, FEI Company, Eindhoven, Netherlands). Three areas of needle surfaces were photographed with 80-120 x magnifications.

Tools of Image J ver. 1.43u (<http://imagej.nih.gov/ij/>) and Adobe Photoshop CS6 (Adobe Systems Nordic AB, Kista, Sweden) were used to determine areas of needle cross-section and tissues and the following needle anatomical parameters (Fig. 1) were calculated: proportions (%) of mesophyll and vascular cylinder from needle area, proportion of intercellular area (for spruce abaxial and adaxial needle sides separately) in the mesophyll, proportions and thicknesses of epidermis and hypodermis, proportions of sclerenchyma, phloem and xylem of the vascular cylinder and average size of resin canals. For spruce, cross-sections of resin canals were not present in all needle cross-sections. For pine, resin canal size, number and proportion per needle area, as well as needle cross-section area has been published by Kivimäenpää et al. (2016). Change in pine needle shape was evaluated by measuring the length of the convex and straight edges (Fig. 1) and calculating their ratio. Change in spruce needle shape was determined by measuring the length and width of needle cross-section (Fig. 1) and calculating their ratio. Number of stomata per needle perimeter (number/mm) from LM images was calculated to study changes in number of stomatal rows. For pine, stomatal density (number/mm) from the needle surface was determined from three

stomatal rows of each SEM image. For spruce, with less continuous stomatal rows, stomatal density (number/mm²) was counted from the surface areas where stomata existed.

Gas exchange and growth measurements

Gas exchange (net photosynthesis, P_n , and stomatal conductance, g_s) was measured from a current year lateral shoot from the uppermost whorl of two spruce seedlings per subplot (in total 32 seedlings) on 4 August 2010 as described by Kivimäenpää et al. (2013). Gas exchange was measured in middle part of one lateral current year shoot from the uppermost whorl of two Scots pine seedlings from both N levels from each subplot (in total 64 seedlings) on 29 August 2012 as described by Kivimäenpää et al. (2016). Measurements were done between 10:00 - 16:00 using LiCOR 6400 XT with opaque conifer chamber using saturating PAR level (1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for spruce and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for pine, determined with light saturation curves), CO₂ concentration 400 ppm and at the prevailing subplot temperature. Gas exchange was related to the total needle area (inside the chamber) as described by Räsänen et al. (2012) for spruce and Kivimäenpää et al. (2016) for pine. Ratio of P_n to g_s was calculated to evaluate water use efficiency.

Four spruce seedlings from each subplot (in total 64 seedlings) were measured for basal stem diameter and height of the main current year shoot at the end of the experiment in 2010. The results of the growth measurements in 2009, after one year of exposure, have been published by Kivimäenpää et al. (2013). The average needle length was measured from 20 randomly selected needles collected from three lateral shoots of the uppermost whorl from one or two seedlings per subplot (in total 28 seedlings) at the end of the growing season.

Basal stem diameter increment from the beginning of the exposures to the end of the exposures (early October) and the height of the main current-year shoot were determined from nine Scots pine seedlings per N level per subplot (in total 144 seedlings) in 2011 and 2012. Pine needle length (average of 10 needle pairs) was measured from the same shoot used for gas exchange measurements.

Statistics

All the results from the spruce experiment were averaged per subplot and those from pine per both N level per subplot. Statistical design of the spruce experiment was split-plot and that of the pine experiment split-split-plot. Linear mixed models ANOVA of SPSS 21 was used to study the main effects ozone, temperature and nitrogen (pine only), and if these factors modified the effects of each other. For spruce, temperature and ozone were fixed factors, and plot a random factor. For pine, temperature, ozone and N level were fixed factors, and subplot and plot random factors. $P \leq 0.1$ was selected as the limit of statistical significance similar to other open-field exposure experiments (Parsons et al. 2008). All interactions $P \leq 0.1$ were further studied by calculating P -values for simple main effects (SME, i.e. post hoc test for interactions) with Bonferroni corrections. Only the significant SMEs are reported.

Results

Anatomy of spruce needles

Elevated temperature did not affect needle length, but reduced the needle cross-section area (Table 3) and mesophyll tissue area (Table S1¹). It also affected needle shape, since it significantly reduced the needle cross-section width by 10 % and non-significantly cross-section length by 4 % (Table 3) that resulted in higher needle cross section length-width ratio under elevated temperature (Table S1). Elevated temperature reduced the thicknesses of the epidermis and hypodermis (Table 3) and their area (Table S1). Proportion of intercellular space in the abaxial side of the needles was higher and resin canals were smaller at elevated temperature compared to ambient temperature treatments (Table 3).

¹ Supplementary data are available with the article through the journal Web site

Opposite to the effect of elevated temperature, elevated ozone increased the thicknesses of epidermis and hypodermis (Table 3). As a result, epidermis and hypodermis thickness in the OT treatment was similar to the control treatment (Table 3). Also the proportion of epidermis and hypodermis was greater in elevated ozone (Table 3). Ozone decreased the proportion of mesophyll tissue and increased the proportion of endodermis (Table 3), but the changes in their areas were not significant (Table S1).

Interactions of ozone and temperature were observed in the stomatal density and proportion of sclerenchyma cells (Table 3). Ozone decreased stomatal density (measured by SEM), but only in the ambient temperature treatment (SME, $P = 0.024$ for O in ambient T). Elevated temperature increased the proportion of sclerenchyma cells in elevated ozone (SME, $P = 0.042$ for T in elevated O), thus, proportion of sclerenchyma was highest in the OT treatment (Table 3).

Gas exchange and growth of spruce

There were significant interaction effects on gas exchange of spruce needles: elevated ozone and temperature alone decreased P_n , g_s and their ratio, but in the OT treatment gas exchange values were even higher compared to all the other treatments (Fig 2). Results of SME tests for interactions were as follows. P_n : $P = 0.015$ for T in ambient O, $P = 0.003$ for T in elevated O, $P = 0.013$ for O in elevated T; g_s : $P = 0.044$ for O in elevated T, $P = 0.088$ for T in elevated O; P_n/g_s : $P = 0.058$ for O in ambient T, $P = 0.091$ for T in elevated O.

At the end of the second growing season, elevated temperature reduced the current year main shoot length by 13 % compared to the ambient temperature treatments (Fig. 3a). The same trend was observed in the basal stem diameter, but the differences were not significant (Fig. 3b).

Anatomy of pine needles

Elevated temperature increased the needle length, and the proportions (Table 4) or areas (Table S2²) of vascular cylinder, phloem and xylem. It also reduced the proportion of mesophyll tissue (Table 4). The reductive effect of elevated temperature on mesophyll proportion was strongest in elevated ozone and lower N availability (SME-test $P = 0.024$ for T in elevated O and -N). Proportion of phloem was increased by temperature only in the higher N level (SME-test $P = 0.003$ for T in +N, Table 4). Elevated temperature also increased the number of stomatal rows (Table 4), but the effect was not significant when the number of stomatal rows was related to the needle perimeter (Table S2).

Ozone increased the proportion (Table 4) and areas (Table S2) of epidermis and hypodermis. Ozone reduced the area (Table S2) and proportions of endodermis (Table 4), but ozone-induced reduction in proportion of endodermis was only seen in lower N level (SME-test $P = 0.013$ for O in -N), as higher N level could compensate the effect of ozone (SME-test $P = 0.028$ for N in elevated O). Area of vascular cylinder was not significantly affected (Table S2), but its' proportion per needle area was reduced by ozone (Table 4). Ozone also increased the areas (Table S2) and proportions of phloem and xylem (Table 4). In addition, ozone increased the proportion of sclerenchyma tissue (Table 4), but the area was not affected (Table S2). Ozone tended to increase the density of stomata per stomatal row, but only in ambient temperature (SME-test $P = 0.061$ for O in ambient T). Needles were on average 0.6 mm shorter in the higher than lower N level (Table 4).

Proportion of intercellular space in the mesophyll tissue and the shape of the needles were not affected by the treatments (Table S2).

Gas exchange and growth of pine

Elevated ozone decreased stomatal conductance by 27 % (Fig. 4a). Pn/gs was not significantly affected by any of the treatments (Fig. 4b).

² ¹Supplementary data are available with the article through the journal Web site

Current year main shoots were 25 % longer in higher N level in 2011 and 22 % longer in 2012 (Fig. 5a, c) than in lower N level. A three-way interaction for the main shoot length was observed in 2011. According to SME tests 1) N increased the shoot length with all other treatment combinations (details of SME not shown), but not under ambient ozone and elevated temperature treatment (SME $P > 0.2$ for N in ambient O and elevated T), 2) elevated temperature increased the shoot length, but only in lower N level and ambient ozone (SME $P = 0.017$ for T in –N and ambient O) and 3) ozone decreased the shoot length in lower N and elevated temperature (SME $P = 0.066$ for O in –N and elevated T). In 2012, elevated temperature increased main shoot length by 26 % (Fig. 5c).

Basal stem diameter increase during the exposure season was 76 % higher in the higher N than in the lower N level in 2011 (Fig. 5 b) and 14 % in 2012 (Fig. 5d). Stem diameter increase was also greater in elevated temperature treatments than ambient temperature treatments, by 32 % in 2011 and by 50 % in 2012. The main reason for significant two- and three-ways interactions in 2011 (Fig. 5b) was that the increase in stem diameter in elevated temperature was not observed in higher N level and ambient ozone (details of SME not shown). In 2012, elevated ozone decreased stem diameter increment by 20 % (Fig. 5d).

Discussion

These two year's exposure studies showed that elevated temperature and elevated ozone modified needle anatomy of Norway spruce and Scots pine seedlings when the buds developed and new shoots grew under exposure conditions. Such findings have not been reported in experiments lasting for only one growing season (e.g. Virjamo et al. 2014). In addition, growth and gas exchange were affected by warming and ozone. Moreover, soil nitrogen availability modified the responses of Scots pine to warming and ozone. The observed alterations are realistic since the level of temperature elevation in this study corresponds to the situation in the near future (Kirtman et al. 2013) and the AOT40 values of elevated ozone exposures are reality in several parts of Central

Europe and Southern Fennoscandia (e.g. Hjellbrekke and Solberg 2015). The level of N fertilization used in this study is in the range recommended for fertilization of pine stands in Finland (Saarsalmi and Mälkönen 2001).

The results from the two separate experiments show that direction in responses to ozone was similar in spruce and pine, but direction in responses to warming was opposite. Despite the fact that the experiments were conducted in different years, the experimental set-up, duration and elevation of ozone and warming exposures were similar, as was the developmental status of the needles for microscopy sampling. Therefore, we think that careful discussion about the reasons for similarities and differences in responses between pine and spruce seedling can be done, especially when differences between the experiments are considered.

Effects of elevated temperature

Most of the elevated temperature effects were opposite in Norway spruce and Scots pine, and based on growth responses, were negative on spruce and positive on pine, including reduced needle cross-section area in spruce, and increased needle cross-section area (Kivimäenpää et al. 2016) and needle length in pine. Our experimental results support the differences in the modelled growth of Norway spruce and Scots pine in Finland under different climate change scenarios (Torssonen et al. 2015) which suggest that pine could be preferred over spruce in forest regeneration in central and southern in Finland in the future climatic conditions. The harmful effects of warming on spruce in our study cannot only be due to warming exposure continuing over the winter, since the negative effects on needle gas exchange (Riikonen et al. 2012) and stem growth (Kivimäenpää et al. 2013) were observed already after the first growing season, while pine benefited from warming during both years. Some of the differences in anatomical and growth responses to warming may be a consequence of the species' adaptation to their natural growth habitat. Elevation of air temperature by 1 °C in the open field exposure conditions might have been a stress to Norway spruce that is adapted to moister, shadier and thus, cooler growing conditions, but not to Scots pine which thrives

in drier, sunny and warmer habitats. In addition, the temperature sums from elevated temperature treatment for pine were close to the species' optimum value for growth, 1445 degree-days, but the sums from both ambient and elevated temperature treatments for spruce exceeded the species' optimum value, 1215 degree-days (Torssonen et al. 2015). It seems that the growing conditions (soil bed for spruce vs. pots for pine) did not influence the results. Even if the pot in long-term can be a growth-limiting factor (Poorter et al. 2012), the pot-grown species had more positive growth responses to warming in our study. Moreover, leaf structure has not been reported to be influenced by pot size (Poorter et al. 2012).

Some of the anatomical responses to elevated temperature suggest that spruce and pine have different leaf water relations under warming. In pine, number of stomata that increased in total and remained constant per needle perimeter, as well as unaltered stomatal conductance and water use efficiency, suggest that warming did not disturb the gas exchange and transpiration rates of needles. The areas of vascular cylinder and xylem and the number of phloem sieve cells have been shown to increase in response to reduced water availability in spruce needles (Sutinen et al. 2006). In this study, the larger area and proportion of vascular cylinder, xylem and phloem may be a sign of increased need of water uptake, nutrient and photosynthetic product transport for faster-growing pine seedlings under warming or drought avoidance due to long-term warming stress. The tissues of vascular cylinder might have been constructed at a cost of photosynthetic mesophyll tissue that was reduced in proportion. Despite the reduced proportion of mesophyll tissue, its' total area was unaffected, and pine needles were capable of maintaining unaltered photosynthesis per needle area under warming (Kivimäenpää et al. 2016). At the seedling level, photosynthesis was probably increased by warming, since the needles were larger, and also the needle total biomass increased in the elevated temperature treatments (unpublished³).

Wider and longer pine needles may be a response analogous to increased leaf area observed in European aspen, a deciduous species, under warming (Hartikainen et al. 2009). The anatomical

³ M.U. Rasheed, A. Kasurinen, M. Kivimäenpää, R. Ghimire, E. Häikiö, P. Mpmah, J.K. Holopainen, T. Holopainen

alterations observed here in pine seedlings were different or opposite to those observed in 25 to 30-year-old Scots pines exposed to warming in closed-top chambers (Luomala et al. 2005). In their study, warming increased intercellular space of the mesophyll, decreased the number of stomata and made needles smaller (thinner) (Luomala et al. 2005). Actually, the responses of the older pines (Luomala et al. 2005) were more similar to Norway spruce seedlings that suffered from warming in our study. The temperature elevation in this study was moderate (ambient + 1 °C) compared to that used in Luomala et al. (2005) (ambient +2.8-6.2 °C). It is possible that the temperature tolerance of pines was exceeded in the study of Luomala et al (2005), and this exceedance could explain the different anatomical responses between their and our study. According to Lilja et al. (2010) the optimal growing temperature for conifer seedlings in Finland is 22/15 °C (day/night). Optimal temperature also depends on species, seedling origin, age and developmental status, and it changes in new growing conditions (Landis et al. 1992).

In spruce, increased intercellular space on the abaxial needle side (facing the IR-heaters) by warming could be regarded as structural acclimation to enhance diffusion of water vapour in the intercellular space, and thinner epidermis and hypodermis to increase cuticular transpiration in order to cool the leaf (Hajibagheri et al. 1983), while the decreased stomatal conductance could indicate a need to reduce stomatal transpiration. Narrower needle shape could be an acclimation mechanism to reduce surface area facing the IR-heaters. Unlike in pine, tissues of vascular cylinder were not increased as a response to higher need of water uptake. The responses in thick-walled tissues of vascular cylinder and epi- and hypodermis may be due to decreased synthesis of cell wall components which is supported by lower concentrations of metabolites of shikimic acid pathways in the same seedlings under warming (Riikonen et al. 2012). Reduced number of stomata, reduced stomatal conductance, and thus lower CO₂ uptake, as well as the lower area of photosynthesizing mesophyll, may be reasons for the reduced net photosynthesis by warming in spruce. Net photosynthesis was probably also decreased at the seedling level, since shoot height was reduced while needle length was unaltered. Pine seedlings under warming had more resin canals

(Kivimäenpää et al. 2016) whereas in spruce resin canals tended to be smaller, which indicate that pine seedlings are likely to have better tolerance to other stresses in the future warmer climate. The size and number of resin canals correlate with accumulation of terpenoids (Björkman et al. 1998) that are essential in defense against biotic stresses (Phillips and Croteau 1999).

Effects of elevated ozone and interactions with temperature

As far as we know, this is the first study showing that moderately elevated ozone concentrations alter proportions of needle tissues of Scots pine and Norway spruce. The current critical level of 5 ppm.h to protect forest trees from ozone (CLRTAP 2004) was exceeded for both species. Increased thickness of epidermis and hypodermis, reported here for both species, has been connected to higher ozone tolerance among pine species (Evans and Miller 1972). Moderately elevated ozone concentrations also increase epidermis thickness of deciduous trees, such as European aspen (Hartikainen et al. 2009) and silver birch (Pääkkönen et al. 1995). Since warming and ozone counteracted the effects of each other in epidermis and hypodermis thickness in spruce needles, warming could make needles more sensitive to ozone or ozone could reduce the cooling of needles via cuticular transpiration in the longer term. Such responses are realistic since our previous study with spruce seedlings showed that warming-induced cellular damage in winter and visible damage in spring were enhanced by ozone (Kivimäenpää et al. 2014).

Some of the observed ozone-warming interactions in the needle structure could be regarded beneficial for needle function. Increased proportion of sclerenchyma in the vascular cylinder indicates that ozone may increase the mechanical support of spruce needles when combined with warming, as well as of pine needles irrespective of warming. Increased areas or proportions of tissues with thick cell walls (epidermis, hypodermis and sclerenchyma) by ozone in the needles of both species may indicate increased synthesis of cell wall constituents under ozone stress (Sandermann 1996). Changes in proportions and areas in the tissues of vascular cylinder indicate

that transport of water and minerals and photosynthates across the endodermis, as well as to and from needles might have been affected by ozone. Transport mechanism of photosynthates is known to be disturbed by ozone stress in hybrid poplar *Populus euramericana* leaves (Landolt et al. 1994). In contrast to Landolt et al. (1994), here the increased areas of phloem and xylem in pine needles and reduced stem diameter growth and decreased needle biomass (unpublished³) may indicate higher need, but also capacity, to transport water and photoassimilates e.g. for defence and repair as a response to ozone stress at the expense of growth (Dizengremel 2001).

A reduction in stomatal conductance, which was observed in both Norway spruce and Scots pine here, is a common response to ozone in tree seedlings and indicates reduction in ozone uptake (Paoletti and Grulke 2005). In addition, an increase in stomatal density as a response to elevated ozone, observed here for pine, has been reported in several deciduous trees (Paoletti and Grulke 2005). In Scots pine, the stomatal responses were similar to those of silver birch (Pääkkönen et al. 1996) where higher stomatal density and lower stomatal conductance were regarded beneficial for ozone detoxification, as ozone was more evenly distributed in the mesophyll. According to Paoletti and Grulke (2005), responses between stomatal densities and conductance do not always correlate because of environmental interactions and species-specific responses. Indeed, here ozone did not increase stomatal density in pine needles under elevated temperature. Moreover, in ozone-exposed spruces both stomatal density and conductance were higher under elevated temperature. These responses in spruce could indicate higher ozone uptake and ozone stress under warming, but enhanced water use efficiency suggests that the response is not necessarily harmful to needle water relations.

Significance of nitrogen

The optimal nitrogen concentrations for growing spruce seedlings in Finland, determined in autumn after the growing season (1.6 – 2.3 %, Rikala 2012), was exceeded, especially in the elevated temperature treatment in this study. It is not likely that needle and shoot-level growth reductions in

spruces under warming were due to high N availability, since N fertilization normally increase needle size (Jokela et al. 1996) and tree growth (Utriainen and Holopainen 2001*b*). Nitrogen is known to modify responses of trees to ozone. For young Norway spruce, ozone (80 ppb) in a chamber exposure was reported to reduce photosynthesis (carbon assimilation), but the reduction was not observed under higher N fertilization level (N concentrations of the current year needles were ca. 0.9 % and 1.9 % at lower and higher N fertilization levels, respectively) (Lippert et al. 1996), which suggests that N compensated for the negative effects of ozone. Slightly elevated ozone in the field exposure (AOT40 indexes 6.9 ppm.h and 2.8 ppm.h in two consecutive years) increased stomatal conductance of Norway spruce seedlings at increased N fertilization level (N concentration in current year needles ca. 1.9 %), but not under N deficiency (ca. 1.0 %) (Utriainen and Holopainen, 2001*b*). This result would indicate that N either compensated the negative effects of ozone, assuming stomatal response was a sign of stomatal dysfunction, or amplified the stimulation of photosynthesis by low ozone exposure. Thus, good N availability in our study might have masked some of the negative effects of ozone in spruce seedlings, assuming the results from studies comparing nitrogen deficiency and optimal fertilization are applicable.

The measured N concentrations from pine needles were optimal for growing pine seedlings in Finland (range 1.3 - 1.8 %, Rikala 2012). The unaltered N concentration in the current-year needles despite the different soil N levels may be due to N allocation to an increased number of needles, based on the greater needle biomass (unpublished³). In pine needles, higher N level counteracted some of the anatomical effects of elevated ozone and elevated temperature, such as decrease in the proportion of mesophyll under elevated ozone and temperature and reduction in proportion of endodermis by ozone, and also enhanced the increase in the proportion of the phloem by elevated temperature. In addition, N counteracted reduced photosynthesis by ozone in pines of this experiment (Kivimäenpää et al. 2016) which is in line with observations on the mesophyll proportion. Moreover, reductive effects of ozone on shoot length were not seen in the higher N level during the first year of exposure. Our results are in contrast to Utriainen and Holopainen (2001*a*)

where higher N availability increased growth losses by ozone in Scots pine. In their study, the differences in N availability levels were larger than here and needle N concentration differences between the treatments (0.8 % vs. 1.3 %) were clear. Greater proportion of the needle phloem may indicate that higher N availability increased transport of photosynthates under warming, but after two exposure seasons warming and N interactions were not observed in shoot and stem diameter growths, which were increased by N availability and elevated temperature irrespective of each other.

In conclusion, the results of the two experiments suggest that Scots pine seedlings may benefit and Norway spruce seedlings may suffer from warming under future climate conditions, and thus give experimental support to the modeling studies, and can be used in choosing tree species for forest regeneration in the future. Anatomical acclimation to warming differs between the species, which seems to be linked mainly to needle water relations, and partly explains the growth responses. The structural defense responses suggest that boreal conifers acclimate to the current and future elevated ozone concentrations without substantial growth losses. The stomatal responses have a central role in the potential longer-term acclimation to conditions with elevated ozone and warming. Responses to warming can be enhanced and those to ozone counteracted in pine seedlings growing in the areas with high N availability, and the changes in the structure and function of the vascular system contribute to this.

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Table 1. Monthly mean (min; max) ozone concentrations (calculated from daily average values) for daytime hours (8 - 22), cumulative ozone exposure index AOT40, and 24-h averages for air temperature and cumulative temperature sums, for the growing seasons 2009 – 2010 in the spruce experiment.

		Ozone (ppb)		Temperature (°C)	
		Ambient	Elevated	Ambient	Elevated
2009	June	24 (16; 34)	32 (18; 46)	15.8 (9.9; 21.7)	17.0 (10.6; 23.1)
	July	25 (14; 33)	36 (15; 62)	17.4 (10.1; 22.2)	18.7 (11.2; 23.0)
	August	24 (15; 33)	34 (16; 53)	16.0 (11.4; 21.1)	17.5 (12.6; 22.7)
	September	25 (13; 42)	32 (16; 53)	11.6 (3.4; 19.3)	12.8 (4.5; 19.8)
	AOT40 (ppm·h)	0.14	4.71		
	Temp. sum (d.d.)			1345	1467
2010	May	34 (17; 50)	45 (18; 67)	12.8 (4.4; 19.9)	13.7. (5.0; 20.8)
	June	29 (18; 39)	42 (22; 62)	14.3 (8.8; 20.3)	15.3 (8.2; 21.4)
	July	30 (16; 42)	44 (23; 66)	22.7 (15.6; 28.1)	23.8 (17.0; 29.6)
	August	26 (10; 42)	38 (11; 64)	17.3 (9.6; 27.0)	18.5 (10.7; 28.7)
	September	19 (14; 28)	25 (18; 33)	11.5 (7.8; 15.4)	12.9 (8.4; 16.1)
	AOT40 (ppm·h)	1.39	13.48		
				1497	1585

Note: Ozone exposure was on 9 June – 30 September 2009 and 5 May- 15 September 2010.

Temperature exposure was on between 9 June 2009 – 15 September 2010, also in winter.

Temperature sum (degree-days above a threshold of 5 °C). Values were obtained from four ambient ozone and elevated ozone plots, and eight ambient and elevated temperature subplots.

Table 2. Monthly mean (min; max) ozone concentrations (calculated from daily average values) for daytime hours (8 - 22), cumulative ozone exposure index AOT40, and 24-h averages for air temperature and cumulative temperature sums, for the exposure periods 2011 – 2012 in the pine experiment.

		Ozone (ppb)		Temperature (°C)	
		Ambient	Elevated	Ambient	Elevated
2011	June	29 (17; 43)	40 (19; 65)	16.6 (10.3; 22.0)	17.5 (11.0; 23.3)
	July	26 (15; 38)	40 (18; 62)	19.8 (14.0; 23.8)	20.8 (14.7; 24.9)
	August	19 (7; 33)	30 (9; 47)	15.9 (13.1; 20.8)	16.8 (14.1; 21.4)
	September	18 (10; 29)	28 (8; 26)	12.0 (7.9; 15.7)	12.6 (9.0; 16.3)
	AOT40 (ppm·h)	0.25	6.29		
	Temp. sum (d.d.)			1287	1391
2012	May	38 (32; 46)	53 (36; 68)	12.8 (9.1; 16.9)	13.4 (10.0; 17.3)
	June	31 (16; 44) *	44 (16; 79) *	14.2 (9.7; 19.1)	15.0 (10.3; 20.2)
	July	26 (16; 37) *	27 (16; 37) *	17.8 (14.1; 24.1)	18.9 (15.3; 25.2)
	August	21 (14; 36) *	31 (17; 48) *	15.2 (10.0; 19.3)	16.4 (11.1; 20.2)
	September	21 (8; 26)	30 (9; 44)	10.5 (5.5; 14.7)	11.5 (6.9; 15.6)
	AOT40 (ppm·h)	0.52	9.58		
				1286	1423

Note: Ozone and temperature exposures were on 31 May – 2 October 2011 and 15 May – 30

September 2012. Temperature sum (degree-days above a threshold of 5 °C). Values were obtained from four ambient ozone and elevated ozone plots, and eight ambient and elevated temperature subplots.

* Due to technical failure there was limited elevated ozone exposure and data logging during 21 June – 6 August 2012. The missing data has been substituted by ambient ozone data from the Finnish Meteorological Institute Kasarmipuisto weather station in the center of Kuopio, 3 km from the field site (Air Quality in Finland; www.ilmanlaatu.fi).

Table 3. Averages (SE) of anatomical features of current year needles of Norway spruce seedlings in August 2010, exposed to elevated ozone and elevated temperature for over two exposure seasons 2009-2010.

	Treatments				Statistics
	C	T	O	OT	P-values
Needle length (mm)	10.4 (0.3)	11.2 (0.5)	10.9 (0.6)	10.4 (0.5)	
Needle cross-section area (mm ²)	0.332 (0.009)	0.311 (0.018)	0.332 (0.099)	0.285 (0.018)	T: 0.038 (↓ 10 %)
Cross-section length (mm)	0.823 (0.01)	0.812 (0.036)	0.809 (0.024)	0.774 (0.045)	
Cross-section width (mm)	0.603 (0.017)	0.558 (0.011)	0.626 (0.014)	0.542 (0.028)	T: 0.004 (↓ 10 %)
Stomatal density (#/mm ²)	166 (5)	149 (5)	143 (7)	149 (8)	O x T: 0.081
Epidermis (µm)	13.5 (0.6)	12.9 (0.2)	14.7 (0.3)	13.7 (0.3)	O: 0.052 (↑ 7 %), T: 0.075 (↓ 6 %)
Hypodermis (µm)	12.5 (0.5)	11.8 (0.4)	13.7 (0.5)	12.2 (0.2)	O: 0.083 (↑ 6 %), T: 0.024 (↓ 8 %)
Epi-and hypodermis (%)	13.6 (0.4)	13.1 (0.3)	14.8 (0.3)	14.8 (0.6)	O: 0.051 (↑ 10 %)
Mesophyll (%)	76.2 (1.2)	77.7 (0.3)	75.9 (1.2)	74.5 (0.5)	O: 0.087 (↓ 2 %)
Abaxial intercellular space (%)	31.3 (2.6)	33.7 (2.4)	30.9 (1.7)	38.0 (2.2)	T: 0.059 (↑ 13 %)
Adaxial intercellular space (%)	36.0 (2.3)	36.3 (1.3)	30.2 (2.6)	37.2 (6.2)	
Resin canal size (µm ²)	6880 (878)	5372 (381)	5737 (648)	4349 (842)	T: 0.095 (↓ 23 %)
Endodermis (%)	2.2 (0.1)	2.1 (<0.1)	2.3 (0.1)	2.5 (0.1)	O: 0.066 (↑ 10 %)
Vascular cylinder (%)	5.2 (0.3)	5.3 (0.2)	5.2 (0.4)	5.9 (0.3)	
Sclerenchyma (%)	8.1 (0.5)	7.8 (1.0)	7.0 (0.5)	8.9 (0.7)	O x T: 0.073
Phloem (%)	4.8 (0.6)	5.1 (0.3)	4.0 (0.5)	4.8 (0.3)	
Xylem (%)	5.5 (0.6)	5.4 (0.2)	5.3 (0.3)	4.5 (0.3)	

Note: C = control, ambient ozone, ambient temperature, T = ambient ozone, elevated temperature, O = elevated ozone, ambient temperature, OT = elevated ozone, elevated temperature, *n* = 4, except *n* = 3 for resin canals in OT. Main effects and interactions at a level *P* ≤ 0.1 for ozone (O) and temperature (T) from Linear Mixed Models ANOVA, and direction of main effects (arrows) with percentual change are shown. See text for SME tests of interactions.

Table 4. Averages (SE) of anatomical features of current year needles of Scots pine seedlings at the end of the growing season 2012, exposed to elevated temperature, elevated ozone and two soil nitrogen levels over two exposure seasons 2011-2012.

	Treatment								Statistics
	C		T		O		OT		P-values
	-N	+N	-N	+N	-N	+N	-N	+N	
Needle length (mm)	54.3 (5.2)	52.4 (3.1)	69.9 (7.1)	64.1 (1.9)	45.9 (4.7)	49.2 (7.4)	54.4 (5.8)	55.5 (6.6)	T: 0.044 (↑ 21 %) N: 0.039 (↓ 1.5 %) O x T: 0.100
Stomatal dens. per row (# mm ⁻¹)	11.7 (0.2)	11.9 (0.1)	12.2 (0.2)	11.9 (0.3)	12.4 (0.3)	12.3 (0.5)	11.9 (0.2)	12.0 (0.2)	
Stomata row number (#)	7.5 (0.4)	8.6 (0.7)	8.8 (0.4)	9.2 (1.2)	8.6 (0.8)	8.6 (0.8)	10.2 (0.8)	9.4 (1.0)	T: 0.047 (↑ 11 %)
Epi- and hypodermis (%)	11.4 (0.8)	11.9 (0.3)	11.5 (0.4)	11.6 (0.6)	13.4 (0.4)	14.1 (0.7)	12.6 (0.5)	12.6 (0.6)	O: 0.041 (↑ 12 %)
Mesophyll (%)	55.4 (1.4)	55.7 (1.5)	54.1 (0.6)	51.9 (2.0)	56.4 (0.9)	53.9 (1.0)	51.5 (2.7)	53.5 (1.1)	T: 0.017 (↓ 5 %) T x O x N: 0.094
Endodermis (%)	5.9 (0.3)	5.6 (0.3)	5.5 (0.1)	5.3 (0.3)	4.8 (0.2)	5.4 (0.2)	5.0 (0.1)	5.3 (0.3)	O: 0.089 (↓ 8 %) O x N: 0.025
Vascular cylinder (%)	22.8 (0.7)	22.5 (0.5)	24.2 (0.7)	24.5 (1.1)	21.0 (0.3)	21.0 (0.6)	22.9 (0.8)	23.0 (0.3)	T: <0.001 (↑ 14 %) O: 0.045 (↓ 6 %)
Sclerenchyma (%)	15.9 (0.4)	19.5 (1.2)	21.5 (2.0)	17.4 (1.5)	21.7 (1.5)	22.2 (1.6)	20.1 (2.5)	20.2 (1.4)	O: 0.047 (↑ 12 %) T x N: 0.094
Phloem (%)	3.1 (0.1)	2.4 (0.2)	2.8 (0.1)	3.1 (0.4)	3.5 (0.2)	3.0 (0.1)	3.5 (0.4)	3.6 (0.1)	O: 0.001 (↑ 17 %), T: 0.087 (↑ 8 %), T x N: 0.010
Xylem (%)	3.6 (0.1)	3.5 (0.1)	3.8 (0.1)	3.9 (0.3)	4.5 (0.3)	4.2 (0.2)	4.6 (0.2)	4.6 (0.3)	O: <0.001 (↑ 17 %), T: 0.075 (↑ 7 %)

Note: C = control, ambient ozone, ambient temperature, T = ambient ozone, elevated temperature, O = elevated ozone, ambient temperature, OT = elevated ozone, elevated temperature, -N lower N level, +N higher N level, $n = 4$. Main effects and interactions at a level $P \leq 0.1$ for ozone (O), temperature (T) and nitrogen (N) from Linear Mixed Models ANOVA, and direction of main effects (arrows) with percentual change are shown. See text for SME tests of interactions.

Figure captions

Fig. 1. Cross-sections of spruce (a) and pine (b) needles with tissues analysed for areas and proportions: epidermis (ep), hypodermis (h), mesophyll (m), rc (resin canals), endodermis (en), phloem (ph), xylem (x), sclerenchyma (sc), vascular cylinder (endodermis and all the tissues within it). Proportion of intercellular space (ics) of mesophyll tissue was reported separately for needle side facing the sky = abaxial (ab) and needle side facing the stem = adaxial (ad) for spruce, and as a whole needle average for pine, where ab = abaxial side and ad = adaxial side. Shape of the spruce needle was determined by calculating the ratio of length (L) to width (W) and that of pine by ratio of convex side length (ab) to straight side length (ad). Number of stomata (arrows) was related to the perimeter of the needles. Scale bar = 0.2 mm in both (a) and (b).

Fig. 2. Net photosynthesis, P_n (a), stomatal conductance, g_s (b) and P_n/g_s (c) of Norway spruce seedlings in August 2010 in control (C), elevated temperature (T), elevated ozone (O) and elevated ozone and temperature (OT) treatments. Values are averages (+SE) of four treatment replicates. Main and interaction effects ($P \leq 0.1$) of Mixed Models ANOVA are shown. See text for SME tests of interactions.

Fig. 3. Length of the current year main shoot (a) and basal stem diameter (b) of Norway spruce seedlings at the end of the growing season 2010 in control (C), elevated temperature (T), elevated ozone (O) and elevated ozone and temperature (OT) treatments. Values are averages (+SE) of four treatment replicates. Main and interaction effects ($P \leq 0.1$) of Mixed Models ANOVA are shown.

Fig. 4. Stomatal conductance, g_s (a) and ratio of net photosynthesis, P_n , to g_s (b) of Scots pine seedlings in 2012 of control (C), elevated temperature (T), elevated ozone (O) and elevated ozone and temperature (OT) treatments and two N availability levels (no added N, and added N; -N, +N). Values are averages (+SE) of four treatment replicates, except $n=3$ for P_n/g_s in O -N treatment. Main and interaction effects ($P \leq 0.1$) of Mixed Models ANOVA are shown. P_n has been published by Kivimäenpää et al. (2016).

Fig. 5. Length of current year main shoot (a, c) and basal stem diameter increase (b,d) of Scots pine seedling at the end of the growing seasons 2011 and 2012 of control (C), elevated temperature (T), elevated ozone (O) and elevated ozone and temperature (OT) treatments and two N availability levels (no added N, and added N; -N, +N). Values are averages (+SE) of four treatment replicates. Main and interaction effects ($P \leq 0.1$) of Mixed Models ANOVA are shown. See text for SME tests of interactions.

Fig. 1.

86x117mm (300 x 300 DPI)

Fig. 2.

278x360mm (300 x 300 DPI)

Fig. 3.

296x418mm (300 x 300 DPI)

Fig. 4.

296x418mm (300 x 300 DPI)

Fig. 5.

278x360mm (300 x 300 DPI)

Supplementary Table 1. Averages (SE) of stomatal density per needle perimeter, ratio of needle cross-section length to width and needle tissue areas of current year needles of Norway spruce seedlings in August 2010, exposed to elevated ozone and elevated temperature for over two exposure seasons 2009-2010.

	Treatments				Statistics
	C	T	O	OT	<i>P</i> -values
Stomatal density (# mm ⁻¹)	1.27 (0.09)	1.31 (0.20)	1.11 (0.22)	1.03 (0.07)	
Cross-section length : width	1.38 (0.04)	1.46 (0.44)	1.29 (0.04)	1.44 (0.08)	T: 0.080 (↑ 8 %)
Epi-and hypodermis (mm ²)	0.054 (0.004)	0.046 (0.002)	0.054 (0.004)	0.049 (0.004)	T: 0.090 (↓ 12 %)
Mesophyll (mm ²)	0.254 (0.007)	0.242 (0.014)	0.253 (0.005)	0.212 (0.012)	T: 0.025 (↓ 10 %)
Endodermis (μm ²)	7370 (535)	6480 (353)	7540 (608)	7150 (786)	
Vascular cylinder (mm ²)	0.017 (0.002)	0.017 (0.001)	0.017 (0.002)	0.017 (0.002)	
Sclerenchyma (μm ²)	1450 (150)	1260 (160)	1210 (150)	1500 (160)	
Phloem (μm ²)	784 (100)	816 (95)	689 (110)	806 (113)	
Xylem (μm ²)	931 (133)	850 (75)	885 (37)	778 (134)	

Note: C = control, ambient ozone, ambient temperature, T = ambient ozone, elevated temperature, O = elevated ozone, ambient temperature, OT = elevated ozone, elevated temperature, $n = 4$. Main effect at a level $P \leq 0.1$ for temperature (T) from Linear Mixed Models ANOVA, and direction of effects (arrows) with percentual change are shown. Effect of ozone (O) and interaction of O and T were not significant.

Supplementary Table 2. Averages (SE) of stomatal density per needle perimeter, ratio for the length of convex to straight needle edge, proportion of intercellular space and needle tissue areas of current year needle of Scots pine seedlings at the end of the growing season 2012, exposed to elevated temperature, elevated ozone and two soil nitrogen levels over two exposure seasons 2011-2012.

	Treatments								Statistics
	C		T		O		OT		P-values
	-N	+N	-N	+N	-N	+N	-N	+N	
Stomatal density per perimeter (# mm ⁻¹)	2.4 (0.1)	2.8 (0.2)	2.8 (0.2)	2.7 (0.3)	2.6 (0.1)	2.7 (0.1)	3.0 (0.1)	2.9 (0.2)	
Convex : straight	1.05 (0.05)	1.10 (0.03)	1.05 (0.06)	1.06 (0.06)	1.05 (0.03)	1.03 (0.03)	1.09 (0.03)	1.02 (0.02)	
Epi- and hypodermis (mm ²)	0.066 (0.004)	0.066 (0.002)	0.068 (0.004)	0.074 (0.002)	0.079 (0.006)	0.079 (0.004)	0.081 (0.006)	0.074 (0.003)	
Mesophyll (mm ²)	0.318 (0.011)	0.314 (0.015)	0.323 (0.023)	0.337 (0.011)	0.337 (0.032)	0.303 (0.023)	0.332 (0.023)	0.316 (0.011)	O: 0.065 (↑ 14 %)
Intercellular space (%)	19.4 (2.9)	17.1 (1.3)	17.7 (1.7)	18.6 (1.6)	19.4 (2.3)	19.8 (3.8)	20.1 (1.7)	17.3 (1.3)	O: 0.062 (↓ 9 %)
Endodermis (mm ²)	0.034 (0.002)	0.032 (0.001)	0.033 (0.002)	0.035 (0.002)	0.029 (0.003)	0.030 (0.002)	0.032 (0.002)	0.031 (0.003)	
Vacular cylinder (mm ²)	0.129 (0.012)	0.153 (0.017)	0.121 (0.012)	0.139 (0.006)	0.141 (0.003)	0.150 (0.013)	0.131 (0.006)	0.160 (0.017)	
Sclerenchyma (mm ²)	0.021 (0.002)	0.033 (0.005)	0.024 (0.004)	0.024 (0.004)	0.030 (0.003)	0.031 (0.005)	0.029 (0.001)	0.032 (0.002)	T:009 (↑ 15 %)
Phloem (μm ²)	3980 (420)	4230 (590)	2890 (450)	4370 (370)	4920 (210)	5240 (720)	3970 (260)	5820 (740)	O: 0.037 (↑ 29 %)
Xylem (μm ²)	4660 (480)	5730 (460)	4240 (590)	5470 (420)	6330 (340)	6970 (850)	5470 (410)	7370 (1260)	T: 0.009 (↑ 25 %)
									O: 0.034 (↑ 30 %)
									T: 0.014 (↑ 23 %)

Note: C = control, ambient ozone, ambient temperature, T = ambient ozone, elevated temperature, O = elevated ozone, ambient temperature, OT = elevated ozone, elevated temperature, -N lower N level, +N higher N level, *n* = 4. Main effects at a level *P* ≤ 0.1 for ozone (O) and temperature (T) and from Linear Mixed Models ANOVA, and direction of main effects (arrows) with percentual change are shown. Main effect of nitrogen (N) or the interactions were not significant.