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Effects of elevated growth temperature and enhanced atmospheric vapour pressure deficit on needle and root terpenoid contents of two Douglas fir provenances

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ABSTRACT

In the present work, we studied the effects of elevated air temperatures, which were above the optimum for photosynthesis, in combination with enhanced atmospheric VPD on two Douglas fir provenances grown under controlled conditions in a climate chamber. Provenance Monte Creek (MC) from the *menziesii-glauca* transition zone, Southern British Columbia, Canada, was derived from a dry environment receiving *ca.* half of the precipitation at its natural site than the interior provenance Pend Oreille (PO) from a mesic site in Northeast Washington State, US. We determined the terpenoid contents in needles and roots of the trees as well as terpene emission from needles and terpenoid synthase activities observing clear provenance-specific patterns. Whereas total terpenoid contents in needles dropped significantly in provenance PO in response to thermal stress, they remained unaffected in MC. The drop in terpenoid content in PO was due to decreased abundance of almost all identified terpenoids with exception of five compounds. Terpene emission was significantly enhanced in thermal-stressed provenance MC but it was unaffected in provenance PO. Oppositely, root terpenoid contents were rather stable in both provenances upon high temperature and enhanced atmospheric VPD. Similarly, we did not observe stress effects on terpenoid synthase activity, which was used as a proxy for the formation of terpenoids. The results indicate that features of the original habitat of the trees determine plant chemotypic properties, for example, thermal stress related responses. The observed decrease of terpenoid levels in needles of PO after long-term exposure to elevated temperature/enhanced atmospheric VPD, might weaken stress-exposed trees. Since terpenoids are essential components of the conifers' defense arsenal against herbivores, decreased terpenoid levels might increase susceptibility of stressed trees to above- and belowground herbivore challenges.

Abbreviations: VPD, vapor pressure deficit; TPS, terpenoid synthase; GC–MS, gas chromatography-mass spectrometry; HSP, heat shock protein; GDP, geranyl diphosphate; FDP, farnesyl diphosphate.

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1. Introduction

Anthropogenic activity caused considerably increased greenhouse gas concentrations in the atmosphere since the beginning of the industrial revolution. In Central Europe, this resulted in 1.3 °C higher mean surface temperatures in the decade from 2004 to 2013 as compared to the pre-industrial level (IPCC, 2013), and further increases by 1.1–6.4 °C are expected until the end of this century (Christensen et al., 2007). In addition to global warming, extreme weather events such as heat waves will likely occur more frequently, for longer durations and at higher intensities in the future (Della-Marta et al., 2007; Hansen et al., 2012; Yao et al., 2013). With a high probability, therefore, elevated temperature will become an important environmental factor influencing plant performance in many ecosystems (Wahid et al., 2007; Peñuelas and Staudt, 2010).

It is well understood that moderately increased temperatures exert growth stimulating effects on trees (Way and Oren, 2010), as seen, for example, in Douglas fir (Ormrod et al., 1999). This effect might be connected to enhanced net CO_2 assimilation, which is at a maximum at 20 °C in Douglas fir (Lewis et al., 2001). In contrast, heat waves exceeding such moderate temperatures by far, will have severe effects on forests, for example, by strongly reducing ecosystem gross primary production and enhancing the mortality of trees (Allen et al., 2010; Ciais et al., 2005; Bastos et al., 2013). Such effects are driven by strongly impaired processes at the biochemical, physiological and the whole plant levels (Saxe et al., 2001; Teskey et al., 2015).

To cope with higher temperatures, plants have evolved a plethora of structural, physiological and/or biochemical adaptations to resist, avoid or tolerate heat stress (Bréda et al., 2006; Weston and Bauerle, 2007). At the biochemical/physiological level, the formation of heat shock proteins (HSPs) is a well-known process linked to heat tolerance in trees (Colombo et al., 1992; Gifford and Taleisnik, 1994; Teskey et al., 2015). For example, photosynthesis may become more tolerant against high temperatures, as some of the HSPs appear to contribute to a stabilization of thylakoid membranes (Horvath et al., 2012; Teskey et al., 2015). Other adjustments of plants to heat include biosynthesis and emission of volatile terpenoids such as the hemiterpene isoprene (Arab et al., 2016) or monoterpenes (Tingey et al., 1980; Peñuelas and Llusià, 2001). Higher temperatures lead to enhanced *de novo* biosynthesis due to increased activity of enzymes involved, e.g. isoprene synthase and monoterpene synthases (Loreto et al., 2006; Loreto and Schnitzler, 2010). As a consequence, considerable biosynthesis and emission of these compounds take place at temperatures above the optimum of net CO_2 assimilation. Fumigation of leaves with terpenoids bit also work with transgenic plants with knocked-out isoprene biosynthesis support the view of enhanced thermotolerance mediated by these compounds (Sharkey and Singsaas, 1995; Loreto et al. 2002; Behnke et al., 2007). It is hypothesized that isoprene and monoterpenes contribute to the integrity of thylakoid membranes. They reduce membrane leakiness at elevated temperatures, stabilize membrane proteins, contribute to the quenching of reactive oxygen species (ROS), thereby improving the thermotolerance of the photosynthetic apparatus (Velikova et al., 2011, 2012; Loreto et al., 1998; Siwko et al., 2007; Vickers et al., 2009). Besides their role in enhancing plants' tolerance at transient temperature increases, terpenoids seem to be involved in tolerance against long-term heat periods (Loreto et al., 1998; Sharkey and Singsaas, 1995). This is indirectly reflected by the general high abundance of stored terpenoids in plants of Mediterranean and (sub)-tropical ecosystems (Jardine et al., 2017; Fasbender et al., 2018; Guidolotti et al., 2019). Moreover, they also fulfil crucial functions against biotic stressors, as they provide defence against pathogens and herbivores (Zeneli et al., 2006; Martin et al., 2003; Gershenzon and Dudareva, 2007; Loreto et al., 2014). For fulfilling the latter function, terpenoids have to be stored in specialized structures in the plant such as the resin ducts of conifer needles (Blanch et al., 2009; Wu and Hu, 1997) or oil glands and leaf hairs in many broadleaves (Singsaas, 2000). Two separated pathways are responsible for the biosynthesis of terpenoids internal plants, besides the plastidic MEP pathway which forms isoprene, monoterpenoids, diterpenoids as well as tetraterpenoids, the cytosolic mevalonic acid (MVA) pathway leads to production of sesquiterpenoids and triterpenoids (Lichtenthaler et al., 1997; Dudareva et al., 2013). Usually, formation of terpenoids is strongly coupled to photosynthesis as intermediates of the Calvin cycle are directly used as precursors in the MEP pathway (Lichtenthaler et al., 1997; Dudareva et al., 2013). However, uncoupling of the two processes occurs when, for example under severe drought stress, photosynthesis is inhibited. Under such conditions C sources different from CO_2, such as carbohydrates, are used for terpenoid biosynthesis (Loreto and Schnitzler, 2010).

Emission of terpenoids can result either from a temperature-dependent release from storage pools or from *de novo* biosynthesis in chloroplasts. Elevated temperatures increase the volatility of terpenoids thereby enhancing emission from pools. On the other hand, biosynthesis might also be stimulated by higher temperatures due to enhancement of enzyme activities (Tingey et al., 1980; Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010).

The actual terpenoid content of leaf tissues is determined by rate of terpenoid biosynthesis and the loss of terpenoids via its emission. Currently there is inconsistent evidence on the effect of elevated temperature on terpenoid content of leaves or roots. Either increased air temperatures over longer periods cause a decrease of leaf or root stored terpenoids due to enhanced release into the environment from storage pools, or this process is (over-)compensated by increased rates of *de novo* biosynthesis. Increased levels of terpenoids might help leaf or root tissue to overcome heat stress periods (Peñuelas and Llusià, 2002, 2003). Whereas most studies on terpenoids have been conducted on aboveground plant parts, data on roots are scarce. To the best of our knowledge, only two studies investigated the effect of elevated temperatures on the terpenoid contents in roots. Both studies dealt with carrots and both demonstrated increased terpenoid contents in roots under elevated temperatures (Rosenfeld et al., 2002; Ibrahim et al., 2006).

The present study was conducted with seedlings of Douglas fir (*Pseudotsuga menziesii*), a tree species naturally distributed from North-Western Canada to New Mexico, US, and from the North American west coast to the eastern parts of the Rocky Mountains (Smith et al., 2009), thereby growing in altitudes from sea level up to 2700 m a.s.l. (Hermann, 1985). Provenance "Monte Creek (MC)" originating from a dry site from the *menziesii-glauca* transition area of the Southern Interior region of British Columbia, Canada, was compared with provenance "Pend Oreille (PO)" from a wetter site of the *glauca* zone of the Okanogan Highlands, WA, USA (see Du et al., 2014; Jansen et al., 2014). We studied the effect of long-term elevated growth temperature in combination with enhanced atmospheric drought, which is considered an important component of "hotter drought" (Allen et al., 2015). We characterized the effect of such stress on terpenoid contents, *in vivo* terpene emissions and *in vitro* terpenoid synthase (TPS) activities in needles as well as terpenoid contents in roots of two Douglas fir provenances. It was hypothesized that (1) long-term exposure to temperatures above the optimum of photosynthesis will decrease needle terpenoid contents in both provenances due to increased terpenoid emissions and impaired leaf TPS activities. We further assume that (2) root terpenoid contents will decrease because reduced photosynthesis will limit the availability of assimilates for terpenoid biosynthesis in the roots. Moreover, we aimed at testing for provenance-specific differences considering the different environmental conditions, mainly differences in annual precipitation, at the provenances' sites of origin.

2. Materials and methods

2.1. Plant cultivation and experimental design

Details of the experimental setup have been described earlier (Du et al., 2014; Jansen et al., 2014). Briefly, three to four years old seedlings of two Douglas fir (*Pseudotsuga menziesii var. glauca*) provenances were investigated in this study. The provenance "Pend Oreille" (PO), which originated from humid environment (Supplemental Table S1) in North Western Washington, USA, was provided by Webster Forest Nursery, Washington State Department of Natural Resources, Olympia, WA, USA. The provenance "Monte Creek" (MC) was provided by Nursery Services Interior BC Timber Sales, Vernon, BC, Canada (Supplemental Table S1); it originated from the transition area of coastal and interior provenances, representing a more arid habitat in the Rocky Mountains near Kamloops, British Columbia, Canada (Supplemental Table S1). The seedlings were placed into two walk-in environmental chambers (KTLK 20000-IV, Nema Industrietechnik GmbH, Netzschkau, Germany) and were grown in 4 l pots on a mixture of commercial potting soil, perlite and sand (1:1:1), which was supplied with long-term fertilizer (3 g L^{-1} substrate) (Osmocote Exact high-end 5–6, 15 N + 9 P + 12 K + 2 Mg, The Scotts Company, LLC, Marysville, OH, USA). The seedlings were watered with tap water every second day and, when exposed to the high temperature treatment, watered daily in order to avoid any water stress. The chambers were illuminated by a mixture of sodium-vapour lamps (NC 1000-00, -01 and -62, Narva, Plauen, Germany, with 6, 8 and 10–11% red light, respectively).

All plants were acclimatized to experimental conditions from February to March 2010 (Supplemental Table S2); the exact experimental design can be found in Du et al. (2014) and Jansen et al. (2014). During the 92 days lasting temperature treatment, the photoperiod lasted for 16 h and the PPFD was 600 μmol m^{-2} s^{-1}, which was above the light saturation point of Douglas-fir. The air temperatures were 20/15 °C (day/night) in the control chamber (C) and 30/25 °C in the thermal-stress chamber (T), relative air humidity was 85 ± 5% during daytime in C and 55 ± 5% in T, and the air VPD amounted to 0.35 ± 0.1 kPa in C and to 1.91 ± 0.2 kPa in T, which were presumed to represent air humid and arid conditions, respectively. Such 10 °C temperature increase during day and night reflects the range of a heat wave, daytime temperatures clearly exceeding the temperature optimum of photosynthesis in Douglas fir. An increase in air temperature is normally accompanied by an increase in VPD and thus both together are components of the "hotter drought" scenario hypothesized to particularly impair tree and forest function in future (Allen et al., 2015). Notably, the trees were kept well-watered during the experiment to avoid occurrence of drought stress due to limited water availability. On day 64 after the start of the treatment, terpenoid emissions from current-year needles were collected and measured afterwards. At the end of the experimental period, from day 76 to 92 after the treatment was started, fine roots and needles of four to five trees of each treatment and provenance were harvested, immediately shock-frozen in liquid N_2 and stored at −80 °C until analyses.

2.2. Determination of emitted terpenes

The terpenes emitted by current-year needles were collected using a glass cuvette (volume 1 L, Heinz Walz GmbH, Effeltrich, Germany) in which the uppermost branch of the seedlings was carefully inserted one day prior to the collection. The cuvette was flushed with synthetic air (21% O_2, 400 ppm CO_2 in N_2, Air Liquide, Düsseldorf, Germany) at a temperature of 24.5 ± 1.5 °C. The cuvette was illuminated with 690 μmol m^{-2} s^{-1} PPFD. All tubes were made of perfluoralkoxy (PFA) (Swagelok, Solon, Ohio, USA) to prevent adhesion on or reaction of terpenes with the tubing material. Collection of terpenes was conducted *via* air sampling glass tubes which contained glass wool for plugging and 20 mg Tenax TA 60/80 and 30 mg Carbotrap B 20/40 (all from Supelco, Bellafonte, PA, USA) as adsorbents. The air from the cuvette was drawn through the air sampling glass tubes for 40 min at a flow rate of 200 ml min^{-1} using an air sampling pump (Analyt-MTC, Müllheim, Germany). Air sampling tubes were then stored in glass vials at 4 °C until terpene analysis (see below). Terpene emission rates were calculated considering dry weight of the needles, sampling time and flow rate; data were background corrected by measurements of the empty cuvette.

2.3. Terpenoid extraction and analysis

Needles and fine roots were homogenized into fine powders by a mortar and pestle. For terpenoid extraction, aliquots of approximately 50 mg frozen plant powder were added to 500 μL methanol. After shaking on thermomixer (Eppendorf AG, Hamburg, Germany) at 30 °C and 1400 rpm for 20 min, the extracts were centrifuged and the supernatants diluted 1:50 (leaves) or 1:5 (roots) with distilled water. We used Stir Bar Sorptive Extraction (SBSE) to adsorb terpenoids in the extract on polydimethylsiloxane (PDMS)-coated stir bars (Twisters®, 0.5 mm PDMS layer thickness, 10 mm in length, Gerstel, Mülheim, Germany). For this purpose, one stir bar was added to each sample extract. Afterwards, samples were shaken at 30 °C and 1400 rpm for 60 min. to allow quantitative adsorption of terpenoids on the stir bars. Finally, the stir bars were removed from the solution, shortly dried with lint free paper tissue and placed into thermodesorption tubes for subsequent GC–MS analysis (Gerstel, Mülheim, Germany).

Analysis was conducted on a gas chromatograph (model 7890A, Agilent, Germany) equipped with a thermodesorption/cold injection system (TDU-CIS) (Gerstel, Germany) and connected to a mass-selective detector (5975C, Agilent, Germany). Details of the method are provided elsewhere (Kleiber et al., 2017). The obtained mass spectra were compared with the National Institute of Standards and Technology (NIST, USA) database for compound identification; in addition, external authentic monoterpene standards were used for this purpose. Terpenoids were quantified with calibration curves of representative terpenoids (*i.e.*, isoprene for hemiterpenes, α-pinene for monoterpenes, linalool for oxygenated monoterpenoids, citronellyl acetate for acetylated monoterpenoids, and α-caryophyllene for sesquiterpenes).

2.4. In vitro determination of terpenoid synthase (TPS) activities

To determine the apparent *in vitro* monoterpenoid and sesquiterpenoid synthase activities, a modified method of Fischbach et al. (2000) was applied. For this purpose, protein extracts containing TPS were mixed with geranyl diphosphate (GDP) or farnesyl diphosphate (FDP), the substrates for monoterpenoid and sesquiterpenoid biosynthesis, respectively. The resulting volatile products were bound to PDMS-coated stir bars (Twister®, 0.5 mm PDMS layer thickness, 10 mm in length, Gerstel, Mülheim, Germany) and subsequently analysed by GC–MS.

For protein extraction, approximately 150 mg frozen homogenized needle material was added into one 2 ml reaction tube after addition of 1 ml plant extraction buffer (50 mM Hepes/KOH pH 7.3, 10% (v/v) glycerol, 2% (w/v) polyvinylpolypyrrolidone) and 10 μl of anti-oxidation solution (0.5 M sodium ascorbate, 0.5 M sodiumdisulfite, 0.5 M dithiothreitol). The solution was shaken on ice for 20 min. After centrifugation, 500 μl of the supernatant were transferred to a NAP-5-Sephadex column (GE Healthcare, Little Chalfont, Great Britain) equilibrated with 3 × 3 ml of terpenoid synthase buffer (TPS buffer) (50 mM MOPS buffer pH 7.3, 10% glycerol and 20 μl anti-oxidation solution per ml MOPS buffer). TPS proteins were eluted with 1 ml TPS buffer.

For the enzyme assay, 440 µl protein extract were transferred into 0.5 ml reaction tubes containing a Twister® and 110 µl of the enzyme assay buffer (1 M $MgCl_2$, 125 mM $MnCl_2$, 2 mM GDP dissolved in MOPS buffer, 2 mM FDP dissolved in MOPS buffer, 10% polyethylene glycol in TPS buffer). Two technical replicates and two controls were prepared with MOPS buffer instead of protein extract and MOPS buffer instead of GDP and FDP to correct for the non-enzymatically formed terpenoid background. The reaction tubes were shaken at 40 °C and 700 rpm for 2 h. Thereafter, Twisters® were shortly washed with distilled water, dried with a lint-free cloth, transferred to thermodesorption tubes and analysed immediately by GC–MS as given above.

2.5. Dry mass determination

Aliquots of approximately 100 mg frozen needle or root powder were dried at 60 °C until the weight remained constant. Concentrations of all parameters were calculated based on dry weight unless indicated otherwise.

2.6. Statistical analysis

For each treatment, four to five biological replicates were used. The software package SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany) was applied for statistical analysis and OriginPro 2016 (Originlab Corporation, Washington, USA) was used for figure generation. The interactions between the two factors "provenance" (PO and MC) and "temperature" (20 °C and 30 °C) on terpenoid contents in needles and roots, terpene emissions as well as terpenoid synthase activity were tested by two-way analyses of variance (ANOVA) followed by Tukey's pairwise multiple comparison test at $P < 0.05$. If required, data were either log- or square-root transformed to ensure normality distribution and equal variance. In addition, Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed using MetaboAnalyst 4.0 (Chong et al., 2018) after log transformation of data. All terpenoid variables obtained from the experiments were used for analysis. Total contents were calculated by the sum of individual components.

3. Results

3.1. Elevated air temperature and atmospheric VPD affect terpenoid contents in a provenance-specific way

In needles of both provenances, 35 terpenoids were identified, whereas only 25 terpenoid species were observed in the roots (Tables 1 and 2). Total needle terpenoid contents ranged from 8 to 36 mg g^{-1} DW (provenance PO) and 13 to 43 mg g^{-1} DW (provenance MC). This was considerably higher than terpenoid contents in the roots, which ranged from 1 to 5 mg g^{-1} DW in both provenances (Fig. 1A and B). Provenance-specific differences in total terpenoid contents were neither observed for needles nor for roots as indicated by two-way ANOVA

Table 1
Terpenoid contents in needles of the Douglas fir provenances Pend Oreille (PO) and Monte Creek (MC) grown at 20 and 30 °C and two-way ANOVA results. Means ± s.e. of 4–5 replicates each are shown. Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature. HT, hemiterpene; MT, monoterpene; MT-O, oxygenated monoterpenoid; MT-A, acetylated monoterpenoid; ST, sesquiterpene; ST-O, oxygenated sesquiterpenoid.

Terpenes [µg g^{-1} DW]		Pend Oreille (PO)		Monte Creek (MC)		$P_{prov.}$	$P_{temp.}$	$P_{prov.*temp.}$
		20 °C	30 °C	20 °C	30 °C			
Isoprene	HT	1 ± 0a	1 ± 1a	1 ± 0a	2 ± 1a	0.076	0.891	0.379
4-Carene	MT	106 ± 17a	26 ± 3b	115 ± 36a	42 ± 9b	0.237	**<0.001**	0.699
3-Carene	MT	14 ± 2a	5 ± 3a	15 ± 6a	16 ± 12a	0.435	0.129	0.345
α-Phellandrene	MT	8 ± 1a	2 ± 1b	9 ± 3a	4 ± 1b	0.130	**0.001**	0.328
α-Pinene	MT	5,912 ± 696a	2,003 ± 315b	5,587 ± 181a	3,865 ± 486ab	0.343	**0.003**	0.184
α-Terpinene	MT	1 ± 0	nd	nd	nd	na	na	na
β-Myrcene	MT	222 ± 33a	63 ± 10b	184 ± 63a	126 ± 24ab	0.809	**0.028**	0.126
β-Phellandrene	MT	90 ± 11a	30 ± 4c	118 ± 31a	56 ± 3b	**0.035**	**<0.001**	0.190
β-Pinene	MT	1,371 ± 261a	543 ± 74b	2,004 ± 323a	1,589 ± 357a	**0.003**	**0.011**	0.167
Camphene	MT	10,614 ± 849a	4,852 ± 628b	9,770 ± 805a	8,123 ± 1138ab	0.347	**0.010**	0.120
γ-Terpinene	MT	19 ± 3a	7 ± 1b	22 ± 6a	12 ± 2ab	0.277	**0.003**	0.345
Limonene	MT	1,503 ± 168a	523 ± 79b	1,464 ± 36a	849 ± 135ab	0.530	**0.003**	0.428
β-Ocimene	MT	272 ± 244a	127 ± 43a	112 ± 97a	475 ± 218a	0.579	0.263	0.274
Sabinene	MT	2 ± 2a	nd	187 ± 186a	14 ± 11a	na	na	na
Tricyclene	MT	2,155 ± 265a	722 ± 107b	2,146 ± 661a	1,319 ± 211ab	0.471	**0.012**	0.457
Borneol	MT-O	14 ± 1a	15 ± 5a	14 ± 9a	14 ± 4a	0.992	0.959	0.893
Isopulegol	MT-O	203 ± 78a	74 ± 34a	99 ± 51a	59 ± 22a	0.254	0.346	0.378
Geranial	MT-O	59 ± 29a	1 ± 1a	261 ± 225a	11 ± 7a	na	na	na
α-Terpineol	MT-O	2 ± 1a	nd	nd	5 ± 5a	na	na	na
Citronellal	MT-O	134 ± 51b	43 ± 23bc	581 ± 231a	29 ± 7c	0.169	**<0.001**	0.094
Citronellol	MT-O	266 ± 56b	350 ± 165ab	786 ± 246a	713 ± 161a	**0.022**	0.973	0.656
Fenchone	MT-O	5 ± 2a	2 ± 1a	13 ± 8a	2 ± 1a	0.552	0.072	0.700
Terpinen-4-ol	MT-O	5 ± 5a	14 ± 7a	24 ± 12a	26 ± 7a	0.081	0.542	0.659
Linalool	MT-O	5.0 ± 2.3a	33 ± 18a	31 ± 13a	22 ± 5a	na	na	na
Geraniol	MT-O	13 ± 8a	2 ± 2a	14 ± 11a	1 ± 1a	na	na	na
Citronellyl acetate	MT-A	239 ± 90a	155 ± 51a	449 ± 132a	459 ± 121a	**0.032**	0.736	0.675
Geranyl acetate	MT-A	464 ± 190a	124 ± 26a	489 ± 149a	174 ± 35a	na	ma	na
Bornyl acetate	MT-A	4,415 ± 566a	2,377 ± 657b	3,306 ± 654a	3,746 ± 413ab	0.825	0.187	**0.049**
Longifolene	ST	17 ± 5a	8 ± 5a	21 ± 6a	10 ± 3a	0.535	0.071	0.778
α-Humulene	ST	116 ± 9a	69 ± 19a	104 ± 28a	51 ± 18a	0.469	**0.025**	0.884
β-Caryophyllene	ST	24 ± 7a	8 ± 5a	22 ± 8a	11 ± 3a	0.972	**0.039**	0.672
β-Elemene	ST	74 ± 18a	44 ± 13a	82 ± 24a	35 ± 12a	0.991	**0.049**	0.650
δ-Elemene	ST	67 ± 7a	16 ± 7b	44 ± 19a	8 ± 4b	0.216	**0.002**	0.517
γ-Cadinene	ST	8 ± 2a	2 ± 1b	5 ± 2a	1 ± 1ab	0.238	**0.010**	0.421
Nerolidol	ST-O	3 ± 2a	3 ± 3a	4 ± 3a	10 ± 8a	na	na	na

nd, not detected; na, not applicable.

Table 2
Terpenoid contents in roots of the Douglas fir provenances Pend Oreille (PO) and Monte Creek (MC) grown at 20 and 30 °C and two-way ANOVA results. Means ± s.e. of 4–5 replicates each are shown. Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature.HT, hemiterpene; MT, monoterpene; MT-O, oxygenated monoterpenoid; MT-A, acetylated monoterpenoid; ST, sesquiterpene; ST-O, oxygenated sesquiterpenoid; DT, diterpene.

Terpenes [μg g^{-1} DW]		Pend Oreille (PO)		Monte Creek (MC)		$P_{prov.}$	$P_{temp.}$	$P_{prov.*temp.}$
		20 °C	30 °C	20 °C	30 °C			
α-Thujene	MT	10 ± 4a	13 ± 2a	21 ± 9a	9 ± 1a	0.423	0.742	0.083
α-Pinene	MT	557 ± 237a	414 ± 93a	1,174 ± 644a	453 ± 143a	0.405	0.328	0.474
α-Fenchene	MT	4 ± 2a	1 ± 1a	2 ± 1a	6 ± 3a	0.346	0.719	0.084
Camphene	MT	19 ± 7a	27 ± 10a	40 ± 20a	15 ± 5a	0.753	0.517	0.214
Octane, 2,6-dimethyl-	MT	127 ± 29a	179 ± 54a	118 ± 4a	165 ± 51a	0.776	0.223	0.949
Sabinene	MT	9 ± 1a	13 ± 5ab	14 ± 3a	6 ± 1b	0.639	0.158	**0.023**
β-Myrcene	MT	30 ± 3c	79 ± 26b	94 ± 34a	31 ± 5b	0.708	0.677	**0.004**
β-Pinene	MT	217 ± 134a	122 ± 43a	133 ± 34a	59 ± 32a	0.497	0.273	0.318
3-Carene	MT	883 ± 141b	1,278 ± 341b	1,886 ± 340a	663 ± 126b	0.486	0.149	**0.009**
Limonene	MT	29 ± 3b	59 ± 18b	85 ± 32a	27 ± 3b	0.636	0.403	**0.006**
m-Cymene	MT	34 ± 7a	57 ± 18a	67 ± 15a	35 ± 5a	0.477	0.721	**0.047**
β-Phellandrene	MT	76 ± 23a	94 ± 25a	114 ± 27a	40 ± 3a	0.743	0.237	0.066
γ-Terpinene	MT	3 ± 1b	5 ± 1b	10 ± 4a	2 ± 0b	0.529	0.074	**0.013**
Terpinolene	MT	24 ± 5b	39 ± 11b	76 ± 28a	22 ± 5b	0.320	0.216	**0.024**
o-Cymene	MT	34 ± 5a	331 ± 12a	40 ± 2a	47 ± 17a	0.315	0.520	0.657
p-Cymene	MT	1 ± 0a	3 ± 1a	3 ± 1a	1 ± 0a	0.963	0.736	**0.020**
cis-p-Mentha-1(7),8-dien-2-ol	MT-O	2 ± 0a	3 ± 1a	3 ± 1a	1 ± 0a	0.991	0.715	**0.018**
p-Mentha-1,5-dien-8-ol	MT-O	1 ± 0a	4 ± 3a	2 ± 1a	1 ± 0a	0.437	0.977	0.118
Geraniol	MT-O	2 ± 0a	3 ± 1a	3 ± 1a	1 ± 0b	0.619	0.399	**0.011**
p-Cymen-7-ol	MT-O	9 ± 2a	17 ± 8a	14 ± 3a	7 ± 2a	0.737	0.552	0.055
Ascaridole	MT-O	40 ± 8a	53 ± 17a	38 ± 2a	72 ± 28a	0.733	0.439	0.783
α-Humulene	ST	1 ± 0a	3 ± 1a	5 ± 2a	2 ± 0a	0.233	0.382	0.052
α-Longipinene	ST	9 ± 2a	11 ± 2a	36 ± 18a	13 ± 1a	0.067	0.401	0.215
(+)-Longifolene	ST	20 ± 8a	21 ± 4a	36 ± 10a	18 ± 2a	0.379	0.236	0.220
Cembrene	DT	29 ± 6a	41 ± 25a	65 ± 16a	24 ± 12a	0.572	0.418	0.138

nd, not detected.

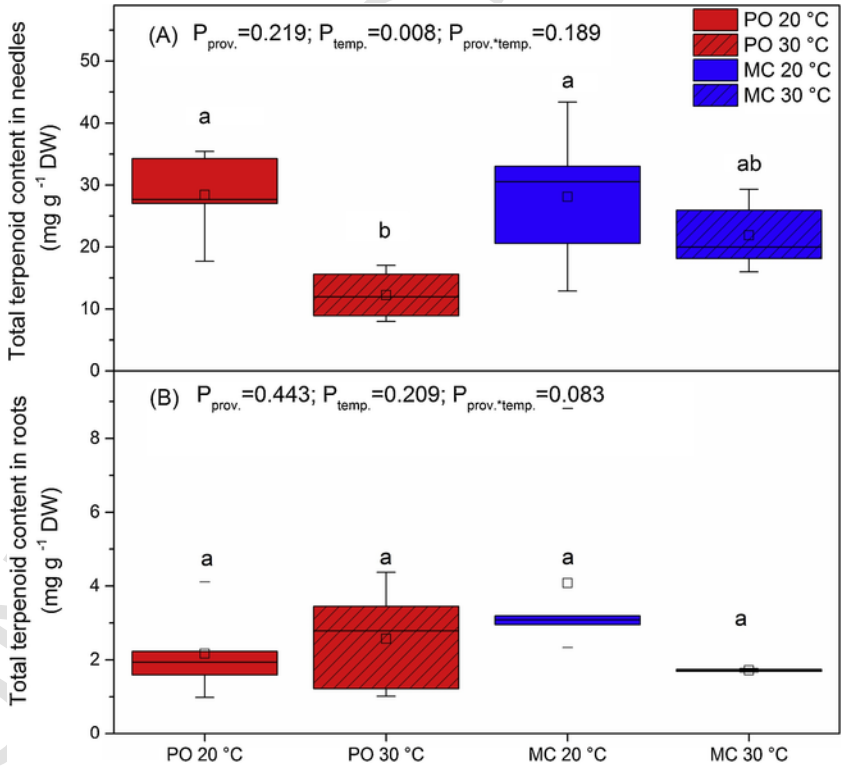

Fig. 1. Effects of elevated temperature on total terpenoid contents in needles (A) and roots (B) of PO and MC provenances. Seedlings were exposed to 20 °C and 30 °C and total terpenoid contents were quantified in the different tissues by GC-MC based on dry weight. Box plots show means (small empty squares) and medians (straight lines, n = 4–5 for each treatment). Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature.

(Fig. 1A and B). However, provenance-specific differences were observed for four terpenoids (*i.e.* β-phellandrene, β-pinene, citronellol and citronellyl acetate) in needles. In the needles of both provenances, camphene was the most abundant monoterpene (37% of total terpenoids), followed by α-pinene (19%), (-)-bornyl acetate (15%), tricyclene (7%) and β-pinene (6%). In roots of both provenances, monoterpene composition was dominated by 3-carene (45%), followed by α-pinene (25%), 2,6-dimethyl-octane (5%), β-pinene (5%) and β-phellandrene (3%) (Tables 1 and 2).

Elevated growth temperature and VPD caused significantly reduced total terpenoid contents in needles of plants belonging to the PO provenance when compared to the contents in control trees grown at 20 °C (Fig. 1A). This effect was caused by a general lower abundance of several terpenoids; we found significantly reduced needle contents of 13 terpenoids (*i.e.* 4-carene, α-phellandrene, α-pinene, β-myrcene, β-phellandrene, β-pinene, camphene, γ-terpinene, limonene, tricyclene, bornyl acetate, *δ*-elemene and γ-cadinene) (Fig. 2, Table 1). In contrast, contents of almost all terpenoids in needles of MC were not significantly affected, although a trend of decreased abundances in seedlings grown at 30 °C became visible. This trend was significant only for 5 terpenoids (*i.e.* 4-carene, α-phellandrene, β-phellandrene and the oxidized monoterpenoid (-)-citronellal) (Fig. 2, Table 1). Moreover, no interaction between provenance and temperature treatment was observed in needles except for bornyl acetate.

In contrast to needles, a non-significant trend towards reduced total terpenoid contents was observed in roots of the MC provenance when the seedlings were grown at elevated air temperature and VPD (Fig. 1B). This observation was caused by a cumulative effect of significantly decreased contents of 7 terpenoids, which were sabinene, β-myrcene, 3-carene, limonene, γ-terpinene, terpinolene as well as geraniol (Fig. 2, Table 2). However, there was no significant effect of elevated temperature on the total terpenoid contents as well as the abundance of individual root terpenoids in provenance PO except for β-myrcene (Fig. 1B, Table 2). In contrast to needles, significant interaction between provenance and temperature treatment was observed for 10 terpenoids in roots (*i.e.* sabinene, β-myrcene, 3-carene, limonene, m-cymene, γ-terpinene, terpinolene, p-cymene, p-mentha-1(7),8-dien-2-ol, geraniol).

To test if the composition of terpenoids differed between (i) tissues, (ii) two provenances and (iii) treatment, we performed sPLS-DA (sparce partial least square differential analysis) based on the contents of all individual terpenoids identified in the needles and roots of the tree seedlings. As expected from the large differences in terpenoid contents, needles and roots of both provenances separated into two clusters (Fig. 3A). The loading plots highlight that (-)-bornyl acetate, tricyclene, 2,6-dimethyl-octane and m-cymene had the greatest influence on the clustering of needle and root tissues (Fig. S1A). Interestingly, PO and MC provenances did not form individual clusters if the terpenoid composition of needles (Fig. 3B) and roots (Fig. 3C) was studied. However, elevated temperature caused a strong shift in needle terpenoid composition of the provenance PO (Fig. 3B). In contrast, no temperature effect on the terpenoid composition was observed for the provenance MC in both tissues (Fig. 3B, C).

3.2. Terpene emissions from needles significantly increased at elevated temperature and VPD in MC but not in PO provenance

Eight different terpenes were identified to be emitted from needles of both provenances (Table 3). Elevated temperature and VPD caused significantly enhanced total terpene emission rates from needles of MC, but not from needles of PO (Fig. 4). This was the result of by trend higher emissions of 3-carene, α-pinene, β-pinene and tricyclene at elevated air temperatures in MC (Table 3). Control plants of MC provenance, mainly emitted isoprene into the atmosphere with emission rates of 567 ± 331 ng m^{-2} s^{-1}. The most strongly released monoterpenes were limonene and β-pinene with emission rates of 118 ± 48 ng m^{-2} s^{-1} and 108 ± 60 ng m^{-2} s^{-1}, respectively. However, under exposure to elevated temperature and VPD, 3-carene dominated the emissions with rates of 195 ± 75 ng m^{-2} s^{-1}, followed by isoprene and α-pinene emissions. Differently from MC, control plants of PO mainly emitted limonene (169 ± 73 ng m^{-2} s^{-1}), isoprene (152 ± 93 ng m^{-2} s^{-1}) and β-phellandrene (24 ± 19 ng m^{-2} s^{-1}). Elevated temperatures changed this pattern, leading to highest emissions of α-pinene (1627 ± 1301 ng m^{-2} s^{-1}), followed by isoprene (851 ± 627 ng m^{-2} s^{-1}) and 3-carene (435 ± 98 ng m^{-2} s^{-1}).

3.3. Effects of elevated temperature and VPD on protein contents and TPS activities in current-year needles of PO and MC provenances

Protein contents in needles of PO amounted to 2.1 ± 0.3 mg g^{-1} FW and 2.6 ± 1.1 mg g^{-1} FW in plants grown under control conditions and elevated temperatures, respectively. Needles of MC contained 1.7 ± 0.2 mg g^{-1} FW (controls) and 2.3 ± 0.5 mg g^{-1} FW (elevated

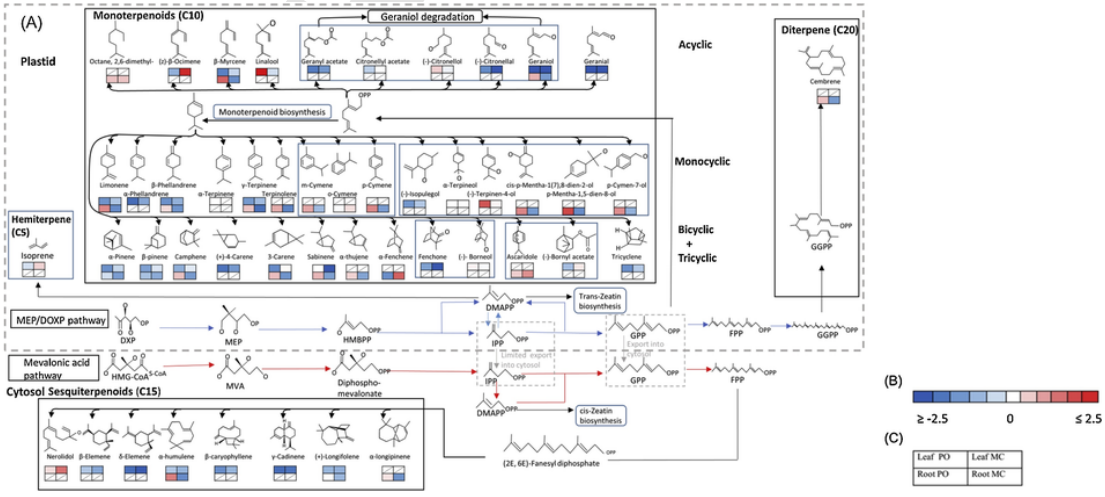

Fig. 2. Temperature- and provenance-specific effects on the terpenoid contents of needles and roots of PO and MC provenances (A). PO and MC were cultivated at 20 and 30 °C for 3 months. (B) Color code indicates log2 fold-changes of terpenoid concentrations of trees grown under 20 °C and 30 °C; red and blue colors indicate increased and decreased concentrations at 30 °C compared to 20 °C, respectively. (C) Order of the four squares below each terpenoid: upper part, fold-changes of needles in PO and MC provenances from left to right; below part, fold-changes of roots in PO and MC provenances from left to right. Empty squares with slash means not detected values at either 20 °C or 30 °C (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

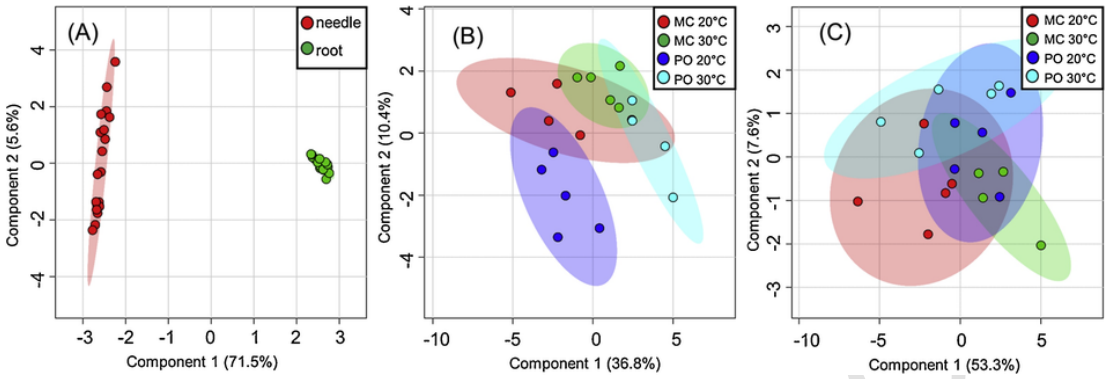

Fig. 3. sparse PLS-DA (sPLS-DA) algorithms based on specific individual terpenoid contents. Trees of the two provenances were exposed to growth temperatures of 20 °C and 30 °C. sPLS-DA was operated using Metabo Analyst 4.0. (A), sPLS-DA based on specific individual terpenoid contents of needles and roots. According to the variance of specific terpenoid contents along PC1 and 2 (capture 77.1% of the total variances), the groups of needles and roots were completely separated. (B) sPLS-DA based on specific individual terpenoid contents of needles in MC and PO provenances under 20 °C and 30 °C. According to the variance of specific terpenoid contents along PC1 and 2 (capture 47.2% of the total variances), the groups of PO-20 and PO-30 were completely separated. (C) sPLS-DA based on specific individual terpenoid contents of roots in MC and PO provenances under 20 °C and 30 °C. According to the variance of specific terpenoid contents along PC1 and 2 (capture 60.9% of the total variances), only a little part of different groups were overlapped.

Table 3
Terpene emissions from needles of the Douglas fir provenances Pend Oreille (PO) and Monte Creek (MC) grown at 20 and 30 °C and two-way ANOVA results. Means ± s.e. of 4 replicates each are shown. Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature. HT, hemiterpene; MT, monoterpene.

Terpene emission [ng m $^{-2}$ s^{-1}]		Pend Oreille (PO)		Monte Creek (MC)		$P_{prov.}$	$P_{temp.}$	$P_{prov.*temp.}$
		20 °C	30 °C	20 °C	30 °C			
Isoprene	HT	567 ± 331	159 ± 159	152 ± 93	851 ± 627	0.712	0.697	0.156
3-Carene	MT	19 ± 14	195 ± 75	nd	435 ± 98	0.303	**<0.001**	**0.027**
α-Pinene	MT	nd	98 ± 79	nd	1627 ± 1301	na	na	na
β-Phellandrene	MT	18 ± 18	7 ± 7	24 ± 19	nd	na	na	na
β-Pinene	MT	108 ± 60	nd	nd	219 ± 82	na	na	na
Camphene	MT	23 ± 23	19 ± 13	12 ± 12	294 ± 214	na	na	na
Limonene	MT	118 ± 48ab	20 ± 13b	169 ± 73ab	370 ± 167a	**0.022**	0.919	0.101
Tricyclene	MT	11 ± 11	7 ± 4	nd	98 ± 73	na	na	na

nd, not detected; na, not applicable.

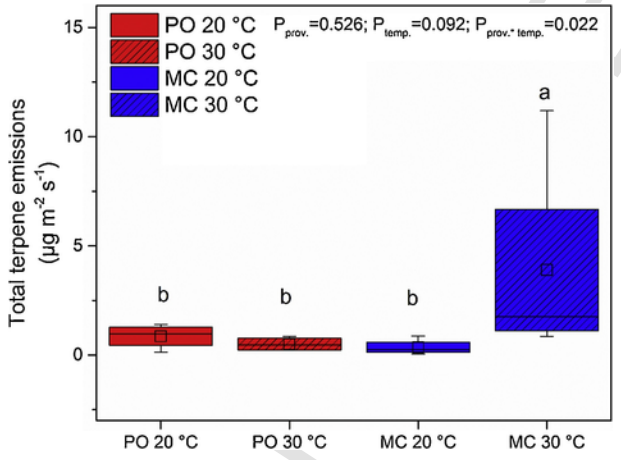

Fig. 4. Effects of elevated temperature on total terpene emissions from current-year needles of PO and MC provenances. PO and MC provenances were exposed to 20 °C and 30 °C and total terpene emissions were calculated by the sum of all the identified emitted terpenes from needles. Box plots show means (small empty squares) and medians (straight lines, n = 4 for each treatment). Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature.

temperature). However, there was no significant effect of temperature on the protein contents of both provenances.

A trend towards reduced total apparent *in vitro* TPS activities was observed in the needles of MC at 30 °C compared to that at 20 °C. However, needle TPS activities of the PO provenance were relatively stable under elevated temperature (Fig. 5A). TPS enzymes of PO grown under control conditions mainly produced trans-geraniol, camphene and (-)-bornyl acetate at activities of 149 ± 16 μkat kg^{-1} protein, 37 ± 14 μkat kg^{-1} protein and 26 ± 5 μkat kg^{-1} protein, respectively (Table 4). In PO plants grown under elevated temperatures, production of geraniol was still dominating (142 ± 45 μkat kg^{-1} protein), followed by the formation of camphene (27 ± 9 μkat kg^{-1} protein) and linalool (22 ± 15 μkat kg^{-1} protein) (Table 4). The biosynthesis of nerolidol was by trend decreased at higher growth temperatures; the production of all other terpenoids was unaffected by growth temperature. Similarly, in the needles of MC, geraniol was the main product of TPS accounting for 45% and 65%, under control conditions and elevated temperatures, respectively (Fig. 5B). Elevated temperatures caused a shift in the terpenoids produced as the share of camphene and α-pinene increased compared to, for example, linalool (Fig. 5B).

4. Discussion

4.1. Provenance-specific effect of air temperature and humidity on leaf terpenoid contents

The total amount of needle terpenoids of the two Douglas fir provenances ranged roughly between 8 and 43 mg g^{-1} DW which is in very

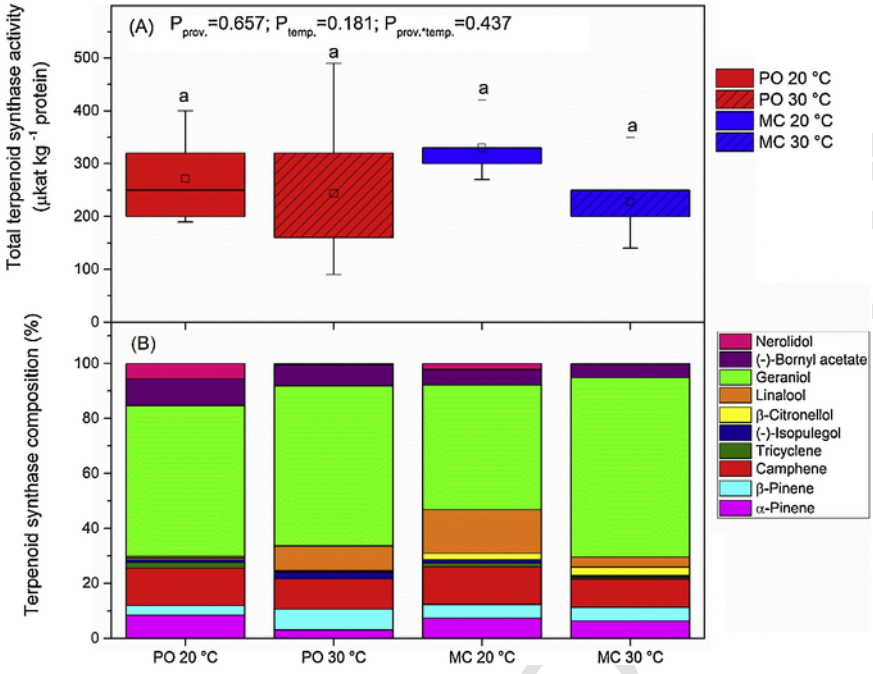

Fig. 5. Effects of elevated temperature on total terpenoid synthase activities (A) and the terpenoids produced by terpenoid synthases (B) in needles of PO and MC. Trees of the two provenances were exposed to growth temperatures of 20 °C and 30 °C.Box plots (A) show means (small empty squares) and medians (straight lines, n = 5 for each treatment). Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature.

Table 4
Terpenoid synthase activities in needles of the Douglas fir provenances Pend Oreille (PO) and Monte Creek (MC) grown at 20 and 30 °C and two-way ANOVA results. Means ± s.e. of 4 replicates each are shown. Different letters denote significant differences at $P < 0.05$ as calculated by two-way ANOVA with Tukey's pairwise multiple comparison test. P_{prov}, P_{temp}, $P_{prov.*temp}$: P-values for the effects of provenance, temperature and the interaction of provenance with temperature. MT, monoterpene; MT-O, oxygenated monoterpenoid; MT-A, acetylated monoterpenoid; ST-O, oxygenated sesquiterpenoid.

Terpenoid synthase activity [μkat kg^{-1}protein]		Pend Oreille (PO)		Monte Creek (MC)		$P_{prov.}$	$P_{temp.}$	$P_{prov.*temp.}$
		20 °C	30 °C	20 °C	30 °C			
α-Pinene	MT	23 ± 11	7 ± 1	24 ± 11	14 ± 3	0.597	0.121	0.714
β-Pinene	MT	9 ± 4	18 ± 5	16 ± 2	11 ± 2	0.996	0.577	0.067
Camphene	MT	37 ± 14	27 ± 9	45 ± 19	23 ± 5	0.861	0.233	0.652
Tricyclene	MT	6 ± 4	nd	4 ± 3	2 ± 1	na	na	na
(-)-Isopulegol	MT-O	3 ± 1	6 ± 5	4 ± 3	1 ± 1	0.531	0.619	0.760
β-Citronellol	MT-O	2 ± 1	1 ± 1	7 ± 3	7 ± 2	na	na	na
Linalool	MT-O	2 ± 1	22 ± 15	52 ± 43	8 ± 5	0.320	0.794	0.154
Geraniol	MT-O	149 ± 16	142 ± 45	149 ± 25	149 ± 28	0.902	0.902	0.919
(-)-Bornyl acetate	MT-A	26 ± 5	19 ± 8	19 ± 7	11 ± 3	0.218	0.222	0.968
Nerolidol	ST-O	15 ± 5	1 ± 1	7 ± 4	1 ± 1	na	na	na

nd, not detected; na, not applicable.

good agreement with published data (2–43 mg g^{-1} DW: Lerdau et al., 1995; Constable et al., 1999; Litvak et al., 2002; Pureswaran et al., 2004; and 1–11 mg g^{-1} FW: Gambliel and Cates, 1995; Zou and Cates, 1995; Giunta et al., 2016; Kleiber et al., 2017). Also the terpenoid composition was comparable to those found in these studies, where the needles were found to mainly contain monoterpenes and acetylated monoterpenoids. Although the natural sites of the provenances used in the present study were close to the *menziesii-glauca* transition zone (Lavender and Hermann, 2014), both provenances showed a terpenoid chemotype typical for interior (*i.e. glauca*) provenances. This chemotype is characterized by an α-pinene/β-pinene ratio < 1, with camphene as the main monoterpene in needles in combination with high levels of bornyl acetate (von Rudloff, 1972, 1975; Lavender and Hermann, 2014; Kleiber et al., 2017).

Long-term exposure to air temperatures above the temperature optimum of photosynthesis (Lewis et al., 2001) in Douglas fir together with increased VPD significantly decreased total terpenoid contents in needles of PO seedlings. This effect was particularly due to decreased levels of 13 individual terpenoids (see Table 1). In contrast, elevated air temperature only slightly (not significantly) affected the terpenoid contents in needles of plants from the MC provenance (only the levels of five terpenoids were significantly reduced). This result suggests that provenance-specific responses to heat stress even though the temperature regime at the origin of both provenances were similar. Obviously, the provenance from the more humid environment (PO) reacted more sensitive to elevated air temperatures in combination with atmospheric drought. This conclusion is in agreement with findings of Du et al. (2014) studying the N metabolism of Douglas fir in the same experiment, where plants from the PO provenance were more sensitive to the stress than those from MC. In good agreement, other data from the same experiment (Jansen et al., 2014) indicated that carbon metabolism in plants of PO was more affected by the stress treatment than

plants of MC. There were strong hints for a metabolic shift from primary metabolism to plant secondary metabolism with enhanced production of defense compounds such as polyols and aromatics. This might suggest that PO invested more C and energy in production of such secondary metabolites at the expense of terpenoids. Moreover, particularly in plants of PO, CO_2 assimilation seemed to be inhibited due to the stress, thus, further limiting the substrate supply for terpenoid biosynthesis. Unfortunately, CO_2 assimilation was not directly measured in this experiment, but reduced photosynthesis is indicated by (i) slightly elevated $\delta^{13}C$ in water soluble organic matter of current years needles and fine roots, (ii) reduced accumulation of above- and belowground biomass (Jansen et al., 2014), and (iii) literature data demonstrating that net photosynthesis of Douglas fir is reduced at elevated VPD (Meinzer, 1982). Moreover, the temperature optimum of photosynthesis was clearly exceeded in our heat stressed plants, thus leading to reduced assimilation rates in the thermally treated plants (Lewis et al., 2001). Elevated needle carbohydrate contents, particularly in thermally treated plants of PO, further demonstrate that carbon allocation between shoot and roots was disturbed. Similar to our results, Snow et al. (2003) also identified reduced levels of some terpenoids (bornyl acetate and geranyl acetate) in current-year needles of an interior provenance of Douglas fir trees when exposed to slightly elevated air temperatures for about two months (+3.5 °C compared to ambient temperatures). In other studies, elevated air temperature did not cause consistent effects on leaf terpenoid contents of the Mediterranean species *Pinus halepensis*, *Pistacia lentiscus*, *Rosmarinus officinalis* and *Globularia alypum* (Llusià et al., 2006). Even enhanced terpenoid contents were demonstrated in needles of conifers but exposure to higher air temperatures lasted for shorter periods of time in those experiments (e.g. *Pinus sylvestris, Picea abies*: Sallas et al., 2003; in *Quercus ilex, Pinus halepensis*: Blanch et al., 2009). We therefore conclude that changes in the terpenoid contents of plants occur species- and provenance-specific on the one hand and strongly depend on the stress regime such as duration and strength of the thermal stress on the other hand. Considering the literature, it seems that short-term exposure to elevated air temperatures rather increases the terpenoid contents whereas long-term exposure leads to a reduction (Tingey et al., 1996; Constable et al., 1999; Litvak et al., 2002; Snow et al., 2003).

The actual needle terpenoid contents are determined by the production rate and/or the loss of terpenoids via emission into the atmosphere (Loreto and Schnitzler, 2010). Biosynthesis of terpenoids is influenced by environmental factors such as light and temperature, and plant internal parameters such as the availability of substrates, the cellular energy and redox status, as well as TPS activities (Bohlmann et al., 1998; Hartikainen et al., 2012; Vanhatalo et al., 2018). The enzymes of the MEP pathway responsible for hemi- and monoterpene biosynthesis (Dudareva et al., 2013), show a temperature optimum around 40 °C (Fischbach et al., 2000; Ghirardo et al., 2010b; Loreto and Schnitzler, 2010) and might even be stimulated if the plants are exposed to heat (Sharkey et al., 2008). In this study, *in vitro* needle TPS activities amounted to approx. 240-280 μkat kg^{-1} protein in PO and to 230-330 μkat kg^{-1} protein in MC. In both provenances, the oxygenated compound geraniol was produced at highest rates, but also the main monoterpenoids of needles such as α-pinene, β-pinene, camphene, and bornyl acetate were synthesized *in vitro*. We tested if the reduced plant internal terpenoid contents in provenance PO could be a result of diminished rates of biosynthesis which might be reflected by reduced TPS activities. However, elevated growth temperature and increased VPD did not decrease the total apparent enzyme activities in PO. Consequently, reduced terpenoid contents in provenance PO cannot be generally explained by lowered TPS activities. Still, we found several consistencies in the data. For example, the enzyme activities indicated a clear trend for reduced rates of α-pinene, camphene, tricyclene and bornyl acetate biosynthesis, which was fully supported by considerable lower contents of these terpenoids in needles of thermal stressed PO plants. Moreover, based on the enzyme activities, linalool biosynthesis seemed to be up-regulated, and indeed, increased linalool levels were observed in PO plants exposed to elevated air temperature and VPD. The trends for the other compounds formed in the enzyme assays were less clear which might at least partially be due to either very low enzyme activities (for formation of isopulegol, β-citronellol, β-pinene) or low abundances of these terpenoids in needles of the PO provenance (nerolidol, geraniol). Similar inconsistencies between TPS activities and terpenoid levels in needles and leaves were found in *Pinus sylvestris* (Vanhatalo et al., 2018) and *Rosmarinus officinalis* (Peñuelas and Llusià, 1997). The observed reduced amounts of many other terpenoids in needles of thermally stressed PO which were not found in the *in vitro* enzyme assays, might be due to emission losses of these compounds from leaf internal storage pools during the initial phases of the experiment. However, since we did not assess this parameter this assumption remains speculative. Moreover, it must be mentioned that our *in vitro* enzyme assays were performed at non-limiting supply of GDP and FDP and, therefore, are indicative for the capacity to synthesize mono- and sesquiterpenoids but not directly for *de novo* biosynthesis *in planta*. Due to insufficient supply with substrate terpenoid synthesis could be limited causing the observed reduction of terpenoid abundance in needles of PO provenance. This assumption is in accordance with impaired carbon metabolism of our plants as shown earlier (Jansen et al., 2014).

In contrast to PO provenance plants, the enzyme activities for the formation of many terpenoids decreased in plants of the MC provenance exposed to thermal stress. This result is counterintuitive when considering the unaffected terpenoid levels in needles of thermal-stressed tree seedlings. The findings of the present study suggest that in MC long-term exposure to air temperatures far above the optimum of photosynthesis of Douglas fir (Meinzer, 1982; Lewis et al., 2001) and at high VPD, limited the substrate availability for terpenoid production and, in consequence, the activities of the enzymes of terpenoid biosynthesis. As mentioned above, this assumption is supported by altered carbon metabolism of the trees in the present experiment (Jansen et al., 2014), particularly due to a metabolic shift towards the biosynthesis of plant secondary metabolites at the expense of a more complete oxidation of carbohydrates (Jansen et al., 2014). Hence, limitation of substrate availability and altered energy status in needles of the trees might have resulted in diminished terpenoid biosynthesis due to warming in MC provenance plants as proposed earlier (Sharkey et al., 2008).

4.2. Terpene emissions are differentially affected in PO and MC

Terpene emission rates of control trees of both provenances ranged around 1 μg $m^{-2} h^{-1}$ (*i.e.* 13 μg g^{-1} DW h^{-1}) which was a little higher than emission rates observed from non-stressed Douglas fir seedlings in other studies (0.1–5 μg $g^{-1} h^{-1}$: Constable et al., 1999; Pressley et al., 2004; Joo et al., 2011; Helmig et al., 2013). The composition of emitted terpenes, however, was very similar to published data on this tree species indicating a strong contribution of α-pinene, 3-carene, limonene and β-pinene making up more than 70% of all terpenes emitted. Isoprene emission contributed to *ca.* 22% to the overall emissions.

We observed clear provenance-specific temperature and VPD effects on the emission of terpenes. Whereas emission of PO was unaffected by the temperature treatment, it increased in MC. Enhanced release of terpenoids from needles of coastal Douglas fir was also demonstrated in a field study on trees grown 4 °C above ambient temperatures (Constable et al., 1999). Even 8-times enhanced terpenoid emissions were observed in another field study on Douglas fir (Joo et al., 2011). Similarly, other conifers emitted more terpenoids at elevated temperature (*Pinus ponderosa* subsp. *scopulorum*: Harley et al., 2014; Constable et al., 1999; *Pinus eliottii*: Tingey et al., 1980); such temperature dependencies were demonstrated for monoterpenes (Tingey et al., 1980),

sesquiterpenes (Duhl et al., 2008) and recently even for the diterpene kaurene (Yáñez-Serrano et al., 2018).

Terpenoid emission into the atmosphere is controlled by physico-chemical characteristics of the terpenoids themselves (e.g. volatility), abiotic (e.g. temperature, light, air humidity) and biotic factors (e.g. herbivory), as well as plant internal factors such as stomatal conductance (Niinemets et al., 2002; Niinemets and Reichstein, 2003; Ghirardo et al., 2012; Vanhatalo et al., 2018). Since δ^{13}C-signatures of needles of the same experimental trees were slightly increased by elevated temperatures and VPD (Jansen et al., 2014), stomatal conductance most likely was rather reduced due to the stress treatment. The unaffected terpene emission rates of PO provenance and the higher rates of terpene emission by plants of MC origin, on the other hand, must be due to other reasons rather than opening of the stomata. Since the abundance of leaf-internal terpene pools did not increase, enhanced emissions most likely resulted from an increased volatility of terpenes at higher temperature (Holopainen and Gershenzon, 2010), or from altered properties of the storing structures (e.g. mesophyll cells or resin ducts) which might have reduced the resistances for terpenoids to be released into the atmosphere (Loreto and Schnitzler, 2010). Moreover, the increased terpenoid emissions under thermal stress and high air VPD might be related to long-distance transport of terpenoids through transpiration from stored terpenoids in other tissue parts, such as resin ducts in bark, wood or roots except needles. Meinzer (1982) discovered that the transpiration rate from current-year twig of Douglas fir was increased under enhanced leaf to air VPD, which could be considered as one indirect hint for such a mechanism.

Although the main compounds abundant in needles were released from both provenances, the composition of emitted terpenoids differed from the composition of needle-stored terpenoids. This finding is in good agreement with many studies on other species (Schindler and Kotzias, 1989; Peñuelas and Llusià, 1997; Llusià and Peñuelas, 1998; Geron et al., 2000; Ormeno et al., 2007; Vanhatalo et al., 2018; Yáñez-Serrano et al., 2018) despite also a close correlation between needle terpenoid content and terpenoid emission rates has been described in Douglas fir (Lerdau et al., 1995). The discrepancy between needle terpenoid levels compared to the emitted terpenoids might arise from different volatilities of the compounds, more than on storing structure within the needles (with different resistances for the volatiles), or a contribution of *de novo* synthesized compounds which has been demonstrated in several conifers (Ghirardo et al., 2010a). In addition, we can also rule out stomatal conductance as a main driver since (1) δ^{13}C-signatures rather indicate slight stomatal closure and (2) an increase in VPD is unlikely to increase stomatal conductance, which could explain the higher emission in MC.

4.3. Root terpenoids

Most of the work on volatile terpenoids in plants was conducted on aboveground plant parts, mainly leaves, whereas the knowledge on root terpenoids is still scarce. Under control conditions the root terpenoid content in MC amounted to approx. 1.5–4 mg g^{-1} DW which was about two times higher than in plants from the PO provenance originating from a wetter environment. In consistence with an earlier study on different provenances of Douglas fir (Kleiber et al., 2017) but also on chemotypes of other species (Kleine and Müller, 2014), provenance-specific patterns were absent as root terpenoid compositions in PO and MC plants were nearly similar. Different from needles, the three most abundant terpenoids in roots were 3-carene, α-pinene and β-pinene, which is in good agreement with root terpenoid contents in Douglas fir seedlings of other provenances (Huber et al., 2005; Kleiber et al., 2017). These obvious differences in terpenoid composition between below- and above-ground tissues became clearly visible in sPLS-DA indicating separated clusters for samples of both plant organs (Fig. 3A).

Exposure of Douglas fir tree seedlings to elevated air temperature and VPD led to provenance-specific changes in terpenoid contents. Whereas trees of PO were widely unaffected by the thermal-stress treatment, there was a non-significant trend to declined total terpenoid contents in roots of MC provenance plants which was mainly driven by significantly reduced contents of seven monoterpenoids. This result was surprising considering the opposite effects found in the needles of the two provenances. It indicates that terpenoid biosynthesis in roots of provenance MC plants are more sensitive to changes in VPD and air temperature (and most likely also to elevated soil temperature which, however, was not monitored in our experiment). Reduced contents of some terpenoids might be due to substrate limitation for terpenoid biosynthesis as suggested from lowered root biomass in heat stressed compared to control plants (Jansen et al., 2014), thus, indicating inhibited allocation of carbohydrates from the shoot to the roots. This assumption is further supported by carbohydrate accumulation in the needles of the trees (Jansen et al., 2014), which is typical for impaired carbon allocation in heat and drought stressed plants often leading to reduced carbon supply to roots (Ruehr et al., 2009; Blessing et al., 2015; Liu et al., 2017; Birami et al., 2018). However, such alterations were observed in both provenances despite only plants of MC were diminished in contents of some terpenoid; hence terpenoid biosynthesis of plants of MC reacted somewhat more sensitive to the stress than that of PO. We cannot provide a final conclusion on such provenance-specific differences. A fascinating aspect on the role of soil bacteria on plant terpenes has been added recently. Salomon et al. (2014, 2016) observed an accumulation of the terpenoid phytohormone abscisic acid and the synthesis of defense related terpenes in leaves of grapevine, which was due to interaction with rhizobacteria. Particularly in agricultural plants, the effects of rhizobacteria on plant health and physiology has been acknowledged (see Lakshmanan et al., 2014) and it is intriguing if terpene biosynthesis as an important part of plant defense would be triggered by such interaction. Moreover, other work reported on the biosynthesis of terpenoid phytohormones (e.g gibberellines, abscisic acid) by beneficial soil microbes (Creus et al., 2004; Piccoli et al., 2011; Egamberdieva et al., 2017; Park et al., 2017; Shahzad et al., 2017) with positive effects on plant performance. Elucidation of the mechanisms involved and the effects on thermally treated Douglas fir provenances was far beyond the scope of our work but should be in the focus of future approaches. In contrast to our study, roots of several *Daucus carota* cultivars even increased the terpenoid contents at elevated growth temperatures (Rosenfeld et al., 2002; Ibrahim et al., 2006). It is challenging to speculate if changes in terpenoid contents under environmental stress allow insight into functions of terpenoids. The role of terpenoids in roots and their release into the soil might be related to plant-plant, plant-mycorrhiza, plant-micobial and plant-animal communication (Ens et al., 2009; De-la-Peña et al., 2012; Trowbridge and Stoy, 2013; Lavender and Hermann, 2014; Kleine and Müller, 2014; Ditengou et al., 2015; Delory et al., 2016; Gfeller et al., 2019). A particular role of root terpenoids might be protection of the belowground plant tissue against soil-abundant herbivores as suggested from experiments in which roots were exposed to the stress hormone methyl jasmonate (Huber et al., 2005) and reviewed by Peñuelas et al. (2014).

Taken together, in a provenance-specific way, the levels of terpenoids in needles (PO) were reduced and terpenoid emission (MC) was increased after long-term exposure to elevated air temperature clearly above the optimum of photosynthesis in Douglas fir and to increased VPD, which partly supports our hypotheses (1) and (2). This was the case although both provenances were of the interior chemotype regarding the terpenoid composition (von Rudloff, 1972, 1975; Kleiber et al., 2017). Long-term adaptation to the different precipita-

tion levels at the site of origin might play a role for the different responses to heat. Reduced terpenoid contents can contribute to a weakening of the tree seedlings exposed to the stress, thus, contributing to a higher susceptibility of the stressed trees against above- and belowground herbivores.

Author contribution statement

JK, IE, AG, HR and JPS designed and planned the experiment. KJ, AK and LVK performed the experiments. QD, AK, IZ, BK and HG analyzed samples in the laboratory and analyzed raw data. QD and JK mainly wrote the manuscript; BK, AG, JPS, HR and IE discussed results and contributed to the writing, all authors commented to the manuscript.

Uncited reference

Rennenberg et al. (2006)

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.103819.

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