Combined Effect of Altitude, Season and Light on the Accumulation of Extractable Terpenes in Norway Spruce Needles

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Abstract: Plants produce specific terpenes, secondary metabolites conferring tolerance to biotic and abiotic stresses. Our study aims to investigate the effects of altitude, light intensity and season on contents of mono- and sesquiterpenes in needles of coniferous Norway spruce (Picea abies). Needles of current shoots representing upper and lower canopy were collected from adult trees growing along an altitudinal gradient (400–1100 m a.s.l.) in summer and autumn. After the extraction in cold heptane, the content of extractable terpenes was determined by gas chromatography coupled with mass spectrometry. Our results show that the total content of terpenes decreases with increasing altitude regardless of canopy position and season. Needles of the upper canopy have a higher total content of terpenes than lower canopy needles, but this difference decreases with increasing altitude in summer. Total content of extractable terpenes increases in autumn when compared to summer particularly in upper canopy needles of trees from high altitudes. Limonene, camphene, α-pinene and myrcene are the most abundant monoterpenes in spruce needles forming up to 85% of total monoterpenes, while germacrene D-4-ol is the most abundant sesquiterpene. Altitude, canopy position and season have a significant interactive effect on most monoterpenes, but not on sesquiterpenes. Terpenoid biosynthesis is thus tightly linked to growth conditions and likely plays a crucial role in the constitution of stress tolerance in evergreen conifers.

Keywords: altitude; canopy position; Picea abies; secondary metabolites; vegetation season; volatile terpene content

1. Introduction

Environmental conditions such as precipitation, temperature, sky turbidity, intensity of solar radiation and/or nutrient availability vary along altitudinal gradients [1,2]. Consequently, physiological and biochemical plant traits change to ensure the adaptation and survival of plant species in different altitudes. Previous studies reported altitude-induced changes in leaf mass per area [3], stomatal density [4], CO₂ assimilation rate and Rubisco carboxylase activity [5,6], and/or production of secondary metabolites [7]. Thus, altitudinal studies enable the identification of the most relevant environmental factors affecting plant communities and mechanisms involved in plant acclimation and adaptation responses to environmental conditions.

Secondary metabolites, including terpenes, are key components for plant defense and may be involved in long-term acclimation and/or local adaptation to both biotic and abiotic stresses [8–10]. Terpenes are widely occurring organic compounds which are produced by plants, particularly conifers. Terpenes are synthesized from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [11] in different plant tissues. In conifers,
monoterpenes and sesquiterpenes are constitutively produced by secretory epithelial cells and stored in high concentrations in resin cells of stems and needles [12,13]. Alternatively, plants store terpenes in small temporary pools in leaf mesophyll which is, for example, typical for beech trees [14].

Generally, terpenes act as herbivore deterrents, antibacterial and anti-mycotic substances [15], pollinator attractants and/or signals in plant–plant communication [16]. Terpenes (isoprene, mono- and sesquiterpenes) may be further involved in plant protection against abiotic stresses including extreme temperatures and/or drought [17,18]. The potential of terpenes to reduce ozone damage [19] and to quench reactive oxygen species [20] has also been demonstrated.

The synthesis and accumulation of secondary metabolites are tightly linked to external factors such as light, temperature or soil water content. The dependence of terpene biosynthesis on altitude is thus likely. However, there are rather few studies investigating the effect of altitude on terpenes, and reports are inconsistent. For example, Demasi et al. [21] reported a negligible effect of altitude on terpene content in lavender, while an increase in monoterpene content with increasing altitude was found in leaves of mastic trees [22] and juniper needles [23]. Effects of vegetation season [24,25], light intensity and temperature [18,26,27] on the biosynthesis and the content of volatile terpenes have also been demonstrated.

Our experiment was designed to investigate the interactive effects of altitude, needle position in the canopy (i.e., light availability) and season on contents of extractable mono- and sesquiterpenes in Norway spruce needles. Norway spruce (Picea abies L. Karst.) is a widely distributed coniferous tree species in the boreal zone and in mountain regions of central Europe. Ongoing drought and the subsequent attack of bark beetles contribute to extensive damage of spruce stands at low altitudes and result in large economic losses. Investigation of protective mechanisms and their capacity, including the formation of secondary metabolites, is thus of great importance. Spruces emit high amounts of monoterpenes and isoprene stored in resin ducts, needles and bark as well as being produced de novo [28]. According to our knowledge, such a complex study of how altitude combined with the aforementioned factors affect the content of terpenes in plants has not been conducted thus far.

2. Materials and Methods

2.1. Site Description

Samples were collected on the southern slope of Hrubý Jeseník Mountains (Czech Republic, Central Europe; 50°2’ N, 17°9’ E) in June and September, hereafter referred to as summer and autumn, respectively. Mature (60–80 years old) Norway spruce (Picea abies L. Karst.) trees naturally growing at low (L; 400 m above sea level, a.s.l.), middle (M; 720 m a.s.l.) and high (H; 1100 m a.s.l.) altitudes were investigated. The basic characteristics, including tree height and the diameter at breast height (DBH), are shown in Table 1. Although the stand density was the same at all sites (~750 trees per hectare), the different forest structures (leaf area index was 7.5, 6.9 and 6.8 m² m⁻² at L, M and H altitudes, respectively) resulted in a lower penetration of solar radiation at the L altitude (transmittance amounted up to 4.3%) when compared with M (4.7%) and H (4.9%). Ambulant measurement of photosynthetically active radiation, using a quantum sensor LI-190 (LI-COR, Lincoln, NE, USA), revealed an intensity of 1450–1630 µmol m⁻² s⁻¹ during sunny days in summer at the level of upper canopy branches, while it was only 60–70, 66–77 and 71–80 µmol m⁻² s⁻¹ at the level of lower canopy branches at L, M and H altitudes, respectively.

Based on the long-term (1985–2015) meteorological datasets, the locality is characterized by an average air temperature decrease of ~2 °C and an increasing monthly sum of precipitation of approximately 10–20 mm per 300 m of altitude (Figure 1A). In the year of investigation, June monthly mean temperatures amounted up to 18 and 12 °C at L and H altitudes, respectively, while these were only 12 and 8 °C in September (Figure 1B). June was characterized by a low monthly precipitation sum amounting up to 20 and 50 mm,
while the relevant sums during September were 120 and 165 mm at L and H altitudes, respectively (Figure 1B).

Table 1. Basic characteristics (means ± standard error) of investigated trees and current-year needles of Norway spruce (P. abies) trees grown at low (L; 400 m a.s.l.), middle (M; 720 m a.s.l.) and high (H; 1100 m a.s.l.) altitudes. Tree characteristics: tree height (H; m), stem diameter at breast height (DBH; cm). Needle characteristics: leaf mass per area ratio (LMA; g m⁻²), leaf water content (LWC; %). Different superscript letters (a–f) indicate significantly different values at p ≤ 0.05.

|                  | L       | M       | H       |
|------------------|---------|---------|---------|
| Tree characteristics |         |         |         |
| H [m]            | 36 ± 0.6 a | 32 ± 0.6 b | 28 ± 0.8 c |
| DBH [cm]         | 47 ± 1.8 a | 45 ± 1.7 a | 43 ± 1.5 a |
| Needle characteristics |      |         |         |
| LMA [g m⁻²]      |         |         |         |
| Upper            | 284 ± 9.4 c | 422 ± 28.1 a | 254 ± 4.6 cd | 338 ± 10.9 b | 225 ± 7.8 d-f | 351 ± 10.3 b |
| Lower            | 219 ± 14.1 d-f | 235 ± 10.1 d-f | 198 ± 10.9 f | 237 ± 14.4 de | 214 ± 11.2 e | 235 ± 18.1 d-f |
| LWC [%]          |         |         |         |
| Upper            | 41 ± 0.7 d | 42 ± 0.4 cd | 37 ± 0.7 e | 43 ± 0.3 cd | 34 ± 1.5 f | 43 ± 0.5 cd |
| Lower            | 46 ± 0.7 a | 44 ± 0.7 bc | 46 ± 1.1 ab | 43 ± 0.6 c | 47 ± 0.9 a | 43 ± 0.6 c |

Figure 1. Annual courses of air temperature (2 m above the ground) and monthly sums of precipitation close to the investigated plots at low (L; 400 m a.s.l.), middle (M; 720 m a.s.l.) and high (H; 1100 m a.s.l.): (A) Long-term (1985–2015) monthly means; (B) monthly means in 2015, year of the investigation.

Soil characteristics also substantially differed along the altitudinal gradient. The contents of organic carbon, nitrogen and potassium (K⁺) significantly increased with increasing altitude; however, the contents of phosphorus, calcium (Ca²⁺) and magnesium (Mg²⁺) decreased with increasing altitude. While C:N ratio was relatively constant along the gradient (ranging between 24.0 and 20.0 g g⁻¹), substantially lower C:P and N:P ratios were reported at low altitudes (8.9 and 0.4 g mg⁻¹, respectively) when compared to M and H altitudes (59.2–87.6 g mg⁻¹ and 2.6–4.5 g mg⁻¹, respectively). See Rajsnerová et al. [6] for further details of growth conditions including soil characteristics of individual localities.

2.2. Needle Sampling, Extraction and Gas Chromatography Analysis

In total five representative trees of average height and overall habits from each altitude (L, M, H) were chosen along the transects approximately 50 m long and 10 m wide. Such sampling design ensured similar growth conditions of selected trees. Their upper (upper canopy) and lower branches (lower canopy) with south and south-west orientation were cut during extended noon hours (10:00–14:00) to minimize the effect of diurnal variation in terpene production previously reported [29,30]. The branches were immediately recut under water and kept in water during the sampling. A mixed sample of current-year needles from three to four shoots was created.
For the determination of terpenes, a modified method according to Schönwitz et al. [31] was used. Approximately 1 g of fresh needles was immediately weighed, frozen in liquid nitrogen and stored at −80 °C until the extraction. Using a pestle, needles were homogenized with a mortar and 5 mL of cold heptane. Samples were ultrasonicated for 5 min and subsequently left in the fridge at 4 °C for 24 h. The extract (1 mL) was pipetted into clear 2 mL vials together with 30 µL of internal standard (1,4-cineole; concentration of 100 µg mL−1). To exclude the possible effect of different water contents in upper and lower canopy needles as well as in needles from different altitudes and seasons, the content of terpenes was recalculated per unit of dry weight. For this purpose, leaf water content (LWC; the leaf fresh minus dry weight divided by leaf dry weight, expressed as a percentage) was determined together with leaf mass per area (LMA; the ratio between leaf dry mass and projected leaf area). The projected area of fresh needles was determined using a portable leaf area meter (Li-3000A, LI-COR, Lincoln, NE, USA) and the needles were subsequently dried to a constant weight at 60 °C for ~48 h.

The gas chromatography (GC) analysis was performed with mass spectrometer (MS) TSQ Quantum XLS triple Quadrupole (Thermo Scientific, Waltham, MA, USA) on a 30 m, 0.25 mm (I.D.), 0.25 µm column (ZB-5MS). To efficiently separate individual terpenes, the following previously tested temperature program was used. Samples (1 µL) were injected in a splitless mode. The inlet pressure of the carrier gas (helium) was 100 kPa at the initial oven temperature and its flow rate was 1.0 mL min−1; the injector temperature was 250 °C. The temperature gradient of the oven began at 35 °C and was kept at this temperature for 6 min followed by an increase to 210 °C at the rate of 10 °C min−1, kept for 1 min and followed by an increase of temperature to 280 °C at the rate of 20 °C min−1. The interface temperature was maintained at 250 °C. Mass spectrometer (electrospray ionization at 50 eV, ion source temperature 200 °C) was performed at full scan between 50 and 200 m/z (scan time 0.15 s).

2.3. Statistical Analysis

Before an analysis of variance (ANOVA), the data for individual parameters were tested for normality using the Kolmogorov–Smirnov test. A three-way fixed-effect ANOVA model was used to analyze the effects of altitude, canopy position, season and their mutual interactions on terpene contents. Subsequently, Fisher’s LSD post hoc test was used to detect significant differences between means of all treatments at p ≤ 0.05. All statistical tests were conducted using Statistica 12 software (StatSoft, Tulsa, CA, USA). To identify the variables that explained a higher proportion of the total variance, which could provide insight into the role of terpenes in spruce needles acclimated to conditions of different altitudes, a multivariate analysis by redundancy analysis (RDA) was performed using Canoco 5 software (Microcomputer Power, Ithaca, NY, USA).

3. Results

The GC–MS analysis revealed in total 20 extractable terpenes and their derivatives in spruce needles (15 monoterpens and 5 sesquiterpenes). All compounds detected are shown in Appendix A Table A1. Limonene, camphene, α-pinene and myrcene are the most abundant monoterpens (forming up to 85% of total monoterpens) while germacrene D-4-ol is the most abundant sesquiterpene (forming up to 94% of total sesquiterpenes) when expressed per needle dry weight. Some monoterpens (3-carene and borneol) were found only in upper canopy needles but not in the shade ones. Contents of some monoterpens were substantially reduced at the highest altitude (camphor, terpinolene, or α-terpineol) or completely disappeared (borneol) (Appendix A).

Multifactorial ANOVA shows significant (p ≤ 0.05) effects of altitude and canopy position on total contents of terpenes, monoterpens as well as sesquiterpenes while the effect of the season is less pronounced. No interactive effect of altitude and canopy position was found, but the combination of altitude, canopy position and season (A × C × S) had a
significant interactive effect on most monoterpenes and thus also on the total content of terpenes in spruce needles (Table 2).

Table 2. Summary of significance levels (p-values of the multifactorial ANOVA—analysis of variance) for the effects of altitude (A), canopy position (C), season (S), and their interactions (×) on amounts of specific mono- and sesquiterpenes extracted from Norway spruce (P. abies) needles. Bold p-values indicate a significant effect at p ≤ 0.05 (n = 5).

| Effect             | A    | C    | S    | A × C | A × S | C × S | A × C × S |
|--------------------|------|------|------|-------|-------|-------|-----------|
| Tricycane          | ≤ 0.001 | ≤ 0.001 | 0.002 | ≤ 0.001 | 0.126 | ≤ 0.001 |          |
| α-pinene           | ≤ 0.001 | ≤ 0.001 | 0.338 | 0.136 | ≤ 0.001 | 0.499 | ≤ 0.001 |
| Camphene           | ≤ 0.001 | ≤ 0.001 | 0.008 | 0.986 | 0.015 | 0.014 | ≤ 0.001 |
| β-pinene           | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | 0.002 | ≤ 0.001 |
| Sabinene           | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | 0.786 | ≤ 0.001 | 0.001 | ≤ 0.001 |
| Myrcene            | 0.003 | ≤ 0.001 | 0.122 | 0.083 | 0.187 | 0.396 | 0.752 |
| Limonene           | ≤ 0.001 | ≤ 0.001 | 0.669 | 0.007 | 0.609 | 0.009 | ≤ 0.001 |
| Eucalyptol         | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | 0.030 | 0.002 | 0.004 | 0.001 |
| 3-carene           | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 |
| Terpinolene        | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 |
| Camphor            | ≤ 0.001 | ≤ 0.001 | 0.011 | ≤ 0.001 | 0.590 | 0.190 | 0.363 |
| Fenchol            | ≤ 0.001 | ≤ 0.001 | 0.003 | 0.984 | 0.030 | 0.662 | ≤ 0.001 |
| Borneol            | ≤ 0.001 | 0.064 | 0.051 | 0.377 | 0.306 | 0.008 | ≤ 0.001 |
| α-terpineol        | ≤ 0.001 | ≤ 0.001 | 0.422 | ≤ 0.001 | ≤ 0.001 | 0.102 | ≤ 0.001 |
| Bornyl acetate     | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | 0.316 | ≤ 0.001 | 0.027 | ≤ 0.001 |
| β-caryophyllene    | ≤ 0.001 | 0.012 | ≤ 0.001 | 0.348 | 0.041 | 0.015 | 0.465 |
| Humulene           | 0.004 | 0.127 | 0.002 | 0.093 | 0.483 | 0.494 | 0.754 |
| Longifolene        | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | 0.377 | ≤ 0.001 | 0.023 | 0.041 |
| β-cadinene         | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | 0.023 | ≤ 0.001 | 0.455 | 0.977 |
| Germacrene D-4-ol  | ≤ 0.001 | ≤ 0.001 | 0.454 | 0.090 | ≤ 0.001 | 0.097 | 0.769 |
| Total              | ≤ 0.001 | ≤ 0.001 | 0.063 | 0.110 | 0.071 | 0.045 | 0.003 |
| Monoterpenes       | ≤ 0.001 | ≤ 0.001 | 0.044 | 0.111 | 0.079 | 0.041 | ≤ 0.001 |
| Sesquiterpenes     | ≤ 0.001 | ≤ 0.001 | 0.726 | 0.189 | 0.004 | 0.146 | 0.718 |

Most terpenes were negatively associated with increasing altitude (Figure 2). This is particularly true for monoterpenes, most notably camphor, α-terpineol, β-pinene, sabinene and α-pinene. Contents of camphor and α-terpineol in upper canopy needles decreased 22 and 12 times, respectively, with increasing altitude in summer, but not in autumn (Appendix A). On the other hand, 3-carene is the only monoterpe positively associated with increasing altitude (Figure 2). 3-Carene was not detected in lower canopy needles and trees from low altitudes. Its content was 2.3 and 1.3 times higher at H than M altitude and trees in autumn and summer, respectively. Similar to 3-carene, the content of bornyl acetate increased with increasing altitude (1.3 times comparing H and L altitudes) but only in upper canopy needles collected at autumn (Appendix A). The accumulation of terpenes was similarly affected by the position of the needles in the upper canopy and the summer period (Figure 2). Under these conditions, terpinolene, longifolene and β-cadinene were more accumulated. On the contrary, higher contents of humulene and β-caryophyllene were found in lower than upper canopy needles in autumn.

The total content of terpenes decreased with increasing altitude regardless of canopy position and season. While the terpene content in lower canopy needles was relatively constant during the season, it significantly increased in upper canopy needles of M and H trees in autumn (Figure 3). Accordingly, terpene content of upper canopy needles significantly decreased with increasing altitude (by 30 and 58% in M and H altitudes when compared to L altitude) in summer, but these differences (non-significant) were only up to 13% in autumn. Significantly higher total terpene contents were found in upper than lower canopy needles in trees of all altitudes and both seasons investigated, but no difference was observed between upper and lower canopy needles of H trees in summer (Figure 3). Evaluating the effect of season, the total content of terpenes was increased in autumn when compared to summer. The most significant increase, by 88%, was found in upper canopy needles from H altitude trees (Figure 2).
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Figure 2. Redundancy analysis (RDA) of the relationship between environmental factors (altitude, canopy position, season) and the content of individual terpenes in summer (June) and autumn (September) in Norway spruce (P. abies) needles. Abbreviations: 3-car = 3-carene, bor = borneol, bora = bornyl acetate, cphe = camphene, cpho = camphor, euc = eucalyptol, fen = fenchol, lim = limonene, myr = myrcene, sab = sabinene, ter = terpinolene, tric = tricyclene, a-pin = α-pinene, a-ter = α-terpineol, b-pin = β-pinene, ger = germacrene D-4-ol, hum = humulene, lon = longifolene, b-cad = β-cadinene, b-car = β-caryophyllene.

Figure 3. Total amount of terpenes in upper canopy (open columns) and lower canopy (filled columns) needles of Norway spruce (P. abies) growing at low (L; 400 m a.s.l.), middle (M; 720 m a.s.l.) and high (H; 1100 m a.s.l.) altitudes. Needles were collected in June (summer; A) and September (autumn; B). Columns represent means, and error bars show estimated standard deviations (n = 5 trees). Identical letters (a–g) indicate homogeneous groups with statistically non-significant differences (p > 0.05).

In spruce needles, the total content of monoterpenes is 12 to 28 times higher than the total content of sesquiterpenes depending on altitude, canopy position and season (Figure 4). Accordingly, changes in monoterpene content caused by changes in altitude, light availability or seasonality determine changes in total terpene content. Limonene, camphene, α-pinene and myrcene are the most abundant monoterpenes forming 78–85% of total monoterpenes, while germacrene D-4-ol is the most abundant sesquiterpene in spruce needles forming 73–94% of total sesquiterpenes. In upper canopy needles, the contents of germacrene-4-ol and total sesquiterpenes significant decreased with increasing altitudes.
The contents are lower by 31 and 52% in M and H altitude when compared to L altitude in summer, respectively. Similarly, lower canopy needles of L trees have almost three-times higher content of total sesquiterpenes when compared to lower canopy needles of M and H trees. However, such trend is substantially less pronounced in autumn and almost negligible in lower canopy needles (Figure 4). On the contrary, monoterpendes content in upper canopy needles of spruce trees from M and H altitudes significantly increased in autumn when compared to summer. It is particularly caused by the accumulation of camphene, limonene, myrcene and eucalyptol (Appendix A). For example, camphene and limonene increased by 16–24% and 150% in M and H altitude, respectively, comparing to L altitude. Even though camphor and sabinene had the greatest response to an altitudinal gradient. The contents of camphor and sabinene increased by 476 and 280%, respectively, in H when compared to L altitude.

**Figure 4.** Total amounts of monoterpenes (A,B) and sesquiterpenes (C,D) in upper (open columns) and lower canopy (filled columns) needles of Norway spruce (*P. abies*) growing at low (L; 400 m a.s.l.), middle (M; 720 m a.s.l.) and high (H; 1100 m a.s.l.) altitudes. Needles were collected in June (summer; left panels) and September (autumn; right panels). Columns represent means, and error bars show standard deviations (n = 5 trees). Identical letters indicate homogeneous groups with statistically non-significant differences (p > 0.05). Contents of most abundant terpenes are shown. Hatching legend for monoterpenes: vertical strokes = limonene, top-down strokes = camphene, dots = α-pinene, crossing strokes = myrcene, empty column = rest of monoterpenes. Hatching legend for sesquiterpenes: bottom-up strokes = germacrene D-4-ol, empty column = rest of sesquiterpenes.

**4. Discussion**

4.1. Terpenes under a Complex Environment

The total amount and composition of terpenes depend on plant genotype, and growth conditions including temperature, water availability or mineral supply, i.e., conditions varying along the altitudinal gradient [1,2,6]. Moreover, terpenes are modulated by the vegetation season and position of needles in the canopy [32]. Investigating these factors, we show, for the first time, interactive effects of altitude-driven growth conditions, light availability due to needle position in the canopies, and season on the extractable terpenes in spruce needles.
In general, total contents and the composition of stored terpenes are in accordance with previously reported values [24–26,33]. In all main genera of conifer trees (*Pinus, Picea* and *Abies*), α-pinene, β-pinene, camphene and limonene are usually the most abundant monoterpenes [34]. However, some minor compounds like cyclic monoterpenes (α- and β-phellandrene) and piperitone, previously detected in spruce needles exposed to drought [35] or enhanced ozone concentration [36], were not found in our study.

4.2. Effect of Season

Regarding seasonal effect, we have found that the total content of terpenes increases from summer to autumn, particularly due to increases in contents of camphene, α-pinene and myrcene. This is in accordance with findings reported earlier [26]. The seasonal variation in terpene abundances can be affected by several factors. Among other things, the differences in terpene content in early autumn (September) could be caused by simply enhanced biosynthesis or indirectly by reduced growth and plant tissue aging [37]. It has been shown that terpene contents vary particularly in buds and young needles in early summer but not in fully developed needles [38]. However, the changes in content and composition of terpenes in evergreen needles during the winter remain unknown and represent a field for further research, especially concerning the formation of frost resistance in plants.

Noticeably, the largest increase in total contents of mono- and sesquiterpenes was found in the upper canopies of trees from high altitude, i.e., trees exposed to most adverse conditions. Recent studies revealed that particularly camphene, α- and β-pinene induce defense against oxidative stress in plants via expression of salicyl acid (SA) and systemic acquired resistance (SAR)-related genes [39] or triggers frost hardening and de-hardening via the glucosylation of sesquiterpenes [40]. Terpenoid biosynthesis thus may play a key role in the modulation of cold stress tolerance, particularly in evergreen conifers.

4.3. Effect of Canopy Position

Solar radiation passing through a forest canopy is exponentially attenuated and leads to the formation of distinct light environments within the canopy [41]. Although the biosynthesis of terpenes is directly linked to intermediates of photosynthesis [42], the effect of light intensity on their biosynthesis remains unclear. Some studies reported an increase [43], while others show small or no effect [26]. Investigation of emission rates of volatile terpenes under standard conditions revealed higher emissions from sun than shade acclimated spruce shoots [18]. However, these differences disappeared in late summer (end of August). In our study, the difference between upper and lower canopy was distinct for both monoterpenes and sesquiterpenes although the difference gradually decreases with increasing altitude in summer. This is due to the decrease in terpene content in upper canopy needles. Noticeably, lower canopy needles do not display such a decreasing trend, especially in higher altitudes.

4.4. Effect of Altitude

Studies conducted to investigate the effect of altitude on terpene content reported inconsistent results. Enhanced production of terpenes in higher altitudes was reported by Said et al. [22] and Giupponi et al. [44], while Martz et al. [23] showed that latitude affects terpene content more than altitude. In accordance with Vokou et al. [45], we found the highest total terpene content under the conditions of the lowest altitude. However, the difference between low and high altitudes is substantially reduced at the end of the vegetation season. Accordingly, we hypothesize an involvement of two temperature-driven mechanisms during the vegetation season. In summer, temperatures at high altitudes (monthly mean air temperature 12 °C) limit photosynthesis [46] and consequently the production of the secondary metabolites (including terpenes) when compared to low altitudes (monthly mean air temperature 18 °C). However, in autumn, chilling temperatures at high altitudes (monthly mean air temperature below 8 °C) likely lead to the accumulation of jasmonates.
in spruce needles inducing subsequently biosynthesis of mono- and sesquiterpenes [31]. Such finding implies the importance of interactive effects between climate conditions, season and needle position within the canopy and indicates nonlinearities in temperature responses.

Temperature is also a key environmental driver influencing an abundance of herbivores and extension of pathogens, i.e., biotic factors affecting the content of terpenes [10,32,47]. Several studies have indeed shown that plants from lower altitudes associated with enhanced temperature are under excessive herbivore pressure [48,49]. It is assumed that terpene content, and particularly the content of 3-carene, may reflect an adaptation to biotic stress conditions. However, such adaptation could be further modulated by other factors particularly water availability affecting the sensitivity of plants to pathogens infestation [50] as well as the accession of terpenes [51].

The altitudinal gradient in the present study is characterized by total precipitation of 753 (L), 891 (M) and 1083 mm per annum (H). Particularly during the summer months, trees experience drought conditions at low altitudes [2,6]. It has been shown that severe drought conditions lead to the accumulation of monoterpenes [52] and total terpenes [51] in needles of coniferous tree species, while their emission through the closed stomata is reduced [53]. Moreover, stomatal closure is hypothesized to upregulate/downregulate specific terpenes under the condition of limited water availability [30]. Contents of camphene, α-pinene and tricyclene were reported to increase substantially in drought-stressed spruce trees [35].

High temperature and limited water availability at low altitudes may result in an enhanced production of reactive oxygen species (ROS) in plants [54]. Generally, ROS inhibit photosynthesis but the impact on the production of secondary metabolites is controversial, as some metabolic pathways, including mevalonate and MEP/DOXP pathways, are elicited by ROS [9,55]. Increased de novo biosynthesis of isoprene under enhanced oxidative stress is thought to result from the stimulated activity of isoprene synthase enzyme [56] and/or induction of the terpene antioxidant pathway [20]. Isoprene and other terpenes contribute to ROS quenching and thus to the protection of photosynthetic apparatus.

5. Conclusions

Altitude and canopy position have significant effect on monoterpenes as well as sesquiterpenes while the effect of the season is less pronounced. Apart from 3-carene, contents of all terpenes decrease with increasing altitude regardless of needle canopy position and season. Needles of the upper canopy have higher contents of terpenes than lower canopy needles, although this difference decreases with increasing altitude in summer. Evaluating the effect of season, the total content of terpenes increases in autumn when compared to summer, particularly at high altitudes. Altitude, canopy position and season thus have a significant interactive effect on most monoterpenes but not on sesquiterpenes. Biosynthesis of terpenes, secondary metabolites markedly contributing to stress tolerance of evergreen conifers, is tightly linked to growth conditions.

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**Appendix A**

**Table A1.** Effect of altitude (low L, middle M, high H), canopy position and season on the contents of individual detected monoterpenes and their derivates (MT) and sesquiterpenes (SQT). Means ± standard deviations are reported (n = 5).

| MT (µg g⁻¹ DW) | Altitude | Summer | Autumn |
|----------------|----------|--------|--------|
|                |          | Upper Canopy | Lower Canopy | Upper Canopy | Lower Canopy |
| 3-carene       | L        | -       | -       | -             | -             |
|                | M        | 2.17 ± 2.75 | -       | 14.73 ± 2.54  | -             |
|                | H        | 5.01 ± 1.53 | -       | 20.37 ± 4.77  | -             |
| Borneol        | L        | 9.04 ± 3.22 | 0.42 ± 0.53 | 4.52 ± 4.54  | 10.06 ± 4.11  |
|                | M        | 0.79 ± 1.10 | -       | 3.77 ± 3.99  | -             |
|                | H        | -       | -       | -             | -             |
| Bornyl acetate | L        | 77.02 ± 15.26 | 39.93 ± 10.86 | 62.53 ± 3.90  | 52.41 ± 3.18  |
|                | M        | 49.93 ± 5.97 | 28.10 ± 5.01 | 66.71 ± 12.03 | 23.34 ± 4.03  |
|                | H        | 40.72 ± 6.33 | 31.69 ± 4.84 | 85.81 ± 9.49  | 38.70 ± 8.49  |
| Camphene       | L        | 641.89 ± 98.85 | 503.86 ± 93.36 | 597.40 ± 78.90 | 572.50 ± 92.09 |
|                | M        | 392.46 ± 57.35 | 386.79 ± 24.55 | 486.20 ± 73.07 | 314.92 ± 62.89 |
|                | H        | 237.91 ± 18.85 | 268.48 ± 51.85 | 489.60 ± 26.79 | 282.78 ± 36.18 |
| Camphor        | L        | 17.43 ± 4.86 | 10.24 ± 3.57 | 17.70 ± 4.11  | 11.43 ± 1.99  |
|                | M        | 4.84 ± 0.72  | 4.78 ± 1.14  | 8.67 ± 0.65   | 5.66 ± 1.49   |
|                | H        | 0.80 ± 0.21  | 1.42 ± 0.33  | 4.58 ± 0.83   | 1.98 ± 0.23   |
| Eucalyptol     | L        | 203.29 ± 26.35 | 145.78 ± 23.15 | 211.03 ± 22.52 | 171.21 ± 21.03 |
|                | M        | 153.89 ± 2.97 | 109.72 ± 8.58 | 201.69 ± 34.47 | 116.81 ± 14.07 |
|                | H        | 70.98 ± 5.45  | 94.06 ± 23.93 | 189.14 ± 36.38 | 120.90 ± 12.16 |
| Fenchol        | L        | 19.17 ± 3.61  | 8.82 ± 1.77  | 15.59 ± 3.57  | 11.63 ± 2.51  |
|                | M        | 10.73 ± 2.66  | 6.51 ± 1.42  | 16.01 ± 1.48  | 6.39 ± 1.35  |
|                | H        | 4.71 ± 0.80  | 2.13 ± 0.37  | 9.90 ± 2.83   | 4.63 ± 1.59  |
| Limonene       | L        | 775.33 ± 204.19 | 399.76 ± 57.19 | 646.94 ± 82.76 | 433.21 ± 66.38 |
|                | M        | 510.75 ± 130.79 | 311.57 ± 45.21 | 593.04 ± 137.33 | 278.41 ± 67.76 |
|                | H        | 381.83 ± 19.07 | 573.21 ± 115.44 | 630.51 ± 87.54 | 294.36 ± 66.69 |
| Myrcene        | L        | 419.36 ± 105.44 | 84.31 ± 8.65  | 540.43 ± 231.41 | 149.09 ± 43.66 |
|                | M        | 537.12 ± 117.06 | 147.06 ± 24.31 | 536.44 ± 134.78 | 153.13 ± 42.65 |
|                | H        | 236.00 ± 67.69 | 54.51 ± 6.03  | 421.96 ± 140.04 | 137.95 ± 27.32 |
| Sabinene       | L        | 37.53 ± 4.92  | 25.44 ± 3.00  | 31.70 ± 6.14  | 28.37 ± 6.57  |
|                | M        | 18.12 ± 3.17  | 22.08 ± 5.20  | 28.97 ± 6.47  | 14.16 ± 2.12  |
|                | H        | 8.49 ± 1.68   | 10.88 ± 2.98  | 32.18 ± 5.55  | 17.18 ± 4.96  |
| Terpinolene    | L        | 3.64 ± 0.58   | 1.18 ± 0.29   | 2.20 ± 0.18   | 0.51 ± 0.12   |
|                | M        | 2.16 ± 0.21   | 0.78 ± 0.12   | 1.79 ± 0.33   | -             |
|                | H        | 0.83 ± 0.07   | 1.21 ± 0.28   | 2.38 ± 0.45   | 0.62 ± 0.15   |
| Tricyclene     | L        | 65.96 ± 10.42 | 49.87 ± 6.64  | 59.53 ± 7.14  | 58.35 ± 10.40 |
|                | M        | 40.13 ± 4.93  | 41.39 ± 3.40  | 46.07 ± 4.77  | 32.62 ± 6.27  |
|                | H        | 24.53 ± 2.06  | 20.10 ± 3.83  | 50.74 ± 1.91  | 29.62 ± 4.21  |
| α-pinene       | L        | 469.85 ± 79.93 | 272.68 ± 44.58 | 341.18 ± 54.41 | 318.33 ± 48.96 |
|                | M        | 232.07 ± 20.54 | 214.72 ± 20.54 | 275.57 ± 18.69 | 169.35 ± 34.20 |
|                | H        | 138.94 ± 5.32 | 144.17 ± 36.82 | 278.62 ± 20.35 | 152.24 ± 21.85 |
| α-terpineol    | L        | 8.07 ± 0.56   | 3.66 ± 1.19   | 6.35 ± 1.03   | 2.20 ± 0.26   |
|                | M        | 4.06 ± 0.81   | 0.98 ± 0.19   | 3.93 ± 1.03   | 1.83 ± 0.36   |
|                | H        | 0.66 ± 0.18   | 1.21 ± 0.17   | 4.02 ± 1.08   | 1.29 ± 0.13   |
| β-pinene       | L        | 96.72 ± 15.40 | 34.66 ± 1.01  | 44.25 ± 5.80  | 34.24 ± 1.39  |
|                | M        | 36.48 ± 7.21  | 21.92 ± 5.98  | 31.03 ± 3.09  | 16.67 ± 4.34  |
|                | H        | 20.26 ± 3.14  | 18.66 ± 3.74  | 42.29 ± 5.64  | 23.15 ± 3.00  |
### Table A1. Cont.

| Altitude | Summer | Autumn |
|----------|--------|--------|
|          | Upper Canopy | Lower Canopy | Upper Canopy | Lower Canopy |
|          |            |            |            |            |
| Germacrene D-4-ol | L | 177.45 ± 50.15 | 107.06 ± 27.79 | 152.33 ± 16.32 | 47.75 ± 5.94 |
|          | M | 115.24 ± 19.37 | 37.48 ± 3.73 | 131.63 ± 32.16 | 42.75 ± 12.43 |
|          | H | 79.14 ± 17.43 | 35.01 ± 6.22 | 106.15 ± 22.00 | 41.26 ± 10.34 |
| Humulene  | L | 6.43 ± 2.38 | 6.79 ± 2.21 | 8.63 ± 2.17 | 10.06 ± 3.23 |
|          | M | 7.62 ± 1.18 | 5.99 ± 1.91 | 9.24 ± 1.92 | 7.22 ± 1.60 |
|          | H | 6.36 ± 1.77 | 3.88 ± 0.54 | 6.75 ± 1.57 | 5.89 ± 1.39 |
| Longifolene | L | 2.64 ± 0.35 | 1.81 ± 0.53 | 1.11 ± 0.07 | 0.97 ± 0.30 |
|          | M | 1.79 ± 0.35 | 0.89 ± 0.27 | 1.17 ± 0.13 | 0.85 ± 0.10 |
|          | H | 0.88 ± 0.07 | 0.63 ± 0.14 | 1.04 ± 0.27 | 0.59 ± 0.09 |
| β-cadinene | L | 2.73 ± 0.25 | 2.45 ± 0.60 | 1.21 ± 0.11 | 1.06 ± 0.17 |
|          | M | 1.95 ± 0.43 | 1.15 ± 0.18 | 1.40 ± 0.12 | 0.75 ± 0.09 |
|          | H | 1.08 ± 0.10 | 0.68 ± 0.11 | 0.89 ± 0.25 | 0.56 ± 0.04 |
| β-caryophyllene | L | 4.97 ± 0.77 | 3.93 ± 1.12 | 6.13 ± 1.34 | 5.67 ± 1.13 |
|          | M | 4.13 ± 0.52 | 2.87 ± 0.64 | 3.05 ± 0.45 | 3.95 ± 0.80 |
|          | H | 4.04 ± 0.83 | 2.32 ± 0.53 | 4.89 ± 1.13 | 4.38 ± 1.10 |

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