Metabolite Composition of Paper Birch Buds after Eleven Growing Seasons of Exposure to Elevated CO\(_2\) and O\(_3\)

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Received: 19 February 2020; Accepted: 12 March 2020; Published: 17 March 2020

Abstract: Research Highlights: Long-term exposure of paper birch to elevated carbon dioxide (CO\(_2\)) and ozone (O\(_3\)) modified metabolite content of over-wintering buds, but no evidence of reduced freezing tolerance was found. Background and Objectives: Atmospheric change may affect the metabolite composition of over-wintering buds and, in turn, impact growth onset and stress tolerance of perennial plant species in spring. Materials and Methods: Low molecular weight compounds of paper birch (Betula papyrifera) buds, including lipophilic, polar and phenolic compounds were analyzed, and freezing tolerance (FT) of the buds was determined prior to bud break after 11 growing seasons exposure of saplings to elevated concentrations of CO\(_2\) (target concentration 560 µL L\(^{-1}\)) and O\(_3\) (target concentration 1.5 \(\times\) ambient) at the Aspen FACE (Free-Air CO\(_2\) and O\(_3\) Enrichment) facility. Results: The contents of lipophilic and phenolic compounds (but not polar compounds) were affected by elevated CO\(_2\) and elevated O\(_3\) in an interactive manner. Elevated O\(_3\) reduced the content of lipids and increased that of phenolic compounds under ambient CO\(_2\) by reallocating carbon from biosynthesis of terpenoids to that of phenolic acids. In comparison, elevated CO\(_2\) had only a minor effect on lipophilic and polar compounds, but it increased the content of phenolic compounds under ambient CO\(_2\) by reallocating carbon from lipophilic compounds to that of phenolic acids. Conclusions: Based on the freezing test and metabolite data, there was no evidence of altered FT in the over-wintering buds. The impacts of the alterations of bud metabolite contents on the growth and defense responses of birches during early growth in spring need to be uncovered in future experiments.

Keywords: Betula papyrifera; bud; carbon dioxide; frost hardiness; global change; metabolome; over-wintering; ozone

1. Introduction

The ongoing climate warming has caused the growing season to begin earlier in northern ecosystems \([1,2]\). This may increase the exposure of vulnerable plant tissues, such as developing buds, to spring frosts \([3,4]\). Perennial plant species can tolerate severe winters by undergoing remarkable structural, biochemical, and genetic adjustments in autumn as a response to decreasing temperature and shortening of photoperiod \([3,5]\). In plant cells, the most prominent biochemical changes involved
in the development of freezing tolerance (FT) include dehydration of cells, accumulation of proteins and carbohydrates, changes in hormone levels and increases in fatty acid desaturation in membrane lipids [5,6]. Development of cold hardiness, early growth and stress tolerance of the newly emerging leaves in spring are energy-demanding processes that are dependent on carbon (C) reserves fixed in the previous year after height growth cessation [7]. Factors that affect the growing season length and photosynthetic leaf area, and thus, accumulation of C reserves during the growing season could also be expected to affect the formation of over-wintering buds and FT in winter. Paper birch (Betula papyrifera) buds possess protective bud scales, embryonic foliage leaves that quickly expand as foliage leaves, and primordial leaves that expand with internodal extension [8]. Healthy buds enable vigorous growth onset and high plant resistance to biotic and abiotic stress factors in spring.

Carbon dioxide (CO$_2$) is the most important anthropogenic greenhouse gas. Concentration of atmospheric CO$_2$ is expected to increase through the next century [9]. An increase in this primary C source of plants will affect their metabolism and growth, especially when other growth resources are abundant [10,11]. Tropospheric ozone (O$_3$) is a global air pollutant and a greenhouse gas. In Northern America and Europe, there is a trend for increasing low and medium range O$_3$ concentrations [12]. Further, the timing of peak surface O$_3$ has been estimated to occur earlier in the spring in the future [13], which may affect the growth onset of different plant species and increase the cumulative O$_3$ uptake during the growing season. Ozone is a strong oxidant, causing accumulation of reactive oxygen species (ROS) and cellular damage inside leaves [14]. Even the current O$_3$ concentrations are known to reduce biomass growth of trees [14]. Several experiments on different plant species have shown that when plants are simultaneously exposed to concentrations of elevated CO$_2$ (eCO$_2$) and elevated O$_3$ (eO$_3$), the positive stimulus effect of eCO$_2$ on growth and photosynthesis is partially negated by eO$_3$ [15–17]. This is believed to be related to reduced stomatal conductance, which reduces O$_3$ flux into leaves under eCO$_2$ [18].

In contrast to a large body of research with effects of eCO$_2$ and eO$_3$ on primary and secondary metabolites in birch leaves [19–22], little work has been addressed on how these gases affect metabolite concentrations and FT of over-wintering buds of birch. According to the earlier studies on paper birch at the Free Air CO$_2$ Enrichment (FACE) experiment in Rhinelander, WI, USA (Aspen FACE), eCO$_2$ and eO$_3$ had great impacts on C gain of the saplings: leaf area, photosynthetic rate and photosynthetic leaf area display duration were higher in eCO$_2$, and lower in eO$_3$ than in the control plants [23–26]. In general, eCO$_2$ delayed, and eO$_3$ accelerated leaf abscission in autumn, but no clear pattern of the effects of the treatments on timing of bud burst was found [25]. Properties of paper birch buds were studied in autumn after eight years of exposure to eCO$_2$ and eO$_3$. It was found that although the size and total C content of the buds was not altered by the treatments, the contents of starch, nitrogen and water was reduced under eO$_3$, but not under eCO$_2$ and eCO$_2+$eO$_3$ treatments [25]. Could the treatment-induced changes in the metabolism and physiology of the paper birch saplings cause carry-over effects that reflect on the bud biochemical composition and FT in the subsequent spring? The results of the few experiments on the effects of eCO$_2$ and eO$_3$ on FT of forest tree species have been variable and seem to be dependent on the plant species. Elevated CO$_2$ reduced FT in the leaves of Eucalyptus pauciflora [27] and Larix decidua [28], whereas in the buds of Betula alleghaniensis [29] and Picea mariana, FT was improved [30]. The mechanisms of how eCO$_2$ might affect FT may include changes in the timing of cold acclimation [27], alterations in the composition of cell walls and membranes [31] and changes in the concentrations of cryoprotective compounds in overwintering organs [32]. Elevated O$_3$ did not affect FT in buds of Betula pendula [33] or Fagus crenata [34]. However, eO$_3$ reduced FT in leaves of Ilex aquifolium, possibly due to membrane dysfunction under eO$_3$ [35]. The overall reduced energy reserves under eO$_3$ [14] may reduce the cryoprotective compounds in the cells of over-wintering organs.

The objective of this experiment was to examine how 11 years of growth under eCO$_2$ and eO$_3$ affects the chemical composition and FT of the overwintering birch buds prior to bud break in spring. Metabolites of the overwintering buds, including lipophilic, polar and phenolic compounds were studied. The potential impacts of the treatment-induced changes on stress tolerance of the buds are discussed.
Based on the existing literature and the results of the earlier studies on paper birch at the Aspen FACE site, we hypothesized that (1) increased C resources under eCO\textsubscript{2} may increase the C allocation to C-based secondary metabolites, but carbohydrate metabolism may not be significantly affected; (2) reduced C resources under eO\textsubscript{3} may result in reduction in carbohydrates and increase in defense-related compounds; (3) changes in metabolite content may alter the FT of the buds: eCO\textsubscript{2} may increase or decrease it, while the impact of eO\textsubscript{3} will be negative; and (4) the interactive effect of eCO\textsubscript{2} and eO\textsubscript{3} will be mainly counteractive.

2. Materials and Methods

2.1. Aspen FACE Site and Plant Material

The Aspen FACE facility is located close to Rhinelander, WI, USA (45.6° N, 89.5° W). In 1997, rooted cuttings of aspen (*Populus tremuloides*), and seedlings of paper birch and sugar maple (*Acer saccharum*) were planted on approximately 32 ha containing 12 experimental rings (30-m diameter): 3 replicates of control (ambient CO\textsubscript{2} (aCO\textsubscript{2}) and ambient O\textsubscript{3} (aO\textsubscript{3})), elevated CO\textsubscript{2} (eCO\textsubscript{2}, target of 560 ppm) and elevated O\textsubscript{3} (eO\textsubscript{3}, target 1.5 \times ambient), or a combination of eCO\textsubscript{2} and eO\textsubscript{3}. The three replicates were blocked across northern, central, and southern regions of the site, i.e., each block contained one replication ring of each treatment. The experiment was a full-factorial design. The present study used birch trees. Fumigation began in the spring of 1998 and continued during daylight hours for 11 growing seasons through 2008, from bud break until leaf fall [36]. In the growing season prior to the sampling for the present experiment (April 3–4, 2009), the exposures were started on May 23 and terminated on October 9, 2008. Carbon dioxide and O\textsubscript{3} were applied daily according to sun angle (about 30 min post-sunrise to pre-sunset). Ozone fumigation was interrupted when maximum temperature was projected to be below 15 °C or when foliage was wet. The monthly average concentrations in the growing season 2008 are shown in Table 1. The temperature conditions between June 2008 and May 2009 are shown in Figure 1. More details on the field site, experimental design and performance are available elsewhere [36,37].

Table 1. Mean of six experimental rings (SD) of hourly concentrations of CO\textsubscript{2} and O\textsubscript{3} at the Aspen Free Air CO\textsubscript{2} Enrichment (FACE) site for the hours from sunrise to sunset during the fumigation season 2008 [36].

|        | CO\textsubscript{2} (µL L\textsuperscript{-1}) | O\textsubscript{3} (nL L\textsuperscript{-1}) |
|--------|---------------------------------------------|---------------------------------------------|
|        | Ambient  | Elevated  | Ambinet  | Elevated  |
| May    | 402 (12) | 520 (71)  | 41 (7)   | 45 (15)   |
| June   | 405 (20) | 538 (62)  | 37 (10)  | 41 (18)   |
| July   | 395 (27) | 531 (69)  | 33 (11)  | 39 (17)   |
| Aug    | 382 (19) | 532 (68)  | 32 (11)  | 41 (19)   |
| Sept   | 397 (12) | 507 (59)  | 30 (13)  | 35 (19)   |
| Oct    | 393 (9)  | 518 (75)  | 28 (7)   | 28 (7)    |
Four birches from each ring were sampled on April 3, 2009, prior to bud break (four samples per ring). Three primary shoots of the lateral branches from the top third of the canopy, each containing four buds, were collected and frozen in liquid nitrogen, freeze dried and homogenized into a powder. Samples of bud powder (59–61 mg) were extracted with 1.0 mL of a cold chloroform/methanol/water mixture (3/5/2, v/v) with ribitol (40 µg/mL) and nonadecanoic acid methyl-ester (20 µg/mL) as internal standards. The extracts were divided into fractions of polar and lipophilic metabolites (for details see) [38]. Then, metabolites of both fractions were transformed into trimethylsilyl derivatives and analyzed by gas chromatography–mass spectrometry (GC-MS). The raw data were processed by TurboMass Gold V.5.4.0 software (Perkin-Elmer, Waltham, MA, USA), and the relative content of metabolites was normalized to the internal standards, and further to 1 g dry mass of bud sample.

Metabolites were identified using the mass spectral and retention time index (RI) libraries: NIST-08 and the Golm Metabolome Database (GMD, Max-Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany; http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html). Mass matches with thresholds of match > 800 (with maximum match equal to 1000) were accepted. RIs of metabolites were calculated according to Van den Dool and Kratz (1963) [39].

Phenolic compounds were studied in polar fractions by high-performance liquid chromatography with diode array detector (HPLC-DAD, Merck-Hitachi, Tokyo, Japan). For their identification, selected set of polar samples were analyzed by HPLC-MS Agilent 1200 (Santa Clara, CA, USA) with a BRUKER micrOTOF-Q-MS detector [40]. Relative contents of individual phenolic compounds were calculated as peak heights per 1 g of dry weight of bud sample.

2.3. Bud Length, Diameter, Dry Mass, Water Content and Freezing Test

Four birches from each ring were sampled on April 4, 2009. Seven primary shoots of the lateral branches from the top third of the canopy, each containing four buds, were collected. One shoot from each birch was used for the determination of bud length and diameter (measured with a digital caliper at the widest point), fresh and dry mass (48 h at 60 °C) and water content (average values for each tree were used in the statistical analyses). For the freezing test, six shoots were placed in a plastic bag with a moist paper tissue, and stored in a cool box with ice. The buds were detached and put in a glass tube and sprayed with distilled water. The tubes were either placed in a growth chamber (+5 °C, control)
or in four air-cooled chambers (WT600/70, Weiss Umwelttechnik GmbH, Reiskirchen-Lindenstruth, Germany) that were adjusted to 0 °C. Test temperatures were: +5, −10, −20, −30, −40 and −196 °C. The temperature in the chambers was lowered by 5 °C h−1. The desired temperatures were maintained for 3 h and were then raised to 0 °C (5 °C h−1). After the freezing exposures, the tips of the buds were split, and subjected to an electrolyte leakage test: 6 mL of distilled water was added in the tubes, which were placed in a shaker for 22 h (175 rpm). The first conductivity measurement was performed (UB-10 Ultrabasic, Denver Instruments, Denver, CO) (L1), and then the samples were heated at 95 °C for 45 min and shaken for 22 h, and the conductivity was measured for the second time (L2). The relative electrolyte leakage (REL) was calculated as REL = (L1/L2) × 100. REL method is based on the principle that plasma membranes in freeze-injured tissues allow increased leakage of electrolytes into the apoplastic water [41], i.e., high REL indicates high freezing damage in the tissues.

2.4. Statistical Analysis

Main effects and interaction of CO2 and O3 were tested using SPSS 25 for Windows (SPSS Chicago, IL, USA) by applying linear mixed model analysis of variance (ANOVA) with CO2 and O3 as fixed factors, and block as a random factor. P values ≤ 0.1 are reported as ‘significant’ to reduce the risk of committing a Type-II error due to the low degree of replication inherent in FACE experiments [42,43]. Significant interactions were studied further by conducting a simple main effects test (SME, i.e., post hoc test for interactions) with Bonferroni corrections (P ≤ 0.05). The normality of the data and homogeneity of variances were checked from residual plots. Data were ln-transformed to meet ANOVA requirements if necessary, and this information is given in Tables 2 and 3. The metabolite data were processed using R software (version 3.4) [44] and MetaboAnalyst (https://www.metaboanalyst.ca) [45]. The data were standardized by Pareto scaling [46] and log-transformation. To identify variables showing the most important variability between treatments, the metabolite data were subjected to Partial Least Squares–Discriminant Analysis (PLS-DA), using the cppls function from the RVAideMemoire package [47]. The most important variables were selected through Variables Importance for Projection (VIP) scores obtained through PLS-DA. Separate analyses were conducted for lipophilic, polar and phenolic compounds. A mixed model ANOVA was performed for the 30 most important lipophilic and polar compounds with a VIP score higher than 1, and for all phenolic compounds (SPSS Chicago, IL, USA). Significant interactions of CO2 and O3 were studied further by conducting the SME.

Table 2. Total relative content of lipophilic, polar and phenolic compounds and the relative content of the major metabolite groups in birch buds collected on April 4, 2009 at the Aspen FACE site.

|                     | C/eCO2 | C/eO3 | C/eCO2+eO3 | Significance | Interaction |
|---------------------|--------|-------|------------|--------------|-------------|
| Lipophilic compounds|        |       |            |              |             |
| Fatty acids         | 1.06   | 1.26  | 1.04       | CO2×O3 0.052 | O3 ↓ (aCO2) |
| Sterols             | 1.04   | 1.27  | 0.97       |              |             |
| Terpenoids          | 1.06   | 1.26  | 1.04       | CO2×O3 0.058 | O3 ↓ (aCO2) |
| Polar compounds     | 0.99   | 0.96  | 1.04       |              |             |
| Amino acids a       | 0.90   | 1.15  | 0.89       |              |             |
| Carbohydrates       | 1.01   | 0.97  | 1.06       |              |             |
| Organic acids       | 1.07   | 0.98  | 1.02       |              |             |
| Phenolic compounds a| 0.95   | 0.89  | 1.00       | CO2×O3 0.047 | CO2 ↑ (aO3) |
| Phenolic acid       | 0.77   | 0.72  | 0.81       | O3 0.082     | O3 ↑ (aCO2) |
| Flavanols a         | 1.11   | 1.05  | 1.12       | CO2 0.088    |             |
| Flavanols           | 0.95   | 0.91  | 1.04       | CO2×O3 0.053 | CO3 ↓ (eO3) |

Note: The ratio of each compound (relative content of metabolites in peak area per 1 g dry weight) between control (C) and eCO2, eO3 and eCO2+eO3 treatments (calculated from untransformed values, each replication per treatment consists of four saplings, n = 3) are shown. P values < 0.1 were considered ‘significant’. Interpretations (statistically significant simple main effects (SME) after Bonferroni adjustment P ≤ 0.05) of significant factor interactions. ↓ decreased content; ↑ increased content; (aCO2), in ambient CO2 level; (eCO2), in elevated CO2 level; (aO3), in ambient O3 level; (eO3), in elevated O3 level; ns, non-significant. a Logarithm transformed for mixed models ANOVA. The ratio of each compound between control and the treatments was calculated using untransformed data.
**Table 3.** Statistically significant metabolites that separated the control (C) and elevated CO$_2$ (eCO$_2$) elevated O$_3$ (eO$_3$) and eCO$_2$ + eO$_3$ treatments, identified from the VIP scores.

| Compound                  | C/eCO$_2$ | C/eO$_3$ | C/eCO$_2$+eO$_3$ | Significance | Interaction          |
|---------------------------|-----------|-----------|-------------------|--------------|----------------------|
| Lip145 Unknown compound   | 1.20      | 1.62      | 1.36              | O$_3$ 0.089  |                      |
| Lip189 Unknown compound   | 1.29      | 1.29      | 0.74              | CO$_2$ × O$_3$ 0.007 | CO$_2$ ↑ (eO$_3$) |
| Lip26 Hexitol             | 0.73      | 0.86      | 0.68              | CO$_2$ 0.020  |                      |
| Lip69 Eicosanoic acid     | 0.86      | 1.00      | 0.80              | CO$_2$ 0.050  |                      |
| Lip152 Dammarane triterpenoid 1 | 1.12      | 1.32      | 1.03              | CO$_2$ × O$_3$ 0.091 | O$_3$ ↓ (aCO$_2$) |
| Lip2 Unknown compound     | 0.43      | 0.77      | 0.27              | CO$_2$ 0.001  |                      |
| Lip105 Triterpenoid 2     | 0.95      | 1.21      | 1.23              | O$_3$ 0.078  |                      |
| Lip138 Unknown compound   | 1.77      | 1.54      | 0.93              | CO$_2$ × O$_3$ 0.058 | CO$_2$ ↑ (eO$_3$) |
| Lip143 Unknown compound   | 2.28      | 1.75      | 1.35              | CO$_2$ × O$_3$ 0.037 | O$_3$ ↓ (aCO$_2$) |
| Pol77 Unknown compound    | 0.96      | 0.94      | 0.87              | CO$_2$ × O$_3$ <0.001 | CO$_2$ ↓ (eO$_3$), O$_3$ ↓ (eCO$_2$) |
| Pol66 Myo-Inositol        | 0.96      | 0.99      | 0.97              | CO$_2$ 0.051  |                      |
| Pol83 Glucose-6-phosphate| 0.67      | 0.65      | 0.92              | CO$_2$ × O$_3$ 0.018 | CO$_2$ ↑ (aO$_3$), O$_3$ ↑ (aCO$_2$) |
| Pol15 Glycerol            | 0.95      | 1.15      | 0.89              | CO$_2$ 0.082  |                      |
| Pol59 Mannitol            | 1.52      | 1.79      | 1.05              | CO$_2$ 0.048, O$_3$ 0.034, CO$_2$ × O$_3$ 0.045 | CO$_2$ ↓ (aO$_3$), O$_3$ ↓ (aCO$_2$) |
| Pol54 D-Galactose         | 0.74      | 0.95      | 0.93              | CO$_2$ × O$_3$ 0.096 | SMEs not sig. |
| Phe16 Unknown compound    | 0.61      | 0.56      | 1.05              | CO$_2$ × O$_3$ 0.006 | CO$_2$ ↓ (eO$_3$), O$_3$↑ (aCO$_2$) |
| Phe13 Proanthocyanidin-dimer, “B” type | 0.81      | 0.86      | 1.58              | CO$_2$ × O$_3$ 0.058 | O$_3$ ↓ (eCO$_2$) |
| Phe14 Quercetin-3-glucopyranoside | 1.09      | 1.05      | 1.29              | CO$_2$ 0.018  |                      |
| Phe17 Quercetin-3-arabinopyranoside | 1.09      | 1.05      | 1.24              | CO$_2$ 0.052  |                      |
| Phe18 Unknown compound    | 1.31      | 0.80      | 1.51              | CO$_2$ 0.002  |                      |
| Phe19 3,4-Di-cafeoyl-quinic acid | 0.67      | 0.91      | 1.38              | O$_3$ 0.097, CO$_2$ × O$_3$ 0.072 | O$_3$ ↓ (eCO$_2$) |
| Phe21 Quercetin + Kaempferol | 1.83      | 1.17      | 0.77              | CO$_2$ × O$_3$ 0.012 | O$_3$ ↑ (eCO$_2$) |

Note: The metabolites are listed according to their contribution to the model in each metabolite group: the most powerful metabolites are listed at the top. The ratio of each compound (relative content of metabolites in peak area per 1 g dry weight) between control (C) and eCO$_2$, eO$_3$ and eCO$_2$ + eO$_3$ treatments (each replication per treatment consists of four saplings, n = 3) are shown. P values for the main effects of eCO$_2$ and eO$_3$ and for their interaction are presented: P values < 0.1 were considered ‘significant’. Interpretations (statistically significant simple main effects (SME) after Bonferroni adjustment P ≤ 0.05) of significant factor interactions. ↓ decreased content; ↑ increased content; (aCO$_2$), in ambient CO$_2$ level; (eCO$_2$), in elevated CO$_2$ level; (aO$_3$), in ambient O$_3$ level; (eO$_3$), in elevated O$_3$ level. * Logarithm transformed for Mixed Models ANOVA. The ratio of each compound between control and the treatments was calculated using untransformed data.
3. Results

3.1. Metabolite Analyses

Altogether, 205 lipophilic compounds, 127 polar compounds and 25 phenolic compounds were quantified in the birch buds. The most abundant lipophilic compounds were terpenoids, steroids and fatty acids, and there were also 120 unidentified lipophilic compounds. The largest groups in polar compounds were carbohydrates, organic acids and amino acids. Twenty-seven unknown polar compounds were found. Phenolic compounds included flavanols, flavonols, phenolic acids (p-coumaroyl- and caffeoyl-quinic acids) and three unknown compounds.

According to the linear mixed model ANOVA, the total content of lipophilic and phenolic compounds was affected by eCO\(_2\) and eO\(_3\) in an interactive manner (Tables 2 and 3). Elevated O\(_3\) caused a reduction in the total lipids under aCO\(_2\), and this was connected with fewer terpenoids under aO\(_3\) (Tables 2 and 3). From the individual lipophilic compounds with the largest significant contribution to the model, eO\(_3\) reduced the contents of an unknown compound 145 and triterpenoid 2 (main effect of O\(_3\)), and also the contents of Dammarane triterpenoid 1 and an unknown compound 143 (under aCO\(_2\), Table 3). The total content of the lipophilic compounds was not affected by eCO\(_2\) (Table 2). From individual compounds, the contents of hexitol and eicosanoic acid and the content of three unknown compounds increased by eCO\(_2\), mostly under eO\(_3\) (Table 3).

The total content of the polar compounds was not significantly affected by the treatments. From individual compounds, four compounds were affected by eCO\(_2\) (increased content of myo-inositol, glycerol (main effect of CO\(_2\)), and glucose-6-phosphate, and decreased content of mannitol (under aO\(_3\)). Elevated O\(_3\) increased glucose-6-phosphate (under aCO\(_2\)) and decreased mannitol (under aCO\(_2\)) (Tables 2 and 3).

The total content of phenolic compounds was increased by eCO\(_2\) under aO\(_3\) (Table 2). This was associated with the increased phenolic acids by eCO\(_2\) under aO\(_3\). The contents of flavonols (main effect of CO\(_2\)) and flavanols (under eO\(_3\) only) were reduced by eCO\(_2\). From individual compounds, eCO\(_2\) reduced the contents of quercetin-3-glucopyranoside, quersetin-3-arabinopyranoside and an unknown compound (Table 3). Elevated O\(_3\) increased the total content of phenolic compounds under aCO\(_2\), and this was related to increased content of phenolic acids. Elevated O\(_3\) affected the individual phenolic compounds mainly under eCO\(_2\) (decreased content of proanthocyanidin (PA) A-dimer, "B" type and caffeoyl-quinic acid, and increased content of quercetin + kaempferol (Tables 2 and 3).

3.2. Bud Dry mass, Length, Diameter, Water Content and Freezing Tolerance

Bud length increased under eO\(_3\), while the bud diameter remained unaffected by the treatments (Table 4). Dry mass and water content (Table 4) of the birch buds were not affected by the treatments. According to the freezing test, the buds were highly freezing tolerant at the time of samplings. No treatment effects on REL were found (Figure 2).

Table 4. Dry mass, water content and length of the buds of paper birch, sampled on April 4, 2009 (mean ± SE, n = 3).

| Parameter            | Control   | eCO\(_2\) | eO\(_3\)  | eCO\(_2\)+eO\(_3\) | Significance |
|----------------------|-----------|-----------|-----------|---------------------|--------------|
| Bud length, mm       | 6.5 ± 0.2 | 6.9 ± 0.5 | 7.2 ± 0.1 | 6.9 ± 0.1           | O\(_3\) 0.089 |
| Bud diameter, mm     | 2.6 ± 0.04| 2.7 ± 0.08| 2.6 ± 0.01| 2.5 ± 0.11          | ns           |
| Dry mass, mg         | 15.7 ± 1.2| 16.6 ± 2.2| 15.8 ± 0.7| 15.0 ± 1.2          | ns           |
| Water content, %     | 38.0 ± 2.8| 39.9 ± 0.8| 39.7 ± 1.0| 41.1 ± 1.1          | ns           |

Note: The trees were exposed to elevated CO\(_2\) (eCO\(_2\)) and elevated O\(_3\) (eO\(_3\)) and a combination of both for 11 growing seasons at the Aspen FACE site (1998–2008). Data were analyzed by applying linear mixed model analysis of variance.
which are typical constituents of bud and leaf surface extracts of birch species [49]. The triterpenoids within the lipophilic compounds. The group consisted primarily of Dammarane type triterpenoids, 2020 Forests 2

that may be important in maintaining membrane fluidity, was increased. lipophilic compounds was small; the content of eicosanoic acid, which is a polyunsaturated fatty acid similar under the control, eC0,

those grown under eO,

may be related to the higher availability of C in paper birches grown under eC0,FACE experiment [23–26,48].

Our results showed that both eC0 (~560 ppm) and eO3 (~1.5 × ambient) induced alterations in the chemical composition of paper birch buds. Gas exposures were terminated at the time of leaf fall in autumn, indicating that these gases have long-lasting impacts on bud chemistry, which may, in part, mediate the growth and defense responses to eC0 and eO3 that have been seen during the Aspen FACE experiment [23–26,48].

Elevated O3 altered C allocation under aC02 by increasing the total concentration of phenolic compounds and decreasing that of lipids in the birch buds. The lower lipophilic compound content of the buds was expressed as a significant reduction in terpenoid content, the most abundant group within the lipophilic compounds. The group consisted primarily of Dammarane type triterpenoids, which are typical constituents of bud and leaf surface extracts of birch species [49]. The triterpenoids are exuded on the inner side of bud scales by glands (trichomes) covering developing tissues in buds [50,51]. Ultrastructural and chemical analysis reveals that glandular trichomes of birches also secrete phenolics [52]. Glandular trichomes may present a primary defence mechanism when the apical growth of juvenile shoots take place, protecting the buds against abiotic and biotic stress factors such as harsh winter and spring weather, air pollutants, herbivory and pathogens [51,53]. Trichomes are formed at the early stages of leaf development, and their formation has been shown to be affected by the growth conditions during the previous year [52,54]. Biosynthesis of lipophilic compounds such as fatty acids and terpenoids is costly compared to other primary and secondary metabolites [55]. Phenolics and terpenoids have different biochemical pathways, and our results suggest that the limited C resources of the birches grown under eO3 have been directed to the synthesis of phenolic compounds at the expense of synthesis of terpenoids [23–25]. This phenomenon was found only under aC02, which may be related to the higher availability of C in paper birches grown under eC02 + eO3 compared to those grown under eO3. Consequently, the content of terpenoids and total phenolic compounds was similar under the control, eC02 and eC02 + eO3 treatments. Overall, the main effect of eC02 on the lipophilic compounds was small; the content of eicosanoic acid, which is a polyunsaturated fatty acid that may be important in maintaining membrane fluidity, was increased.

The effects of eC02 and eO3 on the polar compounds in the birch buds were small. However, the treatments induced some alterations in carbohydrate metabolism. Both eC02 and eO3 reduced the
content of mannitol under the single exposures, and eCO₂ increased that of myo-inositol. Mannitol and myo-inositol are common polyols in plants partaking in a variety of metabolic pathways, and they have a role in resistance to both biotic and abiotic stresses by acting as osmoprotectants and antioxidants [56,57].

In general, both eCO₂ and eO₃ have increased the concentration of various phenolic compounds in the leaves of different tree species [58]. It is often assumed that the surplus of C under eCO₂ is directed to synthesis of secondary compounds along the phenylpropanoid pathway in leaves [11,59]. Under eO₃, antioxidant activity of phenolic compounds may provide some protection against ROS induced by eO₃ [60]. In our experiment, chronic O₃ exposure seems to have induced some potential antioxidant compounds possibly as a response to increased oxidative stress in the leaf cells during bud formation. This supports an earlier study by Peltonen et al. (2006), who found increased surface exudate phenolics in silver birch buds in autumn under eO₃ [61]. In our experiment, the O₃-induced increase in total concentration of phenolic compounds was mainly caused by increased phenolic acids that included neochlorogenic acid and dicaffeoyl-quinic acids. Chlorogenic acids are known to be effective antioxidants [62] and they are also known to increase pathogen resistance [63]. As expected, eCO₂ increased the total concentration of phenolic compounds in buds, but only under aO₃. This corresponded to an accumulation of phenolic acids, although the concentrations of flavanols and flavonols were reduced. Similar findings have been reported for leaves of silver birch [64] and several other tree species [11]. It is known that leaf phenolics may act as defence mechanisms against herbivores and pathogens [20]. In general, an increase in the concentration of phenolics in the birch buds may provide some protection for newly emerging leaves against herbivores and pathogens, as well as increased detoxification capacity against O₃ stress [20]. However, when the effects of eCO₂ and eO₃ on insect-mediated canopy damage were studied in the aspen and birch stands at the Aspen FACE site, it was found that canopy damage was markedly higher under eCO₂, while the opposite trends were apparent under eO₃ [48].

In our experiment, eCO₂ prevented the effects of eO₃ on the total concentration of phenolic compounds and lipids. In other words, the O₃-derived induction of the accumulation of phenolic compounds and reduction in lipophilic compounds disappeared under eCO₂. This is in accordance with several studies on the leaves of different tree species, although whether eCO₂ and eO₃ function independently or in an interactive manner depends on the particular chemical constituent and tree species studied [11,20,64]. Our results are consistent with the prediction that contents of C based secondary compounds will increase in cases where carbohydrates accumulate in excess of growth demands [59,65], which was likely to be occurring at the AspenFACE site under eCO₂. However, Couture et al. (2017) found that the effect of eCO₂ on the phytochemistry of paper birch leaves collected at the AspenFACE site during several growing seasons was minimal, which may indicate acclimation of the saplings to growth under eCO₂ [66].

The freezing tests showed that the buds were highly freezing tolerant at the time of sampling in April 2009. This was probably caused by the freezing events that occurred before the samplings in Rhinelander. No effects of treatments on the FT of buds was found. The results of the metabolite analyses largely support the results of the freezing test. Our earlier study on paper birch at the AspenFACE site showed some O₃-induced alterations in C metabolism in birch buds in November. This was manifested as reduced starch storage under eO₃, although the concentration of soluble sugars was unaltered by the treatments [25], possibly indicating lower energy resources available during the winter [67]. However, in the present experiment, we did not find major treatment-induced alterations in the content or composition of carbohydrates. Accumulations of monosaccharides, sucrose and raffinose in cells are known to be involved in FT of over-wintering plant parts [68]. Further, we found only minor treatment-induced alterations in the fatty acid composition, possibly indicating that the fatty acid desaturation in membrane lipids, which allows maintaining functional membrane fluidity at low temperature [6], was not significantly altered by the treatments. We found no treatment effects on amino acids, which alone or as constituents of several compounds are
known to accumulate as a response to low temperatures, such as dehydrins and polyamines [5]. Further, the water content of the buds, which is often connected with FT, was similar in the treatments [5]. In plant cells, cold stress leads to overproduction of ROS that are highly reactive and cause damage to proteins, lipids and carbohydrates, which ultimately results in oxidative stress. Both phenolic acids [69,70] and terpenoids [71] are known to accumulate in plant cells during cold stress and are suggested to have ROS scavenging capacity. The biological significance of the shift from terpenoids to phenolic acids under eO$_3$ remains unclear.

5. Conclusions

In conclusion, our results showed that the 11-year exposure of paper birch to eCO$_2$ and eO$_3$ modified the chemical composition of the over-wintering buds. Elevated CO$_2$ had only a minor effect on the lipophilic and polar compounds, but it did alter the phenolic content of the buds. Elevated O$_3$ reduced C allocation to biosynthesis of terpenoids and increased it to biosynthesis of phenolic acids, and these changes were evident mainly under aCO$_2$. We were not able to show that eCO$_2$ or eO$_3$ affected FT of the over-wintering buds in spring. The alterations of bud metabolite contents may impact the growth and defense responses of birches during early growth in spring. However, the biological relevance of the alterations of bud metabolite contents should be further studied in future experiments.

Author Contributions: J.R. performed samplings, data analysis, manuscript writing and acquired funding, M.K. participated in data analysis and writing the manuscript, V.O. performed metabolome analyses and edited the manuscript, A.S. conducted the PLS-DA analyses and edited the manuscript, P.M. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Academy of Finland (project number 127256) and the Natural Resources Institute Finland (Luke). Aspen FACE was funded by US Department of Energy; USDA Forest Service, Northern Global Change Program; and the USDA Forest Service, Northern Research Station, and by USDA NRI Grant #2004-36102-14782.

Acknowledgments: We are grateful to Hanna Ruhanen, Marja-Leena Jalkanen and Mervi Ahonpää for their skillful assistance with conducting the freezing tests. We thank Ronald Teclaw for collecting the bud samples and D. Bronson for reviewing the paper.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References
1. Barichivich, J.; Briëffa, K.R.; Myneni, R.B.; Osborn, T.J.; Melvin, T.M.; Ciais, P.; Piao, S.; Tucker, C. Large-scale variations in the vegetation growing season and annual cycle of atmospheric CO$_2$ at high northern latitudes from 1950 to 2011. *Glob. Chang. Biol.* 2013, 19, 3167–3183. [CrossRef]
2. Liu, Q.; Piao, S.; Janssens, I.A.; Fu, Y.H.; Peng, S.; Lian, X.; Ciais, P.; Myneni, R.B.; Peñuelas, J.; Wang, T. Extension of the growing season increases vegetation exposure to frost. *Nat. Commun.* 2018, 9, 426. [CrossRef] [PubMed]
3. Wisniewski, M.; Nassuth, A.; Arora, R. Cold Hardiness in Trees: A Mini-Review. *Front. Plant Sci.* 2018, 9, 1394. [CrossRef] [PubMed]
4. Ma, Q.; Huang, J.-G.; Hänninen, H.; Berninger, F. Divergent trends in the risk of spring frost damage to trees in Europe with recent warming. *Glob. Chang. Biol.* 2019, 25, 351–360. [CrossRef]
5. Welling, A.; Palva, E.T. Involvement of CBF transcription factors in winter hardiness in birch. *Physiol. Plant.* 2006, 127, 167–181. [CrossRef]
6. Buchanan, B.; Gruissem, W.; Jones, R.L. *Biochemistry and Molecular Biology of the Plants*; John Wiley & Sons: Hoboken, NJ, USA, 2002; 1367p.
7. Landhäusser, S.M. Aspen shoots are carbon autonomous during bud break. *Trees* 2011, 25, 531–536. [CrossRef]
8. Macdonald, A.D.; Mothersill, D.H.; Caesar, J.C. Shoot development in *Betula papyrifera*. III. Long-shoot organogenesis. *Can. J. Bot.* 1983, 62, 437–445. [CrossRef]
9. IPCC. *Climate Change 2014 Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Pachauri, R.K., Meyer, L.A., Eds.; IPCC: Geneva, Switzerland, 2014; 151p.
10. Körner, C. Plant CO₂ responses: An issue of definition, time and resource supply. *New Phytol.* 2006, 172, 393–411. [CrossRef] [PubMed]

11. Lindroth, R.L. Impacts of Elevated Atmospheric CO₂ and O₃ on Forests: Phytochemistry, Trophic Interactions, and Ecosystem Dynamics. *J. Chem. Ecol.* 2010, 36, 2–21. [CrossRef] [PubMed]

12. Paolletti, E.; De Marco, A.; Beddows, D.C.S.; Harrison, R.M.; Manning, W.J. Ozone levels in European and USA cities are increasing more than at rural sites, while peak values are decreasing. *Environ. Pollut.* 2014, 192, 295–299. [CrossRef]

13. Schnell, J.L.; Prather, M.J.; Josse, B.; Naik, V.; Horowitz, L.W.; Zeng, G.; Shindell, D.T.; Faluvegi, G. Effect of climate change on surface ozone over North America, Europe, and East Asia. *Geophys. Res. Lett.* 2016, 43, 3509–3518. [CrossRef]

14. Wittig, V.E.; Ainsworth, E.A.; Naidu, S.L.; Karnosky, D.F.; Long, S. Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: A quantitative meta-analysis. *Glob. Chang. Biol.* 2009, 10, 396–424. [CrossRef]

15. Bernacchi, C.J.; Leakey, A.D.B.; Headly, L.E.; Morgan, P.B.; Dohleman, F.G.; McGrath, J.M.; Gillespie, K.M.; Wittig, V.E.; Rogers, A.; Long, S.P.; et al. Hourly and seasonal variation in photosynthesis and stomatal conductance of soybean grown at future CO₂ and ozone concentrations for 3 years under fully open-air field conditions. *Plant Cell Environ.* 2006, 29, 2077–2090. [CrossRef] [PubMed]

16. Talhelm, A.F.; Pregitzer, K.S.; Kubiske, M.E.; Zak, D.R.; Campany, C.E.; Burton, A.J.; Dickson, R.E.; Hendrey, G.R.; Isebrands, J.G.; Lewin, K.F.; et al. Elevated carbon dioxide and ozone alter productivity and ecosystem carbon content in northern temperate forests. *Glob. Chang. Biol.* 2014, 20, 2492–2504. [CrossRef] [PubMed]

17. Riikonen, J.; Lindsberg, M.-M.; Holopainen, T.; Oksanen, E.; Lappi, J.; Peltonen, P.; Vapaavuori, E. Silver birch and climate change: Variable growth and carbon allocation responses to elevated concentrations of carbon dioxide and ozone. *Tree Physiol.* 2004, 24, 1227–1237. [CrossRef] [PubMed]

18. Paolletti, E.; Grulke, N.E. Does living in elevated CO₂ ameliorate tree response to ozone? A review on stomatal responses. *Environ. Pollut.* 2005, 137, 483–493. [CrossRef] [PubMed]

19. Saleem, A.; Loponen, J.; Pihlaja, K.; Oksanen, E. Effects of long-term open-field ozone exposure on leaf phenolics of European silver birch (*Betula pendula* Roth). *J. Chem. Ecol.* 2001, 27, 1049–1062. [CrossRef]

20. Valkama, E.; Koricheva, J.; Oksanen, E. Effects of elevated O₃, alone and in combination with elevated CO₂, on tree leaf chemistry and insect herbivore performance: A meta-analysis. *Glob. Chang. Biol.* 2007, 13, 184–201. [CrossRef]

21. Oksanen, E.; Riikonen, J.; Kaakinen, S.; Holopainen, T.; Vapaavuori, E. Structural characteristics and chemical composition of birch (*Betula pendula*) leaves are modified by increasing CO₂ and ozone. *Glob. Chang. Biol.* 2005, 11, 732–748. [CrossRef]

22. Peltonen, P.A.; Vapaavuori, E.; Heinonen, J.; Julkunen-Tiitto, R.; Holopainen, J. Do elevated atmospheric CO₂ and O₃ affect food quality and performance of folivorous insects on silver birch? *Glob. Chang. Biol.* 2010, 16, 918–935. [CrossRef]

23. Karnosky, D.F.; Pregitzer, K.S.; Zak, D.R.; Kubiske, M.E.; Hendrey, G.R.; Weinstein, D.; Nosal, M.; Percy, K. Scaling ozone responses of forest trees to the ecosystem level in a changing climate. *Plant Cell Environ.* 2005, 28, 965–981. [CrossRef]

24. King, J.S.; Kubiske, M.E.; Pregitzer, K.S.; Hendrey, G.R.; McDonald, E.P.; Giardina, C.P.; Quinn, V.S.; Karnosky, D.F. Tropospheric O₃ compromises net primary production in young stands of trembling aspen, paper birch and sugar maple in response to elevated atmospheric CO₂. *New Phytol.* 2005, 168, 4–12. [CrossRef] [PubMed]

25. Riikonen, J.; Kets, K.; Darbah, J.; Oksanen, E.; Söber, A.; Vapaavuori, E.; Nelson, N.; Kubiske, M.; Karnosky, D. Carbon gain and bud physiology in *Populus tremuloides* and *Betula papyrifera* grown under long-term exposure to interacting elevated CO₂ and O₃. *Tree Physiol.* 2008, 28, 243–254. [CrossRef] [PubMed]

26. Talhelm, A.F.; Pregitzer, K.S.; Giardina, C.P. Long-term leaf production response to elevated atmospheric carbon dioxide and tropospheric ozone. *Ecosystems* 2012, 15, 71–82. [CrossRef]

27. Loveys, B.R.; Egerton, J.J.G.; Ball, M.C. Higher daytime leaf temperatures contribute to lower freeze tolerance under elevated CO₂. *Plant Cell Environ.* 2006, 29, 1077–1086. [CrossRef]
28. Martin, M.; Gavazov, K.; Körner, C.; Hättenschwiler, S.; Rixen, C. Reduced early growing season freezing resistance in alpine treeline plants under elevated atmospheric CO2. 2010. *Glob. Chang. Biol.* 2010, 16, 1057–1070. [CrossRef]

29. Wayne, P.M.; Reekie, E.G.; Bazzaz, F.A. Elevated CO2 ameliorates birch response to high temperature and frost stress: Implications for modeling climate-induced geographic range shifts. *Oecologia* 1998, 114, 335–342. [CrossRef]

30. Bigras, F.J.; Bertrand, A. Responses of *Picea mariana* to elevated CO2 concentration during growth, cold hardening and dehardening: Phenology, cold tolerance, photosynthesis and growth. *Tree Physiol.* 2006, 26, 875–888. [CrossRef]

31. Beerling, D.J.; Terry, A.C.; Mitchell, P.L.; Callaghan, T.V.; Gwynn-Jones, D.; Lee, J.A. Time to chill: Effects of simulated global change on leaf ice nucleation temperatures of subarctic vegetation. *Am. J. Bot.* 2001, 88, 628–633. [CrossRef]

32. Jach, M.E.; Ceulemans, R.; Murray, M.B. Impact of greenhouse gases on the phenology of forest trees. In *The Impact of Carbon Dioxide and Other Greenhouse Gases on Forest Ecosystems*; Karnosky, D.F., Ceulemans, R., Scarascia-Mugnozza, G.E., Innes, J.L., Eds.; CABI Publishing: Oxfordshire, UK, 2001; pp. 193–223.

33. Riikonen, J.; Kontunen-Soppela, S.; Vapaavuori, E.; Tervahauta, A.; Tuomainen, M.; Oksanen, E. Carbohydrate concentrations and freezing stress resistance of silver birch buds grown under elevated temperature and ozone. *Tree Physiol.* 2013, 33, 311–319. [CrossRef]

34. Yonekura, T.; Yoshidome, M.; Watanabe, M.; Honda, Y.; Ogawa, I.; Izuta, T. Carry-over effects of ozone and water stress on leaf phenological characteristics and bud frost hardiness of *Fagus crenata* seedlings. *Trees* 2004, 18, 581–588. [CrossRef]

35. Ranford, J.; Reiling, K. The effect of winter stress on *Ilex aquifolium* L. previously fumigated with ozone. *Environ. Pollut.* 2007, 145, 171–178. [CrossRef] [PubMed]

36. Kubiske, M.E.; Foss, A.R.; Burton, A.J.; Jones, W.S.; Lewin, K.F.; Nagy, J.; Pregitzer, K.S.; Zak, D.R.; Karnosky, D.F. Supporting 13 Years of Global Change Research: The History, Technology, and Methods of the Aspen Face Experiment; Gen. Tech. Rep. NRS-153; Forest Service, Northern Research Station; Publication Series: General Technical Report (GTR) Station: Northern Research Statio; U.S. Department of Agriculture: Newtown Square, PA, USA, 2015; 50p. [CrossRef]

37. Dickson, R.E.; Lewin, K.F.; Isebrands, J.G.; Coleman, M.D.; Heilman, W.E.; Riemenschneider, D.E.; Sober, J.; Host, G.E.; Zak, D.R.; Hendrey, G.R.; et al. *Forest Atmosphere Carbon Transfer Storage-II (FACTS II)—The Aspen Free-Air CO2 and O3 Enrichment (FACE) Project in an Overview*; General Technical Report NC-214; USDA Forest Service North Central Research Station: Rhinelander, WI, USA, 2000.

38. Ossipov, V.; Ossipova, S.; Bykov, V.; Oksanen, E.; Koricheva, J.; Haukioja, E. Application of metabolomics to genotype and phenotype discrimination of birch trees grown in a long-term open-field experiment. *Metabolomics* 2008, 4, 39–51. [CrossRef]

39. Van den Dool, H.; Kratz, P. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* 1963, 11, 463–471. [CrossRef]

40. Ossipov, V.; Klemola, T.; Ruohomäki, K.; Salminen, J.-P. Effects of three years’ increase in density of the geometrid *Epirrita autumnata* on the change in metabolome of mountain birch trees (*Betula pubescens* ssp. *czerepanovii*). *Chemoecology* 2014, 24, 201–214. [CrossRef]

41. Ritchie, G.A. Measuring cold hardiness. In *Techniques and Approaches in Forest Tree Ecophysiology*; Lassoie, J., Hinckley, T.M., Eds.; CRC Press: Boca Raton, FL, USA, 1991; pp. 557–582.

42. Filion, M.; Dutilleul, P.; Potvin, C. Optimum experimental design for free-air carbon dioxide enrichment (FACE) studies. *Glob. Chang. Biol.* 2000, 6, 843–854. [CrossRef]

43. Parsons, W.F.J.; Lindroth, R.L.; Bockheim, J.G. Decomposition of *Betula papyrifera* leaf litter under the independent and interactive effects of elevated CO2 and O3. *Glob. Chang. Biol.* 2004, 10, 1666–1677. [CrossRef]

44. R Core Team. *A Language and Environment for Statistical Computing*: R Foundation for Statistical Computing: Vienna, Austria, 2018; Available online: [http://www.R-project.org/](http://www.R-project.org/) (accessed on 29 March 2019).

45. Chong, J.; Xia, J. MetaboAnalystR: An R package for flexible and reproducible analysis of metabolomics data. *Bioinformatics* 2018, 27, 4313–4314. [CrossRef]
Forests 2020, 11, 330

46. Wiklund, S.; Johansson, E.; Sjöström, L.; Mellerowich, E.J.; Edlund, U.; Shockcor, J.P.; Gottfries, J.; Moritz, T.; Trygg, J. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. *Anal. Chem.* 2008, 80, 115–122. [CrossRef]

47. Hervé, M. RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R Package Version 0.9-71. 2019. Available online: https://CRAN.R-project.org/package=RVAideMemoire (accessed on 29 March 2019).

48. Couture, J.; Meehan, T.; Kruger, L.; Lindroth, R. Insect herbivory alters impact of atmospheric change on north temperate forests. *Nat. Plants* 2015, 1, 15016. [CrossRef]

49. Pokhilo, N.D.; Uvarova, N.I. Isoprenoids of various species of the genus *Betula*. *Chem. Nat. Comp.* 1988, 24, 273–285. [CrossRef]

50. Wollenweber, E.; Dietz, V.H. Occurrence and distribution of free flavonoid aglycones in plants. *Phytochemistry* 1981, 20, 869–932. [CrossRef]

51. Lapinjoki, S.P.; Elo, H.A.; Taipale, H.T. Development and structure of resin glands on tissues of *Betula pendula* Roth, during growth. *New Phytol.* 1991, 117, 219–223. [CrossRef]

52. Valkama, E.; Salminen, J.P.; Koricheva, J.; Pihlaja, K. Changes in leaf trichomes and epicuticular flavonoids during leaf development in three birch taxa. *Ann. Bot.* 2004, 94, 233–242. [CrossRef]

53. Samuels, L.; Kunst, L.; Jetter, R. Sealing plant surfaces: Cuticular wax formation by epidermal cells. *Annu. Rev. Plant Biol.* 2008, 59, 683–707. [CrossRef]

54. Valkama, E.; Koricheva, J.; Salminen, J.P.; Helander, M.; Saloniemi, I.; Saikkonen, K.; Pihlaja, K. Leaf surface traits: Overlooked determinants of birch resistance to herbivores and foliar micro-fungi? *Trees* 2005, 19, 191–197. [CrossRef]

55. Gershenzon, J. Metabolic costs of terpenoid accumulation in higher plants. *J. Chem. Ecol.* 1994, 20, 1281–1328. [CrossRef]

56. Stoop, J.M.H.; Williamson, J.D.; Pharr, D.M. Mannitol metabolism in plants: A method for coping with stress. *Trends Plant Sci.* 1996, 1, 139–144. [CrossRef]

57. Patel, T.K.; Williamson, J.D. Mannitol in Plants, Fungi, and Plant–Fungal Interactions. *Trends Plant Sci* 2016, 21, 486–497. [CrossRef]

58. Holopainen, J.K.; Virjamo, V.; Ghimire, R.P.; Blande, J.D.; Julkunen-Tiitio, R.; Kivimänpää, M. Climate change effects on secondary compounds of forest trees in the northern hemisphere. *Front. Plant Sci.* 2018, 9, 1445. [CrossRef]

59. Koricheva, J.; Larsson, S.; Haukioja, E.; Keinanen, M. Regulation of woody plant secondary metabolism by resource availability: Hypothesis testing by means of meta-analysis. *OIKOS* 1998, 83, 212–226. [CrossRef]

60. Hernández, I.; Alegre, L.; Van Breusegem, F.V.; Munné-Bosch, S. How relevant are flavonoids as antioxidants in plants? *Trends Plant Sci.* 2009, 14, 125–132. [CrossRef]

61. Peltonen, P.A.; Julkunen-Tiitio, R.; Vapaavuori, E.; Holopainen, J.K. Effects of elevated carbon dioxide and ozone on aphid oviposition preference and birch bud exudates phenolics. *Glob. Chang. Biol.* 2006, 12, 1670–1679. [CrossRef]

62. Grace, S.C.; Logan, B.A.; Adams, W.W. Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. *Plant Cell Environ.* 2002, 21, 513–521. [CrossRef]

63. Nicholson, R.L.; Hammerschmidt, R. Phenolics compounds and their role in disease resistance. *Annu. Rev. Phytopathol.* 1992, 30, 369–389. [CrossRef]

64. Peltonen, P.A.; Vapaavuori, E.; Julkunen-Tiitio, R. Accumulation of phenolic compounds in birch leaves is changed by elevated carbon dioxide and ozone. *Glob. Chang. Biol.* 2005, 11, 1305–1324. [CrossRef]

65. Bryant, J.P.; Chapin, F.S.; Klein, D.R. Carbon nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 1983, 40, 357–368. [CrossRef]

66. Couture, J.J.; Meehan, T.D.; Rubert-Nason, K.F.; Lindroth, R. Effects of elevated atmospheric carbon dioxide and tropospheric ozone on phytochemical composition of trembling aspen (*Populus tremuloides*) and paper birch (*Betula papyrifera*). *J. Chem. Ecol.* 2017, 43, 26–38. [CrossRef]

67. Sauter, J.; Wellenkamp, S. Seasonal changes in content of starch, protein and sugars in the twig wood of *Salix caprea*. *Holzforschung* 1998, 52, 255–262. [CrossRef]

68. Levitt, J. *Responses of Plants to Environmental Stress Vol. 1: Chilling, Freezing, and High Temperature Stress*; Academic Press: New York, NY, USA, 1980; p. 510.

69. Solecka, D.; Kacperska, A. Phenylpropanoid deficiency affects the course of plant acclimation to cold. *Physiol. Plant.* 2003, 119, 253–262. [CrossRef]
70. Pennycooke, J.C.; Cox, S.; Stushnoff, C. Relationship of cold acclimation, total phenolic content and antioxidant capacity with chilling tolerance in petunia (Petunia × hybrida). *Environ. Exp. Bot.* **2005**, *53*, 225–232. [CrossRef]

71. Kaplan, F.; Kopka, J.; Sung, D.Y.; Zhao, W.; Popp, M.; Porat, R.; Guy, C.L. Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. *Plant J.* **2007**, *50*, 967–981. [CrossRef] [PubMed]

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