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Metabolite Composition of Paper Birch Buds after Eleven Growing Seasons of Exposure to Elevated CO_2 and O_3

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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 $\mu L\ L^{-1}$) and O_3 (target concentration 1.5 × 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 O_3 by increasing the content of phenolic acids, while the content of flavonols was reduced. 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

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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_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_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_2 may increase or decrease it, while the impact of eO_3 will be negative; and (4) the interactive effect of eCO_2 and eO_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_2 (aCO_2) and ambient O_3 (aO_3)), elevated CO_2 (eCO_2, target of 560 ppm) and elevated O_3 (eO_3, target 1.5 × ambient), or a combination of eCO_2 and eO_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_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_2 and O_3 at the Aspen Free Air CO_2 Enrichment (FACE) site for the hours from sunrise to sunset during the fumigation season 2008 [36].

	CO_2 (µL L^{-1})		**O_3 (nL L^{-1})**	
	Ambient	**Elevated**	**Ambient**	**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	387 (12)	507 (59)	30 (13)	35 (19)
Oct	393 (9)	518 (75)	28 (7)	28 (7)

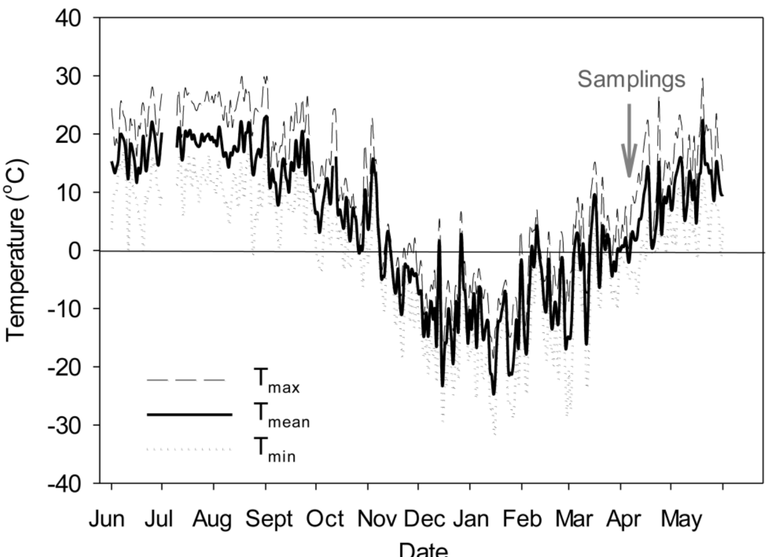

Figure 1. Maximum, minimum and mean air temperatures measured daily at the Aspen FACE site from June 2008 to May 2009. The date of the sampling is shown by the arrow (April 4, 2009).

2.2. Metabolome Analysis

For the metabolome analyses, 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 CO_2 and O_3 were tested using SPSS 25 for Windows (SPSS Chicago, IL, USA) by applying linear mixed model analysis of variance (ANOVA) with CO_2 and O_3 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 ($P \leq 0.05$). 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 CO_2 and O_3 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/eCO_2	C/eO_3	C/eCO_2+eO_3	Significance	Interaction
Lipophilic compounds	1.06	1.26	1.04	CO_2*O_3 0.052	O_3 ↓ (aCO_2)
Fatty acids	0.91	1.02	1.01	ns	
Sterols	1.04	1.27	0.97	ns	
Terpenoids	1.06	1.26	1.04	CO_2*O_3 0.058	O_3 ↓ (aCO_2)
Polar compounds	0.99	0.96	1.04	ns	
Amino acids [a]	0.90	1.15	0.89	ns	
Carbohydrates	1.01	0.97	1.06	ns	
Organic acids	1.07	0.98	1.02	ns	
Phenolic compounds [a]	0.95	0.89	1.00	CO_2*O_3 0.047	CO_2 ↑ (aO_3) O_3 ↑ (aCO_2)
Phenolic acid	0.77	0.72	0.81	O_3 0.082 CO_2*O_3 0.015	CO_2 ↑ (aO_3)
Flavonols [a]	1.11	1.05	1.12	CO_2 0.088	
Flavanols	0.95	0.91	1.04	CO_2*O_3 0.053	CO_2 ↓ (eO_3)

Note: 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 (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 $P \leq 0.05$) 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; 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 [a]	1.20	1.62	1.36	O_3 0.089	
Lip189	Unknown compound [a]	1.29	1.29	0.74	$CO_2 \times O_3$ 0.007	CO_2 ↑ (eO_3)
Lip26	Hexitol	0.73	0.86	0.68	CO_2 0.020	
Lip69	Eicosanoic acid [a]	0.86	1.00	0.80	CO_2 0.050	
Lip152	Dammarane triterpenoid 1 [a]	1.12	1.32	1.03	$CO_2 \times O_3$ 0.091	O_3 ↓ (aCO_2)
Lip2	Unknown compound	0.43	0.77	0.27	CO_2 0.001	
Lip105	Triterpenoid 2 [a]	0.95	1.21	1.23	O_3 0.078	
Lip138	Unknown compound	1.77	1.54	0.93	$CO_2 \times O_3$ 0.058	CO_2 ↑ (eO_3)
Lip143	Unknown compound [a]	2.28	1.75	1.35	$CO_2 \times O_3$ 0.037	O_3 ↓ (aCO_2)
Pol77	Unknown compound [a]	0.96	0.94	0.87	$CO_2 \times O_3$ <0.001	CO_2 ↑ (aO_3), O_3 ↑ (aCO_2) CO_2 ↓ (eO_3), O_3↓ (eCO_2)
Pol66	Myo-Inositol [a]	0.96	0.99	0.97	CO_2 0.051	
Pol83	Glucose-6-phosphate [a]	0.67	0.65	0.92	$CO_2 \times O_3$ 0.018	CO_2 ↑ (aO_3), O_3↑ (aCO_2)
Pol15	Glycerol [a]	0.95	1.15	0.89	CO_2 0.082	
Pol59	Mannitol [a]	1.52	1.79	1.05	CO_2 0.048, O_3 0.034, $CO_2 \times O_3$ 0.045	CO_2 ↓ (aO_3), O_3 ↓ (aCO_2)
Pol54	D-Galactose	0.74	0.95	0.93	$CO_2 \times O_3$ 0.096	SMEs not sig.
Phe16	Unknown compound	0.61	0.56	1.05	$CO_2 \times O_3$ 0.006	CO_2 ↓ (eO_3), O_3 ↑ (aCO_2)
Phe13	Proanthocyanidin-dimer, "B" type [a]	0.81	0.86	1.58	$CO_2 \times O_3$ 0.058	O_3 ↓ (eCO_2)
Phe14	Quercetin-3-glucopyranoside	1.09	1.00	1.29	CO_2 0.018	
Phe17	Quercetin-3-arabinopyranoside	1.09	1.05	1.24	CO_2 0.052	
Phe18	Unknown compound [a]	1.31	0.80	1.51	CO_2 0.002	
Phe19	3,4-Di-caffeoyl-quinic acid [a]	0.67	0.91	1.38	O_3 0.097, $CO_2 \times O_3$ 0.072	O_3 ↓ (eCO_2)
Phe21	Quercetin + Kaempferol	1.83	1.17	0.77	$CO_2 \times O_3$ 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 $P \leq 0.05$) 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. [a] 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.

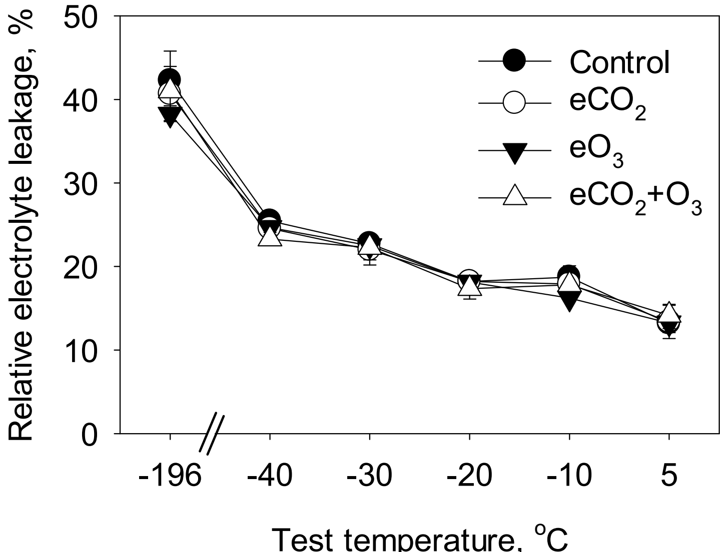

Figure 2. Effect of elevated CO_2 (eCO_2), elevated O_3 (eO_3) and eCO_2 + eO_3 on the relative electrolyte leakage (REL) in the paper birch buds after the freezing test. The buds were sampled on May 4, 2009, after 11 growing seasons of the exposure treatments (1998–2008). Mean values (± SE, n = 3) are presented.

4. Discussion

Our results showed that both eCO_2 (~560 ppm) and eO_3 (~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 eCO_2 and eO_3 that have been seen during the Aspen FACE experiment [23–26,48].

Elevated O_3 altered C allocation under aCO_2 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 eO_3 have been directed to the synthesis of phenolic compounds at the expense of synthesis of terpenoids [23–25]. This phenomenon was found only under aCO_2, which may be related to the higher availability of C in paper birches grown under eCO_2 + eO_3 compared to those grown under eO_3. Consequently, the content of terpenoids and total phenolic compounds was similar under the control, eCO_2 and eCO_2 + eO_3 treatments. Overall, the main effect of eCO_2 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 eCO_2 and eO_3 on the polar compounds in the birch buds were small. However, the treatments induced some alterations in carbohydrate metabolism. Both eCO_2 and eO_3 reduced the

content of mannitol under the single exposures, and eCO_2 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 eCO_2 and eO_3 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 eCO_2 is directed to synthesis of secondary compounds along the phenylpropanoid pathway in leaves [11,59]. Under eO_3, antioxidant activity of phenolic compounds may provide some protection against ROS induced by eO_3 [60]. In our experiment, chronic O_3 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 eO_3 [61]. In our experiment, the O_3-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, eCO_2 increased the total concentration of phenolic compounds in buds, but only under aO_3. 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 O_3 stress [20]. However, when the effects of eCO_2 and eO_3 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 eCO_2, while the opposite trends were apparent under eO_3 [48].

In our experiment, eCO_2 prevented the effects of eO_3 on the total concentration of phenolic compounds and lipids. In other words, the O_3-derived induction of the accumulation of phenolic compounds and reduction in lipophilic compounds disappeared under eCO_2. This is in accordance with several studies on the leaves of different tree species, although whether eCO_2 and eO_3 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 eCO_2. However, Couture et al. (2017) found that the effect of eCO_2 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 eCO_2 [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 effect 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 O_3-induced alterations in C metabolism in birch buds in November. This was manifested as reduced starch storage under eO_3, 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.

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