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Stem and crown growth of Japanese larch and its hybrid F₁ grown in two soils and exposed to two free-air O₃ regimes

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ABSTRACT

Ozone (O₃) pollution and soil infertility may negatively affect boreal forests across the Northern Hemisphere. Impacts to economically and ecologically important larches (*Larix* sp., Pinaceae) are particularly concerning. Using a free air O₃ enrichment (FACE) system, we investigated the effect of two-year elevated O₃ exposure ($\approx 66 \text{ nmol mol}^{-1}$) on Japanese larch (*L. kaempferi*) and its hybrid larch F₁ (*L. gmelinii* var. *japonica* $\times$ *L. kaempferi*) planted directly into either fertile brown forest soil (BF) or BF mixed with infertile volcanic ash soil (VA). Overall, photosynthetic pigmentation and the growth performance of the stem and crown were reduced in both taxa exposed to elevated O₃. Furthermore, hybrid larch, in both O₃ treatments, performed better than Japanese larch. This finding contradicts findings of prior experiments with potential experimental artifacts of O₃ exposure facilities and root restrictions. Elevated O₃ also disproportionately inhibited stem diameter growth and caused an imbalance in chlorophylls *a/b* and chlorophylls/carotenoids ratios. Hybrid and Japanese

larches grown in BF and VA had a significantly lower drop of stem diameter over the run of stem height (from base to top) when exposed to elevated O₃, compared to ambient O₃. This finding indicates altered stem shape under elevated O₃. Among eleven response variables, there were no significant interactions between O₃ treatment and taxa. There was also no significant interaction of soil condition and taxa, suggesting that the two larches shared a similar response to O₃ and soil type. Understanding the performance of hybrid larch in relation to its parent species has ramifications for breeding success in an soil-degraded and O₃-polluted environment.

Keywords: crown, ozone, O₃ biomonitoring, stem shape, abiotic stress, trioxygen

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1. INTRODUCTION

Tropospheric ozone (O₃) is a widespread air pollutant of increasing global significance as it is toxic to plants at species-specific thresholds (Cooley and Manning 1987; Chappelka and Samuelson 1998; Broberg et al. 2015). Ozone level was approximately 10 nmol mol⁻¹ during the pre-industrialization era, about 20 nmol mol⁻¹ up to 1930, and has since continued to increase exponentially in the Northern Hemisphere (Marenco et al. 1994). O₃ pollution is a particularly severe issue in East Asia (Yamaji et al. 2006; Feng et al. 2015; Kim et al. 2015; Koike et al. 2013) and the Mediterranean (Sicard et al. 2013; Kalabokas et al. 2013; Paoletti et al. 2014; Kopanakis et al. 2015). In these regions, sensitive cultivated and wild plant taxa are particularly vulnerable to the adverse effects of ambient O₃ phytotoxicity (Yamaguchi et al. 2011; Oksanen et al. 2013; Saitanis et al. 2015; Osborne et al. 2016).

Soil degradation is also having global ramifications for plant viability (Lal and Steward 1990; Chen et al. 2002; Schroeter et al. 2005; St.Clair and Lynch 2010). Importantly, phosphorus (P) scarcity threatens long term sustainability due to expected increases in demands and decreases in the stocks of phosphate rocks (Vance et al. 2003; Cordell et al. 2009; Van Vuuren et al. 2010; Cooper et al. 2011; Dawson and Hilton 2011). In the long term, soil fertility will be more dependent on qualitative properties of litter, as occurs in undisturbed forests where soils are poor in inorganic nutrients, and thus soil fertility depends on litter deposition (Cuevas and Medina 1986, 1988). However, elevated CO₂ and O₃ levels negatively affect litter quality, with likely implications to soil fertility (Loladze 2002; Deng et al. 2015; Agathokleous et al. 2016a; Shi et al. 2016). Although it is known that soil affects the responses of plants to other applied treatments (Körner 2011; Sigurdsson et al. 2013), the

interaction of O₃ with soil type is underexplored, with the majority of investigations conducted using potted seedlings (Agathokleous et al. 2016).

Larches are deciduous trees within the genus *Larix* (Pinaceae) with high economic and ecological value as they are primary components of Siberian and Canadian boreal forests, and have a wide range across the Northern Hemisphere (Farjon 1990; Abaimov et al. 2000). Larches hold an important role in Eurasia as maintainers of the CO₂ balance (Osawa et al. 2010). Japanese larch (*Larix kaempferi* (Lamb.) Carr.) is a light-demanding pioneer species. As a major plantation species in Japan, significant efforts towards optimum breeding have been conducted (Kurinobu 2005). Consequently, the hybrid larch F₁ was developed by crossing Dahurian larch (*Larix gmelinii* var. *japonica* (Maxim. ex Regel) Pilg.) with Japanese larch (Miyaki 1990). Properties of high growth, stem straightness and resistance to bark gnawing by voles contribute to the viability of hybrid larch as a plantation taxon. In Hokkaido, the northernmost island of Japan, 400 ha of hybrid plantations are established annually (Kurinobu 2005; Kita et al. 2014).

However, breeding of hybrid larch has thus far occurred without taking into consideration elevated O₃ levels. This hybrid has been found sensitive to elevated O₃ levels, experiencing greater O₃-induced damage than its parental species (Ryu et al. 2009; Koike et al. 2012; Kam et al. 2015; Wang et al. 2015). Additionally, under combined conditions of elevated O₃ and CO₂ levels, the negative consequences of O₃ have been shown to dominate (Koike et al. 2012). These studies used: a) open-top chambers with plants directly planted in the ground (Wang et al. 2015); b) open-top chambers with plants in pots (Koike et al. 2012); or c) free-

air O₃-concentration-enrichment (FACE) systems with plants grown in pots (Kam et al. 2015). The effect of O₃ on larch exposed to the natural environment (i.e. without chamber effects), and without root limitation (i.e. planted directly in the ground) remains unknown. Given the economic and ecological importance of larches in Eurasia, studies on the future challenges of this hybrid larch are important to inform future afforestation practices.

The aims of this study were to investigate how two years of free-air O₃ enrichment influences a) the growth and photosynthetic pigmentation of Japanese larch in comparison to its hybrid when grown pot-free, and b) how O₃ impacts differ between two types of soil; fertile brown forest soil (Dystric Cambisols) (BF) and BF mixed with infertile, immature volcanic ash plus pumice soil (Vitric Andosols) (VA). We further aimed to quantify the magnitude of difference among experimental groups to provide practical insights for these ecologically and economically important taxa. We hypothesized that in these fast-growing taxa, O₃ effects on growth would differ from previous findings derived from OTC experiments or pot-grown plants in FACE systems. This prediction is based on the assumption that epigeous growth depends on hypogeous growth and root expansion was restricted in potted experiments. Based on previous findings (Pretzsch et al. 2010; Carriero et al. 2015), we hypothesized that stem diameter growth would be reduced or inhibited by elevated O₃ and that this would occur primarily in the lower third of the stem. Altered stem shape caused by uneven stem growth is a critical issue as this pathological condition may reduce plant survival under windy conditions (Pretzsch et al. 2010; Carriero et al. 2015). Plants grown in infertile soils often allocate resources to root symbionts at the expense of photosynthetic products to overcome nutrient deficiencies (Hermans et al. 2006; Smith and Read 2008). We therefore hypothesized

that larch responses to O₃ would be affected by soil fertility through shifts in photosynthesis and nutritional demands.

2. MATERIALS & METHODS

2.1. Experimental area and meteorological conditions

The experiment was conducted in the FACE system of Sapporo Experimental Forest of Hokkaido University, located at Sapporo, Japan (43°04' N, 141°20' E, 15 m a.s.l.), in the years 2014-2015. The snow-free period lasted from early May to mid-November. Temperature, wind speed, relative humidity, sunshine and precipitation were continuously monitored by the Japan Meteorological Agency (2016) in Sapporo (WMO, ID: 47412, 43°03.6'N 141°19.7'E) (Table 1). The photosynthetic photon flux density (PPFD) was recorded by a HOBO Pendant data logger (UA-002-64, Onset Computer, Co., MA, USA) located in the center of each experimental plot at a height of two meters. During the period June-October (2014 and 2015), an observation was recorded every 5 min. For both years, the PPFD in the experimental plots exceeded 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (*i.e.* light compensation point of photosynthesis, Koike (1988)) from 07:00 to 17:00. The daily 10-hr mean (07:00-17:00) for the experimental plots (n=6) was $362.9 \pm 14.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in 2014 and $354.3 \pm 25.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ in 2015).

2.2. Experimental Design

The O₃ treatments were ambient O₃ (AOZ) and elevated O₃ (EOZ), with three plot replicates for each treatment randomly scattered throughout the experimental forest. Each plot (diameter=6.5 m) contained three soil sub-plots from which BF and VA sub-plots were used

in the present study. Each sub-plot covered an area of $\approx 42\%$ of the total plot area. For both BF and VA sub-plots, 30 cm of soil was removed and placed back. BF is the native soil in this experimental forest. To create VA, volcanic ash soil from the Tomakomai Experimental Forest of Hokkaido University, Tomakomai, Japan, was mixed with the existing BF. Volcanic ash soil, which is poor in P and N content and has high porosity, is widely distributed throughout Hokkaido island, Japan. The pH of soil sub-plots was 5.69 ± 0.11 for BF and 5.09 ± 0.09 for VA. These soils have been widely used in research (Koike et al. 2015) and are considered free from toxic components. Details of chemical and physical properties of BF and VA can be found in Eguchi et al. (2008).

Two-year old seedlings of Japanese and hybrid larches were directly planted into the soils on May 15th, 2014. These seedlings were cultivated by the Forestry Research Institute of Hokkaido Research Organization in Bibai city, ≈ 60 km from Sapporo. Agrochemicals or fertilizers were not used during the experiment.

The FACE system was calibrated to maintain EOZ approximately twice the ambient O_3 concentrations, with a maximum of 80 nmol mol^{-1} . Ozone generated from pure oxygen was diluted with ambient air in a pressurized tank and released into the rings by 2.5-m teflon tubes suspended vertically 2.5 m above ground, and two horizontal Teflon tubes fixed around the plots elevated to 0.5 and 1.5 m. An SM70 Fixed Ozone Monitor (Aeroqual Ltd., Auckland, NZ) operated at the center of each EOZ plot, and a Hioki LR5042-20 (HIOKI E.E. Corporation, Nagano, JP) recorded O_3 data in two-minute intervals. Plants were exposed to EOZ from August 15th to October 26th in 2014 and from April 24th to October 26th in 2015,

during daylight hours with a PPFD higher than $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ (07:00-17:00). The mean 10-hr O_3 levels for EOZ plots were $60.1 \text{ nmol mol}^{-1}$ in 2014 and $72.1 \text{ nmol mol}^{-1}$ in 2015, which are translated to accumulated exposure over a threshold of 40 nmol mol^{-1} (AOT40) values of 8.4 and $49.1 \mu\text{mol mol}^{-1} \text{ h}$ in 2014 and 2015. Although a critical AOT40 level of 8-15 $\mu\text{mol mol}^{-1} \text{ h}$ has been proposed for sensitive forest trees in Japan (Kohno et al. 2005), AOT40 values higher than $30 \mu\text{mol mol}^{-1} \text{ h}$ occur in Japan (Hoshika et al. 2012). Background O_3 concentrations, for the same hours of the corresponding months, were 20.4 and $33.8 \text{ nmol mol}^{-1}$ in 2014 and 2015, respectively, as continuously monitored by an O_3 monitor (Model 202, 2B Technologies, Boulder CO, USA).

2.3. Data collection

2.3.1. Pre-treatment assessment

At the time of planting, initial measurements of plant height (cm) and stem basal diameter (mm) of the lignified stem at the base were taken from each plant. Plant height means were $16.68 (\pm 0.34 \text{ se})$ and $14.57 (\pm 0.32 \text{ se}) \text{ cm}$ for hybrid larch and Japanese larch, respectively. Basal diameter means were $2.26 (\pm 0.06 \text{ se})$, $2.16 (\pm 0.06 \text{ se}) \text{ mm}$ for hybrid larch and Japanese larch, respectively.

2.3.2. Post-treatment assessment

Late in the second growing season (2015), O_3 visible foliar injury was assessed on a continuous scale of 0-100 % for the foliage of each seedling. Each seedling was observed from above and four cardinal directions and the total foliar injury was estimated as percentage of injured needles per plant. Observations were done for both AOZ and EOZ plants.

Needles of similar maturity, randomized for O₃ symptom intensity, were destructively sampled from each plant to assess chlorophyll-related characteristics as potential indicators of physiological activity (Gottardini et al. 2014). Collected needles were stored in a deep freezer until pigments analysis. For each plant extract, needles were immersed in dimethyl sulfoxide (DMSO) solvent and incubated at 65 °C for 4 h under dark conditions, until shadowed thalli became visible. After incubation, each extract was covered with aluminum foil until its measurement. For the extracts, disposable 4.5 mL tubes were used (Nunc CryoTube Vials, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Measurements were taken at 415, 435, 470, 648, 665 nm (GeneSpec III; Hitachi Genetic Systems; MiraiBio, Alameda, CA). The ratio 435/415 (OD₄₃₅/OD₄₁₅) was calculated as a potential index of phaeophytinization, which shows the relative proportion of *Chl_a* and phaeopigment *a* (Ronen and Galun 1984). The total carotenoids content (*TCar*) was calculated according to Lichtenthaler (1987). As we performed the extraction with dimethyl sulfoxide as opposed to acetone, *TCar* should be interpreted for relative differences among experimental groups rather than on absolute values which may slightly differ. The chlorophyll *a* content (*Chl_a*), chlorophyll *b* content (*Chl_b*) and total chlorophyll content (*TChl*) were estimated according to Barnes et al. (1992). The fractions *Chl_a*/*Chl_b* and *TChl*/*TCar* were also calculated.

Growth measurements (cm) were taken at the end of the active growth period in the second growing season (2015). Crown thickness (from the point to which the first lateral shoot is attached on the stem to the highest point of the crown), and mean crown spread (distance between the two farthest points of the crown, as observed from above) were measured

alongside height, using measuring tape. The middle of the stem was marked following plant height measurements. Diameter of the lignified stem was measured at the base, middle and top using a digital a caliber, by taking the mean of two crosswise measurements at each stem level. Stem volume was also calculated for each plant as:

$$Volume = \pi \times \frac{\left(\left(\frac{diameter}{2}\right)^2\right)}{10} \times height \text{ cm}^3$$

where diameter is the mean of two diameter measurements at the base of the stem and π is set at 3.14.

Statistical Analysis

The level of significance for all statistical analyses was set at $\alpha=0.05$. The data of pre-treatment plant height and diameter and post-treatment *Chl_a*, *Chl_b*, *TChl*, *Chl_a/Chl_b*, *TCar* *OD₄₃₅/OD₄₁₅*, *TChl/TCar*, plant height, crown thickness, mean crown spread and stem volume were transformed using the maximum likelihood approach of Box-Cox transformation to approximate the Gaussian fit (Box and Cox 1964). General Linear Models (GLMs) were conducted on transformed data of each pigment-related response variable with Ozone, Soil and Taxa as fixed factors and randomized by plot (Kuehl 1999). Covariate-adjusted GLMs were performed on transformed data for growth-related response variables (Senturk and Muller 2009). The baseline data of plant height were used as a covariate for post-treatment plant height, crown thickness, mean crown spread and stem volume, whereas the baseline data of basal diameter were used as a covariate for post-treatment mean crown spread, stem volume and diameters of stem. For testing diameter across the stem levels (low, middle, upper), baseline-basal-diameter-adjusted GLM was applied using stem level as the

within effects dependent variable. Type 6 Sums of Squares, which uses the σ -restricted coding of effects in order to provide unique effect estimates even for lower-order effects (straightforward computing method), was employed. Post hoc Tukey's HSD test was performed when required.

For the variables that GLMs revealed Ozone as a significant factor, simple linear regression analysis was conducted between each pigment-related variable (predictor) and each growth-related variable (criterion). The gain in plant height and stem basal diameter was calculated by subtracting the pre-treatment score from the post-treatment score. Values of r within the arbitrary segments [0.00, 0.10), [0.10, 0.30), [0.30-0.50), [0.50-0.70), [0.70-0.90) and 0.90+, indicate trivial, low, moderate, large, very large and nearly perfect correlation magnitude, respectively (Hopkins 2000).

Visible injury data were subjected to arcsine transformation before performing a Generalized Linear Mixed Model (GLMM) with identity link function; random and main, two-way and three-way fixed factors specified as in GLMs (Bolker et al. 2008). The variance component was selected for random effect covariance type. An *a priori* decision was made to exclude the two-way effects from the full factorial terms (full: Ozone + Soil + Taxa + Ozone $\times$ Soil + Ozone $\times$ Taxa + Soil $\times$ Taxa + Ozone $\times$ Soil $\times$ Taxa + (Plot)), despite their statistical significance, and include only effects with biological importance. Plot was not significant (Estimate = 17.8, se = 18.3, Z = 0.975, p=0.330, 95% CI [2.4, 133.0]) and excluded to simplify the model; the corrected Akaike Information Criterion (AIC_c) was smaller without Plot. The final model was Ozone + Soil + Taxa + Ozone $\times$ Soil $\times$ Taxa.

The unbiased Cohen's δ was estimated based on balanced real replicates (Hedges and Olkin 1985) so as to quantify the magnitude of difference when the predictor was statistically significant. For covariate-adjusted response variables, δ was estimated by subtracting the baseline effect from the post-treatment effect (Agathokleous et al. 2016c). Estimations of δ were calculated according to Agathokleous et al. (2016b, 2016c). Values within the arbitrary segments [0.00, 0.50), [0.50, 1.50), [1.50-3.00) and 3.00+, indicate neutral, small, moderate and large magnitude of difference, respectively (Agathokleous et al. 2016b, modified from Cohen 1988). Absolute δ values in the interval [0.50-1.50] indicate educational significance while δ values >1.50 indicate practical significance (Agathokleous et al. 2016b, modified from Wolf 1986).

Furthermore, δ was converted to overlapping coefficient (OVL), a potential measure of bioequivalence under the name proportion of similar response (Reiser and Faraggi 1999), using the formula

$$OVL = 2\Phi(-|\delta|/2)$$

where, Φ is the cumulative distribution function of the standard normal distribution and δ the population δ . This coefficient represents the common area under two probability density curves and indicates the agreement between two distributions (Reiser and Faraggi 1999).

Log-transformed height of the plant at which stem diameter was measured was used to plot height against stem diameter (n=3 for each taxon in each soil, O₃ treatment and stem level); the equations are shown in Fig 3. The data of each Ozone group were pooled from taxa and soils, for each stem level, and the slopes of AOZ and EOZ were tested ($\alpha=0.05$) using

comparison of regression lines slopes from two independent samples based on pooled variance (Howell 2010).

For data presentation purposes, the untransformed values are presented. Data processing and statistical analyses were conducted using MS EXCEL 2010 (Microsoft ©), PASW Statistics 18 (formerly SPSS Statistics, IBM ©) and STATISTICA v.10 (StatSoft Inc. ©) software.

3. RESULTS

3.1. Visible injury

Larch needles were discoloured in EOZ but not AOZ treatments. Discolouration was observed as yellowish flecks initiating from the top and progressing towards the base of the needles. The average values of needle areas affected by O₃ visible injury per plant (%) in EOZ plants were 93 (± 3), 83 (± 7), 73 (± 3) and 84 (±5) for hybrid larch×BF, hybrid larch×VA, Japanese larch×BF and Japanese larch×VA, respectively. The value of the corrected AIC_c was 118.548. In the corrected model ($F=81.5$, $P<0.001$), only the predictor Ozone had a significant ($F=564.2$, $P<0.001$) fixed effect. Soil ($F=0.02$, $P=0.895$), Taxa ($F=1.5$, $P=0.239$), and Ozone×Soi×Taxa ($F=1.3$, $P=0.318$) were not significant. The coefficient of AOZ was -67.42 ± 5.68 se, Wald t statistic (t) = -11.87 , $P<0.001$.

3.2. Pigments and plant size

Ozone was not a significant factor for OD₄₃₅/OD₄₁₅ (Table 2, Fig 1). However, EOZ had a small negative effect on Chl_a/Chl_b ($\delta=-1.20$, OVL=0.550) and a moderate negative effect on plant height ($\delta=-1.80$, OVL=0.362), crown thickness ($\delta=-2.26$, OVL=0.259), mean crown spread ($\delta=-2.28$, OVL=0.255) and stem volume ($\delta=-2.89$, OVL=0.148) (Fig 1-2).

296 Furthermore, EOZ had a large negative effect on Chl_a ($\delta=-6.58$, $OVL=0.001$), Chl_b ($\delta=-5.86$,
297 $OVL=0.002$), $TChl$ ($\delta=-6.54$, $OVL=0.001$), $TCar$ ($\delta=-3.49$, $OVL=0.081$) and $TChl/TCar$
298 ($\delta=-3.46$, $OVL=0.084$). For Chl_a , Chl_b , $TChl$, $TCar$ and $TChl/TCar$, 10 % (maximum) of the
299 groups (AOZ and EOZ) overlap and there is a >99 % chance that a random observation from
300 EOZ would reveal a lower score than a random observation from AOZ (area under the
301 receiver operating characteristics (AUC); Ruscio and Mullen 2012).

302 The effect of soil type was significant only for Chl_a/Chl_b (Table 2, Fig 1) to which VA caused
303 a small positive effect ($\delta=0.83$, $OVL=0.678$).

304 Taxa differed in all the variables except the three pigmentation ratios (Chl_a/Chl_b ,
305 OD_{435}/OD_{415} , $TChl/TCar$), with hybrid larch having superior performance to Japanese larch
306 in all the measured characteristics (Table 2, Fig 1-2). In AOZ, hybrid larch and Japanese
307 larch had a difference of small magnitude in $TCar$ ($\delta=1.19$, $OVL=0.550$). A moderate
308 difference between species existed for Chl_b ($\delta=1.48$, $OVL=0.458$), mean crown spread
309 ($\delta=2.15$, $OVL=0.283$), Chl_a ($\delta=1.55$, $OVL=0.438$) and $TChl$ ($\delta=1.58$, $OVL=0.431$). Similarly,
310 in AOZ, they had a difference of large magnitude in plant height ($\delta=3.53$, $OVL=0.078$),
311 crown thickness ($\delta=3.70$, $OVL=0.064$) and stem volume ($\delta=4.08$, $OVL=0.041$). In EOZ,
312 hybrid larch and Japanese larch had a difference of small magnitude in Chl_b ($\delta=1.13$,
313 $OVL=0.573$) and $TChl$ ($\delta=1.43$, $OVL=0.474$) and a difference of moderate magnitude in all
314 the other characteristics including plant height ($\delta=1.72$, $OVL=0.391$), crown thickness
315 ($\delta=2.05$, $OVL=0.304$), mean crown spread ($\delta=1.61$, $OVL=0.420$), stem volume ($\delta=1.67$,
316 $OVL=0.404$), Chl_a ($\delta=1.64$, $OVL=0.412$) and $TCar$ ($\delta=1.66$, $OVL=0.406$).

317 Ozone×Soil was significant only for Chl_b and Chl_a/Chl_b , whereas only for Chl_a/Chl_b there
318 was a difference of interest in multiple comparisons. A moderately higher Chl_a/Chl_b was
319 observed in EOZ×VA compared with EOZ×BF ($\delta=-1.65$, OVL=0.410).

320 The interactions Soil×Taxa and Ozone×Taxa were insignificant, for all the pigment and plant
321 size-related variables.

322 Ozone×Soil×Taxa was significant only for stem volume. Hybrid larch had a smaller stem
323 volume in EOZ×BF compared with AOZ×BF ($\delta=-7.53$, OVL<0.001) and in EOZ×VA
324 compared with AOZ×VA ($\delta=-6.80$, OVL=0.001) with large differences in magnitude.
325 Similar large differences were observed for Japanese larch, which had a smaller stem volume
326 in EOZ×BF compared with AOZ×BF ($\delta=-3.03$, OVL=0.130) and in EOZ×VA compared
327 with AOZ×VA ($\delta=-6.83$, OVL=0.001). Finally, hybrid larch had a greater stem volume than
328 Japanese larch in AOZ×BF ($\delta=5.45$, OVL=0.006), AOZ×VA ($\delta=3.04$, OVL=0.129) and
329 EOZ×VA ($\delta=2.41$, OVL=0.229), of large, large and moderate magnitude, respectively.
330 Hybrid larch stem volume was 29% larger in EOZ×BF, however high RSD (35.5% and
331 28.4%) masked the difference.

332 GLM analysis of diameters across the stem (Fig 3) revealed Ozone ($F=102.13$, $P<0.001$),
333 Taxa ($F=5.30$, $P<0.05$) and Stem level ($F=9.02$, $P<0.001$) but not Soil ($F=0.01$, $P=0.924$) as
334 significant predictors. Stem diameter was higher in AOZ than in EOZ and higher in hybrid
335 larch compared with Japanese larch. Stem diameter was also higher at the lower part
336 compared with the middle part of the stem, and at the middle part compared with the upper
337 part of the stem. From second order interaction, only Ozone×Stem level ($F=46.38$, $P<0.001$)

and Taxa×Stem level ($F=9.70$, $P<0.001$) were significant. Stem diameter was decreased in EOZ treatments at each level. Only the lower part of the stem was different between taxa, where Japanese larch had lower stem diameter than hybrid larch. Ozone×Soil ($F=1.84$, $P=0.195$), Ozone×Taxa ($F=0.75$, $P=0.401$), Soil×Taxa ($F=0.27$, $P=0.611$), Soil×Stem level ($F=0.10$, $P=0.905$) were insignificant. There were no significant effects for third and fourth order interactions: Ozone×Soil×Taxa ($F=2.33$, $P=0.148$), Ozone×Soil×Stem level ($F=2.52$, $P=0.098$), Ozone×Taxa×Stem level ($F=1.72$, $P=0.196$), Soil×Taxa×Stem level ($F=0.48$, $P=0.621$) and Ozone×Soil×Taxa×Stem level ($F=1.43$, $P=0.256$). The slope between the height of the stem, at which diameter was measured, and the stem diameter was lower in EOZ compared to AOZ ($T=-3.61$, $P<0.001$).

The simple linear regressions (Fig 4-5) showed that all the examined pigment-related variables can effectively predict scores on all the growth-related variables ($P < 0.05$, adjusted $R^2 < R^2$). Chl_a/Chl_b had a moderate correlation with all the growth-related variables except stem basal diameter with which it had a large correlation, and $TChl/TCar$ had a large correlation with plant height and mean crown spread (Fig 5). All the other correlations of $TChl/TCar$ and the other pigment-related variables with growth-related variables were very large or near-perfect.

4. DISCUSSION

The O₃-induced visual injury was high in both soil types, and of similar magnitude for both larch taxa. This is consistent with the lower Chl_a , Chl_b , $TChl$, $TCar$ and $TChl/TCar$ of both taxa in EOZ than in AOZ which was of large magnitude. Importantly, despite the large

differences in chlorophyll contents, the difference of hybrid larch and Japanese larch in terms of Chl_a/Chl_b between EOZ and AOZ was small, with 55% of the groups overlapping and an 80% chance that a haphazard observation from EOZ will reveal a lower score than a haphazard observation from AOZ. Chlorophyll production indicates nutritional, photosynthetic and productive efficiency of plants and relates to a unique plant defensive system against insect herbivory through the catalysis of chlorophyll by chlorophyll chlorophyllidohydrolase to produce chlorophyllide and phytol (Curran et al. 1990; Hu et al. 2015). Therefore, chlorophyll molecules are of utmost importance for plant health. Loss of chlorophyll may relate to chlorophyll oxidation or altered regulation of pigment biosynthesis (Caregnato et al. 2013), where the synthesis of new chlorophyll *a* is inhibited or the synthesis of chlorophyll *b* is increased as compared with uninjured needles (Knudson et al. 1977). The present Chl_a/Chl_b findings are consistent with higher sensitivity of chlorophyll *a* than chlorophyll *b* to O_3 in C3 plants reported in previous studies (Knudson et al. 1977; Saitanis et al. 2001; Neufeld et al. 2006; Feng et al. 2008). However, they are inconsistent with other studies where a higher sensitivity of chlorophyll *b* than chlorophyll *a* to O_3 was found in C3 plants in the framework of a rearrangement of the pigments ratio as an adaptive defense mechanism within the chloroplasts to protect PSII reaction centers from photoinhibition (Fernandez et al. 1993; Döring et al. 2013; Pellegrini 2014). The structure of a chlorophyll molecule is characterized by a porphyrin head and a phytol tail with O_3 -sensitive double bonds, however, while chlorophyll *a* has a $-CH_3$ group, chlorophyll *b* has an $-CHO$ group at carbon atom 3 of pyrrole ring II. Despite their similar structure, chlorophylls *a* and *b* have different locations, light absorption spectra and tasks within the photosystems, and these

dissimilarities may define their difference in sensitivity. The present and previous findings (Manninen et al. 1999; Saitanis et al. 2001; Feng et al. 2008; Agathokleous et al. 2016c; Gao et al. 2016) indicate that Chl_a/Chl_b is a more efficient indicator of O_3 stress when O_3 injury is high than when O_3 injury is low, since a reduction of Chl_a associates with an increase in foliar injury (Knudson et al. 1977; Neufeld et al. 2006; Caregnato et al. 2013; Gottardini et al. 2014). Contrastingly, O_3 sensitivity of $TChl/TCar$ was 1.9 times higher (δ) than that of Chl_a/Chl_b . Such observation is consistent with higher sensitivity of $TChl$ than $TCar$ found in C3 plants (Agathokleous et al. 2014, 2016c; Pellegrini 2014). Alterations in $TChl/TCar$ indicate malfunctions in plant physiological and phenological status caused by stress-induced photooxidation through breakdown of chlorophylls and a need for photoprotection (Peñuelas et al. 1995; Pellegrini 2014). In addition to the higher sensitivity of $TChl/TCar$ compared with Chl_a/Chl_b , the correlations of $TChl/TCar$ with the growth-related variables were large or very large whereas those of Chl_a/Chl_b were moderate in four out of five cases. We thus postulate that $TChl/TCar$ may be a more sensitive O_3 biomarker than Chl_a/Chl_b and suggest further investigation of this index for O_3 pollution assessment studies of deciduous coniferous species. It is also worthwhile to test whether Chl_a/Chl_b increases under low stress in the framework of biological plasticity with the central player of Chl_b , and decreases under high oxidation damage in Chl_a (Calabrese and Blain 2009; Pellegrini 2014).

Physiological malfunctions indicated by pigmentation assessment reflected in plant growth through strong intercorrelations. Similarly to pigment-related traits, EOZ moderately suppressed plant height, crown thickness, mean crown spread and stem volume. However, heterosis was observed in the hybrid larch. Such a heterosis was previously observed in stem

and crown of the hybrid larch *Larix decidua* (Mill.) x *L. leptolepis* (Sieb and Zucc.) Gord. (Matyssek and Schulze 1987). Hybrid larch heterosis, observed in this study, was maintained in EOZ despite EOZ negatively affected hybrid growth too. Although vertical growth has been extensively studied, horizontal growth is poorly understood in open-grown tree species (Pretzsch et al. 2015). Tree crown size is an important index both for forest and urban trees showing the epigeous space a tree occupies, which relates to the total leaf area and biomass (Binkley et al., 2013; Forrester 2014; Pretzsch et al. 2015). An EOZ-induced decrease in crown spread and thickness shows smaller crown size along with smaller epigeous space occupied by a tree. Such a decrease may further show decreased light use efficiency, as the latter may decrease with decreasing epigeous tree size (Gspaltl et al. 2013). Cachectic crown size in forest trees may have long term implications for forest ecosystems. Changes in epigeous competition between species of different O₃ sensitivity, undergrowth light intensity, crown faunal composition and carbon sequestration may be affected. In urban environments, suppressed crown size has implications of decreased particulate pollution absorption, reduced shading and a greater risk of branches breaking in high winds (Pretzsch et al. 2015). With EOZ, stem diameter was reduced at each stem level across taxa and soils. However, plants in EOZ had a significantly lower drop of stem diameter over the run of stem height (from base to top) compared to plants in AOZ. This drop indicates a disproportional loss of stem diameter from the base to the top of the stem and may be attributed to a shorter exposure of the upper stem to EOZ than the middle and base. Disproportional inhibition of stem diameter growth across different heights indicates more warped growth and a possible susceptibility to winds. An O₃-induced reduction in stem diameter at the lower third of the

stem was reported in a plantation of the O₃-sensitive Oxford clone of poplar (*Populus maximoviczii* Henry x *berolinensis* Dippel) in a 6-year open-field study (Carriero et al. 2015). A lower increment or failure to increase in stem diameter also occurred mostly at the lower third of the stem of mature European beech trees (*Fagus sylvatica* L.) after a 7-year treatment with elevated O₃ levels (see Pretzsch et al. 2010 for discussion and references). The findings of this study support conclusions by Pretzsch et al. (2010) that a decline in diameter increment at the lower third of the stem should not be interpreted as an overall growth decline due to overestimations.

The present findings of an EOZ-induced diameter suppression of both hybrid and Japanese larches contradict studies with potential experimental artifacts of O₃ facilities and root restrictions. For example, in a study using a FACE system with 60 nmol mol⁻¹ daytime exposure over a growing season, no significant effects of O₃ were found on growth of the stem basal diameter and stem volume of potted hybrid and Japanese larch seedlings (Kam et al., 2015). Contrastingly, in an OTC study where potted seedlings were exposed to 60 nmol mol⁻¹, 7 h per day over a growing season, there was a significant O₃-induced reduction of diameter growth increment and height of potted seedlings of hybrid larch but not its parental species (Koike et al. 2012). However, the whole plant dry mass and the shoot/root ratio of all taxa were unaffected (Koike et al. 2012). Another OTC study on hybrid larch seedlings directly planted in the soil (i.e. no root limitations) with the same O₃ treatment as in Koike et al. (2012), found an O₃-induced reduction in final stem dry mass after two growing seasons of exposure to O₃ (Wang et al. 2015). However, O₃-induced reductions in plant height and stem diameter in the first growing season did not translate to early or late in the second (Wang

et al. 2015). Thus, under experimental conditions with OTCs or potted plants in FACE systems, O₃ appears to influence plant growth differently with altered conditions. The present contradictory results in a system without containment artifacts and root restrictions reveal the importance of free air studies in natural soils to understand how co-occurring environmental changes will impact forest tree species.

Soil, independently from Taxa and Ozone, was a significant factor only for *Chl_a/Chl_b*. In contrast to the EOZ-induced decrease in *Chl_a/Chl_b*, VA caused an increase in *Chl_a/Chl_b*, showing higher *Chl_a* than *Chl_b*. This increase, was upon prevention of the EOZ-induced imbalance in *Chl_a/Chl_b* by VA, which was the only difference of interest in the Soil×Ozone interaction. *Chl_a/Chl_b* is an efficient indicator of the light environment of plants (Dale and Causton 1992; Lei et al. 1996), and its increase associates with acclimation to high light conditions and low N availability (Kitajima and Hogan 2003). Since the light environment was similar for both soils in this experiment, the increase in *Chl_a/Chl_b* in VA may be attributed to the lower N content in VA than in BF (Eguchi et al. 2008). It is also possible that Mg content differed between soil types. As Mg is a central atom in chlorophyll porphyrin ring, such a potential difference might have affected chlorophyll biosynthesis.

The complexity of using *Chl_a/Chl_b* as a measure of the O₃ impact on plants in *in vitro* studies is demonstrated. Attention should be paid to the numerous factors influencing *Chl_a/Chl_b*, and particularly site-specific soil, which may lead to erroneous conclusions. OD₄₃₅/OD₄₁₅ is an ineffective biomarker of O₃ injury in these taxa. *TChl/TCar*, may be more accurate in

assessing O₃ effects on the studied taxa as it is more sensitive to EOZ than *Chl_a/Chl_b* and consistently related to O₃ alone.

Diameter across stem height could be utilized as a low-cost assessment tool of O₃ stress in large trees especially in remote areas where access to equipment and facilities is prohibitive. Still, diameter across stem height and crown size should be taken into account by urban plant pathologists and local authorities in risk analysis (Lorenzini and Nali 2014). It is also recommended to avoid interpretation of O₃ impact on overall growth based on a decline in diameter increment at the lower third of the stem.

5. CONCLUSIONS

The present findings provide evidence for sustained heterosis in hybrid larch under elevated O₃ levels. It is concluded that hybrid larch is no more susceptible to elevated O₃ levels than Japanese larch for the growth and health traits tested. These results differ from previous studies conducted with the same taxa and at the same experimental area but with potential experimental artifacts.

In contrast to the tested hypothesis of a soil-dependent response of larch to O₃, there was no relevant difference except in *Chl_a/Chl_b*. It is recommended including tests with more infertile soil in future studies.

Long-term studies and breeding programmes which account for improvement to O₃-tolerance capacity are required. It is suggested to consider stem shape as well as vertical and horizontal growth when making comparative viability assessments of rehabilitation and plantation

species. Superior growth, stem form and branching traits in changing environment should be an additional goal in practice due to the ecological and economic importance of these traits.

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735 **Captions**

736 **Table 1** Means $\pm$ s.e. of the main meteorological conditions at Sapporo, Japan, for the months
737 June-October, of the years 2014-2015.

738 **Table 2** Results of the GLM statistical analysis. All the pigment-related data subjected to
739 general linear models randomized by plot. Data obtained from Japanese larch and a hybrid
740 larch F₁ seedlings grown in brown forest soil (BF) or BF mixed with volcanic ash soil (VA)
741 and exposed to ambient air (AOZ) or O₃-enriched air (EOZ) (O₃≈66 nmol mol⁻¹) for two
742 growing seasons. Each treatment condition is consisted of three replicates.

743 **Fig. 1** Means ($\pm$ se) of chlorophyll *a* content (*Chl_a*), chlorophyll *b* content (*Chl_b*), total
744 chlorophyll content (*TChl*), *Chl_a/Chl_b* ratio, ratio of optical densities 435 to 415
745 (OD₄₃₅/OD₄₁₅), total carotenoid content (*TCar*) and *TChl/TCar* ratio. Data obtained from
746 Japanese larch (light shading) and a hybrid larch F₁ (dark shading) seedlings grown in brown
747 forest soil (BF) or BF mixed with volcanic ash soil (VA) and exposed to ambient air (AOZ)
748 or O₃-enriched air (EOZ) (O₃≈66 nmol mol⁻¹) for two growing seasons. Different uppercase
749 letters show statistically significant differences within O₃×Soil interaction (Taxa pooled),
750 according to Tukey's HSD posthoc test. The significance of the main factors and O₃×Soil
751 interaction is indicated by ns (non-significant, $P>0.05$), * ($P<0.05$) ** ($P<0.01$) or ***

($P < 0.001$). The interactions $O_3 \times \text{Taxa}$, $\text{Soil} \times \text{Taxa}$ and $O_3 \times \text{Soil} \times \text{Taxa}$ were insignificant for all the response variables. Each value of the eight means resulted from three replicates.

Fig. 2 Means ($\pm$ se) of plant height, crown thickness, mean crown spread and stem volume. Data obtained from Japanese larch (light shading) and a hybrid larch F_1 (dark shading) seedlings grown in brown forest soil (BF) or BF mixed with volcanic ash soil (VA) and exposed to ambient air (AOZ) or O_3 -enriched air (EOZ) ($O_3 \approx 66 \text{ nmol mol}^{-1}$) for two growing seasons. Different lowercase letters indicate statistically significant differences among the means, when $O_3 \times \text{Soil} \times \text{Taxa}$ was significant, and different uppercase letters show statistically significant differences within $O_3 \times \text{Soil}$ (Taxa pooled), according to Tukey's HSD posthoc test. The significance of the main factors is indicated by ns (non-significant, $P > 0.05$), * ($P < 0.05$) ** ($P < 0.01$) or *** ($P < 0.001$). The interactions $O_3 \times \text{Soil}$, $O_3 \times \text{Taxa}$ and $\text{Soil} \times \text{Taxa}$ were insignificant for all the response variables. Each value of the eight means resulted from three replicates.

Fig. 3 Means ($\pm$ se) of diameter across stem as measured at the base (Lower), middle (Middle) and top (Upper) of the stem. Data obtained from Japanese larch (JL, dashed lines) and a hybrid larch F_1 (HL, solid lines) seedlings grown in brown forest soil (BF, lower figure) or BF mixed with volcanic ash soil (VA, upper figure) and exposed to ambient air (AOZ) or O_3 -enriched air (EOZ) ($O_3 \approx 66 \text{ nmol mol}^{-1}$) for two growing seasons. Different uppercase letters A, B and C show statistically significant differences among stem levels (other treatments pooled). Different lowercase letters between means of taxa in each stem level show statistically significant differences within the interaction $O_3 \times \text{Stem level}$ (Soil and Taxa

pooled). Different uppercase letters L, M, N and O above means of taxa in each stem level show statistically significant differences within the interaction Taxa×Stem level (O3 and Soil pooled). Differences are marked according to Tukey's HSD posthoc test. The significance of the main factors and the interactions O3×Stem level and Taxa×Stem level is indicated by ns (non-significant, $P>0.05$), * ($P<0.05$) ** ($P<0.01$) or *** ($P<0.001$). The remaining second order interactions and the third and fourth order interactions were insignificant. Each mean is the product of three replicates.

Fig. 4 Regression analysis of chlorophyll *a* content (Chl_a), chlorophyll *b* content (Chl_b) and total chlorophyll content ($TChl$) with each growth-related variable. Each data point is the average of the plants in each research condition (N=24). The regressions were tested at an α level of 0.05.

Fig. 5 Regression analysis of total carotenoid content ($TCar$), Chl_a/Chl_b ratio and $TChl/TCar$ ratio with each growth-related variable. Each data point is the average of the plants in each research condition (N=24). The regressions were tested at an α level of 0.05.

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794 **Table 1**

	2014	2015
Daily average air temperature (°C)	18.60 ±2.04	17.92 ±2.05
Daily maximum air temperature (°C)	23.08 ±2.03	22.22 ±2.03
Daily minimum air temperature (°C)	14.96 ±2.21	14.50 ±2.22
Daily wind speed (m s ⁻¹)	3.40 ±0.16	3.32 ±0.23
Daily relative humidity (%)	69.6 ±1.69	70.2 ±2.33
Total sunshine duration (h)	181.96 ±11.04	157.54 ±4.59
Total precipitation (mm)	132.6 ±24.23	111.6 ±24.85

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Table 2

	Ozone	Soil	Taxa	Ozone×Soil	Ozone×Taxa	Soil×Taxa	Ozone×Soil×Taxa
<i>Plant height</i>	<i>F</i> =67, <i>P</i> <0.001	<i>F</i> =0.06, <i>P</i> =0.807	<i>F</i> =32, <i>P</i> <0.001	<i>F</i> =0.38, <i>P</i> =0.547	<i>F</i> =0.15, <i>P</i> =0.703	<i>F</i> =0.38, <i>P</i> =0.546	<i>F</i> =0.04, <i>P</i> =0.844
<i>Crown thickness</i>	<i>F</i> =89, <i>P</i> <0.001	<i>F</i> =0.30, <i>P</i> =0.591	<i>F</i> =42, <i>P</i> <0.001	<i>F</i> =0.62, <i>P</i> =0.443	<i>F</i> =0.02, <i>P</i> =0.894	<i>F</i> =0.40, <i>P</i> =0.536	<i>F</i> =0.30, <i>P</i> =0.592
<i>Mean crown spread</i>	<i>F</i> =54, <i>P</i> <0.001	<i>F</i> =0.01, <i>P</i> =0.907	<i>F</i> =19, <i>P</i> <0.001	<i>F</i> =0.14, <i>P</i> =0.711	<i>F</i> =0.01, <i>P</i> =0.938	<i>F</i> =0.19, <i>P</i> =0.669	<i>F</i> =0.01, <i>P</i> =0.938
<i>Stem volume</i>	<i>F</i> =221, <i>P</i> <0.001	<i>F</i> <0.01, <i>P</i> =0.975	<i>F</i> =42, <i>P</i> <0.001	<i>F</i> =1.8, <i>P</i> =0.200	<i>F</i> =0.53, <i>P</i> =0.477	<i>F</i> <0.00, <i>P</i> =0.958	<i>F</i> =4.79, <i>P</i> <0.050
<i>Chl_a</i>	<i>F</i> =633, <i>P</i> <0.001	<i>F</i> =1.13, <i>P</i> =0.304	<i>F</i> =17, <i>P</i> <0.001	<i>F</i> =1.33, <i>P</i> =0.266	<i>F</i> =0.04, <i>P</i> =0.855	<i>F</i> =0.22, <i>P</i> =0.649	<i>F</i> =0.11, <i>P</i> =0.750
<i>Chl_b</i>	<i>F</i> =333, <i>P</i> <0.001	<i>F</i> =0.21, <i>P</i> =0.655	<i>F</i> =11, <i>P</i> <0.010	<i>F</i> =5.2, <i>P</i> <0.050	<i>F</i> <0.01, <i>P</i> =0.957	<i>F</i> =2.94, <i>P</i> =0.106	<i>F</i> =0.153, <i>P</i> =0.701
<i>TChl</i>	<i>F</i> =557, <i>P</i> <0.001	<i>F</i> =0.38, <i>P</i> =0.544	<i>F</i> =15, <i>P</i> <0.010	<i>F</i> =2.2, <i>P</i> =0.156	<i>F</i> =0.03, <i>P</i> =0.867	<i>F</i> =0.7, <i>P</i> =0.422	<i>F</i> =0.01, <i>P</i> =0.940
<i>Chl_a/Chl_b</i>	<i>F</i> =13, <i>P</i> <0.010	<i>F</i> =7.8, <i>P</i> <0.050	<i>F</i> =0.23, <i>P</i> =0.636	<i>F</i> =6.6, <i>P</i> <0.050	<i>F</i> =1.18, <i>P</i> =0.182	<i>F</i> =3.3, <i>P</i> =0.087	<i>F</i> =0.66, <i>P</i> =0.430
<i>TCar</i>	<i>F</i> =161, <i>P</i> <0.001	<i>F</i> =4.3, <i>P</i> =0.055	<i>F</i> =16, <i>P</i> <0.001	<i>F</i> =0.19, <i>P</i> =0.669	<i>F</i> =0.07, <i>P</i> =0.794	<i>F</i> =1.2, <i>P</i> =0.282	<i>F</i> =1.55, <i>P</i> =0.230
<i>OD₄₃₅/OD₄₁₅</i>	<i>F</i> =0.32, <i>P</i> =0.582	<i>F</i> =0.85, <i>P</i> =0.370	<i>F</i> =1.91, <i>P</i> =0.186	<i>F</i> =0.24, <i>P</i> =0.631	<i>F</i> =0.15, <i>P</i> =0.700	<i>F</i> =3.39, <i>P</i> =0.084	<i>F</i> =1.23, <i>P</i> =0.285
<i>TChl//TCar</i>	<i>F</i> =82, <i>P</i> <0.001	<i>F</i> =3.25, <i>P</i> =0.090	<i>F</i> =0.56, <i>P</i> =0.464	<i>F</i> =0.47, <i>P</i> =0.503	<i>F</i> =0.17, <i>P</i> =0.690	<i>F</i> =2.95, <i>P</i> =0.106	<i>F</i> =0.23, <i>P</i> =0.635

Fig 1

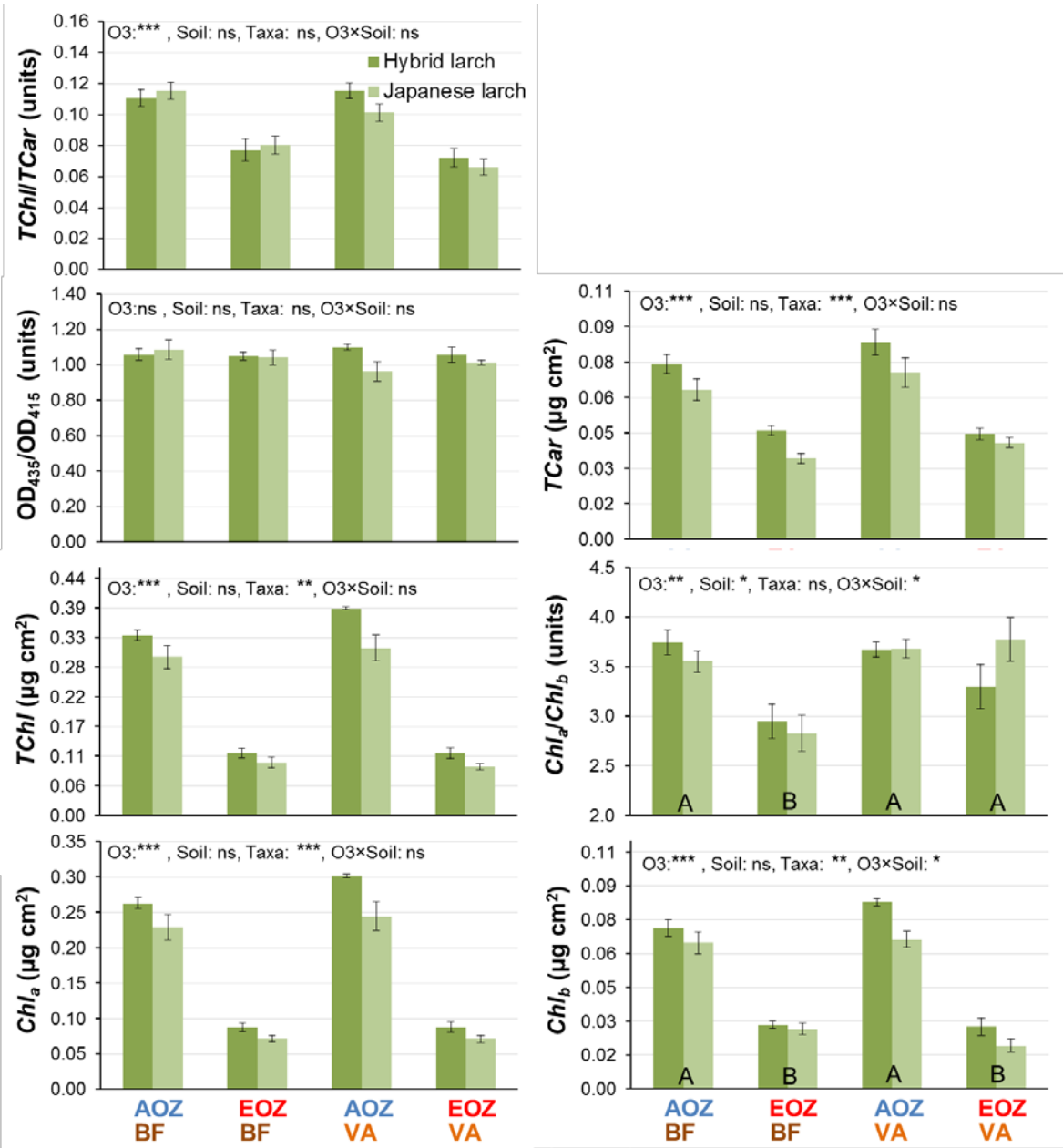


Fig 2

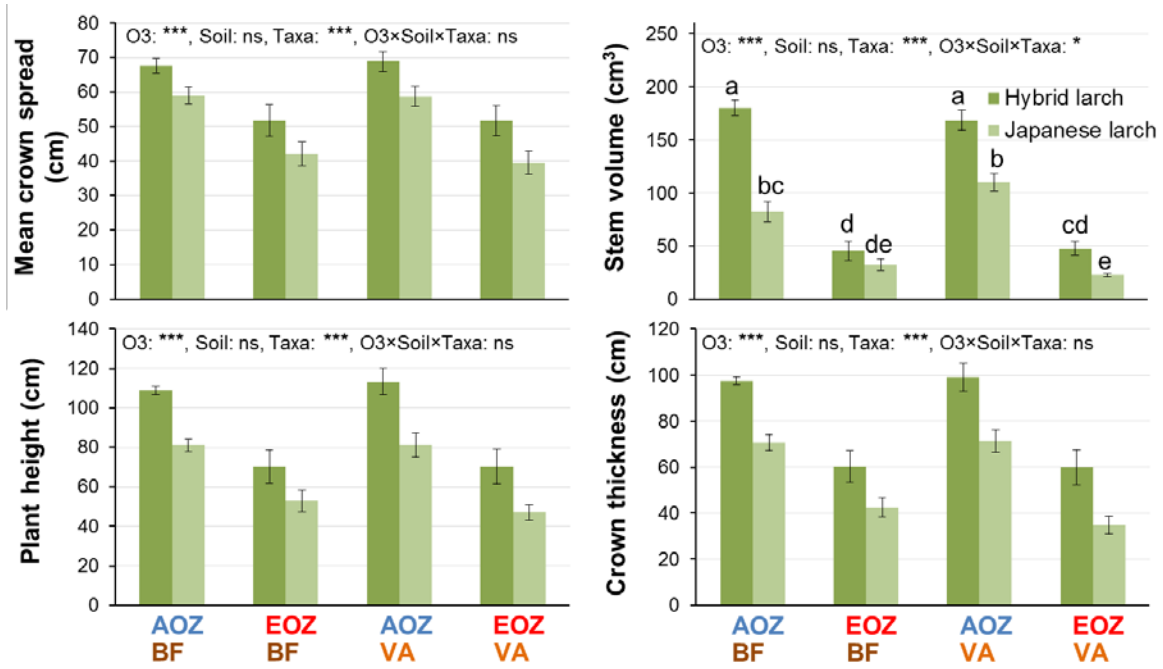


Fig 3

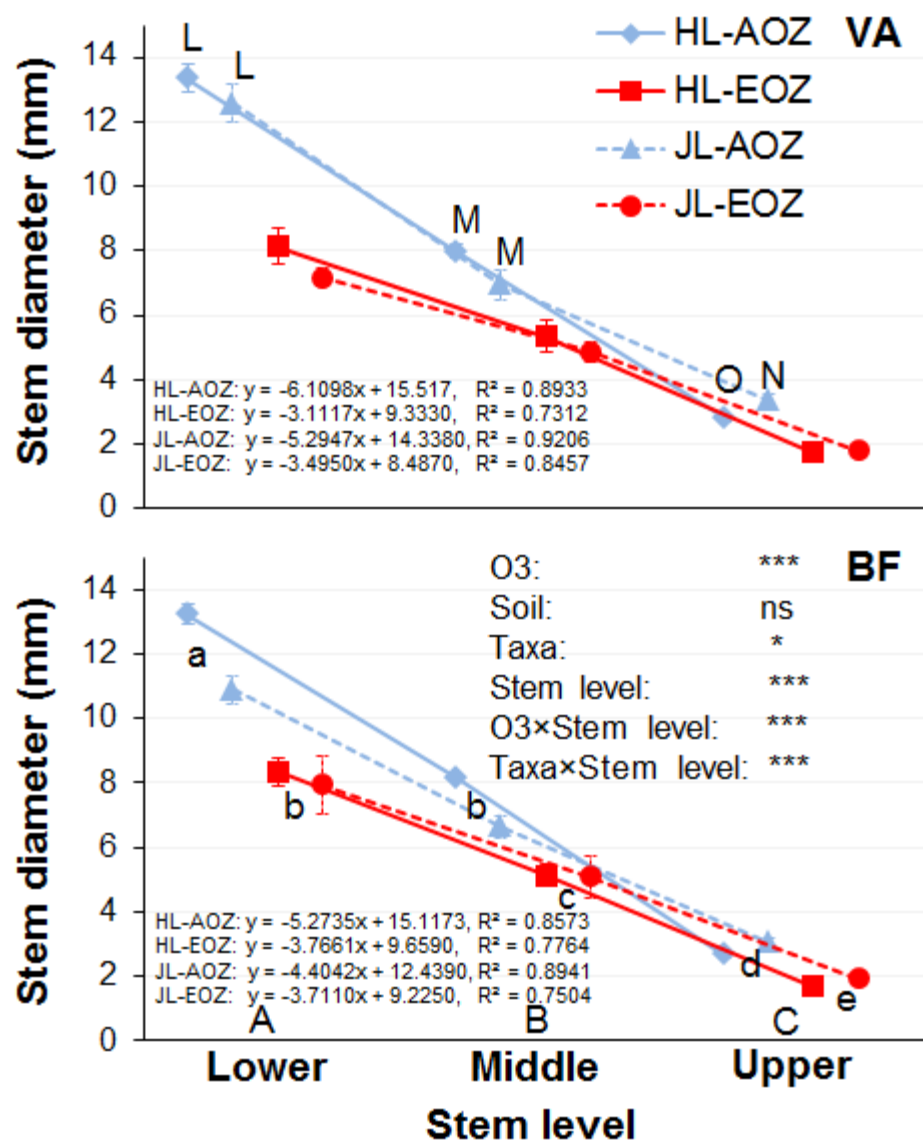


Fig 4

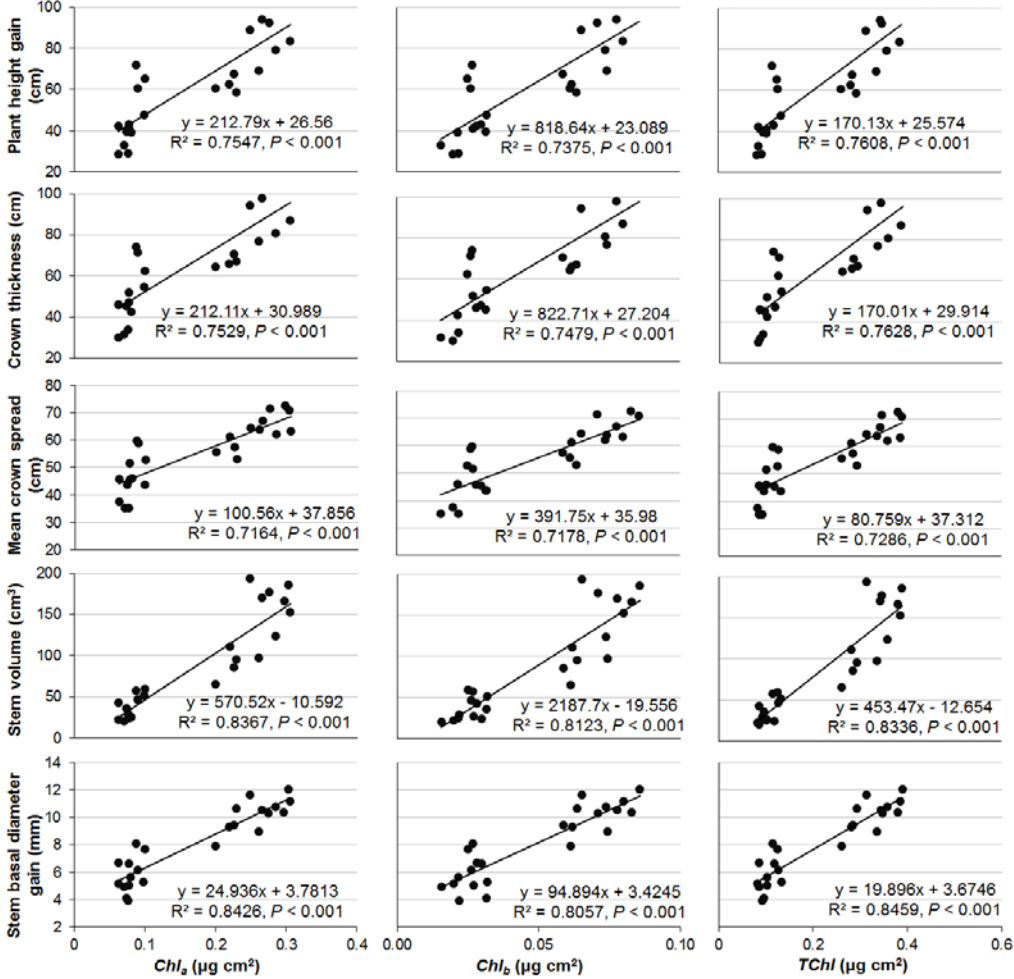


Fig 5

