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Research paper

Ozone-induced hormonal effects underpin germination dynamics in temperate tree species

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ABSTRACT

Tropospheric ozone is a secondary air pollutant that negatively affects plant growth, but its impact on seed germination remains poorly understood despite the occurrence of ozone episodes in early spring. This study investigated the direct effects of ozone exposure on non-dormant seed germination, together with the impact on the key phytohormones that govern germination. We exposed seeds of five widely distributed temperate tree species (*Pinus sylvestris, Betula pendula, Alnus glutinosa, Acer pseudoplatanus*, and *Picea sitchensis*) to elevated (+ 60 ppb) or ambient atmospheric ozone concentrations over 27-day germination periods. Elevated ozone led to a significant delay and reduction in germination (by 17%) in *P. sylvestris*, with the same but weaker trends in *B. pendula* and *P. sitchensis*, and no detectable effects in *A. glutinosa* or *A. pseudoplatanus*. At the end of the trial, ozone altered the balance of the key germination-regulating hormone abscisic acid (ABA; 1.5-fold change) in ungerminated *P. sylvestris* seeds, as well as the levels of hormones that influence ABA transcription (auxin; 9-fold change) and plant responses to abiotic stress (jasmonic acid; 3-fold change; salicylic acid; 2-fold change). These phytohormonal effects provide a suggested mechanism through which ozone can induce changes in seed germination, with species sensitivity likely depending on oxidant uptake, antioxidant capacity and physical seed characteristics. Our results underscore ozone's potential role as a selective pressure by differentially affecting seed germination dynamics.

1. Introduction

Tropospheric, or ground-level ozone is a major air pollutant with numerous detrimental effects on plants. Ozone induced reduction in photosynthetic activity (Dizengremel, 2001; Grulke and Heath, 2020) can lead to decreases in whole-plant biomass (Wittig et al., 2009) and shifts in carbon allocation, either to roots or to shoots (Agathokleous et al., 2016; Grantz et al., 2006). Formed through a series of complex chemical reactions in UV light, ozone is produced from precursor emissions such as volatile organic compounds (VOCs), nitrogen oxides (NO_x), methane (CH_4) and carbon monoxide (CO) (Atkinson, 2000). Critical levels for ozone have been established for trees, based on reductions in plant growth (Emberson, 2020; Mills et al., 2017). Ozone concentrations exceeded critical thresholds for trees in 87.3% sites globally, between 2010 and 2014, leading to an estimated > 5% biomass loss (Mills et al., 2018a). In the UK, ozone levels have followed an upward trend since 2017, with 2022–2024 having the highest annual mean background concentrations recorded to date (daily 8-hour mean, $\mu g/m^3$; UK GOV, 2025).

Ozone-induced reduction in photosynthetic assimilate can alter key processes in the reproductive development of plants (Black et al., 2000). Examples include reductions in the number and weight of reproductive structures (Hayes et al., 2012; Price and Treshow, 1972), decreases in pollen germination and tube growth (Sénéchal et al., 2015) and accelerated onset of flowering (Duque et al., 2021). Ozone has also been documented to affect plant seed and fruit yields: one meta-analysis of 128 peer-reviewed studies found that even ambient ozone exposure (33 parts per billion – ppb) decreased seed number (by 16%), fruit number (by 9%) and fruit weight (by 22%) compared to clean air (Leisner and Ainsworth, 2012). Whilst these effects are well documented in key

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crop species such as *Brassica* spp. (Roberts et al., 2022; Stewart, 1998), they are less explored in tree species. In a rare exception, the Aspen-FACE experiment evidenced a 20% reduction in seed number in paper birch (*Betula papyrifera)*, with trees exposed to elevated ozone for 9 years (Darbah et al., 2008).

Despite increasing evidence of ozone's impact on reproductive output in adult plants (Leisner and Ainsworth, 2012), much less attention has been paid to how ozone exposure affects germination dynamics. There are three potential pathways for ozone to affect seed germination: 1) through maternal-mediated effects, where parental plants are exposed to elevated ozone, thereby affecting seed characteristics as they develop (Darbah et al., 2008); 2) direct effects, such as where seeds in soil are exposed to acute ozone episodes (Abeli et al., 2017); and, 3) indirect effects, such as ozone effects on seed pathogen communities in the soil. Darbah et al. (2008) explored the effect of maternal seed development under elevated ozone on seed germination percentage (GP; the proportion of seeds which germinate) in *Betula papyrifera*, noting a 60% decrease compared to controls. Similarly, Landesmann et al. (2013) exposed a weed community to elevated ozone over a 4-year period, noting a dose-dependent effect in *Spergula arvensis* seeds subsequently germinating in clean air. These findings give an indication of the legacy of maternal-mediated effects on seed development under elevated ozone, rather than germination under ozone, as the seeds were withdrawn from ozone fumigation prior to germination.

To address the question of how seed germination may be affected *during* ozone episodes, Abeli et al. (2017) exposed the seeds of alpine plant species to elevated ozone, noting a delay in first germination and mean germination time, reduced GP and increased proportion of dead (mouldy) seeds. These effects were not consistent however, as some species displayed enhanced germination rates following exposure to 125 ppb for 10 days. To our knowledge, only one study has investigated the effect of elevated ozone on tree species' seed germination. Seeds from multiple provenances of *Pinus sylvestris* and *Picea* sp. were exposed to elevated ozone over two growing seasons in a pot-based free-air ozone enrichment experiment (Prozherina et al., 2009). Elevated ozone caused a reduction in germination percentage in four of the five *Pinus sylvestris* provenances, and one of the four *Picea* spp. provenances (Prozherina et al., 2009).

The process of germination begins with the uptake of water by imbibition, initiating metabolic activation and embryo expansion, culminating in radicle emergence through penetration of the endosperm (if present) and testa (seed coat; Bewley et al., 2013). Germination can only occur in non-dormant seeds, as dormant seeds do not have the capacity to germinate under otherwise favourable environmental conditions (Baskin and Baskin, 2004). Dormancy breaking and/or germination can be determined by the ratio of abscisic acid (ABA) and gibberellins (GA) (Cadman et al., 2006; Kucera et al., 2005). Environmental factors can influence both the balance of, and sensitivity to, these two hormones. ABA synthesis dominates the dormant and non-dormant yet non-germinating state through suppression of endosperm rupture. In contrast, GA synthesis induces dormancy release and the progression of germination by promoting endosperm rupture and counteracting ABA's inhibitory action (Finch-Savage and Leubner-Metzger, 2006).

Recent research has revealed how additional phytohormones may also play a critical role in preventing germination. Increases in auxin, a key hormone regulating plant growth, has been found to impair germination rates in *Arabidopsis* (Liu et al., 2013). Indeed, auxin controls the expression of the ABA transcription factors through interdependency with the ABA signalling pathway (Liu et al., 2013). The stress-response hormone jasmonic acid (JA) also influences seed germination in *Arabidopsis* (Dave et al., 2016) and crops such as barley (*Hordeum vulgare*) (Xie et al., 2007). Pan et al. (2023) evidenced the substantial crosstalk between JA and other phytohormones, such as GA and ABA. JA activates ABA through degradation of jasmonate domain proteins releasing ABA transcription factors, therefore delaying germination. Finally, recent research has revealed a synergistic interaction between JA and auxin pathways, suggesting further crosstalk leading to interaction with ABA to modulate germination responses (Mei et al., 2023).

Whilst several studies highlight that ozone may alter germination dynamics in some species (Abeli et al., 2017; Prozherina et al., 2009), the underlying physiological mechanisms remain poorly understood. It has been hypothesised that the relationship between ozone-induced reactive oxygen species (ROS) and plant hormones is a key mechanism in determining seed germination (Abeli et al., 2017; Sudhakar et al., 2011). This hypothesis centres on the role of ROS in seed development, as ROS must rise to an optimal "oxidative window" to trigger the required hormonal signalling required for germination, although excessive ROS levels damage proteins, lipids, and DNA, leading to seed ageing (Wang et al., 2025). Hydrogen peroxide (H_2O_2), a critical ROS produced within plant cells following ozone exposure, is known to upregulate ABA catabolism, and has been shown to alleviate the dormancy of barley (*Hordeum distichum;* Wang et al., 1998), rice (*Oryza sativa;* Naredo et al., 1998) and apple (*Malus domestica;* Bogatek et al., 2003). Sudhakar et al. (2011) note how ABA was reduced in ozone treated seeds, suggesting that action of hydrogen peroxide most likely led to the observed increase in GP. Furthermore, ethylene, which has been found to increase in seeds in response to ozone exposure (Wilkinson and Davies, 2010), can inhibit ABA action and promote germination in seeds (Kucera et al., 2005). Many of these insights originate from research evaluating ozone's utility as a pre-treatment technique for enhancing germination in commercial crops, where exposure is typically short-lived. Pandiselvam et al. (2020) recently reviewed the existing research in this field, noting a highly variable effect between species, dose strength, duration and accompanying stratification and incubation techniques.

The potential impact of ozone on germinating seeds of tree species remains understudied (Abeli et al., 2017), with most studies focusing on parental exposure or crop species (e.g. Darbah et al., 2008; Pandiselvam et al., 2020). This is surprising given the springtime ozone maximum phenomenon (Monks, 2000), with ozone episodes tending to occur between April-May in the UK (UK GOV, 2025) and northern Europe (Crespo-Miguel et al., 2024). Furthermore, the hormonal mechanisms underlying ozone-induced changes in germination remain largely unresolved, with no study to date characterising the full suite of interacting phytohormones that regulate germination under ozone stress.

Consequently, the aim of this study was to test potential direct effects of ozone episodes on seed germination and phytohormonal dynamics of a range of temperate tree species. We asked 1) whether ozone could alter the time-course of germination and the key components of germination dynamics for a population of pre-stratified and assumed non-dormant seeds: germination proportion (GP), germination rate and time to 50% germination; and 2) whether ozone influenced the level of key phytohormones governing germination by comparing phytohormone profiles, after ozone fumigation, between elevated ozone and control conditions and within developmental stages, including those seeds that remained ungerminated. Our investigations can indicate whether ozone can impact the recruitment of tree species in field conditions (Larson et al., 2015), with important implications for forest restoration (Perring et al., 2022) and potentially provide mechanistic insight into any observed germination response. We hypothesised that ozone would reduce the GP and delay the time course of germination in all the species tested, in addition to upregulating the concentration of key phytohormones inhibiting germination, such as ABA.

2. Methodology

2.1. Species and seed stock

In order to maximise the number of species tested for ozone sensitivity in their germination, within the constraint of the elevated ozone exposure facility, two study trials were conducted, with seeds sown in the cabinets and ozone exposure started on the 13th February and the

5th April 2024 respectively, with both experimental periods lasting 27 days. Species selected for experimentation were *Picea sitchensis* (Trial 1), *Betula pendula* (Trial 1 and 2), *Pinus sylvestris* (Trial 1 and 2), *Alnus glutinosa* (Trial 2) and *Acer pseudoplatanus* (Trial 2). These species were chosen to represent contrasting phylogenetic groups. Further, most tree species have seeds that are classified as physiologically dormant and our chosen species are representative of this dormancy class (Baskin and Baskin, 2004), which implies that appropriate pre-treatment e.g. stratification (Gosling, 2007) can remove dormancy. Seeds were sourced from commercial growers for both trials, with trial 1 species from Forestart, (Shropshire, UK) and trial 2 species from Maelor Forest Nurseries (Wrexham, UK). Seeds fumigated in trial 1 were cold stratified (6-weeks at 4°C; Gosling, 2007), whereas seeds for trial 2 were pre-stratified by the grower using the same methodology. Both sets of seeds were stored at 5°C until use. Before each trial, seeds were checked for the presence of an embryo by gently squeezing them between the fingers. Any seeds found to be empty shells were discarded.

2.2. Experimental conditions

Experiments were carried out using six fumigation chambers, with three replicate chambers receiving elevated ozone and three an ambient air control, at the UKCEH Ozone exposure facility (53° 14' 19.6404" N, 4° 1′ 7.4856" W) Abergwyngregyn, near Bangor, North Wales, UK. The chambers (22 cm x 45 cm x 56 cm) were situated in a temperature-controlled laboratory, and humidity of the ambient air was maintained using an air humidifier (target 20°C and 55% RH; Probreeze, PB−12-UK). Lighting was consistent between chambers, with three overhead lights controlled on 12-hour day-night cycles. Ozone was supplied using an ozone generator (Clearwater, CD1500P), which was supplied with oxygen (O_2) concentrated ambient air. Ozone treatment was randomly assigned to three of the chambers (1, 4 & 6), where ozone enriched air was then injected into ambient air and drawn into each chamber in a negative pressure system. A stable ozone enrichment target of + 60 ppb was maintained in the treatment chambers throughout the fumigation period. This target was selected to represent concentrations characteristic of high ozone episodes (Mills et al., 2012), and higher concentrations have been recorded during springtime ozone maxima in areas of western and northern Europe (France, Switzerland - May 2008; Kalabokas et al., 2017). The remaining ozone chambers (2, 3 & 5) were designated as controls, supplied with non-ozone enriched ambient air. Ozone dose was manually controlled by a PTFE tap on the ozone injection pipe and ozone concentrated air was further mixed using fans within each chamber. Ozone concentration inside each chamber was sampled in rotation every 30 min, at the end of a 5-minute period, logged by an ozone analyser (Envirotech; 49i Thermo Scientific) controlled by LabView software (Version 2012, National Instruments).

A high-ozone concentration was achieved for designated ozone chambers in both trials 1 and 2, 74 ± 24 ppb and 83 ± 25 ppb respectively (Table 1). The control (low ozone) treatment was maintained at ambient air concentrations - 14 ± 6 ppb in trial 1 and 19 ± 6 ppb in trial 2. Temperature and relative humidity were monitored in the rear of each chamber using iButton data loggers (DS1923-F5, Maxim Integrated, San Jose, California, United States), logging at 10-minute intervals. These were monitored to assess any chamber variation and whether there was indication of a germination response to these variables. Temperature and relative humidity differed between the trials, though differences within trials were small (average difference of 0.3 °C and 1.4% RH in trial 1, 0.9 °C and 1.2% RH in trial 2; Table 1).

Table 1
Ozone concentrations and climatic conditions for the ozone and control treatments (n = 3) in the trials 1 and 2.

Trial	Ozone treatment	Ozone concentration (ppb) mean ± SD	Temperature (°C) mean ± SD	Relative humidity (%) mean ± SD
1	High	74 ± 24	20.9 ± 0.82	53.3 ± 8.29
1	Low	14.5 ± 6	21.2 ± 0.85	54.7 ± 8.33
2	High	83.8 ± 25	22.6 ± 0.56	49.7 ± 6.69
2	Low	19.2 ± 6	23.5 ± 0.57	50.9 ± 6.07

Seeds were placed on the surface of seed trays filled with a fine sand, coarse sand and vermiculite mix (1:1:1), with groups of seeds of the same species placed within each well of the seed trays (Table S1). Seed numbers differed between trials and species due to seed availability and seed size; however, within a given species and trial, the number of sown seeds was the same per well across ozone and control cabinets (Table S1). Each tray was situated within a water bath, which was refilled when required to the same level in each cabinet, approximately 3 cm below the level of the seeds. Seed trays were partitioned into 4 segments, with each segment containing 2 rows of 4 wells, with individual species randomly allocated to one of the four segments, and the appropriate number of seeds sown per well at the start of each trial period.

2.3. Measurements

2.3.1. Germination assessments

We classified a seed as 'germinated' once embryo elongation occurred and the radicle protrusion through the endosperm and seed coat was visible (Finch-Savage and Leubner-Metzger, 2006). Every morning during each trial, germinated seeds were removed from the seed trays and frozen using liquid nitrogen, then stored in a −80 °C freezer. On the occasion that a daily check was not possible, those seeds with longer radicles compared to radicle length of conspecifics in the same chamber were estimated to have germinated on the previous day.

2.3.2. Phytohormonal profiling

Phytohormonal profiling was conducted exclusively on *Pinus sylvestris* and *Acer pseudoplatanus* seeds from Trial 2, selected for their larger seed size to provide sufficient mass for analysis. For each germinated seed, the absolute radicle length was also measured and grouped into short (<10 mm), medium (10–20 mm) or long (>20 mm). This grouping represented relatively equal proportions of the radicle length observed in the experiment. The remaining ungerminated seeds of each species, in each cabinet, at the end of the trial were also collected. Upon completion of Trial 2, germinated seeds and ungerminated seeds from each cabinet, separately for each of the two focal species, were bulked by developmental stage (short, medium, long radicle length, and ungerminated) and powdered using liquid nitrogen and a pestle and mortar, then stored at −80 °C for further analysis. In other words, for each of *Pinus sylvestris* and *Acer pseudoplatanus*, we aimed to have sufficient plant material for three replicates per developmental stage per ozone treatment for hormone analysis. As seed collection occurred either upon germination during Trial 2 or at the end of Trial 2 for ungerminated seeds, hormonal profiles were compared between ozone and control-treated seeds within the same developmental stage. Thus, developmental stage refers to seed status at the point of collection rather than representing a longitudinal time series of germination progression.

The phytohormones analysed included the ethylene precursor 1-aminocyclopropane−1-carboxylic acid (ACC); the cytokinins (CK), *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), and isopentenyladenine (iP); the auxins indole−3-acetic acid (IAA) and its oxidised form (oxIAA); the gibberellins GA_1, GA_3, and GA_4; and the stress-response hormones salicylic acid (SA), ABA, and jasmonic acid (JA). These represent several major hormones that interact to regulate both the transition from dormancy to non-dormancy (not investigated here) and the progress of germination once a seed is in the non-dormant state (the focus of our investigation) (Kucera et al., 2005; Finch-Savage and Leubner-Metzger, 2006; Mei et al., 2023). This targeted phytohormone analysis quantified endogenous hormone concentrations but did not determine whether any alterations potentially induced by ozone resulted from altered biosynthesis, catabolism or other regulatory

mechanisms.

Phytohormones were extracted following Albacete et al. (2008) and Martínez-Andújar et al. (2021), with some modifications. Briefly, 100 mg of freeze-dried seed material were suspended in 1 mL of cold (−20 °C) extraction mixture composed of methanol/water (80/20, v/v) for 30 min at 4 °C. Solids were separated by centrifugation (20000 g, 15 min, 4 °C) and re-extracted for 30 min at 4 °C in an additional 1 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus C18 cartridges (SepPak Plus, Waters, Milford, MA, USA) to remove interfering lipids and part of the plant pigments and evaporated at 40 °C under vacuum to near dryness. The residue was dissolved in 1 mL of methanol/water solution (20/80, v/v) using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 μm pore size nylon membrane (Millipore, Bedford, MA, USA). A total of 10 μL of filtered extract were injected into a U-HPLC-MS system consisting of a Vanquish U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface equipped with a 96-microwell plate autosampler and capillary pump. Mass spectra were obtained in the negative mode using Xcalibur software version 4.3 (ThermoFisher Scientific, Waltham, MA, USA). Chromatographic separation was performed on an UltraCore 5 SuperC18 column (50 × 2.1 mm, 5 μm; Avantor ACE) at 30 °C, using methanol + 0.1% acetic acid (A) and water + 0.1% acetic acid (B) at 0.3 mL min^{-1} . The gradient was: 0–1 min, 0% B; 1–3 min, 30% B; 3–6 min, 95% B; 6–7 min, 95% B; 7–10 min, 0% B; followed by re-equilibration to 14 min. Full-scan spectra were acquired from *m/z* 90–500 at high resolution. Exact *m/z* values, retention times, R^2 values and LOD/LOQ parameters are provided in Table S2.

For the quantification of the plant hormones, calibration curves were constructed for each analyte (1, 10, 50, 100 and 400 μg L^{-1}) using external standards. All hormone standards were purchased from OlChemIm (Czech Republic). To ensure quantitative robustness, a mixture of deuterium-labelled analogues corresponding to the hormones analysed in this study ($[^2H_4]$ACC, $[^2H_5]$tZ, $[^2H_5]$tZR, $[^2H_6]$iP, $[^2H_5]$IAA, $[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_2]GA_4$, $[^2H_4]$SA, $[^2H_6]$ABA and $[^2H_6]$JA; OlChemIm Ltd., Czech Republic) was periodically injected as a quality-control reference to monitor instrument drift. These isotope-labelled standards were not added to individual samples. QC injections consistently yielded recoveries of 92–95%, validating the use of external calibration curves and indicating minimal matrix interference. Because baseline noise in Orbitrap full-scan mode was negligible, limits of detection (LOD) and quantification (LOQ) were determined experimentally from the lowest concentrations providing reliable detection and quantification. For most hormones, LOD was < 1 ppb and LOQ was 1 ppb, whereas tZ and ACC were reliably quantified down to 10 ppb, although both were detectable at 1 ppb. tZR showed the highest LOQ (50 ppb), consistent with its lower ionization efficiency in negative mode, but was still detected at concentrations above this threshold in all samples. Calibration curves showed excellent linearity (R^2 = 0.8456–0.9924) across the calibration levels 1–400 ppb (Table S2).

2.4. Statistical analysis

2.4.1. Time course of germination – Time-to-event analysis

To assess whether ozone could alter the time course of germination, we used time-to-event analysis (TTE) following Onofri (2023), through use of the drc and drcte R packages (Onofri, 2023; Ritz et al., 2015) in R studio (R Core Team, 2020). We incorporated uncertainty in our selected time-to-event models by considering 'censoring', where the exact time of germination is unknown and only the interval between observations is known. This is achieved through considering the interval extremes as the independent variable and the daily observed counts as the response.

Analyses were conducted in two stages. First, for each species and trial separately, time-to-event curves were modelled independently for each cabinet (ozone chamber), treating cabinets as independent replicates (total n = 6, n control = 3 and ozone = 3). Cabinet-level model fits provided germination-parameter estimates which were used for subsequent between-treatment comparisons. Second, to test for an overall treatment effect on the time-course of germination for each species in each trial, treatment-level curves were fitted by pooling cabinets across each treatment (control vs ozone) and compared using likelihood-based tests. Trials were analysed separately because species sets differed between trials, and incubation temperatures and relative humidities (Table 1) varied slightly, such that pooling could confound ozone effects with hidden treatments.

2.4.2. Nonlinear regression model selection and fitting

To identify the most appropriate nonlinear regression model to represent the time course of germination, the Akaike Information Criterion (AIC) value was used as the primary criterion. The selection process considered various sigmoidal regression models, fitted separately to each cabinet and species, with germination counts aggregated across the eight wells per species per cabinet. Three-parameter distributions were selected for testing due to the parameterisation of a non-germinating fraction, or asymptote, making them well-suited for biological seed studies where some seeds may never germinate (Brown and Mayer, 1988). The model with the lowest corresponding AIC value for each species in each cabinet can be seen in Table S3. In assessing which model to apply for each species, a 'majority vote' was applied where the model with the largest count across the chambers was selected. The Weibull model (Eq. 1) was the most representative for all species and trials except *Betula pendula* in trial 1, therefore, we decided to apply this model type universally to enable direct comparison between species and trials. As a plateau had not been reached by the end of the study in *A. psuedoplatanus*, an alternative Weibull model (W2.3) was selected for this species. This model type provided the lowest AIC values for half the cabinet TTE curves in this species (Table S3) and produced more biologically-reasonable and interpretable parameter values.

Parametric Weibull TTE models were fitted at the cabinet level for each species (Cabinet Models; Eq. 1), using a Cumulative Distribution Function (CDF; Eq. 2, Onofri, 2023) that describes the probability P that the TTE is equal to or lower than any given time.

$$G(t) = d\ \exp(-\exp(-b[\log(t) - \log(e)]\,)) \quad (1)$$

$$G(t) = \mathrm{P}(T \le t) \quad (2)$$

In these equations, G*(t)* represents the proportion of seeds germinated at time *t*, the coefficient *e* is the median germination time (i.e., where 50% of seeds have germinated); coefficient *b* is the shape parameter; and coefficient *d*, given the curve flattens out, is the maximum proportion of germinated seeds (upper asymptote). Parameters are reported under the W1.3 parameterisation, in which increasing cumulative germination curves correspond to negative values of *b* due to the sign convention of the Weibull formulation used for model fitting.

To test for differences between treatment groups over the course of germination, Weibull models were then fitted across grouped control and ozone chambers for each species (Treatment Models). Likelihood Ratio Tests (LRTs) were then conducted for these two fitted models, where the logarithm of the likelihood for the 'expanded' model was compared with the 'reduced' model, where all curves have been pooled into one common curve for all treatment levels (interpretation at a significance level of 0.05).

2.4.3. Germination characteristics comparison - Cabinet Model Parameters

To investigate which components of the germination curves differed between treatments, values for *e*, *b* and *d* were extracted from the chamber-level Weibull model fits for each treatment per species (n = 3). We used a linear model to test for differences in germination curve parameters between ozone and control treatments. To account for potential variation in environmental conditions among chambers, we

expanded the linear model to include temperature and relative humidity as covariates:

$$\text{germination model parameter} \sim \text{treatment} + \text{temperature} + \text{relative humidity} \quad (3)$$

Treatment effects were tested using the F-test for the treatment term in the fitted linear model. Cases where $p < 0.05$ were considered to be indicative of a meaningful shift in *b*, *d* or *e* under ozone exposure. The normality of the linear model residuals and homogeneity of group variances was assessed using Shapiro-Wilk and Bartlett's test, respectively, with only the *e* parameter values in *P. sitchensis* violating assumed normality of model residuals. Due to the low statistical power of such tests on small sample sizes, visual inspection of Quantile-Quantile and residuals vs. fitted values were also used to assess the normality of residuals and homoscedasticity. Where visual inspection indicated violations of linear model assumptions, Mann-Whitney U tests were conducted to compare parameter values from control and ozone treatments. Indeed, we present Mann-Whitney U results in the main manuscript as the effect estimates are bounded and expressed as the median differences between ozone and control at the cabinet level; we make reference to ANOVA results when appropriate.

2.4.4. Phytohormone profiling assessment – mixed effects models and estimated marginal means contrasts

We tested how hormone concentrations varied with ozone treatment and radicle size class while accounting for shared chamber environments. For each species, linear mixed-effects models were fitted to individual phytohormone concentrations, with fixed effects for treatment (Control vs Ozone), size (ungerminated, short, medium, long), and their interaction (treatment * size), and a random intercept for chamber to account for block-level dependence.

Models were estimated in R studio (R Core Team, 2020) using lme4::lmer() (Bates et al., 2015). Model assumptions were verified using simulation-based residual diagnostics (DHARMa package; Hartig, 2024) and visual checks, including assessments of residual homoscedasticity and normality, as well as tests for singular fits. Where homoscedasticity of model residual variance was violated, suggesting differences in residual spread among size classes, nlme::lme() (Pinheiro and Bates, 2023) was used as a heteroscedastic alternative allowing class-specific residual standard deviations (*weights = varIdent(~1 | size)*). Model support was evaluated using Akaike Information Criterion (AIC) under REML estimation and by likelihood-ratio tests after refitting both models with maximum likelihood. Visual assessments of model residual variance were also repeated.

Estimated marginal means (EMMs) were obtained for each treatment × size combination using emmeans R package (Lenth, 2025) to produce model-adjusted means. Pairwise contrasts were performed within each size class to test for Ozone–Control differences, with *p*-values adjusted for multiple comparisons using the Benjamini–Hochberg correction. Concentrations were analysed on a log $(1 + y)$ scale to stabilise variance, and back-transformed estimates were used for reporting and plotting.

3. Results

3.1. Time-course of germination

Germination progress for every species generally followed an S-shaped trend over 28 days, with minimal germination recorded before Day 5. Visual inspection of the treatment-level TTE curves indicates that ozone exposure tended to slow the germination rate (as estimated by the shape parameter *'b'*), delay the germination midpoint (*'e'*) and reduce the maximum germination percentage (*'d'*) in the species tested (Table S4). The time course of germination for *A. psuedoplatanus* differed from the other species, with a high rate of germination still occurring up to the end of the study period, indicating that its maximum germination percentage (GP) had not been reached. Consequently, biological interpretation of potential reductions in GP in this species was unfeasible.

The varied impact of ozone exposure on the germination dynamics was reflected in Likelihood Ratio Tests (LRT) of the treatment-level models, suggesting a significant treatment effect in some species and trials but not others (Fig. 1). Specifically, in trial 2 for *P. sylvestris*, the high LRT value (42.83, $p < 0.001$) demonstrated a clear difference between the ozone and control germination curves (Fig. 1a). Conversely, in trial 1 for *P. sylvestris*, the LRT value (4.55, $p = 0.21$) was not sufficient to reject the null hypothesis, indicating no significant effect of ozone on the course of germination in that trial (Fig. 1b). Findings between trials were also not consistent for *B. pendula*; the trial 1 LRT produced a significant difference (LRT = 9.13, $p = 0.028$; Fig. 1c), suggesting slightly faster germination rate under control conditions (Table S4). However, in trial 2 *B. pendula* showed no strong treatment effect (LRT = 3.17, $p = 0.37$; Fig. 1d). Thus, for the two species included in both trials, within-species germination percentage varied between the trials, as indicated by the treatment-level model *d* parameter estimates (Table S4). In their single trial *A. pseudoplatanus, P. sitchensis* and *A. glutinosa* each showed no major differences in the time course of germination between treatments (LRT = 2.371, $p = 0.499$, LRT = 3.75, $p = 0.29$ and LRT = 3.82, $p = 0.28$ for each species respectively; Fig. 1e-g).

3.2. Comparison of germination model parameters

In *P. sylvestris* (Trial 2), ANOVA revealed a significant difference in parameter *d* (maximum GP) between ozone and control ($p = 0.02$; Table S5), consistent with the LRT. Meanwhile, *b* (germination curve's shape) and *e* (median germination time) did not differ significantly between treatments ($p = 0.48$, 0.83, respectively). The same was true for *P. sylvestris* in Trial 1, in accordance with the LRT results. No significant treatment effects on the model parameters were found for *B. pendula* in Trial 1, despite the significant LRT result. This discrepancy may indicate that while the overall curve shapes differ slightly between ozone and control, individual chamber-level parameter means were not sufficiently distinct to reach significance. For *B. pendula* in Trial 2, similarly, no parameter was statistically altered by ozone (all $p > 0.20$), aligning with the nonsignificant LRT.

In further agreement with the LRT results, none of *A. pseudoplatanus, P. sitchensis* and *A. glutinosa* had significant differences in parameter values between the treatments. Whilst there was a marginally significant ozone effect on maximum GP (*d*) in *A. pseudoplatanus* ($p = 0.05$), inspection of the germination curves suggested that ozone-treated seeds had not yet plateaued by the end of the 28-day trial. As such, this difference in *d* may reflect premature curve fitting rather than a true biological reduction in maximum germination. Thus, neither the overall LRT analysis nor the chamber-level approach found a clear ozone effect for these species. Mann-Whitney U tests comparing chamber-level parameter estimates between treatments yielded closely aligned results (Table 2). Whilst all species parameters yielded non-significant effects, *P. sylvestris* (Trial 2) and *P. sitchensis* showed a biologically important ozone-related reduction in parameter d (17% and 15% respectively).

3.3. Seed phytohormones

In *P. sylvestris,* mixed-effects models (Table S6) revealed significant interactions between ozone and radicle size class for auxin (IAA; $p = 0.02$ and oxIAA; $p = 0.01$), together with marginal interactions for ABA ($p = 0.09$), GA_3 ($p = 0.06$), and SA ($p = 0.07$). A further significant interaction was found in the CK-iP ($p < 0.01$).

Pairwise contrasts confirmed ozone-induced increases in these hormones, compared to controls, in ungerminated *P. sylvestris* seeds (Figs. 2–5; Table S7). Specifically, within the stress hormones tested, ABA showed a marginally significant 1.5-fold increase under elevated

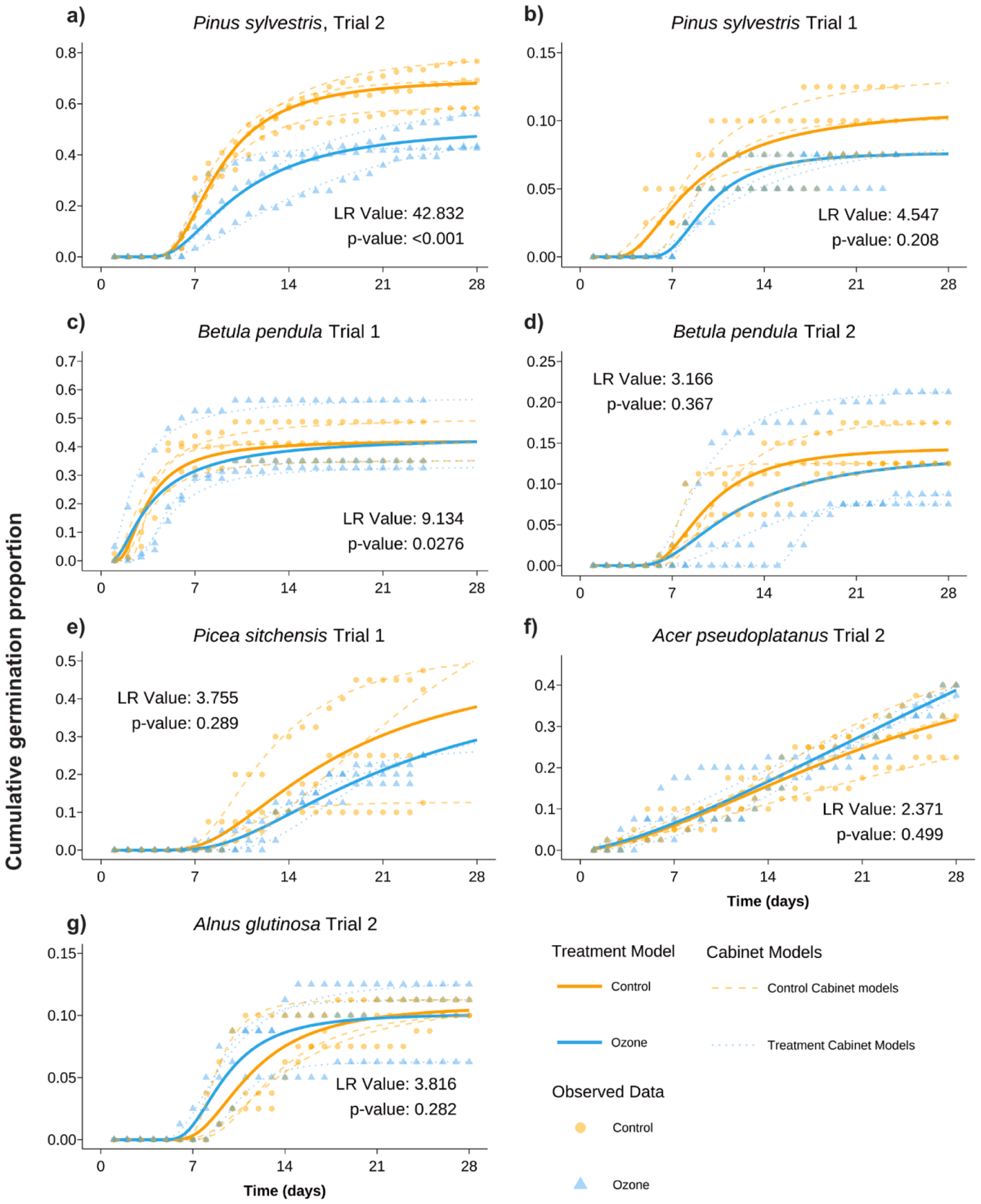

Fig. 1. Effects of ozone exposure on the time-course of seed germination across species and trials. Panels show treatment-level time-to-event models germination curves and raw data for each species: **a)** *Pinus sylvestris* Trial 2, **b**) *Pinus sylvestris* Trial 1, **c**) *Betula pendula* Trial 1, **d**) *Betula pendula* Trial 2, (**e**) *Picea sitchensis* Trial 1 (**f**) *Acer pseudoplatanus* Trial 2, (**g**) *Alnus glutinosa* Trial 2. All models were fitted with 3-Parameter Weibull Distributions. Orange solid line – overall control chambers model, orange dotted lines – individual ozone chambers models, orange circles – control chambers actual data; blue solid line – overall ozone chambers model, blue dashed lines – control chambers models, blue triangles – ozone chambers actual data. The LR and p-values give the likelihood ratio test value and accompanying significance of whether separate orange and blue solid curves are justified ($p \leq 0.05$) or whether one single curve best describes germination (where $p > 0.05$).

Table 2
Effect of ozone treatment on fitted germination parameters (median germination time = *e*, maximum germination percentage = *d*, curve shape = *b*), tested by Mann–Whitney U tests across species and trials. Estimates represent the difference between ozone and control treatments (ozone − control). Positive values indicate higher parameter estimates under ozone. For example, a change in *d* of −0.17 corresponds to an approximately 17% reduction in the proportion of seeds germinating under ozone exposure.

Species	Trial Number	df	estimate	Upper CI	Lower CI	*p*
Median Germination time (*e*)						
Acer pseudoplatanus	2	5	20.15	30.3	−1.47	0.2
Alnus glutinosa	2	5	−2.57	1.46	−4.13	0.4
Betula pendula	1	5	1.18	1.78	−1.31	0.7
Betula pendula	2	5	2.64	9.13	−1.95	0.4
Picea sitchensis	1	5	3.59	7.69	−8.63	0.7
Pinus sylvestris	1	5	2.05	4.73	−0.15	0.2
Pinus sylvestris	2	5	2.29	5.94	−1.88	0.7
Maximum Germination Percentage (*d*)						
Acer pseudoplatanus	2	5	0.49	0.63	0.13	0.1
Alnus glutinosa	2	5	−0	0.02	−0.05	0.7
Betula pendula	1	5	−0.02	0.22	−0.17	0.7
Betula pendula	2	5	−0.04	0.09	−0.1	0.7
Picea sitchensis	1	5	−0.15	0.36	−0.73	0.7
Pinus sylvestris	1	5	−0.02	0.01	−0.06	0.2
Pinus sylvestris	2	5	−0.17	0.03	−0.37	0.2
Growth rate (*b*)						
Acer pseudoplatanus	2	5	−0.02	0.75	−0.79	1
Alnus glutinosa	2	5	−0.5	3.23	−3.38	1
Betula pendula	1	5	0.12	2.29	−1.51	1
Betula pendula	2	5	−0.52	5.64	−14.21	1
Picea sitchensis	1	5	0.32	3.7	−2.94	1
Pinus sylvestris	1	5	−1.1	1.6	−7.17	0.7
Pinus sylvestris	2	5	1	1.6	−2.41	0.7

ozone ($p = 0.085$; Figs. 2b, 5), while SA increased approximately 2-fold under ozone ($p = 0.026$; Fig. 2h, Fig. 5). Likewise, JA had a 3-fold increase in ozone treated seeds, although not significantly different ($p = 0.18$; Fig. 3h; Fig. 5). IAA and oxIAA, were markedly higher under ozone exposure (10- and 9-fold increases respectively; $p = 0.018$ & $p < 0.001$; Fig. 2d, f; Fig. 5). CK likewise increased greatly, with a 3.5-fold increase in iP ($p = 0.002$; Fig. 4d; Fig. 5). Gibberellins ($GA_{1,3,4}$) showed consistent though non-significant directional responses in ungerminated seeds, with 1.15, 1.4 and 1.43-fold increases in ozone treated seeds ($p = 0.6, 0.13, 0.15$ respectively; Fig. 3b, d, f; Fig. 5). Other hormones, including the ethylene precursor ACC (Fig. 4b; Fig. 5) showed generally small effect sizes with no significant change in ungerminated seeds.

For *P. sylvestris* in other size classes, there was a weaker impact of ozone on hormone concentrations. Importantly, the impact was in the opposite direction to the hormone profiles in ungerminated seeds under ozone and control conditions. Thus, there was a consistent reduction in mean estimates of GA_1 and GA_3 concentrations post-germination, for instance a 40% reduction in mean GA_3 concentration in germinated seeds with short radicle lengths after ozone exposure ($p = 0.03$; Fig. 3d). With further reductions of approximately 60% in ABA and the ethylene precursor ACC in long and medium radicle lengths respectively ($p = 0.05$, Fig. 2b; $p = 0.04$, Fig. 4b).

Despite *A. pseudoplatanus* exhibiting hormonal responses to ozone that were similar to those in *P. sylvestris,* treatment and size class interactions were weaker, with only GA_1 showing a near-significant interaction effect ($p = 0.1$). However, pairwise contrasts revealed the same directional change in ABA in ungerminated seeds (2.17-fold increase, $p = 0.039$; Fig. 2a; Fig. 5). IAA also exhibited a near-significant 3.8-fold increase with ozone treatment ($p = 0.089$; Fig. 2c), with a non-significant 2-fold increase in oxIAA ($p = 0.2$; Fig. 2e; Fig. 5). Remaining hormones, including gibberellins, SA, and JA, did not show large directional changes or significant differences between treatments in ungerminated seeds. In the germinated seeds of *A. pseudoplatanus*, an ozone-related increase in gibberellins was detected in those seeds with short radicle lengths (GA_1 - 4.4-fold, $p = 0.03$, GA_4 – 35% increase, $p = 0.06$; Fig. 3a, e; Fig. 5).

4. Discussion

This study has shown consistent trends in the effect of elevated ozone on germination in *P. sylvestris*, *P. sitchensis* and *B. pendula*, marked by a delay in median germination time and reduction in the maximum germination percentage (e.g. 17% in *P. sylvestris*). Phytohormonal analyses, using seeds from trial 2, revealed that ozone exposure altered the balance of key germination regulating hormones in ungerminated seeds, providing novel insights into the ozone-induced alteration in seed germination dynamics observed in this study.

Our results for *P. sylvestris* align with previous findings in temperate tree species (Prozherina et al., 2009), where *P. sylvestris* was observed to be the more susceptible to an ozone-induced reduction in germination percentage than *Picea sitchensis* and *Picea obovata*. The observed delay in median germination time similarly replicates ozone-induced delays previously reported in alpine plants (Abeli et al., 2017). Collectively, these findings indicate that ozone stress may trigger a conserved response in germination phenology across sensitive plant taxa.

We found an ozone-induced alteration of the balance of ABA in the ungerminated seeds of *P. sylvestris* and *A. pseudoplatanus* (Fig. 5). As the key determinant of the inhibition of germination, the upregulation of ABA shifts the ratio of ABA:GA, tipping the physiological balance in favour of maintenance of the non-germinating state (Cadman et al., 2006; Finch-Savage and Leubner-Metzger, 2006). This shift represents a plausible mechanistic pathway for the ozone-induced delay in median germination time, and reduction in germination percentage observed in *P. sylvestris*. As the maximum germination proportion (GP) had not been reached in *A. pseudoplatanus* by the end of the trial, the ultimate effects of the corresponding ABA shift on germination dynamics could not be determined.

A strong ozone signal was also detected in the levels of auxin (IAA) in both species, in particular in its oxidised form (oxIAA) in *P. sylvestris* (Fig. 6). In the model species *Arabidopsis*, increased auxin signalling and biosynthesis has been found to reduce germination rates (Liu et al., 2013). Liu et al. (2013) report an interdependency between auxin and ABA, where auxin acts upstream of the dominant ABA transcription factor ABSCISIC ACID INSENSITIVE 3 (ABI3) by recruiting auxin response factors that control ABI3 expression. The interdependency between ABA and auxin found in *Arabidopsis* is also suggested by our results for *P. sylvestris and A. pseudoplatanus*, with an upregulation of both hormones corresponding to enhanced non-germination under elevated ozone (Fig. 6).

The role of cytokinin (CK) in seed germination is nuanced. CK acts antagonistically to ABA, where ABA suppresses CK biosynthesis and CK interferes with ABA signal transduction, without necessarily reducing ABA levels (Wang et al., 2011). It is therefore surprising to find the upregulation of both ABA and CK (iP) in the ungerminated seeds of *P. sylvestris,* as one could expect the upregulation of ABA to reduce endogenous CK production and consequently may warrant further investigation. However, the documented drop in GP and delay in germination timing in this species indicates ABA-action dominance over CK under elevated ozone. Future research could use transcriptomics or qPCR measurement to determine whether increased biosynthesis, inhibited catabolism or other regulatory mechanisms underpin these changes to CK (and auxin) in tree seeds following ozone exposure.

A strong upregulation in the stress-response hormones jasmonic acid (JA) and salicylic acid (SA) was detected in ungerminated seeds in *P. sylvestris* under elevated ozone, but not in *A. pseudoplatanus.* This response in the likely ozone-sensitive species *P. sylvestris* is

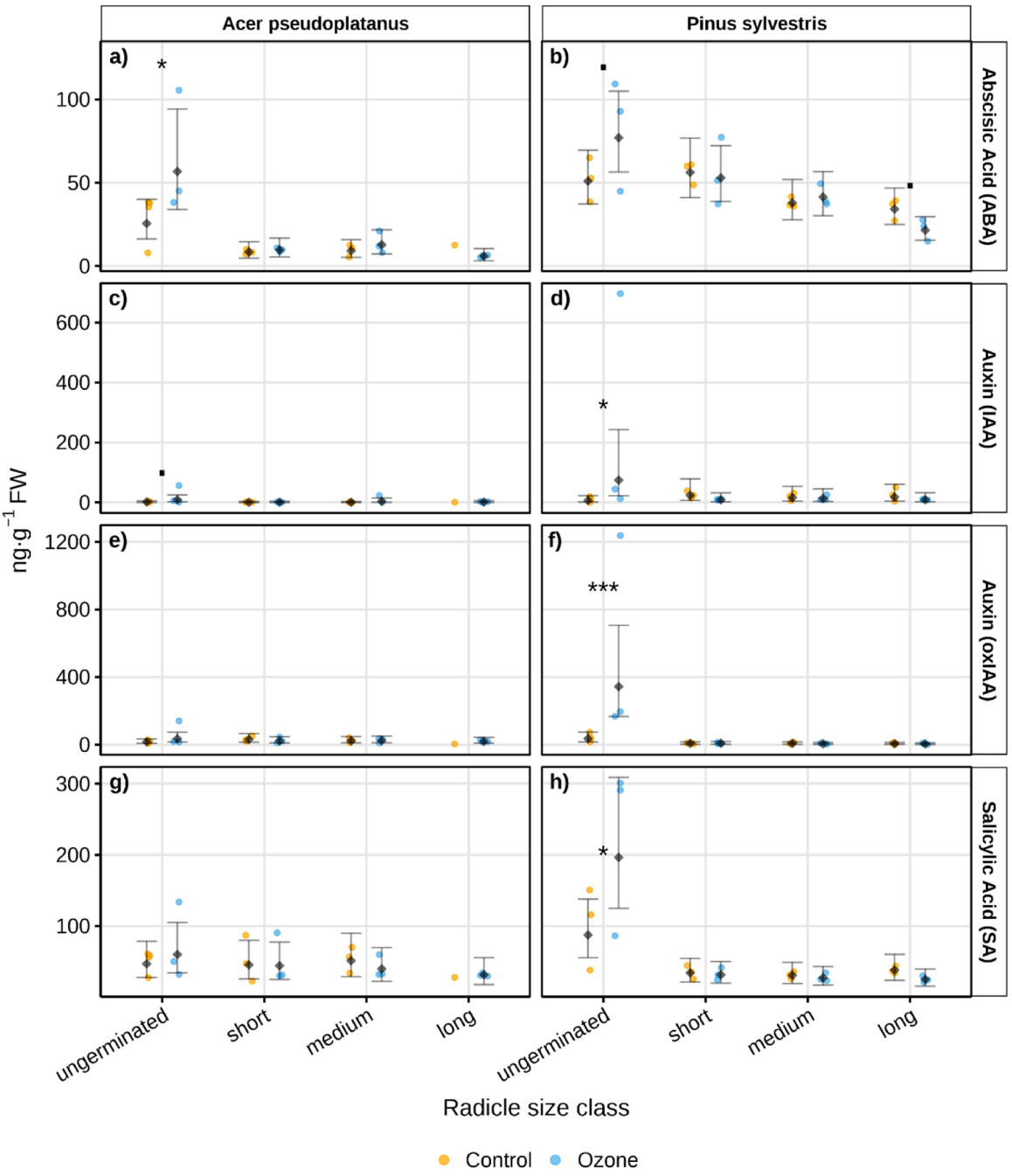

Fig. 2. Modelled mean concentrations (grey points ± 95% CIs) and raw observations (coloured points) of abscisic acid (ABA; **a-b**), auxin (IAA and oxIAA; **c-f**) and salicylic acid (SA; **g-h**) in *Acer pseudoplatanus* and *Pinus sylvestris* presumed non-dormant seeds across four radicle size classes. Ozone (blue) and control (orange) treatments were analysed using linear mixed-effects models. Asterisks indicate significant or near significant ozone vs control differences within size classes based on pairwise contrasts of estimated marginal means (***: $p < 0.001$; *: $p < 0.05$; **.**: $0.05 \leq p < 0.1$). Where a size-class x ozone treatment cell had fewer than two observations, the modelled mean and CI were not drawn and only the raw data are displayed.

understandable, given these two hormones act together in controlling the protective signalling pathway in plants, directing defence against pathogens (Caarls et al., 2015). JA may also play an active role in delaying germination (Dave et al., 2016) as a promoter of ABA (Pan et al., 2023) and could be acting concurrently with the Auxin–ABA pathway to prevent germination in seeds of *P. sylvestris* under elevated ozone (Fig. 6).

Previous studies have suggested that the interaction between ROS and phytohormones is key in ozone-induced alteration of germination dynamics (Abeli et al., 2017; Sudhakar et al., 2011). The potential role of ozone in this interaction is nuanced, with a dose-dependent effect documented in species such as tomato (Sudhakar et al., 2011), where low ozone concentrations may induce the build-up of ROS such as H_2O_2, which in turn inhibits ABA formation/signalling, thereby enhancing germination. Conversely, high ozone exposure can reduce germination rate, as has been found in wheat (Wu et al., 2006). Our study found a similar detrimental effect, with ozone inducing an upregulation in ABA and other germination inhibitors, reducing germination. Indeed, the germination enhancing effect of short-lived (e.g. 20 min) ozone treatments (e.g. Sudhakar et al., 2011; Pandiselvam et al., 2020), has not been replicated in experiments that apply ozone concentrations found in the natural environment (over multiple weeks). Such longer-term

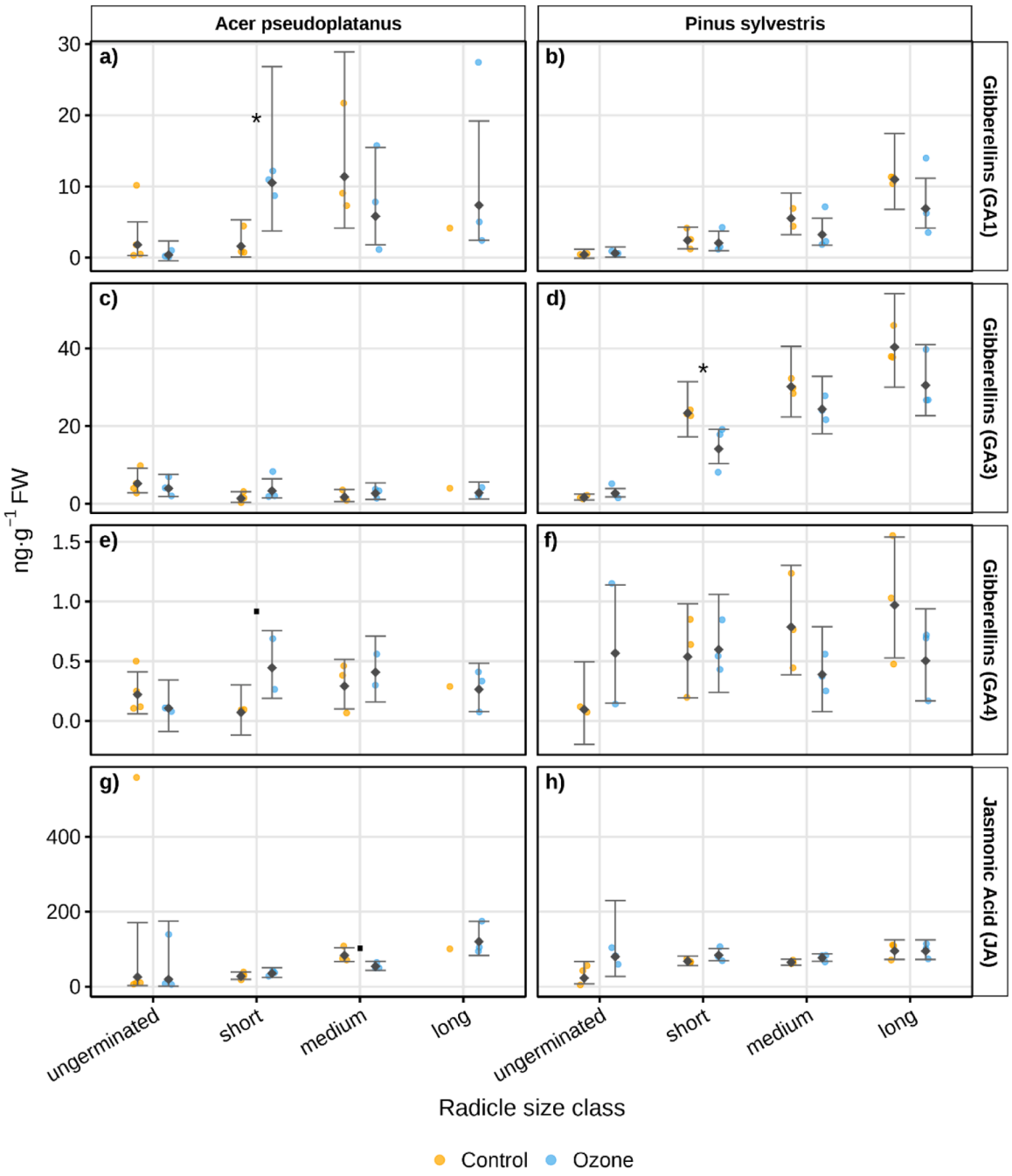

Fig. 3. Modelled mean concentrations (grey points ± 95% CIs) and raw observations (coloured points) of gibberellins (GA_1, GA_3, GA_4; **a-f**) and jasmonic acid (JA; **g-h**) in *Acer pseudoplatanus* and *Pinus sylvestris* presumed non-dormant seeds across four radicle size classes. Ozone (blue) and control (orange) treatments were analysed using linear mixed-effects models. Asterisks indicate significant or near significant ozone vs control differences within size classes based on pairwise contrasts of estimated marginal means (* $p < 0.05$;. $0.05 \leq p < 0.1$). Where a size-class x ozone treatment cell had fewer than two observations, the modelled mean and CI were not drawn and only the raw data are displayed.

studies have instead generally found a delay in mean germination time and a reduction in germination percentage in sensitive species (Abeli et al., 2017; Prozherina et al., 2009). This response could illustrate an evolutionary adaption that inhibits germination in stressful conditions, such as high ozone, to avoid loss of offspring during establishment.

As the seeds used in this study were pre-stratified, we considered them non-dormant, and therefore already within the "oxidative window" where the hormone signals for germination have been triggered (Leymarie et al., 2012; Wang et al., 2025). Within the context of prolonged ozone exposure of non-dormant seeds at concentrations found in the natural environment (Kalabokas et al., 2017), ozone-induced ROS build-up may exceed the oxidative window for germination, therefore seeds begin to fail to germinate and age - through the degradation of membrane phospholipids, proteins and nucleic acids (Kurek et al., 2019). However, quantification of ROS build-up in ozone-treated seeds would be required to substantiate this explanatory framework.

Seeds can be protected from the potentially harmful effects of excessive ROS through their antioxidant systems (De Queiroz et al., 2023). Indeed, the trend of increased SA in ungerminated *P. sylvestris* seeds under elevated ozone indicates activation of plant defence mechanisms, with SA acting as an effective scavenger of ROS (Kaya et al., 2023; Saleem et al., 2021). Thus, the action of ozone through ROS

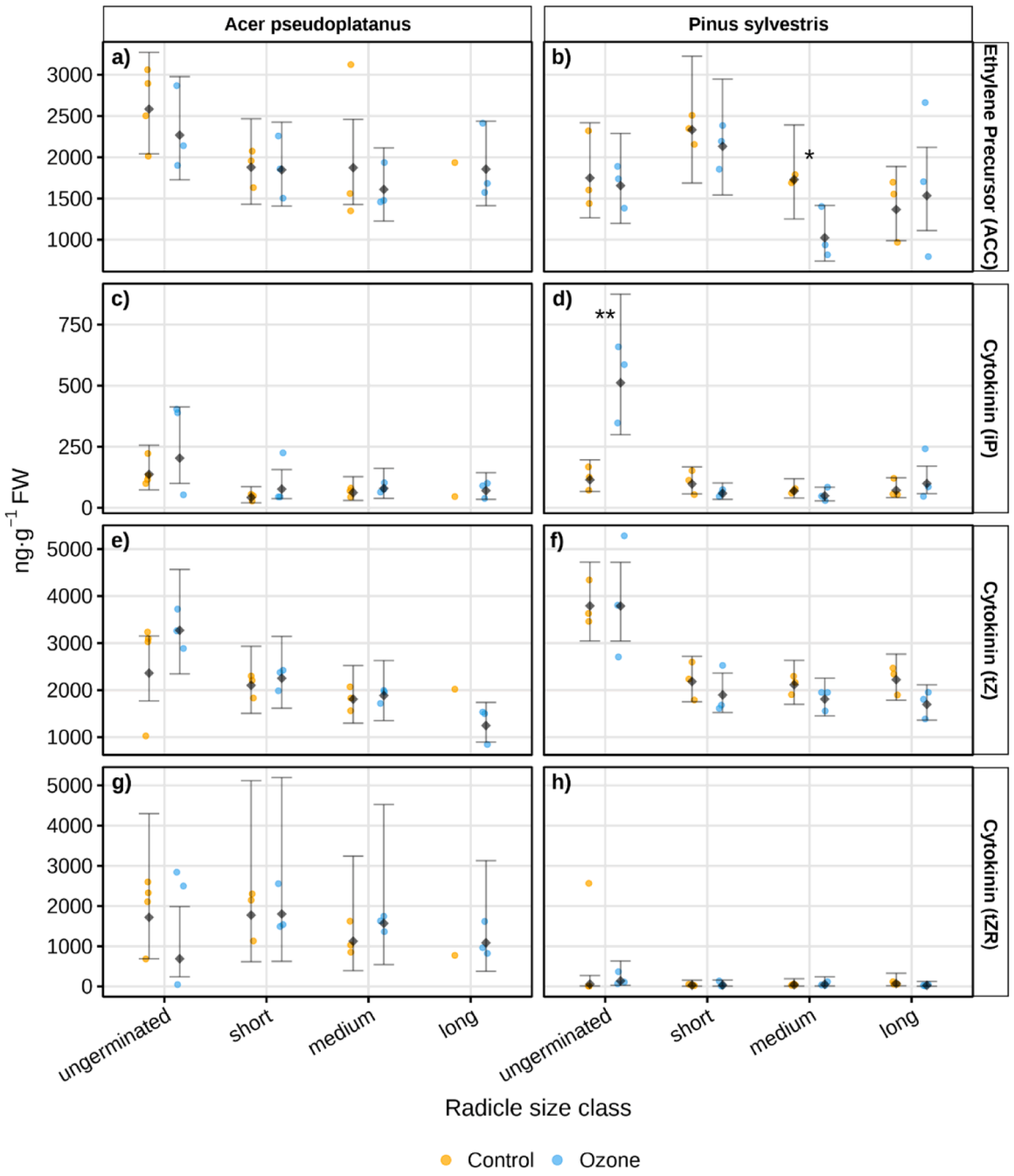

Fig. 4. Modelled mean concentrations (grey points ± 95% CIs) and raw observations (coloured points) of ethylene precursor (ACC; **a-b**) and cytokinins (iP, tZ, tZR; **c-h**) in *Acer pseudoplatanus* and *Pinus sylvestris* presumed non-dormant seeds across four radicle size classes. Ozone (blue) and control (orange) treatments were analysed using linear mixed-effects models. Asterisks indicate significant ozone-versus-control differences within size classes based on pairwise contrasts of estimated marginal means (**: $p < 0.01$; *: $p < 0.05$). Where a size-class x ozone treatment cell had fewer than two observations, the modelled mean and CI were not drawn and only the raw data are displayed.

accumulation depends on the capacity of the antioxidant system of the individual and species in question (e.g. ascorbate–glutathione in *Pinus pinea;* Tommasi et al., 2001). This phenomenon could explain the mixed response of species' germination dynamics to ozone (Abeli et al., 2017), depending on interaction between the efficiency of the species' antioxidant system and the dosage and duration of the exposure period.

To determine further underlying causes of species-specific responses to ozone exposure, answers may be found by tracing the physical pathway of the gas into the seed. As a gaseous pollutant, ozone must pass through the seed coat, which is typically semi-permeable to allow for water uptake and gas exchange (Beresniewicz et al., 1995). In some seeds, most of the gas exchange occurs in the micropylar region (Wagner, 1974), which is key to radicle protrusion and germination (Finch-Savage and Leubner-Metzger, 2006). Once inside the seed, gas can diffuse through gas-filled intracellular spaces (Cloetens et al., 2006). Modelling in oilseed rape has identified such spaces in the seed coat and hypocotyl (Verboven et al., 2013). Consequently, further research could examine differences in the structural barriers to gas diffusivity between species' seed structures to explain of the variability of response to elevated ozone.

Across treatments, germination percentage (GP) varied between trials in both *P. sylvestris* and *B. pendula*, with higher overall GP in Trial 2

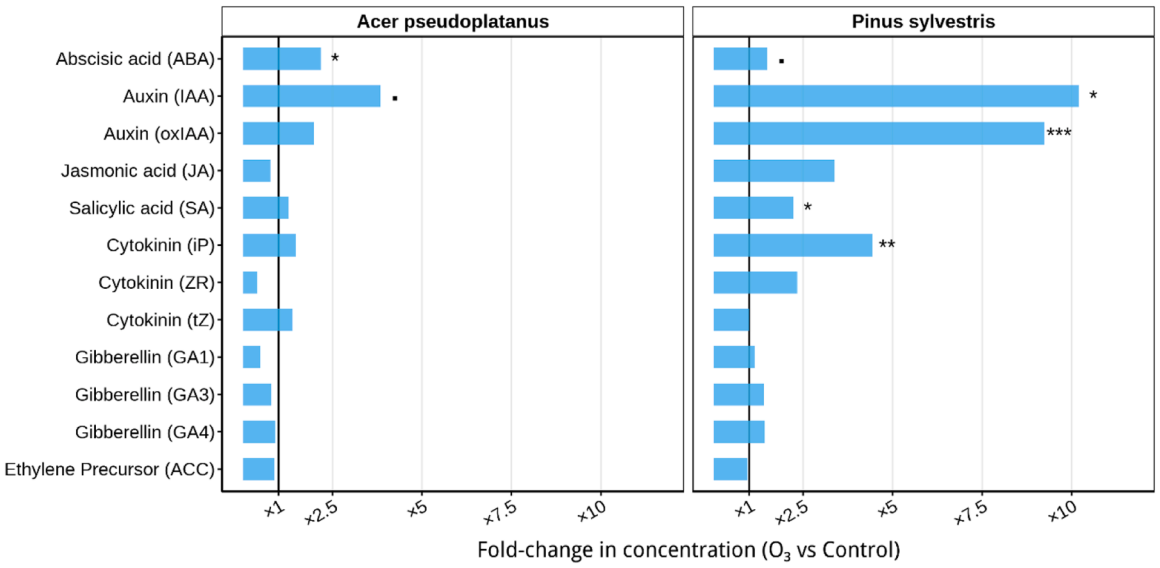

Fig. 5. The fold-change in model-adjusted estimated marginal means of phytohormone concentrations in ungerminated *Acer pseudoplatanus* and *Pinus sylvestris* presumed non-dormant seeds under elevated ozone relative to controls (×1 = no change; values <1 indicate reductions). Asterisks denote significant or near significant treatment effects (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; .: $0.05 \leq p < 0.1$).

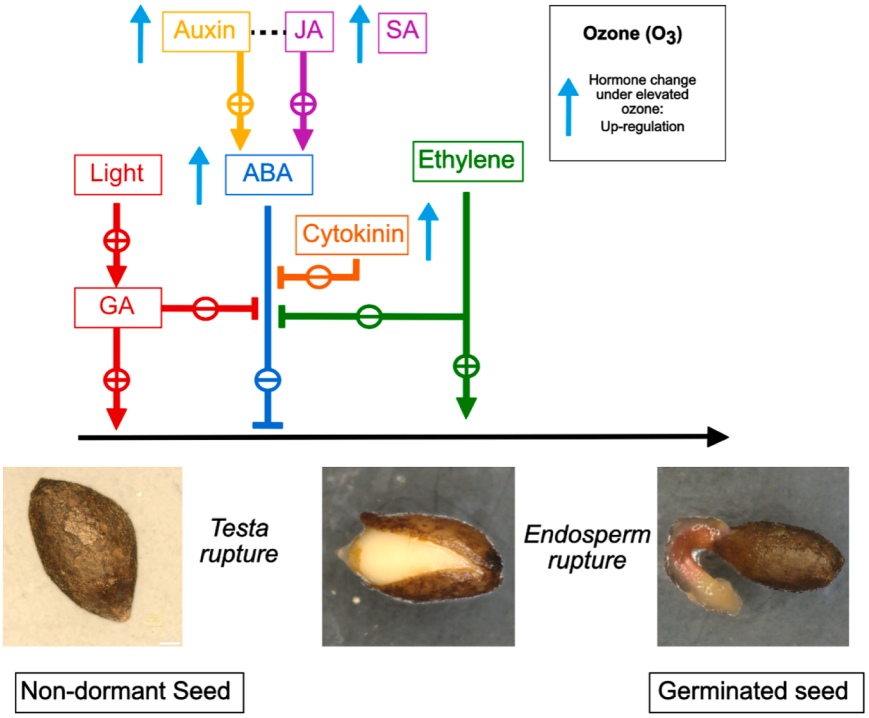

Fig. 6. Ozone (O_3) effects on phytohormone concentrations in ungerminated, presumed non-dormant *P. sylvestris* seeds - conceptual model illustrating how exposure to ozone influences the relative balance of key germination controlling hormones. Under clean-air conditions, the light/gibberellin (GA) pathway promotes testa and endosperm rupture, while abscisic acid (ABA) inhibits endosperm rupture, and ethylene counteracts ABA's inhibitory effects. Auxin promotes expression of ABA transcription factors (e.g. *ABI3*), while jasmonic acid (JA) enhances ABA signalling and JA and auxin may act synergistically. Phytohormone profiling indicates elevated ozone exposure upregulates ABA, shifting the ratio of ABA:GA, tipping the physiological balance in favour of maintenance of non-germination. This shift represents a plausible mechanistic pathway for the delayed median germination time, and reduction in germination percentage documented in *P. sylvestris.* Elevated levels of auxin (IAA & oxIAA), JA and salicylic acid (SA) indicate hormonal crosstalk that may upregulate ABA and trigger antioxidant responses. Schematic adapted from the model system in tobacco from Kucera et al. (2005). Coloured arrows indicate the hormone's influence on the progression of germination, where '+' sign and the arrowhead '→' illustrates promotion and the '-' sign and stop 'ꟾ' demonstrating inhibition. Microscopy imagery taken of *P. sylvestris* © Authors.

for *P. sylvestris* and higher overall GP in Trial 1 for *B. pendula*. This between-trial variation for both species may reflect differences in seed source and trial environmental conditions, including differences in temperature and humidity. Although pre-stratification followed the same general protocol in both trials, it was conducted in different settings and by different practitioners, so variation in the efficacy of pre-stratification cannot be ruled out. In *P. sylvestris*, but not *B. pendula,* ozone reduced GP in both trials, but the magnitude of this effect differed

and was only statistically significant in Trial 2. The difference in ozone response significance between *P. sylvestris* trials therefore appears to reflect the detectability of the ozone response, rather than a reversal in the direction of the effect. Higher overall germination success in Trial 2, combined with greater replication within each chamber, may have increased the ability to distinguish ozone effects from background variation among replicates.

The limited replication per treatment group (n = 3) constrained the statistical power of the present study, particularly in the context of high intraspecific variability between replicates in germination responses (Fig. 1), as has been found elsewhere (Abeli et al., 2017). Although chamber was included as a random effect in the models, it did not notably improve model fit. Moreover, intraspecific phenotypic variation in seed traits (Amimi et al., 2023) and ozone sensitivity (Landesmann et al., 2013) likely played a larger role in driving the observed chamber TTE curve variability. While low replication is common in elevated ozone experiments due to spatial and logistical constraints, it limits the precision of effect estimates and increases the risk of Type II errors. As a result, trends such as reduced germination proportion and delayed median germination time under ozone did not reach statistical significance, despite moderate effect sizes and consistent directional responses (Fig. 1; Tables S3 & S4). These patterns, though not conclusive, may still reflect ecologically relevant effects.

The changes in germination dynamics found in this study have potential implications for plant recruitment and community structure, as germination and subsequent emergence are a key determinant of the maintenance of plant populations (Larson et al., 2015). A key evolutionary mechanism in this process is natural selection acting on shifts in germination phenology, where earlier or later germination confers advantages or disadvantages depending on the factors influencing plant mortality and reproductive success (Donohue et al., 2010). The promotion of non-germination documented in *P. sylvestris* in this study and others (Prozherina et al., 2009), suggests ozone can also act as an environmental stressor delaying germination. Consequently, ozone-induced delays in germination could exert selective pressure on sensitive species. If earlier germination is advantageous, such delays may reduce growth and ultimately fecundity. Conversely, if conditions are unfavourable, delayed germination due to ozone may decrease seed mortality, conferring a selective advantage. These findings support the possibility of ozone exposure influencing community composition (Landesmann et al., 2013), as well as having potential consequences for restoration trajectories (Perring et al., 2022).

Given the effect of ozone on plant germination, it is crucial to understand the cause of species-specific responses and its underlying mechanisms. Varied response of the seeds of tree species to ozone exposure is in accordance with the wealth of literature documenting variable sensitivity to ozone stress between plant taxa (Wittig et al., 2009). However, evidence from this study hints at an inversion of the effect documented in the growth of adult trees, with greatest sensitivity in the seeds of gymnosperm species compared to angiosperms. Clearly, the generalisability of this effect warrants investigation with a greater number of species of different phylogenetic lineages.

5. Conclusion

This study investigated whether tropospheric ozone episodes could influence the time-course of germination and key germination indices in selected temperate tree species, addressing a notable knowledge gap regarding ozone's direct effects on seed germination under documented springtime concentrations. Phytohormonal analyses revealed that ozone exposure altered the balance of key germination regulating hormones in ungerminated seeds, particularly through upregulation of ABA, auxin–ABA interactions, and by stimulating stress hormones (JA and SA) in *P. sylvestris*. These effects provide mechanistic insight into the ozone-induced delay in germination and higher non-germinating fraction observed in *P. sylvestris*, resulting in approximately 17% lower germination success. *B. pendula* and *P. sitchensis* exhibited the same, albeit weaker, trends of delayed germination and reduced germination proportion while no significant emergent germination effects were observed in *A. glutinosa* or *A. pseudoplatanus*, although the latter showed hormonal trends consistent with *P. sylvestris*. Our findings provide evidence of species-specific vulnerability to ozone stress when undergoing germination, particularly among gymnosperms. This underscores ozone's potential ecological role as a selective pressure influencing germination dynamics and forest community composition. We support a dose-dependent ozone-ROS-phytohormone causal framing to explain observed germination dynamics, whereby the action of ozone through ROS accumulation depends on the capacity of the antioxidant system of the individual and species in question. Consequently, future research should explore how seed ROS build-up, antioxidant capacity, physical characteristics and phytohormonal regulation interact to determine species sensitivity to ozone.

Author contributions

JAW designed this study with guidance from MP, LJ, JRH and AS. Discovery Science funding was obtained by TP, supported by JAW and AS. JAW conducted both experimental trials with support from MP and prepared seeds for phytohormonal profiling. Phytohormone profiling was conducted by PMM. JAW designed and conducted the analysis with guidance from AS and MP and prepared the figures and tables, in addition to writing the first draft of the manuscript, with feedback from MP, LJ, JRH, FH and AS. All authors contributed to the final manuscript development.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2026.106434.

Data availability

Seed germination data for trial 1 has been deposited with the Environmental Information Data Centre, UK Centre for Ecology and Hydrology: https://doi.org/10.5285/e0226c12-f07a-48a6-8bef-9ae9e0a7d249. Trial 2 data will be deposited with the EIDC; at this time these data and all scripts are available upon request.

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