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Research Paper

Impact of rising CO_2 and temperature on grass phenology, physiology, and pollen release patterns in northern latitudes

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ABSTRACT

Climate change has complex effects on vegetation, including native grasses and those used as fodder plants. Like many other plant species, grasses respond to climate change by altering their phenology and physiological behavior, leading to changes e.g. in growth, reproduction and metabolic processes. Our study is the first to explore how *Phleum pratense* and *Alopecurus pratensis* respond to rising CO_2 and temperatures projected for northern latitudes for two growing seasons. We investigated growth, phenology, pollen release, and physiological parameters in plants cultivated under these conditions, simulated within environmentally controlled chambers.

Treatment with elevated temperature reduced the number of generative tillers and, consequently, decreased both the number of inflorescences and the season pollen integrals. Pollen release from *P. pratense* started up to 17 days earlier, and the daily peak concentration of released pollen was observed 1–2 h earlier in chambers with elevated temperatures when compared to the present climate conditions. Similar effects were noted in *A. pratensis*. Elevated CO_2 (EC) increased net photosynthesis of *P. pratense*, but this effect was reduced under elevated temperature (ET), suggesting an antagonistic interaction. In *A. pratensis*, both elevated CO_2 and temperature had an additive effect on increasing net photosynthesis, with the highest rate observed under the combined ETEC treatment. The elevated temperature or CO_2 did not affect the plant biomass.

Our findings propose that the rising temperatures in northern latitudes decrease the flowering of studied grasses and shift the seasonal and daily start of the pollen release. Changes in tiller proportions, reduced pollen integrals, and fewer inflorescences suggest that a warmer climate may negatively impact reproductive success, ecological fitness, and allergenic burden of these grasses.

1. Introduction

Human activity has caused unprecedentedly extensive, rapid, and partially irreversible changes in our climate impacting all species, including plants. Rising temperature and carbon dioxide (CO_2) concentration affect plant's physiological processes, such as gas exchange and growth (Albert et al., 2011; Wang et al., 2015; Zhang et al., 2021). Throughout evolution, plants have evolved a diverse range of physiological and structural mechanisms to acclimate and adapt to environmental change, thereby enhancing photosynthesis, growth, and reproductive capacity, while withstanding stress and avoiding damage (Larcher, 2003). Acclimation to elevated CO_2 levels, for example, leads to an increase in the net photosynthesis of plants, and changes in stomatal density and aperture to reducing transpiration and helping them to conserve water (Ainsworth and Rogers, 2007; Larcher, 2003; Xu et al., 2016). Increased photosynthesis also stimulates plant growth, increasing their biomass (Lenka et al., 2021; Zhu et al., 2016).

The effects of climate change parameters can be assessed separately, but evaluating their combined effects (additive, synergistic, or antagonistic) is highly necessary. The interaction between elevated CO_2 levels

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and temperature is complex and can vary depending on plant species and environmental conditions. While elevated CO_2 can enhance photosynthesis and promote plant growth, the simultaneous rise in temperature can influence these processes either positively or negatively, depending on whether temperatures exceed the plant's optimal growth range (Hatfield and Prueger, 2015; Kimball, 2016). Higher temperatures can also affect water use, increasing transpiration and decreasing water availability, which potentially offset the positive effects of elevated CO_2 on plant growth (Cai et al., 2016; Jumrani et al., 2017; Song et al., 2016; Wang et al., 2016; Zhang et al., 2021). Moreover, an antagonistic effect between warming and drought has been observed, while a synergistic effect between CO_2 elevation and drought suggests that the interaction of these factors is highly dependent on water availability. Under well-watered conditions, elevated CO_2 and temperature enhanced plant growth more than each factor alone (Xu et al., 2014).

Reproductive traits of plants, including flowering timing and pollen production, exhibit high sensitivity to environmental fluctuations (Luschkova et al., 2022). In a recent meta-analysis assessing interactions between warming and other global change drivers (Zhou et al., 2023), increased temperature alone was shown to advance the timing of leaf emergence and first flowering, whereas elevated CO_2 alone had no effect on these events. However, when both factors were combined, the on-set of first flowering was further advanced, indicating a synergistic effect. The study suggested that also other parameters, such as, precipitation and nutrient availability, which are expected to change in the future depending on the region, may have interactive effect with increased temperature on plant phenology.

Future climate scenarios indicate changes in the timing and magnitude of male flowering, i.e., pollen seasons. The Shared Socioeconomic Pathways (SSP) 5–8.5-scenario, the so-called worst-case scenario, projects that pollen season may start up to 40 days earlier and last longer in some species (Zhang and Steiner, 2022). Changes in pollen seasons of different taxa have already been observed in Northern Hemisphere (Anderegg et al., 2021; Ziska et al., 2019), as well as increases in pollen load (Gehrig and Clot, 2021). However, the intensity of the phenological response is highly specific to each species and also depends on the bioclimatic region. It has been speculated that while warm springs lead to an earlier onset of the growing season, mild winter conditions can also cause a delay in spring phases in grasslands (Yu et al., 2010). Nevertheless, earlier flowering seasons appear to bet the more common response. For grasses, increasing temperatures have been shown to advance the onset of pollen seasons, both experimentally (Jung et al., 2021) and in long-term studies conducted in Northern Europe (Denmark) (Ahlstrand et al., 2023) and Mediterranean region (Italy) (Ghitarrini et al., 2017). On the other hand, increased CO_2 have been shown to increase timothy pollen production in experimental study (Albertine et al., 2014).

Climate change -induced global warming is estimated to have the greatest impact in northern high latitudes (IPCC, 2021) and the warming rate is much faster than the global average (Rantanen et al., 2022). This brings challenges also to the perennial plants in the region, as the changing climate affects their ability to survive across multiple years and through different seasons. For this reason, the responses of perennial plants to climate change can be very different compared to annual plants (Höglind et al., 2013; Jing et al., 2013). Understanding the changes in flowering and growth is crucial for comprehensively assessing plant adaptation and survival under future conditions.

The Poaceae family, i.e., grasses, includes over 10,000 species and is known for its high allergenic potential (Frisk et al., 2023). Among these species, perennial timothy (*Phleum pratense* L.) and meadow foxtail (*Alopecurus pratensis* L.) occur naturally in northern latitudes, including Europe, Asia and Northern America, and are an important forage grasses in cool climates and essential for livestock (Stewart et al., 2011; Wenick et al., 2008). Meadow foxtail (*A. pratensis*) is a common as wild but less studied grass species compared to commonly cultivated timothy grass (*P. pratense*). *P. pratense* and *A. pratensis* are both winter-hardy and need certain temperature and/or photoperiod prior flowering (Heide, 1986; Höglind et al., 2013). As these species have different flowering times, with meadow foxtail flowering in early summer and timothy later (Bock et al., 2013; Frisk et al., 2023), also their phenological responses to climate change, especially warmer spring temperatures may differ.

The main objective of this study was to evaluate the impact of increasing temperature and CO_2, both individually and in combination (as simulated in environmentally controlled growth chambers) on growth, physiology and flowering patterns of *P. pratense* and *A. pratensis*. We addressed the following questions:

1) Do elevated temperature and CO_2 levels change the timing of pollen release and contribute to the amount of produced pollen?

2) How do plant physiological processes, such as photosynthesis respond to elevated temperature and CO_2, and do the studied species have potential to acclimate to these changes?

3) Do temperature and CO_2 interact in their effects of measured parameters?

2. Material and methods

2.1. Growth experiment

The study examined effects of climate change factors, namely temperature and CO_2 concentration, on two grass (Poaceae) species, timothy (*Phleum pratense*) and meadow foxtail (*Alopecurus pratensis*), over two growing seasons (Fig. 1A). The growth experiment of *P. pratense* was conducted 2020—2021 and *A. pratensis* 2021—2022. The seeds of both grass species were collected from nature in Turku (SW Finland, 60.4517°N, 22.2669°E), sown in 9 cm×9 cm plastic pots with garden soil (mix of peat, fine and coarse sand and plant-based compost; NPK 15–10–16, pH 6.2, Kekkilä, Finland). A total of 320 pots were pre-grown at the Botanic Garden of the University of Turku (Turku, Finland) through the principal growth stage BBCH 1 (leaf development, main shoot) until they were at least in the five-leaf stage (BBCH code 15). The growth stages were named using the extended BBCH-scale for cereals (Meier, 2018). During the pre-cultivation the seedlings were thinned to one seedling per pot and grown in the natural light of mid-June – late July (length of day in Turku) in green-house. Seedlings were grown at temperatures ranging from 19 to 31 °C. The plants were transferred to vernalization conditions of a light rhythm 8:16 light:dark, first with gradually decreasing temperature (from 24° to 7 C° in 12 days) and then six weeks at 6°C. Plants were watered in need.

After six weeks vernalization, the pots were moved for treatments to the University of Eastern Finland (UEF), Kuopio. The pots (approx. 72–80 pots per treatment) were randomly placed into four growth chambers (1270 mm×750 mm, Fitotron®, type: HGC 1014, Weiss Technik), which were programmed to replicate growing conditions (light rhythm, temperature) in Turku (Finland) in the year 2020 and those predicted for the year 2100 (RCP8.5 scenario). The elevated temperature was approx. four degrees higher than the current ambient temperature, and the chamber program followed the average daily temperature curve of the month (May to August) (Fig. 1B). The elevated CO_2 was set to 800 ppm (current ambient concentration 400 ppm). Temperature programs were applied continuously day and night within the chambers, and CO_2 supplementation was maintained for 24 h per day. The elevated CO_2 levels were automatically controlled by a computer using a pulse modulation controller that managed the solenoid valves, which periodically supplied CO_2 (Woikoski Oy, Finland) to each chamber individually. The concentration of CO_2 in each chamber was measured using a PP-Systems EGM4 analyzer. Based on the results, an algorithm calculated the new pulse duration for the feed valves. The photoperiod of the chambers imitated the natural rhythm of daylight in different months (Fig. 1C).

The four different programmed temperature and CO_2 treatments were: 1) control (ambient temperature, ambient CO_2, later referred as

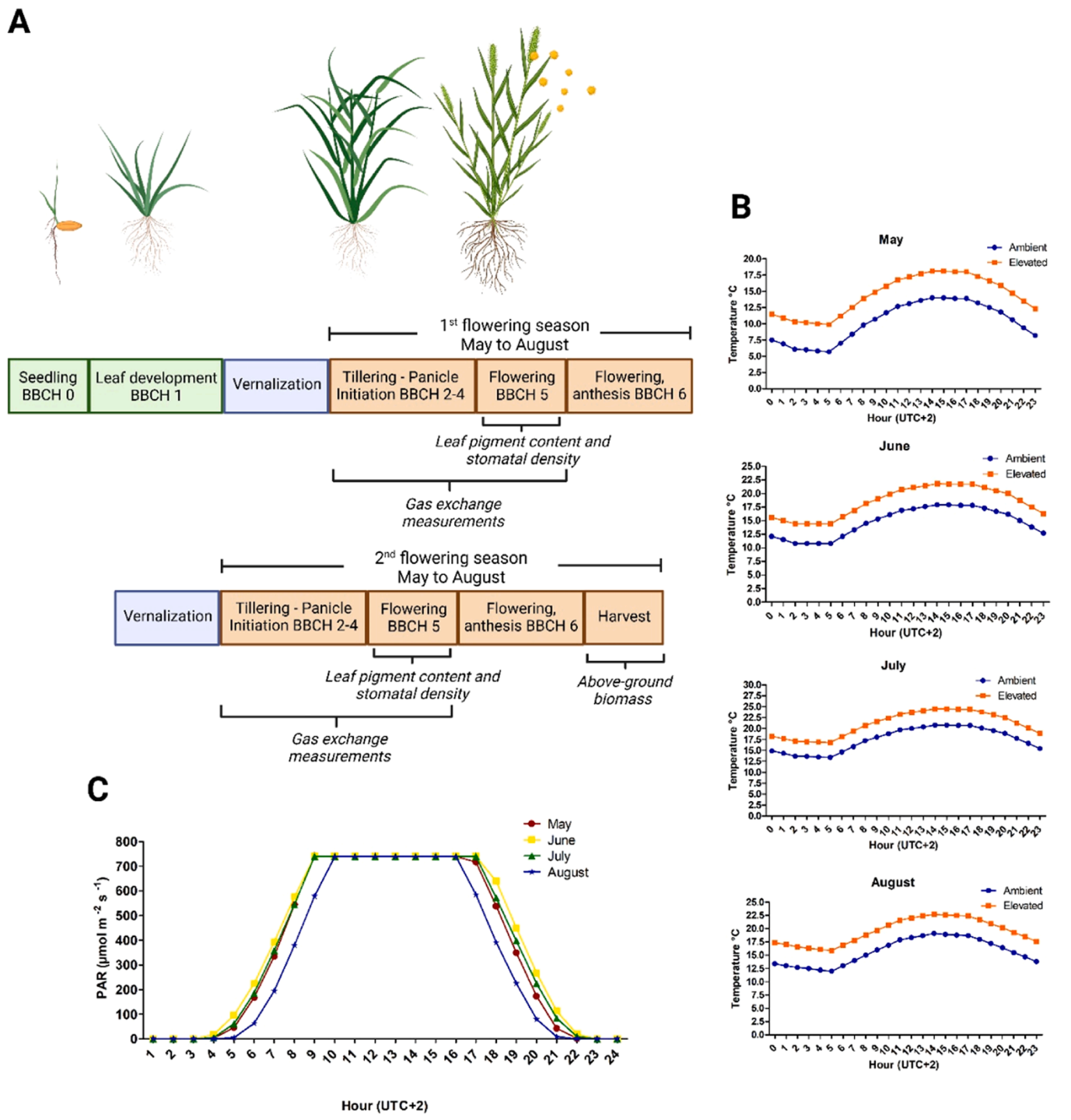

Fig. 1. A) Timeline for growth phases and respective measurements. B) Average daily temperature curve of the month (May to August). The ambient temperature line shows the 2020 temperatures in Turku (Finland) and the elevated line the predicted temperatures for the year 2100 under the RCP8.5 scenario. C) Month-specific photoperiod of the programs. PAR = photosynthetically active radiation. Figure created with BioRender.com.

ATAC), 2) ambient temperature, elevated CO_2 (ATEC), 3) elevated temperature, ambient CO_2 (ETAC) and 4) elevated temperature, elevated CO_2 (ETEC, full climate change treatment). The maximum PAR (photosynthetically active radiation, 400–700 nm) level was 740 µmol m^{-2} s^{-2} at the canopy height and RH was programmed to follow a daily cycle, ranging from 49 % to 93 %, with the highest RH occurring during the night and early morning, and the lowest during the day. Each month had a specific RH program tailored to its conditions. The light source in the chamber was LED grow lights (Valoya B100 NS1 (3 pcs) and G2 (3 pcs), Valoya Oy, Helsinki, Suomi) positioned alternately on the ceiling. A mesh fabric (SEFAR NITEX Switzerland) was placed between the lamps and plant canopy top (about 5 cm below lamps and 25 cm above plant canopy top) to create diffuse light environment in the chambers. From pre-cultivation the pots were fertilized once a week (0.1 % dilution of Kekkilä Garden fertilizer, NPK 17–4–25 %, Kekkilä Oy, Eurajoki, Finland) and adequate water supply of seedlings was ensured by monitoring them daily throughout the experiment and providing manual watering. To prevent the effects of uneven distribution of environmental variables, such as radiation intensity, the pots were rotated twice a week during pre-cultivation and subsequent

vernalization as well as during chamber treatments until the plants began to flower.

The plants were grown for two weeks in the May program, one month in both the June and July programs, and 15 days (*P. pratense* 1st flowering season), 28 days (*P. pratense* 2nd flowering season), 0 days (*A. pratensis* 1st flowering season) and 14 days (*A. pratensis* 2nd flowering season) in the August program. At the end of the first flowering season, the plants were transferred to vernalization conditions, first with gradually decreasing temperature in 7 days to 6 °C for six weeks (8:16 light:dark, PAR level 70–80 μmol m^{-2} s^{-2} at the canopy height). For the vernalization of *A. pratensis*, the PAR level was lowered (30–40 μmol m^{-2} s^{-2}) to enhance the vernalization. The second flowering season followed this six-week vernalization period and was carried out like the first one.

2.2. Phenology, growth and water content

Visual appearance of both *P. pratense* and *A. pratensis* was observed throughout the growth experiments, and the start of flowering (BBCH stage 55) and pollen release (BBCH stage 61) was recorded. After each flowering season (BBCH stage 69), the tiller types (*P. pratense*: non-reproductive vegetative tillers (VEG), reproductive generative tillers (GEN), and elongated vegetative tillers (ELONG); *A. pratensis*: GEN and VEG), and the number and lengths of the inflorescences were observed.

Above-ground biomass of plants was determined after the second flowering season of both species. From each treatment, above-ground part (stem, leaves, and inflorescences) of 10 randomly selected plant individuals were first weighed (fresh weight, FW), and then the plant parts were dried at 60 °C for 3 days and weighed again (dry weight, DW). The water content of the samples was determined using the formula (100-(DW/FW*100)).

2.3. Pollen particle concentrations

Pollen concentration in the chambers was measured with PS2 pollen sensors (Shinyei Technology Co., LTD, Japan), located in one side of the chamber at the height of top of the plants. The PS2 draws sample air continuously at a flow rate of about 0.7 l/min. Inside the sensor, light is scattered by the particles in the sample and detected by two receptors, one as such (signal P) and the other through a polarization filter (signal S). Over time, the signals accumulate into a two-dimensional P–S pattern, which enables identifying the pollen type and discriminating pollen from other particles. Based on the sensor pulses, hourly averages of the pollen concentrations (#/m^3) were calculated by summing the counts in the P–S pattern for each hour and dividing it by the sampled volume (0.042 m^3). Also, season pollen integrals were calculated by summing the counts in the P–S pattern over the whole flowering season. False counts during the chamber doors were open were not included. The measurements were carried out during both flowering seasons of *A. pratensis* in all treatment and during the second flowering season of *P. pratense* in the ATAC and ETEC treatments. During the other flowering seasons and treatments, the instruments were not available for the chamber measurements.

2.4. Physiological measurements

Physiological measurements were conducted over two flowering seasons for *A. pratensis*, while, due to logistic reasons, the data for *P. pratense* were collected only during the second flowering season.

For gas exchange, net photosynthesis (A_{net}), stomatal conductance (g_s), water-use efficiency (WUE, A_{net}/g_s), transpiration (T_r), and intercellular CO_2 concentration (C_i) was measured from the youngest fully developed leaves before the flag leaf stage (BBCH scale: 25–45), from the flag leaf once developed (BBCH scale: 50–55) and from two tillers per plant. Measurements were performed on leaves once a week for 5–6 weeks (*P. pratense*), for 2–3 weeks in the 1st flowering season of *A. pratensis*, and for 4 weeks in the 2nd flowering season of *A. pratensis*, and once after the flag leaves were developed. Gas exchange was measured using a LICOR 6400XT system (LI-COR inc., Lincoln, Nebraska, USA). True leaf area in the leaf cuvette was measured and measurement data corrected accordingly. Stomatal ratio was set to 1. Measurements were conducted at saturating light (1300 μmol m^{-2} s^{-1}) that was based on light saturation curves, at treatment CO_2 concentrations (400 or 800 ppm), temperature (the highest daily temperature of the month), and RH approx. 40 %.

Chlorophyll (Chl) and carotenoid concentrations and stomatal density were determined from flag leaves only (BBCH scale: 50–55). The content of chlorophyll a (Chl a), chlorophyll *b* (Chl b), carotenoid (Car) and total of chlorophyll (Chl a +b) was determined per pigment using the method described by Lichtenthaler (1987). Approximately 50 mg pieces of flag leaves were weighed, frozen in liquid nitrogen (-196 °C), and stored in a freezer (-80 °C) until analyzed. For pigment analyses leaf pieces were extracted into 10 ml of 95 % ethanol in the dark at + 6 °C for 24 hours. The extracts were analyzed with a spectrophotometer (Shimadzu UV-2401PC, Shimadzu Corporation, Kyoto, Japan) at 664 nm, 649 nm, and 470 nm. Chlorophyll and carotenoid concentrations were determined using the following formulas:

$$\text{Chlorophyll a } (Chl_a) = 13.36\ A_{664} - 5.19\ A_{649},$$

$$\text{Chlorophyll b } (Chl_b) = 27.43\ A_{649} - 8.12\ A_{664},$$

$$\text{Chlorophyll a+b } (Chl_{a+b}) = 5.24\ A_{664} - 22.24\ A_{649},$$

$$\text{Carotenoids (Car)} \quad = \frac{(1000 \quad A_{470} - 2.13 \quad C_a - 97.64 \quad C_b)}{209}.$$

The stomatal density was determined from the flag leaves (BBCH scale: 50–55) by making a glue replica using the method implemented by Hartikainen et al. (2012). For the analysis, about 1 cm long piece of leaf was glued to a microscope slide with super glue (Loctite Super Glue, Henkel Norden AB, Bromma, Sweden). The glue was allowed to dry about 10 seconds, after which the leaf was carefully removed from the slide. Glue replicas were made on the top (adaxial) and bottom (abaxial) surfaces of flag leaves. Samples were analysed with an inverted fluorescence microscope (Zeiss Observer. Z1, Carl Zeiss Microlmaging GmbH, Göttingen, Germany) and photographed (Zeiss AxioCam MRm, Carl Zeiss Microlmaging GmbH, Jena, Germany) at 10 x objective magnification. Three pictures were taken of each sample and the number of stomata was analyzed with ImageJ.

Due to the low number of generative tillers and inflorescences in the ETEC treatment in 2nd flowering season of *A. pratensis*, no physiological measurements were performed on those flag leaves.

2.5. Statistical analyses

For statistical analyses of physiological measurements, the results were divided into two groups: measurements before the flag leaves (net photosynthesis, stomatal conductance, transpiration, WUE) and measurements from the onset of flag leaves (net photosynthesis, stomatal conductance, transpiration, WUE, pigment-specific analyses, stomatal density). Both groups were checked separately for data and residual normality by Shapiro-Wilk test and for homogeneity of variances by Levene's test. To test the main effects of CO_2 (C) and temperature (T) and the interaction between the CO_2 and temperature (C × T) we used two-way ANOVA. Interactions ($p < 0.05$) were further studied by comparing Simple Main Effects (SME) with the Sidak test, i.e. examining effects of C in both levels of T, and effects of T in both levels C. One-way ANOVA with Tukey's test used for flag leaves of *A. pratensis* in the second season due to low number of statistical replicates. ANOVA and SME results are shown in Supplementary Excel. Statistical analyses were performed using IBM SPSS Statistics v.27 (IBM, Armonk, NY, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Phenology and growth

3.1.1. Tiller types

In the first flowering season of *P. pratense* the number of vegetative tillers (VEG) was significantly increased by elevated temperature (ET) and decreased by elevated CO_2 (EC) (Fig. 2A). In the second flowering season of *P. pratense*, the number of generative tillers (GEN) was decreased and numbers of elongated vegetative tillers (ELONG) and vegetative tillers increased by ET. Treatments did not affect the tillering of *A. pratensis* during the first flowering season (Fig. 2B). During the second flowering season, the number of vegetative tillers was decreased by ET. In the interaction, EC counteracted the decreasing effect of ET.

3.1.2. Number of inflorescences

Elevated temperature reduced the numbers of inflorescences of *P. pratense* when compared to plants grown under current temperature (1st flowering season: ATAC N = 286, ATEC N = 286, ETAC N = 248, ETEC N = 228; 2nd flowering season: ATAC N = 421, ATEC N = 505, ETAC N = 162, ETEC N = 176). In the first flowering season of *A. pratensis*, elevated CO_2 increased the number of inflorescences (ATEC N = 398, ETEC N = 367) compared to treatments with ambient CO_2 levels (ATAC N = 343, ETAC N = 317). Considerably low numbers of inflorescences were observed in the second flowering season of *A. pratensis* (N = 5—41).

3.1.3. Length of inflorescences

In the 1st flowering season of the *P. pratense*, ET treatments shortened the length of inflorescences (Fig. 3A). In the 2nd flowering season, EC increased the length of inflorescences while ET shortened them (Fig. 3B). Moreover, the interaction between temperature and CO_2 (C × T) was marginally significant (0.068). In the first flowering season of *A. pratensis* EC in ambient temperature increased the length of the inflorescences whereas ET and EC together shortened them (Fig. 3C). In the second flowering season, ET resulted in a clear reduction in inflorescence length and the effect was enhanced by CO_2 (3D).

3.1.4. Biomass and water content

Biomass or water content of *P. pratense* did not change under different treatments (Fig. S1A, B). During the second flowering season of *A. pratensis* ET treatments decreased water content of plants (Fig. S1D).

3.1.5. Timing of pollen release

ET treatments (ETAC and ETEC) induced the pollen release of *P. pratense* to begin 17 days earlier in the 1st flowering season and 14 (ETEC) and 9 (ETAC) days earlier in the 2nd flowering season (Table S1). A similar phenomenon was observed with *A. pratensis* as the release of pollen started 19 days earlier in ETEC and 18 days earlier in ETAC during the 1st flowering season, and 14 and 11 days earlier, respectively, in the 2nd flowering season, when compared to plants grown in ATAC.

3.2. Pollen measurement

In all cases when the PS2 sensors were available, the release of pollen started first in the ETEC treated plants, followed by ETAC, ATEC and ATAC treatment (Fig. 4, Figs. S2 and S3). For example, during the first flowering season of *A. pratensis* (Fig. 4), the start of pollen release was observed by the PS2 sensor 33, 35, 45, and 46 days after the growing season started in ETEC, ETAC, ATEC, and ATAC, respectively. In this case, the pollen season lasted 14 days in ETEC, ATEC, and ATAC but 12 days in ETAC. It must be noted that the start of pollen release observed with the measurements differs from that observed visually (Table S1).

The pollen concentration was lowest during the afternoon and early evening (approximately between 12–20 hours) and peaked in the early morning (Fig. 5 and Fig. S4). In Fig. 5 the mean and the standard error of mean of the pollen concentration of *A. pratensis* are given for each hour of day, averaged over the days when release of pollen was observed by the PS2 sensor (number of days 12 or 14). The peak concentration was observed between 04 and 06 h in ETEC and ETAC treatments and between 05–07 hours in ATEC and ATAC treatments. Based on the observations, the elevated temperature is associated with the earlier timing of daily pollen release, but the elevated CO_2 concentration has no or negligible effect.

In general, the ET and EC treatments decreased the season pollen integrals compared to the control. However, in the 2nd flowering season of *A. pratensis*, elevated CO_2 alone increased the pollen integrals compared to the control (Table 1).

3.3. Physiological measurements

3.3.1. Gas exchange measurements

3.3.1.1. 2nd flowering season of Phleum pratense. Before the flag leaf stage. EC treatments increased net photosynthesis of *P. pratense*,

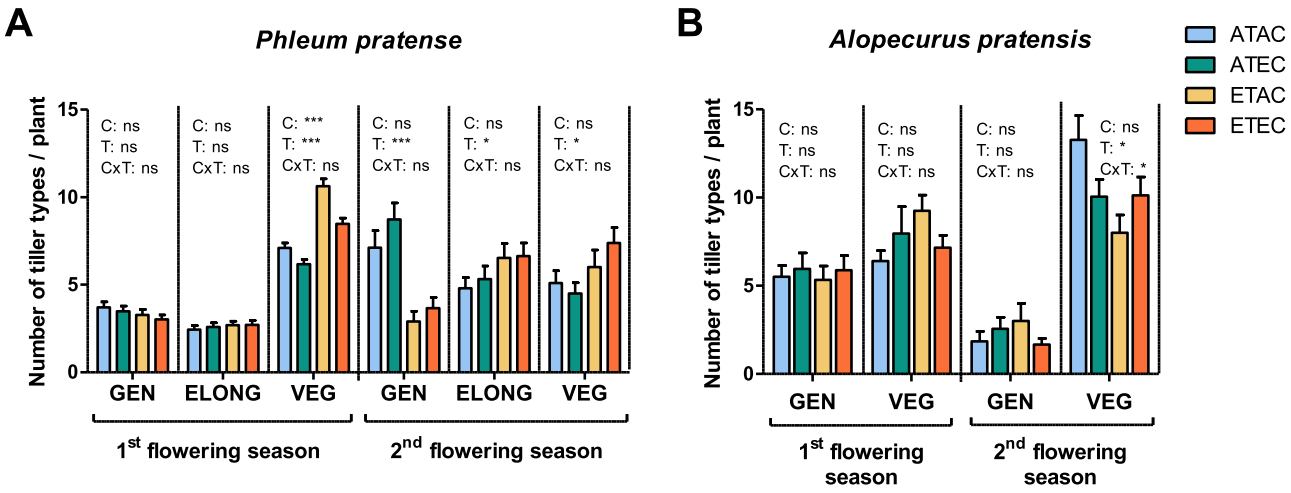

Fig. 2. A number of tiller types in A) *Phleum pratense* and B) *Alopecurus pratensis*. Fig. Shows mean + SE. ANOVA results are shown for each factor: C: CO_2 main effect, T: temperature main effect, and C × T: interaction between CO_2 and T. ns = non-significative effects. *p < 0.05, **p < 0.01, ***p < 0.001 (N = 20–80). GEN = generative tillers, ELONG = vegetative elongating tiller, VEG = vegetative tiller, ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration.

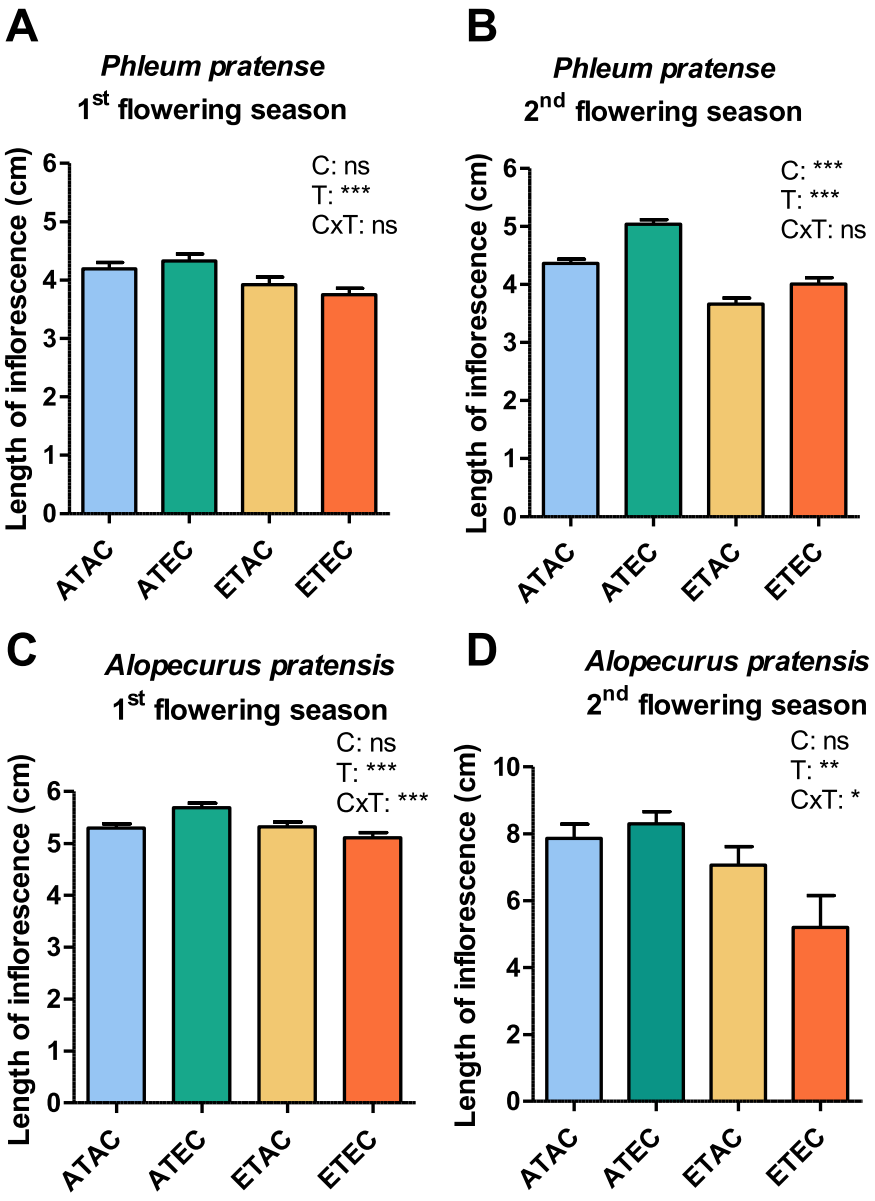

Fig. 3. Length of inflorescences in different treatments in *Phleum pratense* (A and B) and *Alopecurus pratensis* (C and D). Fig. Shows mean + SE. ANOVA results are shown for each factor: C: CO_2 main effect, T: temperature main effect, and C × T: interaction between CO_2 and T. ns = non-significative effects. *p < 0.05, **p < 0.01, ***p < 0.001 (N = 5–505; all inflorescences were measured). ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration.

although the effect was less pronounced when also temperature was elevated (Fig. 6A). Net photosynthesis of *P. pratense* was 87 % higher in the ATEC treatment compared to the control (ATAC) treatment. In the ETAC treatment, net photosynthesis was 13 % higher, and in the ETEC treatment, it was 38 % higher than in the control (ATAC). EC treatments increased water-use efficiency (Fig. 6C) and intercellular CO_2 concentration (Fig. 6E). No significant differences between treatments were observed in stomatal conductance or transpiration rate (Fig. 6B, D).

Flag leaves. EC treatments increased net photosynthesis while ET led to a reduction in the photosynthesis rate (Fig. 6F). Although the interaction (C × T) was not statistically significant, the data suggest an antagonistic effect, where the negative impact of ET on net photosynthesis counteracts the positive effect of EC concentration. ET treatments decreased stomatal conductance (Fig. 6G) while EC treatments significantly increased intercellular CO_2 concentration (Fig. 6J). No significant differences were observed in water-use efficiency or transpiration rate (Fig. 6H, I).

3.3.1.2. 1st flowering season of Alopecurus pratensis. Before the flag leaf stage. Both EC and ET had an additive effect increasing a net photosynthesis of *A. pratensis*, with the ETEC treatment resulting in the highest photosynthesis rate (Fig. 7A1). EC treatments increased water-use efficiency (Fig. 7C1), while ET increased transpiration rate (Fig. 7D1). EC and ET treatments increased intercellular CO_2 concentration (Fig. 7E1). No significant differences were observed in stomatal conductance (Fig. 7B1).

Flag leaves. ET treatment increase a net photosynthesis, and the effect of ET was enhanced by EC (Fig. 7F1). EC treatments significantly increased intercellular CO_2 concentration (Fig. 7J1). No significant differences were observed in stomatal conductance, water-use efficiency, or transpiration rate (Fig. 7G1, H1, I1).

3.3.1.3. 2nd flowering season of Alopecurus pratensis. Before the flag leaf stage. In the 2nd flowering season, the interaction between temperature and CO_2 concentration (C × T) was statistically significant. Elevated CO_2

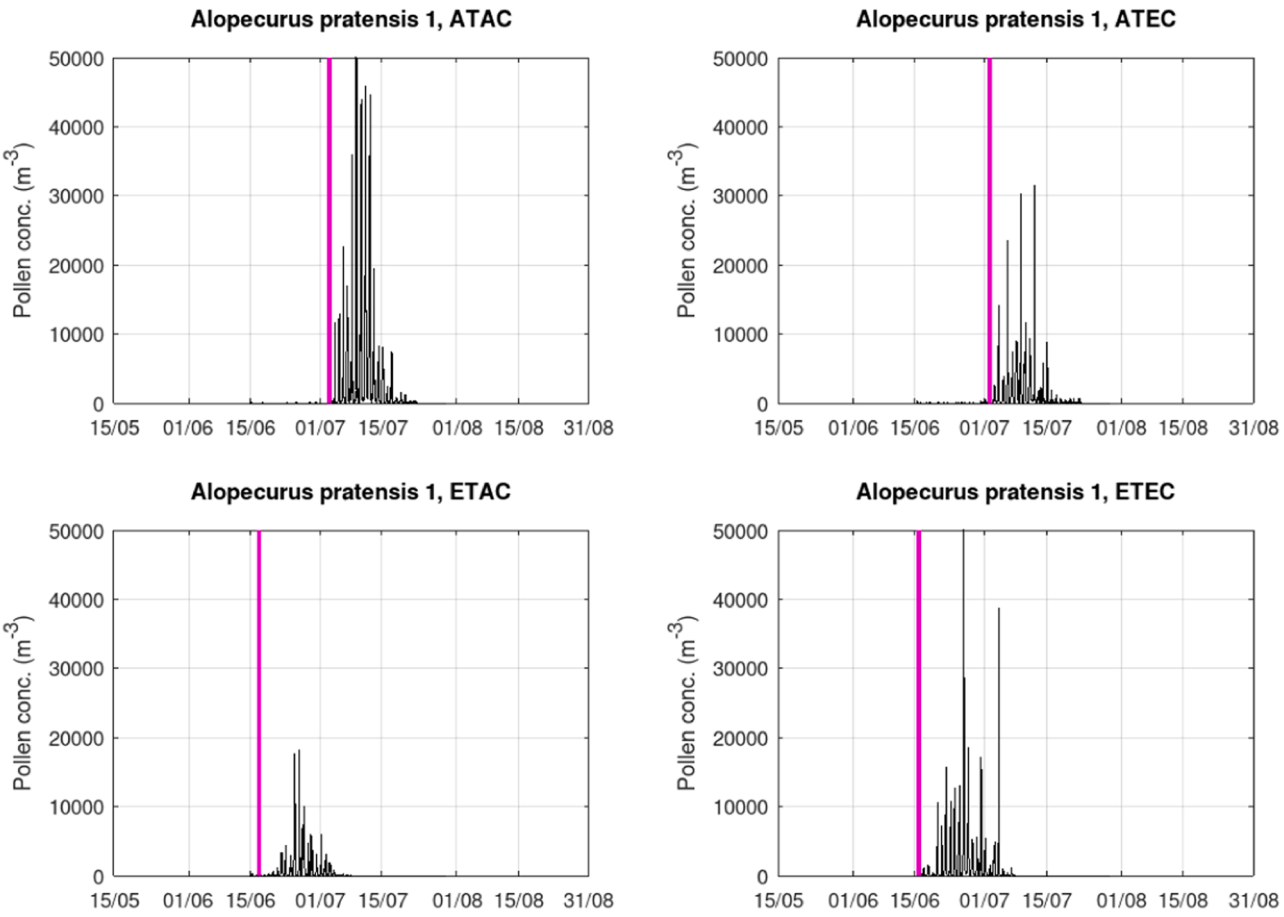

Fig. 4. Hourly average of pollen concentration and visually determined start of pollen release (purple line) in 1st flowering season of *Alopecurus pratensis* in different treatments (ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration).

independently enhanced net photosynthesis, as did elevated temperature independently. In contrast, the combination of elevated CO_2 and temperature (ETEC treatment) resulted in the lowest net photosynthesis Fig. 7A2). ET treatments increased water-use efficiency, while EC treatments increased intercellular CO_2 concentrations (Fig. 7C2, E2). No significant differences were observed in stomatal conductance or transpiration rate (Fig. 7B2, D2).

Flag leaves. ATEC treatment increased net photosynthesis, stomatal conductance, transpiration and intercellular CO_2 concentration of *A. pratensis* (Fig. 7F2-J2). Both ATEC and ETAC treatments decreased water-use efficiency. Because there were so few inflorescences in the ETEC treatment, no data are available for this treatment.

3.4. Leaf pigment contents

In *P. pratense* flag leaves the interaction between temperature and CO_2 concentration (C × T) was statistically significant in Chl b: ET increased Chl b levels under ambient CO_2 conditions, but not under EC conditions (Fig. S6A). Moreover, EC treatments decreased carotenoid levels. Elevated temperature, CO_2 or combined elevated T+CO_2 did not affect Chl a or Chl a +b pigment contents.

In first flowering season of *A. pratensis* ET treatments increased amount of chlorophyll a and b (Fig. S6B). The significant interaction (C × T) for Chl b and a+b indicated the effect of ET was less pronounced under elevated CO_2. Treatment effects on leaf pigments were not significant during the second flowering season (Fig. S6B).

3.5. Stomatal density

In 2nd flowering season of *P. pratense* the interaction between temperature and CO_2 concentration (C × T) was statistically significant, and SME test revealed that ET increased stomatal density under ambient CO_2 conditions, but not under EC conditions (Fig. S7A). No significant changes in stomatal density were observed on *A. pratensis* (Fig. S7B, C).

4. Discussion

4.1. Changes at the seasonal and daily onset of pollen release

Reproductive traits of plants, including critical processes like flowering and pollen production, are particularly vulnerable to environmental fluctuations. The combined impact of global warming and increased CO_2 levels, which enhances plant growth through a fertilization effect, can significantly alter the timing, duration, and intensity of flowering, potentially shifting and extending the growing season (Luschkova et al., 2022). For example, temperature alone has been shown to accelerate leaf emergence and the onset of flowering, while elevated CO_2 on its own does not significantly impact these phases. However, when combined, temperature and CO_2 have a synergistic effect, advancing the first flowering (Zhou et al., 2023).

Regarding pollen seasons, Ziska et al. (2019) have demonstrated an extension in the seasonal duration and an increase in seasonal cumulative pollen or annual pollen loads for various pollen types across the Northern Hemisphere. In North America, the overall pollen season now starts approximately 20 days earlier and lasts longer. Change in the start

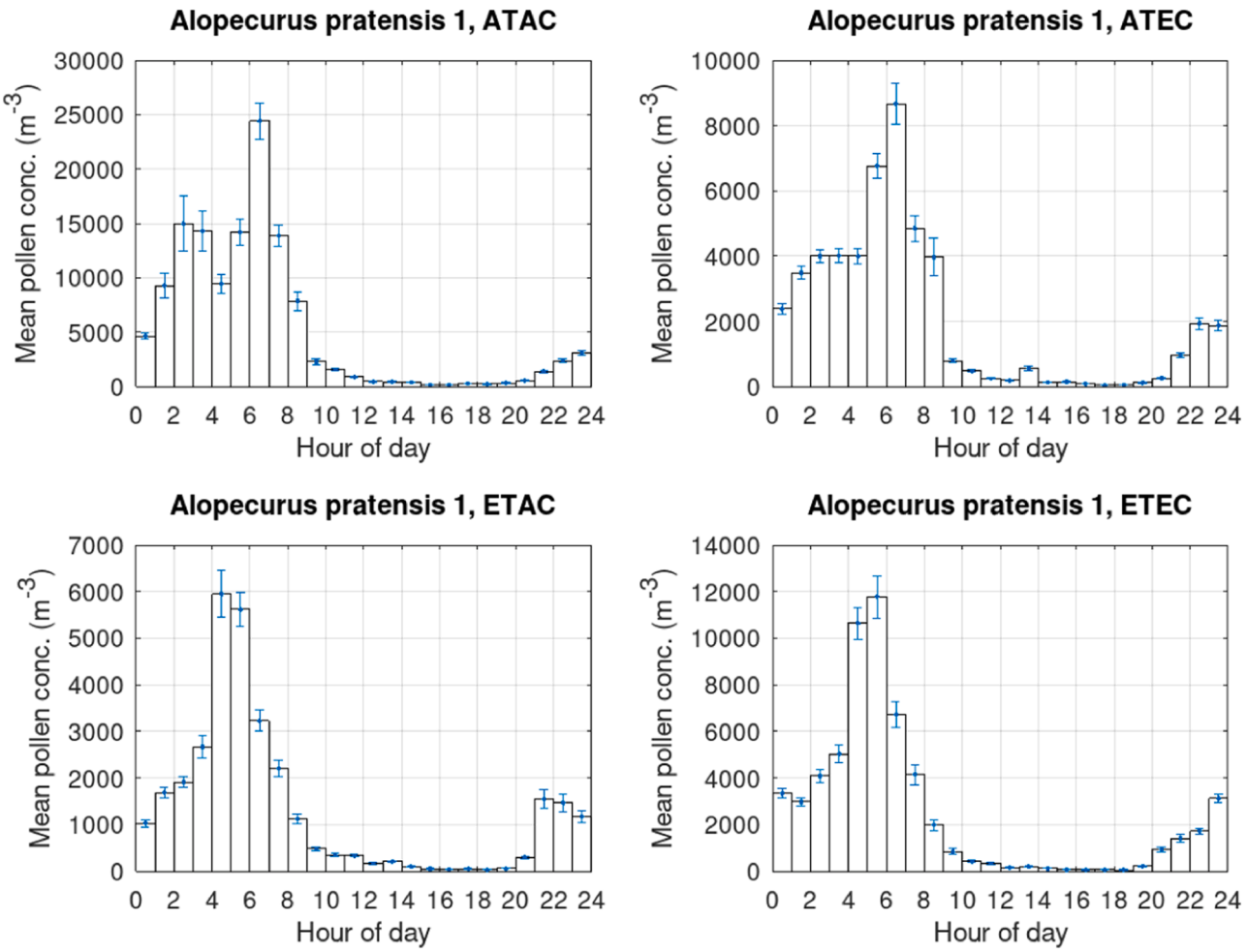

Fig. 5. Mean pollen concentration (histogram bars) and its standard error of mean (error bars) in 1st flowering season of *Alopecurus pratensis* for each hour of day, averaged across the observed pollen season, in all four different treatments (ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration).

Table 1
Season pollen integrals (number of pollen grains) in each treatment (ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration). N/A = not available.

		ATAC	ATEC	ETAC	ETEC
Phleum pratense	1st flowering season	N/A	N/A	N/A	N/A
	2nd flowering season	3205	N/A	N/A	2024
Alopecurus pratensis	1st flowering season	75,108	29,190	16,806	39,734
	2nd flowering season	4007	5410	1562	1687

and duration of the pollen season may become even more pronounced if climate change progresses according to the SSP5–8.5 projection, as modeled by (Zhang and Steiner, 2022). Most of the research has focused on trees that produce allergenic pollen and flower early in spring and subsequently are among those plants that are thought to be most sensitive to warming climate. Grasses and other plants that flower in summer have been studied less. Earlier timing of grass pollen season has been observed and it has been linked with warmer spring temperatures (Ahlstrand et al., 2023; Ghitarrini et al., 2017). Our results from controlled growth chamber experiments confirm this, as elevated temperature significantly advanced the onset of pollen seasons of both studied grass species and the interaction between elevated temperature and CO_2 exhibited synergistic. The shift toward an earlier and prolonged pollen season carries significant implications for public health, particularly for individuals with allergies such as hay fever, asthma, and respiratory conditions. Our study adds the evidence suggesting that climate change is likely to exacerbate these health issues by advancing of allergy seasons.

The timing of daily pollen release varies across species and is influenced by factors like weather and climate. Interestingly, in our study, the concentration of studied grass pollen in chambers peaked early in the morning and was at its lowest in the afternoon. Earlier studies like Emecz (1962) and Ogden and Hayes (1969), align with our study showing anthesis peaks in *P. pratense* and *A. pratensis* before 8 a.m. and in their study all measurements were performed at plant-base level. Noteworthy, in our study, the elevated temperature caused the daily pollen release to start earlier but the elevated CO_2 concentration had no or minimal effect. Given that future climate warming is likely to further advance the timing of pollen release, it is important to consider this when providing recommendations on ventilation and outdoor activities to minimize pollen exposure.

Contrary to our findings, studies from Central Europe have reported different patterns of pollen release by roof level measurements. For example, the highest grass pollen concentrations have been observed between 9 a.m. and 6 p.m., with a peak around midday (Suarez-Suarez et al., 2023). Similarly, Pérez-Badia et al. (2011) found that only small

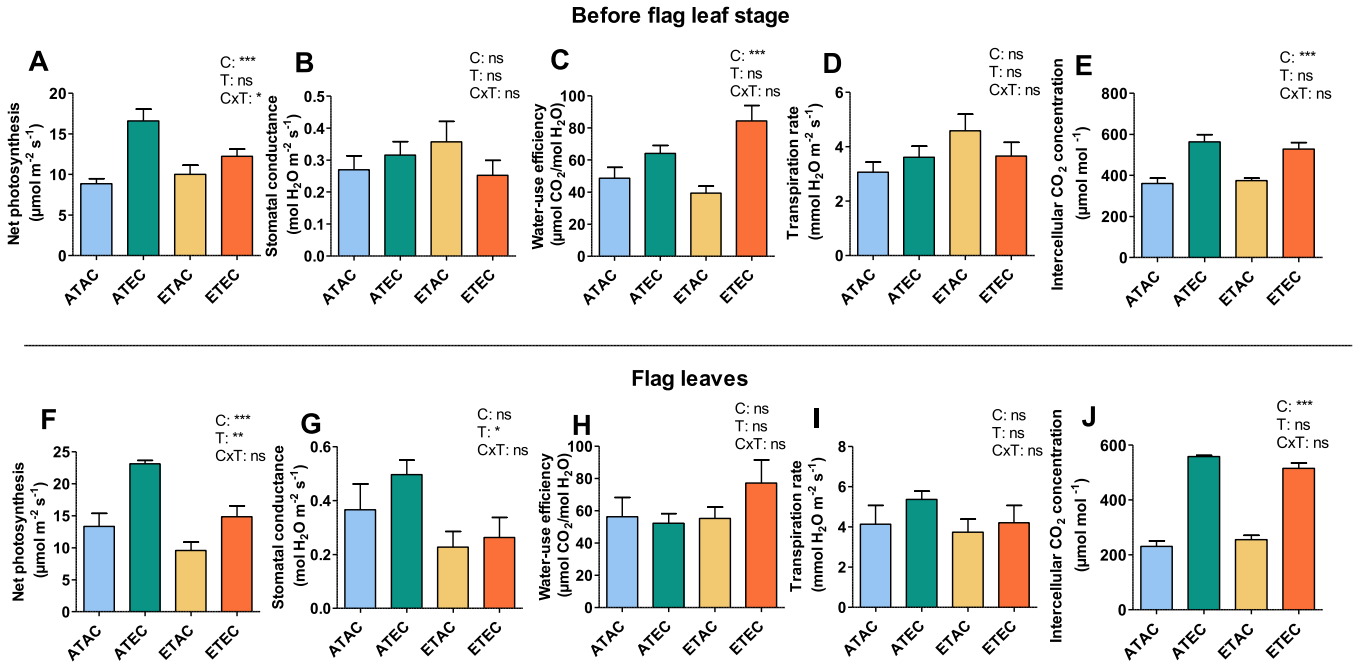

Fig. 6. Gas exchange of *Phleum pratense* before flag leaf stage and from flag leaves in 2nd flowering season. A and F) Net photosynthesis (A_{net}, μmol CO_2 m^{-2} s^{-1}), B and G) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), C and H) water-use efficiency (WUE, A_{net}/g_s, μmol CO_2/mol H_2O), D and I) transpiration rate (Tr, mmol H_2O m^{-2} s^{-1}), E and J) intercellular CO_2 concentration (Ci, μmol mol^{-1}). Fig. Shows mean + SE. C: CO_2 main effect, T: temperature main effect, and C × T: interaction between CO_2 and T. ns = non-significative effects. *p < 0.05, **p < 0.01, ***p < 0.001 (N = 7). ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration.

amounts of most pollen types were present in the air during the early morning, with grass pollen levels specifically rising after 10 a.m. and showing minor peaks at 12 p.m. and 10 p.m. In the line with this, Simoleit et al. (2016) observed grass pollen concentration peaking between 8 a.m. and 10 p.m., with the highest levels occurring around midday or in the afternoon, and the lowest concentrations recorded at night or early morning—patterns that are inconsistent with our present study.

Wind is a key factor in the reproduction of wind-pollinated plants and the spread of pollen. In our study, there was constant air movement in the chambers, but in outdoor studies, wind significantly affects pollen release and transport. Meteorological variables may contribute to differences, but the study design also naturally plays a role. In chamber experiments, controlled climatic conditions eliminate the effects of weather on plants. In outdoor measurements, the distance from the pollen source is longer and the meteorology may strongly affect the residence time between the source and monitor. Also, the monitoring height affects the time at which pollen is detected in the monitor. In the study by Pérez-Badia et al. (2011), the monitor was placed on the roof, and on the other hand, in the study by Simoleit et al. (2016), at a height of 5 m. In our study, the pollen sensors were placed near the grasses, minimizing the distance pollen had to travel to reach the monitors, resulting in almost immediate detection upon release. However, distance between plants and sensors may not be the only explanatory factor. Other environmental and experimental conditions, such as chamber airflow and microclimatic conditions, could also play a significant role in the observed differences.

4.2. Impact of climate factors on plant growth, physiology and pollen production

The effect of climate change in terms of warming and/or increasing CO_2 on plant growth and physiology, especially leaf gas exchange, has been widely studied (Zhang et al., 2021). Research on the Poaceae family has primarily focused on species used as human food, such as rice, and grasses used as fodder crops. In studies on fodder grasses, the emphasis has been more on observing biomass and root development, particularly the effects of nitrogen fertilization in combination with changes in temperature, CO_2, and drought conditions. Previous studies have shown that elevated CO_2 increases aboveground biomass in species like barley and rice (Gardi et al., 2022; Zhang et al., 2022) but not that of timothy (Piva et al., 2013) as was also shown in our study. Although the biomass of the studied grasses did not change in this study, elevated temperature had a significant impact on tiller development and the number of inflorescences in *P. pratense*.

During the 1st flowering season, the relative proportion of vegetative tillers in *P. pratense* was higher compared to generative ones, and it further increased with the elevated temperature. The proportion of generative tillers during the 2nd flowering season was lower in chambers with elevated temperature, while ELONG and VEG tillers increased as a result of the warming. The decrease in generative tillers may have occurred partly because the vegetative tillers increased due to the warming, redirecting carbon from the growth of generative tillers to vegetative ones. Elevated temperature also reduced the number of inflorescences and shortened them in *P. pratense*. The lower season pollen integrals observed in ETEC chamber in *P. pratense* may be explained by the lower number of generative tillers and inflorescences in plants. The changes in the proportion of tiller types, as well as the reduction in season pollen integrals and inflorescence number, suggest that these climate factors may negatively affect reproductive success and ecological fitness of the studied grass species. A similar decrease in pollen production was observed in a recent study, where the pollen concentration of orchard grass (*D. glomerata*) decreased as a result of warming (and urbanization) (Jetschni et al., 2023). Additionally, a long-term study in Switzerland found that the intensity of the grass pollen season did not increase over the past 50 years, despite climate change during this period (Gehrig and Clot, 2021). However, the intensity of the pollen season for some tree pollen taxa were found to have increased.

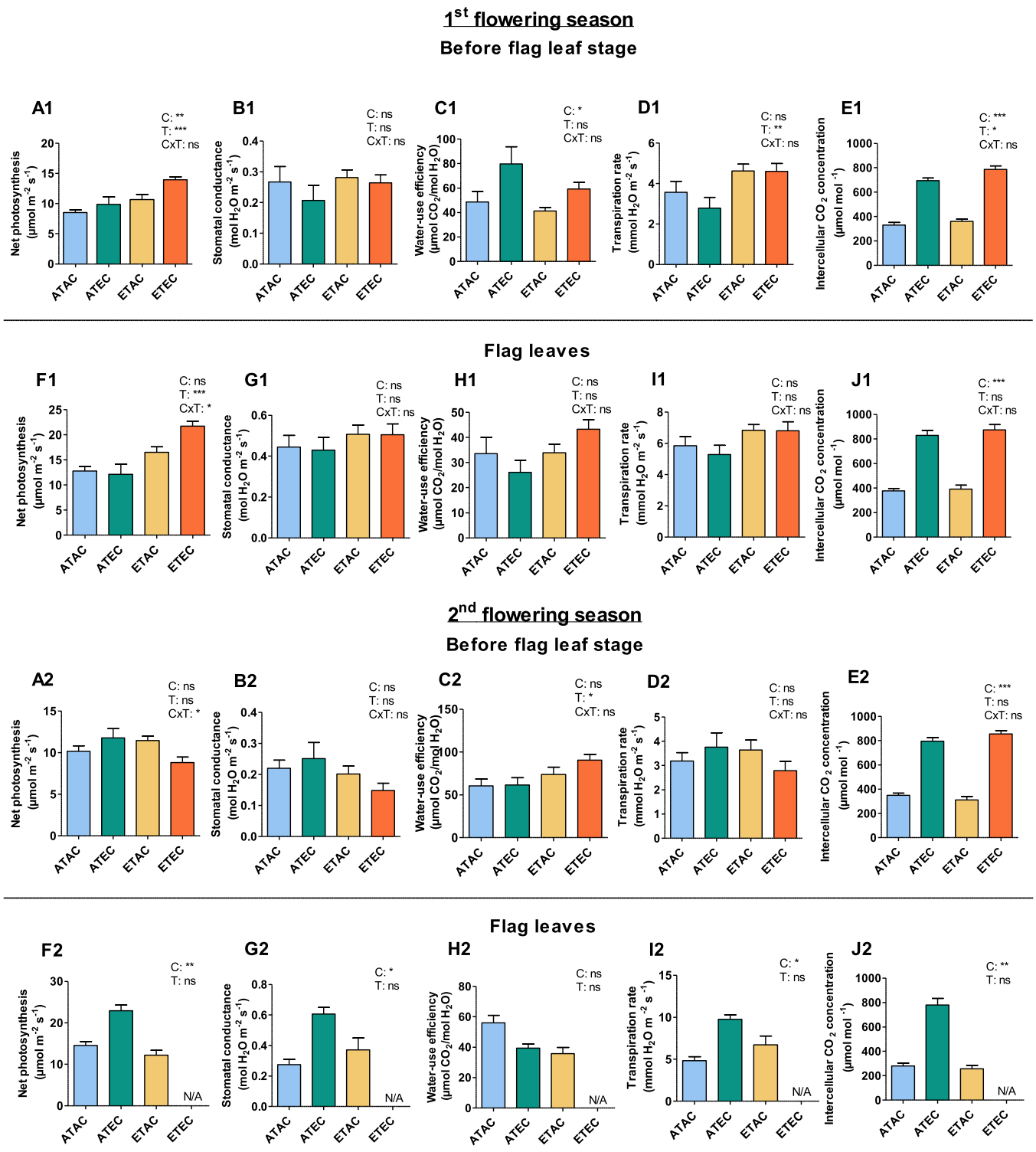

Fig. 7. Gas exchange of *Alopecurus pratensis* before flag leaf stage and from flag leaves in 1st and 2nd flowering season. A and F) Net photosynthesis (A_{net}, µmol CO_2 m^{-2} s^{-1}), B and G) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), C and H) water-use efficiency (WUE, A_{net}/g_s, µmol CO_2/mol H_2O), D and I) transpiration rate (Tr, mmol H_2O m^{-2} s^{-1}), E and J) intercellular CO_2 concentration (Ci, µmol mol^{-1}). Fig. Shows mean + SE. ANOVA results are shown for each factor: C: CO_2 main effect, T: temperature main effect, and C × T: interaction between CO_2 and T. ns = non-significative effects. In Figs. F2-J2, a one-way ANOVA was used. In those figures, the statistical difference 'C' refers to the difference between ATEC and ATAC, while 'T' refers to the difference between ATAC and ETAC based on Tukey's tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (N = 7 in 1st flowering season and N = 5–7 in 2nd flowering season). ATAC = ambient temperature, ambient CO_2 concentration, ATEC = ambient temperature, elevated CO_2 concentration, ETAC = elevated temperature, ambient CO_2 concentration, ETEC = elevated temperature, elevated CO_2 concentration. N/A = not available.

In our study, during the second flowering season of both plants, thrips were observed in all chambers, especially in those with elevated temperature. Despite the biological pest control, the thrips may have had time to injure the plants and thus possibly contribute to the formation of *A. pratensis* inflorescences.

The rising concentration of CO_2 in the atmosphere may also directly influence plant growth by providing plants more resources for photosynthesis. In the short term, acclimation of plants to elevated CO_2 levels induces C3 plants to increase their net photosynthesis and decrease respiration (Long et al., 2004). The impact of the interaction between elevated CO_2 and temperature on photosynthesis in *P. pratense* and *A. pratensis*, both belonging to the Pooideae C3 temperate subfamily specifically adapted to cold seasonal environments, has not been thoroughly documented. In our study, elevated CO_2 significantly increased net photosynthesis of *P. pratense* compared to the control, although this effect was less pronounced under elevated temperature. The interaction between CO_2 and temperature suggested an antagonistic effect, where the negative impact of temperature on photosynthesis counteracted the positive effect of CO_2. Similar results have been observed in timothy grass (*P. pratense*) (Piva et al., 2013), boreal grass, *Phalaris arundinacea* (Ge et al., 2011) and rice, which is also classified as a grass (Poaceae) and a C3 plant (Zhang et al., 2022). Interestingly, the combined increase of temperature and CO_2 enhanced net photosynthesis in *A. pratensis* in 1st flowering season, however, a similar effect was not observed in the 2nd flowering season.

Intercellular CO_2 concentration of leaves (Ci) is a crucial factor in photosynthesis. In our study, C_i in the plants followed the CO_2 concentration of the chambers, meaning that growing in chambers with elevated CO_2 (ATEC, ETEC) significantly increased C_i compared to ATAC and ETAC. In other studies, elevated CO_2 has been found to increase the intercellular CO_2 concentration in *Salvia verbenaca* and *Eucalyptus grandis* (Costa et al., 2023; Javaid et al., 2022), which is in line with our results. A high C_i value indicates that the plant has plenty of CO_2 available for photosynthesis. This can promote photosynthetic efficiency as observed in our study: however, it did not unexpectedly result in an increase in biomass production. This may indicate that carbon was allocated e.g. for root growth or biosynthesis of secondary compounds that were not studied in this project.

In this study, gas exchange was measured before and from flag leaves. Photosynthesis in the flag leaves was higher than before the flag leaves stage. Flag leaves are the last leaf to emerge in a cereal plant during its growth and they are metabolically active and have great photosynthetic capacity (Adachi et al., 2017). Photosynthetic pigments concentrations have been shown to explain warming-induced changes in photosynthesis in boreal grass, *Phalaris arundinacea*, but not those by elevated CO_2 (Ge et al., 2011). This is in line with our *A. pratensis* results as elevated temperature increase chlorophyll a, and b but elevated CO_2 did not affect photosynthetic pigments.

Plants can acclimate to climate change by adjusting their metabolism. For example, if the weather becomes drier, plants can close stomata on their leaves, reducing transpiration and conserving water. Elevated CO_2 alone has been suggested to reduce stomatal conductance (Ainsworth and Rogers, 2007; Lammertsma et al., 2011). In study by Zhang et al. (2022) stomatal conductance increased in response to elevated temperature, while elevated CO_2 alone and in combination with elevated temperature led to a decrease. In our study, elevated temperature decreased stomatal conductance of *P. pratense*. It also increased stomatal density under ambient CO_2, but this increase did not occur under elevated CO_2 conditions. The net effect of elevated CO_2 and temperature on stomatal density was probably an antagonistic interaction, where their combined influence negates their individual effects Changes in stomatal density may partially compensate for variations in stomatal conductance. An increase in stomatal density might indicate a greater need for transpiration to cool the leaf under warming conditions, or it could enhance CO_2 uptake in potentially stressful environments. This adaptive strategy allows the plant to maintain photosynthetic efficiency while managing water loss, reflecting a complex balance between optimizing gas exchange and conserving water under fluctuating environmental conditions.

4.3. The importance of this study and future perspectives

Many studies have been carried out by separately examining the effect of changes in temperature and CO_2. The interactions between elevated CO_2 and elevated temperature are not so well known, especially in the species studied here. This long-term multifactorial experiment revealed intricate effects of these factors on tillering, phenology, pollen release and physiology, and how they may contribute to the acclimation of these grass species to climate change. The RCP8.5 climate scenario was chosen for this study to ensure that the potential impacts of elevated CO_2 and temperature on the selected grass species would be clearly observable. By using this scenario, we aimed to understand the maximum potential effects of rising temperature and CO_2 on grass phenology and physiology, providing valuable insights for preparing for future climate conditions.

This experimental setup enables us to study the plants during two flowering seasons in monthly programs in controlled growth chambers. Growth chambers are environments, where factors such as temperature, gases, humidity, and nutrient levels can be adjusted: plants can be exposed to specific conditions precisely and repeatably. In chamber studies, there are fewer confounding variables, such as extreme weather, animals, or insects, than in field studies, which could affect plant growth. On the other hand, chambers do not correspond to real field research. The use of growth chambers may not provide an accurate representation of how ecosystems will respond to changes in CO_2. Next, the study should be repeated outdoors in experimental conditions where temperature, CO_2 and precipitation can be controlled, and the size of the pot is not limiting the growth. As climate change also alters precipitation and water availability, future studies should evaluate the interaction of these factors together with temperature. In this study, both species are perennial rhizoidal grasses, whose 1st-year flowering is largely based on the root system. In outdoor study, pre-cultivation would also take place in treatments and the plants would adjust more naturally to treatment temperatures after vernalization. The outdoor research would also enable a regular monthly cycle throughout the year, allowing plants to grow for a longer period after flowering before vernalization. In this study, a limited and shorter time was allocated for growth after flowering, which may impact the flowering in the following year.

5. Conclusions

Overall, our multifactorial experiment showed the individual and combined effects of temperature and CO_2 on phenology, physiology and release of pollen. Changes were seen especially in photosynthetic efficiency and intercellular CO_2 concentration, but the studied species responded somewhat differently. Elevated temperature advanced the start of pollen season as well as the daily timing of the pollen release in both species. However, the number of generative tillers and inflorescences decreased, as did the season pollen integrals. These findings imply that future climate conditions may negatively impact the reproductive success and ecological fitness of these species. The overall exposure to grass pollen could be lower, but the impact of climate change on the allergenicity of these species needs to be addressed separately. Nevertheless, further studies are needed to address how predicted increase in precipitation for northern regions or other factors not studied in the chambers might influence the results.

CRediT authorship contribution statement

Tarleena Tossavainen: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Minna Kivimäenpää:** Writing – review &

editing, Supervision, Methodology, Conceptualization. **Annika Saarto:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Marjut Roponen:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Sanna Pätsi:** Writing – review & editing, Investigation. **Mika Komppula:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Anna-Mari Pessi:** Writing – review & editing. **Maria Louna-Korteniemi:** Writing – review & editing, Investigation. **Ari Leskinen:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Tiina Heinonen:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Maria-Viola Martikainen:** Writing – review & editing, Supervision, Investigation.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marjut Roponen, Mika Komppula, and Annika Saarto report financial support that was provided by Research Council of Finland. If there are other authors, they 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.2024.105995.

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