Daylength influences the response of three clover species (*Trifolium* spp.) to short-term ozone stress

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Long photoperiods characteristic of summers at high latitudes can increase ozone-induced foliar injury in subterranean clover (*Trifolium subterraneum*). This study compared the effects of long photoperiods on ozone injury in red and white clover cultivars adapted to shorter or longer daylengths of southern or northern Fennoscandia. Plants were exposed to 70 ppb ozone for six hours during the daytime for three consecutive days. Simultaneously, the daylength in the growth rooms was altered to long-day (10 h light; 14 h dim light) and short-day (10 h light; 14 h darkness) conditions. Thermal imaging showed that ozone disrupted leaf temperature and stomatal function, particularly in sensitive species, in which leaf temperature deviations persisted for several days after ozone exposure. Long-day conditions increased visible foliar injury (30%–70%), characterized by chlorotic and necrotic areas, relative to short day conditions in all species and cultivars independently of the photoperiod in the region they were adapted to.

Introduction

During the last century, the global background levels of tropospheric ozone increased from about 10 ppb to 30–40 ppb (Ashmore 2005, Fuhrer 2009). In northern Fennoscandia, ozone levels usually vary between 30 and 40 ppb with higher, acute, short-term exposures up to 50–80 ppb (Manninen *et al.* 2009). These levels have been shown to negatively affect plant development. Future projections suggest that the occurrence of years with high cumulative ozone exposures could increase in Europe, particularly in northwestern regions (Fuhrer 2009). Tropo-spheric ozone is highly dependent on meteorological conditions, such as climate change. European modeling studies predict a considerable increase in tropospheric ozone levels, even at constant anthropogenic precursor emissions (Meleux *et al.* 2007).

Detrimental effects of ozone on vegetation are well-known and are often seen as reductions in plant growth, biomass, leaf area and seed yield, as well as early senescence and visible damage to leaves (Ashmore 2005). Despite the general negative effects of ozone, ozone sensitivity has been found to vary considerably between species, with some species displaying

extensive ozone damage and others being barely affected at the same external ozone concentration (Bermejo *et al*. 2003, Gimeno *et al*. 2004a, 2004b, Scebba *et al*. 2006, Hayes *et al*. 2007).

In comparative studies of ozone sensitivities, the genus *Trifolium* has been ranked as ozone sensitive as it readily develops visible foliar injury and other symptoms typical of ozone stress (Pihl Karlsson *et al*. 1995a, Bermejo *et al*. 2003, Gimeno *et al*. 2004a). Clover species have therefore been used as ozone bioindicators and red clover (*T. pratense*), white clover (*T. repens*) and, in particular, subterranean clover (*T. subterraneum*) have been used as experimental plants to determine critical levels for plant ozone effects within the United Nations Economic Commission for Europe (UNECE) Convention on Long-range Transboundary Air Pollution (CLRTAP) (Pihl Karlsson *et al*. 1995b, 2003, 2004, Karlsson *et al*. 2009b).

Plant ozone sensitivity depends on many factors, including plant constitutive and induced defense, stomatal ozone uptake, plant functional type, climatic factors and nutrient availability (Musselman *et al*. 2006, Bassin *et al*. 2007). Studies also show that plants in northern regions can be more ozone sensitive than plants at lower latitudes despite lower ambient ozone levels (De Temmerman *et al*. 2002b, Karlsson *et al*. 2009a). It has been suggested that the conditions of summers at higher latitudes, characterized by moderate temperatures, moist climate and long days, are favorable for stomatal opening, thereby increasing stomatal ozone uptake and hence ozone damage (Pleijel *et al*. 2000, Karlsson *et al*. 2009a, 2009b). It has also been proposed that the shorter summer nights in the northern part of Europe provide a too short period of overnight recovery of ozone damage between naturally occurring daytime ozone exposures (De Temmerman *et al*. 2002a).

A recent study showed that daylength can affect ozone sensitivity and the development of ozone-induced foliar injury in subterranean clover (*T. subterraneum*) (Vollsnes *et al*. 2009). The plants were given an identical ozone dose, but differed in the severity of foliar injury due to the different photoperiods given after ozone exposure. Long-day conditions characterized by a bright day and dim light during the night, similar to summers at high latitudes, increased foliar

injury and disrupted leaf temperature regulation and stomatal function (Vollsnes *et al*. 2009). Several studies suggest a role of the photoperiod in plant responses to oxidative stress (Foyer and Noctor 2009). In *Arabidopsis thaliana*, photoperiod was found to affect antioxidant status (Becker *et al*. 2006) as well as the response to photorespiratory H_2O_2 stress (Queval *et al*. 2007), with a short photoperiod inducing defense-related genes and a long photoperiod resulting in lesion development and the activation of programmed cell death (PCD). The foliar injuries induced by ozone stress resemble PCD and the hypersensitive response (HR) of plant-pathogen interactions (Zuccarini 2009). Photoreceptors, in particular phytochrome, appear to be involved in determining the outcome of the pathogenic response (Kangasjärvi *et al*. 2012).

The impact of daylength, CO_2 levels and H_2O_2 on plant function in different genotypes of *Arabidopsis thaliana* has been examined under short- and long-day conditions (Queval *et al*. 2012). CO_2 and H_2O_2 were found to interact with daylength and photoreceptor pathways, indicating close networking between carbon status, light and redox state in plant environmental responses. The daylength effect on ozone-induced leaf injuries in subterranean clover was recently shown to be related to phytochrome regulation and the active and inactive forms of phytochrome (P_{fr} and P_r, respectively) during the dark period (Eriksen *et al*. 2012). The severity of leaf injuries was photoreversible when given short night breaks of alternating red and far red light during the dark period. Red light given as the last night break resulted in long-day conditions and in significantly more severe injuries than a far red light night break, which maintained the long-night and short-day conditions (Eriksen *et al*. 2012).

One expected result of climate change is an expansion of species across latitudes to more northern areas (Saikkonen *et al*. 2012). Focus has been on threats to native biodiversity and economic losses caused by invading species. Less attention has been paid to photoperiodism and its important role in temperate and polar zones for phenological timing. Photoperiod provides a precise and consistent signal related to latitude and the fixed variation in daylength during the year (Körner and Basler 2010, Saik-

konen *et al*. 2012). Our recent studies have shown daylength regulation of ozone sensitivity for subterranean clover plants (Vollsnes *et al*. 2009). An important question is whether daylength regulation of ozone sensitivity also occurs in other plants or clover species.

In the present study, we examined the relationship between ozone and daylength in two cultivars each of red and white clover, to determine whether daylength-dependent ozone responses also occur in other clover species than subterranean clover. In Europe, subterranean clover is generally found in the Mediterranean region extending north to the UK and the Netherlands and can, as such, not be considered well-adapted to northern latitudes (Tutin *et al*. 1968). In the present paper, two commercial cultivars each of red clover and white clover adapted to the southern and northern parts of Fennoscandia were used. These clover species display the same ozone-induced visible foliar injury as subterranean clover (Futsaether *et al*. 2009, Pihl Karlsson *et al*. 1995a). The relationship between ozone injuries and daylength could therefore be studied in cultivars of the same species used in areas at different latitudes with different photoperiods. For comparison, the number of daylight hours (time from sunrise to sunset) is around 17, 20.5 and 24 hours for southern (~55°N), central (~63°N), and northern (> 66.56°N) Fennoscandia, respectively, for the four weeks around the summer solstice. We hypothesize that the detrimental effects of a long photoperiod on the ozone response will be more pronounced in cultivars commonly grown in southern Fennoscandian regions where summer photoperiods are shorter, as compared with northern cultivars adapted to longer photoperiods.

The red and white clover cultivars used as experimental plants in this study are important agricultural crop plants in Norway, used in the production of fodder and in pastures. The Norwegian climate and landscape are well-suited to milk and meat production. The yearly use of seed in Norway for these crop plants is 130 tonnes for red clover and 40 tonnes for white clover (J.A. Repstad, Head of Seed Products at Felleskjøpet, Norway, pers. comm.). Red and white clovers are also among the species belonging to the semi-natural vegetation in Fennoscandia. The annual subterranean clover was used as

a comparison in this study as it has been used as a model plant (Vollsnes *et al*. 2009, Eriksen *et al*. 2012) and has its natural habitat at lower latitudes with shorter photoperiods in the growth season. The ozone response was evaluated by assessing the severity of visible foliar injury and changes to leaf temperature regulation.

Material and methods

Plant material and cultivation

Commercial seeds of three species of clover were used: (1) red clover (*Trifolium pratense*) cv. Nordi (Leüthens Frø AS, Trondheim, Norway) and cv. Bjursele (Felleskjøpet, Holstad, Norway), (2) white clover (*T. repens*) cv. Milkanova (Leüthens Frø AS) and cv. Norstar (Leüthens Frø AS) and (3) subterranean clover (*T. subterraneum*, Svalöf Weibull AB, Svalöf, Sweden). The red and white clover cultivars were chosen based on their use as crop plants in Norway, with two cultivars grown in more southern climates in Norway (Nordi and Milkanova) and the other two in more northern climates (Bjursele and Norstar).

Seeds were germinated in trays containing sandy peat soil (Herbia Plantejord, Nordic Garden AS, Stokke, Norway) and kept in an environmentally controlled growth room at 20 ± 1 °C and > 60% relative humidity (RH). The light conditions followed a 16 h light (210 ± 20 μmol m^{-2} s^{-1} at plant height, provided by 400 W Kolorarc daylight lamps MBID/400/T/H, General Electric Company, Fairfield, CT, USA) and an 8 h dark cycle. Fourteen days after sowing, 24 uniformly developed seedlings of each cultivar were selected and transplanted individually into pots (upper diameter 12 cm, lower diameter 8.6 cm, height 8.5 cm; 12 C, OS pots, Rosti AS, Farum, Denmark) containing sandy peat soil (Herbia Plantejord). Ozone treatment started 17 days thereafter (i.e. 31 days after sowing) when all plants were in the vegetative state.

Ozone exposure

Plants were divided into four treatments in a factorial design with daylength [long day (LD)

or short day (SHD)] and ozone (ozone-free or ozone-supplemented air) as variables. For comparison purposes, the experiment followed the procedure used in our previous studies (Futsaether *et al.* 2009, Vollsnes *et al.* 2009, Eriksen *et al.* 2012). The experiment was designed such that factors other than photoperiod that can influence ozone injuries, e.g. different ozone dose, soil water content, air humidity and the CO_2 level in the air, did not affect the results. A total of 60 plants were arranged randomly in six (three replicates, with and without ozone) Perspex ozone exposure chambers ($l \times w \times h = 420$ mm $\times$ 320 mm $\times$ 400 mm) providing good mixing of air. The experiment was performed twice with different plants (60 each time), one week apart (two repetitions, called runs in the statistical analyses), giving 2×3 true replicates of each cultivar $\times$ treatment group. The plants were positioned in the predefined exposure chambers shortly before the ozone exposure started. The exposure chambers were kept in an environmentally controlled growth room (30 m^2) with diffuse daylight and an artificial light source (400 W Kolorarc daylight lamps) providing 240 ± 40 μmol m^{-2} s^{-1} at plant height, 19 ± 2°C and > 65% RH inside the chambers.

Plants were exposed to six hours of charcoal-filtered air (~1–3 ppb ozone) or charcoal-filtered air supplemented with ozone providing a 70 ± 10 ppb ozone level from 10:00 to 16:00 for three consecutive days. This ozone level is realistic for short-term ozone exposure observed during the growing season in northern Fennoscandia (Pihl Karlsson *et al.* 1995a, Pleijel *et al.* 2006, Manninen *et al.* 2009). Ozone was generated by passing pure oxygen through an ozone generator (model GSG 001.2, Sorbios GmbH, Berlin, Germany). Ozone concentrations inside the exposure chambers were logged continuously by an ozone analyzer and adjusted manually when needed (Photometric O_3 Analyzer, Model 400, Advanced Pollution Instrumentation Inc., San Diego, CA, USA).

Long and short day treatments during and after ozone exposure

To ensure that all plants had the same physi-ological status and morphology at the start of ozone exposure, daylength treatments were not commenced until after the first day of ozone exposure (31 days after sowing). During the three-day ozone exposure period, plants were moved to one of two separate environmentally controlled growth rooms (20 ± 1 °C, RH > 60%) after ozone exposure. One growth room provided long day conditions (LD) with ten hours bright light and 14 hours dim light and the other provided short day conditions (SHD) with ten hours bright light and 14 hours darkness. After the end of the ozone exposure period, the plants were kept in these rooms constantly. Daytime light (240 ± 20 μmol m^{-2} s^{-1}) was provided by 400 W Kolorarc daylight lamps. The dim night-time light given during LD treatment was provided by one fluorescent lamp (~1 μmol m^{-2} s^{-1} at plant height, LUMA, standard LRS, 65W/L53, Enkeltfärg VV, Karlskrona, Sweden) with an Red:Far-red (R:FR) ratio of 6.6 (Franklin 2008). This dim light exposure was chosen to trigger the phytochrome regulation observed to be involved in determining ozone injury severity in subterranean clover (Eriksen *et al.* 2012), while being too low to affect photosynthesis and biomass production. The high R:FR ratio ensured that phytochrome was predominantly in the active far-red absorbing conformation (P_{fr}). Plants were kept under SHD and LD conditions for seven nights starting the night after the first ozone exposure. The two daylength treatments included the same amount of high light for plant biomass production.

Assessment of ozone-induced visible injury

At harvest eight days after the start of ozone exposure, all trifoliate leaves were counted and given individual ozone injury scores from 0 to 4 as follows: 0, no visible ozone injury; 1, < 25%; 2, 25%–50%; 3, 50%–75% and 4, 75%–100% of the lamina injured. Visible leaf injuries were characterized as chlorotic and necrotic spots (*see* fig. 3 in Vollsnes *et al.* 2009). None of the control plants had visible ozone-induced leaf injuries, indicating that the background ozone level in the growth rooms was low. For each plant,

the number of visibly injured leaves was divided by the total number of leaves to calculate the fraction of injured leaves. The sum of the injury scores for all trifoliate leaves of a plant was calculated to give the injury score per plant. Finally, the score per injured leaf was calculated from the injury score per plant divided by the number of injured leaves for each plant.

Thermal imaging

Thermal imaging was used to determine leaf surface temperatures and hence provide an indirect measure of the effects of ozone on leaf transpiration and stomatal function (Jones 2004, Vollsnes *et al*. 2009). A ThermoVision™ A40M infrared camera (FLIR Systems, Danderyd, Sweden) with a 320×240 pixel resolution was used to take 1564 infrared (IR) images of the leaves. A maximum spatial resolution of 0.2×0.2 mm² (minimum absolute pixel size) was obtained using a 200 μm close-up lens. The IR camera was attached to a tripod and fixed 10 cm above the leaf. The leaf was held as horizontally as possible without unduly manipulating or disturbing the leaf to avoid influencing the leaf temperature measurements. A small, rectangular, black painted aluminum strip with known emissivity and coupled to a temperature sensor was used as a temperature reference in each image for determining the background temperature $\overline{T}_{background}$.

Leaf surface temperatures were recorded during as well as for five days after the ozone exposure period. Thermal imaging was performed in the mornings before ozone exposure, i.e. one hour after the light was turned on, and in the late afternoons after ozone exposure. After the last ozone exposure, plants were imaged during the morning and afternoon of days four and five, and during the mornings of days six, seven and eight after the start of ozone exposure. This was done to determine if ozone effects on leaf temperature were temporary or persisted even after terminating the ozone exposure. Prior to imaging, plants were watered by placing the bottom of the pots in water for at least ten minutes and then left to drain for at least ten minutes each morning to avoid closed stomata due to water shortage during ozone exposure and ther-

mal imaging. The second trifoliate leaf of each plant was imaged, as all plants had a minimum of two fully expanded trifoliate leaves at the start of ozone exposure.

Image processing

The temperature for each leaf lamina pixel i ($T_{leaf,i}$) was extracted from the infrared images as described by Vollsnes *et al*. (2009). Leaf emissivity was set to 0.95 (Jones 2004). The background temperature $\overline{T}_{background}$ for each leaf was subtracted from the leaf lamina temperature $T_{leaf,i}$ giving the background corrected temperature $\Delta T = T_{leaf,i} - \overline{T}_{background}$ for the ith leaf lamina pixel.

The effects of ozone and daylength on leaf temperature were determined by analyzing the frequency distribution of the background corrected temperature ΔT for all pixels of a leaf lamina. Ten feature variables of the ΔT leaf distribution were used to obtain a good description of the distribution (Gonzalez *et al*. 2004). The mean, the median, the minimum and the most common (mode) values of the background-corrected temperature distribution were calculated. The temperature range across the leaf surface was also calculated. The variance and skewness of the leaf temperature distribution captured the shape of the distribution. The degree of homogeneity and randomness of the leaf temperature distribution was described by the three variables uniformity, smoothness and entropy. These ten variables formed the multivariate leaf temperature data set.

Statistical analysis

The effects of daylength on the fraction of visibly ozone damaged leaves, the ozone injury scores per plant and the ozone score per injured leaf were analyzed by Generalized Linear Models in SPSS (IBM SPSS Statistics, ver. 20). The data were analyzed with two start models. The first model included the independent factors species (red clover, white clover and subterranean clover), daylength (levels LD and SHD), run (first and second week repetition) and all interactions between them. The second model included the independent factors cultivar (Bjursele, Nordi,

Norstar, Milkanova, subterranean clover), daylength, run and the interactions between them. Factors without a statistically significant effect (starting with the interactions) were considered irrelevant, and removed from the models.

Because the dependent factor "fraction of injured leaves" was calculated by dividing the number of visibly injured leaves with the number of leaves on the same plant, only the number of injured leaves was influenced by the ozone and daylength treatments. Therefore, the statistical analysis using the number of visibly injured leaves as dependent factor was conducted using Poisson's regression (a Generalized Linear Model (GLM) with a Poisson distribution, log-link, and the number of leaves on each plant as the offset). To avoid violation of the normality assumptions required by ANOVA, the "ozone score per plant" was square-root transformed and "ozone score per injured leaf" was squared. Four outliers with ozone score per injured leaf equal to zero (all in plants with SHD treatment) were omitted from the analyses. Bonferroni pairwise comparisons of marginal means were performed to establish whether the responses of cultivars or species differed significantly in the resulting models describing the variability in visible ozone injures among treatments.

For subterranean clover and each of the four cultivars (Nordi, Bjursele, Milkanova and Norstar), we generated data sets of multivariate leaf temperature measurements for both long day (LD) and short day (SHD) treatment along with controls for comparison. Each data set for each daylength treatment was based on six individual ozone exposed plants and six individual controls and considered in three versions to examine time trends in the data: (1) afternoons after ozone exposure (in total $n = 24$ measurements based on days 2 and 3, i.e. two measurements at different times on each plant), (2) mornings following exposure (in total $n = 36$ measurements based on days 2, 3 and 4, i.e. three measurements at different times on each plant), and (3) after the ozone exposure period (in total $n = 72$ measurements based on the afternoon of days 4 and 5, mornings of days 5–8, i.e. six measurements at different times on each plant).

The collection of 30 classification problems (effect of ozone on 5 species/cultivars × 2 daylength treatments × 3 periods of time) was analyzed separately by the pattern recognition technique Linear Discriminant Analysis (LDA) (Fisher 1936, Mardia *et al*. 1979). LDA was used to classify leaves (samples) as either controls or ozone exposed. For each of the 30 data sets, the associated fraction F_c of correctly classified samples (leaves) was computed by comparing the LDA computed classification to the ground-truth, whether samples (leaves) were actually controls or ozone exposed. If the LDA prediction and the ground-truth for a particular sample corresponded, then the LDA classification of that sample was considered to be correct. The statistical significance of the LDA classification was assessed as follows. By random permutation of the class labels (control and ozone exposed), the LDA modeling was repeated a large number K (= 10 000) of times resulting in corresponding (dummy) fractions F_1, ..., F_K of randomly correct classifications for each of the 30 data sets. For each data set, a significance value (p) was computed as the ratio L/K, where the number (L) corresponds to the number of times the genuine fraction F_c, ..., F_k for $r = 1$, ..., K (the dummy fractions).

Principal component analysis (PCA) (Jolliffe 2002) was used as a graphical framework to illustrate the effects of ozone and daylength on the leaf temperature ΔT distributions variables. In PCA, linear combinations of the many original variables are transformed into fewer orthogonal latent variables, i.e. principal components (PCs), to focus the dominant variance pattern of the data set. The multivariate leaf temperature data sets of each cultivar were analyzed separately. The relationships between leaves of different ozone and daylength treatment groups are visualized in score plots and clustering of similar observations is shown to visualize structures in the data. The relationship between the original leaf temperature distribution variables and the principal components are visualized in biplots. In the biplot, the original variables are plotted relative to the principal components. By comparing the location of the original temperature variable relative to the principal component axes, it is possible to assign meaning to the principal components. The clustering of the data (leaf samples) in the PCA score plots can therefore be

understood and interpreted in terms of the original temperature variables.

Multivariate statistical calculations were performed in MATLAB® (ver. 7.9, R2009b, The MathWorks, Inc., Natick, MA, USA) in combination with the MATLAB-compatible PLS-Toolbox® (ver. 5.0.3, Eigenvector Research Inc., Wenatchee, WA, USA).

Results

Ozone-induced visible foliar injury

Ozone-induced visible foliar injuries differed significantly between the three clover species (Tables 1 and 2). Red clover plants had a significantly smaller fraction of injured leaves than subterranean clover, as well as lower ozone scores per plant and ozone scores per injured leaf than white and subterranean clovers (Table 1). White clover had significantly lower ozone scores per plant than subterranean clover, but the fraction of injured leaves and the ozone score per

injured leaf (severity of the foliar injuries) did not differ significantly between these two species (Table 1). The ozone score per injured leaf was close to 3 for both species (Table 1), indicating that approximately 50% of the leaf surface of injured leaves had chlorotic and necrotic spots caused by ozone.

The effects of ozone on the two cultivars of the same species were similar (Fig. 1). There were no significant differences (Bonferroni-corrected $p > 0.05$) between the fraction of injured leaves or the ozone scores per plant of the two red clover cultivars. Likewise, there were no significant differences between the fraction of injured leaves and ozone score per injured leaf for the two white clover cultivars (Bonferroni-corrected $p > 0.05$). However, as Norstar plants had more leaves than Milkanova plants, the ozone score per plant was greater for Norstar than for Milkanova (Bonferroni-corrected $p < 0.001$).

Daylength influenced the development of ozone-induced visible foliar injuries measured as score per plant and score per injured leaf in all species and cultivars (Tables 2, 3 and 4, Fig. 1).

Table 1. The main effects of species on the fraction of ozone injured leaves, ozone injury scores per plant and per injured leaf for three clover species. For red and white clover, the results of the two cultivars were combined, giving $12 \times 2 = 24$ plants. Results are given as mean ± S.E. Different superscripts in the same row indicate significant differences between species. The Bonferroni significance levels for the pairwise comparisons of marginal means of each species pairs are given to the right (Rc = red clover, Wc = white clover, Sc = subterranean clover).

Main effects	Species			Bonferroni-corrected p		
	Red clover ($n = 24$)	White clover ($n = 24$)	Subterranean clover ($n = 12$)	Rc *vs.* Wc	Rc *vs.* Sc	Wc *vs.* Sc
Fraction injured leaves	0.27 ± 0.02[a]	0.37 ± 0.03[ab]	0.46 ± 0.03[b]	0.112	0.007	0.458
Score per plant	5 ± 1[a]	16 ± 2[b]	22 ± 2[c]	< 0.001	< 0.001	0.008
Score per injured leaf	1.8 ± 0.2[a]	2.8 ± 0.2[b]	2.9 ± 0.1[b]	< 0.001	< 0.001	0.817

Table 2. The results of Generalized Linear Models with a maximum of seven independent factors (species, daylength, run and the interactions between these three). Given are the p values for effects of species, daylength and run (repetition number) on the three dependent variables describing ozone-induced visible foliar injuries on the clovers studied. Only factors with $p < 0.05$ were kept in the models. For each analysis, $n = 60$ (fraction of injured leaves and ozone score per plant) or $n = 56$ (ozone score per injured leaf without outliers). ns = not significant ($p \geq 0.05$).

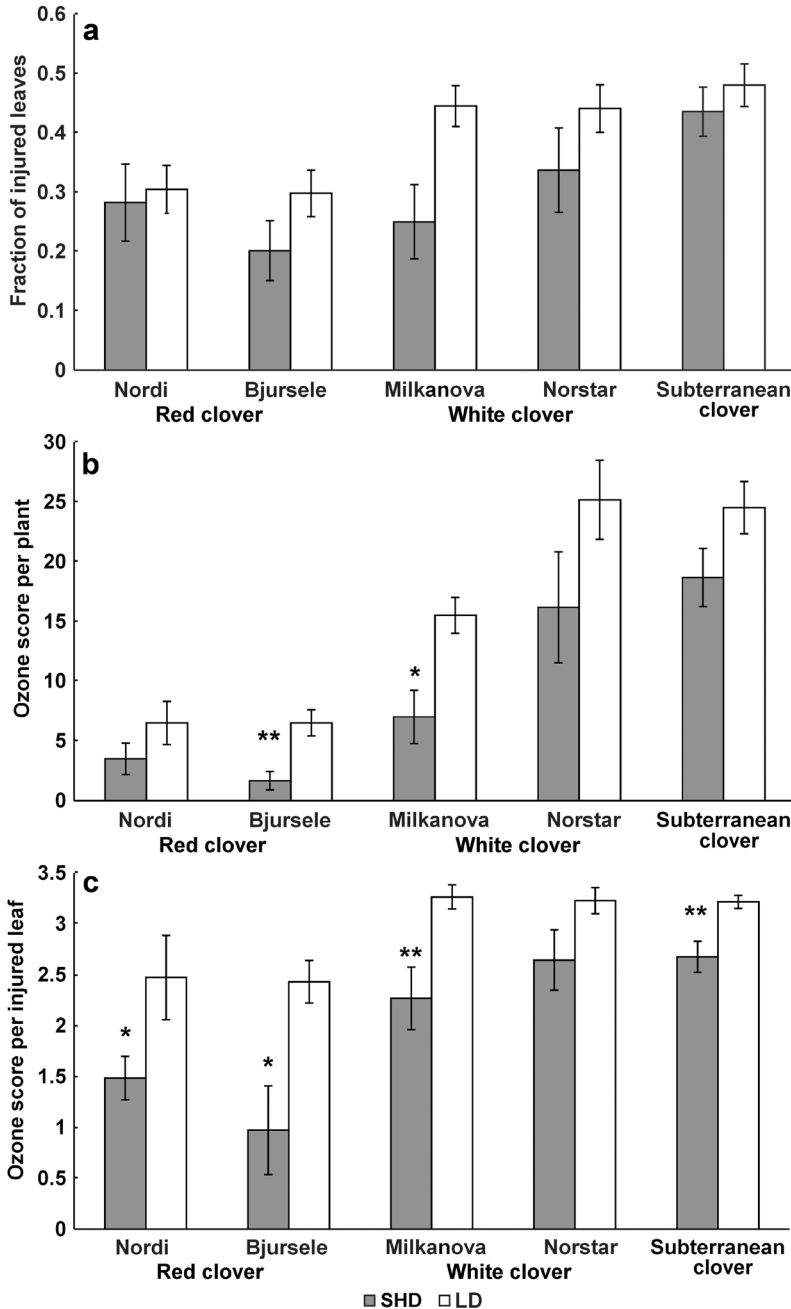|	Likelihood ratio χ^2	Effect of species	Effect of daylength	Effect of run	Effect of interactions
Fraction of injured leaves	16.0	0.010	ns	0.012	ns
Ozone score per plant	79.1	< 0.001	< 0.001	< 0.001	ns
Ozone score per injured leaf (without outliers)	41.0	< 0.001	< 0.001	ns	ns

Fig. 1. Ozone-induced visible foliar injury at harvest in the red clover cultivars Nordi and Bjursele, the white clover cultivars Milkanova and Norstar, and subterranean clover given either short day (SHD) or long day (LD) treatment. (**a**) The fraction of injured trifoliate leaves, (**b**) the ozone score per plant, and (**c**) the ozone score per injured leaf for the cultivar. Plants were exposed to ~70 ppb ozone for 6 h midday for three consecutive days and harvested five days after terminated exposure. Shown are mean ± S.E., n = 6. Significant difference between SHD and LD treatments (*t*-test performed on transformed data [*see* Statistical analysis]) are indicated as follows: * $p < 0.05$, ** $p < 0.01$.

There were no significant daylength × cultivar or daylength × species interactions in the Generalized Linear Models analyses, indicating that the cultivars and species had similar responses to daylength, namely that long days caused more ozone-induced visible injuries. Long days during and after ozone exposure resulted in significant 53% (score per plant) and 77% (score per injured leaf) increases in visible injuries relative to short days (Table 3). For both red clover cultivars, long-day treatment significantly increased the ozone score per injured leaf (Fig. 1c) relative to short-day treatment. The ozone score per plant increased only for the cultivar Bjursele (Fig.

1b). Likewise, long-day treatment significantly increased the ozone score per plant, and the ozone score per injured leaf for the southern white clover cultivar Milkanova (Fig. 1). For the northern cultivar Norstar, however, none of the ozone-induced visible foliar injury measures were significantly affected by daylength. In subterranean clover, injured leaves of long-day treated plants were significantly more severely injured than short-day treated plants (Fig. 1c), as reported in a previous study (Vollsnes *et al.* 2009).

Ozone-induced leaf temperature changes

The effects of ozone on the leaf temperature distribution varied between species and cultivars (Table 5). The two red clover cultivars displayed the same leaf temperature response to ozone. The response was dependent on the daylength treatment, as also observed for visible injury development (Fig. 1). Under short-day conditions, leaf temperature distributions of ozone

exposed and control leaves were not significantly different during as well as after the exposure period (Table 5), indicating that ozone exposure did not affect leaf temperature. Under long-day conditions, however, leaf temperatures of ozone exposed and control leaves were significantly different in the afternoons after exposure, but not in the mornings following exposure. Thus, leaf temperature was restored to the level of control leaves after an ozone-free night. There was no significant difference between exposed and control leaves after the exposure period, indicating recovery of leaf temperature to the level of the controls.

The two white clover cultivars displayed different sensitivities with regard to ozone-induced temperature changes. In Milkanova, leaf temperatures of ozone exposed and control leaves were significantly different in the afternoon shortly after exposure but not in the mornings following exposure (Table 5). This indicates that leaf temperature was altered by ozone but was restored after an ozone-free night. After the exposure period, there was no significant difference between exposed and control leaves, indicating

Table 3. The main effects of daylength on the fraction of ozone injured leaves, ozone injury scores per plant and per injured leaf in clover plants. Comparisons of means (*t*-test) of long day and short day treated plants were performed on transformed data (*see* Statistical analysis) to obtain normal distributions.

	Long day $n = 30$ (mean ± SE)	Short day $n = 30$ (mean ± SE)	*p*
Fraction of injured leaves	0.38 ± 0.02	0.30 ± 0.03	na*
Ozone score per plant	16 ± 2	9 ± 2	0.004
Ozone score per injured leaf	2.9 ± 0.1	1.9 ± 0.2	0.00001

* not applicable, data were not normally distributed and there was no effect of daylength in the Generalized Linear Model analysis.

Table 4. The results of Generalized Linear Models with a maximum of seven independent factors (cultivar, daylength, run and the interactions between these three). Given are the *p* values for the effects of cultivar, daylength and run (repetition number) on the three dependent variables describing ozone-induced visible foliar injuries on the clovers studied. Only factors with $p < 0.05$ were kept in the models. For each analysis, $n = 60$ (fraction of injured leaves and ozone score per plant) or $n = 56$ (ozone score per injured leaf without outliers). ns = not significant ($p \geq 0.05$).

	Likelihood ratio χ^2	Effect of cultivar	Effect of daylength	Effect of run	Effect of interactions
Fraction of injured leaves	16.4	0.048	ns	0.013	ns
Ozone score per plant	106.8	< 0.001	< 0.001	< 0.001	0.022 (cul × r)*
Ozone score per injured leaf (without outliers)	041.5	< 0.001	< 0.001	ns	ns

* cul × r = cultivar × run interaction.

that the effect of ozone on leaf temperature was temporary. These results were not affected by the daylength treatment. In Norstar, however, leaf temperatures of exposed and control leaves were significantly different in the afternoons after exposure, the mornings following exposure as well as for five days after exposure was terminated for both daylength treatments (Table 5), reflecting the high sensitivity of this cultivar. Leaf temperature regulation was perturbed by ozone and was not restored to the level of control leaves even after ozone exposure was terminated. This is also illustrated in the score plot of the principal component analysis (Fig. 2). The PCA scores of ozone exposed and control leaves formed separate clusters during ozone exposure (Fig. 2a and c) as well as after the exposure period (Fig. 2b and d).

Leaf temperature regulation of subterranean clover was also very sensitive to ozone exposure, which agrees with previous observations (Vollsnes *et al.* 2009). The temperature distributions of ozone exposed and control leaves were significantly different during as well as after ozone exposure was terminated for both daylength treatments (Table 5), indicating that ozone altered leaf temperature and that the recovery of leaf temperature to the level of the controls did not occur even after exposure was terminated.

By comparing the PCA score plots (Fig. 2a–d) with the biplot (Fig. 2e), it is possible to compare the scores of Norstar leaves with the original temperature distribution variables. The score clusters of exposed leaves were shifted in the direction of the first quadrant relative to the control leaf clusters (Fig. 2a–d). This quadrant is associated with temperature distributions that are less skewed (shorter tail to the right) with higher values of the mean, median, mode and minimum temperature (Fig. 2e). Thus, exposed leaves had warmer temperature distributions than control leaves which had cooler and right-skewed temperature distributions. This pattern was the same for all cultivars in which ozone affected leaf temperature (Fig. 3). Since leaf temperature increases with decreasing transpiration (Kümmerlen *et al.* 1999, Prytz *et al.* 2003, Jones 2004), the warmer leaf temperature distributions of exposed leaves indicate that leaf transpiration was overall lower in exposed relative to control leaves.

Discussion

The ozone sensitivity of the species used in the present study was previously ranked as *T. subterraneum* > *T. repens* > *T. pratense* in studies using moderate ozone concentrations (~30–70 ppb) (Pihl Karlsson *et al.* 1995a, Futsaether *et al.* 2009). The results of the present study support this ranking. Subterranean clover leaves showed the most severe visible foliar injury and leaf temperature disruptions that persisted for several days after exposure was terminated. The white clover cultivars, in particular Norstar, showed a

Table 5. Probability values (*p*) for the differences between the leaf temperature distributions of ozone exposed and control leaves, determined using LDA and repeated random permutations of class labels. Exposed and control leaf temperatures were compared in the late afternoons after ozone exposure (days 2 and 3, *n* = 24), the mornings following exposure (mornings of days 2, 3 and 4, *n* = 36) and after the ozone exposure period (afternoon of day 4, morning and afternoon of day 5 and morning of the days 6–8, *n* = 72). Plants were exposed to ~70 ppb ozone for 6 h midday on days one, two and three and given either short (SHD) or long day (LD) treatment. ns = not significant (*p* ≥ 0.05).

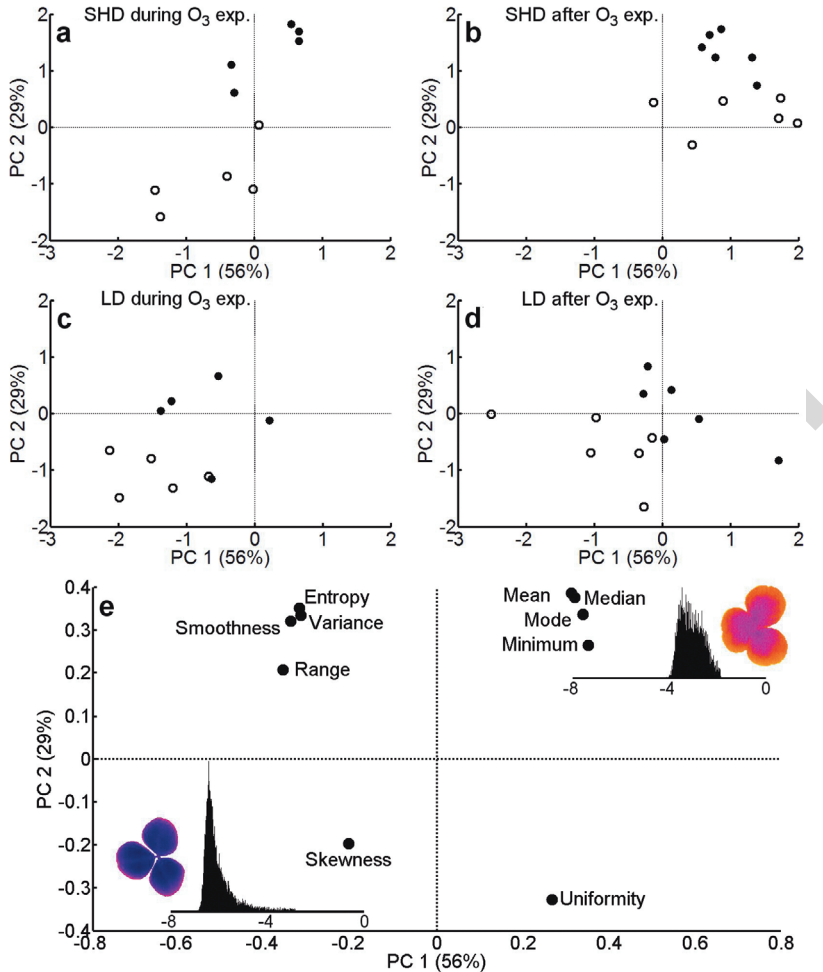| Species	Cultivar	Afternoons after exp.		Mornings following exp.		Period after exp.	
		LD	SHD	LD	SHD	LD	SHD
Red clover	Nordi	0.045	ns	ns	ns	ns	ns
	Bjursele	0.0004	ns	ns	ns	ns	ns
White clover	Milkanova	0.045	0.038	ns	ns	ns	ns
	Norstar	0.002	0.049	0.01	0.0006	0.002	0.0003
Sub. clover		0.0001	< 0.0001	0.0001	0.0001	< 0.0001	< 0.0001

Fig. 2. Principal component analysis (PCA) of the temperature distributions of control (circles) and ozone-exposed (black dots) leaves of the northern white clover cultivar Norstar. Plants were exposed to ~70 ppb ozone for 6 h midday on days 1–3 and given either short (SHD) or long day (LD) treatment. (**a–d**) The mean PCA scores ($n = 6$ leaves) for each treatment group: (**a** and **c**) the mean PCA scores during the ozone exposure period (days 2 and 3 and the morning of day 4) for short day (SHD) and long day (LD) conditions, respectively; (**b** and **d**) the mean PCA scores after the ozone exposure period (afternoon of day 4 and days 5–8) for short day (SHD) and long day (LD) conditions, respectively. Principal components PC1 and PC2 accounted for 56% and 29% of the variance in the data set, respectively. (**e**) The relationship between original leaf temperature distribution variables and the principal components. Thermal images of representative leaves (orange = warm, purple = cold, *see* color bar in Fig. 3) and the corresponding frequency histograms of the leaf temperature distributions are also shown. The scale gives the leaf temperature below the ambient background temperature ($\Delta T = T_{leaf} - \overline{T}_{background}$) such that –4 implies a leaf temperature 4 °C lower than the ambient temperature.

higher degree of foliar injury and temperature disruptions than the red clover cultivars.

Recent studies on subterranean clover indicate that daylength can affect plant responses to oxidative stress caused by ozone, increasing foliar injury under long day conditions, and regulated through the photoreceptor phytochrome (Vollsnes

et al. 2009, Eriksen *et al.* 2012). This study examined whether an ozone-daylength effect is found in other clover species and cultivars, as well as if such an effect is more pronounced in cultivars used at more southern latitudes, where summer photoperiods are shorter, as compared with northern cultivars adapted to longer photoperiods.
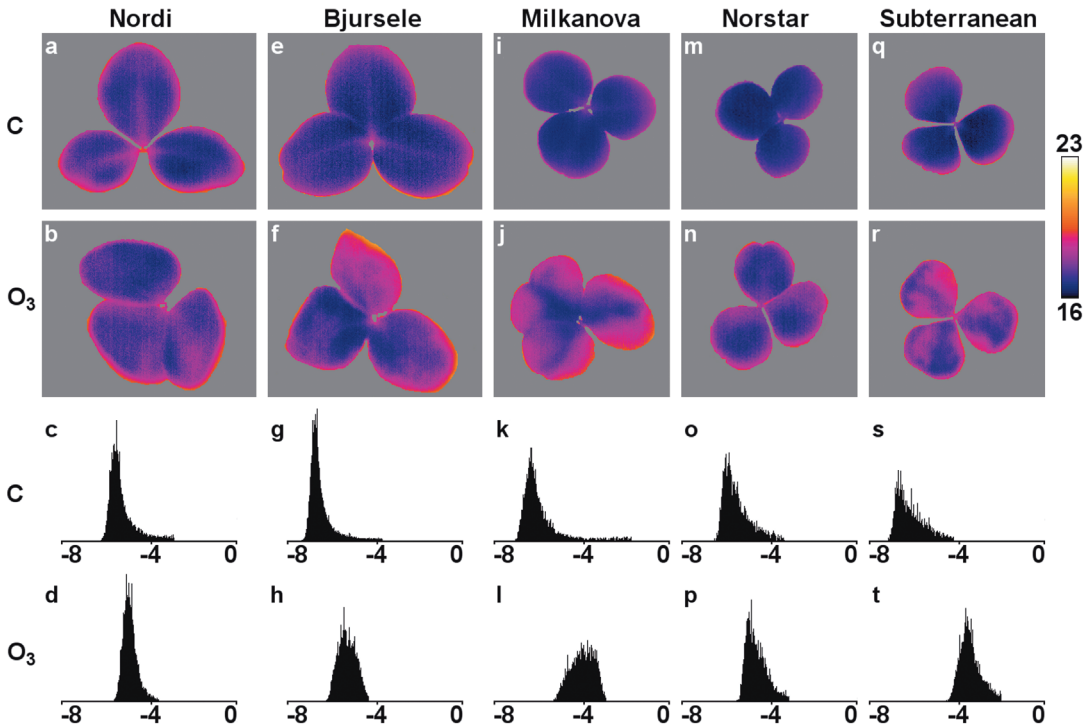
Fig. 3. Thermal images taken in the late afternoon shortly after ozone exposure of control (C) and ozone exposed (O₃) leaves of plants under the long-day treatment (rows one and two). The color bar gives the temperature scale (°C). The corresponding relative frequency histograms of the leaf temperature distributions are shown in rows three and four. The scale gives the leaf temperature below the ambient background temperature ($\Delta T = T_{leaf} - \overline{T}_{background}$) such that –4 implies a leaf temperature 4 °C lower than the ambient temperature. The columns show second trifoliate leaves and corresponding histograms of the red clover cultivars Nordi (**a–d**) and Bjursele (**e–h**), the white clover cultivars Milkanova (**i–l**) and Norstar (**m–p**), and subterranean clover (**q–t**), respectively.

In concurrence with our earlier studies on subterranean clover (Vollsnes *et al*. 2009, Eriksen *et al*. 2012), in comparison with short days, long days increased the ozone-induced visible injuries in the clovers studied in the present experiment. No significant interactions between daylength and cultivars or species were found, implying that the daylength response of the species and cultivars did not differ significantly. Further, we found that daylength affected visible foliar injury severity and leaf temperature regulation in both red clover cultivars despite the differences in latitudes where they are commonly grown. Thus, the intensified effects of a long photoperiod on ozone damage were not restricted to species or cultivars used at central to southern Fennoscandian latitudes but were also found in species and cultivars bred for northern climatic conditions. This is contrary to

the hypothesis of a more pronounced daylength effect in southern species and cultivars. Thus, a daylength-dependent response to ozone may be quite general and not restricted to species or cultivars originating from southern locations.

Ozone sensitivity depends on factors related to plant status such as constituent and inductive defense capacities, repair processes, plant development stage and stomatal ozone uptake, as well as external factors such as climate and external ozone exposure (Musselman *et al*. 2006, Scebba *et al*. 2006, Bassin *et al*. 2007). In this study, external ozone exposure, climate (apart from daylength) and plant development stage were the same and could not be the cause of the observed variations in ozone sensitivity. Constitutive defense capacities can vary between clover cultivars and species (Scebba *et al*. 2006) and may account for some of the differences in ozone sen-

sitivity observed between species and cultivars in this study. The less-sensitive red clover cultivars may have had higher constitutive defense levels than the more ozone-sensitive subterranean clover and white clover cultivars. As the daylength treatment commenced after the first ozone exposure, constitutive defense capacity could not be affected by the daylength treatment. Rather, it is more likely that the inductive response activated after ozone exposure was affected by the daylength treatment. It has recently been shown that a long period of growth under either a long or a short photoperiod resulted in different levels of constitutive defense against oxidative stress in *Arabidopsis thaliana* (Queval *et al*. 2007). This study of *Arabidopsis cat2* mutants established that daylength can determine the response to H_2O_2 produced by photorespiration; under short-day conditions, glutathione and defense genes were strongly up-regulated, whereas under long day conditions, H_2O_2 led to cell death. It appears that photoperiod signaling determines whether the result will be cell death or defense and stress resistance (Queval *et al*. 2007, Foyer and Noctor 2009, Queval *et al*. 2012). In *Arabidopsis*, long photoperiods result in lower induced defense against oxidative stress as compared with short photoperiods. Likewise, all examined clover species in our studies (Futsaether *et al*. 2009, Vollsnes *et al*. 2009, Eriksen *et al*. 2012) were more severely injured by ozone under long- rather than short-day conditions, suggesting that one of the effects of a long photoperiod may be a weakened induced defense capacity. Thus, photoperiod appears to play an important role in determining the outcome of oxidative stress caused by ozone.

This study demonstrates the importance of photoperiodism for the negative effect of ozone. In the future, Fennoscandia is expected to be subjected to higher concentrations of tropospheric ozone as well as warmer climate (Meleux *et al*. 2007, Fuhrer 2009). The distribution limits of many natural and semi-natural plant species are expected to shift towards higher latitudes, with a longer photoperiod during the growth season (Saikkonen *et al*. 2012). Agricultural practices are also expected to change, such that cultivars and species may be used at higher latitudes than at present. The importance of photoperiod for the negative effects of ozone demonstrated in the present study may also apply to other natural, semi-natural and agricultural plant species, causing unexpectedly severe reductions in crop yield and quality, as well as damaging effects on natural vegetation. More knowledge is needed on the effect of photoperiod on plant ozone responses. This information may be useful for calculating the negative effects of ozone on agriculture (i.e. in the UNECE Convention on Long-range Transboundary Air Pollution) as well as in breeding for agricultural and forestry plant cultivars to be used at higher latitudes.

Stomatal closure and reduced stomatal conductance due to ozone occurs in many but not all species and appears to be coupled to inhibition of carbon assimilation and possibly direct effects on stomata (Paoletti and Grulke 2005, Grulke *et al*. 2007, Wittig *et al*. 2007). Ozone exposure can also result in sluggish stomatal responses, characterized by slower stomatal closing and opening and failure to reach pre-injury stomatal conductance levels (Paoletti 2005, Paoletti and Grulke 2005), possibly due to altered guard cell ion transport and impaired stomatal function (Torsethaugen *et al*. 1999, Paoletti and Grulke 2005). In this study, exposed leaves of all plants, except for the red clover cultivars given the short day treatment, were warmer after ozone exposure, indicating an ozone-induced reduction of transpiration. This is in agreement with reduced stomatal conductance observed in other studies. An ozone-free night was sufficient for the recovery of stomatal function in the less ozone sensitive cultivars. However, in the more sensitive subterranean clover and white clover cultivar Norstar, overnight recovery of stomatal function did not occur, implying impaired stomatal opening and failure to reach pre-exposure transpiration levels. Indeed, in these plants, leaf temperature disruptions persisted for several days after exposure was terminated, implying more long-lasting reductions in stomatal function.

Conclusions

Long-day conditions increased leaf ozone injury relative to short-day conditions in three clover species, indicating that an ozone-daylength

effect was not restricted to a particular clover species. Daylength-dependent ozone responses occurred in cultivars commonly used in southern as well as in northern Fennoscandia, implying that also plants adapted to northern photoperiods can be adversely affected by a long photoperiod. This study should thus inspire more studies of the link between photoperiod and the outcome of oxidative stress, knowledge that is particularly valuable to the future of agriculture and forestry at high latitudes.

References

Ashmore M.R. 2005. Assessing the future global impacts of ozone on vegetation. *Plant Cell Environ*. 28: 949–964.

Bassin S., Volk M. & Fuhrer J. 2007. Factors affecting the ozone sensitivity of temperate European grasslands: an overview. *Environ. Pollut*. 146: 678–691.

Becker B., Holtgrefe S., Jung S., Wunrau C., Kandlbinder A., Baier M., Dietz K.-J., Backhausen J.E. & Scheibe R. 2006. Influence of the photoperiod on redox regulation and stress responses in *Arabidopsis thaliana* L. (Heynh.) plants under long- and short-day conditions. *Planta* 224: 380–393.

Bermejo V., Gimeno B.S., Sanz J., de la Torre D. & Gil J.M. 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. *Atmos. Environ*. 37: 4667–4677.

De Temmerman L., Vandermeiren K., D'Haese D., Bortier K., Asard H. & Ceulemans R. 2002a. Ozone effects on trees, where uptake and detoxification meet. *Dendrobiology* 47: 9–19.

De Temmerman L., Pihl Karlsson G., Donnelly A., Ojanperä K., Jäger H.J., Finnan J. & Ball G. 2002b. Factors influencing visible ozone injury on potato including the interaction with carbon dioxide. *Eur. J. Agron*. 17: 291–302.

Eriksen A.B., Vollsnes A.V., Futsaether C.M. & Kruse O.M. 2012. Reversible phytochrome regulation influenced the severity of ozone-induced visible foliar injuries in *Trifolium subterraneum* L. *Plant Growth Regul*. 68: 517–523.

Fisher R.A. 1936. The use of multiple measurements in taxonomic problems. *Ann. Eugenic*. 7: 179–188.

Foyer C.H. & Noctor G. 2009. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid. Redox Signaling* 11: 861–905.

Franklin K.A. 2008. Shade avoidance. *New Phytol*. 179: 930–944.

Fuhrer J. 2009. Ozone risk for crops and pastures in present

and future climates. *Naturwissenschaften* 96: 173–194.

Futsaether C.M., Vollsnes A.V., Kruse O.M., Otterholt E., Kvaal K. & Eriksen A.B. 2009. Effects of the Nordic photoperiod on ozone sensitivity and repair in different clover species studied using infrared imaging. *Ambio* 38: 437–443.

Gimeno B.S., Bermejo V., Sanz J., de la Torre D. & Elvira S. 2004a. Growth response to ozone of annual species from Mediterranean pastures. *Environ. Pollut*. 132: 297–306.

Gimeno B.S., Bermejo V., Sanz J., de la Torre D. & Gil J.M. 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. *Atmos. Environ*. 38: 2295–2303.

Gonzalez R.C., Woods R.E. & Eddins S.L. 2004. *Digital image processing using MATLAB*. Pearson Education Inc., New Jersey.

Grulke N.E., Paoletti E. & Heath R.L. 2007. Comparison of calculated and measured foliar O_3 flux in crop and forest species. *Environ. Pollut*. 146: 640–647.

Hayes F., Jones M.L.M., Mills G. & Ashmore M. 2007. Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. *Environ. Pollut*. 146: 754–762.

Jolliffe I.T. 2002. *Principal component analysis*, 2nd ed. Springer-Verlag, Berlin.

Jones H.G. 2004. Application of thermal imaging and infrared sensing in plant physiology and ecophysiology. *Adv. Bot. Res*. 41: 107–163.

Kangasjärvi S., Neukermans J., Li S., Aro E.-M. & Noctor G. 2012. Photosynthesis, photorespiration, and light signalling in defence responses. *J. Exp. Bot*. 63: 1619–1636.

Karlsson P.E., Pleijel H. & Simpson D. 2009a. Ozone exposure and impacts on vegetation in Nordic and Baltic countries. *Ambio* 38: 402–405.

Karlsson P.E., Pleijel H., Danielsson H., Pihl Karlsson G., Piikki K. & Uddling J. 2009b. Evidence for impacts of near-ambient ozone concentrations on vegetation in southern Sweden. *Ambio* 38: 425–431.

Körner C. & Basler D. 2010. Phenology under global warming. *Science* 327: 1461–1462.

Kümmerlen B., Dauwe S., Schmundt D. & Schurr U. 1999. Thermography to measure water relations of plant leaves. In: Jähne B., Haußecker H. & Geißler P. (eds.), *Handbook of computer vision and applications*, vol. 3, Systems and Applications. Academic Press, London, UK, pp. 763–781.

Manninen S., Huttunen S., Tømmervik H., Hole L.R. & Solberg S. 2009. Northern plants and ozone. *Ambio* 38: 406–412.

Mardia K.V., Kent J.K. & Bibby J.M.1979. *Multivariate analysis*. Academic Press, London.

Meleux F., Solmon F. & Giorgi F. 2007. Increase in summer European ozone amounts due to climate change. *Atmos. Environ*. 41: 247–264.

Musselman R.C., Lefohn A.S., Massman W.J. & Heath R.L. 2006. A critical review and analysis of the use of exposure- and flux-based ozone indices for predicting vegetation effects. *Atmos. Environ*. 40: 1869–1888.

Paoletti E. 2005. Ozone slows stomatal response to light and

leaf wounding in a Mediterranean evergreen broadleaf, *Arbutus Unedo*. *Environ*. *Pollut*.134: 439–445.

Paoletti E. & Grulke N.E. 2005. Does living in elevated CO_2 ameliorate tree response to ozone? A review on stomatal responses. *Environ*. *Pollut*. 137: 483–493.

Pihl Karlsson G., Sellden G., Skärby L. & Pleijel H. 1995a. Clover as an indicator plant for phytotoxic ozone concentrations: visible injury in relation to species, leaf age and exposure dynamics. *New Phytol*. 129: 355–365.

Pihl Karlsson G., Karlsson P.E., Danielsson H. & Pleijel H. 2003. Clover as a tool for bioindication of phytotoxic ozone — 5 years of experience from southern Sweden — consequences for the short-term critical levels. *Sci*. *Total Environ*. 301: 205–213.

Pihl Karlsson G., Karlsson P.E., Soja G., Vandermeiren K. & Pleijel H. 2004. Test of the short-term critical levels for acute ozone injury on plants — improvements by ozone uptake modelling and the use of an effect threshold. *Atmos*. *Environ*. 38: 2237–2245.

Pihl Karlsson G., Pleijel H., Sild E., Danielsson H., Selldèn G., Ericson L. & Skärby L. 1995b. Clover Sweden — a national three year study of the effects of tropospheric ozone on *Trifolium subterraneum* L. *Water Air Soil Pollut*. 85: 1503–1508.

Pleijel H., Berglen Eriksen A., Danielsson H., Bondesson N. & Selldén G. 2006. Differential ozone sensitivity in an old and an modern Swedish wheat cultivar – grain yield and quality, leaf chlorophyll and stomatal conductance. flux response relationship for wheat. *Environ*. *Exp*. *Bot*. 56: 63–71.

Pleijel H., Danielsson H., Pihl Karlsson G., Gelang J., Karlsson P.E. & Selldén G. 2000. An ozone flux response relationship for wheat. *Environ*. *Pollut*. 109: 453–462.

Prytz G., Futsaether C.M. & Johnsson A. 2003. Thermography studies of the spatial and temporal variability in stomatal conductance of *Avena* leaves during stable and oscillatory transpiration. *New Phytol*. 158: 249–258.

Queval G., Neukermans J., Vanderauwera S., Van Breusegem F. & Noctor G. 2012. Day length is a key regulator of transcriptomic responses to both CO_2 and H_2O_2 in *Arabidopsis*. *Plant Cell Environ*. 35: 374–387.

Queval G., Issakidis-Bourguet E., Hoeberichts F.A., Vandorpe M., Gakière B., Vanacker H., Miginiac-Maslow M., Van Breusegem F. & Noctor G. 2007. Conditional oxidative stress responses in the *Arabidopsis* photorespiratory mutant *cat2* demonstrate that redox state is a key modulator of daylength-dependent gene expression, and define photoperiod as a crucial factor in the regulation of H_2O_2-induced cell death. *Plant J*. 52: 640–657.

Saikkonen K., Taulavuori K., Hyvönen T., Gundel P.E., Hamilton C.E., Vänninen I., Nissinen A. & Helander M. 2012. Climate change-driven species' range shifts filtered by photoperiodism. *Nature Clim*. *Change* 2: 239–242.

Scebba F., Giuntini D., Castagna A., Soldatini G. & Ranieri A. 2006. Analysing the impact of ozone on biochemical and physiological variables in plant species belonging to natural ecosystems. *Environ*. *Exp*. *Bot*. 57: 89–97.

Torsethaugen G., Pell E.J. & Assman S.M. 1999. Ozone inhibits guard cell K^+ channels implicated in stomatal opening. *PNAS* 96: 13577–13582.

Tutin T.G., Heywood V.H., Burges N.A., Moore D.M., Valentine D.H., Walters S.M. & Webb D.A.1968. *Flora Europaea*, vol. 2. Cambridge University Press, Cambridge.

Vollsnes A.V., Eriksen A.B., Otterholt E., Kvaal K., Oxaal U. & Futsaether C.M. 2009. Visible foliar injury and infrared imaging show that daylength affects short-term recovery after ozone stress in *Trifolium subterraneum*. *J*. *Exp*. *Bot*. 60: 3677–3686.

Wittig V.E., Ainsworth E.A. & Long S.P. 2007. To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance in trees? A meta-analytic review of the last 3 decades of experiments. *Plant Cell Environ*. 30: 1150–1162.

Zuccarini P. 2009. Tropospheric ozone as a fungal elicitor. *J*. *Biosci*. 34: 125–138.