Visible foliar injury and infrared imaging show that daylength affects short-term recovery after ozone stress in *Trifolium subterraneum*

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Abstract

Tropospheric ozone is a major air pollutant affecting plants worldwide. Plants in northern regions can display more ozone injury than plants at lower latitudes despite lower ozone levels. Larger ozone influx and shorter nights have been suggested as possible causes. However, the effects of the dim light present during northern summer nights have not been investigated. Young *Trifolium subterraneum* plants kept in environmentally controlled growth rooms under long day (10 h bright light, 14 h dim light) or short day (10 h bright light, 14 h darkness) conditions were exposed to 6 h of 70 ppb ozone during daytime for three consecutive days. Leaves were visually inspected and imaged *in vivo* using thermal imaging before and after the daily exposure. In long-day-treated plants, visible foliar injury within 1 week after exposure was more severe. Multivariate statistical analyses showed that the leaves of ozone-exposed long-day-treated plants were also warmer with more homogeneous temperature distributions than exposed short day and control plants, suggesting reduced transpiration. Temperature disruptions were not restricted to areas displaying visible damage and occurred even in leaves with only slight visible injury. Ozone did not affect the leaf temperature of short-day-treated plants. As all factors influencing ozone influx were the same for long- and short-day-treated plants, only the dim nocturnal light could account for the different ozone sensitivities. Thus, the twilight summer nights at high latitudes may have a negative effect on repair and defence processes activated after ozone exposure, thereby enhancing sensitivity.

Key words: Daylength, leaf temperature, ozone, principal component analysis, thermal imaging, thermography, transpiration, *Trifolium*, visible foliar injury.

Introduction

Tropospheric ozone is one of the major air pollutants worldwide reducing crop productivity as well as the growth and diversity of natural vegetation (Krupa *et al.*, 2001; Ashmore, 2005). During the last century, ambient ozone levels have more than doubled and are predicted to increase even faster for the next half century (Stevenson *et al.*, 2000). Ground level ozone was once regarded as a regional problem but is now recognized as a global air pollution problem as it or its precursors can be transported from urban to rural regions, even intercontinentally, thereby increasing areas where vegetation is considered to be at risk (Collins *et al.*, 2000; Derwent *et al.*, 2004).

The effects of ozone on plants are often seen as reductions in photosynthesis, plant growth, and yield, as well as premature senescence and visible foliar symptoms such as chlorotic flecking, necrosis, and bronzing (Pell *et al.*, 1997; Skårby *et al.*, 1998; Benton *et al.*, 2000; Krupa *et al.*, 2001). Stomata are the primary sites of ozone uptake and regulation of ozone flux into the intercellular space (Long and Naidu, 2002). Reduced transpiration and stomatal conductance have been observed after ozone exposure and may represent an ozone avoidance mechanism (Andersen, 2003; Black *et al.*, 2007). Once inside the leaf, ozone generates reactive oxygen species, causing oxidative tissue...
damage and triggering oxidative signalling (Pell et al., 1997; Kangasjärvi et al., 2005).

Negative effects of ozone on crops, semi-natural and natural vegetation are found in northern Europe even though summer daytime levels of ozone are much lower than in southern and central Europe (Pleijel et al., 2000; De Temmerman et al., 2002a; Timonen et al., 2004). For example, a European field study found that Solanum tuberosum developed visible foliar injury at lower ozone levels in Scandinavia than in central Europe (De Temmerman et al., 2002a). Two hypotheses have been suggested to date to account for the higher ozone sensitivity at northern latitudes. Both hypotheses relate to differences in summer climatic conditions between northern and southern Europe. The northern growing season is characterized by long summer days, short nights, and often a wet climate, with moderate temperatures providing favourable conditions for stomatal opening. Thus, stomata are often open for a greater number of hours per day. The first hypothesis therefore suggests that the ozone uptake potential is larger at higher latitudes (Pleijel et al., 2000). The second hypothesis, suggested on the basis of an artificial neuronal network analysis of ozone-induced visible foliar injuries on potato plants across Europe, is that the summer nights at high latitudes are too short to allow for repair and recovery from oxidative stress resulting in chronic ozone injury (De Temmerman et al., 2002a, b). A third characteristic of summers at high latitudes is that the summer nights are never completely dark. Given that a long photoperiod has recently been found to increase leaf lesion severity caused by oxidative stress (Queval et al., 2007), it is possible that northern summer night light conditions may affect ozone sensitivity. However, this third hypothesis has not been investigated.

Thermal imaging has successfully been used to visualize non-destructively the in vivo foliar stress responses before the appearance of visible symptoms (Chaerle and Van Der Straeten, 2001). In thermal imaging, the leaf’s emitted infrared radiation (8–14 µm) is detected and transformed into leaf temperature. As leaf temperature is regulated by transpiration, infrared imaging has been used to study temporal and spatial patterns of transpiration occurring across a leaf surface (Kümmelen et al., 1999; Chaerle and Van Der Straeten, 2000, 2001; Prytz et al., 2003; Jones, 2004). Using thermal imaging, plant–pathogen and plant–herbicide interactions were found to induce localized hot-spots or cool-spots on leaves prior to the appearance of visible injury. These spots were suggested to reflect stomatal closure or cell death (Chaerle et al., 2001, 2003, 2004; Lindenthal et al., 2005).

The primary objective of the current study was to investigate the third hypothesis for increased ozone sensitivity at high latitudes, namely that dim light during night-time (long day conditions) can affect plant ozone sensitivity. The secondary objective was to determine whether thermal imaging can be used to detect, characterize, and monitor early in vivo ozone-induced foliar injury, as has been possible in studies of plant–pathogen and plant–herbicide interactions. Three separate experiments were conducted to determine whether: (i) dim night-time light had any effect at all on ozone sensitivity as assessed by visible leaf injuries; (ii) dim night-time light and the order of leaf emergence had any effect on leaf injury severity; and (iii) thermal imaging was suitable for characterizing in vivo ozone-induced foliar injury. Plants of different ages were used to determine whether a dim night-time light effect on ozone sensitivity occurred consistently regardless of plant age. The clover species Trifolium subterraneum L. was investigated as it is very ozone sensitive, developing characteristic necrotic spots, is used as an ozone bioindicator, and has been used as an experimental plant within the United Nations Economic Commission for Europe International Co-operative Programme (UNECE ICP Crops) (Pihl Karlsson et al., 1995a, b; Sild et al., 1999; Bermejo et al., 2003; Gimeno et al., 2004a, b).

**Materials and methods**

**Plant material and cultivation prior to ozone treatment**

Experiment I: daylength effects on visible leaf injury: Ten Trifolium subterraneum L. (subterranean clover, Svalöf Weibull AB, Svalöf, Sweden) seeds were cultivated per pot (12 C, 650 ml, OS Plastic A/S, Denmark) in a 10:1 (v/v) mixture of sandy peat soil (Huminal, Hydro, Oslo, Norway) and perlite (Planteperlite, L.O.G, Oslo, Norway) and kept in an environmentally controlled growth room at 20°C, relative humidity (RH) >60%, and a 16 h light/8 h dark cycle where light was provided by high intensity discharge lamps (400 W Kolorarc daylight KRC400/T/H, General Electric Comp., Fairfield, CT, USA), and daylight giving a photosynthetic photon flux density (PPFD) of approximately 600 µmol m⁻² s⁻¹ at plant height. PPFD of all light sources was measured using a Li-250 light meter (LiCor, Inc., Lincoln, NE, USA). Ozone treatment started 52 d after sowing.

Experiment II: leaf age and severity of visible injury: T. subterraneum seeds were sown in trays with vermiculite (Agra- RHP 2–4 mm, L.O.G, Norway) and kept in an environment controlled growth room at 20°C, RH >60%, and a 16 h light (240±8 µmol m⁻² s⁻¹) and 8 h dark cycle where light was provided by high intensity discharge lamps (400 W Kolorarc daylight KRC400/T/H, General Electric Comp., Fairfield, CT, USA) and daylight giving a photosynthetic photon flux density (PPFD) of approximately 600 µmol m⁻² s⁻¹ at plant height, 400 W Kolorarc daylight) and 8 h dark cycle.

Thirteen days after sowing, 60 uniformly developed seedlings were selected and transplanted individually into transparent plastic pots (Unipak 5012, 520 ml, Superfos Randers A/S, Randers, Denmark) wrapped in aluminium foil. The pots were filled with modified full-strength Hoagland II nutrient solution (pH 4.7; Hoagland and Arnon, 1938) where 20 µM FeSO₄·7H₂O and 1.4 µM Na₂-EDTA, 0.4 µM CuSO₄, and 0.1 µM Na₃MoO₄·H₂O were used instead of Fe-EDTA, 0.3 µM CuSO₄, and 0.5 µM H₂MoO₄·H₂O, respectively. The nutrient solution was changed regularly. Ozone treatment started 21 d after sowing.
Experiment III: infrared imaging of leaves: Seeds were sown as in Experiment II. PPFD at plant height was 200±50 μmol m⁻² s⁻¹. Seventeen days after sowing, when all plants had developed at least one trifoliate leaf, 12 seedlings were transplanted individually into transparent plastic pots (UniPak 5101, 120 ml) covered with aluminium foil, and filled with modified Hoagland II solution. Ozone treatment started 31 d after sowing.

Ozone exposure

Plants were divided into four different treatments in a factorial design with ozone (ozone-free or ozone-supplemented air) and daylength [long day (LD) or short day (SD)] as variables. Plants were arranged randomly in six (three replicates) exposure chambers (Perspex; length, width, height = 420×320×400 mm³) with RH >75% and 20 °C, and exposed to 6 h of either charcoal-filtered air (CFA) or charcoal-filtered air supplemented with ozone giving 70±7 ppb ozone (CFA+O₃) during midday for three consecutive days. The exposure chambers were kept in an environmentally controlled growth room (30 m²) with RH >75% and 20 °C (daytime light and 14 h darkness. Daytime light (200±50 μmol m⁻² s⁻¹) was provided by 6 h of either charcoal-filtered air (CFA) or charcoal-filtered air supplemented with ozone giving 70±7 ppb ozone (CFA+O₃) during midday for three consecutive days. The exposure chambers were kept in an environmentally controlled growth room (30 m²) with diffuse daylight and an artificial light source (400 W Kolorarc dayligh lamps) giving 240±20 μmol m⁻² s⁻¹ at plant height inside the chambers.

Ozone was generated by passing pure oxygen at a rate of 0.7 l min⁻¹ through an ozone generator (model GSG 001.2, Sorbios GmbH, Berlin, Germany). The ozone concentrations inside the exposure chambers were monitored by an ozone analyser (Photometric O3 Analyzer-Model 400, Advanced Pollution Instrumentation Inc., San Diego, CA, USA). The accumulated ozone dose over the threshold was 550 mol m⁻³ at plant height inside the chambers.

LD and SD treatments

After the daily ozone exposure, plants were transferred to and randomly distributed within two separate environmentally controlled growth rooms (20±1 °C, RH >60%), one providing LD conditions with 10 h bright light and 14 h dim light, and the other providing SD conditions with 10 h bright light and 14 h darkness. Daytime light (200±50 μmol m⁻² s⁻¹) was provided by 400 W Kolorarc lamps. The dim night-time light (0.6–1.1 μmol m⁻² s⁻¹ at plant height) was provided by one fluorescent lamp (Luminette 58W/840, Aura, Karlskrona, Sweden). After the ozone treatment period, plants were cultivated under SD or LD conditions until harvest. Daylength treatment lasted for eight nights.

Assessment of ozone-induced injury and harvest

Experiment I: At harvest, 6 d after ozone exposure (i.e. 60 d after sowing), the total shoot dry mass (105 °C) in each pot was determined. Fully expanded trifoliate leaves were divided into two categories: (i) leaves without visible ozone injury; and (ii) ozone-injured leaves (at least five tiny chlorotic/necrotic spots).

Experiment II: The shoot and root dry masses of each plant were determined at harvest 6 d after ozone exposure (29 d after sowing). All leaves were assessed visually for severity of ozone injury using the following scale: 0, no visible ozone injury; 1, <50%; 2, >50% of lamina injured. Leaves were sorted according to order of emergence; the unifoliate being the oldest leaf, the trifoliate leaf number one being the second oldest leaf, etc.

Experiment III: Imaging was performed in the morning before and in the evening after the daily midday ozone exposure as well as in the morning 1 d after ozone treatment. The fourth and fifth trifoliate leaves of 12 plants were imaged, as all plants had a minimum of six fully expanded trifoliate leaves at the start of ozone exposure. Three replicates from each of the four treatment groups were positioned randomly in separate exposure chambers.

A total of 168 infrared images of leaves were captured using a ThermoVision™ A40M infrared camera (FLIR Systems, Danderyd, Sweden) with a 320×240 pixels uncooled microbolometer focal plane array, a spectral range of 7.5–13 μm, and a thermal sensitivity of 0.08 °C at 30 °C ambient temperature. A 200 μm close-up lens was mounted on the camera, giving a maximum spatial resolution of 0.2×0.2 mm² (minimum absolute pixel size). The infrared camera was mounted vertically ~10 cm above the leaf. Each leaf was positioned in the camera’s field of view to focus the image and to maximize the image area covered by the leaf.

Digital images (in the visible range) of the same leaves were captured using a Canon EOS 20D camera (3504×2336 pixels) equipped with a Canon 100 mm macro lens EF. Images were saved in the Raw Image Format.

On the last day of ozone exposure (33 d after sowing), the fourth and fifth trifoliate leaves were given 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 lamina injured. Shoot and root dry masses of each plant were determined at harvest 5 d after ozone exposure (38 d after sowing).

Image processing

The temperature for each leaf lamina pixel \( T_{\text{leaf},i} \) was extracted from the infrared images using ThermaCAM™ Researcher (v 2.8 Professional, FLIR Systems) and MATLAB® (Rev 7.5, 2007a, The MathWorks, Inc., Natick, MA, USA). Leaf emissivity was set to 0.95 (Idso et al., 1969; Jones, 2004). As the background temperature varied somewhat between imaging sessions, the average background temperature \( T_{\text{background}} \) for each leaf was determined by averaging the temperature in the area surrounding the leaf in the infrared images. The background temperature was subtracted from the leaf lamina temperature \( T_{\text{leaf},i} \), giving the background corrected temperature \( \Delta T_i = T_{\text{leaf},i} - T_{\text{background}} \) for the \( i \)th leaf lamina pixel.

The distribution of the background-corrected temperature \( \Delta T \) for all pixels of a leaf lamina was used to assess whether ozone exposure and daylength treatment affected...
leaf temperature. Thirteen characteristics of the \( \Delta T \) distribution were calculated: mean, median, mode, minimum, maximum, range, standard deviation, variance, skewness, kurtosis, smoothness, uniformity, and entropy (Gonzalez et al., 2004).

**Statistical analysis**

The effects of ozone and daylength on biomass and visible leaf damage were analysed by paired two sample t-tests, and one-way and two-way analysis of variance (ANOVA; significance level \( P \leq 0.05 \)). To avoid pseudoreplication, data from plants or pots with the same daylength treatment within each ozone exposure chamber were averaged such that the variance analyses were performed with three replicates corresponding to the three replicate exposure chambers. The impacts of ozone and daylength on leaf \( \Delta T \) distribution characteristics the mornings before and evenings after exposure were evaluated by analyses of variance on repeated measures (ANOVAR). The fourth and fifth trifoliate leaves were studied separately. The full model included the factors ozone treatment, daylength treatment, the interaction between these two factors, and the day number, starting with the first day of ozone exposure. All statistical calculations were conducted in SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

The multivariate statistical method principal component analysis (PCA) was used as a graphical framework to analyse the effects of ozone and daylength on the 13 characteristics (variables) of the leaf temperature \( \Delta T \) distributions (Næs et al., 2002). In PCA, the information in the data is projected onto a small number of new variables called principal components (PCs), which are linear combinations of the original data. The different PCs are orthogonal to each other, and are estimated to give, in decreasing order, the best description of the variance in the data. The first few PCs contain most of the relevant information in the data. The PCs are described by the loadings for the variables (13 \( \Delta T \) distribution characteristics) and the scores for the samples, the individual leaves. The data are modelled in terms of significant factors, plus errors or residuals. Calculations were performed in MATLAB® combined with PLS-Toolbox® (Rev 3.0, 2008, Eigenvector Research Inc., Wenatchee, WA, USA).

**Results**

**Daylength effects on visible leaf injury (Experiment I)**

In plants exposed 8 weeks after sowing, a significant effect of daylength on ozone-induced leaf injuries was found at harvest. A total of 51% of the leaves on LD-treated plants displayed visible ozone injury compared with 35% for SD-treated plants (Fig. 1A). No significant effects of ozone or daylength were found on shoot dry mass per plant (Table 1), most probably due to the short time interval between exposure and harvest.

**Leaf age and severity of leaf injury (Experiment II)**

In plants exposed 3 weeks after sowing, leaf ozone injury was again more pronounced in LD- than in SD-treated plants, the former having a significantly higher injury score per injured leaf (Fig. 1B).

Leaves were assessed for injuries according to order of emergence. The three oldest leaves, which were fully developed at the time of exposure, had higher injury scores after LD than after SD treatment (Fig. 2). These differences were significant for the unifoliate and the second trifoliate leaves. As the mean score for these leaves was >1.5, most leaves had severe visible injury. Within each daylength treatment, the three oldest leaves displayed approximately the same degree of visible injury.

The mean score for the third trifoliate leaf was <0.5 (Fig. 2). Thus, most of these leaves did not display visible injury, most probably because only half were fully developed on the last day of ozone exposure. Of the third trifoliate leaves fully developed during exposure, 62% were injured in SD-treated plants whereas as many as 83% were injured in LD-treated plants, indicating that newly developed leaves were more susceptible to visible injury under LD treatment.

**Fig. 1.** Effects of daylength on ozone-induced visible leaf injury. (A) Fraction of injured leaves per pot for plants exposed 52–54 d after sowing (Experiment I, \( n=9 \)). (B) The mean injury score per injured leaf (0, no injury; 2, >50% of lamina injured) for plants exposed 21–23 d after sowing (Experiment II, \( n=15 \)). (C) The mean injury score per leaf (0, no injury; 4, 75–100% of lamina injured) for the fourth and fifth trifoliate leaves of plants exposed 31–33 d after sowing (Experiment III, \( n=6 \)). Short day (SD), grey; long day (LD), white. Mean ± SE, *\( P \leq 0.05 \).
Table 1. The dry masses of control and ozone-exposed plants at harvest 6 d (5 d in Experiment III) after ozone exposure for Experiments I–III where SD and LD designate short and long day treatment, respectively.

| Experiment | Control | Ozone |
|------------|---------|-------|
|            | SD      | LD    | SD      | LD    |
| Shoot DM (g) | 0.35±0.06 | 0.28±0.05 | 0.23±0.04 | 0.26±0.03 |
| Root DM (g)  | 0.11±0.01 | 0.11±0.01 | 0.10±0.01 | 0.10±0.01 |
| Shoot DM (g) | 0.044±0.004 | 0.045±0.004 | 0.038±0.003 | 0.039±0.002 |
| Root DM (g)  | 0.11±0.01 | 0.14±0.01 | 0.12±0.02 | 0.08±0.01 |

Mean ± SE, n=3.

Fig. 2. Effects of daylength on the mean visible ozone injury score per leaf for the four oldest leaves exposed to ozone 21–23 d after sowing. Short day (SD), grey; long day (LD), white. Score 0 and 2 denote none and maximum ozone injury, respectively. Mean ± SE, n=15, *P ≤0.05.

None of the plants had a fully developed fourth trifoliate leaf during ozone exposure and none of the leaves younger than the third trifoliate leaf was visibly injured. Thus, the severity of injury depended on the extent of leaf development during exposure.

The shoot and root dry masses at harvest were not significantly affected by ozone or daylength treatment (Table 1).

Visible injury and infrared images of leaves (Experiment III)

In plants exposed 1 month after sowing, 80% of the fourth and fifth trifoliate leaves displayed visible ozone-induced injury after the third day of ozone exposure. LD-treated plants were again more severely injured than SD-treated plants (Fig. 1C). Again, no significant effects of ozone or daylength were found on shoot or root dry weight per plant (Table 1).

Representative visible and infrared leaf images of a control and two exposed LD-treated plants on the evening after the third ozone exposure are shown in Fig. 3. The exposed fifth trifoliate leaf in Fig. 3B displayed slight injury (score 1, <25% injury) with some spots on the central parts of the leaflets and some discoloring along the leaf edges. The severely injured fourth trifoliate leaf (Fig. 3C, score 3, 50–75% injury) displayed extensive discoloring along the leaf edges, and spots and necrotic areas scattered throughout the leaf. The infrared images of the control (Fig. 3D) and exposed leaves (Fig. 3E, F) were clearly different. The central regions of the control leaflets (Fig. 3D) were cooler than the edges, whereas the temperature across the exposed leaves was more homogeneous, with some cooler patches scattered across the leaf (Fig. 3E, F). These patches only occasionally coincided with discoloured areas in the visible images (Fig. 3B, C). The uniformity of the leaf temperature, particularly of the only slightly injured leaf in Fig. 3B, E, indicated that temperature modifications on the exposed leaves were not restricted to areas displaying visible injury.

The relative frequency histograms of the background-corrected leaf lamina temperature distribution ($\Delta T=T_{\text{leaf}}-T_{\text{background}}$) of the control leaf (Fig. 3G) were asymmetric, with a tail to the right capturing the higher temperatures along the leaf edges. The exposed leaf temperature distributions (Fig. 3H, I) were much narrower, were more symmetric, and were closer to the background air temperature, indicating that the exposed leaves were warmer than the control leaf. The mean temperature of the control leaf (Fig. 3D, G) was 4.7 °C less than the background temperature. In contrast, the mean temperatures of the exposed leaves were 1.4 °C (Fig. 3E, H) and 2.8 °C (Fig. 3F, I) lower than the background temperature.

Effects of ozone and daylength on leaf temperature distributions

The multivariate statistics technique PCA enabled the relationship between temperature $\Delta T$ distribution characteristics and the infrared leaf images to be visualized (Fig. 4). Positive PC1 was associated with temperature distributions with relatively high variance, range, smoothness, and entropy. High smoothness captures roughness whereas low smoothness is associated with homogeneity (Gonzalez et al., 2004). Entropy is a measure of randomness. Negative PC1 was associated with high median, mean, mode, and $\Delta T$ uniformity, and a high minimum leaf temperature. Thus, leaves with negative PC1 scores were warmer, with temperatures closer to the background temperature, and temperatures across the leaf were more homogeneous as can be seen by comparing the infrared images along PC1 (Fig. 4).

Variation along PC2 accounted for other aspects of the shape of the distribution. Positive PC2 scores were associated with high kurtosis and skewness. Kurtosis captures the ‘peakedness’ of the temperature distribution, where a high kurtosis distribution has a sharper peak and flatter tail (positive PC2, Fig. 4) and a low kurtosis distribution is more rounded with wider shoulders (negative PC2, Fig. 4). Skewness is a measure of the asymmetry of the distribution, where positive skewness designates a longer tail to the right (positive PC2, Fig. 4).

Figure 5A shows the PCA score plot for SD- and LD-treated plants the morning after the first ozone exposure.
and the first night. Leaf temperature distributions of control and exposed SD-treated plants were clustered around the origin, generally overlapping. LD-treated plants, however, showed very clear differences between leaf temperature distributions of ozone-exposed and control plants. The exposed and control distributions had separated into two distinct groups along PC1. The exposed distributions had more negative PC1 scores, indicating, by comparing with Fig. 4, that exposed leaves were warmer with more uniform leaf temperatures.

For comparison, the mean PCA scores for each treatment group (combination of daylength and ozone) on the evenings shortly after ozone exposure and the following mornings are shown in Fig. 5B. Scores for LD-treated plants exposed to ozone formed a group along negative PC1 that was clearly separated from the scores of control and ozone-exposed SD-treated plants, which were clustered together around the origin. Thus, ozone exposure resulted in persistently higher and more homogeneous leaf temperatures in LD- than in SD-treated plants (see Fig. 4). In addition, ozone exposure did not affect leaf temperature of SD-treated plants.

Grouping the data according to severity of visible ozone injury did not produce separable clusters, again indicating a lack of correspondence between visible injury and leaf temperature response. In addition, the scores of the fourth and fifth trifoliate leaves could not be separated, indicating that they responded similarly to ozone and daylength treatments.

**Analysis of variance of the leaf temperature distributions**

ANOVAR supported the PCA results, but did not reveal a clear pattern in the data as provided by the PCA biplot (Fig. 4). The variables (minimum, range, mean, variance, entropy, and smoothness) along PC1 in the PCA (Fig. 4),
separating LD ozone-treated plants from the others (Fig. 5), were also significantly affected by the interactions between ozone treatment and daylength in the ANOVAR (Table 2). This interaction effect was particularly evident in the evening after ozone exposure, indicating that the effects of the night-time light conditions were present for several hours into the day. The variables described by PC2 (kurtosis, skewness, and maximum, Fig. 4) were, however, not significantly affected by the interaction day-length × ozone in the ANOVAR.

**Discussion**

The present study shows for the first time that LD conditions significantly increased the severity and percentage of subterranean clover leaves showing ozone-induced injury. This increase occurred consistently, regardless of plant age during ozone exposure. The daylength treatments did not differ in daytime climatic and light conditions, daytime ozone exposure, nights in ozone-free air, and the time between ozone exposures. Thus, the ozone exposure of plants under LD or SD conditions was the same. The daylength treatments differed only by their night-time light conditions, with either dim night-time light (LD) or a dark night (SD). Therefore, this study implies that overnight recovery in darkness and not ozone uptake was critical for the lower ozone sensitivity seen in *T. subterraneum* under SD conditions. Repair and defence processes occurring overnight in darkness were sufficient to reduce visible foliar injury and leaf temperature disruptions.

Infrared imaging showed that ozone exposure resulted in persistently higher leaf temperatures in LD-treated plants relative to SD-treated and control plants. This disruption of leaf temperature regulation occurred already the morning after the first ozone exposure and the first dim night, indicating an early response to ozone triggered by the dim night. As leaf temperature reflects transpiration (Jones, 1999, 2004; Kümmersen et al., 1999; Prytz et al., 2003), the results suggest that ozone reduced transpiration in LD- but not SD-treated plants. In darkness, however, plants appeared to be able to recover stomatal function and thereby sufficient transpiration.

The temperatures across leaves of LD-treated plants exposed to ozone were also quite uniform compared with exposed SD-treated and control plants. Hence, ozone-induced changes to leaf transpiration affected the temperature of the entire leaf and were not only restricted to specific patches or regions. This is in contrast to visible foliar injury which occurred as distinct spots on the leaf interior and were not only restricted to specific patches or regions. This is in contrast to visible foliar injury which occurred as distinct spots on the leaf interior surface or discoloured areas along the edges, and occurred on SD-treated as well as LD-treated plants. Specific hot- or cool-spots as have been observed after viral and fungal infection (Chaerle et al., 2004) were not found in the present study. The few occasional cool patches scattered across the leaf or along the leaf edges, possibly signifying decreased leaf integrity, seldom coincided spatially with visible injuries.

**Fig. 4.** Biplot showing the PCA loadings of the characteristics of the temperature distributions ΔT of leaves subjected to ozone exposure (31–33 d after sowing) and two different daylength treatments. PC1 and PC2 accounted for ~85% of the variance in the data set, with PC1 and PC2 capturing 66.4 and 18.3%, respectively. Infrared images and the corresponding histograms of the temperature distributions of some example leaves are shown to visualize the variance in the data set. The temperature scale of all infrared images is the same and stretches from white (warm), via yellow to blue (cold) (compare Fig. 3).
It is therefore possible that leaf temperature changes and visible foliar injuries are results of different mechanisms. Leaf temperature changes may reflect direct effects of ozone or ozone-generated reactive oxygen species on guard cell ion transport (Torsethaugen et al., 1999; McAinsh et al., 2002), whereas visible foliar injury is due to necrotic or programmed cell death (Pell et al., 1997; Van Breusegem and Dat, 2006). Ozone can alter guard cell cytosolic calcium concentrations, potentially affecting calcium-based signaling processes and hence stomatal ability to respond to environmental perturbations (Pei et al., 2000; McAinsh et al., 2002). It has also been shown that ozone can directly affect guard cells by inhibiting inward K⁺ channels, thereby impairing stomatal opening but not the ability of guard cells to close stomata (Torsethaugen et al., 1999). In the current study, exposed LD-treated leaves were warmer than SD and control leaves, but remained cooler than the background air temperature. Thus, leaves were most probably transpiring, but to a lesser extent, suggesting reduced stomatal opening. As altered leaf temperature regulation first became apparent the morning after the first ozone exposure and dim night, it is possible that LD treatment impaired stomatal reopening after night-time.

As changes in light intensity, quality, and photoperiod influence a multitude of plant processes, there are several possible mechanisms by which the dim night-time light may alter ozone-induced visible damage and leaf temperature regulation. Phytochromes are known to be involved in daylength responses as well as in regulating circadian rhythms (Han et al., 2007; Song and Noh, 2007). It is therefore possible that phytochrome-mediated responses are involved. In a recent study, a long photoperiod possibly involving phytochrome-dependent pathways reduced the induction of defence-related genes upon oxidative stress in Arabidopsis, thereby increasing leaf lesion severity (Queval et al., 2007). Thus, it would be interesting to investigate whether phytochrome is involved in altering ozone sensitivity in relation to daylength.

Infrared imaging enabled leaf temperature to be measured non-invasively and repeatedly without disturbance or contact with the leaf. Infrared imaging provided the spatial pattern of the leaf temperature, not only a single temperature averaged over the entire leaf. Thus, it could be shown that the combination ozone–daylength affected the temperature of the entire leaf surface, not only specific regions, as in the case of visible foliar injury. By analysing the characteristics of the leaf temperature distributions using the multivariate statistics technique PCA, differences induced by the interaction between ozone and daylength could for the first time be characterized and visualized (Fig. 4). Furthermore, ozone-induced leaf temperature disruptions in LD-treated plants could be detected and characterized at an early stage, already the morning after the first ozone exposure and the first dim night. Detecting and illustrating these ozone-induced temperature modifications merely by visual inspection of the infrared images or by using univariate statistics (Table 2) would not have been possible. Thus, the combination of multivariate statistics and infrared imaging provided new insights into the leaf

![Fig. 5. (A) PCA score plot highlighting the scores of individual leaves of short-day and long-day-treated plants the morning (32 d after sowing) after the first ozone exposure day and the first night with either SD or LD conditions. (B) Mean PCA scores for each treatment group on the evenings after ozone exposure and the following mornings. Open symbols, controls; filled symbols, ozone exposed. Circles and solid lines, short day; squares and dashed lines, long day.](https://academic.oup.com/jxb/article-abstract/60/13/3677/528459/3684)

### Table 2. Interactions (ANOVAR) between ozone and daylength treatment on leaf temperature distributions of the fourth and fifth trifoliate leaves on four mornings starting the first day of exposure (31–34 d after sowing), and evenings of the three consecutive days of fumigation

|                | Morning |      | Evening |      |
|----------------|---------|------|---------|------|
|                | Fourth  | Fifth| Fourth  | Fifth|
| Minimum        | *       | –    | *       | –    |
| Range          | *       | –    | *       | –    |
| Mean           | –       | –    | –       | –    |
| Variance       | –       | X    | –       | X    |
| Entropy        | X       | X    | *       | *    |
| Smoothness     | –       | *    | –       | –    |
| Maximum        | –       | –    | X       | –    |
| Kurtosis       | –       | –    | –       | –    |
| Skewness       | –       | –    | –       | –    |

*P < 0.05; ×0.05<P<0.10; –P >0.10.
ozone response that would have been difficult to obtain otherwise.

In conclusion, dim night-time light, giving LD conditions, significantly increased ozone-induced visible foliar injury of subterranean clover plants. In addition, ozone-induced leaf temperature changes were detected in LD- but not SD-treated plants. These temperature changes were observed already the morning after the first ozone exposure and dim night, suggesting that the dim night-time light impaired defence capacity and overnight repair of ozone damage. Thus, the results show that light conditions of summer nights at high latitudes can affect plant ozone sensitivity. Furthermore, this study supports a role for multivariate image analysis in monitoring, characterizing, and classifying early plant stress responses.

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