Elevated air humidity increases UV mediated leaf and DNA damage in pea (*Pisum sativum*) due to reduced flavonoid content and antioxidant power

Sheona N. Innes[1,2], Louise E. Arve[3], Boris Zimmermann[4], Line Nybakken[2,5], Tone Melby[1], Knut Asbjørn Solhaug[2,5], Jorunn E. Olsen[1,2], and Sissel Torre[1,2]

[1] Faculty of Biosciences, Norwegian University of Life Sciences, 1430 Ås, Norway
[2] CERAD, Norwegian University of Life Sciences, 1430 Ås, Norway
[3] The Norwegian Food Safety Authority, 2831 Brumundal, Norway
[4] Faculty of Science and Technology, Norwegian University of Life Sciences, 1430 Ås, Norway
[5] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, 1430 Ås, Norway

Abstract

Growth in high relative air humidity (RH, > 85%) affects plant morphology and causes diminished response to stomatal closing signals. Many greenhouses are prone to high RH conditions, which may negatively affect production and post-harvest quality. UV radiation induces stomatal closure in several species, and facilitates disease control. We hypothesised that UV exposure may trigger stomatal closure in pea plants (*Pisum sativum*) grown in high RH, thereby restoring stomatal function. The effects of UV exposure were tested on plants grown in moderate (60%) or high (90%) RH. UV exposure occurred at night, according to a disease control protocol. Lower stomatal conductance rates were found in UV-exposed plants, though UV exposure did not improve the rate of response to closing stimuli or desiccation tolerance. UV-exposed plants showed leaf curling, chlorosis, necrosis, and DNA damage measured by the presence of cyclobutane pyrimidine dimers (CPD), all of which were significantly greater in high RH plants. These plants also had lower total flavonoid content than moderate RH plants, and UV-exposed plants had less than controls. Plants exposed to UV had a higher content of cuticular layer uronic compounds than control plants. However, high RH plants had a higher relative amount of cuticular waxes, but decreased proteins

and uronic compounds. Plants grown in high RH had reduced foliar antioxidant power compared to moderate RH. These results indicate that high RH plants were more susceptible to UV-induced damage than moderate RH plants due to reduced flavonoid content and oxidative stress defence.

Keywords: Stomata, transpiration, ultraviolet, CPD, plant cuticle, HPLC, infrared spectroscopy.

1 Introduction

Ultraviolet (UV) radiation has the highest energy per photon of the portion of the solar spectrum reaching the surface of the earth. The electromagnetic spectrum of UV radiation reaching the earth's atmosphere can be divided into vacuum UV (<200 nm), UV-C (200 to 280 nm), UV-B (280 to 315 nm), and UV-A (315 to 400 nm), though the stratospheric ozone layer absorbs all of vacuum- and UV-C, as well as much of the UV-B radiation [1, 2]. UV radiation is biologically active at low doses and may induce signalling cascades that trigger a range of photomorphogenic responses in plants. However, at high or chronic doses, UV radiation is a stressor and may cause damage to DNA, protein- and membrane lipids, and the photosynthetic apparatus [3]. In the natural environment plants rarely show signs of UV-induced damage, and while many previous studies have focused on plant responses to excessively high UV radiation doses or dose-durations (e.g. Jansen et al., 1998 [4] and refs therein), a shift has been seen in the last decade to more realistic experimental design and focus on UV-induced changes in morphology, physiology, metabolics, and gene expression [2, 3]. UV radiation induces photomorphogenic responses in plants via the UVR8 photoreceptor pathway [1, 5] and the most well documented photomorphogenic response to UV radiation is the biosynthesis of UV-screening compounds, such as flavonoids and anthocyanins [6] through transcription of genes encoding the chalcone synthase (CHS) enzyme, a key enzyme in the phenylpropanoid pathway [7].

UV radiation has also been found to affect plant water relations through effects on stomatal movement, though the magnitude and direction of such effects are dependent on several factors, and reported results are often contradictory [4, 8-11]. While Eisinger et al. [12] reported that the peak of the stomatal opening action spectrum in *Vicia faba* leaves lies in the UV range with a major peak at 280 nm, Tossi et al. [13] proposed a signalling model for stomatal closure in response to UV-B radiation, involving both abscisic acid (ABA)-dependent and -independent pathways. In both

pathways, exposure to UV-B resulted in stomatal closure, though this effect may be species-dependent [9, 14]. The increased ABA concentration frequently associated with UV-B exposure is often a stress-related response, wherein both drought and UV-B tolerance are enhanced [14]. Indeed, several authors have reported increased drought tolerance upon exposure to UV-B radiation [8, 15, 16]. Furthermore, UV-B radiation has been shown to induce thickening of the plant cuticle and cuticular wax [17, 18], though the correlation between increased cuticular thickness and cuticular water loss is questionable [19].

In greenhouse production the environment may be closely regulated for optimal growth. However, in northern latitudes during winter high relative air humidity (RH) is almost unavoidable due to a trade-off between ventilation and energy saving. It has previously been shown that continuous growth of plants in high RH (>85%) has a strong impact on plant transpiration, photosynthesis, growth and desiccation tolerance [20-27]. High RH normally induces stomatal opening, and long term high RH results in larger stomata that are unable to close when exposed to environmental closing signals, such as darkness, drought and ABA [21, 23, 28, 29]. The reasons for the loss of functionality of stomata developed in high RH have been hypothesised to involve changes is the guard cell wall flexibility or altered ABA level and signalling, though other signals are also likely to be involved [30-33]. Environmental changes that trigger stomatal movements, like changes in RH and/or temperature, have been shown to improve stomatal function in high RH [27, 33]. Furthermore, high RH has been reported to increase cuticular transpiration and soften epicuticular waxes [34]. However, how RH affects the wax structure and/or thickness or the chemical composition of the cuticle is inconclusive and species-dependent [35, 36]. Cuticular water loss via diffusion is generally considered negligible [37]. However, under conditions of stomatal closure, cuticular transpiration accounts for the majority of water loss and becomes increasingly important [35].

Many greenhouses have cladding material that either does not transmit UV-B radiation, while at least partially transmitting UV-A radiation, or does not transmit UV radiation at all. Given that UV has been shown to have a role in plant signalling, photomorphogenesis and plant water relations, the use of artificial UV radiation may prove beneficial in the control of plant growth. UV radiation has furthermore been found to have positive effects in the control of plant pathogens, such as powdery mildew [38, 39] and *Botrytis cinerea* [40, 41], and could therefore be an important tool in plant production systems. We decided to expose plants to UV during the dark period, as UV-

exposure in darkness is more efficient in control of powdery mildew since fungal photolyase needs UV-A or blue light for repair of DNA damage [42]. In addition we used unscreened UV-B tubes with a spectral range of UV slightly below 280 nm, as UV wavelengths below 300 nm are necessary for control of powdery mildew [43]. This UV radiation was used to test the effects of a UV exposure protocol which can also be used to control powdery mildew.

It is important to understand the positive and negative effects UV radiation has on the specific plant species both during production and post-harvest, as responses to UV radiation vary between species [4]. We therefore investigated the role of UV radiation on plant growth, transpiration and flavonoid content as well as UV induced damage in a background of moderate and high air humidity in *Pisum sativum*. Exposure to UV radiation affects stomatal movements, plant cuticle structure and chemical composition and could therefore play an important role as a trigger to improve stomatal closure in response to closing signals and desiccation tolerance in plants developed in high RH. Hence we also investigated the effect on stomatal responses and the chemical composition of cuticula. We hypothesised that exposure to UV radiation may contribute towards combatting the negative effects of plant grown at high RH and improving stomatal function and desiccation tolerance.

2 Materials and methods

2.1 Plant material and growth conditions

Pea plants of the wild type pea, *Pisum sativum L.*, cv Torsdag were used in this study. The plants were germinated in 12 cm pots containing peat (L.O.G. Gartnerjord, Rakkested, Norway). The plants were grown in a greenhouse with polyacrylic walls and glass roof at 20℃, with 80% relative air humidity (RH), and 20 h daily supplementary light of 100 µmol m^{-2} s^{-1} (PAR: 400-700 nm) supplied by high pressure sodium lamps (HPS, Osram NAVT- 400W, Munich, Germany) at the Norwegian University of Life Sciences, Ås, Norway (N 59° 40.120', E 10° 46.232'). The plants were grown during August and September in 2014 and 2015, during which time the plants received between 10 and 16 h of daylight [44]. The plants were kept in the greenhouse until they were approximately 10 cm tall.

The plants were then transferred to four environmentally controlled growth chambers for experimental treatments. A factorial 2x2 design (two RH levels: 60% and 90%, and two UV

radiation levels: UV-exposure and no-UV control) was used with 5-8 plants per treatment grown in five repeated experiments. The chambers were maintained at 20°C and 60% or 90% RH throughout the experiment by a PRIVA system (Priva, Ontario, Canada). The plants received 150 ± 10 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR: 400-700 nm) from HPS lamps, as measured at the top of the canopy using a LI-Cor Quantum sensor attached to a LI-250 Light Meter (Li-Cor Inc., Lincoln, NE, USA), for a 20 h photoperiod each day. This gave a daily light integral (DLI) of 10.8 ± 0.7 mol m^{-2} d^{-1}, just slightly higher than the recommended DLI for the best integrated quality of pea plants [45]. UV radiation (Fig. 1) was provided by unscreened fluorescent tubes (Q-panel UV 313, Q-Lab Corporation, Ohio, USA) at 0.15 W m^{-2} UV-B for 40 minutes every night in the middle of the dark period, according to a method adapted from Suthaparan et al. [38] for control of powdery mildew by UV-B. UV radiation was measured at the top of the canopy using a Skye SKU 430/SS2 UVB Sensor connected to a Skye SpectroSense2 Meter (Skye Instruments Ltd, Llandrindod Wells, Powys, UK), which was calibrated using an Optronic OL756 Spectroradiometer (Optronic Laboratories, Inc., Florida, USA). The Green weighting spectrum for DNA damage [46], normalized to 1 at 300 nm, was used to estimate biologically effective UV-B ($UV\text{-}B_{BE}$) at 0.22 W m^{-2}. Measurements are specified for UV-B here, as measurements were taken using a UV-B sensor.

The plants were watered daily and fertilized twice a week using a 50/50 mixture of YaraLiva Calcinit calcium nitrate solution and Kristalon Indigo (both Yara Norge AS, Oslo, Norway), with EC level 1.5 mS cm^{-1}. The plants were subjected to experimental conditions for 15 days before plant growth parameters were measured and further sampling began. Plant height was measured from the rim of the pot to the shoot apical meristem, and the number of leaves (as petiole, leaflets and a tendril) per plant were counted for each plant when the plants were harvested at the end of four of the experiments.

2.2 Water relations

2.2.1 Detached leaf desiccation

After 15 days of growth in the chambers, one fully expanded, undamaged leaflet was sampled from the third or fourth leaf from the base of five plants from each treatment. The analysis was repeated in all five of the experimental rounds. The leaflets were detached 1 h before the end of the light period, placed adaxial side-down on a clean workbench, and weighed after 0 and 180

minutes. The test was performed in a room with 40% RH, 20°C, and 15 µmol m^{-2} s^{-1} irradiance at the surface of the leaves. The relative water content at time 0 was set to 100% and the relative water loss after three hours was calculated (weight after 180 mins/original weight*100).

2.2.2 Stomatal conductance measurements: time series in the dark

Stomatal conductance measurements were repeated in time series on plants that were transferred to a different, dark environment (40% RH, 20°C, darkness) during the light period. Three plants from each treatment were transferred to a dark environment 1 h before the start of the dark period. Conductance rates were recorded on leaflets from the third and fourth leaves from the base of the plants immediately, 1 h, 3 h and 8 h post transfer. The analysis was performed in two replicate experiments.

2.3 Plant injury quantification

2.3.1 Visible symptoms of leaflet injury

Visible plant injuries in the form of leaflet curling, leaflet chlorosis and leaflet necrosis were quantified by counting the number of leaflets >10 mm showing visible injuries on each plant. Leaflets were considered chlorotic/necrotic when >30% of the surface of the leaflet indicated chlorosis/necrosis.

2.3.2 Cyclobutane pyrimidine dimer (CPD-DNA) quantification

DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) were quantified by enzyme-linked immunosorbent assay (ELISA) using OxiSelect UV-Induced DNA damage kits for CPD Quantification (Cell Biolabs, Inc., USA). Fully expanded, undamaged leaflets from the fourth leaf from the base of three plants per treatment were sampled 1 h before the start of the dark period and immediately placed in liquid N_2, followed by storage at -80°C. Frozen tissue (100 ± 0.5 mg) was disrupted from each leaflet sample in a Tissue Lyzer (Mixer Mill Type MM301, Retsch GmbH, Haan, Germany). DNA was extracted using a DNeasy Plant Minikit (QIAGEN GmbH, Hilden, Germany) in a darkened room, with a yellow filter over the light. Standards were prepared according to ELISA protocol. DNA samples were diluted to 0.75 µg ml^{-1} using a cold phosphate-buffered saline (PBS) solution. Samples were then converted to single-stranded DNA by heating to 95°C for 10 min, followed by 10 min on ice. ELISA assay protocol was followed for the reaction

between DNA and anti-CPD antibody solution. The absorbance of the reaction mixture was measured on a microplate reader (Biochrom Asys UVM 340 with KIM, UK) with 450 nm as the primary wavelength. The analysis was performed in two replicate experiments.

2.3.3 Chlorophyll fluorescence

Maximal photosystem II (PSII) efficiency (variable fluorescence [Fv]/maximum fluorescence [Fm]) was measured on fully expanded, visibly undamaged leaflets from the fifth leaf from the base of the plant. This was performed using a portable chlorophyll fluorometer (Plant Efficiency Analyzer, Hansatech Instruments, Norfolk, UK) using excitation light of approximately 3500 μmol photons m^{-2} s^{-1} (PAR: 400-700 nm) after dark adaptation. This analysis was performed in one of the experimental rounds. Undamaged leaflets were used specifically to determine any PSII core damage arising as a direct result of UV exposure.

2.3.4 Leaflet morphology from cross-sections

Leaflet cross-sections were examined to determine RH or UV-induced changes to leaflet morphology. Leaflets from the fully expanded sixth leaf from the base of four plants were detached and cut into approximately 3x3 mm pieces, which were immediately submerged in fixation medium (1.2% glutaraldehyde, 2% paraformaldehyde, 0.1% (v/v) Tween 20 in 0.01 M sodium phosphate buffer, pH 7.2) and stored at 4°C. The samples were dehydrated through a graded ethanol series before being infiltrated with resin LR White (Electron Microscopy Sciences, Hatfield, PA, USA) in a further graded series, with a progressively increasing ratio of LR White resin to ethanol. The sections were then placed in an embedding mould with 100% LR White, which was polymerized overnight at 50°C. Samples embedded in LR White blocks were sectioned using a Micro Star diamond knife (Micro Star Technologies, Huntsville, TX, USA) on a Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Cross sections (2 μm thick) were mounted onto slides and stained using Stevenel's Blue. Coverslips were sealed onto the slides using Depex mounting medium before the slides were viewed using a Leica DM 5000 B light microscope connected to a Leica DFC 425 digital microscope camera with a Leica 10445929 0.5x video objective. Leica Application Suite v4.3.0 software (all Leica Microsystems GmbH, Wetzlar, Germany) was used for image capture and analysis. The analysis was performed in one of the experimental rounds.

2.4 Protective compound analyses

2.4.1 Flavonoid quantification by HPLC

Fully expanded leaflets from the fifth leaf from the base of 4-5 plants per treatment were detached and placed immediately in liquid N_2 for storage before freeze-drying. Samples were freeze-dried using a Telstar LyoQuest (Telstar, Terrassa, Spain). 20 mg of dried, crushed plant material was extracted five times with 600 µg of methanol (MeOH) before the MeOH was evaporated under vacuum and the dried residue was frozen. The residue was redissolved in MeOH and water (200 + 200 µl) before being centrifuged, poured through syringe filters and sealed into HPLC vials. Phenolic acids and flavonoids were analysed by HPLC (Agilent, Series 1100, Germany), which consisted of a binary pump (G1312A), a thermostated autosampler (G1329A), a thermostated column oven (G1316A) and a diode array detector (G1315B). The metabolites were separated using an ODS Hypersil C18 (4.6 x 50 mm) HPLC column (Thermo Scientific, Waltham, Massachusetts, USA). The samples were and eluted (flow rate 2 ml min^{-1}) using a MeOH:water gradient [47]. The auto-injection volume was 20 µl and all runs were performed at 30°C. Identification of metabolites was completed by comparison of retention times and UV spectra with commercial standards. The analysis was repeated in three of the experimental rounds.

2.4.2 Chemical composition of the cuticle

Cuticular chemical composition was analysed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). A pair of fully expanded leaflets were removed from the third leaf from the base of five plants per treatment and air dried in a warming cupboard at 60°C. Epicuticular wax was removed from one leaflet from each pair of leaflet samples. This was performed by washing each leaflet twice in warm (40°C) chloroform for 60 s per wash. Each leaflet sample was measured at three different positions on both adaxial and abaxial sides (6 measurement points per leaf). On both ab- and adaxial sides, two measurement points on each leaflet were basal and close to either side of the midrib. The third measurement was distal and close to the midrib. Samples were measured using a Vertex 70 FTIR spectrometer (Bruker Optik, Germany) with the single-reflection attenuated total reflectance (SR-ATR) accessory. The ATR IR spectra were recorded with 32 scans using the horizontal SR-ATR diamond prism with 45° angle of incidence on a High Temperature Golden Gate ATR Mk II (Specac, United Kingdom).

Spectra were recorded in the region between 7000-600 cm^{-1} with a spectral resolution of 4 cm^{-1}. Each spectrum was recorded as the ratio of the sample spectrum to the spectrum of the empty ATR plate. The penetration depth of the infrared light in ATR-FTIR measurements is 0.5–5 μm, depending on the wavelength [48]. Thus, the FTIR spectra of leaves predominantly contain information on leaf cuticle, while the underlying epidermal cells contribute to a lesser degree. The analysis was performed during one of the experimental rounds.

2.4.3 Antioxidant power in leaves

Antioxidant power in whole leaflets was determined using an OxiSelect Ferric Reducing Antioxidant Power (FRAP) Assay Kit (Cell Biolabs, Inc., CA, USA). Studies analysing antioxidant capacity using several methods (e.g. FRAP and 2,2-diphenyl-1-picrylhydraziyl (DPPH)) have indicated significant correlation between methods [49-51], leading Clarke et al. [51] to conclude the use of one method to be sufficient. As a result of this, only FRAP was used to analyse antioxidant capacity in this study. Fully expanded leaflets from the sixth leaf from the base of three plants per treatment were removed and immediately placed in liquid N_2, followed by storage at -80°C. Tissue samples were weighed out (10 mg) and homogenised in 1 mL cold Assay Buffer. The absorbance of the reaction mixtures were measured on a microplate reader (Biochrom Asys UVM 340 with KIM, UK) using 540 nm as the primary wavelength. One leaflet from three separate plants in each treatment was sampled, and three technical replicates from each leaflet were analysed (total n = 36 including biological and technical replicates). Samples were measured against Iron (II) standards. The results were converted to relative amounts with moderate RH antioxidant power normalized to 100%. The analysis was performed during one of the experimental rounds.

2.5 Data analysis

Significant differences between means were determined for all data using generalised linear models (GLM) and two-way ANOVAs followed by Tukey`s HSD post-hoc tests. Data were tested for normality using Normal-Quantile plots and Shapiro-Wilk Normality tests, and for homoscedasticity using Levene's Test for equality of variances. Differences with $p < 0.05$ were considered significantly different, unless otherwise stated in the text. Statistical tests were

performed in Minitab 16.2 (Minitab 16.2.2, windows version, State College, PA, USA) and RStudio version 1.0.44 (© 2009-2016 RStudio, Inc.).

For the analyses of infrared spectral data, the spectral region of 4000–600 cm^{-1} was selected, and processed using multiplicative signal correction (MSC). The processed spectra were analysed initially using principle component analyses (PCA) to determine which treatment variables could explain the highest proportions of the data. Mann-Whitney *U* tests were then used to calculate the statistical significance of differences in the PCA principal component scores between samples. Partial least-squares–discriminant analysis (PLS-DA) was conducted in order to evaluate the effect of RH or UV exposure on samples. The optimal number of components (i.e., PLS factors) of the calibration models (A_{Opt}) was determined using full cross-validation. Since the majority of models had 4 as an optimal number of components, 4 components were used in all PLS-DA models in order to compare models and avoid over-fitting. The PLS coefficient of determination (R^2) between the taxa was used to evaluate the calibration models. Biochemical similarities between individual leaf samples were estimated by variability test based on Pearson correlation coefficients (PCC) for spectral region of 1900–700 cm^{-1}. All spectroscopy processing methods and data analyses were performed using The Unscrambler X 10.3 (CAMO Software, Oslo, Norway), as well as functions and in-house developed routines written in MATLAB 2014a. 8.3.0.532 (The MathWorks, Natick, MA, USA).

3 Results

3.1 Effect of RH and UV on stomatal function and conductance in pea plants

After 15 days of growth in experimental conditions in the chambers, water loss from detached leaves after three hours under a common RH environment (40% RH) was significantly affected by both RH level and UV exposure (Fig. 2A). No significant interaction was found between RH and UV exposure. Leaves grown in high RH lost 40-50% more water than leaves grown in moderate RH, both with and without UV radiation. Moreover, at both RH levels, leaves exposed to UV radiation lost significantly more water than leaves not exposed to UV (Fig. 2A).

Initial time course measurements of stomatal conductance after transfer of plants to the 40% RH environment and darkness indicated that plants grown in high RH had significantly higher instantaneous stomatal conductance rates than plants grown in moderate RH (Fig. 2B). Moreover,

plants that had been exposed to UV had significantly lower conductance rates than those not exposed to UV. No significant interaction was found between RH and UV exposure. After 1 h, plants grown in high RH without UV still had significantly higher instantaneous conductance than any other treatment (Fig. 2B). After three hours there were no longer any significant differences seen in conductance rates between the treatments, though conductance rates remained between 70 and 110 mmol m^{-2} s^{-1} even after eight hours in darkness. No significant interaction was found between RH and UV exposure.

3.2 Visible injury and CPD-DNA damage

Exposure to UV radiation, caused some leaf curling in moderate RH (Fig. 3A), but had a severely damaging effect with extensive leaf curling on the plants when grown in high RH (Fig. 3). RH and UV exposure had a significant interaction in visible injury and quantified CPD-DNA ($p < 0.05$). Plants exposed to UV radiation showed no chlorosis or necrosis, and very little UV-induced CPD-DNA damage when grown in moderate RH, yet plants grown in high RH had severe visible damage when exposed to UV radiation (Fig. 3). Damage to high RH + UV plants included severe leaf curling (Fig. 3A), leaf chlorosis (Fig. 3B), some leaf necrosis (Fig. 3C), and a significant amount of CPD-DNA damage (Fig. 3D). Structurally, neither RH nor UV radiation affected cellular leaf morphology (Fig. 3E).

3.3 Effect of RH and UV on plant growth and photosynthesis

Growth of pea was significantly affected by RH, but not by UV radiation (Fig. 4). No significant interaction was found between RH and UV exposure. Plants grown in high RH were, on average, 10% taller than plants grown in moderate RH ($p < 0.01$), regardless of UV exposure. A similar result was seen in the number of leaves per plant, with plants grown in high RH having a greater number of leaves than plants grown in moderate RH ($p < 0.03$).

Maximal photosystem II efficiency, (Fv/Fm) were measured on leaves with no visible damage and the results indicated no damage to the photosynthetic apparatus in any of the treatments. All treatment measurements were between 0.83 and 0.85, within the optimal range [52], and no differences between the treatments were found (data not shown).

3.4 Plant protective compounds

3.4.1 Flavonoid content in whole leaves

As expected from previous studies of pea leaves, quercetin-glycosides were the most prominent flavonoid compounds present (Table 1). UV exposure under high RH significantly reduced phenolic acid concentration ($p < 0.01$, Table 1). However, one group of phenolic acids, the chlorogenic acids, showed an opposite trend upon UV exposure in high RH, as well as an increase in chlorogenic acids in high RH plants compared to moderate RH plants (Table 1). Due to opposite trends in phenolic and chlorogenic acids, total phenolic acid concentration showed no significant effect of either RH or UV exposure (Table 1). RH had a significant effect ($p < 0.01$) on total quercetin-glycoside concentration, strengthened by exposure to UV, resulting in –UV plants grown in moderate RH having significantly higher concentrations of quercetin-glycosides than UV-exposed plants grown in high RH (Table 1). Kaempferol-glycosides were found in very low concentrations, and neither RH nor UV affected their concentration (Table 1). Total flavonoid concentrations reflect the pattern seen in quercetin-glycoside concentrations, as the concentration of the latter was so much greater than kaempferol-glycosides (Table 1). No significant interaction was found between RH and UV exposure for any of the flavonoids.

3.4.2 Chemical composition of the leaf cuticle

The infrared spectra of control leaves (detached leaves, dried and left intact) showed characteristic signals related to alkyl groups, which are predominant functional groups in the long-chain chemical constituents of cuticular waxes (Figure 3a). The spectra showed alkyl-related vibrational bands at 2914 and 2846 cm^{-1} (C-H stretch in alkyl groups), 1472 cm^{-1} (CH_2 bending), 1462 and 1365 cm^{-1} (CH_3 deformations) [53, 54].

The spectra of chloroform-washed leaves were devoid of these signals, signifying that the cuticular waxes were removed by the washing treatment (Fig. 5a). The spectra of washed leaves were dominated by the strong signals related to carbonyl groups characteristic for uronic acids and esters, such as glucuronic and galacturonic acids and esters of pectin: a carboxylic ester band at 1735 cm^{-1} (C=O stretch in esters), and two carboxylate bands at 1605 cm^{-1} (COO^- antisymmetric stretch) and 1420 cm^{-1} (COO^- symmetric stretch) [54]. There was lower absorbance of the 1735 cm^{-1} band than the band at 1605 cm^{-1}, indicating that the majority of uronic compounds are in acid or salt form, such as galacturonic acid, and not in ester form, such as methyl esters, which are

common groups in pectic polysaccharides. The remaining principal feature in the FTIR spectra of washed leaves was strong absorbance in 1200-900 cm^{-1} region related to stretching and bending of C-O-C and C-OH bonds characteristic for cuticular saccharides, including monosaccharides, such as arabinose, xylose, mannose, glucose, galactose and uronic acids, as well as polysaccharides, predominantly pectin, hemicellulose and cellulose [54]. Finally, the spectra showed distinctive bands at 1515 and 830 cm^{-1} associated with the vibrations of aromatic rings of phenolic compounds [54, 55].

Principle component analyses of FTIR spectral data indicated strong effects of both UV and RH on leaf cuticle chemical composition (Fig. 5). UV treatment correlated with PC1, while RH correlated with PC2 (Fig. 5b). PC1 loadings indicated that plants exposed to UV radiation had a higher relative content of uronic acids and phenolics, and lower content of cellulose and non-uronic based hemicellulose, than plants not exposed to UV (Fig. 5C). PC2 loadings indicated that high RH plants had a higher relative amount of cuticular waxes and lower amounts of proteins (probably cell wall glycoproteins) and uronic compounds than moderate RH plants (Fig. 5C). Mann-Whitney U tests, based on the PCA principal component scores between samples, confirmed that the UV effect on total chemical composition of leaves was significant at both moderate and high RH.

PLS-DA results indicated that the effect of UV on cuticle chemical composition was stronger in both control and washed leaves from high RH-grown plants compared to moderate RH-grown plants (Table 2, +UV vs -UV). This effect was stronger on the adaxial than on the abaxial side of the leaves. The effect of RH on cuticle chemical composition was stronger in plants exposed to UV compared to plants not exposed to UV (Table 2, 60% vs 90%). This was seen in both control and washed leaves on both adaxial and abaxial leaf sides (Table 2). Variability analyses based on Pearson Correlation Coefficients (PCCs) indicated that growth in high RH resulted in a more uniform cuticular chemical composition between individual leaves compared to moderate RH, irrespective of UV treatment (Table 3).

3.4.3 Antioxidant power of whole leaves

Total antioxidant capacity of whole leaves was tested using a FRAP assay (Fig. 5). Plants grown in high RH had significantly lower total antioxidant capacity that plants grown in high RH

(p = 0.0133). No effect of UV radiation was found, no significant interaction was found between RH and UV exposure (Fig. 6).

4 Discussion

4.1 UV radiation induced damage in plants grown at high RH, but did not affect growth or photosynthetic capacity

Night-time exposure to UV during growth caused plant injuries. There was no visible leaf damage in either RH treatment that had not received UV radiation, yet there was leaf curling in leaves exposed to UV radiation (Fig. 3A), with significantly more in high RH than moderate RH. More severe damage, in the form of chlorosis, some necrosis, and significant CPD-DNA damage was found in UV-exposed leaves from high RH, but not in any of the other treatments (Fig. 3B-D). This clearly shows that growth over time in high RH makes plants more susceptible to UV-induced stress than growth in moderate RH. CPD-DNA damage is repaired by blue light-dependent photolyase [56], and Li et al. [57] found repair of 83% of CPD after 2 h irradiation with white light. The presence of a significant amount of CPDs may be due to the low amount of blue light present in HPS lamps (approximately 5%). Additionally, exposure to UV radiation during the night, as opposed to simultaneous exposure to daylight and UV, may have further decreased the plants' ability to repair DNA damage [56].

In spite of the visible and CPD-DNA damage caused by exposure to UV radiation, neither RH nor UV radiation had an effect on the maximum efficiency of photosystem II in pea plants, indicating that UV radiation did not induce stress on photosystem II in either RH treatment. Furthermore, no significant differences in cellular leaf structure were seen as a result of either RH or UV radiation (Fig. 3E).

Taller pea plants with more leaves in high RH as compared to moderate RH is similar to previous findings in *Rosa hybrida* [58], *Gossypium hirsutum* [59], and several foliage species [60]. However, while previous findings have shown a reductive effect of UV radiation on plant height [39, 61, 62], exposure to UV radiation during the night had no significant effect on plant height in this experiment (Fig. 4A). Roro et al. [63] showed that UV-B induced reduction in stem elongation in pea was mediated through a reduction in bioactive gibberellin (GA), which acts on cell division and cell elongation in the subapical meristem. In the present experiment, a lack of UV effects on

growth may be due to differences in experimental growth conditions, such as light and temperature [3], or the time and dose of UV radiation exposure.

4.2 Flavonoid content and antioxidant power are reduced in high RH leaves

The results indicated a trend towards decreased phenolics and flavonoids in response to high RH and UV exposure, most prominently in the 'strong antioxidant' [64] quercetin-glycoside (Table 1). This may be due to the light conditions during growth. According to Siipola et al. [65], attenuation of solar blue light resulted in a greater reduction in leaf flavonoid content than attenuation of UV radiation in pea plants. Similarly to the lack of CPD-DNA damage repair by photolyases described above, the light provided by HPS lamps may have had insufficient blue light for flavonoid accumulation. This, coupled with UV radiation received during the dark period, as opposed to in combination with other light, indicates some support for flavonoid accumulation being more dependent on light, rather than UV radiation. Enzymes involved in the synthesis of several flavonoids are found to be highly responsive in plants exposed to a wide range of environmental stresses [66], including drought stress [8]. In our experiment, RH was shown to affect flavonoid accumulation: plants grown in high RH had significantly lowered flavonoid content than plants grown in moderate RH (Table 1). This indicates a possibility that high RH is construed as well-watered conditions by the plant, thereby diminishing the need for flavonoid accumulation. Such a scenario is compounded by the results of the FRAP assay (Fig. 6), which showed a decrease in total antioxidant power in leaves grown in high RH. Antioxidant power, measured in the FRAP assay as the power of a sample to reduce a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous (Fe^{2+}) form [67], indicates the ability of the sample to scavenge excess ROS, which have the potential to cause oxidative damage. Taken together, these results show that plants grown in high RH were more susceptible to oxidative damage by UV radiation due to decreased leaf flavonoids, and total antioxidant power, and may explain the increased visible damage and presence of CPD-DNA in high RH +UV plants (Fig. 3).

4.3 UV exposure increases content of phenolic and uronic compounds in leaf cuticles, while high RH increases epicuticular wax

The FTIR-based chemical characterization of leaf cuticles was in accordance with the published data [68-71]. These analyses indicated an increase in content of phenolic and uronic

462 compounds, as well as decrease in content of cellulose and non-uronic based hemicellulose (such
463 as arabinans and xyloglucans), in leaves exposed to UV radiation at both RH levels (Fig. 5). It is
464 important to note that, although the total concentration of phenolic compounds in whole leaves of
465 UV exposed plants decreased (Table 1), the phenolic content in the leaf cuticular layer actually
466 increased (Fig. 5). Therefore, in leaves grown with high UV exposure the epidermis probably has
467 better UV protection by phenolics than control group leaves.

468 However, the main difference in cuticular chemistry between the control and UV-exposed
469 leaves is not in the phenolic content, but rather in the content of uronic compounds. In leaves
470 grown with UV exposure, the cuticular layer had higher content of uronic compounds than in
471 control group leaves. Uronic acids and esters are the principal components of plant cuticles [71].
472 They are embedded in cuticle layer either as monosaccharides, such as glucuronic and galacturonic
473 acids, or as monomer units incorporated in backbone chains in pectic polysaccharides, such as
474 pectin, and side chains in hemicellulosic polysaccharides, such as arabinogalactans and xylans [71,
475 72]. It has been proposed that UV exposure of plant tissue leads to pectin degradation into methane
476 and galacturonic acid in the plant cell wall [70, 73]. Though this mechanism potentially leads to
477 production of superoxide, it may also have a beneficial effect by: 1) release of methane and
478 superoxide as stress-signalling molecules, and 2) accumulation of uronic acid as a precursor in the
479 biosynthesis of ascorbates [74, 75]. Ascorbates can have an essential role in stress mitigation as they
480 act as reducing agents, protecting plants against oxidative stress. The amount of reactive oxygen
481 species (ROS) may increase dramatically under increased UV irradiation and lead to high level of
482 oxidative stress. Therefore, high content of uronic acids, either as free chemicals or as monomers
483 in pectic polysaccharides, may have great protective potential as a build-up of antioxidant
484 precursor chemicals [76]. Moreover, constrained generation of ROS in cuticles and the outer cell
485 wall, where ROS concentration can be regulated by ascorbate biosynthesis, is favoured when
486 compared with considerably more harmful intracellular build-up of ROS.

487 Growth in high RH was seen to increase content of cuticular waxes, and decrease the
488 content of proteins and uronic compounds. It is difficult to assess whether this observation was
489 due to higher production of waxes or lower production of proteins and uronic compounds in plants
490 grown under high RH. Previous studies have indicated that high RH may and may not affect wax
491 coverage and morphology [36]. Should this be the case, the content of proteins and uronic acids is
492 decreased in plants grown in high RH. This may cause a reduction in potential antioxidant power

in the cuticle and upper epidermis, which reflects the situation found in whole leaves grown in high RH (Fig. 6).

4.4 UV radiation did not improve stomatal function in plants produced at high RH but reduced conductance in intact plants and increased water loss in detached leaves

We hypothesised that exposure to UV radiation may trigger stomatal closure in pea plants grown in high RH, and thereby re-establish stomatal function. Indeed, our results showed that pea exposed to UV had lower instantaneous conductance rates immediately after removal from light conditions than plants developed without UV (Fig. 2B). While Jansen and Van den Noort [11] reported that UV exposure may induce stomatal opening or stomatal closure, dependent on the metabolic state of the guard cells, several other studies have previously reported stomatal closure as a response to UV radiation [13, 77, 78]. This takes place either through an increase in ABA concentration, or via regulation by the UVR8 photoreceptor in a signalling cascade involving COP1 and HY5 in *Arabidopsis* in a NO-dependent mechanism [13]. In this study, the degree of stomatal closure due to UV exposure was similar in both moderate and high RH (Fig. 2B). As previously shown [21, 32], plants developed in continuous high RH had higher instantaneous conductance rates than plants developed at lower RH (Fig. 2B). Previous studies on *R. hybrida*, *Arabidopsis thaliana*, *Vicia faba* and *T. virginiana* have shown that stomata developed in continuous high RH are unable to close when exposed to environmental closing signals, such as darkness or exogenous ABA treatment [21-24, 31]. However, the results presented here show closure of stomata in a dark, low RH environment, given sufficient acclimation time, in all treatments (Fig. 2B).

Stomatal conductance was higher in plants grown in high RH compared to moderate RH, showing agreement with previous findings in other species [21, 23]. Though stomatal closure was eventually induced in plants grown in high RH (significant reduction after three hours), exposure to UV did not improve the response time. We hypothesised that due to the stomatal closure response induced by UV exposure [13], UV radiation would improve stomatal responsiveness after growth in high RH. Despite UV-exposed plants having lower instantaneous conductance than plants not exposed to UV, UV exposure resulted in a decreased rate of responsiveness to closing stimuli, indicating that exposure to UV does not improve stomatal responsiveness.

Finally, we found that pea leaves developed in high RH lost more water during a three hour desiccation test than leaves from moderate RH (Fig. 2A). This shows that the stomata do not close properly in leaves from high RH in response to desiccation alone. Furthermore, despite lower instantaneous conductance rates, UV-exposed detached leaves lost more water than controls at both RH levels, indicating no improvement in stomatal closure as a result of UV exposure.

4.3 Conclusions

The present study shows that in pea plants grown in continuous high RH, stomata are more open and less responsive to closing stimuli. The hypothesis that UV exposure would trigger stomatal movement and thereby increase responsiveness has been refuted. While plants grown in both moderate and high RH and exposed to UV had lower instantaneous stomatal conductance rates, the rate of responsiveness to closing stimuli was not improved. Furthermore, plants grown in continuous high RH were more susceptible to UV-induced damage than when grown in moderate RH. This was due to a reduction in leaf flavonoid content and a reduction in leaf antioxidant power, though the mechanisms behind this remain undetermined. UV radiation is a potentially powerful tool in protected plant production but background humidity conditions need to be taken into consideration.

Conflict of interest

There are no conflicts to declare

Acknowledgments

We would like to thank Ida Kristin Hagen for taking care of the plants throughout the experiments. This research was supported by The Norwegian Research Council (Grant 223268/F50 (CERAD)) and VeksthusDynamikk (190395).

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6 Tables

Table 1 Concentration of phenolic compounds in pea leaves grown under moderate (60%) or high (90%) relative humidity RH, with (+UV) or without (-UV) UV radiation. Means ± SE, *n* = 4-5 for three replicate experiments, total *n* = 12-15. Different letters indicate significantly different values.

	60% RH		90% RH	
	-UV	+UV	-UV	+UV
Phenolic acids (mg g^{-1})	0.74 ± 0.04 ^{ab}	0.58 ± 0.06 ^{ab}	0.76 ± 0.06 ^{a}	0.54 ± 0.03 ^{b}
Chlorogenic acid (mg g^{-1})	0.12 ± 0.02 ^{b}	0.11 ± 0.01 ^{b}	0.14 ± 0.01 ^{ab}	0.18 ± 0.01 ^{a}
Total Phenolic acids (mg g^{-1})	0.85 ± 0.05 ^{a}	0.69 ± 0.06 ^{a}	0.91 ± 0.07 ^{a}	0.73 ± 0.04 ^{a}
Quercetin-glycosides (mg g^{-1})	16.17 ± 0.67 ^{a}	14.90 ± 0.54 ^{ab}	13.42 ± 0.93 ^{ab}	12.57 ± 0.74 ^{b}
Kaempferol-glycosides (mg g^{-1})	0.03 ± 0.004 ^{a}	0.03 ± 0.002 ^{a}	0.03 ± 0.003 ^{a}	0.02 ± 0.006 ^{a}
Total flavonoids (mg g^{-1})	16.20 ± 0.67 ^{a}	14.92 ± 0.54 ^{ab}	13.45 ± 0.93 ^{ab}	12.59 ± 0.74 ^{b}

Significance based on two-way ANOVA followed by post-hoc Tukey HSD analyses.

Significance level: $p \leq 0.05$.

Table 2 Comparison of ATR-FTIR data for chemical composition of leaf surfaces (dried leaves) of pea plants grown under moderate (60%) and high (90%) relative humidity (RH) and exposed (+UV) or not exposed (-UV) to UV radiation. Three measurements were taken on each of ad- and ab-axial sides of each leaf, taken on five leaflets from different individuals in each treatment. R^2 values, shown for adaxial and abaxial sides of control (detached, dried) and chloroform-washed leaves, indicate the degree of difference between the different treatments.

		+UV vs –UV (R^2)		**60% vs 90% RH (R^2)**	
Leaf side	**Wash treatment**	**60% RH**	**90% RH**	**+UV**	**-UV**
Adaxial	Control	0.82	0.90	0.86	0.74
	Washed	0.74	0.85	0.95	0.89
Abaxial	Control	0.68	0.77	0.87	0.83
	Washed	0.67	0.83	0.91	0.76

Table 3 Comparison of variability values for dried leaflets of pea plants grown in moderate (60%) or high (90%) relative humidity (RH) and either exposed (+UV) or not exposed (-UV) to UV radiation. Three measurements were taken on each of ad- and ab-axial sides of each leaf, taken on five leaflets from different individuals in each treatment. Variability values, calculated from Pearson correlation coefficients, indicate the degree of variability between individual leaves based on leaf side (ad- or abaxial), wash treatment for each leaf side (control or chloroform-washed, RH with UV radiation notwithstanding), RH, and between +UV and –UV for each RH level. Partial least-squares–discriminant analysis (PLS-DA) was performed based on ATR-FTIR data.

Leaf side	**Wash treatment**	**RH**	**Variability**				
			Leaf side	**Wash treatment**	**RH**	**+UV**	**-UV**
Adaxial	Control	Moderate	105	84	104	78	76
		High			38	18	16
	Washed	Moderate		120	152	89	76
		High			39	18	31
Abaxial	Control	Moderate	85	80	108	68	105
		High			36	12	21
	Washed	Moderate		84	107	70	77
		High			36	12	29

Pearson correlation coefficient (PCC): 1900–700 cm^{-1}

Variability = (1-PCC*) × 10^{-4}

7 Figure legends

Fig. 1 Spectral power distribution (SPD) for Q-panel UV 313 lamps (Q-Lab Corporation, Ohio, USA) measured in W m^{-2} nm^{-1}. Adapted from Q-Lab Corporation. UV-A, UV-B and UV-C regions are indicated.

Fig. 2 Water loss and stomatal conductance in pea plants grown under moderate (60%) and high (90%) relative humidity (RH) with (+UV) or without UV radiation. A) Water loss from detached leaves 3 h after detachment and transfer to a common environment (40% RH, 15 µmol m^{-2} s^{-1}). Means ± SE are shown, *n* = 5 for five replicate experiments, total *n* = 25; B) Stomatal conductance measurements in a time course after removal to a dark environment (40% RH). Means ± SE are shown, *n* = 3 for two replicate experiments, total *n* = 6. Different letters indicate statistically significant differences ($p \leq 0.05$) as analysed by analysis of variance followed by Tukey`s HSD test.

Fig. 3 Injuries (A-D) and cross sectional leaf anatomy (E) in pea plants grown under moderate (60%) and high (90%) relative humidity (RH) with (+UV) or without UV radiation. A) The number of leaflets showing leaf curling; B) the number of leaflets showing leaf chlorosis; C) the number of leaflets showing necrosis; and D) CPD-DNA damage in leaflets. Means ± SE, *n* = 3 in two replicate experiments, total *n* = 6. Different letters indicate significantly different values ($p \leq 0.05$) as analysed using ANOVA followed by Tukey's HSD test. E) Cross sections of leaflets embedded in LR-White stained with Stevenel's Blue taken using a 40x objective.

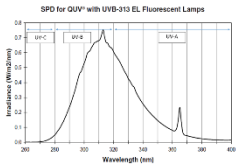
Fig. 4 A) Plant height; and B) number of leaves on plants growing in moderate or high RH, with or without UV radiation. Means ± SE are shown, *n* = 5-8 for four replicate experiments, total *n* = 24-27. Different letters indicate significantly different values as analysed using GLM followed by Tukey's HSD test.

Fig. 5 A) Averaged and preprocessed FTIR spectra of control (blue) and chloroform-washed (red) pea leaves grown under 60% relative humidity (RH) and exposed to UV radiation; B) Principle component analysis (PCA) score plot of FTIR spectral data set comprising measurements on the adaxial side (representative of results for both leaf sides) of the control leaves, with depiction of growth conditions (with (+UV) or without (-UV) UV radiation under moderate (60%) or high (90%) RH). The vectors approximate

the increase in relative amount of wax (W), phenolics (Ph), pectin (Pe), proteins (P), hemicellulose (H) and cellulose (C). The percent variances for the first five principal components (PCs) were 64.40, 15.06, 6.92, 4.34, and 2.28. C) Loading plots on the first two PCs of the PCA.

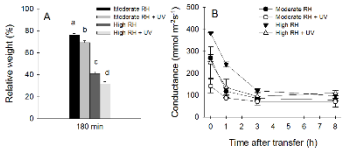
Fig. 6 Relative antioxidant capacity of whole leaves from pea grown in moderate (60%) or high (90%) RH with (+UV) or without UV radiation and tested using a FRAP assay. The values for the other treatments were normalized to the value for the moderate RH antioxidant capacity ± relative SE. Three technical replicates were measured from three leaflets from separate individual plants per treatment. This was performed in a single experiment * Indicates significant difference due to RH (RH: $p < 0.05$).

907 Fig 1



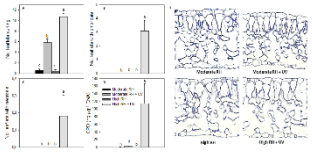
908

909 Fig 2



910

911 Fig 3



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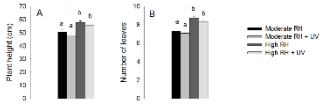
913 Fig 4



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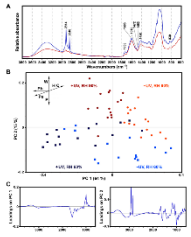
915 Fig 5



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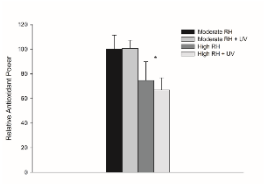
917 Fig 6


Relative Antioxidant Power
120
100
80
60
40
20
0
Moderate RH
Moderate RH + UV
High RH
High RH + UV
*

918