Environmental and Experimental Botany 241 (2026) 106290

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Research paper

Linking stomatal function with photosynthetic light reactions and stress response in faba bean

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ARTICLE INFO

Keywords:
Phenotyping
Leaf temperature
Regulations of photosynthesis
Ozone tolerance

ABSTRACT

Faba bean (*Vicia faba* L.) is a key protein crop, but its cultivation and yield stability are hindered by a number of environmental stresses. Stomata regulate gas exchange between the plant and atmosphere, playing a central role in photosynthesis and mediating plant responses to a wide range of environmental stressors. This study aimed to investigate variations in photosynthetic regulation in faba bean, and to examine leaf temperature and the response to short-term acute ozone (O_3) exposure as proxies for stomatal function. Here, we used a high-throughput plant phenotyping (HTPP) platform to screen 196 faba bean genotypes for photosynthetic and stomatal function under controlled conditions. A subset of extreme genotypes, identified based on relative leaf tempreture from the initial screening, was exposed to a 450 ppb O_3 treatment. Our results revealed strong positive relationship between photosynthetic efficiency and relative leaf temperature. A three-fold difference in relative leaf temperature was observed among genotypes. The O_3 treatment caused signicantly less damage in genotypes with higher leaf temperature compared to those with lower leaf temperature ($p < 0.001$). By combining a HTPP platform with elevated O_3 stress treatment, we identified faba bean genotypes with contrasting stomatal responses to the O_3 exposure. Our results advance understanding of the regulation mechanisms of photosynthetic light reactions and the role of stomatal function in modulating faba bean responses to environmental stressors.

1. Introduction

Faba bean is a globally important cool-season grain legume crop, valued for its high-protein, nutrient-rich seeds and its versatility as a source of food and animal feed (Duc, 1997; Khazaei and Vandenberg, 2020). In addition, its ability to fix atmospheric nitrogen reduces dependence on synthetic fertilizers, positioning it as a key species in low-input and environmentally sustainable cropping systems (Klippenstein et al., 2022). However, the cultivation of faba bean remains limited due to its susceptibility to a number of abiotic stresses such as water deficit (e.g., Khan et al., 2010; Muktadir et al., 2020), extreme temperatures (e.g., Bishop et al., 2016; Zhou et al., 2018), salinity (e.g., Bimurzayev et al., 2021; Tavakkoli et al., 2024), elevated tropospheric ozone (O_3) levels (e.g., Otieno et al., 2022), and a wide range of biotic stresses (e.g., Rubiales and Khazaei, 2022). Increasing climate variability and environmental constraints emphasize the need to develop faba bean cultivars with improved resilience to adverse environmental factors (Mouritzen et al., 2025). Such improvements depend on a better understanding of morpho-physiological traits related to stress adaptation in this crop. Phenotyping diverse faba bean germplasm collections for stress-relevant physiological traits accelerates the identification of pre-breeding resources for use in crop improvement programs aiming to ehance stress resilience (Adhikari et al., 2021) and provides critical insights into the mechanisms underlying stress adaptation in this species.

Stomata are central regulators of plant responses to many abiotic and

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https://doi.org/10.1016/j.envexpbot.2025.106290
Received 29 August 2025; Received in revised form 13 November 2025; Accepted 1 December 2025
Available online 2 December 2025

biotic stresses, coordinating gas exchange, transpiration, and resistance to pathogens. Stomata control the exchange of water vapour and CO_2 between leaves' internal air spaces and the atmosphere. Thus, adjusting stomatal opening to the growing environment is crucial for plant growth and production. In many environmental stress scenarios, such as water stress or pathogen infection, more closed stomata may mitigate the adverse stress effects, but this concurrently decreases photosynthetic carbon assimilation by limiting CO_2 flux into leaves (e.g., Salmon et al., 2020). Stomata are one of the main gateways for the entry of some pathogens inside leaf tissues. They are thus intimately linked to plant immune reactions and biotic stress resistance (Waszczak et al., 2018). Overall, under diverse environmental conditions responsiveness and control of stomatal aperture become critical for favourable carbon gain without excessive water loss as well as pathgen resistance. As water evaporation requires energy, leaves cool down with transpiration. As transpiration rate depends on stomatal conductance, differences in leaf temperature are correlated with differences in stomatal conductance. Leaf temperature has shown strong correlation with stomatal function in various plant species (e.g., Jones, 1999; Chaerle et al., 2007; Sirault et al., 2009) including legumes (Reynolds-Henne et al., 2010), and faba bean (Khan et al., 2010; Khazaei et al., 2013). Phenotypic variation in leaf temperature under uniform environmental conditions may be a target for crop improvement programes aiming to balance crop productivity and resilience.

Photosynthesis is tightly regulated at multiple levels, including adjustments in light harvesting and carbon metabolism. One key protective mechanism is the rapidly reversing component of non-photochemical quenching (NPQ), which allows plants to dissipate excessive light energy as heat under high light or stress conditions, preventing damage to photosynthetic apparatus. The magnitude and dynamics of NPQ, including its relaxation after transitions to lower light were shown to directly affect plant growth and production (Kromdijk et al., 2016; De Souza et al., 2022). The functional link between the regulation of photosynthesis and stomatal function has been described in various plant species (e.g., Stirbet et al., 2024; Murchie and Lawson, 2013), but it has not been systematically characterised in faba bean. More emphasis has been put on stomatal morphology and function and their role in stress response in this species (e.g., Darwish and Fahmy, 1997; Khazaei et al., 2013; Müllers et al., 2022).

Ozone (O_3) serves as a model environmental stressor that induces formation of reactive oxygen species (ROS), key signaling molecules involved in plant development and in responses to abiotic and biotic stresses (e.g., Kangasjärvi et al., 2005; Iyer et al., 2012; Krasensky et al., 2017). O_3 gas enters leaf tissues though stomata, triggering immune reactions that under acute O_3 exposures may cause programmed cell death. O_3 may be used to rapidly and uniformly impose stress for screening large numbers of genotypes for stomatal function (e.g., Waszczak et al., 2024). This approach allows screening large germplasm collections in a high-throuput manner, that is not feasible with gas exchange measurements. The phenotypic varation in response to acute O_3 exposures in faba bean remain poorly studied.

In this study, we used high-throughput plant phenotyping (HTPP) tools to study the variation of leaf temperature and photosynthetic regulatory processes in 196 faba bean genotypes of different origins, using HTPP of diverse chlorophyll fluorescence parameters and leaf temperature. We also assessed the effect of acute O_3 exposure on subset of faba bean genotypes with contrasting leaf temperatures.

2. Materials and methods

2.1. Plant material

One hundred ninety-six faba bean genotypes were used in this study (Table S1). All faba bean genotypes were selfed for at least one generation in an insect-proof greenhouse. Seeds harvested from single plants were used for this study.

2.2. Experiment 1: screening faba bean germplasm

2.2.1. Growing conditions

Experiment 1 was conducted in the climate-controlled glasshouse of the University of Helsinki, Viikki campus, Finland, using a randomized complete block design with four replicates in December 2023. Seeds of all genotypes were inoculated with *Rhizobium leguminosarum* biovar. *viciae* (faba bean strain, Elomestari Oy, Tornio, Finland) before sowing. Seeds were sown in black plastic pots (8 × 8 × 8 cm, apploximately 0.6 L) containing a 1:1 mixture of peat and vermiculite (Karkea Ruukutusseos, WR8014, Kekkilä Oy, Vantaa, Finland) containing all essential nutrients. Each replicate was placed on a separate greehouse table. Soil moisture was maintained at field capacity with automatic table irrigation (three times per week) ensuring that all plants were under well-watered growing conditions. The photoperiod was set to 14 h of light and 10 h of darkness, with a day/night temperature regime of 21 °C/15 °C (±2 °C), and relative humidity was maintained at 60 %. The main light source was high pressure sodium lamps (400 W, Philips, Netherlands). Photosynthetic photon flux density (PPFD) at the canopy level was approximately 250 µmol m^{-2} s^{-1}.

2.2.2. HTPP phenotyping

Phenotyping based on multiple types of images was conducted on 4-week-old seedlings of 196 faba bean genotypes using PlantScreen SC Mobile System (PSI, Czech Republic) that was located in the darkened compartment of the same growth room, assuring minimal changes in temperature and humidily during the imaging process. Four individual seedlings per line were phenotyped. For each imaging session, twenty four randomly picked plants of different genotypes were arranged on a single tray, thermal and chlorophyll fluorescence (CF) images were acquired from a top view. Thermal images were captured immediately after loading the trays into the phenotyping chamber using the inbuilt InfraTec VarioCam thermal camera (1024 × 768 pixels). The measurments were peformed in darkness. The climate control systems of the imaging space assured constant ambient temperature across the visual field. High-resolution grayscale images were analyzed in ImageJ, with relative leaf temperature expressed as grayscale intensity in defined leaf areas. For this, the images were converted to 8-bit grayscale, the areas of equal shape and area were selected over the representative leaf images, and "Measure" command was used to calculate the mean gray value of these areas. To enable comparisons between trays, individual plant values were normalized to the total signal of all 24 plants corresponding to different randomized genotypes within the same tray.

After thermal imaging, plants were adapted to darkness for 30 min, then CF imaging was performed with the inbuilt 1.4-megapixel TOMI-2 camera (PSI). The imaging protocol included the measurments of Fo and Fm (dark-adapted minimal and maximal fluorescence, respectively), then a stepwise light ramp produced by the device's LED array (cool white, 6500 K) and applied in 1.5-min steps of PPFD 200, 400, 600, 800, 1000, and 1200 µmol m^{-2} s^{-1} , which was followed by a 7.5-min dark relaxation period. The adaptation times were chosen as a compromise between achieving a reasonable quasi-steady state of response and maintaining high screening throughput (Kalaji et al., 2014). Saturating pulses (800 ms, ~3000 µmol m^{-2} s^{-1} at canopy level) were triggered at the end of each light step to determine Photosystem II (PSII) quantum yields and NPQ. Maximum PSII efficiency (Fv/Fm) was calculated as (Fm − Fo)/Fm. NPQ was calculated as (Fm − Fm′)/Fm′ (Horton and Ruban, 1992), ETR as [(Fm'-Fs)/Fm'] * PAR * 0.5 * 0.84, where Fm' is maximal flurescence and Fs is steady-state fluorescence for the given light intensity (Genty et al., 1989). NPQ relaxation rate was assessed by measuring partially relaxed NPQ after 1.5 min of darkness following the end of the light ramp. Data were processed using FluorCam 10 software (PSI).

2.3. Experiment 2: acute ozone treatment

Based on results of the experiment 1 and seed stock avaiability, four genotypes from the lowest (#713, #138, #247, and #024) and three from the highest (#191, #020, and #088) relative leaf temperature groups were selected for the acute O_3 exposure experiment.

2.3.1. Growing conditions and ozone exposure

The plants were grown in the same plastic pots and medium as above, in the Aralab chambers (FITOCLIMA BIO, S600/D1200) under white LED light (LED TUBE T5, 4000 K, Ledvance) at 16 h of light (7:00–23:00) and 8 h of darkness photoperiod, with a day/night temperature regime of 21 °C/19 °C (±1 °C), day/night relative humidity 70 / 60 %, and PPFD ~220–250 μmol m^{-2} s^{-1} at the canopy level. The seedlings were exposed to elevated O_3 treaments 18–21 days after sowing. For this, they were moved to ozonation chamber with the same day length, light intensity, temperature and relative humidity on the night before the treatment and in the following morning, after 2 h of light (7:00–9:00), exposed to O_3 gas (450 ppb) for six hours (9:00–15:00). The experiment was performed twice with four replicates.

2.3.2. Thermal imaging

The non-stressed seedlings described in 2.3.1 were imaged with a thermal camera (382 ×288 pixels, 0.04 °C temperature resolution, PI-450, Optris, Germany) just before O_3 stress experiments.

2.3.3. Quantification of ozone-induced damage

For quantification of O_3-induced damage in visible spectrum, all developed leaves were detached and scanned with a flatbed scanner (Epson Perfection V750 Pro). The color balance of the scanned images was changed to make the damaged areas more visible: cyan-red 30 % towards red and magenta-green 30 % towards magenta. Sizes of the discolored areas were then measured and compared to total leaf sizes with Fiji distribution package of ImageJ 2 (Rueden et al., 2017; Schindelin et al., 2012). The areas counted as undamaged had three main color profiles: light green (approximate color values r 61–65, g 56–60, b 6–9), mid-green (approximate color values r 44–48, g 44–48, b 4–7), and dark green (approximate color values r 25–29, g 41–45, b 9–12), while the areas counted as damaged had two main color profiles: light brown (approximate color values r 56–60, g 40–44, b 10–13) and red (approximate color values r 46–50, g 19–23, b 16–19). Alternatively, the cut leaves were stored for three days in cold conditions (+4 °C) moisturized with wet paper towels prior to scanning to further visualize the damage. No color correction was necessary for images of cold-stored leaves, and the color profiles were defined as: undamaged light green (approximate color values r 52–56, g 62–66, b 26–30) and green (approximate color values r 32–36, g 47–53, b 5–10), damaged grey (approximate color values r 34–38, g 38–41, b 15–17), and black (approximate color values r 10–14, g 13–17, b 2–5). For detection of O_3-induced damage in near-infrared (NIR) spectrum, the detached leaves were imaged using the NIR system of the IMAGING-PAM M-Series (Walz) that employes reflection and backscattering of the 780 nm light beam. For false-colour imaging of Fv/Fm following the O_3 exposure, the leaves were placed on moist background immediately after detachment and acclimated to darkness for 10 min, after which Fv/Fm was determined with the IMAGING-PAM M-Series (Walz) using Walz measurment routines.

2.3.4. Stomatal morphology

Stomatal density and size were measured on the middle part of the abaxial surfaces of fully-expanded leaflets from non-stressed seedlings using the impression method. The stomatal imprints were taken from the same plants as were used for thermal imaging. Impressions were taken with Xantopren and its activator (Heraeus Kulzer GmbH, Germany), after which a thin layer of clear nail polish was applied to the impressions to create replicas. These replicas were then used for microscopic observations (Leica DMLB microscope with an attached ICC50W camera, Ernst Leitz Wetzlar GmbH, Heerbrugg, Switzerland). The number of stomata was calculated from at least three microscopic fields at 1218 × 914 μm (250x magnification), and stomatal size was measured at 500 × magnification and converted to μm on eight stomata on the same leaflet samples as were used for stomatal density measurments. The stomatal area was calculated as the outer length of each stomamultiplied by its width.

2.4. Statistical analysis

Even though the layout in the greenhouse experiment followed a complete blocks design, the plants were randomized ignoring blocks during HTPP, thus a simpler one-way ANOVA model was used for data analysis. Principal component analysis (PCA) was done using genotype means. Figures were created in R version 4.5.1 (R Core Team, 2025) with packages ggplot2 (Wickham, 2016), ggpmisc (Aphalo, 2025), segmented (Muggeo, 2025) and smatr (Warton et al., 2012). Segmented-linear-regression models with one change point and the slope of the left side segment constrained to zero were fitted using genotype means as shown in Fig. 3. Lines in Figure S3 were fitted by standardized major axis regression.

3. Results

3.1. Experiment 1

3.1.1. Variation in relative leaf temperature and photosynthetic performance in faba bean germplasm

Highly significant phenotypic variation ($p < 0.001$) in relative leaf temperature was observed among the studied 196 faba bean genotypes (Fig. 1 and Table S2). A three-fold difference in relative leaf temperature was observed between genotype #713, that had the lowest relative leaf temperature, and genotype #191, that had the highest relative leaf tempreture.

The measurements of photosynthetic light reactions in the 196 faba bean genotypes revealed large variation in Photosystem II (PSII) quantum yields (QY), electron transfer rates (ETR) thorugh PSII and NPQ levels at different light intensities. These traits and the raw CF parameters used to calculate them are presented in Table S2. The NPQ and ETR across different tested light intensities are shown in Fig. 2.

Substantial differences in the dynamics of NPQ and ETR between the genotypes with lower versus higher relative leaf temperature were detected (Fig. 2). Across different light intensities, NPQ was lower in most of the coldest (low relative leaf temperature) genotypes and higher in the warmest (high relative leaf temperature) genotypes (Fig. 2A). A reverse trend was observed in ETR, i.e., in the colder genotypes this parameter was overall higher than in the warmer genotypes (Fig. 2B).

3.1.2. Relationships between relative leaf temperature and photosynthetic performance in faba bean germplasm

The above results suggested a link between the relative leaf temperature – a proxy for stomatal conductance, and photosynthetic light reactions that was likely related to limitation of photosynthesis by CO_2 supply. We performed PCA on relative leaf temperature and 22 selected photosynthetic traits derived from CF imaging (Table S2) across the genotypes. The results are presented in Fig. 3A. The two dimensions explained 79 % of the total variance.

The vector of relative leaf temperature closely aligned with NPQ vectors at lower light intensities (200, 400, and 600 μmol m^{-2} s^{-1}), suggesting a strong positive correlation that decreased under higher light intensities. This indicates that the genotypes with the higher relative temperature exhibited higher NPQ. In the fitted linear spline models, variation in relative leaf temperature was able to explain nearly 50 % of the variation in NPQ observed accross genotypes (Fig. 3B). As expected, NPQ increased with increasing irradiance (Fig. 3B). Linear R^2

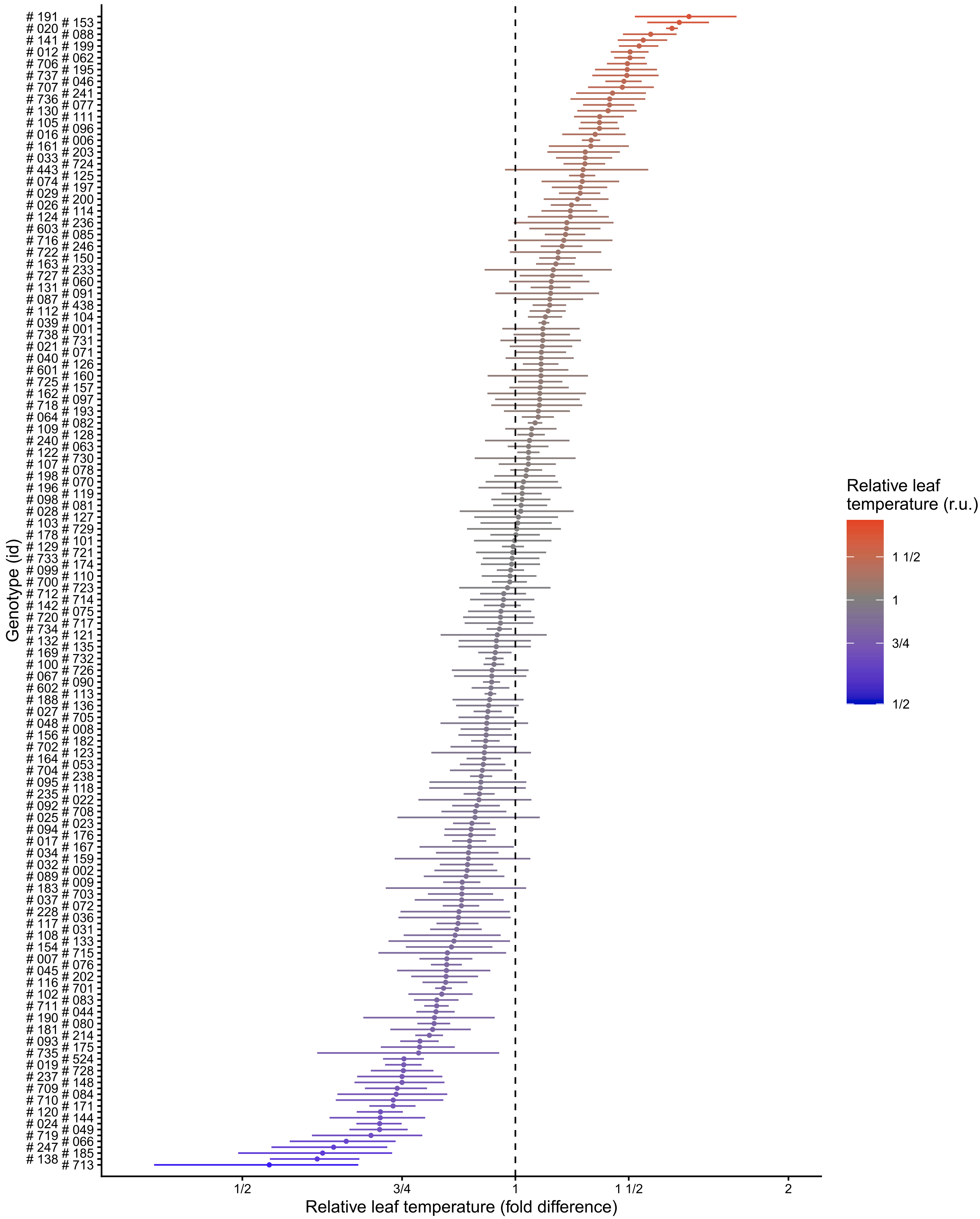

Fig. 1. Relative leaf temperature in 196 faba bean genotypes. The curve describes a cummulative empirical distribution. The genotypes are ordered along the *y*-axis by increasing relative leaf temperature, but equidistant. Colors indicate relative leaf temperature, ranging from highest (red) to lowest (blue) emphasizing the same information shown on the *x*-axis. The error bars indicate ±1 S.E.

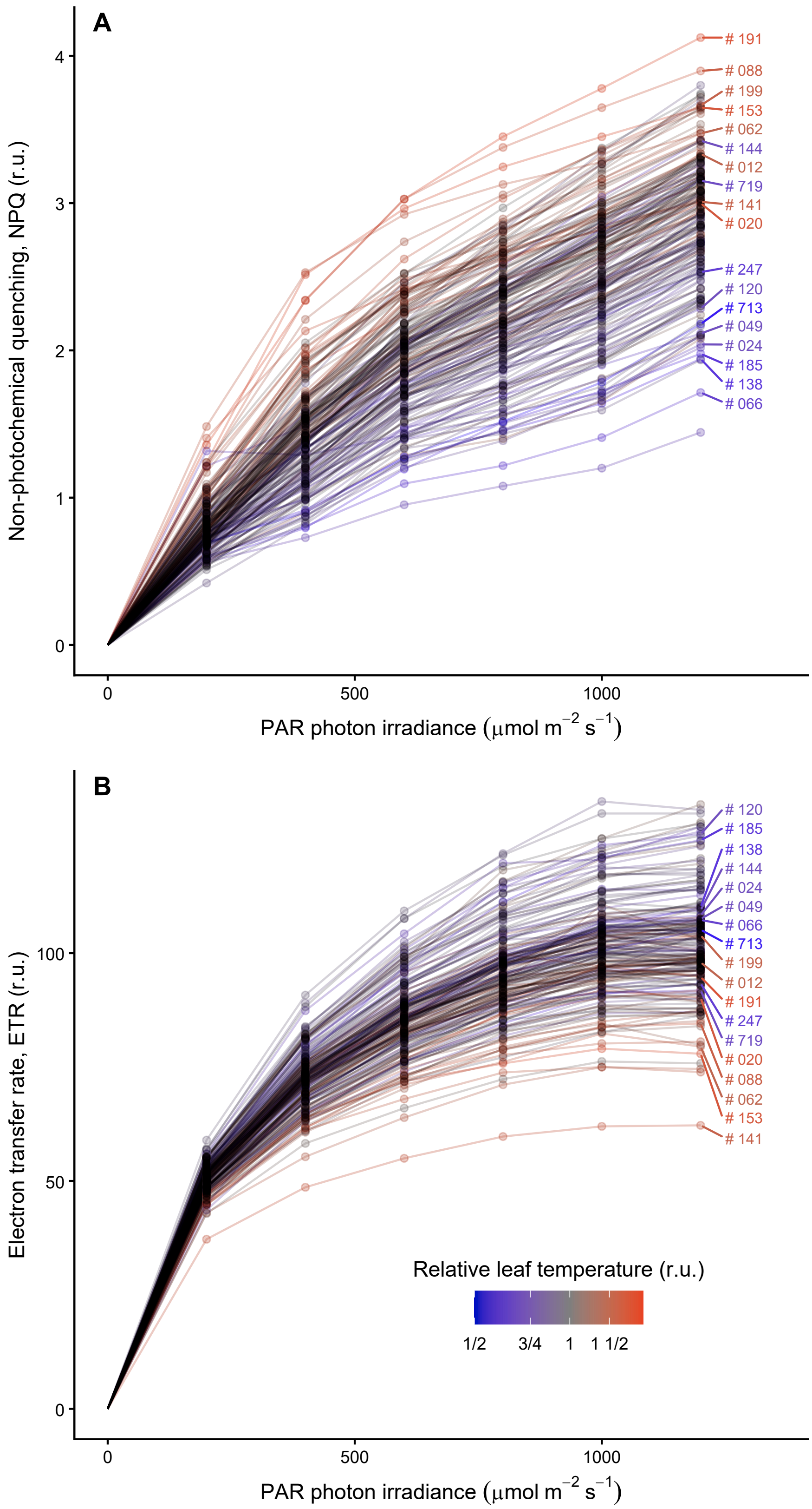

Fig. 2. Variation of photosynthetic traits across the tested genotypes. NPQ (A) and ETR (B) were calculated under different light intensities in the 196 genotypes. Relative leaf temperature of individual genotypes is shown with the same color-coding as in Fig. 1. Colors indicate relative leaf temperature, ranging from highest (red) to lowest (blue). The lines with the most extreme values of relative leaf temperature are labeled.

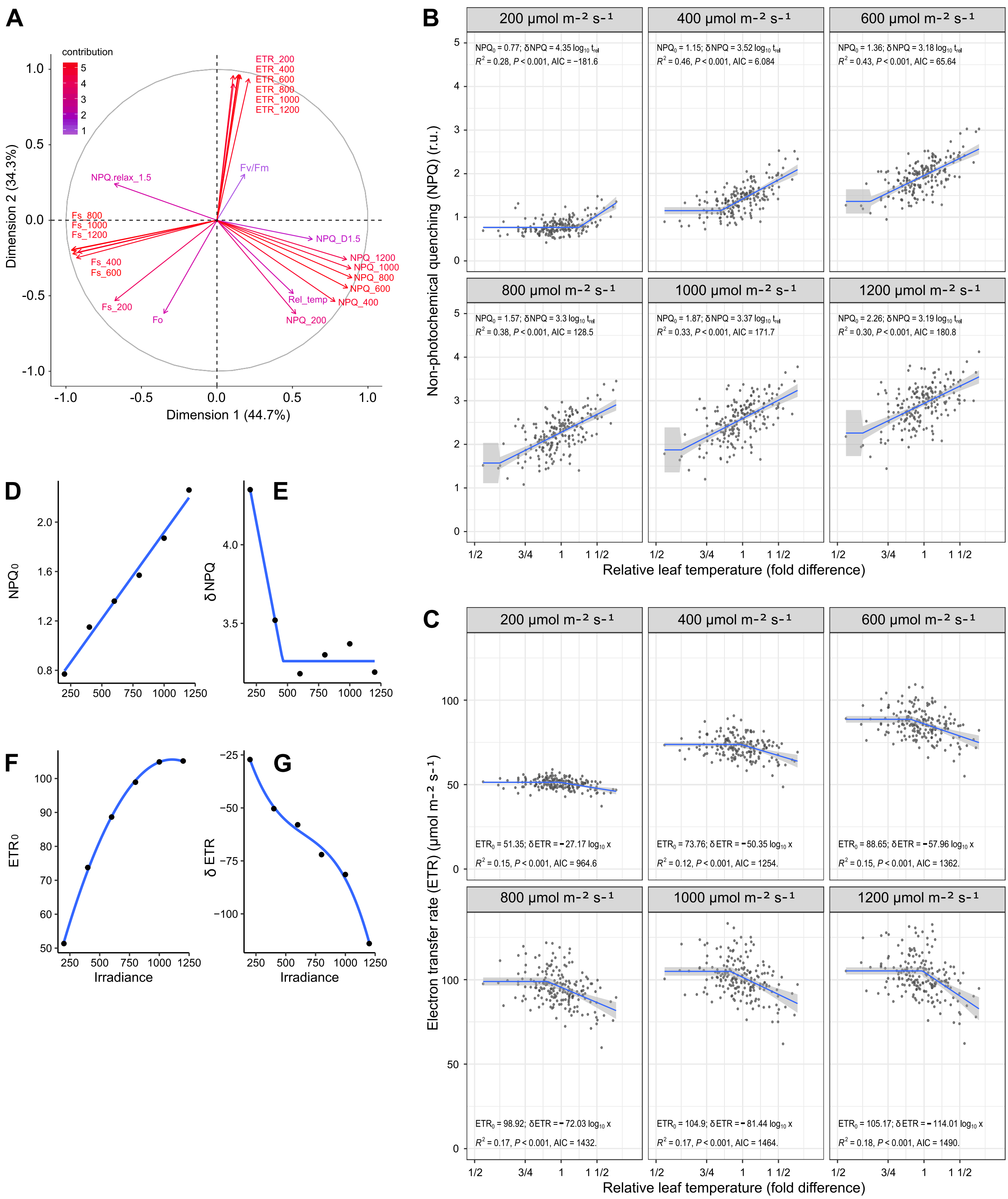

Fig. 3. Relationship between photosynthetic functions and relative leaf temperature across 196 faba bean genotypes. (A) PCA of 23 phenotypic traits including relative leaf temperature, as well as QY, ETR and NPQ measured under different light levels. NPQ_D1.5 is the value of NPQ following 1.5 min of dark relaxation after the light ramp; NPQ_realx_1.5 is the ratio of NPQ_D1.5 over NPQ at 1200 µmol m^{-2} s^{-1}; Fo is minimal and Fm is maximal dark-adapted CF; Fs is steady-state light-adapted CF; (*see* Table S2). (B) Regression NPQ on relative leaf temperature at different light intensities. (C) Regression ETR on relative leaf temperature at different light intensities. NPQ_0 and ETR_0 are the baseline values in the lines with low relative leaf temperatures, δNPQ and δETR are the slopes for NPQ and ETR on log_{10} (relative leaf temperature). (D), (E), (F), and (G) Parameters estimates from (B) and (C), plotted vs. photon irradiance.

between NPQ and leaf temperature increased with increasing irradiance until 400 and 600 µmol m^{-2} s^{-1} and then decreased. The maximum corresponds to PAR photon irradiance approximately two times higher than that during growth, suggesting that at these light intensities stomatal limitations on photosynthetic light reactions were strongest or more consistent among genotypes (Fig. 3B).

In contrast to NPQ, the PCA vectors corresponding to ETR at all light intensities and the commonly used parameter Fv/Fm (the maximal yield of PSII photochemistry in the dark-adapted state) negatively correlated with the relative leaf temperature, indicating that higher leaf temperatures were associated with lower photosynthetic efficiency across all light intensities and in dark-adapted state (Fig. 3C). However, relative leaf temperature was able to explain only between 10 % and 16 % of the variation in ETR among genotypes (Fig. 3C). The partially relaxed NPQ (NPQ_relax_1.5) showed the same trend (Fig. 3A). This indicated that higher relative leaf temperature may be associated with slower NPQ relaxation, which may reflect metabolic alterations imposed by lower stomatal conductance, resulting from smaller, fewer or more closed stomata.

Overall, relative leaf temperature strongly and positively associated with NPQ, but weakly and negatively with actual photosynthetic performance (ETR and Fv/Fm) and NPQ relaxation rate. This indicated that under stomatal limitations to photosynthesis during brief exposure to high light faba bean seedlings activated photoprotection at the cost of photosynthetic efficiency. The change points separating the flat and sloped segments of the regression lines (Fig. 3B and C) likely indicate a change in the limiting factor. We hypothesize that these change points mark the onset of stomatal limitation to photosynthesis, i.e., the level of stomatal closure beyond which photosynthetic NPQ or ETR can no longer be maintained at their baseline values. Notably, with increasing light intensity, the change points shift along the x-axis towards lower relative leaf temperatures, suggesting that stomatal constraints on photosynthesis become more pronounced under higher PAR (Fig. 3B and C). These light-dependent shifts differed between NPQ and ETR, indicating distinct mechanisms underlying their dependence on PAR and relative leaf temperature (Fig. 3D-G).

3.2. Experiment 2

3.2.1. Leaf temperature is linked to ozone stress response

To test whether the differences in relative leaf temperature, assumed to reflect differences in stomatal conductance, correlated with altered tolerance to O_3, which is also assumed to depend on stomatal conductance, we performed O_3 gas fumigation in three selected warm and four cold faba genotypes from experiment 1. Thermal imaging of the non-stressed plants confirmed the expected differences in leaf temperature among the selected extreme genotypes #713 and #191 (approximately 1 °C), although some genotypes identified in experiment 1 as cold (#247 and #024) did not follow the expected leaf temperature trend (Figure S1). Acute O_3 exposure led to development of lesions in the colder, but not in the warmer genotypes identified in experiment 1. O_3 lesions comprised large fraction of leaf area in colder genotypes (Fig. 4A-C), while there was significantly less damage in genotypes with high relative leaf temperature (Fig. 4A and B, Figure S2A). No damage was detected in control plants that were not exposed to O_3 (Figure S2B).For the same extreme genotypes, the density and size of stomata were measured in the abaxial epidermis. The correlations between relative leaf temperature and stomatal area (Figure S3A) and between relative leaf temperature and stomatal density (Figure S3B) were both moderate ($r = 0.57$, $r = 0.62$) and inconclusive ($P > 0.10$).

4. Discussion

This study explored phenotypic diversity of relative leaf tempreture and photosynthetic parameters in faba bean. We demonstrated the link between stomatal function and stress response by exposing faba bean genotypes with extreme relative leaf temperature to the acute O_3 stress. The relationship between O_3 tolerance and stomatal function has been

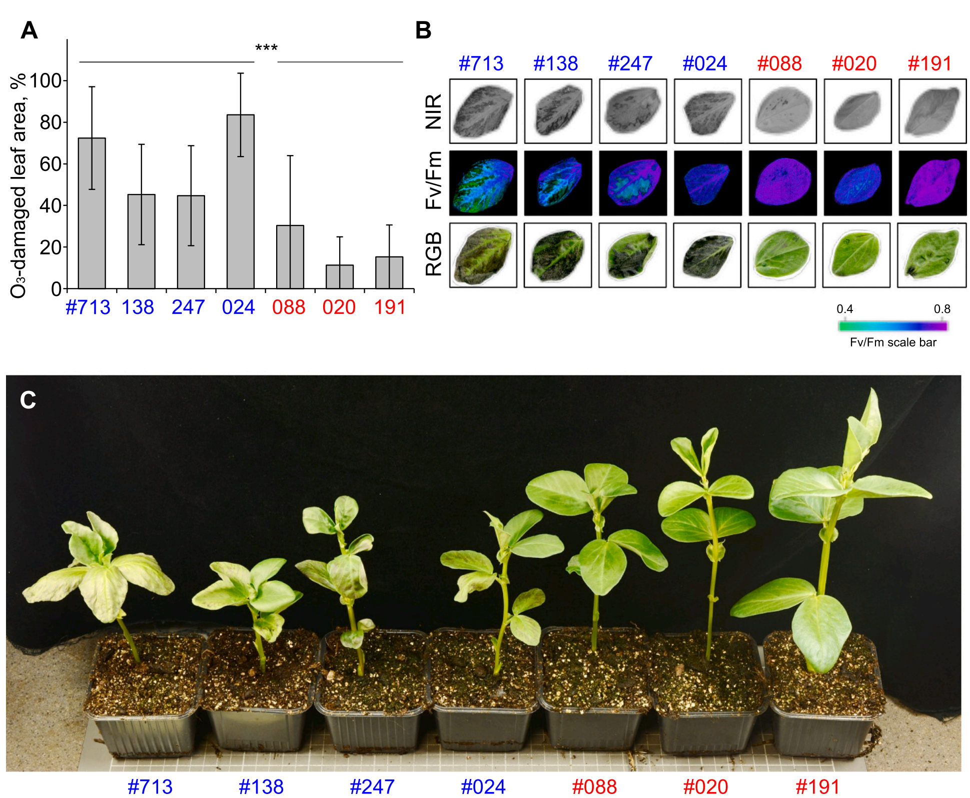

Fig. 4. Tolerance to acute O_3 exposure coincides with higher relative leaf temperature. (A) O_3 lesions comprised large fraction of leaf area in colder genotypes. *** indicates a significant difference ($p < 0.001$) between the mean of genotypes #713, #138, #247, #024 and the mean of genotypes #088, #020, #191 using *t*-test. The quantification is based on pooled data from two whole-experiment replicates, with four plants per genotype in each, but ignoring genotypes for the *t*-test. (B) Near-infrared (NIR) scans and photosynthetic yield (Fv/Fm) measurements were made in O_3-treated leaves. After three days of cold storage, the same leaves were scanned with an RGB scanner. Results for non-stressed leaves are presented in Fig. S2. (C) A photo of representative seedlings after the O_3 exposure.

reported in model plant species such as *Arabidopsis thaliana* (e.g., Waszczak et al., 2024), but remians less explored in crops (Yang et al., 2025). The combination of the above approaches allowed us to validate the link between leaf temperature and response to acute O_3 exposure, as proxies for stomatal function, and photosynthesis in *V. faba*. To our knowlegde, this is the first systematic study of these correlations across stomata-related phenotypes, photosynthesis and acute O_3 stress tolerance in faba bean.

Our results revealed a strong relationship between relative leaf temperature and photosynthetic light reactions. The genotypes with warmer leaves exhibited higher NPQ and lower photosynthetic ETR, while the opposite pattern was observed in genotypes with colder leaves. The relationships of ETR and NPQ with relative leaf temperature showed change points likely attribuitable to irradiance-dependent thresholds in photosynthesis-limiting factors. These patterns are consistent with pronounced stomatal limitations to photosynthesis in faba bean under irradiances doubling or more those during growth, whereby restricted CO_2 availability leads to downregulation of photochemistry and upregulation of protective energy dissipation. Such limitations likely constrain carbon assimilation under fluctuating environmental conditions and may affect growth and yield potential (e.g., Harrison et al., 2020). Interestingly, the strongest correlation was observed at PPFD 400 and 600 μmol m^{-2} s^{-1}, which is approximately two times higher than the growth light to which the seedlings were acclimated. At even higher irradiances the correlation was weaker because of increased variation among genotypes, as ETR reached a plateau in its response to irradiance.

The observed strong carbon limitation of photosynthesis and its variation among faba bean genotypes makes this species a promising model for studying the role of stomatal regulation in maintaining a favourable balance between water use and photosynthesis. In particular, the role of the light environment needs to be considered when examining water stress responses, and analyses of photosynthesis, including CF traits. In the short term, light directly drives photosynthetic electron transfer, regulates stomatal opening and is the source of energy for evapotranspiration. In a longer time frame, light irradiance and spectrum influence plants' acclimation and stress tolerance (Aphalo and Sadras, 2022). Thus, illumination conditions may prove instrumental in such studies and in genotype screening. As faba bean cultivation expands into regions with variable climate regimes, a whole-plant physiological perspective will be essential for guiding the future breeding and production strategies. The physiological diversity revealed in this study provides a foundation for tailoring variety selection to regional growing conditions.

To our knowledge, this is the first systematic study in faba bean to characterize NPQ dynamics under varying light intensities across a broad genetic panel. The combination of CF and thermal imaging - especially when implemented using HTPP platforms - offers a promising path forward for screening complex physiological traits. These tools were shown to significantly enhance selection efficiency by providing quantitative, non-invasive indicators of performance under various stress conditions in this specis (e.g., Pedruzzi et al., 2020; Chen et al., 2023; Yang et al., 2024; Poque et al., 2025). Our results establish a physiological framework for identifying and developing more efficient, stress-resilient faba bean cultivars under controlled growing conditions.

Our results point to a possible role of stomatal function in the reponse to short-term acute O_3 exposure in faba bean. Genotype #713, which had the lowest leaf temperature, showed more damage after acute ozonation, whereas genotype #191, with the highest leaf temperature, showed less damage. Reactions of plants to O_3 have been addressed in diverse plant species (Yang et al., 2025). The effects of O_3 on plants depend on both O_3 concentration and duration of exposure. Chronic low-level exposure typically reduces photosynthesis, inhibits growth, and accelerates senescence without visible tissue damage. In faba bean such exposures only led to moderate decrease in photosynthesis and respiration (Aben et al.,1990), and changes in transpiration (Turcsanyi et al., 2000). In contrast, acute exposure to higher dose of O_3 as used in the present study can induce programmed cell death and visible lesions in sensitive plants (Vainonen and Kangasjärvi, 2015). O_3 enters plant tissues through stomata and generates ROS in the apoplast, thereby mimicking pathogen-induced immune response (Kangasjärvi et al., 2005; Iyer et al., 2012; Krasensky et al., 2017) and triggering immune reactions leading to cell death (Shapiguzov et al., 2012; Waszczak et al., 2018). The magnitude of response depends on the effective dose of O_3 within leaves and thus on stomatal aperture. Accordinlgy, we observed higher sensitivity to O_3 of the faba bean genotypes with lower relative leaf temperature. This suggests that genotypes with higher stomatal conductance may be more susceptible to O_3- triggered damage, and potentially, to certain pathogens. Several pathogens, including powdery or downy mildew and rust fungi, bacteria and nematodes (e.g., Wu and Liu, 2022; Meddya et al., 2023; Hou et al., 2024), use stomata as entry points into host tissue. The relationship between the size of stomata and leaf temperature is consistent with the relationship between the size of stomata and stomatal conductance reported by Khazaei et al. (2013), while O_3 damage was observed in genotypes with cool relative leaf temperatures. Our results therefore suggest that O_3 sensitivity could serve as a useful tool for indirectly screening large faba bean germplasm for tolerance or resistance to other stressors in addition to O_3 itself. It is worth noting that in studies of Arabidopsis accessions exposed to acute O_3 fumigation differences in O_3 sensitivity could not be fully explained by variation in stomatal function, suggesting the involvement of additional processes (Brosché et al., 2010). Our experiments were conducted under climate-controlled growth conditions and controlled O_3 stress treatments. However, under field conditions, a number of environmental variables such as light intensity, temperature, relative humidity, and wind speed will greatly influence physiological traits, particularly gas exchange parameters and leaf/canopy temperature (*reviewed in* Miguel Costa et al., 2013; Xu and Li, 2022). It is important to note that O_3 screening under controlled growing conditions may not fully reflect plant response to environemtal stresors exposure in field conditions, as diurnal fluctuations in ozone concentration, co-occurring stresses (e.g., drought or high vapor pressure deficit) that may influence stress response (Wilkinson et al., 2012). Experiments should be carried out under field growing conditions to validate controlled climate results and to better understand the genotype by environment interactions for such traits, which can provide valuable insights for breeders. Future studies should examine whether leaf temperature can serve as a practical field-based indicator of stress responses in faba bean under variable environmental conditions. Moreover, the screening methods described in this work under grenhouse and crontrolled environment chambers are useful tools for pre-screening large germplasm collections under controlled conditions for physiological and genetic studies.

Genetic control of photosynthesis-related traits such as photosynthetic rate, stomatal conductance and Fv/Fm has been shown to have complex inheritance in faba bean (Khazaei et al., 2019). The HTPP method described in this study, based on the non-invasive measurement of multiple physiological parameters, will pave the way for screening a large diversity of faba bean genotypes and mapping populations to identify genomic regions governing stomatal function and photosynthesis-related traits in this species. Similar studies have previously used low-throughput phenotyping approaches to investigate stomatal morphology and function (Khazaei et al., 2014; Mandour et al., 2023). Using the identified faba bean genotypes with constracting stomatal function from this study, we are developing mapping populations to investigate the genetic and physiological basis of stomatal traits and photosynthetic activity. In parallel, the combination of thermal imaging and O_3 screening offers a novel route to assess stress sensitivity at large scale, linking stomatal regulation with immune responses (Kemppinen et al., 2025). This integrated approach will accelerate understanding of the mechanisms underlying the role of stomata in plant responses to environmental stresses.

5. Conclusions

In this proof-of-concept study, we combined high-throughput phenotyping with uniform stress treatment to screen a large faba bean germplasm. The results revealed interactions between stomatal function and photosynthetic light reactions, and allowed to characterize faba bean genotypes for their sensitivity and tolerance to acute O_3 exposure. The described genetic resources and phenotyping approaches provide foundation for future physiological and molecular studies and screens in faba bean.

CRediT authorship contribution statement

Matleena Punkkinen: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tuomo Laine:** Investigation. **Alexey Shapiguzov:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis. **Hamid Khazaei:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Satu Engström:** Investigation. **Pedro J. Aphalo:** Writing – review & editing, Software, Resources, Methodology.

Funding

The work was supported by the Research Council of Finland, Academy projects, funding decisions 363375 (Fabagen) and 346140 (TreeBio).

Declaration of Competing Interest

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

Acknowledgment

We thank Leena Grönholm for her kind assistance during the greenhouse experiments. We are also grateful to Varvara Shapiguzova for her help with the quantitative analysis of relative leaf temperature. We thank Prof. Alan Schulman for helpful suggestions on the study. Additionally, we thank the Nordic Genetic Resource Center (NordGen) and Prof. Wolfgang Link (Georg-August-Universität Göttingen) for providing the faba bean seeds.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2025.106290.

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