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Reducing the Evaporative Demand Improves Photosynthesis and Water Use Efficiency of Indoor Cultivated Lettuce

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Abstract: Currently, climate change is affecting considerably the availability of freshwater for agriculture, increasing the need for the optimization of crop water use efficiency. Attempts to use VPD (vapor pressure deficit) modulation to reduce water consumption have been made. However, the effects of VPD on leaf stomatal and hydraulic traits, and on possible tradeoffs between photosynthetic carbon gain and transpiration, are rarely reported. We analyzed photosynthesis (gas-exchange, photochemistry) stomatal and hydraulic-related traits of green (G) and red (R) butterhead lettuce (*Lactuca sativa* L.) grown under low and high VPD (LV, HV) in a controlled environment. Our results showed that plants developed a higher number of small stomata under LV, allowing better regulation over opening/closing mechanisms and thus increasing net photosynthesis by 18%. LV plants also achieved better performance of the photosystem II and a more efficient water use (increments in Φ PSII and iWUE by 3% and 49%), resulting in enhanced plant growth and reduced need for irrigation. Significant differences between G and R plants were limited to a few traits, and the physiological response under the two VPDs did not show cultivar-specific response. We discuss the role of VPD management as necessary to maximize crop water use by harmonizing photosynthesis and transpiration.

Keywords: *Lactuca sativa* L. *capitata*; indoor agriculture; mean transpiration rate; net assimilation rate; photochemistry; photosynthetic light curves; stomatal traits; vapor pressure deficit (VPD)



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1. Introduction

Plant transpiration rate is driven by changes in atmospheric conditions and especially by changes in vapor pressure deficit (VPD) [1,2]. High VPD (high evaporative demand) is a major cause for enhanced transpiration rate and can provoke excessive water consumption and photosynthetic limitation in agriculture, which is particularly critical under arid and semiarid climate [3]. Normally, VPD values beyond 1 kPa are potentially stressful for crops, determining reduced stomatal conductance, impairing plant photosynthesis and causing plant water deficit, even when roots are well irrigated [4]. Indeed, under high VPD the transpiration rate increases, provoking water stress; thus, plants typically close their stomata to reduce the water loss and avoid tension on the xylem, resulting in reduced conductance and photosynthesis [5].

Leaf anatomy has a central role in photosynthetic dynamic acclimation to the environment. Indeed, the pathways for both CO₂ uptake and water loss pass through different anatomical tissues, characterized by different resistance and conductance to water and gas diffusion [6–8]. Within leaf anatomical traits, stomata are considered the “gatekeepers” of gas exchanges and impose the greatest resistance on gas and water flows responding to environmental stimuli by changing the rates of both flows [9,10]. Noted previously, stomatal closure can prevent excessive transpiration and therefore uncontrolled water deficit under high evaporative demand, thus maintaining plant water balance [11,12]. However, the degree of stomatal responsiveness cannot overcome the limits imposed

by their anatomical structure [7]. Recent research has pointed out that under long-term exposure to different air VPDs, leaves of the same species (*Rosa hybrida* L.) can develop a different morpho-anatomical structure [13]. For example, concerning stomatal traits, small stomata, often associated with high frequency, have been found in *Vicia faba* L. subjected to high evaporative demand [14], whereas in *Vigna radiata* L., low frequency of big stomata was found under high evaporative demand [15]. Recently, other research has demonstrated that in *C. ruber* and *B. spectabilis* smaller stomata close faster, thus providing a strategy in case of environmental stressors [16,17]. Since it is known that stomata with different size, morphology and frequency present a changed opening/closing pattern, it follows that especially under high VPD, stomatal anatomy plays a central role in the acclimation to environmental changes and in regulating crop water use and water balance [6,18,19]. More specifically, increases in VPD reduce crop yield and productivity and this effect can be partially mediated by stomata acclimation, depending on their traits [20]. Since VPD mediates stomatal anatomical development influencing their opening/closing, optimal water and CO₂ flow rates inside the leaves can be achieved by modulating its level; hence, different VPD levels during cultivation modify the entire carbon gain–WUE (water use efficiency) relationship [3].

WUE is recognized as one of the most important traits influencing crop yield and productivity, especially in water-limited environments [16,21]. Improving crop WUE is fundamental in agriculture since crop production has been threatened by water shortage for centuries and currently water is always a more limited resource. In this context, a few studies have shown that precise VPD control can improve crop yield and production in controlled environment agriculture while saving, at the same time, irrigation water [21,22]. For instance, Zhang et al. [22], alleviated the heat stress and the high VPD in greenhouse tomato production during summer by lowering the VPD with a micro-fog system. The treatment significantly enhanced biomass and photosynthesis, resulting in 12% increments in the marketability of tomato and reducing the overall water use [22]. Moreover, a recent study highlighted that, apart from the average of daily VPD, the extent of the fluctuations in VPD during the day influences lettuce physiological performance [23]. More specifically, the authors showed that with the same daily average VPD, drastic fluctuations in VPD determine decreases in stomatal conductance and net CO₂ assimilation rates. In contrast, moderate fluctuations do not cause reduction of these parameters, also leading to increased leaf lamina expansion and plant growth. Thus, the fine regulation of environmental parameters to achieve stable VPD values becomes crucial to control plant growth.

The development of agricultural technologies that allow for indoor cultivation environments has been fundamental to study the effects of VPD levels and duration on crops with the aim toward extending the crop growing season, maximizing the productivity and quality, and minimizing the input (i.e., water resource) [24]. In the last decade, the role of VPD in reducing plant water demand has been explored further, but it is not completely understood. It is clear that research on VPD regulation cannot disregard the investigations on structure-mediated control of gas and water exchange through the plant. Attempts to identify different morpho-anatomical development driven by atmospheric VPD and its effect on photosynthetic carbon gain and WUE have been seldom reported in few species (*Solanum lycopersicum* L.; *Rosa hybrida* L.) [25,26]. Moreover, the coordinated effects of leaf anatomical development and changing VPD on WUE, also incorporating the tradeoff between photosynthetic carbon gain versus transpiration, are rarely reported.

The aim of this paper was to identify the mechanisms linking VPD modulation to evaporative demand and photosynthetic capacity from a morpho-physiological perspective. Lettuce is one of the most widely cultivated leafy vegetables in controlled environment agriculture. Therefore, a growth chamber experiment was performed growing green and red butterhead lettuces (*Lactuca sativa* L. var. *capitata*) under controlled conditions with two different VPD levels to evaluate plant response in terms of plant biomass, photosynthesis, morpho-anatomical development, transpiration and stomatal regulation. Three questions were addressed: (1) Is stomata differentiation influenced by VPD? (2) Are photosynthetic

CO₂ uptake, stomatal conductance, plant growth and yield influenced by VPD, and how are such possible variations linked to stomatal traits? (3) Does VPD affect the tradeoff between plant carbon gain and cumulative water transpired?

2. Materials and Methods

2.1. Plant Material and Growth Chamber Conditions

The experiment was conducted in March–May 2019 at the Department of Agricultural Science of the University of Naples “Federico II” on butterhead green (G) and red (R) Salanova® lettuce (*Lactuca sativa* L. var. *capitata*) cultivars. Plants were taken from a local provider (Azienda Agricola Punzi; <https://www.punzi.it/?lang=en>; accessed on 1 January 2019) at the stage of 4 true leaves and were transplanted into a growth chamber (KBP-6395F, Termaks, Bergen, Norway). Two consecutive trials in the same growth chamber were performed on 18 lettuce plants (9 green and 9 red Salanova) grown into 10 cm (diameter) trays. The two cycles were identical for environmental conditions and agricultural practices but were conducted at two different VPD levels. More specifically, light was provided by an RGB LED panel (K5 XL750 series, Santa Rosa, CA, USA) set to dispense a PPFD of 315 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the canopy level, which under 12 h photoperiod resulted in 13.6 daily light integral (DLI). Temperature was set at 24 °C and relative humidity was changed to achieve a VPD of 0.7 kPa (low VPD; LV) for the first trial and 1.7 kPa (high VPD; HV) for the second, following Amitrano et al. [15]. VPDs were calculated from the T and RH values recorded every 15 min by two mini data loggers (Testo174H Testo, Titisee-Neustadt, Germany) (Figure S1). Pots were covered with white polyethylene film to minimize evaporation losses from the substrate (1:1, peat:perlite) and were watered until holding capacity and weighed daily to replace the water loss, according to Latha and Reddy [21] (see Section 2.4). Moreover, to improve the uniformity of light intensity and humidity at the canopy level, pots were rotated daily on the chamber shelf. In both cultivation trials, all analyses and sample collection were carried out following the same procedures in terms of days after transplanting, time of the day and replicates.

2.2. Determination of Plant Growth Parameters

Daily, from transplant to harvest, a picture from the top of each plant was captured and images were used to measure the total plant area (PA) through digital image analysis using the color threshold in the RGB channel (ImageJ; Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, 1997–2018). The morphological parameters (fresh and dry biomass) were determined at harvesting, namely 23 days after transplanting (DAT). The dry biomass was measured after oven-drying the samples at 60 °C for at least 3 days, up to constant weight. These measurements were used to reconstruct the daily growth curves in terms of fresh weight (FW) and dry weight (DW) as: $W_d = A_d W_H / A_H$, where W_d is the weight of the day, A_d is the plant area of the day and W_H and A_H are the weight and area at harvesting [27].

2.3. Stomatal Traits Analysis

At 23 DAT, one fully expanded leaf was collected from 6 plants, taking care to select homogeneous leaves. The median region of the lamina was dissected and three peels per leaf were gently stripped from the abaxial surface with soft tip tweezers. For each peel, 5 fields were observed under a light microscope (BX51, Olympus, Hamburg, Germany) at 20× magnification (field area 0.237 mm²) and the number of stomata was counted. Stomatal frequency was expressed as the number of stomata per mm². Stomatal index was computed using the following formula: $(S_N / S_N + E_N) \times 100$, where S_N is the number of stomata and E_N the number of epidermal cells in the microscopic field [28]. The size of 10 stomata per field was measured at 40× magnification, considering both the guard cell major (pole to pole) and minor axes to calculate the area of an imaginary ellipse, following Sorrentino et al. [29]: $\pi a b$, where a is the semimajor axis and b is the semiminor axis.

2.4. Determination of Gravimetric Indices

Plant transpiration was measured by a standardized gravimetric approach of daily pot weighing with an electronic balance, as described by previous research [21]. Weight of all pots was recorded at the same time every day. Daily plant transpiration was estimated as the difference of pot weightings. Daily water loss due to transpiration was completely replenished. Irrigation amount during the whole growing period was estimated from the sum of the daily transpiration. From these data the following indexes were calculated as described by Latha and Reddy [21]: (1) the cumulative water transpired (CWT), as the amount of water added daily to each pot after bringing back to 100% of substrate holding capacity (mL); (2) the leaf area duration (LAD), as $[(A1 + A2)/2 \times 23]$, where A1 and A2 are the initial and final total plant area and the number 23 refers to the duration of the experiments in days ($\text{cm}^2 \text{ day}^{-1}$); (3) the mean transpiration rate (MTR), as the ratio of CWT and LAD, expressed as $\text{mL cm}^{-2} \text{ day}^{-1}$; (4) the net assimilation rate (NAR), as the ratio of dry biomass (DB) and LAD, expressed as $\text{g cm}^{-2} \text{ day}^{-1}$ and 5) the water use efficiency (gWUE), as the ratio of the dry matter accumulation over 23 DAT and the total water transpired over the same period.

2.5. Leaf Gas Exchanges

Leaf gas exchanges were measured at the end of the cultivation cycle (23 DAT), before harvesting, with a portable photosynthesis system (LCA 4; ADC BioScientific Ltd., Hoddesdon, UK) equipped with a broadleaf chamber (cuvette window area, 6.25 cm^2). Measurements were performed from 10 a.m. to 2 p.m. on a fully expanded leaf from all plants, after steady state and equilibration. All the measurements were conducted at ambient CO_2 concentration (about $400 \mu\text{mol}$) and constant temperature (about 23°C), at ambient RH, in order to avoid any external perturbation in VPD. VPD was monitored but not controlled during the gas exchanges so that any differences in transpiration and conductance were due to VPD during plant growth. From gas-exchange measurement the following instantaneous parameters were measured: net photosynthesis (iP_N ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration (iTr ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), stomatal conductance (ig_s ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and intrinsic water use efficiency ($iWUE$), calculated as the amount of carbon gain in photosynthesis rate (iP_N) per unit of transpiration rate (iTr) [30]. Light response curves (P_N -I curves) were also determined on 5 plants per cultivar, to describe the net CO_2 assimilation by a plant leaf (P_N) as a function of an increase in the photosynthetic photon flux density (I) from the total absence of light to a high level of light, e.g., $2000 \mu\text{mol (photon) m}^{-2} \text{ s}^{-1}$. After 10 min exposure to darkness, the PPFD was increased by 0, 50, 200, 400, 800, 1000, 1500 and $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$, under ambient CO_2 concentration and constant conditions in the leaf chamber. Leaves were exposed to each irradiance until the photosynthetic rate was stable for more than 90 s, as reported in Hermann et al. [31]. A maximum radiation intensity of $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ was chosen, following Tsormpatsidis et al. [32], and this intensity was sufficiently large to achieve $P_{N\text{max}}$. A complete light response curve per plant was created in approximately 20 min. From light response curves, I_{max} , defined as the point beyond which no significant change in P_N occurs, and I_{comp} , the light compensation point defined as the point at which CO_2 uptake balances CO_2 released by respiration, were calculated following the model described by Lobo et al. [33]. I_{max} and I_{comp} are considered more appropriate and realistic parameters to compared I_{sat} or $I_{\text{sat}(n)}$ and $P_{g\text{max}}$, for representing the photosynthetic potential of plants since their magnitude is always between the range of real measurements. Indeed, in the other model $P_{g\text{max}}$ is used to define the point beyond which there is no significant change in P_N , but $P_{g\text{max}}$ is obtained when I is infinite; thus $P_{g\text{max}}$ is an abstraction, which forces the existence of I_{sat} or, more appropriately, $I_{\text{sat}(n)}$ (light saturation point at a specific percentile), which is also an abstraction [33,34].

2.6. Measurements of Chlorophyll “a” Fluorescence

Fluorescence emission measurements were performed the same day of gas exchanges on the same leaves. A portable Opti-Sciences fluorometer (ADC Bioscientific Ltd., Hoddesdon, UK), was used for the measurements. Leaf disc regions were dark-adapted with clips for 30 min, prior to fluorescence measurements. To assess the status/efficiency of photosystem II, measurements were conducted in the light and the following parameters were calculated: (i) overall photochemical quantum yield of the PSII (Φ_{PSII}), evaluated according to Genty et al. [35]; (ii) electron transport rate (ETR) according to Krall and Edwards [36] and (iii) nonphotochemical quenching (NPQ) according to Bilger and Björkman [37]. Fluorescence analyses were conducted at steady-state photosynthesis under a light intensity of about $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, with a saturation pulse duration of 0.8 s, by keeping the orientation of the leaf relative to the actinic light source when taking Φ_{PSII} measurements.

2.7. Statistics

The statistical analyses were all carried out using IBM SPSS Statistics (SPSS, Chicago, IL, USA) software. The influence of the two different independent factors (VPD and cultivar) on the dependent variables was tested by applying a two-way analysis of variance (ANOVA) and data are reported in Supplemental Materials. Data were then subjected to one-way analysis of variance (ANOVA), and mean values were separated according to the Tukey test with $p \leq 0.05$.

3. Results

3.1. Growth Curves

Biomass production (both fresh and dry weight) and total plant area varied significantly among the treatments (Figures 1 and 2). During the early growth stages (up to 15 DAT), no significant changes were detected in PA and FW among the four conditions (LVG, LVR, HVG, HVR) (Figure 2a,b). At 15 DAT, PA and FW were significantly higher for LVR and LVG than HVR and HVG ($p < 0.05$) (Figure 2a,b). Thus, 15 days can be recognized as a threshold after which the plants begin to develop differently depending on the VPD. At harvest (23 DAT), PA and FW were significantly higher for LVR than LVG, HVR and HVG ($p < 0.05$). The same trend was found for DW (Figure 2c); however, the threshold after which plants began to develop differently depending on the VPD started at 9 DAT, when the highest values were found in LVG and followed by LVR, which was higher than HVG, and the lowest values were observed in HVR ($p < 0.05$). At 23 DAT, the dry weight was the highest in both LVR and LVG, followed by HVG, and was the lowest in HVR ($p < 0.05$).

3.2. Stomatal Features

The general structure of the stomata was not damaged (Figure 3); however significant changes in quantitative traits were found among treatments (Table 1). Concerning stomatal frequency, LVR plants showed more frequent stomata than LVG, which in turn had significantly higher values than HVG and HVR ($p < 0.05$), whereas stomatal size showed significant differences among all treatments. More specifically, the smallest stomata were detected in LVR plants that showed significantly higher values than LVG, followed by HVR and HVG ($p < 0.05$). Stomatal index was higher in LV plants and lower in HV plants with no differences among cultivars. Overall, under low VPD, SF and SI were enhanced by 32% and 71%, and SS reduced by 22% compared to high VPD (Table S1).

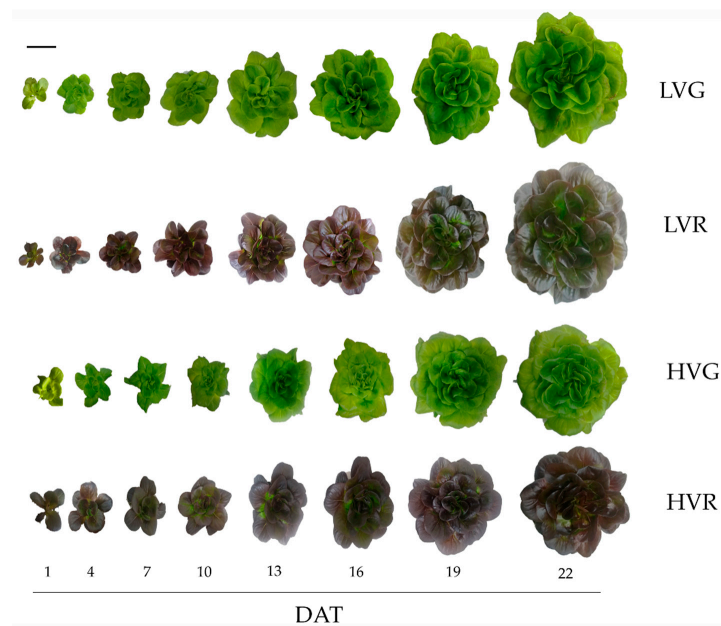


Figure 1. The phenotype of a representative green (G) and red (R) plant from low (LV) and high (HV) VPD condition during the growth cycle. The bar on the top left refers to 10 cm.

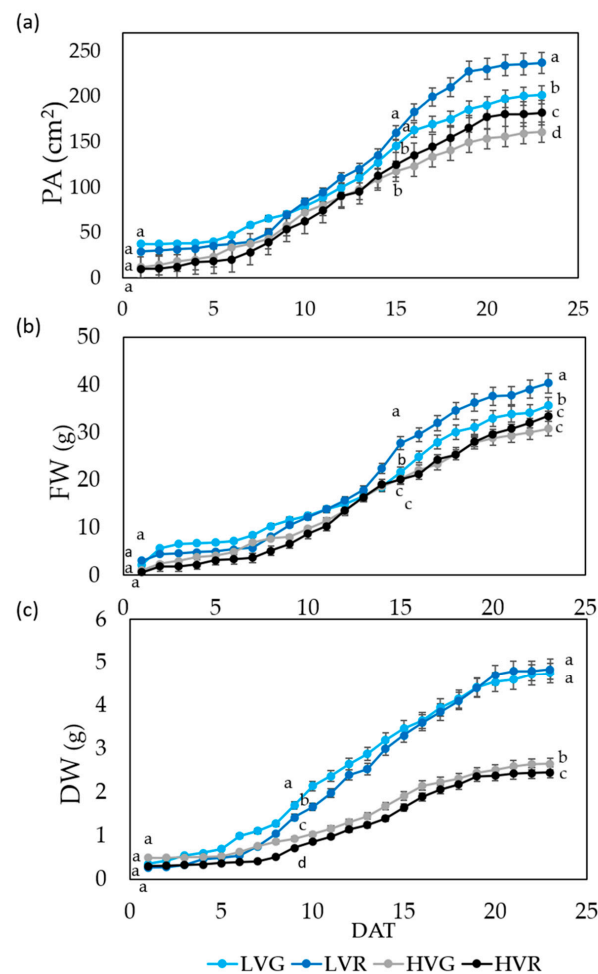


Figure 2. Growth curves of green (G) and red (R) lettuces under low (LV) and high (HV) VPD in terms of plant area (a), fresh weight (b) and dry weight (c). Mean values ($n = 9$) \pm standard errors are shown. Different letters correspond to significantly different values among treatments within a given date, according to Tukey's HSD ($p \leq 0.05$).

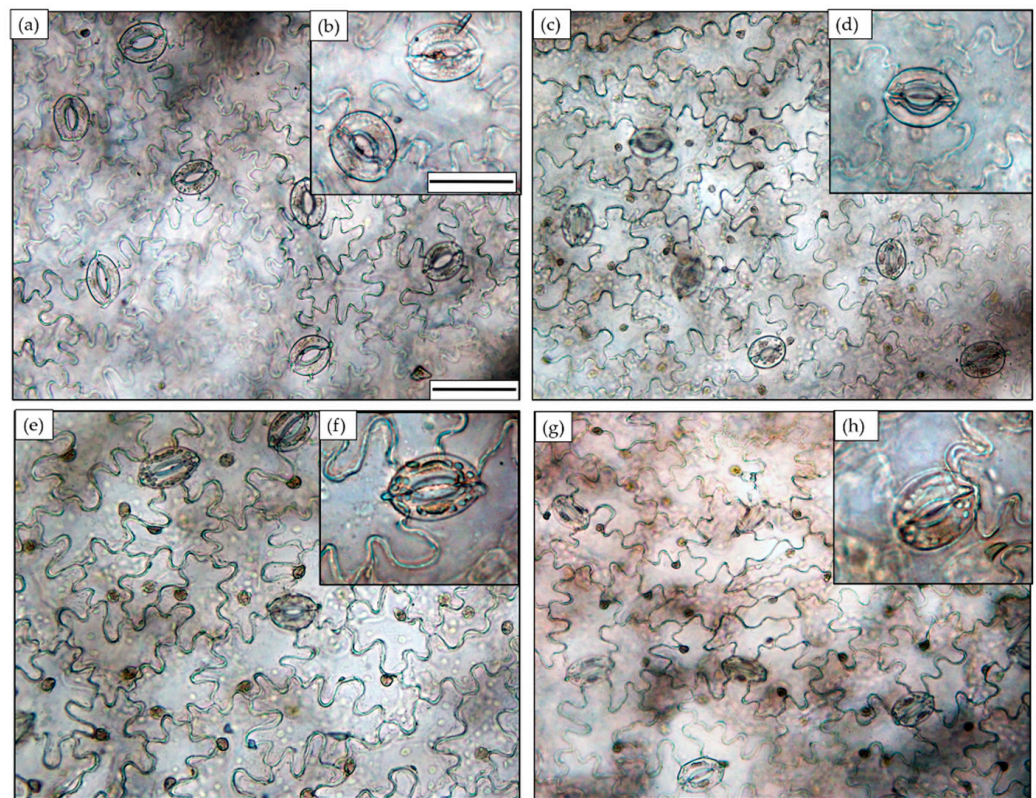


Figure 3. Lettuce stomatal morphology of LVG at 20 \times (a) and 40 \times (b), LVR at 20 \times (c) and 40 \times (d), HVG at 20 \times (e) and 40 \times (f), HVR at 20 \times (g) and 40 \times (h). See Section 2.3 for further details. Images and related details are at the same magnification in the different treatments; bars = 20 microns.

Table 1. Effect of VPD (LV and HV) on stomatal traits of green (G) and red (R) plants: stomatal frequency (SF), stomatal size (SS) and stomatal index (SI) are shown. Mean values ($n = 9$) \pm standard errors are shown. Different letters correspond to significantly different values, according to Tukey's HSD ($p \leq 0.05$).

Treatments	SF	SS	SI
	(n mm ²)	(μ m ²)	(%)
LVG	86.01 \pm 4.50 b	179.3 \pm 7.52 c	12.65 \pm 0.67 a
LVR	93.72 \pm 3.93 a	148.8 \pm 6.08 d	13.67 \pm 0.50 a
HVG	64.19 \pm 2.99 c	208.3 \pm 3.86 a	7.68 \pm 0.33 b
HVR	71.89 \pm 3.20 c	192.8 \pm 4.71 b	7.64 \pm 0.31 b

3.3. Daily Transpiration and Hydraulic-Related Traits

As reported in Figure 4, gravimetric indexes showed significant differences among treatments. The gravimetric transpiration (gTr) (Figure 4a) presented highest values in HVG and HVR and lowest in LVG and LVR ($p < 0.05$). CWT (Figure 4b) presented highest values in HVG and HVR followed by LVR and lowest values in LVG ($p < 0.05$). gWUE (Figure 4c) presented highest values in LVG, followed by LVR and lowest values in both HVG and HVR ($p < 0.05$). LAD (Figure 4d) presented highest values in LVG and LVR and lowest in HVG and HVR ($p < 0.05$). MTR (Figure 4e) presented highest values in HVG, followed by HVR, which in turn presented higher values than LVR and lowest values in LVG ($p < 0.05$). NAR (Figure 4f) presented highest values in LVG and LVR and lowest values in HVG and HVR ($p < 0.05$).

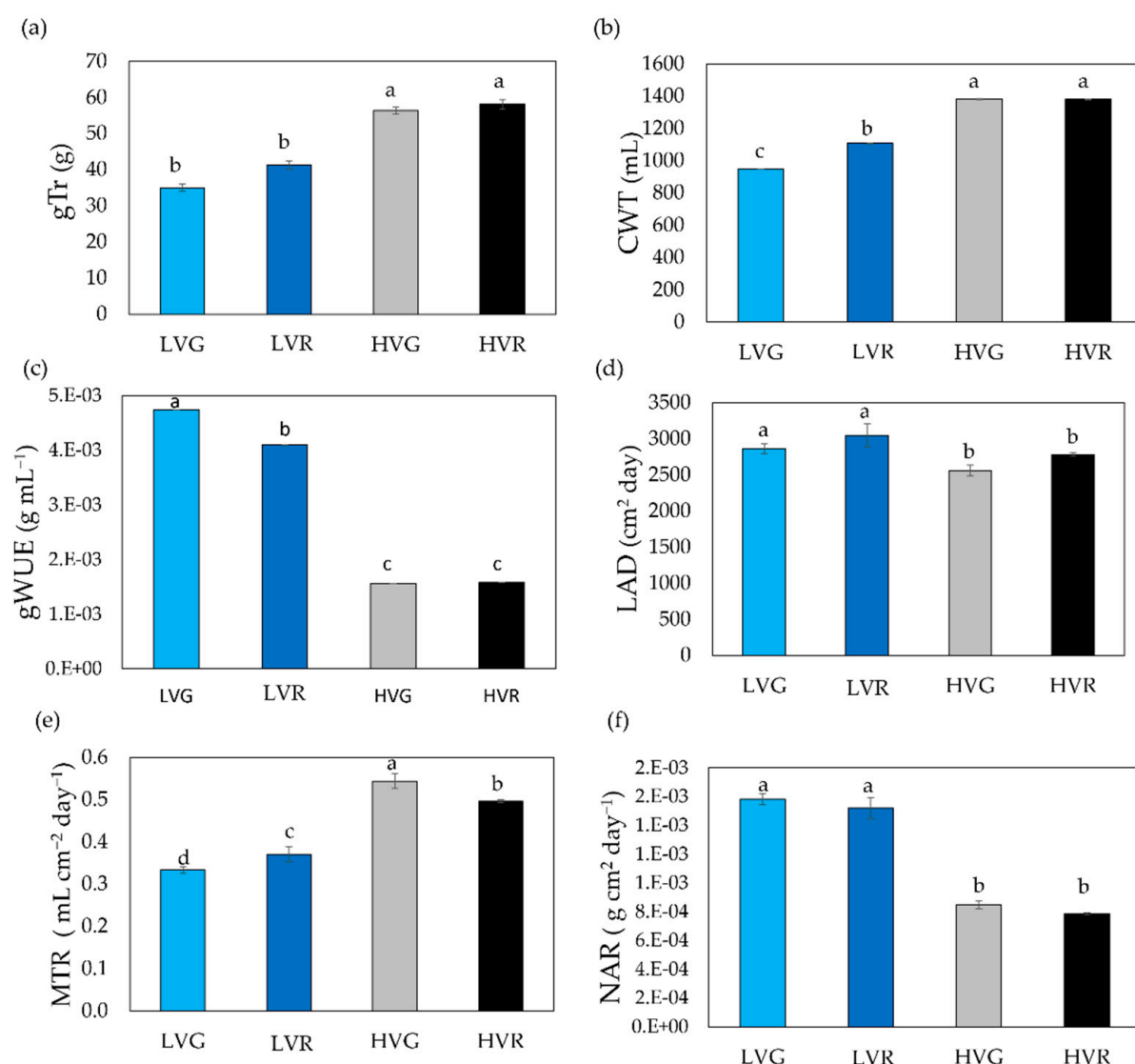


Figure 4. Gravimetric indexes of green (G) and red (R) lettuces under low (LV) and high (HV) VPD: transpiration (gTr; (a)), cumulative water transpired (CWT; (b)), water use efficiency (gWUE; (c)), leaf area duration (LAD; (d)), mean transpiration rate (MTR; (e)) and net assimilation rate (NAR; (f)). Mean values ($n = 9$) \pm standard errors are shown. Different letters correspond to significantly different values among treatments, according to Tukey's HSD ($p \leq 0.05$).

Overall, plant daily transpiration was reduced by 50% under low VPD, cumulative water transpired by 33% and mean transpiration rate by 48%. Gravimetric water use efficiency, leaf area duration and net assimilation rate were enhanced by 64, 10 and 50% under low VPD (Table S2).

3.4. Leaf Gas Exchange, Chlorophyll Fluorescence and Photosynthetic Light Curves

Physiological parameters showed high variation among treatments, as reported in Table 2. More specifically, concerning the chlorophyll fluorescence analysis, Φ PSII was the highest in LVR followed by LVG, which in turn was higher than HVR. Lowest Φ PSII values were found in HVG ($p < 0.05$). NPQ was the highest in HVG, followed by HVR, which in turn was higher than LVG. Lowest NPQ values were found in LVR ($p < 0.05$). ETR was the highest in LVG, followed by all the other conditions where no significant differences were detected. Overall, under low VPD, Φ PSII and ETR were enhanced by 2.9 and 12%, whereas NPQ was reduced by 9% (Table S3). Concerning gas-exchange analysis, iP_N , ig_s and $iWUE$ were enhanced in LVG and LVR compared to HVG and HVR, with no cultivar-specific

differences ($p < 0.05$). Conversely, iTr was enhanced in HVG and HVR compared to LVG and LVR, with no differences between cultivars.

Table 2. Effect of VPD (LV and HV) on physiological parameters of green (G) and red (R) plants. Quantum yield of PSII (Φ_{PSII}), nonphotochemical quenching (NPQ), electron transport rate (ETR) for instantaneous values of gas exchange. Net photosynthesis (iP_N), stomatal conductance (ig_s), transpiration (iTr), water use efficiency ($iWUE$). Mean values ($n = 9$) \pm standard errors are shown. Different letters correspond to significantly different values among treatments within a given date, according to Tukey's HSD ($p \leq 0.05$).

Treatments	Φ_{PSII}	NPQ	ETR	iP_N ($\mu\text{mol CO}_2$ $\text{m}^{-2}\text{s}^{-1}$)	ig_s ($\text{mmol H}_2\text{O}$ $\text{m}^{-2}\text{s}^{-1}$)	$iWUE$ (iP_N/iTr)	iTr ($\text{mmol H}_2\text{O}$ $\text{m}^{-2}\text{s}^{-1}$)
LVG	0.70 ± 0.007 b	1.18 ± 0.48 c	55.96 ± 2.81 a	7.43 ± 0.64 a	0.18 ± 0.006 a	5.11 ± 0.79 a	1.59 ± 0.1 b
LVR	0.71 ± 0.003 a	0.79 ± 0.48 d	45.17 ± 0.69 b	7.74 ± 0.27 a	0.20 ± 0.005 a	4.55 ± 0.30 a	1.68 ± 0.07 b
HVG	0.67 ± 0.002 d	1.46 ± 0.58 a	45.67 ± 3.90 b	5.89 ± 0.14 b	0.07 ± 0.004 b	2.87 ± 0.24 b	2.15 ± 0.18 a
HVR	0.68 ± 0.009 c	1.28 ± 0.52 b	43.72 ± 0.44 b	6.50 ± 0.28 b	0.08 ± 0.004 b	3.52 ± 0.32 b	1.99 ± 0.14 a

Overall, under low VPD, iP_N , ig_s and $iWUE$ were enhanced by 22, 63 and 49%, whereas an opposite trend was recorded for iTr with a reduction of 26% (Table S3).

Photosynthetic light curves, shown in Figure 5, increased until a saturating light intensity of about $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ in both VPD conditions. Leaf P_N was higher under low VPD compared to high VPD plants at all light intensities above $250 \mu\text{mol m}^{-2} \text{s}^{-1}$. No differences among cultivar were detected. Moreover, Figure 5 shows how low VPD plants presented a lower I_{max} and a higher I_{comp} , compared to high VPD with no differences among cultivars.

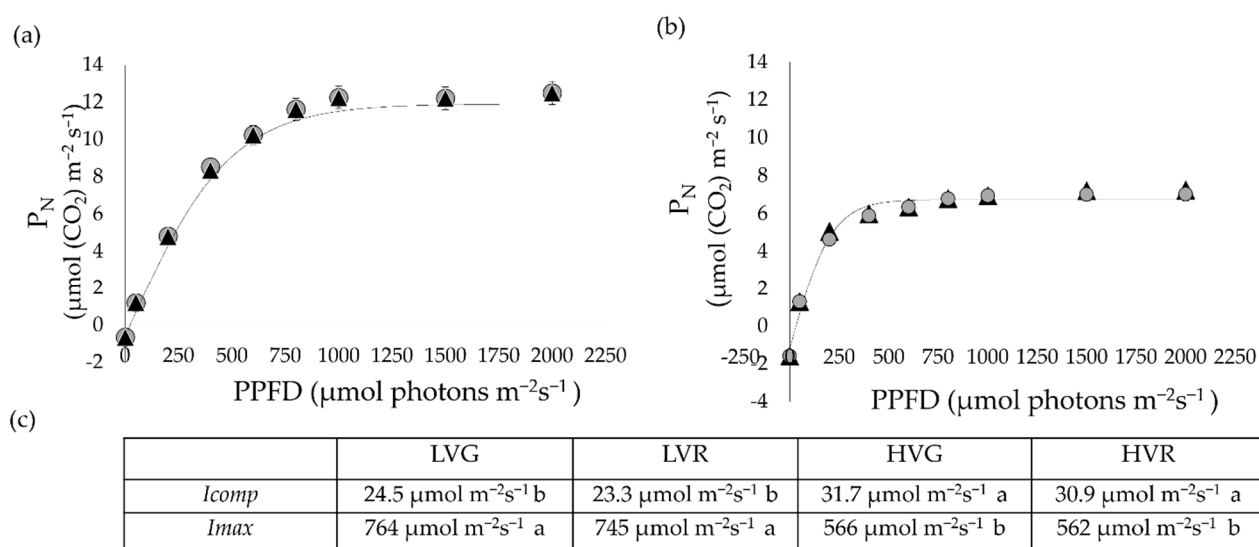


Figure 5. Effect of VPD (LV and HV) on green (G) and red (R) lettuce photosynthetic light curves: (a) LVG (black triangles) and LVR (gray circles); (b) HVG (black triangles) and HVR (grey circles) and (c) derived parameters (I_{comp} , I_{max} , c), according to the model by Lobo et al. [33]. Mean values ($n = 9$) \pm standard errors are shown. Different letters correspond to significantly different values among treatments within a given date, according to Tukey's HSD ($p \leq 0.05$).

4. Discussion

4.1. VPD Changes Stomatal Traits Influencing Crop Physiology

In the present study, we found differences in stomatal traits between the two VPD environments (LV and HV). More specifically, LV lettuces developed a higher stomatal frequency but a smaller stomatal size (Figure 3). A higher frequency of pores under low VPD has been found in different species (tomatoes, rose), almost always leading to enhanced stomatal conductance and photosynthesis [38,39]. Indeed, leaves with high

stomatal frequency allow better control over their opening and closing mechanism [8,40,41]. Furthermore, small stomatal size is known to facilitate the pore aperture, allowing faster ion fluxes, leading to rapid increase in the guard cell turgor, thus improving stomatal conductance [42]. For example, Lawson and Blatt [9] found that bean plants, with smaller and more frequent stomata per unit area, open and close their stomata more rapidly while maintaining unchanged photosynthesis. Likely, the same mechanism can be used to explain why LVG and LVR plants, which developed a more efficient stomata structure, were able to maintain better physiological and hydraulic performance compared to HVG and HVR plants, also allowing a higher stomatal conductance. Indeed, excessive transpiration due to high air VPD determines water loss from the leaves. Under this condition, stomata passively close, responding to a reduced leaf water content and turgor, leading to reduced pore conductivity and photosynthetic rates [43,44]. This probably happened in HV Salanova lettuces since the high evaporative demand increased transpiration from the whole leaf tissue while stomatal conductance and the net photosynthesis were reduced (Figure 3, Table 1). The high transpiration and low stomatal conductance in HV plants was indeed attributed to stomatal closure acting as a safety mechanism in HV leaves with less efficient stomata traits (lower frequency of larger stomata). Since CO₂ enters the leaves through the stomata, stomatal closure or a reduction in stomatal conductance will also decrease the availability of CO₂ in the plant, consequently reducing photosynthesis [45,46]. Similar to our results, other studies conducted on tomatoes in controlled environment agriculture found differences in stomatal conductance under the same light intensity but different air VPD, due to different relative humidity [10,22]. For example, Li et al. [10] explored the coordinated effect of soil moisture and VPD on greenhouse-grown tomatoes. In this research, they found that the low-VPD condition reduced the water stress by mitigating the force driving the water movements and by preventing the loss of turgor; these mechanisms maintained the stomata opened and enhanced at the same time the CO₂ uptake with increments in photosynthetic rates, as happened here for LV Salanova plants. Conversely, in a study on *T. virginiana*, it was observed that prolonged exposure to a low VPD make the stomata insensitive to closing stimuli such as desiccation, high VPD, darkness and especially abscisic acid (ABA); this also occurred when the prolonged low VPD was maintained around a single leaf of the plant during growth [47]. ABA is a phytohormone playing a fundamental role in reducing transpiration by provoking stomatal closure [48]; however, its participation in stomata response to VPD is still under debate [49].

In the present study, it is interesting to notice that LVR plants significantly increased their stomatal frequency compared to LVG (Figure 3); however, no changes in photosynthesis or conductance were detected between the two genotypes (G, R). Carin Murphy et al. [13,26] proposed that under high VPD, stomata in leaves were “diluted” with leaf expansion, so that a higher leaf area would lead to a lower frequency of stomata. However, here the highest plant area was found in LVR compared to LVG (Figures 1 and 2). Indeed, so far, contrasting results in stomatal frequency with leaf expansion have been found in different species and cultivars exposed to different environmental conditions [13]. Moreover, the reduced stomatal index in HV plants suggest that these plants developed smaller leaves with more epidermal cells compared to LV, at the same time reducing the frequency of its stomata. The lack of differences in photosynthesis and conductance between G and R lettuces may be due to the fact that they do not represent different varietal types, only differing in pigmentation [50]. Previous studies have demonstrated that the most remarkable difference between these differently pigmented lettuces lies on the phytochemical content [51].

4.2. Tradeoff between Water Transpired and Plant Carbon Gain

In our study, WUE (both iWUE and gWUE) was enhanced in LV plants together with NAR (net assimilation rate). Usually, NAR is associated to variations in stomatal and mesophyll conductance, which influence WUE and overall plant growth [21]. The current study clearly demonstrated that LV plants presented a morphology of their stomata leading

to higher conductance, thus positively influencing plant photosynthesis, NAR and plant growth (Figures 2 and 4; Table 1). However, variation in stomatal traits between G and R cultivars did not influence NAR.

In contrast, all the traits associated with transpiration, both physiological (iTR) and gravimetric (gTr, MTR and CWT), were reduced in LV plants. The reduction of these traits is a positive outcome for lettuce, indicating that LV plants were able to reduce the water requirement, and thus enhancing the WUE [52]. Recent genetic studies are attempting to develop “trait-based” breeding utilizing WUE. Under a favorable environment, plants increase WUE either by enhancing photosynthetic carbon gain or reducing transpiration; the first strategy is adopted by plants and referred to as “capacity type” and the second as “conductance type” [52,53]. In our study, the wide difference between LV and HV net photosynthesis (Table 1, Figure 5) suggests that both tested genotypes probably belong to the “capacity type”. However, fluxes of CO₂ and H₂O through the mesophyll are tightly linked, usually allowing for balance between carbon gain and water loss—a balance which is influenced by environmental factors and especially by VPD [54,55]. Moreover, from the analysis of photosynthetic light curves it is possible to understand the adaptive mechanisms of the species under different environment, plant stress resistance and productivity [34]. Following the model by Lobo et al. [33], the photosynthetic light curves showed a remarkable difference between LV and HV lettuces, indicating a higher photosynthetic capacity in LV plants. In fact, under low VPD, lettuce net photosynthesis was always higher above the light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Similar results have been reported in a recent paper by Jiao et al. (2019) [56], where the photosynthetic capacity of tomato plants under low VPD, already higher compared to tomato plant grown under high VPD, were enhanced even more by adding CO₂ to the cultivation. In our study, the different photosynthetic levels of the two VPD-grown lettuces were related to a difference in photochemistry. Indeed, fluorescence emission analysis highlighted better performance of the photosynthetic apparatus under low VPD with differences in the quantum yield and electron transport rate, indicating a better condition of the photosystem II and probably a higher capability of converting light energy at the reaction centers. Indeed, in absence of strong physiological disorders, ΦPSII is ubiquitously considered a good indicator of plant health status [57,58]. However, the PSII is also a delicate component of the photosynthetic apparatus and environmental stresses like drought, high irradiance or heat have been correlated to reductions in the PSII efficiency [59]. ΦPSII often declines together with P_N under water stress, suggesting that the mechanisms of CO₂ uptake and the electron transport chain are tuned [60]. Conversely, the same species under moderate drought did not show any impairment in PSII and the photosynthetic performance was mainly regulated by the stomata [61]. Direct correlation of PSII status with different VPDs is not explored much in literature; however, in our study, ΦPSII was reduced in HVG and HVR plants together with reduction in net photosynthesis and stomatal conductance while NPQ was enhanced. The increase in NPQ indicates the occurrence of photoprotective mechanisms in HV lettuces, probably trying to counteract the negative effect of high evaporative demand. Indeed, NPQ is an important mechanism for plants to protect the photosystem and optimize plant growth and survival [62]. In both L and H VPD plants ΦPSI was higher in R cultivar compared to G one, conversely NPQ was reduced in R compared to G (Table 1). Once again, these differences between the two cultivars did not reflect any changes in photosynthesis (Table 1; Figure 5) but may indicate better status of the photosystem II in R plants. Moreover, R Salanova achieved a higher fresh biomass under L VPD and a higher dry biomass under H VPD, compared to its G counterparts (Figure 2b,c). Similar results were found by El-Nakhel et al. [63], where red Salanova increased the biomass by 22% compared to green one, showing that red Salanova reached maturity earlier than the green one. Considering the significant increments in biomass and ΦPSI but not in photosynthesis in R plants, it may be possible that once again the development of R and G plants was different even if all of the plants were the same age.

5. Conclusions

The present study suggested that stomatal features, photosynthesis, transpiration and water use efficiency are strictly interconnected and influenced by different atmospheric VPDs. High VPD levels significantly constrained plant growth and stomatal development, therefore photosynthesis and water use efficiency decreased. Lowering the VPD values offset the negative effects on plant growth and physiology and maximized water use efficiency, probably as a result of two processes: (1) Low VPD moderates plant water stress by reducing the excessive transpiration faced under high VPD conditions. (2) Under low VPD, lettuce leaves develop stomatal traits allowing for better regulation of gas exchanges, ultimately influencing the whole plant growth and yield. Even though in a previous study these two cultivars (G and R) showed differences in growth and phytochemicals [42], here only differences in a few traits, including growth, were detected and the physiological response under L and H VPD did not show cultivar-specific response. In this study, by reducing the atmospheric VPD, a copious reduction in plant water requirements together with improvements in plant productivity were achieved. Thus, improving the VPD for coupling water transport and carbon dioxide acquisition represents a potential to reduce irrigation demand and improve photosynthetic performance, regulating the whole evaporative demand of cultivation in a protected environment. The regulation of VPD in controlled environment agriculture is therefore necessary to maximize crop water use efficiency by harmonizing the photosynthetic improvements and the transpired water savings. However, a recent study by Inoue et al. [23] showed that the range of VPD fluctuations during the day, due to control systems (fogging/fan-and-pad systems) in a controlled environment, can change photosynthetic and growth performance. Such a phenomenon should be taken into account to evaluate the effect of different management of control systems in order to reduce the VPD range of fluctuation, as a means to counteract the negative effects of high VPD levels. Further studies would be desirable to analyze the relations between crop anatomical traits and physiological acclimation when plants are subjected to different VPD fluctuations and high and low VPDs. Understanding such mechanisms would help in designing a strategy of VPD control based on the minimization of fluctuations, with the final goal to achieve efficient agricultural production in a controlled environment.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11071396/s1>, Figure S1: Climatic data of temperature (triangles) and relative humidity (circles) under low (gray circles and triangles) and high (black circles and triangles) VPDs. Data are daily means of T and RH measurements taken every 15 min, Table S1: Effect of VPD and cultivar on Salanova stomatal frequency (SF) and stomatal size (ss), Table S2: Effect of VPD and cultivar on hydraulic-related traits: gravimetric transpiration (gTr), gravimetric water use efficiency (gWUE), cumulative water transpired (CWT), net assimilation rate (NAR), leaf area duration (LAD) and mean transpiration rate (MTR), Table S3: Effect of VPD and cultivar on crop physiology: quantum yield of PSII (Φ PSII), nonphotochemical quenching (NPQ), electron transport rate (ETR), instantaneous net photosynthesis (iPn), instantaneous stomatal conductance (igs), instantaneous water use efficiency (iWUE) and instantaneous transpiration (iTr).

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