Ph.D. Thesis in Sustainable Agricultural and Forestry Systems and Food Security

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Characterization of plant water flows in Controlled environment -PLANT SMART SENSORS

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XXXIII Cycle

“Always do your best.
What you plant now, you will harvest later”
Og. Mandino

Preface

The work presented in this thesis was mostly carried out at the Department of Agricultural Sciences of the University of Naples Federico II, over a period of three years from January 2018 to May 2021, under the supervision of Prof. Veronica De Micco and the co-supervision of Prof. Stefania De Pascale. The project was funded by the Italian Ministry of University and Research (MIUR), in the framework of PON projects for research and innovation (Programma Operativo Nazionale Ricerca e Innovazione 2014-2020). The industrial project partner was an aerospace company: “Kayser Italia srl”. A period of six months was spent at the company premises under the supervision of Dr. Michele Balsamo (both in presence and remotely due to covid-19 pandemic). Furthermore, seven months were spent at the Controlled Environment Agriculture Center (CEAC) of the University of Arizona, under the supervision of Prof. Murat Kacira. Other collaborations included: Prof. Youssef Rouphael and Prof. Giovanni Battista Chirico, at the Department of Agricultural Sciences of the University of Naples Federico II, Prof. Carmen Arena at the Department of Biology of the University of Naples Federico II. Moreover, during the Ph.D. course, a project was submitted and funded in the framework of the EPPN (European Plant Phenotyping Network) 2020 transnational access, supported by the European Union's Horizon 2020 Research and Innovation program, under grant agreement No 731013. Therefore, experiments at the IPK-Leibniz institute of plant genetics and crop plant research (Gatersleeben, Germany) were performed with the support of Dr. Astrid Junker.

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Introduction

The present thesis project "Characterization of water flows in Controlled Environment -PLANT SMART SENSORS" has a multidisciplinary core and aimed towards the creation of synergies between the world of scientific research and the industry. By applying research results to technological development, this research targeted at innovation in the Agrotechnology and Aerospace sectors. Indeed, the introduction of new technologies is pivotal for controlled environment production on Earth to feed a growing population as well as for human permanence in Space in long-term missions where plants are used to regenerate resources (e.g. oxygen, water) and as source of fresh high-nutritious food. The realization of these systems must be based on a precise knowledge of plant morpho-anatomical development and its physiological behavior in closed growth systems, which are strongly influenced by numerous environmental factors including the relative humidity or more specifically the Vapour Pressure Deficit (VPD). In a protected environment (e.g. in Space greenhouses, vertical farm, indoor growing-modules), the control of relative humidity represents a significant problem, which has often been neglected. For instance, in conditions of poor aeration, too high humidity can occur with consequent low values of VPD which reduces the plant transpiration, slowing or stopping the water flow through the SPAC (Soil-plant-atmosphere-continuum), and ultimately blocking the photosynthesis, yield and biomass production.

Even though there have been many studies regarding the VPD control, alone and/or in combination with other environmental factors, certain points are still unclear or controversial, providing contrasting results in different or even in the same species. This happens mainly due to the complex interactions between many microclimatic factors and plant physiological behaviour at different phenological stages.

In a context of climate change, the efficient regulation of VPD can be applied to greenhouse and indoor-module production in order to enhance crop productivity, improve WUE and reduce total water consumption to design irrigation strategies, considering the balance between the amount of water saved and the quantity used to regulate the VPD.

The regulation of the VPD and related environmental parameters need to be designed according to the species and its adaptive plasticity at morphophysiological levels. Thus, the characterization and modeling of water flows in model plants in different growth chamber scenarios (from small modules intended for the spatialization for Space applications, up to structures that can be used in protected cultivation on Earth), as well as the real-time monitoring of the water status of plants, become fundamental for the management of precision agriculture both in support of Space exploration and for the sustainability of urban agriculture. To date, most of the research has focused on either specific physiological/structural aspect at the single-plant level, or on cultivation management or even on technological aspects, with only a few interlinks of knowledge.

The aim of this thesis is to develop knowledge to help filling this gap to improve the understanding of VPD effects on crop productivity, with the creation of synergies among different expertise (e.g., plant physiology, crop science, engineering). To do so, it is fundamental to study the complexity of plant morpho/physiological responses, since without a deep knowledge of mechanisms behind plant responses to the environment it is difficult to determine how and to which extent plants can adapt to any changes in the environmental conditions.

The application of a multidisciplinary approach in research will allow crop production in a sustainable way, even in harsh environments, where a "climate smart-agriculture" becomes necessary to improve crop yield and quality. The present thesis is organized as follows:

Chapter 1 is a review which presents the current state of knowledge on how VPD influences plant morpho-physiological traits in controlled environment agriculture. The study has been published as a review article in *Annals of Applied Biology* (Amitrano et al., 2019 https://doi.org/10.1111/aab.12544). It covers main important aspects of VPD influence on plant growth, morpho-anatomical development, and physiology, emphasizing the possible interaction between VPD and other microclimatic factors in protected cultivation. Furthermore, the rewiew identifies and discusses future research areas, which should be explored further, based on needed synergies among different expertise from biological and horticultural fields.

Chapter 2 presents evidence that the modulation of relative humidity (RH) together with other important cultivation factors such as light (presence/absence), can influence morpho-anatomical development and improve antioxidant content, even at the early stages of plant life cycle (germination, seedling establishment). The combined effect of RH and light was studied during the germination and seedling development of *Vigna radiata* L. (mung bean), a species widespread throughout the world also due to the high nutritional value of its edible sprouts. A manuscript reporting these data has been published in *Plants* (Amitrano et al., 2020a https://doi.org/10.3390/plants9091093).

In **Chapter 3**, the role of leaf anatomical traits (e.g. leaf mesophyll features, stomata and vein traits) in photosynthetic acclimation to short- and long-term changes in VPD was examined in *Vigna radiata* L. adult plants. In this study, we underlined the key role of leaf structure in photosynthetic acclimation to air VPD. The long-term exposure to different VPD levels determined a pre-acclimation at the leaf morpho-anatomical level which influenced the extent of leaf physiological plasticity, changing plant ability to acclimate to any changes in the surrounding microclimate. This different leaf anatomy-related capacity of pre-acclimating becomes

therefore fundamental in the present climate-change scenario due to its key role in the adaptation process under changing environmental conditions. A manuscript reporting these data has been published in *Environmental and Experimental Botany* (Amitrano et al., 2021a https://doi.org/10.1016/j.envexpbot.2021.104453).

In **Chapter 4**, the effect of VPD on morpho-physiological traits also incorporating the trade-off between transpiration and carbon gain was evaluated in two cultivars of Salanova lettuce (*Lactuca sativa* L.) with green and red leaves, in a growth-chamber experiment. Low-VPD turned out to significantly improve growth, stomata development and hydraulic-related traits which led to higher photosynthesis and a reduced water consumption compared to the high-VPD condition. A manuscript reporting these data was published in *Agronomy* (Amitrano et al., 2021b https://doi.org/10.3390/agronomy11071396).

Chapter 5 represents a clear interlink of knowledge between plant scientists, engineers, mathematician and modelists. In this study, published in *Sensors* (Amitrano et al., 2020b https://doi.org/10.3390/s20113110), we used experimental data, based on morpho-anatomical analyses of lettuce plants, to run the Energy Cascade Model (MEC), a model already used to predict biomass production and photosynthetic efficiency in advanced life support systems studies (Space-oriented research). Here, the modification of the model is discussed together with possible improvements and applications.

Chapter 6 focuses on how to modulate the micro-environment, and in particular the VPD levels, in protected cultivation to improve plant antioxidant content in crops. More specifically, the exposure of the same lettuce cultivars mentioned in previous chapters to high VPD determined an improved phytochemical content in lettuce leaves, especially in the red cultivar. Here we discussed a further possibility to use short-term high VPD treatments as a mild stress to boost the phytochemical production in lettuce plants. A Manuscript reporting these data has been published in Horticulturae (Amitrano et al., 2021c http://doi.org/10.3390/horticulturae7020032).

Chapter 7 is a deep focus on how the VPD drives the coordination among morpho-anatomical traits in leaves of the above-mentioned lettuce cultivars, also exploring the variability of traits along the leaf lamina. More specifically, the attention is focused on how stomata and vein develop within lettuce leaves and how these traits are coordinated with leaf size under different VPDs. Results from this study suggest that VPD triggers a different response in lettuce plants in terms of balance of leaf

traits and highlight the possibility of further exploring the microenvironment (combined influence of light and VPD) to adjust the development of stomata and vein densities, thus providing optimal water and gas fluxes through the leaves.

In **Chapter 8**, the experiments conducted during the period spent at the Controlled Environment Agriculture Center of the University of Arizona (UA-CEAC) are reported. The experiments reported here were conducted on the same species of the previous chapters (Salanvoa lettuce with green and red leaves) in a multi-layer vertical farm to test the interaction between VPD and other microclimatic factors on plant morpho-physiological development. More specifically two experimental trials are reported (E1 and E2). In E1, the interaction between VPD levels (low and high) and increasing DLI (Daily Light Integral - 8.6, 12.9, 15.5) was tested to study morpho-physiological changes and to determine the optimal combination of DLI and VPD for lettuce growth. In E2, a sudden salt stress was applied to the cultivation and then CO_2 enrichment was provided, based on the hypothesis that the CO_2 enrichment would mitigate the salt stress, modifying the plant carbon gain/water balance. We evaluated whether the mechanisms of salt stress mitigation due to CO_2 enrichment were different under high and low VPD conditions, depending on the different morpho-anatomical leaf structure.

Chapter 9 reports on experiments conducted at the IPK-Leibniz institute of plant genetics and crop plant research (Gatersleeben, Germany) in the framework of the EPPN2020 transnational access (https://eppn2020.plant-phenotyping.eu/EPPN_Transnational_Access). A report with obtained results is showed in this chapter. These experiments concern the application of high-throughput phenotyping combined with morpho-anatomical analyses on Salanova green and red plants acclimated to a VPD level and then subjected to short-term changes in the VPD. The project submitted to the EPPN transnational access and winner of the grant is presented in Appendix 1.

Chapter 10 and **11** report on the possible industrial applications after the collaboration with the partner company “Kayser Italia srl” (http://www.kayser.it/). Chapter 10 is a study for the definition of scientific and technical requirements for the realization of a miniaturized phenotyping growth chamber to grow microgreens or small crops in Space. The structure of the chamber is based on the “Kubik” incubator, an incubator facility of the European Space Agency with the shape of a cube of about 40 cm that has been operating aboard the International Space Station for more than 12 years, carrying different life science experiments. In the chapter, technical and scientific requirements are listed and a preliminary schedule for the project realization is provided. At the end of the chapter, open issues are also discussed. In Chapter 11, the set-up of a prototype miniaturized cultivation

chamber for use in Space is described and the results of validation tests, carried out at Kayser Italia with brassica microgreens (*Brassica rapa* subsp. *sylvestris* var. *esculenta*) under different air relative humidities (VPD), are reported.

In **Appendix 1**, the project submitted to the EPPN transnational access (PHEW- Automated phenotyping platform to improve lettuce water use efficiency under different VPD and watering regimens) and winner of the grant is presented.

In **Appendix 2,** a brief recap on the activities conducted during the Ph.D. program is presented.

Chapter 1:

Vapour pressure deficit: The hidden driver behind plant morpho-functional traits in controlled environments

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MINOR REVIEW

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Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments

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1.1 Abstract: Currently, climate change is threatening agriculture possibilities to feed a growing population, making necessary the implementation of worldwide cultivable lands also through the improvement of highly intensified cropping systems such as greenhouse and indoor growing modules. To ameliorate plant performance and reach the potential yield in controlled environment, these systems should be based on the fine control of all microclimatic factors among which Vapour Pressure Deficit (VPD) plays a major role. Vapour Pressure Deficit represents a driver for transpiration in plants and it mainly changes with temperature and relative humidity. Through VPD control, it is possible to regulate the entire evaporative demand of an indoor cultivation. VPD control is also crucial in determining specific plant structure, physiological behaviour, thus improving plant growth and productivity. The regulation of VPD in controlled environment should be managed dynamically, since VPD can modulate morphological and physiological processes, which adapt to the new environmental conditions and, in turn, change the environment itself, thus requiring the continuous update of VPD according to phenological stages. In this review, we aim to revise VPD influences on plant growth and development, taking into account both anatomical and physiological modifications. We summarise available information in the scientific literature regarding the wide variety of VPD effects on different plant traits and species. Additionally, we elucidate the importance of VPD regulation in protected agriculture, alone or in combination with other microclimate factors.

The review concludes by proposing future research areas which should be explored further, based on needed synergies among different expertise from biological and horticultural fields.

Keywords: ABA, Controlled Environment Agriculture (CEA), functional anatomical traits, leaf conductance and resistance, photosynthesis, plant water relations, stomatal regulation, transpiration, vein traits

1.2 Rationale: In the coming years, the main challenges for modern agriculture will be the needs to enhance the production of fresh edible products to meet the requirements of the increasing global population (projected to reach 10 billion by 2050) (Truong et al., 2017), to facilitate out-of-season harvest, and to allow food production in cities (urban farming) or even in extreme ecosystems, like polar and desert regions as well as extra-terrestrial environments (Anderson et al., 2017; McCartney and Lefsrud, 2018). Nowadays, ongoing climate changes affect all aspects of global food security (production, availability and access) (IPCC 2017). Moreover, high-input, resource-intensive farming systems, which have caused massive deforestation, limited water supply, depleted soil and raised levels of greenhouse gas emissions, are responsible for a drop in food production and in its distribution (Schmidhuber and Tubiello, 2007). Climate change, due to the increase in atmospheric CO_2 concentration, determines a rise in air temperature and consequently in evapotranspiration (Jagadish et al., 2016), which is ultimately impacting crop production especially in arid and semi-arid regions. Indeed, these conditions affect plant primary production, resulting in decreasing stomatal conductance and gas-exchange rates (Bisbis et al., 2018). Elevated CO_2 also acts on the ecosystem nutrient cycle, decreasing the uptake of nutrients in soil, which may indirectly limit plant growth and development. According to some climate models (Soden and Schwarzkopf, 2005; Wentz, 2007), global warming will exacerbate problems already existing, leading to important changes in atmospheric water vapour content and precipitation amount (Perdomo et al., 2017). It is well established, that plants absorb water from soil due to a gradient of water potential (from roots to leaves) driven by Vapour Pressure Deficit (VPD), which, from a physics perspective, is the difference between the saturation vapour pressure (100% Relative Humidity) and the real vapour pressure in the air, at a given temperature. However, VPD is sensed by plants as the difference between the vapour pressure in the leaf and the one in the atmosphere (Wheeler and Stroock, 2008). In vascular plants, VPD is a driver for transpiration, which is actively regulated by stomatal movements (opening/closing) (Sulman et al., 2016). Since VPD increases when temperature (T) rises, climate warming conditions are expected to upraise its level (Will et al., 2013). Higher VPD will intensify plant physiological stress under water shortage by either increasing plant water loss or limiting carbon fixation, depending on the anisohydric or isohydric behaviour adopted (McDowell et al., 2008; Sade et al., 2012; McDowell and Allen, 2015; Anderegg et al., 2015). The productivity of crop and forestry ecosystems strongly depends on the temporal and spatial distribution of water (precipitation and evaporation), as well as, especially for crops, on the availability of freshwater resources for irrigation.

Optimizing the use of water for crop production will be a challenge for assuring an eco-friendly and sustainable agriculture, especially in arid and semi-arid areas (Medrano et al., 2015). To this purpose, the development of Controlled Environment Agriculture (CEA) systems is pivotal. These systems are based on the modification of the natural environment, to create optimal environments for cultivation, as well as buffering against external weather conditions. Protected cultivation can be achieved in greenhouses or indoor growing modules, through the management of microclimate factors (e.g. T, light intensity, quality and photoperiod, CO_2 concentration and VPD levels), which concern both the aerial and the below-ground organs (i.e., roots). More specifically, VPD regulation in controlled environment represents a strategy to achieve a valuable crop production (in terms of yield and product quality) in a more sustainable way by enhancing resources use efficiency (e.g. saving water and nutrients) and allowing the cultivation of agricultural and horticultural crops all year-round. Protected cultivation has spread throughout the world in the last decades and goes along with technological innovation which helps improving the CEA state-of-the-art (Giacomelli, 2007). Technological research in agriculture goes in the direction of sustainability and automation (mechanization and robotic systems), to maintain specific settings in indoor growing modules avoiding human subjectivity and monitoring plant growth remotely, also acquiring accurate data for scientific and applied purposes. In this context, CEA has been sponsored by National Aeronautics and Space Administration (NASA) and European Space Agency (ESA) in Space-oriented research, especially for edible plant cultivation as bio-regenerators in life support systems (LSSs) (Wheeler, 2010; De Micco et al., 2011; Arena et al., 2014). Bio-regenerative life support systems (BLSSs) are closed-artificial-environments where plants could regenerate air by producing O_2 and removing CO_2, purify water, recycle wastes, and overall provide fresh food for the crew (Wheeler et al., 2003; Paradiso et al., 2014). Even though first studies on Controlled Ecological Life Support Systems (CELSSs) date back to the 1950s, nowadays, with new advents in technology, the challenge is to create the best conditions to optimize the growth of higher plants in protected cultivation, considering all the potential detrimental constraints of confined environments (e.g. resources availability, nutrient recycling confinement, etc.), also taking into account the difficulties in their control. Environmental factors and plant responses are strictly interconnected; therefore, changes in any environment and/or cultivation factors, trigger a cascade of modifications at plant physiological and morpho-anatomical levels, affecting plant behaviour especially in terms of water and gas fluxes, which in turn re-modify the environmental parameters. For these reasons, particular relevance in technological and sustainable agriculture has been given to remote sensing monitoring and its fine, prompt, control (Ren and Martynenko, 2018).

In this review, we focus our attention on VPD and its regulation to improve plant physiological processes as carbon metabolism and water use efficiency (WUE) in controlled environments. We first give an overview of water fluxes through plants focusing on hydraulic conductance and structure-mediated resistance in the Soil-Plant-Atmosphere-Continuum (SPAC). Then, we consider how the

differences in VPD, consequent to changes in Relative Humidity (RH) and air T, may affect plant growth, structure, plant-water relations, photosynthesis, yield and product quality. We focus the attention on above-ground organs, even though we are aware of the importance of root architecture and soil. In addition, we highlight some approaches used so far to regulate VPD in protected cultivation, concluding with summaries of future challenges aiming to understand the potential modulation of photosynthesis, crop performance and product quality through the regulation of VPD in an interactive controlled environment.

1.3 Implications of VPD for sinks and sources: VPD is strictly related to air RH, which is expressed as the ratio between the pressure of water vapour in the air and the pressure of water vapour held in a saturated environment: "e/e_s". At plant physiological level, VPD represents the difference between water pressure inside the leaf and the one in the atmosphere, which has a big impact on plants evapotranspiration (ET) and so, on water flows within plants. VPD is usually expressed in kPa and it increases when T rises and RH decreases (Will et al., 2013). High VPD, together with high irradiance conditions, enhance leaf transpiration and, therefore, water loss from tissues. To reduce water loss, plants adopt several mechanisms also relying on stomatal closure, therefore declining decreasing carbon fixation due to reduced CO_2 diffusion towards the chloroplasts, with negative consequences on photosynthetic activity. The result is a decrease in plant growth and productivity, which represents a major issue for crop production (Zhang et al., 2015; Sinclair et al., 2017). On the other hand, it has been demonstrated that under a reduced VPD environment, leaf gas exchanges and physiological WUE (An/E) are enhanced (Zhang et al., 2015; 2018). Indeed, in low VPD environment plants enhance photosynthetic carbon gain by promoting stomatal opening. This condition should also allow a more severe loss of water through stomata; however, when the atmosphere is saturated with water vapour (high RH conditions) plant ET demand is reduced and consequently, WUE improved. Therefore, in plants there is often an interplay between photosynthetic carbon gain and WUE (Li et al., 2017). In the last decades, research has focused on the concept of photosynthesis and carbon gain (source) being influenced by environmental parameters (particularly by VPD) and controlling plant growth and its structure (sink). CO_2 uptake and photosynthetic rate have always been recognized as the driving forces for plant growth, which was only considered a consequent outcome (Korner, 2015). On the other hand, recent research contradicts this theory providing a new concept in which sinks control sources, so that growth (morphological development), influenced by environmental conditions (VPD), does control photosynthesis. According to this theory, under water shortage the first negative consequence is the inhibition of meristematic cell formation, which is more sensitive to turgor losses, and would limit carbon uptake through a complex signalling between sources and sinks (Korner, 2015; White et al., 2016). Currently, through VPD control is possible to regulate the atmospheric evaporative demand, thus influencing tissue formation, growth

and photosynthesis. Uncertainty remains in finding the best strategies to improve plant productivity and concurrently reduce water loss, because plant responses to environmental conditions are often species and/or cultivars-specific and all the molecular and physiological mechanisms which regulates source-sink relations have not yet been completely unravelled.

1.4 Implications of VPD for plant water relations: Water moves from soil to atmosphere through plants in what is known as Soil-Plant-Atmospheric-Continuum (SPAC), following a negative gradient of water potential. Water enters the roots and moves through the hydraulic vascular system up to the leaves, which are the terminal part of the SPAC and are characterized by a lower water potential. Only a small part (about 5%) of the whole water, which flows through the plant, remains in plants for metabolic demand and storage, while the remaining part returns to the atmosphere in the process of ET which takes place through stomata. As water is transpired through stomata, other water enters the roots, maintaining the continuity of water flux from soil to atmosphere. The flux of water from soil to atmosphere, following a gradient of water potential and passing through a series of resistances, was first hypothesized by Van Den Honert in 1948 and is still valid. Further studies (Nardini, 1999; Sack and Scoffoni, 2012; Xiong et al., 2015a; Xiong et al., 2018) have elucidated the link between conductance and resistance in stomata, mesophyll, and leaves, highlighting the relationships with leaf gas exchanges, in response to different environmental conditions. Leaves represent a conspicuous portion of the entire plant hydraulic resistance being, in fact, also the headquarters of carbon gain (Nardini, 1999). Not only stomatal conductance (g_s) and its kinetics, but also mesophyll conductance (g_m) and leaf hydraulic conductance (K_{leaf}) contribute to determine photosynthetic rates (Flexas et al., 2013) and may act as constraint to photosynthesis (Xiong et al., 2018). K_{leaf} is formed by two components which are strictly related to leaf anatomical structure: the first is referred to the flow inside the xylem (K_x), the second to the flow outside (K_{ox}) (Fig. 2). Both components should be considered in the regulation of WUE and leaf gas exchanges. The K_{ox} mainly depends on vein architecture and particularly on Vein Length per unit Area (VLA), on the structure of mesophyll (e.g. three-dimensional pattern of intercellular spaces) and physiological mechanisms such as the activity of aquaporins (Terashima et al., 2006). Leaves with higher VLA usually presents higher K_{leaf} since they are characterized by a more branched vascular pattern, which better vascularizes throughout the mesophyll, thus decreasing the horizontal pathways for water flow towards epidermis. Such a structural organization, allows greater stomatal density and conductance, bringing to higher gas exchanges per unit area (Sack and Scoffoni, 2013). Conversely, the inside component (K_x) mainly depends on vein density and the morphological traits of the xylem elements (Xiong et al., 2017; Sack and Scoffoni, 2012). Veins are indeed at the basis of the hydraulic structural anatomy of leaves and form a network for the transportation of water, nutrients, and carbon. The roles of leaf vessels, mesophyll thickness and density, and the whole pattern of intercellular spaces in the hydraulic

efficiency have been recognized (Sack, 2006), but uncertainty remains in their role in influencing photosynthetic capacity. Brodribb and co-workers (2007) demonstrated that the photosynthetic performance is related to the closeness of the vein to the evaporative surface and this relation depends on the different conductance and resistance of tissues that water encounters during its flow. Although literature presents contrasting theories, it is gathering more and more support that the majority of hydraulic resistance in leaves (about 50-80 %) is located outside the xylem (Cochard, 2004). The main hydraulic resistance located outside the vascular system would smooth possible hydraulic failure in leaves induced by damages like wall breakdown, cavitation, or vessel embolism at the stem level (Nardini et al., 2003). This outcome is consistent with experiments demonstrating a reduced impact on the whole hydraulic conductance after cavitation events (Nardini, 2001; Nardini et al., 2003). Outside the xylem, spongy tissue is believed to present the highest resistance, due to the major airspace presence and the lowest connection among cells, which decrease the area available for water flows; whereas the bundle sheath (BS) is considered to contribute only in little part to the R_{ox} (outside-xylem resistance), although this last assumption depends on the presence/absence of suberification in secondary cell walls (Buckley et al., 2015). According to these studies, the structural properties of leaf conductive systems are strictly linked to the functional processes of photosynthesis and water transportation, highlighting the pivotal role of leaf and mesophyll anatomy in determining the sites of evaporation, as well as those of major resistances and conductance within leaves. However, the development of vein traits as well as xylem and vascular bundles cells is not only influenced by environmental conditions but also varies a lot within and across species and is linked to intrinsic factors (also tracing back to biogeographical origin) (Brodribb and Feild, 2010; Sack and Scoffoni, 2013; Brodribb et al., 2017). In the light of these considerations, a better understanding of hydraulic architecture in crop species and its response to VPD is require for modelling water fluxes aiming to a fine control of protected cultivation.

1.5 Implications of VPD for stomata functioning and gas exchanges: VPD plays a fundamental role in the process of stomatal opening. When evaporative demand is high (high VPD conditions), plants carry out several adjustments to counteract dehydration. Stomata guard cells lose turgor and, as a consequence, stomata close to avoid excessive water losses (Sinclair et al., 2017). This mechanism impacts on CO_2 diffusion determining a drop in photosynthetic efficiency, CO_2 concentration and photosystem yield. Conversely, when plants grow under low VPD conditions, usually stomatal conductance increases. This phenomenon mitigates stomatal limitations for CO_2diffusion from the atmosphere to the sub-stomatal cavity, resulting in an increment of intercellular CO_2 concentration that, in turn, promotes CO_2 fixation (Flexas et al., 2013; Zhang et al., 2017; Qingjie et al., 2018). Under low T and high RH%, thus low VPD conditions, plants not only increase stomatal opening, but their leaves tend to develop larger stomata (Aliniaeifard et al., 2014; Arve et al., 2017).

Stomatal density is instead more sensitive to atmospheric CO_2 concentration and usually correlates negatively with guard-cell size (Franks and Beerling, 2009). The relationship between stomatal size and density can influence the whole stomatal function and the speed of their response, therefore needs to be taken into account since they contribute to regulate all water and gas fluxes throughout plants, ultimately regulating photosynthesis. The relations between functional anatomical leaf traits and photosynthesis have been largely demonstrated. For instance, Ogughi et al. (2005) found out that photosynthetic capacity in plants not only depends on stomata traits, but also on other structural traits such as the mesophyll thickness, the amount and distribution of intercellular spaces, the number of chloroplasts and their disposition inside the cell. For example, photosynthetic capacity is enhanced by thick mesophyll and by the exposition of chloroplasts towards the intercellular spaces (Oguchi et al., 2005). However, modifications in leaf tissue thickness and chloroplast distribution are subsequently influenced by environmental parameters such as light intensity and quality, T and RH% (e.g. VPD itself), and their interaction (Tosens et al., 2012). Stomatal responses in relation to the regulation of VPD should be studied further, since there are still knowledge gaps especially in the stomata and gas exchanges response to low VPD under water shortage, as well as in the mechanisms that maintain water balance in the above conditions (Qingjie et al., 2018).

1.6 Implications of VPD for ABA-mediated stomatal reaction: ABA (Abscisic Acid) is a plant hormone involved in many developmental processes as well as in stress responses. A correlation between VPD and the level of ABA in leaves has been found: together with increasing VPD, ABA tends to accumulate in leaves, whereas when VPD values go down, a metabolic catabolism of ABA occurs (Bauerle, 2004; McAdam and Brodribb, 2016; Fanourakis et al., 2016). Okamoto and colleagues (2009) found in *Arabidopsis thaliana* an increment in the level of the gene CYP707A, encoding for the enzyme ABA 8'-hydroxylase, in response to high RH%. The mechanisms that involve this gene is the principal ABA catabolic pathway in higher plants (Okamoto et al., 2009), which provokes a drop in both the mobile and local forms of the hormone. Furthermore, this hormone could act as a middle signal between stomatal movements and VPD (Bunce, 1996; Tardieu, 1998). Aliniaeifad and co-workers (2014) found a decrease in ABA levels in leaves of fava bean plants grown under low VPD (about 0.23 kPa), compared to the same plants grown under a moderate VPD (about 1.17 kPa). When plants grown under moderate VPD were transferred in low VPD conditions, the level of ABA decreased sharply, confirming the key effect of VPD on the regulation path of this hormone. It is noteworthy that in a study conducted in herbaceous angiosperms (*Pisum sativum argenteum* mutant and *Dahlia hybrida 'Cinderella'*) changes in cell turgor, obtained by external pressure, changed ABA levels and thus stomatal response to VPD (McAdam and Brodribb, 2015). This phenomenon raised the hypothesis that ABA synthesis under regulated VPD only concerns stomata guard cells and highlights the concept of stomatal reaction to VPD as ABA-mediated.

However, the mechanism of VPD-induced stomatal closure is still unclear and the debate about the role of the hormone in this process remains uncertain.

1.7 Implications of VPD for plant growth, yield and product quality: Despite the importance of VPD regulation for plant growth and development, research conducted so far on VPD regulation and on related effects, has been limited to few species (mainly tomato and bean). The main focus has been to study the effects of RH% and T on the development of specific organs or metabolic processes, mostly in greenhouses. In almost all studies reported, plant development, growth rate and biomass allocation changed after modifications in VPD. For instance, Zhang et al. (2017) found that tomato plants grown in greenhouse under a low VPD of about 1-2 kPa at midday, increased the epigeal biomass production compared to the root biomass and, at the same time, improved fruit development. They also found a conspicuous increment in plant elongation, stem diameter and leaf length. In the former study, the lower VPD condition was realized in greenhouse, by means of artificial humidification systems, which were activated when the evaporative demand exceeded the optimal ranges. This outcome is in agreement with other research in which tomato plantlets grown under low-VPD condition increased plant growth compared to other plants developed in high-VPD environment (Zhang et al. 2017). For example, greenhouse cucumber provided with a fog cooling system, showed a higher dry matter content as well as a high relative growth rate under low VPD, derived from a RH around 86% (Matsuo, 2015). Furthermore, increments in plant extension rate under a transient low VPD, without changing T, were also found in a C4 species *Miscanthus x Gigantheus* (Clifton-Brown, 1999). This increase seemed to be related to an increment in water potential which enhanced turgor-driven cell enlargement due to reduced transpiration rate (Clifton-Brown and Jones 1999). Although increasing crop productivity is the major challenge under controlled environments, the demand for premium quality agricultural products is on the rise, compelled by the increasing interest of consumers and nutritionists in functional and sensorial food (Kyriacou and Rouphael, 2018). Concerning the implications of VPD for enhancing the quality traits under controlled environments, several authors have demonstrated that the use of fogging system as a tool to increase RH% in screenhouses and greenhouses, was able to improve lycopene, organic acids as well as antioxidant activity of several tomato cultivars (Leonardi et al., 2000; Leyva et al., 2015). On the other hand, high VPD inside greenhouses is usually accompanied by extreme T and solar radiation, inducing oxidative stress. As a consequence, detrimental effects on yield, yield components and quality attributes, especially sugars, mineral composition and carotenoids happens (Rosales et al., 2011). The main mechanism behind the deterioration of fruit quality under high VPD could be attributed to lower chlorophylls, thus reducing photosynthetic activity and translocation of photosynthates to the sinks (Xu and Chang, 2007). It has been demonstrated that adjusting VPD at low levels (800 ml of transpired water per plant per day), it is possible to improve the fruit quality

particularly in terms of sugar content. Indeed, the quality of horticultural products is a complex *"dynamic composite of their physicochemical properties and evolving consumer perception, which embraces organoleptic, nutritional and bioactive components"* (Kyriacou and Rouphael, 2018) that can be achieved by modulating microclimatic factors, including VPD, taking into account that different climatic factors (air and root-zone temperature, light conditions and CO_2 enrichment) can act in synergic or antagonistic way on different quality components.

1.8 Strategies for VPD regulation in controlled environment: implications for water saving:

VPD regulations can be an efficient strategy to achieve the challenging goal of sustainable agricultural production, namely increasing crop production per unit area and at the same time reducing water consumption (Deng et al., 2006). Indeed, the irrigation management has been recognized as the most efficient means for improving crop WUE (Kang and Zhang, 2004). In the field, transpiration rates and air VPD have a daily cycle, being usually lowest at dawn and gradually increasing from midday till the end of the day (Devi, 2018), reaching a maximum around 15.00 h (Fletcher et al., 2007), whereas in controlled environment systems it is possible to regulate 24 h VPD values, operating on T and RH levels. In protected cultivation wind-speed is generally lower than field so RH% results higher; moreover, its control is one of the main issues for cultivation in controlled environment, since RH is influenced not only by T, but also by canopy density and ventilation. Given that VPD is directly proportional to air T and inversely proportional to RH, it is feasible to set up different VPD conditions in closed environment by using humidifier/dehumidifier and reduced or increased air T. However, techniques used until now to maintain suitable levels of RH (e.g. fog/humidification systems) consume a certain amount of water, any reduction in transpiration rates result in a reduction of water demand and so in water used for irrigation. Thus, the balance between irrigation and the input of water to lower the VPD should be optimised. Some strategies have been tried to reach this purpose; for example, Zhangh et al. (2018) demonstrated that the mechanism could be maximised by increasing plant density in growth chamber/greenhouse and at the same time, improving fogging systems efficiency. Talbott et al. (2003) increased RH by using atomizing nozzles to supply fine water mist. In other studies (Fletcher et al., 2007; Ben-Asher et al., 2013; Matsuo, 2015), dehumidifier systems plus silica gel, were used to set different RH treatments and thus varied VPD value in protected chambers. These strategies should always be controlled by RH% and T sensors in order to constantly monitor the climatic conditions inside the growth chambers. In greenhouses, however, the most used strategies are cooling and heating systems: from simple shade methods to pad and fan or foggy systems for lowering the T and heat pump (water heater) or hot ventilation for warming. However, it could happen that water from these systems seep outside with a consequence of not having a stable VPD. For this reason, Leyva et al. (2015) tested a system based on both fogging and complementary plastic sheeting (deployed simultaneously with the

fogging system) to keep stable the microclimate condition provided by fogging. Additionally, Zhang et al. (2015) tested a micro-fog system which activated automatically when the VPD was higher than a threshold value (0.5 kPa). It worked through nozzles connected to a water pump and tubes with the goal to alleviate the evaporative request during the hottest months of the year, which correspond the spring-summer growing seasons. Setting up precise T and RH%, and control their range constantly, becomes necessary to maintain the wanted value of VPD. Sensors used so far, in fact, do not measure the actual leaf VPD, but give an insight of the whole VPD surrounding crops as an indirect measure derived by T and RH values. These sensors should be positioned properly: within or as close as possible to the crop canopy in order to provide a good indication of the actual leaf VPD, taking into account the entire ET of the culture.

1.9 Interaction between VPD and other microclimate factors: In controlled environment, whether a greenhouse or an indoor growing module, there is a mutual influence among all microclimate factors. These microclimate factors not only represent the matter and source energy for plant physiology, but also interact with each other to modify the total microclimate, thus modulating plant morphological, physiological, and biochemical processes, acting on specific pathways. Therefore, not only T and RH, but also other parameters should be controlled, or at least monitored, such as: light conditions in terms of intensity, quality and photoperiod CO_2 enrichment, water availability, wind speed, etc. For instance, it is recognised that in simulated artificial environment lighting system intensity indirectly affects air VPD by modifying T, through thermal heating. In addition, changes in light quantity and quality influence stomatal kinetics. Stomata are closed in the darkness, when biological activity is minimal, and opened in full light, allowing the essential need for gas exchanges (Shimazaki et al., 2007). However, stomatal movements also respond to light quality which cause morphological and physiological plant responses, acting on cryptochromes and phytochromes. More specifically, stomatal opening is stimulated by blue wavelengths (Doi et al., 2015) alone, and in combination with red wavelengths (Shimazaki et al., 2007; Arena et al., 2016). Blue light acts activating the H^+ ATPase and driving the potassium uptake through channels, while red light acts mostly indirectly, by decreasing the intercellular CO_2 concentration. At the same time, light effect interacts with atmospheric CO_2 concentration and induces morphological changes in leaf anatomical traits (e.g. stomatal density and size), resulting from the alteration of cell division and differentiation processes during lamina expansion and leading to changes in epidermal cells size (Woodword, 1987). Such structural modifications in turn modify water fluxes in plant, thus affecting physiological response e to VPD variations. Under changing light intensity, g_s kinetics can limit CO_2 assimilation impacting on WUE (McAusland et al., 2016). In order to maximize both WUE and gas exchanges in crops species, with faster stomatal responses (often linked to the occurrence of higher frequency of smaller stomata) are preferred, especially under artificial environments where plant do not

experience long term fluctuations which would face in natural environment, and which would generate leaves adapted to sun or shade. Moreover, in some species it has been found that stomatal and mesophyll conductance as well as the entire leaf hydraulic conductance (K_{leaf}), respond to different light intensities, VPD, CO_2 and to drought stress (Fletcher et al., 2007; Xiong et al., 2015a, 2015b). However, these responses seem to be species-specific. Indeed, in contrast, Tazoe et al. (2009) found that *Triticum estivum* L. cv. Yecora 70 g_m, was independent from light intensity. Additionally, under controlled environment canopy density could drop CO_2 levels even below the atmospheric concentration. Given that, in general, net photosynthesis and crop species productivity improve as the amount of CO_2 in the environment increases, therefore, the enrichment in CO_2 concentration significantly increases crop yield (Thongbai et al., 2010). However, in recent times, with the improvement of technology, strategies which allow VPD regulations in protected cultivation and, at the same time, allow the maintenance of higher CO_2 concentration have been developed (Ohyama et al., 2008; Stanghellini, 2008).

1.10 Conclusions and challenges ahead: The atmospheric VPD plays a crucial role in regulating all water movements through the SPAC; therefore, its precise regulation in controlled agriculture becomes pivotal for the optimisation of plant growth and physiology. Even though, there have been many studies regarding VPD control, alone and/or in combination with other factors, certain points are still unclear or controversial, providing contrasting results in different or even in the same species. Moreover, in a context of climate change, the efficient regulation of VPD can be applied to greenhouse/indoor module production in order to enhance crop productivity, improve WUE and reduce total water consumption to design irrigation strategies, considering the balance between the amount of water saved and the quantity used to regulate VPD. The regulation of VPD and related environmental parameters need to be designed according to the species and its adaptive plasticity at morpho-physiological levels. To date, most of the research has focused on either specific physiological/structural aspect at the single-plant level, or on cultivation management or even on technological aspects, with only a few interlinks of knowledge. To bridge this gap and to achieve a comprehensive understanding of VPD effects on plant growth and on indoor crop productivity, developing at the same time new technologies for its fine control, an integrated perspective with the creation of synergies among different expertise (e.g. plant physiologist, crop scientists, engineers as well as farmers and stakeholders), is needed. Indeed, the fine modulation of VPD in CEA, will allow crop production in a sustainable way even in harsh environments, where a “climate smart-agriculture” become necessary to meet the requirements of the numerous world population. However, the possibility to control VPD is also related to the technological development of automatized and computerized systems, based on remote automatized sensors for the control of the environment and of plant status, especially in the sight of plant cultivation in extreme environments.

However, further detailed investigations especially on: (a) understanding gas exchanges direct and indirect (e.g. anatomical-induced) responses in low VPD environment under water shortage, (b) understanding interactions between environmental and cultivation factors in regulating plant-response to VPD, (c) evaluating how plant responses to VPD change during crop cycle, (d)understanding clearly if ABA-synthesis, under regulated VPD, concerns only stomata guard cells or the whole-plan regulation, explored, (e) finding the most suitable species-specific strategies to improve plant productivity and reduce water loss through the fine regulation of VPD, (f) finding sustainable irrigation methods to improve plant WUE through the VPD modulation, are required.

1.11 References

Aliniaeifard, S., Malcolm Matamoros, P., van Meeteren, U. (2014). Stomatal malfunctioning under low VPD conditions: induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? *Physiologia Plantarum,* 152, 688-699.

Aliniaeifard, S., van Meeteren, U. (2014). Natural variation in stomatal response to closing stimuli among Arabidopsis thaliana accessions after exposure to low VPD as a tool to recognize the mechanism of disturbed stomatal functioning. *Journal of Experimental Botany,* 65, 6529-6542.

Anderegg, W.R.L., Flint, A., Huang, C.-y., Flint, L., Berry, J.A., Davis, Frank W., . . . Field, C.B. (2015). Tree mortality predicted from drought-induced vascular damage. *Nature Geoscience,* 8, 367-371.

Anderson, M. S., Barta, D., Douglas, G., Motil, B., Massa, G., Fritsche, R., Hanford, A. (2017). Key Gaps for Enabling Plant Growth in Future Missions. In *AIAA SPACE and Astronautics Forum and Exposition*, 5142.

Arena, C., De Micco, V., Macaeva, E., Quintens, R. (2014). Space radiation effects on plant and mammalian cells. *Acta Astronautica,* 104, 419-431.

Arena, C., Tsonev, T., Doneva, D., De Micco, V., Michelozzi, M., Brunetti, C., Centritto, M., Fineschi, S., Velikova, V., Loreto, F. (2016) The effect of light quality on growth, photosynthesis, leaf anatomy and volatile isoprenoids of a monoterpene-emitting herbaceous species (*Solanum lycopersicum* L.) and an isoprene-emitting tree (*Platanus orientalis* L.). *Environmental and Experimental Botany*, 130, 122-132.

Arve, L.E., Kruse, O.M., Tanino, K.K., Olsen, J.E., Futsaether, C., Torre, S. (2017). Daily changes in VPD during leaf development in high air humidity increase the stomatal responsiveness to darkness and dry air. *Journal of Plant Physiology,* 211, 63-69.

Bauerle, W.L., Whitlow, T.H., Setter, T.L., Vermeylen, F.M. (2004). Abscisic acid synthesis in Acer rubrum L. leaves—a vapor-pressure-deficit-mediated response. *Journal of the American Society for Horticultural Science,* 129, 182-187.

Ben-Asher, J., Garcia y Garcia, A., Flitcroft, I., Hoogenboom, G. (2013). Effect of atmospheric water vapor on photosynthesis, transpiration and canopy conductance: A case study in corn . *Plant, Soil and Environment,* 59, 549-555.

Bisbis, M.B., Gruda, N., Blanke, M. (2018). Potential impacts of climate change on vegetable production and product quality – A review. *Journal of Cleaner Production,* 170, 1602-1620.

Brodribb, T.J., Feild, T.S. (2010). Leaf hydraulic evolution led a surge in leaf photosynthetic capacity during early angiosperm diversification. *Ecological Letters,* 13, 175-183.

Brodribb, T.J., Feild, T.S., Jordan, G.J. (2007). Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiology,* 144, 1890-1898.

Brodribb, T.J., McAdam, S.A., Carins Murphy, M.R. (2017). Xylem and stomata, coordinated through time and space. *Plant, Cell and Environment,* 40, 872-880.

Buckley, T.N., John, G.P., Scoffoni, C., Sack, L. (2015). How Does Leaf Anatomy Influence Water Transport outside the Xylem? *Plant Physiology,* 168, 1616-1635.

Bunce, J.A. (1996). does transpiration control stomatal responses to water vapour pressure deficit? *Plant, Cell and Environment,* 19, 131-135.

Clifton-Brown, J.C., and Jones, M.B. (1999). Alteration of transpiration rate, by changing air vapour pressure deficit, influences leaf extension rate transiently in Miscanthus. *journal of experimental botany,* 50, 1393–1401.

Cochard, H., Nardini, A., Coll, L. (2004). Hydraulic architecture of leaf blades: where is the main resistance? *Plant, Cell and Environment,* 27, 1257–1267.

De Micco, V., Arena, C., Vitale, L., Aronne, G., Virzo De Santo, A. (2011). Anatomy and photochemical behaviour of Mediterranean Cistus incanus winter leaves under natural outdoor and warmer indoor conditions. *Botany,* 89, 677-688.

Deng, X.-P., Shan, L., Zhang, H., Turner, N.C. (2006). Improving agricultural water use efficiency in arid and semiarid areas of China. *Agricultural Water Management,* 80, 23-40.

Devi, M.J., Reddy, V.R. (2018). Transpiration response of cotton to vapor pressure deficit and its relationship with stomatal traits. *Frontiers in Plant Science,* 9

Doi, M., Kitagawa, Y., Shimazaki, K. (2015). Stomatal Blue Light Response Is Present in Early Vascular Plants. *Plant Physiology,* 169, 1205-1213.

Fanourakis, D., Bouranis, D., Giday, H., Carvalho, D.R., Rezaei Nejad, A., Ottosen, C.O. (2016). Improving stomatal functioning at elevated growth air humidity: A review. *Journal of Plant Physiology,* 207, 51-60.

Fletcher, A.L., Sinclair, T.R., Allen, L.H. (2007). Transpiration responses to vapor pressure deficit in well watered 'slow-wilting' and commercial soybean. *Environmental and Experimental Botany,* 61, 145-151.

Flexas, J., Scoffoni, C., Gago, J., Sack, L. (2013). Leaf mesophyll conductance and leaf hydraulic conductance: an introduction to their measurement and coordination. *Journal of Experimental Botany,* 64, 3965-3981.

Franks, P.J., Beerling, D.J. (2009). Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. *Proc Natl Acad Sci U S A,* 106, 10343-10347.

Giacomelli, G., Castilla, N., Van Henten, E., Mears, D., Sase, S. (2007). Innovation in Greenhouse Engineering. *International Symposium on High Technology for Greenhouse System Management: Greensys,* 801, 75-88.

Grafton, R.Q., Williams, J., Perry, C. J., Molle, F. Ringler, C., Steduto, P., Udall, B., Wheeler, S.A., Wang, Y., D. Garrick10, R.G.A. (2018). The paradox of irrigation efficiency. Higher efficiency rarely reduces water consumption. *Science,* 36, 748-750.

IPCC (2017) Meeting Report of the Intergovernmental Panel on Climate Change Expert Meeting on Mitigation, Sustainability and Climate Stabilization Scenarios (Shukla PR, Skea J, van Diemen R et al.). IPCC Working Group III Technical Support Unit, Imperial College London, UK.

Jagadish, S.V., Bahuguna, R.N., Djanaguiraman, M., Gamuyao, R., Prasad, P.V., Craufurd, P.Q. (2016). Implications of High Temperature and Elevated CO2 on Flowering Time in Plants. *Frontiers in Plant Science,* 7, 913.

Kang, S., Zhang, J. (2004). Controlled alternate partial root-zone irrigation: its physiological consequences and impact on water use efficiency. *Journal of Experimental Botany,* 55, 2437-2446.

Korner, C. (2015). Paradigm shift in plant growth control. *Curr Opin Plant Biol,* 25, 107-114.

Kyriacou, M.C., Rouphael, Y. (2018). Towards a new definition of quality for fresh fruits and vegetables. *Scientia Horticulturae,* 234, 463-469.

Leonardi, C., Guichard, S., Bertin, N. (2000). High vapour pressure deficit influences growth, transpiration and quality of tomato fruits. *Scientia Horticulturae,* 84, 285-296.

Leyva, R., Constán-Aguilar, C., Sánchez-Rodríguez, E., Romero-Gámez, M., Soriano, T. (2015). Cooling systems in screenhouses: Effect on microclimate, productivity and plant response in a tomato crop. *Biosystems engineering,* 129, 100-111.

Li, Y., Li, H., Li, Y., Zhang, S. (2017). Improving water-use efficiency by decreasing stomatal conductance and transpiration rate to maintain higher ear photosynthetic rate in drought-resistant wheat. *The Crop Journal,* 5, 231-239.

Matsuo, S., Suzuki, M., Iwasaki, Y., Fujita, T., Umeda, H. (2015). The optimum air-conditioning system design of water-saving greenhouse and its effects on growth in plants. *CIGR Journal*.

McAdam, S.A., Brodribb, T.J. (2015). The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiol,* 167, 833-843.

McAdam, S.A., Brodribb, T.J. (2016). Linking Turgor with ABA Biosynthesis: Implications for Stomatal Responses to Vapor Pressure Deficit across Land Plants. *Plant Physiology,* 171, 2008-2016.

McCartney, L., Lefsrud, M. (2018). Protected Agriculture in Extreme Environments: A Review of Controlled Environment Agriculture in Tropical, Arid, Polar, and Urban Locations, 455-473.

McAusland, L., Vialet-Chabrand, S., Davey, P., Baker, N.R., Brendel, O., Lawson, T. (2016). Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *New Phytologyst,* 211, 1209-1220.

McDowell, N., Pockman, W.T., Allen, C.D., Breshears, D.D., Cobb, N., Kolb, T., Yepez, E.A. (2008). Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytologyst,* 178, 719-739.

McDowell, N.G., Allen, C.D. (2015). Darcy's law predicts widespread forest mortality under climate warming. *Nature Climate Change,* 5, 669-672.

Medrano, H., Tomás, M., Martorell, S., Flexas, J., Hernández, E., Rosselló, J., Bota, J. (2015). From leaf to whole-plant water use efficiency (WUE) in complex canopies: Limitations of leaf WUE as a selection target. *The Crop Journal,* 3, 220-228.

Nardini, A., Tyree, M.T. (1999). Root and shoot hydraulic conductance of seven Quercus species. *Annals of Forest Science,* 56, 371-377.

Nardini A. (2001) Are sclerophylls and malacophylls hydraulically different? *Biologia Plantarum* **44,** 239–245.

Nardini, A., Salleo, S., Raimondo, F. (2003). Changes in leaf hydraulic conductance correlate with leaf vein embolism in Cercis siliquastrum L. *Trees,* 17, 529-534.

Oguchi, R., Hikosaka, K., & Hirose, T. (2005). Leaf anatomy as a constraint for photosynthetic acclimation: differential responses in leaf anatomy to increasing growth irradiance among three deciduous trees. *Plant, Cell & Environment*,*28*(7), 916-927.

Ohyama, K., Kozai, T., Toida, H. (2008). Greenhouse Cooling with Continuous Generation of Upward-Moving Fog for Reducing Wetting of Plant Foliage and Air Temperature Fluctuations: A Case Study. *Acta Horticulturae*, 321-326.

Okamoto, M., Tanaka, Y., Abrams, S.R., Kamiya, Y., Seki, M., Nambara, E. (2009). High humidity induces abscisic acid 8'-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in Arabidopsis. *Plant Physiology,* 149, 825-834.

Paradiso, R., De Micco, V., Buonomo, R., Aronne, G., Barbieri, G., De Pascale, S. (2014). Soilless cultivation of soybean for B ioregenerative LifeSupport Systems: a literature review and the experience of the MELiSSA ProjectFood characterisation Phase I. *Plant Biology*, *16*, 69-78.

Perdomo, J.A., Capo-Bauca, S., Carmo-Silva, E., Galmes, J. (2017). Rubisco and Rubisco Activase Play an Important Role in the Biochemical Limitations of Photosynthesis in Rice, Wheat, and Maize under High Temperature and Water Deficit. *Frontiers in Plant Science,* 8, 490.

Qingjie, D., Dalong, Z., Xiaocong, J., Xiaoming, S., Jianming, L. (2018). Effects of atmospheric and soil water status on photosynthesis and growth in tomato. *Plant, Soil and Environment,* 64, 13-19.

Ren, D., Martynenko, A. (2018). With the rapid development of agricultural science andtechnology, automation has become the main driving force for agriculture modernization. The applications of robotics and automation technology in agriculture include remote. *International Journal of Robotics and Automation*, 3, 33.

Rosales, M.A., Cervilla, L.M., Sanchez-Rodriguez, E., Rubio-Wilhelmi Mdel, M., Blasco, B., Rios, J.J., Ruiz, J.M. (2011). The effect of environmental conditions on nutritional quality of cherry tomato fruits: evaluation of two experimental Mediterranean greenhouses. *Journal of the Science of Food and Agriculture,* 91, 152-162.

Sack, L., and Frole, K. (2006). Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology,* 87, 483-491.

Sack, L., Scoffoni, C. (2012). Measurement of leaf hydraulic conductance and stomatal conductance and their responses to irradiance and dehydration using the Evaporative Flux Method (EFM). *Journal of Visualized Experiments*.

Sack, L., Scoffoni, C. (2013). Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. *New Phytologyst,* 198, 983-1000.

Sade, N., Gebremedhin, A., Moshelion, M. (2012). Risk-taking plants: anisohydric behavior as a stress-resistance trait. *Plant signaling & behavior*, *7*, 767-770.

Schmidhuber, J., Tubiello, F.N. (2007). Global food security under climate change. *Proceedings of National Accademy of Science U S A,* 104, 19703-19708.

Shimazaki, K., Doi, M., Assmann, S.M., Kinoshita, T. (2007). Light regulation of stomatal movement. *Annual Review in Plant Biology,* 58, 219-247.

Sinclair, T.R., Devi, J., Shekoofa, A., Choudhary, S., Sadok, W., Vadez, V., . . . Rufty, T. (2017). Limited-transpiration response to high vapor pressure deficit in crop species. *Plant Science,* 260, 109-118.

Soden, B.J., Jackson, D.L., Ramaswamy, V., Schwarzkopf, M.D., Huang, X. (2005). The radiative signature of upper tropospheric moistening. *Science,* 310, 841-844.

Stanghellini, C., and Kempkes, F. (2008). Steering of Fogging: Control of Humidity, Temperature or Transpiration? *International Workshop on Greenhouse Environmental Control and Crop Production in Semi-Arid Regions,* 797, 61-67.

Sulman, B.N., Roman, D.T., Yi, K., Wang, L., Phillips, R.P., Novick, K.A. (2016). High atmospheric demand for water can limit forest carbon uptake and transpiration as severely as dry soil. *Geophysical Research Letters,* 43, 9686-9695.

Talbott, L.D., Rahveh, E., Zeiger, E. (2003). Relative humidity is a key factor in the acclimation of the stomatal response to CO2. *journal of experimental botany,* 54, 2141±2147.

Tardieu, F., and Simonneau, T. (1998). Variability among species of stomatal control under fluctuating soil water status andevaporative demand: modelling isohydric and anisohydric behaviours. *journal of experimental botany,* 49, 419-432.

Tazoe, Y., von Caemmerer, S., Badger, M.R., Evans, J.R. (2009). Light and CO2 do not affect the mesophyll conductance to CO2 diffusion in wheat leaves. *journal of experimental botany,* 60, 2291-2301.

Terashima, I., Hanba, Y.T., Tazoe, Y., Vyas, P., Yano, S. (2006). Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO2 diffusion. *journal of experimental botany,* 57, 343-354.

Thongbai, P., Kozai, T., Ohyama, K. (2010). CO2 and air circulation effects on photosynthesis and transpiration of tomato seedlings. *Scientia Horticulturae,* 126, 338-344.

Tosens, T., Niinemets, U., Vislap, V., Eichelmann, H., Castro Diez, P. (2012). Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in Populus tremula: how structure constrains function. *Plant Cell and Environment,* 35, 839-856.

Truong, S.K., McCormick, R.F., Mullet, J.E. (2017). Bioenergy Sorghum Crop Model Predicts VPD-Limited Transpiration Traits Enhance Biomass Yield in Water-Limited Environments. *Frontiers in Plant Science,* 8, 335.

Van den Honert, T.H. (1948). Water transport in plants as a catenary process *Discussions of the Faraday Society,* 3 146-153.

Wentz, F.J., Ricciardulli, L., Hilburn, K., Mears, C. (2007). How much more rain will global warming bring? *Science,* 317, 223-235.

Wheeler, R.M., Mackowiak, C.L., Stutte, G.S., Yorio, N.C., Ruffe, L.M., Sager, J.C., Prince, R.P., Peterson, B.V., Goins, G.D., Berry, W.L., Hinkle, C.R., Knott, W.M. (2003). Crop production for advanced life support systems. Observations from the Kennedy SpaceCenter Breadboard Project. *NASA Technical Memo-randum* 211184, 58.

Wheeler, R.M. (2010). Plants for human life support in space: from Myers to Mars *Gravitational and Space Research,* 23, 25-33.

Wheeler, T.D., Stroock, A.D. (2008). The transpiration of water at negative pressures in a synthetic tree. *Nature,* 455, 208-212.

White, A.C., Rogers, A., Rees, M., Osborne, C. P. (2015). How can we make plants grow faster? A source–sink perspective on growth rate. *Journal of Experimental Botany*, *67*, 31-45.

Will, R.E., Wilson, S.M., Zou, C.B., Hennessey, T.C. (2013). Increased vapor pressure deficit due to higher temperature leads to greater transpiration and faster mortality during drought for tree seedlings common to the forest-grassland ecotone. *New Phytologyst,* 200, 366-374.

Woodword, F.I. (1987). Stomatal numbers are sensitive to increases in CO2 from pre-industrial levels. *Nature,* 327.

Xiong, D., Douthe, C., Flexas, J. (2018). Differential coordination of stomatal conductance, mesophyll conductance, and leaf hydraulic conductance in response to changing light across species. *Plant, Cell and Environment,* 41, 436-450.

Xiong, D., Liu, X., Liu, L., Douthe, C., Li, Y., Peng, S., Huang, J. (2015a). Rapid responses of mesophyll conductance to changes of CO2 concentration, temperature and irradiance are affected by N supplements in rice. *Plant, Cell and Environment,* 38, 2541-2550.

Xiong, D., Yu, T., Zhang, T., Li, Y., Peng, S., Huang, J. (2015b). Leaf hydraulic conductance is coordinated with leaf morpho-anatomical traits and nitrogen status in the genus Oryza. *Journal of Experimental Botany,* 66, 741-748.

Xu, B.J., Chang, S.K. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science,* 72, S159-166.

Zhang, D., Du, Q., Zhang, Z., Jiao, X., Song, X., Li, J. (2017). Vapour pressure deficit control in relation to water transport and water productivity in greenhouse tomato production during summer. *Scientific Reports,* 7, 43461.

Zhang, D., Jiao, X., Du, Q., Song, X., Li, J. (2018). Reducing the excessive evaporative demand improved photosynthesis capacity at low costs of irrigation via regulating water driving force and moderating plant water stress of two tomato cultivars. *Agricultural Water Management,* 199, 22-33.

Zhang, D., Zhang, Z., Li, J., Chang, Y., Du, Q., Pan, T. (2015). Regulation of Vapor Pressure Deficit by Greenhouse Micro-Fog Systems Improved Growth and Productivity of Tomato via Enhancing Photosynthesis during Summer Season. *PLoS One,* 10, e0133919.

Chapter 2

Light and Low Relative Humidity Increase Antioxidants Content in Mung Bean (*Vigna radiata* L.) Sprouts

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Light and Low Relative Humidity Increase Antioxidants Content in Mung Bean (*Vigna radiata* L.) Sprouts

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2.1 Abstract: In the last decades, there has been a growing interest in the production of sprouts, since they are a highly nutritious food, particularly suitable for indoor farming in urban areas. Achieving sprout production in indoor systems requires an understanding of possible alterations induced by the microclimate. The aim of this study was to analyze the combined effect of presence/absence of light and high/low air relative humidity (RH) on mung bean sprouts. Morpho-anatomical development and functional anatomical traits in hypocotyl were quantified. The content of antioxidants, soluble sugars, and starch were measured for nutritional and functional purposes. Different RH regimes mainly induced morpho-anatomical modifications, while the presence/absence of light changed the content of antioxidant compounds. Increments in stele diameter at high RH suggest a higher water uptake and conductivity, compared to the low RH treatment; low RH and light-induced anatomical traits improving plant water transport (reduced number of cortical layers) and increased the production of antioxidants. The overall results suggested that RH and light, already at the early stages of development, affect the plant's nutritional value. Therefore, the combination of light and low RH allows the production of antioxidant-rich mung bean sprouts to be used as a food supplement.

Keywords: air relative humidity; antioxidants; controlled environment agriculture (CEA); morpho-anatomical traits; light

2.2 Introduction: Agricultural practices are changing with the introduction of new technologies for the remote monitoring and controlling of environmental conditions, such as intelligent sensors, robots, proximal/remote sensing [1,2]. These new technologies applied to agriculture are helping to meet the nutritional requirements of an increasing population, minimizing environmental impacts and coping with the scarcity of resource availability, also exacerbated by climate changes [3]. Part of this change is happening in urban areas, where over 54% of the world population is concentrated [4] and which consumes most of the world's energy and resources while producing the majority of greenhouse gases [5]. In this context, controlled environment agriculture (CEA) and, more specifically, urban farming (e.g., rooftop greenhouses, indoor-growing modules) is receiving increasing attention as a method to improve food security, enhance sustainability, and contribute to saving transportation and logistics costs [4,5]. Currently, we are witnessing a growing interest in urban agriculture, which has been well documented by backyard food production, local-sustainable-organic restaurants, and the rise of plant-based-diets, suggesting that people desire to "get closer to their food" [6–8]. Plant-derived fresh food not only has high nutritional value but also plays a role as a stress mitigator for citizens who are subjected to urbanicity effects [9–11]. However, the possibility of growing edible crops as sources of fresh and high nutritional food directly in houses and restaurants relies on technological advancements in CEA, as well as on the development of cultivation protocols to increase crop yield while enhancing the nutritional properties of edible plant parts. In this context, sprouts and microgreens have recently gained popularity as easy-to-produce (e.g., small size, without soil and external inputs as fertilizers and pesticides), healthy gastronomic ingredients [12,13]. Indeed, compared to mature-leaf crops, they contain a higher amount of bioactive compounds, antioxidants, minerals, and phytonutrients [14]. Their consumption has been associated with a reduced risk of cancer, respiratory problems, osteoporosis, and muscle atrophy, which are frequent diseases associated with obesity and malnutrition [15–17]. Among these, *Vigna radiata* L. (mung bean) presents a high nutritional value of its sprouts, whose consumption has been related to antioxidant, anti-inflammatory, antidiabetic, antihypertensive, and antitumor effects [18,19]. Significant challenges in indoor agriculture, include the control of relative humidity (RH) and the demand for power supply, needed to artificially maintain a precise microclimate and especially light regimes to grow different plant species [20–22]. Through humidity management, it is possible to reduce water footprint, influencing the rates of water loss by crop transpiration. In addition to saving water, humidity control is important for crop performance because it influences many physiological processes from germination to senescence, such as cell expansion, photosynthesis, and growth [23,24]. Although RH control in CEA has often been overlooked due to technical

constraints [23], modulation of lighting systems and different RH regimes can increase crop growth performance and enhance food nutritional properties [25]. Sprouts' production partly overcomes these problems because they require small production facilities, shallow substrate, and very short time (4–8 days) to reach a commercial edible size [26]. Furthermore, sprouts cultivation needs low or no demand for photon flux: indeed, most seeds do not require light for sprouting and they might even be inhibited by light (e.g., Phacelia and Allium). However, other species (e.g. Begonia, Primula) need light to germinate [27]. Generally, sprouts used as food-supplement are grown in total darkness and under high levels of relative humidity, to reduce the risk for drying between watering periods [28].

Even though sprouts are usually grown etiolated, lighting systems in indoor growing modules can be modulated to achieve compound-specific improvements in sprouts' quality and sometimes to decrease the level of anti-nutrients [13], especially during the phase of seed germination [29]. Even though lightening systems can increase production costs [21], changes during the early stages of germination lead to increments in some useful metabolites such as flavonoids, phenols, vitamins and other phytochemicals [30,31]. Many studies have tested the effect of light intensity and quality on sprout phytochemical content, with contrasting results depending on the PPFD (photosynthetic photon flux density) and on the specific wavelengths [18,32,33]. However, the general tendency is to have a higher quantity of antioxidants in light-grown sprouts compared to dark-grown ones [34–37], even if sprouts grown in the light can be smaller compared to those grown in the dark [38].

Concerning the implications of RH on sprout production, several authors have demonstrated that increasing humidity during the cultivation could enhance quality traits. For example, Leyva et al. [39] found an increment in antioxidant content in many tomato cultivars grown in the presence of fogging systems. However, cultivation under different RH regimes causes modifications in the organization of crop tissues, cell size, and density [40,41], which may, in turn, affect physiological mechanisms, resource use, and tissue/organ allocation of bioactive compounds.

So far, most of the research has been conducted on adult plants, while only a few studies have been performed aiming to control RH and other environmental factors to enhance sprout growth and phytochemical production. However, the optimization of cultivation protocols already at the first stages of plant development is required not only to optimize sprout growth and enhance the production of useful secondary metabolites but also to achieve fine control of environmental parameters for sustainability issues (i.e., energy consumption). The aim of this study was to analyze the combined influence of two environmental factors namely presence/absence of light and high/low RH, on morpho-anatomical development and production of nutritious secondary metabolites in sprouts of *V. radiata* L. "green azuki", a plant species in the legume family, also known as mung bean. For these reasons, we evaluated sprouts development through morpho-anatomical analyses

and their antioxidant content (i.e., anthocyanins and polyphenols) as well as the amount of soluble sugars and starch, considered as important nutritional traits.

2.3 Results

2.3.1 Sprouts Development: The total length of sprouts, the length of roots and hypocotyls as well as the fresh weight were influenced by RH and LR as main factors, but not by their interaction (RH × LR) (Table 1). Both length and fresh weight significantly ($p \leq 0.001$) increased under high RH, independently from the presence/absence of light. Moreover, sprouts grown in the dark were always significantly more elongated ($p \leq 0.001$) and presented a higher fresh biomass ($p \leq 0.05$) compared to those grown under light. Conversely, dry weight was neither significantly influenced by RH and LR as main factors, nor by their interaction (Table 1).

Table 1. Analysis of variance and means comparison for growth traits in mung bean sprouts under low and high RH and light or dark LR as well as under the four different combinations of RH and LR (LowRH-L; LowRH-D; HighRH-L; HighRH-D). Mean values and statistical significance are shown. NS, *; **, ***, Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences, according to Duncan's multiple-range test ($p \leq 0.05$).

Treatments	Hypocotyl length (cm)	Root length (cm)	Total length (cm)	Fresh weight (g)	Dry weight (g)
Relative humidity (RH)					
Low	2.53 b	0.88 b	3.74 b	0.22 b	0.044 a
High	3.47 a	1.93 a	3.95 a	0.46 a	0.044 a
Light regime (LR)					
Light	2.69 b	1.18 b	3.55 b	0.32 b	0.048 a
Dark	3.32a	1.62 a	4.13 a	0.37 a	0.046 a
Interaction					
LowRH × light	2.26 a	0.63 a	3.40 a	0.20 a	0.047 a
HighRH × light	3.12 a	1.73 a	3.71 a	0.44 a	0.049 a
LowRH × dark	2.80 a	1.13 a	4.08 a	0.25 a	0.042 a
HighRH × dark	3.83 a	2.12 a	4.19 a	0.49 a	0.042 a
Significance					
RH	***	***	***	***	NS
LR	***	***	***	*	NS
RH × LR	NS	NS	NS	NS	NS

2.3.2 Morpho-Anatomical Analysis of Hypocotyls: Microscopy analysis evidenced that the hypocotyls maintained tissue integrity, without evident signs of stress in all the combinations of RH and LR (Figure 1). However, the expansion of the cortical cylinder (in terms of thickness) and stele (in terms of diameter) were significantly influenced by both RH and LR treatments as main factors as well as by their interaction (RH × LR) (Table 2). The sprouts grown in the dark at a low relative humidity (LowRH-D) were characterized by the widest cortical cylinder (TCC), which was significantly thicker ($p \leq 0.05$) compared to the other conditions (Table 2). As regards the stele, the smallest diameter (SD) was found in LowRH-L sprouts, with significantly lower values ($p \leq 0.01$) compared to all the other treatments (Table 2). Moreover, the number of cell layers in the cortical cylinder (CL-

CC) was not influenced by RH as the main factor; however, light alone and in interaction with RH, had a significant effect on CL-CC ($p \leq 0.05$). Conversely, no significant differences among treatments were found in the number of cell layers in the stele (CL-S). Furthermore, the number of cells per unit area in both cortical cylinder and stele (NC-CC and NC-S) were significantly influenced ($p \leq 0.001$ and $p \leq 0.05$ respectively) by microclimate interaction (RH × LR) (Table 2, Figure 1). Both NC-CC and NC-S were significantly higher in LowRH-D than HighRH-L sprouts, which showed in turn significantly higher values than sprouts developed under the other two conditions. The number of phenolic bodies (NPB) was significantly influenced by both RH and LR treatments as main factors ($p \leq 0.001$) as well as by their interaction (RH × LR) ($p \leq 0.05$) (Table 2). Moreover, the diameter of phenolic bodies (DPB) was significantly influenced by LR treatment as main factor ($p \leq 0.001$) and by the interaction (RH × LR) ($p \leq 0.01$) (Table 2). More specifically, LowRH-L presented the highest values of both NPB and DPB, which instead were significantly reduced in HighRH-D.

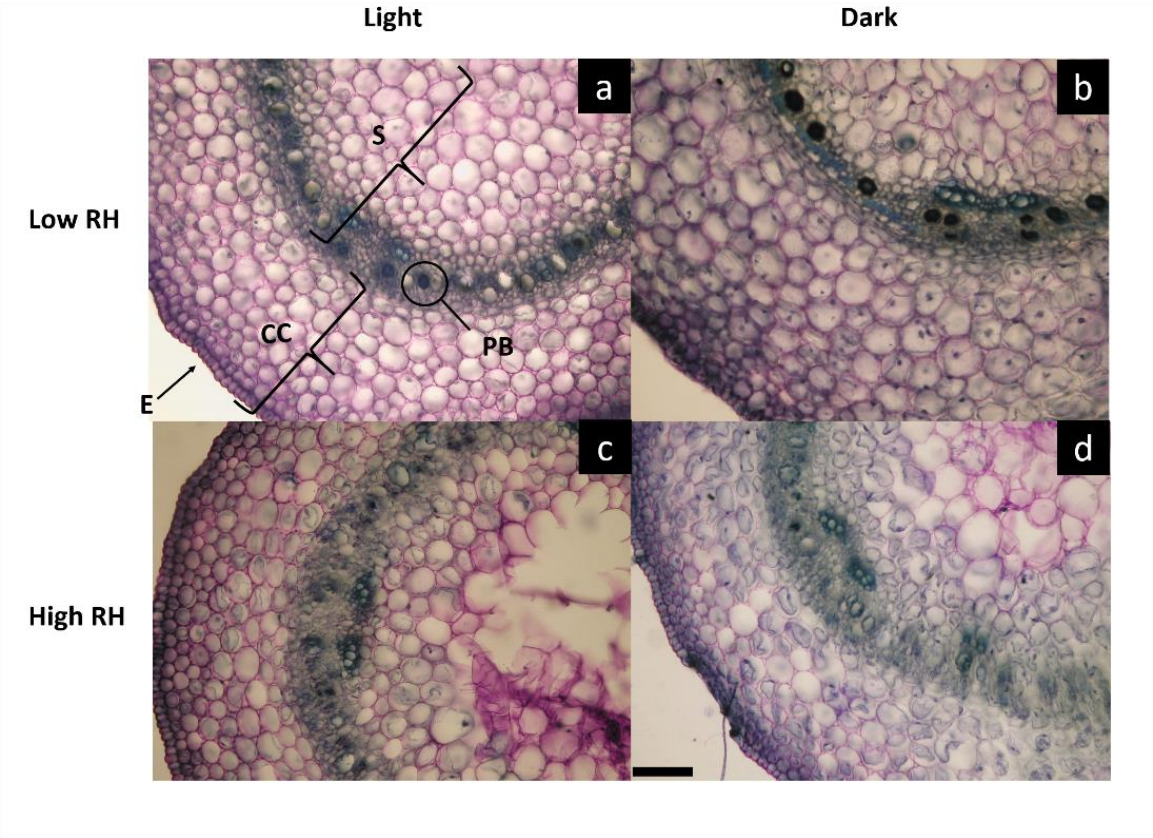

Figure 1. Light microscopy views of cross-sections of hypocotyls of mung bean sprouts grown under the four different combinations of RH and LR: a) LowRH-L; b) LowRH-D; c) HighRH-L; d) HighRH-D, High RH in dark regime). Stele (S), Cortical cylinder (CC), Epidermis, (E) and Phenolic bodies (PB). Images are at the same magnification; scale bar = 100 µm.

Table 2. Analysis of variance and means comparison for the thickness of cortycal cylinder (TCC), stele diameter (SD), total diameter (TD), number of cell layers in the cortical cylinder (CL-CC) and in the stele (CL-S), number of cells per unit area in the cortical cylinder (NC-CC), and stele (NC-S), number of phenolic bodies (NPB), Diameter of phenolic bodies (DPB) in mung bean sprouts under low and high RH and light or dark LR as well as under the four different combinations of RH and LR (LowRH-l; LowRH-D; HighRH-L; HighRH-D). Mean values and statistical significance are shown. NS, *; **, ***, Non-significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences, according to Duncan's multiple-range test (p =0.05).

Treatments	TCC (µm)	SD (µm)	TD (µm)	CL-CC (n)	CL-S (n)	NC-CC (n mm^{-2})	NC-S (n mm^{-2})	NPB (n mm^{-2})	DPB (µm)
Relative humidity(RH)									
Low	963.75a	1418.13b	2381.88 a	10.05 a	16.37a	25.35 a	21.10 a	40.07 a	4.0 a
High	817.14b	1554.28a	2371.43 a	10.02 a	17.82a	23.77 a	18.02 b	27.54 b	3.75 a
Light regime (LR)									
Light	828.46b	1429.33b	2257.80 b	9.8 b	17.45a	29.67 a	23.10 a	40.76 a	4.67 a
Dark	952.43a	1543.07a	2495.51 a	10.27 a	6.75 a	19.45 b	16.02 b	26.85 b	3.07 b
Interaction									
LowRH × light	886.56b	1322.80b	2209.36 b	9.50 b	16.75a	35.45 a	25.50 a	45.43 a	5.1 a
HighRH × light	770.38c	1535.87a	2306.25 b	10.10ab	18.15a	23.90 b	20.70 b	36.09 b	4.25 b
LowRH × dark	1040.94a	1513.46a	2554.40 a	10.60a	16.50a	15.25 c	16.70 c	34.71 b	2.9 c
HighRH × dark	863.92bc	1572.69a	2436.62 a	10.06ab	17.50a	23.65 b	15.35 c	18.99 c	3.25 c
Significance									
RH	***	***	NS	NS	NS	NS	***	***	NS
LR	**	***	***	*	NS	***	***	***	***
RH × LR	*	**	*	*	NS	***	*	*	**

2.3.3 Content of Anthocyanins and Polyphenols, and FRAP Assay: For anthocyanins and polyphenols, both RH and LR as main factors ($p \leq 0.001$) and their interaction (RH × LR) ($p \leq 0.05$) elicited significant changes (Table 3). The antioxidant activity was significantly influenced by both RH and LR as main factors ($p \leq 0.001$) and their interaction (RH × LR) ($p \leq 0.001$), as well (Table 3). The concentration of anthocyanins and polyphenols, as well as an antioxidant activity are shown in Figure 2. The highest content of polyphenols and anthocyanins occurred in sprouts grown at low RH in the presence of light (LowRH-L); such contents significantly decreased in HighRH-L sprouts, which in turn showed significantly higher values than LowRH-D ($p \leq 0.05$) (Figure 2 a,b). Finally, LowRH-D sprouts showed significantly higher values than HighRH-D ones where the lowest contents were detected (Figure 2 a,b). The antioxidant capacity showed the same trends among treatments. The highest antioxidant activity occurred in LowRH-L sprouts, which showed significantly higher values than HIghRH-L and LowRH-D sprouts ($p \leq 0.001$) (Figure 2c). The lowest values, significantly reduced compared to all the other treatments, were detected in HighRH-D sprouts (Figure 2c). The content of anthocyanins and polyphenols were positively correlated with antioxidant capacity ($r = 0.757$, $p \leq 0.001$ for anthocyanins and antioxidant capacity and $r = 0.821$, $p \leq 0.001$ for polyphenols and antioxidant capacity).

Table 3. Analysis of variance and means comparison for functional metabolites and antioxidant activity of mung bean sprouts the four different combinations of RH and LR (LowRH-L; LowRH-D; HighRH-L; HighRH-D). Data are expressed on a fresh weight basis. Mean values and statistical significance are shown. NS, *; **, ***Non significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p \leq 0.05$).

Treatments	**Anthocyanins** (mg g^{-1})	**Polyphenols** (mg GAE mg^{-1})	**Antioxidant activity** (µmol TE/mg^{-1})	**Soluble sugars** (µmol(glucose)Eq. g−1)	**Starch** (µmol (glucose) Eq. g^{-1})
Relative humidity (RH)					
Low	0.85 a	0.022 a	1.01 a	0.24 b	0.047 a
High	0.68 b	0.013 b	0.57 b	0.53 a	0.027 b
Light regime (LR)					
Light	0.93 a	0.026 a	1.02 a	0.33 b	0.046 a
Dark	0.60 b	0.011 b	0.55 b	0.44 a	0.028 b
Significance					
RH	***	***	***	***	*
LR	***	***	***	**	*
RH × LR	*	*	***	**	*

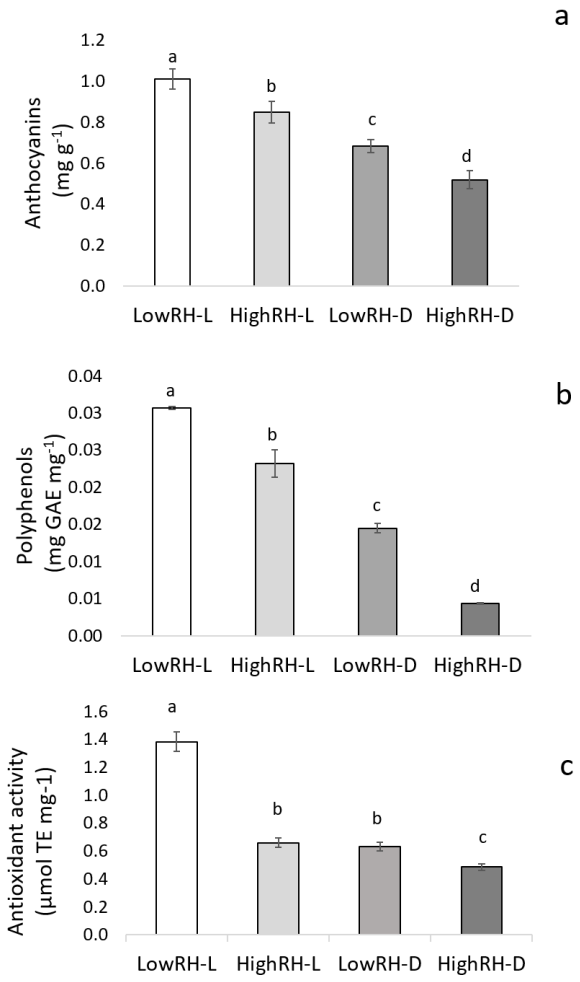

Figure 2. Concentration of anthocyanins (a), polyphenols (b) and antioxidant activity (c) in mung bean sprouts grown under the four different combinations of RH and LR (LowRH-L; LowRH-D; HighRH-L; HighRH-D). Mean values and standard errors are reported; different letters refer to statistically significant differences; $p \leq 0.05$).

2.3.4 Soluble Sugar and Starch Quantification: The content of soluble sugars was significantly influenced by RH and LR as main factors ($p \leq 0.001$ and $p \leq 0.01$ respectively), and by their interaction (RH × LR) ($p \leq 0.01$) (Table 3). Starch content was significantly influenced by both RH and LR as main factors ($p \leq 0.05$), and by their interaction (RH × LR) ($p \leq 0.05$) as well (Table 3). Their content showed an opposite trend among treatments (Table 3, Figure 3). More specifically, HighRH-D sprouts showed significantly higher values of soluble sugars than LowRH-D sprouts ($p \leq 0.01$), which, in turn, were characterized by significantly higher values compared to sprouts developed in the presence of light, independently from humidity (Figure 3a). Regarding starch content, sprouts grown under LowRH-L conditions showed significantly higher values than HighRH-L sprouts ($p \leq 0.05$), which, in turn, were characterized by significantly higher values than sprouts developed in the dark, independently from humidity levels (Figure 3b).

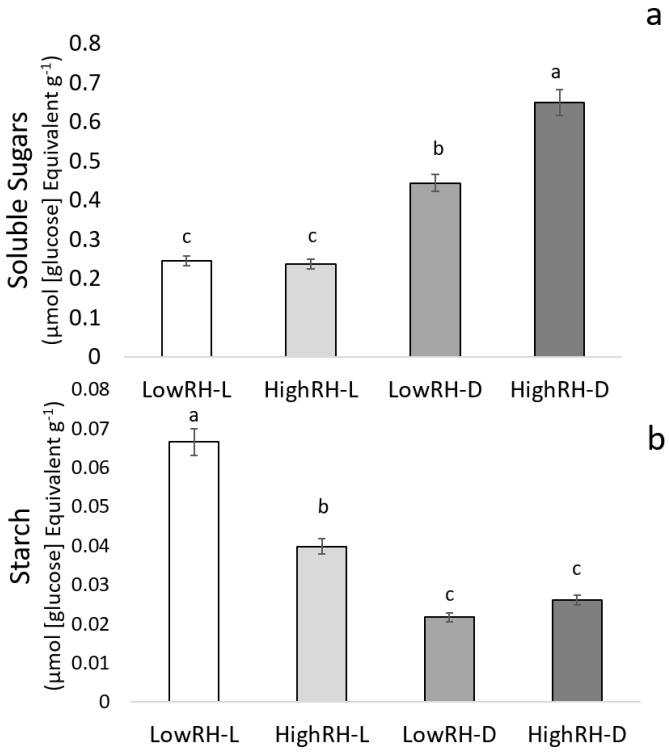

Figure 3. Soluble sugar (a) and starch (b) amount in mung bean sprouts grown under the four different combinations of RH and LR (LowRH-L; LowRH-D; HighRH-L; HighRH-D). Both soluble sugars and starch are expressed on a fresh weight basis. Mean values and standard errors are reported; different letters refer to statistically significant differences; $p \leq 0.05$.

2.4 Discussion: During seed germination, complex biochemical and physiological processes occur, resulting in wide changes in sprout morphology and biochemical composition [42]. This process is triggered by the imbibition of water and is controlled by the whole complexity of environmental parameters [43]. In the present study, the two main factors applied during sprouting (RH and LR), had significant effects on mung bean sprouts growth, morpho-anatomical development, antioxidant capacity, amount of soluble sugars and starch. More specifically, the level of humidity under which sprouts were grown, mostly influenced their morphological development. Indeed, the increase in RH from 60% to 90% lead to sprout lengthening. Furthermore, this elongation was not followed by any increment in total hypocotyl diameter, as well as in the number of cell layers in the cross-section. These results suggest that increased elongation at high RH was either due to enhanced apex proliferation or turgor driven cell-enlargement in the longitudinal direction. The humidity-driven increase in fresh weight (more than doubled under high RH compared to low RH) supports the second hypothesis. The higher turgor-driven cell enlargement is also consistent with the observed lower number of cells per stele unit area, which could be explained by an increase in cell size and unaltered dry weight among treatments. Similar results were found by McIntyre et al. [44] in the apex

growth of potato tubers; these authors hypothesized that high humidity may have increased water potential and cell turgor of sprouts, due to the reduction in their rate of transpiration.In mung bean, the interaction among factors (RH and LR) elicited changes in quantitative anatomical traits in terms of the relative ratio between cortical cylinder and stele, and the number of cells per unit area (thus consequently in cell size) in both tissues (Table 2). The differences in hypocotyl anatomical traits could be the result of adjustment strategies to different environmental conditions. For instance, the occurrence of decreased root diameters under reduced water availability is considered a way to increase water and nutrient uptake by maximizing the absorptive surfaces [45,46]. Furthermore, the reduced number of cortical layers is known to be an acclimation to drought; indeed, under drought the reduced number of cell layers shortens the radial pathway available for water transport, favoring a quick radial flow [47,48]. These results highlight the pivotal role of anatomy in controlling water movement through the soil-plant system and the relationship between xylem anatomy and hydraulic conductivity [49]. Two of the major anatomical features that distinguished categories of root anatomy are indeed the stele diameter and the arrangement of the cortical cells [50,51]. Changes in the size of the cortical cylinder and not in that of the stele has been explained by assuming the cortex acting as a “buffer zone”, partially isolating the stele from environmental stresses such as drought [52,53]. Our results are in agreement with the tendency of having a larger “buffer zone” under conditions of low humidity. The presence of light, and more specifically its intensity and quality, acts on sprout antioxidant content. In the last decades, many studies have dealt with the effects of lighting on crop quality, with very different results depending on photosynthetic photon flux density (PPFD) or light wavelength quality. Although sprouts are commonly produced in the absence of light, it has been demonstrated that even a low amount of PPFD may induce positive outcomes during germination. For example, Pérez-Balibrea et al. [34] found in light-grown broccoli sprouts an increment in phenolic compounds (by 61%) compared to sprouts grown in the dark. In contrast, Qian et al. [54] found in Chinese kale sprouts grown under blue light, the highest levels of total phenolics and anthocyanins, as well as the strongest antioxidant capacity. Phenolics are the products of secondary plant metabolism, which provide essential functions in growth and reproduction [55]. Epidemiological and experimental studies demonstrated that phenolic compounds in the human diet may provide health benefits associated with reduced risk of chronic diseases [56]. In our study, the presence of light enhanced the content of both anthocyanins and phenolics, as also visualized through microscopy (i.e., phenolic bodies), especially under low humidity growing sprouts. These sprouts (LowRH-L), which exhibited the smallest length, also presented the highest antioxidant capacity. It is known that light improves the phenolic content by promoting the production of malonyl CoA and coumaroyl CoA, which participate in the synthesis of phenolic compounds [57]. Therefore, it is not surprising that, in our study, a significant increase in total phenolics was found in light-grown sprouts compared to the dark ones. Furthermore, antioxidants and polyphenols are often synthesized by plants as a defense mechanism in response to abiotic and biotic stresses [58–60]. Under natural conditions, plants grown

in semi-arid climate enhance the production of secondary metabolites to counteract the exposure to high levels of solar radiation, high temperature, and low water availability [61]. The secondary metabolite products may contribute to preventing damages caused by reactive oxygen species (ROS) during the environmental stresses. This is consistent with the increase in the levels of polyphenols and anthocyanins in sprouts grown at low RH, notwithstanding the light regime. Moreover, many studies have reported an enhanced content of polyphenols and anthocyanins in sprouts or microgreens cultivated under light [13,35,62]. Indeed, the exposure to light may be considered the key stimulus for the synthesis of anthocyanins [32,63]. Consistently, in our experiment, the light regime increased the anthocyanin content in mung bean sprouts, which in turn resulted in a bright red color of the hypocotyls. In addition, we found a high correlation between the variation of total polyphenols and anthocyanins, and the antioxidant capacity. Antioxidant capacity, which gradually increases during germination [55] is an important quality index reflecting the synergetic effect of multiple antioxidants, including phenolic compounds. A similar tendency was also found by Gan et al. [64] in a study on mung bean sprouts, where phenolic compounds and ascorbic acid increased along with increments in antioxidant capacity. Indeed, phenolic compounds have been proved to contribute more than other antioxidants to the antioxidant capacity [65]. Moreover, Qian et al. [54] observed a significant increment in the antioxidant capacity in sprouts grown under different light treatments compared to dark, in accordance with the variation tendency of anthocyanin content. In the very early stages of development, the presence of light, determines a rise in antioxidant compounds, together with the onset of photosynthetic activity. Furthermore, it is known that the germination process leads to catabolism and degradation of main storage compounds, often accompanied by an increase in simple sugars, which are an important energy source for seeds during germination and early growth of plants [64,66]. However, the accumulation of sugar can change with environmental conditions or can be subjected to a different allocation [67]. For example, Gill et al. [68] found in seedlings of *Sorghum bicolor* L. grown in the presence of light, an elevated content of soluble sugars in comparison to dark-grown seedlings, especially under stressful conditions (Heat, Cold, NaCl treatments). However, in our study, soluble sugars content significantly decreased in sprouts grown in the presence of light. This could be explained by a simultaneous increase in the synthesis of starch. It is well known that light and consequently, day length and circadian rhythm have a significant effect on starch degradation and synthesis. It has demonstrated that starch breakdown was faster in plants growing in long days and during the night [69].

In conclusion, the overall results suggested that *V. radiata* sprouts are largely responsive to changes in environmental conditions in terms of tissue development, biomass allocation, and antioxidant production. Our results indicated that under light and low RH sprouts have evolved morpho-functional traits to cope with low RH conditions as a mechanism to elude water loss in the very early stages of development. Furthermore, sprouts grown in the presence of light, especially at low RH,

increased polyphenols and anthocyanins contents as well as improved antioxidant capacity, compared to dark conditions. Synthesis and accumulation levels of antioxidant compounds mostly depend on genotype; however, they are also largely affected by environmental factors (microclimate). Therefore, by manipulating the environment, it is possible to obtain antioxidant-rich sprouts with health-promoting properties for consumers [70,71. In our study, the combination of light-low RH was the most effective for the production of antioxidant-rich mung bean sprouts to be used as a food supplement. This could open new market opportunities in the niche of healthier and vegan food [72]. The main outcome of this study is that a fine control of all environmental variables already at the early stages of plant development should be a priority not only for optimizing plant growth but also for favoring the synthesis of useful metabolites in controlled environment agriculture production.

2.5 Materials and Methods

2.5.1 Experimental Design and Plant Material: The experiment was conducted on *V. radiata* L. by placing 120 seeds in Petri dishes (30 seeds per petri dish), layered with filter paper, and imbibed with distilled water till paper capacity. The experiment was performed in three replicates. *V. radiata* seeds were purchased from a local retailer and showed 100% germination. Petri dishes were incubated, open, in two cycles, in a multi-layers walk-in climatic room at the Department of Biology of the University of Naples Federico II (General Impianti S.A.S., Naples, Italy), at a fixed temperature of 23 ± 2 °C, under four combinations of two relative humidity (RH) and light regimes (LR) at the sprouts level: a) 60 ± 2% RH, 1.2 kPa (Vapour Pressure Deficit; VPD), 150 ± 20 µmol photons m^{-2} s^{-1} in the Photosynthetic Active Radiation (PAR) region (LowRH-L); b) 60 ± 2 % RH, in the dark (LowRH-D); c) 90 ± 2% RH, 0.3 kPa (VPD), 150 ± 20 µmol photons m^{-2} s^{-1} in the PAR region (HighRH-L); d) 90 ± 2 % RH, in the dark (HighRH-D). The chosen RH treatments were considered the minimum and maximum levels of the RH range to be maintained to avoid water stress or the spreading of plant diseases, respectively [39]. In LowRH-L and HighRH-L treatments, light was provided by white fluorescent tubes (Sylvania luxline plus -T8, F36W/840, Cool white deluxe, Germany), with a 12 h photoperiod (8 a.m.– 8 p.m.) which resulted in a 6.4 DLI (daily light integral), considered low light intensity for micro-scale vegetable production [13,28]. VPD values were calculated from the corresponding instantaneous T and RH values monitored by mini-sensors (Testo 174H, Testo, Germany) and sampled every 15 min. The indoor air temperature and air-current speed were controlled by a heat pump-based air conditioning system with three inlet vents and one outlet vent on the same side wall; whereas RH was maintained at 60% by means of a dehumidifier (ADH-1000, Airrex Portable dehumidifier, Hephzibah Co. Ltd., Nam-gu, Incheon, Korea). Seedlings were watered every day with fresh distilled water up to water holding capacity of the paper) and harvested after 8 days, before the appearance of the first two leaves, when they reached a commercial edible size standard marketable size, according to Lal and Shanmugasundaram [26]. At the end of the growth period, the percentage of germination was detected and the growth of the 90 sprouts per

each treatment was quantified by measuring epicotyl, hypocotyl and root elongation on digital images (taken during the light hours) through ImageJ 1.45 software (U.S. National Institutes of Health, Bethesda, MD, USA). The fresh weight (FW) and the dry weight (DW) were measured on 30 sprouts per treatment (10 sprouts per replicate). Once tested the homogeneity of the samples, biochemical and morpho-anatomical analyses were performed on 6 and 5 sprouts per treatment, respectively, randomly taken. All these analyses were carried out considering the single sprout as one replicate. For the biochemical assays, the sampling was carried out at 9:00 a.m. in the morning.

2.5.2 Morpho-Anatomical Analyses: Hypocotyls from the six sprouts per treatment were dissected under a reflected light microscope (SZX16; Olympus, Germany) and immediately fixed in F.A.A. (5 mL 40% formaldehyde, 5 mL glacial acetic acid, 90 mL 50% ethanol) for several days. The hypocotyl region was chosen for the analyses because the sprout nutritional quality is mainly influenced by hypocotyl length and anatomical traits. Moreover, in sprout production for human consumption, the main aim is to produce germinated seedlings which have not yet developed true leaves; thus, the primary interest was focused on the hypocotyl region [27]. Thin cross-sections (5 µm thick) of hypocotyls were cut by means of a sliding microtome. The sections were stained with 0.5% Toluidine blue in water [73], mounted with mineral oil for microscopy, and observed under a light microscope (BX60; Olympus, Tokyo, Japan). Digital images were captured using a digital camera (XC50; Olympus) and were analyzed through an image analysis software (AnalySIS 3.2, company, Tokyo, Japan) to quantify anatomical traits. More specifically, the following traits were measured (in four replicates per section): the thickness of cortical cylinder (TCC), stele diameter (SD), total diameter (TD), calculated as the sum of TCC and SD, number of cell layers in the cortical cylinder (CL-CC) and stele (CL-S), and number of cells per unit area in the cortical cylinder (NC-CC) and stele (NC-S). Furthermore, on the same thin cross-sections, phenolic bodies were detected because they appeared colored in dark blue [74]. The presence of phenolic compounds was quantified in terms of the number of phenolic bodies per unit area (NPB) (in four areas per section) and the diameter of phenolic bodies (DPB) (in 3–7 phenolic bodies per section). We also checked for the presence of abnormal lignification, which could have affected the taste and texture of sprouts grown under the light regime, through epi-fluorescence microscopy (BX60, Olympus) with specific settings (Mercury lamp, band-pass filter 330–385 nm, dichromatic mirror 400 nm and above, barrier filter 420 nm and above) for the detection of the UV-induced fluorescence of lignin and other phenolic compounds [75–77]. Under such settings, lignin emits light blue fluorescence, suberin produces white-violet fluorescence, and simple phenolics (those stained in dark blue with Toluidine blue) appear yellow-orange fluorescent. Since no abnormal phenomena of either lignification nor suberization were detected, we decided to quantify only the content of polyphenols and anthocyanins through biochemical assays.

2.5.3 Polyphenol and Anthocyanin Content: Both polyphenols and anthocyanins were determined spectrophotometrically on each of the five sprouts per condition. For polyphenols determination, the procedure reported by Singelton and Rossi [78], modified by Costanzo et al. [79], was used. Briefly, 200 mg of the fresh sample from each hypocotyl were ground in methanol at 4 °C and then centrifuged at 11,000 rpm for 5 min. Pellets were discarded, while supernatants were mixed with 1:1 (v/v) 10% Folin Ciocâlteu and 1:5 (v/v) 700 mM Na2CO3 solution. Samples were then incubated at 4 °C for 2 h. The absorbance was determined at 765 nm (Cary 100 UV-VIS, Agilent Technologies, Santa Clara, CA, USA) and the concentration was expressed as gallic acid equivalents (GAE mg mg-1) using the regression equation between the different concentration of gallic acid standard and the absorbance at 765 nm. Anthocyanin levels were determined following Neff and Chory [80], by incubating the sprouts overnight in 150 mL of methanol, acidified with 1% HCl. Anthocyanins were determined by measuring the absorbance at 530 and 657 nm of the aqueous phase. More specifically, anthocyanins were calculated by subtracting one-fourth of the absorbance at 657 nm from the absorbance at 530 [81], to take into account the overlapping with chlorophylls whose increase in absorbance is different at the two wavelengths when in acidic methanol solution.

Therefore, the relative amount of anthocyanins per seedling (mg g-1) was calculated as follows (Equation (1)):

Acy = (A 530 – (0.25 * A 657))* extraction volume (mL)/1000 * g (FW) (1)

2.5.4 FRAP Assay: The antioxidant capacity was evaluated through the Ferric reducing/antioxidant power (FRAP) assay on each of the five sprouts per condition, following the procedure reported in George et al. [82], modified by Motta et al. [83]: 250 mg of the fresh sample from each hypocotyl were ground in liquid nitrogen and treated with methanol/water (60/40, v/v) solution. Samples were then centrifuged, and the supernatant was collected for the assay. The assay was carried out by adding 2.5 mL of acetate buffer, pH 3.6, 0.25 mL of TPTZ (4,6-tripyridyl-s-triazine, Fluka Chemicals, Switzerland) solution (10 mM) in 40 mM HCl, 0.25 mL of FeCl3·6H2O solution (12 mM), and 150 mL of the supernatant obtained from the above extraction. After 30 min of incubation at room temperature, the absorbance of the formed product (ferrous tripyridyl triazine complex) was read spectrophotometrically at 593 nm. Results were expressed as micromoles of Trolox equivalents (TE) per mg, obtained from the standard curve between 20 and 800 µM Trolox.

2.5.5. Soluble Sugar and Starch Quantification: Soluble sugar and starch were extracted from each of the five sprouts per condition and quantified following the anthrone method [84]. Briefly, 100 mg of the fresh sample from each hypocotyl were firstly ground to powder, then sugars were extracted in 2.5 N HCl and their concentration was determined by the anthrone reaction (ACS reagent 97%, Sigma–Aldrich, Saint Louis, USA) and sulfuric acid. When anthrone reacts with carbohydrates, in a hot bath, a green-colored product emerges. Its absorbance was read

spectrophotometrically at 630 nm. Once sugars suspended in the supernatant were removed and analyzed, pellets were evaporated to dryness and used for starch extraction by means of perchloric acid solution. In hot acidic medium, starch is hydrolyzed to glucose and dehydrated to hydroxymethyl furfural. Subsequently, a standard curve with different glucose concentrations was prepared, and results were expressed as µmol glucose equivalent g^{-1}. For starch concentration, the value obtained from the standard curve was multiplied for 0.9, as 0.9 g of starch yield as 1 g of glucose on hydrolysis [85,86].

2.5.6. Statistical Analysis: Results were subjected to statistical analysis using SPSS® statistical software (SPSS, Chicago, IL, USA). The influence of the two different categorical independent factors (i.e., relative humidity, RH – 2; light regimes, LR – 2), and their possible interaction, on each of the continuous dependent variables were studied by applying two-way analysis of variance (ANOVA). In the case of rejection of the null hypothesis, the Duncan and Student–Newman–Keuls (SNK) post-hoc tests were used ($p \leq 0.05$). Whenever the interaction between RH and LR was significant, data were subjected to one-way ANOVA and multiple comparison tests were performed with Duncan and SNK coefficients, considering as significant level of probability $p \leq 0.05$. The Kolmogorov–Smirnov and Shapiro–Wilk tests were performed to check for normality and a Levene's test of homogeneity was used to determine if samples had equal variance. Finally, to check for correlations among anthocyanins, polyphenols, and FRAP assay, a Pearson rank correlation coefficient was calculated.

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2.6 References

1. King, B.; Wong, K. The 2017 CGIAR Inspire Challenge: Innovation Strategies for Digital Agriculture; publisher: city, country. 2017.

2. Amitrano, C.; Chirico, G.B.; De Pascale, S.; Rouphael, Y.; De Micco, V. Crop Management in Controlled Environment Agriculture (CEA) Systems Using Predictive Mathematical Models. Sensors 2020, 20, 3110.

3. Derkzen, M.L.; Nagendra, H.; Van Teeffelen, A.J.; Purushotham, A.; Verburg, P.H. Shifts in ecosystem services in deprived urban areas: Understanding people's responses and consequences for well-being. Ecol. Soc. 2017, 22.

4. Weber, C.F. Broccoli microgreens: A mineral-rich crop that can diversify food systems. Front. Nutr. 2017, 4, 7.

5. Taylor, R.; Carandang, J.; Alexander, C.; Calleja, J. Making global cities sustainable: Urban rooftop hydroponics for diversified agriculture in emerging economies. OIDA Int. J. Sustain. Dev. 2012, 5, 11–28.

6. Jim, C.Y. Green-space preservation and allocation for sustainable greening of compact cities. Cities 2004, 21, 311–320.

7. Adeyemi, O.; Grove, I.; Peets, S.; Domun, Y.; Norton, T. Dynamic modelling of lettuce transpiration for water status monitoring. Comput. Electron. Agric. 2018, 155, 50–57, doi: 10.1016/j.compag.2018.10.008.

8. Mark, J. Digging for the Roots of the Urban Farming Movement. Gastron. J. Food Cult. 2011, 11, 87–91.

9. Ulrich, R.S.; Simons, R.F.; Losito, B.D.; Fiorito, E.; Miles, M.A.; Zelson, M. Stress recovery during exposure to natural and urban environments. J. Environ. Psychol. 1991, 11, 201–230.

10. Lewis, C.A. Human health and well-being: The psychological, physiological, and sociological effects of plants on people. Hortic. Hum. Life Cult. Environ. 1994, 391, 31–40.

11. Lederbogen, F.; Kirsch, P.; Haddad, L.; Streit, F.; Tost, H.; Schuch, P.; Wüst, S.; Pruessner, J.C.; Rietschel, M.; Deuschle, M.; et al. City living and urban upbringing affect neural social stress processing in humans. Nature 2011, 474, 498–501.

12. Ebert, A.; Wu, T.; Yang, R. Amaranth sprouts and microgreens—A homestead vegetable production option to enhance food and nutrition security in the rural-urban continuum. In Proceedings of the Regional Symposium on Sustaining Small-Scale Vegetable Production and Marketing Systems for Food and Nutrition Security (SEAVEG 2014), Bangkok, Thailand, February 2014; pp. 25–27.

13. Kyriacou, M.C.; Rouphael, Y.; Di Gioia, F.; Kyratzis, A.; Serio, F.; Renna, M.; De Pascale, S.; Santamaria, P. Micro-scale vegetable production and the rise of microgreens. Trends Food Sci. Technol. 2016, 57, 103–115, doi:10.1016/j.tifs.2016.09.005.

14. Adhikari, B.; Dhungana, S.K.; Ali, M.W.; Adhikari, A.; Kim, I.D.; Shin, D.H. Resveratrol, total phenolic and flavonoid contents, and antioxidant potential of seeds and sprouts of Korean peanuts. Food Sci. Biotechnol. 2018, 27, 1275–1284, doi:10.1007/s10068-018-0364-7.

15. Alekel, D.L.; Germain, A.S.; Peterson, C.T.; Hanson, K.B.; Stewart, J.W.; Toda, T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. Am. J. Clin. Nutr. 2000, 72, 844–852.

16. Tada, O.; Yokogoshi, H. Effect of different dietary protein composition on skeletal muscle atrophy by suspension hypokinesia/hypodynamia in rats. J. Nutr. Sci. Vitaminol. 2002, 48, 115–119.

17. Faienza, M.F.; D'Amato, G.; Chiarito, M.; Colaianni, G.; Colucci, S.; Grano, M.; Corbo, F.; Brunetti, G. Mechanisms involved in childhood obesity-related bone fragility. Front. Endocrinol. 2019, 10, 269.

18. Amitrano, C.; Vitale, E.; De Micco, V.; Arena, C. Light fertilization affects growth and photosynthesis in mung bean (Vigna radiata) plants. J. Environ. Account. Manag. 2018, 6, 295–304.

19. Tang, D.; Dong, Y.; Ren, H.; Li, L.; He, C. A review of phytochemistry, metabolite changes, and medicinal uses of the common food mung bean and its sprouts (Vigna radiata). Chem. Cent. J. 2014, 8, 4.

20. Chance, E.; Ashton, W.; Pereira, J.; Mulrow, J.; Norberto, J.; Derrible, S.; Guilbert, S. The Plant—An experiment in urban food sustainability. Environ. Prog. Sustain. Energy 2018, 37, 82–90.

21. Zhang, Y.; Kacira, M. Enhancing resource use efficiency in plant factory. In Proceedings of the International Horticultural Congress IHC2018: III International Symposium on Innovation and New Technologies in Protected 1271, August 2018; pp. 307–314.

22. Lefers, R.M.; Bettahalli, N.S.; Fedoroff, N.V.; Ghaffour, N.; Davies, P.A.; Nunes, S.P.; Leiknes, T. Hollow fibre membrane-based liquid desiccant humidity control for controlled environment agriculture. Biosyst. Eng. 2019, 183, 47–57.

23. Forney, C.F.; Brandl, D.G. Control of humidity in small controlled-environment chambers using glycerol-water solutions. HortTechnology 1992, 2, 52–54.

24. Amitrano, C.; Arena, C.; Rouphael, Y.; De Pascale, S.; De Micco, V. Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. Ann. Appl. Biol. 2019, 175, 313–325, doi:10.1111/aab.12544.

25. Charlebois, S. Microgreens with Big Potential; Wilton Consulting Group. pp. 1–12. Retrieved from https://static1.squarespace.com/static/59a566808419c2c20ebc2768/t/5bec6f7840ec9a4b55d39143/1542221690715/Microgreens+with+big+ potential_CaseStudy.pdf. 2019.

26. Lal, G.; Shanmugasundaram, S. Mungbean Sprout production. In Training Manual; Asian Vegetable Research and Development Center: Shanhua, Taiwan, 2001.

27. Miles, A.; Brown, M. Teaching Organic Farming and Gardening: Resources for Instructors; University of California Farm and Garden: Santa Cruz, CA, USA, 2007.

28. Price, T.V. Seed Sprout Production for Human Consumption—A Review. Can. Inst. Food Sci. Technol. J. 1988, 21, 57–65, doi:10.1016/s0315-5463(88)70718-x.

29. Samuolienė, G.; Urbonavičiūtė, A.; Brazaitytė, A.; Šabajevienė, G.; Sakalauskaitė, J.; Duchovskis, P. The impact of LED illumination on antioxidant properties of sprouted seeds. Cent. Eur. J. Biol. 2011, 6, 68–74.

30. El-Adawy, T.; Rahma, E.; El-Bedawey, A.; El-Beltagy, A. Nutritional potential and functional properties of germinated mung bean, pea and lentil seeds. Plant Foods Hum. Nutr. 2003, 58, 1–13.

31. Silva, L.R.; Pereira, M.J.; Azevedo, J.; Gonçalves, R.F.; Valentão, P.; de Pinho, P.G.; Andrade, P.B. Glycine max (L.) Merr., Vigna radiata L. and Medicago sativa L. sprouts: A natural source of bioactive compounds. Food Res. Int. 2013, 50, 167–175, doi: 10.1016/j.foodres.2012.10.025.

32. Izzo, L.; Arena, C.; De Micco, V.; Capozzi, F.; Aronne, G. Light quality shapes morpho-functional traits and pigment content of green and red leaf cultivars of Atriplex hortensis. Sci. Hortic. 2019, 246, 942–950.

33. Paradiso, R.; De Micco, V.; Buonomo, R.; Aronne, G.; Barbieri, G.; De Pascale, S. Soilless cultivation of soybean for Bioregenerative Life-Support Systems: A literature review and the experience of the MELiSSA Project—Food characterisation Phase I. Plant Biol. (Stuttg) 2014, 16, 69–78, doi:10.1111/plb.12056.

34. Pérez-Balibrea, S.; Moreno, D.A.; García-Viguera, C. Influence of light on health-promoting phytochemicals of broccoli sprouts. J. Sci. Food Agric. 2008, 88, 904–910, doi:10.1002/jsfa.3169.

35. Brazaityte, A.; Sakalauskiene, S.; Samuoliene, G.; Jankauskiene, J.; Virsile, A.; Novickovas, A.; Sirtautas, R.; Miliauskiene, J.; Vastakaite, V.; Dabasinskas, L.; et al. The effects of LED illumination spectra and intensity on carotenoid content in Brassicaceae microgreens. Food Chem. 2015, 173, 600–606, doi:10.1016/j.foodchem.2014.10.077.

36. Ma, M.; Wang, P.; Yang, R.; Gu, Z. Effects of UV-B radiation on the isoflavone accumulation and physiological-biochemical changes of soybean during germination: Physiological-biochemical change of germinated soybean induced by UV-B. Food Chem. 2018, 250, 259–267, doi:10.1016/j.foodchem.2018.01.051.

37. Raicu, D.; Livardariu, O.; Maximilian, C.; BIra, A. The evaluation of the effect of LED-s irradiation on wheat sprouts (Triticum aestivum L.). Rom. Biotechnol. Lett. 2018, 10.

38. Yuan, M.; Jia, X.; Ding, C.; Zeng, H.; Du, L.; Yuan, S.; Liu, J. Effect of fluorescence light on phenolic compounds and antioxidant activities of soybeans (Glycine max L. Merrill) during germination. Food Sci. Biotechnol. 2015, 24, 1859–1865.

39. Leyva, R.; Constán-Aguilar, C.; Sánchez-Rodríguez, E.; Romero-Gámez, M.; Soriano, T. Cooling systems in screenhouses: Effect on microclimate, productivity and plant response in a tomato crop. Biosyst. Eng. 2015, 129, 100–111, doi: 10.1016/j.biosystemseng.2014.09.018.

40. Amitrano, C.; Chirico, G.B.; De Pascale, S.; Rouphael, Y.; De Micco, V. Application of a MEC model for the irrigation control in green and red-leaved lettuce in precision indoor cultivation. In Proceedings of the 2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor), Portici, Italy, 24–26 October 2019; pp. 196–201.

41. Du, Q.; Liu, T.; Jiao, X.; Song, X.; Zhang, J.; Li, J. Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. J. Exp. Bot. 2019, 70, 4949–4962, doi:10.1093/jxb/erz238.

42. Lin, P.-Y.; Lai, H.-M. Bioactive compounds in legumes and their germinated products. J. Agric. Food Chem. 2006, 54, 3807–3814.

43. Sousa, C.; Lopes, G.; Pereira, D.M.; Taveira, M.; Valentão, P.; Seabra, R.M.; Pereira, J.A.; Baptista, P.; Ferreres, F.; Andrade, P.B. Screening of antioxidant compounds during sprouting of Brassica oleracea L. var. costata DC. Comb. Chem. High Throughput Screen. 2007, 10, 377–386.

44. McIntyre, G.I.; Quick, W.A. Control of sprout growth in the potato. Effect of humidity and water supply. Can. J. Bot. 1984, 62, 2140–2145.

45. Eissenstat, D.M. Costs and benefits of constructing roots of small diameter. J. Plant Nutr. 1992, 15, 763–782.

46. De Micco, V.; Aronne, G. Morpho-Anatomical Traits for Plant Adaptation to Drought. In Plant Responses to Drought Stress: From Morphological to Molecular Features; Springer: Berlin, Germany, 2012; pp. 37–61, doi:10.1007/978-3-642-32653-0_2.

47. Fahn, A. Some anatomical adaptations of desert plants. Phytomorphology 1964, 4, 93–101.

48. Peña-Valdivia, C.B.; Sánchez-Urdaneta, A.B.; Meza Rangel, J.; Juárez Muñoz, J.; García-Nava, R.; Celis Velázquez, R. Anatomical root variations in response to water deficit: Wild and domesticated common bean (Phaseolus vulgaris L). Biol. Res. 2010, 43, 417–427.

49. Rodriguez-Gamir, J.; Intrigliolo, D.S.; Primo-Millo, E.; Forner-Giner, M.A. Relationships between xylem anatomy, root hydraulic conductivity, leaf/root ratio and transpiration in citrus trees on different rootstocks. Physiol. Plant 2010, 139, 159–169, doi:10.1111/j.1399-3054.2010.01351.x.

50. McDonald, M.; Galwey, N.; Colmer, T. Similarity and diversity in adventitious root anatomy as related to root aeration among a range of wetland and dryland grass species. Plant Cell Environ. 2002, 25, 441–451.

51. Gu, J.; Xu, Y.; Dong, X.; Wang, H.; Wang, Z. Root diameter variations explained by anatomy and phylogeny of 50 tropical and temperate tree species. Tree Physiol. 2014, 34, 415–425, doi:10.1093/treephys/tpu019.

52. Kondo, M.; Aguilar, A.; Abe, J.; Morita, S. Anatomy of nodal roots in tropical upland and lowland rice varieties. Plant Prod. Sci. 2000, 3, 437–445.

53. Lux, A.; Luxová, M.; Abe, J.; Morita, S. Root cortex: Structural and functional variability and responses to environmental stress. Root Res. 2004, 13, 117–131.

54. Qian, H.; Liu, T.; Deng, M.; Miao, H.; Cai, C.; Shen, W.; Wang, Q. Effects of light quality on main health-promoting compounds and antioxidant capacity of Chinese kale sprouts. Food Chem. 2016, 196, 1232–1238, doi:10.1016/j.foodchem.2015.10.055.

55. Guo, X.; Li, T.; Tang, K.; Liu, R.H. Effect of germination on phytochemical profiles and antioxidant activity of mung bean sprouts (Vigna radiata). J. Agric. Food Chem. 2012, 60, 11050–11055, doi:10.1021/jf304443u.

56. Boivin, D.; Lamy, S.; Lord-Dufour, S.; Jackson, J.; Beaulieu, E.; Côté, M.; Moghrabi, A.; Barrette, S.; Gingras, D.; Béliveau, R. Antiproliferative and antioxidant activities of common vegetables: A comparative study. Food Chem. 2009, 112, 374–380.

57. Kim, J.-H.; Baek, M.-H.; Chung, B.Y.; Wi, S.G.; Kim, J.-S. Alterations in the photosynthetic pigments and antioxidant machineries of red pepper (Capsicum annuum L.) seedlings from gamma-irradiated seeds. J. Plant Biol. 2004, 47, 314–321.

58. Nikolova, M.T.; Ivancheva, S.V. Quantitative flavonoid variations of Artemisia vulgaris L. and Veronica chamaedrys L. in relation to altitude and polluted environment. Acta Biol. Szeged. 2005, 49, 29–32.

59. Arena, C.; Turano, M.; Mele, B.H.; Cataletto, P.; Furia, M.; Pugliese, M.; De Micco, V. Anatomy, photochemical activity, and DNA polymorphism in leaves of dwarf tomato irradiated with X-rays. Biol. Plant. 2017, 61, 305–314.

60. De Micco, V.; Arena, C.; Aronne, G. Anatomical alterations of Phaseolus vulgaris L. mature leaves irradiated with X-rays. Plant Biol. (Stuttg) 2014, 16, 187–193, doi:10.1111/plb.12125.

61. Selmar, D.; Kleinwachter, M. Stress enhances the synthesis of secondary plant products: The impact of stress-related over-reduction on the accumulation of natural products. Plant Cell Physiol. 2013, 54, 817–826, doi:10.1093/pcp/pct054.

62. Hara, M.; Oki, K.; Hoshino, K.; Kuboi, T. Enhancement of anthocyanin biosynthesis by sugar in radish (Raphanus sativus) hypocotyl. Plant Sci. 2003, 164, 259–265.

63. Steyn, W.; Wand, S.; Holcroft, D.; Jacobs, G. Anthocyanins in vegetative tissues: A proposed unified function in photoprotection. New Phytol. 2002, 155, 349–361.

64. Gan, R.-Y.; Wang, M.-F.; Lui, W.-Y.; Wu, K.; Corke, H. Dynamic changes in phytochemical composition and antioxidant capacity in green and black mung bean (Vigna radiata) sprouts. Int. J. Food Sci. Technol. 2016, 51, 2090–2098, doi:10.1111/ijfs.13185.

65. Vinson, J.A.; Dabbagh, Y.A.; Serry, M.M.; Jang, J. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. J. Agric. Food Chem. 1995, 43, 2800–2802.

66. Shi, H.; Nam, P.K.; Ma, Y. Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (Glycine max) germination. J. Agric. Food Chem. 2010, 58, 4970–4976.

67. Meier, H.; Reid, J. Reserve polysaccharides other than starch in higher plants. In Plant Carbohydrates I; Springer: Berlin/Heidelberg, Germany, 1982; pp. 418–471.

68. Gill, P.K.; Sharma, A.D.; Singh, P.; Bhullar, S.S. Changes in germination, growth and soluble sugar contents of Sorghum bicolor (L.) Moench seeds under various abiotic stresses. Plant Growth Regul. 2003, 40, 157–162.

69. Lu, Y.; Gehan, J.P.; Sharkey, T.D. Daylength and Circadian Effects on Starch Degradation and Maltose Metabolism. Plant Physiol. 2005, 138, 2280–2291.

70. Kyriacou, M.C.; Rouphael, Y. Towards a new definition of quality for fresh fruits and vegetables. Sci. Hortic. 2018, 234, 463–469, doi:10.1016/j.scienta.2017.09.046.

71. Lei, W.Q.; Khanizadeh, S.; Vigneault, C. Preharvest ways of enhancing the phytochemical content of fruits and vegetables. Stewart Postharvest Rev. 2007, 3, 1–8.

72. Brückner, B.; Schonhof, I.; Kornelson, C.; Schrödter, R. Multivariate sensory profile of broccoli and cauliflower and consumer preference. Ital. J. Food Sci. 2005, 17.

73. Feder, N.; O'brien, T. Plant microtechnique: Some principles and new methods. Am. J. Bot. 1968, 55, 123–142.

74. Schoenwaelder, M.E. The biology of phenolic containing vesicles. Algae 2008, 23, 163–175.

75. Fukazawa, K. Ultraviolet microscopy. In Methods in Lignin Chemistry; Springer: Berlin/Heidelberg, Germany, 1992; pp. 110–121.

76. Ruzin, S.E. Plant Microtechnique and Microscopy; Oxford University Press: New York, NY, USA, 1999; Volume 198.

77. De Micco, V.; Aronne, G. Combined histochemistry and autofluorescence for identifying lignin distribution in cell walls. Biotech. Histochem. 2007, 82, 209–216.

78. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 1965, 16, 144–158.

79. Costanzo, G.; Iesce, M.R.; Naviglio, D.; Ciaravolo, M.; Vitale, E.; Arena, C. Comparative Studies on Different Citrus Cultivars: A Revaluation of Waste Mandarin Components. Antioxidants 2020, 9, 517, doi:10.3390/antiox9060517.

80. Neff, M.M.; Chory, J. Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. Plant Physiol. 1998, 118, 27–35.

81. Meng, X.; Xing, T.; Wang, X. The role of light in the regulation of anthocyanin accumulation in Gerbera hybrida. Plant Growth Regul. 2004, 44, 243–250.

82. George, B.; Kaur, C.; Khurdiya, D.; Kapoor, H. Antioxidants in tomato (Lycopersium esculentum) as a function of genotype. Food Chem. 2004, 84, 45–51.

83. Motta, C.M.; Simoniello, P.; Arena, C.; Capriello, T.; Panzuto, R.; Vitale, E.; Agnisola, C.; Tizzano, M.; Avallone, B.; Ferrandino, I. Effects of four food dyes on development of three model species, Cucumis sativus, Artemia salina and Danio rerio: Assessment of potential risk for the environment. Environmental Pollut. 2019, 253, 1126–1135.

84. Marshall, J. Drought and shade interact to cause fine-root mortality in Douglas-fir seedlings. Plant Soil 1986, 91, 51–60.

85. Sharma, D.; Pathak, D.; Atwal, A.; Sangha, M. Genetic variation for some chemical and biochemical characteristics in cotton seed oil. J. Cotton Res. 2009, 23, 1–7.

86. Katoch, R. Analytical Techniques in Biochemistry and Molecular Biology; Springer Science & Business Media: Berlin, Germany, 2011.

Chapter 3

Leaf morpho-anatomical traits in *Vigna radiata* L. affect plant photosynthetic acclimation to changing vapor pressure deficit

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Leaf morpho-anatomical traits in *Vigna radiata* L. affect plant photosynthetic acclimation to changing vapor pressure deficit

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3.1 Abstract: Plants adaptation to environmental constraints such as high VPD, involve long-term and transitory changes in photosynthesis, transpiration, and stomatal conductance. The exposure to different VPDs is also recognized to induce structural changes in leaves; nevertheless, the functional interlinks between different leaf anatomical development and plant acclimation to changing environmental conditions, have not been completely understood. The present work aims to unravel whether plants developed under different conditions of vapour pressure deficit show different ability to adapt to short-term changes in the VPD levels. *Vigna radiata* L. plants were cultivated in growth chambers under low and high VPD conditions up to the adult stage, characterized by the presence of at least 4 trifoliate leaves. Then, part of the plants was transferred to the opposite VPD condition. Completely life span compound leaves were subjected to anatomical, eco-physiological and biochemical analyses to quantify parameters linked with hydraulic conductivity and photosynthetic efficiency. Results showed that plants developed in low VPD after the transplanting under high VPD conditions, although presenting a coordination in stomata and vein density, which is considered still inadequate for the acclimation under the increased evaporative demand, showed a certain extent of plasticity. Therefore, they managed to adapt to the new environmental condition, probability due to the presence of smaller stomata and higher VLA (leaf vein density). Whereas, after the transplant, plant developed under high VPD, did not show the same plasticity in adaptation, due to their less

efficient anatomical structure, in terms of stomata number and density, mesophyll composition and vein density. These results highlight the key role of leaf anatomical structure in photosynthetic apparatus acclimation to air VPD.

Keywords: leaf anatomy, leaf vein density, long/short-term acclimation, mesophyll resistance, plant hydraulics, photosynthesis, relative humidity, stomatal conductance, stomata chloroplasts, stomatal size, and density.

3.2 Introduction: One of the major challenges for plant development is to invest resources to construct leaves capable of enhancing photosynthetic carbon gain, thus efficiently converting carbon into biomass. At the same time, however, leaves should be able to provide enough water in order to supply evapo-transpiration losses (Murphy et al., 2014). Hence, any plant makes a compromise between photosynthetic rates and the efficiency of water use. A central role to achieve this compromise is played by the balance among different components of leaf anatomy: chloroplasts in palisade parenchyma cells, headquarter of photosynthesis; stomatal size and density in the epidermis, which control the intake of carbon dioxide as well as the amount of water loss (Fanourakis et al., 2014); the percentage of intercellular spaces, recognized as sites of major resistance to water flows (Buckley et al., 2015; Sack et al., 2015) and leaf vein density and architecture, which are critical for water and nutrient transport (Scoffoni et al., 2015). Across plants species and evolutionary lineages, leaves are enormously diverse in size, shape and in their anatomy (Sack et al., 2015). Although it is not already ascertained if structure is the bottleneck of function or the other way around, it is certain that sink and source activities cannot be decoupled (Dingkuhn et al., 2007; Korner et al., 2015). Indeed, differences in leaf anatomy reflect changes in function in term of net-CO2 assimilation, yield and water transport (liquid and vapour). For instance, the efficiency of water transport in leaves (leaf hydraulic conductance; Kleaf) depends on the complexity of the whole leaf conductance and resistance that water faces during its way from the xylem trough the mesophyll to the site of evapotranspiration (stomata) (Xiong et al., 2017). Eventually, Kleaf, Mesophyll conductance (g_m) and stomatal conductance (g_s) are strictly constrained by leaf vein density, chloroplasts distribution in cells (Tosens et al., 2015) and stomatal size and density, anatomical traits which often develop in harmony. Indeed, the number and density of vessels (transport system) and stomata (evaporative site) allow the maintenance of a balance between water use, carbon gain and evaporation under different environmental conditions (Brodribb & Jordan 2011). The extent to which all these anatomical parameters constraint plant function, severely depends on the species and sometimes this variation is poorly understood. Although leaf structure is responsible for a considerable portion of the whole conductance and resistance of water in plants, environmental conditions, and in particular the Vapour Pressure Deficit (VPD), establish the whole rate of transpiration (Carins Murphy et al., 2014). Plants grown under low VPD (high air relative humidity,

RH) usually exhibit high rates of photosynthesis as well as enhanced stomatal and mesophyll conductance (Amitrano et al., 2019). Conversely, high VPD (low RH) exposed-plants, subjected to high evaporative demand, are less efficient from a physiological point of view and this limitation depends on both stomatal and non-stomatal factors (Shibuya et al., 2017). Recently, several publications have described VPD effects on plant growth and development, and it is now recognized that VPD can induce changes in stomatal size and density, mesophyll anatomy and vein density (Bongi and Loreto 1989; Fanourakis et al. 2013; Du et al., 2019). Therefore, changes in leaf anatomical traits may account for changes in the photosynthetic response. However, a certain amount of intra-species variation due to environmental pressure is often observed (Carins Murphy et al., 2014) and over time inconsistent results have also been found. For instance, in a recent paper, smaller stomata and lower density were found in Arabidopsis thaliana (Lake & Woodward 2008) plants exposed to high VPD compared to low-VPD, whereas Torre et al. (2003) found in Rosa, under a similar environmental condition, smaller stomata but a higher density. Furthermore, Fanourakis et al. (2012) did not observe in Rosa hybrida L. a relation between xylem anatomy and the plant response to air humidity, while they discovered a correlation between stomata anatomical features and sensitivity to RH. Giday et al. (2013) confirmed this correlation, showing that stomata traits, and in particular their size, contribute to determine their function. In the field, VPD increase linearly with the evaporative demand and follows a daily cycle, being lower at sunrise and higher (exciding 2 kPa) during the hottest hours (Fletcher et al., 2007; Leonardi et al., 2000). Currently, changes in climate are enhancing temperature, provoking periods of high VPD (Will et al., 2013). In arid and semi-arid environments, where extreme weather conditions hamper agro-forestry development, temperature rise represents a big issue. Moreover, climate change will very likely lead to extreme weather events and shifts in seasonal cycles (Mann et al., 1996; Regonda et al., 2005). In this context, understanding crop anatomical development and its plasticity in stress adaptation under VPD gradients will be important to cope with those changing conditions. To date, there are no clear indications on morphological or physiological traits that could be strengthened, in a sort of plant “hardening off”, to ameliorate plant growth under climate changing conditions. This can be attributed to a missing knowledge in the links between morphological development and physiological responses (Barbieri et al., 2012).

Our paper deals with the effects of long- and short-term exposure to high and low VPD in controlled environment on plant structure and function. Our main goal was to test the adaptive plasticity showed by *Vigna radiata* L. plants, which developed in a growth chamber under low and high VPD condition, after transplanting under the opposite VPD conditions. We hypothesised that plants grown under high VPD, were limited by their leaf anatomical structure, thus their ability to adapt after the transplanting would be impaired. To do so, we analysed leaf functional and anatomical traits (parenchyma, stomata, and veins) as well as the performance of photosynthetic apparatus (photochemistry, gas exchanges, sugar and starch content), before and after the transplanting. Our

findings could help supporting the idea that leaf anatomical structure plays a key role in photosynthetic apparatus acclimation to air VPD.

3.3 Materials and methods

3.3.1 Experimental design and plant material: Two experimental growth chambers were simultaneously used and set up with the same environmental condition namely, air temperature 23 ± 0.03 °C, Photosynthetic Photon Flux Density (PPFD) at the top of the canopy 200 ± 20 µmol photons m^{-2} s^{-1} ,16h photoperiod, but contrasting VPD: a VPD of 0.57 kPa (RH 80.96 ± 0.11), serving as the Low VPD condition; and a VPD of 1.46 kPa (RH 48.15 ± 0.15), serving as the High VPD condition. T and RH were monitored by mini-sensors (Testo 174H, Testo, Germany) placed in the center of each chamber and installed at approximately 30 cm above the ground. VPD was calculated from the corresponding instantaneous T and RH values sampled every 15 minutes and recorded in the data-logger included in the sensors. The species tested in the study was *Vigna radiata* L. Wilczek, a short-duration legume crop commonly known as “green azuki”. Azuki seeds were germinated to primary roots on wet filter paper for five days in the dark at 25°C. Soon after the germination, 20 seedlings per VPD condition were transferred on standard gardening soil into 10 cm diameter pots and irrigated at field capacity for 30 days until the plants reached the adult stage with at least 4 trifoliate leaves. Afterwards, ten plants for condition were transferred to the opposite growth chamber and kept under the opposite VPD up to 4 days. The transplants from low to high VPD is referred in the text and figures as High VPD tr.; whereas from high to low VPD as Low VPD tr. Before the transplanting, growth measurements in terms of plant height, leaf number and length of leaflets were recorded every 5 days; while morpho-anatomical characterization of completely life span trifoliate leaves (concerning stomata, leaf lamina and leaf vessels) as well as eco-physiological analyses (gas exchange and photochemistry), soluble sugar and starch content, were analyzed before and after the transplanting. Morpho-anatomical analyses reported in the text refer to those performed before the transplanting because the leaves were already at a mature stage and no lamina expansion occurred after transplanting.

3.3.2 Leaf traits: Leaf functional traits were evaluated following Cornelissen et al. (2003) on 6 leaflets per VPD condition. Initially, leaf area (LA) was calculated from pictures of leaves by means of an image analysis software (imageJ; Rasband, W.S., U.S. NIH, Bethesda, Maryland, USA, 1997-2012). Then, fresh weight (FW) was recorded, and leaves were promptly Saturated submerging the petiole of leaf blades in distilled water for 48 h in the dark and re-weighted (SW); while Dry weight (DW) was obtained by oven-drying leaves at 75 °C for 48 h. These parameters were used to evaluate: the water status of the leaves, measured as relative water content (RWC%) and expressed

as percentage of [(FW/DW)/(SW/DW)]; the leaf dry matter content (LDMC) considered a substitute for leaf tissue density (Ryser & Urbas 2000) and expressed as (DW/SW) in gg^{-1}; and specific leaf area (SLA), calculated as the ratio between LA and DW ($cm^2\ g^{-1}$) which is the inverse of leaf specific mass and used as proxy for sclerophylly (Witkosky & Lamont 1991).

3.3.3 Leaf lamina: To accomplished morpho-anatomical analyses of leaf lamina, leaflets were sampled from 6 plants and promptly fixed in FAA solution (40% formaldehyde, glacial acetic acid, 50% ethanol, 5:5:90 by volume). Each leaf was dissected in order to obtain sub-samples of about 5x5 mm, dehydrated in a series of ethanol up to 95% and then embedded in the acrylic resin (JB4, Polysciences, Germany). Resin-embedded leaves were cut in thin cross-sections of about 5 µm by means of a rotary microtome, and stained with 0.5% Toluidine blue in water, as reported in Feder & O'Brien 1968. The sections were observed under a light microscope (BX60; Olympus), imaged through a camera (CAMEDIA C4040, Olympus) and analyzed using the imaging software AnalySIS 3.2 in order to characterized leaf lamina by measuring the thickness of upper and lower epidermis as well as the thickness of palisade and spongy parenchyma and the density of both parenchyma in terms of quantity of intercellular spaces. All measurements were performed in three positions per cross-section, being careful to avoid veins or any other form of interference.

3.3.4 Leaf venation: To determine vein traits, 6 leaflets per condition were chemically cleared with 5% NaOH in aqueous solution and bleached in EtOH dilution series, following Berlyn and Miksche (1976). In order to highlight even the smallest veins, cleared leaves were covered in 1% safranin in EtOH and gently rinsed with 100% EtOH before being covered in 1% fast green and rinsed again. Firstly, whole leaves were imaged for the measurements of the whole leaf area (cm^2), perimeter (cm), length (cm), width (cm) and major VLA (major Leaf vein density per unit area) ($cm\ cm^{-2}$), also known as Vein density. Secondly, using a light microscope (BX60; Olympus) top, middle and bottom thirds of each leaf were captured and imaged as reported for leaf lamina. From those pictures, minor VLA ($mm\ mm^{-2}$), total VLA ($mm\ mm^{-2}$), number of secondary veins (number per mm2) and free vein endings per area (number per mm^2) were measured using image J software (National Institutes of Health, Bethesda, MD, USA), following the standard procedure as reported in Sack and Scoffon (2013).

3.3.5 Stomatal traits: Stomatal traits were determined on lamina abaxial peels, taken centrally in each leaflet avoiding the midrib as well as the margin. For each leaflet (6 per VPD conditions), measurements were averaged from 5 measurements obtained from 3 different peels. Stomatal number as well as size in terms of length and width (µm) of guard cells were recorded. Furthermore,

both stomatal density (number per μm^2) and stomatal index were calculated. Stomatal index (%) was calculated using the following formula: [(S/S+E) 100], where S is the number of stomata and E the number of epidermal cells in microscopic view filed (Paul et al., 2017). From the same peels, 30 stomata per condition were selected for the identification of chloroplasts in stomata guard cells. This analysis was performed in order to obtain a strong correlation of leaf structure to function, and in particular to correlate the number of chloroplasts in guard cells to stomatal conductance and thus, to the entire photosynthetic performance (Zeiger et al., 2001; Lawson et al., 2009). For each stomata, guard cell area was identified and data were reported as density of chloroplasts for the surface of guard cell. Soon after, peels on the slides were stained with Lugol's dye solution, an iodine solution which reacts with starch, producing a deep dark colour. In this way it was possible to detect and count starch masses still present in stomata guard cells.

3.3.6 Gas exchanges: Leaf gas exchange rates were measured before and after the transplanting, by means of an infrared gas analyzer (IRGA) from Qubit Systems Inc. (Kingston, Ontario, Canada). The Qubit Systems S151 IRGA CO2 analyzer have a non-dispersive infrared technology (NDIR) to measure CO2 levels in a flowing gas stream. Furthermore, other environmental parameters such as T and RH were strictly monitored and recorded by sensors in the systems. 3 leaflets per plant were sealed into the chamber and exposed to a LED light with a fluence of 800 µmol m^{-2} s^{-1}. This PPFD was light-saturating for the genotype and it was controlled using a portable pulse amplitude modulated fluorometer, equipped with a light sensor (Fluorpen, FP 100 max). Values of photosynthesis (A_N), were expressed in µmol m^{-2} s^{-1}, as rates of CO_2 exchange per unit time per unit leaf area. Moreover, total conductance to H_2O (gH_2O) and to CO_2 (gCO_2) were measured as derived parameters and expressed as mol m-2 s-1, while the photosynthetic Water Use Efficiency (pWUE) was expressed as the ratio of water used for plant metabolism to water lost through transpiration (A_N/ E).

3.3.7 Photochemistry: Soon after gas exchanges measurements, chlorophyll "a" fluorescence analysis was carried out by means of a portable pulse amplitude modulated fluorimeter (Fluorpen, FP 100 max), to allow a non-invasive evaluation of plants photosynthetic performance. Measurements were performed under growth environmental condition, taking care of obscuring leaves for at least 30 minutes in order to allow a complete re-oxidation of the PSII reaction centers and to measure the maximal PSII photochemical efficiency (Fv/Fm), expressed following Kitajima and Butler (1975). For measurements in the light ΦPSII (the PSII quantum yield) was calculated according to Genty et al. (1989) and later used to deduce ETR (electron transport rate) following the method of Krall and Edwards (1992). Furthermore, non-photochemical quenching, a value which

express the dissipation of the excess of absorbed light was calculated according to Bilger and Bjorkman (1990).

3.3.8 Sugar and starch quantification: Once eco-physiological analyses were performed, soluble sugar and starch were extracted and quantified in the same leaflets, following the anthrone method (Marshall, 1986). Soluble sugars were first extracted with aqueous ethanol, using anthrone reagent (ACS reagent 97%, Sigma-Aldrich) dissolved in sulfuric acid. Anthrone reacts with carbohydrates giving a green-colored product, whose absorbance was read spectrophotometrically at 630 nm. Once sugars were removed, pellets were evaporated to dryness and used for starch extraction, performed with a perchloric acid solution. In hot acidic medium, starch is hydrolyzed to glucose and dehydrated to hydroxymethyl furfural. This compound reacts with anthrone forming the green coloured product. A standard curve with increasing glucose concentration was prepared and results were expressed as: µmol glucose equivalent g^{-1}. For starch concentration, the value obtained from the standard curve was multiplied for 0.9, as 0.9 g of starch yield as 1 g of glucose on hydrolysis (Sharma and Sangha, 2009; Katoch, 2011)

3.3.9 Statistical analysis: Data collected before the transplant were assessed by comparing the relative changes between plants grown under low and high VPD with unpaired t-tests, performed with SPSS 13 statistical package (SPSS, Chicago, IL, USA). The Kolmogorov–Smirnov and Shapiro–Wilk tests were performed to check for normality. Concerning data collected before and after the transplant (gas exchange and photochemistry measurements, as well as soluble sugars and starch), to test the effects of VPD, transplant (TR) and their interaction, a two-way ANOVA was performed. Correlation and regression analysis were performed with SPSS 13. Finally, a multivariate analysis such as: principal component analysis (PCA) and a hierarchical cluster analysis (HCA), were performed using Past3 statistical software. PCA was used as a method to find trends among variables, transforming the original set of variables in a multiple-variable system where 2 Principal components (PCs) which carry the maximum information were recognized (Yu, 2005). Whereas the aim of the clustering process was to group a set of data into clusters, so that data within the same group are as similar as possible. For HCA, the paired group (UPGMA) and Euclidean distances were used for clustering. Results of HCA were displayed as a tree-shaped dendrogram, where the horizontal distance between clusters represented data dissimilarities. Variables used as input for both types of multivariate analyses were standardised to zero mean and unit variance, as suggested in Iezzoni and Pritts (1991).

3.4 Results

3.4.1 Growth: Figure 1 shows the time course of plant growth, recorded every 5 days during the whole experimental trial. Statistically significant differences were found in growth between Low and High VPD plants. Since 5 DAS, in fact, plants in High VPD environment were shorter than Low VPD plants (Fig.1, a). This significant difference ($p < 0.05$) was not present at 15 DAS, where High VPD plants managed to reach the same height of Low VPD, to promptly derive again later. Indeed, after this point (15 DAS), also leaf length and number of leaves (Fig. 1 b,c) of the two treatments begin to be different, until they stabilized up to the final values. In the end, Low VPD plants showed the highest height, number of leaves and leaf expansion. These data are in agreement with the total leaf area (LA) showed in table 1, which resulted more expanded in Low VPD plants.

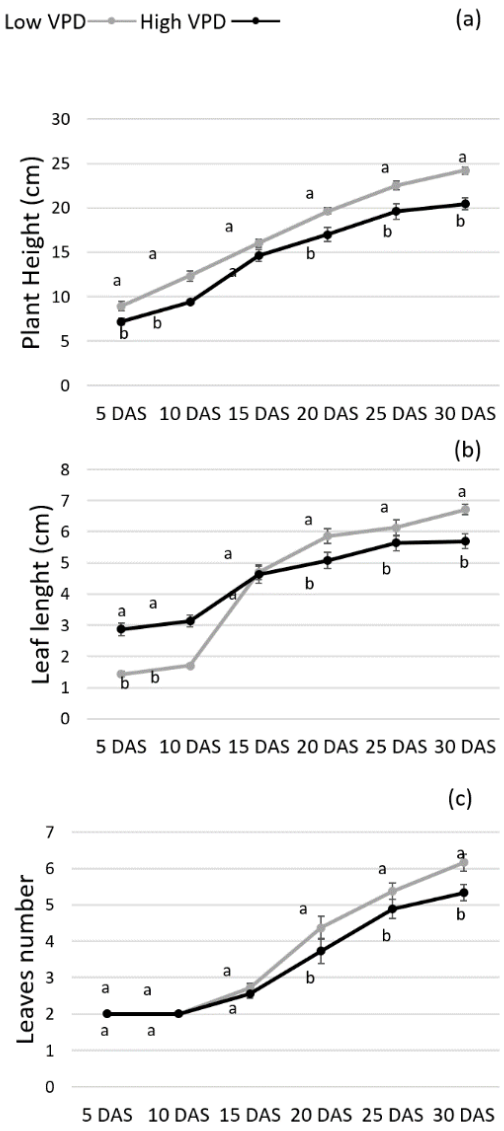

Figure 1. Evolution of whole-plant growth (plant height (a), leaf length (b) and number of leaves (c)) of Vigna radiata L. plants at 5, 10,15,20,25 and 30 Days After Sowing (DAS). Each point in the curve represents mean ± standard error. Different letters correspond to statistically significant differences between treatments of each DAS ($p < 0.05$).

3.4.2 Morpho-anatomical analyses: Substantial variation was found between treatments in leaf traits, tissue anatomy, leaf venation as well as stomatal traits. Averaging values for each condition across the two VPDs, High VPD leaves resulted less expanded with a variation of LA by 3.25-fold and an RWC% by 4.51-fold (Table 1). These leaves also appeared less branched with lower values of total, major and minor VLA (Table 1) and with a variation in number of free endings vein by 6.8-fold. Concerning leaf tissue anatomical traits, except for epidermis (both lower and upper) and palisade thickness which were statistically similar between condition, the thickness of the spongy parenchyma and the percentage of intercellular spaces in both parenchyma, followed the same trend resulting higher in the High VPD condition. In addition, High-VPD-grown-plants presented a lower P/S ratio, fewer but bigger stomata (lower length and width) and a lower stomata density and index, compared to Low VPD (Table 2). Furthermore, in stomata guard cells of High VPD plants, a lower number of chloroplasts but a higher number of starch masses were found.

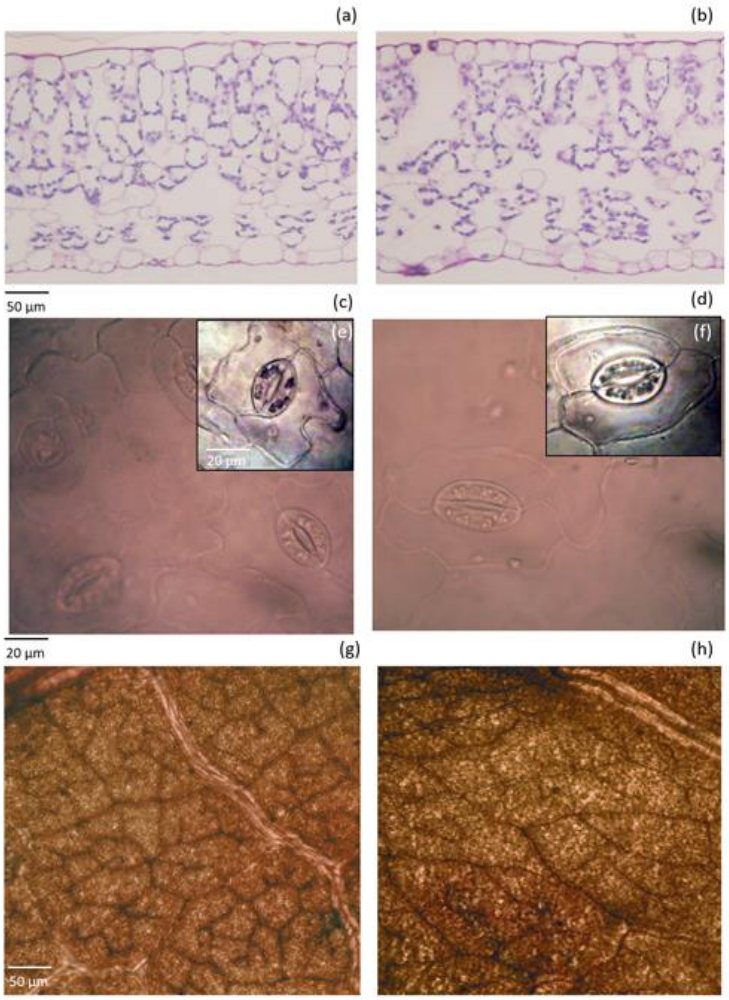

Figure 2. Diversity in leaf morphology of Vigna radiata L. plants grown under Low and High VPD, emphasizing leaf lamina (thickness of mesophyll and intercellular space density) (a,b), number and dimensions of stomata as well as chloroplasts (c,d) and number of starch per guard cell (e), vein traits (number of vein orders, vein length per unit area, VLA) (e,f). Images (a,b,g,h) are at the same magnification; scale bar = 50 µm and images (c,d,e,f) are at the same magnification; scale bar= 20 µm.

Table 1. Mean, standard error (se) and significant difference (s) of Leaf functional and vessel traits of plants grown under Low and High VPD. In parentheses, the unit of measure of each parameter is given.

	Low VPD mean ± se	s	High VPD mean ± se	s
LA (cm^2)	10.02 ± 0.48	a	6.77 ± 0.20	b
SLA (cm^2)	512.37 ± 29.58	a	457.48 ± 28.19	b
RWC (%)	90.88 ± 2.42	a	86.37 ± 0.68	b
LDMC (gg^{-1})	0.087 ± 0.01	a	0.074 ± 0.01	b
Leaf Perimeter (cm)	18.00 ± 1.11	a	19.58 ± 2.53	a
Leaf Length (cm)	6.80 ± 1.01	a	5.39 ± 0.26	b
Major VLA (cm cm^{-2})	422.22 ± 32.93	a	126.08 ± 8.32	b
Minor VLA (mm mm^{-2})	1.48 ± 0.29	a	0.26 ± 0.04	b
Total VLA (mm mm^{-2})	4.57 ± 0.14	a	0.71 ± 0.03	b
Free vein endings per area (n mm^{-2})	8.58 ± 0.27	a	1.78 ± 0.37	b

Table 2. morpho-anatomical traits of leaf lamina of Low and High VPD plants. Mean, standard errors (se) and significant differences (s) are reported. In parentheses, the unit of measure of each parameter is given.

	Low VPD mean ± se	s	High VPD mean ± se	s
Spongy thickness (µm)	90.88 ± 3.31	b	105.48 ± 2.25	a
Palisade thickness (µm)	107.14± 3.27	a	102.31 ± 3.14	a
P/S thickness ratio	1.23 ± 0.063	a	0.98 ± 0.035	b
Upper Epidermis thickness (µm)	21.53 ± 0.79	a	23.84 ± 1.00	a
Lower Epidermis thickness (µm)	24.66 ± 0.75	a	24.067 ± 1.14	a
Spongy intercellular spaces (%)	37.93 ± 1.76	b	50.71 ±1.08	a
Palisade intercellular spaces (%)	22.38 ± 1.81	b	33.69 ± 2.67	a

Table 3. stomatal traits of plants grown under Low and High VPD. For each parameter, mean, standard error (se) and significant differences (s) are given. In parenthesis the unit of measure of each parameter is given.

3.4.3 Eco-physiological analyses: As reported in table 4 and 5, gas exchange and photochemistry measured parameters were influenced by VPD and transplant (TR) as main factors, as well as by their interaction (VPD x TR). However, their interaction had only a slight effect ($P < 0.05$). More

	Low VPD mean ± se	s	High VPD mean ± se	s
Stomatal number (µm)	41.60 ± 1.27	a	36.00 ± 0.66	b
Stomatal lenght (µm)	23.96 ± 0.27	b	28.28 ± 0.35	a
Stomatal width (µm)	14.28 ± 0.22	b	17.73 ± 0.25	a
Stomatal density (µm^2)	0.11 ± 0.0032	a	0.09 ± 0.0017	b
Stomatal index (%)	45.38 ± 0.96	a	42.44 ± 0.51	b
Chloroplasts per guard cell (%)	63.65 ± 0.06	a	29.57 ± 0.02	b
Starch masses per guard cell (n µm^{-2})	5.28 ± 1.29	b	11.98 ± 0.56	a

specifically, leaf gas exchanges measured on High VPD plants showed that net photosynthetic rates (A_N), stomatal conductance and instantaneous WUE (table 4) decreased significantly ($P<0.01$) compared to the opposite condition. Moreover, all photochemical parameters measured, exception made for the thermal dissipation (NPQ), resulted lowered in Low-VPD-exposed plants (table 5). After 4 days of transplanting, when Low VPD plants were moved into a High VPD environment (High VPD tr.) they changed their behavior, beginning to act exactly like high VPD plants before transplanting (High VPD). Indeed, they showed a reduced photosynthesis and conductance as well as WUE and photochemical parameters, except for NPQ (table 5). Conversely, High VPD plants moved to Low VPD environment (Low VPD Tr.) did not change their behavior, maintaining photosynthetic and photochemical rates similar to those before the transplant (table 4,5).

Table 4. Eco-physiological analysis in terms of gas exchange of plant grown under low and high VPD before and after the transplanting. Data are shown as means ± standard error. Different letters within the same column denote statistically significant differences between treatments. A two-way ANOVA was performed to test the effects of VPD, transplant (TR) and their interaction (VPD x TR). (***P<0.001, **P<0.01, *P<0.05, NS non significant).

	A_N (μmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	gH_2O (mol m^{-2} s^{-1})	gCO_2 (mol m^{-2} s^{-1})	pWUE (A_N/E)
VPD					
Low	9.63 ± 0.31a	1.51 ± 0.46b	0.15 ± 0.04 a	0.90 ± 0.25a	5.70 ± 0.14a
High	6.48 ± 0.81b	1.62 ± 0.26a	0.98 ± 0.20 b	0.61 ± 0.13b	4.92 ± 0.11b
TR					
presence	6.71 ± 0.87b	1.47 ± 0. 53b	0.12 ± 0.02a	0.78 ± 0.17a	5.83 ±0.13a
absence	9.17 ± 0.34a	1.69 ± 0.022a	0.12 ± 0.05a	0.72 ± 0.32a	4.39 ±0.18b
Low VPD	12.02 ± 0.77a	1.52 ± 0.06b	0.15 ± 0.043a	0.093 ± 0.0026a	6.82 ± 0.59a
High VPD	6.33 ± 0.23b	1.84 ± 0.07a	0.10 ± 0.037b	0.064 ± 0.0023b	4.84 ± 1.14b
High VPD Tr	6.77 ± 0.25b	1.42 ± 1.28b	0.09 ± 0.008b	0.087 ± 0.012a	5.00 ± 0.34ab
Low VPD Tr	6.66 ± 0.30b	1.55 ± 2.87ab	0.10 ± 0.018b	0.057 ± 0.006b	4.58 ±1.92b
VPD	***	**	***	***	***
TR	***	*	NS	NS	*
VPD x TR	***	*	*	*	*

Table 5. Eco-physiological analysis in terms of photochemistry of plant grown under low and high VPD before and after the transplanting. Data are shown as means ± standard error. Different letters within the same column denote statistically significant differences between treatments. A two-way ANOVA was performed to test the

effects of VPD, transplant (TR) and their interaction (VPD x TR). (***P<0.001, **P<0.01, *P<0.05, NS non significant).

	Fv/Fm	Qy	ETR	NPQ
VPD				
Low	0.77 ±0.19a	0.32 ± 0.17a	107.52 ± 5.77a	2.63 ± 0.48b
High	0.74 ± 0.29b	0.23 ± 0.03b	80.08 ± 10.47b	3.14 ± 1.05a
TR				
presence	0.75 ± 0.27a	0.27 ± 0.047a	91.00 ± 16.06b	2.55 ± 0.56b
absence	0.76 ± 0.3a	0.28 ± 0.048a	96.60 ± 16.16a	3.23 ± 0.96a
Low VPD	0.78 ± 0.0039a	0.33 ± 0.0058a	110.32 ± 1.97a	2.80 ± 0.11b
High VPD	0.74 ± 0.0087b	0.25 ± 0.0081b	82.88 ± 2.72b	3.64 ± 0.34a
High VPD Tr	0.76 ± 0.0030ab	0.23 ± 0.0095b	77.28 ± 11.09b	2.64 ± 0.17b
Low VPD Tr	0.74 ± 0.0061b	0.26 ± 0.0069b	99.72 ± 2.41ab	2.45 ± 0.15b
VPD	***	***	***	*
TR	NS	*	*	**
VPD x TR	*	*	*	*

3.4.4 Sugar ad Starch content: Similarly, soluble sugar and starch content before and after transplanting were influenced by VPD, TR and their interaction (VPD x TR) (Table 6). VPD alone, enhanced the soluble sugars content, while did not elicit significant changes on starch content. Conversely, the effect of transplant positively influenced the soluble sugars content and negatively the presence of starch (table 6). In figure 3 is evident how azuki plants inverted their values before and after the transplanting. For instance, High VPD plants presented a lower amount of soluble sugars but more starch before transplanting (Fig. 3 a,b) and more sugar and a lower content of starch when moved to the opposite VPD condition (Fig. 3 c,d). However, after the transplanting levels of sugar and starch decreased in both VPD condition, never reaching initial values presented before transplanting.

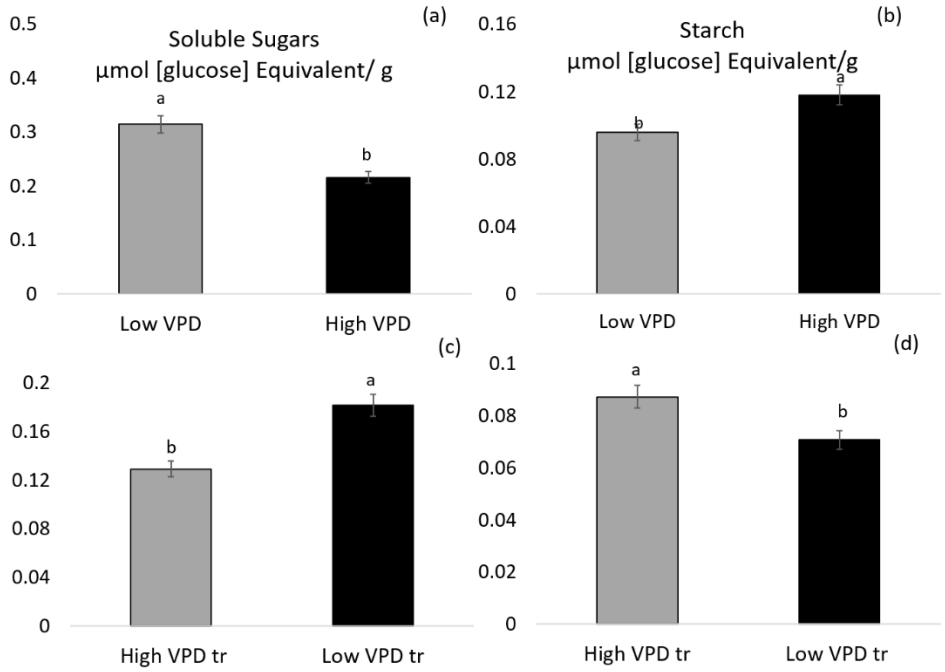

Figure 3. Comparison of soluble sugar and starch content in *Vigna radiata* L. plants grown under Low and High VPD before (a,b) and after (c,d) the transplanting. Values represent mean ± standard error. Different letters correspond to statistically significant differences between treatments ($p < 0.05$).

3.4.5 Correlation and regression analyses: Correlation and linear regression analyses were used to investigate relationships between anatomical traits of *Vigna radiata* L. plants developed under low and high VPDs. The purpose of correlation analysis was to examine the strength and the direction of the relationships, whereas the regression analysis was used to evaluate the relative impact on the predictor variable on the outcome. Pearson correlation coefficient and R^2 were calculated for all relationships. Our data (Fig. 4) revealed a striking positive correlation under all VPD conditions, exception made for stomata length and number (Low VPD Pearson correlation coefficient: 0.73, R^2: 0.58, P:0.01; High VPD Pearson correlation coefficient: 0.76, R^2:0.53, P:0.01). More specifically, Stomatal density and VLA showed a positive correlation in both Low (Pearson correlation coefficient: 0.95, R^2: 0.90, p:0.01) and High VPD (Pearson correlation coefficient: 0.96, R^2: 0.93, p:0.001). The same positive correlation was found between the number of free vein endings and VLA: Low VPD (Pearson correlation coefficient: 0.93, R^2: 0.87, p: 0.01) and High VPD (Pearson correlation coefficient: 0.90 R^2:0.81, p:0.01) and between the number of mesophyll cells and stomata: Low VPD (Pearson correlation coefficient: 0.95, R^2: 0.91, p: 0.01) High VPD (Pearson correlation coefficient:0.94 R^2:0.89, p:0.01). Notwithstanding the positive correlation, High VPD plants always

showed reduced values of stomata density and number, VLA, free vein endings but bigger stomata compared to Low VPD.

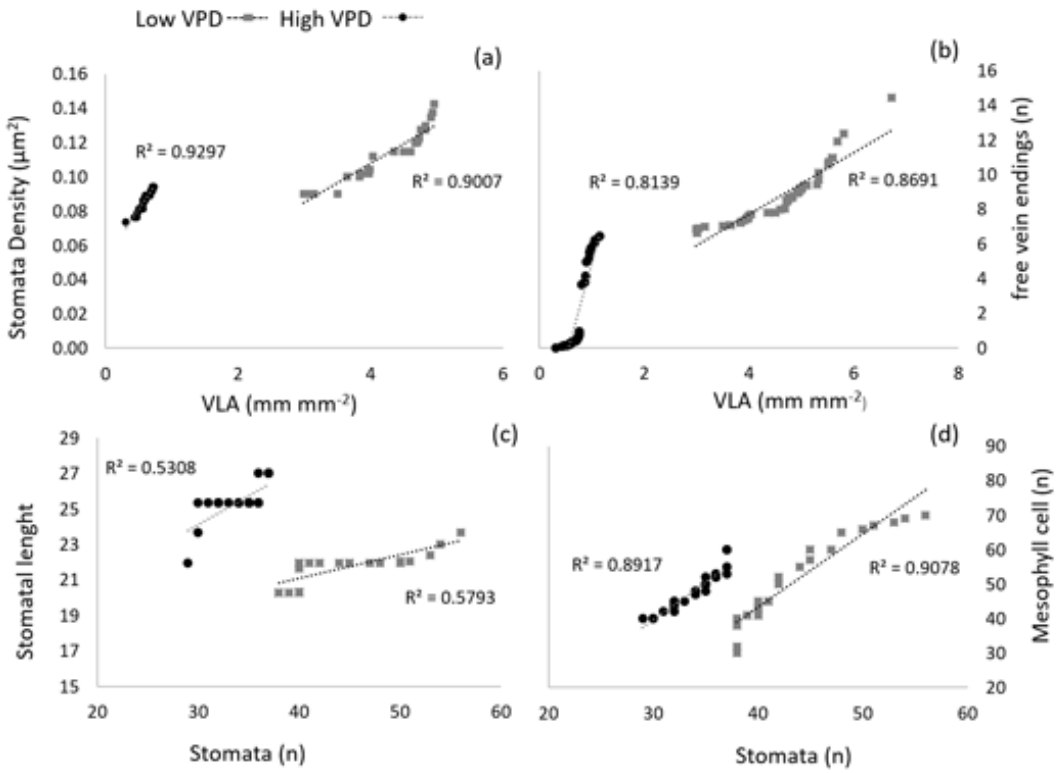

Figure 4. Relationships between Stomatal density and VLA (a), number of free vein endings and VLA (b), Stomatal length and number (c), and mesophyll cell and stomata number (d). in *Vigna radiata* L. plants grown under Low (grey squares) and High (black circles) VPD. Regression lines and r^2 values are shown.

3.4.6 Principal component analysis and Cluster analysis: Multivariate analysis (PCA) was applied to physiological traits (gas exchange and photochemistry) of *Vigna radiata* L. plants grown under high and low VPD, before and after the transplant. The PCs were associated with Eigen values of 8.34 and explained a cumulatively 92.72% of total variance, with PC1 accounting for 65.01 % and PC2 for 27.71 % (Fig. 5,a). PC1 was highly positively correlated with WUE, A_N, Qy, ETR, NPQ, gH_20, gCO_2, Fv/Fm; while PC2 positively correlated with E, and NPQ. The PCA scatter-plot clearly separate the High and Low VPD treatments, in the first and second quadrant respectively. Furthermore, the matrix revealed a strong clustering of plant grown in high VPD before and after (Low VPD tr.) the transplant, also confirmed by the HCA (Fig. 5,b). Cutting the dendrogram at the distance value of 4.5, according to the agglomeration schedule, 2 clusters appeared: the first one on the right containing Low VPD alone and another for both High VPD and the two transplants. However, cutting the dendrogram at distance 3, identified only 2 main clusters: Low VPD, clearly

separated from High VPD tr., and a single cluster for plant subjected to High VPD, before and after (Low VPD tr.) the transplant.

Table 6. Soluble sugars and starch two-way ANOVA for the effects of VPD, Transplant (TR) and their interaction (VPD x TR). (***P<0.001, **P<0.01, *P<0.05, NS non-significant).

	Soluble sugars (µmol[glucose]Eq.g^{-1})	Starch (µmol[glucose]Eq.g^{-1})
VPD		
Low	0.32 ± 0.028 a	0.091 ± 0.017 a
High	0.24 ± 0.046 b	0.094 ± 0.026 a
TR		
presence	0.30 ± 0.010 a	0.078 ± 0.025 b
absence	0.28 ± 0.012 b	0.11 ± 0.023 a
VPD	***	NS
TR	***	*
VPD x TR	***	*

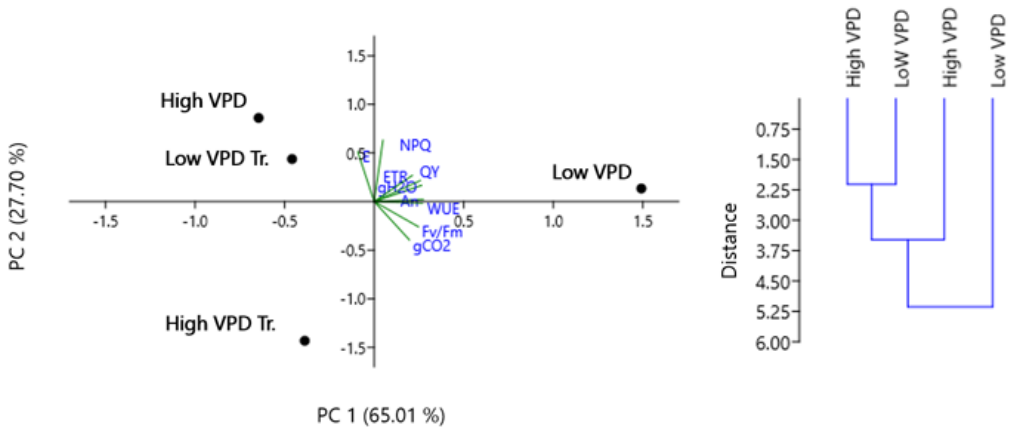

Figure 5. Principal component analysis (PCA) loading plot and scores and hierarchical cluster analysis (HCA) of physiological traits (gas exchange and photochemistry) in *Vigna radiata* L. plants grown under Low and High VPD before and after the transplanting.

3.5 Discussion: Our study proves that the exposure to different air VPD induced, in the same species, an opposite leaf anatomical development, regarding stomata, leaf vein and mesophyll structure. Changes in leaf anatomy have a key role in the photosynthetic response to long-term VPD, particularly influencing CO_2 exchange rate and stomatal conductance. Furthermore, we observed its role under short-term VPD exposure and the extent to which plant structure established the limits of plants physiological acclimation to changes in the VPD environment. Indeed, plants face environmental changing by the simultaneous interaction of both anatomical and physiological traits

(De Micco et al., 2019), but plasticity in plant physiological adaption cannot exceed plasticity in plant anatomy (Brodribb 2009; Xiong et al., 2017). From our results, it is evident how plants exposed to high VPD developed a less efficient anatomical leaf structure which reflects a less efficient physiological performance. Changes in VPD during growth have been shown to affect water available for plants, thus impacting leaf morphology as well as biomass allocation (Giday et al., 2013). In our study, plant at High VPD decreased LA, SLA and RWC % (Table 1), whereas biomass partitioning to the leaves was not affected. A lower leaf area is known to decrease the transpiration rate during growth, also preventing a good nutrient uptake and water distributions. Furthermore, in these plants the entire leaf anatomical structure has developed in the sense of a greater resistance to water flow. Indeed, the significant increment of spongy tissue and intercellular spaces (in both spongy and palisade parenchyma) as well as a significant reduction of the P/S ratio (Table 2), are evidence of a decreased water flux through the tissues and can therefore be used as proxies of mesophyll resistance. Spongy parenchyma and intercellular spaces are indeed recognized as the sites of highest resistance to water flows in leaves, due to the major presence of airspaces and the lowest cell connection, which decrease the fluxes of water in tissues (Amitrano et al., 2019). In our study, plants at High VPD also appeared less branched, with a lower number of free vein endings, compared to Low VPD-grown plants (Table 1). Leaf vein density has a critical role in water supply (Sack and Frole, 2006; Brodribb et al. 2007) and it is also fundamental in determining the balance between water supply and evaporation through stomata (Liu et al., 2015; Brodribb and McAdam 2017). For this reason, the density of leaf veins and stomata have been considered “coordinated” during leaves development. According to this statement, Du et al. (2019) found in two tomato cultivars (Zhongza and Jinpeng) grown under high VPD a different coordination of vein and stomatal density. Zhongza, under long-term high VPD maintained proportional decreases in stomata and vein density also reducing g_s; whereas Jinpeng did not change vein density while reducing the stomatal density, maintaining a higher gs. A coupled coordination among stomata and vein density has been hypothesized not to be able of maintaining water balance and at the same time accommodate the increased evaporative demand under high VPD (Sack and Holbrook 2006; Carins Murhpy et al., 2014, 2017). Our results showed that in Vigna radiata L. plants the exposure to long-term High and Low VPD resulted in a positive correlation between stomatal density and vein density (VLA) and also in the number of free endings vein and VLA (Fig. 4). More specifically, under high evaporative demand, plants presented not only a smaller number of vein and vein density but also a reduced stomata density, but bigger stomata. This phenomenon could be attributed to smaller leaf sizes and/or to cell enlargement. However, although synchronized changes to both epidermal and mesophyll cell size are required to coordinate vein and stomatal density (Du et al., 2019), the decreased stomatal index in high VPD plants (Table 3) and the coordination among mesophyll cell and stomata numbers, are sign that any changes are due to morpho-anatomical and physiological adaption to environmental condition and not to cell sizes. When plants were transplanted to the

opposite chamber, those that developed in Low VPD when exposed to High VPD (High VPD tr.), as expected, reduced their photosynthetic performance, showing lowered values of A_N, g_s, pWUE, Fv/Fm etc... compared to those before the transplant but not compared to High VPD plants (Table 4). Indeed, under high VPD, these plants were subjected to a high evaporative demand, although having a coupled positive coordination between stomatal and vein density which can cause a reduction in leaf water content, due to the excessive evaporative demand, changed their behaviour and carried out several adjustments like stomata closing to counteract dehydration. This could be attributed to the presence of several and smaller stomata which could react faster to any change in the surrounding environmental conditions. Indeed, the regulation of plant gas-exchanges and particularly of stomatal aperture to any short-term variation in VPD, is limited by leaf structural traits (Liu et al. 2015; Brodribb et al., 2017). The occurrence of smaller stomata in leaves can allow a better control of gas exchange (De Micco et al., 2019) and it has also been associated with a better adaptation to water stress (Wilmer and Fricker 1996). Furthermore, smaller stomata, due to the greater area of their membrane surface compared to their total volume, have faster response times compared with larger stomata. Moreover, in combination with a higher stomatal density, the presence of smaller stomata may allow leaves to adapt rapidly under favourable conditions (enhancing their stomatal conductance), and to faster reduce conductance when conditions become unfavourable (Drake et al., 2013; de Boer et al., 2016). Another explanation of their prompt adaptation could be the presence of more chloroplasts per guard cell in their stomata. Several studies have been carried out on these organelles. Besides being involved in blue-light signalling and response (Frechilla et al., 1999; Zeiger, 2000), starch storage (either produced from carbon assimilated in the guard cell chloroplasts, or imported from the mesophyll) and production of osmotically active sugars; chloroplasts play a role in detecting external drought conditions and therefore in activating stomata closure by interacting with the hormonal system, thus functioning as a control points of water flow through leaves (Tallman & Zeiger, 1988; Talbott & Zeiger, 1993, 1998; Zeiger et al., 2002). In our study, the major presence of chloroplasts could have activated a more rapid response of stomata closing, either by photosynthesis or by a conversion of starch into soluble carbohydrates. In this way guard cell chloroplasts may cause the osmotic potential to become more negative and therefore increase their turgor by osmosis (Meidner & Mansfield, 1968). Indeed, anatomical analyses on Low VPD plants confirmed that these leaves present fewer starch masses in their guard cell. On the contrary, Low VPD tr. plants, even though transplanted in a chamber under more favourable environmental conditions, showed a lower plasticity in their adaption. Being unable to adapt to the new surrounding environment, their photosynthetic performances remained basically unchanged compared to those before the transplant. The cluster division that occurs in figure 5 supports this statement, from both PCA and HCA analyses is clear how High VPD and Low VPD tr. plants were always group together, clustering together. These results show that leaves water flow and gas-exchange are strictly constrained by anatomical traits such as: leaf vein density,

chloroplasts distribution, stomatal size and density, un the end being modulated by climate. Scoffoni et al. (2016) demonstrated that a strong coordination occurs between hydraulic and photosynthetic performance in related species of rain forests and that this coordination is strictly connected to leaf anatomical development and especially vein architecture. The leaf hydraulic conductance K_{Leaf} is known to be a major bottleneck in the whole plant water transport pathway and cannot disregard changes in leaves structure. These changes in turn influence photosynthesis and carbon allocation (Sack et al., 2006; Sack et al., 2016). In our study, Low VPD plants presented a higher level of soluble carbohydrates, which sharply decrease after the transplant (High VPD tr.). This is consistent with the gas-exchange data and could primarily be attributed to a decrease in A_N after the transplant and/or to the degradation of starch. High VPD is also known to cause reduction in rubisco activity, affecting carboxylation efficiency and provoking reductions in sucrose content (Lawlor and Fock, 1977). Indeed, in our experiment soluble sugars are always reduced under high VPD conditions (High VPD and High VPD tr.; fig. 3a,c). Concerning starch content, Zrenner and Stitt (1999) stated that short-term high VPD effects are similar to those reported for long duration of water stress, indicating that in leaves at RWC <80% the decreased cell volume leads to an increased metabolic pool of phosphate which is available for phosphorolytic starch degradation. Afterwards, another study showed a decline in starch content of P. juliflora when its leaves were exposed to VPD > 3kPa, indicating that high VPD affects starch mobilization (Shirke et al., 2004). Our results are consistent with these studies; under short-term High VPD conditions (Low VPD tr.; fig. 3d) plants showed a consistent decline in leaves starch content. These results may offer a further prove of the involvement of high VPD to photosynthetic carbon metabolism. Indeed, these plants also presented a reduced A_N whose inhibition, triggered by high VPD, could lead to the down-regulation of carbon metabolism and the export of soluble carbohydrates.

3.6 Conclusions: The present study emphasized the fundamental role of plasticity in leaf anatomical traits in photosynthetic response to changing VPD. From our results is evident how after the transplanting, High VPD tr. Showed a certain extent of plasticity and managed to adapt to the new environmental condition. Whereas Low VPD tr., although placed in a chamber subjected to “better” environmental conditions (compared to those in which they developed), could not adapt due to their impaired and less efficient anatomical structure. In this study we confirmed our hypostasis that plants grown under high VPD, were limited by their leaf anatomical structure, thus their ability to adapt after the transplanting would be impaired, and we underlined the key role of leaf anatomical structure in photosynthetic apparatus acclimation to air VPD. Finally, different leaf anatomical structures become fundamental in the present climate-change scenario due to their role in the adaptation process under changing environmental conditions.

3.7 References

Amitrano C, Arena C, Rouphael Y, De Pascale S, De Micco V. 2019. Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. Annals of Applied Biology.

Amitrano C, Vitale E, De Micco V, Arena C. 2018. Light fertilization affects growth and photosynthesis in mung bean (Vigna radiata) plants. Journal of Environmental Accounting and Management 6, 295-304.

Barbieri G, Vallone S, Orsini F, Paradiso R, De Pascale S, Negre-Zakharov F, Maggio A. 2012. Stomatal density and metabolic determinants mediate salt stress adaptation and water use efficiency in basil (Ocimum basilicum L.). J Plant Physiol 169, 1737-1746.

Bilger W, Björkman O. 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of Hedera canariensis. Photosynthesis research 25, 173-185.

Bongi G, Loreto F. 1989. Gas-exchange properties of salt-stressed olive (Olea europea L.) leaves. Plant Physiology 90, 1408-1416.

Bouda M, Windt CW, McElrone AJ, Brodersen CR. 2019. In vivo pressure gradient heterogeneity increases flow contribution of small diameter vessels in grapevine. Nature communications 10, 1-10.

Brodribb TJ. 2009. Xylem hydraulic physiology: The functional backbone of terrestrial plant productivity. Plant Science 177, 245-251.

Brodribb TJ, Feild TS, Jordan GJ. 2007. Leaf maximum photosynthetic rate and venation are linked by hydraulics. Plant Physiol 144, 1890-1898.

Brodribb TJ, Jordan GJ. 2011. Water supply and demand remain balanced during leaf acclimation of Nothofagus cunninghamii trees. New Phytologist 192, 437-448.

Brodribb TJ, McAdam SAM. 2017. Evolution of the Stomatal Regulation of Plant Water Content. Plant Physiol 174, 639-649.

Buckley TN, John GP, Scoffoni C, Sack L. 2015. How Does Leaf Anatomy Influence Water Transport outside the Xylem? Plant Physiol 168, 1616-1635.

Carillo P, Cirillo C, De Micco V, Arena C, De Pascale S, Rouphael Y. 2019. Morpho-anatomical, physiological and biochemical adaptive responses to saline water of Bougainvillea spectabilis Willd. trained to different canopy shapes. Agricultural Water Management 212, 12-22.

Carins Murphy MR, Jordan GJ, Brodribb TJ. 2014. Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. Plant Cell Environ 37, 124-131.

Carins Murphy MR, Jordan GJ, Brodribb TJ. 2016. Cell expansion not cell differentiation predominantly co-ordinates veins and stomata within and among herbs and woody angiosperms grown under sun and shade. Ann Bot 118, 1127-1138.

Cirillo C, De Micco V, Arena C, Carillo P, Pannico A, De Pascale S, Rouphael Y. 2019. Biochemical, Physiological and Anatomical Mechanisms of Adaptation of Callistemon citrinus and Viburnum lucidum to NaCl and CaCl2 Salinization. Front Plant Sci 10, 742.

Cornelissen J, Lavorel S, Garnier E, Diaz S, Buchmann N, Gurvich D, Reich PB, Ter Steege H, Morgan H, Van Der Heijden M. 2003. A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. Australian journal of Botany 51, 335-380.

de Boer HJ, Price CA, Wagner-Cremer F, Dekker SC, Franks PJ, Veneklaas EJ. 2016. Optimal allocation of leaf epidermal area for gas exchange. New Phytologist 210, 1219-1228.

De Micco V, Amitrano C, Stinca A, Izzo LG, Zalloni E, Balzano A, Barile R, Conti P, Arena C. 2019. Dust accumulation due to anthropogenic impact induces anatomical and photochemical changes in leaves of Centranthus ruber growing on the slope of the Vesuvius volcano. Plant Biol (Stuttg).

De Micco V, Arena C, Vitale L, Aronne G, Virzo De Santo A. 2011. Anatomy and photochemical behaviour of Mediterranean Cistus incanus winter leaves under natural outdoor and warmer indoor conditions. Botany 89, 677-688.

Dingkuhn M, Luquet D, Clément-Vidal A, Tambour L, Kim HK, Song Y. 2007. Is plant growth driven by sink regulation? Implications for crop models, phenotyping approaches and ideotypes. Frontis, 155-168.

Drake PL, Froend RH, Franks PJ. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. J Exp Bot 64, 495-505.

Du Q, Liu T, Jiao X, Song X, Zhang J, Li J. 2019. Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. J Exp Bot 70, 4949-4962.

Fanourakis D, Carvalho SMP, Almeida DPF, van Kooten O, van Doorn WG, Heuvelink E. 2012. Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biology and Technology 64, 64-73.

Fanourakis D, Giday H, Milla R, Pieruschka R, Kjaer KH, Bolger M, Vasilevski A, Nunes-Nesi A, Fiorani F, Ottosen CO. 2015. Pore size regulates operating stomatal conductance, while stomatal densities drive the partitioning of conductance between leaf sides. Ann Bot 115, 555-565.

Fanourakis D, Heuvelink E, Carvalho SM. 2013. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. J Plant Physiol 170, 890-898.

Feder N, O'brien T. 1968. Plant microtechnique: some principles and new methods. American journal of botany 55, 123-142.

Fletcher AL, Sinclair TR, Allen LH. 2007. Transpiration responses to vapor pressure deficit in well watered 'slow-wilting' and commercial soybean. Environmental and Experimental Botany 61, 145-151.

Frechijia S, Zhu J, Talbott LD, Zeiger E. 1999. Stomata from npq1, a zeaxanthin-less Arabidopsis mutant, lack a specific response to blue light. Plant and Cell Physiology 40, 949-954.

Genty B, Briantais J-M, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA)-General Subjects 990, 87-92.

Giday H, Kjaer KH, Fanourakis D, Ottosen CO. 2013. Smaller stomata require less severe leaf drying to close: a case study in Rosa hydrida. J Plant Physiol 170, 1309-1316.

Iezzoni AF, Pritts MP. 1991. Applications of principal component analysis to horticultural research. HortScience 26, 334-338.

Katoch R. 2011. Analytical techniques in biochemistry and molecular biology: Springer Science & Business Media.

Korner C. 2015. Paradigm shift in plant growth control. Curr Opin Plant Biol 25, 107-114.

Krall JP, Edwards GE. 1992. Relationship between photosystem II activity and CO2 fixation in leaves. Physiologia Plantarum 86, 180-187.

Lake J, Woodward F. 2008. Response of stomatal numbers to CO2 and humidity: control by transpiration rate and abscisic acid. New Phytologist 179, 397-404.

Lawlor D, Fock H. 1977. Photosynthetic Assimilation of 14CO2 by Waterstressed Sunflower Leaves at Two O2 Concentrations and the Specific Activity of Products. journal of experimental botany 28, 320-328.

Lawson T. 2009. Guard cell photosynthesis and stomatal function. New Phytol 181, 13-34.

Leonardi C, Guichard S, Bertin N. 2000. High vapour pressure deficit influences growth, transpiration and quality of tomato fruits. Scientia Horticulturae 84, 285-296.

Liu HT, Gong XY, Schäufele R, Yang F, Hirl RT, Schmidt A, Schnyder H. 2016. Nitrogen fertilization and δ18O of CO2 have no effect on 18O-enrichment of leaf water and cellulose in Cleistogenes squarrosa (C4)–is VPD the sole control? Plant, Cell & Environment 39, 2701-2712.

Mann ME, Park J. 1996. Greenhouse warming and changes in the seasonal cycle of temperature: Model versus observations. Geophysical Research Letters 23, 1111-1114.

Marshall J. 1986. Drought and shade interact to cause fine-root mortality in Douglas-fir seedlings. Plant and Soil 91, 51-60.

McAusland L, Vialet-Chabrand S, Davey P, Baker NR, Brendel O, Lawson T. 2016. Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. New Phytol 211, 1209-1220.

Meidner H, Mansfield TA. 1968. Physiology of stomata. Physiology of stomata.

Miksche JP, Berlyn GP. 1976. Botanical microtechnique and cytochemistry: Iowa State University Press.

Paul V, Sharma L, Pandey R, Meena R. 2017. Measurements of Stomatal Density and Stomatal Index on Leaf/Plant Surfaces. Manual of ICAR Sponsored Training Programme for Technical Staff of ICAR Institutes on “Physiological Techniques to Analyze the Impact of Climate Change on Crop Plants”, 27.

Pons TL, Jordi W, Kuiper D. 2001. Acclimation of plants to light gradients in leaf canopies: evidence for a possible role for cytokinins transported in the transpiration stream. journal of experimental botany 52, 1563-1574.

Regonda SK, Rajagopalan B, Clark M, Pitlick J. 2005. Seasonal cycle shifts in hydroclimatology over the western United States. Journal of climate 18, 372-384.

Ryser P, Urbas P. 2000. Ecological significance of leaf life span among Central European grass species. Oikos 91, 41-50.

Sack L, and Frole, K. 2006. Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. Ecology 87, 483-491.

Sack L, Buckley TN, Scoffoni C. 2016. Why are leaves hydraulically vulnerable? journal of experimental botany 67, 4917.

Sack L, Scoffoni C. 2013. Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. New Phytol 198, 983-1000.

Sack L, Scoffoni C, Johnson DM, Buckley TN, Brodribb TJ. 2015. The Anatomical Determinants of Leaf Hydraulic Function. 255-271.

Sack L, Tyree MT, Holbrook NM. 2005. Leaf hydraulic architecture correlates with regeneration irradiance in tropical rainforest trees. New Phytol 167, 403-413.

Scoffoni C, Chatelet DS, Pasquet-Kok J, Rawls M, Donoghue MJ, Edwards EJ, Sack L. 2016. Hydraulic basis for the evolution of photosynthetic productivity. Nat Plants 2, 16072.

Scoffoni C, Kunkle J, Pasquet-Kok J, Vuong C, Patel AJ, Montgomery RA, Givnish TJ, Sack L. 2015. Light-induced plasticity in leaf hydraulics, venation, anatomy, and gas exchange in ecologically diverse Hawaiian lobeliads. New Phytol 207, 43-58.

Sharma D, Pathak D, Atwal A, Sangha M. 2009. Genetic variation for some chemical and biochemical characteristics in cotton seed oil. J. Cotton Res. Dev 23, 1-7.

Shibuya T, Kano K, Endo R, Kitaya Y. 2017. Photosynthetic Properties and Response to Drought in Cucumber Seedlings Acclimatized to Different Vapor-pressure-deficit Levels. The Horticulture Journal 86, 334-339.

Shirke PA, Pathre UV. 2004. Influence of leaf-to-air vapour pressure deficit (VPD) on the biochemistry and physiology of photosynthesis in Prosopis juliflora. journal of experimental botany 55, 2111-2120.

Talbott LD, Zeiger E. 1993. Sugar and organic acid accumulation in guard cells of Vicia faba in response to red and blue light. Plant Physiology 102, 1163-1169.

Tallman G, Zeiger E. 1988. Light quality and osmoregulation in Vicia guard cells: evidence for involvement of three metabolic pathways. Plant Physiology 88, 887-895.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. Journal of the American Society for Horticultural Science 128, 598-602.

Tosens T, Nishida K, Gago J, Coopman RE, Cabrera HM, Carriquí M, Laanisto L, Morales L, Nadal M, Rojas R. 2016. The photosynthetic capacity in 35 ferns and fern allies: mesophyll CO 2 diffusion as a key trait. New Phytologist 209, 1576-1590.

Velikova V, Arena C, Izzo LG, Tsonev T, Koleva D, Tattini M, Roeva O, De Maio A, Loreto F. 2020. Functional and Structural Leaf Plasticity Determine Photosynthetic Performances during Drought Stress and Recovery in Two Platanus orientalis Populations from Contrasting Habitats. International Journal of Molecular Sciences 21, 3912.

Velikova V, Tsonev T, Tattini M, Arena C, Krumova S, Koleva D, Peeva V, Stojchev S, Todinova S, Izzo LG. 2018. Physiological and structural adjustments of two ecotypes of Platanus orientalis L. from different habitats in response to drought and re-watering. Conservation physiology 6, coy073.

Wei H, Kong D, Yang J, Wang H. 2020. Light Regulation of Stomatal Development and Patterning: Shifting the Paradigm from Arabidopsis to Grasses. Plant Communications 1, 100030.

Will RE, Wilson SM, Zou CB, Hennessey TC. 2013. Increased vapor pressure deficit due to higher temperature leads to greater transpiration and faster mortality during drought for tree seedlings common to the forest-grassland ecotone. New Phytol 200, 366-374.

Wilmer C, Fricker M. 1996. Stomata (2nd edn). London: Chapman and Hall.

Witkowski E, Lamont BB. 1991. Leaf specific mass confounds leaf density and thickness. Oecologia 88, 486-493.

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JH, Diemer M. 2004. The worldwide leaf economics spectrum. Nature 428, 821-827.

Xiong D, Douthe C, Flexas J. 2018. Differential coordination of stomatal conductance, mesophyll conductance, and leaf hydraulic conductance in response to changing light across species. Plant Cell Environ 41, 436-450.

Yu P. 2005. Applications of hierarchical cluster analysis (CLA) and principal component analysis (PCA) in feed structure and feed molecular chemistry research, using synchrotron-based Fourier transform infrared (FTIR) microspectroscopy. Journal of agricultural and food chemistry 53, 7115-7127.

Zeiger E. 2000. Sensory transduction of blue light in guard cells. Trends in Plant Science 5, 183-185.

Zeiger E, Talbott LD, Frechilla S, Srivastava A, Zhu J. 2002. The guard cell chloroplast: a perspective for the twenty-first century. New Phytologist 153, 415-424.

Zrenner R, Stitt M. 1991. Comparison of the effect of rapidly and gradually developing water-stress on carbohydrate metabolism in spinach leaves. Plant, Cell & Environment 14, 939-946

Chapter 4:

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

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

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4.1 Abstract: Nowadays, climate change is impacting considerably the availability of freshwater for agriculture, increasing the need for the optimization of crop water-use efficiency. Attempts to use VPD modulation to reduce water consumption have been made. However, the effects of VPD (Vapor Pressure Deficit) on leaf stomatal and hydraulic traits, and on possible trade-offs 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 controlled environment. Our results showed that plants developed a higher number of small stomata under LV, allowing a better regulation over opening/closing mechanisms thus increasing net-photosynthesis by 18%. LV plants also achieved a better performance of the photosystem II and a more efficient water use (increments in ΦPSII and iWUE by 3% and 49%), resulting in an enhanced plant growth and a reduced need for irrigation. Significant differences between G and R plants were limited to few traits and the physiological response under the two VPDs did not show cultivar-specific response. We discussed 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).

4.2 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 semi-arid 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 CO_2 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]. As already mentioned, stomatal closure can prevent the excessive transpiration and therefore the 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 CO_2 flow rates inside the leaves can be achieved by modulating its level; hence, different VPD levels during cultivation modify the whole 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 nowadays 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 the average of daily VPD, the extent of the fluctuations in VPD during the day influences the 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 CO_2 net-assimilation rates. On the opposite, moderate fluctuations do not cause reduction of the above-mentioned 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, allowing the cultivation in indoor environments has been fundamental to study the effects of VPD levels and duration on crop with the aim to extend 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 trade-off 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 CO_2 uptake, stomatal conductance, plant growth and yield influenced

by VPD and how such possible variation is linked to stomatal traits? (3) Does VPD affect the trade-off between plant carbon gain and cumulative water transpired?

4.3 Materials and Methods

4.3.1 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) at the stage of 4 true-leaves and 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, California, United States) set to dispense a PPFD of 315 µmol m^{-2} s^{-1} at the canopy level, which under 12h 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 minutes by two mini data loggers (Testo174H Testo, Germany) (Figure S1). Pots were covered with white polyethylene film to minimize evaporation losses from the substrate (1:1, peat:perlite) and were watered till holding capacity and weighted every day to replace the water loss according to Latha and Reddy [21](see paragraph 2.4). Moreover, to improve the uniformity of light intensity and humidity at the canopy level, pots were daily rotated on the chamber shelf. In both cultivation trials, all the analyses and sample collection were carried out following the same procedures in terms of days after transplanting, time of the day and replicates.

4.3.2 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 RGB channel (ImageJ; Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 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].

***4.3.3 Stomatal Traits Analysis*:** At 23 DAT, one fully expanded leaf was collected from 6 plants, having care of selecting 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 (BX60, Olympus) at 20x magnification (field area 0.237 mm^2) and the number of stomata was counted. Stomatal frequency was expressed as the number of stomata per mm^2. Stomatal index was computed using the following formula: ($S_N/S_N + E_N$) x 100 , where S_N is the number of stomata and EN the number of epidermal cells in the microscopic field [28].The size of 10 stomata per field was measured at 40x 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] as: π a b, where a is the semi-major axis and b is the semi-minor axis.

4.3.4 Determination of Gravimetric Indexes: 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 × 23], where A1 and A2 were the initial and final total plant area and the number 23 refers to the duration of the experiments in days (cm^2 day^{-1}); 3) the mean transpiration rate (MTR), as the ratio of CWT and LAD, expressed as mL cm^{-2} day^{-1} ; 4) the net assimilation rate (NAR), as the ratio of dry biomass (DB) and LAD, expressed as g cm^{-2} 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.

4.3.5 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 am to 2 pm 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 µmol) 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; µmol CO_2 m^{-2} s^{-1}), transpiration (iTr; mmol H_2O m^{-2} s^{-1}), stomatal conductance

(ig_s; mmol H_2O m^{-2} 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. 2,000 µmol (photon) $m^{-2}s^{-1}$. After 10 minutes exposure to darkness, the PPFD was increased by 0, 50, 200, 400, 800, 1000, 1500, 2000 µmol m^{-2} 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 90s, as reported in Hermann et al. [31] A maximum radiation intensity of 2000 µmol m^{-2} s^{-1} was chosen following Tsormpatsidis et al. [32], and this intensity was high enough to achieve $P_N$max. A complete light response curve per plant was created in approximately 20 min. From light response curves, *Imax*, defined as the point beyond which no significant change in P_N occurs, and *Icomp,* 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]. *Imax* and *Icomp* are considered more appropriate and realistic parameters to compared Isat or Isat(n) and Pgmax, for representing the photosynthetic potential of plants since their magnitude is always between the range of real measurements. Indeed, in other model Pgmax is used to define the point beyond which there is no significant change in P_N, but Pgmax is obtained when I is infinite; thus Pgmax is an abstraction, which forces the existence of Isat or better Isat(n) (light saturation point at a specific percentile), which is also an abstraction [33,34].

4.3.6 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 discs 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 (ΦPSII) was evaluated according to Genty et al. [35]; ii) electron transport rate (ETR) according to Krall and Edwards [36]; iii) non-photochemical quenching (NPQ) according to Bilger and Bjorkman [37]. Fluorescence analyses were conducted at a steady-state photosynthesis under a light intensity of about 400 µmol m^{-2} 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 ΦPSII measurements.

4.3.7 Statistics: The statistical analyses were all carried out using the software IBM SPSS Statistics (SPSS, Chicago, IL, USA). 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 Tukey test with $p \leq 0.05$.

4.4 Results

4.4.1 Growth Curves: Biomass production (both fresh and dry weight) and total plant area varied significantly among the treatments (Figures 1,2). 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 2 a, b). At 15 DAT, PA and FW were significantly higher for LVR and LVG than HVR and HVG ($P<0.05$) (Figure 2 a, 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 here the threshold after which plants begin to develop differently depending on the VPD starts at 9 DAT, were highest values were found in LVG followed by LVR which was higher than HVG and lowest values 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 ($p < 0.05$).

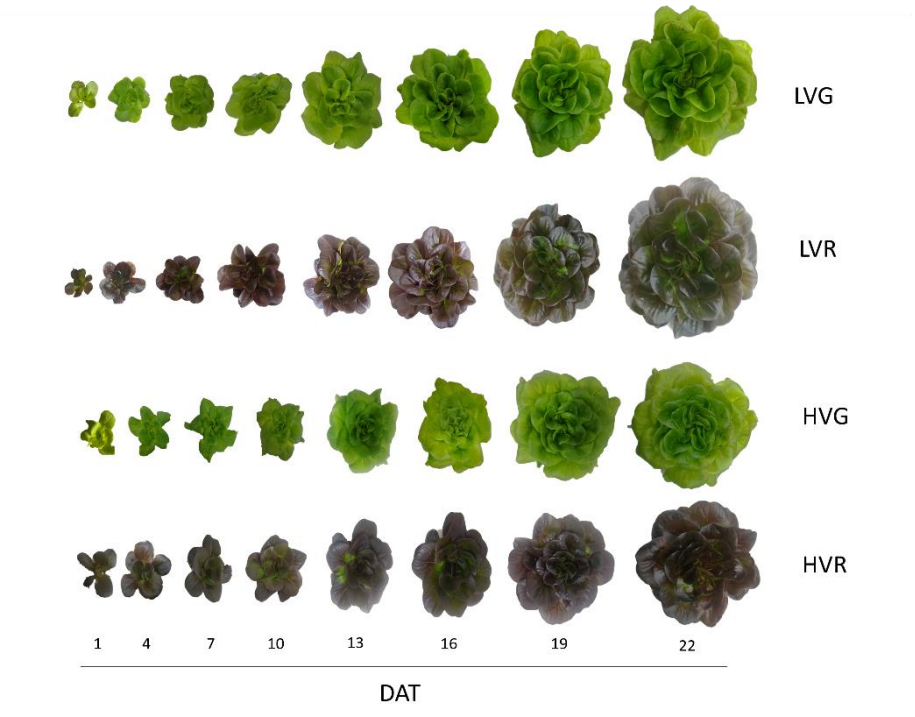


Figure 1: The phenotype of a representative green (G) and red (R) plant from low (LV) and high (HV) VPD condition during the growth cycle.

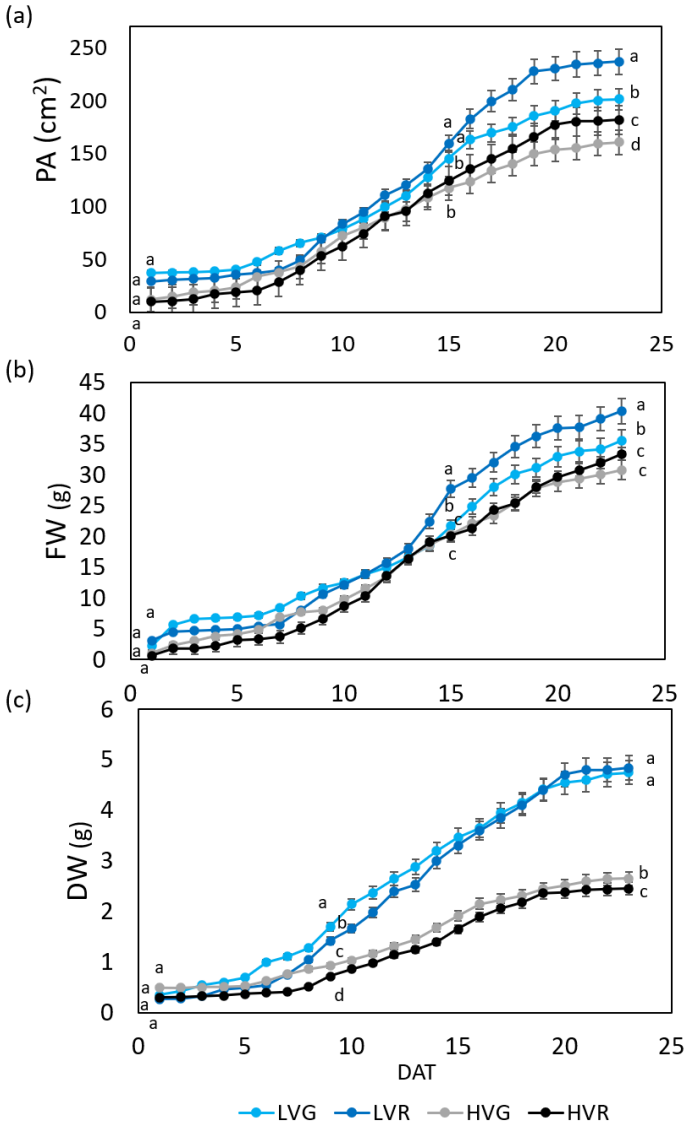


Figure 2: Growth curves of green (G) and red (R) lettuces under low (LV) and high (HV) VPD in terms of plant area (a), fresh weight (b) and dry weight (c). Mean values (n = 9) ± standard errors are shown. Different letters corresponded to significantly different values, among treatments within date,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

4.4.2 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 which 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).

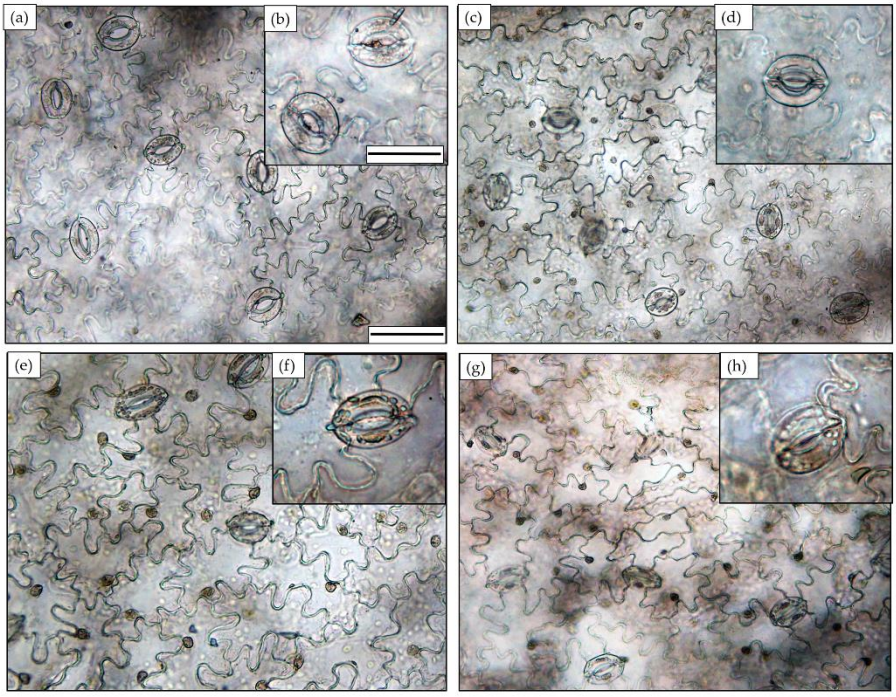


Figure 3 Lettuce stomatal morphology of LVG at 20x (a) and 40x (b), LVR at 20x (c) and 40x (d), HVG at 20x (e) and 40x (f), HVR at 20x (g) and 40x (h). See paragraph 2.3 for further details. In the table (i) stomatal frequency (SF) and size (SS) among treatments are shown. Values are reported as means ± SE (n = 9). Significant differences were compared using Tukey test. Images and related details are at the same magnification in the different treatments; bars = 20 micron.

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) ± standard errors are shown. Different letters corresponded to significantly different values, among treatments within date, according to Tukey's HSD ($p \leq 0.05$).

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

4.4.3 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 ($p < 0.05$).

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

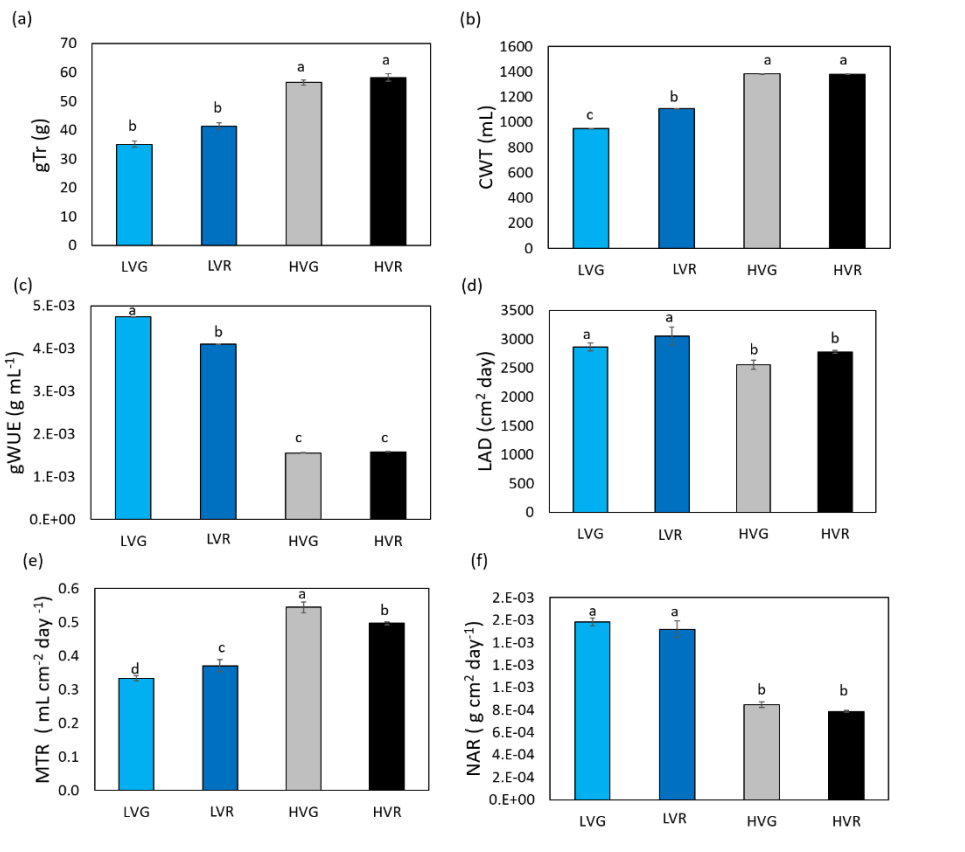


Figure 4: Gravimetric indexes of green (G) and red (R) lettuces under low (LV) and high (HV) VPD: transpiration (gTr; a), cumulative water transpired (CWT;b), water use efficiency (gWUE;c), leaf area duration (LAD;d), mean transpiration rate (MTR;e), net assimilation rate (NAR;f). Mean values (n = 9)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

4.4.4 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, ΦPSII was the highest in LVR followed by LVG, which in turn was higher than HVR. Lowest ΦPSII values were found in HVG ($p < 0.05$).

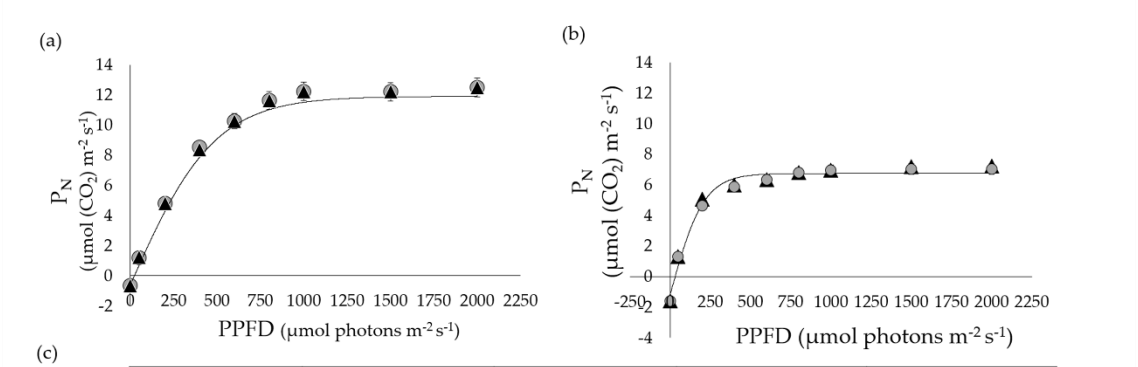
NPQ was the highest in HVG, followed by HVR, which in turn was higher than LVG. Lowest NPQ values were found in LVR ($p < 0.05$). ETR was the highest in LVG, followed by all the other conditions where no significant differences were detected. Overall, under low VPD ΦPSII and ETR were enhanced by 2.9% 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.

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).

Table 2. Effect of VPD (LV and HV) on physiological parameters of green (G) and red (R) plants: Quantum yield of PSII (ΦPSII), non-photochemical quenching (NPQ), electron transport rate (ETR), instantaneous values of gas-exchange: Net photosynthesis (iP_N), stomatal conductance (igs), transpiration (iTr), water use efficiency (iWUE). Mean values (n = 9) ± standard errors are shown. Different letters corresponded to significantly different values, among treatments within date, according to *Tukey's HSD* ($p \leq 0.05$).

	ΦPSII	NPQ	ETR	iP_N ($\mu mol\ CO_2\ m^{-2}s^{-1}$)	ig_s ($mmol\ H_2O\ m^{-2}s^{-1}$)	iWUE (iP_N/iTr)	iTr ($mmol\ H_2O\ m^{-2}s^{-1}$)
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

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



	LVG	LVR	HVG	HVR
Icomp	24.5 µmol $m^{-2}s^{-1}$ b	23.3 µmol $m^{-2}s^{-1}$ b	31.7 µmol $m^{-2}s^{-1}$ a	30.9 µmol $m^{-2}s^{-1}$ a
Imax	764 µmol $m^{-2}s^{-1}$ a	745 µmol $m^{-2}s^{-1}$ a	566 µmol $m^{-2}s^{-1}$ b	562 µmol $m^{-2}s^{-1}$ b

Figure 6: Effect of VPD (LV and HV) on green (G) and red (R) lettuce photosynthetic light curves: a) LVG (black triangles) and LVR (grey 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. [27]. Mean values (n = 9) ± standard errors are shown. Different letters corresponded to significantly different values, among treatments within date, according to *Tukey's HSD* (p ≤0.05).

4.5 Discussion

4.5.1 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 the 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 a better control over their opening and closing mechanism [8,40,41]. Furthermore, small stomatal size is known to facilitate the pore aperture, allowing a 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 their 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 a 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 a 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 bigger stomata). Since CO_2 enters the leaves through the stomata, stomatal closure or a reduction in stomatal conductance will also decrease the availability of CO_2 in the plant, consequently reducing photosynthesis [45,46]. According to our results, other studies conducted on tomatoes in controlled environment agriculture, have 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 growing 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 CO_2 uptake with increments in photosynthetic rates, as happened here for LV Salanova plants. Differently, in a study on *T. virginiana* it has been observed that a prolonged exposure to a low VPD make the stomata insensitive to closing stimuli like desiccation, high VPD, darkness and especially the abscisic acid (ABA); and this happened also 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 bring to a lower frequency of stomata. However, here the highest plant area was found in LVR compared to LVG (Figure 1,2). 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 cell number 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 pigmentated lettuces lies on the phytochemical content [51].

4.5.2 The Trade-off Between Water Transpired and Plant Carbon Gain: In our study WUE (both iWUE and gWUE) was enhanced in LV plants together with NAR (net assimiliation rate). Usually, NAR is associated to variations in stomatal and mesophyll conductance, which influence WUE and the overall plant growth [21]. The current study clearly demonstrated that LV plants presented a morphology of their stomata bringing to a higher conductance, thus positively influencing plant

photosynthesis, NAR and plant growth (Figure 2,4; Table1). However, variation in stomatal traits between G and R cultivars did not influence NAR.

Differently, 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 trying 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 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 CO_2 and H_2O through the mesophyll are tightly linked, usually allowing a balance between carbon gain and water loss, 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 μmol m^{-2} 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 adding CO_2 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 a 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 CO_2 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 indicate 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 6) but may indicate a 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 their G counterparts (Figure 2 b,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 the plant were of the same age.

4.6 Conclusions: The present study suggested that stomatal features, photosynthesis, transpiration, and water use efficiency are strictly interconnected and are 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 offsets the negative effects on plant growth and physiology and maximizes 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 a better regulation of gas-exchanges, ultimately influencing the whole plant growth and yield. Even though in 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, reducing the atmospheric VPD 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 the cultivation in 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 controlled environment, can change photosynthetic and growth performance. Such a phenomenon should be taken into account to evaluate the effect of different managements of control systems in order to reduce VPD range of fluctuation, as a mean 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

designing a strategy of VPD control based on the minimization of fluctuations with the final goal to achieve an efficient agricultural production in controlled environment.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: climatic data of temperature (triangles) and relative humidity (circles) under low (grey circles and triangles) and high (black circles and triangles) VPDs. Data are daily means of T and RH measurements taken every 15 minutes, 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), mean transpiration rate (MTR), Table S3: effect of VPD and cultivar on crop physiology: quantum yield of PSII (ΦPSII), non-photochemical quencing (NPQ), electron transport rate (ETR), instantaneous net-photosynthesis (iPn), instantaneous stomatal conductance (igs), instantaneous water use efficiency (iWUE), instantaneous transpiration (iTr).

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4.7 References:

1. Belko, N.; Zaman-Allah, M.; Diop, N.; Cisse, N.; Zombre, G.; Ehlers, J.; Vadez, V. Restriction of transpiration rate under high vapour pressure deficit and non-limiting water conditions is important for terminal drought tolerance in cowpea. *Plant Biology* **2013**, *15*, 304-316.

2. Medina, S.; Vicente, R.; Nieto-Taladriz, M.T.; Aparicio, N.; Chairi, F.; Vergara-Diaz, O.; Araus, J.L. The plant-transpiration response to vapor pressure deficit (VPD) in durum wheat is associated with differential yield performance and specific expression of genes involved in primary metabolism and water transport. *Frontiers in plant science* **2019**, *9*, 1994.

3. Zhang, D.; Jiao, X.; Du, Q.; Song, X.; Li, J. Reducing the excessive evaporative demand improved photosynthesis capacity at low costs of irrigation via regulating water driving force and moderating plant water stress of two tomato cultivars. *Agricultural Water Management* **2018**, *199*, 22-33.

4. Shamshiri, R.R.; Jones, J.W.; Thorp, K.R.; Ahmad, D.; Che Man, H.; Taheri, S. Review of optimum temperature, humidity, and vapour pressure deficit for microclimate evaluation and control in greenhouse cultivation of tomato: a review. *International agrophysics* **2018**, *32*.

5. Grossiord, C.; Buckley, T.N.; Cernusak, L.A.; Novick, K.A.; Poulter, B.; Siegwolf, R.T.; Sperry, J.S.; McDowell, N.G. Plant responses to rising vapor pressure deficit. *New Phytologist* **2020**, 226, 1550-66.

6. Amitrano, C.; Arena, C.; Rouphael, Y.; De Pascale, S.; De Micco, V. Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. *Annals of Applied Biology* **2019**, 10.1111/aab.12544, doi:10.1111/aab.12544.

7. Brodribb, T.J. Bringing Anatomy Back into the Equation. *Plant Physiol* **2015**, *168*, 1461, doi:10.1104/pp.15.01021.

8. Athanasiou, K.; Dyson, B.C.; Webster, R.E.; Johnson, G.N. Dynamic acclimation of photosynthesis increases plant fitness in changing environments. *Plant Physiology* **2010**, *152*, 366-373.

9. Lawson, T.; Blatt, M.R. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant physiology* **2014**, *164*, 1556-1570.

10. Li, Q.; Wei, M.; Li, Y.; Feng, G.; Wang, Y.; Li, S.; Zhang, D. Effects of soil moisture on water transport, photosynthetic carbon gain and water use efficiency in tomato are influenced by evaporative demand. *Agricultural Water Management* **2019**, *226*, 105818.

11. Buckley, T.N.; John, G.P.; Scoffoni, C.; Sack, L. How Does Leaf Anatomy Influence Water Transport outside the Xylem? *Plant Physiol* **2015**, *168*, 1616-1635, doi:10.1104/pp.15.00731.

12. Vialet-Chabrand, S.R.; Matthews, J.S.; McAusland, L.; Blatt, M.R.; Griffiths, H.; Lawson, T. Temporal dynamics of stomatal behavior: modeling and implications for photosynthesis and water use. *Plant physiology* **2017**, *174*, 603-613.

13. Carins Murphy, M.R.; Jordan, G.J.; Brodribb, T.J. Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. *Plant Cell Environ* **2014**, *37*, 124-131, doi:10.1111/pce.12136.

14. Aliniaeifard, S.; Malcolm Matamoros, P.; van Meeteren, U. Stomatal malfunctioning under low VPD conditions: induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? *Physiol Plant* **2014**, *152*, 688-699, doi:10.1111/ppl.12216.

15. Amitrano, C.; Arena, C.; Cirillo, V.; De Pascale, S.; De Micco, V. Leaf morpho-anatomical traits in *Vigna radiata* L. affect plant photosynthetic acclimation to changing vapor pressure deficit. *Environmental and Experimental Botany* **2021**, 104453.

16. De Micco, V.; Amitrano, C.; Stinca, A.; Izzo, L.G.; Zalloni, E.; Balzano, A.; Barile, R.; Conti, P.; Arena, C. Dust accumulation due to anthropogenic impact induces anatomical and photochemical changes in leaves of Centranthus ruber growing on the slope of the Vesuvius volcano. *Plant Biol (Stuttg)* **2019**, 10.1111/plb.12966, doi:10.1111/plb.12966.

17 Carillo, P.; Cirillo, C.; De Micco, V.; Arena, C.; De Pascale, S.; Rouphael, Y. Morpho-anatomical, physiological and biochemical adaptive responses to saline water of Bougainvillea spectabilis Willd. trained to different canopy shapes. *Agricultural Water Management* **2019**, *212*, 12-22, doi:10.1016/j.agwat.2018.08.037.

18. Bertolino, L.T.; Caine, R.S.; Gray, J.E. Impact of stomatal density and morphology on water-use efficiency in a changing world. *Frontiers in plant science* **2019**, *10*, 225.

19. Pautov, A.; Bauer, S.; Ivanova, O.; Krylova, E.; Yakovleva, O.; Sapach, Y.; Pautova, I. Influence of stomatal rings on movements of guard cells. *Trees* **2019**, *33*, 1459-1474.

20. López, J.; Way, D.A.; Sadok, W. Systemic effects of rising atmospheric vapor pressure deficit on plant physiology and productivity. *Global change biology* **2021**, *27*, 1704-1720.

21. Latha, P.; Reddy, P. Determination of water use efficiency in groundnut by gravimetric method and its association with physiological parameters. *Indian journal of plant physiology* **2005**, *10*, 322.

22. Zhang, D.; Zhang, Z.; Li, J.; Chang, Y.; Du, Q.; Pan, T. Regulation of Vapor Pressure Deficit by Greenhouse Micro-Fog Systems Improved Growth and Productivity of Tomato via Enhancing Photosynthesis during Summer Season. *PLoS One* **2015**, *10*, e0133919, doi:10.1371/journal.pone.0133919.

23. Inoue, T.; Sunaga, M.; Ito, M.; Yuchen, Q.; Matsushima, Y.; Sakoda, K.; Yamori, W. Minimizing VPD Fluctuations Maintains Higher Stomatal Conductance and Photosynthesis, Resulting in Improvement of Plant Growth in Lettuce. *Frontiers in plant science* **2021**, *12*, 458.

24. Amitrano, C.; Chirico, G.B.; De Pascale, S.; Rouphael, Y.; De Micco, V. Application of a MEC model for the irrigation control in green and red-leaved lettuce in precision indoor cultivation. In Proceedings of 2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor); pp. 196-201.

25. Du, Q.; Liu, T.; Jiao, X.; Song, X.; Zhang, J.; Li, J. Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. *J Exp Bot* **2019**, *70*, 4949-4962, doi:10.1093/jxb/erz238.

26. Carins Murphy, M.R.; Jordan, G.J.; Brodribb, T.J. Differential leaf expansion can enable hydraulic acclimation to sun and shade. *Plant, Cell & Environment* **2012**, *35*, 1407-1418.

27. Amitrano, C.; Chirico, G.B.; De Pascale, S.; Rouphael, Y.; De Micco, V. Crop Management in Controlled Environment Agriculture (CEA) Systems Using Predictive Mathematical Models. *Sensors* **2021**, *20*, 3110.

28. Bertolino, L.T.; Caine, R.S.; Gray, J.E. Impact of stomatal density and morphology on water-use efficiency in a changing world. *Frontiers in plant science* **2019**, *10*, 225.

29. Sorrentino, M.C.; Capozzi, F.; Amitrano, C.; De Tommaso, G.; Arena, C.; Iuliano, M.; Giordano, S.; Spagnuolo, V. Facing metal stress by multiple strategies: morphophysiological responses of cardoon (Cynara cardunculus L.) grown in hydroponics. *Environmental Science and Pollution Research* **2021**, 1-11.

30. Xu, Z.; Zhou, G. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *Journal of experimental botany* **2008**, *59*, 3317-3325.

31. Hermann, H.A.; Schwartz, J.M.; Johnson, G.N. From empirical to theoretical models of light response curves-linking photosynthetic and metabolic acclimation. *Photosynthesis research* **2020**, 145, 5-14.

32. Tsormpatsidis, E.; Henbest, R.; Battey, N.H.; Hadley, P. The influence of ultraviolet radiation on growth, photosynthesis and phenolic levels of green and red lettuce: potential for exploiting effects of ultraviolet radiation in a production system. *Annals of Applied Biology* **2010**, *156*, 357-366.

33. Lobo, F.d.A.; De Barros, M.; Dalmagro, H.; Dalmolin, Â.; Pereira, W.; De Souza, E.; Vourlitis, G.; Ortíz, C.R. Fitting net photosynthetic light-response curves with Microsoft Excel—a critical look at the models. *Photosynthetica* **2013**, *51*, 445-456.

34. Korsakova, S.; Plugatar, Y.; Ilnitsky, O.; Karpukhin, Y. A research on models of the photosynthetic light response curves on the example of evergreen types of plants. **2019**.

35. Genty, B.; Briantais, J.-M.; Baker, N.R. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1989**, *990*, 87-92.

6. Krall, J.P.; Edwards, G.E. Relationship between photosystem II activity and CO2 fixation in leaves. *Physiologia Plantarum* **1992**, *86*, 180-187.

37 Bilger, W.; Björkman, O. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of Hedera canariensis. *Photosynthesis research* **1990**, *25*, 173-185.

38. Fanourakis, D.; Heuvelink, E.; Carvalho, S.M. A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. *J Plant Physiol* **2013**, *170*, 890-898, doi:10.1016/j.jplph.2013.01.013.

39. Lu, N.; Nukaya, T.; Kamimura, T.; Zhang, D.; Kurimoto, I.; Takagaki, M.; Maruo, T.; Kozai, T.; Yamori, W. Control of vapor pressure deficit (VPD) in greenhouse enhanced tomato growth and productivity during the winter season. *Scientia Horticulturae* **2015**, *197*, 17-23.

40. Franks, P.J.; Beerling, D.J. Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. *Proc Natl Acad Sci U S A* **2009**, *106*, 10343-10347, doi:10.1073/pnas.0904209106.

41. Kiani-Pouya, A.; Rasouli, F.; Rabbi, B.; Falakboland, Z.; Yong, M.; Chen, Z.-H.; Zhou, M.; Shabala, S. Stomatal traits as a determinant of superior salinity tolerance in wild barley. *Journal of Plant Physiology* **2020**, *245*, 153108.

42. Lawson, T.; Vialet-Chabrand, S. Speedy stomata, photosynthesis and plant water use efficiency. *New Phytologist* **2019**, *221*, 93-98.

43. Patanè, C. Leaf area index, leaf transpiration and stomatal conductance as affected by soil water deficit and VPD in processing tomato in semi arid Mediterranean climate. *Journal of Agronomy and Crop Science* **2011**, *197*, 165-176.

44. Wieland, F. Water Transport and Energy. *Plant, cell & environment* **2017**, *40*, 977-994.

45. Flexas, J.; Medrano, H. Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Annals of botany* **2002**, *89*, 183-189.

46. Hassiotou, F.; Evans, J.R.; Ludwig, M.; Veneklaas, E.J. Stomatal crypts may facilitate diffusion of CO2 to adaxial mesophyll cells in thick sclerophylls. *Plant, Cell & Environment* **2009**, *32*, 1596-1611.

47. Rezaei Nejad. A.; van Meeteren U. The role of abscisic acid in disturbed stomatal response characteristics of *Tradescantia virginiana* during growth at high relative air humidity. *Journal of Experimental Botany* **2007***,* 58, 627–636.

48. Lake, J.A.; Woodward F.I. Response of stomatal numbers to CO2 and humidity: control by transpiration rate and abscisic acid." *New Phytologist* **2008***,* 179, 397-404.

49. Aliniaeifard, S.; van Meeteren, U. Can prolonged exposure to low VPD disturb the ABA signalling in stomatal guard cells?." *Journal of Experimental Botany* **2013**, 64, 3551-3566.

50. El-Nakhel, C.; Petropoulos, S.A.; Pannico, A.; Kyriacou, M.C.; Giordano, M.; Colla, G.; Troise, A.D.; Vitaglione, P.; De Pascale, S.; Rouphael, Y. The bioactive profile of lettuce produced in a closed soilless system as configured by

combinatorial effects of genotype and macrocation supply composition. *Food Chem* **2020**, *309*, 125713, doi:10.1016/j.foodchem.2019.125713.

51. Amitrano, C.; Rouphael, Y.; De Pascale, S.; De Micco, V. Modulating Vapor Pressure Deficit in the Plant Micro-Environment May Enhance the Bioactive Value of Lettuce. *Horticulturae* **2021**, *7*, 32.

52. Impa, S.; Nadaradjan, S.; Boominathan, P.; Shashidhar, G.; Bindumadhava, H.-y.; Sheshshayee, M. Carbon isotope discrimination accurately reflects variability in WUE measured at a whole plant level in rice. *Crop Science* **2005**, *45*, 2517-2522.

53. Farquhar, G.D.; Lloyd, J. Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In *Stable isotopes and plant carbon-water relations*, Elsevier: 1993; pp. 47-70.

54. Sorrentino, G.; Haworth, M.; Wahbi, S.; Mahmood, T.; Zuomin, S.; Centritto, M. Abscisic acid induces rapid reductions in mesophyll conductance to carbon dioxide. *PLoS One* **2016**, *11*, e0148554.

55. Haworth, M.; Killi, D.; Materassi, A.; Raschi, A.; Centritto, M. Impaired stomatal control is associated with reduced photosynthetic physiology in crop species grown at elevated [CO2]. *Frontiers in plant science* **2016**, *7*, 1568.

56. Jiao, X.C.; Song, X.M.; Zhang, D.L.; Du, Q.J.; Li, J.M. Coordination between vapor pressure deficit and CO2 on the regulation of photosynthesis and productivity in greenhouse tomato production. *Sci Rep* **2019**, *9*, 8700, doi:10.1038/s41598-019-45232-w.

57. Maxwell, K.; Johnson, G.N. Chlorophyll fluorescence—a practical guide. *Journal of experimental botany* **2000**, *51*, 659-668.

58. Sorrentino, M.; Capozzi, F.; Amitrano, C.; Giordano, S.; Arena, C.; Spagnuolo, V. Performance of three cardoon cultivars in an industrial heavy metal-contaminated soil: Effects on morphology, cytology and photosynthesis. *Journal of hazardous materials* **2018**, *351*, 131-137.

59. Baker, N.R. Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu. Rev. Plant Biol.* **2008**, *59*, 89-113.

60. Chaves, M.; Oliveira, M. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *Journal of experimental botany* **2004**, *55*, 2365-2384.

61. Montanaro, G.; Dichio, B.; Xiloyannis, C. Shade mitigates photoinhibition and enhances water use efficiency in kiwifruit under drought. *Photosynthetica* **2009**, *47*, 363.

62. Ghorbanzadeh, P.; Aliniaeifard, S.; Esmaeili, M.; Mashal, M.; Azadegan, B.; Seif, M. Dependency of Growth, Water Use Efficiency, Chlorophyll Fluorescence, and Stomatal Characteristics of Lettuce Plants to Light Intensity. *Journal of Plant Growth Regulation* **2020**, 1-17.

63. El-Nakhel, C.; Giordano, M.; Pannico, A.; Carillo, P.; Fusco, G.M.; De Pascale, S.; Rouphael, Y. Cultivar-specific performance and qualitative descriptors for butterhead Salanova lettuce produced in closed soilless cultivation as a candidate salad crop for human life support in space. *Life* **2019**, *9*, 61.

Chapter 5:

Crop Management in Controlled Environment Agriculture (CEA) Systems Using Predictive Mathematical Models

sensors

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Article

Crop Management in Controlled Environment Agriculture (CEA) Systems Using Predictive Mathematical Models †

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5.1 Abstract: Proximal sensors in controlled environment agriculture (CEA) are used to monitor plant growth, yield, and water consumption with non-destructive technologies. Rapid and continuous monitoring of environmental and crop parameters may be used to develop mathematical models to predict crop response to microclimatic changes. Here, we applied the energy cascade model (MEC) on green- and red-leaf butterhead lettuce (*Lactuca sativa* L. var. *capitata*). We tooled up the model to describe the changing leaf functional efficiency during the growing period. We validated the model on an independent dataset with two different vapor pressure deficit (VPD) levels, corresponding to nominal (low VPD) and off-nominal (high VPD) conditions. Under low VPD, the modified model accurately predicted the transpiration rate (RMSE = 0.10 Lm^{-2}), edible biomass (RMSE = 6.87 g m^{-2}), net-photosynthesis (rBIAS = 34%), and stomatal conductance (rBIAS = 39%). Under high VPD, the model overestimated photosynthesis and stomatal conductance (rBIAS = 76–68%). This

inconsistency is likely due to the empirical nature of the original model, which was designed for nominal conditions. Here, applications of the modified model are discussed, and possible improvements are suggested based on plant morpho-physiological changes occurring in sub-optimal scenarios.

Keywords: crop modelling; energy cascade model (MEC); Lactuca sativa L. var. capitata; controlled environment agriculture (CEA); precision horticulture

5.2 Introduction: Environmental control is a key factor to increase plant productivity in controlled environment agriculture (CEA) [1]. Recently, increased interest has been directed towards plant production in closed facilities (e.g., plant factories, vertical farms, indoor-growing modules) [2–4]. However, with the introduction of advanced monitoring and control technologies, it becomes necessary to properly discern plant/microclimate interaction, modulate environmental parameters, and manage cultivation factors. Indeed, in protected agriculture, environmental factors and plant responses are strictly interconnected: alteration of the microclimate can induce modifications in plants (both at morphological and physiological levels), affecting plant behavior especially in terms of transpiration and CO_2/O_2 exchanges, which in turn re-modify the surrounding environment [5]. Recently, a technological “revolution” in agriculture is on-going, pushed by advances in sophisticated technologies such as robots, aerial/proximal sensors and more broadly by the internet of the things (IoT) [6]. This “revolution”, based on the automation of processes and the remote monitoring of systems, would allow the realization of smart farming, with a more lucrative, efficient, and sustainable production [7]. Remote sensing technologies allow the early monitoring of plant responses to environmental stresses such as: drought, salinity, and heat due to high temperature or excessive solar radiation. Currently, the remote sensing of plant physiological behavior is often based on reflectance indices, including photochemical reflectance index (PRI), normalized difference vegetation index (NDVI), leaf area index (LAI), and water index (WI) [8–10]. However, these indexes are mostly used in the field and their use for the remote monitoring of the photosynthetic process under controlled conditions is still limited. Indeed, in protected cultivations, crop monitoring mostly relies on sensors controlling environmental parameters, and on plant gas-exchange and fluorescence analyses [10]. As we progress in adopting technological advancement, issues related to sensor loss of control or breakage may be experienced. Such phenomena would be responsible for modifications occurring at the plant level during the cultivation cycle, such as alterations in plant growth, morphogenesis, and development. In this context, mathematical models, which mimic the behavior of real systems, can be adopted to monitor, simulate, predict, control, and facilitate the understanding of crop behavior in protected cultivation under both nominal (optimal) and off-nominal (sub-optimal) conditions [11]. Until now, most of the controlling tasks in CEA have been designed to maintain specific set-points, neglecting the effects of environmental perturbations on crops [12].

However, evaluating crop status especially under off-nominal conditions represents an added value to forecast possible growth reductions and prevent yield losses by a real-time fine-tuning of environmental control and water management (irrigation schedule), according to different plant phenological stages. The aerial (moist/dry) control and, more specifically, the Vapor Pressure Deficit (VPD) regulation is listed amongst the main critical issues for crop production in CEA [13]. Indeed, VPD is one of the main drivers for plant transpiration: it affects crops during growth, changing plant morpho-physiological development, especially impacting water fluxes in the soil–plant–atmosphere continuum (SPAC), and thus the availability of water during the cultivation [14]. Given that plant transpiration is recognized to be a convenient indicator of its water status, real-time sensor information is a fundamental pre-requisite for the precision irrigation management of crops [15]. Furthermore, in small-indoor-growing modules, like those used for cultivation in Space, the regulation of plant water fluxes can be easily disrupted due to difficulties in the control of VPD in limited volumes. Therefore, a proper irrigation management is even more recommended. These difficulties in VPD control in protected cultivation can determine a rise in air temperature and consequently in evapotranspiration, which impact crop production, also resulting in decreasing stomatal conductance and photosynthetic rates [16]. Transpiration in plants is influenced by environmental conditions and regulated by stomatal opening/closing [17]. High VPD values (1.7–2 kPa) intensify plant physiological stress, especially under water shortage, by increasing plant water loss and decreasing carbon fixation, thus negatively influencing crop growth and productivity, which represents a major issue for production in CEA [18]. Furthermore, together with increasing VPD and water stress, ABA hormone tends to accumulate in leaves and its concentration is negatively correlated with the stomatal conductance, further exacerbating leaf transpiration [19]. Nowadays, there are numerous models which simulate different photosynthetic and plant productivity processes, often focusing on very specific aspects of plant physiology, such as: protection of photosynthetic apparatus through the non-photochemical quenching, mesophyll conductance to CO_2, genotype-environment interactions [20–23]. Among these models, the energy cascade model (MEC) has already been tested to implement crop growth in small prototypes for bioregenerative life support systems (BLSSs) studies and in a lunar/Martian greenhouse [12,24]. This “explanatory” model is therefore considered suitable to predict both biomass, photosynthesis, transpiration, and energy balance in closed systems. Moreover, such a model could easily be applied by stakeholders operating in controlled agriculture facilities for food production. Indeed, the model, requiring just a few environmental and cultivation parameters as inputs, can help forecasting changes happening during the cultivation after the modification of environmental factors, thus being possibly implemented as decision support system.

In the present study, we report an application of the MEC model on butterhead lettuce (*Lactuca sativa* L. var. *capitate*) cultivated under controlled conditions, also presenting a modification to the original model. More specifically, we introduced additional components to include the variation of

canopy quantum yield of PSII (CQY) and carbon use efficiency (CUE) during leaf development. Such parameters, which represent respectively the moles of carbon fixed per mole of photons absorbed and the ratio of net carbon gain to gross carbon assimilation during growth, are critical to the model for the calculation of the net photosynthesis and the biomass production. Furthermore, we applied the modified version of the model on green- and red-leaf lettuce grown in a climatic chamber under two VPD scenarios, namely low and high VPD, corresponding to nominal and off-nominal conditions, respectively. This latter trial allowed the portrayal of the model parameters for nominal and off-nominal scenarios for green- and red-leaf plants, since non-identical behavior often occurs for different cultivars/varieties even under the same growth conditions.

5.3 Materials and Methods

5.3.1 The Original MEC Model: The original energy cascade model was developed for wheat by Volk et al. [25] and then calibrated for other crops like lettuce, rice, soybean, sweet potato and tomato [26]. It is an explanatory model, composed by multivariate equations whose coefficients have been determined through curve fitting of experimental data [27]. The input variables of the first version of the model were the light intensity (photosynthetic photon flux density; PPFD) and the photoperiod. All the model outputs, which mostly concerned the biomass and the growth rate, were function of these two parameters.

The original MEC model was based on an “energy cascade” with three fundamental steps:

the absorption of PPFD by the canopy;

the absorbed energy (A) used in the photosynthetic process to convert carbon into sucrose;

the conversion of sucrose into biomass.

Such a simple model required only three crop parameters, namely (i) the time of canopy closure (tA), (ii) the time of senescence onset (tQ), and (iii) the time of harvesting (tM). Eventually, the model was modified to add the following climatic parameters: air temperature, relative humidity, carbon dioxide concentration, dark period, and plant density, in order to improve the accuracy and robustness of the model [16]. In 2012, Boscheri et al. [12] implemented a modified version of the MEC model, for a multi-crop Lunar greenhouse prototype. The version modified by Boscheri et al. [12] also included a crop transpiration component, used to predict water and plant nutrient consumption.

The main model algorithm components were arranged according to twelve equations, sequentially computed at each time step to calculate the key variables as listed below.

The canopy quantum yield (CQY, mol−1) is defined by an empirical equation as function of the time t, expressed in days after emergence:

$$CQY = CQYMAX \qquad \text{for } t \leq tQ$$

$$CQY = CQYMAX - (CQYMAX - CQYMIN)\,(t - tQ)/(tM - tQ) \quad \text{for } tQ < t \le tM \qquad (1)$$

where CQYMAX and CQYMIN are crop-specific parameters, while tQ and tM are time of the onset of senescence and time of harvesting, respectively.

The carbon use efficiency (CUE), is expressed similarly to CQY, according to the following relation:

$$CUE = CUEMAX \quad \text{for } t \le tQ$$

$$CUE = CUE_{MAX} - (CUE_{MAX} - CUE_{MIN})\,(t - tQ)/(tM - tQ) \quad \text{for } tQ < t \le tM \qquad (2)$$

where CUE_{MAX} and CUE_{MIN} are crop specific parameters, although CUE has been often assumed to be constant for most crops (i.e., $CUE_{MAX} = CUE_{MIN}$), tQ and tM are time of the onset of senescence and time of harvesting, respectively.

The parameter A is the fraction of PPFD absorbed by the top of the canopy and is assumed to increase with time t according to a power law equation, up to a maximum value A_{MAX} at time t = tA, when canopy closure is established:

$$A = A_{MAX} \cdot (t/tA)n \quad \text{for } t \le tA$$

$$A = A_{MAX} \quad \text{for } t > tA \qquad (3)$$

where tA is the time of canopy closure and n is a crop dependent exponent, which is considered to be equal to 2.5 for lettuce [27].

The daily carbon gain (DCG, mol C m^{-2} d^{-1}) is computed as follows:

$$DGC = 0.0036\ H \cdot \alpha \cdot PPFD \qquad (4)$$

where H is the photoperiod, $\alpha = CUE \cdot A \cdot CQY$, and 0.0036 is a constant used to convert µmol to mol and hours to seconds.

The daily oxygen production (DOP, mol O2 m^{-2} d^{-1}) is then given by a fraction (oxygen production fraction, OPF) of DGC:

$$DOP = OPF \cdot DGC \qquad (5)$$

where OPF is expressed in mol O2 mol−1 C and is a crop-specific parameter.

The crop growth rate (CGR, g m−2 d−1) is given by:

$$CGR = MWc \cdot DCG/BCF \qquad (6)$$

where MWc = 12 g mol^{-1} is the carbon molecular weight, while BCF is a crop specific parameter representing the biomass carbon fraction.

Thus, the total edible biomass (TEB), expressed as specific dry weight (g m^{-2}), was calculated by integrating the crop growth rate, multiplied by the fraction of CGR allocated to edible biomass (XFRT):

$$\mathrm{TEB} = \int_{tE}^{tM} \mathrm{XFRT} \cdot \mathrm{CGR} \cdot \mathrm{dt} \quad (7)$$

where tM is the time of harvesting, tE is the time of the onset of edible biomass formation and XFRT represents a partitioning coefficient for the edible biomass, which combines the effects of determinacy and temperature on storage organ growth rates [26].

The gross photosynthesis (P_G, μmol CO_2 m^{-2} s^{-1}) is computed as follows：

$$P_G = \beta \cdot PPFD \quad (8)$$

where β = A·CQY and PPFD is the photosynthetic photon flux density (μmol m^{-2} s^{-1}).

The net photosynthesis (P_N, μmol CO_2 m^{-2} s^{-1}) is computed by accounting for the carbon use efficiency in the photoperiod:

$$P_N = [H \cdot \alpha/24 + \beta\ (24 - H)/24] \cdot PPFD \quad (9)$$

where H is the photoperiod, α = CUE·A·CQY, β = A·CQY and PPFD is the photosynthetic photon flux density (μmol m^{-2} s^{-1}).

The stomatal conductance (g_S, mol m^{-2} s^{-1}) for planophile-type canopies (such as lettuce) is calculated according to Monje et al. [28]

$$g_S = (1.717 \cdot T - 19.96 - 10.54 \cdot VPD) \cdot P_N/[CO_2] \quad (10)$$

where T (°C) is the mean air temperature during light cycle and [CO_2] is the air carbon dioxide concentration expressed as μmol CO_2 mol^{-1}.

The canopy surface conductance for water vapor (gc, mol m^{-2} s^{-1}) is defined as follows:

$$g_c = g_A \cdot g_S/(g_A + g_S) \quad (11)$$

where g_A = 2.5 mol m^{-2} s^{-1} is the aerodynamic conductance and gs the stomatal conductance.

The daily canopy transpiration (DTR, L m^{-2} d^{-1}) is also calculated as follows:

$$DTR = 3600 \cdot H \cdot (MWW/\rho W) \cdot gc \cdot (VPD/PATM) \quad (12)$$

where 3600 is a conversion constant from second to hours, MWW = 18 g mol^{-1} is the molecular weight of water, ρW = 100 g L^{-1} is the water density, and PATM (kPa) is the total atmospheric pressure, which was used to convert vapor pressure to mole fraction.

5.3.2 Limitations of the Original MEC Model Formulation: According to Equations (1)–(12), the original energy cascade model, used for advanced life support systems (ALSs) studies, predicted the biomass production and the photosynthetic rate based on three parameters: (i) the canopy light absorption (A), (ii) the crop quantum yield of PSII (CQY), and (iii) the carbon use efficiency (CUE). The physical and biological trends of these parameters were: a linear increase in PPFD absorption till the canopy closure; a constant CQY until the onset of senescence, followed by a linear decrease till the end of the cycle, and a constant CUE throughout the life cycle (Figure1, orange lines). However, for lettuce and a few other crops like sweet potato, which are harvested before the occurrence of senescence (tQ), the CQY, as the CUE, were assumed to be constant during the entire growth cycle prior harvesting (Figure 1, blue lines).

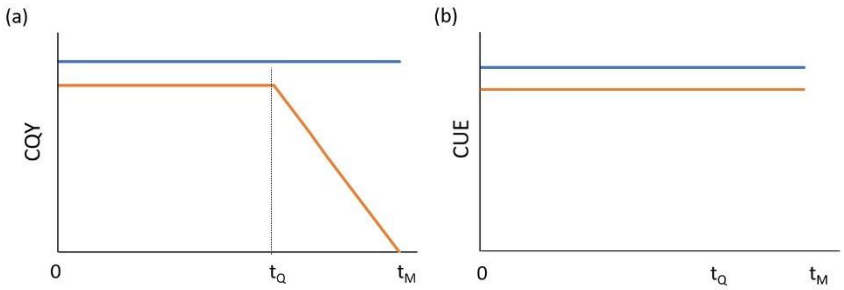

Figure 1. Original MEC model time profile of CQY (a) and CUE (b) for lettuce (blue lines), which was considered to be constant throughout the whole crop cycle; and for other crops (orange lines), in which CQY (a) was considered to be constant until the onset of senescence (tQ), to linearly decrease till the time of harvesting (tM), while CUE (b) was considered to be constant.

5.3.3 Experiments to Retrieve CQY Temporal Pattern: The canopy quantum yield (CQY) represents the moles of carbon fixed per mole of photons absorbed [29–31] and can be assessed in different ways such as:

Dividing the daily P_G (mol C $m^2 \cdot d^{-1}$) by the total absorbed photons (mol $m^2 \cdot d^{-1}$) [32,33];

From the initial slope of saturation-photosynthetic curves [29];

By means of a fluorimeter, an instrument which measures the proportion of the light absorbed by the chlorophyll associated with the photosystem II (PSII), thus indicating the efficiency of the carbon fixation and of the overall photosynthesis [34].

In this study, we designed a calibration experiment for assessing the CQY temporal pattern in butterhead 'Salanova' lettuce (*Lactuca sativa* L. var. *capitata*), by means of a fluorimeter. The experiment was performed from 10 July 2019 to 6 August 2019, at the Department of Agricultural Sciences (University of Naples Federico II). Two weeks after sowing, the plants of butterhead lettuce cultivars, green- and red-leaf Salanova® (Rijk Zwaan, Der Lier, The Netherlands) were transplanted into 10 cm pots filled with a peat:soil mixture (1:1 volume ratio) and exposed to solar light. Air temperature (T) was kept at 23 °C and plants were irrigated at 2-day intervals in order to reach the container capacity (till the beginning of drainage). Chlorophyll "a" fluorescence analyses were performed by means of a portable fluorimeter (ADC Bioscientific) on 3 expanded leaves per 4 green- and 4 red-lettuce plants every day, in order to highlight the leaf-age-driven variations in CQY, according to Genty et al. (1989) [35]. Fluorescence analyses were conducted at steady-state photosynthesis under a light intensity of about 400 µmol m^{-2} 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 CQY measurements. The leaves were chosen from three different positions within the lettuce head (top-t, medium-m, and bottom-b) in order to gain representative data for retrieving the general temporal pattern of CQY throughout the canopy.

5.3.4 Experiments in a Controlled Environment Growth Chamber to Validate the Model: Nine green- and nine red-leaf Salanova lettuce plants were grown in a controlled environment growth chamber (KBP-6395F, Termaks, Bergen, Norvegia) (Figure 2), in two consecutive trials. In the first trial, 1-week old plants were transplanted into 10 cm pots filled with peat:perlite substrate (1:1 volume ratio) and incubated with an average VPD of 0.69 kPa, corresponding to nominal conditions of Low VPD. In the second trial, 9 green- and 9 red-leaf lettuce plants were incubated with an average VPD of 1.76 kPa, corresponding to off-nominal conditions of High VPD. The two different VPDs were achieved by keeping air temperature (T) constant at 24 °C, while changing the relative humidity (RH) accordingly. Temperature and RH were monitored and recorded every 10 min by means of mini sensors equipped with a data logger (Testo 174H). All other microclimate parameters and agricultural practices were the same in the two consecutive experiments. The lighting system was an RGB LED panel, with a light intensity of 315 PPFD µmol m^{-2} s^{-1} at the canopy level (12 h photoperiod; 13.6 daily light integral, DLI). Daily rotation of the trays was performed to ensure homogenous light and humidity across the shelf surface. Plants were daily weighted to assess the loss of water by transpiration (DTR) and were re-watered to field-capacity. Evaporation losses from the substrate were minimized by covering the substrate with a plastic film. Plant growth was assessed by imaging, measuring canopy total area every day, and counting the number of leaves.

Furthermore, dry weight was recorded at the beginning and at the end of both trials. These measurements were used to reconstruct the daily total edible biomass (TEB). Changes in leaf temperature were monitored with an infrared thermometer on three leaves per plant (H-1020; Helect). These measurements were averaged and used instead of T in Equation (10) of the MEC model, to obtain more precise information of the leaf-to-air VPD which influences stomata conductance the most. After 23 days, on fully developed leaves, eco-physiological analyses in terms of gas exchanges (LCA 4; ADC BioScientific Ltd., Hoddesdon, UK) and chlorophyll “a” fluorescence (through the above-reported portable fluorimeter), were performed. Gas-exchange analyses were carried out on fully expanded leaves, using 9 replicates per condition to assess plants physiological behavior (PN and gs) in response to different VPD conditions. During the measurements PAR, RH and carbon dioxide concentrations were set at ambient value and the flow rate of air was set to 400 mL s^{-1}. P_N and g_s values were averaged and also used to evaluate the corresponding model prediction performances.

Figure 2. Climatic growth chamber with sensors used for the two cultivation trials (Low and High VPD) of green and red-leaf lettuce.

5.3.5 Model Structure and Parameter Identification: The results of the experiments conducted to retrieve CQY temporal pattern suggested the opportunity to redesign the MEC model by modifying the temporal variability of some key parameters, without increasing the model complexity. Indeed, fundamental model outputs like TEB and P_N, directly depend on these key parameters, which temporal patterns are influenced by the VPDs levels (nominal and off-nominal) and often result to be cultivar-specific [13]. Based on Equations (1)–(12), the three variables A, CQY, and CUE were reduced to the variable $\alpha = CUE \cdot A \cdot CQY$ and $\beta = A \cdot CQY$. The developmental stages observed for CQY were then assumed to be valid also for α and β, i.e., under the assumption of functional similarity between the variables involved, as also suggested by other studies [36–39]. The key temporal parameters, explaining the different development for α and β, were set equal to those

observed for CQY. The other parameters, identifying the minimum and maximum values for α and β, were calibrated by minimizing the root mean square error (RMSE) of the predicted DTR with respect to the corresponding observations over the entire simulation period, with the generalized reduced gradient optimization algorithm. The calibrated model was then validated against TEB measured over the entire simulation period. Simulated and modelled gs and PN were also compared on day 23 after transplanting (DAT), the day of the experiment when gas exchange analyses were performed to experimentally determine PN and gs. Calibration and validation were performed for each of the four examined scenarios: green lettuce under nominal VPD conditions (G-N); green lettuce under off-nominal conditions (G-ON); red lettuce under nominal VPD conditions (R-N); red lettuce under off-nominal conditions (R-ON).

The statistical performance indices for CQY, DTR and TEB were the linear correlation between predictions and observations (r), the average difference between prediction and observation (BIAS), the root mean square error (RMSE) and the ratio of performance to deviation (RPD). BIAS and RMSE were computed as follows:

$$BIAS = \frac{\sum_{j=1}^{N}\left(\mathrm{X}_{p,j} - \bar{X}_{\mathrm{o,j}}\right)}{N} \tag{13}$$

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{j=1}^{N}\left(\mathrm{X}_{p,j} - \bar{X}_{\mathrm{o,j}}\right)^{2}}{N}} \tag{14}$$

where N is the number of simulation days considered for the computation of the performance index, $\mathrm{X}_{p,j}$ is the prediction on the j-th day, $\bar{X}_{\mathrm{o,j}}$ is the observation day on the j-th day, averaged among the 9 sample plants for each examined scenario. Meanwhile, the RPD was calculated as the ratio of the standard deviation of the experimental data to the standard error of the model predictions. Although considered redundant by some authors [40], it has been suggested that RPD > 3 are good for screening purpose, RPD > 5 are suitable for quality control and RPD values > 8 are considered optimal for every kind of analytical application [41]. The model performance for PN and gs were assessed by the relative BIAS, i.e., the BIAS normalized by the corresponding average measured value on the 23rd DAT.

5.4 Results

5.4.1 Model Equations and Parameters: Results from fluorescence analyses on green- and red-leaf lettuce plants are reported in Figure 3. In both cultivars, top leaves always presented the highest values followed by medium leaves, while the lowest values were recorded in the bottom leaves.

Based on the analyzed experimental data, in green-leaf plants, we distinguished three stages in CQY temporal patterns:

A period of CQY monotonically increasing, starting from the initial leaf lamina development till the beginning of the maturity stage (tMi).

A period of stationary CQY, during plant maturity.

A period of CQY monotonically decreasing, during senescence.

Furthermore, for the red-pigmented lettuce, the first phase was preceded by a period of stationary CQY.

In the light of these considerations, instead of Equation (1), we designed a new mathematical relation to analytically describe the temporal evolution of CQY, consistently with the experimental data. The first stage was modelled by a linear equation, that fits the observed data between the initial time of development (tD) and maturity (tMi). The second stage was modelled by constant value, with CQY = CQY_{MAX}, from tMi till end of maturity (tM). The third stage was modelled by a linear decreasing equation, between tMi and the time tS, corresponding to the end of the senescence period, with CQY = CQYS. The third stage is not relevant from a practical perspective, since it is beyond the time of harvesting, which coincides with tM. Moreover, for the red-leaf cultivar, the initial period of stationary CQY was modelled by a constant value, with CQY = CQY_{MIN}, from the first day after transplanting till tD. This stage is only relevant for the red lettuce, with tD = 8 days, since green lettuce does not present this “adaptation” period (i.e., tD = 1). As illustrated in Figure 3, the other relevant times tMi and tM resulted to be equal to 16 and 23 for all examined scenarios and leaves.

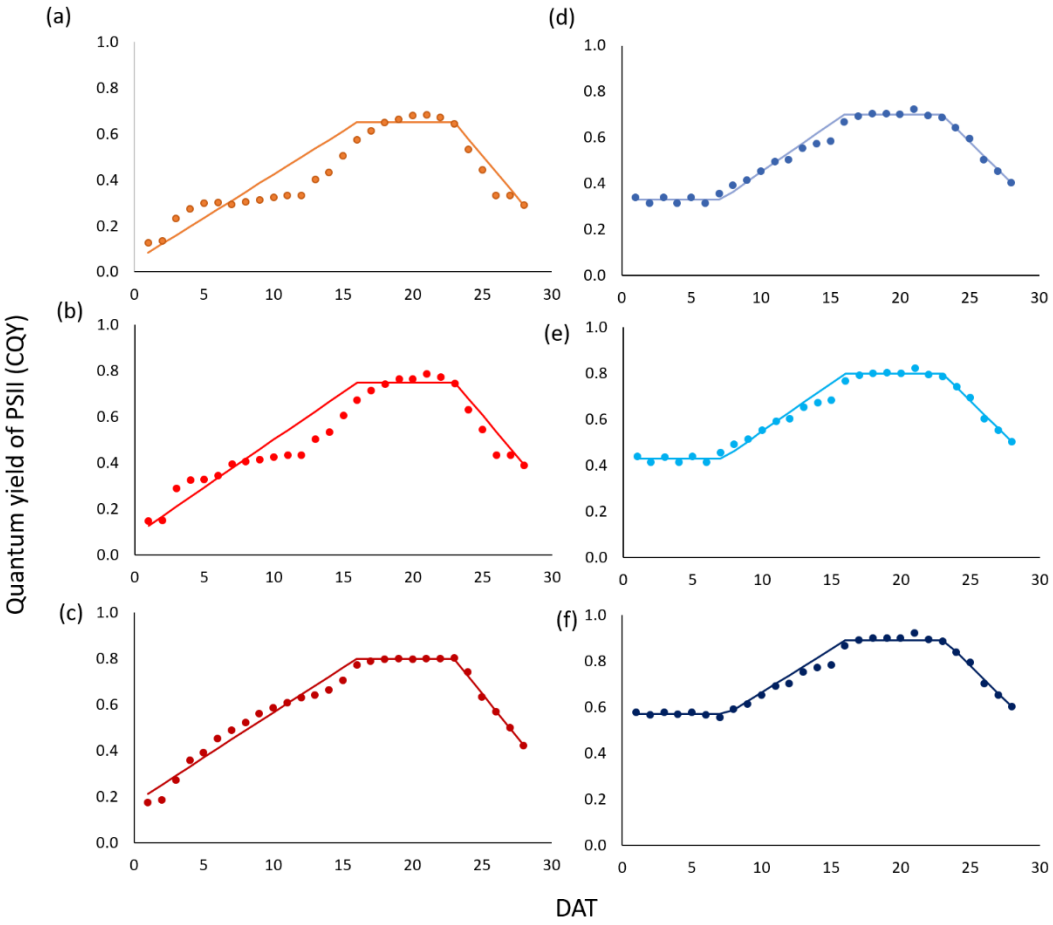

Figure 3. Profiles of CQY for top (a,d), medium (b,e) and bottom (c,f) leaves in the green- (a–c) and red-leaf (d–f) lettuce cultivars; model simulations (line) and experimental data (dots) are reported. Three different phases are identified: (1) a linear increasing; (2) a constant maturity; (3) a decreasing senescence plus an initial phase of stationary CQY for red-leaf plants. All data referred to 30 days after transplanting (DAT).

As shown in Table 1, predicted CQY BIAS was close to zero, while RMSE varied from 0.028 to 0.072 for green- and 0.020–0.021 for red-leaf lettuce. However, the linear correlation between observed and predicted CQY was high in all conditions, always being larger than 0.92. Furthermore, the values for RPD always showed values around 5, indicating the robustness and reliability of CQY prediction model.

Table 1. BIAS, root mean square error (RMSE), linear correlation (r) and ratio of performance to deviation (RPD) for CQY of PSII of green- (G) and red-leaf (R) lettuce cultivars for top-t, medium-m, and bottom-b leaves.

	BIAS	RMSE	r	RPD
G-t	−0.029	0.072	0.93	5.60
G-m	−0.027	0.065	0.96	5.61
G-b	−0.03	0.028	0.99	5.32
R-t	−0.05	0.021	0.99	5.44
R-m	−0.05	0.021	0.99	5.43
R-b	−0.05	0.020	0.99	5.23

In accordance with the assumptions stated in Section 2.5, variables α and β were modelled to change in time according to the following relation:

$$\chi = \chi_{MIN} \quad \text{for } t \le tD$$

$$\chi = \chi_{MIN} + (\chi_{MAX} - \chi_{MIN})\,(t - tD)/(tMi - tD) \quad \text{for } tD \le t < tMi$$

$$\chi = \chi_{MAX} \quad \text{for } tMi \le t < tM \quad (15)$$

where χ denotes the generic variable (either α or β), while χMIN and χMAX the corresponding minimum and maximum values. Parameters αmin, αmax, βmin and βmax were derived from experimental gas-exchange and chlorophyll "a" fluorescence measurements performed in the growth chamber experiment. These parameters were therefore differentiated for the nominal and off-nominal scenarios and for green and red lettuce cultivars, since these cultivars showed different behaviors under the same growth conditions. Afterwards, these parameters were calibrated by minimizing the RMSE with respect to the measured DTR values. Table 2 presents the complete list of model parameters, including: parameters defined by the experimental setting (E), those set according to literature data (L1 and L2), those calibrated by means of the CQY experiments (C1), and those calibrated by means of the DTR measurements during the chamber growth experiments (C2).

Table 2. Parameters used to validate the modified MEC model.

Parameter	Definition	Value	Source
H	Photoperiod (hours)	12	E
PPFD	Photosynthetic photon flux	315	E
BCF	Biomass carbon fraction	0.4	L1
XFRT	Fraction of DCG allocated to edible biomass	0.95	L1
OPF	Oxygen production fraction (mol O2) (mol C)$^{-1}$	1.08	L1
gA	Aerodynamic conductance for water vapor transfer	2.5	L2
tD	Red lettuce initial time of development (days)	8	C1
tMi	Initial time of maturity (days)	16	C1
tM	Time of harvesting (days)	23	C1
αmin	G-N	0.007	C2
	R-N	0.007	C2
	G-ON	0.003	C2
	R-ON	0.003	C2
αmax	G-N	0.017	C2
	R-N	0.021	C2
	G-ON	0.010	C2
	R-ON	0.011	C2
βmin	G-N	0	C2
	R-N	0.022	C2
	G-ON	0.049	C2
	R-ON	0.036	C2
βmax	G-N	0.045	C2
	R-N	0.028	C2
	G-ON	0.056	C2
	R-ON	0.060	C2

In the source column: C1 = calibrated with CQY experiments as illustrated in Section 2.3, C2 = calibrated with chamber growth experiment as illustrated in Section 2.4; E = experimental setting, L1 from [15], and L2 from [13].

5.4.2 Model Performance: From our results, it is evident how DTR, TEB, g_S, and P_N followed similar trends in green- and red-leaf lettuce cultivars grown under both nominal and off-nominal conditions, but with different absolute values and magnitude of changes during the cultivation cycles. Figure 4 shows the observed and predicted DTR values after model calibrations. The irregular pattern of DTR with time, is due to high sensitivity of DTR to slight perturbations in VPD levels during the diurnal hours of experiment. This sensitivity was higher under off-nominal conditions. Indeed, the model was able to reproduce the observed DTR under nominal conditions better than under off-nominal conditions. As reported in Table 3, under nominal conditions, predicted DTR BIAS was almost null, while RMSE was 0.09 L m^{-2} and 0.10 L m^{-2} for green- and red- leaf lettuce, respectively, i.e., less than 20% of the average DTR observed during the entire experiment. Under off-nominal condition, DTR BIAS was still low (max 0.04 L m^{-2} for red-leaf lettuce) but the RMSE increased to 0.21 L m−2 for the green lettuce and to 0.35 for the red lettuce. These RMSE values were still acceptable, since they are below the 30% of the observed DTR. The linear correlation between observed and predicted DTR was almost always high (larger than 0.70), except for the R-ON scenario which exhibited a linear correlation equal to 0.56. However, the RPD values varied from 5.10 to 6.40 for nominal conditions and 5.25 to 5.34 for off-nominal conditions. Being always higher than 5, the RPD overall indicates a good quality of the model predictions.

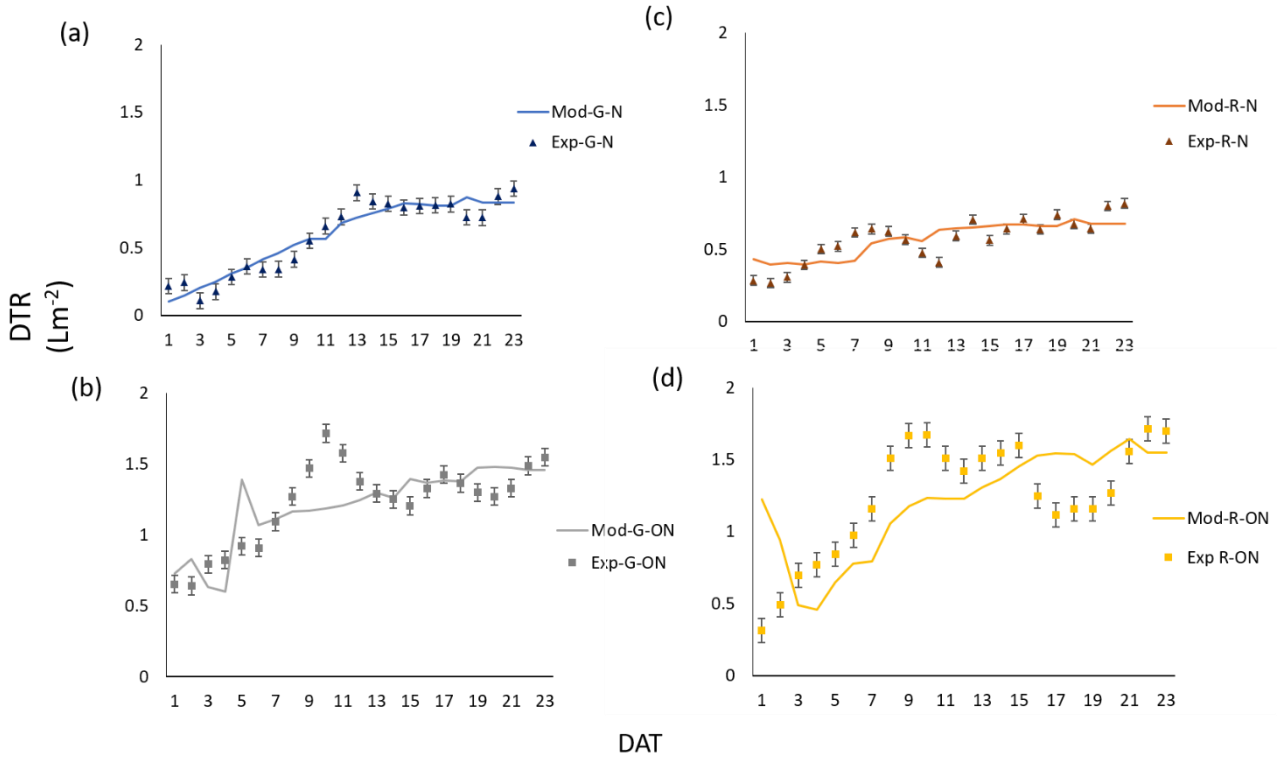

Figure 4. Profiles of DTR (Daily transpiration) for green- (G) (a,b) and red-leaf (R) (c,d) plants under nominal (N) (a,c) and off-nominal (ON) (b,d) scenarios; model simulations (line) and experimental data (dots) are reported.

Table 3. BIAS, root mean square error (RMSE), linear correlation (r) and ratio of performance to deviation (RPD) for Daily Transpiration Rate (DTR) of green- (G) and red-leaf (R) lettuce cultivars grown under nominal (N) and off-nominal (ON) conditions.

	BIAS ($L\ m^{-2}$)	**RMSE** ($L\ m^{-2}$)	**r**	**RPD**
G-N	0.001	0.09	0.95	5.10
G-ON	−0.012	0.21	0.71	5.25
R-N	0.000	0.10	0.74	6.40
R-ON	−0.040	0.35	0.56	5.34

The calibrated model was validated with the observed total edible biomass (TEB), representative of the lettuce daily growth. Total edible biomass was also influenced by VPD conditions: plants under nominal condition developed more biomass than those grown under off-nominal scenarios, both in green- and red-leaf lettuce cultivars (Figure 5). The temporal evolution of TEB was more regular and less sensitive to the perturbations of the experimental settings. The TEB predictions curves fully reflected what was expected by lettuce grown under those different VPD conditions, showing an almost linear increment in biomass till the time of harvesting (Figure 5). Furthermore, the predicted growth curves accurately simulated the lettuce biomass accumulation. In Table 4, BIAS, RMSE, r and RPD for the TEB are reported. The linear correlation coefficient (r) was always close to 1, BIAS varied in the range 0.19–1.11 under nominal conditions, and 0.12–0.40 under off-nominal conditions, whereas RMSE and RPD values varied in the range 4.46–6.87 and 4.87–5.00 under nominal conditions and 2.98–3.60 and 4.88–4.96 under off-nominal conditions, indicating a good reliability of model predictions. Overall, the results showed that the TEB prediction errors were always below 10%.

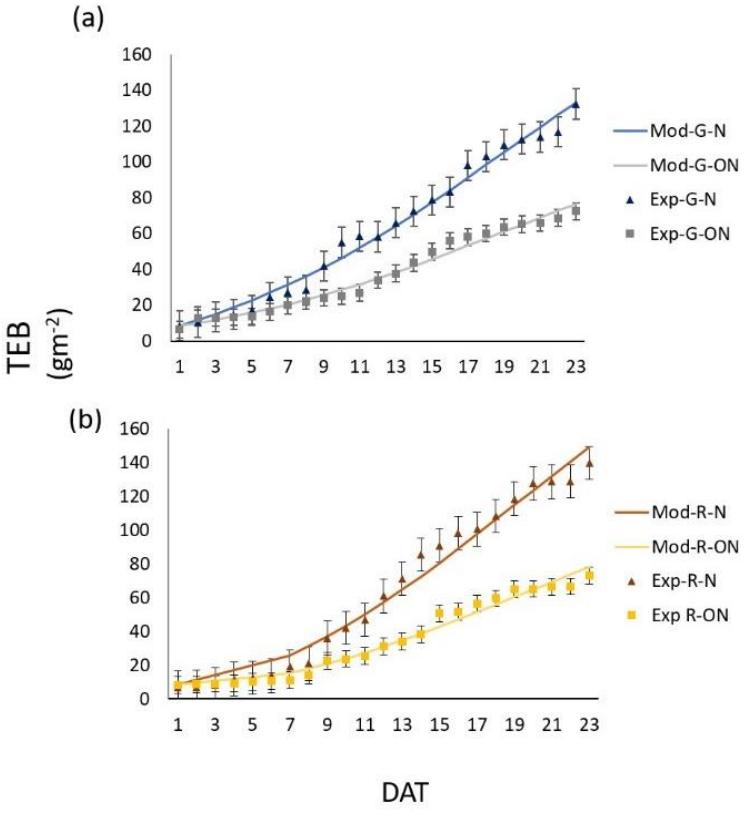

Figure 5. Theoretical (line) and experimental (dots) profiles of TEB (Total edible biomass) for green- (G) (a) and red-leaf (R) (b) plants under nominal (N) and off-nominal (ON) scenarios; model simulations (line) and experimental data (dots) are reported.

Table 4. BIAS, root mean square error (RMSE), linear correlation (r) and ratio of performance to deviation (RPD) for Total Edible Biomass (TEB) of green- (G) and red-leaf (R) lettuce cultivars grown under nominal (N) and off-nominal (ON) conditions.

	BIAS ($g\ m^{-2}$)	**RMSE** ($g\ m^{-2}$)	**r**	**RPD**
G-N	0.19	4.46	0.99	4.87
G-ON	−0.12	2.98	0.99	4.88
R-N	1.11	6.87	0.99	5.00
R-ON	0.40	3.60	0.98	4.96

Figure 6 shows the box-plot distribution of the stomatal conductance (g_S) as well as of the net photosynthesis (P_N). Stomatal conductance and P_N were significantly higher under nominal conditions compared to off-nominal, in both butterhead cultivars. Unfortunately, these data are only available for the 23rd DAT, when leaf gas-exchange analyses were actually performed. As illustrated in Table 5, g_S relative BIAS (rBIAS) was equal to 39% and −0.1% in green- and red-leaf lettuce, respectively; P_N relative BIAS (rBIAS) was instead equal to 34.1% and −10.7%. Under off nominal conditions, the predicted g_S and P_N were much less accurate: rBIAS for g_S was 68.2% and 48.6%

for green- and red-leaf lettuce respectively, while rBIAS for P_N was 75.9% and 70.9%. These larger overestimations of g_S and P_N testifies the limit of some empirical formulations (e.g., Equation (10)) adopted by the MEC model to reproduce the impact of off-nominal conditions on the stomatal conductance.

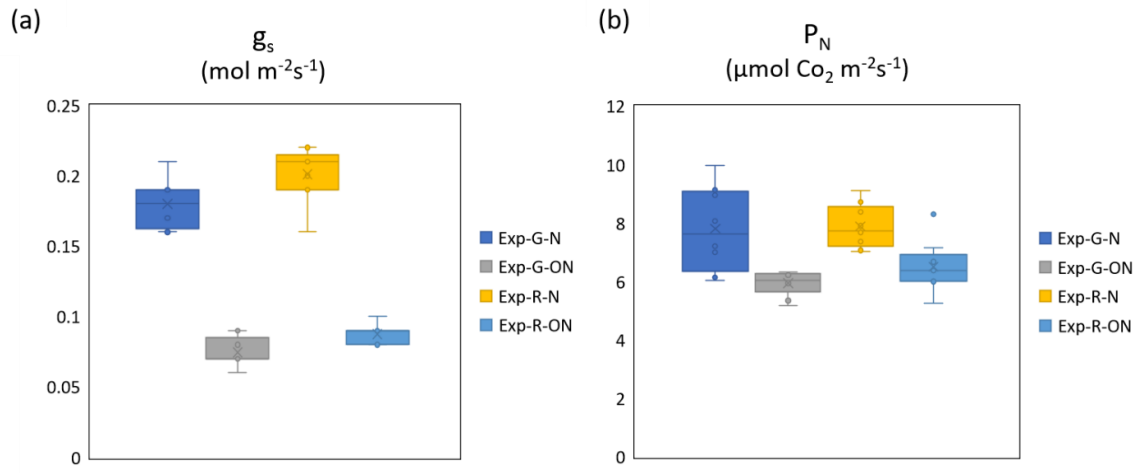

Figure 6. Box-plot distribution of g_s (Stomatal conductance) (a) and of P_N (Net-photosynthesis) (b) for green (G) and red (R) plants under nominal (N) and off-nominal (ON) scenarios. Experimental data referred to 23 DAT.

Table 5. Relative BIAS (rBIAS) and model predictions of stomatal conductance (g_s) and net photosynthesis (P_N), for green- (G) and red-leaf (R) lettuce cultivars grown under nominal (N) and off-nominal (ON) conditions. All data are referred to 23 DAT.

	DAT	rBIAS g_s	Predicted g_s (mol m^{-2} s^{-1})	rBIAS P_N	Predicted P_N (µmol CO_2 m^{-2} s^{-1})
G-N	23	39.4%	0.26	34.1%	9.80
G-ON	23	68.2%	0.13	75.9%	10.38
R-N	23	−0.1%	0.21	−10.7%	7.79
R-ON	23	48.6%	0.13	70.9%	11.12

5.5 Discussion and Conclusions: The proposed version of the MEC model, validated against experimental data on green- and red-leaf lettuce cultivars, grown under both nominal (low VPD) and off-nominal (high VPD) scenarios, proved to be reliable in predicting crop growth and transpiration rate. All previous versions of the MEC model considered CQY and CUE to be constant during plant growth [12,24,26,27]. This assumption for CQY and CUE constant behavior is not consistent with recent literature in which contrasting results on the topic are reported. For instance, in rice, Xu et al. (2019) [39] observed a decline in photosynthetic rate, dark respiration and quantum yield according to leaf aging. In the latter study, both parameters rapidly increased to a maximum around 15 days, to linearly decline as a response to plant aging. Similar findings were also reported in a study on

Rhododendron maximum L., were the decline of CQY during leaf aging was exacerbated by the exposure to high light intensity [42]. The issue is even more complex for CUE, since the carbon use efficiency has been less characterized for horticultural plants and little information exists for lettuce under different environmental conditions [31]. Although many models still rely on a fixed value of CUE set around 0.5 [43,44], this topic has been questioned and more studies contrasting this theory have been reported. For example, Winzeler et al. (1976) [45] showed that CUE of barley increased during the early phases of the growing cycle, while a decrease was reported during the second half of the cultivation period. In forest species, Amthor (2000) [46] showed that CUE is reported to vary sharply with aging, within and among different species and environmental conditions, due to different respiratory needs for growth and maintenance [37,46]. Indeed, it should be noted that CUE represents how efficiently a plant incorporates carbon into biomass and can be defined as follows:

$$CUE = DCG/PG = (PN - RD)/(PN + RN) \quad (16)$$

where RD and RN are the daylight and night respiration, respectively.

Thus, a constant CUE would indicate that plants always present a constant positive respiration rate and that changes in photosynthetic activity would determine limited variations in growth and respiration, both these scenarios being quite unlikely [36]. Many studies have found that changes in the respiration rate during the plant growth cycle are species/cultivar-specific and maintain similar trends as net photosynthesis [38,39,47]. Furthermore, the situation can be different for the same plant species under different environmental conditions. Plant growth under near-optimal conditions have been reported to have smaller changes in CUE than plant grown under limited conditions, because the relative growth rate, higher under optimal conditions, would minimize the effect of maintenance respiration coefficients on the carbon use efficiency [36]. Given these uncertainties in the determination of CUE and the observed variability for CQY, in the present study, we suggested a modified version of the MEC model structure, by aggregating the variables A, CUE, CQY into two variables (α and β) and by assuming for these two variables the same temporal patterns observed for CQY till maturity, under the assumption of physiological similarity. Thus, the number of model parameters to be calibrated was reduced to four. The calibration was performed against the DTR data, while the remaining TEB g_s and P_N experimental data were used for validation. In the present study, we proved that in lettuce a temporal pattern exists in CQY changing during plant aging. Furthermore, our findings highlighted differences between green- and red-leaf lettuce plants. More specifically, a first stage of stationary CQY was observed just for the red 'Salanova' lettuce. This period could be attributed to the time required by this red-pigmented cultivar to adapt to the new environmental condition after transplanting. Indeed, green and red Salanova lettuce, although belonging to the same species, have been proven to have a different behavior even under the same environmental conditions. For instance, under both nominal and off-nominal conditions, red-leaf lettuce exhibited highest values of net photosynthesis, stomatal conductance, as well as a higher

value of edible biomass [13]. Therefore, it was interesting to observe that all leaves of both green and red lettuce plants, notwithstanding the position inside the canopy level (top, medium, bottom), exhibited the same timing for CQY variation, except for the initial time of development (tD). The time tD in red lettuce corresponds with the time of canopy closure (tA), which was also observed to be equal to eight days for both green and red Salanova. Thus, it is feasible that the red cultivar requires an initial time interval for adapting after the transplant. However, red plants completely recover from their initial "delay" by reaching CQY_{MAX} at time tMi = 16 days, as it occurs for green-leaf lettuces, also showing the highest values of net photosynthesis, stomatal conductance and edible biomass, overall suggesting a better physiological performance [48]. In our study, the experimental data and particularly TEB, g_s and P_N assumed the highest values under nominal condition, as a result of the lower evaporative demand. Under a high VPD (dry air), the evaporative demand increases, and plants try to counteract dehydration by closing their stomata, thus decreasing photosynthetic rates and stomatal conductance [5,49,50]. Indeed, under high VPD conditions, transpiration rates were enhanced, and a plant might lose water from tissues with negative consequences on the whole plant–hydraulic system. Thus, these plants require more water to reach the field-capacity, compared to those grown under nominal-conditions. Generally, the cultivation of crops under high VPD results in yield drop-off [51] and often in quality loss [52–55], which are considered major problems for crop production. This calibrated model was able to reproduce the observed transpiration and biomass growth, under both nominal and off-nominal conditions. This capability of prediction could represent an added value for the cultivation management in CEA because it may allow the prediction of any yield loss, consequent to sudden changes in the microenvironment. Furthermore, the reliability in the model prediction concerning the daily transpiration rates could allow the set-up of a precise irrigation schedule, according to changes in the environmental condition, similarly to what is done in other agricultural sectors, by developing decision support systems (DSS) based on the optimal combination of sensors and prediction models [56]. However, the model tended to overestimate stomatal conductance and photosynthesis under off-nominal conditions. A feasible technical explanation to these overestimations is that the empirical model was initially calibrated only for nominal conditions and that a "big leaf" approach is used to calibrate the model equations. A plant, especially when grown in a sub-optimal environment, triggers a cascade of biological processes leading to the development of leaves with different anatomical traits (especially those linked with conductance and hydraulics), thus influencing plant photosynthetic performance and the whole physiological behavior [57–60]. Therefore, in this specific case, overestimation of stomatal conductance and photosynthesis, while maintaining comparable values of transpiration under off-nominal conditions compared to nominal ones, can be explained by the lack of consideration of structural plasticity (e.g., mesophyll density and vein distribution) which can differentially establish the limits of different physiological processes [5]. In light of the above results, by applying this implemented version of the model to cultivation trials, it was possible to simulate variations in

environmental parameters which can be due to sensor failure, power loss, and other problems related to environmental control. The present modified version of the MEC model can simulate crop growth, photosynthesis, and transpiration over a different range of environments and is therefore suitable to be implemented in decision support systems (DSS) for forecasting variations triggered by anomalies in the environmental control. However, the model still has a “big-leaf” approach and can therefore overestimate some processes happening at the crop morpho-physiological level. To increase the functionality of the model, a further step could be to modify the relation used to calculate gs and PN by considering morpho-physiological modifications that would affect plant gas exchanges under off-nominal conditions.

Author Contributions: All authors listed have made a substantial contribution to the work. The study was designed by C.A.M., S.D.P. and V.D.M. C.A.M. performed the experiment and analyses. G.B.C. designed the model and performed the numerical simulations. Y.R. and S.D.P. contributed to the implementation of the research. C.A.M. and V.D.M. wrote the main part of the manuscript. All authors provided critical feedback to the research, contributed to specific parts of the text, supervised the final draft and approved the definitive manuscript.

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Abbreviations

ALSs	Advanced life support systems
AMAX	Maximum fraction of PPFD absorbed by the canopy
BCF	Biomass carbon fraction
BIAS	Average difference between prediction and observation
BLSSs	Bioregenerative life support systems
CEA	Controlled environment agriculture
CGR	Crop growth rate (g m^{-2} d^{-1})
CO_2	Carbon dioxide (ppm)
CQY	Canopy quantum yield
CUE	Carbon use efficiency
DCG	Daily carbon gain (mol C m^{-2} d^{-1})
DLI	Daily light integral
DOP	Daily oxygen production (mol O_2 m^{-2} d^{-1})
DTR	Daily canopy transpiration (mm d^{-1})
g_A	Aerodynamic conductance (mol m^{-2} s^{-1})
g_c	Canopy conductance (mol m^{-2} s^{-1})
g_s	stomatal conductance (mol m^{-2} s^{-1})
H	Photoperiod
IoT	Internet of things
MEC	Energy cascade model
MWc	Carbon molecular weight (12 g mol^{-1})
MWW	Water molecular weight (18 g mol^{-1})

O_2	Oxygen (ppm)
P_{ATM}	Atmospheric pressure (kPa)
P_G	Gross photosynthesis (µmol CO2 m^{-2} s^{-1})
P_N	Net photosynthesis (µmol CO2 m^{-2} s^{-1})
PPFD	Photosynthetic photon flux density (µmol photon m^{-2} s^{-1})
PSII	Photosystem II
RD	Day respiration (µmol m^{-2} s^{-1})
RH	Relative humidity (%)
RMSE	Root mean square error
RN	Night respiration (µmol m^{-2} s^{-1})
SPAC	Soil-plant-atmosphere-continuum
T	Temperature (°C)
tA	Time at canopy closure
tD	Initial time of development
tE	Onset of edible biomass
TEB	Total edible biomass (g m^{-2})
tM	Time of harvesting
tMi	Initial time of maturity
tQ	Time of the onset of senescence
TS	Time of senescence
VPD	Vapour pressure deficit (kPa)
XRTF	Partitioning coefficient for the edible biomass
ρW	Water density (100 g L^{-1})
α	product of A, CQY and CUE
β	product of A and CQY

5.6 References

Story, D.; Kacira, M. Design and implementation of a computer vision-guided greenhouse crop diagnostics system. Mach. Vis. Appl. 2015, 26, 495–506, doi:10.1007/s00138-015-0670-5.

Guzmán, C.H.; Carrera, J.L.; Muñoz, H.A.D.; Berumen, J.; Ortiz, A.A.; Guirette, O.; Arroyo, A.; Brizuela, J.A.; Gómez, F.; Blanco, A.; et al. Implementation of Virtual Sensors for Monitoring Temperature in Greenhouses Using CFD and Control. Sensors 2018, 19, 60, doi:10.3390/s19010060.

Ruan, J.; Wang, Y.; Chan, F.T.; Hu, X.; Zhao, M.; Zhu, F.; Shi, B.; Shi, Y.; Lin, F. A Life Cycle Framework of Green IoT-Based Agriculture and Its Finance, Operation, and Management Issues. IEEE Commun. Mag. 2019, 57, 90–96, doi:10.1109/mcom.2019.1800332.

Zhang, D.; Zhang, T.; Ji, J.; Sun, Z.; Wang, Y.; Sun, Y.; Li, Q.; Zhang; Sun Estimation of Solar Radiation for Tomato Water Requirement Calculation in Chinese-Style Solar Greenhouses Based on Least Mean Squares Filter. Sensors 2019, 20, 155, doi:10.3390/s20010155.

Amitrano, C.; Arena, C.; Rouphael, Y.; De Pascale, S.; De Micco, V. Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. Ann. Appl. Boil. 2019, 175, 313–325, doi:10.1111/aab.12544.

King, B.; Wong, K. The 2017 CGIAR Inspire Challenge: Innovation strategies for digital agriculture. 2017.

Tzounis, A.; Katsoulas, N.; Bartzanas, T.; Kittas, C. Internet of Things in agriculture, recent advances and future challenges. Biosyst. Eng. 2017, 164, 31–48, doi:10.1016/j.biosystemseng.2017.09.007.

Gamon, J.A.; Peñuelas, J.; Field, C. A narrow-waveband spectral index that tracks diurnal changes in photosynthetic efficiency. Remote. Sens. Environ. 1992, 41, 35–44, doi:10.1016/0034-4257(92)90059-s.

Peñuelas, J.; Pinol, J.; Ogaya, R.; Filella, I. Estimation of plant water concentration by the reflectance water index WI (R900/R970). Int. J Remote Sens. 1997, 18, 2869–2875.

Sukhova, E.; Sukhov, V. Connection of the Photochemical Reflectance Index (PRI) with the Photosystem II Quantum Yield and Nonphotochemical Quenching Can Be Dependent on Variations of Photosynthetic Parameters among Investigated Plants: A Meta-Analysis. Remote Sens. 2018, 10, 771, doi:10.3390/rs10050771.

Jones, H.; Cavazzoni, J.; Keas, P. Crop Models for Varying Environmental Conditions. SAE Tech. Pap. Ser. 2002, 1, 2520, doi:10.4271/2002-01-2520.

Boscheri, G.; Kacira, M.; Patterson, L.; Giacomelli, G.; Sadler, P.; Furfaro, R.; Lobascio, C.; Lamantea, M.; Grizzaffi, L. Modified energy cascade model adapted for a multicrop Lunar greenhouse prototype. Adv. Space Res. 2012, 50, 941–951, doi:10.1016/j.asr.2012.05.025.

Amitrano, C.; Chirico, G.B.; De Pascale, S.; Rouphael, Y.; De Micco, V. Application of a MEC model for the irrigation control in green and red-leaved lettuce in precision indoor cultivation. In Proceedings of the 2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor) Institute of Electrical and Electronics Engineers (IEEE), Portici, Italy, 24–26 October 2019; pp. 196–201.

Kupper, P.; Sõber, J.; Sellin, A.; Lõhmus, K.; Tullus, A.; Räim, O.; Kull, O. An experimental facility for free air humidity manipulation (FAHM) can alter water flux through deciduous tree canopy. Environ Exp Bot. 2011, 72, 432-438.

Sulman, B.N.; Roman, D.T.; Yi, K.; Wang, L.; Phillips, R.P.; Novick, K.A. High atmospheric demand for water can limit forest carbon uptake and transpiration as severely as dry soil. Geophys. Res. Lett. 2016, 43, 9686–9695, doi:10.1002/2016gl069416.

Adeyemi, O.; Grove, I.; Peets, S.; Domun, Y.; Norton, T. Dynamic modelling of lettuce transpiration for water status monitoring. Comput. Electron. Agric. 2018, 155, 50–57, doi:10.1016/j.compag.2018.10.008.

Bisbis, M.B.; Gruda, N.; Blanke, M. Potential impacts of climate change on vegetable production and product quality–A review. J. Clean. Prod. 2018, 170, 1602–1620, doi:10.1016/j.jclepro.2017.09.224.

Sulman, B.N.; Roman, D.T.; Yi, K.; Wang, L.; Phillips, R.P.; Novick, K.A. High atmospheric demand for water can limit forest carbon uptake and transpiration as severely as dry soil. Geophys. Res. Lett. 2016, 43, 9686–9695, doi:10.1002/2016gl069416.

McDowell, N.G.; Allen, C.D. Darcy's law predicts widespread forest mortality under climate warming. Nat. Clim. Chang. 2015, 5, 669–672.

Franks, P.J. The Effect of Exogenous Abscisic Acid on Stomatal Development, Stomatal Mechanics, and Leaf Gas Exchange in Tradescantia virginiana. Plant Physiol. 2001, 125, 935–942, doi:10.1104/pp.125.2.935.

Yin, X.; Van L.H. Crop Systems Dynamics: An Ecophysiological Simulation Model for Genotype-by-Environment Interactions; Wageningen Academic Publishers: Wageningen, The Netherlands, 2005; doi:10.3920/978-90-8686-539-0.

Ebenhöh, O.; Houwaart, T.; Lokstein, H.; Schlede, S.; Tirok, K. A minimal mathematical model of nonphotochemical quenching of chlorophyll fluorescence. Biosystems 2011, 103, 196–204, doi:10.1016/j.biosystems.2010.10.011.

Knauer, J.; Zaehle, S.; De Kauwe, M.G.; Haverd, V.; Reichstein, M.; Sun, Y. Mesophyll conductance in land surface models: Effects on photosynthesis and transpiration. Plant J. 2019, 101, 858–873, doi:10.1111/tpj.14587.

Sukhova, E.; Khlopkov, A.; Vodeneev, V.; Sukhov, V. Simulation of a nonphotochemical quenching in plant leaf under different light intensities. Biochim. Biophys. Acta BBA Bioenerg. 2019, 1861, 148138, doi:10.1016/j.bbabio.2019.148138.

Cavazzoni, J. Using explanatory crop models to develop simple tools for Advanced Life Support system studies. Adv. Space Res. 2004, 34, 1528–1538, doi:10.1016/j.asr.2003.02.073.

Volk, T.; Bugbee, B.; Wheeler, R.M. An approach to crop modeling with the energy cascade. Life Support Biosphere Sci. Int. J. Earth Space 1995, 1, 119–127.

Jones, H.; Cavazzoni, J. Top-Level Crop Models for Advanced Life Support Analysis. SAE Tech. Pap. Ser. 2000, doi:10.4271/2000-01-2261.

Cavazzoni, J. Crop-Specific Parameters for Use in Modified Energy Cascade Models–Report to SIMA; The State University of New Jersey, New Jersey Rutgers: New Brunswick, NJ, USA, 2001.

Monje, O. Predicting Transpiration Rates of Hydroponically-Grown Plant Communities in Controlled Environments. Ph.D. Thesis, All Graduate Theses and Dissertations 6754, Utah State University, Logan, UT, USA, 1998.

Lambers, H.; Scheurwater, I.; Mata, C.; Nagel, O.W. Root respiration of fast-and slow-growing plants, as dependent on genotype and nitrogen supply: A major clue to the functioning of slow-growing plants. In Inherent Variation in Plant Growth. Physiological Mechanisms and Ecological Consequences; Backhuys Publishers: Kerkwerve, The Netherlands, 1998; pp. 139–157.

Logan, B.A.; Demmig, A.B.; Adams, W.W., III; Grace, S.C. Antioxidants and xanthophyll cycle-dependent energy dissipation in Cucurbita pepo L. and Vinca major L. acclimated to four growth PPFDs in the field. J. Exp. Bot. 1998, 49, 1869–1879.

Frantz, J.M.; Bugbee, B. Acclimation of Plant Populations to Shade: Photosynthesis, Respiration, and Carbon Use Efficiency. J. Am. Soc. Hortic. Sci. 2005, 130, 918–927, doi:10.21273/jashs.130.6.918.

Frantz, J.M.; Ritchie, G.; Cometti, N.N.; Robinson, J.; Bugbee, B. Exploring the Limits of Crop Productivity: Beyond the Limits of Tipburn in Lettuce. J. Am. Soc. Hortic. Sci. 2004, 129, 331–338, doi:10.21273/jashs.129.3.0331.

Klassen, S.P.; Ritchie, G.; Frantz, J.M.; Pinnock, D.; Bugbee, B.; Schepers, J.; VanToai, T. Real-Time Imaging of Ground Cover: Relationships with Radiation Capture, Canopy Photosynthesis, and Daily Growth Rate. In Nitrification Inhibitors-Potentials and Limitations; American Society of Agronomy and Soil Science Society of America: Madison, WI, USA, 2015; pp. 1–14, doi:10.2134/asaspecpub66.c1.

Maxwell, K.; Johnson, G. Chlorophyll fluorescence—A practical guide. J. Exp. Bot. 2000, 51, 659–668, doi:10.1093/jexbot/51.345.659.

Genty, B.; Briantais, J.-M.; Baker, N.R. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta BBA Gen. Subj. 1989, 990, 87–92, doi:10.1016/s0304-4165(89)80016-9.

Van Iersel, M.W. Carbon use efficiency depends on growth respiration, maintenance respiration, and relative growth rate. A case study with lettuce. Plant Cell Environ. 2003, 26, 1441–1449, doi:10.1046/j.0016-8025.2003.01067.x.

DeLucia, E.H.; Drake, J.E.; Thomas, R.B.; Gonzalez-Meler, M. Forest carbon use efficiency: Is respiration a constant fraction of gross primary production? Glob. Chang. Boil. 2007, 13, 1157–1167, doi:10.1111/j.1365-2486.2007.01365.x.

Zufferey, V. Leaf respiration in grapevine (Vitis vinifera ‘Chasselas’) in relation to environmental and plant factors. Vitis 2016, 55, 65–72.

Xu, J.; Lv, Y.; Liu, X.; Wei, Q.; Qi, Z.; Yang, S.; Liao, L. A general non-rectangular hyperbola equation for photosynthetic light response curve of rice at various leaf ages. Sci. Rep. 2019, 9, 9909, doi:10.1038/s41598-019-46248-y.

Minasny, B.; McBratney, A. Why you don’t need to use RPD. Pedometron 2013, 33, 14–15.

Yi, J.; Sun, Y.; Zhu, Z.; Liu, N.; Lu, J. Near-infrared reflectance spectroscopy for the prediction of chemical composition in walnut kernel. Int. J. Food Prop. 2016, 20, 1633–1642, doi:10.1080/10942912.2016.1217006.

Nilsen, E.T.; Stetler, D.A.; Gassman, C.A. Influence of Age and Microclimate on the Photochemistry of Rhododendron Maximum Leaves ii. Chloroplast Structure and Photosynthetic Light Response. Am. J. Bot. 1988, 75, 1526–1534, doi:10.1002/j.1537-2197.1988.tb11226.x.

Medlyn, B.E.; Dewar, R.C. Comment on the article by R. H.; Waring, J.J. Landsberg and M. Williams relating net primar production to gross primary production. Tree Physiol. 1999, 19, 137–138, doi:10.1093/treephys/19.2.137.

Kerkhoff, A.J.; Enquist, B.J.; Elser, J.; Fagan, W.F. Plant allometry, stoichiometry and the temperature-dependence of primary productivity. Glob. Ecol. Biogeogr. 2005, 14, 585–598, doi:10.1111/j.1466-822x.2005.00187.x.

Winzeler, H.; Hunt, L.A.; Mahon, J.D. Ontogenetic Changes in Respiration and Photosynthesis in a Uniculm Barley 1. Crop. Sci. 1976, 16, 786–790, doi:10.2135/cropsci1976.0011183x001600060012x.

Amthor, J. The McCree–de Wit–Penning de Vries–Thornley Respiration Paradigms: 30 Years Later. Ann. Bot. 2000, 86, 1–20, doi:10.1006/anbo.2000.1175.

Hover, J.M.; Gustafson, F.G. Rate of Respiration as Related to Age. J. Gen. Physiol. 1926, 10, 33–39, doi:10.1085/jgp.10.1.33.

El-Nakhel, C.; Giordano, M.; Pannico, A.; Carillo, P.; Fusco, G.M.; De Pascale, S.; Rouphael, Y.; Nakhel, E.-; Pascale Cultivar-Specific Performance and Qualitative Descriptors for Butterhead Salanova Lettuce Produced in Closed Soilless Cultivation as a Candidate Salad Crop for Human Life Support in Space. Life 2019, 9, 61, doi:10.3390/life9030061.

Ryan, A.C.; Dodd, I.C.; Rothwell, S.; Jones, R.; Tardieu, F.; Draye, X.; Davies, W.J. Gravimetric phenotyping of whole plant transpiration responses to atmospheric vapour pressure deficit identifies genotypic variation in water use efficiency. Plant Sci. 2016, 251, 101–109, doi:10.1016/j.plantsci.2016.05.018.

Jiao, X.-C.; Song, X.-M.; Zhang, D.-L.; Du, Q.-J.; Li, J. Coordination between vapor pressure deficit and CO2 on the regulation of photosynthesis and productivity in greenhouse tomato production. Sci. Rep. 2019, 9, 8700, doi:10.1038/s41598-019-45232-w.

Zhang, D.; Zhang, Z.; Li, J.; Chang, Y.; Du, Q.; Pan, T. Regulation of Vapor Pressure Deficit by Greenhouse Micro-Fog Systems Improved Growth and Productivity of Tomato via Enhancing Photosynthesis during Summer Season. PLoS ONE 2015, 10, e0133919, doi:10.1371/journal.pone.0133919.

Gent, M.P. Effect of Degree and Duration of Shade on Quality of Greenhouse Tomato. HortScience 2007, 42, 514–520, doi:10.21273/hortsci.42.3.514.

Xu, B.; Chang, S.K.C. A Comparative Study on Phenolic Profiles and Antioxidant Activities of Legumes as Affected by Extraction Solvents. J. Food Sci. 2007, 72, S159–S166, doi:10.1111/j.1750-3841.2006.00260.x.

Leyva, R.; Aguilar, C.C.; Sánchez-Rodŕiguez, E.; Romero-Gámez, M.; Soriano, T. Cooling systems in screenhouses: Effect on microclimate, productivity and plant response in a tomato crop. Biosyst. Eng. 2015, 129, 100–111, doi:10.1016/j.biosystemseng.2014.09.018.

Rouphael, Y.; Kyriacou, M.C.; Petropoulos, S.A.; De Pascale, S.; Colla, G. Improving vegetable quality in controlled environments. Sci. Hortic. 2018, 234, 275–289, doi:10.1016/j.scienta.2018.02.033.

Chirico, G.B.; Pelosi, A.; De Michele, C.; Bolognesi, S.F.; D'Urso, G. Forecasting potential evapotranspiration by combining numerical weather predictions and visible and near-infrared satellite images: An application in southern Italy. J. Agric. Sci. 2018, 156, 702–710, doi:10.1017/s0021859618000084.

Murphy, M.C.; Jordan, G.J.; Brodribb, T.J. Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. Plant Cell Environ. 2013, 37, 124–131, doi:10.1111/pce.12136.

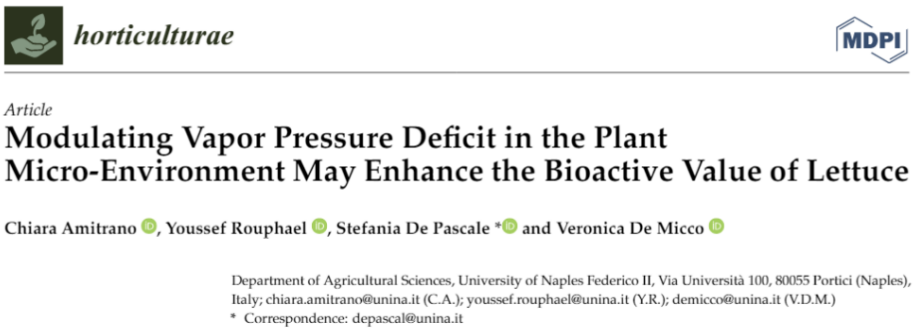
Buckley, T.N.; John, G.P.; Scoffoni, C.; Sack, L. How Does Leaf Anatomy Influence Water Transport outside the Xylem? Plant Physiol. 2015, 168, 1616–1635, doi:10.1104/pp.15.00731.

Sack, L.; Scoffoni, C.; Johnson, D.M.; Buckley, T.N.; Brodribb, T.J. The Anatomical Determinants of Leaf Hydraulic Function; Springer Science and Business Media LLC: Berlin, Germany, 2015; pp. 255–271.

Du, Q.; Liu, T.; Jiao, X.; Song, X.; Zhang, J.; Li, J. Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. J. Exp. Bot. 2019, 70, 4949–4962, doi:10.1093/jxb/erz238.

Chapter 6

Modulating Vapor Pressure Deficit in the Plant Micro-Environment May Enhance The Bioactive Value of Lettuce



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6.1 Abstract: Growing demand for horticultural products of accentuated sensory, nutritional, and functional quality traits has been driven by the turn observed in affluent societies toward a healthy and sustainable lifestyle relying principally on plant-based food. Growing plants under protected cultivation facilitates more precise and efficient modulation of the plant microenvironment, which is essential for improving vegetable quality. Among the environmental parameters that have been researched for optimization over the past, air relative humidity has always been in the background and it is still unclear if and how it can be modulated to improve plants' quality. In this respect, two differentially pigmented (green and red) Salanova® cultivars (*Lactuca sativa* L. var. *capitata*) were grown under two different Vapor Pressure Deficits (VPDs; 0.69 and 1.76 kPa) in a controlled environment chamber in order to appraise possible changes in mineral and phytochemical composition and in antioxidant capacity. Growth and morpho-physiological parameters were also analyzed to better understand lettuce development and acclimation mechanisms under these two VPD regimes. Results showed that even though Salanova plants grown at low VPD (0.69 kPa) increased their biomass, area, number of leaves and enhanced Fv/Fm ratio, plants at high VPD increased the levels of phytochemicals, especially in the red cultivar. Based on these results, we have discussed the role of high VPD facilitated by controlled environment agriculture as a mild stress aimed to enhance the quality of leafy greens.

Keywords: Air humidity (RH); *Lactuca sativa* L. var. *capitata*; Controlled Environment Agriculture (CEA); bioactive compounds; leaf gas exchange; minerals profile; genetic material

6.2 Introduction: Air humidity (RH), and more specifically the Vapor Pressure Deficit (VPD), is one of the most important microclimate factors affecting plant transpiration rate in Controlled Environment Agriculture (CEA). Consequently, VPD affects all physiological and biochemical processes associated with the transpiration, such as: water balance, cooling, gas-exchange, and ion translocation, thus affecting plant growth and productivity [1,2]. It is well established that plants grown under a reduced VPD (high RH) enhance carbon gain by opening their stomata, usually improving at the same time, dry matter production [3]. Moreover, plants enhance growth under high RH levels, as long as the transpiration rate is still enough to support the uptake and distribution of essential macronutrients (Ca^{2+}, Mg^{2+}, K^{+}) and phytohormones (auxin, cytokinin) [4]. Furthermore, in lettuce, high air humidity, especially during night, appears to prevent Ca^{2+} deficiency, a common physiological disorder known as tipburn, which negatively affects the nutritional quality and marketability of the product [5]. Under high VPD levels (low RH), plants try to avoid dehydration and water loss by closing their stomata, which negatively affect photosynthetic efficiency, thus determining a major reduction in plant growth and yield [6,7]. Nevertheless, high VPD in indoor cultivation has proven to enhance vegetable quality, for example increasing ascorbate, lycopene, β-carotene, rutin, and caffeic acid concentrations in greenhouse tomato, often connected to high irradiance during sunny hours when greenhouses are subjected to high VPD [6,8]. In greenhouse cherry tomato cv. *Naomi*, Rosales et al. [9] found an increment in ascorbic acid synthesis in plants grown under high VPD levels (2-3 kPa), probably due to the occurrence of oxidative stress [10,11]. This is consistent with other “controlled” stress like drought or salinity that, if moderately applied to plants, can increase product quality [12,13]. For instance, Favati et al. [14] found improved quality of tomato fruit subjected to deficit irrigation, in particular due to the enhancement of ascorbic acid and β-carotene. Moreover, controlled drought stress increased the levels of carotenoids in edible organs of pepper and carrot as well as the levels of sugars in tomato and cucumber fruits [12]. Notwithstanding the positive outcomes of recent research, little is known about the effects of VPD modulation on leafy greens nutritional and functional quality. Indeed, humidity is one of the most difficult environmental factors to control in CEA (instrumentally and economically), thus often being neglected by growers [6]. However, over the past two decades there has been a growing demand for high quality horticultural products [13,15], with consumers always looking for fresh and high nutritional food [16]. Bioactive compounds, also known as phytochemicals, are already present in leafy green vegetables and especially in lettuce, where red-leaved cultivars present very high content of vitamin C, polyphenols and antioxidant activities compared to their green counterparts [17,18]. Phytochemicals-rich-food are in great demand due to their ability to reduce the risk of cardiovascular diseases, some forms of cancer, and stimulate cognitive health against age-related problems [19]. Even though the genetic material (i.e., genotype) is the principal factor in determining how much phytochemicals a plant will accumulate during its life cycle, the influence of microclimatic factors affecting greenhouse and indoor growing modules vegetables, cannot be neglected. Several

scientific papers have been published regarding genotype, and microclimate (e.g., air and root zone temperature, light quantity, and quality) effects on the quality of controlled environments vegetables [20,21], whereas the effects of VPD on leafy greens quality is still poorly explored.

In light of the foregoing, the aim of the current study was to assess how the modulation of VPD influences the nutritional and functional quality of green and red-leaved lettuce (*Lactuca sativa* L. var. *capitata*). For this purpose, a growth chamber experiment under controlled climatic conditions was conducted, growing plants under two different VPDs (0.6 kPa and 1.7 kPa), considered respectively low- and high- VPD. The development of lettuces in terms of anatomical structure of the leaf lamina, plant growth, as well as some plant physiological responses (Fv/Fm ratio and chlorophyll content) were examined. Treatments were compared in terms of leaf colorimetry coordinates, antioxidant activity, minerals profile, polyphenols, and total ascorbic acid content.

6.3 Materials and Methods

6.3.1 Experimental Design, Lettuce Genotypes, and Controlled Growing Conditions: The experiment was carried out on two butterhead Salanova® lettuce cultivars (*Lactuca sativa* L. var. *capitata*), with green and red leaves. Two-week old transplants were purchased from a local provider and grown at the Department of Agricultural Sciences (University of Naples Federico II, Italy) in two consecutive cycles, in a growth-chamber (KBP-6395F, Termaks, Bergen, Norwey) equipped with a Light-Emitting Diode (LED) panel unit (K5 Series XL750, Kind LED, Santa Rosa, CA, USA), with an emission wavelength range of 400–700 nm. The two cultivation cycles were identical in terms of agricultural practices and microclimatic conditions (light intensity, quality, photoperiod, air, and zone temperature), except for the VPD levels. More specifically, the first cycle was performed under an average VPD of 0.69 kPa and the second under a VPD of 1.76 kPa. The two VPDs were achieved keeping air temperature (T) at 24 ± 1° C and changing the RH accordingly. RH and T were controlled by the growth chamber and monitored inside the chamber by means of mini-sensors (Testo 174 H), equipped with a data-logger which collected data every 15 min. In each cycle, 9 green and 9 red Salanova lettuces were transplanted into plastic trays (14 × 19 × 6 cm: W × L × D) on peat:perlite substrate (1:1 v/v). Daily rotation of the trays was performed to ensure homogenous light and humidity across the shelf surface. Plants were grown for 23 days under a red-green-blue (RGB) light of 315 µmol m^{-2} s^{-1}, 12 h photoperiod (13.6 Daily Light Integral; DLI). All plants were fertigated to field capacity with a modified Hoagland solution (8.2 mM N-NO3−, 2.0 mM S, 2.7 mM K+, 5.8 mM Ca^{2+},1.4 mM Mg^{2+}, 1.0 mM NH^{4+}, 15.0 µM Fe, 9.0 µM Mn, 0.3µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo), resulting in an electrical conductivity of 1.4 dS m^{-1} and a pH of 5.8.

6.3.2 Plant Growth Parameters, Biomass Production, and Leaf Colorimetry: Harvesting of all experimental units was performed 23 days after transplanting (DAT). Before harvesting, each plant was photographed from the top and digital images were used to assess plant total area (PA) through ImageJ 1.45 software (U.S. National Institutes of Health, Bethesda, MD, USA). The number of leaves

(LN) was counted for all plants, which were then weighted to determine the above-ground fresh biomass (FB). For the dry biomass (DB) determination, samples of fresh leaf tissues (about 15 g per plant) were oven-dried at 70 °C for 3 days, until they reached a constant weight. On the harvesting day, leaf color was measured on the upper part of three representative leaves per plant, using a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd., Osaka, Japan). The meter was calibrated with the standard white plate before measurements. Leaf chromaticity was performed following the Commission Internationale de l'Eclairage and expressed as: lightness (L*), b* (+b* yellowness) used to calculate chroma (C* = (a*2 + b*2)1/2) and Hue angle (H° = arctan (b*/a*)).

6.3.3 Anatomical Analyses of Leaves: At 23 DAT, one complete life-span leaf per plant was collected from the median part of the canopy and promptly stored in F.A.A. fixative solution (40% formaldehyde, glacial acetic acid, 50% ethanol, 5: 5: 90 by volume). Each leaf was dissected to remove the apical and basal portions, while keeping the median region of the lamina. 5 × 5 mm portions of the leaf lamina were dehydrated in an ethanol series (50, 70, and 95%) and embedded in the JB4 acrylic resin (Polysciences, USA). Thin cross sections (5 µm thick) were cut by means of a rotary microtome, stained with 0.025% toluidine blue [22] and mounted with mineral oil for microscopy. Sections were analyzed under the BX60 transmitted light microscope (Olympus, Germany), and digital images were collected and analyzed through the Olympus AnalySIS software (AnalySIS 3.2, Olympus). The following functional anatomical traits were quantified: upper and lower epidermis thickness (UET; LET) (µm); palisade parenchyma thickness (PT) (µm); spongy parenchyma thickness (ST) (µm); total leaf lamina thickness (LT) (µm) and percentage of intercellular spaces (IS) (%). All the thickness measurements were taken in 6 positions along the lamina, avoiding veins and damaged areas. The IS was measured as percentage of area occupied by intercellular spaces over a given surface of parenchyma, in three regions of the leaf lamina, as reported in [23].

6.3.4 Mineral Composition in Leaf Tissue: Dried material was used for the evaluation of mineral leaf composition in terms of cations (K^+, Mg^{2+}, Ca^{2+}, Na^{2+}), anions (NO_3^-, SO_4^{2-}, PO_4^{3-}) and acids (malate, tartrate, citrate, isocitrate). Dried leaves (0.25 g per replicate) were suspended in 50 mL of ultrapure water (Milli-Q, Merk Millipore, Darmstadt, Germany), frozen and then shook for 10 min in a water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80 °C. The mixture was then centrifuged at 6000 rpm for 10 min (R-10M, Remi Elektrotechnik, India) and the supernatant, was filtered to 0.45 µm, and stored at −20 °C until analysis. Anions and cations were separated and quantified by ion chromatography equipped with a conductivity detection (ICP 3000 Dionex, Thermo fisher Scientific Inc., MA, USA).

6.3.5 Extraction and Quantification of Total Ascorbic Acid, Polyphenols, Lipophylic, and Hydrophilic Antioxidant Activities: All phytochemical analyses were performed on 9 green and 9 red Salanova lettuces (one leaf per replicate). Total ascorbic acid (TAA) was assessed

spectrophotometrically based on the protocol of Kampfenkel, Montagu, and Inze [24]. The phenolic content (PH) was determined using the Folin-Cicolteau procedure [25] using gallic acid (Sigma Aldrich Inc, St Louis, MO, USA) as a standard. The hydrophilic antioxidant activity (HAA) was measured using N, N-dimethyl-p-phenylenediamine (DMPD) method [26], whereas the lipophilic antioxidant activity (LAA) was measured following the ABTS method [27].

6.3.6 Soil Plant Analysis Development Index and Chlorophyll a Fluorescence Emission: At 12 and 23 DAT (middle and final point of experiments), the Soil Plant Analysis Development (SPAD) index was measured on 9 fully expanded leaves per condition by means of a portable chlorophyll meter SPAD-502 (Konica Minolta, Japan), avoiding major veins, leaflet margins, and damaged areas. On the same dates, measurements of leaf chlorophyll "a" fluorescence emission, were performed on the same leaves to calculate the maximum quantum efficiency of PSII photochemistry (Fv/Fm) on 30′ dark-adapted leaves, with a portable fluorometer, equipped with a light sensor (ADC BioScientific Ltd., Hoddesdon, United Kingdom).

6.3.7 Statistics: Data were initially subjected to a two-way analysis of variance (ANOVA). Interactions between cultivar and VPD (C x VPD) were further addressed through specific one-way ANOVA and treatment means were compared using Duncan's multiple range test performed at $p \leq 0.05$ using the SPSS 20 software package (IBM, Armonk, NY, USA). Moreover, multivariate analysis was used to perform an agglomerative hierarchical cluster analysis (HCA) of the data sets. For HCA, the paired group (UPGMA) and Euclidean distances were used for clustering. Results of HCA were displayed as a tree-shaped dendrogram, where the horizontal distance between clusters represented data dissimilarity, and a heat-map, through the web tool (Clustvis; https://biit.cs.ut.ee/clustvis/).

6.4 Results

6.4.1 Plant Growth Parameters, Biomass Production, and Leaf Colorimetry: As presented in Table 1, cultivar and VPD had a significant effect on Salanova plant area (PA), leaves number (LN), fresh biomass (FB), dry biomass (DB), as main factors and in interaction. More specifically, red cultivar (R) presented higher values of all growth parameters (PA, FB, DB) enhanced by 10, 11, and 4%, with an exception made for LN. At the same time, 0.69 kPa increased all growth parameters (PA, LN, FB, FB) by 17, 12, 15, and 47%, always showing highest values in red cultivar (0.69 R), followed by 0.69 G, 1.76 R, and 1.76 G. Leaf colorimetry parameters were also influenced by C and VPD as main factors and by their interaction (C × VPD). In this case, b*, leaf brightness (L*) and Chroma were higher in G cultivar by 91, 54, and 23% and Hue in R cultivar (82%). Whereas 0.69 kPa elicited increments in b* Chroma and Hue (41, 83 and 23%), while 1.76 kPa enhanced L* (32%). Concerning the interaction, the three colorimetry coordinates had a completely different trends among treatment, with increments in L* in 1.76 G followed by 0.69 G, 1.76 R, and 0.69 G. b* and

chroma values incremented in 0.69 G followed by 1.76 G, 0.6 R, and 1.76 R; whereas Hue values increased in 0.69 R followed by 1.76 R and 0.69 G.

Table 1. Growth analyses consisting of plant area (PA), leaf number (LN), fresh biomass (FB), dry Biomass (DB), and leaf colorimetry coordinates (L*, Chroma and Hue angle) in green (G) and red (R) lettuce plants grown under the two Vapor Pressure Deficit (VPD) levels (0.69 and 1.76 kPa).

	PA (cm^2 $Plant^{-1}$)	LN (no. $Plant^{-1}$)	FB (g $Plant^{-1}$)	DB (g Plant−1)	b *	L *	Chroma	Hue
Cultivar								
G	196 ± 1.02 b	51.9 ± 0.72 a	33.2 ± 0.50 b	3.60 ± 0.03 a	40.3 ± 1.21a	49.4 ± 0.46 a	28.1 ± 0.44 a	107 ± 4.46 b
R	214 ± 0.37 a	49.3 ± 0.81 b	36.8 ± 0.29 a	3.75 ± 0.02 a	3.62 ± 0.78 b	22.7 ± 0.47 b	21.5 ± 0.45 b	195 ± 3.34 a
VPD								
0.69 kPa	224 ± 1.01 a	53.9 ± 0.76 a	37.9 ± 0.48 a	4.80 ± 0.06 a	28.8 ± 1.16 a	34.0 ± 0.33 b	42.3 ± 0.62 a	171 ± 3.15 a
1.76 kPa	186 ± 0.33 b	47.3 ± 0.77 b	32.1 ± 0.31 b	2.55 ± 0.03 b	17.1 ± 0.88 b	38.1 ± 0.34 a	7.24 ± 0.66 b	132 ± 2.02 b
Int.								
0.69 G	211 ± 0.17 b	53.4 ± 0.24 a	35.6 ± 0.17 b	4.75 ± 0.02 a	44.5 ± 0.81 a	48.5 ± 0.42 b	46.2 ± 0.74 a	106 ± 0.44 c
0.69 R	237 ± 0.03 a	54.4 ± 0.40 a	40.4 ± 0.22 a	4.85 ± 0.03 a	9.25 ± 0.80 c	19.5 ± 0.31 d	9.84 ± 0.73 c	236 ± 0.88 a
1.76 G	180 ± 0.20 d	50.4 ± 0.25 b	30.8± 0.23 d	2.46 ± 0.01 b	36.2 ± 0.70 b	50.3 ± 0.77 a	38.3 ± 0.68 b	109 ± 0.28 c
1.76 R	192 ± 0.21 c	44.2 ± 0.49 c	33.4 ± 0.07 c	2.66 ± 0.01 b	-2.00 ± 0.16 d	25.9 ± 0.13 c	4.60 ± 0.11 d	154 ± 2.18 b
Sig.								
C	***	*	***	NS	***	***	***	***
VPD	***	***	***	**	***	***	***	***
C × VPD	***	***	***	***	*	***	*	***

All data are expressed as mean ± standard error. ***, **, *, NS refer to p ≤ 0.001, 0.01, 0.05 and Non-significant, respectively. Lower case letters indicate the significant differences of the interaction.

6.4.2 Morpho-Anatomical Analyses: As shown in Figure 1 the morpho-anatomical structure of the leaf lamina was not different among the four different combinations of cultivar and VPD. Cultivar and VPD alone showed no significant differences on Salanova lettuces morpho-anatomical parameters, with an exception made for LET where G cultivar showed an increment of 13% (Table 2). However, their interaction (C × VPD) elicited a significant difference in the upper and lower epidermis thickness (UET and LET). More specifically, UET was the highest in 0.69 G and the lowest in 1.76 G, while no significant differences were found in R cultivars between 0.69 and 1.76 kPa. Differently, LET was the highest in 0.69 R, followed by 0.69 G and was the lowest in 1.76 with no significant differences between G and R.

Table 2. Morpho-anatomical analyses consisting of upper epidermis thickness (UET), lower epidermis thickness (LET), palisade thickness (PT), spongy thickness (ST), lamina thickness (LT) and percentage of intercellular spaces (IS) in green (G) and red (R) lettuce plants grown under the two VPD levels (0.69 and 1.76 kPa).

	UET (µm)	LET (µm)	PT (µm)	ST (µm)	LT (µm)	IS (%)
Cultivar						
G	23.4 ± 0.66 a	16.7 ± 1.03 a	97.3 ± 4.69 a	148 ± 6.29 a	287 ± 8.86 a	46.2 ± 2.87 a
R	22.4 ± 1.05 a	14.8 ± 0.65 b	94.2 ± 3.71 a	149 ± 11.8 a	282 ± 11.3 a	45.3 ± 2.05 a
VPD						
0.69 kPa	22.9 ± 0.64 a	15.4 ± 0.79 a	95.5 ± 5.06 a	146 ± 7.57 a	281 ± 8.76 a	45.6 ± 1.89 a
1.76 kPa	22.8 ± 1.11 a	16.1 ± 0.74 a	95.9 ± 5.31 a	151 ± 9.15 a	287 ± 13.4 a	46.8 ± 2.50 a
Int.						
0.69 G	24.2 ± 0.63 a	16.2 ± 0.79 ab	94.5 ± 3.10 a	144 ± 3.56 a	280 ± 4.85 a	44.2 ± 1.96 a
0.69 R	22.7 ± 0.64 ab	17.2 ± 0.79 a	99.8 ± 3.93 a	152 ± 8.02 a	293 ± 11.73 a	44.9 ± 1.33 a
1.76 G	21.7 ± 0.70 b	14.6 ± 0.48 b	96.5 ± 3.19 a	147 ± 5.47 a	281 ± 8.02 a	48.1 ± 1.83 a
1.76 R	23.1 ± 0.82 ab	15.1 ± 0.52 b	92.1 ± 4.24 a	151 ± 7.56 a	282 ± 10.82 a	45.6 ± 1.35 a
Sig.						
C	NS	*	NS	NS	NS	NS
VPD	NS	NS	NS	NS	NS	NS
C × VPD	*	*	NS	NS	NS	NS

All data are expressed as mean ± standard error. ***, **, *, NS refer to p ≤ 0.001, 0.01, 0.05, and Non-significant, respectively. Lower case letters indicate the significant differences of the interaction.

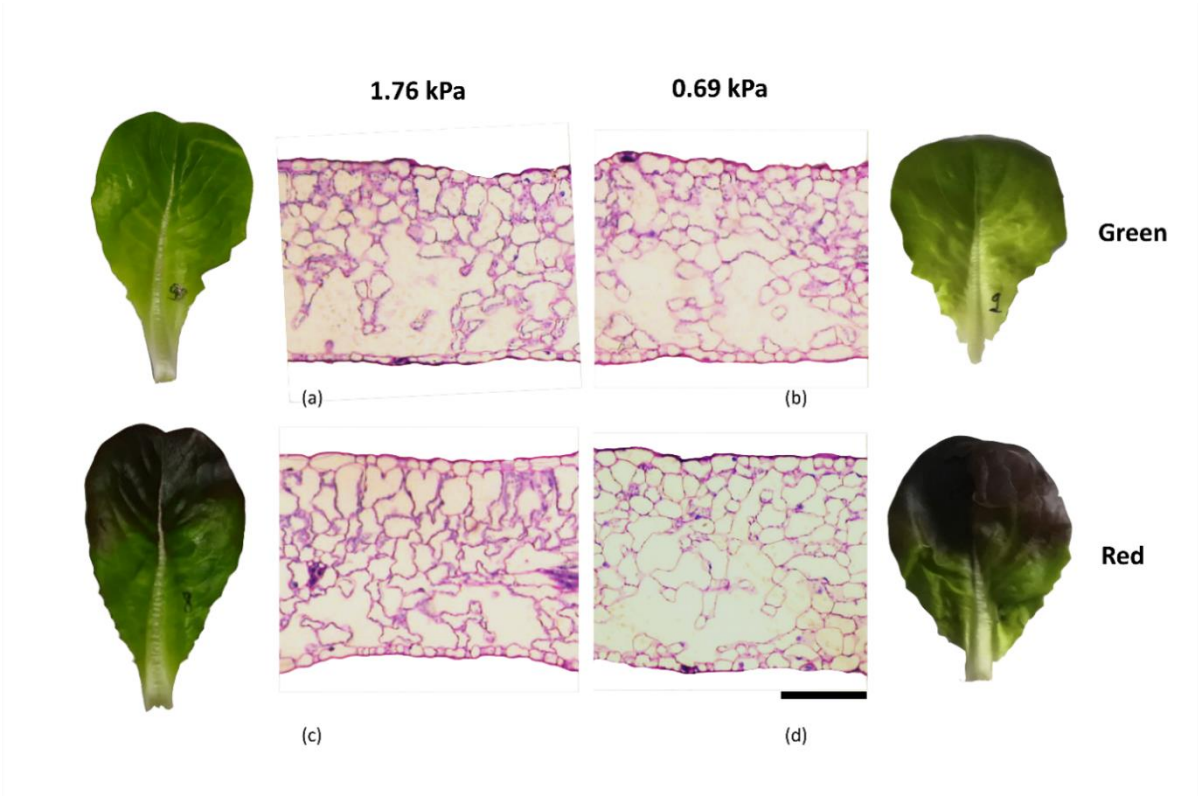

Figure 1. Light microscopy views of cross-sections of green (a,b) and red (c,d) leaf lamina of lettuces grown under the two VPD levels 1.76 (a,c) and 0.69 (b,d). Bar = 100 µm.

6.4.3 Mineral Composition: Results from ion chromatography are showed in Table 3. Mineral content varied among treatments. More specifically, red cultivar enhanced the content of NO_3^-, $Ca^{2+}$and malate by 22, 24, and 50%, whereas green cultivar enhanced the content of K^+, tartrate,

and isocitrate by 20, 45, and 26%. No significant differences among cultivar were detected in the other minerals and organic acids. Differently 0.69 kPa enhanced the content of PO_4^{3-}, Ca^{2+}, malate and tartrate by 24, 19, 53, and 25%, whereas 1.76 kPa enhanced the content of NO_3^-, SO_4^{2-}, and K^+ by 9, 47, and 46%. No significant differences between 0.69 and 1.76 kPa were found in the other minerals and organic acids. Concerning the interaction (C x VPD), no significant changes were found in Na^{2+}, Malate and Citrate. Whereas NO_3^-, SO_4^{2-}, and K^+ followed the same trend with highest values in 1.76 G and no significant differences among the other treatments (0.69 G, 1.76R, 0.69 R). Furthermore, PO_4^{3-} showed highest content under 0.69 both G and R, followed by 1.76 R and 1.76 G; Ca^{2+} content increased under 0.69 R, not showing any significant differences among other treatments; Mg^{2+} content increased under 0.69 R, followed by 1.76 G, 1.76 R, and 0.69 G; tartrate content was more elevated in 0.69 G, followed by 1.76 G, 1.76 R, and 0.69 R and isocitrate content presented highest values in G, with no significant differences between 0.69 and 1.76 kPa, followed by 1.76 R and 0.69 R.

Table 3. Minerals in leaves of green (G) and red (R) lettuce plants grown under the two VPD levels (0.69 and 1.76 kPa).

	NO_3^- (mg/kg FW)	PO_4^{3-} (g/kg DW)	SO_4^{2-} (g/kg DW)	K^+ (g/kg DW)	Ca^{2+} (g/kg DW)	Mg^{2+} (g/kg DW)	Na^{2+} (g/kg DW)	Malate (g/kg DW)	Tartrate (g/kg DW)	Citrate (g/kg DW)	Isocitrate (g/kg DW)
Cultivar											
G	4013 ± 711 b	6.92 ± 0.73 a	2.01 ± 0.25 a	58.2 ± 2.36 a	14.7 ± 0.86 b	3.46 ± 0.74 a	3.36 ± 0.40 a	56.5 ± 4.82 b	3.64 ± 0.28 a	12.2 ± 1.86 a	0.23 ± 0.03 a
R	4911 ± 625 a	7.44 ± 0.67a	1.78 ± 0.29 a	46.6 ± 5.54 b	18.3 ± 2.44 a	3.74 ± 0.64 a	4.11 ± 0.98 a	84.8 ± 8.60 a	1.98 ± 0.25 b	14.5 ± 1.31 a	0.17 ± 0.03 b
VPD											
0.69 kPa	4513 ± 746 b	8.19 ± 0.36 a	1.53 ± 0.21b	42.6 ± 1.56 b	18.3 ± 1.15 a	3.56 ± 0.76 a	3.80 ± 0.56 a	92.6 ± 8.19 a	3.21 ± 0.26 a	13.7 ± 1.41 a	0.18 ± 0.03 a
1.76 kPa	4911± 553 a	6. 24 ±1.39 b	2.25 ± 0.37a	62.2 ± 7.15 a	14.7 ± 1.87 b	3.64 ± 0.59 a	3.65 ± 0.66 a	43.3 ± 1.86 b	2.41 ± 0.28 b	12.9 ± 2.45 a	0.21 ± 0.03 a
Int.											
0.69 G	3246 ± 556 d	8.18 ± 0.27 a	1.26 ± 0.13 b	37.5 ± 0.48 b	15.6 ± 0.27 b	3.04 ± 0.64 c	3.09 ± 0.24 a	76.5 ± 4.28 b	4.65 ± 0.22 a	13.4 ± 0.74 a	0.23 ± 0.02 a
0.69 R	3980 ± 380 c	8.21 ± 0.19 a	1.80 ± 0.15 b	47.7 ± 2.16 b	21.1 ± 1.76 a	4.08 ± 0.25 a	4.52 ± 0.64 a	108.6 ± 7.83 a	1.77 ± 0.08 c	14.2 ± 1.34 a	0.15 ± 0.01 b
1.76 G	4080 ± 310 b	5.65 ± 0.91 b	2.75 ± 0.23 a	78.9 ± 3.77 a	13.9 ± 1.19 b	3.87 ± 0.21 ab	3.62 ± 0.32 a	31.5 ± 1.09 c	2.62 ± 0.12 b	10.9 ± 1.49 a	0.23 ± 0.01 a
1.76 R	4942 ± 490 a	6.84 ± 0.96 ab	1.75 ± 0.28 b	45.5 ± 6.77 b	15.6 ± 1.36 b	3.41 ± 0.77 bc	3.69 ± 0.68 a	55.1 ± 1.54 b	2.19 ± 0.33 bc	14.9 ± 1.93 a	0.19 ± 0.03 ab
Sig.											
C	***	NS	NS	*	*	NS	NS	**	***	NS	*
VPD	*	*	**	***	*	NS	NS	***	**	NS	NS
C × VPD	***	*	**	***	*	**	NS	NS	***	NS	*

All data are expressed as mean ± standard error. ***, **, *, NS refer to $p \leq 0.001$, 0.01, 0.05 and Non-significant, respectively. Lower case letters indicate the significant differences of the interaction.

6.4.4 Antioxidant Activities and Phytochemicals: Antioxidant activity and phytochemical content were influenced by C, VPD, and their interaction (Table 4). Cultivar had a significant effect on TAA, PH, and LAA, with increments in the red cultivar by 27, 12, and 40% compared to the green one; whereas VPD had a significant effect on TAA, PH, and HAA, with increments in the 1.76 kPa plants by 22, 47, and 8% compared to the low VPD condition. However, the interaction (C ×VPD) was always significant. More specifically, TAA content resulted enhanced in 1.76 R followed by 1.76 G, 0.69 R, and 0.69 G. Differently, PH and LAA showed a common trend, with highest values in 1.76 R and 0.69 R; these values were significantly higher than those detected in 1.76 G which in turn showed significantly higher values than 0.69 G. HAA showed highest values once again in 1.76 R which was not significantly different from 0.69R; the latter showed intermediate values between 1.76R and 1.76G, while the lowest values were found in 0.69G that was significantly different from all the other conditions.

Table 4. Total Ascorbic Acid (TAA), Phenols (PH), Hydrophilic antioxidant activity (HAA) and lipophilic antioxidant activity (LAA) in leaves of green (G) and red (R) lettuce plants grown under the two VPD levels (0.69 and 1.76 kPa).

	TAA mg100 g^{-1} FW	**PH** mg GA eq. 100g^{-1} DW	**HAA** mmol AA eq. 100 g^{-1} DW	**LAA** mmol trolox eq. 100g^{-1} DW
Cultivar				
G	76.6 ± 5.22 b	9.21 ± 0.24 b	14.9 ± 0.52 a	30.2 ± 1.73 b
R	97.7 ± 3.74 a	10.3 ± 0.48 a	16.6 ± 0.36 a	42.3 ± 0.70 a
VPD				
0.69 kPa	63.7 ± 3.65 b	8.08 ± 0.21 b	10.3 ± 0.57 b	36.1 ± 1.20 a
1.76 kPa	111 ± 6.86 a	11.9 ± 0.54 a	11.2 ± 0.25 a	36.5 ± 1.77 a
Int.				
0.69 G	52.5 ± 2.93 d	5.83 ± 0.14 c	9.71 ± 0.41 c	28.9 ± 1.01 c
0.69 R	75.1 ± 1.44 c	10.3 ± 0.13 a	10.8 ± 0.32 ab	43.3 ± 0.38 a
1.76 G	101 ± 4.57 b	6.77 ± 0.19 b	10.4 ± 0.21 b	31.5 ± 1.45 b
1.76 R	120 ± 4.59 a	10.3 ± 0.71 a	11.6 ± 0.09 a	41.4 ± 0.64 a
Sig.				
C	**	***	NS	***
VPD	***	*	*	*
C × VPD	*	*	*	*

All data are expressed as mean ± standard error. ***, **, *, NS refer to p ≤ 0.001, 0.01, 0.05 and Non-significant, respectively. Lower case letters indicate the significant differences of the interaction.

6.4.5 Soil Plant Analysis Development Index and Chlorophyll a Fluorescence Emission: Results from SPAD and Fv/Fm are showed in Table 5, separated for data (12 and 23 DAT). At 12 and 23 DAT, C and VPD had a significant effect as main factors and in interaction on SPAD index, showing enhanced values in R cultivar (49, 47%) and under 0.69 kPa (17, 4%). Differently, at both 12 and 23 DAT, cultivar did not elicit significant differences in Fv/Fm, whereas VPD had a significant effect with enhanced values under 0.69 kPa (2, 5%). Concerning the interaction, at both 12 and 23 DAT, SPAD index showed higher values in R cultivar under 0.69 kPa, followed by 1.76 R and 1.76

G and 0.69 G, with no differences among them. Differently, Fv/Fm ratio presented significantly higher values in 0.69 kPa with no differences between cultivars, followed by 1.76 kPa, again with no differences between cultivars.

Table 5. Soil Plant Analysis Development (SPAD) index and Fv/Fm in leaves of green (G) and red (R) lettuce plants grown under the two VPD levels (0.69 and 1.76 kPa) at 12 and 23 DAT (days after transplanting).

	SPAD Index		**Fv/Fm**	
	12 DAT	**23 DAT**	**12 DAT**	**23 DAT**
Cultivar				
G	28.07 ± 0.74 b	27.9 ± 1.46 b	0.77 ± 0.10 a	0.80 ± 0.07a
R	41.85 ± 0.91 a	41.1 ± 1.17 a	0.77 ± 0.30 a	0.80 ± 0.12a
VPD				
0.69 kPa	36.70 ± 0.85 a	35.2 ± 1.00 a	0.78 ± 0.11 a	0.82 ± 0.12a
1.76 kPa	30.51 ± 0.69 b	33.8 ± 1.63 b	0.76 ± 0.20 b	0.78 ± 0.13b
Int.				
0.69 G	28.5 ± 0.57 c	28.5 ± 0.54 c	0.79 ± 0.06 a	0.82 ± 0.02 a
0.69 R	44.9 ± 0.57 a	41.8 ± 0.46 a	0.78 ± 0.06 a	0.82 ± 0.01 a
1.76 G	27.6 ± 0.35 c	27.3 ± 0.92 c	0.75 ± 0.13 b	0.79 ± 0.06 b
1.76 R	38.2 ± 0.69 b	33.4 ± 0.71 b	0.77 ± 0.07 ab	0.78 ± 0.07 b
Sig.				
C	***	*	NS	NS
VPD	***	***	*	***
C × VPD	***	*	*	*

All data are expressed as mean ± standard error. ***, **, *, NS refer to p ≤ 0.001, 0.01, 0.05 and Non-significant, respectively. Lower case letters indicate the significant differences of the interaction.

6.4.6 Hierarchical Clustering of Functional and Nutritional Aspects of Green and Red Salanova: A heat map providing an integrated overview of the effects of cultivar and VPD on the physiological and qualitative traits of Salanova lettuce is displayed in Figure 2. In the left dendrogram, the heat map identified two main clusters, separated by the different cultivar (0.69G and 1.76G on one cluster and 0.69R and 1.76R on the other); furthermore, as visible in the upper dendrogram also variables grouped together. More specifically, our results indicated that 0.69 R separated from the other treatments because of its highest positive relation with the cations content (Na^{2+}, Ca^{2+}), Hue and malate content, and negative relation with isocitrate content and L*. Whereas, 1.76 R separated from the other treatments mainly due to its antioxidant content, especially NO_3^-, HF, TAA, and its negative relation with chroma. Differently, 0.69 G presented a higher positive variation of tartrate, isocitrate and the colorimetry parameter chroma. Finally, 1.76 G separated from the others because of its higher accumulation of SO_4^{2-} and K^+ and negative variation of citrate.

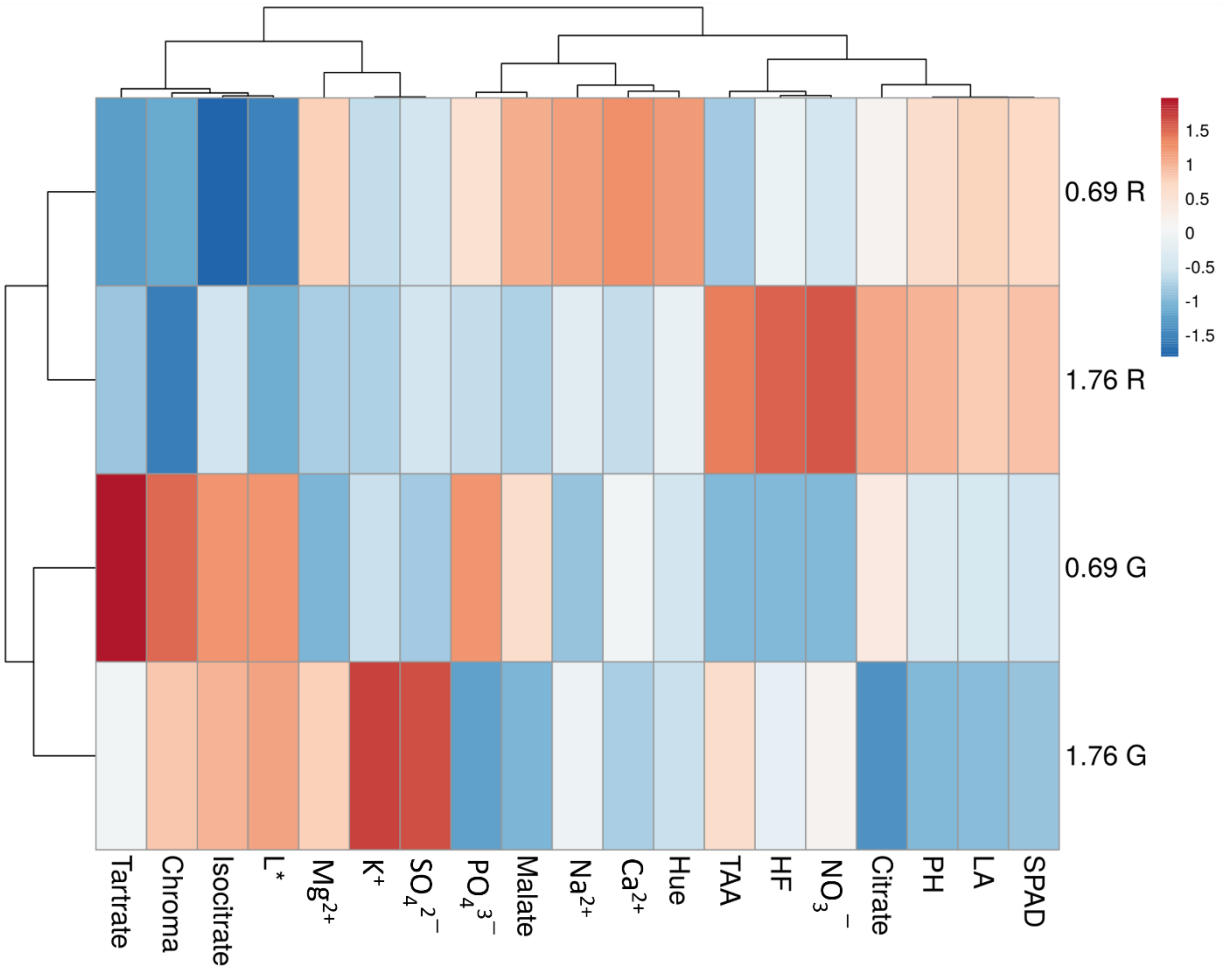

Figure 2. Heat map of qualitative and physiological aspects of green (G) and red (R) lettuce plants grown under the two VPD levels (0.69 and 1.76 kPa).

6.5 Discussion: Modulating the microclimate in indoor module-cultivation can positively affect crop morpho-physiological development, also leading to differences in appearance and in product quality, especially influencing the content of plant secondary metabolites [1,13,28]. In general, high VPD can limit plant growth and dry matter accumulation, reducing yield and photosynthesis, which are major constrains for crop production [29]. Our results are consistent with this general statement, always showing a lower biomass, number of leaves and canopy area in plants exposed to 1.76 kPa. Moreover, lettuce developed under a low-VPD environment (0.69 kPa), apart from increased growth and biomass, also presented a higher Fv/Fm and chlorophyll content (SPAD index) both at 12 and 23 DAS, overall suggesting a better performance of the photosynthetic apparatus. Indeed, a high content of photosynthetic pigments in plants is often associated with high Fv/Fm values [30] and even small increases in the photosynthetic rates are known to cause wide improvements in crop biomass and yield [31,32]. In low-VPD-exposed S. lycopersicum plants, a higher photosynthesis, mostly due to a better regulation of stomatal closure, and consequently high values of Fv/Fm, have been found in correlation with improved yield and biomass [29]. Fv/Fm values lower than 0.8, which is a threshold level for unstressed plants, are common in plants facing the onset of photodamage [33]. Indeed, the chlorophyll “a” fluorescence parameter Fv/Fm reflects the PSII (photosystem II)

maximum quantum efficiency and consequently has been widely used as a screening for early stress detection in plants and for improvements in crop production in CEA [34,35]. For example, several research found a decrease in Fv/Fm in different tomato [36] cultivars subjected to heat stress or a combination of heat and drought stresses [37]. In the present study, plants at 1.76 kPa always presented values lower than 0.8, suggesting that plants may sense the dry air, characteristic of high VPD as a mild-stress, similarly to what happens in conditions of heat stress or drought. However, microscopy observation in lettuce samples did not evidence VPD-induced differences in lamina thickness and intercellular spaces patterns. It is known that VPD levels can bring to a different morpho-anatomical development of leaf lamina, changing the whole mesophyll structure, thus changing the resistance/conductance to water vapor and CO2 within the leaf [38,39]. From these different morpho-anatomical characteristics depend on the photosynthetic rates and the whole plant physiological behavior. Indeed, although some intra- and inter- species variation is observed, physiological responses cannot overcome plant morpho-anatomical structure [40]. In the present study, the reductions in Fv/Fm, photosynthetic pigments, yield, and biomass were not due to VPD-driven changes in morpho-anatomical structure of leaf lamina probably because the microclimate around the developing leaves under the two VPD treatments was not enough different to induce any differential cell differentiation leading to different mesophyll structure. Therefore, the observed reductions in growth and photosynthetic traits were likely linked with the oxidative stress which typically occurs under unfavorable environmental conditions, and which can change crop quality [41]. Several authors have demonstrated that mild to moderate stress stresses could produce higher quality products, especially crop rich in phytochemicals, depending on: the type of stress (environmental, nutritional, etc.), the time of exposure, the intensity of application, as well as the crop species/cultivars [12]. For instance, Da Ge et al. [42], found in maize grains subjected to water stress, increments in Ca^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+}. Additionally, El-Nakhel et al. [43] reported that mineral eustress (half strength nutrient solution) was able to boost the phenolic and carotenoids profile in butterhead Salanova in particular in the red-pigmented ones. Minerals are essential elements in human diet, necessary as co-factors for several enzyme activities [44], and leafy greens are among the prime sources of these nutrients [45]. In our study, 1.76 kPa incremented the concentration of K^{+}, which is involved as a carrier ion, transporting solutes and hormones in xylem and phloem, other than be involved in enzyme activation, osmotic potential, and synthesis of protein [46]. However, Salanova lettuces at high VPD also presented a high nitrate content, especially in red cultivar. Since leafy greens are usually harvested at vegetative growth stages, and the edible parts can accumulate relatively large amounts of nitrate, these crops have been found to be the major source of nitrate uptake by humans. In the present study, however, nitrate concentration in both Salanova cultivars, were inferior to the European Commission regulation No 1258/2011 [47] which set the NO3 content for protected-grown lettuce at 5000 mg NO_3 kg^{-1} per fresh weight [48]. The lower concentration of nitrates has been associated with yellowish leaves characterized by a decreased hue angle and

increased L*, b*, and chroma [49]. In our study, although presenting a higher content of nitrates, 1.76 VPD lettuces decreased b* and L* producing fewer dark leaves but with vivid colors (increased chroma). The analysis of color is an important consideration for edible food, since the most common property to measure quality of any material is its appearance and consumers can easily be influenced by a fruit or vegetable color which they consider inappropriate [50]. Furthermore, different research also reported similar relationships between total N/NO_3 concentrations, chlorophyll content and chromaticity parameters (especially L*), so much to suggest the use of colorimeter reader or SPAD meter to predict the total content of chlorophyll and nitrate in a time-saving non-destructive analytical method [51]. Our results did not show such correlation with L*, however red cultivar presented an enhanced SPAD index as well as a highest nitrate content (Tables 3 and 4), compared to green one. The highest chlorophyll concentration (SPAD index) of red lettuces might seem odd and could be explained by the highest content of nitrates in this cultivar. Moreover, red lettuces also showed a highest content of phytochemicals, compared to green cultivar, especially under 1.76 VPD (Table 5). Many studies have demonstrated that red-pigmented-leafy-green-cultivars contained highest amounts of metabolites compared to their green counterparts [52]. Just to mention a few, El-Nakhel et al. [43] found in red Salanova lettuces higher quantities of phenolic compounds compared to green Salanova plants. Other studies also found an enhanced quantity of ascorbic acid in red-pigmented lettuce leaves [52,53]. Both phenolic compounds and ascorbic acid are potent antioxidants which confers valuable nutritional properties to vegetables [52,54]. Ascorbic acid, like other vitamins, cannot be synthesized by humans endogenously, so it represents an essential dietary component [55]; thus, ascorbic acid-rich-lettuces could represent an added value for the marketability of the products. It is interesting that increments in ascorbate, polyphenols, and antioxidant capacity were reported in lettuce grown under various types of stress. For instance, in lettuces subjected to moderate stress (heat shock, chilling, high light intensity), Oh et al. [56] found a two/three-fold increase in the total phenolic content and a significant increase in the antioxidant capacity, with no adverse effects on the general plant growth. In our study, lettuces exposed to high VPD always enhanced their phytochemical content, compared to those exposed to low VPD, probably sensing the surrounding environment as a mild stress not able to induce permanent structural changes neither cell shrinkage, but still enough to modulate chlorophyll content, Fv/Fm ratio and biomass which resulted reduced under the 1.76 kPa treatment. Levels of antioxidant molecules, such as ascorbate metabolites, phenolic compounds, and α-tocopherol, higher in high VPD, can indicate a defense against oxidative stress [41]. In this study, we examined the total amount of phenolic compounds in leaves; however, as a future perspective it would be valuable to focus on individual phenolic components to have a more comprehensive idea of plant phytochemical's synthesis in response to VPD. The most common effect of low humidity rates on crops is to induce leaf water stress, since under this environmental condition the uptake of water from the soil is not enough to cope with the high transpiration rates [6,57]. Indeed, when subjected

to high VPD, plants begin to dehydrate and start to physically translocate a larger volume of soil water through the plant system, which can also exacerbate the stress if in interaction with other adverse environmental conditions like high EC rates, bringing to the accumulation of additional salts within the plant [58]. Still, the use of these mild-stress during cultivation techniques has proven to increase tomato fruit dry matter content [59], which is an important parameter in improving yield and nutritional quality [60] also increasing sugar content, the ratio of sugar:acids [61], and the synthesis of secondary metabolites and antioxidants [9,62]. There is evidence that many antioxidants play a key role in plant adaptation to abiotic and biotic stresses [56,63]. Additionally, a significant part of antioxidants produced by plants in response to stress is secondary metabolites, including some simple and complex phenolic compounds derived primarily via the phenylpropanoid pathway [64]. As a number of these are phytochemicals with health-promoting qualities in the human diet, in the light of the above results, it would be feasible to use VPD, among other mild environmental stresses, to enhance the phytochemical content of lettuce or other common leafy vegetable. To date there are no clear indications on how to use high VPD levels, in a sort of plant “hardening off”, to ameliorate the nutraceutical value of leafy greens. A next step could be to grow plants under optimal conditions and then subject them to short periods of high VPD to promptly increase their antioxidant levels, without reducing plant photosynthesis and consequently crop production.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, C.A. and V.D.M.; methodology, C.A., Y.R. and V.D.M.; formal analysis, C.A.; investigation, C.A.; resources, Y.R., S.D.P., V.D.M.; data curation, CA and VDM; writing—original draft preparation, CA; writing—review and editing, C.A., Y.R., S.D.P., V.D.M.; supervision, V.D.M.; funding acquisition, Y.R., S.D.P., V.D.M. All authors have read and agreed to the published version of the manuscript.

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6.6 References

1. Gruda, N. Impact of Environmental Factors on Product Quality of Greenhouse Vegetables for Fresh Consumption. Crit. Rev. Plant Sci. 2005, 24, 227–247, doi:10.1080/07352680591008628.

2. Bakker, J. Physiological disorders in cucumber under high humidity conditions and low ventilation rates in greenhouses. ISHS Acta Hortic. 1984, 257–264, doi:10.17660/ActaHortic.1985.156.31.

3. Amitrano, C.; Arena, C.; Rouphael, Y.; De Pascale, S.; De Micco, V. Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. Ann. Appl. Biol. 2019, 175, 313–325, doi:10.1111/aab.12544.

4. Krug, H. Gemuseproduktion: Ein Lehr-und Nachschlagewerk fur Studium und Praxis; Verlag Paul Parey: Singhofen, Germany, 1986.

5. Collier, G.F. Tipburn of Littuce. In Horticultural Reviews; Wiley Blackwell: Hoboken, NJ, USA, 1982; Volume 4, pp. 49–65.

6. Leonardi, C.; Guichard, S.; Bertin, N. High vapour pressure deficit influences growth, transpiration and quality of tomato fruits. Sci. Hortic. 2000, 84, 285–296, doi:10.1016/s0304-4238(99)00127-2.

7. Sinclair, T.R.; Devi, J.; Shekoofa, A.; Choudhary, S.; Sadok, W.; Vadez, V.; Riar, M.; Rufty, T. Limited-transpiration response to high vapor pressure deficit in crop species. Plant Sci. 2017, 260, 109–118, doi:10.1016/j.plantsci.2017.04.007.

8. Dorai, M.; Papadopoulos, A.; Gosselin, A. Influence of electric conductivity management on greenhouse tomato yield and fruit quality. Agronomie 2001, 21, 367–383, doi:10.1051/agro:2001130.

9. Rosales, M.A.; Cervilla, L.M.; Sanchez-Rodriguez, E.; Rubio-Wilhelmi, M.M.; Blasco, B.; Rios, J.J.; Soriano, T.; Castilla, N.; Romero, L.; Ruiz, J.M. The effect of environmental conditions on nutritional quality of cherry tomato fruits: Evaluation of two experimental Mediterranean greenhouses. J. Sci. Food Agric. 2011, 91, 152–162, doi:10.1002/jsfa.4166.

10. Davey, M.W.; Montagu, M.V.; Inze, D.; Sanmartin, M.; Kanellis, A.; Smirnoff, N.; Benzie, I.J.J.; Strain, J.J.; Favell, D.; Fletcher, J. Plant L-ascorbic acid: Chemistry, function, metabolism, bioavailability and effects of processing. J. Sci. Food Agric. 2000, 80, 825–860.

11. Rosales, M.A.; Ruiz, J.M.; Hernández, J.; Soriano, T.; Castilla, N.; Romero, L. Antioxidant content and ascorbate metabolism in cherry tomato exocarp in relation to temperature and solar radiation. J. Sci. Food Agric. 2006, 86, 1545–1551, doi:10.1002/jsfa.2546.

12. Wang, Y.; Frei, M. Stressed food—The impact of abiotic environmental stresses on crop quality. Agric. Ecosyst. Environ. 2011, 141, 271–286, doi:10.1016/j.agee.2011.03.017.

13. Bisbis, M.B.; Gruda, N.; Blanke, M. Potential impacts of climate change on vegetable production and product quality—A review. J. Clean. Prod. 2018, 170, 1602–1620, doi:10.1016/j.jclepro.2017.09.224.

14. Favati, F.; Lovelli, S.; Galgano, F.; Miccolis, V.; Di Tommaso, T.; Candido, V. Processing tomato quality as affected by irrigation scheduling. Sci. Hortic. 2009, 122, 562–571, doi:10.1016/j.scienta.2009.06.026.

15. Kyriacou, M.C.; Rouphael, Y. Towards a new definition of quality for fresh fruits and vegetables. Sci. Hortic. 2018, 234, 463–469, doi:10.1016/j.scienta.2017.09.046.

16. Amitrano, C.; Arena, C.; De Pascale, S.; De Micco, V. Light and Low Relative Humidity Increase Antioxidants Content in Mung Bean (Vigna radiata L.) Sprouts. Plants 2020, 9, 1093, doi:10.3390/plants9091093.

17. Llorach, R.; Martinez-Sanchez, A.; Tomas-Barberan, F.A.; Gil, M.I.; Ferreres, F. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. Food Chem. 2008, 108, 1028–1038, doi:10.1016/j.foodchem.2007.11.032.

18. El-Nakhel, C.; Pannico, A.; Graziani, G.; Kyriacou, M.C.; Giordano, M.; Ritieni, A.; De Pascale, S.; Rouphael, Y. Variation in Macronutrient Content, Phytochemical Constitution and In Vitro Antioxidant Capacity of Green and Red Butterhead Lettuce Dictated by Different Developmental Stages of Harvest Maturity. Antioxidants 2020, 9, 300, doi:10.3390/antiox9040300.

19. Slavin, J.L.; Lloyd, B. Health benefits of fruits and vegetables. Adv. Nutr. 2012, 3, 506–516, doi:10.3945/an.112.002154.

20. Gil, M.I. Preharvest factors and fresh-cut quality of leafy vegetables. Acta Hortic. 2016, 57–64, doi:10.17660/ActaHortic.2016.1141.6.

21. Rouphael, Y.; Kyriacou, M.C.; Petropoulos, S.A.; De Pascale, S.; Colla, G. Improving vegetable quality in controlled environments. Sci. Hortic. 2018, 234, 275–289, doi:10.1016/j.scienta.2018.02.033.

22. Feder, N.; O'Brien, T. Plant microtechnique: Some principles and new methods. Am. J. Bot. 1968, 55, 123–142.

23. De Micco, V.; Amitrano, C.; Stinca, A.; Izzo, L.G.; Zalloni, E.; Balzano, A.; Barile, R.; Conti, P.; Arena, C. Dust accumulation due to anthropogenic impact induces anatomical and photochemical changes in leaves of Centranthus ruber growing on the slope of the Vesuvius volcano. Plant Biol. 2019, 22, 93–102, doi:10.1111/plb.12966.

24. Kampfenkel, K.; Van Montagu, M.; Inzé, D. Effects of iron excess on Nicotiana plumbaginifolia plants (implications to oxidative stress). Plant Physiol. 1995, 107, 725–735, doi:10.1104/pp.107.3.725.

25. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 1999, 299, 152–178.

26. Fogliano, V.; Verde, V.; Randazzo, G.; Ritieni, A. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. J. Agric. Food Chem. 1999, 47, 1035–1040, doi:10.1021/jf980496s.

27. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 1999, 26, 1231–1237, doi:10.1016/s0891-5849(98)00315-3.

28. Gruda, N. Do soilless culture systems have an influence on product quality of vegetables? J. Appl. Bot. Food Qual. 2009, 82, 141–147.

29. Zhang, D.; Du, Q.; Zhang, Z.; Jiao, X.; Song, X.; Li, J. Vapour pressure deficit control in relation to water transport and water productivity in greenhouse tomato production during summer. Sci. Rep. 2017, 7, 43461, doi:10.1038/srep43461.

30. Carillo, P.; Cirillo, C.; De Micco, V.; Arena, C.; De Pascale, S.; Rouphael, Y. Morpho-anatomical, physiological and biochemical adaptive responses to saline water of Bougainvillea spectabilis Willd. trained to different canopy shapes. Agric. Water Manag. 2019, 212, 12–22, doi:10.1016/j.agwat.2018.08.037.

31. Parry, M.A.; Reynolds, M.; Salvucci, M.E.; Raines, C.; Andralojc, P.J.; Zhu, X.-G.; Price, G.D.; Condon, A.G.; Furbank, R.T. Raising yield potential of wheat. II. Increasing photosynthetic capacity and efficiency. J. Exp. Bot. 2011, 62, 453–467, doi:10.1093/jxb/erq304.

32. Zhu, X.-G.; Long, S.P.; Ort, D.R. Improving photosynthetic efficiency for greater yield. Annu. Rev. Plant Biol. 2010, 61, 235–261, doi:10.1146/annurev-arplant-042809-112206.

33. Ogaya, R.; Peñuelas, J.; Asensio, D.; Llusià, J. Chlorophyll fluorescence responses to temperature and water availability in two co-dominant Mediterranean shrub and tree species in a long-term field experiment simulating climate change. Environ. Exp. Bot. 2011, 71, 123–127, doi:10.1016/j.envexpbot.2010.10.016.

34. Sharma, D.K.; Andersen, S.B.; Ottosen, C.O.; Rosenqvist, E. Wheat cultivars selected for high Fv/Fm under heat stress maintain high photosynthesis, total chlorophyll, stomatal conductance, transpiration and dry matter. Physiol. Plant. 2015, 153, 284–298.

35. Baker, N.R.; Rosenqvist, E. Applications of chlorophyll fluorescence can improve crop production strategies: An examination of future possibilities. J. Exp. Bot. 2004, 55, 1607–1621.

36. Poudyal, D.; Rosenqvist, E.; Ottosen, C.-O. Phenotyping from lab to field—Tomato lines screened for heat stress using Fv/Fm maintain high fruit yield during thermal stress in the field. Funct. Plant Biol. 2019, 46, 44–55, doi:10.1071/fp17317.

37. Nankishore, A.; Farrell, A.D. The response of contrasting tomato genotypes to combined heat and drought stress. J. Plant Physiol. 2016, 202, 75–82, doi:10.1016/j.jplph.2016.07.006.

38. Du, Q.; Liu, T.; Jiao, X.; Song, X.; Zhang, J.; Li, J. Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. J. Exp. Bot. 2019, 70, 4949–4962, doi:10.1093/jxb/erz238.

39. Tosens, T.; Niinemets, U.; Vislap, V.; Eichelmann, H.; Diez, P.C. Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in Populus tremula: How structure constrains function. Plant Cell Env. 2012, 35, 839–856, doi:10.1111/j.1365-3040.2011.02457.x.

40. Murphy, M.R.C.; Jordan, G.J.; Brodribb, T.J. Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. Plant Cell Environ. 2014, 37, 124–131, doi:10.1111/pce.12136.

41. Lihavainen, J.; Keinänen, M.; Keski-Saari, S.; Kontunen-Soppela, S.; Sõber, A.; Oksanen, E. Artificially decreased vapour pressure deficit in field conditions modifies foliar metabolite profiles in birch and aspen. J. Exp. Bot. 2016, 67, 4367–4378, doi:10.1093/jxb/erw219.

42. Da Ge, T.; Sui, F.G.; Nie, S.; Sun, N.B.; Xiao, H.; Tong, C.L. Differential responses of yield and selected nutritional compositions to drought stress in summer maize grains. J. Plant Nutr. 2010, 33, 1811–1818, doi:10.1080/01904167.2010.503829.

43. El-Nakhel, C.; Giordano, M.; Pannico, A.; Carillo, P.; Fusco, G.M.; De Pascale, S.; Rouphael, Y. Cultivar-specific performance and qualitative descriptors for butterhead Salanova lettuce produced in closed soilless cultivation as a candidate salad crop for human life support in space. Life 2019, 9, 61, doi:10.3390/life9030061.

44. Soetan, K.; Olaiya, C.; Oyewole, O. The importance of mineral elements for humans, domestic animals and plants—A review. Afr. J. Food Sci. 2010, 4, 200–222.

45. Gupta, K.; Wagle, D. Nutritional and antinutritional factors of green leafy vegetables. J. Agric. Food Chem. 1988, 36, 472–474, doi:10.1021/jf00081a016.

46. Peuke, A.D.; Jeschke, W.D.; Hartung, W. Flows of elements, ions and abscisic acid in Ricinus communis and site of nitrate reduction under potassium limitation. J. Exp. Bot. 2002, 53, 241–250, doi:10.1093/jexbot/53.367.241.

47. Statement on possible public health risks for infants and young children from the presence of nitrates in leafy vegetables. EFSA J. 2010, 8, 1935, doi:10.2903/j.efsa.2010.1935.

48. Colla, G.; Kim, H.-J.; Kyriacou, M.C.; Rouphael, Y. Nitrate in fruits and vegetables. Sci. Hortic. 2018, 237, 221–238, doi:10.1016/j.scienta.2018.04.016.

49. Navarro, A.; Elia, A.; Conversa, G.; Campi, P.; Mastrorilli, M. Potted mycorrhizal carnation plants and saline stress: Growth, quality and nutritional plant responses. Sci. Hortic. 2012, 140, 131–139, doi:10.1016/j.scienta.2012.03.016.

50. Jha, S.N. Colour Measurements and Modeling. In Nondestructive Evaluation of Food Quality; Springer: Berlin/Heidelberg, Germany, 2010; pp. 17–40.

51. Tuncay, O. Relationships between nitrate, chlorophyll and chromaticity values in rocket salad and parsley. Afr. J. Biotechnol. 2011, 10, 17152–17159.

52. Kim, D.E.; Shang, X.; Assefa, A.D.; Keum, Y.S.; Saini, R.K. Metabolite profiling of green, green/red, and red lettuce cultivars: Variation in health beneficial compounds and antioxidant potential. Food Res. Int. 2018, 105, 361–370, doi:10.1016/j.foodres.2017.11.028.

53. Baslam, M.; Morales, F.; Garmendia, I.; Goicoechea, N. Nutritional quality of outer and inner leaves of green and red pigmented lettuces (Lactuca sativa L.) consumed as salads. Sci. Hortic. 2013, 151, 103–111, doi:10.1016/j.scienta.2012.12.023.

54. El-Nakhel, C.; Petropoulos, S.A.; Pannico, A.; Kyriacou, M.C.; Giordano, M.; Colla, G.; Troise, A.D.; Vitaglione, P.; De Pascale, S.; Rouphael, Y. The bioactive profile of lettuce produced in a closed soilless system as configured by combinatorial effects of genotype and macrocation supply composition. Food Chem. 2020, 309, 125713, doi:10.1016/j.foodchem.2019.125713.

55. Li, Y.; Schellhorn, H.E. New developments and novel therapeutic perspectives for vitamin C. J. Nutr. 2007, 137, 2171–2184, doi:10.1093/jn/137.10.2171.

56. Oh, M.M.; Carey, E.E.; Rajashekar, C.B. Environmental stresses induce health-promoting phytochemicals in lettuce. Plant Physiol. Biochem. 2009, 47, 578–583, doi:10.1016/j.plaphy.2009.02.008.

57. Grange, R.; Hand, D. A review of the effects of atmospheric humidity on the growth of horticultural crops. J. Hortic. Sci. 1987, 62, 125–134, doi:10.1080/14620316.1987.11515760.

58. Tack, J.; Singh, R.K.; Nalley, L.L.; Viraktamath, B.C.; Krishnamurthy, S.L.; Lyman, N.; Jagadish, K.S. High vapor pressure deficit drives salt-stress-induced rice yield losses in India. Glob. Chang. Biol. 2015, 21, 1668–1678, doi:10.1111/gcb.12803.

59. Ho, L.; Grange, R.; Picken, A. An analysis of the accumulation of water and dry matter in tomato fruit. Plant Cell Environ. 1987, 10, 157–162.

60. Boyer, J.S. Plant productivity and environment. Science 1982, 218, 443–448, doi:10.1126/science.218.4571.443.

61. Bertin, N.; Guichard, S.; Leonardi, C.; Longuenesse, J.; Langlois, D.; Navez, B. Seasonal evolution of the quality of fresh glasshouse tomatoes under Mediterranean conditions, as affected by air vapour pressure deficit and plant fruit load. Ann. Bot. 2000, 85, 741–750, doi:10.1006/anbo.2000.1123.

62. Rosales, M.A.; Ríos, J.J.; Cervilla, L.M.; Rubio-Wilhelmi, M.M.; Blasco, B.; Ruiz, J.M.; Romero, L. Environmental conditions in relation to stress in cherry tomato fruits in two experimental Mediterranean greenhouses. J. Sci. Food Agric. 2009, 89, 735–742, doi:10.1002/jsfa.3500.

63. Burritt, D.J.; Mackenzie, S. Antioxidant metabolism during acclimation of Begonia × erythrophylla to high light levels. Ann. Bot. 2003, 91, 783–794.

64. Dixon, R.A.; Paiva, N.L. Stress-Induced Phenylpropanoid Metabolism. Plant Cell 1995, 7, 1085, doi:10.1105/tpc.7.7.1085.

Chapter 7

Vapour pressure deficit drives the balance of hydraulic-related anatomical traits in lettuce leaves

7.1 Introduction: Plant traits are the morpho-anatomical, physiological, phenological and biochemical characteristics which can be measured at the single-organism level (Kattge *et al.*, 2020). These traits depend on the genetic properties of the species, reflecting their evolutionary lineage, and are influenced by the surrounding environment (Valladares F, 2007; Violle *et al.*, 2007). Recently, plant traits have been used in ecology studies and vegetation modelling since they can act as proxies to predict how plants will perform when subjected to different environmental stimuli, how they will affect other trophic levels, and how they will influence the entire ecosystem (Garnier and Navas, 2012; Wright *et al.*, 2017). Among plant traits, leaf size has a central role in plant acclimation to environmental conditions (Conesa *et al.*, 2020; Ramírez-Valiente *et al.*, 2010; Yates *et al.*, 2010). Variation in leaf size has been found along climatic gradients, often with increments in lamina expansion in humid habitats (Niinemets *et al.*, 2007; Royer *et al.*, 2005). Differently, small leaves are more easily found in dry environments, since they can react to high irradiance reducing transpiration costs (Sack and Scoffoni, 2013). Indeed, the thickness of the boundary layer increases with leaf size, so it would be difficult for bigger leaves to reduce the heat loss in a dry environment (Wang *et al.*, 2020). Therefore, irradiance during plant growth and the evaporative demand (expressed as the air vapour pressure deficit; VPD) to which plant are subjected, are strictly connected, affecting not only the whole plant-water-relationships, but also the development of leaf size and other plant anatomical traits (Scoffoni *et al.*, 2015). For these reasons, leaves under high light may suffer from risks caused by high VPD and dehydration, and the plasticity of their leaf water-related traits can contribute to maintain an efficient photosynthesis under limiting environmental conditions (Wang *et al.*, 2020). Furthermore, developing small leaves in dry environment optimize the whole plant resources allocation. Narrower leaves save “constructional costs” attributed to more expanded vascular and cell-wall fractions, with competitive advantage in harsh environments where carbon and water availability are already inadequate (Carins Murphy *et al.*, 2012; Niinemets *et al.*, 2007). Furthermore, over the years, leaf size has been positively or negatively correlated with other leaf morpho-anatomical traits linked to plant hydraulics, such as: stomatal density and vein density, often with controversial results. Just to mention a few studies, Gupta (1961a) was one among the firsts to show that in five Solanaceae plants, the average number of stomata per unit area as well as vein

density were negatively correlated to the leaf area (Gupta, 1961b). Carins Murphy *et al*. (2012) proposed that in species with high leaf plasticity, which promptly adapt to changes in environmental conditions, stomatal and vein density should be "diluted" during leaf expansion, thus allowing coordinated changes in vein and stomatal densities. These coordinated changes would allow the maintenance of a high physiological efficiency (photosynthesis, conductance, water use efficiency) of the species under sub-optimal environmental conditions (Brodribb *et al.*, 2007). However, with regard to the relations among leaf size, vein and stomatal density showed variations at high and low VPDs. Carins Murphy *et al.* (2014) found that leaf anatomical traits of *Toona ciliata* were independent from leaf size under high and low VPD, while other researches evidenced an enhanced stomatal density but reduced vein density in *Rosa hybrida* under high VPD (Torre *et al.*, 2003) or reduced stomatal and vein densities coordinated with a reduced leaf area in *Vigna radiata* under high VPD (Amitrano *et al.*, 2021). Differences were even found in different cultivar of *Solanum lycopersicum* with reduced stomatal density and leaf area and no differences in vein density in Jinpeg cv., and reduced stomatal density, enhanced vein density and no differences in leaf area in Zhongza cv. (Du *et al.*, 2019).

Generally, besides some intra- and inter-species variation, leaf morpho-anatomical traits are different in sun and shade leaves, usually with the following general pattern under favorable environmental conditions: leaves developed under sun have less expanded but thicker lamina, higher stomatal density and a well-structured palisade tissue, often showing thin grana stacks in their chloroplasts, compared to shade leaves (Martins *et al.*, 2014; Mathur *et al.*, 2018). Although vein density has been much less studied so far than other traits, there is evidence that sun leaves evolve higher vein density than shade leaves (Uhl and Mosbrugger, 1999; Brodribb *et al.*, 2007; Carins Murphy *et al.*, 2012;).

All these traits, however, have been poorly explored in crops, where only a few studies can be found, mostly in greenhouse trials of tomato (Du *et al.*, 2019; Zhang *et al.*, 2018) and little is known about lettuce morpho-anatomical development under different environmental conditions. In this study we compare the leaf plasticity in size, density of stomata and vein of 2 butterhead lettuces with green and red leaves (*Lactuca sativa* L. var. *capitata*) grown under different VPDs. Furthermore, after developing the first two leaves, lettuce begins to form the rosette pattern that it grows in, creating in the same leaf, shade and sun areas subjected to a different boundary layer, relative humidity and overall, to different microclimatic condition, till developing into a compact head. Thus, we hypothesised that if humidity was the main factor in triggering stomatal and vein density, the highest density should be found in the shaded parts of the leaf, vice versa if light was the main factor, the highest density should be found in the light-exposed parts.

In the light of the above, the main objectives of this study were to: i) understand if vein and stomatal densities are diluted with leaf size in lettuce, ii) explore if, in the same leaf, the areas subjected to different microclimate and sun/shade conditions will prime the development of different patterns of

morpho-anatomical traits, iii) to gain insight and data to be further applied into a developmental model. Assessing the impact of different air humidity and irradiances on leaf anatomical characteristics and hydraulic-related traits, will be an important starting point to evaluate the species capacity to acclimate to the ongoing climate change conditions.

7.2 Materials and methods

7.2.1 Plant material and growth under controlled conditions: The study was conducted on 2 butterhead Salanova® lettuces (*Lactuca sativa* L. var. capitata) with green and red leaves. Lettuce was chosen because it is the most common leafy green crops in controlled environment and to our knowledge, no studies have investigated the development of leaf-anatomical traits in correlation to micro-enviromental conditions before. Seeds were provided by Rijk Zwaan (Rijk Zwaan, Der Lier, The Netherland) and showed 100 % germination. The experiment was carried out in a growth chamber (KBP-6395F, Termaks, Bergen, Norwey) under a photoperiod of 12h. Light was provided by and RGB LED panel with an intensity of 315 PPFD µmol m^{-2} s^{-1} at the canopy level. 18 lettuces were grown for 23 days in two different trials under the same temperature (T) of 24/19 ◦C (day/night) but different air relative humidity (RH), resulting in two different VPDs. The first trial at an average VPD of 0.69 kPa (Low VPD) and the second trial at an average VPD of 1.76 kPa (High VPD). Temperature and humidity were monitored for the duration of the experiment using small sensors equipped with a data logger (Testo 174H). Daily rotation of the trays was performed to ensure homogenous light and humidity conditions across the shelf surface. Plants were daily watered to field capacity. All the analyses were carried out on 10 leaves per each VPD condition. A list of all the measured traits is reported in table 1.

7.2.2 Leaf size and other leaf functional traits: Leaf functional traits were evaluated following Cornelissen *et al.* (2003). Firstly, leaves were scanned to calculate Leaf Size (LS; lamina area in mm^2) using ImageJ software (national Institutes of Health, Bethesda, Maryland, USA). Then, the Fresh Weight (FW) of each leaf was recorded and the leaf petiole was submerged in distilled water in the dark for 48h and then re-weighted to calculate the Saturation Weight (SW); Dry weight (DW) was obtained by oven-drying leaves at 60 °C for 72 h, until they reached a constant weight. These parameters were used to evaluate: the water status of the leaves, measured as relative water content (RWC %) and expressed as percentage of (FW-DW)/(SW-DW); the leaf dry matter content (LDMC) considered a proxy for leaf tissue density (Ryser and Urbas, 2000) and expressed as (DW/SW) in gg^{-1}; Leaf Mass per Area (LMA), calculated as the ratio between DW and LS (g mm^{-2}) which is used as proxy for sclerophylly (Witkowski and Lamont, 1991); and the hypothetical thickness of a single layer of H_2O on the leaf area, Equivalent Water thickness (EWT), calculated as (FW-DW)/LS and expressed in mg mm^{-2} (Elsherif *et al.*, 2019).

7.2.3 Leaf vein traits: To accomplish morpho-anatomical analyses on the entire leaves, each leaf was visually divided into three parts called bottom, b; middle, m; apex, a (**Figure 1**). To determine vein traits, the entire leaves were chemically cleared with 5% NaOH in aqueous solution and bleached in EtOH dilution series, following Miksche and Berlyn (1976). In order to highlight even the smallest veins, cleared leaves were covered in 1% safranin in EtOH for 10 minutes and gently rinsed with 100% EtOH before being covered in 1% fast green in ETOH for a few seconds and rinsed again with 100% EtOH. By means of a light microscope (BX51; Olympus) equipped with a camera (EP50; Olympus), bottom, middle and apex of each leaf was imaged in 5 fields of view at a magnification of 4x (image area 24 mm^2). From those pictures, vein density (VLA) and free vein endings per area (FEV) were calculated using image J software, as follow (Sack and Scoffoni, 2013): VLA as the ratio of the sum of vein lengths of 4° veins and higher and the difference between the area of the image and the area occupied by the 2° veins, expressed in mm mm^{-2} per leaf size; FEV as the ratio of the number of free vein endings and the difference between the area of the image and the area occupied by the 2° veins, expressed in mm^2 as number per leaf size.

7.2.4 Stomatal traits: Stomata traits were determined by microscopic measurements of leaf abaxial peels, taken centrally in each region of the leaf avoiding the midrib as well as the margin. For each leaf, measurements were averaged from 5 measurements obtained from 3 different peels of the apex, middle and bottom (a, m, b) region of each leaf. Each field view was set at 20x magnification (field area of 0. 95 mm^2) Stomatal density (SD) was measured as number of stomata per leaf size expressed in mm^2 using ImageJ. Stomatal length (SL) was determined considering the guard cell length (pole-to-pole) (µm), of 5 stomata per field, at a magnification of 40x (filed area of 0. 15 mm^2). Moreover, density of epidermal cells (ED) was quantified at 20x magnification. ED was expressed as n mm^2 and then multiplied per leaf size. All the measurements were performed in three fields per 5 leaf samples, being careful to avoid main veins or tissue defects. Vein, stomatal and epidermal cell densities were quantified by multiplying the density of veins, stomata, and epidermal cells (mm^2) by leaf size as reported in Carins Murphy et al., 2014. Nevertheless, their densities per mm^2 is also reported in table 4 and Figure S1.

7.2.5 Relationships between leaf size and leaf hydraulic-related traits: The coordination between leaf size and leaf hydraulic-related traits (stomatal and vein density) was assessed by plotting the stomatal density against 1/leaf size and vein density against 1/√leaf size as reported in Carins Murphy *et al.* 2014 (i.e. quantified as the deviation from a proportional deviation). The coordination between the epidermal cell density per leaf and leaf size was tested for proportionality

in the same way. Furthermore, correlations between vein and stomatal density were also calculated: firstly, the correlations between vein density and stomatal density were reported for green and red plants grown under low and high VPD. Secondly, within the single leaf, correlations among vein and stomatal densities were also calculated in the three different positions (a, m, b).

7.2.6 Statistical analysis: Leaf morpho-anatomical traits data of green and red Salanova plants grown at low and high VPD were assessed by a two-way analysis of variance (anova) considering the VPD and the cultivar as main factors. A three-way anova was performed on stomata and vein traits reported in Table 4, considering VPD, cultivar and position within the leaf (a, apex; m, medium; b, bottom) as main factors. The Kolmogorov–Smirnov and Shapiro–Wilk tests were performed to check for normality and Tukey post-hoc test was used for means separation ($p < 0.05$). All statistical analyses were performed with SPSS 13 statistical package (SPSS, Chicago, IL, USA).

Table 1. A list of measured traits and their measurement unit.

Trait	Acronym	Measurement unit
Leaf size	LS	mm^2
Leaf mass per area	LMA	$g\ mm^{-2}$
Leaf dry matter content	LDMC	gg^{-1}
Relative water content	RWC	%
Equivalent water thickness	EWT	$g\ mm^{-2}$
Stomatal density	SD	$n\ mm^{-2}$
Stomatal length	SL	µm
Stomatal width	SW	µm
Epidermal cell density	ED	$n\ mm^{-2}$
Vein density	VLA	$mm\ mm^{-2}$
Free Vein Endings	FEV	$n\ mm^{-2}$

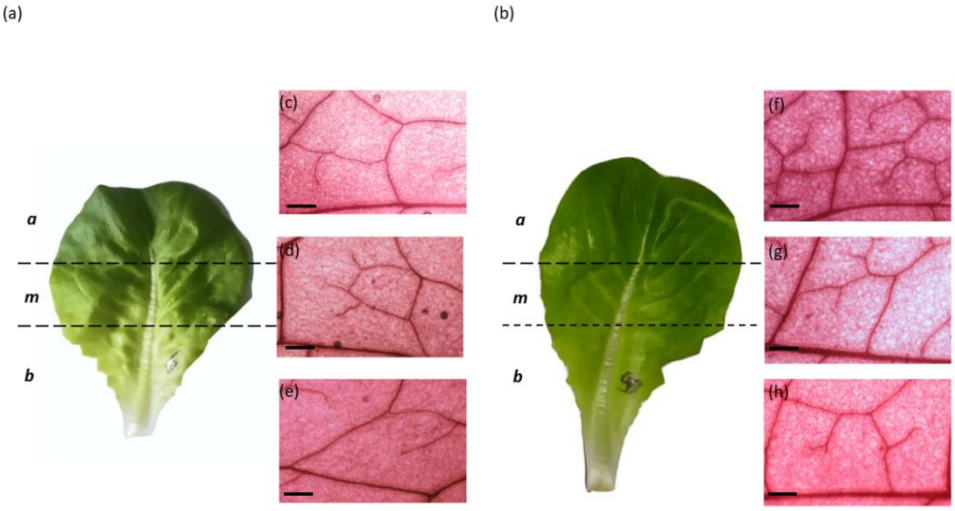

Figure 1. Schematic representation of leaf division into three portions: bottom, middle and apex, from a representative plant grown under (a) high and (b) low VPD. Representative micrographs of leaf veins for both conditions are shown. Scale bars: 500 µm.

7.3 Results

7.3.1 Variation in VPD influence leaf size and other leaf functional traits of green and red Salanova: VPD had a substantial effect on LS, LMA, LDMC and RWC ($p < 0.001$) as main factor ($p < 0.001$) (**Table 2**). Differently, the cultivar and the interaction between main factors (VPD x C) had a significant effect only on RWC ($p < 0.05$, $p < 0.01$, respectively) (**Table 2**). More specifically, leaves from plants grown under low VPD were 22% more expanded of those grown under HV (**Figure 2a; Table 2**). These leaves also presented increased LMA LDMC and RWC (51%, 53%, and 59 % more than HV respectively). Concerning the RWC, significant differences were also found between G and R cultivar, with increments in R by 9%. No significant differences were detected among treatments concerning EWT.

Table 2. Leaf traits in terms of leaf size (LS), leaf mass per area (LMA), leaf dry matter content (LDMC), relative water content (RWC), equivalent water thickness (EWT) of green and red Salanova plants grown under low and high VPD, with no differences within the leaf parts. All data are reported as mean ± se of 9 replicates. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

	LS (mm^2)	LMA ($mg\ mm^{-2}$)	LDMC ($mg\ mg^{-1}$)	RWC (%)	EWT ($mg\ mm^{-2}$)
VPD (V)					
LV	316.79 ± 7.75 a	0.51 ± 0.03 a	0.13 ± 0.008 a	12.28 ± 0.48 a	3.06 ± 0.13 a
HV	247.47 ± 2.48 b	0.25 ± 0.01 b	0.06 ± 0.002 b	4.96 ± 0.35 b	3.55 ± 0.09 a
Cultivar (C)					
G	278.99 ± 6.08 a	0.38 ± 0.02 a	0.09 ± 0.006 a	8.22 ± 0.42 b	3.26 ± 0.10 a
R	285.17 ± 4.15 a	0.37 ± 0.02 a	0.09 ± 0.006 a	9.02 ± 0.41 a	3. 35 ± 0.12 a

Interaction					
LVG	310.07 ± 8.86a	0.52 ± 0.03a	0.13 ± 0.007a	10.74 ± 0.52b	3.15 ± 0.13a
LVR	323.51 ± 6.64a	0.51 ± 0.03a	0.13 ± 0.009a	13.82 ± 0.44a	2.98 ± 0.12a
HVG	247.91 ± 3.29 b	0.25 ± 0.01b	0.06 ± 0.002b	5.72 ± 0.32c	3.38 ± 0.07a
HVR	247.04 ± 1.66 b	0.25 ± 0.01b	0.06 ± 0.002b	4.99 ± 0.37c	3.72 ± 0.11a
Significance					
VPD	***	***	***	***	NS
C	NS	NS	NS	*	NS
VPD x C	NS	NS	NS	**	NS

7.3.2 Variation in VPD influence leaf stomatal and vein traits of green and red Salanova: VPD had a significant effect on stomata and vein traits influencing SD, SL, ED, VLA and FEV as main factor ($p < 0.001$) (**Table 3**). Differently, the cultivar and the interaction between main factors (VPD x C) had a significant effect on SD, SL, FEV ($p < 0.05$) and VLA ($p < 0.01$). LV plants presented more stomata and veins per unit area, with values 38 and 36 % higher than HV, respectively (**Figure 2b,c; Table 3**). LV also elicited the development of more FEV and ED (10 and 50 % more than HV) but smaller stomata (11 % reduction in length of guard cells compared to HV). Concerning SD, SL, VLA and FEV, significant differences were also found between G and R cultivar, with higher values in R cultivar SD by 2 %. Differently, SL, VLA and FEV were higher in G cultivar by 6, 27 and 24 % compared to R one. No significant differences were detected among treatments concerning SW.

Table 3. Stomata and vein traits in terms of stomatal density (SD), stomatal length (SL), stomatal width (SW), Epidermal cell density (ED), Vein density (VLA), free vein endings (FEV) of green and red Salanova plants grown under low and high VPD, with no differences within the leaf parts. All data are reported as mean ± se of 9 replicates. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

	SD ($n\ mm^{-2}$)	SL (µm)	SW (µm)	ED ($n\ mm^{-2}$)	VLA ($mm\ mm^{-2}$)	FEV ($n\ mm^{-2}$)
VPD (V)						
LV	126641 ± 607a	20.28 ± 0.63 b	15.91 ± 0.40 a	$1.93E^{08}$ ± 9.84 E^{06}a	980.50 ± 10 a	5932.94 ± 46 a
HV	77963 ± 349b	22.55 ± 0.59 a	15.93 ± 0.17 a	$1.71E^{08}$ ±5.15 E^{06}b	617.87 ± 19 b	2913.53 ± 16b
Cultivar (C)						
G	111253± 486 b	22.11 ± 0.42 a	16.03 ± 0.37 a	$1.71E^{08}$ ± 5.87 E^{06}a	894.93 ± 29 a	5047.78 ± 35a
R	113325 ± 471 a	20.72 ± 0.38 b	15.92 ± 0.21 a	$1.82E^{08}$ ± 5.77 E^{06}a	703.44 ± 34 b	3798.68 ±27b
Interaction						
LVG	123269 ± 635 b	20.31±0.43b	16.15 ± 0.56a	1.94 E^{08}±6.69E^{06}a	1112.66 ± 40 a	6752.63±3a
LVR	140130 ± 580 a	19.76 ± 0.40c	15.73 ± 0.24a	1.92 E^{08}±6.30E^{06}a	848.34 ± 24 b	5113.25 ± 40b
HVG	73550 ± 338 c	22.06 ± 0.41a	15.91 ± 0.18a	1.71 E^{08}± 5.06E^{06}b	677.21 ± 19 c	3342.93 ± 17c
HVR	82376 ± 361 c	21.27 ± 0.36a	15.91 ± 0.17a	1.71 E^{08}± 5.25E^{06}b	558.54 ± 20 c	2484.12 ± 15c
Significance						
VPD	***	***	NS	***	***	***
C	*	*	NS	NS	**	*
VPD x C	*	*	NS	NS	**	*

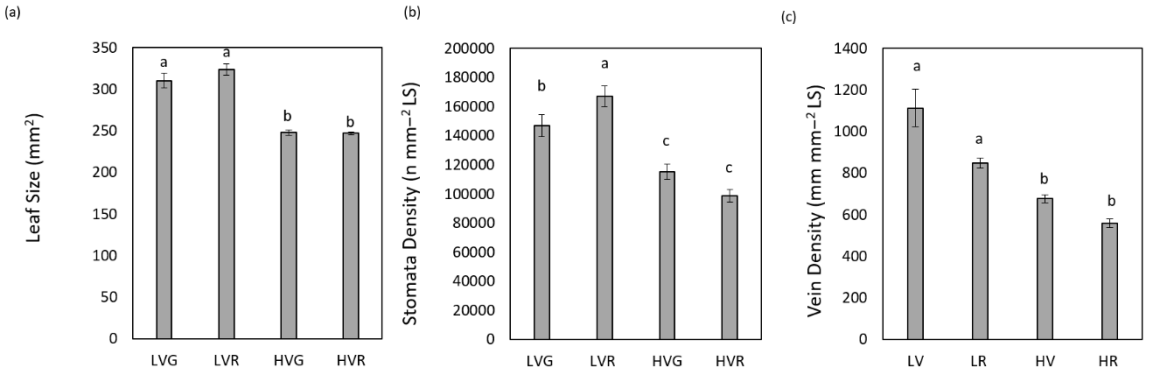

Figure 2. (a) Leaf size, (b) stomatal density and (c) vein density of green and red Salanova lettuces grown under low and high VPD. Mean values ± standard errors are shown. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

7.3.3 Relationships between leaf morpho-anatomical traits: As presented in **figure 3 (a, b)** both vein and stomatal densities values plotted against leaf size deviate from the proportional relationships (broken line). Only the Epidermal cells density maintained a proportional relationship with leaf size in all the conditions (LVG, LVR, HVG, HVR) (**Figure 3c**). Moreover, there was a strong positive relationship between vein and stomatal density in all the conditions (**Figure 4**). At low VPD a significant correlation between vein and stomata in both G and R plants (R^2= 0.96, and 0.88, $p < 0.001$) was found with was based on lower values of both parameters compared to high VPD where the relation was highly significant too (R^2= 0.87 and 0.93, $p < 0.001$) (**Figure 4**).

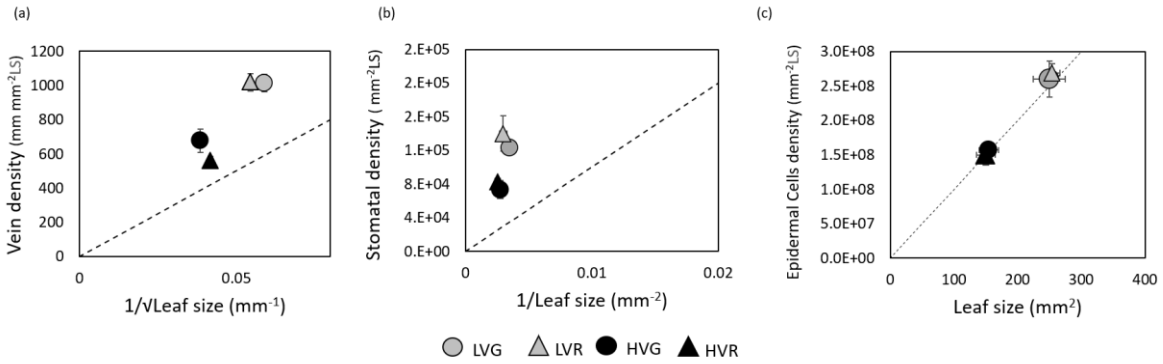

Figure 3. (a) Vein density and 1√leaf size, (b) stomatal density and 1/leaf size and (c) total epidermal cell number per leaf and leaf size of green (circles) and red (triangles) Salanova lettuces grown under low (grey) and high (black) VPD. Mean values ± standard errors are shown. Broken line represents the proportional relationships.

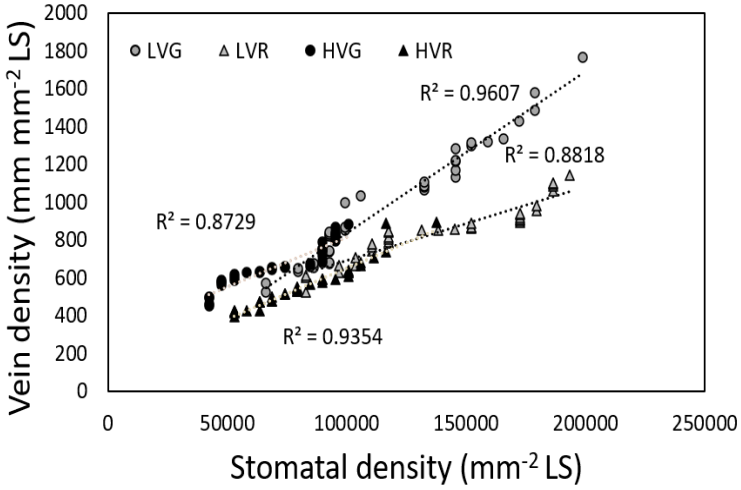

Figure 4. Vein density and stomatal density of green (circles) and red (triangles) lettuces grown under low (grey) and high (black) VPD. Regression lines and R^2 values are also shown.

7.3.4 Variation in VPD influence stomatal and vein distributions within leaves: The distribution of veins and stomata within the lamina changed with the VPD, the cultivar and the position within the leaf (**Table 4**). More specifically, results from ANOVA showed that VPD and position as main factors influenced SD, SL, FEV and VLA ($p < 0.001$). Differently, the cultivar as main factor only influenced the vein traits ($p < 0.01$). Concerning stomatal density (SD), highest values were found at the apex part of Low VPD plants in both G and R cultivars (LVGa and LVRa), followed by HVGa and HVRa and then by LVGa and LVRa, which in turn were higher than HVGm and HVRm. Lowest values were found in HVGb and HVRb. Differently, stomatal length (SL), showed ad opposite trend, being higher under HVGb and HVRb, followed by HVGm, HVRm, HVGa and HVRa (with no differences between them). Lowest values were found in LVGa and LVRa. Free vein endings showed higher values at LVGa and LVRa followed by LVGm and LVRm and by LVGb and LVRb. Lowest values were found in HVGb and HVRb. Differently, vein density was enhanced in the bottom part of L VPD leaves (LVGb and LVRb), which in turn were higher than LVGm and LVRm and LVGa and LVRa. Lowest values were found in HVGa and HVRa. Moreover, the vein and stomatal densities of the three leaf portions displayed positive significant relationships (F**igure 5,6**). However, the degree of these relationships varied among treatments (**Figure 5,6**). Increasing the VPD induced a decrease in vein and stomatal density in HVGb ($R^2 = 0.66$), HVRb ($R^2 = 0.92$), HVGm ($R^2 = 0.89$), HVGm ($R^2 = 0.83$), HVGa ($R^2 = 0.94$), HVRa ($R^2 = 0.30$). Whereas, under low VPD stomatal and vein density increased and were still strongly correlated: in LVGb ($R^2 = 0.87$) LVRb ($R^2 = 0.86$), LVGm ($R^2 = 0.85$), LVGm ($R^2 = 0.92$), LVGa ($R^2 = 0.92$), LVRa ($R^2 = 0.75$). Moreover, moving from b to a, leaves presented reduced vein density and increased stomatal density both under LV and HV (**Figure 6**).

Table 4. Stomatal and vein traits in terms of stomatal density (SD), stomatal length (SL), stomatal width (SW), Epidermal cell density (ED), Vein density (VLA), free vein endings (FEV) from plant grown under low and high VPD with differences within the leaf parts: b, bottom; m, medium; a, apex. All data are reported as mean ± se. Different letters indicate significant differences, according to Tukey test ($p < 0.05$).

	SD ($n\ mm^2$)			SL (µm)			FEV ($n\ mm^{-2}$)			VLA ($mm\ mm^{-2}$)		
VPD (V)												
LV	76.13 ± 2.43 a			20.73 ± 0.35 b			18.790 ± 1.07 a			3.64 ± 0.11 a		
HV	56.06 ± 2.71 b			22.13 ± 0.33 a			10.19 ± 0.58 b			2.67 ± 0.21 b		
Cultivar (C)												
G	64.92 ± 2.38 a			21.44 ± 0.30 a			16.05 ± 0.84 a			3.31 ± 0.24 a		
R	66.43 ± 2.75 b			21.43 ± 0.37 a			16.80 ± 0.81 a			3.13 ± 0.09 b		
Position (P)												
a	95.02 ± 3.10 a			22.05 ± 0.32 a			9.77 ± 0.95 c			2.84 ± 0.11 c		
b	56.11 ± 2.48 b			21.68 ± 0.34 b			12.49 ± 0.73 b			3.19 ± 0.07 b		
m	45.65 ± 2.12 c			20.54 ± 0.35 c			19.21 ± 0.79 a			3.63 ± 0.30 a		
Interaction (VxCxP)	b	m	a	b	m	a	b	m	a	b	m	a
LVG	50.80 ± 1.75 e	62.15 ± 1.76 c	110.65 ± 3.78 a	20.21 ± 0.30c	20.47 ± 0.40c	18.84 ± 0.12d	12.94 ± 0.71c	21.77 ± 0.98b	30.61 ± .54a	4.64 ± 0.13a	3.77 ± 0.11b	2.91 ± 0.15c
LVR	49.04 ± 2.11 e	62.39 ± 2.09 c	111.95 ± 3.09 a	20.85 ± 0.45c	20.02 ± 0.28c	17.41 ± 0.52d	13.01 ± 0.93c	21.81 ± 0.92b	31.55 ± .32a	4.01± 0.14 a	3.38 ± 0.06b	3.13 ± 0.06c
HVG	39.04 ± 2.90 f	51.73 ± 2.89 d	78.08 ± 1.23 b	28.42 ± 0.32a	23.50 ± 0.33b	24.06 ± 0.32b	8.06 ± 0.87d	10.33 ± 0.55cd	12.61 ± 0.36c	3.06 ± 0.08c	2.85 ± 0.06cd	2.65 ± 0.88d
HVR	39.44 ± 1.73 f	52.39 ± 3.19 de	79.38 ± 4.32 b	27.42 ± 0.32a	23.47 ± 0.35b	23.86 ± 0.32b	8.10 ± 0.66d	10.05 ± 0.46cd	12.05 ± 0.56c	2.92 ± 0.07c	2.76 ± 0.06cd	2.71 ± 0.12d
Significance												
VPD	***			***			***			***		
C	NS			NS			**			**		
P	***			***			***			***		
V x C x P	NS			*			*			**		

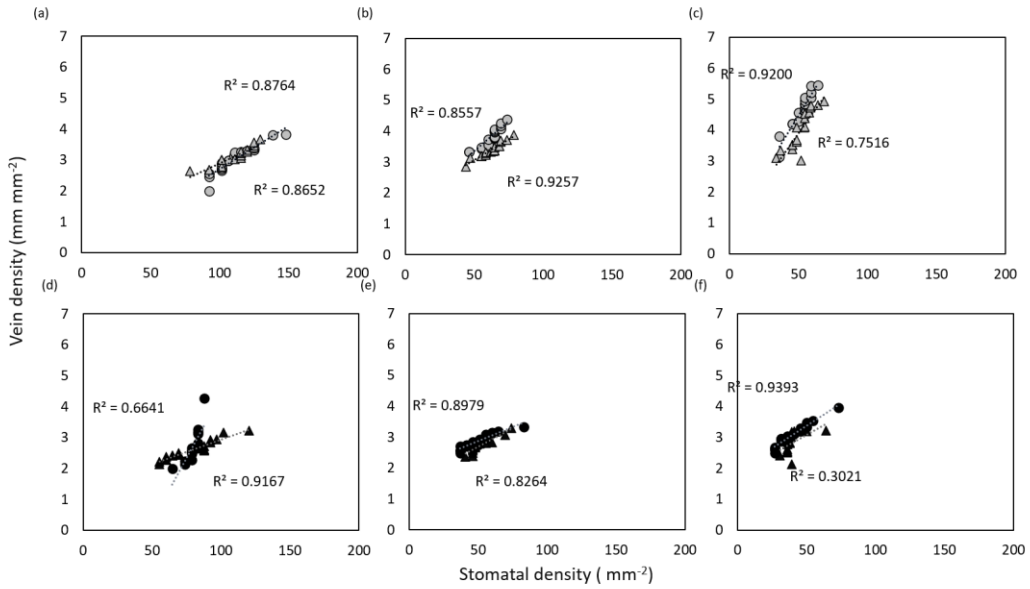

Figure 5. (a), (b), (c) vein and stomatal density relationships of green (circles) and red (triangles) lettuce grown under low VPD (grey), in the bottom (a), middle (b) and apex part (c) of the leaf. (d), (e), (f), vein and stomatal density relationships of green (circles) and red (triangles) lettuce grown under high VPD (black), in the bottom (d), middle (e) and apex (f) part of the leaf. Regression lines and R^2 values are also shown.

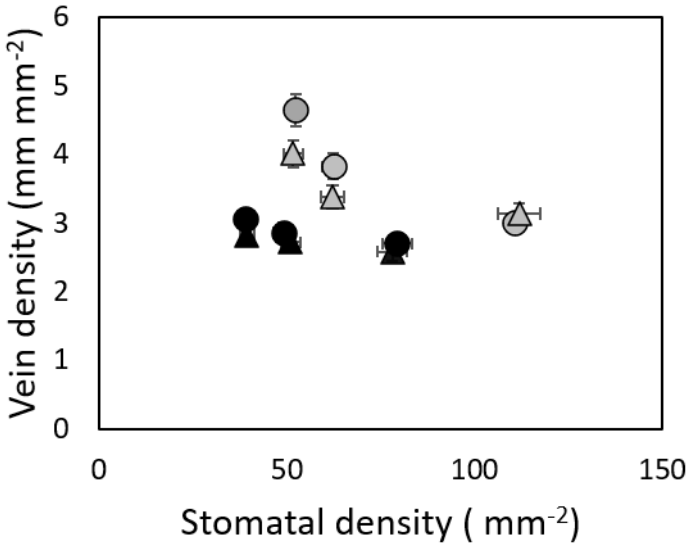

Figure 6. Mean values ± standard errors of vein and stomatal density relationships of green (circles) and red (triangles) lettuce grown under low (grey) and high (black) VPD.

7. 4 Discussion

7.4.1 Response of stomatal and vein densities and coordination with leaf size under different VPDs: So far only a few studies have analyzed how variations in VPD influence stomatal and vein development in the same species, often reporting contrasting results. Moreover, very few information can be found on crop species. More specifically, little is known on the direction of response or coordination (positive/negative) among stomatal and vein densities and their correlation with leaf size in crops. Here, we found increased leaf size under LV compared to HV with no differences between cultivars (**Figure 2**), clearly indicating that leaf size is strictly dependent on VPD. LV leaves also presented increased values of RWC, indicating a better water use. Usually VPD, rainfall and temperature influence leaf size and the global trend is to develop smaller leaves in drier environments (Wright *et al.*, 2017). Under dry air (high VPD), plants with smaller leaves, and a thinner boundary layer easily reduce their heat loads and water demand (Li *et al.*, 2013). Moreover, under a dry environment the relative water content of leaves is commonly reduced (Amitrano *et al.*, 2021; Parkash and Singh, 2020). According to our findings, other greenhouse and indoor trials have also reported smaller leaf size in crops subjected to high VPD levels compared to low VPDs (Jiao *et al.*, 2019; Lu *et al.*, 2015). Furthermore, in *Toona ciliate*, Carins Murphy *et al.* (2012) observed that vein and stomatal densities decreased along with increasing leaf size, especially for smaller leaves developed under high irradiance conditions, suggesting a “dilution” of these leaf anatomical traits with increasing leaf size. However, the same authors in another trial on *T. ciliata* grown under the same light intensity and different VPDs found these anatomical traits to be independent of leaf size (Carins Murphy *et al.,* 2014). Our results are in agreement with this last research, showing no proportional coordination among stomatal density and leaf size as well as vein density and leaf size (**Figure 3**). In the last decades, other studies have supported this idea; for instance, Scoffoni *et al.* (2011) found an independence of minor vein density from leaf size in 10 different species of moist and dry habits. This was confirmed by Sack and Scoffoni (2012) in a study on more than 100 dicotyledonous species. Moreover, here we found a coordination between vein and a stomatal density under both VPD conditions, and more specifically increased density of both anatomical traits in plants under low VPD, the same plants which presented a higher leaf size. An adequate balance between stomata and vein is always required since leaf venation must be enough to supply water to stomata and replenish the water loss due to transpiration in order to maintain an adequate physiological function (Brodribb *et al.*, 2007; Schneider *et al.*, 2017; Scoffoni *et al.*, 2011). Probably, the coordinated development of vein and stomatal densities under both VPDs is an adaptation mechanism of the species trying to maintain the water balance under favorable (LV) and less favorable (HV) environment.

Moreover, in our study there were no significant differences in epidermal cell size between treatments; however, epidermal cell density per leaf area increased under low VPD and was

positively correlated with leaf size both under LV and HV (**Figure 3c**), meaning that epidermal cell number is proportional to changes in leaf area. This suggests that plant regulate the construction of vein and stomata under different VPD conditions, not by increasing the epidermal cell size but actually by developing more cells. Furthermore, we observed a higher stomatal and vein densities at low VPD (higher leaf size; **Figure 2, Table 4, Figures S1**), further supporting that these anatomical traits are not diluted with VPD-induced increasing leaf size. Moreover, in our study there was a reduction in stomatal length under low VPD (**Table 3**). Similarly to our results, smaller stomata have been previously associated with high densities (Franks and Beerling, 2009). However, Giday *et al.* (2013) found that stomatal size and density are not correlated in rose. Size, other than density, is an important trait, since it influences the opening/closing reaction of stomata in response to environmental conditions (Arve *et al.*, 2017). When stomata increment their volume, the surface area to volume ratio decrease and this has been listed as the main reason for their slower response (Drake *et al.*, 2013). However, smaller stomata usually show shorter response times and are able to optimize water fluxes under limiting water availability (Giday et *al.,* 2013). In our study other measured leaf traits (LMA, LDMC) were enhanced in LV (**Table 2**). These traits varied strongly with light, temperature, CO_2 concentration and water stress (Poorter *et al.*, 2009). In particular LMA depends on both LDMC and leaf thickness so much that dividing LMA by LDMC often provides a good estimation of leaf thickness (Vile *et al.*, 2005). Moreover, changes in LDMC have been related to water availability. Therefore, a correlation with other traits (stomatal and vein densities and leaf conductance) has been found (Griffith *et al.*, 2016). The same correlation happens here with higher LDMC associated to higher stomatal and vein density in LV plants. The coordination between leaf hydraulic-related traits (stomatal density and size, vein densities) and the coordination between these anatomical traits and other leaf traits under different VPDs is fundamental because it represents a clear indication of how environmental conditions play a role in the adaptation of leaf anatomical traits.

7.4.2 Acclimation of anatomy to sun and shade within the same leaf: In this study we have examined hydraulic-related traits of lettuce plants along the whole leaves, from the bottom to the apex. Lettuces grow as a rosette, creating in the same leaf different microclimatic conditions, with only the apex part exposed to light. It is known that sun and shade leaves balance different anatomical traits to acclimate to high and low irradiance. Shade leaves are subjected to lower evaporative demand and are in need to maximize light capture, absorption, and processing (Mathur *et al.*, 2018). From a morpho-anatomical point of view, leaves developed under shade are usually thinner, with lower stomatal and vein densities than sun leaves (Martins *et al.,* 2014; Brodribb and Jordan 2011). Our results showed that under different VPDs, lettuce plants maintained a coordinated relationship between stomatal and vein densities. Furthermore, while stomata increased in the part exposed to light (from bottom to apex) independently of the VPD, veins were always denser in plant

exposed to low VPD, along the whole leaf (increasing from the apex to the bottom) (**Table 4**). This probably means that light has a greater influence on stomatal development and relative humidity on vein development, since in both condition stomata incremented in the apex (light exposed) part of the plants, whereas vein incremented in the bottom part and were always more at low VPD (higher relative humidity). Indeed, in the bottom and middle parts of the leaf, covered by other leaves, the microclimatic condition is characterized by a higher relative humidity (Low VPD) and a thicker boundary layer. In dense forests the penetration of light through the canopy it is quite difficult and under such condition lower plants receive and absorb very little light. Different position in the canopy have proved to change anatomical and hydraulic-related traits in trees (Sellin *et al.*, 2019), along a gradient which affect the whole plant photosynthetic capacity and yield (Niinemets *et al.*, 2015). In agreement with our results, high density of stomata has been found in sun leaves of different species (tomatos, sorghum, coffe) (Gay and Hurd, 1975; Jiang *et al.*, 2011; Martins *et al.*, 2014); however very little is known about vein development within leaves and environmental conditions.
The combined influence of light and VPD should be explored further since it could be responsible for the different degree of coordination between these traits. The adjustment of these hydraulic-related traits with microclimatic conditions is fundamental to provide an optimal water and gas fluxes throughout the entire plant (Sellin *et al.*, 2019; Taneda and Tateno, 2011).

7.5 Conclusions: The results of this study suggested that the VPD triggers a different response in lettuce plants in terms of balance of leaf traits. Vein and stomatal densities showed coordinated response under high and low VPD with increments in densities with decreasing VPD. Furthermore, we found a coordination between vein density and leaf size as well as stomatal density and leaf size, thus we can affirm that, in lettuce plants, both stomatal and vein densities are not diluted with leaf size as was hypothesised before by other authors in other species. In the present study, we also suggested that irradiance has a predominant role (compared to VPD) in trigger the formation of stomata but not of veins. Indeed, in the apex part of the leaf, the only one exposed to light, stomatal density was the highest despite the differences in VPD. However, the same relationship does not apply to vein density which was higher under low VPD especially in the bottom part of the leaves, while maintaining the same pattern of stomata density (i.e. increments in the apex followed by middle and bottom part). Nevertheless, positive relationships were always found in vein and stomatal densities from different part of the leaf. Consequently, the allocation of veins and stomata during leaf development is strictly dependent from the microclimatic conditions. Further research is therefore needed to understand the developmental basis for these anatomical traits and their coordination in crops. The plasticity in hydraulic-related traits with microclimatic conditions plays a critical role to provide an optimal water and gas fluxes and helping plants adapt to environmental changing.

7.6 References:

Amitrano C, Arena C, Cirillo V, De Pascale S, De Micco V. 2021. Leaf morpho-anatomical traits in Vigna radiata L. affect plant photosynthetic acclimation to changing vapor pressure deficit. Environmental and Experimental Botany, 104453.

Arve LE, Kruse OM, Tanino KK, Olsen JE, Futsaether C, Torre S. 2017. Daily changes in VPD during leaf development in high air humidity increase the stomatal responsiveness to darkness and dry air. J Plant Physiol **211**, 63-69.

Brodribb TJ, Feild TS, Jordan GJ. 2007. Leaf maximum photosynthetic rate and venation are linked by hydraulics. Plant Physiol **144**, 1890-1898.

Carins Murphy MR, Jordan GJ, Brodribb TJ. 2012. Differential leaf expansion can enable hydraulic acclimation to sun and shade. Plant, Cell & Environment **35**, 1407-1418.

Carins Murphy MR, Jordan GJ, Brodribb TJ. 2014. Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. Plant Cell Environ **37**, 124-131.

Conesa MÀ, Muir CD, Molins A, Galmés J. 2020. Stomatal anatomy coordinates leaf size with Rubisco kinetics in the Balearic Limonium. AoB PLANTS **12**, plz050.

Cornelissen J, Lavorel S, Garnier E, Diaz S, Buchmann N, Gurvich D, Reich PB, Ter Steege H, Morgan H, Van Der Heijden M. 2003. A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. Australian journal of Botany **51**, 335-380.

Drake PL, Froend RH, Franks PJ. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. J Exp Bot **64**, 495-505.

Du Q, Liu T, Jiao X, Song X, Zhang J, Li J. 2019. Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. J Exp Bot **70**, 4949-4962.

Elsherif A, Gaulton R, Shenkin A, Malhi Y, Mills J. 2019. Three dimensional mapping of forest canopy equivalent water thickness using dual-wavelength terrestrial laser scanning. Agricultural and Forest Meteorology **276**, 107627.

Franks PJ, Beerling DJ. 2009. Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. Proc Natl Acad Sci U S A **106**, 10343-10347.

Garnier E, Navas M-L. 2012. A trait-based approach to comparative functional plant ecology: concepts, methods and applications for agroecology. A review. Agronomy for Sustainable Development **32**, 365-399.

Gay A, Hurd R. 1975. The influence of light on stomatal density in the tomato. New Phytologist **75**, 37-46.

Giday H, Kjaer KH, Fanourakis D, Ottosen CO. 2013. Smaller stomata require less severe leaf drying to close: a case study in Rosa hydrida. J Plant Physiol **170**, 1309-1316.

Griffith DM, Quigley KM, Anderson TM. 2016. Leaf thickness controls variation in leaf mass per area (LMA) among grazing-adapted grasses in Serengeti. Oecologia **181**, 1035-1040.

Gupta B. 1961a. Correlation of Tissues in Leaves: 1. Absolute Vein-islet Numbers and Absolute Veinlet Termination Numbers. Annals of Botany **25**, 65-70.

Gupta B. 1961b. Correlation of Tissues in Leaves: 2. Absolute Stomatal Numbers. Annals of Botany **25**, 71-77.

Jiang C-D, Wang X, Gao H-Y, Shi L, Chow WS. 2011. Systemic regulation of leaf anatomical structure, photosynthetic performance, and high-light tolerance in sorghum. Plant Physiology **155**, 1416-1424.

Jiao XC, Song XM, Zhang DL, Du QJ, Li JM. 2019. Coordination between vapor pressure deficit and CO2 on the regulation of photosynthesis and productivity in greenhouse tomato production. Sci Rep **9**, 8700.

Kattge J, Bönisch G, Díaz S, Lavorel S, Prentice IC, Leadley P, Tautenhahn S, Werner GD, Aakala T, Abedi M. 2020. TRY plant trait database–enhanced coverage and open access. Global change biology **26**, 119-188.

Li D, Liu H, Qiao Y, Wang Y, Cai Z, Dong B, Shi C, Liu Y, Li X, Liu M. 2013. Effects of elevated CO2 on the growth, seed yield, and water use efficiency of soybean (Glycine max (L.) Merr.) under drought stress. Agricultural Water Management **129**, 105-112.

Lu N, Nukaya T, Kamimura T, Zhang D, Kurimoto I, Takagaki M, Maruo T, Kozai T, Yamori W. 2015. Control of vapor pressure deficit (VPD) in greenhouse enhanced tomato growth and productivity during the winter season. Scientia Horticulturae **197**, 17-23.

Martins SC, Galmes J, Cavatte PC, Pereira LF, Ventrella MC, DaMatta FM. 2014. Understanding the low photosynthetic rates of sun and shade coffee leaves: bridging the gap on the relative roles of hydraulic, diffusive and biochemical constraints to photosynthesis. PLoS One **9**, e95571.

Mathur S, Jain L, Jajoo A. 2018. Photosynthetic efficiency in sun and shade plants. Photosynthetica **56**, 354-365.

Miksche JP, Berlyn GP. 1976. *Botanical microtechnique and cytochemistry*: Iowa State University Press.

Niinemets Ü, Keenan TF, Hallik L. 2015. A worldwide analysis of within-canopy variations in leaf structural, chemical and physiological traits across plant functional types. New Phytologist **205**, 973-993.

Niinemets Ü, Portsmuth A, Tena D, Tobias M, Matesanz S, Valladares F. 2007. Do we underestimate the importance of leaf size in plant economics? Disproportional scaling of support costs within the spectrum of leaf physiognomy. Annals of Botany **100**, 283-303.

Parkash V, Singh S. 2020. A review on potential plant-based water stress indicators for vegetable crops. Sustainability **12**, 3945.

Poorter H, Niinemets U, Poorter L, Wright IJ, Villar R. 2009. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytol **182**, 565-588.

Ramírez-Valiente JA, Sánchez-Gómez D, Aranda I, Valladares F. 2010. Phenotypic plasticity and local adaptation in leaf ecophysiological traits of 13 contrasting cork oak populations under different water availabilities. Tree Physiology **30**, 618-627.

Royer DL, Wilf P, Janesko DA, Kowalski EA, Dilcher DL. 2005. Correlations of climate and plant ecology to leaf size and shape: potential proxies for the fossil record. American Journal of Botany **92**, 1141-1151.

Ryser P, Urbas P. 2000. Ecological significance of leaf life span among Central European grass species. Oikos **91**, 41-50.

Sack L, Scoffoni C. 2012. Measurement of leaf hydraulic conductance and stomatal conductance and their responses to irradiance and dehydration using the Evaporative Flux Method (EFM). J Vis Exp.

Sack L, Scoffoni C. 2013. Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. New Phytol **198**, 983-1000.

Schneider JV, Habersetzer J, Rabenstein R, Wesenberg J, Wesche K, Zizka G. 2017. Water supply and demand remain coordinated during breakdown of the global scaling relationship between leaf size and major vein density. New Phytologist **214**, 473-486.

Scoffoni C, Kunkle J, Pasquet-Kok J, Vuong C, Patel AJ, Montgomery RA, Givnish TJ, Sack L. 2015. Light-induced plasticity in leaf hydraulics, venation, anatomy, and gas exchange in ecologically diverse Hawaiian lobeliads. New Phytol **207**, 43-58.

Scoffoni C, Rawls M, McKown A, Cochard H, Sack L. 2011. Decline of leaf hydraulic conductance with dehydration: relationship to leaf size and venation architecture. Plant Physiology **156**, 832-843.

Sellin A, Taneda H, Alber M. 2019. Leaf structural and hydraulic adjustment with respect to air humidity and canopy position in silver birch (Betula pendula). Journal of plant research **132**, 369-381.

Taneda H, Tateno M. 2011. Leaf-lamina conductance contributes to an equal distribution of water delivery in current-year shoots of kudzu-vine shoot, Pueraria lobata. Tree Physiology **31**, 782-794.

Torre S, Fjeld T, Gislerød HR, Moe R. 2003. Leaf anatomy and stomatal morphology of greenhouse roses grown at moderate or high air humidity. Journal of the American Society for Horticultural Science **128**, 598-602.

Uhl D, Mosbrugger V. 1999. Leaf venation density as a climate and environmental proxy: a critical review and new data. Palaeogeography, Palaeoclimatology, Palaeoecology **149**, 15-26.

Valladares F GE, Gómez JM. 2007. Ecological limits to plant phenotypic plasticity. The New Phytologist 176: 749-763.

Vile D, Garnier E, Shipley B, Laurent G, Navas M-L, Roumet C, Lavorel S, Díaz S, Hodgson JG, Lloret F. 2005. Specific leaf area and dry matter content estimate thickness in laminar leaves. Annals of Botany **96**, 1129-1136.

Violle C, Navas ML, Vile D, Kazakou E, Fortunel C, Hummel I, Garnier E. 2007. Let the concept of trait be functional! Oikos **116**, 882-892.

Wang J-H, Cai Y-F, Li S-F, Zhang S-B. 2020. Photosynthetic acclimation of rhododendrons to light intensity in relation to leaf water-related traits. Plant Ecology, 1-14.

Witkowski E, Lamont BB. 1991. Leaf specific mass confounds leaf density and thickness. Oecologia **88**, 486-493.

Wright IJ, Dong N, Maire V, Prentice IC, Westoby M, Díaz S, Gallagher RV, Jacobs BF, Kooyman R, Law EA. 2017. Global climatic drivers of leaf size. Science **357**, 917-921.

Yates MJ, Verboom GA, Rebelo AG, Cramer MD. 2010. Ecophysiological significance of leaf size variation in Proteaceae from the Cape Floristic Region. Functional Ecology **24**, 485-492.

Zhang D, Jiao X, Du Q, Song X, Li J. 2018. Reducing the excessive evaporative demand improved photosynthesis capacity at low costs of irrigation via regulating water driving force and moderating plant water stress of two tomato cultivars. Agricultural Water Management **199**, 22-33.

7.7 Supplemental material:

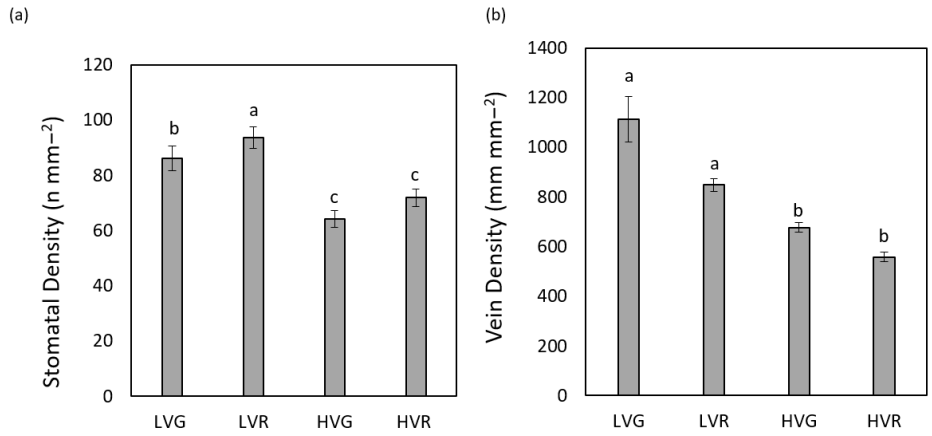

Figure S1. (a) stomatal density and (c) vein density of green and red Salanova lettuces grown under low and high VPD. Mean values ± standard errors are shown. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

Chapter 8

The interaction of VPD with other environmental parameters drives the morpho-anatomical development and the photosynthesis of lettuce plants in a vertical farm

8.1 Introduction: In controlled environment agriculture (CEA) crop growth is regulated by the combination of many environmental factors (e.g. temperature, relative humidity, CO_2, light, nutrient solution). Among these factors the vapor pressure deficit (VPD) has a fundamental role. It combines the effects of temperature (T) and relative humidity (RH) and influences the whole transpiration rates of a cultivation (Devi, 2018). VPD has also been recognized to change the physiological behavior of plants, in terms of gas-exchange, photosystems activity and the leaf internal structure (Amitrano et al., 2019). Although over the years there have been contrasting results (often due to species-specific dynamics), the general trend is to have high rates of photosynthesis and stomatal conductance associated to a reduced transpiration in low VPD environment. Many evidence have suggested that under low VPD, plants increase their biomass and yield, also developing a morpho-anatomical structure of their leaves which allows them to better react to environmental changes. For example, in our previous study on *V. radiata (*Chapter 3*),* plants developed under high VPD and then transferred under low VPD were indeed limited in their ability of acclimating to the new and more favorable environmental conditions by their leaf anatomical structure (coordinated reduction in stomatal and vein density) (Amitrano et al., 2021). There are many studies showing that high VPD reduces stomatal conductance, thereby affecting photosynthesis and growth (Wong, 1993; Bunce, 1996; Ottosen et al., 2002; Bunce, 2003; Ben-Asher et al., 2013); recent research has correlated this physiological reduction to the leaf anatomical development, which is impaired by the high VPD condition. For example, a reduced number of stomata was associated to a smaller dimension in various cultivars of *Rosa hybrida* L. plants subjected to high compared to low VPD (Fanourakis et al., 2013). Similar results were obtained in *Vicia faba* L. plants by Aliniaeifard et al. (2014). However, there is still a lack of information on the combined effects of VPD with other environmental factors. Among these factors, we focused our attention on light intensity, CO_2 enrichment and salinity stress which are easy to control and modulate in CEA. Moreover, there is proof that the modulation of all these parameters alone, affects plant morphological development, thus having an impact on photosynthesis, yield and production. For instance, it is ascertained that light intensity is a key factor

which can modify crop growth and physiological functions (Feng et al., 2019). The variation of light intensity has conspicuous effects on leaf external and internal morphology, its physiological behavior and secondary metabolites production (Kong et al., 2016). Usually, high-light grown plants presents high rates of net-photosynthesis (P_N) correlated to a reduction in specific leaf area (SLA), increments in leaf lamina thickness and cuticle thickness (Fanourakis et al., 2016; Fanourakis et al., 2019). When plants are subjected to salt stress, due to high E.C. (Electric Conductivity) of the substrate, their yield is almost always reduced, depending on the severity of the stress (Buck et al., 2004). Moreover, a few studies have found in tomato plants (*Solanum lycopersicum* L.) a correlation between salt stress and light intensity, pointing out that when light is lower than 200 µmol m^{-2} s^{-1} there is no effect on plant growth up to a nutrient solution EC of 8 dS (considered a very high concentration). When tomato plants are grown at a higher light intensity (400-1000 µmol m^{-2} s^{-1}) plant growth is reduced by the same EC value (Xu et al., 1995; Buck et al., 2004). Altered morphology and reduced growth rate are known to occur under salinity in many crops with consequent reduction in the economic yield (Hendawy and Khalid, 2005). In basil, Bernstein et al. (2010) found a reduction in plant height, root length and leaf area, with increasing salinity.

It is known that increasing atmospheric CO_2 will affect plant growth, including mitigation of stress impact, especially drought; such effects vary considerably between group-species (AbdElgawad et al., 2015). Indeed, atmospheric CO_2 enrichment increases carbon assimilation and improves water economy, resulting in reductions in stomatal conductance (g_s) (Heath, 1998). Crops and herbaceous species are believed to display large reductions in g_s under elevated CO_2 (Morison, 1998). However, in addition to carbon dioxide, stomata respond to a variety of environmental conditions and endogenous factors (e.g. light quantity and quality, VPD, ABA, air pollutants, temperature) so there are still some uncertainties about the capacity for acclimation which may moderate the stomata response to CO_2 (Morison 1998). Moreover, CO_2, as the other factors listed till now, can have an impact on leaf anatomical development. For instance, in two tropical species (*Jatropha grossypifola* L and *Alternanthera crucis* Moq.), Rengifo et al. (2002) found increases in both bundle sheath and leaf thickness due to thicker palisade and spongy parenchymas; however, these effects disappeared with time, suggesting that there was an acclimation in leaf anatomy proceeding with the experiment. Manipulating the indoor climate such as humidity, temperature and CO_2 levels may offset the negative effect of high salinity on yield and fruit quality (Dorai et al., 2001).

In this chapter, we present two experiments (E1, E2) performed in a multi-layer vertical farm to understand the interaction between VPD and other environmental factors (light intensity, salt stress and CO_2 enrichment). More specifically E1 focuses on the interaction between 2 levels of VPDs and 3 different light intensities to understand how it affects crop morpho-anatomical development, growth, and photosynthesis, and select the most appropriate conditions for cultivation in an indoor system. Whereas E2 focuses on how the modulation of aerial environmental parameters (CO_2 and

VPD) can offset the negative effect of sudden increments in salinity in controlled environment agriculture, always focusing on the structure-to-function relationships and looking at the short-term dynamic acclimation of lettuces subjected to a changing environment (EC, CO_2).

8.2 Material and Methods

8.2.1 Experimental design: Both experiments were conducted at the Controlled Environment Agriculture Center of the University of Arizona (UA-CEAC) in a multi-layer vertical farm research facility (**Figure 1**). The controlled growth room has a total cultivation area of 22.5 m^2 and contained two hydroponic growth racks, each composed of 3 layers of growing beds; each rack had an independent 1135L nutrient solution tank. The experiments were conducted on green and red butterhead salanova lettuces (*Lactuca sativa* L. var. *capitata*). Lettuce seeds were germinated in rockwool cubes employing an ebb and flow hydroponic culture technique in a greenhouse under ambient conditions. Lettuce seedlings were transplanted onto the deep-water culture hydroponic bed 14 days after sowing. For the experiments, 2 racks with three growing beds of 1.2 m × 2.4 m each, were used to hold 78 plants each, with a plant density of 21 plants m^2 for a total of 468 plants. Every hydroponic bed had independent LED lighting system (Eclipse F3 LED bar, www.illumitex.com) located at 0.4 m above the bed level. The spectral composition of the light was set in the PF lighting control software at 80% of red, 15% of blue and 5% of green. Detailed description of the facilities and crop system can be found in Caplan (2018). The environmental conditions were monitored and controlled for the entire duration of the experiments. The aerial zone parameters monitored were temperature, relative humidity from which VPD values were calculated, CO_2 concentration, and PAR. The root zone parameters were monitored in terms of electrical conductivity (EC), pH, and dissolved oxygen (**Figure 2**). In both experiments the photoperiod was 12 hours (8am-8pm) and the high VPD condition was achieved by means of a dehumidifier (90L/D Parkoo; Guangdong, China). The duration of each cycle was 23 days.

E1: E1 was conducted on green (G) and red (R) Salanova lettuces to understand the relationships between VPD levels (low, L = 0.7 kPa; high, H = 1.4 kPa) and increasing Daily Light Integral (DLI 1, 2, 3):

DLI 1. 8.6 (200 µmol photon $m^{-2}s^{-1}$ PPFD, photosynthetic photon flux density);

DLI 2. 12.9 (300 µmol photon $m^{-2}s^{-1}$ PPFD);

DLI 3. 15.5 (360 µmol photon $m^{-2}s^{-1}$ PPFD).

78 plants per layer (39 green and 39 red) were grown in two different cycles under low and high VPD to study morpho-physiological changes and to determine the optimal DLI and VPD for lettuce growth.

E2: In E2, green (G) and red (R) Salanova lettuces were grown in two cycles under low and high VPD under a light intensity of 300 µmol $m^{-2}s^{-1}$ (12.9 DLI) in two rack (Control;C and Stress;S). After 10 days after the transplant into the vertical farm, EC was enhanced in one rack (S) from 1.8 to 3.5 µS cm^{-1} by adding NaCl, producing a sudden salt stress. 5 days after the salt stress, CO_2 injection started in the whole room, in order to bring CO_2 concentration from 400 to 1200 ppm. We hypothesized that The CO_2 enrichment would mitigate the salt stress, modifying the plant carbon gain/water balance and that these mechanisms would be different in high and low VPD plants, depending on their different morphological structure.

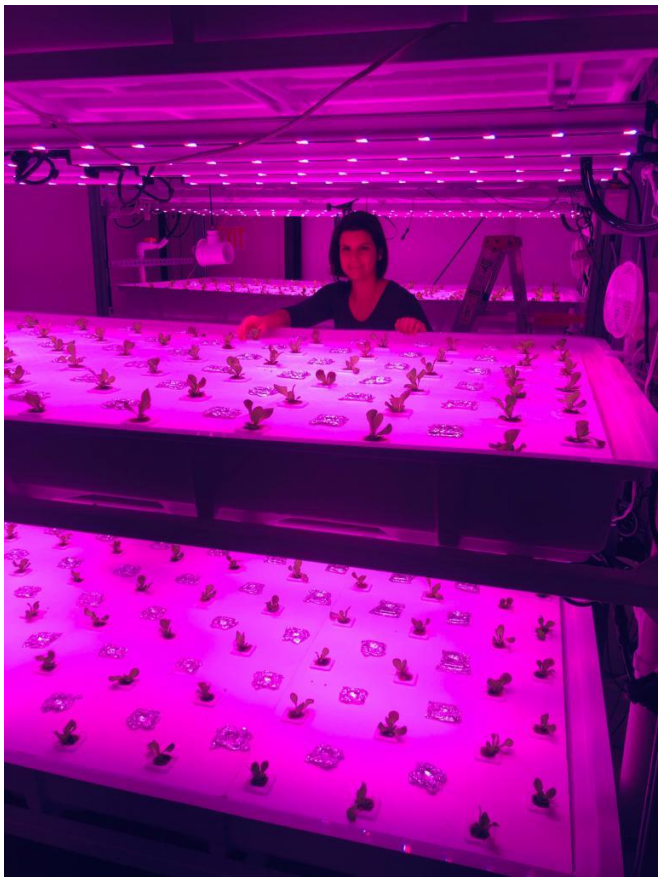

Figure 1: Lettuce transplanting in the multi-layers vertical farm at the UA-CEAC.

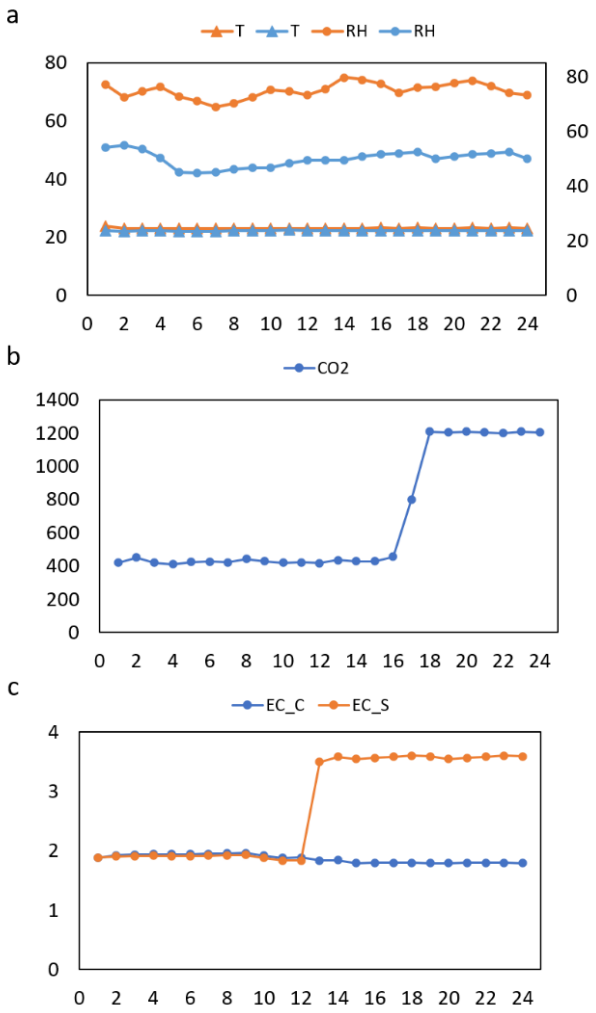

Figure 2: Environmental data in terms of a) temperature (triangles) and relative humidity (circles) under L (orange lines) and H (blue lines) VPD; b) CO_2 enrichment in E2; c) Electric Conductivity for control (C; blue line) and stress (S; orange line) racks.

***8.2.2 Growth measurements**:* At the beginning of each experiment, 5 plants (in excess after transplanting) were used to calculate the fresh and dry biomass as well as the plant area. Fresh biomass (FB) was calculated by removing the roots and weighing the samples by means of a weighing scale. To calculate the dry biomass (DB), samples were oven dried at 60°C for at least 3 days, till they reached a constant weight. Plant area was calculated by imaging each plant on a white board and then calculating the area of the epigeal part by means of digital image analysis (Image J software; national Institutes of Health, Bethesda, Maryland, USA). These initial data were used to compare the plant homogeneity of the two growth cycles. The last day of experiment 20 plants per condition were harvested to repeat the growth measurements (number of leaves, fresh and dry weight of the epigean plant organs, plant area). In E2, every 6 days, at least 5 plants per treatment

were harvested and the growth analyses were repeated to follow the growth in the two racks subjected to the different EC values.

8.2.3 Leaf morpho-functional traits: Leaf functional traits were evaluated at the end of the growth period on 6 plants per treatment following Cornelissen et al. (2003). Firstly, leaves were scanned to calculate Leaf Size (LS; lamina area in mm^2) using ImageJ software. Then, FW of each leaf was recorded, and the leaf petiole was submerged in distilled water in the dark for 48h and then re-weighted to calculate the Saturation Weight (SW); whereas, DW was obtained by oven-drying leaves at 60 °C for at least 3 days, until they reached a constant weight. These parameters were used to evaluate: the water status of the leaves, measured as relative water content (RWC %) and expressed as percentage of (FW−DW)/(SW−DW); the leaf dry matter content (LDMC) considered a proxy for leaf tissue density (Ryser and Urbas, 2000) and expressed as (DW/SW) in gg^{-1}; Leaf Mass per Area (LMA), calculated as the ratio between DW and LS (g mm^{-2}) which is used as proxy for sclerophylly (Witkowski and Lamont, 1991); Specific Leaf area (SLA), calculated as the ratio between LS and DW (mm^2g^{-1}) (Witkowski and Lamont,1991).

8.2.4 Gas-exchanges: In E1 Gas-exchange measurements were carried out at the end of the growth period, with LI-6400 (LI-COR Biosciences, Lincoln, NE, USA). Measurements were taken at ambient CO_2 concentration (400 µmol) and at a photosynthetic active radiation of 1000 µmol $m^{-2}s^{-1}$, which based on previous experiments (chapter 4) was considered the saturation irradiance for the species. Measurements were performed from 10 to 2 am on a fully expanded leaf per 10 plants per treatment. In E2, the same measurements were carried out before and after the CO_2 injection. The following parameters were calculated: P_N (net-photosynthesis), the CO_2 assimilation, measured in µmol CO_2 $m^{-2}s^{-1}$; g_s (the stomatal conductance), measured in mol H_2O $m^{-2}s^{-1}$; and Tr Transpiration, measured in mmol H_2O $m^{-2}s^{-1}$.

8.2.5 Morpho-anatomical analyses: At the end of each experiment, 1 leaf per 6 plants per treatment were sampled from the plant and promptly fixed in the FAA fixative solution (40 % formaldehyde, glacial acetic acid, 50% ethanol, 5:5:90 by volume). Samples were transferred to the PWA lab (Plant and Wood anatomy lab) at the Department of Agricultural Sciences of the University of Naples “Federico II” in order to assess any differences in leaf morpho-anatomical developments among treatments. Each leaf was dissected to obtain 3 pieces of about 10x10 mm of the leaf lamina, in order to accomplish the quantification of:

i) morpho-anatomical traits of leaf lamina tissues;

ii) stomatal traits;

iii) vein traits.

8.2.5.1 Morpho-anatomical analyses of leaf lamina: 5 × 5 mm portions of the leaf lamina were dehydrated in an ethanol series (50, 70 and 95%) and embedded in the JB4 acrylic resin (Polysciences, USA). Thin cross sections (5 µm thick) were cut by means of a rotary microtome, stained with 0.025% toluidine blue and mounted with distilled water (Feder and O'brien, 1968). Sections were analyzed under the BX510 light microscope (Olympus, Germany) and digital images were collected and analyzed through the digital camera Olympus EP50 and Olympus CellSens 3.2 software, to quantify palisade parenchyma thickness (PT) (µm) and spongy parenchyma thickness (ST) (µm). All the thickness measurements were taken in 3 positions along the lamina, avoiding veins and damaged areas.

8.2.5.2 Stomatal traits: Stomatal traits were determined by microscopic measurements of leaf abaxial peels, taken centrally in each part of the leaf lamina, avoiding the midrib as well as the margin. For each leaf, measurements were averaged from 5 measurements obtained from 3 different peels of each leaf. Each field view was set at 50x magnification (field area of 0.15 mm^2) Stomatal density was measured as number of stomata per mm^2 of the leaf area using ImageJ Software. Stomatal length and width were determined considering the length and width (µm) of 5 stomata per field. To calculate stomatal area in E2, the size of 5 stomata per field both guard cell minor and major axes were used to calculate the area of an imaginary ellipse as reported in Sorrentino et al. (2021).

8.2.5.3 Vein traits: To determine vein traits a portion of each leaf lamina was chemically cleared C_3H_6O in aqueous solution and bleached in EtOH dilution series, following Berlyn and Miksche (1976). In order to highlight even the smallest veins, cleared leaves were double stained with safranin and fast greens. More specifically, leaves were kept submerged in 1% safranin in EtOH for 10 minutes, gently rinsed with 100% EtOH, submerged in 1% fast green in EtOH for a few seconds and rinsed again with 100% EtOH. Using a light microscope (BX51; Olympus) equipped with the EP50 camera, each leaf was imaged in 5 field of view at a magnification of 4x (field area of 0.033 mm^2). From those pictures minor vein density (VLA) was calculated using image J software, as the ratio of the sum of vein lengths of 4° veins and higher and the difference between the area of the image and the area occupied by the 2° veins, expressed in mm mm^{-2} (Sack and Scoffoni, 2013; Amitrano et al., 2021).

8.2.6 Statistical analyses: The statistical analyses were all carried out using the software IBM SPSS Statistics (SPSS, Chicago, IL, USA). In E1 the influence of the different independent factors (VPD, cultivar and light intensity) on the dependent variables was tested by applying a three-way analysis of variance (ANOVA) and mean values were separated according to Tukey test with $p < 0.05$. In E2 the influence of the different independent factors (VPD, cultivar, salt stress) on the dependent variables was tested by applying a three-way ANOVA. Also in E2, gas exchanges and leaf traits data (collected before and after the CO_2 injection) were subjected to a four-way ANOVA,

also considering the data (before and after the injection) as main factor. Being the interactions between factors significant, in this chapter, the results of one-way ANOVA are presented.

8.3 Results E1

8.3.1 Growth: The combined effect of VPD, cultivar and light intensity elicited significant changes in Salanova plants (**Figure 3**). More specifically, on average L VPD conditions induced the development of more leaves per plant (**Figure 3a**), at the intermediate and high DLI. Plants at L and H VPD followed the same trend, presenting higher values at light intensity 2, followed by 3 and 1. Overall, LR2 presented the highest number of leaves followed by LG2 and LR3, LG3, HG2 and HR2, HG3, HR3, LG1, LR1. Lowest values were found in HG1 and HR1.Similarly, plant area (**Figure 3b**) was always higher in L compared to H VPD plants and values decreased with increasing light intensity. Highest values were found in LG1 and LR1 followed by LR2, LR3, LG2 and HR1, HG1, HG2, HR2. Lowest values were recorded in HG3 an HR3.Both fresh and dry weight (**Figure 3c,d**) increased with light intensity level (from 1 to 3). For FW, however, no significant differences were found between light intensity 1 and 2 and between L and H VPD. In light intensity 3, the only significant difference was found between LG and HG plants with reductions in HG by 5%. Concerning DW, L VPD plants presented slightly highest values compared to H VPD. However, these differences were not always significant. The significant differences between L and H plants were found in dry weight between LG1 and HG1, LR2 and HR2, LG3 and HG3 with 13% reduction and LR3 and HR3 22% reduction.

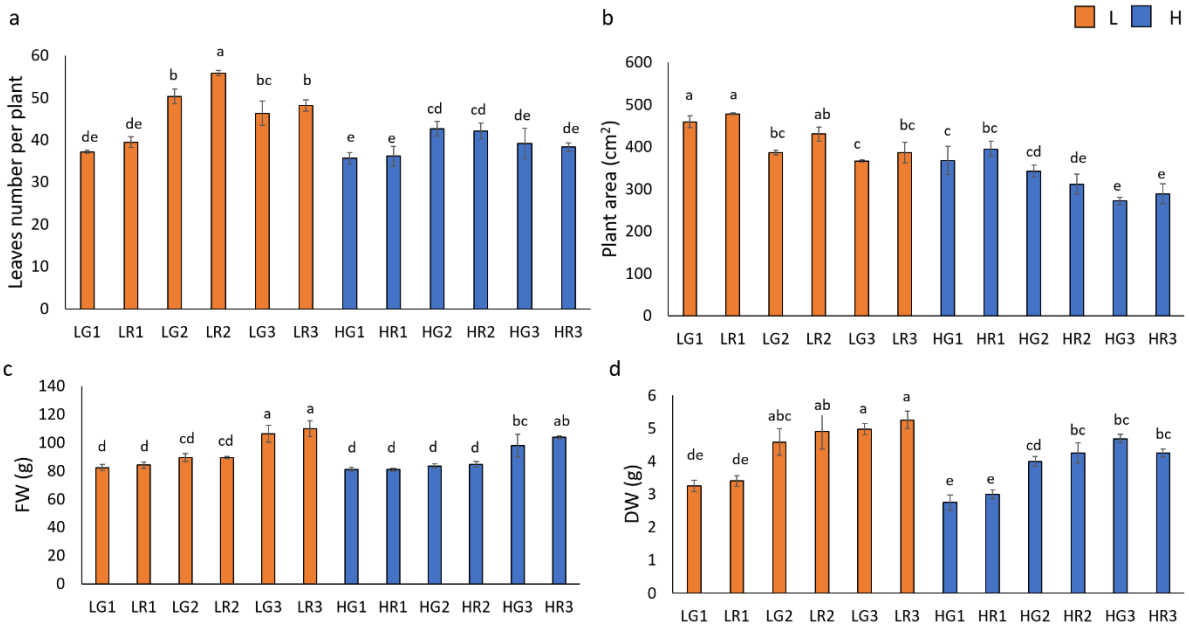

Figure 3: Growth parameters in terms of a) leaves number per plant, b) plant area, c) fresh weight and d) dry weight of Salanova Green (G) and Red (R) plants grown under the three different light intensities (1: 8.6 DLI;

2: 12.9 DLI; 3: 15.5 DLI) at low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.3.2 Morpho-functional traits: Leaf morpho-functional traits were influenced by the interaction of VPD, light intensity and cultivar (**Figure 4**). Leaves from both green and red plants resulted more expanded (**Figure 4a**), when grown under low VPD; however, this trend changed when exposed to different light intensities. Indeed, the lowest light intensity (G1, R1) determined an increment in leaf area: leaf area decreased together with increments in light intensity under L VPD. However, when exposed to the highest light intensity (G3 and R3) no differences in leaf-area were recorded between L and H VPD. Overall, LG1 and LR1 presented the highest leaf area followed by LG2 and by LR2 and HR1. Lowest values were found in HR2. Conversely LMA (**Figure 4b**), was highest in H plants and incremented together with increments in light intensity in both VPD condition. Highest values were found in HR3 and HG3, followed by LG3, LR3, HG2, HR2, HG1, HR1, LG2 and LG3; whereas lowest values in LG1 and LR1.Concerning LDMC (**Figure 4c**), in agreement with LMA, its values where higher under H VPD notwithstanding the different light intensities. However, L and H VPD plants showed a different trend with varying light intensity. L VPD plants presented a higher LDMC in G3 and R3 and lower values, with no significant differences among them in the other conditions (G and R1, G and R2). Differently, H VPD plants presented higher LDMC in both G1, R1, G3 and R3 (with no differences) and lowest values in G2 and R2. Overall, highest values were found in HR1, HG3 and HR3 and lowest in LG1, LR1, LG2, LR2.

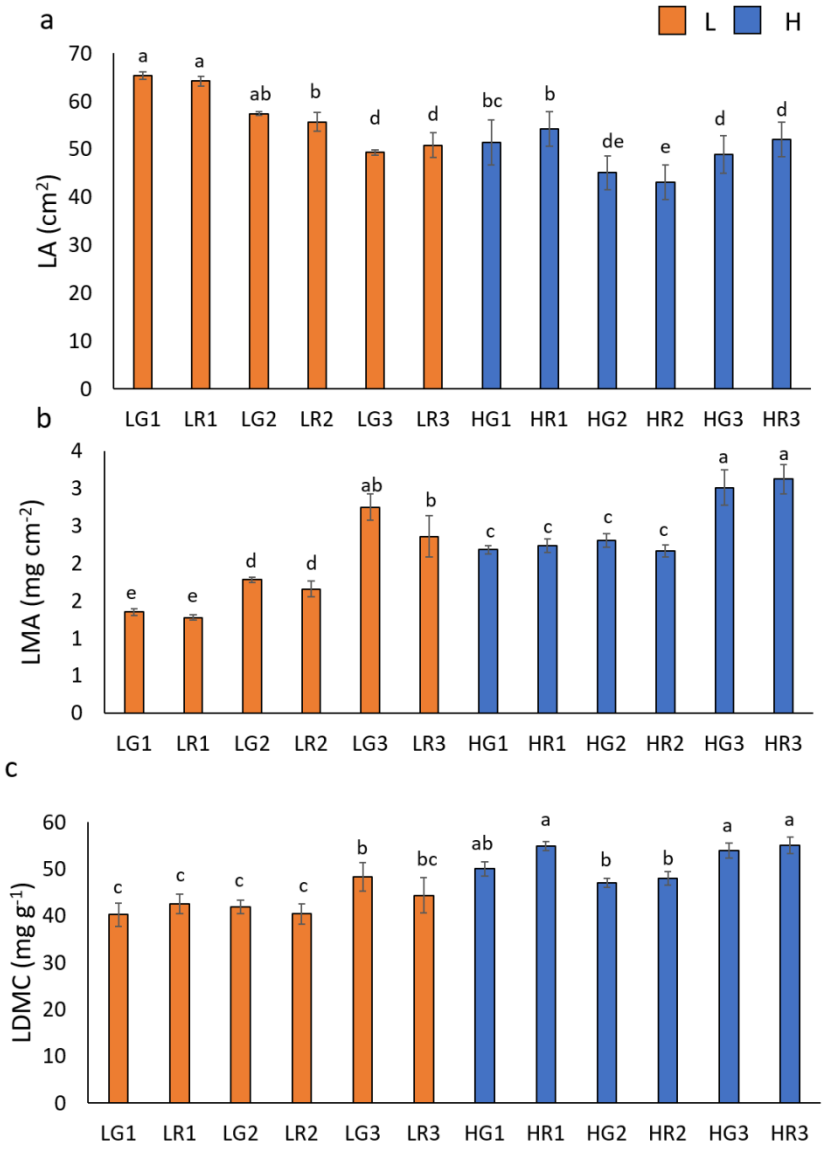

Figure 4: Leaf morpho-functional traits in terms of a) LA, leaf area; b) LMA, leaf mass per area; c) LDMC, leaf dry matter content of Salanova Green (G) and Red (R) plants grown under the three different level intensities (1: 8.6 DLI; 2: 12.9 DLI; 3: 15.5 DLI) at low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.3.3 Morpho-anatomical traits

8.3.3.1 Leaf lamina: The thickness of palisade and spongy parenchymas was influenced by the interaction of VPD and light intensity and cultivar (**Figure 5**). Palisade and spongy thickness followed an opposite pattern of variation among treatments. Palisade parenchyma thickness (**Figure 5a**) was enhanced in L VPD compared to H VPD, moreover it increased according to increasing light intensity. Overall, highest values were found in LG3 followed by LR3, LG2 and LR2, LG1 and LR1, HG3, HR3, HR2, HG2; and lowest values in HG1 and HR1. Conversely, spongy parenchyma thickness (**Figure 5b**) was highest in H VPD and decreased according to increasing light intensity.

Overall, highest values were found in HG1 and HR1, followed by LG1 and LR1, HG2 and HR2, HR3 and HG3, LG2 and LR2; lowest values in LG3 and LR3.

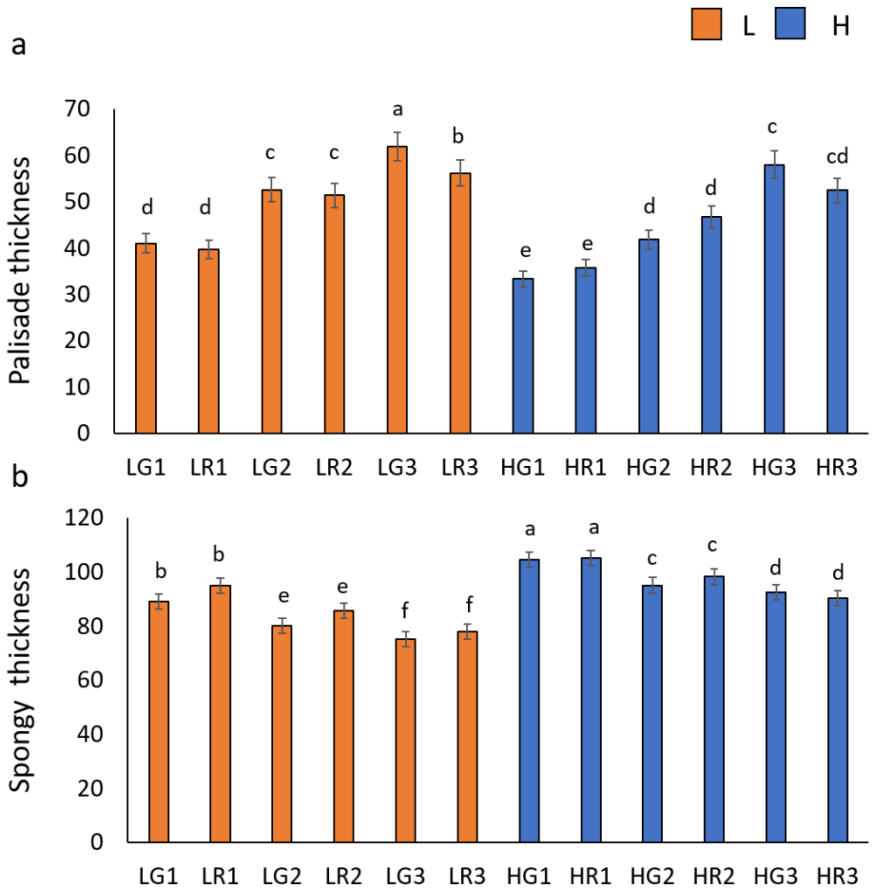

Figure 5: Leaf lamina thickness in terms of a) palisade thickness; b) Spongy thickness of Salanova Green (G) and Red (R) plants grown under the three different level intensities (1: 8.6 DLI; 2: 12.9 DLI; 3: 15.5 DLI) at low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.3.3.2 Stomata: Stomatal density and size were influenced by the interaction of VPD and light intensity and cultivar (**Figure 6**). Changes in stomatal density (**Figure 6a**) were mostly influenced by light intensity; indeed, under low light (1) no differences were detected between L and H VPD whereas under medium light (2) L VPD plants increased significantly the density of stomata, compared to H-VPD. Ultimately, under high light (3) the highest stomata density was found in H VPD compared to L VPD. Overall, highest values were found in HG3, HR3, followed by LG3 and L33, then by LG2 and LR2, HG2 and HR2, and lowest values in G1 and R1 notwithstanding the VPD.Many significant differences were found in stomatal length (**Figure 6b**). More specifically, L VPD plants presented higher values compared to H VPD. Stomata length decreased together according to increasing light intensity in both L and H VPD plants. Overall, highest values were found in LG1 and LR1, followed by LG2 and LR2, HG1 and HR1, HG2 and HR2, LG3 and LR3. Lowest values were

found in HG3 and HR3. Stomatal width did not change among treatments, exception made for HG1 and HR1 where the highest values were detected.

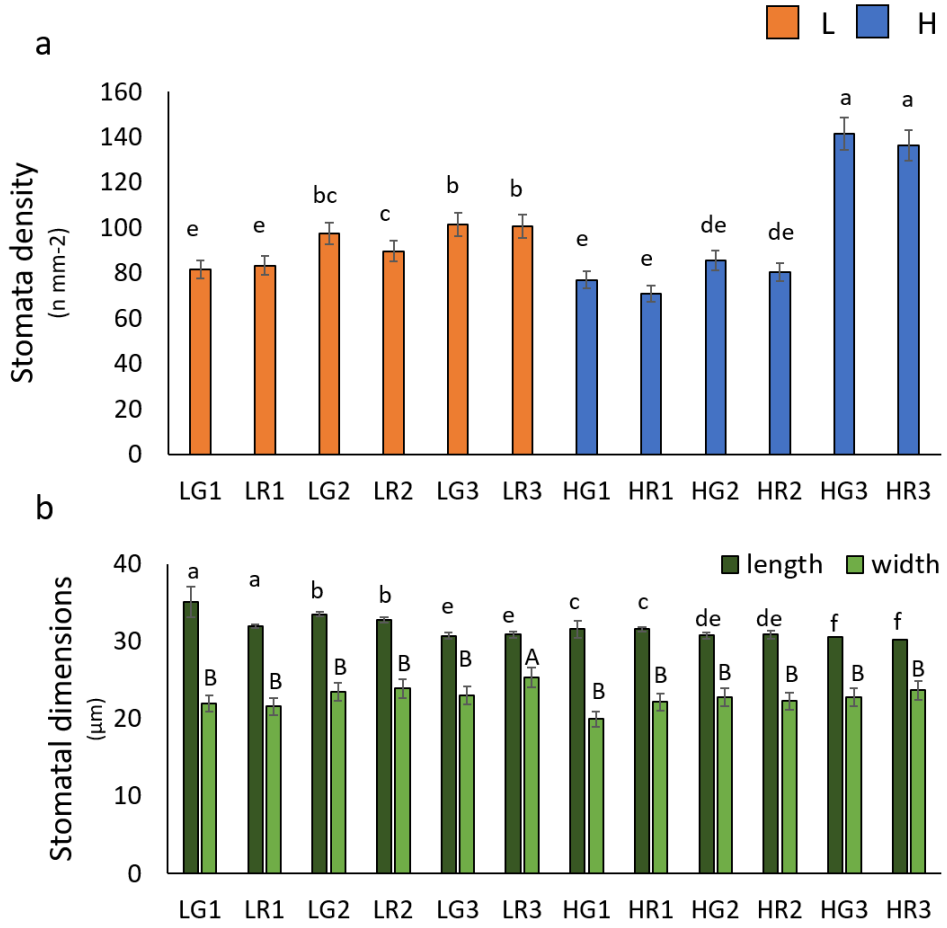

Figure 6: stomatal traits in terms of a) stomatal density; b) Stomatal length and width of Salanova Green (G) and Red (R) plants grown under the three different level intensities (1: 8.6 DLI; 2: 12.9 DLI; 3: 15.5 DLI) at low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.3.3.3 Veins: Vein density was influenced by the interaction of VPD, light intensity and cultivar (**Figure 7**). Changes in vein density were influenced by light intensity; indeed, under low light (1) no differences were detected between L and H VPD whereas under medium light (2) L VPD plants increased significantly the density of stomata, compared to H VPD. Ultimately, under high light conditions (3), vein density was higher in H VPD than L VPD. Overall, highest values were found in HG3 and HR3 plants, followed by LG3 and LR3, LG2 and LR2, HG2 and HR2, and lowest values in G1 and R1 notwithstanding the VPD.

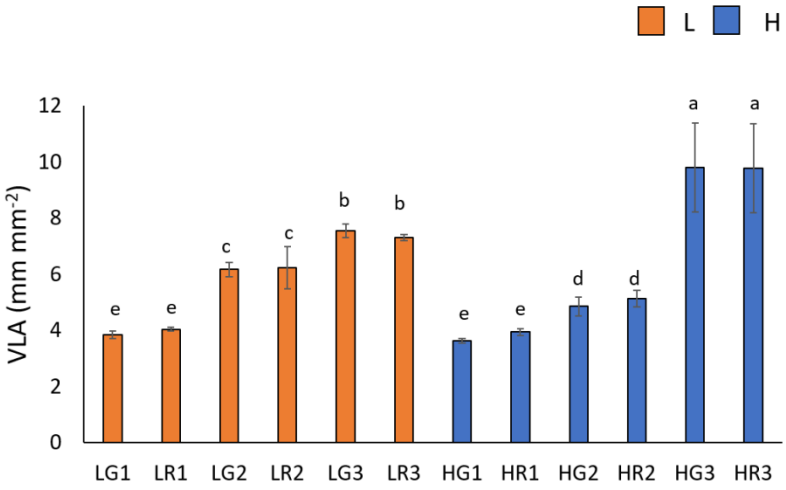

Figure 7: Leaf vein density of Salanova Green (G) and Red (R) plants grown under the three different level intensities (1: 8.6 DLI; 2: 12.9 DLI; 3: 15.5 DLI) at low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.3.4 Gas-exchanges: VPD, light intensity and cultivar strongly influenced the gas-exchange capacity (**Figure 8**). Net-photosynthesis (PN) (**Figure 8a**) was always higher in L compared to H VPD plants, notwithstanding the light intensity. Moreover, in L VPD plants photosynthesis was always enhanced in red Salanova compared to green one. Concerning the light intensity, in both L and H VPD plants, there was a linear decrease in net-photosynthesis together with decreasing light intensity. Overall, highest values were found in LR3, followed by LG3, LG2 and LR2, LR1 and HG3 and HR3, HG2 and HR2. Lowest values were found in HG1 and HR1.Similarly, stomatal conductance (gs) (**Figure 8b**) was enhanced in L VPD plants compared to H VPD. In L plants stomatal conductance was enhanced in red plants only grown at the highest light intensity (R3), whereas no significant differences were found at the other light intensities between G and R plants. Conversely, in H plants g_s was enhanced in red plants only when grown at the lowest light intensity (R1). Concerning the light intensity, g_s decreased at the lowest light intensity (1) in both green and red plants grown under L and H VPD (L and H G1, R1). Differently from net-photosynthesis, there were no significant differences between plants grown at 2 and 3 light intensity, exception made for LR3. Overall, LR3 presented highest values followed by LG3 and LG2 and LR2 then by LG1, LR1, HG2, HR2, HG3, HR3 which were in turn higher than HR1. Lowest values were found in HG1.By contrast transpiration (**Figure 8c**) was enhanced by the high VPD condition, exception made for the low light intensity (1) where no significant differences were found between H and L plants. Differently, significant differences were found between light intensities 2 and 3 with increments in transpiration under the high light condition (3) both in H and L plants. Concerning the cultivar, no significant differences were found between green and red plants. Overall, highest values were found in HG3

and HR3, followed by HG2 and HR2, then by LG3 and LR3 in turn higher than LG2 and LR2. Lowest values were found in G1 and R1 both and L and H VPD.

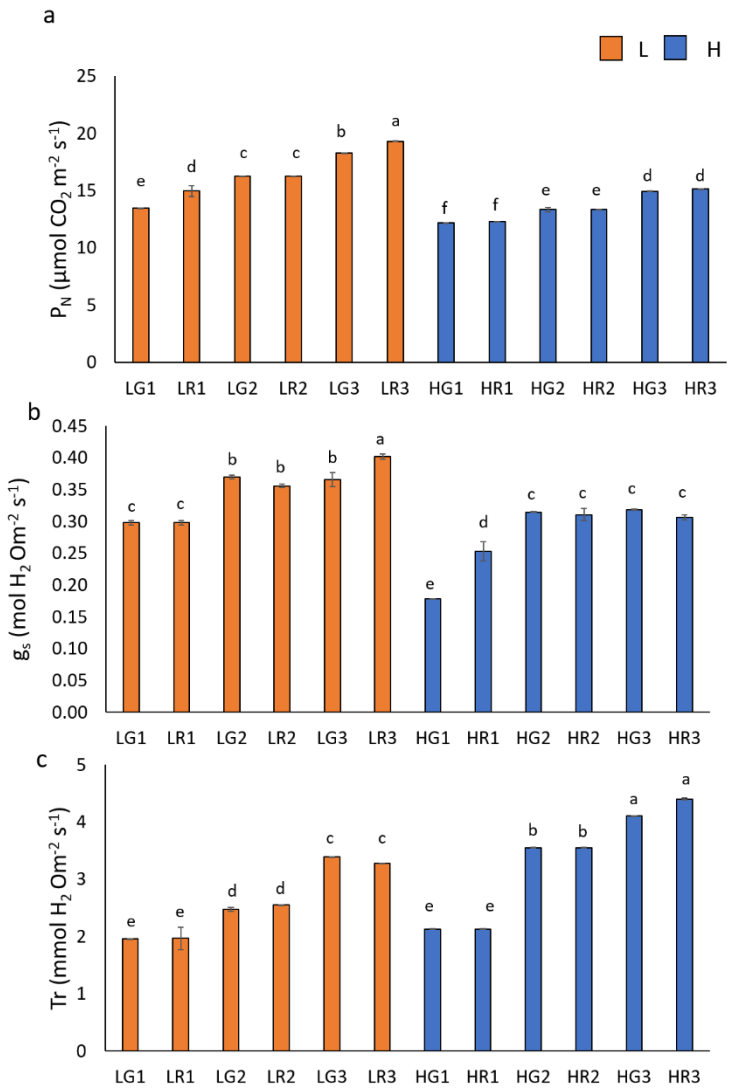

Figure 8: Leaf gas-exchange in terms of a) net-photosynthesis (PN); b) stomatal conductance (gs); Transpiration (Tr) of Salanova Green (G) and Red (R) plants grown under the three different level intensities (1: 8.6 DLI; 2: 12.9 DLI; 3: 15.5 DLI) at low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.4 Results E2

8.4.1 Growth: Growth dynamics in terms of fresh and dry weights (FW and DW) is represented in **Figure 9** and was strongly influenced by L (**Figure 9a,c**) and H VPD (**Figure 9b,d**) in combination with the modulation of the other environmental factors. For both FW (**Figure 9a,b)** and DW (**Figure 9c,d)** it is evident how the curves started to separate at about 12 DAS after the EC stress began in rack 2 (S). However, plants at L and H VPD behave differently with more evident decrease in fresh

and dry weight under L VPD compared to H VPD. It is interesting to notice how after about 16 DAS, when the CO_2 injection began, all the plants boosted their growth again reaching almost the same value of rack 1 (C). This process was more evident under H VPD where HS plants reached the same values of FW and DW of HC plants. Concerning the different cultivars (Green and Red plants), no significant differences were found between G and R among the treatments.

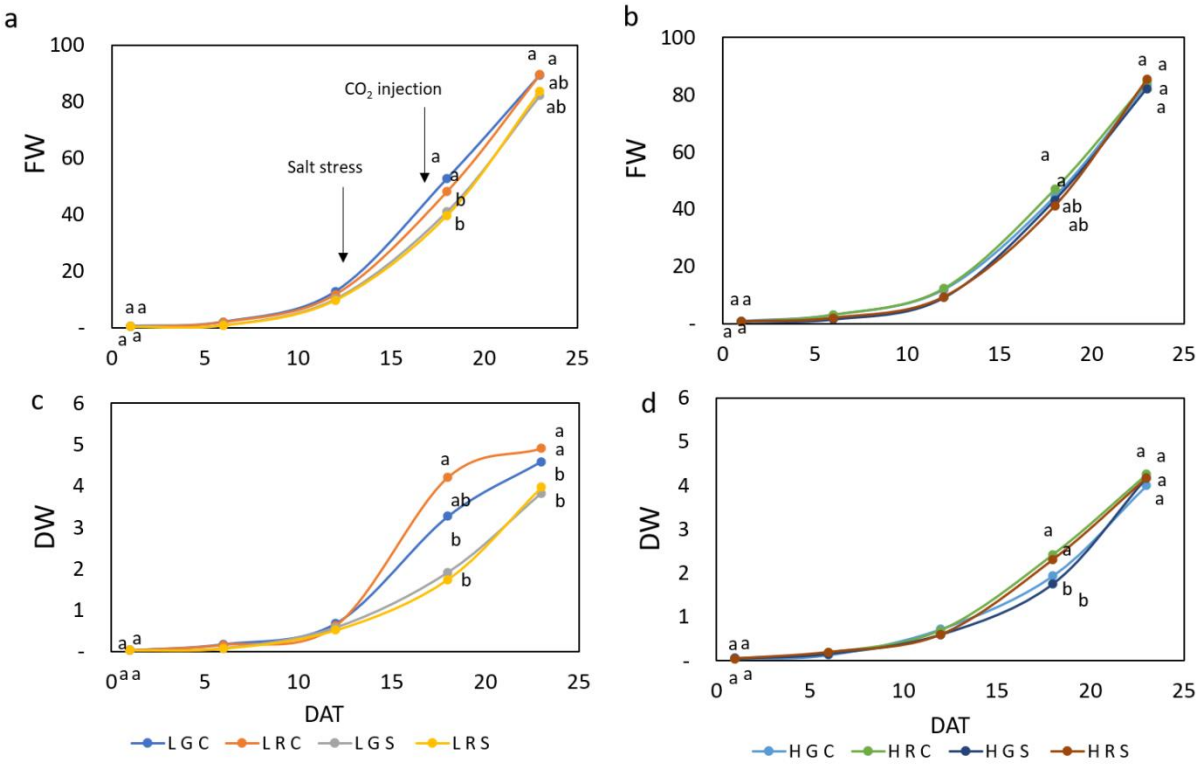

Figure 9: Leaf growth curves in terms of a,b) fresh weight (FW); c,d) dry weight (DW); of Salanova Green (G) and Red (R) plants from Control (C) and Stressed (S) rack, grown under low (L) (a,c) and high (H) (b,d) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.4.2 Morpho-functional traits: Leaf morpho-functional traits in terms of LA, SLA and RWC before and after the CO_2 injection are showed in **Figure 10** and were strongly influenced by L and H VPD in combination with the modulation of the other environmental factors. After CO_2 injection, leaf area (**Figure 10a**) was always higher in control compared to stressed plants in both L and H VPD. Under both VPDs, leaf area was higher in GC and RC plants. Overall, highest values were found in L VPD GC and RC plants after the CO_2 injection and lowest in LGS, HGS and HRS before the CO_2 injection. No significant differences were found between G and R cultivars in both L and H VPD, exception made for LRS before CO_2 injection which presented higher values compared to LGS. Similarly to LA, SLA (**Figure 10b**) values were higher after CO_2 injection both in L and H VPD and were higher in control compared to stress plants under both VPDs before and after the injection. However, no differences were detected in HRC, LGS and LRS before and after CO_2 and in HC and HS after the injection. Overall highest values were found in LCG and LRC after CO_2 injection and lowest in HGS

and HRS before CO_2.Conversely, there were no differences in RWC (**Figure 10c**) before and after the injection. LVPD plants always presented higher RWC compared to HVPD and highest values were found in C compared to S plants. Overall, highest values were found in LGC and LRC and lowest in HGS and HRS.

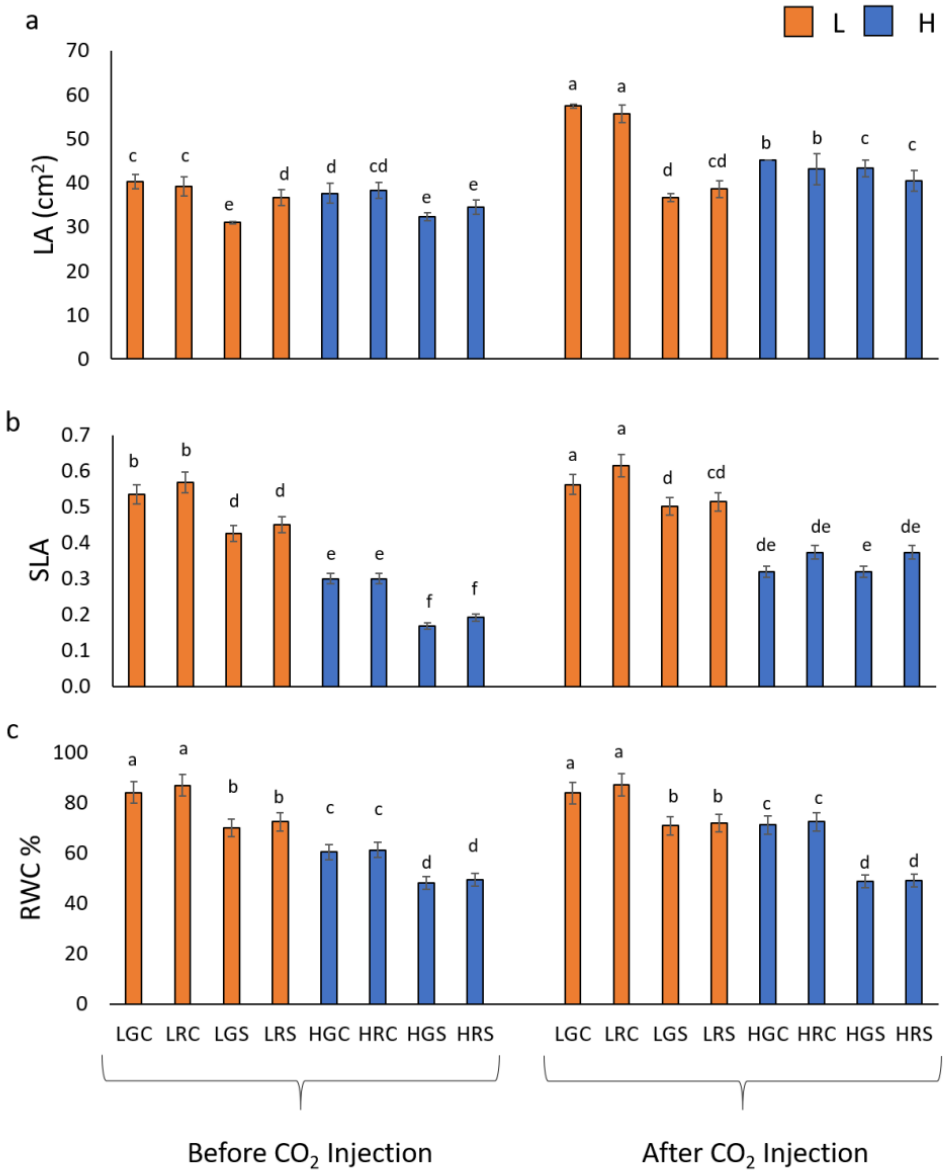

Figure 10: Leaf morpho-functional traits in terms of a) leaf area (LA); b) specific leaf area (SLA); c) relative water content (RWC) of Salanova Green (G) and Red (R) plants from Control (C) and Stressed (S) rack, grown under low (L) (a,c) and high (H) (b,d) VPD before and after the CO_2 injection. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.4.3 Morpho-anatomical traits

8.4.3.1 Leaf lamina: Leaf morpho-anatomical traits in terms of leaf lamina thickness (palisade and spongy parenchyma thickness) are showed in **Figure 11** and were strongly influenced by L and H VPD in combination with the modulation of the other environmental factors. In palisade thickness

(**Figure 11a**), LVPD values were always higher than H VPD. Under L VPD, higher values were found in GS and RS compared to GC and RC. Overall, highest values were found in LRS and lowest in HGC.Concerning spongy thickness (**Figure 11b**), L VPD values were higher than H VPD only in GS and RS; whereas the opposite happened in GC and RC. Under L VPD, palisade thickness presented highest values in RS, followed by GS, RC and lowest in GC. No differences were detected in HVPD. In Low VPD highest values were found in R compared to G plants in both C and S. Overall, highest values were found in LRS and lowest in LGC.

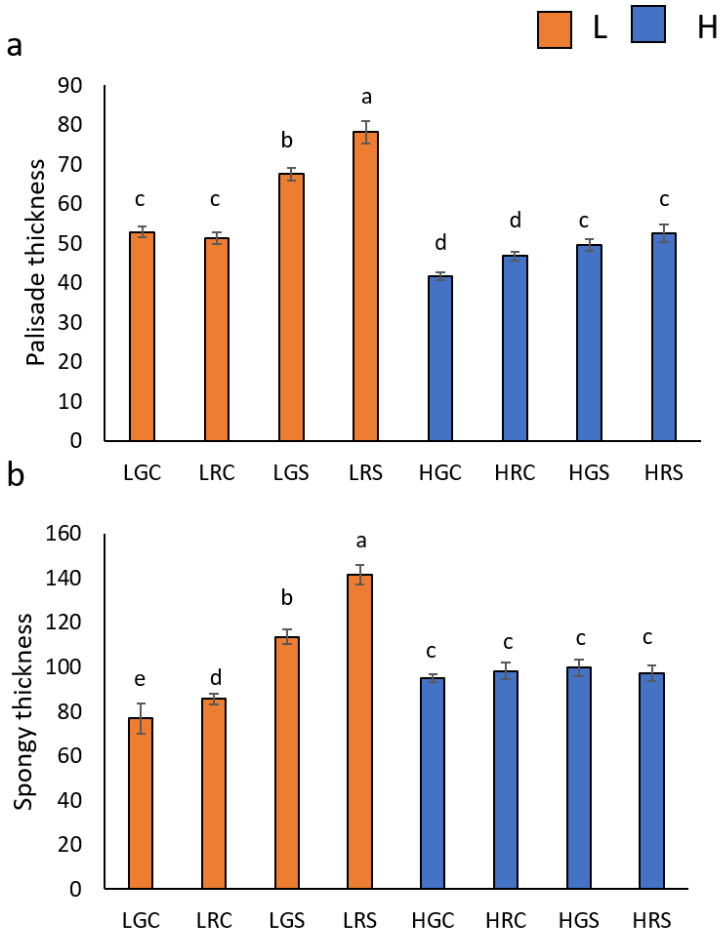

Figure 11: Leaf lamina thickness Leaf lamina thickness in terms of a) palisade thickness; b) of Salanova Green (G) and Red (R) plants from Control (C) and Stressed (S) rack, grown under low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.4.3.2 Stomata: Leaf morpho-anatomical traits in terms of stomatal traits (stomatal density and area) are showed in **Figure 12** and were strongly influenced by L and H VPD in combination with the modulation of the other environmental factors. Concerning stomatal density (**Figure 12a**), LVPD always showed a higher density of stomata than H VPD. Under L VPD, the stomata density was higher in GS and RS, whereas under H VPD the density was higher in GC and RC. Overall, highest values were found in LGS and lowest in HRS. In both L and H VPD, highest values were found in G compared to R plants in both C and S. Differently from stomata density (always higher in L VPD), under L VPD the C plants presented a higher stomatal area (**Figure 12b**) but in S plants this relationship changed with larger stomata found under H VPD. Overall, highest values were detected in HGS and HRS and lowest in LGC and LRC. No differences between G and R plants were detected.

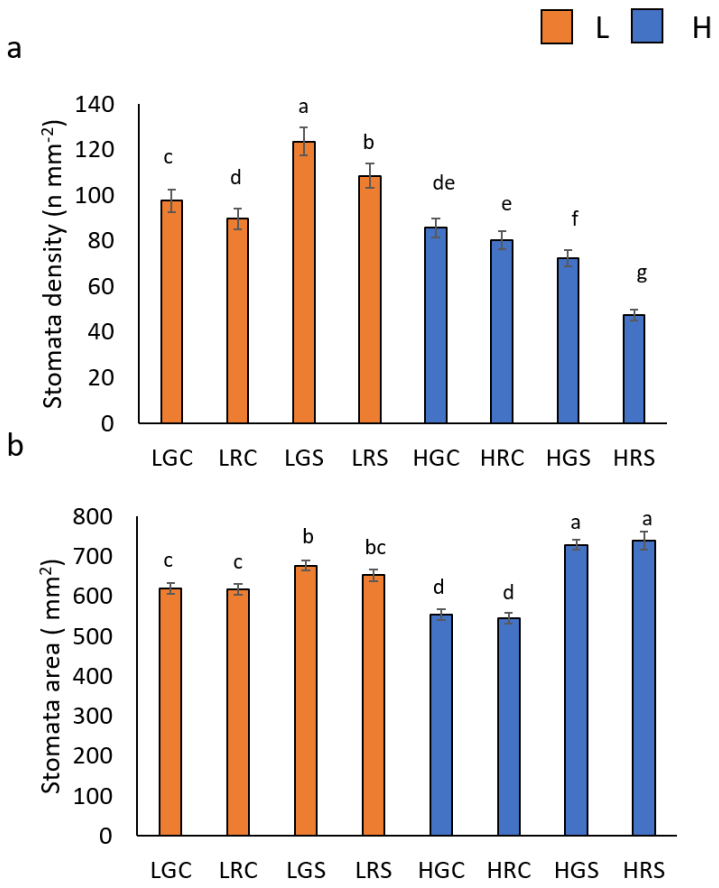

Figure 12: Stomatal traits in terms of a) density; b) area of Salanova Green (G) and Red (R) plants from Control (C) and Stressed (S) rack, grown under low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.4.3.3 Veins: Leaf morpho-anatomical traits in terms of vein density are showed in **Figure 13** and were strongly influenced by L and H VPD in combination with the modulation of the other environmental factors. L VPD always showed a higher density of veins than HVPD. Under L VPD, the density was higher in GS and RS, whereas under H VPD the density was higher in GC and RC. Overall, highest values were found in LGS and LRS and lowest in HGS and HRS. In both L and H VPD no differences were detected between G and R plants in both C and S.

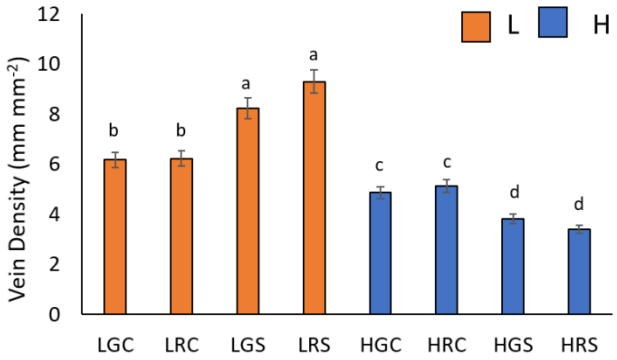

Figure 13: Vein density of Salanova Green (G) and Red (R) plants from Control (C) and Stressed (S) rack, grown under low (L) and high (H) VPD. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.4.4 Gas-exchanges: Leaf gas-exchange traits in terms of P_N, g_s and Tr before and after the CO_2 injection are showed in **Figure 14** and were strongly influenced by L and H VPD in combination with the modulation of the other environmental factors. Net-photosynthesis (**Figure 14a**) was always higher after CO_2 injection both in L and H VPD (compared to before the injection), and It was higher under L VPD both in C and S plants. Overall, highest values were found in L VPD GC and RC plants after the CO_2 injection and lowest in HRS before the CO_2 injection. Significant differences were found between G and R cultivars in LC plants before the injection where LRC presented higher values than LGC (12% reduction), in HS plants before the injection where HGS presented higher values than RGS (16% reduction) and in LS plants after the injection where LRS presented higher values than LGS (3% reduction). Stomatal conductance (**Figure 14b**) was always higher before CO_2 injection both in L and H VPD, except from HGS and HRS whose values did not change before and after the injection. It was always higher under LVPD both in C and S plants. Overall, highest values were found in L VPD GC and RC plants before the CO_2 injection and lowest in HGS before and after the CO_2 injection (71% reduction). Statistically significant differences were found between G and R

cultivars in LC plants before and after the injection where LGC presented higher values than LRC, in HC and HS plants before the injection where HRC and HRS presented higher values than HGC and HGS and after the injection where HRS presented higher values than HGS (2% reduction). Transpiration rates (**Figure 14c**) were always higher before CO_2 injection both in L and H VPD. Higher values were found under H VPD both in C and S plants before and after the injection. Overall, highest values were found in HGS and HRS before the CO_2 injection and lowest in LGC and LRC after the CO_2 injection. No significant differences were found between G and R cultivars, except from LS plants before the injection where LGS presented higher values than LRS (16% reduction).

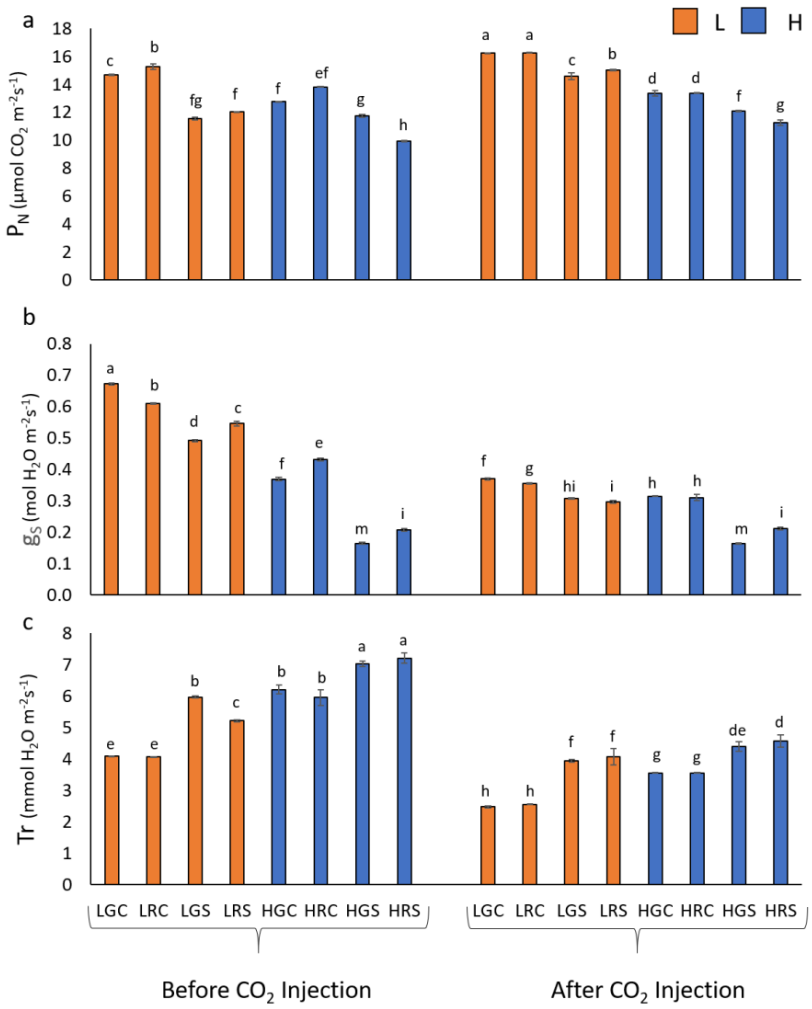

Figure 14: Leaf gas-echange in terms of a) net-photosynthesis (P_N); b) stomatal conductance (g_s); c) Transpiration (Tr) of Salanova Green (G) and Red (R) plants from Control (C) and Stressed (S) rack, grown under low (L) (a,c) and high (H) (b,d) VPD before and after the CO_2 injection. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

8.5 Discussion: Advances in vertical farming have promoted research in plant science for the production of high-yield crops in crowded cities. Most research so far has been addressing CEA

system efficiency, considering the optimization of space, energy- and water-use without discussing how the microenvironment affect plants morpho-anatomical development, thus their capacity of acclimation and finally their quality and yield. Here, we studied lettuce morpho-anatomical development and photosynthesis in a vertical farm under two VPD levels (0.7 kPa; 1.4 kPa) in combination with different environmental factors. Light intensity is certainly one of the most studied environmental factors in CEA, with the ultimate aims of improving crop yield and quality, and at the same time trying to consume less energy. In E1, the interaction between light intensity and VPD elicited significant changes in the morpho-physiological development of Salanova plants. It has been reported, plants grown at high light intensity presented enhanced net-photosynthesis; however, the application of strong-light intensity, that exceed a certain ppfd (depending on the species) often results in decreased P_N (Yao et al., 2017). In our study (E1), the highest level of light (3) increased net-photosynthesis under both low- and high-VPD. This is probably due to plasticity in leaf morphology under different light intensities. Indeed, plants can modify their morphology, anatomy and physiological traits to cope with light stresses (Aleric and Katherine Kirkman, 2005). Here, high light elicited changes in leaf morphology leading to leaves with smaller leaf lamina but thicker parenchymas, increased LMA and LDMC under high compared to medium and low light intensity. Smaller leaves are usually found under high light intensity because plants do not need to increase the leaf surface to capture more light and smaller leaves reduce faster their heat loads (Wang et al., 2020). According to our study, Wang et al. (2020) found smaller leaves with higher LMA in *Rhododendron* plants grown under high light intensity; similar results were found by Kong et al. (2016) on *M. bodinieri*. LMA and LDMC are key traits in plant growth and important indicators of plant adaptive strategies (Westoby, 1998; Grime, 2006), which has been used widely in plant ecology, agronomy, and forestry, but less in plant physiology. However, recent studies have demonstrated that LMA is often positively correlated with net-photosynthesis, relative growth rate and biomass. Indeed, in our study plant at high light increased biomass, LMA and photosynthesis, with highest increments under low VPD, exception made for LMA, which resulted higher in high-VPD exposed plants. Increase in LMA and LDMC can also be influenced by plant internal anatomy, especially by increments in parenchyma tissue thickness (Marques et al., 1999) but also by cell wall thickening (Takashima et al., 2004) or cell size and intercellular spaces (Kong et al., 2016). The thickening of “sun leaves” can depend on several morphologies on the internal structure, such as: i) increments in cell layers in the mesophyll (Arens, 1997); ii) cell elongation in palisade tissue (Gauhl, 1976). For example, a combined increase in the number of cell layers in mesophyll and an elongation of palisade cell was observed in *A. Thaliana* sun leaves (Weston et al., 2000). Here, the highest LMA of high-VPD exposed plants can be ascribed to a wider spongy parenchyma compared to low -VPD which increased the overall lamina thickness (**Figure 5**). It is known that plant exposed to high VPD often increase the spongy parenchyma, also showing a higher density of intercellular spaces. This structure (with a high proportion of spongy parenchyma rich in intercellular spaces) is recognized to

give the major resistance to gas and water flows, because of the lowest cells connection which decrease the area available for the flow (Amitrano et al., 2019). Indeed, in the present study, plants at high VPD presented the highest transpiration and the lower photosynthesis (**Figure 8c**). Plants at high VPD always showed a reduced palisade parenchyma thickness compared to low VPD plants. It is recognized that the palisade tissue allows an enhanced photosynthesis. Indeed, in E1 palisade thickness was enhanced by the higher light intensity in both low and high VPD plants, probably due to a compensation mechanism to maintain a high volume of photosynthetic tissue even in smaller leaves. Furthermore, other changes in leaf internal organization were visible among light intensities and VPDs. For example, hydraulic-related anatomical traits of stomata and veins varied a lot among VPDs and light intensities. These traits are drivers of leaf hydraulics (Sack, 2006; Brodribb and Jordan, 2011); since their balance drives the maintenance of the normal leaf physiological functions, their study is fundamental to understand plant behavior under a changing environment (Amitrano et al., 2021; Chapter 3). The developmental pattern of stomata and vein traits under different light intensity and VPDs is not uniform across species and is therefore considered highly species-specific (Amiard et al., 2005). Here, the highest vein density found under high light and under high evaporative demand, could be a sign of plant trying to increase the water supply to the leaves to meet the high traspirational demand under high light and high VPD condition. Similar results were found in Wang et al. 2020 where *Rhododendrons* plants not only increased vein density under high light intensity, but also stomatal density. This once again is in accordance with our study where the development of stomatal and vein traits seems to be coordinated since lettuce plants showed increased stomatal density under high light and especially under high evaporative demand. Usually, sun leaves of different species tend to develop higher vein and stomatal densities compared to shade leaves (Brodribb and Jordan 2011; Scoffoni et al., 2015). The lower vein density under low light was explained by Sack and Scoffoni (2013) as a way for plants to reduce construction cost and potentially enhance the light interception in shade. In the present study this correlation is maintained under low light intensity, where no differences between low and high VPD plants were found; but not under medium and high light intensities (2, 3) where is the low VPD condition to develop the majority of stomata and veins. This is in accordance with our previous study on *V. radiata* plants where stomatal and vein density always resulted enhanced under the low VPD condition (Chapter 3). In Chapter 3, it is showed that this strict coordination between density of stomata and veins was the reason for mantaining high photosynthetic performance of the species and also explained plant acclimation to changing conditions of VPD (Amitrano et al., 2021; Chapter 3). Moreover, in the present study, larger stomata were found under the lowest light intensity (**Figure 6**). This is a clear sign that plants grown under this irradiance do not need to promptly adjust and close the stomata to avoid the excess of water loss. Indeed, it is known that developing smaller stomata helps plant to cope with stressful condition (e.g. drought, pollution, high VPD), since the opening/closing of smaller stomata is faster (De Micco et al., 2019). Overall, all these morpho-anatomical parameters drive the photosynthetic

acclimation of the species. As presented in **figure 8,** net-photosynthesis and stomatal conductance was higher under low VPD, notwithstanding the growth irradiance and the different coordination of stomata and vein. Although having the highest vein and stomatal density and a coordination of stomata and vein under high VPD and high irradiance the photosynthesis and stomatal conductance are lower if compared with low VPD. This could be a sign that plants are facing limiting conditions (transpiration enhanced by VPD and by high light) and develop more stomata and vein to counteract such stressful condition.

Leaving out the morphological development under different lights and VPD which drives the photosynthetic acclimation of lettuce plants, and focusing on lettuce biomass production, interesting results are reported. Here, we noticed that under low light intensity, lettuces presented more expanded plants with bigger leaves and although presenting a reduced dry weight, compared to the other light conditions maintained a high fresh weight, equal to light intensity 2, often considered an optimum for the species. The maintenance of a higher biomass, at least comparable to the medium light intensity can be explained by the more expanded leaves, considered a key trait to intercept more light or to the internal tissue organization (e.g. highest spongy parenchyma thickness compared to the other light intensities; **Figure 5**). Moreover, transpiration under low light is reduced also under the high VPD condition, which can be an advantageous for the species in indoor cultivation.

In E2 we focus on *if* and *how* the CO_2 enrichment could offset the negative effect of sudden changes in salinity (high EC) changing the structure-to-function relationships of lettuces developed at different VPDs (L and H) under a constant light intensity of 300 µmol $m^{-2}s^{-1}$. It is known that elevated atmospheric CO_2 can stimulate plant growth, metabolism and development by providing additional carbon to the plant (also known as fertilization effect), especially under favorable nutrient and water conditions (AbdElgawad et al., 2016). Furthermore, the role of CO_2 has also been discussed with regard to stress mitigation but the interaction of elevated CO_2 with other environmental parameters, especially after sudden stressful conditions, are less documented in the literature. Understanding such interaction is necessary in a future high-CO_2 scenario (Feng et al., 2014; Xu et al., 2015) but also in precision agriculture. For instance, negative effects of drought were reduced under elevated CO_2 in barely and alfalfa (Erice et al., 2007; Robredo et al., 2011). Also in barley, the oxidative stress induced by salinity was mitigated by elevated CO_2 (Perez-Lopez et al., 2008). In E2, from growth curves (**Figure 9**) is evident how sudden increments in salinity in S rack brought to a reduction in the whole FW and DW, especially under low VPD, also confirmed by reduction in LA and SLA in green and red stressed compared to green and red control plants (**Figure 10**). Reduction in growth is common under elevated EC. In lettuce subjected to increasing EC, Ünlükara et al. (2008) found a severe reduction in fresh and dry biomass and number of leaves. However, in E2 after the CO_2 injection it was possible to notice a boost in growth, especially under high VPD conditions in terms

of FW and DW which managed to reach similar values to non-stressed plants (**Figure 9b,d**), a difference that could be attributed to the reduced growth (FW and area) of green and red control high VPD plants, also confirmed in E1. In Eucalyptus, an ameliorative effect of elevated CO_2 after drought stress was observed by Atwell et al. (2007) so much that in plants growing under ambient conditions the drought stopped their growth after 20 weeks from germination, whereas those in elevated CO_2 increased their biomass by 40 % after 20 weeks. High electrical conductivity of the nutrient solution decreases the possibility of water absorption by plants, also decreasing photosynthesis, cell division and protein synthesis resulting in a lower leaf area (Cometti et al., 2013). Cometti et al. (2012) found in lettuces grown in hydroponic and subjected to high EC and to high temperature, a greater reduction in growth and photosynthesis compared to those subjected to high EC and optimal temperature, which was ascribed to a reduced capacity of absorbing water under elevated temperature. In our study, we observed a general decrease in both low and high VPD lettuces subjected to high EC (LGS, LRS, HGS and HRS); however, in lettuces subjected to high VPD and high EC (HGS and HRS) these reductions were more accentuated, especially in water content (RWC), SLA (**Figure 10**) and stomatal conductance (**Figure 14**). These differences were ascribed to a different anatomical structure of L and H leaves under control and stress conditions. A clear evidence is reported in **Figure 12,** showing how stomata morphology followed a different development under L and H VPD in stressed plants. Indeed, while the density of stomata was reduced under S compared to C plants for HVPD, in LVPD the density was enhanced by 4-6 folds in green and red stressed plants compared to green and red control plants. Concerning stomatal dimensions, even though both L and H VPD plants developed bigger stomata under high EC (**Figure 12b**), the enhanced density of low VPD stressed plants likely enable the maintenance of higher conductance compared to high VPD stressed plants and the same mechanism likely happened in control plants (**Figure 14b**). These results were confirmed by the different development of vein density under L and H VPD under salt stress. Once again, green and red stressed plants showed increments in vein density under low VPD and decrease in high VPD compared to non-stressed plants. Reduction in stomatal density with increasing salinity levels are common in different species (Orsini et al., 2011; Waqas et al., 2017) and together with a reduced aperture of the pores are the most common causes of reduction in photosynthesis and conductance under salt and drought stresses (Kelly et al., 2016; Domec et al., 2017). Conversely, leaf vein traits are still less studied and their development under salt stress has not been explored much. However, to the best of our knowledge this different VPD-driven acclimation mechanisms of lettuces under salt stress (compared to control) has never been reported before and has probably a great influence over photosynthesis. Most CO_2 enrichment experiments have been carried out at ambient VPD, showing increments in net-photosynthesis and decrements in stomatal conductance and transpiration rates, mainly attributed to stomatal closing with the increase in intercellular CO_2 concentration within the leaves (Aasamaa and Sõber, 2011). Our results are in agreement with these studies showing

decreased g_s and Tr after CO_2 enrichment in both L and H VPD, however g_s was significantly higher in low VPD stressed plants compared to high VPD stressed plants and Tr was significantly higher in high VPD stressed plants compared to low VPD stressed plants (**Figure 14c,d**). In agreement with our study, Jiao et al. (2019) found increments in net-photosynthesis in low and high VPD plants grown with CO_2 enrichments; however, in low VPD plants exposed to high CO_2 the stomatal limitation decreased and g_s was always higher than high VPD. In accordance with our previous study (Chapter 4), only a few significant differences were found between green and red lettuces especially in gas-exchange analysis. For example, the higher P_N in low VPD stressed red compared to green plants at the final point of analysis can be ascribed to a higher palisade thickness in red plants. It is generally accepted that the palisade tissue prevails in the mesophyll of dorsiventral leaves, making a major contribution to their photosynthesis (Zheng et al., 2017). Moreover, g_s was higher in low VPD green compared to red control plants in accordance with the higher stomatal density in these plants.

8.6 Conclusions: In this chapter we studied how the interaction between VPD and other environmental parameters affect red and green salanova lettuces grown in a vertical farm, from a morpho-physiological point of view. In E1 the interaction between VPD and light intensity provoked a different morpho-anatomical development of lamina, stomata and veins under L and H VPD. For example, it is interesting to notice that under high light, H VPD plants developed a higher density of veins and stomata, probably to counteract the negative effect of high VPD, enhanced even more by the high transpiration under high irradiance. Moreover, the different anatomical development of leaves brought, under the lowest light, to a fresh biomass and net-photosynthesis not so different from those developed under the medium light (considered an optimum for the species). This can be considered a positive outcome to reduce transpiration lowering the irradiance in controlled environment. In E2 we discussed how morpho-anatomical traits of stomata and veins drive a different acclimation in low and high VPD under control and salt stress and then exposed to short-term CO_2 enrichment. Interesting results showed that under salt stress L and H VPD followed a different developmental pattern of functional traits of leaves. While L VPD plants enhanced their stomata and vein density compared to control, H VPD plants under salt stress reduced even more these traits. This resulted in enhanced photosynthetic rates after CO_2 injection for L plants and lowered for H plants, compared to their values before the injections. However, overall, both plants in L and H VPD were able to overcome the salt stress, managing at the end of the growth to reach values comparable with control.

To conclude, the morpho-anatomical development of lettuces under different environmental conditions plays a fundamental role in plant photosynthetic acclimation; therefore further explorations to quantify intercellular spaces or distribution of chloroplasts in the palisade parenchyma would help better understand the mechanisms underlying plant carbon gain can help in support our

hypothesis. Overall, our findings may contribute to the development of sustainable cultivation strategy in indoor cultivation and, at the same time, improve knowledge about the ecological adaptation of lettuce to environmental stresses, more and more common in the current climate change scenario.

8.7 References

Aasamaa, K., and Sõber, A. (2011). Responses of stomatal conductance to simultaneous changes in two environmental factors. *Tree Physiology* 31(8)**,** 855-864.

AbdElgawad, H., Farfan-Vignolo, E.R., de Vos, D., and Asard, H. (2015). Elevated CO2 mitigates drought and temperature-induced oxidative stress differently in grasses and legumes. *Plant Science* 231**,** 1-10.

AbdElgawad, H., Zinta, G., Beemster, G.T., Janssens, I.A., and Asard, H. (2016). Future climate CO2 levels mitigate stress impact on plants: increased defense or decreased challenge? *Frontiers in plant science* 7**,** 556.

Aleric, K.M., and Katherine Kirkman, L. (2005). Growth and photosynthetic responses of the federally endangered shrub, Lindera melissifolia (Lauraceae), to varied light environments. *American Journal of Botany* 92(4)**,** 682-689.

Aliniaeifard, S., Malcolm Matamoros, P., and van Meeteren, U. (2014). Stomatal malfunctioning under low VPD conditions: induced by alterations in stomatal morphology and leaf anatomy or in the ABA signaling? *Physiol Plant* 152(4)**,** 688-699. doi: 10.1111/ppl.12216.

Amiard, V., Mueh, K.E., Demmig-Adams, B., Ebbert, V., Turgeon, R., and Adams, W.W. (2005). Anatomical and photosynthetic acclimation to the light environment in species with differing mechanisms of phloem loading. *Proceedings of the National Academy of Sciences* 102(36)**,** 12968-12973.

Amitrano, C., Arena, C., Cirillo, V., De Pascale, S., and De Micco, V. (2021). Leaf morpho-anatomical traits in Vigna radiata L. affect plant photosynthetic acclimation to changing vapor pressure deficit. *Environmental and Experimental Botany***,** 104453.

Amitrano, C., Arena, C., Rouphael, Y., De Pascale, S., and De Micco, V. (2019). Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. *Annals of Applied Biology*. doi: 10.1111/aab.12544.

Arens, N.C. (1997). Responses of leaf anatomy to light environment in the tree fern Cyathea caracasana (Cyatheaceae) and its application to some ancient seed ferns. *Palaios* 12(1)**,** 84-94.

Atwell, B.J., Henery, M.L., Rogers, G.S., Seneweera, S.P., Treadwell, M., and Conroy, J.P. (2007). Canopy development and hydraulic function in Eucalyptus tereticornis grown in drought in CO2-enriched atmospheres. *Functional Plant Biology* 34(12)**,** 1137-1149.

Ben-Asher, J., Garcia y Garcia, A., Flitcroft, I., and Hoogenboom, G. (2013). Effect of atmospheric water vapor on photosynthesis, transpiration and canopy conductance: A case study in corn . *Plant, Soil and Environment* 59(No. 12)**,** 549-555. doi: 10.17221/346/2013-pse.

Bernstein, N., Kravchik, M., and Dudai, N. (2010). Salinity-induced changes in essential oil, pigments and salts accumulation in sweet basil (Ocimum basilicum) in relation to alterations of morphological development. *Annals of Applied Biology* 156(2)**,** 167-177.

Brodribb, T.J., and Jordan, G.J. (2011). Water supply and demand remain balanced during leaf acclimation of Nothofagus cunninghamii trees. *New Phytologist* 192(2)**,** 437-448.

Buck, J., Kubota, C., and Wu, M. (Year). "Effects of nutrient solution EC, plant microclimate and cultivars on fruit quality and yield of hydroponic tomatoes (Lycopersicon esculentum)", in: *VII International Symposium on Protected Cultivation in Mild Winter Climates: Production, Pest Management and Global Competition 659*), 541-547.

Bunce, J.A. (1996). does transpiration control stomatal responses to water vapour pressure deficit? *Plant, Cell and Environment* 19**,** 131-135.

Bunce, J.A. (2003). Effects of water vapor pressure difference on leaf gas exchange in potato and sorghum at ambient and elevated carbon dioxide under field conditions. *Field Crops Research* 82(1)**,** 37-47.

Caplan, B.A. (2018). Optimizing Carbon Dioxide Concentration and Daily Light Integral Combination in a Multi-Level Electrically Lighted Lettuce Production System.

Cometti, N.N., Bremenkamp, D.M., Galon, K., Hell, L.R., and Zanotelli, M.F. (2013). Cooling and concentration of nutrient solution in hydroponic lettuce crop. *Horticultura Brasileira* 31(2)**,** 287-292.

Cornelissen, J., Lavorel, S., Garnier, E., Diaz, S., Buchmann, N., Gurvich, D., et al. (2003). A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. *Australian journal of Botany* 51(4)**,** 335-380.

De Micco, V., Amitrano, C., Stinca, A., Izzo, L.G., Zalloni, E., Balzano, A., et al. (2019). Dust accumulation due to anthropogenic impact induces anatomical and photochemical changes in leaves of Centranthus ruber growing on the slope of the Vesuvius volcano. *Plant Biol (Stuttg)*. doi: 10.1111/plb.12966.

Devi, M.J., Reddy, V.R. (2018). Transpiration response of cotton to vapor pressure deficit and its relationship with stomatal traits. *Frontiers in Plant Science* 9 (1572). doi: 10.3389/fpls.2018.01572.

Domec, J.C., Smith, D.D., and McCulloh, K.A. (2017). A synthesis of the effects of atmospheric carbon dioxide enrichment on plant hydraulics: implications for whole-plant water use efficiency and resistance to drought. *Plant, Cell & Environment* 40(6)**,** 921-937.

Dorai, M., Papadopoulos, A., and Gosselin, A. (2001). Influence of electric conductivity management on greenhouse tomato yield and fruit quality. *Agronomie* 21(4)**,** 367-383.

Erice, G., Aranjuelo, I., Irigoyen, J.J., and Sánchez-Díaz, M. (2007). Effect of elevated CO2, temperature and limited water supply on antioxidant status during regrowth of nodulated alfalfa. *Physiologia Plantarum* 130(1)**,** 33-45.

Fanourakis, D., Bouranis, D., Giday, H., Carvalho, D.R., Rezaei Nejad, A., and Ottosen, C.O. (2016). Improving stomatal functioning at elevated growth air humidity: A review. *J Plant Physiol* 207**,** 51-60. doi: 10.1016/j.jplph.2016.10.003.

Fanourakis, D., Heuvelink, E., and Carvalho, S.M. (2013). A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. *J Plant Physiol* 170(10)**,** 890-898. doi: 10.1016/j.jplph.2013.01.013.

Fanourakis, D., Hyldgaard, B., Giday, H., Aulik, I., Bouranis, D., Körner, O., et al. (2019). Stomatal anatomy and closing ability is affected by supplementary light intensity in rose (Rosa hybrida L.). *Horticultural Science* 46(2)**,** 81-89.

Feder, N., and O'brien, T. (1968). Plant microtechnique: some principles and new methods. *American journal of botany* 55(1)**,** 123-142.

Feng, G.-Q., Li, Y., and Cheng, Z.-M. (2014). Plant molecular and genomic responses to stresses in projected future CO2 environment. *Critical Reviews in Plant Sciences* 33(2-3)**,** 238-249.

Feng, L., Raza, M.A., Li, Z., Chen, Y., Khalid, M.H.B., Du, J., et al. (2019). The influence of light intensity and leaf movement on photosynthesis characteristics and carbon balance of soybean. *Frontiers in plant science* 9**,** 1952.

Gauhl, E. (1976). Photosynthetic response to varying light intensity in ecotypes of Solanum dulcamara L. from shaded and exposed habitats. *Oecologia* 22(3)**,** 275-286.

Grime, J.P. (2006). *Plant strategies, vegetation processes, and ecosystem properties.* John Wiley & Sons.

Heath, J. (1998). Stomata of trees growing in CO2-enriched air show reduced sensitivity to vapour pressure deficit and drought. *Plant, Cell & Environment* 21(11)**,** 1077-1088.

Hendawy, S., and Khalid, K.A. (2005). Response of sage (*Salvia officinalis* L.) plants to zinc application under different salinity levels. *J. Appl. Sci. Res* 1(2)**,** 147-155.

Jiao, X.C., Song, X.M., Zhang, D.L., Du, Q.J., and Li, J.M. (2019). Coordination between vapor pressure deficit and CO2 on the regulation of photosynthesis and productivity in greenhouse tomato production. *Sci Rep* 9(1)**,** 8700. doi: 10.1038/s41598-019-45232-w.

Kelly, J.W., Duursma, R.A., Atwell, B.J., Tissue, D.T., and Medlyn, B.E. (2016). Drought× CO 2 interactions in trees: a test of the low-intercellular CO 2 concentration (Ci) mechanism. *New Phytologist* 209(4)**,** 1600-1612.

Kong, D.-X., Li, Y.-Q., Wang, M.-L., Bai, M., Zou, R., Tang, H., et al. (2016). Effects of light intensity on leaf photosynthetic characteristics, chloroplast structure, and alkaloid content of Mahonia bodinieri (Gagnep.) Laferr. *Acta Physiologiae Plantarum* 38(5)**,** 120.

MARQUES, A.R., GARCIA, Q.S., and FERNANDES, G.W. (1999). Effects of sun and shade on leaf structure and sclerophylly of Sebastiania myrtilloides (Euphorbiaceae) from Serra do Cipó, Minas Gerais, Brazil. *Boletim de Botânica da Universidade de São Paulo***,** 21-27.

Morison, J.I. (1998). Stomatal response to increased CO 2 concentration. *Journal of Experimental Botany***,** 443-452.

Orsini, F., Accorsi, M., Gianquinto, G., Dinelli, G., Antognoni, F., Carrasco, K.B.R., et al. (2011). Beyond the ionic and osmotic response to salinity in Chenopodium quinoa: functional elements of successful halophytism. *Functional Plant Biology* 38(10)**,** 818-831.

Ottosen, C., Mortensen, L., and Gislerod, H. (2002). Effect of relative air humidity on gas exchange, stomatal conductance and nutrient uptake in miniature potted roses. *Gartenbauwissenschaft* 67(4)**,** 143-147.

Perez-Lopez, U., Robredo, A., Lacuesta, M., Mena-Petite, A., and Muñoz-Rueda, A. (2008). "Does Elevated CO2 Mitigate the Salt Effect on Photosynthesis in Barley Cultivars?," in *Photosynthesis. Energy from the sun*. Springer), 1529-1533.

R Shamshiri, R., Kalantari, F., Ting, K., Thorp, K.R., Hameed, I.A., Weltzien, C., et al. (2018). Advances in greenhouse automation and controlled environment agriculture: A transition to plant factories and urban agriculture.

Rengifo, E., Urich, R., and Herrera, A. (2002). Water relations and leaf anatomy of the tropical species, Jatropha gossypifolia and Alternanthera crucis, grown under an elevated CO 2 concentration. *Photosynthetica* 40(3)**,** 397-403.

Robredo, A., Pérez-López, U., Miranda-Apodaca, J., Lacuesta, M., Mena-Petite, A., and Muñoz-Rueda, A. (2011). Elevated CO2 reduces the drought effect on nitrogen metabolism in barley plants during drought and subsequent recovery. *Environmental and Experimental Botany* 71(3)**,** 399-408.

Ryser, P., and Urbas, P. (2000). Ecological significance of leaf life span among Central European grass species. *Oikos* 91(1)**,** 41-50.

Sack, L., and Frole, K. (2006). Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology* 87(2)**,** 483-491. doi: https://doi.org/10.1890/05-0710

Sack, L., and Scoffoni, C. (2013). Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. *New Phytol* 198(4)**,** 983-1000. doi: 10.1111/nph.12253.

Scoffoni, C., Kunkle, J., Pasquet-Kok, J., Vuong, C., Patel, A.J., Montgomery, R.A., et al. (2015). Light-induced plasticity in leaf hydraulics, venation, anatomy, and gas exchange in ecologically diverse Hawaiian lobeliads. *New Phytol* 207(1)**,** 43-58. doi: 10.1111/nph.13346.

Sorrentino, M.C., Capozzi, F., Amitrano, C., De Tommaso, G., Arena, C., Iuliano, M., et al. (2021). Facing metal stress by multiple strategies: morphophysiological responses of cardoon (Cynara cardunculus L.) grown in hydroponics. *Environmental Science and Pollution Research***,** 1-11.

Takashima, T., Hikosaka, K., and Hirose, T. (2004). Photosynthesis or persistence: nitrogen allocation in leaves of evergreen and deciduous Quercus species. *Plant, Cell & Environment* 27(8)**,** 1047-1054.

Ünlükara, A., Cemek, B., Karaman, S., and Erşahin, S. (2008). Response of lettuce (Lactuca sativa var. crispa) to salinity of irrigation water. *New Zealand Journal of Crop and Horticultural Science* 36(4)**,** 265-273.

Wang, J.-H., Cai, Y.-F., Li, S.-F., and Zhang, S.-B. (2020). Photosynthetic acclimation of rhododendrons to light intensity in relation to leaf water-related traits. *Plant Ecology***,** 1-14.

Waqas, M., Yaning, C., Iqbal, H., Shareef, M., Rehman, H., and Yang, Y. (2017). Paclobutrazol improves salt tolerance in quinoa: Beyond the stomatal and biochemical interventions. *Journal of Agronomy and Crop Science* 203(4)**,** 315-322.

Westoby, M. (1998). A leaf-height-seed (LHS) plant ecology strategy scheme. *Plant and soil* 199(2)**,** 213-227.

Weston, E., Thorogood, K., Vinti, G., and López-Juez, E. (2000). Light quantity controls leaf-cell and chloroplast development in Arabidopsis thaliana wild type and blue-light-perception mutants. *Planta* 211(6)**,** 807-815.

Witkowski, E., and Lamont, B.B. (1991). Leaf specific mass confounds leaf density and thickness. *Oecologia* 88(4)**,** 486-493.

Wong, S.-C. (1993). "Interaction between elevated atmospheric concentration of CO 2 and humidity on plant growth: comparison between cotton and radish," in *CO2 and Biosphere*. Springer), 211-221.

Xu, H.-l., Gauthier, L., and Gosselin, A. (1995). Effects of fertigation management on growth and photosynthesis of tomato plants grown in peat, rockwool and NFT. *Scientia Horticulturae* 63(1-2)**,** 11-20.

Xu, Z., Jiang, Y., and Zhou, G. (2015). Response and adaptation of photosynthesis, respiration, and antioxidant systems to elevated CO2 with environmental stress in plants. *Frontiers in plant science* 6**,** 701.

YAO, X.-y., LIU, X.-y., XU, Z.-g., and JIAO, X.-l. (2017). Effects of light intensity on leaf microstructure and growth of rape seedlings cultivated under a combination of red and blue LEDs. *Journal of* integrative agriculture 16(1), 97-105.

Zheng, L., and Van Labeke, M. C. (2017). Long-term effects of red-and blue-light emitting diodes on leaf anatomy and photosynthetic efficiency of three ornamental pot plants. Frontiers in Plant Science, 8, 917.

Chapter 9

Project PHEW - Automated phenotyping platform to improve lettuce water use efficiency under different VPD and watering regimens: Report with preliminary results

9.1 General context and scope of the project: Nowadays, about 50% of the global yield loss are due to climatic changes. Increasing VPD (depending on temperature and relative humidity) and drought are among the principal environmental stressors. The aim of this high-throughput phenotyping experiment was to study how stomatal regulation and water use affect carbon gain and biomass allocation in lettuces acclimated to different environmental conditions (VPDs) inducing the development of a different leaf anatomical structure, and then subjected to short-term changes in the VPD. To fulfil this purpose, two lettuce cultivars, already characterized under different VPDs in terms of growth, phytochemical composition, and photosynthesis in previous experiments (Chapters 4,5,6,7,8), were characterized using an automated high-throughput phenotyping facility, under two different VPDs (low and high) in combination with two watering regimes (well-watered and low-watered) and then subjected to sudden changes in the VPD. An Infrared camera was used to estimate plant-water relationships, RGB imaging was applied to track changes in morphological and colorimetric parameters and a fluorescence camera was used to assess any changes in the photosystem II performance. At the end of the experimental trials, leaf samples were collected, chemically fixed, and transferred to the PWA (Plant and Wood Anatomy) laboratory of the University of Naples “Federico II” to analyze the leaf morpho-anatomical development in terms of stomata and leaf lamina traits through light microscopy (see paragraph 9.2.4 for details). Data from phenotyping and microscopy have been integrated to understand mechanisms of lettuce dynamic acclimation to sudden changes in the surrounding environment with reference to different pre-acclimation conditions and to evaluate possible changes in lettuce productivity in the sight of warmer and drier climates. In this chapter detailed material and methods are reported and preliminary results are discussed as useful to implement the knowledge about scientific and technical issues to design automated controlled growth chambers in support of sustainable precision farming on Earth and in Space exploration applications.

9.2 Material and Methods:

9.2.1 Experimental Design: Seeds of green and red Salanova lettuces (*Lactuca sativa* L. var. *capitata*) were provided by the Dutch company Rijk Zwaan and showed 100 % germination. For each trial, 80 green and 80 red seeds were germinated in 10 cm pots on soil (red substrate 2, Klasmann-Deilmann GmbH, Geeste, Germany) in a walk-in growth-chamber under controlled conditions (23/19°C, 75 % RH, 315 μmol photons m^{-2} s^{-1}, 12h photoperiod).

Light was provided by halide lamps (Venture Lighting Europe Ltd., Rickmansworth, Hertfordshire, England). After 10 days, 64 green and 64 red plants were moved to the phenotyping growth chamber (PGC) for small plants and placed in the LemnaTec carriers. To avoid evaporation from the soil and to provide a uniform background for the phenotyping cameras, the soil surface of all the pots was covered with a blue rubber mat (**Figure 1**). A few days prior the experiments, soil water content corresponding to 100 % field capacity (FC) was determined by weighing soil-filled pots after full watering and after drying for 3 days at 80°C as reported in Junker *et al.*, 2015. Once in the phenotyping chamber, half of the plants were irrigated at 100 % FC and are referred to as WW (well-watered) and the other half at 30 % FC, LW (low-watered). Irrigation took place through an automatic weighing/watering station with a pump irrigating into the bottom container, to avoid any interference with lettuce growth.

The first trial was conducted at a VPD of 0.7 kPa (L) and the second at 1.4 kPa (H). After 12 days of cultivation in the PGC, the environmental conditions in the chamber were switched and plants were kept for 5 days at the opposite VPD (LH and HL) to test the short-term acclimation, following the approach reported in Chapter 3 (Amitrano *et al.*, 2021). VPD conditions were obtained by keeping T fixed and changing the RH % accordingly.

The other environmental conditions in the phenotyping chamber were kept at: 23/19°C, 315 μmol photons m^{-2} s^{-1}, 12h photoperiod.

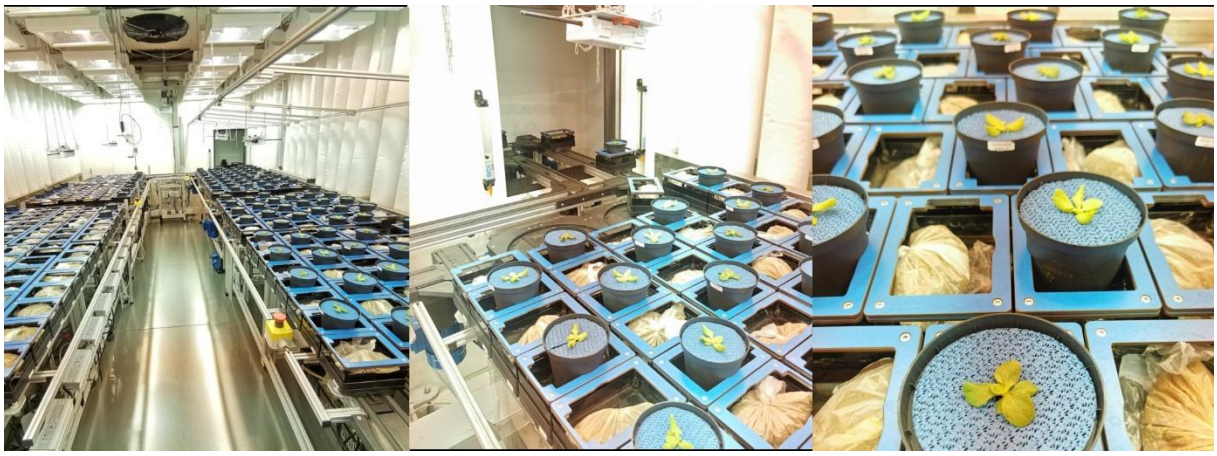

Figure 1: green and red Salanova lettuces in the LemnaTec carriers of the small-plant phenotyping chamber at the IPK-Gatersleben. Pot surface was covered with a blue rubber mat.

9.2.2 Image acquisition and analysis: All images were automatically acquired using the LemnaTec system. All plants were imaged daily from the top view using three imaging procedures VIS, NIR, FLUO (**Figure 2**). VIS images were acquired in the visible light spectrum (~390–750 nm) using a Basler (Basler AG, Ahrensburg, Germany) Pilot piA2400-17gc (RGB) camera with a resolution of 2454 × 2056 pixels. A fluorescence imaging system (FLUO, excitation: 400-500 nm, emission: 520–750 nm) using a Basler Scout scA1400-17gc and (RGB) camera with a resolution of 1624 × 1234 pixels, allowed the quantification of static fluorescence signals of plants. Near-infrared imaging (NIR) was performed in the wavelength range between 1450–1550 nm using a Nir 300 PGE (AlliedVisionTechnologiesGmbHformer VDS Vosskühler GmbH, Stadtroda, Germany) (monochrome camera) sensor with a resolution of 320 × 254 pixels.

On days 12 and 17 after the transfer in PGC (before and after the switch in environmental conditions), imaging of chlorophyll a fluorescence was performed using FluorCam imaging fluorimeters (Photon Systems Instruments, Brno, Czech Republic). Measurements of ΦPSII were made after light adaptation of the plants in adaptation tunnel and after an illumination period in the FluorCam-chamber as indicated in the results section. Duration of the saturating light pulse to induce Fm was 800 ms with an intensity of 4100 µmol m^{-2} s^{-1} (white light) in the system for small plants. After one hour of lights turned off, dark-adapted plants were subjected to eight saturated light pulses (800 ms; 4,100 µmol m^{-2} s^{-1} white light) over the course of 145.8 seconds. After the first saturating light pulse, light was turned off (five seconds) to measure F0, followed by the second saturating light pulse and actinic light to measure maximum fluorescence (Fm) followed by ten seconds dark relaxation to measure the Kautsky kinetics. For the Fm' quenching analysis, white light and actinic light were turned on for 120 seconds and supplemented by six saturating light pulses every 20 s followed by another ten seconds dark relaxation measurements.

The image analysis was performed by means of the IAP (Integrated Analysis Platform) software 9 in different steps: image acquisition, pre-processing, feature extraction and post-processing. From

image analysis, 9 traits were extracted, classified as geometric, color-related (including VIS, FLUO, NIR-related traits) and photochemistry traits (Fv/Fm, ΦPSII, NPQ). All the selected traits are summarized in **Table 1**.

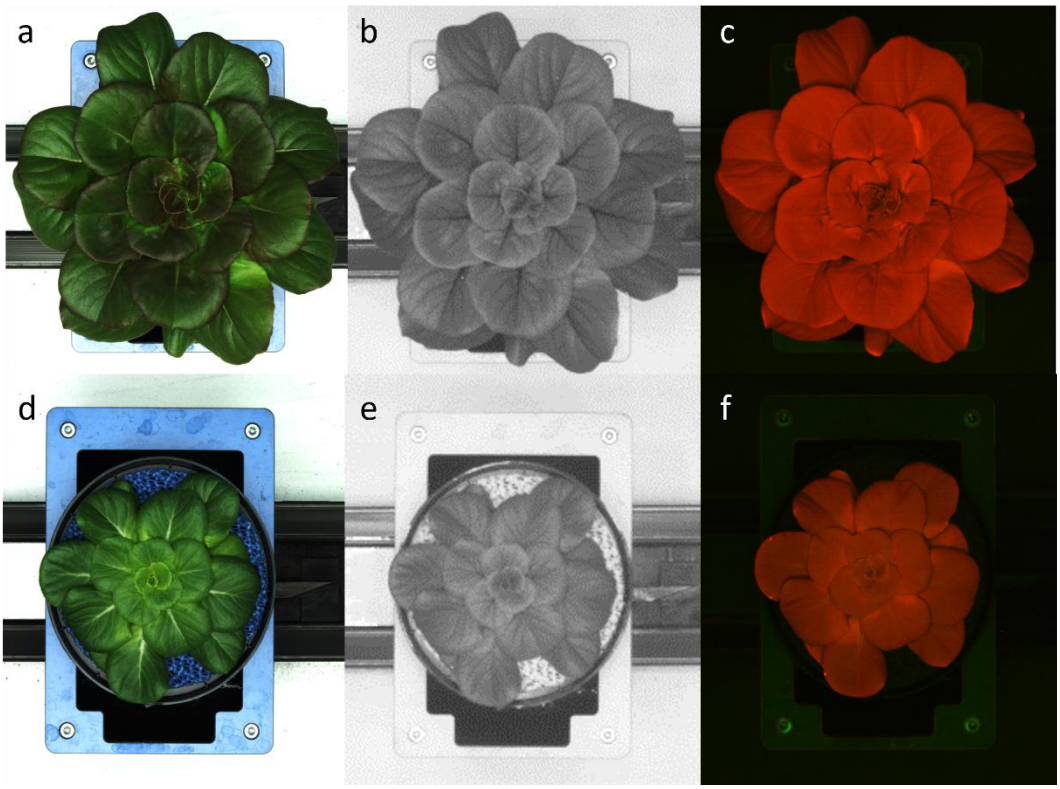

Figure 2: the phenotype of a representative plant under WW (a,b,c) and WL (d,e,f) conditions: images acquired with the three different cameras sensors used in the automated HT screening system for small plants: imaging in the visible light spectrum (VIS) (a,d), fluorescence imaging (FLUO) (b,e), and imaging in the NIR light spectrum (c,f).

Table 1. List of selected traits, name, description code and category are specified.

Trait Name	**Trait Description**	**Trait code**	**Trait category**
Top Area	Projected plant pixel area from top view. It may serve as a measure of plant size and correlates with plant biomass.	TA	Architectural
Convex hull Area	Smallest geometrical object without concave parts that covers whole plant. Provide information on plant size	CHA	Architectural
Yellow to green	Proportion of yellow color plant pixels divided by the count of green color pixels. Measure of amount of senescent tissues (yellow).	Y2G	Physiological and Color-related

Lab color a	Average color in the a* range (green to red) of the L* a* b* color space. Small values indicate green and high values indicate red. Indicator of level of stress.	Lab_a	Physiological and Color-related
Lab color b	Average color in the b* range (blue to yellow) of the L* a* b* color space. Small values indicate blue and high values indicate yellow. High values are Indicator of level of stress.	Lab_b	Physiological and Color-related
Intensity mean	Average intensity of the fluorescence reflection based on the color of each pixel (pure red highest intensity, yellow lowest intensity). Provides a relative measure of photosynthetic health by detecting senescence, necrosis and chlorosis.	Int	Physiological and Color-related
Quantum yied of Photosystem II (PSII)	The operating efficiency of PSII photochemistry, F_q'/F_m', calculated from $(F_m'–F')/F_m'$. It gives the proportion of absorbed light that is actually used in PSII photochemistry and can be used to estimate the rate of electron transport through PSII with knowledge of light absorptance by the leaf and photosystems.	ΦPSII	Physiological
Maxium efficiency of Photosystem II (PSII)	Ratio of variable to maximum fluorescence—the quantum efficiency of open photosystem II centres	Fv/Fm	Physiological
Non-photochemical quecncing	Non-photochemical processes that dissipate excitation energy.	NPQ	Physiological

9.2.3 Manual trait acquisition: Plants were harvested at 27 DAS and fresh and dry weight determination was performed on all plants at a single plant level by cutting the shoot part of each, weighing for the fresh biomass, and then transferring it into a paper bag and drying it for 3 days at 80 °C. All the weighing was done using a fine-scale balance.

9.2.4 Light microscopy analysis: On harvesting days, 1 fully expanded leaf from 6 plants per treatment was collected in FAA fixative (40% formaldehyde, glacial acetic acid, 50% ethanol, 5:5:90 by volume) and brought back at the PWA (plant and wood anatomy) laboratory of the University of Naples for light microscopy analysis. To accomplished morpho-anatomical analyses of leaf lamina, each leaf was dissected in order to obtain sub-samples of about 5x5 mm, dehydrated in a series of ethanol up to 95% and then embedded in the acrylic resin JB4 (Polysciences, Germany). Resin-embedded leaves were cut in thin cross-sections of about 5µm by means of a rotary microtome, and stained with 0.5% Toluidine blue in water, as reported in Feder and O'brien, 1968. The sections were observed under the BX51 light microscope (Olympus, Germany) and digital images were collected and analyzed through the digital camera Olympus EP50 and Olympus CellSens 3.2 software in order to characterized leaf lamina by measuring the thickness of upper (UET) and lower epidermis (LET) as well as the thickness of palisade (PT) and spongy (ST) parenchymas. All measurements were performed in three positions per cross-section, being careful to avoid veins or any other form of interference. Stomatal traits were determined on lamina abaxial peels, taken centrally in each leaflet avoiding the midrib as well as the margin. Peels were analysed under the abpve-mentioned microscope and for each leaflet, measurements were averaged from 5 measurements obtained from 3 different peels. Stomatal density (SD) was calculated as number of stomata per mm^2, measured at 20x magnification, while stomatal area (SA), expressed in μm^2 was

measured at 50x magnification in 10 stomata per leaf, considering both the guard cell major (pole to pole) and minor axes to calculate the area of an imaginary ellipse, as reported in Sorrentino *et al.*, 2021.

9.2.5 Statistical analyses: The statistical analyses were all carried out using the software IBM SPSS Statistics (SPSS, Chicago, IL, USA). The influence of the different independent factors (VPD, cultivar and water) on the dependent variables was tested by applying a three-way analysis of variance (ANOVA). In the case of significant interactions, data were then subjected to one-way analysis of variance (ANOVA), and mean values were separated according to Tukey test with $p < 0.05$.

9.3 Results

9.3.1 Manually acquired traits: **VPD, cultivar and water regimens had a significant effect over fresh and dry weights:** Both fresh and dry weights (**Figure 3**) followed the same trend with significant differences between low VPD (L) and high VPD (H) and the two watering regimens. Plant weights were increased at low VPD (L) in both WW and LW. Moreover, both weights were enhanced in WW plants with increases from 66-77 % compared to LW. No significant differences were found between G and R plants under WW and LW.

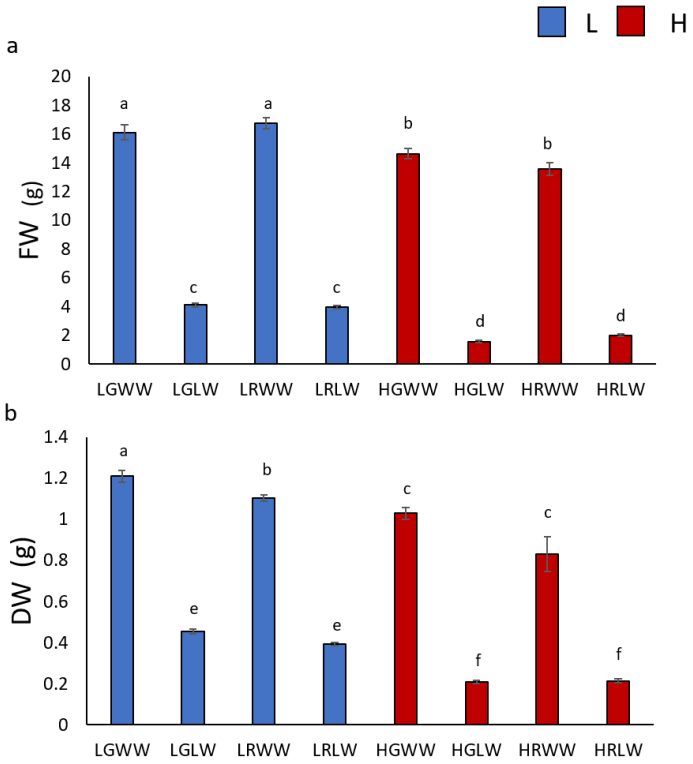

Figure 3: Growth measurements in terms of a) fresh weight (FW) and b) dry weight (DW) of Salanova Green (G) and Red (R) plants grown with the two different water regimens (WW, well watered, and LW, low-watered) under low (L) and high (H) VPD. Mean values and standard errors are shown. Different letters correspond to significant differences according to Tukey test ($p < 0.05$).

9.3.2 Phenotyping traits: In **Figure 4** the expansion in terms of area of green (**Figure 4a**) and red (**Figure 4b**) lettuce plants is showed over the entire growth period, with a focus on the last day of cultivation (histograms). From the Figures it is evident how on day 6 WW and LW plants started to show differences in growth. On the same day, also differences between L and H treatments were more evident, especially in LW plants. According to biomass data, plant area increased at low VPD (L) in both WW and LW. Moreover, area was enhanced in WW plants. From day 12 to the end of growth period, the environmental condition in the chamber changed to the opposite VPD; however, only H R WW plants (now HL R WW) managed to reach values similar to L R WW (now LH R WW) showing no statistically significant differences (**Figure4b** histogram). The same behavior was not showed by green plants (**Figure 4a** histogram).

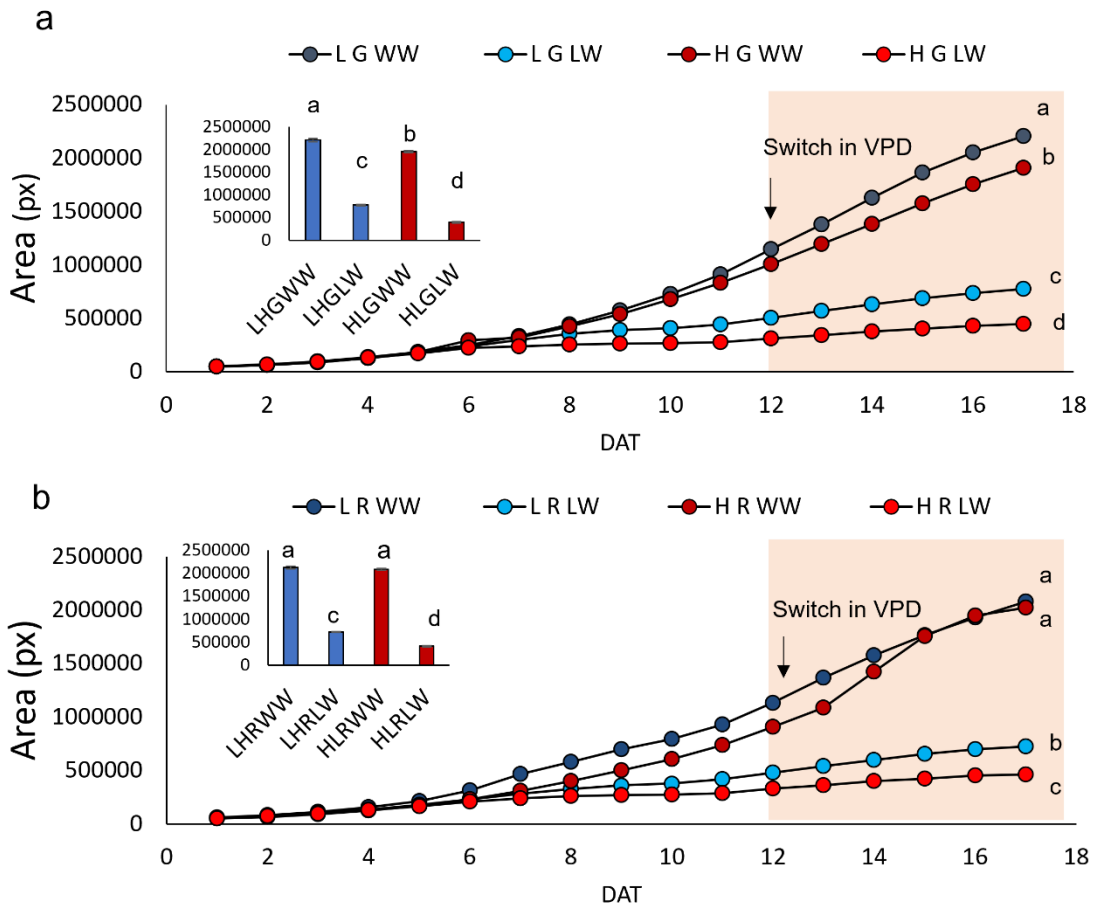

Figure 4: Plant area of a) Salanova Green (G) and b) Salanova Red (R) plants grown with the two different water regimens (WW, well watered, and LW, low-watered) under low (L) and high (H) VPD. Mean values and standard errors are shown. Different letters correspond to significant differences according to Tukey test ($p < 0.05$). Both curves calculated over the whole growth period and histograms concerning the last day of cultivation are showed.

In **Figure 5** phenotyping results from FluorCam imaging fluorimeters are showed, in terms of Fv/Fm (**Figure 5a**), ΦPSII (**Figure 5b**) and NPQ (**Figure 5c**). Fv/Fm values were always higher under L VPD notwithstanding the cultivar (G and R) and the water regimens (WW and LW). Overall highest values were found in L WW plants compared to L LW (reduction of 1.2-3.6 % compared to WW). LH plants showed Fv/Fm values reduced compared to L (2.4 % reduction), but still higher or at least comparable to H and HL. H VPD plants showed lowest Fv/Fm values, especially under LW (reduction of 2.5-3.7 % compared to WW). HL plants showed higher values compared to H plants (increments of about 2 %). Concerning the differences in cultivar, compared to L WW, G WW plants showed higher values under LH and lower H and HL; whereas no differences were found between LGWW and LRWW. Differently, G LW plants showed higher values under LH and lower under L and HL, whereas no differences were detected in H compared to R LW. ΦPSII values (**Figure 5b**) were higher under L VPD in all the condition, except for G LW where no differences were detected among L and LH plants. Overall highest values were found in L WW plants compared to L LW (reduction of 16 % compared to WW). LH plants showed values reduced compared to L, except under G LW, but still higher or at least comparable to H and HL. H VPD plants showed reduced ΦPSII values compared to LVPD, especially under LW (reduction of 20 % compared to L G LW). HL plants showed higher values compared to H, except for GWW where no statistically significant differences were found between H, LH and HL. NPQ values (**Figure 5c**) were always enhanced under LW condition, in order in HL, H, LH and L plants. Concerning the cultivar R plants always showed higher NPQ values than G plants.

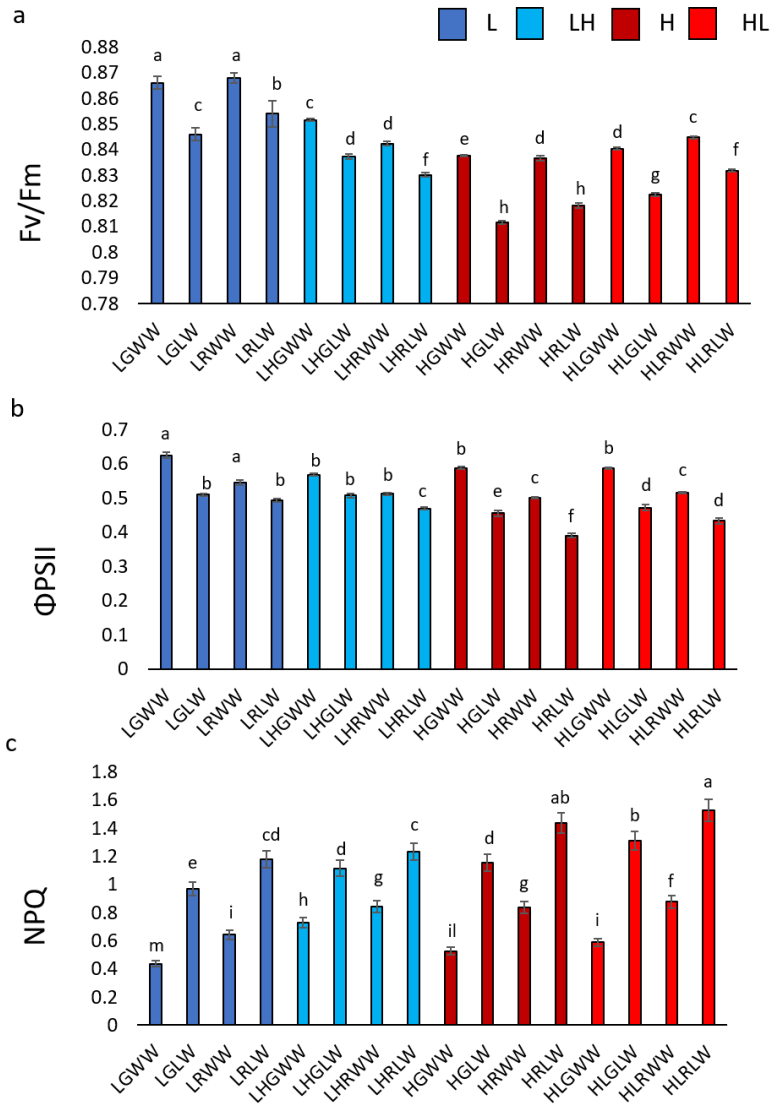

Figure 5: Photochemical parameters in terms of a) Fv/fm, b) ΦPSII and c) of Salanova Green (G) and b) Salanova Red (R) plants grown with the two different water regimens (WW, well watered, and LW, low-watered) under low (L) and high (H) VPD. Mean values and standard errors are shown. Different letters correspond to significant differences according to Tukey test ($p < 0.05$).

In **Table 2** phenotyping results from VIS, NIR and FLUO cameras are showed. As main factors VPD, Cultivar and Water always showed a significant influence over phenotyping data ($p < 0.001$ and $p < 0.01$). Also, the interactions among factors were significant. More specifically, CHA was enhanced under WW and in G cultivar; whereas Y2G, Lab_a, Lab_b and Int were enhanced under LW and Y2G, Lab_a and Int increased in G cultivar while Lab_b in R.

Concerning the VPD, for CHA, Y2G and Lab_a highest values were found in LH followed by HL, L and H. Instead, Int and Lab_b presented higher values in H followed by LH, HL and L. The interaction

among main factors (VPDxCxW) varied more along treatments resulting statistically significant with $p < 0.001$ for Y2G and Int, $p < 0.01$ for CHA and $p < 0.05$ for Lab_a and Lab_b.

Overall, the interaction among factors elicited many changes in lettuce plants among different conditions. CHA was highest in LH G WW and lowest in H G LW, Y2G was highest in LH R LW and lowest in L (with no differences among cultivar and water regimens) and H G WW, LH G WW, LH G LW, HL G WW and HL G LW. Lab_a was enhanced in LH R LW and HL R LW and was reduced in LH G WW and HL G WW, LG WW and L G LW. Lab_b presented highest values in both LH G WW and HL G WW and lowest values in both L G LW and HL R LW. Int was enhanced in H G LW and reduced in L G WW, L R WW and HL G WW.

Table 2: Selected phenotyping traits in terms of Convex Hull Area (CHA), Yellow to Green (Y2G), Lab color a (Lab_a), Lab color b (Lab_b) and intensity mean (Int) of Salanova Green (G) and b) Salanova Red (R) plants grown with the two different water regimens (WW and WL) under low (L) and high (H) VPD before (L, H) and after (LH, HL) the changes in environmental conditions. The effect of VPD, color and water as main factors is also showed.

	CHA	Y2G	Lab_a	Lab_b	Int
VPD					
L	941798.98 **c**	0.042 **d**	109.42 **d**	146.77 **d**	0.67 **d**
H	705215.38 **d**	0.088 **a**	109.82 **c**	150.07 **a**	0.77 **a**
LH	1654841.65 **a**	0.058 **c**	111.72 **a**	149.36 **b**	0.70 **b**
HL	1359831.96 **b**	0.066 **b**	111.30 **b**	147.14 **c**	0.68 **c**
Color					
G	1191242.25 **a**	0.07 **b**	102.49 **b**	158.35 **a**	0.70 **b**
R	1141166.60 **b**	0.12 **a**	118.15 **a**	138.93 **b**	0.72 **a**
Water					
WW	1791806.08 **a**	0.041 **b**	107.85 **b**	143.21 **b**	0.66 **b**
LW	539037.90 **b**	0.084 **a**	113.27 **a**	153.46 **a**	0.75 **a**
Interaction					
L G WW	1405583.59 **d**	0.00019 **f**	98.32739 **g**	142.68 **d**	0.63 **i**
L G LW	596828.85 **l**	0.00014 **f**	98.36892 **g**	133.41 **g**	0.69 **f**
L R WW	1236601.09 **e**	0.00107 **f**	106.88379 **e**	143.73 **d**	0.63 **i**
L R LW	535383.27 **l**	0.00007 **f**	105.28172 **f**	138.44 **f**	0.72 **d**
H G WW	1048050.03 **g**	0.00738 **f**	115.75758 **d**	150.91 **c**	0.70 **e**
H G LW	325733.25 **n**	0.3520 **a**	116.35123**cd**	166.75 **a**	0.83 **a**
H R WW	1133181.74 **f**	0.16603 **b**	116.82561**cd**	139.96 **e**	0.74 **c**
H R LW	311675.43 **n**	0.15656**bc**	117.40012**bc**	166.44 **a**	0.82 **b**
LH G WW	2600019.98 **a**	0.00004 **f**	97.94399 **g**	165.63 **a**	0.66 **h**
LH G LW	875100.85 **h**	0.03 **f**	107.47380 **e**	152.86 **b**	0.72 **cd**
LH R WW	2370652.71 **b**	0.014352**d**	118.26453 **b**	138.24 **f**	0.68 **g**
LH R LW	783619.59 **i**	0.19924 **ab**	122.11828 **a**	149.39 **c**	0.73 **cd**
HL G WW	2235648.24 **c**	0.00008 **f**	98.02747 **g**	165.27 **a**	0.62 **i**
HL G LW	442973.22 **m**	0.00061 **f**	107.67352 **e**	149.51 **c**	0.72 **cd**
HL R WW	2312109.92 **b**	0.13095 **cd**	117.47618**bc**	140.30 **e**	0.65 **h**
HL R LW	446109.03 **m**	0.12650 **cd**	121.01447 **a**	134.72 **g**	0.74 **c**
Significance					
VPD	***	***	***	***	***
C	***	***	***	**	***
W	***	***	***	***	***
VPDxCxW	**	***	*	*	***

9.3.3 Morpho-anatomical traits: VPD, cultivar and water regimens had a significant effect over morpho-anatomical traits (stomata and lamina traits): More specifically, both stomatal density (SD**;** **Figure 6a**) and stomatal area (SA; **Figure 6b**) varied a lot under different VPDs, cultivar and water. SD was enhanced under L compared to H VPD plants, except for RWW condition where no significant differences were found between L and H plants. Moreover, SD was always higher under WW compared to LW with increments in L WW by 15-16% compared to L LW and by 32 % in H WW compared to H LW. Concerning the cultivars, differences were found only in H plants with increments in R WW compared to G WW by 16 % and in G LW compared to R LW by 12 %. SA was always enhanced in LW compared to WW plants with increments in L by 19-22 % and in H by 31-32 %. Moreover, SA was higher under H compared to L VPD among all treatments. Concerning the cultivars, no statistically significant differences were found between G and R among treatments.

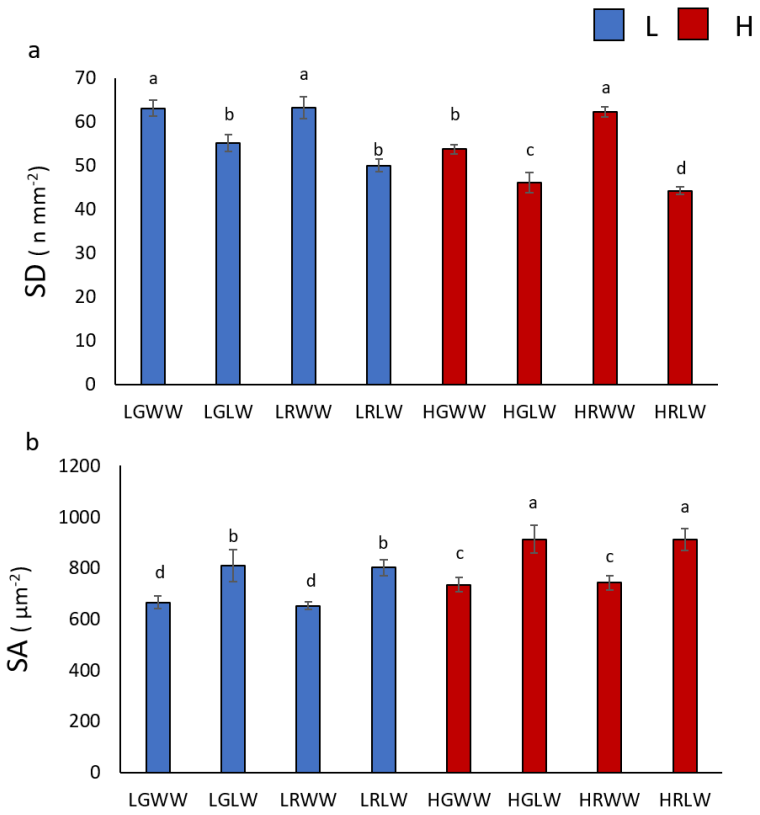

Figure 6: Stomata traits in terms of a) stomatal density (SD) and b) stomatal area (SA) of Salanova Green (G) and b) Salanova Red (R) plants grown with the two different water regimens (WW, well watered, and LW, low-watered) under low (L) and high (H) VPD. Mean values and standard errors are shown. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

Concerning the leaf lamina organization (**Figure 7**). No significant differences were found concerning the upper epidermis thickness (UET). Differently, the lower epidermis thickness (LET) was always

enhanced under L compared to H VPD plants, except for RWW condition where no significant differences were found between L and H plants. Moreover, SD was higher under L compared to H VPD plants, except for RWW where no significant differences were found between H and L. Moreover, while UET was enhanced by 11 % under LW in L plants, the same difference was not showed in H plants where LW plants maintained similar or lower values compared to H WW. Also in LET, concerning the cultivars, the only difference between G and R plants was found in H R WW which showed higher values than H G WW. Many differences were found concerning the thickness of palisade (PT) and spongy (ST) parenchymas. PT followed a different trend under L and H VPD. More specifically, in L VPD plants the thickness was enhanced by 25-29 % in LW, compared to WW; whereas in H VPD plants PT was reduced by 29-31 % under LW plants. ST followed a similar trend compared to PT with increments in the thickness in L VPD by 32-36 % in LW compared to WW and reduction in thickness in H VPD plants LW compared to WW by 35-45 %. Concerning the cultivars, in PT no differences were found except for L R LW that was enhanced by 16 % compared to L G LW. In ST H R WW was enhanced by 22 % compared to H G WW.

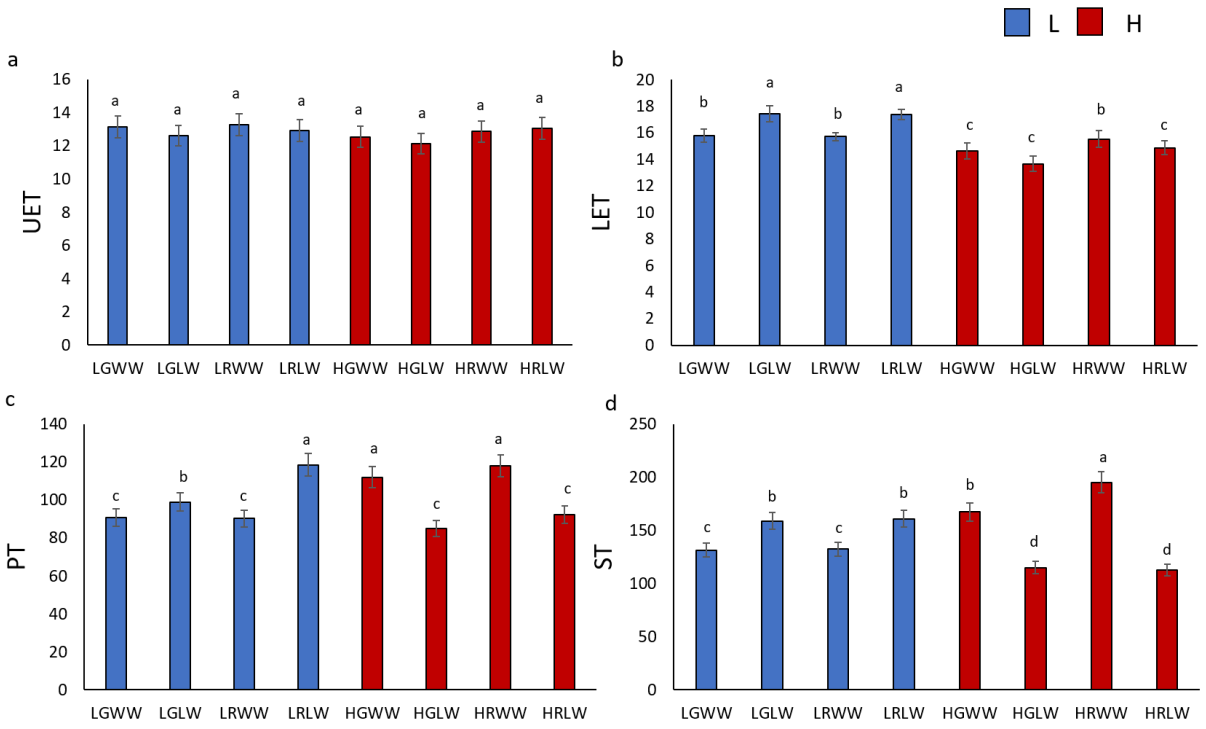

Figure 7: Leaf lamina traits in terms of a) upper epidermis thickness (UET), b) lower epidermis thickness (LET), c) palisade parenchyma thickness (PT) and d) spongy parenchyma thickness of Salanova Green (G) and b) Salanova Red (R) plants grown with the two different water regimens (WW, well watered, and LW, low-watered) under low (L) and high (H) VPD. Mean values and standard errors are shown. Different letters correspond to statistically significant differences according to Tukey test ($p < 0.05$).

9.4 Discussion and Conclusions: Climatic change is increasing the frequency and severity of drought events, also enhancing temperature with a general increment of VPD, which limits crop production worldwide. In this context, many high-throughput phenotyping experiments have

investigated the responses of plants to various environmental stresses like drought, salinity, temperature (Campbell *et al.*, 2015; Hairmansis *et al.*, 2014; Kim *et al.*, 2020). However, to the best of our knowledge, no one has investigated, through high-throughput phenotyping, the combined effect of VPD (low and high) and different water regimens (well-watered and low-watered) to detect the early stress signals in lettuce plants during the first stages of development, with a focus on plant morpho-physiological acclimation to changing VPD. In previous studies (Dodig *et al.*, 2019; Yang *et al.*, 2009), some RGB parameters including area and convex hull area (**Figure 4**; **Table 2**) have been used to predict plant biomass in many crops (especially corn, barley, and wheat). Here, biomass data manually detected at the end of the experiment (**Figure 3**), are in good agreement with plant area detected by RGB cameras (**Figure 4**; **Table 2**), showing the highest area and weight (both fresh and dry) in well-watered plants under low VPD and the lowest in low-watered plants under high VPD. Indeed, reducing VPD by humidification significantly increases biomass allocation in leaves and fruits (Zhang *et al.*, 2017). Interestingly, in the present study after the switch in environmental conditions, only the red cultivar at high VPD (now HL R WW) managed to reach values of plant area similar to the red cultivar at low VPD, under well-watered conditions (**Figure4b**). The short-time exposure to low VPD probably boosted their development in term of area expansion, but it is still not enough to improve fresh and dry weight, which in high VPD plants resulted still lower than the low VPD condition. Increments in area and in biomass in Salanova red cultivar over the green one has been reported before (Chapter 4) and explained as the red Salanova reached maturity earlier than the green one, phenomenon also reported by El-Nakhel *et al.* (2019). However, in the present study, at high VPD, the red cultivar under well watered conditions also showed a higher stomatal density if compared to the green one. This probably explain the improvements in leaf area when red plants developed at high VPD where subjected to short term low-VPD (more favorable environmental conditions). A higher stomatal density is known to improve the carbon gain and to maintain the carbon gain/water loss homeostasis in plants (Lawson and Blatt, 2014). Differently, the lack of increment in fresh and dry weight in HL R WW plants (along with area) could probably be ascribed to the higher development of spongy tissue presented in these plants, compared to the other treatments. Spongy parenchyma, is indeed richer in intercellular spaces with cells which develop a slightly thinner cell walls, compared to palisade parenchyma (Fan *et al.*, 2013). Moreover, in the present study, the chlorophyll a fluorescence imaging revealed many differences between low and high VPD plants before and after the switch in conditions that can serve as proxy for stress symptoms (Chen *et al.*, 2014; Janka *et al.*, 2018). Indeed, fluorescence parameters, including Fv/Fm, are used to determine the status of crops in response to various stresses even before symptoms become apparent (Humplík *et al.*, 2015; Tschiersch *et al.*, 2017) and are key indicators of the growth and photosynthetic efficiency (Baker and Rosenqvist, 2004). From photochemistry data it is evident how the efficiency of photosystem II is reduced in water-stressed plants. After the switch in environmental conditions, both Fv/Fm and ΦPSII showed the same trend with reduction passing

from low to high VPD (L to HL) and enhancement from high to low VPD (H to LH) ; however this trend was more evident in Fv/Fm where values were always statistically significant (**Figure 5**). Vice versa the NPQ, indicator of light energy dissipated into heat to preserve the integrity of photosystem II (Ruban, 2016), followed an opposite trend, being higher under LW and high VPD (H and LH). In our study, however, Fv/Fm values were always higher than 0.8 (even in LW plants), considered a threshold for stressed plants (Ogaya *et al.*, 2011). Also in rice, the parameter Fv/Fm detected through high-throughput phenotyping, did not change significantly in early response of drought stress (Kim *et al.*, 2020). However, other phenotyping traits reveal an early stress in LW plants, especially under high VPD. For example, the Y2G (yellow to green) incremented in low-watered plants under H and LH VPD compared to well-watered plants (**Table 2**), revealing a stressful condition. Y2G is considered one of the color-related traits most sensitive to drought stress which can also reveal wilting symptoms and senescence (Neumann *et al.*, 2015). In accordance with Y2G, the same plants showed enhanced Lab_b. High value of this trait indicate yellow color and Lab_b is therefore a proxy for stress level. Moreover, different studies (Dodig *et al.*, 2019; Ibraheem *et al.*, 2012) have found a relationship between increments in Lab_b and decrease in dry weight in different genotypes. The same relationship is reported in our study, especially for H plants. The main response of plants to drought and high evaporative demand was undoubtedly a reduction in plant area, probably resulting in a decreased water content of the plants. Moreover, a previous study on rice found a strong correlation between the plant area and NIR intensity (indicator of plant water content) (Kim *et al*., 2020). In accordance with this study, here the Int trait is lower in HVPD plants and in LW plants, indicating a lower water content in these lettuces, compared to the other treatments. Moreover, as expected, the Lab_a trait is enhanced in R plants since high value of this traits indicate the color red and small values indicate the color green. Lab_a is also enhanced under LW, indicating an increased redness of water-stressed plants. It is also interesting to see how lettuces react to the shift in the environmental conditions according to their leaf anatomical structure. For instance, low VPD plants, especially under limiting water, developed a thicker palisade mesophyll. This trait together a high stomatal density (compared to H plants) is probably the reason for the maintenance of higher photochemical efficiency even after the switch in environmental condition (LH). Indeed, looking at physiological related triats, LH plants maintained Fv/Fm and ΦPSII values always higher or at least comparable to H and LH plants.

In the present climate change context, very rapid shift in the environmental condition are predicted (Nelson *et al.*, 2014). Thus, understanding the mechanisms of crop morpho-physiological acclimation is necessary to gain knowledge to improve cultivation technique both in the field and in controlled environment agricultureThe analysis of the present data showed that there are strong relationships between all the measured parameters (both manually and remotely), confirming the

potential of this type of instrumentation for monitoring and controlling the progress of crops in a controlled environment and detecting the early-stress symptoms.

9.5 References:

Amitrano C, Arena C, Cirillo V, De Pascale S, De Micco V. 2021. Leaf morpho-anatomical traits in Vigna radiata L. affect plant photosynthetic acclimation to changing vapor pressure deficit. Environmental and Experimental Botany, 104453.

Baker NR, Rosenqvist E. 2004. Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. Journal of experimental botany 55, 1607-1621.

Campbell MT, Knecht AC, Berger B, Brien CJ, Wang D, Walia H. 2015. Integrating image-based phenomics and association analysis to dissect the genetic architecture of temporal salinity responses in rice. Plant Physiology 168, 1476-1489.

Chen D, Neumann K, Friedel S, Kilian B, Chen M, Altmann T, Klukas C. 2014. Dissecting the phenotypic components of crop plant growth and drought responses based on high-throughput image analysis. The plant cell 26, 4636-4655.

Dodig D, Božinović S, Nikolić A, Zorić M, Vančetović J, Ignjatović-Micić D, Delić N, Weigelt-Fischer K, Junker A, Altmann T. 2019. Image-derived traits related to mid-season growth performance of maize under nitrogen and water stress. Frontiers in plant science 10, 814.

El-Nakhel C, Giordano M, Pannico A, Carillo P, Fusco GM, De Pascale S, Rouphael Y. 2019. Cultivar-specific performance and qualitative descriptors for butterhead Salanova lettuce produced in closed soilless cultivation as a candidate salad crop for human life support in space. Life 9, 61.

Fan X-X, Xu Z-G, Liu X-Y, Tang C-M, Wang L-W, Han X-l. 2013. Effects of light intensity on the growth and leaf development of young tomato plants grown under a combination of red and blue light. Scientia Horticulturae 153, 50-55.

Feder N, O'brien T. 1968. Plant microtechnique: some principles and new methods. American Journal of Botany 55, 123-142.

Hairmansis A, Berger B, Tester M, Roy SJ. 2014. Image-based phenotyping for non-destructive screening of different salinity tolerance traits in rice. Rice 7, 1-10.

Humplík JF, Lazár D, Fürst T, Husičková A, Hýbl M, Spíchal L. 2015. Automated integrative high-throughput phenotyping of plant shoots: a case study of the cold-tolerance of pea (Pisum sativum L.). Plant methods 11, 1-11.

Ibraheem NA, Hasan MM, Khan RZ, Mishra PK. 2012. Understanding color models: a review. ARPN Journal of science and technology 2, 265-275.

Janka E, Körner O, Rosenqvist E, Ottosen C-O. 2018. Simulation of PSII-operating efficiency from chlorophyll fluorescence in response to light and temperature in chrysanthemum (Dendranthema grandiflora) using a multilayer leaf model. Photosynthetica 56, 633-640.

Junker A, Muraya MM, Weigelt-Fischer K, Arana-Ceballos F, Klukas C, Melchinger AE, Meyer RC, Riewe D, Altmann T. 2015. Optimizing experimental procedures for quantitative evaluation of crop plant performance in high throughput phenotyping systems. Frontiers in plant science 5, 770.

Kim SL, Kim N, Lee H, Lee E, Cheon K-S, Kim M, Baek J, Choi I, Ji H, Yoon IS. 2020. High-throughput phenotyping platform for analyzing drought tolerance in rice. Planta 252, 1-18.

Lawson T, Blatt MR. 2014. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. Plant Physiology 164, 1556-1570.

Nelson GC, Valin H, Sands RD, Havlík P, Ahammad H, Deryng D, Elliott J, Fujimori S, Hasegawa T, Heyhoe E. 2014. Climate change effects on agriculture: Economic responses to biophysical shocks. Proceedings of the National Academy of Sciences 111, 3274-3279.

Neumann K, Klukas C, Friedel S, Rischbeck P, Chen D, Entzian A, Stein N, Graner A, Kilian B. 2015. Dissecting spatiotemporal biomass accumulation in barley under different water regimes using high-throughput image analysis. Plant, Cell & Environment 38, 1980-1996.

Ogaya R, Penuelas J, Asensio D, Llusià J. 2011. Chlorophyll fluorescence responses to temperature and water availability in two co-dominant Mediterranean shrub and tree species in a long-term field experiment simulating climate change. Environmental and Experimental Botany 71, 123-127.

Ruban AV. 2016. Nonphotochemical chlorophyll fluorescence quenching: mechanism and effectiveness in protecting plants from photodamage. Plant Physiology 170, 1903-1916.

Sorrentino MC, Capozzi F, Amitrano C, De Tommaso G, Arena C, Iuliano M, Giordano S, Spagnuolo V. 2021. Facing metal stress by multiple strategies: morphophysiological responses of cardoon (Cynara cardunculus L.) grown in hydroponics. Environmental Science and Pollution Research, 1-11.

Tschiersch H, Junker A, Meyer RC, Altmann T. 2017. Establishment of integrated protocols for automated high throughput kinetic chlorophyll fluorescence analyses. Plant methods 13, 1-16.

Yang Y, Timlin D, Fleisher D, Kim S-H, Quebedeaux B, Reddy V. 2009. Simulating leaf area of corn plants at contrasting water status. Agricultural and Forest Meteorology 149, 1161-1167.

Zhang D, Du Q, Zhang Z, Jiao X, Song X, Li J. 2017. Vapour pressure deficit control in relation to water transport and water productivity in greenhouse tomato production during summer. Sci Rep 7, 43461.

Chapter 10

Scientific and technical requirements for a miniaturized phenotyping chamber to grow microgreens in Space

10.1 Introduction:

BACKGROUND: This project is set within the framework of the Ph.D. project "Plant Smart Sensors" of the University of Naples "Federico II", Department of Agricultural Sciences, funded by the MIUR with funds from the PON research and innovation 2014-2020, in collaboration with Kayser Italia Srl. The project presents a multidisciplinary character and is aimed at the creation of synergies between the world of scientific research and the industrial reality.

GOAL: The main objective of this project is to provide scientific and technical inputs for the realization of a miniaturized growth chamber for plant phenotyping to grow microgreens in Space. Major interest has been given to controlling environmental parameters (temperature, relative humidity, carbon dioxide) through sensors and to imaging tecnhiques for plant growth and water-status. The results of this research are aimed at the innovation and at increasing the competitiveness of systems for aerospace and agrotechnology sectors.

ACRONYMS AND ABBREVIATIONS

VPD	VAPOUR PRESSURE DEFICIT
ET	EVAPO-TRANSPIRATION
CEA	CONTROLLED ENVIRONMENT AGRICULTURE
RH	RELATIVE HUMIDITY
T	TEMPERATURE
BLSSs	BIO-REGENERATIVE LIFE SUPPORT SYSTEMS
3D	THREE-DIMENSIONAL
LA	LEAF AREA
SLA	SPECIFIC LEAF AREA
LDMC	LEAF DRY MATTER CONTENT
RWC	RELATIVE WATER CONTENT
A_N	NET PHOTOSYNTHESIS
C_I	SUB-STOMATIC CONCENTRATION OF CO_2
G_S	STOMATIC CONDUCTANCE
G_W	WATER CONDUCTANCE
WUE	WATER USE EFFICIENCY
E	EVAPORATION
ΦPSII	QUANTUM YIELD OF PSII

F_v/F_m MAXIMUM PHOTOCHEMICAL EFFICIENCY

ETR ELECTRON TRANSPORT RATE

NPQ NON-PHOTOCHEMICAL QUENCHING

VLA VEIN LEAF DENSITY

10.2 Glossary:

VAPOUR PRESSURE DEFICIT

Vapour pressure deficit, commonly known as VPD, represents the difference (deficit) between the amount of moisture in the air and how much moisture the air can hold when it is saturated. Once air becomes saturated, water will condense to form clouds, dew, or films over leaves. Therefore, the regulation of VPD important for plant transpiration.

EVAPO-TRANSPIRATION

The term evapo-transpiration considers the sum of evaporation from the soil and plant transpiration from leaves to the atmosphere. Evaporation accounts for the movement of water to the air from sources such as the soil and the canopy.

CONTROLLED ENVIRONMENT AGRICULTURE

Controlled environment agriculture or CEA is a technology-based approach for indoor food production. The aim of CEA is to provide protection and maintain optimal growing conditions throughout the development of crops. Production takes place within an enclosed growing structure such as greenhouses, growth chambers, vertical farms and more. CEA optimizes the use of resources such as water, energy, space, capital, and labor.

IMAGING PHENOTYPING

Imaging phenotyping is a non-invasive plant imaging technology which is useful to study and characterize plant morphology and physiology. It operates through different types of cameras aimed at different purposes in chambers with dedicated illumination and automatic sensors. Depending on the setup and the configuration of the cameras, these cabins are equipped with a plant lifting, watering and rotation station.

10.3 General description

10.3.1 Scientific Background: improving crop cultivation in controlled environment is pivotal to ensure food security in a growing population scenario and the perspective of cultivating plants in extreme environments such as onboard Space stations, or the Moon or other planets (i.e. Mars) during either exploratory or colonization missions. Nowadays, ongoing climate changes affect all aspects of food security (production, availability, and access) (climate change and food security, 2008). This is impacting crop production in the various regions of the World in different ways and according to some climate models (Wentz e al 2007) global warming will change the atmospheric water vapour content and the precipitation patterns. As Earth warms, it is likely to have a competition for water resources. Water demand will increase with a contemporary shrink of water supplies, thus affecting the behavior of plants that will face drought conditions and water shortage.
Plants are indeed sensitive to changes in water availability, concerning both the amount of water present in the soil/substrate and its flux toward the atmosphere, which is driven by vapor pressure deficit (VPD) and regulated by stomata (Sulman et al., 2016). The vapor pressure deficit is the difference between the pressure of water vapor in the air (e) and the pressure of water vapor in a saturated atmosphere (e_s), at a given temperature; it could be expressed by the following formula: "$e - e_s$". The VPD is usually expressed in kPa and it increases with the rise of temperature and the reduction of RH %. Plant photosynthetic capacity and water use efficiency (WUE), or more broadly water flows through plants, are deeply influenced by environmental conditions; thus, optimizing the environmental parameters and the use of water for crop productions will be a challenge for assuring a sustainable agriculture. Uncertainness remains in finding the best strategies to ameliorate plant productivity and reduce water consumption by regulation of plant water flows, considering that some ways of reacting to environmental conditions might be specie-specific.

10.3.2 Technological background: to cope with these challenges, the development of Controlled Environment Agriculture (CEA) systems is fundamental and should be based on the modification of the natural environment to create optimal conditions for cultivation. Protected cultivation can be achieved through the monitoring and control of the indoor climate, which concerns both the aerial and the roots compartments. The main goal is to maximize crop production in terms of quantity and quality, allowing the cultivation of field crops throughout the year and, at the same time, reducing the frequency of disease and pests. All these strategies will also contribute to save resources and to recycle nutrients and water in the scenario of a circular economy. Protected cultivation has spread throughout the world in the last decades and goes along with technological innovation which helps improve the CEA state-of-the-art (Giacomelli et al., 2007). Technological research in agriculture goes in the direction of sustainability and automation (mechanization and robotic systems). In this way, it is possible to maintain a specific setting in the growth chambers avoiding human interference

and monitoring plant growth remotely, acquiring accurate data for scientific studies. In this context, controlled environment agriculture has been sponsored by NASA and ESA in Space-oriented research, especially for the cultivation of edible plants as bio-regenerators in life support systems (LSSs) (Wheeler 2010). Bio-regenerative life support systems (BLSSs) are closed-artificial-environments in which plants could regenerate air, through the production of O_2 and the removal of CO_2, purify water, recycle waste, and overall provide fresh food for the crew. Furthermore, the presence of plants on-board, in such a confined environment, has been proven to bring psychological benefits to the astronauts (Stankovic et al., 2018; Goemaere et al., 2019)). Even though these studies date back to the 1950s, nowadays with new advents in technology, the challenge is to create the best conditions for the growth of higher plants in protected cultivations, considering all the potential detrimental constraints of Space environment such as: microgravity, radiation, non-suitable lighting, and the reduced volume available for cultivation in specific mission scenarios. This harsh environment could affect plant growth, modifying some physiological traits and among them relevance is given to the water uptake and transport through the plant. Furthermore, under microgravity conditions, it is possible to have the formation of a quiescent layer on the leaf. This phenomenon would limit the water evaporation and therefore increase the leaf temperature.

10.3.3 Justify the need for the hardware: In this context, the goal of the present study is to give technical and scientific inputs for the development of a "miniaturized growth chamber", equipped with all the technology necessary to grow microgreens at different T° and RH% (thus VPD) conditions. At the same time, this chamber will be able to monitor, through the installation of different types of cameras for imaging phenotyping, changes in water fluxes under the above environmental conditions.

Species: The selected species for the project are *Microgreens* of different botanical families since they are highly suitable for cultivation in growth chamber. However, the hardware can be implemented for other small crop plants (lettuces, micro-tomatoes etc.). Microgreens are easy to cultivate, have a short growth cycle, require minimal volumes and horticultural inputs. Moreover, compared to their mature counterparts they contain higher amounts of phytochemicals and minerals and lower nitrates. Therefore, although plants at this stage of development have not a significant role in resource regeneration, they are interesting as complementary food to be easily produced onboard to integrate crew diet.

Expected results: the results are expected to provide a satisfying description of the growth and development of microgreens and/or small crops in different conditions of temperature and relative humidity. Furthermore, by a comparison of analysis under High and Low VPD, our knowledge of water fluxes through plants in small growth chamber will improve. This set the first stone for its future application either in ground-based experiments or in bioregenerative systems for orbital stations, under micro-gravity conditions.

10.4 Requirements Definition and Analysis

10.4.1 Scientific requirements: follow a list of scientific requirements for *microgreens* growth, considering optimum environmental conditions. However, in section 10.5.2 (technical requirements), the environmental parameters (temperature, humidity, photoperiod) are expressed in a wider range in order to consider also possible environmental stress events. In this way, the growth chamber could be used not only for optimal growth but also for testing the growth and development of the microgreens and/or small crops under sub-optimal or stress conditions.

SPECIES
• *Brassicaceae* • *Asteraceae* • *Chenopodiaceae* • *Lamiaceae* • *Apiaceae* • *Amarillydaceae* • *Amaranthceae* • *Cucurbitaceae*

SIZE
• Height: around 12 cm • To be cultivated in 7-10 cm diameter trays. • High density (1-6 seeds cm^2) • Seed-to-eat: up to 15 days

SUBSTRATE
• Peat

TEMPERATURE
• Optimum: 19/24°C ±1

LIGHT
• Light: intensity of 400-700 nm (PAR- Photosynthetically Active Radiation); quality, specific λ, such as mix of RED (660 nm) and BLUE (470 nm) light • Optimum light intensity: 300 µmol photons $m^{-2}s^{-1}$ • Day-night cycle: 12 h light/ 12h dark

HUMIDITY
• 70-80 (±2) % (optimum condition)

GAS CONTROL
• CO_2 around 350-450 µmol mol^{-1} • O_2 ambient level • Ethylen scrub

WATER SUPPLY
• To provide every day considering the environmental condition which influence evapo-transpiration.

10.4.2 Technical requirements: a list of technical requirements for the growth chamber resulting from the scientific requirements follows.

SYSTEM	CODE	CHARACHTERISTICS
CHAMBER For 2 trays (CH)	CH-01	**Size:** Growth volume x 2 trays (sub-volume of growth chamber): • Height: 20 ± 2 cm for plant • Width 25x25 cm • 1 cm space between trays Height of growth chamber should consider the space for lighting and field-of-view of the cameras.
	CH -02	Note: the dimension requirements are also related to the constrains given by the system utilization on Space platforms.
	CH-03	**Coverage:** The chamber walls could be covered with a aluminum foil to increase plant illumination.
	CH-04	**Partitioning in nutrient compartment-air:** ground equipped with square trays of 10±2 cm and eventually a scale (only for experiments on ground, not for the experiments in µgravity) (weighing lysimeter for the measurement of ET_A (Plant-soil evapotranspiration) **Partitioning in sub-systems:** see below
SUBSYSTEMS		
ENVIRONMENTAL CONTROLS (EC)	EC -01	**Light:** • LED lights: White LED (400-700 nm) or a mix of RED (660nm) and BLUE (470nm) LED in ratio 2:1 or 3:1 • PAR Intensity (300±10 PPFD µmol photons m^{-2} s^{-1}) • Cycle 12 h light • Possible testing conditions for the illumination level and the day-night cycle should be considered.
	EC -02	**Humidity:** • 30-50 % stress condition • 70-80% optimum condition

		By replicating the experiment 2 times having T ° fixed (for example at 23°C) but changing humidity range 2 conditions of VPD shall be obtained
	EC-03	**Temperature** • 24/19 °C (±1 °C) considering optimum for growth • 30/10 °C (±1 °C) stress condition
	EC -04	**CO_2:** Optimum around 350-450 µmol mol^{-1} NOTE: If CO_2 cannot be regulated, it shall at least monitored with a sensor
	EC -05	**Fan:** Internal fan for air circulation
EQUIPMENT FOR INVESTIGATIONS (EI)		Plant imaging phenotyping Cameras:
	EI -01	• **visible light imaging** for growth analysis (above-ground plant morphology, leaf area, color, growth dynamics)
	EI -02	• **fluorescence imaging** for photosynthetic efficiency is feasible by the combination of adapted illumination and filter wheels, filtering the light reaching the sensor of the camera. High intensity blue LEDs can be used for the excitation of Green Fluorescence Protein (GFP), while high intensity red LEDs are suitable for the excitation of chlorophyll molecules.
	EI -03	Measurements of water status: • **Thermal imaging Long-wavelength infrared (LWIR)** infrared cameras to measure plant and leaf temperature, which is indicative of plant water use behavior, including transpiration and leaf stomatal conductance. Water taken up by the roots is lost at level of the leaves by transpiration through:
	EI -04	• **Near Infrared (NIR)** for quantification of water distribution and dynamics
	EI -05	• **Hyperspectral imaging** for detection of water content and leaf pigments, such as chlorophyll and anthocyanins. Cameras shall be placed at the top of the plants. Cameras shall be supported, if possible, by side-view, especially for 3D analysis. Considering that the chamber provides space for 2 trays, every position shall be equipped with different cameras (i.e. RGB, IR, Hyperspectral etc..) and the trays shall rotate in order to acquire data of every type for each plant.
IRRIGATION SYSTEM (IS)	IS -01	**Automatic water supply system**: in relation to scale (weighing Lysimeter) in order to catch changes in soil moisture due to Evapotranspiration, and to replenish water to avoid water deficit.

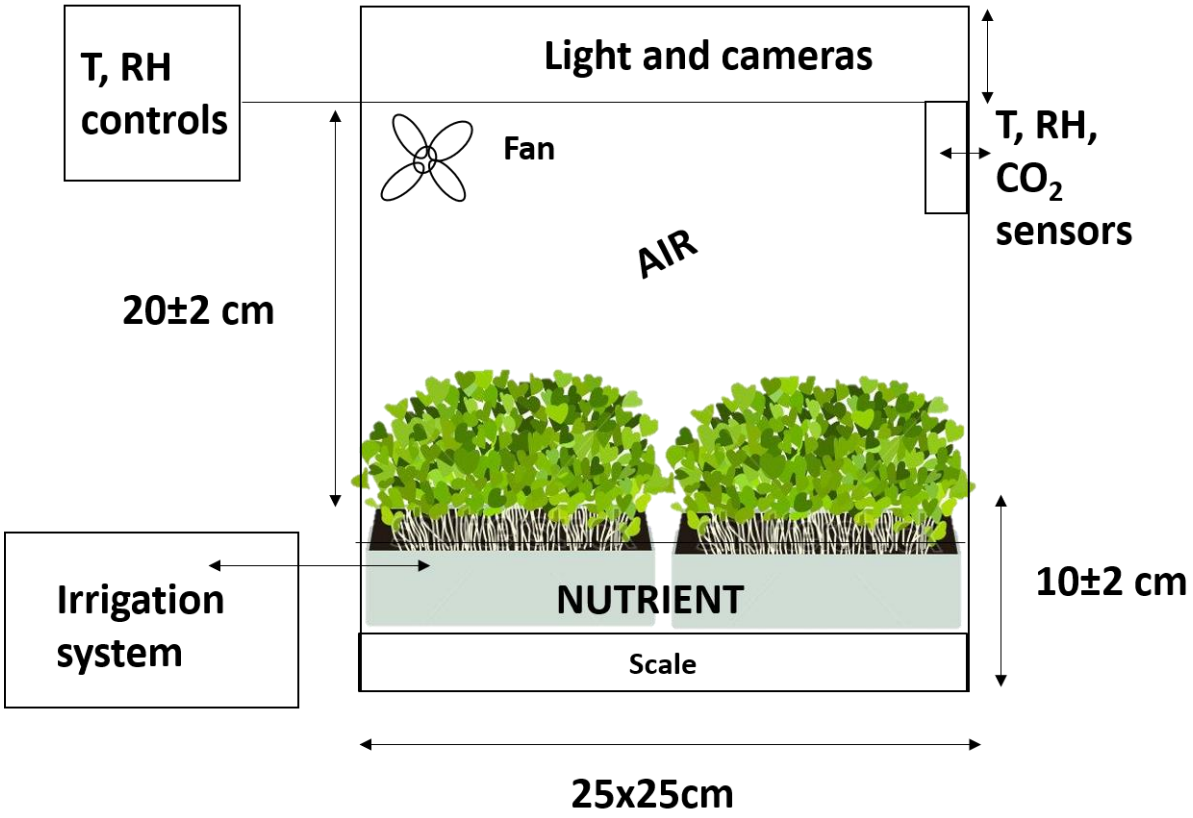

Figure 1: Scheme of growth chamber prototype with specifics and possible collocation of every sub-system.

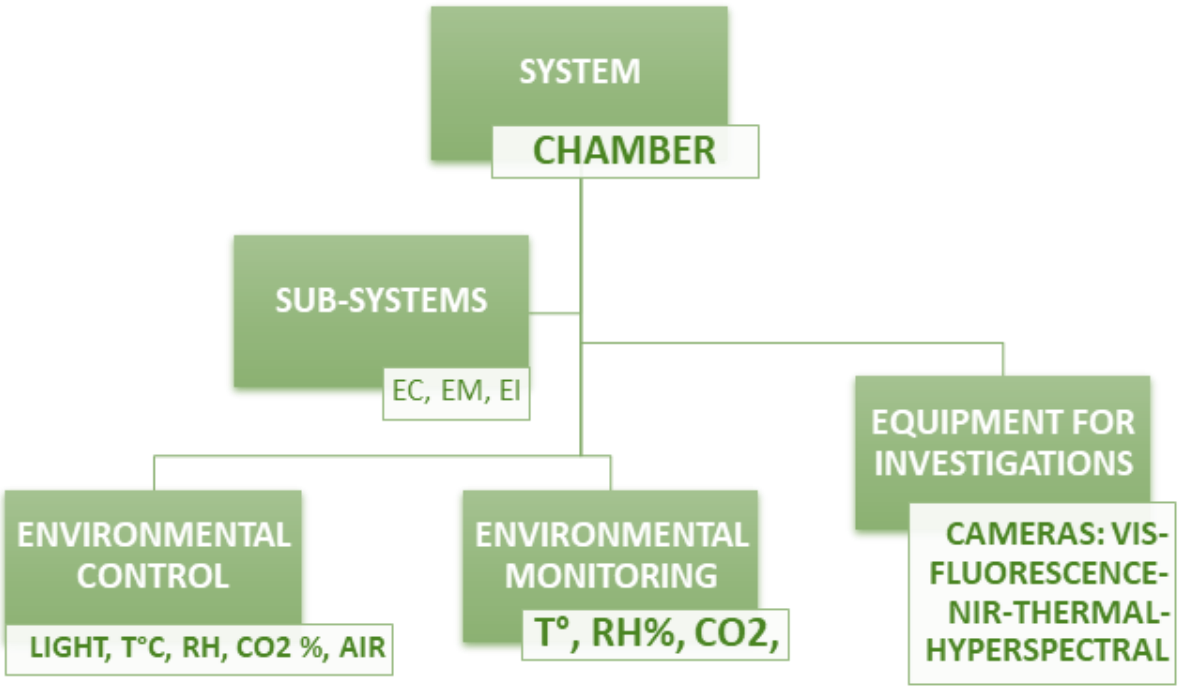

Figure 2: Hierarchical structure of the growth chamber prototype showing how the system is related with its sub-systems.

10.5 Suggested hardware components:

1. ***Environmental control:***

   a. Light panel: on account of the difficulties to find a light LED panel with all the requirement necessary for the hardware (dimension, λ, intensity - see pag 9. Subsystem-light), as reported in Illieva et al. (2010) it is suitable to shape a specific panel using single LED spot: Cree® XLamp® XR Family LED.

   b. Temperature and humidity sensors for the temperature and Rh control: provided that in terms of technological innovation, technology in microclimate sensors is in the vanguard, for the hardware it is appropriate to have sensors like Texas Instruments HDC2080 Humidity & Temperature Digital Sensor. They are small, with wide range of T and RH detected and high levels of accuracy.

   c. CO_2 sensor for the CO_2 gas control: provided that in terms of technological innovation, technology in microclimate sensors is in the vanguard, for the hardware it is appropriate to have sensors like S8 Miniature 10,000ppm CO2 Sensor. Because of its small dimension, measurement range and accuracy.

2. ***Equipment for investigations:***

   A. Lysimeter: to realize ex-novo using a scale or load cells underneath every pot, connecting them with a data logger to have measurement in continuous and to water supply system.

   B. Visible light imaging: CCD camera like Allied Vision Technologies (AVT) Marlin F146B CCD Camera reported in Fahalgren et al. (2015)

   C. Fluorescence imaging: using CCD cameras for visible light imaging + LED panel which provide flash of actinic light 3000 µmol photons m^{-2} s^{-1} (Blue light) + saturating pulse with high intensity LED (red light)

   D. Thermal imaging: Varioscan 3200 ST (Jenoptik, Germany) as reported in Kana et al. (2008)

   E. IR imaging: LEPTON 3.5, 160×120, 57° WITH SHUTTER

   F. Hyperspectral imaging: xiSpec - Hyperspectral Cameras with USB3 Vision

Visible light imaging and Fluorescence imaging will occupy the same position in the chamber, since require the same type of cameras. The other cameras will occupy the three remaining positions. Plants can be located in a carousel; in this way they will undergo on a regular basis every type of cameras.

10.6 Data Analysis: Different types of cameras, listed above, will monitor in continuum plants inside the chamber. All the data, resulting from the monitoring, will be simultaneously acquired and then processed to have a clearer view of results. This process will be realized through means of specific software for imaging analysis. In particular data resulting from Visible light imaging (i.e. acquired by CCD cameras) will provide a view of plant growth and development. Its morphology, architecture, total biomass, leaf area. Thermal imaging (long wavelength infrared-LWIR) will provide measurements of plant leaf temperature, which are indicative of plant water use behavior, including transpiration and leaf stomatal conductance, because evaporation of water cool leaf surface while under drought stress plants close stomata resulting in increased leaf temperature. Hyperspectral cameras will provide information about water content and leaf pigment concentrations, while data resulting from fluorescence imaging will evaluate the "health" status of the photosynthetic apparatus.

10.7 Scientific analysis to support the development of the hardware: In the table below are reported all the scientific analysis and their relative frequency, which will be performed in ground-based experiments on microgreens growing inside the smart growth chamber, for a better understanding of the growth and development of microgreens inside the chamber. Attention will be given to plant-water relationships, with scientific analysis aimed at exploring water fluxes in plants. This is fundamental in order to compare results coming from this analysis with that from imaging cameras and, in this way, having a sort of "control" of the equipment in the hardware.

TYPE OF ANALYSIS	TIME POINTS
GROWTH height, fresh and dry biomass, number of true leaves	At the end of the cycle
PHYSIOLOGY • Gas exchanges (A, Ci, E, g_s, WUE) • Photochemistry (ΦPSII, F_v/F_m, ETR, NPQ)	At the end of the cycle
ANATOMY • Tissue thickness • Stomata (dimension, frequency, opening/closing) • VLA (Leaf vein density)	At the end of the cycle

NUTRITIONAL ANALYSIS • Minerals and organic acids • Antioxidant • Total proteins • Soluble carbohydrates	At the end of the cycle

10.8 Financial projections: The financial projections for the realization of the hardware are highlighted in the table below. It is evident from the table that the major costs are related to the technologies required for investigations. The table account for projected online purchase but another amount should be considered for shipping, material, insurance costs and realization of some tools ex-novo. Also, the expenditures related to the design and development are excluded here.

EQUIPMENT	COST
LIGHT: Cree® XLamp® XR Family LED (20 or more)	~ 4 € EACH. (80 €)
T AND RH SENSORS: Texas Instruments HDC2080 Humidity & Temperature Digital Sensor	~ 6 €
CO2 SENSOR: S8 MINIATURE 10,000PPM CO2 SENSOR	~ 72 €
VISIBLELIGHT IMAGING: Allied Vision Technologies (AVT) Marlin F146B CCD Camera	~86 €
IR IMAGING: Lepton 3.5, 160x120, 57°	~ 80 €
THERMAL IMAGING Varioscan 3200 ST (Jenoptik, Germany)	TO BE DEFINED
FLUORESCENCE IMAGING (SEE PAG. 11 EQUIPMENT FOR INVESTIGATIONS)	TO BE DEFINED
HYPERSPECTRAL IMAGING XISPEC - HYPERSPECTRAL CAMERAS WITH USB3 VISION	TO BE DEFINED
LYSIMETER	TO BE DEFINED

10.9 Schedule for a future project: The project for the plant miniaturized growth chamber realization is foreseen to be conducted in different phases. In a first phase it will be conducted a

literature and market review in order to acquire knowledge on the protocols to be applied and on the components of the facility to be developed. Documentation on scientific requirements and specifics will be produced in order to move to next phase. Then, a preliminary design of the facility will be conducted. Upon successful review of the preliminary design, it will be conducted a critical design phase, and the production of breadboards needed to validate some critical elements. Then, it will start the procurement phase of the components, followed by assembly and testing. The project follows the MAIT (Manufacturing, Assembly, Integration and Testing) plan.

The following is an indicative schedule for this initiative:

1. Start of the project
2. Scientific requirements analysis
3. Design phase
4. Procurement
5. Manufacturing
6. Assembly
7. Integration
8. Testing

Upon approval of such a project a detailed schedule will be created by the assigned project team to include all tasks and deliverables.

10.10 Findings and recommendations: The findings of this study show that this initiative will be highly beneficial to the technological innovation in plant science and has a high probability of success. Key findings are as follows:

Technology:

- Some technologies (i.e. water system irrigation) were not discussed thoroughly in this study. However, the level of knowledge and development achieved in this field is wide and it will be easy to utilize a commercial irrigation system.
- For the project existing technologies will be mainly utilized, which lowers project risks.
- Once in place, this existing technology is simple to operate and maintain for a relatively low cost.

Organization:

- No new facilities are required for the realization of the hardware.

Financial:

- The majority of the fund wills be needed in the first years of operation for design of the hardware and tests.
- Only a small part of the funds will be allocated for maintenance.
- Costs will be amortized in next years, since the hardware, being equipped with a breakthrough technology, could be utilize many times for plant studies with different purposes.

Scientific:

- The realization of the hardware will allow breakthrough in plant scientific community and will broaden the state-of-art of CEA. Thanks to this hardware, we will have a detailed view of plant behavior in controlled environment and especially of plant-water relations under changing environmental conditions. Scientific analysis on microgreens/plants (see section above), performed to better understand water fluxes through plants, will support the analysis of data coming from automatic phenotyping cameras playing a significant role for the results of this study.

10.11 Appendix 1. Open Issues: The main open issue of the project concerns the future spatialization of the hardware. As mentioned before, Space is a harsh environment in which some factors could interact with plant life, altering plant growth and development. Among these factors, microgravity and ionizing radiation are the major constraints to plant development. For instance, ionizing radiation could have a negative impact on plants, lead them to ROS production, damaging proteins and nucleic acids and reducing growth. However, at low doses radiation could even help plant development, leading to that phenomenon known as "hormesis", which regards the improvement of nutritional values and the stimulation of growth. Focusing on water flows, in microgravity, plants could be subjected to the formation of quiescent layer on the leaf. This phenomenon could limit water evaporation and increasing leaf temperature with consequent causes on plant physiology.

Considering the above aspects, the realization of a "miniaturized growth chamber" in which plant could grow under a controlled environment, and in specific T, RH, VPD conditions, becomes fundamental. In fact, water flow, from soil through plant to the atmosphere is severely influenced by environmental parameters. Thus, being able to understand plant behavior under different VPD conditions on ground-based experiments, could help understanding the effects of Space environment on the same plants and on water fluxes through plants.

However, in order to have a complete spatialization of the hardware, some changes are needed. Concerning the size, in projecting the hardware it was already considered the minimum dimension for accommodating 2 trays or 4 plants of a small crop species. However, it is feasible to reduce the

volume available for the microgreens/plants to have a smaller hardware. Thus, the number of cameras and/or their dimension should be reduced as well. Furthermore, the main issue will consist in the support for the growth. It is true that common soils cannot be shipped from earth to the ISS; it could in fact be a source of contamination. So, for plants growth on board, in order to reduce contamination as far as possible other supports for cultivation should be considered, such as: rock wool, aeroponic, pillows etc.

In the end, a future spatialization of the hardware presented in this study is therefore possible, but some aspects of Space environment should be considered in the process of development and some changes should be provided.

10.12 References

1. Brown, Molly E., and Chris C. Funk. "Food security under climate change." (2008).
2. Wentz, Frank J., et al. "How much more rain will global warming bring?." *Science* 317.5835 (2007): 233-235.
3. Sulman, Benjamin N., et al. "High atmospheric demand for water can limit forest carbon uptake and transpiration as severely as dry soil." *Geophysical Research Letters* 43.18 (2016): 9686-9695.
4. Giacomelli, Gene, et al. "Innovation in greenhouse engineering." *International Symposium on High Technology for Greenhouse System Management: Greensys2007 801*. 2007.
5. Wheeler, Raymond M. "Plants for human life support in space: from Myers to Mars." *Gravitational and Space Research* 23.2 (2010).
6. Stankovic, B. (2018). Plants in Space. *Into Space: A Journey of How Humans Adapt and Live in Microgravity*, 153-170.

   Goemaere, S., Van Caelenberg, T., Beyers, W., Binsted, K., & Vansteenkiste, M. (2019). Life on mars from a Self-Determination Theory perspective: How astronauts' needs for autonomy, competence and relatedness go hand in hand with crew health and mission success-Results from HI-SEAS IV. *Acta Astronautica*, *159*, 273-285.
7. Ilieva, Iliana, et al. "Plant experiments with light-emitting diode module in Svet space greenhouse." Advances in Space Research 46.7 (2010): 840-845.
8. Fahlgren, Noah, Malia A. Gehan, and Ivan Baxter. "Lights, camera, action: high-throughput plant phenotyping is ready for a close-up." Current opinion in plant biology 24 (2015): 93-99.
9. T. Instruments, *HDC2080 Low power humidity and temperature digital sensors,* Texas Instrumets.
10. Kaňa, Radek, and Imre Vass. "Thermoimaging as a tool for studying light-induced heating of leaves: correlation of heat dissipation with the efficiency of photosystem II photochemistry and non-photochemical quenching." *Environmental and Experimental Botany* 64.1 (2008): 90-96.

Chapter 11

Set-up of a prototype miniaturized chamber for cultivation in Space and validation experiments with microgreens

11.1 Introduction: As presented in Chapter 10, the possibility of growing plants in Space in miniaturized growth chambers, relies on the fine tuning of all the environmental parameters which requires a deep understanding of their influence over plant growth and development. In this chapter, we present a first prototype of a growth chamber and the results of experimental trials performed not only to assess its functionalities but also to evaluate its possible accommodation inside the ESA Kubik facility (that is permanently located inside the Columbus module of the ISS). Such prototype of a miniaturized plant growth chamber has been realized by Kayser Italia Srl and has hosted experiments with microgreens under different VPDs.

Generally, the difficulties to build such a growth chamber for Space application are related to the fact that, especially in the case of orbital stations or spaceships, there are few resources, such as volume and power, for whatsoever application on the Space platforms (satellites or orbital stations such as ISS). Therefore, the growth chamber should be as miniaturized as possible, as low power as possible and should have an optimum water utilization. In this sense, the prototyping is quite valuable activity as it allows the designers to perform the design optimization and risk mitigation by directly testing the critical aspects.

11.1.1 The Kubik facility: *Kubik* (**Figure 1**) is a miniature laboratory in the shape of a cube of about 40 cm^3 (external dimensions), that has been working aboard the International Space Station for more than 12 years. This small laboratory works at controlled temperature and has hosted a wide range of life science experiments loaded in the EU- Experiment Unit, a small pocket that hosted samples of bacteria, fungi, human cells, rotifers, and small animals such as worms, and plant seedings. KUBIK can run experiments at the same time with different gravity levels (microgravity or fractional gravity), providing researchers a platform to study the effects of weightlessness or other environmental factor as Space radiations. Till today more than 39 experiments from ESA and national Space agencies have been performed in *Kubik*. (http://www.bioreactorexpress.com/16-kubik)

Figure 1: The *Kubik* facility.

11.2: Prototype chamber set-up: The prototype chamber, realized by Kayser Italia Srl (**Figure 2,3**) is a plexiglass cube box of about 20 cm^3, which can monitor Temperature (T), control relative humidity (RH %), control illumination in terms of quality, intensity, and photoperiod. To allow microgreens growth, the prototype has been equipped with RGB dimmable LED light strips (Eseye 5050). In its final configuration, illumination was provided by white light with a wavelength emission spectrum of 400–700 nm. Different tests were carried out to understand the best combination of T, RH and light intensity levels. Indeed, in very small environments (like the prototype chamber) a high light intensity can provoke sudden rise in temperature, thus injuring plant development.

The humidity control exploits quite innovative technology adopted recently by the Kayser Italia Srl. Such a technology requires less volume and power expenditure with respect to the traditional technological solutions based on the cooling chillers. Moreover, functioning of this new technology is independent of the gravity level under which it is working, that is a great advantage compared to the already cited cooling-chillers-based humidity controllers.

The ambient temperature is maintained by having a convective flow of air with a desired temperature all around the growth chamber prototype as it would be performed in the KUBIK.

Different tests were performed with this prototype with the two-fold objective of assessing the technology and of having a first scientific feedback. More specifically, the following goals were pursued:

- Testing the innovative technical solution for the humidity control.
- Understanding the interdependency of temperature, humidity and illumination to design holistic and low-power (as much as possible) environmental control for the future growth chamber suitable for the Space applications.
- Obtaining precise data concerning microgreens biomass and water transpired in such conditions.

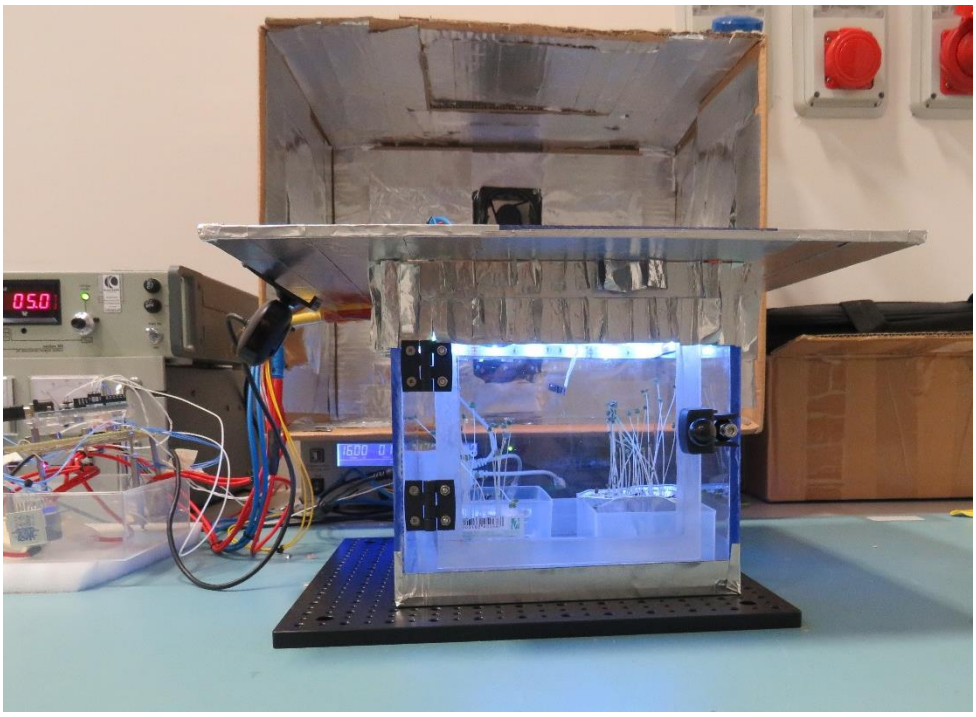

Figure 2: The prototype growth chamber with microgreens.

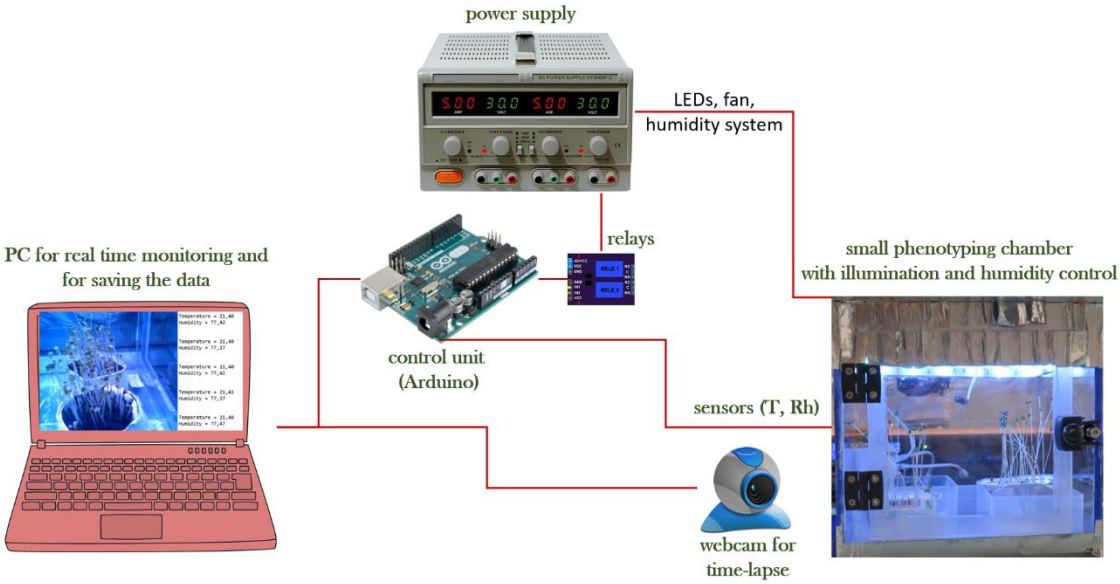

Figure 3: Set-up scheme of the miniaturized growth chamber with sensors.

11.2.1 Functional assessment: In order to show the functioning of the growth-chamber prototype, here are described some of the tests performed by Kayser Italia Srl, in order to assess its functionality. The results of such tests provide insights fundamental to decide the set-up of the environmental parameters to fulfill the cultivation requirements.

More specifically, three tests of the duration of three days were conducted in the empty prototype to monitor different RH, T and light intensity conditions. In **Figure 4** the variation of RH and T with LED lights at the maximum intensity (100 µmol photon m^{-2} s^{-1}) and then with lights off are showed over a period of 12h each. As expected, with the light on at the maximum intensity, reached by the LED strips, the T rises at a level of about 30 °C, considered too high for microgreens growth.

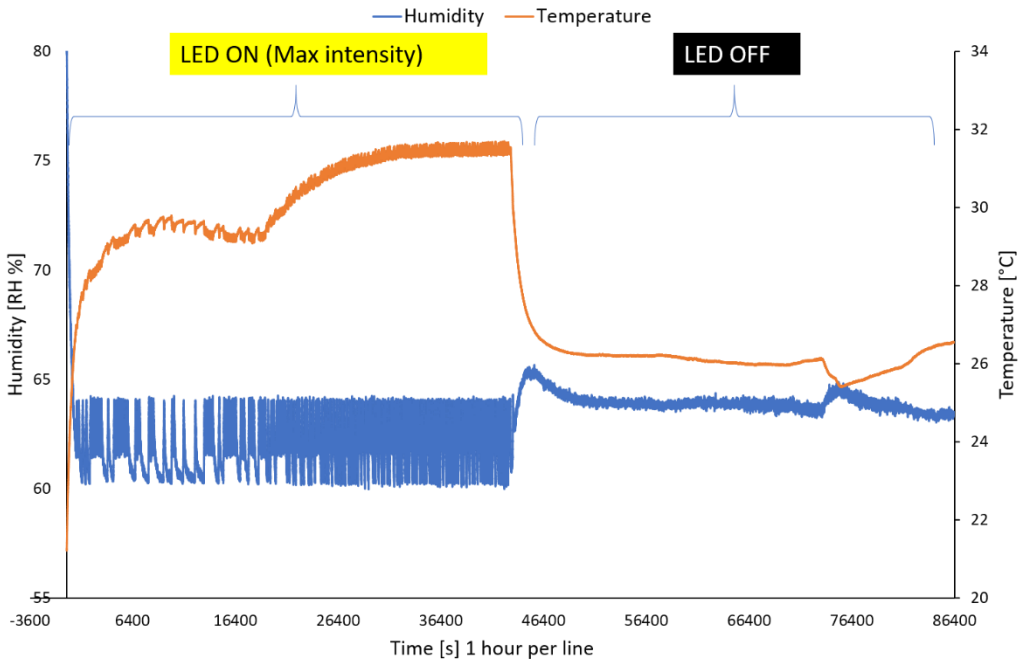

Figure 4: RH % and T changes at changing light intensity (LED on with maximum intensity and LED off)

Conversely, as showed in **Figure 5**, when lowering light intensity (50 µmol photon m^{-2} s^{-1}), the temperature remains at acceptable levels around 25 ° C. It is interesting to notice that at both light intensities the system is capable of keeping RH % constant.

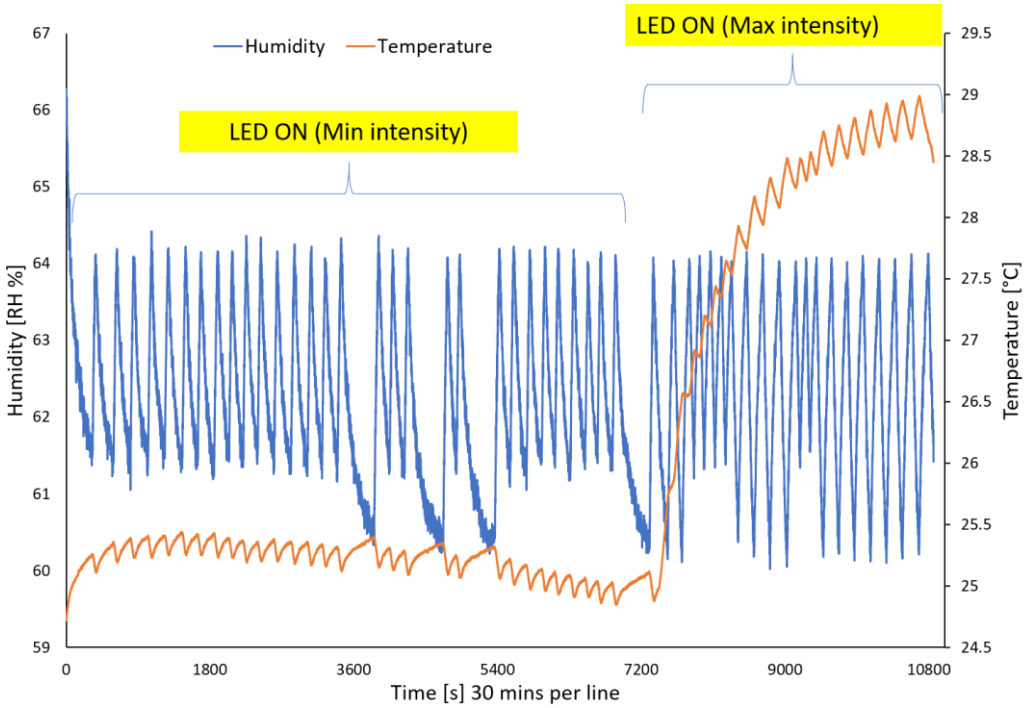

Figure 5: RH % and T changes at changing light intensity (LED on with minimum intensity and LED on with maximum intensity)

11.3 Material and methods

11.3.1 Experimental design and analysis: 5 experimental trials were realized growing *Brassica rapa* subsp*. sylvestris* var*. esculenta* microgreens under different VPDs. This species was chosen because of its good nutritional properties and its common use as microgreens. Seeds were purchased from AgriBioShop (https://www.agribioshop.it/) and showed 100 % germination. All the plant material was delivered from the Department of Agricultural Science (The University of Naples) to Kayser Italia Srl. In two different configurations, to test the best shipping method as a shipping test for the future hardware spatialization.

The two delivery methods were:

1) Trays already containing soil and seeds sealed in vacuum bags.

2) Bags with seeds separated from the soil (to sow before the start of the trials).

Shipping took at least 5 days.

All the trials were performed over a period of 10 days, microgreens were cultivated in small round trays (8.5x8.5cm) (**Figure 2**). In all the trials, the light intensity was 50 µmol photon m^{-2} $s^{-1}$and Temperature was 24 ± 2 °C.

- The first trial (T1) was conducted at a RH of 75% resulting in a VPD of 0.74 kPa, using delivery method 1.
- The second trial (T2) was conducted at a RH of 52% resulting in a VPD of 1.43 kPa, using delivery method 1.

- The third trial (T3) was conducted at a RH of 75% resulting in a VPD of 0.74 kPa, using delivery method 2.
- The fourth trial (T4) was conducted at a RH of 52% resulting in a VPD of 1.43 kPa, using delivery method 2.
- The fifth trial (T5) was conducted at a RH of 90% resulting in a VPD of 0.29 kPa, using delivery method 2.

Trays with microgreens were weighted every day and daily water loss was completely replenished. The cumulative water transpired (CWT) was calculated at the end of the growth as the amount (mL) of water added daily after bringing back to filed capacity. Before harvesting (occurring after 10 days), the seedlings length was measured (cm); then the above-ground fresh weight (FW) was recorded and the DW was measured after oven drying the samples for at least 3 days at 60 °C.

11.4 Results and discussion: The cumulative water transpired (CWT) was higher for T2 and T4 (1.4 kPa) and lower for T1, T3 (0.74 kPa) and especially T5 (0.29 kPa), where lowest values were found (**Figure 6**). CWT is a trait associated with transpiration, and its reduction in microgreens exposed to low VPD is a positive outcome indicating that these plants were able to reduce the water requirement, at the same time enhancing the efficiency of water use. No significant differences were found between the two different delivery systems at low (T1 and T3) and high (T2 and T4) VPD, indicating that the evapo-transpiration was not affected by the shipping.

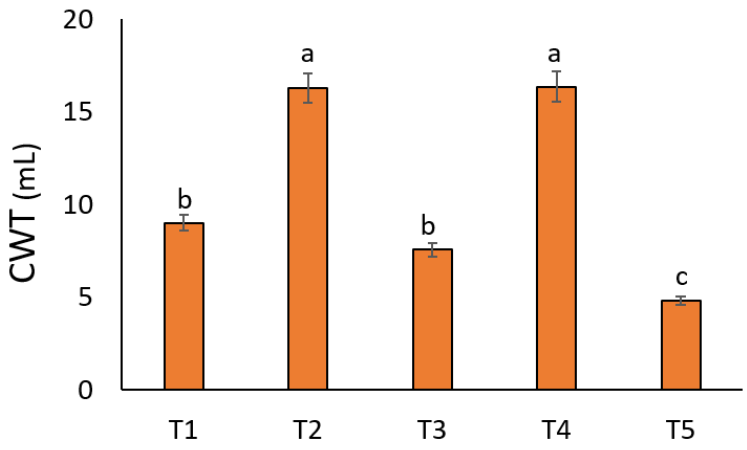

Figure 6: Cumulative water transpired (CWT; mL) of microgreens grown in T1, T2, T3, T4, T5 trials.

Fresh and dry weights (showed in **figure 7**) presented significant differences between the two different delivery methods. More specifically, in FW and DW both at low (T1) and high (T2) VPDs

the delivery method 1 showed reduced values compared to the delivery method 2 (T3 and T4). This is a consistent sign that seed germination was negatively affected by the first delivery method and that the delivery method 2, which keep the seeds separated from the soil since before the starting of experiments, is a better shipping method than the first configuration. Overall, highest values of both FW and DW were achieved by T5 (the lowest VPD condition; 0.29 kPa), meaning that the high RH condition does not injury plant growth. Indeed, too high RH (low VPD condition) can lead to a wide array of problems; most important of which, is the infection and development of plant diseases by a variety of fungi and bacteria species. This puts crops at risk of yield loss and low-quality production. However, these results indicate that, at least for microgreens (10 days after the germination), the lowest VPD condition (T5, 0.29 kPa) is the best way to consistently increase the edible biomass. Indeed, no symptoms of diseases were visually present in T5.

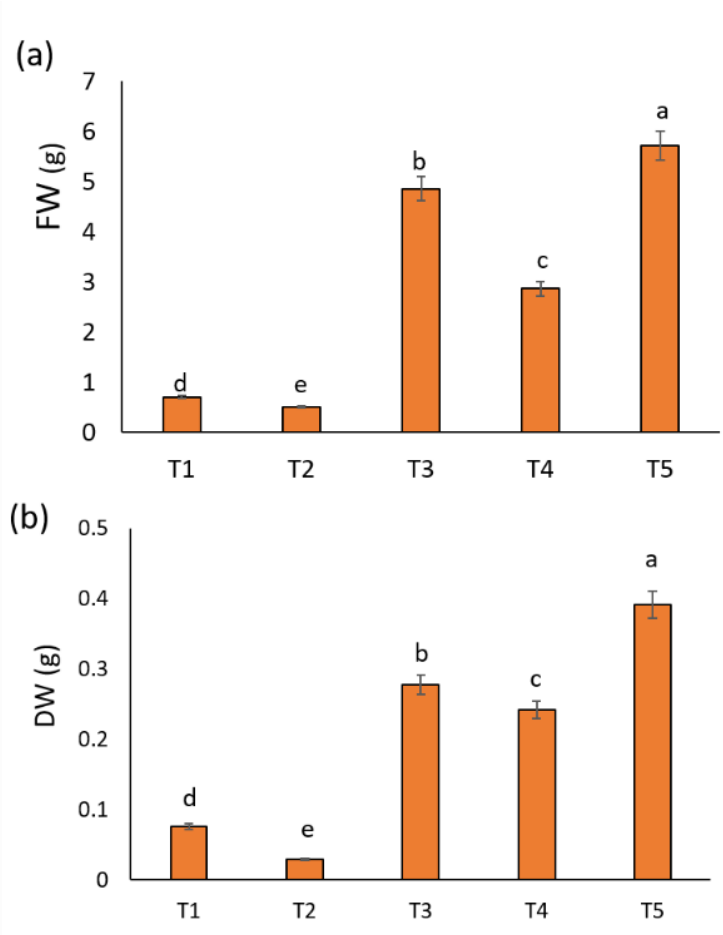

Figure 7: Fresh (FW) and dry (DW) weights (g) of microgreens grown in T1, T2, T3, T4, T5 trials.

In accordance to the biomass data (**Figure 7**), elongation data showed in **Figure 8**, exhibited highest values in T5 (0.29 kPa), confirming the increments in biomass under this environmental condition. Moreover, differently from biomass, slight differences, although still significant, were present

between the two delivery methods. In particular T2 and T4 did not present significant differences, this indicate that method 1 reduces germination but does not impact elongation, once the seeds are germinated. Overall, the shortest microgreens were developed under the high VPD (T2 and T4) notwithstanding the delivery method.

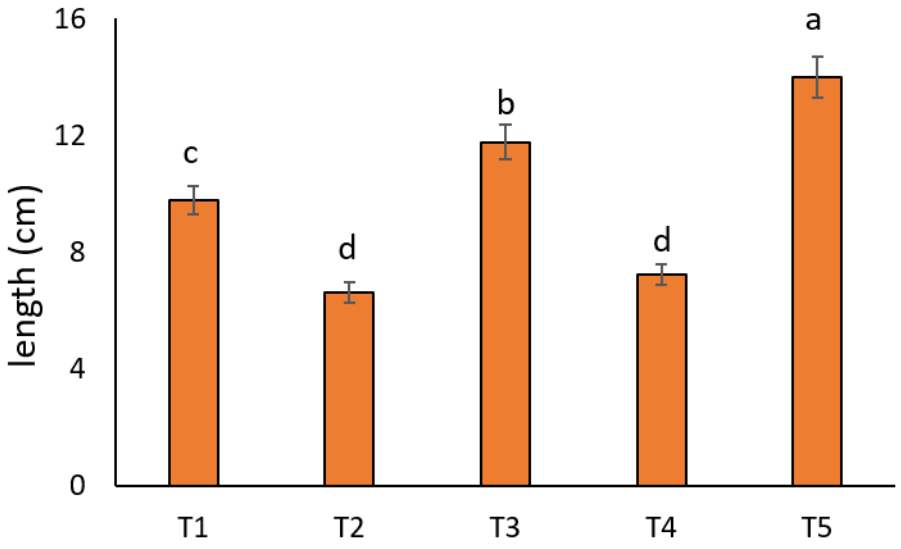

Figure 8: Seedling's length in T1, T2, T3, T4, T5 trials.

11.5 Conclusions and Open issues: The present study showed how both the humidity control and the delivery methods have severe influence on microgreens growth in miniaturized growth chambers for Space farming. It was interesting to notice that microgreens were not affected by the lowest VPD condition (0.29 kPa), showing no disease symptoms while enhancing their biomass and length. In general, the low VPD condition boosted microgreens growth in terms of weights (FW and DW) and length. Moreover, the hardware responded well to the combination of humidity, temperature, and light intensities. However, there are still some implementations to be done before testing the hardware in its final configuration for growth in Space. One of the main constraints is represented by light intensity. Indeed, as showed above, in the configuration of high light intensity, the prototype growth chamber cannot maintain a stable temperature, which becomes too high for microgreens growth (optimum for microgreens around 24 °C). On the other hand, optimal light intensity for microgreens growth is around 300 µmol photons m^{-2} s^{-1}; thus, lowering the light intensity too much would provoke alteration of growth, especially concerning the elongation of hypocotyls as a compensation mechanism to reach more light. To try to overcome these problems, active thermal dissipation technologies or thermal controls could be implemented in order to reach the desired temperature target. Also, different configurations of the ventilation subsystem shall be tested

especially in terms of position of the fans, trying to affect plant growth as little as possible. From the technical viewpoint the tests performed so far has provided some important insights and technical data for the improvement of the thermal control and for the integration of various subsystems.

Conclusions

This thesis project was funded by the Italian Ministry of University and Research (MIUR) in the framework of PON projects for research and innovation. The project was conducted in collaboration with Kayser Italia srl. and the Controlled Environment Agriculture Center of the University of Arizona (UA-CEAC). The aim of the project was dual: to further increase scientific knowledge on plants' responses to microclimatic conditions in controlled environment with special emphasis on plant traits related to water use, and to apply gained knowledge to create synergies between the world of scientific research and the industrial reality targeted at the innovation in the agrotechnology and aerospace sectors.

Over the past three years, as reported in the previous chapters, we focused on unraveling how plant physiology and anatomy are linked, to allow a deeper understanding of mechanistic functioning of crop plants under different environmental conditions. We studied the effects of VPD in controlled environment alone and in combination with other environmental factors/stresses, to test how the morpho-anatomical development of the species could affect their physiology, therefore their productivity, thus ultimately influencing the microenvironment. Experiments in controlled environment have given us the possibility to simulate sudden changes in the environmental conditions, thus testing the plasticity in adaptation of the selected crops. In this way, we were also capable of studying the effects of environmental changes on plants and to understand the morpho-physiological mechanisms that allow plants to respond to short-term changes in the microenvironment. Indeed, VPD regulation has been extensively studied in the past in crop production, especially in greenhouse trials where the relative humidity modulation allows an increased crop production. However, the innovative aspect of this thesis relies in its multidisciplinary approach, going beyond production and marketability in terms of biomass and yield but investigating deeper into the structure to function relationships to understand crop acclimation to a changing environment.

Valuable results were achieved thanks to experiments carried out during the Ph.D. program on species suitable for plant production in controlled environment, with a particular attention to the study of green and red lettuces; the latter chosen due to the contribution of its antioxidant compounds to plant food. It was addressed that the modulation of VPD (in the sense of lowering its levels) is a reliable tool to improve plant growth in term of photosynthesis and production. However, it was also concluded that if developed under different VPDs even the same cultivar develops a different morpho-anatomical structure providing the plants a different capability to acclimate to short-term changes in the microenvironment. Moreover, an overall conclusion is that, while searching for an optimal VPD in controlled environment, careful consideration needs to be placed for balancing morphological, physiological, and anatomical responses of plants and to look for the interaction with

other environmental conditions. Indeed, in Chapter 8 and 9 we explored the interaction between the VPD and other environmental factors (CO_2, light intensity, salt stress, drought) and we always found a different effect on the morpho-anatomical and physiological development of lettuces. More specifically, in Chapter 9 we had the possibility to monitor plant development in a high-throughput phenotyping facility thanks to the application and winning of a grant in the framework of the EPPN (European Plant Phenotyping Network) 2020 Transnational Access (Please find the project *PHEW* winner of the grant in Appendix 1). Applying high-throughput phenotyping allowed us to detect the early stress signals of plants developed under high VPD and water scarcity before symptoms became visible. Another interesting result was achieved in chapter 5 where we discussed how high VPD is sensed by lettuces as a mild-stress provoking enhancement in their phytochemical content, so we proposed the use of short-term high VPD levels to enhance lettuce quality. Overall, the regulation of VPD and all the environmental parameters need to be designed according to the species, considering its adaptive plasticity at the morpho-physiological level. Understanding the structure to function relationships of plants under different VPDs becomes fundamental for the management of precision agriculture both in support of Space exploration and for the sustainability of urban agriculture.

Finally, additional activities have been conducted including the collaboration with other research project, conferences and more broadly education and outreach. The most relevant activities carried out during the Ph.D. program are listed in Appendix 2.

Appendix 1

PHEW - Automated phenotyping platform to improve lettuce water use efficiency under different VPD and watering regimens: Submitted project

A1. Description of feasibility:

The facility considered for the project is the IPK Lemna Tec Scanalyzer for small plants (LTA) in Gatersleben, Germany. This phenotyping platform allows the growth of small plants (about 10 cm diameter and 40 cm height) under controlled environmental conditions (temperature range 10 to 40°C, relative humidity: 40 to 75 %, illumination: 120 -500 $\mu molem^{-2}s^{-1}$ photosynthetic photon flux density, ppfd). The non-invasive image acquisition is carried out with three different camera systems taking images from the top and several side views in the visible wavelength range (390-750 nm), near-infrared (1450-1550 nm) and fluorescence (excitation: 400-500 nm, emission: 520-750 nm); a 3D Laser Scanner PlantEye (PhenoSpex, Heerlen, Netherlands) and a FluorCam device (Photon Systems Instruments (PSI), Brno, Czech Republic) are also part of the system. These multi-sensor setups support the assessment of around 200 traits describing the dynamics in plant development and biomass formation, plant architecture, relations to plant water content (NIR) or levels of fluorophores as well as plant photosynthetic efficiency. Plants can be sequentially moved through all positions as groups of eight carriers (blocks) or as single carriers (full rotation mode). For imaging, carriers are transported to three fixed image stations and pass through a watering/weighing station.

The feasibility of the project was assessed in collaboration with the managers and operators of the phenotyping platform: Dr. Thomas Altman and Dr. Astrid Junker which are in charge of the IPK, Gatersleben. After reading the first draft of the project, they encouraged us to submit the proposal to the present call for EPPN2020 transnational access projects, and schedule the experiments proposed here for the second half of the year 2020, upon the project approval.

More specifically, the following aspects were discussed:

- Feasibility of the phenotyping platform
- Project schedule
- Mutual interest and impact of the project
- Plant material and growth conditions

The PHEW project consist of 2 consecutive trials, where well-watered and low-watered lettuce plants will be exposed to short-term changes in air VPD (please refer to **figure 1**, description of the work). Following the platform scientists' suggestions, we are planning to perform the "pre-cultivation"

of the plants outside the phenotyping platform, in another phyto-chamber with the same climatization ability. 100 red and 100 green 'salanova' lettuces will be cultivated on a substrate automatically maintained at 100% and 50 % field capacity at the watering/weighing station, where a peristaltic pumps supply water or nutrient solution either as a predefined fixed volume or as an individually calculated amount (as the difference of a carrier weight to a pre-defined target weight). When the plants are ready for the first high-throughput (HT) phenotyping analysis, they will be transferred to the LTA platform and subjected to the automated plant transport and imaging system (the IPK LemnaTec Scanalyzer systems for small plants). Lettuce plants will be kept there and subjected to the shift in the environmental conditions to test their reaction to short-term exposure in changing environment, tillthey will be ready for the second HT phenotyping measurements preferably at the completion of the growth cycle. Samples of leaves will be taken before and after the shift in the environmental conditions and will be brought back to the University of Naples Federico II to perform morpho-anatomical analyses, whereas, with the training given by the platform operators, the IAP (Integrated Analysis Platform) open-source software for HT plant image analyses (Klukas et al., 2014) will be used for image-based plant feature extraction. The two kinds of data (i.e. from destructive and image-based methodologies) will be integrated and the consequent multidisciplinary project results will be ready for dissemination activities.

A2 Excellence: description of the work:

A2.1 Background: Sustainable agriculture is shifting from emphasizing production per unit area towards maximizing production per water consumed (Zhang et al., 2017). Plants absorb water through the roots, which flows from the soil to the atmosphere in what is known as SPAC (soil-plant-atmosphere continuum). The main drivers of water flow are the water potential gradient in the liquid phase, and the difference between the leaf and the atmospheric vapour pressure deficit (VPD), in the gas phase. In vascular plants, VPD represents the main driver for transpiration (Amitrano et al., 2019) and affects plant growth and photosynthesis. Since VPD increases when T rises and RH decreases, climate warming conditions are expected to increase its level (Will et al., 2013). VPD values higher than 2 kPa induced by climate change will intensify plant physiological stress especially under water shortage, by either increasing plant water loss or limiting carbon fixation, depending on the anisohydric or isohydric behaviour adopted and on the water availability in the soil or substrate (McDowell et al., 2008; McDowell and Allen, 2015). In this scenario, the improvement of controlled environment agriculture (CEA) systems are fundamental. These systems (greenhouses, vertical farms, indoor growing modules) protect the crops from unfavourable outdoor climate conditions and pests, also offering the opportunity to modify the indoor climate to create an environment which is optimal for crop growth and production (quality and quantity) (Van Henten et al., 2006). Protected cultivation has spread throughout the world in the last decades

and goes along with technological innovation in the direction of sustainability and automation (and robotic systems), for the remote management of plant growth. These applications are pivotal in the Aerospace sector, where plants are being tested in Biorigenerative life support systems (BLSSs) for the sustenance of human life in extra-terrestrial environment, providing oxygen and food, thus for deep-Space exploration and planet colonization (Wheeler and Stroock, 2008; Wheeler, 2010).
To ameliorate plant performance and reach the potential yield in controlled environment, all the microclimatic factors should be precisely controlled. Among them, vapour pressure deficit (VPD) plays a major role. While atmospheric VPD mediates water flow and constrains water productivity to a large extent, the potential to reduce plant water consumption and improve water productivity by regulating VPD is still uncertain and has received far less attention from growers than traditional methods of irrigation. Nevertheless, it is known that under low VPD (high RH and low T) conditions, plants open their stomata allowing photosynthetic carbon gain, thus enhancing growth, leaf gas exchange and physiological water use efficiency (Zhang et al., 2015; Zhang et al., 2018). Conversely, high VPD (low RH) exposed-plants, subjected to high evaporative demand, are less efficient from a physiological point of view and this limitation depends on both stomatal and non-stomatal factors (Shibuya et al., 2017). Furthermore, atmospheric drought is very common under high VPD conditions, especially in arid and semi-arid areas which can lead to changes in water uptake, thus to water stress. Indeed, increasing plant water use efficiency (WUE) is becoming a key issue in semiarid areas, where crop production relies on the use of large volumes of water. Improving WUE, in a climate-change-scenario is mandatory for securing environmental sustainability of food production in these areas. Recently, it has been recognized that different VPDs can induce changes in leaf anatomical structure, i.e. stomatal size and density, mesophyll organization, vein density and distribution (Bongi and Loreto, 1989; Fanourakis et al., 2013; Du et al., 2019). Therefore, changes in leaf anatomical traits, induced by high VPD, may be responsible for the limited growth and photosynthetic response. Indeed, structural traits establish the limits for physiological adjustment since important metabolic processes are constrained by the physics of the plant structure like mesophyll, stomata and vascular architecture which place a physical limit to plant function that cannot be exceeded (Brodribb, 2009).

A2.2 The need for plant phenotyping: Assessing the plant status continuously and with non-destructive methods is fundamental to gain useful information for the managing of crop systems. Plant adaptation to different VPD conditions, rely on the development of structural and functional traits which affect plant hydraulics and photosynthesis; furthermore, their plasticity in adaptation is affected by complex interactions among multiple environmental factors which are also influenced by management/cultivation techniques (De Micco and Aronne, 2012; Amitrano et al., 2019).The ability to cope with either short-term or prolonged stress is also related to plant behaviour in terms of water potential control. Species with anisohydric behaviour would be more adapted to

moderate water deficit and less dependent on changes in soil water content, since they show large declines in leaf midday water potential during drought because they maintain higher stomatal conductance (g_s) and photosynthesis (A_{net}) rates than isohydric species (McDowell and Allen, 2015).

Evaluating plant physiology is fundamental to understand plant growth potential and is generally done by measuring several traits as indicators, at different scales from single plants to large populations. Therefore, it is extremely long and demanding to carry out analyses on the whole canopy, hence it results necessary to sample a sufficient number of replicates to include the whole variability and avoid bias, as well as to perform destructive analyses. Indeed, in recent years while significant progress has been made in molecular and genetic approaches, the quantitative analysis of plant phenotypes, structure and function has become a major bottleneck. The utilization of automated phenotyping platform can overcome these problems.

Through automatic watering/weighing station and image technique, it is indeed possible to monitor plant growth, evapo-transpiration and the dynamic responses under stress, in real-time and on the whole canopy (Li and Kubota, 2009). The combination of visible imaging, primarily used to measure plant architecture (biomass, area, colour, growth dynamic, leaf and root architecture); thermal infrared which use plant temperature to detect difference in stomatal conductance as a measure of the plant response to the water content and transpiration rate; and fluorescence imaging, used to predict photosynthetic status (quantum yield, non-photochemical quenching, leaf health status), are necessary to achieve a comprehensive understanding of plant health status and functioning, with the final aim of developing sustainable plant production with high yield and limited resource consumption.

A2.3 Expected impact of the work: The characterization of water flows in plants and the real-time monitoring of plant water status in controlled environment could solve a very sensitive problem in the field of agro-technlogy in controlled environments, in support of Space exploration with wide effects also on Earth for sustainable precision farming. The atmospheric VPD plays a crucial role in regulating all water movements through the SPAC; therefore, its precise regulation in controlled agriculture becomes pivotal for the optimisation of plant growth and physiological efficiency. Even though there have been many studies regarding VPD control, alone and/or in combination with other factors, certain points are still unclear or controversial, providing contrasting results in different or even within the same species. This happens mainly due to the complex interactions between many microclimatic factors and plant physiological behaviour at different phenological stages. In a context of climate change, the efficient regulation of VPD can be applied to greenhouse/indoor module production in order to enhance crop productivity, improve WUE and reduce total water consumption to design irrigation strategies, considering the balance between the amount of water saved and the quantity used to regulate VPD. The regulation of VPD and related environmental parameters need

to be designed according to the species and its adaptive plasticity at morphophysiological levels. To date, most of the research has focused on either specific physiological/structural aspect at the single plant level, or on cultivation management or even on technological aspects, with only a few interlinks of knowledge.To bridge this gap and to achieve a comprehensive understanding of VPD effects on crop productivity, an integrated perspective with the creation of synergies among different expertise (e.g., plant physiologist, crop scientists, engineers as well as farmers and stakeholders), is needed. Indeed, the fine modulation of VPD in CEA, monitored through plant phenotyping systems, will allow crop production in a sustainable way even in harsh environments, where a "climate smart-agriculture" becomes necessary to ameliorate the quality of crop and to enhance the production, in order to meet the requirements of the increasing world population. Without a deep knowledge of mechanisms of plant response to environmental change it is difficult to determine where to carry out changes to cultivation protocols.

The present study is planned to guarantee the measurement and therefore the control in real-time of environmental humidity, the adaptation of plant to different environmental conditions and their response to water stress. Results will be useful to enhance the efficiency of water use in plant system aimed at the sustainable management of water resource both in large units, compatible with cultivation and production and in smaller unit which can allow the growth on-board in Space environment. Moreover, results can be used to design models for forecasting variations triggered by anomalies in the environmental controls and be adopted to set-up a precise irrigation schedule. This type of tools can also contribute to manage crop production throughout the year and to cultivate in extreme environments, under the expected climate change scenarios. In addition to its scientific impact, the real-time monitoring of crops, along with the development of Smart Systems, represent the first step to achieve some of the objectives of the 7th Environment Action Programme (EAP) and especially: i) "To protect, conserve and enhance the Union's natural capital" through promotion of "...environmentally beneficial agricultural practices", providing information to achieve "...sustainable agriculture… resource efficiency and productivity"; ii) "To turn the Union into a resource-efficient, green and competitive low-carbon economy". Several EU policy objectives will also be interested, such as within the common agriculture policy (CAP) which support farmers and improve agricultural productivity, ensuring a stable supply of affordable food and help tackle climate change while allowing the sustainable management of agroecosystems.

A2.4 Previous work: Currently the team is involved with a PhD project (innovative industrial doctorate) sponsored by MIUR (Italian Ministry of University and Research), in collaboration with Kayser Italia and the University of Arizona (UoA), for the study of plant responses to changing VPD in controlled environment and the evaluation of "smart sensors" for the fine control of relative humidity. The purpose of the research is to acquire fundamental knowledge for the cultivation of higher plants in CEA, calibration of sensors for environmental control and design of

cultivation indoor-modules. To date, multiple specific objectives have been pursued through numerous experiments on different crops in diverse cultivation systems. More specifically, plant response to environmental conditions have been studied with particular attention to the morpho-functional aspects of plant development (microscopy analysis for the quantification of anatomical and cytological characteristics and eco-physiological measurements). Currently an experiment is ongoing at at the Controlled Environment Agricultural Centre (CEAC) of the University of Arizona in a multi-layer hydroponic vertical farm to conduct engineering and science-based research to address challenges and help advancing technology and crop production applications in indoor growing modules. Furthermore, the team is involved in various projects concerning the growth of plants in bioregenerative systems in Space also within the MELiSSA project (Micro-Ecological Life Support System Alternative programme), with particular reference to: 1) soilless cultivation techniques; 2) effect of environmental microclimatic factors (light, T, RH) 3) effect of Space factors (radiation and confined environment) on growth. Those studies concerned aspects of biological, agronomic and technological nature in order to evaluate the possible realization of a complete biological cycle aimed at the production of fresh food and the regeneration of resources on-board Space stations.

A3 Objectives and work plan: The main aim of this project is to maximize the resource use efficiency with particularly attention to the water use efficiency of lettuce plants grown in CEA, under different environmental scenarios, in order to optimize the yield and the quality of edible products. By doing so, a fundamental part will be to analyze the crop plasticity in adaptation to changing VPD, alone and in relation to different water availability. Plant phenotyping will be critical to pursue this goal, because it will allow to identify early stress signals appearing during growth. Furthermore, any changes in the environmental parameters arises a complex process, because the plant itself represents a dynamic sub-system. Indeed, plants implement morpho-functional traits which allow them to adapt to variation in climatic conditions, thus modifying the efficiency of resource use. Even a minimal change in environmental parameters can lead to significant variations in the physiological behavior of the plant, which must be taken into account in order to provide an optimal and dynamic control of the environment itself. The utilization of real-time automated phenotyping platform will be an added value to deeply understand changes happening in the “plant sub-system”, according to the phenological stage and the variations in the environmental parameters.

Specific objectives of the proposal will therefore be: i) to evaluate the responses of different types of lettuce to the variation of parameters such as VPD and water availability, individually and in combination; ii) to obtain the data necessary to define, with the development of a mathematical model, the combination of optimal environmental parameters which enhance resource use efficiency for each type of lettuce; iii) to sample for morpho-anatomical analysis (stomatal density and

dimensions, major and minor vein density (VLA), leaf lamina structure) in order to analyze the possible correlations of such data with between image-derived parameters; iv) finding the most suitable species-specific strategies to improve plant productivity and reduce water loss through the fine regulation of VPD; v) finding sustainable irrigation methods to improve plant WUE through the VPD modulation.

100 Green and 100 red 'salanova' lettuces will be growth and subjected to high throughput phenotyping in 2 consecutive trials. Plants of this cultivar achieve the adult stage still maintaining a size compatible with the selected facility (specifically 10 cm diameter). In the first trial, well-watered (100% field conditions) and low-watered (50% field-conditions) plants will be grown under a "Low VPD" condition (about 0.7 kPa), obtained by a precise modulation of air T and RH; whereas, in the second trial, both well-watered and low-watered plants will be grown under a "High VPD" condition (about 1.7 kPa). After about15 days of growth, the first HT phenotyping analysis will be performed. Soon after the first HT phenotyping analyses, the environmental condition will be promptly changed to reach the opposite VPD condition and after another 5 days of growth a second HT phenotyping analysis will be performed to test the short-term acclimation of lettuces under changing VPD environment. Samples for morpho-anatomical analyses will be collected before and after the short-term exposure. After the experiments, all environmental data as well as phenotyping and morpho-anatomical data will be analyzed and integrated together.

The following diagram provides the detailed project schedule and research activities:

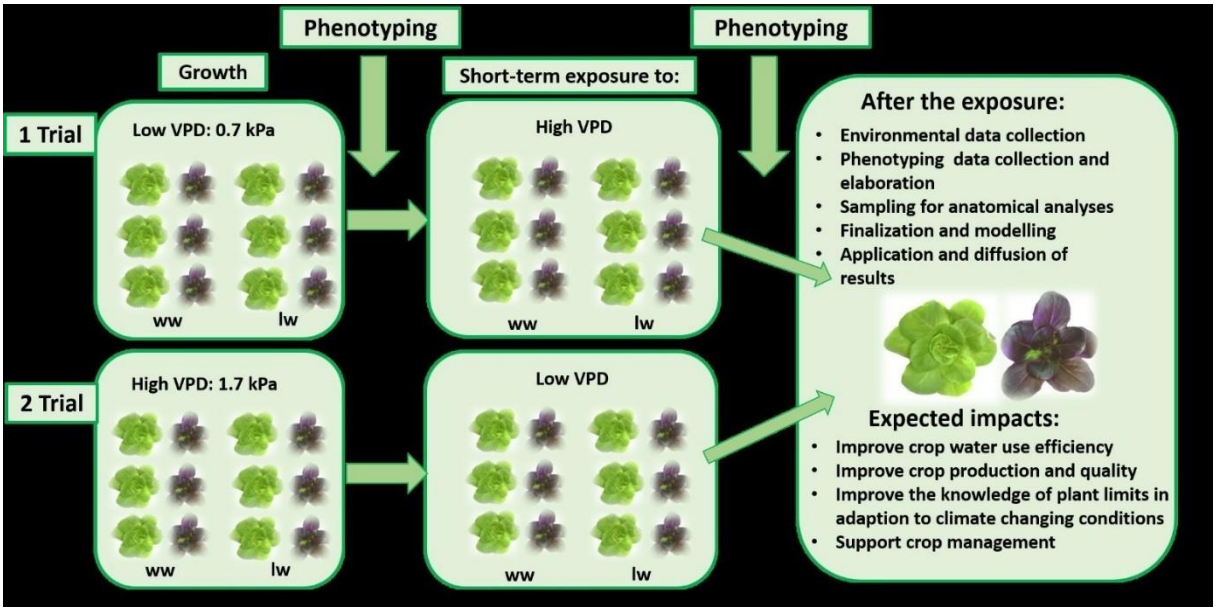

Fig. 1 Summary diagram of the experiments to be conducted at the phenotyping facility. Two consecutive trials are presented. ww and lw indicate well-watered and low-watered plants, respectively. Analyses to perform after the experiments and expected impacts are listed in the last section.

A4 Expected outcomes: The realization of the proposed project will provide a better understanding of crop production under climate changing conditions. The utilization of real-time phenotyping sensors represents a step forward in the evaluation of the growth and health of crops under limiting environmental conditions and is therefore a prerequisite for a sustainable management. Indeed, the early detection of stressors will be fundamental in order to understand where and when to apply countermeasures in the system, in term of a different management of environmental and cultivation factors (i.e. irrigation, environmental control, etc.). To achieve this goal, a synergy between different expertise (horticulture scientists, biologists, engineers) is required.

For the dissemination of the projects results and data sharing, the following actions are planned:

- Developing dissemination material and preparation of a reports with the main results achieved by the project written in a user-friendly way in order to disseminate them throughout the scientific community and public outreach;
- Preparation of scientific papers (to be published in peer-reviewed journals) and other article types in order to disseminate innovation and science highlights;
- Participation to conferences in order to promote networking;
- Data sharing in scientific database, available for the community (e.g. TRY Plant Trait Database).

A5 References*:*

Amitrano, C., Arena, C., Rouphael, Y., De Pascale, S., and De Micco, V. (2019). Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. *Annals of Applied Biology*. doi: 10.1111/aab.12544.

Amitrano, C., Chirico, G. B., De Pascale, S., Rouphael, Y., & De Micco, V. (2019, October). Application of a MEC model for the irrigation control in green and red-leaved lettuce in precision indoor cultivation. In *2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor)* (pp. 196-201). IEEE.

Bongi, G., and Loreto, F. (1989). Gas-exchange properties of salt-stressed olive (Olea europea L.) leaves. *Plant physiology* 90(4)**,** 1408-1416.

Boscheri, G., Kacira, M., Patterson, L., Giacomelli, G., Sadler, P., Furfaro, R., ... & Grizzaffi, L. (2012). Modified energy cascade model adapted for a multicrop Lunar greenhouse prototype. *Advances in Space Research*, *50*(7), 941-951.

Brodribb, T.J. (2009). Xylem hydraulic physiology: The functional backbone of terrestrial plant productivity. *Plant Science* 177(4)**,** 245-251. doi: 10.1016/j.plantsci.2009.06.001.

De Micco, V., and Aronne, G. (2012). Morpho-Anatomical Traits for Plant Adaptation to Drought. 37-61. doi: 10.1007/978-3-642-32653-0_2.

Du, Q., Liu, T., Jiao, X., Song, X., Zhang, J., and Li, J. (2019). Leaf anatomical adaptations have central roles in photosynthetic acclimation to humidity. *J Exp Bot* 70(18)**,** 4949-4962. doi: 10.1093/jxb/erz238.

Fanourakis, D., Heuvelink, E., and Carvalho, S.M. (2013). A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. *J Plant Physiol* 170(10)**,** 890-898. doi: 10.1016/j.jplph.2013.01.013.

Giacomelli, G., Castilla, N., van Henten, E., Mears, D., & Sase, S. (2007, October). Innovation in greenhouse engineering. In *International Symposium on High Technology for Greenhouse System Management: Greensys2007 801* (pp. 75-88).

Klukas, C., Chen, D., Pape, J.M. (2014). Integrated analysis platform: an open-source information system for high-throughput plant phenotyping. *Plant Physiol.* 165, 506–518.doi:10.1104/pp.113.233932

Li, Q., and Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environmental and Experimental Botany* 67(1)**,** 59-64.

McDowell, N., Pockman, W.T., Allen, C.D., Breshears, D.D., Cobb, N., Kolb, T., et al. (2008). Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? *New Phytol* 178(4)**,** 719-739. doi: 10.1111/j.1469-8137.2008.02436.x.

McDowell, N.G., and Allen, C.D. (2015). Darcy's law predicts widespread forest mortality under climate warming. *Nature Climate Change* 5(7)**,** 669-672. doi: 10.1038/nclimate2641.

Shibuya, T., Kano, K., Endo, R., and Kitaya, Y. (2017). Photosynthetic Properties and Response to Drought in Cucumber Seedlings Acclimatized to Different Vapor-pressure-deficit Levels. *The Horticulture Journal* 86(3)**,** 334-339. doi: 10.2503/hortj.MI-154.

Wheeler, R.M. (2010). Plants for human life support in space: from Myers to Mars *Gravitational and Space Research* 23(2)**,** 25-33.

Wheeler, T.D., and Stroock, A.D. (2008). The transpiration of water at negative pressures in a synthetic tree. *Nature* 455(7210)**,** 208-212. doi: 10.1038/nature07226.

Will, R.E., Wilson, S.M., Zou, C.B., and Hennessey, T.C. (2013). Increased vapor pressure deficit due to higher temperature leads to greater transpiration and faster mortality during drought for tree seedlings common to the forest-grassland ecotone. *New Phytol* 200(2)**,** 366-374. doi: 10.1111/nph.12321.

Zhang, D., Du, Q., Zhang, Z., Jiao, X., Song, X., and Li, J. (2017). Vapour pressure deficit control in relation to water transport and water productivity in greenhouse tomato production during summer. *Sci Rep* 7**,** 43461. doi: 10.1038/srep43461.

Zhang, D., Jiao, X., Du, Q., Song, X., and Li, J. (2018). Reducing the excessive evaporative demand improved photosynthesis capacity at low costs of irrigation via regulating water driving force and moderating plant water stress of two tomato cultivars. *Agricultural Water Management* 199**,** 22-33. doi: 10.1016/j.agwat.2017.11.014.

Zhang, D., Zhang, Z., Li, J., Chang, Y., Du, Q., and Pan, T. (2015). Regulation of Vapor Pressure Deficit by Greenhouse Micro-Fog Systems Improved Growth and Productivity of Tomato via Enhancing Photosynthesis during Summer Season. *PLoS One* 10(7)**,** e0133919. doi: 10.1371/journal.pone.0133919.

Appendix 2

A2.1 List of publications

A list of papers published during the Ph.D. program is reported. Papers marked with an asterisk correspond to those reported in the thesis chapters.

1. Sorrentino, M. C., Capozzi, F., **Amitrano, C.,** Giordano, S., Arena, C., & Spagnuolo, V. (2018). Performance of three cardoon cultivars in an industrial heavy metal-contaminated soil: Effects on morphology, cytology and photosynthesis. *Journal of hazardous materials*, *351*, 131-137.
2. **Amitrano, C.,** Vitale, E., De Micco, V., & Arena, C. (2018). Journal of Environmental Accounting and Management. *Journal of Environmental Accounting and Management*, *6*(4), 295-304.
3. ***Amitrano, C.,** Arena, C., Rouphael, Y., De Pascale, S., & De Micco, V. (2019). Vapour pressure deficit: The hidden driver behind plant morphofunctional traits in controlled environments. *Annals of Applied Biology*, *175*(3), 313-325.
4. Damiano, N., Bonfante, A., Cirillo, C., **Amitrano, C.,** Erbaggio, A., Brook, A., & De Micco, V. (2019, October). Retrospective Reconstruction of the Ecophysiological Grapevine Behaviour Through the Analysis of Tree-Ring Series to Validate an Approach to Extract Data from Space-Born and UAV Techniques. In *2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor)* (pp. 191-195). IEEE.
5. **Amitrano, C.,** Chirico, G. B., De Pascale, S., Rouphael, Y., & De Micco, V. (2019, October). Application of a MEC model for the irrigation control in green and red-leaved lettuce in precision indoor cultivation. In *2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor)* (pp. 196-201). IEEE.
6. De Micco, V., **Amitrano, C.,** Stinca, A., Izzo, L. G., Zalloni, E., Balzano, A., Arena, C. (2020). Dust accumulation due to anthropogenic impact induces anatomical and photochemical changes in leaves of Centranthus ruber growing on the slope of the Vesuvius volcano. *Plant Biology*, *22*, 93-102.
7. ***Amitrano, C.,** Chirico, G. B., De Pascale, S., Rouphael, Y., & De Micco, V. (2020). Crop Management in Controlled Environment Agriculture (CEA) Systems Using Predictive Mathematical Models. *Sensors*, *20*(11), 3110.
8. ***Amitrano, C.,** Arena, C., De Pascale, S., & De Micco, V. (2020). Light and Low Relative Humidity Increase Antioxidants Content in Mung Bean (Vigna radiata L.) Sprouts. *Plants*, *9*(9), 1093.
9. Cirillo, V., D'Amelia, V., Esposito, M., **Amitrano, C.,** Carillo, P., Carputo, D., & Maggio, A. (2021). Anthocyanins Are Key Regulators of Drought Stress Tolerance in Tobacco. *Biology*, *10*(2), 139.
10. ***Amitrano, C.,** Rouphael, Y., De Pascale, S., & De Micco, V. (2021). Modulating Vapor Pressure Deficit in the Plant Micro-Environment May Enhance the Bioactive Value of Lettuce. *Horticulturae*, *7*(2), 32.

11. ***Amitrano, C.,** Arena, C., Cirillo, V., De Pascale, S., & De Micco, V. (2021). Leaf morpho-anatomical traits in Vigna radiata L. affect plant photosynthetic acclimation to changing vapor pressure deficit. *Environmental and Experimental Botany*, *186*, 104453.
12. Sorrentino, M. C., Capozzi, F., **Amitrano, C.,** De Tommaso, G., Arena, C., Iuliano, M., ... & Spagnuolo, V. (2021). Facing metal stress by multiple strategies: morphophysiological responses of cardoon (Cynara cardunculus L.) grown in hydroponics. *Environmental Science and Pollution Research*, 1-11.
13. Cirillo, C., Arena, C., Rouphael, Y., Caputo, R., **Amitrano, C.,** Petracca, F., & De Micco, V. (2021). Counteracting the negative effects of copper limitations through the biostimulatory action of a tropical plant extract in grapevine under pedo-climatic constraints. *Frontiers in Environmental Science*, *9*, 76.
14. De Micco V., **Amitrano, C.,** Vitaglione P., Ferracane R., Pugliese M.G., Arena C. (2021) Effect of light quality and ionising radiation on morphological and nutraceutical traits of sprouts for astronauts' diet. *Acta Astronautica*.
15. **Amitrano, C**., Rouphael, Y., Pannico, A., De Pascale, S., De Micco, V. (2021) Reducing the Evaporative Demand Improves Photosynthesis and Water Use Efficiency of Indoor Cultivated Lettuce. *Agronomy*, *11*, 1396.

A2.2 Projects

A list of projects joined during the Ph.D. program is reported; those marked with an asterisk correspond to those reported in the thesis chapters.

1. 2017-... Research Agreement funded by Cantine Astroni s.r.l. for the monitoring of phenology and morphology, and the characterization of some morpho-biometric and biochemical parameters of *Vitis vinifera* L. leaves as indicators of the vegetative-productive balance and the functionality of grafted and free footed grape plants of the Piedirosso vine, reared at the sites at Tenuta Jossa (Camaldoli, Na, IT).(PI: Prof. V. De Micco).

2. 2018-... Research Agreement funded by RADICIRPINE DI CANONICO & SANTOLI S.R.L.S. on "Morpho-anatomical investigations for the evaluation of theoretical conductivity and hydraulic safety in *Vitis vinifera* L.". (PI: Prof. V. De Micco).

3. 2018-... Research Agreement funded by CAT SERVIZI ALLE IMPRESE S.R.L.S. on "Evaluation of the applicability of an integrated approach to environmental sustainability in viticulture with the use of indicators from the Footprint Family. (PI: Prof. V. De Micco).

4. 2019-...“Sustainable models of cultivation of the *Greco* grape: efficiency of use of resources and application of indicators of the 'Footprint family' - GREASE ". PSR Campania 2014 - 2020 Submeasure 16.1 action 2 - D.R.D. n. 339/2017. (PI: Prof. V. De Micco).

5. 2019-... ASI Project (Italian Space Agency): REBUS - In-situ REsource Bio-Utilization for life support in Space; Call for research for future missions of human space exploration - Thematic area Bio-regenerative systems. Participation in WP1300 “Effect of ionizing radiations on plants” (PI: Prof. S. De Pascale).

6. 2020-… ASI (Agenzia Spaziale Italiana) project: MICROX2 - Microgreens x Microgravity' (MICROx2). Partecipation in WP1300 “Definition of environmental parameters (T and RH%) for microgreens production in Space. (PI: Prof. S. De Pascale).

7. *****2020. EPPN2020 Transnational Access: “PHEW- Automated phenotyping to improve lettuce water use efficiency under different VPD and watering regimens”. (PI: Chiara Amitrano).

8. 2020-...NASA AES Habitation Systems. “Microgravity crop production: Meeting the challenges of water/nutrient delivery management,and providing diet diversity for the International Space Station.” (PI: Prof. M. kacira).

A2.3 Conferences

A list of conferences joined during the Ph.D. program is reported in chronological order. Poster, oral communication and received grant are specified in green.

1. **C. Amitrano**, V. De Micco, E. Vitale, C. Arena "Low doses of ionising radiation and the modulation of light quality improve nutritional traits in soybean seedlings." Plant Biology Europe (PBE), Copenhagen, June 18-21-2018 -Poster. Winner of the PBE travel Grant.

2. C. Arena, L.G. Izzo, T. Tsonev, E. Vitale, **C. Amitrano**, S. Fineschi, V. Velikova, F. Loreto "Modulation of light spectrum affects the physiology of beet (*Beta vulgaris* L.) plants irradiated with heavy ions. Plant Biology Europe, Copenhagen, June 18-21- 2018 -Poster.

3. **C. Amitrano**, V. De Micco, C. Arena "Plants for Space Life: Testing the capacity of two edible species to cope with ionising radiation." Society of Experimental Botany, Firenze 3-6 July 2018 -Poster.

4. **C. Amitrano**, C.Arena, L. G. Izzo, A. Stinca, R. Barile, P. Conti, V. De Micco " Morpho-anatomical and physiological responses of *Robinia pseudoacacia* L. plants to anthropogenic dust deposition in the Vesuvius National Park" 113 Italian Botanical Society (SBI) Congress, Fisciano (SA) 12-15 September 2018 -Poster. Winner of the SBI full Grant.

5. V. De Micco, **C. Amitrano**, E. Vitale, G. Aronnee, C. Arena "Morpho-functional plant traits conferring radioresistance: living in extreme conditions by transforming constraints in opportunities".113 Italian Botanical Society (SBI) congress, Fisciano (SA) 12-15 September 2018 - Oral communication.

6. V. De Micco, C. Cirillo, C. Arena, G. Battipaglia, A. Erbaggio, R. Caputo, Y. Rouphael, **C. Amitrano**, E. Vitale, F. Niccoli, A. Brook, A. Bonfante "Evaluating the effect of a biostimulant on growth performance and productivity of *Vitis vinifera* 'Aglianico' through a multidisciplinary approach tracing functional traits in the continuum soil-plant-atmosphere" EGU, Vienna, Austria, 7-12 April 2019 -Oral communication.

7. **C. Amitrano**, F. Niccoli, G. Battipaglia, C. Cirillo, A. Bonfante, A. Erbaggio, Y. Rouphael, V. De Micco "Functional grapevine anatomy: linking traits of tree rings and leaves to eco-physiological behaviour in response to pedo-climatic and cultivation factors" TRACE (tree rings in archeology, climatology and ecology), San Leucio, Caserta, 7-10 May 2019 -Poster.

8. **C. Amitrano**, A. Pannico, Y. Rouphael, S. De Pascale, C. Arena, V. De Micco "Anatomical and eco-physiological responses of salanova green and red lettuce (*Lactuca sativa* L.) plants under different VPD conditions" SBI, Padova, 4-7 September 2019 -Poster.

9. **C.Amitrano**, G.B. Chirico, S. De Pascale, Y. Rouphael, V. De Micco “Application of a MEC model for the irrigation control in green and red-leaved lettuce in precision indoor cultivation” MetroAgriFor, Portici (NA), 24-26 October 2019 -Poster.

10. N. Damiano, A. Bonfante, C. Cirillo, **C. Amitrano**, A. Erbaggio, A. Brook, V. De Micco “Retrospective reconstruction of the eco-physiological grapevine behaviour through the analysis of tree-ring series to validate an approach to extract data from space-born and UAV techniques” MetroAgriFor, Portici (NA), 24-26 October 2019- Awarded as the best Poster of the session.

11. **C. Amitrano**, G. B. Chirico, Y. Rouphael, S. De Pascale, V. De Micco. “Using explanatory crop models to help decision support system in controlled environment agriculture (CEA)”. EGU 2020, 4-8 May 2020 -Oral communication.

12. **C. Amitrano**, Y. Rouphael, S. De Pascale, A. Pannico, V. De Micco. “Plant acclimation to relative humidity modifies the relationship between leaf structure and function in lettuce crop”. Italian Botanical Society (SBI) 2020, 9-11 September 2020. -Oral communication.

13. **C. Amitrano**, V. De Micco, G.B. Chirico, Y. Rouphael, S. De Pascale, K.C. Shasteen, M. Kacira. “Application of the energy cascade model (MEC) on lettuce crop grown in controlled environment agriculture at two different scales: a small growth chamber and a vertical farm. Melissa conference 2020. 3-5 November 2020. -Oral communication.

14. **C. Amitrano**, C. Arena, P. Vitaglione, M. Pugliese, V. De Micco. “Light quality and X-ray treatments improve the nutraceutical properties of mung bean (*Vigna radiata* L.) sprouts. Società Italiana per la Ricerche sulle Radiazioni. 10-12 November 2020 -Oral communication.

A2.4 Courses

A list of courses and summer/winter schools joined during the Ph.D. program both at the University of Naples “Federico II” and other institutions is reported in chronological order.

University of Naples Federico II

1. Data analysis, Prof. Paolo Nasta –13/03 29/05 2018, 56h, 6CFU.
2. NMR (Nuclear magnetic resonance) spectroscopy applied to life science, Prof. Pierluigi Mazzei – 11/04 6/06 2018, 18h, 6CFU.
3. Advanced English, Prof. Dianna Pickens – 1/03 1/06 2018, 70h, 9CFU.
4. Vegetable crop production, Prof. Youssef Rouphael – 6/03 31/05, 70h, 9CFU.
5. Science communication: How to communicate your science, Dr. Eleonora Vitagliano –28/05 1/06, 30h, 6CFU.
6. How to write a scientific paper, Prof. Dianna Pickens and Prof. Domenico Carputo – September-November 2018, 70h, 9 CFU.

Others

1. International Summer School: “Radiation- induced effects with particular emphasis on genetics, development, teratology, cognition as well as space-related health issues”, Belgian Nuclear Research Centre, SCK.CEN, Mol (Belgium), Prof. Sarah Baatout - 12-21/03 2018, 48h, 8CFU.
2. International Summer School of “Functional Plant Traits” Porquerolls, (France), Prof. Eric Garnier - 19-24/05 2019, 27h, 6CFU.
3. International Summer School of “Plant Phenotyping” Metaponto, ALSIA-Agrobios (IT), Prof. Sanità di Toppi - 3-5/07/2019, 27h, 6CFU.
4. Sensors and Controls – Principles of electric circuits. Selection, interfacing, and calibration of digital and analog sensors to measure physical variables. Optical electrochemical and piezoelectric biosensors. Basic bioprocess control. Controlled Environment Agriculture Center, the University of Arizona. Prof: Murat Kacira – January-March 2020, 70h, 9CFU.
5. FoodE Winter School "Sustainability Assessment of City-Region Food Systems" Dr. Martí Rufí-Salís and Dr. Fabio De Menna– 24-26/02/2021, 27h, 6CFU.

A2.5 Education and Outreach

Teaching support activities

-2018-2019 Teaching support activities (n. 16 hours) during the laboratory training classes within the course of “General and Systematic Botany” (Prof. V. De Micco - CdL Agricultural, Forest and Environmental Sciences) on the following topics:

- Preparation of histological preparations and use of the light microscope.
- Determination of the botanical origin of flours through the morphological analysis of amyloplasts.
- Observation and classification of plant tissues through observation under a light microscope.
- Use of botanical guides for species classification.

Teaching support activities (n. 16 hours) during the seminaries within the course of “Elements of Biology and Plant Biology” (Prof. V. De Micco - CdL Food Technologies) on the following topics:

- Space plant biology and extra-terrestrial cultivation.
- Applied botany in crop science

-2018-2019 Support to student examination (about 20 h)

-2020-2021 Support to student examination (about 20h)

-20 May 2020 Seminary “The importance of Botany for crop production” during Prof. De Micco “General and Systematic Botany” (Prof. V. De Micco - CdL Agricultural, Forest and Environmental Sciences)

-25 May 2020 Seminary “The importance of Botany for crop production” during Dr. Ludovica Oddi class “Plant biodiversity and Conservation” (CdL Environmental Biology, University of Torino)

Student competitions

Participation at the student competition “Urban Farm 2021” organized by the University of Bologna (Prof. Francesco Orsini, Dr. Giuseppina Pennisi), as Team leader of the *Agrivolution* Team, which scored first at the first two challenges. https://site.unibo.it/urban-farm/en/teams/teams-2021/view.

Winner of the grant fort he partecipation at the UCSA Lab Village 2021 (http://www.striano.gov.it/content/ucsa-lab-village-2021-idee-ed-azioni-l%E2%80%99economia-circolare-nel-territorio-vesuviano-proroga) with the project *Circular Agriculture.*

Others

- Co-founder of *Giovani per La Scienza* a scientific divulgation group of young scientists (phD students, post-doc fellows) at the Depaertment of Agricultural Science of the University of Naples Federico II.

- Member of the scientific organization of the 2020 CEAC (Controlled Environment Agriculture Center) of the University of Arizona (Tucson, AZ, USA) annual short course (2-6/03/2020)

-Member of the organization committee of the International Summer school in “Wood and Charchoals in Mediterraneean Forest Ecology: anatomical identification and functional traits to interpret

the past and current climate changes", Department of Agricultural Science, University of Naples "Federico II" (21-25/06/2021)

Collaboration as Referee 2020-2021: referee for the following journals: Scientia Horticolturae (Elsevier publishing), Scientific reports (Clarivate Analytics), Biosystem Engineering (Elsevier publisghing), Plant Biology (Blackwell publishing), Peer J (O'Reilly and SAGE), Plant Biology (Blackwell publishing)

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Per aspera ad astra!