

# UniversiTà degli STudi di Napoli Federico II



## Microclimate Factors Modulation Through the Use of Shading Nets and Anti-insect Screens Impacts the Performance and Product Quality of Protected Crops

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# Index

List of PublicationsII
List of TablesIII
List of Figures VI
General Introduction1
Biochemical, Physiological, and Productive Response of Greenhouse Vegetables to Suboptimal Growth Environment Induced by Insect Nets
Improved Porosity of Insect Proof Screens Enhances Quality Aspects of Zucchini Squash Without Compromising the Yield
Divergent Leaf Morpho-Physiological and Anatomical Adaptations of Four Lettuce Cultivars in Response to Different Greenhouse Irradiance Levels in Early Summer Season
Shading Affects Yield, Elemental Composition, and Antioxidants of Perennial Wall Rocket Crops Grown From Spring to Summer in Southern Italy
Pearl Grey Shading Net Boosts the Accumulation of Total Carotenoids and Phenolic Compounds that Accentuate the Antioxidant Activity of Processing Tomato123
Between Light and Selective Shading: Morphological, Biochemical and Metabolomics Insight into the influence of Blue Photoselective Shading on Vegetable Seedlings153
Conclusions
Appendix
Dataset on the Effects of Anti-Insect Nets of Different Porosity on Mineral and Organic Acids Profile of Cucurbita pepo L. Fruits and Leaves
Other publications

## **List of Publications**

- Formisano, L., Miras-Moreno, B., Ciriello, M., Zhang, L., De Pascale, S., Lucini, L., and Rouphael, Y. Between Light and Selective Shading: Morphological, Biochemical and Metabolomics Insight into the influence of Blue Photoselective Shading on Vegetable Seedlings. *Paper submitted to Frontiers in Plant Science on 6th March 2022, under review*.
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- 4. **Formisano, L.**, Ciriello, M., El-Nakhel, C., De Pascale, S., and Rouphael, Y. Dataset on the Effects of Anti-Insect Nets of Different Porosity on Mineral and Organic Acids Profile of *Cucurbita pepo* L. Fruits and Leaves. *Data* **2021**, *6*, 50, doi:10.3390/data6050050.
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# III

## **List of Tables**

### Chapter 2

### Chapter 3

### Chapter 4

Table 1. Rocket yield and dry matter content as affected by cropping season and shading
degree
Table 2. Colorimetric parameters as affected by cropping season and shading degree.108
Table 3. Macroelement content in perennial wall rocket leaves as affected by cropping           season and shading degree         110
Table 4. Effect of the interaction between gropping season and shading degree on

### Chapter 6

<b>Table 1.</b> Photosynthetically active radiation (PAR) during the growing season outside(Control) and under shading nets
Table 2. Air temperature during the growing season outside (Control) and under shading nets.
<b>Table 3.</b> Effects of shading nets on yield and yield parameters
Table 4. Effect of shading nets on total soluble solids (TSS), dry matter, CIELab colorimetric parameters, and fruit size.         .134
<b>Table 5.</b> Effect of shading nets on mineral accumulation in fruits. Data are expressed asmg 100 g <sup>-1</sup> fw137
<b>Table 6.</b> Effect of shading nets on lutein, lycopene, $\beta$ -carotene, and total carotenoids accumulation in fruits. Data are expressed as mg 100 g <sup>-1</sup> fw
<b>Table 7.</b> Effect of shading nets on phenolic compounds accumulation in fruits. Data areexpressed as $\mu g \ 100 \ g^{-1} \ fw.$ .142
<b>Table 8.</b> Effect of shading nets on DPPH, ABTS, and FRAP antioxidant activities. Dataare expressed as mmol Trolox equivalents kg <sup>-1</sup> dw143

**Table 4.** Effects of shading and light quality on minerals accumulation of zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus L.*), tomato (*Solanum lycopersicum L.*), and eggplant (*Solanum melongena L.*) seedlings. Data are mean values ± standard error, *n*=3......172

**Table 5.** Effects of shading and light quality on the accumulation of pigments of zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus L.*), tomato (*Solanum lycopersicum L.*), and eggplant (*Solanum melongena L.*) seedlings. Data are mean values ± standard error, *n*=3......174

### Appendix

## **List of Figures**

### Chapter 2

### Chapter 3

<b>Figure 1.</b> Hourly air temperature ( <b>A</b> ) and soil temperature ( <b>B</b> ) recorded inside the high tunnels covered with nets and without nets
<b>Figure 2</b> . Hourly air relative humidity recorded inside the high tunnels covered with nets and without nets
<b>Figure 3.</b> Average PPFD (Photosynthetic Photon Flux Density) values recorded inside the high tunnels covered with nets and without nets and outside the high tunnels44
<b>Figure 4.</b> Effects of anti-insect nets on the cumulative number of fruits ( <b>A</b> ) and yield ( <b>B</b> ) per plant at different days after transplant (DAT)46
<b>Figure 5.</b> Minimum and maximum relative air humidity ( <b>A</b> ) and air temperature ( <b>B</b> ) recorded outside the high tunnels during the growing season at the experimental site56
<b>Figure 6.</b> Arrigoni Biorete <sup>®</sup> 50 Mesh AirPlus with Harlene HT <sup>®</sup> HDPE (high density polyethylene) monofilament ( <b>A</b> ) and Arrigoni Biorete <sup>®</sup> 50 Mesh with standard HDPE monofilament ( <b>B</b> ). Scale bars are 0.5 mm in <b>A</b> and <b>B</b>
Supplementary Figure 1: Mean temperatures registered from April to July (2015-2018)

### Chapter 4

by the meteorological station of Battipaglia (Salerno, Italy)......58

### Chapter 6

**Figure 1**. Heatmap analysis summarizing the results of yield, mineral, and quality parameters of *Solanum lycopersicum* L. fruits grown under different shade treatments (Control, White net, and Pearl grey net)......145

### **Chapter 7**

**Supplementary Figure 2**. Red:blue ratios of plastic film (No shading), black shading net (Black net) and blue photoselective shading net (Blue net)......157

Figure 4. (A) Venn diagram summarizing the discriminant metabolites down and up accumulated under blue and black nets compared to the unshading plants, as resulted from the variable importance in projection (VIP) analysis (VIP score 1.3). (B) Metabolic processes impaired by shading (blue and black net). Metabolites resulted as discriminant from the VIP analysis, and their fold-change values were elaborated using Viewer Dashboard the Omic of the PlantCyc Pathway Tool software (www.pmn.plantcyc.com). The large dots represent the average (mean) of all log Foldchange (FC) for metabolites, and the small dots represent the individual log FC for each metabolite. The x-axis represents each set of subcategories, while the y-axis corresponds to the cumulative log FC. Nucleo: nucleosides and nucleotides; FA/Lipids: fatty acids and lipids; Amines: amines and polyamines; Carbohyd: carbohydrates; Secondary met: secondary metabolism; Cofactors: cofactors, prosthetic groups, electron carriers, and vitamins: Cell-structures: plant cell structures; Metab reg: metabolic 

## **General Introduction**

The interest in premium-quality fruits and vegetables is an ever-increasing topic among consumers, nutrition specialists, and researchers. The theme of quality lies at the heart of the political and entrepreneurial debate regarding the agri-food sector's competitiveness, posing a challenge to the consumer due to the quality concept's multidimensional nature. In 1971, Lancaster<sup>1</sup>, a forerunner in defining quality attributes, explicitly addressed what consumers perceive; while Steenkamp<sup>2</sup> pointed out that perceived quality can differ from real quality. According to Steenkamp<sup>2</sup>, quality is often based on incomplete information driven by specific cues that change with environment and context. Schreiner et al<sup>3</sup>. hypothesized that quality is idealized by the products' intrinsic factors and social, economic, and marketing factors. A deep investigation of quality perception is necessary, first from a business perspective to reduce the risk of failure in launching new products, and second from a *policy-maker* perspective to understand the links between food quality, safety, and security<sup>4</sup>.

It is noteworthy that the ongoing changes in consumer needs correlate with changes in lifestyle, gender roles, and gastronomic trends<sup>5</sup>. The economic boom in the '50s caused a high demand for agri-food goods, sweeping agriculture and compelling increasingly mechanized food production. The stagnation in consumption caused by the oil crisis in the 1970s emphasized the key role of the consumer in sales strategies. Satisfying consumer expectations was the key to an increase in consumption. In the 1980s, the demand for food became more differentiated, and food was no longer a simple physiological need but rather the answer to a newborn need for gratification. The trend was to extend quality to all production levels in order to meet the changed needs of a consumer who became more attentive to organoleptic and nutritional quality. However, the primary intrinsic attributes of fruits and vegetables, such as color, size, and absence of visual defects, were still disproportionately associated with quality. This awareness has long led to preserving appearance at the expense of nutraceutical value and flavor<sup>5</sup>. With the 'consumer-oriented' revolution of the 1990s, a new model of quality perception emerged<sup>6</sup>. The consumer started playing a central role in the production chain, knowing the nutritional aspects of foods and the positive implications on human health and longevity from daily consumption of fruits and vegetables7.

In 430 BC, Hippocrates, the father of medicine said: '*Let food be your medicine and medicine your food*.' By embracing this statement, the consumer acquired a new perception of quality no longer relegated to the aesthetic aspect but also including the food's content of bioactive phytochemicals, combining food security with gastronomic pleasure<sup>4</sup>. Vegetables are rich in water and essential macronutrients, low in protein and fat and are an excellent source of vitamins, minerals, and beneficial compounds with

antioxidant activity such as polyphenols<sup>8</sup>. Polyphenols play a key role in the visual appearance of fruits and vegetables, the determination of flavor, and have health-promoting properties<sup>9</sup>. Recent studies have revealed that phenolic compounds protect cells during the early development of cancer<sup>10</sup>, exert a significant antioxidant activity with beneficial effects on the vascular and nervous system, reduce the impact of dementia and Alzheimer's and Parkinson's diseases<sup>11</sup> and possess antibacterial, hypocholesterolemic, and hypotriglyceridemic activities<sup>12,13</sup>.

# Agricultural Challenges in the Era of Climate Change: the Role of Insect Screens in Reducing the Gap Between Potential and Actual Yield

The recent climate change is challenging agriculture, endangering the food supply for future generations<sup>14</sup> by reducing the productivity and quality traits of vegetables<sup>15</sup>. Agriculture will have to feed the world's growing population in the near future, which is expected to reach 9.7 billion in the coming decades<sup>16</sup>. On average, farmers worldwide harvest about 50% of their potential yield (i.e., the yield they would achieve under optimal growing conditions)<sup>17</sup>. The gap between potential and actual yield is due to abiotic factors such as excessive solar radiation and heat stress, which account for 60-70% of the losses, and biotic factors, which contribute 30-40%<sup>15-20</sup>. Global warming has encouraged the diffusion of harmful insects and the introduction of alien species in Mediterranean areas that can cause detrimental yield losses and quality degradation of fruits and vegetables if not properly managed<sup>20</sup>. However, more than ever, growers and consumers are more attentive towards "green" strategies to contain biotic stressors such as insects. The strict regulations imposed by the European Union for synthetic insecticides and the growing consumer demand for pesticide-free vegetables, have pushed agriculture to implement integrated pest management strategies<sup>21</sup>. From this perspective, insect nets are an effective and eco-friendly method, achieving 90% effectiveness in excluding specific harmful pests<sup>22,23</sup>. However, the low porosity (percentage of the ratio of open net area and total net area), which is required to ensure high exclusion performance, results in high static pressure drop and reduced airflow, causing a temperature rise that may affect the yield and quality of crops<sup>19,23-25</sup>. High temperatures (a) disrupt plant morphological, physiological, and metabolic processes by altering nutritional quality and flavor<sup>19,24,26</sup>; (b) interfere with primary metabolism by inhibiting bud and root growth, impairing fruit set, damaging fruit, and stimulating leaf abscission<sup>27,28</sup>; (c) alter enzyme activity, modify chloroplast proteins<sup>29</sup>, and promote the production of high amounts of reactive oxygen species (ROS), resulting in impaired photosynthetic pigment biosynthesis<sup>30-32</sup> that reduces photosystem II activity<sup>33</sup>; (d) affect secondary metabolism, inducing physiological alterations and influencing vegetable appearance, flavor, carbohydrate content, and aromatic and antioxidant compounds<sup>21</sup>. In asparagus (Asparagus officinalis L.), high temperatures induced rapid opening of heads and wilting of turion tips, increased fibrousness, and resulted in imbalances in calcium

assimilation<sup>34</sup>. In broccoli (*Brassica oleracea* L.), temperatures around 25 °C induced premature ripening, discoloration, and head deformation<sup>35</sup>. In lettuce, temperatures above 15-18 °C increased physiological disorders, such as head drop, tipburn, and leaf chlorosis<sup>36</sup>. In addition, lower sugar accumulation has been observed for pea (*Pisum sativum* L.), tomato (*Solanum lycopersicum* L.), melons (*Cucumis melo* L.), and watermelons (*Citrullus lanatus* L.)<sup>34,37,38</sup>. However, recent studies revealed that under moderate heat stress plants improve quality traits<sup>36</sup>. In onion (*Allium cepa* L.), increased sulfur compounds (important for flavor) were observed under high temperatures<sup>39</sup>. Similarly, heat stress increased antioxidants in tomatoes, especially ascorbic acid<sup>38</sup>. In summary, high temperatures in protected environments impact the quality of vegetables, both positively and negatively, due to the antioxidant responses to oxidative stress<sup>21</sup>.

To date, the main goal of manufacturers and researchers was to improve the intrinsic characteristics of insect nets to optimize airflow without affecting the exclusion efficiency. Based on these considerations, Chapter 2 represents a literature review wherein we critically analyzed the effects of thermal stress induced by insect nets from the plant perspective. We discussed the technical aspects of insect nets, the characterization of airflow through screened openings, and the description of the morpho-physiological and biochemical impacts of heat stress on plant growth and yield with a focus on antioxidant responses to heat-induced oxidative stress. Therefore, a first experiment was conducted to evaluate the effects of microclimate induced by two types of insect nets with different porosity on zucchini squash (*Cucurbita pepo* L.) productive and qualitative performance (Chapter 3). These findings can provide a helpful resource to extend the knowledge about airflow reduction and thermal increase on yield and quality attributes of fruit vegetables.

Shading Nets: an Effective Tool to Improve Horticultural Production in the Mediterranean Environment

Solar radiation is among the most critical abiotic factors in greenhouse crops, as it affects photosynthesis, water and energy balance, and crop growth and development. Accordingly, solar radiation management in protected environments is undoubtedly one of the essential strategies in horticulture. Shading nets are an efficient solution to protect plants from heat. In protected crops, modulation of light intensity using shading nets represents an innovative approach in manipulating the microclimate that influence functional and bioactive characteristics and preserve postharvest freshness and quality of vegetables. Shading nets reduce the amount of radiation that reaches the crops, affecting the air, soil and plant temperature, relative humidity, and even the radiation direction<sup>41</sup>, thus attracting an increment of research interest<sup>42</sup>. Based on these considerations, we conducted three trials on model crops of Mediterranean horticulture,

such as lettuce (*Lactuca sativa* L.), arugula (*Diplotaxis tenuifolia* L.), and tomato (*Solanum lycopersicum* L.) to evaluate the effects of different shading nets on their yield and quality. Chapter 4 assessed the adaptive mechanisms of four different lettuce cultivars grown under suboptimal microclimatic conditions. Lettuce is generally grown in the winter and spring seasons, being a species well adapted to low temperatures and low light intensity. However, the high demand for year-round products has led to the off-season (spring-summer) cultivation of lettuce in protected environments where high light intensity and high temperatures pose a challenge to its production, affecting growth and yield and causing quality losses. To this end, we evaluated the effects of a white shading net on the microclimate, production, quality, and adaptive mechanisms of four lettuce cultivars in off-season cycle (spring-summer) in the greenhouse. Particularly, growth and yield parameters, leaf gas exchanges, chlorophyll fluorescence, and morpho-anatomical leaf traits (*i.e.*, leaf mass area, stomatal density, and epidermal cell density) were determined. To the best of our knowledge, this was the first research to investigate these aspects.

Today, Southern Italy is the leader in the production of fresh arugula, one of the most popular leafy vegetables in the world, appreciated by consumers for its bitter taste and richness in beneficial phytonutrients such as vitamin C, glucosinolates, and flavonoids. A second experiment was conducted to investigate the effect of two shading nets with different degrees of light extinction (50% and 79%) on the yield, mineral composition, and antioxidants of perennial wall arugula for fresh market, grown in a greenhouse in three different cycles during spring-summer season in southern Italy (Chapter 5). Minerals and total phenolics were determined by ion chromatography coupled to an electrical conductivity detector and Folin–Ciocalteu method, respectively.

Tomato stands as one of the most consumed vegetables worldwide due to its low-calorie content and high fiber, minerals, and phenolic compounds, making it a high-quality functional food. However, environmental stresses such as excessive solar radiation can affect its quality attributes. Chapter 6 evaluated the influence of shading nets on the yield and phytochemical profile of tomato fruits grown in summer in a hot Mediterranean climate. For this purpose, two different shading nets with varying shading factors were used (white net: 30% shading factor; pearl grey net: 40% shading factor). Mineral and organic acid content (by ion chromatography-IC), phenolic profile (by ultra-high performance liquid chromatography-UHPLC coupled with an Orbitrap high-resolution mass spectrometry-HRMS), carotenoid content (by high-performance liquid chromatography with diode array detection-HPLC-DAD), and antioxidant activities DPPH, ABTS, and FRAP (by UV-VIS spectrophotometry) were determined.

# *Photoselective Shading Nets: an Innovative Approach for the Modification of the Light Spectrum in Horticulture*

In recent decades, in addition to the conventional shading nets, photoselective colored shading nets can be available on the market that combine the effect of physical protection with filtration, reducing the intensity of solar radiation and modifying the spectral quality<sup>43</sup>. The reviewed literature showed that manipulation of the light spectrum through photoselective nets may impact significantly on the production and accumulation of bioactive compounds in bell pepper (Capsicum annuum L.)<sup>44-51</sup>, tomato<sup>52-</sup> <sup>58</sup>, lettuce<sup>59-61</sup>, fresh herbs<sup>58,62-65</sup>, spinach (Spinacia oleracea L.)<sup>66,67</sup>, cucumber (Cucumis sativus L.)68, red beet (Beta vulgaris L.)69, turnip (Brassica rapa L.)70, and amaranth (Amaranthus caudatus L.)<sup>71</sup>. Ilić et al.<sup>72</sup> and Santana et al.<sup>45</sup> reported increases in leaf area index in bell pepper grown under colored photoselective shading nets, while an additional study reported yield increases under red nets<sup>73</sup>. Ledonè et al.<sup>48</sup> evaluated the efficiency of white, yellow, green, and red shading nets on bell pepper yield, showing that green nets reduced yield while yellow and red nets resulted in opposite results, also coupled with better fruit quality. Fallik et al.44 reported that colored shade nets, compared to non-photoselective black nets, significantly increased the yield (higher fruit weight), quality, and shelf life of bell pepper. Modification of the light spectrum also provided benefits in summer lettuce crops, increasing leaf area, head weight, and diameter, as well as resulting in advanced production, better leaf coloration and tenderness<sup>59</sup>. In cucumber, the colored photoselective nets increased transpiration rate, stomatal conductance, and net CO<sub>2</sub> assimilation rate<sup>68</sup>.

To date, no contributions are available in the literature that investigated the effects of colored photoselective nets on nursery vegetable seedlings. Pre-conditioning techniques in nurseries are crucial to obtain sturdy plants with appropriate morphology and high levels of organic reserves, vital attributes to ensure high vigor during the establishment period<sup>74</sup>. However, high planting densities reduce seedling quality due to changes in key morphological parameters such as the height and diameter of the stem<sup>75</sup>. Indeed, blue light depletion caused by self-shading can result in excessive stem and shoot elongation, forcing producers to use chemical size regulators. Based on these assumptions, we conducted an experiment aimed at evaluating the effects of the intensity and quality parameters, colorimetric indices, mineral and pigment concentration and metabolic reprogramming of zucchini squash, watermelon, tomato, and eggplant (*Solanum melongena* L.) seedlings for nursery production in a Mediterranean environment.

The data presented below provide a useful contribution to extending the knowledge of the effects of insects and shading nets on the yield and quality of leafy and fruit vegetables in a protected environment under a warm Mediterranean climate.

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## Biochemical, Physiological, and Productive Response of Greenhouse Vegetables to Suboptimal Growth Environment Induced by Insect Nets

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Simple Summary: Global warming jeopardizes agriculture, which must satisfy the demands of the world's expanding population for both staple and high-quality products while ensuring increased sustainability. Environmental and regulatory pressure has prompted farmers to convert their production strategies towards sustainable agriculture systems by introducing integrated pest management strategies. Insect nets are a suitable tool for pest control but require careful assessment of their effects on the generated microclimate. The low porosity that is mandatory for proper exclusion, results in suboptimal airflow and in temperature rise with detrimental effects on crop production and quality. The biochemical and morpho-physiological changes induced by high-temperature impact vegetable crop performance and product quality and represent a significant challenge for the most impoverished populations of the earth, which count on horticultural products as their main source of nourishment. Conversely, latest research demonstrated that high temperatures may also induce oxidative stress-related improvement in certain vegetable quality attributes.

Abstract: Environmental pressure poses a major challenge to the agricultural sector, which requires the development of cultivation techniques that can effectively reduce the impact of abiotic stress factors affecting crop yield and quality (e.g., thermal stress, wind, and hail) and especially of biotic factors, such as insect pests. The increased consumer interest in premium quality vegetables requires the implementation of sustainable integrated pest management (IPM) strategies toward insect pressure, promoted by cultivation under protected environments. In this respect, insect nets represent an excellent, eco-friendly solution. This review aims to investigate the side effects of using insect screens in agriculture. Particular attention is dedicated to the impact on growth, yield and quality of vegetables, focusing on the physiological and biochemical mechanisms of response to heat stress induced by insect screens. The performance of insect nets depends on many factors, foremost on the screen mesh, with finer mesh being more effective. However, finer mesh nets impose high-pressure drop and impede airflow by reducing ventilation, which results in elevated temperature detrimental to crop growth and yield. High temperature (heat stress) tends to impact (i) plant morpho-physiological attributes, (ii) biochemical and molecular properties through changes in primary and secondary metabolism, (iii) enzymatic activity, chloroplast proteins, photosynthetic and respiratory processes, (iv) flowering and fruit setting, (v) accumulation of reactive oxygen species (ROSs) and (vi) the biosynthesis of secondary biomolecules endowed with antioxidant capacity.

**Keywords:** Protected cultivation; Insect-proof screen; Airflow; Heat stress; Biochemical and physiological responses; Functional quality

### 1. Introduction

Nowadays, quality concept has radically evolved, conceiving a "consumer-oriented" revolution; consumers are perceptive about the nutritional aspects of food and demanding concomitantly a captivating high-quality product. According to the consumer, the functional quality is related to the bioactive phytochemical content; the novel quality concept is supported by consumer interest in the health aspect of food and culinary satisfaction<sup>1</sup>. A product with a high sensory profile and nutritional value is safe, appealing, and sustainable. Interestingly, vegetables are highly rich in water and macronutrients, low in protein and lipids and are an excellent source of vitamins and minerals, conveying significant benefits such as compounds with antioxidant potential (vitamin C, carotenoids, and phenolics) when included in daily diets<sup>2</sup>. Phenols and polyphenols are natural compounds endowed with reinforcing health repercussions. Recent studies revealed that phenolic compounds safeguard cells during early cancer development (skin, lung, stomach, esophagus, duodenum, pancreas, liver, breast, and colon)<sup>3</sup>. They also exert considerable antioxidant activity with beneficial effects on the vascular and nervous systems, thus reducing the impact of dementia, Alzheimer, and delineated also Parkinson's diseases<sup>4</sup>. They are by having antibacterial, hypotriglyceridemic activities<sup>5,6</sup>. Nonetheless, hypocholesterolemic and the accumulation of antioxidant molecules is affected by preharvest factors such as genotype, cultivation technique, maturation stage and climate (e.g., heat, drought, and salinity)7,8.

On average, farmers worldwide harvest about 50% of their potential yield (*i.e.*, the yield they would achieve under optimal growth conditions)<sup>9</sup>, where abiotic factors induce about 60–70% of yield loss, while the other 30–40% is due to biotic factors. These latter stresses are a challenge to the agricultural sector and require the development of cultivation techniques that reduce the impact of environmental factors, like wind, hail, excessive radiation, and especially insect damage and thermal stress<sup>10</sup>. The climatic conditions in protected environments foster insect development, such as whiteflies, thrips, and aphids, which cause direct crop damage and transmit phytopathogenic organisms (bacteria, viruses, or fungi), jeopardizing vegetable yield and quality, unless adequately managed<sup>11</sup>. Formerly, farmers widely used insecticides for insect control, but in present-day a turnaround is discerned. Researchers have developed more efficient and selective insecticides with reduced environmental impact and introduced biological pest management methods.

On the other hand, the relentless consumer demand for pesticide-free vegetables and the increased insect resistance to pesticides make insect control more strenuous. The greatest challenge for agriculture is to contain insect attacks by implementing economically and ecologically sustainable integrated pest management (IPM) strategies. From this perspective, physical barriers are a successful method for reducing chemical



insecticides in protected environments<sup>12</sup>. Increasing consumer interest in organic foods and the stricter regulation of chemicals has increased the marketability of anti-insect nets as an insecticide alternative. Farmers are continuously searching for eco-friendly solutions to combat insect pressure in protected environments, and anti-insect nets propound an excellent "green" solution. Their performance depends on many factors, like screen mesh, where small-hole nets are more efficacious<sup>13</sup>. However, small-hole nets are characterized by a high-pressure drop<sup>14</sup>, resulting in high airflow resistance, decreased ventilation and a detrimental increase in temperature affecting crop growth and yield<sup>15</sup>.

The sedentary status of plants triggers them to adapt to different environmental stresses. The effect of thermal stress depends on plant tolerance and its ability to adapt quickly to suboptimal conditions, duration, and intensity. Genotype- and environment-dependent adaptive mechanisms ensure their ability to survive and produce under extreme conditions<sup>16</sup>. Plants have a complex set of sensors in different cellular compartments to activate their defense mechanisms as response to thermal stress. These sensors regulate responses to tolerance development. Thermal stimulus-induced response activation is enabled by the interaction of cofactors and signaling molecules capable of activating thermal stress-sensitive genes such as phytohormones, nitric oxide (NO), sugars (as signaling molecules), and Ca-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPK/MPKs)<sup>17</sup>. For example, the increase in membrane fluidity is associated with the activation of signaling cascades coupled to an increase in Ca<sup>2+</sup> influx, with consequent cytoskeletal reorganization leading to osmolytes and antioxidants production in response to thermal stress<sup>18</sup>.

Although stress-induced responses are usually not univocal, life-cycle modification, protective morpho-physiological barriers activation (avoidance or acclimation mechanisms), and molecular response (tolerance mechanisms) are typical plant reactions to heat stress. Common examples of avoidance and acclimation mechanisms include reducing the absorption of solar radiation by changing leaf orientation (paraheliotropism); reducing water loss by controlling stomatal density; reducing leaf size or abscission; altering membrane phospholipids<sup>16</sup>. In fact, plants exposed to high thermal stress, activate their adaptive response by modifying their morphophysiological, biochemical, and molecular properties<sup>15,18</sup>, thus a makeover of primary and secondary metabolism go on the run. Such stress alters photosynthetic and respiratory processes<sup>19-21</sup>, impairs flowering and fructification<sup>22,23</sup>, reduces enzymatic and chloroplastic activity<sup>24,25</sup>, and promotes reactive oxygen species (ROSs) accumulation<sup>26</sup>. As illustrated by Almeselmani et al.<sup>16</sup>, high temperatures activate the transcription of heat stress-responsive genes, resulting in the synthesis of signaling molecules; osmoprotectants; non-enzymatic antioxidant compounds such as ascorbate (AsA), glutathione (GHS), tocopherol and carotene; enzymatic antioxidant compounds

such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POX), and glutathione reductase (GR).

Research demonstrated the effectiveness of fine-meshed screens in excluding harmful insects, in addition to the detrimental reduction in airflow due to their use. Hence, resulting in suboptimal growth environment, which leads to critical temperature increases and adverse effects on production. To date, the main aim of research was to improve airflow by enhancing the intrinsic netting characteristics and to improving growth conditions without affecting exclusion efficiency. However, due to the "antioxidant response" to oxidative stress, high temperatures can alter the intrinsic and extrinsic quality of vegetables, both positively and negatively. A recent study showed the effectiveness of insect nets in enhancing the quality of zucchini squash without affecting yield and, at the same time, ensuring early production27. To the best of our knowledge, despite relevant available research papers on improved airflow of insect nets and their high-temperature effects on the production and quality of horticultural crops, the reviewed literature showed a shortfall of information in this field of research. The few available contributions suggest that further studies are required to relate the suboptimal growth environment of insect nets to the quality of the produced vegetables, regardless of their exclusion efficiency.

This review is an investigation and a critical analysis of the side effects of agriculture insect screens. The following topics are discussed: i) the technical aspects of insect nets; ii) the airflow characterization through screened openings; iii) the description of the morpho-physiological and biochemical effects of heat stress on plant growth and yield with a view in particular to the antioxidant responses to heat-induced oxidative stress. A literature review was conducted, integrating peer-reviewed papers, books, technical journals and conference proceedings published by 2020, including technical and physical aspects of insect nets and plants' response to high-temperature oxidative stress.

### 2. Technical Aspects of Anti-insect Nets

The increasing consumer interest in fresh, sustainable, and high quality year-round horticultural products, requires the implementation of integrated pest management (IPM) strategies. From this perspective, agro-textiles are a valuable tool for pest management, pollinator confinement, and pesticide reduction. Farmers can rely on different types of insect nets that differ in manufacturing (material, texture, porosity, weight, number of meshes), radiometric (color, shading, transmissivity), physical, and mechanical properties<sup>28</sup>. For these purposes, farmers are uncertain about the best suitable nets, raising several questions. Which materials and technical features are ideal for successful exclusion? How do insect nets work? What are the drawbacks of nets?

A net is a fabric obtained by processing plastic fibers by weaving or non-weaving methods<sup>28</sup>. Woven nets are characterized by regular holes in which air flows due to the connection of vertical warp and horizontal weft threads. In contrast, in a non-woven net,



the fabric is produced by a different process such as extrusion or micro-perforation. The weaving process produces most insect nets available on the market; round or flat plastic monofilaments made of high-density polyethylene (HDPE) or polypropylene (PP), are woven on looms. In agreement with the National Greenhouse Manufacturers Association (NGMA), polyamide (nylon) or multifilament nets in steel and brass or polyethylene and acrylic are marketed but they have several drawbacks compared to HDPE nets<sup>29</sup>. Steel and brass nets are very resistant and durable but they are expensive and relegated to the industrial and hobby sectors, while polyamide nets are lightweight but very weak.

Depending on the texture, as mentioned by Castellano et al.<sup>28</sup>, three types of insect nets are marketed: Italian, English, and Raschel textures. Italian texture (flat woven net) is produced by overlapping weft and warp threads in orthogonal arrangement; the warp threads are spaced to allow the passage of a weft thread between them, which results in a rigid and stable net. However, when the number of threads per cm<sup>2</sup> is reduced, net stability decreases and fabric frays when cut. Whereas, the English texture is a revised and improved version of the Italian one. Two pairs of warp threads are twisted and trapped with weft threads avoiding net fraying. English nets are more stable, resistant, and non-deformable. Moreover, a complex structure characterizes Raschel textured nets; the warp threads are knotted to create longitudinal chains that twist and incorporate weft threads. Raschel and English texture are a valuable solution for insect-proof screens; however, they are recommended for anti-hail and windproof nets where higher tension and resistance are required.

The weft and warp threads form a regular hole's pattern, called mesh, which is the square hole formed at the intersection of a warp and weft thread, varying from 0.2 to 3.1 mm, depending on the insect size to be excluded<sup>28</sup>. Insect nets available on the market are described by mesh number, representing the number of holes per inch in each direction<sup>30</sup>. The insect's exclusion is based on avoiding insect thorax passage ("prison effect")<sup>31</sup> and, theoretically, a net is efficient when the holes are smaller than the thorax width of the insect to be excluded. This parameter also depends on the insect sex<sup>32</sup>. Table 1 shows the average thorax width of "key insects" and the hole size and mesh number required for their effective exclusion from greenhouses. The hypothetical exclusion efficiency does not necessarily coincide with real effectiveness, achieving up to 90% control of a designated pest<sup>33</sup>; for example, due to the shape of thrips (Frankliniella. occidentalis) bodies, they can penetrate through small holes of widespread commercial nets<sup>34</sup>. The reason that small holes do not ensure total exclusion is correlated to the 3D arrangement of the threads. Usually, nets are considered flat structures, but they are three-dimensional, and their effectiveness depends on several factors like the threads' thickness, width, and length of the hole, and its geometry<sup>34</sup>. Warp threads are usually closer together than weft threads, forming a hole with a rectangular geometric structure;

the overlapping of warp and weft threads alters the geometric structure of the hole, allowing easy access of the insect<sup>34</sup>.

Manufacturers do not have specific tools to evaluate insect nets efficiency. Therefore, several laboratory experiments were carried out to assess the exclusion efficiency of different types of nets in calm conditions and at different air velocities and temperatures<sup>35-37</sup>. In recent years, the agro-textile industry has tested and marketed innovative nets with improved airflow, due to thinner threads, without affecting exclusion performance. A recent experiment carried out by Formisano et al.<sup>27</sup> investigated the effects of suboptimal growth environment induced by two 50 mesh nets with different porosities (Arrigoni Biorete<sup>®</sup> 50 mesh and Arrigoni Biorete<sup>®</sup> 50 mesh AirPlus) on the production and quality attributes of *Cucurbita pepo* L. in controlled growing conditions. The improved porosity of the 50 mesh AirPlus net, due to a thinner HDPE filament (Arrigoni Harlene HT<sup>®</sup>), resulted in increased quality traits of zucchini squash without compromising yield. The 50 mesh AirPlus net led to an improvement in the inner microclimate, with lower soil and air temperatures and relative humidity. A comparable study on cucumber showed the positive effects of insect-proof screens with different porosities in containing cucumber beetles in high tunnels while providing adequate ventilation<sup>38</sup>.

The durability and mechanical stability of the nets are essential parameters, and fabrics with complex textures confer enhanced mechanical characteristics, increasing durability. However, durability does not depend exclusively on the number and structure of the threads; several elements, as environmental factors (temperature), chemical treatments, dirt, and UV radiation, affect the mechanical and physical characteristics of plastic threads, leading to premature net deterioration. It is found that UV radiation plays a crucial role in the lifetime and performance of nets<sup>39</sup>; hence, manufacturers use additives to increase the UV stability of HDPE plastic polymers. The longevity of nets is directly related to their resistance to UV radiation, which is expressed in the amount of Kilolangley (kly) and represents the number of years required to reduce the net tensile strength by 50%. For example, a net with 600 kly in a Mediterranean climate region (100–130 kly) potentially has a lifetime of 5–6 years<sup>28</sup>.

Insect nets are usually made with transparent or white fibers; however, the industry has recently tested multifunctional nets supplying protection and photoselection by adding colored and UV-absorbing additives to HDPE polymers. Many authors reported that light modulation using photoselective nets induces a "barrier effect" against pests while reducing the incidence of viral diseases affecting horticultural crops. Antignus et al.<sup>40</sup> reported that UV-absorbing plastic screens were effective in decreasing the dispersion rate of pests in greenhouses. Whiteflies detect solar radiation in a specific light spectrum, and their findings showed that the lack of UV radiation in greenhouse interferes with the flight and orientation of insects. Further studies conducted by Legarrea et al.<sup>41</sup> investigated the impact that UV-absorbing nets had on the visual cues



of two beneficial predators (*Orius levigatus* and *Amblyseius swirksii*). The results obtained showed that the lack of UV radiation created a favorable environment for *Orius levigatus*, in contrast to what occurred with *Amblyseius swirksii*. In a comparative study, Ben-Yakir<sup>42</sup> evaluated the impact of colored photoselective nets (yellow, red, and pearl ChromatiNets<sup>™</sup>) on the containment of aphids and aleyrodids involved in the transmission of the potato virus Y (PVY), cucumber mosaic virus (CMV) in pepper, and the tomato yellow leaf curl virus (TYLC). Specifically, yellow and pearl nets reduced aphid and whitefly infestation up to three-fold compared to red and conventional black nets. Similarly, yellow and pearl nets reduced the incidence of CMV, PVY, and TYLC, up to ten-, three-, and four-fold, respectively.

Over the last two decades, various pest management methods were implemented, such as insecticide-treated insect nets. Studies on cucumber (*Cucumis sativus* L.) and African eggplant (*Solanum macrocarpon* L.), demonstrated the efficacy of pyrethroid-treated nets in the management of aphids and Lepidoptera, although providing lower efficacy in containing tiny insects such as whiteflies (*Trialeurodes vaporarium*) and thrips (*Frankliniella occidentalis*)<sup>43,44</sup>. In a recent trial, Arthurs et al.<sup>45</sup> tested the exclusion performance of two-colored modern long-lasting insecticide net (LLIN) with a larger mesh size (32 holes cm<sup>-2</sup>) compared to a conventional thrips exclusion screen. The results showed lower thrips penetration in yellow-treated nets than in black ones. However, while insecticide-treated nets resulted in considerable airflow increase, larger hole size did not guarantee total thrips exclusion.

Insect nets are commonly used in agriculture, and their effectiveness is proved by many studies, representing an excellent eco-sustainable solution to limit the use of human health harmful pesticides, exposing producers and workers to lower risks. The requests of the globalized market have driven technicians, producers, and researchers to consider insect nets as multifunctional tools that provide high exclusion efficiency, environmental and economic eco-sustainability and that ensure high yields and highquality products. In previous decades, research has focused on improving airflow to limit the detrimental impact of excessive temperatures in the warm Mediterranean regions. High temperatures, if critical thresholds are not exceeded, can ensure an early production and an improvement in the quality of vegetables, such as a higher antioxidant build-up. Despite extensive research on plant response to high temperatures, few studies have examined the possible improvement in quality caused by the insect nets as well as the most appropriate porosity level to ensure a balance between production, quality and efficiency of exclusion.

Turnet an effer	Screen hole size		 Average thorax width⁴ (μm)					
Insect species	Microns	Mesh	Male	Female	Male	Female	Male	Female
Frankliniella occidentalis²	192	132	190.6	258.0	184.4	245.5	215	
Bemisia argentifolii	239						239	
Trialeurodes vaporarium	288						288	8
Aphis gossypii	340	78	486.3		355		355	5
Bemisia tabaci	462 <sup>3</sup>	52	241.7	277.5	215.8	261.3		
Myzus persicae					433.8			
Liriomyza trifolii	640	40			562.5	653.8	608	
Reference	46		3	35		32	46	

**Table 1.** Hypothetical exclusion efficiency<sup>1</sup> of insect nets for the control of a designated pest, hole size and mesh number of widespread insect nets, and average thorax width of "key insects".

<sup>1</sup>An insect net is theoretically effective when the width of its pores is equal or less than the thorax width of the insect to be excluded. <sup>2</sup>Thrips (*Frankliniella occidentalis*) are very thin and can pass through common nets. <sup>3</sup>Thoracic width and hole size are not the only parameters to predict the efficacy of insect exclusion, hole geometry and the way in which holes were formed are crucial elements as well. <sup>4</sup>In this table, the thorax width was measured in dorsal view.



#### 3. Airflow characterization of screened openings

In protected environments, in order to ensure optimal growth conditions, it is necessary to provide adequate ventilation, especially in warm Mediterranean regions. High solar radiation and insufficient ventilation cause rapid harmful rise in air temperature and humidity, exposing crops to severe stress affecting all growth stages and crop production<sup>16</sup>. For sufficient air exchange, vents should be 15% to 25% of the total area and should cover the entire length of the greenhouse for balanced air distribution<sup>30</sup>. The air flowing through the greenhouse moves according to a pressure gradient. The air exchange process occurs either by natural (passive) or forced ventilation<sup>47</sup>, each aimed at replacing warm indoor air with cooler air from outside. With natural ventilation, the airflow through the vents is triggered by temperature differences and wind pressure, but mainly wind contributes to air renewal<sup>48</sup>. The airflow drives insects through the openings and, therefore, insect nets are usually mounted on greenhouse openings like doors and vents<sup>30</sup>. The exclusion performance depends on the mesh and hole geometry<sup>13,32</sup>. Fine-meshed nets, despite their theoretical better exclusion efficiency, they have the disadvantage of low porosity (percentage of the ratio between open net area and total net area). Consequently, a high static pressure drop occurs<sup>14</sup>, leading to inadequate air exchange and rising temperature and humidity<sup>49</sup>.

Despite the availability of advanced solutions to increase net porosity without reducing mesh size, thereby improving air exchange in protected environments, it is still necessary to estimate the pressure drop that occurs through screened openings<sup>30</sup>. From a physical perspective, the air is a viscous and compressible fluid with a variable velocity, which moves according to either the laminar or turbulent regime. Viscous forces govern the movements in a laminar flow while in turbulent flow, inertial forces are also involved. Considering air as an incompressible fluid (constant density), the only variable that discriminates from the turbulent and laminar flow is the Reynolds number (*Re*). For insect net, the Reynolds number is defined as follows:

$$R_e = \frac{ud}{v}$$

where:

u =flux velocity [m/s]; d =thread diameter [m];

v = kinematic viscosity [m<sup>2</sup>/s];

It is a dimensionless parameter that physically expresses how the inertial and viscous forces acting on a fluid particle move at u velocity. When air flows through a screened opening, the flow rate decreases significantly with pressure drop that occurs from the inside out. Therefore, a prediction of the total pressure drop through insect-proof screens is necessary to ensure their correct sizing and, consequently, sufficient air exchange without compromising the exclusion efficiency. The total pressure drop  $\Delta P_T$  is the sum of the pressure drop caused by unscreened opening and insect screen<sup>49</sup> and is given by:

$$\Delta P_T = \Delta P_o + \Delta P_s$$

where:

 $\Delta P_o$  = pressure drop across unscreened opening [Pa];  $\Delta P_s$  = pressure drop across screen [Pa];

The pressure drop generated by insect nets can be assessed both through a "coefficient of discharge" included in the Bernoulli's equation<sup>50-52</sup> and by the motion equation of a fluid through a porous medium (Forchheimer equation)<sup>53,54</sup>. Supposing that air moves by turbulent flow (*Re*>150), it is possible to quantify the pressure drop and the airflow through an unscreened opening using Bernoulli's equation. A fluid movement through an opening is subjected to a contraction, causing in the flow an effect known as *vena contracta* (*V*<sub>c</sub>), which represents the fluid flow point where the section is minimal, the velocity is uniform, and the static pressure is equal to the surrounding air<sup>55</sup>. The ratio between the *vena contracta* and the total area of a hole (*A*) defines the *contraction coefficient* (*C*<sub>c</sub>):

$$C_c = \frac{A_c}{A}$$

As a result of hole contraction, the velocity in the *vena contracta* is lower than ideal velocity ( $V_i$ ); the equation that correlates the two velocities is defined as *velocity coefficient* ( $C_v$ ):

$$C_{v} = \frac{V_{C}}{V_{i}}$$

Outside and inside the net, we have respectively:

$$\frac{\rho}{2} * V_0^2 + P_0 = \frac{\rho}{2} * V_i^2 + P_i$$

where: V =fluid velocity [m/s]; P =static pressure [Pa];  $\rho =$ fluid density [Kg/m<sup>3</sup>];

For the ideal fluid, without friction, the velocity is different from the real one; assuming the external velocity as zero, we obtain the equation that relates the ideal (or theoretical) velocity to the static pressure variation:



$$V_i = \sqrt{2 * \frac{P_0 - P_i}{\rho}}$$

The continuity equation, describing the airflow through an opening, can be defined as follows:

$$Q = A_c * V_c = C_c * A * C_v * V_i = C_c * A * C_v * \sqrt{2 * \frac{P_0 - P_i}{\rho}}$$

The multiplication between the *contraction coefficient* and the *velocity coefficient* is defined as *discharge coefficient* ( $C_d$ ), expressing the resistance that a specific opening offers to the airflow<sup>48</sup>.

Therefore:

$$Q = C_d * A * \sqrt{2 * \frac{P_0 - P_i}{\rho}}$$

Experiments were carried out to determine the *discharge coefficients* of the openings, as well as the nets. The *discharge coefficient* of vents ranged from  $0.60-0.90^{56,57}$  as a function of the sharp edge, whereas it ranged from 0.05 to 0.5 as a function of net porosity<sup>58,59</sup>. The flow resistance is often expressed by the *pressure loss coefficient* (*K*), correlated to the *discharge coefficient* by the following relationship:

$$K = \frac{1}{C_d^2}$$

Based on previous observations, the pressure drop through an unscreened opening is given by the equation below:

$$\Delta P_o = \frac{1}{2} K \rho V^2$$

Moreover, several researchers developed correction functions to adjust the pressure loss value by correlating the *pressure loss coefficient* to the aspect ratio (L/H) of the openings<sup>60</sup> and considering the influence of flaps<sup>48</sup>. Usually, insect nets have an ideal Reynolds number below 150, which results in a laminar flow<sup>61</sup>; therefore, it is known that the *pressure loss coefficient* is a function of both porosity and Reynolds number<sup>62</sup>.

In the literature, numerous researches have linked the *K* coefficient to different porosity values with different *Re* values<sup>48,63,64</sup>. Net resistance to airflow can be evaluated by the physical laws governing the movement of a fluid through porous media. From this viewpoint, nets are assumed as solid porous structures consisting of interconnected holes. On a small scale, the pressure drop is usually expressed by Forchheimer's equation:

$$\frac{\partial P}{\partial x} = \frac{\mu}{K} v + \rho \frac{Y}{K^{1/2}} |v| v$$

The infinitesimal pressure drop is the sum of a linear term, reflecting the flow resistance generated by the viscosity  $\mu$  and the *specific permeability K* of the porous medium, and a quadratic term depending on the permeability of medium *K* and the *inertial factor* (*Y*) (relative to pore characteristics)<sup>53</sup>. Different *K* and *Y* values were reported by Miguel<sup>53</sup> and Valera<sup>4</sup> and were classified based on screen porosity.

As cited by Succi and Vulpiani<sup>65</sup>, fluid flow in porous media is dominated by a high prevalence of dissipative over convective processes. Therefore, at low Reynolds number (*Re*<1), the flow can be described by Darcy's law (linear term of Forchheimer's equation); in particular, the non-linear term can be ignored, and the flow velocity shows a linear trend with pressure loss:

$$\frac{\partial P}{\partial x} = \frac{\mu}{K} v$$

With Reynolds' number over the unit (1<*Re*<100), non-linear effects cannot be ignored<sup>61,65</sup>.

The applicability of Bernoulli and Forchheimer's equation is dependent on Reynolds' number. At *Re*>150, the pressure drop can be determined by the *discharge coefficient* of Bernoulli's equation, whereas laminar flow rate (*Re*<150) by Forchheimer's equation. Teitel<sup>66</sup> and Kittas et al.<sup>50</sup> demonstrated that the variations in pressure drop obtained with the two mentioned methods were relatively small. On the other hand, at *Re*>8 the pressure drop can be determined by the *discharge coefficient*<sup>66</sup>, although it is not constant at all values of the Reynolds number according to Teitel and Shklyar<sup>14</sup>.

Insect nets are effective ecological solutions in regulating pests. However, as shown in published literature, low-porous nets drastically decrease the ventilation rate, resulting in higher relative humidity and temperature gradients in protected environments (**Table 2**). As mentioned by Ajwang et al.<sup>67</sup> the airflow improvement can be achieved by adequately sizing the screened openings according to the pressure drop produced by the net. A correction factor, relative to net porosity, was proposed by Perez-Parra et al.<sup>68</sup> to improve the ventilation area. However, as suggested by Fatnassi et al.<sup>69</sup>, it is not always possible to compensate the pressure drop by increasing the screened area, therefore a forced ventilation system is required in this case.



**Table 2.** Evaluation of anti-insect screens with different discharge coefficient (Cd), porosity ( $\epsilon$ )and mesh size on temperature difference ( $\Delta T$ ) and humidity between the inside and outside ofthe greenhouses under real conditions and with computational fluid dynamics (CFD) simulationmodels.

Experimental conditions	Treatments	Effect on microclimate	Reference
Simulation model	Evaluation of a model to predict the effect of screen area/opening area ratio on $\Delta T$ (inside/outside). Net radiation and wind velocity were set to 500 Wm <sup>-2</sup> and 1 ms <sup>-1</sup> , respectively.	For a screen area/opening area ratio of one, the nets with a discharge coefficient of 0.1 and 0.5 resulted in a $\Delta$ T of 0.75 °C and 4.5 °C, respectively.	58
Multi-span greenhouse	Effect on inner temperature and humidity of two insect screens with different porosities ( $\epsilon$ =0.5 and $\epsilon$ =0.6)	Anti-insect nets with porosity of 0.5 and 0.6 resulted in 2.5 and 2-fold increase in $\Delta T$ , respectively, compared to the unscreened greenhouse.	50
Four-span greenhouse	Effect on inner temperature and humidity of two insect screens with different porosities ( $\epsilon$ =0.2 and $\epsilon$ =0.4) mounted on the roof and side openings of a four-span greenhouse.	Anti-insect nets with porosity of 0.2 and 0.4 resulted in 3 and 2-fold increase in air temperature and humidity, respectively, compared to the unscreened greenhouse.	70
Greenhouse	Effect of anti-thrips net (Cd=0.22) on air temperature in a greenhouse in the tropical region with small plants and low transpiration rate.	Unripe plants (low transpiration rate) grown under the anti-thrips net, led to a temperature increase of 5 °C. Differently, mature plants (high transpiration) under anti-thrips net showed a temperature of 3 °C.	67

### Table 2. Cont.

Greenhouse	Effects of insect nets with different porosities (53, 34, 33 and 19%) on vertical temperature distribution in greenhouses with tomato crops at two different growth stages and two densities.	Fine net porosity resulted in a higher air temperature. The highest temperature peak was recorded at the eave's height of the greenhouse. Taller plants and higher plant density resulted in lower air temperature at all vertical points.	71
Greenhouse	Effect of anti-thrips net $(C_d=0.22)$ on air temperature in a greenhouse in the tropical region with small plants and low transpiration rate.	rate) grown under the anti-thrips net, led to a temperature increase of 5 °C. Differently, mature plants (high transpiration) under anti- thrips net showed a temperature of 3 °C.	67
Greenhouse	Effects of insect nets with different porosities (53, 34, 33 and 19%) on vertical temperature distribution in greenhouses with tomato crops at two different growth stages and two densities.	Fine net porosity resulted in a higher air temperature. The highest temperature peak was recorded at the eave's height of the greenhouse. Taller plants and higher plant density resulted in lower air temperature at all vertical points.	71
CFD simulation model	Evaluation of anti- <i>Bemisia</i> ( $\varepsilon$ =0.41) and anti-thrips ( $\varepsilon$ =0.2) nets positioned on the roof alone and roof and side openings of a multi-span greenhouse on the inner microclimate.	Both nets led to a significant increase in temperature, as compared to unscreened control. Specifically, unscreened control, anti- <i>Bemisia</i> , and anti-thrips nets resulted in $\Delta$ T of 2.4, 7.1 and 5.1 °C, respectively.	72
Greenhouse	Effects of different mesh sizes of nets (40, 52 and 78 mesh) on microclimate and air exchange rates in the humid tropics.	The 78 and 52 mesh nets increased air temperature of 1-3 °C. In addition, the 78-mesh net determined an increase in humidity of about twice as much as observed with the 40-mesh net, while 52-mesh nets led to a rise of 50%.	73
Mono-span greenhouse	Influence of different vent opening positions (side only, roof only and combined roof and side openings) and anti- aphid insect screens on microclimate.	The combined application of roof and side openings resulted in a reduction of the air temperature in the greenhouse compared to the roof or side vents alone.	74



# 4. Morphological, Physiological, and Biochemical Response of Plants Under Heat Stress

### 4.1. Effect of Heat Stress on Growth and Yield

It is well documented that very intense solar radiation and thermal stress negatively affect crop physiology, with significant yield and quality losses in cereals, legumes and vegetables<sup>7,18</sup>. High temperatures affect all growth stages, especially germination and reproduction. Common and early effects caused by high temperatures are necrosis; leaf elongation (hyponastia); drying and burning of leaves, branches, twigs, and stems; fruit discoloration and damage; leaf abscission; poor germination and rooting; loss of turgidity and cell size reduction leading to a decrease in total biomass<sup>22,75</sup>. The plant can also manifest programmed cell death (PCD), causing leaves, flowers and fruits to fall and, in extreme cases, the whole plant to die<sup>76</sup>. Germination, mostly the development of the embryo axis and its emergence, is particularly sensitive to temperature fluctuations. Short exposure to high temperatures can lead to a reduction in the percentage of seed germination or a total inhibition, as well as poor vigor and reduced plant, rootlets and plumules growth<sup>77</sup>.

Considerable high temperature effects were recorded in several crops, affecting their quantitative and qualitative characteristics. In leguminosae such as common bean (Phaseolus vulgaris L.) and peanuts (Arachis hypogea L.), high temperatures reduced yield<sup>78,79</sup>; similarly, in tomato (Lycopersicum esculentum Mill.), Camejo et al.<sup>80</sup> reported significant yield reduction due to defects in embryo fertilization and meiosis. In many cultivated species, the effects of heat stress are more evident in reproductive development than in vegetative growth. All plant tissues are susceptible to high temperatures, and a few degrees increase during anthesis can lead to significant yield losses<sup>2</sup>. According to Zinn et al.<sup>81</sup>, high temperatures shorten the number of days to anthesis, hampering optimal nutrients accumulation for embryo development. Further studies on tomato, snap bean, and zucchini showed tapetum degeneration and pollen sterility caused by PCD and endoplasmatic reticulum malformations<sup>82,83</sup>. Under heat stress, it is likely that the under-regulation of sucrose synthetase and pollen vacuolar invertases occurs, as verified in tomato and cowpea<sup>84</sup>. A further relevant effect induced by high temperature is the abscission of reproductive organs due to increased levels of abscisic acid (ABA) and ethylene (ET), combined with altered or reduced auxin (AUX) biosynthesis85.

### 4.2. Plant Physiological Response to Heat Stress

Heat stress affects a range of physiological processes that are essential for the proper functioning of cell structures. High temperatures hamper water and nutrient uptake and impair most physiological and photosynthetic functions, leading to reduced productivity and economic return<sup>86</sup>. The proper functioning of metabolic processes in plant tissues requires adequate tissue hydration. High temperatures, however, lead to a rapid reduction in the water content of leaf tissue and soil; a decrease in root conductance as in tomatoes<sup>87</sup>, mass, and growth<sup>7</sup>; the activity of critical enzymes, such as nitrate reductase<sup>88</sup>, which is essential for nutrient uptake; and source and sink activity<sup>89</sup>.

Photosynthesis is the most sensitive to heat stress among all plant physiological processes. Complex reactions leading to CO<sub>2</sub> reduction involve thylakoid reactions (specialized internal chloroplastic membranes) and carbon-fixing reactions. Foliar mesophyll cells are rich in chloroplasts, with pigments for light absorption (chlorophylls). In chloroplasts, light energy is captured by two distinct photosystem units (PSI and PSII) and used to trigger electron transfer to reduce NADP<sup>+</sup> and oxidize H<sub>2</sub>O. Therefore, under heat stress, an optimal performance of cell membranes might support a better photosynthetic and respiratory efficiency. However, high temperatures have shown to affect cell structures negatively, thus photosynthesis as well. Specifically, they alter the structure of chloroplasts<sup>25</sup>; reduce the enzymatic activity of ribulose 1,5biphosphate carboxylase (RuBisCo), its regeneration as shown in cotton plants<sup>90</sup> and RuBisCo activase<sup>87,91</sup>; induce the closure of stomata by decreasing the CO<sub>2</sub> availability, and consequently the activity of RuBisCo<sup>92</sup>, which is recognized to have a low affinity toward  $CO_2$  compared to  $O_2^{93}$ ; reduce carbon fixation with oxygen evolution and generate reactive oxygen species (ROS)<sup>80,94</sup>. Notably, damage to photosynthetic pigments was observed, probably due to lipid peroxidation of chloroplasts and thylakoids, reduction or stop of PSII activity, and reduction of electron flux and maximum PSII quantum efficiency (Fv/Fm ratio)<sup>20,21</sup>. Chlorophyll's lower accumulation is due to its reduced biosynthesis, degradation or effects of either due to the deactivation of crucial enzymes such as 5aminolevulinate dehydratase as studied in cucumber<sup>95,96</sup>. Camejo et al.<sup>80</sup> also observed an increase in the chlorophyll a/b ratio and a decrease in the chlorophyll/carotenoid ratio of heat-tolerant tomato cultivars.



### 4.3. Biochemical Response to Heat Stress: the Role of Antioxidant Compounds

In response to heat stress, plants maintain their physiological function through selfregulating mechanisms (*i.e.*, homeostasis) by producing and accumulating a wide variety of osmoprotectants (*i.e.*, "compatible solutes") to restore osmotic pressure<sup>97</sup>. The cells have numerous compounds like proline, glycin-betaine, betaine, soluble sugars, sugar alcohols or tertiary and quaternary ammonium compounds, ubiquitin, dehydrins and late embryogenesis abundant (LEA) proteins<sup>7,98</sup>. These compounds also prevent the deactivation of critical enzymes such as RuBisCo under high temperature, scavenging free radicals and stabilizing subcellular structures<sup>20,99-101</sup>. In addition to compatible solutes, several authors also agree that soluble sugars, such as glucose and sucrose, play a direct role in heat stress tolerance by regulating carbon allocation, acting as signal molecules<sup>102,103</sup>, protecting pollen cells by enhancing their quality as in tomato<sup>104</sup> and acting as antioxidants and ROS scavenger at high concentrations<sup>105,106</sup>.

Thermal stress produces harmful reactive oxygen species (ROS, *e.g.*, compounds with high oxidizing activity and a strong tendency to donate oxygen atoms to other substances)<sup>7</sup>, triggering a "chain" reaction which can be stopped by antioxidant compounds. ROS can be divided into two main categories: free radicals, such as hydroxyl radical (OH•), nitroxide radical (NO•), superoxide anion (O2•·), and singlet oxygen (O•); non-radical species such as hydrogen peroxide (H2O2) and ozone (O3) <sup>107</sup>. ROS production occurs mainly in chloroplast reaction centers, peroxisomes, and especially in the mitochondria by enzymatic and non-enzymatic pathways<sup>107</sup>, by photo-oxidation reactions, Haber-Weiss and Fenton reactions, mitochondrial electron transport chain reactions and during photo-inhibition<sup>108,109</sup>. The superoxide radical anion (O2•·) does not possess high reactivity. It is not able to pass through the mitochondrial membrane, and its formation occurs spontaneously during cellular respiration by cytochrome oxidase that releases partially reduced intermediate compounds including O2•· and H2O2.

Even though H<sub>2</sub>O<sub>2</sub> is not a radical species and does not cause any immediate risk to cell structures, it is involved in the synthesis of reactive ROS. Its formation can also occur due to the enzyme superoxide dismutase (SOD) from two molecules of superoxide anion. The hydroxyl radical (OH•) production, which has a high reactivity towards biomolecules, causing considerable cellular damage, is based on H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>• use in Haber-Weiss and Fenton reactions:

$$O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$$
(Haber-Weiss reaction)  
Fe<sup>2+</sup> + H<sub>2</sub>O<sub>2</sub>  $\rightarrow OH^{\bullet} + OH^- + Fe^{3+}$ (Fenton reaction)

Overexposure to ROS causes oxidative stress that leads to the activation of many cellular antioxidant systems. These are activated to avoid any damage to proteins, enzymes, lipids, photosynthetic pigments, and other cellular components. Oxidative
damage results in protein denaturation and membrane instability, lipid peroxidation, photosynthetic reaction center damage, thylakoid membrane electron leakage, impairment, reduced biosynthesis and reduced accumulation of metabolites, carbohydrates, enzymatic activity and osmotic imbalance<sup>26</sup>. Oxidative stress is, therefore, the natural expression of a damage that occurs when pro-oxidant factors (abiotic and biotic pressures) exceed the endogenous antioxidant defenses.

One of the most frequent oxidative alterations occurs in lipids, causing a "chain mechanism" (lipoperoxidation) in the polyunsaturated fatty acids of membrane The reaction chain produces reactive compounds such phospholipids. malondialdehyde (MDA), able to react with free amino groups of proteins, phospholipids, and nucleic acids, inducing molecular structural alterations<sup>110</sup>. The reaction ends when no more oxygen is available or by the action of antioxidants that donate an atom of hydrogen or an electron, forming non-radical inactive species. However, ROS also acts as a molecular signal, enabling complex metabolic reactions by which the plant activates thermal stress defenses. Mittler et al.<sup>111</sup> highlighted the vital role of ROS in promoting transcription and translation processes in chloroplasts, necessary to develop defenses against high temperature-induced oxidative stress. Environmental stresses prompt ROS production in plants that react by modulating their antioxidant metabolism<sup>76</sup>. Plants undergo high oxidative stress due to harmful ROS under thermal stress and synthesize a wide range of antioxidants, which lead to an increased stress tolerance. The ROS removal is necessary for cell survival, and several studies have shown that antioxidant compounds of enzyme and non-enzyme origin are widely produced in all cell structures under stress conditions<sup>107,111</sup>.

Effective plant defense chemicals are non-enzymic low-weight antioxidant compounds (*i.e.*, "scavengers"), such as glutathione (GHS), ascorbic acid (AsA),  $\alpha$ -tocopherol, phenolics, carotenoids, anthocyanins, plant steroids and flavonoids<sup>112</sup>. Their mode of action is based on altering cellular metabolic functions, stabilizing membranes, and defending photosynthetic and respiratory functions from ROS, synergistic acting with other enzymatic antioxidants and phytohormones. The AsA exerts a protective action against peroxide, superoxide and hydroxide radicals, and singlet oxygen. At the same time,  $\alpha$ -tocopherol protects the cell membrane against lipid peroxidation. The GSH and its oxidized form glutathione disulfide (GSSG), are abundantly present in the cytosol, the nucleus, and mitochondria. GHS is a cofactor of several antioxidant enzymes (*e.g.*, glutathione peroxidase and glutathione transferase), eliminates hydroxyl radicals and singlet oxygen and contributes to the regeneration of vitamins C and E<sup>113</sup>.

The role of antioxidant compounds in the plants' adaptation to heat stress was studied in several plant species. Tomato and watermelon plants grown under high temperatures showed a higher accumulation of soluble phenols than observed in plants grown under optimal conditions<sup>114</sup>. The increased accumulation and reduced oxidation



of phenols were probably due to the increased enzyme activity of phenylalanine ammonia-lyase (PAL) and a lower activity in high temperatures induced by polyphenol oxidase (PPO) and peroxidases (POX). Wahid et al.<sup>112</sup> reported that the accumulation of anthocyanins caused a decrease in the osmotic leaf potential to maximize absorption and prevent water loss through transpiration, as well as acting as a UV screen. In a recent trial on zucchini grown under anti-insect nets, thermal stress increased the content of hydrophilic and lipophilic antioxidant activity, total phenols and total ascorbic acid<sup>27</sup>. Camejo et al.<sup>94</sup> underlined the photoprotective activity of carotenoids such as xanthophyll and terpenoids such as tocopherol in the stabilization of thylakoid membranes. At the same time, zeaxanthin produced by hydroxylation of  $\beta$ -carotene performed similar functions in Arabidopsis<sup>115</sup>. Enzymatic antioxidants are usually considered the most effective anti-ROS tools<sup>116</sup>.

The first defense system of the plant is the SOD, which catalyzes the dismutation of the toxic superoxide anion O<sub>2</sub>• to molecular oxygen and H<sub>2</sub>O<sub>2</sub>:

$$20_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

The hydrogen peroxide produced will act as a substrate for CAT and APX. The CAT is an oxidoreductase of hydrogen peroxide and catalyzes the dismutation of H<sub>2</sub>O<sub>2</sub> to water and oxygen:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

However, the antioxidant compounds play a crucial role in activating the ascorbateglutathione (AsA-GHS) cycle involved in ROS detoxification<sup>76</sup>.

The ascorbate-glutathione cycle (AsA-GHS) or Foyer-Halliwell-Asada pathway (**Figure 1**), includes a series of chemical cascade reactions, described below:

First, the APX catalyzes the reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O utilizing ascorbate as a specific electron donor:

$$2H_2O_2 + AsA \xrightarrow{APX} 2H_2O + 2MDHA$$

The monodehydroascorbate (MDHA), is regenerated by monodehydroascorbate reductase (MDHAR):

$$NADH + H^+ + 2MDHA \xrightarrow{MDHAR} NAD^+ + 2AsA$$

However, monodehydroascorbate, if not rapidly reduced, breaks down into ascorbate and dehydroascorbate (DHA). Dehydroascorbate (DHA) is reduced to ascorbate and oxidized glutathione (GSSG) by dehydroascorbate reductase (DHAR):

$$2GSH + DHA \xrightarrow{DHAR} GSSG + AsA$$

After eliminating the harmful hydroperoxide, the GSSG must return to its reduced form (GSH) to reacquire its antioxidant activity; this is achieved by an NADPH-dependent enzyme known as glutathione reductase (GR) through the following reaction:

 $GSSG + NADPH + H^+ \xrightarrow{GR} 2GSSG + NADP^+$ 



**Figure 1**. Enzymatic and non-enzymatic active antioxidants in plant defense and the Foyer-Halliwell-Asada cycle (also known as the AsA-GHS cycle) with its intermediates are reported. The Foyer-Halliwell-Asada cycle starts with the reduction of hydrogen peroxide in water by ascorbate peroxidase (APX). Abbreviations: SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; MDHA, monodehydroascorbate reductase; DHA, dehydroascorbate reductase; GHS, reduced glutathione; GSSG, glutathione disulphide.

# 4.4. Heat Stress Impact on Product Quality

Thermal stress influences the morpho-physiological aspects of vegetables, thus undermining quality and causing significant economic loss. However, recent studies have shown that plants under moderate heat stress can exhibit better-quality features<sup>117</sup>. In protected environments, thermal stress induces physiological alterations and affects vegetables' appearance, flavor, carbohydrate content and aromatic and antioxidant compounds.

For example, if white asparagus is exposed to thermal stress, the rapid opening of the heads induces purple coloration, thus reducing their quality and economic value; moreover, an increase in fibrousness, wilting of shoot tips and imbalances in calcium assimilation were also observed<sup>118,119</sup>. Studies on onions revealed an increase in sulfur compounds (important for flavor) as the temperature increased, as well as bulb splitting<sup>119,120</sup>. Similarly, carrot cultivars exposed to high temperatures showed a better and more intense taste and an increased terpenes content but a carotene reduction<sup>2</sup>. In broccoli, temperatures around 25 °C, caused head deformation, premature ripening and discoloration<sup>122</sup>. However, as reported by Mølmann et al.<sup>123</sup>, high temperatures induced a higher accumulation of anthocyanins, glucosinolates, phenols and flavonoids that led



to a less sweet taste than in broccoli that had been exposed to lower temperatures (12 °C). Similar findings arose in Chinese cabbage<sup>124</sup>. In the case of lettuce, temperatures above 15–18 °C determined a higher incidence of physiological disorders such as loose heads, tipburn and leaf chlorosis. In contrast, a higher accumulation of bitter compounds and vitamins C and E but a lower accumulation of carotene were recorded<sup>117,119,125,126</sup>. Similarly, in tomatoes, heat stress led to an increase in vitamin C content and antioxidant compounds, contrasted by a decrease of lycopene content and macronutrients such as magnesium, calcium, and potassium. Also, for peas, tomatoes, melons and watermelons, a lower sugar content was observed<sup>119,124,126</sup>.

Several studies have shown a relationship between the expression of antioxidant enzymes, temperature, and genetic tolerance to heat stress. The scientific literature suggests explicitly that antioxidant activity increases over a range of certain temperature levels. Chakrabortty and Pradhan<sup>127</sup> reported that catalase, ascorbate peroxidase and superoxide dismutase enzymes increased up to 50 °C. On the other hand, the activity of peroxidase and glutathione reductase demonstrated a decrease in the temperature range of 20–50 °C.

Temperature is not the only variable to play an important role in enzymatic antioxidant activation and expression. Studies on field crops indicate that the expression of antioxidant enzymes increases in heat-resistant species at all stages of growth. For example, there was a higher accumulation of GHS, and GHS/GSSG ratio<sup>128</sup>, GST (glutathione S-transferase), POX, APX, CAT, SOD and GR<sup>129,130</sup>.

#### 5. Conclusions

Scientists and producers are being motivated by climate change and consumers' appreciation of healthy foods to broaden their vision of conventional production processes, and this is prompting them to adopt multidisciplinary approaches to improve productivity, including varietal selection, pest control and stress reduction. The introduction of insect protection measures has provided a safe tool for the environment, offering the best defense against harmful insects as well as new alien species as part of the attempts to increase greening and environmental sustainability. Nowadays, growers have a wide range of insect nets available that differ in manufacturing and performance, helping them to choose the most suitable ones for their purposes. However, the use of anti-insect nets demands careful assessment of the effect they have on the microclimate, particularly in the warm regions of the Mediterranean, where the radiation surplus causes a rapid and detrimental increase in temperature that will ultimately has to be overcome to avoid a significant drop in production or, in exceptional circumstances, the total loss of production. In a region imperiled by global warming, there is an urgent need to draw the attention of engineers, producers, and researchers to find the right compromise between insect protection and favourable climatic conditions for plant

# 31 Chapter 2

growth. Researchers have focused most of their attention on improving the airflow of anti-insect nets to avoid detrimental increases in temperature and suboptimal growth environments while continuing to exclude insects and not affecting the quality of the final product. However, most of these researches were conducted in a simulated environment using computational fluid dynamics (CFD) models, ignoring the real growth conditions and their interaction with crops presence. The reviewed literature showed that high temperatures induce high adaptive responses in edible vegetables. Plants' defense mechanisms of producing antioxidant compounds against harmful ROS are an excellent quality boost for vegetables until certain threshold. In the light of these considerations, we believe that it is necessary to investigate these aspects to develop mathematical models that can predict the performance of insect nets in real conditions to be able to correlate it with vegetables quality. These models would make it possible to develop versatile insect nets that can provide physical protection, improve airflow and increase the quality of vegetables by accumulating secondary metabolites and maintaining yield in its average.



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# Chapter 3

# Improved Porosity of Insect Proof Screens Enhances Quality Aspects of Zucchini Squash Without Compromising the Yield

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Abstract: In a global climate change environment, assuring optimal growing conditions is a difficult challenge, compromising the food supply for a rapidly rising population. The climatic conditions in the protected environment lead to high temperatures and fast insect development, impacting productivity and vegetables qualitative attributes. Consumers' interest in healthy food requires sustainable tools to manage biotic and abiotic factors and, from this perspective, antiinsect nets represent an excellent "green" solution. For this purpose, our goal was to compare two different anti-insect nets on microclimate, production, and qualitative traits of Cucurbita pepo L. fresh fruits. The experiment was conducted in three separate polyethylene high tunnels, with 50 mesh anti-insect nets of different porosities being installed on the openings of two tunnels, while the third tunnel was a control without nets. Microclimate measurements, as well as yield, physiological, and phytochemicals variables, were assessed. The 50 mesh net led to a decrease in marketable yield (22.5%), fruit number (18.0%), CO<sub>2</sub> net assimilation rate (6.0%), and transpiration rate (29.5%). Total soluble solids, antioxidant activities and total ascorbic acid concentration had an opposite trend. The 50 mesh AirPlus net improved quality aspects of zucchini fruits by increasing total ascorbic acid, total phenols, and antioxidant compounds, with no negative impact on yield.

**Keywords:** *Cucurbita pepo* L; Anti-insect nets; Mesh density; High tunnel; Air temperature; Airflow; Qualitative parameters



#### 1. Introduction

Recent climate changes are severely affecting agriculture and endangering food supply for future generations<sup>1</sup>, especially in countries with lower socioeconomic resources and a higher risk of poverty, thus introducing new challenges for food production. The agricultural sector supplies about 50% of the nourishment needed by the world increasing population, which is expected to reach 9.7 billion in the coming decades<sup>2</sup>. However, the effective yield achieved is only 50%<sup>3</sup> of what would be potentially obtainable, due to biotic and abiotic factors undermining agricultural production, notably insects and photothermal stress<sup>4</sup>. In a global climate change environment, assuring optimal growing conditions is an arduous challenge, especially in warm Mediterranean areas where high temperatures, water, and insects' proliferation are limiting factors, reducing productivity and qualitative vegetable traits<sup>5</sup>. The everincreasing interest of consumers in healthful food has led to a "consumer-oriented" revolution and new quality perceptions. Quality is no longer relegated exclusively to food appearance but also includes its bioactive phytochemical content, combining healthfulness with gastronomic pleasure<sup>6</sup>. The consumer ascribes to food a supporting role for human wellness due to the content of beneficial bioactive compounds. Accordingly, the market is tailored to the requests of an increasingly informed and health-conscious consumer, orienting growers towards high quality and eco-sustainable production. On the other hand, it is well established that the climatic conditions in high tunnels may facilitate the rapid development of pests7, requiring eco-friendly tools for their containment<sup>8</sup> and physical barriers represent an excellent "green" solution.

The exclusion performance of anti-insect nets depends on geometrical and structural hole patterns<sup>9</sup>, where an appropriate net selection can achieve up to 90% control of a designated pest<sup>10</sup>. Today, the manufacturers have the knowledge to develop and produce various kinds of agro-textiles with different mechanical, physical, and radiometric features, satisfying the specific requirements of growers<sup>11</sup>. Aside from the aspects outlined above, most anti-insect nets for agricultural use are manufactured by a weaving process. Several vertical and horizontal warp and weft plastic threads are overlapped and woven, making a regular structure variable in size, according to the insect to be excluded and with a specific porosity (percentage of the ratio between open net area and total net area)11. From a commercial viewpoint, anti-insect nets are classified by mesh number, describing the number of openings per linear inch<sup>12</sup>. The performance of insect exclusion screens is founded on avoiding insect thorax passage through net mesh ("prison effect")<sup>13</sup> and, theoretically, small hole nets are more effective. However, the lower porosity of fine mesh nets, leads to a high static pressure drop<sup>14,15</sup>, resulting in inadequate air exchange and reduced ventilation<sup>16</sup>, hence exposing crops to abiotic stress that affects crop growth and production, while representing a barrier for pollinators<sup>17</sup>.

# 41 Chapter 3

Consequently, a high differential in temperature and relative humidity occurs between the indoor and outdoor growing environment<sup>18,19</sup>.

It is well acknowledged that high temperatures induce morpho-physiological, molecular, and biochemical modifications in plants<sup>4,20</sup> as an adaptive response to heat stress. High temperatures interfere with primary metabolism (photosynthetic and respiratory processes)<sup>21</sup>, inhibit bud and root growth, stimulate leaf abscission, impair fruit set, damage fruits<sup>22</sup>, and decrease root assimilation efficiency<sup>23</sup>. Additionally, heat stress alters enzymatic activity, modifies chloroplast proteins<sup>24,25</sup> and enhances soluble sugar accumulation<sup>26-28</sup>. Furthermore, heat promotes the production of high quantities of reactive oxygen species (ROS), resulting in a lower biosynthesis of photosynthetic pigments<sup>29,31</sup> which reduces the activity of Photosystem II<sup>32</sup>. Blooming and fruit set are similarly sensitive to high temperature stress, as evidenced by studies on zucchini squash<sup>33-34</sup> and tomato<sup>32,35</sup>. Not lastly, high temperatures influence secondary metabolism by stimulating biosynthesis and the accumulation of antioxidant compounds as observed in watermelon and tomato<sup>36</sup>.

Zucchini (Cucurbita pepo L.) is a vegetable that is gaining popularity in Europe, representing a resource for the horticultural chain, ranking fourth among retail vegetables. Among the European countries, Italy has a greenhouse production of 218,950 tons<sup>37</sup> and an annual per capita consumption of 9 kg<sup>38</sup>. Moreover, the increased consumer demand in the national and international market for fresh fruits available allyear-round has made zucchini greenhouse cultivation increasingly popular. Based on our knowledge and the examined literature, there is an evident lack of research assessing the impacts of the microclimate induced by anti-insect nets on the production and quality attributes of zucchini squash. Recent and interesting comparable studies on cucumber have highlighted the positive effects induced by insect exclusion screens in containing cucumber beetles (Acalymma vittatum Fabr.) in high tunnels while ensuring adequate ventilation<sup>39</sup>. Undoubtedly, the few contributions available were mainly focused on evaluating the effects of insect exclusion on crops yield with no emphasis on the interaction between the microclimate and the qualitative and quantitative vegetables response. Based on the considerations mentioned above, the presented research was aimed at assessing the influence of the microclimate change induced by two 50 mesh anti-insect nets with different porosity on the qualitative and quantitative aspects of zucchini fruits. As far as we know, this is the first research investigating these aspects, establishing a basis for future studies.



# 2. Results

#### 2.1. Microclimate Parameters

**Figures 1A, B and 2** show, respectively, the hourly air temperature, the hourly soil temperature and relative humidity inside the high tunnels. As regards air temperature, both nets recorded higher values when compared to the control, especially during the warmer part of the day (from 10:00 to 15:00). However, during the early hours of the day (from 6:00 to 10:00), the 50 mesh AirPlus net showed an average lower air temperature (–10%) than 50 mesh net (**Figure 1A**). Similarly, the soil temperature throughout the day was lower in the high tunnels covered with 50 mesh AirPlus net compared to the 50 mesh one (**Figure 1B**). In particular, from 8:00 to 20:00, it observed an increase of 5 and 14% of the soil temperature under the 50 mesh AirPlus net and the 50 mesh, respectively, compared to the unscreened control (**Figure 1B**). The daily trend of the relative air humidity showed, from 10:00 to 23:00, that the 50 mesh AirPlus net recorded a lower value than both the 50 mesh net and the control (**Figure 2**). The greenhouse cover film affected the PPFD (photosynthetic photon flux density) resulting in an average reduction of 30% compared to the outside (**Figure 3**). Of note, the use of the nets reduced light radiation by only 5% compared to the control without nets.



**Figure 1**. Hourly air temperature (**A**) and soil temperature (**B**) recorded inside the high tunnels covered with nets and without nets.





**Figure 2**. Hourly air relative humidity recorded inside the high tunnels covered with nets and without nets.



**Figure 3**. Average PPFD (Photosynthetic Photon Flux Density) values recorded inside the high tunnels covered with nets and without nets and outside the high tunnels.

# 2.2. Influence of Anti-insect Nets on Yield and Yield Components

The yield and yield components of zucchini squash produced under the different anti-insect nets are presented in **Table 1** and **Figure 4**. The yield and the number of fruits per plant were influenced by treatments, whereas the mean weight of the fruits showed no significant difference (**Table 1**). In particular, the yield and number of fruits grown in the 50 mesh net-covered high tunnel decreased by 23% and 18%, respectively, in comparison to unscreened control. Interestingly, the 50 mesh anti-insect net resulted in an earlier production of 10 and 17-days, compared to the 50 mesh AirPlus net and control, respectively (**Figure 4A**, **B**). Furthermore, up to 80 days after transplant, the 50 mesh net resulted in improved productivity, regarding both yield and number of fruits, while an opposite trend was observed in the following days until the end of the cycle. In fact, after 80 DAT (days after transplant) lower production was evident in 50 mesh nets-treated plants compared to AirPlus 50 mesh net and control (**Figure 4A**, **B**).

		1		
_	Yield	Fruit Number	Mean Weight	
Treatments	(kg fw Plant <sup>-1</sup> )	(no. Plant <sup>-1</sup> )	(g fw Fruit <sup>-1</sup> )	
No-net	$4.00 \pm 0.17$ a	27.20 ± 0.41 a	$147.10\pm4.25$	
50 mesh	$3.09 \pm 0.06 \text{ b}$	$22.32 \pm 0.26$ b	$138.40\pm4.05$	
50 mesh AirPlus	$3.55 \pm 0.18$ ab	26.64 ± 0.35 a	$133.40 \pm 6.56$	
Significance	*	***	ns	

Table 1. Effects of anti-insect nets on yield, number of fruits per plant, and mean fruit weight of
zucchini squash.

Means within each column followed by different letters are significantly different ( $p \le 0.05$ ) according to Duncan's multiple range test. ns, \*, \*\*\* non-significant or significant at  $p \le 0.05$ , and 0.001, respectively. All data are expressed as mean ± standard error, n=3.





**Figure 4**. Effects of anti-insect nets on the cumulative number of fruits (**A**) and yield (**B**) per plant at different days after transplant (DAT).

# 47 Chapter 3

#### 2.3. Influence of Anti-insect Nets on Physiological and Biochemical Parameters

With the exception of soil plant analysis development (SPAD) index, all the analyzed physiological parameters showed significant differences between the different treatments. The leaf net CO<sub>2</sub> assimilation rate (A<sub>CO2</sub>), transpiration (E), and maximum quantum efficiency of PSII photochemistry ( $F_v/F_m$ ) showed a significant decrease in plants treated with 50 mesh net, compared to the 50 mesh AirPlus net and control. Specifically, in plants covered by 50 mesh nets, average values of A<sub>CO2</sub>, E, and F<sub>v</sub>/F<sub>m</sub> were, respectively, 5.77, 29.6, and 6.76% lower than those recorded in the control (**Table 2**). An opposite trend was observed for stomatal resistance ( $r_s$ ) and intrinsic water use efficiency (WUEi) that recorded the highest values in the 50 mesh treatment (**Table 2**).



**Table 2**. Effects of anti-insect nets on Soil Plant Analysis Development Index (SPAD index), net photosynthesis (Aco<sub>2</sub>), stomatal resistance (r<sub>s</sub>), transpiration (E), intrinsic water use efficiency (WUEi), and chlorophyll fluorescence of zucchini squash.

Treatmont	SPAD Index	ACO <sub>2</sub>	ľs	Ε	WUEi	Fluorescence
Treatment		(µmol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	(m² s mol-1)	(mol H <sub>2</sub> O m <sup>-2</sup> s <sup>-1)</sup>	(µmol CO <sub>2</sub> mol <sup>-1</sup> H <sub>2</sub> O)	Fv/Fm Ratio
No-net	$47.19\pm0.59$	12.31 ± 0.16 a	$6.65\pm0.15~b$	3.01 ± 0.29 a	$4.23 \pm 0.28 \text{ b}$	$0.74 \pm 0.00$ a
50 mesh	$45.62\pm0.61$	$11.60 \pm 0.15$ b	$7.40 \pm 0.32$ a	2.12 ± 0.26 b	$5.83 \pm 0.62$ a	$0.69\pm0.02~b$
50 mesh AirPlus	$46.16\pm0.73$	12.26 ± 0.13 a	$6.13 \pm 0.18$ b	3.81 ± 0.29 a	3.33 ± 0.29 b	$0.72 \pm 0.01$ a
Significance	ns	**	**	**	**	**

Means within each column followed by different letters are significantly different ( $p \le 0.05$ ) according to Duncan's multiple range test. ns,<sup>\*\*</sup> non-significant or significant at  $p \le 0.01$ , respectively. All data are expressed as mean ± standard error, n=3.

# 49 Chapter 3

#### 2.4. Fruit Juice pH, Total Soluble Solids, and Dry Matter

Total soluble solids (TSS) and dry matter (DM) of the fruits showed significant differences among the treatments (**Table 3**), while no difference was found for the fruit juice pH (6.35, on average). The total soluble solids showed an increment of 47.9% in fruits grown under 50 mesh net with respect to the control (**Table 3**). Similarly, both nets resulted in a significant increment in DM content of the fruits (+19.7%, on average) compared to untreated control (**Table 3**).

#### 2.5. Analysis of Total Ascorbic Acid, Total Phenols, and Antioxidants Activities

The anti-insect nets significantly affected the total ascorbic acid content, total phenols content and the antioxidant activities (**Table 3**). In particular, hydrophilic antioxidant activity (HAA) and ABTS antioxidant activity of fresh zucchini fruits ranged from 9.93 to 10.58 mmol ascorbic acid eq. 100 g<sup>-1</sup> dw and from 17.4 to 23.1 mmol Trolox eq. 100 g<sup>-1</sup> dw, respectively. Both antioxidant activities were significantly higher in the fruits of plants grown under nets. Similarly, the total ascorbic acid content in the fruits of nets-protected plants was on average 9.7% higher than that recorded in the untreated control (**Table 3**). In contrast, the total phenols content increased by 18.9% only in plants grown under 50 mesh AirPlus compared to control.



**Table 3**. Effects of anti-insect nets on dry matter (DM), pH, total soluble solids (TSS) content, hydrophilic antioxidant activity (HAA), ABTSantioxidant activity (ABTS AA), total phenols (expressed in dry weight, dw) and fruit total ascorbic acid (TAA; expressed in fresh weight, fw) ofzucchini squash.

Treatments	DM	- рН -	TSS	HAA	ABTS AA	Total Phenols	TAA
	(%)		(°Brix)	(mmol Ascorbic ac. eq. 100g <sup>-1</sup> dw)	(mmol Trolox 100g <sup>-1</sup> dw)	(mg Gallic ac. eq. 100g-1 dw)	(mg Ascorbic ac. 100g <sup>-1</sup> fw)
No-net	$4.06\pm0.17~b$	$6.31\pm0.03$	$2.88\pm0.09~b$	9.93 ± 0.05 b	$17.43 \pm 0.66$ b	$165.96 \pm 4.74 \text{ b}$	17.54 ± 0.29 b
50 mesh	$4.84 \pm 0.27$ a	$6.42\pm0.03$	$4.26 \pm 0.06$ a	10.50 ± 0.12 a	22.02 ± 0.07 a	$170.98 \pm 3.43 \text{ b}$	19.46 ± 0.13 a
50 mesh AirPlus	$4.88 \pm 0.01$ a	$6.32\pm0.05$	$3.08 \pm 0.01$ b	10.58 ± 0.14 a	23.10 ± 1.32 a	197.31 ± 2.45 a	19.01 ± 0.61 a
Significance	*	ns	***	*	**	**	*

Means within each column followed by different letters are significantly different ( $p \le 0.05$ ) according to Duncan's multiple range test. ns, \*, \*\*, \*\*\* non-significant or significant at  $p \le 0.05$ , 0.01 and 0.001, respectively. All data are expressed as mean ± standard error, n = 3.

#### 3. Discussion

Anti-insect nets are a sustainable and efficient approach for insect exclusion in protected environments<sup>8</sup>. However, small hole nets lead to detrimental increased temperature and relative humidity<sup>40,41</sup>. The aim of our research was the assessment of anti-insect nets with different porosity on the induced microclimate and on the productive and qualitative performance of zucchini squash plants. In the cultivation area where the experiment was conducted, due to the distinct climatic conditions (warm spring-summer and constant wind), zucchini plants were particularly vulnerable to early attacks by insects and pathogenic fungi. Therefore, especially in early growth stages, when the plants are particularly susceptible, phytopathogenic adversities can quickly lead to the death of young and still poorly lignified plants. For this reason, fungicide treatments (one with penconazole and two more with wettable sulfur) were carried out in all the tunnels, at the same time and the same dosage, to eliminate any variability resulting from their use. Likewise, at the beginning of the test (0 DAT), in order to eliminate any wintering insects, a selective insecticide treatment based on pirimicarb was carried out in all the tunnels (screened and unscreened) by foliar spray application. Subsequently, it was decided to carry out careful monitoring of the biotic pressure through the use of chromotropic traps placed in all the tunnels, intervening with insecticide treatments when the intervention threshold was exceeded. In this regard, in the screened tunnels, even if a certain biotic pressure was present, the intervention thresholds never exceeded during the entire crop cycle; in contrast in the unscreened tunnel, five insecticide treatments with potassium salts of fatty acids C14-18 were necessary to maintain the biotic charge at levels comparable to the screened tunnels, and thus neutralize any variability caused by the different grade of insect attacks. This finding confirms inter alia the effectiveness of anti-insect nets in controlling biotic pressure in the present experiment. The potassium salts of fatty acids are readily degraded via photochemical processes without leaving residues on the vegetation<sup>42,43</sup>; in contrast, they are selectively active on target pests by dissolving the waxes present in the insect cuticle causing their death by dehydration<sup>44,45</sup>.

Our findings demonstrated an evident influence of nets on inner microclimate. The higher air and soil temperature and relative air humidity recorded are in agreement with previous comparable studies<sup>19,46</sup>. It is noteworthy that the lower temperature of air and soil, as well as relative air humidity, were recorded using the 50 mesh AirPlus net. The improved performance of the 50 mesh AirPlus net is due to the employment of a thinner high density polyethylene (HDPE) filament (Arrigoni Harlene HT<sup>®</sup>, Uggiate Trevano (CO), Italy) resulting in a higher porosity at the same mesh number. Teitel and Shklyar<sup>14</sup> demonstrated that increased porosity is associated with reduced static pressure drop, and thus an improvement in airflow and microclimatic parameters. Both nets significantly reduced light transmission compared to the unscreened control; however, this decrease in PPFD around 60 µmol m<sup>-2</sup> s<sup>-1</sup> did not actually affect plant growth.



Nevertheless, agreeing with Klose and Tantau<sup>47</sup>, the lower spacing between adjacent threads does not necessarily imply lower light transmission; probably, soil dust accumulated on the nets and the structure of the threads, were involved in masking light radiation and hence reducing PPFD. The AirPlus 50 mesh net showed a yield and number of fruits in range with the typical greenhouse production of zucchini squash<sup>48</sup>, in contrast the 50 mesh net recorded values below the reference standards. Indeed, the heat stress caused by lower porosity of 50 mesh net affected the physiological activities of zucchini plants, reducing the yield and the number of fruits. In support of our data, different investigations on tomatoes reported adverse effects of heat stress on radical conductance<sup>49</sup>, source and sink activities<sup>50</sup>, and carbon transport to the vegetative apex<sup>51</sup>. However, low fruit number is probably due to fertilization and embryo development defects under high-temperature conditions, as found in previous works on tomato<sup>32,35</sup> and zucchini<sup>33</sup>. Other studies on zucchini revealed that high temperatures are also related to the production of immature and "attached-flowers" fruits, leading to a reduced yield<sup>34</sup>. In fact, at the beginning of the growing cycle, due to the lower recorded soil and air temperatures, anti-insect nets positively influenced plant growth by rising the temperature to an average, convenient for early production. As the growing period advanced (June/July), the opposite trend occurred as temperatures rose and caused adverse conditions to zucchini production. Heat stress caused flowers and fruit drop, leading to a lower total yield, mainly in plants grown using the 50 mesh net.

The anti-insect nets also affected photosynthesis and transpiration. Notably, the 50 mesh net caused lower net CO<sub>2</sub> assimilation (Aco<sub>2</sub>) and maximum quantum efficiency of Photosystem II (F<sub>v</sub>/F<sub>m</sub>), attributable to a reduced biosynthesis of photosynthetic pigments and photosystem II activity or both effects combination. High temperatures altered the permeability and structure of cell membranes and reduced the activity of several enzymes<sup>25</sup> and the regenerative ability of 1.5-bisphosphate ribulose carboxylase (RuBisCo)<sup>49,52</sup>, leading to a reduced carbon fixation, and thus affecting the adjustment capacity of the photosystem II<sup>53</sup>. Furthermore, heat stress impaired and disrupted the oxygen-evolving complex<sup>54</sup>, resulting in the production of potentially harmful reactive oxygen species<sup>29,30</sup>, affecting the biosynthesis of chlorophyll pigments and reducing the photosynthesis. Moreover, Tewari and Tripathy<sup>55</sup> demonstrated that under high-temperature conditions, chlorophyll biosynthesis in *Cucumis melo* L. plants was reduced by 60% due to the deactivation of the 5-aminolevulinate dehydratase enzyme involved in pyrrole biosynthesis. Similarly in tomato, Camejo et al.<sup>32</sup> reported a reduction in chlorophyll/carotenoid ratio content.

Additionally, transpiration is the principal leaf cooling system, and the stomata play a fundamental role in its regulation, offering a low resistance way for gas diffusion through the leaf. Under optimal water and high sunlight levels, the leaf's demand for CO<sub>2</sub> is highest, and thus stomatal resistance reduces while the transpiration rate increases. However, at high-temperature levels, this process is impaired, as occurred in 50 mesh net treatment. The lower CO<sub>2</sub> request, as a result of damaged photosynthetic apparatus and the reduced biosynthesis of photosynthetic pigments, resulted in a decrease in the transpiration rate and an increase in the stomatal resistance and intrinsic water use efficiency (WUEi), through which the plants attempted to minimize water loss by closing the stomata and decreasing transpiration. Further explanations were provided by Taiz et al.<sup>56</sup> and, probably, the reduced transpiration derives from low internal airflow, leading to higher resistance of the air boundary layer at the leaf surface, or from the accumulation of ABA (abscisic acid) in the leaves in response to high temperatures.

Plants in addition to synthesizing primary compounds as proteins, lipids, carbohydrates, and acids, produce a wide range of secondary metabolites indirectly involved in growth and development, as well as relevant defensive properties<sup>56</sup>. Scientific studies<sup>57,58</sup> highlighted that plants' phenolic compounds protect human cells during the first stages of cancer development and exhibit an elevated antioxidant activity that exerts beneficial actions on vascular and nervous systems<sup>59</sup>, mitigating the side effects of certain diseases including dementia, Alzheimer's and Parkinson's<sup>60,61</sup>. Phenolic compounds contribute to an increase in quality of vegetables, which is related to intrinsic (genotype) and extrinsic (environment) factors<sup>62</sup>. Zucchini squash fruits have a high water and macronutrients content as well as a low protein and fat content. Additionally, they have a high content of hydrophilic (vitamin C, niacin, vitamin B-6, riboflavin, and thiamine) and lipophilic (vitamin E,  $\beta$ -carotene, vitamin A, and vitamin K) antioxidant compounds<sup>63</sup>. Many studies have pointed out a positive quality change induced by heat stress, attributable to increased antioxidants compounds as a defensive response to ROSs accumulation<sup>23,31</sup>. According to our experiment, both anti-insect nets induced an increase of TAA, compared to USDA<sup>63</sup> values, and an increase of antioxidant activities. In contrast, the total phenols content was significantly higher in fruits cultivated under the 50 mesh AirPlus net treatment. Investigations on watermelon and tomato revealed that antioxidant molecules produced at high temperatures represent a mechanism of resistance to heat stress<sup>36</sup>; meanwhile, Wahid et al.<sup>23</sup> suggested that they might provide an additional control function of the leaf's osmotic potential to reduce water loss through transpiration, which is supported by our results. However, the lower accumulation of the total phenols occurring in the 50 mesh net treatment could be the result of plants being unable to adapt rapidly to the high thermal stress, leading to an inhibition of phenolics biosynthesis.

Moreover, anti-insect nets also influenced both TSS and DM contents of fruits. As for TSS, compared with the 50 mesh treatment, higher amounts were recorded in fruits, reflecting the higher heat stress induced by the net, making our results aligned with different researches. Indeed, some studies evidenced an increased production of primary metabolites like proline, glycine betaine, and especially of soluble solids in plants exposed to heat stress<sup>23</sup>, in order to improve the protein and cellular membrane stability and to regulate the osmotic potential, representing an indicator of thermal stress. Carbohydrates such as sucrose, the main photosynthesis product, regulate plant



development and allow carbon allocation and sugar signaling, as suggested by Roitsch and Gonzalez<sup>26</sup>. Furthermore, an antioxidant action of sugars<sup>28</sup> and ROS scavenger function<sup>27</sup> was shown.

# 4. Materials and Methods

# 4.1. Growth Conditions, Treatments, and Experimental Design

The present experiment was carried out in 2019 growing season at the greenhouse complex at the experimental farm "Torre Lama" of the University of Naples, situated in Bellizzi (Salerno, southern Italy; latitude 43°31' N, longitude 14°58' E, altitude 60 m). The main physical and chemical soil characteristics at the experimental site were clay loam texture (46% sand, 24% silt, and 30% clay), electrical conductivity (EC): 0.16 dS m<sup>-1</sup>, pH: 7.7, total nitrogen (N): 0.11%, and organic matter: 1.21% (w/w). The Olsen phosphorus and exchangeable potassium were 88 and 980 mg kg<sup>-1</sup>, respectively. The quality of the irrigation water was characterized by high bicarbonate content. The concentrations of ions expressed as mg L-1 were calcium (86); chloride (9); magnesium (20); sodium (7); potassium (und.); sulfate (9); nitrate (4.5); and bicarbonate (285). The values of pH and EC were 7.5 and 0.43 dS m<sup>-1</sup>, respectively. Water was provided by a drip irrigation system consisting of a main polyethylene pipeline (32 mm diameter and 2 atm operating pressure) with a series of semi-compensating dripping wings (16 mm diameter and 60 cm interpolation). The growing system was made of three single high tunnels 30 m long, 7.2 m wide, and 2.8 and 4.5 m high at the eaves and ridges, respectively, each covered with a polyethylene film applied to the greenhouse gables, roof, and the lower part of the side walls (up to a height of 0.6 m above the ground). The high tunnels were irradiated by natural sunlight while relative humidity and temperature were managed through natural ventilation. Figure 5 show minimum and maximum relative air humidity and air temperature recorded outside the high tunnels during the growing season at the experimental site.

Seeds of parthenocarpic zucchini squash (*Cucurbita pepo* L.), variety Zufolo F1 (Olter, Piacenza, Italy) were germinated in vermiculite on 14 March 2019. Seedlings were transplanted on 1 April, at the two true-leaf stages in three single rows with a plant distance of 1.6 and 0.6 m inter- and intra-rows, respectively, giving a density of 1 plant m<sup>-2</sup>.

At transplant, a foliar spray insecticide treatment with pirimicarb at the dose of 2.3 g L<sup>-1</sup> (Pirimor 17.5, Adama, Grassobbio (BG), Italy) was carried out inside the tunnels to eliminate any wintering insects. During the experiment, additional five insecticide foliar spray treatments with potassium salts of fatty acids C14–18 (soft soap) at the dose of 15 mL L<sup>-1</sup> (Acaridoil 13 SL, Agrowin Biosciences, Bergamo, Italy) were applied inside the unscreened tunnel when the intervention threshold (number of insect/trap) was reached by monitoring the insects' count through chromotropic traps. The last insecticide treatment was carried out at 78 DAT. Moreover, at 25 DAT powdery mildew

(*Sphaerotheca fuliginea*) protection was performed inside all the tunnels with penconazole foliar spray treatment at the dose of 0.5 mL L<sup>-1</sup> (Topas 10 EC, Syngenta, Milano, Italy). Subsequently two foliar spray treatments with wettable sulfur at the dose of 1.5 g L<sup>-1</sup> (Wettable Sulfur, Bayer, Milano, Italy) were carried out.

The experimental treatments consisted of two 50 mesh size anti-insect nets differing in porosity and permeability to air, and an unscreened control treatment. The study was conducted to compare the influence of the two anti-insect nets that covered the sidewalls and ventilation openings of the two tunnels, whereas the third tunnel was used as a control (unscreened). The anti-insect nets features were as follows: (1) Biorete® 50 mesh (Arrigoni S.p.A, Uggiate Trevano, Italy; Ø warp-weft: 0.23/0.23; warps-wefts per cm: 20/10; hole dimension:  $0.27 \times 0.79$  mm; permeability to air: 36%; ventilation reduction: 32%; shade factor: 13); (2) Biorete<sup>®</sup> 50 mesh AirPlus (Arrigoni S.p.A, Uggiate Trevano, Italy; Ø warp-weft: 0.17/0.17; warps-wefts per cm: 20/11.7; hole dimension:  $0.33 \times 0.68$ mm; permeability to air: 47%; ventilation reduction: 30%; shade factor: 11). The improved air permeability was achieved by using UV-stabilized high-density polyethylene (HDPE) monofilament (Arrigoni Harlene HT®) that resulted in thinner and more resistant net and leading to an increased hole size for the same mesh number (Figure 6). Treatments were arranged in a completely randomized design where the three treatments were arranged in a cross section within the three tunnels to remove the variation due to the uneven conditions (experimental error) across tunnels. In particular, each horizontal strip that covers the three tunnels was a block of the completely randomized design that contains all three treatments. A total of 150 plants were transplanted in each high tunnel (50 plants for each tunnel cross section).





**Figure 5**. Minimum and maximum relative air humidity (**A**) and air temperature (**B**) recorded outside the high tunnels during the growing season at the experimental site.



**Figure 6**. Arrigoni Biorete<sup>®</sup> 50 Mesh AirPlus with Harlene HT<sup>®</sup> HDPE (high density polyethylene) monofilament (**A**) and Arrigoni Biorete<sup>®</sup> 50 Mesh with standard HDPE monofilament (**B**). Scale bars are 0.5 mm in **A** and **B**.

# 4.2. Microclimate Measurements

Two WatchDog A150 data loggers (Spectrum Technologies Inc, IL, USA; ±0.6 °C/±3% Temp/RH accuracy) separated by 10 m, were located in the midpoint of each high tunnel and placed at a height of 0.5 m above ground level, to record air temperature and relative humidity. The soil temperature was recorded by negative temperature coefficient (NTC) HI141BH thermo logger with external sensor (Hanna instruments<sup>®</sup>, Woonsocket, RI, USA; ± 0.5 °C accuracy) placed in the middle of each high tunnel at a depth of 6 cm. Outside climatic data were measured using a meteorological station Davis Pro2<sup>™</sup> Plus Stations 6163 (Davis Instruments, CA, USA), located 20 m away from the high tunnels. All external sensors were placed at a height of 7 m above ground level. The climatic data were collected at an interval of 30 min. Mean temperatures from April to July (2015–2018) were presented in **Figure S1**, in order to show the redundancy of the mean temperatures among the recent four years during the same period and same zone of our experimental site. These data were collected by the meteorological station of Battipaglia (Salerno, Italy).

Twenty PPFD (photosynthetic photon flux density) measurements were recorded between 11:00 and 13:00 h, inside and outside the high tunnels, using a handheld spectral radiometer (MSC15, Gigahertz-Optik, Turkenfeld, Germany) at 0, 50, and 99 days after transplant.



Mean temperature 35 30 Temperature °C 25 20 15 10 une une une une une April April April April lune lune April April May Mav May ylut ylut ylut ylut ylut April Ma/ May Mav Month 2016 2017 2018 2015 .

**Supplementary Figure 1**: Mean temperatures registered from April to July (2015-2018) by the meteorological station of Battipaglia (Salerno, Italy).

#### 4.3. Yield and Fruit Quality Measurements

The experimental trial was conducted from 30 May to 17 July 2019. The fruits of six plants per plot were harvested three times per week when they reached 12 cm in length (marketable fruits). For each plant, right after harvesting, the fresh weight and number of the fruits were recorded. Deformed or undersized fruits were considered unmarketable.

After 102 DAT, eight representative fruits per plot, free of disease symptoms or visible defects, were sampled and analyzed for quality parameters. The mesocarp of each fruit was homogenized in a Waring® blender (2 l capacity; Model HGB140, CA, USA) for 1 min and then filtered. The extracted juice was measured by a digital refractometer Atago N1 (Atago Co. Ltd., Tokyo, Japan) to determine the total soluble solids (TSS) content expressed as °Brix at 20 °C. A pH meter (HI-9023; Hanna instruments®, Woonsocket, RI, USA) was used for determining the fruit juice pH. One hundred grams of the fruit juice was dried in a forced-air oven at 80 °C for 72 h until reaching constant weight, for dry matter (DM) percentage determination. Sections of sampled fruits were immediately placed in liquid nitrogen and then stored at –80 °C for further qualitative analysis.

# 4.4. Soil Plant Analysis Development Index (SPAD), Leaf Gas Exchange, and Chlorophyll Fluorescence

At 99 DAT, measurements of the SPAD index were performed on fully expanded leaves of six plants per plot using a portable chlorophyll meter SPAD-502 (Minolta Corp. Ltd., Osaka, Japan). A single average SPAD value for each replicate was obtained by measuring twenty leaves randomly.

On the same date, measurements of gas exchange and fluorescence emission were conducted between 11:00 and 13:00 h on the youngest fully expanded leaves. A portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., Hoddesdon, UK) equipped with a broadleaf chamber was used to determine the net CO<sub>2</sub> assimilation rate (Aco<sub>2</sub>), stomatal resistance (r<sub>s</sub>), and transpiration (E). PPFD, relative humidity (RH), and CO<sub>2</sub> concentrations were set at ambient values (700 ± 50 µmol m<sup>-2</sup> s<sup>-1</sup>, RH 55 ± 5%, and 365 ± 5 ppm, respectively) and the flow rate of air was 400 mL s<sup>-1</sup>. Intrinsic water use efficiency (WUEi) was calculated as Aco<sub>2</sub>/E ratio.

Modulated chlorophyll fluorescence was performed on six plants per plot on darkadapted (for at least 10 min) leaves, using a portable fluorometer  $F_v/F_m$  Meter (Opti-Sciences Inc, Hudson, NH USA). The ground fluorescence signal, F<sub>o</sub>, was induced on 10' dark-adapted leaves, by a blu LED internal light of 1–2 µmol m<sup>-2</sup> s<sup>-1</sup>. The maximal fluorescence intensity in the dark-adapted state (F<sub>m</sub>) was induced by a 1s saturating light pulse of 3000 µmol m<sup>-2</sup> s<sup>-1</sup>. The maximum quantum efficiency of open Photosystem II (PSII), Fv/Fm, was calculated as (F<sub>m</sub>-F<sub>o</sub>)/F<sub>m</sub>, according to Kitajima and Butler<sup>64</sup>.

# 4.5. Analysis of Total Ascorbic Acid, Total Phenols, and Antioxidants Activities

Total ascorbic acid (TAA) was assessed by spectrophotometric detection of fresh fruit material as described by Kampfenkel et al.<sup>65</sup>. TAA was measured by UV–VIS spectrophotometry (Hach DR 4000; Hach Co., Loveland, CO, USA). The solution absorbance was measured at 525 nm.

The Folin–Ciocalteu procedure<sup>66</sup> was used for evaluating the total phenolic content. A sample of lyophilized material was extracted in 60% methanol/water (w/v) with gallic acid as standard. UV–VIS spectrophotometer was used to measure the absorbance at 765 nm.

Two hundred milligrams of lyophilized zucchini fruits underwent different extraction procedures in order to quantify the antioxidant activity. The hydrophilic fraction (HAA) was measured as described by Fogliano et al.<sup>67</sup>. Whereas, a wider fraction of the antioxidant activity (ABTS AA) was measured by the method of Pellegrini et al.<sup>68</sup>, where 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay was used. The absorbance of HAA and ABTS AA solutions were measured at 505 and 734 nm, respectively, by UV–Vis spectrophotometry.



# 4.6. Statistical Analysis

All experimental data were analyzed by ANalysis Of VAriance (ANOVA) using the software package SPSS 10 for Windows, 2001 (SPSS Inc., Chicago, IL, USA). After the verification of the normality through the test of Shapiro–Wilk, Duncan's Multiple Range Test (DMRT) was performed at  $p \le 0.05$  on each of the significant measured variables.

# 5. Conclusions

The increasing consumer attention towards healthy foods has driven growers to research alternative eco-sustainable agronomic practices to chemical insecticides. In this perspective, anti-insect nets represent a valid eco-friendly solution. Although small hole nets are more effective, their high resistance to airflow reduces ventilation, resulting in a detrimental increase in temperature and humidity, representing a critical issue in the warm Mediterranean region. The results obtained suggest that the different porosity of tested insect nets modulates the yield and its precocity as well as the quality aspects of zucchini fruits. Particularly, the 50 mesh AirPlus resulted in an overall improved qualitative and physiological parameter without any reduction in yield and number of fruits. Its intrinsic highest porosity led to a better air exchange rate, resulting in an increased dry matter, antioxidant activities, total phenols, total ascorbic acid, CO2 assimilation rate, and transpiration. Based on the results obtained, it appears promising to evaluate the effects of 50 mesh AirPlus on yield and quality attributes of other greenhouse fruit vegetables. However, the 50 mesh net could be an excellent tool for growers to achieve earlier production in autumn-winter or late-winter crop cycles when higher temperatures are appreciated.

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# Divergent Leaf Morpho-Physiological and Anatomical Adaptations of Four Lettuce Cultivars in Response to Different Greenhouse Irradiance Levels in Early Summer Season

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Abstract: Lettuce (Lactuca sativa L.) is a winter-spring leafy vegetable, but the high demand for fresh products available year-round requires off-season production. However, the warm climate of the Mediterranean areas can impair the summer production of lettuce, thus requiring the adoption of genotypes tolerant to high irradiance as well as useful agronomic strategies like shading net installations. The aim of our research was to assess the leaf morpho-physiological and anatomical changes, in addition to productive responses, of four lettuce cultivars ('Ballerina', 'Maravilla De Verano Canasta', 'Opalix', and 'Integral') grown under shading and non-shading conditions to unveil the adaptive mechanisms of this crop in response to sub-optimal microclimate (high irradiance and temperature) in a protected environment. Growth and yield parameters, leaf gas exchanges, chlorophyll fluorescence and morpho-anatomical leaf traits (i.e., leaf mass area, stomatal density, and epidermal cell density) were determined. Under shading conditions, the fresh yields of the cultivars 'Ballerina', 'Opalix' ('Oak leaf') and 'Integral' ('Romaine') increased by 16.0%, 26.9% and 13.2% respectively, compared to non-shading conditions while both abaxial and adaxial stomatal density decreased. In contrast, 'Canasta' under non-shading conditions increased fresh yield, dry biomass, and instantaneous water use efficiency by 9.6%, 18.0% and 15.7%, respectively, while reduced abaxial stomatal density by 30.4%, compared to shading conditions. Regardless of cultivar, the unshaded treatment increased the leaf mass area by 19.5%. Even though high light intensity and high temperature are critical limiting factors for summer lettuce cultivation in a protected environment, 'Canasta' showed the most effective adaptive mechanisms and had the best production performance under sub-optimal microclimatic conditions. However, greenhouse coverage with a white shading net (49% screening) proved to be a suitable agricultural practice that ensured an adequate microclimate for the off-season growth of more sensitive cultivars 'Ballerina', 'Oak leaf' and 'Romaine'.

**Keywords:** *Lactuca sativa* L.; Sub-optimal conditions; Greenhouse; Leaf gas exchange; Fv/Fm ratio; LMA; Stomata



#### 1. Introduction

Lettuce (*Asteraceae; Lactuca sativa* L.,) is one of the most used and popular leafy vegetables globally, but its nutritional value is underestimated for its high water content (about 95%)<sup>1,2</sup>. Lettuce is an essential source of minerals (*e.g.*, potassium, calcium, phosphorus, magnesium, iron and zinc), which help maintain the correct hydro-saline balance of the human body, other than being rich in fibers, bioactive compounds, vitamins and carotenoids that are beneficial molecules for the human health<sup>3,4</sup>. Being a species adapted to low temperatures and low light intensity, lettuce is generally grown in winter and spring seasons. However, the high demand for year-round products has led to off-season cultivation of lettuce (spring-summer) in protected environments<sup>5</sup>. Extending lettuce cultivation into off-season production, where the demand for fresh products is higher, ensures growers better prices with significant economic benefits<sup>6</sup>.

High temperatures and high irradiance are typical of Mediterranean summers; such conditions are a limiting factor for agriculture, especially in sensitive crops such as lettuce, as they lead to morpho-physiological alterations that induce crop yield losses and quality impairments (e.g., head closure, rib discoloration, tipburn)<sup>7-11</sup>. Considering that the optimal temperatures for lettuce growth range from 18 to 28 °C, hightemperature stress combined with a long day induces alterations in water relations, photosynthetic activity, osmolyte accumulation and hormone production<sup>12,13</sup>. Other than leading to quality degradation<sup>14</sup>, these changes lead to a lower marketable yield of lettuce, which is affected by dry matter and water content<sup>15</sup>. To avoid these adverse effects, off-season lettuce production requires adequate crop protection from high solar radiation. In this perspective, shading nets, due to their ability to reduce light intensity, modulate light diffusion and hence reduce temperature, are effective at extending the growing season and improving the quality of horticultural products<sup>16,17</sup>. During summer, shading nets are widely used in Mediterranean areas to create a suitable microclimate for crop production, consequently reducing photoinhibition and improving water use efficiency and crop uniformity<sup>16,18</sup>.

Light fosters lettuce growth only in a specific range of light intensities<sup>19</sup>. Several studies have shown that lettuce grown in summer with light intensity over 600  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> had reduced biomass, leaf area and chlorophyll content<sup>19,20</sup>. These reductions were mainly ascribed to a low instantaneous saturation point, with evidence of oxidative processes (photoinhibition) at 800  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, as reflected by the lower F<sub>v</sub>/F<sub>m</sub> values compared to other plants that would grow well at levels of light intensity higher than 600  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> <sup>5</sup>. To cope with oxidative damage under high-light stress, plants have evolved complex adaptive mechanisms, including short and long-term responses<sup>21,22</sup>. Within hours of the stressful event, plants reduce their photosynthetic activity by closing the stomata, changing the orientation of leaves (heliotropism) and rearranging chloroplasts parallel to the light direction (avoidance response)<sup>21,23-26</sup>. In the long-term,

light stress triggers morpho-physiological changes in the plant, such as a reduction in chlorophyll content and leaf area and an increase in leaf mass area (LMA)<sup>23,27,28</sup>. As observed by Zha et al.<sup>29</sup> in *Lactuca sativa* L., small and thick leaves (higher LMA) have better adaptability to high light intensity by reducing water loss and improving light utilization. Several authors have reported that smaller and thicker leaves show improved heat exchange efficiency, which prevents rapid temperature rise and the consequent water loss under high light conditions<sup>30-33</sup>. Moreover, higher biomass investment in the leaf, as generally found in thicker leaves with higher LMA, has been correlated with an enhanced photosynthetic capacity<sup>28</sup>. However, the morphophysiological response to light intensity may differ among cultivars due to their genetic background<sup>28</sup>.

In response to external stimuli, plants also change the density and size of stomata to ensure a rapid improvement of water use efficiency under sub-optimal growth conditions<sup>34-37</sup>. In general, high light triggers stomatal development<sup>38</sup>, while heat stress has an opposite effect<sup>39-40</sup>. Summer cultivation in the Mediterranean environment couples the effects of excessive light and heat with a detrimental effect on the productivity of not suited crops, such as lettuce. The high demand for evapotranspiration that characterizes this environment implies that the balance between water loss and leaf cooling is a key aspect for plants to thrive, which is partially mediated by the plasticity of stomatal patterning<sup>41</sup>. Muir<sup>42</sup> has observed that high light intensity increased the adaxial stomatal density, which is more exposed to heating, to prevent harmful water loss<sup>38</sup>. The alteration of the stomatal density also impacts the plant's growth rate<sup>35</sup>. However, under the same climatic conditions, water use efficiency shows considerable intraspecific variability<sup>43</sup>. Indeed, plant adaptation to sub-optimal conditions depends on the genotype, environment and their mutual interaction<sup>14</sup>. Several studies have shown that lettuce cultivars with red leaves have better tolerance to high solar radiation than cultivars with green ones, which are more susceptible to photooxidation<sup>44,45</sup>. The high anthocyanin content of red cultivars would probably act as an antioxidant, shielding solar radiation and leading to better adaptability to high light conditions<sup>44,46</sup>. The high genetic variability of lettuce represents an important resource for studying the responses of this crop to different environmental constraints, which will enable conscious breeding programs focused on increasing its adaptability in the modern climate change scenario<sup>11</sup>.

The genetic variability in leaf morphology and pigmentation combined with the most advanced shading technology could be exploited to extend the growing season of lettuce in regions where high light intensity and high temperatures are limiting factors. For this purpose, the morpho-physiological and productive responses of four lettuce cultivars ordinarily grown in open field ('Ballerina', 'Canasta', 'Oak leaf' and 'Romaine') were evaluated under shading and non-shading conditions to identify the most suitable



genotype for cultivation under sub-optimal early summer conditions in a passively ventilated greenhouse. Even though lettuce is one of the most globally consumed vegetables, its susceptibility to the extreme environmental conditions of warm Mediterranean areas severely limits its off-season cultivation. To date, few studies have focused on the adaptive mechanisms of lettuce grown under suboptimal microclimatic conditions like summer greenhouse cultivation. Based on these considerations, it is interesting to understand how different lettuce cultivars respond to extreme conditions in both shaded and unshaded greenhouses by activating specific adaptive mechanisms. As far as we know, this is the first research investigating these aspects, and our results could be useful for both growers and breeders, paving the way for future work.

## 2. Results

# 2.1. Biometric and Yield Parameters in Response to Different Greenhouse Irradiance Conditions

As shown in Table 1 all biometric and yield parameters were affected by the interaction between cultivar (CV) and greenhouse irradiance conditions (GIC) factors. Regarding the leaf number, greenhouse irradiance conditions did not result in a univocal response among cultivars. Specifically, for 'Canasta' and 'Romaine' was observed a reduction in the leaf number under the shading net by 7.7% and 16.8%. In contrast, the shading net increases this parameter (leaf number) in 'Ballerina' and 'Oak leaf' by 6.5% and 8.5%, respectively. Leaf area and fresh yield increased in all cultivars grown under the shading net, except 'Canasta' for which these parameters did not change vs. non-shading conditions. Particularly, 'Ballerina', 'Oak leaf' and 'Romaine' increased leaf area by 14.8%, 58.7% and 18.2% and fresh yield by 16.0%, 26.9% and 13.2%, respectively. In contrast, 'Canasta' recorded the highest fresh yield ( $285.7 \text{ g plant}^{-1}$ ) in the unshaded treatment. With respect to dry biomass, both shaded and unshaded treatment did not result in any significant difference in all cultivars. In contrast, 'Canasta' showed a 15.2% reduction of dry biomass under the shading net. Finally, under shading 'Ballerina', 'Canasta', 'Oak leaf' and 'Romaine' decreased leaf dry matter by 14.9%, 7.1%, 16.1% and 10.8%, respectively.

	Leaf Number	Leaf Area	Fresh Yield	Dry Biomass	Leaf Dry Matter
Source of Variance –	(no. plant <sup>-1</sup> )	(cm <sup>2</sup> )	(g plant <sup>-1</sup> )	(g plant <sup>-1</sup> )	(%)
Cultivar (CV)					
'Ballerina'	33.39 ± 0.57 b	3729 ± 119 b	222.7 ± 7.67 c	13.15 ± 0.09 c	5.94 ± 0.22 a
'Canasta'	30.67 ± 0.61 c	3829 ± 66 b	273.2 ± 5.82 a	13.83 ± 0.54 b	$5.05 \pm 0.10$ b
'Oak leaf'	31.17 ± 0.61 c	2331 ± 246 c	173.1 ± 9.21 d	8.30 ± 0.16 d	$4.84 \pm 0.20$ c
'Romaine'	38.67 ± 1.70 a ***	4204 ± 168 a ***	241.3 ± 7.08 b ***	14.59 ± 0.31 a ***	6.07 ± 0.18 a ***
Greenhouse Irradiance Conditions (GIC)					
Unshaded	$34.08 \pm 1.48$	$3269 \pm 264$	$217.7 \pm 14.40$	$12.69 \pm 0.85$	$5.84 \pm 0.18$
Shaded	$32.86 \pm 0.69$	$3777 \pm 186$	$237.4 \pm 8.04$	$12.24 \pm 0.68$	$5.12 \pm 0.16$
<i>t</i> -Test	ns	ns	ns	ns	*
CV × GIC					
'Ballerina' × Unshaded	32.33 ± 0.58 c	3472 ± 69 d	$206.2 \pm 4.02$ e	$13.24 \pm 0.18$ b	6.42 ± 0.05 a
'Ballerina' × Shaded	$34.44 \pm 0.40 \text{ b}$	3986 ± 14 b	239.2 ± 2.51 c	$13.05 \pm 0.03$ b	$5.46 \pm 0.06$ bc
'Canasta' × Unshaded	31.89 ± 0.11 c	3950 ± 47 bc	285.7 ± 1.80 a	14.97 ± 0.29 a	$5.24 \pm 0.08$ c
'Canasta' × Shaded	29.44 ± 0.59 d	3709 ± 72 cd	260.7 ± 3.00 b	12.69 ± 0.25 b	$4.87 \pm 0.07 \text{ d}$
'Oak leaf' × Unshaded	29.89 ± 0.29 d	$1802\pm108~{\rm f}$	152.6 ± 0.83 g	$8.04 \pm 0.17$ c	5.27 ± 0.08 c
'Oak leaf' × Shaded	32.44 ± 0.40 c	2860 ± 101 e	193.7 ± 0.50 f	$8.57 \pm 0.17$ c	$4.42 \pm 0.10 \text{ e}$
'Romaine' × Unshaded	42.22 ± 1.31 a	3854 ± 132 bc	226.4 ± 5.05 d	14.53 ± 0.61 a	6.41 ± 0.13 a
'Romaine' × Shaded	35.11 ± 0.11 b ***	4555 ± 6 a ***	256.2 ± 1.60 b ***	14.64 ± 0.31 a ***	5.72 ± 0.14 b

 Table 1. Effect of cultivar and greenhouse irradiance conditions on leaf number, leaf area, fresh yield, dry biomass, and leaf dry matter in Lactuca sativa L.

Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters within columns indicate significant mean differences compared by DMRT (*p* = 0.05). ns, \*, \*\*, and \*\*\* denote nonsignificant or significant effects at *p* ≤ 0.05, 0.01, and 0.001, respectively.



#### 2.2. Macronutrients Accumulation in Response to Greenhouse Irradiance Conditions

As observed for the biometric parameters, total nitrogen, nitrate, and macronutrient contents were affected by the CV × GIC interaction (**Table 2**). Except for 'Romaine', the total nitrogen concentration in the leaves of 'Ballerina', 'Canasta' and 'Oak leaf' increased under the shading net by 13.1%, 9.7% and 14.7%, respectively. The same trend was observed for nitrate content which increased under shading net for all cultivars, except for 'Romaine'. Notably, the highest increase in nitrate was recorded in 'Ballerina' (+14.9%). For all cultivars, there was a significant increase in phosphorus content when the shading net was used. The same trend was observed for potassium in 'Romaine' and 'Oak leaf', which increased by 13.5% and 32.1%, respectively, while for 'Ballerina' and 'Canasta', GIC treatment did not affect potassium build-up. 'Oak leaf' showed a significant increase in sodium (23.8%) and magnesium (44.0%) in the shaded treatment compared to the unshaded one. In contrast, the unshaded treatment increased calcium content by 35.2%, 83.5%, 16.7% and 24.1% in 'Ballerina', 'Canasta', 'Oak leaf' and 'Romaine', respectively. On the other hand, sulfur content increased in all cultivars except for 'Oak leaf' in the unshaded treatment.

	Total N	NO <sub>3</sub>	Р	К	
Source of Variance	(%)	(mg kg <sup>-1</sup> FW)	(mg g <sup>-1</sup> DW)	(mg g <sup>-1</sup> DW)	
Cultivar (CV)					
'Ballerina'	3.32 ± 0.10 c	$2017 \pm 69 \text{ b}$	$4.24 \pm 0.17 \text{ c}$	41.25 ± 0.66 c	
'Canasta'	3.90 ± 0.10 a	2279 ± 69 a	$5.03 \pm 0.41$ a	39.17 ± 0.64 d	
'Oak leaf'	$3.65 \pm 0.12 \text{ b}$	2214 ± 50 a	$4.74 \pm 0.19 \text{ b}$	52.56 ± 3.31 a	
'Romaine'	3.40 ± 0.03 c	1744 ± 39 c	4.19 ± 0.12 c	45.45 ± 1.37 b	
	***	***	***	***	
Greenhouse Irradiance Conditions (GIC)					
Unshaded	$3.41 \pm 0.07$	$1963 \pm 56$	$4.07\pm0.07$	$41.71\pm0.86$	
Shaded	$3.72 \pm 0.09$	$2165 \pm 77$	$5.03 \pm 0.18$	$47.50 \pm 2.37$	
<i>t</i> -Test	*	*	***	*	
CV × GIC					
'Ballerina' × Unshaded	3.12 ± 0.06 d	1877 ± 24 c	3.87 ± 0.11 e	40.33 ± 1.15 def	
'Ballerina' × Shaded	3.53 ± 0.06 c	$2156 \pm 60 \text{ b}$	4.61 ± 0.07 c	42.16 ± 0.18 de	
'Canasta' × Unshaded	$3.72 \pm 0.10$ b	$2143 \pm 60 \text{ b}$	4.13 ± 0.01 de	38.64 ± 0.89 f	
'Canasta' × Shaded	$4.08 \pm 0.06$ a	2416 ± 39 a	5.93 ± 0.16 a	39.69 ± 1.00 ef	
'Oak leaf' × Unshaded	3.40 ± 0.05 c	2104 ± 13 b	$4.33 \pm 0.13$ cd	45.29 ± 1.27 c	
'Oak leaf' × Shaded	$3.90 \pm 0.05 \text{ ab}$	2324 ± 4 a	$5.14 \pm 0.07 \text{ b}$	59.84 ± 0.38 a	
'Romaine' × Unshaded	3.41 ± 0.06 c	1727 ± 82 d	3.94 ± 0.06 e	42.58 ± 0.52 d	
'Romaine' × Shaded	3.38 ± 0.02 c	1762 ± 22 cd	4.44 ± 0.07 c	$48.32 \pm 0.95$ b	
	**	*	***	***	

Table 2. Effect of cultivar and greenhouse irradiance conditions on total nitrogen and macronutrients accumulation in Lactuca sativa L.



Table 2. Cont.

	Ca	Ca Mg		Na	
Source of Variance	(mg g <sup>-1</sup> DW)	(mg g <sup>-1</sup> DW)	(mg g <sup>-1</sup> DW)	(mg g <sup>-1</sup> DW)	
Cultivar (CV)					
'Ballerina'	11.11 ± 0.77 a	$3.68 \pm 0.09 \text{ b}$	$1.48 \pm 0.13$ a	$2.11 \pm 0.07 \text{ b}$	
'Canasta'	8.27 ± 1.14 b	3.22 ± 0.19 c	1.63 ± 0.09 a	$1.40 \pm 0.12$ c	
'Oak leaf'	$10.40 \pm 0.42$ a	3.16 ± 0.28 c	$1.25 \pm 0.06 \text{ b}$	2.07 ± 0.11 b	
'Romaine'	10.80 ± 0.60 a ***	4.22 ± 0.16 a ***	1.08 ± 0.07 b ***	3.57 ± 0.14 a ***	
Greenhouse Irradiance Conditions (GIC)					
Unshaded	$11.66 \pm 0.27$	$3.40 \pm 0.17$	$1.50\pm0.09$	$2.30 \pm 0.21$	
Shaded	$8.63 \pm 0.53$	$3.74 \pm 0.18$	$1.21\pm0.07$	$2.27 \pm 0.28$	
<i>t</i> -Test	***	ns	*	ns	
$CV \times GIC$					
'Ballerina' × Unshaded	12.78 ± 0.28 a	$3.56 \pm 0.05 \text{ b}$	1.74 ± 0.11 a	$2.25 \pm 0.02 \text{ b}$	
'Ballerina' × Shaded	$9.45 \pm 0.38$ c	$3.79 \pm 0.15 \text{ b}$	$1.21 \pm 0.06 \text{ b}$	$1.98 \pm 0.05 \text{ bc}$	
'Canasta' × Unshaded	$10.70 \pm 0.26$ bc	$3.45 \pm 0.28 \text{ bc}$	$1.80 \pm 0.06$ a	$1.67 \pm 0.07 \text{ c}$	
'Canasta' × Shaded	5.83 ± 0.67 d	2.98 ± 0.20 cd	$1.45 \pm 0.11 \text{ b}$	$1.14 \pm 0.02 \text{ d}$	
'Oak leaf' × Unshaded	$11.20 \pm 0.28$ b	2.59 ± 0.17 d	$1.24 \pm 0.08$ b	$1.85 \pm 0.07 \text{ c}$	
'Oak leaf' × Shaded	9.60 ± 0.39 c	$3.73 \pm 0.17 \text{ b}$	$1.26 \pm 0.12 \text{ b}$	$2.29 \pm 0.09 \text{ b}$	
'Romaine' × Unshaded	11.96 ± 0.41 ab	3.98 ± 0.10 ab	$1.22 \pm 0.06 \text{ b}$	3.44 ± 0.19 a	
'Romaine' × Shaded	9.64 ± 0.51 c	$4.47 \pm 0.24$ a	$0.94 \pm 0.05 \text{ c}$	$3.69 \pm 0.20$ a	
	**	**	*	**	

Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters within columns indicate significant mean differences compared by DMRT (*p* = 0.05). ns, \*, \*\*, and \*\*\* denote nonsignificant or significant effects at *p* ≤ 0.05, 0.01, and 0.001, respectively.

# 2.3. SPAD Index, Chlorophyll Fluorescence Emission, and Leaf Mass Area (LMA) in Response to Greenhouse Irradiance Conditions

As reported in **Table 3**, the SPAD index measured at different days after transplant (8, 14 and 21 DAT) were affected by the CV × GIC interaction. At 8 DAT, all cultivars showed SPAD index reduction in the shaded treatment. At 14 DAT, the same trend was observed only for 'Canasta' and 'Oak leaf'. Moreover, at 21 DAT, the highest SPAD index values were recorded in 'Ballerina' (36.77) in shaded treatment and 'Oak leaf' (24.82) in unshaded treatment, whereas the other two cultivars showed no significant difference between shaded and unshaded treatments.

Fluorescence and leaf mass area (LMA) values showed significant differences only for the means values of both factors (CV and GIC) (**Table 3**). Regardless of the cultivar, shading net increased the  $F_v/F_m$  ratio by 10.7% and reduced the LMA by 19.6%. The latter parameter showed significant cultivar-dependent response ('Ballerina' > 'Romaine' > 'Canasta' > 'Oak leaf').



	SPAD			Fluorescence	LMA
Source of Variance	8 DAT	14 DAT	21 DAT	Fv/Fm ratio	(g DW m <sup>-2</sup> )
Cultivar (CV)					
'Ballerina'	27.73 ± 0.85 a	31.81 ± 0.28 b	35.51 ± 0.59 a	$0.79 \pm 0.02 \text{ ab}$	47.88 ± 1.06 a
'Canasta'	27.53 ± 1.09 a	31.97 ± 0.70 b	35.82 ± 0.14 a	0.79 ± 0.02 a	37.63 ± 1.76 c
'Oak leaf'	20.79 ± 0.73 b	22.85 ± 0.57 c	22.47 ± 1.07 c	$0.78\pm0.02~b$	26.19 ± 2.20 d
'Romaine'	28.23 ± 0.53 a	33.40 ± 0.38 a	34.05 ± 0.35 b	$0.80 \pm 0.02$ a	42.60 ± 1.90 b
	***	***	***	*	***
Greenhouse Irradiance Conditions (GIC)					
Unshaded	$27.77 \pm 0.97$	$30.88 \pm 1.21$	$32.22 \pm 1.31$	$0.75\pm0.00$	$42.01 \pm 2.32$
Shaded	$24.37 \pm 0.93$	$29.13 \pm 1.34$	$31.70 \pm 2.05$	$0.83 \pm 0.00$	$35.14 \pm 2.65$
<i>t</i> -Test	*	ns	ns	***	***
CV × GIC					
'Ballerina' × Unshaded	29.52 ± 0.52 a	32.34 ± 0.21 bc	34.25 ± 0.29 c	$0.74 \pm 0.00$	$49.88 \pm 1.22$
'Ballerina' × Shaded	25.94 ± 0.34 c	$31.28 \pm 0.28$ cd	36.77 ± 0.22 a	$0.84 \pm 0.01$	$45.87\pm0.34$
'Canasta' × Unshaded	$29.88 \pm 0.47$ a	$33.40 \pm 0.37$ ab	35.60 ± 0.18 b	$0.76 \pm 0.01$	$41.47\pm0.53$
'Canasta' × Shaded	25.18 ± 0.41 c	30.54 ± 0.54 d	36.05 ± 0.09 ab	$0.83\pm0.01$	$33.79 \pm 0.72$
'Oak leaf' × Unshaded	22.35 ± 0.45 d	24.04 ± 0.23 e	24.82 ± 0.38 d	$0.73 \pm 0.01$	$30.48 \pm 2.37$
'Oak leaf' × Shaded	19.23 ± 0.25 e	$21.66 \pm 0.43$ f	$20.12 \pm 0.20$ e	$0.82 \pm 0.00$	$21.91 \pm 0.36$
'Romaine' × Unshaded	29.33 ± 0.38 a	33.74 ± 0.69 a	34.22 ± 0.52 c	$0.76 \pm 0.01$	$46.21 \pm 2.14$
'Romaine' × Shaded	$27.12 \pm 0.17$ b	33.06 ± 0.38 ab	33.88 ± 0.55 c	$0.84 \pm 0.00$	$38.99 \pm 0.67$
	*	*	***	ns	ns

 Table 3. Effect of cultivar and greenhouse irradiance conditions on SPAD index, fluorescence (Fv/Fm ratio), and leaf mass area (LMA) in

 Lactuca sativa L.

Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters within columns indicate significant differences compared by DMRT (*p* = 0.05). ns, \*, and \*\*\* denote nonsignificant or significant effects at *p* ≤ 0.05 and 0.001, respectively

# 2.4. Instantaneous Water Use Efficiency and Morpho-anatomical Leaf Traits in Response to Greenhouse Irradiance Conditions

The CV × GIC interaction did not result in any variation in leaf gas exchanges (Aco<sub>2</sub>,  $g_s$  and E), which were affected exclusively by the mean cultivar effect (data not shown). In contrast, the instantaneous water use efficiency (WUEi) was affected by the CV × GIC interaction, where the difference was only significant in 'Canasta', +15.7% in the unshaded treatment in comparison to shaded (**Figure 1**).



**Figure 1.** Effect of Cultivar (CV) and Greenhouse Irradiance Conditions (GIC) on instantaneous water use efficiency (WUEi) in *Lactuca sativa* L. Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters indicate significant differences compared by DMRT (*p* = 0.05). ns and \*\*\* denote nonsignificant or significant effect at *p* ≤ 0.001, respectively.



**Figure 2** shows illustrative microscopy images of the abaxial side of lettuce leaves in the shaded and unshaded treatment for each cultivar. Morpho-anatomical leaf traits (*i.e.,* stomatal cell density, undulated epidermal cell density and stomatal index of abaxial and adaxial side of leaves) were affected by the interaction CV × GIC (**Figure 3**).

On the abaxial side of the leaves of 'Ballerina', 'Oak leaf' and 'Romaine', shaded treatment led to a significant reduction in stomatal and epidermal cell density while the opposite trend was observed in 'Canasta' (**Figure 3B, C**). In contrast, the stomatal index decreased in shaded treatment for 'Ballerina' and 'Oak leaf' by 20% and 6.7%, respectively, while no significant effect was found for this parameter in 'Canasta' and 'Romaine' (**Figure 3A**).

Regarding the leaves' adaxial side, except for 'Canasta', all cultivars showed the highest stomatal cell density in the unshaded treatment (Figure 4B). In addition, 'Ballerina' and 'Romaine' increased epidermal cell density when cultivated without shading nets (Figure 4C). The latter parameter increased in 'Canasta' by 9% in the shaded treatment, while no significant effect was observed in 'Oak leaf'. Shading net application (shaded treatment) resulted in the lowest stomatal index for all cultivars compared to the unshaded treatment (Figure 4A).



**Figure 2**. Illustrative microscopy images of the abaxial side of *Lactuca sativa* L. leaves in shaded and unshaded treatment (20×). Ballerina unshaded (**A**) and shaded (**B**); Canasta unshaded (**C**) and shaded (**D**); Oak leaf unshaded (**E**) and shaded (**F**); Romaine unshaded (**G**) and shaded (**H**).





**Figure 3**. Effect of Cultivar (CV) and Greenhouse Irradiance Conditions (GIC) on morpho-anatomical traits of abaxial side of *Lactuca sativa* L. Stomatal index (**A**), stomatal cell density (**B**), and epidermal cell density (**C**). Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters indicate significant differences compared by DMRT (*p* = 0.05). ns, \*\*, and \*\*\* denote nonsignificant or significant effect at *p* ≤ 0.01 and 0.001, respectively.



**Figure 4**. Effect of Cultivar (CV) and Greenhouse Irradiance Conditions (GIC) on morpho-anatomical traits of adaxial side of *Lactuca sativa* L. Stomatal index (**A**), stomatal cell density (**B**), and epidermal cell density (**C**). Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters within columns indicate significant mean differences compared by DMRT (*p* = 0.05). ns, \*\*, and \*\*\* denote nonsignificant or significant effects at *p* ≤ 0.01 and 0.001, respectively.



# 2.5. Leaf Pigments and Total Ascorbic Acid Accumulation in Response to Greenhouse Irradiance Conditions

As shown in **Table 4**, the CV × GIC interaction resulted in differences in chlorophyll and carotenoid content. Regardless of greenhouse irradiance conditions, chlorophyll *a*, *b*, total and carotenoid contents for 'Oak leaf' and 'Romaine' were unchanged. Chlorophyll *a* and total chlorophyll content in 'Ballerina' increased by 15.69% and 14.38%, respectively, under shaded conditions. In contrast, under the same irradiance conditions (shaded) chlorophyll *b* and total chlorophyll content in 'Canasta' decreased by 28.00% and 16.72%, respectively. For both cultivars ('Ballerina' and 'Canasta'), carotenoid content increased when grown under shaded conditions (**Table 4**).

Relative to total ascorbic acid, the cultivar 'Ballerina' recorded a 36.16% increase when grown under shading net whereas 'Canasta', 'Oak leaf', and 'Romaine' exhibited no significant difference between treatments (**Table 4**).

Source of Variance	TAA	Chlorophyll a	Chlorophyll b	Total Chlorophylls	Carotenoids
Cultivar (CV)					
'Ballerina'	$10.02 \pm 0.72$ b	15.06 ± 0.50 ab	9.17 ± 0.25 a	24.23 ± 0.75 a	$5.98 \pm 0.35$ b
'Canasta'	13.67 ± 0.47 a	15.61 ± 0.42 a	9.09 ± 0.69 a	24.70 ± 1.06 a	7.07 ± 0.25 a
'Oak leaf'	7.39 ± 0.71 c	13.97 ± 0.32 b	$8.09 \pm 0.28$ b	$22.06 \pm 0.58$ b	7.01 ± 0.16 a
'Romaine'	6.25 ± 0.52 c	11.74 ± 0.23 c	6.67 ± 0.20 c	18.41 ± 0.37 c	5.99 ± 0.14 b
	***	***	***	***	***
Greenhouse Irradiance Conditions (GIC)					
Unshaded	$9.25 \pm 0.66$	$14.03\pm0.52$	$8.61 \pm 0.42$	$22.64 \pm 0.93$	$6.07 \pm 0.19$
Shaded	$9.42 \pm 1.18$	$14.16\pm0.51$	$7.90 \pm 0.36$	$22.06 \pm 0.85$	$6.96 \pm 0.18$
<i>t</i> -Test	ns	ns	ns	ns	**
CV × GIC					
'Ballerina' × Unshaded	8.49 ± 0.28 c	13.96 ± 0.16 b	$8.64 \pm 0.19 \text{ bc}$	$22.60 \pm 0.34$ b	5.21 ± 0.10 e
'Ballerina' × Shaded	11.56 ± 0.39 b	$16.15 \pm 0.18$ a	$9.70 \pm 0.04$ ab	25.85 ± 0.22 a	$6.74 \pm 0.07 \text{ bc}$
'Canasta' × Unshaded	12.81 ± 0.29 ab	16.39 ± 0.48 a	10.57 ± 0.36 a	26.96 ± 0.69 a	$6.58 \pm 0.18$ c
'Canasta' × Shaded	14.52 ± 0.54 a	14.84 ± 0.23 ab	7.61 ± 0.19 cd	$22.45 \pm 0.28$ b	7.57 ± 0.22 a
'Oak leaf' × Unshaded	$8.65 \pm 0.41$ c	13.99 ± 0.60 b	$8.40 \pm 0.44$ bc	22.39 ± 1.03 b	$6.68 \pm 0.06 \text{ bc}$
'Oak leaf' × Shaded	6.13 ± 0.87 cd	13.94 ± 0.39 b	7.79 ± 0.31 cd	$21.73 \pm 0.70$ b	7.34 ± 0.14 ab
'Romaine' × Unshaded	7.04 ± 0.10 cd	11.78 ± 0.23 c	6.84 ± 0.21 d	18.61 ± 0.02 c	5.80 ± 0.18 de
'Romaine' × Shaded	$5.46 \pm 0.84$ d	11.71 ± 0.46 c	6.49 ± 0.35 d	18.21 ± 0.81 c	6.18 ± 0.16 cd
	***	**	***	***	*

**Table 4.** Effect of cultivar and greenhouse irradiance conditions on total ascorbic acid (TAA) and leaf pigments accumulation in Lactucasativa L. Data are expressed as mg  $g^{-1}$  DW

Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test (DMRT) for CV and by *t*-Test for GIC. Different letters within columns indicate significant mean differences compared by DMRT (*p* = 0.05). ns, \*, \*\*, and \*\*\* denote nonsignificant or significant effects at *p* ≤ 0.05, 0.01, and 0.001, respectively



# 3. Discussion

# 3.1. Leaf Morpho-Anatomical Adaptations and Productivity of Lettuce Under Excessive Irradiance and Heat Conditions

The present work was aimed to assess the morpho-physiological and anatomical responses of four lettuce cultivars grown during summer in a protected environment. Interestingly, varying response to the different greenhouse irradiance conditions (shaded and unshaded) was exhibited among cultivars. 'Canasta' showed the best production performance under unshaded conditions due to the activation of cultivar-specific adaptive mechanisms, whereas 'Ballerina', 'Oak leaf' and 'Romaine' were best suited to shaded treatment (**Table 1**).

Irradiance plays a critical role in plant growth, and light intensity above the saturation point leads to yield loss and quality degradation<sup>5</sup>. Confirming the results of previous studies on leafy vegetables<sup>44,47-50</sup>, the use of shading net increased fresh yield in 'Ballerina', 'Oak leaf' and 'Romaine' due to the lower temperature and solar radiation intensity, thus resulting in microclimate improvements for these lettuce cultivars (400–600  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) (**Supplementary Figure 1**)<sup>5,47</sup>.



**Supplementary Figure 1**. Photosynthetic Photon Flux Density (PPFD), average air temperature, and air relative humidity recorded during the growing season at the experimental site under shaded and unshaded conditions.



This result is attributable to a better hydration state of the shaded plants, reflected by the increase in leaf fresh weight, decrease in dry matter % and unaltered dry biomass (Table 1). The different microclimate conditions recorded between the shaded and unshaded sub-compartments of the greenhouse did not influence the water use efficiency (WUE) of these cultivars, revealing their inability to optimize water loss under high irradiance conditions (unshaded treatment) (Figure 1). Therefore, it was necessary for these cultivars to reduce leaf area to overcome the excessive evaporative demand of the unshaded condition, which accounted for the yield loss reported at the end of the growth cycle (Supplementary Figure 2). In contrast, 'Canasta' showed an opposite response compared to the other cultivars, improving the productive performance in unshaded treatment, probably thanks to the improved WUE, which is relevant to conserve water resources in the Mediterranean environment<sup>43</sup>. The different response to the unshaded condition between 'Canasta' and the other cultivars in terms of WUE seems to be in line with the adaptations that occurred in leaf stomatal traits. Except for 'Canasta', all cultivars under high light conditions (unshaded treatment) increased stomatal density both on the abaxial and the adaxial leaf side, confirming the findings reported in the literature<sup>36,37,42,51</sup>. Indeed, in response to changes in light intensity, mature leaves act as stress sensors and induce stomatal density changes in newly formed leaves (long-term response), allowing the plant to adapt to adverse environmental conditions38,51-53.



**Supplementary Figure 2.** Interaction between Cultivar (CV) and Greenhouse Irradiance Conditions (GIC) on plant growth trend quantified through growth index (cm<sup>3</sup> plant<sup>-1</sup>) at different days after transplant. Data are mean values  $\pm$  standard error, *n*=3. Mean comparisons were performed by Duncan's Multiple Range Test for CV and by *t*-Test for GIC. Different letters within columns indicate significant mean differences. \*\* and \*\*\* denote significant effects at *p* ≤ 0.01 and 0.001, respectively.

As previously suggested<sup>54</sup>, lower stomatal densities are beneficial for plant growth and productivity under unfavorable environmental conditions. The lettuce cultivar 'Canasta' reduced the abaxial stomatal density, thus improving WUE and yield (**Figure 3B**). Considering that epidermal cell density was significantly lower in the abaxial side of 'Canasta' leaves under unshaded condition (**Figure 3C**), which indicates a higher cell expansion compared to the shaded condition, it is possible that the lower stomatal density resulted from a "dilution effect" performed by epidermal cells on stomata<sup>55</sup>. This was further confirmed by the unchanged stomatal index, which indicates that stomatal initiation has not been affected by the two different irradiance conditions (**Figure 3A**). These results, combined with the increase in leaf number, dry biomass, and unchanged leaf area, suggest that 'Canasta', differently from other cultivars, is better adapted to high irradiance conditions (unshaded treatment). As suggested by Zhou et al.<sup>56</sup>, the light saturation point for some lettuce cultivars could be more than 800 µmol m<sup>-2</sup> s<sup>-1</sup>,



confirming once again the high genetic variability of this species. Regardless of cultivar, unshaded plants univocally increased LMA (**Table 3**) as an additional adaptive response to light stress<sup>28</sup>. LMA (ratio of dry biomass to leaf area) is a crucial ecological trait in plant adaptation to the environment<sup>28</sup>. Generally, under low light conditions, plants increase leaf area to intercept more light (lower LMA). On the other hand, under high irradiance conditions, plants increase dry biomass per unit leaf area (higher LMA) to improve photosynthetic capacity<sup>28</sup>. In our study, the worst performance recorded by 'Oak leaf' (lower leaf area, fresh yield, and dry biomass) was also associated with the constitutively lowest LMA, suggesting that this cultivar is not well adapted to excessive light and temperature as imposed in our experiment<sup>28</sup>.

# 3.2. Fluorescence, Total Ascorbic Acid, and Carotenoids Content of Lettuce Under Excessive Irradiance and Heat Conditions

In agreement with previous studies<sup>29</sup>, the Fv/Fm ratio varied as a function of light intensity, decreasing in plants exposed to high light intensity (unshaded treatment), probably due to photoinhibition (**Table 3**). However, independently of the cultivar, no changes in the main physiological and yield parameters were observed, suggesting that the decrease in Fv/Fm is not solely attributable to high light photoinhibition. In fact, as observed by Lichtenthaler and Burkart<sup>23</sup>, a minor reduction of the Fv/Fm ratio does not necessarily indicate the onset of photoinhibition processes, but it could be related to other mechanisms of chlorophyll fluorescence quenching, such as heat emission and the establishment of a pH gradient. It is noteworthy that unshaded treatment increased chlorophyll a, b, and total chlorophyll leaf content in all cultivars, probably to prevent the onset of harmful photoinhibition damage (**Table 4**). Our results are not in agreement with the reviewed literature suggesting that chlorophyll content in plant leaves decreases under high light conditions due to chloroplast formation inhibition<sup>5,23</sup>. This highlights how morpho-physiological and anatomical adaptive mechanisms have allowed plants to adapt efficiently to high irradiance stress (unshaded treatment).

In contrast with several studies on *Lactuca sativa* L.<sup>2,29</sup>, the total ascorbic acid content did not increase in 'Canasta', 'Romaine' and 'Oak leaf' in unshaded treatment. while it was significantly reduced in 'Ballerina'. This discordance could be due to a different genotypic response of cultivars to high irradiance intensity. As well as total ascorbic acid, carotenoids content did not show a univocal response in lettuce grown under unshaded conditions. Specifically, the decreased carotenoid contents in 'Ballerina' and 'Canasta' are in agreement with Gerganova et al.<sup>57</sup>. In contrast, 'Oak leaf' and 'Romaine' maintained the content of this crucial bioactive molecule unchanged, probably as a defense system to high irradiance intensity, because these pigments act as photoselective filters<sup>58</sup>. The current results are not in line with the findings of Rouphael et al.<sup>59</sup>, where 'Red Oak leaf' and 'Baby Romaine' demonstrated significantly lower carotenoid

concentrations when grown under lower irradiance in a controlled environment. The same authors reported that the variation of some carotenoids could be in part attributed to the head structure of the different cultivars.

## 3.3. Leaf Ions Accumulation of Lettuce Under Excessive Irradiance and Heat Conditions

The dynamics driving nitrate and mineral accumulation in vegetables are complex because of their influence by the environment × genotype interaction<sup>2</sup>. As expected, high irradiance intensity (unshaded treatment) reduced nitrate content in 'Ballerina', 'Canasta' and 'Oak leaf' because nitrate reductase is more efficient at high light intensity<sup>59</sup>. However, the lower nitrate content could also be attributed to the improved activity of other crucial enzymes such as glutamate synthetase and glutamine synthetase and the inhibition of asparagine synthetase involved in nitrate stabilization and transport processes<sup>60</sup>. In addition, the same cultivars showed a negative correlation between nitrate accumulation and leaf dry matter, as pointed out by Reinink et al.<sup>61</sup> in Lactuca sativa L. It is noteworthy that 'Romaine' did not change in nitrate content under shaded conditions, probably due to a lower constitutive concentration dependent on genotype<sup>2</sup>. Similarly, total nitrogen content showed the same nitrate trend, as supported by the literature review<sup>19</sup>. Like nitrogen, phosphorus is a key element for plant growth and productivity, playing a pivotal role in cellular processes, membrane maintenance and energy molecules biosynthesis<sup>62</sup>. Our results showed a univocal response of cultivars to phosphorus accumulation, decreasing at high light intensity (unshaded treatment). Since phosphorus is essential for maintaining the photosynthetic machinery  $(PSII)^{63}$ , its lower values, regardless of cultivar, would be justified by the lower  $F_v/F_m$ ratio obtained in unshaded plants.

In contrast, in all cultivars, leaf calcium content increased under unshaded conditions. This higher calcium accumulation could be due to plants' lower growth rate under high light conditions (unshaded treatment), except for 'Canasta', which grew faster (**Supplementary Figure 2**). Calcium is a poorly mobile element, and therefore higher growth speed might have reduced for 'Ballerina', 'Oak leaf' and 'Romaine' the translocation of calcium<sup>11</sup>. However, in addition to maintaining membrane and cell wall structure, calcium acts as a signal molecule, promoting the activation of specific adaptive mechanisms that help plants adapt to various abiotic stresses (*e.g.*, high light and high temperature)<sup>64</sup>. In our experiment, the higher concentration of calcium in unshaded plants could result from the fact that calcium had helped improve plants' resistance under light stress. Specifically, it is interesting to note that 'Canasta' showed the highest calcium accumulation (+85%) under unshaded condition, indicating a better adaptation to light stress and improved production performance (greater fresh yield and dry biomass)<sup>64</sup>.



## 4. Materials and Methods

#### 4.1. Experimental Design, Plant Material, and Growth Conditions

The experimental trial was conducted during the early summer season 2020 in a glass greenhouse located at the Department of Agriculture (DIA) of the University of Naples Federico II (Portici, Italy; 40°49' N, 14°15' E, 72 m a.s.l.). The experimental protocol included a white shading net with a 49% light screening factor (2681BL Prisma MDF; Arrigoni S.P.A, Uggiate Trevano, Como, Italy) and an unshaded treatment in factorial combination with four lettuce (Lactuca sativa L.) cultivars with different morphology of leaves (Figure 5). The glasshouse was split into independent compartments of 15 m length and 5 m width each, representing the shaded and unshaded treatments. Plants of each cultivar were randomized in each compartment. Each compartment contained 4 experimental units (one for each cultivar) including six plants (24 plants per compartment). Lettuce cultivars 'Ballerina' (Butterhead lettuce, Rijk Zwaan, De Lier, The Netherlands), 'Maravilla De Verano Canasta' hereafter 'Canasta' (Butterhead lettuce, Pagano Domenico and Figli, Scafati, Salerno, Italy), 'Opalix' hereafter 'Oak leaf' (Leaf lettuce; Enza Zaden, Enkhuizen, The Netherlands) and 'Integral' hereafter 'Romaine' (Cos lettuce; Syngenta, Basel, Switzerland) were transplanted on June 16 into pots (15 × 15 cm, 1.8 L) filled with a 2:1 substrate (v/v) of peat and perlite. The pots were covered with a fine layer of perlite to prevent water evaporation from the substrate. Plants were arranged in double rows with a distance of 35 and 25 cm inter- and intra-rows, respectively, for a density of 11.5 plants m<sup>-2</sup>. Seedlings were irrigated with nutrient solution (NS) provided by a drip irrigation system consisting of a 16 mm polyethylene main pipeline equipped with 2 L h<sup>-1</sup> drippers. The Hoagland NS had the following composition: 8.0 mM nitrate, 0.7 mM phosphorus, 2.5 mM potassium, 3.0 mM calcium, 1.0 mM sulfur, 0.7 mM magnesium, 1.0 mM ammonium, 1 mM sodium, 1 mM chlorine, 20  $\mu$ M iron, 9  $\mu$ M manganese, 0.3  $\mu$ M cupper, 1.6  $\mu$ M zinc, 20  $\mu$ M boron and 0.3  $\mu$ M molybdenum. The pH and EC of the NS were  $6.0 \pm 0.2$  and  $1.2 \pm 0.1$  dS m<sup>-1</sup>, respectively. Relative humidity and temperature were recorded continuously using WatchDog A150 data loggers (Spectrum Technologies Inc., Aurora, IL, USA; 3%/0.6 °C RH/Temp accuracy) at canopy level at different points of the greenhouse. Climate data were collected at a 30-min interval. Periodic measurements of Photosynthetic Photon Flux Density (PPFD) were recorded from 7:30 am to 6:30 pm using a handheld spectral radiometer (MSC15, Gigahertz-Optik, Turkenfeld, Germany). Average temperature, relative humidity and PPFD trend recorded during the growing season at the experimental site are shown in **Supplementary Figure 1**.



**Figure 5**. Illustrative picture of *Lactuca sativa* L. genotypes used in the experiment at transplant. 'Ballerina' (**A**), 'Canasta' (**B**), 'Oak leaf' (**C**), and 'Romaine' (**D**).

## 4.2. Growth, Yield, and Sampling

At 25 days after transplanting (DAT), the plants were harvested, weighed for fresh yield determination (g plant<sup>-1</sup>) and separated into leaves and stems. Leaf area was quantified by digital image analysis with ImageJ v1.52a software (U.S. National Institutes of Health, Bethesda, MD, USA). A subsample of leaf tissue was immediately stored at –20 °C for total ascorbic acid and pigment analysis. All harvested tissues were oven-dried at 70 °C until constant weight (~72 h) for dry biomass (g plant<sup>-1</sup>) and leaf dry matter (%) determination. Dried leaves were ground with an MF10.1 cutting-grinding head mill (IKA®, Staufen im Breisgau, Baden-Württemberg, Germany) and sieved with MF0.5 sieve (0.5 mm hole size; IKA®, Staufen im Breisgau, Baden-Württemberg, Germany) for total nitrogen and minerals determination.

#### 4.3. Plant Growth Index and Soil Plant Analysis Development (SPAD) Index

At 8, 14 and 21 DAT on three plants per plot, the plant growth trend was quantified through the growth index (cm<sup>3</sup> plant<sup>-1</sup>) according to the following equation:

$$GI = \pi \left(\frac{D}{2}\right)^2 Ht$$
(1)

where D is the width as the average of two perpendicular measurements and Ht is the plant height measured from the soil level to the plant highest point (**Supplementary Figure 2**).

Contextually, green index (SPAD) measurements were taken on young fully expanded leaves with a handheld Minolta Chlorophyll Meter SPAD-502 (Minolta Camera Co. Ltd., Osaka, Japan). A single average SPAD value for each replicate was obtained by measuring ten leaves per plot.



# 4.4. Leaf Gas Exchange and Maximum Quantum Efficiency of Photosystem II

On July 9 (24 DAT) between 11:00 am and 2:00 pm, leaf gas exchange measurements and fluorescence emission were performed on healthy fully expanded leaves of three plants per plot. CO<sub>2</sub> net assimilation rate (Aco<sub>2</sub>;  $\mu$ mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>), stomatal conductance (gs; mmol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>) and transpiration (E; mmol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>) were measured using a Li-6400 portable leaf gas exchange analyzer (LI-COR Biosciences, Lincoln, NE, USA). The measurements were performed at ambient CO<sub>2</sub> concentration and photosynthetic active radiation of 1000  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, as set in the leaf gas exchange analyzer chamber. Instantaneous water use efficiency (WUEi) was calculated as Aco<sub>2</sub>/E.

On the same date, on 10 min dark-adapted leaves, chlorophyll fluorescence measurements were taken with a portable fluorometer ( $F_v/F_m$  Meter, Opti-Sciences Inc., Hudson, NH, USA) on the same leaves used for leaf gas exchange measurements. According to Kitajima and Butler<sup>65</sup>, the maximum quantum efficiency of PSII ( $F_v/F_m$ ) was calculated as ( $F_m$ - $F_0$ )/ $F_m$ , where  $F_0$  was the ground signal induced by a blue LED internal light of 1–2 µmol photons m<sup>-2</sup> s<sup>-1</sup> and  $F_m$  was the maximal fluorescence level in the induced darkness by one second of saturating light pulse of 3000 µmol photons m<sup>-2</sup> s<sup>-1</sup>.

#### 4.5. Total Nitrogen and Minerals Determination

Total nitrogen content was determined according to the Kjeldahl method described by Bremner<sup>66</sup>. Briefly, one g of finely ground dry plant sample was mixed with 7 mL of 96% H<sub>2</sub>SO<sub>4</sub> and 10 mL of 30% (*w/w*) H<sub>2</sub>O<sub>2</sub>, then was mineralized in a DK 20 Heating Digester (Velp<sup>®</sup> Scientifica, Usmate Velate, Monza Brianza, Italy). The mineralized sample was distilled in a UDK 140 distiller (Velp<sup>®</sup> Scientifica, Usmate Velate, Monza Brianza, Italy) by adding 33% of NaOH. Ammonia was trapped in H<sub>3</sub>BO<sub>3</sub> by steam distillation and titrated with 0.1 N H<sub>2</sub>SO<sub>4</sub>. All reagents were purchased from Carlo Erba Reagents Srl (Milan, Italy).

Mineral content in lettuce leaves was determined through ion chromatography (ICS-3000, Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA) according to the method described by Rouphael et al.<sup>67</sup>. Briefly, 250 mg of ground dried leaves were extracted in 50 mL of ultrapure water (Arium<sup>®</sup> Advance EDI pure water system; Sartorius, Goettingen, Lower Saxony, Germany), incubated at 80 °C in a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) for 10 min, centrifuged at 6,000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, Mumbai, India) and then filtered by a syringe filter with a 0.45 µm pore size (Whatman International Ltd., Maidstone, Kent, UK). For anions (NO<sub>3</sub><sup>-</sup>, PO4<sup>3-</sup> and SO4<sup>2-</sup>) determination, an IonPac AG11-HC 4 × 50 mm guard column and an IonPac AS11-HC 4 × 250 mm analytical column were used. For cations (K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and Na<sup>+</sup>) determination, an IonPac CG12A 4 × 250 mm guard column and an IonPac CS12A 4 × 250 mm analytical column were used. All columns were purchased from Thermo Scientific<sup>™</sup> Dionex<sup>™</sup> (Sunnyvale, CA, USA).

Except nitrate expressed as mg kg<sup>-1</sup> of fresh weight (FW), all minerals were expressed as mg g<sup>-1</sup> of dry weight (DW). Total nitrogen was expressed as a percentage (%). Minerals and total nitrogen were analyzed in triplicate.

#### 4.6. Morpho-Anatomical Leaf Traits Determination

The LMA was evaluated on nine leaves per treatment as the ratio between leaf DW and leaf area. The number of epidermal cells and stomata were determined on the abaxial and adaxial sides of the same leaves used for leaf gas exchange and LMA measurements, as described by Cirillo et al.<sup>68</sup>. Briefly, leaf impressions were made using cyanoacrylate glue on a microscopy slide. Four images per impression were taken with an optical microscope (Leitz Laborlux 12 microscope, Leica, Wetzlar, Germany) at 20× magnification and were analyzed using ImageJ v1.52a software (U.S. National Institutes of Health, Bethesda, MD, USA) to determine the number of stomata (SN) and epidermal cells (ECN). The following equation was used to calculate the stomatal index expressed as a percentage:

Stomatal index = 
$$\frac{\text{SN}}{\text{SN}+\text{ECN}} \times 100$$
 (2)

Stomatal density and epidermal cell density were calculated as the ratio between the number of cells, and the area photographed for each image (0.241 mm<sup>2</sup>).

#### 4.7. Total Ascorbic Acid and Leaf Pigments Determination

Total ascorbic acid determination was performed as described by Kampfenkel et al.<sup>69</sup>. Four hundred milligrams of frozen sample were extracted with 0.8 mL of 6% trichloroacetic acid (TCA). The extract was incubated for 15 min at –20 °C, whereafter 1.2 mL of 6% TCA was added. The homogenate was centrifuged at 4000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, Mumbai, India). The absorbance was measured at 525 nm through a UV-Vis spectrophotometer ONDA V-10 Plus (Giorgio Bormac s.r.l, Carpi, Italy).

Pigments (chlorophyll *a*, *b* and carotenoids) of lettuce leaves were determined as described by Wellburn<sup>70</sup>. Briefly, 500 mg of fresh sample was extracted in ammonia acetone, pestled in a ceramic mortar, and centrifuged at 2,000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, Mumbai, India). Chlorophyll *a*, chlorophyll *b* and carotenoid contents were determined through a UV-Vis spectrophotometer ONDA V-10 Plus (Giorgio Bormac s.r.l, Carpi, Italy) with an absorbance of 647, 664 and 470 nm, respectively.

Chlorophyll a, chlorophyll b, total chlorophylls, carotenoids, and total ascorbic acid were expressed as mg 100 g<sup>-1</sup> DW as suggested by Kováčik<sup>71</sup>.



# 4.8. Statistics

The Shapiro–Wilk and Kolmororov–Smirnov procedures were performed to verify that the data had a normal distribution, and the Levene, O'Brien and Bartlet tests were conducted to verify the homogeneity of variances. Data were subjected to two-way analysis of variance (ANOVA) using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, Illinois, USA). The mean effect of CV and GIC was compared according to one-way analysis of variance and *t*-Test, respectively. Significant statistical differences were determined by Duncan's multiple-interval test for the CV × GIC interaction and the CV factor at the level of p < 0.05

# 5. Conclusions

High light intensity and high temperatures in Mediterranean regions pose a challenge to off-season lettuce production (spring-summer season), affecting growth and yield and resulting in quality losses. In this perspective, the combination of shading and genotypes tolerant to sub-optimal summer conditions is mandatory for off-season lettuce production. Our results showed that different genotypes revealed diverse responses to adverse microclimatic conditions. Among the four genotypes, 'Canasta' increased fresh yield and WUE in unshaded treatment (Figure 6). This was correlated to specific morpho-anatomical adaptations of this cultivar, such as reduction of stomatal and epidermal cells density. This highlights the better suitability of 'Canasta' to extreme summer conditions, thus presenting it as a promising genotype for off-season production and breeding programs. Nonetheless, the white shading net (49% screening) proved useful in creating an adequate microclimate during the early summer season, ensuring the growth of the more sensitive cultivars 'Ballerina', 'Oak leaf' and 'Romaine'. Even though more light has been shed on the adaptive aspects of lettuce grown at high light intensity, future research should be focused on the secondary metabolism response as an additional defense system for plants to adapt to sub-optimal growing conditions successfully.



**Figure 6.** Schematic graphical representation of the productive and adaptive response of *Lactuca sativa* L. cv 'Canasta' grown under shaded and unshaded conditions in early summer season.



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### Chapter 5

### Shading Affects Yield, Elemental Composition, and Antioxidants of Perennial Wall Rocket Crops Grown From Spring to Summer in Southern Italy

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Abstract: Shading nets have been increasingly drawing research interest, as they allow us to improve the environmental conditions for greenhouse-grown crops. The effects of two shading nets (50% and 79% shading degree), plus an unshaded control, on yield, mineral composition, and antioxidants of perennial wall rocket (Diplotaxis tenuifolia L.-D.C.) grown under tunnels in southern Italy were determined. The shading application resulted in a yield decrease, compared to the unshaded control, except for the highest production under 50% shading in July. The highest yield was recorded in the April-May and May-June and the lowest in July. Similar trends were recorded for plant dry weight, leaf number per rosette and mean weight, but the latter showed the highest value under 79% light extinction in July. The rocket leaves were brighter in the summer cycles than in the spring ones. Leaf nitrate was highest in spring and under 79% shading. Potassium, phosphorus, calcium, and magnesium showed the highest values in spring and in the unshaded control. The lipophilic antioxidant activity showed the highest values under the 79% shading net in the spring cropping seasons, whereas in July it did not significantly differ from 50% light extinction. The hydrophilic antioxidant activity always attained the highest values in the unshaded control. The unshaded leaves had the highest total phenol accumulation when grown in April–May and the lowest in July. The total ascorbic acid content was always highest in the unshaded control leaves compared to the shading treatments. Fifty percent crop shading is, therefore, an effective sustainable tool for increasing the yield of perennial wall rocket leaves in July, when the light intensity under the plastic tunnel exceeds the plant requirements, also resulting in a mineral composition that is not significantly different from that of the unshaded crops.

**Keywords:** *Diplotaxis tenuifolia* L. (D.C.); Sustainable management; Shading nets; Cropping seasons; Leaf production; Minerals; Phenols; Ascorbic acid; Antioxidant activity



### 1. Introduction

*Diplotaxis tenuifolia* L., commonly named perennial wall rocket, is spread worldwide, oriented both to the fresh salad market and the baby leaf industry<sup>1</sup>, appreciated by consumers for its bitter flavor, and rich in beneficial phytonutrients such as vitamin C, glucosinolates and flavonoids<sup>2</sup>.

Perennial wall rocket needs proper levels of light intensity and air temperature to encourage plant growth as well as leaf yield and phytochemical content<sup>3-5</sup>. In order to modulate the aforementioned environmental factors, different strategies can be adopted, among which are the use of shading nets, which can contribute to improving the plant growing conditions, thus leading to more vigorous plants, higher yields and better quality produce<sup>6,7</sup>.

Shading nets are characterized by different mechanical, physical and optical properties<sup>8</sup>, which allow for the modulation of light and temperature levels around crops. Interestingly, the shading nets can concurrently influence the quality and quantity of sunlight radiation, taking into account that some of them, such as the grey- or black-colored nets, do not alter the spectral composition of light, but just reduce its intensity<sup>9-1</sup>. The photoselective screens increase the diffused radiation, normalize excessive levels of light, temperature, humidity, and wind velocity<sup>12</sup> which allow for the greater efficiency of vegetable production in protected cultivation<sup>13</sup>. In addition, photoselective nets improve the quality of vegetables at harvest<sup>14</sup> and at the post-harvest stage<sup>15-17</sup>.

An experiment carried out by Jin et al.<sup>18</sup> showed the effects of light conditions on wild and salad rocket: compared to high light intensity (80–120  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>), under low light intensity (20–30  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) the plants had larger leaf area, a 40% lower antioxidant content, and reduced levels of glucosinolate, quercetin, isorhamnethin, kaempferol, and cyanidin. Francke<sup>19</sup> reported, in *Diplotaxis tenuifolia* and *Eruca sativa*, a higher N and K accumulation under reduced light conditions, whereas P and Ca were higher in the unshaded control.

Recent research revealed that lettuce grown without shading had a lower content of flavonoids if compared with shade net treatments<sup>20</sup>. Otherwise, photoselective nets did not affect the glucosinolate content in turnips (*Brassica rapa* subsp. *rapa* L.), a parameter probably related to genotype and planting date<sup>21</sup>.

Ombodi et al.<sup>22</sup> reported that shading nets led to 15–40% light extinction and caused significant losses in sweet pepper hybrid yields under a plastic tunnel: the production of Karpia F<sub>1</sub> decreased from 8.5 to 6.0 kg m<sup>-2</sup>, that of Karpex F<sub>1</sub> decreased from 7 to 6 kg m<sup>-2</sup>. Other authors showed that organic pepper benefited from the application of shading nets with light extinctions of 25% and 35%, compared to the unshaded control<sup>23</sup>.

Rocket is one of the few C<sub>3</sub>–C<sub>4</sub> *Brassicaceae* species<sup>24</sup>, and better assimilates CO<sub>2</sub> at irradiance levels of 600–900  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, *i.e.*, about 30–40% of the sunlight radiation commonly recorded in the late spring–summer growing season in Mediterranean

### 101 Chapter 5

areas<sup>20</sup>. Indeed, when the radiance energy exceeds the optimal genotype threshold for net photosynthetic assimilation, photo-inhibition is activated along with stress reactions, such as stomatal closure, cell division, leaf expansion and reproductive development<sup>25</sup>. Contrarily, the low irradiation level elicits changes in plant morphological and chemical features, leading to broader and thinner leaves, a less dense canopy and phytochemical content modulation. In the latter respect, the light and temperature inside the greenhouse should encourage the leaves of perennial wall rocket to achieve an appropriate shape, with petioles not excessively long in comparison with the blades, and an appreciable concentration of phytochemicals<sup>26,27</sup>. The aforementioned environmental parameters also affect the crop performance depending on the cropping season, which, in a previous work, influenced the dry matter and macronutrient content as well as the vitamin C, phenols and total glucosinolate concentration in leaves of soilless-grown rocket<sup>26</sup>.

The use of shading nets is one of the strategies aimed at protecting plants from exceeding values of radiation and temperature during the spring–summer crop cycles of *Diplotaxis tenuifolia* L. In the latter respect, the purpose of this research was to investigate the effect of two shading nets characterized by different light extinction levels (50% and 79%, plus an unshaded control) on the yield, mineral composition and antioxidants of perennial wall rocket oriented towards the fresh market, grown in a greenhouse in four different spring–summer cycles in southern Italy.



### 2. Results and Discussion

### 2.1. Meteorological Parameters

The trends of mean Photosynthetic active radiation (PAR), temperature and humidity in the greenhouse are shown in **Figure1a–c**. The PAR generally exceeded the 400  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> level and sometimes even 500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> (with 527  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> as a maximum value) in the June–July and July cropping seasons in the unshaded control, which was 2.84-fold and 4.32-fold higher on average compared to 50% and 79% shading, respectively. In April–May and May–June crop cycles, the mean PAR ranged between 350 and 410  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> in the unshaded control, whereas it showed a 64.6% and 76.8% decrease corresponding to the 50% and 79% light extinction rates, respectively.

### 103 Chapter 5



**Figure 1**. Seasonal trends corresponding to 50% and 79% shading nets and unshaded control under tunnels of: (**a**) photosynthetic active radiation (PAR); (**b**) temperature; (**c**) relative humidity. The dates reported on the x axis correspond to the end of each cropping season in the unshaded control, from the first (13 May) to the fourth (31 July).



The mean daily temperature increased from the transplant to the beginning of the last cropping season in July: in the unshaded plots, it was 5.93% and 15.01% higher compared to 50% and 79% shading respectively. The reduction in PAR values obtained in the current experiment are consistent with previous studies<sup>28-30</sup>.

Unlike the trends of PAR and temperature, humidity values inside the greenhouse remained steady and did not vary between the crops under different shading nets.

### 2.2. Plant Growth and Yield

The main effects of the two experimental factors applied in the present research are shown in **Table1**. The crop cycle was longest in May–June and under 79% shading, and shortest in July; the yield variables examined generally showed a decreasing trend both from the first cropping season to the fourth, and from the unshaded control to 79% shading.

	Cycle Length	Yield		Mean Weight	Total Dry Matter
Experimental Treatment	(days from transplant)	(t ha-1)	Number of leaves per rosette	(g)	(g m <sup>-2</sup> )
Cropping season					
April-May	$27.0 \pm 3.6 \text{ b}$	10.43 ± 1.61 a	$101.2 \pm 16.2$ a	$0.72 \pm 0.06 \text{ b}$	78.3 ± 13.3 a
May-June	31.7 ± 3.1 a	9.99 ± 3.54 a	79.9 ± 17.8 b	$0.86 \pm 0.15$ a	85.3 ± 34.2 a
June-July	$25.0 \pm 2.6 \text{ bc}$	7.65 ± 1.65 b	88.5 ± 16.8 b	$0.60 \pm 0.10$ c	61.6 ± 16.6 b
July	24.0 ± 3.6 c	4.29 ± 1.19 c	60.6 ± 15.4 c	$0.50 \pm 0.08 \text{ d}$	46.7 ± 7.8 c
Shading degree (%)					
Unshaded control	24.3 ± 3.9 b	12.16 ± 3.41 a	106.1 ± 14.3 a	$0.80 \pm 0.24$ a	104.1 ± 29.0 a
50	26.0 ± 3.6 b	8.42 ± 1.21 b	$84.0 \pm 7.5 \text{ b}$	0.71 ± 0.11 b	61.6 ± 9.7 b
79	30.5 ± 3.3 a	6.31 ± 1.49 c	72.7 ± 17.5 c	0.62 ± 0.11 c	42.2 ± 11.4 c

**Table 1**. Rocket yield and dry matter content as affected by cropping season and shading degree.

Within each column, means followed by different letters are significantly different according to the Duncan test at  $p \le 0.05$ 





The interaction between the cropping season and the shading degree was significant on the yield parameters and plant dry matter (**Figures 2a-d**).

**Figure 2**. Interaction between cropping season and shading degree on: (a) leaf yield; (b) leaf number per rosette; (c) mean leaf weight; (d) total dry weight. Values followed by different letters are significantly different according to the Duncan test at  $p \le 0.05$ . Lowercase letters refer to the comparison between the shading treatments within each cropping season, and capital letters refer to the comparison between cropping seasons within each shading treatment.

The shading application resulted in a yield decrease, compared to the unshaded control, over the first three cycles from April to the end of June, whereas the crops grown in July showed a production increase under 50% shading (**Figure 2a**); 79% light extinction always caused the worst performance, but in April–May it did not significantly differ from the May–June cropping season. The highest yield of the unshaded control was recorded in the April–May and May–June crop cycles, and the lowest in July; 50% shading led to the highest yield production in April–May and May–

June crop cycle, followed by June–July and July crop cycle respectively; the yield corresponding to 79% light extinction was highest in April–May and lowest in July.

The number of leaves per rosette (**Figure 2b**) decreased both with increasing the shading and when delaying the crop season, except for the July cycle, when the leaf number did not significantly change from the unshaded control up to 50% shading. In the unshaded control, the highest leaf number was recorded in the April–May crops, and the lowest in the July ones, with no differences between the intermediate cycles. Under 79% shading, the cycles April–May and June–July showed the highest number of leaves. No differences arose between the cropping seasons at 50% shading. The highest differences between 50% and 79% shading were recorded in the July cycle.

The mean leaf weight (**Figure 2c**) did not show significant differences between the shading treatments and the unshaded control in the April–May cycle; it was highest in the unshaded control in May–June and June–July; in the July cycle, shading led to higher mean leaf weight compared to the control. Both in the unshaded control and under the shading treatments, the leaves harvested in May–June attained the highest mean weight, though the latter was not significantly different from that recorded in April-June regarding 79% light extinction.

The highest dry weight content (**Figure 2d**) was recorded in the unshaded rocket leaves in all the cropping seasons, except for July when it was not significantly different from that associated to 50% shading. The control resulted in the highest dry weight content in May–June, 50% shading in April–May and May–June, and 79% light extinction in the April–May cycle.

In the present investigation, increasing shading caused a decreased yield, except for the crop cycle in July, characterized by the highest light intensity (**Figure 1a**), which was better affected by 50% shading compared to the unshaded control. Presumably, the light intensity recorded in July exceeded the perennial wall rocket light requirements and, therefore, the crops benefited from a 50% light reduction. Indeed, the excessive irradiation, over 600–900 µmol m<sup>-2</sup> s<sup>-1</sup>, elicits a leaf temperature increase in C<sub>3</sub> plants, leading to a photoinhibition effect<sup>31</sup> as well as imbalances in rubisco activity<sup>32</sup>, electron transport<sup>33</sup>, and stomatal and mesophyll conductance<sup>34</sup>. In this respect, Santamaria et al.<sup>35</sup> recorded a 50% increase in dry matter in rocket plants with a light intensity reduction from 20 to 10 klux.

As reported by Padulosi and Pignone<sup>36</sup>, rocket is a cool-season crop that shows a shorter cycle with an increase in day length and temperature, consistently with what was recorded in our study. However, the 79% shading level applied in the present research always caused a dramatic reduction in the sunlight radiation entering the greenhouse, whose intensity proved to be under the optimal light needs of *D. tenuifolia* plants. In a previous study, based on a comparison among different leafy vegetable species, Wolff and Coltman<sup>37</sup> highlighted that the crops positively benefited from



shading up to 30%–47%, with lettuce showing a 36% yield increase and head and Chinese cabbage a 23% and 21% augmentation, respectively. Kavga et al.<sup>38</sup> reported that, in a comparison between lettuce and rocket crops grown under a 25% shading net, only the rocket crop showed a yield loss up to 50% compared to the unshaded control. In contrast, Ilić et al.<sup>30</sup> recorded an increased yield of *Lactuca sativa* L. grown under different 50% shading nets; specifically, the leaf area index, the total fresh weight, the leaf number per plant and stem length increased under shading in comparison with the unshaded control, suggesting the existence of a light-dependent mechanism by which the plants regulate the leaf size.

In another study carried out by Caruso et al.<sup>23</sup> on organic pepper, 25% and 35% light extinction resulted in yield increases of 13.5% and 8.1%, respectively, compared to the unshaded control, as a consequence of the 19.4% and 11.3% enhancements of the fruit numbers per plant.

### 2.3. Leaf Color Parameters and Chemical Composition

In the present study the rocket leaves were brighter in the summer cycles than in the spring ones, as reflected by the higher L\* values shown in **Table 2**. No significant differences arose between the shading nets and the control.

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Treatment	L*	a*	b*	
Cropping season				
April-May	$41.1 \pm 1.0 \text{ b}$	$-14.5 \pm 0.9$	$23.1 \pm 1.3$	
May-June	41.1 ± 1.5 b	$-15.3 \pm 1.2$	$21.7 \pm 1.4$	
June-July	$42.9 \pm 0.5$ a	$-14.6 \pm 0.5$	$22.6 \pm 2.1$	
July	$43.4 \pm 0.5$ a	$-15.1 \pm 0.9$	$21.8 \pm 1.1$	
		ns	ns	
Shading degree (%)				
Unshaded control	$42.6 \pm 1.6$	$-14.8 \pm 1.1$	$22.0 \pm 2.4$	
50	$42.2 \pm 2.3$	$-14.6 \pm 1.0$	$22.6 \pm 1.3$	
79	$41.6 \pm 1.7$	$-15.2 \pm 1.0$	$22.7 \pm 1.5$	
	ns	ns	ns	

Table 2. Colorimetric parameters as affected by cropping season and shading degree.

L\*: lightness, from black to white (0 to 100); a\* and b\*: chroma components (-60 to + 60) from green to red and from blue to yellow, respectively. Within each column, ns: no statistically significant difference; means followed by different letters are significantly different according to the Duncan test at  $p \le 0.05$ .

109 Chapter 5

The a\* and b\* colour components were not significantly affected by either the cropping season or the shading net. Our findings are in agreement with the results achieved by Ilić et al.<sup>30</sup> in a previous study aimed to compare the performance of photoselective shading nets on lettuce visual quality attributes in a summer cycle.

As no significant differences arose between the April-May and May-June cropping seasons regarding the macroelement contents as well as the antioxidant compounds and activity, only the results relevant to the April-May crops have been reported in the **Tables 3**.



	us 5 K5 (	ary weight	, exception in	os that is expres	see us g kg	or mean weigh		
Treatment	NO <sub>3</sub>	Ν	К	Р	S	Ca	Mg	Na
Cropping season								
April-May	6863 ± 488 a	$4.50\pm0.36$	47.6 ± 6.3 b	3.02 ± 0.31 a	$7.37\pm0.36$	29.3 ± 1.4 a	3.56 ± 0.15 a	$3.15\pm0.27$
June-July	6615 ± 303 ab	$4.56\pm0.41$	$50.3 \pm 4.8 \text{ ab}$	$2.84 \pm 0.25$ ab	$7.25\pm0.33$	27.8 ± 1.5 ab	$3.37 \pm 0.12$ ab	$3.24 \pm 0.11$
July	6404 ± 305 b	$4.65\pm0.43$	53.4 ± 3.1 a	2.71 ± 0.23 b	$7.18\pm0.28$	$25.5 \pm 3.0$ b	$3.14 \pm 0.32 \text{ b}$	$3.30\pm0.18$
		ns			ns			ns
Shading degree (%)								
Unshaded control	6218 ± 182 b	$4.70\pm0.35$	55.5 ± 1.6 a	3.08 ± 0.14 a	$7.34 \pm 0.33$	29.2 ± 1.9 a	3.50 ± 0.20 a	$3.30\pm0.19$
50	6594 ± 158 b	$4.58\pm0.44$	$51.6 \pm 2.0$ b	$2.85 \pm 0.11$ ab	$7.28\pm0.23$	27.7 ± 1.0 ab	$3.39 \pm 0.10$ ab	$3.22 \pm 0.11$
79	7070 ± 336 a	$4.44\pm0.20$	$44.0 \pm 4.7 \text{ c}$	$2.65 \pm 0.07 \text{ b}$	$7.20\pm0.36$	25.5 ± 3.9 b	$3.20 \pm 0.42 \text{ b}$	$3.18\pm0.42$
		ns			ns			ns

**Table 3.** Macroelement content in perennial wall rocket leaves as affected by cropping season and shading degree. Data are expressedas g kg<sup>-1</sup> of dry weight, except for NO3 that is expressed as g kg<sup>-1</sup> of fresh weight.

ns: not statistically significant. Values followed by different letters are statistically different according to Duncan test at  $p \le 0.05$ .

### 111 **Chapter 5**

Among the macronutrients analyzed (**Table 3**), total nitrogen, sulfur and sodium in perennial wall rocket leaves were not affected by either the cropping season or the shading degree. On the other hand, nitrate showed decreasing values from spring to summer seasons, but increasing concentrations with crop shading enhancements. The potassium content was higher in the leaves grown in summer compared to the spring ones and was increasingly inhibited from the unshaded control to 79% light extinction. Phosphorus, calcium, and magnesium showed the highest values in the spring cropping season and without shading.

In agreement with the study of Tindall et al.<sup>39</sup>, who found that 25 °C was the suitable temperature for the optimal mineral uptake, in the present investigation, the moderate temperatures recorded in April and May led to higher mineral contents compared to summer cropping seasons, except for K. Gregory<sup>40</sup> also reported the increase in NO<sub>3</sub>, Ca, P and Mg at temperatures ranging between 20 and 30 °C, and, in this respect, the plant mineral uptake is affected by the soil temperature, which elicits changes in the root physiology and architecture. Moreover, the air temperature influences the growing relationships between shoots and roots and, accordingly, the photosynthate translocation pattern<sup>34</sup>.

In contrast to our results, which are relevant to perennial wall rocket leaves, Stagnari et al.<sup>41</sup> found, in a greenhouse-grown lettuce, rising trends in mineral contents from the unshaded control to 85% PAR reduction, by 1.18-fold for Ca, 1.26 for P, 1.67 for Mg, and 2.89 for K. Díaz-Pérez<sup>42</sup> recorded an increasing content of N, P, K and Na with a shading degree increase from 0 to 80% in bell peppers, but the other elements' content decreased. Zhao and Oosterhuis<sup>43</sup> also showed the benefit of shading on leaf mineral content: cotton plants grown under 63% light reduction accumulated much more minerals in the leaves, especially N, P, and S, in comparison with the unshaded control. In a study carried out by Chen et al.<sup>44</sup>, the fruit content of N, P, K, and Mg increased under 60% shading, whereas the Ca level decreased. In a further study, Stagnari et al.<sup>45</sup> reported contrasting effects of a green shading net on red turnips, resulting in a lower dry weight of roots and leaves, but an increase in the concentration of soluble and structural carbohydrates, as well as of K, Mg and Zn.

*D. tenuifolia* has a physiological tendency to accumulate nitrate, which is a potential health risk to consumers at high concentrations<sup>46</sup>, and therefore related recommendations are reported in the European Union Regulation N. 1258/2011. However, Steinmetz and Potter<sup>47</sup> reported that high antioxidant contents can inhibit the formation of carcinogenic compounds. In agreement with the results of the present research, the accumulation of NO<sub>3</sub> in plant tissues was enhanced under reduced light intensity in previous investigations on rocket<sup>46</sup> and spinach<sup>48</sup>. In fact, nitrate reduction to nitrite and the ultimate conversion into organic compounds is achieved by the nitrate



reductase enzyme complex, whose synthesis, induction and reducing power through photosynthesis is positively correlated with sunlight intensity<sup>49</sup>.

### 2.4. Antioxidant Compounds and Activity

In this study, the interactions between the cropping season and the shading degree were significant both on antioxidant activity and compounds (**Table 4**). Indeed, lipophilic antioxidant activity (LAA) showed the highest values under the 79% shading net in the first and second cropping seasons, whereas in the July cycle it was not significantly different when compared to 50% light extinction; the unshaded leaves had the lowest LAA, except for the first cropping season, which did not differ from the second one.

The hydrophilic antioxidant activity (HAA) always attained the highest values in the unshaded control, but in April–May it was not significantly different from that recorded in June–July; the lowest HAA was detected in the last cropping season.

The results of the present research are consistent with those obtained by Colonna et al.<sup>50</sup>, who found that LAA was higher under low PAR (200–400  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) compared to high PAR (800–1200  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) conditions. Contrastingly, Jin et al.<sup>18</sup> reported that the full light conditions caused the increase in rocket antioxidant activity compared to the lower light intensity. Indeed, light intensity is stressful to plants when its values are either above or below the optimal threshold related to the specific crop system requirements. In this respect, in the present investigation, LAA showed the highest levels both in July when the sunlight exceeded the perennial wall rocket demands and under the 79% shading, which caused an excessive PAR reduction inside the tunnels.

The total phenols did not show unequivocal trends as a function of the cropping season or the shading degree. Indeed, the unshaded leaves had the highest accumulation of these antioxidants when grown in April–May and the lowest in the July cropping season; controversial trends in the four cropping seasons were related to the two shading degrees.

The total ascorbic acid (TAA) content was always highest in the unshaded control; within each of the shading treatments, the highest values were recorded in July in the unshaded leaves, in April–May in the 50% shaded ones and in June–July in those grown under 79% light extinction.

Many factors affect the antioxidant content, such as air temperature, harvesting time<sup>51</sup> and light. In the latter respect, Luthria et al.<sup>52</sup> found that the UV radiation range between 290 and 400 nm better influences the phenolic acid concentration in tomato fruits than the 380–400 nm range. Indeed, optimal light conditions and UV, in particular, are reportedly essential for optimizing the concentration of phenolic compounds, partly because phenolic compounds have a strong capacity for UV radiation absorption<sup>53</sup>.

In a study carried out by Cano and Arnao<sup>54</sup>, the lipophilic antioxidant activity of lettuce leaves was directly related to the efficiency of photosynthesis; in fact, the youngest leaves showed a lower LAA value. In the present study, the LAA was higher in the summer crop leaves, which were subjected to the most intensive sunlight radiation and in leaves under the 79% shade level, maybe due to the fact that the photosynthetic activity was distributed among fewer leaves.

Polyphenols constitute a heterogeneous natural substance, whose accumulation in plants is reportedly genotype dependent<sup>55</sup>, noted for their beneficial effects on human health. Recent studies have shown the positive effect of phenolic compounds in constraining carcinogenic cell development<sup>56-57</sup>. Furthermore, their strong antioxidant capacity reduces the side effects associated with various diseases of the nervous system<sup>58-60</sup>. The phenol content recorded in the present experiment under the shading net treatments is comparable with the results of Ilić and Fallik<sup>20</sup>. During a summer cycle of *Lactuca sativa* L., photoselective shading nets did not improve the total phenol content compared to the unshaded control. Oh et al.<sup>61</sup> reported the negative effect of the 40 to 50% PAR reduction on the accumulation of phenolic compounds, in contrast with a similar experiment in which the plants were subjected to a high light intensity<sup>62,63</sup>. In previous research carried out by Wang et al.<sup>64</sup>, the total phenolic and flavonoid contents were significantly affected by the shading application.

Ascorbic acid is a major vitamin and antioxidant in vegetables<sup>65</sup>, and in the present investigation its content was higher in the control than in the shaded rocket leaves, confirming the positive correlation of this compound with the light intensity, consistently with the reports of Kosma et al.<sup>66</sup>. The latter authors reported that the 27% shading in greenhouse-grown lettuce elicited the highest leaf ascorbic acid content, because it presumably enhanced the plant's photosynthetic performance compared to 53% and 74% shading. Indeed, the synthesis of ascorbic acid is encouraged under the optimal light conditions relevant to the specific crop and growing season, as previously reported in tomatoes<sup>67</sup>, and in *Arabidopsis thaliana*, where a positive correlation between the ascorbic acid accumulation and the light intensity was found in plants grown under 50-µmol photons m<sup>-2</sup> s<sup>-1</sup> and 250-µmol photons m<sup>-2</sup> s<sup>-1</sup> light intensity<sup>68</sup>.

In a previous study<sup>23</sup>, the ascorbic acid content in pepper fruits was positively correlated with the shading degree, increasing by 31.1% from the unshaded control to 35% shading treatments



	ΤΑΑ		Total phanals	TAA
Treatment	LAA	ПАА	Total phenois	IAA
	(mmol Trolox 100 $g^{-1}$ d.w.)	(mmol AA 100 g <sup>-1</sup> d.w.)	(mg gallic acid 100 g <sup>-1</sup> d.w.)	(mg 100 g <sup>-1</sup> f.w.)
Cropping season (CS)				
April-May	14.76 ± 0.72 c	$7.28 \pm 0.89$ a	$2.45 \pm 0.14$	79.60 ± 12.00 a
June-July	$18.42 \pm 1.38 \text{ b}$	$6.32 \pm 0.75$ b	$2.52 \pm 0.13$	65.97 ± 6.72 b
July	20.14 ± 0.75 a	$6.71 \pm 0.60 \text{ b}$	$2.30 \pm 0.11$	82.08 ± 16.00 a
-	*	*	ns	*
Shading degree (SD, %)				
Unshaded control	$14.84 \pm 0.76$ c	8.83 ± 0.19 a	2.42 ± 0.15 ab	119.70 ± 8.30 a
50	17.87 ± 1.19 b	$7.41 \pm 0.48 \text{ b}$	$2.23 \pm 0.07 \text{ b}$	57.12 ± 4.55 b
79	$20.61 \pm 0.88$ a	4.08 ± 0.13 c	2.62 ± 0.12 a	50.81 ± 3.37 b
SD x CS				
Control × April-May	13.32 ± 0.33 c	$8.98 \pm 0.54$ a	2.85 ± 0.11 ab	123.90 ± 2.77 b
Control × June-July	13.71 ± 1.06 c	9.02 ± 0.21 a	2.53 ± 0.14 abc	90.12 ± 4.23 c
Control × July	17.51 ± 0.53 b	$8.47 \pm 0.11$ a	1.88 ± 0.02 d	145.10 ± 5.56 a
50 × April-May	13.61 ± 0.72 c	9.06 ± 0.22 a	$2.12 \pm 0.14$ cd	72.13 ± 5.72 d
50 × June-July	18.66 ± 0.57 b	$6.03 \pm 0.41$ c	$2.12 \pm 0.10$ cd	47.21 ± 3.97 ef
50 × July	21.33 ± 0.96 a	$7.13 \pm 0.40 \text{ b}$	$2.44 \pm 0.02 \text{ bc}$	52.03 ± 4.99 ef
79 × April-May	17.36 ± 0.65 b	$3.81 \pm 0.02 \text{ d}$	2.38 ± 0.26 c	42.77 ± 3.61 f
79 × June-July	$22.90 \pm 0.48$ a	3.93 ± 0.16 d	2.90 ± 0.16 a	60.60 ± 5.11 de
79 × July	$21.57 \pm 0.60$ a	4.51 + 0.22 d	$2.58 \pm 0.13$ abc	49.06 + 3.92 ef

**Table 4**. Effect of the interaction between cropping season and shading degree on antioxidant compounds and activity of perennial wall rocket leaves.

LAA: lipophilic antioxidant activity; HAA: hydrophilic antioxidant activity; TAA: total ascorbic acid; ns: no statistically significant difference; \* statistically significant at  $p \le 0.05$ . Within each column, means followed by different letters are significantly different according to the Duncan test at  $p \le 0.05$ . Mean values ± standard deviations have been reported.

### 3. Materials and Methods

### 3.1. Growing Conditions and Experimental Protocol

Research on the perennial wall rocket (*Diplotaxis tenuifolia* (L.) D.C.) cultivar Nature was carried out in the experimental fields of the Department of Agricultural Sciences of Naples University Federico II in Portici (Naples, southern Italy, 40°49' N, 14°15' E, 72 m a.s.l.) in 2019. The trial was conducted under three tunnels, each of them 5.0-m wide, 30-m long, 2.0- and 3.5-m tall at wall and roof, respectively, covered with a thermal polyethylene film, in a sandy loam soil (76% sand, 17% silt, 7% clay), with a pH of 6.9 and an electrical conductivity of 512 mS cm<sup>-1</sup>, from 19 April to 31 July.

In each crop cycle, continuous measurements of PAR, air temperature and relative humidity were performed, both under shading nets and in an unshaded control. In addition, periodic measurements of PAR were taken four times during the daily light period between sunrise and sunset in order to check the net shading degree.

The rocket rosettes were arranged in four rows per bed, mulched with a biodegradable film, with a 20-cm spacing both along and between the rows, with a density of 14.3 rosettes per m<sup>2</sup>.

The experimental protocol was based on the comparison between two shading nets (Frangisole 50, 50% light extinction, and Frangisole Iron 90, 79% light extinction; both nets were provided by Arrigoni S.p.A, Uggiate Trevano, Italy) plus an unshaded control, each of them corresponding to a tunnel, in factorial combination with four crop cycles (April–May; May–June; June–July; July). A randomized complete block design was used with three replications, and the experimental unit had a 6.4-m<sup>2</sup> surface area.

The first crop cycle began on 19 April and ended on 13 May, 15 May and 20 May in the unshaded control, 50% and 79% shading treatments, respectively. The second crop cycle ended on 11 June in the control, on 13 and 17 June in the plots under 50% and 79% shading, respectively. The third cycle lasted until July 3 in correspondence of the unshaded control, 4 and 8 July corresponding to 50% and 79% shading respectively. The fourth crop cycle ended on 24, 26 and 31 July in the control, 50% and 79% shaded treatments, respectively.

The perennial wall rocket crops were managed through the following sustainable farming practices: organic fertilization prior to transplant with N,  $P_2O_5$  and  $K_2O$  (at a rate of 38, 10 and 30 kg ha<sup>-1</sup>, respectively); 15-µm-thick MaterBi biodegradable black mulching; protection against fungal diseases and pests with copper oxychloride and azadirachtin treatments, respectively; drip irrigation when the soil available water at 10 cm depth dropped to 80%, based on the crop evapotranspiration<sup>69</sup>; N,  $P_2O_5$  and  $K_2O$  supply by fertigation at a dose of 112, 30 and 90 kg ha<sup>-1</sup>, respectively.



At harvest, the rocket leaves at the marketable stage were cut to 12- to 15-cm lengths, at 3 to 5 cm above the soil surface, so as to safeguard the vegetative apex and allow for a more efficient re-growth<sup>27</sup>.

At each harvest time, on random samples taken in all the experimental plots, yield and colorimetric determinations were performed, while mineral composition and antioxidant compound activity were measured in the laboratory.

### 3.2. Dry Weight

The assessment of leaf dry weight was done after the dehydration of the fresh samples, at 70 °C until a constant weight was reached, in a forced-air oven.

### 3.3. Leaf Colorimetric Parameters

The leaf color parameters L\*, a\* and b\* were measured on the central area of the upper surface of 10 leaves per replicate by means of a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd., Osaka, Japan)<sup>27</sup>.

### 3.4. Mineral Elements

The content of, P, K, Na, Ca, NO<sub>3</sub>-N, Mg and S was measured in leaf dry tissues ground in a Wiley Mill and then sieved through an 841-micron mesh. To prepare the samples, 250 mg of leaf tissue powder suspended in ultrapure water (50 mL) (Milli-Q, Merck Millipore, Darmstadt, Germany) underwent three freeze-thaw cycles in liquid nitrogen and was then shaken in a water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80 °C for 10 min. The resulting mixture was managed according to the procedure of Rouphael et al.<sup>70</sup> and the determinations of the mineral elements were performed in compliance with the same method<sup>70</sup>.

For the determination of the total nitrogen concentration, the Kjeldahl method as described by Bremner<sup>71</sup> was used, and the results were expressed as the percentage of N in the plant sample.

### 3.5. Antioxidant Compounds and Activity

The total phenolic content in methanolic extracts was assessed using the Folin–Ciocalteu method with gallic acid as a standard. Five hundred mg of freeze-dried material was extracted in 60% methanol (10 mL), placed on a shaker for 15 min and then centrifuged for 5 min 4,000× g. One hundred  $\mu$ L of the supernatant was combined with 500  $\mu$ L of Folin–Ciocalteau's reagent (Sigma-Aldrich Inc., Milano, Italy) and 400  $\mu$ L of 7.5% sodium carbonate/water (*w*/*v*). After 30 min of incubation in the dark at room temperature, the solution absorbance was measured at 765 nm by an ultraviolet–visible spectrophotometer, expressing the results as mg gallic acid (Sigma-Aldrich Inc.) per 100 g of dry weight.

The total ascorbic acid (TAA) was determined by a spectrophotometric method as described by Kampfenkel et al.<sup>72</sup>, by reducing the dehydroascorbate to ascorbic acid upon the sample preincubation with dithiothreitol. The solution absorbance was measured at 525 nm, expressing the results as mg ascorbic acid per 100 g fresh weight.

Lipophilic antioxidant activity (LAA) was determined according to Re et al.<sup>73</sup>, and the hydrophilic antioxidant activity (HAA) in compliance with Fogliano et al.<sup>74</sup>.

### 3.6. Statistical Processing

The data were analyzed by the two-way analysis of variance using the SPSS software version 21, and the Duncan multiple range test was performed for mean separations at a 0.05 probability level. The data, expressed as percentages, were subjected to angular transformation before processing.

### 4. Conclusions

From a study carried out in southern Italy on perennial wall rocket (*Diplotaxis tenuifolia* L.-D.C.) grown under tunnels, it arose that the unshaded crops showed the highest yield from mid-April to late June, whereas the application of a 50% shading net produced a beneficial effect on the leaf production in July, in relation with the highest PAR and temperature values, whereas the 79% shading proved to limit the plant light requirements in any cropping season. The unshaded crops generally showed higher mineral accumulation compared to the 79% light extinction, but the mineral accumulation was not significantly different from that elicited by the 50% shading. Interestingly, the latter treatment resulted in the highest content of selenium, a microelement acting as an effective antioxidant. The ascorbic acid and the connected hydrophilic antioxidant activity were best affected by the highest light intensity, whereas the opposite trend was shown by the phenols and lipophilic antioxidant activity. The shading nets proved to be an interesting tool within sustainable horticultural systems, though the optimal degree of light extinction for achieving the highest yield and produce quality depends on the cropping season.



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123 Chapter 6

### Chapter 6

### Pearl Grey Shading Net Boosts the Accumulation of Total Carotenoids and Phenolic Compounds that Accentuate the Antioxidant Activity of Processing Tomato

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**Abstract:** Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetables worldwide due to its low caloric intake and high fiber, minerals, and phenolic compounds, making it a highquality functional food. However, fruit quality attributes can be affected by pre-harvest factors, especially environmental stresses. This research aimed to evaluate the influence of two shading nets (white net-30% and pearl grey net-40% shading degree) on the yield and phytochemical profile of tomato fruits grown in summer under the Mediterranean climate. Mineral and organic acid content (by ion chromatography-IC), phenolic profile (by ultra-high performance liquid chromatography-UHPLC coupled with an Orbitrap high-resolution mass spectrometry-HRMS), carotenoid content (by high-performance liquid chromatography with diode array detection-HPLC-DAD), and antioxidant activities DPPH, ABTS, and FRAP (by UV-VIS spectrophotometry) were determined. Tomato fruits grown under the pearl grey net recorded the highest values of total phenolic compounds (14,997 µg 100 g<sup>-1</sup> of fresh weight) and antioxidant activities DPPH, ABTS, and FRAP, without affecting either fruit color or marketable yield. The reduction of solar radiation through pearl grey nets proved to be an excellent tool to increase the phytochemical quality of tomato fruits during summer cultivation in a Mediterranean environment.

Keywords: Solanum lycopersicum L.; Shading screens; Industrial tomato; UHPLC/HRMS; HPLC-DAD; Lycopene; Chlorogenic acid; Rutin; FRAP; ABTS



### 1. Introduction

Providing a comprehensive definition of vegetable quality nowadays is an everincreasing meticulous task. Historically, the primary goal of the horticultural supply chain was to ensure food security by breeding 'high yielding' genotypes, considering quality as something exclusively related to visual attributes such as size, shape, and color<sup>1-3</sup>. However, a changed socio-economic and cultural context have accelerated the transition to a 'consumer-oriented' model, where the consumer is aware and informed about the nutraceutical value of vegetables<sup>1,4</sup>. The irreconcilable contrast between the frenetic rhythms imposed by modern times and the desire for a healthy lifestyle has drawn attention to the importance of a nourishing diet as a lifeline<sup>5</sup>. A healthy lifestyle diet based on the consumption of large portions of fruits and vegetables, such as the Mediterranean diet, is a powerful weapon for reducing the incidence of pathological disorders through a regular intake of natural boosters such as vitamins, minerals, and phytonutrients<sup>6</sup>.

Native to South America, tomato (Solanum lycopersicum L.) is a staple food of healthy dietary regimen, as well as an essential raw ingredient of recipes and processed products, appointing it among the most consumed foods worldwide<sup>3,7,8</sup>. The premiumquality organoleptic properties of ripe tomato fruits are due to the interaction of soluble sugars (glucose, fructose, and sucrose) and organic acids (citric and malate), which give a perfect mix of sweetness, acidity, and tastiness<sup>2,3,9</sup>. The intense red coloration, known to influence consumer perceptions, is attributed to lycopene, the most abundant carotenoid and accounting for about 80% of the total pigments<sup>10,11</sup>. As a determinant of the visual quality of fruits, lycopene is known to be beneficial for human health, since carotenoids cannot be synthesized ex novo by humans, but must necessarily be introduced through the diet<sup>3,12</sup>. A recognized beneficial action exerted by lycopene, related to its antioxidant activity, has been highlighted in several studies showing a negative correlation between its intake and the incidence of chronic diseases<sup>10,13</sup>. However, it is worth noting that tomatoes also contain other pigments such as  $\alpha$ carotene,  $\beta$ -carotene, and lutein, which contribute equally to the nutritional value<sup>3</sup>. The low caloric value and well-recognized benefits of carotenoids complete the richness in fiber, minerals, and phenolic compounds of tomatoes, making it an excellent functional food<sup>7</sup>. In addition, Slimestada and Verheulb<sup>14</sup> reported about 100 phenolic compounds in tomatoes, of which the most abundant flavonoids are quercetin and kaempferol derivatives (rutin and naringenin), while for phenolic acids it is chlorogenic acid<sup>12,15</sup>. The high bioactivity of phenolic compounds bestows them potent antioxidant activities that can trigger anti-inflammatory, anti-atherogenic, anti-tumor, hepatoprotective, antiviral, and cardioprotective responses; attributes that are increasingly desired in foods<sup>2,16</sup>.

It is well established in literature that most quality traits of tomatoes can shift according to preharvest factors, such as genotype, harvest, and ripening stage, growing

### 125 Chapter 6

conditions, and especially environmental stresses<sup>3,11,17</sup>. Wang et al.1<sup>8</sup> documented that microclimatic factors, remarkably, light and temperature, affect the phytochemical profile of fresh horticultural products, resulting in an ongoing modification of their nutritional quality. However, many authors agree that environmental factors which are most likely to affect the nutritional value of tomatoes are temperature and light<sup>3,15,19,20</sup>. Pressman et al.<sup>21</sup> and Sato et al.<sup>22</sup> showed that yield parameters (number and weight of fruits) are negatively affected by average temperatures above 29 °C due to pollination or fruit set defects. Moreover, Spicher et al.<sup>23</sup> and Lu et al.<sup>24</sup> did not detect structural damage to the photosynthetic apparatus during the vegetative development stage at temperatures near 38 °C.

Light is an essential abiotic component for plant growth, as it provides energy for photosynthesis and is crucial for many physiological processes and qualitative aspects<sup>25.</sup> Quality traits of tomato fruits, such as the content of vitamin C, carotenoids, and phenols, are firmly conditioned by light intensity and duration<sup>3</sup>. The available literature review shows that the phytochemical content of tomato fruits under high light radiation is ambiguous. For example, tomatoes grown under high light intensity led to high flavonoid content, probably due to increased UV-B radiation<sup>3</sup>. Conversely, it has been reported that high light intensity can impair lycopene accumulation with repercussions on intrinsic quality attributes of tomato fruits<sup>11,26</sup>.

In Mediterranean areas, shading nets are extensively used for reducing solar radiation in summer crop cycles, thereby minimizing the occurrence of cracking and discoloration in tomato fruits as they provide a mixture of diffuse and unmodified natural light from which plants benefit<sup>19,27</sup>. Solar radiation is the main parameter influenced by the shading nets and depends on the design properties such as the number of meshes per cm and the shade factor<sup>19,28-30</sup>. The aim of our work was to evaluate the influence of shading nets on the yield and particularly on the phytochemical profile of tomato fruits grown in midsummer in a Mediterranean climate. For this purpose, two different shading nets with varying shading factors were used (white net: 30% shading factor; pearl grey net: 40% shading factor), which can be of additional technique and advancement to modulate the qualitative attributes of tomato.



### 2. Materials and Methods

### 2.1. Plant Material, Experimental Design, and Growth Conditions

The trial was conducted in spring-summer 2021 at 'Raffaele Tamburrino' farm, located in Villa Literno (Caserta, Italy, 10 m above sea level). Tomato (Solanum lycopersicum L.) Quorum F1 seedlings (ISI sementi S.p.A., Fidenza, Italy) were transplanted at the phenological stage of three true-leaves on 3 June 2021, arranged in a double row at a density of 3.5 plants per m<sup>2</sup>. The experimental protocol was based on comparing two shading nets supplied by Arrigoni S.p.A (Uggiate Trevano, Como, Italy) plus an unshaded control, each corresponding to a plot of 240 m<sup>2</sup> (experimental unit) which was randomized in three replicates. Net characteristics were as follows: (1) 2633BL Prism LDF (hereafter 'white net'; shading factor: 30%; air permeability: 44%); (2) 2633GP Prism LDF (hereafter 'pearl grey net'; shading factor: 40%; air permeability: 44%). Fertilization, irrigation, and control of phytopathogens were carried out according to the standard agricultural practices of the cultivation area. Specifically, one month before transplanting, disc harrowing and soil leveling were performed. Water was supplied through a drip irrigation system every two or three days. Nutrient management was performed by fertigation with 150 kg ha<sup>-1</sup> of N, 40 kg ha<sup>-1</sup> of P<sub>2</sub>O<sub>5</sub>, and 220 kg ha<sup>-1</sup> of K<sub>2</sub>O. Phosphorus was entirely supplied during soil preparation operations (pre-transplanting), while nitrogen and potassium were supplied before crop establishment (30% and 55% for N and K<sub>2</sub>O, respectively) and the remainder during the crop cycle. The crop was protected against Phytophthora infestans, Tuta absoluta, Aphids spp., Bemisia tabaci, Trialeurodes vaporariorum, and Tetranychus urticae. Climatic parameters, such as relative humidity, air temperature, and photosynthetically active radiation (PAR), were continuously recorded using WatchDog A150 dataloggers (Spectrum Technologies Inc., Aurora, IL, USA; ± 0.6 °C/±3% Temp/RH accuracy) placed at 0.5 m above ground level. During the experiment, the average air temperature was 28.1 °C, 27.4 °C, and 26.8 °C for the white net, pearl net, and control, respectively.

#### 2.2. Fruit Harvest, Yield, and Fruit Quality Measurement

The experimental trial lasted a total of 91 days (3 June to 1 September). At harvest (91 days after transplanting, DAT), fruits of 15 representative plants were sampled for each replicate, avoiding border plants. The fruits were counted, weighed, and separated into two groups: marketable (ripe and free of visible defects) and unmarketable (misshapen, undersized, and green) fruits. The equatorial and polar diameters were determined on the marketable fruits using digital caliper (±0.02 mm accuracy; RS PRO, Sesto San Giovanni, Milan, Italy). A representative sample of the marketable fruits was blended in a Waring<sup>®</sup> blender (2 L capacity; Model HGB140, McConnellsburg, PA, USA) for 1 min and filtered to determine the juice quality. From the extracted juice, the total soluble

solids (TSS) content, expressed as °Brix at 20 °C, was determined using an Atago N1 portable digital refractometer (Atago Co. Ltd., Tokyo, Japan). An aliquot of fruit juice (approximately 100 g) was dried in a ventilated oven at 70 °C until a constant weight was reached to determine the percentage of dry matter. The dried fruit material was then blended with a KM13 rotating blade grinder (Bosch, Gerlingen, Germany) and stored for mineral and organic acid analysis.

A part of the marketable fruits was immediately frozen at –80 °C and underwent a freeze-drying cycle (Alpha 1–4 Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for further qualitative analysis.

### 2.3. Determination of Fruit Color Using CIELab Color Space

Twenty marketable fruits per replicate were selected to determine colorimetric indices using a Minolta Chromameter CR-400 portable colorimeter (Minolta Camera Co. Ltd., Osaka, Japan). For each fruit, two colorimetric measurements were made (on two opposite sides of the fruit) of the indices L (brightness, 0 to 100), a\* (greenness, –60 to +60), and b\* (yellowness, –60 to +60). Chroma ('colorfulness' quantitative attribute) and Hue angle (qualitative color attribute in the relative amounts of redness and yellowness) were calculated as described by the International Commission of Illumination (CIE):

Chroma =  $[(a^*)^2 + (b^*)^2]^{0.5}$ Hue angle =  $\tan^{-1} b^*/a^*$ 

### 2.4. Mineral Content Determination

The determination of cations (K, Mg, and Na), anion (P), and organic acids (malate and citrate) was carried out by ion chromatography according to the protocol described in detail by Formisano et al.<sup>31</sup>. Briefly, 250 mg of dried and finely ground fruits were mixed with 50 mL of ultrapure water, extracted for 10 min in a water bath at 80 °C, and then centrifuged at 6,000 rpm for 10 min. Twenty-five  $\mu$ L of the supernatant, filtered through a 0.45 µm syringe filter, was injected into an ion chromatographic system coupled with an electrical conductivity detector (ICS 3000, Thermo Scientific™ Dionex<sup>™</sup>, Sunnyvale, CA, USA). The isocratic separation of the cations was performed using 25 mM methanesulfonic acid as eluent (Sigma Aldrich, Milan, Italy) using an analytical column IonPac® CS12A (4 × 250 mm) equipped with an IonPac® CG12A precolumn (4 × 250 mm) and a CERS500 autoregenerating suppressor. The separation of organic acids and the anion P was carried out in gradient mode with potassium hydroxide (5 mM-30 mM, flow rate of 1.5 mL min<sup>-1</sup>) using an IonPac<sup>®</sup> ATC-HC anion trap (9 × 75 mm), an IonPac<sup>®</sup> AG11-HC guard column (4 × 50 mm), an IonPac<sup>®</sup> AG11-HC IC column ( $4 \times 50$  mm), and a DRS600 auto-regenerating dynamic suppressor. All analytical columns, precolumns, traps, and suppressors were purchased from Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup> (Sunnyvale, CA, USA). The concentrations of the minerals and



organic acids in fruits were expressed as mg 100 g<sup>-1</sup> of fresh weight (fw). Each treatment was analyzed in triplicate.

# 2.5. Determination of the Polyphenol Profile by Ultra-High Performance Liquid Chromatography (UHPLC) and Orbitrap High-Resolution Mass Spectrometry (HRMS) Analysis

Polyphenols profile detection and quantification were performed according to the protocol described in detail by El-Nakhel et al.<sup>32</sup>. Briefly, 5 µL of the extracted samples according to the procedure described by Vallverdú-Queralt et al.<sup>33</sup>, were analyzed using a Dionex Ultimate 3000 ultra-high-pressure liquid chromatography (UHPLC) system (Thermo Fisher Scientific<sup>TM</sup>, Waltham, MA, USA) coupled to an Orbitrap high resolution mass spectrometry (HRMS) (Thermo Fisher Scientific<sup>TM</sup>, Waltham, MA, USA). The chromatographic separation of polyphenols was carried out with a Luna Omega PS (1.6 µm, 50 × 2.1 mm, Phenomenex, Torrance, CA, USA) thermostated column (T = 25 °C). The mobile phase consisted of a two-phase solution: water (phase A) and acetonitrile (phase B). Both mobile phases contained 0.1% formic acid (v/v). An ESI source (Thermo Fisher Scientific<sup>TM</sup>, Waltham, MA, USA) was used in negative ion mode (ESI–), setting two scan events (Full ion MS and All ion fragmentation, AIF) for all compounds of interest. Data processing was performed with Quan/Qual Browser Xcalibur software, v. 3.1.66.10 (Thermo Fisher Scientific<sup>TM</sup>, Waltham, MA, USA). Polyphenols were expressed as  $\mu$ g 100 g<sup>-1</sup> fw.

### 2.6. Spectrophotometric Determination of ABTS, DPPH, and FRAP Antioxidant Activities

The ABTS<sup>+</sup> antioxidant activity was performed as described by Re et al.<sup>34</sup>. The solution of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate radical (ABTS<sup>+</sup>) in water was obtained using the classical method of ABTS incubation in darkness at 23 °C for 16 h with potassium peroxydisulfate. After incubation, the stock solution was diluted with ethanol (1:88) until reaching an absorbance of  $0.700 \pm 0.050$  at 734 nm. A 0.1 mL aliquot of each sample that was previously filtered and diluted (1:10) with 70% methanol, was mixed with 1 mL of ABTS<sup>+</sup> solution and stored at ambient temperature for 2.5 min. The absorbance was immediately recorded at 734 nm.

The radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the protocol proposed by Brand-Williams et al.<sup>35</sup>. A 1 mL aliquot of DPPH solution (4 mg 10 mL<sup>-1</sup> of 96% methanol) was added to 200  $\mu$ L of the studied extract, mixed, and incubated at ambient temperature for 10 min. The absorbance was recorded at 517 nm.

The determination of the ferric reduction antioxidant power (FRAP) assay was performed following the protocol described by Rajurkar and Hande<sup>36</sup> with minor modifications. This assay is based on the fact that antioxidants reduce ferric ions to ferrous ions, creating a blue complex (Fe<sup>2+</sup>/2,4,6-tris(2-pyridyl)-s-triazine, TPTZ) with an

absorption peak at 593 nm. Briefly, 150  $\mu$ L of each sample was mixed with 2.850 mL of FRAP solution (1.25 mL of 10 mM TPTZ solution in 40 mM HCl + 1.25 mL of 20 mM FeCl<sub>3</sub> in water + 12.5 mL of 0.3 M acetate buffer, pH 3.6) and incubated for 4 min. The absorbance at 593 nm was then read.

The absorbances of the ABTS, DPPH, and FRAP assays were recorded by UV-VIS spectrophotometer (Shimadzu, Japan). The results were expressed as mmol Trolox equivalents kg<sup>-1</sup> dw. All analyses were performed in triplicate.

### 2.7. Carotenoids Determination

Carotenoids were quantified by high-performance liquid chromatography with diode array detection (HPLC-DAD) according to the protocol of Salomon et al.<sup>37</sup>. Briefly, 0.1 g of lyophilized tissue was macerated with 1 mL of ultra-pure water and 5 mL of ethanol/n-hexane (60:50, v/v) and then sonicated and centrifuged (15 min at 4,000 rpm). After removing the solvent phase by vacuum dry centrifugation, the pellet was subjected to two vacuum extraction/centrifugation cycles. A mixture of methanol and methyl-t-butyl ether (MTBE) (1:1, v/v) was added to the completely dried pellet and analyzed by the HPLC-DAD technique. Calibration curves were constructed using commercial  $\beta$ -carotene and lutein standards purchased from Sigma-Aldrich (Milan, Italy). Results were expressed as mg 100 g<sup>-1</sup> fw. All analyses were performed in triplicate.

The lycopene content of the fruits was determined by spectrophotometry according to the protocol described by Sadler et al.<sup>38</sup>. Lycopene quantification was performed by measuring the absorbance of the hexane extract at 472 nm, using pure lycopene (Sigma-Aldrich, Milan, Italy) to construct the calibration curve. Lycopene content was expressed as mg 100 g<sup>-1</sup> fw. All analyses were performed in triplicate.

### 2.8. Statistical Analysis

All data were analyzed with IBM SPSS Statistics software (SPSS Inc., Chicago, IL, USA) version 26.0 for Windows 10 and are presented as mean  $\pm$  standard error, *n*=3. All mean effects were subjected to one-way ANOVA analysis. Statistical significance was determined with Tukey's HSD test at the *p* = 0.05 level. All plant responses to shading treatments were summarized via a color heatmap generated using the web tool ClustVis (https://biit.cs.ut.ee/clustvis/; accessed on 9 December 2021). The Euclidean distance was used as a measure of similarity and hierarchical clustering with complete linkage heatmaps, and the data were normalized and visualized using a false color scale (red = increase in values; blue = decrease in values)<sup>39</sup>.



### 3. Results and Discussion

### 3.1. Microclimatic Parameters

Shading nets significantly reduced PAR compared to the unshaded control (**Table 1**). In June, the mean PAR in the open field (control) was 1247  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> in contrast to the mean PAR observed under white and grey shading nets with a mean value of 871 and 703  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, respectively. In July, the mean PAR of the control was 1271  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, approximately 2.0% higher than in June. In August, the mean PAR was the lowest, with the control averaging 1127  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> (**Table 1**).

 Table 1. Photosynthetically active radiation (PAR) during the growing season outside (Control) and under shading nets.

Treatment	June	July	August
Control	1247 ± 5.49 a	1271 ± 7.02 a	1127 ± 7.54 a
White net	871 ± 6.66 b	889 ± 6.43 b	786 ± 3.38 b
Pearl grey net	703 ± 13.30 c	727 ± 3.18 c	633 ± 3.53 c
Significance	***	***	***

\*\*\* significant at  $p \le 0.001$ . Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.

In the present study, the effect of the temperature was separated from that of the solar radiation (**Table 1** and **2**). The highest mean temperature was recorded in July under the white net (29.3 °C), while the lowest was recorded in June under the pearl grey net (26.8 °C). However, regardless of the mean PAR values and the degree of shading, the difference in temperature recorded outside and under the shade nets was not significant (**Table 2**).

Table 2. Air temperature durin	g the growing season outside (	Control) and under shading net
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Treatment	June	July	August
Control	$25.9 \pm 0.37$	$27.5 \pm 0.32$	$26.9\pm0.94$
White net	$27.1 \pm 0.06$	$29.3 \pm 0.31$	$28.0\pm0.23$
Pearl grey net	$26.8 \pm 0.53$	$28.2\pm0.74$	$27.1 \pm 0.20$
Significance	ns	ns	ns

ns non-significant according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.

#### 3.2. Yield and Yield Parameters

Tomato is one of the most consumed vegetables worldwide and represents one of the driving crops for many countries economy, due to its dual use as a fresh and processed product (*e.g.*, pasta, sauce, peeled tomatoes, juice, ketchup)<sup>8,10</sup>. To date, world tomato production is estimated at 180 million tons, with China alone having a total production of approximately 63 million tons, followed by India (~19 million tons), Turkey (~13 million tons), the United States of America (~11 million tons), Egypt (~7 million tons), and Italy (~5 million tons)<sup>40</sup>. However, it is well known that tomato yield is strongly influenced by environmental factors (such as humidity, temperature, and solar radiation), genotype, and preharvest factors (growing practices)<sup>19,41</sup>.

In our study, the number of total fruits per plant showed a significant decrease compared to the control when the plants were under shading (Control > White net > Pearl grey net; Table 3). Probably, high light intensity conditions induced an ecophysiological response to mitigate stress, increasing the number of fruits but reducing the transpiring surface (diameter of the fruit) compared to shaded conditions. Consequently, the reduction in the fruit number induced by shading did not affect the weight of the fruit (**Table 3**). Total, marketable, and unmarketable fruits weight (kg pt <sup>1</sup>) did not show significant differences in shaded plants compared to the control, in contrast to the findings of Angmo et al.<sup>42</sup>, who reported an increase in total marketable fruit weight in the open field compared to shaded conditions, which could be attributed to different environmental conditions, genetic material, and cultural practices<sup>43-45</sup>. In the present experiment, we adopted a processing tomato cultivar that was neither tied nor defoliated, in contrast to the methods used by the authors mentioned above, and in part, could have determined different production responses. Shading nets resulted in a considerable increase in marketable fruit weight of 47.5%, compared to the control, justifying the non-significant difference in total fruit weight per plant (Table 3).



		Yield			Fruit number		Mean
Treatment	Total	Marketable	Unmarketable	Total	Marketable	Unmarketable	marketable fruits weight
	(kg pl-1)	(kg pl-1)	(kg pl-1)	(n° fruits pl-1)	(n° fruits pl-1)	(n° fruits pl-1)	(g)
Control	$2.58\pm0.15$	$2.25\pm0.02$	$0.33 \pm 0.06$	351.50 ± 9.41 a	268.11 ± 1.66 a	83.39 ± 2.06 a	$8.50 \pm 0.75 \text{ b}$
White net	$2.56\pm0.24$	$2.12\pm0.08$	$0.44 \pm 0.05$	251.47 ± 5.55 b	$182.98 \pm 0.77$ b	$68.48 \pm 1.96$ b	11.58 ± 0.76 a
Pearl grey net	$2.20\pm0.07$	$1.89\pm0.08$	$0.32 \pm 0.04$	184.88 ± 4.75 c	141.13 ± 1.09 c	43.75 ± 1.55 c	13.37 ± 0.36 a
Significance	ns	ns	ns	***	***	***	*

Table 3. Effects of shading nets on yield and yield parameters.

ns, \*, and \*\*\* non-significant or significant at  $p \le 0.05$  and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3. Pl = plant.

### 3.3. Quality Attributes of Fruits

The growing interest in high-quality food products has forced growers to meet the changing needs of increasingly demanding consumers. In the past, the marketable quality of vegetables relied primarily on visible characteristics, but now sensory and organoleptic characteristics have become a primary parameter driving consumer choice<sup>2,46</sup>. In tomatoes, one of the sensory attributes that determine the organoleptic quality of the fruit is the content of soluble solids (glucose, fructose, and sucrose), which, combined with organic acids and amino acids, represents approximately 75% of dry matter<sup>46</sup>. In the literature, it is known that fruit sweetness is strongly influenced by genetic material<sup>26</sup>. Almeida et al.<sup>20</sup> have studied the effects on the accumulation of total soluble solids (TSS) of five genotypes of tomatoes under different environmental conditions. The authors found that the TSS content ranged among the genotypes from 5.6 to 7.2 °Brix, and according to the environmental conditions from 3.8 to 8.9 °Brix. In our experiment, we found that light radiation affected this crucial qualitative parameter (Table 4). Davies et al.<sup>47</sup> highlighted the evidence of a direct relationship between solar radiation and sugar content in tomatoes. Our results confirm this correlation as fruits exposed to direct solar radiation (Control) showed the highest value of TSS (7.43 °Brix; Table 4), confirming what was reported by Ilić et al.<sup>48</sup> in a similar experiment. The higher TSS content in the control fruits was probably attributable to the lower water assimilation capacity of the fruits, which also justified the high dry matter content (8.71%; Table 4)<sup>49</sup>. However, it is worth considering that an increase in the amount of solar radiation received by the plant may increase photosynthesis, and thus carbohydrates in the fruit<sup>8</sup>. On the other hand, the direct correlation between solar radiation and TSS is not univocal, as different results are found in the literature, again highlighting how genotype plays a crucial role in the adaptation to different environmental conditions<sup>10,48</sup>.

Another qualitative aspect that can influence consumer choice is color, since a wellcolored fruit is qualitatively superior. Practically, the color of the fruit depends on the physical and biochemical changes that occur naturally during the growth and ripening stages or after harvest<sup>50</sup>. Among the CIELab colorimetric parameters, only the L (brightness) parameter varied significantly in response to shading, with the highest value obtained in fruits grown under white shading nets (**Table 4**). For definition, L is "an approximate measure of brightness, which is the property according to which any color can be considered equivalent to a member of the greyscale, between black and white"<sup>51</sup>. The increase in L under shaded conditions agrees with the findings of Messina et al.<sup>52</sup>. However, the same authors also reported a decrease in a\* values (less intense red color) and an increase in b\* values (more intense yellow color) that we did not observe in our study (**Table 4**).



Similar to TSS content and color, fruit size and shape are also essential quality traits. Although shape is primarily determined by genetic background, size also depends on the interaction of the latter with the environment<sup>2</sup>. From a physiological point of view, the increase in fruit size depends on the enlargement of the pericarp due to the production of new cells during the anthesis process and the growth and expansion of cells that last until the fruit ripening<sup>2</sup>. According to Angmo et al.<sup>42</sup>, compared to the control, we observed an average increase in the equatorial and polar diameter of fruits of 8.60 and 10.50%, respectively, when grown under shading nets (**Table 4**). The larger fruit size recorded under shading nets accounted for the higher average marketable fruit weight (**Table 3**), attributed to the higher water content in the fruit (lower dry matter) that resulted in a dilution effect on TSS (**Table 4**).

**Table 4.** Effect of shading nets on total soluble solids (TSS), dry matter, CIELab colorimetricparameters, and fruit size.

Treatment	TSS	Dry Matter	т	.*	<b>L</b> *	
Treatment	(°Brix)	(%)	L	ď	D	
Control	7.43 ± 0.30 a	8.71 ± 0.32 a	36.35 ± 0.12 b	$28.80 \pm 0.90$	$23.27\pm0.64$	
White net	$5.40\pm0.06~b$	$7.63 \pm 0.14$ b	38.12 ± 0.24 a	$29.49\pm0.20$	$23.50\pm0.16$	
Pearl grey net	$5.30 \pm 0.25$ b	$7.25 \pm 0.20$ b	37.32 ± 0.26 b	$28.21 \pm 0.31$	$23.76\pm0.35$	
Significance	**	*	*	ns	ns	

Tuestasest	Charama	II	Equatorial diameter	Polar diameter
Treatment	Chroma	Hue angle	(mm)	(mm)
Control	37.03 ± 0.08 ab	$218.94 \pm 0.59$	24.52 ± 0.21 c	33.16 ± 0.01 b
White net	37.70 ± 0.22 a	$218.55 \pm 0.21$	26.26 ± 0.11 b	36.55 ± 0.17 a
Pearl grey net	36.89 ± 0.03 b	$220.11 \pm 0.62$	$27.00 \pm 0.04$ a	36.71 ± 0.13 a
Significance	*	ns	***	***

Table 4. Cont.

ns, \*, \*\*, and \*\*\* non-significant or significant at  $p \le 0.05$ , 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.
#### 3.4. Mineral Content of Fruits

Minerals, like other macromolecules (carbohydrates, proteins, and fats), are required to preserve some physical and biochemical processes essential for life<sup>53</sup>. Currently, mineral deficiency in the human diet is a severe problem for industrial and developing countries<sup>54</sup>. Given the high intake of tomatoes, the potential contribution of tomato fruit to the mineral intake of human diet is of high importance<sup>55</sup>. It is well established that the most abundant mineral in tomato fruit is potassium<sup>54</sup>. Potassium plays a crucial role in maintaining cellular homeostasis, nerve impulse conduction and muscle contraction, and the glycogenesis process<sup>53,56</sup>. In plants, potassium is an activator of enzymatic processes and contributes significantly to the photosynthetic process<sup>57</sup>. Among the macronutrients reported in **Table 5**, potassium was the most abundant mineral in the fruit and was affected by shading treatment with highest value recorded under white net. Although potassium is crucial for color determination, the change in its content was not coupled with a perceived change in color (a\*) (**Table 4**). This result could be partially related to the optimal potassium content (361.43-445.65 mg 100 g<sup>-1</sup> fw)<sup>57</sup>.

Phosphorus is the main component of bones and is involved in many metabolic processes (kidney function and cell growth); it has a buffering action and is involved in the formation of high-energy compounds (adenosine triphosphate) and in phospholipid synthesis<sup>53,58</sup>. Similarly to potassium, the phosphorus content was significantly affected by growth conditions (**Table 5**). The higher value (14.88 mg 100 g<sup>-1</sup> fw) recorded in fruits grown under unshaded conditions would help to better explain the higher TSS obtained from the same treatment. Indeed, Lavon et al.<sup>59</sup> showed a positive correlation between this essential macroelement and TSS content in tomato fruits.

Although magnesium deficiency in the human diet is rarely a determinant of pathological states (WHO;<sup>60</sup>), this mineral is crucial as it is a component of bones and teeth and is an active component of different enzymatic systems<sup>53</sup>. In our study, the magnesium content was not affected by shading (**Table 5**). Although Milenković et al.<sup>19</sup> observed a reduction in magnesium in tomato fruits exposed to direct solar radiation, our results do not show the same trend. This discordance could be attributable not only to the different genetic material, but also to the different light conditions.

Tomato acidity is a crucial component of the organoleptic quality of fruits<sup>61</sup>. Interactions between reducing sugars and organic acids are essential to confer sweetness, tartness, and flavor intensity to fruits<sup>2,3,9</sup>. The main organic acids in tomato fruits are malic and citric acids, but the perception of acidity is mainly due to the latter, which is the most abundant organic acid<sup>2,61</sup>. Shading treatments reduced the citrate content in fruits by 23.7%, compared to the control that showed the highest values (140.36 mg 100 g<sup>-1</sup> fw). The higher citrate content in the fruits of the control could probably be attributable to a higher source:sink ratio during the pre-ripening phase, increasing the respiration rate of the fruits. Therefore, a higher respiration rate could



have promoted glycolysis and increased citrate production<sup>61</sup>. However, it should be noted that changes in fruit water content were observed between treatments (**Table 3**), which may have interfered with acidity due to a dilution/dehydration effect<sup>61</sup>.

Treatment	Р	К	Mg	Na	Malate	Citrate
Control	14.88 ± 0.94 a	$412.08 \pm 4.76$ b	$13.04 \pm 0.53$	$6.84\pm0.72$	34.15 ± 1.91 a	140.36 ± 7.84 a
White net	7.63 ± 0.41 b	$445.65 \pm 1.08$ a	$12.49 \pm 0.43$	$5.29 \pm 0.17$	$26.78 \pm 0.74 \text{ b}$	109.90 ± 1.20 b
Pearl grey net	9.21 ± 0.15 b	361.43 ± 3.58 c	$11.23 \pm 0.30$	$6.14 \pm 0.32$	29.89 ± 1.02 ab	104.33 ± 2.92 b
Significance	**	***	ns	ns	*	**

Table 5. Effect of shading nets on mineral accumulation in fruits. Data are expressed as mg 100 g<sup>-1</sup> fw.

ns, \*, \*\*, and \*\*\* non-significant or significant at  $p \le 0.05$ , 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.



#### 3.5. Fruit Pigments

The relevance of the quanti-qualitative profile of carotenoids in tomato fruits is mainly attributable to their dual function. In fact, while these biomolecules determine the coloration of ripe fruits, they are crucial in the human diet due to their recognized antioxidant activity<sup>2,62</sup>. Furthermore, it is important to note that the body cannot synthesize these valuable pigments, making their intake through plant consumption mandatory<sup>63</sup>. In plants, carotenoids are used to capture light and protect the photosynthetic apparatus from excessive solar radiation, attract pollinators, and facilitate seed dispersal<sup>2,62</sup>. The data reported in **Table 6** show that, compared to the control, shading treatments resulted in the highest biosynthesis of total carotenoids in fruits. Although there is a wide variability in carotenoid content in the literature, our results are in agreement with the findings of Flores et al.<sup>64</sup> in red tomato fruits, which showed that the most abundant carotenoid was lycopene, followed by  $\beta$ -carotene, and lutein. Compared to shaded conditions, the ~40.0% reduction in the lycopene content in control fruits confirms that excessive radiation exerts an inhibitory effect on the biosynthesis and accumulation of this critical pigment<sup>3,65,66</sup>. Leyva et al.<sup>3</sup> noted that the decrease in lycopene content which is found in our work as well, could be attributed to direct solar radiation and not air temperature, since Helves et al.<sup>67</sup> observed that fruit surface temperatures of 30 °C trigger the degradation of this pigment. These conditions could have occurred in our case under control unshaded conditions.

Lycopene is a crucial intermediate in the biosynthesis of many carotenoids such as  $\beta$ carotene and xanthophylls such as lutein<sup>63</sup>. Consequently, it is not surprising that high solar radiation (Control) resulted in an average reduction in  $\beta$ -carotene (-43.4%) content, compared to shaded conditions. Not least, it is interesting to note that although lycopene is responsible for the red color of tomatoes<sup>68</sup>, the significant differences in lycopene content between treatments did not affect the colorimetric parameter a\* (**Table 4**). This result could be related to the direct correlation between lycopene content and fruit size (equatorial diameter and polar diameter; **Table 4**), which probably influenced the colorimetric analyses.

Treatment	Lutein	Lycopene	$\beta$ -carotene	Total carotenoids
Control	$0.022 \pm 0.001$ b	$1.666 \pm 0.061 \text{ b}$	$0.358 \pm 0.012 \text{ b}$	$2.046 \pm 0.074 \text{ b}$
White net	$0.024 \pm 0.000$ ab	2.881 ± 0.053 a	0.623 ± 0.013 a	3.528 ± 0.065 a
Pearl grey net	$0.027 \pm 0.001$ a	$2.828 \pm 0.080$ a	0.643 ± 0.018 a	3.498 ± 0.099 a
Significance	*	***	***	***

**Table 6**. Effect of shading nets on lutein, lycopene,  $\beta$ -carotene, and total carotenoids accumulation in fruits. Data are expressed as mg 100 g<sup>-1</sup> fw.

\* and \*\*\* significant at  $p \le 0.05$  and 0.001. Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.

#### 3.6. Phenolic Compounds and Antioxidant Activity of Fruits

In their natural habitats, plants are threatened by a large number of potential enemies, and to defend themselves, they produce a wide range of heterogeneous protection compounds (pigments, signaling molecules, and aromas) known as 'secondary metabolites', which play an important role in their survival<sup>69</sup>. Secondary metabolites are classified on the basis of their chemical structure and biosynthetic pathways. They can be divided into three groups: terpenoids, phenolic compounds and flavonoids, and sulfur-containing compounds and nitrogen-containing alkaloids<sup>70</sup>. Present in most fruits and vegetables, secondary metabolites show beneficial effects on human health<sup>71</sup>. They have well-established anticancer, antiaging, anti-diabetic, and anti-obesity activity, in addition to their protection against Alzheimer's and cardiovascular diseases<sup>70</sup>. Synthesized through the shikimate biochemical pathway, phenolic compounds represent the most abundant type of secondary metabolites in plants<sup>72</sup>. Their biosynthesis begins from non-oxidative deamination of phenylalanine, mediated by the enzyme Phenylalanine Ammonia-Lyase (PAL), leading to the formation of cinnamic trans acid as a key intermediate at the base of secondary products derived from phenylpropanoid (flavonoids and isoflavonoids, coumarins, lignins, esters of hydroxycinnamic acid, and phenolic compounds)72. The accumulation of these compounds varies between organisms, tissues, and growth stage, and can be influenced by environmental conditions, because gene expression levels that encode key enzymes in the phenylpropanoid biosynthesis pathway are affected by environmental stressors (light, temperature, and nutritional deficits)<sup>73</sup>.

The UHPLC analysis identified 20 phenolic compounds that could be classified into the following categories: phenolic acid derivatives, flavonoid derivatives, and hydroxycinnamoyl quinic acid derivatives (**Table 7**). The different light intensity conditions that characterized the treatments in the present experiment influenced the total content of phenolic compounds, calculated as the sum of all the detected individual phenolic compounds. Specifically, the Pearl grey shade net resulted in the highest



accumulation of total phenolic compounds (14,997  $\mu$ g 100 g<sup>-1</sup> fw), followed by the Control (12,377  $\mu$ g 100 g<sup>-1</sup> fw) and the White shade net (9,869  $\mu$ g 100 g<sup>-1</sup> fw). Regardless of the treatment, the largest contribution to total phenolic compounds resulted from flavonoid derivatives (7,776  $\mu$ g 100 g<sup>-1</sup> fw, on average), as reported by Bertin and Génard<sup>2</sup>, followed by phenolic acid derivatives (4,097  $\mu$ g 100 g<sup>-1</sup> fw, on average) and lastly hydroxycinnamoyl quinic acid derivatives (541  $\mu$ g 100 g<sup>-1</sup> fw, on average). Although flavonoids are 'semi-essential' compounds, having no well-defined nutritional function, they are crucial for protecting antioxidant compounds from oxidative degradation in humans and plants<sup>74</sup>.

As reported by Slimestad and Verheul<sup>8</sup> and Abreu et al.<sup>75</sup>, regardless of treatments, rutin was the most abundant flavonoid (Table 7). In contrast, Bertin and Génard<sup>2</sup> reported that the most abundant flavonoid in tomatoes was naringenin, demonstrating how genotype, cultural practices, environmental conditions, and even analytical determination techniques can influence the content of these compounds. Rutin is considered one of the best natural antioxidants currently known that can exert important pharmacological activities, acting as antibacterial, anti-inflammatory, antiallergic, antiviral, antiprotozoal, and antitumor. It has also marked cytoprotective, vasoactive, antiplatelet, hypolipidemic, and antihypertensive activities<sup>76</sup>. The highest rutin values were obtained using a Pearl grey shading net (4,414  $\mu$ g 100 g<sup>-1</sup> fw), similarly to the other flavonoid derivatives (kampferol-3-diglucoside > naringenin > rutin-O-pentoside > kaempferol-3-O-rutinoside > naringenin-C-diglycoside > apigenin-C-hexoside-hexoside > naringenin-C-hexoside > quercetin-O-dihexoside > genistin), except for rutin-Ohexoside, which showed the highest value under the White shading net and Control (Table 7). The reviewed literature shows that the increase in flavonoids (mainly rutin) is promoted by intense solar radiation<sup>77,78</sup>, which is not in line with the results obtained in this experiment. The total flavonoid content of fruits grown under pearl grey net was 59.9% higher than that recorded in the unshaded Control (Table 7). The reason behind this can be partly explained by the fact that most of the reviewed works did not separate the effect of temperature from that of solar radiation, while in our study, the only significantly different parameter was PAR (Table 1 and Table 2). Furthermore, it should be considered that the response of the plant to a stressor depends not only on the genotype, intensity, and magnitude of the stressor, but also on the stage of development of the plant and the organs involved<sup>2</sup>. For example, despite evidence in the literature that water stress can increase the levels of phenolic compounds, Atkinson et al.<sup>79</sup> observed that the greatest accumulation of flavonoids was recorded in tomato leaves following water stress, while in fruits no changes were observed. Similarly, Abreu et al.<sup>75</sup> showed, in line with our results, that shading increased the phenolic content in tomato fruits, compared to the Control, that resulted in a greater accumulation of these compounds only in the leaves. This result was probably attributable to the fact that the

#### 141 Chapter 6

leaves were directly exposed to the stressor (high direct solar radiation). Not least, the obtained up-regulation of flavonoids could result not only from the limitation of solar radiation recorded in August under the pearl grey net (**Table 1**), but also from ecophysiological responses induced by the different microclimatic conditions that lead to the plant to modify its primary metabolism (fewer but larger fruits; **Table 3**). As argued by Campa et al.<sup>80</sup>, low light intensity would have triggered the production of phenolic compounds with which plants would have counterbalanced the reduction in antioxidant enzyme activity. In contrast with flavonoids trend, the highest hydroxycinnamoyl quinic acid derivatives values were obtained under Pearl grey net (685 µg 100 g<sup>-1</sup> fw) and in the Control (633 µg 100 g<sup>-1</sup> fw), while the lowest were obtained under White net (305 µg 100 g<sup>-1</sup> fw) (**Table 7**). In particular, the values of tricaffeoylquinic and dicaffeoylquinic acids in the more shaded conditions (pearl grey) were 53.30 and 44.50% higher than the average of the other treatments.

In contrast to what was observed for flavonoid derivatives and hydroxycinnamoyl quinic acids, the content of phenolic acid derivatives was lower in fruits harvested under pearl grey shading net (**Table 7**). As reported in the literature, chlorogenic acid is the main non-flavonoid phenolic compound found in tomato fruits that possess high antioxidant, antibacterial, anti-inflammatory, antiviral, antimicrobial, hepatoprotective, cardioprotective, and neuroprotective properties<sup>2,8,81,82</sup>. The highest value of this crucial antioxidant compound was found in the unshaded Control (3,363 µg 100 g<sup>-1</sup> fw) while the lowest was found in the Pearl grey net treatment (1,799 µg 100 g<sup>-1</sup> fw) (**Table 7**). However, Botella et al.<sup>83</sup> reported that homovanillic acid-O-hexoside was found to be the second most prevalent compound, with the highest values (11.51 µg g<sup>-1</sup> fw) recorded in fruits grown under the white net. The lowest values of coumaric acid O-hexoside were found in the White net treatment, whereas the lowest ones of ferulic and caffeic acids were found in the Control (**Table 7**).

In contrast to what was observed with flavonoid and hydroxycinnamoyl quinic acid derivatives, the heterogeneity of phenolic acid derivatives among treatments emphasizes that irradiation and/or shading strongly influenced the biosynthesis of this class of phenolic compounds. Probably, different light conditions could have unequivocally influenced the assignment of phenolic substrates to individual branches of the phenylpropanoid pathway<sup>73</sup>.



		<u>.</u>		
Phenolic Compounds	Control	White net	Pearl Grey net	Significance
PHENOLIC ACID DERIVATIVES				
Chlorogenic acid	3,363 ± 105 a	2,470 ± 47 b	1,799 ± 46 c	***
Homovanillic acid-O-hexoside	939 ± 38 b	1,151 ± 24 a	956 ± 23 b	**
Caffeic acid-O-hexoside	418 ± 19 a	372 ± 5 b	343 ± 11 b	**
Coumaric acid-O-hexoside	74 ± 5 a	56 ± 1 b	81 ± 2 a	**
Ferulic acid	$20 \pm 0$ c	48 ± 2 b	61 ± 3 a	***
Ferulic acid-O-hexoside	$19 \pm 1 b$	34 ± 2 a	21 ± 2 b	*
Caffeic acid	$16 \pm 1 b$	27 ± 1 a	25 ± 1 a	**
Total phenolic acid derivatives	4,848 ± 164 a	4,157 ± 80 b	3,287 ± 78 c	***
FLAVONOID DERIVATIVES				
Rutin	2,944 ± 101 b	2,481 ± 48 c	4,414 ± 112 a	***
Kampferol-3-diglucoside	1,979 ± 82 b	1,578 ± 24 c	3,245 ± 80 a	***
Naringenin	1,199 ± 50 b	450 ± 15 c	1,851 ± 51 a	***
Rutin-O-pentoside	333 ± 11 b	$387 \pm 4 b$	732 ± 20 a	***
Rutin-O-hexoside	167 ± 6 a	161 ± 2 a	140 ± 6 b	*
Kaempferol-3-O-rutinoside	105 ± 5 c	$164 \pm 10 \text{ b}$	225 ± 4 a	***
Naringenin-C-diglycoside	$72 \pm 6 b$	$78 \pm 4 b$	225 ± 10 a	***
Apigenin-C-hexoside-hexoside	34 ± 1 c	57 ± 2 b	84 ± 1 a	***
Naringenin-C-hexoside	37 ± 1 b	30 ± 1 c	66 ± 1 a	***
Quercetin-O-dihexoside	$14 \pm 0 b$	12 ± 0 c	21 ± 1 a	***
Genistin	$10 \pm 0 b$	10 ± 1 b	21 ± 0 a	***
Total flavonoid derivatives	6,896 ± 263 b	5,407 ± 107 c	11,025 ± 276 a	***
HYDROXYCINNAMOYLQUINIC ACID DERIVATIVES				
Dicaffeoylquinic Acid	505 ± 28 a	266 ± 3 b	557 ± 22 a	***
Tricaffeoylquinic Acid	128 ± 7 a	39 ± 3 b	128 ± 5 a	***
Total hydroxycinnamoyl quinic acid derivatives	633 ± 35 a	$305 \pm 5 b$	685 ± 26 a	***
Total phenolic compounds	12,377 ± 460 b	9,869 ± 183 c	14,997 ± 378 a	***

Table 7. Effect of shading nets on phenolic compounds accumulation in fruits. Data are expressed as µg 100 g<sup>-1</sup> fw.

\*, \*\*, and \*\*\* non-significant or significant at  $p \le 0.05$ , 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.

Different spectrophotometric assays were carried out for the determination of antioxidant activity of tomato fruits. Specifically, we evaluated the free radical scavenging activity DPPH, the free radical scavenging activity by ABTS decolorization, and the ferric reducing antioxidant capacity FRAP (**Table 8**). Our findings showed significant antioxidant activity in fruits grown under pearl grey net, probably related to the higher content of total phenolic compounds (**Table 7**)<sup>75</sup>.

In detail, DPPH antioxidant activity increased as the degree of shading increased, with the highest (40.72 mmol Trolox eq. kg<sup>-1</sup> dw) and lowest (32.21 mmol Trolox eq. kg<sup>-1</sup> dw) values obtained in fruits shaded with the pearl grey net and in the control, respectively. On the contrary, the ABTS assay did not show the same trend, with the lowest value (35.33 mmol Trolox eq. kg<sup>-1</sup> dw) obtained in fruits shaded with the white net and the highest value (43.70 mmol Trolox eq. kg<sup>-1</sup> dw) obtained with the pearl grey net.

The FRAP activity showed the highest value (34.38 mmol Trolox eq. kg<sup>-1</sup> dw) in fruits shaded with the pearl grey net, while no significant differences were observed between the unshaded control and the White net treatment.

Furthermore, the correlation coefficient between the content of total phenolic compounds and antioxidant activities was highly significant, especially for the FRAP ( $R^2 = 0.74$ ) and ABTS ( $R^2 = 0.99$ ) assays. The correlation coefficient between the DPPH assay and the total phenolic compounds ( $R^2 = 0.37$ ) was less significant than that of the FRAP and ABTS assays (**Table 8**). These discrepancies could be due to synergistic effects between phenolic compounds and other chemical components that can contribute to the total antioxidant activity or the type of assay used<sup>84,85</sup>. The lower value of DPPH found in control plants (**Table 8**) could be attributed to the lower content of total carotenoids, especially lycopene (**Table 6**).

Treatment	DPPH	ABTS	FRAP
Control	32.21 ± 0.40 c	39.18 ± 0.09 b	27.51 ± 0.31 b
White net	35.54 ± 0.37 b	35.33 ± 0.30 c	27.64 ± 0.17 b
Pearl grey net	40.72 ± 0.22 a	43.70 ± 0.58 a	34.38 ± 0.81 a
Significance	***	***	***

 Table 8. Effect of shading nets on DPPH, ABTS, and FRAP antioxidant activities. Data are expressed as mmol Trolox equivalents kg<sup>-1</sup> dw.

\*\*\* significant at  $p \le 0.001$ . Different letters within each column indicate significant differences according to Tukey's HSD test (p = 0.05). All data are expressed as mean ± standard error, n=3.



#### 3.7. Cluster Heatmap of Yield and Quality Parameters of Fruits

A heatmap was performed for all the above parameters to provide a detailed overview of the yield parameters, minerals, pigments, and antioxidant activity of tomato fruits under different shading treatments.

Heatmap analysis of the aggregated data identified two main clusters corresponding to the control and shaded treatments (White net and Pearl grey net), respectively (**Figure 1**). The separation between the two clusters was mainly due to crucial carotenoids (such as  $\beta$ -carotene and lycopene) and total carotenoids. Two separate sub-clusters (White net and Pearl grey net) were defined under the second cluster indicating that shading was the main clustering factor, while unshaded treatment was the second.

The analyzed parameters were separated into three main clusters, each subdivided into secondary sub-clusters (**Figure 1**). Clusterization of the analyzed parameters shows that the Control improved the citrate, phosphorus, and malate content of the fruits compared to the results observed under the white net and Pearl grey. On the contrary, the Control reduced the mean marketable fruit weight and the content of  $\beta$ -carotene, lycopene, and total carotenoids. The Pearl grey net treatment was characterized by increased antioxidant capacity, total flavonoids derivatives, and lutein.



**Figure 1**. Heatmap analysis summarizing the results of yield, mineral, and quality parameters of *Solanum lycopersicum* L. fruits grown under different shade treatments (Control, White net, and Pearl grey net).



#### 4. Conclusions

The increasing consumer demand for healthy foods with high nutritional value has prompted researchers and producers to focus on production techniques to ensure high yields and premium quality products. In warm Mediterranean regions, high light and high temperatures pose a challenge to tomato production, especially affecting fruit quality and nutraceutical values. The use of shading nets (white and pearl grey) did not significantly affect the average temperature of the growing environments compared to the open field (Control). This result allowed us to separate the effect of temperature from solar radiation and to understand more deeply the influence of light on the quality attributes of tomato fruits. Compared to the control, shading reduced, on average, total fruits (-37.93%) without affecting total yield as a result of higher average fruit weight (+46.76%). The higher shade and the better diffusion of light with the pearl grey net led to a more significant accumulation of all compounds with antioxidant activity. Compared to the Control, we observed a 70.96% increase in total carotenoids, mainly due to lycopene (+ 69.74%), and in the content of total phenolic compounds (+ 21.17%), with the most outstanding contribution given by rutin (4,414  $\mu$ g 100 g<sup>-1</sup> fw), Kampferol-3-diglucoside (3,245  $\mu$ g 100 g<sup>-1</sup> fw), and naringenin (1,851  $\mu$ g 100 g<sup>-1</sup> fw). Finally, the use of the pearl grey net is a valuable tool for the eco-sustainable production of tomatoes, able to preserve and improve the quality attributes of the fruits. The promising results obtained in this study pave the way for future investigations to evaluate the qualitative responses induced by the pearl grey net in other vegetables.

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# Chapter 7

# Between Light and Selective Shading: Morphological, Biochemical and Metabolomics Insight into the influence of Blue Photoselective Shading on Vegetable Seedlings

Abstract: High nursery densities reduce seedling quality due to the competition for light. High light intensity, shading, and blue light depletion activate morpho-physiological and metabolomic responses in plants, resulting in size modification to gain an advantage over neighboring plants. Our research aimed to unravel the effects of light intensity and quality on nursery seedlings at the morphological and biochemical levels. To this aim, the effect of black shading and blue photoselective shading nets were investigated in terms of morphometric, ionomic, and untargeted metabolomics signatures in Cucurbita pepo L., Citrullus lanatus L., Solanum lycopersicum L., and Solanum melongena L. seedlings. Plant height, diameter, sturdiness index, leaf area, specific leaf area, shoot/root ratio, and mineral content (by ion chromatography-IC) were evaluated. In Cucurbita pepo L and Citrullus lanatus L., the blue net reduced the shoot/root and chlorophyll a/b ratios and increased stem diameter and total chlorophyll. The black net increased plant height, stem diameter, and sturdiness index in Solanum lycopersicum L. and Solanum melongena L. At the same time, unshading conditions reduced leaf area, specific leaf area, shoot/root ratio, and total chlorophyll. The blue net improved the sturdiness index and quality of Cucurbita pepo L. and Citrullus lanatus L. Such impact on morphological parameters induced by the different shading conditions was corroborated by a significant modulation at the metabolomics level. Untargeted metabolomics phytochemical signatures of the selected plants, and the subsequent multivariate analysis coupled to pathway analysis, allowed highlighting a broad and diverse biochemical modulation. Metabolomics revealed that both primary and secondary metabolism were largely affected by the different shading conditions, regardless of the species considered. A common pattern arose to point at the activation of plant energy metabolism and lipid biosynthesis, together with a generalized down accumulation of several secondary metabolites, particularly phenylpropanoids. Our findings indicate an intriguing scientific interest in the effects of selective shading and its application on other species and different phenological stages.

**Keywords:** shading screen; plantlets; sturdiness index; red:blue ratio; metabolomics; plant metabolism; lipid biosynthesis, phenylpropanoids

#### 1. Introduction

Nursery activities are the "backbone" of modern agricultural production systems, in addition to asserting additional assets for social and economic sustainability<sup>1-3</sup>. In the agricultural scene, the horticultural and nursery sectors have grown over the years due to their distinctive dynamism, the ongoing technological upgrading, and investment in new growing techniques to meet the increasing demand for high-quality seedlings (*i.e.*, healthy, vigorous, and balanced development) and adaptability to different climates and soils. The adaptability of seedlings to changing environmental conditions is the cornerstone of nursery production. Drought, soil salinity, temperature, humidity, and sub-optimal nutrient levels are examples of environmental pressures that imperil seedling establishment, performance, and survival in their natural habitats<sup>4</sup>. Preconditioning nursery techniques are crucial to produce robust plants with adequate morphology and high levels of organic reserves. These latter attributes are critical to ensure increased vegetative vigor during seedling establishment<sup>4</sup>. Direct morphological parameters (such as plant height, stem diameter, root length, dry weight, and leaf area), derived (such as the sturdiness index, shoot/root ratio, and leaf area ratio), and physiological parameters (such as mineral and chlorophyll content) are usually used for seedling quality assessment<sup>5</sup>. For example, a lower sturdiness index (*i.e.*, the ratio of stem height to stem diameter) reduces seedling lodging, while a low shoot/root ratio reduces mortality rates when grown in drought environments<sup>4,6</sup>. Manas et al. 6 reported that higher shoot dry weight (high content of photosynthetic reserves) increased vigor and survival of seedlings after transplanting. At the same time, Grossnickle<sup>7</sup> pointed out that high leaf area (excessive shoot growth) could lead to severe transplant shock as a consequence of water imbalances between shoot and root. Finally, a thicker stem and a larger root system increased resistance to transplant shock<sup>8</sup>.

In plants, vegetative growth and development depend on division, cell elongation, directional growth, and branching<sup>9</sup>, where light is one of the environmental parameters that can drive many of these processes<sup>10</sup>. Plants are light-dependent and therefore have evolved sophisticated photoreceptors that control specific biochemical and physiological aspects to maximize photosynthetic performance by adapting to a specific light environment<sup>11,12</sup>. Usually, plants under high light intensity have higher photosynthetic activity, thicker roots, and long shoots. In contrast, plants increase leaf size under shading conditions, show a higher chlorophyll content, and lower their light compensation point to balance the reduced photosynthetic activity<sup>13–15</sup>.

However, modern nursery techniques based on high planting density can reduce seedling quality due to unwanted changes in key morphological parameters<sup>16</sup>. In highdensity seedlings, tight spaces cause a strenuous struggle for light, a scenario that triggers photo-morphogenetic adaptations to increase competitiveness among plants<sup>12,17</sup>. To manage the challenging relationship between neighbors, plants can rely on two strategies: react (avoidance) or adapt (tolerance)18. Plants, through photoreceptors, detect shading as a reduced intensity and/or changes in light quality<sup>17,18</sup>. For example, depletion of blue radiation is an indicator of effective shading to which plants respond by elongating the stem and increasing the angle of incidence of the leaves (hyponastia) to take advantage of neighboring plants<sup>17</sup>. Stimulus-response induction is mediated by cryptochrome, a phototropin photoreceptor involved in the uptake of blue light and contributes largely to plant shape<sup>11</sup>. Blue light depletion caused by self-shading can result in excessive stem and shoot growth, an undesirable aspect for nursery seedlings. Thus, forcing producers to use chemical size regulators. Although morphological responses to blue light are genotype-dependent and can differ even among genotypes<sup>19</sup>, increasing blue radiation through alternative non-chemical methods could be a viable and environmentally sustainable aid to reduce nursery seedling size. However, the application of the blue spectrum in seedling cultivation has rarely been studied or documented in scientific manuscripts and very little is known about the metabolic changes associated with planting exposition to the blue net. Considering the direct linkage between light and essential processes (not limited to photosynthesis) and its connection to the carbon and nitrogen fluxes<sup>20</sup>, studying the metabolic processes underlying selective shading is crucial in understanding the profound impact of shading in crops. In this sense, the hypothesis-free comprehensive profiling provided by untargeted metabolomics may provide a holistic overview of the different biochemical processes triggered by selective shading, thus providing valuable insights into the metabolic reprogramming induced in crops.

Based on these assumptions, the objective of our research was to evaluate the effects of light intensity and quality through the use of black shading nets and blue photoselective shading nets on morphometric and quality parameters, colorimetric indices, mineral and pigment content. At the same time, metabolic reprogramming was investigated through untargeted metabolomics in zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus* L.), tomato (*Solanum lycopersicum* L.), and eggplant (*Solanum melongena* L.) seedlings for nursery production in the Mediterranean environment. To our knowledge, this is the first work that has investigated these aspects and will be of prime interest to seedling producers.

### 2. Materials and Methods

#### 2.1. Experimental Design, Plant Material, and Technical Characteristics of the Nets

The experimental trial evaluated the intensity and quality of light on nursery seedlings. It was carried out in spring-summer 2021 at "Vivai Giuseppe Bene" nursery farm, located in Poggiomarino (Naples, Italy, 40°79' N, 14°53' E, 46 m.s.l.). The

experiment protocol was based on comparing a blue photoselective shading net, a commercial black shading net (as control), and a transparent plastic film in ethyl vinyl acetate. The shading nets were in factorial combination with zucchini squash seedlings (Cucurbita pepo L. cv. San Pasquale, Pagano Domenico & Figli, Scafati, Italy), watermelon (Citrullus lanatus L. cv. Crimson Sweet, Pagano Domenico & Figli, Scafati, Italy), tomato (Solanum lycopersicum L., cv. OR Grandborghese, Four-Blumen Vegetable seeds, Piacenza, Italy), and eggplant (Solanum melongena L., cv. Mirabelle F1 - Seminis, Milan, Italy), sown in polystyrene plug trays (experimental unit) (*Cucurbitaceae*: 60 plants/tray; Solanaceae: 180 plants/tray). The experimental design was randomized into three replicates. Seeds were sown on June 29 2021, covered with a thin layer of vermiculite and placed in a germination chamber for 36 h (until seed coats cracked and the shoots just started to emerge). On July 2, the trays were moved under the nets. The characteristics of the nets were as follows: (1) ChromatiNet® Blue (hereafter "Blue net"; shading factor: 40%; red:blue ratio = 1; Ginegar Plastic Products LTD, Kibbutz Ginegar, Israel); (2) 2635NE Agri LDF black (hereafter "Black net"; shading factor: 40%; red:blue ratio=1.4; Arrigoni S.p.A, Uggiate Trevano, Italy); (3) Sunlux 200 EVO plastic film (hereinafter "No shading"; shading factor: 20%; red:blue ratio = 1.4; Comagri S.r.l., Grumello del Monte, Italy). The red:blue ratio of the nets and the degree of light extinction were evaluated using a portable spectral radiometer (MSC15, Gigahertz-Optik, Turkenfeld, Germany) (Supplementary Figure 1), while photosynthetically active radiation (PAR) was continuously recorded using WatchDog A150 dataloggers (Spectrum Technologies Inc., Aurora, IL, USA) (Supplementary Figure 2).



**Supplementary Figure 1.** Effects of shading nets on photosynthetically active radiation (PAR). Data are mean values  $\pm$  standard error, *n*=3. All mean effects were subjected to one-way ANOVA analysis. Statistical significance was determined with Tukey's HSD test at the *p* = 0.05 level.



**Supplementary Figure 2.** Red:blue ratios of plastic film (No shading), black shading net (Black net) and blue photoselective shading net (Blue net)

#### 2.2. Sampling and Determination of Morphometric and Quality Indices of Seedlings

Seedlings were sampled when they reached their marketable size (at two true leaves for zucchini squash and watermelon and three true leaves for tomato and eggplant). Specifically, zucchini squash, watermelon, tomato, and eggplant were sampled at 14, 19, 21, and 27 days after sowing, respectively. Twenty defect-free plants per experimental unit were harvested (avoiding border plants), weighed, and separated into leaves, stems, and roots. Plant height (cm plant-1) was measured, and leaf area (cm<sup>2</sup> plant-1) was assessed by digital image analysis using ImageJ v1.52a software (U.S. National Institutes of Health, Bethesda, MD, USA). A leaf tissue subsample was immediately stored at -20  $^{\circ}$ C for pigment determination, while another subsample was immediately frozen at –80 °C and subjected to а freeze-drying cycle (Alpha 1-4 Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for metabolomic analyzes. The diameter of the stem was measured using a digital caliper (±0.02 mm accuracy; RS PRO, Sesto San Giovanni, Italy). The roots were gently cleaned in water, spread on graph paper and measured in length (cm plant-1). All tissues collected were oven dried at 70 °C to constant weight (~72 h) to determine dry weight (mg plant-1). The dried leaves and stems were ground with an MF10.1 cutting head mill (IKA®, Staufen im Breisgau, Germany) and sieved with an MF0.5 sieve (hole size 0.5 mm; IKA<sup>®</sup>, Staufen im Breisgau, Germany) for mineral determination. Then, derived quality indices such as shoot/root ratio, sturdiness index (stem height/root collar diameter), and specific leaf area (LAR, cm<sup>2</sup> mg<sup>-1</sup> plant<sup>-1</sup>; leaf area/total dry weight) were calculated.

#### 2.3. Soil Plant Analysis Development (SPAD) Index and Leaf Color Determination.

At harvest, SPAD index (greenness index) was measured on twenty young and fully expanded leaves of each experimental unit using a portable chlorophyll meter (SPAD-502, Minolta Camera Co. Ltd., Osaka, Japan) and CIELab colorimetric coordinates by a Minolta CR-300 colorimeter (Minolta Co. Ltd., Osaka, Japan) calibrated with a corresponding Minolta standard.

#### 2.4. Mineral Determination

The determination of cations (K, Ca, and Mg) and anions (Nitrate and P) in zucchini squash, watermelon, tomato, and eggplant seedlings was assessed by ion chromatography according to the method described in detail by Formisano et al.<sup>21</sup>. Briefly, 0.25 g of finely ground dry material was mixed with 50 mL of ultrapure water (Arium<sup>®</sup> Advance EDI pure water system, Sartorius, Goettingen, Germany), placed in a shaking water bath for 10 min (100 rpm; Julabo, Seelbach, Baden-Württemberg, Germany), and centrifuged for 10 min (6,000 rpm, R-10M centrifuges, Remi Elektrotechnik Ltd., Mumbai, India). A 0.25 µL aliquot of the supernatant was filtered and processed by anionic chromatography coupled to an electrical conductivity detector (ICS-3000, Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA). Columns, pre-columns, and self-regenerating suppressors were purchased from Thermo Scientific™ Dionex™ (Sunnyvale, CA, USA). Cations separation was performed isocratically using 25 mM methanesulfonic acid as eluent (Sigma Aldrich, Milan, Italy). Anions separation was performed in a gradient mode (5 mM-30 mM KOH with a 1.5 mL min<sup>-1</sup> flow). The integration and quantification of minerals was performed using Chromeleon<sup>™</sup> 6.8 Chromatography Data System (CDS) software (Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA), comparing the peak areas of the samples with those of the standards. Anions and cations concentrations were expressed as g kg-1 dry weight (dw), except for nitrate, which was expressed as mg kg<sup>-1</sup> fresh weight (fw). Each treatment was analyzed in triplicate.

#### 2.5. Pigments Determination

Pigments (total chlorophyll, a, b, and carotenoids) were determined as described by Formisano et al.<sup>22</sup>. Briefly, 0.5 g of fresh leaves were extracted in ammonia acetone, crushed in a ceramic mortar, and centrifuged at 2,000 rpm for 10 min using an R-10 M centrifuge (Remi Elektrotechnik Limited, Mumbai, India). The contents of chlorophyll a, chlorophyll b, and carotenoids were determined by UV-Vis spectrophotometry (ONDA V-10 Plus, Giorgio Bormac srl, Carpi, Italy) with an absorbance of 647, 664, and 470 nm, respectively. Total chlorophylls were calculated as chlorophyll a + chlorophyll b. In addition, the chlorophyll a/chlorophyll b ratio was calculated. Total chlorophylls and carotenoids were expressed as mg  $g^{-1}$  fw.

#### 2.6. Metabolomics Analysis

The untargeted metabolomics profiling of the four seedling species, namely zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus* L.), tomato (*Solanum lycopersicum* L.), and eggplant (*Solanum melongena* L.), was carried out by extracting 0.5 g of dried leaves in 5 mL of extraction solvents, composed by 80% v/v methanol + 20% v/v ultrapure water and acidified with 0.1% formic acid (Merck KGaA, Darmstadt,

Germany). The samples were subsequently homogenized using a Polytron<sup>®</sup> PT1200 E (Kinematica AG, Malters, Switzerland) homogenizer and centrifuged at 8,000× g for 15 min. The supernatants were filtered with a 0.22 mm syringe filter and transferred in glass vials ready to be injected (volume of 6 µL) into the ultra-high-pressure liquid chromatography coupled to a quadrupole time of flight mass spectrometer (UHPLC-QTOF-MS; Agilent Technologies, Stevens Creek Blvd, Santa Clara, CA, USA) as previously reported<sup>23</sup>. In detail, the chromatographic separation was achieved by using an Agilent InfinityLab Poroshell 120 pentafluorophenyl (PFP) column (2.1 × 100 mm, 1.9 µm) (Agilent Technologies, Stevens Creek Blvd, Santa Clara, CA, USA) and a binary mixture of water and acetonitrile acidified with 0.1% (v/v) formic acid as mobile phase (LC-MS grade, VWR, Milan, Italy). The data analysis after the samples acquisition was carried out using Agilent Profinder B.10.0 (Agilent Technologies, Stevens Creek Blvd, Santa Clara, CA, USA) in order to align and annotate the features according to the 'findby-formula' algorithm against the PlantCyc 12.6 database<sup>24</sup> retaining only those compounds putatively annotated within 75% of replications in at least one condition<sup>25</sup>. Monoisotopic accurate mass was used together with the entire isotopic profile, achieving level 2 of confidence in annotation<sup>26</sup>.

#### 2.7. Statistical Analysis

Data from each species were subjected to one-way analysis of variance (One way ANOVA) using IBM SPSS Statistics software (SPSS Inc., Chicago, IL, USA) version 26.0 for Windows 11 and presented as mean  $\pm$  standard error, n = 3. Statistical significance was determined using Tukey's HSD test at the p = 0.05 level. All seedling responses to changing light intensity and quality on morphometric and quality indices, minerals, colorimetric parameters, and pigment accumulation were summarized via color heatmaps generated using the web-based tool ClustVis (https://biit.cs.ut.ee/clustvis/; accessed January 3, 2022). The Euclidean distance was used as a measure of similarity and hierarchical clustering with full link heatmaps, and the data was normalized [ln(x + 1)] and displayed using a false color scale (red = increase in values; blue = decrease in values)<sup>27</sup>.

The chemometric interpretation of the metabolic features was conducted with Mass Profiler Professional B.15.1 (Agilent Technologies, Stevens Creek Blvd, Santa Clara, CA, USA), as previously described in our work<sup>23</sup>. Using this software, the raw metabolomic data set was transformed and normalized and then used for fold-change analysis. For this purpose, supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA), using SIMCA 16 (Umetrics, Malmo, Sweden), was performed considering all the species together and only the nets as a factor. Subsequently, the OPLS-DA model was validated, and model fitness parameters (goodness of fit:  $R^2Y$ ; goodness of prediction:  $Q^2Y$ ) were inspired through permutation test (n = 100) and Hotelling's T2 (95% and 99% confidence limit for the suspect and strong outliers, respectively). Then, the variable importance in projection (VIP  $\geq$  1.3) used adopted to identify discriminant metabolites among different treatments for the four species and the resulted compounds were subjected to a fold-change (FC) to better understand the differences among treatments compared to the unshading plants. After that, VIP markers were uploaded into the Omic Viewer Pathway Tool of PlantCyc (Stanford, CA, USA) to identify the pathways and processes affected by treatments.

#### 3. Results and Discussion

#### 3.1. Effects of Light Intensity and Quality on Morphometric and Seedling Quality Indices

Light plays a pivotal role in regulating physiological and critical processes in plants<sup>10,11,28</sup>. Through complex mechanisms, plants capture light reaching their leaves and activate molecular pathways to acclimate to specific light environments<sup>29</sup>. However, the productive performance also depends on light quality, which can trigger particular gene expressions that have a different impact on plant survival<sup>4,5,12,19</sup>. The morphometric indices in **Table 1** show a significant effect of light intensity and quality on plant height. Except for zucchini squash (*Cucurbita pepo* L.), shading treatments (Black and Blue net) increased, on average, watermelon (*Citrullus lanatus* L.), tomato (*Solanum lycopersicum* L.), and eggplant (*Solanum melongena* L.) seedlings size by 24.36, 35.91%, and 28.04%, respectively, compared to the unshaded treatment (No shading).

**Table 1.** Effects of shading and light quality on morphometric indices of zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus L.*), tomato (*Solanum lycopersicum L.*), and eggplant (*Solanum melongena L.*) seedlings. Data are mean values ± standard error, *n*=3.

Creation	Treatment -	Plant height	Plant height Leaf area Shoot dry weight R		Root dry weight	Shoot/root ratio
Specie		(cm plant-1)	(cm <sup>2</sup> plant <sup>-1</sup> )	(mg plant-1)	(mg plant-1)	Shoot/root ratio
	No shading	2.364±0.018 b	43.675±0.330 a	356.167±9.076 a	108.433±3.795	3.674±0.352
Zucchini squash	Black net	2.821±0.014 a	43.477±0.135 a	340.233±2.747 a	100.067±1.486	3.811±0.007
	Blue net	2.338±0.019 b	42.254±0.147 b	304.300±7.199 b	101.433±0.837	3.458±0.024
	Significance	***	**	**	ns	ns
	No shading	2.820±0.091 c	20.561±0.690 b	321.867±1.068 a	86.200±1.914 b	4.201±0.117 a
Watermelon	Black net	3.752±0.087 a	21.904±0.609 b	296.100±5.575 b	77.100±1.415 c	3.980±0.052 a
	Blue net	3.262±0.045 b	24.893±0.323 a	327.033±1.281 a	100.567±0.437 a	3.404±0.055 b
	Significance	***	**	***	***	***
	No shading	5.309±0.123 c	7.421±0.069 c	119.133±2.811 b	44.067±0.606 b	2.694±0.023 b
Tomato	Black net	7.915±0.077 a	11.776±0.053 a	148.933±6.438 a	52.933±1.386 a	2.820±0.059 ab
	Blue net	6.516±0.095 b	10.568±0.030 b	133.267±3.689 ab	44.900±0.361 b	2.962±0.055 a
	Significance	***	***	*	***	*
	No shading	3.589±0.011 c	17.587±0.613 c	127.833±2.210 b	71.567±0.940 a	1.807±0.073 c
Eggplant	Black net	5.046±0.086 a	27.274±0.504 b	157.233±3.830 a	61.100±1.206 b	2.985±0.119 a
	Blue net	4.145±0.031 b	31.425±0.335 a	163.900±2.743 a	64.700±0.351 b	2.622±0.033 b
	Significance	***	***	***	***	***

Different letters within columns indicate significant mean differences according to Tukey's HSD test (p = 0.05). ns, \*, \*\* and \*\*\* denote non-significant or significant effects at  $p \le 0.05$ , 0.01, and 0.001, respectively.

# 163 **Chapter 7**

The increase in plant height in our experiment is a typical phenotypic response to the so-called "shade avoidance syndrome" (Figure 1)<sup>17</sup>. In the shade, plants detect light depletion through specific photoreceptors such as phytochromes<sup>18</sup>, and they trigger morphological changes that promote stem elongation through a complex network of hormones and transcriptional regulators<sup>30-33</sup>. As reported by Casal<sup>34</sup> and Ballaré and Pierik<sup>35</sup>, under shading, the active state of phytochrome B (Pfr) is converted to the inactive state (Pr). This conversion releases the negative feedback of phytochrome B on phytochrome interacting factors (PIFs), leading to auxin and gibberellin production that results in cell elongation, thus ensuring better light accessibility to plants. Similarly, plants' changes in the spectral light quality are detected as a warning signal of future competition. The literature has well documented that the depletion of blue light, or its limited availability, can prompt stem elongation due to an attenuation of the cryptochrome-PIFs interaction<sup>11,36,37</sup>. In the present investigation, increasing the percentage of blue light in the light spectrum by photoselective blue net (Black net: R/B = 1.4, Blue net: R/B = 1; Supplementary Figure 1) decreased the height of plants, compared to the Black net (Table 1). Similarly to phytochromes, the effects of blue light on cryptochromes generate signals that suppress gibberellin and auxin synthesis, affecting gene expression involved in elongation repression<sup>38</sup>. Our results are consistent with previous studies in tomato<sup>39-43</sup>, cucumber (Cucumis sativus L.)<sup>44,45</sup>, broccoli (Brassica oleracea var. italica), kohlrabi (Brassica oleracea Gongylodes)<sup>46</sup> and pepper (Capsicum annuum L.)<sup>45</sup> grown under Led light.



**Figure 1.** Illustrative picture of the effects of light intensity and quality on seedling height of *Cucurbita pepo* L. (**A**), *Citrullus lanatus* L. (**B**), *Solanum lycopersicum* L. (**C**), and *Solanum melongena* L. (**D**).

# 165 **Chapter 7**

The leaf area showed divergent trends between *Cucurbitaceae* and *Solanaceae* (Table 1). The unshading condition reduced leaf area in tomato and eggplant compared to the shading treatments. Probably, under high light intensity, plants reduced leaf expansion to catch less light and limit any damage of the photosystem. The reduction in leaf area also explains the lower shoot dry weight registered for the same species (Table 1). Shoot dry weight reflects the net gain from photosynthesis and its accumulation is mainly driven by the source:sink of the photosynthesis. Generally, high shoot dry weight indicates a better growth potential<sup>6</sup>. However, Grossnickle<sup>7</sup> suggested that a high leaf weight could lead to an increased transplant stress under suboptimal conditions (e.g., drought and heat), because the root system might not provide sufficient water to the leaves to maintain an adequate water balance during the establishment phase. The different responses observed for leaf area and shoot dry weight in Cucurbitaceae could be derived from their less permanence in the nursery (12-15 days for Cucurbitaceae vs 20-30 days for Solanaceae) and the genotypic effect (Table 1). In zucchini squash, regardless of light intensity, the ratio R/B = 1.4 (No shading and Black net) increased leaf area and shoot dry weight, that is consistent with the findings of Hernández and Kubota<sup>42</sup>, who reported an increase in shoot dry weight due to a higher allocation of dry weight to the leaves. In contrast, as in eggplant, the highest leaf area in watermelon was obtained under the Blue net (R/B = 1). Our results are in agreement with the reviewed literature, where Liu et al.<sup>43</sup> reported that an R/B = 1 ratio promoted leaf expansion in tomato by improving light absorption, while Lian et al.<sup>44</sup> and Kim et al.<sup>45</sup> reported similar results in Lilium (Lilium oriental 'Pesaro') and Chrysanthemum (Dendranthema grandiflorum Kitam 'Cheonsu').

Except for zucchini squash, shading increased the leaf area ratio (LAR; **Table 2**). As Freschet et al.<sup>46</sup> reported, the LAR increased under shading due to the increased leaf area rather than the dry weight of the leaf. This result is confirmed in tomato and eggplant, where leaf areas were, on average, 50.54 and 66.88% higher than that in the unshading condition.

C	Treatment	Stem diameter	Stem diameter Root lenght		Leaf area ratio
Specie		(cm plant <sup>-1</sup> ) (cm plant <sup>-1</sup> )		- Sturdiness index	(cm <sup>2</sup> mg <sup>-1</sup> plant <sup>-1</sup> )
	No shading	0.442±0.001 ab	11.042±0.081 c	5.416±0.022 b	0.123±0.002 b
Zucchini squash	Black net	0.436±0.003 b	12.077±0.100 a	6.682±0.410 a	0.128±0.001 b
	Blue net	0.456±0.007 a	11.519±0.029 b	5.249±0.047 b	0.139±0.003 a
	Significance	*	***	**	**
	No shading	0.433±0.003 b	11.043±0.536 b	6.267±0.134 c	0.064±0.002 b
Watermelon	Black net	0.404±0.006 c	12.995±0.173 a	9.974±0.189 a	0.074±0.001 a
	Blue net	0.477±0.002 a	11.633±0.394 ab	7.175±0.159 b	0.076±0.001 a
	Significance	***	*	***	**
	No shading	0.262±0.003	11.331±0.176 a	20.304±0.927 c	0.062±0.001 b
Tomato	Black net	0.266±0.004	10.876±0.426 a	30.508±0.854 a	0.079±0.003 a
	Blue net	0.261±0.000	9.371±0.173 b	26.993±0.043 b	0.079±0.002 a
	Significance	ns	**	***	**
	No shading	0.232±0.006 b	10.048±0.075 b	15.600±0.170 b	0.137±0.003 b
Eggplant	Black net	0.250±0.003 a	10.929±0.137 a	20.312±0.115 a	0.174±0.007 a
001	Blue net	0.261±0.002 a	11.314±0.187 a	15.914±0.155 b	0.192±0.003 a
	Significance	**	**	***	***

**Table 2.** Effects of shading and light quality on quality indices of zucchini squash (*Cucurbita pepo L.*), watermelon (*Citrullus lanatus L.*), tomato (*Solanum lycopersicum L.*), and eggplant (*Solanum melongena L.*) seedlings. Data are mean values ± standard error, n=3.

Different letters within columns indicate significant mean differences according to Tukey's HSD test (p = 0.05). ns, \*, \*\* and \*\*\* denote non-significant or significant effects at  $p \le 0.05$ , 0.01, and 0.001, respectively.

## 167 Chapter 7

Regarding the effects of light quality on the root system, it should be noted that blue light promoted root growth in watermelon, resulting in a lower shoot/root ratio (**Table 1**). The shoot/root ratio is a crucial index for seedlings as it correlates with their survival<sup>4</sup>. In general, reducing the shoot/root ratio reduces the plant mortality rate at transplant establishment<sup>4</sup>. An inadequately developed root system cannot provide enough water to large shoots, making plants unsuitable for active growth<sup>52</sup>. In zucchini squash, the intensity and quality of light did not affect root dry weight and, consequently, shoot/root ratio. While in shading treatments, root length increased, on average, by 6.85%, compared to the unshading condition (**Table 2**). A different situation was observed for *Solanaceae*. In tomato, the Black net promoted root growth (> root dry weight), while the same trend was not found in eggplant, where the Blue net lowered the shoot/root ratio in shading conditions (**Table 1**). However, the lowest shoot/root ratio (1.807) was recorded under unshading conditions due to the higher root dry weight (**Table 1**).

In addition to the root system and plant height, the diameter of the stem plays a crucial role in seedling survival and growth. A larger stem diameter reduces transplant stress by improving water transport and uptake<sup>8,53</sup>. Compared to the Black net, the Blue net increased the stem diameter in zucchini squash and watermelon, while no effect was observed in tomato (**Table 2**). The lowest value was obtained in the No shading treatment in eggplant, which justified the lower shoot dry weight (**Table 1** and **Table 2**). As Grossnickle and MacDonald<sup>8</sup> indicated, the divergent results revealed that the relationship between big stem diameter and seedling survival is not universal. The effects of blue light on stem diameter increase were previously reported in mature plants of canola (*Brassica napus* 'Modena')<sup>54</sup> and cress (*Lepidium sativum* L.)<sup>28</sup> grown under Led light.

The different responses of plants to the quantity and quality of light on the height and diameter of the stem were mirrored in the sturdiness index (**Table 2**). In nursery production, a lower sturdiness index indicates a better-quality plant and is an indirect parameter for evaluating the seedlings survival rate and growth performance<sup>8</sup>. In our study, regardless of family and species, unshading conditions and the Blue net increased plant compactness (lower sturdiness index) compared to the Black net (**Table 2**). The increased plant compactness was directly related to the plant height reduction and stem diameter increase obtained in the above treatments (**Table 1** and **Table 2**).

The perception of the world around us is determined by the mutual interaction between physical stimuli and sensory responses. Color is one of the most important sensory attributes, influencing consumer choice and decision and being a predictor of sensorial quality attributes in food<sup>55</sup>. However, color is also crucial in the nursery production of premium quality seedlings, characterized by high compactness and vivid colors. Except for zucchini squash, the findings in Table 3 showed a significant influence of the different treatments on the CIELab colorimetric parameters and the Spad index. However, the species' response to the change of the intensity and quality of light was not univocal. In watermelon, the effects of the Blue net on the morphometric and qualitative parameters were coupled with an increase in the Spad index and a reduction in b<sup>\*</sup>, compared to the other treatments (Black net and No shading; **Table 1** and **Table 2**). However, the Blue net led to the lowest  $L^*$  (46.475) while the highest  $L^*$  (47.459) was obtained in the No shading treatment. The same increasing trend was observed for L<sup>\*</sup> and b<sup>\*</sup> in zucchini squash in the No shading treatment (**Table 3**). In *Solanaceae*, the highest SPAD index was obtained in the No shading treatment. However, this finding was not associated with improved morphometric and qualitative indexes of plants grown under the same conditions (Table 1 and Table 2). However, the most negative a\* values were recorded under the Black net. The lowest L\* in tomato (47.749) was recorded in the No shading treatment, while in eggplant (43.879) it was recorded in the Blue net treatment.

# 169 Chapter 7

 Table 3. Effects of shading and light quality on Spad index and CIELab colorimetric parameters of zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus L.*), tomato (*Solanum lycopersicum L.*), and eggplant (*Solanum melongena L.*) seedlings. Data are mean values ± standard error, n=3.

Specie	Treatment	SPAD index	L*	a*	b*
	No shading	41.102±0.564	45.861±0.308 a	-16.977±0.272	24.034±0.346 a
Zucchini squash	Black net	41.593±0.483	42.325±0.368 b	-16.017±0.126	21.625±0.189 b
	Blue net	42.212±0.054	41.576±0.380 b	-16.248±0.366	21.469±0.255 b
	Significance	ns	***	ns	***
	No shading	47.733±0.044 b	47.459±0.056 a	–15.696±0.056 b	24.374±0.107 a
Watermelon	Black net	47.421±0.061 b	46.935±0.134 b	−14.850±0.104 a	21.779±0.146 b
	Blue net	48.932±0.128 a	46.475±0.092 c	–14.797±0.023 a	21.245±0.018 c
	Significance	***	***	***	***
	No shading	43.369±0.477 a	47.749±0.202 b	–15.596±0.085 a	25.400±0.211 b
Tomato	Black net	37.055±0.486 b	49.749±0.172 a	–17.784±0.035 c	30.038±0.080 a
	Blue net	37.157±0.127 b	49.943±0.061 a	–17.405±0.059 b	29.895±0.044 a
	Significance	***	***	***	***
	No shading	39.955±0.068 a	44.750±0.236 b	–13.294±0.129 a	21.855±0.178 b
Eggplant	Black net	37.336±0.075 b	45.848±0.078 a	–15.623±0.018 c	25.464±0.693 a
	Blue net	36.352±0.087 c	43.879±0.122 c	-14.301±0.122 b	22.203±0.064 b
	Significance	***	***	***	**

Different letters within columns indicate significant mean differences according to Tukey's HSD test (p = 0.05). ns, \*\*, and \*\*\* denote non-significant or significant effects at  $p \le 0.01$  and 0.001, respectively.

#### 3.3. Effects of Light Intensity and Quality on Mineral and Pigment Accumulation in Seedlings

The change in intensity and quality of light affects the hormonal pathways of signal molecules involved in transmitting light signals to the roots, which regulates the uptake of nutrients in seedlings<sup>56</sup>. Except for tomato, the unshading condition reduced nitrate (on average, -60.27, -20.82, -34.26%, in zucchini squash, watermelon, and eggplant, respectively), compared to shadings conditions (**Table 4**). Under unshading conditions, the demand for sugars and organic nitrogen is high (higher photosynthetic activity), and vacuolar nitrate is exchanged for soluble sugars and organic acids. Moreover, under shading conditions, nitrate may be a readily available vacuolar osmoticum<sup>13</sup>. This could explain the reduction of nitrate in our study under unshaded conditions. Compared to the No shading treatment, the Blue net reduced nitrate by 18.57% in tomato. Similarly, Li et al.<sup>20</sup> and Ohashi-Kaneko et al. <sup>57</sup> reported nitrate reduction in plants exposed to blue light. A similar trend was observed in zucchini squash and eggplant (**Table 4**).

Phosphorus is an essential macronutrient involved in photosynthesis, energy metabolism, respiration, and maintenance of cellular structures. It drives enzyme activation, stimulates root and stem development, and constitutes ATP and nucleic acids (DNA and RNA)<sup>58</sup>. Xu et al.<sup>59</sup> reported that phosphorus utilization efficiency increases with high light intensity within a threshold, beyond which adverse effects on nutrient uptake were observed. However, as observed in our study, the species do not have a univocal response (**Table 4**). In zucchini squash under unshading treatment, phosphorus decreased by 5.57%, compared to Black net. On the contrary, an opposite trend was observed in tomato and eggplant (+13.39 and +102.45%, respectively), compared to the Black net.

Potassium is the most abundant inorganic cation in plants that performs a wide range of metabolic functions such as osmoregulation, cell homeostasis and takes role in enzymatic activation and protein synthesis<sup>60</sup>. The No shade treatment significantly increased potassium in eggplant (on average, +13.14%) compared to shading treatments, while in tomato, the highest potassium values were obtained under the Blue net (**Table 4**). Probably, blue radiation directly influenced potassium uptake. In fact, it was reported in the literature that blue light can regulate stomatal opening and, consequently, promote nutrient uptake through transpiration-induced mass flow<sup>61,62</sup>. Watermelon did not show significant differences for potassium between treatments, while in zucchini squash, the highest value (34.934 g kg<sup>-1</sup> dw) was obtained in No shading treatment (**Table 4**).

Like potassium, the highest calcium was obtained in the No shading condition in zucchini squash. In contrast, shade provided the highest calcium values in watermelon and tomato compared to the No shading treatment (**Table 4**). Under shading treatments, the highest magnesium content in watermelon, tomato, and eggplant was obtained (**Table 4**). Specifically, watermelon and eggplant showed an increase in magnesium
# 171 | Chapter 7

under the Blue net, while there was no difference between shading nets in tomato. However, it should be noted that the increase in magnesium under shading treatments in tomato and eggplant was well correlated with the increase in total chlorophyll (**Table 5**). Mg is the central atom of the chlorophyll a and b porphyrin ring of green plants<sup>63</sup>.

Emocio	Treatment	Nitrate	Р	K	Ca	Mg	
Specie	Treatment	(mg kg-1 fw)	(g kg-1 dw)	(g kg-1 dw)	(g kg-1 dw)	(g kg <sup>-1</sup> dw)	
	No shading	95.494±0.612 c	1.661±0.010 b	34.934±0.076 a	4.009±0.063 a	1.906±0.022 b	
Zucchini squash	Black net	282.574±5.331 a	1.759±0.020 a	34.391±0.122 b	3.741±0.030 b	2.039±0.039 a	
	Blue net	198.165±1.745 b	1.762±0.017 a	33.899±0.117 c	3.645±0.022 b	1.952±0.007 ab	
	Significance	***	**	***	**	*	
	No shading	17.315±0.712 b	0.555± 0.008 b	26.681±0.206	5.982±0.168 c	1.757±0.018 c	
Watermelon	Black net	21.312±0.418 a	0.579±0.003 ab	26.598±0.117	7.156±0.047 b	1.935±0.031 b	
	Blue net	22.426±0.165 a	0.591± 0.005 a	26.675±0.150	9.268±0.243 a	2.168±0.050 a	
	Significance	***	**	ns	***	***	
	No shading	81.002±2.047 a	0.686±0.005 b	16.148±0.363 b	3.842±0.133 b	1.495±0.032 b	
Tomato	Black net	74.137±2.800 ab	0.605±0.004 c	16.807±0.217 b	6.842±0.042 a	1.913±0.012 a	
	Blue net	65.958±1.625 b	0.780±0.023 a	18.503±0.121 a	6.597±0.040 a	1.999±0.030 a	
	Significance	**	***	**	***	***	
	No shading	133.763±8.286 c	2.814±0.054 a	40.201±0.539 a	3.336±0.066 a	1.929±0.058 b	
Eggplant	Black net	237.072±9.772 a	1.390±0.035 c	35.203±0.634 b	3.049±0.094 ab	2.097±0.065 b	
201	Blue net	169.909±4.223 b	2.289±0.007 b	35.884±0.386 b	2.708±0.106 b	2.628±0.045 a	
	Significance	***	***	***	**	***	

**Table 4**. Effects of shading and light quality on minerals accumulation of zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus* L.), tomato (*Solanum lycopersicum* L.), and eggplant (*Solanum melongena* L.) seedlings. Data are mean values ± standard error, *n*=3.

Different letters within columns indicate significant mean differences according to Tukey's HSD test (p = 0.05). ns, \*, \*\* and \*\*\* denote non-significant or significant effects at  $p \le 0.05$ , 0.01, and 0.001, respectively.

# 173 Chapter 7

Plants are endowed with sophisticated photoreceptors that transduce the light signal. Chlorophylls (a and b) absorb photons in the blue and red regions and drive metabolic processes by "collecting" energy<sup>59</sup>. It is not surprising that changing the intensity and quality of light affected pigment biosynthesis. Regardless of light intensity, the light quality modification (Blue net) increased total chlorophyll in zucchini squash, watermelon and eggplant, compared to the No shading and Black net treatments (Table 5). Plants adapt their chlorophyll pigment content to the light spectrum, and our results are in line with previous findings in lettuce<sup>60</sup> and cucumber<sup>41</sup>. Similarly, Hogewoning et  $al.^{61}$  reported an increase in total chlorophyll in cucumber under R/B = 1 ratio, the same as the Blue net used in our experiment. In watermelon, the increase in total chlorophyll under the Blue net showed the same trend as the Spad index (**Table 3**), as previously reported by Son et al.60 in lettuce. In tomato, the highest chlorophyll content was recorded in shading treatments (Table 4). Probably, tomato plants felt the reduction in light intensity (but not quality) and produced more photosynthetic pigments to absorb more light energy. Although chlorophyll content is reported in the literature to be positively associated with photosynthetic capacity and indirectly with productivity<sup>60</sup>, our results do not correlate positively with shoot dry weight (Table 1). However, for nursery seedlings, this result could positively correlate with survival during plant establishment, in addition to providing a productive boost to adult plants.

Common adaptations to irradiation include an increase in the chlorophyll a/b ratio, a parameter that is proposed as a biological assay to evaluate the light environment<sup>62</sup>. However, **Table 5** does not show a clear trend among species for this parameter. Under the Blue net, in zucchini squash and eggplant, chlorophyll a/b ratio decreased as total chlorophyll increased, which is attributable to an increased chlorophyll b production under blue light (data not shown). In contrast, in tomato grown under shading, we observed an increase in chlorophyll a/b, compared to the No shading condition. Watermelon did not show significant differences in chlorophyll a/b or carotenoid for both intensity and quality of light.

Carotenoids are accessory pigments that capture light and transfer energy to chlorophylls and have photoprotective and antioxidant functions<sup>59</sup>. In zucchini squash and eggplant, carotenoids increased as the light intensity increased (on average, +33.52 and +9.48%, respectively), compared to shading conditions. Our results reflect carotenoids role in protecting leaves from excessive light. Carotenoids probably protected the photosynthetic machinery from high light intensity under No shading treatment<sup>63</sup>. Not least, in tomato, the Blue net reduced carotenoids by 4.92 and 4.13%, compared to the Black net and the No shading treatments, respectively.

**Table 5**. Effects of shading and light quality on the accumulation of pigments of zucchini squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus L.*), tomato (*Solanum lycopersicum L.*), and eggplant (*Solanum melongena L.*) seedlings. Data are mean values ± standard error, n=3.

Enorio	Treatment	Total Chlorophyll	Chlorophyll a/b	Carotenoids	
Specie	I reatment	(mg g <sup>-1</sup> fw)	(mg g <sup>-1</sup> fw)	(mg g <sup>-1</sup> fw)	
	No shading	1.741±0.022 c	1.378±0.030 a	0.237±0.006 a	
Zucchini squash	Black net	1.885±0.002 b	1.308±0.017 a	0.210±0.004 b	
	Blue net	2.074±0.002 a	1.125±0.025 b	0.145±0.002 c	
	Significance	***	***	***	
	No shading	1.397±0.009 b	1.838±0.005	0.345±0.001	
Watermelon	Black net	1.359±0.006 b	1.884±0.045	0.346±0.002	
	Blue net	1.524±0.015 a	1.774±0.036	$0.349 \pm 0.002$	
	Significance	***	ns	ns	
	No shading	1.040±0.026 b	1.768±0.032 b	0.363±0.002 a	
Tomato	Black net	1.161±0.006 a	1.967±0.014 a	0.366±0.001 a	
	Blue net	1.138±0.007 a	1.982±0.021 a	0.348±0.003 b	
	Significance	**	***	**	
	No shading	1.395±0.004 c	1.691±0.020 a	0.335±0.003 a	
Eggplant	Black net	1.450±0.011 b	1.642±0.003 a	0.317±0.003 b	
	Blue net	1.529±0.017 a	1.539±0.007 b	0.296±0.005 c	
	Significance	***	***	***	

Different letters within columns indicate significant mean differences according to Tukey's HSD test (p = 0.05). ns, \*\* and \*\*\* denote non-significant or significant effects at  $p \le 0.01$ , and 0.001, respectively.

### 175 Chapter 7

# 3.4. Cluster Heatmap of the Effects of Light Intensity and Quality on Morphometric and Quality Indices, Minerals, Colorimetric Parameters, and Pigments Accumulation in Seedlings.

Heat maps were made to provide a detailed view of the seedlings' morphometric, quality, mineral, color, and pigment parameters under different light treatments (light intensity and quality). In general, a different response was observed between families (*Solanaceae* and *Cucurbitaceae*) and between species.

Except for watermelon (**Figure 2B**), heatmaps analyses of aggregate data in zucchini squash (**Figure 2A**), tomato (**Figure 2C**), and eggplant (**Figure 2D**) identified two main clusters corresponding to the high light intensity treatment (No shading) and shading treatments (Black net and Blue net) (**Figure 2**). Two separate sub-clusters (Black net and Blue net) were defined under the second cluster indicating that shading was the main clustering factor, while light spectrum modification was the second.

In zucchini squash, blue net reduced leaf area, chlorophyll a/b ratio, shoot and root dry weight, shoot/root ratio, sturdiness index, increased stem diameter, specific leaf area, and total chlorophyll content (**Figure 2A**). Similarly, the blue net increased stem diameter, total chlorophyll, calcium and magnesium, and carotenoids in watermelon while reducing shoot/root ratio and chlorophyll a/b ratio (**Figure 2B**). In contrast to the findings of *Cucurbitaceae*, in tomato and eggplant, the blue net had less effect on size reduction (**Figure 2**). In tomato, an increase in stem diameter was observed under black shading net, leading at the same time to the rise in height and thus to a higher sturdiness index (**Figure 2C**). In tomato, unshading conditions resulted in lower leaf area, lower chlorophyll a/b ratio, lower specific leaf area, lower total chlorophyll content (**Figure 2C**). In eggplant, the black net increased plant height and high sturdiness index. High light intensity reduced shoot/root ratio and shoot dry weight, leaf area, specific leaf area, root length, total chlorophyll, and stem diameter while increasing chlorophyll a/b ratio (**Figure 2D**).

176



**Figure 2**. Heatmap analysis summarizing the results of morphometric and quality indices, minerals, colorimetric parameters, and accumulation of pigments of *Cucurbita pepo* L. (A), *Citrullus lanatus* L. (B), *Solanum lycopersicum* L. (C), and *Solanum melongena* L. (D) seedlings. Original values are ln(x + 1)-transformed. Columns with similar annotations are collapsed by taking the mean inside each group. The rows are centered; unit variance scaling is applied to the rows. Both rows and columns are clustered using Euclidean distance and complete linkage.

### 177 Chapter 7

#### 3.6. Effects of Light Intensity and Quality on Metabolic profile of Seedlings

The metabolic profile of *Cucurbitaceae* (zucchini and watermelon) and *Solanaceae* (tomato and eggplant) seedling was obtained by using an untargeted metabolomics approach to better understand the effect of shading on the physiological process. More than 4000 metabolites were detected through UHPLC-QTOF-MS analysis. To identify a general trend in plant response to light modulation, only the shading conditions were considered as a factor for supervised multivariate statistics, and all the species were investigated together for the metabolomics analysis. The entire dataset was analyzed using the supervised orthogonal partial least squares discriminant analysis (OPLS-DA) resulting in a clear separation of samples in the score plot based on the net shading (**Figure 3**). In fact, the first latent vector t[1] clearly indicated that shading triggered a specific metabolic signature different from the unshading plants. Moreover, the second latent vector t[2] showed that shading plants presented a distinctive metabolic profile depending on the net (blue or black).



**Figure 3.** Score plot of orthogonal projection to latent structures discriminant analysis (OPLS-DA) supervised modeling carried out on untargeted metabolomics profiles of zucchini, watermelon, tomato, and eggplants leaves and considering the light quality and intensity as a factor.

178

Therefore, as suggested by the morphometric and quality indices of seedlings, the metabolic profiles indicated a precise modulation of the leaf at the molecular level when changing light quality and intensity, which corroborates morphological changes. In this sense, Wang et al.<sup>69</sup> reported the modulation of the biochemical fingerprint of tea plants under different light intensity, in particular under three supplemental intensities of blue light.

Once confirmed that shading strongly modulated leaf metabolic profile regardless of the plant species, the discriminant metabolites that explain the separation of profiles in the score plot were selected by the variable importance in projection (VIP) analysis. Venn diagrams show that most compounds overlap for black and blue net, indicating a shared effect of shading (Figure 4A) according to previous studies that pointed out light quality and intensity as an essential factor in plant metabolism<sup>20,69</sup>. However, 45 and 24 compounds were down and up accumulated, respectively, in the sole presence of the black net. In comparison, 25 and 45 compounds were down and up accumulated, respectively, exclusively in the presence of the blue net. Regardless of the specific metabolites, both black and blue net presented a high ratio of down/up accumulated compounds since black net decreased the biosynthesis of 153 compounds while increased the biosynthesis of 86 while for the blue net 133 compounds decreased and 107 increased. Looking at the specific metabolites, the most discriminant markers were those related to terpenes and phenylpropanoids possessing the highest VIP score and indicating their strong implication in plant response to light intensity and quality, as previously reported<sup>69</sup>. Nevertheless, several classes of metabolites including primary and secondary metabolism were found to be discriminant in plant response. The influence of blue quality and intensity on plant metabolism has been previously confirmed through the metabolomics and transcriptomic approach that revealed that low-intensity blue, medium-intensity blue light, and high-intensity blue light triggered a reprogramming in essential physiological processes and secondary metabolism<sup>69</sup>. Moreover, it has been reported that shading alters nitrogen and carbon metabolism, which explains the changes observed at the biochemical level and is supported by the nitrate concentration data under shading<sup>20</sup> (Table 4).



**Figure 4.** (**A**) Venn diagram summarizing the discriminant metabolites down and up accumulated under blue and black nets compared to the unshading plants, as resulted from the variable importance in projection (VIP) analysis (VIP score 1.3). (**B**) Metabolic processes impaired by shading (blue and black net). Metabolites resulted as discriminant from the VIP analysis, and their fold-change values were elaborated using the Omic Viewer Dashboard of the PlantCyc Pathway Tool software (www.pmn.plantcyc.com). The large dots represent the average (mean) of all log Fold-change (FC) for metabolites, and the small dots represent the individual log FC for each metabolite. The x-axis represents each set of subcategories, while the y-axis corresponds to the cumulative log FC. Nucleo: nucleosides and nucleotides; FA/Lipids: fatty acids and lipids; Amines: amines and polyamines; Carbohyd: carbohydrates; Secondary met: secondary metabolism; Cofactors: cofactors, prosthetic groups, electron carriers, and vitamins; Cell-structures: plant cell structures; Metab reg: metabolic regulators.

180

Considering the chemical diversity of VIP compounds, these 238 metabolites were further analyzed by classifying them into the plant biosynthetic pathways (**Figure 4B**). **Figure 4B** depicts the biochemical reprogramming triggered by light intensity and quality in plant leaves regardless of the species. Overall, shading seemed to positively modulate those pathways related to primary metabolism (*i.e.*, amino acids, nucleotides, and carbohydrate biosynthesis) while compromising secondary metabolism. However, those molecules involved in several essential processes as phosphoenolpyruvate or cabamoyl-aspartate increased under shading while citrate and isocitrate decreased as a common response. Previously studies reported that energy metabolism was affected by shading. In particular, Li et al.<sup>20</sup> observed a decrease in sugar content and suggested a lower need for energy under shading conditions that lead to changes in carbon flux from the synthesis of glucose to a feedback mechanism by shifting stored glucose to amino acid metabolism instead of normal carbon metabolism.

On the other hand, according to our results, Lakshmanan et al.<sup>70</sup> observed an increase in the flux of metabolic pathways after blue light treatment in Arabidopsis Thaliana, including the biosynthesis of lipids. Our findings revealed that fatty acids and lipid biosynthesis was positively regulated by the blue net rather than the black net. In agreement with our results, Wang et al.<sup>69</sup> observed that blue light promoted lipid biosynthesis, mainly sterols and sphingolipids that are membrane structural components and might act as signal molecules. Notably, compounds classified into "cofactors, carriers, and vitamin biosynthesis" were modulated by black and blue 6-methoxy-3-methyl-2-all-trans-decaprenyl-1,4-benzoquinol, 3shading. demethylubiquinol-9, 3-demethylubiquinol-9 and 3-nonaprenyl-4-hydroxybenzoate decreased under shading while 3,4-dihydroxy-5-all-trans-decaprenylbenzoate increased, pointing out modulation of ubiquinone pathway and respiratory electron transport in this response. In contrast, thiamine and thiamine diphosphate were positively regulated by the blue net and negatively modulated by the black net. Moreover, several compounds related to the biosynthesis of chlorophylls upstream (*i.e.*, Mg-protoporphyrin, haematoporphyrin, uroporphyrin) were positively modulated under shading, according to the physiological measures including the uptake of Mg and the literature, while chlorophyll degradation products (protochlorophyll a) decreased44,63.

On the other hand, secondary metabolism biosynthesis was strongly repressed by both the blue and black net. This repression is reflected in the marked down accumulation of nitrogen-containing compounds, which was the most affected class of secondary metabolites. This might be explained by the modulation in amino acid metabolism, phenylpropanoids and terpenes being more marked for black shading<sup>20</sup>. Despite this, blue net provokes an accumulation of precursors of N-containing metabolites and the accumulation of some phenylpropanoids (dalnigrein 7-O- $\beta$ -D-

## 181 Chapter 7

apiofuranosyl-(1-6)- $\beta$ -D-glucopyranoside, amorphigenin, cyanidin 3-O-(6"-O-malonyl)- $\beta$ -glucoside, 4-hydroxycoumarin) according to previous results. In fact, light intensity and shading regulate the expression of the genes and the activity of enzymes involved in the biosynthesis of flavonoids, anthocyanin, catechins, and flavanols<sup>20</sup>. In particular, blue light affects the synthesis of flavonoids even if this modulation (positive or negative) depends on plant species but also the light intensity<sup>69</sup>. In addition, flavonoid metabolism is also influenced by the TCA cycle and the biosynthesis of carbohydrates and amino acids, indicating a complex network between primary and secondary metabolism under shading rather than a direct effect on the specific expression of key genes<sup>20</sup>. In contrast, terpenoids seemed to be shading-specific modulated and seemed to be particularly altered by shading, as suggested by the VIP analysis. Precursors such as mevalonate and squalene, and their final products as sterol and carotenoids and terpene hormones as brassinosteroids were specifically modulated, being this effect stronger under blue shading in agreement with the general modulation of lipids under shading.

#### Conclusions

Light drives many vital processes in plants, which show different morphophysiological responses to varying light intensity and quality as an adaptation. For example, shading increases leaf area and pigment content, while high light intensity increases photosynthetic activity and shoot growth. However, changing light quality also induces adaptive changes in plants. Due to self-shading, blue light depletion in high-density plants reduces seedling quality (less compactness), driving producers to use chemical size regulators. In our work, we demonstrated that the response of plants to changing light intensity and quality is species-specific. Moreover, the untargeted metabolomics approach allowed us to identify a common pattern across species in response to shading. Considering that light controls essential biochemical and physiological processes, our results highlighted that both primary and secondary metabolism, together with phytohormone profile, were largely affected by shading, resulting in a biochemical modulation much broader than photosynthesis and phytohormone profiles. These common patterns included plant energy metabolism and lipid biosynthesis and included a down accumulation of secondary pathways, particularly regarding phenylpropanoids.

The morphological changes induced by the different shading conditions corroborate the shift in metabolomic signatures we observed, indicating that a set of biological processes are modulated by shading. The comprehension of the mechanisms involved pivotally supports the implementation of photoselective shading in dedicated applications, towards the definition of more resilient crop production. Such information is of general relevance and is even more important in cropping systems under less favorable intense light conditions, where photoselective shading could represent a sustainable approach. Notwithstanding, it is also important to consider that further information is advisable in the future on this subject, including the effect on different crops and/or at different plant growth stages.

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# Chapter 8

# Conclusions

Scientists, extensions specialists, food nutritionists, and farmers are motivated by climate change and consumer appreciation for healthy foods to broaden their vision on conventional production processes, especially in the warm regions of the Mediterranean, where light, high temperatures, and biotic pressure (e.g., insects, fungal, and bacterial disease) pose challenges for horticulture. This is encouraging to embrace multidisciplinary approaches to improve productivity, including new growth goals, pest control strategies, and stress reduction tools in eco-friendly perspectives. The introduction of insect nets in agriculture has provided a practical, green, and ecosustainable tool for pest management. However, most research on improving the airflow of insect nets has been conducted using simulated computational fluid dynamics models without considering the interaction of crops with the nets under real growing conditions. The results presented in Chapter 3 helped shed light on the effects of insect nets on microclimate and quanti-quantitative traits in zucchini squash (Cucurbita pepo L.), suggesting that different porosity modulates fruit marketable and early yield as well as nutritional quality of the product. In particular, the better air exchange of the net with higher porosity (AirPlus<sup>®</sup> 50 mesh) improved fruit quality (higher dry matter content, antioxidant activities, total polyphenols, and total ascorbic acid) and physiological parameters (increased CO<sub>2</sub> assimilation rate and transpiration) without any negative impact on yield and number of fruits. On the other hand, the net with lower porosity could be an efficient tool for anticipating production in fall-winter or late-winter crop cycles when lower temperatures are appreciated.

Light drives many life processes in plants, which manifest different morphophysiological responses as the intensity of light changes to adapt to a specific light environment. High light intensity and high air temperatures in Mediterranean regions pose a challenge for the production of model crops such as lettuce (*Lactuca sativa* L.), arugula (*Diplotaxis tenuifolia* L.), and tomato (*Solanum lycopersicum* L.). From this perspective, shading nets reduce the percentage of light and change its direction, resulting in greater light penetration into the canopy. In hot Mediterranean summers, the growth and yield of lettuce (*Lactuca sativa* L.) are strongly influenced by high light intensity and high temperatures, which makes a careful selection of genotypes tolerant to sub-optimal summer conditions and the use of shade nets mandatory. The results shown in Chapter 4 demonstrated that different lettuce genotypes respond differently to varying light intensities. The Canasta lettuce cultivar increased fresh yield and intrinsic (*i.e.*, physiological) water use efficiency in the unshaded treatment, related to specific morpho-anatomical adaptations, such as reduced stomatal and epidermal cell density. However, the white shading net proved helpful in providing an appropriate microclimate during the early summer season, ensuring the growth of the more sensitive cultivars Ballerina, Oak leaf, and Romaine.

The light and temperature inside the greenhouse should encourage the leaves of perennial arugula (*Diplotaxis tenuifolia* L.) to reach an appropriate shape, with petioles that are not excessively long compared to the blades and an appreciable concentration of phytochemicals. However, the optimal degree of light extinction for maximum yield and product quality depends on the growing season. The above environmental parameters also influence crop performance depending on the growing season. The study presented in Chapter 5 on the perennial arugula showed that shading nets are an interesting tool within sustainable horticultural systems. Specifically, a 50% crop shading degree is a sustainable tool to increase perennial arugula leaf yield in July characterized by high environmental pressure when the light intensity exceeds the plant's needs while also achieving a mineral content not significantly divergent from unshaded crops. Ascorbic acid and hydrophilic antioxidant activity were more affected by higher light intensity.

In tomato (*Solanum lycopersicum* L.), fruit quality is influenced by environmental factors such as photothermal stress. As shown in Chapter 6, white and pearl grey non-photo selective shading nets did not significantly affect total yield. However, reducing solar radiation through pearl grey nets seems to be a promising tool to increase the phytochemical quality of tomato fruits during summer cultivation in a Mediterranean environment. The improved light diffusion of the pearl grey shading net increased total carotenoids and phenolic compounds. The promising results obtained pave the way for future investigations to evaluate the quality responses induced by pearl grey netting in other vegetables.

Changes in the quantity and spectral quality of light can induce morphophysiological adaptations in seedlings for nursery production. For example, in high-density plantings, blue photo selective nets can improve the compactness of nursery plants, limiting the use of chemical size regulators. In Chapter 7, we observed a species-specific response to changing the intensity and quality of light. High light intensity and photo selective blue net reduced size in all species studied, compared to the non-photo selective black shading net. In watermelon (*Citrullus lanatus* L.), the blue net reduced the shoot/root ratio compared to eggplant (*Solanum melongena* L.) and tomato (*Solanum lycopersicum* L.), which showed the same result under unshaded conditions. In zucchini squash (*Cucurbita pepo* L.), no significant shoot/root ratio variation was observed between treatments, while the blue net had the lowest carotenoid content.

Our results highlighted that both primary and secondary metabolism, together with phytohormone profile, were largely affected by shading, resulting in a biochemical modulation much broader than photosynthesis and phytohormone profiles. These common patterns included plant energy metabolism and lipid biosynthesis and included a down accumulation of secondary pathways, particularly regarding phenylpropanoids.

The morphological changes induced by the different shading conditions corroborate the shift in metabolomic signatures we observed, indicating that a set of biological processes are modulated by shading. The promising results obtained pave the way for future investigations to evaluate the qualitative responses induced by blue photo selective nets in nursery seedling production.

# Appendix



# Dataset on the Effects of Anti-Insect Nets of Different Porosity on Mineral and Organic Acids Profile of *Cucurbita pepo* L. Fruits and Leaves

Luigi Formisano, Michele Ciriello, Christophe El-Nakhel, Stefania De Pascale, and Youssef Rouphael\*

**Abstract:** The growing interest in healthy foods has driven the agricultural sector towards ecofriendly implementation to manage biotic and abiotic factors in protected environments. In this perspective, anti-insect nets are an effective tool for controlling harmful insect populations concomitantly with reducing chemicals' interference. However, the low porosity of nets necessary to ensure high exclusion efficiency for a designated insect leads to reduced airflow, impacting the productivity and quality attributes of vegetables. The evidence presented in this dataset pertains to the content of total nitrogen, minerals (*i.e.*, NO<sub>3</sub>, K, PO<sub>4</sub>, SO<sub>4</sub>, Ca, Mg, Cl, and Na), and organic acids (*i.e.*, malate and citrate) of zucchini squash (*Cucurbita pepo* L. cv. Zufolo F1) in leaves and fruits grown with two anti-insect nets with different porosities (Biorete® 50 mesh and Biorete® 50 mesh AirPlus), is and analyzed by the Kjeldahl method and ion chromatography (ICS3000), respectively. Data of total nitrogen concentration, macronutrients, and organic acids provide indepth information about plants' physiological response to microclimate changes induced by antiinsect nets. The evidence reported in this dataset supports the research article "Improved Porosity of Insect Proof Screens Enhances Quality Aspects of Zucchini Squash without Compromising the Yield".

Dataset: https://zenodo.org/record/4749122#.YJqTLrUzbZR

Dataset License: CC BY-NC-ND

Keywords: Zucchini squash; Insect-proof screens; Protected environment; Ion chromatography

190

### 191 Appendix

#### 1. Summary

Agriculture supplies more than half of the food for feeding the world's rapidly growing population, but less than 50% of the total production is harvested (*i.e.*, yield potential) due to abiotic and biotic factors such as photothermal stress, pathogenic fungi and insects<sup>1,6</sup>. Guaranteeing ideal growing conditions is mandatory, especially in warm Mediterranean areas where the constraints imposed by climate change are causing severe yield and quality losses<sup>1</sup>. Growers and consumer awareness for environmentally sustainable agricultural systems drive the agricultural sector to implement agronomic strategies relying on integrated pest management (IPM). In the past, farmers have used chemical pesticides indiscriminately for pest control with detrimental impacts on the ecosystem<sup>7</sup>. However, the severe limitations on the use of chemical insecticides and the increasing consumer demand for organic and pesticide-free vegetables have pushed growers towards ecologically and economically sustainable alternatives8. In this framework, anti-insect nets represent an effective and "green" solution for the containment of harmful insects in protected environments, achieving 90% effectiveness in excluding designated harmful pests9. On the other hand, their exclusion performance depends on the weft and warp thread arrangement and, consequently, on the holes' geometry and structure; hence, this performance decreases when the holes' size increases<sup>10,11</sup>. However, low porosity (percentage of the ratio of open mesh area to total mesh area) causes a high-pressure drop, which reduces airflow with a consequent increase in temperature in the growing environment, which is detrimental for crop growth<sup>4,12,13</sup>. It is well-known that heat stress induces molecular, biochemical, and morphological changes in plants as an adaptive response to adverse conditions<sup>14,15</sup>. Several studies have highlighted the critical role of Ca and K in stress signaling and the regulation of growth and developmental processes<sup>16</sup>. High temperatures affect the photosynthetic process (primary metabolism)17,18, which is strictly related to leaf macronutrient concentrations such as nitrogen (N), which is the main constituent of proteins involved in the C<sub>3</sub> cycle (Calvin–Benson), magnesium (Mg), sulfur (S), and phosphorus (P)<sup>19</sup>. The latter plays a crucial role in cellular processes, stabilizing cell membranes, contributing to the synthesis of energy molecules such as ATP and ADP and nucleic acids<sup>20</sup>. On the other hand, the high consumer demand for fresh vegetables all year round has encouraged growers to expand their production potential in protected environments. In the last decades, zucchini squash (Cucurbita pepo L.) has gained popularity in the European horticultural markets and has become one of the most demanded and consumed vegetables. In the Italian horticultural market, a consumption of about 9 kg per capita is estimated<sup>21</sup>, with a greenhouse production of more than 200 tons per year<sup>22</sup>. At present, research has focused mainly on the exclusion efficiency of anti-insect nets, ignoring the impact induced on the inner microclimate of growing environments and thus on the production and quality of Cucurbitaceae. This data



descriptor reports a dataset acquired about total nitrogen and ion chromatographic analysis on leaves and fruits of *Cucurbita pepo* L. to integrate our previous study aimed at assessing the effects of the microclimate induced by two anti-insect nets with different porosities (Biorete<sup>®</sup> 50 mesh and Biorete<sup>®</sup> 50 mesh AirPlus; Arrigoni S.P.A, Uggiate Trevano, Como, Italy) on the qualitative–quantitative performance of zucchini squash<sup>1</sup>. Our goal was to investigate how the suboptimal microclimate induced by two anti-insect nets could affect the mineral profile of zucchini leaves and fruits, as well as the content of malate and citrate, which are organic acids crucial for the taste and flavor of food and represent important phytochemicals for biological processes<sup>23</sup>.

### 2. Data Description

The data release is stored Zenodo on (https://zenodo.org/record/4749122#.YJqTLrUzbZR). The dataset has two spreadsheets named "leaves" and "fruits", each corresponding to zucchini leaves and fruits, respectively. Both worksheets have the same data layout, distributed in thirteen columns (from letters A to M) and ten rows. Specifically, the first column (A) indicates the used treatments: "No-net" (i.e., control without anti-insect net; from A2 to A4 columns); "50 mesh AP" (i.e., treatment with Biorete® 50 mesh AirPlus; from A5 to A7 columns); "50 mesh", (i.e., treatment with Biorete<sup>®</sup> 50 mesh; from A8 to A10 columns). The second column (B) reports the number of replicates for each treatment. The third column (C) reports the total nitrogen content determined by the Kjeldahl method, expressed as g kg<sup>-1</sup> dw. From column D to column M, the concentrations of NO<sub>3</sub>, K, PO<sub>4</sub>, SO<sub>4</sub>, Ca, Mg, Cl, Na, malate, and citrate, respectively, were determined by ion chromatography and expressed as g kg<sup>-1</sup> dw. The data from our dataset were subjected to statistical analysis and are reported in Table 1 and Table 2

# 193 Appendix

**Table 1**. Total nitrogen, minerals, and organic acids of zucchini squash leaves grown in a protected environment with anti-insect nets.All data are expressed as the mean  $\pm$  standard error, n=3.

Treatments-	Total N	NO <sub>3</sub>	К	PO <sub>4</sub>	$SO_4$	Ca	Mg	Cl	Na	Malate	Citrate
	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)
No-net	$22.40\pm0.39a$	$0.12\pm0.01$	$31.68 \pm 1.12$	$2.82\pm0.43b$	$4.49\pm0.30a$	$17.50 \pm 0.67a$	$4.84 \pm 0.32a$	$15.53 \pm 0.53a$	$0.27\pm0.03c$	$21.30 \pm 0.99b$	12.31 ± 1.03a
50 mesh AP	$17.45\pm0.52c$	$0.21\pm0.04$	$29.71 \pm 1.71$	$9.72 \pm 0.51a$	$1.74\pm0.14b$	$14.96 \pm 0.53b$	$5.12 \pm 0.09a$	$4.33\pm0.19b$	$1.84 \pm 0.08a$	31.70 ± 1.21a	$9.12\pm0.32b$
50 mesh	$19.93\pm0.91\mathrm{b}$	$0.23\pm0.04$	$27.07\pm0.65$	$9.66 \pm 0.76a$	$2.62\pm0.31b$	$10.47\pm0.50c$	$4.11\pm0.05b$	$2.91\pm0.28c$	$1.20\pm0.04b$	$24.41 \pm 1.45b$	$8.18\pm0.85b$
Significance	**	ns	ns	***	***	***	*	***	***	**	*

ns, \*, \*\*, \*\*\* non-significant or significant at  $p \le 0.05$ , 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p = 0.05).



**Table 2.** Total nitrogen, minerals, and organic acids of zucchini squash fruits grown in a protected environment with anti-insect nets. All data are expressed as the mean ± standard error, *n*=3.

Treatments	Total N	NO <sub>3</sub>	К	PO <sub>4</sub>	SO <sub>4</sub>	Ca	Mg	Cl	Na	Malate	Citrate
	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)	(g kg <sup>-1</sup> dw)
No-net	$36.04\pm0.94\mathrm{b}$	$0.20\pm0.16$	$44.12\pm0.14$	$18.79\pm0.70$	$2.51 \pm 0.29$	$4.54\pm0.14a$	$4.14\pm0.28$	$8.69\pm0.74a$	$2.87\pm0.16$	$25.52 \pm 1.22a$	$2.43 \pm 0.32$
50 mesh AI	P35.65 ± 1.08b	$0.19\pm0.12$	$41.63 \pm 0.91$	$18.32 \pm 1.12$	$2.40\pm0.12$	$2.94\pm0.21\mathrm{b}$	$4.34\pm0.14$	$6.43\pm0.27b$	$3.18\pm0.17$	$21.21\pm0.16b$	$3.19\pm0.18$
50 mesh	$39.71 \pm 0.61a$	$0.21\pm0.05$	$41.36 \pm 1.39$	$18.62 \pm 2.08$	$2.84\pm0.46$	$2.07\pm0.04c$	$3.61\pm0.20$	$5.75\pm0.10b$	$2.81 \pm 0.20$	$15.37 \pm 1.19 \mathrm{c}$	$2.23\pm0.34$
Significance	e *	ns	ns	ns	ns	***	ns	**	ns	***	ns

ns, \*, \*\*, \*\*\* non-significant or significant at  $p \le 0.05$ , 0.01 and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p = 0.05).

#### 3. Methods

#### 3.1. Experimental Design and Plant Samples Collection

The experiment was conducted in 2019 at "Torre Lama" experimental farm of the University of Naples (Bellizzi, Salerno, Italy; latitude 43°31' N, longitude 14°58' E, altitude 60 m). Experimental treatments consisted of two 50-mesh anti-insect nets, with different porosity and air permeability (Biorete® 50 mesh and Biorete® 50 mesh AirPlus; Arrigoni S.p.A, Uggiate Trevano, Italy) that covered the sidewalls and ventilation openings of the two tunnels, while the third tunnel was used as an unscreened control. Zucchini seedlings (*Cucurbita pepo* L. cv. Zufolo F1; Olter, Piacenza, Italy) were transplanted on 1 April 2019, in three single rows with a density of 1 plant m<sup>2</sup>. The experimental trial lasted until 17 July 2019. Marketable fruits (minimum length of 12 cm) were harvested three times a week starting 60 days after transplant (DAT).

At 102 DAT, 30 marketable fruits per treatment were harvested, cut in half, and placed in a ventilated oven at 80 °C until a constant weight was reached (~5 days). The specific time for fruit harvesting (102 DAT) was chosen because the production of zucchini squash fruit was more uniform and representative in all the growing tunnels (screened and unscreened), as also supported by the literature [24,25]. At the end of the experiment (17 July 2019, 107 DAT), 20 fully expanded leaves per plot were harvested and placed in a ventilated oven at 70 °C for 3 days. The dry plant material (leaves and fruits) was ground in a MF10.1 Wiley Laboratory mill, IKA® (Staufen im Breisgau, Baden-Württemberg, Germany) and sieved with an MF0.5 sieve (0.5 mm hole size; IKA®, Staufen im Breisgau, Baden-Württemberg, Germany) for total nitrogen, minerals, and organic acid determination.

#### 3.2. Total Nitrogen Determination

Total nitrogen content in zucchini squash leaves and fruits was determined according to the Kjeldahl method with minor modifications<sup>26</sup>.

In detail, one gram of ground dry sample was weighed and mixed in a 250 mL borosilicate glass tube (Ø42 × 300 mm; Velp® Scientifica, Usmate Velate, Monza Brianza, Italy) with 7 mL of 96% sulfuric acid (H<sub>2</sub>SO<sub>4</sub>; Carlo Erba Reagents Srl., Milan, Italy), and antifoam catalyst (3.5 g K<sub>2</sub>SO<sub>4</sub> + 0.1 g CuSO<sub>4</sub> × 5H<sub>2</sub>O; Velp® Scientifica, Usmate Velate, Monza Brianza, Italy) and 10 mL of 30% hydrogen peroxide (Carlo Erba Reagents Srl., Milan, Italy). The tubes were placed on a heating digester (DK 20 Heating Digester; Velp® Scientifica, Usmate Velate, Monza Brianza, Italy) for 30 min at 420 °C. As a result of the digestion phase, ammonium sulphate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] was produced:

analyte + H<sub>2</sub>SO<sub>4</sub> 
$$\rightarrow$$
 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + CO<sub>2</sub> + SO<sub>2</sub> + H<sub>2</sub>O (1)

After the digestion phase, the tubes with mineralized samples were cooled and then distilled in a UDK 140 distiller (Velp<sup>®</sup> Scientifica, Usmate Velate, Monza Brianza, Italy)



by adding 33% sodium hydroxide (NaOH; Titolchimica, Pontecchio Polesine, Italy) (distillation phase). Under these conditions, the ammonium ion was transformed into ammonia:

$$(NH_4)_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + 2H_2O + 2NH_3$$
<sup>(2)</sup>

Ammonia was trapped in boric acid (H<sub>3</sub>BO<sub>3</sub>; Honeywell Riedel-de haën, Charlotte, USA) by steam distillation and collected in an Erlenmeyer flask:

$$NH_3 + H_3BO_3 \rightarrow NH_4H_2BO_3 + H_3BO_3$$
(3)

A methyl red ( $C_{15}H_{15}N_3O_2$ ) and bromocresol green ( $C_{21}H_{14}Br_4O_5S$ ) indicator (HACH, Loveland, Colorado, USA) were added to the distilled solution and titrated with 0.1 N sulfuric acid (Carlo Erba Reagents Srl., Milan, Italy) until the acquisition of reddish color by the solution (pH around 5.0). The titration volume was used to calculate the percentage of total nitrogen that was converted to g of nitrogen per kg of dry weight (dw). Each treatment was analyzed in triplicate.

### 3.3. Minerals and Organic Acid Determination

In accordance with Rouphael et al.<sup>27</sup>, and in order to determine cations (K, Ca, Mg, Na), anions (NO<sub>3</sub>, SO<sub>4</sub>, PO<sub>4</sub>, Cl), and organic acids (malate and citrate), 250 mg of finely ground dried leaves and fruits were weighed on a PI-314.1 analytical balance (Denver Instruments, Denver, Colorado, USA), then placed in centrifuge tubes (Corning<sup>®</sup>, New York, USA) and mixed with 50 mL of ultra-pure water prepared through an Arium® Advance EDI pure water system (Sartorius, Goettingen, Lower Saxony, Germany). The samples were frozen and thawed in liquid nitrogen three times and immersed in a SW22 shaking water bath (80 °C, 100 rpm, 10 min; Julabo, Seelbach, Baden-Württemberg, Germany) and then centrifuged with an R-10M centrifuge (6,000 rpm, 10 min; Remi Elektrotechnik Ltd., Mumbai, Maharashtra, India). The supernatant was filtered with a specific syringe filter (0.45 µm pore size; Whatman International Ltd., Maidstone, Kent, UK), and processed by ion chromatography coupled to an electrical conductivity detector (ICS3000, Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup>, Sunnyvale, CA, USA), using a sample injection volume of 25 µL. Isocratic separation of cations (K, Ca, Mg, Na) was performed using a 4  $\times$  250 mm analytical column (IonPac CS12A, Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup>, Sunnyvale, CA, USA) equipped with an IonPac CG12A precolumn (4 × 250 mm; Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup>, Sunnyvale, CA, USA) and an electrolytically self-regenerating suppressor (CERS500; 4 mm, Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA). The eluent consisted of 25 mM methanesulfonic acid (Sigma Aldrich, Milan, Italy), prepared with ultrapure water. The separation of anions (NO<sub>3</sub>, SO<sub>4</sub>, PO<sub>4</sub>, Cl) and organic acids (malate and citrate) were performed in gradient mode using an IonPac® ATC-HC anion trap (9 × 75 mm; Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup>, Sunnyvale, CA, USA), an IonPac<sup>®</sup> AG11-HC guard column (4 × 50 mm; Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA), an IonPac® AG11-HC IC column (4 × 50 mm; Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup>, Sunnyvale, CA,

### 197 Appendix

USA), and a self-regenerating dynamic suppressor (DRS600; 4 mm, Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA) using 5 mM–30mM potassium hydroxide (KOH) with a flow of 1.5 mL min<sup>-1</sup>. All columns were kept at 30 °C. Integration and quantification of minerals and organic acids were performed using z Chromeleon<sup>™</sup> 6.8 Chromatography Data System (CDS) Software (Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA), by comparing the peak areas of samples with those of the standards. Multistandard solutions (anionic and cationic) were prepared as combinations of individual Ion Chromatography certificate standard solutions (Thermo Scientific<sup>™</sup> Dionex<sup>™</sup>, Sunnyvale, CA, USA). Concentrations of anions, cations, and organic acids in leaves and fruits were expressed as g kg<sup>-1</sup> dw. Each treatment was analyzed in triplicate.

#### 3.4. Statistic

The evidence presented in this dataset was analyzed with IBM SPSS Statistics (SPSS Inc., Chicago, IL, USA) software version 26.0 for Windows 10 (Microsoft Corporation, Redmond, Washington, USA). All data are presented as the mean  $\pm$  standard error, *n*=3. The mean effects of total nitrogen, minerals, and organic acids were subjected to Oneway ANalysis Of VAriance (ANOVA). Statistical significance was determined with Duncan's Multiple Range Test (DRMT) at *p* < 0.05 level.



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# 201 Appendix

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