University of Naples Federico II

Department of Biology

PhD Thesis

Improving photosynthetic efficiency and plant growth in controlled environments: the role of light quality, biostimulant application and ionising radiation

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Index

General Introduction

1.1 Light quality and higher plants

Light is one of the most important ecological factors affecting the development of higher plants on Earth (Whitelam and Halliday, 2007). Plants exhibit remarkable plasticity in response to light environments (Ouzounis *et al.*, 2015). In their natural habitats, plants are exposed to continuous light spectral variations depending on weather conditions, time of day, season and latitude and in turn, they need to modulate and adjust all physiological processes in response to the different light perception.

The three light dimensions, namely photoperiod (light duration), quantity (intensity) and quality (spectral distribution) significantly affect plant growth, development and physiology (Figure 1.1.1) (Ouzounis *et al.*, 2015; Arena *et al.*, 2016; Yang *et al.*, 2017).

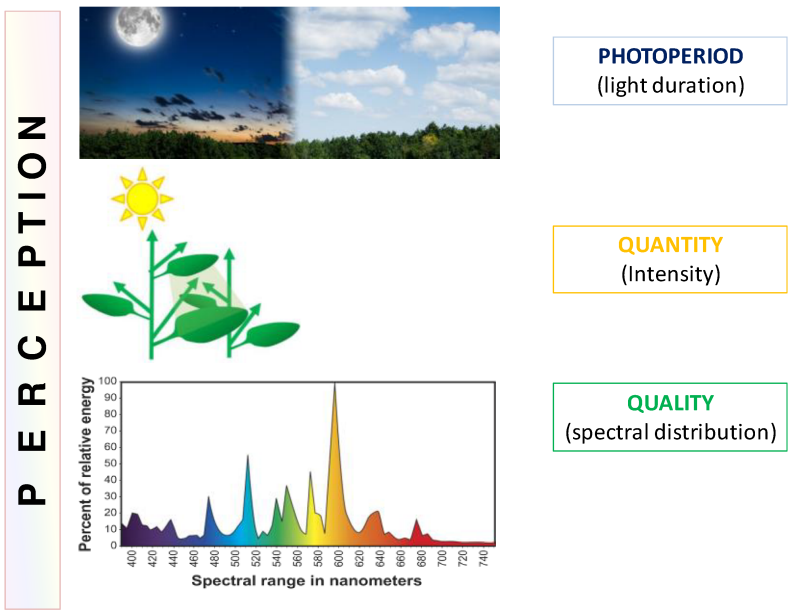

Figure 1.1.1: The three dimensions of light.

In particular, light quality exerts a key role in inducing specific responses, as not only leaves and green cotyledons, but also internodes, hypocotyls and roots may differently perceive the light environment (van Gelderen et al., 2018). The top of the

canopy or the upper part of plants are subjected to the entire light spectrum arriving on the Earth's surface, and, in turn, are adapted to high levels of irradiance. Conversely, the lower layers of the canopy or the lower of plants have to cope with shady environments which receive a different light quality. The wavelengths of visible light are the most used by plants. Generally, blue and red wavelengths are absorbed at the top of the canopy, while green and far-red wavelengths are reflected and transmitted to the lower layers of vegetation (Fiorucci and Fankhauser, 2017).
The leaf behaviour mimics the canopy. More specifically the blue and red wavelengths are quickly absorbed by the harvesting pigment complexes in the top layers of leaf, while the green wavelength penetrates further into the leaf and drives photosynthesis in the deep mesophyll parenchyma. The absorption of red, blue and green wavelengths differently stimulate the photosynthetic process within the leaf and canopy profile, contributing to the whole plant carbon gain and crop yield (Smith *et al*., 2017).

Different spectral regions selectively activate specific photoreceptors. The main families of photoreceptors, namely phytochromes, cryptochromes, phototropins, and UVR8, induce highly overlapping sets of genes, indicating the presence of shared signalling components (Ouzounis *et al*., 2015).
In this sense, light quality regulates several responses (Olle and Viršilė, 2013) relative to germination (Barrero *et al*., 2012), stomatal opening, (Goins *et al*., 1997), leaf anatomical structure (Liu *et al*., 2011), photosynthetic pigment production and Rubisco expression (Fan *et al.,* 2013), as well as disease resistance (Wang *et al.* 2010). The spectrum composition also affects leaf gas exchanges (Trouwborst *et al*., 2016), biomass production (Hernandez and Kubota, 2016) and synthesis of metabolites, such as phytochemicals and volatile organic compounds (Ohashi-Kaneko *et al*., 2006; Arena *et al.,* 2016) which are implicated in plant defences against abiotic stresses (i.e., temperature, water stress, ionising radiation, and nutrient lack (Gill e*t al*., 2010; Tuteja *et al.,* 2011; Bian *et al*., 2015; Arena *et al*., 2019).

Most of the literature on the plant physiological and structural response to light quality derives from experiments performed in growth chambers using Light Emitting Diodes (LED) technology, which allows to obtain a single or a combination of specific light wavelengths on plants. Generally, the adjustment of light quality at a specific growth stage should be considered as a strategic tool for improving crop

yield, nutritional quality, or plant physiological performance in controlled growth environment.

1.2 Modulation of light spectrum and plant growth in closed environments: application in sustainable agriculture and Space farming

As in nature, also under controlled conditions, light is the main factor driving the fundamental process at the basis of primary production: the photosynthesis.

In the last years, the possibility to grow higher plants, especially crops, in controlled environments is gaining increasing attention. Controlled or semi-controlled environments reduce the extreme variability of plant responses to the multiple environmental factors occurring in the field. Plants are grown in dedicated chambers setting specific temperature, relative humidity, photoperiod and light intensity conditions.

In controlled environments, the **light quality** may be manipulated by the selection of specific wavelengths to obtain proper light configuration, exclusive for each crop (Figure 1.2.1).

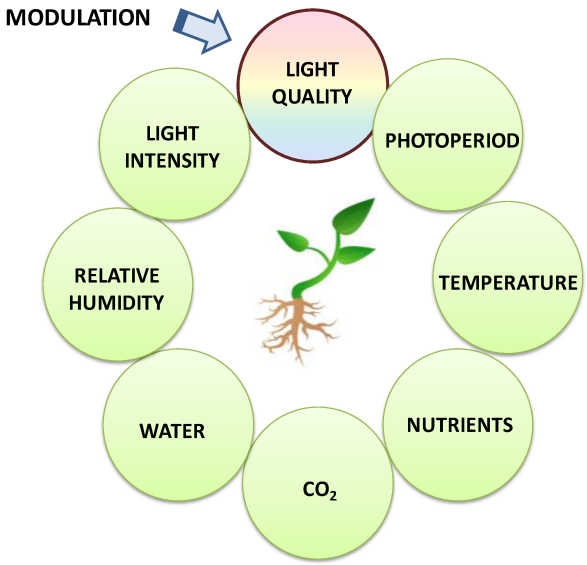

Figure 1.2.1: In controlled environments, plants are grown managing water and nutrient availability and selecting temperature, relative humidity, CO_2 concentration, light intensity and photoperiod. A specific light quality regime can be obtained modulating the light spectrum by Light-Emitting Diodes (LEDs) technology.

The opportunity to select specific light quality regimes, using the light emitting diodes (LEDs) technology, has revolutionised scientific research and crop production. LEDs are sustainable and highly efficient light sources, characterised by small size, long life, cool emitting temperature and reduced consumption compared to other light sources. Many studies showed important benefits linked to their use, especially in regions where the natural light source is not sufficient for plant growth (Singh et al., 2015).

Several studies have been carried out on higher plants, testing monochromatic lights or mixing different wavelengths of the spectrum. These studies deeply contributed to enlarge the knowledge on plant responses, especially crops, to specific wavelengths but also highlighted the importance to adopt the modulation of the light spectrum as a '**valid tool**' to grow plants in controlled environments. In particular, special attention is paid to the wavelength(s) most efficiently at physiological level in inducing a better photosynthetic performance in wide cultivated species (Singh *et al.*, 2015; Taulavuori *et al.*, 2017; Shamshiri *et al.*, 2018).

The selection of appropriate light wavelengths, **alone or in combination with other additional variables** during plant growth, may deeply affect plant physiological performance and organ development not only improving photosynthesis but also enhancing biomass production, food quality (i.e., modification of nutraceutical compounds and antioxidants) and the tolerance against abiotic stresses (Ohashi-Kaneko *et al.*, 2006; Gill *et al.*, 2010; Tuteja *et al* 2011; Bian *et al.*, 2015; Arena *et al.*, 2016).

Figure 1.2.2: Examples of plant growth in controlled environments.

The use of light quality to produce plants with specific nutritional and physiological traits represents an ecological and sustainable cultivation practice to be

applied in indoor cultivation systems, such as growth chambers, greenhouses and vertical farms around the world (Figure 1.2.2). Currently, the formulation of innovative agricultural protocols at low ecological impact is becoming a priority to meet the increasing food demand of human population. In this context, the use of **light quality as 'natural fertiliser' alone or combined with other eco-friendly practice such as the employment of biostimulants** could be an excellent solution to:

- enhance crop productivity for a reliable food supply;
- preserve the overexploitation of soil and natural resources;
- avoid the overuse of agrochemicals.

The use of light quality as practice for improving photosynthetic efficiency for sustainable crop production meet several of 'the Sustainable Developmental Goals (DSGs) required by the '2030 Agenda for Sustainable Development', which explores the potential solutions overcoming the present and future global challenges (UN 2015, 2017).

Producing fresh food not only in extreme environments on Earth, but also in Space is another important challenge for the research in view of long-term extraterrestrial missions. The possibility of prolonging space missions, and consequently the permanence of humans in space, depends on the possibility of providing them with an adequate supply of fresh foods to meet their nutritional requirements. In extreme environments, technology has to substitute for the Earth's natural conditions in order to allow plants to grow. Artificial biospheres and greenhouses will be essential for future human space exploration (Wheeler, 2011; Zabel 2016; Carillo *et al.*, 2020).

The light spectrum modulation is widely applied to cultivate crops in extreme environments characterised by ecological constraints (i.e., elevated or low temperature, high level of irradiance, or ionising radiation), which do not permit plant survival.

Figure 1.2.3: Antarctic Greenhouse, EDEN ISS Project. (https://eden-iss.net)

Cold or hot deserts are examples of extreme Earth environments (Figure 1.2.3). Many Space-oriented experiments on Earth (see the EDEN ISS project in Antarctica, https://eden-iss.net) reproduce the constraints to which plants could be exposed in extraterrestrial environments, developing plant cultivation technologies for safe food production in Space.

In particular, the studies concerning the regulation of the photosynthetic process under different light quality regimes are crucial for the key role that plants may exert within the Bioregenerative Life Support Systems (BLSSs). A BLSS (Figure 1.2.4) is an example of a closed environment reproducing an artificial ecosystem, in which higher plants regenerate water and air through transpiration and gas exchanges and produce fresh food for the crew (Wolff *et al.*, 2013; Wheleer, 2017; Zabel, 2018; Arena *et al.*, 2019).

The energetic input for a such artificial ecosystem is represented by the light. Then, the selection of proper light sources is essential for the improvement of the photosynthetic process and specific light quality regimes can make the difference for plant cultivation onboard the International Space Station (ISS) and future Space platforms (Figure 1.2.5). It is noteworthy that the volume at disposal for plant growth on ISS is limited and does not allow to produce enough fresh food to supplement entirely the astronaut diet; thus the experimentation on ISS may be considered a first step for implementing plant growth systems (Carillo *et al.*, 2020). In these limited volumes, manipulating opportunely the light quality would allow increasing the plant edible biomass and nutraceutical compounds.

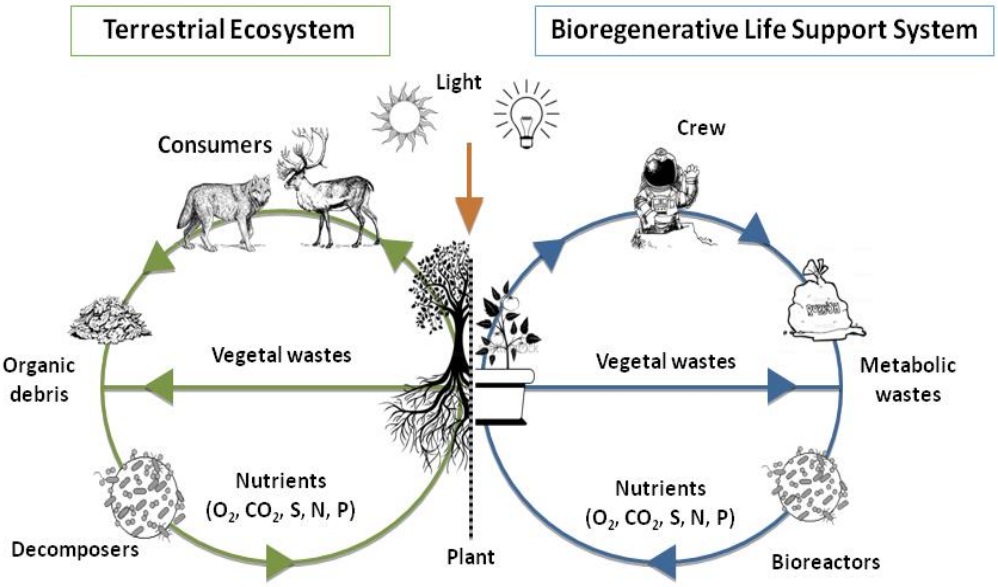

Figure 1.2.4: Analogies between a terrestrial ecosystem and a Bioregenerative Life Support System (Arena et al., 2012).

Figure 1.2.5: Plants growing onboard the International Space Station in view of the realisation of Extraterrestrial platforms on Moon and Mars (NASA.gov).

Despite the controlled environments of ISS or future Space platforms, Space remains a complex habitat, completely different from the Earth. Space can be considered a novel environment where plants are subjected to multiple stressors which exert direct or indirect effects on tissue structure and functional processes. During the Earth evolution many ecological factors, including solar radiation and gravity, may have exerted a fundamental role in determining the phenotype of higher plants (Bateman et al., 1998). In the further evolution of higher plants in Space, it is expected that the plants would face again environmental conditions similar to those of remote past on the Earth, such as reduced gravity and increased ionizing radiation

(De Micco et al., 2011). These new levels of gravity and ionizing radiation (IR) are known to cause alterations in various aspects of plant growth.

Currently, the exposure to ionizing radiation (IR) represents the main constraint affecting organism survivor in Space, including plants (Arena *et al.*, 2014). IR may act at molecular, morpho-structural and physiological level (Arena *et al.*, 2014, De Micco *et al.*, 2011; De Micco *et al.* 2014; Arena *et al.*, 2017). The cosmic radiation consists of a wide variety of high-energy protons and atomic nuclei. Several experiments demonstrated that the effects of IR on plants depend on radiation quality (high or low Linear Energy Transfer – LET), delivered dose, type of exposure (acute or chronic), and the intrinsic traits of the target organism (i.e. species, cultivar, development stage, structure of organs and tissues, and genetics) (De Micco *et al.,* 2011). Generally, plant response to ionising radiation is dose-dependent: permanent damage at high doses, harmful consequences at intermediate levels and stimulatory effects at low doses are expected (Arena *et al.*, 2014). Particular attention is paid to non-lethal doses of heavy ions utilized by breeders in agriculture for improving specific traits in crop species such as early maturity, high yield, and better fruit quality (Tanaka *et al.*, 2002; Honda *et al.*, 2006; Hou *et al.*, 2008; Xie *et al.*, 2008; Kharkwal, 2012; Dong *et al.,* 2016; Jo *et al.,* 2016; Oladosu *et al.*, 2016). Not-lethal doses of ionizing radiation may, therefore, exert a positive outcome, transforming a constraint in an opportunity. In this view, understanding the effects of IR on higher plants, especially on the photosynthetic process and plant nutraceutical properties, is a challenge not only for the possible utilisation of higher plants on BLSSs, but also to improve the characteristics of cultivated plants on the Earth.

To date, ionising radiation and light quality have been evaluated as two independent factors. In the perspective of plant cultivation in Space, or in extreme Earth environments their interaction on edible plants should be investigated.

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PhD project, aim and planning

The PhD project is focused on the role of light quality in regulating the photosynthetic machinery of higher plants in controlled environments. In particular, the project explores, for the first time, if and how specific light wavelengths during growth may modify plant physiological behaviour and phytochemical production in response to biostimulant application or exposure to ionising radiation. Among different variables affecting plant growth, biostimulants application was selected with the specific aim to improve the overall plant physiological performance in terms of primary and secondary metabolism in the context of sustainable agricultural practices. The ionising radiation was chosen as a space stress environmental factor in the view of experiments finalised to plant cultivation in Space.

This Project provides new insights with important ecological implications. Light quality has a key role in influencing the primary production in the field as well as in controlled environments. The Ground-based and the Space-oriented experiments proposed in this thesis are strictly linked to each other connected by the possibility to develop suitable cultivation systems overcoming all the constraints factors enforced by unfavourable or extreme environments, on Earth as well as in Space (Figure 2.1).

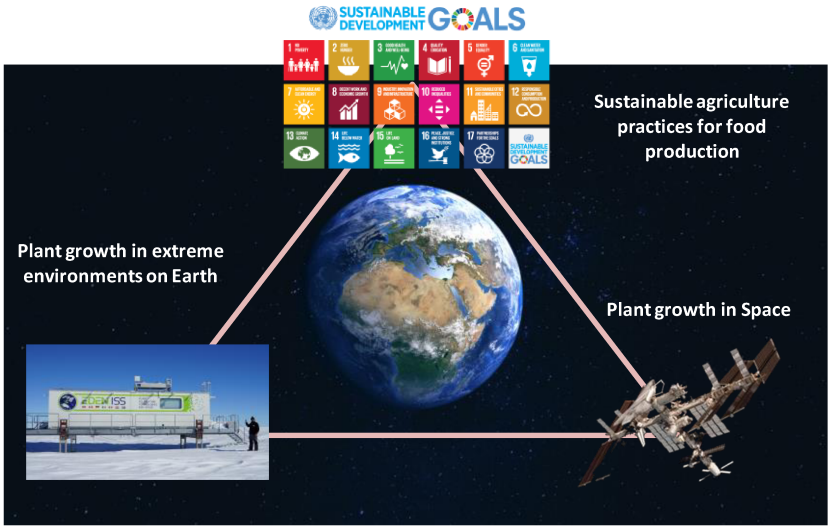

Figure 2.1: Ground-based and Space-oriented experiments provide the common outcome of developing suitable cultivation systems overcoming all the constraints factors enforced by unfavourable or extreme environments, on Earth as well as in Space.

The first step of the project was the selection of crops, chosen on the basis of specific functional and structural criteria: high demand for food, short life span, fast growth, elevated productivity, high nutraceutical value. The selection identified four model plants cultivated worldwide: soybean (*Glycine max* L.), spinach (*Spinacia oleracea* L.), chard (*Beta vulgaris* L. cv cicla), and tomato (*Solanum lycopersicum* L. cv. 'Microtom' and cv. 'Piennolo'). These species are also considered functional foods for the high production of phytochemicals and antioxidants.

The experiments were carried out in controlled and semi-controlled environments to reduce the extreme variability of plant response to the multiple environmental factors occurring in the field. Plants were grown in dedicated growth chambers under specific temperature, relative humidity, photoperiod, and light intensity conditions. Only the light spectrum was opportunely modulated to obtain specific light quality regimes during plant development and study the contribution of specific wavelengths in promoting photosynthetic performance.

The outcomes of these experiments were utilised in the subsequent trials to test how light quality combined with growth-promoting agents, i.e., biostimulants, or ionising radiation (namely, heavy ions) may modify photosynthesis and antioxidant production.

A downscaling investigative approach was adopted (Figure 2.2), considering the structure to function relationships between plant and growth environment. The plant behaviour was monitored from germination to a fixed developmental stage, depending on target species through non-invasive analyses (biometrical determinations; leaf gas exchanges; chlorophyll *a* fluorescence emission). At the end of the experiments, destructive analyses were carried out on harvested plants to evaluate changes in the total biomass production, morphology and anatomy, and leaf functional traits. Finally, the collected samples were further processed to assess variations in the secondary metabolism and the occurrence of plants' antioxidant response as a result of the beneficial interaction between light quality and these additional factors or as a countermeasure to potential stress.

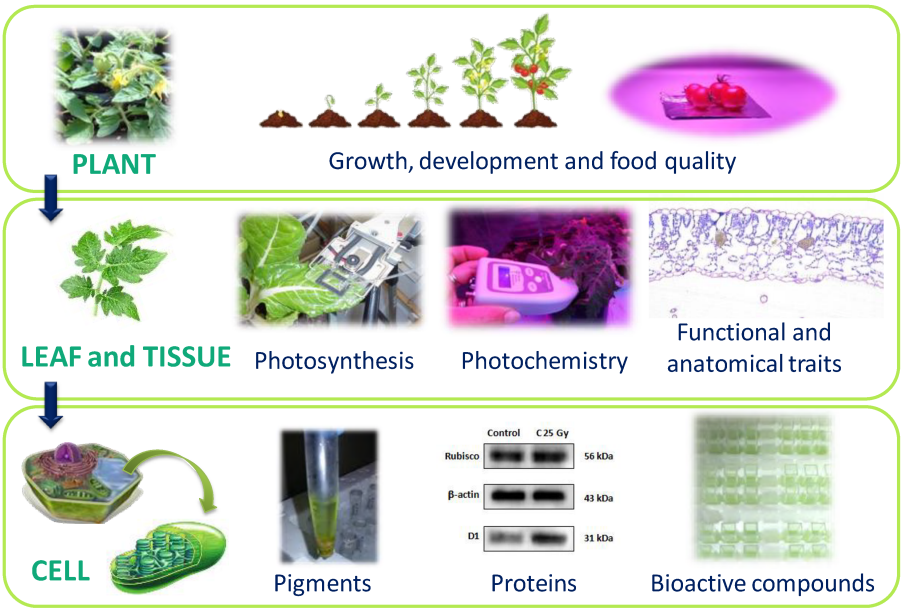

Figure 2.2: Multidisciplinary down-scaling investigative approach, from plant to leaf and cell.

The thesis is organised in chapters composing three content sections. The first explores the effect of light quality on two tomato cultivars. The second is dedicated to the response of spinach and soybean plants to the interaction between different light quality regimes and different biostimulants (beneficial soil microorganisms and protein hydrolysates). The third section studies the interplay between light quality and heavy ions (Calcium, Carbon and Titanium) on two crops selected by the International Space agencies, namely chard and tomato.

The last chapter provides a general conclusion.

The work presented in this thesis was carried out in the period from January 2018 to April 2020 at the Department of Biology of the University of Naples Federico II. Prof. Carmen Arena supervised the work during these years. A period of three months was spent working with Prof. Violeta Velikova at the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, in Sofia, Bulgaria.

A continuous collaboration with GSI Helmholtzzentrum für Schwerionenforschung Institute (Darmstadt, Germany) allowed obtaining irradiated samples for the studies on heavy ions on plants and scientific support for the specific research topic “Plant and Space”.

Section I – Modulation of light spectrum: study cases of two tomato cultivars.

In this section are presented two studies on the effect of light quality on different tomato cultivars.

The first research is focused on the effect of light quality on the photomorphogenesis of tomato plants during the early developmental stages to assess the most suitable wavelengths in guaranteeing the highest photosynthetic rates.

The second study extended the investigations of the light spectrum modulation to the whole life cycle of tomato from seed to fruit in order to provide insights not only on photosynthesis but also on fruit nutraceutical traits.

Chapter I – The role of monochromatic red and blue light in tomato early photomorphogenesis and photosynthetic traits.

Luigi Gennaro Izzo, Bruno Hay Mele, Luca Vitale, Ermenegilda Vitale, Carmen Arena.

Environmental and Experimental Botany, 179 (2020) 104195.

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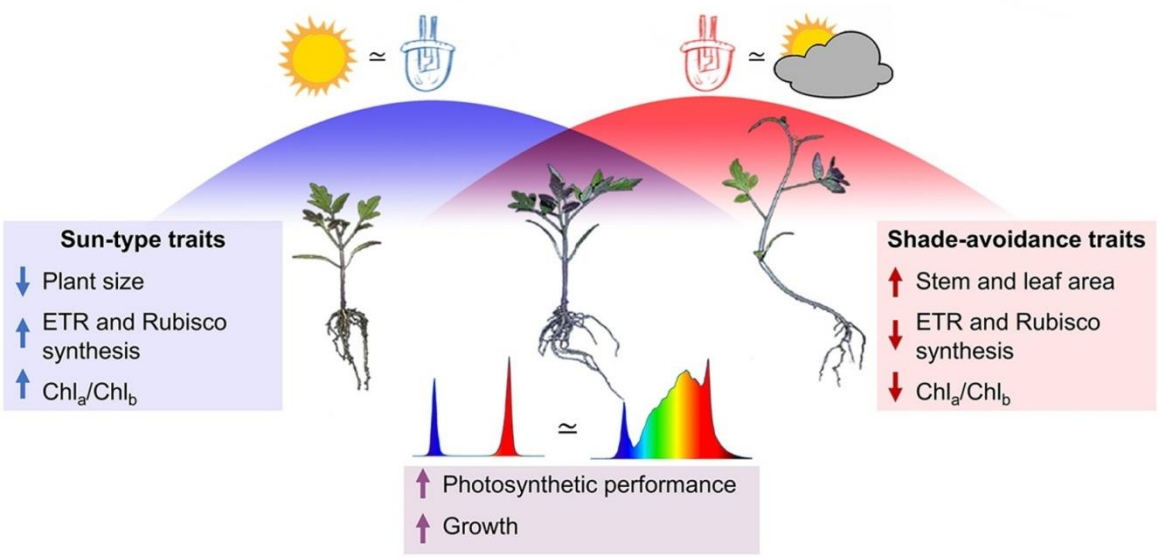

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The role of monochromatic red and blue light in tomato early photomorphogenesis and photosynthetic traits

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ABSTRACT

Plant development and physiology are strongly influenced by the environmental light spectrum that triggers and controls several functional and structural response in plants. However, few studies investigated the effect of monochromatic light during plant photomorphogenesis from seed imbibition up to seedling development. The present research aimed to assess the mechanisms engaged by plants to optimize light harvesting and utilization of different wavelengths during the early photomorphogenesis in tomato, a high-value crop cultivated worldwide. Seeds were germinated in a growth chamber under four light treatments (100 % red light, R; 100 % blue light, B; 60 % red 40 % blue light, RB; white light, W) and seedlings were grown up to sixteen days. Hypocotyl and cotyledon development were measured during the early stages of growth. Chlorophyll fluorescence measurements, D1 and Rubisco protein expression, as well as chlorophyll and carotenoids content, were determined on the first true leaves to assess in the early growth stage the efficiency of photosynthetic apparatus. Tomato early photomorphogenesis was strongly influenced by light quality. The seedling growth under red-blue and white light determined comparable responses, enhancing both photosynthesis and biomass production compared to monochromatic treatments. The pure R light stimulated hypocotyl elongation, cotyledon expansion, plant height, and leaf area, but produced seedlings with reduced photosynthetic capacity as indicated by the lowest Rubisco content and photochemical efficiency, and the highest thermal dissipation. Monochromatic blue light induced in seedlings the highest Rubisco amount, more compact size and reduced biomass, but similar level of pigments and photochemical efficiency compared to other light treatments. Our data indicate that the lack of blue or red light negatively affects early tomato development, in term of morphology and physiology. However, blue wavelengths resulted more critical than red ones for the functionality of the photosynthetic apparatus.

1. Introduction

Light is one of the main factors driving plant growth, being both an energy source and a developmental signal. Recently, considerable attention has been paid to research with light-emitting diodes (LEDs) as sole-source lighting for plant cultivation in controlled environments to increase the efficiency of crop production (Gómez and Izzo, 2018). LED technology enables proper investigation on the role of light quality in plant growth due to the possibility to select specific wavelengths and light spectra (Massa et al., 2008). Several studies have shown that particular wavelengths can activate different photoreceptors that prime complex signaling, ultimately resulting in precise physiological and biochemical responses (da Silva and Debergh, 1997; Ohashi-Kaneko et al., 2007; Arena et al., 2016; Huché-Thélier et al., 2016; Amitrano et al., 2018; Chinchilla et al., 2018). Previous experiments showed that the red region of the light spectrum has the highest quantum efficiency for leaf photosynthesis (McCree, 1973). However, plants grown under monochromatic red light exhibited a low maximal photochemical efficiency, unresponsive stomata and a low photosynthetic capacity compared to plants grown with additional blue light (Hogewoning et al., 2010). Besides, red light promotes skotomorphogenesis gene expression, negatively regulating photomorphogenesis (Jiao et al., 2007).

Blue photons drive the photosynthetic reaction less efficiently than red photons because their high energy is not fully utilized. An excess of blue wavelengths suppress growth; plants produce smaller, thicker and darker green leaves compared to plants grown without blue light.

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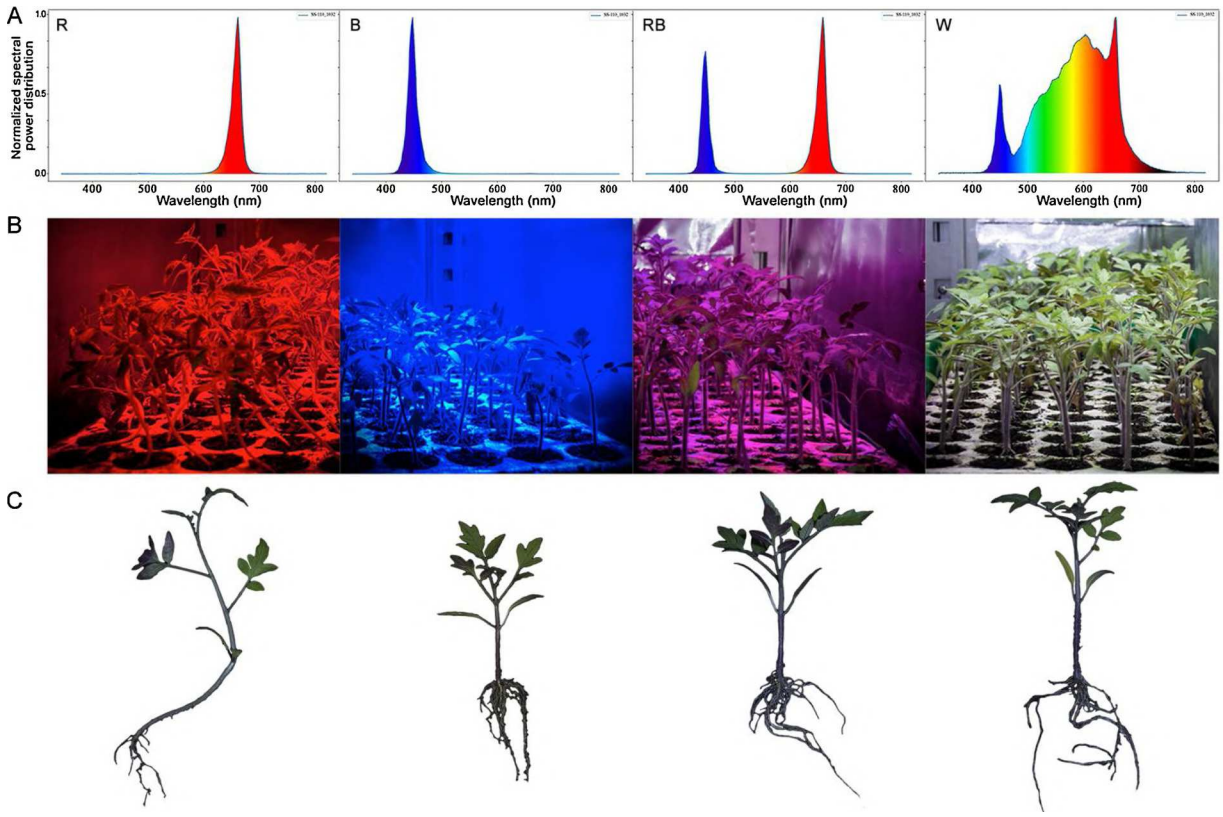

Fig. 1. A) Light spectra of different treatments: red light (R) with a maximum peak emission at 664 nm, blue light (B) with a maximum peak emission at 446 nm, a mixture of red (R: 60 %) and blue (B: 40 %) light (RB), and broad-spectrum white light (W); B) Plants growing in the growth chamber under different light quality; C) Tomato seedlings grown under different light treatments.

Generally, only a low intensity of blue is needed in a light spectrum for fully functional photosynthesis (McCree, 1973). The blue light is considered a growth regulator because is involved in several plant critical responses such as phototropism, photomorphogenesis, stomatal opening, chloroplast development, and leaf expansion (Christie and Briggs, 2001; Whitelam and Halliday, 2007; Briggs, 2007). In indoor and in supplemental greenhouse lighting the blue light has less or no growth-inhibiting effects, thus a little amount of blue is always included in the light spectrum.

Plants grow under monochromatic light generally presented physiological disorders such as a reduced photosynthesis and biomass, because they are lacking important signal for growth and development (Trouwborst et al., 2016; Yang et al., 2018; Izzo et al., 2019). Indeed, the use of monochromatic light can induce severe alterations in photomorphogenesis because of an unbalanced activation of photoreceptors which mediate the light-dependent development of plants (Landi et al., 2020). Over the past three decades, LED technology has been extensively used for investigating the effects of light quality on plant development and functioning, with particular attention to photosynthesis and photomorphogenesis (Graham et al., 2019). The influence of specific wavelengths on plant growth has been mainly tested on seedlings already developed under broadband light sources (Brown et al., 1995; Nanya et al., 2012; Fan et al., 2013; Kim et al., 2014; Wollaeger and Runkle, 2014; Kong et al., 2018). Conversely, to date, few studies focused on the effect of monochromatic light on seedling development from seed imbibition/germination, that represents one of the most sensitive steps.

The comparison of different findings has clarified how several factors deeply influence the plant response to light quality, some related to plant characteristics (e.g. species, cultivar, stage of development) and other associated to the specific monochromatic wavelengths (Lobiuc et al., 2017; Graham et al., 2019; Kong et al., 2019). Although available results are often contrasting and not easily comparable, there seems to be common agreement that red and blue photons within the incident spectrum are necessary for proper plant development (Hogewoning et al., 2010; Wang et al., 2016; Izzo et al., 2018; Clavijo-Herrera et al., 2018).

An open question is if the monochromatic light delivered to plants since germination may elicit seedlings with specific phenotypes, modulating some physiological traits involved in photomorphogenesis and photosynthetic apparatus development. The modulation of the early photomorphogenesis represents a valuable tool to achieve plants of the best quality for horticultural production, and light quality is one of the most effective means to adjust plant morphology in controlled environment production (Demotes-Mainard et al., 2016; Mah et al., 2018). In this context, light quality could be used to manipulate plant architecture and induce favourable characteristics in terms of agricultural requirements. For example, the increasing plant elongation can benefit the harvesting of microgreens, and the grafting of rootstock or inhibiting stem elongation can produce compact plants to be used in vertical farming.

Among crops, tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated species all over the world and constitutes a source of health-promoting compounds (Dorais et al., 2008; Rigano et al., 2016; Francesca et al., 2020). In recent years, considerable attention has been given to the cultivation of tomato in a controlled environment since it has been demonstrated that this species is particularly responsive to the different light spectral composition (Xiaoying et al., 2012; Wollaeger and Runkle, 2014; Yang et al., 2018; Arena et al., 2016). To our knowledge, no studies have investigated the effects of monochromatic red or blue light on tomato photomorphogenesis from seed imbibition up to seedling development. Considering the importance of this crop, the manipulation of the light spectrum to improve the early morphogenesis could contribute to obtaining better plants from a physiological

point of view and thus more efficient for food production in the indoor cultivation. In this work, we investigated seedling responses to light quality during the early stages of tomato development in which photomorphogenesis plays a critical role for plant growth.

More specifically, we aimed to understand 1) how monochromatic blue and red light may affect leaf light interception and photochemical behavior of seedlings and 2) how the different red and blue amount may drive plant development at the early stage.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *Solanum lycopersicum* L. cv. 'Piennolo' were surface sterilized in a 3% sodium hypochlorite solution for 3 min, and then thoroughly washed with distilled water. Seeds were sown in polystyrene trays for seedlings with pots (5 cm in diameter, 10 cm in depth) filled with horticultural grade substrate (70 % peat and 30 % perlite by volume) and placed in a growth chamber under four different lighting treatments (Fig. 1B). Sixty-five seeds were sown in each tray, and the seedlings were grown with a temperature regime of 24/18 °C (day/night) and relative humidity (RH) of 60–80 %. Seedlings were watered regularly in order to overcome the losses for evapotranspiration and to avoid water stress.

2.2. Lighting treatments

Four different light regimens were provided by a LED lighting system equipped with high-power LED chips and dimmers to adjust light intensity. The different light quality treatments were: red light (R) with maximum peak emission at 664 nm, blue light (B) with maximum peak emission at 446 nm, a mixture of red (R: 60 %) and blue (B: 40 %) light (RB), and a broad-spectrum white light (W) (Fig. 1A). All treatments were kept with a 12 h photoperiod at the same light intensity expressed as photosynthetic photon flux density (PPFD) of 190 ± 5 µmol m^{-2} s^{-1}. PPFD was determined on a 12-point light map within each treatment compartment and was measured daily above the plant canopies with a spectroradiometer (SS-110; Apogee Instruments Inc., Logan, UT). Photosynthetic photon flux density has been kept constant at the plant canopy height throughout the experiment by adjusting the intensity and the distance of light sources.

2.3. Measurements of germination and plant morphological parameters

The percentage of seed germination under different light quality treatments was evaluated on sixty-five samples *per* treatment. Seeds were considered to have germinated when the root protruded the seed coat. The final percentage germination (FPG) was calculated 4 and 7 days after sowing (DAS), according to the formula:

$$FPGnDAS = \frac{\text{Number of germinated seeds after nDAS}}{\textit{Total number of seeds}} x\ 100$$

Hypocotyl length (HL), cotyledon length (CL), and cotyledon area (CA) were measured on five seedlings *per* treatments at 8, 10, and 12 DAS. Plant height (PH), internodal distance (IND), and leaf area (LA) were measured at 16 DAS. HL, CL, PH, and IND were measured with a ruler, whereas CA and LA were determined through digital photos analyzed with ImageJ (Image Analysis Software, Rasband, NIH). Plant biomass, divided in roots, stem, and leaves, was measured at 16 DAS by drying vegetable material in a oven at 70 °C until reaching of constant weight. Leaf mass per area (LMA) was calculated as the ratio of leaf dry weight to leaf area and expressed as g cm^{-2}, according to Cornelissen et al. (2003).

2.4. Chlorophyll a fluorescence measurement

Chlorophyll *a* fluorescence measurements were carried out on seedlings at 16 DAS in the growth chamber at PPFD of 190 ± 5 µmol photons m^{-2} s^{-1}, which represented the plant growth irradiance, by a portable PAR-FluorPen FP 100-MAX-LM (Photon System Instruments, Czech Republic) as described in Izzo et al. (2019).

The PSII maximal photochemical efficiency (F_v/F_m) was determined on 30 min dark-adapted leaves. The basal fluorescence (F_o) was induced by an internal blue-light (1 − 2 µmol m^{-2}·s^{-1}) whereas the maximal fluorescence in the dark (F_m) was induced by 0.8 s saturating light pulse of 3000 µmol m^{-2}·s^{-1}. The quantum yield of PSII electron transport rate (Φ_{PSII}) was determined by means of an open leaf-clip suitable for measurements under ambient light and calculated according to Genty et al. (1989). Non-photochemical quenching (NPQ) was calculated following Bilger and Bjorkman (1990). After, on the same seedlings used for the point measurements, the Rapid Light Curves (RLCs) were also applied to evaluate the seedlings photosynthetic capacity at different PPFD, ranging from 0 to 1500 µmol photons $m^{-2}$$s^{-1}$. RLCs were used to calculate other photosynthetic descriptors: the maximum electron transport rate (ETR_{max}), alpha (α) (i.e. the initial slope of the curve that represents the rate of photon conversion under lower irradiances) (Runcie and Riddle, 2004), and saturating light intensity (Ek = ETR/α) (Ralph et al., 2002). These parameters were estimated using the exponential function of Webb et al. (1974) on the ETR curves.

The fluorescence point measurements as well as the RCLs were performed on five plants *per* light treatment.

2.5. Pigments quantification

Chlorophylls and carotenoids were extracted in ice-cold 100 % acetone from five leaves taken from five plants *per* treatment at 16 DAS. The extracts were centrifuged at 5000 rpm in a Labofuge GL (Heraeus Sepatech, Hanau, Germany) for five minutes, and the supernatant absorbance was determined using a spectrophotometer (UV–vis Cary 100, Agilent Technologies, Palo Alto, California, USA). Content of Chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b), total chlorophylls (Chl), carotenoids (Car), Chl_a/Chl_b, and Car/Chl were calculated according to Lichtenthaler (1987).

2.6. Assays for Rubisco and D1 content: protein extraction, SDS-PAGE and western blotting

Leaf protein extraction was carried out on five leaves per treatment at 16 DAS following the procedure of Sorrentino et al. (2018). Leaf tissues (500 mg), were finely ground in a mortar with liquid N_2. The powder was suspended in 10 % TCA/acetone solution, centrifuged at 16,000 *g* for 3 min at 4 °C and then washed first in methanol (80 %) and after in acetone (80 %). After drying (50 °C for 10 min), the pellet was re-suspended in 1:1 phenol (pH 8.0)/SDS buffer and centrifuged at 16,000 x g for 3 min. The upper phenol phase was treated with methanol containing 0.1 M ammonium acetate, stored overnight at −20 °C and centrifuged again. The obtained pellets were washed once with 100 % methanol and once with 80 % acetone and then suspended in an SDS sample buffer.

SDS-PAGE (10 %) was performed by using Dual Color Protein Standard (Bio-Rad Laboratories S.r.l., Segrate, Milano, Italy) as a marker, and Laemmli loading buffer added to samples to follow the protein separation. Western blot analysis on samples was performed using a blocking solution (100 mM Tris − HCl pH 8.0, 150 mM NaCl, 0,1% Tween 20, 5% BSA) and specific primary antibodies (Agrisera) to reveal different proteins: namely Rubisco (anti-*rbc*L, rabbit polyclonal serum), D1 (anti-*psb*A, hen polyclonal), and Actin (anti-ACT, rabbit polyclonal serum). Actin was utilized as loading control. The immunorevelation was carried out using the kit for chemiluminescence (Westar supernova, Cyanagen Srl, Bologna, Italy) by ChemiDoc System

(Bio-Rad). Densitometry analysis was performed using ImageJ software (Rasband, W.S., U.S. NIH, Bethesda, Maryland, USA, 1997–2012).

2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) through the Sigma-Stat 3.5 software (Jandel Scientific, San Rafael, CA, USA). The Holm-Sidak test was applied for all multiple comparison tests with a significance level of $P < 0.05$. Data are reported as mean values ± standard error.

To explore the overall data, we used the R environment for statistical computing and graphics R Core Team (2018). We first selected variables of interest for each treatment, physiological parameters and plant part, ($4 \times 2 \times 2$), then calculated the arithmetic mean ($n = 3$), and finally used the scale function to centre the data around the mean and scale it by using the standard deviation.

The transformed data were visualized using a heatmap (heatmap function). The heatmap clustering was a two-step process: 1) we calculated the Euclidean distance among samples; 2) we used the distance values to iteratively find the maximum possible distance between points belonging to two different clusters ("complete" method).

3. Results

3.1. Seedling development and growth

The exposure of seeds to monochromatic red light initially led to an increase ($P < 0.05$) of the germination rate: at 4 DAS the germination under R treatment was 71 %, while it was 51 %, 39 % and 29 % for seeds under B, RB and W light, respectively. At 7 DAS, the germination reached the 90–95 % in all light treatments with no significant difference among light quality regimens.

The different light quality regimens during the early growth stage significantly affected seedling development in tomato (Fig. 1B,C). Hypocotyl length was highest ($P < 0.01$) in plants grown under monochromatic red light and lowest ($P < 0.01$) in plants under blue light at 8, 10, 12 DAS, showing an increase of HL up to 114 % in red ($P < 0.01$) compared to blue light (Fig. 2). In addition, plants grown under W light showed significantly longer ($P < 0.05$) hypocotyl than RB plants with an increase of 20 % ($P < 0.05$) (Fig. 2). Cotyledon length in R plants was higher ($P < 0.01$) compared to other treatments at 8 DAS, 10 and 12 DAS (Fig. 2). Cotyledon area was lowest ($P < 0.01$) under blue light and resulted comparable among R, RB and W light at 8, 10, and 12 DAS (Fig. 2).

Sixteen days after sowing, plant height and internodal distance significantly differed among light quality treatments and were highest ($P < 0.05$) in R plants and lowest ($P < 0.05$) in B plants. Leaf area was comparable in plants grown under R, RB and W light, while it was significantly lower in plants developed under monochromatic blue light ($P < 0.05$) (Table 1).

As regards plant biomass, seedling grown under monochromatic red light, R, showed the lowest fresh and dry root weight ($P < 0.05$), while in RB and B plants root biomass was comparable to W plants. Conversely, R light increased stem fresh weight compared to other treatments, while B light showed the lowest value ($P < 0.05$). As regards dry weight of stem, no differences were found among R, RB and W light, whereas blue light reduced ($P < 0.05$) DWS. In terms of leaf biomass, fresh weight of leaves was the highest and the lowest ($P < 0.05$) in W in B plants, respectively. Differently, dry weight of leaves in RB plants was higher ($P < 0.05$) compared to R and B and was comparable to W plants (Table 1). In addition, RB and B treatments increased the leaf mass per area compared to W plants, whereas R treatment determined the lowest LMA ($P < 0.05$) (Table 1).

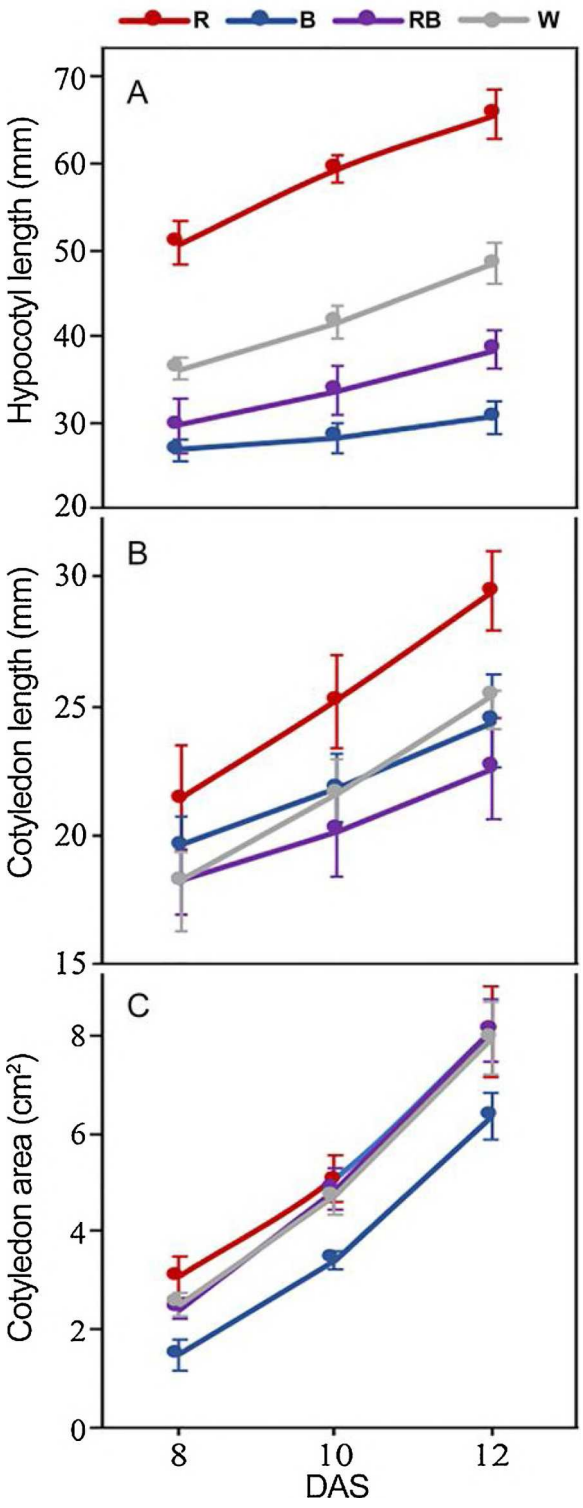

Fig. 2. Hypocotyl length (A), cotyledon length (B), and cotyledon area (C) of *S. lycopersicum* seedlings grown under 100 % red light (R), 100 % blue light (B), 60 % red and 40 % blue light (RB), and white light (W). Each value represents the mean ± standard error; $n = 5$.

3.2. Chlorophyll fluorescence emission, photosynthetic pigment content and Rubisco analysis

Sixteen days after sowing, the analyses of photochemical parameters significantly varied among light quality treatments. R plants exhibited the lowest ($P < 0.01$) value of Φ_{PSII} and F_v/F_m, (Fig. 3A, C)

Table 1
Morphology and biomass (mean ± standard error; $n = 5$) at 16 DAS of *S. lycopersicum* grown under red (R), blue (B), red and blue (RB), and white (W) light. Different letters indicate statistically significant differences among treatments ($P < 0.05$).

Parameter	Units	R	B	RB	W
PH	cm	11.6 ± 0.47 a	5.48 ± 0.36 d	7.38 ± 0.16 c	9.40 ± 0.32 b
INT	cm	2.90 ± 0.24 a	1.61 ± 0.17 c	2.32 ± 0.18 b	2.50 ± 0.07 b
LA	cm^2	19.3 ± 1.31 a	13.9 ± 0.75 b	18.3 ± 0.40 a	19.3 ± 0.63 a
FWR	g	0.11 ± 0.01 c	0.23 ± 0.04 b	0.33 ± 0.03 a	0.31 ± 0.02 a
FWS	g	0.83 ± 0.05 a	0.28 ± 0.02 d	0.44 ± 0.03 c	0.53 ± 0.04 b
FWL	g	0.50 ± 0.02 b	0.44 ± 0.03 c	0.53 ± 0.01 ab	0.55 ± 0.03 a
DWR	mg	10.1 ± 1.25 c	20.5 ± 2.27 b	25.0 ± 2.12 a	21.1 ± 1.46 ab
DWS	mg	41.3 ± 1.68 a	20.0 ± 1.65 b	33.9 ± 1.97 a	33.4 ± 2.11 a
DWL	mg	56.1 ± 3.27 b	63.4 ± 4.00 b	85.2 ± 3.84 a	76.3 ± 3.94 ab
LMA	$g\,m^{-2}$	29.2 ± 2.35 c	45.5 ± 1.97 a	46.7 ± 2.14 a	39.5 ± 0.96 b

PH = plant height; INT = internodal distance; LA = leaf area; FWR = fresh weight of roots; FWS = fresh weight of stem; FWL = fresh weight of leaves; DWR = dry weight of roots; DWS = dry weight of stem; DWL = dry weight of leaves; LMA = leaf mass per area.

and the highest ($P < 0.05$) NPQ compared to the other treatments (Fig. 3B). Plants grown under monochromatic blue light also showed a reduced ($P < 0.05$) Φ_{PSII} and an increased ($P < 0.05$) NPQ compared to W and RB. No difference was found in maximal photochemical efficiency of PSII among B, RB, and W plants, while red light significantly reduced ($P < 0.05$) the F_v/F_m ratio. Plants grown under W and RB light showed no difference in Φ_{PSII}, NPQ, and F_v/F_m (Fig. 3).

The photochemical response of Rapid Light Curves (RLCs) evidenced significant differences among seedlings grown under different light quality regimens, in particular at saturating irradiance. More specifically, while alpha did not vary among light treatments, Ek and ETR_{max} was the lowest ($P < 0.01$) in R and the highest ($P < 0.05$) in RB seedlings, respectively. Plants grown under monochromatic B treatment showed Ek and ETR_{max} values comparable to W plants.

Light quality also influenced pigment content of tomato seedlings. Chl_a content was higher ($P < 0.05$) in RB compared to R and B plants with the lowest ($P < 0.05$) value in R plants (Table 2). Conversely, R and B plants showed higher ($P < 0.01$) Chl_b content compared to W and RB plants. The highest ($P < 0.05$) total chlorophylls and carotenoids content, and Chl_a/Chl_b ratio ($P < 0.01$) were found in RB plants, while the lowest ($P < 0.05$) values were measured in R plants. The carotenoid/chlorophylls ratio was higher ($P < 0.05$) in B and RB compared to R and W plants (Table 2).

The western blot carried out on leaf tissue of *Solanum lycopersicum* (Fig. 4C) indicated an increase ($P < 0.01$) of Rubisco level in RB and B compared to W and R plants. The highest and lowest Rubisco content were found in RB and R seedlings, respectively ($P < 0.01$) (Fig. 4A). As regards D1 protein, no significant difference in the expression was found among light treatments (Fig. 4B).

3.3. Heat map analysis

A heat map synthesizing the response of the measured parameters provided an integrated view of the effect of light quality on the development of *S. lycopersicum* seedlings (Fig. 5). Clustering the measures at each time step produced four groups corresponding to the four light quality treatments, although the original time sequence order is not preserved. Thus, we will refer to each cluster with the name of the corresponding light treatment.

The RB and W clusters are the closest to each other in terms of measured parameter responses, and are equidistant from cluster B. At the same time, cluster R is considerably separated from the other three clusters: red light reduced DWL, DWR, FWR, Φ_{PSII}, and F_v/F_m, and increased PH, FWS, DWS, and IND compared to B, RB, and W, contributing to separate the R cluster from the others.

Monochromatic R and B treatments showed opposite responses differing in most of the measured parameters, but they both showed an increase of NPQ and Chl_b compared to dichromatic (RB) and multichromatic (W) treatments. RB and W light showed a similar response of most of the measured parameters, except for the content of carotenoids, Chl_a, and Rubisco, which were lower in W compared to RB plants. Besides, RB and B plants showed similar content of carotenoids, Chl_a, and Rubisco.

4. Discussion

4.1. Effects of light quality on seedling development and growth

Light is one of the main environmental factors controlling seed germination, and appropriate light conditions are essential for seed germination in many plant species (Oh et al., 2006). In our study, monochromatic red light promoted tomato seed germination compared to other light quality regimens only at the beginning of the lighting treatment, whereas this stimulatory effect was lost after seven days. This result is consistent with findings of other authors, who demonstrated a positive response of seed germination to red light in *Brassica napus* (Tehrani et al., 2016). It is noteworthy that in our experiment the blue light at the beginning did not produce in tomato an inhibition of germination as observed in *Hordeum vulgare* and *Lolium rigidum* (Goggin et al., 2008; Gubler et al., 2008; Xu et al., 2009). It may be supposed that, in our case, as the seeds were shielded by a thin layer of soil, the reduced penetration of blue wavelength through the soil may have limited its inhibitory effect, usually caused by blue-light induced ABA accumulation (Xu et al., 2009).

The heat map carried out in this work provides a broad view of morphological and physiological traits and enabled the identification of phenotypic variation patterns associated with growth under different light quality treatments. Differently from the seed stage, the diverse light quality strongly influenced the early seedling development in tomato modifying the capacity of light perception through hypocotyl elongation and the cotyledons expansion. The heatmap analyses clearly evidenced a net separation between the B and R clusters, indicating an opposite response of B and R seedlings on the basis of both morphological and physiological traits. Indeed, the RB and W clusters are closest to each other showing similar characteristics, except for photosynthetic pigment content and Rubisco synthesis.

Monochromatic red light, favoring the biomass allocation to the stem, produced tomato seedlings very tall with poor root biomass. Monochromatic blue light sorted an opposite effect inhibiting hypocotyl and stem elongation and inducing in the seedlings a more compact size with a reduced leaf area, roots, stem, and leaves production compared to R and other light treatments. Similar results were found in the same species by Dieleman et al. (2019) who reported a more compact size of plants developed under pure blue light. The plant growth under monochromatic blue light is less affected than under pure red light, considering that blue wavelengths can activate both cryptochromes and phytochromes, resulting in the lack of shade avoidance syndrome, likely due to a balanced cryptochrome/phytochrome stimulation (Landi

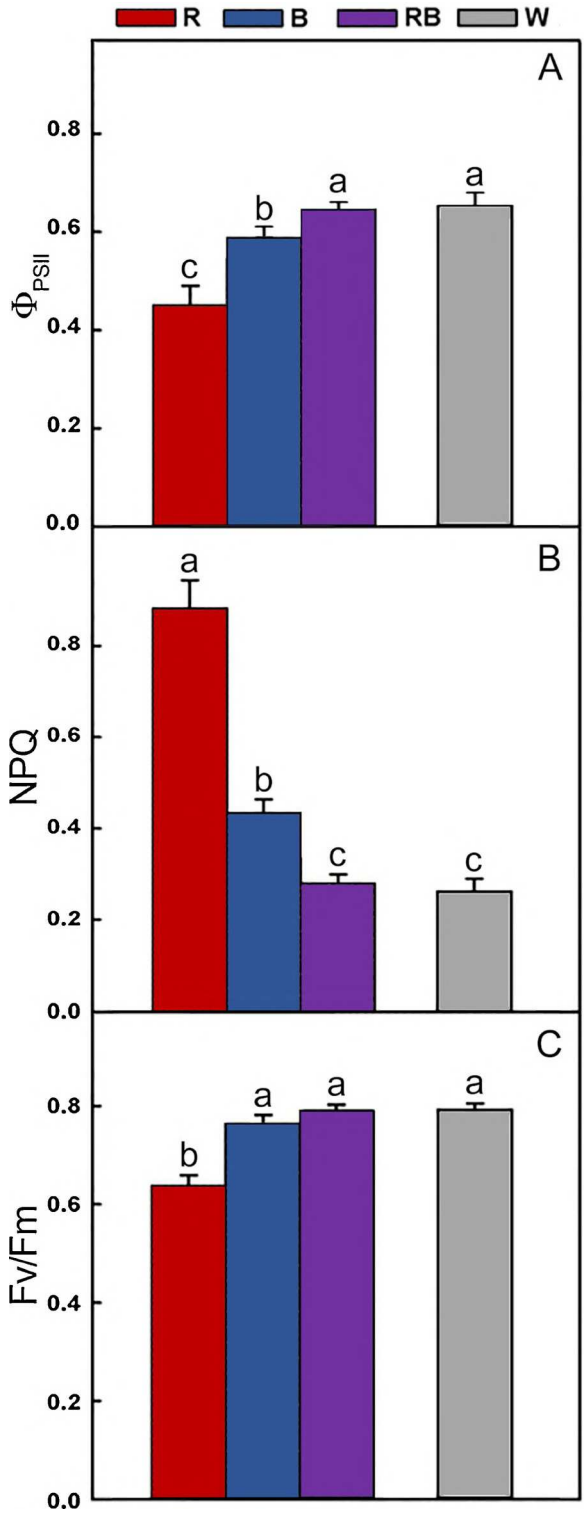

Fig. 3. Photosystem II quantum yield (Φ_{PSII}) (A), non-photochemical quenching (NPQ) (B) and maximal photochemical efficiency (F_v/F_m) (C), in *S. lycopersicum* plants grown under different light treatments: R = 100 % red light; B = 100 % blue light; RB = 60 % red and 40 % blue light; W = white light. Each value represents the mean ± standard error; $n = 5$. Different letters indicate statistically significant differences among treatments at $P < 0.05$.

et al., 2020).

Our results are in line with previous works, in which it is reported that increasing blue light negatively affects plant elongation and leaf area, inhibiting cell division and expansion (Dougher and Bugbee, 2004; Liscum et al., 1992; Nanya et al., 2012). Conversely, plants grown under red light showed typical shade-avoidance characteristics, namely increased hypocotyl elongation, internodal distance and leaf surface. In R plants, although leaf area was found comparable to RB and W plants, leaf dry weight resulted lower, indicating the development of thinner leaves with a reduced biomass, that, in turn, determined a reduced LMA in R leaves compared to B and RB ones. The negative effects induced by monochromatic red light have been attributed to high stimulation of phytochrome due to the absence of far-red light, which directly affects plant growth (Landi et al., 2020).

The reduced LMA may not be a favorable trait for tomato seedlings which result smoother and more exposed to phytophagy attacks (Turner, 1994). Moreover, LMA is positively correlated to increasing solar radiation and plants with high LMA are more resistant to a wide range of environmental stress factors (Ogaya and Peñuelas, 2007). In our experiment, the reduction of leaf area in seedlings grown under monochromatic B light and RB significantly influenced LMA. The higher LMA in these leaves, compared to R and W ones might be related to high doses of blue light, which is known to trigger sun-type responses at the chloroplast level, including elevated resistance to photoinhibition and high photosynthetic capacity (Lichtenthaler et al., 1980).

This hypothesis is consistent with changes in Rapid Light Curves (RLCs) profile and photosynthetic pigment content. In R plants, ETR_{max} saturated for values of irradiance lower compared to other light treatments where the blue wavelengths are present in different percentage. R leaves also exhibited a lower Chl_a and carotenoids content compared to RB and W, while a higher Chl_b. The increase in chlorophyll *b* may represent a strategy to enhance light harvesting, in accordance with the phytochrome-mediated response of shade avoidance due to environments with reduced blue light (Franklin and Whitelam, 2005). Accordingly, the ratio between Chl_a and Chl_b significantly decreased in R as a strategy adopted by plants, which modulated the antenna complex to increase light harvesting in a limiting light environment. However, this response is species-specific considering that other species have shown an increase in chlorophyll and carotenoid content under monochromatic red light (Vitale et al., 2020). Similar to plants grown under R light, monochromatic blue light increased Chl_b content, however Chl_a and Chl_a/Chl_b were comparable to W light. The extension of the antenna complex with increased Chl_b in plants grown under blue light, might be due to a lack of specific wavelengths, including red light. Chlorophyll and carotenoid production are stimulated by blue light through cryptochrome activation (Weller et al., 2001). As expected, we found that light spectra including blue wavelengths enhanced the production of photosynthetic pigments compared to red light, as also reported by Hogewoning et al. (2010) and Dieleman et al. (2019). However, B seedlings showed no shade-avoidance characteristics, but rather typical sun-type traits in line with the high energy of blue photons, especially if considering the compact morphology of plants with thicker leaves (higher LMA) and a reduced leaf area (LA) and internodal distance (IND).

The seedlings grown under treatments with different amount of blue wavelengths, namely B, RB, and W light developed short and straight stems, likely due to a phototropin-mediated positive phototropism triggered by blue light (Christie, 2007). Conversely, plants grown under pure red light developed elongated and tortuous stems, likely due to the lack of blue-driven phototropic stimuli.

Although high doses of blue light reduce plant growth and biomass production, because their energy is not fully utilized in photosynthesis, it is well known that at least a low percentage of blue light is necessary in supplementing red light for optimal plant growth (Hoenecke et al., 1992; Cope and Bugbee, 2013; Snowden et al., 2016; Cope et al., 2014; Hernandez and Kubota, 2016). In the present study, the combination of 60 % red and 40 % blue light increased biomass production compared to monochromatic treatments and was comparable to broad-spectrum white light (19 % blue). Overall, RB and W lights showed similar plant responses in terms of growth and morphology of the different organs

Table 2
ETR curve-derived parameters and total pigment content in *S. lycopersicum* grown under red (R), blue (B), red and blue (RB), and white (W) light. Dara are mean ± standard error; $n = 5$. Different letters indicate statistically significant differences among light treatments ($P < 0.05$).

Parameter	Units	R	B	RB	W
Ek	μmol photons m^{-2} s^{-1}	0.25 ± 0.01 a	0.26 ± 0.01 a	0.26 ± 0.01 a	0.25 ± 0.01 a
ETRmax	μmol electrons m^{-2} s^{-1}	182.08 ± 11.73 c	245.34 ± 5.96 b	269.46 ± 3.52 a	243.59 ± 3.91 b
alpha (α)		704.28 ± 34.32 c	950.11 ± 38.32 b	1059.15 ± 62.47 a	857.98 ± 40.02 b
Chl_a	mg g^{-1}	0.25 ± 0.02 c	0.43 ± 0.02 b	0.49 ± 0.04 a	0.40 ± 0.04 ab
Chl_b	mg g^{-1}	0.17 ± 0.01 a	0.16 ± 0.01 a	0.14 ± 0.01 b	0.14 ± 0.01 b
Chl_{tot}	mg g^{-1}	0.42 ± 0.01 c	0.59 ± 0.01 ab	0.63 ± 0.02 a	0.55 ± 0.02 b
Car	mg g^{-1}	0.13 ± 0.01 d	0.25 ± 0.01 b	0.27 ± 0.01 b	0.19 ± 0.02 c
Chl_a/Chl_b		1.55 ± 0.06 c	2.68 ± 0.08 b	3.61 ± 0.09 a	2.60 ± 0.08 b
Car/Chl_{tot}		0.31 ± 0.01 b	0.43 ± 0.01 a	0.43 ± 0.01 a	0.34 ± 0.01 b

E_k = saturating light intensity; ETR_{max} = maximum electron transport rate; *alpha* = rate of photon conversion under lower irradiances; Chl_a = chlorophyll *a*; Chl_b = chlorophyll *b*; Chl_{tot} = total chlorophylls; Car = carotenoids.

but produced differences in chlorophylls, carotenoids, and Rubisco content which were lower in W compared to RB plants. As stated by Mitchell and Stutte (2015), there is no single light-quality recipe that serves all species and every stage of the plant growth. However, a right combination of red and blue LEDs can typically drive photosynthesis and regulate vegetative growth in most species. In addition, as suggested by Cope and Bugbee (2013), it is likely that the optimal light spectrum for plants changes with age, as plants need to balance leaf area expansion (to maximize light harvesting) with stem elongation and reproductive growth, especially during early seedling development. Considering that the early development of tomato seedlings was very sensitive to light quality, manipulating the spectral light composition during the early photomorphogenesis could represent a promising tool to achieve high quality seedlings on a short-time scale.

4.2. Photosynthetic performance in relation to different light quality

Our data indicate that photosynthetic apparatus of seedlings at early stage of development is very sensitive to light quality, and in particular to monochromatic red and blue light treatments.

The heatmap analysis evidenced a net separation within four clusters based not only on morphological characteristics, but also on photosynthetic attributes. In particular, the monochromatic red and blue light reduced PSII photochemical efficiency in seedlings and enhanced thermal dissipation of excess light energy through non-photochemical quenching. The plant growth under the mixed RB light treatment produced similar photochemical responses of plants grown under W light, addressing the great part of the electron transport to photochemistry (elevated Φ_{PSII}) and reducing thermal dissipation (low NPQ). However, even if both red and blue monochromatic light treatments are less effective than RB and W light in driving photochemistry, our data suggest that the plant growth under R more than under B wavelength determined a stressful condition for the photosynthetic apparatus, as indicated by the highest reduction of PSII maximal photochemical efficiency (F_v/F_m) in R seedlings. Our results suggest that blue wavelengths are more critical than red ones because they stimulate more the photosynthetic electron transport capacity, according to the higher ETR_{max} in B compared to R plants. These results are consistent with data

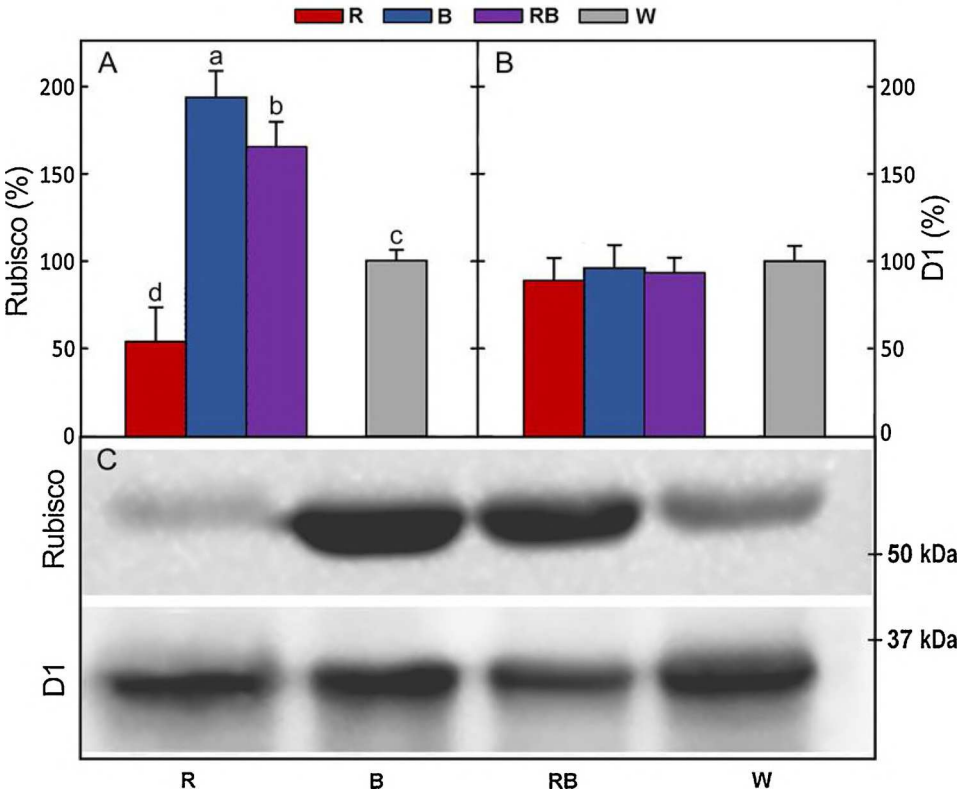

Fig. 4. Densitometry of Rubisco (A) and D1 (B) proteins from western blot analysis (C) of *S. lycopersicum* plants grown under different light treatments. R = 100 % red light; B = 100 % blue light; RB = 60 % red and 40 % blue light; W = white light. Each value represents the mean ± standard error ($n = 5$) considering W light as 100 %. Different letters indicate statistically significant differences among treatments at $P < 0.05$.

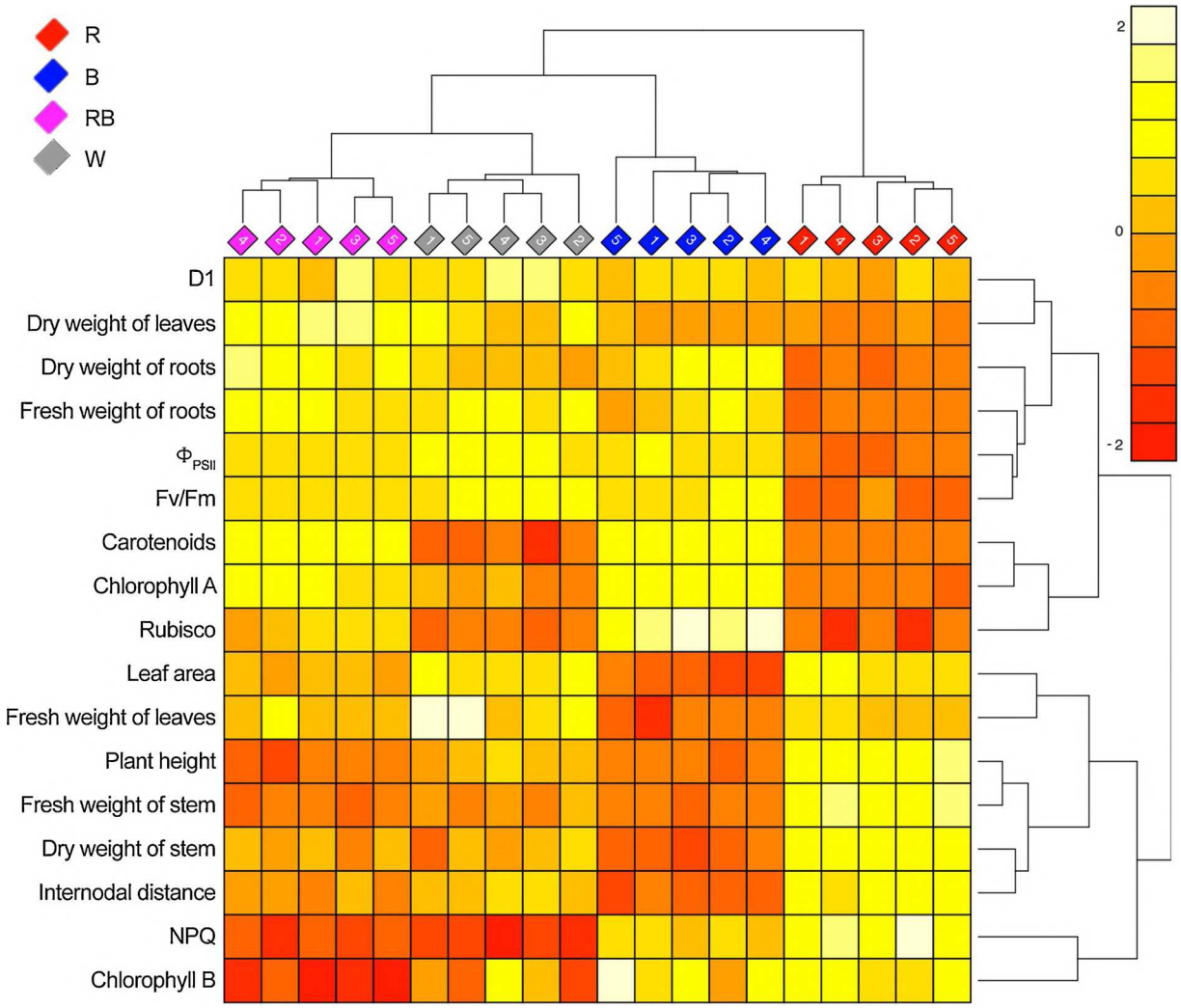

Fig. 5. Cluster heat map analysis summarizing *S. lycopersicum* responses to light quality treatments (R = 100 % red light, B = 100 % blue ligh, RB = 60 % red and 40 % blue light, W = white light). Results are visualized using a false color scale with pale yellow indicating an increase and red a decrease of the response parameters.

reported by Miao et al. (2016) in cucumber leaves. These authors found a reduction of photochemical efficiency under red light, which limited the electron transport rate both in PSII and in PSI, increasing the non-photochemical quenching.

The addition of blue to red light significantly promoted photosystems activity and linear electron transport in cucumber, while the pure blue light (100 %) did not impair the photosystems activity and electron transport capacity. Yang et al. (2018) demonstrated in tomato that the combination of red and blue light was beneficial for the photosynthetic efficiency, whereas monochromatic blue light reduced photosynthetic efficiency, enhancing the cyclic electron flow and NPQ to protect photosystems from photoinhibition. In our experiment, the growth under monochromatic blue light did not induce a decline in F_v/F_m compared to RB and W light treatments, indicating that the photosynthetic apparatus of tomato seedlings was not damaged by high-energy blue wavelengths. The moderate intensity of monochromatic blue light (190 µmol photons $m^{-2}s^{-1}$) experimented by tomato seedlings during growth was useful to drive photosynthesis without inducing photoinhibition.

The morphological "sun-leaf" traits induced by different percent of blue light during growth is consistent with the photochemical behavior of seedlings at saturating irradiance. In fact, whereas under lower irradiance the rate of photon conversion (alpha) did not differ among treatments, at saturating light intensity, B and RB seedlings, which received 100 % and 40 % of blue wavelengths respectively during the early development, reached the maximum electron transport rates (ETR_{max}) at saturating light intensities (Ek) higher compared to R and W seedlings. This result indicates that blue light may act as a regulator of the electron transport activity in tomato seedlings and that this capacity is pronounced at elevated irradiances.

At sub-saturating irradiance used in our study, the blue wavelengths are not too much to induce a strong rise of thermal dissipation processes, because the most part of light energy is used in photochemistry. Conversely, plants grown under monochromatic red light developed morphological traits typical of shade leaves, proving to suffer for the inadequate light supply to photosystems that limited the photochemical efficiency and determined a strong rise of NPQ. Thus, at sub-saturating light irradiances, monochromatic blue and red light cannot be considered equally effective at driving photosynthesis for tomato seedlings.

The addition of blue light to red (in our case R:B = 60:40) was effective in promoting the quantum yield of PSII electron transport compared to monochromatic blue and red light, further reducing in seedlings the need for thermal dissipation. The RB light regimen induced in seedlings the same photochemical behavior of W light, indicating that R and B wavelengths are both requested for an optimal functioning of the photosynthetic apparatus. The higher photosynthetic efficiency was in agreement with plant growth, since the higher biomass production was achieved in RB and W plants, while monochromatic light negatively affected growth of tomato seedlings according to a reduced photochemical efficiency, especially under red light.

In our experiment, the immunoblotting analysis on leaf tissue of *S. lycopersicum* seedlings grown under different light spectra indicated that the amount of D1 protein was not affected by light quality, in contrast with F_v/F_m reduction found in seedlings grown under monochromatic red light. Our results are in contrast with Kato et al. (2015), who demonstrated that monochromatic blue and red light influenced D1 degradation, that was faster under red compared to blue light according to F_v/F_m decline. As no change in D1 level was found in R seedlings, we suppose that the decrease in F_v/F_m was not due to a D1 photodamage, rather than to a down-regulation mechanism by which seedling reduced the photochemical efficiency and increased the thermal dissipation to prevent irreversible injuries to reaction centers. However as in the D1 degradation are involved some specific proteases (i.e. FtsH and Deg, see Kato et al., 2015) enhanced by blue more than red light, it cannot be excluded that the moderate blue and red light intensity experimented by seedling during the growth was not enough to activate the D1 cleavage.

Conversely to D1 protein, Rubisco amount varied significantly among seedlings grown under different light quality treatments, showing in R and RB seedlings the highest level of expression. Previous researches demonstrated that blue light typically induces sun-type chloroplasts (Lopez-Juez and Hughes, 1995; Walters and Horton, 1995), and increasing doses of blue light enhance photosynthetic rate per unit leaf area (Goins et al., 1997; Hernández and Kubota, 2016; Hogewoning et al., 2010; Terfa et al., 2013; Yorio et al., 2001). In well-watered tomato plants, blue light is expected to promote CO_2 uptake in plants by suppressing signaling of ABA-induced stomatal closure (Inoue and Kinoshita, 2017). As a consequence, an improvement of photosynthesis is expected in B plants, as also suggested by the higher Rubisco content and photochemical activity compared to R plants. Besides, blue light is involved in adjustments of photosynthetic apparatus and influences the biochemical properties of photosynthesis (Senger and Bauer, 1987; Leong and Anderson, 1984), inducing a higher Rubisco content and activity per unit leaf area compared to plants grown under red light (Eskins et al., 1991).

Blue light increases the transcription level of the gene *rbc*S encoding for the small subunit of Rubisco (Sawbridge et al., 1994). Accordingly, we found that in tomato seedlings different amount of blue light stimulated the Rubisco expression, which increased under RB and more under B compared to W and R plants. Our results are also consistent with studies performed on other species (Fluhr and Chua, 1986; Poyarkova, 1973). However, further studies are needed to deepen the relation between the changes in the photosynthetic apparatus and gas exchange under monochromatic red or blue light.

Hogewoning et al. (2010) found that photosynthetic rate under blue and red light increases with increasing blue light percentage from 0 to 50 %. In our study, plants grown under RB light (40 % blue light) increased biomass production, which is likely due to a higher photosynthetic capacity as a combination of greater photochemical efficiency and higher level of Rubisco, which might ultimately lead to higher CO_2 assimilation rates. Accordingly, the lowest Rubisco content together with reduced capability of photosynthetic apparatus to utilize the absorbed light led to the lowest biomass accumulation under monochromatic red light. Without the “mitigating effect” of red light, the pure blue light, even inducing the highest Rubisco expression levels, did not lead to a photochemical efficiency comparable to RB and W plants.

The highest amount of Rubisco in B seedlings was consistent with the highest maximum electron transport rates (ETR_{max}) at saturating irradiance, indicating a balance between the electron transport capacity and Rubisco synthesis. However, this balance did not imply a more efficient photosynthetic carbon fixation, because the pure blue light determining a strong reduction of leaf area, limited light interception and harvesting. This reduction has been also associated with a decrease in the size and number of leaf epidermal cells, compromising the overall plant growth (Dougher and Bugbee, 2004).

In conclusion, our findings suggest that red and blue wavelengths have considerable effects on various plant responses during early photomorphogenesis. The growth under red and blue light primes regulation mechanisms implicating a fine interaction between photosynthesis and plant morphological traits, which ultimately determine the plant growth. The pure red light induced shade-avoidance responses in seedlings increasing hypocotyl and cotyledon elongation, pigment content, and leaf area. These changes resulted in a strong decline of photochemical activity, compared to RB treatment and more complete W light spectra. Monochromatic blue light induced a reduction of the overall plant size affecting plant growth and, in a less severe manner, photochemistry. The lack of specific wavelengths such as blue or red light severely impaired plant development, in term of both morphology and physiology. However, blue light resulted more essential than red light for the functionality of photosynthetic apparatus, resulting in seedlings with higher physiological performance. Being seedling development very sensitive to red and blue wavelengths, light recipe opportunely designed may represent an effective tool in improving such phenological phase on a short-time scale.

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CRediT authorship contribution statement

Luigi Gennaro Izzo: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Bruno Hay Mele:** Investigation, Data curation, Writing - review & editing. **Luca Vitale:** Investigation, Writing - review & editing, Supervision. **Ermenegilda Vitale:** Investigation, Writing - review & editing. **Carmen Arena:** Conceptualization, Methodology, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Chapter II – Different light spectral composition regulates the photosynthetic capacity and fruit antioxidant properties of *Solanum lycopersicum* L. cv. 'Microtom' in controlled environment.

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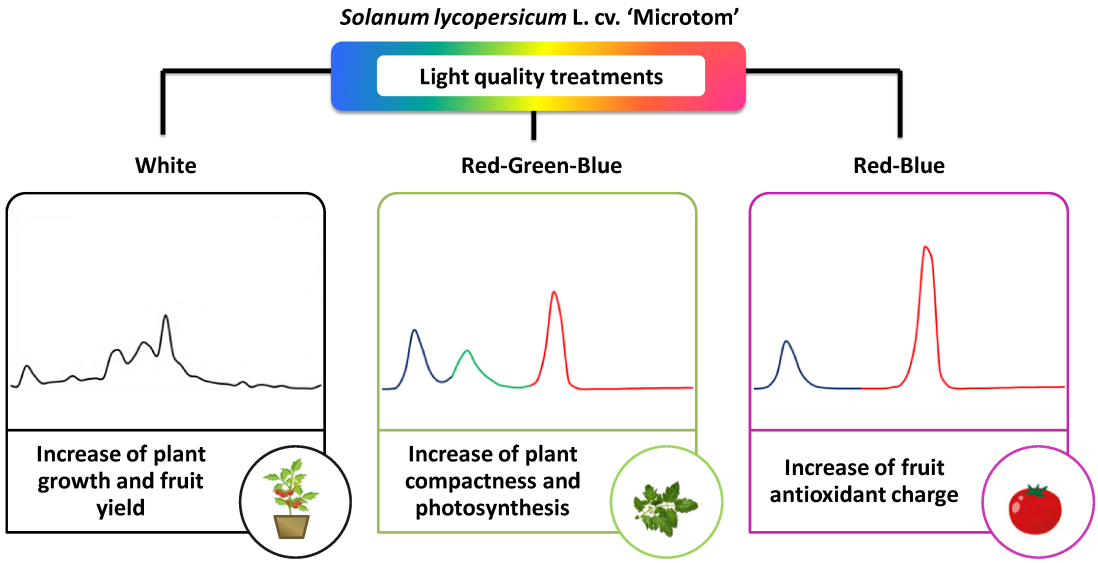

Different light spectral composition regulates the photosynthetic capacity and fruit antioxidant properties of *Solanum lycopersicum* L. cv. 'Microtom' in controlled environment.

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Abstract

- Light quality plays an essential role in setting plant structural and functional traits, including antioxidant compounds. This paper aimed to assess how manipulating the light spectrum during growth may regulate the photosynthetic activity and fruit bioactive compound synthesis in *Solanum lycopersicum* L. cv. 'Microtom' to improve plant physiological performance and fruit nutritional value.
- Plants were cultivated under three light quality regimes: Red-Green-Blue (RGB), Red-Blue (RB) and white (W), and growth was monitored from seed to fruit ripening. Leaf functional traits, photosynthetic efficiency, Rubisco and D1 protein expression, and antioxidant production in fruits were analysed.

- Compared to W, RGB and RB regimes reduced plant height and increased leaf number and specific leaf area, enhancing plant compactness. Despite reduced biomass, the RGB regime significantly improved photosynthesis and stomatal conductance, favouring rubisco synthesis and carboxylation rate compared to RB and W regimes. The RB light produced plants with fewer flowers and fruits with the highest polyphenol content, antioxidant capacity and SOD and CAT activities.
- The high percentage of the green wavelength in the RGB regime promoted photosynthesis but reduced plant reproductive capacity compared to W and RB. The RB regime increased the fruit nutritional value, induced the highest production of health-promoting antioxidants.

Key words: D1 protein, gas exchanges, leaf functional traits, light quality, photochemistry, Rubisco

Abbreviations: AsA, ascorbic acid; B, blue; CAT, catalase; cv, cultivar; ETR, electron transport rate; FRAP, ferric reducing antioxidant power; G, green; LA, leaf area; LDMC, leaf dry matter content; LEDs, light emitting diodes; F_v/F_m, maximum PSII photochemical efficiency; V_{cmax}, maximum rate of Rubisco carboxylation; g_m, mesophyll conductance to CO_2 diffusion; A_N, net CO_2 assimilation; NPQ, non-photochemical quenching; PPFD, photosynthetic photon flux density; ΦPSII, quantum yield of PSII electron transport; R, red; RWC, relative water content; SLA, specific leaf area; g_s, stomatal conductance; SOD, superoxide dismutase; W, white.

Introduction

Meeting global demand for healthy fresh food production for the increasing population represents a crucial challenge currently. In the last decades, extensive cultivation areas have been overexploited with intensive impoverishing of natural resources and an increase of cultivation techniques not ever sustainable (FAO 2017). Generally, open field cultivation is increasingly threatened by the risks and uncertainties associated with biotic and abiotic stresses, such as pest attacks,

drought, and frost, exacerbated by the ongoing climate changes (Pandey *et al.* 2017). These circumstances have impelled new cultivation approaches to guarantee sustainable plant crop production (Dutta-Gupta2017; FAO 2019). The cultivation under controlled conditions in greenhouses and closed systems has emerged as a feasible alternative, as it optimises the plant growth environment by minimising the interactions with the external factors (Dutta-Gupta 2017; Amitrano *et al.* 2018; Pennisi *et al.* 2019).

The application of specific light quality regimes during plant growth is gained an increasing interest in Controlled Environment Agriculture (CEA), an innovative approach to controlling plant development and productivity (Niu & Masabni 2018; Izzo *et al.* 2020).

The growth under specific light wavelengths allows obtaining plants with improved morphological, anatomical, and physiological traits (Arena *et al.* 2016; Yang *et al.* 2017). In circumstances in which unfavourable outdoor conditions limit the open field cultivation, the manipulation of light quality by light-emitting diodes (LEDs) technology offers the possibility to select the growth regime more appropriate to specific requirements of the different crops, representing an effective tool to improve crop productivity and food quality in indoor cultivation.

The possibility to produce fresh food in extreme environments, such as Space platforms, has increased the interest in evolving light protocols promoting plant photosynthesis and yield (Gomez & Izzo 2018). NASA has supported the development of LED-based plant growth systems since the late 1980s for space-oriented research on the International Space Station (ISS), to support future colonies on the Moon and Mars (Massa *et al.* 2006; Wheeler 2010), with important implications also on Earth linked to the possibility to cultivate plants in extreme terrestrial environments like hot and cold deserts.

Knowledge about plant physiological and morphological responses to LED lighting increased noticeably during the last years; however, as the light requirements depend are specie-specific, it is not easy to define a standard behaviour.

For instance, red and blue wavelengths are most efficiently utilised for photosynthesis (Vitale *et al.* 2020). Red light influences the photosynthetic

apparatus development, biomass accumulation, and stem elongation (Urbonavičiūte *et al*. 2007; Wang *et al*. 2009), as well as the level of soluble sugars (Cui *et al*. 2009) and fruits antioxidant compounds, like carotenoids and phenols (Panjai *et al*. 2017).

The blue light is mainly involved in vegetative growth regulation, early photomorphogenesis, and stomata control (Chen *et al*. 2014; Singh *et al*. 2015; Izzo *et al*. 2020). A high proportion of blue wavelengths within the light spectrum, being more energetic, may cause light avoidance phenomena in chloroplasts, reducing photosynthesis (Loreto *et al*. 2009; Pallozzi *et al*. 2013) and increasing the antioxidant production (i.e., lettuce, spinach) (Lester 2006; Ohashi-Kaneko *et al*. 2007; Hasan *et al*. 2017) and protein biosynthesis (Li & Pan 1994; Hasan *et al*. 2017) in some leafy vegetables.

Finally, green light penetrating deeply in the leaf mesophyll layers and reaching the lower and inner canopy levels promotes photosynthesis and plant carbon gain in the deepest chloroplasts (Terashima *et al*. 2009), improving crop productivity and yield (Smith *et al*. 2017). Besides, the green wavelengths are also involved in seed germination and plant flowering (Wang & Folta 2013) and increasing the antioxidant properties of vegetables and sprouts, contributing to the high food quality (Bantis *et al*. 2016; Samuolienė *et al*. 2011).

Based on previous evidence, the modulation of the light spectrum, by selecting and combining different proportions of red, blue, green wavelengths may produce in selected crops attractive characteristics, namely high photosynthetic rates, fruit yield or edible biomass and nutraceutical compounds, promising in the context of sustainable agriculture in greenhouses and small volumes such as those at disposal on the ISS.

This study aimed to evaluate the effects of three different light quality regimes, white (W), red-green-blue (RGB), and red-blue (RB) light on *Solanum lycopersicum* L. cv. 'Microtom' growth, photosynthetic performance, and fruit antioxidant properties. Specific attention is paid to photosynthetic process regulation, including the expression levels of PSII D1 protein and Rubisco in response to light quality treatment to understand the mechanisms allowing the plant to improve productivity. Among different and more productive tomato cultivars, 'Microtom' was chosen in

our experiment for a series of characteristics, such as short life cycle, compact size, fast growth, which makes this cultivar ideal for indoor cultivation at high plants density, conversely to other tomato landraces requiring wide spaces (Okazaki & Ezura 2009; Saito *et al*. 2011; Shikata *et al*. 2016). For its good traits, 'Microtom' is widely used in breeding programs and space-oriented experiments to provide fresh food to the space crew (Saito *et al*. 2011; De Micco *et al*. 2014, Arena *et al*. 2019; Colla *et al*. 2007).

Materials and Methods

Plant material and growth conditions

Seeds of *Solanum lycopersicum* L. cv. 'Microtom', provided by Holland Online Vof (Amsterdam, The Netherlands), were sown in 3.0 L pots filled with peat soil. Plants were cultivated in a climatised chamber under three different light regimes: white light (W) obtained by using fluorescent tubes (Lumilux L360W/640 and L360W/830, Osram, Germany); red-green-blue (RGB, Red 33%, Green 33%, Blue 33%) and red-blue (RB, Red 66%, Blue 33%) supplied by light-emitting diodes (LEDs) (LedMarket Ltd., Plovdiv, Bulgaria) with the following emission peaks: 630 nm red, 510 nm green, 440 nm blue. An SR-3000A spectroradiometer was used to the spectral composition of the three light sources (Fig. 1) with 10 nm resolution (Macam Photometrics Ltd., Livingston, Scotland, U.K.). 'Microtom' plants were grown for 90 DAS (days after sowing) until fruit ripening under the following environmental conditions: photosynthetic photon flux density (PPFD) 300 ± 23 μmol photons m^{-2} s^{-1} for each light treatment, day/night air temperature 24/18 °C, relative air humidity 60-70%, and a photoperiod of 12 h.

Plants were irrigated to pot capacity with tap water at a two-day interval to reintegrate the water loss for evapotranspiration. Every two weeks, plants were fertilised with Hoagland's solution.

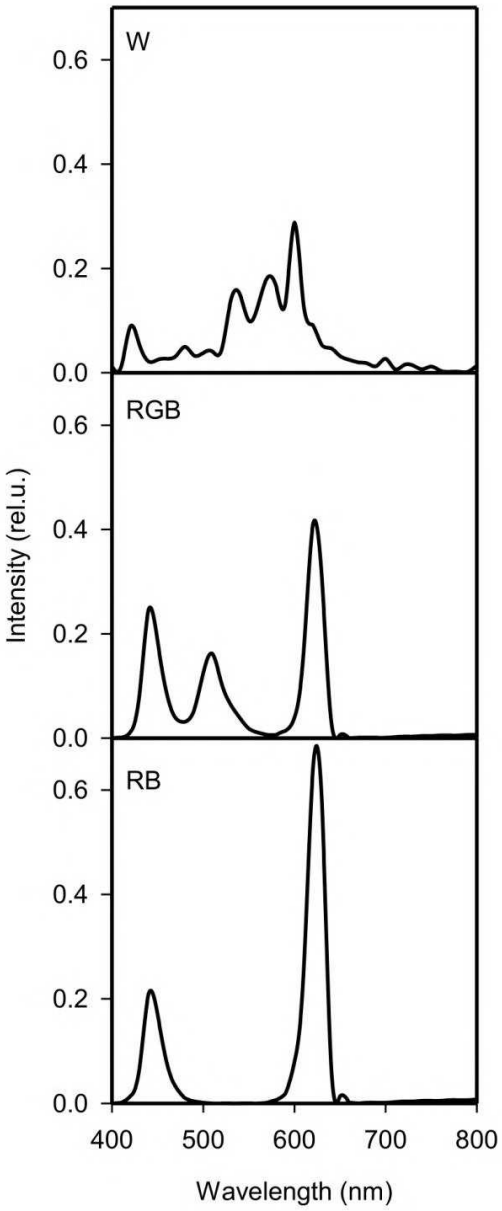

Figure 1: Spectral distributions in the relative energy of the fluorescent tubes and LEDs panels recorded for W (white fluorescent light), RGB (R 33%, G 33%, B 33%) and RB (R 66%, B 33%) treatments at the top of the plant canopy.

Measurements of plant growth and leaf functional traits

Measurements of plant height (cm, considering the main stem), leaf number, fruit number, fruit weight (g FW per plant), and plant total biomass (g FW per plant) were carried out at 100 DAS, as well as the ratios leaf biomass/total biomass and fruit biomass/total biomass. The flower number was monitored starting from 40 up

to 70 DAS until the first fruits' appearance, considering for each plant the sum of flowers measured within the range 40-70 DAS.

The determination of leaf functional traits (leaf area, LA; specific leaf area, SLA; leaf dry matter content, LDMC; relative water content, RWC), flavonoids and Nitrogen Balance Index (NBI) were assessed at 50 DAS before flowering according to Cornelissen *et al.* (2003) on fully expanded leaves. LA (cm^2) was measured by acquiring digital images and using the program ImageJ 1.45 (Image Analysis Software, NIH, USA). SLA was determined as the ratio of leaf area to leaf dry mass and expressed in $cm^2\ g^{-1}$. LDMC was calculated as dry leaf mass to saturated fresh mass and reported in $g\ g^{-1}$. RWC was expressed as a percentage of the ratio (fresh leaf mass – dry leaf mass)/(saturated leaf fresh mass – dry leaf mass). The saturated fresh mass was obtained by submerging the petiole of leaf blades in distilled water for 48 h in the dark at 15°C, whereas the dry mass was determined after oven-drying leaves at 75°C for 48 h.

Measurements of plant growth and leaf functional traits were evaluated on 5 different leaves from 5 plants per light regime.

Gas exchange and chlorophyll a fluorescence measurements

Gas exchange and chlorophyll a fluorescence measurements were carried out on five fully-expanded leaves per each light treatment at 50 DAS. The net CO_2 assimilation (A_N) and the stomatal conductance (g_s) were measured using a portable leaf gas exchange system (LCpro+, ADC BioScientific, UK). The apical leaflet of each compound leaf was clamped into the gas exchange system cuvette for measurements at 1000 μmol $m^{-2}\ s^{-1}$ PPFD, 25 ± 2 °C leaf temperature and 50-60% relative humidity. The photosynthesis and the stomatal conductance were calculated as indicated in von Caemmerer & Farquhar (1981). The mesophyll conductance to CO_2 diffusion (g_m) was determined using the variable J method (Loreto *et al.* 1992), whereas the maximum rate of Rubisco carboxylation (V_{cmax}) was estimated as proposed by Farquhar, von Caemmerer & Berry (1980).

After gas exchange measurements, chlorophyll *a* fluorescence was assessed by a Fluorescence Monitoring System (FMS, Hansatech Instruments, King'Lynn, UK).

The background fluorescence signal, F_0, was induced on 20 min dark-adapted leaves, by an inner light of about 2–3 μmol photons m^{-2} s^{-1}, at a frequency of 0.5 kHz. Previous experiments demonstrated that 20 minutes are sufficient to obtain complete re-oxidation of PSII reaction centres (Shahzad *et al.* 2020). The maximum fluorescence level (F_m) in the dark-adapted state was determined with a 1s saturating light pulse of about 6000 μmol photons m^{-2} s^{-1}. The maximum PSII photochemical efficiency (F_v/F_m) was calculated as $(F_m-F_0)/F_m$. Under illumination at plant growth irradiance, the steady-state fluorescence (F_s) was measured, and maximum fluorescence (F_m') in the light-adapted state was determined by applying a saturating pulse of 0.8 s with over 6000 μmol photons m^{-2} s^{-1}. The quantum yield of PSII electron transport (Φ_{PSII}) was calculated as $(F_m'-F_s)/F_m'$ according to Genty *et al.* (1989), while the non-photochemical quenching (NPQ) was expressed as $(F_m-F_m')/F_m'$ as reported in Bilger & Björkman (1991).

Photosynthetic proteins D1 and Rubisco and pigments content

After chlorophyll fluorescence and gas exchange measurements, the same leaves were collected to perform the protein extraction following the procedure of Wang *et al.* (2006) modified by Arena *et al.* (2019). Protein extracts were quantified with the Bradford assay (1976) and subjected to an SDS-PAGE (12%). The Western Blot analysis started treating the leaf samples with a blocking solution (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween20, 10% Milk). In order to reveal the selected proteins, samples were then incubated with the respective primary and secondary antibodies (Agrisera, Vännäs, Sweeden): anti-PsbA (chicken, 1:5000 v/v) and goat anti-chicken for D1 protein, anti-RbcL (rabbit, 1:10 000 v/v) and goat anti-rabbit for Rubisco. Immuno-revelation was carried out using the kit for chemiluminescence (ECL Western Blotting Analysis System, Ge Healthcare) with the Chemidoc system (Bio-Rad Laboratories). The software Quantity One (Bio-Rad) was used for the densitometric analysis to obtain quantitative information associated with the individual bands. The density value of each band was expressed in percentage and represented as a bar diagram assuming the bar of W light as control (100%).

The determination of photosynthetic pigments content, total chlorophylls (a+b) and carotenoids (x+c) was performed according to Lichtenthaler (1987). Briefly, leaf samples of known area were homogenised in ice-cold 100% acetone utilising a mortar and pestle. The extracts were centrifuged at 3200 *g* for 5 min in a Labofuge GL (Heraeus Sepatech, Hanau, Germany). The absorbance was measured by spectrophotometer (UV-VIS Cary 100; Agilent Technologies) at wavelengths of 470, 645 and 662 nm, and pigment concentration was expressed as μg cm^{-2}.

2.5 Fruit antioxidant characterization

The effect of different light quality regimes on the antioxidant properties of 'Microtom' fruits was evaluated by grinding fresh samples (0.250 g) in liquid nitrogen. The ascorbic acid (AsA) content, superoxide dismutase (SOD) and catalase (CAT) activities were determined as previously described in Arena *et al.* (2019).

The AsA concentration was evaluated with the Ascorbic Acid Assay Kit II (Sigma-Aldrich, St. Louis, MO, USA) based on the ferric reducing/antioxidant and ascorbic acid (FRASC) assay. Antioxidants contained in the sample are involved in reducing Fe3+ into Fe2+, resulting in a coloured product. After the addition of ascorbate oxidase, any ascorbic acid is oxidized and quantified by measuring the absorbance at 593 nm with a spectrophotometer (UV-VIS Cary 100; Agilent Technologies). The AsA concentration was determined using a standard curve and expressed in mg L^{-1}, as reported in Costanzo *et al.* (2020).

The SOD Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the SOD activity by measuring inhibition of the nitroblue tetrazolium (NBT) reduction into blue formazan. The absorbance of the blue colour generated during the colourimetric reaction was read at 440 nm with a spectrophotometer (UV-VIS Cary 100; Agilent Technologies). The volume of the sample that caused the 50% inhibition in blue formation was defined as a unit of SOD activity.

The CAT activity was assessed through the Catalase Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). The colourimetric decomposition reaction of H_2O_2 into H_2O and O_2 was spectrophotometrically (UV-VIS Cary 100; Agilent Technologies) followed by monitoring the decreasing absorbance at 520 nm. The amount of enzyme capable

of decomposing 1 μmol of H_2O_2 per minute at pH 7.0 and 25°C was considered a CAT activity unit.

The total antioxidant capacity was assessed as described in George *et al.* (2004) by the Ferric Reducing Antioxidant Power assay (FRAP). Briefly, the samples (0.250 g) were treated with methanol/water solution (60:40, v/v) and centrifuged at 20 817 *g* for 15 min at 4°C. The extracts were mixed with the FRAP reagents (300 mM acetate buffer pH 3.6, 1:16.6 v/v; 10 mM tripyridyltriazine, TPTZ, in 40 mM HCl, 1:1.6 v/v; 12 mM $FeCl_3$, 1:1.6 v/v) and incubated for 1 h in the dark. Then, the absorbance was read at 593 nm by a spectrophotometer (UV-VIS Cary 100; Agilent Technologies). The antioxidant capacity was calculated using a Trolox standard curve and expressed as μmol Trolox equivalents (μmol Trolox eq. g^{-1} FW).

The total polyphenols were determined by the procedure reported in Costanzo *et al.* (2020). Briefly, samples (0.02 g) were extracted with aqueous 80% methanol at 4°C (for 30 min) and then centrifuged at 12 851 *g* for 5 min. The soluble fraction was mixed with 10% Folin-Ciocalteu solution, 1:1 v/v, and after 3 min, 700 mM Na_2CO_3 solution was added to the resulting mixture (5:1, v/v). Samples were incubated for 2 h in the darkness. The absorbance was measured at 765 nm spectrophotometrically (UV-VIS Cary 100; Agilent Technologies). The total polyphenol content was calculated using a gallic acid standard curve and expressed as mg Gallic Acid Equivalents (GAE) 100 g^{-1} FW.

Statistical analysis

Results were analysed using SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA). The effect of the different light quality treatments on the investigated parameters was assessed by applying a one-way analysis of variance (ANOVA). The Student-Newman-Keuls test was applied for all pairwise multiple comparison tests with a significance level of $p \leq 0.05$. The Kolmogorov–Smirnov and Shapiro–Wilk tests were performed to check for normality. Data are reported as mean values ± standard error (n=5). The overall parameters for leaves and fruits were visualized by two heatmaps (heatmap function). The heatmaps were plotted using the ClustVis program package (https://biit.cs.ut.ee/clustvis/online) and clustering both rows and

columns with Euclidean distance and average linkage. In the heatmaps, the numeric differences are evidenced by the colour scale: red and blue indicate increasing and decreasing values, respectively.

Results

Biometric measurements and leaf functional traits

Different light quality regimes affected significantly plant morphological attributes and leaf functional traits (Fig.2 and Table 1).

Figure 2. Representative *S. lycopersicum* L. cv. 'Microtom' plants cultivated under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes.

RGB and RB treatments reduced ($p<0.05$) plant height and increased ($p<0.05$) leaf number compared to W light treatment. On the other hand, plants grown under the RGB regime developed the lowest ($p<0.05$) number of flowers and fruits as well as a reduced ($p<0.05$) fruit and total biomass than W and RB plants, which showed comparable values.

The growth under the three light regimes also induced a different partitioning of fresh biomass among, more specifically, plants cultivated under RGB and RB light regimes invested more biomass ($p<0.01$) into leaves (high leaf biomass/total biomass ratio) compared to W plants. Conversely, W and RB plants showed higher ($p<0.05$) partitioning of biomass in fruits (high fruit biomass/total biomass). Under RGB treatment, LA significantly decreased ($p<0.05$) compared to W and RB light regimes. An opposite behaviour was observed for SLA and LDMC: W plants

showed a lower ($p<0.05$) SLA and a higher ($p<0.05$) LDMC compared to those grown under RGB and RB which exhibited comparable values. Lastly, RWC was not affected by different light quality treatments.

Table 1. Morphological parameters and leaf functional traits of *S. lycopersicum* L. cv. 'Microtom' plants cultivated under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. Data are mean (n=5) ± standard error. Different letters indicate statistically significant differences among light treatments ($p<0.05$) according to one-way ANOVA.

	Light quality regimes		
	W	RGB	RB
Morphological parameters			
Height (cm)	15.67±0.66 [a]	11.27±0.37 [b]	12.17±0.56 [b]
Leaf number	22.67±0.97 [c]	30.00±0.84 [b]	37.67±1.85 [a]
Flower number	50.00±2.76 [a]	36.67±1.32 [b]	47.00±3.33 [a]
Fruit number	18.67±1.93 [a]	10.00±0.63 [c]	14.67±1.49 [b]
Fruit weight (g)	34.79±0.66 [a]	19.26±0.68[b]	28.74±0.26[a]
Plant total biomass (g)	58.55±4.58 [a]	40.44±0.82 [b]	52.31±0.68 [a]
Leaf biomass/total biomass	0.17±0.005 [b]	0.28±0.002 [a]	0.24±0.007 [a]
Fruit biomass/total biomass	0.58±0.008 [a]	0.47±0.004 [b]	0.57±0.008 [a]
Leaf functional traits			
LA (cm^2)	14.06±0.156 [a]	10.85±0.044 [b]	15.62±0.044 [a]
SLA ($cm^2\ g^{-1}$)	321.52±2.611 [b]	399.45±2.936 [a]	409.45±2.936 [a]
RWC (%)	81.97±0.265 [a]	78.84±0.419 [a]	82.90±0.419 [a]
LDMC ($g\ g^{-1}$)	0.10±0.001 [a]	0.08±0.001 [b]	0.08±0.001 [b]

LA: Leaf area, SLA: specific leaf area, RWC: relative water content, LDMC: leaf dry matter content

Gas exchange and fluorescence emission measurements

RGB light regime determined a significant increase ($p<0.05$) of A_N and g_m compared to W and RB treatments (Fig. 3A, C). Conversely, different light quality regimes did not affect g_s (Fig. 3B). Consistent with A_N, V_{cmax} was higher ($p<0.05$) in RGB than W and RB plants. The lowest value of V_{cmax} was measured in RB plants (Fig. 3D).

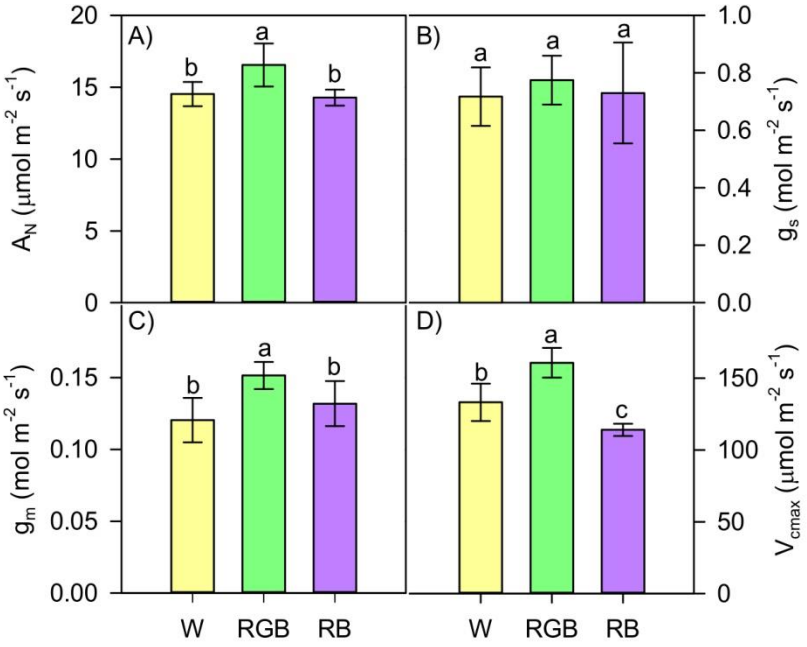

Figure 3. A) Net CO_2 assimilation (A_N), B) stomatal conductance (g_s), C) mesophyll conductance (g_m), D) maximum rate of Rubisco carboxylation (V_{cmax}) in *S. lycopersicum* L. cv. 'Microtom' plants grown under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. Data are mean ± standard error (n=5). Different letters indicate statistically significant differences among light treatments ($p<0.05$) according to one-way ANOVA.

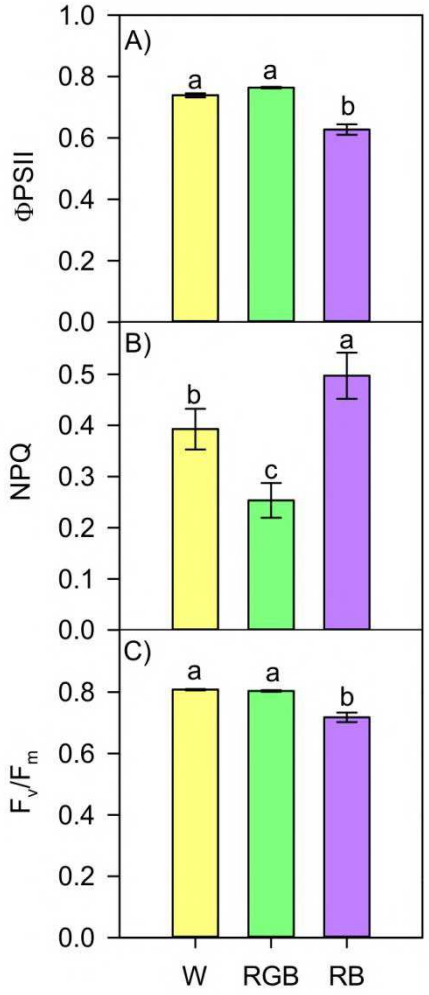

Figure 4. A) Quantum yield of PSII electron transport (Φ_{PSII}), B) non-photochemical quenching (NPQ), and C) maximum PSII photochemical efficiency (F_v/F_m) in *S. lycopersicum* L. cv. 'Microtom' plants cultivated under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. Data are mean ± standard error (n=5). Different letters indicate statistically significant differences among light treatments (p<0.05) according to one-way ANOVA.

The values of Φ_{PSII} and F_v/F_m were lower (p<0.01) in RB compared to RGB and W plants (Fig. 4A, C). Consistently, RB plants also showed a higher (p<0.05) NPQ compared to RGB and W plants (Fig. 4B). In particular, plants grown under the RGB regime exhibited the lowest (P<0.01) NPQ.

Photosynthetic proteins and leaf pigments content

The plant cultivated under RB light significantly reduced (p<0.05) the content of D1 protein and Rubisco compared to W and RGB light regimes. No difference in D1 protein amount was found between W and RGB plants. On the contrary, plants grown under RGB light showed the highest (p<0.01) Rubisco amount among light treatments (Fig. 5).

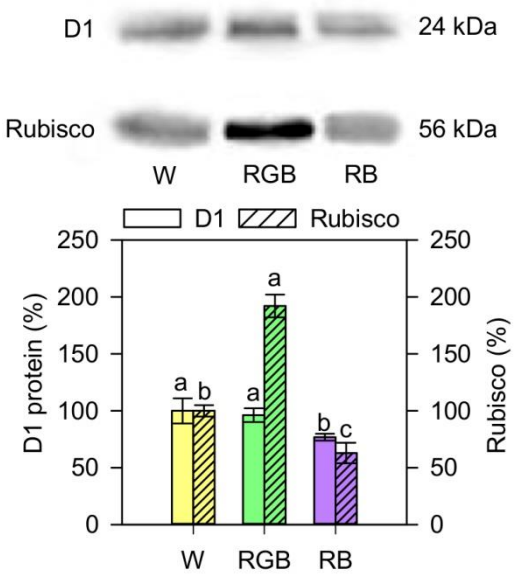

Figure 5. Western Blot and densitometric analysis of PSII D1 protein and Rubisco in *S. lycopersicum* L. cv. 'Microtom' plants grown under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. The density value of each band was expressed as a percentage and represented as a bar diagram assuming the bar of W light regime as 100%. Data are mean ± standard error (n=3). Different letters indicate statistically significant differences among light regimes (p<0.05) according to one-way ANOVA.

The total chlorophyll and carotenoid contents significantly decreased (p<0.05) in RGB and RB compared to W plants. The lowest (p<0.01) concentration of photosynthetic pigments was found in plants grown under RB light regime (Fig. 6A, B). An opposite trend was observed for Chl *a/b* ratio, which resulted higher (P<0.05) in RGB and even more in RB compared to W plants (Fig. 6C).

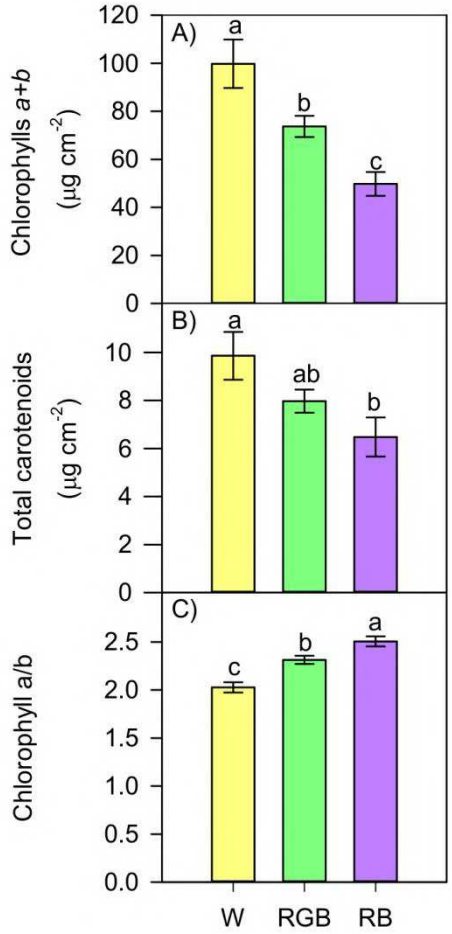

Figure 6. A) Total chlorophylls (*a+b*), B) total carotenoids, C) ratio between chlorophyll a and b (Chl a/b) in *S. lycopersicum* L. cv. 'Microtom' plants grown under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. Data are mean ± standard error (n=5). Different letters indicate statistically significant differences among light regimes ($p<0.05$) according to one-way ANOVA.

Antioxidant determination in fruits

The plant cultivation under RB light regime strongly affected the antioxidant properties of fruits. Indeed SOD and CAT activities, as well as the antioxidant capacity significantly increased ($p<0.01$, $p<0.001$ respectively) in RB compared to W and RGB fruits (Fig. 7A, B, C). SOD and CAT activities did not differ between W and RGB fruits, conversely to the antioxidant capacity, which was higher ($p<0.001$) in RGB than W fruits. Furthermore, the total polyphenol content also increased ($P<0.001$) in RB compared to W and RGB fruits, reaching a concentration about 9 times higher than that found under the other two light regimes (Fig. 7E). On

the other hand, the RB light regime did not promote the AsA content, which decreased ($p<0.05$) in RB compared to W and RGB fruits (Fig. 7D).

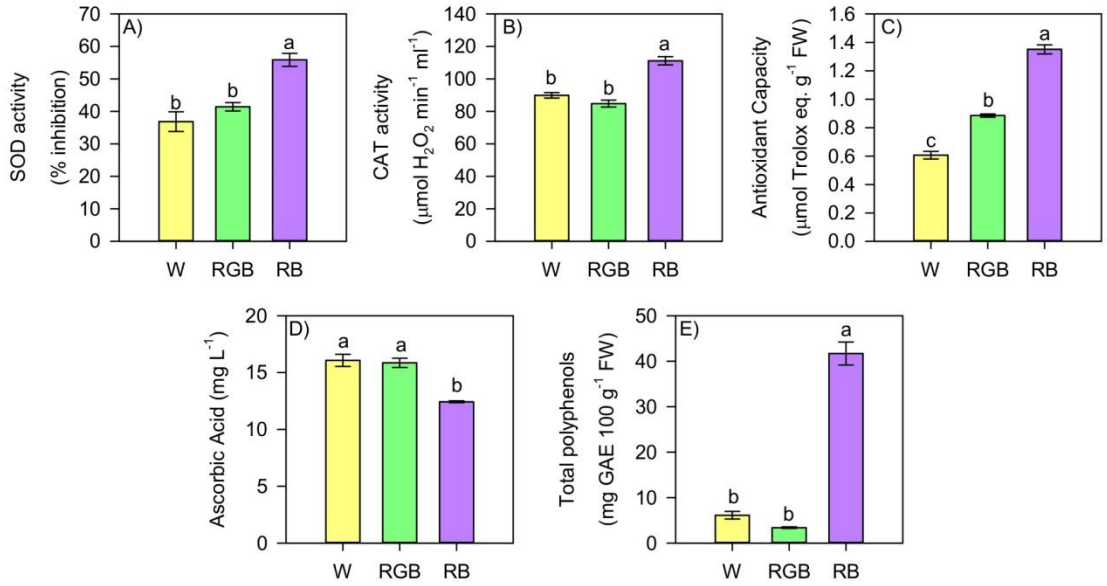

Figure 7: A) SOD activity, B) CAT activity, C) antioxidant capacity, D) ascorbic acid concentration, E) total polyphenols in *S. lycopersicum* L. cv. 'Microtom' fruits developed on plants grown under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. Data are mean ± standard error (n=5). Different letters indicate statistically significant differences among light regimes ($p<0.05$) according to one-way ANOVA.

Heatmap analyses

An overview of the morphological, photosynthetic and functional traits of 'Microtom' plants in response to W, RGB and RB light regimes is displayed in Fig. 8A.

The heatmap separated W and RB from RGB plants, evidencing how the green wavelength to light spectrum effectively promotes gas exchanges and carbon fixation. Conversely, under W light growth regime are clustered plants with high biomass and flower number, high photochemistry, photosynthetic pigment content and D1 protein amount. RB light regime grouped plants with high SLA, leaf number and chlorophyll a/b ratio.

Fig. 8B summarises the fruit traits, including the antioxidant properties. RB fruits were separated from RGB and W fruits. In particular, W light regime induced higher

fruit production and fruit biomass. Conversely, the RB light regime clustered fruits with higher antioxidant charge due to higher values of CAT and SOD activities, polyphenols and total antioxidant capacity.

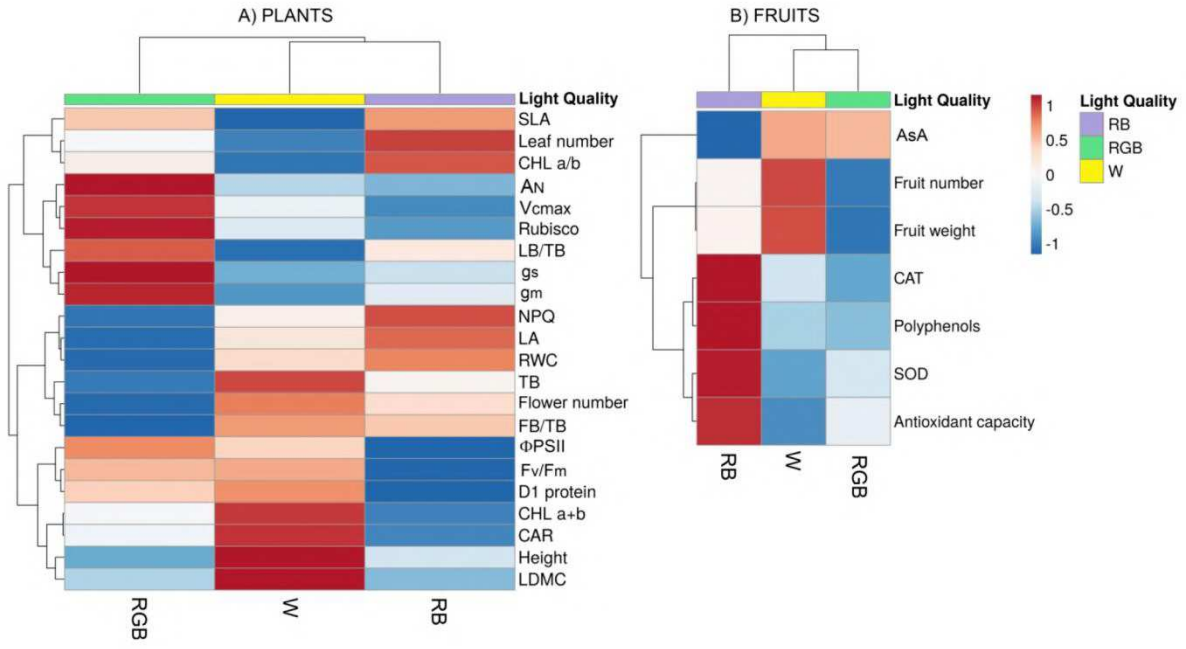

Figure 8: Cluster heatmap analysis summarising plant morphological, physiological and biochemical traits (A) and fruit characteristics (B) of *S. lycopersicum* L. cv. 'Microtom' plant cultivated under white fluorescent (W), red-green-blue (RGB, R 33%, G 33%, B 33%) and red-blue (RB, R 66%, B 33%) light regimes. The colour scale shows numeric differences within the data matrix: red and blue indicate increasing and decreasing values. Parameters are clustered in the rows; sample groups are clustered in the Light Quality factor columns.

Discussion

Our study showed that different light quality regimes strongly affect the photosynthetic and morphological traits in 'Microtom' plants and the antioxidant capacity of fruits and confirm that the modulation of the light spectrum may be a valuable tool in inducing specific traits in this cultivar, especially in indoor cultivation environments.

According to earlier studies performed on tomato, RB and RGB light quality regimes significantly reduced the stem elongation compared to W light (Xiaoying *et al.* 2012; Arena *et al.* 2016; Dieleman *et al.* 2019; Izzo *et al.* 2020). The higher percentage of blue light composing RB and RGB treatments may be responsible for the more

compact size observed in these plants because blue wavelengths can negatively affect the plant elongation by inhibiting cell division and expansion (Dougher & Bugbee 2004; Nanya *et al.* 2012). However, the 'Microtom' cultivar is intrinsically characterised by a compact size, and the enhancement of this typical trait may advantage its cultivation in a high plant density condition or restricted volumes.

It is also speculated that the different light spectra provided by the three LEDs regimes could have altered the size and structural plant features by altering the plant hormones balance (i.e., auxins, cytokinins, gibberellins), affecting plant growth (Kurepin *et al.* 2012). It is noteworthy that plants architecture strongly depends on hormonal balance (Guo *et al.* 2020).

Generally, more compact size is also associated with reduced total biomass. In our case, only under the RGB regime, the dwarf growth was accompanied by a decrease in total biomass because the lowest fruit biomass was produced in RGB plants. The growth under RGB regimes induced more biomass partitioning toward leaves and less toward fruits compared to W plants, and it could explain the better photosynthetic performance in RGB plants which invest more in photosynthetic structures. In RB plants, we cannot observe the same response, despite the comparable leaf biomass. It may be argued that the green component provided in RGB regimes at the same percentage of R and B wavelengths (33%) penetrating further into the leaf and in the lower leaf layers of plant than red or blue light, would increase leaf photosynthesis to a greater extent than the only red or blue light (Terashima *et al.* 2009). The lower fruit number in RB and RGB than W plants may depend on the far-red portion of the spectrum supplied only by the W regime. The ratio red/far red regulates the phytochromes involved in plant morphogenetic responses (Casal & Casal 2000). A higher proportion of far-red promotes stem extension, biomass production, and fruit yield and stimulate dry mass partitioning to fruits by increasing fruit sink strength in tomato plants (Kalaitzoglou *et al.* 2019; Ji *et al.* 2020).

The different light quality regimes also affected leaf functional traits indicating that leaf structural adjustments were necessary to allow plant acclimation to the light environment.

Plants grown under RGB and RB lights reduced the LDMC and increased the SLA compared to W plants, indicating a variation of potential relative growth rate (Meziane & Shipley 1999). Both SLA and LDMC are involved in the trade-off between quick biomass production (high SLA, low LDMC species) and efficient conservation of nutrients (low SLA, high LDMC species) (Poorter & de Jong 1999) thus, a stimulated SLA by RGB and RB suggests a more efficient growth strategy, under these specific light quality regimes.

Light-induced modifications of leaf structure strongly affected gas exchange and photosynthetic carbon gain (Johkan 2012; Arena *et al.* 2016; Vitale *et al.* 2020).

Despite similar values of SLA and LDMC, RGB and RB plants did not show the same photosynthetic behaviour. More specifically, RGB light seems to be more efficient for carbon assimilation than W and RB. The higher A_N in RGB plants was not due to the difference in stomatal conductance (g_s), but rather to an increase of mesophyll conductance (g_m), indicating a reduced limitation to CO_2 diffusion in mesophyll cells compared to W and RB plants.

Thinner leaves, as well as less dense tissues in RGB plants (low LDMC), may reduce the limitations to the CO_2 diffusion in the mesophyll (Niinemets *et al.* 2009; Tomás *et al.* 2013), leading to a higher amount of the CO_2 available at the carboxylation sites, which, in turn, led to the significant increase of the maximum rate of Rubisco carboxylation (V_{cmax}) and net CO_2 assimilation. This hypothesis is consistent with the highest level of Rubisco found in RGB plants.

The lack of significant differences in the stomatal conductance between RGB and RB plants suggested that A_N decline in RB compared to RGB plants was not due to stomatal limitation but rather to other causes such as an impairment of Rubisco activity.

It is noteworthy that a decreased capacity of ribulose-1,5-bisphosphate (RuBP) carboxylation or regeneration may be associated with lower photosynthetic performance (Onoda *et al.* 2005). We assume that the decline of Rubisco expression may be responsible for reducing of the V_{cmax} and A_N observed in RB compared to W and RGB plants. There is a close relationship between Rubisco amount and CO_2 assimilation, and light quality exerts a critical role in photosynthetic regulation.

However, the plant responses were generally species-specific and depending on light quality treatments and different proportion among the utilised wavelengths. Consistent with our results, Miao *et al.* (2016) demonstrated in cucumber plants that the RB treatment (R:B 8:1) determined no change in g_s but decreased V_{cmax} and photosynthesis compared to W light. Other authors observed stimulation of stomatal conductance and photosynthesis in spinach plants grown under RB light (R: B 3:2) (Vitale *et al.* 2020) and an increase of Rubisco expression and photochemistry in tomato seedlings (Izzo *et al.* 2020).

In our case, the addition of green to red and blue wavelengths had positive effects on 'Microtom' photosynthetic machinery, especially on Rubisco expression, compared to the W and RB treatments. Our results contrast with the findings of Wang *et al.* (2009) and Su *et al.* (2014), who reported a decline in Rubisco expression and activity under red and green wavelengths with a consequent reduction of photosynthesis. The green light has an essential role in controlling plant development and photosynthesis because it enters more in-depth into the leaf mesophyll and canopy layers, driving photosynthesis where other wavelengths, in particular, red and blue, are limiting (Folta 2005; Terashima *et al.* 2009; Smith *et al.* 2017). In Microtom, the cultivation under RGB and RB regimes enhanced the intrinsic plant compactness, creating denser layers of leaves than W plants. In this circumstance, the high percentage of green light in the RGB regime may have been a reliable driver for photosynthesis, allowing RGB plants to obtain a high photosynthetic carbon gain than W and RB plants, cultivated at a lower and no percentage of green wavelength, respectively. The high proportion of red in RB regime did not favoured photosynthesis probably because it negatively affected the Rubisco and D1 protein amount, implicated in photosynthetic reactions.

Compared to W and RGB, the cultivation of 'Microtom' plants under the RB regime determined a different partitioning of absorbed light energy by photosynthetic apparatus, allocating the reductive power of the electron transport chain in non-radiative dissipation mechanisms (an increase of NPQ), rather than in photochemistry (reduced values of F_v/F_m and Φ_{PSII}). Furthermore, the lowest amount of photosynthetic pigment content (both total chlorophylls and carotenoids) also

evidences in RB plants a lower capability of light energy harvesting and conversion in photochemical reactions (Chen *et al*. 2014).

However, it cannot be excluded that RB plants down-regulated the photosynthetic pigment content to reduce light absorption, thus avoiding photodamages to PSII in a condition of limited photosynthesis. The higher chlorophyll a/b ratio (Chl a/b) also indicates in RB plants an adjustment of the light-harvesting system, and more specifically, a reduction of Chl b mainly active and responsible for light absorption of high-energy blue wavelengths (Wang *et al*. 2009).

The maintenance of the PSII activity is strictly related to the pigment concentration and the turnover of the D1 protein encoded by the psbA gene. The decline of F_v/F_m ratio in RB compared to W and RGB plants may indicate a slowdown of D1 turnover resulting from the imbalance between its degradation and replacement (Miao *et al*. 2016). The synthesis *ex-novo* of D1 protein is crucial for alleviating photoinhibition and maintaining high photosynthetic capacity in plants (Yamamoto 2001). Studies performed on cyanobacteria demonstrated that low intensity of blue light produced an accelerated degradation of the *psbA* protein. This response is amplified with the increase of blue light intensity and is reversed by red light (Tsinoremas *et al*. 1994).

Bian *et al*. (2018) found that continuous RB light growth regime induced in lettuce plants oxidative stress responsible for the downregulation of *PsbA* expression and photosynthesis decline. Consistent with the findings of Liu *et al*. (2019), in our experiment, the addition of green to red and blue wavelengths seems to exert a positive effect on the D1 expression, which levels were comparable with those found in W plants, leading to a similar PSII photochemical efficiency.

The plant growth under different light quality modified the chemical composition and antioxidant properties of tomato fruits affecting the production of bioactive molecules. Based on this evidence, the manipulation of the light spectrum may be a valuable tool to regulate the synthesis of useful metabolites for the human diet. In particular, RB light strongly enhanced the antioxidant properties of 'Microtom' fruits, despite the production of a lower number of berries per plant than W. The total polyphenol content increased 9-fold in RB compared to W fruits. Accordingly, the antioxidant capacity also increased in RB and RGB fruits compared to W, with the

highest values under RB regime. Our findings agree with previous studies on the same species, which demonstrated the stimulatory role exerted by RB light on the total polyphenols and antioxidant capacity (Xie *et al.* 2016). Recently, Panjai *et al.* (2017) demonstrated that continuous red light and short-term exposure of UV radiation affected the postharvest ripening of green tomatoes stimulating the total flavonoid and phenolic amount and hydrophilic and lipophilic antioxidant activity. It is noteworthy that wavelengths in the range of red, blue and UV-light have a strong effect on the accumulation of polyphenols, enhancing the antioxidant capacity and the reactive oxygen species (ROS) scavenging potential in tomato fruits (Castagna *et al.* 2014; Xie *et al.* 2016; Panjai *et al.* 2017). In other crops, other compounds, such as carotenoids or vitamins, act as non-enzymatic defences driving the antioxidant capacity (Racchi 2013; Hasan *et al.* 2017; Ntagkas *et al.* 2019; Xie *et al.* 2019). Among antioxidants, AsA is considered one of the ROS powerful scavengers. Ascorbic acid (AsA) is ubiquitous in plants, including fruits (Racchi 2013). Signals for light regulation are perceived in fruits, and the positive effect of light on AsA concentration seems to be influenced much more by light intensity rather than quality. It has been recently demonstrated that light drives the AsA production in tomato fruits (Ntagkas *et al.* 2019). Tomato berries stored in the darkness did not produce AsA, conversely detached green fruits (photosynthetically active) accumulated ascorbate when exposed to light (300-600 μmol m^{-2} s^{-1}) and mature red fruits (non-photosynthetically active) did not respond to light stimulation (Ntagkas *et al.* 2019). The exposure of detached tomato berries for days to pure blue light resulted in about 10% AsA increase than white, red or green wavelengths (Ntagkas *et al.* 2019). Xu *et al.* (2014) evidenced that blue light was efficient in determining the increase of AsA synthesis in postharvest strawberries. The monochromatic blue wavelength alone or in combination with red, also stimulated the AsA content in leafy vegetables (Ohashi-Kaneko *et al.* 2007; Li & Kubota 2009; Ma *et al.* 2014). In our study, 'Microtom' RB fruits showed a consistent reduction of AsA content than W and RGB fruits. This result may depend on the R: B ratio (60:40) used in our study, which was not adequate for AsA content stimulation in fruits. On the contrary RB berries showed a significant increase of antioxidant enzymes SOD and CAT than

W and RGB fruits. It cannot be excluded that the reduction of photosynthetic activity and electron transport rate would have favoured the ROS production within the whole pant tissues responsible for the activation of the scavenging system also in fruits (Racchi 2013). Thus, the reduction of the AsA content in RB 'Microtom' fruits and the increase of the SOD and CAT activity may be linked to the emergence of signals in leaves due to light-mediated mechanisms transmitted to nearby fruits, influencing their metabolism and quality.

Probably the propagation of light-mediated signals, have induced distant responses in RB 'Microtom' fruits, determining a reduction in the AsA content, used by ascorbate peroxidase (APX) as a co-factor during scavenging of H_2O_2, and a concomitant rise of polyphenols, total antioxidant capacity, SOD and CAT activity, definitely improving RB fruit antioxidant properties.

The heatmap clustered W and RB from RGB plants based on different physiological attributes, evidencing for RGB plants the best photosynthetic performance in terms of gas exchange and Rubisco amount. Conversely, W regimes effectively promoted the high plant total biomass and reproductive structures (flower and fruit number).

Concerning the fruits, the heatmap visualization showed that the RB light regime greatly influenced the antioxidant production, except for AsA, suggesting the RB as the best regime to guarantee fruits with a higher nutraceutical value, despite low number.

Overall results indicate that the photosynthetic apparatus of 'Microtom' grown under RGB treatments use light more efficiently than RB treatment. In fact, under the RGB growth regime, plants showed an improvement in the photosynthetic performance, evidencing of the critical role of the green portion of the spectrum. RGB light treatment induced a more compact architecture, better efficiency of light conversion at reaction centres and higher photosynthesis compared to W and RB light treatments. The increase of A_N under RGB light treatment is likely the result of an improved mesophyll conductance due to changes in leaf structure and the up-regulation of Rubisco responsible for the rise of maximum carboxylation efficiency in RGB compared to W and RB plants.

However, despite the reduced photosynthetic performance, RB light regime induces an increase of antioxidant charge in 'Microtom' fruits.

This study provides valuable information for developing appropriate light cultivation protocols for tomato in controlled-environment agriculture (CEA) and enhancing the antioxidant power in fruits with implications not only for cultivation indoor but also in extreme environments as Space orbiting stations.

Author's contribution:

Conceptualization: V.V., T.T. and C.A.; Investigation: E.V., T.T., G.C., R.P., C.A; Data curation: E.V and C.A. ; Formal analysis: T.T. and E.V.; Funding acquisition: V.V. and C.A.: Writing-original draft: E.V. and C.A; Writing-.review and editing: E.V., V.V., T.T., G.C., R.P., C.A

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Section II – The relationship between light quality and biostimulants in improving cultivation strategies.

During the century, increasing human population and economic development will continue to press on agricultural systems. Several agricultural practices, such as the use of new arable lands, overexploitation of soil and natural resources and chemical fertilisers and pesticides utilisation, will increase in the next future to meet the increasing food demand. All these methods negatively impact human health and ecosystems (EEA 2019, FAO 2019).

Currently, soil characteristics, biodiversity and agriculture itself are severely compromised by climate change. Modifications in rainfall regime, humidity, temperature, frequency and magnitude of extreme events, such as hot waves, are strictly linked to the distribution and abundance of pest species and pollinators, influencing crop growth, phenology and yield (EEA 2019, FAO 2019, IPCC 2019). In addition to not foreseeable changes in environmental conditions, the overexploitation of the resource endangers the large-scale production of many vegetables and fruits, making it necessary to apply alternative and innovative agricultural strategies to limit the impact on the environment (Figure 1).

In this context, Controlled Environment Agriculture (CEA) and, more specifically, urban farming (e.g., greenhouses, indoor-growing modules, vertical farms) is receiving increasing attention as a method to improve food security, enhance sustainability and product quality, and contribute to reducing the logistics costs (Figure 1)(Benke and Tomkins, 2017; van Iersel, 2017; Shamshiri *et al.*, 2018).

In indoor cultivation, such as in the open field, the application of biostimulants represents an eco-friendly solution increasingly used to replace common chemical fertilizers (Rouphael and Colla, 2018). Du Jardin, (2015) provided the best definition of biostimulant: “A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content”.

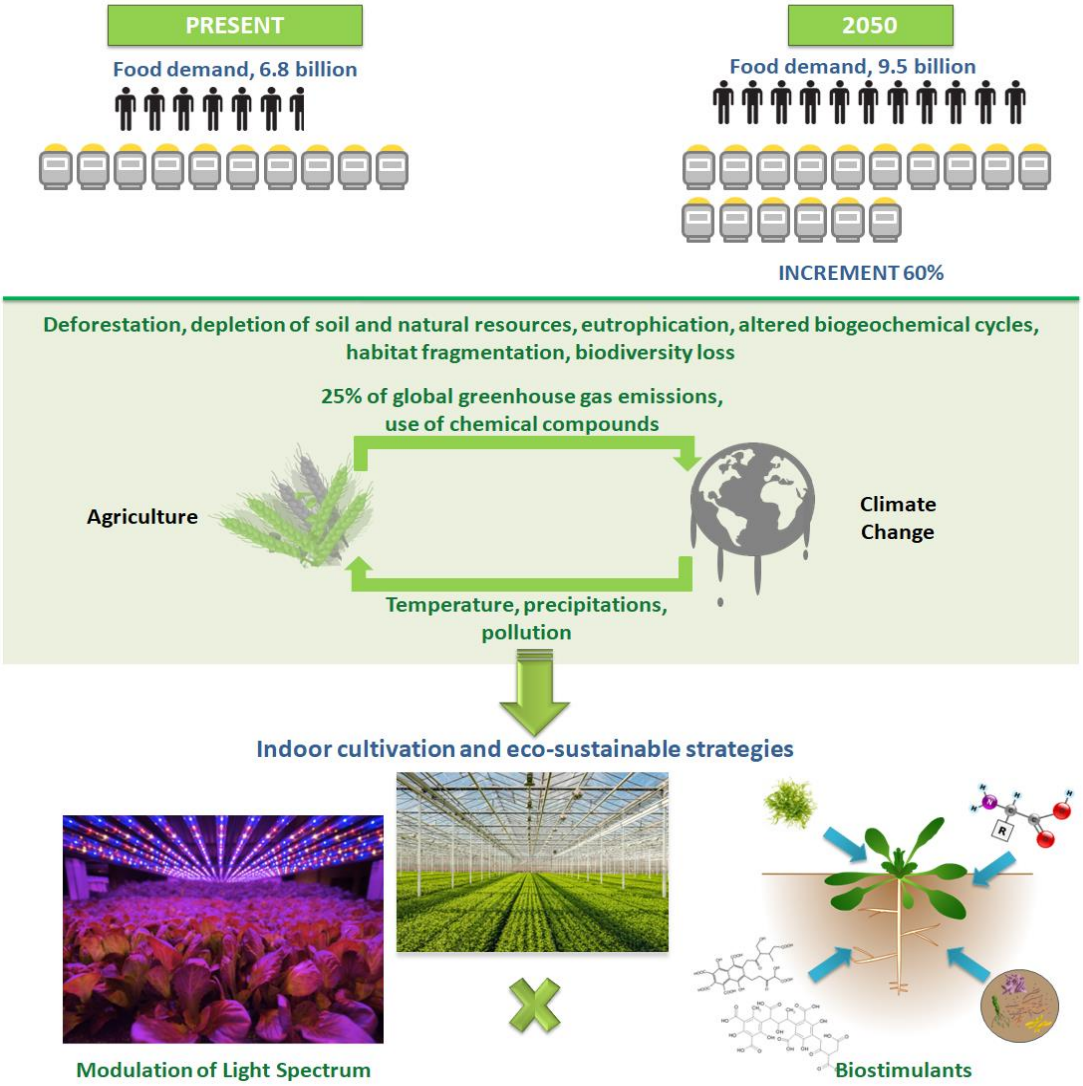

Figure 1: The estimated population by 2050 will result in a 60% increase in global food demand. Agricultural practices have a significant environmental impact and contribute to the ongoing climate change, which in turn affects the quantity and quality of global food production. The indoor cultivation and the adoption of sustainable practices, such as the modulation of the light spectrum and the application of biostimulants, are proposed as interesting solutions for the coming decades (Rouphael and Colla, 2018; EEA 2019; FAO 2019; Pennisi *et al.*, 2019).

Different types of biostimulants, e.g., beneficial soil microorganisms (bacteria, fungi), seaweeds, higher plant extracts, humic and fulvic acids, protein hydrolysates (PHs), are currently applied to crops (Figure 2).

Their effects on plants mainly depend on species intrinsic properties and application strategies, namely at soil, seed or leaf level.

A fascinating new approach is to combine the application of biostimulants with specific light quality regimes during plant growth. The joined effect of two potential

benefits could enhance the whole plant performance, resulting in a higher crop yield. However, at present, only a few studies have explored this possibility.

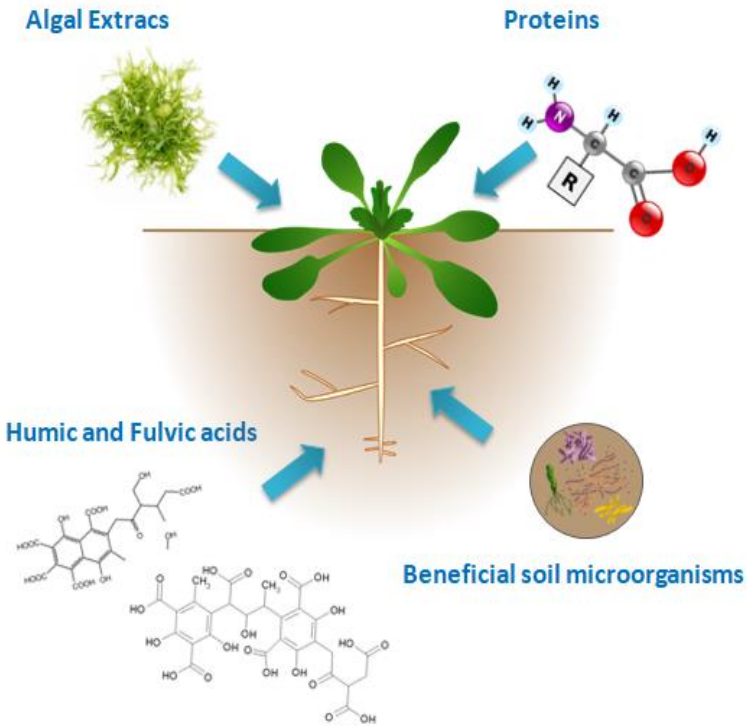

Figure 2: Different type of biostimulants and application strategy.

This section includes two papers focused on applying specific light quality regimes and different kinds of biostimulants during plant development and exploring the outcomes of their interplay on growth, photosynthetic performance, and nutraceutical properties in two of the most cultivated open-field crops: spinach and soybean.

In these studies, two kinds of biostimulants were employed: beneficial soil microorganisms and protein hydrolysates (PHs), testing two different application strategies: providing biostimulant directly on soil or pretreating seeds with biostimulants.

The first paper assesses whether the modulation of the light spectrum may influence the plant-microbe interaction in a reduced volume of soil, contributing to improving the plant yield.

The second study evaluates the possibility to produce functional food in soilless cultivation, combining specific light quality regimens with pre-treatment of soybean seeds with increasing concentration of amino acid-based biostimulant.

These studies represent an innovative contribution and may open new scenarios on the potentiality of the interplay between light quality and biostimulants in indoor cultivation.

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Chapter III – Effect of different light quality and biofertilizers on structural and physiological traits of spinach plants.

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Effects of different light quality and biofertilizers on structural and physiological traits of spinach plants

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Abstract

In this work, the effects of light quality and beneficial microbes (biofertilizer) supply on structural and ecophysiological traits of spinach were investigated. Plants were grown under four light quality regimens: white light (WL), red-blue (RB), red-green (RG), and red (R) light, with or without the addition of biofertilizer. RG and R plants without biofertilizer showed morphological traits typical of shaded plants as wide leaf lamina and high photosynthetic pigment content. These plants also exhibited a higher photosynthetic capacity compared to WL and RB plants. The improved photosynthesis in RG plants was due to both morphological and physiological adjustments allowing a better utilisation of light energy, whereas in R plants it has been attributed to a reduced photorespiration rate. Biofertilizer application under WL improved plant performance enhancing photosynthesis. The high carbon gain compensates the costs of symbiosis. Biofertilizer application under R light favouring too much the microbial root colonisation, removed the benefits of symbiosis. The interaction of light quality and biofertilization significantly affects the root–microbe relationship.

Additional key words: antioxidants; gas exchange; light manipulation; photochemistry; plant–microbe interaction.

Introduction

Agroecosystems require high-energy inputs to reach a high level of productivity, deeply affecting climate and environment (Clark and Tilman 2017). For this reason, it is crucial to shift towards a sustainable agriculture to preserve natural resources and reduce the impact on the environment. Indoor cultivation by sustainable innovative tools might represent a promising solution to reduce the deleterious effects of extensive crop production on the ecosystems.

Light manipulation, through light-emitting diode (LED) technology, is becoming one of the most valuable approaches in controlled-environment agriculture. The LED technology offers many advantages over traditional forms of lighting including high luminous efficiency, reduced energy consumption and cost, and low heat production (Singh *et al*. 2015, Izzo *et al.* 2019, Paradiso *et al.* 2019). Moreover, the LED light systems allow managing the light spectrum composition defining specific light regimes useful for plant growth and development.

Light spectrum composition affects plant growth influencing plant anatomy, morphology, and physiology (Ye *et al.* 2017, Zheng and Van Labeke 2017). In particular, red and blue wavelengths are efficiently used by photosynthetic apparatus and are fundamental for the plant healthy growth. Red light determines changes in shoot/stem ratio or shoot/root ratio, plant structure, and photosynthesis (Schuerger *et al.* 1997, Amitrano *et al.* 2018). Blue light is essential for chlorophyll biosynthesis, stomatal opening, chloroplast development and maturation, as well as synthesis of photosynthetic enzymes (Heo *et al.* 2002, Urbonaviciute *et al.* 2007, Hernández and Kubota 2016, Wang *et al.* 2016). The addition of green light can further increase plant biomass under certain circumstances (Kim *et al.* 2004, Johkan *et al.* 2012). Some studies have demonstrated that also green light has an essential role in controlling plant development and photosynthesis, because it penetrates deeper into the leaf mesophyll and canopy layers, driving photosynthesis where other wavelengths (*i.e.*, red and blue) are limiting (Terashima *et al.* 2009, Folta 2005, Smith *et al.* 2017). Green light has been rarely mixed to red and blue wavelengths for leafy vegetable production (Arena *et al.* 2016, Hristozkova *et al.* 2017). For this reason, further investigations are needed to assess if the beneficial effects of green light are the result of a

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Abbreviations: Anth – anthocyanin; Car – carotenoid; C_c – chloroplast CO_2 concentration; DAS – days after sowing; ETR – electron transport rate; ETR/P_{gmax} – electron sink processes other than carbon assimilation; FRAP – ferric reducing antioxidant power; g_m – mesophyll conductance; g_s – stomatal conductance; I – inoculated; LDMC – leaf dry mass content; LMA – leaf mass per area; LT – leaf thickness; NI – noninoculated; P_{gmax} – light-saturated gross photosynthetic rate; P_{Nmax} – light-saturated net photosynthetic rate; TP – total polyphenols; TSC – total soluble carbohydrates; Φ_{NO} – quantum yield of nonregulated energy dissipation; Φ_{NPQ} – quantum yield of regulated energy dissipation; Φ_{PSII} – effective quantum yield of PSII photochemistry.
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direct effect on photosynthesis or rather is responsible for other light-mediated morphogenic mechanisms.

Light quality may also enhance the production of bioactive compounds, improving the nutraceutical properties of some crop species. More specifically, the selection of specific wavelengths influences the biosynthesis of polyphenols (*e.g.*, phenols, flavonoids, anthocyanins, *etc.*) (Victório *et al.* 2015, Ye *et al.* 2017) and other antioxidant compounds (*e.g.*, ascorbic acid, tocopherols, carotenoids, *etc.*) (Samuolienė *et al.* 2016) with valuable effects on human health.

Besides light quality, the addition of beneficial microorganisms to soil is a conventional practice to improve plant productivity as it influences the availability and the uptake of macro and micronutrients (Ahemad and Kibret 2014, Nascente *et al.* 2017) or the synthesis of natural growth regulators (*i.e.*, hormones) (Spaepen and Vanderleyden 2011). Furthermore, microorganisms offer to plant the protection from pathogens through antimicrobials production, trigger the accumulation and/or release of secondary metabolites, and stimulate the induction of systemic resistance (Compant *et al.* 2005, Mhlongo *et al.* 2018). All these aspects contribute to the overall plant health status and represent an attractive alternative to the use of synthetic chemicals for sustainable agriculture with benefits on both human health and the environment.

It is noteworthy that light extent and quality directly or indirectly influence microbial growth. Bacteria and fungi perceive the environmental light conditions through light-sensing proteins and modulate their growth in response to light (Purschwitz *et al.* 2007, Hristozkova *et al.* 2017). Some studies show that blue wavelengths inhibit bacteria and fungi growth (De Lucca *et al.* 2012) whereas red wavelengths promote the formation of arbuscular mycorrhizal fungi (AMF) (Cruz 2016). Light may also indirectly affect microbial growth as it stimulates the production of photosynthetic exudates that represent a source of readily available nutrients for microorganisms (Doornbos *et al.* 2012). Thus, any influence of light on plant metabolism or microorganism growth may influence plant–microorganism interactions. These relationships are species-specific for plants and microorganisms and might depend on the applied light quality regimen (Hristozkova *et al.* 2017). Current knowledge on the combined effects of light quality and beneficial microorganisms on plant growth is limited (Alsanius *et al.* 2019); more research on this topic might help to maximise the plant productivity for food provisioning by setting-up specific growth protocols.

Among crops widely utilised in human nutrition, spinach (*Spinacia oleracea* L.) responds to different light quality regimens with changes in plant development and nutritional properties (Matsuda *et al.* 2007, 2008; Ohashi-Kaneko *et al.* 2007, Agarwal *et al.* 2018). This leaf vegetable is also sensitive to the beneficial microorganism biofertilisation as increases edible biomass, bioactive compound content, and resistance to stress (Çakmakçı *et al*. 2007, Zuccarini and Savé 2016, Khalid *et al.* 2017).

In this paper, we assessed the relationship between different light quality regimes and the application of beneficial microorganism in spinach plants. In particular, we analysed plant growth, photosynthetic behaviour, functional leaf traits and bioactive compound production under different light quality treatments, with or without the addition of plant growth-promoting microorganisms (PGPM) on the soil. The information acquired from this study will contribute to the knowledge on the light–plant–microbe interaction and can be used to develop sustainable growth protocols for leafy crops by maximising the indoor cultivation.

Materials and methods

Plant material and experimental set-up: Seeds of spinach plants (*Spinacia oleracea* L.) were sown in 0.5-L plastic pots filled with a mixture of sterilised sandy soil and perlite substrate (3:1, v/v) and placed inside a growth chamber, equipped with a LED lighting system, under four different light regimes: broad-spectrum white light (WL), red-blue (RB, emission peaks at 620 and 660 nm, emission peak at 460 nm, 60:40), red-green (RG, emission peaks at 620 and 660 nm, emission peaks at 500 and 530 nm, 60:40), and 'pure' red (R, emission peaks at 620 and 660 nm) light. All plants were subjected to the same growth conditions: PPFD of 350 μmol(photon) m^{-2} s^{-1} at the top of the canopy, 25/15°C day/night temperature, 50/70% day/night relative humidity, and photoperiod of 12 h. Temperature and humidity were monitored by a digital thermo-hygrometer (*HC520 Digital Thermo-Hygrometer*, *Cheerman*, Guangdong, China), and the irradiance was measured by the *Li-Cor190R* quantum sensor (*Li-Cor*, Lincoln, Nebraska, USA). Plants were watered to field capacity to reintegrate water lost by evapotranspiration and fertilised every week with a complete nutritive solution composed by micronutrients, nitrogen, phosphorus, and potassium ($N:P_2O_5:K_2O$, 20:20:20 g L^{-1}) (*Poly-Feed GG*, *Haifa Italia*, Bologna). A commercial biofertilizer (*RadiNET*, *Micosat F*®, *C.C.S. Aosta s.r.l.*, Aosta, Italy) containing mainly arbuscular mycorrhizal fungi (AMF) (*Glomus* genus, *Rhizophagus irregularis*), saprophytic fungi (*Pochonia chlamydosporia*, *Tricoderma* genus), and a reduced amount of rhizosphere bacteria (*Bacillus* and *Streptomyces* genus) was applied to soil at sowing and every week for three weeks. In each application, 0.6 g of biofertilizer was dissolved in 10 ml of deionized water. For each light regime (WL, RB, RG, R), five plants were treated with biofertilizer (inoculated plants – I) and five plants without (noninoculated plants – NI); ten plants for each light regime in total.

Biometrical measurements and functional leaf trait determinations: Green leaf area per plant was measured every 20 d, acquiring the images by a digital camera and measuring leaf expansion by *Image J* software (Wayne Rasband NIH, http://imagej.nih.gov/ij/index.html).

Plant biomass was determined at 100 d after sowing (DAS) drying roots and shoots in a forced-air oven at 75°C up to constant mass. Functional leaf traits were monitored at harvest time (100 DAS) on five noninoculated and five inoculated plants by each light growth treatment. The leaf area – LA [cm^2], leaf mass per area – LMA [g cm^{-2}], leaf

dry mass content – LDMC [g g^{-1}], and leaf thickness – LT [μm], were determined according to Cornelissen *et al.* (2003).

Photosynthetic pigment content: Total chlorophylls and carotenoids were determined at 100 DAS on five different leaf samples from each light treatment according to Lichtenthaler (1987). Pigments were extracted from samples using mortar and pestle in ice-cold (4°C) 100% acetone and centrifuged at 5,000 rpm for 5 min (*Labofuge GL*, *Heraeus Sepatech*, Hanau, Germany). The absorbance of supernatants was quantified by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Santa Clara, CA, USA) at 470, 645, and 662 nm and pigment concentration expressed in mg 100 g^{-1}(FM).

Polyphenols, anthocyanins, antioxidant capacity and carbohydrates determination were carried out on five different leaves (one leaf per plant) collected at 100 DAS. Polyphenols were determined as reported in Arena *et al.* (2019). Samples (0.02 g) were ground in liquid nitrogen, incubated with methanol at 4°C, and centrifuged at 11,000 rpm for 5 min. The supernatant was extracted and mixed with 1:1 (v/v) 10% Folin-Ciocâlteu and 1:5 (v/v) 700 mM Na_2CO_3 solution. Samples were incubated at 4°C for 2h. The absorbance was quantified by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 765 nm. The total polyphenols concentration was calculated and expressed as gallic acid equivalents [mg(GAE) 100 g^{-1}(FM)] using a regression equation between gallic acid standards and A_{765}.

Total anthocyanins content was determined on 0.05-g sample leaves ground in liquid nitrogen, treated with methanol 1% HCl solution, and stored overnight at 4°C. After the addition of 1:0.6 (v/v) ultra-pure water and 1:1.6 (v/v) chloroform, samples were centrifuged at 11,000 rpm for 5 min. Supernatant was extracted from each sample adding 1:1 (v/v) 60% methanol 1% HCl 40% ultra-pure water solution. The absorbance was measured spectrophotometrically (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 530 and 657 nm. The relative amount of anthocyanin was expressed as [($A_{530} - 0.33A_{657}$) 100 g^{-1}(FM)] (Mancinelli *et al.* 1975).

The ferric reducing antioxidant power (FRAP) assay was performed to determine the total soluble antioxidant capacity according to method described by George *et al.* (2004) and modified by Motta *et al.* (2019). Samples (0.250 g) were ground in liquid nitrogen, treated with 60:40 (v/v) methanol/water solution and centrifuged at 14,000 rpm for 15 min (4°C), collecting supernatants for the assay. The FRAP reagent (1:16 300 mM acetate buffer pH 3.6; 1:1.6 of 10 mM TPTZ in 40 mM HCl; 1:1.6 of 12 mM $FeCl_3$) was added to each sample extract and the mixture incubated in the darkness for 1 h. The sample absorbance was read by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 593 nm. Total antioxidant capacity was quantified and expressed as mmol Trolox equivalents [mmol(TE) 100 g^{-1}(FM)] using a Trolox standard curve.

Total soluble carbohydrates content was determined on five different leaf samples (0.01 g) of each treatment following the anthrone method reported by Hedge and Hofreiter (1962). The absorbance was measured at 630 nm by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA). The amount of total soluble carbohydrates in the extracts was calculated using a glucose standard curve and expressed as glucose equivalents [g(GE) 100 g^{-1}(FM)].

Photosynthetic characteristics and Chl fluorescence parameters: Gas exchange and fluorescence measurements were simultaneously performed on fully expanded leaves by means of *LI-6400* (*Li-Cor*, Lincoln, Nebraska, USA) integrated with *LI-6400-40* leaf chamber fluorometer. Light-response curves (LRC) were carried out illuminating the leaves with red plus blue LEDs at 25°C, 360 μmol(CO_2) mol^{-1}, and 50% air relative humidity (RH) to determine the light-saturated net photosynthesis. Net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). At each irradiance level, the steady-state fluorescence yield (F_s) and the maximal fluorescence yield in the light-adapted state (F_m') were measured applying a 0.8 s-saturating flash of 8,000 μmol(photon) m^{-2} s^{-1}, and the effective quantum yield of PSII photochemistry (Φ_{PSII}) (Genty *et al.* 1989), the regulated (Φ_{NPQ}) and the nonregulated (Φ_{NO}) energy dissipation (Kramer *et al.* 2004) were calculated. Electron sink processes other than carbon assimilation (ETR/P_{gmax}) were evaluated by the ratio between the electron transport rate (ETR) and light-saturated gross photosynthetic rate (P_{gmax}) (Krall and Edwards 1992). Maximal quantum yield of PSII photochemistry (F_v/F_m) was measured at the end of each LRC on 30-min dark-adapted leaves measuring the minimal fluorescence of the dark-adapted state (F_0) and maximal fluorescence yield of the dark-adapted state (F_m), applying a saturation pulse of 8,000 μmol(photon) m^{-2} s^{-1}. Mesophyll conductance (g_m) was determined at 360 μmol(CO_2) mol^{-1} by the variable *J* method (Loreto *et al.* 1992), assuming that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration and that chlorophyll fluorescence gives a reliable estimation of the quantum yield of electron transport. Mitochondrial respiration in the light (R_L) and the CO_2-compensation point in the absence of day respiration (T^*) were estimated according to Laisk and Oja (1998) by performing P–C_i response curves at three different light intensities and using only the points on the linear portion of the curves. g_m was used to calculate the concentration of CO_2 at the sites of carboxylation (C_c).

Arbuscular mycorrhizal fungi (AFM) colonization assay: A set of plants was used for AMF colonization assay in WL and R plants. Pieces of roots were cleared in 10% KOH and stained with 0.05% aniline blue in vinegar 5% (v/v), according to Vierheilig *et al.* (1998) and Vierheilig and Piché (1998). Images were acquired by light microscopy (*Nikon Eclipse E1000*, *Nikon Instruments Inc.*, Melville, New York, USA) using a digital camera (*Nikon DXM1200F Microscope Camera*, *Nikon Instruments Inc.*,

Melville, New York, USA). The root colonization was expressed as % considering the ratio between the number of root fragments showing colonization and the total number of root fragments observed; the root infection was expressed as number of vesicles presenting on a cm of root fragment.

Statistical analysis was performed by *Sigma-Plot 12.0* software package (*Jandel Scientific*, San Rafael, CA, USA). Data were analysed by two-way *ANOVA* followed by the *Duncan*'s test for multiple comparison procedures. The results are reported as mean (n = 5) ± standard deviation. Differences were considered statistically significant at $p \leq 0.05$. *Shapiro-Wilk*'s and *Kolmogorov-Smirnov*'s tests were performed to check for normality. The correlations between selected parameters were investigated using *Pearson*'s correlation test.

Results

Root colonization by AMF: Spinach roots resulted colonized by AMF (Fig. 1). Roots of plants grown under monochromatic red light showed a higher microbe infection compared to plants grown under white light (Table 1).

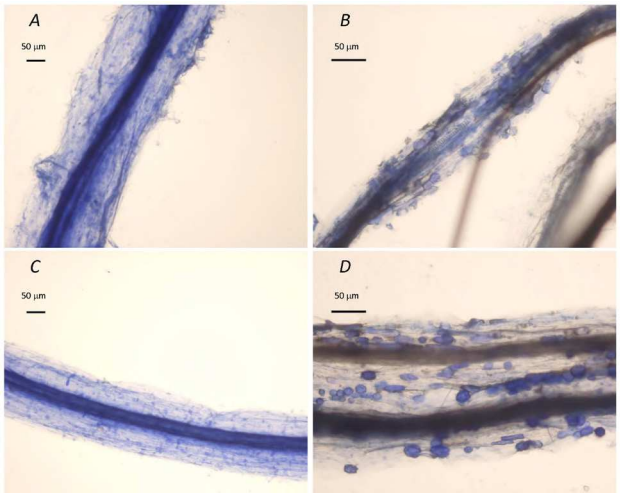

Fig. 1. Microbe infection in spinach roots. Noninoculated plants under white light, WL (*A*); inoculated plants under WL (*B*); noninoculated plants under pure R light, R (*C*); inoculated plants under pure R light, R (*D*). 10× magnification – *A*,*C*; 20× magnification – *B*,*D*. Scale bar = 50 µm.

Plant growth and leaf functional traits: The diverse light treatments significantly affected the plant morphology (Fig. 1S, *supplement*). The total biomass did not change under different light treatments compared to WL in both NI and I plants (Fig. 2*A*). The combination light treatments × biofertilisation produced a rise of the biomass in I compared to NI plants only under RG light treatment. Conversely to dry shoot mass (Fig. 2*B*), the interaction light quality × biofertilizer affected root dry mass production (Fig. 2*C*). Among all light treatments, RB light promoted root biomass accumulation in noninoculated plants. The addition of biofertilizer to light treatments induced a significant rise of root biomass in WL and RG plants compared to respective noninoculated samples. The shoot/root ratio was the lowest in RB noninoculated plants compared to other light treatments. The biofertilizer application under different light quality treatments did not induce an increase of the root/shoot ratio, compared to noninoculated plants, except for RB plants that show a slight significant increase (Fig. 2*D*). As regards leaf functional traits, plants grown under RG and R light treatments showed leaves with greater area, lower LMA and LT compared to WL and RB plants (Table 2). These latter were characterised by high values of LMA and LDMC. The interaction biofertilizer × light treatment affected only R plants (I-R) where leaves with lower LA and higher LMA were found compared to noninoculated ones.

Photosynthetic and Chl fluorescence parameters: Light quality influenced leaf gas exchanges in both noninoculated (NI) and inoculated (I) plants (Fig. 3). The light-saturated net photosynthetic rate (P_{Nmax}), stomatal (g_s) and mesophyll (g_m) conductance to CO_2 were higher in RB, RG, and R compared to WL plants, reaching a maximum under pure R light treatments (Fig. 3 *A*–*C*). In inoculated plants, the highest values were observed under RG treatment. The addition of biofertilizer significantly increased P_{Nmax}, g_s, and g_m in WL and RB plants, whereas significantly reduced these parameters under pure R treatment. The concentration of CO_2 at carboxylation sites (C_c) was higher under RG and R compared to WL and RB light growth regimes in both NI and I plants (Fig. 3*D*). The noninoculated R plants showed the highest C_c value. The interaction light × biofertilizer was significant only for R plants showing a reduction of C_c in I compared to NI plants.

The effective quantum yield of PSII photochemistry (Φ_{PSII}) increased under RB, R, and RG light treatment

Table 1. Root colonization [%] and root infection [number of vesicles per cm] in noninoculated (NI) and inoculated (I) plants. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. *Capital letters* indicate significant differences between WL and R light treatments in inoculated (I) plants ($p \leq 0.05$). *Asterisks* represent different levels of significance ($^{***}p \leq 0.001$). WL – white light; R – pure red light; L – light treatment; B – biostimulant; L × B – interaction light × biostimulant.

Parameters	WL		R		*ANOVA*		
	NI	I	NI	I	L	B	L × B
Colonization [%]	0	72.00 ± 4.80^{B}	0	94.00 ± 7.17^{A}	***	***	***
Infection [vesicles cm^{-1}]	0	33.95 ± 5.30^{B}	0	111.27 ± 37.93^{A}	***	***	***

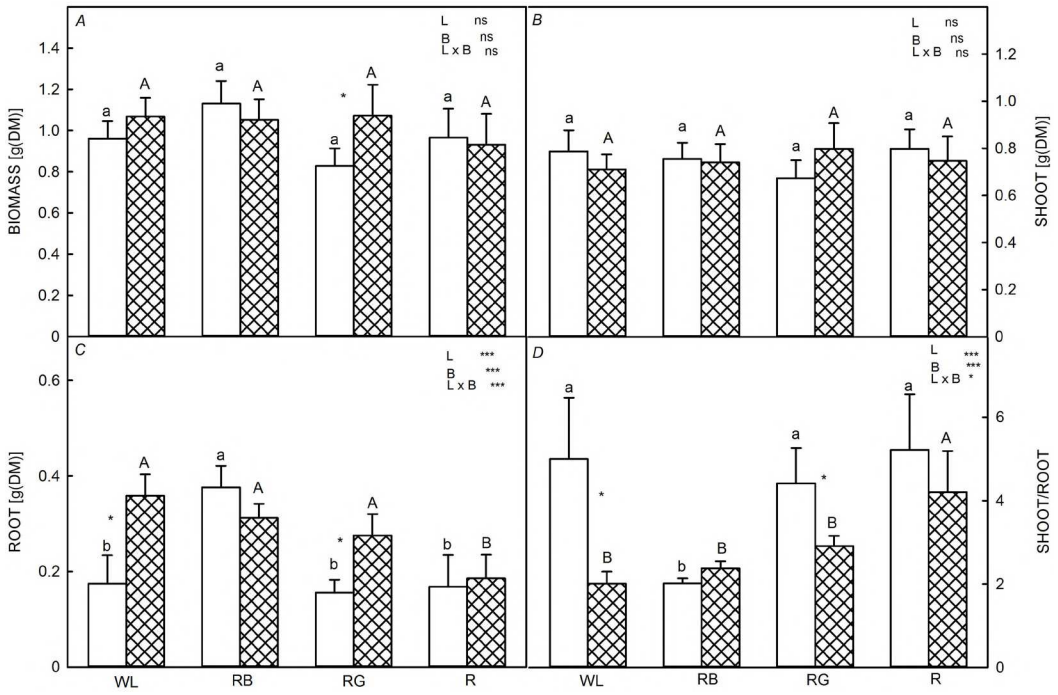

Fig. 2. Total biomass (*A*), shoot (*B*), and root (*C*) biomass, and shoot/root ratio (*D*) in noninoculated (*white bar*) and inoculated (*full bar*) plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represents different levels of significance ($^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B); ns – not significant.

compared to WL (Fig. 4*A*). The interaction light × biofertilizer determined a remarkable increase of Φ_{PSII} in WL plants and a significant reduction in R plants. The quantum yield of regulated energy dissipation (Φ_{NPQ}) showed an opposite trend, inducing a decrease of Φ_{NPQ} in WL and an increase in R plants (Fig. 4*B*). A significant increase of nonregulated energy dissipation (Φ_{NO}) was observed only under R treatment regardless of biofertilizer application (Fig. 4*C*). The pure R treatment determined a reduction of ETR/P_{gmax} ratio compared to the other light regimes (Fig. 4*D*). An interaction light × biofertilizer was found only for Φ_{PSII}. In particular, under WL the application of biofertilizer increased Φ_{PSII}, while it decreased Φ_{PSII} under R light.

Bioactive compounds: Plants grown under RG and R light treatments showed a significant increase of total chlorophyll and carotenoid content, compared to WL and RB plants, with the highest value in R plants. Within R light treatment, the application of biofertilizer induced a reduction of photosynthetic pigment content in I compared to NI plants (Table 3). The total polyphenol content was significantly reduced in RB, RG, and R compared to WL plants (Table 3); the application of biofertilizer increased polyphenol content only in RG inoculated plants compared to noninoculated but did not affect the anthocyanins content that was the same for both NI and I RG plants. Conversely, the addition of biofertilizer decreased anthocyanin amount in WL and RB plants compared to noninoculated samples (Table 3). The soluble antioxidant capacity was higher in RG and R compared to WL and RB plants in both inoculated and noninoculated plants. The total soluble carbohydrate content was lower in RG and R compared to WL and RB plants in both inoculated and noninoculated plants, with the lowest values for R plants (Table 3).

Correlation among the investigated leaf parameters: P_{Nmax} was positively correlated to g_s (r = 0.891) and g_m (r = 0.799), Φ_{PSII} (r = 0.841), photosynthetic pigments (r = 0.560), and antioxidant capacity (r = 0.635), and was negatively correlated to Φ_{NPQ} (r = – 0.856) (Table 4). This latter was negatively correlated to LMA (r = – 0.610) and LDMC (r = – 0.653). g_m was negatively correlated to LMA (r = – 0.838) and LMDC (r = – 0.783). Φ_{PSII} was negatively correlated to Φ_{NPQ} (r = – 0.826) and anthocyanins content (r = – 0.379), and positively correlated to soluble antioxidant capacity (r = 0.569) (Table 4). This latter was positively correlated to photosynthetic pigment content (r = 0.544) and negatively correlated to total polyphenol amount (r = – 0.403) and Φ_{NPQ} (r = – 0.759) (Table 4).

Discussion

Plant growth, photosynthesis, and bioactive compound production: The manipulation of the light spectrum allows to obtain specific physiological responses in spinach plants associated with the modulation of photosynthesis and the synthesis of bioactive compound. Our data indicate that

Table 2. Leaf area (LA), leaf mass per area (LMA), leaf dry mass content (LDMC), and leaf thickness (LT) determined in noninoculated (NI) and inoculated (I) spinach plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represent different levels of significance (*** $p \leq 0.001$, * $p \leq 0.05$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B). ns – not significant.

Leaf traits	WL		RB		RG		R		*ANOVA*		
	NI	I	NI	I	NI	I	NI	I	L	B	L × B
LA [cm^2]	5.52 ± 1.32^{a}	5.47 ± 1.38^{A}	4.88 ± 0.74^{b}	4.55 ± 0.65^{B}	11.09 ± 0.54^{c}	10.49 ± 2.62^{C}	25.67 ± 5.20^{d*}	11.32 ± 6.38^{C}	***	*	ns
LMA [g cm^{-2}]	0.012 ± 0.002^{a}	0.011 ± 0.001^{A}	0.013 ± 0.001^{a}	0.013 ± 0.001^{A}	0.0048 ± 0.000^{b}	0.0055 ± 0.001^{B}	0.0020 ± 0.000^{c*}	0.0046 ± 0.001^{B}	***	*	ns
LDMC [g g^{-1}(DM)]	0.14 ± 0.02^{a}	0.14 ± 0.01^{A}	0.16 ± 0.02^{a}	0.15 ± 0.00^{A}	0.086 ± 0.01^{b}	0.091 ± 0.01^{B}	0.086 ± 0.01^{b}	0.093 ± 0.01^{B}	***	ns	ns
LT [µm]	0.082 ± 0.013^{a}	0.077 ± 0.001^{A}	0.085 ± 0.002^{a}	0.089 ± 0.000^{A}	0.056 ± 0.002^{b}	0.059 ± 0.005^{B}	0.023 ± 0.002^{c*}	0.049 ± 0.010^{B}	***	*	ns

the RB light treatment, without biofertilisation, induced a partitioning of biomass toward roots compared to the other light regimes, confirming the positive influence of blue light on root development (Canamero *et al.* 2006) and the requirement of blue light for the optimal growth of spinach plants (Yorio *et al.* 2001, Agarwal *et al.* 2018). Generally, the absence of blue light or its insufficient or excessive amount, determined shade-avoidance responses, causing a reduction of total biomass and an imbalance in plant development (Chang *et al.* 2016, Yorio *et al.* 2001, Hernández and Kubota 2016, Agarwal *et al.* 2018). In our experiment, plants grown without blue light (RG and R plants) developed typical traits of a shade-avoidance syndrome (*i.e.*, higher leaf area and elongated shoots and petioles) but did not reduce their biomass. These plants showed morphological, physiological, and biochemical adjustments favouring the carbon gain. Beside a greater leaf area per plant, the changes included thinner leaves characterised by lower LMA and LDMC values and high chlorophylls and carotenoid content compared to the other light treatments. These specific traits may be associated with a more efficient light harvesting and CO_2 distribution inside the leaf and may have favoured the photosynthesis.

Leaf structure is an important determinant in controlling photosynthesis because it influences the light distribution within leaf as well as the CO_2 diffusion at the carboxylation sites. The light wavelengths are selectively absorbed and distributed inside the leaf. Red or blue light is largely absorbed by chloroplasts near leaf surface, whereas green penetrating deeper than red or blue light, drives the photosynthesis deeply in the mesophyll (Terashima *et al.* 2009). We hypothesise that the development of thin leaves under RG and R light treatments allowed light to penetrate deeper in the leaf layers. The reduced content of anthocyanins and polyphenols in plants grown under green wavelengths also may be associated with a more light penetration within leaf tissues since these compounds act as a natural filter against the light (Steyn *et al.* 2002, Landi *et al.* 2015). The reduced LMA and LDMC in RG and much more in R leaves contributed to alleviation of the limitations to the CO_2 diffusion in the mesophyll (Niinemets *et al.* 2009). We assumed that the higher photosynthetic rate of RG and R plants was due to the higher stomatal and mesophyll conductance. In fact, the reduced leaf thickness and tissue density shortening the pathway of CO_2 diffusion toward chloroplasts, likely helped gas exchange. The plant growth under green light (RG) developed leaves with higher Φ_{PSII} and carbohydrate content compared to pure R leaves, indicating that the green wavelength drives a higher utilisation of radiant energy in photochemistry. The improved CO_2 diffusion in R compared to RG leaves was the main reason for the stimulation of photosynthesis in these plants. Such elevated CO_2 concentration in the chloroplasts significantly reduced the photorespiration, according to Φ_{PSII} decline and ETR/P_{gmax} ratio value near to the theoretical threshold of 4–5. The decrease of photorespiration determined the rise of Φ_{NO} and the decline of Φ_{NPQ}, exposing plants to the risks of reactive oxygen species (ROS) accumulation (Agarwal *et al.* 2018), this could be a reason by which in

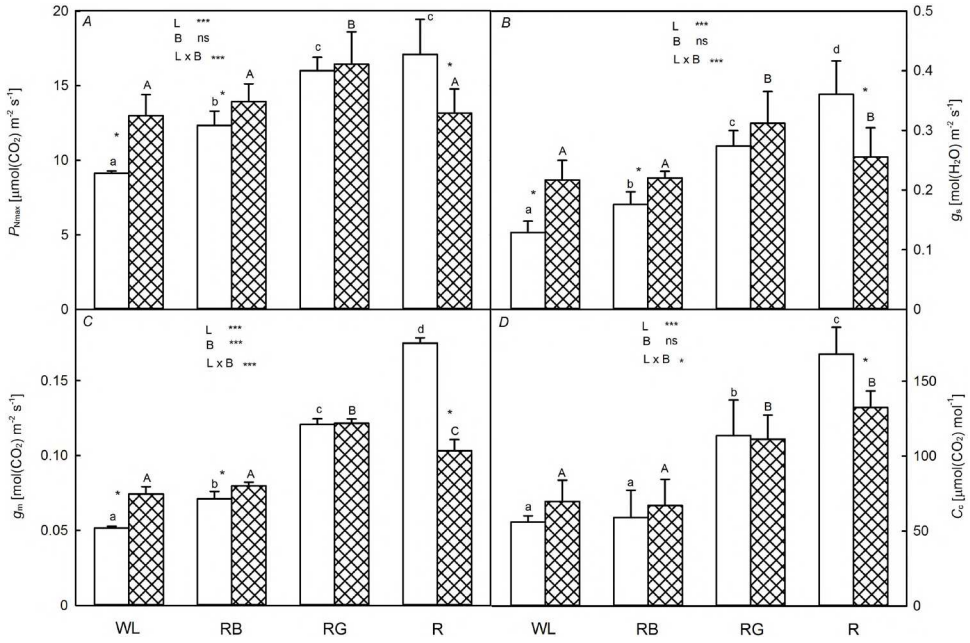

Fig. 3. Light-saturated net photosynthetic rate (P_{Nmax}) (*A*), stomatal conductance (g_s) (*B*), mesophyll conductance (g_m) (*C*), chloroplast CO_2 concentration (C_c) (*D*) in noninoculated (*white bar*) and inoculated (*full bar*) plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represents different levels of significance ($^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B); ns – not significant.

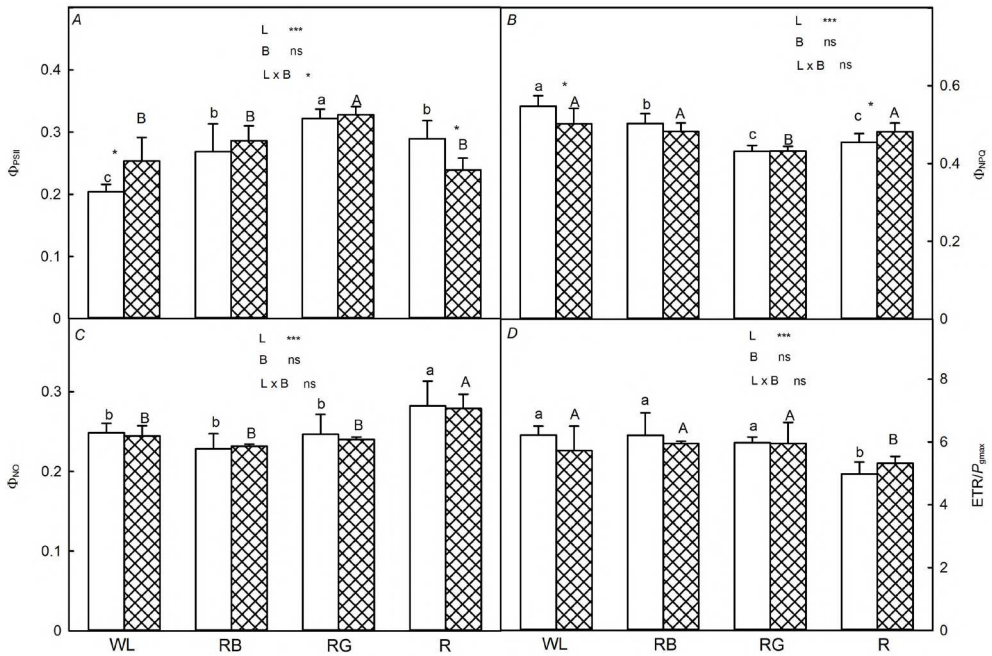

Fig. 4. Effective quantum yield of PSII photochemistry (Φ_{PSII}) (*A*), quantum yield of regulated energy dissipation (Φ_{NPQ}) (*B*), quantum yield of nonregulated energy dissipation (Φ_{NO}) (*C*), electron sink processes other than carbon assimilation (ETR/P_{gmax}) (*D*) in noninoculated (*white bar*) and inoculated (*full bar*) plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represents different levels of significance ($^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B); ns – not significant.

Table 3. Total chlorophyll, Chl (*a+b*); total carotenoids, Car (*x+c*); total polyphenols, TP; relative content of anthocyanins, Anth; soluble antioxidant capacity, FRAP; and total soluble carbohydrates, TSC; determined in noninoculated (NI) and inoculated (I) spinach plants. WL – white light; RB – red + blue light; RG – red + green light; R – pure red light. Data are means ± SD (n = 5). Results were analysed by two-way *ANOVA* followed by *Duncan*'s multiple range test. Significant differences ($p \leq 0.05$) were indicated by *small letters* between noninoculated (NI) and *capital letters* between inoculated (I) plants. Significant differences ($p \leq 0.05$) between NI and I plant groups inside each light treatment are indicated with an asterisk (*). The number of asterisks in *ANOVA* represent different levels of significance (*** $p \leq 0.001$) among light treatment (L), biostimulant (B), and the interaction light × biostimulant (L × B). ns – not significant.

Parameter	WL		RB		RG		R		*ANOVA*		
	NI	I	NI	I	NI	I	NI	I	L	B	L × B
Chl (*a+b*) [mg 100 g^{-1}(FM)]	112.40 ± 11.91^{a}	103.84 ± 23.86^{A}	95.77 ± 25.24^{a}	101.74 ± 12.70^{A}	202.67 ± 25.33^{b}	195.71 ± 30.69^{B}	422.80 ± 86.30^{c*}	181.73 ± 32.43^{B}	***	***	***
Car (*x+c*) [mg 100 g^{-1}(FM)]	22.64 ± 3.65^{a}	21.80 ± 3.47^{A}	21.04 ± 4.30^{a}	21.74 ± 2.47^{A}	41.02 ± 5.75^{b}	38.65 ± 5.75^{B}	84.48 ± 18.97^{c*}	35.77 ± 4.66^{B}	***	***	***
TP [mg(GAE) 100 g^{-1}(FM)]	141.08 ± 20.54^{a*}	115.20 ± 9.92^{A}	106.06 ± 13.41^{b}	90.79 ± 14.71^{BC}	86.38 ± 10.32^{c}	98.56 ± 17.06^{AB}	87.69 ± 8.65^{c*}	53.68 ± 10.59^{D}	***	***	***
Anth [Anth 100 g^{-1}(FM)]	90.30 ± 10.12^{a*}	70.83 ± 9.72^{B}	87.44 ± 10.03^{a}	72.82 ± 2.93^{B}	37.75 ± 7.54^{c}	39.78 ± 19.24^{C}	77.56 ± 14.44^{a}	79.86 ± 16.46^{AB}	***	ns	ns
FRAP [mmol(TE) 100 g^{-1}(FM)]	0.27 ± 0.03^{a}	0.34 ± 0.06^{A}	0.31 ± 0.05^{a}	0.39 ± 0.02^{A}	0.65 ± 0.07^{b}	0.74 ± 0.14^{B}	0.60 ± 0.16^{b}	0.55 ± 0.10^{C}	***	ns	ns
TSC [g(GE) 100 g^{-1}(FM)]	23.96 ± 3.38^{a}	21.80 ± 1.34^{A}	26.76 ± 1.63^{a}	27.04 ± 4.97^{B}	7.63 ± 5.15^{b}	9.19 ± 2.57^{C}	3.14 ± 1.07^{c}	3.40 ± 1.36^{D}	***	ns	ns

RG and R plants increased significantly the antioxidant capacity in order to counteract efficiently the oxidative stress. Changes in the light spectrum are also responsible for changes in the amounts of polyphenols. The absence of blue wavelengths in RG and R treatments led to a drastic reduction of polyphenols content in spinach plants, emphasising the critical role exerted by blue light on the synthesis of the secondary metabolites.

Compared to WL, considered as control, the growth under red-blue (RB) light improved both stomatal and mesophyll conductance, stimulating photosynthesis. This result is consistent with previous studies on tomato and oriental plane (Arena *et al.* 2016). Other authors reported no benefits of blue light on photosynthesis in spinach (Yorio *et al.* 2001, Agarwal *et al.* 2018) or a reduced photosynthesis and mesophyll conductance in other species (Loreto *et al.* 2009, Pallozzi *et al.* 2013). As no difference was found between WL and RB in leaf functional traits affecting g_m (*i.e.*, LT, LMA, and LDMC) (Tomás *et al.* 2013), we supposed that other factors were responsible for the high photosynthetic performance of RB compared to WL plants.

The high Φ_{PSII} in RB plants may indicate an enrichment of electron transport, likely mediated by an enhancement of cytochrome *f* (Cyt *f*) complex. Matsuda *et al.* (2007) reported an increase in Cyt *f* content in spinach plants grown under a blue light intensity of 300 μmol(photon) m^{-2} s^{-1}, similar to that utilised in our study. From biochemical point of view, the plant growth under RB light induced a reduction of polyphenols content compared to WL. This result was in contrast with previous studies on spinach plants (Agarwal *et al.* 2018) and other crops (Tomás *et al.* 2013).

Generally, polyphenols contribute to the second antioxidant system and are engaged when primary antioxidants are exhausted, such as under stress circumstances. Their synthesis, driven by blue light, is mediated by cytochrome P_{450} and lead to ROS accumulation (Lobiuc *et al.* 2017). Based on this statement, we supposed no stress condition for spinach plants at blue light intensity utilised in our study. Also, the content of anthocyanins and total antioxidant capacity in RB plants comparable to WL control seems to suggest the absence of stress due to prolonged growth under blue light.

Light quality and plant-microorganism interaction: Beneficial microorganisms (PGPM) such as fungi and bacteria added to plant growth mean may improve productivity and tissue nutraceutical value eventually potentiating the effect of specific light wavelengths. At present, little information is available on the interplay between light quality and beneficial microbes on plant physiological performance. Our data demonstrated that arbuscular mycorrhizal fungi (AMF) infected the spinach roots. The results are in agreement with other studies demonstrating that AMF belonging to *Glomus* genus infect spinach roots (Zuccarini and Savé 2016, Khalid *et al.* 2017) and the higher root infection occurred with rhizobacterial inoculation (Khalid *et al.* 2017). The inoculation of spinach plant under white light regime

Table 4. Coefficients of correlation (*Pearson*'s test) among the leaf investigated parameters: light-saturated net photosynthetic rate, P_{Nmax}; stomatal conductance, g_s; mesophyll conductance, g_m; chloroplast CO_2 concentration, C_c; effective quantum yield of PSII photochemistry,Φ_{PSII}; regulated energy dissipation, Φ_{NPQ}; nonregulated energy dissipation, Φ_{NO}; electron sink processes other than carbon assimilation, ETR/P_{gmax}; total chlorophyll, Chl (*a*+*b*); total carotenoids, Car (*x*+*c*); leaf area, LA; leaf mass per area, LMA; leaf dry mass content, LDMC; leaf thickness, LT; total polyphenols, TP; relative content of anthocyanins, Anth; soluble antioxidant capacity, FRAP; and total soluble carbohydrates, TSC. Data are means ± SD (n = 5). The significant *Pearson*'s correlation coefficients are reported in bold (p<0.05).

	P_{Nmax}	g_s	g_m	C_c	Φ_{PSII}	Φ_{NPQ}	Φ_{NO}	ETR/P_{gmax}	Chl (*a*+*b*)	Car (*x*+*c*)	LMA	LDMC	LT	TP	FRAP	Anth	TSC
P_{Nmax}	1	**0.891**	**0.799**	**0.584**	**0.841**	**–0.856**	0.103	**–0.350**	**0.562**	**0.550**	**–0.610**	**–0.653**	**–0.537**	–0.181	**0.635**	–0.196	**–0.510**
g_s		1	**0.806**	**0.754**	**0.563**	**–0.727**	**0.394**	**–0.617**	**0.637**	**0.613**	**–0.696**	**–0.708**	**–0.645**	–0.224	0.537	–0.073	**–0.590**
g_m			1	**0.898**	**0.512**	**–0.671**	**0.507**	**–0.545**	**0.908**	**0.902**	**–0.838**	**–0.783**	**–0.824**	–0.302	**0.710**	–0.009	**–0.755**
C_c				1	0.192	**–0.491**	**0.737**	**–0.713**	**0.853**	**0.837**	**–0.869**	**–0.817**	**–0.831**	**–0.434**	**0.619**	0.089	**–0.797**
Φ_{PSII}					1	**–0.826**	–0.339	0.207	0.256	0.251	**–0.350**	**–0.402**	–0.266	–0.026	**0.569**	**–0.380**	–0.212
Φ_{NPQ}						1	**–0.153**	0.111	**–0.404**	**–0.386**	**0.584**	**0.630**	**0.458**	0.201	**–0.759**	0.231	**0.489**
Φ_{NO}							1	**–0.745**	**0.578**	**0.568**	**–0.586**	**–0.519**	**–0.583**	–0.330	0.269	0.296	**–0.654**
ETR/P_{gmax}								1	**–0.546**	**–0.535**	**0.457**	**0.463**	**0.465**	0.305	–0.163	–0.274	**0.523**
Chl (*a*+*b*)									1	**0.996**	**–0.781**	**–0.665**	**–0.832**	–0.296	**0.544**	0.191	**–0.713**
Car (*x*+*c*)										1	**–0.761**	**–0.636**	**–0.824**	–0.292	**0.521**	0.178	**–0.692**
LMA											1	**0.915**	**0.927**	**0.438**	**–0.702**	–0.118	**0.895**
LDMC												1	**0.714**	**0.424**	**–0.695**	–0.136	**0.794**
LT													1	**0.361**	**–0.589**	–0.090	**0.856**
TP														1	**–0.403**	–0.153	**0.477**
FRAP															1	–0.026	**–0.671**
Anth																1	–0.184
TSC																	1

promoted the growth enhancing the dry mass production mainly in the roots. The PGPM addition improved nutrient uptake, phosphorus solubility, and hormones production, while host plant sustained symbiotic costs by supplying photosynthates for microbe metabolism and growth. In WL inoculated plants (WL-I), P_{Nmax} was upregulated likely to compensate for the costs of symbiosis (Kaschuk *et al.* 2009). The improved CO_2 uptake was attributed to the rise of both g_s and g_m as well as to an increase of ETR. The enhanced electron transport capacity may be the consequence of a high nutrient availability promoted by beneficial microorganisms (Walker *et al.* 2014). Consistent with studies of Khalid *et al.* (2017) on spinach, we found in WL inoculated plants a lower polyphenol and anthocyanin content and a high antioxidant capacity compared to noninoculated samples. In our opinion, the high antioxidant capacity might balance the low polyphenol amount, increasing the scavenging potential of the inoculated plants.

The interaction with light quality changes the relationships between plants and microorganisms. Several studies demonstrated the importance of light for the symbiotic functioning of PGPM, and in particular for AMF. Hristozkova *et al.* (2017) showed for the first time the influence of light quality on mycorrhizal symbiosis formation in tomato, indicating how the phenotypic plasticity was affected by light spectral composition. In our study, a different phenotypic plasticity was found in several key traits of inoculated plants confirming that different light quality regimes strongly change the plant's plastic responses to beneficial microorganisms. In our experiment, red light strongly promoted the root colonization by microbes, enhancing the development of arbuscular mycorrhizal fungi, in particular *Glomus* (Cruz 2016). Conversely, blue wavelengths seemed to inhibit bacteria and fungi growth (De Lucca *et al.* 2012). If the interaction light quality × microorganisms is favourable under WL and RB light treatments, it becomes null or negative in RG and R plants, respectively. It is likely to suppose that in inoculated R plants the energetic costs of the symbiosis became too elevated for the high AMF colonisation. Thus, the decrease of photosynthesis was likely due to the strong microorganisms' carbon demand. Under RG treatment, the inoculated plants were able to pay for the energy cost of symbiosis, increasing photosynthesis thanks also to beneficial properties of the green wavelength. The RG plants, investing more carbon in aboveground biomass compared to shoots, improved the nutrient uptake by the roots. The interplay of RB light and microorganisms increased photosynthetic capacity maybe for the low symbiosis cost; we based our hypothesis on the statement that blue light exerts an inhibitory effect on microbes' growth and development. However, it cannot be excluded that the lower carbon allocation to root, observed in RB plants, might indicate also an efficient nutrient and water transport *via* fungi (Kothari *et al.* 1990). In this case, the blue light might have promoted a higher nitrate reductase activity improving the nitrate assimilation in these plants, as found by other authors (Agarwal *et al.* 2018).

Conclusions: Light quality influences the phenotypic plasticity of spinach plants, inducing changes in morphology and physiology. The green wavelength promotes the plant carbon gain enhancing the photosynthetic rates and reducing the limitation to CO_2 diffusion. The exposure to pure red increases the photosynthesis promoting the light harvesting and improving the CO_2 diffusion to carboxylation sites that reduce significantly the photorespiration. Light modulation also affects the secondary metabolites synthesis and the antioxidant capacity. The interaction between light quality and microorganism-based biofertilizer alters the spinach phenotypic plasticity affecting the plant responses to microbes. In particular, the growth under pure red light promotes the root colonisation by microorganisms raising the costs of symbiosis. Under this condition, the interaction of plant–microorganisms becomes unfavourable.

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Chapter IV – The interplay between light quality and biostimulant application affects the antioxidant capacity and photosynthetic traits of soybean (*Glycine max*. L. Merrill).

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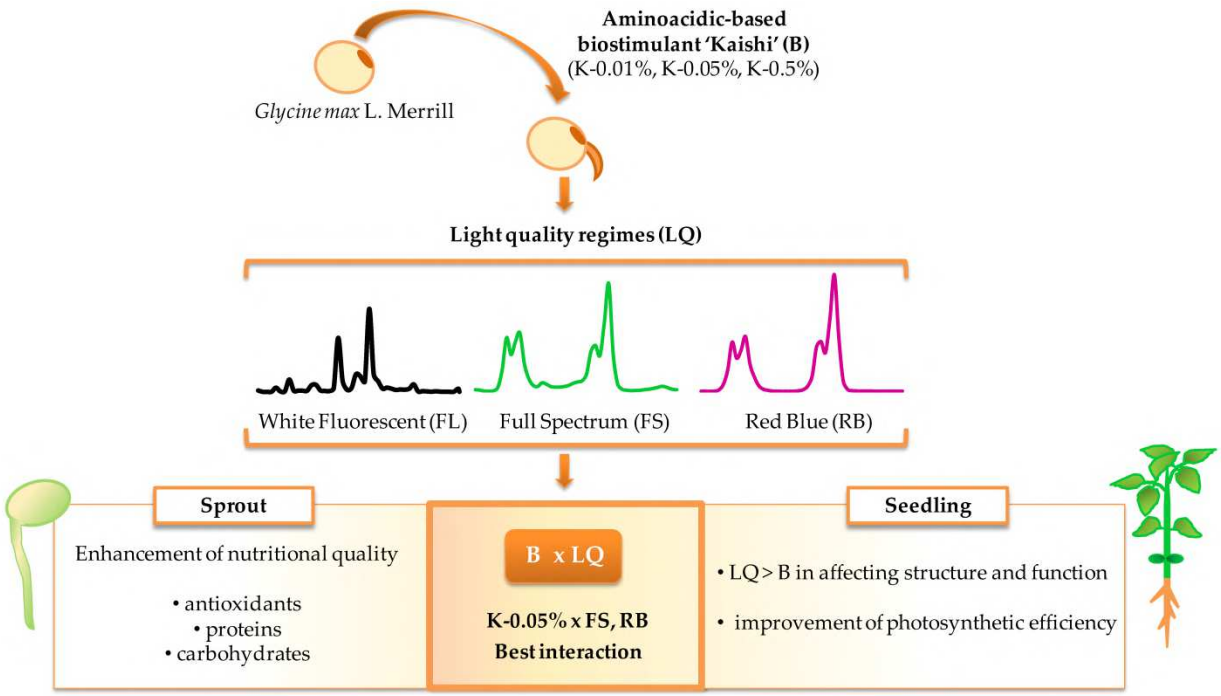

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The Interplay between Light Quality and Biostimulant Application Affects the Antioxidant Capacity and Photosynthetic Traits of Soybean (*Glycine max* L. Merrill)

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Abstract: This paper evaluates the combined effect of biostimulant and light quality on bioactive compound production and seedling growth of soybean (*Glycine max* L. Merrill) plants. Germinated seeds pre-treated with different concentrations (0.01%, 0.05%, 0.5%) of an amino acid-based biostimulant were grown for 4 days at the dark (D), white fluorescent light (FL), full-spectrum LED (FS), and red-blue (RB) light. Potential changes in the antioxidant content of sprouts were evaluated. Part of the sprouts was left to grow at FL, FS, and RB light regimes for 24 days to assess modifications in plants' anatomical and physiological traits during the early developmental plant stage. The seed pre-treatment with all biostimulant concentrations significantly increased sprout antioxidant compounds, sugar, and protein content compared to the control (seeds treated with H_2O). The positive effect on bioactive compounds was improved under FS and RB compared to D and FL light regimes. At the seedling stage, 0.05% was the only concentration of biostimulant effective in increasing the specific leaf area (SLA) and photosynthetic efficiency. Compared to FL, the growth under FS and RB light regimes significantly enhanced the beneficial effect of 0.05% on SLA and photosynthesis. This concentration led to leaf thickness increase and shoot/root ratio reduction. Our findings demonstrated that seed pre-treatment with proper biostimulant concentration in combination with specific light regimes during plant development may represent a useful means to modify the bioactive compound amount and leaf structural and photosynthetic traits.

Keywords: sprout bioactive compounds; light quality modulation; amino acids based biostimulant; PSII photochemical efficiency

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1. Introduction

In the last decades, agricultural practices are changing to meet the increasing market demand in response to the nutritional requirements of a growing population. This high production of food is leading to an overexploitation of the resources, especially of the soil, also exacerbated by climate changes [1]. In this context, new cultivation techniques in agriculture that minimize environmental impacts and cope with the lack of resource availability should be desirable as well as the possibility to produce functional food out of the soil or on suitable substrates.

Light modulation in terms of quality, intensity, and duration deeply influences plant morphogenesis, photosynthesis, and growth. Currently, the manipulation of the light quality as a tool to obtain specific physiological and morphological traits is largely used

in controlled environment agriculture (CEA), an innovative approach in which crops are cultivated indoor (greenhouses, growth chambers, vertical farms) to optimise their cultivation for food, pharmaceutical, and nutraceutical applications and save resources [2,3]. The light modulation approach allows an in-depth understanding of the photosynthetic responses to different light wavelengths [4,5] as well as the plant potentiality to produce bioactive compounds induced by diverse light quality treatments [6]. Several studies have been carried out on different crops, testing monochromatic light or mixing different wavelengths. Bian et al. [7] reported that the accumulation of phytochemicals in vegetable crops, such as lettuce, cucumber, tomato, radish, and spinach, depended on light quality and intensity. Light quality affects mainly carotenoids, phenolics, and vitamin C [8]. Among the visible spectra, red and blue lights are essential for photosynthesis and have often been used in plant research and for commercial production. It was previously reported that red and blue LEDs effectively enhance plant growth and secondary metabolites synthesis, and these effects are species-specific [9–11]. Despite the contrasting results, the primary outcomes agree that red and blue wavelengths are absorbed in the top of the leaves/canopy and are the most used regions of the light spectrum driving the photosynthetic process, biomass accumulation, shoot elongation, root development, stomata opening/closing regulation mechanism, pigment and polyphenol synthesis [7,12–15]. However, the green component penetrating deeper in the leaf tissues and canopy layer promotes the CO_2 fixation in regions not sufficiently reached by blue and red lights [16]. Overall, the choice of specific wavelengths matching plant photoreceptors can determine plant morphology, physiology, and metabolism, allowing us to define suitable light fertilization protocols [17].

As light quality modulation, the application of biostimulants may also be considered as an innovative eco-friendly and promising strategy replacing the common chemical fertilizers [18,19]. Biostimulants of different origins exist, including bacteria, fungi, seaweeds, higher plant extracts, protein hydrolysates (PHs) [20]. The composition, as well as the application strategies (at seed, soil, or leaf level), may influence their mode of action and the effects on crops. Several classes of biostimulants are highly used to improve seed germination, root system development, nutrient absorption, growth, productivity, and tolerance to environmental stresses [21–23]. Currently, the use of biostimulants in agriculture is increasing due to the need for low impact and more sustainable agricultural management approaches [24]. Among available classes, the biostimulants based on protein hydrolysates and the products containing amino acids are particularly worthy of attention because they enhance plant yield and quality in terms of growth, phytochemical content, N-uptake, and tolerance to many abiotic stresses [21,23,25,26]. Recent researches have specifically demonstrated that biostimulants can improve the nutritional traits of some plant-derived foods by enhancing the accumulation of secondary metabolites and phytonutrients in different parts of the plant [27].

Based on experimental evidence, the biostimulants application as well as the light quality manipulation are key aspects to be addressed in the next years for sustainable agricultural management approaches. Nowadays, only a few studies investigated the joined effect of light spectrum modulation and biostimulant showing responses depending on species and its phenotypic plasticity [19,28]. This paper aimed to explore the potential beneficial effects of the biostimulant application under different light quality regimes on plant bioactive compounds, seedling development, and photosynthesis. Soybean (*Glycine max* L. Merrill) was selected as a model species as it is largely desired in the marketplace for the high nutritional properties of seeds and sprouts [29]. In the present work, we treated soybean seeds with increasing doses of a new amino acid-based biostimulant (B) and tested the best concentration for promoting seed germination and bioactive compound synthesis in sprouts. Thereafter, the biostimulant pre-treated seeds were exposed to specific light quality (LQ) regimes (white fluorescent, FL; full-spectrum, FS, and red-blue, RB) to assess if the interaction biostimulant × light quality (B × LQ) may enhance the sprout nutritional value and photosynthetic activity of seedlings during the early developmental stage improving the overall seedling growth performance.

The outcomes of this study may be useful for the development of new protocols for the cultivation of soybean on a broad scale in the context of sustainable agriculture and to improve soybean sprout quality.

2. Results

2.1. Effect of Biostimulant on Seed Germination

One-way ANOVA analysis demonstrated that different concentrations (0.01, 0.05 and 0.5%) of the Kaishi biostimulant (K-) did not affect neither the germination percentage (G%) nor days to 50% emergence (E_{50}) compared to control (treated with H_2O). The G% values were 86 ± 4.4^a, 80 ± 5.0^a, 83 ± 3.3^a, 83 ± 6.2^a for H_2O, K-0.01%, K-0.05% and K-0.5%, respectively. The observed E_{50} values were: 1.9 ± 0.3^a, 2.0 ± 0.1^a, 2.1 ± 0.2^a, 2.0 ± 0.4^a for H_2O, K-0.01%, K-0.05% and K-0.5%, respectively.

2.2. Effect of Biostimulant and Light Regimes on Sprout Bioactive Compounds, Proteins, and Sugars

Figure 1 shows an overview of the qualitative traits of soybean sprouts in response to different biostimulant concentrations (K-0.01, K-0.05, and K-0.5%) and light quality regimes (dark, FL, FS, and RB).

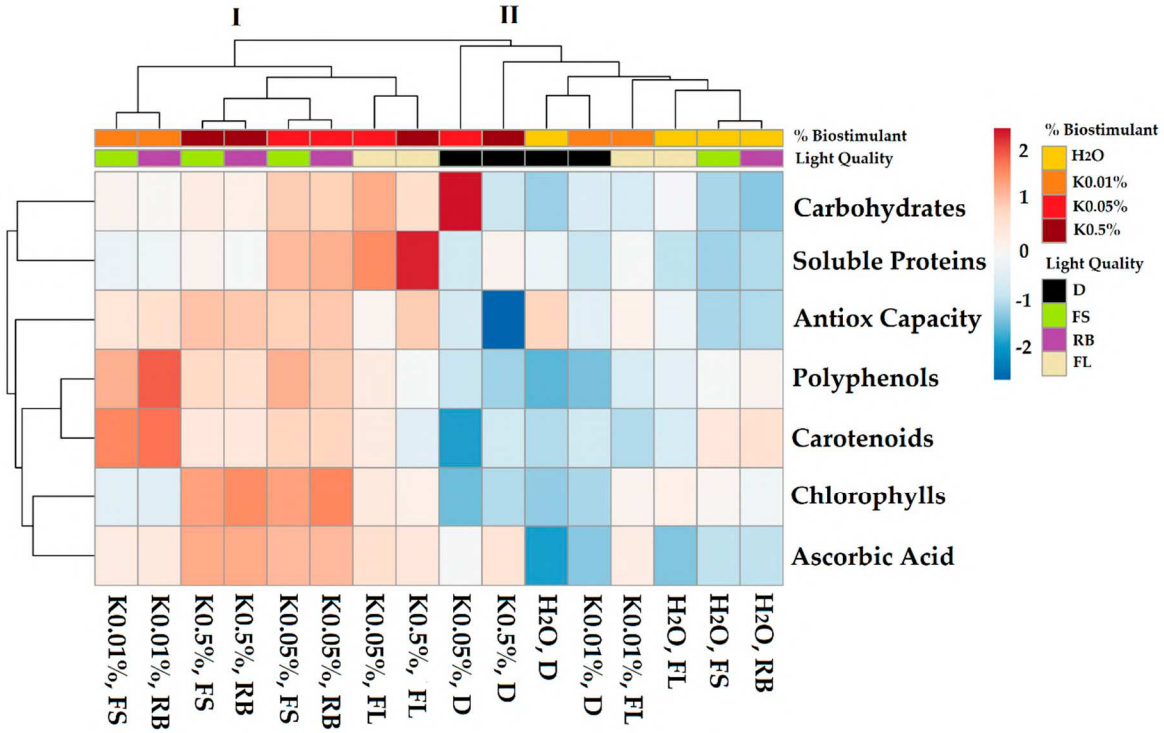


Figure 1. Cluster heatmap analysis summarizing qualitative traits of soybean sprouts (8 DAS) in response to different concentrations of biostimulant (K-0.01, K-0.05, and K-0.5%) and different light quality regimes (dark-D, white fluorescent-FL, full-spectrum-FS and red-blue-RB). Seeds treated with H_2O served as a control. Numeric differences within the data matrix are shown by the color scale: red and blue indicate increasing and decreasing values, respectively. Parameters are clustered in the rows; sample groups are clustered in the columns by the two independent factors Biostimulant and Light Quality.

The heatmap established two main clusters (I and II), which strongly depended on the applied B and LQ regimes. Cluster II included all sprouts grown under dark, all control (H_2O irrespective of the light regime), and K-0.01% × FL sprouts. Conversely, cluster I incorporated the remaining part of the testing groups. Cluster I showed higher values of biochemical compounds compared to cluster II. In particular, within cluster I, the subcluster composed of K-0.05% × FS and K-0.05% × RB sprouts, was characterized by a higher level of nutraceutical traits.

The effects of biostimulant and light quality as independent factors and their interaction on bioactive compounds of soybean sprouts were reported in Table 1.

Table 1. Analysis of variance and means comparison for bioactive compounds in soybean sprouts in response to different biostimulant (B) concentrations (K-0.01, K-0.05, and K-0.5%) and light quality (LQ) regimes (D, FL, FS, and RB) as well as under 16 different combinations of B × LQ. Different letters within each column indicate significant differences according to Student-Newman-Keuls multiple comparison tests ($p < 0.05$). Asterisks (*) represent the level of significance for main factors (B, LQ) and their interaction (B × LQ): NS-not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Seeds treated with H_2O served as a control.

	Bioactive Compounds						
	TAC	**TPC**	**CHL**	**CAR**	**CARB**	**AsA**	**SP**
B							
H_2O	1.48 b	0.77 c	0.33 b	0.034 a	59 c	7.5 c	55 c
K-0.01%	1.59 a	0.92 a	0.30 b	0.038 a	65 b	12 b	60 b
K-0.05%	1.62 a	0.95 a	0.44 a	0.037 a	82 a	15 a	72 a
K-0.5%	1.58 a	0.88 b	0.44 a	0.035 a	69 b	15 a	70 c
LQ							
D	1.40 b	0.62 c	0.21 c	0.028 c	67 a	9.7 c	59 c
FL	1.60 a	0.83 b	0.40 b	0.023 b	71 a	12 b	72 a
FS	1.64 a	1.02 a	0.45 a	0.042 a	68 a	14 a	63 b
RB	1.64 a	1.04 a	0.46 a	0.042 a	67 a	14 a	63 b
Interaction							
H_2O × D	1.72 a	0.56 e	0.20 c	0.028 b	57 c	5.3 d	61 c
K-0.01% × D	1.47 bc	0.59 e	0.22 c	0.029 b	62 c	7.4 c	54 d
K-0.05% × D	1.41 bc	0.69 d	0.17 c	0.024 b	92 a	12 b	56 d
K-0.5% × D	1.02 d	0.64 e	0.23 c	0.031 b	60 c	14 b	64 c
H_2O × FL	1.51 b	0.79 d	0.39 b	0.031 b	67 b	7.2 c	54 d
K-0.01% × FL	1.58 b	0.74 d	0.38 b	0.028 b	62 c	13 b	63 c
K-0.05% × FL	1.56 b	0.93 c	0.42 b	0.038 ab	80 b	14 b	81 b
K-0.5% × FL	1.74 a	0.85 c	0.40 b	0.033 b	75 b	14 b	89 a
H_2O × FS	1.34 c	0.85 c	0.38 b	0.038 ab	57 c	8.8 c	51 d
K-0.01% × FS	1.65 a	1.11 b	0.31 b	0.048 a	69 b	13 b	60 c
K-0.05% × FS	1.74 a	1.11 b	0.56 a	0.043 a	77 b	16 a	76 b
K-0.5% × FS	1.77 a	1.02 b	0.56 a	0.038 ab	71 b	17 a	65 c
H_2O × RB	1.35 c	0.88 c	0.35 b	0.039 ab	55 c	8.8 c	52 d
K-0.01% × RB	1.68 a	1.25 a	0.30 b	0.049 a	67 b	14 b	62 c
K-0.05% × RB	1.76 a	1.05 b	0.59 a	0.043 a	77 b	17 a	77 b
K-0.5% × RB	1.76 a	0.99 b	0.59 a	0.038 ab	69 b	17 a	63 c
Significance							
B	***	***	***	NS	***	***	***
LQ	***	***	***	***	NS	***	***
B × LQ	***	***	***	**	***	***	***

TAC: total antioxidant capacity (μmol TE g^{-1} FW); TPC: total polyphenol content (mg GAE g^{-1} FW); CHL: total chlorophylls (mg g^{-1} FW); CAR: total carotenoids (mg g^{-1} FW); CARB: total carbohydrates (mg GE g^{-1} FW); AsA: ascorbic acid (ng μL^{-1}); SP: soluble proteins (mg BSA eq g^{-1} FW).

The content of the bioactive compounds in soybean sprouts was influenced by B and LQ as main factors and their interaction (B × LQ). The only exception was the total carotenoid content, which was not affected by B, and carbohydrate amount was not affected by different LQ (Table 1). Regardless of the LQ regimes, among B treatments, K-0.05% and K-0.5% increased ($p < 0.001$) the chlorophyll content (Table 1). An increase in soluble protein level ($p < 0.001$) was found only in sprouts pre-treated with K-0.01% and K-0.05% showing the highest value at K-0.05% (Table 1). Compared to control, all B concentrations promoted ($p < 0.001$) ascorbic acid and total polyphenols content, as well as the antioxidant capacity ($p < 0.01$) and carbohydrates ($p < 0.01$) amount. In particular, carbohydrates reached the highest value ($p < 0.001$) at K-0.05% (Table 1).

Compared to sprouts exposed to darkness (D), those developed under FL, FS, and RB light regimes displayed greater ($p < 0.001$) antioxidant capacity, polyphenol, chlorophyll, carotenoid, ascorbic acid, and protein content independently from the biostimulant concentration (Table 1). In particular, the total polyphenols, chlorophylls, carotenoids, and ascorbic acid reached the highest values ($p < 0.001$) under FS and RB compared to FL light regime, which showed the highest ($p < 0.001$) protein concentration (Table 1).

As regards the interaction, all combinations B × LQ were significant (Table 1). In particular, K-0.01/0.05/0.5% × RB and K-0.01/0.05/0.5% × FS promoted TAC compared to H_2O × RB and FS. Conversely, K-0.01/0.05/0.5% × D reduced TAC compared to H_2O × D. The combinations K-0.05% × FS and K-0.05/0.5% × RB significantly increased CHL. Among all interactions, K-0.01% × RB induced the greatest TPC, while K-0.05% × D the highest CARB value. The interaction of K-0.05 and K-0.5% × FS and RB produced the highest AsA content, while K-0.5% × FL was the most effective in increasing the SP content.

2.3. Influence of Biostimulant and Light Quality on Seedling Morpho-Anatomical and Physiological Parameters

Figure 2 summarises the physiological and morphological traits of soybean seedlings in response to different B concentrations (K-0.01, K-0.05, and K-0.5%) and LQ regimes (FL, FS, and RB).

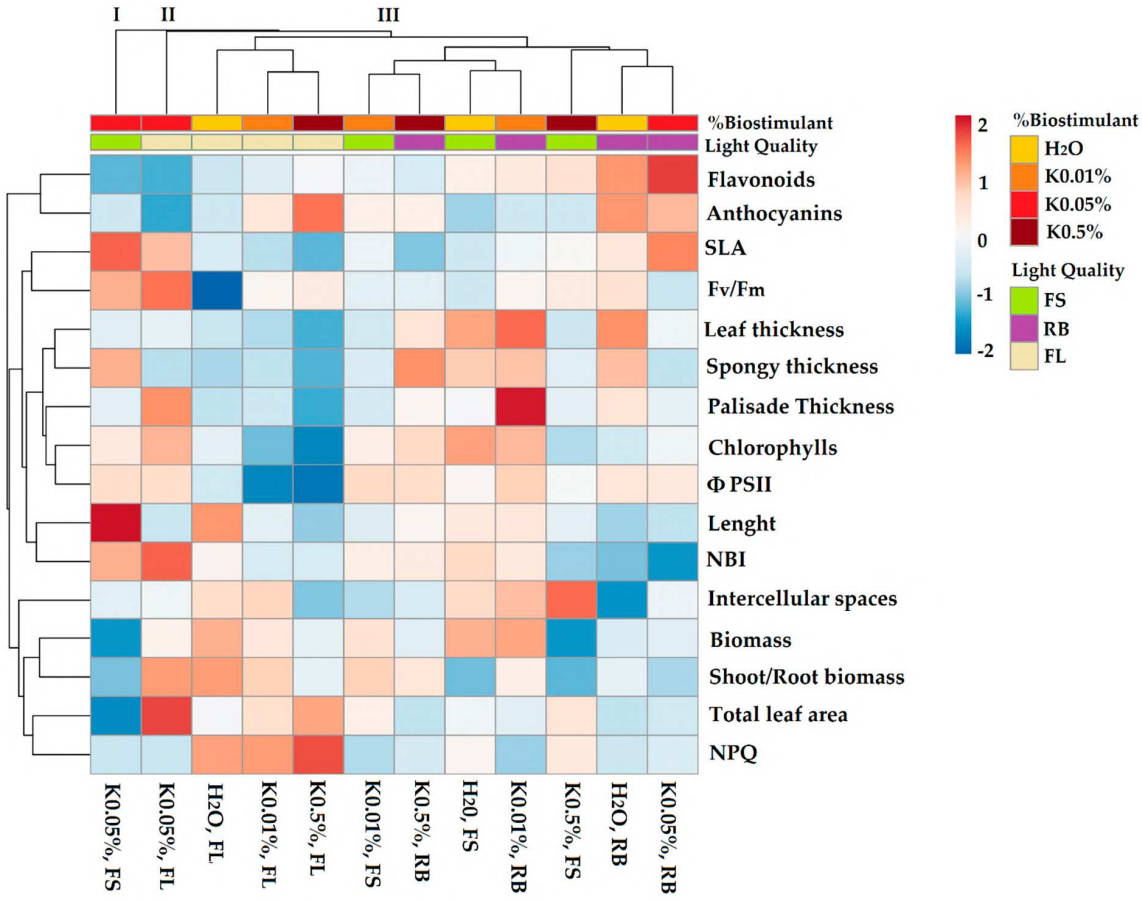


Figure 2. Cluster heatmap analysis summarizing physiological and morpho-anatomical parameters of soybean seedlings (at 24 DAS) in response to different concentrations of biostimulant (K-0.01, K-0.05, and K-0.5%) and different light quality regimes (white fluorescent-FL, full-spectrum-FS and red-blue-RB). Seeds treated with H_2O served as a control. Numeric differences within the data matrix are shown by the colour scale: red and blue indicate increasing and decreasing values, respectively. Parameters are clustered in the rows; sample groups are clustered in the columns by the two independent factors, biostimulant and light quality.

The heatmap established three main clusters. The first cluster (I) included K-0.05% × FS seedlings, while the second (II) only contained K-0.05% × FL seedlings. The third cluster (III) was divided into two subclusters. One, on the left, included all the seedlings developed under FL light (H_2O, K-0.01, K-0.5%). The second, on the right, incorporated the other testing groups. The generation of clusters I and II identified the B as the main discriminant factor compared to LQ, suggesting that the concentration K-0.05% significantly affected both soybean physiological and morphological traits. Within cluster III, LQ acted as the main discriminant factor compared to the biostimulant application and separated FL from FS and RB seedlings. Within the FL group, the separation of control from biostimulant-treated seedlings was evident. Conversely, within the second subcluster, no clear division between FS and RB seedlings occurred. Cluster I was characterised by higher values of SLA, photochemical PSII efficiency, NBI, plant length, total leaf area. Cluster II displayed a higher shoot/root biomass ratio and NPQ. Cluster III grouped seedlings with elevated values of leaf thickness (spongy and palisade), intercellular spaces, and pigment content.

2.3.1. Morphological Traits and Leaf Anatomy

Analysis of variance revealed that different B concentrations did not affect as main factors total plant leaf area, plant length, total plant biomass, and shoot/root biomass allocation, but significantly ($p < 0.001$) modified SLA (Table 2). Conversely, LQ influenced the morphological leaf traits and biomass partitioning. In particular, FS and RB seedlings showed a reduced ($p < 0.05$) leaf area and shoot/root biomass allocation ($p < 0.001$) and an increase in SLA ($p < 0.05$) (Table 2) when compared to FL. No significant interaction B × LQ was found in morphological parameters except for SLA ($p < 0.05$) (Table 2).

Table 2. Analysis of variance and means comparison for morphological parameters and anatomical traits in soybean seedlings in response to different biostimulant (B) concentrations (K-0.01, K-0.05, and K-0.5%) and light quality (LQ) regimes (FL, FS, and RB) as well as under 12 different combinations of B × LQ. Different letters within each column indicate significant differences according to Student-Newman-Keuls multiple comparison tests ($p < 0.05$). Asterisks represent the level of significance for main factors (B, LQ) and their interaction (B × LQ): NS-not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Seeds treated with H_2O served as a control.

	Morphological Parameters					**Anatomical Traits**			
	TLA	**SLA**	**Lenght**	**Biomass**	**S/R**	**LT**	**PT**	**ST**	**IS**
B									
H_2O	20 a	221 b	40 a	0.37 a	1.47 a	140 a	71 a	48 a	14 a
K-0.01%	22 a	215 b	38 a	0.37 a	1.67 a	134 b	74 a	45 a	15 a
K-0.05%	21 a	286 a	40 a	0.35 a	1.41 a	130 c	73 a	45 a	14 a
K-0.5%	23 a	199 b	36 a	0.35 a	1.37 a	127 c	68 b	45 a	14 a
LQ									
FL	25 a	214 b	38 a	0.37 a	1.72 a	124 c	69 b	41 b	14 a
FS	20 b	240 a	41 a	0.35 a	1.26 b	132 b	70 b	47 a	15 a
RB	19 b	237 a	37 a	0.36 a	1.45 b	143 a	76 a	49 a	13 b
Interaction									
H_2O × FL	21 a	210 b	45 a	0.38 a	1.89 a	125 c	66 d	41 b	17 ab
K-0.01% × FL	24 a	198 b	40 a	0.37 a	1.73 a	124 c	67 d	42 b	20 ab
K-0.05% × FL	29 a	271 a	35 a	0.36 a	1.88 a	131 bc	82 b	41 b	14 b
K-0.5% × FL	27 a	179 c	34 a	0.35 a	1.41 a	117 d	62 e	39 b	10 c
H_2O × FS	21 a	206 b	40 a	0.38 a	1.11 a	147 a	71 cd	51 a	17 ab
K-0.01% × FS	22 a	224 b	37 a	0.37 a	1.73 a	127 c	68 cd	43 b	11 bc
K-0.05% × FS	15 a	299 a	49 a	0.33 a	1.13 a	129 bc	70 cd	52 a	13 b
K-0.5% × FS	23 a	232 b	37 a	0.37 a	1.10 a	126 c	70 cd	44 b	20 a
H_2O × RB	19 a	248 b	34 a	0.36 a	1.41 a	149 a	75 c	51 a	8.3 c
K-0.01% × RB	20 a	226 b	41 a	0.39 a	1.56 a	152 a	87 a	51 a	18 a
K-0.05% × RB	19 a	291 a	35 a	0.35 a	1.22 a	132 bc	70 cd	42 b	14 b
K-0.5% × RB	18 a	185 c	39 a	0.39 a	1.62 a	139 b	72 cd	54 a	12 b
Significance									
B	NS	***	NS	NS	NS	***	***	NS	NS
LQ	*	*	NS	NS	***	***	***	***	**
B × LQ	NS	*	NS	NS	NS	***	***	***	***

TLA: total leaf area (cm^2); SLA: specific leaf area ($cm^2 g^{-1}$); Length: total seedling length (cm); Biomass: total seedling biomass (g DW); S/R: shoot/root biomass allocation; LT: leaf thickness (μm); PT: palisade thickness (μm); SP: spongy thickness (μm); IS: intercellular spaces (%).

The interactions K-0.05% × FL, K-0.05% × FS, and K-0.05% × RB induced the highest ($p < 0.001$) SLA values (Table 2).

The anatomical analysis of soybean leaves (Figure 3) evidenced a dorsiventral structure, with mesophyll composed of two layers of palisade cells, spongy parenchyma, and the presence of intracellular spaces.

All anatomical traits were significantly influenced ($p < 0.001$) by B and LQ as main factors, as well as by their interaction (B × LQ), except for the spongy thickness and intercellular space percentage, which were unaffected by B application (Table 2).

The leaf thickness decreased ($p < 0.01$) as the B concentration increased compared to the control. Among B treatments, K-0.5% determined the development of seedlings with the thinnest ($p < 0.05$) palisade parenchyma (Table 2).

Seedlings developed under RB light regime were characterized by a thicker ($p < 0.001$) palisade tissue, irrespectively from B concentration. The plant growth under FS and RB increased ($p < 0.001$) the spongy tissue thickness when compared to the FL regime. Consistently, FS and even more RB light determined the development of thicker ($p < 0.001$) leaves compared to FL. Finally, RB light induced a consistent decrease ($p < 0.01$) of the percentage of intercellular spaces accompanied by a more compact mesophyll organization (Table 2, Figure 3).

The interaction B × LQ determined the development of thickest leaves in particular for H_2O × FS and H_2O × RB as well as for K-0.01% × RB. This latter combination also determined the highest palisade thickness. The interactions H_2O × RB and K-0.5% × FL produced in seedlings the most significant IS reduction.

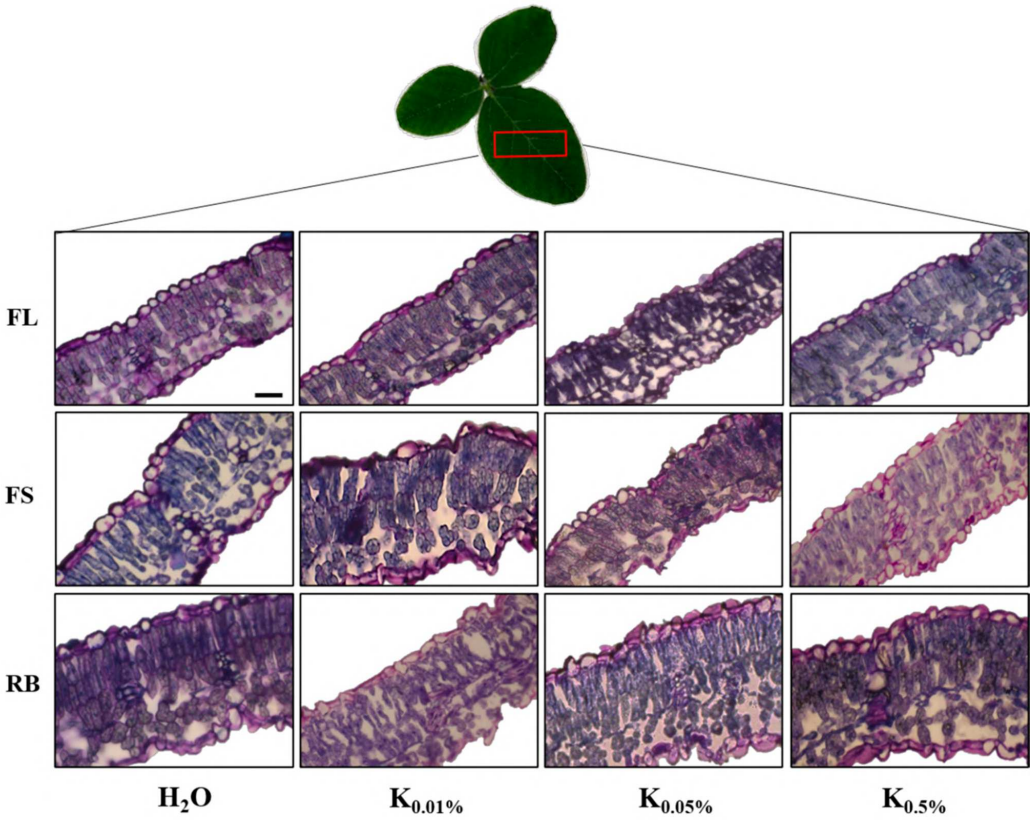


Figure 3. Cross-sections (stained by Toluidine blue) of leaf lamina of soybean seedlings at 24 DAS sprouted from seeds pre-treated with different concentrations of biostimulant Kaishi (K-0.01, K-0.05, and K-0.5%) and grown under different light quality regimes: white fluorescent-FL, full-spectrum-FS and red-blue-RB. Seeds treated with H_2O served as a control. Scale bar: 50 μm.

2.3.2. Pigments, Nitrogen Balance Index, and PSII Photochemistry

The effects of biostimulant and light quality and their interaction on pigments and functional traits of soybean seedlings were shown in Table 3.

Table 3. Analysis of variance and means comparison for pigments and functional traits in soybean seedlings in response to different biostimulant (B) concentrations (K-0.01, K-0.05, and K-0.5%) and light quality (LQ) regimes (FL, FS, and RB) as well as under 12 different combinations of B × LQ. Different letters within each column indicate significant differences according to Student–Newman–Keuls multiple comparison tests ($p < 0.05$). Asterisks (*) represent the level of significance for main factors (B, LQ) and their interaction (B × LQ): NS—not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Seeds treated with H_2O served as a control.

	Pigments			**Functional Traits**			
	CHL	**FLAV**	**ANTH**	**NBI**	**Φ_{PSII}**	**NPQ**	**F_v/F_m**
B							
H_2O	37 a	1.32 a	0.208 a	28 a	0.45 b	1.46 b	0.741 b
K-0.01%	36 a	1.28 a	0.207 a	29 a	0.44 b	1.29 c	0.748 b
K-0.05%	38 a	1.27 a	0.205 a	30 a	0.51 a	1.12 d	0.765 a
K-0.5%	34 b	1.29 a	0.211 a	27 a	0.41 c	1.59 a	0.750 b
LQ							
FL	34 b	1.23 b	0.210 a	29 a	0.36 b	1.74 a	0.753 a
FS	37 a	1.28 b	0.204 b	29 a	0.49 a	1.26 b	0.751 a
RB	37 a	1.38 a	0.211 a	26 b	0.51 a	1.10 c	0.747 a
Interaction							
H_2O × FL	35 a	1.23 b	0.205 bc	29 ac	0.40 c	1.88 b	0.726 b
K-0.01% × FL	32 b	1.25 b	0.213 ab	26 bc	0.28 d	1.89 b	0.749 ab
K-0.05% × FL	40 a	1.15 b	0.198 c	35 a	0.51 ab	1.09 d	0.784 a
K-0.5% × FL	30 b	1.29 b	0.222 a	27 bc	0.26 d	2.11 a	0.754 ab
H_2O × FS	40 a	1.32 ab	0.202 bc	31 ab	0.46 b	1.39 c	0.735 ab
K-0.01% × FS	37 a	1.28 ab	0.207 bc	29 ac	0.52 ab	1.02 d	0.743 ab
K-0.05% × FS	37 a	1.16 b	0.203 bc	33 ab	0.51 ab	1.09 d	0.773 ab
K-0.5% × FS	34 a	1.35 a	0.203 bc	28 bc	0.45 b	1.51 c	0.754 ab
H_2O × RB	34 a	1.43 a	0.218 ab	24 c	0.50 ab	1.10 d	0.762 ab
K-0.01% × RB	40 a	1.33 ab	0.202 bc	30 ac	0.53 a	0.97 d	0.751 ab
K-0.05% × RB	33 a	1.49 a	0.216 ab	22 c	0.49 ab	1.19 d	0.735 ab
K-0.5% × RB	37 a	1.24 b	0.209 bc	30 ac	0.51 ab	1.15 d	0.741 ab
Significance							
B	*	NS	NS	NS	***	***	*
LQ	*	***	**	*	***	***	NS
B × LQ	***	***	***	***	***	***	*

CHL: chlorophylls (r.u); FLAV: flavonoids (r.u); ANTH: anthocyanins (r.u); NBI: nitrogen balance index; Φ_{PSII}: effective quantum yield of PSII; NPQ: non-photochemical quenching; F_v/F_m: maximum PSII photochemical efficiency.

As the main factor, the B application showed a significant effect ($p < 0.05$) on chlorophyll content. On the other hand, LQ as the main factor or in combination with biostimulant (B × LQ) determined significant changes ($p < 0.001$) on chlorophylls, flavonoids, and anthocyanins (Table 3). More specifically, the concentration K-0.5% reduced ($p < 0.05$) chlorophyll content compared to control, K-0.01%, and K-0.05% (Table 3).

The applied LQ regimes differently modulated the leaf pigment composition. Namely, FS and RB increased ($p < 0.05$) seedling chlorophylls compared to FL light, while FS reduced ($p < 0.05$) the anthocyanin amount compared to FL and RB regimes. On the other hand, RB enhanced ($p < 0.001$) flavonoid leaf concentration compared to FL and FS regimes (Table 3).

The most significant interactions were K-0.01 × FL and K-0.5% × FL, which negatively affected the seedling chlorophyll content.

LQ significantly affected ($p < 0.001$) nitrogen balance index (NBI) alone or in combination with biostimulant (B × LQ). In particular, RB was the only light regime inducing a decline ($p < 0.05$) of NBI (Table 3).

The effective quantum yield (Φ_{PSII}) and the non-photochemical quenching (NPQ) were significantly influenced ($p < 0.001$) by B and LQ as main factors, as well as by their interaction (B × LQ). In contrast, the maximum PSII photochemical efficiency F_v/F_m was affected only by B and B × LQ interaction ($p < 0.05$) (Table 3).

The K-0.05% concentration determined the highest ($p < 0.001$) Φ_{PSII} and F_v/F_m, and the lowest ($p < 0.001$) NPQ values. Regardless of B concentration, Φ_{PSII} significantly increased ($p < 0.001$) in FS and RB compared to FL seedlings (Table 3). Conversely, NPQ decreased ($p < 0.001$) under FS and even more under RB compared to FL light regime (Table 3).

All B × LQ interactions for functional traits were significant (Table 3). In particular, K-0.05% × FL significantly increased the NBI compared to H_2O × RB and K-0.05% × RB. Moreover, K-0.5% × FL determined the highest NPQ, whereas K-0.05% × FL interaction promoted the highest Φ_{PSII} value within the FL regime. K-0.05% × FL produced a significant increase in F_v/F_m compared to H_2O × FL.

3. Discussion

This study evaluated for the first time the interaction between B application and LQ on *Glycine max* L. Merrill, a species widely consumed around the world as a source of protein-rich foods and beverages. The seed pre-treatment with the amino acid-based biostimulant Kaishi and the LQ regime during plant growth, as single factors or in interaction, strongly influenced the bioactive compound synthesis in sprouts producing an enrichment of antioxidant capacity, protein, and carbohydrate amount compared to the controls. On the other hand, during the seedling development, the growth under different LQ regimes acted as the main factor in modifying some leaf functional and anatomical traits influencing the seedling photosynthetic behaviour.

3.1. Effects of Biostimulant Seed Pre-Treatment and Light Quality on Sprout Bioactive Compounds

In the last decades, modern agricultural practices have emphasised the use of biostimulants to improve crop yield in a sustainable way. However, the observed effects strongly depend on species, kind of biostimulant, and application method [30–35].

The pre-treatment with different agents generally decreases seed dormancy and improves the metabolic processes occurring before radicle emergence. In particular, protein hydrolysates may modify the number of amino acids stored into the seeds as nutrients and energetic reserve, affecting germination and plant development [36–42].

Conversely to these findings, the amino acid-based biostimulant used in our study did not promote germination or days to 50% emergence compared to control. We suppose that the specific doses used for seed pre-treatment did not satisfy the seed metabolic activity for the germination process. On the other hand, the pre-treatment proved effective after germination since it enhanced the sprout nutritional traits and plant development in combination with specific LQ regimes.

However, we cannot outline a B dose-dependent trend for nutraceutical compounds because, in all treated samples, the bioactive molecule amount (e.g., antioxidants, chlorophylls, carotenoids) and carbohydrate and protein content were higher than control. K-0.05% seemed to be the most appropriate concentration to obtain healthier sprouts. Our results were in part consistent with Kim et al. [43], who demonstrated that seeds soaked with increasing concentrations of persimmon fruit powder produced sprouts proportionally richer in amino acids, ascorbic acid, and polyphenols. The amino acid-based biostimulants promoted polyphenol production [44] by stimulating nitrogen metabolism enzymes involved in the synthesis of these compounds [45]. In our study, the growth of soybean sprouts under specific LQ regimes (RB and FS) enhanced the positive effect of B on phytochemicals compared to FL and continuous darkness.

Chlorophylls and carotenoids were stimulated under FS and RB, suggesting the crucial role of red and blue wavelengths in the synthesis of photosynthetic pigments [46,47], while the FL regime produced sprouts richer in proteins than the other light regimes. Consistent

with Mastropasqua et al. [48], our data demonstrated that soluble proteins increased in sprouts grown under light compared to the dark and identified the FL light regime as the most effective in inducing the protein synthesis into greening cotyledons. Even if the highest protein content was found in K-0.5% × FL sprouts, the concentration of K-0.05%, increasing the soluble proteins under all light regimes compared to control and K-0.01%, evidenced a positive interplay between this specific concentration and light quality. Among antioxidants, the AsA content raised under FS and RB light regimes, especially when joined with K-0.05 and K-0.5%. The beneficial effect of these concentrations on seed metabolism was probably helped the increasing percentage of blue wavelength in the FS and RB light regimes (respectively 37% and 40%) compared to FL (12%). Consistent with this hypothesis, previous studies demonstrated that blue light promoted the expression of genes involved in the modulation of the ascorbic acid [48–50]. LQ regimes also alter the metabolism of phenolic compounds, which are generally more abundant in light- than in dark-grown sprouts [43,46,49]. The higher percentages of red and blue light may have stimulated soybean polyphenol synthesis as observed in several crop species [48], enhancing the expression of several related genes [51].

However, big differences may occur depending on plant species or degree of light exposure. Our data indicated that the interactions K-0.01, K-0.05, and K-0.5% × FS and RB were the most effective in favouring the polyphenol production in soybean sprouts.

The increased amount of AsA, polyphenol, and pigment content contributed to the high antioxidant capacity observed in FS and RB compared to the dark and FL sprouts at all B concentrations. These results highlighted that the interplay of B × LQ is a powerful means to obtain higher quality food.

Finally, soybean sprouts developed under dark generally contained more carbohydrates than those exposed to light, regardless of the LQ regime [48,49]. During germination, especially in photosynthetically active sprouts exposed to light, lipids, proteins, and carbohydrates are metabolized to gain energy for growth and several biological functions [48,49,52]. In our study, none of the LQ regimes affected the carbohydrate content compared to dark. Conversely, B at all tested concentrations increased the carbohydrate content compared to control, with the highest stimulation at K-0.05%. We assumed that the short period of light exposure (in our case, four days) was not adequate to induce mobilization of carbohydrates in soybean sprouts when photosynthesis was not yet started.

3.2. Effects of Biostimulant Seed Pre-Treatment and Light Quality on Photosynthesis and Early Plant Development

Previous research carried out on soybean plants demonstrated that seed treatment with different concentrations of fish-derived PHs positively affected the plant's vital processes, increasing plant biomass, phenolic compounds, and chlorophyll [53]. The seed pre-treatment with Kaishi biostimulant did not affect plant biomass or morphological traits, except SLA. The specific concentration of K-0.05% inducing the highest SLA, under all LQ regimes, appeared the most appropriate to improve plant productivity [54].

Our data proved that the effect of LQ on leaf morphological traits was stronger than those of the B seed pre-treatments. The growth of seedlings under FS and RB reduced the total leaf area and shoot/root biomass ratio but increased SLA compared FL light regime, indicating the development of the smallest plants with a higher investment in leaves and roots biomass. This aspect may be physiologically advantageous for plants because the higher SLA implicates higher photosynthetic yield, while a more developed root system may favor the plant water and nutrient supply. Our results were consistent with other studies reporting the efficiency of RB light in inducing higher plant yields, dwarf growth, and root expansion [13,55–58].

Considerable changes in leaf anatomical characteristics occurred in soybean plants subjected to B treatments. In Paradiso et al. [59], the seed inoculation with plant-growth-promoting microorganisms (PGPMs) determined plants with thicker leaves characterized by larger intercellular spaces. These anatomical traits significantly improved the PSII photochemical efficiency resulting in more efficient photosynthesis and growth. In our

study, the seed pre-treatment with Kaishi decreased the leaf thickness. The different response may be likely due to the diverse origin of the applied biostimulant: in the first case, a mixture of PGPMs, in our study, a protein hydrolysate. However, the seed pre-treatment with K-0.05%, consistent with higher SLA, improved the PSII photochemistry in soybean seedlings compared to control and other treatments, resulting in an investment of the absorbed light in photochemical reactions (higher Φ_{PSII} and F_v/F_m) rather than in photoprotective processes (lower NPQ).

The growth under FS and RB increased the photosynthetic efficiency and reduced the need for thermal dissipation processes [60] compared to the FL regime. This result may be ascribed to the higher red: blue ratio of FS and RB regimes than FL, which positively affected the photosynthetic apparatus [61]. It could also be suggested that the better photosynthetic efficiency in FS and RB seedlings may be due to the anatomical modifications induced by red and blue wavelengths preferentially absorbed in the upper leaf tissues [12,13,19,62]. According to previous findings, higher proportions of red and blue light in FS and RB regimes have led to the thickening of palisade and spongy tissues resulting in denser leaves than those of plants grown under FL light [63]. Leaf thickness significantly influences the space availability for chloroplast development [64]. Indeed, a denser palisade tissue generally contains more chloroplasts and chlorophylls involved in light-harvesting and photochemical reactions [16,65,66], improving the photosynthetic efficiency.

Even if the seed pre-treatment with Kaishi biostimulant did not produce any effects on pigments, the FS and RB light regimes differently modulated chlorophylls, anthocyanins and flavonoids, engaged in leaf photoprotection [7,14,67]. The RB light regime stimulated flavonoid synthesis. Following our results, it has been demonstrated that the RB LED regime enhanced the expression level of flavonoid-related genes compared to fluorescent light, leading to the increasing of these compounds [68].

The anthocyanin level decreased under FS compared to FL and RB leaves. It is noteworthy that anthocyanin production is activated by blue light and UV and in some species is augmented by far-red addition. We supposed that the effect of the high percentage of blue light inducing anthocyanin accumulation in RB leaves might be slowed down by the presence of green wavelength in FS leaves [69]. The anthocyanin level comparable between FL and RB was probably due to the different proportions among light spectrum wavelengths. This aspect needs to be further clarified.

Interestingly, the NBI decrease in RB seedlings compared to FL and FS indicated that the high flavonoid production needed a high carbon demand to produce carbon-based secondary compounds. This result suggested the occurrence of a significant trade-off between secondary and primary metabolism under RB light regimes [70–73].

Overall, our results indicated that seed pre-treatment with biostimulant Kaishi exerted positive outcomes on soybean, especially when combined with specific light growth regimes. The seed pre-treatment was not helpful for the germination process but alone or joined with LQ regimes significantly improved bioactive compounds in sprouts. The interplay between K-0.05% × FS and K-0.05% × RB favoured important physiological traits such as higher SLA and PSII photosynthetic efficiency linked to plant productivity.

The heatmap separated the controls (H_2O) from most of the sprouts pre-treated with Kaishi (B) and all dark (D) sprouts from most of the sprouts exposed to the light quality regimes (LQ), indicating that both biostimulant and light have a significant role during sprouting. The best interactions B × LQ were K-0.05% × FS and K-0.05% × RB since they displayed the highest bioactive compounds' content. Concerning the seedlings, the heatmap visualization showed that only K-0.05% greatly influenced the physiological and morphological traits regardless of the specific LQ regime. The seed pre-treatment with different B concentrations was particularly useful under the FL regime, which separated control from biostimulant-treated seedlings. Most of the differences were lost under FS and RB light regimes, suggesting that the biostimulant effect was less critical than LQ in inducing changes in plant structure and function. Moreover, the interaction B × LQ signifi-

cantly affected leaf anatomy and pigment content in seedlings, with positive implications on the photosynthetic process.

4. Materials and Methods

4.1. Seed Pre-Treatment and Germination

The biostimulant (Kaishi, AMM n°1171296) used in this study is manufactured and distributed by Sumi Agro France (251 rue de Faubourg Saint Martin, 75010 Paris, France, www.sumiagro.fr (accessed on 9 March 2018). Kaishi is a biostimulant with a unique liquid formula containing L-amino-acids of vegetal origin extracted through an enzymatic hydrolysis process and applied in biological agriculture.

Dry soybean seeds (*Glycine max* L. Merrill, Bulgarian variety) were soaked for 4 h in biostimulant solutions with different concentrations: 0.01%, 0.05%, and 0.5% (the following abbreviations were used throughout the whole text K-0.01%, K-0.05%, K-0.5%). Distilled water (H_2O) served as a control.

Solutions were prepared by adding to the biostimulant (liquid formula) different amounts of distilled water necessary to obtain the desired concentrations (*v/v*). Each seed was soaked in 1 mL of solution for 4 h. After, seeds were carefully placed in Petri dishes supplied with a double layer of filter paper wetted with distilled water and incubated in the dark at 24 ± 2 °C. The double layer of filter paper was maintained wetted by adding distilled water when necessary.

The effect of the Kaishi treatment on germination was evaluated after 4 days of incubation in the dark when a constant count of germinated seeds was obtained. The germination percentage (G%) and the days to 50% emergence (E_{50}), which indicates the rapidity in terms of days to obtain 50% germination, were calculated as reported in Noman et al. [74], using the following formulas:

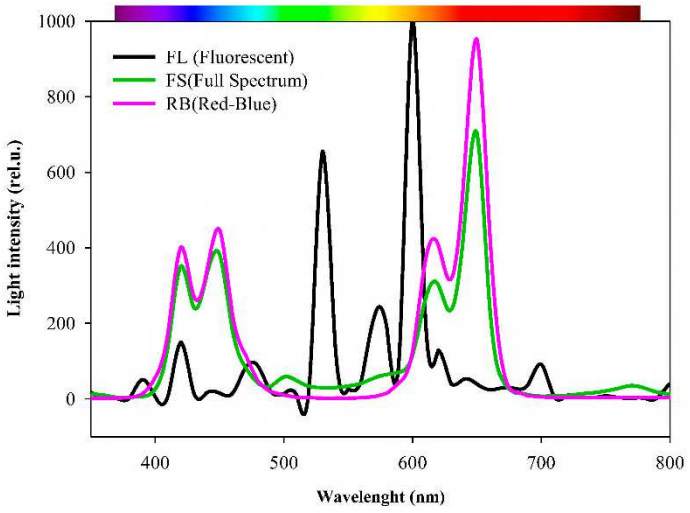
$$G\% = (\text{Number of germinated seeds}/\text{Total number of seeds}) \times 100, \quad (1)$$

$$E_{50} = t_i + (N/2 - n_i)(t_j - t_i)/(n_j - n_i), \quad (2)$$

where N = final number of germinated seeds; n_i = number of seeds emerged by count at time t_i when $n_i < N/2$; n_j = number of seeds emerged by count at time t_j when $N/2 < n_j$. The germination test was performed on 50 seeds per biostimulant concentration for a total of 200 seeds and repeated four times.

4.2. Growth Conditions

For the light treatments, three growth chambers with different light quality regimes were used. The white fluorescent light (FL) was supplied by a combination of fluorescent tubes (Lumilux L36W/640 and L36W/830, Osram, München, Germany); full-spectrum (FS) was obtained by a combination of far-red, red, yellow, green, blue, UV-A and white light-emitting diodes (LEDs), and red-blue (RB, red 60%-blue 40%) derived from (LEDs) (LedMarket Ltd., Plovdiv, Bulgaria). The spectral composition of the light regimes was determined by an SR-3000A spectro-radiometer at 10 nm resolution (Macam Photometrics Ltd., Livingston, Scotland, U.K.), as reported in Figure 4. Sprouts and plants were grown under controlled conditions: light intensity of photosynthetic photon flux density (PPFD) 360 µmol photons m^{-2} s^{-1} for each light treatment, day/night air temperature 24/18 °C, relative air humidity 60–70%, and a photoperiod of 14 h.



(**a**)

	Light Regime		
	FL	FS	RB
UV (350-390nm)	0.6%	0.61%	-
Blue (390-500nm)	12.43%	37.03%	40%
Green (500-560nm)	25.04%	6.57%	-
Yellow (560-600nm)	14.24%	3.38%	-
Red (600-700nm)	45.48%	48.79%	60%
Far-Red, IR (700-800nm)	2.21%	4.13%	-
Photon flux density: 360 µmol $m^{-2}s^{-1}$			
Irradiance Range: 350-800nm			

(**b**)

Figure 4. Light spectra used in the experiment (**a**). Spectral data and energy percentage of different light quality regimes. FL (white fluorescence tubes); FS (full-spectrum, LED); RB (red-blue, LED) (**b**).

At 4 DAS, 40 sprouts germinated from control (H_2O) and biostimulant pre-treated seeds (K-0.01%, K-0.05%, and K-0.5%) were carefully placed in Petri dishes supplied with a double layer of filter paper wetted with distilled water. Then they (10 sprouts for each biostimulant concentration × light treatment) were moved to the growth chambers for further 4 days under dark (D), white fluorescent light (FL), full-spectrum (FS), and red-blue (RB). At 8 DAS, when they reached the size for the market demand, sprouts were collected for biochemical analyses.

A cohort of 15 germinated seeds from control (H_2O) and each biostimulant concentration (K-0.01%, K-0.05%, and K-0.5%) was transplanted in plastic 1.0 L pots filled with tap water and left to grow until the achievement of the V1 stage (fully developed trifoliated leaves) under three light quality regimes: FL, FS, and RB (5 sprouts × light treatment).

The pots were refilled with tap water to field capacity when necessary. At 24 DAS, the seedlings were subjected to measurements of photosynthetic activity, leaf anatomy, and leaf functional attributes.

4.3. Analyses on Soybean Sprouts

Generally, sprouts used as food supplements are grown in total darkness [48]. Here, to assess the possible interaction B × LQ, the sprouts germinated from pre-treated seeds with increasing biostimulant concentrations (K-0.01%; K-0.05% and K-0.5%) were grown in continuous darkness (D), and also under the 3 different light quality regimes (FL, FS, RB).

The sampling for the biochemical analyses was carried out at 9:00 a.m. in the morning.

Biochemical Analyses

Biochemical analyses were carried out on 10 sprouts for each biostimulant concentration × light treatment. Each single sprout equates one replica.

The antioxidant capacity of soybean sprouts was determined by ferric reducing antioxidant power (FRAP) assay according to the method reported by George et al. [75], modified by Vitale et al. [19].

Briefly, samples (0.250 g) were ground in liquid nitrogen, mixed with 60:40 (*v/v*) methanol/water solution, and centrifuged at 14.000 rpm for 15 min (4 °C). FRAP reagents (300 mM acetate buffer pH 3.6; 10 mM tripyridyltriazine (TPTZ), 40 mM HCl and 12 mM $FeCl_3$) were added to the extracts of each sample in 16.6:1.6:1.6 (*v/v*), respectively. After 1 h in darkness, the absorbance at 593 nm was measured with a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). Trolox (6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid) was used as the standard, and total antioxidant capacity was quantified and expressed as μmol Trolox equivalents per mg of fresh weight (μmol TE g^{-1} FW).

Total polyphenols were determined as reported in Arena et al. [76]. Powdered samples (0.200 g) were extracted in methanol at 4 °C and centrifuged at 11.000 rpm for 5 min. Extracts were mixed with 1:1 (*v/v*) 10% Folin–Ciocâlteu reagent and, after 3 min, with 5:1 (*v/v*) 700 mM Na_2CO_3 solution. Samples were incubated for 2 h in darkness. Then, the absorbance at 765 nm was measured with a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). The total polyphenol content was calculated and expressed as mg of gallic acid equivalents per g of fresh weight (mg GAE g^{-1} FW) from the calibration curve using gallic acid as standard.

The ascorbic acid (AsA) content was determined using the Ascorbic Acid Assay Kit (MAK074, Sigma-Aldrich, St. Louis, MO, USA), following the procedure reported by Costanzo et al. [77]. Briefly, 10 mg of sample was homogenized in 4 volumes of cold AsA buffer and then centrifuged at 13.000 rpm for 10 min at 4 °C. The liquid fraction was mixed with AsA assay buffer to a final volume of 120 μL. The assay reaction was performed by adding the kit reagents to the samples. In this assay, the AsA concentration was determined by a coupled enzyme reaction, which develops a colorimetric (570 nm) product proportionate to the amount of ascorbic acid contained in the sample. The concentration of ascorbic acid in the samples was referred to as a standard curve and expressed in ng μL^{-1}.

Total chlorophylls and carotenoids were determined according to Lichtenthaler [78]. Pigments were extracted from powered samples (0.200 g) in ice-cold 100% acetone and centrifuged at 5.000 rpm for 5 min (Labofuge GL, Heraeus Sepatech, Hanau, Germany). The absorbance of supernatants was measured by spectrophotometer (Cary 100 UV-VIS, Agilent Technologies, Santa Clara, CA, USA) at wavelengths of 470, 645, and 662 nm and pigment concentration expressed as mg per g^{-1} of fresh weight (mg g^{-1} FW).

Total carbohydrates content was determined following the anthrone method reported by Hedge and Hofreiter [79]. Briefly, powered samples (10 mg) were mixed with 2.5 N HCl in which carbohydrates are first hydrolyzed into simple sugars. The concentration was estimated by the anthrone reagent dissolved in ice-cold H_2SO_4. In a hot acid medium, glucose is dehydrated to hydroxymethyl furfural that forms with anthrone, a green-coloured product with an absorption maximum at 630 nm. The absorbance was measured by a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). The number of total carbohydrates was calculated using a glucose standard curve and expressed as mg glucose equivalents per g^{-1} of fresh weight (mg GE g^{-1} FW).

Total soluble protein content was determined according to Bradford [80] and Im et al. [81]. Powdered samples (0.200 g) were homogenized in 0.2 M potassium phosphate buffer (pH 7.8 + 0.1 mM EDTA). Samples were centrifuged at 10.000 rpm for 20 min at 4 °C. The supernatant was added to the dye reagent, and the absorbance was read at 595 nm using a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). The total soluble protein content was calculated from a calibration curve using bovine serum albumin (BSA) as standard and expressed as mg BSA equivalents per g^{-1} FW (mg BSA eq g^{-1} FW).

4.4. Analyses on Soybean Seedlings

Morphological parameters, leaf functional attributes, leaf anatomy determinations, and chlorophyll *a* fluorescence emissions analysis were carried out at 24 DAS on 15 seedlings for each biostimulant concentration and 20 seedlings for each light quality regime. One seedling equates one replica.

4.4.1. Morphological Parameters and Leaf Functional Attributes

The total leaf area and the total plant length were measured by digital images analyzed by ImageJ software (Image Analysis Software, Rasband, NIH, Bethesda, Maryland, USA).

The biomass of shoot and root was determined on dry weight bases after oven-drying the samples at 75 °C for 48 h.

The Specific leaf area (SLA) was estimated following Cornelissen et al. [82] as the ratio of leaf area to leaf dry mass (DW) and expressed in $cm^2\ g^{-1}$ DW. Leaf area (LA) was measured using ImageJ software (Image Analysis Software, Rasband, NIH, Bethesda, Maryland, USA) and expressed in cm^2.

The relative chlorophyll, flavonoid, and anthocyanin content, as well as the nitrogen balance index (NBI), were determined by a plant pigment meter (Dualex, Force-A, Paris, France) equipped with a leaf-clip sensor and expressed in relative units.

4.4.2. Leaf Anatomy

The leaf anatomical analyses were performed by collecting leaf segments from each middle leaflet of the first fully expanded trifoliate leaves. After sampling, each segment was fixed in the fixative solution (40% formaldehyde/glacial acetic acid/50% ethanol, 5/5/90 *v/v/v*) at 4 °C and processed for inclusion according to the standard histological protocols for light microscopy [83].

After, tissue cross-sections of 3 µm thickness were stained with 0.025% Toluidine Blue in citrate buffer 0.1M, pH 4. All images were acquired by a light microscope (Axioskop Zeiss, Oberkochen, Germany) equipped with a digital camera (AxioCam MRc5, Zeiss, Oberkochen, Germany) using the same magnification (20×) and analysed through the AxioVision software (Carl Zeiss AG, White Plains, NY, USA) and the ImageJ software (Image Analysis Software, Rasband, NIH, Bethesda, Maryland, USA). More specifically, three fields of view and three different positions per image of each section were stored and used to determine the total leaf thickness (µm) and the thickness (µm) of the palisade and spongy tissues within the mesophyll. All measurements were carried out carefully, avoiding veins. Finally, the incidence of intercellular spaces was expressed as a percentage (%) of tissue occupied by intracellular spaces over a given surface considering three portions along the leaf lamina.

4.4.3. Chlorophyll a Fluorescence Emission Analysis

Chlorophyll *a* fluorescence measurements were performed using the IMAGING-PAM M-series, chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The minimum (F_0) and maximum (F_m) fluorescence were determined in 30-min dark-adapted seedlings and were used to calculate the maximum quantum yield of photosystem II (PSII) as:

$$F_v/F_m = (F_m - F_0)/F_m. \quad (3)$$

Then plants were exposed to actinic light (800 µmol photons $m^{-2}s^{-1}$) for 7 min and every 40 s saturating pulses (10,000 µmol photons $m^{-2}s^{-1}$) with duration 0.8 s were applied to determine the steady-state (F′) and maximum (F_m′) fluorescence in light-adapted state. The effective quantum yield of PSII, Φ_{PSII}, was determined as described in Genty et al. [84], by the formula:

$$\Phi_{PSII} = (F_m' - F)/F_m', \quad (4)$$

The non-photochemical quenching NPQ was calculated as indicated in Bilger and Björkman [85] as:

$$NPQ = (F_m - F_m')/F_m'. \quad (5)$$

4.5. Statistical Analysis

The overall parameters were visualized by a heatmap (heatmap function). The heatmap was plotted by using the ClustVis program package (https://biit.cs.ut.ee/clustvis/online (accessed on 16 April 2021)) and clustering both rows and columns with Euclidean distance and average linkage. In heatmaps, the numeric differences are evidenced by the colour scale: red and blue indicate increasing and decreasing values, respectively.

All data were analysed using the SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA). A one-way ANOVA was performed on the dataset for seed germination.

The influence of the two different independent factors, namely biostimulant concentration (B) and light quality treatment (LQ), and their possible interaction were analyzed by two-way ANOVA. The Kolmogorov–Smirnov test was used to check the normality. The Student–Newman–Keuls (SNK) test was applied for all pairwise multiple comparison tests with a significance level of $p < 0.05$). Whenever the interaction between B and LQ was significant, data were subjected to one-way ANOVA and multiple comparison tests were performed with the SNK coefficient.

5. Conclusions

The seed pre-treatment with Kaishi biostimulant, the different light quality regimes, and their interaction significantly modified the bioactive compound level in soybean sprouts and morpho-anatomical traits and photosynthetic efficiency in seedlings. More specifically, while germination was unaffected, the seed pre-treatment increased the sprout antioxidant charge and the protein and carbohydrate content producing a richer food than control. The beneficial effects of biostimulant were improved in sprouts grown under FS and RB light regimes than FL and D, with the most critical effect at K-0.05% for both FS and RB light growth conditions. In seedlings, the effect of seed pre-treatment was evident only for the concentration of K-0.05%, which promoted higher SLA and PSII photochemical efficiency compared to control. Compared to FL, the positive effect of the biostimulant was enhanced in seedlings grown under FS and RB light regimes. The present study provides evidence that seed pre-treatment with Kaishi biostimulant and the plant growth under FS and RB regimes is a practical approach to obtain, in a sustainable way, sprouts with a more elevated nutritional value and seedling with high photosynthetic efficiency.

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Section III – The Interaction between light quality and ionising radiation: constraint or opportunity?

Plant growth in Space is constrained by multiple stressors such as altered gravity and high radiation levels, and technical limits related to the narrow volumes. The effects resulting from ionising radiation (IR) exposure represents one of the most significant constraints for the survival of organisms in Space, because IR may trigger damages at the molecular, morpho-structural and physiological level, compromising the success of the space missions (Cucinotta e Durante, 2006; Durante and Cucinotta 2008; Arena *et al.,* 2014; Durante, 2014).

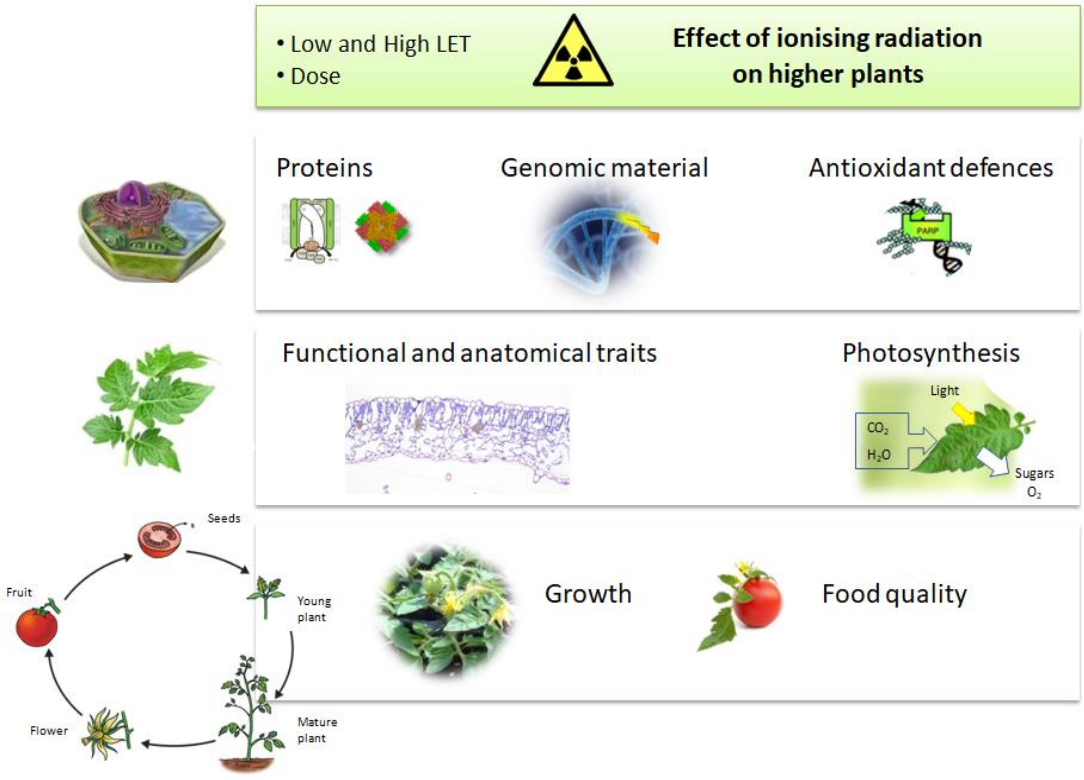

Figure 1: Schematic representation of the outcomes of ionising radiation on plants at different organization levels.

Primarily long-manned missions represent a strong risk factor for the increased exposure of organisms to space radiation. Several space experiments or ground-based studies explored the outcomes of the exposure of seeds, higher plants and photosynthetic microorganisms to low-Linear Energy Transfer (LET) and high-LET

ionising radiation. Generally, ionising radiation may have different effects on plant metabolism, growth, and reproduction, depending on plant developmental stage, physiological and morphological traits, and genetic characteristics (Arena *et al.*, 2014b; De Micco *et al.*, 2011). Moreover, depending on the dose or radiation type (low or high-LET), ionising radiation exerts detrimental outcomes at high doses, harmful consequences at intermediate levels, and stimulatory effects at shallow doses.

Many studies show that plants are much more radioresistant than animals due to differences in cell structure and metabolism (Arena *et al.*, 2014; Medina *et al.*, 2015). Plant cells present some traits such as thickened cell walls, cuticle, hairs (pubescence), phenolic compounds and often polyploidy, which can help them counteract the detrimental effects of ionising radiation (Real *et al.*, 2004, De Micco *et al.*, 2014, 2014b). Thus, ionising radiation, even if hazardous for mammals, may exert positive effects on plants. It is well known that specific doses of ionising radiation can improve plant defence against stressors, stimulating the production of antioxidants. Figure 1 reports the main targets of plant exposure to low and high-LET ionising radiation.

In this chapter the outcomes of high-LET ionising radiation (carbon, titanium and calcium heavy ions) will be discussed with particular attention on the stimulation effects sorted at very low doses on plant physiological traits and bioactive compound production.

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Chapter V – Suitability of *Solanum lycopersicum* L. 'Microtom' for growth in Bioregenerative Life Support Systems: exploring the effect of high-LET ionising radiation on photosynthesis, leaf structure and fruit traits.

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RESEARCH PAPER

Suitability of *Solanum lycopersicum* L. 'Microtom' for growth in Bioregenerative Life Support Systems: exploring the effect of high-LET ionising radiation on photosynthesis, leaf structure and fruit traits

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Keywords

Antioxidants; heavy ions; leaf anatomy; space ecosystem; tomato fruits.

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ABSTRACT

- The realisation of manned space exploration requires the development of Bioregenerative Life Support Systems (BLSS). In such self-sufficient closed habitats, higher plants have a fundamental role in air regeneration, water recovery, food production and waste recycling. In the space environment, ionising radiation represents one of the main constraints to plant growth.
- In this study, we explore whether low doses of heavy ions, namely Ca 25 Gy, delivered at the seed stage, may induce positive outcomes on growth and functional traits in plants of *Solanum lycopersicum* L. 'Microtom'. After irradiation of seed, plant growth was monitored during the whole plant life cycle, from germination to fruit ripening. Morphological parameters, photosynthetic efficiency, leaf anatomical functional traits and antioxidant production in leaves and fruits were analysed.
- Our data demonstrate that irradiation of seeds with 25 Gy Ca ions does not prevent achievement of the seed-to-seed cycle in 'Microtom', and induces a more compact plant size compared to the control. Plants germinated from irradiated seeds show better photochemical efficiency than controls, likely due to the higher amount of D1 protein and photosynthetic pigment content. Leaves of these plants also had smaller cells with a lower number of chloroplasts. The dose of 25 Gy Ca ions is also responsible for positive outcomes in fruits: although developing a lower number of berries, plants germinated from irradiated seeds produce larger berries, richer in carotenoids, ascorbic acid and anthocyanins than controls.
- These specific traits may be useful for 'Microtom' cultivation in BLSS in space, in so far as the crew members could benefit from fresh food richer in functional compounds that can be directly produced on board.

INTRODUCTION

Sustainable human space exploration, including long-duration manned missions to the Moon and Mars, has some essential requirements, among which one of the most challenging is habitat management, given the unfeasibility of providing enough resources from Earth for astronauts' survival (Vernikos *et al.* 2016). With the goal of achieving self-sustenance of humans in space, in the last decades, many space agencies, including NASA, ESA and JAXA, have been promoting the development of Bioregenerative Life Support Systems (BLSS), also known as Closed Ecological Life Support Systems (CELSS). These are self-sustaining artificial ecosystems, where plants play a fundamental role in supporting air regeneration, water and waste recycling, food production, as well as providing psychological support to the crew (Lasseur & Savage 2001; Arena *et al.* 2012, 2014a; Giacomelli *et al.* 2012). The choice of specific plant species for cultivation in BLSS is a critical issue that goes beyond plant survival, pointing more and more towards high growth efficiency, productivity and resource use efficiency. Apart from criteria typically based on technical requirements for cultivation (*e.g.* high tolerance to abiotic stresses such as osmotic imbalance, high yields, high ratio of edible/non-edible biomass, compact size in the case of volume constraints; Salisbury *et al.* 1997; Tibbitts & Henninger 1997; Wheeler 2017) and nutritional value of plant-derived fresh food (De Micco *et al.* 2012; Paradiso *et al.* 2012), the choice of species must take into account the plant ability to cope with the harsh space environmental conditions due to ionising radiation and altered gravity (Kiefer & Pross 1999; He *et al.* 2006; De Micco *et al.* 2011, 2014a; Arena *et al.* 2014b). Microgravity, being considered less constraining in the case of missions targeted to the long-term permanence in planetary outposts, radiation remains the main stressor to plant survival for space exploration. The space radiation environment consists of a wide variety of ion species with a continuous range of energies. The major galactic cosmic ray (GCR) particle types include hydrogen (H), helium (He), carbon (C), oxygen (O), neon

(Ne), silicon (Si), calcium (Ca) and iron (Fe) (Norbury *et al.* 2016). Among solar energetic particles (SEP), Ca ions are involved in several solar events and astrophysical processes (Bakaldin *et al.* 2015). In particular, the stable isotopes ^{48}Ca and ^{40}Ca have long half-lives and are used in studies on particle physics (Chen *et al.* 1997), or as radioisotope tracers in clinical investigations for understanding Ca homeostasis in health and disease (Neer *et al.* 1978; Smith *et al.* 1985).

Many space-oriented ground-based investigations (performed with single ion beams at fixed energies) or experiments performed directly in space have demonstrated that ionising radiation may have several outcomes (De Micco *et al.* 2011). The diverse radiation-induced plant behaviour depends on radiation quality (high or low Linear Energy Transfer – LET), dose and type of exposure (acute or chronic), as well as on the intrinsic traits of the target organism (*i.e.* species, cultivar, development stage, structure of organs and tissues, and genetics; De Micco *et al.* 2011). Plant response to ionising radiation is dose-dependent: permanent damage at high doses, harmful consequences at intermediate levels and stimulatory effects at low doses are expected (Arena *et al.* 2014a).

Experiments on seeds irradiated with heavy ions, or gamma or X-rays, often provide contrasting results: the inhibition of seed germination and seedling growth is frequently ascribed to the formation of free radicals in irradiated seeds (Kumagaia *et al.* 2000; Kovács & Keresztes 2002), whereas the improvement in germination is due to increased tegument porosity and faster water uptake (Hammond *et al.* 1996).

Among physiological processes, photosynthesis is particularly sensitive to ionising radiation. Alterations to photosynthesis may be due either to radiation-induced modifications in the leaf structural traits affecting gas exchange, or to a direct effect on light-harvesting complexes (LHC), electron transport carriers, enzymes of the carbon reduction cycle and the oxygen-evolving complex (Arena *et al.* 2013, 2017a). Threshold doses can be established between damage and positive outcomes: for instance, low doses of gamma rays (2, 4 and 8 Gy), delivered at the seed stage, have been reported to promote photosynthesis, respiration, electron transport rate (Vlasyuk 1964; Kim *et al.* 2004) and to increase the content of chlorophylls and carotenoids in leaves (Marcu *et al.* 2013). Conversely high doses of ionising radiation negatively influence photosynthesis, through the inhibition of the chlorophyll, LHC synthesis (Abe *et al.* 2002; Kovács & Keresztes 2002; Palamine *et al.* 2005) and photosystem II (PSII) D1 protein turnover (Giardi *et al.* 1997; Esposito *et al.* 2006).

Exposure to high doses of ionising radiation may increase the production of reactive oxygen species (ROS) also in tissues of adult plants, thus compromising not only the photosynthetic apparatus but also whole cells, by triggering membrane lipid peroxidation and protein modifications (Foyer & Mullineaux 1994). However, it is noteworthy that the maintenance of a low free radical concentration may represent a mechanism to improve plant tolerance to multiple stresses, with ROS acting as a signal for the activation of cell-protective response and defence pathways (Foyer & Mullineaux 1994; Donahue *et al.* 1997; Foyer & Noctor 2005).

The potential use of irradiation with heavy ions for improving specific traits in crops, such as the traits 'dwarf' or 'semi-dwarf', has been repeatedly applied (Mei *et al.* 1994, 1998; Honda *et al.* 2006; Jo *et al.* 2016; Oladosu *et al.* 2016). A further important aspect for species selection involves the nutritional plant traits: the space environment may predispose the crew to diseases (*i.e.* osteoporosis, muscle atrophy) that may be reduced by applying nutritional countermeasures *via* functional compounds (Bergouignan *et al.* 2016). In a futuristic view, such compounds would be furnished by plant-derived fresh food produced directly in space and characterised by possible radiation-induced improvement of the nutritional value (*e.g.* increased content of antioxidant compounds). Indeed, plants may perceive ionising radiation during cultivation in BLSS on board spaceships or in planetary greenhouses as a stimulus to produce antioxidants and secondary metabolites in order to protect their tissues. The potential use of irradiation with heavy ions (*i.e.* carbon, oxygen, argon, neon) for improving specific traits in crops, such as early maturity, high yield, radio-resistance and better fruit quality, has often been applied in other not space-oriented experiments, providing valuable results for ground-based research (Tanaka *et al.* 2002; Honda *et al.* 2006; Hou *et al.* 2008; Xie *et al.* 2008; Kharkwal 2012; Dong *et al.* 2016; Jo *et al.* 2016; Oladosu *et al.* 2016). The occurrence of low doses of radiation during space cultivation would become a 'natural' factor to be considered not as a constraint for plants but rather as a benefit. Often during space flights plants experience non-lethal doses of cosmic radiation, which contain the potential agents responsible for induction of crop changes. At present, knowledge in this field is scarce (Kharkwal 2012).

This study may be considered a pilot experiment, in which we aimed to assess the effect of Ca ions, in particular the stable isotope ^{48}Ca, delivered to dry seeds at a dose of 25 Gy, on the development, eco-physiological and morpho-functional traits of leaves and fruits of *Solanum lycopersicum* L. 'Microtom' (tomato) plants. This specific isotope has been used until now only for physics research and clinical studies and, unlike other heavy ions (*i.e.* carbon and iron), its effects on plants are unknown for both ground-based and space-oriented experiments. The dose of 25 Gy was considered to represent a threshold to obtain phenotypic alterations without compromising plant health status, based on available information from other *Solanaceae* species (Masuda *et al.* 2004; Jo *et al.* 2016). Our hypothesis was that such doses of ^{48}Ca ions may induce favourable outcomes for photosynthesis, biomass production and antioxidant production in tomato fruits.

MATERIAL AND METHODS

Plant material, irradiation procedure and experimental design

The species *S. lycopersicum* L., cv. 'Microtom' was selected as a model crop in this experiment because it has specific traits such as dwarf size, growth at high plant density (up to 1357 plants·m^{-2}) and short life cycle (70–90 days from sowing to fruit ripening). In addition, it does not require hand pollination (Okazaki & Ezura 2009; De Micco *et al.* 2014b) and contains a high amount of the antioxidant melatonin in different organs, including the seeds (Stürtz *et al.* 2011).

Dry seeds of 'Microtom', provided by Holland Online Vof (Amsterdam The Netherlands) from the same production batch, were transported to Germany and divided in two groups, namely control and treated seeds. The latter were irradiated with Ca heavy ions [isotope ^{48}Ca; E: 200 MeV·u^{-1}

(monoenergetic), LET: 180 keV·μm^{-1}; dose rate 1 Gy·min^{-1}] at a dose of 25 Gy. The irradiation was performed using a pencil beam in a spread out Bragg peak (SOBP), in the heavy-ion synchrotron (SIS) at GSI Helmholtzzentrum für Schwerionenforschung (Darmstadt, Germany). This dose, largely below the threshold for occurrence of DNA damage (Kazama *et al.* 2011), was chosen to assess possible stimulatory effects on plant development and fruit production. The experiment was performed twice.

After irradiation, irradiated and control seeds were transferred to the Department of Biology at the University of Naples Federico II (Naples, Italy). Throughout the transfers, both groups of seeds were assessed in the same environmental conditions to avoid any bias due to different pre-germination conditions other than irradiation. Then, both cohorts of seeds were planted and cultivated in the same experimental conditions until the completion of the plant life cycle (from *seed-to-seed*).

Plants germinated from both irradiated and control seeds were monitored and compared until fruit ripening. Changes in plant growth and development were estimated through biometric and leaf anatomical analyses. The functionality of the photosynthetic apparatus was evaluated using fluorescence emission measurements, determination of photosynthetic pigment content, D1 protein of PSII and Rubisco levels. The antioxidant capacity of leaves was evaluated through the determination of ascorbic acid (AsA) content, superoxide dismutase (SOD) and catalase (CAT) activity. The SOD and CAT activity, AsA, carotenoids, polyphenols and anthocyanin content were analysed in fruits. All data were evaluated in the light of plant acclimation to ionising radiation, analysing integrated structural–functional mechanisms leading to the correct functioning of the photosynthetic process for the production of biomass with high nutritional value.

Germination, plant cultivation and biometry

Irradiated seeds ($n = 40$) and controls ($n = 40$) were placed into sterile Petri dishes on disks of filter paper and incubated in the dark at 20 °C. Germination percentage and rate were monitored daily; seeds were considered germinated when the emerging root was longer than the seed maximum diameter. The final percentage germination (FPG) was calculated 4 and 7 days after sowing (DAS), according to the following formula:

$$\mathrm{FPG_{nDAS}} = \frac{\text{Number of germinated seeds after nDAS}}{\text{Total number of seeds}} \times 100$$

Seedlings from irradiated and control seeds were transferred into 15-cm diameter pots, filled with peat-based compost (peat:soil, 1:1 v:v) and placed in a growth chamber under controlled conditions of temperature (25 ± 1 °C), relative humidity (RH 60 ± 10%) and light (photosynthetic photon flux density, PPFD, 155 ± 5 μmol·photons·m^{-2}·s^{-1}). Plants were cultivated up to fruit ripening, and during the whole life cycle (90 days), plants were irrigated at 2-day intervals to replenish the water lost to evapotranspiration.

Biometric analyses were performed once a week on ten plants (five control plants, C; and five plants from irradiated seeds, I) throughout the crop cycle in order to measure the following parameters: plant height (considering the main stem), plant total leaf area, number of leaves, flowers and fruits. For leaf area, images of the leaf lamina of each leaf per plant were obtained with a digital camera (Nikon Coolpix A300, Nikon Europe) and analysed with ImageJ software (Rasband, W.S., U.S. NIH, Bethesda, MD, USA, 1997-2012). At the end of the plant life cycle, dry plant biomass was determined by separating plants into leaves, stem and roots, which were weighed after oven-drying at 75 °C for 48 h.

Fluorescence emission measurements and photosynthetic pigment content determination

Chlorophyll *a* fluorescence measurements and pigment extraction were carried out on five fully expanded leaves from five control (C) and five irradiated (I) plants at 30 DAS. Chlorophyll *a* fluorescence measurements were conducted using a pulse amplitude modulated fluorometer (Junior-PAM; Walz, Effeltrich, Germany), equipped with a monitoring leaf-clip JUNIOR-B (Walz). On 30 min dark-adapted leaves, the background fluorescence signal, F_0, was induced using internal light provided by a blue LED of about 2–3 μmol·photons·m^{-2}·s^{-1}, at a frequency of 0.5 kHz. For this species, 30 s is sufficient to obtain complete re-oxidation of PSII reaction centres (De Micco *et al.* 2014b). The maximum fluorescence level in the dark-adapted state (F_m) was measured with a 1-s saturating light pulse (8000 μmol·photons·m^{-2}·s^{-1}) at a frequency of 10 kHz; the maximum PSII photochemical efficiency (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_0)/F_m$. Measurements in the light were carried out by exposing each leaf to a PPFD of 420 μmol·photons·m^{-2}·s^{-1} for 5 min; this PPFD was chosen because it falls within the range of maximum quantum yield for 'Microtom' plants (De Micco *et al.* 2014b). The steady-state fluorescence signal (F_t) and maximum fluorescence ($F_{m'}$) under illumination were measured, setting the measuring light at a frequency of 10 kHz; $F_{m'}$ was determined after a 1 s saturating light pulse (8000 μmol·photons·m^{-2}·s^{-1}). The quantum yield of PSII electron transport (Φ_{PSII}) and non-photochemical quenching (NPQ) were expressed according to Genty *et al.* (1989) and Bilger & Björkman (1990).

After fluorescence analyses, leaves were collected from plants for determination of chlorophylls and carotenoids following the procedure reported in De Micco *et al.* (2014a) and Arena *et al.* (2014a) for 'Microtom' and dwarf bean, respectively. More specifically, pigments were extracted with a mortar and pestle in ice-cold 100% acetone, centrifuged at 3200 *g* for 5 min in a Labofuge GL (Heraeus Sepatech, Hanau, Germany). The absorbance of the supernatants was quantified with a spectrophotometer (UV-VIS Cary 100; Agilent Technologies, Palo Alto, CA, USA) at 470, 645 and 662 nm, according to Lichtenthaler (1987). Total chlorophyll and carotenoid concentrations were expressed in mg·g^{-1} FW.

Protein extraction and Western blot analysis

Protein extraction from leaves was carried out on six fully expanded leaves sampled from three control and three irradiated plants at 30 DAS, according to Wang *et al.* (2006) and Bertolde *et al.* (2014), using 0.3 g dry plant material for each sample. Protein extracts were quantified using the Bradford

colorimetric assay (BioRad protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Milan, Italy), measuring absorbance at 595 nm with the UV-VIS Cary 100 spectrophotometer. An SDS-PAGE (12%) was performed by using Dual Color Protein Standard (Bio-Rad Laboratories) as marker and Laemmli loading buffer added to samples to follow protein separation. Western blot analysis on leaf samples was performed using a blocking solution (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 5% BSA) and primary antibodies (Agrisera, Vännäs, Sweden) to reveal different proteins: Rubisco (anti-RbcL, rabbit polyclonal serum), D1 (anti-PsbA, hen polyclonal) and Actin (anti-ACT, rabbit polyclonal serum) as loading control (Arena *et al.* 2017b). Immuno-revelation was performed using the kit for chemiluminescence (Westar Supernova, Cyanagen) with the ChemiDoc System (Bio-Rad Laboratories). Densitometric analysis was performed to obtain quantitative information associated with individual bands using Quantity One 1-D Analysis Software (Bio-Rad Laboratories). The protein β-actin was used as loading control. The value of each band was normalised to the corresponding β-actin band. Density values are expressed in arbitrary units and represented as bar diagrams that are pixel volumes of protein bands.

Leaf anatomy

Anatomical analyses were performed on six fully expanded leaves sampled from six control and six irradiated plants at 70 DAS. More specifically, one of the two middle leaflets per each compound leaf was cut and immediately submerged in the chemical fixative FAA (40% formaldehyde/glacial acetic acid/50% ethanol, 5/5/90 v/v/v). After 2 weeks of fixation, subsamples were obtained by dissecting a 5 x 5 mm region of the leaflet lamina under a reflected light microscope (SZX 16; Olympus, Hamburg, Germany) and used for preparation of thin sections.

The subsamples were dehydrated in an ethanol series (50%, 70%, 95% ethanol) and embedded in acrylic resin JB4® (Polysciences, Warrington, PA, USA). Cross-sections (5-μm thick) were cut using a rotatory microtome. Sections were divided in two groups: the first stained with 0.025% toluidine blue in 0.1 M citrate buffer, pH 4, to obtain general staining of all structures; the second stained in 0.02% red ruthenium solution in order to better highlight hemicelluloses and pectins (Jensen 1962; Reale *et al.* 2012). Stained sections were mounted with Canadian balsam and observed under a transmitting light microscope (BX60; Olympus). Images were collected with a digital camera (Camedia C4040; Olympus) at different magnifications and analysed with the software Olympus AnalySIS 3.2.

Leaf lamina thickness, as well as palisade parenchyma thickness and spongy parenchyma thickness, were measured in six positions along the lamina, avoiding veins. The incidence of intercellular spaces in the spongy parenchyma was measured as the percentage of tissue occupied by intercellular spaces over a given surface in four regions along the mesophyll. The cell area and shape of the upper and lower epidermis, palisade and spongy parenchyma were quantified in 15 cells per each tissue per section. More specifically, cell shape was defined by the following indices: cell aspect ratio (maximum width/height ratio of a rectangle circumscribing the cell); cell convexity (fraction of the cell's area and its convex area; De Micco *et al.* 2008; Van Buggenhout *et al.* 2008).

Trichome and stomatal frequency ($n \cdot mm^{-1}$) were calculated by counting, respectively, the number of stalks and stomata present along the section on both the upper and lower epidermis in four positions along the lamina and referring these numbers to the length of the section analysed. The frequency of chloroplasts per palisade and spongy parenchyma ($n \cdot mm^{-2}$) was calculated by counting the number of chloroplasts present in a given region of the palisade and spongy tissues, respectively, in four positions along mesophyll, and referring these to the area of the region analysed. The frequency of calcium oxalate crystals ($n \cdot mm^{-1}$) was calculated by counting, respectively, the number of crystals present along the section in the mesophyll in four positions along the lamina and referring these numbers to the length of the section analysed.

Leaf and fruit antioxidants: AsA content, SOD and CAT activity

For the determination of AsA content, SOD and CAT activity, five fully expanded leaves of plants from irradiated and control seeds were collected. Samples used for AsA extraction (1.0 g fresh leaf or fruit) were washed in distilled water, dried with filter paper and immediately frozen at −80 °C; the frozen tissues were ground under liquid nitrogen with a pestle and mortar to a fine powder and used for analysis. AsA concentration was determined using the Ascorbic Acid Assay Kit II (Sigma-Aldrich, St. Louis, MO, USA). In this method, AsA concentration is measured using the ferric reducing/antioxidant and ascorbic acid (FRASC) assay. The reduction of Fe^{3+} to Fe^{2+} by antioxidants present in the sample (1.0 g fresh leaf) results in a colorimetric product. The simultaneous addition of ascorbate oxidase to samples oxidises the AsA in sample tissue, allowing measurement of the AsA concentration. The AsA concentration was quantified with a spectrophotometer (UV-VIS Cary 100; Agilent Technologies) at 593 nm, referred to a standard curve and expressed in $ng \cdot \mu l^{-1}$.

The CAT activity was measured on leaf and fruit extracts, following the protocol provided by the Catalase Assay Kit (Sigma-Aldrich), based on a colorimetric method in which the decomposition reaction of H_2O_2 into H_2O and O_2 is spectrophotometrically followed as the decrease at A_{520}. A unit of CAT activity is defined as the amount of enzyme which decomposes 1 μmol H_2O_2 for 1 min at pH 7.0 and 25 °C.

The SOD activity was determined with a SOD Assay Kit (Sigma-Aldrich) with a colorimetric method based on the transformation reaction of colourless nitroblue tetrazolium (NBT) into blue formazan, after reduction with superoxide anion O_2^-. SOD activity was determined by measuring inhibition of the NBT reduction into blue formazan. The blue colour developed in the reaction is measured at 440 nm. The volume of the sample that causes 50% inhibition in appearance of colour is considered as a unit of SOD activity.

Determination of carotenoids, anthocyanins and polyphenols in fruits

For the determination of carotenoids, anthocyanins and polyphenols in fruits, five fruit samples were collected from five irradiated and five non-irradiated plants. For carotenoid

analysis, pigments were extracted from samples (~0.15 g) with a mortar and pestle in ice-cold 100% acetone, centrifuged at 3200 *g* for 5 min in a Labofuge GL (Heraeus Sepatech). Absorbance of the supernatants was quantified in a UV-VIS Cary 100 spectrophotometer at 470, 645 and 662 nm, according to Lichtenthaler (1987). Total carotenoid concentrations were expressed in $mg \cdot g^{-1}$ FW.

To determine anthocyanin content, fruit samples (~0.25 g) were ground with liquid N_2, treated with methanol 1% HCl solution and stored in a dark refrigerator to allow the extraction. A mixture of 1.0:0.6 (v/v) ultra-pure H_2O (MilliQ®) and 1.0:1.6 (v/v) chloroform was added to each sample. Samples were centrifuged (Eppendorf 5804R; Hamburg, Germany) at 12850 *g* for 5 min. The supernatant was extracted from each sample by adding 1:1 (v/v) 60% methanol 1% HCl: 40% ultrapure H_2O (MilliQ®) solution. The absorbance was quantified in a UV-VIS Cary 100 spectrophotometer at 530 and 657 nm. The total anthocyanin content was expressed in $\mu g \cdot g^{-1}$ FW.

For polyphenol determination, the procedure reported by Porzio *et al.* (2018) was followed. Fruit material (~0.2 g) was homogenised with liquid N_2 and samples were treated and incubated with methanol, mixed and incubated in a dark refrigerator. Samples were centrifuged (Eppendorf 5804R) at 12,850 *g* for 5 s. The supernatant was extracted and 1:1 (v/v) 10% Folin Ciocâlteu and 1:5 (v/v) 700 mM Na_2CO_3 solution were added to each sample. Samples were incubated in a dark refrigerator for 2 h. The absorbance was quantified in a UV-VIS Cary 100 spectrophotometer at 765 nm. The total polyphenol concentration was calculated and expressed as gallic acid equivalents ($mg \cdot GAE \cdot g^{-1}$ FW) using the regression equation between gallic acid standards and A_{765}.

Statistical analysis

Statistically significant differences among control and irradiated plants were assessed with a *t*-test based on a significance level of $P < 0.05$. Shapiro-Wilk and Kolmogorov-Smirnov tests were performed to check for normality. Percentage data were transformed through the arcsine function before statistical analysis. The data reported are average ± SE per each treatment. The package Sigma-Plot 12.0 (Jandel Scientific, San Rafael, CA, USA) was used for graphical and statistical data processing.

RESULTS

Effect of Ca ions on germination, plant growth and fruit ripening

The exposure of seeds to Ca ions at a dose of 25 Gy initially led to a slowing down in the germination rate: at 4 DAS only 50% of irradiated seeds germinated compared to the control ($P < 0.05$); at 7 DAS, the germination of the irradiated seeds increased up to 83.3% and no significant differences were observed compared to controls (100%).

Both controls (C) and plants germinated from irradiated seeds (I) completed the whole plant cycle up to fruit ripening at 90 DAS. Plants germinated from Ca-irradiated seeds showed a significant reduction ($P < 0.01$) in plant height compared to controls during the whole life cycle (Fig. 1A), whereas no significant difference in the number of leaves was detected (Fig. 1B). From the start of plant development, C plants had a significantly larger leaf area than I plants, and both plant groups were characterised by a similar progressive increase in leaf area up to 70 DAS (Fig. 1C). After 70 DAS, leaf area in I plants increased with a steeper slope compared to C plants, leading to similar leaf area per plant at the end of the cycle. At harvesting, C plants had a significantly higher ($P < 0.05$) number of flowers, and a significantly higher ($P < 0.05$) number of smaller fruits (Table 1). The dry biomass was significantly lower ($P < 0.05$) in Ca-irradiated compared to control plants (Table 1).

Effect on leaf anatomy

Microscopy analysis of the leaf sections showed regular anatomical organisation in both C and I plants (Fig. 2).

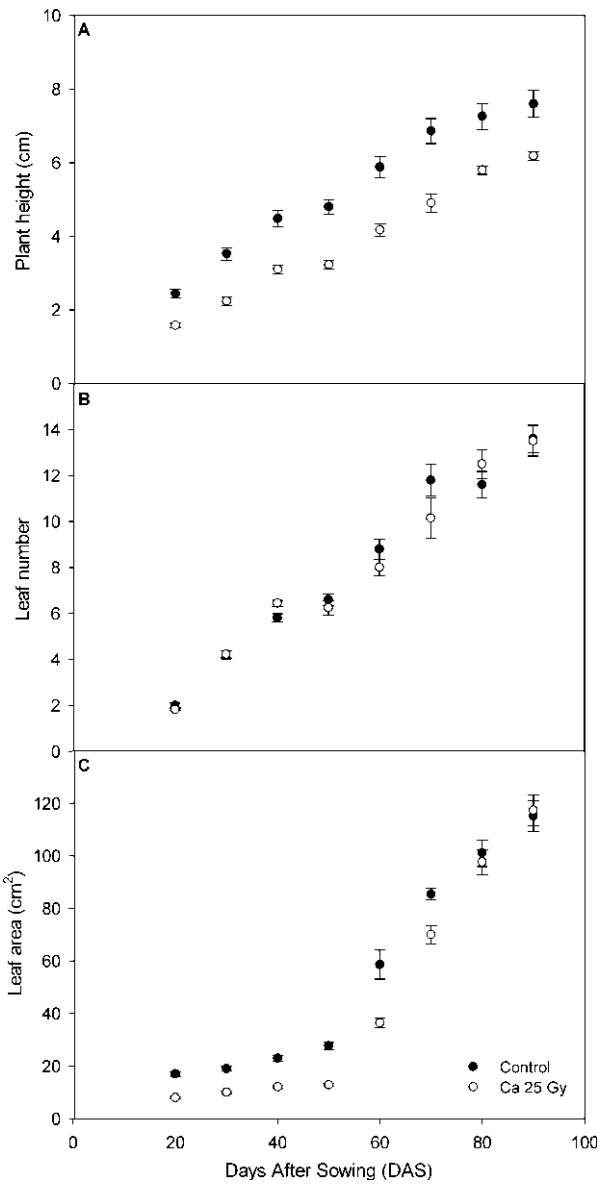

Fig. 1. Plant height (A), number of leaves (B) and leaf area (C) of *Solanum lycopersicum* L. 'Microtom' plants grown from Ca ion-irradiated and control seeds. Mean values ± SE. Different letters indicate significant differences ($P < 0.05$).

Table 1. Number of flowers and fruits, fruit size and plant dry biomass in *Solanum lycopersicum* L. 'Microtom' plants grown from irradiated (Ca 25 Gy) and control seeds. Mean values (n = 5) ± SE. Different letters indicate significant differences ($P < 0.05$)

	control	Ca 25 Gy
Flower (*n*)	16.20 ± 0.84^a	8.00 ± 0.34^b
Fruit (*n*)	23.00 ± 1.00^a	11.00 ± 0.52^b
Fruit diameter (cm)	0.156 ± 0.011^a	0.242 ± 0.017^b
Dry biomass (g)	1.90 ± 0.07^a	1.00 ± 0.04^b

Irradiation did not induce structural alterations in leaves from controls, and these were characterised by a typical dorsiventral structure (Fig. 2B). The whole lamina, as well as palisade and spongy tissues, did not show significant differences in terms of thickness (Table 2). In the two plant groups (C and I), the percentage of intercellular spaces was similar (Table 2). However, the presence of significantly smaller cells in all leaf tissues ($P < 0.05$), except spongy parenchyma, in I plants compared to C plants (Table 3) suggests that leaves of plants from irradiated seeds are made up of a higher number of smaller cells. As regards elongation (aspect ratio) and turgidity (convexity), irradiation did not trigger an unequivocal response in the different tissues (Table 3). Trichomes and stomata were homogeneously distributed along the lamina, without significant differences between C and I plants in both the upper and lower epidermis (Table 2). The number of chloroplasts was significantly reduced by irradiation ($P < 0.05$) only in the palisade parenchyma (Table 2). Leaves from I plants had a significantly higher number of calcium oxalate crystals in the mesophyll as compared to the C plants ($P < 0.05$; Table 2, Fig. 2).

Calcium ion effects on PSII photochemistry and photosynthetic proteins

The I plants had values of Φ_{PSII}, ETR and F_v/F_m that were significantly higher ($P < 0.05$) than those of C plants (Fig. 3A–C). In contrast, Ca irradiation caused a significant decrease in NPQ in I compared to C plants (Fig. 3C). A significant increase in total chlorophylls and carotenoids was found in leaves of I compared to C plants ($P < 0.05$; Table 4). Western blot (Fig. 4A) and densitometric analysis showed a significant rise ($P < 0.05$) in D1 (PsbA) protein level in I compared to C plants (Fig. 4B), but there were no differences in Rubisco amount between the two treatments (Fig. 4C).

Leaf and fruit antioxidant response

The AsA content and CAT activity were not significantly different in leaves from the two plant groups, while SOD activity was significantly higher ($P < 0.05$) in leaves from I than C plants (Table 4). The same analyses performed on fruits showed a significant increase in AsA content ($P < 0.05$) and SOD activity in fruits of I plants compared to controls, but no significant differences in CAT activity (Table 5). I plants also had a significantly higher ($P < 0.05$) content of total carotenoids ($x + c$) and anthocyanins compared to C plants (Table 5). No differences in total polyphenol concentrations were found between the two treatments (Table 5).

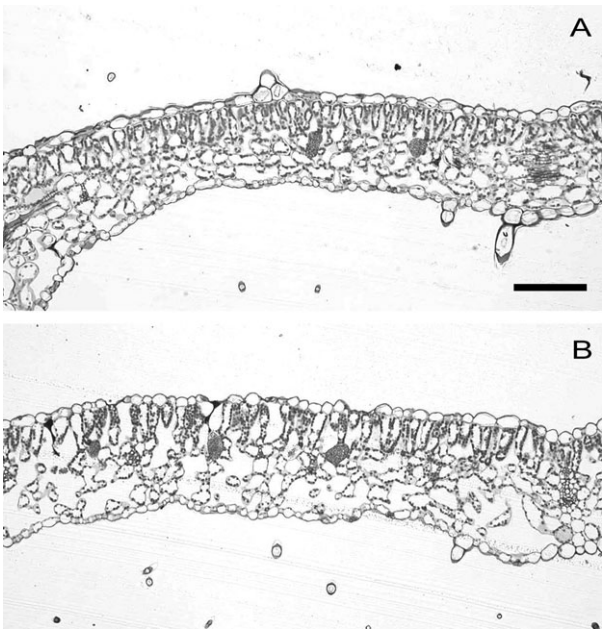

Fig. 2. Transmitted light microscopy views of cross-sections of leaf lamina of *Solanum lycopersicum* L. 'Microtom' plants grown from non-irradiated control seeds (A) and Ca ion-irradiated seeds (B). The images are at the same magnification. Bar = 100 μm.

Table 2. Anatomical functional traits of leaves of *Solanum lycopersicum* L. 'Microtom' plants grown from irradiated (Ca 25 Gy) and control seeds. Mean values ± SE. Different letters indicate significant differences ($P < 0.05$)

	control	Ca 25 Gy
Tickness (μm)		
Lamina	162.76 ± 3.22^a	158.36 ± 4.25^a
Palisade parenchyma	55.79 ± 1.28^a	54.46 ± 1.20^a
Spongy parenchyma	79.15 ± 2.51^a	76.09 ± 3.25^a
Intracellular spaces (%)	54.8 ± 1.38^a	54.4 ± 1.90^a
Number of stomata ($n \cdot mm^{-1}$)		
Upper epidermis	1.96 ± 0.31^a	1.96 ± 0.34^a
Lower epidermis	4.54 ± 0.56^a	4.21 ± 0.33^a
Number of trichomes ($n \cdot mm^{-1}$)		
Upper epidermis	0.67 ± 0.25^a	0.34 ± 0.14^a
Lower epidermis	1.54 ± 0.30^a	1.43 ± 0.30^a
Number of chloroplasts ($n \cdot mm^{-2}$)		
Palisade parenchyma	17.33 ± 0.52^a	15.33 ± 0.41^b
Spongy parenchyma	9.89 ± 0.46^a	9.72 ± 0.35^a
Number of crystals ($n\ mm^{-1}$)	3.22 ± 0.42^a	4.54 ± 2.51^b

DISCUSSION

The irradiation of seeds of *S. lycopersicum* 'Microtom' with Ca ions at 25 Gy, although causing an initial slowdown in germination rate and plant growth, did not prevent achievement of the complete life cycle. Following the exposure to ionising radiation, germination rate may be affected, depending on intrinsic species characteristics (Wei *et al.* 1995; Zimmerman *et al.* 1996; Hase *et al.* 1999; Kumagaia *et al.* 2000). In the case of 'Microtom', the dose of 25 Gy of Ca ions was not sufficient to produce cytotoxic effects. Irradiation delivered to dry seeds, which are more resistant than other phenological stages, prevents

oxidative stress. The seed structure and high levels of compounds like melatonin, for instance, could play an important role in the seed antioxidant defences, protecting the endosperm and reproductive tissue from biological and environmental injuries (Manchester *et al.* 2000; Okazaki & Ezura 2009; Stürtz *et al.* 2011). However, the occurrence of some radio-induced effects is unavoidable.

Plants that developed from irradiated seeds were characterised by a reduction in some morphological traits, reproductive parameters and thus yield (*e.g.* plant height, number of flowers and fruits, total biomass), but radiation-triggered beneficial effects were also detected, such as the increase in antioxidant compounds in fruits.

Table 3. Cell area and shape (aspect ratio and convexity) in the different tissues of leaves of *Solanum lycopersicum* L. 'Microtom' plants grown from irradiated (Ca 25 Gy) and control seeds. Mean values ($n = 90$) ± SE. Different letters indicate significant differences ($P < 0.05$)

	control	Ca 25 Gy
Upper epidermis		
Area (μm^2)	317.36 ± 15.10[a]	205.47 ± 8.60[b]
Aspect ratio	1.70 ± 0.05[a]	1.40 ± 0.03[b]
Convexity	1.24 ± 0.01[a]	1.25 ± 0.01[a]
Palisade parenchyma		
Area (μm^2)	678.70 ± 24.18[a]	589.47 ± 17.88[b]
Aspect ratio	3.28 ± 0.05[a]	3.27 ± 0.07[a]
Convexity	1.16 ± 0.01[a]	1.19 ± 0.01[b]
Spongy parenchyma		
Area (μm^2)	417.30 ± 25.2[a]	399.45 ± 19.62[a]
Aspect ratio	1.54 ± 0.03[a]	1.69 ± 0.05[b]
Convexity	1.29 ± 0.00[a]	1.26 ± 0.01[b]
Lower epidermis		
Area (μm^2)	159.96 ± 12.8[a]	106.68 ± 9.16[b]
Aspect ratio	1.44 ± 0.04[a]	1.46 ± 0.04[a]
Convexity	1.24 ± 0.01[a]	1.25 ± 0.01[a]

Table 4. Total chlorophyll, carotenoids and ascorbic acid (AsA) content and activity of catalase (CAT) and superoxide dismutase (SOD) in leaves of *Solanum lycopersicum* L. 'Microtom' plants germinated from irradiated (Ca 25 Gy) and control seeds. Mean values ($n = 5$) ± SE. Different letters indicate significant differences ($P < 0.05$)

	control	Ca 25 Gy
Total chlorophylls (mg g^{-1} FW)	3.58 ± 0.23[a]	4.02 ± 0.15[b]
Total carotenoids (mg g^{-1} FW)	0.48 ± 0.02[a]	0.60 ± 0.02[b]
AsA content (ng μl^{-1})	16.70 ± 0.88[a]	15.47 ± 0.18[a]
CAT activity ($\mu mol\ H_2O_2\ min^{-1}\ ml^{-1}$)	85.61 ± 3.99[a]	87.07 ± 1.64[a]
SOD activity (inhibition %)	39.12 ± 0.48[a]	51.06 ± 2.65[b]

As reported by Kiong *et al.* (2008), survival of plants to maturity depends on the nature and extent of chromosomal damage, which increases with increasing doses of radiation, leading to reduced germinability and a decrease in plant growth. However, it cannot be excluded that there will be some species-dependent sensitivity to heavy ion irradiation. In Komai *et al.* (2003), carbon ions did not affect germination rates or flowering in *Spinacia oleracea* plants at doses up to 15 Gy, but a dose of 25 Gy caused morphological aberrations. In our experiment, Ca ion irradiation at 25 Gy caused the formation of a reduced number of flowers, and consequently fruits, compared to controls. However, irradiation still allowed completion of the life cycle. This is crucial for achieving the formation of new seeds for long-term plant cultivation in radiation-exposed cultivation chambers, being the stable production of fresh food to complement crew nutritional needs – one of the challenges for manned space exploration (Giacomelli *et al.* 2012). In 'Microtom', Ca ion irradiation of seeds induced limited stem elongation, and reduced total biomass compared

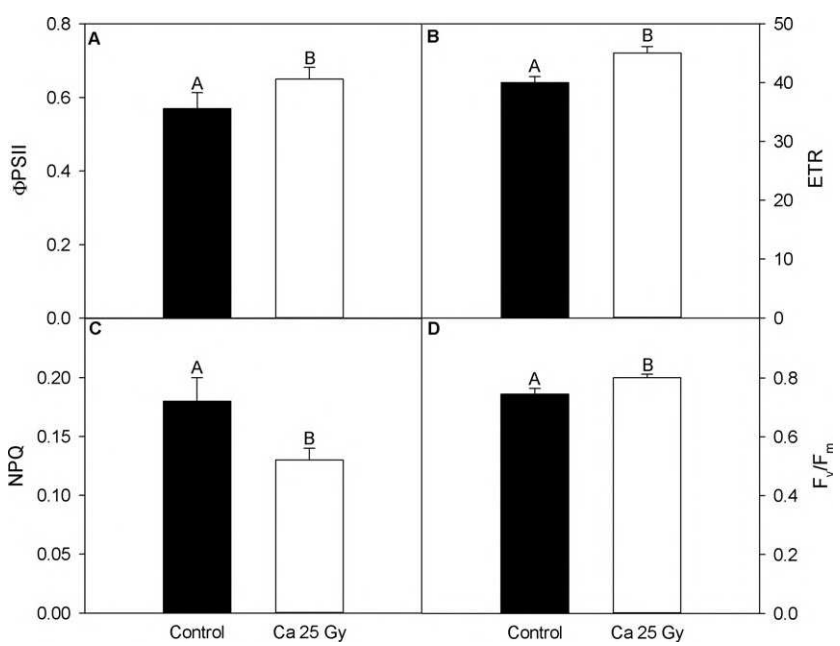

Fig. 3. Quantum yield of PSII electron transport (ΦPSII, A), linear electron transport rate (ETR, B), non-photochemical quenching (NPQ, C) and maximum PSII photochemical efficiency (F_v/F_m, D) in *Solanum lycopersicum* L. 'Microtom' plants grown from irradiated and control seeds. Mean values ± SE. Different letters indicate significant differences ($P < 0.05$).

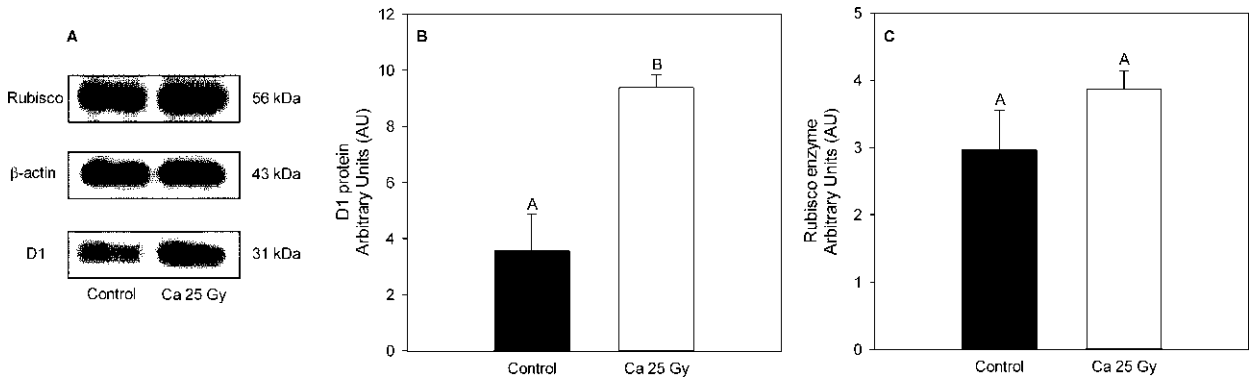

Fig. 4. Western blot (A) and densitometric analysis of D1 protein (B) and Rubisco enzyme (C) in *Solanum lycopersicum* L. 'Microtom' plants grown from irradiated and control seeds. The bar diagrams represent pixel volumes of D1 protein and Rubisco in samples. Each band was normalised to the appropriate β-actin band. Mean values ± SE. Different letters indicate significant differences ($P < 0.05$).

Table 5. Superoxide dismutase (SOD) and catalase (CAT) activity, ascorbic acid (AsA), carotenoids, polyphenols and anthocyanin content of fruits of *Solanum lycopersicum* L. 'Microtom' plants germinated from irradiated (Ca 25 Gy) and control seeds. Mean values ($n = 5$) ± SE. Different letters indicate significant differences ($P < 0.05$).

	control	Ca 25 Gy
Total carotenoids (mg g^{-1} FW)	29.08 ± 4.87^a	59.73 ± 7.96^b
Total polyphenols (mg GAE g^{-1} FW)	0.149 ± 0.007^a	0.147 ± 0.004^a
Total anthocyanins (μg g^{-1} FW)	132.28 ± 9.53^a	170.14 ± 4.96^b
AsA content (ng μl^{-1})	30.44 ± 0.68^a	38.98 ± 1.27^b
CAT activity (μmol H_2O_2 min^{-1} ml^{-1})	92.70 ± 3.99^a	85.12 ± 2.17^a
SOD activity (inhibition rate %)	23.75 ± 1.15^a	35.90 ± 1.78^b

to non-irradiated controls. This is in agreement with the induction of dwarf growth reported as a response to irradiation with high and low LET ionising radiation, also in tomato (Mei *et al.* 1994; Nechitailo *et al.* 2005; De Micco *et al.* 2014b). The more compact size can be considered a positive trait for plants to be grown in reduced volumes in BLSS (Monje *et al.* 2003; De Micco *et al.* 2009). Hence, the irradiation of 'Microtom' seeds would increase the intrinsic dwarf growth of this cultivar, fulfilling one of the most important requirements for plant selection for space cultivation in mission scenarios where the volume availability is a technical constraint (Stutte *et al.* 1999; Paradiso *et al.* 2014).

After 70 DAS, plants from irradiated seeds did not show differences in leaf number, leaf area and most leaf anatomical traits compared to the control, indicating that 25 Gy Ca ions do not induce structural aberrations responsible for lowering physiological performance in 'Microtom' plants. Traits such as stomatal frequency and incidence of intercellular spaces were not affected by irradiation, suggesting that no structure-mediated alterations in resistance to gas exchange are triggered by irradiation. However, the radiation-induced formation of smaller cells in the palisade tissue would indicate increased photosynthetic capacity, according to the common paradigms (Wilson & Cooper 1969), which together with higher chlorophyll content, would compensate for the lower number of chloroplasts. Smaller palisade cells were also found in leaves of 'Microtom' plants grown from seeds irradiated with specific doses of X-rays, which, however, did not induce the same trends and extent of variations in the other tissues (De Micco *et al.* 2014b).

The lack of unique radiation-induced trends in terms of variations in size and shape of cells in different tissues of leaves from plants grown from seed irradiated with low-LET ionising radiation is thus confirmed for irradiation with Ca ions. The differences in plasticity of anatomical traits in the different tissues may depend on several factors, including the degree of cell differentiation, tissue age as well as specific mechanisms for tissue-autonomous developmental control (Kovács & Keresztes 2002; Zaka *et al.* 2002; Kozuka *et al.* 2011). In leaves of 'Microtom' plants grown from irradiated seed, an increased amount of calcium oxalate crystals was observed in the mesophyll. These crystals are widespread among plants species and have several roles, including in regulation of the calcium pool (Nakata 2003), plant protection against herbivores (Franceschi & Nakata 2005), detoxification from oxalate and/or heavy metals and light reflection (Webb 1999; Franceschi & Nakata 2005; Nakata 2012; He *et al.* 2014).

Several studies have demonstrated that ascorbate degradation could be the first source of oxalate required for the formation of crystals, where it represents the organic part that binds Ca ions in many species, including tomato (Loewus 1999; Franceschi & Nakata 2005; Tooulakou *et al.* 2016; Truffault *et al.* 2017). It has been hypothesised that crystals of calcium oxalate produced under drought conditions may act as a dynamic carbon pool because of the degradation of oxalate in H_2O_2 and CO_2 (Tooulakou *et al.* 2016). It has been demonstrated that Ca at toxic concentrations within cells is incorporated in calcium oxalate crystals (Franceschi 1989; Mazen *et al.* 2004). Consistent with previous studies, we consider that the increase in number of crystals in 'Microtom' plants from irradiated seeds may represent a defence mechanism adopted by the leaves to ameliorate the stress conditions induced by Ca ion irradiation.

Plants of 'Microtom' seemed to efficiently face the radiation-induced stress through adjustments at the physiological level. Indeed, the significant increase found in total chlorophylls and carotenoids in leaves of plants from seeds irradiated with Ca ions is in agreement with several studies reporting an increase in pigment content associated with a stimulation in photosynthesis after exposure to gamma rays (Kim *et al.* 2004; Alikamanoğlu *et al.* 2007; Marcu *et al.* 2013). Changes in photosynthetic pigment content and composition may be due

to adjustments within PSII complexes (Kim *et al.* 2004), supporting the hypothesis that the rise in photosynthetic pigments, induced by Ca ions, may promote the PSII light harvesting capacity and activity. This assumption is supported by the significant increase in F_v/F_m, Φ_{PSII} and ETR values in plants grown from irradiated seeds compared to controls.

The better photochemical performance of 'Microtom' plants after seed irradiation is consistent with the higher level of PSII D1 expression. D1 protein synthesis has been reported as negatively affected by ionising radiation in rice plants grown from irradiated seed (Mei *et al.* 1994; Palamine *et al.* 2005). The new synthesis and replacement of labile D1 protein is a primary event in the PSII repair cycle to restore crop productivity (Yokthongwattana & Melis 2006). We hypothesise that the specific dose used in this study, being non-lethal for plants, had a stimulatory effect on D1 protein synthesis and, in turn, on functionality of the photosynthetic apparatus. The higher photosynthetic efficiency in irradiated plants, compared to controls, suggests that the chloroplast functionality was not affected by possible oxidative stress. This could be due either to occurrence of the safety action of plant enzymatic and non-enzymatic scavenger machinery against free radicals (Zaka *et al.* 2002) or the high radio-resistance of this species, making the specific dose of Ca ions used in this experiment ineffective. The stability of chloroplasts in 'Microtom' plants grown from irradiated seed is also confirmed by the lack of differences in Rubisco expression in comparison with control plants. The Rubisco protein is very sensitive to ROS injury, being degraded through radio-induced H_2O_2 accumulation (He *et al.* 2004).

As consequence of seed exposure to the Ca 25 Gy dose, 'Microtom' leaves showed high SOD activity, but no variations in CAT activity and ascorbate levels compared to not-irradiated seed. Based on these results, we hypothesise that the increase in SOD activity is likely sufficient to maintain the ROS concentration at levels that are not dangerous to the cell but can be useful for signalling purposes. Indeed, low levels of ROS may act as a signal for activation of stress response and defence mechanisms (Donahue *et al.* 1997; Knight & Knight 2001). The studies of Zaka *et al.* (2002) conducted on plants grown in radiation-contaminated areas of Kazakhstan after the Chernobyl disaster, demonstrated that exposure to low chronic doses of ionising radiation enhanced the activity of some antioxidant enzymes, especially SOD, minimising the harmful effect of ionising radiation and inducing plant radio-resistance.

The irradiation of 'Microtom' seeds strongly affected fruit characteristics: even if no changes in CAT activity were detected, higher values of SOD activity and a larger amount of ascorbic acid, chlorophylls, carotenoids and anthocyanins were induced by Ca ion irradiation. The intake of food rich in antioxidant molecules (*i.e.* carotenoids, ascorbic acid, anthocyanins) may reduce the biological damage caused by chronic exposure of astronauts to space radiation. Hence, the focus of radioprotection studies has shifted to testing the radioprotective potential of plants and herbal products as a supplement to the human diet (Waldren *et al.* 2004; Jagentia 2007). Some antioxidants, such as SOD and A, C and E vitamins, have demonstrated protective roles against hematopoietic syndrome fatalities (Weiss & Kumar 1988; Weiss *et al.* 1995). The modulation of endogenous antioxidants, such as SOD, may be useful in specific radiotherapy protocols (Weiss & Landauer 2000, 2003), whereas ascorbic acid (vitamin C) reduces the frequency of mutations in human–hamster hybrid cells and mice cells exposed to high-LET carbon ions (Sarma & Kesavan 1993; Konopacka *et al.* 1998; Waldren *et al.* 2004).

Other studies suggest that carotenoids may also regulate DNA repair processes (Cooke *et al.* 1998; Fillion *et al.* 1998; Torbergsen & Collins 2000) and reduce the level of lipid peroxidation (Ben-Amotz *et al.* 1998). Phenolic compounds also have a key role in radioprotection (Emerit *et al.* 1997; Arora *et al.* 2005). Anthocyanins, present in various fruits and vegetables, especially edible berries, have several biomedical functions: lipid peroxidation prevention, DNA integrity protection, cardiovascular disorder prevention and inflammatory response mediators (Zafra-Stone *et al.* 2007).

In conclusion, the overall results suggest that low doses of ionising radiation delivered at the seed stage do not impair plant growth while stimulating the production of specific functional compounds in 'Microtom' fruits, namely carotenoids, ascorbic acid and anthocyanins. The high radio-resistance of tomato 'Microtom', together with the stimulatory effect of low doses of Ca ions on the content of compounds useful in the human diet in fruits make this cultivar particularly interesting for cultivation in BLSS in space. The enrichment of astronauts' diet with such fruits would act as a food countermeasure to counteract the risk of oxidative damage caused by the chronic exposure to cosmic radiation during long duration missions (Halliwell 1996; Arora *et al.* 2005; Maurya *et al.* 2006) in absence of re-supply from Earth.

The results of this study represent a starting point for further investigations aiming to verify the potential of heavy ions in promoting favourable traits in *Solanum lycopersicum* and other plant crop species suitable for growing as food in space. However, despite much progress in this field, to define a standard behaviour on the effect of ionising radiation on plants still remains a challenge. A strong degree of uncertainty resides in the radiation quality, delivered dose and species. Moreover, further studies focusing on the effect of ionising radiation on plant metabolism and on more sensitive target tissues, such as meristems, are desirable to explore the possibility that the constraints of ionising radiation may be harnessed as a benefit for crop production in space.

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Chapter VI – Light spectral composition influences structural and eco-physiological traits of *Solanum lycopersicum* L. cv. 'Micro-tom' in response to high-LET ionizing radiation.

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Light spectral composition influences structural and eco-physiological traits of *Solanum lycopersicum* L. cv. 'Microtom' in response to high-LET ionizing radiation

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Abstract: This study evaluated if specific light quality (LQ) regimes (white fluorescent, FL; full-spectrum, FS; red-blue, RB) during plant growth modifies morphological and photosynthetic traits of *Solanum lycopersicum* L. 'Microtom' plants irradiated at the dry seed stage with 25 Gy ^{48}Ca ions (IR). The irradiation reduced plant size while increased leaf dry matter (LDMC) and relative water content (RWC) compared to control. FS and RB light regimes determined a decrease in plant height and a rise of RWC compared to FL plants. The irradiation under FS, and RB regimes favoured the development of dwarf plants and improved the leaf water status. Under the FL regime, irradiated plants showed reduced photosynthesis and stomatal conductance. The opposite behavior was observed in RB irradiated plants in which gas exchanges were significantly stimulated. RB regime enhanced Rubisco expression in irradiated plants also inducing anatomical and functional adjustments (i.e., increase of leaf thickness and incidence of intercellular spaces). Finally, ^{48}Ca ions did not prevent fruit ripening and the achievement of the 'seed-to seed' cycle, irrespective of the LQ regime. Overall, the present study evidenced that RB light regime was the most effective in optimising growth and photosynthetic efficiency of 'Microtom' irradiated plants. These outcomes may help to develop proper cultivation protocols for the growth of dwarf tomato in Controlled Ecological Life Support Systems (CELSS).

Keywords: indoor cultivation, heavy ions, light quality, photosynthesis, Rubisco expression

1. Introduction

The cultivation of crops in controlled conditions guarantees food production throughout the year, overcoming the limitations of environmental constraints. In the last years innovative lighting sources, based on light-emitting diodes (LEDs), have been more

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and more used in plant cultivation both to enhance plant photosynthesis and to regulate photomorphogenic responses, including bioactive compounds synthesis [1,2].

The manipulation of the light spectrum is a wide applied methodology to reach specific goals in plant cultivation, since different wavelengths may act on both structural and functional traits. However, it is not easy to extrapolate a common behavior because the extent of these responses is species-specific. Generally, the red (R) and blue (B) wavelengths are the most efficiently for photosynthesis, contributing to the whole plant carbon gain, and drive morphogenesis from the early stages [3]. The B wavelength perceived by plants through cryptochromes controls many processes such as stem elongation, phototropism, chloroplast movement within cells, stomatal opening, and elicits the biosynthesis of secondary metabolites such as flavonoids [4,5]. The R light plays the most important role in the development of photosynthetic apparatus and influences morphogenesis by light-induced transformations in the phytochrome system. However, monochromatic R or B lights do not satisfy normal plant growth requirements and the absence of one of the two wavelengths produces photosynthetic inefficiencies: only a suitable proportion of these two components of the light spectrum allows an optimal plant growth and development. For instance, percentages of B wavelengths greater than R often result in lower photosynthetic rates and, therefore, a lower carbon investment in biomass production whereas the lack of B results in abnormal growth and dysfunction of photosynthetic process [3].

In the last decades, many studies are focused on finding new technologies or growth protocols to improve plant production and food quality under controlled conditions. The use of LED technology in the building of an optimal light environment for indoor plant growth, by selecting specific wavelengths to elicit plant photomorphogenic, biochemical or physiological responses, is an effective solution to achieve the target of improving crop yield and quality [6]. This technology is promising for applications in vertical farming industry and to support off-grid agriculture. A futuristic application regards crop production under controlled conditions in extreme environments such as Space, where LED technology would favor efficient plant growth in Controlled Ecological Life Support Systems (CELSS). In the sight of long-duration Space exploration missions, plants in CELSS would be crucial to perform all the roles they carry out on Earth for food production and resource regeneration (i.e., carbon dioxide removal, oxygen production, water recovery, and waste recycling).

However, in Space, high doses of ionizing radiation (IR) may endanger plant growth and photosynthesis [7]. Generally, the manned Space outposts are shielded against IR and only low not lethal doses reach the indoor environment. Thus, in a hypothetical cultivation of plants in Space, it may be supposed that plants will be exposed to low doses of IR, which, as for light quality, is used by plant breeders to improve some specific traits in plants. Plants irradiated with carbon, oxygen, argon, or neon ions at low doses have shown valuable responses in terms of early maturity, high yield, better fruit quality [8-14]. Recently, Arena and colleagues [15] have demonstrated, for the first time, the effectiveness of ^{48}Ca ion, in improving the development, eco-physiological and morpho-functional traits of leaves and fruits of tomato plants. The authors showed an improved photosynthetic efficiency in plants derived from irradiated seeds with 25 Gy ^{48}Ca ions. Indeed, through photosynthesis, the plant produces photoassimilates for vegetative and reproductive organs, while the morpho-anatomical and biochemical traits contribute to define the photosynthetic capacity of the leaves and also promote a greater investment of carbon in the reproductive organs with a consequent increase in fruit yield.

In this paper we assessed through a multidisciplinary approach, if the interplay between specific light quality regimes during plant growth may enhance in *Solanum lycopersicum* L. 'Microtom' plants, the positive outcomes obtained by seed exposure to low doses of Ca heavy ions in terms of morphological and functional traits with a specific focus on the photosynthetic apparatus.

2. Results

2.1 Effect of ionizing radiation and light quality on plant growth and leaf functional traits

Seed irradiation with Ca ions at a dose of 25 Gy did not affect the germination percentage that reached 100% in both control and irradiated plants. All plants completed the growth life cycle producing ripe fruits at 120 DAS.

Plant morphological traits were significantly affected by irradiation (IR) and light quality (LQ) as main factors, as well as by their interaction (IR x LQ) (Figure 1, Table 1).

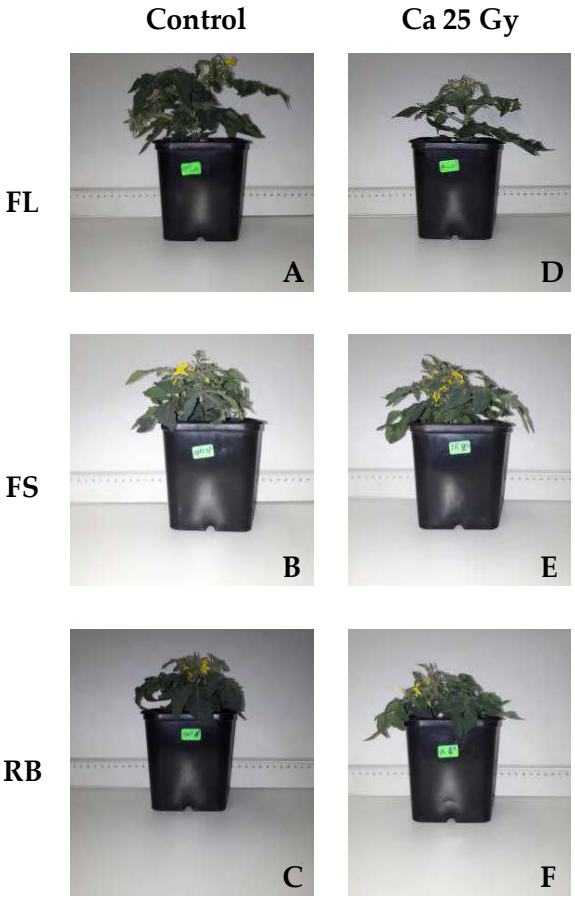

Figure 1: Plants of *S. lycopersicum* L. 'Microtom' from control (A-C) and irradiated seeds at Ca 25 Gy (D-F) and grown under FL, FS, and RB light quality regimes.

Table 1: Analysis of variance and means comparison for morphological traits of 'Microtom' plants in response to ionizing radiation (IR) (Ca 25 Gy), light quality (LQ) regimes (FL, FS and RB), and 6 different combinations of IR x LQ. Different letters within each column indicate significant differences according to Student-Newman-Keuls multiple comparison test ($p<0.05$). NS-not significant; *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

	Height	TLA	Leaves	Flowers	Fruits
IR					
C	7.1 a	304 a	10 b	57 a	24 a
IR	5.5 b	271 b	11 a	51 b	23 a
LQ					
FL	7.7 a	323 a	11 a	50 b	25 a
FS	5.7 b	276 b	11 a	50 b	26 a
RB	5.4 b	263 b	11 a	62 a	20 b
Interaction					
CxFL	9.0 a	343 a	11 b	66 a	26 a
CxFS	6.2 b	288 bc	11 b	49 b	27 a
CxRB	6.0 b	281 bc	9.5 b	55 b	19 a
IRxFL	6.4 b	303 b	11 b	35 c	24 a
IRxFS	5.2 c	264 cd	11 b	50 b	25 a
IRxRB	4.9 c	245 d	13 a	68 a	21 a
Significance					
IR	***	***	*	*	NS
LQ	***	***	NS	***	**
IR x LQ	*	*	***	***	NS

Height: plant height (cm); TLA: total plant leaf area (cm^2); Leaves: number of leaves per plant (n $plant^{-1}$); Flowers: number of flowers per plant (n $plant^{-1}$); Fruits: number of fruits per plants (n $plant^{-1}$).

Irradiated plants showed a higher ($p<0.05$) number of leaves, but a reduced ($p<0.05$) number of flowers, total leaf area and plant height compared to control. The growth under FS and RB regimes determined a reduction ($p<0.001$) in plant height and total leaf area compared to FL, irrespectively from irradiation. RB plants also showed the highest ($p<0.001$) flower number and the lowest ($p<0.01$) fruit number (Table 1) compared to FL an FS light regimes.

The interaction IR x LQ was significant for all growth traits except fruit number (Table1). Plant height was significantly lower in irradiated plants under FS and RB regimes compared to all the other conditions, with maximum value in C x FL plants. Irradiated plants under FS and RB showed lower TLA than under FL regime, while C x FL plants exhibited the highest TLA among all treatments. In IR x RB plants, the number of leaves was higher than all the other treatments, while the number of flowers was comparable to C x FL plants and significantly higher compared to the other conditions.

IR significantly influenced leaf functional traits with exception of specific leaf area (SLA), while LQ had a significant effect only on leaf area (LA). The interaction IR x LQ was significant for LA and relative water content (RWC) (Table 2).

Table 2: Analysis of variance and means comparison for functional and anatomical traits of 'Microtom' plants in response to ionizing radiation (IR) (Ca 25 Gy), light quality (LQ) regimes (FL, FS and RB) and 6 different combinations of IR x LQ. Different letters within each column indicate significant differences according to Student-Newman-Keuls multiple comparison test ($p<0.05$). NS-not significant; *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

	Functional traits				Anatomical traits				
	LA	SLA	LDMC	RWC	LT	PT	ST	IS	SD
IR									
C	9.4 b	210 a	0.11 b	60 b	231 a	99 a	100 a	31 a	215 a
IR	13 a	219 a	0.12 a	66 a	228 a	94 a	97 a	33 a	221 a
LQ									
FL	13 a	212 a	0.12 a	54 b	188 c	74 c	81 c	24 c	213 a
FS	10 b	217 a	0.11 a	66 a	221 b	92 b	94 b	32 b	222 a
RB	9.8 b	214 a	0.11 a	69 a	280 a	123 a	120 a	40 a	217 a
Interaction									
CxFL	9.6 b	219 a	0.11 a	55 d	200 bc	80 bc	86 bc	28 b	181 a
CxFS	9.1 b	203 a	0.11 a	62 c	214 b	92 b	92 b	30 b	234 a
CxRB	9.5 b	207 a	0.11 a	62 c	280 a	125 a	120 a	36 b	229 a
IRxFL	17 a	205 a	0.12 a	53 d	175 c	68 c	76 c	21 c	246 a
IRxFS	12 b	231 a	0.11 a	69 b	228 b	93 b	96 b	33 b	210 a
IRxRB	10 b	222 a	0.12 a	75 a	280 a	121 a	119 a	48 a	206 a
Significance									
IR	***	NS	*	**	NS	NS	NS	NS	NS
LQ	***	NS	NS	NS	***	***	***	***	NS
IR x LQ	***	NS	NS	*	NS	NS	NS	*	NS

LA: Leaf area (cm^2); SLA: Specific leaf area ($cm^2\ g^{-1}$); LDMC: Leaf dry matter content ($g\ g^{-1}$); RWC: Relative water content (%); LT: lamina thickness (μm); PT: palisade thickness (μm); ST: spongy thickness (μm); IS: intercellular spaces (%); SD: Stomata density ($n\ mm^{-2}$).

IR determined the development of leaves characterized by higher ($p<0.001$) LA, LDMC and RWC ($p<0.01$) compared to control under all light quality regimes (Table 2). Considering LQ as main factor, FL plants showed higher and lower values ($p<0.001$) of LA and RWC, respectively, compared to FS and RB. The interactions IR x LQ showed that IRxFL plants were characterized by the highest value of LA, while IR x RB plants exhibited the highest RWC among different IR x LQ combinations.

The light quality regimes and the irradiation treatment did not determine any qualitative change in leaf anatomical features of 'Microtom', with leaves showing the distribution of tissues typical of the dorsiventral structure (Figure 2).

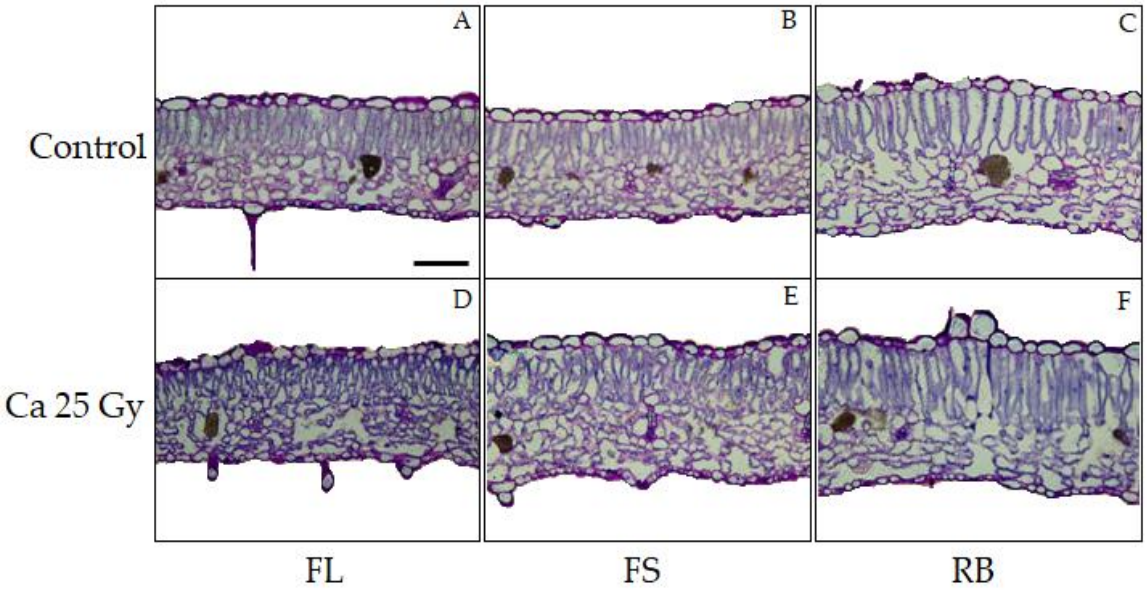

Figure 2: Light microscopy views of cross sections of leaf lamina of *S.lycopersicum* L. 'Microtom' in control (A-C) and irradiated (Ca 25 Gy) (D-F) plants grown under FL, FS and RB light regimes. Images are at the same magnification; scale bar = 100 μm.

IR as main effect did not induce alterations in the analyzed quantitative anatomical traits compared to control (Table 2). Conversely, LQ significantly influenced leaf anatomical traits except for stomata density. In particular, plants grown under RB regime showed leaves with higher incidence of intercellular spaces (IS) ($p<0.01$) and thicker palisade and spongy tissues compared to FS plants which in turn showed significantly higher values of such parameters than FLs (Table 2, Figure 2).

The interaction IR x LQ was significant only for IS (Table 2). In particular, IRxRB and IRxFL plants showed respectively higher and lower IS values compared to plants grown in the other conditions (Table 2).

2.2 Effect of ionizing radiation and light quality on photosynthetic gas exchanges

The photosynthetic light response curves of 'Microtom' plants are shown in Figure 3.

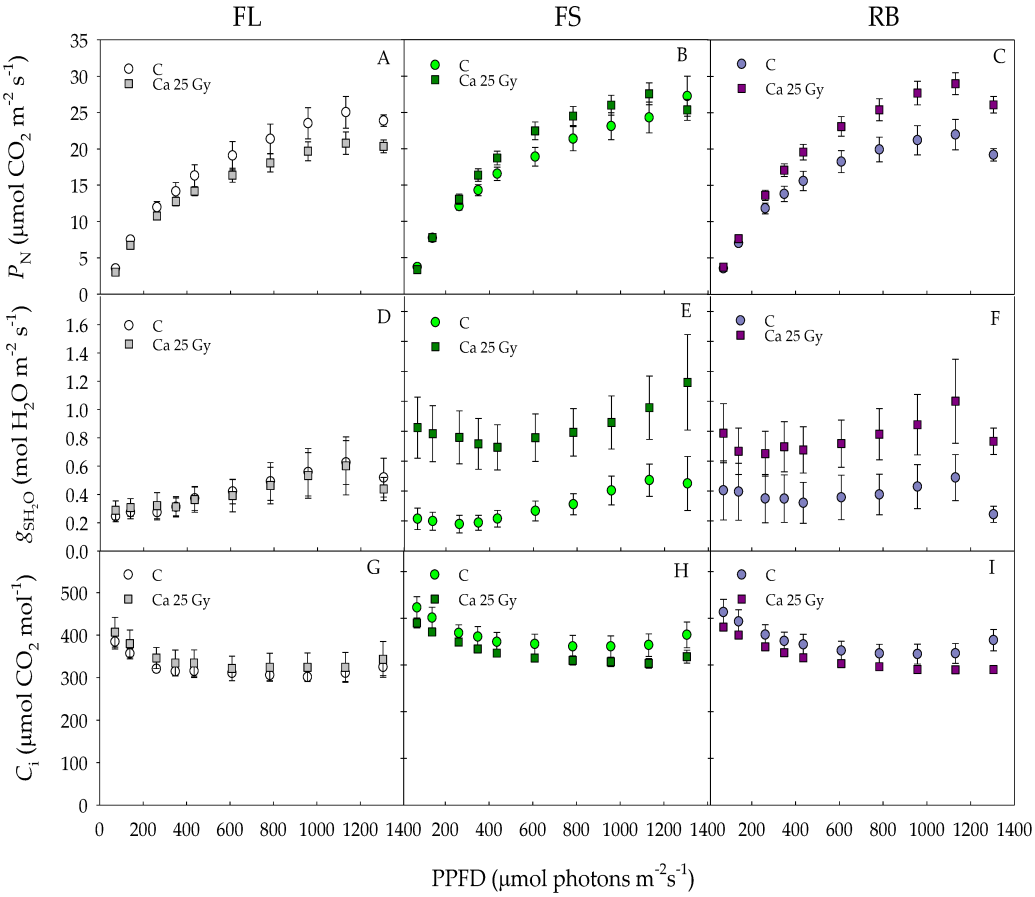

Figure 3: Net photosynthetic rate (P_N) (A-C), stomatal conductance (g_s) (D-F), intercellular CO_2 concentration (C_i) (G-I) of *S. lycopersicum* L. 'Microtom' in control (C) and irradiated (Ca 25 Gy) plants grown under FL, FS and RB light regimes. Data are reported as mean values ± standard error (SE).

Photosynthetic rates (P_N) were similar in control and Ca-ion irradiated plants up to about 400 PPFD in all light regimes (Figure 3A, B, C). Above this value, P_N values increased in control more than Ca-irradiated plants when grown under FL light, while P_N values were significantly higher in Ca-irradiated than control plants under FS and RB regimes.

IR increased stomatal conductance in FS and RB plants compared to not irradiated control while it had no significant effect under FL regime (Figure 3 D, E, F). Intercellular CO_2 concentration (C_i) was not significantly influenced by irradiation in FL conditions, while determined a higher increase in control than Ca-irradiated FS and RB plants leading to significant differences at values of PPFD higher than 1200 (Figure 3 G, H, I).

Table 3 showed the effect of IR, LQ and IR x LQ on light response curve derived parameters.

Table 3: Analysis of variance and means comparison for light response curve derived parameters of 'Microtom' plants in response to ionizing radiation (IR) (Ca 25 Gy), light quality (LQ) regimes (FL, FS and RB) and 6 different combinations of IR x LQ. Different letters within each column indicate significant differences according to Student-Newman-Keuls multiple comparison test ($p<0.05$). NS-not significant; *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

	P_{Nsat}	I_{sat}	Φ_{CO2}
IR			
C	23 a	508 a	0.047a
IR	25 a	518 a	0.050a
LQ			
FL	23 b	525 a	0.044b
FS	26 a	517 a	0.051a
RB	25 a	497 a	0.052a
Interaction			
CxFL	25 b	558 a	0.045 b
CxFS	24 b	509 a	0.048 a
CxRB	22 c	457 a	0.049 a
IRxFL	20 c	492 a	0.042 b
IRxFS	27 a	524 a	0.053 a
IRxRB	29 a	537 a	0.055 a
Significance			
IR	NS	NS	NS
LQ	*	NS	**
IR x LQ	***	NS	*

P_{Nsat}: light saturated CO_2 uptake (μmol CO_2 $m^{-2}s^{-1}$); I_{sat}: light saturation point (μmol photons $m^{-2}s^{-1}$); Φ_{CO2}: quantum yield of photosynthesis (μmol CO_2 $mmol^{-1}$ photons).

IR did not significantly influence P_{Nsat}, I_{sat} or Φ_{CO2}. Conversely, LQ significantly influenced both P_{Nsat} and Φ_{CO2} which resulted higher ($p<0.01$) in FS and RB than FL plants. The interaction IR x LQ was significant only for P_{Nsat} and Φ_{CO2}. In particular, higher values were reached in IR x FS and IR x RB plants compared to the other treatments.

2.3 Effect of ionizing radiation and light quality on biochemical compounds, proteins and Rubisco

The total antioxidant capacity was influenced by LQ as main factor and by the interaction IR x LQ, while polyphenol and flavonoid contents were significantly influenced only by IR and LQ as main factors (Table 5).

Table 5: Analysis of variance and means comparison for total antioxidant capacity, polyphenol, flavonoid, protein content and Rubisco amount of 'Microtom' plants in response to ionizing radiation (IR) (Ca 25 Gy), light quality (LQ) regimes (FL, FS and RB) and 6 different combinations of IR x LQ. Different letters within each column indicate significant differences according to Student-Newman-Keuls multiple comparison test ($p<0.05$). NS-not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

	TAC	TPC	FLAV	PROT	RUB
IR					
C	2.9 a	0.92 a	20 a	0.30 b	9.7 b
IR	2.8 a	0.83 b	18 b	0.41 a	15a
LQ					
FL	2.6 b	0.83 b	18 b	0.27 c	14 a
FS	3.0 a	0.87 b	19 b	0.34 b	10 b
RB	2.9a	0.92 a	20 a	0.45 a	13 a
Interaction					
CxFL	2.6 b	0.86 bc	19 bc	0.31 c	13 b
CxFS	2.9 ab	0.91 b	20 b	0.28 c	6 d
CxRB	3.1 a	0.99 a	21 a	0.30 c	10 c
IRxFL	2.7 b	0.79 c	17 c	0.23 d	14 b
IRxFS	3.2 a	0.83 bc	18 bc	0.41 b	13 b
IRxRB	2.6 b	0.85 bc	18 bc	0.59 a	17 a
Significance					
IR	NS	***	***	***	***
LQ	***	**	**	***	**
IR x LQ	***	NS	NS	***	*

TAC: total antioxidant capacity (μmol TE g^{-1} FW); TPC: total polyphenol content (mg GAE g^{-1} FW); FLAV: total flavonoids (mg CE g^{-1} FW); PROT: total proteins (μg BSA eq mg^{-1} FW); RUB: Rubisco amount (arbitrary units).

IR itself reduced ($p<0.001$) the content of polyphenols and flavonoids compared to controls (Table 5). Irrespective of IR, the RB and FS regimes determined significantly higher ($p<0.05$) total antioxidant capacity than FL, while the amount of polyphenols and flavonoids was significantly higher ($p<0.01$) in RB than both FS and FL regimes. The interaction IR x LQ showed that the combinations C x RB and IR x FS induced higher antioxidant capacity than other conditions while C x RB promoted the highest polyphenol and flavonoid content compared to all combinations.

Total proteins and Rubisco amount were significantly influenced by IR and LQ as main factors as well as by their interaction IR x LQ (Table 5, Figure 4).

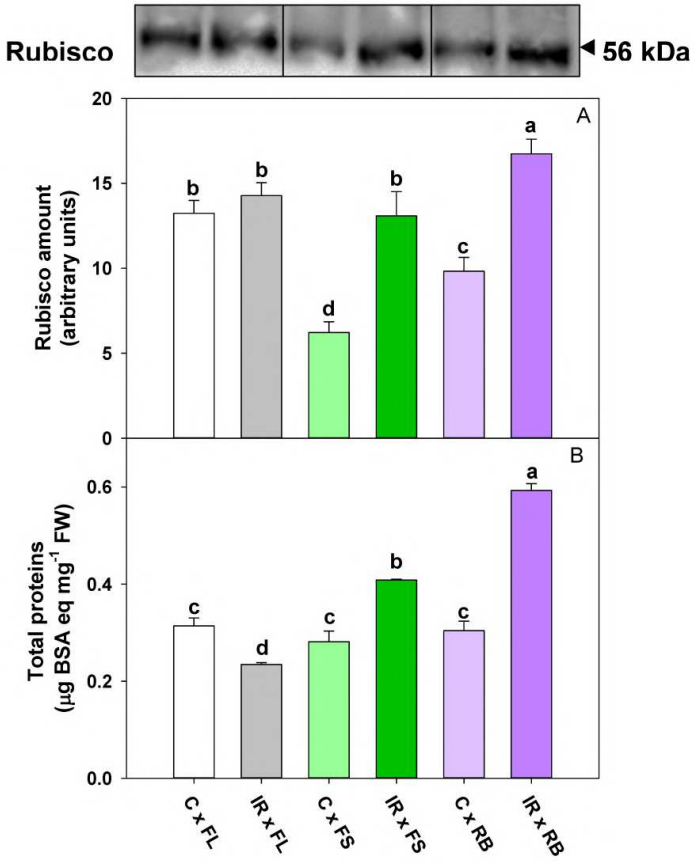

Figure 4: Western blot and densitometric analysis of Rubisco (A), total protein content (B), in leaves of *S.lycopersicum* L. 'Microtom' plants from non-irradiated control (C) and irradiated seeds (IR) (Ca 25 Gy) and grown under FL, FS and RB light regimes. Mean values ± SE. Different letters indicate significant differences according to Student-Newman-Keuls multiple comparison test ($p<0.05$).

In particular, independently of the LQ regime, IR promoted ($p<0.001$) the protein content and Rubisco amount compared to control (Table 5). At the same time, RB showed higher ($p<0.001$) content of proteins than FS plants which in turn had higher values ($p<0.001$) than FLs (Table 5). The Rubisco amount instead was significantly lower ($p<0.01$) in FS than both FL and RB plants (Table 5).

In particular, IR x FL showed a reduced content of total proteins compared to control plants grown under all light regimes which in turn were characterized by lower values than IR x FS and than IR x RB (Table 5, Figure 4B). The amount of Rubisco was comparable among C x FL, IR x FL, IR x FS, which showed significantly lower and higher values than IR x RB and the other two conditions respectively (Table 5, Figure 4A).

2.4 Heatmap analysis

Figure 5 summarizes the morphological and physiological traits of 'Microtom' plants in response to ionizing radiation (IR) (Ca 25 Gy) and light quality (LQ) regimes (FL, FS, RB).

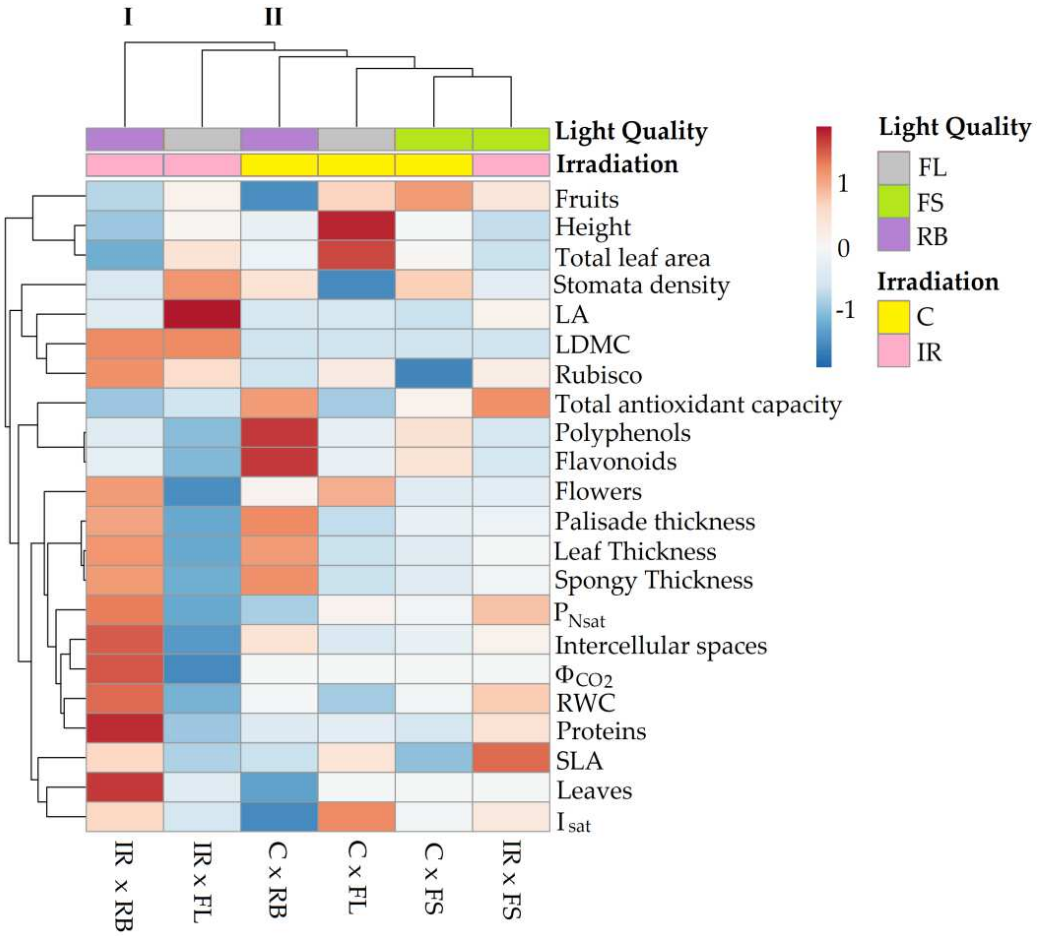

Figure 5: Cluster heatmap analysis of morphological and physiological parameters of 'Microtom' plants in response to ionising radiation (IR) (Ca 25 Gy) and light quality (LQ) regimes (FL, FS, RB). Numeric differences within the data matrix are shown by the colour scale: red and blue indicate increasing and decreasing values, respectively. Parameters are clustered in the rows; sample groups are clustered in the columns by the two independent factors, IR and LQ.

The heatmap established two main clusters. The first cluster (I) included IR x RB plants, while the second (II) incorporated the other treatments.

The interaction C x FL induced higher values of plant height, leaf area, flower number and I*sat*, whereas IR x FL induced greater LA and stomata density.

C x FS plants produced the highest number of fruits, while under IR x FS treatment higher SLA and antioxidant capacity were observed.

C x RB plants were characterised by a rise in polyphenol and flavonoid content. Finally, the separation of IR x RB plants from the other clusters highlighted that the interplay between Ca-ions 25 Gy and RB light regime significantly affected morpho-anatomical and physiological traits of 'Microtom' plants. Indeed, increased RWC, leaf thickness, intercellular spaces, light response curve parameters were observed in IR x RB plants.

3. Discussion

This study showed that the interplay between ionizing radiation (Ca-heavy ions) and light quality regimes elicits specific structural and eco-physiological responses in 'Microtom' plants irradiated at the dry seed target stage, which should be taken into account when designing the cultivation protocols for this species in BLSSs in Space.

The IR represents the principal constraint in the extra-terrestrial environment and may affect plant growth and development at different phenological stages [16]. Exposure

to IR generally compromises the germination rate depending on dose, type of radiation, and plant species [15,17-20]. Our data demonstrate that the irradiation of dry seeds with Ca ions at 25 Gy does not impair the germination process nor prevent the complete life cycle 'from seed- to- seed', confirming previous studies which indicate the dry seed as the most radioresistant stage in Microtom plants [15,16,21]. The intrinsic radioresistance of seeds takes into account not only the anatomical structure but also the presence of compounds such as melatonin, and the scarcity of water which may avoid the occurrence of oxidative stress due to radiolysis phenomena, preserving the endosperm from severe damage [15,22-24].

Tomato plants sprouted from Ca-ion irradiated seeds showed alterations in some morphological traits such as plant height and total leaf area compared to control which determined in irradiated plants a more compact structure and dwarf growth as observed by other authors in several crops [15,25-27]. Possible alterations during cell division may induce dwarf growth [18,19]. The "dwarf effect" was emphasized when irradiated plants were grown under specific light quality regimes, and in particular under FS and RB. The high percentage of blue light in FS and RB light regimes, respectively 37% and 40%, might have further influenced the plant size. Indeed, blue wavelengths are recognized to affect cell division and expansion, resulting in reduced stem elongation and leaf area [28-32]. The interplay of heavy ions and light quality regimes significantly affected flower formation. Plants sprouted from irradiated seeds and grown under the FL regime reduced the flower number compared to control according to previous findings on the same tomato cultivar [15]. This negative effect is overturned under FS and RB light regimes, indicating a positive role exerted by red and blue wavelengths on anthesis. It has been previously demonstrated that the B/R ratio <1.0, as in the present study, would promote flowering during tomato seedling growth [29]. The finding that cultivation of Microtom at specific wavelengths is capable to counteract the negative effect of radiation on flowering is promising for Space cultivation in so far as the completion of the seed-to-seed cycle is essential for fruit production onboard and for the production of new viable seeds for the next generations.

Changes observed in leaf functional traits provide further evidence of the high 'Microtom' responsivity to both IR and light growth environment. Plants developed from Ca-ion irradiated seeds showed higher LDMC which positively affected the RWC compared to control. Ca-ions may have induced a more significant investment of photosynthates towards sclerenchyma tissues, increasing the tissue density and facilitating nutrient and water retention [33,34]. At the same time, the higher RWC in plants grown under FS and RB light regimes may depend on the stimulatory effect exerted by the blue light on the root system [35,36]. We hypothesize that a more significant expansion of roots may have positively influenced the water absorption from the soil and, therefore determined the higher RWC in these plants. The combination IR x RB was particularly effective in promoting RWC, suggesting a more performing use of water resource that may result particularly advantageous, considering that water recycling and saving is essential within the BLSSs.

Qualitative leaf anatomical traits were not altered by irradiation, suggesting that Ca-ions at low doses were insufficient to induce apparent structural modifications [15]. From a quantitative viewpoint, leaf thickness and intercellular spaces were significantly influenced by LQ. More specifically, FS and even more RB light treatments induced the development of thicker leaves characterized by a higher percentage of intercellular spaces. The increase in the incidence of intercellular spaces might be in part responsible for the increase in RWC given that airspaces in the mesophyll are the sites of highest resistance to water flow in leaves [37-39]. The increase in intercellular spaces, associated with the increased RWC, may have significantly reduced water losses and improved the photosynthetic efficiency of irradiated seeds under FS and RB light regimes. IR generally impairs photosynthesis and stomatal conductance, irrespectively from the kind of radiation and

dose [40-44]. Consistent with other studies, the photosynthetic metabolism of Microtom was affected by ionizing radiation.

Moreover, the most novel result is the reversal of the effect of ionizing radiation when plants are grown under different light quality regimes. Under FL regime, irradiated plants decreased photosynthesis compared to control; conversely, an opposite trend was observed under the RB regime. The higher photosynthetic rates can be ascribed to the high stomatal conductance of RB compared to FL plants, due to the stimulation of blue wavelengths on stomatal opening [45-49]. Furthermore, the increased incidence of intercellular spaces (IS%) within the mesophyll, may have also favored the CO_2 diffusion to carboxylation sites, increasing the photosynthetic rate and Rubisco activity. Consistent with high photosynthetic rates, FS and RB irradiated plants exhibited a greater amount of Rubisco than controls. It has been demonstrated that the higher quantity of blue wavelengths, such as those in FS and RB regimes, elicits a positive effect on Rubisco expression and plant photosynthetic capacity [3,50]. Irrespective of the LQ regime, total proteins did not vary among control plants but changed among irradiated ones. The lowest value was found in IR x FL plants, as IR may have induced the proteins' degradation or inhibited their synthesis [51,52]. Conversely, IR x FS and IR x RB plants showed a greater protein content likely due to the positive effect exerted by blue and red wavelengths on the protein synthesis [32,53-55]. It is also reasonable to assume that the increase in Rubisco amount significantly contributed to the total protein rise under these treatments.

The interaction IR x LQ significantly affected also the leaf antioxidant properties. As expected, the highest portion of blue light in the RB regime determined the increase in the total antioxidant capacity, flavonoids and polyphenols [32,36,56], but in combination with ionizing radiation, it induced a reduction in antioxidants. An increase in polyphenols and flavonoids in irradiated plants is expected to counteract the radio-induced oxidative damages [7,21,26,57,58]. In our case, the decrease in antioxidants in irradiated plants may indicate that the dose of Ca 25 Gy is not dangerous for plants.

'Microtom' plants showed no detrimental effect at the dose of IR used in this study. The heatmap clearly separated IR x RB plants from the others, evidencing that if irradiated plants are grown under specific light regimes, such as RB, a beneficial effect in terms of gas exchanges can be obtained. Moreover, the interplay between LQ and IR significantly modulates the tomato plant's morphological responses, further affecting the intrinsic dwarf habitus of this cultivar. Besides high photosynthetic gain, the dwarf growth is one of the most desirable requirements for cultivation in a high plant density condition or slim volumes, such as those available in Space systems [15,26,59,60]. The plant bioactive compounds such as antioxidants, polyphenols and flavonoids decreased in irradiated 'Microtom' plants compared to control, especially in combination with RB growth regime, indicating that these plants do not perceive the dose of ionizing radiation used in our study as a potential stress.

4. Materials and Methods

4.1 Plant material, irradiation procedure and experimental design

Dry seeds of *Solanum lycopersicum* L. cv 'Microtom', provided by Holland Online Vof (Amsterdam, The Netherlands), from the same production batch, were divided into two groups: "control" group and "irradiated" group. For this latter, seeds were irradiated with Ca heavy ions [isotope ^{48}Ca; E: 200 MeV·u-1 (monoenergetic), LET: 180 keV·μm-1; dose rate 1 Gy·min-1] at a dose of 25 Gy. The irradiation procedure was performed at GSI Helmholtzzentrum für Schwerionenforschung (Darmstadt, Germany), using a pencil beam in a spread-out Bragg peak (SOBP), in the heavy-ion synchrotron (SIS). The dose of 25 Gy, under the threshold for occurrence of DNA damage [61], was chosen to not prevent the plant development but rather to obtain a stimulatory effect on plant growth and physiology. Non-irradiated seeds served as a control.

Control and irradiated seeds were sown in 1.2 L pots filled with soil (86% peat, 9% sand, 3% quartz sand, 2% perlite) and stored in the dark until germination. Afterwards,

seedlings were transferred in a growth chamber under three different light quality regimes (5 pots per each light treatment): white fluorescent (FL), full-spectrum (FS), and red plus blue (RB) light. FL was supplied by a combination of fluorescent tubes (Lumilux L36W/640 and L36W/830, Osram, München, Germany), FS was obtained by the combination of far-red, red, yellow, green, blue, UV-A and white light emitting diodes (LEDs), while RB (red 60% - blue 40%) derived from LEDs (LedMarket Ltd., Plovdiv, Bulgaria). The spectral composition of the light regimes was determined by a SR-3000A spectro-radiometer at 10 nm resolution (Macam Photometrics Ltd., Livingston, Scotland, UK) as reported in Figure 6.

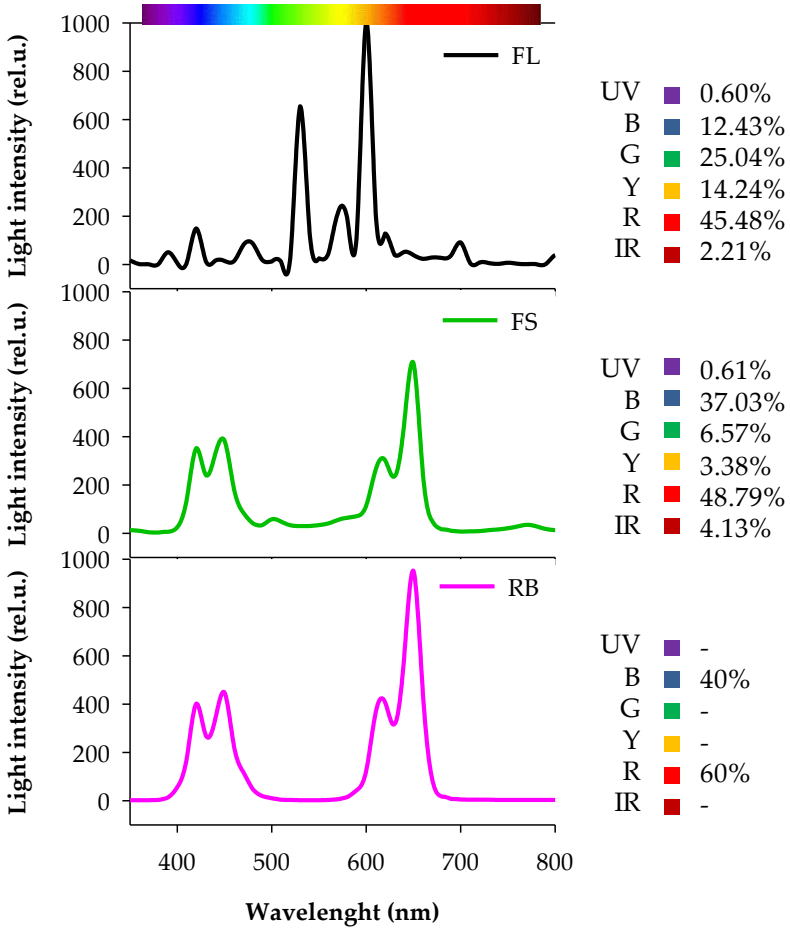

Figure 6: Light spectra used in the experiment, spectral data and energy percentage of different light quality regimes. FL, white fluorescent tubes; FS, full-spectrum, LED; RB, red-blue, LED. Photon flux density: 360 µmol $m^{-2}s^{-1}$. Irradiance Range: 350-800nm. UV, ultra-violet (350-390nm); B, Blue (390-500nm); G, Green (500-560nm); Y, Yellow (560-600nm); R, Red (600-700nm); IR, Far-Red (700-800nm).

In the growth camber, the photosynthetic photon flux density (PPFD) was kept at 360 µmol photons m^{-2} s^{-1} at the top of the canopy for all LQ regimes with photoperiod of 14h. The relative air humidity was 60-70% and day/night air temperature 25/20°C ±2°C. Plants were irrigated weekly to field capacity to reintegrate water lost by evapotranspiration and fertilized weekly with half-strength Hoagland's solution.

Plant growth was followed until 120 days after sowing (DAS) once fruit ripening was completed. Control and irradiated plants, grown under different light quality regimes, were compared in terms of leaf functional and anatomical traits, photosynthetic efficiency, pigment content, biochemical compounds, proteins and Rubisco amount.

All morphological, anatomical and physiological measurements were conducted at 70 DAS, except flower and fruit number, which were monitored from the appearance up to 120 DAS, considering for each plant the sum of flowers and fruits produced and harvested.

4.2 Seed germination, biometrical measurement and leaf functional traits

The germination test was performed on 10 seeds per treatment and repeated three times for a total of 30 control seeds and 30 irradiated seeds.

The germination percentage (G%) was calculated as:

$$G\% = (\text{Number of germinated seeds}/\text{Total number of seeds}) \times 100 \quad (1).$$

The effect of irradiation and light quality on biometric characteristics (plant height, total plant leaf area, leaf, flower, and fruit number) was evaluated on five plants per treatment. The leaf functional traits (leaf area, LA; specific leaf area, SLA; leaf dry matter content, LDMC; relative water content, RWC) were estimated according to Cornelissen et al. [62]. Leaf area was measured by using the Image J software (Image Analysis Software, Rasband, NIH, Bethesda, Maryland, USA) and used to calculate SLA ($cm^2\ g^{-1}$) as the ratio between leaf area to leaf dry mass. LDMC ($g\ g^{-1}$) was estimated as leaf dry mass to saturated fresh mass; RWC was calculated as: (leaf fresh mass – leaf dry mass) /(leaf saturated fresh mass – leaf dry mass). The saturated fresh mass was obtained by submerging the petiole of leaf blades in distilled water for 48h in the dark at 5°C whereas the dry mass was determined after oven-drying leaves at 75°C for 48h.

The total green leaf area per plant was measured acquiring the images by a digital camera and measuring leaf expansion by Image J software (Image Analysis Software, Rasband, NIH, Bethesda, Maryland, USA).

4.3 Anatomical analyses

The leaf anatomical analyses were carried out on three fully expanded leaves collected from three different plants per treatment before flowering. Briefly, segments from the middle leaflets of compound leaves were cut and immediately submerged in the fixative solution FAA (40% formaldehyde/glacial acetic acid/50% ethanol, 5/5/90 v/v/v). Subsamples (5 mm^2) were dissected from the leaflet lamina, flattened on a microscope slide, mounted with water and observed under a transmitting light microscope (BX51; Olympus, Germany) to determine the stomatal density (n mm^{-2}). Leaf lamina thickness, palisade and spongy parenchyma thickness were determined on another group of subsamples (5 mm^2) which were dehydrated in ethanol series (50%, 70%, 95% ethanol) and embedded in the acrylic resin JB-4 (Sigma Aldrich). Cross-sections of 5 µm thickness were obtained by a rotatory microtome and stained with 0.5% toluidine blue in water [63]. The sections were observed under a transmitted light microscope (BX51; Olympus) and images were collected with a digital camera (EP50; Olympus) and analysed with the software Olympus CellSens 2.3.

Leaf lamina, palisade parenchyma and spongy parenchyma thickness, were measured in three positions along the lamina, avoiding veins. The incidence of intercellular spaces in the spongy parenchyma was measured as the percentage of tissue occupied by intercellular spaces over a given surface along the mesophyll.

4.4 Gas exchange measurements

Leaf gas exchanges measurements were performed at 70 DAS by means of a portable leaf gas-exchange system (LCpro+, ADC BioScientific, UK) on ten fully expanded leaves per treatment (two leaves per plant). The apical leaflet was clamped into cuvette and measurements were carried out at leaf temperature of 25±2°C, relative humidity of 50-60% and ambient CO_2 (400 µmol mol^{-1}). Light response curves (LRC) were performed by exposing leaflets to white light ranging from 70 to 1200 µmol $m^{-2}\ s^{-1}$ PPFD. The net CO_2 assimilation (P_N), stomatal conductance (g_s) and intracellular CO_2 concentration (C_i) were

calculated by the software operating the gas-exchange system following the equations of von Caemmerer and Farquhar [64]. In LRC, gas-exchange parameters were recorded after reaching the steady-state (about 5-10 min) for each PPFD step. Parameters derived by light response curves (P_{Nsat}, I_{sat}, and Φ_{CO2}) were calculated following Abe et al. [65].

4.5 Total protein and Rubisco amount

After gas exchange measurements, five leaves (one leaf per plant) per treatment were collected for protein extraction and Rubisco amount determination. The protein extraction was carried out according to Wang et al. [66] using 0.3 g of fresh material for each sample. The extracts were quantified by the Bradford assay [67] (BioRad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Milan, Italy) measuring the absorbance at 595 nm (spectrophotometer UV-VIS Cary 100; Agilent Technologies, Palo Alto, CA, USA) and using the bovine serum albumin (BSA) as standard. SDS-PAGE (10%) was performed following the procedure reported in Arena et al. (2019), using Pro-liner 3-colour (Cyanagen Srl, Bologna, Italy) as a marker, and Laemmli loading buffer added to samples to follow the protein separation.

Western blot analysis was performed using a blocking solution (100mM Tris-HCl pH 8.0, 150mM NaCl, 0,1% Tween 20, 2,5% BSA). To reveal Rubisco, samples were incubated with the respective primary antibody (Agrisera, Vännäs, Sweeden) anti-RbcL (AS03037, 1:10 000 v/v). Anti-Rabbit IgG (H&L), HRP conjugated (AS09602, 1:6000 v/v) was used as secondary antibody. The immunorevelation was carried out using the kit for chemiluminescence (Westar supernova, Cyanagen Srl, Bologna, Italy) by ChemiDoc System (Bio-Rad). The software Image Lab (Bio-Rad Laboratories, Hercules, California, USA) was used for the densitometric analysis to obtain quantitative information associated with the individual bands. Density values are expressed in arbitrary units and described as bar diagrams representing the pixel volumes of protein bands.

4.6 Leaf biochemical analyses

The leaf biochemical analyses, namely total antioxidant capacity, total polyphenols, total flavonoids were performed on five fully expanded leaves (one leaf per plant) per treatment.

The antioxidant capacity was determined using the ferric reducing antioxidant power (FRAP) assay according to the method reported by George et al. [68], modified by Vitale et al. [69]. Samples (0.250 g) were ground in liquid nitrogen, mixed with 60:40 (v/v) methanol/water solution, and centrifuged at 14.000 rpm for 15 min (4°C). FRAP reagents (300 mM acetate buffer pH 3.6; 10 mM tripyridyltriazine (TPTZ), 40 mM HCl and 12 mM FeCl3) were added to the extracts of each sample in 16.6:1.6:1.6 (v/v), respectively. After 1 h in darkness, the absorbance at 593 nm was measured with a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard and total antioxidant capacity was quantified and expressed as μmol Trolox equivalents per gram of fresh weight (μmol TE g^{-1} FW).

Total polyphenols were estimated following Arena et al. [15]. Powdered samples (0.02g) were extracted in methanol at 4°C and centrifuged at 11.000 rpm for 5 min. Extracts were mixed with 1:1 (v/v) 10% Folin–Ciocâlteu reagent and, after 3 min, with 5:1 (v/v) 700 mM Na2CO3 solution. Samples were incubated for 2 h in darkness. Then, the absorbance at 765 nm was measured with a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). The total polyphenol content was calculated and expressed as mg of gallic acid equivalents per gram of fresh weight (mg GAE g^{-1} FW) from the calibration curve using gallic acid as standard.

Total flavonoid content was evaluated according to Moulehi et al. [70] and Sun et al. [71]. Powered sample (0.02g), diluted with aqueous 80% methanol, was mixed with 3:1 (v/v) 5% $NaNO_2$ (sodium nitrite). After 6 min, 10% $AlCl_3$ (aluminium chloride) and NaOH

(1M) were added to the mixture. Lastly, the mixture was adjusted with distilled water. The absorbance was read at 510 nm. Total flavonoid content was calculate using a catechin standard curve and expressed as mg catechin equivalent per gram of fresh weight (mg CE g^{-1} FW).

4.7 Statistical analysis

Statistical analysis was performed by using the SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA). The effect of IR on germination were evaluated by t-test based on a significance level of $p < 0.05$. The influence of the two different independent factors, namely ionizing radiation (IR) and light quality treatment (LQ), and their possible interaction on biometrical, anatomical and functional traits were analyzed by two-way ANOVA. The Kolmogorov-Smirnov test was used to check the normality. The Student-Newman-Keuls (SNK) test was applied for all pairwise multiple comparison tests with a significance level of $p<0.05$. Whenever the interaction between IR and LQ was significant, data were subjected to one-way ANOVA and multiple comparison tests were performed with SNK coefficient.

The overall parameters were visualized by a heatmap (heatmap function). The heatmap was plotted by using the ClustVis program package (https://biit.cs.ut.ee/clustvis/online) and clustering both rows and columns with Euclidean distance and average linkage. In heatmaps, the numeric differences are evidenced by the color scale: red and blue indicate increasing and decreasing.

5. Conclusions

This study demonstrated that different light spectral composition modifies the morphological and physiological attributes of 'Microtom' plants sprouted from seeds irradiated with Ca-ions at 25 Gy, inducing dwarf growth and ameliorating the plant water relationships. The completion of the life cycle was observed in all plants, irrespective of light regimes. In particular, the RB treatment enhanced the compact architecture in irradiated plants, representing a valuable trait for the limited volumes at disposal for plant cultivation in Space. The RB light growth regime also improved the photosynthetic performance of irradiated plants by modulating stomatal conductance and Rubisco content exerted by blue light. At the anatomical level, the occurrence of more intercellular spaces in RB irradiated plants likely improved the mesophyll conductance and induced an increase of Rubisco expression. This study suggested that specific LQ regimes modify functional attributes in 'Microtom' irradiated plants, favouring photosynthesis. This result is particularly encouraging in 'Microtom' cultivation on board of Controlled Ecological Life Support Systems (CELSS) as a food supplement for astronaut diet in long-term Space missions.

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Chapter VII – Light quality regime during growth affects the photosynthetic behavior and antioxidant properties of *B. vulgaris* L. exposed to high-energy heavy ions: implications for cultivation in Space.

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Light quality regime during growth affects the photosynthetic behavior and antioxidant properties of *B. vulgaris* L. exposed to high-energy heavy ions: implications for cultivation in Space.

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Abstract

This study aimed to evaluate the effects of different light quality regimens (RGB- Red 33: Green 33: Blue 33; RB- Red 66: Blue 33; W- white light) on *B. vulgaris* plants sprouted from seeds irradiated with Carbon (C) and Titanium (Ti) heavy ions to assess if the growth under specific wavelengths may modify the photosynthetic behaviour and bioactive compound synthesis of irradiated plants. Gas exchanges were affected by light quality and ionising radiation; under W light, net photosynthesis (A_N) and water use efficiency (iWUE) declined in C- and Ti-ion irradiated plants compared to control. Conversely, under RGB and RB regimes, A_N and iWUE did not vary between irradiated and control plants. Photosynthetic

pigment content was affected by light but not by ionising radiation, lower under RGB and RB regimes than W.
Total carbohydrate and protein content amount decreased in control and C-ion plants under RB compared to RGB and W regimes, while it increased in Ti-ion plants. Total protein content was affected by light quality only in C-ion plants, which declined under RGB and RB compared to the W regime. The antioxidants are modified by both light quality and heavy-ion treatment. Polyphenols significantly decreased in Ti-ion irradiated plants compared to control and C-ion plants irrespective of light growth regime. Anthocyanin content did not vary between irradiated and control plant groups under RGB and RB, but it declined under the W regime in Ti-ion plants. C-ion irradiated plants showed higher antioxidant capacity than control and Ti-ion plants under W and RGB light regimes. In Ti-ion plants RGB and RB stimulate the antioxidant capacity compared to W.

Keywords: Antioxidants, *Beta vulgaris* L., ionising radiation, light quality, photosynthesis, Space Closed Ecosystem.

Abbreviations: FRAP, ferric reducing antioxidant power; iWUE, intrinsic water use efficiency; LEDs, light emitting diodes; Fv/Fm, maximum PSII photochemical efficiency; A_N, net CO_2 assimilation; NPQ, non-photochemical quenching; PPFD, photosynthetic photon flux density; RGB, red-green-blue; RB, red-blue; Φ_{PSII}, quantum yield of PSII electron transport; gs, stomatal conductance; W, white light.

Introduction

The realisation of Bio-regenerative life Support Systems (BLSSs) is a crucial step in the view of future long-term human-crewed missions in Space. Transit-vehicles, space stations, and platforms on Moon and Mars will include these self-sustaining artificial ecosystems based on the balance between heterotrophs (humans and microorganisms) and autotrophs (plants or algae). In particular, higher plants significantly contribute to restoring the resource in closed environments, regenerating and purifying air through CO_2 absorption and O_2 emission and

transpiration as well as producing fresh food supplies to the crew (Wolff et al., 2013; Wheleer, 2017; Zabel, 2018; Arena *et al*., 2019).

Space is a totally different environment compared to Earth and many environmental factors may constraint the plant surviving in the extraterrestrial environment such as altered gravity, interaction between microgravity and fluid-dynamics, modified conditions of pressure, temperature, etc. Currently, the exposure to ionizing radiation (IR) represents one of the major limits for the survival of life forms, because it may trigger changes at molecular, morpho-structural and physiological level, compromising the success of the space missions (Arena *et al*., 2014).

Regarding autotrophs, the maintenance of a healthy and efficient photosynthetic apparatus is necessary to ensure plants the important role of resource regenerators in Space.

Currently, it is known that photosynthetic apparatus may be damaged by IR at different steps (De Micco *et al*., 2011). Changes in the antenna complex, reaction centres, electron transport rate, respiration, leaf structure (Kim *et al*., 2004; Palamine *et al*., 2005; Arena *et al*., 2013; Marcu *et al*., 2013; Park *et* al., 2015; Arena *et al*., 2017, 2019), biomass (Nechitailo *et al*. 2005; Jan *et a*l., 2012; Ali *et al*., 2016; Yadav *et al*., 2016) and variations in sugar and starch metabolism (Hwang *et al*., 2014) are also reported in response to low and high LET ionising radiation. However, radio-induced oxidative stress triggers the production of a large variety of compounds with antioxidant functions (Zaka *et al*., 2002; Esnault *et al*., 2010; Kim *et al*., 2011; Arena *et al.,* 2019), which may defend plants from harsh conditions and at the same time enhance their nutritional properties. IR can affect plant growth, photosynthesis and secondary metabolite production, causing different responses which range from the inhibition to the promotion of qualitative traits, depending on the species, phenological stage, dose and radiation quality (De Micco *et al*., 2011; Jan *et al*., 2012; Arena *et al*., 2014; De Micco *et al*., 2014, 2014b). Understanding the effects of IR on higher plants is a prerequisite for Space biology, not to mention endpoints and applications in other research fields, such as environmental protection (Caplin and Willey, 2018) and breeding programs aimed to improve specific traits in selected cultivars (Oladosu *et al*., 2016). Even though the biological effects of different

components of the galactic cosmic rays were largely investigated on plants and animals, very little is known concerning Titanium heavy ions exclusively tested in animal models to evaluate the oncologic risk linked to manned space missions. These studies evidenced that Titanium ions induce oxidative stress and genomic alterations associated with several health risks (Jangiam et al., 2015; Rithidech et al., 2016; Li *et al.*, 2018).

Within the BLSSs, radio-induced effects on plants could be influenced by other environmental factors. Thus, defining agricultural practices as well as micro-environmental parameters for selected species represents an essential aspect. In particular, the illumination source affects the inputs and outputs of the whole system (Zabel, 2018). In the last years, light-emitting diodes (LEDs) revolutionised lighting technology for crop production. Compared to traditional light sources, LEDs are more suitable as characterised by non-thermal photon emission, greater longevity and energy-saving properties (Hasan *et al*., 2017). LED illumination also makes possible the modulation of light intensity and spectral composition during the whole plant life cycle, selecting suitable growth protocols for crop species (Yeh and Chung, 2009; Amitrano *et al.,* 2018). Higher plants show a higher plasticity in response to light in terms of intensity, quality and duration (Ouzounis *et al.*, 2015). Spectral composition affects several plant processes (Olle and Viršilė, 2013), among them germination (Barrero *et al.*, 2012), stomatal opening, (Goins *et al.,* 1997), chloroplast ultrastructure and leaf anatomy (Singh *et al.*, 2015; Liu *et al*., 2011), pigment production (Fan et al., 2013; Amitrano et al., 2018) resistance to diseases (Wang *et al.*, 2010) photosynthesis (Arena *et al.,* 2016; Trouwborst *et al.*, 2016; Izzo *et al.,* 2020) and biomass production (Li and Kubota, 2009; Ouzounis et al., 2015; Sams *et al.*, 2016; Hernandez and Kubota 2016). Furthermore, light quality stimulates the synthesis of phytochemicals (Ohashi-Kaneko *et al*., 2006), which in turn can improve the nutritional quality of crops (Hasan *et al.*, 2017) and strengthen plant defence against abiotic stress, such as high temperature, nutritional deficiency and heavy metals (Gill *et al.*, 2010; Tuteja *et al.,* 2011; Bian *et al*., 2015; Arena *et al* 2016). For these reasons, the modulation of the light spectrum emerged as a promising tool to cultivate plants in controlled conditions, providing encouraging

results for sustainable terrestrial agriculture and Space research (Massa *et al*., 2008; Avercheva *et al*., 2014; Mitchell *et al.*, 2015; Amitrano *et al.,* 2018).

To date, ionising radiation and light quality have been investigated as two independent factors. In the perspective of plant cultivation in Space, their interaction on edible plants should be investigated. This work aims to assess, for the first time, how acute exposure of high LET ionising radiation affects the growth and development of *Beta vulgaris* L. var. *cicla*, obtained from dry seeds irradiated with two components of the galactic cosmic rays, namely Carbon (C) and Titanium (Ti) heavy ions, provided at the dose of 10 Gy. In addition, as a second novel aspect, the growth process was performed under specific wavelength combinations to study the interplay between light quality and ionising radiation and to test if a specific light spectrum may modify plant response to C and Ti heavy ions.

Material and Methods

Plant material, irradiation procedure and experimental design

Beta vulgaris L. var. cicla (white chard) is a widely consumed crop, considered a functional food because of the high content of its secondary metabolites, associated with antitumoral activity (Ninfali and Angelino, 2013). Moreover, other characteristics (compactness, edible biomass productivity), make chard one of the selected crops for the introduction in the Space Greenhouses, designed as Closed Ecological Life Support Systems (Zabel, 2018).

Dry chard seeds (n=150) were divided into control (n=50) and treated groups (n=100). The 50 seeds of treated groups were irradiated with Carbonium [isotope ^{12}C; E: 300 MeV/u (monoenergetic), LET: 13keV/µm; dose rate 1Gy/min] and 50 seeds with Titanium [isotope ^{50}Ti; E: 1000 MeV/u (monoenergetic), LET: 108keV/µm; dose rate 1Gy/min] at the dose of 10 Gy. Seeds were collected into T25-flasks and the irradiation was performed using a pencil beam in a spread-out Bragg peak (SOBP), at the heavy-ion synchrotron (SIS) at the GSI Helmholtz zentrum für Schwerionenforschung GmbH, (Darmstadt, Germany).

For C ions the dose of 10 Gy, being below the threshold for occurrence of DNA damage, could be considered not lethal for plant development (Kazama *et al.*, 2011)

and even induce stimulatory effects in chard plants. We hypothesised that at the same dose, Ti ions may not prevent plant growth and/or lead to similar responses.

All the seeds were maintained in the same storage and transport facilities to avoid any bias due to different pre-germination conditions. Irradiated and control seeds were then transferred to the laboratory and placed in Petri dishes on three layers of filter paper to follow the germination process.

Both germination and plant cultivation took place in a climatised room under three different light regimens:

- W (White), provided by fluorescent tubes (Lumilux L360W/640 and L360W/830, Osram, Germany);
- RGB (Red 33%, Green 33%, Blue 33%) and RB (Red 66%, Blue 33%) provided by red, green and blue LEDs (Octa Light LTD, Bulgaria) as reported in Arena et al. (2016).

The spectral composition (Figure 1) was measured by a SR-3000A specroradiometer at 10nm resolution (Macam Photometrics Ltd., Livingston, Scotland, UK).

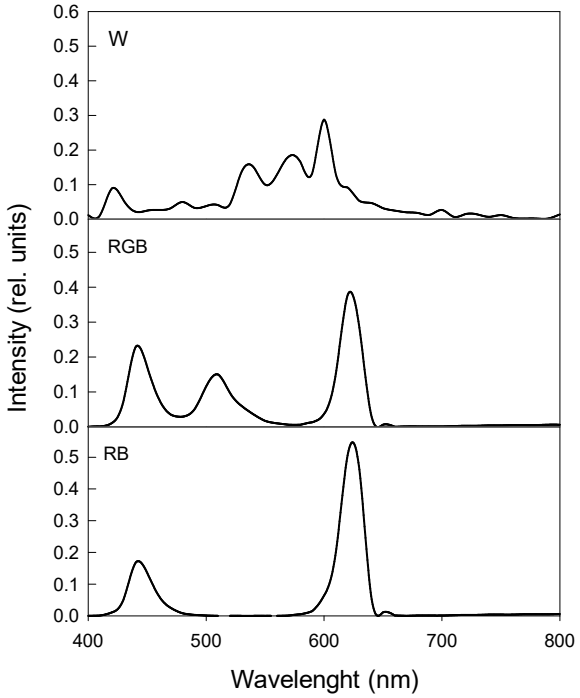

Figure 1: Spectral distributions in the relative energy of the fluorescent tubes and LEDs. Spectral scans were recorded for W (White light), RGB (Red 33%, Green 33%, Blue 33%) and RB (Red 66%, Blue 33%) at the top of the canopy.

The total photosynthetic photon flux density (PPFD) was the same in each light treatment (300 ±12 μmol photons m^{-2} s^{-1}). All plants were kept under air temperature of 25/15 °C (day/night), relative humidity 60–70%, and a 12 h photoperiod. Every two weeks, plants were fertilised with tap water and Hoagland's solution to pot capacity to reintegrate water lost by evapotranspiration.

The plant growth was followed up to 90 days after sowing (DAS) at the Plant Physiology and Genetics Institute of Bulgarian Academy of Science (Sofia, Bulgaria). Gas exchanges and fluorescence emission measurements were carried out on fully expanded leaves to assess how radiation may have affected photosynthetic apparatus functionality and how the plant growth under different light quality may have influenced plant photosynthetic behaviour. In addition, at the end of the vegetative cycle, biometrical measurements, leaf functional traits, photosynthetic pigments, total carbohydrates content and antioxidant amount were also detected on mature plants as a proxy for carbon allocation, and the nutritional status of plants. These analyses were performed at the Department of Biology of University Federico II of Naples (Italy).

Germination, biometrical measurements and leaf functional traits

The percentage of seed germination under different light quality treatments was evaluated on fifty samples per treatment. Seeds were considered to have germinated when the root protruded the seed coat. The germination percentage (GP) was calculated 7 days after sowing (DAS), according to the formula:

$$GP_{7DAS}\frac{Number of germinated\ seeds\ after\ 7\ days}{Total\ number\ of\ the\ seeds}\ 100$$

After germination, 10 seedlings from the control, 10 seedlings from C and 10 seedlings from Ti irradiated seeds were sown in 3.0 L pots filled with peat soil.

At the end of the experimental period of 60 days after sowing (DAS), total biomass (TB) and shoot biomass (SB) were determined on five plants for each treatment weighting and the whole and the shoot portion, respectively. The TB and SB were expressed as g fresh weight per plant (g FW $plant^{-1}$).

Leaf gas exchanges and chlorophyll a fluorescence emission measurements

Leaf gas exchanges were measured at 60 DAS on fully expanded leaves by a portable gas-exchange system (LCpro+, ADC BioScientific, UK). The middle part of the leaf was clamped into the 6.25 cm^2 gas-exchange cuvette and exposed to a constant flow (300 µmol s^{-1}) of synthetic air (79% N_2, 21% O_2 and 400 µmol mol^{-1} CO_2). Measurements were carried out at 25 ± 2°C leaf temperature and 500 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The relative humidity in the leaf chamber was set at 50–60%. The intrinsic water use efficiency (iWUE) was calculated as a ratio between photosynthesis (A_N) and stomatal conductance (gs). All gas-exchange parameters were recorded after reaching a steady-state, usually 7–10 min for each measurement and calculated by the equations of von Caemmerer and Farquhar (1981) with the software operating within the gas-exchange system.

Chlorophyll *a* fluorescence measurements were carried out by means of a Fluorescence Monitoring System (FMS, Hansathech Instruments, King's Lynn, UK). The determination of minimum (F_o) and maximum (F_m) fluorescence was carried out on 20 min dark adapted leaves. The maximum quantum yield of PSII photochemistry (F_v/F_m) was determined as $(F_m - F_o)/F_m$. The measurements in the light were carried out on leaves adapted to PPFD of 500 µmol m^{-2} s^{-1}. A saturating pulse of 0.8 s with > 6000 µmol photons m^{-2} s^{-1} was applied in order to determine the maximum ($F_{m'}$) and the steady-state (F_s) fluorescence in light adapted condition. The quantum yield of PSII electron transport (Φ_{PSII}) was calculated according to Genty *et al.* (1989) as: $\Phi_{PSII} = (F_{m'} - F_s)/F_{m'}$. The non-photochemical quenching (NPQ) was calculated as $NPQ = (F_m - F_{m'})/F_{m'}$ (Bilger and Björkman, 1991).

Photosynthetic pigments and antioxidants determination

Photosynthetic pigment and antioxidant content were determined on five fresh leaves, collected from different plants, for each experimental condition.

The determination of the photosynthetic pigment content, namely total chlorophylls (a+b) and carotenoids (x+c), was performed according to Lichtenthaler (1987). Leaf samples of known area were treated with ice-cold 100% acetone by means of a mortar and pestle. The extracts were centrifuged at 5000 rpm for 5 min in a Labofuge

GL (Heraeus Sepatech, Hanau, Germany). The absorbance was measured by spectrophotometer (UV-VIS Cary 100; Agilent Technologies) at wavelengths of 470, 645 and 662 nm and pigment concentration was expressed as μg cm^{-2}.

The total polyphenol content was evaluated following the procedure of Ainsworth and Gillespie (2007) with some modifications as reported in Arena *et al.* (2019). Fresh samples were ground in liquid nitrogen and, extracted with methanol at 4°C and centrifuged at 11.000 rpm for 5 min. The soluble fraction was mixed with 10% Folin-Ciocalteau solution, 1:1 v/v and after 3 min 700 mM Na_2CO_3 solution was added to the resulting mixture (5:1, v/v). Samples were incubated for 2 h in the darkness. The absorbance was measured at 765 nm with a spectrophotometer (UV-VIS Cary 100; Agilent Technologies). The total polyphenol amount was expressed as mg of Gallic Acid Equivalents g^{-1} FW (mg GAE g^{-1} FW) using a gallic acid standard curve.

The anthocyanin content was determined following Arena et al. (2019). Fresh samples were ground with liquid nitrogen, treated with methanol 1% HCl solution and stored overnight at 4°C. After adding 1:0.6 (v/v) ultra-pure water and 1:1.6 (v/v) chloroform, samples were centrifuged at 11.000 rpm for 5 min. After mixing the supernatants with 1:1 (v/v) [60% (methanol 1% HCl) 40% ultra-pure water] solution, the absorbance was meanured with a pectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA) at 530 and 657 nm. The relative amount of anthocyanin was expressed as (A_{530}-1/3A_{657}) g^{-1} FW (Mancinelli *et al.*, 1975).

To evaluate the antioxidant capacity, the Ferric Reducing Antioxidant Power (FRAP) assay was performed on fresh leaves samples grinded with liquid nitrogen according to George *et al.* (2004). Briefly, the samples (0.250g) were treated with methanol/water solution (60:40, v/v) and centrifuged at 14.000 rpm for 15 min at 4°C. The extracts were mixed with the FRAP reagents (300 mM acetate buffer pH 3.6, 1:16.6 v/v; 10 mM tripyridyltriazine, TPTZ, in 40 mM HCl, 1:1.6 v/v; 12 mM $FeCl_3$, 1:1.6 v/v) and incubated for 1 h in the dark. Then, the absorbance was read at 593 nm by a spectrophotometer (UV-VIS Cary 100; Agilent Technologies). The antioxidant capacity was calculated using a Trolox standard curve and expressed as μmol Trolox equivalents (μmol Trolox eq. g^{-1} FW).

Total soluble carbohydrate content and protein quantification

Total soluble carbohydrates were assessed on five leaves samples for each treatment, following the anthrone method as reported by Hedge and Hofreiter (1962). The absorbance was measured spectrophotometrically at 630 nm (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). The amount of soluble carbohydrates in the extracts was expressed as mg Glucose equivalents g^{-1} FW (mg Glu eq g^{-1} FW) using a Glucose standard curve.

Protein extraction was carried out on fresh leaf samples ground in liquid nitrogen according to Wang *et al*. (2006). Total protein content was quantified by Bradford colourimetric assay (1976), measuring spectrophotometrically (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA) the absorbance at 595 nm, and expressed as μg BSA equivalents g^{-1} FW (bovine serum albumin, Sigma Aldrich) using a BSA standard curve.

Statistical Analysis

All experimental data and graphical data processing were performed with Sigma-Plot 12.0 software (Jandel Scientific, San Rafael, CA, USA). A two-way ANOVA was performed on data collected considering C- and Ti-heavy ions treatments (HI) and the light quality regimes (LQ) as main factors. The Kolmogorov-Smirnov test was used to check the normality. The Student-Newman-Keuls test was applied for all pairwise multiple comparison tests with a significance level of $p<0.05$. The summary of Two-Way ANOVA analysis is shown in Table 1.

The overall parameters were visualized by a heatmap (heatmap function). The heatmap was plotted by using the ClustVis program package (https://biit.cs.ut.ee/clustvis/online) and clustering both rows and columns with Euclidean distance and average linkage. In the heatmap, the numeric differences are evidenced by a colour scale: red and blue indicate increasing and decreasing values, respectively.

Table 1. Two-way analysis of variance in *B. vulgaris* plants in response to irradiation with C and Ti heavy ions (HI) and light quality (LQ) regimes, and their interaction (HI x LQ). NS-not significant; *p<0.05; **p<0.01; ***p<0.001.

Parameters	Factors		
	HI	LQ	HI x LQ
TB	*	*	*
SB	ns	*	ns
A_N	***	**	***
g_s	***	***	**
iWUE	ns	*	*
ΦPSII	ns	**	ns
NPQ	*	***	*
Fv/Fm	ns	ns	ns
CHL	***	***	*
CAR	***	***	***
CARB	ns	***	***
TP	***	ns	ns
TPC	***	ns	ns
ANTH	***	***	ns
TAC	***	***	***

TB: total biomass; SB: shoot biomass; A_N: net CO_2 assimilation; g_s: stomatal conductance; iWUE: intrinsic water use efficiency; Φ_{PSII}: quantum yield of PSII electron transport; NPQ: non-photochemical quenching; F_v/F_m: maximum PSII photochemical efficiency; CHL: total chlorophylls; CAR: total carotenoids; CARB: total carbohydrates; TP: total proteins; TPC: total polyphenols; ANTH: anthocyanins; TAC: total antioxidant capacity.

Results

Germination and plant biomass

Growth under different light quality regimes affected both the germination and the biomass accumulation in control and C, and Ti ions irradiated plants (Table 2).

Compared to W and RGB, the RB regime reduced significantly ($p<0.01$) GP, TB, and SB in the control plants. Within the C-irradiated plant group, GP, TB and SB were unaffected by light regimes, and GP reached 100%. Within the Ti-irradiated plant group, no difference was found in TB and SB depending on different light quality, but RGB plants showed a decrease ($p<0.05$) of GP compared to W and RB ones.

Table 2: Germination percentage (GP), total biomass (TB), and shoot biomass (SB) of *B. vulgaris* plants sprouted from Control and irradiated Carbon (C-10Gy) and Titanium (Ti-10Gy) seeds and grown under white fluorescent (W), red-green-blue (RGB) and red-blue (RB) light quality regimes. Data are mean (n=5) ± standard error. Different lowercase letters indicate statistically significant differences among light treatments within same plant group, uppercase letters indicate differences between Control and irradiated plants under the same light quality regime, according to two-way ANOVA ($p<0.05$).

		GP %	TB (g FW)	SB (g FW)
Control	W	$75\pm2.2^{a,B}$	$30.693\pm3.423^{a,A}$	$24.759\pm3.861^{a,A}$
	RGB	$50\pm3.2^{a,B}$	$28.443\pm4.706^{a,A}$	$24.598\pm4.023^{a,A}$
	RB	$25\pm2.5^{b,C}$	$16.739\pm1.983^{b,A}$	$13.706\pm1.787^{b,A}$
C 10 Gy	W	$100\pm0.0^{a,A}$	$28.271\pm2.007^{a,A}$	$22.306\pm1.848^{a,A}$
	RGB	$100\pm0.0^{a,A}$	$20.670\pm4.067^{a,A}$	$17.012\pm3.419^{a,A}$
	RB	$100\pm0.0^{a,A}$	$26.524\pm1.896^{a,B}$	$21.168\pm1.229^{a,A}$
Ti 10 Gy	W	$100\pm0.0^{a,A}$	$29.727\pm2.922^{a,A}$	$22.272\pm2.534^{a,A}$
	RGB	$60\pm2,4^{b,B}$	$22.837\pm2.721^{a,A}$	$18.967\pm2.966^{a,A}$
	RB	$75\pm3,1^{a,B}$	$29.388\pm4.585^{a,B}$	$22.108\pm3.023^{a,A}$

The comparison among control and irradiated plants evidenced that TB and SB were not affected by heavy-ion treatments under W and RGB light regimes. Moreover, C-ion irradiated plants reached the full GP regardless of the light regime, unlike control and Ti-ion plants. Finally, under RB light regime Ti- and C-ion irradiated plants increased ($p<0.05$) TB and SB compared to control. No difference under the RGB growth light regime was evidenced (Table 2).

Gas exchanges and chlorophyll fluorescence emission measurements

The heavy ion irradiation treatments and the growth under different light quality regimes strongly affect the photosynthetic performance of B. vulgaris plants. Within the control plant group, the application of RGB and RB light regimes determined lower ($p<0.001$, $p<0.05$) A_N and gs values compared to W plants, but no difference in iWUE (Figure 2 A-C). The decline of A_N and g_s in these plants was accompanied by the significant reduction ($p<0.05$) of Φ_{PSII} and the rise ($p<0.05$) of NPQ (Figure 2 D, E). Within the C-ion plant group, A_N did not vary among light regimes, but the plant growth under RGB and RB light determined the decline ($p<0.001$) of g_s and the increase($p<0.05$) of iWUE compared to W (Figure 2 A-C). These plants also showed lower ($p<0.05$) ΦPSII and higher ($p<0.05$) NPQ values

than W regimes (Figure 2 D, E). In the Ti-ion plant group, the RGB regime significantly improved ($p<0.05$) A_N and g_s compared to W and RB, and iWUE compared to W (Figure 2A-C). The RB regime reduced ($p<0.05$) Φ_{PSII} and enhanced ($p<0.05$) NPQ compared to W and RGB (Figure 2 D, E). Finally, no significant difference was detected in Fv/Fm among light quality regimes nor heavy ion irradiation treatments (Figure 2F).

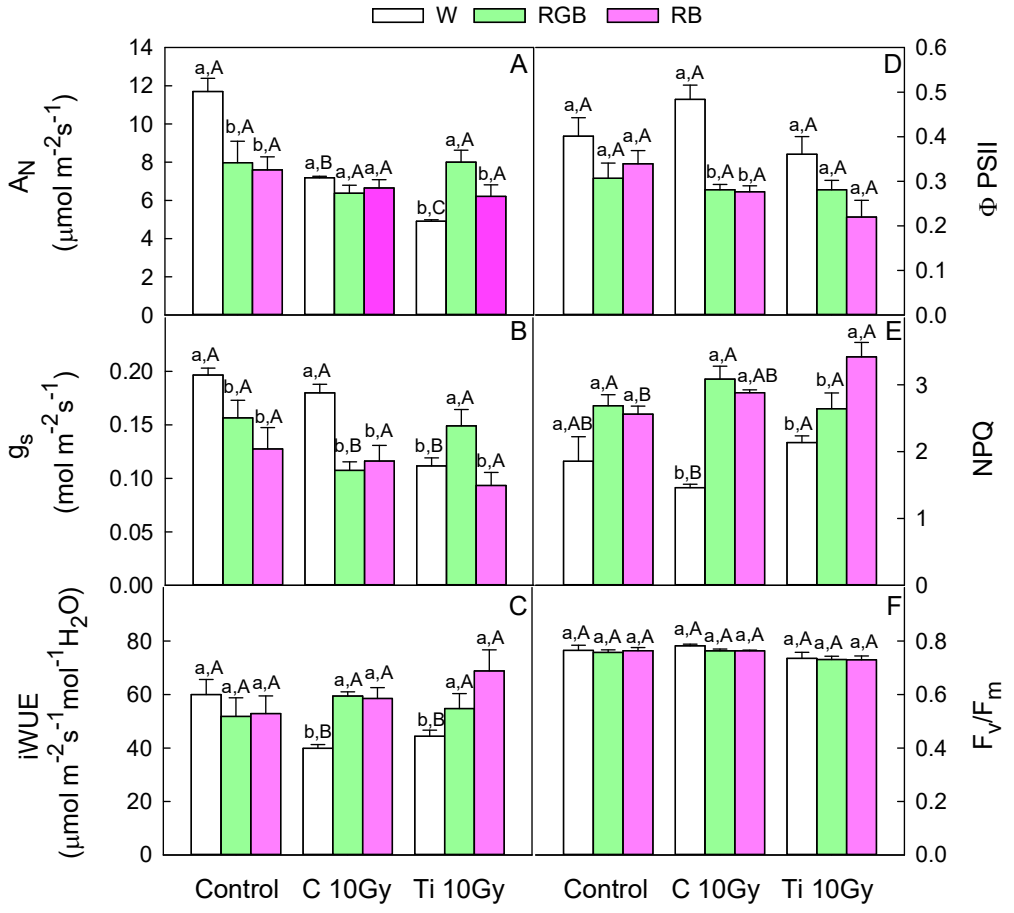

Figure 2: (A) Net CO_2 assimilation, A_N; (B) stomatal conductance, g_s; (C) intrinsic water use efficiency, iWUE; (D) quantum yield of PSII electron transport, Φ_{PSII}; (E) non-photochemical quenching, NPQ; (F) maximum PSII photochemical efficiency, F_v/F_m of *B. vulgaris* plants sprouted from Control and irradiated Carbon (C-10Gy), and Titanium (Ti-10Gy) seeds and grown under white fluorescent (W), red-green-blue (RGB) and red-blue (RB) light quality regimes. Data are mean (n=5) ± standard error. Different lowercase letters indicate statistically significant differences among light treatments within same plant group, uppercase letters indicate differences between Control and irradiated plants under the same light quality regime, according to two-way ANOVA ($p<0.05$).

The comparison among control and irradiated plants evidenced that under the W light regime, a remarkable decline of A_N ($p<0.01$) and iWUE ($p<0.05$) was observed in C-and Ti-ion irradiated plants compared to control, whereas the stomatal conductance (g_s) only in Ti- ion plants was significantly reduced ($p<0.001$) compared to C-ion irradiated plants and control. Conversely, under RGB and RB light regimes, A_N and iWUE did not vary among irradiated and control plants, except RGB C-ion irradiated plants, which showed lower ($p<0.05$) g_s values than control and Ti-ion irradiated plant groups. Φ_{PSII} was not affected by heavy ion irradiation under all light quality regimes (Figure 2D). Conversely, NPQ showed the lowest ($p<0.05$) value in C-ion irradiated plants under W light, no difference in under RGB regime between irradiated and control plants, while higher ($p<0.05$) NPQ value in Ti-ion irradiated plants than control and C-ion groups (Figure 2E).

Plants nutritional traits and bioactive compound

Light quality and heavy-ion treatments exert substantial changes in photosynthetic pigment content, total protein and carbohydrate amount, and antioxidants deeply affecting tissues' nutritional properties.

As an independent factor, light quality determined significant differences in not irradiated control plants compared to those subjected to C-and Ti-ion irradiation treatment (Figure 3). Within the control plant group, RGB and RB regimes induced a significant decline ($p<0.001$) of total chlorophylls, carotenoids and carbohydrates compared to W light (Figure 3A-C) and no difference in total protein content (Figure 3D). In C-ion irradiated plant group, similar results were observed for total chlorophylls carotenoids and total proteins, which exhibited lower values ($p<0.01$) under RGB, and RB compared to the W regime. Conversely, only the RB regime affected the total carbohydrate content, determining in these plants a significant drop ($p<0.01$) compared to W and RGB light treatments. Within the T-ion irradiated plant group, the RB light regime sorted the most critical effects; more specifically, compared to W light, it induced a decrease ($p<0.05$) of total chlorophyll and carotenoid content, this latter more pronounced ($p<0.05$) than those found at RGB

regime, and a rise ($p<0.05$) of total carbohydrates (Figure 3A-C). Conversely, no change in protein content among the different light regimes was found (Figure 3D).

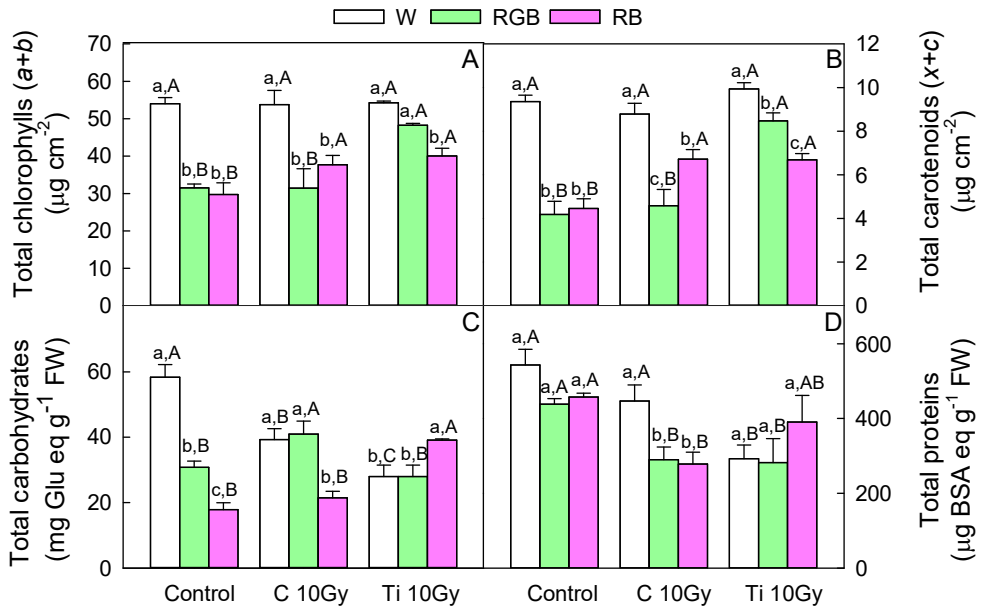

Figure 3: (A) Total chlorophylls, (B) total carotenoids, (C) total carbohydrates, (D) total proteins of *B. vulgaris* plants sprouted from Control and irradiated Carbon (C-10Gy), and Titanium (Ti-10Gy) seeds and grown under white fluorescent (W), red-green-blue (RGB) and red-blue (RB) light quality regimes. Data are mean (n=5) ± standard error. Different lowercase letters indicate statistically significant differences among light treatments within same plant group, uppercase letters indicate differences between Control and irradiated plants under the same light quality regime, according to two-way ANOVA ($p<0.05$).

The comparison among control and irradiated plants within the same light quality regimes evidenced that under W light total chlorophyll and carotenoid did not vary (Figure 3A, B). Conversely, C-ion, and even more Ti-ion irradiated plants showed a significant decline ($p<0.05$) of total carbohydrates compared to control (Figure 3C). Ti-ion irradiated plants also exhibited a lower ($p<0.05$) total protein content than control and C-ion plant groups (Figure 3D). The growth under RGB light regimes promoted ($p<0.001$) in Ti-ion plants the chlorophyll and carotenoid levels and in C-ion plants the total carbohydrate content only, compared to control. Moreover, the RGB growth regime decreased ($P<0.05$) the total protein content in both irradiated plant group compared to control. Under the RB regime, C- and Ti-ion plant groups showed higher ($p<0.05$) pigment content and lower ($p<0.05$) protein

level than control. A higher ($p<0.01$) total carbohydrate amount was found only in Ti-ion plants compared to control and C-ion plants.

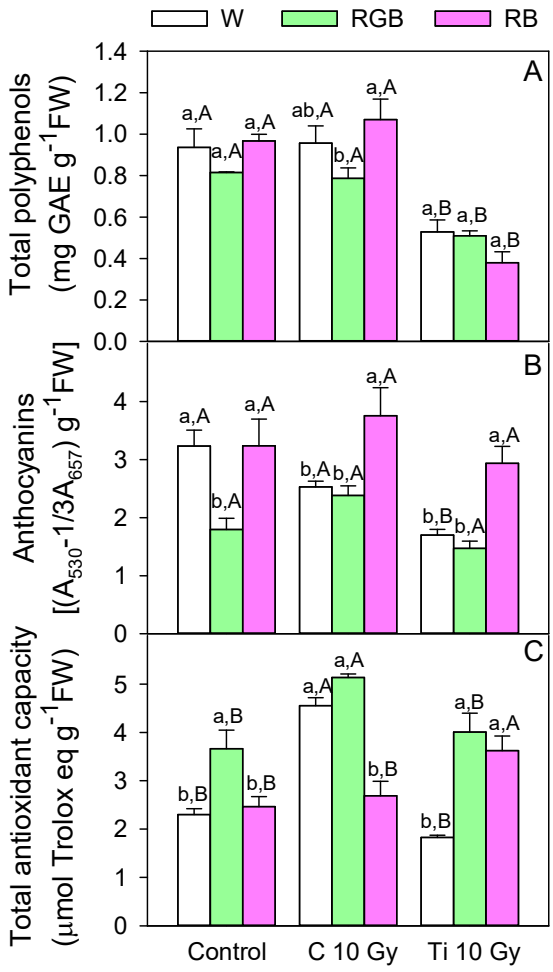

Figure 4: (A) Total polyphenols, (B) total anthocyanins, (C) total antioxidant capacity of *B. vulgaris* plants sprouted from Control and irradiated Carbon (C-10Gy), and Titanium (Ti-10Gy) seeds and grown under white fluorescent (W), red-green-blue (RGB) and red-blue (RB) light quality regimes. Data are mean (n=5) ± standard error. Different lowercase letters indicate statistically significant differences among light treatments within same plant group, uppercase letters indicate differences between Control and irradiated plants under the same light quality regime, according to two-way ANOVA ($p<0.05$).

The total antioxidant capacity of irradiated plants was also influenced by light quality regimes (Figure 4C). A significant increase of antioxidant capacity was observed in the C-ion plant group under W ($p<0.001$) and RGB ($p<0.01$) light regimes compared to the control and Ti-ion plant group. Under RB light, Ti-ion

irradiated plants' total antioxidant capacity was higher ($p<0.01$) than control and C-ion plant groups.

The antioxidant charge of plants is modified by both light quality and heavy-ion treatment. Within the control and Ti-ion irradiated plant groups, the total polyphenol content did not change with the different light quality regimes; conversely, it showed a significant reduction ($p<0.05$) under RGB, compared to W and RB regimes (Figure 4A). In the control plant group, the anthocyanin content was lower ($p<0.05$) under RGB than W and RB regimes, while it increased in C-and Ti-ion plant groups under RB compared to W and RGB (Figure 4B). Finally, the total antioxidant capacity in the control group was higher ($p<0.05$) under RGB compared to W and RB light regimes, whereas it reached the lowest ($P<0.01$) value in the C-ion plant group under RB light and in the Ti-plant group under W light (Figure 4C).

The comparison among control and irradiated plants under the same light growth regimes evidenced that total polyphenols significantly decreased ($p<0.01$) in Ti-ion irradiated plants under all applied light regimes compared to control and C-ion plants (Figure 4A). Under W and RGB light growth regimes, the irradiated plants did not evidence differences in anthocyanin content than non-irradiated controls. Conversely, under the W regime, Ti-ion irradiated plants evidenced a significant anthocyanin reduction ($p<0.05$) compared to control, and C-ion treated plants (Figure 4B). The total antioxidant capacity of irradiated plants was also influenced by light quality regimes (Figure 4C). A significant increase of antioxidant capacity was observed in the C-ion plant group under W ($p<0.001$) and RGB ($p<0.01$) light regimes compared to the control and Ti-ion plant group. Under RB light, Ti-ion irradiated plants' total antioxidant capacity was higher ($p<0.01$) than control and C-ion plant groups.

Heatmap analysis

An overview of all measured parameters in response to heavy ions irradiation (C and Ti) and three light quality regimes (W, RGB and RB) is reported in Figure 5.

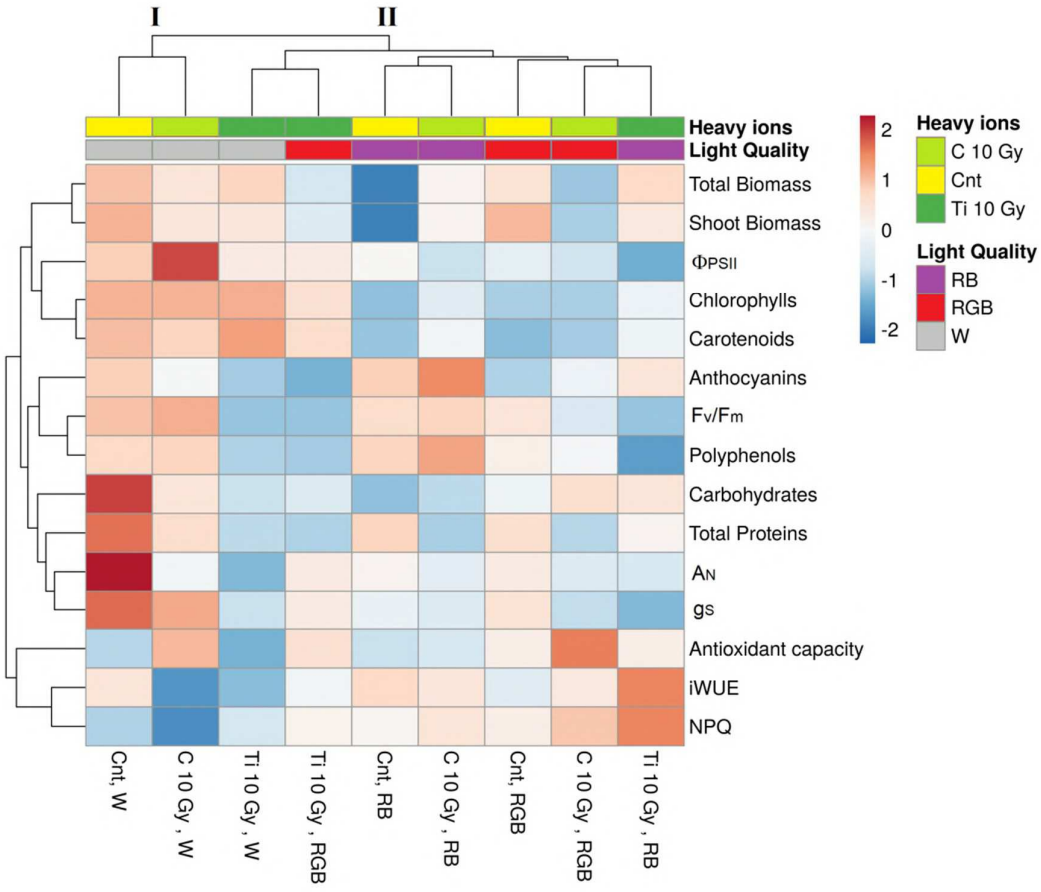

Figure 5: Cluster heatmap analysis summarising morphological and eco-physiological parameters of *Beta vulgaris* L. cv *cicla* plants grown under different light quality regimes (W, RGB and RB), from not irradiated (Control) and irradiated seeds with Carbon (C) and Titanium (Ti) heavy ions at the dose of 10 Gy. Numeric differences within the data matrix are showed by the colour scale: light blue and dark blue indicate increasing and decreasing values, respectively. Parameters are clustered in the rows; sample groups are clustered in the columns by the two independent factors, Heavy ions and Light Quality.

The heatmap provided two main clusters. The first cluster (I) included plants sprouted from control and C ion-irradiated seeds grown under W light regime. The second cluster (II) was divided into two sub-clusters: the first one contained Ti-ion irradiated plants grown under W and RGB regimes; the second firstly grouped control and C ion-irradiated plants grown under RB light, and lastly control and C ion-irradiated plants exposed to RGB regime with Ti-ion plants grown under RB light. The heatmap indicated that control and C-ion irradiated plants showed a similar response for different parameters irrespective of the light quality regime. In

particular, under W light, they exhibited the highest values of biomass, pigments, PSII photochemical efficiency and antioxidant compounds.
Among Ti-ion plants, those exposed to RB light were characterized by higher values of iWUE and NPQ than W and RGB plants. Ultimately, the heatmap highlighted that the selected light quality treatments exerted different responses in *B. vulgaris* plants irradiated with C and Ti-ions.

Discussion

This work demonstrated that low doses of carbon and titanium heavy ions provided at seed stage may modify the *B. vulgaris* eco-physiological response (i.e., photosynthesis and bioactive compounds) compared to not irradiated control depending on different light quality regimes during growth.
These results may have an impact on controlled environment agriculture, especially in an extreme environment, such as Space. *B. vulgaris* is a widely consumed crop around the world (Ninfali and Angelino, 2013), and has been also introduced in the Closed Ecological Life Support Systems (CELSSs) designed for Space missions (Zabel, 2018). Despite shielding countermeasures on platforms, in the extra-terrestrial environment the development of plants is constrained by ionising radiation, which induces a wide range of physiological responses depending mainly on dose, radiation quality and plant phenological stage (De Micco *et al.*, 2011). In the view of space cultivation, the seed germination could represent a critical step. Previous researches demonstrated that the irradiation with C ions at the dose of 10 Gy, determined in rice seeds a significant reduction in germination rate (Sjahril *et al.,* 2018), whereas no variation was found in spinach seeds, for doses up to 15 Gy (Komai *et al.*, 2003). Conversely, there is no evidence about the effect of Ti ions on seeds germination. In *B. vulgaris* the seed irradiation with C-ions induced the total germination compared to control and Ti-ion irradiated seeds irrespectively from light quality regimes, likely suggesting that C ions being more energetic, are more powerful in inducing a seed tegument porosity which in turn may determine a higher water permeability favouring germination (Hammond *et al.*, 1996; Arena *et al.*, 2019). In control plants, the reduction of germination under RGB and RB light

regimes may be likely ascribed to the presence of a higher amount of blue wavelenghts (33%) which inactivated the phytocrome A involved in seed germination (Barrero *et al*., 2014)

In not-irradiated plants, the total and shoot biomass were reduced under RB compared to W and RGB regime. This is not a surprising result because several experiments have been demonstrated that plant biomass, may be enhanced under RB and RGB light growth regimes, but also reduced depending on the species (Kim *et al*., 2006; Arena *et al*., 2016; Amitrano *et al.*, 2018). Probably the complete lack of green wavelength in RB treatment has negatively affected the biomass accumulation. A very interesting result is that when irradiated seeds were grown under RB treatment an opposite behaviour was obtained since the total biomass was significantly improved. Such an increase was not due to the partitioning of carbon allocation in shoot, but rather in root biomass. The root implementation could be a valuable trait in improving the nutrient and water absorption in these plants. A reduction of biomass, associated with a more compact size, is generally common in irradiated plants (Thiede *et al*., 1995; Nechitailo *et al.* 2005; Honda *et al.*, 2006; De Micco *et al.* 2014b; Jo *et al.*, 2016; Arena *et al.,* 2019). In our case, we do not observe any changes of total biomass in irradiated plant compared to control neither under conventional W light nor RGB regime. It may be hypothesised that the irradiation provided at seed stage at the doses of 10Gy is not sufficient to induce significant changes in growth attributes.

Heavy ions as main factor or in combination with light quality regimes deeply affect the photosynthetic activity in *B. vulgaris*. It is widely demonstrated that ionising radiation generally impair photosynthesis (A_N), stomatal conductance (g_s) and water use efficiency (WUE), irrespectively from the kind of radiation and dose (Thiede *et al*., 1995; Ursino *et al*., 1977; Jia and Li, 2008; Moghaddam *et al.*, 2011; Fan *et al.*, 2014). These mechanisms are strictly interconnected because the CO_2 uptake in photosynthesis and the water loss in transpiration follow the same route through stomata (Jones, 2004). In control plants, the photosynthetic gas exchanges were very sensitive to LQ. In particular, RGB and RB light regimes determined a strong reduction of A_N and gs compared to W. The seed irradiation with C-ion seems

to offset the effect of light quality on A_N, which resulted comparable under all light regimes. On the other hand, the seed irradiation with Ti-ion in combination with RGB and RB regimes stimulated A_N, compared W light. It may be argued that in C-ion irradiated plants, the high percentage of red and blue wavelengths have improved the stomatal control in RGB and RB plants, determining a reduction in stomatal conductance, which have enhanced the iWUE. However, the occurrence of stomatal limitations ascribed to the potentially detrimental effects of C heavy ions on photosynthetic machinery cannot be excluded. The interplay Ti-ion irradiation and RGB and RB regimes may have had a stimulatory effect on A_N and gs compared to W, with the consequence also in this case of an enhancement of iWUE. There is general evidence that blue light, acting on the guard cells, induces the stomatal opening, improving conductance and consequently the photosynthetic process (Akoyunoglou, 1984; Sæbø, 1995; Brown *et al.,* 1995; Goins *et al.*, 1997). In response to changing light, the leaves of irradiated plants adopted adjustments in morphology and stomatal movements to improve the intrinsic water use efficiency. More specifically, RB wavelengths alone or with the addition of green light that penetrates deeper inside the canopy (Sun *et al*., 1998; Brodersen and Vogelmann, 2010), might have induced changes in leaf thickness facilitating the CO_2 diffusion (Kim *et al*., 2006; Terashima *et al.*, 2011; Smith *et al.*, 2017; Zhang and Van Labeke, 2017). Moreover, irradiated plants have likely optimized the stomatal opening process, enhancing the balance between CO_2 and water loss. The PSII photochemistry was affected by light quality but not by IR. However, the seed irradiation with the different ions induced a diverse partitioning of light energy compared to control plants. In RGB and RB C-ion irradiated plants the reduction of quantum yield of PSII electron transport (Φ_{PSII}) was consistent with the A_N decline, and NPQ rise, indicating that the photosynthetic apparatus diverted the light energy in thermal dissipation mechanisms when carbon assimilation is reduced (Yang et al., 2017).

Conversely, under W regime, besides the A_N decline, the Φ_{PSII} remained still elevated, suggesting the occurrence of photochemical processes other than photosynthesis (i.e, photorespiration, Mehler reaction), which contributed to

avoiding photoinhibitoty and photooxidative damages to photosystems. This response suggests a mechanism aimed to optimise the PSII efficiency and transfer to the other photochemical processes the excess of light energy (Guidi *et al.*, 2019). The efficiency of the different regulation mechanisms of absorbed light is confirmed by the absence of variation in the maximum quantum efficiency of PSII (F_v/F_m) among the light quality regimes. The control and Ti-ion irradiated plants were characterized by a similar photochemical behaviour, except under RB light regime, which maximises the thermal dissipation process at photosystem level as the main safety valve against putative photoinhibitory damages (Demmig-Adams *et al.*, 2014; Hamdani *et al.*, 2018).

The reduction of photochemical reactions in control and C-ion irradiated plants under RGB and RB compared W light regimes is consistent with photosynthetic pigment decline. The down-regulation of chlorophyll and carotenoid biosynthesis represents a safely strategy to avoid the excessive light capture. As this result is common in control and C-ion irradiated plants, it is likely that it cannot be attributed to C-ions buth rater to light quality. Indeed, the red wavelengths, being more photosynthetically efficient, usually determine photosynthetic pigment reduction in different species (Sood *et al.*, 2005; Fan *et al*., 2013; Amitrano *et al.*, 2018; Hamdani *et al.*, 2018; Liu *et al*., 2018; Zhang and Van-Labeke, 2017). In Ti-ion irradiated plants the effects of red light on photosynthetic pigments are reverted, because no variation occourred among light quality.

Our study indicates that the LQ was the main determinant in inducing changes in carbohydrates, while IR in proteins. C and T-ion irradiated plants exhibited a reduced carbohydrate and protein production compared to control. Previous studies performed on different species exposed to gamma rays demonstrated that, depending on dose and the plant phenological stage, the carbohydrate and protein levels may decrease, remain unchanged, or increase (Thiede *et al*., 1995; Jan *et al*., 2012; Stajner *et al*., 2007; Kiong *et al*., 2008; El-Beltagi *et al*., 2011). Generally, the higher dosage of gamma irradiation breaks the seed proteins and produces more amino acids. This may inhibit the protein synthesis and thus induce a total proteins content decline in plants (Hameed *et al*., 2008; Kiong *et al*., 2008). RB may induce a lower amount of

sugars (Chen *et al*., 2014), or an enhancement of sugars and proteins in various species (Zhang *et al.*, 2010; Li *et al.*, 2012; Amitrano *et al.*, 2018). It is likely that the intrinsic characteristics of the specific heavy ions might have induced a different fashion under RB regime, which exerted a positive stimulation only if it is applied to plants irradiated with Ti ions.

Ionising radiation as main factor strongly affects TPC, ANTH and TAC, depending on ion type. While C ions did not affect the concentration of anthocyanins and polyphenols, Ti ions determined a reduction of these compounds. In order to cope with the oxidative stress induced by ionising radiation and mitigate the risk of disease, a diet rich in polyphenols is essential for the crew on-board space platforms. Usually, phenolic compounds exert a screening function against high levels of solar radiation, protecting cell structures from photoinhibitory damages (Lattanzio *et al*., 2008; De Micco *et al*., 2014, 2014b; Bian *et al*., 2015). In the same way, they counteract the detrimental effects of ionising radiation (Fan *et al*., 2005; He *et al*., 2011; Arena *et al*., 2013; De Micco *et al*., 2014, 2014b).

The trend of polyphenols and anthocyanins in IR irradiated plants results controversial because some crops showed an enhancement followed exposures to gamma and X rays or carbon heavy ions (Fan *et al*., 2005; Moghaddam *et al.*, 2011; He *et al.*, 2011; De Micco *et al*., 2014), other a decline (De Micco *et al.*, 2014, 2014b). The different response depends on the radiation quality and dose. In our study, Ti ions induced a decline in polyphenols content irrespectively from the light quality regime during plant growth. Conversely, anthocyanins strongly depend on both IR and LQ. RB regimes determined a significant rise of the anthocyanins content in both C and Ti ions irradiated plants. It is known that the biosynthesis anthocyanins, is typically associated with blue light, but can be also stimulated also by red and blue wavelengths and green light (Bian *et al.*, 2015; Lekkham *et al.*, 2016). The anthocyanins content improves the nutritional properties of many leafy crops (Agarwal *et al.*, 2018; Lobiuc *et al.*, 2017; Livadariu *et al*., 2019) and may be an attractive trait for the irradiated *B. vulgaris* plants. Finally, C ions irradiation determined a consistent increase of the total antioxidant capacity maybe due to the stimulated production of several different compounds characterized by the

antioxidant properties as found in other studies performed on different species, such as lettuce, irradiated with UV and gamma rays (Fan *et al*., 2005; Alothman *et al*., 2009; Sallam and Anwar, 2017). In Ti ion-irradiated plants the antioxidant response was potentiated under RGB and RB regimes confirming that red and blue light with supplemental green could positively act on the synthesis of biochemical compounds which potentially serve to improve the total antioxidant capacity of chard plants, reinforcing the tolerance in stress conditions and the total nutrient quality (Ohashi and Kaneko, 2007; Samuolienè *et al*., 2011; Hasan *et al*., 2017; Livadariu *et al*., 2018).

Conclusions

Control and C-ion irradiated plants showed a similar response for different parameters irrespective of the light quality regime. In particular, under W light, they exhibited the highest values of biomass, photosynthetic pigment content, PSII photochemical efficiency and antioxidant compounds.

Among Ti-ion plants, those exposed to RB light were characterized by higher iWUE and NPQ than W and RGB plants. The overall results demonstrated that it is possible to join ionising radiation with light quality regimes during growth to obtain in leafy vegetables some suitable characteristics, especially in terms of bioactive compounds beneficial for human diet on Earth such in Space in the view of possible utilization of *B. vulgaris* as fresh food to complement the astronaut diet.

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General Conclusions

The PhD project investigated how different light quality regimes during plant growth may modify the photosynthetic response and help the bioactive compound production in different crops cultivated in controlled environments and subjected to a positive biostimulant application) or negative (ionising radiation) inputs. The finality of the project is to provide new insights on the possibility of grown plants in indoor cultivation even in extreme environments, including Space platforms, where there is the need to counteract ionising radiation and optimize the photosynthetic performance in narrow growth volumes.

To this purpose, the experiments have been focused on widely consumed crops such as tomato, spinach, soybean and chard, considered important functional foods and characterized by a high yield and fast growth. The performed studies may be grouped in three different sections, based on different pursued aims.

Section (I) explored the effect of light quality on photosynthetic apparatus; Section (II) evaluated the relationship between light quality and biostimulant application; Section (III) studied the interaction between light quality and ionising radiation.

The overall results highlighted that plants are highly responsive to light quality regimes during growth, which strongly affects plant responses to additional factors, namely biostimulants and ionising radiation.

The specific outcomes deriving from the three experimental parts of the PhD project are reported below.

I) The performed experiments suggest that red and blue wavelengths, as monochromatic lights or combined in different light regimes, deeply influence the interaction between photosynthesis and plant morphological traits, not only during early developmental stages but rather during the whole life cycle.

The pure red wavelength induced shade-avoidance responses in tomato or spinach, increasing plant elongation, leaf area and pigment content. However, if these changes resulted in a strong decline of photochemical activity in tomato, the exposure to pure red light promoted light-harvesting and increased the photosynthesis in spinach plants.

Blue light, alone or combined with red and green light, mainly influences photochemical responses and induces a more compact plant growth size.
The addition of green to red and blue wavelengths elicits adjustments of leaf structure and/or increase of Rubisco amount, improving the photosynthetic performance in both tomato and spinach plants, confirming the importance of the green portion of the spectrum in the carbon assimilation processes.

II) The application of microorganisms or amino acid-based biostimulants is a practical and valid approach to improving photosynthetic efficiency and enhancing the production of phytochemical compounds in spinach and soybean, especially joined with proper light quality regimes during plant growth.
However, the positive outcomes greatly depend on the specific wavelengths provided by plants during development and the kind of applied biostimulant.
Indeed in spinach plants, while the growth under red-blue light promoted biomass and photosynthesis, the pure red regime resulted in unfavourable interaction plant–organisms since it strengthened the root colonization by microorganisms and increased the energetic cost of symbiosis.

III) Low doses of ionising radiation delivered at the seed stage do not impair plant growth but can be considered a positive factor in stimulating the photosynthetic performance and the production of specific functional compounds.
Our results demonstrate that light quality, especially the RB regime, can improve the photosynthetic process in plants sprouted from irradiated seeds through a fine-tuning between structure and function. However, besides much research toward this topic, it is still challenging to define the doses at which the hormetic effect can be expected for different crops under a specific light quality regime. In particular, the regulation of photosynthesis strongly depends on the intrinsic radioresistance of the species and the relative biological effectiveness of specific ion.

The overall experiments provided evidence for the most suitable light treatments to be adopted in synergy with other abiotic factors such as biostimulant or ionizing radiation to maximize the photosynthesis and bioactive compound

production in crops widely used human diet. The outcomes of this research may have implications not only for developing sustainable protocols for indoor cultivation but also for plant growth in extreme environments on Earth and Space, such as the orbiting stations.

Future study perspectives may implicate different issues:

- the evaluation of the positive outcomes of specific light quality treatments on plants subjected to different kinds of abiotic stress, such as water or saline stress, to verify if and how by and an enhancement of the photosynthetic performance, crops will be able to overcome the unfavourable environmental constraints, maintaining high carbon gain.
- the use of light quality to improve the commercial value of crops in terms of bioactive compounds exporting the positive outcomes of the interplay between light quality and biostimulant in controlled environments and open field.
- extending the study of the interaction light quality/ionizing radiation to other crop species, suitable to be grown in slim volumes, and other heavy ions constituting the cosmic radiation to maximize plant cultivation in the Space environment.

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