Department of Agricultural Sciences

University of Naples Federico II

Ph.D. Thesis in Agricultural and Food Sciences

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Improving plant physiological performance and growth by increasing the efficiency of lighting systems

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"…whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved."

Charles R. Darwin, The origin of species

Preface

The work presented in this thesis was carried out in the period from December 2015 to November 2018 at the Department of Agricultural Sciences and at the Department of Biology of the University of Naples Federico II. Principle supervisor was Prof. Giovanna Aronne and Co-supervisor was Dr. Carmen Arena. A period of six months was spent working with Dr. Celina Gómez in the Environmental Horticulture Department at the University of Florida. The project was funded by the Italian Space Agency (ASI) and the European Space Agency (ESA) within the framework of MELiSSA (Micro-Ecological Life Support System Alternative).

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Introduction

Plant cultivation in controlled environment has been grown considerably for commercial vegetable production in addition to research purposes in plant science. In a controlled environment, as well as in nature, light is a main factor affecting plant growth, development, and photosynthetic performance. Therefore, the yield and the quality of plant products are highly dependent on the amount of available light and its spectral composition.

Different types of electric lamps, such as fluorescent, high-pressure sodium, and light-emitting diode (LED) are used as a sole source or supplementary source in lighting systems for plant production which energy requirement can be very high. Although most light sources consist of fluorescent lamps due to the low investment costs, LEDs are undoubtedly expanding the technological frontier to increase the efficiency of lighting systems in controlled environment for plant cultivation.

The development of LED-based plant-growth systems has been firstly supported by NASA since the late 1980s, and the first use of LEDs to grow plants in space in 1995 paved the way for the development of the Vegetable Production Chamber (VEGGIE) on the International Space Station. As regards application on Earth, during the last decade the use of LEDs has been gradually increasing worldwide due to their small size, long life, narrow wavelength emission and cool emitting temperature compared with other light sources. These characteristics of LED technology are enabling to design and develop high-performance growth chambers, reducing energy consumption and optimizing cultivation volume in multi-tiered high-density growing systems. Furthermore, LED lamps allow growers to fine-tune the intensity and the spectral quality of the light source to fulfil plant requirements with an accurate species-specific approach in maximizing plant production.

Light interaction with plants is not limited to photosynthesis. In addition to light intensity and quality, plants perceive also light direction which is essential in phototropic responses. Light is a major influential stimulus on plant tropisms,

together with gravity force, and both compete and interact with each other. Considering plant cultivation in altered-gravity environment such as on the ISS, the moon or mars, light plays an unique role as an external stimulus in shaping the plant in a three-dimensional space through photomorphogenesis and phototropism. However, little is known about the interaction between plant tropisms, especially considering tropic responses of roots, and only recently advances in knowledge have been made thanks to the opportunities to experiment in absence of gravity on the ISS combining the use of LED technology.

In this context, a deep understanding of plant responses to the different characteristics of light is needed and the peculiarities of LED technology provide promising opportunities for study and research in the field of plant science.

The study and research activities carried out during this Ph.D. program were focused on plant responses to spectral composition of light by using LED technology. More specifically, the studies considered species suitable for plant production in controlled environment, with particular attention to red-leaf or reddish-leaf plants due to their contribution of antioxidant compounds to plant food.

Given that the general aim of this Ph.D. was to improve plant cultivation in Space, in addition to studies specifically focused on the effect of light on plant growth, part of the research was dedicated to interactions between light and altered gravity. To perform experiments in altered-gravity conditions it was necessary to use specific facilities such as the International Space Station (ISS), the Large Diameter Centrifuge, and the Random Positioning Machine.

For the purposes of these studies, I submitted two projects within the ASI and ESA educational calls. The first, ROOTROPS, was aimed to unravel the interaction between light quality and altered gravity on root orientation. The project was submitted to ESA within the "*SpinYourThesis*" Call and is now in negotiation phase. The second, MULTITROP, was the only winner of the ASI YiSS call 2016 and was performed on the ISS. Due to technical constraints (necessity to use a refurbished hardware) the experiment had to be performed in the dark. MULTITROP was therefore aimed to investigate on chemotropism-hydrotropism interactions in absence of gravity stimulus. The experiment was successful and we look for another flight opportunity to include light as an additional stimulus.

Aims and content of each chapter of the thesis are reported hereby.

Chapter 1 is a review which presents the current state of knowledge on the LEDs applications in plant production and has been published as a review article (Gómez & Izzo, 2018). It covers main important aspects of using light emitting diode technology in controlling the plant growth and development, emphasizing the advantages of using LED light in various horticulture production systems, including future opportunities for the expansion of the vertical farming industry, applications for space-based plant growth systems, and potential solutions to support off-grid agriculture.

Chapter 2 presents evidence on how the modulation of light spectrum can improve productivity and food quality in controlled environment. The effects of light quality on green- and red- leaf cultivars of *Atriplex hortensis*, a plant with a high nutritional value currently revalued for leafy vegetable production, were compared. Particular attention was given to the responses of plant pigments to different light treatments aiming at defining optimal light conditions to enhance the production of antioxidant pigments that increase the nutritional value of plant food. A manuscript reporting these data has been published in *Scientia horticulturae* (Izzo et al., 2019).

In *Chapter 3* the effect of small changes in light quality and intensity on the growth and physiological responses of two lettuce cultivars were evaluated. Short-term end-of-day light treatment turned out to effectively modify physiological and morphological plant responses that may ultimately lead to higher yields without increasing the daily amount of light, thus increasing the efficiency of lighting treatment. An article reporting this study has been published (Chinchilla et al., 2018).

In *Chapter 4* the role of blue and red light on morphological, physiological, and anatomical responses of reddish-leaf lettuce was evaluated performing a dose-response curve between 100% blue light and 100% red light. Blue-light dose turned out to significantly affect lettuce growth. The study has been reported at the “69th International Astronautical Conference” in Bremen and is currently under revision for publication on *Acta astronautica* journal.

Chapter 5 focuses on tropic responses of plant roots to gravity and other stimuli to further investigate how not-gravitropic stimuli can direct root growth in altered-gravity conditions. Gravity, nutrient and water interaction of stimuli for root

orientation in microgravity was evaluated reporting a part of the results of the MULTITROP project. As the senior of the three University students in the applicant team, I played a major role in all the activities carried out during the pre-flight, flight and post-flight phases of the experiment. The MULTITROP experiment was performed on the ISS, all aims were achieved and results regarding both methods for seed species selection and root tropism are presented. Some of the results have been reported in two publications: one is available as proceeding of the “2nd Symposium on Space Educational Activities”, and the other presented at the “69th International Astronautical Conference” in Bremen and is currently under revision for publication on *Acta astronautica* journal.

As last chapter a general conclusion is given. In addition, two *Appendices* are reported. The first presents ROOTROPS project that aims to evaluate the variations in tropic effects of different light wavelengths and light intensity in orienting root growth under hypergravity and simulated microgravity conditions.

In the second Appendix an outlook on the activities conducted during the Ph.D. program is presented.

Chapter 1

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Review

Increasing efficiency of crop production with LEDs

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Increasing efficiency of crop production with LEDs

1.1. Abstract

Light-emitting diode (LED) technology is paving the way to increase crop production efficiency with electric lamps. Users can select specific wavelengths to elicit targeted photomorphogenic, biochemical, or physiological plants responses. In addition, LEDs can help control the seasonality of flowering plants to accurately schedule uniform flowering based on predetermined market dates. Research has shown that the monochromatic nature of LEDs can help prevent physiological disorders that are common in indoor environments, and help reduce incidence of pest and disease pressure in agriculture, which could ultimately increase crop production efficiency by preventing crop losses. Furthermore, a significant attribute of LED technology is the opportunity to reduce energy costs associated with electric lighting. Studies have shown that by increasing canopy photon capture efficiency and/or precisely controlling light output in response to the environment or to certain physiological parameters, energy efficiency and plant productivity can be optimized with LEDs. Future opportunities with LED lighting include the expansion of the vertical farming industry, applications for space-based plant growth systems, and potential solutions to support off-grid agriculture.

Abbreviations: CO_2: carbon dioxide; DE: day extension; DLI: daily light integral; EOD: end-of-day; HPS: high-pressure sodium; ICL: intracanopy lighting; ISS: international space station; LED: light emitting diode; LD: long-day; NASA: national aeronautics and space administration; NI: night interruption; PAR: photosynthetically active radiation; PD: pre-dawn; PPF: photosynthetic photon flux; RQE: relative quantum efficiency; SD: short-day; UV: ultraviolet; UV-B: ultraviolet-B; VF: vertical farms

1.2. Introduction

The use of light-emitting diodes (LEDs) for plant lighting has revolutionized the greenhouse and controlled-environment industry. Initial interest in LEDs as a radiation source for plants centered on the opportunity to improve light sources for space-based plant growth systems [1–4]. Within the last two decades, horticultural researchers have proven that LEDs can serve as an energy-efficient replacement for incandescent lamps to control photoperiodic responses in flowering plants. Studies have also demonstrated that LEDs are viable alternatives to fluorescent lamps for sole-source lighting in growth rooms, and are currently major competitors of high-pressure sodium (HPS) lamps for supplemental lighting in greenhouses. A myriad of recent studies describe the many advantages of using LEDs for plant production, which range from the application of narrowband radiation to serve as cues that drive specific photomorphogenic, biochemical, or physiological plants responses, to applications for pest and disease management, and reductions in energy consumption from plant lighting. A review of key studies focused on increasing the efficiency of crop production with LEDs, and discussion of current and potential applications follows.

1.3. Monochromatic LEDs for plant production

1.3.1. Light-quality control of plant growth and development

A valuable attribute of using LEDs for plant lighting is the option to select specific wavelengths for a targeted plant response [5]. Broadband red light (600 to 700 nm), which typically promotes dry mass gain, stem elongation, and leaf area expansion of many plants species, has the highest relative quantum efficiency (RQE) for driving single-leaf photosynthesis [6,7]. Initial LED plant-lighting research in the 1990s proved that plants could grow and complete their life cycle with red LEDs alone, but growth and development was significantly improved when red LEDs were supplemented with small proportions of blue light [8–12]. Because blue LEDs were not widely available at the time, initial studies were conducted using red LEDs (660 nm) supplemented with blue fluorescent lamps.

Relative quantum efficiency curves indicate that broadband blue light (400 to 500 nm) is 25 to 35% less efficient that red light in driving single-leaf photosynthesis [6,7]. Cope et al. [13] described the potential factors that limit the RQE of blue light for photosynthesis: (1) approximately 20% of blue photons are absorbed by non-photosynthetic pigments (e.g. anthocyanins), which result in energy lost as heat and/or fluorescence; and (2) some blue photons are absorbed by accessory pigments (e.g., carotenoids), which can be 10 to 65% less efficient than chlorophyll molecules at transferring light-energy to the photosynthetic reaction center [13]. However, studies have shown that up to a species- or cultivar-specific threshold, increasing the proportion of blue light can increase single-leaf photosynthetic capacity and efficiency [14,15]. Increasing blue light often inhibits cell division and expansion, reducing leaf area (i.e., radiation capture) and stem elongation and increasing leaf thickness in most plant species. Bugbee [16] suggested that the reduction in radiation capture is the primary reason for reduced growth (dry mass gain) in response to higher blue light. Blue light is also known to affect leaf stomatal aperture, regulate chloroplast development, and control photomorphogenic and phototrophic plant responses primarily through the action of cryptochrome and phototropin photoreceptors [17–19]. Several studies indicate that 5 to 20% of blue light within the total photosynthetic photon flux (PPF) is needed to improve growth and development and minimize shade-avoidance responses (e.g., elongated internodes, petioles, and hypocotyls, larger, thinner leaves, decreased chlorophyll production, and early flowering) in controlled environments [10,20–24].

A general conclusion from sole-source light-quality research suggests that plant responses to LEDs are species- and sometimes cultivar-specific, and greatly depend on the stage of plant development, light intensity, duration of treatment, or other environmental interactions [23]. Dissolved chlorophyll pigments absorb light most effectively in the red and blue regions of the photosynthetically active radiation (PAR) spectrum (400 to 700 nm). Therefore, early LED systems were equipped with red and blue LEDs alone. However, because other accessory pigments (e.g., carotenoids) efficiently absorb much of the light that is poorly absorbed by chlorophyll, plants can use most of the light within PAR for photosynthesis [25]. Thus, commercial fixtures for plant production now include LEDs with peak

wavelengths beyond red and blue. In fact, white LED fixtures are increasingly being used in growing environments because they help overcome some of the complications involving LED color selection and, depending on the desired growth characteristic, may minimize unwanted responses from the range of possible plant responses to narrowband red and blue light [26]. White LED fixtures can be produced either by combining LEDs with different peak wavelengths or, more commonly, by using blue LEDs with a phosphor coating. At the expense of efficiency, the phosphor absorbs some fraction of the photons emitted by the blue LEDs and re-emits light with longer wavelengths through luminescence, generating white light [27]. The components of the phosphor coating will typically dictate the percentages of red, green, and blue light available for plant growth with broadband white LEDs.

Although green (500 to 600 nm) and far-red light (700 to 800 nm) are often disregarded as useful wavebands for photosynthesis because of their minimal absorption by chlorophyll pigments, studies suggest that they can have positive direct and indirect effects in plant growth and photosynthesis. Because red and blue photons are efficiently absorbed by chlorophyll, most red and blue light is absorbed within a few cell layers from the leaf surface, while green photons can penetrate deeper into the leaf [28]. Accordingly, Sun et al. [29] found that red and blue light drive CO_2 fixation primarily in the upper palisade mesophyll of the chloroplast, while green light drives CO_2 fixation in the lower palisade. Similarly, Terashima et al. [30] reported that with high PPF, once the upper chloroplasts of individual leaves are saturated by white light, additional green light can increase photosynthesis by penetrating deeper into the leaf and driving CO_2 fixation of inner chloroplasts that are not light-saturated by white light. Green light has also been shown to penetrate deeper into the foliar canopy than red and blue light, and can therefore increase whole-plant photosynthesis by stimulating CO_2 fixation of inner- and lower-canopy leaves [31–33]. What's more, depending on species, the RQE of absorbed broadband green light can be comparable with that of red, and higher than that of blue [6,7]. Another useful feature of green LEDs, particularly when used to create white light with narrowband red and blue LEDs, is that it can allow for a better visual

assessment of plant-status and true-leaf color, something that is typically hard to do when plants are irradiated with purple light from red and blue LEDs only.

Far-red wavelengths can regulate phytochrome-mediated morphological and developmental plant responses. In an effort to promote radiation capture and survival under a low red-to-far red spectra (i.e., similar to shade), plants develop a shade-avoidance response resulting in stem elongation and larger, thinner leaves. Park and Runkle [34] found that supplementing red and blue LEDs with far-red increased plant growth indirectly through leaf expansion, and directly through an increase in whole-plant net assimilation, defined as the rate of increased dry mass per unit leaf area. Zhen and van Iersel [35] evaluated the potential of enhancing photosynthesis in plants grown under red + blue or broadband white LEDs supplemented with far-red LEDs. The authors found that far-red light, which preferentially excites photosystem I, can increase the photosynthetic efficiency of shorter-wavelengths that over-excite photosystem II; their findings prove that different wavelengths of light can have synergistic effects that improve the overall rate of photochemistry and CO_2 assimilation [35]. Both of these studies suggest that adding far-red to fixtures with monochromatic LEDs could improve photosynthetic light-use efficiency and increase crop growth in controlled environments.

Except for studies evaluating photoperiodic control of flowering plants, most horticultural research focused on plant growth-responses to LEDs have used a constant spectral environment throughout the day, and typically, during an entire crop cycle. However, dynamic control of LED-light quality can provide the opportunity to change the spectral environment overtime, which may be required to optimize growth and development throughout a plant's life cycle. Several studies have demonstrated that end-of-day (EOD) (i.e., light applied at the end of the photoperiod) far-red can be used as an effective non-chemical means to control plant morphology in a number of crops [36–42]. Moreover, short-term exposure to pre-dawn (i.e., light applied before the start of the photoperiod) or EOD light-quality treatments can have significant effects on plant growth and morphology [43–46]. Although the mechanisms that drive biomass increase under short exposure to PD or EOD-light are unknown, they may be related to hormonal changes that affect the circadian rhythm of plants and induce instantaneous changes in stomatal

conductance and transpiration, which have been shown to strongly respond to light quality [15,47,48].

As stated by Mitchell and Stutte [26], there is no single light-quality recipe that serves all species and every stage of plant growth. However, a compromise between red and blue LEDs can typically drive photosynthesis and regulate vegetative growth of most plants. As suggested by Cope and Bugbee [20], it is likely that the optimal light spectrum for plant growth and development changes with plant age, as plants need to balance leaf area expansion (to maximize radiation capture) with stem elongation and reproductive growth. A thorough understanding of the energy balance needed by plants to regulate growth throughout their life cycle is essential to the development of LED light sources for plant applications. Furthermore, it is important to consider that plants grown indoors are typically exposed to a light spectrum that depends on the electric-lamp type used. In contrast, greenhouse-grown plants develop under broad-spectrum sunlight and sometimes receive supplemental lighting from a specific spectra provided by electric lamps. Thus, if LEDs are used to supplement sunlight, additional blue light may not be as critical as it seems to be for indoor production; that is because sunlight's broad spectrum contains significant amounts of blue light at midday, which may be sufficient for normal plant growth and development [49]. In addition, because supplemental lighting typically constitutes only a fraction of the total irradiance received by plants, mostly during light-limited periods, photomorphogenic and physiological disorders that have been reported for plants grown under narrowband lighting in growth chambers (see *Controlling physiological disorders*) are potentially less likely to occur in greenhouse production using narrowband supplemental lighting.

1.3.2. Controlling seasonality of flowering plants

Similar to growth and development, flowering responses to light quality are species- or cultivar-specific and are primarily determined by the duration of the continuous dark period within a day, also known as the critical night length [50]. Plants are typically classified into response groups based on how that critical night length affects flower regulation. Day-neutral plants flower regardless of photoperiod,

assuming other environmental and cultural factors are not limiting. Short-day (SD) plants flower most rapidly when uninterrupted dark periods are longer than some species-specific critical night length. In contrast, flower induction of long-day (LD) plants is most rapid when dark periods are shorter than a critical duration. Therefore, when the photoperiod is short, longer days (i.e., shorter nights) from day-extension lighting with electric lamps can induce flowering of LD plants or inhibit flowering of SD plants to enable vegetative growth. Similarly, night interruption (NI) or PD lighting can be effective at interrupting the dark period and thus, promoting LD photoperiodic responses [51,52]. However, NI has been shown to induce flowering in LD plant more effectively than day extension or PD lighting [50].

In addition to a critical night length, light quality is essential to control the seasonality of flowering plants. Red and far-red light-absorbing phytochromes are the primary photoreceptors that regulate flowering of photoperiodic species, although blue light, which is also weakly absorbed by phytochromes, has been shown to regulate flowering at higher intensities (e.g., 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) than the typical <2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ required from red and/or far-red light [53–56].

In the floriculture industry, commercial growers typically extend or truncate the photoperiod to accurately schedule uniform flowering of most photoperiodic-sensitive species based on a predetermined market date (e.g., mother's day, Easter, Christmas). Because of their high far-red photon emission, incandescent light bulbs used to be the lamp of choice for low-intensity photoperiodic lighting. However, with the advancement of LEDs, incandescent bulbs were quickly replaced as photoperiodic lamps because of their short-life span, and more importantly, because they were being phased out of production in many countries due to their electrical inefficiency [57,58]. Compact fluorescent lamps are more energy efficient and longer lasting than incandescent bulbs. However, their spectra has little or no effect on regulating flowering [58]. The narrow bandwidth of LEDs makes precise control of light quality possible, which has significantly broadened our understanding of how different wavebands regulate flowering. Compared to incandescent bulbs, LEDs provide significant advantages such as reducing energy and maintenance costs, accelerating flowering, or preventing excessive stem elongation in some plant species.

In a coordinated greenhouse grower trial, Meng and Runkle [59] compared LED lamps emitting primarily red and far-red radiation with incandescent bulbs to create LDs with NI; the authors confirmed that LEDs were as effective as incandescent bulbs at regulating flowering of several herbaceous ornamental crops. However, research has shown that not all LED lamps are effective at regulating flowering; their effectiveness depends on their spectral composition. Craig and Runkle [60] found that a balanced combination of red and far-red radiation from LEDs promotes flowering of several LD plants. In contrast, red-enriched radiation with LEDs works best at delaying flowering in SD plants [61]. Furthermore, Meng and Runkle [62,63] reported that NI with low far-red radiation from LEDs may not be perceived as a LD; the authors suggested that LD plants can be classified into far-red-dependent and far-red-neutral varieties based on their flowering responses to far-red radiation. Meng and Runkle [62] also found that cool-white LEDs and warm-white LEDs have a similar effectiveness at regulating flowering than red or blue + red LEDs. Relatively few studies have explored the efficacy of green radiation at regulating photoperiodic flowering. Under SDs, NI or DE (day extension) with low or high intensity green LEDs were shown to inhibit flowering of SD plants [64–66]. However, similar to blue-light flowering responses, the degree of flowering regulation with green LEDs seems to depend on intensity and/or treatment duration, and are most likely species-specific. Meng and Runkle [55] suggested that because green radiation can exert an inhibitory flowering effect in some species similar to that of low-intensity red light, a combination of green and red LEDs could be more effective at inhibiting flowering of SD plants than either waveband alone. Lastly, flowering responses to light quality also seem to be dependent on daily light integral (DLI), which refers to the cumulative number of photons within PAR received during a 24h period. Kohyama et al. [67] found that adding far-red to red + white radiation in NI promotes flowering of some ornamental species under a low DLI (≤6 $mol \cdot m^{-2} \cdot d^{-1}$) but not under a DLI <12 $mol \cdot m^{-2} \cdot d^{-1}$.

1.3.3. Controlling physiological disorders

The monochromatic nature of LEDs can lead to physiological disorders in some plant species or cultivars that are typically not present when plants are grown under

broadband light. For example, intumescence, a cultivar-specific physiological disorder that is characterized by abnormal outgrowth of cells on plant surfaces (typically induced by abiotic stress), was first associated with a lack of ultraviolet (UV; 300 to 400 nm) and far-red radiation in the spectral environment [68,69]. Others have found that UV radiation can prevent intumescence development on susceptible cultivars of tomato (*Solanum lycopersicum*) and ornamental sweet potato (*Ipomoea batatas*) [70–72]. Similarly, far-red and/or blue light have been shown to mitigate intumescence injury in tomato and cowpea (*Vigna unguiculata*) in UV-deficient light environments [5,69,73–75]. Due to the damage in the photosynthetic tissue, intumescence not only impairs the physiological processes of plants, but also negatively affects the overall aesthetic quality of plant products, which is a significant concern with ornamental crops grown under sole-source lighting with narrowband LEDs.

As previously mentioned, red light alone can result in suboptimal growth and development of many plant species [8,10–12,14]. Research has shown that 100% red light can lead to physiological disorders that can result in undesirable stem elongation and/or deficient chlorophyll biosynthesis, leading to low photosynthetic rates due to a dysfunctional photosynthetic apparatus [15,76,77]. The mechanisms behind the suboptimal growth under monochromatic red light are not yet fully understood and seem to be species-specific, as some plants have been shown to produce higher biomass under 100% red light compared to a combination of wavelengths [75,78,79].

1.3.4. Reducing incidence of pest and disease pressure

Irradiating plants with specific wavebands of LEDs has potential for reducing pest pressure and suppressing plant disease in production environments, which could ultimately increase crop production efficiency by preventing crop losses. The role of light quality in plant disease resistance has been correlated with light-induced signaling pathways that interact with plant-defense regulatory mechanisms. Effects of light quality on secondary metabolite accumulation (e.g., flavonoids) have been associated with plant immunity, disease development, and insect interactions [80–82]. A large number of studies have reported increases in light-quality-induced

secondary metabolite as means to increase nutritional attributes of plant products [83]. However, fewer studies have focused on evaluating the level of plant protection in response to light-induced, metabolite-based resistance.

Ultraviolet LEDs, namely those with peak wavelengths in the ultraviolet-B (UV-B; 320 to 290 nm) region of the spectrum, can effectively control powdery mildew caused by *Sphaerotheca aphanis* in strawberry (*Fragaria* × *ananassa*) [84] and *Podosphaera pannosa* in rose [85]. Similarly, blue LEDs have been shown to inhibit the development of *Botrytis cinerea* in detached tomato leaves and, when used as PD and EOD treatments, can reduce incidence of black leaf mold (*Pseudocercospora fuligena*) in greenhouse-grown tomato [86,87]. Green LEDs have been proven to be effective for controlling strawberry anthracnose (*Glomerella cinglata*) [88], leaf spot disease (*Corynespora cassiicola*) in perilla (*Perilla frutescens*) [89], and cucumber anthracnose (*Colletotrichum orbiculare*) and gray mold (*Botrytis cinerea*) [90]. It has also been reported that red LEDs can induce resistance to powdery mildew caused by *Podosphaera pannosa* in roses [91], *Sphaerotheca fuliginea* in cucumber (*Cucumis sativus*) [92], and downy mildew in basil caused by *Peronospora belbahrii* [93].

The use of narrowband LEDs is also a promising approach for increasing the attractiveness, specificity, and adaptability of conventional insect traps. Adding UV, green, and/or yellow LEDs to insect traps has been shown to increase the capture efficacy of fungus gnats (*Bradysia difformis*), greenhouse whitefly (*Trialeurodes vaporariorum*), oriental fruit fly (*Bactrocera dorsalis*), biting midges (*Culicoides brevitarsis*), red flour beetle (*Tribolium castaneum*), sweet photo weevil (*Euscepes postfasciatus*), and cotton bollworm (*Helicoverpa armigera*), among others, compared to non-LED-supplemented traps [94–103].

A variety of factors can influence the effectiveness of light quality to reduce incidence of pest and disease pressure, including the specific peak wavelength of LEDs, light intensity, and time of exposure. However, with the advancement of LED technologies, there is an increasing interest in using light quality as an integral component of pest management programs that can reduce the dependence on environmentally hazardous pesticides. Although research has shown that LEDs can suppress some diseases and reduce pest pressure that are of economic importance

in major crops, consideration should be placed to the indirect effects that applying this light-quality treatments will have on plant growth and development.

1.4. Reductions in energy consumption

The cost of electricity to provide electric light in controlled and semi-controlled (greenhouse) environments is high. In 2014, Nelson and Bugbee [104] published an economic analysis comparing electric costs of using multiple lighting technologies, including ten types of LED fixtures. The authors concluded that the cost per photon delivered from LEDs was higher than that of all traditional horticultural lamps (e.g., HPS, cool-white fluorescent, metal halide), and that at the time, the best HPS and LED fixtures had nearly identical efficiencies ($\mu mol \cdot J^{-1}$) [104]. More recently, Wallace and Both [105] compared the energy efficiency of various LED and HPS lamps and also concluded that the best HPS and LED fixtures had similar efficiency. However, electrical efficiency of LEDs continues to increase, and as the technology improves and the capital cost for purchasing LED equipment decreases, the cost per photon will likely continue to decrease. Moreover, if canopy photon capture efficiency is maximized, lighting system efficiency of LEDs can be significantly increased by capitalizing from “precision lighting”.

1.4.1. Intracanopy lighting (ICL)

The relative coolness (i.e., low radiant heat output) of LED surfaces allows for high flexibility in lamp placement and resulting light distribution within plant canopies. The ability to focus radiation close to plant canopies means that less energy is needed to achieve target PPFs than if a hot light-source is located further away from the crop surface. In an effort to increase the efficiency of irradiation by allowing direct light into the inner canopy of crop stands, Frantz et al. [31,106] performed initial proof-of-concept studies with ICL using 15-watt fluorescent lamps; the authors demonstrated that by maintaining irradiance of the inner foliar canopy above the light-compensation point, ICL-grown cowpea could yield 50% of the edible biomass using only 10% of the total input energy compared to traditional top lighting. Subsequently, when LEDs became readily available for research, Massa et al. [107]

validated previous ICL studies using LED “lightsicles” that were individually energized at different vertical planes to keep pace with plant growth. Others have confirmed that ICL with LEDs can prevent a decrease in photosynthesis and premature senescence of lower-canopy leaves grown with sole-source lighting [107] or supplemental lighting [108–111]. Moreover, Gómez and Mitchell [112] reported significant energy savings from supplemental lighting when using ICL with LEDs compared to HPS lamps. In contrast, Dueck et al. [108] reported an increase in energy consumption when growing tomatoes with intracanopy supplemental lighting with LEDs. However, the higher energy consumption was attributed to the higher heating requirements with LEDs compared to HPS, as ‘waste’ thermal energy from HPS lamps typically helps offset winter heating costs in greenhouses [113].

1.4.2. Targeted lighting

Because ‘waste’ heat is removed remotely from the photon-emitting surface of LEDs, lamps can be placed close to crop surfaces without overheating or scorching plants. Moreover, because LEDs and their fixtures can be designed to cast narrow beams of light, targeted lighting can be applied by selectively switching on LEDs positioned directly above individual plants as they grow. Poulet et al. [21] reported that targeted, close-canopy lighting of lettuce using red and blue LEDs reduced energy consumption per unit dry mass by 32 or 50% compared to total coverage sole-source lighting using either broadband LEDs or red + blue LEDs, respectively.

1.4.3. Dynamic control of LEDs

An underutilized property of LED fixtures is their ability to precisely control PPF with dimming in response to the environment or to certain physiological parameters. As described by van Iersel [114], controlling the intensity of the light output of LEDs can be accomplished using one of two methods: (1) current control or pulse width modulation (i.e., control of the frequency at which LEDs are turned on and off; typically thousands of times per second); or (2) duty cycle control (i.e., fraction of time the LEDs are energized during each on/off cycle).

Pinho et al. [115] evaluated dynamic lighting as a way to control LED supplemental lighting; their system automatically compensated for variation of sunlight PPF at plant canopy level. The authors used an on-off switching algorithm in order to maintain a constant PPF with LEDs and reported a 20% reduction in energy consumption compared to HPS lamps [115]. Similarly, Clausen et al. [116] and Schwend et al. [117] reported 25% and 21% reduction in energy consumption, respectively, when sensor-based dynamic LED lighting was adjusted based on the environment. More recently, van Iersel and Gianino [118] demonstrated that by adjusting the duty cycle of LEDs based on the ability of plants to use light efficiently, an adaptive LED light controller can reduce the energy costs of supplemental LED lighting by preventing the PPF at canopy level from dropping below a user-defined threshold. In practice, their system allows for supplemental lighting with LED fixtures to automatically provide more light when there is little sunlight and dim as the amount of sunlight increases [118]. Early trials with this adaptive system showed that energy consumption can be reduced by 60% with only a 10% decrease in crop biomass, as compared to timer-controlled LED fixtures [119]. In a separate set of studies, van Iersel et al. [120,121] focused on adjusting PPF based on the physiological properties of crops, rather than on changing light intensities. They showed that a biofeedback system that relies on a chlorophyll fluorometer and a quantum sensor to measure the quantum yield of photosystem II and PPF, respectively, can determine the electron transport rate, compare that value to a user-defined threshold, and then change the light output of the LED light (either by changing the duty cycle or current) to maintain a range of different electron transport rates in a variety of species [120,121]. Similarly, Carstensen et al. [122] used a remote-sensing approach with a spectrophotometer to sense the dynamics of chlorophyll fluorescence emission from a plant canopy; the authors developed a model that appears to be indicative of the light-use efficiency and light-induced stress of plants [122]. As shown in these studies, dynamic control of LED lighting can help optimize energy efficiency and plant productivity with LEDs.

1.5. Future of LEDs

1.5.1. Vertical farms (VFs)

Commercial VFs produce high-value plant products in multi-tiered, high-density growing systems. As suggested by others [123], LEDs are adequate candidates for sole-source photosynthetic lighting in VFs because fixtures typically have low power density per unit growth area ($kW \cdot m^{-2}$) and can deliver high light intensities with low radiant heat delivered to crops. Initial efforts to produce high-value crops in warehouse-based plant factories used water-cooled HPS lamps; however, the high-energy consumption needed to produce with HPS lamps negated economic viability [26]; follow-up research used fluorescent lamps, which became standard in controlled environments [124]. However, LEDs are now widely used in VFs in Asia and are gaining popularity in other countries, where commercial VFs produce a variety of leafy greens, young plants, and low-profile fruit crops. Kozai et al. [125] includes a comprehensive review of the of the many potential applications of LEDs in urban agriculture. Akiyama and Kozai [126] described the impact of LED fixture design (lamp and plant spacing) on the spatial distributions of PPF in a simulated VF. Ibaraki [127] showed that depending on canopy structure, LEDs can be used to control the direction of light and reduce the distance between lamps and plants, thus maximizing light-use efficiency in terms of irradiance ($W \cdot m^{-2}$) and PPF ($\mu mol \cdot m^{-2} \cdot s^{-1}$). According to Hayashi [128], improvements in cost reduction, energy efficiency, quality, intensity, and flexibility of LED fixtures have driven VF research in Japan, the Netherlands, England, Taiwan, South Korea, and the U.S. In addition, future projects are targeting applied research to support relevant VF concepts in the Middle East, Central and South America, and Africa. Currently, the VF industry in North America is considered to be in its initial stage, with many entrepreneurs and growers investing in different lighting technologies given the lack of an acceptable business model or standard method of implementing LED technologies [129]. However, some expect that the increasing number of horticultural-grade LED manufacturers will establish models to help improve production efficiency with standardized light-quality formulas and suggested methods for minimizing production costs [130]. One critical aspect to consider is the cost-effectiveness of

VF, particularly when used to produce food crops. Most Asian and European countries that have successfully adopted VF with LEDs have land and/or environmental limitations for producing high-quality fresh food, which, coupled with food-safety concerns, justify the high sale-value in those regions of the world. However, research has shown that although some U.S. consumers are willing to pay premium prices for locally-grown produce, the average consumer is hesitant to purchase costly food grown in VFs [131]. Moreover, Yano et al. [132] showed that consumers believe that LEDs could negatively affect the nutritional attributes and overall taste desirability of plant products. There is a need to improve public perception about VF in an effort to establish a reliable consumer base that will drive the industry forward.

1.5.2. Space farming

The development of LED-based plant growth systems has been supported by NASA since the late 1980s for research in the International Space Station (ISS), to evaluate bioregenerative life-support systems, and to support future colonies on the Moon and Mars [133,134]. The first use of LEDs to grow plants in space in 1995 [135] paved the way for the development of the Vegetable Production Chamber (VEGGIE), which has demonstrated the feasibility of supporting a space garden in the ISS [136,137]. Mitchell et al. [138] estimated that up to 50 m^2 of cropping area are needed to sustain one crewmember on a mission, which highlighted a challenging energetic cost for the NASA Biomass Production Chamber [139]. In this context, LEDs play a key role at enabling energy-affordable food production in controlled environments intended for life support in space [21,107,112,140]. Furthermore, the small size of LEDs contribute to reducing the equivalent system mass of a lighting system, which can attenuate the overall cost of a space mission [141]. Another attractive feature of LEDs for space applications is that their long lifetime and reliability can significantly reduce maintenance costs and astronaut labor requirements for plant growth systems [142]. In addition, the solid-state electronics of LEDs ensure safety and affordable risk management strategies that are highly important in manned space missions [2]. The influence of the space-flight

environment on plant growth has been highlighted in several studies [143–148], which indicate that there is a critical need to conduct research that will support the goal of providing efficient plant-based bioregenerative life support systems in extreme environments (e.g., altered gravity, ionizing radiations, ultradian rhythms). The implementation of LEDs in the ISS laboratories allows evaluations of important questions in fundamental biology aiming at improving our knowledge about plant production in space [149,150].

1.5.3. Off-grid plant production

Improvements in robustness and cost reduction of LEDs have made access to electric lighting a reality for rural communities that used to solely depend on fuel-based lighting [151]. The low-energy requirement of LEDs in combination with photovoltaics has led to the development of solar-powered LED systems, which may offer significant opportunities for off-grid agricultural applications [152].

1.6. References

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Chapter 2

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Original paper

Light quality shapes morpho-functional traits and pigment content of green and red leaf cultivars of *Atriplex hortensis*

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Light quality shapes morpho-functional traits and pigment content of green and red leaf cultivars of *Atriplex hortensis*

2.1. Abstract

Recently the value of red-leaf species as food plants is increasing due to their high content in antioxidant compounds, mainly anthocyanins. Most work has been done on the modulation of light quality to maximise the production of antioxidant compounds in reddish leafy vegetables that mostly adjust the amount of foliar anthocyanins as a reaction to several environmental factors, including light. The aim of this study was to compare the effect of light quality on green- and red-leaf cultivars with a focus on the influence that different light wavelengths have on morpho-functional traits and pigment content. We selected as model plant *Atriplex hortensis* considering that cultivars with either fully-red or fully-green leaves are available. This species is characterized by the presence of betacyanins, an anthocyanin-homologue pigment known for its antioxidant properties. Plants were grown under four lighting treatments: 100% white light (W), 100% red light (R), red/blue light 50/50 % (RB), and red/green/blue light 33/33/33 % (RGB). All treatments provided a daily light integral (DLI) of 10.8 $mol \cdot m^{-2} \cdot day^{-1}$ over a 12-h photoperiod. Results showed that, in both green and red cultivars, light quality determines changes in morpho-functional traits and the combination of red and blue wavelengths enhances productivity and betacyanin content. In red plants, betacyanin content was two order of magnitude larger than in green plants, was significantly modulated by light quality, and increased according to the increasing percentage of blue wavelengths within the light spectrum. In the framework of enhancing antioxidant compounds in plant food through the adjustment of light spectrum, fully red plants should be considered as more promising than green cultivars.

2.2. Introduction

In the last few years, red leaf plants have aroused intriguing scientific debates, not only investigating their evolutionary and ecological significance [1-5], but especially to assess their value as food plants for their nutritional contribute based on the high content in antioxidant compounds [6-9]. In most species, the red colour is due to the accumulation of anthocyanins. This red pigment is well-known for its antioxidant properties and therefore considered as an additional nutritional value of red-leaf plants [10-12]. Previous studies indicate that anthocyanins carry out several functions in plants, representing the "nature's Swiss army knife" [13]. A role in photoprotection is widely accepted, although this hypothesis is not always applicable and several exceptions are mostly explained by considering plant/herbivores interactions or complex responses due to multiple stresses [13-14]. Betalains, originally called 'nitrogenous anthocyanins' [15], are a class of water-soluble compounds exclusive to the order Caryophyllales [16] occurring in the seeds, fruits, flowers, leaves, stems, and/or roots of species from a wide range of natural environments [17-20]. Within betalain pigments, betacyanins are known as anthocyanin homologues because of similar histological location, similar array of red colours and similar antioxidant properties; moreover, they are inducible by similar environmental cues, including light, UV radiation and abiotic stressors such as drought, low temperatures and salinity [21]. In the framework of food processing, betacyanins are reported to be more stable than anthocyanins in a broader range of pH and thermal treatments [22]. From a chemical and physical point of view, both anthocyanins and betalains, generally located in vacuoles of epidermal and/or mesophyll cells in leaves, strongly absorb light in the ultraviolet, blue and green wavelengths [21,23-25]. Interestingly, layers of cells with red pigments as anthocyanins or betacyanins can act as a spectral filter with peculiar properties in terms of absorption and diffusion, influencing the intensity and the quality of light through the mesophyll [26].

Plant responses to light quality are studied by using light-emitting diode (LED) technology, and understanding the effect of different light wavelengths on productivity and quality traits of crop species is an advanced research field. Thanks

to previous research LED technology is currently applied to increase the efficiency of crop production in controlled environment [27] . The ability to control the spectral composition of LED-light sources enables to develop efficient lighting systems customised to satisfy specific crop requirements [28]. Recently, the continuous technological advancement of LEDs has made this light source even more efficient and affordable to develop sustainable plant factories with artificial lighting (PFALs) [29] and also enabling plant cultivation in Space in the sight of long-term manned missions to Moon or Mars [30,31].

Several studies have shown that the spectral composition of light radiation can activate different photoreceptors that prime complex signalling, ultimately resulting in specific physiological and biochemical responses [32-34]]. Previous studies reported that red light has the highest relative quantum efficiency (RQE) in driving single-leaf photosynthesis and typically promotes biomass production and leaf area expansion, while blue light is less efficient than red light [35,36]. A significant amount of blue photons are absorbed by: a) non-photosynthetic pigments (e.g. anthocyanins) which results in energy loss as heat and/or fluorescence, and b) accessory pigments (e.g. carotenoids) which are less efficient than chlorophyll at transferring light-energy to the photosynthetic reaction centres [37]. Blue light affects several plant responses including stomatal opening, chloroplast movements, leaf expansion, shoot elongation, enzyme synthesis, and phototropism [38-43]. The blue-light induced reduction of leaf lamina expansion [44-46] might be ascribed to inhibition of cell division or enlargement found as response to blue light [47-49].

As regards green light, the RQE is comparable with that of red, and higher than that of blue light [35,36]. Green light can penetrate deeper through the canopy and into the leaf than red and blue light and can therefore increase CO_2 fixation of inner chloroplasts enhancing whole-plant photosynthesis [50,51]. However, a general conclusion from sole-source light-quality research suggests that plant responses to light quality are species- and sometimes cultivar-specific [52]. In addition, considering cultivars characterised by different leaf colour (e.g. green vs red leaf), plant responses to light quality can be affected by the spectral adjustment imposed by anthocyanin layers in leaves [26]. As for pigment production, it has been

demonstrated that also leaf functional traits and leaf anatomy are affected by light quality regimens [53]. Considering that anatomical characteristics of leaves influence photosynthesis and radiation capture [54,55], a better understanding of how different light wavelengths affect the leaf anatomy could give insights to comprehend plant developmental and physiological responses to light quality.

Most of the experiments testing the effects of light quality on plant growth refers to green- or reddish-leaf species [56-61], while studies on the effects of the different spectral composition of light radiation on plants characterised by fully red leaves are scarce [62]. In reddish plants, the accumulation of considerable levels of anthocyanins is typically a reaction to environmental conditions (mainly temperature and light). Leaf reddening (from green to red) is a continuum phenomenon that can be modulated by adjusting mainly light intensity and also light quality. More specifically, reddening is due to an increase in anthocyanins content according to the photon flux density and to the amount of blue wavelengths within the light spectrum [63-65]. Most recently, studies on the use of reddish plants as food products are aimed at identifying the best light wavelength combination to maximise the production of anthocyanins and other beneficial antioxidant compounds [58,63]. Son and Oh [66] reported a positive effect of providing a mixture of blue and red LEDs on both crop quality and yield in green and reddish cultivars of lettuce.

Although leaf reddening is a natural and widespread reaction of plants to several conditions, only a limited number of species have evolved genotypes whose leaves are constantly and uniformly red (purple) independently from specific environmental conditions. Considering that in red leaf plants the quantity of anthocyanins is naturally much higher than their green relatives [26], further research on the interactions between light quality and red leaf species is needed to maximise the accumulation of antioxidant compounds as anthocyanins aiming at healthy food production. In this context, the possibility to select species capable to adapt and benefit from the light spectrum modulation gives the opportunity to improve plant food production. Indeed, plants grown under different light spectra are reported to modulate the synthesis of various beneficial antioxidant compounds, including anthocyanins [67,68]. The hypothesis is that high-energy wavelengths stimulate

anthocyanin production to protect photosynthetic tissues, thus chloroplasts, from energizing wavelengths that commonly induce inhibition phenomena of the photosynthetic apparatus [69].

The general aim of this work was to investigate the effect of different light qualities on morpho-functional traits and pigment content in both fully-red and fully-green plants of the same species using the green cultivar to perform a comparative analysis. We selected *Atriplex hortensis* L. (Order Caryophyllales, family Amaranthaceae) as model plant because it is one of the few species including cultivars with either fully-red or fully-green plants. We hypothesised that both cultivars are affected by light-quality treatments and that betacyanin content, which is already high in the red cultivar, may increase in response to increasing blue light.

2.3. Materials and Methods

2.3.1. Plant material and growing conditions

Atriplex hortensis was used as model plant. This species is reported to have a high nutritional value [70] and is currently revalued for leafy vegetable production and suitable for controlled environment horticulture [71]. As most species belonging to order Caryophyllales, *A. hortensis* is characterized by the presence of betacyanins [16], an anthocyanin-homologue pigment known for its antioxidant properties [21] and often simply referred to as anthocyanins [72-75]. Within *A. hortensis* two cultivars are available: one with fully-red leaves (var 'Rubra') the other with fully-green leaves (var 'Alba'). Seeds of the two cultivars were incubated on agar medium (1%) in Petri dishes at 18 °C for 5 days. Seedlings were subsequently transplanted into plastic pots (350 mL individual volume) filled with horticultural grade substrate composed of 70% peat and 30% perlite by volume. Throughout the experiment, plants were sub-irrigated with plain water supplemented with a water-soluble fertiliser (4N-2P-4K; Plagron®, Weert, The Netherlands) every 5 days.

Plants were grown on 28 × 61 × 60 cm (length x width x height) compartments inside a growth chamber. Average ambient day and night air temperature of the chamber was set at 20 °C. However, radiation from the lamps raised the ambient temperature during the photoperiod, which was uniformly maintained at 21±1 °C by installing

cooling fans. Set point for relative humidity (RH) was 60 to 80%. An additional shielded temperature and RH sensor was placed in each treatment compartment to provide real-time data monitoring and to ensure that ambient temperature differences among treatments were ≤1 °C.

2.3.2. Lighting treatments

Plants were grown under four light spectra: 100% white light (W), 100% red light (R), red/blue light 50/50 % (RB), and red/green/blue light 33/33/33 % (RGB). All treatments were performed using LED light sources. As regards white light, we used phosphor-coated white LEDs with a colour temperature of 4700K (neutral white) for the full-spectrum treatment. Wavelength emission spectra were: 400–700 nm for W, 660 ± 5nm for R, 460 ± 5nm for B, and 530 ± 5nm for G. All treatments provided a daily light integral (DLI) of 10.8 $mol \cdot m^{-2} \cdot day^{-1}$ over a 12-h photoperiod. Photosynthetic photon flux density was measured at mid-canopy height using a portable PAR-FluorPen FP 100-MAX-LM (Photon System Instruments, Czech Republic). Light mixing (≤5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) within treatments was minimised by properly using reflecting and darkening sheets. Within each treatment, pots were daily rotated randomly to minimise difference in microclimate within the experimental area. One-month-old plants were used for the analyses that included: biometry, leaf anatomy, chlorophyll *a* fluorescence emission and leaf pigment content (chlorophylls, carotenoids and betacyanins).

2.3.3. Quantification of morphological traits

Plant height, number of leaves, internode length (considering the first internode from the base of the stem), total leaf area, fresh weight and dry weight of leaves were measured in 5 randomly-selected plants per treatment for both cultivars. Total leaf area was measured through digital photos of leaves captured with a camera (Canon 60D equipped with a Canon EF 24-105mm f/4L IS II USM lens) and analysed with Image J 1.45 (National Institutes of Health, Bethesda, Maryland, USA). For the measurement of dry weight, leaves were oven-dried to a constant mass at 70 °C.

2.3.4. Microscopy and digital image analysis

5 fully-expanded leaves (at the second node from the base of the stem) from 5 plants per treatment for both cultivars were collected for microscopy analyses, immediately fixed in FAA (40% formaldehyde: glacial acetic acid: 50% ethanol - 5: 5: 90 by volume) and stored at 4°C. Each leaf was dissected to obtain subsamples (approximately 5x5 mm) from the median portion of the lamina. Subsamples were dehydrated in an ethanol series (up to 95%) and embedded in the JB4® acrylic resin (Polysciences, Warrington, PA, USA) for sectioning. Semi-thin cross sections (5 µm) of the leaf were cut with a rotative microtome. Sections were stained with 0.025% Toluidine blue in 0.1M citrate buffer at pH 4 [76], mounted with distilled water and observed under a transmission light microscope (BX60, Olympus, Tokyo, Japan). Images were collected through a digital camera (CAMEDIA C4040, Olympus, Tokyo, Japan) and analysed with AnalySIS 5.0 (Olympus, Tokyo, Japan).

Leaf anatomy characterization was carried out by measuring the thickness of leaf mesophyll and epidermis in 5 regions of the leaf cross-section. Stomata of abaxial and adaxial epidermis were counted along the whole section. Stomatal density was calculated as number of stomata per unit of length ($n \cdot mm^{-1}$). Whenever palisade and spongy tissues were not clearly distinguishable, due to low elongation of palisade cells, we separated lower and upper mesophyll by a virtual line across the leaf vascular bundles. The percentage area occupied by chloroplasts in the lower and upper mesophyll was calculated per unit mesophyll surface using digital photos analysed with ImageJ.

2.3.5. Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence measurements were carried out using a portable PAR-FluorPen FP 100-MAX-LM (Photon System Instruments, Czech Republic) on 5 fully-expanded leaves from 5 plants per treatment for both cultivars. Leaves and plants were selected as for anatomical analyses. The ground fluorescence (F_o) was induced by an internal blue-light (1-2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on 30 min dark-adapted leaves. The maximal fluorescence in the dark (F_m) was induced by 0,8 s saturating light pulse of 3000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The PSII maximal photochemical efficiency (F_v/F_m) was calculated as the ratio of variable fluorescence to maximal fluorescence, where

$F_v=F_m-F_o$. The measurements in the light were carried out into the growth chamber. The quantum yield of PSII electron transport rate (QY) was determined by means of an open leaf-clip suitable for measurements under ambient light, according to Genty *et al.* [77]. Non-photochemical quenching (NPQ) was calculated following Bilger and Bjorkman [78], according to the following formula: NPQ = $(F_m/F_{m'})-1$, where $F_{m'}$ represent the maximal fluorescence in light-adapted leaves.

2.3.6. Determination of pigment content

5 leaves from 5 plants per treatment for both cultivars were used for the determination of pigment content. Leaves and plants were selected as for previous analyses. Total chlorophylls and carotenoids were extracted in ice-cold 100% acetone. The extracts were centrifuged at 5000 rpm in a Labofuge GL (Heraeus Sepatech, Hanau, Germany) for 5 minutes and then the absorbance of supernatants was determined by a spectrophotometer (UV-VIS Cary 100, Agilent Technologies). Pigment content was calculated according to Lichtenthaler [79].

The extraction and quantification of betacyanins were accomplished by method as described by Schliemann *et al.* [80]. Samples were frozen in liquid N_2, and extracted with 80% aqueous methanol containing 50 mM ascorbate. The extracts were centrifuged at 15000 g for 10 min. The absorbance of supernatants was determined at 538 nm by a spectrophotometer (UV-VIS Cary 100, Agilent Technologies) and betacyanin concentration was determined using the extinction co-efficient described by Wyler *et al.* [81].

2.3.7. Statistical analysis

The influence of the two different categorical independent variables (i.e. Atriplex cultivar - 2 - and light quality - 4 -), and their possible interaction, on each of the continuous dependent variables were studied by applying two-way analysis of variance (ANOVA). In case of rejection of the null hypothesis the Student–Newman–Keuls (SNK) post-hoc test was used ($p<0.05$). The normality and homogeneity of the variances of the datasets were assessed by the Shapiro Wilk's test and Levene's test, respectively. If needed, the variables were logarithmic transformed to achieve the normality and homogeneity of the variances. Data expressed as percentage

were transformed through arcsine function. The Sperman's rank correlation coefficient was calculated to evaluate the relation between the betacyanin content and light quality with $p<0.05$. All data were processed using Microsoft Excel and STATISTICA ver. 8.0 (StatSoft, Inc. 2008).

2.4. Results

Light quality affected morphological traits of green and red plants of *A. hortensis* (Table 1). With the exception of total leaf area and leaf dry weight parameters, plants of both cultivars grown in white and RGB light developed similar height, number of leavers and internode length. RGB induced a decrease in total leaf area of plants especially in green cultivar. The combination of red and blue light maximised plant growth in both cultivars, while red light negatively affected dry biomass production especially in red plants. Under R treatment, red plants, compared to green plants, developed leaves with similar biomass production but showed a tendency to develop larger lamina, therefore resulting tenderer.

Table 1 Effects of cultivar and light quality on morphological traits of *Atriplex hortensis* plants

Cv	Light quality	Height (cm)	Leaves$^{n.s.d.*}$ (n.)	Internode (cm)	Total leaf area (cm^2)	Fresh Weight leaves (g)	Dry Weight leaves (g)
Green	R	14.9±1.6b	11.2±0.5	2.24±0.20ab	28.1±1.7cd	0.96±0.07cd	0.13±0.00ce
	RB	22.4±1.8a	14.4±0.7	2.72±0.18a	42.1±3.7ab	1.54±0.09a	0.26±0.01a
	RGB	18.2±1.9b	13.6±0.7	2.33±0.22ab	24.6±2.5d	0.99±0.05bcd	0.13±0.00ce
	W	17.6±1.4b	14.4±0.4	2.13±0.12ab	39.5±2.6abc	1.30±0.09b	0.21±0.01b
Red	R	13.9±0.7b	13.2±0.5	1.82±0.07b	36.3±3.3abc	0.88±0.09d	0.07±0.00d
	RB	23.4±1.5a	14.8±0.5	2.77±0.12a	37.4±3.8abc	1.22±0.08bc	0.15±0.01c
	RGB	17.6±0.7b	14.4±0.4	2.15±0.11ab	31.8±1.3bcd	1.11±0.04bcd	0.11±0.00e
	W	17.9±1.0b	14.8±0.5	2.14±0.14ab	44.4±2.8a	1.14±0.04bcd	0.16±0.01c

Each value represents the mean ± SE, n=5. Different letters indicate significant differences among the combination of light quality and cultivar. The light quality treatments of red, red-blue, red-green-blue, and white for the two cultivars (G: green and R: red) are showed as R, RB, RGB and W, respectively. **not statistically different*

Plants of *A. hortensis* developed shortly petiolate and opposite leaves, characterised by a widely triangular blade with a sagittate or cordate base and entire or sparely toothed margins (Fig. 1A, B). Anatomical analysis showed that leaf blades

have a dorsiventral structure generally characterised by a palisade and a spongy tissue with a few intercellular spaces in plants developed under W, RB and RGB light regimes (Fig. 1C, D). The palisade tissue often consisted of two layers of elongated cells. In plants developed under R light, cell elongation in the palisade parenchyma was reduced; this phenomenon was very clear in red leaves where it was not possible to distinguish palisade from the spongy parenchyma cells (Fig. 1E, F).

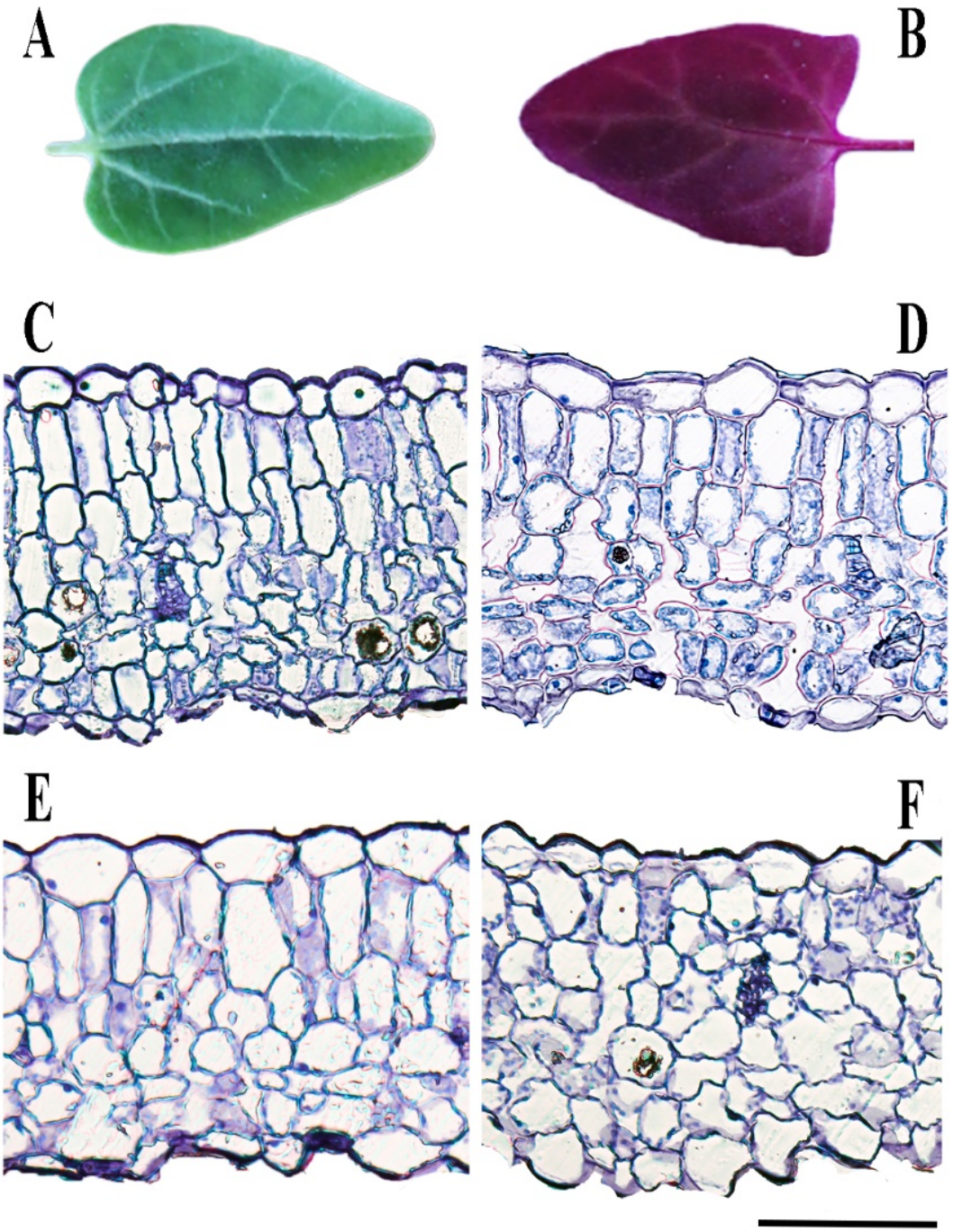

Fig. 1. Leaf morpho-anatomical traits of green and red plants of *A. hortensis*. Top view of green (A) and red (B) leaves. Light microscopy views of cross-sections of green (C) and red (D) leaves under white light; cross-sections of green (E) and red (F) leaves under red light. Images of leaf cross-sections are at the same magnification. Leaf sections from plants developed under RB and RGB light are not shown because they were not different from those shown in A and B. Bar = 100 µm.

Red leaves accumulate betacyanins in the vacuoles of the epidermal cells. Quantitative anatomical analysis showed that leaf thickness and epidermis thickness were similar in both cultivars and were not affected by light treatments (Table 2).

Table 2 Effects of cultivar and light quality on anatomical parameters of *Atriplex hortensis* leaves

Cv	Light quality	Leaf thickness$^{n.s.d.*}$ (µm)	Epidermis thickness$^{n.s.d.*}$ (µm)	Stomatal density (abaxial) (n. mm^{-1})	Stomatal density (adaxial) (n. mm^{-1})	Chloroplasts in lower mesophyll (%)	Chloroplasts in upper mesophyll (%)
Green	R	231.2±19.8	48.5±4.2	18.8±2.3a	2.63±0.81a	5.17±0.55a	6.34±0.46b
	RB	223.5±24.8	53.4±5.0	21.6±4.4a	0.66±0.15b	4.00±0.61a	7.96±0.33a
	RGB	253.6±45.0	48.2±5.4	14.6±3.2a	0.52±0.18b	5.67±0.51a	7.76±0.31a
	W	196.8±19.3	38.4±3.2	20.2±5.6a	0.74±0.13b	5.98±0.74a	8.90±0.62a
Red	R	155.3±20.7	43.8±3.4	6.45±1.5b	0.83±0.39b	3.40±0.32b	4.55±0.23c
	RB	270.8±21.4	50.1±5.5	4.87±1.2b	0.69±0.17b	3.69±0.33b	6.17±0.19b
	RGB	274.2±41.6	61.5±4.0	5.26±1.8b	0.37±0.07b	3.78±0.34b	5.29±0.31bc
	W	171.6±17.7	46.2±3.7	7.81±2.1b	0.69±0.15b	3.89±0.12b	5.70±0.40bc

Each value represents the mean ± SE, n=5. Different letters indicate significant differences among the combination of light quality and cultivar. The light quality treatments of red, red-blue, red-green-blue, and white for the two cultivars (G: green and R: red) are showed as R, RB, RGB and W, respectively. **not statistically different*

Leaves of *A. hortensis* are clearly hypostomatic (Table 2) and red leaves developed about three times less stomata than green leaves. Within each cultivar, the number of stomata on the abaxial lamina was not affected by lighting treatments and was higher in green-leaf plants compared to red-leaf plants. Adaxial stomatal density was similar in all treatments with the exception of green leaves under R light that showed the highest value.

In the lower portion of the mesophyll, the amount of chloroplasts was higher in the green cultivar compared to red cultivar and was not affected by light quality (Table 2). In the upper mesophyll of green leaves, the lowest content of chloroplasts was

an effect of the red-light treatment. This reduced quantity of chloroplasts was similar to the highest value measured in red leaves grown under RB, RGB and W light conditions.

Light use efficiency of plants at different light qualities, investigated with chlorophyll a fluorescence emission measurement, showed that green plants performed better than red plants (Table 3). More specifically, green plants grown under W, RGB and RB light showed the highest values of PSII maximal photochemical efficiency (F_v/F_m) and quantum yield of PSII electron transport (QY). In both green and red plants, the lowest performance was measured in R treatment. Moreover, under red light the PSII maximal photochemical efficiency of green plants was reduced to values similar to the highest measured in red plants. In red leaves, non-photochemical quenching (NPQ) was significantly higher than in green leaves only in plants grown under R and RGB light regimens. In both cultivars, the highest value of NPQ was recorded in plants grown under R light.

Table 3 Effects of cultivar and light quality on maximal PSII photochemical efficiency, quantum yield of PSII electron transport, non-photochemical quenching of *Atriplex hortensis*

Cv	Light quality	F_v/F_m	QY	NPQ
Green	R	0.73±0.00b	0.34±0.02d	2.49±0.32b
	RB	0.80±0.00a	0.70±0.00a	0.40±0.03d
	RGB	0.81±0.00a	0.66±0.01a	0.85±0.21d
	W	0.82±0.00a	0.68±0.00a	0.46±0.02d
Red	R	0.66±0.01d	0.26±0.02e	5.14±0.46a
	RB	0.70±0.00c	0.51±0.01c	1.66±0.45bcd
	RGB	0.75±0.00b	0.50±0.02c	2.16±0.41bc
	W	0.75±0.00b	0.59±0.01b	1.12±0.12cd

Each value represents the mean ± SE, n=5. Different letters indicate significant differences among the combination of light quality and cultivar. The light quality treatments of red, red-blue, red-green-blue, and white for the two cultivars (G: green and R: red) are showed as R, RB, RGB and W, respectively.

Leaf pigment analysis showed that total content of chlorophylls and carotenoids was almost double in green leaves compared to red (Table 4). Green plants under white

light produced chlorophyll content similar to red leaf plants under any lighting treatment. Regarding carotenoids, their amount did not change among lighting treatments within each cultivar. As expected, red leaves contained much more betacyanins than green leaves with an average amount differing by two orders of magnitude. In green leaves light quality did not affect the betacyanin content. Differently, in red leaves betacyanin accumulation varied according to different lighting treatments with the highest and lowest values measured under RB (50% blue light) and R treatment (0% blue light) respectively. Moreover, we also found a positive correlation ($p<0.05$) between betacyanin content and the percentage of blue wavelengths within the light spectrum.

Table 4 Effects of cultivar and light quality on leaf pigment contents of *Atriplex hortensis*

Cv	Light quality	Total Chlorophylls ($mg\ g^{-1}$)	Total Carotenoids ($mg\ g^{-1}$)	Total Betacyanins ($\mu g\ g^{-1}$)
Green	R	0.33±0.04ab	0.08±0.00ab	0.36±0.03e
	RB	0.45±0.04a	0.10±0.01a	0.37±0.07e
	RGB	0.37±0.03ab	0.10±0.01a	0.30±0.03e
	W	0.29±0.01bc	0.07±0.00ab	0.14±0.01e
Red	R	0.21±0.07bc	0.04±0.01bc	15.8±1.92d
	RB	0.22±0.04bc	0.04±0.00bc	39.2±2.31a
	RGB	0.16±0.01c	0.04±0.00bc	28.6±2.46b
	W	0.13±0.01c	0.03±0.00c	23.5±2.35c

Each value represents the mean ± SE, n=5. All measurements Different letters indicate significant differences among the combination of light quality and cultivar. The light quality treatments of red, red-blue, red-green-blue, and white for the two cultivars (G: green and R: red) are showed as R, RB, RGB and W, respectively.

2.5. Discussion

Atriplex hortensis is often cultivated as vegetable for human nutrition and is known for its nutritional value and ease of growth. However, specific studies to evaluate *A. hortensis* growth and its betacyanin accumulation under different light spectra have not been carried out yet. In our experiment, both green and red cultivar of *A. hortensis* showed similar morphological responses to light quality proving that this species is suitable to be used as model system to compare the effects of different light wavelengths on fully-red and fully-green plants.

Although there is no single light spectrum to optimise plant growth for all species, it is widely accepted that the combination of red and blue LEDs maximises

photosynthesis and growth of most green plants [82]. We found that RB light maximises plant growth and biomass production not only in the green cultivar of *A. hortensis,* but also in red plants. These results suggest that similar wavelength combinations can be used for controlled environment horticulture of both green and red plants.

Worst performances were obtained under R light conditions, showing that a red light syndrome occurs in *A. hortensis* as reported for other species [83]. Thus, monochromatic red light turned out to be inappropriate for efficient production of *Atriplex hortensis*. Previous studies have shown that supplementing red with blue light can increase photosynthetic capacity and efficiency of plants, and at least a small percentage of blue wavelengths within light spectrum is required to prevent any dysfunctionality of the photosynthetic apparatus [45,83]. Supplementing red with blue light proved to be effective in improving plant performances also in *Atriplex hortensis* resulting in higher biomass production compared to monochromatic red light. Differently, adding green to red and blue light negatively affected plant growth. This could be due to the fact that RGB treatment has a reduced amount of red photons compared to RB, wasting light energy in wavelengths with a lower RQE [36]. Moreover, a possible explanation of a reduced plant growth could be the fact that smaller leaves were developed under RGB limiting radiation capture of plants, thus reducing biomass production.

As regard leaf anatomy, it has been reported that supplementing monochromatic red LEDs with blue fluorescent lamps increases leaf thickness and chloroplast area per cell in *Betula pendula* Roth [85]. Similarly, leaf thickness was higher in cotton (*Gossypium hirsutum* L.) grown with red and blue LEDs compared to 100% red LEDs [86]. Arena *et al*. [53] found that leaf thickness was reduced in tomato leaves grown under red and blue LEDs compared to those grown under white fluorescent lamps. In contrast, in the same study, leaf thickness of oriental plane (*Platanus orientalis* L.) was highest under red and blue LEDs [53]. Moreover, it is known that leaf thickness is a key factor determining space availability for chloroplast development [55]. In our case, although we found no differences in leaf thickness, the amount of chloroplasts varied among lighting treatments, especially as regards

upper mesophyll. Both cultivars of *A. hortensis* grown under monochromatic red light showed a reduction in chloroplast amount that could explain the lower photochemical efficiency and biomass production compared to other treatments. A more detailed anatomical analysis showed that the cell elongation in the upper mesophyll layers is a continuous phenomenon depending on the interaction with light spectrum composition. The development of one layer of long cells (single-layered palisade tissue), two layers of less elongated cells (double-layered palisade tissue) or a multi-layered roundish cells (absence of palisade tissue) can be interpreted as a plastic adaptation of the mesophyll structure to light quality: the best light combination for plant growth determined the development of a typical dorsiventral leaf anatomy (with two clearly distinct tissues and functions) while the worst light treatments led to a single unspecialised tissue structure. A less distinct dorsiventral symmetry found in R treatment leads to a more uniform distribution of intercellular spaces within the whole mesophyll and to a reduced difference in terms of stomatal density between the two epidermises. Accordingly, a more uniform distribution of chloroplasts between the upper and lower mesophyll can be considered as a further evidence of a less specialised leaf morpho-functional structure determined by monochromatic red light. The formation of a mesophyll without a clear difference between palisade and spongy tissues caused by R light treatment can be also associated with the larger number of stomata found in the adaxial epidermis. Accordingly, Li *et al*. [86] reported the highest stomatal density (SD) in cotton plants under 100% red LEDs. However, it is also reported that increasing blue light enhances the number of stomata per unit leaf area [87].

Green plants are typically reported to perform better than red plants in terms of quantum yield of PSII and photosynthesis [60,88,89]. Similarly, the green cultivar of *A. hortensis* performed better than red cultivar in terms of plant growth and biomass production in any light condition. Moreover, we found that the amount of chloroplasts was larger in green than in red leaves that is consistent with the larger content of chlorophyll and the higher photochemical efficiency found in green leaves. Better performance of green plants could also be linked to enhanced gas exchanges and carbon assimilation processes according to their larger number of stomata in the

abaxial epidermis. Monochromatic red light is known to be a harmful condition for photosynthetic systems altering chloroplast functionality [83]. Both varieties of *A. hortensis* were clearly stressed by red light treatment according to the results obtained by chlorophyll a fluorescence analysis, showing a reduction in the PSII photochemical efficiency (F_v/F_m and QY), and higher values of non-photochemical quenching (NPQ). This treatment turned out as the most unfavourable for plant growth and it is conceivable that the symptoms are due to a lack of key spectral components within the incident spectrum, such as blue wavelengths, which represent a significant part of chlorophyll absorption spectrum [36]. Conversely, treatments with a combination of different wavelengths showed the best results in terms of light utilization.

In *A. hortensis*, the content of chlorophylls and carotenoids, although different between cultivars, was not affected by light quality. Consequently, light spectrum modulation cannot be considered as an effective tool to enhance chlorophyll and carotenoid production in this species under low photosynthetic photon flux. It has been previously reported that chlorophyll concentration in soybean and radish is predicted by both absolute and relative amount of blue light [90]. Thus, it has to be considered that, instead of spectral composition of light spectrum, the total amount of incident blue light could also affect pigment composition of *Atriplex hortensis* if considering PPF values higher than those evaluated in our study. On the other hand, light treatments are reported to have a dramatic effect on the accumulation of anthocyanins in several species including those characterised by reddish leaves [56,91-93]. Generally the increase of anthocyanin amount in leaves is expected as an additional photoprotective strategy carried out by photosynthetic apparatus under high light [14]. In our experiment, the light intensity during growth is maintained in the range of maximal quantum yield for *Atriplex* species to avoid photodamages at photosystem level, thus the significant increase of betacyanins in red than in green plants can be interpreted as an early signal in response to a possible stress, since these leaves are likely more sensitive to changes in environmental conditions [94]. Although a few studies have evaluated the potential photoprotective role of betacyanins in leaves, it is typically reported that after high-

light exposure a smaller declines in the quantum efficiency of PSII happens in betacyanic than in green leaves [21,95,96]. In our case, we evaluated the betacyanin production in relation to light quality while maintaining the same irradiance between treatments; therefore, the light intensity was not a stress condition and did not induce photoprotection phenomena. As expected, we found a much higher content of betacyanins in red than in green plants. The two orders of magnitude difference between green and red leaves proved that the red cultivar could be considered as a valuable crop even if its productivity in terms of biomass accumulation is slightly lower compared to green plants. Interestingly, our data showed also that the amount of betacyanins in red plants of *A. hortensis* is significantly affected by light spectrum modulation. Several works showed that the accumulation of anthocyanins is typically related to the amount of blue light and that anthocyanin content is enhanced with increasing blue light [63,64,97-100]. Consistently, we found that the betacyanin content increased according to the increasing percentage of blue wavelengths within the incident light spectrum. Nevertheless, further studies are necessary to better define the phenomenon and to understand if it is also linked to the absolute quantity of blue light in addition to its proportion with respect to the other light wavelengths. Overall, this phenomenon gives useful insights to fine tune the technical requirements for controlled environment cultivation to enhance betacyanins in plant food.

Light quality treatments similar to those used in our experiment modulate anthocyanins accumulation also in reddish lettuce with the potential of increase the quantity up to four times more than the lowest mean value [56]. The total chlorophyll and carotenoid amounts in green leaves were always lower compared to red leaves under all light-quality treatments. On the contrary, the betacyanin content follow the opposite behaviour being higher in red than green leaves. Beside the inherent characteristics due to different cultivars, it is noteworthy that the different light-quality treatments induced a fine modulation of betacyanin levels. More specifically, in our species the best light treatment (RB) induced an increase of betacyanins that was twice compared to the minimum amount found in red cultivar under R light and a hundred times compared to green plants. In our species, the best light treatment

(RB) induced an increase of betacyanin content that was twice compared to the lowest amount found in red cultivar under R light and a hundred times larger compared to the amount found in green plants. Moreover, it is remarkable that the largest amount obtained in red *A. hortensis* was ten times higher than that found in reddish lettuce [56]. Consequently, fully-red plants should be considered as a suitable biological system for healthy food production due to their content in red pigments that is characteristically much higher compared to reddish plants and it can easily enhanced through appropriate lighting treatments.

2.6. Conclusions

This study support that light-spectrum modulation using LED technology is a reliable tool to increase the efficiency of plant cultivation. *Atriplex hortensis* turned out to be a model species to compare the effect of light quality on green- and red-leaf plants. Results on *A. hortensis* suggest that similar lighting treatments can be used to improve both green- and red-plant cultivation in controlled environment. In both cultivars, light quality determined changes in morpho-functional traits and the combination of red and blue wavelengths enhanced plant growth. In red plants, light quality significantly modulated the content of betacyanins in leaves that increased according to the percentage of blue wavelengths within the light spectrum. In addition, betacyanin content was two order of magnitude larger in red than in green plants. In the framework of enhancing antioxidant compounds in plant food through adjustment of light spectrum, fully-red plants should be considered as more promising than green or reddish cultivars.

2.6. Conclusions

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2.7. References

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Chapter 3

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Article

Growth and Physiological Responses of Lettuce Grown under Pre-Dawn or End-Of-Day Sole-Source Light-Quality Treatments

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Growth and physiological responses of lettuce grown under pre-dawn or end-of-day sole-source light-quality treatments

3.1. Abstract

The objective of this study was to evaluate growth and physiological responses of 'Cherokee' and 'Waldmann's Green' lettuce (*Lactuca sativa*) exposed to small changes in light quality and intensity within a 24-h period. Three pre-dawn (PD; 0600 to 0700) and three end-of-day (EOD; 2100 to 2200) treatments were evaluated in the study, each providing 50 ± 2 μmol·m^{-2}·s^{-1} of either blue, red, or broadband white light from light-emitting diodes (LEDs). To account for the main daily light integral (DLI), broadband white LEDs provided 210 ± 2 μmol·m^{-2}·s^{-1} from 0700 to 2200 or from 0600 to 2100 for the PD or EOD treatments, respectively. A control treatment was included which provided 200 ± 2 μmol· m^{-2}·s^{-1} of white light from 0600 to 2200. All treatments provided a DLI of 11.5 mol·m^{-2}·day^{-1} over a 16-h photoperiod. Regardless of cultivar, no treatment difference was measured for hypocotyl length or leaf number. However, plants grown under EOD-blue or PD-white had up to 26% larger leaves than those grown under PD-red and 20% larger leaves than control. In addition, plants grown under EOD-blue produced up to 18% more shoot fresh mass compared to those grown under control, EOD-red, or PD-red. Contrasts for gas-exchange data collected during the main photoperiod showed that light quality was not significant within PD or EOD for any of the parameters evaluated. However, regardless of light quality, stomatal conductance (g_s) and transpiration (E) were up to 34% and 42% higher, respectively, for EOD-grown plants compared to control. Our results suggest that 1 h of low intensity EOD-blue light has the potential to promote lettuce growth by increasing leaf area and shoot fresh mass when the main DLI from sole-source lighting is provided by broadband white LEDs.

3.2. Introduction

Traditional horticultural lamps (e.g., high-pressure sodium, cool-white fluorescent, metal halide) are useful at providing adequate daily light integral (DLI) indoors.

However, light-emitting diodes (LEDs) offer unique opportunities for exploring light-quality effects on plant growth, development, and metabolism. A useful feature of LEDs is their inherent capability to provide accurate spectral control in growing environments by producing narrow-spectrum light. This allows plant photoreceptors to perceive light cues that can control morphology and improve product quality. Numerous plant species have been evaluated under LED lighting with favorable results in production and flowering control [1]. However, to date, most sole-source light-quality research focused on plant growth-responses to LEDs have used a constant spectral environment throughout the day, and typically, during an entire crop cycle.

On a clear-sky day, early morning tends to be rich in blue light (400 to 500 nm), whereas the low sun angle from late afternoon will result in a low red (600 to 700 nm) to far-red (FR; 700 to 800 nm) spectrum [2]. However, regardless of season and cloud cover, midday sunlight has similar distributions of broadband blue, green (500 to 600 nm), and red light [3]. It is likely that cues received from the changing spectral distribution of sunlight have contributed to evolutionary responses of plants in the natural environment. Therefore, constant spectral environments within a 24-h period might not maximize the full potential of plant growth and development under sole-source lighting.

Most studies evaluating changes in light-quality within a 24 h-period have focused on the phytochrome (phy)-mediated responses to red and FR light. Kasperbauer [4] reported that a brief (5-min), low-intensity (17 $\mu mol \cdot m^{-2} \cdot s^{-1}$) treatment of end-of-day (EOD; at the end of the main photoperiod) FR light increased internode length of tobacco (*Nicotiana tabacum*) seedlings compared to untreated controls. Several follow-up studies have demonstrated that EOD-FR can be used as an effective non-chemical means to control plant morphology in a number of crops [5–11]. Moreover, Zahedi and Sarikhani [12] showed that flower induction of strawberry (*Fragaria ananassa*) can be accelerated in 12-week-old plants treated with EOD-FR light.

An alternative evaluation to changes in spectral distribution over time has focused on the pre-dawn (PD; before the start of the main photoperiod) or EOD effects of light within the photosynthetically active radiation (*PAR*) range, namely blue and red light. Sung and Takano [13] reported that shoot fresh mass, leaf area, and stem

diameter of cucumber (*Cucumis sativus*) seedlings grown under electric lamps (unknown type) were significantly higher when plants were PD-irradiated with blue fluorescent lamps compared to those treated with PD-red light from fluorescent lamps or untreated controls. In addition, the authors reported that when seedlings were exposed to PD-blue light, transpiration (*E*), photosynthetic rate (*A*), abaxial stomatal density, and stomatal opening of leaves increased compared to untreated controls [13]. Similarly, Hanyu and Shoji [14] reported a 20% increase in total dry mass of spinach (*Spinacia oleracea*) grown under white fluorescent lamps with a 30-min treatment of PD-blue compared to that of plants irradiated with PD-red or untreated controls. The same study also reported that the total dry mass of spinach treated with 30-min EOD-red light was higher compared to that of plants treated with EOD-blue light [14]. Using LEDs, Jishi et al. [15] reported higher growth (leaf area expansion and shoot fresh and dry mass) of lettuce treated with PD-blue compared to plants grown under a constant 14-h photoperiod of 50% blue + 50% red light. Recent work by Kuno et al. [16] also showed that PD-blue alone or in combination with EOD-red light can increase biomass production of lettuce compared to a constant spectrum throughout the day. It has been proposed that the growth increase in response to PD-blue is related to a rapid induction of stomatal opening caused by the high sensitivity of stomata to blue light [15,17]. Accordingly, others have suggested that inducing an early stomatal aperture might allow plants to reach their maximum stomatal aperture faster during the photoperiod and thus, may increase carbon fixation by reducing diffusional limitation to CO_2 uptake early in the day [18–21].

Although some of these studies suggest that potential production advantages can be achieved by manipulating PD or EOD-light in controlled environments, most of the significant findings are based on treatment comparisons with different photoperiods or DLIs, which may have introduced confounding effects in the results. In order to understand the physiological factors that drive plant responses to spectral changes over time, the objective of this study was to evaluate growth and gas-exchange responses of lettuce to changes in blue, red, or white light within a 24-h period, maintaining photoperiod and DLI constant. We hypothesized that PD-blue light would increase growth and physiological activity compared to other PD or EOD

treatments due to an increase in photosynthetic activity during the light period induced by early stomatal aperture.

3.3. Materials and methods

3.3.1. Plant material and growing conditions

Seeds of 'Cherokee' and 'Waldmann's Green' lettuce (Johnny's Selected Seeds, Winslow, ME, USA) were germinated on moist filter paper inside a Petri dish placed under ambient laboratory conditions for 48 h. Seedlings were subsequently transplanted into 48-cell plug trays (100 mL individual cell volume) filled with horticultural grade substrate composed (by volume) of 60% peat and 40% perlite (Sunshine Mix #4, Sun Gro Horticulture, Agawam, MA, USA). Each tray was cut into six sub-sections of four cells, each with one plant, which were randomly placed under one of seven light-quality treatments. Throughout the experiment, plants were sub-irrigated as necessary with tap water supplemented with a water-soluble fertilizer (Jack's Professional® General Purpose, 20N-4.4P-16.6K; J.R. Peters Inc., Allentown, PA, USA) to provide the following (in $mg \cdot L^{-1}$): 15 N-NO3, 7.5 P, 15 K, 0.11 Mg, and micronutrients.

3.3.2. Lighting treatments

All treatments provided a DLI of 11.5 $mol \cdot m^{-2} \cdot day^{-1}$ over a 16-h photoperiod, measured with a spectroradiometer (SS-110; Apogee Instruments Inc., Logan, UT, USA) at mid-canopy height. Three PD (0600 to 0700) and three EOD (2100 to 2200) light-quality treatments were evaluated in the study, each providing 50 ± 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of either blue, red, or broadband white light from LEDs (RAY66; Fluence Bioengineering, Austin, TX, USA). The main DLI was provided by white LEDs emitting 210 ± 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 0700 to 2200 or from 0600 to 2100 for the PD or EOD treatments, respectively. A control treatment was included which provided 200 ± 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of white light from 0600 to 2200. An illustration of the treatments is shown in Figure 1. The blue and red LEDs had peak wavelengths of 446 or 664 nm, respectively; the broadband white LEDs had three main peaks: blue (446 nm), orange (599 nm) and red (664 nm).

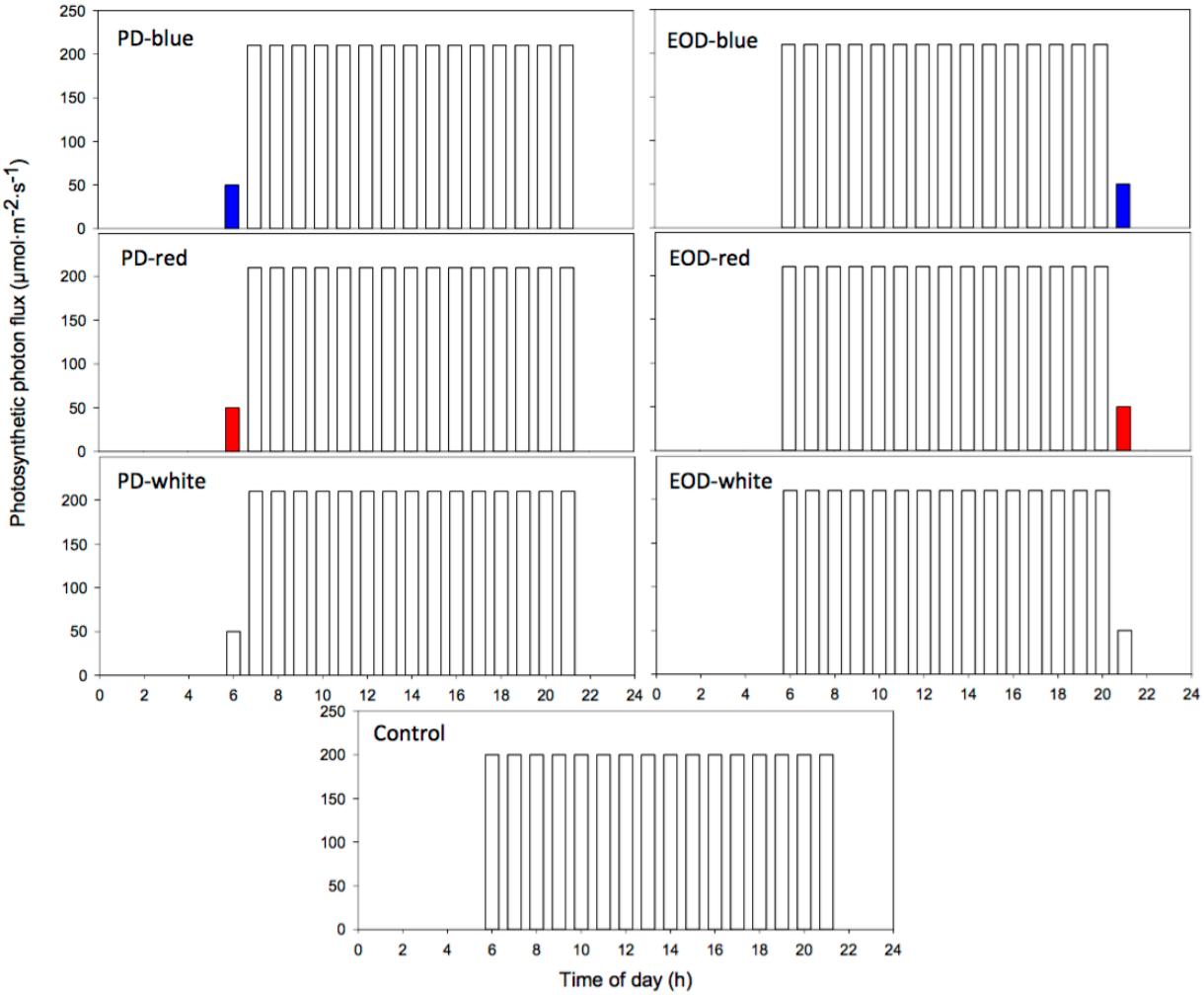

Figure 1. Illustration of the light quality, intensity, and photoperiod used throughout the study. pre-dawn; EOD = end-of-day. PD = pre-dawn; EOD = end-of-day.

Plants were grown on 41 x 61 x 183 cm compartments within multilayer shelves placed inside a walk-in growth chamber (C6 Control System with ECoSys Software; EGC, Chagrin Falls, OH, USA); each compartment was a replicate of a treatment. Before starting the experiment, a light map was generated to determine the maximum photosynthetic photon flux (*PPF*) for each treatment (no plants present) using a spectroradiometer. Light output to achieve our target *PPF* was controlled with a dimmer (Solunar; Fluence Bioengineering) connected to a backup battery (BE425M-LM; APC, West Kingston, RI, USA). Light pollution (<5 µmol·m^{-2}·s^{-1}) within treatments was minimized by covering the sides and back of the shelves with a double layer of 0.3-mm-thick black and white polyethylene film (white side facing the plants). A 215 x 200 cm black and white polyethylene film curtain was used to prevent light pollution between the two opposite shelves (black side facing the

plants). Within each treatment, sub-trays were randomly rotated daily to minimize location effects within the experimental area.

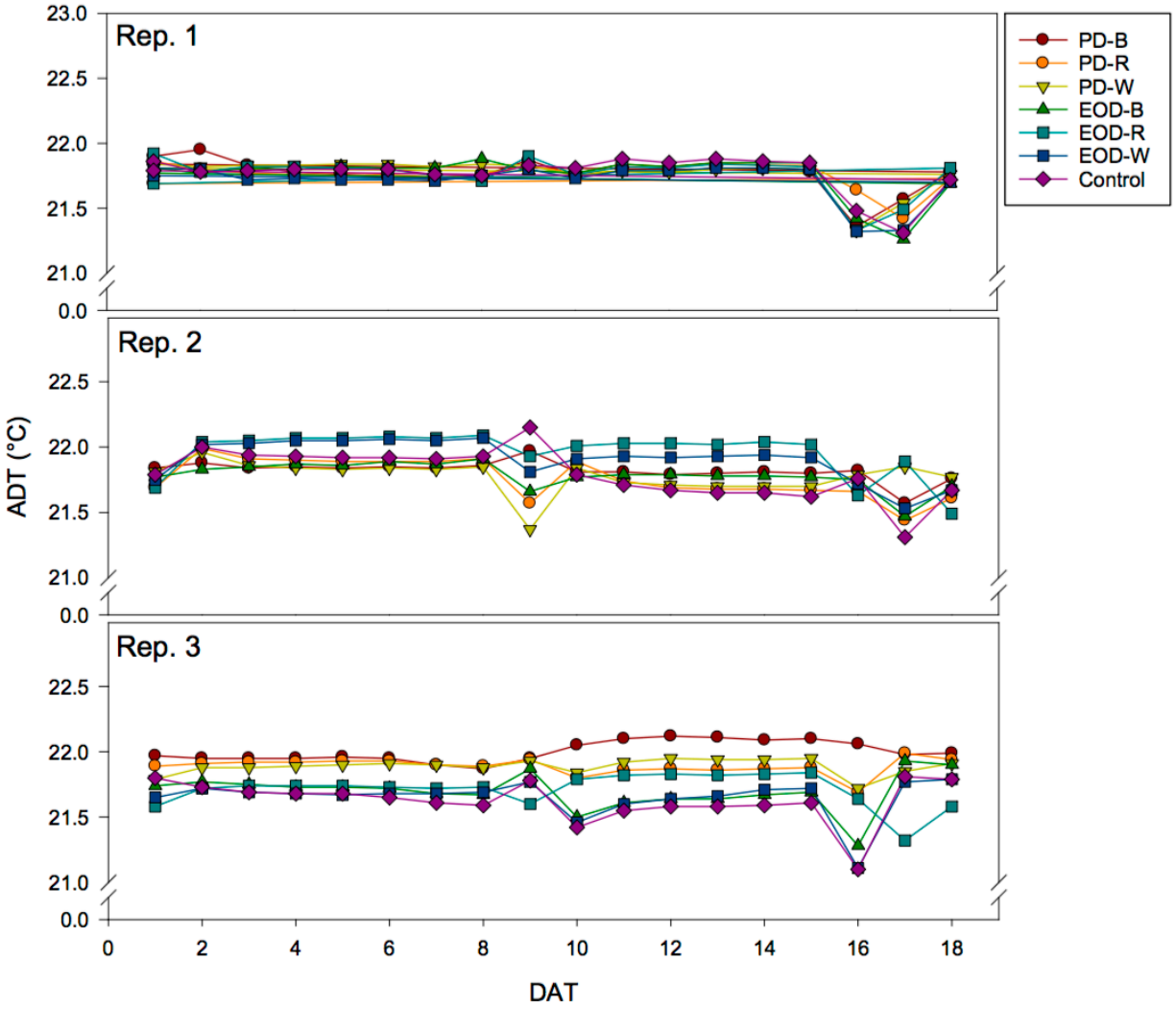

Figure 2. Average daily near-canopy air temperature (ADT) measured during each experimental replication. PD = pre-dawn; B = blue; R = red; W = white EOD = end-of-day; DAT = day after transplanting.

3.3.3. Data collection and plant measurements

The average ambient day (from 0600 to 2200) and night (from 2200 to 0600) air temperature of the chamber was set at 20 and 21 °C, respectively. However, radiation from the lamps raised ambient temperature during the photoperiod, which was uniformly maintained at ~22 °C by installing cooling fans (AC Infinity AXIAL 1238; City of Industry, CA, USA) as needed. The set points for ambient CO_2 and relative humidity (RH) were 405 ppm and 60 to 80%, respectively. Near-canopy air

temperature was monitored using fine-wire thermocouples [Type K, 5SC Series, 0.25 mm diameter; OMEGA Engineering Inc., Norwalk, CT, USA] placed directly under a leaf from a plant located at the center of each treatment and interfaced to a data logger (CR1000; Campbell Scientific, Logan, UT, USA) (Figure 2). To avoid partial shading of the plants, the thermocouples were not shielded. An additional shielded temperature and RH sensor (RC-4HA/C; Elitech, Milpitas, CA, USA) was placed at the center of each treatment compartment to provide real-time data monitoring and to ensure that ambient temperature differences among treatments were ≤1 °C.

From four days to one day prior to harvest, a portable gas-exchange system (LI-6400XT; LI-COR, Lincoln, NE, USA) was used to measure survey photosynthetic rate (A), stomatal conductance (g_s), and transpiration (E) on six randomly selected plants per cultivar per treatment. Data were collected at three different times of measurement (ToM): PD (0600 to 0700), EOD (2100 to 2200), and during the main photoperiod (0900 to 1500). The reference CO_2 concentration, leaf temperature, RH, and flow rate inside the chamber were 405 μmol· mol^{-1}, 22 °C, approx. 60%, and 500 mL·min^{-1}, respectively. Measurements were conducted under ambient *PPF* (without the use of an external light source), ensuring leaf exposure to the target intensity from each treatment. Additionally, prior to harvest, SPAD index was measured with a chlorophyll meter (SPAD-502; Konica Minolta Sensing Inc., Osaka, Japan) on three different points on a leaf. Gas-exchange data and average SPAD index were collected for the youngest fully expanded leaf of each plant.

Six plants per treatment were destructively harvested. Immediately following harvest, hypocotyl length was measured with a ruler. The number of leaves (>1 cm) per plant was counted and total leaf area was measured using a leaf area meter (LI-3000A; LI-COR). Shoot fresh mass was measured using an electronic balance. Subsequently, shoots were oven-dried to a constant mass at 70 °C for dry mass determination. Specific leaf area (SLA) was calculated by dividing leaf area by shoot dry mass.

Three replications were conducted over time following the same procedures as previously described. Each experimental replication was terminated 18 d after

treatment initiation. All treatments were re-randomized within the chamber before the start of each replication.

3.3.4. Data analysis

Response data were analyzed using generalized linear mixed model procedures as implemented in SAS PROC GLIMMIX (SAS/STAT 14.2; SAS Institute, Cary, NC, USA) using a normal distribution function for all response variables, except leaf number, which was modeled on the Poisson scale. Experimental repeat and its interaction with treatment x cultivar were considered to be random effects. For plant growth responses, there was no interaction between cultivar and treatment, except for shoot fresh mass (Table 1). Therefore, data are presented as main effects. Similarly, for physiological measurements the cultivar ⇥ treatment interaction was not significant ($P > 0.15$), and hence treatments were compared at the main effect level using linear contrasts. Because gas-exchange data collected during the PD and EOD ToM include ToM as a confounding effect, all treatment contrasts were made for data collected during the photoperiod ToM.

3.4. Results

3.4.1. Growth responses

No treatment difference was measured for hypocotyl length or leaf number (Table 1). Plants grown under EOD-blue or PD-white had ~25% larger leaves than those grown under PD-red. However, based on SLA, plants grown under PD-white produced thinner leaves compared to those grown under PD-red (434 vs. 349 m^2_{leaf} per g_{leaf} dry mass, respectively). In addition, plants grown under EOD-blue produced up to 18% more shoot fresh mass compared to those grown under control, EOD-red, or PD-red. Similarly, although not significant, shoot dry mass of plants grown under EOD-blue was 14% higher than that of plants grown under control, EOD-red, or EOD-white, and 10% higher compared to that of plants grown under PD-blue, PD-red, or PD-white.

Regardless of treatment, hypocotyls were 0.6 cm longer and shoot dry mass was 0.1 g higher for ‘Waldmann’s Green’ compared to ‘Cherokee’ (data not shown). In

contrast, based on SLA, 'Cherokee' produced significantly thinner leaves than 'Waldmann's Green' (435 vs. 344 m2leaf per gleaf dry mass, respectively), but 'Waldmann's Green' produced ~2 fewer leaves per plant. Although the number of leaves per plant was significantly higher for 'Cherokee', the significant difference in leaf thickness, as indicated by SLA, is most likely responsible for the cultivar differences in shoot dry mass.

Table 1. Hypocotyl length (HL), leaf area (LA), specific leaf area (SLA), leaf number, shoot fresh mass (FM), and shoot dry mass (DM) of lettuce plants grown in a controlled environment under one of seven light treatments z.

Treatment	HL (cm)	LA (cm^2)	SLA ($m^2 \cdot g^{-1}$)	Leaf No.	FM (g)	DM (g)
PD-B	4.2 a [w]	104 ab	413 ab	7.1 a	3.6 ab	0.26 a
PD-R	3.9 a	86 b	349 b	7.0 a	3.5 b	0.26 a
PD-W	3.9 a	107 a	434 a	7.1 a	3.6 ab	0.26 a
EOD-B	4.5 a	108 a	397 ab	6.9 a	4.0 a	0.29 a
EOD-R	4.2 a	90 ab	378 ab	7.0 a	3.5 b	0.25 a
EOD-W	3.8 a	92 ab	376 ab	6.9 a	3.6 ab	0.25 a
Control	4.0 a	90 ab	381 ab	6.8 a	3.4 b	0.25 a
Treatment	NS	**	*	NS	**	NS
Cultivar [y]	***	***	***	***	NS	***
Treatment × Cultivar	NS	NS	NS	NS	*	NS

[z] Three pre-dawn (PD) (0600 to 0700) and three end-of-day (EOD) (2100 to 2200) light treatments were evaluated in the study, each providing 50 ± 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of either blue (B), red (R), or white (W) light; the main photoperiod provided 210 ± 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of W light from 0700 to 2200 or from 0600 to 2100 for the PD or EOD treatments, respectively. A control treatment was included which provided 200 ± 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of W from 0600 to 2200; all treatments provided a DLI of 11.5 $mol \cdot m^{-2} \cdot day^{-1}$ (n = 36). y The cultivars evaluated were 'Cherokee' and 'Waldmann's Green'. w Means within columns followed by the same letter are not different based on the least significant difference test $P \leq 0.05$.; ***, **, *, NS indicate statistical significance at $P \leq 0.001$, 0.01, and 0.05, and not significant, respectively.

3.4.2. Physiological responses

No treatment difference was measured for SPAD index (data not shown). Means for survey A, g_s, and E at different ToM are presented in Table 2. Initial contrasts showed that A and g_s measured in plants grown under control were unaffected by ToM. However, E was 22% higher when control-grown plants were measured in the PD relative to the EOD ToM. Initial contrasts also indicated that for all treatments except control, A, g_s, and E were significantly higher when data were collected during the main photoperiod ToM compared to the PD or EOD ToMs, which is most

likely attributed to the differences in *PPF* at the different ToMs (210 μmol·m^{-2}·s^{-1} for the main photoperiod vs. 50 μmol· m^{-2}·s^{-1} for the PD or EOD).

Table 2. Survey photosynthesis (*A*), stomatal conductance (g_s), and transpiration (*E*) measured for lettuce plants grown in a controlled environment under one of seven light treatments z.

Contrast No.	Time of Measurement (ToM) y	Treatment	*A* (μmol·m^{-2}·s^{-1})	g_s (mmol·m^{-2}·s^{-1})	*E* (mmol·m^{-2}·s^{-1})
1	Pre-dawn	Control	8.6	297	5.0
2	Pre-dawn	PD-B	3.0	219	3.7
3	Pre-dawn	PD-R	2.8	183	3.3
4	Pre-dawn	PD-W	2.9	174	3.4
5	End-of-day	Control	8.8	226	3.9
6	End-of-day	EOD-B	3.2	227	4.2
7	End-of-day	EOD-R	3.1	175	3.0
8	End-of-day	EOD-W	3.2	186	3.3
9	Main photoperiod	Control	7.7	304	4.5
10	Main photoperiod	PD-B	8.8	350	5.4
11	Main photoperiod	PD-R	7.9	380	6.2
12	Main photoperiod	PD-W	8.2	313	5.0
13	Main photoperiod	EOD-B	9.2	396	6.2
14	Main photoperiod	EOD-R	9.1	408	6.2
15	Main photoperiod	EOD-W	9.2	397	6.4
Initial contrasts					
1 vs. 5			NS	NS	**
1 vs. 9			NS	NS	NS
5 vs. 9			NS	NS	NS
2, 3, 4 vs. 10, 11, 12			***	**	***
6, 7, 8 vs. 13, 14, 15			***	***	***
Treatment contrasts within the main photoperiod ToM					
Control vs. PD					
9 vs. 10, 11, 12			NS	NS	*
9 vs. 10			NS	NS	NS
9 vs. 11			NS	NS	**
9 vs. 12			NS	NS	NS
Waveband within PD					
10 vs. 11			NS	NS	NS
10 vs. 12			NS	NS	NS
11 vs. 12			NS	NS	NS
Control vs. EOD					
9 vs. 13, 14, 15			NS	**	***
9 vs. 13			NS	*	**
9 vs. 14			NS	**	**
9 vs. 15			NS	*	***
Waveband within EOD					
13 vs. 14			NS	NS	NS
13 vs. 15			NS	NS	NS
14 vs. 15			NS	NS	NS

z Three pre-dawn (PD) (0600 to 0700) and three end-of-day (EOD) (2100 to 2200) light treatments were evaluated in the study, each providing 50 ± 2 μmol·m^{-2}·s^{-1} of either blue (B), red (R), or white (W) light; the main photoperiod provided 210 ± 2 μmol·m^{-2}·s^{-1} of W light from 0700 to 2200 or from 0600 to 2100 HR for the PD or EOD treatments, respectively. A control treatment was included which provided 200 ± 2 μmol·m^{-2}·s^{-1} of W from 0600 to 2200; all treatments provided a DLI of 11.5 mol·m^{-2}·day^{-1} (n = 36). y EOD and PD data were collected during from 2100 to 2200 and 0600 to 0700, respectively. Main photoperiod data were collected between 0900 and 1500. ***, **, *, NS indicate statistical significance at the P < 0.001, 0.01, and 0.05, and not significant, respectively.

Contrasts for data collected within the main photoperiod ToM showed that light quality did not affect any of the gas-exchange parameters evaluated within PD or EOD (Table 2). In addition, *A* and g_s of plants grown under PD were not different than control; however, *E* was 38% lower in control plants relative to those grown under PD-red. Similarly, there were no differences for *A* between EOD and control plants measured during the main photoperiod ToM; however, regardless of light quality, g_s and *E* were up to 34% and 42% higher, respectively, for EOD-grown plants compared to control.

3.5. Discussion

Our results suggest that 1 h of low intensity EOD-blue light has the potential to promote lettuce growth by increasing leaf area and shoot fresh mass when the main DLI from sole-source lighting is provided by broadband white LEDs (Table 1). Although significant effects in plant growth and morphology from short-term exposures to PD or EOD light-quality treatments have been reported, our findings do not correspond with those of others who indicate that PD-blue or EOD-red light can increase biomass production of plants [13–16,22]. As shown by others, leaf stomatal features are greatly affected by blue light [2,23]. Accordingly, Goto [17] suggested that a possible explanation for the reported increases in plant growth under PD-blue relate to the significant effect that blue light has on leaf stomatal development and aperture. Similarly, Jishi et al. [15] proposed that the increase in plant growth with low-intensity PD-blue light is caused by changes in stomatal aperture at the end of the dark period (i.e., PD), which might induce a premature "awakening" of the photosynthetic apparatus that could increase the overall photosynthetic activity of plants by minimizing limitations to CO_2 diffusion early in the day. Although it is likely that blue light is perceived as a cue for plants to recognize the onset of the light period, our results do not show higher physiological activity under PD-blue compared to other treatments (Table 2). Similar to our findings, Auchincloss et al. [24] concluded that changes in PD-stomatal opening and conductance do not increase daytime *A* of sunflower (*Helianthus annuus*). Interestingly, our results do indicate that, regardless of light-quality, short-term

exposure to EOD-light stimulates daytime g_s and E, which may have increased the photosynthetic efficiency of plants during the light period by promoting the absorption of water and ions from roots, or by stimulating efficient water, ion, and hormone transport through plants.

Only one of the three experiments reported by Jishi et al. [15] accounted for photoperiod and DLI when comparing PD or EOD blue and red light; their results showed a higher leaf area in lettuce produced under a 14-h photoperiod of 7-h PD-blue + 7-h EOD-red compared to other treatments that combined blue and red light during the main photoperiod. To our knowledge, all other studies reporting higher growth under PD-blue or EOD-red have photoperiod or DLI as a confounding effect. Therefore, no direct comparison can be made with our findings, as light-quality responses to spectral changes over time cannot be distinguished with day length responses in those studies. However, as shown by others, plant responses to light-quality are not only species, but sometimes cultivar-specific, and may depend on the background environment and on the plant developmental stage [25]. Therefore, the results from those studies might also reflect specific environmental conditions and plant-specific responses to light-quality.

The significant increase in leaf area and shoot fresh mass with EOD-blue compared to control might be related to the relative absorption of blue light by the active [far-red-absorbing (Pfr)] and inactive [red-absorbing (Pr)] forms of phy, which are known to regulate the perception of day length in plants and can affect leaf area expansion (Table 1). Although blue radiation is best absorbed by cryptochrome (cry) and phototropin (phot) photoreceptors, it is also weakly absorbed by phy [26]. Meng and Runkle [27] reported that phy-mediated responses can be controlled with moderate-intensity (~30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) blue light due to the secondary absorption peak of phy in the blue region of the spectrum. Furthermore, Liu et al. (2012) showed that phy B controls the expression of both *ERECTA* and *EXPANSIN* family genes, which regulate cell expansion in leaves. Others have shown that blue radiation can be perceived as a long-day photoperiodic response in plants [28,29], and increases in leaf area expansion under long photoperiods have been reported in many plant species [30]. Therefore, if phy-regulated photoperiodic stimuli from EOD-blue extends into the dark period, plants may perceive EOD-blue as day length

extension, possibly stimulating leaf area expansion. Larger leaves from EOD-blue most likely increased the radiation capture of plants and may have directly affected the increase in shoot fresh mass measured in our study

Similar to our findings, Hanyu and Shoji [14] reported a negative growth response to PD-red (Table 1). The mechanisms that affect plant growth in response to PD-red are not well understood but could be associated with changes in the relative proportion of Pfr to the total amount of phy (i.e., phy photoequilibrium) at the end of the dark period. During the day, Pfr suppresses genes involved in elongation and growth; however, at night, Pfr slowly converts into the inactive Pr, which increases the expression of genes involved in elongation and growth [31,32]. Red light applied at the end of the dark period might interrupt the conversion of Pfr to Pr, leading to a reduction in cell elongation.

In conclusion, PD blue light was not effective at promoting growth or physiological activity of lettuce plants. However, short-term EOD light can be useful to modify physiological and morphological plant responses that may ultimately lead to higher yields without increasing DLI. In the wake of indoor farming technologies, implementing solutions that can increase production efficiency without negatively affecting plant growth is a major advantage. Controlling light-quality with LEDs is a readily available tool to increase production efficiency indoors and can contribute to the establishment of better production practices for indoor farming.

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Chapter 4

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Gas exchange and leaf anatomy of lettuce in response to blue and red LEDs as a sole-source lighting

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Gas exchange and leaf anatomy of lettuce in response to blue and red LEDs as a sole-source lighting

4.1. Abstract

The sustainability of long-duration manned missions in space relies on plant-based Bioregenerative Life Support Systems (BLSSs). Providing optimal light conditions in closed environments is crucial for proper design and optimization of space-based plant growth chambers. Light-emitting diodes (LEDs) are a promising electric light source for BLSSs research due to their capability to control spectral output to meet specific crop requirements. In addition, controlling light quality permits direct stimulation of plant photoreceptors that can improve yield and nutritional attributes of food crops. The objective of this study was to quantify the effects of blue light on growth and morphology, physiology, and anatomical features of red lettuce (*Lactuca sativa* cv. *'Outredgeous'*) grown under different red-to- blue-light ratios. Five treatments were evaluated in this study: 100% red; 7% blue + 93% red; 26% blue + 74% red; 66% blue + 34% red; 100% blue. Treatment comparisons indicate that except for 100% blue, the increasing percentages of blue light resulted in decreased edible biomass production. In addition, stomata density decreased under monochromatic blue or red light, but no significant difference was found in stomatal limitation of photosynthesis.

4.2. Introduction

Space exploration of the solar system to establish extra-terrestrial human settlements is a challenge of the 21st century. Long-term manned missions to Mars will require consumables (food, water, oxygen) that become more expensive to launch as mass increases [1]. Resupplying consumables is inadequate and risky, while *in-situ* resource utilization currently cannot fully achieve crew requirements. Thus, bioregenerative life-support systems (BLSSs) are needed for the colonization of the Moon or Mars [2].

The Micro-Ecological Life-Support System Alternative (MELiSSA) project led by the European Space Agency (ESA), promotes the concept of a closed loop ecosystem. MELiSSA is organized in different compartments based on micro-organisms degrading and transforming organic waste into usable elements, which along with carbon dioxide from the crew, can feed higher-plants and algae, and in return provide, food, oxygen, and water to astronauts [3].

Growing plants in space requires a deep understanding of plant growth in controlled-environments [4]. In this context, LEDs play a key role at enabling both accurate research and energy-affordable food production intended for life support in space [5]. The first use of LEDs to grow plants in space in 1995 paved the way for the development of Veggie and the Advanced Plant Habitat (APH), which are currently supporting plant production as a dietary supplement aboard the International Space Station (ISS) [6,7]. Compactness of LEDs and their reliable long lifetimes contribute to reducing the equivalent system mass of a lighting system. In addition, the maintenance costs and astronaut labor requirements for plant growth systems are lower [1]. Furthermore, LED solid-state electronics ensure safety and affordable risk-management strategies that are critical in space missions [8].

Previous studies indicate that a combination of blue and red LEDs is suitable for plant cultivation in controlled environments [9]. Red light typically promotes dry mass gain and has the highest relative quantum efficiency (RQE) for driving single-leaf photosynthesis [9,10]. Although blue light is photosynthetically less efficient than red light, it has important photomorphogenic and phototropic effects on plants [11,12].

Lettuce is a candidate species for space agriculture because of its fast growth and compactness, in addition to its sensitivity to light quality [13,14]. ‘Outredgeous’ lettuce was the first crop tested by NASA in the VEGGIE growth chamber on the ISS [15]; the cultivar was selected because of its nutritional value due to a high concentration of anthocyanins [16].

Research has shown that supplementing blue with red light inhibits hypocotyl elongation and increases biomass production of lettuce [17,18]. However, monochromatic red light has been shown to sometimes increase dry mass of lettuce [19]. Therefore, the optimal sole-source light recipe under a combination of blue and red LEDs requires further investigation.

The objective of this study was to perform a dose-response curve evaluating morphological traits of lettuce shoots and roots between 100% blue light and 100% red light. The results of this study would be useful in suggesting optimal growth conditions for lettuce cultivation in a controlled environment under blue and red LEDs.

4.3. Materials and methods

4.3.1. Plant material and growing conditions

Seeds of 'Outredgeous' lettuce (*Lactuca sativa* L.) (Johnny's Selected Seeds, Winslow, MN, USA) were pre-germinated until radicle emergence and subsequently transplanted into 48-cell plug trays filled with Greens GradeTM Arcillite (Profile Products LLC; Buffalo Grove, IL, USA). Controlled-release fertilizer (Nutricote 14N-4P-14K; 90-day release; Florikan, Sarasota, FL, USA) was used at a 2.5 g·L-1 rate. Seedlings were propagated inside a walk-in growth chamber (C6 Control System with ECoSys Software; EGC, Chagrin Falls, OH, USA) (Fig. 1) where constant ambient temperature and relative humidity (RH) were set at 21 °C, and 60% to 70%, respectively. Carbon dioxide concentration in the growth chamber was maintained at 1200ppm to simulate growing conditions of closed environment such as bioregenerative life support systems or International Space Station. CO_2 and RH in the growth chamber were monitored throughout the experiment with a data logger (DL1; ECG). During the course of the experiment, plants were sub-irrigated as necessary with tap water.

4.3.2. Lighting treatments

Five light treatments were evaluated in the study: 100% red (R); 7% blue + 93% red (7B); 26% blue + 74% red (26B); 66% blue + 34% red (66B), and 100% blue (B) (Fig. 1). All treatments provided a photosynthetic photon flux (PPF) at mid-canopy height of 200 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ measured with a spectroradiometer (SS-110; Apogee Instruments Inc., Logan, UT, USA) and a 16-h photoperiod. Target PPF was achieved controlling light output with dimmers (Solunar; Fluence Bioengineering, Austin, TX, USA).

Plants were grown on two opposite compartments within multilayer shelves placed inside a growth chamber. Each compartment was a replicate of a treatment. For each treatment replication, near-canopy air temperature was monitored using fine-wire thermocouples (Type K, 5SC Series, 0.25 mm diameter; OMEGA Engineering Inc., Norwalk, CT, USA), interfaced to a data logger (CR1000; Campbell Scientific, Logan, UT, USA). Within each compartment, plants were randomly rotated daily to minimize the location and edge effects.

4.3.3. Growth and morphological measurements

Six plants per treatment were destructively harvested 21 days after treatment initiation. Shoots and roots were separated by cutting the plants to the root collar. The number of leaves (length >1 cm) per plant was counted (NL). Shoot fresh mass (SFM) was measured using an electronic balance.

Root morphology was measured by scanning the roots using a Perfection-4990 scanner (Epson, Suwa, JA) interfaced to WinRHIZO (Regent Instrument Inc., Québec, CA). Excess substrate was removed by gently submerging the roots in water. After all roots were washed, roots for one plant were separated in a 10 W × 15 L cm polycarbonate container filled with a thin layer of water and laid on a horizontal plane to be scanned. Total root length (TRL) and number of root tips (NRT) were calculated from the scanned images using WinRHIZO.

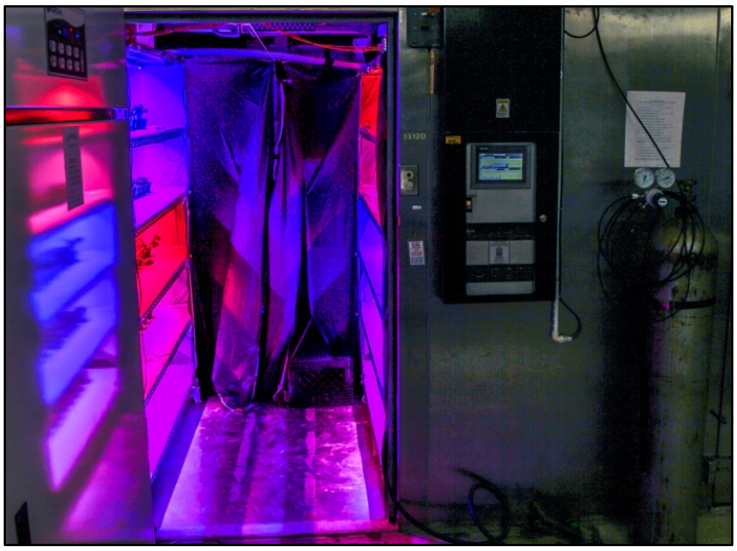

Fig. 1. Walk-in growth chamber at the Environmental Horticulture Department, University of Florida

4.3.4. Physiological measurements

Three days prior to harvest, a portable leaf gas exchange system (LI–6400XT; Li–Cor, Lincoln, NE, USA) was used to measure photosynthetic rate (*A*) and stomatal conductance (g_s). The reference PPF, leaf temperature, RH, and CO_2 concentration inside the cuvette were maintained at the same set-points of the growth chamber.

4.3.5. Anatomical leaf traits measurement

Epidermal imprints of the adaxial surface of leaves were made following the procedure described by Wilson et al. [20]. Images of the imprints were captured with a digital camera mounted on an optical microscope (DP71; Olympus Inc., Tokyo, JP). Stomata were counted using ImageJ (National Institutes of Health, Bethesda, MD, USA) and stomata density (SD) was calculated as the number of stomata per unit area.

4.3.6. Data analysis

Six plants were grown in each compartment and treatments were replicated two times. Data were compared by using one-way analysis of variance (ANOVA). Multiple-comparison tests on the main-effect means were performed using Tukey's post-hoc test ($P<0.05$). All data were processed using Microsoft Excel and STATISTICA ver. 8.0 (StatSoft, Inc. 2008).

4.4. Results

4.4.1. Growth and morphology

Shoot fresh mass (SFM) gradually decreased with increasing blue light, except for 100% blue, for which shoot fresh mass was similar to that of plants grown under 26B (Table 1). Similarly, number of leaves (NL), total root length (TRL) and number of root tips (NRT) decreased with higher blue light. However, plants grown under monochromatic red light produced smaller roots and fewer root tips than 7B (Fig. 2).

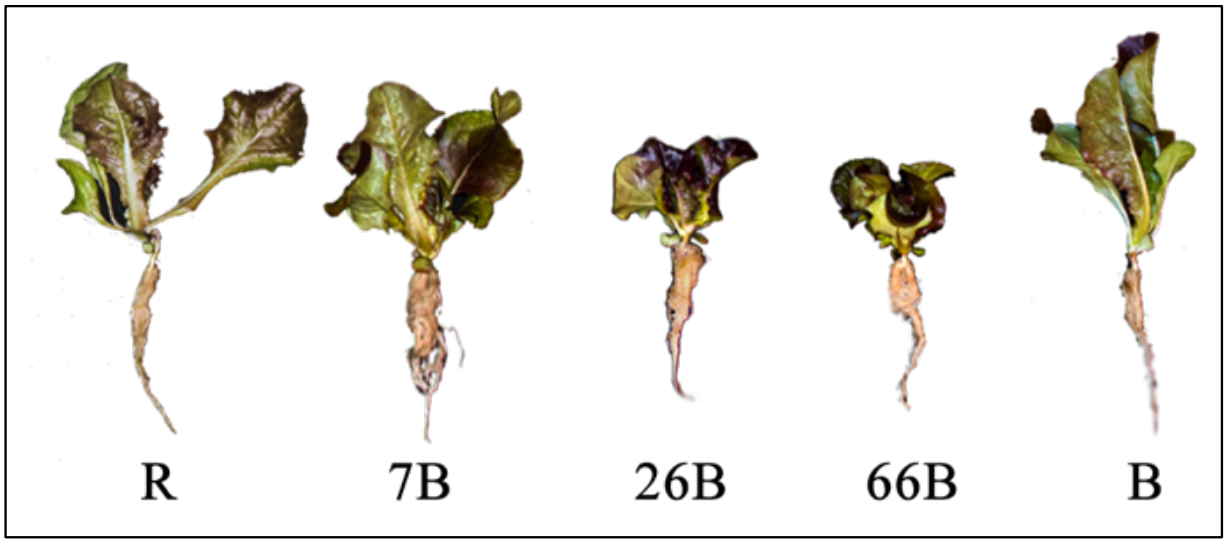

Fig. 2. 'Outredgeous' lettuce plants grown under different blue-light doses. R = red; B = blue.

4.4.1. Physiology

Photosynthetic rate (*A*) increased with increasing blue light, but only R treatments was significantly lower compared to other lighting treatments (Fig. 3). Similarly, stomatal conductance (g_s) showed higher values with increasing blue light, except for 100% blue light that was lower than 66B (Fig. 4).

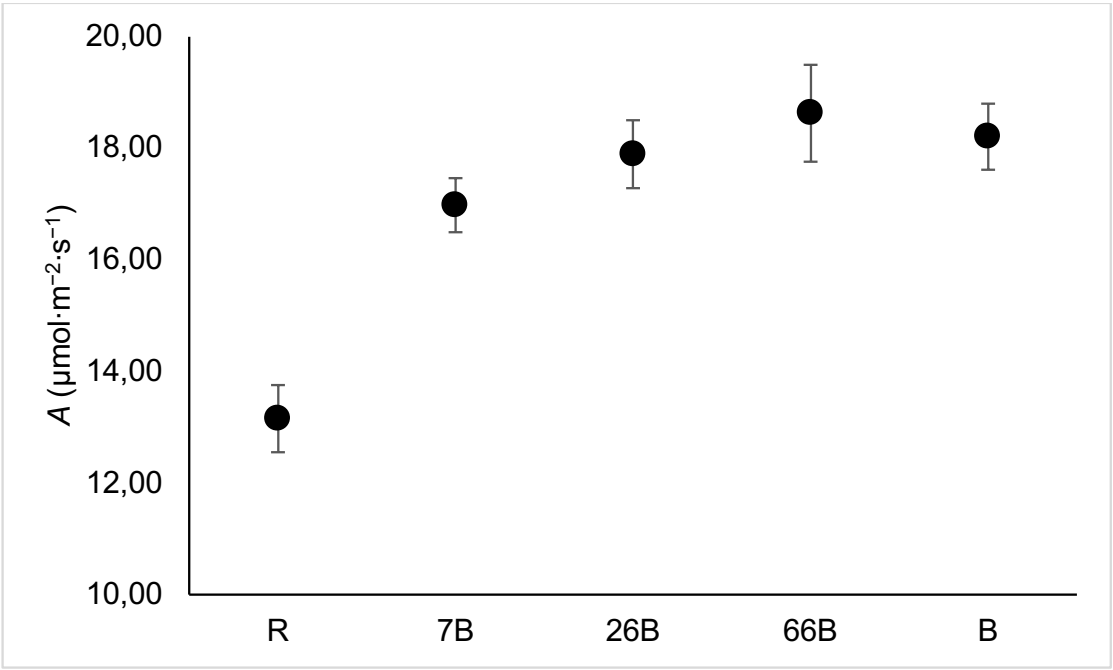

Fig. 3. Effect of different blue-light doses on photosynthetic rate (A). Vertical bars denote 0.95 confidence interval..

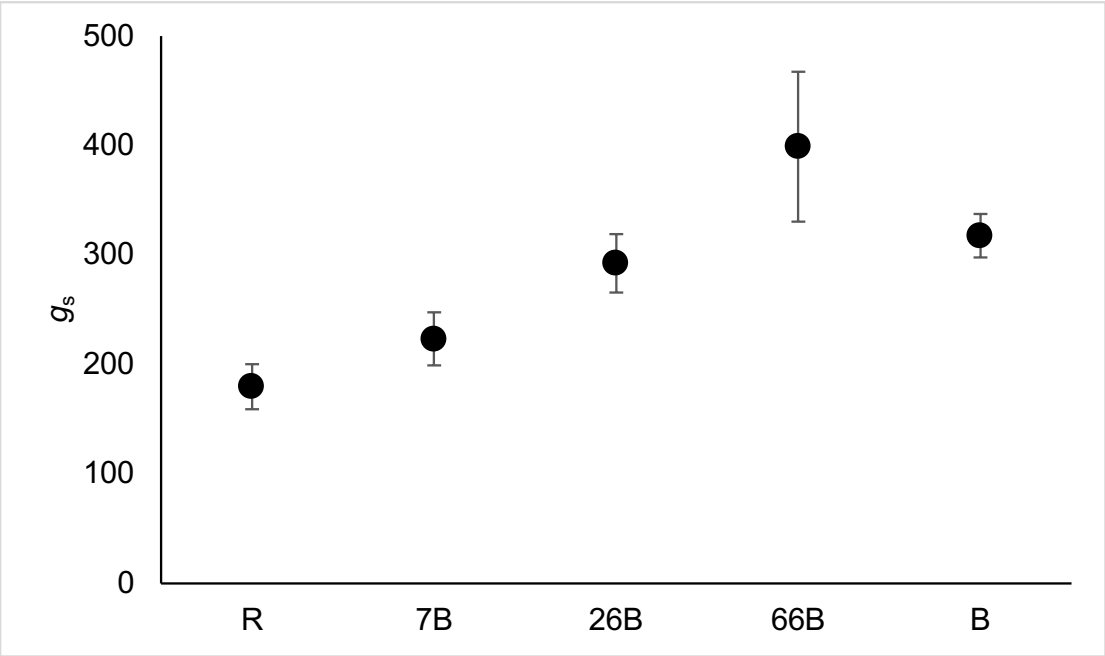

Fig. 4. Effect of different blue-light doses on stomatal conductance (g_s). Vertical bars denote 0.95 confidence interval.

4.4.1. Anatomy

Monochromatic B or R had lower SD compared to treatments with a combination of blue and red LEDs (Fig. 5).

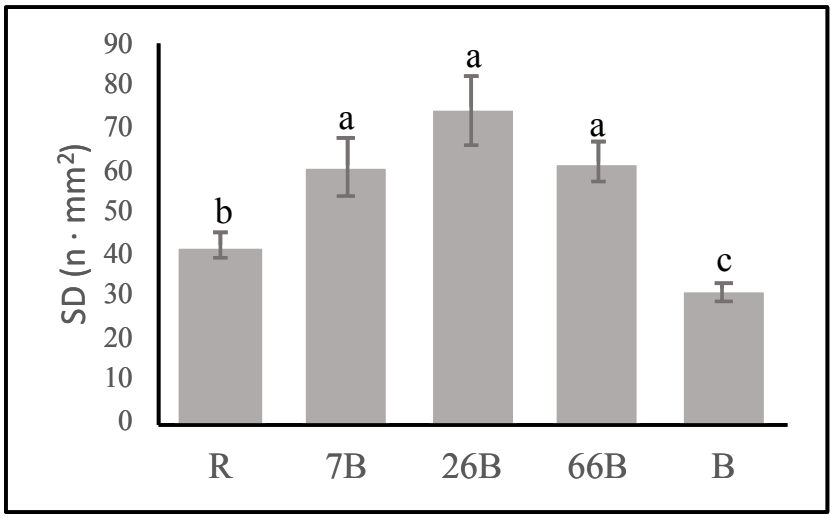

Fig. 5. Effect of different blue-light doses on adaxial stomatal density (SD). The results are shown as mean ± SE; different letters indicate differences at P < 0.05.

4.5. Discussion

As shown in Table 1, we found a general growth reduction (SFM and NL) in response to increasing blue light, except for 100% blue. Others have reported

similar reductions in fresh mass and leaf number in response to higher blue light [19,21,22].

Relative quantum efficiency curves indicate that blue light is up to 35% less efficient than red light in driving photosynthesis [23]. Therefore, it is likely that photosynthesis per unit of radiation capture decreased with increasing blue light (Fig. 3). However, plants grown under B had similar or higher SFM compared to those grown under 26B and 66B, respectively. Hernández and Kubota [22] found similar responses in cucumber seedlings. However, no description of the mechanisms resulting in the unpredictable response to 100% blue light have been identified.

The highest SFM was found in monochromatic red light and could be related to the highest leaf area produced in those plants (data not shown). Others have reported that large leaves under 100% red light are typically the result of a shade-avoidance response induced by the lack of blue light, which inhibits cryptochrome activation in plants [9,11,24–26]. Cryptochromes have been shown to regulate several physiological responses in plants, including stomatal opening, root development, and programmed cell death, and are most likely key to optimal plant functioning in controlled environments [27].

Similar to the trend measured for SFM, root morphology was affected by the increasing proportion of blue light (Table 1). We found that 7B resulted in the highest TRL and NRT. Responses to light quality on root morphology have been mostly reported for micropropagation studies conducted *in vitro*. A decrease in root development under monochromatic red light has been associated with reduced levels of cytokinins [28]. Therefore, the combination of blue and red LEDs has been shown to promote root formation compared to monochromatic red light [29]. Similarly, it has been reported that increasing blue light can induce a significant decrease in root fresh and dry mass and reduce the shoot to root ratio [30,31]. More and longer roots under 7B may increase the ability of plants to absorb water and nutrients and could potentially have resulted in more growth if plants were to be grown for longer periods of time, beyond the timeframe of this study.

Hogewoning et al. [9] found that plants grown under 100% red light have unresponsive stomata, which negatively affect gas-exchange responses such as stomatal conductance (g_s). Our findings show significant differences in g_s (Fig. 4)

Stomatal conductance increased with increasing blue light up to 66% blue that showed the highest value of g_s. The lighting treatment with 100% blue did not follow the trend and showed a similar value compared to 26B treatment. However, net photosynthesis was lowest in R and steadily increased with higher blue light (Fig. 3). This could be partly explained by an increase in steady-state g_s under high blue light [18,24,27]. Blue-light responses, which control the capacity of stomata to regulate conductance, are perceived by phototropins that activate a signaling cascade resulting in fast stomatal aperture under background red light [33]. Although SD under monochromatic red or blue light was lower than other treatments, g_s was negatively affected only in R (Fig. 5). It is possible that blue-light induced stomatal aperture counteracted the reduction in SD under B and thus prevented lowering of g_s. In addition, we found that plants grown under a combination of blue and red light had higher SD than those grown under monochromatic light, which may suggest that a combination of blue and red light is necessary to maximize stomatal development in lettuce.

In general, the 7B was found to be the best treatment for lettuce cultivation in a controlled environment under blue and red LEDs. Although monochromatic red light resulted in the highest production of edible fresh biomass, poor root development and reduced gas exchanges may lead to unfavorable plant growth at the harvest stage.

4.6. Conclusions

Adjusting the light spectrum with LEDs is a major tool for optimizing efficiency of plant compartments in bioregenerative life-supports systems for space. Our findings indicate that the spectral quality from LEDs can significantly affect lettuce growth in controlled environments. Plants increase gas exchange with increasing blue light, but overall growth was reduced. While searching for the optimal light recipe for plant growth and development, careful consideration needs to be placed for balancing morphological, physiological and anatomical responses of plants.

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Chapter 5

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MULTITROP: an educational project on root tropism interactions

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MULTITROP: the challenge of using refurbished hardware for an educational and scientific experiment on the ISS

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MULTITROP: an educational and scientific project on root tropism interactions in microgravity

5.1. Abstract

In 2017, ASI (Agenzia Spaziale Italiana) promoted YiSS (Youth ISS Science), a call for educational and scientific experiments to be performed on ISS during the VITA (Vitality, Innovation, Technology and Ability) mission, with astronaut Paolo Nespoli. A requirement of the call was to select the Experiment Unit within a set of nine previously used for other experiments in microgravity. The EUs were designed, developed and flight-certified by Kayser Italia.

Among the 13 projects submitted for the competition, MULTITROP (MULTITROPism: interaction of gravity, nutrient and water stimuli for root orientation in microgravity) was the winner. The project was ideated by scientists of the Department of Agricultural Sciences of the University of Naples Federico II, in collaboration with University and High School students. In addition to the educational aims, the experiment had a scientific goal in plant Space biology. It aimed to disentangle the role of gravity from two other stimuli for root orientation: hydrotropism and chemotropism. ASI has funded and coordinated the programme, also providing access to the Space resources thanks to a bilateral agreement with NASA.

MULTITROP was performed in a BIOKON container equipped with two Experiment Units previously flown for the YING (Yeast In No Gravity) experiment, supported by ESA (European Space Agency) in 2009. The hardware was refurbished and re-adapted by Kayser Italia to fulfil the new mission requirements.

One lesson learned was that to plan a new experiment with refurbished HW, scientists should evaluate not only the HW details but also the specific environmental conditions expected during the pre-flight and flight operations. Data from the ISS experiment confirmed that chemotropism has a stronger effect compared to hydrotropism in microgravity. Altering gravity on Earth by using a

uniaxial clinostat showed similar results, but only when root axis was perpendicular to rotation axis. Scientific outcomes contributed to a better understanding of root tropism interactions which will have possible application in improving Earth and Space agriculture in controlled environments.

5.2. Introduction

In 2017, ASI promoted the YiSS program, that provided access to the Space resources thanks to a bilateral agreement with NASA. Peculiarity of the call was the requirement to perform the experiment by using hardware belonging to ASI and used for previous experiments in microgravity. The original hardware was designed, developed and flight-certified by Kayser Italia that was also responsible for its new refurbishment. Among the 13 proposals submitted for the competition, the experiment “MULTITROPism: interaction of gravity, nutrient and water stimuli for root orientation in microgravity” (MULTITROP) was the winner. Activities were distributed in a period of about 28 months (from September 2016 to December 2018) and organized in four phases: a) pre-submission phase; b) pre-flight phase; c) flight phase; d) post-flight phase. At present, the experiment has been successfully executed on the ISS and only few more final on-ground tests need to be performed. Overall both educational and scientific goals have been fully accomplished.

5.2.1. Educational aims and activities

A principal aim of MULTITROP was to enhance young people’s interest in Space biology. The experiment was conceived by scientists at the Department of Agricultural Sciences of the University of Naples Federico II in collaboration with three students from the Department of Agricultural Sciences of the University of Naples Federico II (two master students and a Ph.D. student, myself), and nine students from the High School ‘Liceo Scientifico Filippo Silvestri’ located in Portici (Napoli), Italy. The proposer working group was named DALiSS team. Further goals for the Ph.D. student were the development of the abilities to conceive, propose

and run a scientific project and also to lead a student group in the laboratory activities.

During the pre-submission phase, students attended seminars on several topics including: a) morpho- functional traits of seed germination and seedling development, b) root tropisms, c) how to plan a scientific experiment. All proposers actively collaborated to fulfil the requirements for submission. They recorded a video clip aimed to introduce the team, describe the experiment and highlight student's feedback on expected results. It is available at the following link:

https://www.youtube.com/watch?v=RK_pn38dFPc

Figure 1. Logo of the MULTITROP experiment.

Throughout the period of the MULTITROP activities, high school students were mainly involved in seminars, simple laboratory tests and numerous dissemination events in addition to the regular school schedule. University students, besides to the above-mentioned activities, were asked to deepen the study of specific scientific issues related to plant Space biology and were involved in most of the numerous experimental and implementation accomplishments.

5.2.2. Theory

Plants react to the variability of the surrounding environmental factors directing growth of their organs on the basis of sensory information. These reactions are defined as tropisms: directional growth responses to a directional stimulus [1]. Tropisms allow plants to adjust their growth in order to best acquire light, water and nutrition, or avoid stressing and damaging situations. A tropic reaction can be active (if based on genetically programmed alterations in the homeostasis of plant growth) or passive (if elicited through harmful effects altering growth) and also positive or negative according to the direction, respectively towards and away from the stimulus causing the tropism [2].

At root level, tropic responses take place in the few millimeters of the root tip. More specifically, most of the external stimuli are perceived in the columella cells located in the central part of the root cap. These ephemeral cells are also the source of the polar auxin flow which moves through the external root tip cells and onwards to the location of tropic curvature in the elongation zone. A uniform auxin distribution causes a straight growth of the root tip, while a differential lateral accumulation of auxin reduces the elongation of the external cells and determines a local bending of the tip. The phenomenon was firstly and independently described by Went (1926) [3] and Cholodny (1940) [4] and is therefore referred to as the Cholodny-Went theory. More recently, the molecular functions of these processes have been studied [e.g. 5]. Moreover, the possibility to disentangle the effect of gravity on root growth orientation by means of experiments in space increased scientific efforts to investigate on the other tropisms and their interactions [6; 7].

5.2.3. Scientific aims

In addition to the educational activities, the experiment had a scientific goal in plant Space biology. The MULTITROP experiment was conceived in this framework and aimed to study the role of hydrotropism and chemotropism in root orientation in absence of the gravity stimulus.

Gravity is considered the principal factor guiding root orientation on Earth. However, several different tropisms have been identified for root growth. In addition to gravitropism, the most extensively studied tropisms include phototropism,

hydrotropism, chemotropism, halotropism and thigmotropism [8]. Nevertheless, scientific evidence suggests that also other environmental factors play a role in directing root growth; among them, there are electric fields, magnetism and sound [9]. Although most research focuses on isolated tropisms and cuts out interactions between different tropisms, it is clear that in natural circumstances the final growth strategy relies on the integration of proportional influences of all tropic signals.

The scientific aim of the MULTITROP experiment was to disentangle the role of gravity from two other stimuli for root orientation: hydrotropism and chemotropism. Considering that gravity is a dominant directional stimulus masking the effects of other tropisms, the relative importance of water and nutrient solution as potential attractive factors in root growth orientation was investigated by performing the experiment in microgravity. Experimental setup consisted of seeds placed in between two substrate diskettes soaked either with pure water or with nutrient solution. Three possible scenarios were hypothesized: a) if roots develop in the substrate with the nutrient solution, chemotropism prevails on hydrotropism; b) if roots develop equally in both types of substrates, hydrotropism prevails on chemotropism; c) if roots develop according to embryo axis, neither water nor nutrients act as a directional stimulus.

5.2.4. The challenge to use refurbished hardware

One of the requirements to apply for the YiSS call was the use of refurbished HW to be chosen from a list of nine available options. After a careful evaluation of the technical description of each HW, the PI considered the YING-B2 as the most suitable to accomplish the scientific requirements of the MULTITROP experiment. It was immediately clear that a set of critical constraints had to be tackled during the pre-flight phase in order to adapt the biological system to the HW features without altering the scientific goals of the experiment. Among others, the main constraints were: a) small volume of the single growth chambers; b) impossibility to select and control a specific temperature during the experiment run; c) no alternative at performing the biological activation of the experiment at the launch site.

5.3. Materials and methods

MULTITROP has been performed in a BIOKON container equipped with two YING-B2 EUs previously flown for the YING experiment supported by ESA in 2009. Both the BIOKON and the YING-B2 units have been designed, manufactured and certified for launch by Kayser Italia.

5.3.1. HW description and constraints

The BIOKON used for the MULTITROP experiment is a passive container (Figure 2 a) which provides a dedicated environment for the execution of life science experiments in microgravity.

This BIOKON is composed of two volumes: a top vented case containing the batteries pack for autonomous operations of the experiment and a lower sealed case containing the two YING-B2 experiment units (Figure 2 b) with the biological samples.

Figure 2. (a) BIOKON container; (b) YING-B2 Experiment Units inside the BIOKON.

The BIOKON used for MULTITROP was flown in 2009 for the DAMA mission carrying the IFOAM experiment. The BIOKON configuration was completely different from the one used for MULTITROP, mainly in terms of battery type and electronics. For the previous experiment, the EUs were interfaced to the BIOLAB facility on the ISS and therefore both their electronic and mechanic interfaces were

not suitable for the MULTITROP experiment, where the EUs interface only to the self-standing BIOKON container in which were placed.

Besides, being the type of biological samples different from the previous experiment, it was necessary to design an ad-hoc tool for adapting the MULTITROP samples inside the culture chambers.

In addition to the small volume of the growth chambers, the main technical constraints and challenge of the HW refurbishment were related to the fact that biological activation would have occurred at the implementation phase (placing the dry seeds between the two wet substrates) and most of the experiment would have run during the launch phase. The first consequence of this circumstance was the need to perform the biological and hardware integration at the launch site, a few hours before the SpX launch. The reason behind this constraint is that YING-B2 EUs have only one reservoir chamber per each culture chamber and this one had to be filled with the chemical fixative required for “blocking” the root growth at experiment completion.

5.3.2. Pre-flight scientific activities

Pre-flight activities aimed at the development of the biological system, the adaptation of the experimental unit and the definition of the duration of the germination and growth phases (to define the exact moment of injection of the fixative).

During this phase we investigated literature and performed tests to find the most appropriate species that fits with the imposed environmental parameters, time requirements, and volume of the experimental unit. Seed germination tests were performed in Petri dish according to Baskin & Baskin [10].

For an accurate choice of the suitable plant species, we applied the method of the Subsequent Exclusion Criteria. To reduce the number of candidate species we applied the following criteria:

1. species of agri-food interest
2. seed size compatibility to HW requirement
3. seed germination rates, timing, and uniformity

4. seedling development compatibility to the expected flight timeline and temperature range

Several tests were made also for the selection of the substrate best suited to the experimental setup. Several tests were conducted concerning the following types of substrates:

- Polyurethane sponge
- Rockwool
- Floral foam
- Perlite
- Cellulose sponge
- Oasis®

During the selection tests we gave priority to the following characteristics of the substrate:

- Retain water under hypergravity conditions experienced during the launch phase (2 to 5 g)
- No leakage between diskettes surfaces;
- Suitable plasticity necessary to shape it or mold it into the form of the EU;
- Possibility to sanitize the substrate in order to avoid contaminations;
- Diffusion of the fixative solution to guarantee the right fixation of the experiment.

As regards the choice of the nutrient solution for the experiment, based on a literature overview, we found evidence for an active, positive chemotropism towards nutrients, for a solution previously used by Frederick & Newcombe (1904) [11]. Laboratory experiments were carried out to verify the seed/substrate interaction and to select the best combination.

Once defined the species, the substrate and the nutrient solution, we performed ground tests in the YING-B2 hardware for the validation of the experiment. The

preliminary results were elaborated and used to define the following protocols which were approved by ASI and NASA:

- Sterilization protocols
- Protocols for the transfer of the material from the laboratory to the launch base and the installation in the capsule of the launch vector
- Protocols for the activities needed to be carried out when the launcher returned (hardware recovery and storage of samples until transfer to the laboratory)

5.3.3. Flight activities

Flight phase on the ISS was preceded by the late access activities aimed to the experiment set up and payload delivery to NASA. The team involved for the late access experiment set up worked at the Space Station Processing Facility of NASA Kennedy Space Center in Cape Canaveral (FL) in the period from 28th November until 15th December 2017 (Figure 3).

Figure 3. From left to right: L.G. Izzo, S. De Francesco, G. Aronne, and L.E. Romano at the NASA Kennedy Space Center.

Hardware assembly protocols were defined during the preflight phase, approved by NASA after the Experiment Simulation Test and used at the NASA KSC laboratory for the ISS experiment and at the laboratory of the Department of Agricultural Sciences at Portici for the entire control test on Earth. The team at the launch site assembled the experimental unit YING-B2 following the procedure listed in the protocol provided by Kayser Italia, and prepared and integrated the biological samples.

Substrate was shaped in diskettes and soaked with water (WOD) or nutrient solution (NOD) in centrifuge tubes (Figure 4).

Figure 4. Substrate diskettes in centrifuge tube

Diskettes were centrifuged in tubes for five minutes to simulate hypergravity level expected during launch in order to avoid leakages.

Seeds were disinfected in 2% (v/v) sodium hypochlorite for 5 minutes, and then rinsed with sterile water. After seed disinfection, diskettes and seeds were placed in the EUs. The seeds and diskettes were placed following a specific order as shown in Figure 5. Once biological integration was completed, EUs were sealed with a metallic plate and then subjected to a Leakage Test using a vacuum chamber.

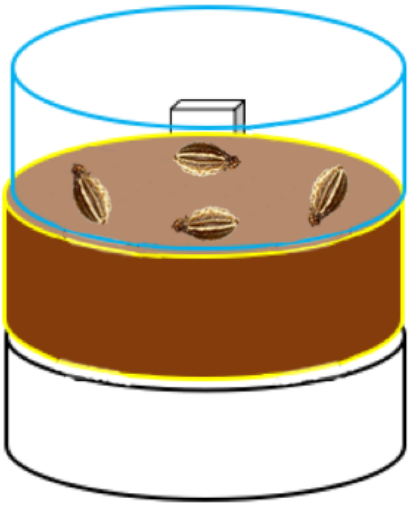

Figure 5. Seed positioning between WOD (blue) and NOD (brown) oasis diskettes.

The MULTITROP payload was launched to the ISS with the SpaceX Crew resupply service mission (CRS) 13. On the ISS, the experiment required little crew time and the only operations conducted by the astronaut were:

- HW downloading from the Dragon;
- Positioning of the hardware in the Node 2 of the ISS;
- Activation of the fixative release mechanism by lowering a switch;
- Storage in Node 2 of the ISS;
- HW uploading into the Dragon before unberth

Splashdown occurred one month after the launch. The payload was retrieved by SpaceX, hand back to NASA, and sent to Italy keeping it under controlled temperature (15±5 °C).

5.3.4. Post-flight activities

After receiving the BIOKON, we proceeded with the de-integration of the YING-B2 experimental flight unit from BIOKON; the recovery of environmental data recorded in flight, and the retrieval of biological samples.

Morphological analysis of seedlings developed in microgravity was performed without dismantling the setup of the biological samples. Because the substrate used for the experiment was not a transparent material, we looked for an alternative method to scan the samples and get a 3D reconstruction of the root growth

orientation within the substrates. After several unsuccessful attempts of non-invasive technique we decided to use the X-ray microtomography to analyze samples. Thanks to the cooperation with CNR ISAFOM of Ercolano (IT) we used a Bruker Skyscan 1272 to perform a high-resolution X-ray microtomography. Subsequently, CTAN and CTVOL software were used to process and analyze the images and obtain a 3D reconstruction of the content of each growth chamber. X-ray microtomography allowed us to observe seed position, seed germination, and root orientation.

In addition to the flight experiment, numerous other tests were carried out in the laboratory as control of the main experiment performed on the ISS. Such tests were always performed in the YING B2 experiment units and incubating the seeds at 22±4 °C for 180h. Two different types of tests were performed in the laboratory: a) YING B2 incubated stationary and vertically and b) YING B2 incubated while continuously rotating on a uniaxial clinostat.

In the first case, normal gravity conditions were tested using two different substrate dispositions. One disposition (CON) followed our standardized scheme of placing the substrate disk with nutrient solution (NOD) at the base of the cultivation chamber and the substrate disk with water (WOD) on top. For the second disposition (COW), we decided to invert this order. The scientific hypothesis was to prove that on Earth roots always grow downward regardless of the position of water and/or nutrient.

Tests performed on the uniaxial clinostat aimed to have a preliminary assessment of multiple tropism interactions under simulated microgravity conditions. Two different setup were tested on the clinostat, placing root axis parallel (CLH) or perpendicular (CLV) to rotation axis of clinostat.

5.3.5. Statistical analysis and data elaboration

Descriptive statistics and statistical inferences were conducted by Microsoft Excel and IBM SPSS, respectively. The Chi-squared test was used to determine whether there was a significant difference between the expected and the observed root development frequencies obtained in diverse conditions. The null hypothesis of the tests was rejected if $p < 0.05$.

5.4. Results

5.4.1. Experiment adaptation to the YING B2 hardware

For the experiment we refurbished the YING B2 EU previously used for yeast cultivation in Space and readapt the hardware to grow seedlings (Figure 6a). The size of the growth chamber was one of the most challenging characteristics of the hardware. After testing several design proposals, we divided the growth chamber volume into two sections to have two different substrate conditions within each growth chamber. This was achieved by using two substrate diskettes that were soaked one with water and the other with nutrient solution (Figure 6b).

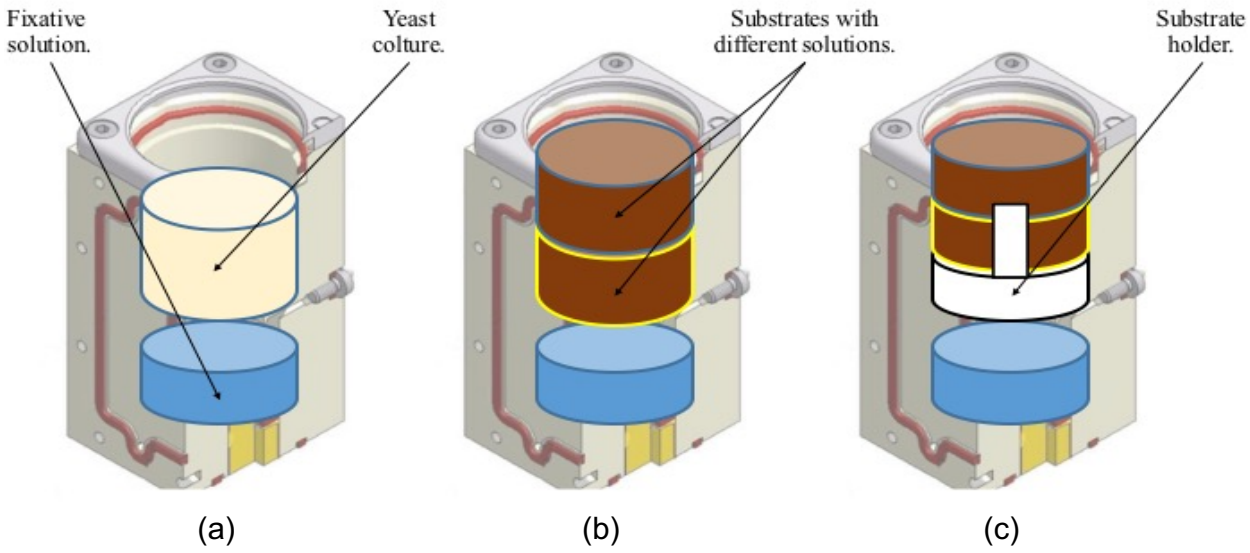

Figure 6. (a) yeast culture, (b) substrate, (c) final setup.

The available volume of fixative solution to be used at the end of the experiment was not sufficient to fill the air volume. An HW solution was adopted to overcome this additional constraint. For a complete wetting of the samples with the fixative solution, we developed a 3D-printed holder to be placed underneath the substrate disks, in order to reduce the air volume of the growth chamber and also facilitate the extraction of the samples after the experimental run (Figure 6c).

In addition, the technical-support partner Kayser Italia did the refurbishment of the hardware that addressed the following aspects:

- Power Supply
- YING-B2 EUs mechanical accommodation inside the BIOKON

- YING-B2 EUs firmware programming for execution of the timeline
- Tool realization for adaptation of the experiment samples to the EU

To implement the above-mentioned modifications, a new battery type has been used according to safety requirements, and a new battery pack, including an electronic circuit protection, has been designed, manufactured and tested against possible leakage and launch loads vibrations.

Then the YING-B2 EUs electronics has been adapted for receiving power from this battery pack and the microcontroller has been re-programmed for the activation timeline requested. Finally, an interface plate has been designed and realized to accommodate the two YING-B2 EUs inside the BIOKON (Figure 7).

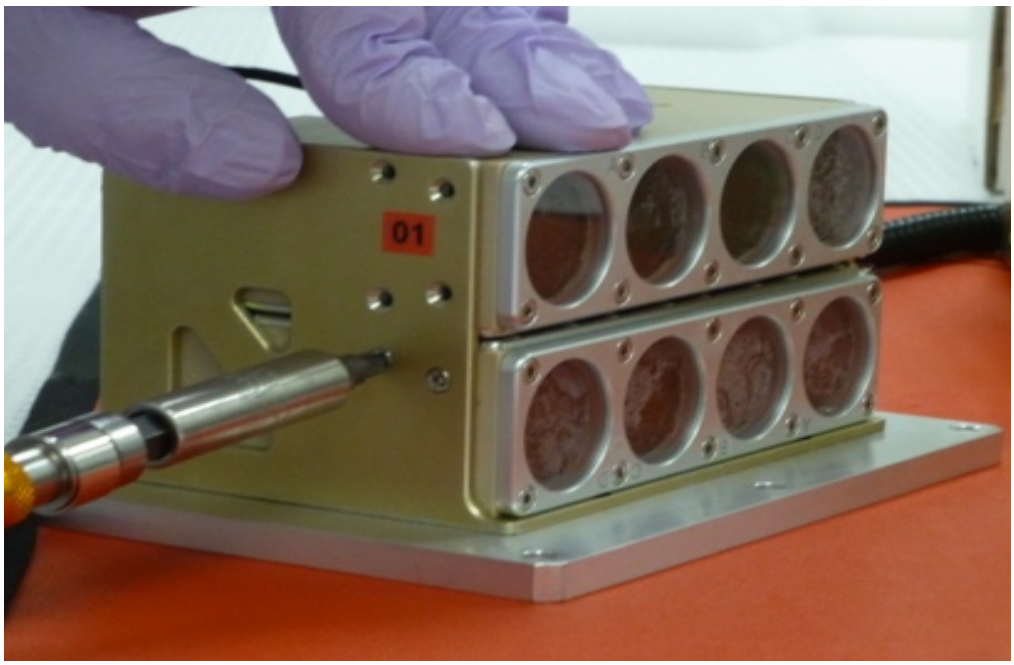

Figure 7. Interface plate for accommodation of the YING-B2 EUs inside the BIOKON.

5.4.2. Substrate selection

Considering the experimental design, the experimental conditions and schedule, and the constraints related to the hardware, the choice of the substrate was fundamental.

Among different substrates, Oasis® turned out to be suitable for the MULTITROP experiment. This substrate exposed to a centrifugal force of 6g loses half of the

water in the first minutes, but holds the remaining amount of water for a long hypergravity exposure with a decrease of only 16% in one hour. In addition, this substrate showed all the required characteristics and was lightweight and easy to handle.

5.4.3. Species selection

Considering the possible application of results from this experiment, we considered the main role of plants in space cultivation systems that is food production. This criterion resulted in considering fifty species of agri-food interest (Table 1). Number of candidate species was reduced to 27 species following the second criterion related to the size of seeds and available volume for growth.

The number of species was reduced to five after analyzing the germination dynamics of seeds focusing on germination rates, timing, and uniformity in seedlings germination.

The last criteria applied considered the effect of the temperature expected on board on seed germination. Results showed that *Daucus carota* L. was the suitable plant species for the experiment, exhibiting few variations in germination dynamics at different temperatures. In addition, four cultivated varieties of *Daucus carota* were tested and *Daucus carota* cv 'Chantenay' by Franchi seed company turned out to be the best to perform the MULTITROP experiment in the YING B2. Data showed that within the temperature range 22±4°C these seeds germinate not earlier than 56h from hydration and reach the target stage in 180±8h. Therefore seeds of *Daucus carota* cv 'Chantenay' fulfilled the biological requirements of the flight experiment by avoiding to germinate before reaching the microgravity conditions and by developing the root up to the target stage before crew availability to inject chemical fixative (HW activation).

Table 1. Candidate species for MULTITROP experiment.

Species	Criterion 1	Criterion 2	Criterion 3	Criterion 4
Allium ampeloprasum	*	*		
Allium cepa	*	*		
Allium sativum	*	*		
Asparagus officinalis	*	*		
Beta vulgaris	*	*		
Brassica oleracea	*	*		
Brassica rapa	*	*		
Capsicum annuum	*	*		
Chenopodium quinoa	*	*		
Cicer arietinum	*			
Cichorium endivia	*	*		
Citrullus lanatus	*			
Cucumis melo	*			
Cucumis sativus	*			
Cucurbita maxima	*			
Cucurbita pepo	*			
Daucus carota	*	*	*	*
Eruca vesicaria	*	*		
Foeniculum vulgare	*	*	*	
Fragaria vesca	*	*		
Glycine max	*			
Hordeum vulgare	*			
Lactuca sativa	*	*		
Lathyrus sativus	*			
Lens culinaris	*	*		
Linum usitatissimum	*	*	*	
Lupinus albus	*			
Medicago sativa	*	*		
Ocimum basilicum	*	*		
Oryza sativa	*			
Petroselinum crispum	*	*		
Phaseolus lunatus	*			
Phaseolus vulgaris	*			
Pisum sativum	*			
Raphanus sativus	*			
Raphanus sativus var. longipinnatus	*	*		
Secale cereale	*			
Sedum graveolens	*	*	*	
Sesamum indicum	*	*		
Solanum lycopersicum	*	*		
Solanum melongena	*	*		
Trifolium repens	*	*		
Trigonella foenum-graecum	*	*	*	
Triticum aestivum	*			
Triticum durum	*			
Triticum monococcum	*			
Vicia faba	*			
Vigna angularis	*			
Vigna radiata	*			
Zea mays	*			
Total	50	27	5	1

5.4.4. Root orientation on Earth and ISS

Data recorded from both Earth and ISS experiments showed that gravity interacts with other tropic stimuli in root development of carrot seedlings. In all tests performed, seedlings developed roots in one of the two diskettes. We never found roots growing at the interface between the two diskettes.

Under normal gravity condition (seeds incubated stationary in 1g) all germinated seeds orientated root growth following the gravitational vector (Table 2). Roots always developed downward regardless of the presence/absence of nutrients in the substrate. As expected gravitropism was dominant on chemotropism ($p < 0,05$).

Table 2. Number of roots developed into the NOD or WOD diskette in the YING B2 assembled placing on bottom either the nutrient diskette (CON) or the water diskette (COW).

	CON	*COW*
NOD	29	0
WOD	0	28
No germination	3	4
Total	32	32

Tests on clinostat were performed using two different setup, CLH (root axis parallel to rotation axis) and CLV (root axis perpendicular to rotation axis). Comparing data sets from the two experimental setups, it was clear that the different arrangements of the EU influenced results (Table 3).

Table 3. Number of roots developed in NOD or WOD diskette under CLH, CLV and ISS conditions, and results of the χ^2test.

Test	Germinated seeds (n)	Roots in NOD (n)	Roots in WOD (n)	χ^2	p-value
CLH	29	15	14	0.069	0.7923
CLV	30	21	9	9.6	0.0021
ISS	27	20	7	12.52	0.0004

In CLH position there was no preferential growth direction and the number of roots developed into the NOD and WOD diskettes was similar. Differently, in CLV position there was a preferential growth towards NOD diskette. In this case, chemotropism turned out to be effective in attracting roots ($p < 0.05$).
Data from the experiment performed in microgravity condition on the ISS showed a preferential growth toward NOD ($p < 0.05$). Thus in the absence of gravity stimulus, chemotropism effectively oriented root growth.

5.5. Discussion

5.5.1. Refurbishment

The use of refurbished hardware to setup the MULTITROP experiment on the ISS resulted overall positive. It was a demanding experience during which the whole team have learned several lessons to be used for future opportunities.
Scientists were ready to spend most of their time to adapt the biological system to the HW characteristics. However, specific constraints of the HW got complicated by the interaction with environmental conditions and timing of pre-flight and flight operations. Before submission, the scientific team was informed of all HW details and had envisioned solutions for the constraints (compatibility between growth chamber volume and seed size, necessity to select seedlings with late hypocotyl development, requirement of late access to the launch site for experiment implementation, etc.).
During the pre-flight phase, the assignment to the specific mission and consequently the definition of all the timing and environmental conditions of the pre- launch, launch, berthing, de-stowage up to the injection of chemical fixative by the crew, generated a series of further constraints. By means of prevision models, we find out that radicles were going to protrude after launch but before the berth and that root growth was going to reach the target stage soon after de-stowing from the cargo vehicle.
Adaptation of the biological system to the constraints related to the prelaunch-launch operations resulted by far the most challenging. The timeline from payload hand-over to de-stowage and experiment deactivation on the ISS (with the steps

ranging from a few hours to a few days), combined with temperature uncertainties, gave hard time to the scientific team and required further collaboration with Kayser Italia and ASI teams to obtain as many as possible additional expected data.

Within the MULTITROP experience, temperature control deserves a special comment. It is well known to plant biologists that even small changes in temperature conditions affect seed germination percentage and timing as well as radicle growth rate [10]. Impossibility to run the experiment within a specific range of temperatures was clearly stated at the beginning of the pre-flight phase. At first sight the scientific team considered this issue resolvable by knowing the range of expected temperature values. However, such a range turned out to be extremely wide and useless to fine-tune seed selection and the timeline for experiment deactivation. The ideal seeds for MULTITROP had to germinate all together, not earlier than three days after payload handover (minimum time interval to guarantee germination in microgravity). Moreover, their radicles had to reach the target elongation not earlier than six and a half days from payload handover (minimum time interval for crew availability to deactivation). For most of the seed species, temperature changes of even few degrees would have resulted in a complete mismatching of these requirements. The selected carrot seeds turned out to adapt quite well to the environmental conditions occurred during the experiment and scientific aims were successfully achieved.

Overall, the use of the Subsequent Excluding Criteria in the selection of the most suitable type of seed resulted spot-on. A similar method was already used to select cultivars for plant cultivation in space [12] and resulted valuable also to select the most suitable seed species for MULTITROP. The first criterion of considering agronomic/food species turned out to be useful also to easily find a wide assortment of high-quality seed stocks on the market and to lower the variability in the temperature response generally wide in the seeds of wild species [13]. In retrospect, the fine adjustments of the biological system to the technical requirements would have been much easier within a framework of controlled temperature conditions.

5.5.2. Root tropism interactions on Earth and ISS

On Earth gravitropism is dominant on other root tropisms and the attractive action of other stimuli is overshadowed by the influence of gravity [14, 15]. Our experiment highlighted that without the dominant effect of gravitropism, other stimuli influence root growth orientation. Data from the experiment on ISS also suggest that chemotropism has a stronger effect compared to hydrotropism in orienting root growth in microgravity. Such results corroborate what observed by Newcombe and Rhodes at the beginning of the last century [11].

Data from the clinostat experiments showed that, roots developed towards nutrients in simulated microgravity, but only in a specific experimental setup. This happened in the case of root axis placed perpendicular to rotation axis of the clinostat. It is interesting that different results were achieved changing the root axis position with respect to the clinorotation axis. Concerns have been raised in using uniaxial clinostat to simulate microgravity in biological experiments [e.g. 16]. In our case testing the effect of clinorotation on two different root positions, a significant aspect has been highlighted reveling that root rotation axis plays a key role in simulating microgravity on Earth. Within this framework, further investigation is needed to have a better understanding of the phenomenon and optimize biological experiments in simulated microgravity.

The efforts made to carry out a scientific experiment despite experimental restrictions and constraints, allowed us to fully achieve not only the educational but also the scientific goals planned for the project. It has been verified that the roots are attracted by the presence of nutrients in the absence of gravity stimulus. From an ecological point of view, it would be interesting to understand the plant mechanisms underlying the first choices in a life stage in which the roots are sustained by the nutrients already present in the seed endosperm.

Scientific outcomes will contribute to a better understanding of root tropism interactions and will have possible application in plant cultivation in controlled environments. Using specific nutrient solutions to adjust plant responses during germination could be a strategy to optimize plant cultivation in Space. This could be true also for nutrient management aiming at reducing waste and increasing efficiency of plant cultivation on Earth.

5.6. Conclusions

From the MULTITROP experience we learned that refurbished HWs can be successfully used for experiments in Space even when their scientific aims are far away from those of the original experiment. However, for the scientific team the challenges to accomplish the mission are not comparable with those commonly experienced with experiments for which a specific HW is developed. To plan a new experiment with refurbished HW, researchers should be able to evaluate not only the HW details, but also other specific environmental conditions expected to occur during the pre-flight and flight operations. Within this scenario, to limit biological constraints, the possibility to control environmental parameters (such as temperature) should be always furnished.

As regards scientific results, among the three hypotheses, data from the ISS experiment confirmed that chemotropism has a stronger effect compared to hydrotropism. Different types of tropisms have now been shown to exist in an increasing number of plant species and, interestingly, species-specific mechanistic differences in the response exist.

Although progress has been made in understanding tropisms, many more questions remain open. Such scientific effort is worth considering that water consumption is a big deal for global agriculture. In addition, a deepen knowledge is needed to optimize plant production in controlled environment also considering the increasing demand of growing plant in Space for future explorations.

5.7. Acknowledgements

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Conclusions

This Ph.D. project was funded by the Italian Space Agency (ASI) within an agreement with the ESA-MELiSSA (Micro-Ecological Life Support System Alternative) programme. This project gave me the opportunity to develop the knowledge, the methodologies and the interest concerning research in plants science carrying forward the studies already started at the end of the master's degree course.

The aim of the MELiSSA project is to develop a Bioregenerative Life Support System for future long-term manned mission in Space. Among the different compartments that which constitute this artificial ecosystem, plant compartment plays a primary role in producing fresh food and oxygen, and in managing wastes. The scholarship founded by ASI and ESA for this Ph.D. program aimed to deepen the knowledge about plant cultivation in a controlled environment, with particular regard to the optimization of lighting systems to improve plant productivity.

The work carried has developed in different phases and has concerned different aspects of research, scientific dissemination, participation in projects and conferences, as well as international collaborations.

Research activities have been focused on plant responses to different characteristics of light, such as intensity, quality and direction, by using LED technology which provides promising opportunities for study and research in the field of plant science.

An in-depth literature review regarding the application of LED technology in plant cultivation systems has been carried out and the critical points of the research in progress were evaluated. This literature survey has been published as a review article on *AIMS Agriculture and Food* journal.

Experiments carried out during the Ph.D. program considered species suitable for plant production in controlled environment, with particular attention to red-leaf or reddish-leaf plants due to their contribution of antioxidant compounds to plant food. The main approaches used to develop the research activities were focused on morphological, physiological and anatomical responses of plants to the different lighting treatments. It was addressed that light spectrum modulation is a reliable tool to increase the efficiency of plant growth in terms of production and quality of food. Fully red plants proved to be more promising than green or reddish cultivars in

improving antioxidant properties of plant food. In addition, it was also concluded that short-term light-quality treatments can effectively modify plant responses during growth and may ultimately increase plant production without using additional amount of light energy.

In the context of optimizing plant cultivation in controlled environment, an overall conclusion is that, while searching for an optimal light recipe, careful consideration needs to be placed for balancing morphological, physiological and anatomical responses of plants through an appropriate use of light features to achieve an optimal plant growth.

In addition to the literature review and the experimental activities, part of the Ph.D. was dedicated to learn how to develop and manage a research project. The involvement was global: from the design idea to the preparation of the proposal, up to carrying out the experimental activities and the data analyses, including the drafting of technical and administrative documents. In particular, these activities concerned two projects in space biology: one funded by the Italian Space Agency (ASI) in which I worked with the role of proposer and leader of the student team, and one funded by the European Space Agency (ESA) with the role of coordinator. Within the opportunities provided by the ASI, MULTITROP project won YiSS competition and gave me the opportunity to develop and perform an experiment in microgravity which flew on the International Space Station in December 2017. Among the activities carried out during the project, I deepened my knowledge and interest about plant tropisms and seed germination dynamics. Numerous tests have been carried out and specific methodologies have been developed to overcome the limits of experimental constraints. The participation in the activities performed at the Kennedy Space Center in Florida for the setup and integration of the experiment has enriched my experience and interest in Space biology research. The project has achieved all the designed objectives, both educational and scientific. The results from this project suggest that chemotropism has a stronger influence on root groth direction compared to hydrotropism.

Thanks to the experience gained from MULTITROP project, I presented as coordinator the ROOTROPS project to the *“SpinYourThesis”* call within the ESA educational activities. The project focuses on the effects of light on root tropisms in

altered gravity, with the aim of deepening the Ph.D. topics related to the interaction between plants and light. Although the project has not been selected for ESA educational activities, the quality and the topic of the proposal have aroused interest in the ESA scientific committee which suggested the application to the *"Continuously Open Research Announcement"* to perform experiment in altered gravity conditions. The project is now in negotiation phase and would become an opportunity to continue the studies faced during the Ph.D. program.

Among all activities, results obtained during these three years of research activities were presented at numerous international conferences. In addition, the topics covered during the Ph.D. program were deepened thanks to the participation in high-level international courses such as the "MELISSA Summer University 2016" at Girona and the "Lighting in greenhouses and vertical farms" at Wageningen University. The opportunities provided by this Ph.D. program allowed me to expand networks, knowledge and familiarity of scientific community collaborating with other institutes and researchers. Overall results contribute to the international scientific debate aimed to improve plant performance and growth by increasing the efficiency of lighting systems for plant cultivation on Earth and in bioregenerative life support systems for space missions.

Appendix 1

ROOTROPS: tackling the roots of bending

Preface

The proposal was submitted to "*SpinYourThesis*" call within the European Space Agency (ESA) educational activities by Luigi Gennaro Izzo as Team Leader of ROOTROPS. Although the committee agreed on the high quality of the proposal, concerns were raised on the feasibility of completing all the experiments within the 2.5 days allocated at the Large Diameter Centrifuge for educational projects. ESA scientific committee therefore advised to apply in the frame of the ESA Continuously Open Research Announcement (CORA) for access to the ground-based facility at ESA European Space Research and Technology Centre (ESTEC).

At present, a revised version of the project has been proposed by the same team supervised by Prof. Aronne. The proposal passed the initial selection step and is currently under negotiation.

Abstract

The movements of plant roots are mostly attributable to tropisms, which are directional-growth responses guided by directional stimuli. Tropisms allow plants to adjust their growth as a function of environmental stimuli aiming at growth optimization and stress avoidance. Gravity and light represent the most influential stimuli on directional-growth of plants, which compete and interact with each other in shaping the plant in a three-dimensional space. According to literature, most of the studies on interactions between gravitropism and phototropism were performed in microgravity conditions considering 1g as the highest level of gravity. Consequently, new investigations have to be carried out in hypergravity to overtake such a limit and obtain a much-extended overview of root responses to altered gravity. By combining different light treatments and gravity levels, the experiment aims to investigate on the role of hypergravity and light as external stimuli whose interaction directs root growth. We hypothesize that as the gravity level increases the root phototropism is attenuated. Physiological consequences of the treatments on the root meristematic tissue and changes in gene expression will be analyzed.

Expected results will give new insights on the evolutionary processes faced by plants during land colonization. Scientific outcomes could have future agronomic applications in plant rooting methods for controlled-environment cultivations, such as those of Bioregenerative Life-Support Systems in Space.

General objectives

During the long past evolutionary pathways, the transition of plants from water to land environments required adaptations for structural support because under 1g conditions air does not provide any support for upright growth of plants. Adaptation to terrestrial environment required also changes in functional features. Rhizoids of early plants were not capable of water uptake playing a role in anchoring function only. Morpho-functional changes occurred in plants during land colonization regarded also adaptations to changes in light quantity and light quality. In aquatic environments, photosynthetic organisms receive a reduced amount of light energy radiated from the sun due to the passage through water. More specifically, blue and red wavelengths (those mainly absorbed by photosynthetic pigments) do not penetrate deep in water. The apparently steady state of morpho-functional traits in higher plants is the result of complex evolutionary processes that took place in the past. Experiments in altered environmental conditions (such as altered gravity) might furnish unexpected insights in the framework of using plant for cultivation in non-terrestrial environments.

Most of the research activities in this field are performed in microgravity while the effect of hypergravity are much less investigated especially if in interaction with other external stimuli as light. The ESA's Large Diameter Centrifuge (LDC) can be used to test and compare the effect of several levels of hyper-gravity therefore giving the possibility to extrapolate data and investigate on plant reactions to a wide spectrum of altered gravity conditions (from 1g to 20g).

Considering that we cannot reduce gravity on Earth and that opportunities for experiments in real microgravity are limited and expensive, alternative solutions are requested for preliminary tests on Earth. In addition to widely used systems like clinostats and RPMs, hyper-gravity is reported to be a promising method, based on

the Reduced Gravity Paradigm (RGP) (van Loon, 2016). Hypergravity conditions are mainly experienced during launch towards the Space and the ISS. In this phase, which lasts a few minutes, gravity level can reach up to 9g. Considering the plant organisms and their growth timing, this short phase could not affect the general growth of a plant, but when focusing on the seed-germination stages and on the roots movements the effects can be noticeable. This happens when the activation of seed germination processes occurs on Earth before the launch, as in the case of MULTITROP, an ASI (Italian Space Agency) experiment performed on the ISS in December 2017, during the increment 54. The hypergravity level experienced by the seeds during germination could modify the normal growth and direction of developing roots. Understanding the mechanisms that guide root growth in hypergravity would support the design of experiments that include plant organisms to be launched on the ISS. In this context the light may act as a powerful tool in counteracting the effects of hypergravity.

Consequently, the knowledge regarding interactions between gravitropism and phototropism in altered gravity must be deepened. It has to be considered that root reaction to light stimuli can be different in different plant species, therefore it is worth to compare evolutionarily and phylogenetically distant taxa.

Finally, looking at the future that foresees the colonization of new planets by man, a better understanding on plant behaviors in altered gravity is mandatory. The scientific outcomes of this experiment could represent an important step to move forward on the intricate and fascinating road that guides man in Space.

Scientific and technical aspects

Plants respond directly to gravity and light. Roots grow downward, or towards the center of Earth, and mostly away from light. Plants growth response to gravity is known as gravitropism, while phototropism represents the growth response to light. Both tropisms are controlled by plant growth hormones. Auxin is the plant hormone involved in these mechanisms and, in high concentrations, retards the growth of root cells. When auxin is distributed symmetrically, all sides of the root grow at the same rate. If the root tip is not faced downward, a higher accumulation of auxin on the

lower side causes a decrease in cell elongation within the central elongation zone of the root, which induces the root bend towards gravity. Root phototropism also functions according to the theory of auxin redistribution. This causes the often-strong gravitropism to have a masking influence, and it is no surprise that the two most recent new forms of root phototropism have been discovered in microgravity (Millar et al., 2010; Vandenbrink et al., 2016).

The scientific objective of ROOTROPS is to investigate the interactions between gravitropism and phototropism during the root's movements in altered gravity. A multidisciplinary approach will be taken to provide a complete description of the early Root Development process. Particular attention will be given to root curvature (tropisms), root morphological, anatomical features (length, number, angles of secondary roots) and cell growth and proliferation status in the apical root meristems (including both cytological and gene expression approaches) of roots developed at different hypergravity levels with different directional light treatments. Regarding light, the effects of different light wavelengths and different light intensity on the root bending processes will be analyzed. A critical point of the experiment is the choice between the candidate species, considering that it is important to consider that not all species have roots showing the same responses to light. Studies by Schaefer (1911) and Hubert and Funke (1937) demonstrated that about half of the tested species (n=166 and n=152 respectively) reacted to unidirectional white light with negative root phototropism whereas about half showed no response at all. In these studies, only handful displays a positive response (reviewed by Kutschera & Briggs, 2012). Species with a strong root phototropism represent a model to study the trade-off between gravitropism and phototropism by investigating the hypergravity levels that overcome and mask the phototropic stimulus.

Experiment description

The equipment, specifically designed and performed for this experiment, will be implemented in the gondolas. As reported in Fig. 1, in each gondola a sealed holder container (HC) will be placed and locked on the base plate. The HC contains 8 sub-chambers, each of which equipped with a growth-substrate container (GSC) and a

lighting system except for the dark treatment (Fig. 1A). A camera will be used to monitor the experiment during the run. The GSCs have the following dimensions: 200mm * 200mm * 50mm (length*width*height) and will be integrated in an Isolating Box (IB) (Fig. 1B).

The GSCs are made of transparent plastic and will be filled with a growth substrate (phytagel and nutrient solution) that allows light to reach the seeds and the seedling roots. In each GSC at least 10 seeds will be placed on the growth substrate (Fig. 1B).

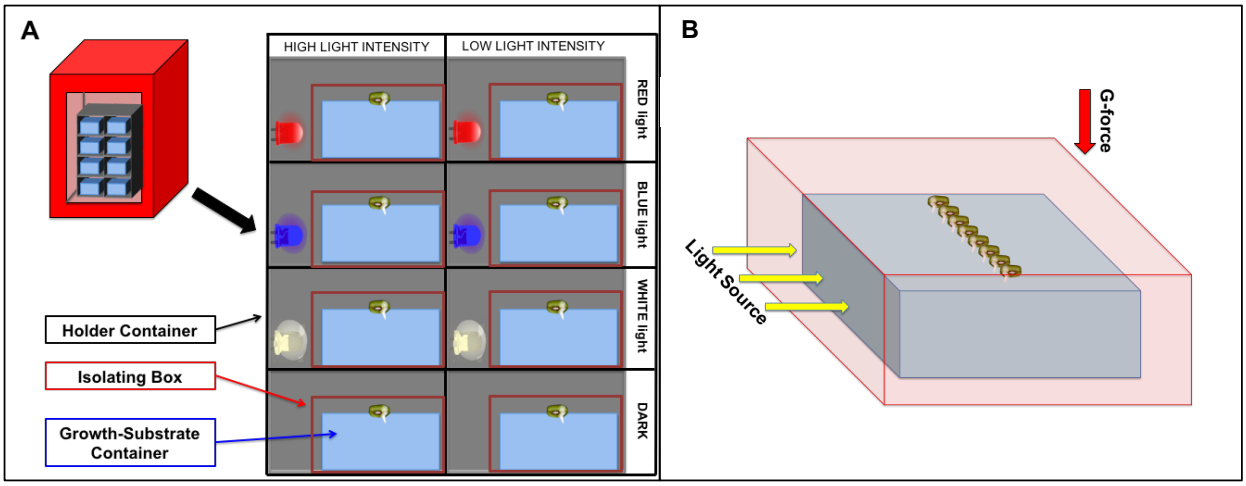

Fig. 1: ROOTROPS experiment set-up. A: Top view of the internal set-up of each gondola. B: Growth-substrate container (GSC) integrated in the Isolating Box. Position of seeds and light source relative to the direction of the G-force.

Proposed species

The interaction between hypergravity and light will be studied in two species: one belonging to the Dicotyledons (*Vigna radiata,* TBC) and one to the Monocotyledons (*Hordeum vulgaris,* TBC). Although these species are so different from a taxonomical point of view, they are similar in size and root growth rate. Moreover, they have been used for former experiments in microgravity and are considered as suitable crop species for future space missions.

Required hypergravity levels

In addition to the 1g static and 1g rotating conditions as controls, our experiment requires four hypergravity levels: 1.3g, 1.7g, 2.3g, 3.0g, to be obtained with two

subsequent experimental accesses. Details of the two units of access to collect seedling samples are reported in the following table. A third unit of access is expected to be performed about two months later, after collecting input from the results of the first and second units of access in order to: 1) validate the data on root curvatures due to the continuous unilateral light treatments and g-force direction selecting the g level with most significant results (tentatively 1.7g/3.0g level), and 2) apply a transcriptional gene expression assay in the root meristems of the most convenient species (tentatively, from *Vigna radiata,* due to bigger root size, it will be easier to collect more and better quality RNA from the root meristems. In such a case, 3-4 Biological replicates are required for qPCR studies).

Lighting treatments

Interactions between hypergravity and light on root tropism will be studied analyzing the effect of different light wavelengths and intensities. More specifically, four different light-quality conditions will be set-up in each gondola: a) White, b) Red, c) Blue, d) Dark. Moreover, two levels of light intensities will be obtained by dimming LED light sources in the separate sub-chambers (Fig.1A).

Data collection

Experiment data of the first and second units of access will come from a) image analysis of the root curvature and growth rate of the seeds, germinating under the different conditions and b) post-sampling microscopy analysis of the germinated seeds by applying immunofluorescence methods and quantitative anatomy techniques.

Seed germination and root development will be monitored during the experiment run by means of a camera.

At the end of each run, images from the above, the front and the lateral views of the samples will be captured to measure the x, y, z root projections of each seedling. Root curvature and growth direction in relation to gravity and light stimuli will be measured by means of image analysis protocols applied to roots of each single seed.

Microscopy analysis will be performed using confocal, epi-fluorescent and light microscopes. Immunofluorescence methods will be mainly aimed to investigate the possible changes in the size of the meristem and the size of the nucleolus which is an indicator of protein production and, in effect, of cell growth. The size of the meristem will be measured by the number of the meristematic cells and the length of meristem from the quiescent center to the beginning of the elongation zone, enabled by the visualization of cell walls by SCRI Renaissance 2200 fluorescent stain (Musielak et al., 2015). The size of the nucleolus will be determined by immunofluorescent labelling of fibrillarin, one of the main nucleolar proteins, performed as previously described in Manzano et al., 2018. Protocols for digital image analysis will be applied to compare a set of anatomical traits such as number, size, shape of cells of different root zones in different treatments.

Experimental data in the third unit of access will come from a) image analysis of the root curvature and growth rate of the seeds germinating under the different conditions and b) post-sampling RNA extraction and transcriptional (qPCR) analysis of the expression of selected genes (known to be affected in previous altered gravity experiments using *Arabidopsis thaliana*) related to meristematic root functions already observed by microscopy analysis in the first and second unit of access samples. Some of the candidate genes monitored will be the orthologous genes of the Arabidopsis AtNUC-L1 (At1g48920) as a cell growth marker, Prolifera PRL (At4g02060) as a cell proliferation marker, Cyclin B1 (AT4G37490) as marker of the G2 cell cycle phase and MET1 (At5g49160) as an epigenetic activity marker using Actin (At3g18780) as a reference gene. Detailed methodology will be adapted from Kamal et al. (2018).

Traditional horticultural lamps (e.g., high-pressure sodium, cool-white fluorescent, metal halide) are useful at providing adequate daily light integral (DLI) indoors. However, light-emitting diodes (LEDs) offer unique opportunities for exploring light-quality effects on plant growth, development, and metabolism. A useful feature of LEDs is their inherent capability to provide accurate spectral control in growing environments by producing narrow-spectrum light. This allows plant photoreceptors to perceive light cues that can control morphology and improve product quality. Numerous plant species have been evaluated under LED lighting with favorable

results in production and flowering control [1]. However, to date, most sole-source light-quality research focused on plant growth-responses to LEDs have used a constant spectral environment throughout the day, and typically, during an entire crop cycle.

Partial-gravity simulation test

According to results from the previous three experimental runs, we plan to setup a further test to investigate the interactions between light and simulated partial gravity on root growth orientation. One species, and one light treatment will be tested under simulated Moon and Mars gravity levels.

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Appendix 2

List of publications

A complete list of articles published during the Ph.D. period is reported following. Articles marked with an asterisk correspond to those reported in the thesis chapters.

1 Arena C., Figlioli F., Sorrentino M.C., Izzo L.G., Capozzi F., Giordano S., Spagnuolo V. 2017. Ultrastructural, protein and photosynthetic alterations induced by Pb and Cd in *Cynara cardunculus* L., and its potential for phytoremediation. *Ecotoxicology and Environmental Safety*, 145: 83–89

2* Chinchilla S., Izzo L.G., van Zanten E., Gómez C. 2018. Growth and physiological responses of lettuce grown under pre-dawn or end-of-day sole-source light-quality treatments. *Horticulturae*, 4: 8

3* Gómez C. & Izzo L.G. 2018. Increasing efficiency of crop production with LEDs. *AIMS Agriculture and Food*, 3 (2): 135–153

4* Izzo L.G., Arena C., De Micco V., Capozzi F., Aronne G. Light quality shapes morpho-functional traits and pigment content of green and red leaf cultivars of *Atriplex hortensis. Scientia Horticulturae*, 246: 942-950

5 De Micco V., Amitrano C., Stinca A., Izzo L.G., Zalloni E., Balzano A., Barile R., Conti P., Arena C. Dust accumulation due to anthropogenic impact on the Vesuvius volcano protects leaves of *Centranthus ruber* from excess light. *Plant Biology* (under revision)

6* Izzo L.G. & Aronne G. 2018. MULTITROP: an educational project on root tropism interactions. *Proc. 2nd Symposium on Space Educational Activities*, Budapest, Hungary

7* Izzo L.G., Mickens M., Aronne G., Gómez. 2018. Gas exchange and leaf anatomy of lettuce in response to red and blue sole-source lighting from LEDs. *Proc. 69th International Astronautical Conference*, Bremen, Germany

8* Aronne G., De Micco V., De Pascale S., Izzo L.G., Romano L.E., De Francesco S., Carrubba E., Valentini G. 2018. MULTITROP: the challenge of using a refurbished hardware for an educational and scientific experiment on the ISS. *Proc. 69th International Astronautical Conference*, Bremen, Germany

Projects

- "MULTI-TROP: interactions for root orientation in microgravity"
  Principal Investigator: Prof. Giovanna Aronne
  Partners: Agenzia Spaziale Italiana and Kayser Italia

- "ROOTROPS: tackling the roots of bending"
  Principal Investigator: Prof. Giovanna Aronne
  Partners: ESA-ESTEC Life & Physical Science (NL), Centro de Investigaciones Biológicas, Plant Cell Nucleolus, Proliferation & Microgravity (ES), e UNCG Biology Department (USA)

- "BIO-IND: *Monitoraggio della biodiversità delle specie vegetali e valutazione di indicatori dello stato di salute in specie modello nel territorio del Parco Nazionale del Vesuvio"*.
  Principal Investigator: Prof. Veronica De Micco
  Partner: Ente Parco Nazionale del Vesuvio

- "WAPS - *Water Across the Plant Systems: effects of microgravity on organ morphological and functional traits"*
  Principal Investigator: Prof.ssa Giovanna Aronne
  Partners: Centre for Interdisciplinary Research in Space (CIRiS), Université Blaise Pascal - Clermont-Ferrand II

Conferences

1. “7th International AgroSpace Workshop: Mars – A Long Way to Go”, Santa Maria in Sperlonga (LT), Italy, 26 - 27 May 2016
2. “MELiSSA Workshop 2016”, Université de Lausanne, Lausanne, Switzerland, 8 - 9 June 2016
3. “17th International Congress on Photosynthesis Research - Photosynthesis in a Changing World”, MECC Congress Centre, Maastricht, The Netherlands, 7 - 12 August 2016
4. “III International Plant Science Conference (IPSC)”, Campus X, Università di Tor Vergata, Italy, 21 - 23 September 2016
5. “MELiSSA Summer University 2016”, Llafranc, Girona, Spain, 11 – 14 October 2016
6. “IV International Plant Science Conference (IPSC)”, Campus Universitario, Università di Parma, Italy, 20 - 23 September 2017
7. “2nd Symposium on Space Educational Activities”, University of Technology and Economics, Budapest, Hungary, 11 - 13 April 2018
8. “AgroSpace-MELiSSA workshop”, Consiglio Nazionale delle Ricerche, Roma, Italy, 16 - 18 May 2018
9. “American Society for Horticultural Sciences annual conference”, Washington, DC, 31 July - 3 August 2018
10. “69th International Astronautical Conference”, Bremen Exhibition & Conference Center, Bremen, Germany, 1 - 5 October 2018

Oral presentations

1. Izzo L.G. Improving Plant Physiological Performance and Growth by Increasing the Efficiency of Lighting Systems. MELiSSA Summer University 2016, Llafranc, Girona, Spain, 11 - 14 October 2016.
2. Muthert L., Izzo L.G., Aronne G. Gravity vs. other external stimuli in seedling root tropisms. IV International Plant Science Conference (IPSC) Campus Universitario, Università di Parma, Italy, September 21th 2017
3. Izzo L.G., Aronne G; "MULTITROP: an educational project on root tropism interactions". 2nd Symposium on Space Educational Activities, 11 - 13 April, Budapest, Hungary
4. Izzo L.G. Light quality management in Space environments. Bulgarian Academy of Sciences, Institute of Plant Physiology and Genetics, Sofia, Bulgaria, 11 May 2018
5. Izzo L.G., Arena C., De Micco V., Aronne G. Light quality influences differently green- and red leaf plant growth. AgroSpace-MELiSSA workshop, Consiglio Nazionale delle Ricerche, Roma, Italy, 16 - 18 May 2018
6. Aronne G., De Micco V., De Pascale S., Izzo L.G., Romano L.E., De Francesco S., Carrubba E., Neri G., Galofaro G., Piccirillo S., Valentini G. MULTITROP: an experiment for the ISS. AgraoSpace-MELiSSA workshop, Consiglio Nazionale delle Ricerche, Roma, Italy, 16 - 18 May 2018
7. Izzo L.G., Mickens M., Aronne G., Gómez C. Gas exchange and leaf anatomy of lettuce in response to red and blue sole-source lighting from LEDs. American Society for Horticultural Sciences annual conference, Washington, DC, 31 July - 3 August 2018
8. Aronne G., Izzo L.G., De Micco V. Plant growth in space: struggling with environmental factors and physical constraints. V International Plant Science Conference, Fisciano Campus Universitario, Italy, 12 - 15 September 2018
9. Izzo L.G., Mickens M., Aronne G., Gómez. Gas exchange and leaf anatomy of lettuce in response to red and blue sole-source lighting from LEDs. 69th International Astronautical Conference, Bremen Exhibition & Conference Center, Bremen, Germany, 1 - 5 October 2018
10. Aronne G., De Micco V., De Pascale S., Izzo L.G., Romano L.E., De Francesco S., Carrubba E., Valentini G. MULTITROP: the challenge of using a refurbished hardware for an educational and scientific experiment on the ISS. 69th International Astronautical Conference, Bremen Exhibition & Conference Center, Bremen, Germania, 1 - 5 October 2018

Posters

1. Izzo L. G., Arena C., Aronne G. Improving Plant Physiological Performance and Growth by Increasing the Efficiency of Lighting Systems. MELiSSA Workshop 2016, Université de Lausanne (UNIL), Lausanne, Switzerland, 8 - 9 June 2016

2. Izzo L. G., Arena C., De Pascale S., De Micco V., Aronne G. Effects of Light Quality on Two Different Cultivars of *Atriplex hortensis* with Red or Green Leaves. 17th International Congress on Photosynthesis Research - Photosynthesis in a Changing World, MECC Congress Centre, Maastricht, The Netherlands, 7 - 12 August 2016

3. Izzo L. G., Hay Mele B., Arena C. The Trade-off Between Red and Blue Light in Optimize the Photosynthetic Performance and Plant Growth. 17th International Congress on Photosynthesis Research - Photosynthesis in a Changing World, MECC Congress Centre, Maastricht, The Netherlands, 7 - 12 August 2016

4. Izzo L. G., Arena C., De Pascale S., De Micco V., Aronne G. Red vs. Green Leaf: Effects of Light Quality on Morpho-Functional Traits. III International Plant Science Conference (IPSC), Campus X, Università di Tor Vergata, Italy, 21 - 23 September 2016

5. Izzo L. G., Arena C., De Micco V., Aronne G. Light quality enhances anthocyanins modulation in red and green cvs of *Atriplex hortensis* L. IV International Plant Science Conference (IPSC), Campus Universitario, Università di Parma, Italy, 20 - 23 September 2017

6. De Micco V., Izzo L. G., Amitrano C., Stinca A., Aronne G., Barile R., Conti P., Arena C. Anthropogenic impact in the Vesuvius National Park: effect of dust accumulation on leaves of *Centranthus ruber* (L.) DC. subsp. ruber. IV International Plant Science Conference (IPSC), Campus Universitario, Università di Parma, Italy, 20 - 23 September 2017

7. Chinchilla S., Izzo L. G., van Zanten E., Gómez C. Growth and physiological responses of lettuce grown under pre-dawn or end-of-day sole-source light-quality treatments.American Society for Horticultural Sciences annual conference, Washington, DC, USA, 31 July - 3 August 2018

8. Amitrano C., Arena C., Izzo L. G., Stinca A., Barile R., Conti P., De Micco V. Morpho-anatomical and physiological responses of *Robinia pseudoacacia* L. plants to anthropogenic dust deposition in the Vesuvius National Park. V International Plant Science Conference (IPSC), Campus Universitario, Fisciano, Italy, 12 - 15 September 2018

Courses and seminars

1. *"Statistica Esplorativa"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, January 2016
2. *"Statistica"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, February - March 2016
3. *"Statistica Applicata ed Avanzata"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, May - July 2016
4. Summer School *"Flowers and Pollinators: field and laboratory techniques to assess functionally for biodiversity conservation"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, September 2016
5. *"How to prepare a research paper and present experimental data"*, Department of Agricultural Sciences, University of Naples Federico II, October - December 2016
6. *"Photoinhibition and Photoprotection in Plant Cell"*, Dott.ssa Arena Carmen, Department of Biology, University of Naples Federico II, Italy, April 13th 2016
7. *"Ecology of forest fire in Turkey"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, January 26th 2016
8. *"Lighting in greenhouses and vertical farms"*, Horticulture & Product Physiology Group, Wageningen University and Research (NL), April 2017
9. "La botanica applicata in campo ecologico ed agronomico", Department of Agricultural Sciences, University of Naples Federico II, Italy, January 11th 2017
10. *"Il nuovo paradigma della sostanza organica: associazioni supramolecolari e implicazioni agroambientali"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, February 8th 2017
11. *"Agricoltura intensiva, fertilità dei suoli e soppressività naturale verso i patogeni tellurici"*, Department of Agricultural Sciences, University of Naples Federico II, Italy, September 13th 2017
12. *"BVOC distribution. Methods of analyses"*, Department of Biology, University of Naples Federico II, Italy, May 2017

Side activities

- Support in the laboratory activities to the students Luuk Muthert (University of Utrecht) and Leone Ermes Romano (University of Naples Federico II) during thesis work regarding root tropism interactions in altered gravity.

- Support in the course of General and Systematic Botany for laboratory exercises at the Department of Agricultural Sciences, University of Naples Federico II, Italy (2017/2018)

- Exhibition of the "MULTI-TROP Experiment: seeds on the International Space Station" at "Notte Europea dei Ricercatori", Department of Agricultural Sciences of the University of Naples Federico II, September 29th 2017

- Interview for RaiNews24 at the headquarters of the Italian Space Agency in Rome during the event "Paolo Nespoli, the VITA mission", July 28th 2017

- Exhibition of the "MULTI-TROP: interactions for the orientation of roots in microgravity" at "Mediterraneo e dintorni - XIV edition", Portici Botanical Garden, May 20th 2017

- Interview for "100 Degrees of Freedom" on RadioKaos Italia to disseminate the MULTITROP project the Ph.D. research work, December 7th 2016.

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