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Improving Controlled Environment Agriculture

(CEA) Efficiency for Targeted Phytonutrient

Production in Space: A Study within the Microx2

Project

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Ph.D. student

Dr. Gabriele Paglialunga

Ph.D. Coordinator

Prof. Roberta Bernini

Thesis Supervisor

Prof. Giuseppe Colla

Co-tutor

Dr. Alberto Battistelli

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Abstract

In the next decade, significant challenges are anticipated in the field of space exploration. In order to support human life in space, it is critical to use diet as a tool to maintain astronauts' health, enhance resilience to space-related stress factors, and ultimately their performance to ensure mission success. To address these needs, the implementation of bioregenerative life support system (BLSS), including *in-situ* food production compartments leveraging advanced agronomic technologies is crucial. The objective of this thesis is to contribute scientific insight focused on optimizing resource utilization in controlled environment agriculture (CEA), aiming to enhance the quality of horticultural species in terms of the accumulation of phytochemical compounds, including vitamins, antioxidants, and prebiotics, which are fundamental for nutrition in space. Most of the work described in this thesis was conducted within the project 'Microgreens x Microgravity' – "Microx2" funded by Italian Space Agency (ASI). The experiments conducted aimed at: 1) identifying the most suitable microgreen species for cultivation in space and for meeting the nutritional needs of the flight crew; 2) defining the environmental parameters, including light intensity, temperature, and relative humidity (VPD), conducive to maximizing resource efficiency in enhancing productive and qualitative performances; 3) defining technical protocols, including substrate selection and management of the nutrient solution, aimed at maximizing resource efficiency and optimizing species-specific productive and qualitative outputs. Through the development and use of a selection algorithm based on objective comparison, the two best microgreens species for cultivation in space were identified: *Raphanus raphanistrum* subsp. *sativus* 'Saxa' and *Brassica oleracea* var. *capitata* f. *sabauda* 'Vertus'. The variability in the productive and qualitative response of the two selected species to the experimental growth conditions tested, underlines the need for precise control of growing conditions and in-depth knowledge of how to manage them in order to produce targeted phytonutrients such as vitamin C. The data generated by Microx2 activities have contributed to defining the hardware and software components for a modular, automatic, and intelligent CEA system designed to support research and production activities within the International Space Station. In addition

to the experiments conducted within the Microx2 project, this thesis presents the activity focused on the accumulation of prebiotic compounds such as inulin, in young *Chicorium intibus* plants, for evaluating its potential production in CEA and to assess the feasibility of using novel agronomic inputs such as plant biostimulants to enhance yields in greenhouse and growth chamber. The work demonstrated how this species is compatible for space farming purposes adapting well to relative short cycles in CEA condition to produce young plants with high prebiotic value and additionally, delved into the impact of employing plant biostimulants under partial or full controlled environmental conditions. Moreover, the thesis described a further research activity that aimed at investigating: a) ways to increase the accumulation of vitamin C in *Eruca vesicaria* (rocket) leaf via management of the light environment and b) the study of the biochemical control of such modification of ascorbate metabolism. The experiment demonstrates the feasibility of managing light resources through straightforward strategies to enhance crop quality. The low-intensity continuous blue light treatment ($48 \mu\text{mol m}^{-2} \text{s}^{-1}$) enabled a doubling of vitamin C pool in hydroponically grown rocket, through enhanced activity of the main biosynthetic pathway rather than the recycling pathway.

KEYWORDS: CEA, BLSS, Microgreens, Phytochemicals, Nutraceuticals, Light management, VPD, Substrate, Nutrient solution.

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1. Introduction

1.1. The new era of space exploration

The future of manned space exploration missions, foreseen for the next decades, points far beyond the Earth's orbit, toward the Moon and Mars [1]. Leading this charge is NASA's Artemis program, serving as the vanguard in propelling humanity towards the Moon and laying the foundation for the monumental leap towards Mars. Within the Artemis program, NASA cultivates partnerships with industry, nations, and academia, fostering a collaborative endeavor aimed at sustainable lunar exploration [2].

In the mission of Artemis I, NASA's groundbreaking Space Launch System propelled the Orion spacecraft, embarking on a remarkable 1.4-million-mile journey beyond the Moon and back. This uncrewed flight test, the initial step in a series of progressively intricate missions, laid the foundation for a sustained presence on the Moon, a strategic imperative for future crewed missions to Mars. Building on the success of the Artemis I mission in 2022, Artemis II is set to demonstrate a broad range of capabilities essential for deep space missions. This upcoming flight test will mark NASA's inaugural crewed mission aboard the Space Launch System rocket and the Orion spacecraft. The launch crew, consisting of Reid Wiese, Victor Glover, Christina Koch and Jeremy Hansen, is scheduled to launch by September 2025. Following the completion of the two Artemis test missions, Artemis III, expected in 2026, signifies the much-anticipated return of humanity to the lunar surface after more than half a century. NASA is set to make history by sending the first humans to explore the region near the lunar South Pole, representing a momentous milestone in the continuous saga of human space exploration. The planning for space exploration within the Artemis program is continually evolving and adapting, with potential realignments being considered given the high complexities and challenges that such an endeavor entail. However, the upcoming stage of human space exploration envisions the establishment and habitation of a lunar gateway, a permanent base on the Moon's surface, and the launch of exploratory crewed missions to Mars [3]. This will open a new era in space exploration [4].

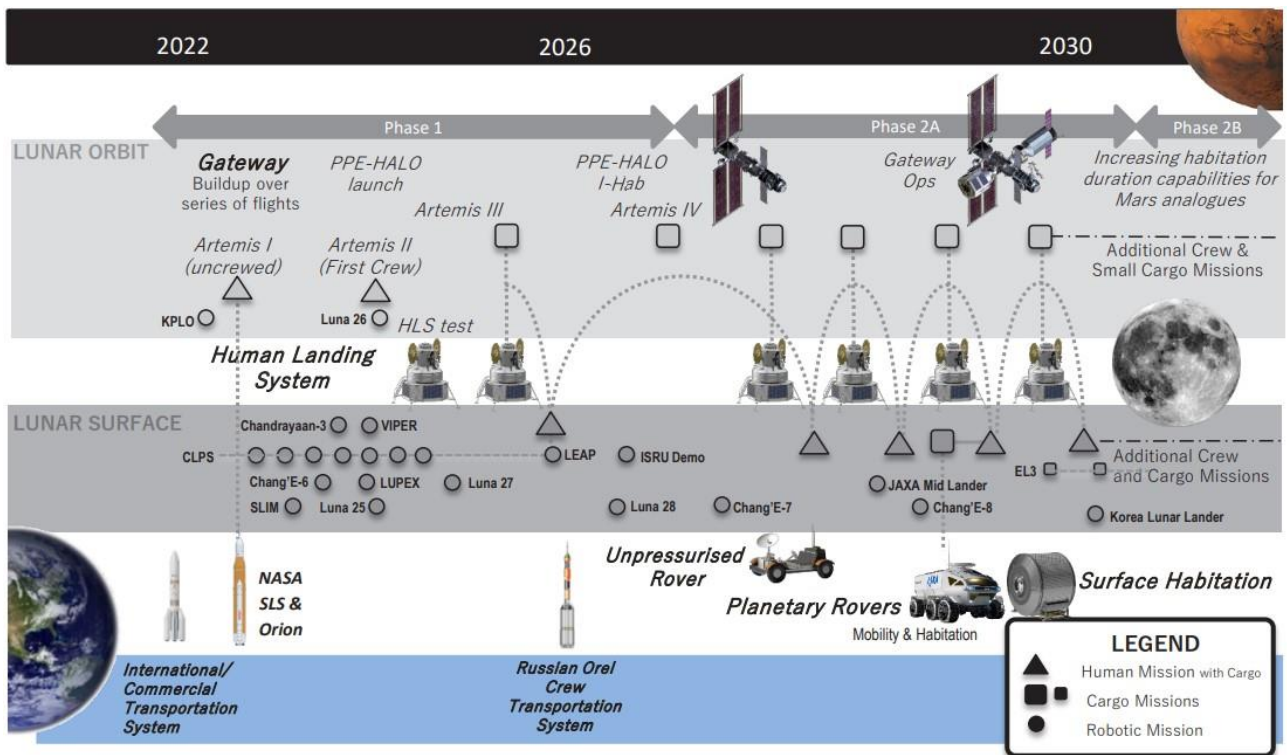


Figure 1. Space exploration roadmap updated to 2022 [5].

1.2. The role of food and nutrition

Nutrition's crucial role in exploration was already evident in maritime and polar journeys throughout history [6, 7]. Astronauts who rely on space food systems for extended periods are particularly vulnerable to the consequences of nutrient deficiency or excess. Moreover, the food provided during short-duration space missions follow Earth's dietary standards, as the duration was deemed short enough to avoid effects of inadequate nutritional intake. However, moving to Long Duration Space Exploration (LDSE) requires that nutrition not only prevent nutrient deficiency, but also serve as a countermeasure against the many negative effects due to the space environment on human physiology [8]. In LDSE, understanding astronaut nutrition, adapting to microgravity, and ensuring a sustainable food system are as critical to mission success as spacecraft mechanics.

1.2.1. Nutrition requirements definition and its evolution

The initial effort to establish nutritional requirements for LDSE took place during a conference convened by NASA in 1991, in preparation for 90-180 days missions for the Space Station Freedom project. The panel proposed terrestrial nutritional requirements but with specific adjustments and recommendations, taking into account documented losses of bone and muscle tissue, as well as assumed heightened stress levels in astronauts. The need to conduct assessments of nutritional status before, during, and after the flight was highlighted, along with the recommendation that nutrient intake preferably occurs through food rather than supplements [9]. The next nutritional guidelines emerged in 1996 for the joint U.S.-Russian Shuttle-Mir and NASA-Mir Programs, tailored for missions lasting up to 360 days on the ISS [10].

In 2005, NASA envisioned a first look at the set of nutritional requirements for missions beyond low-Earth orbit [11]. In 2016, nutrition experts convened to update these requirements for missions up to 1 year, specifically targeting planned exploration missions like Orion, Lunar Gateway, and Artemis. The requirements were established based on terrestrial nutritional standards, with a few exceptions [12]. As reported by Smith et al., (2021) [13] in the second volume of “Human Adaptation to Space: the role of food and nutrition”, the standard Earth nutritional requirements are primarily designed to mitigate nutritional deficiencies and not specifically to prevent chronic diseases, even though these diseases have nutritional underpinnings. Despite ongoing discussions and initial attempts to expand current nutritional requirements to address diseases, more information is needed before full implementation even on Earth. For space travel, particularly on LDSE, nutrition must not only prevent nutrient deficiency but also act as a countermeasure against various negative effects on human physiology due to the space factors.

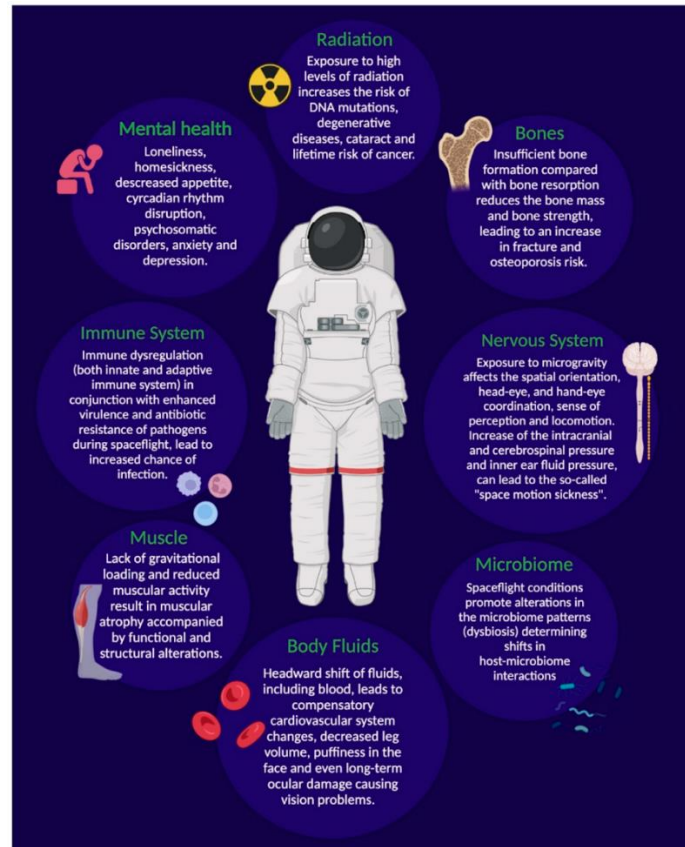
1.2.2. Space stressors

The space environment presents a multitude of challenges to human health. Physiological and psychological changes are associated with spaceflight. The loss of homeostatic balance and the onset of oxidative stress due to the space environment constitute a primary aspect requiring solutions.

First concern is the radiation risk that represents the major showstopper for manned exploration and colonization of the Solar system. Galactic cosmic rays (GCR) exposures during LDSE will be about 10 times higher than exposures during Low Earth Orbit (LEO) missions. The onset of chronic effects due to prolonged exposure (>0.1 Gy/year) to GCR represents a problem. Moreover, acute effects from solar particle events (SPE) (total dose >0.7) and combined effect of CGR and other space stressors underlines the need to assess radiation risk for exploratory-class missions beyond low-Earth orbit and develop countermeasures including radioprotectors, radio-mitigators and anti-radiation therapies to allow a safe and stable human presence in space [14, 15]. The spacecraft environmental factors including, cabin pressure, gas composition, temperature and humidity are other factors that influence homeostatic and oxidative balance, as well as short, long-term health and performances. Concentrations of CO₂ on the ISS, approximately 10 times higher than those on Earth, may affect behavior, cognition, sleep, and have implications for bone health, metabolism, cerebral blood flow, and ocular pathologies. The anticipated increase in extravehicular exploration activities (EVA) raises the level of exposure to hypobaric conditions, potentially leading to an elevated risk of vision impairment issues, sensorimotor impairment, alterations in cardiovascular and immune function, and anorexia [16]. Microgravity poses another concerning factor with the potential to impact the physiological state of astronauts. The body systems affected by microgravity include the neurovestibular, cardiovascular, musculoskeletal, bone metabolic, and immuno-hematological systems [17]. Exposure to microgravity results in muscle volume loss and diminished performance, particularly affecting the legs, with reductions in maximal force and power production observed during both short- and long-duration spaceflights [18]. Bone loss occurs during spaceflight, primarily affecting weight-bearing bones. Initial observations were made in Skylab astronauts [19], followed by further documentation after Mir [20] and ISS missions [21], with an average bone loss ranging from 1% to 1.5% per month of spaceflight [16]. LDSE heightens the risk of depression and dietary challenges. Neurological incidents, including significant weight loss, were observed in Mir astronauts. ISS astronauts also experienced cognitive decline and weight

loss during prolonged missions. Risk of depression increases with mission duration. The MARS-500 project, with six individuals isolated for 520 days, showed measurable impacts on brain microstructure, indicating changes in myelin plasticity during isolation [13].

Figure 2. Biological features of spaceflight. as reported in [22]



1.2.3. Diet countermeasures

The identification of specific nutrients and nutraceuticals that could provide protective or reparative mechanisms against the challenges posed by microgravity, radiation, and psychological stressors during space missions is crucial to design space diets and appropriate food production systems [23]. The goal is to leverage nutritional strategies to enhance the health and resilience of astronauts, contributing to comprehensive well-being strategies for extended space exploration missions. To give insights, the role of phytochemicals such as vitamins and polyphenols in maintaining antioxidant

health have been reviewed by several authors [8, 24–26]. The protective effect of Vitamin A, C, and E against γ -rays was investigated in vitro and the potential to reduce DNA damages before and after irradiation was shown [27]. In addition, the radioprotective potential of both vitamin A and C were successfully tested in mice [28, 29]. These compounds also have a synergistic effect by enhancing the antioxidant potential when administered together [30]. Moreover, vitamin A, C and E are involved in several fundamental physiological processes in the human body such as immune function, bone health, eye health, and are related to the body's response to the space environment [8]. Naturally occurring polyphenols like resveratrol, quercetin, curcumin, and catechins have shown antioxidant and anti-inflammatory effects along with anti-diabetic properties that play a key role against oxidative stress, apoptosis, inflammation, and mitochondrial dysfunction [24, 25]. Interestingly, resveratrol has also been associated with the preservation of muscle health, which usually suffers in extraterrestrial conditions like a Martian gravity analog [31]. Carotenoids including lutein and zeaxanthin, show potential protection against retinal damage and age-related macular degeneration [32]. Hence nutritional countermeasures in space with antioxidant dense plant-based food, provide a great opportunity for LDSE, whereas the main concern is to counteract the potential for long-term oxidative damage in humans under these conditions.

In addition to phytochemicals, several minerals including calcium (Ca), iron (Fe), copper, Cu, zinc (Zn), selenium (Se) and manganese (Mn) have shown direct or indirect radioprotection. Ca is recognized as an effective inhibitor of radiation-induced lipid peroxidation [33]. Fe, Cu, Zn, Se and Mn are crucial for enzyme and protein functions that actively remove free radicals and their products. The administration of these metals has been linked to increased survival rates in irradiated mice, attributed to the induction of tissue metallothionein [34]. Finally, another relevant group of nutraceuticals is that of prebiotics, which might be a promising tool to improve immune function in space exploration missions and warrants further investigation of space exploration. Gut microbiota is a major player in immune tolerance and inflammation and may change during the spaceflight [22]. Fiber including cellulose, hemicellulose, pectin, lignin and in particular fructans prebiotics

such as inulin, present in plants, are the strongest and more positive effectors of gut microbiota [35]. Recent findings demonstrated that the supplementation of fructooligosaccharides and inulin-based prebiotics improve muscle function [36]. Furthermore, considering the impact of the brain-gut microbiota axis on psychology, there are promising indications that administering prebiotics could indirectly enhance mental well-being [37–39]. This could be particularly crucial as a stress countermeasure in space [40].

1.2.4. Space food standards

Nutrition is just one aspect of the food system; as missions extend and alternative food systems are contemplated, considerations must extend to various factors. Acceptability, safety, shelf life, and resource requirements are equally crucial, necessitating integrated solutions for spaceflight. Presently, there is no food system designed to meet the challenges of extended exploration missions [8]. The acceptability of the food system in space is crucial to ensure adequate caloric intake during missions. Astronauts must find the provided foods acceptable, as they would not benefit from nutritious foods they are unwilling to consume [41]. Variety, choice, quality after storage, and menu fatigue over time can influence the acceptability of the food [42]. Despite these constraints, foods must meet nutritional requirements and receive a high sensory acceptability score from a panel of evaluators, including astronauts. Addressing behavioral factors influencing consumption in a resource-limited environment remains a challenge. Safety is a paramount concern, involving potential hazards from physical, chemical, and microbiological sources. In the confined environment of a spacecraft, where medical capabilities are limited, a case of foodborne illness could have severe consequences. Such an occurrence not only poses a risk of mission loss but also elevates the potential for loss of human lives, particularly as the spacecraft moves farther away from Earth. The safety of the current long-term shelf-stable food system is overseen on Earth through processing, packaging, and microbiological testing, requiring substantial resources that are unavailable in current or planned spacecraft [13]. Moreover, during storage, some nutritional and quality factors in spaceflight foods degrade, reaching unacceptable levels within 1-3 years [43]. The current shelf life is sufficient

for missions to the ISS, but for longer missions it will be challenging to prevent nutritional and quality degradation [44].

Last but not least, as reported below (paragraph 1.4.1.), the first challenge of developing an efficient space food production system is limited resources. These resources include mass, volume, power, water and crew time. While there is a common belief that generating food in space would be more resource-efficient than relying on pre-packaged options, when mission extends in duration and distance, many efforts still need to be made to address this aspect.

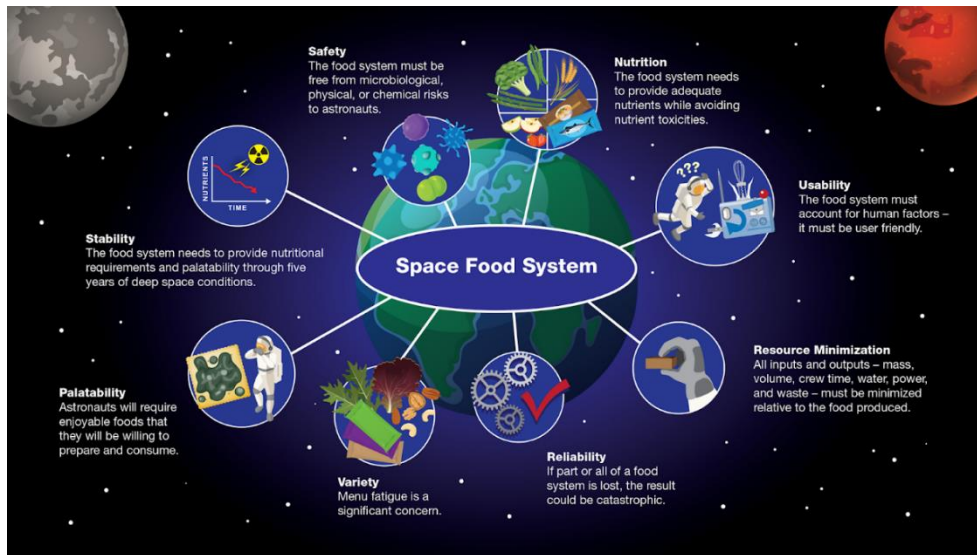


Figure 3. Space food requirements as reported in [1]

1.3. Bio-regenerative Life-Support Systems (BLSS)

The new era of LDSE, characterized by missions exceeding 30 days, diverge significantly from current extended missions in LEO. LDSE missions will require high-level of crew autonomy, as regular resource resupply will become logistically and economically disadvantageous due to distance from Earth. Moreover, the primary source of nutrition for the crew aboard the ISS relies on processed and prepackaged space food which has been demonstrated to not meet the shelf-life requirements of LDSE scenarios [43]. Moving toward LDSE missions, it will be vital to grow *in-situ*, healthy plant food intended for astronaut consumption and to regenerate resources during space

flights and extraterrestrial colonization. In this context the development of Bio-regenerative Life-Support Systems (BLSS) is crucial to provide food recycle vital resources and ultimately replace the need for resupply from Earth [45, 46]. Undoubtedly, a BLSS offers potential advantages over a prepackaged food system to support LDSE. At the core of the BLSS concept, alongside fresh food production, lies the potential of a biological-based system for the bio-regeneration of gasses, absorbing environmental carbon dioxide and releasing oxygen, and recycling of waste materials, such as gray water, within a closed habitat system [47, 48]. As a final noteworthy advantage of a plant-based BLSS, the potential psychosocial benefits of engaging in agricultural activities should not be underestimated in the unfamiliar space environment; growing plants foster a connection to Earth [49]. Global initiatives explored bioregenerative closed system studies for human life support in space. Programs like BIOS (Russia, 1972) [50], NASA's Controlled-Ecological Life Support System (CELSS) (USA, 1978) [51], Micro-Ecological Life Support System Alternative (MELiSSA) (EUROPE, 1987) [52], the Closed Ecology Experiment Facilities (CEEF) (Japan, 1994) [53] and the Lunar Palace 1 (China, 2013) [54], have significantly contributed to the development of new technologies and findings for bioregenerative systems, bringing about a major breakthrough in the advancement of knowledge for Earth purposes as well [55].

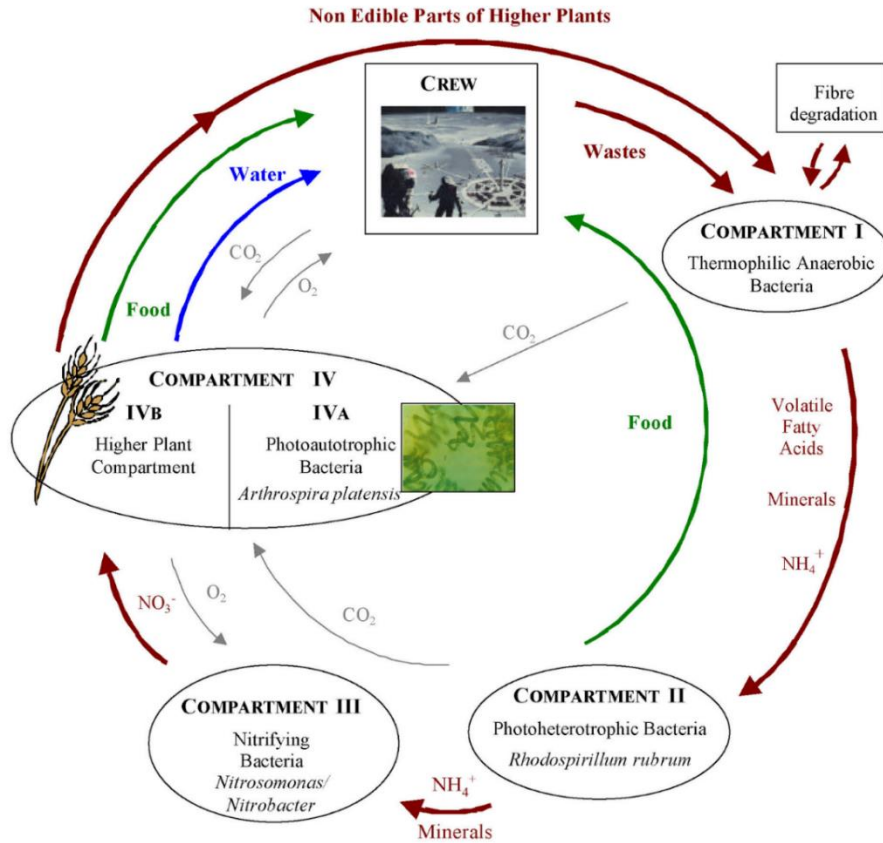


Figure 4. MELiSSA loop diagram as reported in [52]

1.4. The Space-farming challenge

Despite the need to integrate food production systems into bio-regeneration for the development of functional and efficient BLSS, meeting the nutritional requirements during LDSE by cultivating plants, remains a considerable challenge. Farming in space will face completely new environmental conditions, technical constraints and require the maximization of resources which influence payload design, and ultimately, impact the cost and feasibility of a mission [1, 56, 57]. Hence, conventional terrestrial cultivation practices and crops need to be adapted to thrive in novel environments with distinct biophysical conditions [58]. The aspects to consider are numerous, and the scenario is highly complex. In addition to the individual impacts of space factors, their interactions can exert diverse outcomes and relevance depending mainly on the context of the mission considered. In the following paragraphs, a brief insight into some of the factors that can complicate aspects related to space horticulture is provided.

1.4.1. Volume and mass constraints

The success of extended space missions relies on the ability to generate substantial and nutritious food within spatial constraints. Cultivating crops within the confined volumes of a spacecraft presents a distinct challenge, demanding meticulous planning and optimization of every square centimeter to achieve maximum food yield. The design of cultivation spaces must be precisely engineered, accounting for spatial restrictions and ensuring the overall system's functionality. Beyond engineering considerations, spatial and mass limitations influence crop choices, substrate, water and fertilizer utilization. From crop selection to layout of the system, the design directly impacts the efficiency of food production in space, emphasizing the importance of the volume resource efficiency [58].

1.4.2. Power limitation

Although technological advancements in recent years have increased the efficiency of plant illumination, heating, ventilation, cooling and irrigation systems, energy expenditure for plant cultivation is still an issue. The cultivation of plant-based food aboard a spaceship necessitates an estimated electric energy consumption exceeding 1100 kWh per day per crew member, contingent on the chosen plant species [59–61]. The ISS, for example, meets its electrical energy demands through solar energy harnessed by photovoltaic cell modules which generate 84–120 kW. The essential ISS's energy requirements range from 75–90 kW for payload and research operation [62–64]. It is evident that an energy gap exists. While power management may not be a significant concern on the ISS since the past plant growth units required only a few hundred Watts due to their limited size primarily designed for research, future food production systems will need a substantial part of the total energy budget of a spacecraft or planetary base. Thus, prioritizing the development of technologies to minimize energy demand is still crucial for the future of plant cultivation in space [65].

1.4.3. Microgravity

The condition of altered gravity or zero g pose significant challenges for space farming. Firstly, microgravity, complicates water and nutrients delivery to plants. In microgravity, the absence of buoyancy-driven convection affects gas exchange (O_2 , CO_2), potentially inducing hypoxia in both leaves and roots [66]. From a technical point of view, the development of functional irrigation and nutrient delivery systems is fundamental. On the other hand, various works have explored the transcriptional [67], metabolic [68], and morphological [69] adaptations of numerous species, including studies on reproductive stages and *seed-to-seed* experiments [70–72]. Observations from this perspective are diverse and species-specific. Moreover, there is a broad variability in different cases, attributable to the effect of various experimental scenarios, especially in Earth-based tests [69, 73]. The initiation of scientific operations on the ISS significantly enhanced opportunities for experimentation under true microgravity conditions, revealing the feasibility, net of specific measures, to produce plant-food in space [74].

1.4.4. Ionizing radiation

Space radiation primarily consists of GCR and SPE, exerting substantial but still poorly understood effects on organisms. During extended space missions, dose rates from GCR may range from 0.4 to 0.8 mGy/day and 1 to 2.5 mSv/day. SPE dose rates could peak at 50 mGy/h inside a shielded vehicle and 250 mGy/h for an astronaut in deep space extra-vehicular activity [75]. The examination of plant responses to ionizing radiation demonstrates notable variations influenced by factors such as the type of radiation, dosage, species, developmental stage, and genetic traits [76, 77]. Generally, plants exhibit greater tolerance to ionizing radiation compared to animals. Low doses of ionizing radiation (1 or 2 Gy) can even induce a positive acute response in plants, stimulating growth and accelerating photosynthetic respiration [78]. It may also induce radioresistance phenomena through the production of phenolic compounds, chlorophyll, and carotenoids [79, 80], which could prove useful from a nutraceutical perspective. The problem could significantly increase when chronic effects due to GCR or acute high

radiation doses, such as those caused by SPE occur. Depending on the mission scenario, the onset of altered mitotic/meiotic capacity, sterility, embryo lethality or mutations could be catastrophic. For this reason, it is necessary to deepen the understanding of the effects of radiation on plants in both the short and long term and implement radioprotection or radio mitigation strategies tailored to the specific mission requirements [81].

1.4.5. High CO₂ levels

The levels of CO₂ in the environment of a spacecraft are typically very high. For instance, on the ISS, despite the active Carbon Dioxide Removal Assembly and Vozdukh systems, the CO₂ concentration rarely drops below 3000 ppm but can reach values greater than 6500 ppm [82]. The challenge becomes even more pronounced when considering missions to the surface of Mars, where the atmosphere is almost entirely composed of CO₂. In these scenarios, in addition to advanced robotic systems such as the Mars Oxygen In-Situ Resource Utilization Experiment (MOXIE) for oxygen regeneration [83], plant-based BLSS to recycle CO₂ are considered [48]. CO₂ plays a crucial role as a significant carbon source in the process of photosynthesis and the overall growth of plants [84]. Multiple studies have suggested that CO₂ enrichment environments typically do not harm plants and can enhance the biomass production of various species demonstrating the potential use of CO₂ treatments to optimize both crop yields and quality [85–87]. However, it is important to note that the range of CO₂ treatments in agricultural experiments or industrial settings on Earth typically falls between 700 and 1000 ppm [88]. The benefits of CO₂ enrichment decline or disappear above a certain level, with common adverse effects on plant growth at concentrations above 1000 ppm [89, 90]. Studies aimed at optimizing growth in experiments simulating the ISS environment have revealed the presence of adverse effects, including chlorosis, necrosis, and stunted growth. Similar symptoms were detected on the cultivar of Chinese cabbage ‘Tokyo Bekana’ grown in the Veggie systems on the ISS. After troubleshooting efforts to identify the causes, the super-elevated CO₂ concentrations on ISS emerged as a candidate condition responsible for the observed symptoms in Tokyo Bekana [91]. A potential solution for food production is to enhance the level of environmental

control in space farming systems. Veggie, for instance, is an open system that shares the same atmosphere as the ISS. Systems like the Advanced Plant Habitat (APH) already exist, offering a higher degree of isolation and environmental control [92]. On the other hand, for the integration of plants into BLSS that are also capable of recycling CO₂, selecting plant species with higher resistance to CO₂ hyper-saturation becomes critical [91].

1.4.6. Reduced atmospheric pressure.

On Earth, plant growth at high altitudes is more limited by temperature than pressure. It is only in space farming context, that atmospheric pressure becomes a challenging independent variable [93]. In the environment of a spacecraft, atmospheric pressure is regulated to maintain it within the survival limits for the flight crew. For instance, on the ISS, atmospheric pressure is maintained at 101 kPa. Despite being demonstrated that in cases of reduced atmospheric pressure, plants react with morphological [94], metabolic [95] and gene expression [96] modulation, in the confined environment of a spacecraft, atmospheric pressure may not be a factor, exceptional case apart, that directly influences plant physiology and growth. In the context of extraterrestrial colonization, the issue takes on greater technical and engineering relevance. Within the confined spaces of extraterrestrial growing systems, challenges arise due to the necessity of maintaining atmospheric pressure in the face of the vacuum of space. For instance, Mars atmospheric pressure is 0,6 kPa which is less than 1/100 of Earth's atmospheric pressure at sea level [97]. As explained by Paul and Ferl (2006) [93], the need to utilize natural light to reduce energy consumption and the launch mass associated with artificial lighting systems requires identifying a material that is transparent enough yet sufficiently robust to contain a plant growth environment sustaining higher pressure than the near vacuum on the Moon or the low-pressure atmosphere on Mars. Moreover, as highlighted by Wheeler et al. (2011) [97], the risk of failure must be calculated in these contexts, and this includes the potential occurrence of events leading to pressure loss. This clearly becomes catastrophic if such events take place in crew-dedicated compartments, but it becomes notably important if they compromise cultivation environments, potentially resulting in food losses.

1.5. Space farming systems

Experiments on plant growth have held significant importance since the first space station programs. In the past 40 years more than 20 plant growth systems were utilized to grow over 40 different plant species in space [98]. To date, systems employed to facilitate the growth and study of plants in space have been marked by notably small volumes. Primarily tailored for researching plants' response to space-related factors, these systems have had a constrained influence on real food production and integration into life support systems. From the initial system, Oasis, implemented on the Salyut space station in 1971, to the more contemporary growth chambers, the cultivation surface area has consistently ranged from 0.001 to 0.170 m² [98]. In addition, over time, space-based plant production systems have undergone significant technological evolution. The optimization of environmental control is one of the aspects that has shown significant improvements in this context. Despite several systems having closed vegetation environments, they relied directly on the cabin atmosphere. With the introduction of the Plant Growth Facility (PGF) system on the Space Shuttle in 1997, the ability to independently control temperature, humidity, and CO₂ was implemented, including the addition of an ethylene filter [99]. A certain level of automation was already tested aboard the MIR space station in 1990 with the SVET system, which was equipped with a microprocessor capable of receiving data from environmental sensors and controlling the lighting system and ventilation. In particular, the improvement of this system with an Environmental Measurement System and Gas Exchange Monitoring System (SVET-GEMS) enabled the monitoring of air and soil conditions, leaf temperature, irradiation, and oxygen levels. This advancement allows for the measurement of photosynthesis and transpiration rates in microgravity, representing a significant improvement over earlier space data acquisition [100–102]. Plant growth chambers in space have faced challenges in managing irrigation under microgravity conditions, leading to the exploration of different substrates and systems. From passive setups in agar to active nutrient delivery methods such as perforated tubing and porous tubes, various approaches have been tested over the years [98, 99]. The lighting systems

have witnessed significant advancements in terms of light intensity, spectrum composition, and modulation capacity. The 1990s saw accelerated light emitting diode (LED) development that replaced fluorescents and high-pressure sodium lamps, offering advantages like high efficiency, compact size, controllability, and variable spectrum [98, 99, 103]. The latest advancements in plant growth experiments on the ISS are represented by Veggie and Advanced Plant Habitat (APH). The Veggie vegetable production system, developed by ORBITEC (Madison, WI) was launched to the ISS in April 2014, Veggie is a low mass, low power, and low crew time-requiring system with a flexible, transparent bellows, LED lighting array, and a passive wicking watering system. It provides stable environmental conditions and has been used for various experiments [104]. From 2014 to 2019, Veggie conducted up to 12 crop experiments not intended for human consumption and 11 crop experiments with edible produce [105]. Ground control experiments are conducted at Kennedy Space Center in Veggie hardware located in ISS environmental simulator chambers [104]. APH represents an advancement in terms of environmental control. APH was delivered to the ISS in parts aboard two commercial resupply missions: OA-7 in April 2017 and SpaceX-11 in June 2017 [106]. The growth chamber provides precise environmental control, including temperature (18-30°C), relative humidity (50-86%), and CO₂ concentration (500-5000 $\mu\text{mol mol}^{-1}$). The lighting system allows adjustable photosynthetic photon flux (PPF) between 0-1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and light cycles can be programmed [106]. The APH communicates with crew via a laptop and receives ground commands from the KSC Experiment Monitoring Area [107]. First space edible chili pepper was grown in the APH system during NASA's Plant Habitat-04 in 2021.

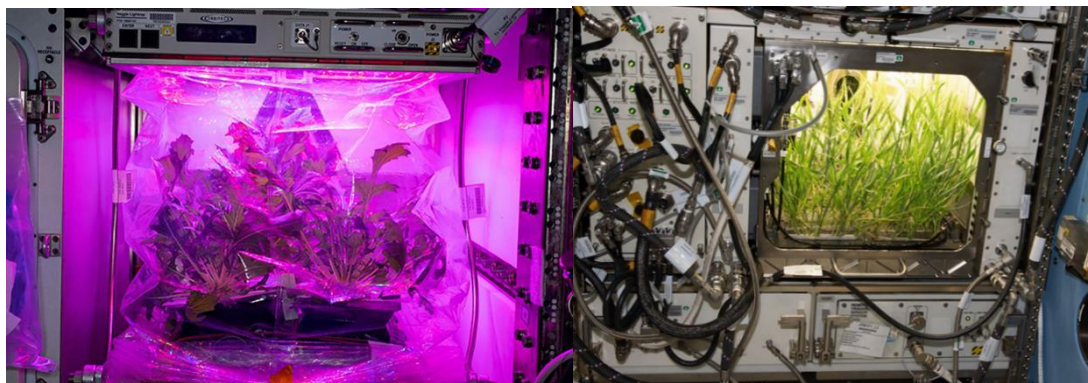


Figure 5. (Left) Veggie system [104]. (Right) APh system [107].

1.6. Species selection criteria

The challenges associated with spatial factors, as mentioned earlier, can be identified with a common thread, however the criteria for selecting the most suitable species for space farming must align not only with the mission context but also with the intended role within a bioregenerative system. In the context of the mission scenario, species selection should take on a different connotation, introducing diversification for specific selection criteria. Species selection is one of the main factors influencing productivity, quality, and resource use efficiency in terrestrial environments and this becomes even more crucial for accomplishing tasks like CO₂ removal, water, and waste regeneration in space. Identifying selection criteria and choosing the most suitable species for space missions is a matter that has engaged the efforts of numerous scientists, with studies being published for over 70 years [108]. The initial attempt to define a list of candidate crops for space farming was held in 1961-62 during the “Biologistics for Space Systems Symposium”. Four main criteria were defined for species selection, including: 1) high photosynthetic efficiency under low artificial light conditions; 2) production of edible parts of plants under low artificial light conditions; 3) resistance to increasing osmotic pressures; 4) absence of pharmacologically active substances. Additionally, plant compactness was considered as an additional characteristic to occupy the least possible volume [109]. In the First Principal Investigators Meeting of NASA’s CELSS a total of 21 criteria included in the two main general categories were considered. Nine of them (energy concentration, nutritional composition, palatability, serving size and

frequency, processing requirements, use flexibility, toxicity, and human experience), fell into the category of human nutrition and convenience (the "use" criteria), and the remaining 12 (proportion of edible biomass, yield of edible plant biomass, continuous vs. determinate harvestability, growth habit and morphology, environmental tolerance, photoperiodic and temperature requirements, symbiotic requirements and restrictions, carbon dioxide-light intensity response, suitability for soilless culture, disease resistance, familiarity with species, and pollination and propagation) were predominantly "cultural considerations". A comprehensive assessment was conducted, evaluating and assigning scores to a total of 115 species based on their suitability for a CELSS [110]. In the proceedings of two NASA workshops on "Use of Higher Plants in CELSS" held at the O'Hare Airport Conference Center Chicago, Illinois, November 1979 and at the Ames Research Center Moffett Field, California March 1980 main criteria for plant selection were described as: A) food production; B) nutrition; C) oxygen production and carbon dioxide utilization; D) water recycling; E) waste recycling. Other criteria including short plants with high leaf density, growth habit, wide range of environmental tolerance, accumulation of toxic compounds, disease resistance, production of irritating air borne pollen and self-pollination were described [110]. Two groups of plants were selected; in the first group of eight plant species including wheat, rice, white potatoes, sweet potato, soybeans, peanuts, lettuce and sugar beets was designated for intensive study since they are species that were commonly utilized as food plants and could provide the major nutritional needs. The second group of six plant species including taro, winged beans, broccoli, strawberries, onion and peas was designated for exploratory study since they were species for which there was less reported information or that have lower nutritive content but high psychological value [111]. As reported by Wheeler (2004) [108], other species lists in the efforts for CELSS program were provided by Nishizaki et al. (1990) [112], Salisbury and Clark (1996) [113], Mitchell et al. (1996) [114]. In preparation for the NASA testbed called BIO-Plex in the late 90's, the concept of Crop Readiness Level (CRL) was introduced. The concept originated from the aerospace engineering practice of employing the Technology Readiness Level (TRL) to monitor the development and maturity

of hardware or engineering components intended for spaceflight. The proposed readiness scale for crop at that time ranged from 0 to 3. Level 0 indicated minimal knowledge of the crop in controlled environments, 1 implied some testing with published results, 2 denoted extensive testing with cultivar trials, and 3 represented thorough testing, cultivar comparisons, and successful scale-up in a closed system [115]. To ensure systematic and consistent development of crops for space-based application, in 2019 a modification to the CRL was proposed to extend the range levels up to 9. In summary Levels 1 and 2 address incompatibilities, while level 3 considers recent experiences and potential issues in testing at ISS simulated environmental conditions. Levels 4 and 5 focus respectively on seed sterilization and flight-like testing. Levels 6 and 7 evaluate chemistry, organoleptic properties, and microbiology. Level 8 marks successful crop establishment in space, and level 9 denotes consumption in space [116]. The Melissa Food Characterization (MFC) project conducted comparative plant growth bench tests to collect preliminary data on various aspects [117]. Initial attempt introduced wheat, red beets and lettuce in the MELiSSA loop for initial hydroponic cultivation tests related to crop yield and carbon exchange modeling. Later during the MFC1 phase (years 2009-2010) 4 crops including durum wheat [118], bread wheat [119], potato [120] and soybean [121] were selected for BLSS experimentation [122]. Different cultivars of each species were screened for their performance in a closed-loop application through parameter ranking. This included the characterization of edible/inedible biomass ratio, nutritional quality, processability, and overall performance under specific conditions of hydroponic cultivation and artificial illumination for the implementation [117]. Beyond the development of BLSS, the implementation of a “salad machine” during the stay inside a spacecraft has been considered [123] even if it is not envisioned as a technology competing with the current atmospheric resupply and regeneration systems. Such units through the use of pick-to-eat and salad crops could offer moderate quantities of fresh, nutritious and flavorful foods to supplement the crew's diet [124]. Moreover, in the context of space experiments conducted so far, BLSS systems have not yet been implemented and remain confined to the ground-testing phase. Meanwhile, small growth systems like Veggie and APH, are

nearing the readiness level for food production and have undergone testing in space experimentation for over 40 years. In this context, the primary criteria for crop selection include a high harvest index, elevated nutritional values, short production cycle, minimal crew-time investment, along with low environmental and agronomic input requirements. Nowadays, several works have investigated the performance of different, pick-and-eat, salads and dwarf crops in space and space-like environment experiments [71, 125–133]. In addition, different selection criteria and methods were recently proposed and have generated various ranking lists of crops [134–137].

1.6.1. Microgreens as potential candidates

Several works investigated microgreens as potential candidate for space farming since they easily fit the criteria mentioned above, such as ease of cultivation, high-volume optimization potential, rich phytochemical composition, fast production and little crew time investment [138–140]. Microgreens are a novel class of salad crops which have gained popularity for their large range of colors, peculiar tastes, and elevated nutraceutical properties [141]. Microgreens are usually harvested between the complete expansion of the cotyledons and the appearance of the second true leaf, ensuring rapid production cycles (7-21 days). In addition to the ability to quickly produce fresh food, the characteristics of a fast production cycle offer significant advantages over longer cycles to reach maturity. This is primarily reflected in the reduced susceptibility to biotic disease. Furthermore, it broadens the possibilities of forming consortia of different genotypes, which can be advantageous both in terms of nutritional and sensory aspects, as well as in disease prevention [138]. Their production can be implemented on various types of substrates with shallow depth, with limited or no nutrient supplementation (in depth description of the various types of substrates and nutrient solution will be discussed in chapter 5 of this thesis). Additionally, they have a lower photon flux requirement (further details related to light intensity requirements will be discussed in chapter 4 of this thesis) compared to long-cycle crops and do not require cultural interventions that go beyond seeding and harvesting. This translates into reduced storage volumes, lower water and energy consumption, ease cultivation and time savings. The high

availability of phytochemicals such as vitamins, minerals, polyphenols and glucosinolates highlights the health benefit potential of microgreens [142]. Moreover, several studies have demonstrated the antioxidant, anti-inflammatory, anti-carcinogenic, anti-obesity, antimicrobial, and anti-diabetic activities of microgreens [143]. Microgreens production systems may be considered a resilient phytochemical factory with the potential to provide a significant nutritional integration in just a small serving size so they can be used to meet dietary needs of the astronauts by reaching targeted daily intakes of specific nutrients and nutraceuticals with the potential to counteract astronauts diet deficiency and space-induced pathologies [138]. A large array of microgreens species has been tested for human consumption (in depth description of the species will be discussed in chapter 3 of this thesis), each boasting a unique spectrum of colors, ranging from green, yellow, red, to crimson or multicolor, flavors that span from neutral to slightly sour, spicy, or bitter, and varied textures, such as juicy, crunchy, or regular [144, 145]. This rich diversity and peculiar characteristics serve to enhance the overall variety, choices, and palatability, effectively preventing menu fatigue and promoting increased consumption and acceptability.

1.6.2. Other functional crops

A pivotal aspect, presenting a significant challenge for both space nutrition and the growth of controlled environment agriculture (CEA) and vertical farming markets, is the selection of species characterized by specific qualities, such as the accumulation of targeted phytonutrients to meet precise nutritional and health needs. Nowadays, the concept of functional foods is gaining more and more ground [146]. The exploitation of genetic diversity in species, focusing on those predisposed to the specific accumulation of certain active compounds, should be a selection criterion of relevant importance. The plant kingdom encompasses a plethora of phytochemical species, each capable of playing significant roles in nutrition and health. A concrete example is highlighted by glucosinolates, such as glucoraphanin, secondary metabolites primarily produced in *Brassicaceae* like *Brassica oleracea* [147]. Their quantity varies significantly among varieties and offers various nutraceutical potentials, including the improvement of cardiovascular health [148] and the

reduction of cancer risk [149]. Similarly, artemisinin, a terpene with diverse therapeutic purposes such as anti-inflammatory, antiallergic, antibiotic, and anticancer properties [150], is characteristic of a limited number of species in the *Artemisia* family [151]. From a qualitative standpoint, for space purposes, there is a general consensus that the selection should prefer varieties with high levels of vitamins, carotenoids, and polyphenols to address primary physiological needs and counteract the strong oxidative pressure on human health. Furthermore, fructans like inulin, with elevated prebiotic potential, could play a fundamental role in space nutrition [152]. This compound is abundant in a few species, including *Cichorium intybus* L. and *Cynara cardunculus*, as described later in chapter 6. The integration into space farming of species selected for specific nutritional and health characteristics must, of course, respect the technical production constraints outlined earlier. The evaluation to produce new functional species in CEA should be undertaken. Moreover, genetic improvement, whether through conventional or transgenic methods, holds the potential to develop new cultivars suitable for the challenging growth conditions in space, enriched with nutraceutical compounds.

1.7. Controlling quality in CEA

The potential benefits of CEA extend beyond space applications, encompassing significant advantages for Earth's food production. These benefits include a substantial increase in crop yields per unit of land and water used compared to traditional agriculture, free from the constraints of weather or pests. CEA also holds promise for reducing the environmental impact of food production by minimizing water usage, fertilizer, and pesticide use. Furthermore, in the context of urban farming, a modern evolution of CEA concepts, the proximity to consumers can decrease transportation emissions and enhance food security [153]. In addition to these advantages, one notable aspect of CEA, when compared to open-field conditions, is its ability to finely modulate environmental parameters and optimize agronomic inputs for better control of quality through the targeted biosynthesis of specific phytonutrients. Firstly, the ability to adjust photoperiod, light intensity, and spectrum composition, especially with recent advancements in LED lights, plays a

pivotal role in ensuring higher yields and nutritional quality of vegetables. For example, the accumulation of ascorbic acid is proportional to light intensity [154]. Several studies have demonstrated increased accumulation of β -carotene and lutein under elevated PPFD [155, 156]. However, careful consideration is needed, as excessive light intensity can lead to photodamage and compromise plant growth. Optimal light intensity should align with the physiological requirements of the plant to maximize energy efficiency and quality without inducing abiotic stress. Spectrum composition is another crucial factor influencing the accumulation of various phytochemical compounds, such as ascorbate, carotenoids, polyphenols, and anthocyanins. By providing the proper light recipe in terms of selected spectral bandwidth, also the accumulation of undesirable compounds such as nitrates and oxalic acid can be limited [157]. Temperature control is essential in CEA and significantly influences the quality of horticultural species. For instance, chilling temperatures (8 °C) can enhance the content of chlorophylls, carotenoids, polyphenols, flavonoids, and glucosinolates in kale plants [158]. Similarly, varying day/night temperatures in lettuce affect anthocyanin content [159]. Air humidity, influenced by factors like plant transpiration, irrigation, and ventilation, remains a challenging parameter to regulate in CEA. Atmospheric Vapor Pressure Deficit (VPD) that can be modulated by environmental control is the driving force of plant transpiration. Precise control of VPD can optimize environmental conditions for plant growth and enhance the quality of leafy greens [160]. The atmospheric composition, particularly CO₂ concentration, is a controllable parameter in CEA influencing crop quality. Elevated CO₂ levels can enhance biosynthesis major and micronutrients in food crops, affecting the accumulation of phytochemicals like flavonoids in various species [161–163]. Moreover, high CO₂ has been shown to increase glucosinolate content in broccoli [164] and enhance lycopene, β -carotene, and ascorbic acid in tomato fruits [165]. To ensure optimal yield and quality, it is crucial to consider the single and synergistic effects of these factors on a species-specific basis, in combination with different agronomic inputs, and make necessary optimizations. Further research is necessary to harness the full potential of CEA by modifying

growth environments to produce targeted phytonutrients and achieve better crop quality.

References are listed in Appendix I of the bibliography section.

2. Aims and scope

The objective of this thesis is to contribute scientific insight focused on optimizing resource utilization in CEA, aiming to enhance the quality of horticultural species in terms of the accumulation of phytochemical compounds, including vitamins, antioxidants, and prebiotics. These compounds hold substantial relevance as nutrient and nutraceuticals, particularly within the sphere of human dietary considerations in the context of space missions. Most of the work described in this thesis was conducted within the project “Systems and technologies for the production of microgreens in the Space ‘*Microgreens x Microgravity*’ - *MICROx2*” carried out with the collaboration and the coordination of the Italian Space Agency (ASI). Agreement n. 2021-2-HH.0. The *MICROx2* project has the overall goal of defining the scientific requirements for the implementation of a Phase A and B study for a flight apparatus aimed at space qualification of a system for in-orbit production of microgreens. These vegetables are intended to integrate astronauts' diets with fresh products rich in nutritional and nutraceutical substances. The project focuses on two main research areas: 1) *FOOD PRODUCTION* and 2) *ENVIRONMENTAL CONTROL*. The major contribution of this thesis pertains to the first research area, that of *FOOD PRODUCTION* which aims to identify and characterize the most suitable microgreens species for the development of an autonomous food production system to sustain the astronauts crew in various conceivable scenarios and on advancing agro-bio-technological knowledge to produce unprocessed fresh food as a functional food to counteract the degenerative processes that astronauts experience in space.

This research, developed in collaboration with various research groups belonging to the Department of Agriculture at the University of Federico II in Naples, was conducted within the scope of the working package (WP) under the responsibility of the team at the Plant Physiology Laboratory of the Institute for Research on Terrestrial Ecosystems (IRET) of the National Research Council (CNR), WP-3900 “*Study of physiological and biochemical control of food quality and safety*”. The experiments conducted aimed at: 1)

identifying, through an objective approach based on specific criteria, the most suitable microgreen species for cultivation in space and for effectively meeting the nutritional needs of the flight crew (chapter 3); 2) defining the environmental parameters, including light intensity, temperature, and relative humidity (VPD), conducive to maximizing resource efficiency in enhancing productive and qualitative performance of the microgreens, with specific reference to the accumulation of phytochemical compounds (chapter 4); 3) definition of technical protocols, including substrate selection and management of the nutrient solution, aimed at maximizing resource efficiency and optimizing species-specific productive and qualitative outputs (chapter 5). Special emphasis is placed on maximizing the accumulation of Vitamin C as the primary quality-driving attribute. Based on experimental results and analyses, this research generated simple models for selecting vegetables and environmental parameters to maximize the efficiency of resource use in the production process (input) and optimize the quality of outputs. The results obtained from these activities contributed to defining choice models and scientific requirements for the design of a flight apparatus, collected in a technical specification which ASI has entrusted to Thales Alenia Space which is responsible for the final design and construction of the system. In addition to the experiments conducted within the *Microx2* project, chapter 6 describes the work contributing to the project "*In situ Resource Bio-Utilization for life support in Space (ReBUS)*" funded by ASI: Protocol ASI no. 1714 of February 19, 2018—no. DC-VUM-2017-080. In this case, the activity focused on the accumulation of prebiotic compounds such as inulin in young *Chicorium intibus* plant, for evaluating its potential production in CEA with the possibility of integration into BLSS and to assess the feasibility of using novel agronomic inputs such as biostimulants to enhance yields. In Chapter 7, it is described a spinoff research activity that aimed at investigating: a) ways to increase the accumulation of ascorbic acid in rocket leaf via management of the light environment and b) the study of the biochemical control of such modification of ascorbate metabolism. Specifically, the aim was to increase the Vitamin C content in *Eruca vesicaria* through the implementation of continuous light treatment within the blue spectrum.

3. Applying productivity and phytonutrient profile criteria in modelling species selection of microgreens as Space crops for astronaut consumption

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3.1. Introduction

Extensive efforts have been devoted to the design and development of cultivation systems in Space, however, only a few studies provide a codified selection method of the best species/cultivars to be used in flight experiments or cultivations in Space. For example, De Micco et al. (2012) [1] developed an objective and repeatable methodology for the selection of soybean cultivars to be used in BLSSs. A specific algorithm considering the relevance of the different plant traits was elaborated to rank the soybean cultivars and to identify the best ones for subsequent cultivation trials. Similarly, Massa et al. (2015) [2] reported a selection of leafy greens vegetables as supplements for astronaut's diet to be grown in the Veggie growth chamber onboard the ISS by using a ranking method based on growth, nutrition, and organoleptic parameters. Dueck et al. (2016) [3] also reported a selection methodology for choosing Space crops using literature data and information provided by growers on horticultural aspects, human nutrition, and psychology, and precluding the selection of crops that are not ready to eat. A further study by Aronne et al. (2020) [4] was aimed to select the best species among 50 candidate crops to conduct an experiment onboard the ISS using refurbished hardware. Authors applied subsequent inclusion/exclusion criteria to select the biological system that best fit to the hardware and timing expected during the prelaunch, launch and flight operations [4]. Given the potential of microgreens as a functional space food there is a need to identify those species/cultivars with satisfactory yield and food quality. Candidate genotypes of microgreens mostly include species from the *Brassicaceae*, *Asteraceae*, *Chenopodiaceae*, *Lamiaceae*, *Apiaceae*, *Amarillydaceae*, *Amaranthaceae*, and *Cucurbitaceae* families [4, 5]. No previous studies have reported a selection of microgreens to be used as functional Space food. In

this context, we developed a selection method for microgreens species, based on an objective comparison of numerous genotypes aiming to identify those with the best growth and nutritional characteristics for cultivation in Space. Herein we describe the algorithm used to generate the ranking list by adopting a two-level selection based on growth and nutritional parameters, and their prioritization.

3.2. Materials and Methods

3.2.1. Literature survey and data elaboration

Species selection was conducted in two phases. For the first round of selection, we conducted a literature survey and evaluated several articles reporting data on microgreens. To obtain a reliable dataset for comparing the different species, we decided to use only data from articles reporting a comparison between several microgreens' species grown under the same conditions. Specifically, we used data provided by Xiao et al. (2012) [6]; Xiao et al. (2016) [7], and Kyriacou et al. (2019) [8]. In this regard, the studies by Xiao et al. (2012) [6] and Xiao et al. (2016) [7] were conducted in unheated greenhouses and under ambient light, whereas Kyriacou et al. (2019) [8] conducted their trials under controlled environment using commercial peat-based substrate fertigated with a quarter-strength Hoagland and Arnon formulation, day/night temperatures of $22/18 \pm 2^{\circ}\text{C}$, 12 h photoperiod, relative air humidity of 65-75% and photosynthetic photon flux density (PPFD) of $300 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$. With this approach we gathered data on 39 genotypes (Table 1) and 25 parameters included in 3 categories (Table 2), that were used to construct a working matrix for the first algorithm elaboration. To compare the 39 genotypes of microgreens, data of each parameter were normalized to fit a range between 0 and 1 (minimum and maximum values respectively). Specifically, as also reported in Massa et al. (2015) [2], average values of each parameter were normalized among cultivars/species so that the minimum value equates to 0 and the maximum value equates to 1 using the following formula: $X_i = [x_i - \min(x)] / (\max(x) - \min(x))$. As regards those parameters for which lower values are most desirable (i.e., sowing density, growth period, seed weight, nitrate, and iron),

the normalized data were inverted so that the minimum value was equal to 1 and the maximum value was equal to 0.

Table 1. List of microgreens genotypes used for the selection.

N	Family	Genus	Species	Variety	Common name
1	Amaranthaceae	<i>Amaranthus</i>	<i>hypochondriacus</i>		Amaranth
2	Apiaceae	<i>Apium</i>	<i>graveolens</i>		Celery
3	Apiaceae	<i>Coriandrum</i>	<i>sativum</i>		Coriander
4	Brassicaceae	<i>Barbarea</i>	<i>verna</i>		Cress
5	Brassicaceae	<i>Brassica</i>	<i>juncea</i>		Brown mustard
6	Brassicaceae	<i>Brassica</i>	<i>napus</i>	<i>napobrassica</i>	Rutabaga
7	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>acephala</i>	Black cabbage
8	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>alboglabra</i>	Chinese kale
9	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>botrytis</i>	Cauliflower
10	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>capitata</i> f. <i>alba</i>	White cabbage
11	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>capitata</i> f. <i>rubra</i>	Red cabbage
12	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>capitata</i> f. <i>sabauda</i>	Savoy cabbage
13	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>gongylodes</i>	Kohlrabi
14	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>italica</i>	Broccoli
15	Brassicaceae	<i>Brassica</i>	<i>oleracea</i>	<i>pekinensis</i>	Napa cabbage
16	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>chinensis</i>	Pak choy
17	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>gemmifera</i>	Brussels sprouts
18	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>narinosa</i>	Tatsoi
19	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>nipposinica</i>	Mizuna
20	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>perviridis</i>	Komatsuna
21	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>rapa</i>	Turnip
22	Brassicaceae	<i>Brassica</i>	<i>rapa</i>	<i>ruvo</i>	Rapini
23	Brassicaceae	<i>Eruca</i>	<i>sativa</i>		Rocket
24	Brassicaceae	<i>Lepidium</i>	<i>bonariense</i>		Peppercress
25	Brassicaceae	<i>Lepidium</i>	<i>sativum</i>		English cress
26	Brassicaceae	<i>Nasturtium</i>	<i>officinale</i>		Watercress
27	Brassicaceae	<i>Raphanus</i>	<i>sativus</i>	<i>longipinnatus</i>	Daikon radish
28	Brassicaceae	<i>Raphanus</i>	<i>sativus</i>		Radish
29	Brassicaceae	<i>Wasabia</i>	<i>japonica</i>		Wasabi
30	Chenopodiaceae	<i>Atriplex</i>	<i>hortensis</i>		Garden orache
31	Chenopodiaceae	<i>Beta</i>	<i>vulgaris</i>		Beet
32	Chenopodiaceae	<i>Spinacia</i>	<i>oleracea</i>		Spinach
33	Fabaceae	<i>Pisum</i>	<i>sativum</i>		Pea
34	Lamiaceae	<i>Ocimum</i>	<i>basilicum</i>	<i>purpurascens</i>	Red rubin basil
35	Lamiaceae	<i>Ocimum</i>	<i>basilicum</i>		Basil
36	Malvaceae	<i>Corchorus</i>	<i>olitorius</i>		Jute mallow
37	Poaceae	<i>Zea</i>	<i>mays</i>		Maize
38	Polygonaceae	<i>Rumex</i>	<i>acetosa</i>		Sorrel
39	Polygonaceae	<i>Rumex</i>	<i>acetosella</i>		Red sorrel

3.2.2. Assignment of priority levels to categories and parameters in the first selection phase

For the selection method we considered the relative importance of the different categories and parameters in the framework of cultivation in Space. Specifically, we assigned values ranging between 1-3 for categories and 1-5

for parameters (Table 2). As regards categories, we set the highest priority ($P = 3$) to “Phytochemicals”, and then descending to “Growth” ($P = 2$) and to “Elements” ($P = 1$). As regards parameters included in the “Growth” category we assigned the highest values to yield ($p = 5$) and dry weight ($p = 4$) and then in descending order growth period ($p = 3$), sowing density ($p = 1$) and seed weight ($p = 1$) (Table 2). As regards the parameters related to quality aspects included in the categories “Phytochemicals” and “Elements” we assigned the specific priority levels considering nutritional requirements, physiological level content, and diet composition defined for Space missions [9, 10]. To quantify the priority values to be attributed to different parameters, three criteria were mainly considered. First, the nutraceutical importance of the compound and its effects due to the intake on human health such as the role in the metabolism, in the absorption of nutrients, the antioxidant and anti-inflammatory power and that of contrasting specific illness of the astronauts. As a second criterion we considered the loss of the putative component of the astronaut physiological level during the flight to select those species that can supply adequate amounts of phytonutrients to counterbalance the loss. Third, the presence of the different compounds in the astronaut diet and the perishability of Space food due to the Spacecraft environment. The low availability or absence of the specific nutraceutical compound in Space food, as well its degradation due to flight environmental factors such as temperature, atmosphere and radiation resulted as an important characteristic to be considered, especially in concomitance of a high nutraceutical role of the component). With this approach we assigned the highest priority level ($p = 5$) to ascorbic acid, β -carotene, tocopherol, and total polyphenols since their relevance for daily consumption was recognized. Lower priority ($p = 2$) was assigned to the remaining variables of the “Phytochemicals” category. As “Elements” category, the highest priority was assigned to calcium ($p = 5$), whereas an intermediate value ($p = 3$) was assigned to phosphorus, potassium, and magnesium. Finally, we assigned a value of $p = 2$ to sodium, sulfur, manganese, copper, and zinc. For both nitrate and iron (inverted value), which were considered as not desirable parameters due to their negative effects on human health, we assigned a value of $p = 3$ (Table 2).

Table 2. Priority levels of categories and parameters used for the first ranking of microgreens.

Category	Category priority (P)	Parameter	Parameter priority (p)
Growth	2	Yield	5
		Dry weight	4
		Growth period	3
		Sowing density	1
		Seed weight	1
Phytochemicals	3	Ascorbic acid (Vitamin C)	5
		Polyphenols	5
		Tocopherol (Vitamin E)	5
		β-carotene	5
		Antioxidant activity	2
		Phyllochinon (Vitamin K)	2
		Lutein	2
		Violaxantin	2
		Chlorophylls	2
Elements	1	Calcium	5
		Phosphorus	3
		Magnesium	3
		Nitrate	3
		Potassium	3
		Iron	3
		Sodium	2
		Sulfur	2
		Manganese	2
		Copper	2
		Zinc	2

3.2.3. Algorithm Calculation

For each microgreens genotype, the formula (1) was used to calculate the score of the individual parameters (s_i) as the product of the normalized value of the parameter (x_i) and its priority level (p_i):

$$(1) s_i = x_i \times p_i$$

The formula (2) was then used to calculate the score of the individual categories (X_i) as the average of the scores of the parameters included in the category:

$$(2) X_i = \mu (s_i)$$

Finally, the formula (3) was used to calculate the score of each genotype of microgreens (S_i) as the sum of the products between the scores of the categories (X_i) and their respective priority levels (P_i):

$$(3) S_i = \sum (X_i \times P_i)$$

Based on the calculated scores, we obtained a first ranking list of microgreens (Results, Table 4).

3.2.4. Second selection phase

The elaboration carried out for the first level of selection provided the ranking list based on the scores of 39 microgreens genotypes. From this ranking list, we selected the 6 top species for the second level of selection, namely coriander, savoy cabbage, daikon radish, red cabbage, white cabbage, and common radish. Differently from the first phase, in the second phase of selection we used an experimental approach based on germination and cultivation tests to gather similar and additional data to those collected from the literature for the first selection.

3.2.5. Germination tests and seed volume

The six top species (i.e., coriander, savoy cabbage, daikon radish, red cabbage, white cabbage, and common radish) were analyzed to evaluate germination rates at different days after sowing (DAS). Seeds of *Brassica oleracea* var. *capitata* f. *sabauda* cv. Vertus, *Brassica*

oleracea var. *capitata* f. *alba* cv. Copenhagen 2, *Brassica*

oleracea var. *capitata* f. *rubra* cv. Cabeza Negra 2, *Raphanus*

sativus var. *longipinnatus* cv. White Icicle, and *Raphanus sativus* cv. Saxa 2 were provided by Pagano Costantino & F.lli Srl (Scafati, Salerno, Italy).

Seeds of *Coriandrum sativum* L. cv. Micro Splits were provided by CN Seeds Ltd., Pymoor, Ely, Cambridgeshire, UK. For each species, the germination tests were conducted in an incubator at $22 \pm 0.5^\circ\text{C}$ using four Petri dishes lined with laboratory filter paper wetted with distilled water. 100 seeds were placed in each Petri dish, for a total of 400 seeds per species. The percentage of germination was evaluated at 3, 5, and 10 days after sowing. For each of

the species tested, seed volume was measured using a 10 mL graduated cylinder containing distilled water. In particular, 100 seeds were submersed in 3 mL of water inside the cylinder and the volume of the seeds was measured as an increase in the total volume.

3.2.6. Cultivation tests - plant material and growth conditions

Cultivation tests were conducted using the same seeds of coriander, savoy cabbage, daikon radish, red cabbage, white cabbage, and common radish used for the germination tests. Based on the size of the different seeds, a sowing density of 4 seeds cm⁻² was adopted for coriander and the genus *Raphanus*, and 5 seeds cm⁻² for the genus *Brassica*. The microgreens seeds were sown in plastic trays (~200 cm²) filled with peat-based substrate (Special Mixture, Floragard Vertriebs-GmbH, Oldenburg, Germany; pH 5.48 and EC 0.282 mS cm⁻¹) and placed in a fully controlled growth chamber (KBP-6395F, Termaks, Bergen, Norway). Seed germination occurred in darkness at 24°C and 100% relative humidity (RH) for six days for coriander and four days for the rest. During germination, the seeds were sprayed with osmotic water (EC = 100 ± 10 µS cm⁻¹), whereas after emergence, plants were fertigated with a quarter-strength Hoagland solution (pH = 5.9 ± 0.2 and EC = 400 ± 25 µS cm⁻¹). Once the microgreens emerged, the artificial light (400-700 nm) provided by light-emitting diode panels (K5 Series XL750, Kind LED, Santa Rosa, CA, USA) was turned on (12h photoperiod), and the growth chamber was set at 24/18 ± 2°C and RH 65 ± 5%. The LED panels were set to deliver an adequate absorption spectrum for photosynthesis, and an average photosynthetic photon flux density (PPFD) of 300 ± 15 µmol m⁻² s⁻¹ at canopy level. Each cultivar cultivation was replicated three times (tray = replicate), and all the trays were rotated daily within the growth chamber to maintain a homogeneous light, temperature, and humidity repartition.

3.2.7. Cultivation tests - colorimetric measurement, harvest and sampling

Just before harvest, the CIELAB color space parameters (L*, a* and b*) of microgreens canopy were measured by taking eight measurements per tray via a portable Minolta Chroma meter (CR-400, Minolta Co. Ltd., Osaka,

Japan). Then a^* and b^* values were used to calculate the hue angle and chroma. For harvest, cotyledonary stage was chosen for all the cultivars, as shown in Figure 1, resulting in 13, 14 and 16 days after sowing (DAS) for *Raphanus* genus, *Brassica* genus and coriander, respectively. All microgreens' cultivars were harvested just above the substrate level by avoiding any impurities, and they were assessed directly for their fresh yield that was expressed in kg m^{-2} . Eight randomly chosen microgreens plantlets per tray were measured to assess hypocotyl length, which was expressed in cm. Part of the harvested material was placed in a forced air oven at 60°C until constant weight, in order to assess the dry weight of each replicate and consecutively calculate dry matter (DM) %. The remaining harvested material was directly placed in liquid nitrogen, and then stored at -80°C to be used successively for the qualitative analysis.



Figure 1. Representative photos of the cotyledonary stage of the different microgreens' species at harvest.

3.2.8. Cultivation tests - non-structural carbohydrates

Measurements of non-structural carbohydrates (NSC) were performed using 10 mg samples of the powder obtained from the freeze-dried material. The extraction was performed in 1 mL of 80% ethanol at 80°C for 45 min under continuous shaking conditions. The extract was centrifuged at $16000g$ for 5 min, soluble sugars (glucose, fructose, and sucrose) were recovered in the

supernatant, and starch was in the pellet. Soluble sugar determination, by spectrophotometric coupled enzymatic assay, was performed, as described in Scartazza et al. (2017) [11]. All sugar assays were performed in dual-wavelength mode (340–405 nm) in a plate reader (Spectrotar Nano BMG, Labatech GmbH, Ortenberg, Germany). The pellet, containing starch, was washed four times with a 50 mM NaAcetate buffer (pH 4.5) and then suspended and autoclaved at 120°C for 45 min in 1 mL of the same buffer. After autoclaving, the sample was incubated at 50°C for 1 h with amyloglucosidase (70 U) and α -amylase (4U) to hydrolyze the starch to glucose. The glucose produced by starch hydrolysis was then measured as described before by spectrophotometric coupled enzymatic assay.

3.2.9. Cultivation tests - pigments content

Neoxanthin, violaxanthin, β -carotene, and lutein content were determined by the extraction of 10 mg powdered freeze-dried microgreens with 2 mL 100% acetone at 4°C under dark conditions using a glass-glass homogenizer. The samples were centrifuged at 16000*g* for 5 min at 4°C and filtered through a 0.2 μ m nylon PPII syringe disposable filter; 15 μ L of the clear extract was used to determine the concentration of pigments by an HPLC U3000 system (Dionex™ ICS-5000; Thermo Fisher Scientific, Waltham, MA, United States), equipped with a C18(2) LUNA (Phenomenex, Bologna, Italy) analytical column (5 μ m, 250 mm $\times$ 4.6 mm) and a related guard column (Phenomenex, Bologna, Italy) maintained at 30°C. All separations were achieved isocratically using, from 0 to 4 min, a mobile phase composed of solution A: 1.75% water, 1.75% methanol, 1.75% dichloromethane, and 94.75% acetonitrile, and from 4.1 to 18 min a mobile phase composed of solution B: 50% acetonitrile and 50% diethyl acetate, with a final re-equilibration of 4 min with solution A. The flow rate was 1 ml min⁻¹ for a total run time of 22 min. The autosampler was maintained at 4°C, the UV detector wavelength was set at 440 nm, and concentrations of pigments were determined against standard curves [12]. Chlorophylls (Chl a and Chl b) were quantified spectrophotometrically using the same ethanolic extracts used for NSC determination, as described in Lichtenthaler and Wellburn (1983) [13].

3.2.10. Cultivation tests - total anthocyanins and total phenolic content

Total anthocyanins were determined by extracting 10 mg of lyophilized powder in 2 ml of 1% HCl in methanol for 1 hour at 65°C. The liquid extract was separated by centrifugation at 16000g for 5 minutes. After centrifugation, the supernatant was separated, and the total anthocyanin content was quantified spectrophotometrically by measuring the absorbance at 530 nm and 657 nm to correct the chlorophylls degradation products. Concentration was expressed as cyanidin-3-glucoside equivalent values and using an extinction coefficient of 30000 mol⁻¹ cm⁻¹. The phenolic component (Total Polyphenol Content, TPC) was quantified according to the protocol described by Usenik et al. (2008) [14] by extracting 10 mg of powder from the lyophilized samples in 2 ml of 100% methanol. After centrifugation at 16000g for 5 min, the supernatant was recovered and used for the spectrophotometric quantification of polyphenols, determining the absorbance at 765 nm. The amount of total polyphenols was then calculated by relating the absorbance of each sample to the calibration curve of gallic acid.

3.2.11. Cultivation tests – total ascorbic acid

For the determination of total ascorbic acid (Tot. Asc. A.), 10 mg of the frozen powder was extracted in an ice-cold glass-glass homogenizer with 2 mL of 3% Metaphosphoric acid (MPA) at 4°C. The mixtures were centrifuged at 16000 × g for 5 min at 4°C. The supernatants were filtered through a 0.2 µm (Whatman) PPII nylon filters. Tris (2-carboxyethyl) phosphine (TCEP) was added as a reducing agent to the final concentration of 5 mmol/L in the filtered extract, which was then incubated for 30 min at 25°C in order to reduce all the dehydro-ascorbic acid to ascorbic acid. After 30 minutes, 5 µl of samples were injected into HPLC for the quantification of Tot. Asc. A. The chromatographic method used is that described in Chebrolu et al. (2012)[15] with minimal changes. Tot. Asc. A. was analyzed using an UltiMate 3000 HPLC System ThermoScientific™ Dionex (Sunnyvale, CA, USA) coupled with a UV/VIS detector (ThermoScientific™ Dionex). The separation was performed using a Phenomenex Luna C18(2) column (250 mm × 4.6 mm i.d. and particle size 5 µm) and the run time was 15 min.

The Tot. Asc A. peak was detected at 254 nm and the processing of the chromatographic peaks was performed using the software version Chromeleon 7.2 (ThermoScientific™ Dionex). The entire chromatographic separation was performed in an isocratic mobile phase consisting of 0.010 mol L⁻¹ of KH₂PO₄, maintained at pH 2.8 and flow rate of 0.7 mL min⁻¹. The quantification was performed by means of a calibration curve of an ascorbic acid standard. All the reagents used are of a high degree of purity for HPLC analysis.

3.2.12. Cultivation tests - nitrate, phosphate, and sulfate

The determination of inorganic anions was performed by the extraction of 10 mg of powdered materials in water at 80°C for 45 min under continuous shaking conditions. The extract was centrifuged at 16000*g* for 5 min and the supernatants were filtered through a 0.2 µm nylon PPII syringe filter prior to injection on an ion chromatography system (Dionex™ ICS-5000; Thermo Fisher Scientific, Waltham, MA, United States) equipped with a conductivity detector, an analytical IonPac AS11-HC column (4 × 250mm) (Thermo Fisher Scientific, Waltham, MA, United States) with a related guard column and an IonPac Anion Trap Column (ATC)-1 (Thermo Fisher Scientific, Waltham, MA, United States). The system was coupled with an ERSTM 500 Electrolytically Regenerated Suppressor (Dionex™ ICS-5000; Thermo Fisher Scientific, Waltham, MA, United States) applying the external water mode configuration, using 100% of methanol. Runs were carried out at 30°C and a flow rate of 1ml min⁻¹ using a sodium hydroxide stepped gradient as reported by Proietti et al. (2019)[16]. The electrical signal was integrated into micro-Siemens (µS). The eluents and the inorganic anion standard solutions were prepared using HPLC-grade reagents (Merck KGaA, Darmstadt, Germany). U3000-HPLC and ICS-5000 chromatography system control, data acquisition, and processing were performed with the software Chromeleon Data System 6.8 (Dionex™ ICS-5000; Thermo Fisher Scientific, Waltham, MA, United States).

3.2.13. Assignment of priority levels to categories and parameters in the second selection phase

Data obtained from the germination, cultivation, and quality tests were entered in the working matrix and used to calculate the ranking list of the 6 top species using a similar procedure as described for the first algorithm elaboration. The calculation of the final scores for each species was performed using the formula (1), (2), and (3) as described previously. In particular, for the second ranking list, the parameters, categories and their respective priority levels used for the calculation are shown in Table 3. Similar to the first selection phase, we assigned the highest priority (value = 3) to the category “Phytochemicals” and then descending values to “Growth” (value = 2) and to “Elements” (value = 1). Overall, data on “Phytochemicals” from the cultivation tests were expressed as the quantity of the specific compound on fresh weight per day per m² of cultivated area. The second phase of the selection process followed a similar approach as the first phase, in which priority levels were assigned to the parameter. The rationale of the prioritization of parameters included in the “phytochemicals” category, was based on the nutraceutical value of the specific compound, the potential loss of components in the astronaut’s physiological level during the flight, and the perishability of the compounds. Furthermore, in this second phase, statistically significant differences in the production of each compound were considered as the driving criterion for the attribution of the priority levels. Highest priority values (p = 5) were assigned to total ascorbic acid and then descending (p = 4) to anthocyanins and total polyphenols. We then assigned p = 3 to lutein and p = 2 to total non-structural carbohydrates and total soluble carbohydrates. The lowest level (p = 1) was assigned to total chlorophylls, β -carotene, violaxanthin, neoxanthin, starch, sucrose, glucose, and fructose. As regards the prioritization of “Growth” parameters, we assigned the highest priority levels to Yield, growth period (inverted), germination, and seed volume (inverted). As for parameters of the “Element” category, we assigned p = 1 for both nitrate and sulfate.

Table 3. Priority levels of categories and parameters used for the second ranking of microgreens.

Category	Category priority (P)	Parameter	Parameter priority (p)
Growth	2	Growth period	5
		Fresh yield	5
		Germination	3
		Seed volume	3
		Hypocotyl length	2
		Dry weight	2
		Sowing density	2
		L*	1
		a*	1
		b*	1
		Chroma	1
		Hue	1
Phytonutrients	3	Total Ascorbic Acid	5
		Anthocyanins	4
		Total polyphenols	4
		Lutein	3
		Total non-structural carbohydrates	2
		Total soluble carbohydrates	2
		Total chlorophylls	1
		β -carotene	1
		Violaxanthin	1
		Neoxanthin	1
		Starch	1
		Sucrose	1
		Glucose	1
		Fructose	1
Elements	1	Nitrate	1
		Sulfate	1

3.3. Results

3.3.1. First ranking list

The ranking list resulting from the first algorithm elaboration using literature data showed score values ranging between 0.956 and 4.687 across microgreens genotypes as shown in Table 4. Results showed that most genotypes belonging to the family *Brassicaceae* are listed at the top of the ranking. However, coriander (*Apiaceae*) resulted in first place with the highest score. From this ranking list we selected the six top species, namely coriander, savoy cabbage, daikon radish, red cabbage, white cabbage, and common radish, for the germination and cultivation tests of the second selection phase.

Table 4. Ranking list of microgreens based on the scores calculated using literature data.

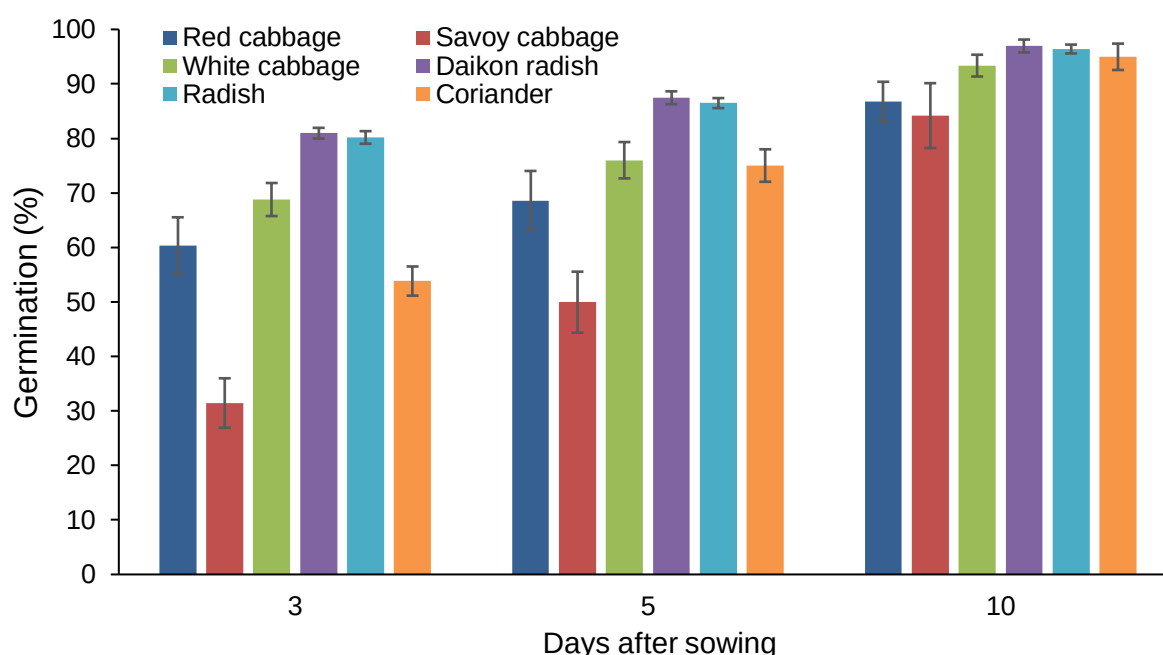
Rank	Species	Common name	Score
1	<i>Coriandrum sativum</i>	Coriander	4.687
2	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>sabauda</i>	Savoy cabbage	4.632
3	<i>Raphanus sativus</i> var. <i>longipinnatus</i>	Daikon radish	4.586
4	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>rubra</i>	Red cabbage	4.541
5	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>alba</i>	White cabbage	4.487
6	<i>Raphanus sativus</i>	Radish	4.375
7	<i>Brassica oleracea</i> var. <i>italica</i>	Broccoli cabbage	4.373
8	<i>Brassica oleracea</i> var. <i>acephala</i>	Black cabbage	4.232
9	<i>Brassica oleracea</i> var. <i>alboglabra</i>	Chinese kale	4.214
10	<i>Brassica oleracea</i> var. <i>botrytis</i>	Cauliflower	4.209
11	<i>Brassica rapa</i> var. <i>ruvo</i>	Rapini	4.203
12	<i>Brassica oleracea</i> var. <i>pekinensis</i>	Napa cabbage	4.053
13	<i>Brassica oleracea</i> var. <i>gongylodes</i>	Kohlrabi	4.035
14	<i>Brassica rapa</i> var. <i>narinosa</i>	Tatsoi	3.829
15	<i>Brassica rapa</i> var. <i>chinensis</i>	Pak choy	3.746
16	<i>Brassica rapa</i> var. <i>perviridis</i>	Komatsuna	3.720
17	<i>Brassica rapa</i> var. <i>rapa</i>	Turnip	3.693
18	<i>Brassica napus</i> var. <i>napobrassica</i>	Rutabaga	3.673
19	<i>Brassica rapa</i> var. <i>nipposinica</i>	Mizuna	3.523
20	<i>Brassica rapa</i> var. <i>gemmifera</i>	Brussels sprouts	3.434
21	<i>Ocimum basilicum</i>	Basil	3.181
22	<i>Ocimum basilicum</i> var. <i>purpurascens</i>	Red rubin basil	3.162
23	<i>Beta vulgaris</i>	Beet	3.024
24	<i>Amaranthus hypochondriacus</i>	Amaranth	2.974
25	<i>Brassica juncea</i>	Brown mustard	2.872
26	<i>Wasabia japonica</i>	Wasabi	2.714
27	<i>Lepidium sativum</i>	English cress	2.701
28	<i>Lepidium bonariense</i>	Peppercress	2.658
29	<i>Eruca sativa</i>	Rocket	2.500
30	<i>Rumex acetosella</i>	Red sorrel	2.376
31	<i>Corchorus olitorius</i>	Jute mallow	2.250
32	<i>Pisum sativum</i>	Pea	2.022
33	<i>Apium graveolens</i>	Celery	1.550
34	<i>Barbarea verna</i>	Cress	1.514
35	<i>Atriplex hortensis</i>	Garden orache	1.272
36	<i>Nasturtium officinale</i>	Watercress	1.209
37	<i>Spinacia oleracea</i>	Spinach	1.050
38	<i>Zea mays</i>	Maize	0.972
39	<i>Rumex acetosa</i>	Sorrel	0.956

3.3.2. Germination and seed volume

Germination was higher than 50% at 3 DAS in all the species analyzed, except for savoy cabbage showing lower values (Figure 2). At 3 DAS, common radish and daikon radish showed the highest percentage values of 80% and 81%, respectively. Successively, the germination percentages showed an increase over time in all species up to 10 DAS, reaching maximum values between 84% (savoy cabbage) and 96% (daikon radish). Seed volume was

highest in coriander (100-Seeds, 1.4 mL), and lowest in red, savoy and white cabbage (100-Seeds, ≤ 0.55 mL), whereas 100-Seeds volume of both daikon and common radish resulted 0.65 mL (Table 5).

Figure 2. Germination of red cabbage, savoy cabbage, white cabbage, daikon radish, common radish, and coriander seeds at 3, 5 and 10 days after sowing.



3.3.3. Harvest and colorimetric analysis

Among the six cultivars of microgreens used for cultivation tests, radish was characterized by the highest fresh yield and dry matter per m^2 (3.66 kg m^{-2} and 0.25 kg m^{-2} , respectively) (Table 5). Whereas coriander yielded the lowest ($\sim 42.3\%$ less than radish) and had the lowest hypocotyl length among all the tested microgreens, but it showed the highest DM (8.41%), that was approximately 27.15% higher than the other species. Coriander and daikon radish exhibited significantly brighter canopy (L) than red cabbage and savoy cabbage (Table 6). In addition, coriander canopy was greener (a^* : -16.27) than the others, as opposed to red cabbage canopy that was leaning more towards red (a^* : -8.65) and blue (b^* : 14.61) and less saturated (Chroma: 16.98) than the other microgreens species. As for Hue, white cabbage and savoy cabbage canopy had significantly higher values than radish.

Table 5. Biometric parameters, and Phosphate and Sulfate content

Source of Variance	100-Seeds volume (mL)	Hypocotyl length (cm)	Fresh yield (kg m ⁻²)	Dry biomass	DM (%)	Phosphate (mg m ⁻² day ⁻¹ FW)	Sulfate
Species							
Daikon radish	0.65	6.23 ± 0.18 a	2.09 ± 0.04 d	0.13 ± 0.01 d	6.33 ± 0.13 b	239 ± 13 b	271 ± 14 b
White cabbage	0.50	5.97 ± 0.02 a	2.43 ± 0.03 c	0.16 ± 0.01 cd	6.44 ± 0.40 b	182 ± 27 b	288 ± 23 ab
Coriander	1.40	4.61 ± 0.12 b	2.11 ± 0.02 d	0.18 ± 0.00 bc	8.41 ± 0.09 a	179 ± 18 b	50.3 ± 3.0 c
Red cabbage	0.55	5.84 ± 0.30 a	2.71 ± 0.04 b	0.18 ± 0.00 b	6.74 ± 0.08 b	218 ± 1 b	293 ± 20 ab
Radish	0.65	6.36 ± 0.23 a	3.66 ± 0.09 a	0.25 ± 0.01 a	6.89 ± 0.15 b	367 ± 14 a	352 ± 5.9 a
Savoy cabbage	0.45	6.63 ± 0.08 a	2.84 ± 0.03 b	0.19 ± 0.00 b	6.67 ± 0.04 b	196 ± 15 b	333 ± 15 ab
Significance		***	***	***	***	***	***

*** Significant at $p < 0.001$. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$)

Table 6. Colorimetric parameters

Source of Variance	L*	a*	b*	Chroma	Hue
Species					
Daikon radish	41.28 ± 2.29 a	-14.27 ± 0.56 d	23.71 ± 0.71 b	27.68 ± 0.88 b	121.0 ± 0.4 ab
White cabbage	37.40 ± 0.95 ab	-12.42 ± 0.14 b	20.26 ± 0.60 c	23.77 ± 0.57 c	121.6 ± 0.5 a
Coriander	42.11 ± 0.99 a	-16.27 ± 0.08 e	27.34 ± 0.34 a	31.82 ± 0.33 a	120.8 ± 0.2 ab
Red cabbage	33.15 ± 0.69 b	-8.65 ± 0.11 a	14.61 ± 0.14 d	16.98 ± 0.13 d	120.6 ± 0.4 ab
Radish	38.27 ± 0.16 ab	-13.7 ± 0.24 cd	24.02 ± 0.31 b	27.66 ± 0.35 b	119.7 ± 0.4 b
Savoy cabbage	34.68 ± 0.51 b	-12.51 ± 0.08 bc	19.98 ± 0.05 c	23.58 ± 0.05 c	122.1 ± 0.2 a
Significance	***	***	***	***	*

*, *** Significant at $p < 0.05$ and 0.001 , respectively. Different letters within each column indicate significant differences according to Duncan Tukey's multiple-range test ($p = 0.05$)

3.3.4. Non-structural carbohydrates

The production of the NSC is shown in (Table 7). The NSC production levels varied significantly ($p < 0.001$) among the six microgreens species. In terms of Total NSC and total soluble carbohydrates, the cultivar Candela showed a lower score compared to the other species, among which the total amount of these compounds did not vary significantly. The production levels

of the soluble fractions (glucose, fructose, and sucrose) resulted significantly different among the different species. Radish, savoy cabbage, white cabbage and red cabbage showed an average increase of 65% of glucose compared to coriander and daikon radish. The highest fructose production was found in red cabbage and savoy cabbage, whereas daikon radish showed the lowest levels. For the sucrose production, coriander showed the best performance, followed by radish and savoy cabbage. On the other hand, white cabbage, red cabbage, and daikon radish did not show significant differences, with an average value of $79.5 \text{ mg m}^{-2} \text{ day}^{-1} \text{ FW}$ representing the lowest value of sucrose production. As regards the quantification of starch, the species with the highest production level was radish that showed an increase of 37%, 52%, 80%, 308% and 429% compared to red cabbage, white cabbage, savoy cabbage, coriander, and daikon radish, respectively.

Table 7. Sugars and starch accumulation, total soluble carbohydrates (TSC), and total non-structural carbohydrates (NSC)

Source of Variance	Glucose	Fructose	Sucrose ($\text{mg m}^{-2} \text{ day}^{-1} \text{ FW}$)	Starch	TSC	NSC
Species						
Daikon radish	$706 \pm 91 \text{ c}$	$160 \pm 22 \text{ d}$	$77.8 \pm 14 \text{ d}$	$105 \pm 25 \text{ d}$	$943 \pm 125 \text{ b}$	$1048 \pm 145 \text{ b}$
White cabbage	$1175 \pm 17 \text{ ab}$	$234 \pm 18 \text{ cd}$	$80.7 \pm 1 \text{ cd}$	$364 \pm 56 \text{ abc}$	$1484 \pm 48 \text{ ab}$	$1982 \pm 192 \text{ a}$
Coriander	$814 \pm 33 \text{ bc}$	$395 \pm 12 \text{ ab}$	$864 \pm 25 \text{ a}$	$136 \pm 8 \text{ cd}$	$2073 \pm 51 \text{ a}$	$2209 \pm 46 \text{ a}$
Red cabbage	$1170 \pm 52 \text{ ab}$	$460 \pm 43 \text{ ab}$	$80.2 \pm 5 \text{ cd}$	$406 \pm 91 \text{ ab}$	$1710 \pm 93 \text{ a}$	$2116 \pm 183 \text{ a}$
Radish	$1433 \pm 143 \text{ a}$	$325 \pm 41 \text{ bc}$	$236 \pm 2 \text{ b}$	$555 \pm 17 \text{ a}$	$1994 \pm 182 \text{ a}$	$2549 \pm 193 \text{ a}$
Savoy cabbage	$1218 \pm 80 \text{ ab}$	$491 \pm 41 \text{ a}$	$136 \pm 3 \text{ c}$	$308 \pm 55 \text{ bcd}$	$1846 \pm 122 \text{ a}$	$2154 \pm 174 \text{ a}$
Significance	***	***	***	***	***	***

*** Significant at $p < 0.001$. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$). TSC = glucose + fructose + sucrose; NSC = TSC + starch

3.3.5. Vitamin C

Microgreens genotypes showed significant different production levels ($p < 0.001$) of Tot. Asc. A. The greatest value of Tot. Asc. A. was found in radish. The lowest level of Tot. Asc. A. was found in the cultivar coriander which showed 81% lower value compared to the best performing cultivar. Savoy cabbage, red cabbage, white cabbage and daikon radish showed intermediate

values with a reduction of 18, 34, 38 and 58%, respectively, when compared to radish.

3.3.6. Total anthocyanin content and total phenolic content

The content of total anthocyanins and TPC is shown in Table 8. The ANOVA analysis showed significant differences ($p < 0.001$) of both parameters among microgreens genotypes analyzed. The total anthocyanins content was not measurable in white cabbage and coriander. Red cabbage showed elevated accumulation of anthocyanins, resulting to be 3, 5 and 10-fold higher compared to radish, savoy cabbage and daikon radish, respectively. The best performing microgreens in terms of TPCs production resulted to be radish with $458 \text{ mg m}^{-2} \text{ Day}^{-1}$. Results also showed that red cabbage and savoy cabbage accumulated intermediate levels of TPC showing a 25%, 68% and 77% increase compared to white cabbage, daikon radish and coriander, respectively (Table 8).

Table 8. Pigments, Total Phenolic Content (TPC) and Total Ascorbic Acid (Tot. Asc.A.) content

Source of Variance	Total chlorophyll	Anthocyanins ($\text{mg m}^{-2} \text{ day}^{-1} \text{ FW}$)	TPC	Tot. Asc. A
Species				
Daikon radish	$30.64 \pm 2.91 \text{ b}$	$1.55 \pm 0.11 \text{ c}$	$200 \pm 9 \text{ d}$	$79.5 \pm 4 \text{ cd}$
White cabbage	$26.36 \pm 2.34 \text{ b}$	N.D.	$268 \pm 22 \text{ c}$	$104 \pm 8 \text{ bc}$
Coriander	$33.74 \pm 2.55 \text{ b}$	N.D.	$189 \pm 6 \text{ d}$	$31.9 \pm 2 \text{ d}$
Red cabbage	$37.05 \pm 0.16 \text{ ab}$	$14.9 \pm 1.13 \text{ a}$	$347 \pm 17 \text{ b}$	$111 \pm 15 \text{ bc}$
Radish	$49.77 \pm 5.50 \text{ a}$	$4.98 \pm 0.85 \text{ b}$	$458 \pm 12 \text{ a}$	$168 \pm 21 \text{ a}$
Savoy cabbage	$31.39 \pm 3.79 \text{ b}$	$2.74 \pm 0.05 \text{ bc}$	$323 \pm 14 \text{ bc}$	$138 \pm 11 \text{ ab}$
Significance	**	***	***	***

, * Significant at $p < 0.01$ and 0.001 , respectively. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$). N.D. = not detected.

3.3.7. Pigments

The content of total chlorophylls resulted to be significantly different only for radish which showed a 57% higher production compared to the other microgreens genotypes (Table 9). None of the lutein, β -Carotene, neoxanthin and violaxanthin content resulted to be statistically different

among the tested species, showing an overall average production of 8.7, 5.9, 2.1 and 37.3 mg m⁻² day⁻¹ FW, respectively (Table 9).

Table 9. Lutein, β -carotene, Neoxanthin and Violaxanthin content

Source of Variance	Lutein	β -Carotene (mg m ⁻² day ⁻¹ FW)	Neoxanthin	Violaxanthin
Species				
Daikon radish	8.51 $\pm$ 1.46	6.29 $\pm$ 0.85	2.24 $\pm$ 0.23	50.3 $\pm$ 10
White cabbage	6.29 $\pm$ 1.59	4.26 $\pm$ 0.41	1.44 $\pm$ 0.01	22.3 $\pm$ 5.5
Coriander	6.74 $\pm$ 0.47	4.92 $\pm$ 0.47	2.10 $\pm$ 0.23	34.8 $\pm$ 3.1
Red cabbage	8.58 $\pm$ 1.26	6.27 $\pm$ 1.32	1.67 $\pm$ 0.28	34.7 $\pm$ 8.1
Radish	10.3 $\pm$ 0.88	6.13 $\pm$ 0.26	2.37 $\pm$ 0.53	49.7 $\pm$ 5.7
Savoy cabbage	11.5 $\pm$ 3.16	7.38 $\pm$ 2.16	2.55 $\pm$ 0.66	31.7 $\pm$ 4.9
Significance	ns	ns	ns	ns

Ns = non significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test (p= 0.05)

3.3.8. Nitrate, phosphate, and sulfate

Nitrate accumulation was not detectable among the cultivars tested, whereas phosphate and sulfate accumulation resulted to be significantly different among microgreens genotypes (P<0.001) (Table 5). The highest level of phosphate was found in radish (367 mg m⁻² day⁻¹ FW) which also showed the highest production of sulfates (352 mg m⁻² day⁻¹ FW). On the other hand, the lowest accumulation of phosphate was found in white cabbage and coriander with an average of 182 and 179 mg m⁻² day⁻¹ FW respectively, whereas red cabbage, savoy cabbage and daikon radish showed intermediate values. For the sulfate content, coriander showed the lowest accumulation with an 86% decrease compared to radish.

3.3.9. Calculation 2nd algorithm

Experimental data gathered from the germination and cultivation tests were used to construct the working matrix for the calculation of the second ranking list including the six top species (i.e., coriander, savoy cabbage, daikon radish, red cabbage, white cabbage, and common radish). Data elaboration was performed using the priority levels of categories and parameters reported in Table 3. Results of the algorithm calculation showed that radish obtained

the highest score (8.409), followed by savoy cabbage (6.661), whereas coriander showed the lowest score (3.957) (Table 10).

Table 10. Ranking list of the 6 top species of microgreens

Rank	Species	Common name	Score
1	<i>Raphanus sativus</i>	Radish	8.409
2	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>sabauda</i>	Savoy cabbage	6.661
3	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>rubra</i>	Red cabbage	6.421
4	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>alba</i>	White cabbage	4.527
5	<i>Raphanus sativus</i> var. <i>longipinnatus</i>	Daikon radish	4.315
6	<i>Coriandrum sativum</i>	Coriander	3.957

3.4. Discussion

Microgreens generally have a higher content of phytonutrients and lower nitrates compared to their mature-leaf counterparts, thus resulting in high quality functional food [6, 17]. Indeed, microgreens provide a significant quantity of phytonutrients that can help astronauts in coping with the stressful conditions of the Space [18, 19].

Since there are many species that have been proposed for microgreens production [18], we developed a method based on an algorithm with the aim of objectively select those microgreens species most suitable for cultivation in Space according to productivity and phytonutrient profile criteria. The selection method consisted of two subsequent rankings elaborated using first literature data and, successively, using data obtained with cultivation tests specifically conducted for this study. The first ranking list included 39 plant species commonly used for the production of microgreens according to the reference literature. Most of the species herein considered belong to the family *Brassicaceae* (26 out of 39) whereas the remaining species belong to *Amaranthaceae* (1), *Apiaceae* (2), *Chenopodiaceae* (3), *Fabaceae* (1), *Lamiaceae* (2), *Malvaceae* (1), *Poaceae* (1), *Polygonaceae* (2). In this regard, although there is a growing interest in microgreens and in the use of new microgreens varieties, no studies presented a comparison between species for the selection of microgreens genotypes based on productivity and phytonutrient profile criteria. Indeed, most studies on microgreens

investigated the effects of environmental conditions or cultivation techniques using one or a few species. For this study, in the first selection phase, we focused on documents providing a comparison between different microgreens species grown under standard conditions. With this approach we gathered literature data on horticultural and nutritional parameters to be included in the working matrix for a comparative analysis between the different species based on the prioritization of the different parameters.

A similar approach based on parameter prioritization has been used by De Micco et al. (2012) [1] and Massa et al. (2015) [2], but with differences from our study mostly due to the specific aims of the selection. Indeed, prioritization of parameters can have a large effect on the final scores of the different species. Nevertheless, the method herein reported provides the possibility of modeling species selection by adjusting the prioritization of productivity and phytonutrient parameters according to specific objectives. In our case, the selection process aimed to ensure that the selected plant food had elevated nutraceutical value.

After the first selection phase we identified six top species with the highest score that were then used for germination and cultivation tests. This restricted list of microgreens allowed us to study each species with more details introducing new parameters to be measured and implemented in the working matrix for the elaboration of the ranking list. For this second selection phase, we chose to include in the working matrix data of “Phytonutrients” parameters expressed as the daily production per m^2 . This approach allowed us to better identify those species with higher production efficiency of phytonutrients, considering there are constraints regarding volume available for cultivation in Space. Furthermore, data on daily production of phytonutrients per m^2 are useful to estimate the surface to be cultivated according to the growth period of each species and the astronauts’ daily dietary requirements.

Ghoora et al. (2020) [20] investigated the contribution of nutrients (NC), compared to the nutrient claims of FDA (U.S. Food and Drug Administration), of ten culinary microgreens with a consumption of 85g (USDA reference amount customarily consumed of green leafy vegetables) of fresh biomass. Authors pointed out that the vitamin’s NC ranged between

28.2–116.4%, 28.5–332% and 24.3–71.8% respectively for ascorbic acid (vitamin C), α -tocopherol (vitamin E) and β -carotene (pro-vitamin A). Since microgreens can efficiently fulfill the daily intakes of vitamins, along with a great contribution in antioxidants, the prioritization of different categories favored the “Phytonutrients” category. However, growth characteristics that describe the general productivity efficiency of the crop, such as yield, dry weight, and growth period, cannot be understated since they impact the overall size of the cropping system, thereby affecting key aspects of Space facilities as total resources used and mass at launch. For these reasons, we assigned the second highest priority level to the “Growth” category. Pinto et al. (2015)[17] investigated the potential contribution of lettuce microgreens to supply the recommended daily intake of minerals. In the study by Pinto et al. (2015) [17], the Ca, P, Mg, K, and Na Estimated Daily Intake (EDI), based on a consumption of 25 g of microgreens, respectively represented only the 3.48, 3.10, 1.76, 1.75, 0.83% of the EFSA RDAs (recommended dietary allowances) and AIs (adequate intakes) mean for men and women. In the study by Ghoola et al. (2020) [20] the NC of macro-elements such as Ca, Mg, K, Na reached respectively 6.2%, 14.4%, 8.7% and 8.4%. In our view, producing food rich in minerals *via* microgreens is less relevant than producing bioactive molecules, since minerals are stable during conservation. Furthermore, even if microgreens can contribute to the mineral supply, the achievement of the recommended daily intake solely through the consumption of microgreens is difficult, while it can be easily obtained using other food sources. Therefore, we assigned to “Elements” category the lowest priority level.

Maintaining proper antioxidant health is a priority for astronauts during spaceflight. Several factors including ionizing radiation, microgravity, exercise and even diet are a source of ROS [21–24]. The role of phytochemicals such as vitamins and polyphenols in maintaining antioxidant health have been reviewed by several authors [9, 25–27]. The protective effect of Vitamin A, C, and E against γ -rays was investigated *in vitro* and the potential to reduce DNA damages before and after irradiation was shown [28]. In addition, the radioprotective potential of both vitamin A and C were successfully tested in mice [29, 30]. These compounds also have a synergistic

effect by enhancing the antioxidant potential when administered together [31]. Moreover, vitamin A, C and E are involved in several fundamental physiological processes in the human body such as immune function, bone health, eye health, and are related to the body's response to the space environment [9]. In order to give priority to a specific nutraceutical component useful for the astronauts' diet, it is necessary to evaluate the deficiencies that may occur during the spaceflight. Smith et al. (2005) [32] analyzed the blood levels of vitamins and minerals in 11 astronauts before and after 128-195 day of spaceflight on board of the International Space Station. They pointed out that vitamin E was affected by the spaceflight since γ -tocopherol significantly decreased after landing. However, no losses were detected for β -carotene, whereas the ascorbate status has not yet been investigated. As regards the stability of the nutraceuticals in Space-stored food, while vitamin A declines but with sufficient concentrations kept after 3 years of storage, the vitamin C content in most fruit products degrades between 32 and 83% [33]. Moreover, Vitamin E has been found to be the most sensitive to irradiation during storage [34]. According to the aforementioned criteria, the highest priority among the parameters of the "Phytonutrient" category, in the first selection phase, was assigned to ascorbic acid, tocopherols, β -carotene and polyphenols. Bone loss is a significant health concern associated with long-duration Space missions and the microgravity environment [35]. The rate of bone mineral loss during spaceflight is estimated to be between 0.5-1% per month [36]. Net rate of bone calcium loss in Mir and Skylab missions resulted in approximately 250 mg every day. Adequate intake of calcium-rich foods or supplements will play a significant role in ensuring the skeletal health of astronauts during prolonged Space missions [32]. In this regard, the highest priority level for parameters included in the "Element" category was assigned to calcium. As regards iron and nitrate parameters included in the "Element" category, data were inverted in order to valorize microgreens genotypes with low accumulation. Low iron and nitrate accumulator species are desirable when selecting crops for BLSSs. The International Space Station (ISS) food system has high levels of dietary iron. The current iron U.S. Dietary Reference Intake (DRI) is 8-10 mg d⁻¹ and crew members on long-duration Space missions

have been observed to consume excessive amounts of iron (20-25 mg iron d⁻¹) [37]. In addition, physiological changes during spaceflight led to an accumulation of iron in storage tissues which can act as an oxidizing agent inducing damages at DNA level [38]. In addition, high amounts of nitrate can cause serious health problems for humans. The accumulation of nitrate in leafy crops has been largely reported [39, 40]. The European Commission Regulation No 1258/2011 establishes the limits for nitrate content in vegetable productions based on species and growing conditions. Importantly, despite microgreens are young plantlets with low incidence of nitrate accumulation [17], the cultivation practices could influence the content of this compound [41, 42].

Overall, according to parameters prioritization discussed above and the algorithm calculation, radish and savoy cabbage are the most suitable microgreens genotypes to be used for cultivation in Space. Nevertheless, with the possibility to adjust the algorithm calculation based on productivity and phytonutrient profile criteria, further research deepening the effects of microgravity on human's metabolism, and the related countermeasures based on specific diets, can provide data and insights to optimize the selection of plant food species intended for cultivation in Space. Horticultural research, on the other hand, can provide strategies for maximizing the production of certain characteristics.

3.5. Conclusions

The selection method of microgreens developed in this study uses estimated scores based on prioritization of parameters and is preferred over a simple listing of plants since it has an argued basis for the given priorities. The method herein reported consists of two subsequent rankings elaborated using first literature data and, successively, using data obtained with cultivation tests specifically conducted for this study. The method also provides the possibility to adjust the algorithm calculation and can be repeated for further selection including new crops or new data. Furthermore, the approach of grouping the different parameters into different categories allow to easily assign higher or lower priorities related to other parameters included in the same group, instead of prioritizing parameters all together. So far, the

flexibility of this selection method provides potential adaptability for selecting new cultivars and/or including other criteria relevant to support astronauts' diet for different aims such as growth chambers on orbital platforms, long-duration travels, or extraterrestrial colonization.

References are listed in Appendix II of the bibliography section.

4. Defining growth requirements of microgreens in space cultivation via biomass production, morpho-anatomical and nutritional traits analysis

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4.1. Introduction

Although microgreens are young plantlets, harvested at the most till the second true leaf stage, several studies demonstrated that the modulation of environmental and agronomic parameters influenced their response in terms of yield and phytochemical [1–4]. Light is the source of energy for plant growth, and it is pivotal to many processes during plant life. In protected cultivation, the setting of an optimal light intensity is fundamental considering that light requirement may change among species and varieties [5]. A proper light modulation is also important for system design and efficiency in facilities dedicated to plant production under fully controlled environmental conditions, like vertical farming [6–8]. In the last decades, literature about plant-light interaction in controlled environment increased considerably, also by virtue of space related studies, after the invention of Light Emitting Diode (LED) technology [9–11]. The modulation of light intensity can have positive effects on plants nutraceutical values [12, 13], even when the intensity is high and could be perceived by plants as stress, inducing them to synthesize antioxidant compounds to mitigate ROS (reactive oxygen species) formation [14, 15]. Excess of light can also affect the photosystems' efficiency and inducing photo-inhibition mechanisms, other than causing the formation of a peculiar plant structure and changing the profiles and ratios of photosynthetic pigments [16]. It was recently demonstrated that it is possible to improve the antioxidant profile in several microgreen species by modulating the photoperiod, light intensity, and wavelength [17, 18]. Therefore, light modulation can be a powerful tool to control performances and quality of microgreens grown in space also considering interactions with other environmental variables. Among environmental control needs, VPD regulation is one of the utmost challenges

to face in indoor agriculture due to its dynamic interaction with crops in the soil-plant-atmosphere *continuum* [19–21]. Recent studies clearly indicate that VPD impacts all physiological and biochemical processes related to transpiration, also affecting the morpho-anatomical development of crops in terms of leaf lamina tissues organization, stomatal and veins dimensions and densities, which in turn modify crops physiology in terms of gas-exchange and water use [22, 23]. The influence of VPD on crops' nutraceutical compounds has been way less studied compared to other environmental factors, but some research has agreed that high VPD levels (1.7 - 3 kPa) enhanced the bioactive value of some crops (tomato, cucumber, lettuce), increasing their ascorbic acid, β -carotene and polyphenolic content [24–26]. However, to the best of our knowledge, there is no information about the effect of different VPDs on microgreens production and nutritional potential nor on the combined effects on these parameters exerted by concomitant variations in light intensity and VPD. Light and VPD are key aspects of the environmental control in Biorigenerative Life Support Systems (BLSSs) being crucial determinants of gas-exchange in leaves and particularly of transpiration. Moreover, the condensation of transpired water, driven by VPD and light conditions, is among the most demanding processes in controlled environment systems in terms on hardware and energy requirements, being even more difficult to control in space environment [27]. Moreover, as reported by Amitrano et al., (2020) [28] on mung bean sprouts, both VPD and light have proven to change biomass allocation, antioxidant production, and morpho-functional traits already at the very early stage of development (e.g. germinated seeds, sprouts). In this experiment, two species (*Brassica oleracea* var. *capitata* f. *sabauda* 'Vertus' and *Raphanus raphanistrum* subsp. *sativus* 'Saxa') were cultivated for microgreen production, testing the combined effect of two vapor pressure deficits (VPD: 0.14 and 1.71 kPa) and two light intensities (150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Microgreen morpho-anatomical development and biomass production were analysed to have an overview of their resource use-efficiency and growth. The innovative approach of the experiment is the strategy aiming to define system baselines, with an experimental protocol including interaction between light and VPD, with the target of achieving the nutritional

quality expected for each species. To do so, the nutritional and nutraceutical potential, in terms of ascorbic acid, phenols, anthocyanin, carotenoids, and carbohydrates, as well as the content of anti-nutritional compounds such as nitrate, sulfate and phosphate were analysed. The results may contribute to the definition of the scientific requirements for the design of a flight apparatus for microgreen production in space.

4.2. Materials and methods

4.2.1. Plant material and experimental design

Two microgreen cultivars belonging to two different species were used: i) cabbage, *Brassica oleracea* var. *capitata* f. *sabauda* ‘Vertus’ and ii) radish, *Raphanus raphanistrum* subsp. *sativus* ‘Saxa’. For simplicity through the text and tables ‘Saxa’ and ‘Vertus’ will be indicated as species. The two species were previously selected as reported in chapter 3 [29]. Seeds were purchased from a local provider and experiments were run at the Department of Agricultural Sciences of the University of Naples Federico II (Naples, IT), in two consecutive cycles. Both cycles were conducted in a two-shelves growth chamber (KBP-6395F, Termaks, Bergen, Norway), equipped with a LED lighting panel (K5 Series XL750, Kind LED, Santa Rosa, CA, USA), with an emission wavelength in a range of 400-700 nm (Figure 1). The two cultivation cycles were identical in terms of light intensity, quality, photoperiod and air temperature, except for the VPD levels. The first cycle was performed under a VPD of 0.14 kPa (defined as Low VPD, LV) and the second under a VPD of 1.71 kPa (defined as High VPD, HV) at ambient CO₂. The two VPDs were achieved keeping air temperature (T) at 24 ± 1°C during the day and 18 ± 2°C during the night and changing the RH accordingly. Both T and RH% were monitored inside the chamber using mini-sensors (Testo 174H, Testo, Germany), equipped with a data-logger collecting data every 15 minutes. 7 g of seeds for ‘Saxa’ and 6 g for ‘Vertus’ were sown on coconut fiber mats into plastic trays (width 14 cm x length 19 cm x depth 6 cm), and fertigated with a quarter-strength modified Hoagland solution (in mM: 2.0 nitrate, 0.25 sulfur, 0.20 phosphorus, 0.62 potassium, 0.75 calcium, 0.17 magnesium, 0.25 ammonium; in µM: 20 iron, 9 manganese, 0.3 copper, 1.6 zinc, 20 boron, 0.3 molybdenum), with an electrical conductivity (EC) of 0.4

$\pm 0.1 \text{ dSm}^{-1}$, and a pH of 6 ± 0.2 . Two light intensities were set in the two chamber shelves, the first of $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photosynthetic photon flux density (PPFD) and the second of $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$, with a red (R), green (G), blue (B) spectrum of R45: G10: B45 (in % of the total emission), and a 12 h photoperiod. Each treatment was replicated in 6 trays and daily rotation of the trays ensured a homogenous exposure to light and humidity. Microgreens were harvested at the appearance of the first two true leaves by cutting at the substrate level. Specifically, cultivation lasted 10 days in cabbage and 11 days in radish. At harvest, biometrical analyses were performed, and a sub-sample of 6 microgreens was stored in a fixative solution for anatomical analyses, while a second sub-sample of 10 g was rapidly frozen, lyophilized and stored at -80°C for biochemical analyses.



Figure.1 Different views (**A, B**) of the climatic growth chamber used for the two experiments on *Brassica oleracea* var. *capitata* f. *sabauda* ‘Vertus’ and radish, *Raphanus raphanistrum* subsp. *sativus* ‘Saxa’ microgreens under Low VPD (LV) and High VPD (HV), at two light intensities (150 and $300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

4.2.2. Colorimetric and biometric measurements

Just before harvesting, the color properties of the microgreen canopy were measured in three different positions of each tray by means of an 8 mm-aperture Minolta CR-400 Chroma Meter (Minolta Camera Co. Ltd., Osaka, Japan). The meter was calibrated with a standard white plate before

measurements. Leaf chromaticity was performed following the Commission Internationale de l'Eclairage and expressed as: greenness (a^*), yellowness (b^*) and lightness (L^*), used to calculate chroma ($\text{Chroma} = (a^{*2} + b^{*2})^{1/2}$) and Hue angle ($H^\circ = \arctan(b^*/a^*)$).

After cutting, the whole harvested material was weighed to determine the fresh weight, expressed in kg m^{-2} , 10 microgreens per tray were used to measure the microgreen length (ML). The dry weight (DW), expressed as g m^{-2} , was obtained after drying the remaining material in a forced-air oven at 65°C until constant weight. Dry matter content (DM%) was also calculated and expressed as a percentage of fresh weight.

4.2.3. Plant anatomical investigation

For the anatomical investigation each microgreen leaf stored in the F.A.A. fixative solution (40% formaldehyde, glacial acetic acid, 50% ethanol, 5:5:90 by volume) was dissected to keep a portion of the median region of the lamina (about $5 \times 5 \text{ mm}$), which was dehydrated in an ethanol series (from 50 to 95%), then embedded in the JB4 acrylic resin (Polysciences, USA). Thin cross sections ($5 \mu\text{m}$ thick) were cut using a rotary microtome (pfm medical, Bio-optical, Germany) and mounted on a microscope slide, then stained with 0.025% toluidine blue and analyzed using a transmitted light microscope (BX51, Olympus, Germany) equipped with a digital camera (Olympus EP50, Olympus, Germany). Digital images were collected and analyzed through the Olympus cellSens 3.2 software, and thickness was measured in: i) the upper epidermis (UET), ii) the lower epidermis (LET), iii) the palisade parenchyma (PT), iv) the spongy parenchyma (ST), and v) the total leaf lamina (TLT) and expressed in μm . Moreover, the intercellular spaces (IS) were measured as the percentage of the area occupied by space among cells over a given surface of parenchyma. All the measurements were taken in 3 positions along the lamina, avoiding veins and damaged areas if present.

4.2.4. Ascorbate, anthocyanins and polyphenols content

Asorbic acid (AsA), dehydroascorbic acid (DHAs) and total Ascorbic Acid (TAsA) content analysis was performed as reported in paragraph 3.2.11.

(chapter 3). Determination of Total anthocyanins (TAcy) and total phenolic content (TPoly) was achieved as reported in paragraph 3.2.10. (chapter 3).

4.2.5. Photosynthetic pigments and carbohydrates content

Photosynthetic pigments including neoxanthin (Neo), violaxanthin (Vio), lutein (Lut), chlorophylls (Chl a+b) and β -carotene (β -Car) were determined as reported in paragraph 3.2.9. (chapter 3). Measurement of carbohydrates content including starch, total soluble carbohydrates (TSolub) and total non-structural carbohydrates (TNsc) was performed as reported in paragraph 3.2.8. (chapter 3).

4.2.6. Anions analysis

Anions analysis (nitrate, sulfate and phosphate) was conducted as reported in paragraph 3.2.12. (chapter 3).

4.2.7. Statistical analyses

To test the influence of the three independent factors, i) Species, ii) VPD, iii) Light intensity, on the dependent variables, a three-way analysis of variance (ANOVA) was performed using the IBM SPSS Statistics software (SPSS, Chicago, IL, USA). When the interactions were significant, a one-way ANOVA was performed, separating the values according to Tukey test with a p value ≤ 0.05 . Moreover, a multivariate analysis was performed as principal component analysis (PCA) on all the calculated traits (biometric, anatomical, and biochemical traits), using the software Past3 (Natural History Museum, University of Oslo, Norway). Variables used as input for multivariate analysis were standardized to zero mean and unit variance

4.3. Results

4.3.1. Growth analysis

The main factors of this investigation (species, VPD and light) had a significant effect, alone and in interaction, on microgreen growth depending on the specific parameter (Table 1, Figure 2). Particularly, the species alone elicited a significant effect ($p \leq 0.001$) on the fresh weight (FW), dry weight

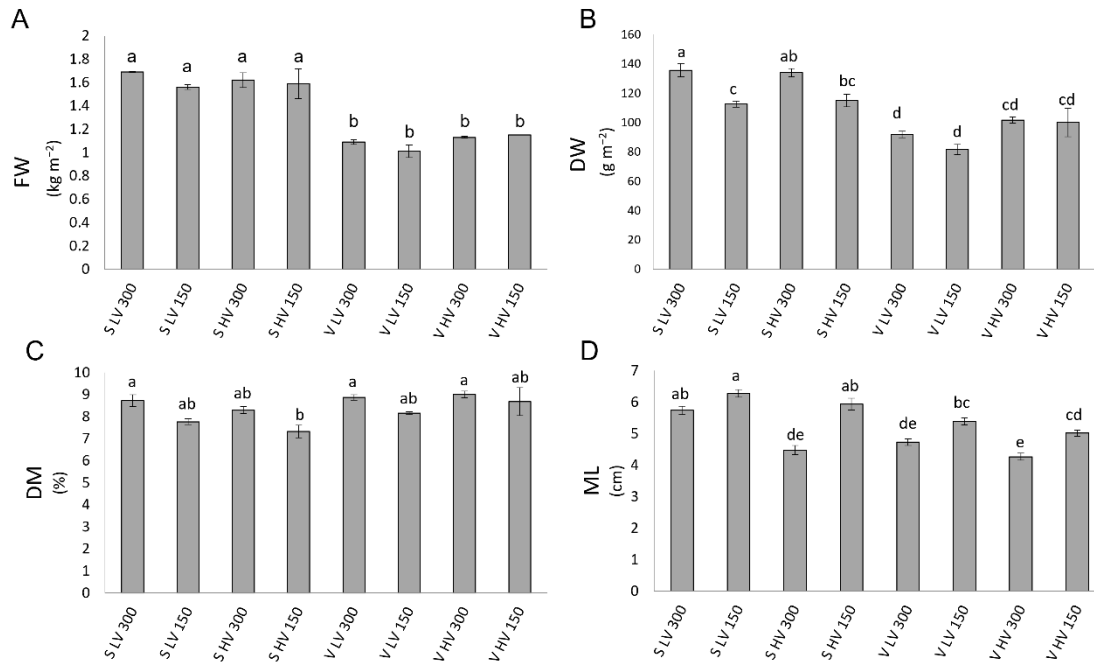
(DW) and microgreen length (ML), always showing higher values in ‘Saxa’ compared to ‘Vertus’. Differently, the VPD had a significant effect ($p \leq 0.001$) only on ML where at lowest values (LV) corresponded a higher elongation. Light elicited significant effects ($p \leq 0.01$) on DW and DM%, with increments of both parameters at 300 PPFD, and on ML ($p \leq 0.001$) with decreases in elongation at 300 PPFD. The interaction among the three factors (SxVxL) showed a significance of $p \leq 0.01$ for DW and ML, but it was non-significant for FW and DM%. Concerning the DW (Figure 2B), highest values were found in S LV 300 and S HV 300, in turn higher than S LV 150 and S HV 150, followed by V HV 150 and 300; lowest values were observed in V LV 150 and 300. For the ML (Figure 2D), highest values were found in S LV 150 and 300 and S HV 150, followed by V LV 150 and V HV 150, which were in turn higher than S HV 300 and V LV 300; lowest values were found in V HV 300.

Table 1. Analysis of variance and means comparison for growth-related traits of Fresh Weight (FW), Dry Weight (DW), Dry Matter Content (DM) and microgreen length (ML) of ‘Saxa’ and ‘Vertus’ microgreens grown under Low VPD (LV) and High VPD (HV), under two light intensities: 150 and 300 PPFD ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

	FW (kg m^{-2})	DW (g m^{-2})	DM (%)	ML (cm)
Species				
Saxa (S)	$1.36 \pm 0.43\text{a}$	$124 \pm 13.5\text{a}$	8.62 ± 0.73	$5.62 \pm 1.03\text{a}$
Vertus (V)	$1.09 \pm 0.11\text{b}$	$91.4 \pm 15.6\text{b}$	8.67 ± 0.82	$4.86 \pm 0.69\text{b}$
VPD				
Low (LV)	1.08 ± 0.32	102 ± 24.4	8.38 ± 0.58	$5.53 \pm 0.83\text{a}$
High (HV)	1.07 ± 0.29	113 ± 18.8	8.32 ± 1.04	$4.93 \pm 0.98\text{b}$
Light				
300 PPFD	1.35 ± 0.26	$113 \pm 24.2\text{a}$	$8.71 \pm 0.50\text{a}$	$4.82 \pm 0.86\text{b}$
150 PPFD	1.32 ± 0.37	$102 \pm 18.6\text{b}$	$7.98 \pm 0.95\text{b}$	$5.66 \pm 0.85\text{a}$
Significance				
Species	***	***	NS	***
VPD	NS	NS	NS	***
Light	NS	**	**	***
SxVxL	NS	**	*	**

NS, *, **, *** stands for non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.
Different letters correspond to significant differences according to Tukey test.

Figure 2. Fresh weight (FW, (A), dry weight (DW, (B), dry matter content (DM%, (C) and microgreens length (ML, (D), of ‘Saxa’ and ‘Vertus’ microgreens grown under Low VPD (LV) and High VPD (HV) at the two light intensities (150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). All data are expressed as mean $\pm$ standard errors. Different letters correspond to statistically significant differences according to Tukey test ($p \leq 0.05$).



4.3.2. Colorimetry analysis

Table 2 shows the results from colorimetry analysis. Here, the effect of the species alone was significant for all the parameters analyzed ($p \leq 0.001$), except for the Hue, always showing higher values in ‘Saxa’ compared to ‘Vertus’. Differently, the VPD had significant effects only on brightness (b^*) ($p \leq 0.05$) with increments in the parameter at HV and on Hue angle ($p \leq 0.001$) with decreases in the parameter at HV. The light intensity was significant for b^* , chroma and Hue ($p \leq 0.001$), and lightness (L^*) ($p \leq 0.01$). The highest light intensity (300 PPFD) determined higher values of b^* , L^* and chroma but lower Hue. Here, the interaction $S \times V \times L$ was significant, only for the Hue value ($p \leq 0.001$) which showed higher values in S HV 150 and V LV 150, followed by S LV at both 150 and 300, which were in turn higher than V HV 150 and V LV 300. Lowest values were found in S HV 300 and V HV 300.

Table 2. Analysis of variance and means comparison for colorimetry-related traits of greenness (a*), yellowness (b*), lightness (L*), Chroma and Hue angle of ‘Saxa’ and ‘Vertus’ microgreens grown under Low VPD (LV) and High VPD (HV) at the two light intensities (150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

	a*	b*	L*	Chroma	Hue
Species					
Saxa (S)	-13.3 $\pm$ 1.24a	20.68 $\pm$ 2.34a	36.6 $\pm$ 5.07a	24.6 $\pm$ 2.53a	123 $\pm$ 1.85
Vertus (V)	-11.7 $\pm$ 0.86b	18.25 $\pm$ 1.73b	34.5 $\pm$ 3.55b	21.7 $\pm$ 1.83b	123 $\pm$ 1.67
VPD					
Low (LV)	-12.3 $\pm$ 1.37	18.97 $\pm$ 2.46b	35.4 $\pm$ 4.76	22.7 $\pm$ 2.75	123 $\pm$ 1.56a
High (HV)	-12.4 $\pm$ 1.24	19.73 $\pm$ 2.12a	35.3 $\pm$ 3.89	23.4 $\pm$ 2.34	122 $\pm$ 1.87b
Light					
300 PPFD	-12.5 $\pm$ 1.43	20.15 $\pm$ 2.01a	36.2 $\pm$ 4.36a	23.7 $\pm$ 2.21a	122 $\pm$ 1.62b
150 PPFD	-12.3 $\pm$ 1.14	19.30 $\pm$ 2.35b	34.7 $\pm$ 4.33b	22.4 $\pm$ 2.73b	123 $\pm$ 1.53a
Interaction					
S LV 300	-13.5 $\pm$ 0.14	20.9 $\pm$ 0.35	38.7 $\pm$ 0.56	24.9 $\pm$ 0.36	123 $\pm$ 0.29ab
S LV 150	-13.2 $\pm$ 0.27	20.2 $\pm$ 0.47	35.0 $\pm$ 1.09	24.2 $\pm$ 0.53	123 $\pm$ 0.26ab
S HV 300	-13.1 $\pm$ 0.13	21.5 $\pm$ 0.29	37.8 $\pm$ 0.69	25.2 $\pm$ 0.29	121 $\pm$ 0.27d
S HV 150	-13.5 $\pm$ 0.22	20.1 $\pm$ 0.37	35.1 $\pm$ 0.71	24.3 $\pm$ 0.41	124 $\pm$ 0.26a
V LV 300	-11.8 $\pm$ 0.14	18.9 $\pm$ 0.25	34.3 $\pm$ 0.74	22.3 $\pm$ 0.28	122 $\pm$ 0.20cd
V LV 150	-11.6 $\pm$ 0.16	17.4 $\pm$ 0.26	34.7 $\pm$ 0.57	20.9 $\pm$ 0.30	124 $\pm$ 0.19a
V HV 300	-11.7 $\pm$ 0.13	19.4 $\pm$ 0.23	34.3 $\pm$ 0.55	22.6 $\pm$ 0.25	121 $\pm$ 0.22d
V HV 150	-11.6 $\pm$ 0.12	18.1 $\pm$ 0.27	34.3 $\pm$ 0.45	21.5 $\pm$ 0.27	123 $\pm$ 0.28bc
Significance					
Species	***	***	***	***	NS
VPD	NS	*	NS	NS	***
Light	NS	***	**	***	***
SxVxL	NS	NS	NS	NS	***

NS, *, **, *** stands for non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.
Different letters correspond to significant differences according to Tukey test.

4.3.3. Anatomical analysis

All the treatments allowed the formation of an ordinary mesophyll structure (Figure 3), however there were significant differences in the parameters analyzed among treatments (Table 3). The main factors had a significant effect, alone and in interaction, depending on the specific parameter. The Species elicited significant differences except for the lower epidermis thickness (LET), always with higher values in ‘Saxa’ but with a different degree of significance: p value ≤ 0.001 for palisade thickness (PT), spongy thickness (ST), and intercellular space percentage (IS), and $p \leq 0.01$ for the upper epidermis thickness (UET) and total lamina thickness (TLT). The VPD significantly influenced PT ($p \leq 0.01$), ST ($p \leq 0.05$), TLT ($p \leq 0.001$) with

increments at LV and IS ($p \leq 0.001$) with reduction at LV. Differently, light intensity significantly influenced PT ($p \leq 0.01$) with increments at 300 PPFD and TLT ($p \leq 0.01$), ST, and IS ($p \leq 0.001$) with increments at 150 PPFD. The interaction among the main factors (SxVxL) significantly influenced all parameters except for LET, with UET and PT ($p \leq 0.05$), ST and IS ($p \leq 0.01$) and TLT ($p \leq 0.001$). ‘Saxa’ developed thicker leaves with more intercellular spaces. Overall, microgreens at LV resulted thicker with less IS% compared to HV and microgreens at 150 PPFD enhanced their total thickness, the thickness of the spongy parenchyma and IS % compared to microgreens grown at 300 PPFD, which however had a thicker palisade tissue.

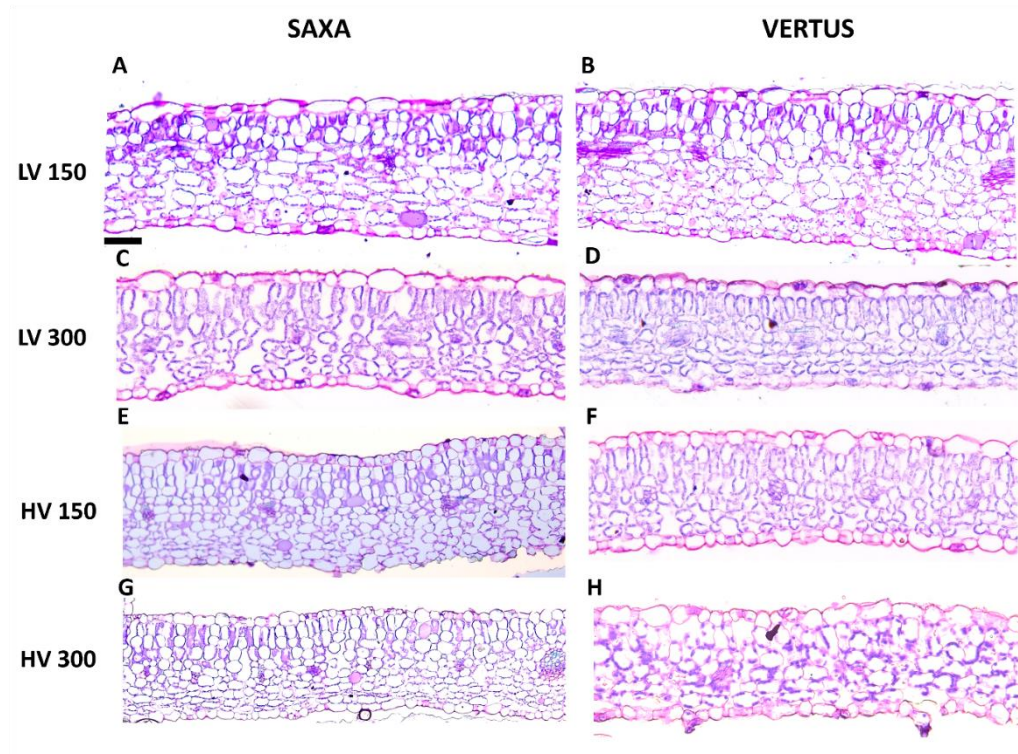


Figure 3. Light microscopy views of leaf lamina cross sections randomly chosen among the many shots of ‘Saxa’ (A, C, E, G) and ‘Vertus’ (B, D, F, H) microgreens under low VPD (LV, (A–D) and high VPD (HV, (E–H) at the two light intensities (150 PPFD, (A, B, E, F) and 300 PPFD, (C, D, G, H). Images are at the same magnification, scale bar = 50 μ m.

Table 3. Analysis of variance and means comparison for anatomical-related traits of Upper Epidermis Thickness (UET), Palisade parenchyma Thickness (PT), Spongy parenchyma Thickness (ST), Lower Epidermis Thickness (LET), Total Lamina Thickness (TLT) and the percentage of Intercellular Spaces (IS) of ‘Saxa’ and ‘Vertus’ microgreens grown under Low VPD (LV) and High VPD (HV) at the two light intensities (150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

	UET (μm)	PT (μm)	ST (μm)	LET (μm)	TLT (μm)	IS (%)
Species						
Saxa (S)	23.1 $\pm$ 5.14a	110 $\pm$ 23.4a	187 $\pm$ 55.2a	20.2 $\pm$ 5.40	340 $\pm$ 63.4a	37.6 $\pm$ 0.71a
Vertus (V)	21.1 $\pm$ 5.12b	97.3 $\pm$ 20.0b	157 $\pm$ 36.8b	19.8 $\pm$ 4.30	295 $\pm$ 41.5b	28.4 $\pm$ 0.62b
VPD						
Low (LV)	22.2 $\pm$ 5.11	107 $\pm$ 23.6a	180 $\pm$ 61.1a	20.6 $\pm$ 5.56a	331 $\pm$ 66.1a	30.4 $\pm$ 1.14b
High (HV)	22.1 $\pm$ 5.32	101 $\pm$ 21.5b	165 $\pm$ 34.8b	19.5 $\pm$ 4.15b	308 $\pm$ 48.4b	35.8 $\pm$ 1.12a
Light						
300 PPFD	22.2 $\pm$ 5.76	120 $\pm$ 25.3a	150 $\pm$ 36.8b	19.8 $\pm$ 5.48	209 $\pm$ 10.7b	21.6 $\pm$ 0.53b
150 PPFD	22.1 $\pm$ 4.76	100 $\pm$ 19.8b	190 $\pm$ 51.4a	20.2 $\pm$ 4.39	301 $\pm$ 53.8a	41.3 $\pm$ 0.53a
Interaction						
S LV 300	24.3 $\pm$ 1.09a	120 $\pm$ 4.01a	162 $\pm$ 5.58b	21.5 $\pm$ 1.41	328 $\pm$ 8.67b	24.4 $\pm$ 0.18f
S LV 150	21.1 $\pm$ 0.67ab	107 $\pm$ 3.83abc	243 $\pm$ 11.2a	19.8 $\pm$ 0.90	391 $\pm$ 12.5a	43.8 $\pm$ 0.32b
S HV 300	22.8 $\pm$ 0.89a	104 $\pm$ 5.61bcd	159 $\pm$ 6.32bc	18.9 $\pm$ 0.82	305 $\pm$ 10.9b	27.5 $\pm$ 0.56e
S HV 150	24.4 $\pm$ 0.91a	111 $\pm$ 2.41abc	172 $\pm$ 3.11b	20.6 $\pm$ 0.69	199 $\pm$ 3.93c	49.6 $\pm$ 0.13a
V LV 300	22.8 $\pm$ 1.19a	118 $\pm$ 3.21ab	128 $\pm$ 4.13c	21.2 $\pm$ 0.88	291 $\pm$ 5.67bc	14.4 $\pm$ 0.18h
V LV 150	20.9 $\pm$ 0.77ab	84.5 $\pm$ 2.44e	166 $\pm$ 4.23b	20.1 $\pm$ 0.90	292 $\pm$ 5.36bc	34.5 $\pm$ 0.22d
V HV 300	18.1 $\pm$ 0.92b	90.6 $\pm$ 3.48de	150 $\pm$ 10.2bc	17.8 $\pm$ 0.91	277 $\pm$ 12.9bc	19.5 $\pm$ 0.15g
V HV 150	21.9 $\pm$ 0.75ab	95.5 $\pm$ 2.65cde	174 $\pm$ 5.14b	20.1 $\pm$ 0.54	311 $\pm$ 5.48b	39.4 $\pm$ 0.17c
Significance						
Species	**	***	***	NS	**	***
VPD	NS	**	*	*	***	***
Light	NS	**	***	NS	**	***
SxVxL	*	*	**	NS	***	**

NS, *, **, *** stands for non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.
Different letters correspond to significant differences according to Tukey test.

4.3.4. Ascorbate, anthocyanins and phenolic content

The effect of the three main factors (species, light intensity and VPD) alone and in interaction on dehydroascorbic acid (DHAs), ascorbic acid (Asa), total ascorbic acid (TAsA), total anthocyanins (TAcy), and total phenolic content (TPoly) are reported in Table 4. ‘Vertus’ microgreens showed significantly higher values than ‘Saxa’ for all variables except for TAcy. The effect of VPD was significant only in DHAs and AsA. The LV treatment increased the content of DHAs and decreased that of AsA. The two light intensity levels differentially modulated the accumulation of AsA ($p \leq 0.01$) and Tot AsA ($p \leq 0.001$), always with higher content at 300 PPFD than at 150 PPFD. The interaction among the main factors (SxVxL) only influenced the DHAs content, determining the highest value in ‘Vertus’ under 300 PPFD and Low

VPD (V LV 300) ($p \leq 0.05$). The TAcy content was not significantly influenced by any of the experimental factors.

Table 4. Analysis of variance and means comparison for dehydro-ascorbate (DHAs), ascorbate (AsA), total ascorbate (TAsA), total anthocyanins (TAcy) and total polyphenols (TPoly) content of ‘Saxa’ and ‘Vertus’ microgreen grown under Low VPD (LV) and High VPD (HV) at the two light intensities: 150 and 300 PPFD ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

	DHAs	AsA	TAsA	TAcy	TPoly
	(mg 100g FW ⁻¹)	(mg 100g FW ⁻¹)	(mg 100g FW ⁻¹)	(mg 100g FW ⁻¹)	(mg 100g FW ⁻¹)
Species					
Saxa (S)	16.6 ± 2.13b	13.1 ± 1.81b	29.7 ± 2.48b	6.04 ± 0.66	145 ± 8.57b
Vertus (V)	37.2 ± 2.76a	24.5 ± 3.06a	61.6 ± 2.06a	5.59 ± 0.33	193 ± 8.94a
VPD					
Low (LV)	31.4 ± 3.9a	15.7 ± 2.09b	47.1 ± 4.97	6.46 ± 0.59	172 ± 10.26
High (HV)	22.4 ± 3.53b	21.8 ± 3.53a	44.2 ± 5.62	5.21 ± 0.38	166 ± 12.28
Light					
300 PPFD	27.8 ± 3.5a	23.1 ± 3.46a	50.9 ± 5.01a	6.37 ± 0.32	175 ± 10.52
150 PPFD	25.9 ± 4.36a	14.4 ± 1.77b	40.4 ± 5.14b	5.30 ± 0.64	163 ± 11.82
Interaction					
S LV 300	21.7 ± 1.27bc	16.5 ± 5.12	38.3 ± 4.77	6.35 ± 0.26	151 ± 2.2
S LV 150	18.6 ± 5.54c	8.95 ± 2.35	27.6 ± 3.18	7.32 ± 2.45	162 ± 34.2
S HV 300	18.2 ± 1.76c	13.6 ± 1.47	31.8 ± 3.12	6.68 ± 1.08	140 ± 6.28
S HV 150	8.02 ± 3.36c	13.1 ± 4.88	21.2 ± 3.98	4.41 ± 0.12	127 ± 7.45
V LV 300	46.4 ± 3.97a	22.4 ± 3.87	68.7 ± 1.02	6.95 ± 0.73	185 ± 11.6
V LV 150	38.9 ± 4.36ab	14.9 ± 2.03	53.9 ± 4.75	5.23 ± 0.09	190 ± 22.1
V HV 300	25.3 ± 2.92bc	40.0 ± 3.21	64.9 ± 0.21	5.54 ± 0.25	225 ± 5.50
V HV 150	38.3 ± 3.06ab	20.6 ± 0.91	58.8 ± 2.46	4.22 ± 0.06	171 ± 17.5
Significance					
Species	***	***	***	NS	***
VPD	**	*	NS	NS	NS
Light	NS	**	***	NS	NS
S x V x L	*	NS	NS	NS	NS

NS, *, **, *** stands for non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.
Different letters correspond to significant differences according to Tukey test.

4.3.5. Photosynthetic pigment and carbohydrates content

The pigments accumulation was mainly driven by the species, determining significant differences between ‘Saxa’ and ‘Vertus’. Indeed, ‘Vertus’ has a higher content of neoxanthin ($p \leq 0.05$), lutein ($p \leq 0.01$), chlorophylls a+b ($p \leq 0.05$) and β -carotene ($p \leq 0.01$) compared to ‘Saxa’ (Table 5). A different level of accumulation of pigments was found for neoxanthin ($p \leq 0.001$),

lutein ($p \leq 0.05$) and β -carotene ($p \leq 0.05$) in response to the VPD, with a higher content of these compounds at LV. Conversely, the different light intensities as well as the interaction among factors did not determine significant variations for any of the pigments analyzed. Concerning the starch, total soluble non-structural carbohydrates (TSolub), and total non-structural carbohydrates (TNsc) higher values were found in ‘Vertus’ microgreens ($p \leq 0.001$) (Table 5). The VPD was statistically significant only in starch ($p \leq 0.05$), with higher values under LV, while the light intensity resulted significant in all the parameters ($p \leq 0.001$), always with increments at 300 PPFD. The three factors interaction resulted to influence only the accumulation of starch ($p \leq 0.01$), that was two-fold higher in the combination V HV 300 compared to V LV 300 and over 10 times greater compared to the averages values of the other experimental conditions.

Table 5. Analysis of variance and means comparison for Neoxanthin (Neo), Violaxanthin (Vio), Lutein (Lut), Chlorophylls (Chl a+b), β -caroten (β -car), Starch, total soluble sugars (TSolub), Total Non-Structural carbohydrates (TNsc) content of ‘Saxa’ and ‘Vertus’ microgreens grown under Low VPD (LV) and High VPD (HV) at the two light intensities:150 and 300 PPFD ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

	Neo (mg 100g FW ⁻¹)	Vio (mg 100g FW ⁻¹)	Lut (mg 100g FW ⁻¹)	Chl a+b (mg 100g FW ⁻¹)	β -Car (mg 100g FW ⁻¹)	Starch (mg 100g FW ⁻¹)	TSolub (mg 100g FW ⁻¹)	TNsc (mg 100g FW ⁻¹)
Species								
Saxa (S)	1.40 $\pm$ 0.10b	27.5 $\pm$ 2.59	4.98 $\pm$ 0.35b	64.3 $\pm$ 5.26b	3.64 $\pm$ 0.25b	54.7 $\pm$ 9.28b	463.9 $\pm$ 46.7 b	518.6 $\pm$ 54.9b
Vertus (V)	1.69 $\pm$ 0.134a	22.9 $\pm$ 1.61	6.60 $\pm$ 0.37a	82.0 $\pm$ 5.14a	4.60 $\pm$ 0.22a	258 $\pm$ 72.3a	1127 $\pm$ 70.7a	1386.3 $\pm$ 128.9a
VPD								
Low (LV)	1.80 $\pm$ 0.12a	27.9 $\pm$ 2.54	6.27 $\pm$ 0.44a	79.0 $\pm$ 6.21	4.44 $\pm$ 0.31a	129 $\pm$ 35.6a	831.9 $\pm$ 111.7	960.8 $\pm$ 139.8
High (HV)	1.28 $\pm$ 0.83b	22.5 $\pm$ 1.55	5.32 $\pm$ 0.37b	67.3 $\pm$ 4.85	3.80 $\pm$ 0.20b	184 $\pm$ 76.1b	759.7 $\pm$ 120.3	944.0 $\pm$ 185.2
Light								
300 PPFD	1.50 $\pm$ 0.11	25.2 $\pm$ 1.0	5.76 $\pm$ 0.39	70.9 $\pm$ 4.9	4.05 $\pm$ 0.25	271 $\pm$ 68.9a	954.6 $\pm$ 116.4a	1226.3 $\pm$ 174.2a
150 PPFD	1.58 $\pm$ 0.14	25.2 $\pm$ 3.04	5.82 $\pm$ 0.47	75.4 $\pm$ 6.54	4.20 $\pm$ 0.30	41.6 $\pm$ 6.44b	637.0 $\pm$ 95.3b	678.7 $\pm$ 99.4b
Interaction								
S LV 300	1.49 $\pm$ 0.15	25.1 $\pm$ 3.25	4.84 $\pm$ 0.61	58.1 $\pm$ 8.35	3.39 $\pm$ 0.33	94.4 $\pm$ 4.81c	628.4 $\pm$ 46.2	722.8 $\pm$ 41.5
S LV 150	1.67 $\pm$ 0.32	35.3 $\pm$ 9.43	6.12 $\pm$ 1.21	81.3 $\pm$ 17.91	4.47 $\pm$ 0.89	42.8 $\pm$ 10.9c	402.1 $\pm$ 83.7	444.9 $\pm$ 90.7
S HV 300	1.03 $\pm$ 0.17	24.6 $\pm$ 1.07	4.34 $\pm$ 0.03	54.4 $\pm$ 1.87	3.14 $\pm$ 0.17	64.2 $\pm$ 11.4c	533.5 $\pm$ 56.6	597.7 $\pm$ 67.9
S HV 150	1.4 $\pm$ 0.07	24.7 $\pm$ 2.62	4.64 $\pm$ 0.12	63.3 $\pm$ 1.09	3.57 $\pm$ 0.1	17.3 $\pm$ 4.07c	291.9 $\pm$ 54.5	309.2 $\pm$ 57.0
V LV 300	1.93 $\pm$ 0.05	26.3 $\pm$ 1.21	6.65 $\pm$ 0.33	82.2 $\pm$ 5.03	4.80 $\pm$ 0.02	319 $\pm$ 53.6b	1355.6 $\pm$ 87.79	1674.9 $\pm$ 34.4
V LV 150	2.12 $\pm$ 0.28	25.0 $\pm$ 2.26	7.47 $\pm$ 0.8	94.4 $\pm$ 9.48	5.11 $\pm$ 0.63	59.3 $\pm$ 5.65c	941.5 $\pm$ 42.1	1000.7 $\pm$ 42.6
V HV 300	1.57 $\pm$ 0.13	24.7 $\pm$ 2.83	7.23 $\pm$ 0.29	88.9 $\pm$ 2.23	4.85 $\pm$ 0.14	608 $\pm$ 79.9a	1300.9 $\pm$ 52.4	1909.5 $\pm$ 45.6
V HV 150	1.13 $\pm$ 0.12	15.8 $\pm$ 2.81	5.06 $\pm$ 0.67	62.6 $\pm$ 3.19	3.63 $\pm$ 0.24	47.2 $\pm$ 16.6c	912.7 $\pm$ 128.2	959.9 $\pm$ 130.6
Significance								

Species	*	NS	**	*	**	***	***	***
VPD	***	NS	*	NS	*	*	NS	NS
Light	NS	NS	NS	NS	NS	***	***	***
S x V x L	NS	NS	NS	NS	NS	**	NS	NS

NS,*,**,*** stands for non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.

Different letters correspond to significant differences according to Tukey test.

4.3.6. Anions content

The anions analysis revealed that the two species differently accumulated nitrate, sulfate, and phosphate (Table 6), with ‘Saxa’ reaching a content of nitrate ($p \leq 0.001$) and phosphate ($p \leq 0.01$) about two-fold higher compared to ‘Vertus’. On the other hand, ‘Vertus’ accumulated more sulfate ($p \leq 0.001$). The effect of VPD resulted statistically significant only for the accumulation of nitrate ($p \leq 0.05$) where the LV led to 52% increment compared to HV. Differently, the light intensity alone was never significant as well as the interaction among factors.

Table 6. Analysis of variance and means comparison for nitrate, sulfate, phosphate content of ‘Saxa’ and ‘Vertus’ microgreens grown under Low VPD (LV) and High VPD (HV) at the two light intensities (150 and 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

	Nitrate (mg 100g FW ⁻¹)	Sulfate (mg 100g FW ⁻¹)	Phosphate (mg 100g FW ⁻¹)
Species			
Saxa (S)	74.9 $\pm$ 6.84a	12.4 $\pm$ 1.19b	32.7 $\pm$ 4.5a
Vertus (V)	36.1 $\pm$ 8.74b	23.6 $\pm$ 1.31a	17.9 $\pm$ 2.13b
VPD			
Low (LV)	66.5 $\pm$ 9.96a	18.4 $\pm$ 2.38	28.2 $\pm$ 5.08
High (HV)	43.7 $\pm$ 8.71b	17.6 $\pm$ 1.79	22.4 $\pm$ 2.74
Light			
300 PPFD	50.3 $\pm$ 8.52	19.3 $\pm$ 2.1	21.0 $\pm$ 2.47
150 PPFD	58.6 $\pm$ 10.8	16.8 $\pm$ 2.05	29.6 $\pm$ 5.04
Interaction			
S LV 300	58.7 $\pm$ 8.49abc	11.9 $\pm$ 1.48	25.5 $\pm$ 5.78
S LV 150	94.6 $\pm$ 18.3a	11.3 $\pm$ 3.19	48.5 $\pm$ 13.2
S HV 300	57.5 $\pm$ 2.64abc	14.8 $\pm$ 2.85	21.6 $\pm$ 3.17
S HV 150	83.5 $\pm$ 4.90ab	11.5 $\pm$ 2.59	35.4 $\pm$ 3.16
V LV 300	66.6 $\pm$ 26.1abc	28.2 $\pm$ 0.62	17.2 $\pm$ 7.78
V LV 150	43.7 $\pm$ 10.2abc	22.3 $\pm$ 3.16	21.5 $\pm$ 2.3
V HV 300	21.3 $\pm$ 4.03bc	22.0 $\pm$ 2.28	19.8 $\pm$ 3.54
V HV 150	12.6 $\pm$ 0.48c	22.1 $\pm$ 2.81	12.9 $\pm$ 0.97
Significance			

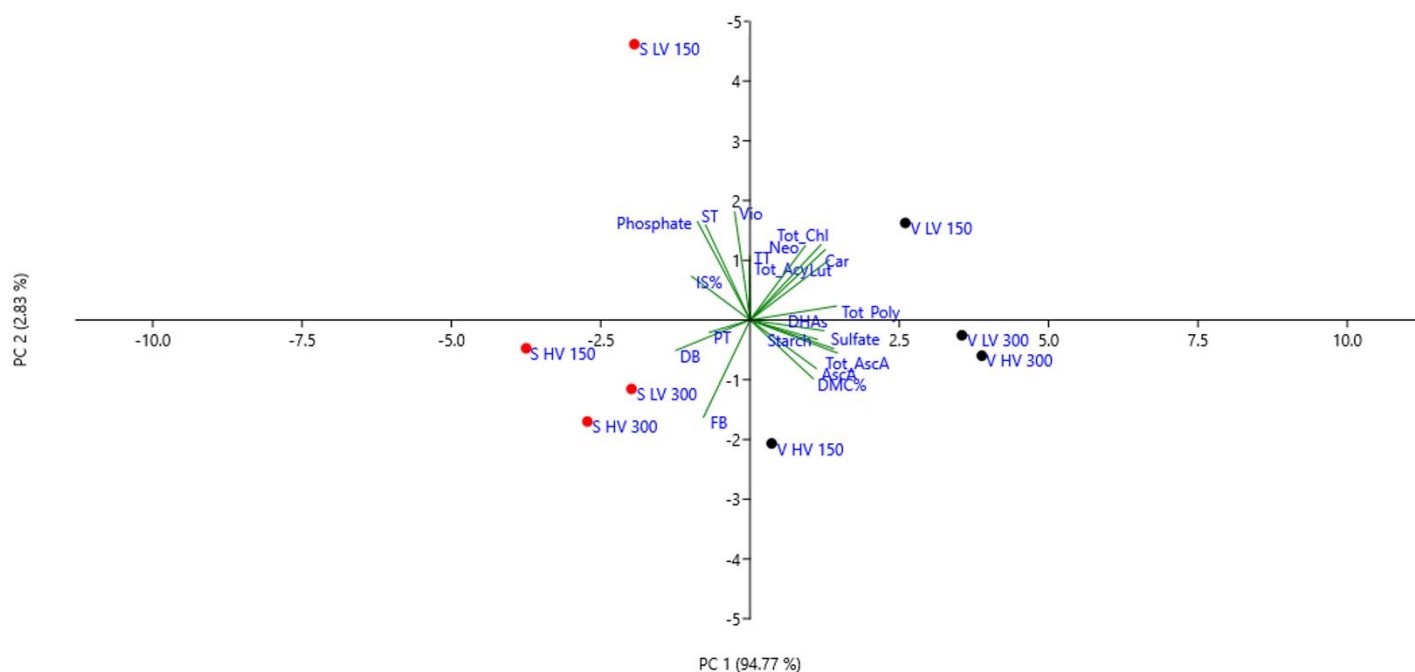
Species	***	***	**
VPD	*	NS	NS
Light	NS	NS	NS
S x V x L	*	NS	NS

NS,*,**,*** stands for non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.
Different letters correspond to significant differences according to Tukey test.

4.3.7. Principal component analysis

The PCA scatter plot reported in Figure 4 shows that the first two components explained a 97.6% of the total variance, with PC1 explaining most of the total variance (94.8%) and PC2 the remaining 2.8%. The first component (PC1) was highly positively correlated with all the variables, except for DW, FW and PT. It is interesting to notice that the scatter plot clearly separated the two species into separated groups; however, both ‘Saxa’ and ‘Vertus’ microgreens at LV and 150 PPFD differently separated creating other two sub-groups.

Figure 4. Principal component analysis (PCA) loading plot and scores of biometric, anatomical, and biochemical traits of ‘Saxa’ and ‘Vertus’ microgreens under Low VPD (LV) and High VPD (HV) at the two light intensities (150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).



4.4. Discussion

So far, different species and cultivars have been tested as possible candidates for food production in space according to some specific requirements, including their nutritional values, resource-use efficiency and suitability to grow in a closed controlled environment [30]. Therefore, it is fundamental to investigate the interaction among environmental variables on microgreens growth, development, and phytochemical production.

4.4.1. Species-related differences

In the present study, the two species tested, *B. oleracea* ‘Vertus’ and *R. raphanistrum* ‘Saxa’, although both belonging to the *Brassicaceae* family, showed a very different growth, morpho-anatomical development, and bioactive compounds level. ‘Saxa’, which was harvested first (10 days after sowing vs. 11 for ‘Vertus’), reached the highest fresh and dry weight as well as the highest elongation (Table 1, Figure 2). This could be explained by the anatomical analysis (Table 3), which highlighted a thicker mesophyll in ‘Saxa’ compared to ‘Vertus’, due to a higher palisade and spongy thickness and also due to a higher percentage of intercellular spaces. Leaf anatomical structure has a fundamental relationship with the plant photosynthetic performance, and usually a thicker and more dense tissue facilitates the transfer of gas and water among mesophyll cells [31]. Moreover, palisade cells are also known to concentrate more chloroplasts compared to spongy cells [32], so one would expect ‘Saxa’ to have a higher photosynthetic pigment content. In our study, however, the content of both chlorophylls and carotenoids, as well as neoxanthin and lutein, was higher in ‘Vertus’ (Table 5). This is probably ascribed to the reduced palisade thickness of ‘Vertus’ leaves, which may force the plant to synthesize more pigments in order to intercept more light, thus increasing the photosynthetic efficiency. ‘Vertus’ also presented the highest content of dehydroascorbate, ascorbate and polyphenols compared to ‘Saxa’ (Table 2). Species-specific differences can play a significant role in determining the content of ascorbate in different plants [24]. Ascorbate (or vitamin C) is a well-known antioxidant and radical scavenger and is an essential dietary nutrient in humans. Its role in human body involves the enhancement of the immune system [33], prevention of

bone density loss [34] and a function as a cofactor in the biosynthesis of collagen, carnitine, and neurotransmitters [35]. Recently, vitamin C has also been reported to have a role as radioprotector [36–38], thus in a space mission context, can help neutralizing free radicals and protecting cells from damage induced by ionizing radiation [39]. Another difference between the two species concerned the accumulation of minerals (Table 6). ‘Saxa’ was found to be a higher accumulator of nitrates and phosphate, while ‘Vertus’ accumulated more sulfate. It is noteworthy that the nitrate content in both plant species was well below the maximum levels allowed in food products by the European Commission regulation No 1258/2011. Therefore, the accumulation of these compounds for the two microgreen species under the above-reported growing conditions does not represent a health concern [40].

4.4.2. Light intensity influence on microgreen development and phytochemical content

Different studies have shown that light intensity has a significant impact on the antioxidant potential of microgreens in terms of anthocyanins and polyphenols content, usually with increments in light corresponding to increments in the phytochemical profile [1, 41]. Here we found that the highest light intensity (300 PPFD) was associated with a higher ascorbate content (Table 4), consistently with previous research demonstrating a progressive ascorbate accumulation in microgreens, proportionally to the light level [42]. However, in our study we did not find a significant variation in the content of anthocyanin or polyphenols in microgreen grown under different light irradiances, nor in the carotenoids and other photosynthetic pigment content. Secondary phytochemicals, such as polyphenols and anthocyanins, are highly desirable in astronaut’s diet since they have several positive impacts on health, including cardiovascular health, neuroprotective function, diabetes management, anti-inflammatory and anti-cancer activity [43] and could help counteract the detrimental effect of space environment [39]. Carotenoids are also essential for human nutrition; incorporating carotenoids into the food supply for space missions could have multiple benefits, including antioxidant protection [44], immune system support [45], and eye health maintenance [46]. The fact that these compounds

(anthocyanins, polyphenols and carotenoids), which are also desirable for nutritional purposes, do not increase with light intensity represents an added value as there is always a trade-off between light intensity and electricity consumption in controlled environment agriculture [47], especially in the sight of space outposts oriented towards eliminating or minimizing superfluous wastes [48]. Moreover, in our study, microgreens at 150 PPFD grew taller than those at 300 PPFD, probably because of a stretching mechanism, typical of plants grown under low light intensity, which lengthen towards the light source in order to enhance their light harvesting capacity [49].

It is worth noting that the lower light intensity did not impact the total fresh weight of microgreens which was still comparable to that produced at 300 PPFD (Table 1). The dry weight, however, as well as the dry matter content, increased under the high light intensity. This is a common response of plants grown under high irradiance and is also consistent with the enhanced thickness of the palisade tissue under 300 PPFD (Table 3). The increase in palisade tissue thickness goes in parallel with the development of more chloroplasts, thus with increments in the photosynthetic rate [50]. Besides, being the site of photosynthesis, chloroplasts also represent a site for starch storage in the form of grains [51]. Indeed, in our study at 300 PPFD, together with the increments in palisade tissue, the total NSC levels increased (Table 5). The two species showed to be accumulators of soluble carbohydrates, since soluble sugars (glucose, fructose, sucrose) dominated the total non-structural carbohydrate content. Starch was always much lower than soluble sugars, but its content varied more in response to the environmental variables tested. In almost all photosynthetic organisms', starch is one important products of photosynthesis and the storage carbohydrates in leaves, allowing the accumulation of carbon and energy without affecting the osmolyte concentration of the cell [52]. Starch is accumulated in chloroplast during the day and degraded at night to support plant needs, with a complex pathway requiring the coordinated actions of several enzymes [53]. Even though we cannot go deeper into the starch kinetics, here, we can hypothesize that, as for other species like *Arabidopsis*, *Lemna*, and mung bean [28, 54, 55], under high irradiance

microgreens allocate photosynthates to starch, as a response to an imbalance between photosynthate production and use. This shows that plant metabolism as early as 10-11 days after germination was already fully dominated by photosynthetic production of assimilates, highlighting the importance of the environmental control on gas exchanges as a tool to govern plant performances even in microgreens production.

4.4.3. VPD levels influence on microgreen development and phytochemical content

The air VPD is the primary factor affecting the transpiration rate of crops in controlled environment, thus affecting plants' photosynthetic rate and the whole development of the leaf lamina [56, 57]. It has been demonstrated that plants grown under high VPD levels reduce the photosynthesis and the biomass production and are often characterized by a lower leaf thickness and a reduction of cell enlargement [58]. Our results partially agree with this statement, since HV microgreens did not show significant difference in growth in term of fresh and dry weight, only resulting shorter than LV microgreens (Table 1, Figure 2). However, HV microgreens showed a reduced tissue thickness and a higher percentage of intercellular spaces, compared to LV (Table 3). As mentioned before, having more intercellular spaces can represent a disadvantage for HV microgreens, already subjected to an enhanced transpiration due to the dryer air (high VPD). Indeed, the wide presence of intercellular spaces reduces the cell connection, decreasing the velocity of both gas and water flows, thus imposing a high mesophyll resistance [57]. In accordance with our results, in a previous study on *V. radiata* sprouts subjected to different levels of relative humidity, sprouts at low RH (high VPD) evolved morpho-anatomical traits which increased the resistance to water flows and did not allow them to elude water loss and allocate biomass [28]. Here, HV condition did not negatively impact microgreen biomass accumulation, but the leaf anatomy developed by HV microgreens characterized by a higher resistance of gas and water flows, would probably lower the photosynthetic and hydraulic efficiency in future plant growth phases.

Furthermore, we observed that HV microgreens had a greater content of ascorbate. To our knowledge there is no previous research on the effect of VPD on microgreens. Our results could be compared with findings from Amitrano et al., (2021) [40] which reported an increase in total ascorbate content in lettuce grown at 1.76 kPa in comparison to 0.69 kPa, due to the mild stress given by the high VPD conditions. Usually, when plants are subjected to mild stresses caused by high temperature, salinity, VPD or drought, they tend to synthesize more antioxidants with scavenger activity, trying to react to the oxidative stress. The production of antioxidants is effective above a threshold depending on the type and intensity of the stress. Exceeded the threshold, plants are not able to react anymore with their biochemical activities and start to perish [59].

Here we also found that the high VPD level reduced the content of nitrates in both species. This effect is useful in the sight of cultivating microgreens for nutritional purposes. Usually, lower content of nitrates is also associated with the qualitative parameters with respect to the yellowish of the leaves, and in particular with enhanced L^* , b^* and chroma and reduced hue [60]. In this study, there were no significant differences between HV and LV microgreens concerning L^* and chroma; however, microgreens at HV showed an enhanced yellowness exemplified by the parameter b^* and by a reduced chroma. For edible products the analysis of colour is an important tool since consumers are easily influenced by the qualitative aspect of vegetables [61]. Furthermore, several studies have found an inverse relationship between b^* and dry weight, using the b^* parameter as a proxy for stress level [62, 63]. LV microgreens with a reduced yellowness in both species achieved a better visual quality, they also maintained high levels of polyphenols, vitamin C and anthocyanins and enhanced the accumulation of pigments such as neoxanthin, lutein and β -carotene, compared to HV (Table 5). These pigments, besides having important nutritional functions, are used as proxies for the photosynthetic performance of the plant. Carotenoids are primarily involved in the protective mechanisms of the photosynthetic apparatus from photo-damages [64]. Lutein and neoxanthin are involved in the xanthophyll cycle and ease the distribution of absorbed light energy in the leaf, therefore are correlated with the photosynthetic activity [65]. Thus, LV microgreens not

only grew taller, raising the phytochemical content, but also had a more efficient functioning, demonstrated by the more suitable anatomical structure and the high content of pigments and if such a trend is maintained, probably adult plants may have a greater photosynthetic performance. However, the combination of more environmental factors together, can have synergistic or antagonistic effects on microgreen growth and phytochemical content.

4.4.4. Microgreens response to microenvironment and general conclusions

The combination of VPD and light intensity influenced the growth, quality, and the morpho-anatomical traits of the two species. As highlighted by the multivariate analysis performed on all the traits analysed (Figure 4), the genetics had a remarkable effect since ‘Saxa’ and ‘Vertus’, at different VPDs and light intensities, separated into two groups mainly driven by the species. However, both microgreens’ species separated into two different sub-groups due to the effect of LV and 150 PPFD. ‘Saxa’ LV 150 was mainly driven by spongy thickness, violaxanthin and sulfate content, while ‘Vertus’ LV 150 due to the remaining pigments content. Both species grown at LV under 150 PPFD achieved the highest height and fresh weight. Even if they reduced the dry weight compared to microgreens grown under 300 PPFD, they managed to achieve a dry matter content (DM%) comparable to those grown under the highest light intensity (Figure 1). DM%, referred to as tissue density, is a good predictor of resource capture and use [66]. Tomlins et al. (2012) [67] found in sweet potato correlation between the dry matter content and the carotenoids content, attributed to textural changes: plants with high β -carotene were also more solid and less tender, thus preferred by consumers. Our results agree with this research since both ‘Saxa’ and ‘Vertus’ LV 150 increased the tissue thickness and synthesize high β -carotene and photosynthetic pigments (Table 6).

In conclusion, it is notable that the effects of environmental factors on the development and the antioxidant potential of microgreens can be complex and dependent on multiple factors. Thus, the modulation of environmental parameters during the early morphogenesis represents a valuable tool to adjust the quality of the products and the plant architecture to fulfil specific

needs like the production of more compact plants with high phytochemical content to use in controlled environment systems [68, 69].

For these reasons, to allow microgreen cultivation in space as food supplements, the development of space-targeted agro-technologies is mandatory, taking in mind the possible alterations induced by space flight environments. Indeed, the cultivation in closed facilities of limited volume under microgravity may change microgreen resource utilization, likely modifying the nutritional properties of the products, with species-specific responses. In the light of the above, choosing a low VPD together with a low light intensity might be a good strategy to save energy, while developing microgreens with a high phytochemical content, important for nutritional values and as good anatomical traits indicators of high resource use efficiency, useful also if some of these species could be grown as adult plants to be used as bio-regenerators or for seeds production, and not only as microgreens for dietary supplements. Indeed, it must be considered that crop systems like microgreens need a high number of seeds, which can represent a significant upload mass influencing mission planning and payload design, especially in short-duration missions [70]. The quantity of seeds to be transported depends on the mission duration, the production capacity of the facilities, the number of crew members, and the average daily consumption of microgreens. Therefore, it is a parameter that needs to be considered specifically for each mission case. Thus, having some plants dedicated to seed production can be an added value; however, in our experiment, the quantity used (6-7 g per trays) can produce a fresh biomass of 1.09 and 1.36 kg and considering 50g per meal it is possible to provide an approximate of 20 meals. Thus, 1 kilogram of seeds is sufficient for a 3-month production representing an efficient volume expenditure.

References are listed in Appendix III of the bibliography section.

5. Substrate and fertigation management modulate microgreens production, quality and resource efficiency

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5.1. Introduction

microgreen production parameters and phytochemicals profiles can be heavily influenced by environmental and agronomical factors [1–3]. To ensure that optimal yield and quality are achieved, it is crucial to consider the effect of these factors on a species-specific basis and make necessary optimizations. Further research is necessary to profit from CEA through modifying growth environments in order to produce targeted phytonutrients in microgreens [2]. Growth substrates, which are ideally derived from the available, renewable, and inexpensive source material, represent one of the main agronomical factors shaping production costs, yield, waste production, and quality attributes of microgreens farming [1]. There are two classes of available media for microgreen cultivation, which differ by their physical, chemical and biological characteristics: (1) organic, i.e., made of natural derived and biodegradable materials; and (2) inorganic, which are usually inert [4]. The effects of cultivation media on the quantity and quality of the produce have been tested by several authors [4–6]. The management of the fertilization procedure is another important pre-harvest factor influencing the quality of the produce [7, 8]. Pre-fertilization of the growing media and/or post-emergence fertigation should be applied according to the species germination time and growth rate [9]. Generally, the common post-emergence NS are based on a modified Hoagland NS with half (50%) or quarter (25%) strength [10–12]. Although fertilization is essential to obtain satisfying productions [9], several authors have shown that the introduction of moderate nutritional stress through mineral nutrient deprivation resulted in an enhanced phytochemical profile and greater quality of microgreens due to the activation of the secondary metabolism of the plant [13–15]. While there is a growing body of literature investigating how to efficiently produce

microgreens on Earth systems, no space-oriented ground-based research on microgreen productivity and quality has been conducted so far. In this paper, the effects of different substrates (coconut fiber and cellulosic sponge) and NS management (quarter-strength Hoagland and half-strength Hoagland/osmotic water) were tested on two different species of microgreens: *Raphanus sativus* cv. Saxa 2 and *Brassica oleracea* var. *capitata* f. *Sabauda* cv. Vertus. The aim of the work was to assess the effects of the interactions among species and growing conditions on (a) productivity and quality; (b) sizing of space facilities devoted to the production of nutraceuticals for the astronaut's wellbeing.

5.2. Materials and methods

5.2.1. Plant material and experimental design

Based on the selection reported in chapter 3 [16], two microgreen species, radish (*Raphanus sativus* cv. Saxa 2) and savoy cabbage (*Brassica oleracea* var. *capitata* f. *Sabauda* cv. Vertus), for simplicity through the text and tables 'Saxa' and 'Vertus' will be indicated as species, both provided by Pagano Costantino & F.lli Srl (Scafati, Salerno, Italy), were sown on two different substrates: coconut fiber (Sisal Fiber, Imola, Italy) and cellulose sponge (Spontex SAS, Colombes, France). The sowing density was 32.500 and 42.500 seeds m⁻² for 'Saxa' and 'Vertus', respectively. The experiment was carried out in a growth chamber (KBP-6395F, Termaks, Bergen, Norway) at the Department of Agricultural Sciences of the University of Naples "Federico II" (Portici, Italy). The LED lighting system (K5 Series XL750, Kind LED, Santa Rosa, CA, United States) had a light spectrum in the range of 400–700 nm and an intensity of $300 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$. Seed germination occurred in the dark, at 24°C and 100% relative humidity (RH). Once the microgreens emerged, the light was turned on (12 h photoperiod), and the growth chamber was set at $24/18 \pm 2^\circ\text{C}$ and RH $65 \pm 5\%$. The microgreens were subjected to two different fertigation strategies that included (1) a quarter concentration of Hoagland' NS (QS; EC: $0.4 \pm 0.05 \text{ mS cm}^{-1}$; pH: 6 ± 0.2) for the entire crop cycle as a control; (2) a Hoagland's NS with half concentration (HS; EC: $0.8 \pm 0.05 \text{ mS cm}^{-1}$; pH: 6 ± 0.2) for the first half of the crop cycle and osmotic water for the remaining part (H₂O; EC: 65.7 μS

cm⁻¹; pH:6.2), henceforth HS/H₂O. The microgreens were distributed in the growth chamber according to a randomized trifactorial experimental design in which the factors were species [SP; ‘Saxa’ and ‘Vertus’], substrate [SU; coconut fiber (Coco) and cellulose sponge (Sponge)] and different management of the NS (QS and HS/H₂O). Each experimental unit was replicated three times ($n = 3$; 24 experimental units). The ‘Saxa’ and ‘Vertus’ microgreens were cut at the substrate level 14 and 16 days after sowing, respectively, weighed for fresh weight (FW) determination, freeze-dried and finely ground for subsequent analysis and for dry weight (DW) and dry matter percentage (DM) determination. An aliquot of the microgreens was immediately frozen at -80°C for qualitative analysis.

5.2.2. Ascorbic acid content

Determination of total Ascorbic Acid (Tot. AscA), was conducted as reported in paragraph 3.2.11. (chapter 3).

5.2.3. Total anthocyanins and total phenolic content

Total anthocyanins (Tot. Ant) and the phenolic component (TPC) were determined as reported in paragraph 3.2.10. (chapter 3).

5.2.4. Pigments content

Pigments content analysis of chlorophylls (a and b), β -carotene, lutein, neoxanthin and violaxanthin, was achieved as reported in paragraph 3.2.9. (chapter 3).

5.2.5. Non-structural carbohydrates

Measurements of non-structural carbohydrates (NSCs) were performed as reported in paragraph 3.2.8. (chapter 3).

5.2.6. Anions content

The analysis of inorganic anions (nitrate, sulfate and chloride) was conducted as reported in paragraph 3.2.12. (chapter 3).

5.2.7. Daily production of total ascorbic acid, microgreens serving, and facilities cultivation surface size

The elaboration of the daily Tot. AsCA production (DTAP) of the system per square meter was conducted based on the length of the growing period, the biomass productivity and the total ascorbate concentration of the microgreens produced. In addition, considering the average requirement (AR) set by EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), (2013) [17] at 90 mg day^{-1} for an adult, it was possible to calculate the cultivation surface size (CSS) needed to produce the AR and the size of the serving that would apport the AR, for each of the factor's combination. DTAP, Serving and CSS were expressed as $\text{mg m}^{-2} \text{ day}^{-1}$, grams and m^2 , respectively.

5.2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 (Chicago, IL, United States). All parameters of the experiments were subjected to the Multivariate analysis following the General Linear Model. Mean values were separated according to Tukey's test with $p = 0.05$. All the parameters analyzed were subjected to Principal Component Analysis (PCA) using Minitab[®] 21.4 statistical software (Minitab LLC, State College, PA, United States).

5.3. Results

5.3.1. Production parameters

The species, the substrate, and their combination highly affected the yield (fresh and dry yield) and dry matter ($\text{SP} \times \text{SU}$; Table 1). For both 'Saxa' (the most productive species) and 'Vertus', the cultivation in Coco substrate increased fresh and dry yield but reduced dry matter. Regardless of SP and SU, QS treatment increased fresh yield and decreased dry matter compared to HS/H₂O throughout the crop cycle (Table 1). Although the $\text{SP} \times \text{NS}$ interaction did not result in significant differences in fresh and dry yields, the lowest dry matter values of the two species were obtained in the QS treatment (Table 1). Moreover, different NS management did not result in significant differences for microgreens grown in Coco for all the parameters shown in Table 1. Differentially, in the Sponge substrate, the use of QS NS,

compared to HS/H₂O, increased fresh yield by 10.46% and decreased dry matter by 6.43%.

Table 1. Average content of fresh weight (FW), dry weight (DW), and dry matter (DM) expressed in kg m⁻², g m⁻² and percentage (%), respectively.

	FW (kg m ⁻²)	DW (g m ⁻²)	DM (%)
Species (SP)			
Vertus (V)	2.45 a	181.51 a	7.60 b
Saxa (S)	1.63 b	141.12 b	8.93 a
Substrate (SU)			
Coco	2.47 a	175.60 a	7.20 b
Sponge	1.61 b	147.03 b	9.33 a
Nutrient solution (NS)			
HS/H ₂ O	1.99 b	160.09	8.45 a
QS	2.09 a	162.54	8.08 b
SP × SU			
S × Coco	2.95 a	198.67 a	6.74 d
S × Sponge	1.95 b	164.34 b	8.45 b
V × Coco	1.99 b	152.53 c	7.65 c
V × Sponge	1.27 c	129.72 d	10.21 a
SP × NS			
S × HS/H ₂ O	2.39	179.39	7.72 c
S × QS	2.50	183.62	7.48 d
V × HS/H ₂ O	1.59	140.78	9.19 a
V × QS	1.67	141.46	8.67 b
SU × NS			
Coco × HS/H ₂ O	2.46 a	176.19 a	7.26 c
Coco × QS	2.48 a	175.01 a	7.13 c
Sponge × HS/H ₂ O	1.53 c	143.98 b	9.64 a
Sponge × QS	1.69 b	150.08 b	9.02 b
Significance			
SP	***	***	***
SU	***	***	***
NS	***	ns	***
SP × SU	***	**	***
SP × NS	ns	ns	*
SU × NS	*	*	***
SP × SU × NS	ns	ns	ns

*, **, *** Significant for $p < 0.05$, 0.01 and 0.001. ns= not significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$).



Figure 1: ‘Saxa’ (A) and ‘Vertus’ (B) microgreens at harvest. Coco: coconut fiber, Sponge: cellulose sponge, 0.25: quarter concentration of Hoagland’s (QS) nutrient solution and 0.5: half concentration of Hoagland’s nutrient solution followed by osmotic water for the second half of the growing cycle (HS/H₂O)

5.3.2. Total ascorbic acid, total anthocyanins and total phenolic content

The values of Tot. AscA, Tot. Ant and TPC are shown in Table 2. ‘Vertus’ accumulated 41.0% more Tot. AscA than ‘Saxa’ (Table 2). The SU determined a significant effect for Tot. AscA, which was greater than 17.4% on Sponge compared to Coco. The NS and the interaction between the factors were not effective on modulating the Tot. AscA accumulation. Tot. Ant content was significantly influenced by the SP × SU and SU × NS interactions (Table 2). On both the substrates, ‘Saxa’ showed higher Tot. Ant level than ‘Vertus’. The use of the Sponge substrate resulted in an overall greater Tot. Ant content. NS significantly influenced the content of Tot. Ant only in combination with the Sponge substrate, where the HS/H₂O solutions induced an increase of 19.0% in comparison to the QS (Table 2). The analysis conducted on the TPC showed significant interaction for the SP × SU combination. The SU had a dominant effect on the TPC: the low accumulating species, ‘Saxa’, cultivated on Sponge overcame ‘Vertus’ when it was cultivated on Coco (Table 2), while the use of Sponge resulted in an

increment of 68.0 and 42.0% compared to Coco, respectively in ‘Vertus’ and ‘Saxa’. The NS factor did not significantly affect TPC.

Table 2. Average content of total AscA (Tot.AscA), total anthocyanin (Tot.Ant) and Total Phenolic Content (TPC) (expressed in mg 100 g⁻¹ fresh weight (FW)).

	Tot. AscA (mg 100 g ⁻¹ FW)	Tot. Ant (mg 100 g ⁻¹ FW)	TPC (mg 100 g ⁻¹ FW)
Species (SP)			
Saxa (S)	91.68 b	7.20 a	177.30 b
Vertus (V)	129.14 a	5.17 b	215.92 a
Substrate (SU)			
Coco	102.60 b	4.62 b	153.94 b
Sponge	120.44 a	7.75 a	239.29 a
Nutrient Solutions (NS)			
HS/H ₂ O	110.91	6.58 a	199.16
QS	111.4	5.79 b	194.07
SP × SU			
S × Coco	78.56	5.16 c	146.69 d
S × Sponge	104.80	9.25 a	207.92 b
V × Coco	123.20	4.09 d	161.19 c
V × Sponge	136.08	6.25 b	270.66 a
SU × NS			
Coco × HS/H ₂ O	103.12	4.75 c	159.15
Coco × QS	102.16	4.5 c	148.73
Sponge × HS/H ₂ O	118.70	8.42 a	239.16
Sponge × QS	122.18	7.09 b	239.41
Significance			
SP	***	***	***
SU	***	***	***
NS	ns	**	ns
SP × SU	ns	***	***
SP × NS	ns	ns	ns
SU × NS	ns	*	ns
SP × SU × NS	ns	ns	ns

*, **, *** Significant for $p < 0.05$, 0.01 and 0.001. ns= not significant. Different letters within each column indicate significant differences according to Tukey’s multiple-range test ($p = 0.05$).

5.3.3. Pigments

The contents of β -carotene and violaxanthin showed significant interaction for the SP × SU and SU × NS combinations, respectively (Table 3). The combination Saxa × Sponge showed a 35.0% higher content of β -carotene than the combination Saxa × Coco. The Sponge × HS/H₂O combination showed a content of violaxanthin higher than that of the combination Coco ×

HS/H₂O and Sponge × QS (Table 3). The other combinations did not show significant effects. The analysis of the other pigments showed significant differences between the two species tested for the content of lutein and chlorophylls (*a* and *b*), with the best performance in the accumulation of all three components in the case of ‘Vertus’. Finally, no significant difference was found for the neoxanthin content.

Table. 3 Average content of pigments (Chlorophylls and carotenoids) expressed in mg 100 g⁻¹ fresh weight (FW).

	Chlorophyll a (mg 100 g ⁻¹ FW)	Chlorophyll b (mg 100 g ⁻¹ FW)	β-Carotene (mg 100 g ⁻¹ FW)	Lutein (mg 100 g ⁻¹ FW)	Neoxanthin (mg 100 g ⁻¹ FW)	Violaxanthin (mg 100 g ⁻¹ FW)
Species (SP)						
Saxa (S)	36.83 b	12.47 b	2.85 b	4.86 b	1.26	22.64
Vertus (V)	54.04 a	19.46 a	3.79 a	6.62 a	1.75	22.57
Substrate (SU)						
Coco	45.73	16.28	3.16	5.48	1.35	20.65
Sponge	47.43	16.58	3.48	6.00	1.66	24.56
Nutrient solution (NS)						
HS/H ₂ O	46.55	16.48	3.28	5.82	1.63	23.90
QS	46.61	16.38	3.36	5.66	1.37	21.31
SP × SU						
S × Coco	32.82	11.25	2.43 a	4.18	1.04	19.64
S × Sponge	40.83	13.69	3.27 b	5.55	1.47	25.65
V × Coco	58.63	21.32	3.88 b	6.78	1.65	21.67
V × Sponge	54.04	19.46	3.69 b	6.45	1.85	23.46
SU × NS						
Coco × HS/H ₂ O	43.83	15.82	2.98	5.27	1.25	18.57 b
Coco × QS	47.62	16.75	3.34	5.68	1.44	22.73 ab
Sponge × HS/H ₂ O	49.26	17.13	3.58	6.37	2.02	29.23 a
Sponge × QS	45.61	16.02	3.38	5.63	1.30	19.89 b
Significance						
SP	***	***	***	***	ns	ns
SU	ns	ns	ns	ns	ns	ns
NS	ns	ns	ns	ns	ns	ns
SP × SU	ns	ns	*	ns	ns	ns
SP × NS	ns	ns	ns	ns	ns	ns
SU × NS	ns	ns	ns	ns	ns	**
SP × SU × NS	ns	ns	ns	ns	ns	ns

*, **, *** Significant for $p < 0.05$, 0.01 and 0.001. ns= not significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$).

5.3.4. Non-structural carbohydrates

The contents of the non-structural carbohydrates (NSC) are reported in Table 4. The content of NSC was high and variable, with several differences likely due to either other factors and/or their partial or total interactions. Regardless of the experimental conditions, the most abundant NSC was glucose followed by starch, fructose, and sucrose (Table 4). Total soluble NSC (Tot. soluble) where the most relevant contributors to the total content of NSC in the microgreens, with some instances where the soluble NSC were close to represent 2.0% of fresh weight of the microgreens. The data highlighted the effect of the interaction $SP \times SU \times NS$ on the content of fructose, sucrose and Tot. soluble. For these components, the modulation of the contents, due to the different nutritional solutions, is clearly visible in the case of the use of the Sponge substrate and ‘Vertus’ species. In the case of fructose and sucrose, the different substrates appear to have an accentuated modulation effect in the latter cultivar with higher quantities accumulated on Sponge (+66.0% in HS/H₂O compared to QS). On the Tot. soluble, the differences due to the interactions of the factors determine different levels of accumulation for each combination of species and substrate, with the highest content found in Vertus $\times$ Sponge and attributable to the interaction with the HS/H₂O solution (Table 4). The total NSC content appears to be influenced by the $SP \times SU$ and $SP \times NS$ combinations. In neither of the two different combinations, however, does the interaction hide the greater concentration of NSC in ‘Vertus’ compared to ‘Saxa’. The $SP \times SU$ combination maintains this difference, while the use of Sponge almost a double increase in the NSC content for both species. The NS component interacted with the species, increasing the productivity in the treatment with HS/H₂O only in the case of ‘Vertus’. The glucose and starch content show no variability in response to the considered factors. Significant variations in glucose were detected between the two SP, SU, and NS, while differences in the starch content were induced only by the SP and SU (Table 4). The higher content of glucose and starch of the two species was found in the ‘Vertus’ cultivar while, as regards the evaluation of the performances between the two different substrates, the higher content was found for the samples grown on Sponge. Finally, HS/H₂O NS determined significantly higher glucose levels than the QS.

Table. 4 Average content of glucose, fructose, sucrose, starch, total soluble and total non-structural carbohydrates expressed in mg 100 g⁻¹ fresh weight (FW).

	Glucose (mg 100 g ⁻¹ FW)	Fructose (mg 100 g ⁻¹ FW)	Sucrose (mg 100 g ⁻¹ FW)	Starch (mg 100 g ⁻¹ FW)	Tot. Soluble (mg 100 g ⁻¹ FW)	Tot NSC (mg 100 g ⁻¹ FW)
Species (SP)						
Saxa (S)	358.7 b	75.3 b	49.6 b	191.0 b	483.5 b	674.4 b
Vertus (V)	781.9 a	376.5 a	89.0a	522.7 a	1247.3 a	1770.0 a
Substrate (SU)						
Coco	458.34 b	172.53 b	47.40 b	257.31 b	678.28 b	935.59 b
Sponge	682.20 a	279.19 a	91.14 a	456.38 a	1052.53 a	1508.91 a
Nutrient solution (NS)						
HS/H ₂ O	603.06 a	244.94 a	74.14 a	355.50	922.14 a	1277.65 a
QS	537.49 b	206.77 b	64.40 b	358.19	808.66 b	1166.85 b
SP × SU						
S × Coco	277.28	66.12 c	35.84 c	96.55	379.23 d	475.79 d
S × Sponge	440.03	84.40 c	63.35 b	285.38	587.78 c	873.16 c
V × Coco	639.41	278.94 a	58.97 b	418.07	977.32 b	1395.39 b
V × Sponge	924.38	473.97 b	118.92 a	627.38	1517.27 a	2144.66 a
SP × NS						
S × HS/H ₂ O	361.95	77.70 c	44.79 c	172.40	484.45 c	656.84 c
S × QS	355.36	72.82 c	54.40 c	209.53	482.57 c	692.10 c
V × HS/H ₂ O	844.17	412.19 a	103.49 a	538.60	1359.84 a	1898.45 a
V × QS	719.62	340.72 b	74.41 b	506.85	1134.75 b	1641.60 b
SU × NS						
Coco × HS/H ₂ O	480.24	177.97 c	45.84 c	267.72	704.05 c	971.77
Coco × QS	436.45	167.08 c	48.97 c	246.90	652.51 c	899.41
Sponge × HS/H ₂ O	725.88	311.92 a	102.44 a	443.28	1140.24 a	1583.52
Sponge × QS	638.52	246.45 b	79.84 b	469.48	964.81 b	1434.29
SP × SU × NS						
S × Coco × HS/H ₂ O	280.52	68.0 d	33.0 c	91.46	381.5 e	472.93
S × Coco × QS	274.04	64.2 d	38.7 c	101.64	377.0 e	478.64
S × Sponge × HS/H ₂ O	443.39	87.4 d	56.6 cd	253.33	587.4 d	840.75
S × Sponge × QS	436.67	81.4 d	70.0 bc	317.42	588.1 d	905.57
V × Coco × HS/H ₂ O	679.95	288.0 c	58.7 cd	443.98	1026.6 c	1470.60
V × Coco × QS	598.87	269.9 c	59.2 cd	392.16	928.0 c	1320.18
V × Sponge × HS/H ₂ O	1008.38	536.4 a	148.3 a	633.23	1693.01 a	2326.29
V × Sponge × QS	840.38	411.5 b	89.6 b	621.53	1341.5 b	1963.02
Significance						
SP	***	***	***	***	***	***
SU	***	***	***	***	***	***
NS	*	***	*	ns	***	*
SP × SU	ns	***	***	ns	***	**
SP × NS	ns	***	***	ns	***	**
SU × NS	ns	**	**	ns	*	ns
SP × SU × NS	ns	**	***	ns	*	ns

*, **, *** Significant for $p < 0.05$, 0.01 and 0.001. ns= not significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$).

5.3.5. Anions content

The nitrate, sulfate and chloride content are shown in Table 5. The nitrate content showed significant differences due to the interaction between the three factors analysed, resulting in higher level in the combination between Saxa $\times$ Coco $\times$ QS. The lowest content was found in the combination Vertus $\times$ Sponge with no differences between the two NS. The sulfate content was on average 22.3 mg 100 g⁻¹ FW, ranging among 13.17 and 32.7 mg 100 g⁻¹ FW in the combination's Saxa $\times$ Coco and Sponge $\times$ HS/H₂O, respectively, (Table 5). Sulfate content was influenced by the interaction between SU $\times$ NS with the effect of the different NS can be seen in the Sponge substrate where the QS result in lower content. Between the substrates higher levels of sulfate is accumulated with the use of Sponge. The range of chloride content varies from a minimum of 25.34 mg 100 g⁻¹ FW in the combination Saxa $\times$ Coco $\times$ HS/H₂O to a maximum of 53.15 mg 100 g⁻¹ FW in the combination Saxa $\times$ Sponge $\times$ HS/H₂O. The chloride content appears to be influenced by the SP $\times$ SU interaction with a greater accumulation in the combination of Saxa grown on Sponge, which presents an increase of 56.0% compared to the average of the other combinations, which have no accumulation difference for this compound (Table 5).

Table 5. Average content of nitrate, sulfate and chloride content (expressed in mg 100 g⁻¹ fresh weight) processed for each test factor.

	Nitrate (mg 100 g ⁻¹ FW)	Sulfate (mg 100 g ⁻¹ FW)	Chloride (mg 100 g ⁻¹ FW)
Species (SP)			
Saxa (S)	60.57 a	19.66 b	40.41 a
Vertus (V)	31.12 b	24.85 a	32.04 b
Substrate (SU)			
Coco	58.09 a	16.13 b	31.52 b
Sponge	33.59 b	28.39 a	40.93 a
Nutrient solution (NS)			
HS/H ₂ O	30.22 b	24.58 a	36.67
QS	61.47 a	19.94 b	35.78
SP $\times$ SU			
S $\times$ Coco	70.1 a	13.17 c	31.24 b
S $\times$ Sponge	51.05 b	26.15 ab	49.58 a

V × Coco	46.1 b	19.08 bc	31.81 b
V × Sponge	16.13 c	30.63 a	32.28 b
SP × SN			
S × HS/H ₂ O	38.90 b	21.5	39.25
S × QS	82.23 a	17.82	41.57
V × HS/H ₂ O	21.53 c	27.65	34.09
V × QS	40.70 b	22.05	29.99
SU × SN			
Coco × HS/H ₂ O	37.80 b	16.49 c	28.57
Coco × QS	78.38 a	15.76 c	34.48
Sponge × HS/H ₂ O	22.63 c	32.66 a	44.77
Sponge × QS	44.55 b	24.11 b	37.08
SP × SU × SN			
S × Coco × HS/H ₂ O	47.82 cd	13.72	25.34
S × Coco × QS	92.35 a	12.63	37.15
S × Sponge × HS/H ₂ O	29.99 de	29.28	53.15
S × Sponge × QS	72.11 ab	23.02	46
V × Coco × HS/H ₂ O	27.79 de	19.26	31.79
V × Coco × QS	64.41 bc	18.9	31.82
V × Sponge × HS/H ₂ O	15.27 e	36.05	36.39
V × Sponge × QS	17.0 e	25.21	28.16
Significance			
SP	***	**	*
SU	***	***	*
NS	***	***	ns
SP × SU	**	***	*
SP × SN	***	ns	ns
SU × SN	***	***	ns
SP × SU × SN	*	ns	ns

*, **, *** Significant for $p < 0.05$, 0.01 and 0.001. ns= not significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$).

5.3.6. Daily total ascorbic acid production, cultivation surface size and microgreens serving

The daily total ascorbic acid production (DTAP) in Saxa × Coco × HS/H₂O was almost twice that of the combination Vertus × Sponge × HS/H₂O (Table 6). However, according to the statistical analysis, differences in the CSS are mainly attributable to the different interaction of SP and SU ($p < 0.05$). The minimal surface needed to produce 90 mg day⁻¹ of ascorbate is achieved by the combination of Saxa × Coco, Saxa × Sponge and Vertus × Coco with an average of 0.59 m², while, in comparison, Vertus × Sponge combination required a significative larger surface. The useful amount of fresh microgreen serving (Serving) for the intake of 90 mg of ascorbate is influenced by the interaction of all the three factors. According to Tukey's test, the intake of the AR of ascorbate through the consumption of microgreens produced by the

combination Saxa $\times$ Coco $\times$ QS required an amount of biomass 1.7 times higher than other tested combinations. The minimal serving useful to achieve the AR is 80 g.

Table. 6 Averages of daily total ascorbate production (DTAP), fresh microgreens biomass to achieve the adequate daily intake of 90 mg of ascorbate (Serving) and the cultivation surface size to produce the AR of 90 mg of ascorbate (CSS) elaborated for each test factor (expressed as mg m⁻² day⁻¹, g and m² respectively).

	DTAP (mg m ⁻² day ⁻¹)	Serving (g)	CSS (m ²)
Species (SP)			
Saxa (S)	155.79	103.34 a	0.60 b
Vertus (V)	132.02	70.02 b	0.72 a
Substrate (SU)			
Coco	160.71	96.35 a	0.58 b
Sponge	127.09	77.02 b	0.74 a
Nutrient solution (NS)			
HS/H ₂ O	143.01	84.40	0.67
QS	144.80	88.96	0.64
SP $\times$ SU			
S $\times$ Coco	165.73	119.32 a	0.57 b
S $\times$ Sponge	145.84	87.36 b	0.63 b
V $\times$ Coco	155.70	73.37 b	0.59 b
V $\times$ Sponge	108.34	66.68 b	0.84 a
SP $\times$ SU $\times$ NS			
S $\times$ Coco $\times$ HS/H ₂ O	191.94 a	102.39 ab	0.49
S $\times$ Coco $\times$ QS	139.51 abc	136.2 a	0.65
S $\times$ Sponge $\times$ HS/H ₂ O	136.88 abc	88.60 b	0.67
S $\times$ Sponge $\times$ QS	154.79 abc	86.12 b	0.59
V $\times$ Coco $\times$ HS/H ₂ O	142.28 abc	78.78 b	0.64
V $\times$ Coco $\times$ QS	169.10 ab	67.96 b	0.54
V $\times$ Sponge $\times$ HS/H ₂ O	100.91 c	67.84 b	0.90
V $\times$ Sponge $\times$ QS	115.77 bc	65.52 b	0.78
Significance			
SP	*	***	**
SU	**	**	***
NS	ns	ns	ns
SP $\times$ SU	ns	*	*
SP $\times$ NS	ns	*	ns
SU $\times$ NS	ns	ns	ns
SP $\times$ SU $\times$ NS	*	*	ns

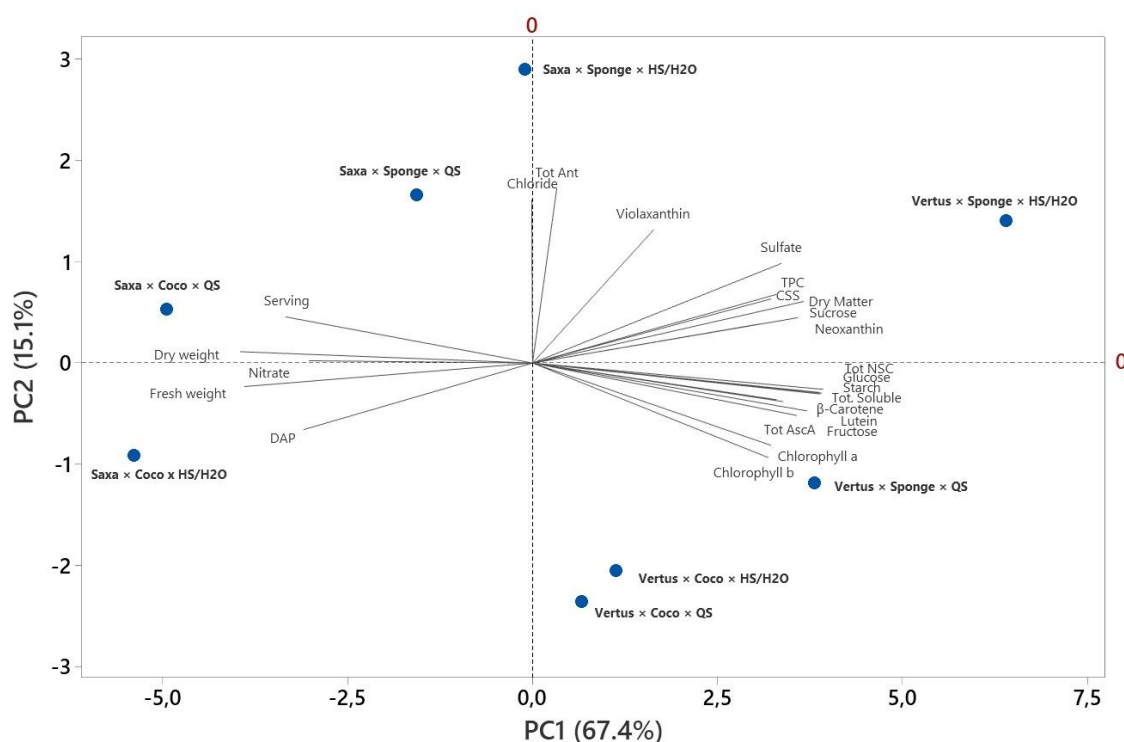
*, **, *** Significant for $p < 0.05$, 0.01 and 0.001. ns= not significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$).

5.3.7. Principal component analysis

Principal component analysis (PCA) has demonstrated to represent an adequate approach of collectively representing sample population divergence over multiple traits of productivity and quality in return for numerous cultivation factors [1]. In the current study, the PCA facilitated a brief view of the affinity among the microgreens species performance, their compositional variables and their daily total ascorbic acid production, cultivation surface size and microgreens serving (Figure 2). The species-dependent effects of the two substrates and nutrient solutions were highly visualized due to the good quality of the PCA loading and score plots as designated by the high percentage of the total variance (82.5%). The first two Principal Components (PCs) were correlated with eigenvalues greater than 1 (data not shown) and explained 82.5% of the cumulative variance, with PC1 accounting for 67.4% and PC2 for 15.1%. The biplot showed that PC1 was positively correlated with dry matter as biometric parameter and most of the quality attributes, including Tot. AscA, TPC, neoxanthin, lutein, β -carotene, glucose, fructose, sucrose, starch, Tot. soluble, Tot NSC, and CSS. PC1 was as well negatively correlated with two components of the biometric parameters (fresh and dry weight), nitrate, DAP and the serving. Moreover, PC2 was positively correlated with total Ant., violaxanthin, sulfate and chloride, while it was negatively correlated with chlorophylls a and b. In this controlled environment study, the score plot of the PCA integrated information on the bioactive profile of the two microgreens species subjected to two different nutrient solutions management and two different substrates: ‘Vertus’ was characterized by higher lutein, β -carotene, chlorophylls, total anthocyanins, total ascorbic acid, TPC, soluble sugars, total NSC, starch. Whereas, ‘Saxa’ was superior in fresh and dry weight, DTAP, serving and nitrate. In particular, ‘Vertus’ cultivated on Sponge substrate and subjected to HS/H₂O was positioned on the positive side of PC1 in the upper right quadrant of the PCA score plot as it delivered high quality microgreens (Figure 2). In addition, the same species cultivated on Sponge substrate and subjected to QS and cultivated on coco fiber with both nutrient solutions was positioned in the lower right quadrant. ‘Saxa’ microgreens grown on Sponge substrate with both nutrient solutions plus grown on coco fiber and subjected

to QS were positioned in the upper left quadrant (Figure 2) and characterized by high serving, dry weight and chloride. Finally, the lower left quadrant depicted the treatment ‘Saxa’ cultivated on Coco and subjected to HS/H₂O. Thus, the effected PCA in this study illustrated a broad view of yield, quality traits and serving of ‘Saxa’ and savoy microgreens.

Figure 2. Principal component analysis loading plot of biomass parameter (fresh weight, dry weight and dry matter), quality parameters (Tot AscA, Tot Ant, TPC, violaxanthin, neoxanthin, lutein, chlorophyll a, chlorophyll b, β -carotene, glucose, fructose, sucrose, total soluble, total NSC, nitrate, sulfate and chloride), daily total ascorbate production (DTAP), microgreens serving to supply the AR of ascorbate (Serving) and the cultivation surface size for the production of ascorbate AR (CSS) of savoy cabbage (SC) and radish (R) microgreens grown on coconut fiber or cellulose sponge and supplied with quarter strength Hoagland nutrient solution (QS) or half strength Hoagland/osmotic water (HS/H₂O).



5.3. Discussion

In order to optimize the yield and nutritional quality of microgreens, the choice of plant species is a critical factor [16, 18, 19]. This is confirmed by the different production responses (fresh and dry yield) of the two microgreen species used, independently of the influence of the substrate and NS management. Interestingly, the yield obtained for ‘Saxa’ (2.45 kg m^{-2}), the most productive species in our experiment, was lower than what was recorded by Kyriacou et al. (2019) [20] for radish Sango (5.97 kg m^{-2}) in a recent work in which 13 different species of microgreens grown on peat were characterized from a productive and phytochemical point of view. This significant difference is attributed to the harvest stage difference (cotyledons vs. two true leaves), in addition to the substrates used in this experiment vs. the peat moss-based substrate used for the screening. In a different study of Izzo et al. (2023) [16] (chapter 2), the same ‘Saxa’ and ‘Vertus’ species were both cultivated on peat-based substrate and demonstrated higher yield. As reported by Kyriacou et al. (2020) [1], the chemical and physical characteristics of the substrates significantly influenced the production response of microgreens. According to the authors’ hypothesis, the lower fresh yield obtained in our experiment could be attributed to a lower air capacity of Sponge and Coco, compared to peat. This characteristic, typical of coherent substrates characterized by a higher micropore content, would hinder root growth and justify the higher dry matter (8.26%, on average) obtained, compared to Kyriacou et al. (2019)[18, 20] (4.10–6.05%). However, for both species of microgreens, Coco ensured a better response to production, confirming what was reported in part by Kyriacou et al. (2020) [1]. In contrast, dry matter percentage did not show the same trend as observed for fresh production, demonstrating that the species response to the substrate for this parameter is not uniform. Relative to the effect induced by the management of different NS, regardless of the cultivar, the mid-crop cycle replacement of the half-strength solution with osmotic water only (HS/H₂O) resulted in significant differences in fresh yield compared to quarter-strength (QS) NS. While QS treated and HS/H₂O treated delivered the same amount of nutrients, the difference in supply and deficiency (particularly of N and P) in the later growth stage (higher rooting and transpiration activity) could

explain this result. Notably, a reduction in fresh production and an increase in dry matter were recorded only for microgreens grown on Sponge, again confirming the influence of the chemical and physical characteristics of the substrates on productivity.

Recently there has been a significant increase in research focusing on the nutritional aspects of microgreens, also oriented on the positive health outcomes that certain species can exert [21–23]. Hence, species selection can be also based on the type of phytonutrient needed to achieve a balanced diet and specific nutritional benefits. Although, we found that the content of certain phytochemicals, such as lutein and chlorophylls, are mainly determined by the plant genotype, the biochemical profile of microgreens can be influenced, in a species-specific way, by the cultivation regimen. A significant interaction between species and substrates on anthocyanins [6], polyphenols and β -carotene [1] contents were already reported. These compounds have relevant nutraceutical functions since their antioxidant potentials can be helpful to counteract the detrimental effect of the space environment [24–26]. In our study, the interaction between species and substrates affected Tot. Ant., TPC, and β -carotene content, but also the accumulation of sulfate and chloride. Brassicaceae are the main contributors of dietary sulfate and 0.5 mg g^{-1} is considered a high concentration of sulfate in food and can negatively affect human health [27, 28]. Although different growing conditions in the experiment affected their content, the sulfate concentration in microgreens was consistently recorded as being lower than 0.5 mg g^{-1} . EFSA has set a safe and adequate intake for chloride to 3.1 g day^{-1} . The content in a 100 g serving of microgreens, obtained under the best chloride accumulator combination, would carry a very low dose of chloride, well below the daily threshold for an adult person, and would therefore not represent a threat for astronaut health.

Anthocyanins content strongly depends on the genetic background [29], but the modulation potential of environmental factors such as light regime and temperature is generally recognized [30]. Our data show that mineral nutrition can be used to modulate anthocyanins content, and that this effect might be prone to interaction with other agronomical factors. In fact, we showed that the HS/H₂O NS regime leads to an increase in anthocyanin content when

combined with the cellulose sponge substrate, representing the first report on significant interaction between substrate and nutrition management on microgreens anthocyanins content. The accumulation of anthocyanins in vegetative tissues represents a general plant reaction to various stressors including nutrient deficiency [31–33]. In our work, the accumulation of anthocyanins is boosted through nutritional stress induced by nutrient deprivation, as already reported in other studies [11, 13, 34]. However, the role of the substrate in contributing to the accumulation of these compounds deserve more investigations. Notably, in the work by Bulgari et al. (2021) [6], the anthocyanins content of red basil was enhanced by vermiculite use (pH 7–8), while this accumulation was lower in coconut fiber (pH 5.5–7). In our study, the cellulose Sponge substrate could be a potential contributor to the nutritional stress due to his alkaline pH (pH 8.49) [1]. The favourable pH of coconut fiber (pH 5.34) could facilitate nutrient absorption, thus mitigating the nutritional stress induced by the HS/H₂O nutrient solution. This, in turn, may lead to a less anthocyanins buildup when compared to the cellulosic sponge substrate.

HS/H₂O NS also resulted in enhanced fructose and sucrose content when combined with the Sponge and ‘Vertus’. The content of these soluble sugar could be an important post-harvest attribute influencing the shelf-life and the taste of the microgreens produce [6, 35]. Our results suggest that, depending on the specific species and substrate, NS management could represent a valuable tool to improve the quality of microgreens by increasing the fructose and sucrose content. The nitrate content is another important parameter for determining the quality of leafy vegetables [36]. Nitrate intake might affect metabolism in humans, thereby producing a decrease in blood pressure [37]. While this might be a positive effect on humans on Earth, it could be detrimental in space where low blood pressure is already induced by microgravity [38]. Although the level reached in microgreens for all the tested treatments is much lower than the threshold defined by the European Commission Regulation No 1258/2011, the use of different conditions can determine a different accumulation level of these compounds. In particular, the lower content was detected in combination with ‘Vertus’ and Sponge. Although in this last combination the different NS did not produce different

results, in all the other combinations the sub-optimal nutrient conditions achieved through the use of HS/H₂O treatments resulted in a lower accumulation of nitrate.

Nutrient deprivation strategies involve withholding or limiting the supply of nutrients to plants for a period of time before they are harvested to trigger the plant secondary metabolome and enhance crop quality [34]. Significant increases in the content of total ascorbic acid, anthocyanins, and phenolic acids were already achieved through nutrient supply deprivation in lettuce microgreens, with a decrease also in the content of nitrates [13]. Our study found significant interactions between the factors that affect the accumulation of phytochemicals in the two tested species. This highlighted the importance of using different species for microgreens production in space and of scientifically planned production conditions for obtaining food products with various nutraceutical characteristics. On the other hand, the variability in the response of the two species to the growth conditions used underlines the need for precise control of environmental conditions and in-depth knowledge of how to manage them. While this may represent a potential complication, it can also be used to increase the system versatility, develop an expert system, or manage targeted phytonutrient production. As stated by Mortazavi et al. (2015)[39], ascorbic acid (AscA) is a potential non-toxic and cost-effective radioprotector that might play an important role in counteracting radiation damage in space. Known as Vitamin C, AscA is a well-known antioxidant and the most widely used vitamin supplement worldwide. In addition to its antioxidant activity, AscA has been shown to have anti-atherogenic and anti-carcinogenic properties [40]. In the context of human space exploration, AscA can help to neutralize free radicals and protect cells from damage induced by ionizing radiation [25]. It is also involved in maintaining the immune system function, which is strongly affected during space travel [41]. AscA is a labile molecule, which can be easily damaged or destroyed by heat, light, and oxygen. It is, therefore, clear that the on-board production of fresh food containing elevated nutraceutical properties is necessary to provide an adequate intake of AscA. In this prospective, AscA could be considered a quality driving attribute on the basis of which the dimensions of closed microgreens cultivation system should be determined to achieve the AscA

daily doses for astronauts. The EFSA recommended daily intake of vitamin C is 90 mg day⁻¹ for adult men, and 75 mg day⁻¹ for adult women. These recommendations are based on the average daily intake that is sufficient to meet the nutritional requirements and to prevent deficiency. The data indicate that microgreens have high concentrations of vitamin C, but the concentration can vary greatly depending on the trial and conditions tested. The efficiency of a production combination on phytonutrients, can be evaluated on the basis of two fundamental variables: (a) the concentration of one or several phytonutrients; (b) the productivity per day and unit area in one or several phytonutrients, which is determined by the previous parameter in combination with the productivity in biomass. A high concentration is important as it contributes to the nutrient productivity of the system and allows for easy daily consumption with a relatively small amount of food.

5.4. Conclusion

The current study comprises a novel report on how different substrates and nutrient solution management can impact the growth and the phytochemicals of ‘Saxa’ and ‘Vertus’ microgreens. Our results indicate that cultivating on coco fiber, a higher fresh weight is obtained, while when applying NS/H₂O total Ant. can be triggered, while Sponge substrate boost TPC. By using the right conditions, the daily recommended AscA intake can be achieved with 80 grams of fresh biomass. The tests conducted showed considerable variability in daily productivity and unit area and, for example, the cultivation of ‘Saxa’ on Coco severely increased the fresh biomass required for the achievements of the AR. In our trial, less than 0.6 m² of cultivation area could ensure the average daily dose when considering the optimal species and substrate combination (‘Saxa’ cultivated on both substrates and savoy cabbage on Coco). Greater area would be required in less optimal production conditions such as those provided by savoy cabbage and Sponge, but this choice could still be viable if other characteristics are targeted.

References are listed in Appendix IV of the bibliography section.

6. Chicory Taproot Production: Effects of Biostimulants under Partial or Full Controlled Environmental Conditions

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6.1. Introduction

Chicory (*Cichorium intybus* L.) is a cultivated species belonging to the *Asteraceae* family, naturally and diffused, and cultivated in many regions of the world. The extensive breeding undergone in centuries has produced several varieties; leaves, stems, and roots of chicory are consumed fresh or cocked, utilized as herbal medicine preparations, or destined for industrial processing [1–3]. Chicory roots are traditionally consumed as vegetable in some regions of Italy and France. Moreover, chicory roots are produced for the extraction of inulin-type fructans, which are well-known prebiotics [4]. The term prebiotic indicates “a substrate that is selectively utilized by host microorganisms conferring a health benefit”, following the definition agreed by The International Scientific Association for Probiotics and Prebiotics (ISAPP) with a consensus statement [5]. At the moment, several molecules are recognized to correspond to this definition, and more are under investigation [6–8]. Several but not all prebiotics or candidate prebiotics are also dietary fiber. Without a doubt, fructans are among the most effective prebiotics and are also dietary fiber. Chicory is the prevalent source of purified fructans for the market [9]. In addition to fructans, other components of the root tissue, such as the cell wall components and various phytochemicals, contribute to the nutritional value of this source of prebiotics [9, 10]. While there is a growing consensus concerning the relevance of an adequate intake of prebiotic for human and animal health, recent work has focused on the importance of using full plant tissues instead of the isolated prebiotic molecules, both in terms of positive health effects and food cycle sustainability [11, 12]. Food containing a high quantity of fructans in addition to dietary fiber and other health-promoting phytochemicals can be a relevant functional complement to the diet of populations that consume low dietary

fiber and prebiotics. An emerging body of evidence indicates that prebiotics can also alleviate the effects of several health threats such as those occurring under malnutrition, dysbiosis, or even in space missions [13]. In view of the recognized and growing evidence on the health benefits that nutritional fiber and prebiotic consumption can exert on human health, it is surprising that the use of chicory taproot as fresh vegetable is so limited. Moreover, in the context of space farming, there has been little consideration for plant species rich in prebiotics, despite the potential for them to serve as high-value functional foods. Exploring this perspective could prove to be interesting, as it may yield substantial nutritional benefits for astronauts undertaking extended space missions.

Chicory roots for industrial inulin extraction are produced in open field conditions from early summer to winter. Roots are harvested from October until February, reaching a total yield of fresh roots up to 50 t ha⁻¹ with an inulin content as high as 15%. Several cultivars are available on the market [14], and there is a large body of literature on agronomical and eco-physiological aspect of chicory cultivation for inulin production in open field conditions [15–17]. Research results demonstrated that inulin accumulation in root tissues can have a great variability under open field conditions due to the unpredictable change in environmental factors [18, 19]. With respect to the traditional cultivation in open field conditions, controlled environment offers the opportunity to manage the environmental factors, to obtain faster crop growth, year-round production, and high-quality standard of vegetables, as reported for example for artichoke [20]. Limited studies are available on the production of fresh chicory roots under controlled conditions.

Plant Biostimulants (PBs), such as natural substances and beneficial microorganisms, could be an interesting tool to improve productivity and quality of vegetables in a sustainable manner, by reducing agrochemical inputs and preventing abiotic stress. According to the EU Fertilising Products Regulation (2019/1009), PBs are defined on the basis of their agricultural functions for the improvement of one or more of the following characteristics: (1) nutrient use efficiency, (2) crop tolerance to abiotic stress, (3) quality traits, or (4) availability of confined nutrients in the soil or rhizosphere. PBs have been specifically designed for seed treatment, foliar spray, or soil drench

application. Foliar spray is gaining significant importance in the market owing to its advantages, including better convenience in terms of application and better absorption than root drench application. Among biostimulant substances, protein hydrolysates (PHs) and seaweed extracts (SWE) have been successfully used as foliar sprays in many horticultural crops [21]. Several authors showed that PHs and SWE were able to stimulate plant primary and secondary metabolisms, resulting in enhanced growth and production as well as higher nutritional quality of horticultural crops [22–26]. Rouphael and coworkers [24] demonstrated that PHs and SWE treatment significantly increase the shoot fresh weight of lettuce plants, in both non-saline and salinity conditions. The same author [24] demonstrated that PHs and SWE application increase yield and dry matters as well as total phenolics and ascorbate content in spinach leaves. According to the results of Choi et al. (2022) [25], foliar and soil treatments of PHs enhanced shoot fresh weight and dry matters of romaine lettuce as well as fruit fresh weight, fruit number, and dry matter of tomato plants. The same work [25] showed that PHs enhanced chlorophyll and carotenoid content, antioxidant activity, and total phenolics of both studied species. Hussain et al. (2021) [26] showed that SWE had a positive effect on tomato yield and the total soluble solid content in tomato fruit, increasing the number of flower clusters and flowers, fruit number, dry weight of leaves, and roots. Scientific literature regarding the use of plant biostimulants is constantly increasing and involving different species and growing conditions. PBs are organic molecules, that can originate from waste organic matter biorefinery cycles. Therefore, it is possible to imagine their production via closed-loop waste recycling in BLSS from non-edible parts of cultivated plants or food scraps [27]. However, to our knowledge, there is no information about the potential benefits of using PBs on chicory for enhancing root yield and the accumulation of fructans. The aims of this study were (i) to assess the feasibility of a controlled environment agriculture (CEA) system for Earth and space applications for chicory production in short and out-of-season cycles, with a variable level of environmental control and foliar applications of biostimulants to produce prebiotic rich roots for fresh consumption; (ii) to ascertain to which extent variable environmental control level can affect the prebiotic content of the fresh chicory root.

6.2. Materials and Methods

6.2.1. Greenhouse Trial

The trial was carried out from 14 December 2020 to 14 April 2021 in a polyethylene unheated greenhouse at the Experimental Farm of Tuscia University (latitude 42°25' N, longitude 12°08' E, altitude 310 m). Chicory (*Cichorium intybus* L. var. Bischoff) seeds from L'Ortolano srl (Cesena, Italy) were used as plant material. Plants were grown under natural light conditions and air temperature ranged from 7 to 27 °C. The soil was a sandy loam soil having a pH of 7.3, organic matter content of 18.8 g/kg, total nitrogen of 1.16 g/kg, assimilable P of 35 mg/kg, and cation exchange capacity of 25.3 meq/100 g. Cation exchange capacity was saturated with the following elements: Ca 67.7%, Mg 13.9%, K 9.8%, Na 8.6%. Treatments were three commercial biostimulant products and an untreated control. The tested products were a vegetal-derived protein hydrolysate (Trainer®—Hello Nature Italia srl, Rivoli Veronese, Italy; VPH), an animal-derived protein hydrolysate (Siapton®—Isagro S.p.A., Milan, Italy; APH), and a seaweed extract (Toggle®—Acadian Plant Health, Nova Scotia, Canada; SWE). The commercial biostimulant Trainer® was a legume-derived protein hydrolysate produced through enzymatic hydrolysis; it contained 190 g/kg of organic carbon and 50 g/kg of organic nitrogen, 310 g/kg of peptides, small amount of free amino acids, and no phytohormones. The commercial Siapton® was a collagen-derived protein hydrolysate produced in high pressure and thermal conditions (140 °C for 30 min at 3.6 atm). It contained 250 g/kg of organic carbon and 85 g/kg of organic nitrogen, 543.5 g/kg of total amino acids, and 79 g/kg of free amino acids. The detailed compositions of Trainer® and Siapton® were reported by Rouphael et al. (2021) [28]. Toggle® was derived from marine plant extract obtained by extraction with water solution containing acids or alkali. It contained 20 g/kg of organic carbon and 7 g/kg of mannitol. Biostimulant treatments were foliarly applied from 19 January every week until the end of the experiment for a total of 8 applications. Every foliar spray treatment was carried out with a 3 L spray bottle. Biostimulant concentrations (2.5 mL/L of VPH, 1.5 mL/L for APH, and 1.5 mL/L for SWE) used in each foliar application were those suggested in the product

labels. Treatments were arranged in a completely randomized block design with 4 replicates for a total of 16 plots having a surface of 2.5 m² each. Before sowing, soil was broadcast fertilized with 1.0 t ha⁻¹ of a granular mineral fertilizer NPK 12-12-17 (Blue Altea[®]—Hello Nature Italia srl, Rivoli Veronese, Italy). Chicory seeds were sown on 14 December 2020 in single rows spaced 20 cm apart. After emergence, seedlings were thinned at 10 cm in order to reach a plant density of 30 plants m². A drip irrigation system was set up between the rows in order to irrigate two rows per drip line. Drip lines had in-line emitters located 0.30 m lines apart and an emitter flow rate of 3.4 L h⁻¹. Weeds were removed by hand hoeing as necessary during the whole experiment. In each biostimulant application date, the soil plant analysis development (SPAD) index and modulated chlorophyll fluorescence were measured. SPAD index was measured on fully expanded leaves by means of a portable chlorophyll meter SPAD-502 (Konica Minolta, Tokyo, Japan). Thirty healthy and fully expanded leaves were randomly measured and averaged to a single SPAD value for each experimental plot. Modulated chlorophyll fluorescence was measured, in dark adapted (for at least 15 min) leaves on three plants per plot, using a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd., King's Lynn, UK) with an excitation source intensity higher than 3000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the sample surface. The minimal fluorescence intensity (F₀) in a dark-adapted state was measured in the presence of a background far-red light to favor rapid oxidation of intersystem electron carriers. The maximal fluorescence intensities in the dark-adapted state (F_m) were measured by 0.8 s saturating pulses (3000 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The maximum quantum yield of the open photosystem II (PSII) (F_v/F_m) was calculated as (F_m–F₀)/F_m. Four plants per plot were sampled at 37 and 66 days after emergence (DAE) to weight the roots and calculate the average growth rate of the fresh biomass. Final harvest was performed 14 April 2021 (113 DAE). Forty plants per plot were harvested and leaves were cut from the taproot in order to weigh them separately and determine the fresh weight of aerial part and roots. Leaves and roots were separately dried in a ventilated oven at 75 °C and in a freeze dryer, respectively, until constant weight to determine the dry weights. Inulin yield per hectare was calculated by multiplying the inulin concentration by root dry biomass and plant density.

6.2.2. Growth Chamber Trial

The plant material was the same used for the greenhouse experiment. Plants were grown at the CNR-IRET, under fully controlled environmental conditions in a growth chamber (Fitotron SGD170 Sanyo Gallenkamp, Birmingham, UK) [29]. The chamber was equipped with two LED lamps (model LX60, Heliospectra AB, Goteborg, Sweden). During the experiment, temperature was set at 20 °C during the day and 15 °C during the night, CO₂ was maintained at 400 ppm, relative humidity at 70%, and photoperiod was 12 h day/12 h night. For germination, two seeds per cell were seeded in a polystyrene seed tray and irrigated with distilled water, under 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically photon flux density (PPFD), provided by cool white 5700 K LED. Germination occurred in six days; thereafter, the seedlings were irrigated with a nutrient solution having a pH of 6.5 and EC of 1.7 mS cm^{-1} as the one selected for leafy plants in the EDEN ISS project [30]. Seedlings were grown in plastic pots (9 × 9 × 20 cm) filled with perlite and irrigated with the same nutrient solution. After the transplant, light intensity was increased to 550 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, with a combination of cool white 5700 K, red, and blue LEDs. Plant density in the chamber was set at 16 plant m^{-2} . Chicory plants cultivated in the growth chamber were foliar treated with the two plant biostimulants used in the greenhouse trial: Trainer[®] (VPH) and Toggle[®] (SWE); the tested rates were those used in the greenhouse trial (2.5 mL/L for VPH, and 1.5 mL/L for SWE). Due to the negative performances in the greenhouse trial, the APH (Siapton[®]) was not used in the growth chamber experiment. A control treatment where leaves were sprayed with only water was also included. Foliar treatments started twenty-eight DAE and were carried out every week, until the end of the experiment for a total of four applications. The three treatments were arranged in a completely randomized design with eighteen plants per treatments.

At 30, 37, and 55 days after planting (DAP), four plants per plot were collected and weighted to determine the fresh biomass. At final harvest, six plants per treatment were collected and the roots were quickly washed, then the leaves were cut from the taproots and the fresh weight of the two organs was measured. The same samples used for the fresh biomass analysis were

dried with a freeze dryer in order to assay the dry matter content and the non-structural carbohydrate content.



Figure 1: (Left) greenhouse trial; (Right) growth chamber trial.

6.2.3. Non-Structural Carbohydrate

For compositional analysis, dried roots from both trials were milled to a fine powder using an MF 10 miller IKA (IKA®-Werke GmbH & Co. KG, Staufen, Germany) and sieved to screen particles until a 0.5 mm grid. For non-structural carbohydrate (NSC) extraction, 20 mg of dried root powder was quenched in 0.5 mL of 100% ethanol at 70 °C in open screw plastic tubes until complete ethanol evaporation was achieved. The solid residue was resuspended in 1 mL of water and extracted at 70 °C for 2 h under vigorous stirring. The extract was centrifuged at 9000× g for 10 min. The supernatant was passed through a nylon filter 0.45 µm PPII syringe filters (Whatman Inc., Maidstone, UK) and subsampled. One aliquot was used for fructans hydrolysis [27] and a second aliquot for the measurements of glucose, fructose, and sucrose. Fructans were hydrolyzed in 60 mM HCL for 2 h at 70 °C with vigorous stirring. Glucose, fructose, and sucrose present in the first aliquot of the extract and fructose and glucose derived by the fructans hydrolysis were measured by high-performance anion exchange chromatography, with pulsed amperometric detection (HPAEC-PAD), equipped with a gold working electrode (1.0 mm in diameter) and an Ag/AgCl reference electrode (Dionex™ ICS-5000, Thermo Fisher Scientific,

Waltham, MA, USA). An analytical CarboPac PA100 column (4 mm × 250 mm) with a relative guard column was used. All runs were carried out at 30 °C using a mobile phase gradient with two aqueous solutions: (A) NaOH 1 mol L⁻¹; (B) Na-acetate 1 mol L⁻¹ at a flow rate 1 mL min⁻¹ as described in [28]. Calculations of inulin concentration and average degree of polymerization (DP_{av}) is as reported in Verspreet et al. [29]:

$$(1) \quad \text{Inulin (\%)} = k (G_f + F_f)$$

$$(2) \quad k = \frac{180 + 162 (DP_{av} - 1)}{180 DP_{av}}$$

$$(3) \quad DP_{av} = \frac{F_f}{G_f} + 1$$

where G_f and F_f are the concentration of glucose and fructose released from fructan and are calculated as follow:

$$(4) \quad G_f(\%) = \frac{180.16 V_{ex} (G_a - G_b - S_a)}{10000 m_s}$$

$$(5) \quad F_f(\%) = \frac{180.16 V_{ex} (F_a - F_b - S_a)}{10000 m_s}$$

where 180.16 is the molecular weight of glucose or fructose, V_{ex} is the volume of the extract (mL), m_s is the sample mass (mg), G_a and G_b are the concentration of glucose (μM) in the nonhydrolyzed and hydrolyzed sample, respectively, F_a and F_b are the concentration of fructose in the nonhydrolyzed and hydrolyzed sample, respectively (μM), and S_a is the concentration of sucrose in the nonhydrolyzed sample (μM).

6.2.4. Statistical Analysis

The statistical analysis was carried out using IBM SPSS Statistics 20 (Chicago, IL, USA). All the parameters of the greenhouse and growth room experiments were subjected to one-way analysis of variance (ANOVA). Mean values were separated according to Tukey's test with $p = 0.05$.

6.3. Results

6.3.1. Greenhouse Experiment

In the greenhouse experiment, seedling emergence occurred nine days after sowing (DAS) and the first true leaf stage was recognized 18 days after emergence (DAE). The start of root thickening was visually observed at 66 DAE when the root fresh weight resulted in an average of 10.5 g. The average growth rate of fresh root biomass from 37 to 66 DAE and from 66 DAE until the end of the experiment (last 47 days), resulted in $0.37 \text{ g} \cdot \text{day}^{-1}$ and $1.60 \text{ g} \cdot \text{day}^{-1}$, respectively. Bolting rate at harvest was not significantly affected by treatments (avg. 51%). Treatments did not significantly affect the SPAD index and maximum quantum yield of PSII of chicory leaves, as shown in Table 1 and Table 2.

Table 1. Effect of biostimulant treatments on SPAD index of chicory leaves during the greenhouse trial.

Treatment	SPAD Index							
	66 DAS	73 DAS	80 DAS	87 DAS	94 DAS	101 DAS	108 DAS	115 DAS
Control	35.3	43.3	38.0	39.9	42.4	45.7	49.5	76.0
VPH	36.4	41.5	41.5	40.6	42.9	46.6	48.5	75.2
APH	35.8	40.4	42.3	39.8	38.4	40.9	46.0	76.8
SWE	35.4	41.7	39.5	41.2	40.2	42.7	47.6	68.3
Significance	ns	ns	ns	ns	ns	ns	ns	ns

VPH = Vegetal Protein Hydrolysate; APH = Animal Protein Hydrolysate; SWE = Seaweed Extract. DAS = Days after sowing. ns = not significant.

Table 2. Effect of biostimulant treatments on maximum quantum use efficiency of PSII (Fv/Fm) of chicory leaves during the greenhouse trial.

Treatment	Fv/Fm							
	66 DAS	73 DAS	80 DAS	87 DAS	94 DAS	101 DAS	108 DAS	115 DAS
Control	0.805	0.772	0.790	0.807	0.791	0.794	0.820	0.793
VPH	0.814	0.762	0.778	0.815	0.769	0.813	0.818	0.783
APH	0.811	0.769	0.783	0.788	0.760	0.789	0.798	0.800
SWE	0.813	0.772	0.789	0.805	0.792	0.805	0.797	0.783
Significance	ns	ns	ns	ns	ns	ns	ns	ns

VPH = Vegetal Protein Hydrolysate; APH = Animal Protein Hydrolysate; SWE = Seaweed Extract. DAS = Days after sowing. ns = not significant.

The maximum quantum yield of PSII ratio (Fv/Fm) of leaves has been largely used as a sensitive marker of plant photosynthetic performance; Fv/Fm significantly decreased in leaves when PSII photoinhibition is induced by stress conditions. In the greenhouse trial, the Fv/Fm values of chicory leaves, displayed in Table 2, were always in the range typically recorded for healthy plants (0.75–0.85).

Table 3 shows the results of plant biomass analysis and inulin content in chicory roots, while the concentrations of glucose, fructose, and sucrose in chicory roots are reported in Table 4. VPH applications determined a statistically significant increase of fresh and dry weight, and diameter of taproot in comparison with the application of APH while SWE and Control treatments gave intermediate values which were not statistically different from both VPH and APH treatments (Table 3). No statistical differences were recorded for leaf fresh and dry biomass, leaf dry matter, root length, root dry matter, shoot/root ratio, and inulin concentration in roots (Table 3). The degree of polymerization of inulin was on average 9.17%, and no differences resulted from the biostimulant treatments (data not shown). Average leaves and root fresh weight of the whole trial were $130 \text{ g} \cdot \text{plant}^{-1}$ and $42 \text{ g} \cdot \text{plant}^{-1}$, respectively, corresponding to a production of fresh leaf and root of $38.98 \text{ t} \cdot \text{ha}^{-1}$ and $12.59 \text{ t} \cdot \text{ha}^{-1}$, respectively. The inulin yield per hectare (Figure 2), was significantly increased by VPH (avg + 29%) compared to Control and APH treatments. The SWE treatment resulted in an intermediate value with no significant differences with VPH, APH, and Control treatment.

Table 3. Effects of plant biostimulants on fresh and dry biomass of leaves and roots, root characteristics, shoot to root ratio, and root inulin concentration in the greenhouse chicory trial.

Treatment	Leaf Fresh Weight	Leaf Dry Matter	Leaf Dry Weight	Root Fresh Weight	Maximum Root Diameter	Root Length	Root Dry Matter	Root Dry Weight	Shoot/Root	Inulin Concentration
	(g plant ⁻¹)	(%)	(g plant ⁻¹)	(g plant ⁻¹)	(cm root ⁻¹)	(cm root ⁻¹)	(%)	(g plant ⁻¹)		(% dry wt.)
Control	134.7	5.89	8.33	42.1 ab	2.19 ab	17.0	15.3	6.44 ab	1.65	44.7
VPH	139.4	6.10	10.02	48.6 a	2.37 a	18.0	15.8	7.98 a	1.65	46.2
APH	117.0	6.44	10.65	35.4 b	2.05 b	16.4	17.0	6.02 b	1.94	47.4
SWE	128.7	6.08	10.67	41.9 ab	2.29 ab	17.0	17.1	7.18 ab	1.74	47.9
Significance	ns	ns	ns	**	*	ns	ns	*	ns	ns

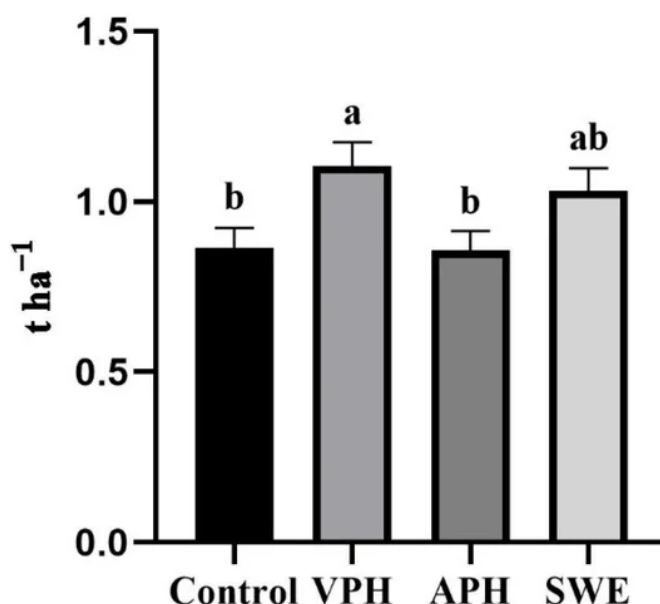
VPH = Vegetal Protein Hydrolysate; APH = Animal Protein Hydrolysate; SWE = Seaweed Extract. *, ** Significant at $p < 0.05$ or 0.01 . ns = not significant. Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p = 0.05$).

Table 4. Effect of biostimulant treatments on glucose, fructose, and sucrose concentration of chicory roots in the greenhouse trial.

Treatment	Soluble Carbohydrate Concentration (% dry wt.)		
	Glucose	Fructose	Sucrose
Control	1.18	0.33	3.00
VPH	1.38	0.30	2.59
APH	1.37	0.32	3.10
SWE	0.92	0.37	2.45
Significance	ns	ns	ns

VPH = Vegetal Protein Hydrolysate; APH = Animal Protein Hydrolysate; SWE = Seaweed Extract. ns = not significant.

Figure 2. Effect of biostimulants on inulin yield per hectare in greenhouse chicory. Bars indicate the standard errors of the mean values. Different letters indicate significant differences according to Tukey's multiple-range test ($p = 0.05$). VPH = Vegetal Protein Hydrolysate; APH = Animal Protein Hydrolysate; SWE = Seaweed Extract.



6.3.2. Growth Chamber Experiment

In the growth chamber harvest occurred 64 days after planting (DAP). At 30, 37, 55 DAP the average weights for fresh taproots were 8.80, 21.57, and 42.24 g, respectively. The taproot biomass growth rates between 0–37 and 37–64 DAP was not statistically different between treatments and resulted in an average of 0.58 and 0.81 g·day⁻¹, respectively. At harvest, no bolting was detected in all chicory plants. No statistical differences were determined by the biostimulant treatments on the selected plant variables (Table 5 and Table 6). In terms of inulin yield, on the hectare basis, the treatments showed no significant differences in comparison with untreated plants, resulting in 1.0 t·ha⁻¹ for VPH and SWE and 0.7 t·ha⁻¹ for the control. The estimated average production resulted in 12.2 t·ha⁻¹ and 6.95 t·ha⁻¹ of fresh leaf and root, respectively. Taproot fresh weight per plant was 43.5 g·plant⁻¹ on average. Average leaf fresh weight was 76.78 g·plant⁻¹. The average percentage of taproot dry matters at the end of the experiments was 25.5%. The related dry

mass production amounted to $1.8 \text{ t} \cdot \text{ha}^{-1}$. No substantial differences were found for S/R ratio that resulted in an average of 0.98. Average inulin content was 57% of the dry matter. Thus, average inulin production resulted in $0.95 \text{ t} \cdot \text{ha}^{-1}$. The average degree of polymerization of the inulin chain resulted in an average of 13.5% with no significant differences among treatments (data not shown).

Table 5. Effects of plant biostimulants on fresh and dry biomass of leaves and roots, root characteristics, shoot to root ratio, and root inulin concentration in growth chamber chicory trial.

Treatment	Leaf Fresh Weight	Leaf Dry Matter	Leaf Dry Weight	Specific Leaf Dry Weight	Root Fresh Weight	Root Dry Matter	Root Dry Weight	Shoot/Root	Inulin Concentration
	(g plant ⁻¹)	(%)	(g plant ⁻¹)	(g cm ⁻²)	(g plant ⁻¹)	(%)	(g plant ⁻¹)		(% dry wt.)
Control	67.63	12.20	10.45	2.93	37.63	25.05	9.45	1.11	54.42
VPH	87.55	12.74	12.74	2.82	46.73	27.02	12.84	0.99	59.57
SWE	73.70	10.76	9.65	3.09	46.00	24.69	11.40	0.85	56.65
Significance	ns	ns	ns	ns	ns	ns	ns	ns	ns

VPH = Vegetal Protein Hydrolysate; SWE = Seaweed Extract. ns = not significant.

Table 6. Effect of biostimulant treatments on glucose, fructose, and sucrose concentration of chicory roots in the growth chamber trial.

Treatment	Soluble Carbohydrate Concentration (% dry wt.)		
	Glucose	Fructose	Sucrose
Control	0.89	0.83	1.63
VPH	1.09	1.33	1.41
SWE	0.92	0.89	1.73
Significance	ns	ns	ns

VPH = Vegetal Protein Hydrolysate; SWE = Seaweed Extract. ns = not significant.

6.4. Discussion

Controlled environment (partial or full) allowed to grow chicory for out-of-season production of health promoting vegetables, with high prebiotic potential. Different works give insights regarding the open field cultivation aimed to the conventional production of inulin, extracted, and used for food and industrial purposes [16, 18, 31]. Less is known about out-of-season cultivation conditions oriented to the production of fresh vegetables and ready to eat products. The choice of suitable environmental and agronomic inputs, in any case, is essential for controlling the production cycle and guaranteeing the quality and quantity of the yield.

For chicory taproot, Van Arkel and coworkers [16] described three growing phases based on changes observed in the pattern of inulin accumulation in field conditions. These phases can be well correlated with the phenological status of the crop and are influenced by environmental factors: phase 1—onset of root thickening and inulin biosynthesis; phase 2—substantial increase of root mass and inulin accumulation but decreasing of the inulin polymerization grade; phase 3—polymerization grade continues to decrease, and the yield remains constant. In our experiments, only phase 1 occurred due to the short cycle adopted. This phase in Van Arkel [16] field condition experiment occurred in one month. According to the growth rate of our experiments, the start of root thickening and the onset of phase 1 occurred in two months for greenhouse and one month in the growth chambers determining different duration of the two experiments. In the greenhouse, the delay of the start or root thickening could be attributable to the low temperature and low light availability of the winter period. In our experiment, sowing time (held in December) largely anticipates the conventional growing season of chicory taproot, that usually starts in early summer in full field conditions. On the other hands, in the growth chamber, thanks to full environmental control, plants experienced constant and largely optimal conditions both in terms of temperature and light availability during the full growth cycle, resulting in a faster growth rate.

The sowing time in the greenhouse and the management of environmental parameters in the growth chamber resulted also to be an important factor that could influence the transition to the reproductive stage in chicory plant.

Despite the fact that chicory inflorescence has high feed quality and is desirable for foraging production [32, 33], in chicory cultivation for root production, bolting is a negative occurrence since it causes lignification of the root, a decrease in root productivity, and might allow seed dispersal, favoring the spread of the species as a weed [34]. Floral induction is mainly driven by low temperature, and flowering requires a long day photoperiod [35]. However, cold requirement depends on the variety, age of the plant, and occurrence of other environmental stresses; high temperature, for example, can devernalize chicory or induce bolting depending on concurring environmental and physiological variables [17]. In the unheated greenhouse, low temperature in early phases led to floral induction and bolting occurred under favorable photoperiod. Moreover, vernalization conditions are unfavorable for a rapid plant growth. In the greenhouse trial, low temperatures up to 7 °C induced vernalization, and fast stem elongation started at the beginning of April, in concomitance of the occurrence of longer photoperiod and high temperatures (up to 27 °C). Hence, greenhouse experiment's growing season resulted to be favorable for chicory flower induction and bolting. No bolting occurred in the growth room since temperatures were higher, allowing a faster development of plants while avoiding vernalization. Reduced bolting can be obtained by genetic improvement and by a high level of temperature control in the greenhouse. Less feasible, although technically possible, appears to be the control of the photoperiod. A few works have shown a correlation between the use of plant biostimulants and early flowering [36, 37] and an increase of flower number [38] in horticultural crops, but no information is reported for chicory. In our trials, we did not observe any effects of tested biostimulants on bolting of chicory plants.

The management of variables related to light input, such as light intensity, photoperiod, and DLI, is a powerful tool to modulate fresh and dry biomass, growth rate, along with the duration of the production cycle of plants. Moreover, especially in growth chambers, a correct use of light inputs results in high use efficiency of resources such as water, light, and energy (WUE, LUE, and EUE), essentials for a sustainable and feasible indoor cultivation. Pennisi et al. (2020)[39] compared three different DLI (14.4, 18, and 21.6

$\text{mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) in chicory cultivar. Plants grown under $14.4 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ resulted in higher leaf area (+81%), leaf fresh biomass (+47%), along with high WUE (33%), LUE (57%), and EUE (120%) when compared to a DLI of $21.4 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$. The fresh biomass and the other use efficiency parameters decrease as the DLI increases. In contrast, plants grown with DLI of $21.4 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ resulted in higher leaf dry matter content. The increase in dry matter content in plants undergoing longer days was previously related to higher carbohydrate production through photosynthesis in lettuce [40]. An increase in photosynthesis due to the optimal light management can result in an increase of photosynthate allocation in sink organs [29]. In chicory taproot plants, an increase of the source potential due to high DLI could hypothetically result in higher inulin yield and DP in roots especially in a short production cycle when the root is harvested during an ongoing active accumulation phase. Our results indicate that in the growth chamber, where the DLI was $23.76 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, leaf and taproot dry matter and inulin content resulted higher than in the greenhouse where the estimated average DLI was $15 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, although no considerable differences were detected for the single root weight. By contrast, leaf fresh biomass resulted higher in greenhouse than in the growth chamber. Taproot fresh biomass production per unit area of the greenhouse resulted to be 2.3-fold higher than in the growth chamber, but this was associated with the higher greenhouse plants' density. However, dry matter content in the growth chamber showed a 1.6-fold increase with respect to the greenhouse chicory, resulting in almost the same inulin yield per square meter. The higher inulin DP obtained in the growth chamber could be attributed to the optimal range of temperature adopted. Low temperatures increase the effect of the hydrolytic activity of fructan exohydrolases (FEH) enzymes, promoting fructans depolymerizations [41]. In the growth room, temperatures were kept at 20 °C during the day and 15 °C at night, and at this temperature range a low FEH activity is expected. This consideration suggests the possibility to modulate the DP in order to achieve the desired chain length in observation of the market objectives [42]. Moreover, growth room production gave a high amount of fresh leaf and taproot biomass. The grow room data demonstrate that a fully controlled environment allows to shorten the production cycle and

to better control quality attributes of chicory taproots. Cultivation in vertical farming systems could represent a feasible and sustainable manner to produce chicory in urban areas coupling the increase of the cultivation surface allowed by vertical farming [43] with the possibility to control production calendar, produce availability, and its quality characteristics.

Plant biostimulants, showed the potential to affect differentially the crop performances from quali-quantitative point of view. The greenhouse production of inulin calculated on the hectare basis resulted to be statistically higher by 29% in the VPH treatment in comparison with Control and APH treatments. The above findings are mainly related to the significant positive effects of biostimulant on root dry matter production. Colla and coworkers [22] demonstrated that bioactive compounds in VPH such as small peptides and amino acids can stimulate plant growth and productivity through the modulation of primary and secondary metabolism. Moreover, several bioassays reported that the tested VPH ‘Trainer[®]’ exerted an auxin-like activity stimulating root growth [44, 45]. On the other hand, APH treatment caused a decrease of (i) the root fresh weight, (ii) the maximum root diameter, (iii) the root dry weight, and iv) the fresh and dry root production per hectare with respect to the application of VPH. Phytotoxicity effects along with suppression of growth in concomitance with the use of animal-derived PHs were already reported by several authors [28, 46–48]. The phenomenon is recognized as “general amino acid inhibition”, and it is due to an excessive leaf uptake of free amino acids leading to intracellular amino acid imbalance [49]. Based on the negative performances of APH under greenhouse trial, only VPH and SWE were considered in the growth chamber experiment. The lack of significant differences among biostimulant treatments under growth chamber conditions is in line with the role of biostimulants to ameliorate plant productivity under limiting growing conditions. In the growth chamber, plants experienced optimal growing conditions, reducing the benefits of biostimulant applications. However, it should also be considered that the small sample size under growth chamber conditions together with the high genetic variability of the chicory open-pollinated cultivar increased the experimental error making difficult to detect significant differences among biostimulant treatments.

Inulin yield is considered the most important criterion in taproot chicory production and breeding [50]. In a conventional spring to autumn production cycle, recorded inulin yields per hectare are ten-fold higher than the one obtained here under different types of controlled conditions [51]. However, conventional productions are aimed to the extraction and isolation of the polymer in order to satisfy food and industrial market requests [16]. Our work was focalized on the production of prebiotics for rich, fresh vegetables throughout a fast and out-of-season production cycle, and we demonstrated the feasibility of this production procedure. The taproot at this production stage can be eaten fresh or cooked, or easily transformed by the food industry to be consumed in various kinds of healthy, ready-to-eat products. The young taproots, harvested much early than in normal field production systems, were rich in prebiotics and valuable for the fresh products market. Furthermore, when different levels of environmental control were applied, we obtained fresh roots of similar size, a couple of which could be considered a reasonable food serving, but with different inulin content. The roots harvested in the growth room trial had higher inulin content than the roots produced under greenhouse conditions. A serving of 100 g of chicory root produced in the growth chamber could provide on average about 14.5 g of inulin, while the same amount of root obtained in the greenhouse would provide almost half of that amount.

There is a fast-rising body of evidence indicating that the daily consumption of prebiotics in addition to fiber can be a powerful option to ameliorate human and animal health [52–54]. According to EFSA, the recommended daily intake of “chicory-type inulin” for human is up to 12 g/day [55]. Recently, it was pointed out that eating the full chicory root can be a better way of prebiotic intake than assuming inulin-based nutritional integrators where inulin is almost pure or at very high concentration [9] since the root also contains cell wall polymers, such as cellulose, hemicellulose, pectin, and lignin, and other phytochemicals. An excessive intake of inulin and fiber can cause discomfort or have detrimental effects on gut functioning; hence, an inulin-rich food should be managed easily to provide the correct amount of prebiotic. Our results on the differences among growth chamber- and greenhouse-produced chicory taproot show that the inulin amount in 100 g of

fresh root can be modulated within a large interval via environmental control of the growing conditions. This opens new and interesting perspectives for developing controlled agriculture systems for Earth- and space-oriented functional food production.

6.5. Conclusions

The results demonstrated the possibility to produce fresh chicory taproot with high prebiotic content in a short growing cycle and out of season both in greenhouse and growth chambers. The controlled environment offers the opportunity to modulate the growing conditions to optimize the inulin yield. The estimation of the necessary surface area in the growth chamber, considering our growth conditions, to produce enough root biomass daily to meet the recommended daily intake of inulin for one person is equivalent to 8.08 m². The application of plant biostimulants showed significant effects only under greenhouse conditions with an increase of inulin yield by 27.8% with foliar sprays of VPH compared with untreated control. The foliar applications of the other two biostimulants (APH and SWE) were not effective to enhance crop growth and inulin yield in comparison with untreated control. In conclusion, the trials also highlighted that controlled-environment agriculture is a suitable way of producing young chicory taproot for fresh consumption and highlighted the potential benefits of this production system in modulating prebiotic content of chicory roots for Earth and space applications. Further studies could identify the potential benefits of PBs for seed treatment or root application and to understand the mode of action of PBs through metabolomics. Finally, in the framework of the “ReBUS” project, the plant material from the roots obtained in our experimental trials was sent to the Department of Cell Biology and Neuroscience, National Institute of Health (Italy), for experimental tests on a murine model of chronic stress. The work is still in progress, but the data are promising and indicate that fructans contained in chicory roots, by supporting microbial communities and/or through the release of metabolites (short-chain fatty acids), contribute to the recovery of various alterations induced by chronic stress. This is observed at both the behavioral and physiological levels, highlighting the

potential of prebiotic-rich vegetables to counterbalance the detrimental effects of stress and support astronauts' performances.

References are listed in Appendix V of the bibliography section.

7. Qualitative traits of hydroponically grown *Eruca vesicaria* L. treated with continuous blue light

7.1. Introduction

The introduction of LED technology has marked a revolutionary shift in agricultural practices, providing researchers with unprecedented precision in adjusting light parameters towards better control over crop production [1, 2]. Notably, LEDs, distinguished by their adaptable spectrum and energy efficiency, have risen to prominence as the preferred lighting technology in Controlled Environment Agriculture (CEA) [3, 4]. Scientists have undertaken thorough investigations to examine the impacts of diverse light treatments on plant production and quality response. These studies have delved into a wide range of light management, encompassing different light intensity levels [5, 6], modifications in light spectral composition [7, 8], alterations in photoperiod durations [9, 10], preharvest supplemental treatments [11, 12] and postharvest interventions during storage [13, 14].

Continuous light (CL) treatment, characterized by exposure to continuous illumination without a normal day-night rhythm, has been the subject of extensive scientific investigation in the context of crop production [15]. This approach is based on the concept of diminishing light intensity while extending the duration of illumination over 24 hours, aiming to achieve a substantial daily light integral (DLI) while concurrently reducing the quantity of photons delivered per unit of time [10]. Scientific studies have explored the impact of CL on plant biomass accumulation, photosynthetic efficiency, and nutritional quality, demonstrating its potential to influence key physiological processes [15–17]. Apart from assessing the physiological response of plants to CL to quantify tangible gains in productivity and quality in CEA, other potential benefits encompass resource efficiency including a reduction in the electric power peaks demanded by the system, as well as a decrease in the size requirements of the illumination and cooling systems [10].

Beyond CL, research exploring the effects of specific light spectra on plant physiology has generated substantial insights into the multifaceted ways light

shapes crop production and quality [18, 19]. Notably, studies have demonstrated that blue light (465 nm) might play a relevant role in modulating plant metabolisms and the subsequent various quality related aspects such as the antioxidant capacities in several plant species [20–22]. Therefore, the ongoing exploration of blue light management in CEA on a species-specific basis holds the potential to provide valuable insights for improving the nutraceutical content and quality of horticultural species.

Among various studies, it has been demonstrated that the pool of ascorbic acid (AsA) in horticultural species can be regulated by the blue light component of the spectrum. In particular, Zhang et al., 2020 [23], showed that low Red:Blue light ratios increase the total ascorbate content in lettuce. Kang et al., 2019 [24], demonstrated that blue light radiation enhances AsA accumulation in Chinese cabbage seedlings by activating gene expression involved in biosynthetic and recycling pathways. End-of-production treatment with a high proportion of blue light irradiation improved AsA accumulation by enhancing the activities of key enzymes responsible for AsA synthesis enhancing the nutritional quality of lettuce [25]. The most effective exogenous precursor of L-ascorbic acid is L-galactono-1,4-lactone, which is directly converted to AsA by the mitochondrial enzyme L-galactono-lactone-1,4-lactone dehydrogenase (GalLdh). AsA pool is also regulated through the AsA recycling pathway, involving the enzymatic activity of monodehydroascorbate reductase (MDHAR) catalyzing the reduction of monodehydroascorbate (MDhA) in AsA, dehydroascorbate reductase (DHAR) catalyzing the reduction of dehydroascorbate (DhAs) in AsA, and ascorbate peroxidase (APX) which is responsible for AsA oxidation in MDhA which can spontaneously dissociate to DhAs. Modulating the Blue light component of the spectrum in combination with the extension of the photoperiod could be a way to increase AsA content in responding species.

Eruca vesicaria (L.), a member of the *Brassicaceae* family, stands out as a food crop with considerable importance, given its substantial commercial value, especially as a widely favored ingredient in fresh salads around the world. Remarkably, this plant has been identified and selected as one of the optimal species for food production in space, as highlighted by Dueck et al., (2016) [26]. Moreover, it demonstrates the capability to thrive and yield in

CEA [27, 28] also with great performance under CL condition [10]. In this study, we chose to implement a CL treatment on *Eruca vesicaria*, specifically focusing on the blue wavelength. This approach aimed to provide 24 hours of constant-intensity blue light (48 PPFD), and 12 hours of full spectrum light (232 PPFD) only during the daylight phase. The first aim was to assess potential quality gains through the light treatment, with a particular emphasis on the accumulation of AsA, considered as the main quality-driving attribute in the context of this thesis.

7.2. Materials and Methods

7.2.1. Plant Materials and Growth Conditions

Rocket, *Eruca vesicaria* (L.) (La Semiorto Sementi, Sarno Salerno, Italy), was grown at the Consiglio Nazionale delle Ricerche-Istituto di Ricerca sugli Ecosistemi Terrestri (CNR-IRET) under fully controlled environmental conditions in a growth chamber (Fitotron SGD170 Sanyo; Gallenkamp, Leicestershire, United Kingdom). The chamber, that was equipped with two LED lamps (model LX60; Heliospectra AB, Gothenburg, Sweden), was divided into two sections (one for treatment and one for control) by a reflective and perforated panel, allowing air circulation and maintaining a homogeneous environment in both sections. Two Seeds were sown in each 2 ml Eppendorf tubes (Eppendorf Srl, Milan, Italy), filled with perlite and vermiculite and whose bottom end was removed. The Eppendorf tubes were positioned in plastic racks (24 × 34 cm) each with 224 seats and positioned longitudinally in the plastic rack, leaving a row of empty seats between each row of Eppendorf tubes, covering a total of 112 seats. Each rack was fixed on the top of a container with a water capacity of 8 L, with half of the tube immersed in water. Seeds were germinated under 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically photon flux density (PPFD), provided for 12 h per day (12-h dark) by cool white 5700 K LED up to 9 days after emergency (DAE) when the treatment starts (time zero). At this stage, one hundred twelve plants were grown in each container at a density of 2,105 plants per m^2 , and the water was substituted with an aerated and complete nutrient solution as the one used by Proietti et al., (2021) [10]. The nutrient solution was renewed every week, ensuring that pH and EC were maintained at 6.5

and 1.7 mS cm^{-1} , without relevant changes. A temperature of $22 \pm 1^\circ\text{C}$ / $25 \pm 1^\circ\text{C}$ night/day, relative air humidity of $70 \pm 1\%$, and CO_2 concentration of 600 ppm were maintained.

7.2.2. Light treatment

The lamps (model LX60; Heliospectra AB, Gothenburg, Sweden) were equipped with four types of dimmable (0-1000) LEDs. The emission characteristics of the different LED types were as follows: blue LEDs (B), 450 nm; red LEDs (R), 660 nm; far red LEDs (FR), 730 nm; and the cool white LEDs (W), 5700 K. The W LEDs had an emission spectrum of B = 26%, green (G) = 45%, R = 27%, and FR = 2%. Two light treatments started from time zero until harvest: 1) control with a photoperiod of 12-h light/12-h dark and PPFD of $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by W, R and B LEDs to obtain a final R:B:G ratio of 1:1:1 and a total of $96 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light; 2) continuous blue light (CBL) treatment with a photoperiod of 24-h that provides a continuum of $48 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light and 12h of the rest of the spectrum at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided entirely by cool white LEDs with an R:B:G ratio of 2:1:2. Both light treatments received the same daily cumulative intensity of photosynthetically active quanta (as daylight integral – $\text{DLI} = 12.10 \text{ mol m}^{-2} \text{day}^{-1}$). The 12 h at 48/0 (CBL /Control) $\mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light is defined as the “night period” while the 12 h at 248/280 (Treatment /Control) $\mu\text{mol m}^{-2} \text{s}^{-1}$ of full spectrum is defined as “day period”.

7.2.3. Plant Measurements and Compositional Analysis

Harvest occurred 20 days after time zero. The total number of plants for each rack was divided into two samples, for each of which fresh weight, dry weight, dry matter percentage, leaf area index (LAI), and ash content were determined. Leaf area was calculated by scanning two subsamples, each weighing 20 g, from each rack that were analyzed using the ImageJ software. Specific leaf dry weight (SLDW) was calculated using 4 leaf discs of 1.90 cm^2 for each rack (8 leaf discs for each treatment) cut from the lamina with a cork borer. Ash content was determined only for the shoot by ignition of the dried powder at 575°C in a furnace (P 300; Nabertherm GmbH, Lilienthal, Germany), with temperature ramping according to the NREL/TP-510-42622

method [29]. For the quantification of non-structural carbohydrates (NSCs), pigments, total phenolic content, total anthocyanins, organic acid, and inorganic anion concentration, fresh samples were collected before the harvest at the end of the “night period” and immediately frozen in liquid nitrogen, ground to a fine powder in a mortar using a pestle under liquid nitrogen conditions and preserved at -80°C until analyses. For AsA analysis and AsA related enzyme activities, plants were sampled at harvest in two times during the photoperiod t: 1) at the end of the “night period”; 2) at the end of the “day period”.

7.2.4. Ascorbate

Asorbic acid (AsA), dehydroascorbic acid (DhAs) and total Ascorbic Acid (TAsA) content analysis was performed as reported in paragraph 3.2.11 (chapter 3).

7.2.5. Non-Structural Carbohydrates

Measurements of non-structural carbohydrates (NSCs) were performed as reported in paragraph 3.2.8. (chapter 3).

7.2.6. Pigments

Pigments content analysis of chlorophylls (a and b), β -carotene, lutein, neoxanthin and violaxanthin, was achieved as reported in paragraph 3.2.9. (chapter 3).

7.2.7. Total Anthocyanins and Total Phenolic Content

Total anthocyanins (Tot. Ant) and the total phenolic comontent (TPC) were determined as reported in paragraph 3.2.10. (chapter 3).

7.2.8. Anions content

The analysis of inorganic anions (nitrate, sulfate, phosphate, malate and citrate) was conducted as reported in paragraph 3.2.12. (chapter 3).

7.2.9. Enzyme activity

Galactono 1-4 lactone dehydrogenase (GalLDH) (EC 1.3.2.3) activity was assayed by monitoring the reduction of Cytochromes c (Cyt c) at 550 nm according to **Zha et al.** (2019) [30] with some modification. 0.3 g leaf tissues were extracted by 2 mL pre-cooled extraction buffer which contained 100 mM PBS (pH 7.4), 0.4 M sucrose, 10% (v/v) glycerol, 1 mM EDTA, 5 mM TCEP, and 1% (w/v) polyvinylpyrrolidone (PVP). The reaction mixture (1 mL) contained 50 mM potassium phosphate (pH 7.8), 1.05 mg/mL Cyt c, 5.6 mM L-Galactono-1,4-lactone, and 0.1 mL enzyme extract. One unit of the enzyme was defined as the activity that oxidized 1 μ mol of L-Galactono-1,4-lactone corresponding to the reduction of 2 μ mol of Cyt c per min.

For the assay of monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4) and dehydroascorbate reductase (DHAR) (EC 1.8.5.1) 0.3 g leaf tissues were homogenized in with 2 mL pre-cooled extraction buffer. The extraction buffer contained 50 mM PBS (pH 7.5), 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.2% (v/v) and 2% (w/v) polyvinylpyrrolidone (PVP). The extract was centrifuged at 4 °C, then the supernatant was used for the spectrophotometric measurement using the method described in Miyake and Asada (1992) [31] with some modification. MDHAR activity was assayed by monitoring the absorbance at 340 nm. The reaction mixture (3 mL) contained 50 mM HEPES-KOH (pH 7.6), 0.5 mM ascorbate, 0.3 mM NADH, 0.5 units ascorbate oxidase, and 0.1 mL enzyme extract. One unit of the enzyme was defined as the amount that oxidized 1 μ mol of NADH per minute. DHAR activity was assayed by monitoring the absorbance at 265 nm. The reaction mixture (3 mL) contained 100 mM HEPES-KOH (pH 7.0), 1 mM EDTA, 2.5 mM GSH, 0.6 mM DHA, and 0.1 mL enzyme extract. One unit of the enzyme was defined as the amount that reduced 1 μ mol of DHA per min. To assay Ascorbate peroxidase (APX) (EC 1.11.1.11) activity, 0.3 g leaf tissues were extracted by the same extraction buffer used for GalLDH. The enzyme activity was determined by monitoring the reduction of ascorbate absorbance at 290 nm [32]. The reaction mixture (3 mL) contained 50 mM potassium phosphate (pH 7.8), 0.5 mM AsA, 0.25 mM H₂O₂, and 0.1 mL enzyme extract. The reaction was triggered by adding H₂O₂. One unit of the enzyme was defined as the amount that oxidized 1 μ mol of AsA per min.

7.2.10. Statistical Analysis

The statistical analysis was carried out using IBM SPSS Statistics 20 (Chicago, IL, USA). All the parameters of the experiment were subjected to one-way analysis of variance (ANOVA).

7.3. Results

7.3.1. Biomass assay

Statistically significant increase of 9.9% in dry matter content was observed in the CBL treatment group compared to the control group after 20 days after time zero (Table 1). Furthermore, the SLDW of vegetables treated with continuous blue light exhibited a significant increase of 19,9 % compared to the untreated samples. No significant differences in fresh weight, dry weight, ash content and LAI between the CBL treated group and the control group were recorded.

Table 1. Means comparison for growth-related traits of fresh weight, dry weight, specific leaf dry weight (SLDW), ash and leaf area index (LAI) after 20 days after time zero of *Eruca vesicaria* grown under CBL and Control.

	Fresh weight (g m ⁻²)	Dry weight (g m ⁻²)	Dry matter (%)	SLDW (g m ⁻²)	Ash (% DW)	LAI
CBL	3471.06	239.17	6.86	40.00	7.07	7.43
Control	3111.47	193.51	6.24	33.36	7.80	6.53
Sign.	ns	ns	***	*	ns	ns

*, *** Significant for $p < 0.05$ and 0.001 . ns= not significant.

7.3.6. Ascorbate

The analysis of ascorbic acid-related parameters, including AsA, Tot.AsA, and DhAs (dehydroascorbic acid), in the CBL group compared to the control both sampled during “night period” (A) and “day period” (B) is summarized in table 6. In the “night period” sample (A) the CBL group exhibited a significant increase in both AsA and Tot.AsA levels compared to the control group, with approximately 69.1% and 72.5% increments, respectively. However, there was no significant difference in DhAs levels between the two

groups. In the “day period” sample (B), the trend was similar, with the CBL treated group exhibiting a significant increase in both AsA and Tot.AsA levels compared to the control group. Specifically, there was an approximate 65.9% increase in AsA and a 62.2% increase in Tot.AsA. No significant difference was observed in the content of AsA, Tot.AsA, and DhAs between the “night period” and “day period” samples for both the treatments and controls (data not shown).

Table 6. Analysis of variance and means comparison for Total Ascorbic Acid (Tot.AsA), Ascorbic Acid (AsA) and Dehydro-Ascorbic Acid (DhAs) content of *Eruca vesicaria* sample grown under CBL and control, collected during night (A) and day (B).

A	AsA	Tot.AsA	DhAs	B	AsA	Tot.AsA	DhAs
(mg 100 g ⁻¹ FW)				(mg 100 g ⁻¹ FW)			
CBL	100.95	109.74	8.79	CBL	91.20	94.15	2.95
Control	59.45	63.59	4.14	Control	55.02	58.09	3.07
sign.	*	*	ns	sign.	*	*	ns

* Significant for $p < 0.05$. ns= not significant.

7.3.2. Non-structural carbohydrates

The results of the NSC analysis are showed in table 2. The analysis revealed no significant differences in the levels of glucose, fructose, sucrose, starch, total soluble sugars, or total non-structural carbohydrates between the CBL treated group and the control.

Table 2. Means comparison for non-structural carbohydrates including glucose (Glu), fructose (Fru), sucrose (Suc), starch, total soluble (Tot. sol.) and total NSC (Tot. NSC) of *Eruca vesicaria* grown under CBL and control.

	Glu	Fru	Suc	Starch	Tot.sol	Tot. NSC
(% DW)						
CBL	12.48	1.85	7.65	5.56	21.98	27.53
Control	10.45	1.52	6.73	5.83	18.70	24.53
Sign.	ns	ns	ns	ns	ns	ns

ns= not significant.

7.3.3. Pigments

The pigments analysis revealed chlorophyll *a*, chlorophyll *b*, total chlorophylls and lutein showed a significant increase in the CBL treatment group respectively of 19.5%, 18.3%, 19.1% and 18.6% compared to the control group (Table 3). The Chla/Chlb ratio, β -carotene, Neoxanthin and Violaxanthin contents did not exhibit significant variations between the groups.

Table 3. Means comparison for pigments content including chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophylls (Tot. Chl), chlorophylls ratio (Chla/Chlb), β -carotene, lutein (Lut), neoxanthin (Neo) and violaxanthin (Vio) of *Eruca vesicaria* grown under CBL and control.

	Chl a	Chl b	Tot. Chl	Chla/Chlb	β -Car	Lut	Neo	Vio
	(mg 100 g ⁻¹ FW)							
CBL	93.06	33.85	126.91	2.75	6.65	9.04	3.04	102.45
Control	77.91	28.6	106.51	2.72	6.06	7.62	2.6	93.76
sign.	*	*	*	ns	ns	*	ns	ns

* Significant for $p < 0.05$. ns= not significant.

7.3.4. Total Anthocyanin and Total Phenolic content

The results for Tot. Ant and TPC are presented in table 4. No significant differences were observed for Tot. Ant and TPC between the CBL treated group and the control group.

Table 4. Means comparison for total anthocyanin (Tot. Ant) and Total Phenolic Content (TPC) of *Eruca vesicaria* grown under CBL and control.

	Tot. Ant	TPC
	(mg 100 g ⁻¹ FW)	
CBL	0.89	105
cont.	0.77	88.2
Sign.	ns	ns

ns= not significant.

7.3.5. Anions

The statistical analysis indicated a notable increase in malate content, whereas a decrease in citrate in the CBL samples (Tab. 5). Specifically, malate demonstrated a 106.1% increase, while citrate showed a decrement of 42.74% in the CBL treatment group compared to the control group. No significant differences in nitrate, sulfate, and phosphate levels were detected.

Table 5. Means comparison for anions content including nitrate, sulfate, phosphate, malate and citrate of *Eruca vesicaria* grown under CBL and control.

	Nitrate	Sulfate	Phosphate	Malate	Citrate
	(ppm)	(ppm)	(ppm)	(mg 100 g ⁻¹ FW)	(mg 100 g ⁻¹ FW)
CBL	4929.72	1010.61	1047.44	470.00	71.00
Control	4502.04	1102.24	1316.87	228.00	124.00
sign.	ns	ns	ns	***	*

*, *** Significant for $p < 0.05$ and 0.001 . ns= not significant.

7.3.7. Enzyme activity assay

The enzyme activity assay results at the end of the night period revealed non-significant differences in GalLdh, DHAR and MDHAR activities between the CL-Blue group and the control group. In contrast, APX activity showed a significant increase, indicating a 75.6% increase in the treated group compared to the control. Samples collected at the end of the day period revealed a significant increase of approximately 20% of GalLdH activity in the CL-Blue samples compared to the control. DHAR and MDHAR activities showed non-significant differences. Notably, even during the day period APX activity exhibited a significant increase, indicating an approximately 101% rise in the CBL group compared to the control. In comparison between night and day, no significant differences were observed for any of the analyzed enzymes, neither in the CBL treatments nor in the control group (data not shown).

Table 7. Analysis of variance and means comparison for enzyme activity of L-galactonolactone dehydrogenase (GalLdh), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase, (MDHAR), ascorbate peroxidase (APX) in *Eruca vesicaria* sample grown under CBL and control, collected during “night period” (A) and “day period” (B).

A	GalLdh	DHAR	MDHAR	APX
(U g ⁻¹ FW)				
CBL	1.11	0.89	3.58	5.74
Control	0.91	0.69	4.74	3.28
Sign.	ns	ns	ns	**

B	GalLdH	DHAR	MDHAR	APX
(U g ⁻¹ FW)				
CBL	1.02	0.69	2.68	5.65
Control	0.85	0.64	2.74	2.81
Sign.	*	ns	ns	*

* Significant for $p < 0.05$. ns= not significant.

7.4. Discussion

In CEA conditions environmental control can be used to modulate productivity and quality of the produce, while optimizing resource use efficiency. Light is a key factor in CEA modulating productivity and quality while representing one of the most relevant costs for the facilities, affecting its economical profitability and environmental footprint [33]. Considering the significant role of Vitamin C as a fundamental nutraceutical to enhance the quality of horticultural species, the possibility to manage light aimed at increasing the AsA content becomes promising [24, 25, 34, 35]. A useful benchmark for ascorbate production efficiency could be the amount of produce providing the EFSA recommended daily dose (90 mg day⁻¹ to an adult person. [36]. Rocket under our continuous blue light treatment reveals a significant increase in Tot. AsA content compared to the controls, during both the night and the day period. With the CBL treatment, ~93 grams of fresh rocket are needed to meet the daily intake of 90 mg of vitamin C while, under control conditions, ~157 grams would be required. To better convey the idea of the gain achieved in terms of ascorbate, we obtained similar levels as those achieved in a previous study conducted by the same research team on rocket under continuous light, although in that case, the DLI was 25.92

mol quanta m² day⁻¹ and with constant PPFD under white LED or red+blue LED [10]. As a protector against the photooxidative damage in plant, the accumulation of AsA is recognized to be proportional to the level of light intensity [37]. The ability to selectively increase its content by adjusting the time of irradiation distribution of specific spectral bandwidth at low intensity levels represents an easy and cost-saving method compared to an approach using a general increase of light intensity.

In our experiment, remarkably, the objective of increasing AsA content by modulating the blue light distribution on a daily basis was achieved without undesirable effects on productivity or other analyzed variables. Indeed, literature presents conflicting findings concerning the impact of continuous light on plant productivity [15]. While different authors demonstrated that continuous light treatments, equivalent to the controls daily light integral, have shown increased shoot fresh and dry biomass of leafy vegetables in CEA [10, 23], other have reported detrimental effect including reduced growth or leaf chlorosis [38–40]. This variability can be attributed primarily to species-specific responses of plant and variations in experimental conditions including light intensity, light spectrum, duration of the treatments and factors influencing growth beyond light exposure. Our experiment represents a new approach regarding the CL for two main reasons: 1) the discontinuity in terms of light intensities during the treatments (PPFD dropped from 248 $\mu\text{mol m}^2 \text{min}^{-1}$ to 48 $\mu\text{mol m}^2 \text{min}^{-1}$ after 12 h and vice versa); 2) the discontinuity in terms of light spectrum during the treatment (shifting from full spectrum to selected blue bandwidths after 12 h and vice versa). It is well known that photosynthetic efficiency and plant growth are regulated by the synergistic effect of multiple light spectra, with red and blue light being particularly crucial for photosynthesis [41]. Red light comprises photons of lower energy, readily utilized by plants during photosynthesis, perfectly fitting with the absorption peak of chlorophylls [42]. Blue light, in addition to imparting energy to photosystems, also promotes photosynthesis by stimulating stomatal opening, allowing higher CO₂ availability at the carboxylation sites [43]. In our experiment the diminished intensity of blue light imposed during the “day period” in the CBL treatment did not reduce fresh and dry weight

compared to the control, on the contrary it slightly increases the dry matter percentage and SLDW.

Moreover, in our experiment, a significant increase in chlorophylls and lutein content has been observed. Findings related to the effect of blue light on pigment concentration are present in literatures [44]. Fan et al., (2013) [45] revealed that blue light was observed to be beneficial for the accumulation of chlorophylls and carotenoids in comparison to red light. However, the concentration of these pigments was higher in seedlings under the red plus blue LED treatment. It was shown that blue light could reverse red light-induced pigment inhibition response [46]. In his work Hogewoning et al., (2010) [47] highlighted that the chlorophyll and carotenoid content per unit leaf area increased with the percentage of blue light up to 50%. Long-term blue light treatment promoted the accumulation of lutein and β -carotene in Chinese cabbage [48]. On the other hands, although various studies demonstrated the influence of blue light on the accumulation of polyphenols and anthocyanins [49, 50], in our work, we did not find significant differences in these compounds attributable to the treatment.

Non-structural carbohydrates represent a substantial portion of the total dry matter (approximately 25%), yet our study did not detect significant differences in glucose, fructose, sucrose, or starch. This finding indicates that non-structural carbohydrates did not contribute to the observed increase in the percentage of dry matter or SLDW in samples treated with CBL. This also indicates that photosynthetic efficiency was not altered due to the treatment supporting the hypothesis that the higher AsA content was not a result of an increased substrate availability due to improved photosynthesis. The total LAI was not affected by the blue light modulation treatment, and this evidence contrasts the hypothesis of a reduced cell expansion in treated plants, causing an increase of AsA just as a result of a greater tissue compactness due to the blue light effect [45, 51–53].

Consistent with the increase in AsA in plants under our CBL conditions, there is also an enhancement in the enzymatic activity of GalLDH in samples collected during the day. The enzyme catalyzes the final step of ascorbate biosynthesis, and several authors confirm its role in increasing the ascorbate pool in response to light stimulation, both in terms of intensity and

photoperiod [30, 37, 54, 55]. The role of light spectrum quality on the accumulation and biosynthesis of ascorbate has been previously assessed in lettuce with spectrum ratios under continuous light set at 75R:25B, 50R:50B, 25R:75B, all with a light intensity of $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [35]. Zha and colleagues [35] demonstrate that blue light (25R:75B) led to an increase in total ascorbate content, primarily attributed to higher activity of recycling enzymes such as MDHAR and DHAR rather than an increased activity in the main biosynthetic pathway.

However, in the Zha's study [35], even though the total ascorbate was significantly increased, the content of ascorbate and dehydroascorbate did not differ among the various treatments. On the contrary, in the recently published work by Bournonville et al., (2023) [56], blue light promoted the accumulation of ascorbate in tomato fruit through the deactivation of PAS/LOV photoreceptors that inhibits the activity of GGP (GDP-L-galactose phosphorylase), which is known as a crucial regulatory step in the main biosynthetic pathway of ascorbate. In our study, APX activity is significantly increased in continuous light treatments with blue light, but in contrast, the dehydroascorbate content does not differ significantly either among the treatments or between night and day. The data suggest that there is a proportional ascorbate recycling activity to counteract oxidation due to APX activity in CBL, which in our case has an efficiency in ascorbate consumption equal to 99.3 mg min^{-1} on the 100 grams basis. However, the analysis of the enzymatic activities of MDHAR and DHAR showed no significant differences between the blue light treatment and the control. It is known that monodehydroascorbate can be spontaneously reduced to ascorbate by ferredoxin present in chloroplasts. Miyake and Asada (1994) [57] demonstrated that monodehydroascorbate is reduced in the thylakoid membranes of spinach by ferredoxin, and the contribution of MDHAR to ascorbate recycling was minimal or even negligible. Furthermore, Morell et al., (1997) [58], hypothesized that in chloroplasts, dehydroascorbate is not accumulated due to the activity of MDHAR and ferredoxin. The latter spontaneously reduces monodehydroascorbate in illuminated chloroplasts without the contribution of enzymes and is then regenerated by Ferredoxin-NADP⁺ reductase. The hypothesis is that ferredoxin might be sufficient to

“counteract” the high activity of APX in plants exposed to continuous light, and therefore, it can reduce the monodehydroascorbate produced to the same levels as in control samples.

The significant increase in malic acid and the decrease in citric acid in CBL group indicate an imbalance within the tricarboxylic acid cycle during the blue irradiation phase of the treatments. The rise in malic acid could, in this case, be an indicator of greater stomatal opening, in line with the observed higher nutrient solution consumption in the CBL treatments (data not shown). It is known that malate is used to counterbalance potassium charges, and this influences stomatal opening [59]. Furthermore, it is known that blue light has a direct effect promoting stomatal opening [60] and that this correlates with the synthesis and translocation of malate in guard cells [61]. In our case, although we were unable to ascertain the difference between the guard cells and the rest of the leaf tissue, the induction of stomatal opening due to blue light during the nighttime phase may have stimulated the biosynthesis of malate at the expense of citrate accumulation, for which the substrate from glycolysis is not available due to the low photosynthetic efficiency of the irradiation. Nevertheless, the increase in transpiration and higher consumption of nutrient solution in the treatment did not lead to an elevation in nitrates, sulfates, or phosphates that could compromise the product quality if they exceed certain concentration limits.

In conclusion, our experiments propose an efficient and simple method to improve the content of AsA in rocket grown under fully controlled conditions. Given the importance of this vitamin C both in terrestrial and space nutrition, the use of continuous blue light proves to be a promising strategy to enhance the quality of vegetables in CEA with no detrimental effect on productivity or other quality variables. Nevertheless, while quantifying the gain in light use efficiency in this manner, it is essential to take into account the potential drawback of increased water consumption associated with the impact of continuous blue light and to verify that the physiological response to blue light, as we used it, is shared by other relevant species but rocket. Further, metabolomic and transcriptomic studies may be useful to better understand the effect of continuous blue light treatment on *Eruca vesicaria* AsA biosynthesis and accumulation.

References are listed in Appendix VI of the bibliography section.

8. General conclusions

In the next decade, significant challenges and advancements are anticipated in the field of space exploration. In order to support human life in space, particularly during long-duration missions, it is of paramount importance to utilize diet as a tool to maintain astronauts' health, enhance resilience to space-related stress factors, and ultimately their performance to ensure mission success. To address these needs, the implementation of bioregenerative life support system (BLSS), including *in-situ* food production compartments leveraging advanced agronomic technologies is crucial. These systems should support the cultivation of plant species with enhanced nutritional and nutraceutical potential, that exert their quality through the high content of specific phytochemicals. Once integrated into the diet, these compounds can meet specific dietary and physiological needs. To effectively achieve this goal, the selection of plant species with defined traits capable of maintaining high productivity under the diverse constraints and demanding growth conditions, inherent to space environments, is paramount. Microgreens have emerged as ideal candidates and are gaining increasing traction in research aimed at developing productive systems capable of providing fresh food with high nutraceutical potential in space. Experimentation with microgreens has been considered in various research programs coordinated by space agencies such as NASA, ESA, ASI, and several international research organizations.

Within the framework of the *Microx2* project, funded by ASI, and this Ph.D. activities, the two best microgreens species for cultivation in space were identified: *Raphanus raphanistrum* subsp. *sativus* 'Saxa' and *Brassica oleracea* var. *capitata* f. *sabauda* 'Vertus' (Chapter 3). The identification was carried out through the development and use of a selection algorithm based on objective comparison. This algorithm is based on subsequent rankings and the prioritization of parameters, and the flexibility of this selection method provides potential adaptability for selecting new cultivars and/or including other criteria relevant for other selection purposes. Vitamin C (ascorbic acid) is one of the most crucial phytochemical constituents for nutrition in space

environments, as such it heavily influenced the selection and enhance the value of plant products intended for consumption in this context. This compound, which acts as a buffer against photooxidative stress in plants, also plays a fundamental role in human physiology and is useful in counteracting oxidative stress triggered by environmental factors that burden human body homeostasis during spaceflight. Ascorbic acid is labile during storage, and this make *in-situ* production particularly relevant in the case of long-term space missions. In addition to this compound, the accumulation of other phytochemical species that enhance the antioxidant capacity of food is significant. Their biosynthesis and accumulation in plants are determined not only by the species but also by the cultivation conditions. Among the most significant aspects affecting the feasibility of space farming for ensuring a reasonable level of crew autonomy from ground supplies, are the available volumes and masses required for the cultivation system to operate. To define the baseline of a cultivation system suitable for meeting specific dietary targets, it is necessary to define the cultivation area required to achieve the objective. The variability in the productive and qualitative response of the two selected species to the growth conditions used, underlines the need for precise control of growing conditions and in-depth knowledge of how to manage them in order to produce targeted phytonutrients such as vitamin C. High levels of VPD and light intensity have resulted in an increase in ascorbate content in both microgreen species. However, this does not translate into a significant improvement in terms of resource efficiency. In fact, a general comparison of the two experimental trials (Chapter 4 and 5), conducted on the two selected species, shows that off-nominal VPD conditions, such as those used in the first trial (Chapter 4), did not lead to satisfactory levels of vitamin C availability. Lower concentrations and lower biomass production were observed compared to the second trial. Conversely, in the second trial (Chapter 5), where the relative humidity (and consequently the VPD) fell within an intermediate range, higher biomass production and vitamin C accumulation were recorded. Under suitable conditions, a daily recommended intake of vitamin C can be achieved with 80 grams of fresh microgreens, requiring less than 0.6 m² of cultivation area. This condition is met when using the species *Raphanus raphanistrum* subsp. *sativus* ‘Saxa’

grown on cellulose sponge substrate, or the species *Brassica oleracea* var. *capitata* f. *sabauda* ‘Vertus’ grown on coconut fiber, both at 300 PPFD. A further choice between these two conditions can be made if the aim is to maximize additional qualitative characteristics such as the content of anthocyanins and polyphenols. In this case, the use of the combination of ‘Saxa’ cultivated on cellulose sponge would be a better choice and the use of fertigation with HS/H₂O would lead to a boost in the accumulation of anthocyanins. However, in the context of exploring the potential of microgreens, future research on the two selected species should focus on their productive and qualitative responses to the characteristic effects of the space environment, such as microgravity, ionizing radiation and high atmospheric CO₂ level. In this way, it will be possible to improve the *crop readiness level* of these species and bring them to actual cultivation in space for definitive use as a food resource. The data generated by *Microx2* activities, combined with the expertise of the various research teams involved in the project, have contributed to defining the hardware and software components for a modular, automatic, and intelligent cultivation system. This system is climate-controlled and designed to support research and production activities within the International Space Station and ASI has granted a Phase A study to build it.

Our experiment with *Eruca vesicaria* demonstrates the feasibility of managing light resources through straightforward strategies to enhance crop quality (chapter 7). The low-intensity continuous blue light treatment (48 $\mu\text{mol m}^{-2} \text{s}^{-1}$) enabled a doubling of vitamin C concentration in hydroponically grown rocket, resulting in a more nutritious product without a significant increase in biomass production, while also avoiding excess energy consumption. This finding is particularly intriguing if its applicability to other species can be confirmed. Nevertheless, we observed higher nutrient solution consumption in the treatment, which could prove counterproductive, especially in space environments where resources like water and fertilizers are severely limited. Therefore, exploring additional cultivation environment management strategies, such as adjusting atmospheric composition or VPD, may be necessary to mitigate transpiration and consequently minimize water losses. From a physiological perspective, based on the analysis of enzymatic

activities, it is hypothesized that the larger pool of ascorbate is a result of enhanced stimulation of the primary biosynthetic pathway rather than increased recycling activity. To delve deeper into understanding the precise mechanism by which blue light, both in terms of relative PPFD percentage and absolute light intensity, impacts ascorbic acid biosynthesis, additional experiments and more comprehensive analytical details are necessary.

In the context of space nutrition, prebiotics have been little considered as functional components useful in countering the effects of space travel. In this thesis, the potential prebiotic production under fully controlled environmental conditions, was studied in the light of the enormous potential of prebiotics to affect the astronaut wellbeing. In fact, they are capable of positively regulating the gut microbiome-host interaction with benefits to several physiological functions that are threatened in space. As elucidated in Chapter 6, *Cichorium intybus* can be grown in space compatible conditions producing taproot with an excellent content of inulin, acknowledged for its exceptional prebiotic properties. The work demonstrated how this species is compatible for space farming purposes adapting well to relative short cycles (70 days from sown to harvest) in a completely controlled environment to produce young plants that accumulate high levels of fructans (14.7 % of the fresh weight). Among the advantages of integrating chicory taproot into the diet, rather than consuming extracted and isolated prebiotics, there is the synergistic effect in modulating the microbiota due to the presence of other fibers that constitute the plant biomass, in addition to the content of other nutraceutical components of plant origin. The consumption of 100 grams of chicory taproot produced under our growth conditions covers the EFSA proposed daily intake of inulin (12 g day⁻¹). This species is well-suited to the demands of space farming, boasting a high harvest index and near-complete edibility (both leaves and roots), which minimizes waste. Under our experimental conditions in the growth chamber, the minimum surface area required to produce the daily intake of inulin is 8 m². In contrast to the production achieved in a greenhouse setting, the application of biostimulants in fully controlled environment did not manifest discernible effects on the analyzed variables. This observation is theoretically linked to the optimal environmental conditions maintained within the growth chamber. However,

in the space environment, characterized by potential exposure to various abiotic stresses, the efficacy of these biostimulants may yield different results. Consequently, additional investigations involving biostimulant application under conditions that replicate the space environment can be still considered. Further experiments within the research activities of the IRET-CNR team are planned to enhance fructan biosynthesis through targeted management of the cultivation environment and agronomic inputs. Additionally, experiments are planned under flight-like growing conditions to assess chicory's potential for integration into the BLSS loops.

Finally, the work carried out in contributing to the scientific understanding of food production in space also provides a contribution to terrestrial horticulture. Globally, an increasing number of companies specializing in CEA and vertical farming are emerging, also thanks on technological advancements derived from research efforts for space. These realities represent an advanced front in addressing the significant challenges that agriculture, essential for humanity's sustenance and development, must confront in the face of the changes our planet is undergoing.

9. Bibliography

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