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Review

Bioregenerative systems to sustain human life in Space: the research on higher plants

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Abstract: Human exploration beyond Low Earth Orbit (LEO) will require technologies regenerating resources like air and water, and producing fresh food while recycling consumables and waste. Bioregenerative Life Support Systems (BLSSs) are artificial ecosystems in which appropriately selected organisms, including bacteria, algae and higher plants, are assembled in consecutive steps of recycling, to reconvert the crew waste into oxygen, potable water and edible biomass. Higher plants are considered the most promising biological regenerators to accomplish these functions, thanks to their complementary relationship with humans, however, cultivation in Space requires the knowledge of their response to Space factors (e.g. altered gravity and ionizing radiation) and specific cultivation conditions (e.g. controlled environment, hydroponic systems). This article summarises the most relevant research on higher plants achieved in view of their cultivation in an extraterrestrial environment.

Keywords: Bioregenerative Life Support Systems (BLSSs); artificial ecosystem; altered gravity; ionizing radiation; controlled environment.

1. Introduction

Despite the complex technologies and the advanced knowledge required to grow plants in such an extreme environment, agricultural systems for Space have been envisaged since 1880, when the novelist Percy Greg wrote about a Space explorer travelling to Mars and bringing plants to be used for waste recycling (Wheeler et al., 2017). Already in the 1920s, the Russian scientist Konstantin Tsiolkovsky described how humans and plants might co-exist in a closed environment and envisioned agricultural modules capable of gathering sunlight and operating at reduced atmospheric pressure, to allow human survival in Space (Tsiolkovsky, 1975). Decades later, the possible role of higher plants in long Space missions was clearly described (Ley, 1948).

Regeneration of resources for Space application focused on physical-chemical processes until the 1950s, when the first reasoning on plants as both a regenerating tool and source of food led to the introduction of algae for life support in Space in the studies of the US Air Force, (Myers, 1954). In the last decades, extensive research has been focused on the development of life support systems (LSSs) for Space, based on living organisms. These Bioregenerative Life Support Systems (BLSSs) are artificial ecosystems in which appropriately selected organisms are assembled by combining their metabolic routes in consecutive steps of recycling, to reconvert the waste produced by the crew into nutritional biomass, oxygen, and potable water (Hendrickx and Mergeay, 2007). Specifically, they are modular systems including sub-units hosting microorganisms, plants and animals able to accomplish different specific functions in a closed regenerative loop (Wheeler, 2010). Many BLSSs containing biological regenerators, such as bacteria, microalgae, higher plants and fish, have been proposed, however higher plants seem to be the most promising bioregenerators.

The National Aeronautics and Space Administration (NASA) initiated bioregenerative research in the 1960s, working on bacteria, but did not include testing of higher plants until about 1980, with the start of the CELSS (Controlled Ecological Life Support System) programme (Wheeler et al. 1996, 2003). Later, NASA's Marshall Space Flight Center (Alabama) designed and built a regenerative life support hardware system for the International Space Station (ISS): the Environmental Control and Life Support System (ECLSS). The ECLSS provides clean air and water to the ISS crew and laboratory animals, as well as technical support for other systems, creating a comfortable environment and minimizing the resupply burden (https://www.nasa.gov/centers/marshall/history/eclss.html). The ECLSS consists of two key components, the Oxygen Generation System (OGS) and the Water Recovery System (WRS). The OGS produces oxygen for breathing and replaces oxygen lost for experimental use, airlock depressurization, module leakage, and carbon dioxide venting. It is capable of generating oxygen at a selectable rate and of operating continuously and cyclically. The WRS provides clean water, meeting stringent purity standards, by recycling crewmember urine, cabin humidity condensate, and extra vehicular activity wastes.

Central to the concept of these systems is the use of photosynthetic organisms and light to regenerate air and to produce fresh food (Wheeler, 2010). This approach can be clearly described by comparing the general metabolic equations for human respiration and plant photosynthesis (Figure 1), where plants (or other photosynthetic organisms) remove CO_2 from the air while generating oxygen (O_2) and producing edible biomass (CH_2O). Higher plants in particular have a complementary interrelationship with humans: in a simplistic vision, plants recycle human waste and provide nutrients to humans, while humans recycle plant waste and provide nutrients to plants. More specifically, plants represent an optimal tool for multiple functions: atmospheric regeneration, by means of CO_2 assimilation and O_2 emission in photosynthesis, wastewater purification through transpiration, and recycling of waste products through mineral nutrition. Furthermore, plants provide fresh food, which would help to preserve the astronaut's wellbeing, and contributes to creating an Earth-like environment, mitigating the psychological stress of the mission (Lasseur et al., 2010).

Despite the notable research work on this topic, still, at the present, all the resources needed for short-term Space missions are brought from Earth, but this will not be possible for longer missions for

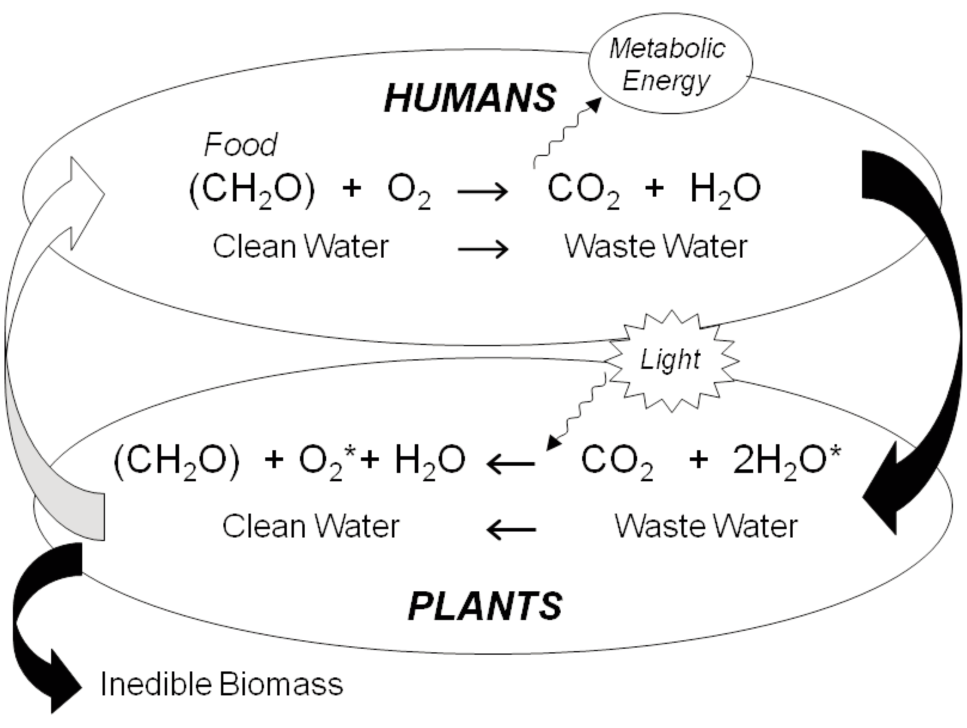

Figure 1. General metabolic equations for human respiration and plant photosynthesis as the basis of the complementary relationship between human beings and higher plants (courtesy of Raymond Wheeler, NASA).

economical and practical reasons. Indeed, it has been calculated that, with the current physical-chemical regeneration systems, each astronaut will need an average of 15 kg of resources per day, including food, water and oxygen. This implies that, even considering a mission to Mars lasting only 500 days, about 7.5 tons of consumables in total will be required per person so that the supply from Earth would be logistically difficult and expensive. In this scenario, maximizing the self-sufficiency of orbital stations and planetary colonies, while minimizing the need for resupply, are imperative goals to enable long term manned missions and human permanence beyond the Low Earth Orbit.

This review summarizes the research activity recently carried out in Europe on higher plant-based BLSSs.

2. Bioregenerative life support systems for Space: the ESA programme MELiSSA

Comprehensive reviews about the history, and the progress and prospect of research on controlled ecological life support around the World have been produced recently (Guo et al., 2017; Wheeler et al., 2017; De Pascale et al., 2021). On the other hand, the relevance of fresh plant food in the accomplishment of a complete and balanced diet to fulfil the astronauts' needs and to preserve human health in Space has been highlighted since the first long term human permanence in Space (reviewed by Lane et al., 2002). Table 1 summarises the key literature and the major advances in relation to the main topics of the research on higher plants for bioregenerative life support in Space.

In Europe, pioneering studies on plant cultivation in atmospherically closed chambers, aiming to quantify crop gas exchange in the specific environment, provided already in the 1980s comprehensive data sets on photosynthesis, respiration, and transpiration in some candidate crops, like wheat (Gerbaud et al., 1988; Andre et al., 1989). Also, some of the first studies on plant growth under hypobaric conditions were carried out in some European countries, such as France (Andre and Massimino, 1992) and Germany (Daunicht and Brinkjans, 1992).

In 1987, the European Space Agency (ESA) initiated the program Micro-Ecological Life Support System Alternative (MELiSSA), still ongoing, to design and test life support concepts, based on ecological principles for materials cycling, for long term manned missions in Space outposts and planet surfaces. The program aims to conceive an artificial bioregenerative ecosystem for resource regeneration including microorganisms and plants, inspired by the reconversion cycle of organic matter in natural lake ecosystems (Mergeay et al., 1988). More specifically, MELiSSA investigates higher plants-, algae- and microorganisms-based technologies for food, water and oxygen production in long-duration Space missions with limited supplies. The driving element of MELiSSA is the recovering of edible biomass, water and O_2 from organic waste (faeces, urine, CO_2 and minerals), using light as an energy source for photosynthesis (Hendrickx et al., 2006). The main objectives of the program are to develop the technologies for a safe and reliable closed-loop regenerative system to sustain human presence in Space and, more generally, to gain knowledge on the global challenges of waste recycling, water provision, and food production in harsh environments on Earth, through biological and bio-physical-chemical coupled processes (https://www.esa.int/Our_Activities/Space_Engineering_Technology/Melissa).

Most of the initial MELiSSA research activity focused on the processing of human waste using microorganisms, including photosynthetic bacteria or cyanobacteria, hosted in specific bioreactors, and providing edible biomass (Lasseur et al., 1996). Later on, the project expanded to include higher plants in a controlled environment compartment devoted to plant cultivation (Waters et al., 2002). In the current layout, the MELiSSA cycle is a loop of five interconnected compartments, each with a specific biotransformation task, colonized by thermophilic anaerobic bacteria, photo-heterotrophic bacteria, nitrifying bacteria, photosynthetic organisms, and the crew as both the first producer (of waste) and the final user (of products) (Figure 2). Photosynthetic organisms are hosted in Compartment IV, consisting of a higher plant cabinet and a cyanobacteria growth unit (Poughon et al., 2009).

For ground demonstration, the MELiSSA Pilot Plant (MPP), a laboratory dedicated to the physical realisation at a pilot-scale of the closed-loop to test the system in terrestrial conditions, operates at the

Table 1. Key literature and major advances of the research on higher plants for bioregenerative life support in Space.

Topic	Content	Authors
Research on Bioregenerative Life Support	History in the different countries around the world; current major achievements and future perspectives	Guo et al., 2017 Wheeler et al., 2017
Selection criteria for candidate crops	Methodology for cultivar selection through an objective and repeatable procedure, based on nutritional and technical requirements	De Micco et al., 2012
Plant biology and crop production in Space	Plant response to the growth in a controlled environment and to space factors; cultivation technologies and strategies to improve resource use efficiency	De Pascale et al., 2021 Wolff et al., 2014
Environmental stimuli influencing the plant behaviour	Gravitropism, phototropism, hydrotropism, chemotropism	Nakamura et al., 2019 Vandenbrink and Kiss, 2019
Space factors		
Ionizing radiation	Anatomical, physiological and morphological effects; possible positive influence on plant growth, yield and reproduction	Esnault et al., 2010
Microgravity	Direct and indirect effects of altered gravity; experimental evidences under both simulated or real microgravity	De Micco et al., 2011 De Micco et al., 2014a Kiss, 2014
Analogs for microgravity simulation	Clinostats, random-positioning machines (RPM), magnetic levitation devices; critical analysis and comparison to results under real microgravity	Kiss et al., 2019
Food diet and human health	Astronaut nutritional requirement, food safety and quality, functional food;	Lane et al., 2002 Cahill and Hardiman, 2020
Growth chambers and cultivation modules	Experiments and evolution of the related hardware for ground and flight research	Porterfield et al., 2003 Zabel et al., 2016

Autonomous University of Barcelona (Spain) (Gòdia et al., 2004). MELiSSA studies, in the MPP and the laboratories of the 14 partners of the MELiSSA community, concern multiple topics, all ultimately aiming at the inter-connection of all the compartments. Also, the ESA developed strategies to transition ground-based testing of agriculture into actual Space flight settings, such as the International Space Station (ISS), and the upgrading of the European Modular Cultivation System (EMSC) to perform testing on the ISS (Wolff et al., 2014). In this scenario, also European companies in the aero-space field, such as Aero Sekur (Rossignoli, 2016) and Thales Alenia (Boscheri et al., 2012) support research and development in Space agriculture through their programs or joined projects.

The development of such complex systems as the BLSSs are, requires strict interactions and a continuous know-how transfer between biologists, agronomists, engineers and medical doctors. In this context, the activity of the team of the University of Naples Federico II (UniNa) concerns the field of plant biology and plant cultivation in Space aimed at the development of BLSSs for human space exploration.

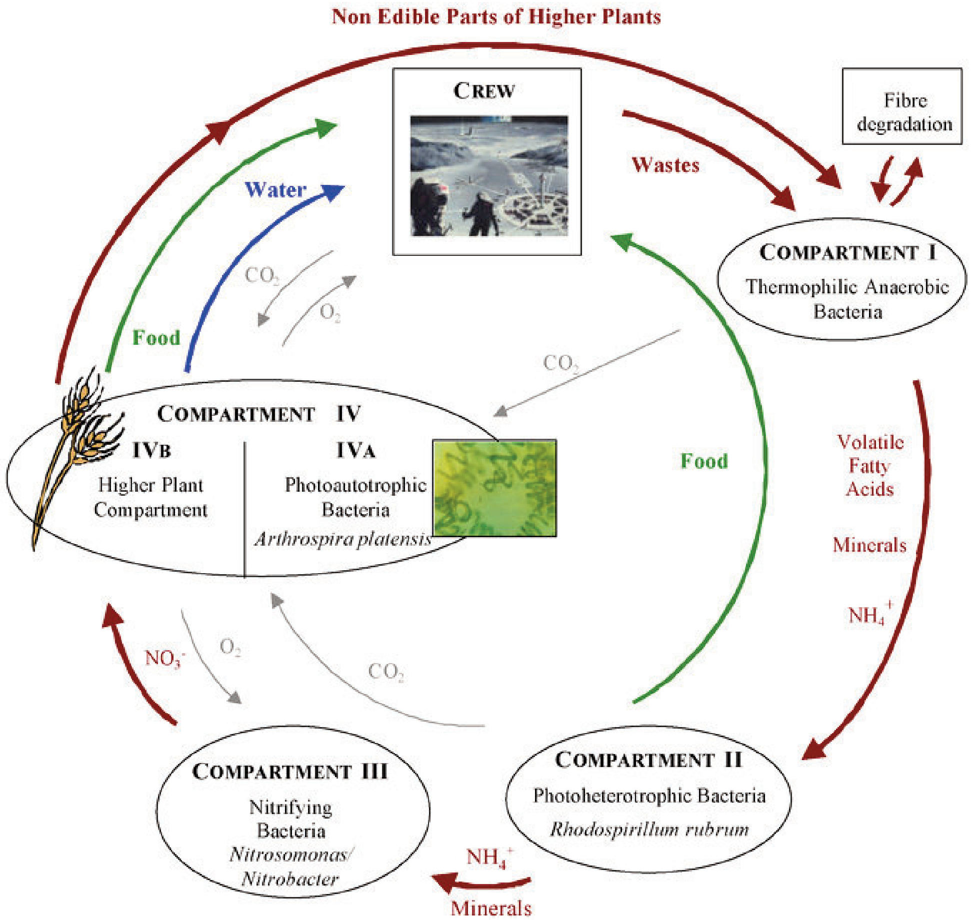

Figure 2. Compartments of the MELiSSA loop, appointed for the following specific functions: organic waste degradation and solubilisation by thermophilic anoxygenic bacteria (CI), carbon compounds removal by photoheterotrophic bacteria (CII), nitrification by nitrifying bacteria (CIII), food and oxygen production by photosynthetic bacteria (CIVa), food, oxygen and water production by higher plants (CIVb), astronauts crew (CV).

Thanks to projects funded by the Italian Space Agency (ASI), ESA and other institutions, the UniNa team has gained relevant expertise on many aspects of plant cultivation in Space, concerning biological, agronomical, and environmental issues related to plant life in the presence of the multiple constraints of the Space environment (Paradiso and De Pascale, 2019). The main topics of the research activity concern the selection of candidate crops/cultivars, the water and nutrient management for cultivation in soilless systems, the plant interactions with beneficial microorganisms and biostimulants, the characterization of substrates (including stimulants of planetary soils in the view of *in-situ* resource utilization), the environmental control in growth chambers for both short term (e.g. "salad machines" onboard Space vehicles) and long term scenarios (i.e. BLSSs in planetary outposts). Besides, the effect of Space factors (e.g. microgravity and ionizing radiation) on plant growth and physiology and the completion of the seed-to-seed cycle are investigated under both real or simulated conditions, and the nutritional aspects of plant fresh food in the crew diet (including the contribution of innovative leafy vegetables such as microgreens) are investigated. Finally, the design of modules for plant cultivation in microgravity is performed based on specific crop requirement of the different crops (e.g. tuberous plant species).

3. The effects of real and simulated Space factors on plant growth

3.1. Altered gravity

Current knowledge on the effects of altered gravity on plant behaviour relies on data collected under both simulated and real microgravity. A relevant contribution to the comprehension of plant responses to altered gravity comes from ground experiments with clinostats and other reduced gravity simulating instruments (e.g. random-positioning machines, magnetic levitation devices). Uniaxial clinostats were historically used to investigate the effects at the early stages of plant development (seedlings) and to plan successive experiments in real microgravity in Space (Aronne et al., 2001, 2003). Later, they were replaced by more efficient three-dimensional clinostats, however these instruments do not really reduce gravity but only constantly change its direction (reviewed by Kiss et al., 2019).

Despite the reduction in the photosystem activity observed in early Spaceflight experiments (e.g. on wheat plants grown onboard the Space shuttle Discovery for 10 days; Tripathy et al., 1996), plant gas exchange, photosynthesis and metabolism were not affected by real microgravity when an efficient environmental control was guaranteed (Stutte et al., 2005; Musgrave, 2007). Similarly, flight experiments have revealed no detrimental effects of gravity or other Space factors on plant morphology in both short and long term experiments, within a single growing cycle (Wolff et al., 2013).

When flight investigations in real microgravity in Space vehicles are possible, experiments are performed in duplicate replicating the experimental layout on Earth to provide clear information on the effects of gravity. For instance, De Micco et al. (2006, 2008) investigated seed and seedling behaviour under both real and simulated microgravity in soybean [*Glycine max* (L.) Merr.]. Results of ground experiments showed that other environmental factors, such as temperature, interfere with microgravity in affecting the plant response, and anatomical modifications under simulated microgravity were not always the same as those elicited by real microgravity. At the cell level, perturbations in the deposition of cellulose were found in seedlings developed in Space at the beginning of the primary cell wall, although this phenomenon seemed to be reversible in the later stage of the secondary wall development.

Among the different environmental stimuli influencing the plant behaviour, including gravity through gravitropism, light through phototropism, and water through hydrotropism, gravitropism is the most investigated, also through Space experiments (most recent reviews by Nakamura et al., 2019; Vandenbrink and Kiss, 2019). Briefly, under microgravity plants still orient their growth toward the light source and following a water potential gradient, however, the response will depend on the time of exposure (Millar et al., 2010). When higher plants are exposed to short term (seconds to hours) microgravity (e.g., in parabolic flights and rockets), they usually exhibit abiotic stress responses: for instance, Ca_2^+-, lipid-, and pH-signalling are rapidly enhanced, then the production of reactive oxygen species (ROS) and other radicals increases, along with changes in metabolism and auxin signalling (Zheng et al., 2015). Under long-term (days to months) microgravity exposure, plants acclimatize to the stress by changing their metabolism and oxidative response and by enhancing other tropic responses.

Referring to root development, gravity is recognised as a dominant stimulus influencing growth on Earth (gravitropism), however, it is known that it often masks other tropisms (Muthert et al., 2020). A recent experiment performed onboard the ISS aimed to disentangle hydrotropism from chemotropism for root development and orientation in absence of the gravity stimulus in carrot (*Daucus carota* L.) (Izzo et al. 2019). Seeds were placed on an inert substrate, imbibed with water or a disodium phosphate solution, and germinated in microgravity and at 1g. In the flight experiment, the radicle protruded from the seed ventral side due to the asymmetric position of the embryo, and the primary root showed a positive chemotropism towards the solution. In the ground experiment, as expected the positive chemotropism was masked by the dominant effect of gravity and roots developed downward regardless of the presence of nutrients in the substrate.

Reduced gravity affects plant biological processes also indirectly, by influencing the surrounding physical environment (De Micco et al., 2014a; Kiss, 2014). For instance, the lack of air convection in microgravity, and the consequent increase of boundary layer thickness, reduce the rate of gas exchange and transpiration, hence the transport of water and solutes within the plant (Porterfield, 2002). Indeed, the presence of a still boundary layer restricts the emission of CO_2 from the cell, causing changes in pH with serious effects on plant physiology and metabolism (Kitaya et al., 2010). The limited convection also retards the water vapour transfer reducing transpiration (which is one of the major driving forces for water transport in the xylem), and this in turn reduces the heat exchange between the leaf surface and the ambient air, with a consequent increase of leaf temperature (Kitaya et al., 2003). In addition, limited water consumption reduces nutrient uptake (Wolff et al, 2013) and can trigger hypoxia in the root zone (Porterfield, 2002). Consequently, a proper control of air movement is essential to guarantee heat/gas exchanges between the plant and the surrounding environment, to promote the growth of healthy plants in microgravity. While in the aerial part of the plant this effect can be reduced by proper forced air ventilation (Musgrave et al., 1997; Kitaya et al., 2003), in the root zone, where the gas and fluid behaviour is altered as well, root hypoxia is still an issue in plant experiments in Space, and it can also result in a reduced uptake and transport of nutrients (Porterfield et al., 2000; Liao et al., 2004).

3.2. Ionizing radiation

Cosmic radiation alters gene expression and affects the plant genome through DNA damage and chromosome mutations, but these effects seem do not impede plant survival (Karoliussen et al., 2013). Plants have been grown in low Earth orbit during several consecutive generations (Sychev et al., 2007), however, it is still unclear if the plant genome remains stable under Space conditions over a long period, and experimental results are often conflicting and not comparable since the plant response depends on many other experimental factors, including genotype and developmental stage, and radiation type and dose (Arena et al., 2014; De Micco et al., 2011).

Recently, Gudkov et al. (2019) collected a large body of data on the effects of ionizing radiation on plant growth and reproduction, and on the changes induced at the genetic level, highlighting instead the large gap in the knowledge of the influence on biochemical and physiological processes.

Due to the shielding of the experimental facilities, which is a strict requirement for manned Space missions, long term exposure to low chronic radiation is considered more relevant than high acute doses (Wolff et al., 2014). Also, based on the few studies performed with chronic exposure (Real et al., 2004), it seems to have a stronger impact on the genetic structure (Kovalchuk et al., 2000), and to reduce the genetic variability, probably as an adaptive process to chronic stress (Esnault et al., 2010). As matter of fact, different mechanisms are involved in the two responses and, while acute exposure affects more the regulation of some oxidative stress-related genes, chronic stress influences genes involved in general stress and nucleic acid metabolism, resulting in adaptive responses, and in photosynthesis and carbohydrate metabolism (Kovalchuk et al., 2007).

Generally, the radiation-induced damage increases with increasing doses and, at the same dose, it depends on the quality of radiation, and high-LET (Linear Energy Transfer) is more hazardous than low-LET radiation in determining genetic mutations (Arena et al., 2017).

Exposure to ionizing radiation increases embryo lethality, and determines dwarf architecture and modification of floral elements (Esnault et al., 2010), even though radiation has also been reported to increase growth (e.g. plant height), yield and reproductive success (e.g. formed seeds) (reviewed in De Micco et al., 2011). However, plant sensitivity or resistance to radiation varies among the species, due to differences in molecular and biochemical mechanisms (Esnault et al., 2010; Poulet et al., 2016). In addition, at the morphological and anatomical levels, the extent of the damage depends on the tissue organization, and complex tissues seem to be less sensitive (De Micco et al., 2014b, c).

At the functional level, ionizing radiation affects photosynthesis at different steps of the process, by impairing electron transport carriers, light-harvesting pigment-protein complexes and enzymes of the carbon reduction cycle, and the light phase seems to be more sensitive than the dark phase (Wheeler, 2003; Wolf et al., 2013; Arena et al., 2013, 2014, 2017). However, the increase in the synthesis of phenolics promoted by the X-rays and their accumulation along chloroplast membranes in the leaf mesophyll (De Micco et al., 2014b) can be interpreted as a protection mechanism of the photosynthetic apparatus (Arena et al., 2014). A higher amount of antioxidant compounds was found also in fruits of tomato plants from seeds exposed to high-LET ionising radiation from heavy ions (Arena et al., 2019).

In the dwarf cultivar of tomato, 'Microtom' (*Lycopersicon esculentum* Mill.), irradiation with increasing doses of X-rays (from 0.3 to 100 Gy), at different phenological phases (seed, vegetative stage, reproductive stage), did not affect seed germination, and plants from irradiated seeds completed the growth cycle, though they showed a more compact size (De Micco et al., 2014c). Dose-depended variations occurred in phenolic content. Sporadic perturbations of leaf structure occurred in the vegetative phase at high doses, however, they did not impair the photosynthetic efficiency. Exposure to X-rays was more harmful on developing leaves than on mature leaves (Arena et al., 2017). The highest doses (50 and 100 Gy) reduced photochemical efficiency in both leaf types, and also exerted mutagenic effects in developing leaves. In 'Microtom', the irradiation of seeds with high-LET radiation with heavy ions (i.e. ^{48}Ca ions at 25 Gy), did not prevent the seed-to-seed cycle, but resulted in smaller plants with bigger fruits, richer in superoxide dismutase (SOD), carotenoids, anthocyanins and ascorbic acid than controls (Arena et al., 2019).

In *Phaseolus vulgaris*, high doses of ionising radiation (50 and 100 Gy) induced a detrimental effect on the photosynthetic apparatus, including a significant decline of photosynthetic pigment content and rubisco activity, which resulted in a reduction of leaf expansion (Arena et al., 2013). As observed for 'Microtom', ionising radiation produced different injuries depending on leaf age and, also in beans, the mature leaves were more resistant than the young ones (Arena et al., 2014).

4. Cultivation in a controlled environment

Crop selection for BLSSs is based on both life support and technical requirements (De Micco et al., 2012). The former is linked with the nutritional needs of humans (e.g., carbohydrate, protein, and fat) as well as to the capability of regenerating vital resources (i.e. air and water). Moreover, the research highlighted the potential of fresh foods as countermeasures to degenerative diseases induced by Space factors and the food-related psychological benefits. Specifically, fresh plant food can be a valuable source of healthy bioactive compounds, such as vitamins and antioxidants (e.g., carotenoids, flavonoids), as well as specific proteins, useful to mitigate the multiple stress effects from long-duration space permanence, including osteoporosis, muscle atrophy, oxidative cytotoxic stress and protein oxidation (Cahill and Hardiman, 2020). In addition, the presence of plants in space outposts is demonstrated to alleviate the detrimental effects of isolation, by creating an Earth-like environment, and the cultivation activity itself is known to provide emotional support (Heer et al., 2020).

On the other hand, the choice of a plant species is also based on biological and technological issues, such as adaptability to the closed environment and hydroponic system, harvest index, food processing and horticultural requirements. The most relevant features are compact size, short cultivation cycle, high resources regeneration efficiency and productivity, resistance to diseases, relatively low light requirements, and tolerance to osmotic stress from NaCl (in view of urine recycling). However, it is worth noting that the requirements for life support and the choice of the crop change depending on the mission scenario, and especially the duration and distance from Earth. Currently, criteria to select plant species and cultivars to grow in Space consider also their content of bioactive components/nutraceuticals in addition to their nourishing content (Kyriacou et al., 2017).

For short-term missions, on-board plant production represents a fresh food integration of the astronauts' diet since crew life is still mainly based on resources taken from Earth. In this case, crops providing vitamins, minerals and bioactive compounds (salads crops and microgreens) are chosen. These requisites take into account the typical constraints of Space missions, including the reduced availability of resources in terms of astronauts' time, volume and energy, and the need for sanitary safety. For long-term missions, for example to Mars, which cannot rely on supply from Earth, staple crops, such as potato, soybean and wheat, are needed to provide the crew with fundamental macromolecules (i.e. carbohydrates, lipids and proteins) and energy.

Plant species selected and tested as candidates for cultivation in Space BLSSs belong to different crop types: cereals, tuberous plants, legumes, fruit and leafy vegetables, and (e.g. wheat, rice, potato, sweet potato, soybean, tomato and lettuce), and their response to the growth in a controlled environment has been tested in Space-oriented ground studies from NASA, ESA, and other national Space agencies (reviewed by Wheeler, 2017; Guo et al., 2017).

For instance, within the ESA project MELiSSA Food Characterization (FC), four candidate crops, bread and durum wheat, potato, and soybean, were chosen, and experiments on cultivars selected for cultivation in BLSSs were carried out in growth chambers with electric lighting, using either hydroponics (i.e. nutrient film technique, NFT) or solid growing media (i.e. substrates), under different environmental conditions (e.g., light intensity, photoperiod, air CO_2 concentration) and using different cultivation protocols (e.g., substrate, recycling nutrient solution composition). In general, results demonstrated good adaptability to hydroponic cultivation in the controlled environment of bread wheat (Page and Feller, 2013), durum wheat (Stasiak et al., 2012), soybean (Paradiso et al., 2012) and potato (Molders et al., 2012). In addition, in soybean further analyses highlighted that the hydroponic cultivation improved the nutritional quality of seeds, and revealed some differences among the cultivars in nutritional composition, suggesting that specific genotypes could be chosen to obtain the desired nutritional features of the product depending on its final use (seeds, sprouts, soymilk, or okara) (Palermo et al., 2012). Finally, within the same project, an objective and repeatable procedure to choose the best cultivar for BLSSs was created by elaborating an algorithm based on the relevance of the most relevant features (De Micco et al., 2012; Paradiso et al., 2014a).

Later on, the FC Phase II investigated the possibility of optimizing the performance of the four crops by exploiting the action of plant growth-promoting microorganisms (PGPMs) in closed-loop hydroponics. Inoculation with a mixture of beneficial species: bacteria, yeasts, mycorrhiza and trichoderma induced changes in the root microbiome composition, varying between crops and over time and enhanced plant growth and productivity (Sheridan et al., 2017). In soybean, these benefits were found to be related to positive changes in leaf functional traits and higher photosynthesis (Paradiso et al., 2017). In soybean also the possibility of applying N-fixing bacteria (namely *Bradyrhizobium japonicum*), exploiting the natural symbiosis of legume crops that occurs in soil, was investigated in closed-loop NFT. Nitrogen fertilizers inhibit rhizobia, however, urea is profitably used in soil, where the urease of telluric microbes catalyzes the hydrolysis to ammonium, which has a lighter inhibitory effect. In Space outposts, urea derived from the crew urine could be used, however, whether the plants can use it as the sole source of N and its effect on root symbiosis in hydroponics were not known. Results showed a low use efficiency of urea in young plants which, however, increased in adult plants implying a possible application in a later growth stage or combination with nitrate in earlier stages (Paradiso et al., 2015). Root symbiosis did not enhance the N nutrition and the plant ability to use urea, because of the ineffective infection process in NFT, where nodules are constantly exposed to the continuous water motion and submerged in low oxygen conditions. Hence, the hypothesis that the presence of a substrate could improve the bacterial performance in hydroponics was investigated, by comparing two systems, NFT and cultivation on rockwool, and two nitrogen sources, nitrate and urea (Paradiso et al., 2014b). As expected, cultivation on rockwool positively influenced root nodulation and plant performance, without affecting the composition of seeds. Urea drastically reduced the seed yield but enhanced the nodulation and increased the seed N content, hence it can be included in the nutrient recipe for soybean to promote bacterial activity if a proper ammonium/nitrate ratio is maintained.

Light is the most relevant factor for plant growth in a controlled environment, as modulation of light, in terms of quantity (intensity), duration (photoperiod) and quality (spectral composition), is a useful tool for enhancing plant gas exchange (i.e. air and water regeneration) and improving the plant growth and yield (Meinen et al., 2018) as well as the product quality (Bian et al., 2015; Proietti et al., 2013). This last aspect is particularly important in those crops in which anti-nutritional factors can accumulate in light sub-optimal conditions (e.g. glycoalkaloids in potato; Paradiso et al., 2019), or in some novel functional foods, such as microgreens (Figure 3), that could be used as a countermeasure to degenerative diseases induced by Space factors, and in which the synthesis of health-promoting bioactive com-

Figure 3. Microgreens species belonging to the botanical families of *Apiaceae* (coriander), *Brassicaceae* (cress, kohlrabi, komatsuna, mibuna, mustard, pak choi, radish, tatsoi), *Lamiaceae* (green and purple basil), *Malvaceae* (jute), and *Chenopodiaceae* (Swiss chard) grown in a phytotron at the facilities of the University of Naples (Italy), in studies on phytochemical composition (Credits Antonio Pannico).

pounds (such as polyphenols and carotenoids) can be driven through specific light recipes (Kyriacou et al., 2017, 2019). Also, since energy limitations in BLSSs can imply abnormal lighting conditions, investigating the light-use efficiency and the light response of different genotypes within each crop is crucial to optimize plant cultivation, by assorting species and cultivars to obtain both adequate amounts of fresh biomass and a good nutritional composition along with resource recycling (Rouphael et al., 2019).

Nowadays, light emitting diodes (LEDs) are the most promising light source for plant cultivation in controlled environments, as they offer many advantages compared to conventional lamps, such as the possibility of tailoring the light spectrum and regulating the light intensity, depending on the specific requirements of the different crops and development stages, and also to promote the biosynthesis of functional compounds (Paradiso and Proietti, 2021). In addition, these lamps show smaller size and higher energy efficiency, with lower heat dispersion, and are safer and more robust than lamps with a filament, pressurized gas, or mercury in glass. The lower heat dispersion does not interfere with a controlled climate and, also thanks to the smaller volume, make it possible to place the lamps close to the plants, in modern inter-lighting and intra-canopy systems. Finally, LEDs equipped with driver chips are suitable for digital control and light protocols (i.e. daily light integral).

At present, increasing attention is given to In-Situ Resources Utilization (ISRU) including the use of native soils, and more specifically of the finer fraction of lunar and Martian regolith. Chemical analyses of lunar regolith samples collected during Apollo missions demonstrated the absence of toxic compounds hazardous for plant, animal or human life (Papike et al., 1982). Instead, both the lunar and Martian regolith contain a discrete amount of minerals which could provide macro and micronutrients essential for plant growth (Nagaoka et al., 2013; Wamelink et al. 2014). However, limited quantities have been registered for nitrogen (NO_3^-, NH_4^+), and phosphorus ($H_2PO_4^-$, HPO_4^{2-}) and sulfur (SO_4^{2-}), as a consequence of the lack of organic matter (Ming and Henninger, 1994).

In-depth studies on lunar and Martian regoliths allowed the development of a series of Standard Lunar Regolith Simulants (SLRS), to be used as analogues in ground experiments (Leshin et al. 2013; Sibille et al., 2005; Stoeser et al., 2008). Previous tests demonstrated the possibility of growing higher plants on these regoliths even without adding nutrients, but for limited periods (Kozyrovska et al., 2006; Zaets et al., 2011; Wamelink et al., 2014), as the lack of organic matter and nutrients from its decomposition, the scarce cohesion of mineral components and the consequently limited water holding capacity are limiting factors (Bourg and Loch, 1995). In addition, regoliths sometimes exhibit non-optimal pH values and the presence of toxic elements (soluble aluminium, heavy metals) (Foy, 1984). Hence, the configuration of a mineral and biological fertile substrate for edible plant growth based on regoliths still represents one of the main challenges in Space biology research.

Recently, in ISS experiments on leafy greens, arcillite was used for growing plants from seeds in plant pillows in the Veggie cultivation module (Massa et al., 2017). Plant pillows are small expandable bags and contain two different sized arcillite substrates (0.6 mm at 100% or mixed 0.6 to 1-2 mm at 1:1 ratio), mixed with a polymer-coated controlled release fertilizer. This system allowed good seed germination and substrate containment; however, the root mat water reservoir did not always provide the proper amount of water, requiring it to be supplemented with time-consuming crew manual watering.

Recent research is focusing on the hypothesis that planetary soils could be used as the cultivation substrate and mission waste (as organic or green compost from both the crew and the crops) could be applied as amendments, fertilizers or biostimulants, to ameliorate physical, chemical and biological properties of these materials. These studies imply the characterization of the geochemistry and mineralogy of stimulants (e.g. Mojave Mars stimulant MMS-1) and of the physico-chemical and hydraulic properties of MMS-1 based mixtures with green compost at different ratios (Caporale et al., 2020), as well as the agronomical and physiological response of model species (i.e. lettuce) to the growth on these substrates in a controlled environment, also considering the response of different genotypes and the nutritional quality (Duri et al., 2020).

5. Growth chambers and cultivation modules for flight and ground experiments

Since the first launch in orbit on unmanned vehicles in the 1960s and crewed missions in 1971, over 50 experiments of plant cultivation have been performed in 21 different growth chambers onboard Space vehicles: Porterfield et al. (2003) gave a detailed review of experiments and related hardware in the years 1960-2000, and Zabel et al. (2016) provided recently an extensive update of the evolution of design and technological solutions of a "salad machine" system that could be used to provide a source of fresh foods for astronauts on Space stations or during Mars transit.

Based on the continuous advance in technology and the increase in knowledge on plant response to the Spaceflight environment, the size and shape of Space plant growth chambers changed and the technologies implemented in the different subsystems developed. This progress also reflects the parallel time succession of the Spacecrafts and the Space stations on which they operated, namely the Soviet-Russian Salyut and Mir, the International Space Station, and the Space Shuttle. The comparative analysis of the facilities, in the timeline of their utilization, highlighted the evolution of each subsystem (i.e. nutrient delivery system, illumination system and atmosphere management system), of the growth area and volume and of the candidate crops, until the last generation NASA modules Veggie and the Advanced Plant Habitat (APH) (Zabel et al., 2016). Veggie is a research platform for food production in Space currently in use on the ISS. It was designed to provide a pick-and-eat diet of salad, and it is capable of growing a wide array of crops (e.g. lettuce, spinach, mizuna, tomato, pepper, green onion, radish, herbs and strawberry). It is equipped with LED lighting and little internal environmental control, for low power usage, low launch mass and stowage volume, and minimal crew time requirements (Massa et al., 2017). It is essentially an open system so that plants grow in the same atmosphere that the crew inhabit, with only slight control, and this makes Veggie an ideal system to study plant-human-microbe interactions in microgravity.

In addition to the extensive work in the design of modules for typical "salad crops", recently research focused on specific modules for edible tuberous plants, (e.g. potato and sweet potato). In this respect, the ESA project Precursor of Food Production Unit (PFPU) aims to design a modular cultivation system for tuberous plants in microgravity. Among the different modules of the PFPU demonstrator, the Root Module is the component accommodating the plant tubers and roots. The prototype has been designed and preliminarily tested on Earth through a step-by-step procedure, including the hydrological characterization of possible cultivation substrates, the set-up of a porous tube water distribution system, and a tuber-to-tuber growing cycle of potato (*Solanum tuberosum* L.) (Paradiso et al., 2020). Among six substrates tested, including both organic and synthetic materials, a cellulosic sponge was selected as the most suitable one, based on the hydrological properties (i.e. air and water transport and retention capacity), and the designed distribution system, integrated with water sensors to drive irrigation and fertigation management, was able to work timely and uniformly in the cellulosic sponge (Figure 4). Most of these growth chambers were used for research on the effects of microgravity on plants, but all are also able to produce small amounts of fresh food.

Figure 4. Leaf and stem development and stolon and tuber formation in potato plants grown on the cellulosic sponge as a degradable organic substrate, during the experiments of the ESA project PFPU at the University of Naples (Italy).

Relevant information on plants for BLSSs are gained in ground experiments, and several plant chambers designed for the specific purpose, equipped with devices for monitoring climatic and cultural parameters and recording data for modelling have been built (Poulet et al., 2016).

The Biomass Production Chamber (BPC), operating at the NASA Kennedy Space Center of Cape Canaveral (Florida) from 1988 to 1998, consists of a 20-m^2 growing area inside an atmospherically closed volume of 113 m^3. Tests performed in the BPC provided baseline values for numerous candidate plant species (e.g. wheat, soybean, tomato, lettuce), in terms of productivity, gas exchanges, evapotranspiration and mineral nutrition, useful for calculation and modelling of BLSSs, as well as valuable indications for future similar studies (e.g. the necessity to control volatile organic compounds to a low level in confined environments). The chamber was decommissioned after nearly 10 years of continuous operation, and many of the results from the crop tests have been reported in the open literature, including biomass yields and gas exchange rates (CO_2 removal and O_2 production), and the full assessment of radiation use efficiencies (as a few examples: Wheeler et al., 1996, 2008).

In Canada, the Controlled Environment Systems Facility at the University of Guelph provides a complete research venue (comprising 24 sealed chambers) suitable for measurement of plant growth, gas exchange, volatile organic compound evolution and nutrient use in a precisely controlled environment. Some hypobaric chambers also allow a variable pressure and are capable of sustaining a vacuum (Bamsey et al., 2009; Dixon et al., 2017).

In Europe, in the MELiSSA Pilot Plant (MPP) at the Autonomous University of Barcelona (Spain), Space-oriented experiments are carried out in the Higher Plant Chamber (HPC), consisting of a sealed glove-box chamber, with 5 m^2 growing area (9 m^3 volume), equipped with a closed hydroponic system and a control system for environmental parameters (Figure 5). The role of the HPC in the BLSS scheme

Figure 5. Higher plant chamber (HPC) of the MELiSSA pilot plant (external and internal view) at the University of Barcelona (Spain) and lettuce plants in subsequent stages of development during the ACSA experiments.

is growing plants, enabling the production of fresh food and the regeneration of water and oxygen for the crew, while recycling waste already elaborated by specific bacteria. The chamber is prearranged for gas, liquid and solid connections with the loop, suitable for staggered plantations. It is equipped with sensors for the measurement of gas exchange and the sampling of the recirculating nutrient solution for the determination of water and nutrient use, in a precisely controlled environment (Peiro et al., 2020).

Among several critical aspects for reliable performance of growth chambers, homogeneous air distribution is one of the most relevant, since it affects the plant gas exchange. For instance, recent tests on lettuce (*Lactuca sativa* L.) showed an irregular plant growth, presumably related to inadequate air distribution, in the MELiSSA Higher Plant Chamber (HPC) in Barcelona (Spain). Hence, the heating, venti-

Figure 6. General view of the laboratory devoted to Space research on higher plants at the University of Naples (Italy) and an internal view of the Plant Characterization Unit (PCU) (Credits Antonio Pannico).

lation and air conditioning (HVAC) subsystem was upgraded based on a detailed computational fluid dynamics (CFD) analysis. After that, a new experiment carried out in optimized airflow conditions revealed good environmental control and a very homogeneous plant growth, in terms of both biometric measurements and mineral composition (Peiro et al., 2020). Overall, the results demonstrated the beneficial effect of an adequate air distribution system in plant growth chambers and the effectiveness of CFD analysis to design and control the gas distribution.

Thank to the ESA project PacMan (Plant characterization unit for a closed life support system - engineering, manufacturing and testing), the last generation chamber Plant Characterization Unit (PCU) has been designed and built recently, as a facility able to measure all the variables required for modelling the higher plant compartment. The PCU works in a Space devoted laboratory, located at the facilities of the University of Naples (Italy). It consists of a completely sealed 2 m^2 sealed chamber, equipped with a closed hydroponic loop and sensing and control systems (Figure 6), for a precise monitoring and control of the environment, including both the hydroponic module (root zone) and the atmospheric module (plant aerial part).

6. Conclusions

Higher plants will play a crucial role in life support systems for human exploration of Space. Extensive research performed in plant biology has demonstrated that plants can adapt to Space conditions, and survive while completing a seed-to-seed cycle. However, the information about the long-term effects of real Space factors on essential plant processes is still limited, and more experimental data from large scale prolonged tests, as well as mechanistic models to predict the performance of plants in the Space environment, are required for their successful integration in BLSSs. Besides, science-driven technological innovation is needed to realize efficient cultivation systems for the Space environment in different mission scenarios. This innovation will contribute, in parallel, to more sustainable agriculture and food production also on Earth.

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