Ph.D. Thesis
in
Sustainable Agricultural and Forestry Systems and Food Security

Effects of ionizing radiation on plants to define crop shielding requirements for future space habitats

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XXXVII Cycle

Preface

The work presented in this thesis was mostly carried out at the Department of Agricultural Sciences of the University of Naples Federico II, over three years from November 2021 to November 2024, under the supervision of Prof. Veronica De Micco and the co-supervision of Prof. Carmen Arena and Prof. Stefania De Pascale. The project was funded by the European Space Agency (ESA) and the Italian Space Agency (ASI) within the MELiSSA (Micro-Ecological Life Support System Alternative) Ph.D. POMP program.
During the Ph.D. course, a visiting period was spent at the Belgian Nuclear Research Centre SCK-CEN (Mol, Belgium) under the supervision of Prof. Nele Horemans and at the MarSimulator facility, developed by the GSBMS (Scientific group of space biology and medicine, Paul Sabatier University of Toulouse III, Toulouse -France) under the supervision of Dr. Veronica Pereda within a project funded by the European Space Agency (ESA) within the Call for Ideas SciSpacE CORA – Ground-Based Facility (GBF) (Ref. I-2023-07518).
Other collaborations included: Prof. Marco Durante and Dr. Walter Tinganelli, at the Biophysics Department, GSI Helmholtzzentrum für Schwerionenforchung, Darmstadt, Germany; Prof. Eugenie Carnero Diaz at Institute of Systematic, Evolution, Biodiversity, Sorbonne University, Paris, France; Prof. Maria Grabriella Pugliese at the Department of Physics “E. Pancini”, University of Naples Federico II, Naples, Italy.

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Introduction

The present thesis *"Effects of ionizing radiation on plants to define crop shielding requirements for future space habitats"* is within ongoing research on the general goal of realizing Bioregenerative Life Support Systems (BLSS) for space exploration. Employing a multidisciplinary approach, this thesis aims to characterize the impact of ionizing radiation exposure on the growth and performance of plants as resource *regenerators* within BLSS frameworks.

Future space exploration presents profound scientific and technological challenges, especially for long-duration missions beyond Low Earth Orbit (LEO) to destinations such as the Moon, Mars, and beyond (De Pascale et al., 2021). As mission durations increase, reliance on Earth for the resupply of essential resources, including food, oxygen, and water, becomes unsustainable. In this context, the BLSS emerged as a cornerstone of sustainable space exploration, offering a closed-loop solution that leverages biological processes to regenerate vital resources (Paradiso & De Pascale, 2021). Plants play a central role in these systems due to their ability to produce oxygen, sequester carbon dioxide, recycle water, and provide a renewable source of fresh food, also rich in bioactive compounds, potentially helpful to counteract space-induced diseases in future space crew (Cazalis, 2021; Kononikhin et al., 2017).

However, the space environment introduces extreme conditions that threaten plant survival and performance, challenging their integration into BLSS. Factors such as exposure to ionizing radiation (IR) can impair physiological processes, reduce productivity, and compromise resource regeneration (De Micco et al., 2022). IR is particularly critical, as plants deployed in space will encounter exposure to Galactic Cosmic Rays (GCRs) and sporadic Solar Particle Events (SPEs) (Simpson, 1983; Zeitlin et al., 2013).

IR refers to electromagnetic waves or high-energy particles possessing enough energy to displace electrons from molecules (Obodovskiy, 2019). Various types of IR (e.g. X-rays, neutrons, protons, or heavy ions), differ in their physical properties such as mass, charge, and energy, which influence their interactions with living organisms (De Micco et al., 2011; Arena et al., 2014 a; Gudkov et al., 2019; Geras' kin, 2024).

IR effects depend on its linear energy transfer (LET). Sparsely ionizing (low-LET) radiation is generally less effective than densely ionizing (high-LET) radiation, inducing several biological effects at the same absorbed dose. In plants, the exposure to low-LET X-rays has been shown to induce a range of effects, including mutations, chromosomal aberrations, and alterations in growth and development (Gupta et al., 2019; Gudkov et al., 2019; Sax, 1963; Arena et al 2014 b; De Micco et al., 2014).

High-energy heavy ions are generally densely ionizing and they are not part of the natural radiation background in space. However, they are part of the galactic cosmic radiation spectrum experienced by crews of human spaceflights. Densely ionizing radiation produces more clustered lesions in the DNA than low-LET radiation, and these DNA lesions are difficult to repair (Furusawa, 2014; Tinganelli & Durante, 2020). For instance, they have proven to be more effective in generating new germplasm in plants (Guo et al., 2024), also leading to significant changes in other plant traits (Kazama et al., 2008; Park et al., 2022; Liu et al., 2013; De Micco et al., 2021a; Arena et al., 2019; Vitale et al., 2021 b). Despite decades of research on plant cultivation in space, the effects of IR remain poorly understood, particularly in scenarios involving high-LET and chronic low-dose exposure (Mousseau & Møller, 2020). Existing studies have primarily focused on short-term experiments with acute doses conducted in Earth-based facilities, often using radiation analogs that do not fully replicate the complex radiation spectra of space (De Micco et al.,2022). For example, X-rays are frequently used due to their comparable effectiveness to low-energy protons commonly encountered in space environments (Durate & Cucinotta, 2008). Heavy ions (also called HZE particles), such as carbon (^{12}C) and iron (^{56}Fe), serve as representative moderate- and high-LET radiation in ground-based experiments to simulate the GCRs spectrum (Schuy et al., 2020; Simonsen et al., 2020). These ions span a range from moderate to high-LET. However, research on plant responses to such radiation remains limited, with existing studies yielding contradictory results (De Micco et al., 2022). Plant radiosensitivity appears to vary widely depending on species, radiation type, and dose. This knowledge gap is particularly pressing as future space missions will increasingly rely on the robust performance of plants under sustained radiation exposure, not only for food production but also for broader bioregenerative functions within BLSS (De Pascale et al., 2021).

The experimental approach applied in this thesis involves controlled irradiation of different plant species, irradiated at different phenological stages, using various radiation types and doses to broaden the knowledge on plant responses. This setup enables the evaluation of effects on biomass production, photosynthetic efficiency, and stress-response pathways. By characterizing the detrimental and stimulatory impacts of IR, the study also aims to identify critical thresholds and underlying mechanisms that could inform strategies to enhance plant performance under space conditions. Particular attention is given to the accumulation of secondary metabolites, such as phenolic compounds and antioxidants, which play a crucial role in mitigating oxidative damage and may enhance the nutritional value of plants.

The thesis is organized as follows:

Chapter 1 is a perspective on the key findings from space-related research on higher plants, emphasizing the critical knowledge gaps that remain to be addressed. It explores the capabilities and limitations of existing experimental facilities designed to simulate space conditions and outlines the requirements for future infrastructure needed to achieve fundamental biological research objectives. The chapter also underscores the importance of fostering interdisciplinary collaboration and establishing global standard operating procedures for analysis and data collection in space-related experiments. The paper has been published in *npj Microgravity* (De Micco et al., 2023; https://doi.org/10.1038/s41526-023-00315-x).

Chapter 2 focuses on the evaluation of different doses of X-rays on the morpho-anatomical and nutritional traits of microgreens of *Brassica rapa* L., irradiated at the stage of germinated seeds. The results showed that X-ray exposure does not induce detrimental effects on growth, while it stimulates the production of antioxidants, improving plant defense and nutritional value. The overall results support the idea of using this species in space as a supplemental functional food. The paper has been published in *Horticulturae* (De Francesco et al.,2023: https://doi.org/10.3390/horticulturae9040452).

Chapter 3 compares the responses of microgreens of *Brassica rapa* obtained from seeds exposed to different radiation sources including low- and high-LET radiation. Seed germination, the morphological, anatomical, and biochemical traits were analyzed. The results reveal that microgreens grown from irradiated seeds demonstrate a remarkable resistance to radiation, with no significant abnormalities adversely impacting their growth. However, the impact of irradiation on the traits of microgreens varied significantly depending on the type and dose of radiation applied. The absence of a consistent trend in the measured parameters highlights the complex interplay between radiation conditions and plant responses.

Chapter 4 highlights the radio-resistance of *Salvia hispanica* (chia) microgreens, suggesting their potential suitability as a candidate for space farming. The findings reveal that chia microgreens develop distinct physiological, anatomical, and biochemical adaptations in response to varying types and doses of heavy irradiation, demonstrating their capacity to withstand the challenges posed by space radiation.

Chapter 5 presents findings from the COMBO-AGR project aimed to explore how chronic ionizing radiation and reduced gravity interact to affect the germination and growth of *Brassica rapa* microgreens. Results from the analysis of harvested microgreens revealed a moderate reduction in growth under combined chronic irradiation and altered gravity, likely linked to oxidative stress concentrated in the root system. These results mark a significant step toward understanding plant adaptation in space and advancing strategies for sustainable food production on orbital and planetary platforms.

Chapter 6 analyses the possible differential responses of green- and red-leaved cultivars of Salanova® lettuce subjected to different doses of heavy ions in terms of growth, anatomical and physiological traits. Additionally, it evaluates the effects of pre-treatment with heavy-ion irradiation on plants later subjected to prolonged exposure to low-dose gamma radiation. The findings emphasize the intricate and dose-dependent nature of plant responses to radiation, revealing notable differences between the two cultivars.

Appendix 1 provides a brief description of the different radiation units.

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Chapter 1

Perspectives for plant biology in space and analogue environments

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PERSPECTIVE OPEN

Perspectives for plant biology in space and analogue environments

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Advancements in plant space biology are required for the realization of human space exploration missions, where the re-supply of resources from Earth is not feasible. Until a few decades ago, space life science was focused on the impact of the space environment on the human body. More recently, the interest in plant space biology has increased because plants are key organisms in Bioregenerative Life Support Systems (BLSS) for the regeneration of resources and fresh food production. Moreover, plants play an important role in psychological support for astronauts. The definition of cultivation requirements for the design, realization, and successful operation of BLSS must consider the effects of space factors on plants. Altered gravitational fields and radiation exposure are the main space factors inducing changes in gene expression, cell proliferation and differentiation, signalling and physiological processes with possible consequences on tissue organization and organogenesis, thus on the whole plant functioning. Interestingly, the changes at the cellular and molecular levels do not always result in organismic or developmental changes. This apparent paradox is a current research challenge. In this paper, the main findings of gravity- and radiation-related research on higher plants are summarized, highlighting the knowledge gaps that are still necessary to fill. Existing experimental facilities to simulate the effect of space factors, as well as requirements for future facilities for possible experiments to achieve fundamental biology goals are considered. Finally, the need for making synergies among disciplines and for establishing global standard operating procedures for analyses and data collection in space experiments is highlighted.

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INTRODUCTION

At the beginning of space exploration, research in space life science was focused on the human body to gain a fundamental understanding on the responses to the stressful space environment, to ultimately prevent health risks and protect astronauts by managing space-induced pathological issues[1,2]. In the last decades, increasing interest has been raised towards plant space biology due to the awareness that cultivation of higher plants in space is a requirement for long-duration human missions, where the regeneration of resources and plant-based food production onboard must be increased at the expense of re-supply from Earth[3].

Plants play a crucial role in Bioregenerative Life Support Systems (BLSS). Artificial ecosystems such as the BLSS are high-technology systems, based on the integration of physico-chemical and biological processes, to support long interplanetary missions[4,5]. The BLSS concept includes several interconnected compartments in which different organisms are used to sequentially recycle resources[6,7]. Within a BLSS, the photoautotrophic compartment enables the production of edible biomass, oxygen, and water as resources for the astronaut, starting from carbon dioxide, wastewater and other wastes. An example of BLSS is the MELiSSA (Micro-Ecological Life Support System Alternative) loop by the European Space Agency (ESA) that aims to sustain astronaut life in space missions to reduce the initial payload and dependency from Earth[8]. The MELiSSA loop is made of interconnected compartments among which the requirements of the higher plant compartment need to be fulfilled at least in part by the outputs of other compartments[9]. As well, the species/cultivar choice and environmental control are dictated by the need to meet the requirements of the other compartments (e.g., oxygen requirements of the animal crew) in addition to the generally accepted requirements including short cultivation cycles, reduced plant size, high harvest index, and resistance to diseases. Increasing effort is posed on plants to produce healthy food specifically designed to sustain crew in long term missions[3,10,11].

Additionally, research in space-plant biology and space-agriculture is required alongside the strong efforts to increase the knowledge on human biology in space with the aim to predict risk factors, prevent disease(s), and implement effective countermeasures to manage health emergencies during missions. Among countermeasures for assuring astronauts' well-being against degenerative diseases and psychological issues, there is the introduction, in their nutrition, of plant-derived fresh food produced directly on board or within a habitat. Further, there may also be benefits for crew mental health by allowing crews to participate in plant cultivation in the living and/or working pressurized modules in space[12,13].

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WHAT ARE THE MAIN CONSTRAINTS TO PLANT CULTIVATION IN SPACE?

From an applied point-of-view, the design and realization of BLSS require a better understanding of plant acclimation and adaptation processes that determine the plant's capability to complete a full life cycle in space (producing viable offspring) for both model plants and crops. As a matter of fact, the altered growth and behaviour of plants in space can alter the input/output balance between the different compartments as well as the potential nutritional value of the derived fresh-food. Indeed, as any other living organism, plants' survival and reproduction strongly depend on interactions with environmental factors (e.g. temperature, light, oxygen/carbon dioxide, water, and volume availability) including the novel space factors (e.g., altered gravity and ionizing radiation). Among different environmental factors, altered gravity and ionizing radiation are recognized to be the main constraints for growth in space conditions. In the following section, we briefly summarize the current knowledge about the effects of microgravity and radiation on plants.

WHAT IS ALREADY KNOWN ABOUT THE EFFECTS OF MICROGRAVITY AND RADIATION

To date, European scientists studying plant space biology have focused on understanding the effects of altered gravity on several plant systems (e.g. different species/cultivars, target organs/tissues). Recently, more attention has been dedicated to the effect of ionizing radiation (IR) so far as it represents a clear challenge for exploratory-class missions[14]. Altered gravity and exposure to IR can induce changes in gene expression, cell proliferation and differentiation, signalling and physiological processes (see precise data and references in the following paragraphs). The expected consequence of these changes would be serious structural and functional alterations at the whole organism level. In addition, since these cellular and molecular changes are detected in early developmental stages, developmental alterations would also be expected. However, altered tissue organization, organogenesis and functioning have not always been detected under spaceflight conditions. For example, none of these effects has been reported to occur when plants have been cultured in space: adult organisms were produced with no evident aberrations and the entire *seed-to-seed* life cycle of plants was successfully achieved under spaceflight conditions[15,16]. Interestingly, this phenomenon is not exclusive to plants. It was first detected and reported by Marco et al. [17] in the fruitfly *Drosophila melanogaster* and defined as "an apparent paradox". Up to now, this paradox has not been resolved and remains an important challenge for space biology research.

Effect of microgravity and altered gravity on plant growth and reproduction

Since 475 million years ago, land plants have evolved under the same 1 *g* gravity level. Early pioneering space experiments have demonstrated that plants are able to survive and grow in space, although morpho-physiological alterations were soon reported in crop species as lettuce and lentil[18,19]. In early research, deficiencies in the experimental setup and in used facilities were sometimes responsible for confusing and contradictory results. For example, nutrient absorption has been reported to increase, decrease or remain unchanged under reduced gravity, likely due to hardware limitations and species-specific responses[20]. Now, however, there is evidence that microgravity in space environment does not prevent plant growth and reproduction but causes serious alterations in plant physiology and development[21]. Plants respond to changes in mechanical inputs, therefore gravity-related research offers a unique opportunity to elucidate the mechanisms of gravity perception whose knowledge is necessary to better understand fundamental processes and the physiological changes in space. Moreover, in exploratory-class long-term missions, plants will face not only a lack of gravity (or residual gravity) during spaceflight (and inside the International Space Station - ISS) but also the lunar gravity (0.17 *g*) and the Martian gravity (0.37 *g*) which are substantially lower to what plants normally experience on Earth. Indeed, in forecasting possible acclimation of plants in environments with altered gravity, thresholds for gravity sensing should be considered. Previous studies indicated that the threshold acceleration perceived by lentil roots in spaceflight can be in the order of 10^{-3} *g* or lower[22], but the effects of altered gravity on cell cycle have been detected at higher levels, intermediate between Moon and Mars gravity, in simulation experiments on Earth[23]. Similarly, the range for the attenuation of phototropism in higher plants occurs in the same range 0.1-0.3 *g*[24].

Plant developmental patterns and gravity perception: from gene expression to cell cycle and organ development. Most studies to reveal the effect of altered gravity on plant development have been focused on the root meristem[25]. Experiments under microgravity simulation and onboard the ISS revealed the disruption of the meristematic competence in seedlings, i.e. the loss of the coordinated progress of cell proliferation and cell growth that characterizes meristematic cells under gravity conditions on Earth[26–28].

An acceleration in the cell cycle was detected in in-vitro cultured *Arabidopsis* MM2d cells grown in simulated microgravity conditions produced in a Random Positioning Machine (RPM), resulting from downregulation of genes involved in the G2/M transition checkpoint and upregulation of genes controlling the G1/S transition. Other phenomena were the downregulation of significant genes for ribosome biogenesis and the corresponding depletion of the levels of nucleolar proteins, the depletion of the nuclear transcription and an increase in chromatin condensation, related to the epigenetic regulation of gene expression[29,30]. Experiments using different levels of reduced gravity and hypergravity indicated modulation of alterations for each level, with Mars gravity inducing milder alterations[23,31]. Indeed, studies on gravitropism and phototropism have shown that the reduced gravity level on Mars of 0.38 *g* should not be a major problem for plant growth[24].

Auxin is a key factor regulating the connection between perceived stimuli and cellular responses, controlling the balance between cell proliferation and cell differentiation, regulating the cell cycle progression and the coordination between cell growth and cell division[32–35]. The role of the LAZY proteins in gravity sensing has been recently described, being crucial players linking gravity perception and gravitropic curvature through the proper redistribution of auxin, the relocation of the auxin efflux carrier PIN-FORMED (PIN) proteins for the tropic response of both roots and shoots[36]. However, the role of auxin and its polar transport in plant growth and development under microgravity conditions is not fully understood and requires further investigation to solve controversial concerns due in part to its complex interaction with cytokinin. For example, it has been shown in *Arabidopsis* that real microgravity does not influence the distribution of auxin in the primary root, whereas it affects that of cytokinin[37]. In contrast, the inhibition of pea hypocotyl growth in microgravity is correlated with an attenuation of polarized auxin transport, a decrease in auxin levels and an increase in cytokinin levels[38].

Parallel transcriptomic experiments exploring how simulated (i.e. clinorotation, RPM and diamagnetic levitation) and real microgravity change gene and protein expression have shown a complex response of plants at early developmental stages (mostly *Arabidopsis* seedlings), involving reprogramming of the gene expression pattern[39]. Specific genes of response to gravity alteration have not been found, while the main and most frequent targets of this gene reprogramming are: genes coding for heat shock-related elements, cell wall remodelling factors, oxidative burst intermediates and components of the general mechanisms of plant defence against stressors, being differently affected at the different spaceflight, lunar and martian *g* levels[40]. The results of several experiments in space using a centrifuge to produce different gravity levels showed a differential response to each level, triggering different adaptive responses, involving changes in the regulation of different sets of genes. Changes in gene expression were lower under Mars gravity compared to microgravity on the ISS[40,41].

Interaction with other factors. Many plant responses are primed by the interaction between gravity and other physical, chemical, or biological factors. In the last decades, orbital platforms enabled comprehensive studies on the mechanisms underlying plant growth in microgravity[42]. Still, there is a need to further investigate the interactions between gravity and other environmental factors including temperature, light, oxygen/carbon dioxide, water availability, electric and magnetic fields, especially in the framework of plant morphogenesis and tropic responses, also considering intra-specific genotypic variability[43,44]. With the exception of a limited number of studies concerning microgravity interactions with magnetic fields, water or chemical stimuli on crops (i.e. flax, cucumber and carrot)[45–47], previous research has mainly focused on the interactions between gravity and light. It has been shown that the sensitivity of plants to light is influenced by microgravity and that phototropic curvature of the shoot and root organs are largely affected by changes in gravity conditions[48] Experiments performed using the EMCS onboard the ISS or the ESA ground-based facilities resulted in discovering novel phototropic responses of plants, both the model *Arabidopsis* and *Brassica oleracea*, proving that the interaction between gravity and light changes according to the magnitude of g-force[49–51]. In this framework, light quality has a prominent role in determining the direction and strength of phototropic responses of shoots and roots, with major differences between blue and red wavelengths under a wide range of gravity levels[51,52]. On the other hand, previous studies also showed that light can control the sensitivity of plants to gravity through phytochrome-regulated pathways, indicating that phytochromes play a key role in integrating multiple environmental stimuli[53].

Gene expression studies with different levels of altered gravity showed that the adaptive response appeared enhanced by red light photostimulation. Red light activates cell proliferation and ribosome biogenesis in pea[54], and in the "Seedling Growth" series of experiments in the ISS, red light caused a concerted upregulation of marker genes for cell proliferation, cell growth and auxin polar transport[55]. Experiments with different levels of gravity (microgravity, Mars gravity and ground control gravity) with and without red light photoactivation have shown that red light restored the auxin distribution patterns which appeared altered under microgravity, while in roots grown at 0.3 *g*, the auxin polar transport was slightly altered, irrespective of photoactivation[56]. The red light was also shown to counteract the decoupling between cell proliferation and growth in root meristems reported in earlier experiments[41].

Gene expression alterations, evaluated by RNA-seq, showed different responses to different gravity levels and modulation of gene expression by red light photoactivation. As an example, Mars gravity level induced an adaptive response, consisting of the activation of environmental acclimation-related transcription factors (WRKY and NACs families), especially in photostimulated samples[41].

It appears clear that plant cultivation in future space missions implies research-based strategies that involve gravity-substituting factors (e.g., light) to counteract the effects of microgravity or partial gravity conditions such as on the Moon and Mars. Nowadays, the technological advancement in cultivation systems is providing effective and affordable tools to control environmental factors for plant growth in space with the possibility of using external cues for both application and research purposes[42]. Gravity might be replaced by specific stimulation in terms of light wavelengths and photon flux density for the regulation of plant growth and development. Prospectively, other factors are expected to play such a role including water, electric and magnetic fields, chemicals, or microorganism, but further investigation is needed.

Seed-to-seed cycle. Nowadays, it is crucial to delve into the mechanisms by which altered gravity conditions can affect plant reproduction and seed viability, in order to develop cultivation strategies for the improvement of plant-based BLSS in future human settlements on the Moon and Mars[57]. The completion of the *seed-to-seed* cycle, in fact, will be essential to produce viable seeds to be used for the cultivation of plants over time without relying on terrestrial supply.

Early studies with plants grown for extended periods in microgravity reported an overall reduction of plant growth and difficulties in the transition to the reproductive stage[58]. Since the first seed production in space by *Arabidopsis thaliana* plants in 1982, a few experiments on plant reproduction have been performed[59]. Overall, previous studies showed that the *seed-to-seed* cycle can be accomplished in most species tested in microgravity, although with reduced quality of embryos and seeds produced by plants due to delayed embryo development, modification in storage reserves, delayed starch use in cotyledons, and decreased cell number in cotyledons[15,58,60–62]. Furthermore, experiments using simulated microgravity (e.g., clinorotation) showed significant alterations during the development of male gametes in several crop species[63].

Given that most studies have investigated the effect of microgravity on early stages of plant development with sporadic studies on plant reproduction, there is a large gap of information to be filled to fully the effects on the growth of plants in the adult stage and on plant reproduction.

Effects of ionizing radiation on plant growth and development

Outside Low Earth Orbit, IR is variable in space and time and can severely constrain organisms' growth[3,14]. IR can cause direct damage to the structures encountered, but also indirect due to the generation of reactive oxygen species (ROS)[64,65]. The oxidative stress due to ROS production may damage important components of plant cells, including lipids and proteins, but especially DNA[66,67]. The degree of direct DNA damage and proper functioning of DNA repair systems determine the consequences of IR exposure for plants at morpho-structural and physiological level[68].

Nevertheless, plants' responses to IR are not fully understood yet. Experiments in space where plants, either the model *Arabidopsis* or crops such as beans and tomato, were exposed to cosmic radiation and on the ground with exposure to low- and high- linear energy transfer (LET) radiation have shown that IR can have positive, null or negative effects on plants, at genetic and morphophysiological levels depending on IR properties and plant intrinsic factors such as type of radiation, its LET, exposure time (acute or chronic), dose, plant species/cultivar, developmental stage at the time of irradiation[69]. The effect of IR is also tissue-specific and depends on tissue architecture: complex tissues in beans and tomatoes seem less sensitive to damage[70,71], and, on the contrary, the meristematic cells are the most sensitive to radiation[72].

High-LET radiation, like protons and heavy ions, is more harmful in inducing genetic mutations compared to low-LET radiation such as X- and γ-rays[73,74]. Concerning the dose of exposure, high doses (>100 Gy for seeds; >50-70 Gy for vegetative stages) can lead to

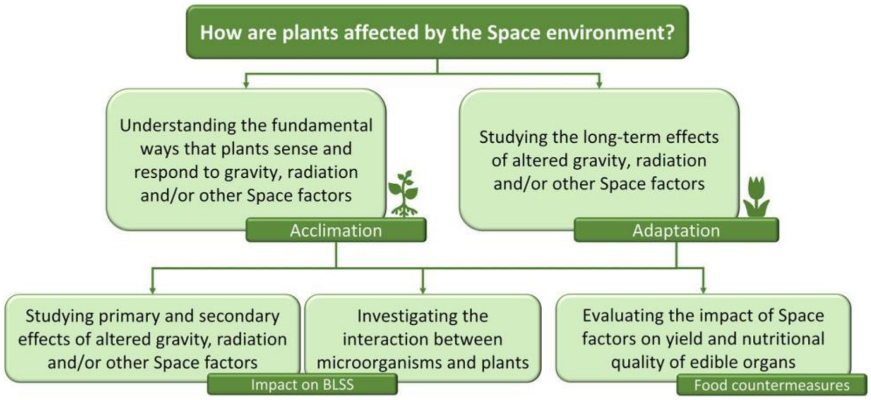

Fig. 1 Schematic view of the main knowledge gaps in plant biology which regard understanding processes of acclimation and adaptation to space factors. Understanding such processes is fundamental to evaluate the impact on the functioning of BLSS and on the value of plant-derived food for the integration of astronauts' nutrition.

harmful outcomes, such as reduced levels of photosynthesis and germination, embryo lethality, loss of apical dominance, dwarf architecture, altered leaf anatomy, and accelerated senescence[67,69,75]. Low doses of IR, on the other hand, appear to induce hormetic response in plants, stimulating germination, growth, photosynthetic and respiration rate, improving the content in chlorophyll, carotenoids, non-enzymatic (ascorbic acid, glutathione, and anthocyanin) and enzymatic antioxidants (ascorbate peroxidase, catalase, and superoxide dismutase) and phenolic compounds, effective in counteracting the oxidant action of ROS, thus increasing plant nutritional value and radioresistance[75–80].

Most of the studies performed on plants have been conducted by irradiating dry seeds with acute doses (due to limitations in the volume and time of irradiation availability)[75,81]. Moreover, most of the studies involving crop species have mainly been focused on using IR to introduce genetic variation and selecting plant cultivars with specific traits[82]. Only a few studies have considered the effects on the yield, nutritional value as well as interaction with other factors. A recent study has indicated that the effect of X-rays delivered to germinated seeds at different doses is strongly influenced by light quality during subsequent cultivation[83].

As a result, little information is available on the variation of radiosensitivity during the different phenological phases in the case of acute exposure and on the effects of chronic exposure. Indeed, it is important to emphasize that resistance to large doses of radiation delivered in an acute way (in shorter times than those necessary for the repair of cellular damage) often does not translate into resistance to chronic exposures for multiple generations and vice versa[84]. Therefore, in the sight of future space exploration, the time is ripe for increasing the efforts to investigate plant responses to chronic low dose-rate and high LET radiation to clarify all the processes and mechanisms behind the radioresistance phenomenon[14].

KNOWLEDGE GAPS IN MICROGRAVITY AND RADIATION RESEARCH IN PLANT BIOLOGY

According to the current scientific knowledge on plants' responses to space factors, in order to successfully achieve the *in-progress* target of crewed missions in space, it is necessary to fill knowledge gaps in plant biology. They can be synthesized in the following five points (Fig. 1).

1 - Understanding the fundamental ways that plants sense and respond to gravity alone or in combination with radiation and other space and environmental/cultivation factors. This point is mainly related to short-term effects and acclimation strategies and includes studies on tropisms and morphogenesis.

2 - Studying the long-term effects of altered gravity, radiation and/or other space environment factors on plants and understanding how plants adapt to this new kind of environment.

3 - Studying primary and secondary effects of altered gravity, radiation and/or other space factors on plant growth and reproduction.

4 - Investigating the interaction between microorganisms and plants (beneficial and pathogenic) under space conditions in light of the realization of cultivation modules in BLSS.

5 - Investigating the effects of space factors on yield and nutritional value and quality (e.g. production of nutraceutical compounds) of edible organs targeting the use of plant-derived fresh food as countermeasures to improve astronauts' health.

Points 3-5 can be investigated in the short-period, thus being mainly targeted to unravelling the acclimation strategies of plants to space factors. The same points can be investigated in the long-term and over multiple generations to evaluate the heritability of varied traits, hence leading to adaptation. In the latter case, constraints to reproduction become crucial to be analyzed.

Overall, the main objective of research activities in plant space biology is to reveal potential acclimation and adaptation mechanisms and processes in the response of plants (crops and model species) to microgravity, partial gravity and variable space radiation in combination with other environmental/cultivation conditions (e.g. airflow, light), through the developmental phases of a whole life cycle. This will allow the resolution of the "apparent paradox" between molecular and cellular effects *versus* organismic and developmental effects by understanding the mechanisms by which plants overcome the impacts occurring at early plant life stages after exposure to spaceflight conditions.

To understand if exposure to adverse space conditions potentially leads to acclimation and adaptation, the long-term responses of plants have to be investigated through sequential studies of plants after different times of exposure to single or multiple space factors, at different phases of the plant development. The acclimation has to be studied during the life cycle of the plant and adaptation has to be studied after several generations of plants exposed to a space environment.

Knowledge of plant-microorganism interactions is also important. Plants naturally attract microorganisms, some detrimental to plant health while others establish symbiotic relationships. Plants also have endophytes (bacteria and fungi) living between plant cells, some of which are transmitted to the following generations. Understanding the effect of the space environment on the relations between microorganisms and plants can help assess risks to future crew food supply or discover opportunities for microorganisms-mediated enhanced crop yield.

Unravelling the plant acclimation and adaptation processes, responsible for producing essentially viable adult individuals, is important not only for our fundamental understanding within plant biology but also for the realization of BLSS.

Indeed, addressing the five points mentioned above would allow to evaluate:

- How the growth processes and regeneration capacity of plants are affected by space factors and thus impact the cultivation requirements in producers' modules of BLSS.
- If reproductive success is achieved and whether multiple generations of plants are possible to obtain, in order to guarantee the possibility to produce seeds for successive cultivation cycles.
- If and how the yield and nutritional value and quality of edible organs are affected and thus impact the astronauts' nutrition.

The two first points regard every plant species, not only crops but also model species such as *Arabidopsis*, while the second and third mainly refer to crop species.

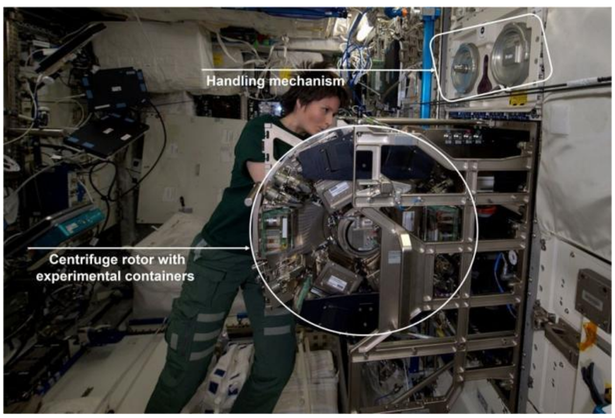

Fig. 2 ESA astronaut Samantha Cristoforetti working with ESA's Biolab facility in the Columbus laboratory on the International Space Station. It appears evident the presence of the centrifuge but the strict limitations of volume available for the experimental containers. Credits: ©ESA/NASA.

FACILITIES IN SPACE AND ON EARTH TO STUDY THE EFFECTS OF MICROGRAVITY AND RADIATION ON PLANTS

The ISS is an important research platform to study not only the effects of reduced gravity but also the long-term consequences of low dose space radiation on plants. The daily dose received in the ISS has been estimated at 0.5 mSv, assessed by physical dosimetry using phantoms[85], which is about 100 times higher than the dose on Earth, and about 2.6 and 1.28 times lower than the dose on the Moon and Mars surface[86,87]. By combining studies on ISS of plants exposed to various space factors (gravity/space radiation) and environmental cultivation conditions (e.g. different airflow or light conditions), additional required knowledge for future space agriculture can be obtained. Long-term experiments and full cycle studies of plants require a minimum cultivation area. Although the growth area has become larger in the newer ISS facilities (compared to systems such as Kubik and Icecubes), they are still considered too small for crops and full life cycles. When a 1 *g* or simulated Moon/Mars gravity exposure is required, the growth area is limited due to the diameter of the centrifuge rotor that has to fit into standard-sized racks on the ISS. An extensive review was done on the space plant growth systems where more than 20 systems are described[42]. The ESA BIOLAB facility on ISS has 4 Advanced Experiment Containers (AEC) on two rotors with a limited growth area per AEC (http://wsn.spaceflight.esa.int/docs/Factsheets/8%20Biolab%20LR.pdf) (Fig. 2) (Table 1). The BIOLAB allows unique experimental equipment to be built inside the AEC, and the available growth area for the plant will depend on the instrumentation required in the AEC to perform the experiment but still remains limited. The NASA systems such as Vegetable Production System (VEGGIE, 2014) and Advanced Plant Habitat (APH, 2017) have increased crop growth area, but they are still relatively small[88–90]. The APH is a closed, controlled growth system with full environmental monitoring and control. The VEGGIE system is simpler, with less control, and designed for more crew interaction (Table 1). For plant experiments where smaller volumes are required, the NASA Advanced Biological Research System (ABRS) is available with two experimental research chambers (growth area 0.053 m^2). The chamber has light and environment control, and one of the two chambers is outfitted with Green Fluorescent Protein Imaging System. The JAXA Plant Experiment Unit (PEU), available on the Kibo laboratory on ISS, is

Table 1. Comparison of key parameters for plant cultivation for ESA Biolab (http://wsn.spaceflight.esa.int/docs/Factsheets/8%20Biolab%20LR.pdf), NASA VEGGIE[88], and NASA APH[86,88].

Parameter	BIOLAB (ESA)	VEGGIE (NASA)	APH (NASA)
Growth area and volume	1. 6 cm × 6 cm × 10 cm 2. 10.8 cm × 15 cm × 13.7 cm	1. 47 cm × 1300 cm^2 2. 41.9 cm × 1300 cm^2	1. 45 cm × 1708 cm^2
Light provision	White adjustable 1.7–11.1 W/m2 PAR, Infra-Red	100–500 μmol m^{-2} s^{-1} Red, Blue, Green	0–1000 μmol m^{-2} s^{-1} White, Red, Blue, Green, Far-red
Temperature range	18–40 °C	ISS cabin equivalent	18–30 °C
Humidity control	Yes	No	Yes
Gas regulation	Yes	No	Yes
Trace gas removal	Yes	No	Yes
Centrifuges (fractional gravity)	2	No	No
Experiment positions	4–6 containers	1 container	4 root modules
Growth substrate/root support	Experiment dependent	Pillows with growth substrate, controlled-release fertiliser and seeds Water reservoir	2 moisture sensors O_2 sensor Water/nutrient delivery system
Observation	Images (top view) Video	ISS camera used by crew	2 colour cameras (top and side view) Infrared camera

equipped with a LED lighting system with red and blue LEDs, a growth chamber (growth area 0.027 m^2), an automated watering system and a CCD camera.

What is further required?

Larger growth areas, where both model plants and crop plants can be cultivated for long-term experiments (during the whole life cycle), are needed to fully understand the plant acclimation and adaptation to the space conditions. Such a facility requires the control of environmental factors (including temperature, light, gasses, relative humidity, airflow, nutrient solution, and watering), and continuous imaging of the plant growth (e.g., visible light images, IR images using thermal cameras, fluorescence images). Environmental and imaging data should as a minimum be partially and periodically downloaded for feedback from the science teams collaborating from the ground. To collect data for the molecular, cell and anatomical studies, plant samples need to be harvested after sequential exposures to reduced gravity, preserved on ISS either by freezing or chemical fixation and brought back to the ground for analyses. In addition, the possibility to make microscopy analyses onboard has become a reality with the ESA/DLR Fluorescence Microscopy Analysis System (FLUMIAS), a high-resolution fluorescence microscope for live-cell imaging that is available on the ISS[91].

A specific point affecting the technical needs of space facilities for plant culture is the possibility of performing comparative studies in spaceflight at different levels of gravity, including the Moon and Mars gravity, as well as the inflight 1 *g* control. The latter is of the highest importance and makes mandatory the implementation of centrifuges in the facilities used for plant cultivation in the ISS. The advanced sophisticated facilities for plant cultivation now available on ISS, such as VEGGIE, APH and the Exposed Roots On-Orbit Test System (X-ROOTS), which have proven successful in the cultivation of a wide range of plant species, are not equipped with centrifuges. ESA in 2018 decommissioned the European Modular Cultivation System (EMCS), a highly useful facility in which different successful experiments were carried out, some of them including pioneering comparative analyses at different levels of gravity in addition to the necessary in flight 1 *g* controls. Later on, in 2020, ESA promoted a discussion group to adapt the ESA Biolab facility to harbour experiments using crop species and encompassing the full plant life cycle while being exposed to different gravity levels. Whatever the final decision adopted, the need for an effective plant cultivation facility in space with the capabilities mentioned above, as a key to gaining knowledge to support human life in space exploration, is becoming more and more urgent within the next years.

APPLICATIONS AND BENEFITS FOR EARTH

Understanding how plants are able to grow and adapt to space conditions will ensure reliable and predictable food supplies for human space exploration and has strong synergies with the United Nations Sustainable Development Goals, global food security and circular economy.

The sophisticated agro-technologies developed for space applications (e.g., innovative lighting, watering and nutrient delivery systems, fine environmental monitoring with automated control, imaging systems to analyze plant health, etc.) bring innovations to agriculture on Earth to improve sustainable plant cultivation and food production. For example, developing volume-saving, highly efficient plant growth controlled environments is beneficial for food and drug production particularly in densely populated urban areas in line with vertical farming technologies, in underground facilities with no natural light source, and in general in extreme environments such as deserts and poles.

Developing smart and safe pest control methods is applicable in confined volumes where aerosols are undesirable and natural predation for the reduction/removal of pests is not possible.

Besides these more human operational and exploration-oriented goals, it should also be stressed that the unique near weightlessness environment, as well as the high levels of ionizing radiation, also provide a research laboratory that cannot be obtained in on-ground laboratories and as such can answer specific and fundamental questions in life sciences. Improving the knowledge on how plants respond to ionizing radiation can provide information applicable in all the fields in which radiation is studied on Earth ranging from breeding programs, decontamination methods and radioecology.

FUTURE PERSPECTIVES AND RECOMMENDATIONS IN THE SHORT AND LONG TERM

Today there is a need for fundamental research that goes beyond the demonstration of plants' ability to acclimate and adapt to the space environment. A multi-parameter facility would help unravel the effects of altered gravity in combination with other factors, either typical of space (i.e. ionizing radiation) or of confined volumes of cultivation chambers. Moreover, by modulating the exposure to specific environmental conditions (e.g., air flow or light), it would be possible to study the direct and indirect effect of the space factors on plants, bridging the knowledge gaps of the acclimation/adaptation mechanisms.

Currently, to study plant development in the space environment through the whole life cycle of a plant, including crops/food plants, is possible only using the ISS research platform: no other active platforms possess capabilities for answering the plant biology research questions listed in this paper. The infrastructure present on ISS can be modernized to achieve the needed goals to point to the sustainability of space exploration with BLSS.

Using the ISS platform for research will be the defining stepping stone into exploratory-class missions deeper into space. International efforts are ongoing to design and develop additional payloads in the frame of the Artemis program (e.g., NASA PRISM solicitation) (https://www.nasa.gov/feature/nasa-releases-prism-call-for-potential-lunar-surface-investigations). The Gateway platform is also currently planned for lunar orbit in the mid-late 2020 s. This should provide opportunities for experiments deeper into space, particularly outside of Earth's magnetic field and the unique radiation environment can be exploited to further develop our understanding of the effect that different types of radiation may have on plants in combination with other space factors.

It is not straightforward to indicate what are the more urgent goals of plant space biology since it has been recognized that space farming is becoming more and more a necessity as long as the roadmap for human space exploration goes beyond LEO (BLEO). A summary of possible goals (targets) within the main knowledge gaps (open fundamental scientific questions) identified in the previous paragraphs is reported in Fig. 3.

The first, second and third knowledge gaps should be addressed in experiments to be performed in the short- and medium-term since they will provide fundamental information that is the basis for the realization of more complex experiments in the long-term. The knowledge obtained in the short- and medium-term will be fundamental to defining requirements and developing new hardware to support long-term exploratory-class human missions. Indeed, in such missions, completely closed BLSS are themselves the "main requirement" although with technological differences depending on the scenarios determining the environmental constraints and mission duration.

In conclusion, to achieve successful space exploration, it is fundamental to apply an integrative approach in space biology merging together the information gained at different biological levels (e.g. molecular, cellular, tissue/organ up to the whole

Open fundamental scientific question	Proposed Targets of Research Activities including ground & Space experiments	Testbed	Space relevance
Relevance in the short- and medium- term			

Understanding the **fundamental ways** that plants sense and respond to gravity, radiation and/or other Space factors	• Molecular responses:
	  • Detecting differential responses of the redundant genes to changing environmental conditions
	  • Integrative space omics research including epigenetics, transcriptional or post-transcriptional regulation, etc.
	• Signal mechanisms, cell proliferation, cell cycle, cell differentiation, organogenesis, whole plant functions
	• Chemical gradients (e.g. phytohormones)
	• Plant posture
	• Plant water relations and gas-exchanges
	• Water/nutrients uptake and transport
	• Short-term traits for radioresistance
	• Effects of multiple external stimuli (light, water, nutrient) on root and shoot orientation in altered gravity
	• Cumulative effects of suboptimal environmental factors
		• Ground
		• LEO
		• BLEO
		• Moon
			• Use of Space as a laboratory
			• Space exploration (life support, food production and mental wellbeing)

Studying **primary and secondary effects** of altered gravity, radiation and/or other Space factors	• Distinguishing between primary and the secondary (e.g. confinement) effect of the altered gravity with specific focus on water flow and gas-exchanges • Develop multiuser and reusable facilities for long-term plant cultivation equipped with systems for the fine control of environmental factors, for plant imaging, and with centrifuges to perform on- board terrestrial, Lunar or Martian gravities	• Ground • LEO • BLEO	• Use of Space as a laboratory
Investigating the interaction between **microorganisms and plants**	• Root mycorrhizal development • Root-microorganism interactions • Interactions between plants and beneficial or pathogenic microbial species	• Ground • LEO • BLEO • Moon • Mars	• Use of Space as a laboratory • Space exploration
Relevance in the medium- and long- term			
Studying the **long-term** effects of altered gravity, radiation and/or other Space factors	• Developing sequential studies on growth and development of plants after different times of exposure to Space conditions and/or at different developmental phases • Plant reproductive biology (e.g. flower induction, sporogenesis, gametogenesis, fertilization, embryogenesis) • Long-term effects (acclimation or adaptive) of ionizing radiation • Mechanisms developed for radioresistance to chronic ionizing radiation • Unravelling whether the interaction between radiation and other Space factors and is antagonistic, null or synergistic per each process • Photosynthesis • Water/nutrient uptake and transport • Plant nutritional value and quality	• Ground • LEO • BLEO • Moon • Mars	• Use of Space as a laboratory • Space exploration - growing crop plants in space for regenerative food supplies - development of protocols
Relevance in the short-, medium- and long- term			
Evaluating the impact of Space factors on **yield and nutritional quality** of edible organs	• Effect of ionizing radiation alone or in combination with other factors on yield and nutritional quality of edible organs	• Ground • LEO • BLEO • Moon • Mars	• Space exploration (development of plant-based food countermeasures)

Fig. 3 Summary of possible goals (targets) within the main knowledge gaps of plant biology research in space with priorities in the timeframe of 2022–2030 and beyond (short-term, 2022–2024; medium-term, 2024–2030; long-term, beyond 2030) using primarily the ISS platform. Other research platforms such as ground, Moon, Mars, LEO and BLEO (beyond LEO) are also included. They represent both the basis for the research on ISS and future research activities post-ISS (e.g. the GATEWAY orbiting the Moon).

individual) as well as integrating knowledge among organisms (producers, consumers and degraders) also to understand the effect of space factors on their capability of networking in the artificial ecosystem as in nature on Earth. Space biology has been historically divided into sub-disciplines dealing with animals/mammals, microbes and plants without much interaction. However, some processes of altered metabolism as those regarding DNA-repair mechanisms as well as ROS and peptide signaling as stress responses are well conserved among species (including animals and plants). This paves the way towards the need for making synergies among disciplines to achieve an integrated picture of common *vs* distinct responses to space factors in different organisms. Possibly, the establishment of global standard operating procedures for space *omics* (including metagenomics) data sets generation and annotation, including but not limited to those generated within the framework of ESA and NASA projects, can allow the expansion of statistical power in space flight experiments by means of the federation of data sets.

Indeed, it has been recognized that to achieve BLSS operating in space and guarantee better protection of human health and well-being in space, an integrated, multidisciplinary approach linking together different branches of life science (e.g. animal and human physiology, plant biology, microbiology, etc.) is needed, as well as the cooperation with physical sciences, technologies and engineering. Therefore, there is a need to bridge and strengthen the interconnections between them also through the design, proposal, and realization of new studies and experiments.

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AUTHOR CONTRIBUTIONS

V.D., G.A., N.C., E.C., R.H., N.H., V.L., J.M., V.P., M.S. and A.K. developed the concept of this perspective paper. V.D., G.A., S.D. and L.I. developed a first structure of the manuscript. V.D. took lead in coordination and writing; G.A., N.C., E.C., S.D., R.H., N.H., L.I., I.L.D., V.L., J.M., V.P., M.S. and A.K. wrote specific parts of the manuscript. All authors provided critical feedback and helped shape the concept and perspectives. All authors revised and approved the submitted version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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Chapter 2

Growth, Anatomical, and Biochemical Responses of the Space Farming Candidate Brassica rapa L. Microgreens to Low-LET Ionizing Radiation

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Article

Growth, Anatomical, and Biochemical Responses of the Space Farming Candidate *Brassica rapa* L. Microgreens to Low-LET Ionizing Radiation

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Abstract: An adequate and balanced diet is fundamental in preserving the health of astronauts from several space-induced diseases. Therefore, the integration of a diet with fresh food, rich in bioactive compounds such as microgreens produced directly onboard, may be useful in space for human nutrition. However, ionizing radiation (IR) in space represents a significant hindrance for organisms, with potential critical outcomes on plant morpho-anatomical, eco-physiological, and biochemical aspects, depending on the plant and IR features (e.g., species, developmental stage, IR dose, and type). In this study, we analyzed the effect of different doses of X-rays (0-control, 0.3, 1, 10, 20, and 30 Gy) on the morpho-anatomical and nutritional traits of microgreens of *Brassica rapa* L., irradiated at the stage of germinated seeds. After the irradiation, microgreens were cultivated in controlled conditions. At harvest, the morpho-biometric traits were analyzed, along with the leaf functional anatomical traits and the phytochemical content of the aboveground biomass. The results showed that X-ray exposure does not induce detrimental effects on growth, while it stimulates the production of antioxidants, improving plant defense and nutritional value. The overall results support the idea of using this species in space as a supplemental functional food.

Keywords: exo-agriculture; food countermeasure; ionizing radiation; low-LET radiation; microgreens; morpho-anatomical traits; phytochemicals; space exploration

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1. Introduction

There is an unbreakable link between human nutrition and health: a varied and balanced diet, combined with a healthy lifestyle, are effective tools for the prevention, management, and treatment of a wide range of pathological diseases, particularly for individuals in specific physiological and psychological conditions due to the exposure to stressful factors. Astronauts belong to this category, as they work in isolation and confinement conditions in space, and are constantly exposed to extreme environmental factors such as ionizing radiation (IR) and altered gravity [1–3].

The integration of a diet with appetible fresh-food from plant origin, being rich in bioactive compounds (e.g., antioxidants, vitamins, minerals, fibers), may be an effective countermeasure toward the occurrence of space-induced diseases in astronauts, which also induce a positive effect on their psychology [4–9].

Given the higher content of phytonutrients in microgreens compared to adult plants, they have become extremely popular in the last decades, being proposed as a supplement functional food for Earth and for space cultivation [10–12]. Microgreens, also called "vegetable confetti", are young and tender greens harvested after 1–3 weeks from germination, developed from seeds of several crop species, with bright colors, delicate textures, and peculiar flavors. The use of microgreens in space could help enhance appetite and preserve the health and homeostasis of astronauts, who are used to relying on pre-packaged food as the one and only source of nourishment. Microgreens have a short, easy to manage production cycle, and need very low input resources for cultivation. Moreover, they are characterized by a high harvest index [10,13,14], making them suitable for direct cultivation onboard during space flights.

Currently, several families are candidates to be exploited in microgreen production. Among these, many species of the Brassicaceae family present high amounts of micronutrients such as polyphenols, ascorbic acid, carotenoids, tocopherols [15–18], selenium, sulfur, calcium, and glycosylates [19–21], which are very effective in neutralizing radiation-induced oxidative damage and the risk of the occurrence of several types of cancer [7,20,22–24]. These secondary metabolites are tightly linked to plant protection and defense pathways, and may be influenced by environmental conditions and agricultural practices [25–27].

Nevertheless, space farming must face and overcome several hurdles related to the same environmental constraints that affect the health of astronauts [28–30]. Among the space factors, ionizing radiation is considered to be the main constraint for exploratory class crewed missions and long-term permanence on Moon and Mars. Indeed, in space, the galactic cosmic rays and solar particle events are responsible for harmful outcomes in both mammals and plants through direct and indirect mechanisms involving DNA and other chemical compounds as the main targets.

Ionizing radiation, interacting with living matter, induces radiolysis processes and the over-production of reactive oxygen species (ROS), thus resulting in oxidative stress to nucleic acids, lipids, and proteins, which are the main targets of ROS [31–35].

In the last decades, many studies have explored the effects of ionizing radiation on the morpho-anatomical and physiological traits of crops with different main purposes [34,36–38]: (i) explore the radio-tolerance of different species; (ii) understand if low levels of ionizing radiation could strengthen plant phytochemical and growth responses; (iii) simulate the effect of a space environment on plants.

Notwithstanding the fact that the impact of IR on plant fitness is not completely unraveled, it has emerged that in the plant response to IR, the radiation features (e.g., dose, exposure time, and type of IR delivered) may act as discriminators along with other traits strictly related to the plant (e.g., species, cultivar as well as phenological stage and type of tissue exposed) [39–43].

The exposure doses measured on the ISS, Moon, and Mars have been estimated to be about 0.5, 1.37, 0.64 mSv/day, respectively, which are about hundreds of times that of an the Earth dose. In the experiments of radiobiology performed on Earth, considering the reduced possibility to recreate all the radiation spectrum and their dose intensity, particle accelerators are used. In these facilities, dose rates of many orders of magnitude higher than those in space are normally used to reduce the duration of the trials and to estimate the possible effects that would occur with lower doses over a longer period of time [44].

In most experiments, the dry seeds were the irradiation target, enhancing the hypothesis of the major radio-resistance of plants, especially in this phenological stage, due to the structural and metabolic traits of the dry seed. However, testing the responses to radiation impact on actively growing tissues, as in the case of meristems during the seed germination, may represent a more useful way to clarify the mechanism behind the radiation-induced alterations in plants.

The breaking of the seed's dormancy period due to imbibition, and subsequent hydration of the integuments, leads to a series of processes such as the activation of metabolic activity, the mobilization of the seed reserve, and the activation of the meristem cell proliferative pathways. At this stage, the young plant is very vulnerable to abiotic stress like IR, potentially compromising the maintenance of genome integrity, essential for meristem function, and consequently for seedling growth and establishment [45–47]. Thus, overcoming this stage may represent a bottleneck in the cultivation of adult plants in bioregenerative life support systems (BLSSs) to successfully support the human inhabitation of Moon and Mars [28,48].

To the best of our knowledge, very few studies have explored the effect of ionizing radiation on imbibed or germinated seeds of different species, but this effect has never been tested on microgreens.

For this purpose, we irradiated germinated seeds of *Brassica rapa* L. subsp. *Sylvestris* var. *Esculenta* microgreens with X-rays at the doses of 0.3, 1, 10, 20, and 30 Gy (i.e., up to tens of times higher than the absorbed dose estimated for long-duration missions as those on Moon and Mars) plus a non-irradiated control (0), in order to evaluate the dose– response curves of plants as well as the possible occurrence of positive or negative effects. X-rays were chosen since they are considered as the reference radiation before exploring other radiation types and because they share a similarity in effectiveness with space-low- energy protons [49]. At the end of the cultivation cycle, morpho-biometric, anatomical, and biochemical analyses were conducted to evaluate the effect of X-rays on the growth and productivity performance of the microgreens to evaluate their possible use in the space environment as a food supplement.

2. Materials and Methods

2.1. Experimental Design

Seeds of *Brassica rapa* L. subsp. *sylvestris* var. *Esculenta* were purchased from a local provider (Bioseme s.c.a.r.l., Piombino, Italy) and before the irradiations, preliminary germination tests were carried out to evaluate the rate of the germination and growth of the radicles in order to establish the most suitable target length of seedlings for irradiation. The tests were conducted in Petri dishes by placing the seeds on three layers of filter paper imbibed with distilled water and incubating them in a growth chamber at 24 ˚C in the dark. Eventually, a radicle length of 0.3 cm, reached after 30 h from the start of germination, was chosen as the target length for the irradiation test, and the seed incubation time and environmental parameters were modulated to have seedlings of the target length at the time of irradiation.

2.2. Irradiation Procedure and Microgreens Cultivation

The irradiation took place at the National Cancer Institute IRCCS Fondazione G. Pascale in Naples (Italy), using a linear accelerator Linac Synergy (Elekta) and carried out at the pre-established doses of 0.3, 1, 10, 20 and 30 Gy X-rays. The instrument delivered 6 MV photon beams with a dose rate of 200 MU/min, with the 3D-CRT (three-dimensional conformal radiotherapy technique), which provided the exposure of the sample to two opposing fields of size 20x20 cm2 at the isocenter. For each dose, two thousand germinated seeds, with 0.3 cm radicle, were placed between two Perspex blocks of 2.5 cm and 5 cm, leaned on filter paper soaked in distilled water.

After the irradiation, germinated seeds were immediately transferred to the laboratory, divided into 5 replicates per dose (400 seeds used per replicates), sowed in pots (12x9x4 cm) filled with standard gardening soil, and placed in a growth chamber under controlled conditions of temperature (24 ± 2°C), relative humidity (RH 70 ± 10%), and light (white LED light of 200 ± 20 μmol photons m−2s−1 photosynthetic photon flux density, PPFD; 12h photoperiod).

During the transfer to and from the irradiation center, the germinated seeds were kept under controlled temperature conditions, together with the not-irradiated control. At 14 days from seeding, microgreens were harvested and divided into groups to perform morpho-biometric, anatomical, and biochemical analyses.

2.3. Morpho-Biometric Analyses and Sampling

At harvest, the survival percentage of microgreens was evaluated for each dose and control as the percentage of seedlings that grow up to the “stage” of microgreens with two expanded leaves after 14 days from sowing [50–52].

On 10 microgreens per replicate, the total length was measured, and at the same time, pictures of the 10 microgreens were taken in order to calculate their leaf area by using ImageJ software (U.S. National Institutes of Health, Bethesda, MS, USA). Moreover, on the same microgreens used to calculate the length and leaf area, the leaf fresh and dry weight were evaluated using a fine scale. The dry weight was obtained after drying the samples in an oven at 60 ˚C until they reached a constant weight.

Microgreens were then sampled for microscopy and biochemical analyses. Then, the fresh and dry biomass of the whole canopy was calculated as the amount of biomass produced per a growing area of 0.018 m^2 and reported as Kg m^{-2} for the fresh biomass and g m^{-2} for the dry biomass.

2.4. Anatomical Analyses

Microgreens collected for microscopy analyses were fixed in the F.A.A. chemical fixative (5 mL of 40% formaldehyde, 5 mL of glacial acetic acid, 90 mL of 50% ethanol) to prevent decomposition phenomena and the loss of chemical-physical properties. Subsequently, the leaf samples (*n* = 3) were dissected in order to obtain sub-samples of leaf lamina of about 5 × 6 mm, which were then subjected to dehydration, infiltration, and inclusion in an acrylic resin (JB4®, Polysciences, Eppelheim, Germany). These sub-samples were sectioned with a rotary microtome, obtaining cross sections, 5 μm thick, which were stained with Toluidine Blue at 0.5% in distilled water (Feder and O'Brien, 1968). Then, the sections were mounted with mineral oil and observed under a transmitted light microscope (BX 51, Olympus, Hamburg, Germany). Digital images were acquired with a camera (Olympus EP50) and the software CellSens 2.3 (Olympus, Tokyo, Japan) was used to analyze and quantify some of the functional anatomical parameters. Specifically, in each section, the total thicknesses of the leaf lamina (TLLT), the upper epidermis thickness (UET), lower epidermis thickness (LET), the palisade parenchyma thickness (PPT), and the spongy parenchyma thickness (SPT) were measured at three points of the section. Furthermore, the adaxial and abaxial stomatal density (SDad; SDab) per linear transect unit as well as the percentage of intercellular spaces in the spongy parenchyma (SPIS) were also measured in three regions of the section.

2.5. Antioxidant, Pigment and Protein Determination

These analyses were performed on 8 microgreens per treatment. The antioxidant capacity was determined using the Ferric Reducing Antioxidant Power (FRAP) assay according to the protocol of George et al. [53], modified by Costanzo et al. [54], which was performed on microgreens previously powdered in liquid nitrogen. The samples were treated with a 60:40 methanol/water solution in a centrifuge at 14,000 rpm for 15 min at 4 °C. The FRAP reagents (300 mM acetate buffer pH 3.6, 1:16.6 *v*/*v*; 10 mM tripyridyl triazine, TPTZ, in 40 mM HCl, 1:11.6 *v*/*v*; 12 mM FeCl3, 1:11.6 *v*/*v*) were combined with the extracts and incubated for 1 h in the dark. Then, the absorbance was read at 593 nm by a spectrophotometer (UV-VIS Cary 100; Agilent Technologies, Palo Alto, CA, USA). The antioxidant capacity was calculated using a Trolox standard curve and expressed as μmol Trolox equivalents g^{-1} FW (μmol Trolox eq g^{-1} FW).

The total content of chlorophylls (*a* + *b*) and carotenoids (*x* + *c*) was assessed according to Lichtenthaler [55]; leaf disks of 0.283 cm^2 area were homogenized in ice-cold 100% acetone using a mortar and pestle. The extracts were centrifuged at 5000 rpm for 5 min in a Labofuge GL (Heraeus Sepatech, Hanau, Germany). Afterward, the sample absorbance was determined using a UV-VIS Cary 100 spectrophotometer from Agilent Technologies at wavelengths of 470, 645, and 662 nm.

The amount of total polyphenols was determined as reported in Vitale et al [55]. Fresh samples were powdered in liquid nitrogen, treated with 80% methanol at 4 °C and centrifuged at 11,000 rpm for 5 minutes. The soluble fraction was mixed with 10% Folin-Ciocalteu solution in a ratio of 1:1 v/v for three minutes and then treated with 700 mM Na2CO3 solution in a ratio of 1:5, v/v. Samples were incubated for 2 h in the darkness. The absorbance was measured at 765 nm with a spectrophotometer (UV-VIS Cary 100; Agilent Technologies) and the content of total polyphenol was expressed as mg of Gallic Acid Equivalents g−1 FW (mg GAE g−1 FW) using a gallic acid standard curve.

The total protein content was determined on five fresh leaf samples ground in liquid nitrogen according to Wang et al. [56]. The protein amount was quantified by Bradford colorimetric assay [57], measuring the sample absorbance at 595 nm by a spectrophotometer (UV-VIS Cary 100, Agilent Technologies, Palo Alto, CA, USA). The protein concentration was expressed as μg BSA equivalents g−1 FW, using a BSA (bovine serum albumin) to build a standard curve.

For the evaluation of the ascorbic acid (AsA) content was used the Ascorbic Acid Assay Kit (MAK074, Sigma-Aldrich, St. Louis, MO, USA), following the procedure reported by Costanzo et al[53]. Briefly, 10 mg of the sample was homogenized in 4 volumes of cold AsA buffer before being centrifuged at 12,850 g for 10 minutes at 4 °C. To a final volume of 120 L, the supernatant was mixed with AsA assay buffer. In this assay, the AsA concentration was determined using a coupled enzyme reaction that produced a colorimetric (570 nm) product equal to the amount of ascorbic acid in the sample. The concentration of ascorbic acid was expressed in ng mL−1 and calculated in relation to a standard curve.

2.6. Statistical analyses

Data were analyzed through one-way analysis of variance (ANOVA) using the SPSS statistical package (SPSS Inc., Chicago, IL, USA) and the SNK (Student-Newman-Kelus)) multiple comparison tests (*p* 0.05). To check for normality, the Kolmogorov–Smirnov test was performed; the arcsine transformation function was applied to percent data before statistical analysis.

3. Results

3.1. Morpho-Biometric Traits

The different doses of X-rays did not induce statistically significant changes in terms of the survival percentage of microgreens. Control and microgreens from seeds irradiated at doses from 0.3 up to 20 Gy showed a survival percentage ranging between 62% and 65%, whilst the 30 Gy microgreens showed a tendency to increase the survival, reaching 71%, which was still not significantly different from the other doses. No morphological aberrations were detected in the microgreens from irradiated seedlings compared tothe controls.

All doses induced a significant increase in fresh biomass (Figure 1a) compared to the control. The highest value was observed at the dose of 30 Gy, whose microgreens were significantly heavier than those at 0.3 and 20 Gy, which in turn showed significantly higher values that at 1 and 10 Gy. In contrast to the dry biomass (Figure 1b), only the dose of 20 Gy induced a significant increase in weight compared to all the other doses and to the control. Concerning the microgreen leaves, the leaf area (Figure 1c), the leaf fresh weight (Figure 1d) and dry weight (Figure 1e) showed the same trend of variation. Compared to the control, all microgreens from the irradiated seeds showed significantly lower values. More specifically, the irradiation induced a significant decrease in all parameters up to 10 Gy, followed by an increase again up to values at 30 Gy comparable to the control (for leaf fresh weight) and to 0.3 Gy (for leaf dry weight and leaf area). No radiation-induced variations in terms of the microgreen length (Figure 1f) were observed.

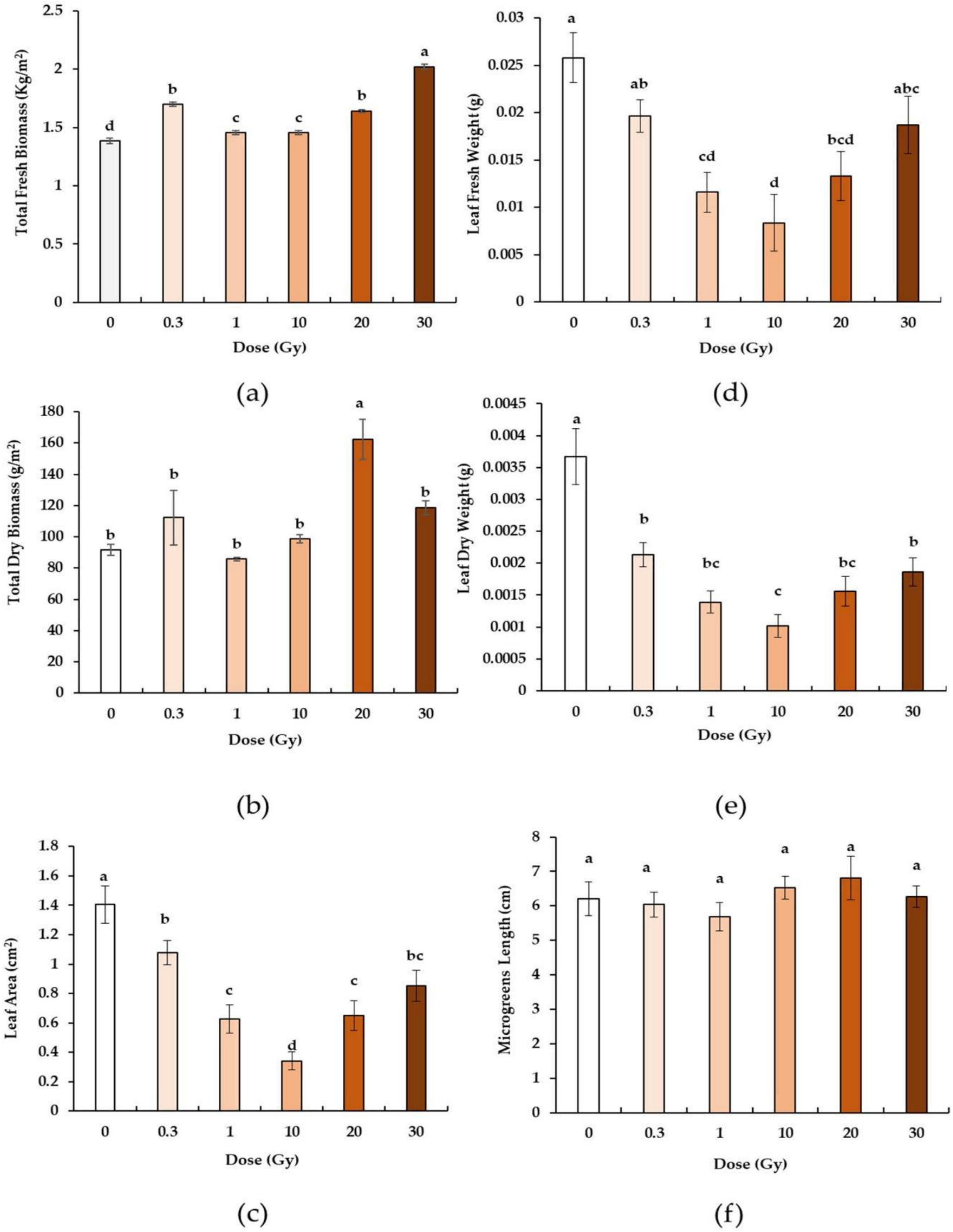

Figure 1. Fresh (**a**) and dry biomass (**b**), leaf area (**c**), fresh (**d**) and dry leaf weight (**e**), and total length of the microgreens (**f**) of *B. rapa* microgreens from the control (0) and irradiated seedlings with increasing doses (0.3, 1, 10, 20, 30 Gy) of X-rays. Mean values and standard errors are shown (n = 5 for biomass; n = 10 for leaf weight, area, and microgreens length). Different letters correspond to significant differences among irradiation doses according to the Student–Newman–Keuls multiplecomparison tests ($p \leq 0.05$).

3.2. Anatomical Traits

The exposure to increasing doses of X-rays did not cause evident aberrations in the organization of tissues of the typical leaf lamina with dorsiventral anatomy (Figure 2). From a quantitative viewpoint, irradiation induced statistically significant changes in all of the analyzed anatomical parameters (Figure 2 and Table 1), except for the upper epidermis thickness (UET), lower epidermis thickness (LET), and abaxial stomatal density (SDab). No unique trend of variation was found in the different analyzed parameters after irradiation. More specifically, the total thicknesses of the leaf lamina (TLLT) and the spongy parenchyma thickness (SPT) were significantly higher at the doses of 1 and 30 Gy compared to all of the others, with 10 Gy showing the lowest values. The palisade parenchyma thickness (PPT) was significantly higher at 1 Gy than the control and 30 Gy microgreens, which in turn showed significantly higher values than all the others.

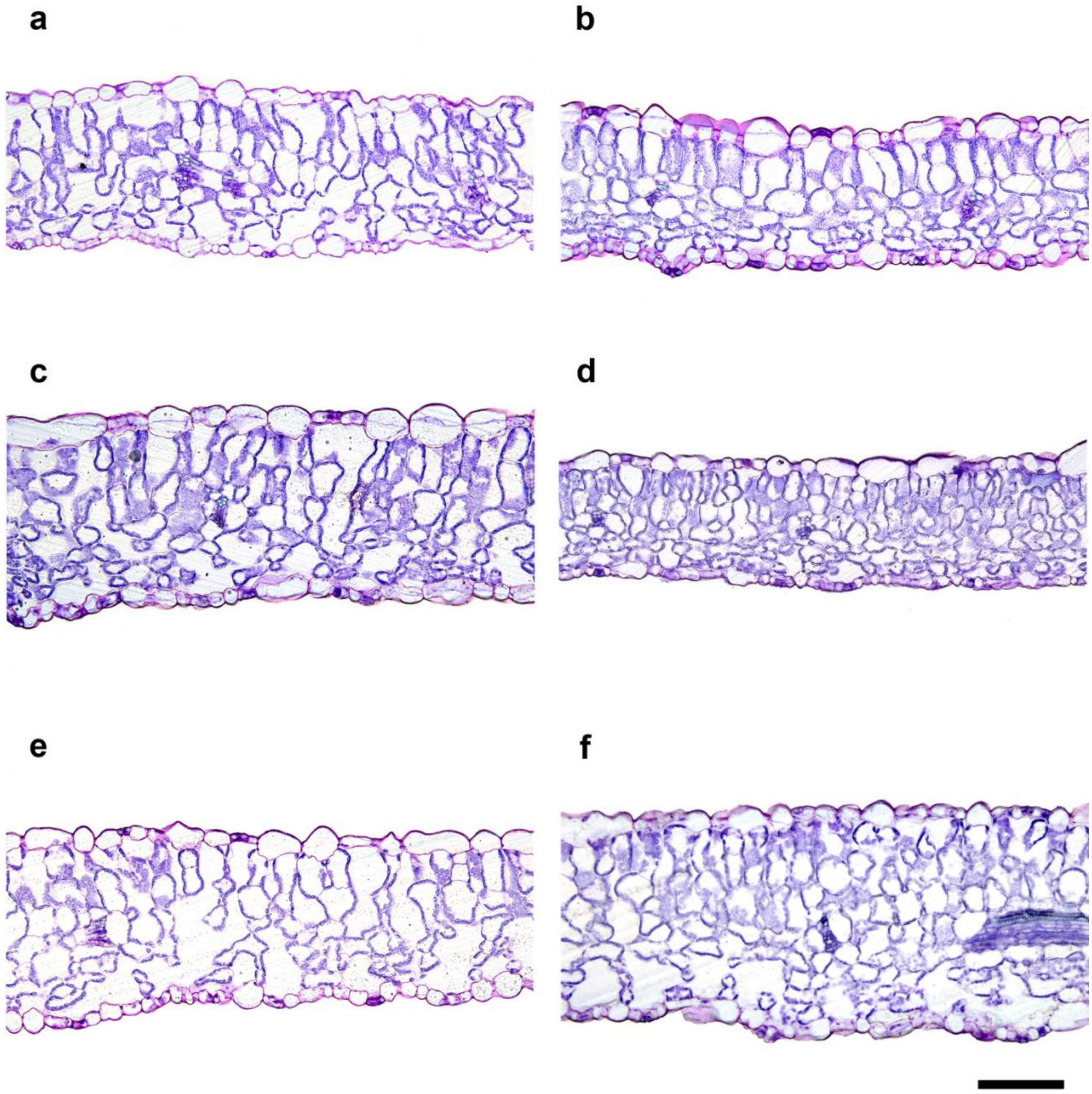

Figure 2. Light microscopy views of cross sections of *B. rapa* leaf lamina from the control (0 Gy, (**a**)) and irradiated seedlings with increasing doses (0.3 Gy, (**b**); 1 Gy, (**c**); 10 Gy, (**d**); 20 Gy, (**e**); 30 Gy, (**f**)) of X-rays. Images are all at the same magnification. Bars = 100 µm.

Table 1. Effect of irradiation on the leaf anatomical traits: upper epidermis thickness (UET), palisade parenchyma thickness (PPT), spongy parenchyma thickness (SPT), lower epidermis thickness (LET), total leaf lamina thickness (TLLT), percentage of spongy parenchyma intercellular spaces (SPIS), and stomatal density of the adaxial and the abaxial surface (SDad; SDab) in the leaves of *B. rapa* microgreens from the control (0) and seedlings irradiated with increasing doses (0.3, 1, 10, 20, 30 Gy) of X-rays. The mean values and standard errors are shown (n = 27 for tissue thicknesses and SPIS%; n = 9 for SD).

	0 Gy	**0.3 Gy**	**1 Gy**	**10 Gy**	**20 Gy**	**30 Gy**	**Sig.**
UET (µm)	21.3 ± 1.07 a	21.2 ± 0.85 a	24.2 ± 1.16 a	20.6 ± 1.06 a	20.2 ± 0.99 a	22.8 ± 0.86 a	NS
PPT (µm)	61.7 ± 1.92 b	55.3 ± 2.40 c	70.8 ± 2.46 a	47.9 ± 1.31 d	52.0 ± 1.55 cd	62.9 ± 2.07 b	***
SPT (µm)	87.4 ± 3.11 b	82.1 ± 2.08 bc	104 ± 2.55 a	77.0 ± 2.13 c	86.5 ± 2.32 b	107 ± 3.19 a	***
LET (µm)	16.7 ± 0.79 a	15.9 ± 0.77 a	17.7 ± 1.10 a	15.6 ± 0.70 a	16.4 ± 0.78 a	18.5 ± 0.76 a	NS
TLLT (µm)	187 ± 3.67 b	173 ± 3.43 c	217 ± 3.96 a	160 ± 2.01 d	175 ± 3.45 c	211 ± 4.47 a	***
SPIS (%)	10.3 ± 1.45 b	8.96 ± 1.84 b	13.6 ± 1.46 a	10.3 ± 1.12 b	14.2 ± 2.02 a	15.0 ± 1.15 a	***
SD ad (n/mm)	3.05 ± 0.48 ab	3.58 ± 0.61 ab	2.09 ± 0.30 b	2.71 ± 0.54 ab	4.27 ± 0.68 a	2.35 ± 0.39 b	*
SD ab (n/mm)	3.66 ± 0.74 a	5.09 ± 1.13 a	3.98 ± 0.55 a	4.89 ± 0.77 a	5.97 ± 0.94 a	4.89 ± 0.81 a	NS

NS, *, and ***, Not significant or significant at $p < 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to the Student–Newman–Keuls multiple comparison tests($p \leq 0.05$).

Concerning the percentage of intercellular spaces in the spongy parenchyma (SPIS), all doses except for 0.3 Gy induced a significant increase compared to the control. In contrast, for the stomatal density on the adaxial lamina (SDad) at 20 Gy, the values were significantly higher than at 1 and 30 Gy.

3.3. Antioxidants, Photosynthetic Pigment, and Proteins

The X-rays elicited a significant effect on all of the analyzed biochemical parameters, with differences depending on the dose. Compared to the control, irradiation induced a significant increase in antioxidant capacity (Figure 3a), with no significant differences among doses. The total polyphenol content (Figure 3b) significantly increased in the irradiated samples starting from 1 Gy compared to the control and 0.3 Gy plants, which exhibited comparable values. The highest polyphenol concentration was found in the 20 Gy plants, while intermediate values were detected at 1, 10, and 30 Gy.

Concerning the production of ascorbic acid, the irradiation at 20 Gy induced a sig- nificant reduction compared to the control, while all of the other treatments showed intermediate values (Figure 3c).

The total content of chlorophylls and carotenoids was also modulated by the radiation treatments, but this modulation was not dependent on the dose (Figure 3d,e). For the chlorophylls, 10 Gy seedlings showed values significantly higher than 0 and 30 Gy, while 0.3, 1, and 20 seedlings exhibited intermediate values (Figure 3c). For the carotenoids, an apparent increase was confirmed at 0.3 and 10 Gy, but it was not significant compared to the control and other doses, except for the dose of 30 Gy, at which the plants exhibited a significantly lower amount of carotenoids compared to other treatments (Figure 3e).

The protein content decreased with increasing doses. More specifically, it was similar in the control, 0.3, and 1 Gy, while it was statistically lower at 20 and 30 Gy (Figure 3f).

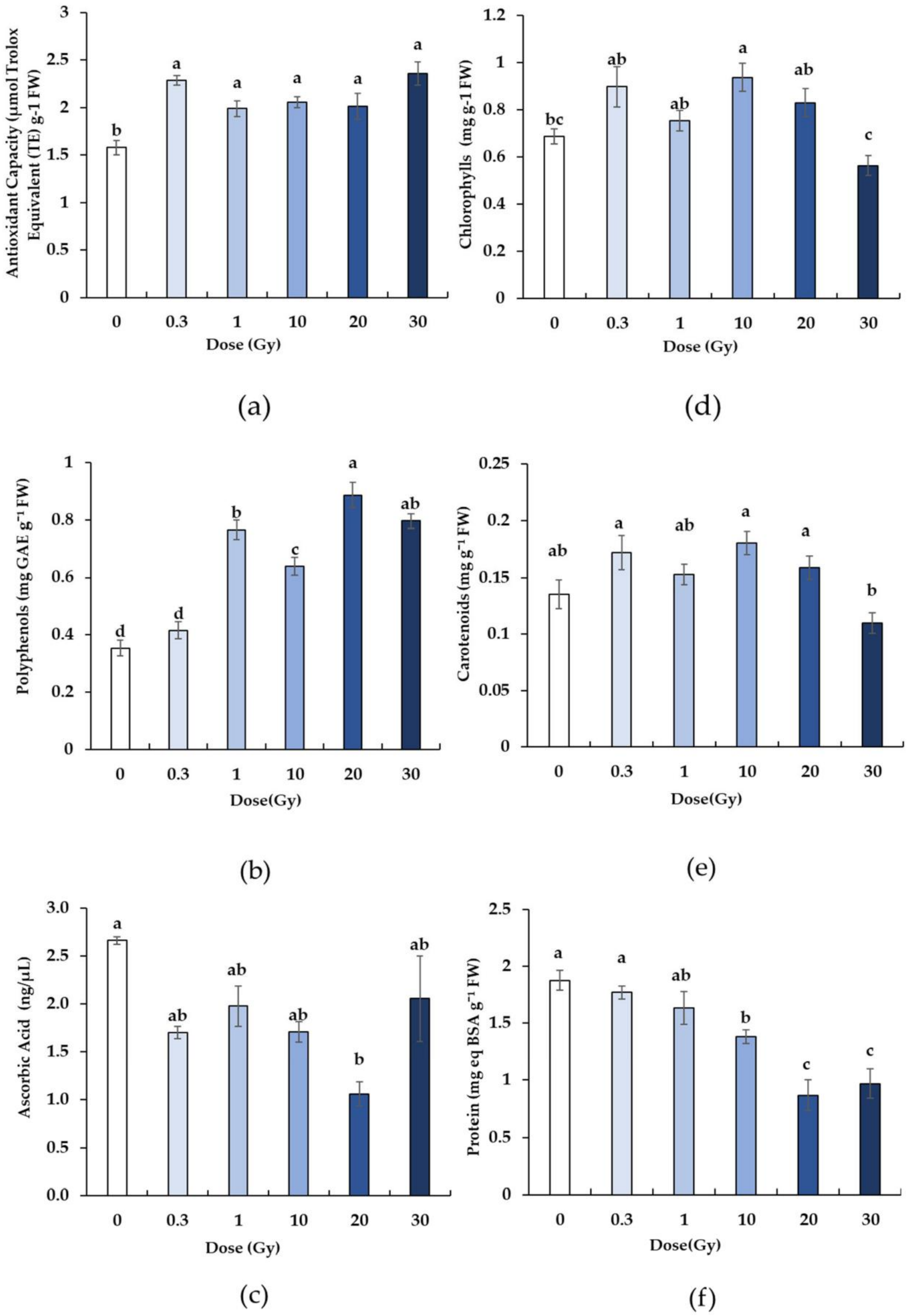

Figure 3. Total antioxidant capacity (**a**) and the content of polyphenols (**b**), ascorbic acid (**c**) chlorophylls (**d**), carotenoids (**e**), and protein (**f**) in *B. rapa* microgreens from the control (0) and irradiated seedlings with increasing doses (0.3, 1, 10, 20, 30 Gy) of X-rays. The mean values and standard errors are shown (n = 8). Different letters correspond to significant differences among irradiation doses according to the Student–Newman–Keuls multiple comparison tests ($p \leq 0.05$).

4. Discussion

This study indicated that *B. rapa* has valuable radio-resistance, since all X-ray doses tested, from 0.3 up to 30 Gy, did not prevent the seedling survival and growth when the target of irradiation was the germinated seed. This stage represents a very delicate phase in the plant life cycle because after the break of dormancy, the plant is characterized by active meristems and morphogenesis, particularly vulnerable to abiotic stress, which may weaken the seedling establishment [59]. In space, the presence of ionizing radiation [29,30,44] may influence early morphogenesis in plants [45–47]. Previous research has demonstrated that low doses of X-rays may generally induce positive effect on the viability, germination, and growth of the seeds, while high doses may reduce or hamper these delicate phases [35,60,61].

Despite the few published works focusing on the effects of IR on plants at the stage of germinated seeds, our results are consistent with previous findings on *Vigna radiata* L. sprouts, in which X-rays did not cause growth aberrations as well as premature senescence or death in irradiated seedlings [62].

In our study, the morpho-anatomical and biochemical responses observed in *B. rapa* microgreens irradiated at the stage of germinated seed were dependent on the dose, but no univocal tendency was found for all of the measured parameters. Specifically, the relationships appeared to be nonlinear, confirming what was found in previous studies [62,63].

In terms of the morphological parameters, despite the length of the microgreens not changing within the different doses, it is noteworthy that the variations observed in the fresh and dry biomass (leaves and hypocotyls) of the microgreens were not in agreement to those found in the leaves alone, indicating that the effect of radiation on biomass accumulation and photosynthate allocation may already be organ-specific in the first stages of development. This result suggests a greater allocation of resources toward the hypocotyls, resulting in a probable greater diameter and robustness. Concerning the whole canopy biomass, here, all doses showed enhanced fresh biomass compared to the control, while the dry biomass high values were observed only at the dose of 20 Gy. In a previous study on tomato cultivar ‘microtom’ irradiated with X-rays [64], 20 Gy was found to be a threshold dose for the occurrence of positive outcomes on plant growth, which became null or negative at higher doses.

Regarding the leaf parameters, increasing X-ray doses induced a decrease in the fresh and dry weight and in the leaf area up to 10 Gy, which would appear as a new threshold dose for these parameters in brassica. A similar reduction in the same parameters was observed by [35] on juvenile leaves of *Phaseolus vulgaris* L. exposed to increasing doses of X-rays, probably highlighting a greater radiosensitivity of the leaves compared to the whole canopy in different species.

The anatomical analysis underlined that exposure to X-rays, particularly at 1 and 30 Gy, induced the formation of a thicker leaf lamina, with leaves at 1 Gy also having a thicker palisade parenchyma tissue. It can be speculated that if such a trend is maintained in the further emerging leaves, adult plants may have a greater photosynthetic gain. With respect to the percentage of intercellular spaces in the spongy parenchyma (SPIS), with the exception of the 0.3 and 10 Gy dose, all of the other doses showed comparable or even significantly higher values compared to the control. The increase in SPIS may respond to the need by irradiated seedlings to better control transpiration, since a higher presence of intercellular spaces indicates reduced cell connection, and consequently slowed flow [65,66].

The effect induced by increasing doses of X-ray on stomatal density was different between the two epidermal layers: for the upper epidermis, it resulted in an increase or the maintenance of stomatal density compared to the control, consistent with the behavior of the intercellular spaces. Conversely, the upper epidermis was not affected by the irradiation, suggesting that stomatal density is a stable trait, in agreement with other recent studies [41,67].

At the biochemical level, X-rays induced positive responses in microgreens, significantly increasing the antioxidant capacity and total polyphenol content compared to the control plants. This may suggest a strategy employed by plants to increase the synthesis of antioxidants to protect plant cells from radiation-induced oxidative stress [36,37], which also had the useful consequence of increasing the nutritional profile of plants. It has been demonstrated that the increase in phenolic compounds, beyond exerting a detoxifying action against free radicals, also acts as a natural screen against radiation including IR, preferentially distributing along photosynthetic membranes [63,64].

Our findings are partially in agreement with previous works [39,62,67–70] in which the irradiation of various plants species with different types and doses of IR determined the maintenance, reduction, or increase in polyphenols compared to the control plants, remarking on the difficulty in highlighting an unequivocal behavior in response to IR. Conversely, the ascorbic acid (AsA) concentration showed a tendency to decrease, even if its reduction was not statistically significant compared to the control, except for the dose of 20 Gy. These data suggest that AsA does not contribute to the rise in total antioxidant capacity observed in response to the increasing X-ray doses. Ascorbic acid is recognized as one of the most powerful "oxidant scavengers "and is quickly used by ascorbate peroxidase (APX) as a co-factor for the detoxification of ROS [71]. The significant decrease in its concentration at 20 Gy could depend on the strong request by the detoxification system at this specific dose.

It is interesting to note that the total chlorophyll and carotenoid content was not negatively affected by radiation. Specifically, the significant increase in the values observed in both parameters at doses of 0.3, 10, and 20 Gy could be the result of a sort of "surviving strategy" by plants to enhance photosynthesis in response to the decrease in the expansion and thickness of the leaf lamina, especially of the palisade parenchyma observed at the same doses. This evidence, also found in other works [41,67], can be considered as a positive characteristic, given the pivotal role of these pigments in the photosynthetic process. Furthermore, being that chlorophyll and carotenoids are also employed in antioxidant activity, their importance extends to the nutritional sphere in protecting the human bodyfrom oxidative stress [72–74]

Medium and high IR doses have been reported to also induce an accumulation of soluble proteins in the tissues of plants grown from irradiated seeds [75–79]. On the other hand, nonlinear effects, both positive and negative, were observed at low IR doses [75,80–82]. In our experiment, the soluble protein content decreased as the irradiation dose increased, suggesting a stressful condition for microgreens. Ionizing radiation may cause protein oxidation, shortening their functional life and altering the processes of protein synthesis, degradation as well as protein profile [32]. As mentioned before, to avoid oxidative stress, plants increase or decrease the content of various secondary metabolites with antioxidant activity such as phenols, terpenoids, and proteins [83].

In conclusion, the results presented in this paper indicated that the responses of *B. rapa* microgreens to X-rays were dose- and parameter-dependent. Irradiation did not induce detrimental effects at the morphological, anatomical, or biochemical level, allowing for the normal growth and establishment of seedlings. However, some variations in functional anatomical traits, if maintained in adult leaves, could lead to variations in the functional efficiency of the photosynthetic process. Nevertheless, at this stage of development, which is primarily aimed at consumption for diet integration, the exposure to X-rays has induced stimulatory outcomes like an overproduction of antioxidants, probably due to the implementation of plant defense. Indeed, the production of these secondary metabolites improved the nutritional profile of microgreens as well as the ability of plant cells to counteract oxidative stress, supporting the hypothesis of using this species for cultivation in space as a supplemental functional food. Further studies are still needed to test the radioresistance of this species as well as to other types of ionizing radiation, especially high-LET radiation and chronic radiation doses as well as further testing their palatability and safety attributes for future crews to confirm the suitability of Brassica microgreens for cultivation in space.

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Chapter 3

Effects of sparsely and densely ionizing radiation on *Brassica rapa* microgreens: a morphological, anatomical, and biochemical study

Chapter 4

Effect of carbon- and iron-ions irradiation on growth and biochemical traits of *Salvia hispanica* L. microgreens

Chapter 5

Combined effects of microgravity and chronic ionizing radiation on Brassica rapa microgreens

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Chapter 6

Effects of heavy ions and chronic ionizing radiation on green and red lettuce

Conclusion

The present thesis project, "Effects of ionizing radiation on plants to define crop shielding requirements for future space habitats”, was funded by the European Space Agency (ESA) and the Italian Space Agency (ASI) within the MELiSSA (Micro-Ecological Life Support System Alternative) Ph.D. POMP program. The project was conducted in collaboration with the Belgian Nuclear Research Centre (SCK-CEN, Belgium), the Paul Sabatier University of Toulouse III (France), and the Biophysics Department of GSI Helmholtzzentrum für Schwerionenforchung (Germany). This work aimed to investigate the interplay between plant physiology and the unique challenges posed by the space environment, where ionizing radiation poses a significant obstacle to the successful implementation of BLSS.

The integration of diverse experimental approaches in this thesis (i.e. controlled acute exposures to both low- and high-LET radiation, and chronic low-dose radiation also combined with microgravity) enabled a deep evaluation of plant responses to the multifaceted challenges posed by the space environment. The findings from Chapters 2 through 6 collectively reveal the intricate nature of radiation effects, varying across species, cultivars, and phenological stages. For example, *Brassica rapa* microgreens, from both irradiated germinated and dry seeds, showed a good tolerance across different radiation sources. Moreover, *Salvia hispanica* exhibited significant physiological and biochemical adaptations under high-LET radiation. Furthermore, the investigation into Salanova® lettuce cultivars demonstrated also the profound influence of cultivar-specific traits on radiation responses, emphasizing the importance of genetic and phenotypic diversity in plant selection for space missions.

The concept of thresholds emerged as a unifying theme across these studies, revealing the dose-dependent nature of plant responses to radiation. Some doses often induced hormetic effects, stimulating growth-promoting pathways and enhancing nutritional and defense traits. However, as certain doses exceeded critical thresholds, the benefits of adaptation gave way to stress, characterized by diminished growth and biochemical function. The findings underscore the importance of identifying and leveraging these thresholds to design radiation exposure protocols that optimize plant resilience and productivity. Additionally, the combined effects of radiation and other stressors, such as microgravity, highlighted the complex interplay of environmental factors in shaping plant responses.

Future research should focus on mapping detailed dose-response curves for a wider variety of plant species and cultivars, elucidating the molecular mechanisms underlying radiation and stress adaptation, and investigating the long-term impacts of chronic radiation exposure. Multi-generational studies of plants grown under space-relevant conditions will also be essential for understanding the sustainability of adaptive responses.

The gained information on radiations' responses of the various crop species will be helpful to evaluate the impact on the regenerative power of plants in BLSS and refine cultivation requirements and shielding opportunities.

Appendix 1

A clear understanding of radiation types and measurement units is essential for interpreting the findings of this thesis.

Radiation Types:

Ionizing radiation is classified based on its Linear Energy Transfer (LET), which describes the energy deposited per unit path length as the radiation interacts with matter.

- **High-LET Radiation**: examples include heavy ions (e.g., carbon, iron) found in Galactic Cosmic Rays (GCRs). High-LET radiation causes dense ionization along its path, leading to severe biological damage. LET is expressed as:

  $$LET = \Delta E / \Delta x \text{ (in keV/}\mu\text{m)}$$ [1]

  where ΔE is the energy deposited, and Δx is the distance traveled in the medium.

- **Low-LET Radiation**: examples include X-rays and gamma rays. These forms of radiation cause more diffuse ionization effects, generally resulting in less severe biological impact compared to high-LET radiation.

Radiation Measurement Units:

Quantitative measurements in radiation dosimetry and protection require precise and universally understandable quantities and units, typically based on the SI system. Key organizations shaping these standards include the International Commission on Radiation Units and Measurements (ICRU), focusing on physical dosimetry, and the International Commission on Radiological Protection (ICRP), which addresses biological effects and radiation protection guidelines. Essential units include:

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[1] An, H., Wen, X., Li, D. T., Wang, Y., Li, C. H., Yang, S. S., ... & Cao, Z. (2020). Detection technology and design analysis of LET spectrum of space radiation particles. *Nucl. Tech*, *43*, 43-51.

1. **Gray (Gy)**: The SI unit for *absorbed dose,* defined as the energy deposited by radiation per unit mass of a material. This unit quantifies the physical impact of radiation on matter, regardless of its type or biological consequences:

   $$D\ (Gy)=E/m$$

   where E is the total energy absorbed (in joules), and m is the mass of the material (in kilograms). One gray equals one joule per kilogram (1 Gy=1 J/kg).[2]

2. **Sievert (Sv)**: In radio protection, Sv is used for measuring the *equivalent dose* and the *effective dose.* The *equivalent dose* accounts for the biological effects of different types of radiation, which vary based on the particle type and energy. It is calculated as H, and incorporates a weighting factor (wR) to account for the biological impact of the radiation type:

   $$H(Sv)=D\times wR$$

   where wR is the radiation weighting factor, which varies depending on the type and energy of the radiation (e.g., 1 for X- and gamma-rays, 20 for alpha particles).

   The *effective dose* gives a measure of the overall risk to the entire body, weighted by the sensitivity of each organ:

   $$E=\sum(Ht\times wT)$$

   where E is the effective dose (in sieverts, Sv), Ht is the equivalent dose for tissue or organ t, wT is the tissue weighting factor, which reflects the sensitivity of the tissue or organ to radiation.[1]

3. **Electronvolt (eV)**: The eV is a measure of particle energy, defined as the energy gained by an electron when it is accelerated through an electric potential difference of 1 volt:

[2] Mattsson, S., & Söderberg, M. (2012). Dose quantities and units for radiation protection. In *Radiation Protection in Nuclear Medicine* (pp. 7-18). Berlin, Heidelberg: Springer Berlin Heidelberg.

$$1\ \text{eV} = 1.602 \times 10^{-19}\ \text{joules}$$

This unit is often used to describe the energy of individual particles in atomic and molecular interactions. In radiation contexts, energies are often expressed in kiloelectronvolts ($1\ \text{keV} = 10^3\ \text{eV}$) or megaelectronvolts ($1\ \text{MeV} = 10^6\ \text{eV}$), as these scales align with the energies involved in ionizing radiation processes.[3]

[3] Del Guerra, A., & Panetta, D. (2019). Fundamentals of Natural and Artificial Radioactivity and Interaction of Ionizing Radiations with Matter. *Nuclear Medicine Textbook: Methodology and Clinical Applications*, 3-19.