UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

Department for Agriculture and Forest Sciences (DAFNE)

PhD. Course
Plant and Animal Sciences (XXXIV)

in co-supervision with ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development

GreenCube: on-ground space environment simulation effects on *Lepidium sativum L.* microgreens

A multidisciplinary approach to study the effects of multiple abiotic stress

Scientific-disciplinary sector **BIO/04**

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A.A. 2020/2021

Cooperation is the essence of progress,
in particular in the Space domain.

Renato Krpoun
Swiss Space Office chief

Abbreviation

BLSS – **B**ioregenerative **L**ife **S**upport **S**ystem
CEH – **C**ontrolled **E**nvironment **H**orticulture
CFD – **C**omputational **F**luid **D**ynamic
CPPS – **C**losed **P**lant **P**roduction **S**ystem
CV – **C**oefficient of **V**ariation
DLI – **D**aily **L**ight **I**ntegral
DLR - Deutsches Zentrum für **L**uft- und **R**aumfahrt
DWC – **D**eep **W**ater **C**ulture
EC – **E**lectrical **C**onductibility
FAO – **F**ood and **A**griculture **O**rganization of the United Nation
G7 – **G**roup of **7**
HPS - High-**P**ressure **S**odium
HVAC – Heating, **V**entilation and **A**ir **C**onditioning
IRP – **I**nternational **R**esource **P**anel
ISS – **I**nternational **S**pace **S**tation
NDS – **N**utrient **D**elivery **S**ystem
NFT – **N**utrient **F**ilm **T**echnique
NIR – **N**ear-**I**nfrared **R**adiation
PAR – **P**hotosynthetic **A**ctive **R**adiation
PFAL – **P**lant **F**actories with **A**rtificial **L**ight
PFD – **P**hoton **F**lux **D**ensity
PPFD – **P**hotosynthetic **P**hoton **F**lux **D**ensity
RH – **R**elative **H**umidity
SD – **S**tandard **D**eviation
SEM – Standard **E**rror **M**ean
SI – **S**ysteme **I**nternational or international system of units
UN – **U**nited **N**ation
UNEP – **U**nited **N**ations **E**nvironmental **P**rogram
VPD – **V**apour **P**ressure **D**eficit
WHO – **W**orld **H**ealth **O**rganization
WFP – **W**orld **F**ood **P**rogram

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Abstract

Space exploration will live soonly a new era of funding and achievements thanks to the Artemis project coordinated by NASA. In this framework, new lymph will be given to BLSSs that are mandatory systems to gain the possibility to live far from Earth's biogeochemical natural cycles.

Unfortunately, the possibility to gain a place in space facilities for research purposes is hard. The exploitation of new forms of space accommodations represented by micro and nano CubeSat is the new frontier of plants in space science. By using these minisatellites and a proper set of remote sensors and actuators it is possible to perform many experiments with remote ground control directly into the harsh space environment and with a relatively low budget.

In this framework, the GreenCube mission will be sent into space on board the maiden flight of the new ESA rocket, the VEGA-C, made by Avio, in mid2022 together with other CubeSats. Into the GreenCube CubeSat, there will be housed cultivation of *Lepidium sativum L.* microgreens that will start sprouting once it reaches the orbit in the *Van Allen belt* at about 6000km altitude.

The cultivation will be held into a hypobaric pressure tank, in real microgravity conditions and exposed to the galactic cosmic weather while an array of white full-spectrum LEDs will illuminate the cultivation process and an automated syringe will water the seeds regularly.

In this contest the necessity to assemble an on-ground test facility capable to perform the same environmental factors was mandatory.

The facility was built-up into the ENEA “Casaccia” Research Centre thanks to the already present *Calliope* ^{60}Co irradiation facility that made it possible to expose samples to low chronic γ radiations and that can host the 2D clinostat that was properly modified to light-up plants solidly with clinorotation. The tests on the microgreens were held to explore the new

possibility of studying multiple stress together belonging to the space environment while being on Earth.

Morphometrical measurements, fluorometric and plant flow cytometry analysis and metabolomics profiling were performed on microgreens after exposure to several stress such as: simulated microgravity, hypobaria and γ radiation, taken singularly or in combination, with the aim to highlight the potential plant response to the space environment.

Probably for the first time, it was possible to cultivate microgreens into an hypobaric cultivation volume during chronic clinorotation and chronic γ rays exposure.

It was developed a new solid method of nuclei extraction for plant flow cytometry that should be performed directly in space facilities like the ISS.

One of the first studies on multiple space stress performed simultaneously was carried on with a multidisciplinary approach.

Plants of *Lepidium sativum L.* cope with simulated space environment and simulated microgravity was the most effective stress on the variance of the measurements done.

1. Introduction

Since 1950 world population is growing constantly. UN and FAO prevision report that the world population will reach the amount of 8.6 billion individuals by 2030, 10.1 billion by 2050 and between 9.4 and 12.7 billion by 2100 (YORK, 2019). New citizens of the world will live in cities and the 2018 UN World Urbanization Prospects revision foresees that the percentage of human beings in the cities will grow from 55% (2018) to 68 % by 2050 (United Nations, 2019). Up to now, there are 548 cities with at least 1 million residents and by 2030 they will be 706. The most populated cities with at least 10 million inhabitants, known as "Megacities", are 33 and they will be 43 by 2030. Among them, Tokyo is the world's most populated city with 37 million citizens, followed by Delhi with 29 million, Shanghai with 26 million, and Mexico City and São Paulo, each with 22 million residents(United Nations, 2019). With the growth of population and urbanization, it is also growing the air pollution that the WHO has declared as the major environmental risk to health. The cumulative effect of air pollution with other factors is the cause of 7 million deaths every year (World Health Organization, 2015, 2020). In comparison, Malaria is the cause of 0.4 million death in 2018, while Covid-19 is about 3 million people around the world (World Health Organization, 2020).

Air pollution is the result of the blindness of the pasts generations on the transformation of raw materials into goods for everyday life. Air Pollution is naturally produced by volcanoes, dust storms, sea salt spray and wildfires around the globe; but it also comes from almost all human activities, from field to plate. Air pollution is the effect of fossil fuel emissions into the atmosphere after a burning process like those for power and heat production. Among these, it is possible to find transport, industries, energy plants, agriculture, domestic heating plants and so on.

Water and soil pollution are, as well as the air, an effect of almost all human activities, but in particular from the agriculture compartment where the use of fertilizers and the land transformation are the most impacting factors

(Environment Programme, 2017). Agriculture derived pollution affects freshwater and aquifers and this reflects on economic activities, health, food security itself and the biodiversity of rivers, oceans and lands (FAO, 2020). To be honest, the food production system is responsible for 25% of the total pollution source(Foster, C., Green, K., Bleda, M., Dewick, P., Evans, B., Flynn A., Mylan, 2006; Roser, 2020).

The G7 asked the IRP to zoom into the problem of the efficiency in the use of the natural resources and the UNEP lastly report a roadmap to change our future before it changes us. As they wrote, "Pollution is not a new phenomenon; it is largely controllable and often avoidable, but considerably neglected" (Environment Programme, 2017). Inger Andersen, the Executive Director of UNEP, also said that "natural resources are vital for our well-being, our housing, and our transportation. Their efficient use is central to a future with universal access to sustainable and affordable energy sources, emissions-neutral infrastructure and buildings, zero-emission transport systems, energy-efficient industries and low-waste societies" (Hertwich, Edgar; Lifset, Reid; Pauliuk, Stefan; Heeren, Niko; Ali, Saleem; Tu, Qingshi; Ardente, Fulvio; Berrill, Peter; Fishman, Tomer; Kanaoka, Koichi; Kulczycka, Joanna; Makov, Tamar; Masanet, Eric; Wolfram, 2020).

Together with the population growth, the pollution is also growing, and with them, there is an increase in food demand. FAO report that during the past 50 years, the food demand is tripled and for the following 30 years the best forecasting scenario set the feed demand to +70% with an additional quantity of about 1 billion tons of cereals and 200 million tons of meat (FAO, 2017c). This production requires much land allocated to agriculture and this is in line with the current trends that from 1970 till 2050 will bring the total arable land to 1732 million hectares with an increase between 2012 and 2050 of 165 million hectares (11%) (FAO, 2017c).

This high demand for food production asks for more soil use for agriculture which is not unlimited and due to environmental changes, caused by human activities, is diminishing year by year (FAO, 2017c). To have a

comprehensive view of the world like it is now and how it could be in the following years, is needed to bring to light another great problem of our time, the nonsense balance between obesity and unnourished people of all ages. Obesity is rising dangerously worldwide, in 2014 the percentage of overweight people aged 18 and over was 40%; in 2015 the overweight children under 5 were 6.2% of the total and if the trend remains stable, it will reach 11% (or 70 million) by 2025. As astonishing as obesity is the undernutrition statement that set the hunger people worldwide at 11% (or 800 million) (FAO, 2017b). WFP also said that the quality of food produced in an unhealthy environment is reflected in a general malnutrition statement, in particular in those countries where the laws about environmental protection are missing (FAO, 2020).

The UN proposal to solve this puzzle of problems arrived in 2015 with the establishment of the Sustainable Developmental Goals (Saini et al., 2016). 17 actions that invite people, countries, and institutions to participate actively in the change of our common future.
These 17 actions are:

1. **No Poverty** - End poverty in all its form everywhere
2. **Zero Hunger** - End hunger, achieve food security and improve nutrition and promote sustainable agriculture
3. **Good health and well-being** - Ensure healthy lives and promote well-being for all of all ages
4. **Quality Education** - Ensure inclusive and equitable quality education and promote lifelong learning opportunities for all
5. **Gender equality** - Achieve gender equality and empower all women and girls
6. **Clean water and sanitation** - Ensure availability and sustainable management of water and sanitation for all
7. **Affordable and clean energy** - Ensure access to affordable, reliable, sustainable, and modern energy for all

8. **Decent work and economic growth** - Promote sustained, inclusive, and sustainable economic growth, full of productive employment and decent work for all
9. **Industry, innovation and infrastructure** - Build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation
10. **Reduce inequalities** - Reduce inequality within and among countries
11. **Sustainable cities and communities** - Make cities and human settlements inclusive, safe, resilient and sustainable
12. **Responsible consumption and production** - Ensure sustainable consumption and production patterns
13. **Climate Action** - Take urgent action to combat climate change and its impacts
14. **Life below water** - Conserve and sustainably use the oceans, sea and marine resources for sustainable development
15. **Life on land** - Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, halt and reverse land degeneration and halt biodiversity loss
16. **Peace, justice and strong institutions** - Promote peaceful and inclusive societies for sustainable development, provide access to justice for all and build effective, accountable and inclusive institutions at all levels
17. **Partnership for the Goals** - Strengthen the means of implementation and revitalize the global partnership for sustainable development

Looking at them it doesn't seem to be so difficult to take part in the active action against bad behaviours. All new projects involved in agriculture, transport, economics and so on, use these actions as a lighthouse toward going.

In this scenario, my proposal to achieve safe development is the use of technologies in agriculture and looking for a downstream application of space

research to ensure a sustainable future and help the world face these major problems.

Indeed, to contrast the environmental pollution and changes, and the continuous demand for healthy food in an unhealthy world, we need to adopt a new form of horticulture in which is possible to cultivate more with less in a safe environment, as it will be needed to do on moon and mars. The application of new technologies will bring new jobs and efficient use of natural resources.
To make this real we need a univocal approach where all the technological upgrades from both sides, Earth and Space, work together toward the main challenge, make the world healthier using a resilient way of work.

Since mankind shifted from hunter/gatherer to farmer, the agriculture trend of technological development is constantly growing. We passed from gathering fruits and berries to domesticating animals and planting seeds then we created a permanent shelter in which we have formed communities and finally we establish a stable food supply. This process is working for thousands of years and today we have gained a level of knowledge that makes it possible to cultivate in the harshest environment on Earth and even in Space.
This kind of cultivation is possible thanks to the Controlled Environment Horticulture (CEH) techniques (Toyoki Kozai, 2018; Geilfus, 2019; Kozai et al., 2019).

1.1. Controlled Environment Horticulture

The term Horticulture comes from two Latin words, *Hortus* and *Cultura*. The first is translatable with “Garden” and the second one with “Managing”. Indeed, “Horticulture” means the practices and techniques operated to manage a garden (Janick and Paris, 2022; Mitchell, 2022).

The term CEH means a set of practices and techniques involved in the management of the cultivation processes inside a “closed” environment. Each vegetable species of the world, like the animal ones, has a set of environmental parameters and biotic connections that together form their

ecological niche. Those parameters have ranges around an optimum level which can be smaller or wider depending on the intrinsic characteristics of each species (Watson et al., 2018). This means that a *"Stenoecia"* species will live into a narrow range of biotic and abiotic parameters, while a *"Euriecia"* species should live into a wider range of environmental factors. Knowing the exact "size" of these parameters and the physiological needs of a species could help farmers cultivate it in a controlled environment ensuring a longer seasonal production, a higher yield, less disease, and a high-quality product.

To implement these features within an agricultural process, farmers need technological facilities that can modulate the environmental factors in favour of the vegetable crop of interest.

One of the last and most valuable examples of a CEH is the EDEN-ISS project of DLR that in 2018 from February to September produced a total amount of 268kg of fresh vegetables into a high-tech controlled environment developed within a container and placed in Antarctica (Zabel et al., 2015; Zabel and Zeidler, 2019).

1.1.1. Structure design

Before all the technical constraints and agricultural tips, a look-through "greenhouse" made of transparent materials needs to be placed in a specific place, depending on the geomorphological characteristics of the area and with a proper orientation that makes the most of the sunlight both for what concern heat and power energy production.

Indeed, one of the first technological implementations from the ancient world is the idea to close, in part or completely, the crops into a greenhouse (Janick and Paris, 2022).

Greenhouses are spread all around the world and help farmers lengthen the seasons and shelter the crops from adverse weather keeping the internal environment stable during the months. Greenhouses exist in a wide range of shapes and measures and differ from each other also by their technological

level. Depending on the need of the species and the effects on the surrounding environment, farmers can choose among a wide range of possibilities.

It is possible to pass from an opened tunnel greenhouse to a completely confined closed Plant growth chamber. Tunnel greenhouses are the simplest way to protect a crop from bad weather conditions and help to maintain stable the temperature inside lengthening the seasons. They are made of a great variety of covering materials. The most used one is a polyethylene film, followed by polycarbonate and glass; among the polyethylene films, there are a series of photoselective transmission mediums for different spectral frequencies (FAO, 2017a). These covering materials are used to protect crops, maintain the right temperature during the day and help light to enter during winter. The use of photoselective materials with NIR reflecting pigments can avoid overheating during summer without blocking PAR absorption by plants (Hemming et al., 2006). The use of other kinds of coverage, like photovoltaic panels, could absorb wavelengths useful for plants' photosynthesis or shade the crop area below and in that case, farmers need an integrated artificial light system. These covering materials are frequently used also in the framed greenhouses in which are normally present a series of windows that can be opened or closed as needed. This process is mandatory in a closed greenhouse to obtain an airflow that helps to maintain the right temperature and humidity levels suitable for different crop production. Among those framed greenhouses it is possible to find a great range of technological implementation involved in almost all the agricultural practices. It is possible to find a mechanical seeder, automatized pesticide and nutrient solution delivery system, remote-controlled artificial light plant, and HVAC. The firsts mentioned above are typical examples of open field technologies applied to a controlled environment horticulture system, while the artificial light system was developed properly for indoor cultivation where the use of specific light sources and spectra helped farmers to face the unstoppable changing seasons and bad weather. As well as artificial light systems, also the HVAC technology helps to maintain a proper air flow inside a closed or semi-closed greenhouse as well as the modulation of temperature and air humidity. These

kinds of greenhouses shall be also built upon existing buildings becoming "rooftop gardens" or inside them turning into plant factories in which the use of the artificial light system is predominant. These kinds of farms are usually developed in height and are known as Vertical Farms. Going deeper into the possible applications of all these technologies, it is possible to cultivate into a completely shaded environment. PFALs are one of the most advanced applications of CEH existing now, the use of the artificial light system able to simulate sunlight gives the chance to stop classically thinking about agriculture.

Indeed, thanks to PFALs, it is possible to cultivate wherever and whenever. There is also among PFALs, a great difference dependent on the crop production and the purpose of the cultivation, and these are the driving forces of the technological features adopted each time. Behind the use of these technologies, there is the conceptual idea of "producing more with less". This means that with efficient use of energy and soil surface, it is possible to harvest a great number of high-quality products. We are shifting now from square meter agriculture to cubic meter horticulture. Vertical farms are spread all over the world and are usually involved in the clean production of vegetables in which, thanks to the controlled environmental factors and the closing of the cultivation area from the outside surrounding environment, insects are left out and pesticides are banned. The stop of the use of pesticides together with the efficient use of water and nutrient solution is among the most successful technology implementation to save our planet and all the "good" insects like bees. As mentioned before, together with the stop of pesticides, the use of a nutrient delivery system (NDS) tailored for a vertical farm, will help with water management and its efficient consumption.

All those systems working together are connected and form the microclimate control system.

1.1.2. Microclimate and environmental control system

As above, all the systems integrated within the structure design of a greenhouse, or a grow chamber are needed to fine-tune the microclimate for plants cultivation.

The microclimate is the set of the environmental parameters that can be managed and modulated to adjust the cultivation environment to make it suitable for the crop going toward a high-quality product (Kozai et al., 2019).

The main environmental parameter which is usually controlled into a greenhouse or a PFAL is temperature. Indeed, the temperature is one of the main factors affecting plants' growth and development. The modulation of temperature value can be performed through many techniques: it is possible to shade the greenhouse with covering films, open or close ventilation system, utilize an HVAC and so on. Each of the possibilities involves a different level of technological facilities. To make the most from the energy consumption, the temperature value should remain stable throughout the day and different for each species or, at least, botanical family. Indeed, each species have a minimum, maximum and optimum value of air temperature in which its development is possible. The growth rate of plants is temperature correlated and typically, rising temperature results in a faster growth at least until the threshold value is exceeded (Kozai et al., 2019). When a threshold value, both for high or low temperature, is exceeded it may cause abiotic stress that can produce a delay in harvest, loss of yield and quality products and in the worst case the death of the plants.

Inside a CPPS temperature should be controlled not only for what concerns the air, but also for leaf surface, substrate, and nutrient solution temperature. The use of a light-bulb lamp, for example, as well as rising air temperature, also rise the leaf surface temperature due to the far-infrared spectral radiation emitted (Kitaya et al., 1998; Kitaya, 2005). This rising in leaf temperature needs to be avoided by facilitating natural convection or forcing it with fans.

The leaf boundary layer resistance is the layer of air in contact with the leaf surface and is responsible for the CO_2 uptake and of the H_2O vapour transport

from stomata. This layer is fundamental for the evapotranspiration rate and so for the photosynthetic rate of plants together with the stomatal conductance. Stomatal conductance is a parameter of plant physiology involved in the gas exchange between the internal substomatal chamber and the air above the stomata. This factor is affected by air velocity (Shibata et al., 1995), light quality and intensity (KIM, 2004), hydration status of the plant(Agurla et al., 2018; Buckley, 2019) and by CO_2 concentration (Engineer et al., 2016).

To have a good performance in PFALs the total control above the air velocity and direction is needed because it affects a wide range of physiological responses (Peiro et al., 2020).

The air velocity to maintain a low resistance of the leaf boundary layer and the stomatal resistance into a CPPS is stated between 0.5 – 1 m/s (Shibata et al., 1995). A CFD study of the airflow inside the CCPS is mandatory to make the most out of the airflow management.

Along with temperature and air velocity, another microclimate-controlled factor is air humidity. Air humidity is represented by the quantity of water as vapour, into a cubic meter of air. It is measured with two principal values: absolute humidity is the water content into the air and is expressed as g/m^3 and RH is expressed as a percentage of the water content into a cubic meter referred to the maximum it can contain. Warmer air can contain more water in form of vapour and so RH decrease with the rise of temperature; on the contrary colder air can contain less water and so, without changes in the absolute water content, RH increase.

As well as for the temperature, air RH has also a range of performance that is always species-dependent. Low air RH can increase the evapotranspiration rate, resulting in a water deficit stress for the plant. On the other hand, a high air RH results in the stop of the evapotranspiration, the close of the stomata and so the halt of the photosynthesis (Ryu et al., 2014; Kaiser et al., 2015).

The best way to visualize air RH and temperature is the VPD. VPD is the difference between the amount of aqueous vapour in the air and the maximum it can contain at the given temperature. As mentioned before, rising temperature means the possibility to contain more water vapour in the air, so at higher temperature VPD increase without increasing the RH. The SI uses kPa as the measurement unit for VPD. VPD is used to better manage the air RH and temperature threshold values during the phenological stage of development of plants, from seedlings to flowers and fruits formation. This helps farmers to have more direct information about the microclimatic status and make decisions easier.

As for the temperature, into PFALs, the best way to modulate RH and so VPD, is the use of an HVAC module. These modules often offer a user-friendly human interface that helps farmers better manage the cultivation process.

In the following sections are presented the more frequently used irrigation/cultivation techniques, lighting systems, nutrients types to explain how, as climatic conditions, they affect crops growth.

1.1.3. Nutrient Delivery System

Into CPPS, the soil is rarely used because it could be a vehicle of algae, fungi and bacteria and its sanitation procedures are complex and expensive. Moreover, the concentration of the nutrients in the medium are often unknown and this could result in a non-homogeneous growth. To avoid these problems, it is frequently used a substrate that does not interact with the plants except for their support at the root level. The control of the irrigation is mandatory to maintain the right amount of moisture among the roots zone and so keep plants well hydrated. Moreover, throughout the irrigation, are brought to the plants the macro and micro-nutrients, and this is called fertirrigation. We will use the term NDS to indicate the diffusion of nutrients throughout the irrigation system into the PFALs where usually is used nutrients free substrate. There is a wide range of irrigation techniques among the so-called *hydroponics*, here we will introduce them.

The simplest hydroponic NDS is the drip system where the nutrient solution is given to the roots by a micro-irrigation system made of drippers. This system uses a very small amount of water and can run cycling the irrigation periods alternating them with drought periods. It can suffer the concentration of nutrients just under the irrigation point and blockage due to the crystallization of nutrients on the drippers. In case of energy shutdown, this system can cause rapid death of plants due to the localized small water content inside the growth medium. This system is frequently used for potted plants sited on a drain vessel.

Outside of PFALs, it is also used for the irrigation of wide areas intended for small plants or arboreous cultivation.

With the use of a drain tray, it is possible to create an NDS based on a small layer of nutrient solution that passes through the vessel. This technique is called NFT and is frequently used in PFALs where is possible to stack more than one shelf of cultivation, one over the other. The use of this technique involved the continuous use of water flow that need to be well oxygenated and is strictly necessary to control pH and EC to maintain the optimum growing conditions. This system often involves plants in a pot with inert substrate inside which are then placed into canals or pipes and sometimes bigger trays where the pots can be suspended over the thin layer, just above the stream flow, or placed on the drain trays. This system could suffer overgrowing of the root system that finds a suitable place where to grow and then needs to be pruned. Moreover, with a power outage or a pump failure, plants could suffer drought till death.

The use of retaining water substrates is preferable for Ebb&Flow irrigation techniques where the potted plants are lined on the drain trays and the nutrient solution cycling floods the cultivation area. In this NDS are usually cultivated small plants due to the height water can reach between two irrigation period that could be not enough to soak the medium properly for big potted plants. Indeed, is necessary to avoid the submersion of roots for a prolonged time because it can result in anoxia stress at the roots level. Water reservoir needs

to be huge due to the great amount of water used for irrigation into wide areas of cultivation where 1 cm of water height over a square meter area, is a total amount of 10L of nutrient solution. As for the NFT, Ebb&Flow systems suffer power outages and pump failures which mean the lack of water for one or more irrigation cycles.

Another major technique used in PFALs is DWC where plants are cultivated directly into the nutrient solution tank or in single sealed vessels where the nutritive solution needs to be well oxygenated and in which is strictly necessary the control and modulation of pH and EC. In DWC, to maintain good aeration of the nutrient solution, it is usually used a micropore stone in which air is inflated and bubbling in the tank continuously.

Aeroponics systems are those in which the plants' pots are suspended into a basin in which the nutrient solution is sprayed all over the substrate firstly and then directly to the roots when they grow out of the substrate. In this way, the nutritive solution is absorbed directly by the root system and so it's mandatory not to have a nutritive solution too much concentrated which could result in salinity stress for plants. This system suffers the clogged mist nozzle and roots may need to be pruned.

In all these systems, the nutrient solution shall be kept in dark conditions avoiding excessive evaporation and light that can enhance algal blooming.

All these systems are usually closed-loop hydroponic irrigation techniques that involve the recycling of water and the renewal of the depleted nutrient solution after one or more cultivation cycles.

To make the most out of the hydroponics cultivation techniques the nutritive solution shall be prepared starting from zero nutrient and neutral pH water. This "pure water" is usually made by osmotic plants that are directly mounted into PFALs architecture and, within it, a widespread system of pipes that is needed for the circulation of the nutrient solution and of the depleted one. These osmotic plants are necessary to make the most out of the fertilizer formulation available on the market, mainly because each brand offers a

different solution for a wide range of diseases or situations to be used in addition to the standard fertilizer and also because with a dosing system it is possible to add only the depleted micro or macro-nutrient.

To be honest, nutrients into CPPSs are not only delivered to plants throughout the irrigation and so by water, but recently the use of slow-release fertilizers have changed the way plants are fed. This new system is widespread among herbs cultivation in pot combined with a drip system or also for leafy vegetables, like lettuce, cultivated on inert substrates over NFT or Ebb&Flow trays. The use of this kind of fertilizer strongly limits the diffusion of nutrients into irrigation systems and reservoir basins which means that the water is less harmful to rivers and aquifers and its disposal is easier (Shaviv, 1993; Mikula et al., 2020; Bi et al., 2021).

As mentioned in the previous sections, the nutrient solution needs to be kept at a proper temperature. This necessity is fundamental to avoid the thermal stress of the root system. To do this, chillers are the most frequently used system to maintain a great quantity of water into an optimum temperature range with minimum fluctuation. The water reservoir, usually kept at a lower temperature in respect to the air, could be also used for thermal inertia of the entire vertical cultivation systems.

So, the control and management systems are necessary to drive the cultivation processes and, moreover than temperature, air RH, VPD, nutrient solution temperature, pH and EC and the cultivation process itself, plants cultivated absolutely need a proper artificial light system.

1.1.4. Light system

Light is the most ancient driving force for plant growth and development together with gravity. Light is involved in a wide range of plants physiological behaviour. Light is fundamental for crop production, moreover if you plan to cultivate all year long. The use of artificial light system in PFALs is compulsory because of the internal shaded areas that naturally creates a multilevel production system.

Light is emitted naturally by Sun, as photons or waves, and its spectrum covers all the physiological necessities of plants that during the aeons have evolved using it. Many studies have been carried on during the last century on how plants perceive light, how they use it, what kind of light is preferable for a given phenological status, how much light they need and so on (Kozai and Zhang, 2016; Bantis et al., 2018; Kozai et al., 2019; Zhang et al., 2020).

For indoor vertical farming, the dogma is "produce more with less" and so is to cultivate more vegetables with less energy consumption possible. To achieve these results, it is necessary a better knowledge of plants physiology to balance energy consumption with food production. Here after are presented typical light systems and is explained how light quality (as a spectrum) and quantity (as intensity) and its duration (known as photoperiod) drive the cultivation process.

First, I need to introduce you to some essential concepts. Light, in agriculture, is composed of photons. These photons are particles that run sinusoidally at the speed of light. The distance between the apexes of the wave determines the wavelength and so, in the eye-visible region of the electromagnetic spectrum, their colour. The biggest is the distance between the apex, the greatest is the wavelength but the lowest is the energy of that particle. The eye-visible region goes from about 390nm to 760nm of wavelength and coincides with the PAR region. PAR region is the portion of the electromagnetic spectrum in which photons are suitable for the photosynthesis of the plant. These photons, depending on the wavelength, have different energy levels (Ruban et al., 2011). Each kind of light source, even the Sun, has different ratios of emitting wavelength. This could be intended as the light spectrum of an emitting source of light and influence mostly the morphology of plants (Decoteau et al., 1997; Chen et al., 2014).

Light intensity indeed, is defined as the quantity of light, as photons, that reach a square meter surface in a second. Its measurement unit is $\mu mol/(m^2 \cdot s)$ and is mainly named as Photosynthetic Photon Flux Density or PPFD. Sun has a light intensity of about 2000 $\mu mol/(m^2 \cdot s)$ while the artificial lights range

from 0 to 1000 μmol/(m^2·s). The intensity of light influences mostly the biomass production of plants.

Light photoperiod is simply the duration of the lighting stimulus on the leaves of plants. In nature, it depends on the season and on the latitude at which the plant is rooted. It influences mostly some phenological behaviour of plants, like flowering and senescence.

The sum of the light intensity given for the total amount of time of the light stimulus during a day is known as DLI and is normally expressed as mol/(m^2·d).

Typically, crystal clear greenhouses use sunlight as the primary light source for their cultivations. Occasionally they are furnished with a series of lamps that help the cultivation process during late season periods or cloudy days. Those lamps are fundamental for the success of the cultivation mostly when the crop is cultivated over the season or even if the crop is not in its characteristic climatic zone. Depending on the technological level and on the application needed, there is a very wide range of lamp choices.

Within greenhouses the most frequently used lamps are HPS. These kinds of lamps have an irregular spectral distribution characterized by many emission peaks. These lamps have an extremely high-power absorption that is converted only for a reduced percentage into photons. The rest of the energy is converted in the longest wavelength such as the infrared region and so directly into heat. These kinds of lamps are widely diffused into the greenhouses as they are cheap but during the last 20 years Light Emitting Diode, also known as LED, have gained great visibility thanks to their high efficiency. Indeed, LEDs convert about 50% of the electrical consumption into photons. Single colour LEDs with their narrow spectrum, are widely used in the research field to find the physiological effect of specific wavelengths on the growth of plants and the combination of different spectrums. A great quantity of studies has been carried out on the combination or the use of single colour LEDs on plant physiology (Bula et al., 1991; Chang and Chang, 2014; Zhen and van Iersel, 2017; Cavallaro et al., 2022). Thus, in this way it is

possible to focus all the energy on the photosynthetic activities, other research found that certain wavelengths not properly linked to the absorption spectrum of the photosynthetic pigments have a measurable effect on plant physiology (Kim et al., 2004). Recently the development of new white LEDs for agricultural purposes has opened new research activity on the full spectrum effect on plant physiology (Li and Kubota, 2009).

1.1.5. Reproducibility

The knowledge about how to pilot and modulate the environmental parameters inside a closed chamber is mandatory to obtain a controlled environment that can be used in any other conditions without being affected by the surrounding.

Finally, the knowledge about how to cultivate a plant through the modulation of the environmental parameters that are part of an ecological niche is the only way possible to build up a colony on another celestial body and is one of the rarest examples of an Earth developed technology applied to space research.

Humankind will always need new land for its development, and this means an exploration campaign that led to the colonization of new areas and probably, soon, other celestial bodies. To colonize the unknown, the most effective way is to bring the confidential environment in which we all already live, with us.

This means biogeochemical processes, living organisms, trophic chain and so on. Trying to face this high challenge, the Bioregenerative Life Support System, known as BLSS, have been studied long as a possible solution.

1.2. Microgreens, a *Nutritional Taste*

In this contest, *Microgreens* are a novel food product that is taking steps into everyday life since the 1980s when this new food product started to appear on chefs' tables of California (Lubow, 2006; Palmer, 2010; Bliss, 2014; Kyriacou et al., 2016; Verlinden, 2020). After their first's appearance, they

are becoming common both in the marketplace as well as homegrown products (Parida, 2020).

A microgreen is a term that defines a plant that has reached the phenological stage of fully developed cotyledons after the germination process from the seeds and the first true leaf is coming through (Verlinden, 2020).

There are at least forty definitions of microgreens in scientific papers (Verlinden, 2020) in which other authors referred to them as tasty, colourful, spicy seedlings that are ready to be eaten after the sprouting process but, these definitions are not referred to their phenological status and so, are not botanically correct.

Indeed, the verb “to germinate” is different from “to sprout”.

In the horticultural field, they are cultivated as “sprouts” and so are defined as fast-growing vegetables just for the fact that they are cultivated for a short time that indeed really depends mainly on the cultivated species' proper characteristics.

They can be colourful or simply greens, with an intense spicy flavour and sometimes with a crunchy consistency.

Quite almost all crop species shall be cultivated as microgreens and their cultivation process is quite simple and doesn’t need a huge amount of water and energy for the growth process (Verlinden, 2020).

At that peculiar phenological stage, their resistance to the surrounding environment is given only by their capability to fast counteract biotic and abiotic stress. This is possible thanks to their high internal concentration of phytochemicals that works as an antioxidant defence system (Delaquis and Mazza, 1995; González-Lamothe et al., 2009; Xiao et al., 2012; De-La-Cruz Chacón et al., 2013; Cavaiuolo and Ferrante, 2014). Many studies report their higher phytonutrient concentration and defence capabilities that differ from species to species and also respond differently to the wide range of external stimuli that have been carried out in several experiments during the last 20 years of research (Verlinden, 2020).

This makes them a potential superfood that has already risen among many industrialized countries of the world (Teng et al., 2021).

In two words, they can be defined as superfoods with a “Nutritional Taste”.

Microgreens are now commercially widespread in some markets around Europe, the USA, and Asia. They are commonly cultivated on fabric substrate and sold fresh and uncut or as seeds to cultivate at home. A proper cultivation procedure is however needed to take out the most from them as their internal beneficial compounds would be depleted by wrong cultivation methods.

To face the rising problem of world hunger, microgreens shall be considered not as a solution but indeed as a starting point for good nutrition in all countries (Brentlinger, 2007; Di Gioia et al., 2021; Nayak et al., 2021). Moreover thinking about the possibility to cultivate them directly in our own house or with minimum technological facilities. Malnutrition, as already stated, is the key problem of the coming years and good information about the quality of microgreens could bring their consumption level higher than now (Nayak et al., 2021).

As well as for general crop production in a controlled environment, microgreens need facilities capable to modulate the environmental parameters to fine-tune the cultivation processes.

The peculiarity of microgreens to be short in height and with fast growth from sowing to harvesting time makes them eligible for multilevel cultivation systems.

PFALs have indeed all the characteristics to be used for microgreens crop production as they have: hydroponics cultivation systems, HVAC systems, artificial light systems, NDSs, and a proper number of professional employees.

However, the high yield obtained within microgreens shall reduce the overall dimensions of these production systems till the design of a cultivation unit that could fit into a kitchen and that can be named “Ortodomestico”

(InHouseHort) as it could be fit into a house and regularly produce a yield of microgreens with a good *nutritional taste*.

Microgreens' growth and developmental process and their chemical composition are indeed directly influenced by several environmental factors such as air temperature and humidity, air velocity, micro and macro elements in the circulating nutrient solution, intensity, spectrum and duration of the light stimulus and, all of these features can be modified at will to obtain specific results (Verlinden 2020).

Plants' Chlorophyll absorbs mainly in the red (663 nm and 642 nm) and blue (430nm and 453 nm) regions of the light spectrum and these wavelengths are the ones that mainly affect their development (Lefsrud et al., 2006b, 2006a, 2007, 2008; Wheeler, 2008; Lin et al., 2013; Chen et al., 2014; Hayashi and Higgins, 2016).

The red light, indeed, is sensed by specific photoreceptors called phytochromes that are capable of generating a series of inputs that start or modify the metabolic pathways related to germination, stem elongation and/or leaf expansion (Pinho et al., 2004, 2012; Pinho, 2008).

On the other hand, Blue light is received by cryptochromes and phytotropins and regulates the phenological processes of de-etiolation, phototropism, the movement of chloroplasts inside the cells, roots growth and, light-induced opening of stomata, redox balance, etc (Kritsky 1984).

Knowledge gained during the last 15 years of research, and even before, had explained how light interacts with biological matter, in a particular vegetable organism, and researchers had started to combine a different kind of light to stimulate and suppress many physiological processes (Zhang et al., 2020).

The effects of LED light in the cultivation of microgreens have been studied on their nutrients compositions (Zhang et al., 2020; Bantis, 2021), on their morphology and yield in different species of the Brassicaceae family, and in particular *Brassica oleracea, B. juncea, B. rapa* (Samuoliene et al., 2013; Brazaitytė et al., 2015, 2021; VAŠTAKAITĖ et al., 2015), pea, broccoli,

mustard, borage, amaranth, parsley, chard, kale (Samuoliene et al., 2013), Valerianella locusta (Wojciechowska et al., 2015), buckwheat (Choi et al., 2015), *Ocimum basilicum* (Lobiuc et al., 2017).

Plant research, as well as microgreens research, is conducted in cultivation chambers designed to support growth and development under a completely controlled environment reducing the environmental stress conditions to which plants may be subject (Rouphael et al., 2018) or fine-tuning those stress to deeply study their effects on plant physiology.

1.3. Microgreens nutritional properties

The very first study that reports microgreens' nutritional contribution was written in 2012 and states that plants cultivated as microgreens have a phytochemical concentration from 4 to 40 times higher than their respective harvested form (Xiao et al., 2012).

Since then a large number of scientific papers were written on this topic analysing the effect of various biotic and abiotic stress on all the developmental stages, from imbibition time to the quantity of light (Zhang et al., 2020).

Light, together with gravity, was indeed one of the first stimulus plants had to cope with on Earth since their appearance, both in oceans as algae as well as on soil as moss and fern till higher plants.

Light may have changed its intensity and spectrum during aeons, but plants had use it as a source of energy since the beginning.

Indeed, light is the driving force of chlorophyll photosynthesis and also has a fundamental role in driving the synthesis and accumulation of other phytochemicals in plants (Zhang et al., 2020). Thanks to the knowledge gained during plants physiology base research, this process is now achievable by using LED lamps equipped with specific wavelengths (Bula et al., 1991).

In particular, in microgreens, research was conducted on the importance of the effect of the quality of red light on the total content of phenolic

compounds and of the antioxidant activity (Samuoliene et al., 2012; Samuolienė et al., 2016); the integration of red, blue and far-red light on antioxidant activity (Brazaitytė et al., 2019, 2021); the integration of ultraviolet light A (UV-A) and its effect on growth, on the synthesis of phenolic compounds, anthocyanins, ascorbate and tocopherol (VAŠTAKAITĖ et al., 2015); the effect of the dosage of blue light on growth, on the content of total phenolic compounds, anthocyanins, ascorbate, flavonoids and antioxidant activity(Vaštakaite et al., 2017).

Growing plants by supplementing Far-red, red, green, blue and UV light could lead to an increase in the synthesis of chlorophyll and so biomass, the contents of phenolic and other antioxidants, glucosinolates, vitamins in various species (Bian et al., 2016; Lobiuc et al., 2017; Kyriacou et al., 2019; Verlinden, 2020) but better results are obtained when optimized ratios of these wavelengths are chosen to obtain better yields (Darko et al., 2014; Dou et al., 2017; Kyriacou et al., 2019; Verlinden, 2020).

1.4. Astronauts diet

Plants are useful in space settlements as they produce oxygen and pure water but, together with these extremely important features, they should be also a good source of food.

A healthy diet is indeed mandatory for crew members as they need to live in a constantly challenging environment that exposes them to stressful conditions like microgravity, unhealthy air, and chronically exposure to ionizing radiations (Stein, 2001; Anderson et al., 2015; Tang et al., 2021).

Up to now, astronauts have eaten quite always pre-prepared foods that were rehydrated directly in space before the consumption.

This approach helps surely for the storage capacity, the expiration time, and the safeness of foods given to the crew as the possibility of an illness must be avoided by all possible means.

Crew members, indeed, need to be always ready to face any sudden problems that may come out during a mission as the help from Earth can only arrive after a long time.

To help cope with these many problems, for the army as well as for astronauts, a diet rich in fruits and vegetables can prevent depression (Tsai et al., 2012; McMartin et al., 2013; White et al., 2013), improve mood (Smith and Rogers, 2014; Conner et al., 2015, 2017), and increase cognitive performance (Nooyens et al., 2011), and it could be right to sustain the astronauts to carry on the many experiments for fresh cultivation of plants in space (Zabel et al., 2016). Indeed, this diet can be achieved not only by lyophilized food but also with fresh food cultivated directly in space (Wheeler, 2010). NASA has already defined the nutritional goals for astronauts and many authors have looked deeply into their menu and diet (Anderson et al., 2015).

In the guidelines has been explicated the astronauts' daily intake quantity for proper nutrition (Anderson et al., 2015) and by comparing those requirements with the data of nutrients concentration in microgreens (Xiao et al., 2012) it should be possible to convert them as a quantity of microgreens, depending on the species, that shall be intake into the astronauts' diet to cover some vitamins and micro and macronutrients allowance.

Lepidium sativum L. is known to have many pharmacological e therapeutical uses(Oszmiański et al., 2013; Asra Jabeen*, 2017; Sharma, 2020) and is frequently used as oil extract from seeds, seedlings, and sometimes also leaves and branches were used for the preparation of traditional medicines in many Asiatic countries (Scartezzini and Speroni, 2000).

In space, astronauts will have to cope with a hazardous environment that Human Research Program by NASA had shortly condensed in the "**RIDGE**", space **R**adiation, **I**solation and confinement, **D**istance from Earth, **G**ravity fields, and hostile/closed **E**nvironment.

Those conditions will affect their health status (Barratt and Pool, 2008; Barratt et al., 2020) bringing them to many characteristic space diseases like

muscular and cardiovascular deficiencies (Thornton et al., 1987; Convertino, 1996; Leach et al., 1996; AKIMA et al., 2000; Meck et al., 2001; Perhonen et al., 2001; Trappe et al., 2009; Gopalakrishnan et al., 2010; Lee et al., 2015), early osteoporosis (Convertino, 1996; Lane & Schoeller, 2000; Sibonga, 2013; S. M. Smith et al., 2012), and eye-related disease (Roberts et al., 2006; Mader et al., 2011; Stenger et al., 2017; Zhang and Hargens, 2018; Lee et al., 2020).

In this contest, a proper diet aimed to face these situations that always happen to space inhabitants is mandatory(Pavez Loriè et al., 2021).

The presence of antioxidants like phenolic acids, flavonoids and carotenoids in astronauts' diet is under study in particular for their bioavailability which is higher in fresh food than the lyophilized food and even worst in manmade pills (Fernández-García et al., 2012; Platel and Srinivasan, 2016; Cosme et al., 2020; Gómez et al., 2021; Tang et al., 2021; Zhang et al., 2021).

1.5. Plants in space

Plants here on Earth are well known to produce, together with algae in the oceans, the oxygen that all humankind and other animals and microorganisms are breathing/consuming every time, every day.

Even on the ISS the air that crew members are breathing right now, is coming from the chlorophyll photosynthesis that was collected and stored before lifting off.

Vegetable organisms are necessary for keeping life safe on Earth and they will be necessary for keeping life safe on other planets or extra-terrestrial colonies.

Indeed, the possibility to cultivate plants in space to produce oxygen by converting carbon dioxide and purifying the water through evapotranspiration was one of the first and it is up to now the first and unique objective of all the national agencies for space activities.

The story of plants cultivation in space began in the 1950' when both the USA and Russia had started their space race. Russia was the first nation to bring humans into space and also the first that brought seeds (Halstead and Dutcher, 1984). Indeed in 1960 onboard the Sputnik 4 some seeds were flown into space and pawned the path for the US Biosatellite II that seven years later housed the cultivation of four red pepper plants (Johnson and Tibbitts, 1968; Salisbury et al., 1997; Porterfield et al., 2003).

In the last 20 years on the ISS, many cultivations of plants both from monocot to dicotyledons had been successfully concluded and recently crew members were authorized to eat the romaine lettuce cultivated inside the VEGGIE system developed by GIOIA DI MASSA (Christine Brown-Paul, 2015; Massa et al., 2017a, 2017b; Khodadad et al., 2020).

Many systems were developed since Sputnik 4 to cultivate plants (Porterfield et al., 2003; Cooper et al., 2011; Poulet et al., 2016; Zabel et al., 2016; Escobar and Nabity, 2017) and up to now the technological level reached is very high, like the Advanced Plant Habitat (Monje et al., 2020) but many details are still missing.

Spaceflight plant research is indeed conducted in cultivation modules specifically designed to allow growth in a reduced gravity environment under completely controlled conditions (Monje et al., 2003). These chambers have limited space and volume to reduce the environmental stress conditions to which the plants may be subjected during in-orbit experiments while providing an adequate air temperature and humidity, the correct concentration of gases (O_2, CO_2, VOCs), enough water and nutrients, optimal light quality, and intensity to not alter the normal plant patterns of growth and development.

Plant cultivation systems had to face problems like lack of convection in microgravity as well as the different distribution of fluids, like water, that in space behave differently in respect to the Earth where gravity intensity is

higher than in space (Yendler et al., 1995; Bingham et al., 1996, 2000; Chamindu Deepagoda et al., 2012, 2014; Jones et al., 2012).

Lacking convection means also a random and at least non-homogeneous distribution of other gases like CO_2 inside the cabin and the cultivation unit as well as non-uniform distribution of temperature and humidity. The lack of convection brought to the low performance of evapotranspiration from stomata and in general from the leaf surface since the layer of humidity is not renewed by the wind.

Nutrient delivery systems in space can be still improved since the necessity of a good quantity of water and air in the roots zone is necessary for a healthy cultivation process. By the way, in the VEGGIE system, water is injected into a substrate directly by crew members and there is not an automatic system for the irrigation of crops produced in space (Massa et al., 2017a).

Recently is in developing a new cultivation system by NASA, the PONDS in collaboration with TUPPERWARE company (Howard G. Levine, 2018).

In these kinds of enclosed controlled environmental systems, it should be possible to assess and characterize the effects of individual or multiple stresses by analyzing the plant responses with phenotyping platforms (Zhao et al., 2019; Yang et al., 2020). These platforms use a variety of imaging techniques able to reveal symptoms of a wide range of stresses at early stages.

Moreover, using such platforms, it is possible to collect data for quantitative studies of complex traits related to the growth, yield and adaptation to biotic and abiotic stress and are recognized as the only tool able to deliver an accurate description of trait expression in multiple-stress environments (Li et al., 2014; Zhao et al., 2019; Cotrozzi and Couture, 2020). Artemis projects, although it does not mention “plant cultivation” on the first page, have many projects involved for plant cultivation (NASA, 2020).

1.6. Plant growth and development in space

Plants are sessile organisms that can't escape from hazardous circumstances but instead, they must cope with many extreme situations coming from the surrounding environments.

They have developed many strategies to face these kinds of problems and academics have reached deep knowledge on the mechanism involved in their response and survival.

The space environment is not the same as the one it is possible to find on Earth. Indeed, here on Earth, some environmental parameters can change from one geographic point of the globe to another, while in space many environmental parameters change completely (i.e. Gravity intensity), others gradually (i.e. Radiation intensity) and some do not change at all (i.e. atmospheric pressure) or can be modulated (light, temperature, humidity, etc).

Plants during aeons have evolved with the same intensity of gravity vector that drove their progressive growth in two main directions, toward gravity with roots and opposite to it with the growth of shoot and canopy.

Fortunately, in space, many authors already stated that there is no physical, chemical or biological process that is so dependent on gravity that plants cannot mature in its absence (De Micco et al., 2014; Levinskikh et al., 1999; Sychev et al., 2007).

The choice of the plants that could be cultivated into space must meet some specific requirements (Medina et al., 2021b, 2021a) like the short growth cycle, a predetermined growth, high yield in respect to sowed seeds, a pour amount of characteristics disease and also a good resistance to them in general, good resistance to the cosmic environment (Medina et al., 2021a, 2021b) and an increased quantity of bioactive compounds that fit with the maintenance of health status of the crew members during the flight and then during their permanence in a possible extraterrestrial colony (Salisbury, 1999; Pavez Loriè et al., 2021), indeed also ornamental plants will be a sure choice as they will help inhabitants to feel at home.

All these requirements are mandatory if the objective is the possibility of persistence of humankind in other planets as well as in space. Indeed, plants thanks to chlorophyll photosynthesis produce oxygen while absorbing carbon dioxide and purifying water in the meanwhile of this process.

The cultivation of plants in space is the foundation of an Advanced Life Support System that may involve many other biological and non-biological compartments that however led to a BLSS (Fu et al., 2016).

Within these kinds of systems, plants together with mechanical, chemical, and other biological entities shall replicate the complexity of the Bio-Geo-Chemical cycles that on Earth recycle and convert matter from one form to another regenerating primary resources.

Plants were already grown in space and performed many cultivation cycles from seed to seeds (Sychev et al., 2007; De Micco et al., 2014)and so they are eligible for space cultivation but, to cultivate them and with the objective of a BLSS, the building of a compartment dedicated to this purpose is mandatory and many engineering aspects shall be considered.

In space like on Earth, light stimulus shall be always on top of the cultivation plane as lacking gravity stimulus and so of gravitropism, shall be substituted by phototropism (Schulze et al., 1992).

Plants shall be cultivated into a dedicated compartment in which a proper ventilation system will be required to help plants' evapotranspiration and the section shall be confined.

Indeed, many studies conducted on Earth report the increase of efficiency of plant photosynthesis as carbon dioxide increases (Taub, 2010; Ainsworth et al., 2020), together with other stringent environmental parameters that must be kept into the "optimum zone" meaning the increase of efficiency of oxygen production and water purification.

The pressure inside the space greenhouse shall be maintained at lower values than on Earth (Ewert et al., 2002; Rygalov et al., 2002; Moghimi Esfandabadi and Bannova, 2019). Indeed, lower values of pressure mean a lower quantity

of air that instead shall be used by crew members in other sections of the space station or of the colony; and together with reduced pressure, a modified partial pressure of other gasses shall enhance the photosynthesis power while storing the carbon dioxide coming from crew compartments. A low-pressure structure is also less in danger in respect to the ones at atmospheric pressure when the outside environment is practically 0 atm.

Indeed, an advanced space greenhouse, both if it is built on the surface of another celestial body or inside one of its caves to help shield the ionizing radiation, will face the problem of differential pressure between the inside of the structure and the outer space (Maggi and Pallud, 2010; Esfandabadi and Bannova, 2019).

To counteract these technical problems of safety, many studies have been carried out on the hypobaric cultivation of plants to understand the effects of low pressure on their physiology and viability.

By controlling not only the atmospheric pressure but also its composition in gas, it should be desirable to reduce N_2 partial pressure and enhance CO_2 percentage. Indeed, this approach will reduce the buffer gas needs, like N_2, and also with the architectural design of the greenhouse itself.

Moreover, the most are fine-tuned the environmental parameters like the light quality, intensity, and photoperiod and the temperature, humidity and air speed velocity, the highest the control on the cultivation inside the space greenhouse.

Ionizing radiations will change gradually as the cultivation will set far from Earth's surface. Indeed, the magnetosphere of Earth keeps humankind safe from a very harsh environment (Furukawa et al., 2020; Held, 2020).

Low Earth Orbit is the definition of a portion of space around the Earth whose borders are from 200km to 1600km from the surface. Inside this band is orbiting right now the ISS with a crew of astronauts on board. In this band, the radiation environment is composed mainly of particles coming from the Sun and by the galactic cosmic rays coming from outer space. This region is

mostly shielded by the Earth magnetosphere and allow us to experience the space environment quite safely making possible a wide range of experiments in many research fields (Reitz, 2008; Allen et al., 2018).

Far from the Earth's surface, the shielding efficiency of the magnetosphere fails, and the solar particle events and the galactic cosmic rays hit human objects with a dose that depends on the orbit and the galactic weather.

For example, booking a trip to Mars, according to the Mars Science Laboratory, means exposure of 0.46Gy/day (Hassler et al., 2014).

Greencube mission ground preliminary tests on plant materials, for this thesis, had experienced a dose rate of 0.1Gy/h.

1.7.Space environment simulation facilities

Space environment, as it is, can be experienced only in space and reaching space test facilities, like the International Space Station, is hard and the reproducibility of those experiments on Earth is dubious (Kiss et al., 2019; Manzano et al., 2020).

Indeed, space is characterized mostly by Ionizing radiations and microgravity (Allen et al., 2018). The first factor is a composition of three events that occur naturally in our solar system, the localized trapped particle belts, the periodic/occasional Solar Particle Events, and the background Galactic Cosmic Rays. All these natural events had a different footprint of energy and radiation types and react differently with the biological tissues or with the unanimated electrical components(Allen et al., 2018). The interaction with the matter, whatever it is, causes a change in the radiation environment changing from the surface of the impact to the layers below.

Just why the nature of these Ionizing radiations is various, the quantity and the effect and so the damages occurring after the exposure to them are very complex to be predicted.

In particular, the Greencube mission will orbit around Earth at a height of about 6000km, just inside the *Van Allen* radiation belts (Santoni et al., 2020).

NASA had started developing a new great chamber to simulate better the cosmic weather to be as close as possible to the real situation. The GCR Simulator can generate a wide spectrum of ion beams and can perform low dose rate exposure like it is in space (Norbury et al., 2016; Simonsen et al., 2020).

Even if a great number of scientific papers were produced for space environment effect on plants, a poor quantity is focused on the possibility to perform them on the ground and simultaneously.

1.7.1. Microgravity

As research in the space environment is worthy but expensive, a simple system to simulate microgravity was developed at the beginning of the plants in space science. The very first experiment performed to simulate microgravity conditions dates back to the early 1800 (van Loon, 2007)and from that date, these systems have evolved to the most recent Random Positioning machines.

These systems, called Clinostat, are mainly composed of a rotating plane (1 or 2 axis of rotation), in which plants are kept in a rotatory perpetual movement that confused the cells in the root apex that are deputed to feel the gravity vector by the sedimentation of statoliths that, in continuous rotation never stop moving.

Many studies have been carried on using clinostats for simulating the microgravity environment (Herranz et al., 2013, 2022; Manzano et al., 2018). There exist many kinds of clinostats and each of them probably recreates a different magnitude of microgravity and so it is hard to compare results from experiments performed on different species but also the same species in different laboratories. Moreover, microgravity generated by clinostat is simulated and not real and this means a possible mismatch between results obtained in the Space and on Earth.

1.7.2. Cosmic Radiation

As already mentioned, radiations in space are a mix of several particles, each with a specific characteristic (Allen et al., 2018). NASA has started developing a new comprehensive facility that shall provide a wide spectrum of radiations simultaneously (Norbury et al., 2016; Simonsen et al., 2020). However, in the present days, experiments that used radiations were constrained by the irradiation facility and by the specific particles it can generate.

Another big constrain is the possibility to perform a small range of radiation energy that instead is often very high. Indeed, a lot of experiments in this field of research are related strongly to high acute exposure to radiation that is however very far from what plants will experience in a real space habitat.

At ENEA "Casaccia" Research Centre in Rome, the Calliope ^{60}Co irradiation facility was developed in 1967 for agriculture research purposes and qualification tests on materials intended for space applications.

The exposure chamber has a dimension of 7.0m * 6.0m floor for 3.9m in height, is one of the hugest exposure chambers in Europe and also in the world and its irradiation intensity varies from 0Gy to 7.3kGy/h (updated January 2022).

Thanks to this facility it was possible to expose the simulation of the cultivation volume of the GreenCube mission to a low chronic dose both in static and clinorotated conditions while kept at 1atm or 0.5atm.

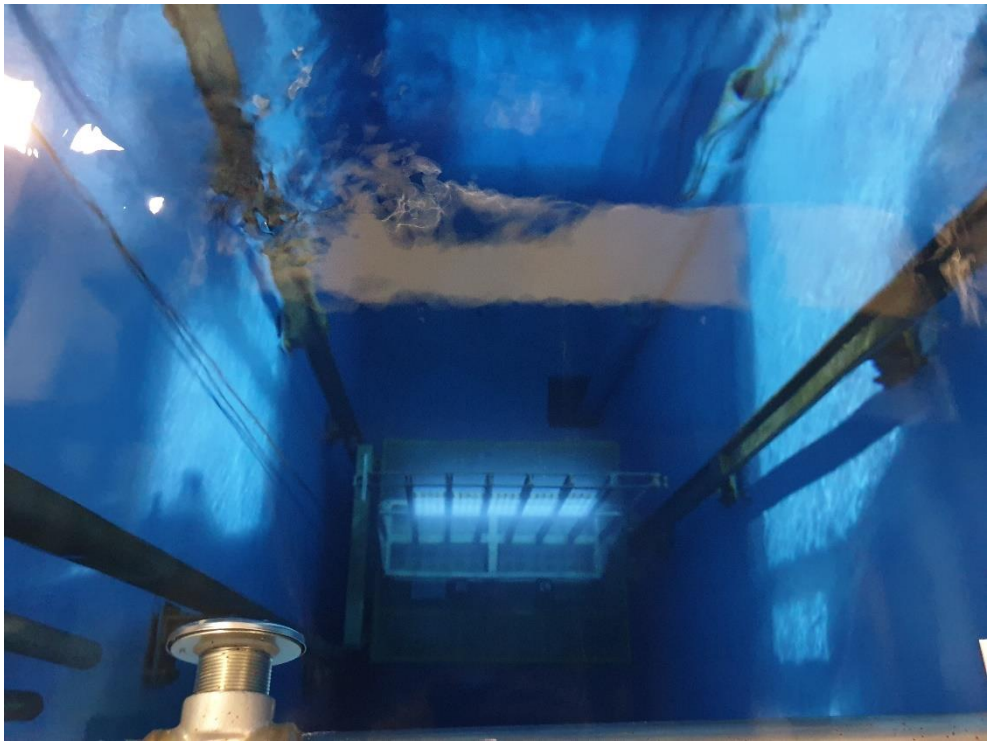

Fig. 1.7.1 ^{60}Co γ irradiation source in BioShield pool

1.7.3. Hypobaria

An advanced space greenhouse, both if it is built on the surface of another celestial body or inside one of its caves to help shielding the ionizing radiation, will face the problem of differential pressure between the inside of the structure and the outer space (Esfandabadi and Bannova, 2019; Moghimi Esfandabadi and Bannova, 2019). To counteract this technical problem of safety, many studies have been carried out on the hypobaric cultivation of plants (Corey et al., 2002). The most are fine-tuned the environmental parameters the highest the control on the cultivation inside the space greenhouse (ISHIGAMI and GOTO, 2008). This means controlling not only the atmospheric pressure, but also its composition in gas with a reduced N_2 partial pressure and an enhanced CO_2 percentage, the light quality, intensity, and photoperiod control, temperature, humidity and airspeed velocity (E. G. Wilkerson et al., 2007; ISHIGAMI and GOTO, 2008).

New forms of space research facilities are gaining momentum in the present times. As mentioned above, reaching the ISS is quite difficult and so to make feasible and cheap the possibility to reach real space environment, many space agencies are now developing new rockets (ArianeGroup, 2018) and payloads services (Chantal Cappelletti Simone Battistini Benjamin K. Malphrus, 2021) to send into space experiments of all kinds (Marzioli et al., 2020; Santoni et al., 2020; Kanapskyte et al., 2021; Trouillefou et al., 2021; Zea et al., 2021; Robson and Cappelletti, 2022).

1.8. Analysis of plant physiology and growth in space

Most of the actual analyses performed in agrobiology laboratories are defined as wet or destructive analyses. This means that to obtain a certain kind of information from a sample it is needed to destroy irreparably the sample itself making it useful for a restricted series of later analyses. Among this kind of analysis, it is possible to list the measurement of the dry weight for what concerns the morphometrical analysis and the extraction of pigments for the spectrophotometric analysis. Even proteomics, metabolomics and other kinds of chemical analysis can be listed among the destructive analysis and also plant flow cytometry that can be used to investigate ploidy and DNA damages of plants exposed to stress (Rutter et al., 2020).

However, plant flow cytometry is rarely performed in space and is instead a good source of data as it can give information about the stress response and effect on cell cycle regulation as it is already stated that microgravity affects cell cycle regulation, and diversification of nuclei number in the different cell cycle phase (Legué et al., 1996; Yu et al., 1999; Matía et al., 2010; Manzano et al., 2013, 2016, 2018, 2020; Medina et al., 2015; Boucheron-Dubuisson et al., 2016).

Is good to know that cutting away a leaf from a plant causes a series of chemical reactions and signals transmission that not only affects the plant itself but also the plants around it (Zhu, 2016; Peck and Mittler, 2020). Using this kind of analysis on a schedule during the growth of plants can bring to a mystification of the results that may be caused by the stress response and not

by the experimental design research. Under highly environmental controlled conditions and with the appropriate combination of image analysis technologies and methods (Chaerle et al., 2007; Zhao et al., 2019) it is possible to identify early symptoms of a wide range of stresses, monitor physiological processes and provide acceptable phenotyping results (Li et al., 2014).

These platforms use a variety of imaging techniques able to reveal symptoms at early stages for a wide range of stresses, to collect data for quantitative studies of complex traits related to the growth, yield and adaptation to biotic and abiotic stress and are recognized as the only tool for delivering an accurate description of trait expression (Cotrozzi & Couture, 2020; L. Li et al., 2014; C. Zhao et al., 2019).

These imaging techniques include visible-light imaging, multispectral and hyperspectral imaging, fluorescence imaging, thermal infrared imaging, and 3D imaging reconstruction.

Visible light imaging provides information on the canopy cover and colour and it is the simplest imaging technology for plant health sensing particularly useful to assess measure aspects of plant architecture such as leaf/cotyledon area, color, growth dynamics, seedling vigor, seed morphology, image based projected biomass (Li et al., 2014); hyperspectral imaging is a rapid technique very useful in the identification of plants with altered leaf pigment status recording the complete reflectance spectrum, from ultraviolet up to short-wave infrared, that is attributed to various plant characteristics (Behmann et al., 2018; Mohd Asaari et al., 2018; Cotrozzi and Couture, 2020; Mishra et al., 2020); fluorescence imaging provides a rapid real time non-invasive screening technique to identify plants with altered metabolism and growth, using specific devices (Multiplex and Dualex, Force-A, France) it is possible to monitor different physiological processes estimating compounds such as flavonols, anthocyanins and chlorophyll (Brazaitytė et al., 2015, 2019; Sytar et al., 2015; Zivcak et al., 2017) via specific fluorescence based indices (Flav, Anth, SFR) obtained from different combinations of the red

(RF) and far-red (FRF) fluorescence signals at the various excitation bands (UV-A 370 nm, Blue 460 nm, green 516 nm and Red 637 nm).

Thermal imaging allows the visualization of infrared radiation at the canopy level useful to detect plant temperature differences in stomatal conductance for the quantification of plant responses to water stress and transpiration rate (Munns et al., 2010; Jones et al., 2018). 3D imaging technique measures 3D plant structure parameters quantitatively with active and passive methods (Fiorani and Schurr, 2013; Kazmi et al., 2014; Li et al., 2014).

The interpretation of the sensor data for stress detection is crucial requiring the use of advanced methods of data handling, image analysis and interpretation with specific software and tools (Lobet et al., 2013) to identify physical and biochemical changes to the plant connected with the experimental condition tested.

The environmental and image sensors have the scope of monitoring the environmental conditions aimed at allowing the microgreens' optimal growth and development. A key role in the monitoring of the microgreens' health status will be played by a combination of multiple sensors such as environmental, visible, thermal, hyperspectral and fluorescence able to obtain data from spectral bands and fluorescence signals to allow the identification and discrimination of the effects of the different LED light regimes and conditions on microgreen growth process and physiology trying to find the best results.

The necessity to perform non-destructive analysis of plant stress is growing fast among research activities (Humplík et al., 2015; Danilevicz et al., 2021). Indeed, it allows to perform more than one analysis on the same tissue and makes it possible to gain information without altering the metabolism of the plants (Lichtenthaler et al., 1986; Krause, 1991; Maxwell and Johnson, 2000; Mohammed et al., 2003; Živčák et al., 2014; Sytar et al., 2015; Ngo et al., 2019; Chowdhury et al., 2021). Using indeed a technological instrument that uses light to analyse something that otherwise needs to be destroyed is a new

frontier for physiological analysis. Moreover, this kind of instrument can be used in a remote control that means no alteration of environmental parameters due to the researcher presence in the climatic chamber or open field.

One of the promising instrumentations that could be used is called Multiplex, from Force-A, France.

Multiplex is a portable fluorimeter able to emit precise wavelength thanks to its LED array that exciting internal pigmentations and recording back the fluorescence emitted by them after the excitation can analyse the internal content of chlorophyll, flavonols, anthocyanins and even the nitrogen concentration. It performs over 250 pulsed and synchronized flashes of its LED array in just one second and then internally calculate an amount of 9 fluorimetric indexes based on its internal standard. It has a rechargeable battery and is simple to carry on.

1.9.CubeSat as a platform for plant biology experiments

This work of thesis is based fundamentally on the development of devices and facilities able to simulate the harsh conditions of space environment here on Earth for the on-ground preliminary tests to study the effects on plant materials of hypobaria, microgravity and cosmic radiations for the GreenCube mission.

GreenCube is a mission proposed by ENEA, “La Sapienza” University of Rome and the University of Naples “Federico II”, financed by Italian Space Agency (ASI). It was proposed to join the maiden flight of the VEGA-C rocket by Avio (Colleferro, Italy) for the European Space Agency into Medium Earth Orbit with a 3U CubeSat (Santoni et al., 2020). It was scheduled for mid-2020 but due to the pandemic situation, its launch was delayed by late-2022. The purpose of the GreenCube mission is to assess microgreens growth into a small, controlled environment with remote operational in an outer space environment, characterized by microgravity and galactic cosmic rays coupled with a hypobaric condition.

GreenCube is a 3U (10*10*30cm) CubeSat developed by La Sapienza University of Rome that house a cultivation chamber in which will be cultivated a total of 45 seedlings of *Lepidium sativum L.* on a cultivation substrate suitable for space application that will be watered by a stepping motor syringe directly at substrate level, avoiding leakage. The seedlings will be light by a LEDs array of full-spectrum LEDs and the internal pressure of the cultivation chamber will be 0.5atm aiming to avoid structural problems due to the external vacuum environment of space.

The cultivation chamber has a control and management system that will operate the watering and the illumination of seedlings during the flight and has also a series of sensors to record data from the cultivation.

The species selected for this mission is *Lepidium sativum L.*, a plant species from the Brassicaceae family that had been studied a lot for its phytonutrient contents. Brassicaceae family is well known to have many phenolics and glucosinolates compounds such as antioxidants, anticarcinogenic and also antimicrobial characteristics (Holst and Williamson, 2004; Krumbein et al., 2005; Schreiner et al., 2009). Species from the Brassicaceae family had a good negative correlation between their daily consumption and the onset of cardiovascular and cancerous disease (Huyskens-Keil et al., 2010; Truzzi et al., 2021).

Lepidium sativum L. has been known to have a good quantity and variety of phytochemical compounds suitable for space missions as they have all the characteristics to be a suitable source of nutraceutical components for human health (Ahmad et al., 2021).

2. Aim of the work

This work of thesis aimed to make effective the possibility to expose Garden Cress microgreens to multiple stress conditions simultaneously and to analyse the effects of those stress on plants' growth and development, using a multidisciplinary approach that takes into account their feasibility directly in space. Indeed, those simulated stress are related to the space environment and are Microgravity, Hypobraria and Chronic radiation.

For this purpose, together with traditional morphological analysis, it was used a non-destructive optical instrument for the quantitative measurement of chemical compounds and a new nuclei extraction method for plant flow cytometry analysis.

Another objective was to lay the foundation for a new comprehensive approach to plant space research that responds to the unavoidable presence of the galactic cosmic environmental factors like radiation, microgravity and hypobaria which affect simultaneously the development of plants in space.

To do this it was necessary to deeply understand the functionality of materials and infrastructure that could be used to achieve the goal of having a low-tech system to simulate the space environment. Most of the work was deputed to the research, realization and assembling of several facilities that match the technological level needed.

This work of thesis was also developed as the on-ground experimental analysis to be paired and compared with the real space experimental conditions that will be experienced by microgreens of *Lepidium sativum L.* that will fly onboard the GreenCube CubeSat during its journey in space.

3. Materials & Methods

Hereafter is reported the core part of this work. It was found a great number of engineering, technical, and technological issues, before the biological ones, intending to recreate the stressful condition of outer space here on Earth trying to be as close as possible to a real situation. Thanks to the facilities housed in the ENEA "Casaccia" Research Centre and thanks to the great expertise of technicians and researchers it was possible to manage those peculiar problems. After this, it was possible to perform the biological experiments.

3.1.System design

Since the lack of a replica, and also a proper number of replicas, of the satellite itself, to perform the biological experiments into a reference system, to recreate an experimental system as close as possible to the real GreenCube CubeSat that will fly on the VEGA-C rocket (Avio, Rome, Italy) in its maiden flight toward space, the following choices were taken.

3.1.1. Cultivation container

Greencube Satellite is a 3U CubeSat made of three 1U units (10*10*10 cm) stacked vertically for a dimension of 10*10*30 cm. To recreate the small environment of the Greencube CubeSat and in particular the cultivation volume it was needed a container capable of maintaining the internal pressure of 0.5atm. It has to be as much as possible close to the dimension of the real cultivation pressure chamber. It was needed the possibility to house the 3D printed cultivation basement, a replica of the real CubeSat, in it. It must be transparent to allow light to reach the seedlings and to house a watering system.

Trying to match as much as possible of those constraints, it was selected a commercial off-the-shelf FoodSaver 1.2L vacuum crystal-clear container for food storage (Fig. 3.1.1).

Fig. 3.1.1 Food Saver vacuum container 1.2L

It has the dimension of 12*10*10cm (h*l*d); it is made to maintain 0.5atm pressure inside; it is easily washable; it is made of Tritan (from Eastmann producer) a special plastic polymer that is robust, crystal-clear and durable, it is BFA-free and with a HAZE grade of 0.6, meaning that is quite transparent; the light percentage absorbed by this plastic material is around 1.8% without altering the spectrum shape of the lighting source.

3.1.2. Cultivation plate

Inside the FoodSaver 1.2L Vacuum food container, it was placed a 3D printed replica of the bottom part of the satellite with the housing for the cultivation substrate. This component has a square shape of 8cm side per 1.2cm height (Fig. 3.1.2).

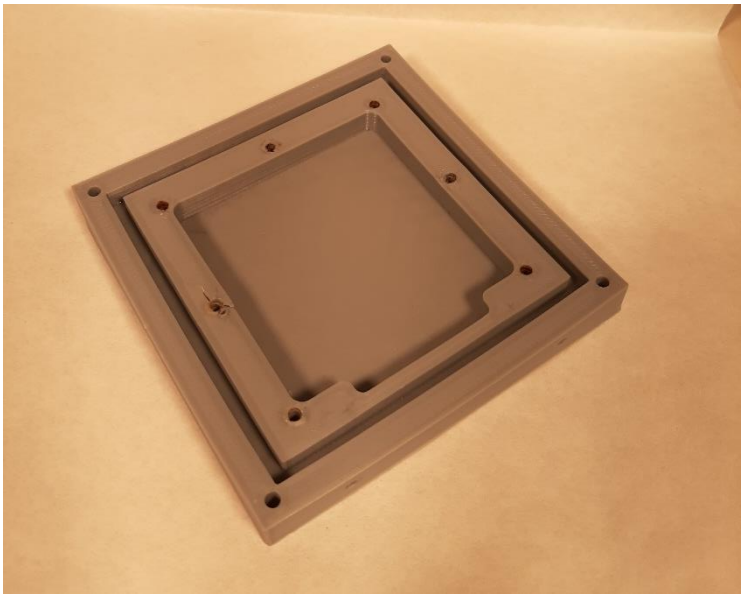

Fig. 3.1.2 3D printed bottom section of GreenCube CubeSat

At the centre of it, there is the place for the cultivation substrate. A plastic grid has been designed to maintain the substrate in place. On this mask, a grid made of fifteen 0.8cm side squares was realized arranged in three by five matrices (Fig. 3.1.4).

The cultivation plate was connected to the bottom of the cultivation container with two Hooks-and-Loops fastener strips. This makes possible the rapid set-up of the experimental trials, it is easy cleaning procedures and the possibility to cultivate using the clinostat in which the cultivation container within the cultivation plate and substrate, were kept in continuous rotation simulating the microgravity.

The cultivation plate and the grid were locked with inox steel screws to avoid the fall of the cultivation substrate during the experimental trials.

3.1.3. Cultivation Substrate

To prevent leakage of liquids and in particular of the nutrient solution during the space flight, was chosen a substrate with great water retention. The *Oasis Floral Foam* is a polyphenolic foam used in horticulture mainly for fresh-cut flowers and has a great imbibition capacity. It is simple to cut in shape. To realize perfectly shaped substrate forms best fitted for the housing in the cultivation plate was designed and 3D printed a plastic mould (Fig. 3.1.3).

Fig. 3.1.3 Oasis Floral Foam and casting mould of substrate

To make it ready for the cultivation cycle the substrate, after the cut-in-shape realization procedure from the main block, was firstly embedded in a 3D test mask and then to allow a better disposition of the seeds in the foam and to favour rooting were realized three pass-through holes for each of the fifteen squares of the grid present on the plastic cover (Fig. 3.1.4).

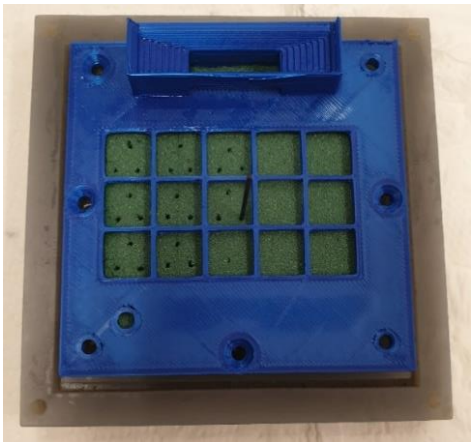

Fig. 3.1.4 Grid and pass-through holes for seeds

This was mandatory to gain the same seeds disposition and helped the seeds to germinate and develop the roots properly.

The maximum water holding capacity of the preformed oasis block was 30 ml.

The substrate was precharged with active charcoal using a suspension of 45mg of active charcoal powder (Sigma) in 30ml of milli-Q water that was poured on the substrate (Fig. 3.1.5).

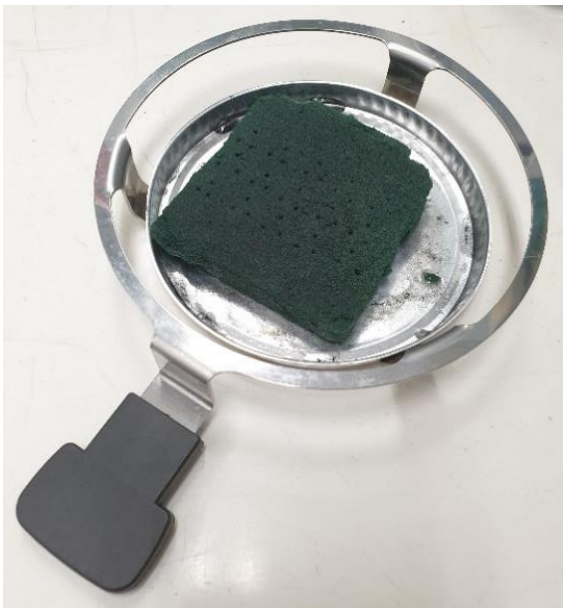

Fig. 3.1.5 Substrate charged with Activated Charcoal

Then it was dried in the oven at 60°C overnight (Fig. 3.1.6).

Fig. 3.1.6 Oven-dried substrate

At the end of the drying process, the substrates were individually into a plastic heat-sealed bag before gamma-ray treatment (Fig. 3.1.7).

Fig. 3.1.7 Substrate stored for gamma sterilization

The sealed plastic bags were gamma-irradiated in the Calliope ^{60}Co irradiation facility at the ENEA "Casaccia" Research Centre with a dose rate of 5kGy/h for 6 hours for a total dose of 30 kGy.

3.1.4. Nutrient Delivery System

The nutrient delivery system inside the CubeSat is made up of a direct-drive stepper motor-driven Luer-lock syringe connected to the irrigation line and controlled by a microcontroller device.
This makes it possible to perform irrigations with a precise and pre-set schedule directly at the substrate surface level. The possibility to replicate this system inside the cultivation containers was hard and it was used an automatic perfusion syringe for medical purposes (Fig. 3.1.8).

Fig. 3.1.8 *Cané* perfusion syringe

Unfortunately, only one syringe was available and finally, single perfusion of 10ml of ¼ strength Hoagland solution (Hoagland and Arnon, 1950) was given at the start of the experiment before closing the cultivation container and

performing the selected stresses. Hoagland solution was made fresh before the beginning of each experimental trial starting from the stock solutions. Distilled sterile water was added to dilute the solution up to a quarter, autoclaved and then filtered by using a 0.2µm syringe sterile filter while pouring on the cultivation substrate with embedded seeds.

The initial quantity of nutrient solution necessary to start adequately the germination process was determined after several preliminary trials (data not shown). 10ml of nutrient solution was enough to support the growth of cress seedlings for seven days inside the cultivation containers. 10ml of nutrient solution were used as irrigation volume for every experimental trials performed.

3.1.5. Light system

The light system is composed of LED strips that mount the same single LEDs that are mounted on the satellite, the LM301H made by Samsung with an 80CRI value and 3500K colour temperature with a characteristics full range spectrum (Fig. 3.1.9).

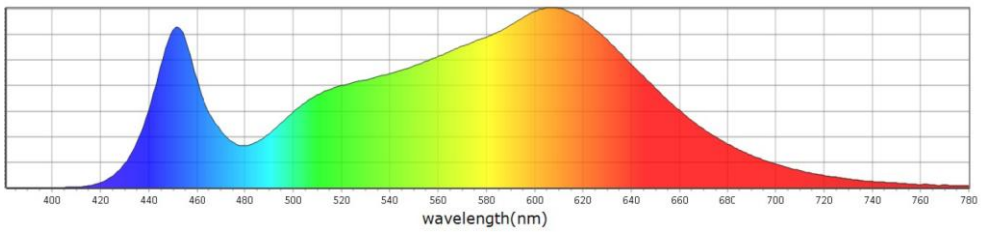

Fig. 3.1.9 LM301H 3500K light spectrum

The lights were custom made by CreScience (Germany) and were attached both to the lighting support system of the clinostat and to the shelf structure used for static experimentation. These two cultivations platforms were both developed and used inside the climatic chamber and into the Calliope ^{60}Co irradiation facility at ENEA "Casaccia" Research Centre to perform static and clinorotated gravity stress while chronically irradiated or not. For the use on the clinostat, a specific upgrade was done to make possible the connection of the electrical device on the rotating shelf while moving. This

was mandatory in these experiments to perfectly replicate the microsatellite conditions in which the light system is solidly stacked with the cultivation plate and is always above it.

More information about the upgraded design of the clinostat is reported in the dedicated section. Light intensity was measured with the spectroradiometer, the UPRTek M350S (The Netherlands) that allows the possibility to check the total PPFD and also the spectrum shape. The light intensity was set at 200µmol/m^2·s with a photoperiod of 12h day/night that was started after 2 days of complete darkness for a total DLI of 8.64mol/m^2·d.

Thanks to the use of crystal-clear containers, it was possible to illuminate the plants while growing, keeping the light system outside the cultivation container and avoiding the deleterious increase in temperature that is hard to modulate during the cultivation process in this model system.

3.2.Species selection

The species for the GreenCube mission was selected by the team from University of Naples "Federico II". It was selected the garden cress, scientifically known as *Lepidium sativum L.*. Garden cress is a dicotyledon species of Brassicaceae family (Table 3.2.1). It is well known for its phytonutrient concentration and medic purposes (Ahmad et al., 2021). They are also characterized by a superficial mucilage that help seeds to absorb water and maintain humidity better (Behrouzian et al., 2014).

	Garden Cress
	Tripartite cotyledonal leaves with a deep green colour. True leaves with long stems.
	Scientific name: *Lepidium sativum L.*
	Days to harvest as microgreens: 9-12 days
	Annual lifespan
	Seeds weight: 1000 seeds as 2.50 g
	Seeds density as microgreens: 1-3 seeds/cm^2

	Taste and flavour: Spicy
	Cultivation challenge: medium
	Optimal growth temperature: 15-24°C

Table 3.2.1 *Lepidium sativum L.* characteristics

3.2.1. Seeds disinfection

For the Greencube mission, the CubeSat itself and seeds inside it may stay months ready on their final configuration inside the rocket waiting for the launch to outer space. It is mandatory to maintain the seeds dormancy and their cleaning till we will link to the flying Greencube CubeSat and start the watering schedule.

To do this, the seeds disinfection procedure aiming to prevent bacterial and fungal spread during the cultivation in space lacks a wet disinfectant like H_2O_2 solution. This is to prevent the possibility of the sprouting phenological stage during the placement of the seeds into the substrate and not when the CubeSat is in its designated orbit.

Fig. 3.2.1 Ozone generatore, 3.5 g/h, *Tommesani*

To disinfect them, finally, it was chosen O_3 gas left flowing through seeds for 15 minutes inside a 50ml Falcon tube (Baskakov et al., 2022). Ozone was produced by Tommesani Ozone producer machine with a flow rate between 3000 and 3500 O_3 mg/h (Fig. 3.2.1).

3.3.Seeds placement

Just before the start of the experimental trials, seeds were externally disinfected with 15 minutes O_3 treatment and then arranged to be placed in the tailormade holes over the substrate. *Lepidium sativum L.* seeds do have not a rounded shape, but instead, have a particular elliptic shape in which is possible to detect the point from which the primary root emerge and that apex is pointed into the substrate. A total of 45 seeds were placed on each substrate. The substrate was then housed inside the cultivation plate and the grid was screwed on it. This cultivation unit made of a cultivation plate, grid, substrate,

and seeds was watered with 10ml nutrient solution previously made a filtered with a 0.2µm sterile filter and placed inside the cultivation container (Fig. 3.3.1).

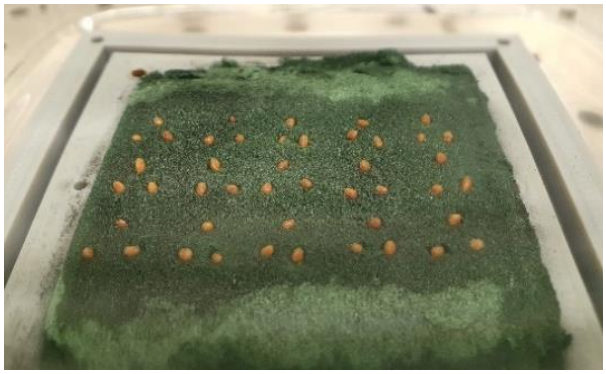

Fig. 3.3.1 Watered seeds ready for experimental trials

All these procedures were performed inside a sterile laminar flux hood before the sealing of the experimental container.

3.4. Experimental design

As already stated, this work of thesis led to the possibility to simulate multiple cosmic environmental factors to analyse the effect of the stresses taken singularly or coupled or even all three simultaneously. Depending on the space environment simulation that was needed, the containers were set at atmospheric or under low pressure, on a static or clinorotated plane and exposed or not to chronic γ irradiation of 0.1 Gy/h inside the Calliope ^{60}Co Irradiation facility at ENEA "Casaccia" Research Center.

To match all these experimental factors, 8 different experimental situations were carried on. All the performed experiments set up with the stress imposed are reported in the following table (Table 3.4.1).

ID	Conditions			ID	Conditions		
C	1atm	Stat	0Gy/h	PG	0.5atm	Clino	0Gy/h
P	0.5atm	Stat	0Gy/h	PR	0.5atm	Stat	0.1Gy/h
G	1atm	Clino	0Gy/h	GR	1atm	Clino	0.1Gy/h
R	1atm	Stat	0.1Gy/h	PGR	0.5atm	Clino	0.1Gy/h

Table 3.4.1 Experimental Conditions ID

Three replicas of each of these experimental conditions were performed.

3.5.Space environment experimental simulation

One of the major challenges of this work was to perform multiple space stress simulations (microgravity, hypobaria, radiations) simultaneously. To achieve this purpose were used several facilities in the ENEA "Casaccia" Research Centre

3.5.1. Simulated Microgravity

To simulate the microgravity effects on plant growth and development during the Greencube space mission a single axis (2D) clinostat was used at ENEA "Casaccia" Research Centre, modified by integrating the lighting system with the rotating plane (Fig. 3.5.1).

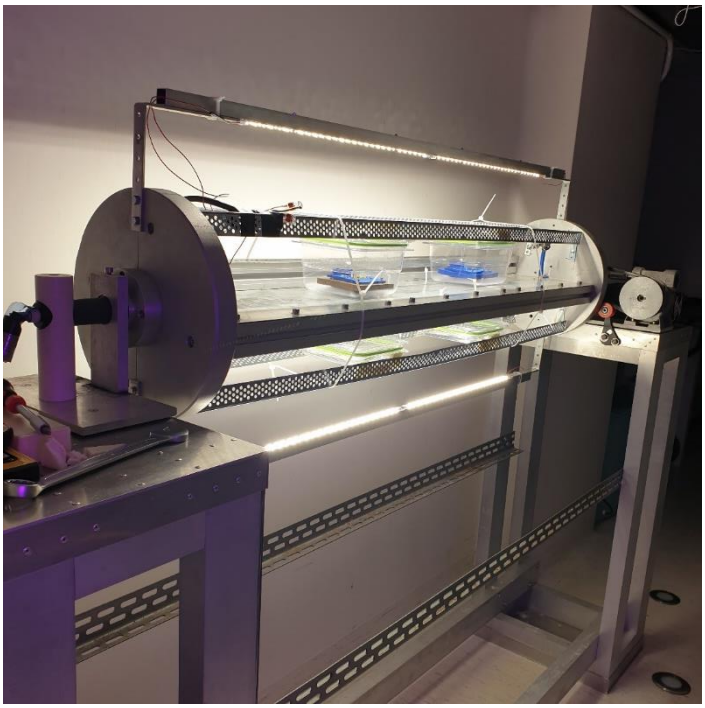

Fig. 3.5.1 Clinostat

To study microgravity effects on plant growth and development during GreenCube spaceflight experiments the light is in solidarity with the cultivation compartment. Cultivation plates (Fig. 3.3.1) were anchored to the cultivation container thanks to the Hooks-and-Loops fastener strips. Cultivation containers were anchored to the cultivation plane with angular aluminium profiles (Fig. 3.5.1).

To know the exact rotation regime (ω; rounds per minute) to simulate microgravity was used the following formula (1):

$$(1)\ \frac{Fc}{g} = \frac{v^2}{r} \times \frac{1}{g}$$

by replacing the velocity *v* with the following equation (2):

$$(2)\ v = 2\pi r\left(\frac{\omega}{60}\right) = \left(\frac{\pi}{30}\right) r\omega$$

The resulting formula is (3):

$$(3)\ \frac{\mathrm{Fc}}{\mathrm{g}} = \frac{\left(\frac{\pi}{30}\right)^2 \times \mathrm{r}^2}{\mathrm{r}} \times \frac{\omega^2}{9.81}$$

Now the formula (4) depends on the round per minute (ω) and by the distance from the rotation axes (*r*)

$$(4)\ \frac{Fc}{g} = 1.12 \times 10^{-3} r\omega^2$$

By replacing the *r* value with 0.01 m (distance of the plant from the rotation axis) and Fc/g with 10^{-5} the ω value is 1.5 rpm (van Loon, 2007).

The static counterpart experimental trials, among which there was the control sample, were performed on a stand provided with the same light system, realized with 3500K 80CRI CreScience FluxStrips.

3.5.2. Hypobaria

To test hypobaria effects on plants during GreenCube spaceflight experiments were used FoodSaver 1.2L vacuum food storage containers. This system has a sealing gasket that avoids the gain of pressure when the pre-calibrated vacuum pump brings the internal pressure to 0.5 atm.

The containers were able to maintain hypobaric conditions for the complete duration of the experiments. As a control, the cultivation plate was closed inside the FoodSaver 1.2L containers without vacuum.

The substrate with seeds was watered with 10 ml of nutrient solution just before the experiment start.

3.5.3. Chronic irradiation

To test the effects on plants of low chronic low dose gamma rays, the cultivation containers were brought into the Calliope ^{60}Co irradiation facility in ENEA "Casaccia" Research Centre.

This irradiation facility produces γ radiations from a ^{60}Co radioactive source. The facility, developed in 1967 was realized for the irradiation of big experimental prototypes and agriculture research purposes. In Fig. 3.5., below, is reported the place in the Calliope facility where were placed the samples during the experimental trials.

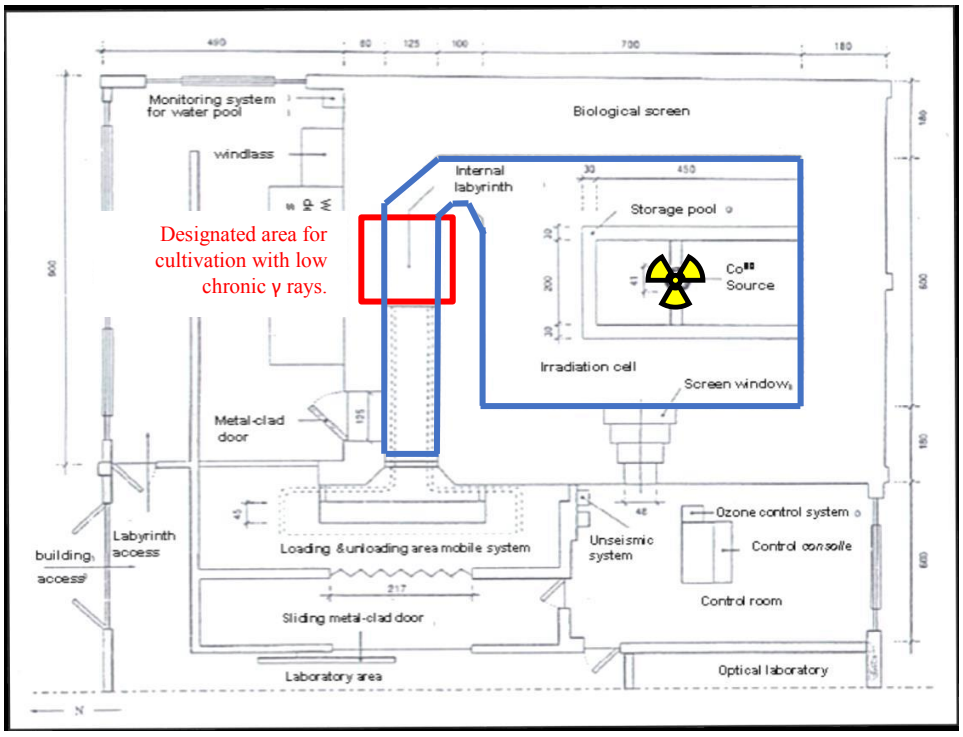

Fig. 3.5.2 Calliope ^{60}Co γ irradiation facility planimetry.
Blue line is the internal border of the radiation dome.

The ^{60}Co Calliope irradiation facilities laboratory head responsible and her team found a spot in which the radiation dose rate is 0.1Gy/h. This dosage was chosen based on the experimental findings that report the experience of a recoverable satellite launched in 2004 in which they calculate an equivalent dose for damages found on plants of about 50Gy of γ radiation for the total duration of the experiment that lasted for 18 days in space (Wei et al., 2006).

To do this, it was used Fricke's absolute dosimeter to precisely measure the intensity of γ rays and so the dose rate that then will be experienced by the plants.

After the shielding of the electrical components like the Meanwell of the LED lights and the DC brushless motor for the movement of the clinostat, with Lead blocks, it was possible to expose the samples to a total dose of about 16Gy during the 7 days of experiment (0.1Gy/h for 24h per day, during 7days experimentation timing).

The total radiation dose taken by the microgreens for these experimental trials was about 16Gy.

3.6. On ground stress analysis

After 7 days of experimentation, samples were collected, and measurements performed.

Measurements were performed just after the conclusion of the experimental procedures. Hereafter are presented instrumentations and techniques used to perform the measurements.

3.6.1. Morphometrical analysis

To evaluate the effect of simulated space stresses on microgreens, as well as to analyse the control treatment, for each sample were recorded the total and cotyledonal fresh weight, while the total dry weight was analysed on 20 seedlings using an analytical balance (Radwag AS 220.X2) and a Thermobalance (Radwag MA 50.X2.IC.A)

The height of the hypocotyl was recorded using a digital calliper (DIGI-MAX-Sigma) and the cotyledonal area was measured with the AM350 portable Leaf Area Meter (ADC Bioscientific Ltd. Hoddesdon UK).

3.6.2. Fluorimetric analysis

Fluorimetric analyses were performed on the adaxial side of the cotyledon using the portable fluorometer Multiplex (A-Force, Orsay, France; Fig. 3.6.1).

Fig. 3.6.1 Multiplex 3A, Force-A, French

Multiplex 3A from ForceA (Orsay, France) is a hand-held fluorimeter that mounts four excitation light sources: UV at 373nm, Blue at 470nm, Green at 516nm and Red at 635nm.; and three photoreceptors: Yellow (590nm), Red (685nm) and Far-red (735nm) (Fig. 3.6.2; Fig. 3.6.2).

It uses the fluorescence re-emitted after excitation to measure indexes that are correlated with the content of various plant pigments.

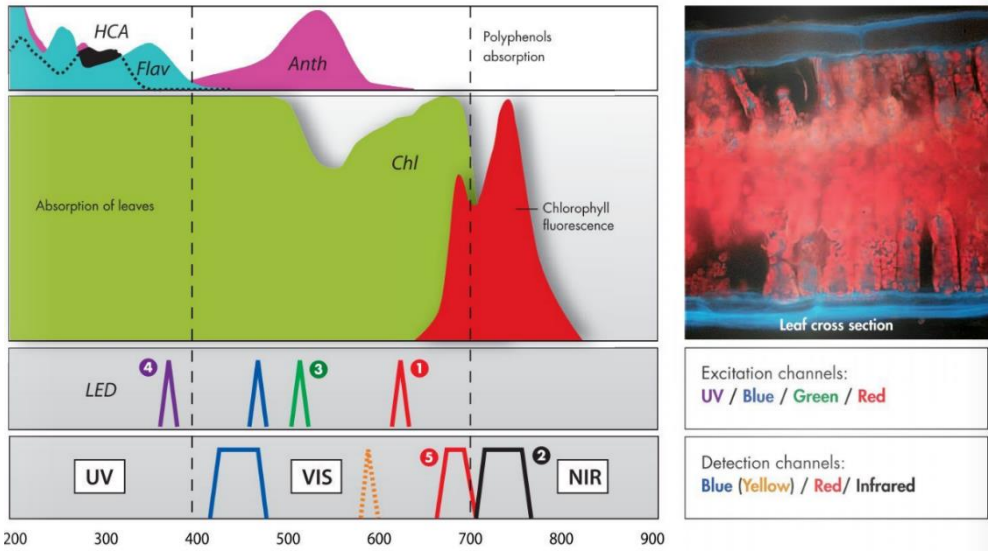

Fig. 3.6.2 Multiplex 3A excitation and detection channels

Multiplex performs up to 250 synchronized flashes per second of the emissions sources per each sample analysis and released a series of nine fluorescent indexes that can be used to compare samples of the same species among different treatments (Gitelson et al., 1999).

Multiplex optical analysis was performed for each sample, and so for each of the microgreens grown during the experimental trials on the adaxial face of the leaves. A great quantity of data had to be analysed and a series of hypotheses were done after data processing work.

It was considered the use of these Multiplex Indexes:

1- SFR_R is related to Chlorophyll's content (Lichtenthaler et al., 1986; Buschmann, 2007)
2- ANTH_RB is related to Anthocyanins' content (Agati et al., 2005)
3- FLAV is related to Flavonols' content (Ounis et al., 2001; Cerovic et al., 2002)
4- NBI_R is related to the Metabolism index (primary vs secondary) (Cartelat et al., 2005; Agati et al., 2013)

These indexes were valued for only the adaxial faces. This choice was made to prove the possibility to use this new kind of analysis in remote conditions in which it will be not possible to analyse the abaxial face of the leaves.

With this piece of work, it is proposed the use of this instrument for plant space applications.

3.6.3. Spectrophotometric measurement of Chlorophyll and Anthocyanin content

To correlate fluorometric qualitative analysis with quantitative analysis, the cotyledonal leaves from 10 samples from each experimental trial were collected and used for cotyledonal pigments extractions; three replicas for each experimental trial were performed.

Extraction procedures

Cotyledons were weighed, collected into 2ml Eppendorf tubes and cryopreserved into liquid nitrogen. At the end of the collection phase cotyledons were rapidly grounded with pestle and 0.5 ml of methanol was added in suspension and the tubes were incubated on ice for 30 minutes with gentle agitation, then 0.5ml of methanol was added again to each tube and incubated on ice for 30 minutes with agitation. After this, tubes were centrifuged at 10000 rpm at 4°C for 10 minutes and the supernatant was collected in another tube.

For the spectrophotometric measurement of Chlorophyll content 100µL of the supernatant were added to 900µL of methanol into a photometric cuvette and then the absorbance was read at 652 nm and 665 nm as stated in the procedure of Sumanta et al. (2014)(Sumanta et al., 2014)For the determination of Chlorophyll content were used the following formulas :

$$ChlA = \frac{(16.52 * A_{665} - 9.16 * A_{652}) * dF * Vf}{CA}$$

$$ChlB = \frac{(34.09 * A_{652} - 15.28 * A_{665}) * dF * Vf}{CA}$$

$$Chl_{tot} = ChlA + ChlB$$

Where,

- A_{665} is the absorbance at 665nm
- A_{652} is the absorbance at 652nm
- dF is th diluition factor
- Vf is the final volume
- CA is the cotyledon area

The same extract was used for chlorophyll and anthocyanins concentration evaluation

To measure the anthocyanins concentration, 1µL of HCl 36% was added at 100 µL of supernatant and then mixed with 900 µL of methanol. Anthocyanin

spectrophotometric measurement was performed reading the solution absorbance at 530nm and then applying the following formula.

$$Anth = \frac{(485/34300) * A_{530} * dF * Vf}{CA}$$

Where,

- A_{530} is the absorbance at 530nm
- dF is the diluition factor
- Vf is the final volume
- CA is the cotyledonal area

3.6.4. Flow Cytometry

Onboard the International Space Station is housed a flow cytometer, the MICROFLOW1 by INO (Fig. 3.6.3), it is usually used to perform clinical analysis of body fluids of crew members (Cohen et al., 2008, 2011; Dubeau-Laramée et al., 2014; Xun et al., 2018; Amalfitano et al., 2020).

Fig. 3.6.3 Microflow1 by INO

This cytometer could be used to perform plant flow cytometry analysis to investigate many aspects of the effect of the space environment on vegetable cells.

Plant flow cytometry could be used to analyse the effects of stress on plants evaluating changes on plant ploidy and cell cycle as well as chromosomal differences with specific Fluorescent In Situ Hybridization techniques (Lucretti et al., 2014).

In this thesis, the cell cycle and the ploidy level of plant nuclei were investigated by flow cytometry after exposure to all the "simulated space stresses" (alone or in combinations) as previously described

Endoreduplication of nuclei was estimated calculating two indexes, the Mean C-Level and the Cycle Value (Barow and Jovtchev, 2007; Pellicer et al., 2021).

The first is calculated as the ratio between the sum of the multiplied quantity of nuclei by their N-level, divided by the total sum of the quantities of the nuclei in the different ploidy levels (following equation).

$$\frac{[(2 * n_{2C}) + (4 * n_{4C}) + (8 * n_{8C}) + (16 * n_{16C}) + (n * n_{nC})]}{(n_{2C} + n_{4C} + n_{8C} + n_{16C} + n_{nC})}$$

Where n_{2C}, n_{4C}, … are the quantity of the nuclei collected in the specific N-level.

The second is calculated as the ratio between the sum of the quantity of the nuclei in the specific N-level multiplied by a progressive number starting from 0, and by the total sum of the quantities of the nuclei at different ploidy levels.

$$\frac{[(0 * n_{2C}) + (1 * n_{4C}) + (2 * n_{8C}) + (3 * n_{16C}) + (n * n_{nC})]}{(n_{2C} + n_{4C} + n_{8C} + n_{16C} + n_{nC})}$$

Where n_{2C}, n_{4C}, … are the quantity of the nuclei collected in the specific N-level.

Another index used, was calculated based on Zedek et al. 2016 (Zedek et al., 2016)z. This Index is used to highlight the difference between the number of nuclei in the G2/M phase and in the G1/S phase. This index is known as RelG2

$$RelG2 = \frac{n_{G2/M}}{n_{G1/S}}$$

Where $n_{G2/M}$ and $n_{G1/S}$ are the quantity of nuclei collected in G2/M phase and G1/S phase respectively.

In this work, it was not used a biological internal standard as in space this very biological standard will be affected as well as the experimental samples. It is wanted to point out the necessity of developing a new method to compare the plant flow cytometry measurements avoiding the use of internal biological standards. It was proposed the use of fluorescent beads to be used as a standard since the new cytometers, like the one it was used, lack many alignment problems suffered by previous analogical instruments. With these new facilities, it will be preferable to find a new form to compare studies from different labs.

The Well-Method

Aiming to push over the study of plants in space it was developed and tested a new solid method of nuclei extraction from plant tissues to speed up flow cytometric analysis that could be used in microgravity conditions.

Using the CytoflexS from Beckman Coulter (California, USA; Fig. 3.6.4) with 96well plate reader option it was possible to perform analysis of a great number of samples.

Fig. 3.6.4 CytoflexS Flow Cytometer from Beckman Coulter with 96 wells plate reader option

Such a method could be even used in microgravity conditions and could push over the study of plants' cell cycle behaviour in space.

Up to now the most used techniques of plant nuclei extraction for flowcytometry is the chopping method. It was refined by Galbraith in 1983 (Galbraith et al., 1983) and is used for quite every kind of cytometric analysis on plant tissues that need nuclei isolation and suspension.

The well-method is briefly composed by the following steps:

1- Choose the plant tissue, even at the level of the single root tip
2- Collection of the plant tissue into a well of the multiwell plate
3- Addition of the buffer solution
4- Homogenization of the samples directly into the well using an ultraturrax probe
5- Addition of the fluorescent stain
6- Flow cytometric analysis

The pros of this new method are the possibility to fast perform a large number of analysis at a single plant level; the amount of sample needed is very small (even less than 1mg), the lack of filtering of the nuclei suspension before running the analysis, and the extremely fast procedure of sample processing.

The CytoflexS, a new technology flow cytometer that fortunately lacks many fluidics problems and allow to perform analysis of cloudy kind of samples, has also a multi-well reader option that allows to fast perform hundreds (max 96 at a time) of analyses per day and can stir the sample-well before the reading, avoiding the sedimentation of nuclei and debris.

This new method, briefly described, was used to extract the nuclei from plant tissues, in particular root apex and cotyledon, to analyse their ploidy level after the cultivation process in all the eight experimental trials presented in this work of thesis.

Three replicas of ten whole microgreens samples for each experimental trial were collected and fixed by incubation for 15 minutes into a 25ml solution of TRIS 1X buffer (Galbraith et al., 2021; Loureiro et al., 2021) containing 1% formaldehyde. Then, samples were drained and rinsed 3 times for 5 minutes into a 25ml solution made only of TRIS 1X buffer. After these steps, from each of the ten samples were collected 10 root tips and 10 cotyledonary leaves, and put separately in the wells.

100µL of LB01 buffer solution (Doležel et al., 1989) with DAPI at 2µg/mL were added per well. Homogenization was performed in each well for 13 seconds at 7000rpm with a MiniTURRAX mounting a T5 probe that perfectly fit into the well. The probe was washed into deionized water and then dried on lab paper every time between a well and the next. After this, further 100µL of LB01 containing the dye were added to each well. The quality of the isolated nuclei was ensured by a rapid sight at fluorescent microscope (Nikon NIKON ECLIPSE TE 2000 S) provided with a UV ext filter.

In each sample, 2-3 µL of a suspension of UV alignment beads (AlignFlow UV standard, ThemoFisher, USA) was added as an internal standard at the density of 2x 10*4 beads/ml.

Data recorded with Cytoflex S were directly analysed by Cytexpert 2.3, gating and exporting statistical data that were organized using Excel and successively processed by using Graph Pad Prism 9.

3.6.5. Metabolomics

For both Polar and Semi-Polar metabolites detection, 15 seedling, with three replicas for sample, were collected into a 50ml Falcon tube frozen in liquid nitrogen and then transferred at -80°C until the analysis. Prior to the analysis, 10 mg for Polar and 3 mg for Semi-Polar fractions of freeze-dried leaves powder from each sample were weighed and extracted with 0.75 mL cold 75% (v/v) methanol with for 1 mg/l daidzein as internal standard for Polar metabolites, as described (Dono et al., 2020); and with 0.25 mL cold 100% (v/v), 1 mL of chloroform spiked with 5 mg/l α-tocopherol acetate as internal standard and 0.25 mL 50 mM Tris buffer (pH 7.5, containing 1 M NaCl) (Dono et al., 2020) for Semi-Polar. Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) conditions were as previously reported for, respectively, Polar (Dono et al., 2022) and Semi-Polar (Frusciante et al., 2022) metabolomes. The identification process were validated comparing chromatographic and spectral properties with authentic standards (when available) and reference spectra, in house database, literature data, and based on the m/z accurate masses, as reported in the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic mass identification, or on the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/), in the case of adduction detection. Quantification of each metabolite was carried by calculating the relative contents to the daidzein (Polar) and α-tocopherol acetate (Semi-Polar) internal standard levels. MS chromatograms were subjected to untargeted metabolomics analysis using the software SIEVE (Thermo Fisher Scientific). Data recorded

were organized using Excel and successively processed by using Graph Pad Prism 9 for statistical analysis.

3.6.6. Statistical analysis

GraphPad Prism 9 (GraphPad Software, San Diego, CA) software was used to perform statistical analysis on the data obtained from the experiments collected and processed with Microsoft Excel. To analyse multiple stress effects, a three-way ANOVA was performed for each of the measured parameters followed by pairwise multiple comparisons adopting the Tukey's test All the results from the statistical analysis are presented as mean ± SE (Standard Error). SE was calculated as $SE = \frac{\sigma}{\sqrt{n}}$ where σ is the Standard Deviation and *n* is the population number.

4. Results

Fundamental for this study and for the Greencube project/mission was the reconstruction here on earth of the space environment to study the simultaneous effects of multiple stresses such as Microgravity, Hypobaria and Radiations on growth and development of Cress microgreens. To reach this objective was necessary where possible to use commercial off-the-shelf (COTS) devices such as the Foodsaver containers and to realize alternatively specific devices such as the 2D clinostat used during the experimental trials (Fig. 3.5.1). This prototype was developed to support the simulated microgravity experimentations thanks to the integrated lighting plant. This system is lightweight and easily transportable. It is best suited for walk-in growth chambers and also for the CALLIOPE ^{60}Co Irradiation facility at ENEA "Casaccia" Research Centre (Fig. 1.7.1; Fig. 3.5.2). For the seeds disinfection was mandatory to avoid earlier germination. The use of O_3 as a disinfectant was a success since inside the cultivation container the presence of mould and fungi wasn't revealed, and seeds can remain dry waiting to reach the orbit.

In the following sections are reported the results from morphometric, fluorometric, flow cytometry, metabolomics and about the possibility to use the multiplex in space as an alternative to the ground traditional chemical analyses.

4.1. Morphometric analysis

Data recorded with the instrumentations were collected into a spreadsheet of Excel 365 software from Microsoft® and then was used the GraphPad Prism 9 statistical analysis software to perform a Three-Way ANOVA to evaluate the presence of driving stress and then it was performed multiple comparisons between the control sample and any other stress condition. These two procedures helped us to evaluate the effect of the stresses taken singularly or simultaneously on the growth of *Lepidium sativum L.* microgreens.

4.1.1. Seedling's fresh weight

In Table 4.1.1 are reported the results of the Three-way ANOVA statistical analysis for *Fresh Weight* measurements. Even if there is the presence of a main factor, in particular *Gravity* whose effect on total variance is statistically significant, the presence of the interactions means that the stresses affect the variance of the measurements by their synergic and antagonistic influences. Being statistically significant the interaction among all the stress is difficult to attribute the final effect on *Fresh Weight* to one of them alone.

Morphometry – Fresh Weight	*%Var*	*P value*
Pressure	0.02	ns
Gravity	7.49	****
Radiation	2.80	ns
Pressure x Gravity	7.70	****
Pressure x Radiation	2.38	****
Gravity x Radiation	1.08	ns
Pressure x Gravity x Radiation	4.33	****

Table 4.1.1 Three-Way ANOVA results for Fresh Weight. ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

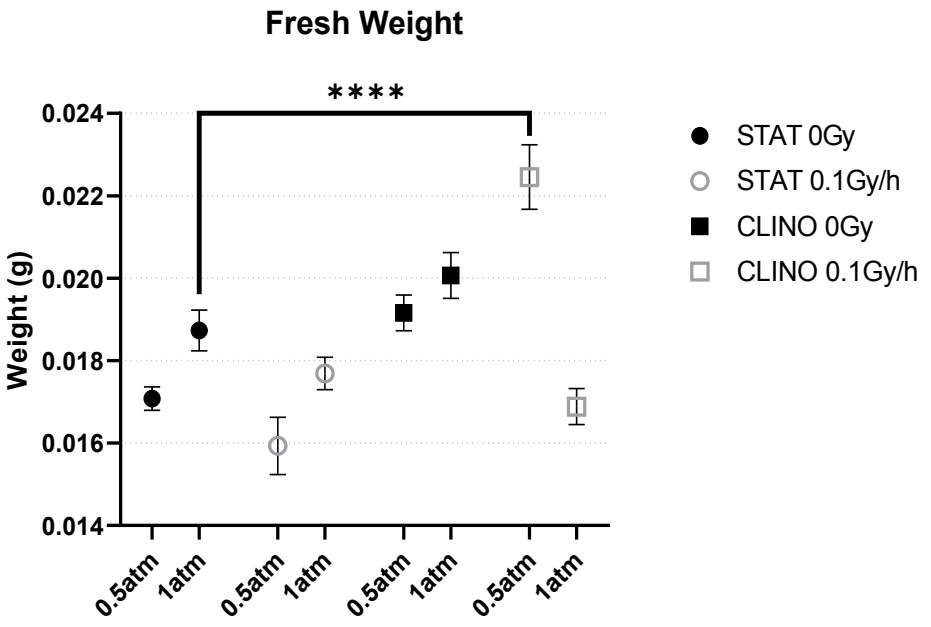

Fig. 4.1.1 Fresh Weight. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Graphically visualizing the data of the *Fresh Weight* in their extended form, expressed as mean ± SEM in Fig. 4.1.1 above, the lowest values were recorded for *PR* samples that were cultivated in hypobaric conditions while irradiated and the highest values were recorded for *GreenCube* simulated conditions. The only significant difference is between the control samples and the *GreenCube* ones.

4.1.2. Cotyledonal fresh weight

In Table 4.1.2 below, are reported the results of the Three-way ANOVA for *Cotyledonal Weight* measurements. Even if there is the presence of the main factor, in particular *Radiation* whose effect on total variance is statistically significant, the presence of the interactions means that the stresses affect the variance of the measurements by their synergic and antagonistic influences. The antagonistic interaction among *Pressure X Gravity* affects the final result of *Cotyledonal Weight* alternating a positive effect on measurements once for 1atm samples and once for 0.5atm samples when cultivated in Static or Clinorotated condition.

Morphometry – Cotyledonal Weight	*%Var*	*P value*
Pressure	0.00	ns
Gravity	1.64	ns
Radiation	11.27	***
Pressure x Gravity	9.90	****
Pressure x Radiation	0.28	ns
Gravity x Radiation	0.99	*
Pressure x Gravity x Radiation	1.84	ns

Table 4.1.2 Three-Way ANOVA results for Cotyledonal Weight. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

In the framework of the Three-Way ANOVA, the test was followed by multiple comparisons using Tukey,s test for mean differences evaluation.

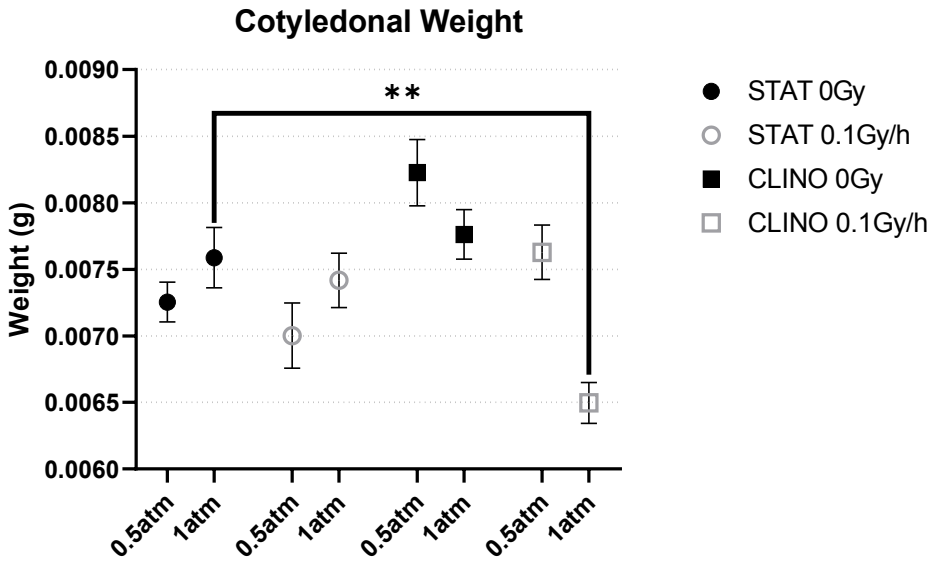

Fig. 4.1.2 Cotyledonal Weight; P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Differently visualizing the data of the *Cotyledonal Weight*, expressed as mean ± SEM in Fig. 4.1.2 above, the highest value was recorded for *PG* samples that were cultivated in hypobaric conditions while clinorotated. The lowest value, instead, was recorded for *PGR* samples that were clinorotated while irradiated. This condition results significantly different from the control samples. This same condition whose also one with the lowest values for *Fresh Weight*.

4.1.3. Cotyledonal Area

In Table 4.1.3 below, are reported the results of the Three-way ANOVA for *Cotyledonal Area* measurements. For this parameter, *Pressure* and *Radiation* stress affect the total variance of the measurements resulting in a positive effect of lower pressure on cotyledonal surface expansion. The interaction of *Gravity X Radiation* results as the major interaction that affects the total variance with an antagonistic effect between the measurements in which these two stress vary. By the way, the presence of a significant interaction between all the stress makes difficult to attribute the final results to a single stress.

Morphometry – Cotyledonal Area	*%Var*	*P value*
Pressure	1.31	**
Gravity	2.87	ns
Radiation	0.55	****
Pressure x Gravity	0.25	ns
Pressure x Radiation	1.16	ns
Gravity x Radiation	11.69	****
Pressure x Gravity x Radiation	5.82	*

Table 4.1.3 Three-Way ANOVA results for Cotyledonal Area. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

In the framework of the Three-Way ANOVA, multiple comparisons using Tukey's test for mean differences evaluation was performed.

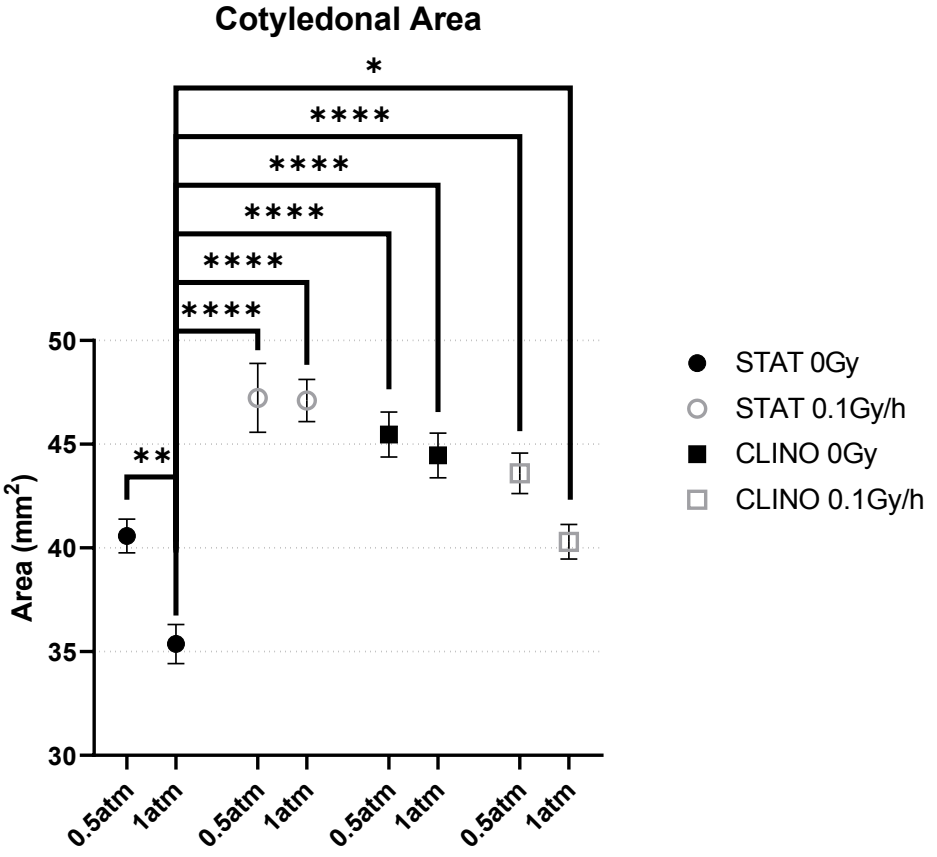

Fig. 4.1.3 Cotyledonal Area. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As it is possible to see in Fig. 4.1.3 above, which reports *Cotyledonal Area* expressed as mean ± SEM, the lowest value was recorded for the control

condition which is significantly different from all the others conditions marking positive effects of the stress on these measured parameters.

4.1.4. Hypocotyl Height

The results of the Three-way ANOVA for *Hypocotyl Height* measurements are reported in Table 4.1.4 below. *Pressure X Radiation* stress affects the total variance of the measurements resulting in values that diverge at 1atm while converging at 0.5atm. The interaction of all the stress together makes it difficult to find the main effect due to one stress only.

Morphometry – Hypocotyl Height	*%Var*	*P value*
Pressure	0.48	ns
Gravity	2.06	****
Radiation	16.95	****
Pressure x Gravity	0.10	ns
Pressure x Radiation	3.27	****
Gravity x Radiation	0.41	ns
Pressure x Gravity x Radiation	0.45	*

Table 4.1.4 Three-Way ANOVA results for Hypocotyl Height. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

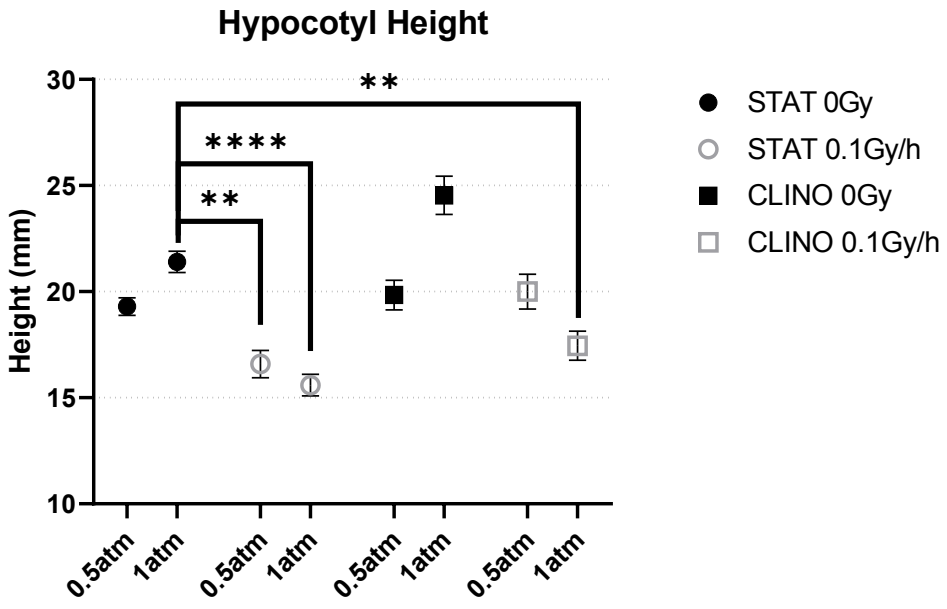

Fig. 4.1.4 Hypocotyl Height. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Fig. 4.1.4, above, reports the statistically significant differences between the control condition and the other ones. In particular, it was found that *Radiation* decreases the hypocotyl height of PR, R and GR conditions which result significantly different in respect to the control condition.

4.1.5. Seedling's dry weight

Looking at Table 4.1.5 below, in which are reported the results of the Three-way ANOVA for Microgreens *Dry Weight* it is possible to find two main effect given by *Pressure* and *Gravity* stress that mainly drives the variance of the samples mean.

Morphometry – Dry Weight	*%Var*	*P value*
Pressure	21.53	****
Gravity	4.28	**
Radiation	3.66	ns
Pressure x Gravity	0.08	ns
Pressure x Radiation	0.66	ns
Gravity x Radiation	0.18	ns
Pressure x Gravity x Radiation	0.81	ns

Table 4.1.5 Three-Way ANOVA results for Dry Weight. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

In the framework of the Three-Way ANOVA, the test was followed by multiple comparisons using Tukey's test for mean differences evaluation.

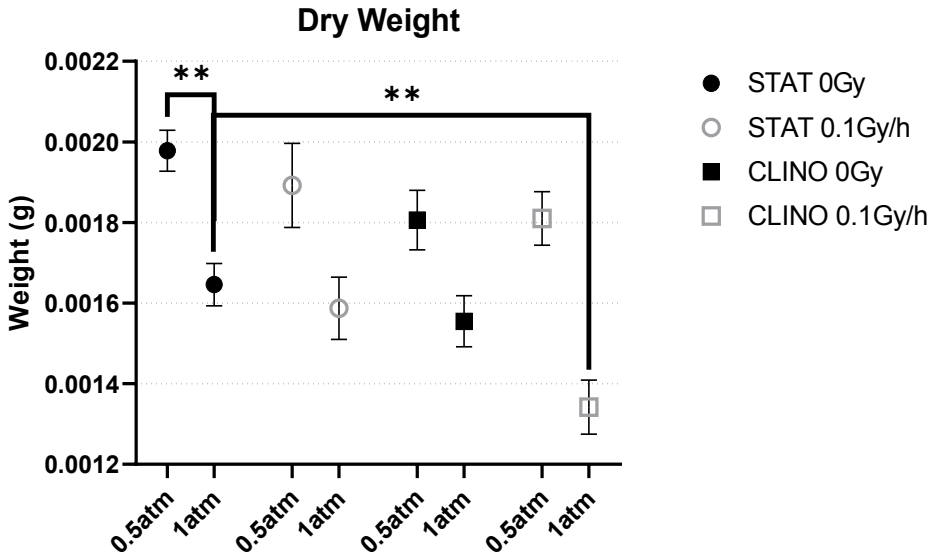

Fig. 4.1.5 Dry Weight. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

In Fig. 4.1.5 above, seedlings' dry weight is reported as mean ± SEM. In particular, it is possible to highlight a positive effect of the combination of lower pressure and static condition that is completely in contrast with the opposite combination which is atmospheric pressure during clinorotation. Chronic exposure to γ rays results in lower values for both static and clinorotated conditions. With respect to the control, it is possible to find significant differences with P and GR conditions.

4.2. Fluorometric analysis

Data collected with the Multiplex instrument were arranged by using Excel 365 software from Microsoft® and then was used the GraphPad Prism 9 statistical analysis software to perform a Three-Way ANOVA to evaluate the presence of driving stress. Successively it was performed multiple comparisons between the control condition and any other stressed condition. These two procedures helped to evaluate the effect of the stresses taken singularly or simultaneously on the growth of *Lepidium sativum L.* microgreens with respect to their chlorophyll, flavonols and anthocyanins content recorded as reflectance indexes.

4.2.1. SFR_R

The Simple Fluorescent Ratio excited with RED was analysed for only the adaxial side of the cotyledon.

This index is proportional to the chlorophyll content inside the leaf epidermis (Buschmann, 2007) and so it could be used to evaluate the metabolic status of plants at the time of analysis.

Fluorometry – SFR_R	*%Var*	*P value*
Pressure	0.79	***
Gravity	2.45	**
Radiation	1.59	ns
Pressure x Gravity	7.48	****
Pressure x Radiation	0.57	*
Gravity x Radiation	1.27	**
Pressure x Gravity x Radiation	8.54	****

Table 4.2.1 Three-Way ANOVA results for SFR_R. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As it is possible to see in Table 4.2.1, SFR_R index variance was driven by the interaction between all the stress making it difficult to assign the effect on variance to only one of the stresses.

As it is done for the morphometrical analysis, hereafter will be analysed the comparison between the control sample and the other stressed samples.

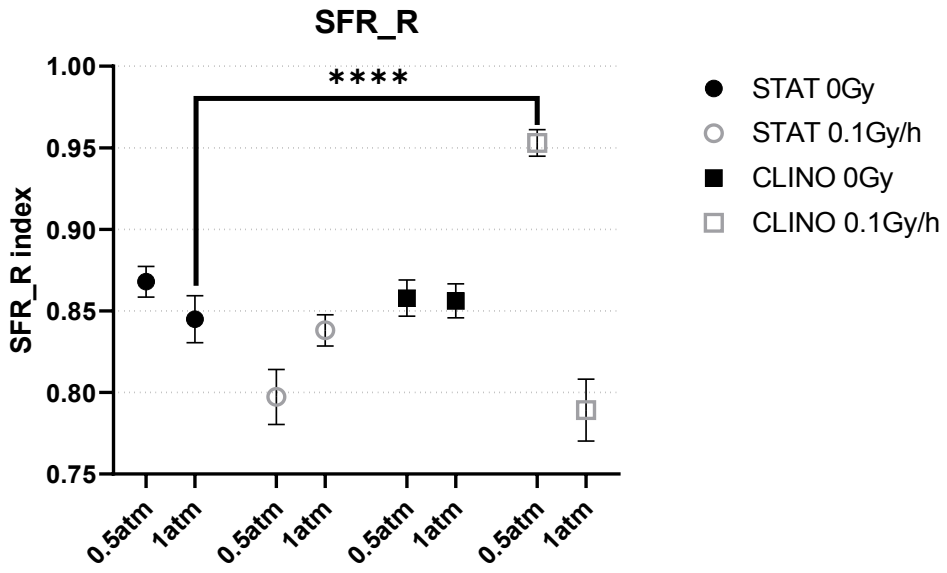

Fig. 4.2.1 SFR_R. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As chlorophyll content in the leaves is proportional to the SFR_R index, it is possible to use this non-dimensional index for plants of the same species, in the same phenological status, and that receive different treatments to compare the effect of those treatments on their internal chlorophyll content.

Data of the *SFR_R* index expressed as mean ± SEM in Fig. 4.2.1 above. The lowest values were recorded for samples cultivated at 0.5atm and chronically irradiated while the highest values were recorded for the GreenCube condition that either resulted significantly different from the control condition and also underline a similarity with the *Fresh weight*.

4.2.2. FLAV

FLAV index was recorded with the use of the Multiplex on the adaxial face of the cotyledonal leaves and it was analysed the index values collected from the analysis of the adaxial face only.

This index is proportional to the flavonols content inside the leaf epidermis (Ounis et al., 2001; Cerovic et al., 2002)and so it can be used to evaluate the metabolic status of plants at the time of analysis.

Fluorometry – FLAV	*%Var*	*P value*
Pressure	0.55	ns
Gravity	10.18	**
Radiation	4.88	***
Pressure x Gravity	0.34	ns
Pressure x Radiation	0.75	*
Gravity x Radiation	20.80	****
Pressure x Gravity x Radiation	0.94	ns

Table 4.2.2 Three-Way ANOVA results for FLAV. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As it is possible to see in Table 4.2.2 the interaction of *Gravity X Radiation* was the principal cause of the total variance of the FLAV index values.

As it is done for the presentation of the morphometrical data, hereafter will be presented the comparisons between the control experiment and all the other experiments in which the stress factors were imposed singularly, coupled or simultaneously.

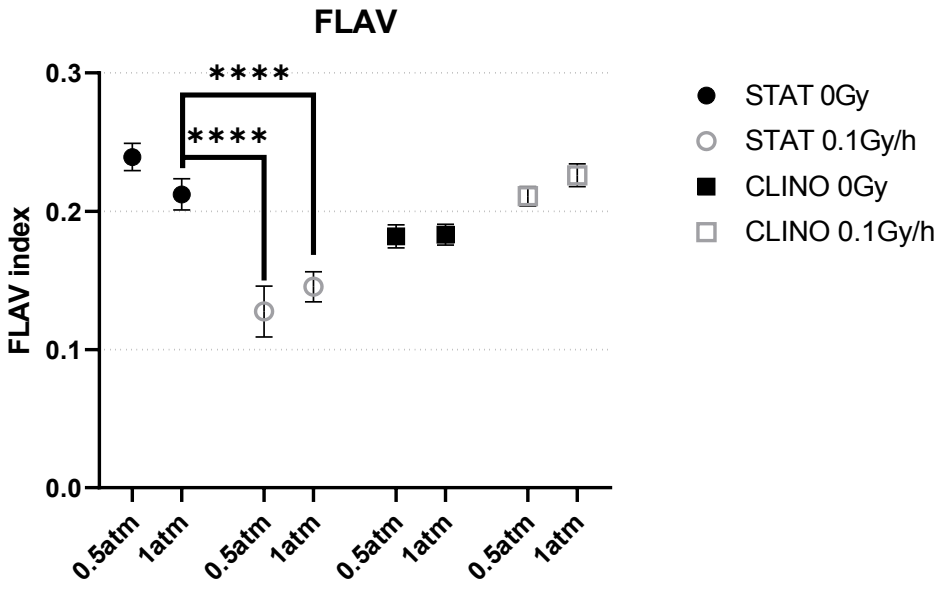

Fig. 4.2.2 FLAV. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As already mentioned, the FLAV index is proportional to the internal content of flavonols in the leaves' epidermis, so it is possible to use this non-dimensional index to analyse the effect of stress on plants of the same species, in the same phenological status and that receive different treatments.

Graphically visualizing the data of the FLAV index expressed as mean ± SEM in Fig. 4.2. above, the highest value was recorded for samples that were simply cultivated at 0.5atm, while the lowest values were recorded for samples cultivated in static conditions in Calliope 60Co facility and so, chronically irradiated and in particular the major detrimental effect was recorded for samples cultivated in hypobaric condition. These two conditions result statistically significantly different from the control.

4.2.3. ANTH_RB

ANTH_RB index was recorded with the use of the Multiplex on the adaxial face of the cotyledonal leaf and it was analysed the index values recorded on the adaxial face.

This index is proportional to the anthocyanins content inside the leaves' epidermis (Agati et al., 2007; Tuccio et al., 2011) and so it can be used to evaluate the metabolic status of plants at the time of analysis.

Fluorometry – ANTH_RB	*%Var*	*P value*
Pressure	0.37	ns
Gravity	28.70	****
Radiation	5.79	****
Pressure x Gravity	0.72	ns
Pressure x Radiation	0.11	ns
Gravity x Radiation	0.00	ns
Pressure x Gravity x Radiation	3.89	***

Table 4.2.3 Three-Way ANOVA results for ANTH_RB. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As it is possible to see in Table 4.2.3, the major effect is attested to *Gravity* stress but there is also the interaction of all the three stress together that influence mutually the total variance of the measurements. This means that

ANTH_RB index values were influenced by the synergic and antagonistic effect of the stresses.

As it is done for the presentation of the morphometrical data recorded, hereafter will be analysed the comparisons between the control experimental condition with all the other stressful conditions.

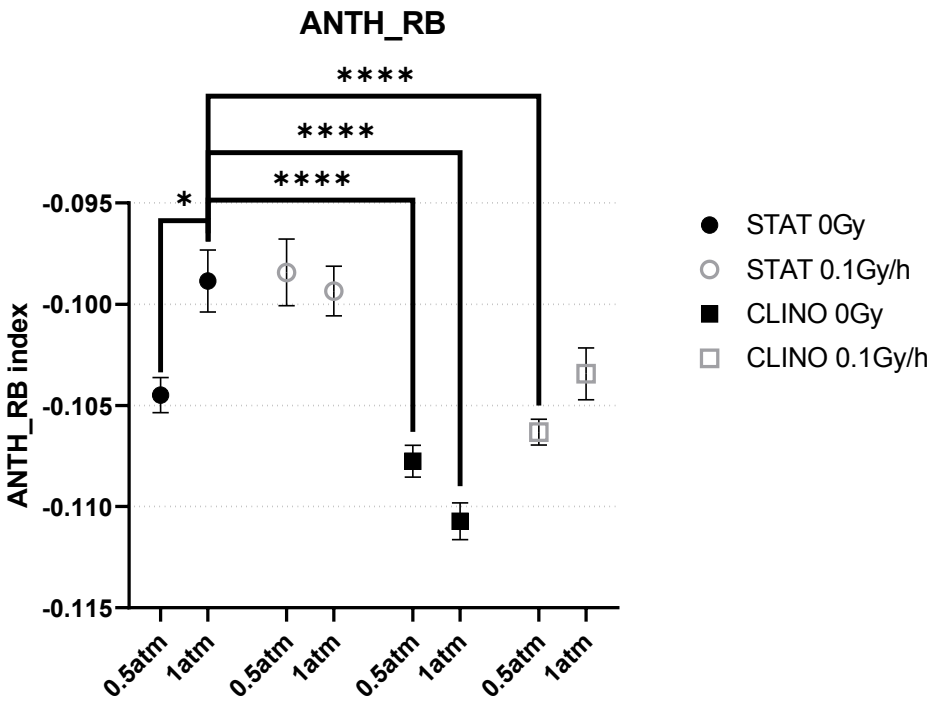

Fig. 4.2.3 ANTH_RB. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As already mentioned, the ANTH_RB index is proportional to the internal content of anthocyanins in leaves, thus it is possible to use this non-dimensional index to underline the effect of stress on plants of the same species, in the same phenological status but that receive different treatment to compare the effect of those treatments on the internal content of anthocyanins.

ANTH_RB indexes data expressed as mean ± SEM, are reported in Fig. 4.2. above. The highest values were recorded for the control condition which results statistically different from P samples and from G and PG samples which result in the lowest values. The GreenCube condition result is also significantly different from the control.

4.2.4. NBI_R

NBI_R index was recorded with the use of the Multiplex on the adaxial face of the cotyledonal leaves and it was analysed the index values recorded on the adaxial face only.

This index is proportional to the fertilization status of the plants (Cartelat et al., 2005) and is calculated by the multiplex as a ratio between the Chlorophyll content and the Flavonols content inside the leaves' epidermis and so it can be used to evaluate the metabolic status of plants at the time of analysis (Agati et al., 2013).

Fluorometry – NBI_R	*%Var*	*P value*
Pressure	0.68	ns
Gravity	3.75	ns
Radiation	2.07	**
Pressure x Gravity	2.72	***
Pressure x Radiation	1.22	**
Gravity x Radiation	15.58	****
Pressure x Gravity x Radiation	2.72	**

Table 4.2.4 Three-Way ANOVA results for NBI_R. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As it is possible to see in Table 4.2.4 there is not a real driving force that affects the total variance of the measurements of the index. Indeed, the presence of the interaction among all the stress means that the variability of the measurements is caused by the synergic and antagonistic effect of the three-stress put together. The major variability is attested to the interaction of *Gravity x Radiation*.

As it is done for the presentation of the morphometrical data recorded, hereafter will be analysed the comparisons of the control samples with the other experimental conditions.

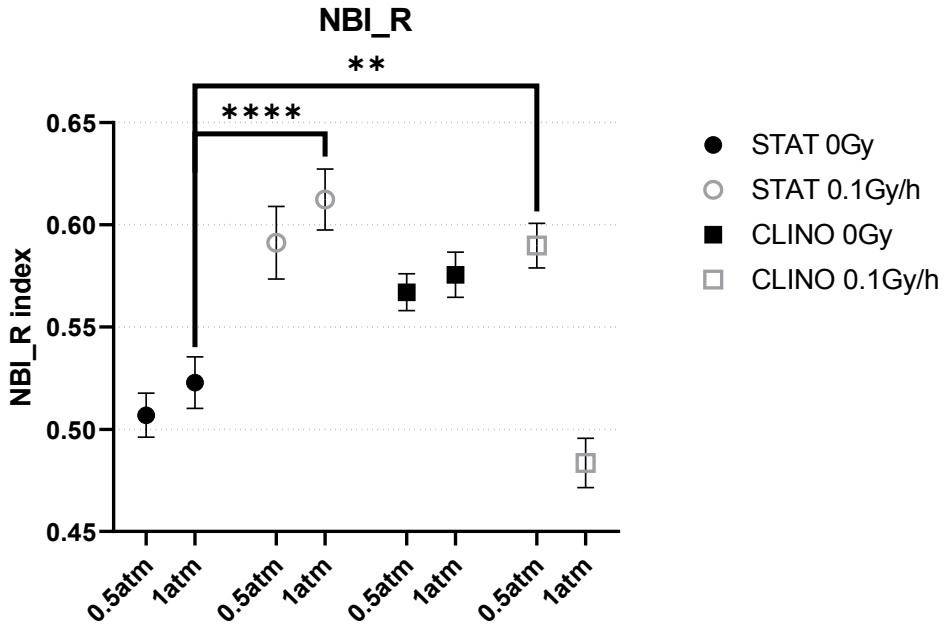

Fig. 4.2.4 NBI_R. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Scientific literature reports that the NBI_R index is proportional to the internal content of nitrogen in leaves (Cartelat et al., 2005), but it can be used to assess the metabolic status of the plants (Agati et al., 2013). It is possible to use this non-dimensional index to compare the effect of these stress on plants of the same species, that were in the same phenological status but that receive different treatments.

In Fig. 4.2., above, are graphically visualized the data of the NBI_R index expressed as mean ± SEM. The lowest value was recorded for samples cultivated in clinorotation while chronically irradiated (GR). The highest value, instead, was recorded for samples that were chronically irradiated. The PGR and R conditions result significantly different from the control condition for the analysis of the adaxial face only.

4.3. Metabolomics

After the metabolomic analyses, about fifty metabolites were detected both for Polar and Semi-Polar analyses. It was possible to find the presence of characteristics alkaloid *of Lepidium sativum L.* species known as Lepidine C

(Fig. 4.3.) and Lepidine (B/D/E/F), these metabolites belonging to the class of imidazole alkaloid and were already detected in Garden cress (Maier et al., 1998).

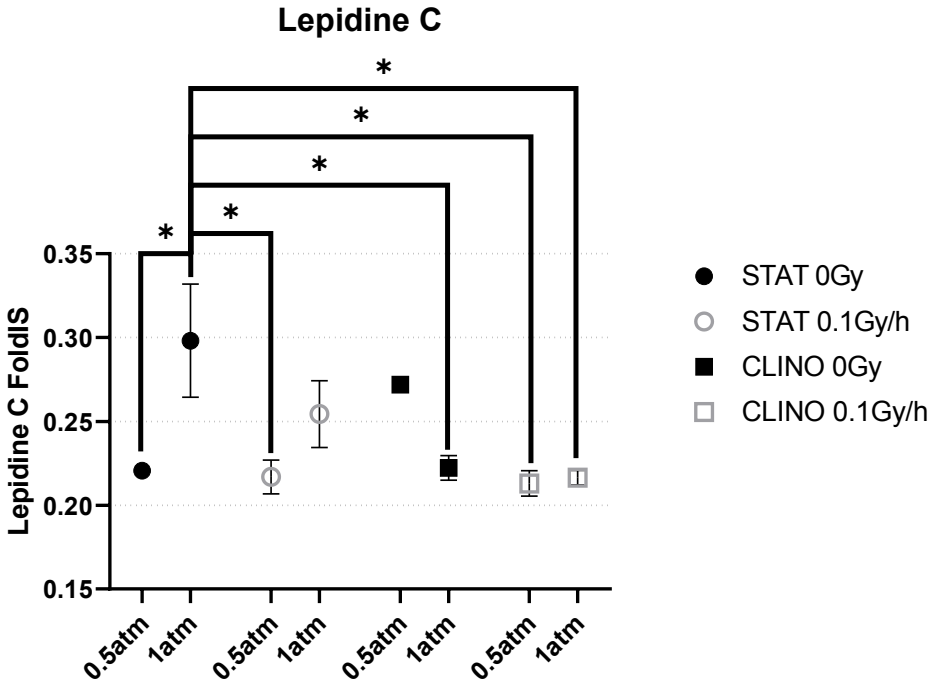

Fig. 4.3.1 Lepidine C. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The highest quantity of *Lepidine C* was recorded for the control condition, while the lowest concentration was detected for GreenCube experimental condition. There is a significant difference between the control condition and the GreenCube one. The interaction between all three imposed stress together is statistically significant and report about 10% of all the variability of the samples (Table 4.3.1).

Metabolomic – Lepidine C	*%Var*	*P value*
Pressure	5.56	ns
Gravity	5.16	ns
Radiation	14.70	*
Pressure x Gravity	30.41	**
Pressure x Radiation	0.20	ns
Gravity x Radiation	0.35	ns
Pressure x Gravity x Radiation	10.18	*

Table 4.3.1 Three-Way ANOVA results for Lepidine C. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Was detected also the metabolite Esculin which belongs to the coumarin classes that were already found in *Lepidium sativum L.* seedlings extract (Hijazin et al., 2019; Abdallah et al., 2020).

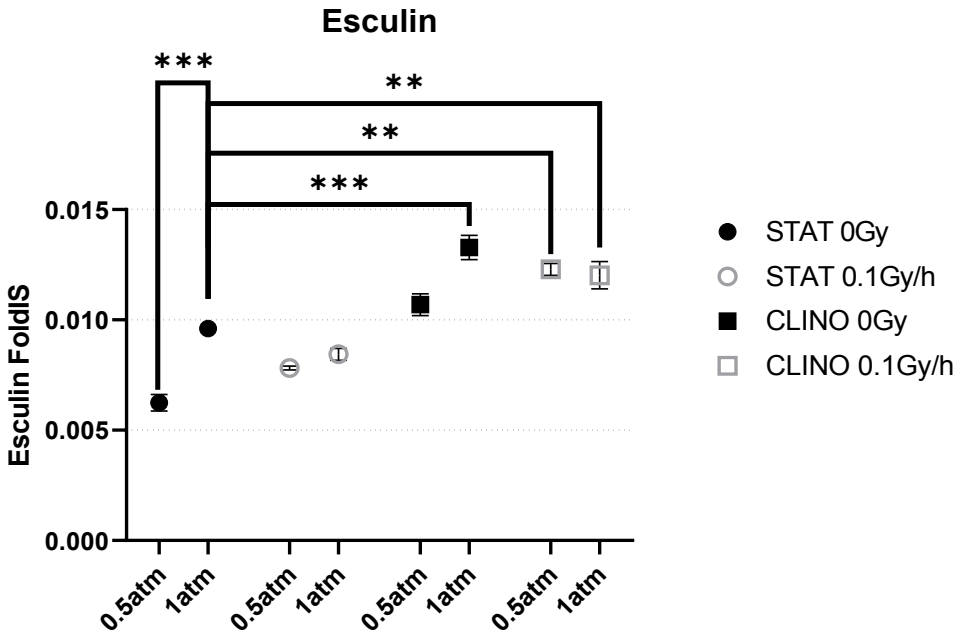

Fig. 4.3.2 Esculin. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The lowest concentration value of Esculin (Fig. 4.3.) was found for the P condition in which the pressure stress was imposed in form of the lower pressure of cultivation. The highest value was recorded for the G trial in which only the microgravity stress was imposed. There is a statistically significant difference between the control condition and the GreenCube trial in which it was the recorded concentration was higher. The interaction between Pressure and Radiation was effective in total variability (about 9%) of the samples' measurements. By the way, Gravity alone is responsible for about 74% of the total variability (Table 4.3.2).

Metabolomic – Esculin	*%Var*	*P value*
Pressure	11.15	****
Gravity	73.57	****
Radiation	0.16	ns
Pressure x Gravity	0.77	ns
Pressure x Radiation	8.81	***
Gravity x Radiation	0.00	ns
Pressure x Gravity x Radiation	0.00	ns

Table 4.3.2 Three-Way ANOVA results for Esculin. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Among the phenolic acid class, the metabolite with the highest concentration in the extracts was Sinapic acid.

Sinapic acid (Fig. 4.3.) is a primary metabolite involved as a precursor of many other metabolites and is widely diffused in many other species of the Brassicaceae family (Cartea et al., 2010; Nguyen et al., 2021).

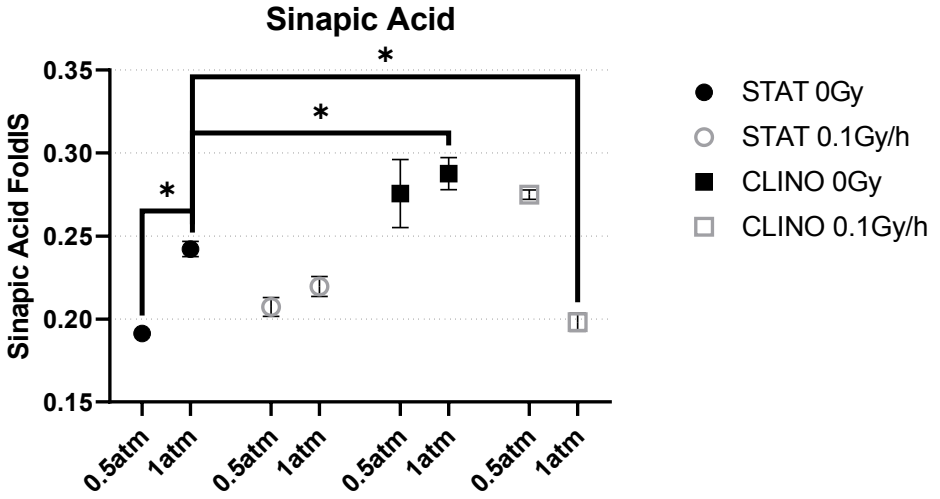

Fig. 4.3.3 Sinapic Acid. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The lowest value of concentration detected was for the P condition and was significative different from the control condition. The highest value was recorded for the G condition with a significant difference with respect to the control. The GreenCube condition, even if it was not significantly different from the control condition, reported one of the highest concentrations among

the sample means. The interactions among the coupled stress (Pressure X Gravity, Pressure X Radiation and Gravity X Radiation) were effective on the total variability of the sample means and were responsible for about 42% (summarized), Gravity stress alone was responsible for about 33% of the total variability (Table 4.3.3).

Metabolomic – Sinapic acid	*%Var*	*P value*
Pressure	0.00	ns
Gravity	33.34	****
Radiation	10.08	**
Pressure x Gravity	17.67	***
Pressure x Radiation	17.52	***
Gravity x Radiation	7.53	**
Pressure x Gravity x Radiation	2.74	ns

Table 4.3.3 Three-Way ANOVA results for Sinapic acid. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The major antioxidant effect shall be attested to the presence of the flavonoid class. It was found the presence of conjugated flavonoids in a concentration higher than their non-conjugated counterparts. In particular non-conjugated Kaempferol was detected and had the higher concentration recorded for the control condition (Fig. 4.3.).

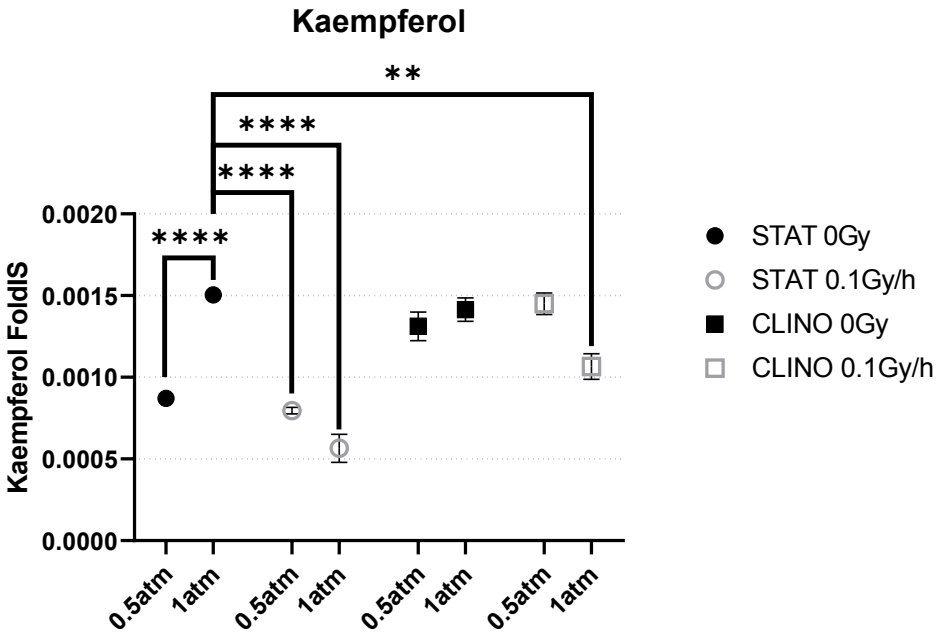

Fig. 4.3.4 Kaempferol. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The results of the Three-Way ANOVA for this metabolite, highlight that the total variability was attested to the presence of significative interactions among the coupled stress (Table 4.3.4) with a major effect due to Pressure X Radiation (about 25%). Control and GreenCube conditions had almost the same concentration of this metabolite (Fig. 4.3.).

Metabolomic – Kaempferol	*%Var*	*P value*
Pressure	0.19	ns
Gravity	30.79	****
Radiation	20.35	****
Pressure x Gravity	6.35	**
Pressure x Radiation	24.73	****
Gravity x Radiation	8.80	***
Pressure x Gravity x Radiation	1.93	ns

Table 4.3.4 Three-Way ANOVA results for Kaempferol. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The same metabolite in the conjugated form, Kaempferol 3-O-glucosyl-rhamnosyl-galactoside (Fig. 4.3.), was not only 10 times more concentrated

than the not conjugated form but the highest values were recorded for the stressed condition while the control condition was among the lowest.

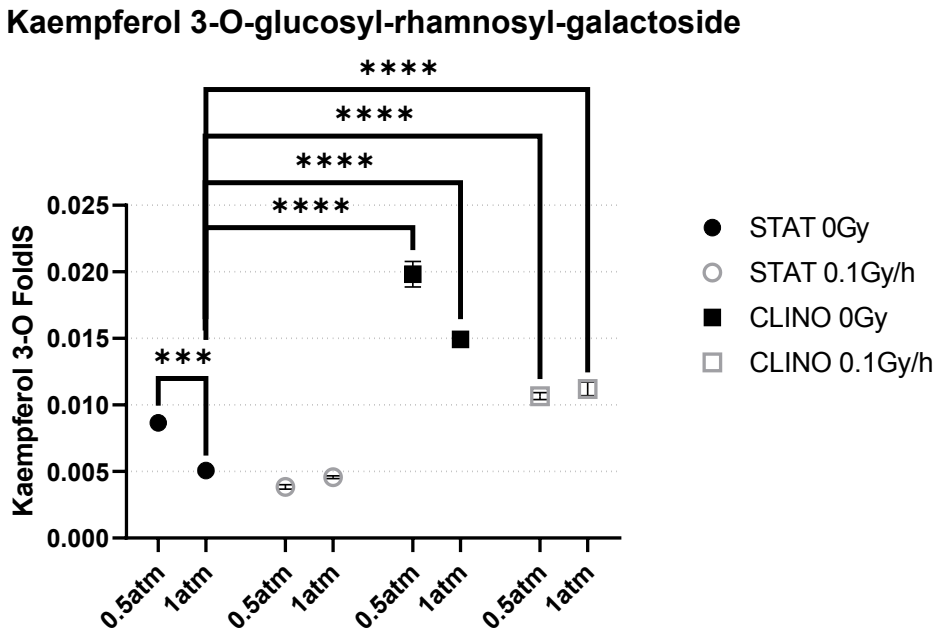

Fig. 4.3.5 Kaempferol 3-O-glucosyl-rhamnosyl-galactoside. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

There is a significant difference between the control group and the GreenCube condition. The total variability of the sample mean was poorly due to the interaction of Pressure X Radiation and Gravity X Radiation (5.4% and 3.3% respectively) but, Gravity alone was responsible for about 68% of the total variability followed by Radiation with about 19% (Table 4.3.5).

Metabolomic – Conjugated Kaempferol	*%Var*	*P value*
Pressure	2.96	****
Gravity	67.80	****
Radiation	18.88	****
Pressure x Gravity	0.13	ns
Pressure x Radiation	5.43	****
Gravity x Radiation	3.28	****
Pressure x Gravity x Radiation	0.07	ns

Table 4.3.5 Three-Way ANOVA results for conjugated Kaempferol. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

It was found the presence of a great quantity of Linolenic acid that belongs to the fatty acid class and was the most abundant metabolite of that class.

Linolenic acid (Fig. 4.3.) report a detrimental trend due to Radiation and Gravity stress that were responsible for about 30% and 18% respectively of the total variation of the sample means (Table 4.3.6), while lower pressure had an overall positive effect on the accumulation of this metabolite and was responsible for about 20% of the total variability. No interactions were detected, and the only significative difference was between the control and the RG condition which was also the one with the lowest concentration.

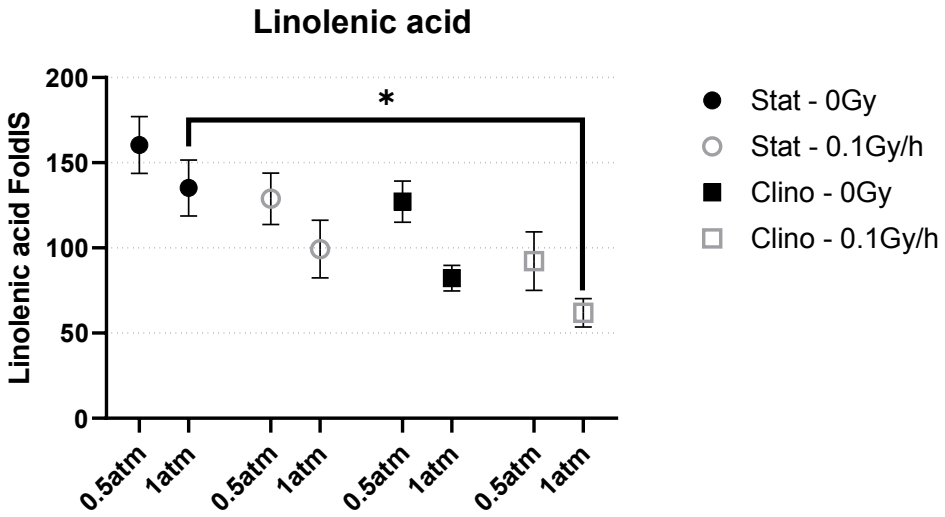

Fig. 4.3.6 Linolenic acid. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Metabolomic – Linolenic acid	*%Var*	*P value*
Pressure	19.94	**
Gravity	30.30	**
Radiation	17.83	**
Pressure x Gravity	0.49	ns
Pressure x Radiation	0.13	ns
Gravity x Radiation	0.17	ns
Pressure x Gravity x Radiation	0.42	ns

Table 4.3.6 Three-Way ANOVA results for Linolenic acid. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Among the carotenoid class, known for their antioxidant capability in stressful conditions, the most abundant metabolite was Lutein known for its beneficial application in eye diseases (Buscemi et al., 2018).

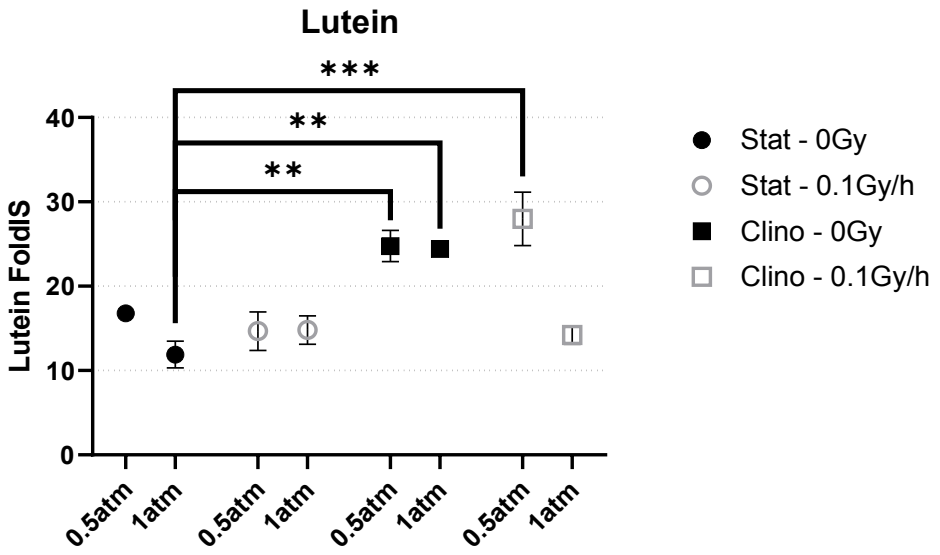

Fig. 4.3.7 Lutein. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Lutein's lowest concentration was recorded for the control condition while the highest concentration was detected in GreenCube seedlings (Fig. 4.3.). Among these two conditions, there is a significant difference. For this metabolite, the total variability was caused by the interaction of the effects of all three stress together, that account for about 14% of the total variance. As for other metabolites, Gravity alone was effective on total variability for about 45% (Table 4.3.7).

Metabolomic – Lutein	*%Var*	*P value*
Pressure	14.42	**
Gravity	44.71	****
Radiation	1.58	ns
Pressure x Gravity	3.53	ns
Pressure x Radiation	2.86	ns
Gravity x Radiation	2.47	ns
Pressure x Gravity x Radiation	13.86	**

Table 4.3.7 Three-Way ANOVA results for Lutein. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

The sum of the metabolites from the class of the chlorophyll (Fig. 4.3.8) was used to highlight the linear regression (Fig. 4.4.3) with the results obtained with Multiplex 3A (SFR_R; Fig. 4.2.1) reflectance investigation analysis.

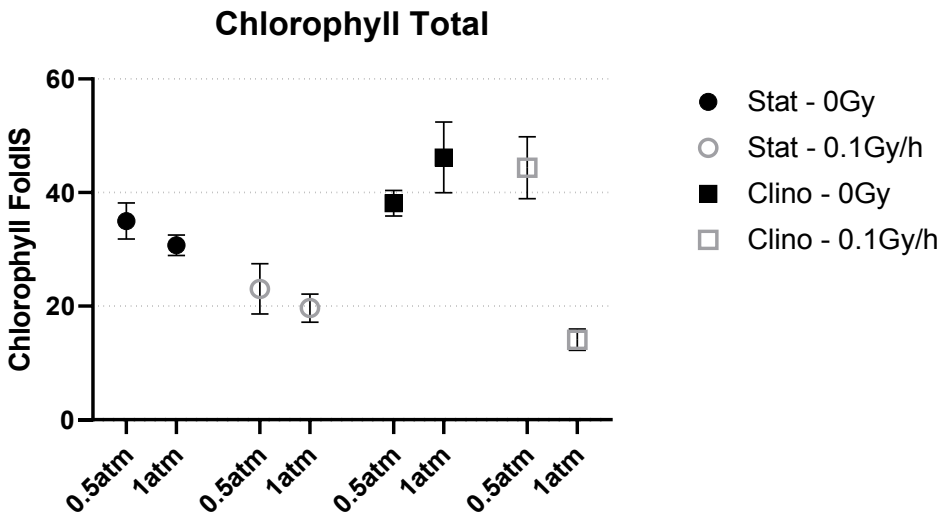

Fig. 4.3.8 Chlorophyll concentration from metabolomic analysis

No statistically significant differences were recorded between the control condition and the experimental trial for the total chlorophyll concentration analysed with metabolomic (Fig. 4.3.8) but a slightly good linear regression was found, as expected, by comparing those concentrations with the Multiplex measurements (Fig. 4.4.3).

It was analysed the presence of vitamins that were found with the detection of ProVitamin A (α-carotene, all-trans-β-carotene and cis-β-carotene; Fig. 4.3.), Vitamin B2 and B3 (Fig. 4.3.), Vitamin E (Fig. 4.3.) and Vitamin K (Fig. 4.3.).

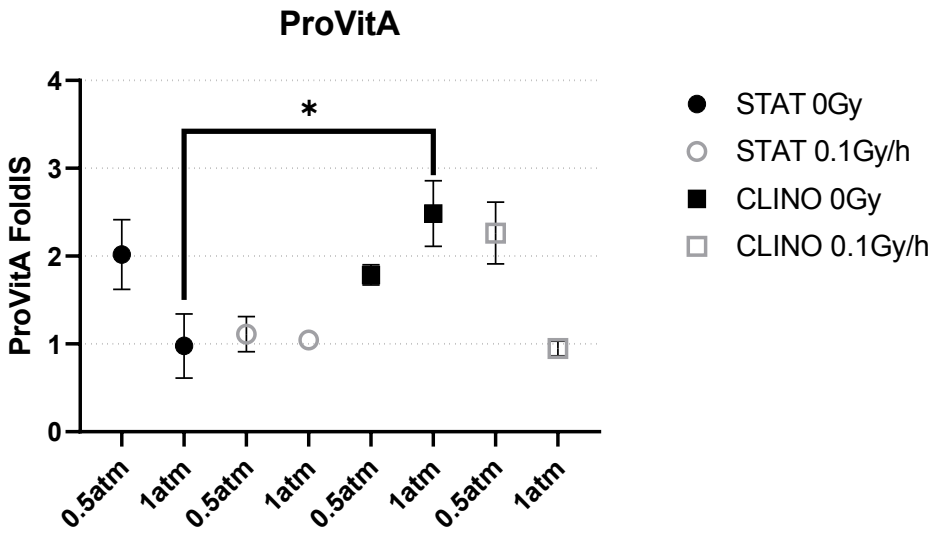

Fig. 4.3.9 Vitamin A. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Vitamin A (Fig. 4.3.) lowest values were recorded for the Control condition, PR, R and GR conditions, while the highest values were recorded for G and GreenCube condition. The total variance of the samples was due to the interaction effect of all the three stress together that accounting for about 28%. In these cases, Gravity alone was responsible for about 17% of the total variance of the sample means (Table 4.3.8).

Metabolomic – Vitamin A	*%Var*	*P value*
Pressure	9.23	*
Gravity	16.79	**
Radiation	11.17	*
Pressure x Gravity	0.75	ns
Pressure x Radiation	3.36	ns
Gravity x Radiation	0.15	ns
Pressure x Gravity x Radiation	27.75	**

Table 4.3.8 Three-Way ANOVA results for Vitamin A. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

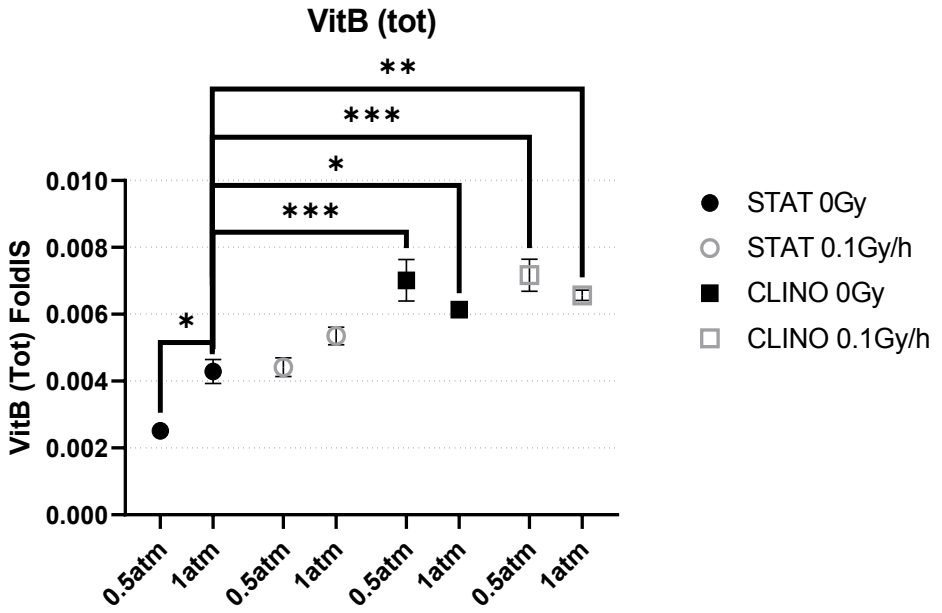

Fig. 4.3.10 Vitamin B. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Vitamin B shows a significant effect of the interaction of Pressure X Gravity and Gravity X Radiation (10.9% and 3.6% respectively), but Gravity alone was responsible for about 66.5% of the total variance (Table 4.3.9). The highest concentration was reached by the GreenCube condition which is significantly different from the control condition. The lowest value was recorded for the P condition (Fig. 4.3.).

Metabolomic – Vitamin B	*%Var*	*P value*
Pressure	0.95	ns
Gravity	66.46	****
Radiation	7.81	**
Pressure x Gravity	10.92	***
Pressure x Radiation	0.20	ns
Gravity x Radiation	3.55	*
Pressure x Gravity x Radiation	0.77	ns

Table 4.3.9 Three-Way ANOVA results for Vitamin B. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

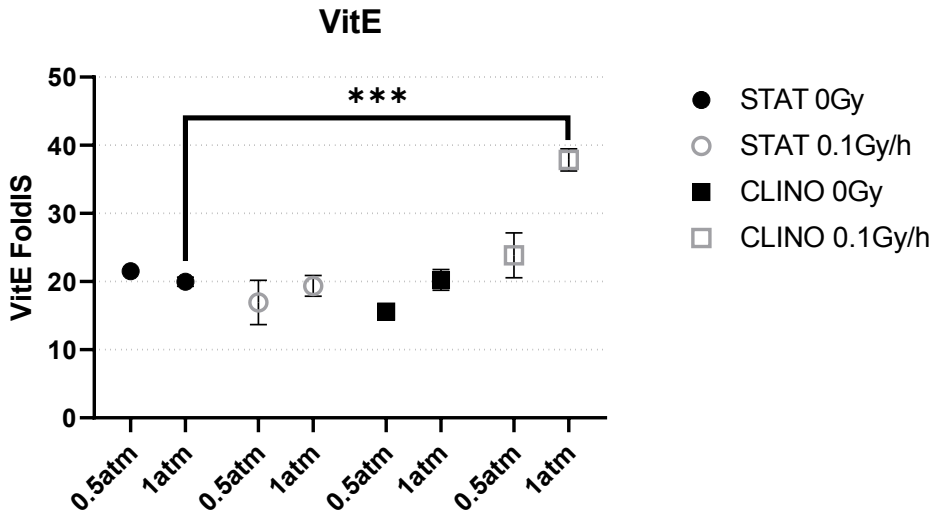

Fig. 4.3.11 Vitamin E. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Vitamin E (Fig. 4.3.) highest value was recorded for the GR condition which is also significantly different from the control. Gravity X Radiation was an effective interaction that account for about 30% of the total variance of the samples' mean (Table 4.3.10).

Metabolomic – Vitamin E	*%Var*	*P value*
Pressure	11.95	**
Gravity	12.26	**
Radiation	13.42	**
Pressure x Gravity	9.89	**
Pressure x Radiation	5.62	*
Gravity x Radiation	30.31	****
Pressure x Gravity x Radiation	0.92	ns

Table 4.3.10 Three-Way ANOVA results for Vitamin E. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

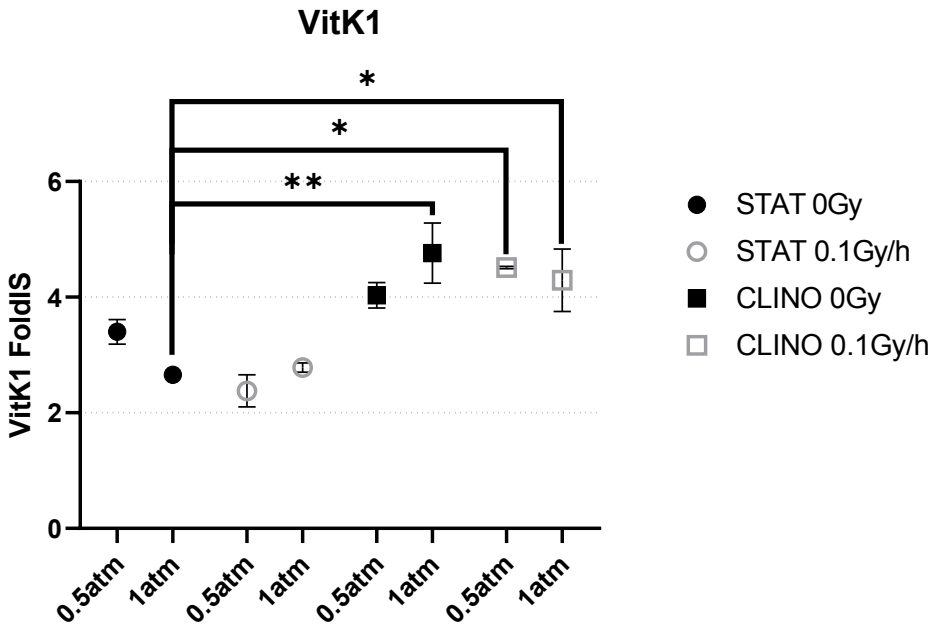

Fig. 4.3.12 Vitamin K. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Vitamin K's highest values were found in stressed conditions with a significant difference between the GreenCube condition and the Control condition (Fig. 4.3.). On Vitamin K a significant effect on total variance was due to the interaction between all the three stress together which is responsible for about 7% of total variance but the only other significant effect was reported for Gravity stress which is responsible for about 68% of the total variability (Table 4.3.11).

Metabolomic – Vitamin K	*%Var*	*P value*
Pressure	0.05	ns
Gravity	68.34	****
Radiation	1.32	ns
Pressure x Gravity	1.22	ns
Pressure x Radiation	0.07	ns
Gravity x Radiation	1.37	ns
Pressure x Gravity x Radiation	7.39	*

Table 4.3.11 Three-Way ANOVA results for Vitamin K. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

4.4. Multiplex as a non-destructive alternative

Spectrophotometric analyses were performed to assess a correlation with Multiplex 3A measurements. This is of fundamental priority to propose and validate the use of a non-destructive optical sensor for space missions in which plants cultivation is involved. The possibility to avoid chemical destructive analysis means the possibility to shorten the times of analysis, have all the samples available for every other kind of analysis and have them available to feed astronauts.

By using a linear regression analysis between SFR_R values and the chemical ones obtained with the chlorophyll extraction by spectrophotometric analysis (Fig. 4.4.1), it was found a linear interaction between them, with good linearity (R^2=0.88, p<0.0001).

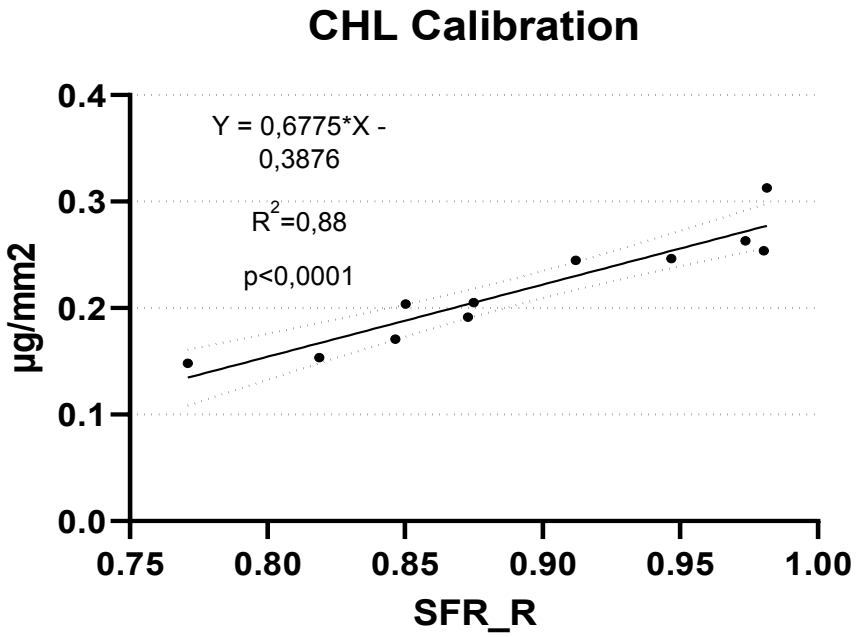

Fig. 4.4.1 Multiplex Chlorophyll calibration to Spectrophotometric analysis

Linear regression analysis was performed also for Anthocyanins concentration (Fig. 4.4.2) resulting in a poorly good correlation but in agreement with multiplex user guides.

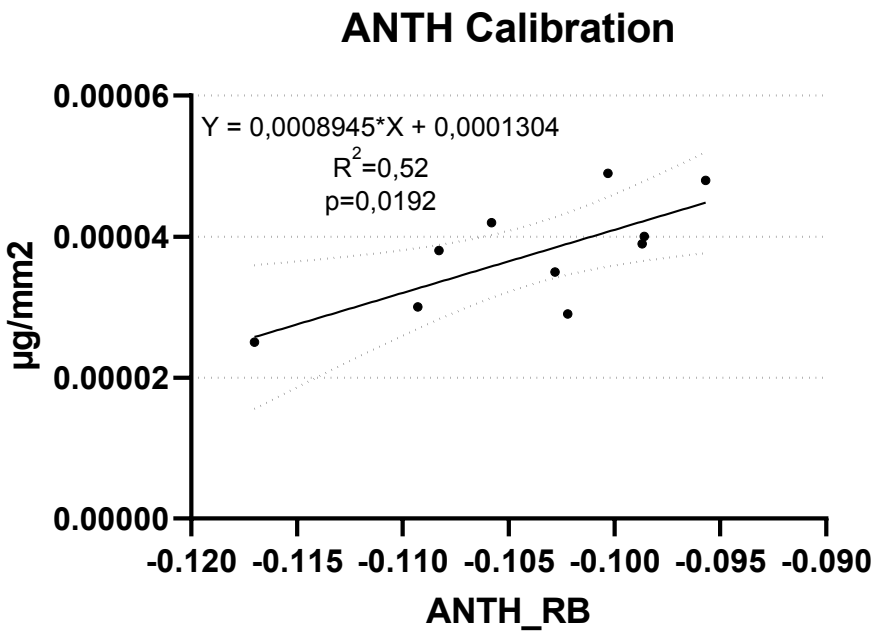

Fig. 4.4.2 Multiplex Anthocyanins calibration to spectrophotometric analysis

Being the range of measurements that were recorded for *Lepidium sativum L.* cotyledonal leaves very narrow in respect to the measurement range of Multiplex, it is possible to say that for these experiments, and so only for *Lepidium sativum L.* cotyledonal leaves, there is a linear function that could be used to assess the chlorophyll content and the anthocyanins concentration of only the Cotyledonal leaves using just the measurements recorded on the adaxial face of those cotyledons, by using the calibration formulas.

This means that in a space application in which cotyledonal leaves needed to be analysed for chlorophyll concentration, or anthocyanins content, those leaves shall be not cut off the plants as indeed measurements can be done directly on fresh living leaves.

The same good correlation trend was found with metabolomic analysis in which metabolites of chlorophyll class, put together, retrieve good linearity with respect to the SFR_R multiplex analysis (R^2=0.6, p=0.0237; Fig. 4.4.3).

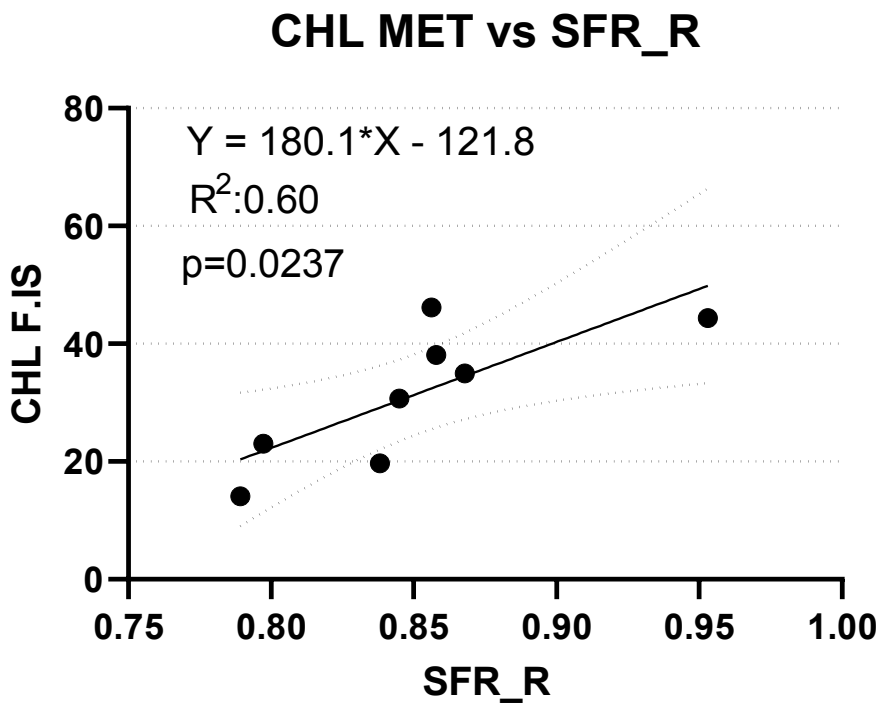

Fig. 4.4.3 Multiplex Chlorophyll calibration to metabolomic analysis

This was expected but confirmed the possibility to use the Multiplex to analyse plants at least for chlorophyll concentration.

4.5.Flow Cytometry

Plant flow cytometry was used to estimate nuclear DNA content and damages after the cultivation process under the 8 different experimental conditions. Among the damages, it could be possible to find DNA ploidy changes and cell cycle anomalies.

Data from three different excitations by three laser beams were recorded and, and the most accurate emission data were obtained using the 405 nm laser (Violet) using a Band Pass filter at 525 nm and DAPI staining. The DNA measurements reporting better CV values and resolution power among the characteristic's ploidy peaks were chosen for the evaluation of external stimula vs cell cycle and DNA ploidy.

4.5.1. The "Well" Method

This new method is solid, fast, and easy to perform.

Analysis was performed on single root tips and cotyledons to highlight ploidy changes and space stress effect on nuclear DNA. The classic chopping method (Galbraith et al., 1983), uses a greater amount of sample material and it's high time consuming thus reducing the number of analyses could be performed during a working day, in respect to our new isolation method.

The analyses were run without filtering the sample, thanks to the wide sample needle istalled on the CytoflexS (200 µm size in diameter) meaning a great advantage in reduction of time to analyse nuclear suspensions one by one. Ten thousand events were recorded per each analysis and a mean of 42% and 57% of the collected events (except the beads) are eligible as nuclei in the 2N, 4N, 8N, and 16N (only roots) phase for root and cotyledons, respectively. The mean CV value of G1/2N nuclei spanned from less than 3% to a large 10%, with an averaged CV of 5% for every single analysis, for both roots and cotyledons.

The alignment UV-beads performed stably for every experimental trial, giving proof of the accuracy of the measurement as they have a 3.27% mean CV with an SD of about 0.4% meaning a change of resolution channel of about ±200 channels on a total resolution of sixteen millions channels.

To analyse the effect of the experimental conditions on nuclei it was chosen to use two calculated indexes that can highlight the effect of stress on the nuclear DNA content.

It was decided to estimate the presence of the endoreduplication using two indexes, the Mean C-Level (Mishiba and Mii, 2000; Engelen-Eigles et al., 2001; Barow and Jovtchev, 2007), and the Cycle Value (Barow and Meister, 2003).

Data collected were analysed and then reported in Table 4.5.1, for root and Table 4.5.2, for cotyledons, respectively. Data are reported as mean values ± Standard Deviation of the indexes in the 8 experimental trials.

These two indexes give an information about the ploidy level of the analysed tissues and can be used to compare the effect of stress on the cell cycle regulation.

Root	*Mean C-Level*	*Cycle Value*
C	4.35±0.22	0.90±0.08
P	4.31±0.45	0.85±0.11
G	4.57±0.57	0.90±0.14
R	4.95±0.44	1.06±0.11
PG	4.25±0.19	0.82±0.06
PR	4.65±0.33	0.95±0.08
GR	4.03±0.44	0.80±0.15
PGR	4.16±0.41	0.81±0.14

Table 4.5.1 Summary of root analyses by flow cytometry

Cot	*Mean C-Level*	*Cycle Value*
C	3.10±0.18	0.54±0.09
P	2.93±0.19	0.46±0.10
G	3.07±0.29	0.52±0.14
R	2.84±0.33	0.41±0.16
PG	2.52±0.27	0.26±0.13
PR	3.00±0.17	0.49±0.08
GR	2.87±0.30	0.43±0.15
PGR	2.70±0.27	0.34±0.13

Table 4.5.2 Summary of cotyledons analyses by flow cytometry

Hereafter are reported the data resulting from the analysis run at cytometer separated by plant organ, roots and cotyledons.

4.5.2. Mean C-Level in roots

The results of the Three-way ANOVA on Mean C-Level variability report a predominant effect of gravity stimulus on the total variability of the samples, followed by the combination of Gravity and Radiation stress (Table 4.5.3).

Flow Cytometry – Mean C-level root	*%Var*	*P value*
Pressure	1.94	ns
Gravity	10.60	**
Radiation	0.67	ns
Pressure x Gravity	0.16	ns
Pressure x Radiation	0.24	ns
Gravity x Radiation	16.89	***
Pressure x Gravity x Radiation	3.30	ns

Table 4.5.3 Three-Way ANOVA results for Mean C-level in roots. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

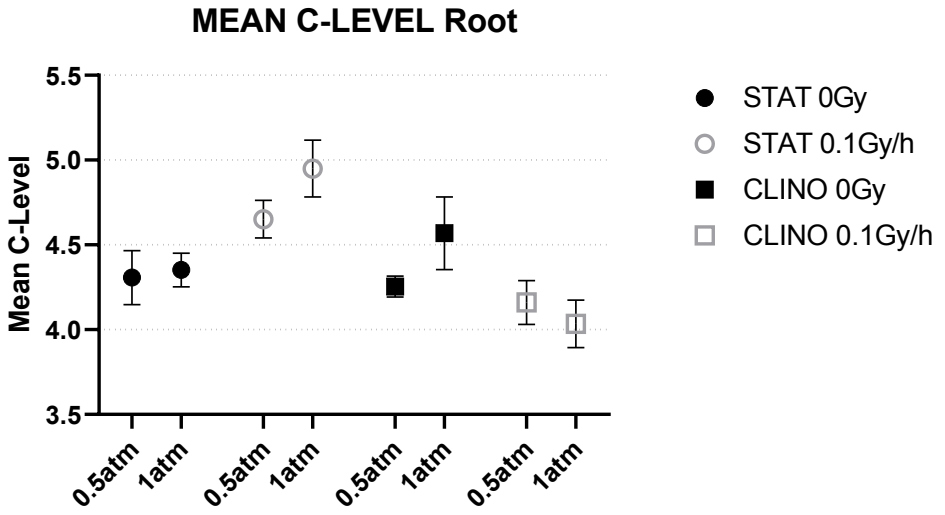

Fig. 4.5.1 Mean C-level in roots. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As it is possible to see in Fig. 4.5., no significant differences were recorded for this index for root apex analyses. The effect of radiation exposure enhances the Mean C-Level indicating a possible endoreduplication effect. This possible effect is recorded also for clinorotated samples but with less emphasis. A detrimental effect was registered instead on the samples exposed to γ rays while clinorotated.

4.5.3. Cycle Value in roots

The results of the Three-way ANOVA on Cycle Value variability report a predominant effect of Gravity stress on the total variability of the samples,

followed by the combination of Gravity and Radiation stress. Also, Pressure alone had a significant effect on total variability (Table 4.5.4).

Flow Cytometry – Cycle value root	*%Var*	*P value*
Pressure	4.84	*
Gravity	15.36	***
Radiation	1.68	ns
Pressure x Gravity	0.87	ns
Pressure x Radiation	0.06	ns
Gravity x Radiation	10.78	**
Pressure x Gravity x Radiation	1.62	ns

Table 4.5.4 Three-Way ANOVA results for Cycle value in roots. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

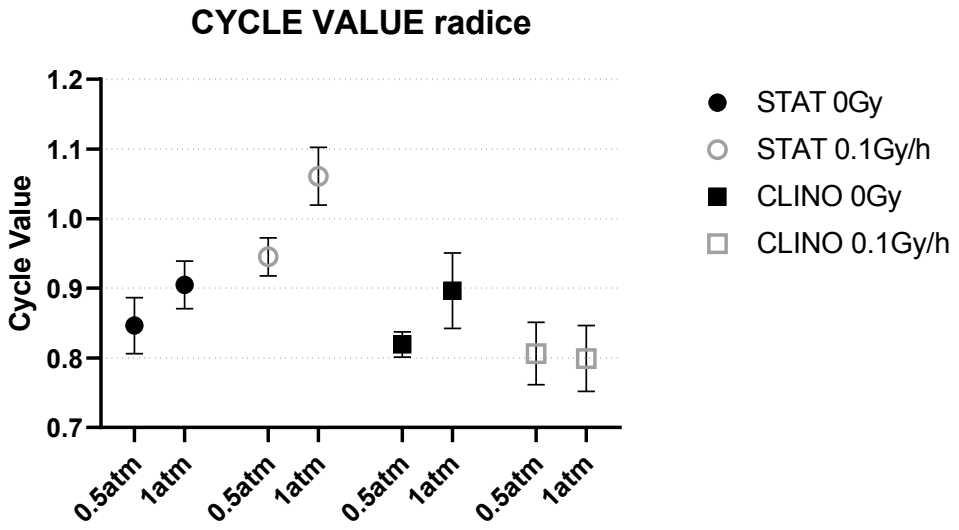

Fig. 4.5.2 Cycle value in roots. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As for the Mean C-Level, in the Cycle Value, no significant differences were found. It is reported an enhancement of the index for samples that were exposed to γ radiations inside the CALLIOPE ^{60}Co irradiation facility in the ENEA "Casaccia" Research Centre, and the effect is higher for the plants maintained at atmospheric pressure than that cultivated at 0.5atm. Samples that were instead chronically clinorotated report a decrease of the index with lower values with respect to the control sample. The combination of radiation and microgravity report a detrimental effect with respect to the control situation for this index.

4.5.4. RelG2 in roots

The results of the Three-way ANOVA of RelG2 (Table 4.5.5) variability report a predominant effect due to pressure change, followed by microgravity stress and the combination of pressure and microgravity.

Flow Cytometry – RelG2	*%Var*	*P value*
Pressure	17.76	***
Gravity	14.79	***
Radiation	0.65	ns
Pressure x Gravity	6.36	*
Pressure x Radiation	0.13	ns
Gravity x Radiation	0.02	ns
Pressure x Gravity x Radiation	0.06	ns

Table 4.5.5 Three-Way ANOVA results for RelG2 in roots. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

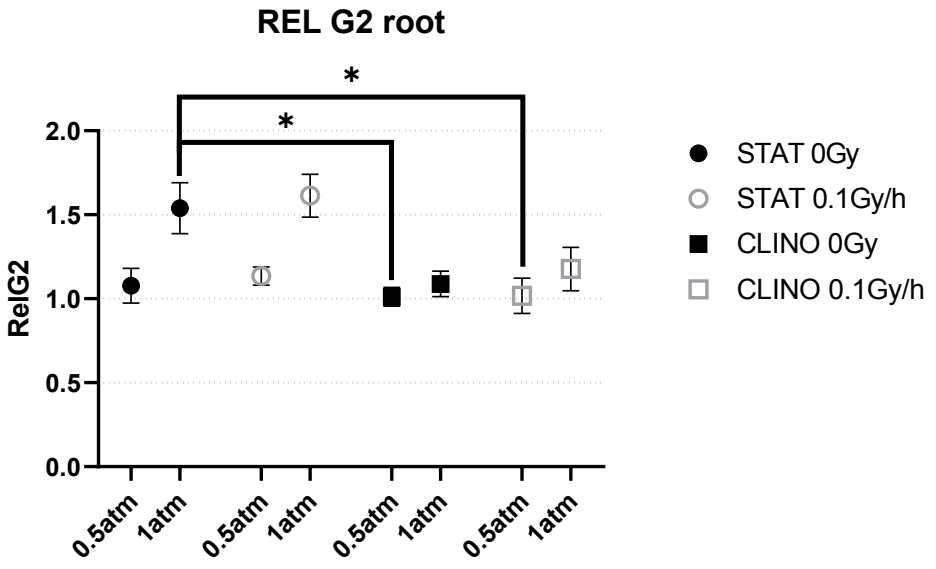

Fig. 4.5.3 RelG2 in roots. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Low pressure seems to drive the nuclei population always to a lower index value with respect to their counterpart at atmospheric pressure.

Data graphed from the analysis (Fig. 4.5.) underline a general decrease of the index, meaning a major quantity of G1/S nuclei in respect to G2/M (+$G1_{4N}$/S) nuclei, for all the trials in respect to the control (*C*).

The only exception was for the *R* trial which instead report an increase in the index with respect to the control trial.

A significant difference was found between the control sample and the GreenCube one.

4.5.5. Mean C-Level in cotyledons

The results of the Three-way ANOVA on Mean C-Level variability for cotyledonal leaves report an effect of pressure and gravity on the total variability of the samples. The interaction pressure x gravity highlights an antagonistic effect between these two stresses (Table 4.5.6).

Flow Cytometry – Mean C-level cotyledons	*%Var*	*P value*
Pressure	9.13	**
Gravity	8.24	**
Radiation	0.80	ns
Pressure x Gravity	8.45	**
Pressure x Radiation	8.55	**
Gravity x Radiation	0.47	ns
Pressure x Gravity x Radiation	0.03	ns

Table 4.5.6 Three-Way ANOVA results for Mean C-level in cotyledons. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

Cotyledonal leaves report a different ploidy level compared to the root apex. These two organs of the plant are completely different.

For this index, it is found a significant difference between the control condition and the GreenCube one with a reduction of the Mean C-level for the latter. It seems that all three stresses had a detrimental effect on this calculated index indeed a significant difference is found when all of them are experienced by the cultivated microgreens (Fig. 4.5.).

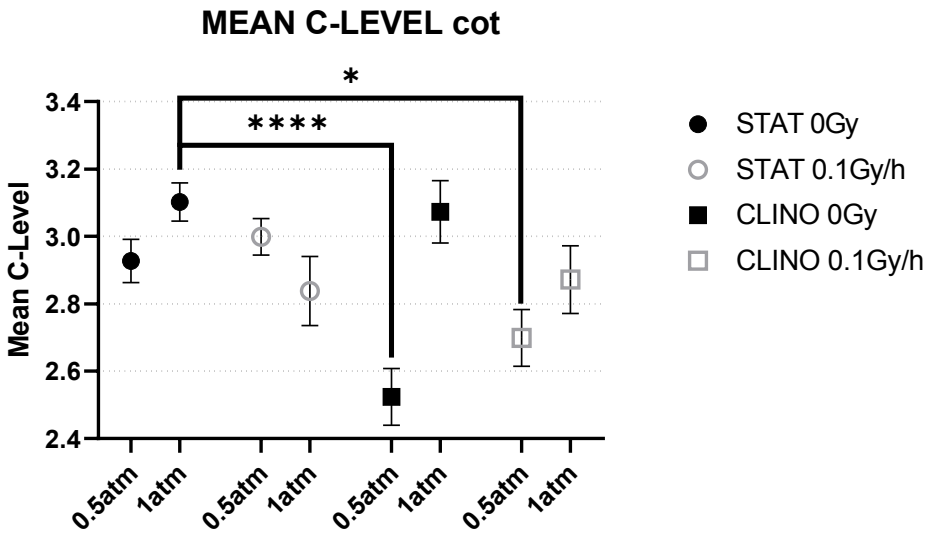

Fig. 4.5.4 Mean C-level in cotyledons. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

4.5.6. Cycle value in cotyledons

The results of the Three-way ANOVA on Cycle value variability for cotyledonal leaves report the same trend of the Mean C-level for cotyledons, an effect of pressure and gravity on the total variability of the samples. The interaction of pressure x gravity underlines an antagonistic effect on the measured index (Table 4.5.7).

Flow Cytometry – Cycle value cotyledons	*%Var*	*P value*
Pressure	9.31	**
Gravity	8.50	**
Radiation	0.79	ns
Pressure x Gravity	8.48	**
Pressure x Radiation	8.27	**
Gravity x Radiation	0.61	ns
Pressure x Gravity x Radiation	0.02	ns

Table 4.5.7 Three-Way ANOVA results for Cycle value in cotyledons. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

As already seen for Mean C-level index on the same plant organ, the cotyledonal leaves, it is found a statistically significant difference between the control condition and the GreenCube one. It is also found a difference between the control and the PG condition. The higher value was recorded for the control condition and all the other stressed samples report lower values

compared to it. An overall detrimental effect on this index is reported when stress was imposed during the cultivation (Fig. 4.5.).

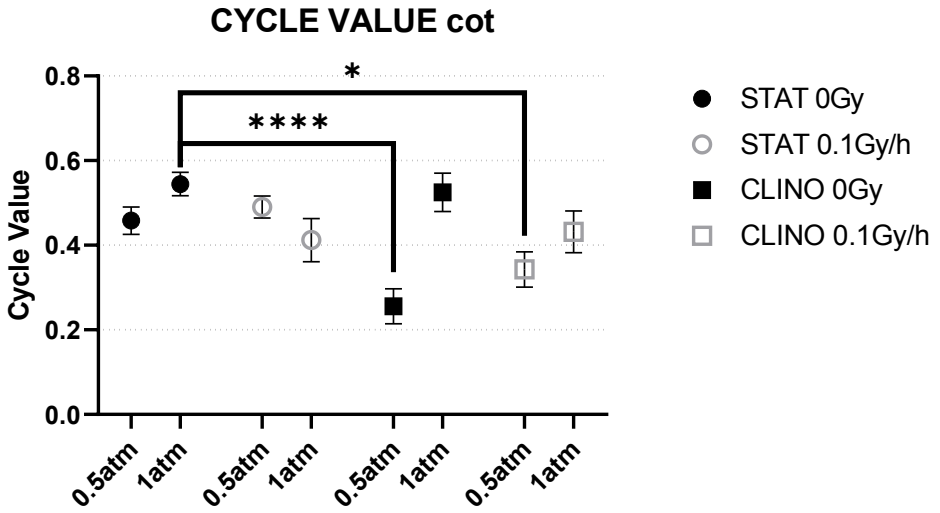

Fig. 4.5.5 Cycle value in cotyledons. P value: ns (P>0,05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

4.5.7. RelG2 in cotyledons

The results of the Three-way ANOVA for RelG2 variability for cotyledonal leaves (Table 4.5.8) reported a similar result with respect to the RelG2 for roots, which could be an effect of pressure and gravity on the total variability of the samples. The interaction of pressure x gravity was added with the interaction of pressure x radiation.

Flow Cytometry – RelG2 cotyledons	*%Var*	*P value*
Pressure	11.09	**
Gravity	5.08	*
Radiation	1.87	ns
Pressure x Gravity	6.12	*
Pressure x Radiation	7.26	**
Gravity x Radiation	0.31	ns
Pressure x Gravity x Radiation	0.00	ns

Table 4.5.8 Three-Way ANOVA results of RelG2 in cotyledons. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

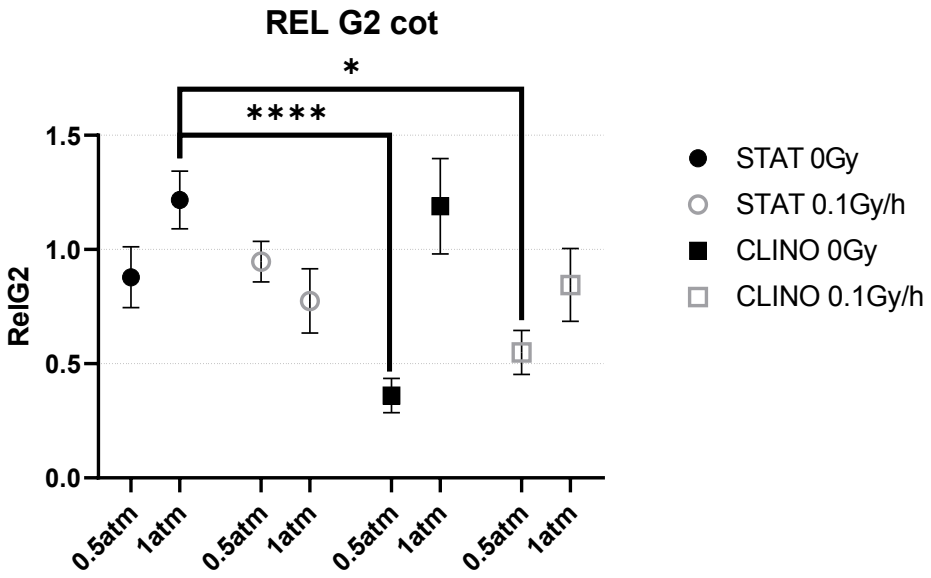

Fig. 4.5.6 RelG2 in cotyledons. P value: ns (P>0.05); * (P≤ 0.05); ** (P≤ 0.01); * (P≤ 0.001); **** (P≤ 0.0001)**

For nuclei extracted from cotyledons, it is possible to see a similar effect of the low pressure on C-Value as it was for root nuclei.

Data graphed from the analysis (Fig. 4.5.) underline a general decrease of the index, meaning a major quantity of G1/S nuclei in respect to G2/M (+$G1_{4N}$/S) nuclei, for all the trials in respect to the control (*C*).

The only exception was for the *G* trial which instead report an increase in the index with respect to the control trial.

A significant difference was found between the control sample and the GreenCube one.

5. Discussion

Greencube mission will launch in late-2022 as part of the maiden flight of Vega-C rocket vector from AVIO (Rome, Italy). The main objective of this mission is to provide an autonomous cultivation system for the outer space environment in which human presence is not essential since the cultivation system is autonomous, and it is fitted into a 3U CubeSat.

Mission length, for what concern the cultivation activities, will start once the CubeSat will reach the scheduled orbit and will last for 2-3 weeks since the water reservoir will be drained. During this period *Lepidium sativum L.* microgreens will grow surrounded by one of the harshest environments possible, the Van Allen belts in outer space. Remote control and environmental sensors will record growth parameters that will be sent to our mission control centre in Rome, at La Sapienza University.

Greencube's mission was presented at European Space Agency as a cooperation work between the La Sapienza University of Rome, the Italian National Agency for New Technology, Energy and Sustainable Economic Development (ENEA), University of Naples “Federico II” and the Italian Space Agency. This last institution was responsible for funding this project.

Lepidium sativum L. was chosen because of its internal chemical composition which is a promising source of nutritional factors and phytopharmaceutical primer (GOKAVI et al., 2004; Mali et al., 2007; Podsędek, 2007; Sharm and Agarwal, 2011). It’s easy to cultivate in hydroponic conditions and is typically short in height with a fast-growing cycle. Studying such a species for space applications led to the objective of creating a list of nutritionally feasible plants to use as a food source for the astronauts in space. Together with the MELiSSA candidate species, Potato, Durum wheat, wheat, and Soybean (Stasiak et al., 2012; Paradiso et al., 2014; De Pascale et al., 2021), Garden cress could be a best suited species for space travel and new settlements.

This work of thesis was carried out aiming to design an artificial environment as close as possible to the real situation of cultivation that GreenCube *Lepidium sativum L.* microgreens will face during space mission orbiting around our globe. To do this, many technological problems were resolved during the test trials, and both the external conditions and internal ones were recreated as close as possible to the real harsh environment satellite will have to face.

The development of a 2D clinostat (Fig. 3.5.1) with the light strictly connected with the cultivation plane was very useful to recreate both the general cultivation conditions of space in which the light system shall be always over the plant cultivation substrate while gravity stimulus is lost (Paul et al., 2012) and the real condition of the GreenCube mission (Santoni et al., 2020).

The use of ^{60}Co Calliope Irradiation Facility as a chronic γ irradiation plant (Fig. 1.7.1; Fig. 3.5.2) was a challenging opportunity that opens the possibility of a new experimental campaign in which it will be possible to expose both plants and cultivation systems to a low chronic γ environment simulating space conditions.

These two facilities together open a new scenario in plant physiology studies on the evaluation of the effects of multiple stress thanks to the possibility to cultivate plants directly in an irradiation facility experimenting with low or high chronic γ radiations. This allow a better simulation of the final environmental condition to study the space stress effect on plant growth and development than all the research done before.

Without the possibility to have several GreenCube satellite devices to support multiple abiotic stress experimental trials, the use of a crystal-clear vacuum container (Fig. 3.1.1) was the most effective compromise to simulate the CubeSat plant growth chamber. Even if lights and climatic control systems were impossible to be fitted inside the containers, as it is for the real mission, the light stimulus reached the plants thanks to their transparency, and the temperature was managed by controlling the surrounding environment. The

possibility to customize and attach the cultivation plate to the bottom of the container made possible the cultivation in a simulated microgravity environment.

Moreover, it is necessary to cultivate plants in hypobaric conditions since the requirements for safety in a space colony state that space greenhouses shall be at a lower pressure than the crew sectors (Escobar and Nabity, 2017; Esfandabadi and Bannova, 2019). The possibility to manage the gas composition of the cultivation environment will be one of the fundamental constraints of the new colonization conditions (Wheeler, 2009).

Experiments like GreenCube and its on-ground preliminary tests are of fundamental importance to improve the knowledge about the effect of real space conditions, harsher than ISS, on plants development (Rajapakse et al., 2009). Recording data from such a small fully controlled environment, both the inside and the outside conditions, it is what we will need soon to cultivate remotely the first plants on Moon and then on Mars. Remote sensing and remote operations are indeed the real future for plant cultivation in space.

This is the very first study in which multiple abiotic stress was performed on the same cultivation trial simultaneously and that proves that is possible to use a non-destructive method of analysis to verify in real time the metabolic status of microgreens without the sacrifice of samples. The compromise between destructive analysis and non-destructive ones will be another step of the plant's cultivation in space together with the calibrations of them and the preliminary studies for the effect understandings that are only their first's steps toward future cultivation techniques, that goes toward the decision making of a plausible presence of artificial intelligence and, however, to a remote-control work condition.

The selection of full-spectrum LEDs was an optimal choice for this kind of mission (Santoni et al., 2020). Indeed, the array of white LEDs is built with more LEDs than that needed, this is for a redundancy safeness aspect in these kinds of CubeSat missions. Having Red, Blue, and White colours for LEDs need to have a redundancy also for them decreasing the space for other

components. Moreover, the use of a full spectrum light gain feasibility as remote imaging analysis shall be achieved without false colour. Full-spectrum light research on plant physiology is now increasing due to the higher knowledge of photosynthesis mechanisms and all the other light-driven physiological activities (Zhang et al., 2020). Plants are indeed evolved during the aeons using all the light spectrum of the Sun that have effects on thousands of biological aspects. Secondly, the presence of a full-spectrum light makes it possible to take photos without colour alterations meaning a better interpretation of the plant health status during the cultivation process.

The Multiplex optical sensor was used to compare the effects of the stresses on plant materials avoiding the destruction of the samples (Harbinson et al., 2012). This approach will be a key feature of the future space mission in which the image post processing of the samples is difficult to be carried out in space. This non-destructive method of analysis, previously tested and calibrated in an on-ground preliminary test will also allow the possibility remotely sense and measure the physiological status of the plant avoiding the astronaut's presence and interference during cultivation and reducing the possibility of pathogens contamination and diffusion.

The instrument was used in this research activity to use a non-destructive method for the evaluation of pigments content and the comparison among the treatments performed. Thanks to the scientific literature it was possible to use this new instrument at all its potentiality and some of the evidence that came out from its utilization will open the way to a new possibility of applications for space non-destructive evaluation of the physiological status of plants.

Analysis of the effect of the simulated space environment on plant DNA is already carried out using a new solid fast and highly reliable nuclei's extraction technique, that could be run directly into space.

This new technique was developed aiming to analyse the effect of stress at a single plant level reducing experiment time without compromising data accuracy performing fast a great amount of analysis and facilitating analases it shall be possible to easily replicate onboard the ISS. Metabolomics analysis,

even if it will never be possible to perform in space, was done aiming to understand how the space environment affects the metabolites pattern. This was mandatory to better predict the phyto-nutraceutical characteristics of plants that will be eaten in space by astronauts and other space inhabitants.

Even if it is not easy to predict the response to all these stresses together and surely the response to them varies depending on the amount of stress experienced. We hope this work will pave the way for new kinds of experiments in which more than a single stress could be studied at the same time for a better understanding of the joint effect as of a real situation in space.

Plants must face many stresses simultaneously in space and this surely affects their growth capabilities. Analysis of whole the data recorded, highlight some expected effect and trend on several parameters.

Dry weight, as resulting from the 3-Way ANOVA (Table 4.1.5), was mostly affected by "Pressure" (about 21.5% of the total variation; ****) and by "Gravity" stress (about 4.3% of the total variation; **).Pressure effects on Dry weight were higher in hypobaric conditions and these results are in accordance with the works of He et al. (He et al., 2003, 2007, 2008; Rajapakse et al., 2009; He and Davies, 2012) that cultivating lettuce and wheat in small and controlled environment found that hypobaria not only enhance biomass production, and so dry mass but also enhance the leaf area and reduce the production of ethylene. As stated for dry weight also for cotyledonal area pressure effects in hypobaric conditions were higher (He et al., 2003, 2007; He and Davies, 2012). The Dry Weight data represents the detrimental effects of the ionizing radiations and simulated microgravity that, when combined, result in the worst effects registered (R<G<GR).

Ethylene is known to be an antagonist in crop production (Fischer, 1950; JACKSON and OSBORNE, 1970; Dimov, 2000), moreover, if cultivation occurs in a small and closed environment (Massa et al., 2016) as it could be in theCubeSat. The integration of active charcoal in the cultivation substrate was a a good solution to mitigate the adverse effects of ethylene (Yu et al.,

2019) registered in the first trials in which it was not used (data not shown). It helps the regular growth of the plant and particularly the roots.

On the other hand, fresh weight is instead quite always lower for 0.5atm seedlings than 1atm ones as already found by Rajapakse et al. 2009 who find an increase in fresh weight for plants cultivated at atmospheric pressure if compared with low pressure cultivated samples (Rajapakse et al., 2009). By the way, in this case, the PGR condition, the GreenCube simulation, results in the highest fresh weight recorded with a significant difference from the control (Fig. 4.1.1).

Cotyledonal leaf weight was affected by the interaction of Pressure X Gravity (Table 4.1.2). In a past study on clinostat, Yamada et al. 1993 found that the Cotyledonal weight is enhanced by clinorotation as it happens in this study (Fig. 4.1.2) (Yamada et al., 1993). The irradiation exposure seems to harm cotyledonal weight, mostly if it is coupled with microgravity (Table 4.1.2).

Hypocotyl height was mainly affected by "Radiation" stress (Table 4.1.4; about 17% of total variation; ****), followed by the combination of "Pressure X Radiation" stress (about 3.3% of total variation; ****).

Hypocotyl Height was negatively affected by hypobaria as reported in Tang et al 2014 (Tang et al., 2014)but is also affected negatively by radiations (Shakoor et al., 1984; Khalil et al., 1986; Al-Salhi et al., 2004; Majeed et al., 2009). Indeed, trials exposed to γ rays are always shorter than samples not exposed with similar stress imposed, and samples that experience the simulated microgravity are longer than the static ones (Fig. 4.1.4). Indeed, as expected, plants cultivated on clinostat are higher than static cultivated plants (Hoson et al., 1992; Poulet et al., 2016), but those cultivated within the Calliope facility are shorter as radiations have a major influence on the growth of seedlings.

However, plants belonging to the Brassicaceae family, such as *Lepidium sativum L.*, are known to have good radioresistance (Kim et al., 2021).

A detrimental effect of hypobaria was also observed for seeds germination timing that was delayed by about 24h in hypobaric conditions with respect to the 1atm trials (data not shown) without altering the total germination rate at 7 days that was always higher than 95% (data not shown).

Chemical compounds, such as photosynthetic pigments, were affected by the imposed stressful conditions.

The highest value of SFR_R was registered for the GreenCube trial which also had the third-highest cotyledonal weight with a pronounced positive effect of simulated microgravity and ionizing radiations (Zeerak et al., 1994; Gautam et al., 1998; Rascio et al., 2001; Jan et al., 2012)that is reflected also in the total fresh weight (highest value).

It seems that the most stressful condition brings a systemic response that enhances the metabolism and the defensive systems since both for Fresh weight (Fig. 4.1.1) and SFR_R (Fig. 4.2.1) multiplex index, the highest values were recorded for the GreenCube condition, the most harmful one.

Indeed, Anthocyanin's index reported as the ANTH_RB Multiplex index (Fig. 4.2.), reports a lower value for the GreenCube condition, while the precursor of anthocyanins, the flavonols, expressed as FLAV Multiplex index (Fig. 4.2.) are above the general mean. It is possible to speculate that in that stressful condition, antioxidant activities consume antioxidant compounds and enhance their production stimulating the pathway of their precursor.

For what concern non-destructive qualitative analysis carried on with the use of Multiplex, Rajapakse et al. 2009 report lower values of anthocyanins for plants of lettuce cultivated in hypobaric conditions as it was found for P condition cultivated microgreens (Fig. 4.2.) (Rajapakse et al., 2009). Rajapakse also found this diminishing of flavonols' quantity for low pressure cultivated plants as well as for the experimental trials performed in this work of thesis that were exposed to γ rays (Fig. 4.2.) (Rajapakse et al., 2009).

On contrary, Rajapakse reports a decrease in chlorophyll content for plants cultivated at low pressure (Rajapakse et al., 2009) which is in contrast with

data recorded for this work of thesis (Fig. 4.2.1) that instead record a positive effect on chlorophyll of low pressure for samples that were cultivated in static and not irradiated conditions, chronically clinorotated and cultivated within the combination of ionizing radiations and clinorotation, the GreenCube condition that was also significantly different from the control samples.

Ionizing radiations effects are reported to be driven mostly by the total dosage but by the function of the dose rate imposed for the given experiment. Indeed, a very low chronic exposure to γ rays, which are the most energetic form of radiation (Mewaldt, 1994), is very different in terms of effects from an acute but high in intensity exposure even if the final total dose is the same. Ionizing radiations can negatively affect photosynthesis (Arena et al., 2013, 2014, 2017), indeed, in trials that were simply exposed to γ rays the SFR_R index (Fig. 4.2.1) were lower than the other conditions. This effect, however, could be explained also by the dilution effect in the cotyledonal leaves that, for R and PR conditions, were indeed the largest among all the trials (Fig. 4.1.3) (BROUGHAM, 1960; Xiong et al., 2015).

The GR condition results to be the one with the lowest Cotyledonal Weight (Fig. 4.1.2) and Dry Weight (Fig. 4.1.5). It had also one of the lowest cotyledonal areas (Fig. 4.1.3) and the lowest SFR_R index mean value (Fig. 4.2.1). All these detrimental effects of simulated microgravity and radiations were reflected in the total fresh weights, which is one of the lowest, and in a short hypocotyl (Fig. 4.1.4). It seems that the combined effect of the imposed stress had a generally negative effect on the development of this trial cultivated at atmospheric pressure.

Protective phytochemicals compounds such as flavonols and anthocyanins in this work of thesis respond as expected, indeed chronically exposed seedlings to γ rays report higher values of ANTH_RB Multiplex index (Fig. 4.2.) that is positively correlated with anthocyanins content. Clinorotated samples report the lower values among all the treatments while the combination of ionizing radiations and clinorotation is found to be at a lower value with respect to the control condition.

As already described in **Results** (**4.4)**, Multiplex could be a powerful instrument to be used in plant space applications. It is portable, rechargeable, it is simple to use. One of the goals of these analyses was to assess if it was possible to use a non-destructive optical analysis to highlight differences among the samples (Kalaji et al., 2014, 2016). The use of Multiplex could be a promising choice in space plant science because it shall avoid the use of chemical analysis *in loco* and so in space, during the experimentations.

It gives a chance to overstep the bottleneck of laboratory "wet" analysis and ensures the possibility to follow a cultivation process, in a closed environment, day by day for the total time of cultivation resulting in a choice-maker for the best time of harvesting. This study demonstrates that Multiplex can be used to find differences among treatments and that they agree with the chemical procedures, even if the samples number is restricted.

By using Multiplex, it could not be needed the presence of a crew member to collect data, and this means a lower risk of pollution of crop production. Moreover, it can be used to follow the trend of some metabolite changes during the plant growth and development without altering the samples themselves and so let them be available for crew consumption. This is of fundamental importance as it will be possible to choose the right harvest time per crop production taking most of the phytonutrients contained in the plant's tissues.

With this work, it is demonstrated that for *Lepidium sativum L.* microgreens it is possible to analyse the adaxial face of cotyledonal leaves only to measure the content of some leaves' pigments. By using the calibration equation, it will be possible to know the internal concentration of Chlorophyll and Anthocyanins of *Lepidium sativum L.* by using multiplex in space.

Fast output analyses are mandatory in space applications and those analyses need to be simple, replicable and cheap.

With this new method, it could be possible to highlight the effect of space stress on plant DNA in a fast e reliable way. A high quantity of analysis shall be done at a single plant level with high certainty.

This method could be performed on-ground as well as in space with minimum equipment and traineeship. Our method needs a very small amount of sample, and even a single root tip can be analysed.

A good yield of nuclei was recorded considering that the extraction procedure was performed on a single root tip whose fresh weight was about 1mg.

Our cytometer can afford to analyse a not filtered sample and the procedure performed directly into the well of a 96 multiwell plate with a homogenizer make it fast and easy.

This method is an innovation in plant flow cytometry and brings the chopping method a step further as it can be used without any particular skills, helping in plant breeding research and many other studies, such as ploidy changes and cell cycle alterations.

Stresses were reported to have a detrimental effect on the cell cycle (Medina and Herranz, 2010; Qi and Zhang, 2020). However, plants, which are sessile organisms, had developed during their evolution a number of adaptations to cope with stress conditions, and many plants respond to environmental stress with endoreduplication. Endoreduplication occurs when in the G2/M phase paired chromosomes do not separate from each other and cytokinesis does not happen (Barow and Jovtchev, 2007; Dolezel, 2007). By doing this, plants enhance their chromosome number and so their total nuclear DNA content, increasing gene numbers together with all their genetic information which could help to face injuries that may occur. Thanks to flow cytometry it is possible and easy to analyse and highlight DNA changes and these kinds of plant self-defence techniques going to simply recording the intensity of fluorescence emitted by the nuclei in suspension with respect to the control standard population or by comparing the number of nuclei extracted in the G2/M phase with respect to the G1/S phase (Galbraith, 2012).

Lepidium sativum L. is a plant species from the Brassicaceae family. Past flow cytometry studies on this plant family, tried to understand the inner nature of their chromosomal segregation (Halldén et al., 1987; JOHNSTON, 2005). Indeed, it was found that monocentric species chromosomal segregation shall be more susceptible to chromosomal aberration than holocentric species (Stear and Roth, 2002; Escudero et al., 2015; Jankowska et al., 2015). *Lepidium sativum L.* was not found to have the target sequence characteristics of Brassicaceae monocentric species (Halldén et al., 1987), but it seems to have other repeated sequences that shall introduce *Lepidium sativum L.* to the monocentric chromosomal segregation species. Our results showed the effect of chronic radiation brought an enhanced endoreduplication value.

Endoreduplication occurs in the stressed seedlings as the Mean C-Level and the Cycle value indexes for root vary and increase in plants that were exposed to a chronic γ rays irradiation environment. Zedek et al. 2016 have reported in their paper the intention to evaluate throughout flow cytometry the effect of γ rays on nuclear DNA content (Zedek et al., 2016). Our results show an increase in the >2N phase for samples that were chronically exposed to γ rays. RelG2, instead, was less predictive in this case as its value for *R* samples is slightly higher than the control expressing however, together with the endoreduplication indexes, an overall effect of γ radiation exposure on the enhancement of the N phase.

Low RelG2 index values, express the possibility that nuclei of clinorotated seedlings remain stacked in the G1/2N phase as an effect of microgravity stated by Yu et al. 1999 who carried out experiments on lentil seedlings grown in space (Legué et al., 1996; Yu et al., 1999). Indeed, also the mean C-Value and the Cycle Level of that samples have values that are closer to the control.

The combined effect of these two stresses, Gravity and Radiation, shows a decrease in the mean C-Value and the Cycle level. RelG2 shows the same block of G1/2N nuclei for the only clinorotated samples. It seems that the low chronic radiation effect was blocked by the clinorotation effect as it slows the

duplication rate of nuclei avoiding the possible problem of radiation on duplication events (Medina and Herranz, 2010).

However, chronic stress can indeed increase the capability of the nuclear system to self-repair itself as the damage intensity is very low (Spampinato, 2017).

Metabolomic analyses were performed aiming to analyse the effect of the stressful space condition on the metabolite concentration in *Lepidium sativum L.* microgreens.

This was mandatory according to the possibility to use vegetable essences as a nutraceutical source in astronauts' diet (New et al., 2000; Cena et al., 2003; Suri et al., 2020; AlAli et al., 2021; McNulty et al., 2021; Smith, 2021).

In particular, the presence of metabolites that shall be classified as antioxidants in *Lepidium sativum L.* seedlings' extract was found to have an osteoprotective effect on a murine model (Oszmiański et al., 2013; Abdallah et al., 2020). In particular, it seems that Linolenic acid, an ω3, which is the most abundant fatty acid in *Lepidium sativum L.*'s extract, shall reduce the bone loss of density (van Papendorp et al., 1995; Vanek and Connor, 2007; Pankova and Tsvetkova, 2015). This metabolite has a lower concentration in simulated space conditions than in Earth control in this work of study (Fig. 4.3.). It was found that lipids biosynthesis is negatively affected by radiation exposure (Abo-State et al., 2019)and seems to be positively affected by lower pressure.

A similar osteoprotective effect was also attested to the presence of Esculin (Zhao et al., 2017; Abdallah et al., 2020), which is also detected in these experiments, where its concentration is enhanced by simulated space stress (Fig. 4.3.). This metabolite was already detected in past metabolomic analyses of the same specie (Hijazin et al., 2019).

Lepidines have benefic effects particular in candidiasis (Gacemi et al., 2020) that are well known among the astronauts' possible infections (Taylor et al., 1973). This class of metabolites seems also to have a beneficial effect being

effective against pathogenic microorganisms such as the *Micrococcus pyogenes* (aka *Staphylococcus aureus*) (Pande et al., 2002).

The decrease of Lepidine in this study (Fig. 4.3.) under stressful conditions is a negative result for its application as an antimicrobial but it was also studied that Lepidine could have an anti-ovulatory effect and a reduced concentration may result in a lower effect on this side for female astronauts (SHUKLA, 2020).

The presence of different metabolites of the Phenolic acids class was also labelled for their healthy effect on bones and muscles for the murine model (Folwarczna et al., 2009). In particular, among this class of metabolites, the presence of Sinapic acid was predominant. This metabolite is widespread among all the Brassicaceae family as part of many metabolic pathways (Nguyen et al., 2021). It was studied for its medical application (Hameed et al., 2016; Pandi and Kalappan, 2021) ranging from antimicrobial activity (Chen, 2016) to antianxiety activity (Yoon et al., 2007), and is also effective as a cardioprotector (Roy and Stanely Mainzen Prince, 2012). Its internal concentration in seedlings' extract showed a positive accumulation with respect to the control for the simulated space conditions (Fig. 4.3.) and this is a positive result thinking about the possibility to use Garden cress as a nutraceutical source for astronauts' diet.

As written before, astronauts suffer from eyes disease. *Lepidium sativum L.* seedlings' extract is plenty of Lutein that belongs to the carotenoid class and is well known to have a positive effect on eye-related misfunction and deterioration (Abdel-Aal et al., 2013; Buscemi et al., 2018; Li et al., 2020; Montesinos et al., 2021 In particular, it was stated a good and positive accumulation of Lutein in stressed plants beside the control situation (Fig. 4.3.), and these positive accumulations are due to the synergic and antagonistic effects of the imposed stresses, in particular microgravity (Table 4.3.7).

Vitamins are a class of metabolites that are essential to living in space. Many studies had been carried out on astronauts' diets attempting to give them the

proper nutrition to cope with the space environment (Smith et al., 2005; Tang et al., 2021). Anderson et al 2015 wrote the guidelines for humans in space which stated the daily allowance for astronauts according to the NASA agency (Anderson et al., 2015). Within these guidelines is often reported as the prepackaged food given to astronauts is not enough for their nutrients intake and that has to be covered with the use of food supplements.

Vitamin K, detected in seedlings' metabolites' extract, was already studied as a countermeasure in bone-related diseases (Vermeer et al., 1998; Stein, 2001; Zwart et al., 2011). This particular vitamin changes concentration in the stressed trials (Fig. 4.3.) caused by the effect of Gravity stress (Table 4.3.11).

Vitamin B also reports a positive enhancement of its concentration in stressed conditions, in particular in the GreenCube one (Fig. 4.3.). Vitamin B is known to have a beneficial effect as an antimicrobial element (Ahgilan et al., 2016).

Vitamin E shall be used in space applications with a therapeutical purpose for cardiovascular (Shekelle et al., 2004) and eye disease (Tanito, 2021). Its low variability and slightly enhancement in simulated space conditions (Fig. 4.3.) for these experimental trials shall be a good point in favour of the choice of *Lepidium sativum L.* seedling for astronauts' diet.

Vitamin A, like vitamin E, is radiation-sensitive (Watkins et al., 2022), and food storage for space applications often loss its internal vitaminic concentration. In Garden cress grown for this experiment, fast grow seedlings accumulate a higher concentration of provitamin A in the stressed conditions (Fig. 4.3.; Table 4.3.8). In particular, a significantly higher concentration was found for plants cultivated in microgravity conditions (Fig. 4.3.). it is well known that pro vitamin A had a positive effect on humans (Jaswir et al., 2011; Michalak, 2022) and its increase in this experiment is another good example of how microgreens shall be used in space for nutrients integration in astronauts' diets.

The use of plants, and in particular Garden cress, in space application shall take into account the complexity of its metabolites pattern.

6. Conclusions

For the GreenCube mission on-ground preliminary test was developed an on-ground comprehensive simulation of space stress. This was made effective thanks to the connection of several facilities able to perform single space stress like chronic radiation, chronic simulated microgravity and chronic hypobaric environment. This was done with the aim to recreate an experimental environment as close as possible to the real situation GreenCube Cubesat will face during its mission orbiting at 6000km altitude.

The possibility to simulate and so to experience all these stresses simultaneously is of fundamental importance because in the following years the main space objective will be to bring a stable colony on the moon where plants must surely cope at least with hypobaria and ipogravity.

Even if on ground preliminary test experimentations are very far from the real situation in space, this piece of work was performed as one of the first experimental activities in which most of the space stresses that plants will have to cope with, and so microgravity, hypobaria and chronic radiations were present simultaneously and affect plants growth and development.

To perform this experiment, was necessary to develop a cultivation system to be used to expose plant samples to different space stresses. In particular, it was developed a light system that rotates solidly with the rotation of the clinostat to better replicate the real space situation in which the artificial light system will always be over the plants' cultivation systems. This system was added of sealed crystal-clear cultivation container that can maintain low pressure for a long period. Finally, the experiment was run into Calliope ^{60}Co irradiation facility at ENEA “Casaccia” Research Centre, where was possible to perform low chronic γ radiation.

In this framework, ENEA could be more than a simple player as it housed experts and facilities that can fine reproduce the harshest space environment to plants, animals (such as insects) and architectonic and electronic infrastructure.

Analyses like morphometric measurements are still one of the major phenotypic analyses that are possible to be carried out in space, both live and by remote sensing. Many of the morphological measurements that were performed during this work of thesis are indeed easy to convert into remote imagery analysis so it will be possible to follow the growth of samples in space without taking time from the crew's programmed schedules and to understand when plants will be ready to be eaten by the crew.

Together with morphometrical analyses, it was suggested the use of a non-destructive optical system for chemical composition analyses that, besides the accuracy of the measurements, could be a good compromise between the loss of possibly eatable vegetables and the fast superficial analysis with the instrument that in the end save the crop at least from the lab destructive analysis.

This new approach proposed for space applications shall have more resonance as it allows to perform analyses directly in space avoiding the problems related to the conservation and transport of the samples from space back to the Earth that surely affect their integrity and accuracy.

Furthermore, in this work of thesis, it was suggested to use a plant flow cytometry approach to analyse the effect of space stress on plants development.

Onboard the ISS has housed a flow cytometer that shall be used for this purpose together with proper training of the crew members dedicated to the experiments and also with the use of the new extraction method, here presented, to run the analysis.

Indeed, as the point was to develop fast and reliable methods to perform analysis in space, plant flow cytometry analysis shall be one of them. This new nuclei’s extraction method shall be performed directly in space conditions, with a small amount of sample, even at a single root level. Further studies in real microgravity of the extraction procedure during parabolic flight shall confirm this sentence.

However, performing plant flow cytometry analysis directly in space needs to detach from some cytometric dogma still under studying. The use of a biological sample with known DNA content cannot be used as a standard in space conditions as it will face the same extreme environmental factors as the samples but may respond to the stress in many different ways, mystifying the results.

Firstly, to face this problem, it was proposed to use a non-biological, incorruptible, and unchangeable standard that will be not affected by the external environment in terms of dimension and fluorescence emission. Surely this approach needs more time and more experiments to be deeply investigated, and a proper alternative must be found to bring over the possibility to perform these kinds of analyses in space. With this work it was paved the way to a new research era of plant flow cytometry.

Metabolomic analysis shows how hard is to find what happens inside plants cells that drive their metabolic pathway when “space stress” were imposed. Besides that, it shows also how microgreens, with their beneficial nutraceutical compounds, will be needed in space journeys aiming to expand and enrich the nutraceutical proposal of the astronauts’ diet to face the space environment.

For sure, we lack deeper analysis at genomic level that must be taken into account to understand which stress and how it enhanced or lowered the metabolite production in a complex space simulation like it was done for this work of thesis.

Among all the measurements done, it seems that *simulated microgravity* had the widest effect on plants' phenotype. And above all, the presence of interactions among the stress imposed during the cultivation of microgreens states that the cultivation of plants in space is affected by a larger quantity of stress and that to better understand how those stress affect their growth, it is needed to perform them simultaneously or, preferable, directly in space environment.

After this work, it is possible to say that *Lepidium sativum L.* can be cultivated as microgreens in the spacecraft environment. Its nutraceutical properties were influenced by these environmental factors but, however it grown into a harsh environment, in hypobaric conditions, during simulated microgravity and chronic γ radiation.

It was noticed a lack of literature on the effectiveness of microgreens beneficial properties on human beings. It's mandatory to underline the necessity to perform accurate studies on this topic in the following years to assess the bioavailability and efficacy of microgreens nutrients on crew members during stressed condition.

Moreover, it wasn't found in scientific literature a comprehensive approach to studying different space stress simultaneously and made difficult to ensure the effect on phenotype to a specific space stress.

Finally, this kind of research comes from the awareness that is mandatory to work in a multidisciplinary environment, surrounded by experts from every field of study. We need to have as many as possible points of view on the same topic. This approach is promising for new experiments and humankind exploration where crew members will be constituted of engineers, biologists, agronomists, psychologists, and human doctors to help to cope with new colonization challenges.

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