In-Situ Resource Utilization (ISRU) for life support in Space

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Science is not a collection of facts; it is a process of discovery.

Robert Zubrin

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General Introduction

During the past century, we have witnessed humanity's great strides which defeating gravity and going beyond Earth, driven by the desire for discovery. Like the European explorations overseas in the XV century led to the discovery of unknown lands, these new goals could lead to a new era of exploration for humankind. The 21st century is an era rich in scientific findings with rapid technological development. Hence space travels and the colonization of other celestial bodies would result no longer an image close to the world of science fiction but would be desirable soon.

With this perspective, many Space Agencies, Research Centers, and Universities are working to establish colonies self-sustaining and independent by Earth, first on the Moon e then on Mars. The establishment of such a complex system is expensive, in addition, it must be considered the commitment to planning and managing the replenishments, which involve other additional costs also. The estimation of launch costs ranges from 10,000$ up to 300,000$ per kg (for the Low Earth Orbit and Mars surface, respectively), clearly would become excessively expensive and complicated to manage. So, it is mandatory the costs reduction through the implementation of Bioregenerative Life Support Systems (BLSSs). To make it, in a future outpost, we should use mostly the native materials and recycle all kinds of waste as far as possible, as proposed in the *In Situ* Resource Utilization (ISRU) approach. In a future long-term settlement, a sustaining and auto-regenerating solution could be permanent agriculture, applicable to the current constraints, which ensures more than the sole food.

Bioregenerative life support systems

The realization of long-term manned space-exploration missions and the permanence of human colonies on orbital stations or planetary habitats will require Bioregenerative Life Support Systems (BLSSs). BLSSs are systems capable of regenerating large parts of the essential resources for humans' survival (i.e. H_2O, O_2, food), solving the constrain of the unfeasible complete re-supply from Earth (Wheeler et al., 2003).

The regeneration of resources for Space application focused on physical-chemical processes until the 1950s, when the first reasoning on plants as both a regenerating tool and source of food led to the introduction of algae for life support in Space in the studies of the US Air Force, (Myers, 1954). In the last decades, extensive studies have focused on the development of systems for life support in Space based on living organisms. These are artificial ecosystems based on the harmonization of compartments involving both living organisms and physical-chemical processes to achieve a safe, self-regulating, and chemically balanced artificial ecosystem to support human life, reconverting the waste produced by the crew into nutritional biomass, oxygen, and potable water (Hendrickx and Mergeay, 2007). Specifically, they are modular systems including sub-units hosting microorganisms, plants, and animals able to accomplish different specific functions in a closed regenerative loop (Wheeler, 2010). Bioreactors have been widely explored to grow microalgae for food, waste treatment, and production of biomaterials. However, among biological components, higher plants are the most promising because, as every photosynthetic organism, they regenerate the atmosphere by CO_2 uptake and O_2 production, can recycle nutrients derived from human wastes and provide not only food tastier and more complex than algae, but also non-nutritive benefits. Indeed, higher plants play a role in psychological support against conditions of isolation, which are recognized to elicit behavioral changes in crew members (Paradiso et al., 2014).

To realize space greenhouse modules, and even more in the case of BLSSs, there is common agreement on the need for the integration of different disciplines and approaches to achieve a deep knowledge of both plant biological processes and crop production techniques under Space constraints (De Micco et al., 2009).

The literature, about Space experiments with plants clearly demonstrated that plants can be cultivated in Space (in microgravity conditions on Low Earth Orbit spacecraft) even achieving the seed-to-seed cycle.

As well, cultivation protocols and procedures are species- and cultivar-specific and need to be defined once the response of plants to cultivation factors is unraveled. Therefore, ground-based experiments are needed for understanding how growth efficiency and yield (considering all plants' outputs as oxygen, biomass, etc.) is affected by cultivation and simulated Space factors, to establish the requirement specifications for all subsystems, including the lighting system and a nutrient delivery system (De Pascale et al., 2021).

In situ resources utilization

The Utilization of extraterrestrial resources, or the In-Situ Resource Utilization (ISRU), is the collection, processing, storing and use of materials encountered during human or robotic space exploration that replace materials that would otherwise be brought from Earth (Sacksteder and Sanders, 2007). The key to survival of a self-sustaining extra-terrestrial colony will be its smart use of in-situ resources that are available. For instance, in a future settlement, it would be possible to fabricate bricks and panels from local materials (ie., Lunar concrete) and use them for constructing habitats, greenhouses, workshops, and storage buildings. Metals could be extracted from local rocks and soil to make beams, wires, and solar power cells. The gases contained in the regolith could also be used in inflatable structures, clothing, and insulation could be made from fabric woven from basalt material (O'Handley et al., 2001). ISRU is viewed as an enabling technology for the exploration and commercial development of space. It is fundamental to any program of extended human presence and operation on other extraterrestrial bodies that we learn how to utilize the indigenous resources (Sanders, 2000). One of the main needs for ISRU processing is to replace life support gases (oxygen, nitrogen, water, etc.) that are lost due to leaks and airlock operations. A closed-loop life support system is required in the colony. However, any closed-loop life support system will have some losses. ISRU products would be used to replace these losses. For instance, carbon dioxide could be extracted from the regolith or from the atmosphere to support plant growth, which in turn would produce oxygen and food. With the chief benefits of being able to reduce the mass, cost, and risk of space exploration while providing capabilities that enable the commercial development of space (Sanders, 2000).

Candidate crops

Plants can adapt to extreme environments on Earth, and model plants have been shown to grow and develop through a full life cycle in microgravity. Growing crops in space is as much about developing the humans' technological capacity to provide plants with adequate growth conditions in the unique environment, as is about the symbiotic relationship between plants and space travelers (Stankovic, 2018). However, when growing plants on extended missions, other requirements become increasingly important. Space travel brings about constraints do not present on Earth. Space, energy, and mass will be limited on board the spacecraft and the spaceflight environment (e.g., microgravity, reduced gravity, radiation) may create problems for plant culture and development. For these reasons, the selection of suitable crops will depend on species performance within the restrictions imposed by recycling ecosystems; and on the ability of harvested parts to meet human needs (Hoff et al., 1982).

Research on the identification of possible species that could support human life in space environments started in the early 1960s, and several experiments have been carried out so far (Chunxiao and Hong, 2008). For selecting promising species to involve in bioregenerative systems were identified general criteria, below it will be mentioned some of the main ones, such as 1) Energy concentration (considering the amount of the major nutrients, particularly energy like the calories in the food consumed); 2) Nutritional composition (plants contain many mono-or poly-functional components important in human nutrition); 3) Processing requirements (number and complexity of processes needed to make the vegetable ready to eat); 4) Harvest index (percentage partitioning between edible and inedible plant parts); 5) Yield (as amount produced per unit growing area per time, affected by light use efficiency and efficiency of cultivation space).

Therefore, we can differentiate two categories of candidate crops. 1) that it will be call "energy crop", consisting of tubers, cereals, and legumes, plants that have a high energy value. 2) that it will be call "salad crop" which unlike the latter have shorter cycles, do not require preparation to be consumed (ready to eat), and have high nutraceutical values.

According to the results of an initial survey of the acceptance of fresh vegetable crop candidates by space station crew members, lettuce (*Lactuca sativa* L.) was the most preferable crop among the various leafy greens tested (Mauerer et al., 2016). Its leaves constitute a nutritious food source, it is well-established in human diets, and when consumed in large quantities it could fulfill the recommended daily intake of most macro- and micro-nutrients (Mou, 2012). The nutritional value and bioactive compounds content of lettuce can be regulated within proper environmental conditions (e.g. light intensity and spectrum, nutrient solution composition, atmospheric CO_2 conditions, etc.), while the great availability of cultivars with very diverse qualities has proven to be the key to this species successful cultivation in space farms (Konstantopoulou et al., 2010; Park et al., 2012; Kang et al., 2013).

Study perspective

Plants grown on Earth require a certain combination of environmental parameters (temperature, humidity, light intensity, concentrations of nutrient salts, and others). The cultivation in bio-regenerative systems will require that we provide Earth-like conditions to ensure plant growth. So, the task is to create an artificial environment (a greenhouse) that is adequate for plant growth at an appropriate rate, and at an acceptable cost in terms of mass, energy, and other resources (Rygalov et al., 2001). A brief digression is indispensable to clarify that we are aware that the term "soil" includes a whole series of characteristics that the simulants (and the respective samples of reference) do not have. Nevertheless, we used consciously to ease the narrative flow.

The employment of the ISRU and the development of plants cultivation protocols soil-based, to apply in a future extraterrestrial settlement, was the focus of this work.

With this aim, step-by-step proceeding, first identifying substrates and their characteristics (strong and weak points) and then focalizing on the gaps to fill. Due to the peculiarity of the project, it was not possible to use native soils. As an alternative to this, simulants were adopted. In this regard, many simulants were mainly developed to test specific aspects like the abrasiveness of dust on the spacesuits or rovers. Observing a scarce use in the agronomic field, therefore, we reviewed the current scientific literature to understand how to use the simulants as substrate for the cultivation (Chapter 1), drawing a line on the applicability points and the future challenges. Although the simulants are lacking in some nutrients (nitrogen, phosphorus, sulfate, and organic matter), and they show high electroconductivity, an alkaline pH, and a texture like the sand, they can be improved through a terraformation process.

For this purpose, using waste materials (like plants residues and the feces of astronauts) may be a suitable solution to enhancement the regolith characteristics and limit costs. These materials can work both as nutrient supply for the plants and as amendments, improving soil structure. So, to test our idea, we have selected two simulants; the first one was a Martian soil simulant (MMS-1), while the second one was a simulant of Lunar

Highlands (LHS-1). In the beginning, we have also selected another simulant, the MGS-1 clay enriched. The preliminary tests highlighted a high inability as growth media (excessive compactness, high concentration of soluble salts, and very alkaline pH), so we preferred to exclude them in the successive experiments We chose as amendments 1) a green compost, as surrogate of future plants residues composting, and 2) manure deriving by monogastric animals, as surrogate of astronauts feces.

We mixed different amounts of simulants and organic amendments to find the better recipe for a suitable growth media, able to guarantee the plants' outliving and taking full advantage of the waste recycling. Following a reverse path, we have also evaluated the effects of cultivation on the different substrates, performing a deep investigation about the simulants and the amendments features, analyzing these last before and after the plant growth.

In Chapter 2 a detailed study on geochemical and mineralogical composition of MMS-1 was performed. To assess how the compost addition may impact the sustainability of space agriculture by exploiting local resources and how change the physico-chemical and hydraulic properties of growing substrates. These last were obtained by mixing MMS-1 and green compost at varying rates (0:100, 30:70, 70:30, 100:0; v:v). We selected and applied a commercial green compost of pruning waste and grass cuttings with a low C/N ratio (to simulate as closely as possible the compost produced in a bioregenerative life support system). Evaluating the potential of these mixtures as a growing medium for two butterhead cultivars of lettuce (*Lactuca sativa* L. var. *capitata*). The Experiment was carried out under growth chamber-controlled conditions, using a modified Hoagland nutrient solution.

While the effects on yield, physiological (net CO_2, Water Use Efficiency, and stomatal resistance) and qualitative traits (mineral composition, polyphenol profile, and antioxidant activity) of two lettuce cultivars with different pigmentations were the focus of Chapter 3.

To make these "soils" an adequate medium for plant growth, in Chapter 4 were evaluated the effects of monogastric manure on Lunar and Martian simulants, using four different rate manure: simulant (0:100, 10:90, 30:70, 50:50; w:w); as target plant was chosen a lettuce cultivar (Grand Rapids) well studied in space research. The agronomic performances of mixtures were evaluated, specifically the lettuce growth and its nutrient uptake; the microbial biomass (C and N); the enzymatic activity; and the changes in nutrient bioavailability due to plants activities. A detailed study on root zone was conducted to increase the understanding of the terraforming process. To try to make possible the exploitation of Mars and Moon regoliths for agricultural purposes in a future BLSS.

Contextually, in Chapter 5 we assessed nutritional parameters of lettuces grown on two simulants (in growth chamber condition). To evaluate the nutraceutical properties such as the antioxidant activity and the presence and the amount of several healthy compounds (i.e., flavonoids and β-carotene) in order to find the best combination that maximizes its nutritional value while ensuring an acceptable yield.

Chapter 1

The Potential for Lunar and Martian Regolith Simulants to Sustain Plant Growth: A Multidisciplinary Overview

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Abstract

Bioregenerative life support systems (BLSS) are conceived of and developed so as to provide food sources for crewed missions to the Moon or Mars. The *in situ* resource utilization (ISRU) approach aims to reduce terrestrial input into a BLSS by using native regoliths and recycled organic waste as primary resources. The combination of BLSS and ISRU may allow sustainable food production on Moon and Mars. This task poses several challenges, including the effects of partial gravity, the limited availability of oxygen and water, and the self-sustaining management of resources. Lunar and Martian regoliths are not available on Earth; therefore, space research studies are conducted on regolith simulants that replicate the physicochemical properties of extra-terrestrial regoliths (as assessed *in situ* by previous missions). This review provides an overview of the physicochemical properties and mineralogical composition of commercially available Lunar and Martian regolith simulants. Subsequently, it describes potential strategies and sustainable practices for creating regolith simulants akin to terrestrial soil, which is a highly dynamic environment where microbiota and humified organic matter interact with the mineral moiety. These strategies include the amendment of simulants with composted organic wastes, which can turn nutrient-poor and alkaline crushed rocks into efficient life-sustaining substrates equipped with enhanced physical, hydraulic, and chemical properties. In this regard, we provide a comprehensive analysis of recent scientific works focusing on the exploitation of regolith simulant-based substrates as plant growth media. The literature discussion helps identify the main critical aspects and future challenges related to sustainable space farming by the *in situ* use and enhancement of Lunar and Martian resources.

Keywords: *in situ* resource utilization, regolith simulants, space exploration, Moon, Mars, extra-terrestrial farming, microgravity, food production.

1 Introduction

Current scientific inventions and technological advancements may allow space travel and, in the far future, the development of bioregenerative life support systems (BLSS) on other celestial bodies (Zubrin and Wagner, 2011; NASA, 2018). From this perspective, the colonization of the Moon or Mars is closer to reality than it is to science fiction.

Studies focusing on various aspects of life on other celestial bodies have helped make it possible to contemplate extra-terrestrial colonization. These include studies on celestial bodies with a gravitational pull different from that on Earth (Hoson et al., 2000), the recycling of oxygen and water (Primm et al., 2018), and other issues related to sustainable food production in controlled environments or BLSS. The provision of terrestrial resources to permanent extra-terrestrial human settlements is not economically sustainable (Verseux et al., 2016), not only because of the high cost and resource/energy requirements, but also due to the difficulty and time needed to plan and execute launches (Llorente et al., 2018). Given the long-term nature of space missions and future space settlements, BLSS also need to be self-sustaining so as to reduce inputs from Earth and to deal with any challenges threatening the success of the missions. An efficient BLSS must be capable of purifying water, revitalizing the atmosphere, and producing food in a closed loop system (Menezes et al., 2015; Foing et al., 2018; Llorente et al., 2018). This can also be accomplished through *in situ* resource utilization (ISRU), which requires the use of native materials (Karl et al., 2018) and waste as primary resources (Menezes et al., 2015). Instead of relying on a closed loop, new materials found on site could be brought into the life support systems, thus making them sustainable and expandable.

Numerous studies have evaluated the feasibility of life on other planets. Some have conducted hydraulic and engineering tests to assess the practicality of building (Gertsch et al., 2008) and manufacturing (Chow et al., 2017; Karl et al., 2018), whereas others have experimented with microbial (Verseux et al., 2016; Kölbl et al., 2017) and plant growth (Gilrain et al., 1999; Kozyrovska et al., 2006; Wamelink et al., 2014) in an extra-terrestrial environment. Space farming based on local resource exploitation (Maggi and Pallud, 2010; Ramírez et al., 2019) is a promising strategy for food production (Ming and Henninger, 1989) on extra-terrestrial habitats, as it can allow water recycling, organic waste composting, and oxygen production or CO_2 consumption (Verseux et al., 2016; Llorente et al., 2018). This would also reduce the launch mass from Earth and the waste generated by human settlement (food cost-cutting). Another important aspect is the psychological comfort plants can provide for astronauts during their long period of isolation (Nechitailo and Mashinsky, 1993; Ivanova et al., 2005; Marquit et al., 2008; Bates et al., 2009).

An ISRU approach for fresh food production is crucial to guarantee sustainability in extra-terrestrial BLSS. Using the local regolith as "soil" for plant growth would be a viable way to grow food, even though "extra-terrestrial soil" is very different from vital and fertile "terrestrial soil" (Certini et al., 2009, 2020; Juilleret et al., 2016). The appropriate term for the surficial unconsolidated fine mineral material on other planetary bodies is regolith, as it lacks living matter and is still very similar to the underlying parent rock. Soil taxonomy defines soil as "*a natural body that comprised solids (minerals and organic matter), liquid, and gases that occur on the land surface, occupies space, and is characterized by one or both of the following: horizons, or layers, that are distinguishable from the initial material as a result of additions, losses, transfers, and transformations of energy and matter or the ability to support rooted plants in a natural environment*" (Soil Survey Staff, 2014). Regolith does not have associated organic matter or a microbiome. In theory, regolith can be classified as soil if it has undergone the same processes that the Earth-based regolith undergoes to become soil (e.g., the presence of oxygen, the influences of wind and water, and activity by living organisms). Likewise, regolith can be defined as soil if it has undergone organic processes. Due to the lack of a standardized method for evaluating regolith efficacy, many published studies claiming to have assessed regolith for plant growth have used microbiome-contaminated regolith, which is in effect a soil.

The Lunar science community uses the word "soil" in an engineering geology sense, identifying "Lunar soil" with the finer-grained fraction of the unconsolidated material (regolith) on the Lunar surface (Heiken et al., 1991). A more complex and informative definition of extra-terrestrial soils (along with attempts of classification) has been provided by Certini et al. (2009, 2020). These native soils can be defined as the (bio)geochemically/physically altered material on the surface of a planetary body that encompasses surficial extra-terrestrial telluric deposits. According to this definition, the soil is a material that retains information about its environmental history, and whose formation does not require the presence of life. In this context, and considering the known geochemical features of extra-terrestrial regoliths, the surface deposits on planetary and other celestial bodies—such as Venus, Mars and Earth-Moon—should be considered soils in a pedological sense (Certini et al., 2009). Moreover, the chemical diversity across *in situ* and regional soils on Mars suggests the existence of many different soil types and processes (Certini et al., 2020). In this review, we frequently use the term “soil” when referring to the surface of the Moon and Mars as a potential crop substrate, and adopt this term when discussing the literature.

The Lunar regolith has been studied on the Moon, and has also been analyzed on Earth using samples brought back by the Apollo missions. The regolith on Mars has been analyzed by rovers and robotic spacecrafts. These studies have elucidated the physical and chemical properties of Lunar and Martian native soils. Nevertheless, there is only a minimal quantity of Lunar material on Earth that is closely guarded, and no samples have been brought back from Mars to date. Therefore, most commercial regolith simulants have been produced by closely replicating the specific physicochemical properties of extra-terrestrial surfaces. Most existing simulants were developed to address specific application fields, and although their chemical interactions or properties related to mechanical abrasion have been assessed to mitigate potential risks (Rickman et al., 2013), their agricultural properties have rarely been evaluated. Naturally, plant growth, morphology, and physiology on the Moon or other planets are expected to be greatly affected by the sterile and nutrient-poor nature of extra-terrestrial soil and the different gravitational and climatic conditions. However, these regolith simulants, although not sterile, may play an essential role in improving our understanding of the environmental phenomena on the Moon and Mars. They may also help solve potential problems related to the exploitation of Lunar and Martian regolith as plant growth substrates.

Table 1. List of the simulants considered in the present work, divided by reference category. In detail the availability on the market and the reference analysis for chemical and mineralogical characteristics.

Category	Simulant Name	Commercialised	Bulk Chemistry	Mineralogy	References
Lunar Dust Simulants	BHLD20	May Be Available	XRF	XRD and SEM	Sun et al., 2017
	CLDS-i	May Be Available	XRF	XRD	Tang et al., 2017
	DUST-Y	No	N/A	N/A	Britt and Cannon, 2019; Cannon and Britt, 2019
	Kohyama Simulant	May Be Available	Not specified	Not specified	Sueyoshi et al., 2008
Lunar Highlands Simulants	LHS-1	Yes	XRF	XRD	https://sciences.ucf.edu/class/wp-content/uploads/sites/23/2019/02/Spec_LHS-1.pdf
	NAO-1	May Be Available	XRF	Not specified	Li et al., 2009
	NU-LHT/1M/2M/3M/1D/2C	May Be Available	Calculated	Not specified	Stoeser et al., 2010a; Zeng et al., 2010
	OB-1	May Be Available	QEMSCAN	EDS	Battler and Spray, 2009
	OPRH2N/H2W	Yes	Calculated	Use a mineral recipe	McKay et al., 1994
	OPRH3N/H3W	Yes	Calculated	Use a mineral recipe	
Lunar Mare Simulants	BP-1	May Be Available	XRF	XRD	Rahmatian and Metzger, 2010; Stoeser et al., 2010b; Suescun-Florez et al., 2015
	CAS-1	May Be Available	XRF	CIPW normative	Zheng et al., 2009
	CSM-CL	May Be Available	XRF	Not specified	van Susante and Dreyer, 2010.
	CUG-1A	May Be Available	XRF	Not specified	He et al., 2010
	FJS-1	Yes	XRF	Not specified	Kanamori et al., 1998; Matsushima et al., 2009
	FJS-2	Yes	XRF	Not specified	
	FJS-3	Yes	XRF	Not specified	
	JSC-1/1A/1AF/1AC/2A	May Be Available	XRF	XRD	McKay et al., 1994; Sibille et al., 2006
	LMS-1	Yes	XRF	XRD	https://sciences.ucf.edu/class/wp-content/uploads/sites/23/2019/02/Spec_LMS-1.pdf
	OPRL2N/L2W	Yes	Calculated	Use a mineral recipe	McKay et al., 1994
	Oshima Simulant	May Be Available	Not specified	Not specified	Sueyoshi et al., 2008

Mars Simulants	JEZ-1	Yes	Calculated	XRD	Cannon et al., 2019
	JMSS-1	May Be Available	XRF	XRD and SEM-EDS	Zeng et al., 2015
	JSC-Rocknest	May Be Available	XRF	XRD	Archer et al., 2018; Clark et al., 2020
	KMS-1	May Be Available	N/A	N/A	Lee, 2017
	MGS-1 /1S/1C	Yes	Calculated	XRD	Cannon et al., 2019
	Y-Mars	May Be Available	XRF	XRD	Stevens et al., 2018
	MMS-1	Yes	WD- and ED-XRF	XRPD	Caporale et al., 2020

All data are from the Planetary Simulant Database and are dated April 2019. May be available= simulant is not commercialised online, but, if requested for scientific purposes, may be available from the producers in small amounts; N/A= Not available information; XRF= X-ray fluorescence; XRD= X-ray diffraction; SEM= Scanning electron microscopy; QEMSCAN= Scanning electron microscope, equipped with up to four light element energy-dispersive X-ray detectors and an electronic processing unit for automated quantitative evaluation of minerals; EDS= Energy dispersive X-ray spectrometry; WD-= Wavelength dispersive; ED-= Energy dispersive; XRPD= X-ray powder diffraction.

This review aims to provide a comprehensive overview of the potential for existing Martian and Lunar simulants to serve as substrates for growing crops in BLSS. First, we assess selected Lunar and Martian regolith simulants on the basis of their physicochemical and mineralogical properties. Second, we describe previously tested strategies and sustainable practices for using these simulants as plant growth media, with emphasis on the main critical aspects and challenges in deploying these systems. In this review, we consulted 74 scientific papers and 10 technical reports on Lunar and Martian regolith simulants published between 1970 and 2021. The main critical aspects of space agriculture are presumed to be related to nutrient availability, air and fluid movements in different gravitational conditions, and potentially toxic elements in the substrates. Potential future challenges include a lack of adequate knowledge about the extra-terrestrial environment and the development of best agronomic practices for the first space colony.

2 An overview of the properties of regolith simulants

This review provides an overview of the petrographic/mineralogical compositions and bulk chemistry of Lunar and Martian regolith simulants developed over the last 3-4 decades, which are mostly available for research purposes. We included simulants listed in the Planetary Simulant Database (https://simulantdb.com/) of the Colorado School of Mines. Of these, we selected 30 simulants whose petrographic/mineralogical and chemical characteristics had been described in 27 scientific papers and included in the Planetary

Simulant Database (Table 1). According to the Planetary Simulant Database classification scheme, the included simulants were divided into four categories: 11 Lunar Mare simulants (McKay et al., 1994; Kanamori et al., 1998; Sibille et al., 2006; Sueyoshi et al., 2008; Zheng et al., 2009; Matsushima et al., 2009; He et al., 2010; van Susante and Dreyer, 2010; Rahmatian and Metzger, 2010; Stoeser et al., 2010b; Suescun-Florez et al., 2015); 6 Lunar Highlands simulants (McKay et al., 1994; Battler and Spray, 2009; Li et al., 2009; Stoeser et al., 2010a; Zeng et al., 2010); 4 Lunar Dust simulants (Sueyoshi et al., 2008; Sun et al., 2017; Tang et al., 2017; Britt and Cannon, 2019; Cannon and Britt, 2019b); and 9 Martian simulants (Zeng et al., 2015; Lee, 2017; Archer et al., 2018; Stevens et al., 2018; Cannon et al., 2019; Caporale et al., 2020; Clark et al., 2020).

2.2 Mineralogy of the Lunar and Martian simulants

There is no universal simulant that comprehensively represents the mineralogy of the Lunar and Martian surfaces. Similar to the Earth's crust, the surficial layers of the Moon and other planets show high heterogeneity and spatial variability. Therefore, it is difficult to create a simulant for every mineralogical combination or potential application. To develop a simulant, it is crucial to find terrestrial rocks with compositions and qualitative and quantitative mineralogical patterns similar to those of Lunar and Martian regoliths. Mineralogical assemblages can be modified to reproduce the general variability on the Moon and Mars. However, simulants comprised of the majority of minerals found in Lunar and Martian surficial regoliths often lack some minor or rare phases (including phosphates, sulfides, and phyllosilicates) that affect ISRU and plant growth. This limitation can be overcome by the exogenous addition of minerals that are deficient in the selected rocks. However, even with this addition, it is difficult to replicate all regolith characteristics in a single simulant (Seiferlin et al., 2008). As a result, many research teams have produced their own simulants over the years (Cannon et al., 2019). In any case, no existing simulants contain moisture or biological components (Gertsch et al., 2008).

The surface morphology of the Moon is dotted with meteorite and micrometeorite impact sites (Gertsch et al., 2008). Samples brought back to Earth by the Apollo missions at the end of the 1970s revealed that the Lunar regolith was a mixture of varying amounts of two primary rocks: 1) the Lunar Mare dark basalt and 2) the lighter-colored, feldspar-rich anorthosite of the Lunar Highlands. These are mixed with an approximately constant proportion of impact melt glass (McKay et al., 1994). Based on the mineralogical composition of this regolith, Earth & Space 2006 and the 2nd NASA/ARO/ASCE Workshop on Granular Materials in Lunar and Martian Exploration (Malla et al., 2006) proposed two compositional end-members of Lunar simulants to be used as an ideal set of root simulants: 1) low-Ti basalt for Lunar Mare and 2) high-Ca anorthosite for Lunar Highlands. Indeed, the Lunar Mare simulants FJS-3, Oshima simulant, FJS-2, OPRL2N, and FJS-1 are 81–100% basaltic (Table S1, Supplementary Material), whereas the anorthositic rocks in the Lunar Highland simulants NU-LHT/1M/2M/3M/1D/2C, OPRH2N/H2W, and OPRH3N/H3W are 43–80% basaltic (Table S1). As shown analytically (Table S1) and synthetically (Figure 1; Table S2), the mineralogical compositions of only 11 Lunar simulants have been characterized.

The mineralogy of the Lunar Mare and Highlands simulants primarily consists of plagioclases, mafic minerals (nesosilicates and inosilicates such as olivine and pyroxene), and glass plus opaque (with a prevalence of glass) (Table S2; Figure 1). On average, compared to Lunar Highland simulants, Lunar Mare simulants are more enriched with mafic minerals (7% vs. 35%, respectively) and oxides (0.2% vs. 2.9%, respectively). However, Lunar Highland simulants contain higher levels of anorthite plagioclases than Lunar Mare simulants (55% vs. 38 %, respectively).

During the period of the Apollo missions, exploration activities on the Lunar surface were seriously hampered by dust. Consequently, an additional type of simulant was proposed, called Lunar Dust. This simulant was created based on the data on Lunar dust collected by the Lunar Soil Characterization Consortium (Taylor et al., 2001; Wallace et al., 2009). Available data on the petrographic characteristics of this simulant indicate a wide range of rock types, ranging from gabbro to anorthosite (Table S1). In accordance with the mineralogy of the Lunar dust regolith, the Lunar Dust simulant is enriched by glass and opaque than the Lunar Mare and Lunar Highland simulants (53% vs. 25% and 33%, respectively), and exhibits lower levels of nesosilicates, inosilicates, and tectosilicates (Table S2; Figure 1).

The Martian surface was shaped by the combined action of the wind (physical erosion) and water (chemical weathering) and lava flows (Zeng et al., 2015; Cannon et al., 2019), all of which contributed to the formation of the Martian "soil" (Bandfield et al., 2011). The data collected by the Curiosity rover over the last decade have shed light on the composition and physical properties of the Martian regolith. Peters et al. (2008) reported that the Martian regolith is classified as a fine-grained and cohesionless rocky soil that is mixed with dust due to planet storms. The surface is covered by a basaltic sand that is mainly composed of plagioclases and mafic minerals (including nesosilicates and inosilicates such as olivine and pyroxene) (Peters et al., 2008; Zeng et al., 2015; Filiberto, 2017). The Martian regolith also contains relatively lower levels of phyllosilicates (smectite and saponite), sulphate salts (such as gypsum, anhydrite, and alunite-jarosite) (McSween and Keil, 2000; Gaillard et al., 2013; McCollom et al., 2013), and the iron oxides (such as magnetite, hematite, and ferrihydrite) (Benison et al., 2008; Peters et al., 2008; Zeng et al., 2015; Cannon et al., 2019) that make Mars "the red planet" (Grotzinger et al., 2014; Hurowitz et al., 2017)**.**

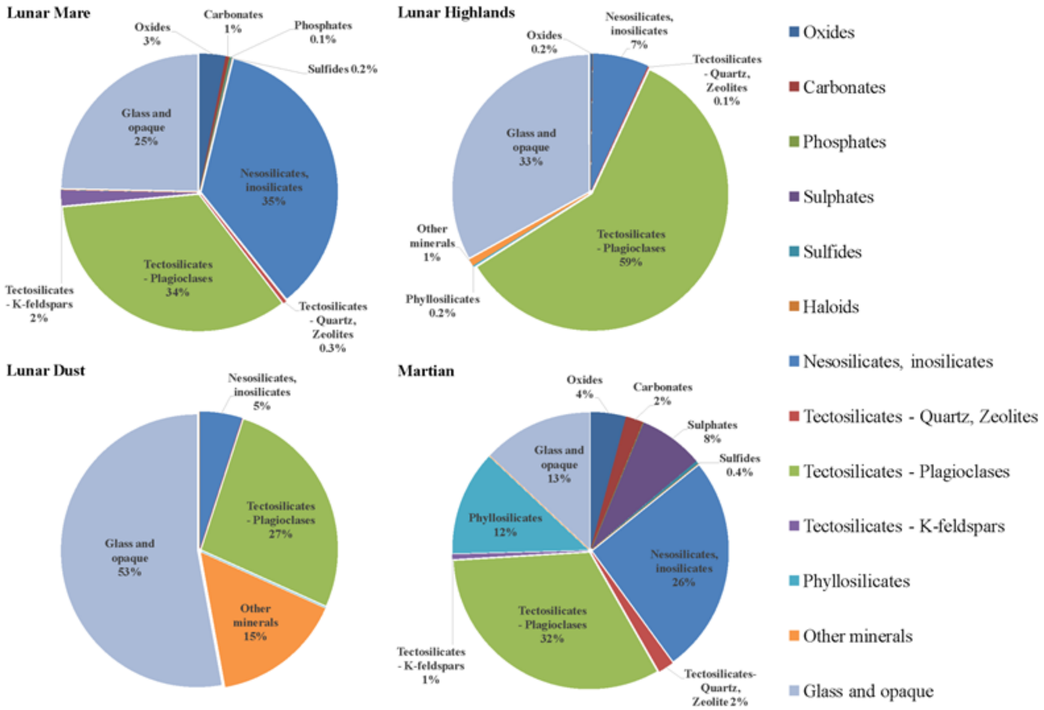

Figure 1. Data of the figure are relative only to simulants (19/30) whose mineralogical composition is reported for more than 73% of the total mass in the literature (Table S1). The mineralogical classes are split as follow: Oxides (Ilmenite; Magnetite; Ti-magnetite; Hematite; Ferrihydrite; Cr-spinel); Carbonates (Calcite; Fe-carbonate; Mg-carbonate); Phosphates (Apatite); Sulphates (Gypsum; Selenite; Mg-sulfate; Anhydrite); Sulfides (Pyrrhotite; Sulfide); Nesosilicates - inosilicates (Olivine + pyroxene + ilmenite; Olivine; Clinopyroxene; Augite; Enstatite; Pyroxene + Hornblende; Pyroxene; Diopside; Hypersthene); Tectosilicates - Quartz, Zeolite; Tectosilicates - Plagioclases (Plagioclase; Anorthite; Albite; Labradorite); Tectosilicates - K-feldspars (Sanidine; K-feldspar; Orthoclase); Phyllosilicates (Smectite; Saponite; Chlorite); Other minerals (undifferentiated). Glass + opaque (Plagioclase glass, basaltic glass, hydrated silica, Volcanic glass; Glass; Opaque + Glass).

2.3 Bulk chemistry and physicochemical properties

The bulk chemistry and physicochemical properties of Lunar and Martian regolith simulants have been analyzed to assess their ability to support extra-terrestrial farming in the future. Studies are primarily focused on the essential nutrients required by plants to complete their life cycle. Plants can obtain large quantities of macronutrients (including N, P, K, S, Ca, and Mg) from the growth media. These, along with C, H, and O (derived from the atmosphere) contribute to over 95% of a plant's entire biomass (when measured as dry matter). Because micronutrients are required in lower quantities, their levels in plant tissues are measured in parts per million. These include Cl, B, Zn, Fe, Mo, Mn, Cu, and Ni.

In the absence of any organic matter, the main chemical compositions and mineralogy of Lunar and Martian simulants are very similar to those of their respective reference samples from the Lunar and Martian surfaces (Mortley et al., 2000; Beegle et al., 2007; Peters et al., 2008; Zeng et al., 2015; Kölbl et al., 2017). The data from rovers show low variability in bulk chemical composition of regolith in the areas where measurements have been acquired (Zeng et al., 2015). Nevertheless, we need to assume that the unexplored areas of the Lunar and Martian surfaces may exhibit different mineralogy and chemical compositions. NASA's Perseverance rover has successfully cored Martian rocks, and data received from this rover will certainly broaden our knowledge of Martian geochemistry. Figure 2 shows the mean chemical compositions (as oxide percentages) of the 30 simulants listed in Table 1. The oxides of manganese (MnO), titanium (TiO), chromium (Cr_2O_3), phosphorus (P_2O_5), and sulfur (SO_3) occur at very low concentrations in each simulant category. Therefore, for the sake of clarity, these are all collated in the "others" category (Figure 2). SiO_2 is the principal constituent in all the simulants. SiO_2 levels are consistently ~45% in the Lunar simulants, and ~39% in the Martian ones. This difference is likely due to the higher occurrence of amorphous materials in Lunar simulants (Figure 1). Likewise, Lunar simulants show higher Al_2O_3 and CaO levels than Martian simulants, whereas the opposite is true for Fe (FeO and Fe_2O_3) and MgO (Figure 2). These trends are explained by higher levels of Ca-plagioclases in Lunar simulants and of Fe-(hydr)oxides (magnetite, hematite, ferrihydrite), nesosilicates (olivine), and inosilicates (pyroxene) in Martian ones. Lunar and Martian simulants also contain significant levels of other nutrients essential for plant growth. These include potassium (average, 1.0%; 0.6% of K_2O), phosphorus (0.3%; 0.4% of P_2O_5), sulfur (0.1%; 3.4% of SO_3), and manganese (0.1%; 0.3% of MnO), which are derived from K-feldspars, phosphates, sulfates, and Mn oxides, respectively (Figure 1). The simulants also contain inorganic carbon in the form of carbonates, and their levels are higher in Martian simulants than in Lunar ones. Simulants also contain non-negligible amounts of sodium (Lunar: 2.8% of Na_2O; Martian: 2.1% from Na-plagioclases) and potentially toxic elements (e.g., Cr as Cr_2O_3), which may induce salt or other abiotic stresses in rhizosphere-competent microorganisms and plants (Caporale et al., 2020).

Simulants lack key nutrients for plants such as N, which is frequently absent in minerals and occurs in biomolecules. Micronutrients such as Mo, Ni, B, Cu, and Zn are generally occluded in accessory minerals in trace concentrations (i.e., in the order of parts per million).

The total amount of the most essential elements may be more than adequate to satisfy the requirements for plant growth in simulants. However, plants generally take up only the bioavailable forms of elements (such as the readily soluble and exchangeable forms), and not the elements occluded in mineral structures that are released only after mineral weathering. For plants, nutrient availability in soil is governed by the pseudo-equilibrium between aqueous and solid phases, rather than by the total nutrient content. Factors such as pH, redox potential, electrical conductivity (EC), texture, type and relative abundance of fine solid particles play a key role in regulating nutrient availability in a plant growth medium (Adamo et al., 2018). Thus, these factors should be assessed when growing plants in regolith simulant-based substrates. Unfortunately, the pH and EC of simulants are not provided in the Planetary Simulant Database. Studies report that the Martian and Lunar simulants have a pH above 6, and have alkaline properties in some cases (Gilrain et al., 1999; Zaets et al., 2011; Wamelink et al., 2014; Caporale et al., 2020; Eichler et al., 2021), suggesting low rates of mineral weathering and cation release. The only data regarding EC in Martian simulants indicate low values of 0.2–0.3 dS m^{-1} (Gilrain et al., 1999; Caporale et al., 2020), suggesting no adverse effect of salinity on plant growth.

Agronomic techniques and crop management also affects nutrient availability and dynamics in a growth substrate. Thus, the addition of sustainable amounts of organic amendments (e.g., compost or manure) or mineral fertilizers to Lunar and Martian simulants may enhance the bioavailability of essential nutrients and provide missing vital nutrients such as N and organic C. These practices can also aid in pH adjustment and have positive effects on microbial rhizosphere activity and nutrient biogeochemical cycles.

Potentially toxic elements such as Al and Cr usually precipitate and are poorly available in alkaline environments (Brautigan et al., 2012); therefore, their presence in non-negligible amounts should not hinder plant growth in simulants. However, analyzing the bioavailability of these elements in the substrates and their levels in plant tissues may help evaluate the potential risk they pose to space crews. Due to the phytochemistry of the cold and oxidizing environment, toxic perchlorate salts occur commonly on the Martian surface in concentrations of 0.5–1% (Oze et al., 2021); however, they were not present in any of the 30 simulants. Perchlorates can be taken up by plants and make their edible parts unsafe to eat. To remediate Martian soils rendered toxic by perchlorates, several papers have proposed a biochemical approach that involves transforming perchlorates into chloride and oxygen (Rikken et al., 1996; Davila et al., 2013).

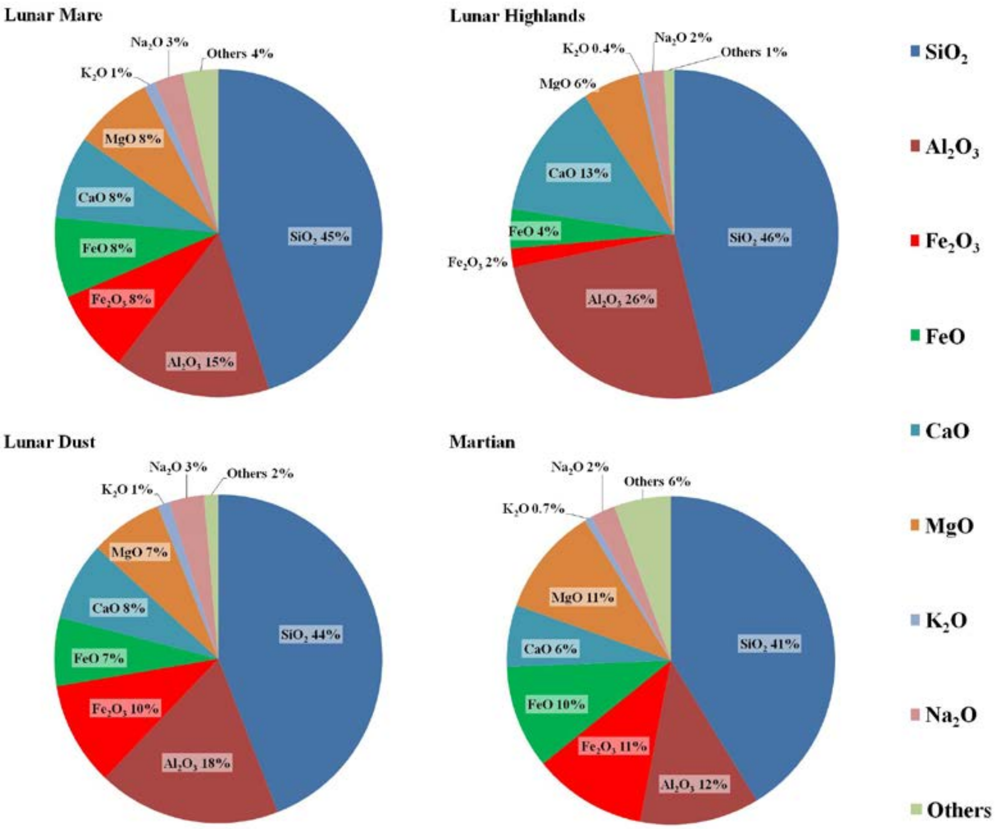

Figure 2. Bulk chemistry of 30 lunar and martian simulants listed in Table 1 (the source of the information used to build the graph and the analytical techniques used to produce chemical data are reported in Table 1 and in the text).

2.4 Physical and hydrological properties

Reduced gravity (e.g., in a spacecraft in orbit) causes changes in crucial hydrological variables and alters some fundamental characteristics of water flow and liquid distribution inside a porous medium. The characteristic retention curve of a porous medium is of paramount importance for water movement and plant growth. In the terrestrial environment, water movement towards root hairs is determined by both gravity, which decreases with water depletion, and capillary forces, which increase as water content decreases. In microgravity, capillary forces exert complete control over liquid distribution in a plant growth medium. By simulating a wetting and drying cycle using conventional unsaturated flow models adapted to microgravity, Jones and Or (1999) showed that the retention curve has narrower pore-size distributions in microgravity. This may be due to particle rearrangement, increased air entrapment, and enhanced hysteresis. The authors also reported a decrease in unsaturated hydraulic conductivities, which can be explained by the modified hydrodynamics in microgravity. Chamindu Deepagoda et al. (2014) used equations to quantify the water retention of substrates in terrestrial conditions and under different gravity conditions. Their analysis concluded that the Lunar simulant was the worst in terms of water retention capacity, likely because of its large pore space (0.52 cm^3 cm^{-3}) and particle size (0.25–1.0 mm).

Maggi and Pallud (2010) evaluated the effect of Martian gravity on soil processes by using a highly mechanistic model. This model had been previously tested for terrestrial crops, and couples soil hydraulics and nutrient biogeochemistry. The net leaching of NO_3^- solutes, gaseous fluxes of NH_3, CO_2, N_2O, NO, and N_2, the depth concentrations of O_2, CO_2, and dissolved organic carbon (DOC), and pH in the root zone were measured in two cropping units under a simulation of the gravitational conditions on Earth and Mars (9.81 *g* and 0.38 *g*, respectively). These units were similarly fertilized and irrigated, but had different initial soil moisture content. The water and nutrient leaching of soil was reduced by 90% under Martian gravity. This enhanced the microbial metabolism, promoted faster decomposition of DOC, and much higher emission of NO, N_2O, N_2, and CO_2. The authors concluded that cultivation on Mars would require less water for irrigation and lower external nutrient supply than on Earth. Unless the soil bulk density is very high, it is expected to have small influences on plant growth. Based on data from the Pathfinder lander, the potential bulk density of Martian soil has been estimated at 1.07–1.64 g cm^{-3} (Moore et al., 1999). The data from the Viking 1 lander suggests a bulk density of 1.15 g cm^{-3} for Martian soil (Moore and Jakosky, 1989), with a volatile loss of 0.1–1.0% by weight at 500 °C (Peters et al., 2008). The bulk density of Lunar soil varies between 1.5–1.7 g cm^{-3} (Taylor, 2007).

Pore size distribution is potentially associated with the percolation process, such that an increase in particle size enhances the percolation process. The percolation threshold changes under reduced gravity, resulting in improved aeration conditions in the early stages of simulations. Although reduced gravity can influence gas and fluid movement, it does not necessarily lead to better rhizospheric conditions. This is because the root zone is primarily influenced by air in pore spaces (Chamindu Deepagoda et al., 2014). Therefore, if the small pore space is filled with air and the substrate is almost water-saturated, conditions could become critical for plant growth and would need to be managed carefully.

Particle size distribution is another critical aspect that influences the potential of a regolith to be a growth medium. On Earth, soils are made of particles (namely clay, silt, and sand particles) of different sizes. The optimal assemblage (loam texture) of certain proportions of sand, silt, and clay-sized particles can maximize the ability of the soil to sustain plant growth. The particle size distributions of Lunar and Martian simulants must be modified to create an optimal particle assemblage (Cannon et al., 2019) that promotes air permeability (to avoid anoxia stress) and geochemical and mechanical interactions to enhance element bioavailability (Beegle et al., 2007; Zeng et al., 2015; Cannon et al., 2019).

Caporale et al. (2020) conducted an interesting study on the mixing of organic compost with the MMS-1 Martian simulant. This study showed how the addition of green compost to the MMS-1 simulant affects the physical and hydrological properties of the mixture. As expected, the bulk density progressively decreased with increasing rates of compost in the mixture, ranging from 1.39 g cm^{-3} (pure simulant) to 0.60 g cm^{-3} (pure compost). The addition of compost to pure simulant proportionally increased the maximum amount of water retained. The retention curves of the pure simulant and a 70:30 (v/v) simulant:compost mixture tended to converge when the matrix suction was approximately 60 cm. In contrast, the retention curve of a 30:70 simulant:compost mixture was always higher than those of the other two substrates. However, the suitability of a substrate for the cultivation of a candidate crop cannot be established solely through analysis of the retention curve. In other words, higher values of the saturated water content do not necessarily translate to better performance. The authors also showed that compost addition to the pure simulant exerted a more significant effect on the macropore region than on the micropore domain. Water held in macropores exceeding 120 μm in diameter was not directly beneficial for root water uptake in lettuce (the plant considered in the study), and even caused root asphyxia. Moreover, the three mixtures considered had a similar distribution of pore sizes with diameters below 50 μm. Based on its hydraulic properties, the 70:30 mixture was best substrate, as the percentage increase in large pores (diameter, 50–120 μm) was more significant in this mixture than in the 30:70 one. In terms of water and nutrient transport processes, all the mixtures were acceptable as growth media in a hydroponic cultivation system, where a timely water supply is guaranteed. However, if the objective is to manage lettuce irrigation to minimize irrigation frequency, the 70:30 mixture was the most promising substrate due to its hydraulic properties. Thus, the concerns change if the aim is to have an optimized collection system with more efficient energy consumption and system usage.

3 Key studies on plant cultivation on regolith simulants: critical aspects of - and solutions for - growing crops on other planets

A good knowledge of the physicochemical and hydraulic properties of Lunar and Martian regolith simulants is of paramount importance in developing and building off-world BLSS based on an ISRU approach, in which native substrates are exploited as plant growth media. Evaluating how simulant properties can influence plant growth, physiology, and health can help overcome deficiencies and critical concerns in a sustainable and effective way. The macro- and micronutrient levels, porosity, and water availability are fundamental parameters in assessing the capability of a substrate to sustain plant growth. Many studies have evaluated substrate efficiency (Ming et al., 1993; Ming and Henninger, 1994; Aglan et al., 1998; Mortley et al., 2000) and water management (Ramírez et al., 2019; Wamelink et al., 2019) over the years. To be integrated with crop production, a good regolith simulant should have certain physical characteristics, including the following: i) optimal water holding capacity to maintain an effective level of humidity after irrigation; and ii) optimal air circulation in the porous medium to allow efficient gas exchange and root and microbial respiration. Plants commonly take up nutrients from the soil solution, either in a dissolved form, through exchange, or through easy release at the solid-water interface. The fluxes of water, air, and nutrients in a growth substrate are closely linked to its physicochemical properties (Brady, 1975).Therefore, particle interactions or aggregation and the consequent formation of a structured substrate are critical for better plant growth. For extra-terrestrial farming, it is well-established that several simulant properties—such as an alkaline pH, a high availability of sodium, the predominance of macro- vs. micropores, and a scant water holding capacity—can influence plant growth, health, and vigor (Wamelink et al., 2014; Caporale et al., 2020). Thus, it is essential to evaluate whether these soil simulants can be exploited to support future colonies.

As reported in section 2.2, plants need macro and micronutrients for optimal growth, and these can be found in both inorganic and organic forms in soil on Earth (Hopkins and Huner, 2008; Fageria, 2009). Terrestrial soil minerals (mainly feldspars and micas) are the main source of K. Calcium, Mg, S, and Fe, are usually abundant in soil, and microelements such as Zn, Cu, Mn, B, Mo, Cl, and Ni originate from both minerals and organic matter. Carbon is absorbed from the atmosphere through photosynthesis (Hopkins and Huner, 2008), and organic matter is the primary source of both N and P. Lunar and Martian soils lack organic matter and biotic activity (Seiferlin et al., 2008), although there is some evidence of a biofilm (Thomas-Keprta et al., 2014; Eigenbrode et al., 2018). It is also worth noting that the Curiosity rover investigations at Gale crater on Mars discovered indigenous N in sedimentary and aeolian deposits (Stern et al., 2015), although its exact concentration and potential use for plant growth is debatable. Thus, Lunar and Martian soils (and, therefore, regolith simulant-based substrates) are potentially deficient in all macro- and micronutrients, which are derived exclusively (N), mostly (P, S), or partly (K, Ca, Mg, Fe, Zn, Cu, Mn, B, Cl, and Ni) from the degradation of organic components. Thus, regolith simulants cannot support sustainable ISRU for crop production without exogenous inputs of inorganic fertilizers or organic matter.

To date, Lunar and Martian simulants have been primarily studied for applications other than agronomy (de Vera et al., 2004; Gertsch et al., 2008; Kölbl et al., 2017; Karl et al., 2018), although they have potential use as a substrate for crop growth (Mortley et al., 2000). Several strategies and treatments can be applied to ameliorate the nutrient deficiency of simulants and enhance their performance as plant growth substrates. The first solution involves the use of a stable organic amendment, not only as a supply of organic carbon, but also to improve the physical features of regolith (Gilrain et al., 1999). The use of this amendment and soil tillage can also help mitigate the effects of microgravity on water leaching (Maggi and Pallud, 2010). Furthermore, selected pioneer plants can be grown initially (Kozyrovska et al., 2006) to improve the root zone during cultivation and provide plant residues for humification at the end of their life cycle.

The selection of candidate pioneer plant species is a key aspect to consider (Gilrain et al., 1999; Mortley et al., 2000; Kozyrovska et al., 2006; Wamelink et al., 2014; Ramírez et al., 2019). Plants produce O_2 and fix CO_2, serve as food for space crews, have a role in water recycling (Maggi and Pallud, 2010; Llorente et al., 2018), and are actively involved in soil structure formation. To improve ISRU, several researchers have proposed the development of microbial consortia (Zaets et al., 2011; Verseux et al., 2016; Llorente et al., 2018) that could improve the mineral uptake of plants.

Table 2. Overview of soil-based space farming experiments.

Ref. soil	Simulant Name	Treatments/ Sterilisation	Nutrient supply	Species	Propagation material	Crop cycle	Measurements	Reference
Moon		Add lunar soil to support/growth medium (a wood pulp product stabilized with acrylonitril resin)/ Sterilized (by steam autoclaving)	Yes	*Allium cepa* L.; *Anacystis nidulans* (Richt) Drouet; *Brassica oleracea* L.; *Capsicum frutescens* L.; *Chenopodium amaranticolor* Coste and Reyn.; *Chlorella pyrenoidosa* Chick; *Citrullus vulgaris* Schrad.; *Citrus limonia* L.; *Cucumis melo* L.; *Cucumis sativus* L.; *Glycine soja* L. Sieb and Zucc.; *Haplopappus gracilis* Nutt. Gray; *Helianthus annuus* L.; *Lactuca sativa* L.; *Lycopersicum esculentum* Mill.; *Lycopodium cernuum* L.; *Marchantia polymorpha* L.; 4 *Nicotiana tabacum* L. var.; *Onoclea sensibilis* L.; *Oryza sativa* L.; *Phaeodactylum tricornutum* Bohlin; *Phaseolus aureus* L.; *Phaseolus vulgaris* L.; *Pinus elliottii* Engelm.; *Pinus lambertiana* Dougl.; *Pinus palustris* Mill.; *Porphyridium cruentum* Ag. Naeg.; *Raphanus sativus* L.; *Saccharum officinarum* L.; *Solanum tuberosum* L.; *Sorghum vulgare* Pers.; *Spinacia oleracea* L.; *Todea barbara* L. Moore; *Triticum vulgare* Vill.; 2 *Zea mays* L. var.	(A); (S); (T); (TC)	Max 30 days	-Seed germination; -Biometric parameters; -Chemical analyses; -Histologic analysis; -Plants color.	Walkinshaw et al., 1970; Walkinshaw and Johnson, 1971
Moon		Add lunar soil to growth medium/ Sterilized (By washed with triple-distilled water and spectroquality chloroform and methanol)	Yes	*Nicotiana tabacum* L.	(TC)	84 days	-Pigment determinations; -Lipid content; -Growth rate; -Total biomass	Weete and Walkinshaw, 1972; Weete et al., 1972

Moon	Analogous made by a mix of sand and rocks	A bacterial consortium was spayed onto seeds/ Sterilized (by heating at 170°C for 2h and autoclaving at 112°C for 40 minutes)	No	*Tagetes patula* L.	(S)	70 days	-Elemental analyses; -Fresh biomass	Zaets et al., 2011
Moon	Analogous made by inert aggregates	No treatments/ Not specified	Yes	*Ipomoea batatas* L.	(T)	120 days	-Biometric parameters	Aglan et al., 1998
Moon and Mars	Not specified	No treatments/ Not specified	Yes	*Ipomoea batatas* L.	(T)	120 days	-Biometric parameters	Mortley et al., 2000
Mars	Analogous collected from desert	Salt stress/ Not sterilized	Yes	*Solanum tuberosum* L.	(T)	134 days	-Biometric parameters; -Stomatal conductance; -Chl.phyll SPAD values	Ramírez et al., 2019
Mars	JSC-1A Mars	Mix simulant with different ratios of leaf compost/ Not sterilized	Yes	*Beta vulgaris* L.	(S)	90 days	-Plants weights; -Substrate phisico-chemical analysis	Gilrain et al., 1999
Moon and Mars	JSC Mars-1A and JSC1-1A Lunar	No treatments/ Not sterilized	No	*Arnica montana* L.; *Sinapsis arvensis* L.; *Urtica dioica* L.; *Cirsium palustre* L.; *Sedum reflexum* L.; *Festuca rubra* L.; *Vicia sativa* L.; *Lupinus angustifolius* L.; *Melilotus officinalis* L.; *Lotus pedunculatus* Cav.; *Solanum lycopersicum* L.; *Secale cereale* L.; *Daucus carota* subsp. *sativus* Hoffm.; *Lepidium sativum* L.	(S)	50 days	-Seed germination; -Biometric parameters;	Wamelink et al., 2014

Moon and Mars	JSC Mars-1A and JSC1-1A Lunar	Mix simulants with organic matter/ Not sterilized	Yes	*Solanum lycopersicum* L.; *Secale cereale* L.; *Lepidium sativum* L.; *Allium ampeloprasum* L.; *Chenopodium quinoa* Willd.; *Pisum sativum* L.; *Raphanus raphanistrum* subsp. *Sativus* L.; *Diplotaxis tenuifolia* L.; *Allium tuberosum* Rottler ex Spreng.	(S)	159 days	-New seeds germination; -Total aboveground dry biomass	Wamelink et al., 2019
Moon	Analogous made by anorthosite rocks	Seeds treatment with bacterial consortia and substrate inoculation with microorganisms/ Not specified	Not Specified	*Tagetes patula* L.	(S)	Not specified	-Microbial activity	Kozyrovska et al., 2006
Mars	JSC Mars-1A; MMS-1; MGS-1; MGS-1P	Add of perchlorate to the simulants/ Not sterilized	Yes	*Lactuca sativa* L.; *Arabidopsis thaliana* L.	(S)	Max 28 days	-Chlorophyll and carotenoid content	Eichler et al., 2021
Mars	MMS-1	Mix simulant with different percentage of compost/ Not sterilized	Yes	*Lactuca sativa* L.	(T)	19 days	-Leaf Gas Exchange; -Biometric parameters; -Plant mineral composition; -Chlorophyll and C vitamin content; -Carotenoids and Polyphenols profile	Duri et al., 2020

(A) Algal cultures; (S) seed and/or spore; (T) transplanting; (TC) tissue cultures.

3.1 Apollo-era plant experiments with Lunar samples

The first studies of plant growth on Lunar regolith were conducted in the early 70s in the Lunar Receiving Laboratory, where small amounts of real Lunar materials (brought from the Moon by the Apollo missions) were mixed with growth media (such as wood pulp product) stabilized with acrylonitrile resin (Walkinshaw et al., 1970; Walkinshaw and Johnson, 1971; Weete and Walkinshaw, 1972; Weete et al., 1972). These studies were designed to determine whether Lunar materials contained any agents capable of generating an epiphytotic disease in representative species of the plant kingdom. The Lunar material was sterilized to avoid external contamination, and the entire laboratory was kept under quarantine conditions. The plant growth substrates containing extra-terrestrial materials were treated as inert media without considering their nutrient content and composition (Table 2). The main parameters monitored were seed germination capacity, growth alteration, phytotoxicity, and disease incidence. Walkinshaw et al. (1970) grew 35 representative plant species in aseptic conditions in different cultivation systems, including algae, seeds, spores, seedlings, gametophytes, and tissue cultures of higher plants. The authors noted the absence of disease agents in the plants tested under experimental conditions, and concluded that the Lunar material could potentially support the growth of a wide range of plant species. Specifically, ferns, liverworts, and tobacco were particularly effective in exploiting the Lunar material as a nutrient source. A year later, Walkinshaw and Johnson (1971) focused on the possible differences in chemical composition among plants grown on Lunar material. The results showed a direct interaction between plant species and Fe, Al, and Ti uptake from the Lunar substrate. Notably, cabbage and Brussels sprouts exhibited higher absorption of Mn.

Based on the findings of Walkinshaw et al. (1970), researchers further investigated the effect of the Lunar material on tobacco plants in terms of their constituent biomolecules and secondary metabolites. A tissue culture experiment over a period of 12 weeks used the Lunar material recovered from Walkinshaw's experiment, which was washed and sterilized before the trial. Weete et al. (1972) found that tissue grown in contact with Lunar material had a higher concentration of total sterols than in the control. They also found differences in absolute and relative fatty acid concentrations. Moreover, the chlorophyll and carotenoid concentrations were higher in treated plants, with chlorophyll *a* being the major pigment present (Weete and Walkinshaw, 1972). According to the review by Ferl and Paul (2010), the Apollo-era plant experiments with Lunar samples provided many insights into the biological impact of the Lunar environment on terrestrial life forms, which were useful for future research in support of Lunar exploration. The modern molecular approaches (-omic sciences) were not available during the Apollo-era plant experiments. However, those studies provided useful preliminary information on how Lunar samples and Earth biota interacted with and affected each other.

3.2 Plant growth experiments on Lunar and Martian simulants

As a part of NASA's Advanced Life Support Program, Aglan et al. (1998) and Mortley et al. (2000), evaluated the response of sweet potato clones grown under microgravity in Lunar and Martian simulant media containing a buried microporous tube system for watering and fertigation. In these tests, the simulants mainly provided mechanical anchorage for the plant roots, and did not cause any adverse or toxic effects in the plants. Therefore, the authors concluded that both simulants showed potential for use as a substrate for crop production.

As a biotechnological approach to plant cultivation in an extreme environment (such as a Lunar base), Kozyrovska et al. (2006) proposed the growing of pioneer plants (*Tagetes patula* L.) in a Lunar rock anorthosite substrate. The simulants contained specific root-colonizing bacteria that could decompose the Lunar silicate rock and release the cations essential for plant growth. This strategy may prove to be a practical necessity in order to support plant growth in a substrate with low nutrient availability. The primary function of the pioneer plants with associated microorganisms is to form a soil with adequate fertility. This soil can then be used to grow plants of a second generation (such as wheat, rice, and soybean, among others) to provide Lunar explorers with fresh sources of vitamins, nutrients, and biomolecules. At the end of the growth cycle, the authors demonstrated that the first-generation plant residues could serve as a supply of green manure for humification and as a potential nutrient source.

The first large-scale controlled experiment evaluating potential plant growth (germination, growth, flowering, and seed formation) on the JSC1-1A Lunar and JSC-1A Martian regolith simulants was conducted by Wamelink et al. (2014). Fourteen different species of wild plants, crops, and nitrogen fixers (see Table 2) were grown for 50 days in isolation under Earth-like light and atmospheric conditions, while only using demineralized water and no fertilizers or substrate amendments. The results indicated that neither simulant was an adequate source of plant nutrients. Nevertheless, the Martian simulant outperformed the Lunar simulant in biomass production, as it had trace levels of ammonium nitrate and carbon and no stressors that could cause a higher pH or low water holding capacity. Tomato and wheat crops performed particularly well on the artificial substrates. Three species flowered, but only two produced seeds. In conclusion, the authors raised several open questions regarding the representativeness of the simulants, their water holding capacity, the availability of N and other nutrients on Mars and the Moon, and the influences of gravity, light, and other extra-terrestrial environmental conditions.

Waste management and efficient resource use are critical aspects of BLSS for both the Moon and Mars. A possible solution for the problem of waste management is composting, which can be incorporated into the agronomic treatment of regoliths as an amendment in line with the ISRU approach. By mixing compost with various regolith simulants, several studies have evaluated the role and potential utility of organic waste in plant cultivation and the management of extra-terrestrial settlements. These investigations not only provide a better approach to the management of residues, but elucidate the effects of organic matter amendment on mineral-based substrates (Gilrain et al., 1999; Wamelink et al., 2019; Duri et al., 2020). To help overcome the chemical constraints on plant growth in pure simulants, Gilrain et al. (1999) conducted preliminary studies in an ALS plant growing system using a variety of proportional combinations of the JSC Mars-1 regolith simulant and a municipal leaf compost. The Swiss chard was used as a candidate crop, and half of the treatments received a modified half-strength Hoagland's solution. Plants grown in compost:simulant ratios of 1:0, 3:1, and 1:1 showed yields that were greater than those in the 1:3 and 0:1 ratios, and control plants irrigated with only water produced similar trends. However, overall plant growth was significantly lower, indicating that nutrient supply by both the compost and regolith simulant was not enough to sustain the entire plant growth cycle. The authors concluded that the compost mainly promoted plant growth by improving the physical features of the regolith that regulate water and/or nutrient availability.

A study reported the growth of 14 different plant species on a Martian soil simulant and, to a lesser extent, on a Lunar soil simulant (Wamelink et al., 2014). As a follow-up experiment, Wamelink et al. (2019) grew 10 different crop species (see Table 2) on the JSC1-1A Lunar and JSC-1A Martian regolith simulants (provided by NASA) containing organic residues from first harvests (fresh mown grass of *Lolium perennne* L.). A nutrient solution was also added to mimic the addition of human feces and urine. The main goal was the production of edible crops and their seeds for a next generation. The authors harvested the edible parts of nine out of ten crops. The biomass production was highest in the Earth control and the Martian soil simulant, but was significantly lower in the Lunar simulant. Only three species (radish, rye, and cress) produced seeds. Radish germination rates were lower in the Lunar simulant than in the Earth control soil and Marian simulant. The authors defined their study as a small step towards the implementation of a sustainable agricultural ecosystem for a Lunar or Martian colony. They further encouraged the search for the optimal organic matter content and physical characteristics of the simulants in future studies.

Recently, two butterhead lettuce (*Lactuca sativa* L. var. capitata) cultivars (green and red Salanova®) have been cultivated in the MMS-1 Mojave Mars simulant mixed with green compost at different rates (simulant:compost ratios, 0:100, 30:70, 70:30, and 100:0; v:v) in a phytotron open gas exchange growth chamber (Caporale et al., 2020; Duri et al., 2020). A detailed characterization of the physicochemical, mineralogical, and hydrological properties of the simulant, compost, and their mixtures was provided. This was the first characterization of MMS-1 in terms of its mineralogical composition (x-ray diffraction) and spectroscopic features (by mid-infrared MIR spectroscopy). MMS-1 was found to be a coarse-textured alkaline substrate mostly composed of plagioclase, amorphous material, and (to a lesser degree) of zeolite, hematite, and smectites. Although it was a source of nutrients for lettuce, it did not supply organic matter, N, and S, and provided very scant amounts of P. As reported above in section 2.3, organic amendment improved the physical properties of the simulant (such as bulk density and water holding capacity). It also lowered the pH of the alkaline simulant, enhanced its cation exchange capacity, organic C and N levels, and the availability of macro- and micronutrients. The red Salanova® lettuce grown in the 30:70 mixture showed the best crop performance, photosynthetic activity, intrinsic water use efficiency, and quality traits (mineral, carotenoid, and phenolic contents). The 70:30 mixture showed a slight decline in lettuce yield and quality; however, the authors concluded that it was a more sustainable choice for space farming, as it exhibited more efficient use of limited resources (e.g., compost). The study by Caporale et al. (2020) found discrepancies between the measured bulk chemistry of the MMS-1 simulant and that provided by the producer. This supported the observations by Cannon et al. (2019), who suggested that the MMS-1 simulant was derived from different source material than the original MMS. In the absence of rigorous documentation by producers of simulants, this shows the need for an adequate characterization of commercial simulants prior to the designing and planning of any scientific experiments.

Over the past two years, the new data collected by rovers has allowed the scientific community to broaden its knowledge of Martian environmental features. For instance, the Martian surface has been found to have a high salt concentration (Ramírez et al., 2019) and high levels of perchlorates (Eichler et al., 2021). To evaluate the impact of abiotic stressors on plant growth and health, Ramírez et al. (2019) tested the responses of 65 potato genotypes grown in Mars-like soil from the La Joya desert in Southern Peru (characterized by high EC, ranging from 19.3 to 52.6 dS m^{-1}). Only 40% of the genotypes survived and yielded crops (0.3–5.2 g tuber $plant^{-1}$). At the end of the study, the authors stated that the selection of tolerant genotypes, appropriate sowing methods, and soil management strategies were crucial for crops to withstand the extreme salinity and yield produce. More recently, Eichler et al. (2021) tested the growth rates of lettuce and *Arabidopsis* plants cultivated on three pure Martian regolith simulants—JSC-Mars-1A, Mars Mojave simulant (MMS), and Mars Global simulant (MGS-1)—enriched with calcium perchlorate (2% w/v). None of these simulants could support plant growth in the absence of nutrient supplementation. However, with the addition of nutrients, both plant species grew on JSC-Mars-1A and MMS, but did not grow on MGS-1. The authors linked this failure to the high alkalinity of MGS-1, and suggested acidifying the simulant to achieve plant growth. Calcium perchlorate-enriched simulants were unable to sustain plant growth, even with nutrient supply.

Fackrell et al. (2021) have developed and characterized five new Martian simulants—Global soil (MBas), Phyllosilicate-smectite (MPSmec), Phyllosilicate-illite/chlorite

(MPChl), Sulfate-rich (MSul) and Carbonate-rich (MCarb)—for applications in space farming tests. These simulants have been found to be mineralogically, chemically, and spectrally comparable to Martian regolith and bedrock (according to available data), and are exploitable for plant growth in future studies on Martian surface analogues. In the conclusion to their work, the authors strongly advised that the fertility and feasibility of a simulant should be assessed not only on the basis of its mineralogical/chemical composition, but also on how physical and (bio)chemical weathering of the substrate affects its nutrient bioavailability over time.

4 Conclusions

This paper was intended to be a comprehensive review of the potential for Lunar and Martian simulants being used as substrates for plant growth. Given the costs associated with shipping to either of these off-world sites, as well as the need to establish sustainable off-world operations, more research in this area is essential. This review analyzed more than 70 articles on the Lunar and Martian regolith simulants used as analogues in terrestrial experiments. We identified their main properties, critical aspects, currently available solutions to enable the growth of higher plants, and the potential challenges in deploying them in life support systems.

The literature review showed that pure regolith simulants may be suitable media for plant growth (at least for limited periods) and function as a source of essential nutrients such as K, Ca, Mg, and Fe. However, they lack organic matter and key macronutrients such as N, P, and S. Furthermore, these simulants generally exhibit numerous features harmful for plant health, such as an alkaline pH, high availability of Na, low cohesion of mineral components, the predominance of macro- vs. micropores, and low water holding capacity. In addition, the Martian regolith sometimes contains toxic perchlorates. Hence, the configuration of a mineral-rich and fertile biological substrate for edible plant growth based on regolith simulants still presents a challenge in space biology research.

In many studies, nutrient deficiency in simulants was overcome by fertigation with nutrient-rich solutions (e.g., Hoagland). However, this agronomic technique is not feasible and sustainable in space agriculture, as the nutritional resources must be carried from the Earth and cannot be produced in BLSS. A promising strategy is the adding of *in situ* recycled organic matter to enrich regolith simulants. This is a sustainable and effective technique to enhance the chemical and biological fertility and physical and hydraulic properties of regolith-based substrates (e.g., permeability and water retention). The organic waste produced by BLSS crews can help recover compounds and allow their use as fertilizers or compost to support plant growth. Thus, future studies should evaluate the efficacy of these treatments. Moreover, consecutive cycles of plant cultivation on the same regolith-based substrate can allow prolonged root exudation and the release of organic acid molecules and CO_2. This can lower the substrate pH, increase mineral weathering rates, enhance nutrient release/availability, promote the aggregation of particles of different sizes (to form a more efficient porous system), and overall contribute to soil improvement.

Many vital aspects of space farming have already been explored in the published experiments on plant growth in simulant-based substrates. Nevertheless, many other challenging factors still need to be considered to assess the true potential of extra-terrestrial farming based on the exploitation and development of *in situ* Lunar and Martian resources. For example, water management and recycling are paramount in sustainable BLSS modules developed and based on the ISRU strategy. Water is another limited resource in space, and we need a better understanding of water movement and fluxes in regolith-based substrate/plant systems under microgravity. In extra-terrestrial soil, water dynamics would regulate the extent of mineral weathering and the rate of organic matter decomposition, thus greatly affecting the biogeochemistry and bioavailability of nutrients and plant growth. Future studies simulating the potential environmental conditions of off-world bases are highly recommended.

Another aspect which needs further investigation is the effect of the space environment (such as different gravity and climatic conditions) on plant physiology (e.g., the biophysical limitations on gas exchange and transpiration), and how this affects plant growth and productivity and substrata properties. In sustainable scenarios of space farming, regolith-based substrates are required to sustain plant growth throughout the plant life cycle, including the complete seed maturation needed for reproduction. Although this may be feasible for microgreens or salad crops, it is more challenging for other candidate species such as potato (a source of carbohydrates) and soybean (a source of proteins), which require more nutrients and resources to produce a sufficiently edible yield than do salad crops.

The presence of–and interactions with–biota (including pathogens, cyanobacteria, plant growth-promoting bacteria, beneficial symbiotic fungi, and worms) and biostimulants will add further complexity to life support systems with extra-terrestrial soils. However, little is known about their effects on: i) mineral weathering rates in the early stages of terraforming; ii) decomposition and recycling of organic plant residues and human excreta; iii) plant nutrition mediated by symbiotic fungi, compared with that mediated by entirely abiotic systems; and iv) protection of plants from environmental stresses. The occurrence of perchlorates in the Mars regolith provides a significant challenge to its use as an agricultural substrate. Thus, further steps—such as water rinses, phytoremediation, volatilization, and chemical reduction by using perchlorate-reducing bacteria—are necessary to make Mars regolith a viable growing substrate.

In conclusion, we encourage research that examines the effects of using *in situ* extra-terrestrial soils on several fundamental environmental functions (other than food security and biomass production) that constitute the primary functions of soil. On Earth, soils are the link between the air, water, rocks, and organisms. Soils are known to affect the climate, atmosphere composition, carbon and nutrient recycling, water quality and maintenance, biotic regulation, buffering, and the transformation of potentially harmful elements and compounds. In future, we should dedicate more attention to the development of pedogenesis on extra-terrestrial surface materials and explore the effects of regolith/simulant weathering on the soil-plant-atmosphere system. Providing ecosystem services by using a regolith-based substrate might be the key to a fruitful and sustainable Lunar or Martian BLSS.

Chapter 2

Geo-mineralogical characterisation of Mars simulant MMS-1 and appraisal of substrate physico-chemical properties and crop performance obtained with variable green compost amendment rates

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Abstract

The configuration of a biologically fertile substrate for edible plant growth during long-term manned missions to Mars constitutes one of the main challenges in space research. Martian regolith amendment with compost derived from crew and crop waste in bioregenerative life support systems (BLSS) may generate a substrate able to extend crew autonomy and long-term survival in space. In this context, the aim of our work was threefold: first, to study the geochemistry and mineralogy of Mojave Mars Simulant (MMS-1) and the physico-chemical and hydraulic properties of mixtures obtained by mixing MMS-1 and green compost at varying rates (0:100, 30:70, 70:30, 100:0; v:v); secondly, to evaluate the potential use of MMS-1 as a growing medium of two lettuce (*Lactuca sativa* L.) cultivars; thirdly, to assess how compost addition may impact on sustainability of space agriculture by exploiting *in situ* resources. MMS-1 is a coarse-textured alkaline substrate consisting mostly of plagioclase, amorphous material and secondarily of zeolite, hematite and smectites. Although it can be a source of nutrients, it lacks organic matter, nitrogen, phosphorus and sulphur, which may be supplied by compost. Both cultivars grew well on all mixtures for 19 days under fertigation. Red Salanova lettuce produced a statistically higher dry biomass, leaf number and area than Green Salanova. Leaf area and plant dry biomass were the highest on 30:70 simulant:compost mixture. Nevertheless, the 70:30 mixture was the best substrate in terms of pore-size distribution for water-plant relationship and the best compromise for plant growth and sustainable use of compost, a limited resource in BLSS. Many remaining issues warrant further investigation concerning the dynamics of compost production, standardisation of supply during space missions and representativeness of simulants to real Martian regolith.

Keywords: Space agriculture, Martian regolith, bioregenerative life support system, *Lactuca sativa* L. var. *capitata*, waste recycling, sustainability.

1 Introduction

The sustainability of long-term manned missions to Mars and planet exploration will depend on the cultivation of edible plants on locally resourced substrates and recycling of water and crew biological waste within a totally autonomous bioregenerative life support system (Häder et al., 2018). This system would make maximum use of resources available on the Martian surface and would initially minimise and eventually eliminate the need for expensive re-supply from Earth. Self-sufficiency for the Mars colony would improve crew safety and chances of long-term survival in the space environment (Silverstone et al., 2003). Moreover, an efficient use of resources from Mars to support crew survival could provide a huge reduction in energy and transport costs. Consequently, the growing of edible plants would allow the use of light radiation to fix carbon dioxide from the atmosphere and replenish oxygen while producing food for space travellers. The water transpired and purified by crop plants can be condensed and recycled to contribute to the system's water balance. Moreover, the presence of growing plants provides humans with a familiar environment while far away from their home planet (Salisbury, 1999).

The surface of Mars is covered by unconsolidated regolith produced by the joint action of impact comminution, physical wind erosion, lava and chemical weathering by oxidants (Murchie et al., 2009), which can be potentially used as growth substrate for edible plants. As revealed by the CheMin instrument on the Mars Science Laboratory (MSL) Curiosity rover, Martian regolith is a subequal mixture of crystalline and amorphous phases (Dehouck et al., 2014; Achilles et al., 2017). Martian regolith appears as a porous medium, mainly consisting of coarse particle-size fractions but also containing finer minerals (Stoker et al., 1993). It can thus present mechanical properties required for the support of plant life. The establishment of a biologically active and fertile substrate - consisting mainly of Martian regolith - to grow edible plants during long-term manned missions to Mars is one of the main challenges faced by current space research studies. All essential nutrients for plant growth appear to be present in sufficient amounts in Martian regolith, with the exception of those arising from organic matter, especially N but also P and S (Wamelink et al., 2014). However, N in reactive forms (ammonium and nitrate) is part of solar wind and may arise from volcanic activity or lightning (Mancinelli and Banin, 2003). Accordingly, the presence of an indigenous source of fixed N on Mars surface in the forms of nitrite and nitrate was discovered by the Sample Analysis at Mars Instrument (SAM) on board NASA's Curiosity rover in Martian rocks and regolith of Gale crater (20-250 mg kg^{-1}). Such a source of N, albeit insufficient to sustain possible crop cultivation on Mars, makes Gale crater a potential landing site for manned missions to exploit for agricultural purposes (Stern et al., 2015, 2017; Calef-III et al., 2016; Sutter et al., 2017; Navarro-González et al., 2019). On the other hand, N was not detected by the robotic spacecraft Mars Pathfinder on the Martian regolith of Chryse Planitia (Foley et al., 2003), indicating a high heterogeneity in elemental composition on the surface of Mars.

The lack of organic matter and thus of a source of organic nitrogen (N), phosphorus (P) and sulphur (S) in Mars regolith could be offset by amendment with compost arising from efficient treatment and recycling of crew sewage and inedible crop waste from concluded plant crop cycles. Efficient waste management/composting of crop residues with processed human excreta can undoubtedly increase chances of producing high-quality crops (De Micco et al., 2009). Compost production might also mitigate methane, nitrous oxide and carbon dioxide generation (Nelson et al., 2008), thus promoting a circular economy system where resource inputs, waste, emissions and energy leakages are minimised by closing energy and material loops. The amendment of Martian regolith with composted organic waste may also improve the water-holding capacity, physico-chemical properties and nutrient availability, as well as partially mitigate the negative effects of regolith alkaline pH and the presence of potentially toxic elements. Moreover, the presence of an organic carbon (C) substrate may stimulate pioneering biological activity at the plant root-regolith interface, which could slowly enhance the fertility of the inert Martian substrate over time and make it more suitable for plant growth.

To date, many crop species have been tested for their potential application in space BLSS (Velayudhan et al., 1995). Criteria for selecting potential crops include yield potential, nutritional value, horticultural and environmental requirements, harvest index, processing requirements and others (Chunxiao and Hong, 2008; El-Nakhel et al., 2019; Rouphael et al., 2019). Besides, the tolerance of candidate crops to toxic compounds, such as perchlorates, and the different attitude of crop species to uptake and translocate such toxicants into edible tissues are key factors to take into account. Occurrence of concerning levels of perchlorates in Martian regolith was proved by the Phoenix (Hecht et al., 2009) and Viking landers (Navarro-González et al., 2010), as well as by the Curiosity rover (Glavin et al., 2013; Sutter et al., 2017). Lettuce is certainly a good candidate since it is a fast-growing and health-promoting leafy vegetable, and a source of vitamin C, phenolic compounds and dietary fibre (Caporale et al., 2014; El-Nakhel et al., 2019; Pannico et al., 2019; El-Nakhel et al., 2020b), required by space crews undergoing significant oxidative and inflammatory stresses during space missions (Kyriacou et al., 2017; Goodwin and Christofidou-Solomidou, 2018).

As actual Martian regolith samples are not available on Earth, scientific experiments on space agriculture for manned missions to Mars can be carried out with commercial simulants of Mars regolith arising from crushed terrestrial rocks, which tend to replicate the geotechnical and compositional features of Martian regolith studied during the past unmanned missions to Mars. However, detailed characterisation studies on the mineralogical, physico-chemical and hydraulic properties of Martian regolith simulants are essential to evaluate the true potential of these materials as plant growth substrates. In this context, the aims of this work were: i) to study in detail the geochemical and mineralogical composition of MMS-1 Mars simulant and the physico-chemical and hydraulic properties of growing substrates obtained by mixing MMS-1 and green compost at varying rates (0:100, 30:70, 70:30, 100:0; v:v); ii) to evaluate experimentally, under growth chamber controlled conditions, the potential of MMS-1 as a growing medium of two butterhead cultivars of lettuce (*Lactuca sativa* L. var. *capitata*), fertigated with a modified Hoagland formulation; iii) to assess how compost addition may impact on the sustainability of space agriculture by exploiting local resources. For experimental purposes we selected and applied a commercial green compost of pruning waste and grass cuttings with a low C/N ratio in order to simulate as closely as possible the compost produced in a bioregenerative life support system from crop residues and processed crew excreta.

2 Materials and methods

2.1 History and declared features of MMS-1 Mars simulant

Fine-grade Mojave Mars Simulant MMS-1 was purchased from The Martian Garden (Austin, Texas, USA), a commercial company selling two Mars simulants: MMS-1 and MMS-2. Mojave Mars Simulant MMS was first developed by the Jet Propulsion Laboratory (JPL, Pasadena, California, USA) from a basaltic flow in the Mojave Desert, mainly to overcome the deficiency in the hygroscopic properties of the JSC Mars-1 simulant. According to the chemical composition provided by the supplier, MMS-1 is an analogous of MMS of JPL, while MMS-2 is a Mars simulant spiked with Fe(III) and Mg oxides and sulphates to offset discrepancies in bulk chemistry between Mars regolith and MMS-1 (Planetary-Simulant-Database, 2019). On the other hand, Cannon et al. (2019) stated that MMS-1 actually arises from the highly altered red cinder material described by Beegle et al. (2007) instead of the Saddleback Basalt of the Mojave desert (source of MMS simulant of JPL).

2.2 MMS-1 Mars simulant characterisation

2.2.1 Major and trace element composition

Fifteen grams of fine-grade MMS-1 simulant were manually ground in an agate mortar and mixed with 2 ml of a 15 % (w/w) Elvacite® 2046 resin/acetone solution (Malvern Panalytical, Malvern, UK). After drying, the powder was poured into aluminium cups and pressed to generate three pellets (4 tons cm^{-2}).

Thereafter, the concentrations of SiO_2, Al_2O_3, Fe_2O_3, CaO, K_2O, MgO, MnO, Na_2O, P_2O_5, and TiO_2 into each pellet expressed as percentages were measured by a Supermini 200 (Rigaku Corporation, Tokyo, Japan) wavelength dispersive X-ray fluorescence (WD-XRF) spectrometer equipped with a Pd tube (50 kV, 4 mA, 200 W) at the laboratory facilities of Innovative Solution Srl (Noci, BA, Italy). The calibration of the instrument and method validation were assessed by using a series of geological standards provided by *Service d'Analyses des Roches et des Minéraux* (SARM, CRPG-CNRS, Vandoeuvre-lès-Nancy, France). To determine the loss on ignition (LOI) 1 g of ground sample was poured into a ceramic crucible and heated to 1000 °C for 12 h.

The concentrations of trace elements (Ba, Cu, Ni, Rb, Sr, Zn and Zr) in each pellet expressed as mg kg^{-1} were measured by a NITON XL3t 900 portable energy dispersive X-ray fluorescence (ED-XRF) spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an Ag target (50 kV, 40 µA, 2 W) at the laboratories of Innovative Solution Srl (Noci, BA, Italy). The detector resolution was <160 eV (Mn Kα). Instrument performance was checked using standard reference materials (ERM-CC141 and NIST 2711a).

2.2.2 Simulant mineralogical composition

Particle-size fractionation following IUSS (formerly known as ISSS) size classes (>200, 20-200, 2-20 and <2 µm) was carried out by wet sieving (>200 µm) and sedimentation cycles in Esenwein cylinders (20-200, 2-20 and <2 µm) after sample dispersion aided by sodium hexametaphosphate. Each particle-size fraction was exhaustively recovered, weighed and stored for subsequent characterisation.

Samples of each simulant particle-size class were back-loaded onto perforated aluminium sample holders to obtain a random orientation of particles, and analysed by X-ray powder diffraction (XRPD) using a Miniflex II (Rigaku Corporation, Tokyo, Japan) X-ray diffractometer equipped with a Cu tube (Cu-Kα X-ray source, 15 mA, 30 kV) at the lab facilities of Innovative Solution Srl (Noci, BA, Italy). For the fine sand fraction (20-200µm) data were acquired between 3° and 120° 2θ, with a counting time of 3 sec per step, while for sand (>200µm), silt (2-20µm) and clay (<2 µm) fractions the range 3-70° 2θ was investigated using a speed of 2°/min; the step scan was always 0.02° 2θ. A continuous spin of the sample was applied to all analysed fractions. The incident beam passed through a 0.3 mm Soller slit, 1.25° divergent slit, a 10 mm mask and a 1.25° antiscatter slit. Analysis of diffraction data was performed by GSAS software (Larson and Von Dreele, 2000) and EXPGUI was the user graphical interface (Toby, 2001).

A combination of Rietveld and reference intensity ratio (RIR) methods was used for quantitative XRPD analysis of the simulant fine sand fraction (Gualtieri, 2000). Similarly, the quantitative analysis of smectite was carried out using the Rietveld method (Castellini et al., 2017). According to Alizai et al. (2012), for samples with weight percentages of clay minerals <10%, the conventional method of Moore and Reynolds (1989) may cause high uncertainties on clay mineral quantification.

2.2.3 Spectroscopic and thermo-gravimetric properties

Attenuated total reflection - Fourier transform infrared (ATR-FTIR) spectroscopy was performed with a Perkin-Elmer FTIR/NIR spectrometer Frontier with ATR accessory at room temperature in the range 650-4000 cm^{-1}. The FTIR spectra of every separated particle-size fractions of MMS-1 simulant were collected as a result of 64 running scans at a spectral resolution of 1 cm^{-1}.

Thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC) thermograms of fine-grade MMS-1 simulant were obtained using a simultaneous thermal analyser (Perkin Elmer STA 6000, Perkin-Elmer, Norwalk, CT, USA) in an air flow(50 mL min^{-1}) with a heating rate of 10 °C min^{-1} and temperature ranging from 30 to 900 °C.

2.3 Compost characterisation

The compost used in the current study was purchased from Vivai Gardea (Villafranca di Verona, Italy), and was obtained through the natural decomposition and slow transformation of plant matter, such as pruning waste and grass cuttings, into mature and stable compost by microorganisms under controlled conditions. The main properties of the compost were measured as follows.

1-2 mg of compost samples (n=3) underwent combustion analysis by a Thermo Scientific Flash EA 1112, equipped with a thermal conductivity detector (TCD), to determine total C, H, N and S element contents. Calibration of the analyser, check of element accuracy and recovery were performed using acetanilide (Sigma Aldrich, 99.5%) standard.

The total content of major (Al, Ca, Fe, K, Mg, Na and P) and trace elements (As, Cd, Co, Cr, Cu, Ni, Pb, V and Zn) was measured in acid-digested compost samples by inductively coupled plasma - optical emission spectrometry (ICP-OES). 0.3 g of each compost sample were placed in 100 ml PFA HP-500 Plus digestion vessels and mixed with 8 ml of concentrated HNO_3 and 2 ml of H_2O_2 and then digested in a CEM Mars Plus microwave oven. The heating program was performed in two steps: the temperature was increased from 25 to 165 °C in 10 min and held at 165 °C for 2 min. Then it was increased from

165°C to 180°C in 6 min and held at 180°C for 10 min. After cooling to room temperature, the digested samples were filtered through ash-free filter papers (Whatman 40) and transferred to 50 ml volumetric flasks and brought to volume with Milli-Q water. The total element concentrations in compost were then measured by ICP-OES technique (Perkin-Elmer 8000 DV, Perkin-Elmer, Norwalk, CT, USA) equipped with a Scott nebulizer system. In all analytical phases, blanks and triplicate samples were used to ensure the quality and reproducibility of the results. Compost was also characterised by ATR-FTIR spectroscopy, TGA and DSC according to the method described in section 2.2.3.

2.4 Characterisation of MMS-1/compost mixtures

2.4.1 Particle-size distribution and main chemical properties

MMS-1 Mars simulant and green compost were mixed at varying rates (0:100, 30:70, 70:30, 100:0; v:v) in order to obtain four mixtures to use as growing substrates for lettuce plants. The simulant:compost mixtures 0:100 and 30:70 are evidently non-sustainable in a BLSS in future manned missions to Mars. In this work these mixtures potentially represent the best scenarios for plant growth in terms of physico-chemical and biological fertility to be compared with more realistic and sustainable mixtures (70:30, 100:0) in space agriculture oriented towards a sustainable use of resources. For physico-chemical characterisation, substrate samples were collected at the start point and at the end of the growing cycle of lettuce plants and then dried to a constant weight. The contents in organic carbon (OC) and organic matter (OM = OC 1.724) were determined by wet digestion using the Walkley-Black procedure. The pH was measured by potentiometry in milliQ water or 1M KCl solution at 1/2.5 substrate/water ratio by HI 1131B pH meter (Hanna Instruments, Woonsocket, Rhode Island, USA), while electrical conductivity (EC) was measured at 1/5 substrate/water ratio by Basic 30 conductivity meter (Crison Instruments, Alella, Spain). Readily bioavailable nutrient concentrations in the substrates were determined in 1 g of sample suspended in 20 ml of ultrapure water (Arium Pro, Sartorius, Göttingen, Germany) and stirred in an orbital lab shaker (KS125 basic, IKA, Staufen, Germany) for 2h. After centrifugation (15 min at 3000 rpm), water extracts were analysed to determine the concentration of cations (Ca^{2+}, K^+, Mg^{2+}, Na^+) and anions [chlorine (Cl^-), nitrate (NO_3^-), phosphate ($H_2PO_4^-$and HPO_4^{2-}; referred to as PO_4 in the next sections and tables) and sulphate (SO_4^{2-})] by ion chromatography (ICS-3000, Dionex Sunnyvale, CA, USA) with a conductivity detector, using an IonPac CG12A pre-column and IonPac CS12A separation column for cations (Dionex Sunnyvale, CA, USA), as well as an IonPac AG11-HC pre-column and an IonPac AS11-HC separation column for anions (Dionex Sunnyvale, CA, USA).

Particle-size distribution was assessed after Na-hexametaphosphate dispersion, sieving and sedimentation cycles previously described (section 2.2.2). Additionally, total carbonates were measured by the calcimeter method. For determination of cation exchange capacity (CEC), the MMS-1 simulant was extracted by a 0.4M $BaCl_2 \cdot 2H_2O$ and $N(CH_2OHCH_2)_3$ solution at pH 8.2; moreover, these extracts were analysed by flame atomic absorption spectroscopy (FAAS, Perkin Elmer AAnalist 700) to measure the concentrations of extractable and plant-available K and Na (after dilution with Ce), Ca and Mg (after dilution with La). All physical and chemical analyses were performed in triplicate.

2.4.2 Physical and hydrological properties

The hydrological properties of the different simulant:compost mixtures were also studied. The experimental protocol consisted in measuring the main physical properties, i.e. saturated hydraulic conductivity (Ks), bulk density, saturated water content and container capacity, and further in determining water retention curves. For Ks determination, substrate mixtures were packed into cylindrical samplers 14 cm long and 8.5 cm in diameter. According to the structural characteristics of pure simulant, pure compost and the other two mixtures, the assemblage in the sampling cylinders was made applying the same pressure by the operator, and thus ensuring a settlement permitting gas and water movements. After transferring the samples to the permeameter, the bottom of the mixture samples was connected to a water source, and samples were allowed to wet at a pressure head of a few centimetres. The level of the water source was progressively increased until the samples were saturated. Ks was then determined using the constant head method (Reynolds et al., 2002). For pure compost core (0:100), Ks was not determined because the compost showed a tendency to float when subjected to water movement. Bulk density (BD) was calculated by dividing the substrate dry mass by the sample volume measured after sample saturation (core method, Grossman and Reinsch, 2002). Except for the pure compost core, shrinkage was small (changes in bulk density between the beginning and end of an experiment were <0.05 g cm-3). After completing determination of Ks, the samples were carefully driven to the scale for the determination of saturated weight. This operation is particularly delicate because during transport the sample must not lose its saturation condition. Once the saturated weight was obtained, the saturated water content (θs) was calculated by dividing the volume of water by the volume of the mixture sample. It is also possible to calculate water content of the substrate at field capacity, which represents the volume of water retained after the end of a drainage process and describes the upper limit of water available for the plant. In this work we measured instead effective container capacity (CC), following the methodology of Klute (1986). In particular, after a 24-hour drainage process driven by gravity under a glass bell with 100% relative humidity, thus avoiding any evaporative process, the gross weight of the sample allowed us to determine the volume of water retained, having subtracted the weight of the container. Division of this value by the sample volume yielded the container capacity. Under this condition the samples reach the state of hydrostatic equilibrium, i.e. the pressure head at the bottom is close to zero.

To determine the water retention curve, the samples were subjected to an evaporation experiment under laboratory conditions. The pure compost sample was not subjected to this experimental procedure due to its swelling and also to the difficulty of ensuring hydraulic contact between the porous capsule and the substrate matrix. After placing the sample in a structure, namely that described by Ciollaro and Romano (1995), the experiment was performed by drying the sample with a small fan at the top to increase the soil evaporation rate. With a load cell under the plate carrying the sample, the weight was measured, while the suction was monitored with the horizontally inserted porous capsule connected to pressure transducers at 3.5 cm, 6.5 cm, 9.5 cm substrate depth. The variables were monitored every 30 minutes throughout the experiment. During the evaporation experiment the temperature fluctuations in the laboratory were ± 1.0 °C. To derive the water retention curve for each mixture, Wind method was applied (Arya, 2002). By spline interpolation of the water retention curve (MATLAB software, MathWorks, Natick, MA, USA), the following additional physical properties were also calculated: air filled porosity (AFP), easily available water (EAW) and buffer capacity (BC), according to Caron et al.

(2007). Further, by using lettuce root water uptake parameters taken from the literature (Taylor and Ashcroft, 1972), a functional comparison was made between the three mixtures in terms of water reservoir, calculating the lettuce buffer capacity (LBC), considered as the volume of water retained between suctions of 2.45 kPa and 19.6 kPa. The latter value of suction represents the mid point between optimal matric potential at which water uptake by lettuce roots begins, and incipient water stress. Finally, using the retention curve data of the three mixtures, it was possible to determine the frequency distribution of pore diameter for each substrate using the algorithms illustrated in Flint and Flint (2002).

2.5 Plant growth assay on MMS-1/compost mixtures

2.5.1 Phytotron growth chamber conditions, plant material, fertigation system and experimental design

A nineteen-day experiment was conducted in a 28 m^2 phytotron open-gas-exchange growth chamber (7 m W × 2.1 m H × 4.0 m D) at the experimental station of the Department of Agricultural Sciences, University of Naples Federico II, located in Bellizzi (Salerno), southern Italy (495543 m E, 4496589 m N; 60 m above sea level). High-pressure sodium lamps were used at a light intensity of 420 μmol m^{-2} s^{-1} photosynthetic photon flux density, with a light/dark regime of 12/12 h corresponding to a temperature of 24/18 °C, respectively. Relative humidity was maintained at 65-75 % by a fog system, while air circulation and dehumidification were provided by two Heating, Ventilation and Air Conditioning (HVAC) systems. This experiment was carried out at ambient CO_2 concentration of 370-410 ppm.

Two butterhead cultivars, namely Green Salanova® and Red Salanova® (RijkZwaan, Der Lier, The Netherlands), of lettuce (*Lactuca sativa* L. var. *capitata*) were transplanted after two weeks from sowing in plastic pots (7 × 7 × 8 cm) filled with one of four growing substrates obtained by mixing MMS-1 Mars simulant and compost at varying rates (0:100, 30:70, 70:30, 100:0; v:v). The plastic pots were placed on propylene gullies with a slope of 1% in order to facilitate the collection of draining solution, since an open loop hydroponic system was adopted. Fertigation was delivered by a drip irrigation system through auto-compensated drippers with a flow rate of 2 L h^{-1}. The nutrient solution consisted of a modified Hoagland formulation: 9.0 mM N–NO_3^-, 2.0 mM S, 1.0 mM P, 4.0 mM K, 4.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+, 15 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo, yielding EC and pH values of 1.5 dS cm^{-1} and 5.8, respectively.

Eight treatments were obtained from a factorial combination of two cultivars and four substrate mixtures. The treatments were arranged in a randomised complete-block design with three replicates, yielding a total of 24 experimental units. Each experimental unit consisted of seven plants, for a total number of 168 lettuce plants, distributed at a density of 15.5 plants m^{-2} (15 cm intra-row × 43 cm inter-row).

2.5.2 Growth analysis and biomass determination

All plants were harvested at 19 days after transplanting. Leaf number and shoot fresh biomass of the aerial part were determined, where leaf area was measured by an area meter (LI-COR 3100C, Biosciences, Lincoln, Nebraska, USA). Shoot and root dry biomass were measured on an analytical balance (Denver Instrument, Denver, Colorado, USA) after sample drying in a forced-air oven at 70 °C until a constant weight was reached (nearly three days). The roots were washed softly with demineralised water in order to determine the dry biomass mentioned above. Shoot dry matter was calculated based on the official method 934.01 of the Association of Official Analytical Chemists (AOAC, 2005). Shoot-to-root ratio was also calculated by using the dry biomass weight.

2.6 Statistical analysis

The experimental design consisted of a factorial combination of the two lettuce cultivars and four different substrate mixtures for a total of eight treatments with three replicates. A randomized complete-block design was adopted, with a total of six plants for each replica (for total of 144 plants). One-way (post hoc test: Duncan’s multiple-range test) and two-way analyses of variance (ANOVA) were performed by the software IBM SPSS Statistics v23 (Armonk, NY, USA). Statistical differences were assumed at $p < 0.05$.

3 Results and discussion

3.1 Chemical and mineralogical composition, spectroscopic and thermo-gravimetric properties of MMS-1 Mars simulant

The content in main element oxides (%) and trace elements (mg kg^{-1}) in fine-grade MMS-1 is provided in Table 1A. The MMS-1 simulant mostly consists of SiO_2 (57.3 % of the total), Al_2O_3 (12.9 %) and Fe_2O_3 (9.1 %), but also contains significant amounts of other element oxides such as CaO, K_2O, MgO, MnO, Na_2O and P_2O_5 (0.1-4.9 %), essential for plant growth and development. Comparing our data with those provided by the MMS-1 supplier (The Martian Garden) or found by Peters et al. (2008) who characterised the analogous MMS Mars simulant produced by JPL, we found a higher content (plus 1-28 %) in SiO_2, Na_2O, P_2O_5 and TiO_2, and a lower content (minus 16-41 %) in Al_2O_3, Fe_2O_3, CaO, MgO and MnO. We also measured a 4.4-fold higher content in K_2O and a greater LOI (18%) including H_2O, SO_3 and Cl. These differences may be justified by the different analytical methodologies adopted (WD-XRF in the present study, acid digestion and ICP-OES in the reference data). Besides, as stated by Cannon et al. (2019), there are discrepancies between MMS-1 simulant (lacking rigorous documentation on its own mineralogical composition) and the original MMS simulant, highlighted by different Vis-NIR spectral properties. An analogous comparison between trace element contents determined by ED-XRF (Table 1A) and average contents of nine whole-rock samples from Saddleback Mountain (Mojave desert of eastern California, USA) determined by ICP-MS (Peters et al., 2008), highlighted higher contents in Rb, Zr (up to 4-fold more) and Ba, Ni, Sr (plus 1-28 %), and lower contents in Cu and Zn (minus 28-46 %). Thus, in terms of chemical classification, the MMS-1 simulant can be considered an intermediate (SiO_2 =57.3%) peraluminous [$(K_2O + Na_2O)/Al_2O_3 = 0.5$] igneous trachy-andesitic rock, having Ba, Zr, Sr and Ni as the most abundant trace elements.

Table 1. A) Content inmain oxides, loss of ignition and trace elements in fine-grade simulant,measured byWD- and ED-XRF, respectively (n=3). B) Content in C, H, N and S in compost measured by elemental analyser and H/C and C/N ratios calculated as atomic ratios (n = 3) and content in major and trace elements in compost, measured by ICP-OES (n = 3). C, H, N and S stand for carbon, hydrogen, nitrogen, sulphur, respectively.

A)

SiO_2	(%)	57.3 ± 1.3
Al_2O_3		12.9 ± 0.1
Fe_2O_3		9.1 ± 1.0
CaO		4.9 ± 0.9
K_2O		2.1 ± 0.4
MgO		4.1 ± 0.3
MnO		0.1 ± 0.04
Na_2O		4.2 ± 0.8
P_2O_5		0.2 ± 0.06
TiO_2		1.1 ± 0.2
Loss of ignition	(%)	4.0 ± 0.2
Ba	(mg kg^{-1})	566 ± 16
Cu		31 ± 4
Ni		116 ± 14
Rb		45 ± 2
Sr		283 ± 2
Zn		52 ± 3
Zr		292 ± 2

B)

C	(%)	29.3 ± 3.0
H		3.1 ± 0.3
N		2.3 ± 0.2
S		0.1 ± 0.03
H/C		1.3 ± 0.1
C/N		14.8 ± 0.6
Al	(g kg^{-1})	4.7 ± 0.3
Ca		12.5 ± 3.6
Fe		4.7 ± 0.5
K		17.2 ± 1.7
Mg		3.9 ± 1.1
Na		3.5 ± 0.1
P		2.6 ± 0.2
As	(mg kg^{-1})	2.9 ± 0.6
Cd		0.2 ± 0.1
Co		2.3 ± 0.3
Cr		7.8 ± 1.1
Cu		97 ± 5
Ni		9 ± 0.6
Pb		24 ± 1.2
V		6.5 ± 0.4
Zn		91 ± 10.3

Figure 1 shows the mineralogical composition of four MMS-1 particle-size fractions. The X-ray Powder Diffraction (XRPD) patterns of all fractions reveal an abundant presence of plagioclases (in many cases of anorthite) with sharp peaks at 0.405, 0.375 and 0.320 nm, along with a large amount of iron oxides (hematite) with characteristic peaks at 0.270, 0.252 and 0.185 nm. A double peak at 1.262 and 1.342 nm is clearly evident in the clay fraction (<2 μm) and ascribed to smectite minerals, very likely to Na-saturated montmorillonite, while the peak in the 1.4 nm region in the silt fraction (2-20 μm) appeared broader. Quantitative analysis of the sand fraction (20-250 µm) also reveals a significant occurrence of amorphous material (probably glass) and zeolites (clinoptilolite), representing 28% and 12% of the fraction, respectively. On the other hand, the content in plagioclases was found to be more than 50 % of the fraction (S1). Sharp and characteristic peaks of plagioclases and hematite were also evident in the XRD diffractogram of the analogous MMS simulant, characterised by Beegle et al. (2007).

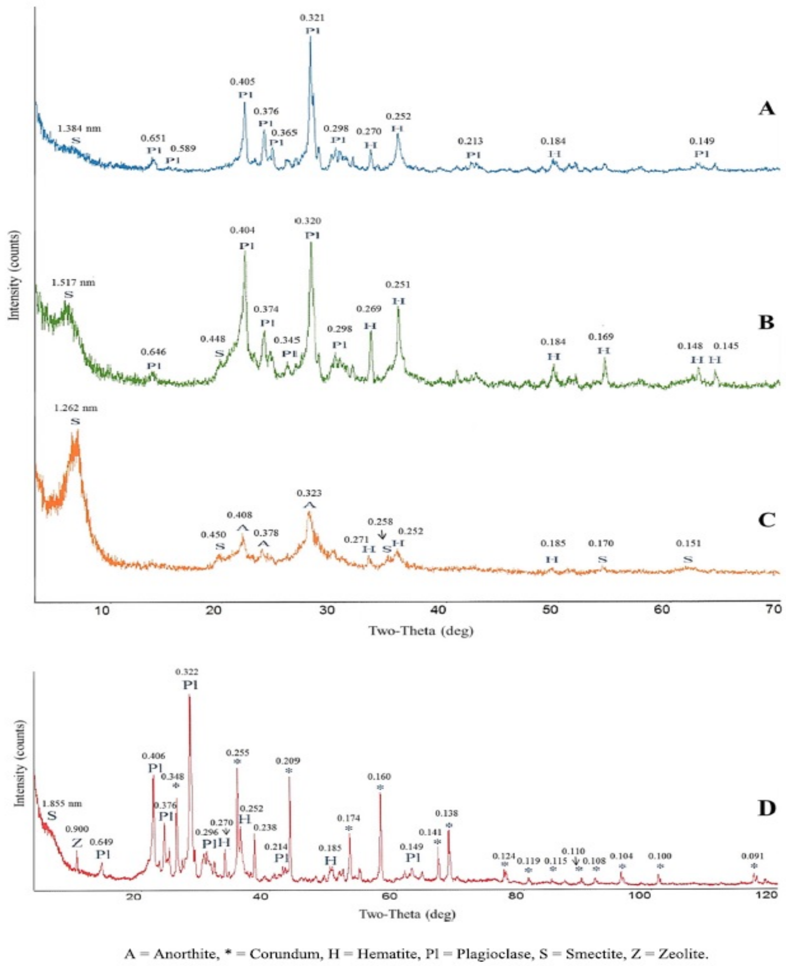

Figure 1. X-ray Powder Diffraction (XRPD) of particle-size fraction >200 µm (A), 20–200 µm (D), 2–20 µm (B) and <2 µm (C) of MMS-1 Mars simulant.

The ATR-FTIR spectra of MMS-1 particle-size fractions are depicted in Figure 2. The spectra of the clay and fine silt fractions clearly differ from those of the two coarser particle-size fractions in: i) the sharp and intense signal at 995-1010 cm^{-1}, which is attributed to the strong Si−O stretching band of smectite minerals; ii) the shoulder at 3635 cm-1, commonly assigned to the −OH stretching band of the smectite montmorillonite; iii) the signal at 790cm^{-1}, which may reveal the presence of Fe in the octahedral sheets of the smectites (Dixon and Weed, 1989). The weak signals at 1600-1650 cm^{-1} and 1400 cm^{-1} reveal the presence of hematite (Feng et al., 2017). The weak signals at 1500 cm^{-1} can be assigned to amorphous calcium carbonates (Cai et al., 2010). To the best of our knowledge, this is the first evidence of MMS-1 characterisation by mid-infrared (MIR)spectroscopy; spectral properties of MMS-1 and analogous MMS simulants were formerly studied in the Vis-NIR spectral range (Beegle et al., 2007; Peters et al., 2008; Cannon et al., 2019).

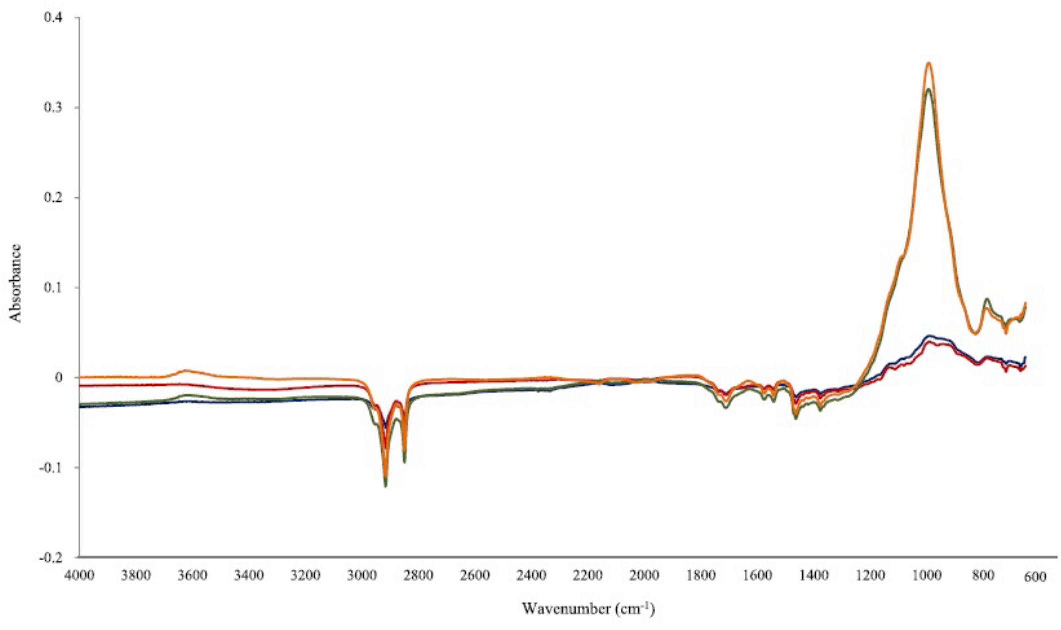

Figure 2. Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR) spectroscopy of particle-size fraction >200 µm (blue line), 20–200 µm (redline), 2–20 µm (green line) and <2 µm (orange line) of MMS-1 Mars simulant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Neither TGA nor DSC curves of fine-grade MMS-1 provide any relevant information on the nature of the Martian simulant (S2A). The principal weight loss in the TGA single-stage-cracking-curve of MMS-1 is around 100 °C, essentially due to moisture loss (Dixon and Weed, 1989).

3.2 Chemical composition, spectroscopic and thermo-gravimetric properties of compost

The elemental composition, H/C and C/N ratios of compost are shown in Table 1B. A comparison between element contents of our compost and data from a systematic review on numerous green-waste composts (Reyes-Torres et al., 2018) reveals a good presence of N (1.7-fold higher than the average value from 33 scientific studies), a medium-low abundance of organic C (22% lower than the average from 17 scientific studies) and a low content in S (equal to the minimum value from 11 scientific studies) (Table 1B). Likewise, the contents of other major elements such as Ca, Fe, K, Mg and P in our compost (Table 4) are 1.1- to 3.0-fold higher than the average values. The contents of Cr and Ni in the compost used in our study (Table 1B) are found to be lower than the average values, while those of Cd and Pb do not exceed maximum values reported in the review study (Reyes-Torres et al., 2018). On the other hand, the contents of other potentially toxic elements for plant growth such as Cu and Zn exceed the maximum values of 2.6 and 1.1, respectively, of the review study (Reyes-Torres et al., 2018), but are within the legal limits on compost amendments set by Italy and other EU countries (Amlinger et al., 2004). The low C/N ratio (Table 1B) indicates a significant presence of low-molecular-weight N-rich materials such as peptides, in relation to C-rich components (e.g. polysaccharides). This improves the availability of N in the rhizosphere and thus promotes its uptake by plant roots (Epstein, 2011). However, the medium-low H/C value (Table 1B) also suggests a significant occurrence of aromatic compounds (Kasiuliene et al., 2018).

ATR-FTIR spectrum of the compost is shown in S3. The broad absorption band around 3000-3500 cm^{-1} is associated to −OH stretching vibrations in alcohols, phenolic or carboxylic acids (Bornemann et al., 2010). The shoulders at 2923 and 2851 cm^{-1} are basically attributed to the symmetric and asymmetric stretching vibrations in $-CH_2$ and −CH groups of aliphatic chains (Bellamy, 1975). The shoulder at 2160 cm^{-1} can be assigned to C≡C stretch vibrations of asymmetrical alkyne groups (Soobhany et al., 2017).

The weak bands around 2000 cm^{-1} can be attributed to overtones of aromatic compounds. Compost shows an intense absorption band in the 1450-1320 cm^{-1} region, which is generally assigned to either aliphatic CH_2 bending vibrations (1450 and 1420 cm^{-1}), or phenolic OH or C−OH deformations or COO− symmetric stretching (Ali et al., 2012). The signal at 1030 cm^{-1} is associated to the C−O stretching of carbohydrates, thereby indicating a significant occurrence of polysaccharides in the compost (Zaccheo et al., 2002). The signal at 873 cm^{-1} is assigned to C=C and C−H vibrations, indicative of the presence of aromatic moieties in the compost (Niemeyer et al., 1992).

The TGA and DSC curves of compost are reported in S2B. The thermal profile of the TGA curve indicates the weight loss as a function of temperature, while the DSC curve further clarifies the behaviour of compost during analysis by correlating weight loss to the sample thermal degradation. The principal weight losses in the compost TGA multistage-cracking-curve are due to moisture loss (around 100 °C), the degradation of hemicellulose and other labile components (200-300 °C), cellulose (250-350 °C) and lignin and other polyphenols (200-500 °C) (Lee et al., 2018). A further weight loss was observed at around 600 °C (between 550 °C and 760 °C), characterising the de-carbonisation of calcite ($CaCO_3$), with loss of CO_2 (Nada et al., 2012). The thermal DSC curve of compost is mainly characterised by two exothermic peaks. The first at around 300 °C may be attributed to the degradation of the most labile compounds, such as carbohydrates, free lipids and amino acids (Fernández et al., 2012). The second exothermic peak at higher temperatures (450-500 °C) might be due to the combustion of the aromatic moiety of the compost, such as lignin and other polyphenols (Plante et al., 2009).

3.3 Particle-size distribution and main chemical properties of MMS-1/compost mixtures

Table 2 shows the main properties and nutrient concentrations of the different growing substrates obtained by mixing MMS-1 simulant and compost at varying rates, before the growing cycle of green and red-pigmented Salanova lettuce. The alkaline pH of MMS-1 simulant, mostly due to the high concentration of exchangeable and total Na, is reduced to sub-alkaline values by compost addition. Moreover, OM content, EC and nutrient concentrations significantly decrease at increasing rates of MMS-1 simulant in the growing substrate, except for Na (Table 2). In particular, the concentrations of K, Cl, NO_3, PO_4 and

SO_4 in the 100% MMS-1 substrate are less than 4 % of the concentrations of the same nutrients in the 100% compost substrate, while Mg and Ca are 13 % and 17 %, respectively, indicating a possible release of the two nutrients from MMS-1 simulant minerals. Therefore, although the pool of available nutrients in the pure Mars simulant is much lower than that of the pure compost, it can contribute to support plant growth (Wamelink et al., 2014), especially if integrated by mineral fertigation and organic amendment. In this regard, the non-statistically different concentrations of Na in the four substrates reveal that large amounts of soluble Na can be easily available from MMS-1 simulant. This pool of bioavailable Na in the MMS-1 simulant, together with alkaline pH and absence of biological fertility, might induce salt stress in plants (Qin et al., 2013). Broad differences in terms of chemical composition of green compost and JSC Mars-1 regolith simulant were also observed by Gilrain et al. (1999), who grew Swiss chard in a mixture of compost/JSC Mars-1 at different ratios.

The particle-size fractionation of pure MMS-1 simulant reveals an abundance of coarse particles, with a predominance of sand (65 % of the total), along with a high presence of fine sand (26 %). As a consequence, the occurrence of fine fractions is very low, with only 6.5 % of fine silt and 2.5 % of clay. An analogous particle-size distribution was also observed by Allen et al. (1998), Peters et al. (2008) and Zeng et al. (2015) in the Mars regolith simulants JSC Mars-1, MMS and JMSS-1, respectively. The pH in 1M KCl is sub-alkaline (~1.0 lower than pH in milliQ water), probably due to the abundance of short-range ordered or variable charge minerals, generally included in amorphous residual materials (S1), or to Al solubilisation from this peraluminous rock. The contents in organic C and total N are negligible (their sum was ~0.03 % of the simulant), probably arising from pioneer biofilms colonising simulant mineral surfaces, which may as well explain the low C/N ratio. The content in total carbonates is moderate (< 3 %) but does not affect element bioavailability in the substrate. The cation exchange capacity is rather low - essentially due to the scarce presence of clay minerals and organic matter - and is mainly saturated by Ca (65 %), followed by Na (16 %), Mg (11 %) and K (8 %). These percentages reveal an abundance of exchangeable Na at the expense of exchangeable Mg and K.

Table 2. Particle-size distribution and main chemical properties of different mixtures (simulant: compost rates: 0:100, 30:70, 70:30, 100:0; v:v), measured before the growing cycle of green and red Salanova lettuces.

Mixture property	Start point			
	Simulant:compost (v:v)			
	0:100	30:70	70:30	100:0
OM (% w/w)	50.5 a	25.9 b	8.7 c	< 0.1 d
pH in milliQ water	8.25 c	8.29 b	8.28 b	8.86 a
Electrical conductivity (dS m^{-1})	3.5 a	1.8 b	0.6 c	0.3 d
Water-soluble Ca (mg kg^{-1} DW)	1242 a	848 b	465 c	212 d
Water-soluble K (mg kg^{-1} DW)	10149 a	7019 b	1967 c	21 d
Water-soluble Mg (mg kg^{-1} DW)	380 a	256 b	131 c	49 d
Water-soluble Na (mg kg^{-1} DW)	204	275	294	216
Water-soluble Cl (mg kg^{-1} DW)	1782 a	1061 b	310 c	8 d
Water-soluble NO_3 (mg kg^{-1} DW)	741 a	439 b	130 c	12 d
Water-soluble PO_4 (mg kg^{-1} DW)	405 a	238 b	61 c	1 d
Water-soluble SO_4 (mg kg^{-1} DW)	263 a	158 b	62 c	9 d
Coarse sand (> 200 µm - g kg^{-1})	–	–	–	650
Fine sand (200–20 µm - gkg^{-1})	–	–	–	260
Silt (20–2 µm - g kg^{-1})	–	–	–	65
Clay (< 2 µm - g kg^{-1})	–	–	–	25
pH in 1 M KCl	–	–	–	7.7
OC (g kg^{-1} DW)	–	–	–	0.2
Total N (g kg^{-1} DW)	–	–	–	0.1
C/N ratio	–	–	–	2.7
Total carbonates (g kg^{-1} DW)	–	–	–	27
Cation exchange capacity (cmol(+) kg^{-1})	–	–	–	7.9
Exchangeable Ca (mg kg^{-1} DW)	–	–	–	1034
Exchangeable K (mg kg^{-1} DW)	–	–	–	248
Exchangeable Mg (mg kg^{-1} DW)	–	–	–	106
Exchangeable Na (mg kg^{-1} DW)	–	–	–	292

Different letters indicate significant differences according to one-way ANOVA, Duncan's multiple-range test ($p = 0.05$). - indicates not available data. DW stands for dry weight. OM indicates organic matter and OC means organic carbon.

3.4 Physical and hydrological properties of MMS-1/compost mixtures

The hydrological properties, directly measured in the cores or derived from the water retention curve, along with the values of packing bulk density, are reported in S4. Even if Ks is usually not of direct importance for water and nutrient uptake by plants, it has some relevance for salt leaching and for changing the composition of the substrate solution (Blok et al., 2019). The outcomes of permeability tests indicate that all the mixtures, if compared to the average values ofplant-growing media, show a medium to high saturated hydraulic conductivity, according to the classification of Kutílek and Nielsen (1994).

A consequence of compost addition to simulant is the increase in Ks, albeit not proportional. As expected, values of bulk density progressively decrease with increasing rates of compost in the mixture (from 1.39 g cm^{-3} of pure simulant to 0.60g cm^{-3} of sole compost). The bulk density of the 30:70 (0.90 g cm^{-3}) and 70:30 (1.14 g cm^{-3}) simulant:compost mixtures does not significantly differ from the weighted average of the two densities with respect to the respective volumes (0.73 g cm^{-3} and 1.15 g cm^{-3}, respectively). The same behaviour is also observed for θs; the relation is quite deterministic, thus giving the chance to determine a linear relationship in order to predict θs of a simulant:compost mixture, with an R^2 equal to 0.99. This function is as follows:

$$\theta s_{(100\text{-}x:C)} = 0.0024 * C + \theta s_{(100:0)}$$

The same also holds for container capacity, which shows the following relation:

$$CC_{(100\text{-}x:C)} = 0.0026 * C + CC_{(100:0)}$$

As in the previous relation, also this empiric relation shows an R^2 equal to 0.99.

Figure 3 represents the experimental values of the volumetric water retention curves of the different mixtures. The results show that the substrates behave differently in terms of water retention at increasing matric suction. In particular, it is evident that the addition of compost to pure simulant proportionally increases the maximum amount of water retained. The retention curves of pure simulant and 70:30 simulant:compost mixture tend to converge when matric suction approximates to 60 cm. On the other hand, the retention curve of 30:70 simulant:compost mixture is always higher than the other two. In fact, the suitability of a substrate for the cultivation of a candidate crop cannot be established with the sole analysis of the retention curve; in other words, higher values of the saturated water content do not necessarily mean better performance. Analysing the hydrological parameters derived from the water retention curve in S4, it is possible to assess the suitability of the mixtures for the cultivation of a staple crop as far as water relations are specifically concerned. We can make a generic evaluation by analysing the AFP, EAW and

BC indexes. To investigate further, we can infer the suitability of the substrates analysed for lettuce, especially when we consider the LBC index. Following Caron et al. (2007), appropriate values of AFP should be between 0.15 and 0.30 $cm^3\ cm^{-3}$; all of the three AFPs are in this range, with the 70:30 mixture showing a borderline value of 0.14 cm3 cm-3. In terms of EAW, normal values lie between 0.20 and 0.30 and all of the substrates show lower values, with the 70:30 mixture having the highest value (0.15). The buffer capacity values rise with increasing percentages of compost in the mixture, ranging from 0.092 $cm^3\ cm^{-3}$ of 100:0 to 0.150 for 30:70.

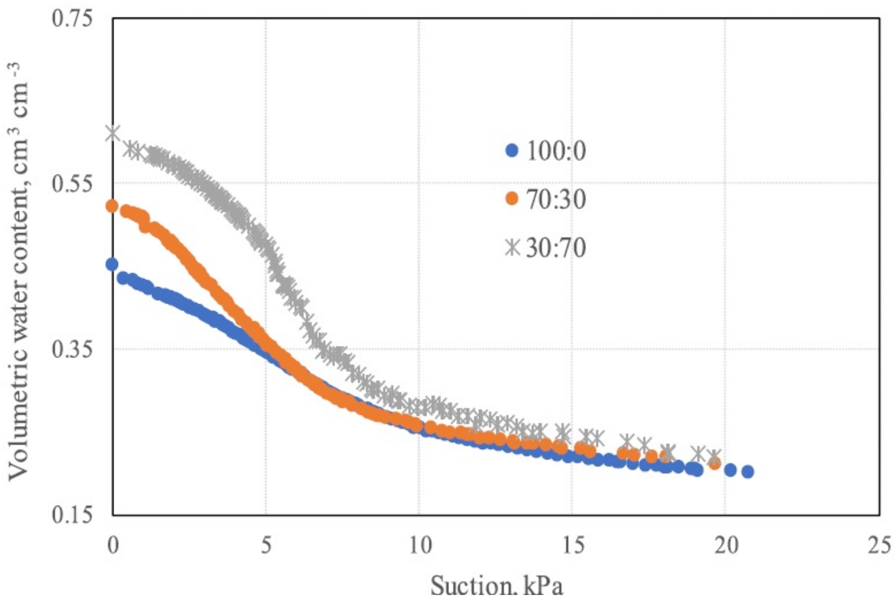

Figure 3. Experimental water retention curves of different mixtures (simulant:compost rates: 30:70, 70:30, 100:0; v:v).

Values of buffer capacity seem very low if compared with container capacity, thus giving a false impression of inadequacy of the substrates with respect to cultivation. Instead, on analysing the LBC index, it may be noted that all the substrates can guarantee, as far as the water relation is concerned, a suitable water state for the profitable cultivation of lettuce. An interesting aspect is that the percentage of LBC with respect to θs increases at increasing rates of compost in the mixtures. Indeed, there is an increase from 44 % of pure simulant to 56 % of 70:30 mixture. This means that compost addition to a mineral-based growing medium enhances the ability of the substrate to supply water to the crop.

Macropores are the primary pathway to conduct the flow of water when the soil is saturated. Deeks et al. (2004) define effective pores or macropores as those having a nominal diameter >50 µm whereas residual pores or micropores are <50 µm, equivalent to 6 kPa of suction. By analysing the frequency distribution of pore diameter of the mixtures, some interesting aspects may be highlighted. The vertical dense dotted line in Figure 4 represents the boundary between macropores and micropores and allows us to better understand what effect is caused by addition of compost to the pure simulant. At first glance it is clear that the compost exerts greater effect on the macropore region than on the micropore domain.

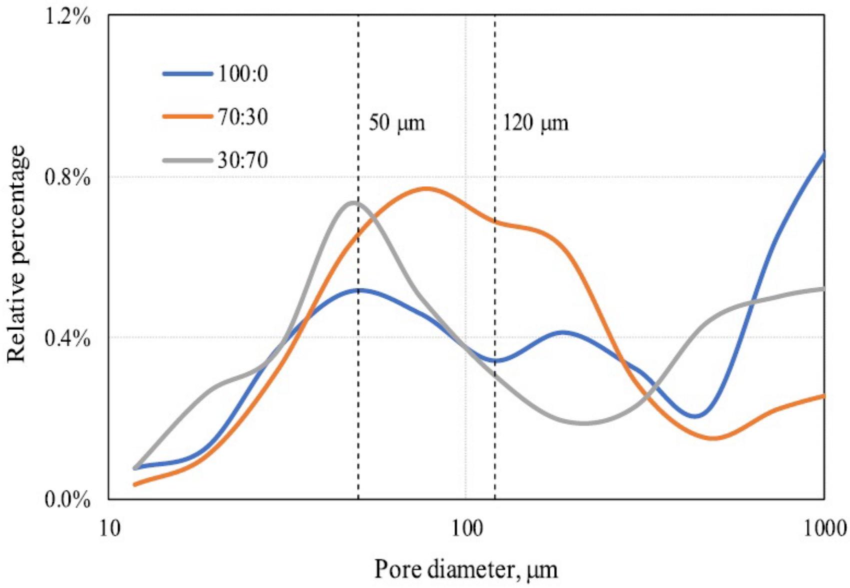

Figure 4. Pore-size distribution of different mixtures (simulant:compost rates: 30:70, 70:30, 100:0; v:v).

Given that water held in macropores exceeding 120 µm in diameter is not directly useful for root water uptake of lettuce, but can even cause root asphyxia, and that the pore size distribution below 50 µm is very similar among the three mixtures considered, it is evident that the best substrate from the water relations is 70:30, because the percentage increase of large pores (diameter between 50 and 120 µm) is greater than that found in the 30:70 mixture. Overall, in terms of water and nutrient transport processes, all selected substrates would be eligible to be used as growth media in a hydroponic cultivation system, where a timely water supply is guaranteed. On the other hand, if the basis is to have an optimised collection system with regard to energy consumption and system usage, the matter changes. If, for example, the objective is to manage lettuce irrigation in order to minimise irrigation frequency, the 70:30 mixture seems the most promising substrate according to its hydraulic properties.

3.5 Agronomic performance of green and red-pigmented lettuce on MMS-1/compost mixtures and nutrient composition in substrates at the end of the growing cycle

The results regarding growth parameters of red and green Salanova lettuces are presented in Table 3. For leaf area, leaf number, shoot and total dry biomass no significant interaction between the two tested factors (cultivar and simulant:compost mixture) is observed, whereas a significant interaction is recorded for the case of shoot dry matter percentage, root dry biomass and root-to-shoot ratio (Table 3). Regarding the effect of lettuce cultivar, the red-pigmented butterhead Salanova exhibits a higher leaf number, leaf area, shoot and total dry biomass than its green counterpart. A better crop performance of red versus green-pigmented butterhead Salanova in terms of fresh and dry biomass production was also reported by Giordano et al. (2019) in a closed soilless cultivation system. Moreover, when averaged over lettuce cultivars, the highest values of leaf area and total dry biomass are recorded in the substrate containing 30 % of MMS-1 and 70 % of compost. Likewise, Gilrain et al. (1999) found a statistically equivalent yield of Swiss chard grown in a mixture of compost and Mars regolith simulant (JSC Mars-1) at volume ratios of 1:0, 3:1 and 1:1, but higher than the yield of plants grown in substrates 1:3 and 0:1. The lettuce plants grown in 100% of MMS-1 produce a lower biomass in comparison to plants amended with compost at varying rates due to the lack of organic matter and biological fertility in the simulant and probably to the significant presence of exchangeable Na which may have caused salt stress in the lettuce plants (Qin et al., 2013). However, the reduction rate in all studied parameters is always moderate (<19 %). This is mostly due to the adequate nutrient supply from modified Hoagland solution, but also to the release of some essential elements, such as Ca, Mg and K (Table 3), from minerals of MMS-1 regolith simulant in contact with plant roots and their own exudates.

Table 3. Biometric data on green and red Salanova lettuces grown in different substrates (simulant:compost rates: 0:100, 30:70, 70:30, 100:0; v:v), measured at the plant sampling.

Source of variance	Leaf area	Leaf number	Shoot dry biomass	Shoot dry matter	Roots dry biomass	Shoot/root ratio	Plant dry biomass
	(cm^2 plant^{-1})	(no. plant^{-1})	(g plant^{-1})	(%)	(g plant^{-1})		(g plant^{-1})
Cultivar (C)							
Green Salanova	1012 b	44 b	2.8 b	5.1 b	0.55 b	5.1 b	3.3 b
Red Salanova	1148 a	54 a	3.4 a	5.5 a	0.58 a	5.8 a	3.9 a
Simulant:compost (v:v) (S)							
0:100	1086 b	50.3 a	3.1 a	5.2 b	0.50 c	6.3 a	3.6 b
30:70	1146 a	50.6 a	3.2 a	5.0 c	0.61 a	5.2 b	3.8 a
70:30	1075 b	49.6 a	3.0 b	5.1 c	0.57 b	5.2 b	3.5 b
100:0	1014 c	47.1 b	2.9 b	5.8 a	0.58 b	5.1 b	3.5 b
C x S							
Green Salanova × 0:100	1027	46.1	2.9	5.2 b	0.46 d	6.2 a	3.3
Green Salanova × 30:70	1072	47.0	2.9	4.8 d	0.62 a	4.8 c	3.5
Green Salanova × 70:30	1010	44.8	2.7	4.9 cd	0.57 b	4.7 c	3.2
Green Salanova × 100:0	939	41.4	2.6	5.4 b	0.57 bc	4.6 c	3.2
Red Salanova × 0:100	1145	54.5	3.4	5.3 b	0.54 c	6.3 a	3.9
Red Salanova × 30:70	1220	54.3	3.5	5.1 bc	0.61 a	5.7 b	4.1
Red Salanova × 70:30	1140	54.4	3.2	5.2 b	0.57 b	5.7 b	3.8
Red Salanova × 100:0	1089	52.8	3.3	6.2 a	0.59 ab	5.5 b	3.8
Significance							
Cultivar (C)	***	***	***	***	**	***	***
Simulant(S)	**	*	***	***	***	***	***
C x S	ns	ns	ns	**	**	***	ns

Levels of significance (p) at two-way ANOVA are categorised as follow: ns=non-significant, *, **, ***=significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to one-way ANOVA, Duncan's multiple-range test (p=0.05).

These findings thus strongly suggest that the 70:30 simulant:compost mixture is the best option and compromise between plant growth performance and sustainable use of the compost, a limited resource in a BLSS of space farming. Accordingly, a relatively good capacity of the Mars regolith simulant JSC Mars-1 to sustain plant growth in the absence of organic or mineral fertilisation was demonstrated by Wamelink et al. (2014) in a large-scale controlled experiment with 14 plant species, whose growth and flowering on JSC Mars-1 simulant were much better than on Moon regolith simulant (JSC1-1A) and even slightly better than on control nutrient-poor river soil. Likewise, sweet potato growth and yield were not adversely affected when grown in the JSC Mars-1 simulant under controlled environment and nutrient supply (Mortley et al., 2000), in comparison to plants grown with the nutrient film technique (NFT).

The below-ground root system (i.e. root biomass), which may improve nutrient uptake/assimilation and translocation leading to higher biomass production, is significantly affected by the interaction between the two tested factors, with the highest values recorded with 30:70 simulant:compost ratio in both green and red Salanova (Table 3). Similarly, a significant interaction is also observed for the shoot-to-root ratio, where a significant decrease is observed in both cultivars, though with a more severe reduction recorded in green Salanova (Table 3). It is noteworthy that the shoot/root ratio in the lettuce plants progressively decreases at increasing regolith content in the growing substrate (Table 3), probably because plants need a more extensive root system in less fertile substrates, characterised by a lower nutrient availability in comparison to compost alone.

Table 4 shows the pH, EC and nutrient concentrations in the different MMS-1/compost substrates after the growing cycle of green and red Salanova lettuces. In comparison to the starting point, the pH in the 100% MMS-1 substrate decreases by ~1.0 and ~0.5 after the green and red Salanova growing cycle, respectively. A lower pH is also observed in the other substrates, probably due to the effect of the constant fertigation with the sub-neutral modified Hoagland solution and possible release of low-molecular-weight organic acids from root exudates. Similarly, at the starting point, EC and nutrient concentrations progressively decrease from pure compost (0:100) to pure simulant (100:0) substrates. However, the constant supply of nutrients from fertigation throughout the crop growing cycle attenuates the differences among the four substrates in comparison to the starting point.

According to the nutrient concentrations in the four mixtures at the end of the cycle (Table 4), Green Salanova lettuce would basically have assimilated more K, Na, Cl, NO_3 and PO_4 from the substrates, while Red Salanova would have taken up more Ca, Mg and SO_4 from the mixtures. A similar trend in nutrient uptake/assimilation of green versus red-pigmented butterhead Salanova was also found by El-Nakhel et al. (2019), who studied the nutritional composition of two lettuce cultivars in closed soilless cultivation.

Table 4. Physicochemical properties of different mixtures (simulant/compost rates: 0:100, 30:70, 70:30, 100:0; v:v), measured after the growing cycle of green and red Salanova lettuces.

Mixture property	End cycle green Salanova				End cycle red Salanova			
	Simulant:compost (v:v)				Simulant:compost (v:v)			
	0:100	30:70	70:30	100:0	0:100	30:70	70:30	100:0
pH in milliQ water	7.79 b	8.1 a	7.69 b	7.72 b	7.92 bc	8.14 ab	7.76 c	8.18 a
EC (dS m^{-1})	2.4 a	1.0 b	0.5 c	0.3 d	2.4 a	1.1 b	0.5 c	0.3 d
Water-soluble Ca (mg kg^{-1} DW)	1505 a	543 b	233 d	310 c	1457 a	549 b	234 c	231 c
Water-soluble K (mg kg^{-1} DW)	7629 a	2791 b	1101 c	62.2 d	7745 a	3030 b	1001 c	45.1 d
Water-soluble Mg (mg kg^{-1} DW)	387 a	139 b	69 c	51 c	390 a	154 b	58 c	39 c
Water-soluble Na (mg kg^{-1} DW)	179 a	149 b	164 ab	108 c	183 a	145 b	153 b	162 b
Water-soluble Cl (mg kg^{-1} DW)	71 a	17 b	3 d	5 c	86 a	4 c	9 b	2 c
Water-soluble NO_3 (mg kg^{-1} DW)	255 a	74 b	32 c	33 c	294 a	84 b	45 c	34 c
Water-soluble PO_4 (mg kg^{-1} DW)	463 a	162 b	67 c	1 d	589 a	231 b	71 c	1 d
Water-soluble SO_4 (mg kg^{-1} DW)	541 a	271 b	218 b	151 c	530 a	237 b	184 c	157 c

Different letters indicate significant differences according to one-way ANOVA, Duncan's multiple-range test (p= 0.05). DWstands for dry weight. EC means electrical conductibility.

4 Conclusions

The development of a regolith-based bioregenerative agriculture coupled to efficient water and waste recycling management for long-term manned missions to Mars is one of the main topics in current space research. The present work dealt with this challenge and demonstrated that a staple crop such as lettuce, a source of vitamin C, phenolic compounds and dietary fibre for space crews, can be profitably grown on a Martian regolith simulant (MMS-1) amended by stable compost which might be produced on board by efficient recycling of crew and crop waste. Although MMS-1 may provide plant nutrients such as Ca, Mg and K, it is devoid of organic matter and biological fertility, and hence of elements such as N, P and S. It is an alkaline and coarse textured substrate, lacking adequate water holding capacity, with very low occurrence of fine particles and minerals exerting colloidal properties. Therefore, it needs to be amended with an organic substrate, a source of essential nutrients to establish a pioneer biological activity in the rhizosphere and to enhance the physico-chemical and hydraulic properties of the regolith simulant, potentially exploitable as plant growing medium. A source of organic matter (electron donor) may be functional to the development of microaerophilic or anaerobic microorganisms belonging to *Dechloromonas*, *Azospira* and *Dechlorospirillum* genera (Sijimol et al., 2015), which were found to be capable of reducing and transforming the toxic perchlorates found on the surface of Mars into chloride and oxygen (Rikken et al., 1996).

The results of our study showed that lettuce plants may be grown in MMS-1 Martian regolith simulant if suitably amended by compost and fertilised. On the other hand, the moderate reduction of all growth parameters when the simulant rate increases in the mixture suggests suitable nutrient supply to plants from modified Hoagland solution and the release of some essential elements, which guarantee low but not negligible lettuce production. Overall, in terms of water and nutrient transport processes, all selected substrates would be eligible to be used as growth media in BLSS. However, the substantial improvement in hydraulic properties and water-plant relations in the 70:30 simulant:compost mixture indicates that the latter is the best option combining satisfactory plant growth performance and sustainable use of compost, a limited resource in BLSS. However, many remaining issues warrant further investigation concerning the dynamics of compost production, standardisation of supply during long-term manned missions and the representativeness of simulants with respect to real Martian regolith. The monitoring of nutrient availability, properties and fertility of the amended Martian regolith simulant over time, in consecutive plant growing cycles, is a crucial task for follow-up studies with a view to assessing the long-term sustainability of space agriculture based on amended substrates available *in situ*.

Chapter 3

Mars Regolith Simulant Ameliorated by Compost as *in situ* Cultivation Substrate Improves Lettuce Growth and Nutritional Aspects

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Abstract

Heavy payloads in future shuttle journeys to Mars present limiting factors, making self-sustenance essential for future colonies. Therefore, *in situ* resources utilization (ISRU) is the path to successful and feasible space voyages. This research frames the concept of planting leafy vegetables on Mars regolith simulant, ameliorating this substrate fertility by the addition of organic residues produced *in situ*. For this purpose, two butterhead lettuce (*Lactuca sativa* L. var. *capitata*) cultivars (green and red Salanova®) were chosen to be cultivated in four different mixtures of Mars MMS-1 simulant:compost (0:100, 30:70, 70:30 and 100:0; v:v) in a phytotron open-gas-exchange growth chamber. The impact of compost rate on both crop performance and nutritive value of green and red-pigmented cultivars was assessed. The 30:70 mixture proved to be the optimal in terms of crop performance, photosynthetic activity, intrinsic water use efficiency and quality traits of lettuce. In particular, red Salanova® showed the best performance in terms of these quality traits especially registering 32% more phenolic content in comparison to 100% simulant. Nonetheless, the 70:30 mixture represents a more realistic scenario when taking into consideration the sustainable use of compost as a limited resource in space farming, still accepting a slight significant decline in yield and quality in comparison to 30:70 mixture.

Keywords: *Lactuca sativa* L.; Mojave Mars Simulant (MMS-1); compost amendment; phytotron open gas exchange growth chamber; ISRU; mineral content; photosynthetic activity; phenolic profile; space mission.

1 Introduction

The NASA has fixed the year 2030 as target date for manned mission to Mars (Benaroya et al., 2013; Menezes et al., 2015; Meyen et al., 2016; Verseux et al., 2016; Llorente et al., 2018). With this announcement, the agency confers a real opportunity to colonize the red planet, a scenario where space farming encompasses the success of such long-lasting space mission. A journey to Mars requires tools inputs and food supply, but loading these latters onto the shuttle involves serious

technical limitations (Menezes et al., 2015; Llorente et al., 2018), not considering that this periodical delivery of inputs is economically and operatively unfeasible (Meyen et al., 2016; Verseux et al., 2016). Self-sustenance is an essential key for the success of future colonies, therefore a better comprehension of *in situ* resources utilization (ISRU) is crucial (Benaroya et al., 2013; Menezes et al., 2015).

Plants can sustain crew survival away from the Earth, by producing fresh food as part of their edible biomass and simultaneously contributing to several ecological services like air purification and water recycling (Loader et al., 1999; Paradiso et al., 2014; Fu et al., 2016; Llorente et al., 2018; El-Nakhel et al., 2019), as well as sustaining psychological wellbeing of space explorers (Bates et al., 2009; Koga and Iwasaki, 2013; Odeh and Guy, 2017). More importantly, a selection of candidate crops for food production is done upon selected criteria (Chunxiao and Hong, 2008; Wheeler, 2017) such as nutritional value, plant size, adaptability to extreme environmental conditions (i.e. different conditions of gravity and temperatures), low input requirements (in terms of nutritional elements, water and light), plant short life cycle and high harvest index (Kuang et al., 2000; Chunxiao and Hong, 2008; Meinen et al., 2018; El-Nakhel et al., 2019). Among various potential candidate species (cereals, vegetables and tubers), lettuce (*Lactuca sativa* L.) is well ranked. Indeed, lettuce leaves are rich in antioxidant compounds and in macro and micro nutrients, which can support the human diet as part of the daily intake (Hoff et al., 1982; Baslam et al., 2013). Nevertheless, the nutritional value of lettuce depends on the cultivar and its interaction with the environment (Rouphael, 2017; Kim et al., 2018; Giordano et al., 2019). Moreover, plants can be a source of health promoting secondary metabolites such as phenols (DellaPenna, 1999; Kim et al., 2016), whose formation and concentration is species and stressors dependent (DellaPenna, 1999; Kennedy and Wightman, 2011). For instance, nutritional chemical eustress like moderate salinity and nutrient deficiency can positively trigger physiological responses improving vegetables nutritional value (Rouphael and Kyriacou, 2018; Rouphael et al., 2018; El-Nakhel et al., 2019).

The Mars surface is composed primarily of mafic rocks, usually basalts (McCollom et al., 2013; Zeng et al., 2015; Filiberto, 2017; Cannon et al., 2019). Basaltic rocks and sediments are composed of varying amounts of olivine, pyroxene, plagioclase, and vitric and lithic fragments. On Mars, these minerals are accompanied by variable amounts of iron oxides and sulfates (Benison et al., 2008), suggesting that basaltic sediments may weather physically and chemically, providing additional insights into the formation of Mars soils and dust. As for the presence of Mars organic matter, very low amounts were detected by the current survey from landers and rovers (Eigenbrode et al., 2018).

To our knowledge, very few works dealt with the cultivation on Mars simulants. Among them we mention Gilrain et al. (1999), Mortley et al. (2000) and Wamelink et al. (2014), with only Gilrain et al. (1999) adopting diverse ratios of simulant and compost. Moreover, there are no data concerning the responsive interaction of plant qualitative traits with Mars simulant substrate. Therefore, in perspective of this framework, the potentialities and limitations of lettuce cultivation on the red planet have to be evaluated. For these reasons, two lettuce cultivars with different pigmentations were selected for a growth chamber experiment, using the Mojave Mars simulant MMS-1 as a hypothetic *in situ* substrate resource amended with a vegetal compost, to simulate the organic waste produced during the journeys on Mars. As demonstrated in a recent complementary study (Caporale et al., 2020), the amendment with green compost enhanced the physicochemical and hydraulic properties of the alkaline and nutrient-poor Mars simulant, concomitantly resolving the disposal issue of organic effluents in future manned missions to Mars. Overall, the data produced in this study represent the first knowledge on the response of plants to a very extreme environment such as that of the Mars simulant, in regards to the nutritional profile (mineral composition, antioxidant compounds and phenolic acids). This set of information is of a major utility for planning future space missions intended to Mars colonization.

2 Materials and methods

2.1 Plant Growth Conditions and Experimental Design

A nineteen-day experiment was carried out in a phytotron open-gas-exchange climate chamber (28 m^2: 7.0 × 2.1 m × 4.0 m; W × H × D), at the experimental farm of the Department of Agricultural Sciences, University of Naples Federico II, Italy. 24/18 °C light/dark, respectively, was the adopted temperature regime, while relative humidity ranged between 65 and 75% and was maintained through a fog system. High pressure sodium (HPS; Master SON-T PIA Plus 400 W, Philips, Eindhoven, The Netherlands) lamps were used to provide a 12 h photoperiod and 420 μmol m^{-2} s^{-1} light intensity at canopy level. Ambient CO_2 concentration (370-410 ppm) was adopted for this experiment, while air dehumidification and circulation were maintained by two heating, ventilating and air conditioning (HVAC) systems.

Green and red Salanova® (Rijk Zwaan, Der Lier, The Netherlands), were the chosen butterhead lettuce cultivars (*Lactuca sativa* L. var. *capitata*). Fourteen days after sowing, these cultivars were transplanted in pots (7 × 8 × 8 cm) filled with one of four different substrate mixtures as follow: 100:0, 70:30 30:70 and 0:100 v:v of MMS-1 simulant and compost, respectively. The Mojave Mars Simulant (MMS-1) was bought from The Martian Garden (Austin, Texas, USA), while the compost of vegetal waste was bought from GARDEA (Villafranca di Verona, Verona, Italy). The latter was sifted through a 2 mm sieve before the preparation of the mixtures. The mineralogical and physico-chemical properties of both mineral and organic substrates of the four mixtures are reported in Caporale et al. (2020) study.

The pots were distributed on propylene gullies, with a resulting density of 15.5 plants m^{-2} (43 cm inter-raw and 15 cm intra-row spacing). The plants were fertigated through a drip irrigation system (open loop) equipped with 2 L h^{-1} auto-compensating drippers. The nutrient solution consisted of a modified Hoagland formulation: 9.0 mM nitrate, 2.0 mM sulfur, 1.0 mM phosphorus, 4.0 mM potassium, 4.0 mM calcium, 1.0 mM magnesium, 1.0 mM ammonium, 15.0 μM Iron, 9.0 μM manganese, 0.3 μM cupper, 1.6 μM zinc, 20.0 μM boron, and 0.3 μM molybdenum. The pH and the electrical conductivity (EC) were 5.8 and 1.5 dS m^{-1}, respectively.

A factorial combination of four different substrate mixtures and two lettuce cultivars with different pigmentations accounted for eight treatments replicated three times. A randomized complete-block design was adopted for this experiment, with a total of 24 experimental units of seven plants each (total of 168 plants).

2.2 Leaf Gas Exchange

A portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., UK) was used to measure the net CO_2 assimilation rate (A_{CO2}), stomatal resistance (r_s) and transpiration rate (E) just before harvesting. Based on Carillo et al. (2019) method, A_{CO2} was divided by E in order to calculate the Intrinsic Water Use Efficiency (WUEi). Fully expanded leaves were chosen to carry the measurements of the leaf gas exchange, and eighteen measurements were done by treatment.

2.3 Fresh Biomass and Sampling

At harvest, shoot fresh weight (g $plant^{-1}$) was determined on five plants per experimental unit. Then leaves were dried for 72 h in a forced-air oven set at 70 °C in order to determine dry matter percentage needed for the calculation of leaf nitrate content expressed per fresh weight. Corresponding roots were washed with distilled water and placed as well in the oven to obtain dry material necessary for mineral analysis. Two plants per experimental unit were directly frozen in liquid nitrogen, lyophilized and stored at -80 °C for phytochemical analysis.

2.4 Total Nitrogen, Nitrate and Mineral Content

Dried leaves and roots were ground in a Wiley mill. For foliar total nitrogen determination, Kjeldahl method was employed Bremner (2016), using 1 g of dried samples. As for foliar and root mineral content determination, 0.25 g of the dried material was analysed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) based on the method adopted by Rouphael et al. (2017).

2.5 Total Chlorophyll and Total Ascorbic Acid Content

Total chlorophyll and total ascorbic acid content (TAA) were assessed by UV–Vis spectrophotometric analysis based on Lichtenthaler and Wellburn (1983) and Kampfenkel et al. (1995) protocols, respectively. Fresh lettuce material was used for both protocols. After extraction, a spectrophotometer (Hach DR 2000, Hach Co. Loveland, CO, USA) was used to measure the absorbance at 647, 664 and 525 nm, in order to determine Chlorophylls a, b and TAA, respectively. Whereas, total chlorophyll was calculated as the sum of chlorophylls a and b.

2.6 Carotenoids Quantification by HPLC-DAD and Polyphenols Analysis by UHPLC-Q-Orbitrap HRMS

As described in Kyriacou et al. (2019), carotenoids were extracted from freeze-dried lettuce material in ethanol enclosing 0.1 % butylated hydroxytoluene (BHT) as an altered method of Kim et al. (2008) and quantified by HPLC-DAD.

As for polyphenols, an UHPLC system (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) was used for quantification and separation. A Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) was used to facilitate the analysis of the mass spectrometry. The details of the polyphenols extraction are mentioned by Kyriacou et al. (2019b).

2.7 Statistical Analysis

A factorial combination of Salavona lettuce cultivars (red and green) with four different substrate mixtures was carried out as experimental design, for a total of eight treatments, in triplicate. A randomized complete-block design was adopted, with a total of 24 experimental units of six plants each (for a total of 144 plants).The obtained data were subjected to analysis of variance (Two-way ANOVA) using the software package SPSS 20. The mean effect of simulant:compost and the interaction between the two factors were performed using Duncan's Multiple Range Test (DMRT) performed at $P \leq 0.05$. Furthermore, Student's *t*-test was used to compare the two cultivars of lettuce.

3 Results

3.1 Yield and Physiological Parameters

As illustrated in Figure 1, fresh yield exhibited a significant interaction ($P \leq 0.05$) between the cultivar (C) and Mars simulant rate in the substrate (S). Both butterhead lettuce cultivars had the highest fresh yield in 30:70 simulant:compost mixture, registering 61.2 and 68.0 g plant^{-1} fresh weight (fw) for green and red Salanova, respectively. Whereas, the lowest fresh yield was recorded for both cultivars in 100 % simulant, ~21% lower than in the 30:70 mixture. The other two substrate mixtures (0:100 and 70:30) showed intermediate fresh yield with a different percentage of reduction between the two cultivars in comparison to the highest fresh weight.

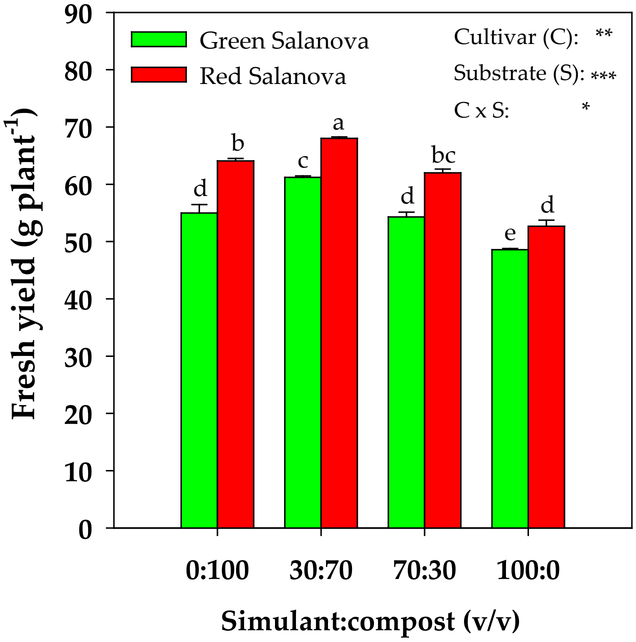

Figure 1. Fresh yield of green and red butterhead Salanova lettuce as influenced by substrate mixtures (four different rates of MMS-1 simulant:compost v:v). Different letters above bars indicate significant mean differences according to Duncan's multiple range tests ($P \leq 0.05$). Vertical bars indicate ± SE of means. *, **, *** Significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

All physiological measurements presented in Figure 2 showed a significant interaction (C × S). As mean effect of the simulant:compost mixture, transpiration rate (E) was the highest in 30:70 mixture (2.6 mol H_2O m^{-2} s^{-1}) and the lowest in both 70:30 and 100:0, with 0:100 being non significantly different in-between these three mixtures (data not shown). It is noteworthy that the cultivar factor had no effect on this physiological parameter. As for net CO_2 assimilation rate (A_{CO2}), green and red Salanova showed the highest values in 30:70 mixture (11.3 and 14.0 μmol CO_2 m^{-2} s^{-1}, respectively) and the lowest values in 100:0 (33 and 32% lower, respectively). Stomatal resistance (r_s) was the highest in 100:0 (6.43 m^2 s mol^{-1}) for green Salanova and in 0:100 and 30:70 (7.63 and 7.48 m^2 s mol^{-1}, respectively) for red Salanova (Figure 2). As for intrinsic Water Use Efficiency (WUEi), the highest values were noted in 30:70 and 70:30 for green Salanova and in 30:70 for red Salanova, while the lowest values were noted in 0:100 and 100:0 for green Salanova and in 70:30 and 100:0 for red Salanova (Figure 2).

3.2 Shoots and Roots Mineral composition

The analysis of shoot and root mineral contents on a dry weight basis (Table 1) showed basically no significant differences between cultivars and no interaction of the two factors C × S. The only exception was the root nitrate concentration, which was significantly higher in green Salanova (42.9 g kg-1 dw), and the shoot SO4 concentration, which was significantly higher in red Salanova (2.5 g kg-1 dw). As well, the interaction C × S was significant ($P \leq 0.05$) only for the root Mg concentration, reaching the highest value of 4.3 g kg-1 dw in 100:0 (100 % simulant) for green Salanova, whereas for the red cultivar the values of all mixtures, except for 0:100 (100% compost), had non-significant different values with an approximate mean of 3.2 g kg-1 dw. In contrast, there were significant differences between substrates. In 100% simulant, shoot and root mineral composition was characterized by the lowest values of nitrate (only shoot), PO4, K and SO4, and by the highest accumulation of Mg and Na. In the same substrate, Salanova shoots exhibited the highest concentration of Ca, which increased gradually with the rise of simulant rate in the substrate (Table 1). In 100% compost, shoot and root mineral composition were characterized by the highest concentrations of Cl and K. The latter concentration reduced gradually with the increase of simulant rate in the substrate, to register a value of 15.5 g kg-1 dw in the roots and 45.2 g kg-1 dw in the shoots (3.8- and 1.7-fold less than the other

3 mixtures, respectively), simultaneously accompanied by an increase of Na content in roots (1.7 g kg-1 dw) and shoots (12.8 g kg-1 dw; 4- and 2-fold, respectively; Table 1).

As for total nitrogen and nitrate expressed on fresh weight basis (Table 2), no significant difference was found neither for the cultivar and substrate factors mean effect nor for their interaction.

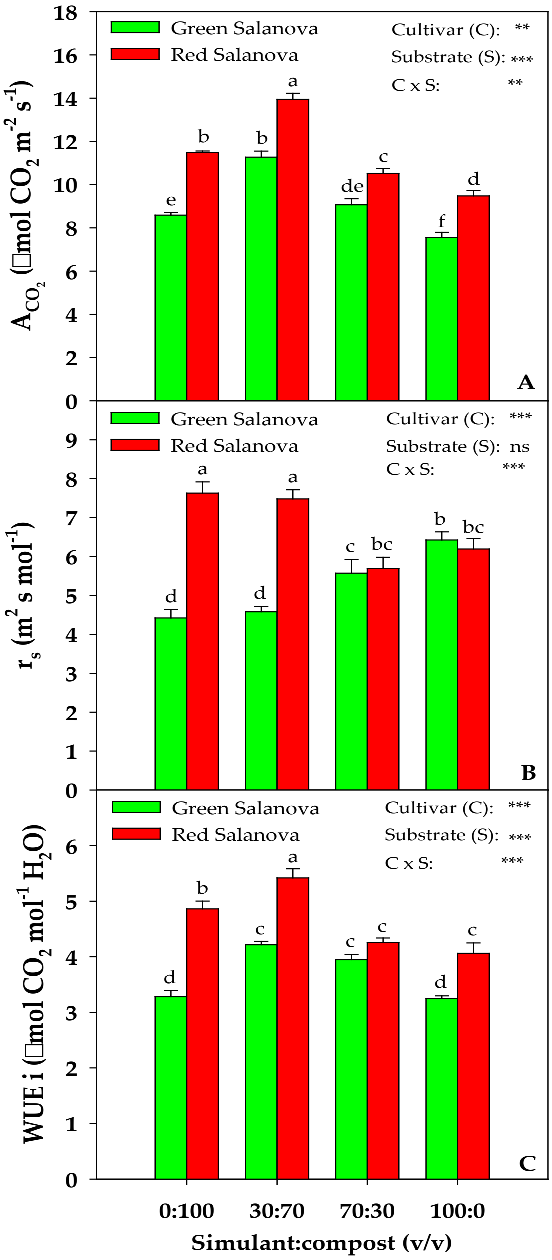

Figure 2. Physiological parameters: net CO_2 assimilation rate [A_{CO2}] (A), stomatal resistance [r_s] (B) and intrinsic Water Use Efficiency [WUEi] (C) of green and red Salanova lettuce as influenced by substrate mixtures (four different rates of MMS-1 simulant:compost v:v). Different letters above bars indicate significant mean differences according to Duncan's multiple range tests ($P \leq 0.05$). Vertical bars indicate ± SE of means. ns,**, *** Non-significant or significant at $P \leq 0.01$ and 0.001, respectively.

3.3 Total Ascorbic Acid, Total Chlorophyll and Carotenoids Content

As reported in Table 2, lutein and β-carotene did not exhibit any interaction between the two factors C × S, with both being significantly more concentrated in the red cultivar, and β-carotene being only influenced by the mean effect of the cultivar. As mean effect of the mixture, lutein was significantly the highest in 70:30 mixture and the lowest in 100% simulant (31.7 % less) (Table 2). Moreover, total chlorophyll showed the same trend as β-carotene, being only influenced by the mean effect of the cultivar, with the red cultivar registering significantly higher content. Total ascorbic acid manifested a significant interaction C × S (Figure 3). Indeed, in 30:70 mixture green and red cultivars behaved differently. Where green Salanova registered the lowest value of 3.0 mg AA 100 g-1 fw and red Salanova showed the highest value of around 87.1 mg AA 100 g-1 fw along with 100 % regolith (Figure 3).

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Table 1. Shoot and root mineral composition of green and red Salanova lettuce as influenced by substrate mixtures (four different rates of MMS-1 simulant:compost v:v).

Source of variance	NO_3 (g kg^{-1} dw)		PO_4 (g kg^{-1} dw)		K (g kg^{-1} dw)		Ca (g kg^{-1} dw)		Mg (g kg^{-1} dw)		Na (g kg^{-1} dw)		Cl (g kg^{-1} dw)		SO_4 (g kg^{-1} dw)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Cultivar (C)																
Green Salanova	27.6	42.9 a	9	5.7	64.7	50.6	7.1	6.2	2.5	2.8	1	5.9	3.3	2.2	1.5 b	8.9
Red Salanova	30.4	28.8 b	10.4	7.5	71.7	44.6	6.2	6	2.5	2.9	1	5.2	3.1	1.9	2.5 a	9.4
Simulant:compost (S)																
0:100	29.4 a	33.3 ab	11.2 a	8.7 a	82.7 a	69.2 a	4.8 c	5.6 ab	2.2 b	2.2 c	0.8 b	2.0 c	6.9 a	2.8 a	2.2 a	9.6 ab
30:70	32.5 a	24.9 b	11.9 a	7.3 a	75.2 b	48.4 b	6.4 b	6.7 a	2.4 b	2.5 bc	0.8 b	2.7 bc	2.0 b	1.7 b	2.3 a	9.3 b
70:30	32.4 a	43.6 a	9.5 b	7.3 a	69.5 c	57.4 b	6.9 b	6.6 a	2.3 b	3.0 b	0.9 b	4.8 b	2.0 b	2.0 b	2.0 a	11.1 a
100:0	21.7 b	41.6 a	6.2 c	2.9 b	45.2 d	15.5 c	8.5 a	5.3 b	3.2 a	3.9 a	1.7 a	12.8 a	1.7 b	1.9 b	1.4 b	6.6 c
C x S																
Green Salanova x 0:100	28	37.3	10.6	6.9	80.2	77.6	5.5	5.6	2.3	2.2 c	0.9	1.9	7.5	3.1	1.7	10.1
Green Salanova x 30:70	32.6	30.7	11.2	6.1	71.3	48	6.4	6.6	2.3	2.2 c	0.8	2.6	1.9	1.5	1.7	8.8
Green Salanova x 70:30	30.4	52.1	8.3	6.6	64.6	59	7.3	6.6	2.3	2.6 bc	1	5	2.2	2.2	1.6	10.5
Green Salanova x 100:0	19.5	51.5	5.8	3.2	42.6	17.8	9.3	5.9	3.2	4.3 a	1.5	14	1.7	2.2	1.1	6.2
Red Salanova x 0:100	30.9	29.2	11.8	10.6	85.3	60.8	4.2	5.6	2.1	2.3 c	0.8	2.1	6.4	2.4	2.8	9.1
Red Salanova x 30:70	32.4	19.1	12.8	8.6	79.2	48.9	6.3	6.9	2.6	2.8 bc	0.7	2.8	2.2	1.8	2.9	9.7
Red Salanova x 70:30	34.4	35	10.8	8	74.5	55.9	6.5	6.7	2.3	3.3 b	0.7	4.5	1.9	1.8	2.4	11.8
Red Salanova x 100:0	23.8	31.7	6.5	2.7	47.8	13.1	7.7	4.7	3.3	3.4 b	1.9	11.6	1.8	1.7	1.7	7.1
Significance																
Cultivar (C)	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns
Substrate (S)	*	*	***	***	***	***	***	*	***	***	**	***	***	**	***	***
C x S	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns

ns,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Cultivar means were compared by *t*-test. Substrate mixture means and interaction were compared by Duncan's multiple-range test ($P = 0.05$). Different letters within each column indicate significant differences.

Table 2. Total nitrogen, nitrate, total chlorophyll, lutein and β-carotene of green and red Salanova lettuce as influenced by substrate mixture (four different rates of MMS-1 simulant:compost v:v).

Source of variance	Total N	Nitrate	Total chlorophyll	Lutein	β-carotene
	(g $100g^{-1}$ dw)	(mg kg^{-1} fw)	(mg $100g^{-1}$ fw)	(mg kg^{-1} dw)	(mg kg^{-1} dw)
Cultivar (C)					
Green Salanova	3.9	1488	10.3 b	85.5 b	262.4 b
Red Salanova	4.0	1528	21.8 a	249.5 a	511.2 a
Simulant:compost (S)					
0:100	3.9	1542	15.4	170.3 b	386.9
30:70	4.0	1609	14.6	164.0 b	379.3
70:30	4.0	1637	16.7	199.4 a	437.3
100:0	3.8	1244	17.6	136.2 c	343.6
C x S					
Green Salanova x 0:100	3.9	1486	10.6	88.7	271.0
Green Salanova x 30:70	4.0	1670	10.2	88.8	262.7
Green Salanova x 70:30	3.9	1591	9.9	112.4	295.2
Green Salanova x 100:0	3.8	1205	10.5	52.1	220.7
Red Salanova x 0:100	4.0	1598	20.1	251.9	502.8
Red Salanova x 30:70	4.1	1548	19.0	239.3	495.8
Red Salanova x 70:30	4.1	1682	23.5	286.3	579.3
Red Salanova x 100:0	3.9	1283	24.7	220.3	466.6
Significance					
Cultivar (C)	ns	ns	***	***	***
Substrate (S)	ns	ns	ns	**	ns
C x S	ns	ns	ns	ns	ns

ns, **, *** Non-significant or significant at $P \leq 0.01$, and 0.001, respectively. Cultivar means were compared by t-test. Substrate mixture means and interaction were compared by Duncan's multiple-range test ($P = 0.05$). Different lowercase letters within each column indicate significant differences ($P \leq 0.05$).

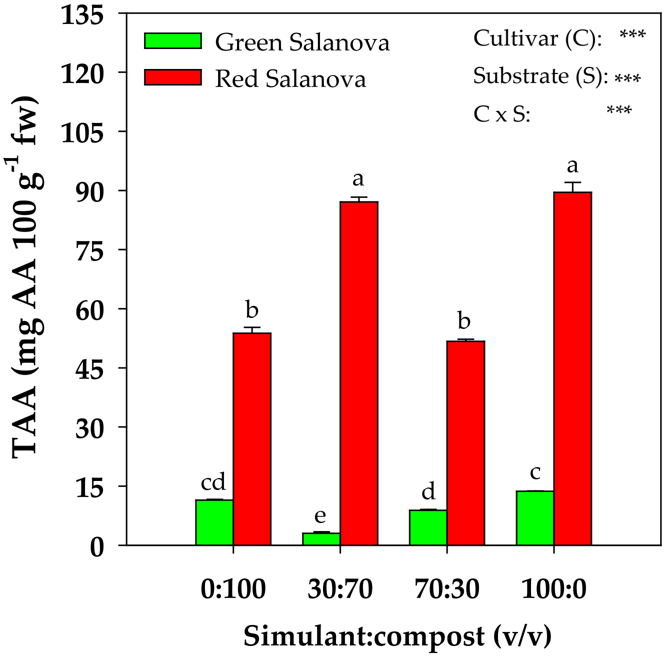

Figure 3. Total ascorbic acid (TAA) content of green and red Salanova lettuce as influenced by substrate mixture (four different rates of MMS-1 simulant:compost v:v). Different letters above bars indicate significant mean differences according to Duncan's multiple range tests ($P \leq 0.05$). Vertical bars indicate ± SE of means. *** Significant at $P \leq 0.001$.

3.4 Polyphenols Content Profile

Polyphenols profile studied in green and red Salanova is presented in Table 3. Among all the detected polyphenols, only quercetin-malonyl-glucoside showed no significant interaction between the two factors C × S. Indeed, the cultivar and substrate mean effect determined the differences, with red Salanova showing a value of 1276 µg g^{-1} dw that is around 52% higher than that of green Salanova. Furthermore, as mean effect of the mixture this phenolic compound was the most concentrated in 100% compost (1335 µg g^{-1} dw) around 63.8% higher than the average registered in the other three mixtures (Table 3). The most abundant polyphenols in both cultivars where feruloyl tartaric acid, rutin, quercetin-malonyl-glucoside, caffeoyl feruloyl quinic acid, coumaroyl quinic acid and chlorogenic acid but in different concentrations. Chlorogenic acid content was not influenced by the substrate mixture in green Salanova (≈ 330 µg g^{-1} dw), while it was the highest in 0:100

and 30:70 mixtures for red Salanova (≈ 4780.5 μg g^{-1} dw) and decreased by 37% in 100% simulant. An opposite trend was noted for feruloyl tartaric acid, whose content in red Salanova was not influenced by the mixture (≈ 978 μg g^{-1} dw), while in the green cultivar the highest content was registered in 100% compost (1099 μg g^{-1} dw). As for coumaroyl quinic acid, the highest content was registered in 100% simulant for green Salanova (562.4 μg g^{-1} dw) and in 30:70 mixture for its red counterpart (890.2 μg g-1 dw). Caffeoyl feruloyl quinic acid and rutin registered the highest content in 100% compost for the green cultivar (577 and 884 μg g^{-1} dw, respectively) and for the red cultivar (692 and 577 μg g^{-1} dw, respectively; Table 3). At the end, this significant interaction between C× S was also obvious for the total polyphenol content. As matter of fact, green Salanova total polyphenol content did not vary statistically among the different mixtures, while red Salanova total polyphenol content decreased gradually with the simulant rate increase (Figure 4).

Table 3. Polyphenol profile of green and red Salanova lettuce as influenced by substrate mixture (four different rates of MMS-1 simulant:compost v:v).

Source of variance	Clorogenic Acid	Caffeic Acid Hexoside	Caffeic Acid	Luteolin -7-Oglucoside	Apigenin Malonil Glucoside	Coumaroyl Quinic Acid	Coumaric Acid	Feruloyl Quinic Acid	Quercetin- 3-O-Galactoside	Dicaffeoyl quinic Acid
	(μg g^{-1} DW)									
Cultivar (C)										
Green Salanova	330 b	9.7	15.1 b	4.1 b	64.8 a	420.6 b	9.5 a	17.8 b	7.7 b	nd
Red Salanova	4156 a	6.8	57.9 a	8.4 a	24.0 b	746.7 a	6.8 b	25.8 a	40.1 a	90.0
Simulant:compost (S)										
0:100	2437 a	12.6 a	34.2 b	5.7 c	100.8 a	534.1 c	8.0 b	21.5 b	23.2 b	134.9
30:70	2534 a	6.8 c	46.4 a	6.6 b	26.0 b	620.8 b	7.7 b	23.7 a	34.4 a	73.5
70:30	2345 a	7.4 b	48.3 a	7.5 a	32.4 b	502.9 c	9.2 a	25.1 a	22.7 b	84.0
100:0	1658 b	6.4 c	17.1 c	5.1 d	18.5 c	676.6 a	7.8 b	16.8 c	15.2 c	67.5
C x S										
Green Salanova x 0:100	138 d	17.8 a	6.1 f	4.5 e	175.5 a	372.3 e	9.5 b	15.3 c	6.2 f	nd
Green Salanova x 30:70	241 d	6.7 b	15.6 de	3.9 fg	30.4 bc	351.5 e	8.9 c	19.6 b	7.0 f	nd
Green Salanova x 70:30	639 d	7.5 b	26.0 c	4.3 ef	31.9 b	396.0 e	10.7 a	21.3 b	10.3 e	nd
Green Salanova x 100:0	302 d	7.0 b	12.7 ef	3.6 g	21.6 cd	562.4 d	8.9 c	15.1 c	7.2 f	nd
Red Salanova x 0:100	4735 a	7.4 b	62.4 b	7.0 c	26.1 bc	696.0 c	6.5 e	27.8 a	40.3 b	134.9 a
Red Salanova x 30:70	4826 a	7.0 b	77.1 a	9.2 b	21.7 cd	890.2 a	6.6 e	27.9 a	61.9 a	73.5 c
Red Salanova x 70:30	4050 b	7.3 b	70.7 a	10.7 a	32.9 b	609.8 d	7.6 d	29.0 a	35.1 c	83.0 b
Red Salanova x 100:0	3014 c	5.8 c	21.5 cd	6.5 d	15.4 e	790.8 b	6.6 e	18.6 b	23.2 d	67.5 c
Significance										
Cultivar (C)	***	ns	***	***	*	***	***	***	***	na
Substrate (S)	**	***	***	***	***	***	***	***	***	***
C x S	***	***	***	***	***	***	*	**	***	***

Table 3. Polyphenol profile of green and red Salanova lettuce as influenced by substrate mixture (four different rates of MMS-1 simulant:compost v:v).

Source of variance	Quercetin -3-O-Glucuronide	Quercetin -3-O-Glucoside	Feruloyl glycoside	Kaempferol -7-O-Glucoside	Rutin	Quercetin Malonyl glucoside	Kaempferolo -3-O-Rutinoside	Feruloyl tartaric Acid	Caffeoyl feruloyl quinic Acid
					(μg g^{-1} DW)				
Cultivar (C)									
Green Salanova	69.3 a	7.4 b	10.0 a	4.1 b	814.0 b	614 b	51.8 b	1064 a	571 b
Red Salanova	52.8 b	34.6 a	7.3 b	9.2 a	866.3 a	1276 a	73.3 a	978 b	656 a
Simulant:compost (S)									
0:100	76.3 a	25.3 a	8.2 c	5.4 c	943.0 a	1335 a	67.4 a	1039 a	634 a
30:70	73.6 a	21.3 c	9.5 b	6.8 b	846.2 b	914 b	60.3 b	1015 b	618 b
70:30	60.2 b	22.8 b	10.8 a	9.1 a	808.1 c	774 b	62.0 b	1014 b	606 c
100:0	34.1 c	14.6 d	6.1 d	5.4 c	763.3 d	757 b	60.5 b	1016 b	596 d
C x S									
Green Salanova x 0:100	101.3 a	5.8 f	8.7 c	4.2 d	883.8 b	865	61.2 b	1099 a	577 e
Green Salanova x 30:70	74.9 b	6.5 ef	11.0 b	3.9 d	782.1 d	631	47.2 c	1051 b	573 ef
Green Salanova x 70:30	68.3 b	10.2 d	12.7 a	4.4 d	825.9 c	535	48.8 c	1054 b	569 f
Green Salanova x 100:0	32.5 d	7.1 e	7.5 d	3.9 d	764.4 d	424	49.8 c	1054 b	566 f
Red Salanova x 0:100	51.2 c	44.8 a	7.7 d	6.7 c	1002.1 a	1805	73.6 a	980 c	692 a
Red Salanova x 30:70	72.2 b	36.1 b	8.0 d	9.6 b	910.3 b	1196	73.4 a	979 c	664 b
Red Salanova x 70:30	52.1 c	35.5 b	8.8 c	13.7 a	790.4 d	1013	75.1 a	975 c	644 c
Red Salanova x 100:0	35.7 d	22.0 c	4.7 e	6.9 c	762.3 d	1090	71.1 a	978 c	625 d
Significance									
Cultivar (C)	ns	***	**	***	ns	***	***	***	***
Substrate (S)	***	***	***	***	***	***	***	***	***
C x S	***	***	***	***	***	ns	***	**	***

ns,*,**, *** Non-significant or significant at P ≤ 0.05, 0.01, and 0.001, respectively; nd, not detected; na, not applicable. Cultivar means were compared by t-Test. Substrate mixture means and interaction were compared by Duncan's multiple-range test (P = 0.05). Different letters within each column indicate significant differences

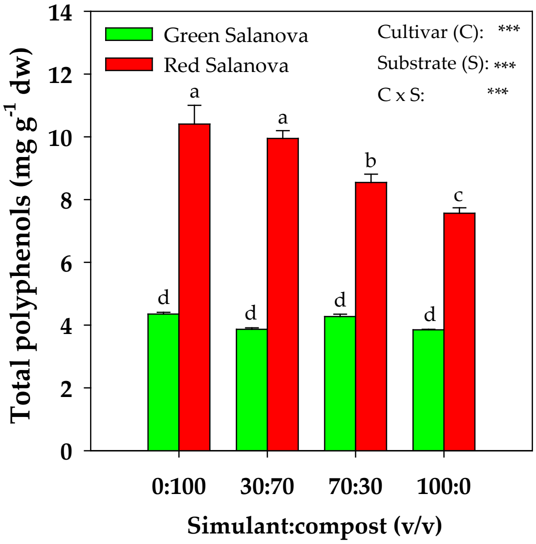

Figure 4. Total polyphenols content of green and red Salanova lettuce as influenced by substrate mixture (four different rates of MMS-1 simulant:compost v:v). Different letters above bars indicate significant mean differences according to Duncan's multiple range tests ($P \leq 0.05$). Vertical bars indicate ± SE of means. *** Significant at $P \leq 0.001$.

4 Discussion

Planet Mars colonization can solely be realized via the adaptation of a bioregenerative life support systems (BLSSs) without an umbilical support from Earth (Rouphael et al., 2019), by using *in situ* resources as much as possible and avoiding any additional reload due to technical and economic constraints (Massa et al., 2006; Verseux et al., 2016). In the present study, the utilization of MMS-1 as plant growth substrate mixed with variable rates of compost was studied to grow two cultivars of lettuce, with the purpose to identify suitable and sustainable simulant:compost rates enabling future colonists to obtain a compromise between yield and nutritional status of the produced vegetables. Caporale and co-workers (2020) characterized the pure and mixed substrates from a physical, chemical, mineralogical and hydraulic point of view. They found that MMS-1 is a coarse-textured alkaline mineral substrate mainly composed of plagioclase and amorphous material with accessory minerals including zeolite, hematite and smectite clays. Although MMS-1 simulant can be a source of nutrients (i.e. Ca, Fe, Mg, K), it lacks organic matter, N, P and S, which can be only supplied through the compost amendment, which in turn enhances the main physical, chemical and hydraulic properties of the plant-growth substrate.

Simulant:compost mixtures had a clear effect on Salanova lettuce yield, with 30:70 mixture revealing the highest registered yield for both cultivars, and 100% simulant revealing the lowest yield. Similarly, a superior yield with the addition of compost to JSC Mars-1 simulant was noticed for Swiss chard (Gilrain et al., 1999). In our case, such yield response can be interpreted by the highest A_{CO2} and WUEi for both cultivars and a low r_s for green Salanova observed in 30:70 mixture, simultaneously with the lowest A_{CO2} and WUEi for both cultivars and a higher r_s for the green cultivar in 100% simulant. The application of organic matter had shown to increase the concentration of chlorophylls a and b (Caporale et al., 2018), and to promote net photosynthesis and water use efficiency (Ouni et al., 2014). Indeed, in this study the best performance was observed in lettuce grown in the three mixtures containing compost that enhanced water and nutrient availability, especially in the mixture with 70% compost (30:70). Our results confirm Rouphael et al. (2019) observations about the better yield performance and higher A_{CO2} and WUEi of red Salanova in comparison to green Salanova. This observation, in extreme environment as the extraterrestrial farming, could be highly handy, because an optimized water use efficiency in an environment with low water availability, and a higher CO_2 assimilation in an abundant CO_2 atmosphere (95%) (Benison et al., 2008; Schuerger et al., 2008; Badescu, 2009; Maggi and Pallud, 2010) could be highly appreciated especially in a BLSSs. Moreover, it was demonstrated that reduced gravity indirectly affect the surrounding environment of the plant, influencing the physiological transport of water and solutes, and gas exchange (Porterfield, 2002). For instance, on Mars, the low gravity (1/3 of Earth's gravity) could interact with the buoyancy-driven thermal convection causing an increase of boundary layer thickness with consequent biophysical limitations on the processes of gas exchange and transpiration in higher plants (Porterfield, 2002).

Simulant:compost mixtures, particularly 100% simulant and 100% compost enhanced accumulation of certain elements in both lettuce cultivars. Only the 30:70 mixture produced a proper accumulation of NO_3, PO_4 and K in Salanova shoots associated with a good repartition between shoots and roots, which explain the higher yield of green and red Salanova obtained in this mixture. All the three mixtures rich in compost showed higher shoot and root accumulation of SO_4 in comparison to 100% simulant, which can be explained by the increasing bioavailability of the anion with the increasing rate of compost in the growth substrate (Caporale et al., 2020). Furthermore, red Salanova significantly accumulated more SO_4 than its green counterpart, and this is coherent with El-Nakhel et al. (2020) findings. High accumulation of PO_4, K and Cl in plants cultivated in 100% compost, and Mg and Ca in plants cultivated in 100% simulant is mostly explained by the abundance of bioavailable fractions of these ions in the mixtures. As described in a complementary study by Caporale et al. (2020), the concentrations of water-soluble K, Cl, NO_3, PO_4 and SO_4 in the 100% MMS-1 simulant substrate were less than 4% of the concentrations of the same nutrients in the 100% compost substrate, while Mg and Ca were 13% and 17% less, respectively, indicating a good bioavailability of the two nutrients even in the pure MMS-1 simulant substrate. Clearly, compost affects plant mineral content (Abd El-Salam et al., 2016; Agegnehu et al., 2016; Paulauskiene et al., 2018). Indeed, Ca, Mg and Na contents showed a lower accumulation in the presence of compost in the mixtures, which might be due to the cation exchange capacity of the compost regulating the release of the elements from the substrates to the plants. On the other hand, although MMS-1 Mars simulant was found to be very rich in Al oxides (Caporale et al., 2020), Salanova plants did not show any Al phytotoxic effect, since this element is poorly soluble and bioavailable in sub-alkaline growth substrates as those of the experiment, whilst it exerts phytotoxicity at highly-acidic pHs with soluble cations undergoing to acid hydrolysis (Kabata-Pendias, 2010). Still, in the 100% simulant, green and red Salanova plants grew respectively less by 20.6% and 22.6% in comparison to the 30:70 mixture. This can be justified by lower NO_3, PO_4 and K shoot concentrations and PO_4 and K roots concentrations compared to other mixtures. Besides a higher content of nutrient, MMS-1 Mars simulant amended with compost, had as well enhanced physical (bulk density and pore-size distribution) and hydraulic (water holding capacity and retention) properties compared to the pure simulant, which may have positively influenced the crop performance (Caporale et al., 2020). In particular, it was evident that the compost addition to the simulant, proportionally increased the amount of water retained by the substrate and enhanced more macropore and micropore domains (Caporale et al., 2020). The decrease of K shoots and roots concentrations were inversely correlated with Na shoot and root concentrations. This behavior can be interpreted as a result of K shortage with Na substituting K in non-specific functions like vacuolar osmotic potential maintenance (El-Nakhel et al., 2019). Accordingly, Caporale et al. (2020) supposed that the consistent bioavailable pool of Na in the MMS-1 simulant, together with alkaline pH and absence of biological fertility, could have induced a salt stress in plants grown in pure simulant substrate. Furthermore, Salanova nitrate content expressed on a fresh weight basis in all four mixtures was within lettuce maximum nitrate limit set by the European Commission Regulation No 1258/2011 for commercialization.

Red Salanova showed a higher content of lutein, β-carotene and total chlorophyll in comparison with the green cultivar, which is in harmony with El-Nakhel et al. (2020a, 2020b) results. Nevertheless, only lutein was ameliorated by the presence of the compost, in mixtures 30:70 and 70:30, respectively. These findings are not fully in line with Thatikunta et al. (2012) and Ouni et al. (2014) who declared that organic matter can increase chlorophyll and carotenoid content. Differently, Lefsrud et al. (2008), Kołton et al. (2014) and Ouzounis et al. (2015) declared that chlorophyll, lutein and β-carotene are mainly influenced by light.

Moreover, total ascorbic acid, other than being more concentrated in red Salanova, it was the highest in mixture 30:70 and 100% simulant for this cultivar, probably because the lower chemical and biological fertility of the two simulant-rich substrates caused a greater oxidative stress in the plants. As for total polyphenols, which were as well highly rich in red Salanova (around 123% more than in the green cultivar) and positively modulated with the increase of the compost percentage in the mixture, while they remained statistically equal in green Salanova among all four mixtures. Such diverse modulation pattern of polyphenols in both cultivars was noted as well in El-Nakhel et al. (2019) work, where green and red Salanova were subjected to a nutrient solution eustress. The antioxidant activity of plants is affected by the amount of organic matter present in the substrate, namely the compost rate in our experiment, due to various factors such as higher K availability since this element is strongly linked to enzymatic activities (Fanasca et al., 2006; Fageria, 2009), the greater abundance of soluble salts (Ding et al., 2018) and micronutrients (Taghipour et al., 2017). As matter of fact, our results showed a positive correlation between the compost rate in the substrate (S) and total polyphenols (r>0.95), confirming the potential qualitative improvement of vegetables due to compost application as reported by Sousa et al. (2005), Saikia and Upadhyaya (2011), Aminifard et al. (2013) and Luján-Hidalgo et al. (2017). The relevant presence of aromatic moieties and hence of stable and humified organic compounds in the compost, evidenced by Caporale et al. (2020) through infrared spectroscopy and thermogravimetric analysis, may have stimulated the production of polyphenolic compounds in lettuce foliar biomass (Schiavon et al., 2010; Canellas et al., 2015).

Overall, red Salanova had a better phytonutrient profile in comparison to its green counterpart notwithstanding the mixture adopted. Such dense bioactive profile was as well proven for red Salanova in previous studies (El-Nakhel et al., 2019, 2020b; El-Nakhel et al., 2019; Giordano et al., 2019; Rouphael et al., 2019). Similarly, the study of Neocleous et al. (2014) showed that red “baby” lettuce exhibited better antioxidant activity in comparison to green “baby” lettuce when subjected to saline stress. Indeed, as declared by Rapisarda et al. (1999) and Rouphael et al. (2012), it is the genotype and the extrinsic stressors that affect the formation of bioactive compounds.

5 Conclusions

Future space missions intended for the colonization of Mars are partnered with economical and mechanical constraints when considering a replenishment from Earth. Such fact could be drastically alleviated by enhancing *in situ* resources utilization, like an opportune exploitation of Mars regolith as main substrate for vegetable production. The physical, chemical and hydraulic attributes of this substrate, also known as Mars soil, can be improved by the addition of organic residues produced *in situ*, which can evoke better quality and higher yield of the produced vegetables. Our work supports the findings of Rouphael et al. (2019) and El-Nakhel et al. (2019) of red Salanova being a candidate crop for space farming. Indeed, this cultivar presented higher yield, photosynthetic activity and bioactive compounds in comparison to its green counterpart. The 30:70 (simulant:compost) mixture demonstrated to be the most convenient mixture in terms of increasing yield, A_{CO2}, WUEi, total ascorbic acid and total polyphenols of the red cultivar. Nevertheless, cultivation on 100% simulant substratum was feasible as well, although yielding around

20% less production and a decrease in shoots of NO_3, PO_4, K and bioactive compounds except for total ascorbic acid. Nonetheless, the 70:30 mixture represents a more realistic scenario when taking into consideration the sustainable use of compost as a limited resource in space farming, still accepting a slight significant decline in yield and quality in comparison to 30:70 mixture. These findings reassure space explorers concerning the utility of Mars regolith as cultivation substrate and demonstrate the importance of using the organic residues produced by any cultivation in space in order to enhance the fertility of this mineral substrate. Nevertheless, future studies regarding cultivations without additive fertigation and solely counting on *in situ* fertility is of major importance to reduce any additional load, and nevertheless testing organic matter from conveniently treated human excrements is worthy.

Chapter 4

The suitability of Lunar and Martian soils for food plant growth. The effects of monogastric-based manure fertilization on lettuce growth and physiology, soil enzymatic activity and nutrient bioavailability

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Abstract

To make feasible the crewed missions to the Moon or Mars, space research is focusing on the development of bioregenerative life support systems (BLSS) based on *in situ* resource utilization (ISRU), allowing to reduce terrestrial input, to exploit native regoliths and to recycle organic wastes. In this regard, the present work aims to assess the agronomic performance of plant growth substrates consisting of MMS-1 (Mars) or LHS-1 (Lunar) simulants mixed with a commercial horse/swine monogastric manure at varying rates (100:0, 90:10, 70:30, 50:50, w/w). Specifically, we evaluated: i) lettuce (*Lactuca sativa* L. cultivar 'Grand Rapids') growth on these substrates (for 30 days in open-gas-exchange climate chamber with no fertilization), plant physiology and nutrient uptake; ii) microbial biomass C and N, enzymatic activity and nutrient bioavailability in the simulant/manure mixtures after plant growth.

A better agronomic performance, in terms of plant growth and physiology, nutrient availability and enzymatic activity, was provided by substrates containing MMS-1, in comparison to LHS-1-based ones, due to the better physicochemical and hydraulic properties, lower alkalinity and availability of Na (assessed in a complementary paper). Amendment with a monogastric-based manure significantly improved the ability of both simulants to sustain plant growth. The best crop growth response was achieved on the 70:30 simulant/manure mixturedue to good availability of nutrients combined with an optimal water availability and air circulation. A 70:30 simulant/manure mixture is also a more sustainable option than a 50:50 mixture for a BLSS developed on ISRU strategy. Matching crop growth performance and chemical, mineralogical and physico-hydraulic characteristics of possible plant growth media for space farming allows a better understanding of the processes and dynamics occurring in the experimental substrate/plant system, potentially suitable for an extra-terrestrial BLSS.

Keywords: Space farming; Mars simulants; organic amendment; sustainable use of resources; extra-terrestrial food production; bioregenerative life support system.

1 Introduction

Research on plants in space is shifting from cell biology to the production of crops, and from small-scale studies on synthetic growth media to investigations on materials relevant to Mars and Moon environments where crop growth is envisaged (Gilrain et al., 1999; Mortley et al., 2000; Wamelink et al., 2014; Caporale et al., 2020; Duri et al., 2020; Fackrell, 2021).

Fresh plants for crew consumption were first conceived within a very fast production timeframe as in the growing of sprouts for their nutritional and nutraceutical properties (e.g., De Micco and Aronne, 2008), but with the drawbacks of high use of resources. The idea of longer space missions and permanence on Mars and the Moon has elicited the concept of Bioregenerative Life Support Systems (BLSS) where plants are grown with the aim of self-sufficiency for inputs and where added functions are oxygen production and crew waste recycling for the recovery of water and nutrients (Wheeler, 2017). An efficient and sustainable BLSS would be developed on the concept of *in situ* resource utilization (ISRU), which requires the use of native materials such as regolith and waste as primary resources (Duri et al., 2022).

In their review of plant research within America, Asia and Europe space missions or ground settings, Zabel et al., (2016) and Wheeler (2017) list more than 20 species grown in different space devices. These include food and ornamental crops, or plants solely devoted to research such as *Arabidopsis*. Edible species are cereals, legumes, and a range of tuber or vegetable crops (Tibbitts and Alford, 1980; Wheeler, 2010) chosen for health and psychological benefits associated with growing plants in confined environments.

Due to palatability and the content of nutraceuticals, salad species have enjoyed popularity to the point that plant-producing installations in space have been referred to as “salad machines” since early efforts, as reviewed by Wheeler et al. (2001). Leafy vegetables also meet other major criteria for the choice of space crops, such as fast growth, high harvest index and minimal area requirements.

Among recent research Khodadad et al. (2020) grew red romaine lettuce (*Lactuca sativa cv ‘Outredgeous,’*) in the “Veggie” growth system (Morrow et al., 2005) on the International Space Station (ISS) in comparison with ground-grown plants. Space and ground leaves showed differences in some nutrients (Fe, K, Na, P, S, and Zn) and total phenolics, but no differences in anthocyanin and ORAC (Oxygen Radical Absorbance Capacity) levels. The growth medium of Veggie chambers is a “pillow” made of solid porous argillite fertilized with nutrient solutions or controlled release fertilizer, therefore all materials sourced on earth. Wheeler et al. (2001) and Wheeler (2017) report that plants have been grown in space on different artificial solid media and nutrient film techniques. Zabel et al. (2016) review growth media for on-orbit plant growth chambers, ranging from solidified agar to perforated tubing wrapped in a wick or porous tubes.

The quest for self-sufficiency and the BLSS concept have raised interest for using growth media materials which may be locally sourced in space missions (Benaroya et al., 2013). Research on such materials is also relevant to future *in-situ* crop growing for instance on Mars and the Moon and is effectively a first step for studying their potential agriculture environment. Gilrain et al. (1999) proposed mixtures of compost and regoliths analogue to Mars surface materials (referred to as simulants) for growing Swiss chard; Mortley et al. (2000) and Wamelink et al. (2014) tested Moon and Mars simulants as plant growth media. After a test on different plants (Fackrell and Schroeder, 2020), Fackrell (2021) grew the legume moth bean (*Vigna aconitifolia*) on Mars simulant with a set of microbial inoculants to provide biologically fixed nitrogen and help acquisition of other nutrients.

As for lettuce, Caporale et al. (2020) grew two varieties of lettuce on a coarse-textured alkaline Mars simulant (i.e., MMS-1) with the addition of organic matter from green compost, which may be produced in space from inedible parts of plants. The MMS-1 simulant was found to contain considerable amounts of Na, and plant nutrients such as Ca, Mg and K, but no organic matter and related macronutrients needed for plant growth, such as N, P and S. Compost amendment complemented the simulant’s ability to provide elements for plant growth, lowered the bulk density and pH of the simulant, and modified its hydraulic properties. Organic amendment also contributes to enrich simulants in microbial biomass useful to support nutrient bioavailability and organic matter break down. This resulted in an improved growth of lettuce up to the dose of 30% green compost (Caporale et al., 2020). This research team also studied the lettuce nutritional profile, content of nutraceuticals, photosynthetic activity, and water use efficiency as a function of different MMS-1 simulant/green compost mixtures (Duri et al., 2020).

Optimization of scarce resources, such as selected plant nutrients and water in space, has required crop design to address allometric relations between aboveground plant parts. The increase of harvest index has been targeted (Wheeler et al., 2001; De Micco et al., 2009) by choosing appropriate species or creating dwarf varieties, like the short life-cycle Apogee and Perigee wheats (e.g., Stoklosa et al. 2011). Allocation of resources to roots, though, has received little attention in space plant research. Roots often represent an investment in inedible parts but at the same time play a key role in acquiring resources and in driving belowground processes (Gregory and Kirkegaard, 2017). In poor growth media ratios of root to shoot mass are typically larger (Agren, 2003; Hermans et al., 2006) and roots have a different architecture and fine root percentage (Yuan and Chen, 2012; Péret et al., 2014) which increase resource acquisition. Therefore, root/shoot ratios and root architecture represent a classical optimization problem and need to be addressed for conditions relevant to crop production in space.

In a companion paper (Caporale et al., 2022, submitted) studies the mineralogical, physical and chemical properties of MMS-1 Mars and LHS-1 Lunar regolith simulants. Authors found that both simulants are alkaline and coarse textured with low water holding. The LHS-1 Lunar simulant shows abundant plagioclases and lower bioavailability of nutrients, total porosity, saturated hydraulic conductivity and water retention but higher bioavailability of potentially toxic elements in comparison with the MMS-1 Mars simulant. To enhance the fertility and physicochemical properties of these simulants, potentially exploitable as plant growth media, these authors amended them with varying rates (0, 10, 30 and 50% in weight) of a commercial horse/swine monogastric-based manure i.e., an analogue of composted plant residues and crew excreta which can be produced during space missions. In that context, the recycling and valorization of limited resources, as organic wastes and water, is of paramount importance. Caporale et al. (2022, submitted) observed that amendment with manure improves physical and chemical properties of simulants, and more so for the lunar; hence, simulant-manure mixtures result in interesting candidate growth media for BLSS. Adding organic matter from monogastric excreta, besides ameliorating physical properties, will also affect the biology of growth media in ways that have not been inquired yet.

The overall object of this paper is to evaluate the agronomic and environmental performances of MMS-1 or LHS-1 simulant / monogastric-based manure mixtures (100:0, 90:10, 70:30, 50:50, w/w), in terms of: i) lettuce (*Lactuca sativa* L. cultivar 'Grand Rapids') growth and nutrient uptake; and ii) microbial biomass C and N, enzymatic activity and nutrient bioavailability in simulant/manure mixtures after plant growth. This aim is relevant for viable production in BLSS but also for insights on the possible exploitation of Mars and Moon regoliths for agricultural purposes.

We specifically tested the following hypotheses:

(i) the Lunar simulant supports the plant growth and quality to a lower extent than Mars simulant due to poorer physical and chemical properties, as assessed by Caporale et al. (submitted) in a companion paper;

(ii) amendment with monogastric-based manure relieves nutritional and physical constraints of pure mineral simulants for plant growth allowing to produce without fertilization as manure enriches them in organic matter and microbial biomass wich is able, in turn, to break down organic matter and make nutrients available. The microbial functionality should improve in terms of activity of the main soil enzymes involved in biogeochemical cycles of nutrients;

(iii) plants growing on amended and pure regoliths modulate their investment in belowground parts and their root morphology and topology according to nutrient availability of growth media, in order to optimize the global harvest index, understand plant behavior and acquire information for crop design in regolith-based regenerative agriculture;

(iv) organic matter supply by monogastric-based manure stimulates root growth and microbial activities in the rhizosphere, with positive outcomes on nutrient bioavailability and geochemistry.

2 Materials and Methods

2.1 Plant material, growth chamber condition, and experimental treatments

Lettuce seedlings (*Lactuca sativa* L. cultivar 'Grand Rapids', West Coast Seeds, Vancouver, British Columbia, Canada) were grown in the nursery using polystyrene trays filled with vermiculite. At the third true leaf stage, plants were transplanted into plastic pots (9 x 9 x 9 cm) filled with different mixtures of simulants and monogastric manure and transferred to the growth chamber.

The experiment was carried out at the experimental farm of the Department of Agricultural Sciences, University of Naples Federico II (Italy) in a walk-in open-gas-exchange climate chamber (28 m^2: 7.0 × 2.1 × 4.0 m; W × H × D). HPS lamps (Master SON-T PIA Plus 400 W, Philips, Eindhoven, The Netherlands) were used to provide 400 µmol m^{-2} s^{-1} light intensity at canopy level with a 16/8-hour photoperiod (light/dark) under ambient CO_2 concentration conditions (370-410 ppm). A day/night air temperature and humidity regime of 22/18 °C and 60/80%, respectively, was provided through two heating, ventilation, and air conditioning (HVAC) systems and a fog system. Plants were irrigated throughout the crop cycle (30 days from transplanting to harvest) with only osmotized water using a drip irrigation system (open loop) equipped with 2 L h^{-1} self-compensating drippers.

Experimental treatments consisted of two different simulants, Mojave Mars Simulant (MMS-1, The Martian Garden, Austin, Texas, USA) and Lunar Highlands Simulant (LHS-1, Exolith Lab, Center for Lunar & Asteroid Surface Science of University of Central Florida, Orlando, Florida, USA), mixed at different rates (100:0, 90:10, 70:30 and 50:50 w:w) with ground monogastric manure (Jolly Pellet, Agraria Di Vita srl, Pistoia, Italy) sieved to 2 mm.

2.2 Morpho and physiological measurements

One day before harvest (30 days after transplanting; DAT) the maximum plant height (H) and average diameter (D_m; as the average of two transverse diameters, where one of the two was the maximum diameter) of canopy of all plants were measured and then used to determine the growth index (GI) by the formula [3,14 x $(D_m/2)^2$ x H]. At the same time, SPAD index and chlorophyll fluorescence were measured using a portable chlorophyll meter (SPAD-502, Minolta Corp. Ltd., Osaka, Japan) and a portable fluorometer (Fv/Fm Meter, Opti-Sciences Inc., Hudson, NH, USA), respectively. According to Kitajima and Butler (1975) the maximum efficiency of Photosystem II (PSII) was calculated as F_v/F_m, with $F_v=F_m-F_0$, where the ground fluorescence signal (F_0) was induced on 10 minutes dark-adapted leaves, by a blu LED internal light of 1–2 µmol m^{-2} s^{-1} and the maximal fluorescence (F_m) was induced by a 1 second of saturating light pulse of 3000 µmol m^{-2} s^{-1}.

At harvest, plants were cut at the soil level and shoot fresh weight (g $plant^{-1}$) and number of leaves (LN) per plant was recorded, while leaf area (LA, cm^2 $plant^{-1}$) was measured using an electronic area meter (LI-COR 3100C biosciences, Lincoln, Nebraska, USA). Harvested tissues were oven-dried at 70°C to constant weight (~72 h) for determination of dry weight (dw, g $plant^{-1}$) and leaf dry matter concentration (DM, %).

After cutting the above-ground part pots were turned on the side and their content was gently extruded. Roots were brushed free of soil and then washed on a 0.5-mm sieve. All soil was then submerged with water in order to collect remaining root fragments by elutriation.

Image analysis of roots was performed with the WinRhizo ArabidopsisV2009c (Regent Instruments Inc., Chemin Sainte-Foy, Canada) image analysis software on root systems placed in a 20 x 25 cm transparent tray with a 5-mm deep layer of water and scanned at 600 dpi by STD4800 Image Acquisition System. The following traits were measured: root surface area (RA, cm^2 $plant^{-1}$), mean diameter (D, mm), root volume (RV, cm^3 $plant^{-1}$), total root length (RL, m $plant^{-1}$), and length separated in 10 diameter classes: from 0.0 to >4.5 mm in increments of 0.5 mm.

After scanning roots were oven dried at 70 °C until constant weight and weighed to obtain the root dry mass (Rdw g $plant^{-1}$).

The specific root surface (SRS, m^2 g^{-1}) was calculated as the ratio of total surface to total length of roots. Indices of allometric relations between above- and below-ground plant parts were calculated as root to shoot biomass ratio (RSw g g^{-1}), and root to leaf area ratio (RLA, m g^{-1}).

2.3 Determination of microbial biomass carbon and nitrogen in manure amended simulants

Microbial biomass carbon (MBC) was measured according to the fumigation-extraction method (Vance et al., 1987). Briefly, 10 g of moist simulant were exposed to $CHCl_3$ for 24 h at 25 °C and then treated with 0.5 M K_2SO_4 for 30 min under orbital shaking at 200 rpm; the suspension was filtered through Whatman 42 filter paper. A non-fumigated control underwent the same procedures described above without the $CHCl_3$ exposure. Organic carbon in the extracts was determined after oxidation with 0.033 M $K_2Cr_2O_7$ at 110 °C for

1.5 h by titration with 0.1 M Mohr salt solution. Results were expressed in mg C kg^{-1} dried samples.

Microbial biomass nitrogen (MBN) was measured on 0.5 M K_2SO_4 extracts according to Brookes et al. (1985) and by the alkaline persulfate oxidation (Cabrera and Beare, 1993), with some modification. Briefly, an aliquot of the extract (1 mL) was diluted to 20 mL with deionized water and placed in falcon tube. Twenty mL of the oxidizing reagent were added. The tubes were placed in an autoclave for 30 min at 120 °C. After that, the tubes were allowed to cool at room temperature and nitrate was determined by UV analysis in a spectrophotometer (PerkinElmer, UV/Vis Lambda 365) at 220 nm. MBN was calculated using a K_{EN} factor of 0.54 (Brookes et al., 1985). The total nitrogen concentration was calculated on calibration curve by using glycine at different concentration of N (0.1, 0.2, 0.4, 0.6, 1, 1.5, 2.5, 5, 7, 10 mg L^{-1}). Results were expressed in mg N kg^{-1} dried samples. All determinations were in triplicate.

2.4 Enzymatic activity assay in manure amended simulants

Enzyme activities were determined within 15–20 d from the collection of the simulant samples stored at 4 °C. Dehydrogenase (DH) was determined with tetrazolium salts (TTC) solution as described by Alef and Nannipieri (1995). The fluorescein diacetate hydrolysis (FDA) was assessed as described by Green et al. (2006). Alkaline phosphatase (PHO) was determined in according to Tabatabai and Bremner (1969). Triplicates were analysed for each activity assay.

2.5 Nutrient bioavailability in simulant/manure mixtures after plant growth

Promptly (i.e., readily soluble) and potentially bioavailable fractions of the main macro and micronutrients were extracted in triplicate from simulant/manure mixtures, after plant growth cycle, by 1 M NH_4NO_3 (solid/solution ratio: 1/25; reaction time: 2 h; BS ISO 19730, 2008) and 0.05 M EDTA at pH 7 (solid/solution ratio: 1/10; reaction time: 1 h; Rauret et al. 2001), respectively; the extracts were then filtered through filter papers (Whatman 42) and analysed by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES, Thermo Scientific iCAP 7400, Waltham, MA, USA).

2.6 Mineral analysis and calculation of plant nutrients uptake

A subsample of dried leaves was ground and sieved to 0.5 mm using a cutting-grinding head mill (MF 10.1, IKA, Staufen im Breisgau, Baden-Württemberg, Germany) for determination of water-extractable cationic (Ca, K, Mg) and anionic (phosphate: PO_4; sulphate: SO_4) nutrient contents in leaves, according to the method described by Pannico et al. (2019). Briefly, 250 mg of dried samples were extracted in 50 ml of ultrapure water, incubated at 80 °C in a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) for 10 min and then filtered by a nylon syringe filter with a 0.45 µm pore size (Phenomenex, Torrance, CA, USA). The content of anions and cations was detected by ion chromatography (ICS-3000, Dionex, California, USA) coupled to an electrical conductivity detector. Plant nutrients uptake (mg plant^{-1}) of each element was calculated using the following formula: Dry biomass (g plant^{-1} dw) x Element concentration (mg g^{-1} dw).

2.7 Statistical Analysis

The experimental design consisted of a factorial combination of the two simulants and four different substrate mixtures for a total of eight treatments with three replicates. A randomized complete-block design was adopted, with a total of 16 experimental units of six plants each (for total of 96 plants). For soil nutrients and enzymatic activity a third experimental factor was introduced since at the end of plant growth the soil was collected in two fractions: *Rhizo*: the soil retained around roots after gently shaking and *Bulk*: the rest of the soil.

The analysis of variance was therefore conducted as two-way or three-way ANOVA using the software package SPSS version 21.0 (SPSS Inc., Chicago, Illinois, USA). When separation of means was required, it was conducted through Duncan's Multiple Range Test (DMRT), performed at $p \leq 0.05$. Relations between selected traits were analyzed through correlation or regression analysis.

3 Results and Discussion

3.1 Biometric parameters of lettuce plant

The mean effect of simulant showed significantly higher values of GI, LN, LA and dry biomass in MMS-1 compared to LHS-1, whereas DM was higher in the Lunar simulant (Table 1). The interaction between simulant (S) and amendment percentage (M) factors was statistically significant for GI, LN, LA, dry biomass, and DM (Table 1). Lettuce plants grown on the MMS-1 mixture with 30% manure recorded significantly higher values of GI, LN, LA and dry biomass compared to pure MMS-1 simulant (52-, 2-, 28- and 12-fold more than pure simulant, respectively) (Table 1). Similarly, within the different LHS-1-based mixtures, both 10% or 30% manure concentrations show significantly higher values of GI, LN, LA and dry biomass than the pure LHS-1 simulant (on average 45-, 2-, 30- and 8-fold more than pure simulant, respectively) (Table 2). In contrast, the leaf DM content, in both simulants, was on average significantly higher by 94% and 112%, respectively, in pure MMS-1 and LHS-1 compared to manure-treated mixtures (Table 1). In particular, this latter parameter, regardless of the simulant, turns out to be inversely correlated to the manure percentage in substrates (R=0.88).

Regolith simulants are extremely poor in both nutrients and organic matter proving to be notably unsuitable for plant growth (Seiferlin et al., 2008), therefore especially under such extreme conditions and in the absence of external nutrient inputs, soil amendment is particularly effective in improving fertility (Caporale et al., 2020; Duri et al., 2020; Sajjad, 2020). In the present experiment, the higher growth of plants cultivated on the Mars simulant was probably due to the worse physico-chemical characteristics of the Lunar substrate, as assessed in the complementary paper (Caporale et al., 2022, submitted). Amendment treatments significantly promoted plant biometric characteristics compared to pure substrates, and in the range 0-30 %, plant growth increased with manure dose. This result was ascribable to the improvement in hydraulic characteristics and nutrient availability driven by the manure supply (Zhang et al., 2021), while the decrease in dry biomass recorded at the 50% dose could be due to a higher electrical conductivity and endowment of phytotoxic elements, such as Na (Duggan and Jones, 2016). Similar results were found in previous work with MMS-1 and increasing doses of a green compost (Duri et al., 2020). The decrease in leaf DM observed as the dose of manure increases may be ascribed to a higher water content in lettuce leaves probably related to greater water availability in the substrate resulting from the higher water holding capacity of the amended simulants (Caporale et al., 2022, submitted). In this regard, the DM content of plants grown under conditions of reduced water availability was found to increase likely as a result of higher accumulation of assimilates required for maintenance of plant metabolism and activation of stress responses (Roitsch, 1999).

Table 1. Foliar biometric parameters in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w.

Source of Variance	Growth index	Leaf number No. plant^{-1}	Leaf area cm^2	Dry biomass g plant^{-1}	Dry matter %
Simulants (S)					
MMS1	1458 ± 266	13.75 ± 1.14	450 ± 79.1	4.33 ± 0.75	16.1 ± 1.77
LHS1	512 ± 109	8.91 ± 0.62	127 ± 28.1	1.38 ± 0.32	18.2 ± 2.19
	***	***	***	***	***
Amendment (%) (M)					
0	31.8 ± 6.09 c	6.69 ± 0.44 d	16.8 ± 4.50 d	0.43 ± 0.07 c	27.7 ± 1.13 a
10	1153 ± 208 b	13.29 ± 1.25 b	355 ± 74.2 b	4.34 ± 0.81 a	16.9 ± 0.63 b
30	1645 ± 311 a	14.08 ± 1.47 a	490 ± 113 a	4.56 ± 1.05 a	13.3 ± 0.54 c
50	1109 ± 352 b	11.25 ± 1.28 c	292 ± 99.0 c	2.09 ± 0.76 b	10.7 ± 0.48 d
	***	***	***	***	***
S x M					
MMS1 x 0	45.0 ± 2.78 de	7.56 ± 0.29 e	26.5 ± 2.7 f	0.58 ± 0.07 e	25.3 ± 0.17 b
MMS1 x 10	1588 ± 153 b	16.00 ± 0.67 b	521 ± 14.2 b	6.10 ± 0.30 b	16.5 ± 0.32 c
MMS1 x 30	2332 ± 105 a	17.33 ± 0.19 a	740 ± 23.7 a	6.88 ± 0.32 a	12.8 ± 0.76 de
MMS1 x 50	1866 ± 218 b	14.11 ± 0.11 c	512 ± 24.1 b	3.77 ± 0.25 c	9.77 ± 0.29 f
LHS1 x 0	18.6 ± 1.80 e	5.83 ± 0.36 f	7.2 ± 0.2 f	0.29 ± 0.02 e	30.1 ± 0.80 a
LHS1 x 10	717 ± 56.2 c	10.58 ± 0.22 d	190 ± 7.2 d	2.58 ± 0.29 d	17.2 ± 1.33 c
LHS1 x 30	958 ± 44.3 c	10.83 ± 0.44 d	240 ± 17.6 c	2.23 ± 0.20 d	13.9 ± 0.76 d
LHS1 x 50	353 ± 39.0 d	8.39 ± 0.20 e	71.8 ± 4.7 e	0.40 ± 0.02 e	11.6 ± 0.48 ef
	***	***	***	***	*

Non-significant (ns). *, **, *** Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test ($P = 0.05$). Different lowercase letters within each column indicate significant differences ($P \leq 0.05$).

Regarding root traits, the mean effect of simulants (Table 2) on Rdw, RA and RL showed significantly higher values in MMS-1 with increases of about 100 to 200 % compared to LHS-1. Differences were not significant for RV and SRS. Manure concentration as a main effect showed significantly highest values at 30 % and lowest at 0 % for Rdw, RA, RL and RV with maximum differences of one order of magnitude. The Specific Root Surface at 10 % was about two-fold that of pure simulant and of other manure concentrations, which did not show significant differences among them.

Interactions between experimental factors were significant for all root traits (Table 2) except diameter (Figure 1). For Rdw, RA, RL, the highest value was found in MMS-1 at 30 % manure, but for RA this was not significantly different than in MMS-1 at 50 %. Values were highest at 30 % in both simulants for RV. For SRS values were highest in LHS-1 at 10 %. Pure simulants always showed the lowest values except for SRS in MMS-1 where values at 0 % and 50 % were not significantly different. For all traits reported in table 5 values recorded in MMS-1 were in most cases statistically higher than those recorded in LHS-1 with equal manure percentage. Root dry mass in our work ranged from 0.05 g in LHS-1 at 0 % manure to 1.46 g in MMS-1 at 30 % manure. Dry mass values in pure simulants are lower than 0.1 g and lower than values reported in the literature for lettuce grown in different systems (substrate, hydroponics or aeroponic - Li et al., 2018) whereas amendment brings root dry mass closer to literature ranges (Aroca et al., 2008; Li et al., 2018). Root traits in lettuce have been reported to vary strongly with genetics and management. Our data are lower than values of about one to two thousand meters plant^{-1} reported by Murakami et al. (2002) for field-grown lettuce, but higher than those found in Li et al. (2018) in soilless systems and using an imaging system of lower resolution.

Root average diameters were higher in LHS-1 than in MMS-1 at all manure concentrations (Figure 1a), and at 0 % manure in both simulants, whereas differences were not significant between 10 %, 30 % and 50 % manure (Figure 1 b). Values of average diameter of 0.5 mm like in LHS-1 are in line with those reported by Li et al. (2018) for different growth systems. Rowse (1974) reported higher values in the uppermost 10 cm soil layer at harvest, while deeper roots were finer on average. Also, irrigation resulted in finer root diameter.

Absolute values of length for very fine roots (diameters smaller than 0.5 mm – Figure. 1 c; d) show large differences between treatments: average values of MMS-1 (Figure 1 c) were 235% higher than those of LHS-1. Regarding the effect of manure, very fine root length increased from 15 to 24 times with amendment reaching the highest value at 30 % manure, and thereafter decreasing so that the length of very fine roots was not significantly different at 10 and 50 % manure levels (Figure 1 d). The percentage of root length allocated to each diameter class is reported in figure S1 of Supplementary Material (appendix). Most of the root length was found in the finest root classes with about 64 to 89 % of roots in the class of diameter up to 0.5 mm (Figure S1 a), about 9 to 29 % in the class of roots with diameters between 0.5 and 1 mm (Figure S1 b) and up to about 4 % in the 1>D<1.5 mm class (Figure S1 c).

Table 2. Root biometric parameters in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w.

Source of Variance	Root dry mass g plant^{-1}	Root length m plant^{-1}	Root surface area cm^2 10^{-2} plant^{-1}	Root volume cm^3 plant^{-1}	Specific root surface m^2 g^{-1}
Simulants (S)					
MMS1	0.84 ± 0.18	158.52± 29.9	17.28 ± 3.1	15.2 ± 2.5	0.23 ± 0.02
LHS1	0.30 ± 0.08	57.21 ± 13.7	8.93 ± 2.2	11.3 ± 2.8	0.30 ± 0.05
	*	**	*	n.s.	n.s.
Amendment (%) (M)					
0	0.07 ± 0.01 d	8.74 ± 1.5 c	1.39 ± 0.2 c	1.85 ± 0.3 d	0.18 ± 0.02 b
10	0.40 ± 0.06 c	116.26 ± 15.5 b	15.11 ± 1.5 b	16.28 ± 1.5 b	0.43 ± 0.04 a
30	1.08 ± 0.13 a	180.67 ± 22.7 a	21.81 ± 1.7 a	21.99 ± 1.5 a	0.23 ± 0.02 b
50	0.71 ± 0.2 b	125.78 ±33.2 b	14.11 ± 3.3 b	12.87 ± 2.6 c	0.22 ± 0.01 b
	**	**	**	**	**
S x M					
MMS1 x 0	0.09 ± 0.01 e	13.15 ± 0.7 f	2.01 ± 0.2 d	2.55 ± 0.4 de	0.21 ± 0.02 c
MMS1 x 10	0.60 ± 0.01 c	163.24 ± 2.2 c	18.88 ± 0.5 b	17.62±1.0 bc	0.32 ± 0.01. b
MMS1 x 30	1.46 ± 0.01 a	243.83 ± 12.9 a	25.44 ± 1.0 a	21.28 ± 0.6 a	0.17 ± 0.03 d
MMS1 x 50	1.18 ± 0.21 b	213.8 ±28.4 b	22.79 ± 3.0 a	19.3 ± 2.5 b	0.21 ±0.01 c
LHS1 x 0	0.05 ± 0.00 e	4.33 ± 0.3 f	0.77 ± 0.0 e	1.17 ± 0.1 e	0.16 ± 0.01 d
LHS1 x 10	0.21 ± 0.21 d	69.29 ± 6.2 e	11.35 ± 1.3 c	14.94 ± 2.1 c	0.54 ± 0.01 a
LHS1 x 30	0.69 ± 0.06 c	117.51±10.9 d	18.18± 1.7 b	22.70 ± 2.2 a	0.28 ± 0.03 bc
LHS1 x 50	0.24 ± 0.02 d	37.71 ± 1.3 f	5.44 ± 0.0 d	6.39 ± 0.0 d	0.24.±0.01 c
	**	**	**	**	**

Non-significant (ns). *, **, *** Significant at P ≤ 0.05, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test (P = 0.05). Different lowercase letters within each column indicate significant differences (P ≤ 0.05).

In the six classes with diameters from 1.5 to 4.5 mm very small percentages were found, and trends of differences between treatments were similar between classes; we therefore grouped roots with diameters from 1.5 to 4.5 mm (Figure S1 d). In the finest root class the percent root length was higher in MMS-1 than LHS-1, and higher with manure added than in pure simulants (Figure S1 a) but differences between treatments were less pronounced than for absolute fine root length values shown in Figure 1 c-d. Plants grown on LHS-1 allocated proportionally more root length to classes with diameter from 0.5 to 4 mm than those grown on MMS-1, with less root length percentage in simulants mixed with manure (Figure S1 b-d). Roots with D>4 mm were only found occasionally therefore data were highly variable and differences between treatments were not significant (Figure S1 e). These roots represent a very small percentage in length (<0.5 %) but a much higher percentage in weight and can account for part of the finding that root mass and length of MMS-1 were almost three-fold than that of LHS-1, but root surface only less than two-fold (Table 2), with the consequence that specific root surface was higher in LHS-1. In general, absolute values shown in Figure 1 and percentages shown in Figure S1 indicate a belowground system made of thicker roots in LHS-1 than in MMS-1 and in pure simulants than in growth media with manure.

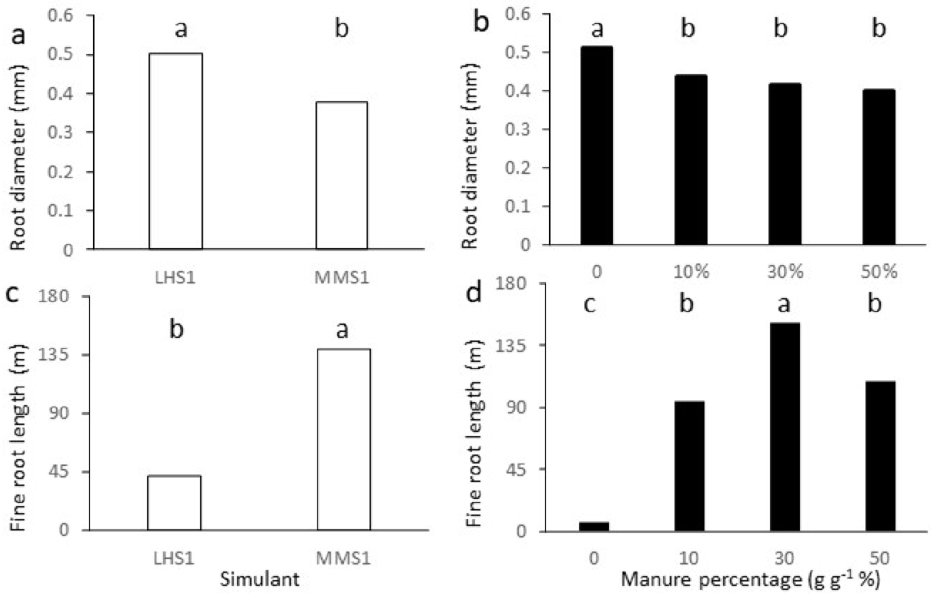

Figure 1. Main effects of simulant (a,c) and manure concentration (b,d) on root diameter (a,b) and length of roots with diameter< 0.5 mm (c,d). Bars with different letters are different for P<0.05 at the post-hoc Duncan's mean separation test.

Manure amendment of MMS-1 and LHS-1 in mixtures used for this experiment resulted in a higher amount of nutrients, an increase in porosity and water retention, a reduction in bulk density and a dilution of toxic substances found in pure simulants (Caporale et al., 2022, submitted). All of such improvements may be invoked to have an effect on our findings of larger, finer root systems in more productive treatments. A higher root length and finer root systems in plants have often been interpreted in terms of response to a high level of nitrogen (Agren, 2003; Lynch et al., 2012); in lettuce enhanced root length density at high N is reported (Jackson and Stivers, 1993; Murakami et al., 2002). Controversial behavior is recorded for phosphorus: P deficiency has been found to promote (Sarker and Karmoker, 1970; López-Bucio et al., 2003), or reduce (Beroueg et al., 2021) root proliferation, depending on species, but often results in finer root systems (e.g. Beebe et al., 2006; Lynch and Brown, 2008). In lettuce under low P Beroueg et al. (2021) reported a higher taproot growth with lower branching although branch diameters were finer.

Lettuce has been found to be very sensitive to compaction of the growth medium, even across narrow ranges of bulk density (1.25 to 1.50 g cm^{-3} Carr and Dodds, 1983) partly overlapping with the wider range of bulk densities in our mixtures spanning from 1.390 to 0.812 in MMS-1 and from 1.792 to 0.869 g cm^{-3} in LHS-1 (Caporale et al., 2022, submitted).

Other relevant differences between Mars and Lunar pure simulants included a lower amount of toxic elements and a higher CSC and content of some nutrients, porosity and water holding for MMS-1 (Caporale et al., 2022, submitted). The LHS-1 simulant, though was shown to have a higher water holding than MMS-1 between suctions of 25 cm and 600 cm of equivalent height of water (Caporale et al., 2022, submitted), where the upper value is the matric potential at which lettuce water uptake starts slowing down due to water stress according to Taylor and Ashcroft (1972). This indicates a higher volume of readily available water for non-limited lettuce growth in LHS-1, which might be expected to reproduce effects of water availability reported in the literature on root proliferation and a higher proportion of fine roots (Rowse, 1974). However, in our case this potential superiority of LHS-1 was not large enough to offset the negative effects on fine root proliferation and overall growth, due to poorer ranking of the Lunar simulant compared to MMS-1 for other physical and all chemical properties. We are unable to attribute final agronomic performance of growth media to any single factor among water availability, porosity, bulk density, concentrations of nutrients and toxic elements, due to their contemporary variation, and to interactive or offsetting effects. Interactions with management also add complexity to the comparison: Caporale et al. (2022, submitted) discuss that the higher water retention between 25 and 600 cm would be meaningful only in case of low frequency-high volume irrigation, whereas it would not give LHS-1 any particular advantage over MMS-1 in case of high-frequency-low volume irrigation strategies as the drip irrigation used in our experiment and other systems likely to be used in space settings.

In our research very high amendment rates result in a reduction of plant aboveground performance in both simulants. This confirms findings of previous research (Duri et al., 2020). Our data show a lower belowground growth as well, and this cannot be directly related to nutrients or physical properties of growth media, except for a reduction of saturated hydraulic conductivity which suggests macropore clogging by organic amendments (Caporale et al., 2022, submitted) and a possible impairment of aerobic processes.

In our data root mass, surface, volume, total and fine root length ranked close to plant leaf area ranking and indicate that maximum lettuce leaf production was obtained with a finer root system. Among allometric relation between above- and below-ground traits (Figure 2) the root to leaf area ratio (Figure 2 a) was higher in plants grown on LHS-1 than on MMS-1, and in pure simulants compared to the corresponding amended treatments. No significant difference was found between manure concentrations within each simulant except for LHS-1 where values at 10 % were lower than at 0, 30 and 50 % manure. Similar trends were found for root length per unit leaf area (Figure 2 b): they show that a higher investment in root surface or length is necessary to produce unit leaf area for plants grown on Lunar rather than Mars simulant at all manure levels, and that amendment increases root efficiency by decreasing root length to leaf area ratios. Root length per unit leaf area (Figure 2 b), though, shows that lowest absolute values, corresponding to highest efficiency of roots, are found at 10 % manure for both simulants although for MMS1 values were not different from those at 30 % manure.

Area ratios or the root length/leaf are ratio are a functional expression of the relative sizes of above- and below-ground exchange surfaces or their proxies (Mortimer, 1992) and in our case (Figure 2 a; b) they provide a framework consistent with the functional equilibrium theory (e.g. Klepper, 1991), where richer belowground environments allow to invest less in root systems per unit aboveground functional unit (e.g. leaf area). While our data indicate that the richer Mars simulant and amended treatments allow a more efficient crop production, though, there is no further decrease of unit root investment with increasing manure dose and in fact the lowest belowground unit investment corresponds to the 10 % manure percentage (and 30 % in MMS-1 as well). This is an indication of limiting conditions emerging at higher amendment doses which limit efficiency and need to be investigated.

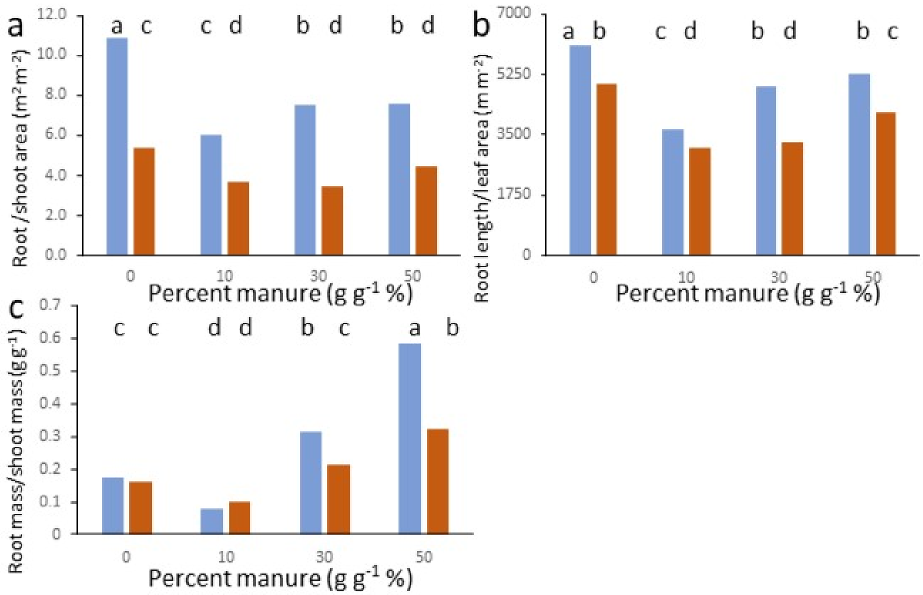

Figure 2. Interaction of simulant and manure concentration on allometric relations; a) root to shoot mass ratio; b) root to leaf surface area; c) root length per unit leaf area. Orange bars: MMS1; blue bars: LHS1. Bars with different letters are different for $P<0.05$ at the post-hoc Duncan’s mean separation test.

The root to shoot mass ratio (Figure 2 c) shows a more hormetic (Agathokleous et al., 2019) pattern than area ratios. Plants grown on LHS-1 had significantly higher ratios than on MMS-1 at manure concentration of 30 % and 50 %, showing a proportionally higher investment in below-ground organs per unit above ground mass produced. Values for both simulants were lowest at 10 % manure, with a mass investment in roots between 0.079 and

0.099 that of shoots, and highest at 50% manure where the ratio reached values between 0.50 and 0.60 in LHS-1 and around 0.30 in MMS-1. From the viewpoint of carbon partitioning our data and represent belowground C allocation ranging from about 8 to almost 60% of shoot mass. Values of 10 to 20 % are common in the literature under different management systems (e.g. Murakami et al., 2002; Li et al., 2018). Values of 30 % or higher - as found in our data at manure concentration of 30 and 50 % - are not uncommon in lettuce (e.g. Aroca et al., 2008), nevertheless they are considered high in view of resource optimization for common terrestrial growth systems (Li et al., 2018). This indicates that maximum production in Mars and Lunar simulants is obtainable at around 30 % manure, but with an excessive carbon cost, corresponding to inefficient allocation compared to a lower production at 10 % manure. Besides functional balance between organs devoted to resource acquisition, the shoot/root mass ratio depends on many functions of roots and shoots like mechanical stability or transport; therefore, physiological balance is better judged based on area or area/length ratios (Mortimer, 1992; Butler et al., 2013). The mass ratio, though remains important for judging efficiency in allocation of assimilates, and especially so in space environments where inputs are scarce. Besides, in our case all allometric ratios (Figure 2a:b:c) indicate a lower efficiency of high manure rates compared to 10 %. Agathokleous et al. (2019) report that root/shoot mass ratio dose-dependance in many instances follows a direct or inverse u-shaped relation as in our data; still an indication of higher production with lower efficiency needs optimization of other management decisions or relief from constraints. Findings from Caporale et al. (2022, submitted) in our growth media point to the need to address problems related to pore space structure: they found an increase in total porosity with amendment, but argue that it is counterbalanced and overwhelmed by macropore clogging as manure content increases in the mixtures, with resulting reductions in saturated hydraulic conductivity (Ks). The maximum positive effect of manure is recorded for MMS-1 at 10% of manure content (Ks=3.82 cm h^{-1}) and for LHS-1 at 30% of manure content (Ks=2.42 cm h^{-1}). Mechanisms underlying high root/shoot ratios at high manure content linked to a reduction in macropore and Ks may be found in hormonal retardation of shoot stomatal behavior and growth as reported when roots are exposed to consequences of waterlogging like low root zone temperatures (Atkin et al., 1973) or poor soil aeration (Lynch et al., 2012).

3.2 Physiological parameters of lettuce plants

Regardless of manure concentration, plants grown on Mars simulant exhibited a significantly higher SPAD index level (avg. 12.4) than Lunar substrate (avg. 10.5), while no significant differences were observed for fluorescence values (Table 3). On the other hand, the mean effect of amendment showed a significantly higher SPAD index value in the 30% treatment compared to pure simulants (13.4 and 9.2, respectively). In contrast, all manure applications recorded significantly higher fluorescence values (F_v/F_m) than the pure simulant. However, no significant interaction between the two tested factors was observed for both physiological parameters under investigation (Table 3).

Table 3. SPAD index and fluorescence in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w.

Source of Variance	SPAD index	Fluorescence Fv/Fm ratio
Simulants (S)		
MMS1	12.40 ± 0.53	0.738 ± 0.02
LHS1	10.51 ± 0.57	0.721 ± 0.03
	***	ns
Amendment (%) (M)		
0	9.27 ± 0.57 c	0.579 ± 0.04 b
10	11.63 ± 0.35 b	0.756 ± 0.01 a
30	13.48 ± 0.53 a	0.803 ± 0.00 a
50	11.42 ± 0.93 b	0.781 ± 0.01 a
	***	***
S x M		
MMS1 x 0	10.12 ± 0.59	0.612 ± 0.03
MMS1 x 10	11.79 ± 0.71	0.744 ± 0.00
MMS1 x 30	14.31 ± 0.48	0.800 ± 0.01
MMS1 x 50	13.36 ± 0.10	0.798 ± 0.01
LHS1 x 0	8.41 ± 0.75	0.547 ± 0.08
LHS1 x 10	11.46 ± 0.30	0.767 ± 0.00
LHS1 x 30	12.66 ± 0.70	0.806 ± 0.00
LHS1 x 50	9.49 ± 0.75	0.765 ± 0.01
	ns	ns

Non-significant (ns). *, **, *** Significant at P ≤ 0.05, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test (P = 0.05). Different lowercase letters within each column indicate significant differences (P ≤ 0.05).

The SPAD index is an effective nondestructive tool for an indirect measurement of chlorophyll content (Li et al., 2018; Dong et al., 2019). The dependence of photosynthesis on chlorophyll molecules as the primary medium of harvesting light energy to drive electron transport reactions has been demonstrated (Croft et al., 2017). In a similar experiment on lettuce grown at different mixtures of MMS-1 and green compost, photosynthetic rate decreased at higher compost concentrations (Duri et al., 2020); this response was consistent with the reduction in SPAD index and dry biomass recorded in our work at the 50 % manure dose. However, our highest biomass treatments (30% manure dose) showed 3-fold lower SPAD values than those recorded on butterhead lettuce grown in NFT (nutrient film technique) under optimal environmental and nutrient availability conditions (El-Nakhel et al., 2019). As well as the SPAD index, the maximum quantum efficiency of PSII (Fv/Fm) is also an indicator of photosynthetic efficiency and plant health (Ferrante and Maggiore, 2007; Cendrero-Mateo et al., 2016). Generally, Fv/Fm values between 0.79 and 0.84 are approximate optimum values among unstressed leaves of many different species, while lower values indicating plant stress (Maxwell and Johnson, 2000). In our experiment, the very low fluorescence values recorded in plants grown on pure simulants indicated the occurrence of photosystem damage due to the severe nutritional stress (Schreiber et al., 1995).

3.3 Extractable nitrogen, carbon, microbial biomass nitrogen and carbon in simulant/manure mixtures after plant growth

A significant increase of Extr N and Extr C in both *Rhizo* and *Bulk* samples of LHS-1 and MMS-1 occurred by increasing manure concentration (Table 4). By comparing the *Rhizo* samples of LHS-1 and MMS-1 no significant differences in terms of Extr N was observed (Table 4), whereas within the *Bulk* samples amended with 30% manure the value of MMS-1 was greater than that of LHS-1 (122.7 ± 5.0 and 94.7 ± 2.6 mg kg^{-1}, respectively). Conversely, in most samples the Extr C was greater in LHS-1 than in MMS-1 in both *Rhizo* and *Bulk* samples (Table 4). MBN and MBC followed the Extr N and Extr C trend: they increased by increasing the manure concentration (Table 4). The values of MBN in both *Rhizo* and *Bulk* samples of LHS-1 upon 30 and 50% manure treatment exceeded those of MMS-1 (Table 4). Instead, the values of MBC in both *Rhizo* and *Bulk* samples of LHS-1 resulted greater compared to those of MMS-1 only if treated with 50 % manure (1258.7 ± 202.1 and 1500.8 ± 165.0 mg kg^{-1}, in *Rhizo* and *Bulk* samples, respectively; Table 4).

Table 4. Extractable nitrogen, carbon, microbial biomass nitrogen and carbon in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w %), separated in rhizo and bulk soil after lettuce growth.

Source of Variance	Extr. N	Extr. C	MBN	MBC
	mg kg^{-1} DW			
Simulants (S)				
MMS1	78.0 ± 56.4	152.6 ± 101.0	77.8 ± 50.3	397.4 ± 350
LHS1	75.0 ± 52.7	185.2 ± 120.2	92.0 ± 67.6	523.2 ± 548
	ns	ns	ns	ns
Amendment % (M)				
0	11.6 ± 2.9 d	32.5 ± 10.6 d	11.4 ± 3.5 d	46.2 ± 17 d
10	39.9 ± 4.3 c	111.3 ± 15.0 c	57.5 ± 9.9 c	152.6 ± 43 c
30	112.6 ± 11.8 b	218.6 ± 48.9 b	110.4 ± 34 b	500.6 ± 152 b
50	141.7 ± 20.9 a	313.1 ± 40.0 a	160.2 ± 20.9 a	1141.8 ± 304 a
	***	***	***	***
Rhizo vs bulk soil (RB)				
RH	81.7 ± 60.9	166.4 ± 110.5	94.9 ± 66.4	450.6 ± 428
BK	71.3 ± 47.0	171.4 ± 113.9	74.8 ± 50.9	470.0 ± 498
	ns	ns	ns	ns
S x M x RB				
MMS1 x 0 x RH	10.3 ± 1.0 f	32.1 ± 7.0 f	12.5 ± 2.2 k	51.0 ± 16 g
MMS1 x 0 x BK	10.4 ± 1.0 f	21.5 ± 8.0 f	12.6 ± 2.8 k	37.0 ± 13 g
MMS1 x 10 x RH	35.0 ± 2.2 e	96.4 ± 10.2 e	69.1 ± 4.0 h	146.4 ± 26 f
MMS1 x 10 x BK	43.1 ± 2.6 d	112.0 ± 15.3 e	59.6 ± 2.8 i	183.8 ± 44 e
MMS1 x 30 x RH	118.7 ± 5.1 b	181.2 ± 25.5 d	92.6 ± 3.4 f	483.6 ± 138 d
MMS1 x 30 x BK	122.7 ± 5.0 b	201.9 ± 35.5 d	78.0 ± 6.5 g	470.0 ± 139 d
MMS1 x 50 x RH	162.7 ± 5.0 a	287.0 ± 29.7 c	163.3 ± 8.6 b	843.0 ± 158 b
MMS1 x 50 x BK	120.2 ± 6.5 b	288.7 ± 32.8 c	134.0 ± 5.9 d	964.5 ± 128 b
LHS1 x 0 x RH	14.1 ± 4.2 g	44.0 ± 7.1 f	10.0 ± 4.7 k	63.4 ± 12 g
LHS1 x 0 x BK	11.5 ± 2.9 g	32.4 ± 7.1 f	10.4 ± 3.5 k	33.6 ± 12 g
LHS1 x10 x RH	37.7 ± 2.0 e	119.7 ± 12.9 e	58.2 ± 1.5 i	140.2 ± 43 f
LHS1 x 10 x BK	43.5 ± 2.6 d	117.4 ± 11.7 e	42.9 ± 1.2 j	140.2 ± 49 f
LHS1 x 30 x RH	114.3 ± 5.2 b	216.0 ± 30.3 d	164.7 ± 5.6 b	618.8 ± 171 c
LHS1 x 30 x BK	94.7 ± 2.6 c	275.3 ± 48.0 c	106.1 ± 4.4 e	429.8 ± 122 d
LHS1 x 50 x RH	160.0 ± 4.9 a	355.0 ± 21.9 a	188.6 ± 5.8 a	1258.7 ± 202 a
LHS1 x 50 x BK	123.7 ± 5.8 b	321.8 ± 34.7 b	154.7 ± 4.4 c	1500.8 ± 165 a
	***	***	***	***

Non-significant (ns). *, **, *** Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test (P = 0.05). Different lowercase letters within each column indicate significant differences ($P \leq 0.05$).

Our results are consistent with literature reports of increases of MBC and MBN following organic amendment (Zhang et al., 2015; Li et al., 2018; Yu et al., 2020). Zhang et al. (2015) found a strong increase of MBC and MBN after horse manure-based amendment and they attributed this response to the readily metabolizable carbon and nitrogen in the applied manure. Also Li et al. (2018) observed an enhancement of MBC and MBN after 1 month from pig and cattle manure application in their field experiments carried on three different soils.

Recently, Yu et al. (2020) reported that pig manure had the best performance among different organic amendments in increasing soil MBC and MBN. In a soilless study Sax and Scharenbroch (2017) found wood chips or compost-based organic amendments of vermiculite, inert substrate used in growing nursery, enhanced chemical and biochemical fertility obtaining an increase of MBC, respiration, TOC and TN.

3.4 Enzymatic activities in simulant/manure mixtures after plant growth

Rhizo and *Bulk* samples of LHS-1 and MMS-1 without manure amendment had no DH activity (Figure 3a). Upon manure addition DH activity increased with manure rate (10, 30 and 50 %) (Figure 3a). At 10 and 30% manure the DH activity was significantly greater in MMS-1 and no significant differences between *Rhizo* and *Bulk* samples were observed (Figure 3a). At 50 % manure concentration the DH activity grew more in LHS-1 compared to MMS-1 reaching 21.5 and 23.8 µg TPF g^{-1} h^{-1} in *Rhizo* and *Bulk* samples, respectively (Figure 3a).

Dehydrogenases are intracellular enzymes involved in redox processes of a wide range of organic molecules and their activity is related to living microbial organisms (Werheni Ammeri et al., 2022). DH activity is strictly correlated with soil microbial biomass and its metabolic activity (Dick, 2011). The activity of these enzymes solely is greater in the rhizosphere because of the presence of the root-microorganism system in which a greater abundance of microorganisms occurs (Caracciolo et al., 2015). In our experiment there were no significant differences between *Rhizo* and *Bulk* soils by increasing manure rates until 50 %.

Most of samples *Rhizo* and *Bulk* MMS-1 and LHS-1 exceeded 90 µg fluorescein g^{-1} h^{-1} (Figure 3b) already at 10 % manure rate in according to findings of Bonanomi et al. (2020) who found an enhancement of the FDA activity upon organic amendments in soil. Although in the literature no significant differences were registered between rhizospheric and non-rhizospheric media after compost amendment (Martínez et al., 2005), *Rhizo* LHS-1 at 10 % rate and *Rhizo* MMS-1 50 % rate manure showed a slightly reduced FDA activity respect to *Bulk* soil (Figure 3b). At manure doses higher than 10%, no further stimulation of FDA activity occurred and at 30 and 50% manure MMS1 *Bulk* samples showed greater activity levels than LHS-1 samples. At 50 % manure addition the *Bulk* sample MMS1 reached the greatest FDA activity level (105.9 µg fluorescein g^{-1} h^{-1}; Figure 3b). Simple simulants with no manure amendment showed FDA activity anyway, although small (Figure 3b) due to microorganisms whose presence is demonstrated by MBC data (Table 4). The greater FDA activity recorded in MMS-1 could be explained by a more intense rhizosphere effect since lettuce plants grew up better in MMS-1 as all biometric parameter highlighted (Tables 1 and 2).

Pure LHS-1 and MMS-1 simulants had an almost-zero AP activity (Figure 3c). Values of AP activity increased with manure percentage in the mixtures (Figure 3c) and this is in agreement with Yang et al. (2018), Gupta et al. (2016) and Liu et al. (2010). In general values were higher in *Rhizo* than in *Bulk* samples and reached 3.3 µmol p-NP g^{-1} h^{-1} at 50% manure rate in MMS-1 (Figure 3c), in coincidence with P demand of plant and microorganisms which could stimulate this enzyme activity (Yang et al., 2018). Zymography studies, based on a relatively new technique to visualise the spatial distribution of potentially active enzymes in soil with 2D images, have highlighted an intense phosphatase activity close to roots (Heitkötter and Marschner, 2018; Ma et al., 2018; Hummel et al., 2021). Phosphatase activity is generally higher in the rhizosphere compared to bulk soil as this enzyme is either directly released by roots or by microorganisms that are stimulated by rhizodeposits (Kuzyakov and Razavi, 2019). Spohn and Kuzyakov (2013) evaluated alkaline and acid phosphatase near the lupine root and they found the alkaline phosphatase was up to 5.4 times greater in *Rhizo* than in *Bulk* soil.

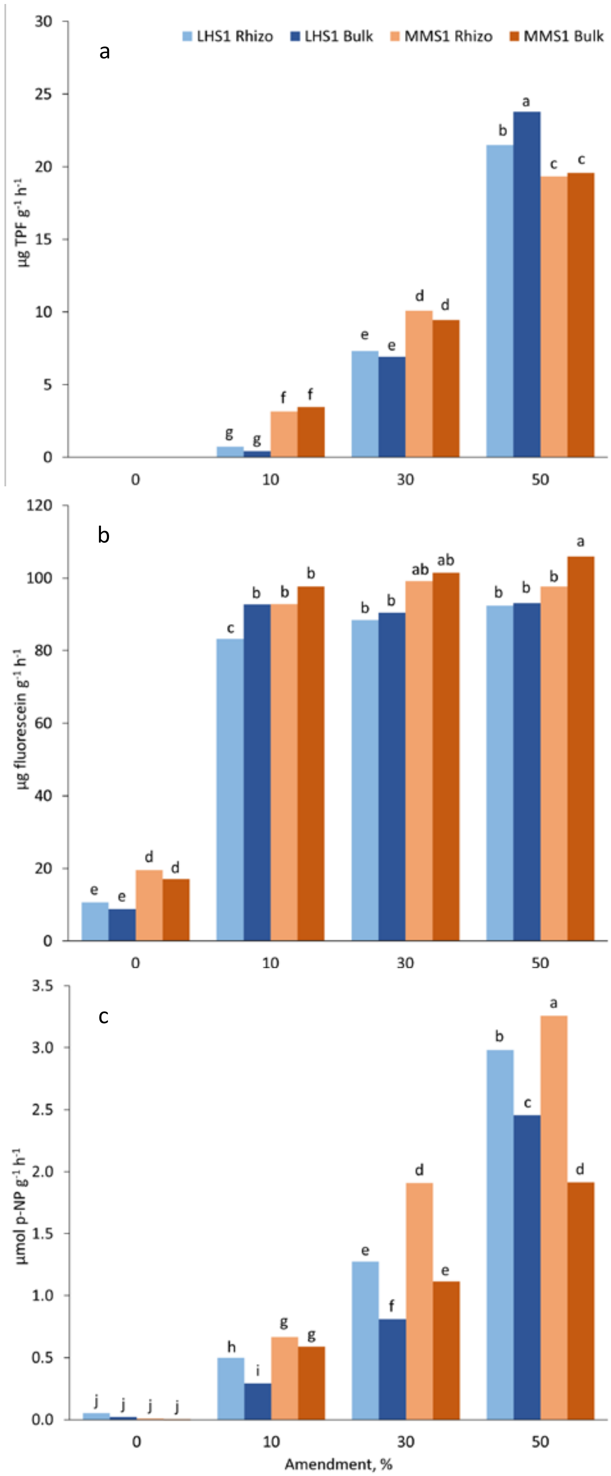

Figure 3. Interaction of simulant, manure concentration and Rhizo vs. Bulk soil on a) dehydrogenase; b) fluorescein diacetate hydrolysis; c) alkaline phosphomonoesterase activity. Bars with different letters are different for $P<0.05$ at the post-hoc Duncan's mean separation test.

3.5 Nutrient bioavailability in simulant/manure mixtures after plant growth

The concentration of the main macro and micronutrients in different MMS-1 or LHS-1/manure mixtures (separated in rhizo and bulk soil after lettuce growth), extracted by 1M NH_4NO_3 to assess the promptly-bioavailable fractions (BS ISO 19730, 2008), and 0.05M EDTA at pH 7 to evaluate the potentially-bioavailable fractions (Rauret et al., 2001), are shown in Tables 5 and Table S1.

The promptly (Table 5) and potentially (Supplementary Material, Table S1) bioavailable fractions of Ca, K, Mg, P and Mn extracted from MMS-1-containing mixtures were significantly higher than those extracted from LHS-1-based mixtures, while the opposite was observed with Fe and Na (and promptly-bioavailable Cu and Zn). In most of the cases, this trend was already recognized and discussed in the companion paper (Caporale et al., 2022, submitted) for nutrient contents of simulants and mixtures at the start point before lettuce growth, and it is mainly due to the higher total nutrient contents in the MMS-1- than LHS-1-based mixtures. Despite mixtures with LHS-1 contain more Ca than Mars simulant-based substrates, they are a lower source of promptly- and potentially-bioavailable Ca for plants and rhizosphere biota; in contrast, they released larger amounts of promptly- and potentially-bioavailable Na in comparison to MMS-1-containing mixtures, and this can also explain the different alkalinity and chemical properties of the two simulants. As recently discussed by Duri et al. (2022) in their review on the potential for Lunar and Martian regolith simulants to sustain plant growth, plants take up only the bioavailable forms of nutrients from a simulant-based growth substrate, not the elements occluded in mineral structures that are released only after mineral weathering. Hence, plants can exploit only a low-to-moderate fraction of the total nutrient contents in a simulant to satisfy their requirements.

Table 5. Concentration (mg kg^{-1} DW) of main macro and micronutrients in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w %), separated in rhizo and bulk soil after lettuce growth, extracted by 1M NH_4NO_3 (n=3).

Source of Variance	Ca	K	Mg	P	Fe	Na	Mn	Cu	Zn
					mg kg^{-1} DW				
Simulants (S)									
MMS1	2215	768	416	11.8	0.69	45.2	1.57	0.15	0.10
LHS1	1102	490	246	8.5	1.45	69.2	0.67	0.17	0.13
	***	***	***	***	***	*	***	*	***
Amendment % (M)									
0	1201 d	90.3 d	156 d	0.10 d	0.04 c	31.3 b	0.24 b	0.04 d	0.03 d
10	1551 c	338 c	227 c	6.08 c	0.84 b	27.5 b	1.48 a	0.14 c	0.10 c
30	1869 b	707 b	388 b	14.9 b	1.52 a	39.4 b	1.39 a	0.19 b	0.14 b
50	2012 a	1382 a	553 a	19.5 a	1.87 a	130 a	1.37 a	0.27 a	0.19 a
	***	***	***	***	***	***	***	***	***
Rhizo vs bulk soil (RB)									
RH	1634	527	303	9.82	1.12	51	1.13	0.16	0.11
BK	1683	731	359	10.5	1.01	63	1.11	0.16	0.12
	ns	**	***	ns	ns	ns	ns	ns	ns
S x M x RB									
MMS1 x 0 x RH	1950	147	260	0.13	0.04	27.3	0.08	0.06	0.02
MMS1 x 0 x BK	2124	187	296	0.13	0.04	36.9	0.06	0.06	0.03
MMS1 x 10 x RH	2258	421	334	7.39	0.19	29.5	2.22	0.11	0.06
MMS1 x 10 x BK	2176	558	375	9.28	0.59	30.8	2.39	0.12	0.09
MMS1 x 30 x RH	2339	671	406	18.1	0.85	29.0	2.01	0.17	0.11
MMS1 x 30 x BK	2183	1106	484	18.8	0.99	38.2	1.94	0.17	0.12
MMS1 x 50 x RH	2358	1254	528	19.5	1.61	66.3	2.00	0.25	0.16
MMS1 x 50 x BK	2330	1801	644	21.4	1.18	103	1.87	0.23	0.17
LHS1 x 0 x RH	330	15.9	45.8	0.09	0.04	29.7	0.45	0.03	0.03
LHS1 x 0 x BK	398	12.0	24.4	0.05	0.03	31.4	0.39	0.03	0.03
LHS1 x10 x RH	796	147	78.4	2.80	1.16	26.7	0.58	0.13	0.08
LHS1 x 10 x BK	976	225	122	4.87	1.42	23.1	0.72	0.19	0.16
LHS1 x 30 x RH	1377	472	289	12.5	2.59	36.8	0.85	0.23	0.18
LHS1 x 30 x BK	1576	579	372	10.4	1.65	53.8	0.77	0.18	0.15
LHS1 x 50 x RH	1663	1092	486	18.1	2.52	163	0.88	0.30	0.22
LHS1 x 50 x BK	1697	1379	553	18.9	2.17	189	0.71	0.28	0.20
	ns	ns	ns	ns	ns	ns	ns	ns	ns
S x M	***	ns	***	***	*	**	***	**	ns

For the sake of clarity, this wide table shows only the mean values, not followed by standard deviations. Non-significant (ns). *, **, *** Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test (P = 0.05). Different lowercase letters within each column indicate significant differences ($P \leq 0.05$).

The amendment of MMS-1 and LHS-1 simulants with increasing rates of monogastric-based manure determined a significant increase of the promptly (Table 5) and potentially (Table S1) bioavailable fractions of the macro and micronutrients. Specifically, the nutrient bioavailable fractions in the 90:10, 70:30, 50:50 simulant/manure mixtures were, respectively, 11-, 24-, 32-fold (Table 5), and 5-, 11-, 14-fold (Table S1), higher than those in the pure simulants (100:0). Likewise, in comparison to pure MMS-1 simulant, Caporale et al. (2020) noted an increase of water-soluble fraction of nutrients, such as Ca, K, Mg, nitrate, phosphate and sulphate, when they amended the simulant with green compost at increasing rates (up to 70% of compost in volume).

For the majority of the nutrients, no statistically significant differences between promptly- or potentially-bioavailable fractions extracted from *Rhizo* soil and those extracted from *Bulk* soil were found. Actually, except for 100:0 treatment, the substrate separation into *Rhizo* vs. *Bulk* soil was very challenging and purely indicative, due to the abundance of root biomass into a relatively small volume of each pot. Nevertheless, a significant depletion of promptly- and potentially-bioavailable K and Mg in the *Rhizo* vs. *Bulk* soil occurred, probably due to a fast uptake rate of two macronutrients by the lettuce plants in the last growth phase. In contrast, there was a significant increase of the promptly-bioavailable Cu in the *Rhizo* vs. *Bulk* soil, maybe due to release of root exudates, rhizosphere pH acidification and enhanced microorganism activity. The interaction among the three factors: simulants (S) x amendment (M) x *Rhizo*/*Bulk* soil (RB), was significant ($p<0.05$) only for the promptly-bioavailable Mn (Table 5), not significant in all the other cases. On the other hand, the interaction between simulants (S) x amendment (M) factors was significant for the majority of nutrients, except promptly and potentially bioavailable K and Zn (Tables 5 and S1).

The monitoring of the promptly- and potentially-bioavailable fractions of nutrients in the simulant/manure mixtures, before (described in the companion paper, Caporale et al., 2022, submitted) and after lettuce growth cycle, evidenced an overall reduction of potentially-bioavailable fractions of the macro and micronutrients, mainly due to plant uptake and bioaccumulation in microbial biomass. The release and mobilization of these nutrients from mineral and organic moieties of substrates, regulated by the intense root and microbial activity and enhanced by water periodic supply, induced an increase of the promptly-bioavailable pool of Ca, Mg and Na at the end of plant growth, in comparison to the start point. Indeed, at least for Ca, this phenomenon may be also due to the release of nuclear Ca^{2+} by plant root, essential to the modulation of the plant growth hormone auxin and establishment of nitrogen-fixing and phosphate-delivering arbuscular mycorrhizal endosymbiosis (Leitão et al., 2019). Unlike Ca and Mg (whose promptly-bioavailable pool raised up to 58%), the promptly-bioavailable fraction of Na at the end of plant growth was on average 5-fold and 7-fold higher than the start point, respectively, in MMS-1 or LHS-1/manure mixtures (100:0 excluded). This abundance of promptly-bioavailable Na might have caused a salt stress in plants (Qin et al., 2013; Caporale et al., 2020; Duri et al., 2020), which can justify, at least in part, the lower growth and agronomic performance of lettuces grown on LHS-1-based vs. MMS-1-based substrates.

3.6 Leaf mineral content and plant nutrients uptake

The mean effect of amendment showed a significant increase of about 12- and 2-fold in phosphate and magnesium concentration, respectively, at the highest manure dose compared to the pure simulant (Table 6). Potassium, Ca, Na and SO_4 contents incurred significant interaction of the tested factors (S x M) (Table 6). In particular, the Martian mixtures showed at the highest manure dose an increase in K, Ca, and SO_4 content by 162%, 154%, and 600%, respectively, compared to the pure MMS-1 simulant, while Na content was significantly higher at doses 30 and 50%. Regarding plants grown on Lunar simulant, K and SO_4 concentration was on average 70% and 248% higher in manure-treated plants than in the untreated substrate, while Na content at the 50 % manure dose was significantly higher compared to all other treatments. In contrast, the Ca content in the Lunar mixtures was significantly higher by 96% at the 30% manure dose compared to the pure simulant (Table 6).

Regardless of amendment factor, the mean effect of the simulant shows significantly higher uptake of all analyzed elements in plants grown on MMS1 (Table S2). In turn, the plant uptake of all elements analyzed was affected by the S × M significant interaction (Table S2). In Martian mixtures, per-plant uptake of PO_4, Mg, Ca and Na was significantly higher at the 30% manure dose (109-, 13-, 23- and 23-fold more than pure simulant, respectively), whereas the amount of K and SO_4 assimilated per plant was significantly higher at the 30% and 50% manure doses (on average 18- and 49-fold more than pure simulant, respectively) (Table S2). Regarding the Lunar simulant, with the exception of Na whose highest values were recorded in all manure-treated mixtures, PO_4, K, Mg, Ca and SO_4 uptake was significantly higher in plants grown on the 10 % and 30 % manure-treated mixtures (on average 66-, 14-, 9-, 14- and 26-fold more than pure simulant, respectively) (Table S2).

Trends in leaf mineral content were consistent with what was discussed in the previous section about plant nutrient bioavailability in different substrates. The increase in mineral element availability in the soil as the manure dose increases was widely demonstrated (Schlegel, 1992; Sun et al., 2014). In our work, PO_4, K, and Mg increased linearly in both simulant mixtures and leaf tissues as the dose of manure applied increased. Specifically, bioavailable Na levels increased more than proportionally in both simulant/manure mixtures, reaching significantly higher contents in LHS-1-based substrates than in MMS-1-based ones; this finding reflected the notably high Na concentration found in lettuce leaves at the highest manure dose. The latter result may explain the reduction in dry biomass recorded at the 50 % manure dose due to the detrimental effects of Na, as reported in several other works on lettuce (Bie et al., 2004; Shin et al., 2020). In addition, as demonstrated in the literature (Cramer, 1997; Lazof and Bernstein, 1998), the high Na content found in LHS-1 at the highest manure dose also resulted in reduced Ca assimilation, further contributing to the severe reduction in dry biomass recorded in plants grown in this simulant mixture.

Table 6. Mineral contents in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w %).

Source of Variance	PO_4	K	Ca	Mg	SO_4	Na	Cl
				g kg^{-1} dw			
Simulants (S)							
MMS1	4.10 ± 0.73	26.53 ± 2.97	6.32 ± 0.60	1.69 ± 0.14	0.72 ± 0.14	1.89 ± 0.20	0.60 ± 0.12
LHS1	3.54 ± 0.59	24.82 ± 1.79	5.69 ± 0.43	2.10 ± 0.11	0.66 ± 0.08	2.17 ± 0.37	1.05 ± 0.15
	ns	ns	*	***	ns	ns	***
Amendment (%) (M)							
0	0.53 ± 0.02 c	16.01 ± 0.33 c	3.65 ± 0.15 c	1.65 ± 0.11 bc	0.21 ± 0.02 c	1.41 ± 0.21 b	1.56 ± 0.15 a
10	3.89 ± 0.15 b	24.50 ± 0.93 b	5.78 ± 0.28 b	1.55 ± 0.10 c	0.67 ± 0.09 b	1.48 ± 0.19 b	0.48 ± 0.07 c
30	4.67 ± 0.21 b	25.94 ± 0.34 b	7.04 ± 0.42 a	1.90 ± 0.19 b	0.72 ± 0.03 b	1.83 ± 0.27 b	0.55 ± 0.12 c
50	6.19 ± 0.64 a	36.25 ± 3.33 a	7.56 ± 0.61 a	2.49 ± 0.11 a	1.14 ± 0.13 a	3.41 ± 0.33 a	0.73 ± 0.10 b
	***	***	***	***	***	***	***
S x M							
MMS1 x 0	0.56 ± 0.02	15.93 ± 0.26 d	3.49 ± 0.21 e	1.44 ± 0.12	0.20 ± 0.02 d	1.21 ± 0.29 d	1.26 ± 0.05
MMS1 x 10	3.83 ± 0.29	23.07 ± 1.19 b	6.29 ± 0.35 bcd	1.35 ± 0.02	0.50 ± 0.11 c	1.47 ± 0.24 cd	0.34 ± 0.07
MMS1 x 30	5.07 ± 0.12	25.32 ± 0.43 bc	6.64 ± 0.67 bc	1.54 ± 0.13	0.78 ± 0.03 b	2.18 ± 0.00 bc	0.30 ± 0.03
MMS1 x 50	6.95 ± 0.82	41.80 ± 3.59 a	8.87 ± 0.18 a	2.45 ± 0.13	1.40 ± 0.11 a	2.70 ± 0.16 b	0.50 ± 0.02
LHS1 x 0	0.51 ± 0.04	16.10 ± 0.68 d	3.80 ± 0.21 e	1.85 ± 0.07	0.23 ± 0.03 d	1.61 ± 0.30 cd	1.85 ± 0.14
LHS1 x 10	3.95 ± 0.15	25.93 ± 0.93 bc	5.27 ± 0.12 d	1.76 ± 0.07	0.85 ± 0.04 b	1.50 ± 0.35 cd	0.61 ± 0.07
LHS1 x 30	4.26 ± 0.18	26.56 ± 0.06 bc	7.44 ± 0.53 b	2.26 ± 0.18	0.67 ± 0.04 bc	1.47 ± 0.48 cd	0.79 ± 0.08
LHS1 x 50	5.43 ± 0.90	30.69 ± 3.39 b	6.25 ± 0.29 cd	2.53 ± 0.21	0.88 ± 0.10 b	4.11 ± 0.18 a	0.95 ± 0.03
	ns	**	***	ns	***	*	ns

Non-significant (ns). *, **, *** Significant at P ≤ 0.05, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test (P = 0.05). Different lowercase letters within each column indicate significant differences (P ≤ 0.05).

4 Conclusions

This study demonstrated that pure Mars (MMS-1) and Lunar (LHS-1) simulants can sustain plant growth (at least leafy vegetables such as lettuce), even in absence of fertilization. These simulants (i.e., an assemblage of terrestrial crushed rocks build up to replicate the physicochemical properties of extra-terrestrial regoliths, assessed *in situ* by previous missions), however, hold several properties that can hinder plant growth, such as alkaline pH, low content of promptly-bioavailable nutrients but high bioavailability of Na, predominance of macro vs. micropores and consequent scant water holding capacity, etc. The amendment of these nutrient-poor and alkaline substrates with stabilized organic matter such as horse/swine monogastric manure at varying rates (100:0, 90:10, 70:30, 50:50, w/w) mitigated these negative features and significantly improved the ability of MMS-1 and LHS-1 simulants to sustain plant growth due also to the enhancement of microbial biomass abundance and activity. The mixture containing 70 % in weight of simulant and 30 % of manure provided the best outcomes in terms of biomass production and plant vigour/health. As well, this mixture is a more sustainable option for a BLSS developed on ISRU strategy than 50:50 simulant/manure growth medium. However, to assess the feasibility of these manure-amended Lunar and Martian soils for plant growth in space settings, these findings need to be validated in follow-up experiments under microgravity. In this context, the diverse water movement and dynamics in the soil/plant system can differently regulate the extent of mineral weathering and the rate of organic matter decomposition, the biogeochemistry and bioavailability of nutrients and consequently, plant growth, physiology and health. Moreover, the monitoring of fertility, properties and terraforming processes occurring in the manure-amended Lunar and Martian soils over time, under consecutive cultivation cycles of different crop species, is of paramount importance to widen scientific knowledge in sustainable space food production systems.

Chapter 5

The growth of lettuce plants on Lunar and Martian soils amended with monogastric-based manure: effect on plant nutritional traits

This chapter has been submitted for publication on Frontier in Nutrition

Abstract

The supplementation of bioactive compounds in astronaut's diets is undeniable, especially in an extreme and inhospitable habitat of future space settlements. The aim of this study was to enhance the Martian and Lunar regolith fertility (testing two commercial simulants) through the provision of organic matter (manure) as established by *in situ* resource utilization (ISRU) approach. In this perspective, we obtained 8 different substrates after mixing Mojave Mars Simulant (MMS-1) or Lunar Highlands Simulant (LHS-1), with four different rates of manure (0, 10, 30 and 50%, w/w) from monogastric animals. Then we assessed how these substrates can modulate fresh yield, organic acids, carotenoids content, antioxidant activity, and phenolic profile of lettuce plants (*Lactuca sativa* L.). Regarding fresh biomass production, MMS-1-amended substrates recorded higher yields than LHS-1-ones; plants grown on70:30 MMS-1/manure mixture produced the highest foliar biomass. Moreover, we found an increase in lutein and β-carotene content by +181% and +263%, respectively, when applying the highest percentage of manure (50%) compared with pure simulants or less-amended mixtures. The 50:50 MMS-1/manure treatment also contained the highest amounts of individual and total organic acids, especially malate content. The highest antioxidant activity for ABTS assay was recorded when no manure was added. The highest content of total hydroxycinammic acids was observed when no manure was added, while ferulic acid content (most abundant compound) was the highest in 70:30 simulant/manure treatment, as well as in pure LHS-1 simulant. The flavonoids content was the highest in pure-simulant treatment (for most of compounds), resulting in the highest total flavonoids and total phenols content. Our findings indicate that the addition of manure at specific rates (30%) may increase the biomass production of lettuce plants cultivated in MMS-1 simulant, while phytochemical composition is variably affected by manure addition, depending on the simulant. Therefore, the agronomic practice of manure amendment showed promising results, however it must be tested with other species or in combination with other factors such as fertilization rates or biostimulants application to verify its applicability in space colonies for food production purposes.

Keywords: *in situ* resource utilization (ISRU), Moon, Mars, Antioxidant Activity, Carotenoids, Phenolic profile

1 Introduction

In recent years there is a growing interest in space exploration and the subsequent establishment of extraterrestrial colonies on the Moon or Mars. In addition to the leading space agencies, such as the National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA), private companies (e.g. SpaceX) are also currently focused on space research (Shammas and Holen, 2019; Sarang et al., 2020). The synergistic collaboration of many countries and experts in various disciplines has resulted in the achievement of new technological milestones, which should allow in the coming decades the realization of full-fledged missions aimed at both exploration and colonization of new planets (Zubrin and Wagner, 2011; Verseux et al., 2016). When planning the establishment of a future outpost, either on the Moon or Mars, it is fundamental to consider the self-sufficiency of the colony as the hypothesis of total resources supply from Earth would be practically unrealistic in regards of both time management and high costs (Massa et al., 2006; Llorente et al., 2018; Zabel et al., 2020). In this regard, indigenous soil-based agricultural systems could be an effective solution to relieve the inputs of a bio-regenerative life support system (BLSS) (Silverstone et al., 2003; Nelson et al., 2008; Maggi and Pallud, 2010; Rouphael et al., 2019) and meanwhile, the *in-situ* resources utilization (ISRU) approach could provide a valuable contribution to cost reduction by using *in-situ* materials and recycling all colony waste products to the maximum extent possible (Sridhar et al., 2000; O'Handley et al., 2001; Rygalov et al., 2001; Benaroya et al., 2013; Duri et al., 2020, 2022). Recently, Cannon and Britt (2019a) evaluated a set of alternatives for the possible development of a self-sufficient community on Mars. The authors assumed the *in-situ* production of basic needs, emphasizing that the diet of colony residents would necessarily have to change by focusing on insect farming for food production rather than plant cultivation, pointing out the differences in terms of growth cycle duration, area devoted and production costs (Cannon and Britt, 2019a). However, this assessment omits the remarkable ecological role that plants may play, since apart from food production they are able to strongly support the BLSS through water recycling, CO_2 fixation, and oxygen production (Wheeler, 2004; Maggi and Pallud, 2010; Llorente et al., 2018), thus being an pivotal part of a biological loop that is not only focused on food production. In addition, there are nutritional aspects of plant derived food products that cannot be neglected, such as their content in fundamental macro and micro-minerals, bioactive compounds such as carotenoids and phenolic acids that are extremely important for a balanced human health (Yoon et al., 2012; Lima et al., 2014).

The Martian and Lunar surface is composed mainly of basaltic rocks and sediments that include varying amounts of different minerals such as olivine, pyroxene, plagioclase, anorthosite, vitric and lithic fragments, iron oxides, and sulfates (Ohtake et al., 2009; Taylor et al., 2010; Grotzinger et al., 2014; Zeng et al., 2015; Kayama et al., 2018; Cannon et al., 2019). Several studies reported that Martian as well as Lunar soils are not suitable for plant cultivation, as they are poor in nutrients (primarily nitrogen) and organic matter, and also lack proper soil structure (Wamelink et al., 2014; Caporale et al., 2020). However, these short comings could be compensated by proper agronomic practices (De Micco et al., 2009; Ming, 2015). Due to the unavailability of real Martian and Lunar regolith samples for agronomic testing, scientific experiments on space cultivation can be only conducted with commercial simulants derived from crushed terrestrial rocks, which tend to replicate the geotechnical and compositional characteristics of regolith studied during the past manned and un-manned missions to the Moon or Mars, respectively (Duri et al., 2022). Over the years various research organizations have developed different versions of extra-terrestrial soil simulants (Rickman et al., 2013; Cannon et al., 2019). To make such media suitable for plant cultivation, the use of organic soil amendments, such as monogastric manure, could be hypothesized. The use of monogastric manure is based on the concept that this organic matter would be more similar to crew excrement, which after properly treated (Harder et al., 2019; Krounbi et al., 2019; Moya et al., 2019) could be adopted to improve the physicochemical characteristics of regolith. Another important aspect is the species selection for food production in BLSS. The selection of candidate crops was usually carried out using specific criteria such as nutritional value, plant size, adaptability to extreme environmental conditions, low resource requirements, short crop cycle and high harvest index (Kuang et al., 2000; Chunxiao and Hong, 2008; Wheeler, 2017; Meinen et al., 2018; El-Nakhel et al., 2019). Among the various candidate species, lettuce (*Lactuca sativa* L.) is highly ranked, as it is a fast-growing leafy vegetable with a high harvest index (>0.9) (Dueck et al., 2016). In addition, its leaves are rich in mineral elements, dietary fiber, carotenoids, vitamin C, and phenolic compounds (Caporale et al., 2014; El-Nakhel et al., 2019; Pannico et al., 2019; El-Nakhel et al., 2020b), which are sorely needed especially for space crews subjected to severe oxidative and inflammatory stresses during space missions (Bates et al., 2009; Kyriacou et al., 2017; Goodwin and Christofidou-Solomidou, 2018; NASA, 2021).

To date, very few works have evaluated the plant cultivation on Martian or Lunar soil simulants (Gilrain et al., 1999; Mortley et al., 2000; Wamelink et al., 2014) and among them only Gilrain et al. (1999), Onsay et al. (2019) and Duri et al. (2020) have adopted different simulant and compost ratios, without any of the abovementioned studies having addressed the effect of manure amendment. Therefore, the purpose of the present work was to evaluate the response in terms of nutraceutical properties of a widely used crop such as lettuce to soil amendment, by testing mixtures at different percentages of extra-terrestrial simulant and monogastric manure. The main aim was to evaluate if manure amendment can compensate the defects of extra-terrestrial regolith, as established in the ISRU approach, to find the best mix that can maximize the nutritional value of lettuce while ensuring an acceptable yield.

2 Materials and Methods

2.1 Plant material, growth chamber condition, and experimental treatments

A pot experiment was carried out in a walk-in open-gas-exchange climate chamber with lettuce plants (*Lactuca sativa* L. cv.'Grand Rapids') grown in different mixtures of extra-terrestrial soil simulants and monogastric manure. Two different simulants were tested: the Mojave Mars Simulant (MMS-1), purchased from The Martian Garden (Austin, Texas, USA) and the Lunar Highlands Simulant (LHS-1) purchased from Exolith Lab (University of Central Florida, Orlando, Florida, USA). Both simulants are coarse-textured alkaline (pH 9 to 10) substrates consisting mostly of plagioclase (anorthite) and amorphous Al and Fe minerals. Although they can be a source of available nutrients, such as Ca, Mg and K, they lack organic C, N, and available P and S, essential for plant growth. As well, they easily release soluble Na, which can induce salt stress in the plants (Caporale et al., 2022b, submitted). The tested treatments were prepared after mixing each simulant with sieved (2 mm) horse/swine monogastric manure (Agraria Di Vita srl, Pescia, Pistoia, Italy) at doses of 0, 10, 30, and 50% (w/w). This manure, characterized by a low C/N ratio, can provide a significant amount of potentially available N for rhizosphere microorganisms and plant roots. At the same time, according to the medium-low H/C value, it would comprise a significant aromatic moiety, ensuring a good stability of the organic matter over time. It is also an important source of nutrients, however it contains significant amounts of Na, which negatively raise its pH to 9.0 and E.C. to ~7 dS m^{-1} (Caporale et al., 2022b, submitted). Plants were transplanted into 9 x 9 x 9 cm pots and arranged in the growth chamber under a day/night air temperature and air relative humidity regime of 22/18°C and 60/80%, respectively, establishing a 16-8 h(light/dark) photoperiod. Growth chamber lighting was provided by high-pressure sodium lamps (Master SON-T PIA Plus 400W, Philips, Eindhoven, The Netherlands) with a photosynthetic photon flux density (PPFD) at the canopy level of 400 ± 10 μmol m^{-2} s^{-1}. The experiment was conducted at ambient CO_2 concentration (370-410 ppm), while air exchange and dehumidification were provided by two HVAC systems. Lettuce plants were irrigated with only reverse osmosis water throughout the crop cycle (31 days).

The experimental design was laid out according to the randomized complete-block factorial design with four manure amendment rates (M) and two extraterrestrial soil simulants (S), with three replicates. Each experimental plot consisted of four plants (total of 96 plants).

2.2 Sample preparation, nitrate and organic acids analysis

At harvest, the fresh biomass (g $plant^{-1}$) of all plants for each treatment was determined. Fresh samples from each plant were split into two subsamples; one of them was instantly frozen in liquid nitrogen, lyophilized and stored at -80°C for further phytochemical analyses, while the remaining sample was used for water content determination after drying in forced-air oven at 70°Cto constant weight (around 72 h). Oven dried samples were then ground with a cutting-grinding head mill at 0.5 mm (IKA, MF 10.1, Staufen, Germany) and used for nitrate and organic acid content determinations (Malate, Tartrate, Oxalate, Citrate and Isocitrate). In brief, 250 mg of ground leaf tissue were suspended in 50 mL of ultrapure water(Arium® Advance EDI pure water system; Sartorius, Goettingen, Lower Saxony, Germany), stirred in shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80°C for 10 minutes, filtered at 0.45 μm and finally analyzed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) as described by Pannico et al.(2019).

2.3 Analysis of Antioxidant activity

For antioxidant activity,200 mg of lyophilized material was extracted with 5mL of methanol (stored at 4°C) and then centrifuged at 400 rpm for 5 min.The supernatant was collected and re-centrifugedafter adding a further 5 mL methanol to the pellet. Two different assays were carried out for antioxidant activity determination: the ABTS-scavenging capacity based on the method described by Re et al.(1999); and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity using the procedure proposed by Brand-Williams et al.(1995) modified, briefly detailed as follow.

For ABTS assay, a stock solution was incubated at temperature of 4°Cfor 16 h (2.5 mL of aqueous ABTS-7 mM and 44 mL of potassium persulfate-2.45 mM). Then this stock solution was diluted (1:88) with ethanol having an absorbance of 0.700 ± 0.050 at 734 nm. The analysis was performed combining0.1 mL of filtered sample and 1 mL of ABTS radical working solution and then monitoring the absorbance after 2.5 min at 734nm). For DPPH assay,1 mL of methanolic $DPPH^{\cdot}$100 µM was added to 200 µL of diluted lettuce extract. The absorbance of DPPH was 0.90 ± 0.02 at 517 nm, while the decrease in absorbance of the resulting solution was monitored after 10 min of incubation at room temperature in the dark at 517 nm.

All determinations were performed in triplicate and the results were expressed as Trolox® equivalent antioxidant capacity (TEAC, mmol Trolox® equivalents kg^{-1} dw).

2.4 Extraction and preparation for carotenoids and phenolic profile assays

The freeze-dried lettuce samples (100 mg) were extracted modifying the method of Kim et al.(2008) as follows. In particular, the samples were mixed in 6 mL of ethanol containing 0.1% BHT, and placed in water bath for 5 min at 85°C.Then, 120 µL of 80% KOH was added to samples and subsequently they were vortexed and returned to water bath for 10 min at the same temperature. At the end, they were placed in ice to stop the reaction and in each solution were added 3 mL of distilled water and 3 mL of hexane. Subsequently, centrifugation was applied to collect the hexane layer and the pellet was re-extracted twice more using hexane. Finally, after all the extraction procedures the hexane layers were combined and dried with nitrogen gas. 1 mL of chloroform was added to recover the residue and filtered with nylon filter (0.2 µm) before the quantification by HPLC-DAD. For the quantification, a Shimadzu HPLC Model LC 10 (Shimadzu, Osaka, Japan) was used, equipped with a reverse phase 250 mm × 4.6 mm, 5 µm Gemini C18 column (Phenomenex, Torrance, CA, USA), after injecting 20 µL of sample. The following A:B gradient: 0–8 min (82:18); 8–12 min (76:24); 12–18 min (39:61); and 18–25 min a linear gradient to equilibration (82:18), was prepared using acetonitrile and ethanol:n-hexane:dichloromethane (1:1:1) (respectively for mobile phase A and B). The absorbance measurements were recorded at 450 nm and expressed in mg kg^{-1} dw. To perform the quantification, a linear calibration curves was carried out using lutein and β-carotene standards at 6 levels of concentration (from 5 up to 100 µg ml^{-1}).

100 mg of lyophilized sample was used for polyphenols quantification (expressing it as µg g^{-1} dw). The extraction procedure involved the use of 5mL of methanol and water solution (60:40, v/v) which was sonicated with the sample for 30 min. Then, the suspension was centrifuged (400 rpm) for 15 min, and filtered with filter paper (0.45 µm) using 10 Ml for mass spectrometry (HRMS-Orbitrap) analysis. UHPLC system (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Dionex Ultimate 3000 Quaternary pump performing at 1250 bar and a thermostated (25°C) Kinetex 1.7 µm biphenyl (100 mm × 2.1 mm) column (Phenomenex, Torrance, CA, USA) was used to carry out the polyphenols determination assay. A volume of 2 µL was injected, using a flow rate of 0.2 mL $min^{-1}$to elute and using 0.1% formic acid in water and 0.1% formic acid in methanol, respectively, to prepare a gradient of A and B in the following way: 0 min-5% B, 1.3 min-30% B, 9.3 min-100% B, 11.3 min-100% B, 13.3 min-5% B, and 20 min-5% B. A Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) was involved to mass spectrometry analysis. An ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) in negative ion mode (ESI-) was used. The following ion source parameters were applied: -2.8 kV spray voltage, sheath gas (N_2> 95%) 45, auxiliary gas (N_2> 95%) 10, capillary temperature 275°C, S-lens RF level 50, and auxiliary gas heater temperature 305°C. The polyphenolic compounds targeted acquisition was carried out on parallel reaction monitoring (PRM) mode, set as follow: microscans at 1, resolution at 35.000,

AGC target at 5e5, maximum ion time at 100 ms, MSX count at 1, isolation window at 1.0 m/z. Input time frame for elution and collision energy (CE) were optimized for each polyphenolic compound. The accuracy and calibration of the Q Exactive Orbitrap LC-MS/MS was checked using a Thermo Fisher Scientific reference standard mixture and setting the mass tolerance window for the two analysis modes at 5 ppm. The linearity of the method both low and high (5 mg kg^{-1}-120 mg kg^{-1}) concentration ranges was assessed using six concentration levels in each calibration range. The low limit of detection (LOD) and low limit of quantitation (LOQ) values for HPLC-DAD analysis of carotenoids were determined for β-carotene, while in the case of LC-MS/MS analysis of polyphenols were based on chlorogenic acid and rutin signal-to-noise levels. LOD and LOQ for each compound were obtained by serial dilutions of stock solution, and the analysis and processing of data were performed using the X calibur software, v. 3.0.63 (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 Statistical Analysis

Data were subjected to two-way ANOVA using IBM SPSS software package (SPSS Inc., Chicago, Illinois, USA). The mean effects of simulants (S) and manure amendment (M) factors were compared according to the unpaired Student's t-Test and one-way analysis of variance, respectively. Significant statistical differences were determined by Tukey-KramerHSD test for the S factor and the S x M interaction at the level of $p \leq 0.05$.

3 Results

3.1 Fresh biomass, nitrate, and organic acids content

A statistically significant interaction was observed between simulant (S) and manure percentage (M) factors, with the highest value recorded in the treatment where MMS-1 was combined with 30% manure; while the Lunar simulant (LHS-1) had the highest yields in the intermediate treatments (10 and 30% of manure) (Figure 1). Moreover, regardless of the amendment treatment, lettuce fresh biomass produced on MMS-1 simulant was on average 3.7 fold higher than on LHS-1 indicating that the specific simulant is more appropriate for lettuce cultivation than LHS-1 substrate.

The simulant mean effect showed significant differences for water, nitrate, malate, citrate and isocitrate content, with the highest values obtained in MMS-1 except for nitrate content which was significantly lower compared to Lunar simulant (-22%) (Table 1). The water content showed a direct correlation with the percentage of amendment (R=0.89) presenting a 17% increase in the 50% manure treatments compared to the non-amended simulants (Table 1). A statistically significant interaction of the two factors (S x M) was observed in the content of all detected organic acids. There was a significantly higher malate, tartrate, and oxalate content in the 50% MMS-1 mixture compared to the rest of the treatments where an increase by 486%, 217% and 67% was recorded, respectively; however, the citrate content did not differ between the various combination of simulants and manure rates (Table 1).

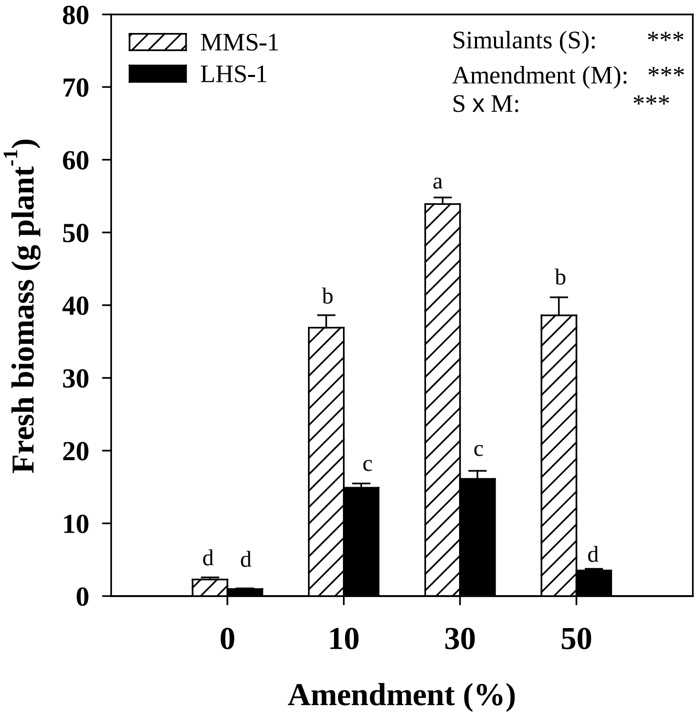

Figure 1. The effect of growth substrate composition on fresh biomass production (g plant^{-1}) of lettuce plants. Different letters above bars indicate significant mean differences according to Tukey's multiple range tests ($P \leq 0.05$). Vertical bars indicate ± SE of means. *** Significant at $P \leq 0.001$.

Regarding the Lunar simulant, malate and tartrate contents were significantly higher (on average +275% and +130%, respectively) in the amended mixtures than in the pure LHS-1.

On the other hand, the isocitrate content was significantly higher in the non-amended simulant and 50% amendment than in the 10% and 30% manure treatment; in contrast, the citrate content was significantly lower in the non-amended LHS-1 compared to the 30% amended mixture (Table 1). Finally, the highest content of individual and total organic acids (except for the case of citrate where no significant differences were observed) was recorded for the highest rate (50%) of manure, regardless of the simulant, while MMS-1 had higher amounts of malate, citrate and isocitrate than the LSH-1 simulant, regardless of the manure rate (Table 1).

Table 1. Water content, nitrate, and organic acids content of lettuce as influenced by growth substrate composition

Source of Variance		Water Content (%)	Nitrate mg kg^{-1} fw	Malate	Tartrate	Oxalate g kg^{-1} dw	Citrate	Isocitrate
Simulants (S)	MMS-1	84.1 ± 1.83 a	12.49 ± 0.58 b	25.36 ± 4.13 a	3.15 ± 0.38	0.77 ± 0.05	12.64 ± 0.28 a	0.268 ± 0.012 a
	LHS-1	81.9 ± 2.21 b	15.91 ± 1.02 a	20.26 ± 2.71 b	2.80 ± 0.27	0.76 ± 0.02	11.81 ± 0.35 b	0.234 ± 0.012 b
Amendment (%) (M)	0	72.3 ± 1.13 d	14.59 ± 1.19	7.05 ± 0.33 d	1.48 ± 0.09 c	0.73 ± 0.04 bc	12.05 ± 0.64	0.265 ± 0.006 ab
	10	83.1 ± 0.63 c	13.19 ± 1.45	20.63 ± 0.86 c	2.95 ± 0.21 b	0.68 ± 0.03 c	12.12 ± 0.39	0.241 ± 0.029 bc
	30	86.8 ± 0.62 b	13.33 ± 1.15	27.96 ± 1.47 b	3.37 ± 0.11 b	0.79 ± 0.02 ab	12.68 ± 0.48	0.220 ± 0.008 c
	50	89.7 ± 0.73 a	15.68 ± 1.66	35.59 ± 4.58 a	4.12 ± 0.41 a	0.85 ± 0.08 a	12.06 ± 0.42	0.278 ± 0.010 a
S x M	MMS-1x0	74.7 ± 0.17	12.74 ± 1.83	7.48 ± 0.44 c	1.54 ± 0.18 c	0.65 ± 0.01 cd	13.30 ± 0.63 ab	0.259 ± 0.009 abc
	MMS-1x10	83.5 ± 0.32	11.75 ± 0.86	20.71 ± 1.35 b	3.04 ± 0.44 b	0.61 ± 0.01 d	12.46 ± 0.62 ab	0.303 ± 0.014 a
	MMS-1x30	87.2 ± 0.76	12.62 ± 0.90	29.37 ± 2.87 b	3.15 ± 0.06 b	0.78 ± 0.04 bc	11.98 ± 0.57 ab	0.214 ± 0.009 cd
	MMS-1x50	90.9 ± 0.94	12.85 ± 1.50	43.86 ± 3.98 a	4.89 ± 0.33 a	1.02 ± 0.06 a	12.83 ± 0.36 ab	0.297 ± 0.006 a
	LHS-1x0	69.9 ± 0.80	16.44 ± 0.57	6.62 ± 0.39 c	1.42 ± 0.04 c	0.80 ± 0.02 b	10.80 ± 0.35 b	0.272 ± 0.005 ab
	LHS-1x10	82.8 ± 1.33	14.64 ± 2.78	20.55 ± 1.35 b	2.86 ± 0.17 b	0.75 ± 0.02 bcd	11.79 ± 0.51 ab	0.179 ± 0.010 d
	LHS-1x30	86.4 ± 1.09	14.05 ± 2.29	26.55 ± 0.81 b	3.59 ± 0.12 b	0.81 ± 0.02 b	13.39 ± 0.57 a	0.225 ± 0.015 bcd
	LHS-1x50	88.4 ± 0.48	18.52 ± 1.88	27.31 ± 4.54 b	3.36 ± 0.35 b	0.68 ± 0.03 bcd	11.29 ± 0.37 ab	0.258 ± 0.009 abc
Significance	S	**	*	**	ns	ns	*	***
	M	***	ns	***	***	***	ns	***
	SxM	ns	ns	*	**	***	**	***

ns,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Cultivar means were compared by *t*-test. Substrate mixture means and interaction were compared by Tukey's multiple-range test ($P = 0.05$). Different letters within each column indicate significant differences

3.2 Antioxidant activity

A significant interaction of the two factors (S x M) was observed for ABTS and DPPH assays, where the non-amended simulants had the highest Trolox content in the case of ABTS, while the non-amended simulants and the combination of MMS-1 x 50% of manure and LSH-1 x 30% of manure recorded the highest activity in DPPH assay (Table 2). The simulant mean effect (S) showed significantly higher ABTS and DPPH assays in lettuce grown on LHS-1 (+3.2% and +10.7%, respectively) compared with those on MMS-1 (Table 2). Similarly, the non-amended simulants showed the highest activity in all the studied assays.

Table 2. Antioxidant activity of lettuce as influenced by growth substrate composition.

Source of Variance		ABTS	DPPH
		mmol trolox kg^{-1}	
Simulants (S)	MMS-1	74.97 ± 3.9 b	56.17 ± 2.1 b
	LHS-1	77.38 ± 4.1 a	62.17 ± 1.3 a
Amendment (%) (M)	0	97.95 ± 1.3 a	66.40 ± 1.6 a
	10	66.46 ± 3.0 c	54.20 ± 2.6 c
	30	68.40 ± 0.7 bc	57.03 ± 2.9 bc
	50	71.90 ± 1.8 b	59.04 ± 0.6 b
S x M	MMS-1x0	95.44 ± 1.1 a	64.81 ± 1.9 ab
	MMS-1x10	60.80 ± 1.6 d	48.90 ± 1.8 d
	MMS-1x30	67.80 ± 1.0 cd	50.89 ± 2.3 cd
	MMS-1x50	75.86 ± 0.7 b	60.07 ± 0.4 ab
	LHS-1x0	100.4 ± 0.9 a	67.99 ± 2.6 a
	LHS-1x10	72.13 ± 3.0 bc	59.50 ± 1.3 b
	LHS-1x30	69.00 ± 1.1 bc	63.16 ± 0.5 ab
	LHS-1x50	67.95 ± 0.6 c	58.02 ± 0.6 bc
Significance	S	*	***
	M	***	***
	SxM	***	**

*,**, *** Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Cultivar means were compared by *t*-test. Substrate mixture means and interaction were compared by Tukey's multiple-range test (P = 0.05). Different letters within each column indicate significant differences

3.3 Carotenoids content

The mean effect of simulants (S) showed a significantly higher concentration of lutein in LHS-1 (236 mg kg^{-1}) compared to MMS-1 (200 mg kg^{-1}), whereas no significant difference was detected in β-carotene content. For both carotenoids, a direct correlation was observed between their content and the amendment percentage of the different mixtures (R>0.97). Moreover, a significant interaction of SxM was found in both carotenoids reaching the highest content at the 50% manure dose (Figure 2). Specifically, the Martian simulant recorded an increase in lutein and β-carotene content at 30% (+78.7% and +141%, respectively) and 50% (+181% and +263%, respectively) amendment compared to non-amended MMS-1 (Figure 2). Regarding LHS-1, the lutein content is on average 132% higher at the two intermediate mixtures and 245% higher at the maximum manure dose with respect to the non-amended simulant. Similarly, the β-carotene content in the Lunar simulant was significantly higher by 206% and 287% at 10% and 50% amendment treatments, respectively, while its concentration at the 30% manure dose was between the latter two mixtures (Figure 2).

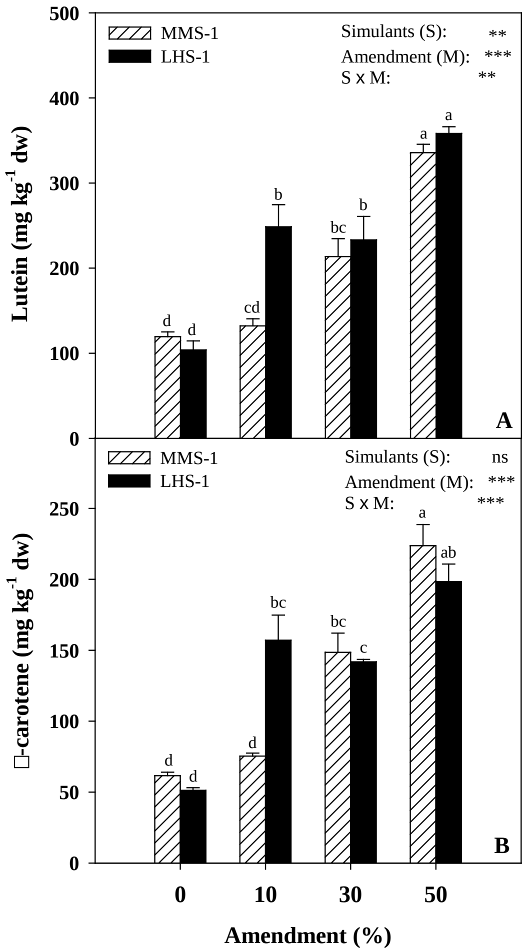

Figure 2. The effect of growth substrate composition on carotenoids content (mg kg^{-1} dw) of lettuce plants. Different letters above bars indicate significant mean differences according to Tukey's multiple range tests ($P \leq 0.05$). Vertical bars indicate ± SE of means. ns,**, *** Non-significant or Significant at $P \leq 0.01$ and 0.001, respectively.

3.4 Phenolic compounds profile and total phenolics composition

Chlorogenic acid was the most prevalent compound among all detected hydroxycinnamic acids, followed by ferulic acid, feruloyl-disinapoyl-gentionbiose and synapoyl-hexose (Table 3a). A significant interaction of SxM factors was found for coumaroyl-diglucoside, ferulic acid, feruloyl-disinapolyl-gentionbiose and synapoyl-hexose. Specifically, coumaroyl-diglucoside and feruloyl-disinapolyl-gentionbiose were the highest in the non-amended MMS-1 simulant, while the content of ferulic acid and feruloyl-disinapoyl-gentionbiose varied among the treatments. The ferulic acid was significantly higher in the pure MMS-1 compared to the 10% and 50% manure mixtures. In contrast, synapoyl-hexose was significantly higher in the 30% and 50% manure Lunar simulant than in pure LHS-1. The mean effect of simulants (S) was significant for coumaroyl-diglucoside and synapoyl-hexose content which was the highest in MMS-1, whereas the opposite trend was found for ferulic acid (highest content in LSH-1 simulant). The mean effect of the amendment (M) revealed an inverse correlation between chlorogenic acid concentration and manure dose in the different mixtures, reaching a 20.7% reduction at the highest amendment percentage compared to the non-amended simulant. The same trend was also observed for coumaroyl-diglucoside, feruloyl-disinapoyl-gentionbiose and total hydroxycinnamic acids, whereas the opposite trend was recorded for synapoyl-hexose which increased in the amended simulant compared to the non-amended one (Table 3a).

Regarding the flavonoids profile, no significant interactions SxM were found in flavonoids as well as total flavonoids and total phenols content. Moreover, the mean effect of simulants (S) showed a significantly higher concentration in the case ofkaempferol-3-diglucoside, quercetin3-sophoroside-7-glucoside and rutin in LHS-1 compared toMMS-1 simulant (Table 3b). Interestingly, the mean effect of the amendment (M) revealed a progressive decrease in the content of most of the detected compounds when manure was added in the tested simulants. The same trend was also observed for total flavonoids and total phenols content, resulting in a reduction at the maximum manure dose of 53.0% and 23.3%, respectively, compared to the non-amended simulant.

Table 3a. Phenolic profiles and total phenolic compositionof lettuce as influenced by growth substrate composition

Source of Variance		chlorogenic acid	coumaroyl-diglucoside	ferulic acid	feruloyl-disinapoyl-gentionbiose	Synapoyl-hexose	Total hydroxycinnamic acids
		μg g^{-1} dw					
Simulants (S)	MMS-1	2545 ± 67	0.155 ± 0.034 a	45.41 ± 2.59 b	6.77 ± 0.33	8.16 ± 0.42 a	2605 ± 66.8
	LHS-1	2639 ± 87	0.083 ± 0.012 b	55.67 ± 4.26 a	6.85 ± 0.49	6.94 ± 0.38 b	2708 ± 90.2
Amendment (%) (M)	0	2951 ± 81 a	0.247 ± 0.045 a	59.57 ± 8.18 a	8.92 ± 0.30 a	6.54 ± 0.29 b	3027 ± 89.0 a
	10	2583 ± 47 b	0.067 ± 0.003 b	41.31 ± 1.89 c	5.67 ± 0.25 c	8.21 ± 0.29 a	2638 ± 46.0 b
	30	2492 ± 56 bc	0.078 ± 0.015 b	55.86 ± 3.24 ab	6.77 ± 0.10 b	8.42 ± 0.68 a	2563 ± 53.0 bc
	50	2341 ± 48 c	0.084 ± 0.012 b	45.40 ± 2.28 bc	5.89 ± 0.21 c	7.04 ± 0.78 ab	2400 ± 47.7 c
S x M	MMS-1x0	2817 ± 102	0.343 ± 0.026 a	43.60 ± 4.97 b	8.34 ± 0.33 a	6.04 ± 0.40 bc	2875 ± 106
	MMS-1x10	2542 ± 94	0.061 ± 0.001 cd	41.35 ± 3.61 b	5.52 ± 0.42 cd	8.56 ± 0.15 ab	2598 ± 91.1
	MMS-1x30	2528 ± 70	0.106 ± 0.016 bc	55.09 ± 5.89 ab	6.89 ± 0.16 b	9.33 ± 0.76 a	2600 ± 64.8
	MMS-1x50	2292 ± 73	0.111 ± 0.001 bc	41.59 ± 3.31 b	6.35 ± 0.10 bcd	8.72 ± 0.22 a	2348 ± 70.1
	LHS-1x0	3086 ± 68	0.151 ± 0.007 b	75.54 ± 7.43 a	9.50 ± 0.13 a	7.03 ± 0.08 abc	3178 ± 73.7
	LHS-1x10	2623 ± 28	0.073 ± 0.002 cd	41.28 ± 2.21 b	5.82 ± 0.33 bcd	7.86 ± 0.53 abc	2678 ± 26.3
	LHS-1x30	2455 ± 96	0.049 ± 0.000 d	56.63 ± 4.15 ab	6.65 ± 0.09 bc	7.51 ± 0.95 abc	2526 ± 92.0
	LHS-1x50	2391 ± 61	0.058 ± 0.006 cd	49.22 ± 0.65 b	5.44 ± 0.11 d	5.36 ± 0.38 c	2451 ± 61.9
Significance	S	ns	***	**	ns	**	ns
	M	***	***	**	***	**	***
	SxM	ns	***	**	**	**	ns

ns,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Cultivar means were compared by *t*-test. Substrate mixture means and interaction were compared by Tukey's multiple-range test ($P = 0.05$). Different letters within each column indicate significant differences

Table 3b. Phenolic profiles and total phenolic composition of lettuce as influenced bygrowth substrate composition

Source of Variance		hyperoside	km 3-diglucoside	kaempferol-3-glucoside	quercetin-3-glucoside	qn 3-sophoroside- 7-glucoside	rutin	luteolin-7-O-glucoside	Total flavonoids	Total phenols
						μg g^{-1} dw				
Simulants (S)	MMS-1	158.8 ± 16.4	3.43 ± 0.5 b	1.45 ± 0.06	17.2 ± 1.7	0.690 ± 0.143 b	3.2 ± 0.4 b	1.63 ± 0.07	186 ± 19	2792 ± 85
	LHS-1	168.5 ± 11.1	4.06 ± 0.6 a	1.36 ± 0.09	18.0 ± 1.2	0.854 ± 0.141 a	3.9 ± 0.5 a	1.60 ± 0.14	198 ± 13	2907 ± 103
Amendment (%) (M)	0	221.5 ± 5.7 a	6.54 ± 0.4 a	1.29 ± 0.13	23.8 ± 0.6 a	1.503 ± 0.102 a	6.2 ± 0.4 a	1.41 ± 0.14	262 ± 6 a	3289 ± 92 a
	10	165.9 ± 11.4 b	3.00 ± 0.2 b	1.46 ± 0.03	17.8 ± 1.1 b	0.720 ± 0.057 b	2.8 ± 0.1 b	1.67 ± 0.04	193 ± 13 b	2831 ± 58 b
	30	163.8 ± 6.9 b	3.03 ± 0.2 b	1.33 ± 0.09	17.4 ± 0.8 b	0.548 ± 0.095 bc	2.9 ± 0.2 b	1.52 ± 0.11	191 ± 8 b	2753 ± 58 bc
	50	103.4 ± 11.7 c	2.41 ± 0.2 b	1.56 ± 0.12	11.5 ± 1.1 c	0.315 ± 0.019 c	2.4 ± 0.2 b	1.84 ± 0.25	123 ± 13 c	2523 ± 58 c
S x M	MMS-1x0	220.9 ± 10.7	5.97 ± 0.5	1.52 ± 0.13	23.8 ± 1.2	1.473 ± 0.099	5.6 ± 0.4	1.64 ± 0.15	261 ± 12	3136 ± 118
	MMS-1x10	163.5 ± 24.8	2.87 ± 0.2	1.50 ± 0.05	17.7 ± 2.4	0.622 ± 0.022	2.7 ± 0.2	1.63 ± 0.06	190 ± 28	2788 ± 119
	MMS-1x30	168.3 ± 14.5	2.82 ± 0.2	1.24 ± 0.12	17.8 ± 1.7	0.339 ± 0.026	2.7 ± 0.2	1.50 ± 0.15	195 ± 17	2794 ± 78
	MMS-1x50	82.47 ± 7.8	2.06 ± 0.1	1.56 ± 0.10	9.7 ± 0.9	0.325 ± 0.002	2.0 ± 0.1	1.75 ± 0.18	100 ± 9	2448 ± 70
	LHS-1x0	222.1 ± 6.9	7.12 ± 0.5	1.06 ± 0.13	23.9 ± 0.6	1.534 ± 0.202	6.8 ± 0.4	1.19 ± 0.13	264 ± 7	3442 ± 72
	LHS-1x10	168.4 ± 5.0	3.12 ± 0.2	1.43 ± 0.01	18.0 ± 0.5	0.819 ± 0.078	3.0 ± 0.2	1.71 ± 0.04	197 ± 5	2874 ± 28
	LHS-1x30	159.3 ± 2.1	3.23 ± 0.4	1.42 ± 0.13	17.0 ± 0.3	0.756 ± 0.022	3.1 ± 0.3	1.55 ± 0.19	186 ± 3	2712 ± 95
	LHS-1x50	124.3 ± 13.8	2.76 ± 0.3	1.55 ± 0.24	13.3 ± 1.2	0.306 ± 0.040	2.8 ± 0.3	1.92 ± 0.51	147 ± 16	2598 ± 78
Significance	S	ns	*	ns	ns	*	**	ns	ns	ns
	M	***	***	ns	***	***	***	ns	***	***
	SxM	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Cultivar means were compared by *t*-test. Substrate mixture means and interaction were compared by Tukey's multiple-range test ($P = 0.05$). Different letters within each column indicate significant differences

4 Discussion

The present study evaluates the possibility of using human excreta (replaced by a surrogate derived from monogastric farm animals) as a soil amendment to improve the physicochemical and structural characteristics of Martian or Lunar regolith for plant production, in preparation fora future manned mission to Mars or Moon, respectively. To improve ISRU protocols and minimize procurement from the Earth, the impact of the amendment rate on the nutritional and functional characteristics of lettuce was evaluated. Although this vegetable provides only limited amount of calories, it is nevertheless a rich source of bioactive compounds, such as carotenoids (mainly lutein and β-carotene) and polyphenols, which counteract the development of chronic diseases and could be helpful to maintain health in space missions (Kim et al., 2016). A long-term space mission inevitably results in a progressive decline in astronauts' mental and physical performance (Bychkov et al., 2021). In this context, dietary supplementation with fresh vegetables rich in nutraceuticals possessing high antioxidant activity could on the one hand minimize pathophysiological effects (Smith et al., 2019; Bychkov et al., 2021) and on the other hand help maintain the mental well-being of individuals forced to live in isolated or extreme environments (Bates et al., 2009; Duri et al., 2022). Considering the above, two commercial simulants (a Martian and a Lunar simulant) were amended with different ratios of manure from monogastric animals aiming to evaluate the possible effects on lettuce cultivation. According to the literature, previous experiment with human excreta as plant fertilizers gave promising results in terms of crop performance and acceptability from farmers (Moya et al., 2019). Therefore, the goal of this experiment was to test whether it is possible to improve the physicochemical parameters of Martian and Lunar simulants through the manure amendment and extrapolate the obtained results to cultivation of lettuce in space conditions using human excreta as soil amendment.

Regardless of treatments, the low yield recorded was attributable to a severe nutritional deficiency as excluding the limited input by the manure, both regoliths simulants were highly lacking in key macronutrients and organic matter (Seiferlin et al., 2008; Caporale et al., 2022, submitted). The higher fresh biomass obtained in plants grown on the Martian simulant could be due to the worse physico-chemical characteristics of the Lunar substrate, such as the lower water retention capacity and higher pH, and/or the higher ammonium nitrate content of the Martian regolith, as assessed in a complementary study - part1 (Caporale et al., 2022, submitted) and discussed aswell by Wamelink et al. (2014). By analyzing the interaction between the tested factors (S x M), the yield reduction observed in plants grown on the substrates containing 50% of manure, especially in the case of LHS-1 simulant, could be due to the increase in electrical conductivity of the substrates by the higher manure content (Duggan and Jones, 2016). This trend was also analyzed and discussed in a complementary study - part 2 (Caporale et al., 2022a, submitted), which assessed the suitability of these eight MMS-1 or LHS-1/manure mixtures for space food production, by matching their physicochemical and hydraulic characteristics with the lettuce growth performance (biometric and physiological parameters), soil enzymatic activity and nutrient bioavailability in the growth media at plant harvest time.

According to Wamelink et al. (2014), the better performance of Martian simulant compared to the Lunar one could be due to better water holding capacity, however the increased rates of manure (>30%) probably exaggerated the water content and had negative effects on lettuce plants growth in both simulants. Similar results were also observed by Petropoulos et al. (2020a) who also associated the differences in water content of lettuce plants to the differences in water holding capacity of the substrates tested. The results of our study are in accordance with the findings of Duri et al. (2020) who also suggested that the amendment of Martian regoliths with 30% of compost was the most realistic in terms of crop performance and compost availability in space conditions, while the 30:70 (Martian regolith:compost) gave the best overall results. Caporale et al. (2020) also suggested the use of 30% of green compost due to larger leaf area and better pore size distribution. However, Duri et al. (2020) also suggested that a genotype dependent response was observed which could justify the differences with our study. Moreover, the fact the abovementioned studies used composts as soil amendments instead of manure could also explain the differences in observed results due to the difference in the physico-chemical properties of the tested amendments. Therefore, it seems the intermediate amounts of manure are the most beneficial since they not only increase the amounts of water that simulants may hold but also increase nutrients availability and retain pH and EC values at acceptable levels for efficient plant growth.

The percentage of manure amendment in the substrates also affected the plants antioxidant activity, showing a clear response to nutritional stress, confirming the findings observed in a previous work (Duri et al., 2020). The non-amended simulants showed the highest antioxidant activity in the case of ABTS assay, while the response to DPPH varied among the tested treatments. Moreover, in all the assays the non-amended simulants had the highest antioxidant activity, regardless of the simulant which further justifies our previous argument regarding the stressful conditions that lettuce plants were subjected to when grown in non-amended substrates. Our hypothesis was also supported by the phenolic acid, flavonoids and total phenols content, since the biosynthesis of these secondary metabolites tends to increase as a response to plant stressors (Dai and Mumper, 2010), while their content is strongly dependent on genotype and agronomic conditions (Zhao et al., 2007; Ordidge et al., 2010). Moreover, the higher ABTS and DPPH values recorded in lettuces grown on LHS-1 mixtures were consistent with the lower fresh biomass yield for the same treatment, confirming the high stress exerted by the Lunar simulant.

In agreement with Kim et al.(2016) the most abundant phenolic acid in lettuce in our study was chlorogenic acid, which indicates that phenolic profile is highly associated with the genotype (El-Nakhel et al., 2020a, 2020b). Moreover, total phenolic acids, total flavonoids and total phenols content was the highest in non-amended simulants which was also reported by Duri et al. (2020) for Red Salanova lettuce plants, whereas the same authors did not record significant differences in total phenols content for Green Salanova plants at the tested rates of simulant:compost. This fining indicates that genotype is also important for plant response to abiotic stressors and various lettuce genotypes should be tested in future studies to make safe conclusions about the potential of cultivating lettuce in Martian or Lunar soils and the possibility to use human excreta as soil amendments.

Nitrate content in leaf tissues is strongly influenced by soil nitrate and ammonium levels (Nazaryuk et al., 2002); nevertheless, in our experiment, the amendment dose did not significantly affect the foliar concentration of this anion. Regarding the effect of the simulant, the lower NO_3 content recorded in MMS-1 is probably due to a dilution effect triggered by both the higher fresh biomass and the greater water content of plants grown on this simulant compared to LHS-1. Very low nitrate levels (on a fresh basis) have also been found in lettuce (Pannico et al., 2020) and *Brassicaceae* (El-Nakhel et al., 2021b) grown under under severe nutrient deficit. However, it seems that nitrate content is also highly depended on the genotype since according to El-Nakhel et al. (2019a) significant differences between two Salanova lettuce cultivars (Green and Red Salanova) showed a different response to nitrate accumulation when grown under controlled conditions. It is also important to facilitate optimum growth conditions, especially regarding light intensity, since leafy vegetables tend to increase its content when grown under suboptimal light conditions (Petropoulos et al., 2011; Rouphael et al., 2019).

Manures have been shown to improve the water retention capacity of substrates (Zhang et al., 2021; Duri et al., 2022). These findings corroborate the higher water content of lettuce plants recorded at high manure doses, probably due to increased water availability as the percentage of amendment increases (Caporale et al., 2022b, submitted). The dose of manure in regolith mixtures also induced an adaptive response by the plants, which resulted in an accumulation of organic acids. These metabolites are involved in different biochemical pathways at cell level, as intermediates of photosynthesis and amino acids biosynthesis (López-Bucio et al., 2000; Osmolovskaya et al., 2018) or as osmoregulators and cell protectors against stress conditions (Petropoulos et al., 2020b). In our experiment, the increased content of malate, tartrate, oxalate, and isocitrate in the 50% substrates is likely due to higher salt stress, as observed in previous work on lettuce (El-Nakhel et al., 2021a) and other leafy vegetables (Petropoulos et al., 2020a). Moreover, the content of specific organic acids such as oxalates is also associated with nitrogen availability and nitrogen form and an increase of oxalates should be expected with increasing nitrogen availability (Petropoulos et al., 2018, 2020c), as was also the case of high amendment rates in our study. The content of organic acids in vegetables not only affects their taste, but also their acceptability, nutraceutical value, and shelf life (Poyrazoğlu et al., 2002; Ayaz et al., 2006; Rodríguez-Bernaldo de Quirós et al., 2010). In addition, they are beneficial to human health by acting as antioxidants due to their ability to chelate metals (Sánchez-Mata et al., 2012).

The increase in lutein and β-carotene achieved in plants grown on the 50:50 mixtures was consistent with findings on lettuce by Kim et al. (2016) following increasing doses of soil amendment. In the same line, Hernández et al. (2021) suggested that reduced nitrogen availability resulted in reduced amounts of carotenoids, whereas high salinity induced the biosynthesis of these compounds. High amounts of carotenoids such as lutein and β-carotene may play a protective role against oxidative stress since they act as reactive oxygen species scavengers and quenchers of free radicals (Rouphael et al., 2019). Although carotenoids content is highly associated with light conditions and light intensity in particular (Kołton et al., 2014; Ouzounis et al., 2015), the application of soil amendments from different sources may also increase their content and improve the quality of lettuce plants (Cruz et al., 2012) and their health beneficial effects (Kim et al., 2016). However, considering the genotypic variation in carotenoids content among the various lettuce genotypes, further studies with multiple genotypes are needed in order to identify those cultivars that could be used in space colonies as health promoting food sources (Mou, 2005).

5 Conclusions

The supplementation of bioactive compounds in astronaut's diets is undeniable, especially in an extreme and inhospitable habitat of future space settlements. The carotenoids content was positively correlated to the increment of monogastric manure in the growth substrate (+ 210% of lutein and + 273% of β-carotene). In contrast, the content of total phenols was lower in amended simulants than in pure ones, while the antioxidant activity was shown to be mainly related to the phenolic content. Our results indicate that the lettuce yields observed in the tested growth substrates are still not sufficient to ensure the self-sustainability of future space settlements. Exclusive input of pure water and manure does not appear to meet the minimum soil fertility requirements necessary to guarantee optimal crop development. However, it must be considered that pedogenesis is regulated by long processes that cannot be accomplished in a single lettuce cycle since, since the formation of fertile soil from disintegrated parental rock requires several chemical and physical alterations and continuous inputs of organic matter. Similarly, in a future extraterrestrial outpost it will be feasible to gradually improve the regolith fertility with repeated cropping cycles and continuous inputs of organic substances composed partly by crew excrements and partly by residues of previous crops. In addition, a start-up phase involving minimal nutrient supplementation and inoculation of plant growth-promoting rhizobacteria (PGPR) should be also investigated in future studies. In this regard, we can consider our results promising, as they demonstrated that by adding 30% of manure to pure regolith, it is possible to complete a lettuce cycle by feeding the plants with only pure water. However, further studies are needed with more lettuce genotypes to explore genotypic variation and make safe conclusions about the potential cultivation of the species in regoliths.

Overall conclusions

In recent years there is a growing interest in space exploration and the subsequent establishment of extraterrestrial colonies on the Moon or Mars. During the PhD period we assessed the influence of extraterrestrial soils (Lunar and Martian), through the use of simulants, on the morphological, physiological and nutritive performance of an important leafy vegetables such as lettuce (*Lactuca sativa* L.). The selection of candidate crop was usually carried out using specific criteria such as nutritional value, plant size, adaptability to extreme environmental conditions, low resource requirements, short crop cycle and high harvest index. Among the various candidate species, lettuce is highly ranked, as it is a fast-growing leafy vegetable with a high harvest index (>0.9). In addition, its leaves are rich in mineral elements, dietary fiber, carotenoids, vitamin C, and phenolic compounds, which are sorely needed especially for space crews subjected to severe oxidative and inflammatory stresses during space missions..

The study of the current literature about the potential use of Lunar and Martian simulants in plants cultivation (Chapter 1) highlighted, a scarce use in this field, and then surfaced that, generally, these simulants present several problems, like an alkaline pH, high Na amount, and low water holding capacity. However, the pure regolith simulants may be suitable media for plant growth for limited periods. Indeed, they perform as a source of nutrients such as K, Ca, Mg, and Fe; elements usually involved in many vital functions of plant life, ranging from root development and functionality, improving the nutrient uptake, and affecting the photosynthesis and the enzymes’ activity also. On the other hand, they are characterized by the abscence of organic matter as well as essential macronutrients such as N, P, and S, entailing lack in many plants' physiological and biochemical processes.

In many studies, the nutrient deficiency was bypassed using nutrient-rich solutions. But in space agriculture, this solution is not sustainable because resources should be delivered continuously from the Earth. The future colonization of extraterrestrial bodies can solely be realized via the adaptation of a BLSSs without umbilical dependence from Earth by using *in situ* resources as much as possible. One promising strategy to overcome this problem can be the reuse of organic waste materials, able to enhance the chemical and biological fertility and physical and hydraulic properties of regolith-based substrates, as well as reduce the inputs (as proposed by the ISRU approach)

Based on the above considerations, there is an urgent need to develop a regolith-based bioregenerative agriculture coupled with efficient water and waste recycling management for long-term manned missions.

As known, the addition of amendments leads to changes in hydraulic and geochemistry properties of soil, thereby also altering the water-plant relations and bioavailability of nutrients. In our case, better plant growth was recorded for lettuces grown on 30:70 mixture (simulant: compost), exhibiting a higher yield, A_{CO2}, WUEi, total ascorbic acid, and total polyphenols content. But with a view to sustainable use of organic residues, the best compromise is the mixture with 30% because it is a more realistic scenario and ensures satisfactory plant growth.

Noteworthy, the results on pure simulant show cultivation feasibility even with a significant reduction of yield (around 20% less) accompanied with a reduced amount of NO_3, PO_4, K, and bioactive compounds except for total ascorbic acid. The reduction of all growth parameters when the simulant rate increases in the mixture suggests that the modified Hoagland solution ensures a suitable nutrient supply.

In the absence of fertigation, MMS-1 and LHS-1 can sustain lettuce outliving (at least for a short time) as demonstrated. Usually, in the soil-plant system are established relationships; these interactions with substrates effects above- and under-ground tissues. So, by improving the efficiency of the substrate and resources, we get an impact on the plants. Indeed, the amendment of these simulants (nutrient-poor and alkaline) with monogastric manure mitigate their negative features, promoting plant growth in a low input regime, as may be needed in the future outpost. The mixture containing 70 % in weight of simulant and 30 % of manure provided the best outcomes in terms of biomass production and plant vigour/health. Even if the lettuce yield is low to ensure the self-sustainability of an extraterrestrial outpost, we can consider it as a positive result because demonstrating that just adding 30% of amendment to pure regolith and using only pure water is possible to complete a lettuce crop cycle. Stressing the concept of the potential use of these simulants as media for the plants' cultivation, especially if conscientiously managed. In an extreme habitat such as a future settlement, nutritional and quality aspects play as important a role as fresh biomass production. Because providing the bioactive compounds in astronauts' diets is undeniably fundamental.

To the best of knowledge, the stressors drive the plants to changes in their metabolism, increasing the formation of phytochemical compounds linked to the secondary metabolism, as also highlighted by our results. Indeed, the nutritional stress to which we subjected the plants, led to the formation of higher content of phenols in plants grew on pure simulant showing an inverse correlation with the percentage of manure in the substrates, while no difference between the two simulants were appreciated. Also, the antioxidant activity showed a marked effect by the substrate's amendment. The activity was lower on the mixtures which were present manure than on pure simulants. Supporting the hypothesis that the biosynthesis of these secondary metabolites tends to increase in response to stressors.

Another focal point in space farming is the choice of plant genotype. As shown, two cultivars butterhead lettuce (Salanova) exhibited different quanti-qualitative performances. Indeed the red cultivar presented a higher yield, photosynthetic activity, and bioactive compounds in comparison to the green one, underlining the effect of genetic response to environmental factors.

In a future extraterrestrial outpost it will be feasible to gradually improve the regolith fertility with repeated cropping cycles and continuous inputs of organic substances composed partly by crew excrements and partly by residues of previous crops. In addition, a start-up phase involving minimal nutrient supplementation and inoculation of plant growth-promoting rhizobacteria (PGPR) should be also investigated in future studies. In this regard, we can consider our results promising, as they demonstrated that by adding 30% of manure to pure regolith, it is possible to complete a lettuce cycle by feeding the plants with only pure water. However, further studies are needed with more lettuce genotypes to explore genotypic variation and make safe conclusions about the potential cultivation of the species in regoliths.

"If you discover a new land, but you behave in the same ways as you did before. And then it becomes just like the land you left behind"

Anonimous

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Appendix

Supplementary Materials of Chapter 1

Table S1. Petrographic and mineralogical composition of lunar and martian simulants (30 in total). Rocks are gathered in 6 petrographic classes, such as: anorthosite, basalt, gabbro, norite, hartzburgite, dunite, while the minerals are summarized in 12 classes, as follow: Oxides (Ilmenite; Magnetite; Ti-magnetite; Hematite; Ferrihydrite; Cr-spinel); Carbonates (Calcite; Fe-carbonate; Mg-carbonate); Phosphates (Apatite); Sulphates (Gypsum; Selenite; Mg-sulfate; Anhydrite); Sulfides (Pyrrhotite; Sulfide); Nesosilicates - inosilicates (Olivine + pyroxene + ilmenite; Olivine; Clinopyroxene; Augite; Enstatite; Pyroxene + Hornblende; Pyroxene; Diopside; Hypersthene); Tectosilicates - Quartz, Zeolite; Tectosilicates - Plagioclases (Plagioclase; Anorthite; Albite; Labradorite); Tectosilicates - K-feldspars (Sanidine; K-feldspar; Orthoclase); Phyllosilicates (Smectite; Saponite; Chlorite); Other minerals (undifferentiated). Glass + opaque (Plagioclase Glass, Basaltic Glass, Hydrated Silica, Volcanic Glass; Washed Volcanic Glass; Glass; Opaque + Glass).

	Lunar Mare Simulants											Lunar Highlands Simulants						Lunar Dust Simulants				Martian simulants								
	FJS-1	FJS-2	FJS-3	OPRL2N	Oshima Simulant	BP-1	CAS-1	CSM-CL	CUG-1A	JSC-1/1A/1AF/1AC/	LMS-1	OPRH2N/H2W	OPRH3N/H3W	NU-LHT/1M/	LHS-1	NAO-1	OB-1	DUST-Y	Kohyama Simulant	BHLD20	CLDS-i	JMSS-1	JEZ-1	JSC-Rocknest	KMS-1	MGS-1	MGS-1S	MGS-1C	Y-Mars	MMS-1
Rocks																														
Anorthosite				10.0							19.8	70.0	80.0	43.4				83.0												
Basalt	100	88.0	81.0	90.0	85.0						7.5	30.0	20.0		0.5			0.6				93.0								
Gabbro																			100											
Norite														30.0																
Hartzburgite														6.1																
Dunite																													3.9	
Mineral Oxides																														
Ilmenite							2.3	1.0		0.1	4.3			0.7	0.4			0.1						1.0						
Magnetite						3.0	3.0										0.3					5.0	1.1		5.8	1.9	1.1	1.1	5.3	
Ti-magnetite										0.4																				
Hematite						2.0																2.0	0.3	1.7		0.5	0.3	0.3		3.0
Ferrihydrite																							2.1			3.5	2.1	2.1		
Cr-spinel										1.1																				
Carbonates																														
Calcite						2.7																		1.5						
Fe-carbonate																							0.8			1.4	0.8	0.8		
Mg-carbonate																							11.0							
Phosphates																														
Apatite							0.7			0.1																				
Sulphates																														
Gypsum																											40.0			
Selenite																													1.4	
Mg-sulfate																							2.4			4.0	2.4	2.4		
Na-sulfate																														
Anhydrite																							1.0			1.7	1.0	1.0	3.6	
Sulfides																														
Pyrrhotite																													2.9	
Sulfide										1.0																				
Nesosilicates, inosilicates																														
Olivine, pyroxene and ilmenite																					10.0									
Olivine						6.2	8.2	8.0	48.0	9.0	11.1				0.2		0.04	0.2					32.0	11.7	31	13.7	8.2	8.2		
Clinopyroxene								4.0		0.1							0.1													
Augite						23.7																							13.1	
Enstatite																													4.2	
Pyroxene + Hornblende																19.2														
Pyroxene									20.0		38.2				0.3			0.4					12.0	11.0	14.2	20.3	12.2	12.2		
Diopside							7.7																							
Hypersthene							14.6																							
Tectosilicates																														
Quartz						1.7				0.2							0.3							2.3						
Zeolite																														12.0
Tectosilicates - Plagioclases																														
Plagioclase								40.0	9.0	37.1					74.4	60.0	43.3			25.0	15.0		16.0	42.9	48.9	27.1	16.4	16.4		
Anorthite							56.3																							57.0
Albite										0.3																			31.1	
Labradorite						60.7																								
Tectosilicates - K-feldspars																														
Sanidine																													1.7	
K-feldspar								2.0		1.4														2.3						
Orthoclase							7.2																							
Phyllosilicates										0.1							0.5						6.0	20.4				40.0	30.5	0.5
Smectite																	0.5						6.0	20.4				40.0		0.5
Saponite																													30.5	
Chlorite										0.1																				
Other minerals																														
undifferentiated																	1.9													
Glass and opaque																														
Plagioclase glass																	2.1													
Basaltic glass																							13.5			22.9	13.7	13.7		
Hydrated silica																							1.8			3.0	1.8	1.8		
Volcanic glass																				20.0										
Glass								44.0	23.0	49.3	24.5			19.7	24.2		53.0	9.0			75.0									
Opaque + Glass																20.8														28.0

Table S2. Data used to build Figure 1. Data are relative only to simulants (19/30) whose mineralogical composition is reported for more than 73% of the total mass in the literature (Table S2). The mineralogical classes are split as follow: Oxides (Ilmenite; Magnetite; Ti-magnetite; Hematite; Ferrihydrite; Cr-spinel); Carbonates (Calcite; Fe-carbonate; Mg-carbonate); Phosphates (Apatite); Sulphates (Gypsum; Selenite; Mg-sulfate; Anhydrite); Sulfides (Pyrrhotite; Sulfide); Nesosilicates - inosilicates (Olivine + pyroxene + ilmenite; Olivine; Clinopyroxene; Augite; Enstatite; Pyroxene + Hornblende; Pyroxene; Diopside; Hypersthene); Tectosilicates - Quartz, Zeolite; Tectosilicates - Plagioclases (Plagioclase; Anorthite; Albite; Labradorite); Tectosilicates - K-feldspars (Sanidine; K-feldspar; Orthoclase); Phyllosilicates (Smectite; Saponite; Chlorite); Other minerals (undifferentiated). Glass + opaque (Plagioclase glass, basaltic glass, hydrated silica, Volcanic glass; Glass; Opaque + Glass).

	Lunar Mare Simulants							Lunar Highlands Simulants				Lunar Dust Simulants			Martian Simulants								
Mineral	**BP-1**	**CAS-1**	**CSM-CL**	**CUG-1A**	**JSC-1/1A/ 1AF/1AC/2A**	**LMS-1**	**mean**	**LHS-1**	**NAO-1**	**OB-1**	**mean**	**BHLD20**	**CLDS-i**	**mean**	**JEZ-1**	**JSC-Rocknest**	**KMS-1**	**MGS-1**	**MGS-1S**	**MGS-1C**	**Y-Mars**	**MMS-1**	**mean**
Oxides	5.0	5.4	1.0	0	1.5	5.5	***3.1***	0.4	0	0.3	***0.2***	0	0	***0***	3.5	2.8	5.8	5.9	3.5	3.5	5.4	3.0	***4.2***
Carbonates	2.7	0	0	0	0	0	***0.5***	0	0	0	***0***	0	0	***0***	11.8	1.6	0	1.4	0.8	0.8	0	0	***2.0***
Phosphates	0	0.6	0	0	0	0	***0.1***	0	0	0	***0***	0	0	***0***	0	0	0	0	0	0	0	0	***0***
Sulphates	0	0	0	0	0	0	***0***	0	0	0	***0***	0	0	***0***	3.4	0	0	5.7	43.4	3.4	5.1	0	***7.6***
Sulfides	0	0	0	0	1.0	0	***0.2***	0	0	0	***0***	0	0	***0***	0	0	0	0	0	0.0	3.0	0	***0.4***
Haloids	0	0	0	0	0	0	***0***	0	0	0	***0***	0	0	***0***	0	0	0	0	0	0	0	0	***0***
Nesosilicates, inosilicates	29.9	30.5	12.1	68.0	9.0	63.1	***35.4***	0.5	19.2	0.1	***6.6***	0	10	***5.0***	44.0	23.9	45.2	34.0	20.4	20.4	17.7	0	***25.7***
Tectosilicates - Quartz, Zeolites	1.7	0	0	0	0.2	0	***0.3***	0	0	0.3	***0.1***	0	0	***0***	0	2.4	0	0	0	0	0	11.9	***1.8***
Tectosilicates - Plagioclases	60.7	56.3	40.4	9.0	37.4	0	***34.0***	74.8	60	42.5	***59.1***	38.5	15	***26.7***	16.0	45.3	48.9	27.1	16.4	16.4	31.8	56.7	***32.3***
Tectosilicates - K-feldspars	0.0	7.2	2.0	0	1.4	0	***1.8***	0	0	0	***0***	0	0	***0***	0	2.4	0	0	0	0	1.7	0	***0.5***
Phyllosilicates	0	0	0	0	0.1	0	***0***	0	0	0.5	***0.2***	0	0	***0***	6.0	21.5	0	0	0	40.0	31.2	0.5	***12.4***
Other minerals	0	0	0	0	0	0	***0***	0	0	2.3	***0.8***	30.8	0	***15.4***	0	0	0	0	0	0	0	0	***0***
Glass and opaque	0	0	44.4	23.0	49.3	31.4	***24.7***	24.3	20.8	54.0	***33.0***	30.8	75	***52.9***	15.3	0	0	25.9	15.5	15.5	4.0	27.9	***13.0***
	100.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

Supplementary Materials of Chapter 2

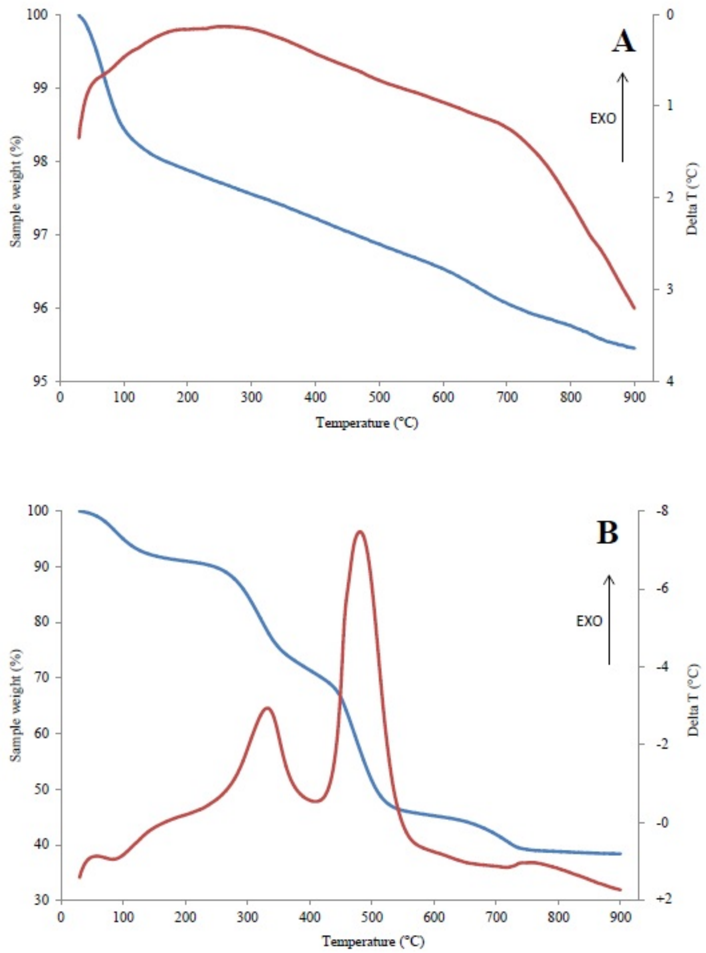
S1. Mineralogical composition of simulant particle-size fraction 20-250 μm, assessed by quantitative X-ray powder diffraction (XRPD) analysis.

Hematite (%)	Plagioclase (Anorthite) (%)	Smectite (%)	Zeolite (Clinoptilolite) (%)	Amorphous Residual (%)
3	57	<1	12	28

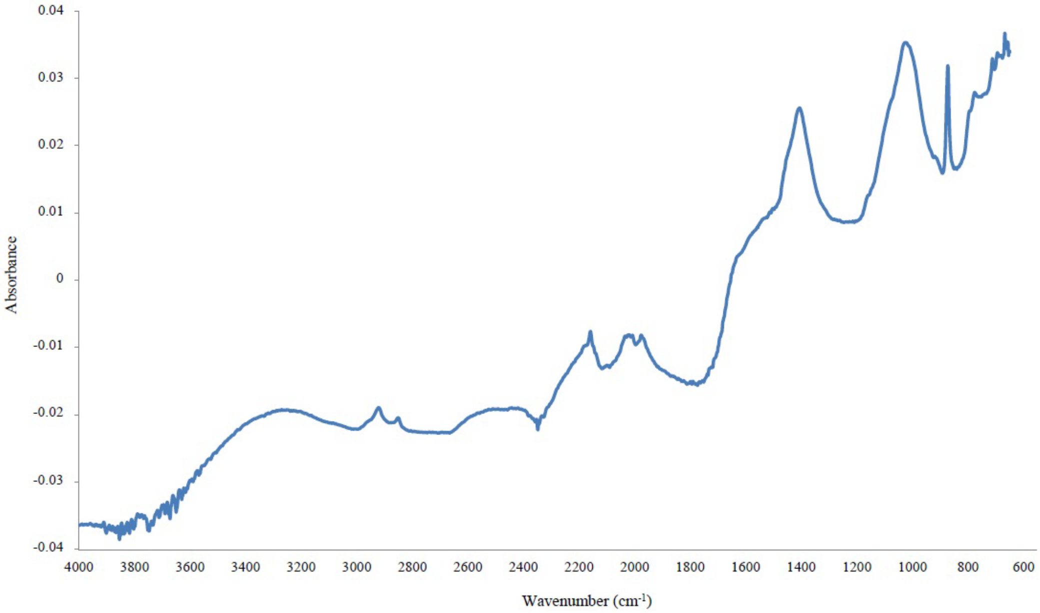
S2. Physical and hydrological properties of different mixtures (simulant:compost rates: 0:100, 30:70, 70:30, 100:0; v:v).

Mixture property	Simulant : Compost (v:v)			
	0:100	30:70	70:30	100:0
Bulk density (g cm^{-3})	0.60	0.90	1.10	1.40
Saturated hydraulic conductivity (cm min^{-1})	-	0.07	0.13	0.05
Saturated water content (cm^3 cm^{-3})	0.70	0.61	0.52	0.45
Container capacity (cm^3 cm^{-3})	0.69	0.59	0.51	0.42
Air filled capacity (cm^3 cm^{-3})	-	0.02	0.01	0.02
Easily available water (cm^3 cm^{-3})	-	0.11	0.15	0.08
Buffer capacity (cm^3 cm^{-3})	-	0.15	0.10	0.09
Lettuce buffer capacity (cm^3 cm^{-3})	-	0.34	0.25	0.20

- indicates that for 0:100 substrate the measurement was not possible due to floating of the material.



S3. Thermo-gravimetric (TGA, blue line) and Differential Scanning Calorimetry (DSC, red line) thermograms of fine-grade MMS-1 simulant (A) and compost (B).



S4. Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR) spectroscopy of compost.

Supplementary Materials of Chapter 4

Table S1. Concentration (mg kg^{-1} DW) of main macro and micronutrients in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w %), separated in rhizo and bulk soil after lettuce growth, extracted by 0.05M EDTA at pH 7 (n=3).

Source of Variance	Ca	K	Mg	P	Fe	Na	Mn	Cu	Zn
	mg kg^{-1} DW								
Simulants (S)									
MMS1	11756	770	772	687	222	119	115	6.03	21.5
LHS1	7981	460	514	556	356	150	45.5	6.00	21.1
	***	***	***	***	***	**	***	ns	ns
Amendment % (M)									
0	2794 d	85.3 d	235 d	124 d	18.0 c	87.8 c	12.9 d	0.72 d	1.13 d
10	6659 c	333 c	418 c	404 c	230 b	93.8 c	81.0 c	4.75 c	10.0 c
30	13981 b	715 b	863 b	934 b	452 a	131 b	109 b	8.42 b	29.0 b
50	16039 a	1326 a	1055 a	1025 a	455 a	226 a	118 a	10.2 a	45.1 a
	***	***	***	***	***	***	***	***	***
Rhizo vs bulk soil (RB)									
RH	9933	521	598	626	297	129	80.0	6.30	21.6
BK	9803	709	687	617	280	140	80.7	5.73	21.1
	ns	***	***	ns	ns	ns	ns	**	ns
S x M x RB									
MMS1 x 0 x RH	5065	159	420	245	14.8	83.2	25.8 f	0.80	2.14
MMS1 x 0 x BK	5244	169	445	240	9.77	88.4	21.2 f	0.70	1.09
MMS1 x 10 x RH	8753	389	555	477	107	93.2	131 c	4.02	9.28
MMS1 x 10 x BK	9135	548	662	491	115	96.9	146 b	4.02	9.72
MMS1 x 30 x RH	15781	678	884	980	353	111	151 ab	9.15	29.0
MMS1 x 30 x BK	15759	1133	1050	994	361	123	158 a	8.55	29.6
MMS1 x 50 x RH	17170	1270	950	1062	417	157	146 b	11.50	47.3
MMS1 x 50 x BK	17139	1815	1206	1006	395	202	143 b	9.48	44.0
LHS1 x 0 x RH	430	8.65	47.4	5.74	23.2	89.9	2.28 g	0.67	0.66
LHS1 x 0 x BK	435	4.29	25.7	4.15	24.1	89.5	2.37 g	0.69	0.64
LHS1 x10 x RH	4607	173	209.2	338	367	93.2	24.4 f	5.38	11.16
LHS1 x 10 x BK	4142	223	246	311	332	91.8	22.9 f	5.58	9.95
LHS1 x 30 x RH	12267	465	703	899	567	136	66.2 e	8.48	28.7
LHS1 x 30 x BK	12117	586	815	862	526	153	62.1 e	7.50	28.8
LHS1 x 50 x RH	15394	1023	1017	1003	526	268	93.1 d	10.4	44.1
LHS1 x 50 x BK	14452	1196	1047	1029	480	276	90.5 d	9.31	45.0
	ns	ns	ns	ns	ns	ns	*	ns	ns
S x M	***	ns	***	***	***	*	***	***	ns

For the sake of clarity, this wide table shows only the mean values, not followed by standard deviations. Non-significant (ns). *, **, *** Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test (P = 0.05). Different lowercase letters within each column indicate significant differences ($P \leq 0.05$).

Table S2. Nutrient uptake in different mixtures of MMS-1 or LHS-1 simulants and manure (simulant/manure rates: 100:0, 90:10, 70:30, 50:50; w/w %).

Source of Variance	PO_4	K	Mg	Ca	SO_4	Na
	mg plant^{-1}					
Simulants (S)						
MMS1	21.2 ± 3.97	121 ± 20.3	7.20 ± 1.160	29.7 ± 5.04	3.48 ± 0.69	8.72 ± 1.61
LHS1	5.54 ± 1.41	35.9 ± 8.7	2.76 ± 0.612	8.40 ± 2.04	1.02 ± 0.27	2.34 ± 0.53
	***	***	***	***	***	***
Amendment % (M)						
0	0.24 ± 0.04 c	6.92 ± 1.1 c	0.68 ± 0.071 d	1.55 ± 0.23 d	0.09 ± 0.01 b	0.56 ± 0.08 c
10	16.9 ± 3.32 b	104 ± 18.3 a	6.38 ± 0.883 b	25.9 ± 5.56 b	2.65 ± 0.44 a	6.55 ± 1.53 b
30	22.2 ± 5.73 a	117 ± 26.2 a	7.74 ± 1.250 a	30.9 ± 6.55 a	3.41 ± 0.88 a	9.10 ± 2.68 a
50	14.1 ± 5.52 b	84.9 ± 33.2 b	5.14 ± 1.883 c	18.0 ± 6.99 c	2.84 ± 1.16 a	5.89 ± 1.89 b
	***	***	***	***	***	***
S x M						
MMS1 x 0	0.32 ± 0.04 d	9.19 ± 1.1 d	0.82 ± 0.053 d	2.01 ± 0.24 e	0.11 ± 0.01 e	0.66 ± 0.10 d
MMS1 x 10	23.6 ± 2.93 b	141 ± 14.1 b	8.22 ± 0.492 b	38.2 ± 0.81 b	3.12 ± 0.82 b	9.11 ± 1.94 b
MMS1 x 30	34.9 ± 1.54 a	174 ± 9.8 a	10.5 ± 0.350 a	45.3 ± 2.25 a	5.35 ± 0.39 a	15.0 ± 0.68 a
MMS1 x 50	26.1 ± 2.94 b	157 ± 15.4 ab	9.26 ± 0.871 b	33.4 ± 2.35 c	5.33 ± 0.70 a	10.1 ± 0.14 b
LHS1 x 0	0.15 ± 0.02 d	4.66 ± 0.4 d	0.54 ± 0.046 d	1.10 ± 0.10 e	0.07 ± 0.01 e	0.47 ± 0.10 d
LHS1 x 10	10.3 ± 1.57 c	67.4 ± 10.0 c	4.53 ± 0.500 c	13.6 ± 1.34 d	2.19 ± 0.28 bc	3.99 ± 1.21 c
LHS1 x 30	9.57 ± 1.29 c	59.2 ± 5.4 c	4.97 ± 0.047 c	16.4 ± 0.73 d	1.47 ± 0.04 cd	3.23 ± 0.93 cd
LHS1 x 50	2.15 ± 0.23 d	12.2 ± 0.6 d	1.02 ± 0.095 d	2.52 ± 0.16 e	0.35 ± 0.02 de	1.66 ± 0.10 cd
	***	***	***	***	***	***

Non-significant (ns). *, **, *** Significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Simulants (S), Amendment (M) and Rhizo vs bulk soil (RB) and interaction were compared by Duncan's multiple-range test ($P = 0.05$). Different lowercase letters within each column indicate significant differences ($P \leq 0.05$).

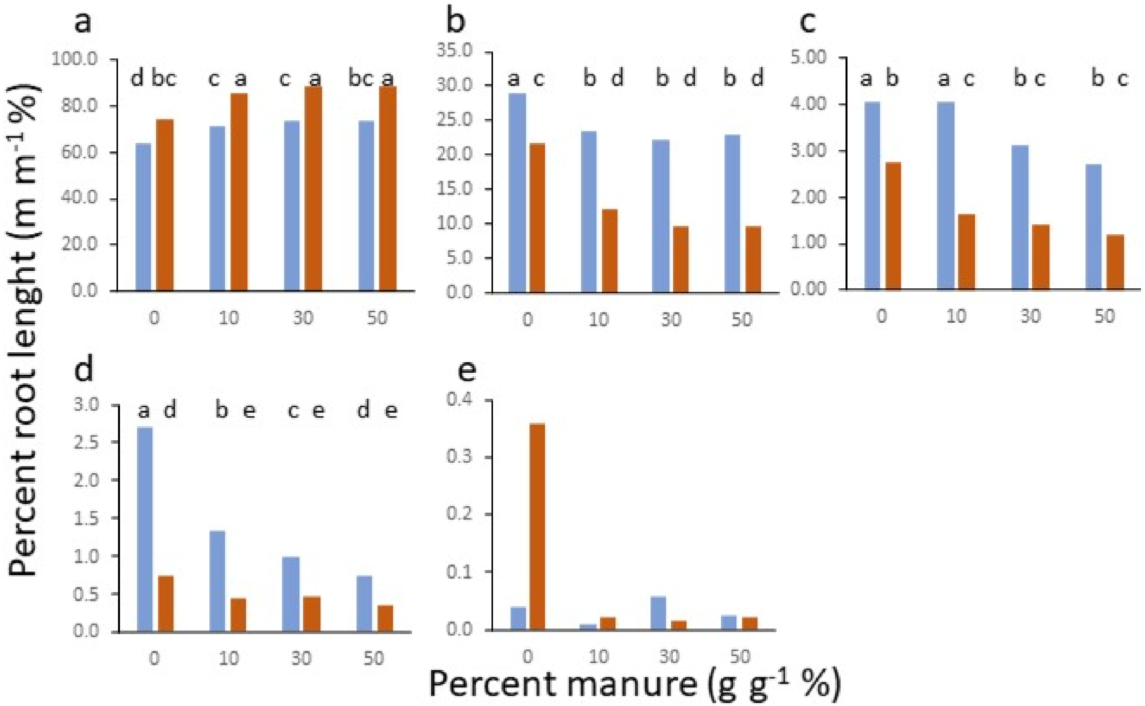


Figure S1. Interaction of simulant x manure concentration on percentage of total root length in each of ten diameter classes: a: D<= 0.5 mm; b: 0.5<D<=1 mm; c 1<D<=1.5 mm; d 1.5<D<=4.5 mm; e D> 4 mm. Orange bars: MMS1; blue bars: LHS1. Bars with different letters are different for P<0.05 at the post-hoc Duncan Multiple Range Test for $p \leq 0.05$.