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Seed Priming with *Ulva lactuca L.* in Cultivars Grown in Martian and Lunar Regolith Analogues

Jéssica Carneiro Oliveira[1,2]*, Renato Crespo Pereira[3], Taylor Sawyer Johnson[4], Rafael Loureiro[2,4]

[1]Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM) and Universidade Federal Fluminense (UFF); [2]Blue Marble Space Institute of Science, Seattle, Washington, 98154, United States; [3]Department of Marine Biology, Fluminense Federal University (UFF), Morro do Valonguinho, s/n. Centro, Niterói, 24001-970, Rio de Janeiro, Brazil; [4]Winston-Salem State University, 601 S. Martin Luther Jr. Winston-Salem, NC 27110, United States

Abstract

As human settlements expand to lunar and Martian bases, optimizing food production in these environments becomes crucial. This study investigates the use of macroalgae, specifically Ulva lactuca L., as an affordable, sustainable approach for seed priming to enhance germination in extraterrestrial soils. The focus was on the germination and growth of Capsicum annuum L. (pepper), Lactuca sativa L. (lettuce), Cicer arietinum L. (chickpea), and Pisum sativum L. (pea) in simulated Martian and lunar regolith. Two concentrations of U. lactuca powder (0.2 and 0.4 g · L^{-1}) were tested under controlled conditions. The study also conducted a qualitative chemical analysis of U. lactuca to identify bioactive components essential for phytohormone formation. The germination and emergence rates of the seeds in the lunar regolith were higher than those in the Martian regolith. Martian regolith's optimal treatment for pea and chickpea seed germination was 0.2 g · L^{-1}, which also favored seedling emergence. In the lunar regolith, optimal germination rates for pea seeds were observed with both treatments and chickpea seeds. The germination percentage of lettuce seeds in the lunar regolith was higher than the control, with 0.2 g · L^{-1}, while there was no significant difference for the other seeds. The study recommends the application of U. lactuca powder as an effective biostimulant for the examined cultivars due to the presence of plant growth regulators (PGRs) that enhance germination and seedling emergence under challenging conditions.

Keywords

regolith simulant • plants in space • seed priming • regolith-based agriculture • biostimulant

Introduction

Space agriculture is critical to the survival of humans outside Earth due to its potential to provide a sustainable source of food and generate income. However, numerous challenges must be faced (Oluwafemi et al., 2018; Cannon Britt, 2019; Verseux et al., 2021). For example, due to the lower luminosity levels, which are crucial for plant development, on Mars and the Moon (Cannon Britt, 2019), lighting for plant growth needs to be supplemented and fully controlled (Ming Brady, 1989; Wamelink et al., 2014; Wheeler, 2017). The high level of cosmic radiation on the Martian surface, which is 17 times higher than on Earth, could significantly impact plant growth and lead to a drastic reduction in plant biomass (Tack et al., 2021; Guo et al., 2017). Another challenge is cultivating plants in a regolith-based system where research is being carried out to optimize the use of In-Situ Resource Utilization (ISRU) (Eichler et al., 2021).

The optimization of ISRU aims to adapt the lunar and Martian environments for plant acclimatization (Eichler et al., 2021). Regolith is unconsolidated material overlying rock and may include dust, broken rocks, and other related materials (Fackrell et al., 2021; Duri et al., 2022). Additionally, regolith contains no associated organic matter or microbiome; therefore, it can only be defined as soil if it has undergone organic processes (Duri et al., 2022). Consequently, it is a stressful substrate for plant growth.

Multidisciplinary studies have explored life-support bioregeneration and food production in extreme space conditions (Miller Ward, 1966; Wheeler et al., 2011; Poulet et al., 2016). For example, the use of marine macroalgae has been studied for their ability to convert carbon dioxide and water into oxygen, as well as research with cyanobacteria fed with Martian analogs (MGS-1) and Mars atmosphere as a source of nutrients to be suitable for photobioreactors of life support systems (Miller Ward, 1966; Verseux et al., 2021). Technologies that aim to measure the gas exchange of the entire plant under the influence of different air velocities allow for understanding the photosynthesis measurements performed by the individual (Poulet et al., 2020).

In space agriculture, the challenges to be overcome to ensure efficient growth of cultivars include the production of successful

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†Corresponding author: Jéssica Carneiro Oliveira

E-mail: jjessicacarneiro@gmail.com

crop cycles or using crops that accumulate a high nutrient content (Wamelink et al., 2019; Llorente et al., 2018).

For future interplanetary nutrition, it is essential to choose crops that require minimum preparation time, provide essential vitamins (B1, K, C), and do not degrade like most bioactive compounds in stored food systems or require processing (Johnson et al., 2021; Poulet et al., 2022). In addition, producing fresh foods may provide crucial nutritional and psychological information for long-term missions and future human settlers in the ideal choice of vegetables with organoleptic properties (Massa et al., 2015; Zhang et al., 2022).

All essential macro and micronutrients for plant growth are present in sufficient amounts, both in lunar and Martian regolith, except the reactive nitrogen (Duri et al., 2022). Nitrogen in absorbable forms (NO_3 and NH_4) is one of the essential minerals necessary for the growth of almost all plants due to the mineralization of organic matter and nitrogen fixation through mutualistic associations between plants and the terrestrial soil microbiome (Stevens et al., 2011). However, organic matter is absent on Mars and the Moon, although both sites contain graphitic carbon (Parnell, 2005), and no microorganism activity has been recorded. The absence of sufficient absorbable nitrogen can be resolved using nitrogen-fixing species (Llorente et al., 2018).

Martian soil exhibits high levels of perchlorate ($NaClO_4$) (Hecht et al., 2009) that are toxic to humans; therefore, cultivation in this type of soil will need specific care (Eichler et al., 2021). Studies have been carried out to reduce the levels of this toxic substance. For example, some organisms have been suggested as potential candidates for Martian regolith, such as plants of woody species, non-woody terrestrial plants, aquatic plants, and potential microbes that carry out phytoremediation through phytoaccumulation, phytodegradation, and rhizodegradation (Misra et al., 2021), but these authors recommend further studies in the area so that agriculture based on ISRU (In-Situ Resource Utilization) become viable.

Plant germination and performance can be altered or impaired under atypical conditions in soils with no organic matter, such as those on Mars and the Moon. To ensure a self-sufficient civilization, it will be necessary to improve the cultivation of food plants through biotechnology, robotics, and agriculture (Wheeler et al., 2011; Wheeler, 2017; Cannon Britt, 2019) to associate biological systems with local natural resources (Verseux et al., 2016; Cannon Britt, 2019). Thus, these food mechanisms and biological processes must be fully understood (Verseux et al., 2016). In the processing of seeds, techniques or specific treatments before sowing provide faster and more uniform germination and the generation of seedlings more tolerant to abiotic stresses, higher productivity, and crop quality (Paparella et al., 2015; Sivritepe Sivritepe, 2016).

Seed priming techniques with phytohormones have been reported as an effective complement to minimize the effects of environmental, stressful conditions on vegetables (El-Aziz Kasim et al., 2016; García et al., 2020; Hamouda et al., 2022). This technique consists of pre-soaking seeds with the help of substances that contain bioactives, such as phytohormones, that aid in breaking dormancy and metabolic stimulation of seeds (Rhaman et al., 2020). There is an improvement in shoot length and fresh root mass of wheat seedlings, as well as a higher percentage of chlorophyll a, b, and total, carotenoid and wheat grain carbohydrate (Shahbazi et al., 2015) and, in tomato species, there is an improvement in germination and growth mainly of the stem, and it can act as an antibacterial agent (Sekhouna et al., 2021).

According to du Jardin (2015), a plant biostimulant is any microorganism or substance that is applied to plants to promote nutrient uptake efficiency, enhance resistance to non-living stresses, and improve the qualitative attributes of the cultivated variety without relying on the addition of nutrients. Biostimulants are considered efficient when applied in minimal amounts to promote plant growth, and many of these are derived from marine macroalgae (du Jardin, 2015; Yakhin et al., 2017).

Species of the cosmopolitan marine green macroalgae *Ulva* spp. are indicated as biostimulants and biorefined products by the presence of bioactive substances (Jaulneau et al., 2011; Hernández-Herrera et al., 2014; Sekhouna et al., 2021; Pappou et al., 2022), as well as macro- and microelements, amino acids, vitamins, phytohormones, betaines and sterols that act in stimulating seed germination (Khan et al., 2009; Hafting et al., 2015; Sekhouna et al., 2021; Pappou et al., 2022). For example, marine macroalgal extracts were used to soak seeds with promising results for organic production in species such as *Solanum lycopersicum*, *Abelmoschus esculentus*, *Ceratonia siliqua, Triticum aestivum*, *Vigna radiata*, *Vigna sinensis*, and *Zea may*s (Ibrahim et al., 2014; Divya et al., 2015; Reis et al., 2020; El Boukhari et al., 2021; Hussein et al., 2021; Zouari et al., 2022).

The use of *Ulva* spp. extracts as a biostimulant is a promising alternative to improve seedlings' metabolic capacity and physiological development when they are under stressful conditions (El Boukhari et al., 2021). These macroalgae can be indicated when implemented in seed priming techniques by hydration (Rhaman et al., 2021) because it contains hormones essential for the development and stimulation of germination in its composition and allows the seeds to be prepared to receive this biostimulant before germination and for the improvement of their metabolic processes (El Boukhari et al., 2020; Rhaman et al., 2021).

The use of dry and crushed marine macroalgae in the form of powder may reduce costs due to the facility of production and low production costs when compared to the extraction of the polysaccharide called ulvan from the *Ulva* species, which has been investigated for use in agriculture (Alves et al., 2013; Reis et al., 2020; Putra et al., 2024). The cost of producing macroalgae extract for agriculture can vary widely depending on the species of macroalgae, the extraction method used, and the production scale since exact numbers for the extraction processes and production of commercially available *U. lactuca* extract proxies were used to estimate the overall cost of production (Alves et al., 2013; Zhang et al., 2022).

In a study by Shahidi et al. (2017), the cost of producing macroalgae extract from *Ascophyllum nodosum* using a cold-water extraction method was estimated to be around $14.42 per kg. Another study by Eom et al. (2019) investigated the cost-effectiveness of using macroalgae extract as a biofertilizer in rice cultivation. The authors reported that the cost of producing the macroalgae extract was around $13.16 per kg when using a hot water extraction method.

Multiple studies have estimated the cost of payloads based on various mission designs and launch vehicles (**Table 1**). For example, Musiolik et al. (2021) estimate the cost per kg to be between $4,000.00 and $40,000.00, depending on the launch vehicle and other factors. Sowers et al. (2020) present a commercial approach to affordable access to lunar resources, including the cost of payloads. They estimate the cost per kg to be between $1,000.00 and $5,000.00 for regular missions and between $5,000.00 and $10,000.00 for long-term missions (used as a Martian proxy). Maruyama et al. (2021) discuss the potential of SpaceX's Starship to reduce the cost of payloads to Mars and the Moon, estimating the cost per kg to be around $200.00 to $300.00, assuming a fully reusable launch system and cost-saving measures.

Assuming a payload cost of $20,000 per kg (the midpoint of the range provided by Musiolik et al. (2021), the cost of using the tested amounts of 0.2 and 0.4 $g \cdot L^{-1}$ of macroalgae extract on missions would be approximately $0.40 and $0.80, respectively.

Assessing the germination and emergence of seedlings from cultivars treated with *U. lactuca* L. is a method to investigate potential crosstalk between the bioactive compounds of marine macroalgae and seed metabolism. (Reis et al., 2020). The germination and emergence of seedlings of cultivars when subjected to the treatment of this macroalgae allows for the assessment of potential effects on seed germination and seedling growth (Hamouda et al., 2022; Hernández-Herrera et al., 2023). By analyzing the presence of bioactive compounds in *U. lactuca* and its phytochemical extracts, as well as conducting hormonal analysis of seeds, we can understand the mechanisms by which these compounds may influence seed metabolism, providing insights into the potential crosstalk between marine macroalgae, bioactive, and seed physiology (Hamouda et al., 2022). To this end, it is essential to analyze the presence of bioactive compounds of *U. lactuca* and its phytochemical extracts for compression of its composition and hormonal analysis of seeds.

Table 1. Estimated cost per kg of macroalgae extract.

Payload Cost	Author	Total Cost per kg
$4,000.00-$40,000.00	Ehlmann et al. (2005)	$50,968.00 - $1,760,000
$1,000.00-$5,000.00 (regular) $5,000.00-$10,000.00 (long-term)	Zuniga et al. (2015)	$14,160.00 - $60,000.00 (Regular) $70,000.00 - $144,200.00 (long-term)
$200.00-$300.00	Heldmann et al. (2022)	$13,360.00 - $21,120.00

Therefore, to improve biosynthetic capacity under adverse conditions, as well as biomass production, techniques should be considered that allow for increased vigor and hormonal indices of these plants (Llorente et al., 2018) to play a role in the development of a self-sustaining civilization (Verseux et al., 2016).

Previous studies have demonstrated the success of the cultivation of viable crops in Martian and lunar regolith-based agriculture (RBA) with nutrient supplementation (Wamelink et al., 2014; 2019; Eichler et al., 2021; Kasiviswanathan et al., 2022), but some of these studies did not consider important aspects, such as complete sterilization of the regolith and, in Martian analogs, the addition of perchlorate to the regolith. Although it is considered a viable solution for a continuous and self-sustaining alternative to *in-situ* cultivation, the use of Martian and lunar regolith as the answer to complete ISRU-dependent missions remains to be determined (Eichler et al., 2021).

In this study, we proposed to conduct experimental verification of the enhancement of seed vigor in sweet pepper (*Capsicum annuum* L.), lettuce (*Lactuca sativa* L.), chickpea (*Cicer arietinum* L.), and pea (*Pisum sativum* L.) under simulations of Martian and lunar regolith. Furthermore, we chose these crop productions of fresh food, which can provide significant nutritional and psychological benefits for long-term missions and future colonizers, aiding in selecting ideal vegetables with desirable organoleptic properties (Massa Wheeler, 2015).

For interplanetary nutrition in the future, it is essential to select crops that do not require processing, have minimal preparation time, provide essential vitamins, and do not degrade like most bioactive compounds in stored food systems (Johnson et al., 2021; Poulet et al., 2022). Furthermore, fresh food can provide significant nutritional and beneficial organoleptic effects in long-duration missions and for future settlers choosing the ideal vegetables with organoleptic properties (Massa Wheeler, 2015). The simulations will be carried out using different concentrations of *Ulva lactuca* powder, and the seed germination and development will be conducted in a fully controlled greenhouse incubator. The simulators will serve as stress indicators. Additionally, we will evaluate the phytochemicals of this marine macroalgae to comprehend the potential crosstalk between different phytohormones in generating a stress response for each treatment conducted.

Material and methods

Collection and obtaining of U. lactuca powder

U. lactuca specimens were collected at Arpoador Beach, Rio de Janeiro, RJ, Brazil (22º 59'S - 43º 11'W) on June 9, 2021. In the laboratory of the Botanical Garden Research Institute of Rio de Janeiro, RJ, Brazil, epiphytes and associated organisms were removed from the algae by washing with running water for their taxonomic identification, according to Koeman (1985), and for the preparation of the biostimulant. The specimens were frozen and later freeze-dried to obtain powder used in vitro experiments. The *U. lactuca* powder was obtained in a ball mill (SOLAB-SL 38 Solab Científica) for 2 minutes until it reached the granulometry (0.9 mm) (Reis et al., 2020). The pH at 0.2 $g \cdot L^{-1}$ was 5.1 (32.5 salinity), and the pH at 0.4 $g \cdot L^{-1}$ was 5.36 (34.75 salinity).

Regolith

Martian regolith

Experiments with the analog of Martian regolith were carried out with the MGS-1 Mars Global Simulant (University of Central Florida, Exolith Lab)—with an average particle size of 90 µm. According to CLASS Exolith Lab, the relative abundances of each element, evaluated by the percentage of wet weight (wt%) detected by X-ray fluorescence, were SiO_2 (42.9), TiO_2 (0.6), Al_2O_3 (12.8), FeO (11.2), MnO (0.1), MgO (14.6), CaO (7.4), Na_2O (1.5), K_2O (0.6) and P_2O_5 (0.1).

Lunar regolith

Experiments with the analog of lunar regolith were carried out with the Off Planet Research H3N (Farside Lunar Highlands Simulant) (University of Central Florida, Exolith Lab). The LHS-1 Lunar Highland Simulator is developed by CLASS Exolith Lab (University of Central Florida) with an average particle size of 50 µm. According to CLASS Exolith Lab, the relative abundances evaluated of each element, detected by the percentage of wet weight (wt%) by X-ray fluorescence, were SiO_2 (51.2), TiO_2 (0.6), Al_2O_3 (26.6), FeO (2.7), MnO (0.1), MgO (1.6), CaO (12.8), Na_2O (2.9), K_2O (0. 5), and P_2O_5 (0.1).

Conditions for seed germination and seedling development

Germination tests were carried out in a Conviron oven (GEN1000 SH) at Winston-Salem State University (WSSU), according to the conditions described in **Table 2**. Illumination was 400 $\mu mol \cdot m^{-2} \cdot s$, using fluorescent lamps. The seeds of each cultivar species (n=10) were previously treated with a 5% sodium hypochlorite solution for 5 minutes. After treatment, seeding was performed in Petri dishes (10 cm in diameter) (n=4 repetitions) containing lunar and martian regolith simulators and 5 mL of distilled water as the main components of the culture medium (control treatment). Each Petri dish was lined with plastic wrap to prevent evaporation.

Table 2. Controlled conditions were maintained in an incubator for seed germination assays in terrestrial soil samples and simulated lunar and Martian regolith.

Conditions	Day	Night
Photoperiod	16:00 h	8:00 h
Temperature	23 °C	18 °C
Relative Humidity	70%	70%
CO^2	400 ppm - 1000 ppm	1000 ppm
Light	~ 400 $\mu mol.m^{-2}.s^{-1}$HPLED	N/A

Experimental design

Seeds (n=10 for each cultivar in each Petri dish) of pepper (*C. annuum*), lettuce (*L. sativa)*, chickpeas (*C. arietinum*), and peas (*P. sativum*) were concentrated in 5 mL of the different concentrations (0.2 and 0.4 $g \cdot L^{-1}$) of *U. lactuca* powder solution, and distilled water used as control, distributed in Petri dishes (n=4 repetitions) content lunar and Martian regolith simulators (substrates). These quantities were adapted from the results obtained by Reis et al. (2020), who also used *U. lactuca* in germination assays. The ground soil (Elite Soil from The Soil Makers Company [Colchester, Connecticut, United States]) was used to compare the physiological behavior of the seeds in the control. The seeds were obtained from Johnny's selected seeds company (Winslow, ME, USA).

Evaluation of seed vigor

Seed vigor was determined according to the Rules for Seed Analysis (Brasil, 2009). Seeds were classified as germinated when primary root protrusion with positive geotropism (1 mm) was observed. At the same time, seedlings were considered normal when they presented essential structures at an ideal stage of development (Brasil, 2009). Over 10 days, the seeds were evaluated daily to determine the production of primary roots and the formation of seedlings at an ideal stage of development (Brasil, 2009). Both measurements were performed daily until the counts stabilized.

To assess vigor, the parameters germination speed and seedling emergence were used, calculated according to the formula proposed by Labouriau Pacheco (1978), which consists of dividing the total number of germinated seeds or seedlings emerged by the number of days elapsed. The formula used to calculate the peak value was based on Czabator (1962). This formula considers the cumulative germination percentages obtained each day during the germination test and the number of days required to reach each cumulative percentage.

Analysis of the qualitative chemical profile of U. lactuca

The qualitative analysis of the chemical profile of *U. lactuca* was obtained in the Metabolomics Core Lab at Winston-Salem State University (WSSU). Two types of extracts of *U. lactuca* were prepared: methanol and aqueous. 5 g of *U. lactuca* powder was suspended in water (500 mL) and incubated for 24 h at 40 °C in an orbital shaker (Corning® LSE™ Compact Centrifuge). The resulting solution was filtered through Whatman number 1 filter paper, and the extracts were stored at 4 °C. The qualitative analysis used the following standard procedure for identifying phytochemical constituents, such as alkaloids, terpenoids, phenol, sugar, saponins, flavonoids, quinines, protein, and steroids (Altemimi et al., 2017). The *U. lactuca* solution was characterized using Fourier Infrared spectroscopy (FT IR) and Gas Chromatography-Mass Spectrometry (GCMS) analysis.

Hormonal analysis of cultivated seeds

The samples were analyzed at Astrobotany Laboratory, Department of Biological Sciences, Winston-Salem State University (WSS), with the aim of understanding the interaction between several hormones in the production of a stress response (Martian and Lunar regolith) in each treatment.

Cytokinin hormones (tZ or cZ), auxins (IAA), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), gibberellins (GA_4 active forms of Gibberellins) of all germinated species were quantified: *C. annuum* (pepper)*, L. sativa* (lettuce), *C. arietinum* (chickpeas) and *P. sativum* (pea). For cytokinins, auxins, and the abscisic acid profile, a fresh weight (FW) of 100 mg was placed in 2 mL microcentrifuge tubes along with a 5 mm zirconium oxide grinding ball. The samples were quickly frozen in liquid nitrogen and stored at -80 °C. Smaller samples were collected in a microcentrifuge tube with methanol (300 µL) in a microcentrifuge tube.

After the measurement of FW of each sample, homogenization was performed before purification. The samples were placed on a Teflon adapter (15 min) to maintain the temperature of -80 °C while ground in an MM 301 (Retsch GmbH) mixer mill at 25 Hz frequency for 2 minutes. After the sample was fully homogenized, 0.5 mL of cold extraction solvent (-20 °C) was added to the tubes (for cytokinins and auxin - 75% methanol and formic acid solution and for abscisic acid - 75% methanol and methylene chloride). Also, 50 µL of internal standards were added, mixed, and left at -20 °C for 1 hour.

The samples were centrifuged at 20,000g at 4°C for 20 minutes. The supernatant was then transferred to new 2mL tubes. The pellet obtained was extracted again using 0.5 mL of solvent (for each phytohormone) for 30 minutes. This process ensures more than 95% solvent extraction.

During the quantification phase of Liquid Chromatography Coupled to Mass Spectrometry (LC-MS Therm Scientific Orbitrap ID-X Tribrid), the dry samples were dissolved in 30 µL of 15% acetonitrile in water and 30 µL of 5% methanol in water. After being dissolved in the solution, the samples were centrifuged at 20,000g at 4 °C for 20 minutes. The supernatant was transferred to automatic sampler vials. An aliquot of 1/10 was injected in LC-MS for metabolite analysis. Calibration patterns were also injected to obtain the calibration curve parameters.

Statistical analysis

To evaluate the effect on seed germination when submitted to treatments with *U. lactuca* (0.2 and 0.4 $g \cdot L^{-1}$), statistical analysis was used to estimate the degree of significance between these different concentrations using the IBM® SPSS® Statistics software. The tests of homogeneity of the variance of the data were previously tested for parametric analyses of the data by the Shapiro-Wilk test. Unifactorial variance analysis (ANOVA) was performed to evaluate significant differences between each parameter of the different treatments in seed experiments. To evaluate the significant differences between the means of the groups from the ANOVA, the Tukey post hoc test was used. The data were defined as mean ± standard deviation, and the confidence interval for the difference of tests was 95% (p= 0.05).

Results

Effects of U. lactuca powder on germination and seedling emergence

Martian regolith analog MGS-1 Mars Global Simulant

Seed germination of cultivars *C. annum*, *P. sativum*, *C. arietinum,* and *L. sativa* was 100% in terrestrial soil but variable under the other treatments and in Martian regolith.

The germination percentages of *C. annum* seeds (**Figure 1A**) were not significantly distinct under the two concentrations: 0.2 and 0.4 $g \cdot L^{-1}$ of *U. lactuca* powder (p= 0.135 and p= 0.645, respectively) when compared with those of the control (MGS Control). However, the seedling emergence of *C. annum* (**Figure 2A**) was significantly different from that of the control (MGS Control) when applied at $0.4 g \cdot L^{-1}$ of *U. lactuca* powder (p= 0.001).

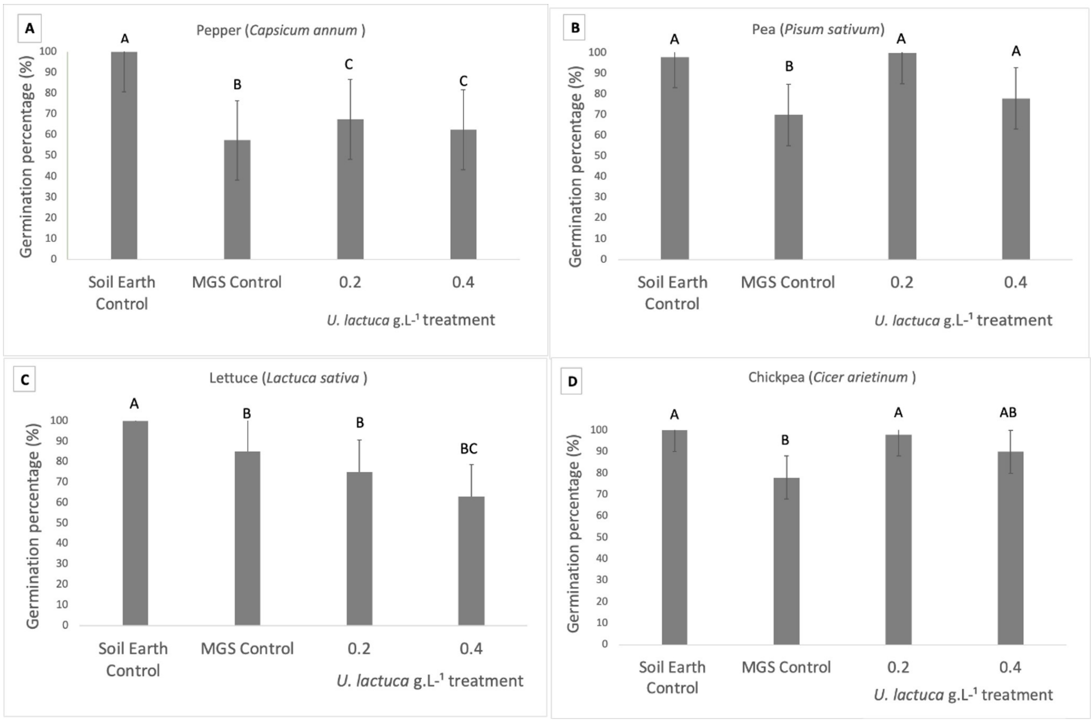

Figure 1. Germination percentage of *C. annum* **(A)**, *P. sativum* **(B)**, *L. sativa* **(C)**, and *C. arietinum* **(D)** in controls (terrestrial soil and Martian regolith MGS-1) and in treatments (0.2 and 0.4 g · L^{-1} of *U. lactuca* powder), n=4/10 subsamples (seeds) for each cultivar. Standard Error (SE) upper limits were not capped since germination was 100% with no distinctive ranges.

When applied at 0.2 g · L^{-1} in *P. sativum* seeds, germination percentage (**Figure 1B**) was higher at 97.5 ± 5% and significantly different (p= < 0.001) compared to the MGS Control (70 ± 8.2 %). Treatments subjected to lower concentrations of *U. lactuca* powder on *P. sativum* seeds grown in Martian regolith obtained a germination percentage about as good as when cultivated in terrestrial soil (97.5 ± 5%). Among the concentrations, 0.2 and 0.4 g · L^{-1} of *U. lactuca* powder, there was a significant difference (p= 0.001) where the concentration in a smaller amount favored the development of germination of *P. sativum* seeds. Regarding seedling emergence (**Figure 2B**), the best concentration for *P. sativum* seeds was also 0.2 g · L^{-1} compared to the control (MGS Control p= < 0.001), and there was a significant difference between treatments.

The percentage of germination of *L. sativa* (**Figure 1C**) submitted to 0.4 g · L^{-1} of *U. lactuca* powder was significantly different (p= 0.025) compared to the control (MGS Control). *Lactuca sativa* seeds germinated well in Martian regolith (85 ± 5.7 MGS Control) without any treatment with *U. lactuca*. When applied 0.2 g · L^{-1}, there was no significant difference (p= 0.470) in relation to the control, as there was no difference between treatments (p= 0.291). Concentrations of 0.2 g · L^{-1} (p= 0.064) and 0.4 g · L^{-1} (p= 0.892) exerted significantly different effects compared to MGS Control on the development of *L. sativa* seedlings (**Figure 2C**).

Capsicum arietinum seeds (**Figure 1D**), when cultivated in Martian regolith and submitted to the treatment of 0.2 g · L^{-1}, had a significantly different percentage of germination in relation to the MGS Control (p= 0.006). Furthermore, the percentage of seed germination (97.5 ± 5 %) was different compared to that in terrestrial soil (100 ± 0 %). The development of *C. arietinum* seedlings (Figure 2D) was significantly different in relation to the MGS Control (p= 0.01) when subjected to lower amounts.

In lunar analog Farside Lunar Highlands Simulant

Seed germination of the cultivars *C. annum*, *P. sativum*, *C. arietinum,* and *L. sativa* was total (100%) in terrestrial soil and very similar in all cultivars, but variable under the other treatments and in lunar regolith, as well as in seedling emergence.

Capsicum annum seeds (**Figure 3A**) did not germinate, with a significant difference between the 0.2 and 0.4 g · L^{-1} treatments and the LHS control (p=1.00 and p= 0.35, respectively). However, when small amounts of *U. lactuca* powder were applied, seed germination was stimulated (90.0 ± 14.1%) compared to the corresponding LHS control (85.0 ± 5.8%).

Regarding seedling emergence (**Figure 4A**), both treatments (0.2 and 0.4 g · L^{-1}) were not significantly different from the control (LHS control p= 0.224 and p= 0.655m, respectively). However, when small amounts of *U. lactuca* powder were

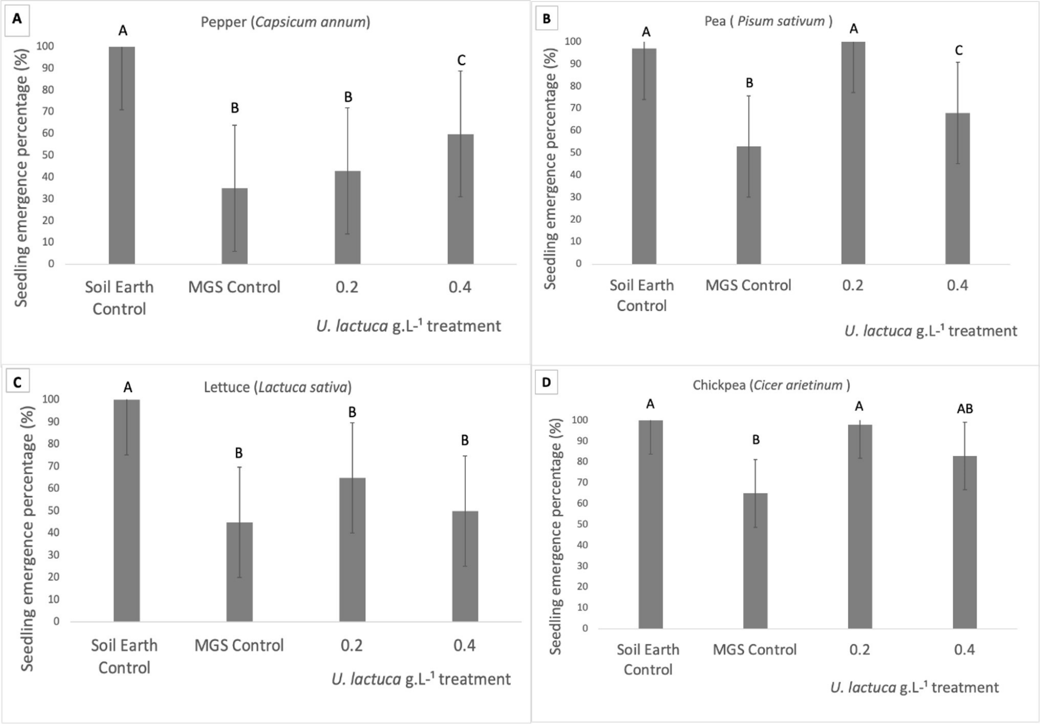

Figure 2. Percentage of the emergence of seedlings of *C. annum* **(A)**, *P. sativum* **(B)**, *L. sativa* **(C)** e *C. arietinum* **(D)** in controls (terrestrial soil and Martian regolith MGS-1) and treatments (0.2 and 0.4 g · L^{-1} *U. lactuca* powder), n=4/10 subsamples (seeds) for each cultivar. Standard Error (SE) upper limits were not capped since germination was 100% with no distinctive ranges.

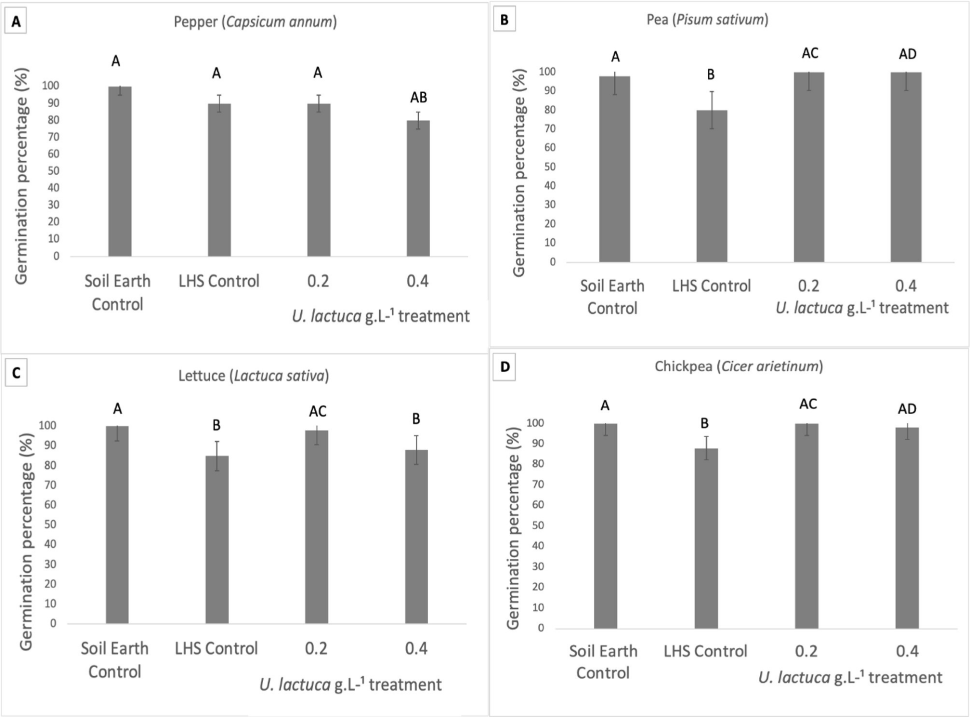

Figure 3. Percentage of germination of *C. annum* **(A)**, *P. sativum* **(B)**, *L. sativa* **(C)**, and *C. arietinum* **(D)** in controls (terrestrial soil and lunar regolith LHS-1) and treatments (0. 2 and 0. 4 g · L^{-1} *U. lactuca* powder), n=4/10 subsamples (seeds) for each cultivar. Standard Error (SE) upper limits were not capped since germination was 100% with no distinctive ranges.

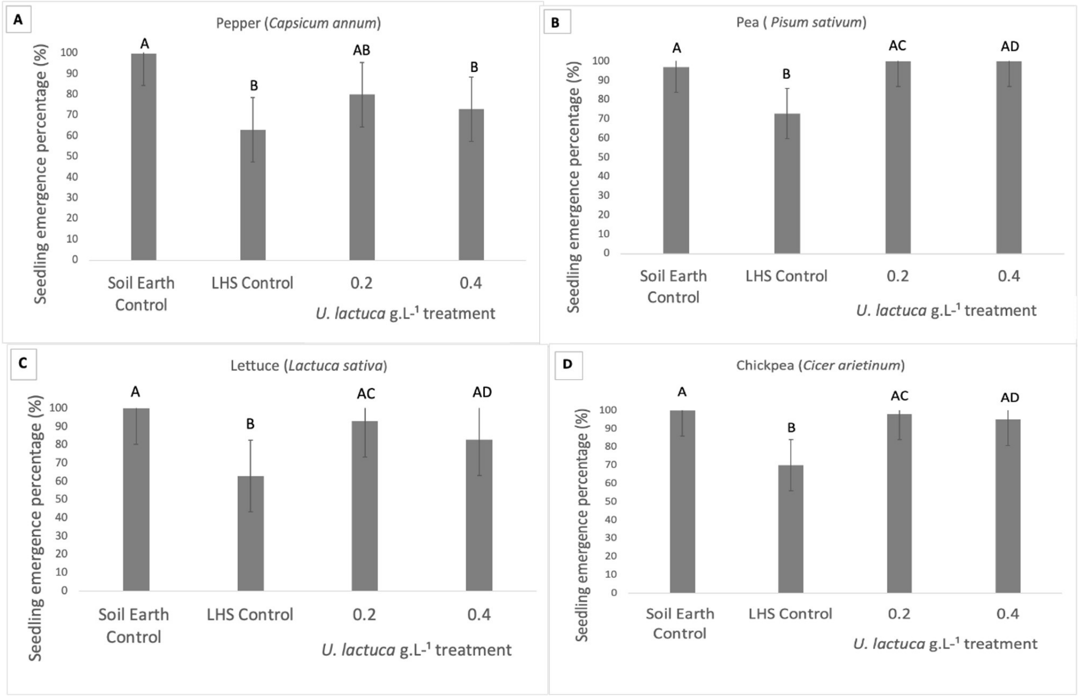

Figure 4. Percentage of the emergence of *C. annum* **(A),** *P. sativum* **(B),** *L. sativa* **(C),** and *C. arietinum* **(D)** in controls (terrestrial soil and lunar regolith LHS-1) and treatments (0. 2 and 0. 4 g · L^{-1} *U. lactuca* powder), n=4/10 subsamples (seeds) for each cultivar. Standard Error (SE) upper limits were not capped since germination was 100% with no distinctive ranges.

applied, the percentage of *C. annum* seedling development was stimulated (80.0 ± 20 %) compared to the corresponding LHS control (63.0 ± 12.5 %).

The seeds of *P. sativum* (**Figure 3B**) had a significant percentage of germination in relation to the control (LHS control) in both concentrations (0.2 $p = < 0.001$ and 0.4 g · L^{-1}; $p = 0.001$). The treatment, when applied in proportions of 0.2 and 0.4 g · L^{-1} of *U. lactuca* powder, seeds grown in lunar regolith resulted in a germination percentage (100 ± 0 %; 100 ± 0 %) considerably as good as seeds grown in terrestrial soil (97.5 ± 5 %).

The results with *P. sativum* seeds were also favorable in relation to seedling emergence (**Figure 4B**), with a significant difference between the control (LHS Control) and the two concentrations of 0.2 and 0.4 g · L^{-1} of *U. lactuca* powder ($p = < 0.001$; $p = 0.001$).

In *L. sativa*, the germination percentage (**Figure 3C**) was significantly different when the seeds were submitted to 0.2 g · L^{-1} of *U. lactuca* powder in relation to the control (LHS Control $p = 0.010$). Regarding the two treatments, 0.2 and 0.4 g · L^{-1} of *U. lactuca* powder, the one with the lowest concentration, had a difference ($p = 0.04$), which suggests that for *L. sativa* seeds, the best treatment is 0.2 g · L^{-1} of *U. lactuca* powder.

The percentage of L. sativa seedling emergence (**Figure 4C**) was significantly different in both concentrations in relation to the control (LHS Control $p = 0.001$ and $p = 0.023$). However, when subjected to lower concentrations, the percentage of seedling emergence was as good as in terrestrial soil (92.5 ± 5%; 100 ± 0%).

The percentage of germination in *C. arietinum* seeds (**Figure 3D**) was significantly different from the control (LHS Control) in both concentrations: 0.2 and 0.4 g · L^{-1} of *U. lactuca* powder ($p = 0.002$; $p = 0.008$, respectively).

The percentage of the emergence of *C. arietinum* seedlings (**Figure 4D**), when compared to the control (LHS Control), was also significantly different in both concentrations (both $p = < 0.001$) and can be considered adequate to treat seeds of this cultivar.

Relationship between germination speed and seedling emergence to lunar and Martian regolith.

The germination speed of cultivar seeds developed in the lunar regolith was lower than in the Martian regolith ($p = 0.035$) (**Figure 5**). The same pattern was observed in the emergence of seedlings, with the seeds in Mars regolith developing more rapidly ($p = 0.023$).

Indicators of abiotic stresses

In all seeds, the antagonist reaction between ABA and GA occurred to promote dormancy breakage and the beginning of germination development. In addition, all seeds had high peaks of phytohormones when exposed to 0.4 g · L^{-1} treatment compared to the control.

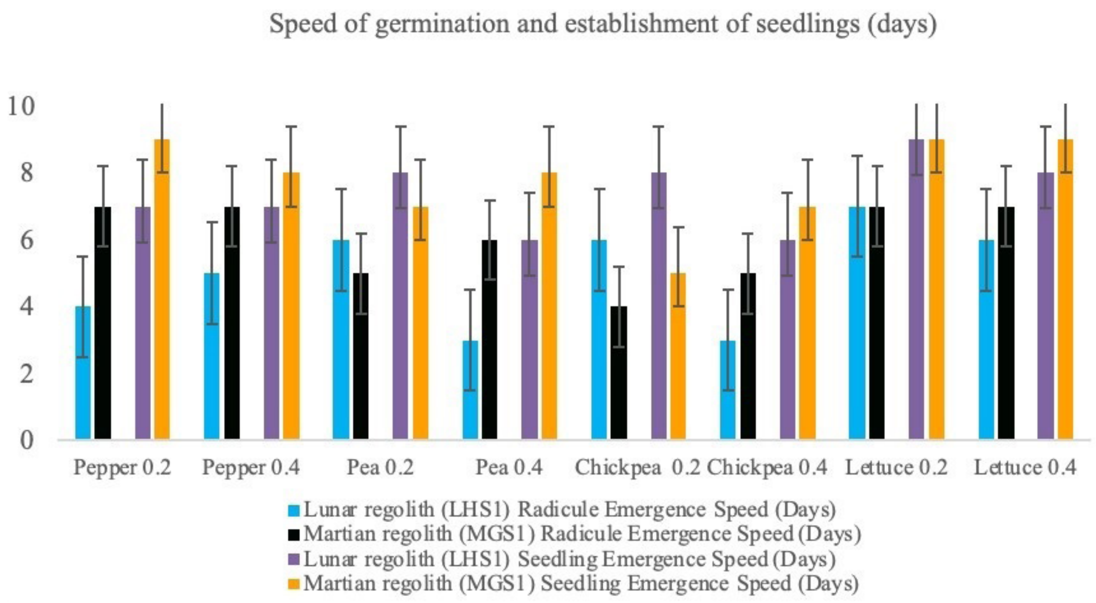

Figure 5. Germination speed and seedling emergence of cultivars developed in lunar and Martian regolith when treatment was applied at concentrations of 0.2 $g \cdot L^{-1}$and 0.4 $g \cdot L^{-1}$.

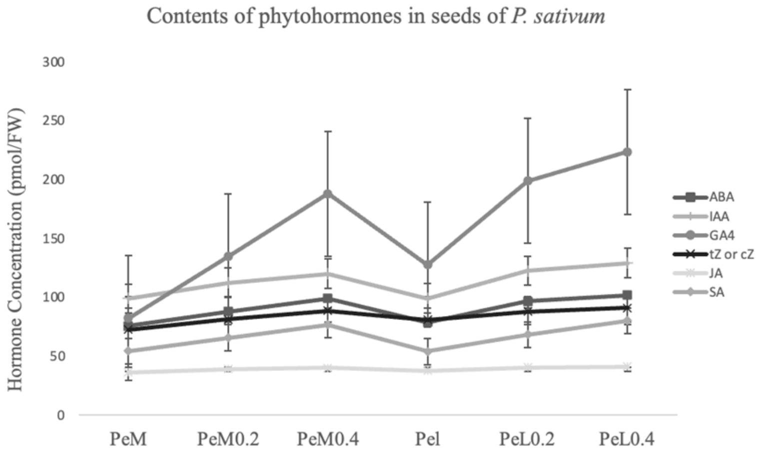

Figure 6. Contents of phytohormones in seeds of *P. sativum* (pmol/FW) with mean and standard deviation. PeM: Martian regolith pea. PeM0.2: Pea Martian regolith concentration of 0.2 $g \cdot L^{-1}$. PeM0.4: Pea Martian regolith concentration of 0.4 $g \cdot L^{-1.}$ PeL: Lunar regolith pea. PeL0.2: Pea lunar regolith concentration of 0.2 $g \cdot L^{-1}$. PeL0.4: Pea lunar regolith concentration 0.4 $g \cdot L^{-1.}$

Pisum sativum seeds (**Figure 6**) had lower ABA peaks in the Martian regolith (75.8 pmol/FW) and lunar regolith (78.9 pmol/FW). However, when exposed to concentrations of *U. lactuca*, the levels of these phytohormones were high, with peaks of GA_4 in Martian regolith (188.1 pmol/FW) and lunar regolith (223.06 pmol/FW) when applied 0.4 $g \cdot L^{-1}$ compared with control (82.3 pmol/FW; 127.8 pmol/FW).

Cicer anuum seeds (**Figure 7**) had peaks of GA_4 in Martian regolith (96.9 pmol/FW) and lunar regolith (102.9 pmol/FW) when applied 0.4 $g \cdot L^{-1}$, compared with control (70.1 pmol/FW; 88.9 pmol/FW). In addition to IAA peaks in Martian regolith (96.9 pmol/FW) and lunar regolith (98.9 pmol/FW) when subjected to a concentration of 0.4 $g \cdot L^{-1}$ compared to control (79.5 pmol/FW; 80.7 pmol/FW, respectively).

Lactuca sativa seeds (**Figure 8**) had IAA peaks in Martian regolith (93.9 pmol/FW) and lunar regolith (97.4 pmol/FW) when 0.4 $g \cdot L^{-1}$ was applied, compared with the control (72.8 pmol/FW; 83.9 pmol/FW). In addition to GA_4 peaks in Martian regolith (89.2 pmol/FW) and lunar regolith (98.9 pmol/FW) when submitted to a concentration of 0.4 $g \cdot L^{-1}$ compared to the control (66.9 pmol/FW; 78 pmol/FW), respectively.

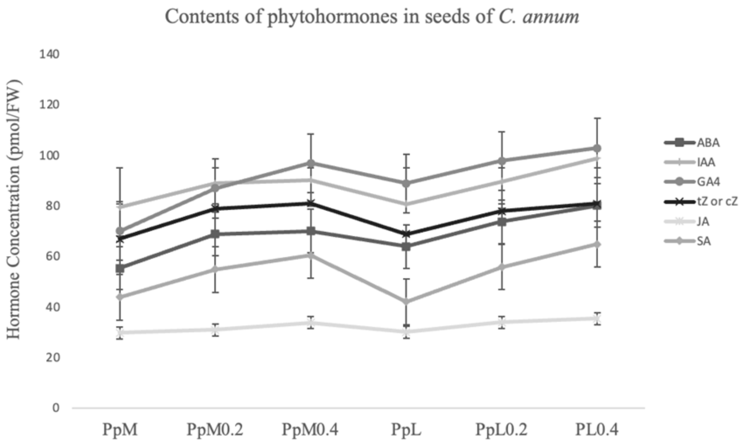

Figure 7. Contents of phytohormones (pmol/FW) in seeds of *C. annum* with mean and standard deviation. Peak hormonal concentration for each type of regolith, Martian, and lunar. Legend: PpM: Pepper Martian regolith. PpM0.2: Martian regolith pepper with a concentration of 0.2 g · L^{-1}.PpM0.4: Martian regolith pepper with a concentration of 0.4 g · L^{-1}. PpL: Lunar regolith pepper. PpL0.2: Lunar regolith pepper with a concentration of 0.2 g · L^{-1}. PpL0.4: Lunar regolith pepper with concentration 0.4 g · L^{-1}.

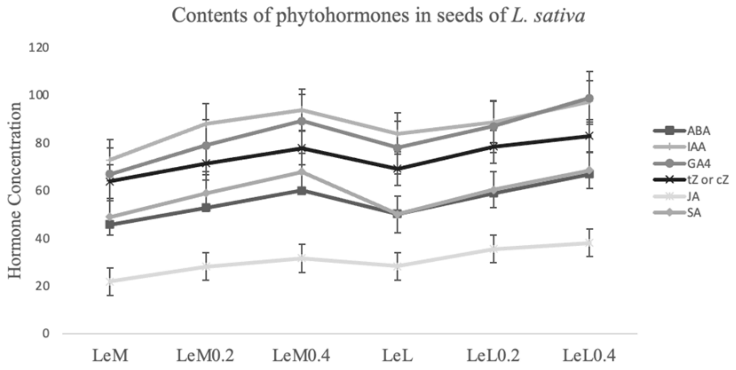

Figure 8. Contents of phytohormones (pmol/FW) in seeds of *L. sativa* with mean and standard deviation. Peak hormonal concentration for each type of regolith, Martian, and lunar. Legend: LeM: Lettuce Martian regolith. LeM0.2: Martian regolith lettuce with a concentration of 0.2 g · L^{-1}.LeM0.4: Martian regolith lettuce with a concentration of 0.4 g · L^{-1}. LeL: Lunar regolith lettuce. LeL0.2: Lunar regolith lettuce with a concentration of 0.2 g · L^{-1}. LeL0.4: Lunar regolith lettuce with a concentration of 0.4 g · L^{-1}.

Cicer arietinum seeds (**Figure 9**) had peaks of phytohormones in both regoliths when submitted to a concentration of 0.4 g · L^{-1}, such as ABA peaks (101.6 pmol/FW) in Martian regolith and lunar regolith (104.8 pmol/FW). In addition, there were peaks of IAA (128.2 and 121.1 pmol/FW) and GA$_4$ (219.7 and 156.9 pmol/FW) in both simulated lunar and Martian regolith, respectively, when subjected to higher amounts of *U. lactuca* powder, compared to the control.

Qualitative chemical analysis of U. lactuca

In the phytochemical analysis of seaweed constituents (**Table 3**), the results of gas chromatography-mass spectrometry (GC-MS) evidenced the presence of alkaloids, terpenoids, flavonoids, fatty acids, and bioactive compounds such as 5-Octadecenal, 1-Trichananol, Neophytadiene, Lactaropallidin, Phytol, Lucenin, and Vincadifformine in *U. lactuca*.

Proposition of the macroalgae U. lactuca as a seed priming technique

The evidence presented here was combined with previous knowledge in the corresponding research area to propose a model regarding the possible interaction of secondary metabolites of *U. lactuca* and its precursors of phytohormones as plant growth regulators (**Figure 10**) to be used in a seed priming technique.

Discussion

The present work evaluated the effect of different concentrations of *U. lactuca* powder in the germination and growth of the cultivars *C. annuum*, *L. sativa*, *C. arietinum,* and *P. sativum.* Terrestrial soil was expected to perform

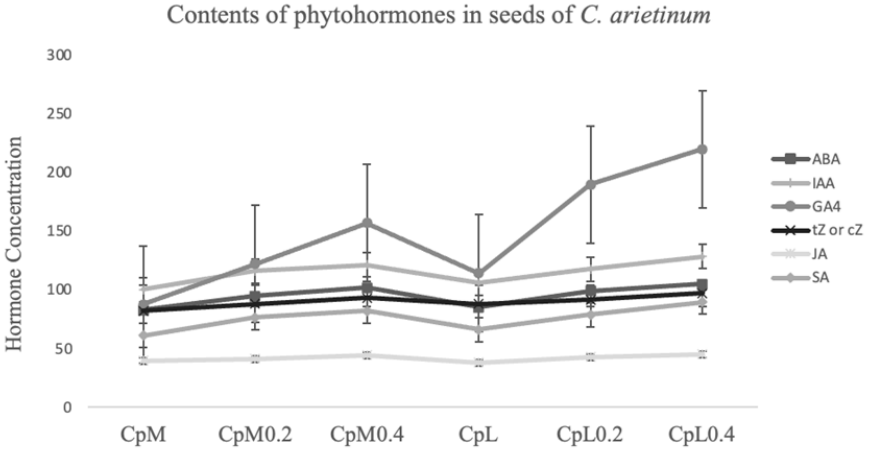

Figure 9. Contents of phytohormones (pmol/FW) in seeds of *C. arietinum* with mean and standard deviation. Peak hormonal concentration for each type of regolith, Martian, and lunar. CpM: Martian regolith chickpea. CpM0.2: Martian chickpea regolith concentration of 0.2 g $g \cdot L^{-1}$. CpM0.4 chickpea Martian regolith concentration of 0.4 $g \cdot L^{-1}$. CpL: lunar regolith chickpea. CpL0.2: lunar regolith chickpea concentration of 0.2 g. $g \cdot L^{-1}$. CpL0.4: chickpea lunar regolith concentration 0.4 $g \cdot L^{-1}$.

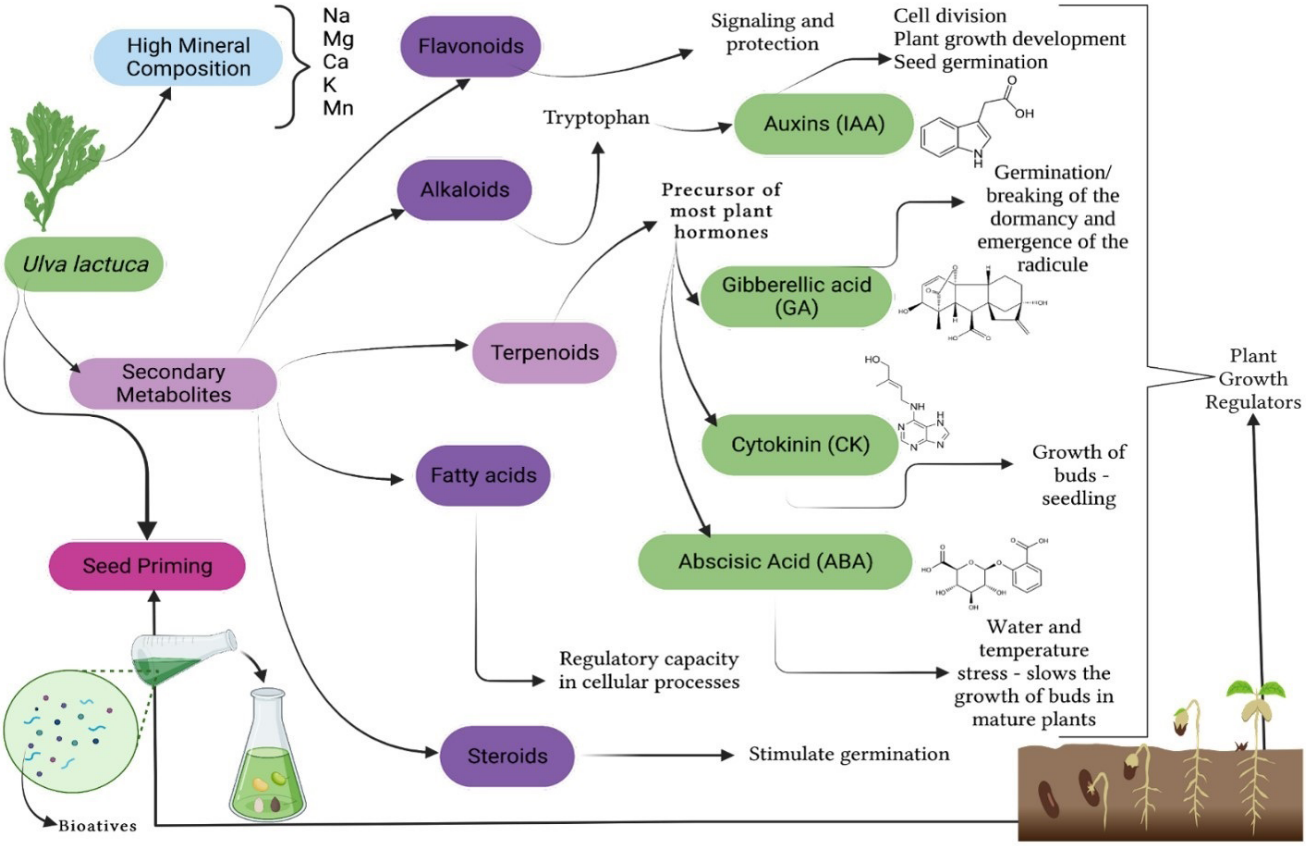

Figure 10. Hypothetical model representing the interaction of secondary metabolites of *U. lactuca* and their phytohormone precursors as plant growth regulators.

Table 3. *U. lactuca* qualitative *analysis.*

Chemical class	Compounds
Alkaloids	Vincadifformin Quinines
Terpenoids	Neophytadiene
	Phytol
	Lactaropallidin
Flavonoids	Lucenin 2
	Quercetin 7.3′,4′-Trimethoxy
Steroids	Digitoxigenin
Fatty acids	5-Octadecenal
	Erucic Acid
	9-Octadecenoic acid
	1-Tricosanol
	n-Hexadecanoic acid
	Myristate Isopropyl
Phenols	Uncharacterized

better than both regoliths, Martian and lunar. These results obtained indicate that the technique of seed priming using the marine macroalgae *U. lactuca* in small amounts (0.2 g · L^{-1}) is considered an excellent biostimulant to increase the percentage of seed germination under stressful conditions, mainly on *C. arietinum* and *P. sativum* in both simulated, Martian and lunar. Our results follow the previous study that documented the same patterns of responses using lower *U. lactuca* concentrations in the treatment of tomato seeds (*Solanum lycopersicum* L.) under stressful conditions and which related the invigoration of seeds to the bioactive substances present in this macroalga (Hernández-Herrera et al., 2016).

Lactuca sativa seeds developed well in lunar regolith when 0.2 g · L^{-1} of *U. lactuca* powder was applied for germination and seedling emergence. In Martian regolith, germination rates were satisfactory for the seeds without any addition of biostimulant (control). However, regarding seedling emergence, it was not satisfactory with the probability of loss of this cultivar.

Macroelements present in marine macroalgae (Hernández-Herrera et al., 2016), as well as in Martian regolith (Caporale et al., 2020; Eichler et al., 2021), may slow or positively affect the osmotic potential of plant seeds (Castellanos-Barriga et al., 2017; Eichler et al., 2021). The matric potential of *L. sativa* seeds may have been influenced by the presence of these macro elements of the Martian regolith, reducing the metabolic capacity of seeds and the development as seedlings in the culture medium due to high regolith toxicity (MGS-1) and because their pH is significantly more alkaline (pH > 9) (Eichler et al., 2021). Under these conditions, the addition of the macroalga did not increase the percentage of seed germination; including the addition of *U. lactuca*, to a greater extent, may affect the yield or formation of radicle and plumule (Mzibra et al., 2018; Reis et al., 2020).

In Martian regolith, the seeds of *C. annuum* performed well concerning seedling emergence when higher amounts of biostimulant (0.4 g · L^{-1}) were applied. The more acidic pH of *U. lactuca* in higher concentration (0.4 g · L^{-1}, pH= 5.36) probably promoted *C. annuum* germination in simulated Martian regolith. Most acid extracts of brown seaweed (*Ascophyllum nodosum* and *Fucus vesiculosus*) and acid extracts of green macroalgae *U. lactuca* were also beneficial for *Vigna radiata* and *Vigno mungo* seeds (Sharma et al., 2012; Castellanos-Barriga et al., 2017) and influenced the length of the shoots, root length, and fresh weight of seed plants denoting with bioactive of these algae.

The differential responses of *Capsicum annuum*, *Lactuca sativa*, *Cicer arietinum*, and *Pisum sativum* to the regolith simulants and *Ulva lactuca* powder may be attributed to their distinct physiological and genetic adaptations. For example, *Capsicum annuum*'s relatively robust root system may render it more capable of exploiting the biostimulant properties of *U. lactuca*. In contrast, the faster metabolic rates observed in *Pisum sativum* may facilitate quicker adaptation to the nutrient stresses imposed by regolith simulants (Kulkarni et al., 2099; Sánchez et al., 2004)

Ulva spp. biostimulants were applied at different concentrations to promote seed germination and root induction in plants that grew under stressful environments or were subjected to stressful conditions (El Boukhari et al., 2021; Zouari et al., 2022). The results were positive for those that were treated at low dosages (e.g., 0.1 and 0.2 %), while high concentrations (e.g., dosages over 1%) inhibited seed performance and seedling development (Hernández-Herrera et al., 2014; Castellanos-Barriga et al., 2017; Reis et al., 2020; Hamouda et al., 2021) according to the methodology used for the preparation of liquid extracts of seaweed. In addition, if applied to seeds before stress occurs, the biostimulant helps the plant to become more tolerant (Borges et al., 2014; Kasim et al., 2022)

Macroalgae of the genus *Ulva* contain elements such as Na, K, Ca, Mg, Cl, and NO_3 (Khan et al., 2009; Craigie, 2011; Tabarsa et al., 2012; Hussein et al., 2021) and, in simulated Martian and lunar regolith, it contains macro elements such as potassium (K_2O), phosphorus (P_2O_5), magnesium (MgO) and calcium (CaO) (Caporale et al., 2020; Eichler et al., 2021). Notably, lunar regolith (LHS-1) promoted a faster seed germination rate than Martian regolith (MGS-1). The fact of its best performance can be explained by its high concentrations of calcium in its composition (12.8 wt%), as it is known to stimulate the production of gibberellins (Taiz et al., 2015). Furthermore, most seeds grown in lunar regolith with lower amounts of *U. lactuca* (0.2 g · L^{-1}) had a higher germination potential than the control.

Radish seeds (*Raphanus sativus*) were pre-soaked with biostimulants based on two macroalgae, *Codium taylorii* and *Pterocladia capillacea,* for two hours before sowing. Using these macroalgae in pre-sowing allowed better metabolic development in everyday and stressful situations (Kasim et al., 2022). Similarly, Hamouda et al. (2022) evaluated the effect of seed priming in different concentrations *of Ulva linza* and *Corallina officinalis* extracts on wheat germination (*Triticum aestivum* L.). They provided a good percentage of germination and seedling emergence in relation to control.

Seed preparation increases metabolic activities in pre-germination and antioxidant activities and accelerates metabolic recovery of stress-degraded membranes (El Boukhari et al., 2021). This technique increases the biomass of the adult plant, contributes to better metabolism performance, and adds bioactive compounds necessary for human food (Hamouda et al., 2022), such as phytohormones that are plant growth regulators (PGRs).

García et al. (2020) identified PGRs in extracts of *U. lactuca* and found higher concentrations of GA_4, highlighting the potential use of this macroalgae as a biostimulant. They were found in species of *Ulva* auxins (indole-3-acetic acid, IAA, and indole-3-butyric acid, IBA), gibberellins (gibberellic acid, GA_3), ABA, cytokines (ribocytic kinetin, KR), and salicylic acid, which may act in the inhibition or acceleration of seed germination (Crouch van Staden, 1992; Gupta Abu-Ghannam, 2011).

Gupta and Abu-Ghannam (2011) mention that the interactions of the different metabolic pathways of phytohormones regulate various plant physiological processes. Phytohormones play an essential role in the regulatory process of plant growth and development. They are essential chemical messengers, allowing plants to develop when subjected to some stress factor (Rhaman et al., 2020), as in the case of regolith. For example, auxins are essential in plant growth and development, promoting cell division, maintaining meristem, organogenesis, cell standardization, and root and stem development. Cytokines regulate the development of roots and stems, while gibberellins and abscisic acid control germination and stimulate germination and dormancy induction (Crouch van Staden, 1992; Gupta&d Abu-Ghannam, 2011).

For germination to occur in the first stages of development, it is essential to have an antagonistic reaction between ABA and GA to promote dormancy breakdown (Taiz et al., 2015; Tuan et al., 2018). In addition, it is known that both cytokinin and auxin interact synergistically to control specific essential processes, such as the maintenance of the meristem (Su et al., 2011). In the analysis of phytohormones of the seeds (pmol/FW), the expected occurred in relation to this balanced reaction between hormones ABA and GA. There was a decline in ABA and an increased GA sensitivity, which favored germination (Taiz et al., 2015; Tuan et al., 2018). However, when treated at higher concentrations ($0.4\ g \cdot L^{-1}$), phytohormone levels increased significantly, which may have caused stress.

Subjecting seeds to high concentrations can extend or delay plant germination time, and radicle and plumule formation can degenerate (Selvam Sivakumar, 2013; Hernandèz-Herrera et al., 2014, 2016; Mzibra et al., 2018). Hormonal peaks significantly differed in control at ideal concentrations ($0.2\ g \cdot L^{-1}$), which probably favored improved hormonal balance and metabolic development of most seeds in this study.

As in our study, previous phytochemical analysis of *U. lactuca* also revealed the presence of alkaloids, terpenoids, flavonoids, and fatty acids (Hernández-Herrera et al., 2014; García et al., 2020). These bioactives are present in the secondary metabolism of macroalgae of the genus *Ulva* (Anjali et al., 2019), and metabolic pathways are essential for forming phytohormones (Taiz et al., 2015). However, their performance in the metabolic pathways of plants remains poorly understood (Gupta et al., 2011). We propose a scheme for the possible action and interaction between the bioactive elements of *U. lactuca* and their action as biostimulants in seeds (**Figure 10**). Seed germination metabolism is directly induced by the response to seaweed application (El Boukhari et al., 2021). One can see the crosstalk between the ability of macroalgae secondary metabolite components, such as phenolic compounds, terpenes, and alkaloids, to influence the biosynthesis of phytohormones (Lepiniec et al., 2006; Dumas et al., 2010; Gupta et al., 2011; Jaulneau et al., 2011; Taiz et al., 2015). These compounds are of interest due to their antioxidant properties that act as influential protectors against biotic and abiotic stresses (Lepinec et al., 2006; García et al., 2020) and can act on seed germination. For example, terpenes are responsible for the biosynthesis of gibberellins, cytokinin, and abscisic acid (Taiz et al., 2015).

Flavonoids modulate auxin transport through direct and indirect interactions with cell transport and regulatory mechanisms and, in addition, are of current interest due to their antioxidant properties, which accumulate in seed coatings of various plant species, including peas, and which function as protective agents against biotic and abiotic stresses (Lepiniec et al., 2006). Alkaloids are essential in tryptophan biosynthesis, a precursor in auxin synthesis (Taiz et al., 2015; Jiang et al., 2022).

The nutritional deficiencies of lunar and Martian regolith can be overcome by producing nutrient-rich solutions. However, this cultivation method is not sustainable for space agriculture, as nutrients must be brought from Earth (Cannon Britt, 2019). A solution to mitigate this problem would be biostimulants based on marine macroalgal extracts because they are used in small quantities (Castellanos-Barriga et al., 2017; Reis et al., 2020; Hamouda et al., 2022) and mixed in regolith simulators with organic matter from *Ulva* spp. Since they contain bioactive substances, they may directly influence the growth of edible plants and the availability of nutrients in regolith-based agriculture (RBA).

Figuring out how to live on the Moon and Mars is the ultimate test of sustainability in agriculture, and learning to grow plants in a hostile environment can maximize the use of valuable and harmful resources, such as water and fertilizers (de Micco et al., 2009; Pickett et al., 2020). In addition, space agriculture will be decisive for the survival of humans outside the Earth, mainly because it is viable as a source of income (Cannon Britt, 2019). Biostimulants offer a potentially new approach to regulating/modifying physiological processes in plants to stimulate growth, reduce stress-induced limitations, and increase productivity (Yakhin et al., 2017; Pardilhó et al., 2022). The raw material of *Ulva* spp. may be available in natural banks and periodic blooms or from integrated multitrophic aquaculture, which turns our focus to this promising source of biostimulants as a nutritional additive to Martian and lunar regolith simulators.

Conclusion

The metabolism of the studied seeds and the emergence of seedlings were directly stimulated by the response of the application of the macroalgae *U. lactuca* because they contain chemicals that participate in the biosynthesis of phytohormones and prove the efficiency in stimulating germination. In priming seed techniques under stressful conditions, we recommend the use of *U. lactuca* in low concentrations (0.2 $g \cdot L^{-1}$) for chickpea seeds (*C. arietinum*) and peas (*P. sativum*) in Martian regolith. Both concentrations were satisfactory for lunar regolith for chickpeas (*C. arietinum*) and peas (*P. sativum*). For lettuce (*L. sativa*), the lowest concentrations were satisfactory. We recommend the lowest concentration (0.2 $g \cdot L^{-1}$) for the best cost-benefit; we propose further investigations and protocols for applying this biostimulant for its proper use, mainly to investigate the use of different concentrations. The use of *Ulva* spp. will be essential to connect biological systems with local natural resources (lunar and Martian regolith) to improve the feeding of interplanetary civilization and increase the chances of a self-sustaining establishment.

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