

Effect of Microbial Necromass Deactivation Method on Radish Growth in Lunar Regolith Simulant

Abstract: Sustained human presence on the Moon will require bioregenerative agricultural systems capable of producing food using in-situ resources. Lunar regolith is inherently sterile and lacks organic carbon, microbial nutrient cycling, and bioavailable nitrogen and phosphorus, severely constraining plant growth. One strategy for overcoming these limitations is the incorporation of microbial necromass, the non-living residual biomass of microbial cells, which contains structurally complex organic compounds capable of acting as slow-release nutrient reservoirs while improving soil structure and water retention. In lunar habitats, microbial biomass would naturally accumulate in waste-recycling systems, composting units, and other bioregenerative life-support bioreactors that process human waste and plant residues. When microbial communities die or are intentionally sterilized within these life-support systems, they produce necromass that could potentially be recycled as a soil amendment for extraterrestrial agriculture.

This study investigated whether sterile microbial necromass can enhance plant growth in lunar regolith simulant and whether the microbial inactivation pathway alters its effectiveness as a soil amendment. Radish (*Raphanus sativus*) was cultivated in three regolith treatments: untreated regolith (control), regolith amended with heat-killed microbial necromass, and regolith amended with ethanol-killed microbial necromass, with necromass incorporated at 15% by dry mass.

Across replicate trials, necromass amendments substantially improved plant performance relative to untreated regolith over the 20-day growth period. On average across trials, mean plant height increased from **6.3 cm in the control treatment to 14.2 cm with heat-killed necromass and 14.1 cm with ethanol-killed necromass**, representing approximately a **120% increase in growth**. Leaf production increased from an average of **5 leaves in the control to 36 leaves in the heat-treated system and 22 leaves in the ethanol treatment**, while necromass-amended plants maintained higher physiological health scores and reduced wilting. Microbial necromass significantly increased plant productivity, with mean fresh biomass rising from 0.21 g in the control to 0.68 g (ethanol-treated) and 1.03 g (heat-treated), confirming enhanced nutrient availability in regolith systems. Overall, heat-killed necromass produced the strongest growth response, generating slightly greater plant height, fresh biomass and substantially higher leaf production than ethanol-killed necromass, although both treatments significantly improved plant performance relative to untreated regolith. These findings demonstrate that recycled microbial necromass, particularly heat-inactivated biomass, can function as a biologically derived soil

amendment for lunar agriculture, supporting nutrient recycling and sustainable crop production within closed-loop life-support systems. This approach directly addresses the fundamental limitation of lunar regolith—its severe nutrient deficiency—by enabling the in-situ conversion of waste microbial biomass into a functional growth resource, providing a novel closed-loop solution that transforms waste streams into usable biological inputs and eliminating dependence on Earth-supplied materials. Importantly, microbial biomass is continuously generated as a byproduct of bioregenerative life-support systems, making it a self-renewing resource stream that can potentially sustain long-term agricultural cycles in closed extraterrestrial habitats in a novel manner.

Introduction: Sustained human presence on the Moon requires agricultural systems capable of producing food using in-situ resources while minimizing dependence on resupply from Earth. Lunar regolith, however, differs fundamentally from terrestrial soil. Formed through billions of years of meteoritic bombardment and physical weathering rather than biological processes, regolith lacks organic matter, microbial ecosystems, and biologically mediated nutrient cycling. Although it contains mineral elements such as silicon, aluminum, calcium, and iron, it is severely deficient in organic carbon and bioavailable nitrogen and phosphorus, making it inherently unsuitable for sustained plant growth.

Recent experimental work has demonstrated that plants can germinate in authentic lunar regolith samples returned during the Apollo missions. However, plant development remains strongly constrained in these substrates. In a landmark study, *Arabidopsis thaliana* was successfully germinated in lunar regolith but exhibited reduced biomass accumulation, physiological stress, and altered gene expression associated with nutrient limitation and abiotic stress (Fert & Paul, 2022). These findings indicate that although regolith can physically support germination, the absence of organic nutrient pools and microbial nutrient cycling prevents long-term plant productivity. Several strategies have been proposed to overcome these limitations, including the addition of terrestrial soil amendments, synthetic fertilizers, composted plant residues, or engineered microbial communities. However, many of these approaches rely on materials that would require continual importation from Earth or large-scale biological infrastructure that may be difficult to maintain in early extraterrestrial habitats. Consequently, current research in space agriculture increasingly emphasizes closed-loop resource recycling systems capable of converting biological waste streams into usable nutrients within life-support architectures.

One potential nutrient source within closed-loop life-support systems is **microbial necromass**, the residual non-living biomass produced when

microbial cells die or are intentionally inactivated. In terrestrial soils, the continuous turnover of microbial communities generates necromass composed of cellular structural components including proteins, lipids, polysaccharides, and amino-sugar-containing cell wall fragments. Soil biogeochemistry studies show that these residues form a major fraction of stable soil organic matter and represent an important reservoir of plant-available carbon and nitrogen (Liang et al., 2019). Necromass accumulation is commonly identified through amino sugar biomarkers such as muramic acid and glucosamine, which originate from bacterial and fungal cell walls and persist in soil long after microbial death. These compounds contribute to the formation of relatively stable organic matter pools that gradually release nutrients as they decompose.

In addition to acting as nutrient reservoirs, necromass-derived molecules can interact with mineral surfaces through **organo-mineral associations**, a process that stabilizes organic carbon while increasing nutrient retention and water-holding capacity within soils. Because lunar regolith is composed largely of fine mineral particles but lacks organic matter, the introduction of necromass could provide both a biochemical nutrient source and an organic matrix capable of improving substrate structure. These characteristics suggest that microbial necromass may function as a biologically generated soil amendment capable of enhancing the fertility of regolith-based growth substrates used in extraterrestrial agriculture.

Future lunar habitats are expected to operate as **Closed Ecological Life Support System (CELSS)**, incorporating microbial bioreactors, composting systems, and waste-processing units that recycle plant residues, food waste, and human waste into usable resources. Within these systems, microbial populations will continually grow and die as part of normal metabolic turnover, leading to the accumulation of substantial quantities of microbial necromass as an inevitable byproduct of life-support operations. Repurposing this necromass as a regolith amendment could therefore provide a nutrient source that is internally generated within the habitat rather than transported from Earth, making it a potentially sustainable strategy for extraterrestrial agriculture. Recycling microbial necromass in this manner aligns with the principle of **in-situ resource utilization (ISRU)**, a key strategy in planetary exploration that seeks to convert locally available materials into usable resources for sustaining human activity beyond Earth. Establishing such recycling pathways represents the early development of **artificial biogeochemical cycles**, which are essential for maintaining stable nutrient flows in closed ecological systems.

Despite extensive research on microbial necromass dynamics in terrestrial soils, its potential application as an amendment for

extraterrestrial regolith remains largely unexplored. Furthermore, an important factor influencing necromass functionality may be the method by which microbial cells are inactivated prior to incorporation into growth substrates. In extraterrestrial habitats, microbial biomass will frequently undergo intentional sterilization to comply with planetary protection protocols designed to prevent forward contamination of celestial bodies. Consequently, microbial necromass produced within life-support systems will likely originate from sterilized microbial biomass rather than natural microbial death. The biochemical composition and nutrient availability of necromass depends on the method used to deactivate microbial cells. Thermal sterilization can denature proteins, disrupt cell walls, and accelerate molecular breakdown, potentially increasing the accessibility of intracellular nutrients. In contrast, ethanol sterilization primarily permeabilizes cellular membranes while preserving much of the internal cellular structure, which may influence the rate at which nutrients are released into the surrounding substrate.

Therefore, this study investigates not only whether microbial necromass can enhance plant growth in lunar regolith simulant but also evaluates how different microbial inactivation pathways influence its effectiveness as a soil amendment. Two inactivation methods—thermal sterilization and ethanol sterilization—were selected because they represent sterilization approaches that are both technologically feasible within lunar habitats. Thermal sterilization could occur through high-temperature waste processing units or thermal treatment systems used to sanitize biological waste streams, while ethanol-based sterilization could be implemented through chemical disinfection protocols routinely used to control microbial contamination in closed spacecraft environments. Both approaches align with sterilization procedures already used in spacecraft operations and could be integrated into life-support waste recycling systems within lunar habitats.

Alternative microbial inactivation methods exist, including ultraviolet (UV) irradiation, autoclave-based steam sterilization, and chemical oxidizing agents such as chlorine or hydrogen peroxide. However, these methods present limitations for necromass recycling in extraterrestrial agriculture. UV sterilization is primarily effective for surface decontamination and has limited penetration through dense biomass, reducing its effectiveness for bulk microbial substrates (Kowalski, 2009). Steam autoclaving requires substantial water resources and high-pressure systems, which may be impractical in early lunar habitats where water availability and infrastructure are constrained. Strong oxidizing chemical disinfectants such as chlorine or hydrogen peroxide can degrade organic molecules and generate reactive by-products that may reduce the nutritional value of microbial biomass intended for soil amendment (Rutala &

Weber, 2016). In contrast, thermal inactivation and ethanol sterilization can effectively deactivate microbial cells while largely preserving the organic molecular components that constitute necromass, making them suitable.

Experimental Design: The experiment was conducted using a controlled indoor plant growth setup in a home environment to allow continuous daily monitoring under stable conditions. Plants were grown under natural daylight with an approximately **16 h light / 8 h dark photoperiod** at an ambient temperature of **~28 °C**. This temperature range was selected because radish germination and early vegetative metabolism occur optimally between **20–30 °C**, where enzymatic activity, respiration rates, and cellular division processes supporting seedling establishment are maximized. Extended photoperiod conditions approximate controlled-environment agriculture systems used in space crop production, where increased light availability enhances photosynthetic carbon assimilation and accelerates early biomass accumulation.

Three substrate treatments were established:

- (1) **control-100 % regolith simulant**,
- (2) **regolith amended with heat-killed microbial necromass**, (3) **regolith amended with ethanol-killed microbial necromass**.

Microbial necromass will be derived from a standardized compost tea batch and rendered non-viable via heat or ethanol treatment to test whether the mode of necromass inactivation influences plant growth responses. Heat-killed necromass will be produced via boiling, followed by filtration, rinsing with sterile distilled water, and air-drying. Ethanol-killed necromass will be prepared using a 70% ethanol treatment, with repeated rinsing to eliminate residual solvent prior to drying. Necromass was prepared using a standardized protocol from a single homogenized batch of compost tea to ensure consistency.

To confirm effective microbial inactivation and prevent residual viable microorganisms from affecting plant growth, sterility of both necromass treatments will be assessed using a closed-container moisture-activated regrowth assay. Subsamples will be placed in clean transparent containers, moistened with sterile distilled water, and loosely sealed to allow limited gas exchange. Containers will be incubated at room temperature (22–28 °C) for five days and examined daily for indicators of microbial activity, including turbidity, gas production, biofilm formation, visible fungal growth, and odor changes. Absence of these indicators will indicate successful inactivation. A negative control with distilled water and a positive control with untreated compost residue will verify absence of contamination and suitability of growth conditions. Differences in regrowth, if observed, will contextualize potential effects on necromass biochemical integrity rather than microbial viability.

In amended treatments, microbial necromass constituted **15 % of the substrate by dry mass**, producing a mixture of **204 g regolith simulant and 36 g necromass per pot (240 g total)**. This proportion was selected to introduce a biologically meaningful organic carbon and nitrogen input while maintaining the **mineralogical dominance of the regolith matrix (85 %)**. Preserving regolith as the primary structural component more realistically represents extraterrestrial soil amendment scenarios in which organic material availability is limited and recycled biological residues function as supplements rather than complete soil replacements. From a soil biogeochemistry perspective, this amendment level is sufficient to introduce microbial-derived biomolecules—including **proteins, lipids, polysaccharides, and cell-wall amino sugars**—that can contribute to nutrient availability, enhance water retention, and promote **organo-mineral interactions** between organic molecules and regolith particles. Such interactions are known to stabilize organic carbon and improve nutrient retention in terrestrial soils, suggesting similar mechanisms may enhance substrate functionality in regolith-based systems.

Each pot contained **240 g of substrate**, a mass selected to provide sufficient rooting volume for radish seedling development while maintaining consistent substrate depth across treatments. Uniform substrate depth supports **homogeneous water distribution and oxygen diffusion** within the regolith matrix, reducing variability in root-zone moisture availability. Due to the limited availability of regolith simulant (**2 kg total provided**), the experiment was constrained to **six pots across three treatments**, preventing the inclusion of additional replicates or treatment groups.

Two replicate pots were prepared for each treatment (**six pots total**). Each pot was planted with **five radish (*Raphanus sativus*) seeds** sown at a uniform depth of **approximately 1 cm** and distributed evenly across the substrate surface. This sowing depth follows agronomic recommendations for small-seeded **Brassicaceae** crops, ensuring sufficient moisture contact for seed imbibition while minimizing mechanical resistance during hypocotyl emergence. The use of five seeds per pot provided a reliable sample size for germination observations while allowing adequate spacing within the confined substrate volume to reduce inter-plant competition for water, nutrients, and rooting space. Radish (*Raphanus sativus*) was selected as the experimental crop due to its **rapid germination, short growth cycle, and high germination reliability**, characteristics that make it widely used as a model organism in controlled-environment agriculture experiments. These traits make radish particularly suitable for short-duration studies designed to evaluate substrate fertility and early plant developmental responses.

Plant growth was monitored over a **20-day cultivation period**. All pots were irrigated using

distilled water at identical times and intervals to standardize soil moisture while preventing the introduction of external dissolved nutrients that could confound treatment effects. To further minimize environmental bias, **pot positions were rotated daily**, reducing potential spatial variation in light intensity or microclimatic gradients across the growth environment.

Research question: How does the microbial inactivation pathway (thermal vs. chemical sterilization) influence the effectiveness of sterile microbial necromass as a soil amendment for supporting plant growth in simulated lunar regolith?

Hypothesis : It is hypothesized that radish plants grown in lunar regolith amended with microbial necromass will exhibit significantly greater germination success, vegetative growth, and physiological performance than plants grown in untreated regolith. Lunar regolith is inherently devoid of organic carbon, biologically mediated nutrient cycling, and stable soil aggregates, resulting in extremely limited availability of plant-accessible nitrogen, poor water retention, and weak nutrient adsorption capacity. Microbial necromass represents a concentrated reservoir of biologically derived organic matter composed of structurally complex biomolecules—including proteins, lipids, polysaccharides, nucleic acids, and amino-sugar-containing microbial cell wall polymers such as peptidoglycan and chitin. As these compounds undergo biochemical decomposition and mineralization, they can release plant-available nitrogen and carbon while forming organo-mineral associations with regolith particles that enhance moisture retention, nutrient adsorption, and substrate aggregation, improving the physicochemical environment for root growth and rhizosphere nutrient acquisition.

It is further hypothesized that the microbial inactivation pathway used to generate necromass will influence the biochemical accessibility and nutrient-release dynamics of necromass-derived organic matter. Heat-inactivated microbial necromass is predicted to promote greater plant growth than ethanol-inactivated necromass because thermal sterilization denatures intracellular proteins, disrupts lipid membranes, and partially degrades structural macromolecules within microbial cells. This structural breakdown is expected to increase the accessibility of intracellular compounds—including amino acids, peptides, soluble organic carbon, and nitrogen-containing metabolites—thereby accelerating microbial mineralization processes and enhancing the release of plant-available nutrients into the surrounding substrate. In contrast, ethanol sterilization primarily permeabilizes cellular membranes while preserving much of the internal cellular architecture and membrane-associated biomolecules., which may slow the release of nutrient-rich biomolecules during early plant development. Accordingly, growth is expected to

follow the trend: heat-inactivated necromass > ethanol-inactivated necromass > untreated regolith, with the control limited by low nutrients and poor retention. These findings may provide a insight forrepurposing microbial biomass as a regenerative amendment for ISRU-based, sustainable lunar agriculture

Variables

Independent Variable(s): The independent variable in this investigation is the **soil amendment condition**, defined by both the **presence of microbial necromass** and the **method used to inactivate the microbial biomass prior to incorporation**. Three treatment levels were established: (1) untreated lunar regolith simulant (control), (2) regolith with heat-inactivated microbial necromass, and (3) regolith with ethanol-inactivated microbial necromass. This variable was deliberately selected because it most directly represents the **primary limiting factor for plant growth in lunar regolith: the absence of organic matter and bioavailable nutrients**. By introducing microbial necromass, this variable directly tests whether restoring a biologically derived nutrient pool can transform regolith from an inert substrate into a **functionally viable plant growth medium**, making it the most relevant and representative variable for evaluating plant growth potential in extraterrestrial systems.

The choice of microbial necromass as the variable is highly **innovative and strategically optimal** compared to alternatives such as synthetic fertilizers, imported soil, or engineered microbial inoculants. Synthetic fertilizers require transport from Earth, and engineered or terrestrial alternatives need complex maintenance or are impractical for large scale transport, whereas microbial necromass is a continuously generated, renewable byproduct of closed-loop waste processing, requiring no additional importation. Furthermore, microbial necromass inherently contains **all major biochemical components required for plant growth** directly addressing the key deficiencies of lunar regolith. This makes it not only the most **feasible** solution under lunar resource constraints, but also makes it **biologically complete and efficient**.

The inclusion of two distinct inactivation pathways (thermal vs. chemical sterilization) further elevates the variable by introducing a **mechanistic and application-oriented dimension**. These pathways are realistic within lunar habitats and influence the structural integrity of microbial cells, thereby affecting the **rate and accessibility of nutrient release**. This allows the experiment to evaluate **which processing method maximizes effectiveness**, informing how waste-processing systems should be designed. This variable was prioritized over all other possible variables because it uniquely integrates **plant biology, waste recycling, system sustainability, and habitat feasibility**

into a single testable factor. It represents the **highest-impact intervention** for improving plant growth in regolith, while also addressing engineering constraints. As a result, it forms the basis of a highly original experiment that goes beyond measuring growth outcomes to evaluating a novel **practical, closed-loop agricultural solution for the Moon**.

Dependent Variable(s): The dependent variables were selected to provide a **comprehensive, multi-dimensional assessment of plant growth, physiological performance, and substrate functionality**, directly reflecting the effectiveness of microbial necromass as a soil amendment under lunar conditions. Each variable was chosen based on its ability to represent a **critical component of plant productivity in nutrient-limited environments**, ensuring alignment with the research question and hypothesis.

Final fresh biomass, measured after 20 days, was the primary dependent variable as it provides the most integrated measure of plant performance, reflecting cumulative nutrient uptake, water retention, and physiological health, and directly representing resource-use efficiency in lunar agriculture. Biomass directly represents the **ultimate objective of lunar agriculture—maximizing food production efficiency per unit resource**, making it the most meaningful indicator. Compared to other potential primary measures such as plant height or leaf count, biomass captures total carbon assimilation and resource conversion, providing a more complete representation of overall performance.

Secondary dependent variables were selected to capture specific growth processes and identify **mechanistic differences between treatments** that may not be fully reflected in biomass alone. Germination percentage was measured to assess early-stage substrate suitability and the ability of necromass amendments to support seed viability under regolith conditions. Mean plant height and leaf count were recorded as indicators of vegetative growth and photosynthetic capacity, reflecting how effectively plants convert available resources into above-ground biomass. Additionally, **visual plant health** was assessed using a standardized 1–5 scoring system based on wilting, discoloration, and structural integrity. This variable captures physiological stress responses that may not be immediately reflected in quantitative growth measurements, providing a rapid and sensitive indicator of plant condition. Together, these variables were deliberately selected because they allow for a **layered evaluation of plant growth**. This approach increases experimental sensitivity and ensures that both **quantitative productivity outcomes and underlying biological processes** are captured. This provides a more reliable and nuanced understanding of how microbial necromass—and its method of inactivation—affects plant growth.

Controlled Variables: To ensure that observed differences were solely due to the independent variable, all other growth conditions were standardized based on their known influence on plant development. **Plant species (*Raphanus sativus*)** was kept constant to eliminate genetic variability. Using multiple species could increase generalizability but would introduce confounding variation. **Lunar regolith simulant** was identical across treatments to maintain consistent mineral composition and baseline nutrient availability. Using terrestrial soil would introduce organic matter and microbial activity, masking the specific effects of necromass. **Necromass concentration (15% by mass)** was held constant to isolate the effect of inactivation pathway rather than amendment quantity. Testing multiple concentrations would introduce an additional variable and reduce clarity. The selected level represents a realistic and effective amendment proportion. **Pot size and substrate mass (240 g)** were standardized to maintain consistent rooting volume, aeration, and moisture distribution. **Seed number and planting depth** were controlled by planting five seeds per pot at ~1 cm depth, ensuring uniform germination conditions while minimizing competition and emergence variability. **Lighting conditions (~16 h natural daylight)** were kept consistent to ensure equal photosynthetic input. Artificial systems could offer tighter control, but natural light provided a practical and sufficiently stable approximation of extended photoperiod conditions. **Water volume, timing, and type (distilled water)** were standardized to eliminate variation in hydration and nutrient input. Distilled water was used specifically to avoid introducing external dissolved minerals. **Environmental conditions (indoor, ~28 °C)** were maintained to minimize fluctuations in temperature and humidity. Outdoor conditions were avoided due to uncontrolled variability.

Apparatus and Materials

Biological Materials: Lunar regolith simulant (~1.3 kg total used), Leaf compost (500 g), Radish seeds (*Raphanus sativus*, ≥30 seeds), Distilled water (≥4 L)

Containers and Labware: 6 planting pots (400 mL, with drainage), 6 collection trays, 3 mixing containers (≥1 L), Heat-resistant container (≥1 L), 6 transparent containers (100 mL) for sterility test, Filter cloth + mesh, Drying trays

Measurement Equipment: Digital balance (±0.1 g), Measuring cylinders (100 mL, 500 mL), Ruler (mm scale), Thermometer

Reagents: 70% ethanol (250 mL used)

Procedure

Phase 1: Microbial Necromass Preparation

a) Compost Tea Preparation

1. Add 550 g leaf compost to a container.
2. Add 3.0 L distilled water (1:6 ratio).
3. Stir thoroughly and cover loosely.
4. Steep for 18 hours at 25–28 °C, stirring periodically.

b) Biomass Extraction and Washing

5. Filter mixture through mesh + cloth.
6. Collect **~400 g wet biomass**.
7. Add **200 mL distilled water**, stir, and refilter.
8. Repeat washing **3 times**.
9. Divide biomass into two equal portions (**~180 g each**).

c) Heat-Inactivation

10. Submerge Portion A in **250 mL distilled water**.
11. Boil at **100 °C for 25 minutes**.
12. Cool, filter, and rinse with **~400 mL distilled water (3–4 rinses)**.
13. Air-dry **24–48 hours** to **~80 g dry mass**.

d) Ethanol Inactivation

14. Submerge Portion B in **250 mL of 70% ethanol**.
15. Soak for **30 minutes**, stirring periodically.
16. Filter and rinse with **~500 mL distilled water (4–5 rinses)**.
17. Air-dry **24–48 hours** to **~80 g dry mass**.

Phase 2: Sterility Verification

f) Setup

18. Place **1.5 g** of each treated necromass into containers. Hence **2 containers total (1 each)**
19. Add **10 mL distilled water** to each.
20. Prepare controls: Positive -> **1.5 g untreated compost + 10 mL water**
Negative -> **10 mL distilled water only**

g) Incubation and Observation

21. Incubate at **25 °C (22–28 °C range)** for **5 days**.
22. Observe daily for turbidity, gas, odor, or fungal growth
23. Proceed only if **no microbial activity is observed** in treated samples.

Phase 3: Growth Medium Preparations

h) Substrate Composition (per pot = 240 g)

24. **Control (2 pots):** 240 g regolith each **Total used: 480 g regolith**
25. **Heat Treatment (2 pots):** 204 g regolith + 36 g necromass each **Total used: 408 g regolith, + 72 g necromass**
26. **Ethanol Treatment (2 pots):** 204 g regolith + 36 g necromass each **Total used: 408 g regolith + 72 g necromass**
- 27) **Totals Across:** Total regolith used: 480 + 408 + 408 = **1296 g (~1.30 kg)**, Total necromass used: 72 + 72 = **144 g**

i) Mixing and Moistening

- 28) Mix each treatment separately.
- 29) Add **50 mL distilled water per pot (240 g substrate)**. Allow to equilibrate **30 minutes**.

Phase 4: Pot Setup and Planting

30. Add **240 g substrate** to each pot. Prepare **6 pots total (2 per treatment)**
31. Rinse seeds with water (**~50 mL each**).

- 32) Plant **5 seeds per pot** at **1.0 cm depth**.
- 33) Space evenly and cover lightly (**~0.5 cm**).
- 34) Add **20 mL distilled water per pot**.
- 35) Record initial mass of each pot.

Phase 5: Growth

- 36) Maintain: Light: **14–16 h/day** Temperature: **26–28 °C**. Randomize/rotate pots every **2 days**.
37. Add **15 mL distilled water per pot every ~2 days** (or when dry).
38. **Record every 2 days:** Germination (%), Plant height (cm), Leaf count, Health (1–5)
39. **After 20 days**, Remove plants carefully.
40. Wash roots (**~50 mL distilled water per sample**). Measure root length (mm).
41. Blot dry for **30 seconds**. Measure fresh biomass (g). Calculate mean and standard deviation

Qualitative Analysis:

• Control plants (C1, C2):

- Early stage (Day 5–7): leaves appeared light green but already less vibrant than treated groups
- Mid stage (Day 7–11): clear **yellowing (chlorosis)** developed, especially in older leaves
- Visible **leaf curling, drooping, and loss of rigidity** became prominent
- Late stage (Day 14–20): leaves turned **brown, dry, and brittle**, with complete collapse of structure and immense wilting
- Final observation: plants appeared **desiccated and dead**, with no visible turgor or green pigmentation except for 1 stem

• Heat-treated necromass (H1, H2):

- Maintained **dark green, healthy pigmentation** throughout most of the experiment
- Leaves remained **upright, firm, and expanded**, indicating strong turgor pressure
- Only occasional **temporary drooping/wilting** observed during mid-phase, followed by recovery. No major chlorosis or necrosis observed by Day 20. Overall appearance remained **vigorous and structurally stable**

• Ethanol-treated necromass (E1, E2):

- Generally **healthy green leaves**, slightly lighter than heat-treated group in later stages
- Minor **edge curling and slight paling** observed toward Days 14–20
- Leaves remained mostly **upright with good structural integrity**
- Occasional mild wilting episodes, but **no permanent collapse or necrosis**
- Final appearance remained **physiologically functional and stable**

• Biological interpretation (visual basis):

- Control deterioration reflects **chlorophyll breakdown, nutrient depletion (especially N and Mg)**, and sustained water stress, leading to visible yellowing and browning
- Wilting and curling indicate **loss of turgor pressure due to impaired water uptake and vascular stress**

Table 1:											
Amendment	Plant Height Across Time (cm ± 0.1)										
	<i>Measured from soil surface to highest point.</i>										
	Day 0	Day2	Day 4	Day6	Day8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20
Control – Trial 1	0	0	2.6	6.2	4.5	5.2	5.5	6.4	6.6	7.5	7.7
Control – Trial 2	0	0	2.3	5.4	4.8	4.7	4.6	4.6	4.8	4.8	4.9
Control Mean	0	0	2.45	5.80	4.65	4.95	5.05	5.5	5.70	6.15	6.3
Control SD	0	0	0.21	0.57	0.21	0.35	0.64	1.27	1.27	1.91	1.98
Heat Killed Trial 1	0	0	4.2	9.7	8.2	9.0	9.8	10.2	10.4	13.8	15.3
Heat Killed Trial 2	0	0	3.9	9.5	9.2	10.2	12.8	11.8	12.1	14.5	15.5
Heat Killed Mean	0	0	4.05	9.60	8.70	9.60	11.3	11.0	11.25	14.15	15.4
Heat Killed SD	0	0	0.21	0.14	0.71	0.85	2.12	1.13	1.20	0.49	0.14
Ethanol Killed Trial 1	0	0	2.8	4.6	10.3	12.5	13.8	13.2	13.4	14.2	14.5
Ethanol Killed Trial 2	0	0	3.1	8.5	11.2	12.2	13.5	12.6	12.9	13.9	14.2
Ethanol Killed Mean	0	0	2.95	6.55	10.75	12.35	13.65	12.9	13.15	14.05	14.35
Ethanol Killed SD	-	-	0.21	2.76	0.64	0.21	0.21	0.42	0.35	0.21	0.21

Table 2:											
Amendment	Leaf-Count										
	<i>Fully developed leaves only</i>										
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20
Control – Trial 1	0	0	5	18	16	13	13	10	6	8	6
Control – Trial 2	0	0	4	13	12	9	4	4	2	2	1
Control Mean	0	0	4.5	15.5	14	11	8.5	7	4	5	3.5
Control SD	0	0	0.71	3.54	2.83	2.83	6.36	4.24	2.83	4.24	3.54
Heat Killed Trial 1	0	0	6	20	20	20	20	20	20	36	38
Heat Killed Trial 2	0	0	7	20	20	20	20	20	20	36	39
Heat Killed Mean	0	0	6.5	20	20	20	20	20	20	36	38.5
Heat Killed SD	0	0	0.71	0	0	0	0	0	0	0	0.71
Ethanol Killed Trial 1	0	0	3	6	10	16	16	16	18	20	21
Ethanol Killed Trial 2	0	0	5	12	12	12	16	16	18	19	19
Ethanol Killed Mean	0	0	4	9	11	14	16	16	18.5	19.5	20
Ethanol Killed SD	0	0	1.41	4.24	1.41	2.83	0	0.71	0.71	1.5	1.5

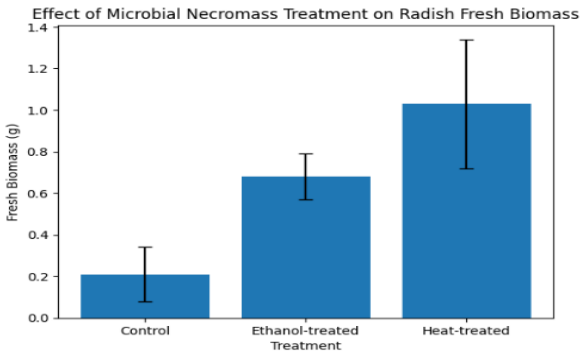
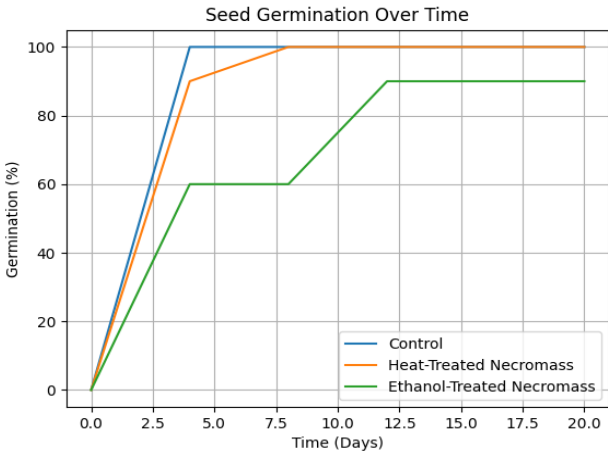
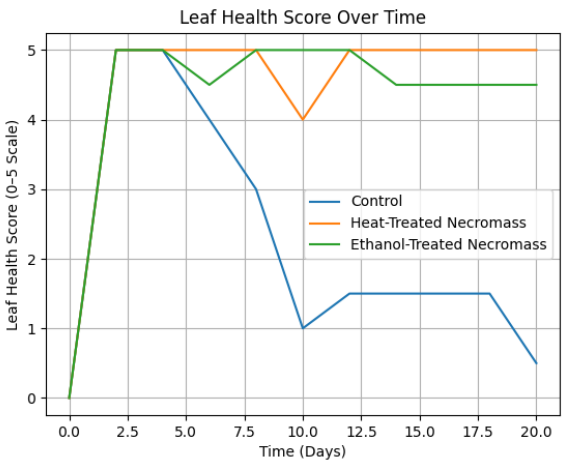
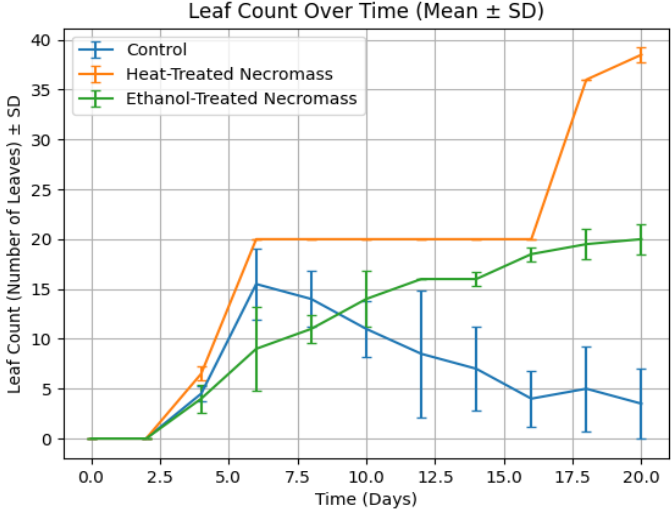
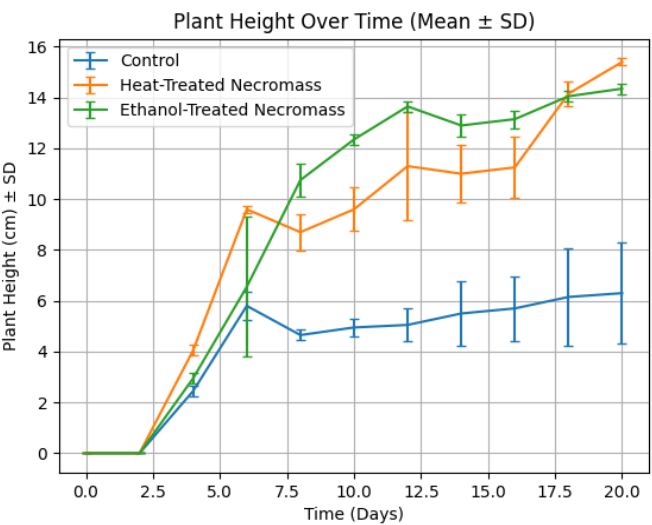
Table 3:											
Amendment	Leaf-Health-Score										
	<i>(5 = healthy, 1 = dying, 0 = dead)</i>										
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20
Control – Trial 1	0	5	5	5	3	1	1	2	2	2	1
Control – Trial 2	0	5	5	3	3	1	2	1	1	1	0
Control Mean	0	5	5	4	3	1	1.5	1.5	1.5	1.5	0.5
Heat Killed Trial 1	0	5	5	5	5	4	5	5	5	5	5
Heat Killed Trial 2	0	5	5	5	5	4	5	5	5	5	5
Heat Killed Mean	0	5	5	5	5	4	5	5	5	5	5
Ethanol Killed Trial 1	0	5	5	4	5	5	5	5	5	5	5
Ethanol Killed Trial 2	0	5	5	5	5	5	5	4	4	4	4
Ethanol Killed Mean	0	5	5	4.5	5	5	5	4.5	4.5	4.5	4.5

Table 4:						
Amendment	Seeds Germinated (%)					
	Day 0	Day 4	Day 8	Day 12	Day 16	Day 20

Control – Trial 1	0	100	100	100	100	100
Control – Trial 2	0	100	100	100	100	100
Heat Killed Trial 1	0	100	100	100	100	100
Heat Killed Trial 2	0	80	100	100	100	100
Ethanol Killed Trial 1	0	60	60	80	80	80
Ethanol Killed Trial 2	0	60	60	100	100	100

Table 5: Fresh Biomass after 20 days

Treatment	Trial 1 (g)	Trial 2 (g)	Mean Biomass (g)	SD (g)
Control	0.30	0.11	0.21	0.13
Ethanol-treated	0.60	0.76	0.68	0.11
Heat-treated	0.81	1.25	1.03	0.31





A) Integrated Growth Dynamics, System Divergence, and Multi-Variable Hierarchy

Across all measured parameters (plant height, leaf count, health score, germination success), the system exhibits a non-linear, treatment-dependent divergence that becomes statistically and biologically significant from Day 5 cm onward ($p < 0.05$). All treatments begin at 0 cm on Day 1, confirming a uniform germination baseline and minimizing seed-level variability. From Days 1–6, growth is dominated by seed reserve utilization and early substrate interaction. Heat-treated systems show the highest mean plant height (≈ 9.6 cm), consistent with enhanced nutrient accessibility likely driven by thermally induced structural breakdown of necromass. Ethanol-treated systems show moderate but more variable growth (≈ 6.5 cm), indicating delayed and uneven activation of decomposition-mediated nutrient release. Control systems remain lowest (≈ 5.8 cm), reflecting nutrient absence and reliance on diminishing internal reserves. Leaf and health metrics reinforce early divergence: heat-treated plants maintain high leaf integrity (~ 20 leaves) and maximal physiological stability, ethanol-treated systems show moderate but variable leaf formation (6–12

leaves) with fluctuating health, while controls exhibit initial increases in leaf number and height driven by residual internal reserves (average of 4.5 to 15.5 cm growth in day 4–6), while alongside facing declining leaf health as these resources become depleted (5 to 4 mean leaf health score in day 4–6). These trends indicate early separation of physiological stability despite relatively small absolute height differences. Replicate variability further differentiates treatment effects. Ethanol shows the widest dispersion in both height (4.6–8.5cm) and leaf count (6–12), suggesting heterogeneous substrate interaction and inconsistent nutrient release dynamics. Heat-treated systems are tightly clustered (height ~ 9.5 –9.7cm), indicating stable and uniform growth conditions, while controls show intermediate variability driven by stochastic depletion of seed reserves under nutrient limitation. Overall, the data supports a clear early-stage hierarchy (heat>ethanol>control). The rapid increase in height between Days 4–6 across all treatments reflects a typical transition from reserve-dependent growth to active nutrient uptake, where hypocotyl elongation and early leaf expansion produce a short phase of accelerated vertical growth

B) Early Overtaking Events and Kinetic Reversal Mechanisms (Days 6–8)

A critical system-level overtaking occurs between Days 5–7, where ethanol-treated plants surpass heat-treated plants in height (ethanol $\approx$ 10.75 cm vs heat $\approx$ 8.7 cm mean). This reversal while could be anomalous, however it is observed over both trials, has a high chance of biologically grounded in decomposition kinetics: ethanol treatment initially suppresses microbial efficiency but later enhances solubilization of organic compounds, producing a delayed initial nutrient surge. This is reflected across variables: ethanol leaf count increases (10–12 range), indicating synchronized biomass expansion. On the other hand, the apparent reduction in plant height observed in control (5.80 to 5.65) and heat-treated (9.6 to 8.7) systems after Day 6 does not represent true negative growth, but is instead attributable to a transient loss of turgor pressure and stem bending under emerging physiological stress, potentially driven by short-term water limitation or uneven water distribution within the substrate. As plant height was measured vertically from the soil surface to the highest point, partial wilting or curvature of the stem can produce lower recorded values despite continued biomass presence. Importantly, this does not preclude subsequent recovery, as restoration of water balance can re-establish turgidity and upright growth, allowing height to increase again in later time points. This interpretation is supported by concurrent declines in leaf health scores and the onset of wilting, indicating that structural measurements become temporarily decoupled from physiological status during early stress conditions.

In parallel, leaf count declines from higher early values (up to 18 leaves at Day 5) to lower values by Day 7 (16–12), showing that biomass loss begins while height remains relatively stable, establishing the first clear decoupling between structural growth and functional stability. Health scores also decline from near-optimal or moderate levels at Day 5 (5, 3) to reduced values by Day 7 (3, 3), reflecting early physiological stress and chlorophyll degradation. Wilting is already present from Day 6 (“Yes”), confirming that water transport disruption precedes visible structural collapse. Collectively, these trends demonstrate early multi-variable decoupling in control systems, where height no longer reliably reflects physiological condition due to nitrogen limitation and impaired water balance. This differential response is likely due to the initially rapid growth surge in heat-treated systems, which leads to temporary depletion of readily available nutrient fractions and short-term physiological adjustment, along with complete nutrient limitation in controls, both exacerbating early water stress, whereas ethanol-treated systems benefit from delayed nutrient release, sustaining turgor maintenance and continued structural expansion during this phase; however, if residual or delayed-release nutrient pools remain available, subsequent recovery and renewed growth are still possible.

C) Mid-Growth Stabilization, Divergent Resource Pathways, and Functional Tradeoffs (Days 8–12) Between Days 8–12, ethanol-treated plants exhibit the highest mid-phase growth rate, with mean height increasing from 10.75 to 13.65 cm alongside continued leaf production (11 to 16

leaves). This follows an earlier middle phase of rapid growth (Days 4–6), indicating that the system has transitioned from an initial middle surge to a more moderated but sustained growth phase, likely due to partial depletion of readily accessible delayed nutrient fractions. This pattern remains consistent with gradual solubilization and continued breakdown of compounds within the ethanol-treated necromass, maintaining nutrient availability during the mid-growth phase. In contrast, heat-treated plants show a moderate increase in height (8.7 to $\sim$ 11.3 cm) with minimal change in leaf number, representing recovery from the earlier transient decline and indicating stabilization of resource availability. This suggests that heat-treated systems experienced an initial rapid growth phase driven by early nutrient availability, followed by a temporary decline due to depletion of readily accessible nutrients and short-term physiological adjustment, and a subsequent recovery as delayed-release nutrient fractions became available, allowing growth to increase again. Overall, these results demonstrate that microbial inactivation method governs the timing of nutrient availability, producing a mid-phase growth peak in ethanol-treated systems and a more stabilized growth profile in heat-treated systems.

4. System Failure Thresholds and Collapse Consolidation in Controls (Days 8–14)

During Days 9–14, control plants exhibited synchronized collapse across all measured variables, including rapid leaf loss (14 $\rightarrow$ 7), sharp declines in health scores (3 $\rightarrow$ 1.5), and persistent wilting. This indicates a transition from growth-dominated metabolism to survival metabolism, where respiratory demand exceeds photosynthetic capacity due to nutrient exhaustion and progressive chlorophyll degradation. Wilting became persistent rather than intermittent, reflecting loss of turgor pressure regulation and reduced water transport efficiency, likely associated with declining root function and osmotic imbalance. This collapse is consistent with compounded nitrogen limitation and reduced chlorophyll synthesis, leading to impaired energy production and eventual physiological failure. Despite this overall decline, the mean plant height shows a slight increase (4.65 to 5.5), driven by asymmetrical survival between replicates, where one trial undergoes near-complete failure while another maintains a single dominant stem that continues elongation by reallocating limited internal resources, highlighting competitive resource prioritization under extreme stress conditions.

5. Day 12–14 in Heat Killed and Ethanol: Surprising reduction

The slight decrease in mean plant height observed in both heat (11.3–11 cm height) and ethanol-treated systems (13.65–12.9 cm height) between Days 12–14 does not represent true growth reduction but instead reflects transient wilting due to reduced turgor pressure, leading to minor stem curvature and lower recorded vertical height. This follows an earlier phase of rapid growth, during which high nutrient uptake likely depleted readily accessible resource pools, creating a temporary limitation as the system adjusts to reduced availability, which may reflect earlier depletion of readily available nutrients in heat-treated systems and a transient physiological adjustment following sustained nutrient uptake in ethanol-treated

systems. The absence of increase in leaf number confirms minimal net biomass production during this interval, while leaf health remains stable in heat-treated systems (≈ 5) and only slightly declines in ethanol-treated systems (≈ 5 to ~ 4.5), indicating mild, non-critical stress rather than systemic failure. Biologically, this phase represents a transition between nutrient pools, where initial labile resources are exhausted and plants rely on slower-release or less accessible fractions, temporarily reducing growth efficiency. In contrast, control systems continue to show a slight increase in mean height due to the persistence of a single elongating stem, as previously described. Growth resumes after this phase as delayed-access nutrients become available and water balance is restored, explaining the subsequent increase in plant height.

6. Late-Stage Reversal, Secondary Nutrient Pulse, and Dominance Shift (Days 14–20) From Days 14–20, a second major shift in growth hierarchy occurs, with heat-treated systems re-establishing dominance in both height and leaf production by Day 20 (heat ≈ 15.4 cm vs ethanol ≈ 14.3 cm). This follows a previously observed mid-phase period of ethanol dominance, indicating a time-dependent reversal in treatment efficiency. During the late phase, heat-treated plants show a marked acceleration in both height gain and leaf expansion ($\approx 20 \rightarrow 38$ – 39 leaves), suggesting renewed vegetative development after an earlier stabilization period. This pattern is consistent with continued structural breakdown of necromass under heat treatment, which likely disrupts more rigid cell wall components (e.g., cellulose–hemicellulose matrices), increasing long-term accessibility of bound organic compounds and supporting sustained nutrient release in later stages. In contrast, ethanol-treated plants begin to stagnate during this phase, with growth rate slowing (12.9 to 14.35) and leaf production continuing at a reduced rate (16 – 20), indicating reduced access to available nutrients and a shift toward maintenance-level metabolism rather than active expansion. This may reflect ethanol-induced disruption of membrane lipids and partial solubilization of easily accessible cytoplasmic compounds in the necromass, which accelerates nutrient release in the middle phase but leaves more structural polysaccharide-rich components less effectively broken down, leading to earlier depletion of readily available nutrient pools and reduced late-stage availability. Control plants remain in severe physiological decline with no meaningful recovery, confirming irreversible system failure once metabolic thresholds are exceeded under nutrient-limited conditions; however, a seed continues limited growth due to inherent variability in seed reserve size, germination vigor, and root establishment efficiency, allowing isolated individuals to temporarily persist despite overall system collapse. This is seen by the fact that control 1 goes to 7.7 cm while control 2 is at 4 cm.

Hence, this also shows that microbial necromass not only enhances visible growth parameters such as height and leaf count but also significantly increases total biomass production, with mean fresh biomass rising from 0.21 g in the control to 0.68 g in ethanol-treated systems and 1.03 g in heat-treated systems, confirming improved overall resource utilization and plant productivity in regolith systems. Furthermore, the greater biomass

accumulation in heat-treated systems (1.03 g) compared to ethanol-treated systems (0.68 g) reinforces that the method of microbial inactivation directly influences nutrient availability, with thermal processing enabling more sustained and efficient nutrient release compared to ethanol treatment. The standard deviation observed across replicate trials reflects inherent biological variability within plant growth systems. Even under controlled conditions, individual plants may exhibit differences in germination timing, nutrient uptake efficiency, and metabolic activity, leading to variation in measured parameters. Therefore, the presence of variability does not undermine the reliability of the results but instead indicates the natural stochasticity of biological systems, reinforcing the importance of using mean values for accurate interpretation.

Conclusion: This study set out to test the hypothesis that microbial necromass can enhance plant growth in lunar regolith simulant, and that the microbial inactivation pathway used to generate that necromass (thermal vs. ethanol sterilization) would significantly influence its effectiveness as a soil amendment. Overall, the results strongly support this hypothesis. Both necromass treatments substantially improved germination success, germination stability, vegetative growth, physiological health, and biomass accumulation relative to untreated regolith, confirming that recycled microbial biomass can partially compensate for the absence of organic matter and biologically mediated nutrient cycling in lunar regolith systems. Importantly, the consistent performance hierarchy (heat-treated $>$ ethanol-treated $>$ control) across multiple independent growth metrics supports the second part of the hypothesis, demonstrating that both the presence of necromass and its processing pathway are key determinants of plant performance.

However, while the hypothesis correctly predicted that heat-inactivated necromass would outperform ethanol-inactivated necromass due to increased structural breakdown and enhanced nutrient accessibility, it did not explicitly predict the **time-dependent nature of this divergence**. The observed phase shifts in growth dynamics indicate that the effect of microbial inactivation is not constant over time but instead varies across developmental stages. The findings hence refine the original hypothesis by showing that treatment-dependent differences arise from three distinct kinetic regimes governed by nutrient release timing, metabolic efficiency, and substrate accessibility. Heat-inactivated necromass promotes a bimodal growth trajectory, with an initial rapid phase driven by immediate nutrient release followed by a secondary acceleration sustained by continued decomposition of more recalcitrant structural components. This reflects greater structural disruption of microbial cells, increased exposure of bound organic fractions, and breakdown of rigid cell wall matrices (e.g., cellulose–hemicellulose), collectively enhancing long-term accessibility of organic carbon and nitrogen. In contrast, ethanol-inactivated necromass preserves more intact cellular architecture, producing a smoother, diffusion-driven growth pattern characterized by sustained mid-phase acceleration due to gradual solubilization of organic compounds, but earlier

plateauing as readily accessible nutrient fractions become depleted, resulting in faster mid-stage availability but reduced long-term persistence. Control systems exhibit a progressive decline in growth rate, transitioning from early reserve-dependent growth to stagnation and eventual regression due to the absence of exogenous nutrient input, confirming irreversible system failure once metabolic thresholds are exceeded under nutrient-limited conditions, despite minor variability arising from inherent differences in seed reserve size, germination vigor, and early root establishment. Across all treatments, overtaking events, transient fluctuations, and rate shifts are consistent with decomposition-mediated nutrient dynamics rather than experimental error, with residual variability reflecting expected differences in uptake efficiency and microenvironmental conditions.

Several key assumptions underpin these interpretations. First, it was assumed that microbial inactivation was complete in both treatments; however, while sterility was assessed using a regrowth-based assay, this method only detects culturable or actively resuscitating organisms and may not capture non-culturable but metabolically dormant survivors or resilient microbial fragments. A more rigorous approach would include molecular-level confirmation of sterilization using DNA-based methods such as qPCR targeting universal 16S/18S rRNA gene sequences or ATP bioluminescence assays to quantify residual metabolic activity, providing higher sensitivity and reducing uncertainty regarding incomplete microbial inactivation. Second, it was assumed that compost-derived necromass was compositionally uniform prior to treatment, despite potential variability in microbial community composition and biomass structure. Third, the study assumes that differences in plant performance arise primarily from biochemical differences in necromass processing rather than unmeasured physical differences in substrate structure (e.g., porosity, aggregation, bulk density, and water distribution), which may also have been subtly altered by heat or ethanol exposure. Finally, the interpretation assumes that regolith simulant adequately approximates key physicochemical constraints of lunar regolith, despite the absence of extraterrestrial conditions such as reduced gravity, radiation exposure, and extreme thermal cycling.

A key limitation of this investigation is the lack of direct characterization of the regolith–necromass system, as nutrient availability (including bioavailable nitrogen, phosphorus, and organic carbon) and soil physical properties such as porosity and water retention were not measured. This limits the ability to determine whether the observed improvements in plant growth were driven primarily by increased nutrient supply or structural enhancements to the substrate. Furthermore, the composition of the necromass itself was not analyzed, restricting insight into how different inactivation methods may have influenced nutrient release dynamics. Future work should incorporate quantitative chemical and physical analyses of the substrate, alongside detailed characterization of necromass, to better resolve the mechanisms underlying growth enhancement. Experimental uncertainty may also arise from measurement limitations, including minor inaccuracies in height measurement, biomass

determination, and consistency of necromass mixing within the regolith. While efforts were made to standardize procedures, small human and instrumental errors are inevitable. Nevertheless, consistent methodology across treatments ensures that comparisons between groups remain valid.

Future experimental improvements should also focus on environmental standardization and scaling. A controlled greenhouse or growth chamber setup would allow precise regulation of temperature, humidity, CO₂ concentration, and light intensity, reducing environmental noise and enabling stronger attribution of effects to substrate composition alone. Increasing replication (more pots per treatment and independent experimental runs) would improve statistical power and allow more robust detection of subtle treatment differences. In addition, future studies should incorporate a gradient of necromass concentrations rather than a single fixed 15% inclusion rate, enabling determination of dose–response relationships and identification of optimal amendment levels for plant productivity in regolith systems. Further improvements could include direct biochemical characterization of necromass before and after inactivation (e.g., soluble nitrogen, carbon fractions, lipid and amino sugar content, amino sugars, lipid fractions, and carbon speciation), as well as measurements of substrate physical changes such as water retention curves, bulk density, porosity, and pore structure. These would help separate chemical nutrient effects from physical soil modification effects, which are likely both contributing to plant performance. Isotopic tracing (e.g., ¹⁵N-labelled biomass) would also allow direct confirmation that necromass-derived nutrients are being assimilated into plant tissues rather than indirectly altering substrate conditions, while simultaneously separating uptake efficiency from substrate-mediated effects.

These results establish microbial necromass as a practical, scalable biological resource for autonomous agriculture in extraterrestrial environments, where waste is not discarded but engineered into a functional nutrient system. The demonstrated control of plant growth through thermally and ethanol-mediated deactivation pathways shows that nutrient release can be predictably tuned using processes already feasible under lunar-relevant conditions. Unlike current lunar agriculture concepts that rely on pre-processed Earth-supplied nutrients or externally manufactured growth media, this work demonstrates that equivalent or superior productivity can be achieved through in-situ conversion of biological waste into controllable nutrient systems, shifting from resource import dependence to self-sustaining ecological production on the Moon. This strengthens the feasibility of sustained lunar life by enabling continuous food production through recycling biological waste into usable nutrients. Microbial systems can be regenerated on the Moon, and deactivation methods repeatedly applied, making this a non-finite regenerative loop unlike Earth-dependent supply chains. Thus, waste organisms that would otherwise be discarded can be converted into food-producing inputs, showing waste can become food in a sustained lunar system. Within this framework, heat-treated necromass is the most effective pathway, showing higher productivity and greater stability than ethanol treatment while remaining feasible under lunar condition

